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Ferroptosis in malignant brain tumors

Edited by
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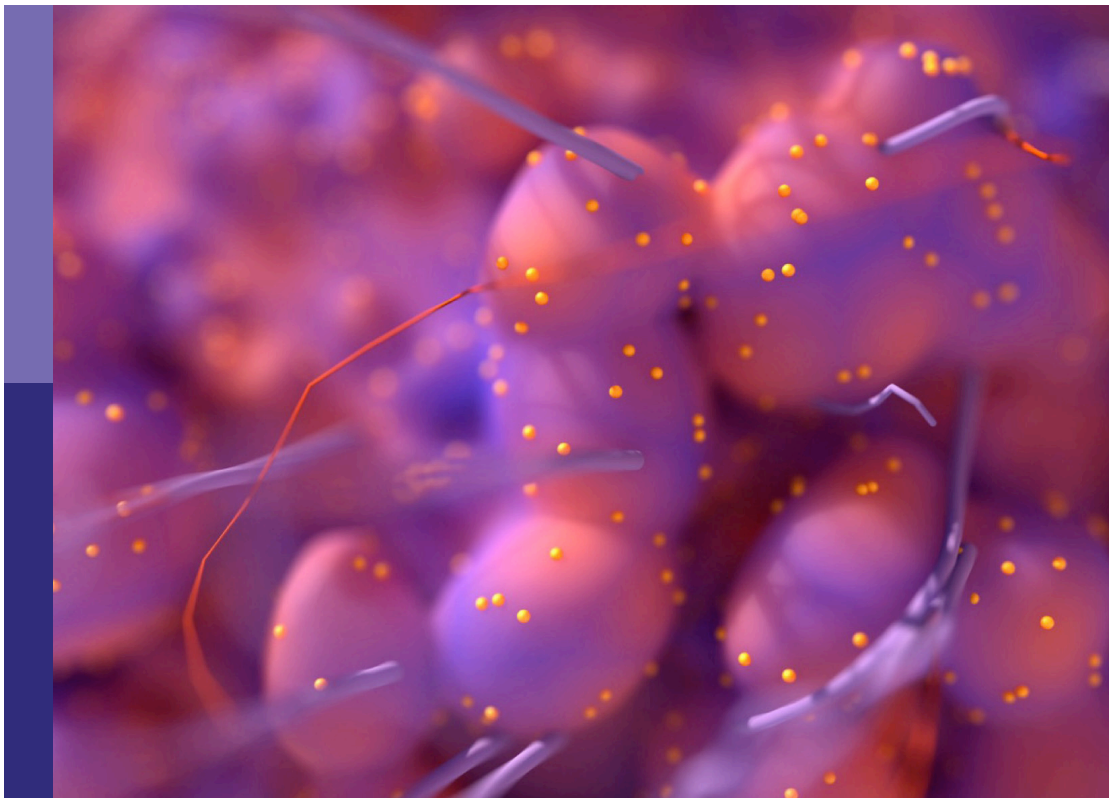

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Ferroptosis in malignant brain tumors

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Editorial: Ferroptosis in malignant brain tumors

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Editorial on the Research Topic
Ferroptosis in malignant brain tumors

Malignant primary brain tumors constitute around 30% of all primary brain tumor diagnoses in the United States. Unfortunately, these type of tumors still have a fatal prognosis despite advancements in the neuro-oncological toolbox. Although a multimodal therapy approach is the current gold standard, malignant primary brain tumors display a complex intratumoral heterogeneity. As a consequence, brain tumors trigger intricate molecular and metabolic shifts within the tumor microenvironment which might be responsible for therapy resistance and tumor relapse. However, our understanding of the molecular composition and orchestration of malignant primary brain tumors is still incomplete for efficient clinical translation. Thus, further investigations into the mechanisms of brain tumor growth are urgently needed.

Recent research has highlighted the relevance of ferroptosis in tumorigenesis – a process of iron-dependent programmed cell death. This novel mechanism also reveals clinical relevance due to its potential to mitigate oxidative stress and treatment resistance. However, the underlying mechanisms and regulators of ferroptosis remain elusive, with limited dedicated research. Consequently, triggering ferroptosis emerges as a promising therapeutic route, particularly for malignant brain tumors, which demand a new treatment paradigm.

This Research Topic aims to provide a comprehensive overview of ferroptosis in brain tumor development, progression, recurrence, and its interplay with the immune and tumor microenvironment, along with its therapeutic prospects. This endeavor culminated in a collection of five original research articles and twelve review articles, contributed by eminent global ferroptosis researchers.

Several manuscripts have illuminated distinct aspects of ferroptosis in malignant gliomas. Zhou et al. investigated the impact of miR-29b-mediated targeting of GPx7 (glutathione peroxidase 7), revealing that GPx7 suppression enhances erastin-induced ferroptosis. Dong et al. uncovered the influence of ferroptosis-related genes on immunity, stemness and prognosis in glioblastoma, suggesting novel prognostic indicators. Fu et al. identified LncRNA (long noncoding RNA) PELATON as a ferroptosis suppressor and prognostic signature, introducing fresh insights into the intricate molecular landscape of

these tumors. Kram et al. showcased an upregulation of ACSL4 (acyl-CoA synthetase long-chain family member 4) and ALDH1A3 (aldehyde dehydrogenase 1A3) proteins during tumor relapses, indicating an increased vulnerability of glioblastoma relapses to ferroptosis. For further understanding of the dynamic progression and structural attributes of necrosis in glioblastomas, Yee et al. conducted a timely study of necrosis development in a mouse glioblastoma model, linking radiographic and histological observations. The extent of necrosis seen among glioblastoma patients was reconstructed using orthotopic xenograft glioma model induced by hyperactivation of the Hippo pathway transcriptional coactivator with PDZ-binding motif (TAZ).

Within the realm of review articles, Zhao et al. meticulously examined the pivotal role of iron transporters in ferroptosis within malignant brain tumors. This exploration seamlessly segued into the realm of PPARγ modulation in further research by the Yakubov team unraveling the intricate interplay between this molecular orchestrator and the ferroptosis process within the context of malignant glioma and tumor-related edema. On the other hand, Ferrada et al. embarked on an exploration of pharmacological avenues to incite ferroptosis, specifically targeting glioblastoma and neuroblastoma. Meanwhile, Chi et al. illuminated the potential benefits and challenges of harnessing ferroptosis in treatments, revealing insights into the distinctive molecular and microenvironmental traits inherent to these separate brain tumors. A detailed analysis of the immunological milieu of gliomas by Wang et al. identified ferroptosis's role within the complex immune microenvironment.

The remaining publications in this Research Topic focus on the current situation, prospects, drug applications and off-target effects of ferroptosis induction in malignant brain tumors. Lu et al. provided insights into the molecular mechanisms of ferroptosis in glioma progression and treatment, while Yin et al. discussed the mechanisms of long non-coding RNAs in glioma ferroptosis. Zhang et al. explored ferroptosis-related ncRNAs in an effort to achieve personalized treatment regimen for gliomas through ferroptosis. Yao et al. proposed a ferroptosis-based drug delivery system for malignant brain tumors. Zhou et al. highlighted the emerging role of ferroptosis as a promising therapeutic target in glioblastoma treatment, particularly in cases that are resistant to conventional therapy. Xie et al. explored autophagy-dependent ferroptosis as a potential treatment for glioblastoma. In addition, Dahlmanns et al. analyzed genetic profiles of ferroptosis in malignant brain tumors and off-target effects of ferroptosis induction, emphasizing the need for precision in harnessing this therapeutic strategy.

Collectively, the collection of research in this Research Topic provides a comprehensive panorama of ferroptosis's multifaceted involvement in malignant brain tumors. These articles accentuate specific molecular targets, such as GPx, long non-coding RNAs, iron transporters, and PPARγ, in the context of distinct brain tumor types. The diverse array of mechanisms, prospective therapeutic pathways, and challenges associated with ferroptosis in malignant brain tumors underscore the imperative need for further research to unlock its full therapeutic potential. This pursuit not only opens avenues for innovative strategies but also holds the potential to reshape the treatment terrain for malignant brain tumors, ushering in renewed optimism for both patients and the neuro-oncological community.

Author contributions

EY: Supervision, Writing – original draft, Writing – review & editing. AG: Writing – review & editing. NS: Writing – review & editing.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genetic Profiles of Ferroptosis in Malignant Brain Tumors and Off-Target Effects of Ferroptosis Induction

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Glioblastoma represents the most devastating form of human brain cancer, associated with a very poor survival rate of patients. Unfortunately, treatment options are currently limited and the gold standard pharmacological treatment with the chemotherapeutic drug temozolomide only slightly increases the survival rate. Experimental studies have shown that the efficiency of temozolomide can be improved by inducing ferroptosis – a recently discovered form of cell death, which is different from apoptosis, necrosis, or necroptosis and, which is characterized by lipid peroxidation and reactive oxygen species accumulation. Ferroptosis can also be activated to improve treatment of malignant stages of neuroblastoma, meningioma, and glioma. Due to their role in cancer treatment, ferroptosis-gene signatures have recently been evaluated for their ability to predict survival of patients. Despite positive effects during chemotherapy, the drugs used to induce ferroptosis – such as erastin and sorafenib – as well as genetic manipulation of key players in ferroptosis – such as the cystine-glutamate exchanger xCT and the glutathione peroxidase GPx4 – also impact neuronal function and cognitive capabilities. In this review, we give an update on ferroptosis in different brain tumors and summarize the impact of ferroptosis on healthy tissues.

Keywords: ferroptosis, neuroblastoma, glioblastoma, erastin, neuron, xCT, brain tumor therapy, off-target effects

INTRODUCTION

Ferroptosis is as an iron-dependent form of cell death, which is different from previously known forms of cell death such as apoptosis, necrosis, or necroptosis. It is characterized by the accumulation of reactive oxygen species (ROS) and lipid peroxidation (1–3). After finding that activating ferroptosis in cancer cells of mice improved the effectiveness of temozolomide treatment – a first-line chemotherapeutic drug against glioblastoma (glioma WHO grade IV) (4, 5) – further investigations revealed the important role of ferroptosis also in human cancer patients.

Glioma is a type of primary brain tumor that is generated from glial cells in the central nervous system. These gliomas are classified by the WHO into low-grade glioma (WHO grade II) and high–grade glioma (WHO grade III/IV), where higher grading is associated with poorer prognosis (6).

Ferroptosis represents an option to improve treatment for patients suffering especially from these more malignant tumors, including glioblastomas, because these are difficult to cure by radiation, resection, or pharmacological treatment alone. Especially because pharmacological treatment is affected by drug resistances (7).

Since the discovery of ferroptosis in 2012 (1) several key molecules have been identified, which are either directly integrated into the ferroptosis process or act as inducers. Current data about key players in ferroptosis and their role in glioma have been reviewed elsewhere (8, 9). The recently launched database ferrDB provides an overview of these regulators and markers in ferroptosis (10).

This review provides an overview of ferroptosis in the therapy of various brain tumors with a focus on ferroptosis gene signatures, which have a strong translational value in predicting patients' prognosis, and of the effects of ferroptosis induction in non-cancerous tissue that is also affected during treatment (**Figure 1**).

PROMISING FINDINGS ON FERROPTOSIS INDUCTION IN NEUROBLASTOMA AND MENINGIOMA

Expanding on the treatment boosting effects of ferroptosis induction in glioma, there are also promising findings in other types of cancer. Neuroblastoma is a highly relevant pediatric cancer in younger children (11), with limited treatment options and therapy resistance if occurring in its high–risk form (12). Induction of ferroptosis to limit tumor growth has been emerging as a striking new concept to treat neuroblastoma.

Ferroptosis can be induced by several small molecules [as reviewed elsewhere (8)] or by inhibition of the glutathione peroxidase GPx4 (13) and glutamate/cystine antiporter system x_c^- (SLC7A11; also referred to as xCT) through the drugs erastin (1), sulfasalazine (14), or sorafenib (15), amongst others.

Recently, treatment with the steroidal lactone withaferin A was found to induce the nuclear factor erythroid 2–related factor 2 (Nrf2) pathway and to inactivate the GPx4 pathway, a duality making this strategy highly effective in treating both neuroblastoma cells and transplanted xenografts in mice (16). In this study the substance was targeted to the tumor site with nanoparticles, which reduces side-effects (17).

Chemosensitization to erastin–induced ferroptosis was also accomplished after knockdown of the iron exporter ferroportin in neuroblastoma SH-SY5Y cells (18).

In about 25% of neuroblastoma cases MYCN is amplified (19). In patient-derived xenografts of these cases, the xCT-driven antioxidant response after sulfasalazine application is increased compared to controls, which leads to an increase in ferroptosis and subsequently limited tumor growth (19). Further studies revealed that the transferrin receptor 1 was upregulated in response to such MYCN amplification, leading to increased GPx4 sensitivity and rendering neuroblastoma cells vulnerable to ferroptosis induction (20). In addition to this genetically mediated sensitization, the inhibition of PKCα stimulated ferroptosis and sensitized neuroblastoma stem cells to etoposide, which is particularly relevant given the central role of stem cells in conferring resistance to therapy (21). Neuroblastoma cell lines also express a very low level of ferritin heavy chain 1, whose reduction leads to a rise in ROS and a higher sensitivity to ferroptosis (22). In meningioma cell lines derived from patients covering WHO Grades I–III, the vulnerability to erastin-induced ferroptosis was increased both by a loss of neurofibromin and by a low level of E-cadherin. The expression of these proteins is driven by the myocyte enhancer factor 2C, making it a promising factor to manipulate during meningioma treatment (23, 24).

In summary, treatment of cancers such as neuroblastoma and meningioma in their advanced stages may be improved by exploiting the role of ferroptosis.

FERROPTOSIS-GENE SIGNATURES IN GLIOMA

Gliomas represent a major form of brain cancer, divided into WHO grades I to IV with glioblastoma being the most devastating form of human brain cancer (6) because it is associated with a low survival, therapy resistance and limited treatment options (25). To overcome these obstacles, genetic studies based on large patient databases have examined the link between gene expression in glioma and overall survival in risk–stratified patient cohorts. In these studies, ferroptosis– and glioma–related genes of interest were identified by screening RNA sequencing data and associated clinical data. These gene–signatures constitute a risk-model, predicting the overall survival of the patients. To avoid overfitting, the models were each constructed in one database, e. g. Chinese Glioma Gene Atlas (CGGA), and validated using other databases, e. g. Repository for Molecular Brain in Neoplasia Data (REMBRANDT) or The Cancer Genome Atlas (TCGA) (26–32). The risk–models are shown and described in **Table 1**. The risk models that are based on the ferroptosis–related genes stratified glioma patients into a low–risk and high–risk cohort.

In high-risk cohorts, the median survival probabilities indicated by Kaplan-Meier curves were significantly decreased. The risk-score was often correlated with clinicopathological features such as the WHO grade or the O–6–Methylguanine–DNA Methyltransferase (MGMT) promotor methylation status, proving the suitability of ferroptosis–related gene expression pattern for patient outcome prediction. Interestingly, functional annotation of the ferroptosis–related genes in the risk–models revealed that often the immune system is involved: Investigation of RNA sequencing data from glioblastoma (TCGA) revealed that the expression levels of ferroptosis suppressors such as CD44, HSPB1 and SLC40A1 correlated with the degree of immunosuppression and were related to survival of patients (34). The expression of these suppressors could also be induced by acetaminophen (34).

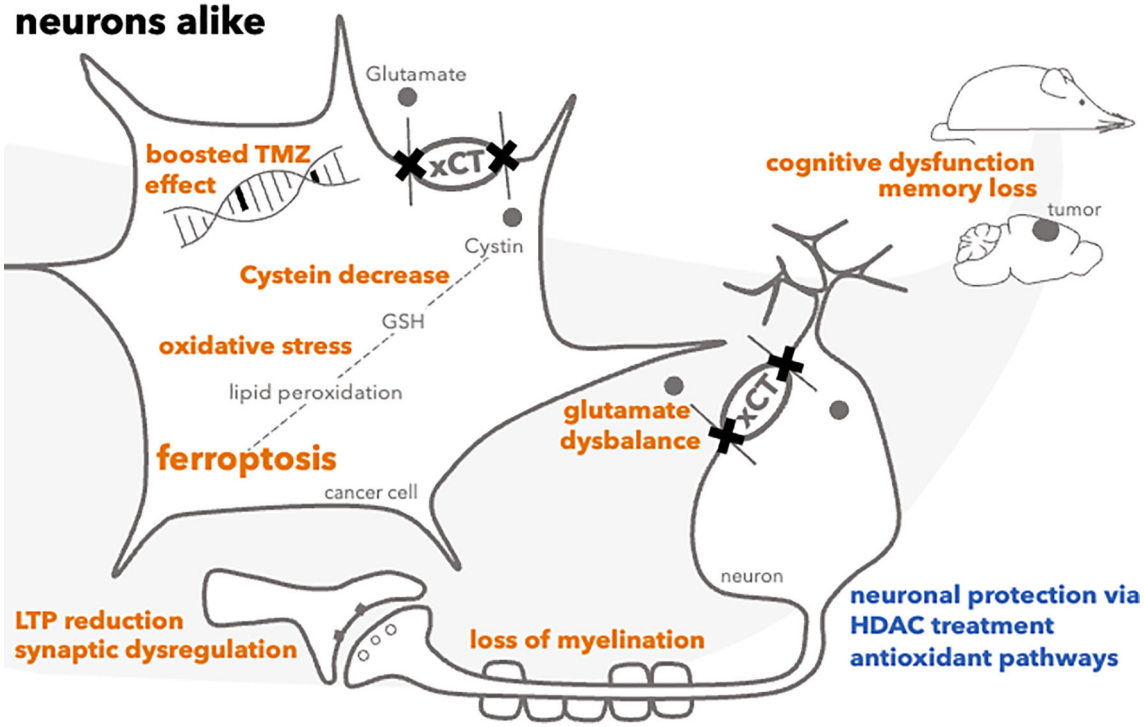

FIGURE 1 | Consequences of ferroptosis induction in cancer cells and neurons.

This bioinformatics-based immunology-ferroptosis-link was substantiated by experimental data showing that glioma GL261 cells during their early ferroptotic stages (induced by RSL3) promoted the activation of dendritic cells, which indicates a vaccination-like effect of the tumor cells on the immune system (35). With this, a link between ferroptosis and immunological responses in the context of glioma is strongly suggested and awaits further experimental clarification.

In Wan et al. the relevance of a link between ferroptosis and metabolism in the context of glioma was suggested based on a database analysis (29). For tumors, their increased metabolic reliance on utilizing amino acids (36) and lipids (37) represents malignancy hallmarks of cancer in general. In glioma, the approach of developing an amino acid-risk score – alike the here-described panels concerning ferroptosis-related genes - revealed that the expression of genes involved in amino acid metabolism is important for glioma patients' survival prognosis (38). In glioma with non-mutated isocitrate dehydrogenase (IDH), branched-chain amino acids such as leucine and isoleucine, and their catabolizing enzyme branched-chain amino acid transaminase 1 (BCAT1), are more expressed – in turn, BCAT1 knock-down in glioma cells reduces the viability of glioma cells (39). Underlining the role of ferroptosis during amino acid regulation in cancer, the induction of ferroptosis eventually inhibited transcription of BCAT2 and the direct inhibition of BCAT2 led to ferroptosis in target cells (40). Additionally, cysteine and glutamate represent important amino acids during ferroptosis induction (41), whose homeostasis is interrupted by blocking xCT to achieve cell death.

In general, cancer cell growth and development are reliant on an increased lipid usage (42, 43). Thus, interfering with these pathways by oxidation of the lipids may boost cancer therapy by exploiting ferroptosis (31, 37). Increased lipid peroxidation is the result of ferroptosis induction and eventually leads to ferroptotic cell death (1). As one of the proteins that was used to generate survival-predicting ferroptosis-related genes panels (**Table 1**), ACSL4, increases the content of omega-6 polyunsaturated fatty acids in cellular membranes and thus regulates how sensitive cells are to ferroptosis (44).

In one ferroptosis–signature panel, the data suggested that a risk–score built up by 19 ferroptosis genes was negatively correlated with the expression of MGMT, which confers resistance to temozolomide (26). However, many different mechanisms have been proposed to be contributing to temozolomide resistance in glioma (5, 45), which makes it difficult to assess their respective translational importance. Interestingly, not only coding RNA but also long non–coding RNA was shown to be predictive regarding overall survival (30).

TABLE 1 | Risk-models using ferroptosis-related genes and their predictive capabilities.

Study	Databases	Genes inside final gene signature	Gene function based on GO/KEGG	Correlation of the signature/ risk model with:	What does the gene signature predict?
(26)	CGGA, TCGA, GSE16011, REMBRANDT	19 genes SAT1, ATP5G3, HSPB1, FANCD2, HMGCR, CBS, GCLC, GCLM, CD44, ALOX12B, ALOX5AP, CISD1, NFE2L2, EMC2, ALOX4, DPP4, AKR1C2, LPCAT3 and NCOA4	Cell death, migration, and immune systems function → tumorigenesis and progression	WHO tumor grades, clinical/ pathological tumor features	Overall survival
(27)	CGGA TCGA	25 genes Protective: BAP1, GLS2, CISD1, PRNP, AKRIC3, TF, ACACA, ACSL6 and MAP3K5 Hazardous: CDKN1A, G6PD, HSPB1, LOX, STEP3, ACSL1, CP, HMOX1, CYBB, ANO6, PCBP1, PGD, AURKA, G3BP1 and TP53	Responses to oxidative stress, nutrient level, and extracellular stimuli; pathways involve fatty acid synthesis, ferroptosis	1p/19q codeletion, IDH1 status, MGMT promoter methylation status, histology, age, WHO grading, PRS type	Overall survival
(31)	CGGA TCGA	12 genes (ferrDB-based) Protective: VDAC2, MAP3K5, DNAJB6, CHMP5 Hazardous: TP63, NFE2L2, MT3, LAMP2, HSPB1, FANCD2, EIF2AK4 and ARNTL	Metabolic processes related to glutamate, immune systems response, and plasma membrane receptor complex	1p/19q codeletion, IDH1 status, MGMT promoter methylation status, radiation therapy	Overall survival
(28)	CGGA TCGA; GSE16011, REMBRANDT	11 genes Associated with a poor prognosis were a -high expression of CD44, FANCD2, HSBP1, MT1G, NFE2L2 and SAT1 -low expression of AKR1C3, ALOX12, CRYAB, FADS2 and ZEB1	Cancer progression by modulation of the immune system function	1p/19q non-codeletion, MGMT promoter methylation status, IDH status, recurrent and secondary tumors	Overall survival
(29)	REMBRANDT, CGGA-693, CGGA-325, TCGA	59 genes, metabolism of -Iron: FANCD2, NCOA4, TFRC, PHKG2, HSPB1, ACO1, FTH1, STEAP3, NFS1, IREB2, HMOX1 and MT1G -Lipid: ACSL4, AKR1C1-3, ALOX15, ALOX5, ALOX12, CARS, CBS, CISD1, CS, DPP4, GPX4, HMGCR, LPCAT3, FDFT1, ACSL3, PEPB1, ZEB1, SQLE, FADS2, ACSF2, PTGS2 and ACACA -Antioxidants: GCLC, SLC7A11, KEAP1, NQO1, ABCC1, CHAC1, GSS, GCLM and NFE2L2 -Energy: GLS2, SLC1A5, GOT1, G6PD, PGD and ATP5G3 Other genes: CD44, HSPB1, CRYABM, RPL8, SAT1, TP53, EMC2 and AIFM2	Metabolism of iron, lipids, antioxidants, and energy	High risk scores: glioma WHO grade IV, IDH wildtype, no codeletion 1p/19q	Overall survival
(30)	TCGA, CGGA, REMBRANDT	15 Long non-coding RNAs: SNAI3-AS1, GDNF-AS1, WDFY3-AS2, CPB2-AS1, WAC-AS1, SLC25A21-AS1, ARHGEF26-AS1, LINC00641, LINC00844, MIR155HG, MIR22HG, PVT1, SNHG18, PAXIP1-AS2, SBF2-AS1	–	Low-risk groups: Radiotherapy was effective High-risk group: Unfavorable immunological situation	Overall survival
(33)	Pubmed-reported ferroptosis-proteins, TCGA GBMLGG, CGGA	8 genes: ALOX5, CISD1, FTL, CD44, FANCD2, NFE2L2, SLC1A5, GOT1	Lipid metabolism, carboxylic acid metabolism	IDH1_p.R132H (6/8), tumor purity (5/8), MGMT methylation (5/8),	-Overall survival -Progression-free survival

In addition to the common prediction of overall survival, one study was able to also accurately predict patients' progression-free survival based on ferroptosis–related proteins (33).

While all presented risk models were capable of stratifying patients into high-risk and low-risk cohorts, the number of ferroptosis–related genes required to create the prognostic model substantially varies from 8 up to 59 included genes (**Table 1**). Redundancies of several genes between different risk models might indicate their general importance.

To evaluate if these genes are exclusively predictive of the outcome prognosis in glioma, we examined their role in comparable gene signature panels in other cancer types: A number of genes that are part of glioma risk–models (CARS, FANCD2, HMGCR, NCOA4 and SLC7A11) (46) and (AKR1C1, CARS1, CBS, CD44, CHAC1, DPP4, FANCD2, GOT1, HMGCR, SLC1A5, NCOA4 and STEAP3) (47) also accurately predicted patients' prognosis in clear renal cell carcinoma. Similarly, survival probability in hepatocellular carcinoma was reliably predicted by a glioma prediction model (ACSL3, ACSL6, ACACA, G6PD, SLC1A5, SLC7A11 and VDAC2) (48) and by a risk model with a strong overlap with the genes in the glioma models (G6PD, HMOX1, LOX, SLC7A11, STMN1/Stathmin 1)

(49). It is, however, unlikely that ferroptosis signatures are similar across all different types of cancer, which is exemplified by a study predicting breast cancer based on a completely different set of ferroptosis–related genes (50). It will be interesting to investigate the minimum number of expressed ferroptosis–related genes in a tumor for the patients' outcome to benefit from ferroptosis induction and to investigate, how the gene expression is systematically distributed across different kinds of tumors.

FERROPTOSIS IN HEALTHY NEURONS AND POTENTIAL SIDE–EFFECTS OF FERROPTOSIS INDUCTION

The functional property of cystine/glutamate exchanger xCT is the uptake of cystine and the extrusion of glutamate – a key molecule of neuronal function, whose homeostasis is key for proper signal transduction and cognitive behavior (39, 51, 52). Because another function of xCT is the stimulation of the antioxidative response of the cell, xCT–inhibitors can induce ferroptosis (1) **(Figure 1)**.

Given the promising preclinical finding of improved temozolomide (Temodal®, Temcad®) chemotherapy outcome through combination with xCT–inhibitory small molecules (4), it appears necessary to also investigate such drugs' potential impact on other cells in the vicinity of the tumor tissue and in the whole body. In particular, diseases of the peripheral nervous system are known side–effects of some chemotherapeutic treatments (53), and also have been linked to ferroptosis (54).

Here, we take a closer look at the impact of xCT interference on neuronal and cognitive function (**Figure 1**)

An investigation of how the xCT inhibitors erastin and sorafenib affect cultured hippocampal neurons in their morphology and their vesicle pool size – a parameter tightly linked to neuronal function – has shown that such treatment could significantly disturb neuronal viability (55). In the hippocampus of xCT–deficient mice, long–term potentiation and long–term memory were impaired (56), which highlights the importance of xCT–driven glutamate homeostasis for cognition. Although a reduction of extracellular glutamate would be expected after xCT–inhibition or deletion, additional extracellular glutamate could not reverse this effect (56). In primary hippocampal cell cultures consisting of both, neurons and glia, extracellular amino acid profiling could not confirm a reduction, but rather an increase in extracellular glutamate after erastin–induced xCT inhibition, suggesting a complex regulatory interplay between different cell types of the brain (55).

Inhibition of xCT led to a myelination defect in organotypic cerebellar slices after a few days of treatment, showing that neuronal function is disturbed also on the axonal level (57).

On a behavioral level, xCT was linked to stress resilience in the ventral hippocampus, because alterations in the histone acetylation status increased xCT expression and in turn recruited other glutamate receptors to modulate glutamate homeostasis (58). Mice with intraperitoneal erastin injections developed iron depositions in several organs such as brain, kidney and spleen, mild cerebral infarction and epithelial changes in the duodenum (59).

Efforts to examine ferroptosis–inhibitory agents to protect against such adverse effects have demonstrated that hippocampal HT22 cells could be protected from ferroptosis with Ajudecunoid C – a chemical isolated from *Ajuga nipponensis* – *via* an activation of an antioxidant response element pathway (60), or with diphenylamine compounds (61). Similarly, spinal cord neurons have been protected from erastin–induced ferroptosis through LipoxinA4–induced activation of the Akt/Nrf2/HO–1 signaling axis (62), which represents a key player in the regulating of ferroptosis and also in glioma treatment (63–65). The impact of erastin on neuronal viability was further counteracted in primary cortical neurons and SH–SY5Y cells by the iron chelator deferoxamine (66). Despite ferroptosis being similar in neurons and cancer cells, class 1 histone deacetylase inhibitors (HDACs) treatment protected neurons from ferroptosis but augmented ferroptosis in HT1080 fibrosarcoma cells (67), thereby providing the best possible outcome. This promising finding now awaits its experimental evaluation in other cell types, for example in different glioma cell lines.

Ferroptosis can also be thwarted on other levels of the ferroptosis–inducing process, for example by selenium–mediated inhibition of the antioxidant glutathione peroxidase 4 (GPx4) (68), which is also implicated in the pathophysiology of glioblastoma (7, 69, 70b). Similar to xCT–deficient mice (56), conditional deletion of GPx4 in adult forebrain neurons resulted in impaired functions of memory and spatial learning (71), and its deletion from dopaminergic midbrain neurons increased anxious behavior (72). These examples from a list of several more ferroptosis–inhibitory agents demonstrate that such drugs, initially intended to counteract neurodegeneration, could also act as support during chemotherapy to protect healthy tissue.

In contrast to erastin, which remains a purely experimental substance, multi–kinase inhibitor and ferroptosis inductor sorafenib has entered human clinical trials that included assessment of neuropsychological effects during cancer therapy. Learning, memory, and executive functions suffered over the course of treatment (73). This is further supported by a study in rats that revealed neurochemical disturbances in the hippocampus during treatment with sorafenib (74). Although the histology of the hippocampus was unaffected in that study, treatment with sorafenib for 28 days strongly decreased levels of several key metabolites such as glutamate, GABA, serine, or choline, which were measured by nuclear magnetic resonance spectroscopy. In contrast, striatum and prefrontal cortex remained rather unaffected (74). In primary rat hippocampus cultures, high–performance liquid chromatography revealed that, already after 24 h of sorafenib treatment, levels of glutamate, serin, and alpha–aminobutyric acid were increased, and levels of glycine, cystine, and phosphoethanolamine were decreased (55). These data illustrate metabolic disturbances in response to sorafenib treatment, which may account for cognitive dysfunction.

In addition to emerging as possible side effects of ferroptosis pathway manipulation, cognitive impairment was also described as a glioblastoma symptom (75). Cognitive impairment often

delays diagnosis and is associated with a reduced overall survival (75), which should be considered when assessing cognitive dysfunction as potential side effects of add–on drugs.

CONCLUSION

The pharmacological therapy of malignant brain tumors is difficult, especially of late–stage glioma with its treatment resistance and recurrences. The novel idea of enhancing treatment outcome through ferroptosis induction continually gains attention. Recent data uncovered a link between ferroptosis–signatures in malignant glioma and overall survival, with many studies using expression of ferroptosis–related genes to accurately predict patients' survival probability. Harnessing ferroptosis to improve tumor therapy will be an appealing approach also in malignant neuroblastoma and meningioma. But interfering with ferroptosis induction also has off–target effects, which may decrease the quality of life. Therefore, the increase in survival probability predicted by ferroptosis–gene-based risk models should be traded of against potential harm through ferroptosis–inducing add–on therapy. Ideally, patients should be screened for ferroptosis-related gene expression - based on a unified set of disease-relevant ferroptosis-related genes - and stratified into high-risk or low-risk cohorts to judge their individual clinical prospects. Future clinical trials may evaluate the benefits versus side effects of ferroptosis inducing cancer treatment enhancement for different patient groups.

In summary, ferroptosis induction is a hope yielding approach to enhance antitumor therapy but requires an intricate balance between attacking the tumor and preserving the different cell types of the healthy tissue.

AUTHORS CONTRIBUTIONS

MD proposed the research. MD and JD both reviewed the literature and collected references. MD, EY, and JD wrote the manuscript and finalized the paper. All authors contributed to the article and approved the submitted manuscript.

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GLOSSARY

ACACA	Acetyl-CoA carboxylase 1
ACO1	Aconitase 1
ACSF2	Acyl-CoA Synthetase Family Member 2
ACSL1/3/4/6	Acyl-CoA Synthetase Long Chain Family Member 1/4/6
AKR1C1-3	Aldo-keto reductase family 1 member C3 1-3
ANO6	Anoctamin 6
AIFM2	Apoptosis Inducing Factor Mitochondria Associated 2
ALOX12B	Arachidonate 12-Lipoxygenase 12R Type
ALOX12	Arachidonate 12-Lipoxygenase 12S Type
ALOX15	Arachidonate 15-lipoxygenase
ALOX5	Arachidonate 5-Lipoxygenase
ALOX5AP	Arachidonate 5-Lipoxygenase Activating Protein
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like protein 1
ABCC1	ATP Binding Cassette Subfamily C Member 1
ATP5G3	ATP Synthase Membrane Subunit C Locus 3
AURKA	Aurora Kinase A
BAP1	BRCA1 Associated Protein 1
CISD1	CDGSH Iron Sulfur Domain 1
CP	Ceruloplasmin
CHAC1	ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1
CHMP5	Charged multivesicular body protein 5
CS	Citrate Synthase
CD44	Cluster of differentiation 44
CRYAB	Crystallin Alpha B
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CBS	Cystathionine Beta-Synthase
CARS	Cysteinyl-TRNA Synthetase 1
SLC7A11	Cystine/Glutamine antiporter xCT or Solute Carrier Family 7 Member 11
CYBB	Cytochrome b(-245) beta subunit
DPP4	Dipeptidyl peptidase 4
Hsp40	DnaJ Heat Shock Protein Family
DNAJB6	Member B6
EMC2	ER membrane protein complex subunit 2
EIF2AK4	Eukaryotic translation initiation factor 2α kinase 4
FANCD2	FA Complementation Group D2
FDFT1	Farnesyl-Diphosphate Farnesyltransferase 1
FADS2	Fatty acid desaturase 2
FTL	Ferritin Light Chain
G6PD	Glucose-6-phosphate dehydrogenase
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit
GCLM	Glutamate-Cysteine Ligase Modifier Subunit
GOT1	Glutamic-Oxaloacetic Transaminase 1
GLS2	Glutaminase 2
GPX4	Glutathione Peroxidase 4
GSS	Glutathione Synthetase
HSPB1	Heat shock protein beta-1
HMOX1	Heme oxygenase 1 gene
IREB2	Iron-responsive element-binding protein 2
KEAP1	Kelch Like ECH Associated Protein 1
LPCAT3	Lysophosphatidylcholine Acyltransferase 3
LAMP2	Lysosome-associated membrane protein 2
HMGCR	HMG-CoA reductase
LOX	Lysyl Oxidase
MT3	Metallothionein 3
MT1G	Metallothionein-1G
MAP3K5	Mitogen-activated protein kinase kinase kinase 5
NQO1	NAD(P)H Quinone Dehydrogenase 1
SLC1A5	Neutral amino acid transporter B(0)
NFS1	NFS1 Cysteine Desulfurase
NFE2L2	Nuclear factor-erythroid 2-related factor 2
NCOA4	Nuclear Receptor Coactivator 4
PEPB1	Phosphatidylethanolamine Binding Protein 1
PGD	Phosphogluconate Dehydrogenase
PHKG2	Phosphorylase Kinase Catalytic Subunit Gamma 2
PRNP	Prion protein
PTGS2	Prostaglandin-Endoperoxide Synthase 2
G3BP1	Ras GTPase-activating protein-binding protein 1
RB1	RB Transcriptional Corepressor 1
RPL8	Ribosomal Protein L8
STEAP3	Six-transmembrane epithelial antigen of the prostate 3
SAT1	Spermidine/Spermine N1-Acetyltransferase 1
SQLE	Squalene Epoxidase
TFRC	Transferrin Receptor
TP53	Tumor protein p53
TP63	Tumor protein p63
VDAC2	Voltage-dependent anion-selective channel protein 2
ZEB1	Zinc Finger E-Box Binding Homeobox 1

(Continued)

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GPX7 Is Targeted by miR-29b and GPX7 Knockdown Enhances Ferroptosis Induced by Erastin in Glioma

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Background: Glioma is a lethal primary tumor of central nervous system. Ferroptosis is a newly identified form of necrotic cell death. Triggering ferroptosis has shown potential to eliminate aggressive tumors. GPX7, a member of glutathione peroxidase family (GPXs), has been described to participate in oxidative stress and tumorigenesis. However, the biological functions of GPX7 in glioma are still unknown.

Methods: Bioinformatics method was used to assess the prognostic role of GPX7 in glioma. CCK8, wound healing, transwell and cell apoptosis assays were performed to explore the functions of GPX7 in glioma cells. *In vivo* experiment was also conducted to confirm *in vitro* findings. Ferroptosis-related assays were carried out to investigate the association between GPX7 and ferroptosis in glioma.

Results: GPX7 was aberrantly expressed in glioma and higher expression of GPX7 was correlated with adverse outcomes. GPX7 silencing enhanced ferroptosis-related oxidative stress in glioma cells and the loss of GXP7 sensitized glioma to ferroptosis induced by erastin. Furthermore, we found that miR-29b directly suppressed GPX7 expression post-transcriptionally. Reconstitution of miR-29b enhanced erastin sensitivity, partly *via* GPX7 suppression.

Conclusions: Our study clarified the prognostic role of GPX7 in glioma and preliminarily revealed the role of GPX7 in ferroptosis, which may be conducive to the exploration of therapeutic targets of glioma.

Keywords: bioinformatics, ferroptosis, glioma, GPX7, miR-29b

INTRODUCTION

Glioma is the most common and lethal tumor of the central nervous system, and glioblastoma (GBM, grade IV) is the most malignant subtype (5-year survival are only about 5.5%) (1). Considering the high recurrence and mortality rates of glioma, it is crucial to investigate its causes and potential molecular mechanisms to find new targets for early diagnosis and personalized treatment.

To defend oxidative stress, the living organisms have evolved several antioxidative mechanisms that prevent cells from damage induced by reactive oxygen species (ROS) (2). Among these mechanisms, the glutathione peroxidase (GPXs) family is a major antioxidant enzyme family (GPX1-GPX8) that reduces various ROS such as hydrogen peroxide and lipid peroxides (3).

In the past, GPX7 was viewed as an important intracellular sensor that detects redox level and transmits ROS signals to multiple biologic processes (4). Recent studies have reported that GPX7 participated in the initiation and progression of tumors. DunFa Peng et al. (5) reported that GPX7 had tumor suppression function in oesophageal adenocarcinomas and was silenced by promoter DNA methylation. Zheng Chen et al. (6) showed that GPX7 was downregulated in gastric cancer and reconstitution of GPX7 suppressed tumor growth in 3D organotypic models. E. Guerriero et al. (7) showed that GPX7 had an overexpression in hepatocellular carcinoma tissues. In glioma, only one bioinformatic study reported that GPX7 was a potential prognostic molecule based on Chinese Glioma Genome Atlas (CGGA) database (8). However, the mechanisms by which GPX7 might exert its actions in the development of glioma remain to be elucidated.

Ferroptosis is a newly discovered type of necrotic cell death caused by the accumulation of lipid-based ROS and has gained growing interest on account of its close relevance to multiple pathological situations (e.g. neurodegeneration, ischemia/reperfusion injuries and malignancies) (9, 10). Several small molecule compounds have been developed to trigger ferroptosis of tumors. Erastin is the most commonly used ferroptosis inducer, which directly suppresses the cystine/glutamate antiporter (system x_c^-) to suppress GSH synthesis (11, 12). GPX4, known as the key enzymatic inhibitor of ferroptosis, can protect biological membranes from peroxidative degradation (10). Yang et al. reported that the inhibition of GPX4 elevated the lipid peroxidation level and induced the ferroptosis of tumors (13). However, we need to take into account the dependence of lipid metabolism and the abundance of GPX4 in the specific tissues. Pharmacological targeting of GPX4 may only achieve partial anti-tumor effects (14). Based on the antioxidative functions and complex roles of GPX7 in tumors, we speculate that GPX7 may participate in glioma development by regulating ferroptosis, which need to be further verified.

MicroRNAs (miRNAs) are endogenous small RNA molecules, which inhibit gene expression by binding directly to complementary sequences located mostly in the 3′-untranslated regions (3′UTR) of mRNAs (15). In cancers, miRNAs can elicit notable effects on cell phenotypes by suppressing the expression of target genes (16). However, to date, whether GPX7 expression is regulated by miRNA has not been reported.

Our study, for the first time, disclosed that GPX7 was targeted by miR-29b and we preliminarily explored the relationship between GPX7 and ferroptosis. Integrating bioinformatic analysis and experimental analysis allow more effective contributions to the promising target of GPX7 in glioma.

MATERIALS AND METHODS

Dataset Selection

The transcript level of GPX7 in glioma was assessed by Oncomine, The Cancer Genome Atlas (TCGA) (17), GSE16011 dataset (from Gene Expression Ominibus database) (18) and the Repository of Molecular Brain Neoplasia Data (REMBRANDT) (19). Apart from that, the protein level of GPX7 was validated by 127 glioma tissue samples obtained from Huanhu Hospital (Department of Neurosurgery, Huanhu Hospital, Tianjin, China; 2017). All cases were primary gliomas and patients received no extra treatments such as radiotherapy and chemotherapy before surgery. All the biopsies were acquired from surgical resection and processed immediately into formalin-fixed, paraffin-embedded tissues to produce the tissue array. The clinicopathological features of patients were concluded in **Table S2**.

Cells and Chemicals

The human glioblastoma cell lines U87, T98G and LN229 were purchased from Beijing Beina Chuanglian Biotechnology Institute and A172 and T98G were purchased from iCell Bioscience Inc. Co. Ltd. (Shanghai, China). Erastin (T1765), RSL3 (T3646), ferrostatin-1 (fer-1, T6500), liproxstatin-1 (lip-1, T2376) and deferoxamine (DFO, T1637) were purchased from TOPSCIENCE (Shanghai, China).

Cell Transfection and Lentivirus Infection

The sense oligonucleotide sequences of siRNAs and miRNAs mimics were concluded in **Supplementary Table S1**. The transfection of siRNA and miRNA mimics was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA). The establishment of stable GPX7 knockdown cell line *via* lentivirus vectors (LV3-shGPX7, the sequence was shown in **Table S1**) was conducted by GenePharma (Suzhou, Jiangsu, China). The siRNAs, miRNA mimics, knockdown lentivirus vectors and pcDNA3.1-GPX7 overexpression plasmid were purchased from GenePharma (Suzhou, Jiangsu, China).

Quantitative Real-Time PCR (qPCR)

The qPCR was performed according to the processes, as previously reported (20).The primers were designed as follows: GPX7 forward, 5′-AGTAGCCCCAGATGGAAAG-3′ and reverse, 5′-TCGCTTCAGTAGGATGAGC-3′; GAPDH forward, 5′-CAATGACCCCTTCATTGACC-3′ and reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′. Relative quantification was analyzed by the $2^{-\Delta\Delta}$Ct method.

Western Blot and Immunohistochemistry (IHC)

Western blot assay was carried out according to the manufacturer's protocol, as previously described (20). The blocked membrane was incubated with antibody against GPX7 (1:500; 13501-1-AP, Proteintech, IL, USA) or β-actin (1:1000; CST, MA, USA) for at least 4 hours. After incubation with second antibody for 60 min, chemiluminescence and exposure were performed.

Immunohistochemistry assay was completed by two experienced pathologists from Pathology Department of Huanhu Hospital. Patients were grouped into high and low expression cohorts grounded on the integrated scores of GPX7 expression, as previously reported (21). The primary antibodies used in IHC were Ki67 (ab16667, abcam, UK), MMP2 (D4M2N, CST, USA), N-cadherin (D4R1H, CST, USA).

CCK-8, Wound Healing, Invasion and Apoptosis Analysis

Cells were seeded in 96-well plates (5×10^3 cells per well) and transfected with siGPX7 or co-transfection of miR-29 mimics and pcDNA3.1-GPX7 plasmid. CCK-8 agent (K009-500; ZETA LIFE, CA, USA) was injected to each well for 30-minute incubation at 0, 24, 48 and 72h time points, respectively. Finally, microplate reader (iD5, Molecular Devices, CA, USA) was employed to measure the absorbance at 450 nm. For wound healing assay, cells were cultured in 12-well plates. Next, the bottoms of wells were scratched with a pipette tip. After being washed twice, cells were cultured within the serum-free medium for 24h. Finally, the wound closure (%) was measured by ImageJ. Transwell and apoptosis assays were performed according to the manufacturer's protocols, as previously described (22).

Glutathione Assay

GSH Detection Kit (Solarbio Co., Beijing, China) was used to detect reduced glutathione (GSH) according to the manufacturer's instructions.

Lipid Peroxidation Assay

The level of lipid peroxidation was detected using BODIPY 581/591 C11 (GLPBIO, CA, USA) according to the manufacturer's instructions.

Cellular Iron Concentration Assay

The iron assay kit (FerroOrange, F374, Dojindo Molecular Technology) was used to detect cellular iron concentration level according to the manufacturer's protocols.

Dual Luciferase Reporter Assay

The wild/mutant-type 3′UTR of GPX7 was inserted into the GP-miRGLO vector (Promega, WI, USA). LN229 and T98G cells were cotransfected with wild or mutant vectors and NC mimic or miR-29a/b/c-3p mimic. The activities of luciferase were measured 48h after transfection according to the manuals of the Dual Luciferase Assay System (Promega, WI, USA).

Immunofluorescence Assay

Cells plated onto poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde for 20 min. The cells were permeabilized with 0.5% Triton X-100 (Sigma, USA) and blocked by 5% BSA for 2 h. The coverslips were incubated with primary antibody and secondary antibody following the manufacturer's protocol. Primary antibodys were GPX7 (1:500; 13501-1-AP, Proteintech, IL, USA), Ki67 (1:1000, ab16667, abcam, UK), MMP2 (1:800, D4M2N, CST, USA) and N-cadherin (1:1000, D4R1H, CST, USA). Secondary antibody was Alexa Fluor® 488-conjugated Anti-Rabbit IgG (H+L) (1:800, Jackson ImmunoResearch Inc, USA). After being mounted with antifade mounting medium with DAPI (ZSGB-BIO, Beijing, China), images were acquired under a confocal microscope (LSM 800, Zeiss, Germany)

Xenograft Model With Nude Mice

BALB/c-A nude mice (female, 4 weeks old) were purchased from Sibeifu Beijing Biotechnology Co. Ltd. (Beijing, China). Animal assays were conducted under the approval of the Animal Care and Used Committee of Tianjin Huanhu Hospital. First, the mice were randomly allocated into four groups and 1×10^7 LN229 cells stably transfected with lentiv-GPX7 or lentiv-NC were inoculated s.c. into the axillary fossa of mice every two groups. When tumor volume reached approximately 50 mm^3, one of every two groups were treated with erastin dissolved in 5% DMSO/corn oil (23) (30 mg/kg intraperitoneal injection, every other day) for 3 weeks. The tumor size was estimated using formula (length × $width^2$/2). 42 days later, the mice were sacrificed, and the tumors were removed for IHC assay.

Bioinformatics Analysis

The differential expression of GPXs was analyzed using GEPIA, a web database containing abundant normal specimens from GTEx database (24). Gene set enrichment analysis (GSEA) (25) was conducted with the false discovery rate (FDR) < 0.25 and normal P value < 0.05 as thresholds. The correlation of GPX7 and immune infiltration in glioma was analyzed in TIMER (https://cistrome.shinyapps.io/timer/) (26, 27), an online web portal which can investigate immune cell infiltration levels using data from TCGA.

For ferroptosis analysis, ferroptosis gene set (contributed by WikiPathways) was downloaded from Molecular Signatures Database (28). Ferroptosis and GO and KEGG gene sets related to redox biology and glutathione metabolism were analysed by GSEA. A well-established model of ferroptosis potential index (FPI) was defined as the enrichment score (ES) of positive components minus that of negative components, which was calculated using single sample gene set enrichment analysis (ssGSEA). The details of FPI model could be obtained from a previous paper (29).

Statistical Analysis

The relationships between GPX7 protein expression and clinical variables of glioma were estimated using *Chi-Square* test. Patients with missing information were excluded from the corresponding analysis. Student's t-test and one-way analysis of variance (ANOVA) were used to test for significant differences between two or multiple groups, respectively. All tests were two-sided. The statistical analysis was performed using R software v3.6.3. and GraphPad Prism software.

RESULTS

Preparation of Datasets

The clinical characterastics of patients from 4 datasets were concluded in **Table 1**. The detailed information of patients in Huanhu cohort were recorded in **Table S2**.

TABLE 1 | The information of patients in 4 datasets.

Characteristic	TCGA	GSE16011	REMBRANDT	Huanhu
Total	703	284	472	127
Age				
≥52	263	133		45
<52	407	143		82
Gender				
Male	386	184	221	77
Female	284	92	126	50
Grade				
I		8	2	
II	248	24	98	39
III	261	85	85	35
IV	161	159	130	53
Histology				
Pilocytic astrocytoma		8		
Astrocytoma	192	29	147	28
Oligodendroglioma	190	52	67	46
Oligoastrocytoma	128	28		
Glioblastoma	160	159	219	53
Mixed glioma			11	
IDH1 mutation				
Yes	91	81		87
No	34	140		40
1p19q codeletion				
Yes		110		48
No		45		29
KPS				
<80	71	82		
≥80	341	182		

GPX7 mRNA Levels in Different Databases

Firstly, the mRNA expression level of GPX7 in GBM was analyzed in Oncomine database. Pooled analysis of six datasets revealed an upregulation of GPX7 in GBM than in normal (**Figure 1A**, $P = 0.038$). This result was also validated in other three datasets (**Figures 1B–D**, $P < 0.001$).

GPX7 Is Related to Clinicopathological Factors of Glioma

In TCGA, GSE16011 and Rembrandt, GPX7 expression was upregulated in tumor with grade IV compared with those with lower grades (**Figure 1E**). For histopathologic type, higher expression of GPX7 was found in the patients with adverse histopathologic type (**Figure 1F**). Besides, GPX7 expression increased among older patients and those with wild-type IDH1 (**Figures 1G, H**). Moreover, some other clinicopathological factors (Karnofsky Performance Status (KPS) and gender) were also analyzed (**Figures 1I, J**). These data indicated that GPX7 high expression predicted adverse malignant phenotypes of glioma.

GPX7 Predicts Worse Survival in Glioma

Patients were grouped into two cohorts based on median expression value. In TCGA, Kaplan-Meier survival analyses showed that glioma patients with GPX7-high had a worse prognosis than that with GPX7-low (**Figure 2A**). And ROC analyses demonstrated that GPX7 was a good predictor of survival (**Figure 2A**). Similar results were also obtained in GSE16011 and Rembrandt datasets (**Figures 2B, C**).

To further explore the independent prognostic value of GPX7, the univariate analysis revealed that GPX7-high correlated significantly with a worse OS (**Figure 2D**). In multivariate analysis, GPX7 was still independently correlated with OS (**Figure 2E**), along with age, grade and KPS. Additionally, we also analyzed the expression levels of other GPX family members in glioma based on GEPIA. We found GPX1, GPX3, GPX4 and GPX8 had relative high expression levels in both LGG and GBM, while GPX2 had low expression level (**Supplementary Figure S1A**). However, in multivariate analyses, none of them was independently correlated with OS (**Supplementary Figure S1B**).

GPX7 Is Associated With Tumor and Immune Related Pathways

GSEA was employed to find the biological functions of GPX7 in glioma. In TCGA, we found some gene sets related to tumorigenesis (e.g. P53 signaling pathway and cell cycle) and immunity (e.g. natural killer cell mediated cytotoxicity and antigen processing and presentation) were enriched in KEGG analysis in the cohorts with GPX7 high expression (**Figure 2F**). For GO terms, tumorigenesis and immunity related gene sets (e.g. negative regulation of cell cycle phase transition, activation of innate immune response and T cell receptor signaling pathway) were also enriched (**Figure 2F**). Meanwhile, similar consequences were also gained in CGGA (**Figure 2G**).

To better understand the roles of GPX7 in the immune microenvironment of glioma, we analyzed the correlations between GPX7 and several common immune cell types in TIMER. As shown in **Figure 2H**, strong positive correlations existed between GPX7 expression and the infiltrations of all six immune cells types in LGG. Meanwhile, GPX7 was also positively correlated with the infiltrations of B cells, neutrophils, macrophages and dendritic cells in GBM.

Protein Expression of GPX7 in Glioma Tissues

Representative IHC slides of specimens from Huanhu cohort with different grades are shown in **Figure 3A**. Some examples of high expression of GPX7 in patients with grade II and low expression in grade III were showed in **Supplementary Figure S1C**. We found that GPX7 had relative high expression levels in samples with grade IV, histologic GBM, IDH1 wild and in samples without 1p19q codeletion, while no association was found between GPX7 and age, Ki67, P53 mutation and MGMT methylation (**Figure 3B** and **Table S2**).

We also detect GPX7 expression in five GBM cell lines using western blot and RT-PCR methods (**Figures 3C, D**). LN229 and T98G were selected for subsequent experiments. Two siRNAs (siGPX7-i and siGPX7-ii) were used to suppress GPX7 expression (**Figures 3E, F**).

GPX7 May be Relevant to Ferroptosis in Glioma

Given that the loss of GPX7 causes the increase of the intracellular ROS concentration and sensitizes cells to excessive

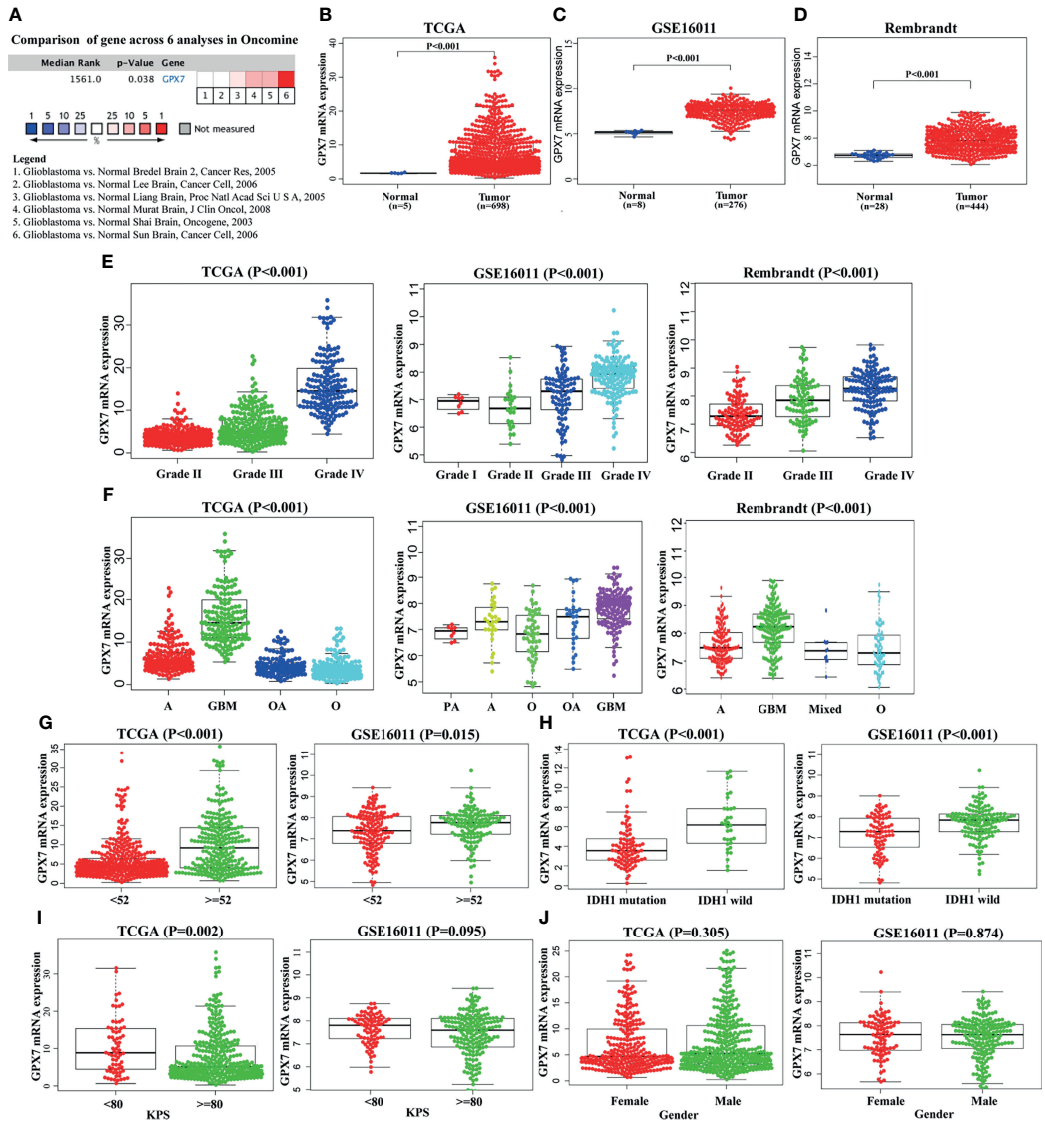

FIGURE 1 | The expression of GPX7 among databases. **(A)** The expression differences of GPX7 between normal and GBM were analyzed across the six analyses in Oncomine database. **(B–D)** The expression differences of GPX7 between normal and GBM in TCGA, GSE16011 and Rembrandt datasets. **(E–J)** The relationships between GPX7 and clinical characteristics among different datasets.

environmental oxygen (30), we explored preliminarily the association between GPX7 and ferroptosis in both TCGA and CGGA. As shown in **Figure 4A** and **Supplementary Figure S2A**, GSEA revealed that ROS metabolic process, response to oxidative stress, response to oxygen radical, glutathione metabolism and ferroptosis gene sets were enriched in cohort with GPX7-high. Then, we employed the ferroptosis potential index (FPI) to represent the level of ferroptosis based on the mRNA data from the two databases. As shown in **Figures 4B, C** and **Supplementary Figures S2B, C**, higher FPI was correlated with advanced tumor grades and shorter survival time in glioma. Moreover, patients with GPX7-high tended to have higher FPI levels (**Figure 4D** and **Supplementary Figures S2D**). These data implied a potential association between GPX7 and ferroptosis in glioma.

GPX7 Silencing Enhances Ferroptosis-Related Oxidative Stress in Glioma Cells

To verify the aforementioned bioinformatic analysis results, ferroptosis-related oxidative stress indicators, such as reduced GSH content, lipid peroxidation and Fe^{2+} concentration, were assessed in glioma cells. The inhibitor (fer-1) and inducer (erastin) of ferroptosis were employed in this research. Firstly, the susceptibility of glioma cells to ferroptosis was evaluated. CCK-8 assay showed that LN229 and T98G cells treated with erastin (10 μM) for 24 h had a significant decline in cell viability,

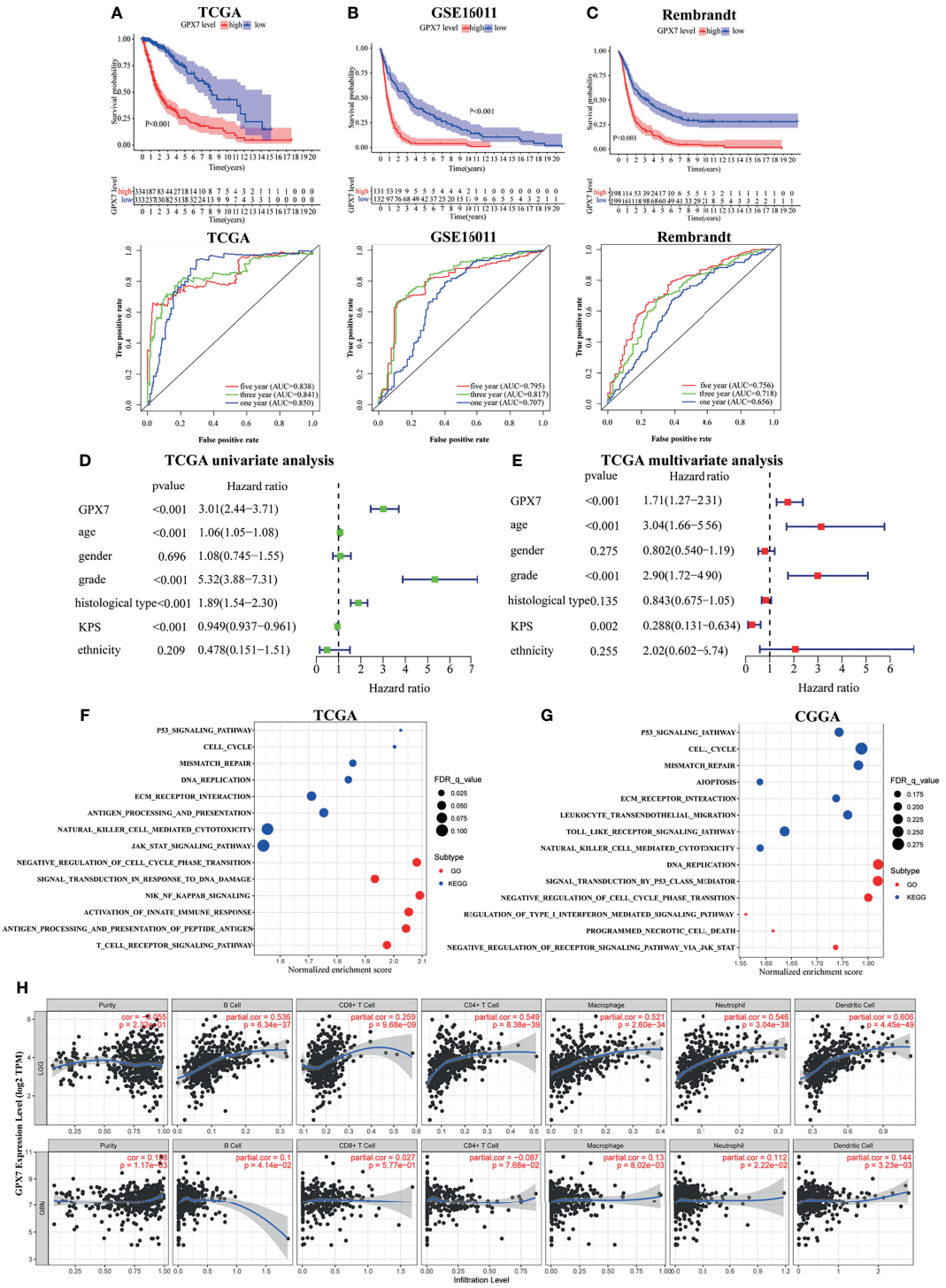

FIGURE 2 | The prognostic values of GPX7 in public databases and GSEA. **(A–C)** Kaplan–Meier analysis and 1, 3 and 5- year ROC curves of survival. **(D, E)** Univariate and multivariate Cox regression in TCGA. **(F, G)** GO and KEGG enrichment analyses using GSEA method in TCGA and CGGA. **(H)** The correlation between GPX7 and immune infiltration in LGG and GBM using the TIMER database.

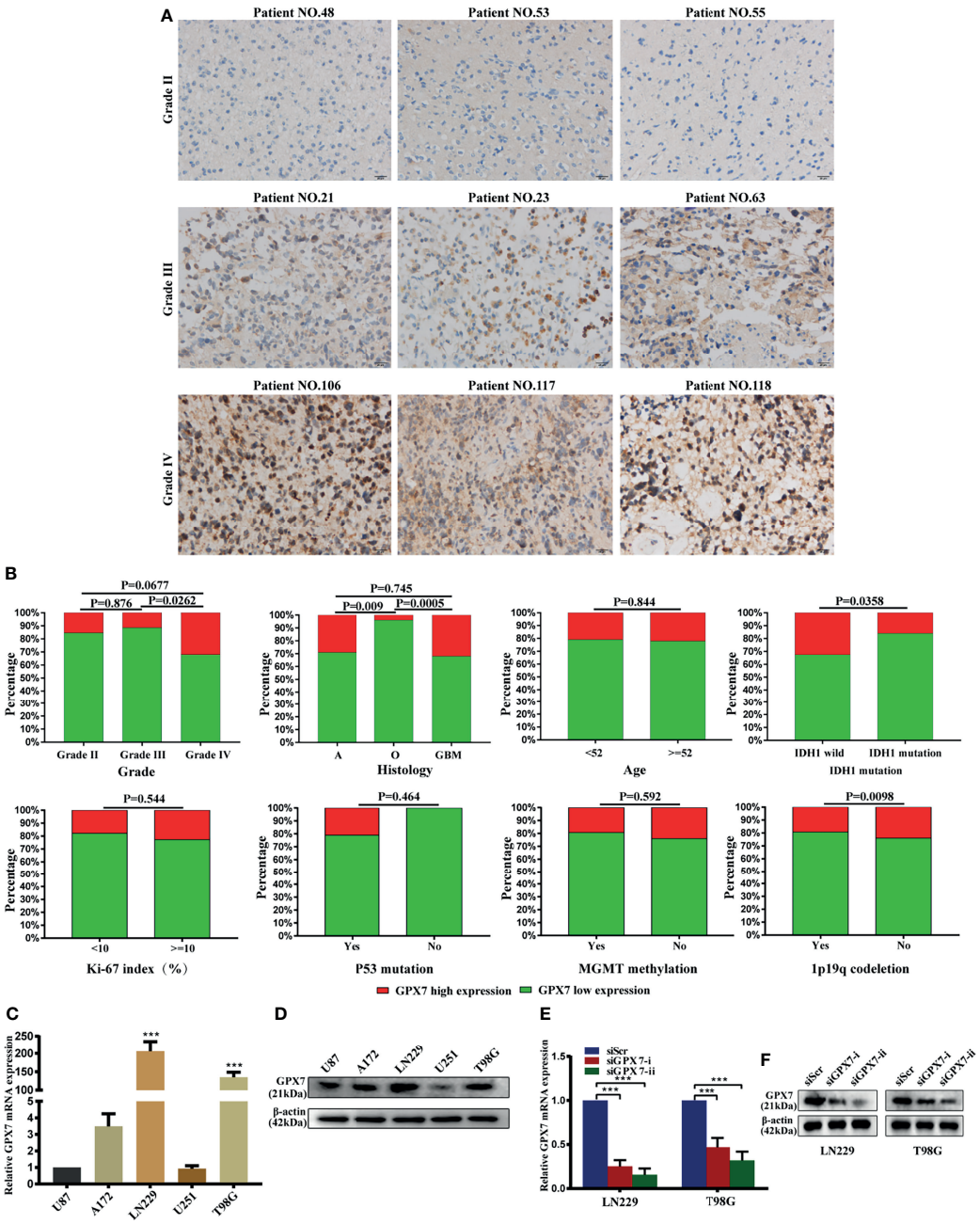

FIGURE 3 | The protein expression of GPX7 in Huanhu cohort. **(A)** Representative IHC slides of specimens with grade II, III and IV. Patients' numbers were recorded in **Table S2**. **(B)** Associations between GPX7 protein level and clinical variables in the Huanhu cohort. **(C, D)** The mRNA and protein levels of GPX7 in five different GBM cell lines using qPCR (with U87 as a control) and western blot. **(E, F)** PCR and western blot were performed to determine the expression of GPX7 after transfection with two siRNAs (siGPX7-i and siGPX7-ii). ***P < 0.001.

which was blocked by fer-1 (**Figure 4E**). Among two siRNAs, we selected siGPX7-ii, which showed more effective inhibition of gene expression (**Figures 3E, F**), for the follow-up experiments. As shown in **Figure 4F**, GPX7 knockdown resulted in reduced GSH depletion in both LN229 and T98G cells, which was aggravated when combined with erastin treatment. The Fe^{2+} level was detected by FerroOrange regent. We found GPX7 deficiency didn't affect iron level, but the combination of GPX7 deficiency and erastin treatment leaded to higher iron level than erastin treatment alone (**Figure 4G**). Lipid peroxidation is a key indicator of ferroptosis which can be detected by C11 BODIPY 581/591 probe. Compared with control group, lipid peroxidation

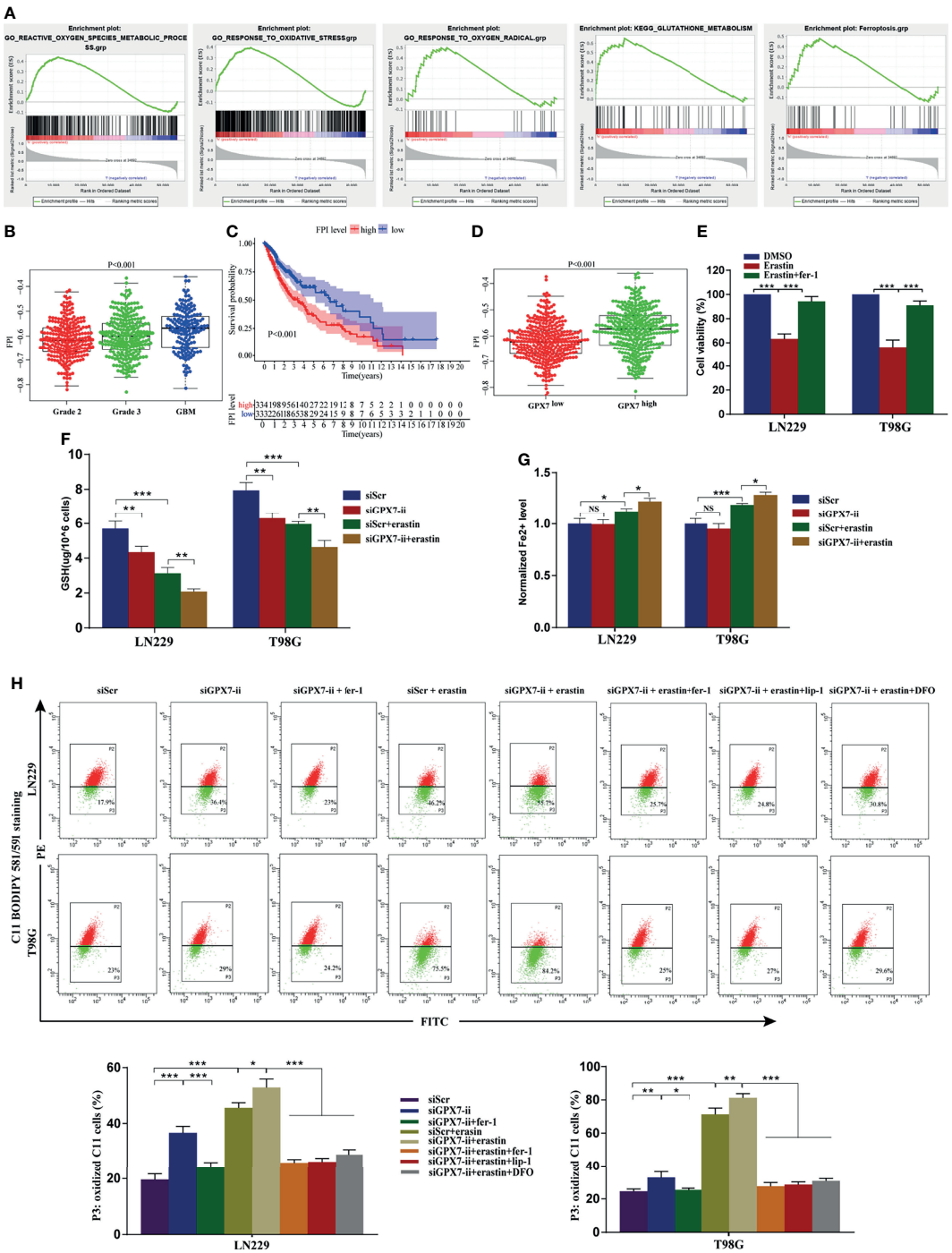

FIGURE 4 | The association of GPX7 and ferroptosis in glioma. **(A–D)** Bioinformatics analysis of the association of GPX7 and ferroptosis based on data from TCGA. **(A)** GSEA of ferroptosis and redox biology related gene sets in cohort with GPX7 high and low. **(B)** The different FPI levels among different WHO grades of glioma. **(C)** Kaplan-Meier analysis of OS according to the FPI level. **(D)** The association of GPX7 expression and FPI level in TCGA glioma samples. **(E)** CCK-8 assay was used to detect the cell viability of glioma cells treated with erastin (10 μM) for 24 h with or without fer-1 (2 μM). **(F)** The reduced GSH level in glioma cells subjected to siGPX7-ii transfection with or without erastin (10 μM) treatment. **(G)** The Fe2+ concentration was measured by FerroOrange probe. **(H)** Lipid peroxidation was detected in glioma cells subjected to siGPX7-ii transfection with or without fer-1 (2 μM), erastin (10 μM), lip-1 (100 nM) and DFO (100 μM) treatment, using C11 BODIPY 581/591 probe on flow cytometry. Within each chart, cells in P3 region in green represent those stained with oxidized dye. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

increased significantly in the GPX7 knockdown group (**Figure 4H**). Fer−1 suppressed the accumulation of lipid peroxidation and GPX7 deficiency could enhance the effect of erastin (**Figure 4H**). We also used three ferroptosis inhibitors (lip-1, fer-1 and DFO) on GPX7 knockdown cells treated with erastin. We found that lip-1, fer-1 and DFO can abolish the increase of lipid peroxidation of GPX7 knockdown cells treated with erastin (**Figure 4H**). These results showed that GPX7 silencing could promote ferroptosis-related oxidative stress induced by erastin in glioma cells *in vitro*.

GPX7 Silencing Synergizes With Erastin to Suppress Glioma Both *In Vitro* and *In Vivo*

Based on the regulatory effect of GPX7 on ferroptosis-related oxidative stress, we then investigated the GPX7 mediated effect on glioma development and sensitivity to erastin. CCK-8, wound healing, transwell and apoptosis flow cytometry assays were conducted to examine proliferative, migratory and invasive abilities of glioma cells with GPX7 knockdown alone or with GPX7 knockdown and erastin (10 μM) cotreatment. As shown in **Figure 5A**, CCK-8 assay revealed that GPX7 knockdown alone did not affect the proliferation of glioma cells. However, the combination of GPX7 knockdown and erastin treatment significantly suppressed the proliferation of glioma cells. This effect was more pronounced with higher erastin concentrations. In addition, GPX7 knockdown and erastin cotreatment significantly inhibited the migratory and invasive abilities (**Figures 5B, C**) and increased apoptosis (**Figure 5D**) of glioma cells, while GPX7 knockdown alone did not exhibit obvious effects. Furthermore, immunofluorescence staining revealed that the combination treatment downregulated the expressions of several indicators related to cell proliferation, migration and invasion, including Ki67, MMP2 and N-Cadherin (**Supplementary Figure S3A**). We also found that lip-1, fer-1 and DFO can suppress the apoptosis rate of GPX7 knockdown cells treated with erastin (**Supplementary Figure S3B**). Additionally, we assessed the combinational effects of GPX7 knockdown and RSL3, another ferroptosis inducing agent which inhibits directly the activity of GPX4 (13). However, the synergistic effects of GPX7 knockdown and RSL3 treatment were not obvious (**Supplementary Figures S2E, F**). These results indicated that GPX7 deficiency enhanced ferroptosis-related oxidative stress, which may not be adequate to exert obvious effects on the malignant phenotypes of glioma cells, but sensitized cells to erastin induced ferroptosis.

Then we explored whether GPX7 silencing promotes erastin-induced ferroptosis *in vivo*. A tumor xenograft model was established by subcutaneously inoculating LN229 cells infected with lentiv-NC or lentiv-GPX7, respectively. As shown in **Figures 5E–G**, the group subjected to the combination of GPX7 knockdown and erastin treatment showed a significant tumor growth inhibition, wheras the mice subjected to GPX7 knockdown alone exhibited no tumor growth suppression. Apart from that, erastin treatment alone can also suppress tumor growth. Furthermore, IHC staining revealed that the combination treatment also downregulated the expressions of Ki67, MMP2 and N-Cadherin (**Figure 5H**). These results indicated that GPX7 knockdown synergizes with erastin to inhibit glioma both *in vitro* and *in vivo*.

GPX7 Is a Direct Target of miR-29 Family

MiRNA is closely related to tumorigenesis, angiogenesis and chemoresistance (15). However, whether GPX7 is regulated by miRNAs is still unknown. In this research, we used TargetScan (31), miRDB (32), Tarbase (33) and mirDIP (34) databases to predict which miRNAs target GPX7. Taking the intersection of predicted results, miR-29 family (miR-29a/b/c-3p) were included (**Figure 6A**). We then used dual luciferase assays to verify our prediction. The seed sequences of miR-29 family that match the 3′UTR of the GPX7 gene were shown in **Figure 6B**. In dual luciferase assays (**Figure 6C**), transfection with miR-29a/b/c-3p mimics inhibited the WT luciferase reporter activity but did not decrease MT luciferase reporter activity. More importantly, qPCR and western blot showed that GPX7 expression was significantly impaired after elevating miR-29a/b/c expression in LN229 and T98G cells (**Figures 6D, E**). The evidence suggests that GPX7 was targeted by miR-29 family. To explore whether all miR-29 family members modulate GPX7 expression in glioma, we mined tumor gene expression profiles in TCGA and CGGA databases and found that only miR-29b-3p expression level was inversely correlated with GPX7 expression in the two databases (**Figures 6F–H**). Given that three miR-29 family members share an identical seed sequence (**Figure 6B**) and usually have similar biological functions (35), therefore, we only selected miR-29b-3p (denoted as miR-29b) for subsequent experiments.

GPX7 Restoration Can Reverse miR-29b Mediated Enhancement of Ferroptosis-Related Oxidative Stress

Given GPX7 being a direct target of miR-29b, we then investigated whether miR-29b also regulates ferroptosis-related oxidative stress in glioma cells. Reduced GSH, Fe^{2+} concentration and lipid peroxidation were measured. Compared with control group, GSH levels (**Figure 7A**) were markedly reduced while Fe2+ concentration wasn't affected in the mimic group (**Figure 7B**). Furthermore, the accumulation of lipid peroxidation was significantly promoted in the mimic group (**Figure 7C**). Interestingly, we found miR-29b mediated changes in the indicators of ferroptosis were abrogated by GPX7 restoration (**Figures 7A, C**). Ferroptosis inhibitors (lip-1, fer-1 and DFO) can abolish the increase of lipid peroxidation of cells subjected to miR-29b mimic transfection and erastin treatment (**Figure 7C**). These data indicated that miR-29b can regulate ferroptosis-related oxidative stress in glioma cells, at least partially *via* GPX7.

MiR-29b Synergizes With Erastin to Suppress Glioma Proliferation, Migration, Invasion and Induce Apoptosis Partially *via* GPX7

The above results stimulated us to explore whether miR-29b can sensitize glioma cells to erastin. Using the same experimental assays employed above, we found that LN229

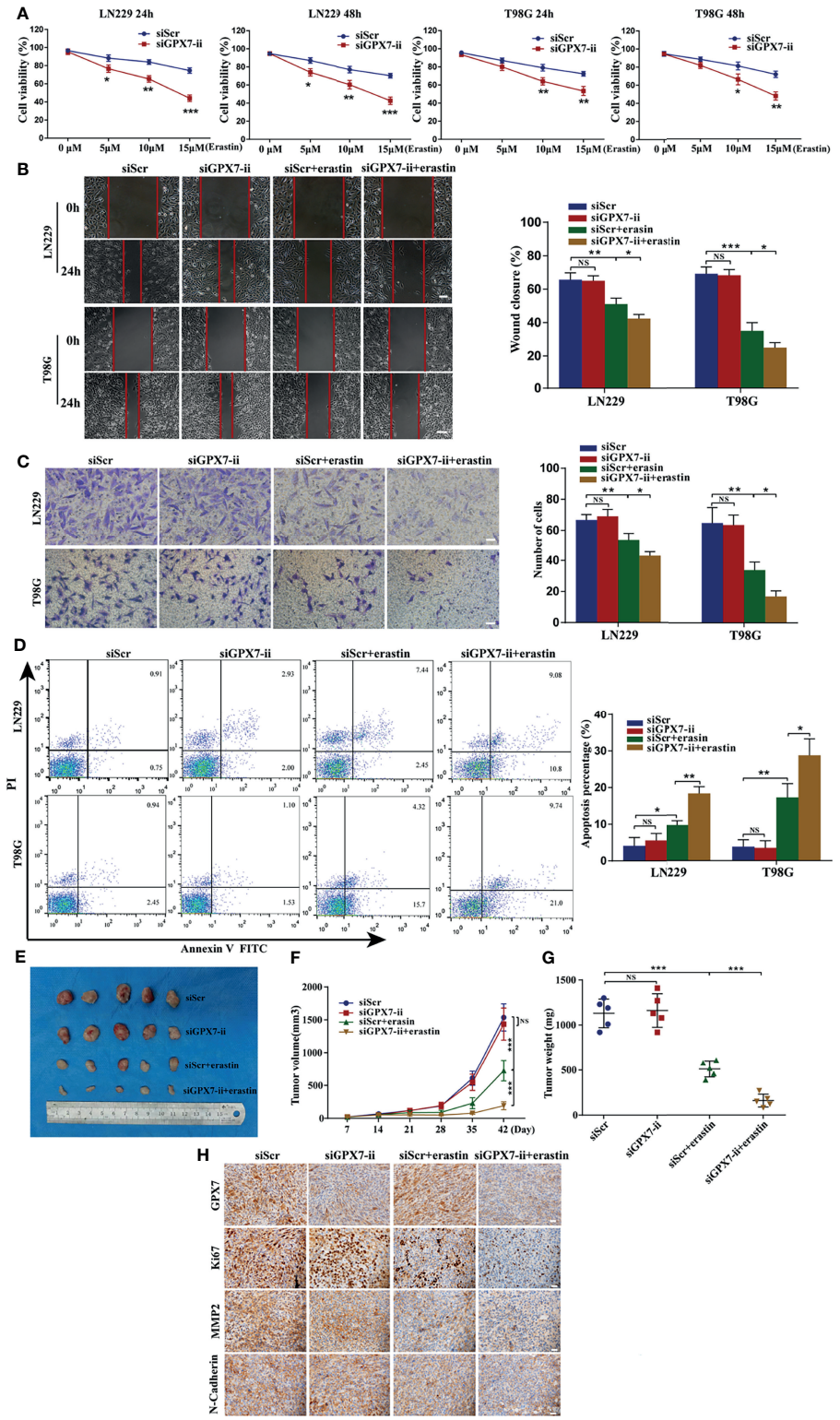

FIGURE 5 | GPX7 silencing sensitizes glioma cells to erastin both *in vitro* and *in vivo*. **(A)** CCK-8 assay was applied to analyze the viability of LN229 and T98G cells treated with different concentrations of erastin following the transfection with siGPX7 and siScr. **(B)** Migration ability of cells was analyzed using wound-healing assay. Scale bar, 250 μm. **(C)** Invasive ability of cells was evaluated by transwell assay. Scale bar, 100 μm. **(D)** FITC annexin V and PI apoptosis assay. **(E)** Knockdown of GPX7 enhanced erastin-induced ferroptosis *in vivo*. The volume of tumors was shown **(F)**, and the tumor weight was measured at the endpoint **(G)**. **(H)** Immunohistochemistry staining of xenograft model-derived tumors for GPX7, Ki67, MMP2 and N-Cadherin. Scale bar = 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

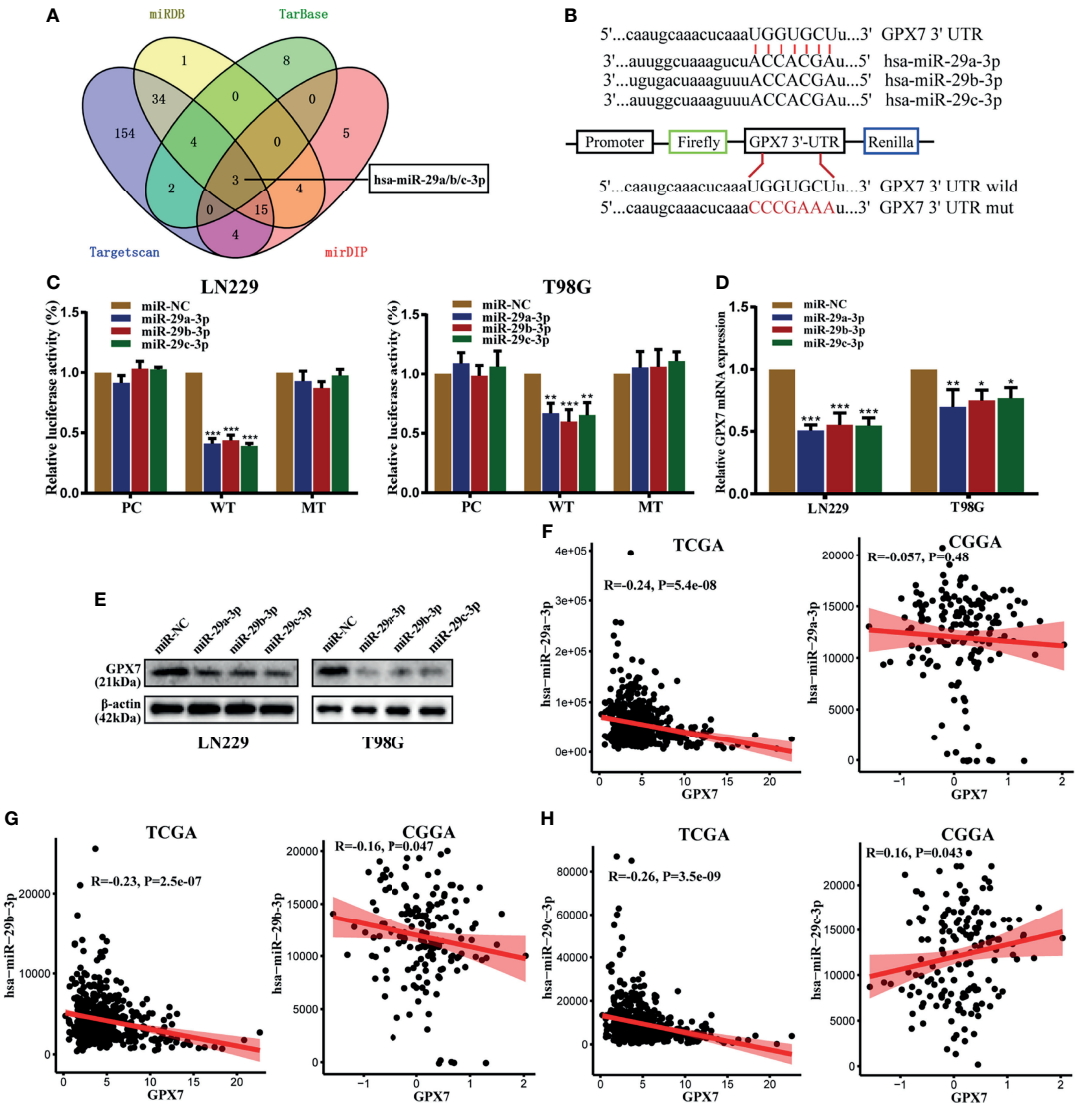

FIGURE 6 | GPX7 is a direct target of miR-29 family. **(A)** The venn diagram of predicted mRNAs in four different databases. **(B)** The seed sequences of miR-29 family that match the 3′UTR of GPX7 gene and the reporter vectors containing wild-type or mutant GPX7 3′-UTR. **(C)** Dual luciferase reporter assays in LN229 and T98G, following cotransfection with miR-29 family and empty vector (pcDNA-3.1) or plasmid containing wild or mutant type 3′UTR of GPX7. **(D, E)** The mRNA and protein levels of GPX7 in cells transfected with miR-29 family mimic or miR-NC. **(F–H)** The correlations between GPX7 expression and miR-29 family in TCGA and CGGA. R: Pearson correlation coefficient. *P < 0.05, **P < 0.01, ***P < 0.001.

and T98G cells with miR-29b mimic transfection and erastin cotreatment attenuated proliferative (**Figure 8A**), migratory (**Figure 8B**) and invasive (**Figure 8C**) abilities and promoted apoptosis rate of glioma cells (**Figure 8D**). Similarly, restoration of GPX7 following transfection with miR-29b mimic can reverse the miR-29b and erastin mediated synergistic inhibitory effects on glioma cells (**Figures 8A–D**). Apart from that, lip-1, fer-1 and DFO can abolish the increase of apoptosis rate of cells cotreated with miR-29b mimic and erastin (**Supplementary Figure S3C**). In summary, these results revealed that miR-29b can sensitize glioma cells to erastin partially *via* GPX7.

DISCUSSION

To date, some, but not all, molecules involved in glioma progression have been identified. In our study, multi-database analyses showed that GPX7 expression was upregulated in glioma and was an independent prognostic factor of glioma patients. GSEA revealed that some tumor-related signaling pathways and immunity-related activities are enriched in the GPX7 high expression group. Additionally, some immune cells were positively correlated with GPX7 expression in glioma.

Ferroptosis is a form of regulated cell death marked by lipid peroxidation (14). Previously, the ferroptosis inducers, for instance

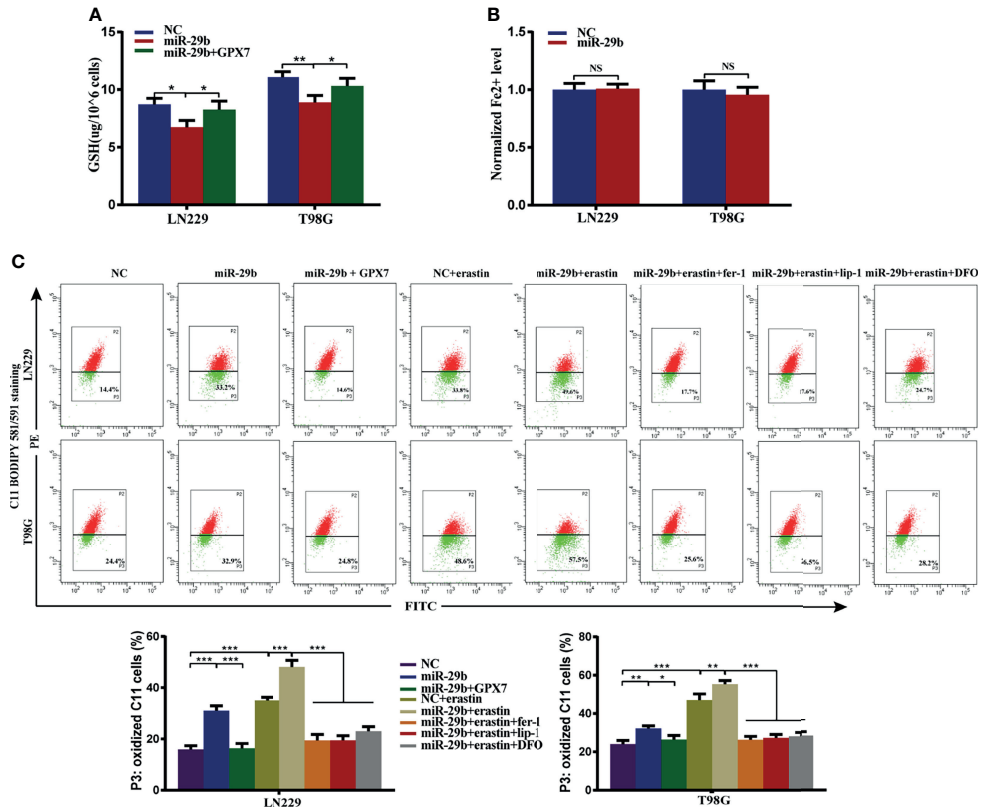

FIGURE 7 | GPX7 restoration can reverse miR-29b mediated enhancement of ferroptosis-related oxidative stress. LN229 and T98G cells were transfected with miR-29b mimic or cotransfected with miR-29b mimic and GPX7 overexpression plasmid. **(A)** The reduced GSH level of cells. **(B)** The Fe2+ concentration was measured by FerroOrange probe. **(C)** Lipid peroxidation was detected in glioma cells treated with or without fer-1 (2 μM), erastin (10 μM), lip-1 (100 nM) and DFO (100 μM). Within each chart, cells in P3 region in green represent those stained with oxidized dye. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant.

erastin, have shown potentials to eliminate malignancies, including glioma (36), melanoma (37) and other tumors (38). In addition, the combination of ferroptosis inducers and other therapies, such as chemotherapeutic agents and radiation, can result in stronger effects (36, 39). However, tumor cells can elevate the expression of other antioxidant genes that results in increased resistance to cell death (40). Yagoda N et al. reported that the expression of VDAC2/3, the targets of erastin on the outer mitochondrial membrane, markedly decreased after 10 h of erastin treatment, which led to erastin resistance (41). Elucidating more factors that regulate ferroptosis will certainly help to apply ferroptosis induction to anti-tumor therapies. In this study, we found GPX7 knockdown promoted lipid peroxidation and decreased the level of GSH. In addition, the combination of GPX7 deficiency and erastin treatment showed a remarkable synergistic effect on the induction of ferroptosis of glioma. Therefore, targeting GPX7 may help reverse the erastin resistance in glioma treatment.

As a peroxide sensor, GPX7 detoxifies peroxides and has been described to play essential roles in diseases. In normal oesophageal squamous epithelial cells, GPX7 knockdown can lead to the increase of intracellular ROS and oxidative DNA damage induced by pH4 bile acids, which increases the risk of oncogenesis (4). In glioma, our work found that GPX7 knockdown alone exerted no direct effect on tumor growth, although ferroptosis-related oxidative stress was promoted. We speculated that an external stimuli of oxidative stress, for instance erastin treatment, may be required for GPX7 targeting therapy of glioma.

Unlike other glutathione peroxidases, protein disulfide isomerase (PDI) and glucose-regulated protein GRP78, instead of GSH, are the main substrates of GPX7 (42, 43). GPX7 may exert antioxidant function through mechanisms different from GPX4, the key regulator of ferroptosis. In our study, we found that the loss of GPX7 resulted in decreased GSH level in glioma cells. This effect may be attributed to the activation of other antioxidant enzymes in the balance between the energy metabolism and oxidative damage resistance.

MiRNA-mRNA regulation has been identified as important regulatory mechanism in ferroptosis of tumors (15, 16, 23, 44). Based on algorithm prediction and experimental validation, our work found that miR-29b could directly inhibit GPX7 post-transcriptionally, exerting similar ferroptosis induction effect on glioma, synergizing with erastin treatment. In previous study, miR-29b was also found to promote oxidative stress in ischemic

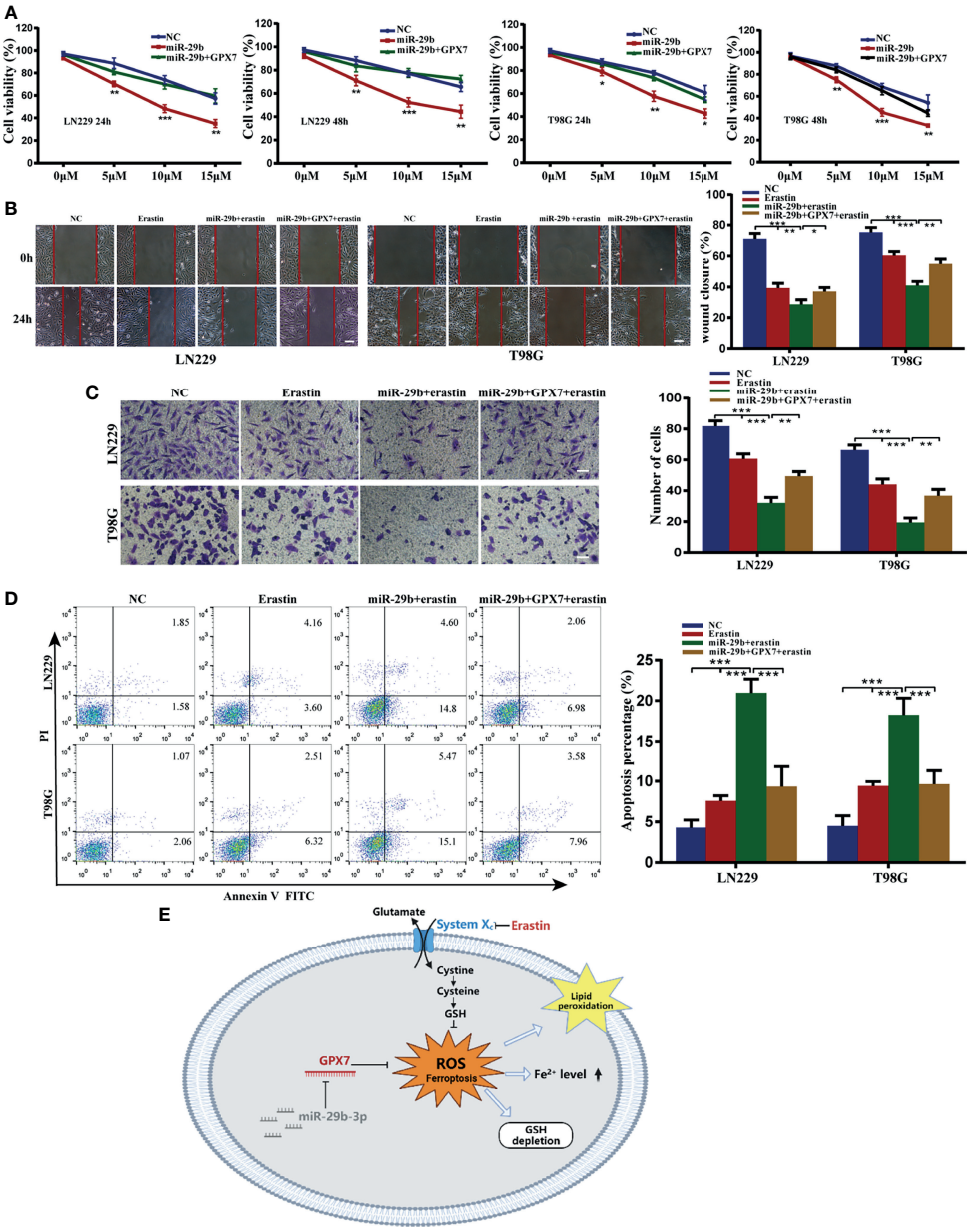

FIGURE 8 | Synergistic inhibitory effects of miR-29b mimic transfection and erastin cotreatment on glioma cells, which can be partially reversed by GPX7 restoration. **(A)** LN229 and T98G cells transfected with miR-29b mimic or cotransfected with miR-29b mimic and GPX7 overexpression plasmid were treated with erastin at different concentrations. Proliferation ability of cells was evaluated using CCK-8. **(B)** Wound healing assay. The concentration of erastin is 10 μM. Scale bar, 250 μm. **(C)** Transwell assay with 10 μM erastin used. Scale bar, 100 μm. **(D)** Annexin V and PI apoptosis assay. The concentration of erastin is 10 μM. **(E)** The schematic diagram of the roles of miR-29b/GPX7 in glioma ferroptosis induced by erastin, which was created on the BioRender.com. *P < 0.05, **P < 0.01, ***P < 0.001.

stroke (45). Therefore, miR-29b in oxidative stress is worthy of further study.

Some limitations were present: first, the survival data of patients in Huanhu hospital were missed. Second, we found GPX7 expression was associated with immune infiltration, but the specific roles of GPX7 in the immunomodulation are still unclear. Third, more investigations should be conducted into whether GPX7 affects the known ferroptosis-related signaling pathways and how GPX7 knockdown enhances the effects of erastin. Lastly, subcutaneous rather than intracranial *in situ* nude mice xenograft model was applied in our study due to the presence of the blood brain barrier which may prevent the penetration of erastin into the tumor. These possibly limit the cogency of our findings.

In summary, multi-center data revealed that GPX7 high expression was associated with poor clinical outcomes. GPX7 knockdown mediated enhancement of ferroptosis-related oxidative stress promoted glioma ferroptosis induced by erastin. Furthermore, miR-29b suppressed GPX7 expression post-transcriptionally in glioma. Reconstitution of miR-29b enhanced erastin sensitivity, partly *via* GPX7 suppression. All in all, suppressing GPX7 could be a valuable strategy for glioma treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Huanhu Hospital of Tianjin Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Care and Used Committee of Tianjin Huanhu Hospital.

AUTHOR CONTRIBUTIONS

Study design: YZ, LX, and HY. Data collection: YZ, HW, and FW. Data analysis and interpretation: YZ, HW, and YY. Writing and review of the manuscript: YZ, XT, and HY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.802124/full#supplementary-material

Supplementary Figure S1 | The expression of other GPX family members in glioma based on TCGA. **(A)** The expression differences of GPXs between normal and glioma were analyzed in GEPIA. **(B)** The multivariate Cox regression regarding GPXs in TCGA. **(C)** Some examples of high expression of GPX7 in patients with grade II and low expression in patients with grade III.

Supplementary Figure S2 | (A–D) Bioinformatics analysis of the association of GPX7 and ferroptosis based on data from CGGA. **(A)** GSEA of ferroptosis and redox biology related gene sets in cohort with GPX7 high and low. **(B)** The different FPI levels among different WHO grades of glioma. **(C)** Kaplan-Meier analysis of OS according to the FPI level. **(D)** The association of GPX7 expression and FPI level in CGGA glioma samples. **(E)** CCK-8 assay was applied to analyze the viability of LN229 and T98G cells treated with different concentrations of RSL3 following the transfection with siGPX7 and siScr. **(F)** FITC annexin V and PI apoptosis assay was applied analyze the apoptosis rate of LN229 and T98G cells treated with RSL3 (1 μM) following the transfection with siGPX7 or siScr.

Supplementary Figure S3 | (A) Immunofluorescence staining of LN229 and T98G cells subjected to siGPX7-ii or siScr transfection with or without erastin (10 μM) treatment for GPX7 (orange), Ki67 (green), MMP2 (red) and N-Cadherin (yellow). The nucleus is stained with DAPI (blue). Scale bar = 50 μm. **(B)** Apoptosis assay was used to analyze the apoptosis rate of cells treated with or without erastin (10 μM), fer-1 (2 μM), lip-1 (100 nM) and DFO (100 μM) following the transfection with siGPX7 and siScr. **(C)** Apoptosis assay was used to analyze the apoptosis rate of cells treated with or without erastin (10 μM), fer-1 (2 μM), lip-1 (100 nM) and DFO (100 μM) following the transfection with miR-29b mimic or NC mimic.

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Ferroptosis-Related Gene Contributes to Immunity, Stemness and Predicts Prognosis in Glioblastoma Multiforme

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Ferroptosis, a recently discovered regulated programmed cell death, is associated with tumorigenesis and progression in glioblastoma. Based on widely recognized ferroptosis-related genes (FRGs), the regulation of ferroptosis patterns and corresponding characteristics of immune infiltration of 516 GBM samples with GSE13041, TCGA-GBM, and CGGA-325 were comprehensively analyzed. Here, we revealed the expression, mutations, and CNV of FRGs in GBM. We identified three distinct regulation patterns of ferroptosis and found the hub genes of immunity and stemness among DEGs in three patterns. A prognostic model was constructed based on five FRGs and verified at the mRNA and protein level. The risk score can not only predict the prognosis but also the degree of immune infiltration and ICB responsiveness by functional annotation. The overall assessment of FRGs in GBM patients will guide the direction of improved research and develop new prognostic prediction tools.

Keywords: ferroptosis, glioblastoma multiforme, immunity, stemness, prognosis

INTRODUCTION

Grade IV glioma, which is termed as glioblastoma multiforme (GBM), is the most lethal glioma (1). Despite advances in the treatment of GBM with surgery, radiation, and chemotherapy, the survival rate of patients remains 18 months (2). Previous investigations have depicted some malignant biological features that contribute to the highly recurrent and drug-resistance of GBM (3). Tremendous research studies have focused on molecular markers that contribute to GBM stemness and immunity (4, 5). Our previous study conducted a comprehensive analysis of the stemness of GBM (6). However, many therapies targeting these molecular markers become less effective in clinical practice. Therefore, the novel and effective prognostic models for the prediction of GBM prognosis and immunotherapy response need to be investigated and clarified.

Ferroptosis is a new type of programmed cell death proposed by Stockwell et al. (7). Research on the significance of ferroptosis in cancer has recently gained momentum, whereas disruption of this process under human intervention may show clinical effects (8, 9). Ferroptosis manifests cell membrane rupture and blebbing, mitochondrial and morphological changes, with the cell nucleus remain intact (10). For instance, downregulation of SLC1A5 provides melanoma cell partial immunity to ferroptosis induction (11). GOT1 inhibition promotes

pancreatic cancer cell death by potentiating the activity of ferroptosis (12). Knockdown of TFRC can inhibit the cell proliferation of BRCA cell lines (13). More and more genes related to ferroptosis have been identified in glioma, such as ACSL4 that protects glioma cells and exerts antiproliferative effects by activating a ferroptosis pathway (14), ATF3 that contributes to brucine-induced glioma cell ferroptosis (15), and COPZ1 that manipulates NCOA4 to regulate the ferroptosis process in GBM (16). These ferroptosis-related genes (FRGs) are closely linked to tumorigenesis and progression. However, whether these genes are associated with the prognostic value and molecular functions of GBM patients has not been elucidated.

Despite its implication in cell death, recent studies also evaluated ferroptosis-associated diseases and their role on immunity. For example, $CD8^+$ T cells suppress tumor growth by inducing ferroptosis and pyroptosis (17). In addition, ferroptosis could release various damage-associated molecular patterns (DAMPs) or lipid metabolites that are involved in the cellular immune response (18). Notably, ferroptosis was associated with tumor immune checkpoints in clear cell renal cell carcinoma (19). These researches explored the mechanisms in ferroptosis and immune microenvironment. However, these studies have not specifically focused on GBM, and the relationship between ferroptosis and immune response in GBM has not been well characterized.

Herein, we integrated data from the public Gene Expression Omnibus (GEO), The Cancer Genome Atlas (TCGA) databases, and the Chinese Glioma Genome Atlas (CGGA) to evaluate the role of FRGs signature in the prognosis in GBM patients. We further identified three distinct regulations of ferroptosis. Comparison of the DEG of three patterns unveiled five key genes involved in immunity and stemness. These genes may have potential value in the regulation of ferroptosis in GBM. Finally, a risk score based on FRGs had been constructed. Within functional annotation, we found that the risk score is not only a good predictive value for survival but also a potential factor for immune checkpoint blockade (ICB) responsiveness. Our study could help to guide the link between ferroptosis and GBM stem cells intensive research in the future and identify new ferroptosis-related targets and immune therapies.

MATERIALS AND METHODS

Data Acquisition

Raw RNA-seq data (FPKM files) and clinical data on GBM were extracted from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/) and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo). After data filtration, GSE13041, TCGA-GBM, and CGGA-325 with 516 GBM tissue samples were gathered in this study for further analysis. Infiltration estimation for all TCGA tumors was collected from TIMER2.0 (http://timer.comp-genomics.org/). Copy number variant (CNV) data and somatic mutations of all genes were downloaded from the UCSC Xena browser (https://xenabrowser.net). CNV differences of all genes were calculated by the chi-square test ($p < 0.05$). The location of the significantly different genes on the chromosomes was shown by the RCircos R package. The protein–protein interaction network was produced by the STRING (https://www.string-db.org) database and was reconstructed *via* Cytoscape software. The protein expressions in human normal tissues and tumor tissues were validated *via* the Human Protein Altas (HPA, https://www.proteinatlas.org/).

Identification of FRGs

Ferroptosis-related genes had been categorized according to the existing literature which contains iron metabolism, oxidant metabolism, lipid metabolism, energy metabolism, and other unclassified factors (9, 20–22). According to the description of FRGs in glioma research (23), 59 genes were incorporated into follow-up studies and were provided in **Supplementary Table 1**. Considering the small number of normal brain tissues in the TCGA, we investigated the expression of the FRGs on the online web server GEPIA (http://gepia.cancer-pku.cn/). Differentially expressed genes (DEGs) were calculated using the R package "LIMMA" ($|\log FC| > 1$ and $p < 0.05$).

Functional Enrichment Analyses

To functionally annotate DEG sets during the analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG), pathway analysis, and Gene Ontology (GO) were performed in R software version 4.0.3 using ClusterProfiler package. To calculate mRNA expression-based stemness index (mRNAsi), we used the OCLR algorithm constructed by Malta's team (24). The mRNAsi was represented using an index between zero to one to signal that the higher the mRNAsi, the greater activity of cancer stem cells. The CytoHubba plugin version 0.1 in Cytoscape version 3.8.2 was employed to identify hub genes, and enrichment analysis was performed using the ClueGO plugin version 2.5.8.

The TIMER, CIBERSORT, QUANTISEQ, Microenvironment Cell Populations-counter (MCP-counter), XCELL, and Estimating the Proportion of Immune and Cancer cells (EPIC) algorithms were used to estimate the abundance of immune cells between the high- and low-risk groups. The "ESTIMATE" R package was used to assess immune infiltration (based on the ImmuneScore, StromalScore, and ESTIMATEScore). The clustering was performed using WGCNA and the module–trait correlations with mRNAsi, EREG-mRNAsi, and ESTIMATEScore. According to the number of the genes, the minModuleSize of the mRNA was set to 50. Gene sets that could predict the responses to immune checkpoint blockade therapy were obtained from the work by Mariathasan. Single-sample gene set enrichment analysis (ssGSEA) was used to estimate immune-related functions in TCGA-GBM patients utilizing gene set variation analysis (GSVA) (25) version 1.40.1.

Tumor immune dysfunction and exclusion (TIDE) (http://tide.dfci.harvard.edu/), a well-established algorithm was employed to predict the clinical response to ICB therapy (26). TIDE is a computational framework developed to evaluate the potential of tumor immune escape from the gene expression profiles of cancer samples. The TIDE score could serve as a surrogate biomarker to predict response to ICB. The SubMap (https://www.genepattern.org/) was employed to validate the reliability of the prediction of TIDE. Mapping result is

represented as a subclass association matrix filled with *p*-values for each subclass association (27).

Construction of a Scoring System and Calculate the Risk Score

Univariate Cox regression analysis was implemented to filtrate the prognostic FRGs. The ConsensusClusterPlus package in R was employed to investigate the detailed information in unsupervised subclasses discovery and to divide samples into appropriate parts for maximum stability (28). Thereafter, we used the R package "glmnet" to conduct least absolute shrinkage and selection operator (LASSO) Cox regression algorithm and development of a potential risk signature. The minimum value of lambda was derived from 1,000 crossvalidations ("1-se" lambda), which corresponding partial likelihood deviance value was the smallest for the risk model (29, 30). At last, coefficients with regression were confirmed by the "cvfit" function with 1,000 repeats. FRG prognostic signature involves five genes. The risk score calculating formula is as follows:

$$Riskscore = \sum_{i=1}^{n} Coef_i * x_i$$

where Coef_i means the coefficients and x_i is the expression value of each FRGs. This formula was used to calculate the risk score for each GBM patient. The predictive ability of prognostic signature for clinical traits and survival was reflected by receiver operating characteristic (ROC) and the area under the curve (AUC).

The independent clinical factor validated by univariate and multivariate Cox regression analyses was enrolled to construct a nomogram for prognosis prediction. Patients with missing data were excluded. The nomogram was performed using the "survival" and "regplot" packages of R 4.1.0 to investigate the probability of 1-, 3-, and 5-year overall survival (OS).

RNA Extraction and Real-Time PCR

For RNA extraction, three GBM tissues and one peritumoral brain edema were collected in the Second Affiliated Hospital of Harbin Medical University. This research was approved by all the patients and the ethics committee of hospital. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. According to the manufacturer's instructions of the Nanodrop ND-2000 spectrophotometer (Thermo Scientific, USA), 2 μg of the total RNA was transcribed into cDNA. SYBR Green PCR kit (Takara, Japan) was used for qRT-PCR. The 2–ΔΔCq method was used to calculate gene transcription level, with β-actin mRNA as control. Data represent the mean ± SD of triplicate real-time PCR. Primers (Tsingke Biotechnology Co., Ltd, Beijing, China) used are displayed in **Supplementary Table 2**. Clinical characteristics of patient cohort are displayed in **Supplementary Table 3**.

Statistical Analysis

All the data were analyzed using the R software version 4.1.0. The OS of the patients with glioma between different groups was analyzed using Kaplan–Meier curves with the log-rank test. Correlations were assessed *via* Spearman's coefficient. Kruskal–Wallis tests were applied for the comparison of gene expression in two or more groups. The landscape of CNV and gene location were visualized by the RCircos R package (31). A $p < 0.05$ was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 9 for rest of the data.

RESULTS

Landscape of FRGs in GBM

A total of 59 FRGs previously reported were included in this study. We first analyzed the expression of these genes in TCGA-GBM and normal tissues. The expression of 59 genes showed significant differences in TCGA-GBM with normal samples (**Figure 1A**). Among them, FANCD2, STEAP3, HMOX1, and other eight genes were upregulated in GBM ($p < 0.001$), whereas ACSL4, GLS2, and PEBP1 were the opposite ($p < 0.001$). We next examined CNV and chromosome location. Chromosome 10 carried the largest number of genes that undergo copy number variation. PGD and SLC1A5, the genes with the highest frequency of copy number loss, were located on chromosomes 1 and 19, respectively (**Figures 1B,C**). After that, we investigated the mutation frequencies of these genes in the TCGA-GBM dataset. As a result, there were 21 FRGs with mutation frequency >1%, and TP53 had the highest mutation frequency which was predominantly missense mutation (**Figure 1D**). Given the high frequency of copy number loss and mutation of TP53, we further explored the gene expression of FRGs between TP53 wildtype and mutant type. Four genes differentially expressed between subgroups were shown, and ABCC1 exhibited an increased expression in the TP53 mutant group (**Figures 1E–H**), which may be associated with malignant progression of TP53 mutant status (32).

The Relationship Between FRGs and Prognosis

Three mRNA-seq datasets that include TCGA-GBM ($n = 161$), CGGA325 ($n = 137$), and GSE12041 ($n = 218$) were integrated to interrogate the prognostic significance of FRGs. According to the previous studies of different metabolic pathways, genes related to ferroptosis were preliminarily divided into five categories. Gene expression, correlation, and prognostics are shown in **Figure 2A**. Among them, AKR1C1, AKR1C3, FDFT1 that involved in lipid metabolism, and NCOA4 that involved in iron metabolism were significantly associated with improved prognosis, which can be regarded as the protective factors. In contrast, the expression of STEAP3, HMOX1 that involved in the iron metabolism, and HSPB1 and SAT1 belong to other categories was associated with poor prognosis, which can be regarded as the risk factors (**Figure 2A**). Next, the Kaplan–Meier survival curve was used, and six genes that include NCOA4, STEAP3, AKR1C1, AKR1C3, FDFT1, and HSBP1 were most significantly related to OS (**Figures 2B–G**), which indicates that they may be vital in predicting patient prognosis.

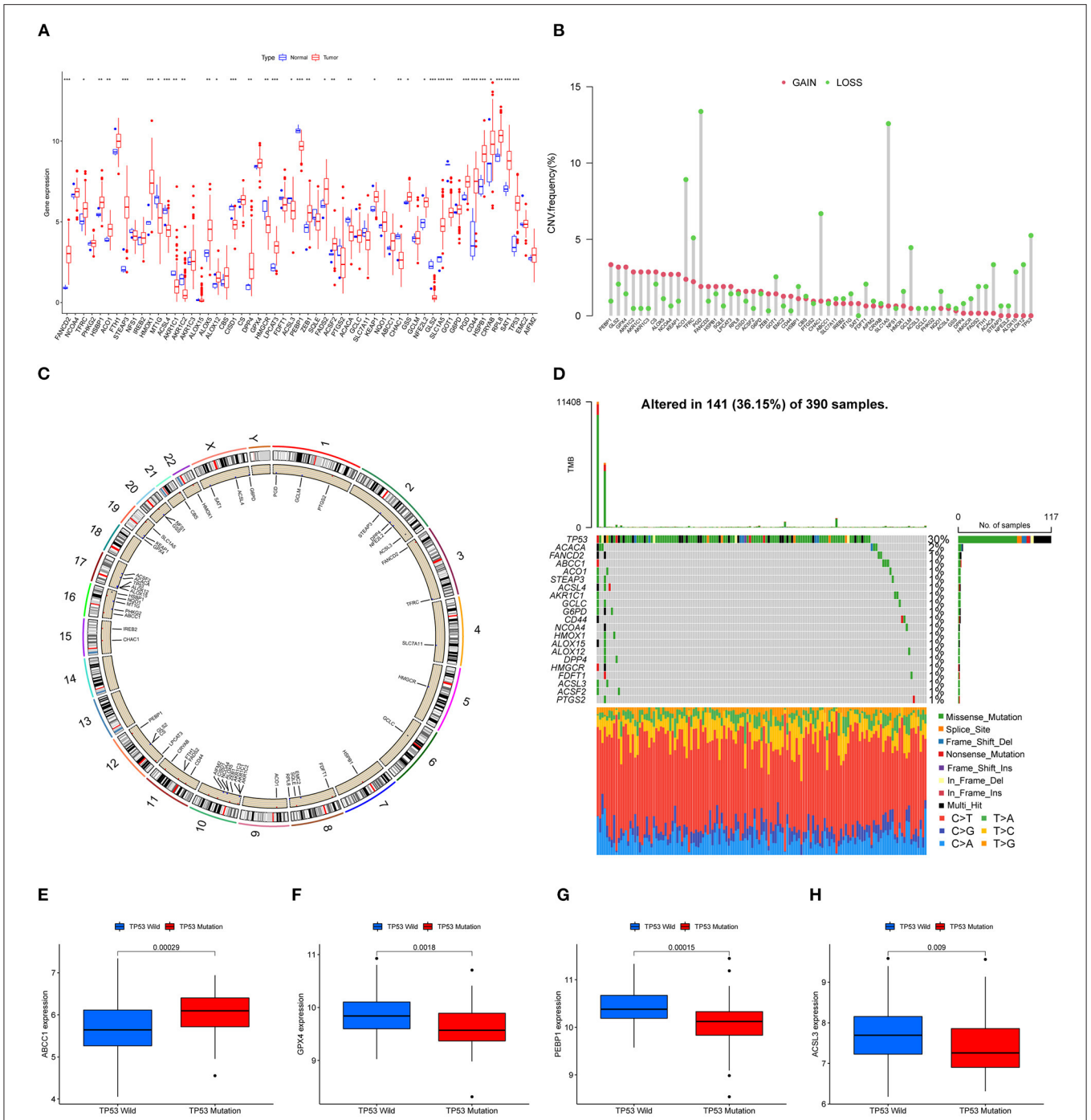

FIGURE 1 | Landscape of FRGs in TCGA-GBM. **(A)** The expression levels of FRGs in the TCGA dataset. Red: upregulated genes; blue: downregulated genes. **(B)** CNV frequency; red: CNV gain; green: CNV loss. **(C)** The CNV distribution of all chromosomes. **(D)** The mutation of FRGs in GBM; green boxes represent missense mutations, orange splicing mutations, blue frameshift mutations, and red nonsense mutations. **(E–H)** Four genes (ABCC1, GPX4, PEBP1, and ACSL3) with significantly different expressions between TP53 wildtype and mutant ($p < 0.01$).

GSVA and ssGSEA Analysis in three Clusters of FRGs

To characterize the functions of these FRGs in GBM, they were clustered for further analysis (**Figure 3A**). The consensus distributions for k (2 to 9) were displayed in the empirical cumulative distribution function (CDF) plots (**Figures 3B,C**); given the consensus matrix, $k = 3$ seemed to be the most suitable choice. Besides, to verify the effectiveness of unsupervised

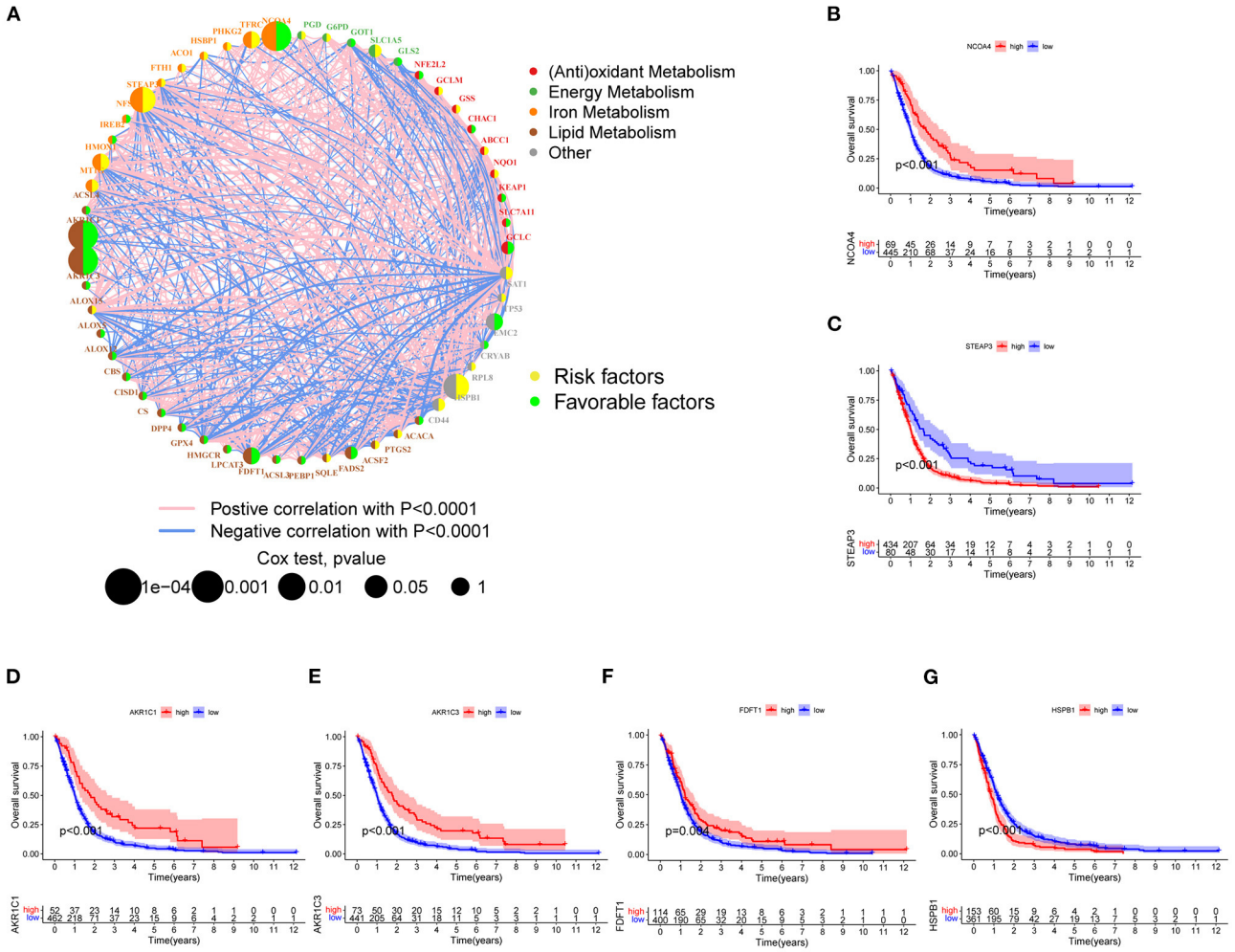

FIGURE 2 | FRGs associated with prognosis. **(A)** The degree distribution of the prognostic-related gene network. The yellow circles indicate high-risk genes, and the green circles indicate low-risk genes. The remaining five colored dots represent five types of FRGs. The blue line indicates a negative correlation, and the pink lines indicate positive correlations with correlation coefficients of $p < 0.0001$. Cox regression analysis (Cox p-value range 0.0001–1). The size of the dot reflects the p-value. **(B–G)** Six FRGs significantly associated with prognosis (NCOA4, STEAP3, AKR1C1, AKR1C3, and HSPB1 $p < 0.001$; FDFT1 $p < 0.01$).

clustering, principal component analysis (PCA) can clearly show the distinction between 3 clusters which proved the accuracy of our selection for k and the effectiveness (**Figure 3D**). K–M analysis found significant differences in OS in the three clusters ($p = 0.008$), and cluster A seemed to have the poorest prognosis. Next, the expression of FRGs in the three clusters and their clinical characteristics were shown in the heatmap (**Figure 3F**). To gain insights into the functional implication, GSVA was performed to analyze the differentially enriched KEGG pathways in two of any three clusters. Samples in cluster A showed prominent enrichment of nod-like receptor (NLR) signaling pathway, apoptosis, amino sugar and nucleotide sugar metabolism, and cytokine–cytokine receptor interaction, etc. (**Figures 3G–I**). Finally, enrichment of immune cell fractions in the tumor immune microenvironment (TIME) was assessed using the ssGSEA algorithm. As a result, most types of immune cells were significantly enriched in cluster A, such as activated CD8 T cell and eosinophil (**Figure 3J**). These findings indicated that different regulatory patterns based on FRGs reflected the mechanisms in tumor growth, apoptosis, and immune infiltration.

Identification of Hub Genes in DEGs Using WGCNA and Functional Annotation

To investigate the specific phenotype-related genes for each regulatory pattern of ferroptosis, we used the "LIMMA" package to identify the DEGs. A total of 1,622 DEGs were picked out in three clusters (**Figure 4A**). Functional enrichment analysis found that the main function of these DEGs enriched in neutrophil activation neutrophil-mediated immunity (BP), collagen-containing extracellular matrix, secretory granule lumen (CC), and actin-binding (MF) (**Figures 4B,C**). For the KEGG pathway, the most significant pathway for these DEG enrichment was cytokine–cytokine receptor interaction.

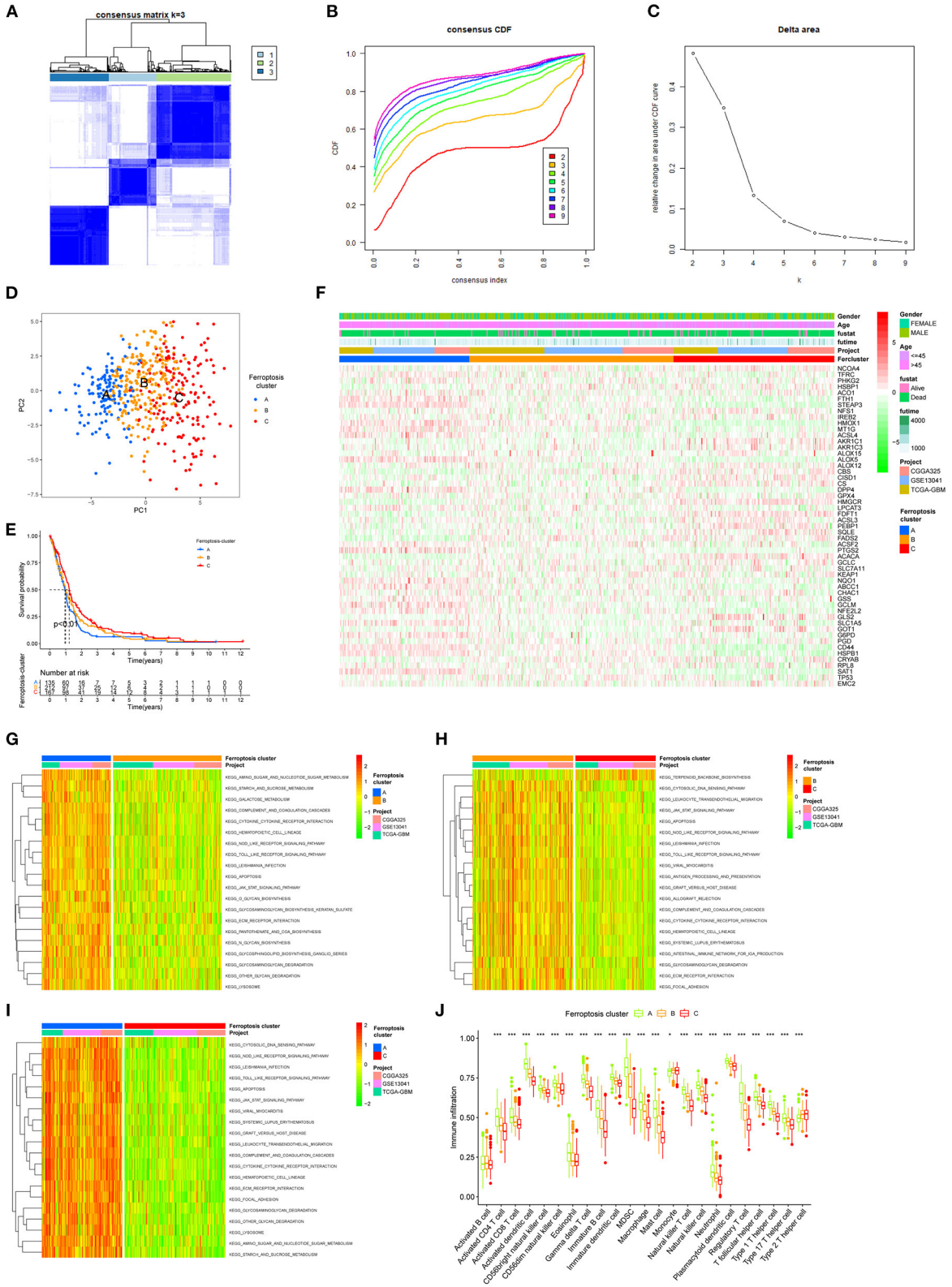

FIGURE 3 | The unsupervised clustering process for FRGs. **(A)** Consensus clustering matrix for k = 3. **(B)** Consensus clustering CDF for k = 2–9. **(C)** Relative change in area under the CDF curve for k = 2–9. **(D)** A PCA plot of unsupervised clustering when k = 3. **(E)** Prognostic differences between the three clusters after merging survival information ($p < 0.01$). **(F)** The heatmap of three clusters and their clinical characteristics. **(G–I)** Visualization for the results of KEGG for DEGs with three clusters. **(J)** The relative enrichment of each immune cell fraction in the TIME with the gene sets using ssGSEA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

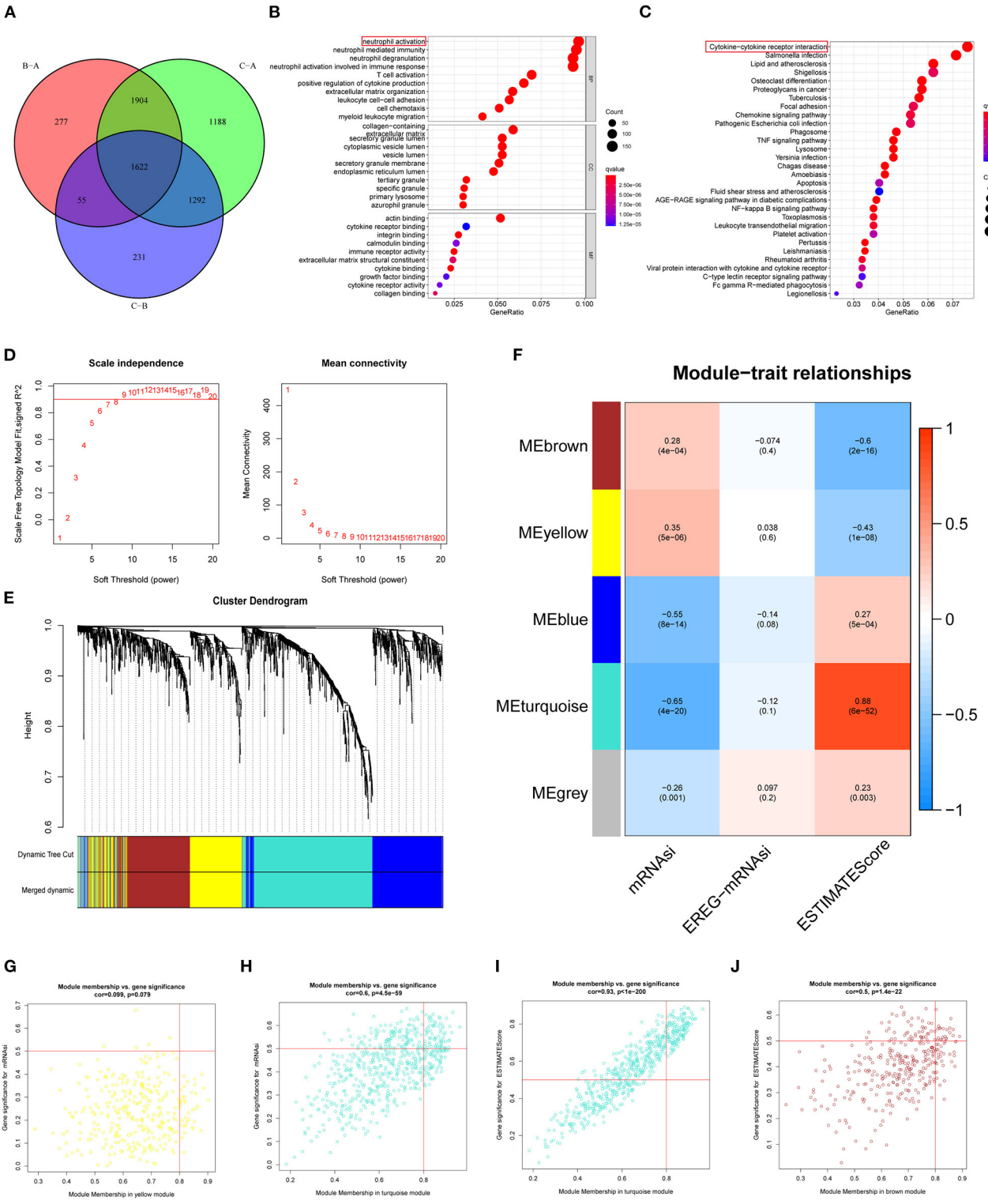

FIGURE 4 | Characteristics of potential traits in different patterns of ferroptosis regulation. **(A)** A Venn diagram of differential genes in three patterns ($p = 0.001$). **(B)** GO analyses of differentially expressed. **(C)** KEGG pathway analyses of differentially expressed. **(D–F)** Hierarchical clustering dendrograms of identified coexpressed genes in modules. The branches of the cluster dendrogram correspond to the different gene modules. Each leaf corresponds to a gene. Each colored row represents a color-coded module, which contains a group of highly connected genes. A total of 5 modules were identified after the merger. **(F)** Correlations between the gene modules and target traits including mRNAsi, EREG-mRNAsi, and ESTIMATEScore. The corresponding *p*-value is increased in size from blue to red. **(G–J)** The four modules most significantly correlated with mRNAsi and ESTIMATEScore. Cor was the coefficient indices and *p* was Pearson's correlation.

Next, WGCNA was performed to structure gene coexpression networks and further identified biologically meaningful modules that corresponded to designate phenotype-related genes, which include stemness indices and the ESTIMATEScore. The most appropriate β was 7, and the relatively balanced scale independence and mean connectivity of the WGCNA were identified (**Figure 4D**). A total of 5 modules (merged dynamic) were identified (**Figure 4E** minModuleSize = 50). To analyze the correlations between merged modules and the immune and stemness phenotypes, module eigengenes (MEs), which could be regarded as representative of the gene expression patterns in a module, were determined and used to calculate the correlations with designated phenotypes. The heatmap in **Figure 4F** revealed the key modules (MEyellow and MEturquoise for the mRNAsi and MEturquoise and MEbrown for the ESTIMATE). The correlation graphs were plotted to select the hub genes in these modules (**Figures 4G–J**). Interestingly, the module turquoise had the most positive correlation with ESTIMATEScore and most negative correlation with mRNAsi. Genes with p.MMturquoise ≥ 0.8 and GS.mRNAsi ≤ -0.5 or GS.ESTIMATEScore ≥ 0.5 were screened out (**Supplementary Table 4**).

The STRING was used to construct PPI networks. In the module turquoise, 71 genes are negatively correlated with mRNAsi, and 134 genes are positively correlated with ESTIMATEScore (GS > 0.5 and MMturquoise > 0.8). According to the confidence, the genes with significant interaction were screened, and the filtered results were imported into the Cytoscape for network visualization. The top 30 of these genes associated with mRNAsi and ESTIMATEScore with the highest combined score in STRING are exhibited (**Figures 5A,B**). CytoHubba and Maximal Clique Centrality (MCC) were used to explore important nodes. Top 10 MCC values were selected, and then, the intersection was taken to get the hub genes in PPI analysis (**Figures 5C,D**). As the result, we found that 5 genes, which include TLR4, TLR8, TNF, CD86, ITGAM, and PTPRC, were the common hub genes in the two modules. These five key genes were analyzed by KEGG and GO enrichment using the "ClueGO" and "CluePedia" plugins for Cytoscape software (**Figures 5E,F** $p < 0.05$). We found that these genes showed the enrichment of GO terms related to microglial cell activation, positive regulation of NIK/NF-kappaβ signaling and interleukin (interleukin-8 production and regulation and positive regulation of interleukin-1 beta production). Collectively, these hub genes may be the key components of the GBM immune and stemness module that contribute to immunoregulatory functions during ferroptosis.

Cluster Analysis of DEG Levels

Previous results revealed the special performance of the DEGs in immunity and stemness of GBM. To further study, the association between these DEGs and FRGs was clustered into three categories according to their correlations in GBM. Given the consensus matrix for the analysis, $k = 3$ seemed to be the most suitable choice (**Figures 6A–D**). Similarly, these clusters also showed significant survival differences (**Figure 6F** $p < 0.001$). The differences in survival obtained by this clustering are consistent with those using FRGs (**Figure 3E**). The expression of DEGs in the clustering of the two methods and their clinical characteristics were shown in the heatmap (**Figure 6E**), and these findings were independent of clinical traits, such as age and sex. For FRGs, we found that most genes show significant differences in DEG clusters (**Figure 6G**). Interestingly, among the FRGs, FTH1, STEAP3, HMOX1, and 13 other genes showed an increased expression in cluster A, which had the worst prognosis.

Construct the FRG Prognostic Signature

Among the genes related to prognosis (**Figure 2A**), 16 FRGs were correlated with the OS of GBM patients. LASSO Cox analysis was performed to establish an FRG prognostic signature. In the crossvalidation process, lambda.Min was regarded as the optimal value (**Figures 7A,B**). A number of 5 FRGs were identified, and corresponding coefficients were calculated. A number of 516 samples were divided into train set (TCGA-GBM and GSE13041) and test set (CGGA-325), and samples were split into high- and low-risk subgroups by the median value of the risk score. Kaplan–Meier survival curves depicted that GBM patients with increased risk scores had worse clinical outcomes (**Figures 7C,D**, $p < 0.001$ in both train and test datasets). Next, we established 3- and 5-year ROC curves and found that the risk score can effectively distinguish GBM patients with different survival statuses in train set (**Figure 7E**, 3-year AUC = 0.706, 5-year AUC = 0.782). The risk score and survival status distributions of the train set are shown in **Figure 7F**. The mortality of patients increased with the increase of the risk score. The expressions of risk genes and protective genes in these 5 genes are shown in the heatmap (**Figure 7G**), TFRC, and STEAP3 as the risk factors increased in the high-risk score group. Conversely, NCOA4, AKR1C1, and AKR1C3 become the protective factors. The risk scores in the ferroptosis cluster and gene cluster are shown in **Figures 7H,I**. Univariate and multivariate Cox regression analyses show the independent prognostic value of this risk score (**Supplementary Figures 1A,B**). A Nomogram model was established which contained risk score, recurrent, age, and gender to assess the survival prediction in GBM patients (**Supplementary Figure 1C**). A Sankey diagram is used to link clustering, scoring, and survival status (**Supplementary Figure 2**). Most of the surviving patients belong to the low-risk group and cluster C. Finally, considering the small number of normal samples in the dataset, we validated the expression of the five key genes by qRT-PCR with the unpaired *t*-test in human GBM tissues. The results of qRT-PCR (**Figures 8A–E**) and dates in GEPIA (**Figure 8F**) were consistent with the expression of protein in HPA (**Figures 8G–K**).

Enrichment Analyses of Immune-Related Functions

The enrichment scores of immune cells and corresponding immune functions and pathways with ssGSEA were quantified for the TCGA dataset. Silico approaches that include TIMER, CIBERSORT, CIBERSORT–ABS, QUANTISEQ, MCPCOUNTER, XCELL, and EPIC computational were employed to quantify the immune cells in high- and low-risk groups (**Figure 9A**). Consequently, the fraction of B cell, $CD8^+$ T cell, and M2 macrophage were significantly increased in the

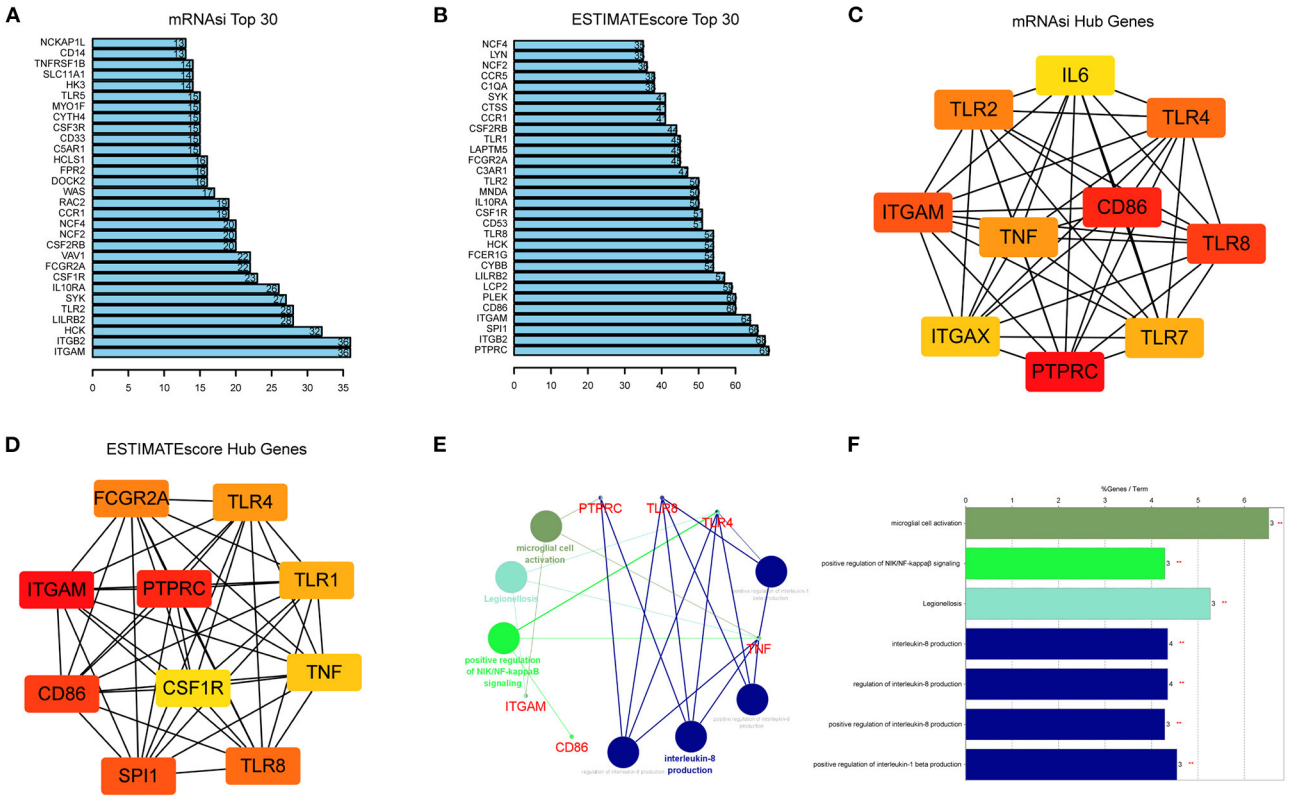

FIGURE 5 | Identification of hub genes from the PPI network. **(A,B)** PPI network obtained using STRING and showing the top 30 highest scoring genes. The bar plots of top 30 key genes of mRNAsi **(A)** and ESTIMATEScore **(B)**. **(C,D)** Top 10 hub genes of mRNAsi **(C)** and ESTIMATEScore **(D)** explored by CytoHubba algorithm in Cytoscape software 3.8.0. and red nodes represent hub genes. **(E,F)** KEGG pathway and GO analysis of five overlapping genes (TLR4, TLR8, TNF, CD86, ITGAM, and PTPRC) using the "ClueGO" and "CluePedia" plugins ($p < 0.05$).

low-risk group. NK cell and T cell regulatory (Tregs) were enriched in the high-risk group. The MHC class I scored higher in the high-risk group. The GSVA method was used to calculate the immune event scores of the high- and low-risk groups. APC coinhibition, HLA, and type I IFN response scored higher in the low-risk group (**Figure 9B**, $p < 0.01$). Next, we explored the relationship between immune checkpoint-related genes and risk score (**Figure 9C**). The expression of CD44, TNFRSF14, and NRP1 in the high-risk group was significantly higher than that in the low-risk group. Given this, we introduced the TIDE algorithm to assess the efficacy of FRG signatures in predicting ICB responsiveness in GBM. Submap was used to compare the prediction results (**Figure 9D**). As a result, different groups in train and test sets showed comparable performance in predicting the GBM response to anti-CTLA4 therapy ($p < 0.05$). Finally, we use GSEA to perform GO enrichment analysis on high- and low-risk GBM patients. The samples of the high-risk group were enriched in positive regulation of transcription from RNA polymerase II promoter in response to stress (GOBP), mitotic G2 M transition checkpoint (GOBP), BHLH transcription factor binding (GOMF) (**Figure 9E**). In the low-risk group, enriched GO terms were cell cortex region (GOCC), negative regulation of amyloid precursor protein catabolic process (GOBP), and oxidoreductase activity acting (GOMF). In conclusion, GBM patients with high- and low-risk scores had different immune-related functions (**Figure 9F**).

DISCUSSION

Malignant glioma remains a considerable threat to human health, and the prognosis of patients with GBM is dismal (33, 34). Recently, regulated cell death has gained considerable attention in cancer, especially ferroptosis (7, 35, 36). Herein, 59 FRGs in GBM were included in this study to investigate the characteristics with expression, OS, and functions. CNV exists as a genetic polymorphism in the human genome, and CNV alters tumorigenesis by deletion or amplification of a copy number of a gene (37, 38). In this study, the highest frequency of FRG PGD and SLC1A5 CNV (loss) was located on chromosomes 1 and 19, respectively. SLC1A5 expression correlated positively with immune cells, such as tumor-infiltrating B cells, $CD4^+$ T in hepatocellular carcinoma, and lower-grade glioma (39). For mutations in FRGs, TP53 mutation is one of the most frequent genetic alterations in primary glioma. Previous studies have shown that TP53 polymorphism is associated with the risk of primary glioma (40). Of note, the expression of ABCC1 was

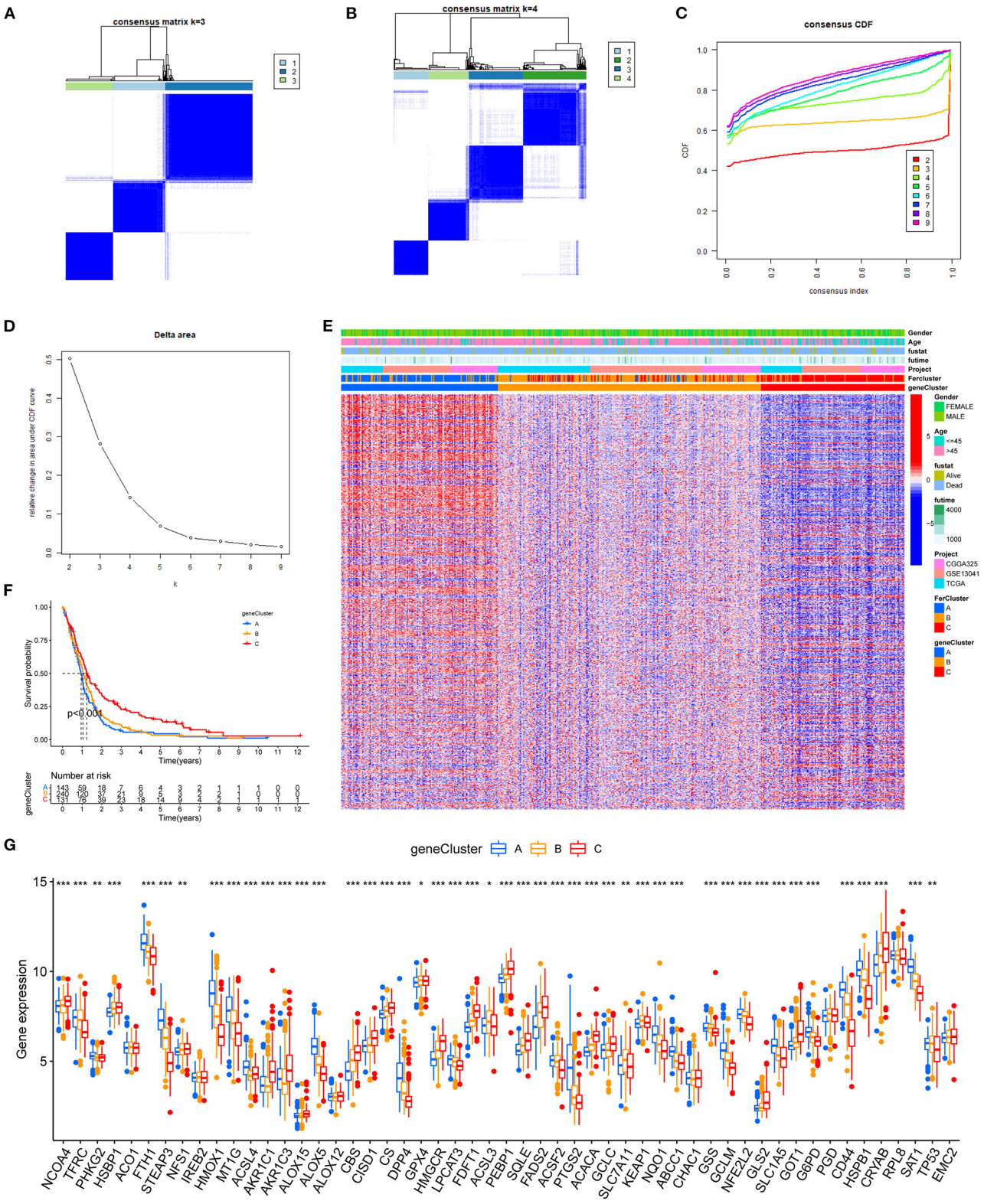

FIGURE 6 | Hierarchical cluster analysis of DEGs. **(A–D)** The consensus clustering matrix for $k = 3$ was determined by CDF for $k = 2$–9. **(E)** Prognostic differences between the three clusters after merging survival information ($p < 0.01$). **(F)** The heatmap of three clusters and their clinical characteristics. **(G)** The expression levels of FRGs in different clusters (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

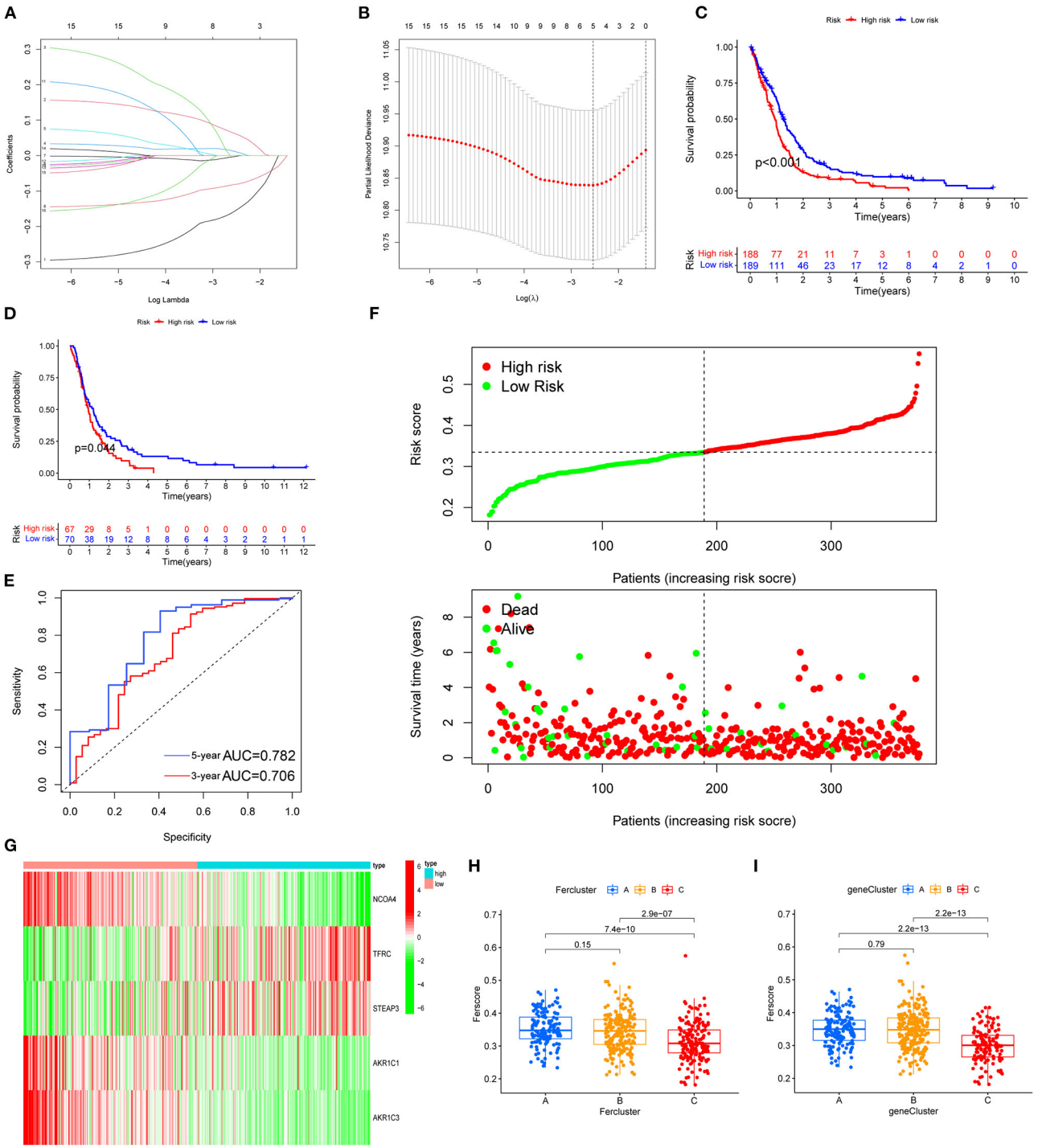

FIGURE 7 | FRG prognostic signature. **(A,B)** The process of building the signature. LASSO regression was performed, calculating the minimum criteria. **(C,D)** Kaplan–Meier curves showed that the high-risk subgroup had worse OS than the subgroup in training set (**C**, $p < 0.001$) and test set (**D**, $p < 0.001$). **(E)** ROC curves showed the predictive efficiency of the risk signature on the 3-year and 5-year survival rates of training set (3-year AUC = 0.706, 5-year AUC = 0.782). **(F)** The distributions of risk scores and the distributions of risk scores and OS status. The green and red dots indicated the alive and dead status, respectively. **(G)** The heatmap based on the expression of the five genes in the high- and low-risk group. **(H,I)** According to the formula, the different clustered samples are scored using the coefficients.

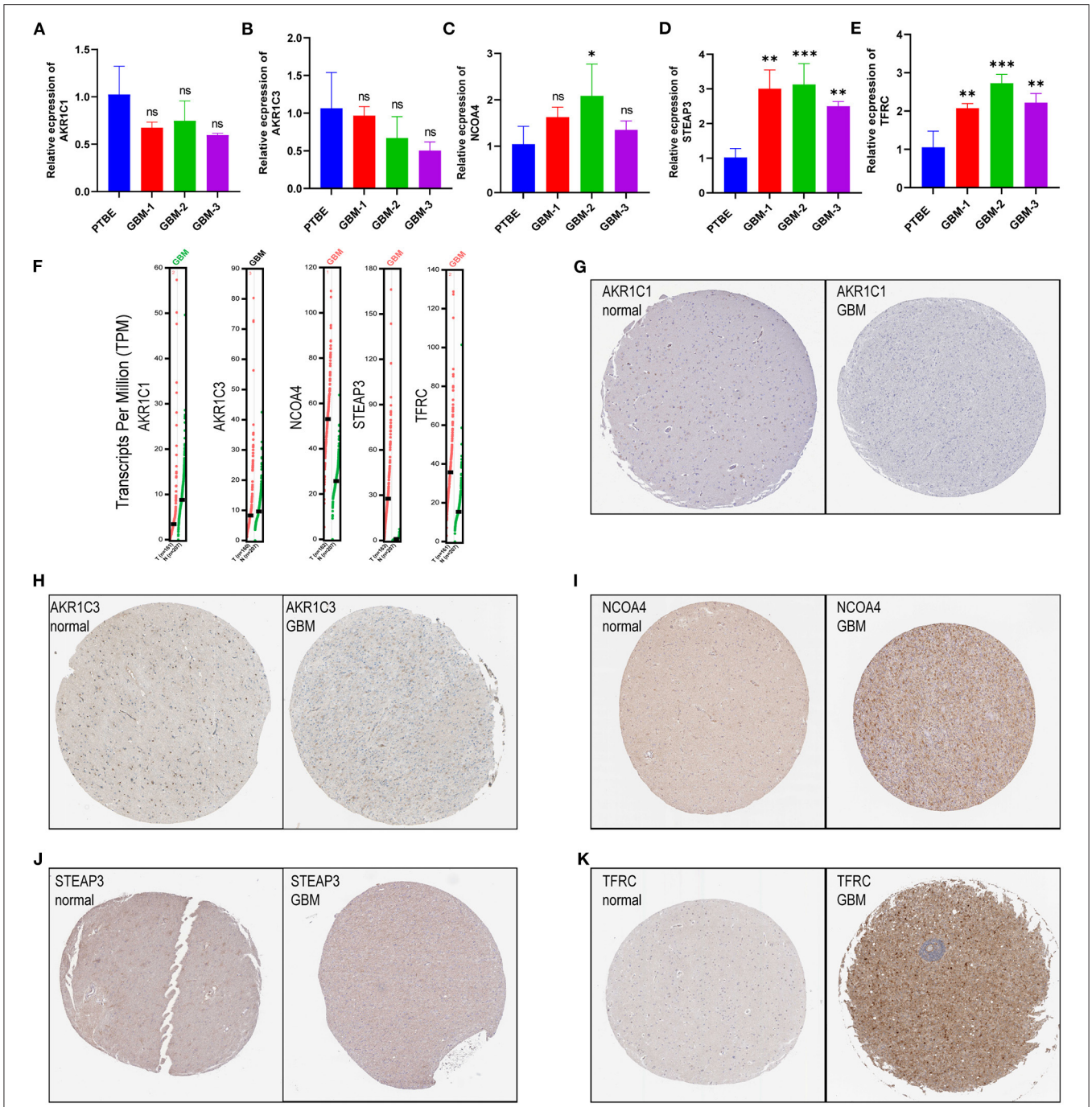

FIGURE 8 | Validation of risk genes **(A–E). (A)** AKR1C1, **(B)** AKR1C3, **(C)** NCOA4, **(D)** STEAP3, and **(E)** TFRC expression in peritumoral brain edema and GBM tissues. **(F)** The expression of prognostic model with five genes (NCOA4, TFRC, STEAP3, AKR1C1, and AKR1C3) in GEPIA. **(G–K)** Validation of five genes with immunohistochemistry from the HPA database.

higher in mutant TP53 whereas the expression of GPX4, PEBP1, and ACSL3 were higher in the wild-type TP53 group. The finding implicated TP53 mutation status was an important link in the regulation of other FRGs.

Unsupervised cluster analysis of the expression values of FRGs identified three distinct patterns in GBM. Cluster A with the worst prognosis showed high enrichment in NLR signaling pathway apoptosis, and amino sugar and nucleotide sugar metabolism. The NLR family of receptors had been recognized as the key roles of immunity and inflammation with GBM (41). Meanwhile, a variety of immune cells, such as activated CD8 T cell and eosinophil, exhibit aggregation in cluster A. Whether a direct mechanism of immune cells on ferroptosis nodes might be of physiological relevance remains elusive.

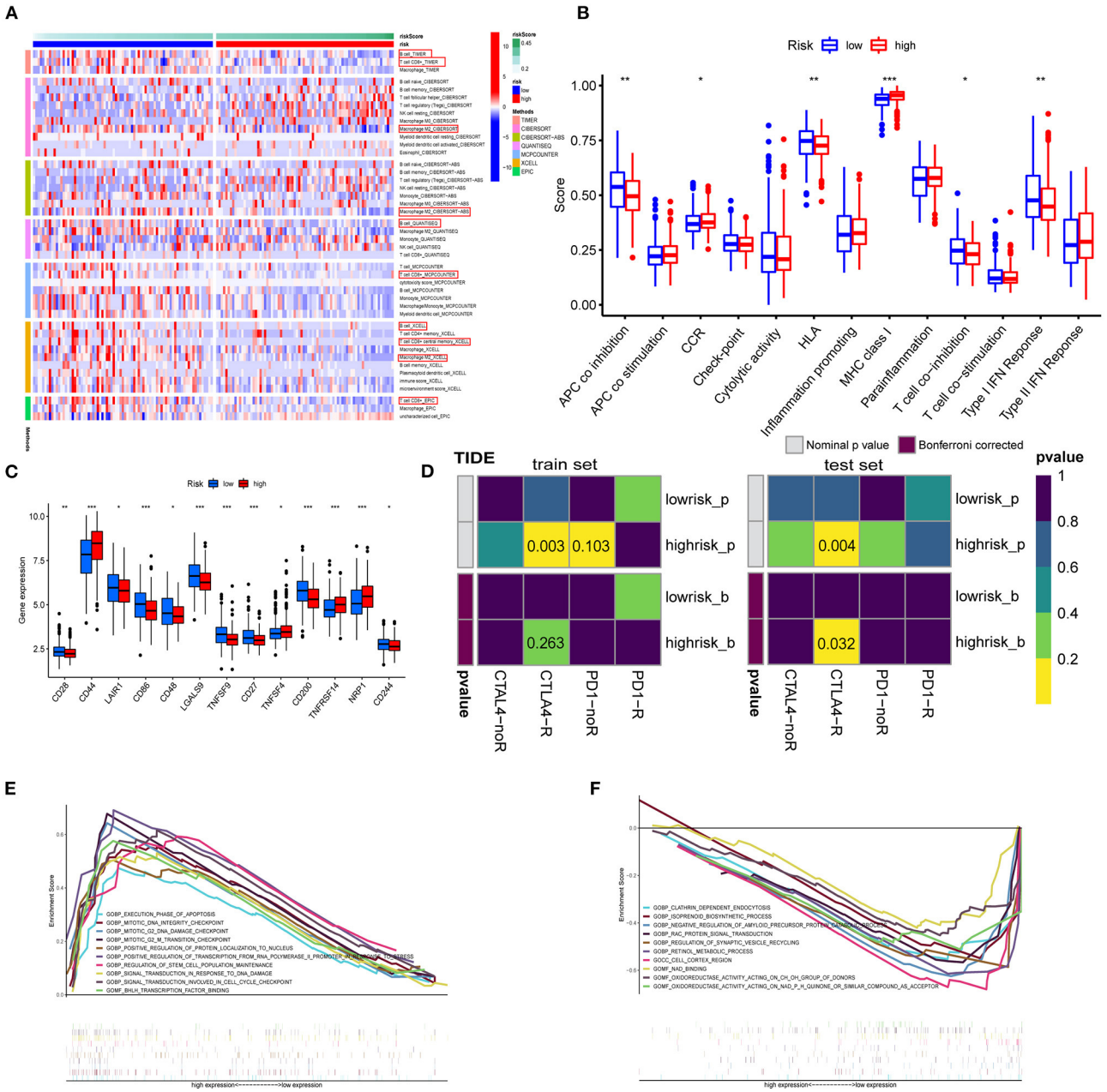

FIGURE 9 | Enrichment analysis of immune-related functions. **(A)** The TIMER, CIBERSORT, CIBERSORT.ABS, QUANTISEQ, MCPCOUNTER, XCELL, and EPIC algorithms were applied for the immune infiltration of the high- and low-risk groups. **(B)** Immune score estimated by single sample GSEA (ssGSEA) in different groups. **(C)** Immune checkpoints in the tumor microenvironment. **(D)** TIDE prediction of the association of genes prognostic signature with ICB responsiveness in train and test sets. **(E,F)** GO analysis for high-risk group **(E)** and low-risk group **(F)** using GSEA 4.1.0.

Recent studies have shown that CD8 T cells may in sensitizing tumor cells toward ferroptosis (42). In addition, 1,622 DEGs were selected from three patterns. GO and KEGG pathway analysis revealed that DEG enrichment was mainly involved in the immunity biological process. Activated neutrophils are induced by the microenvironment of GBM (43). Meanwhile, some immune-associated lncRNAs in glioma were verified to be closely related to cytokine–cytokine receptor interaction (44). Functional annotation of the hub genes identified by WGCNA illuminated the potential regulatory mechanisms by which of FRGs regulate on the immune and stemness phenotypes. Recent studies suggest a possible negative regulation between stemness and immune activation (45). Glioblastoma multiforme stem cells, characterized by self-renewal and therapeutic resistance, play

vital roles in GBM (6). Using the app CytoHubba in Cytoscape, we filtered 5 hub genes in both immune and stemness PPI networks. TLRs are expressed on both immune and tumor cells, which play dual roles in countering cell proliferation, migration, invasion, and glioma stem cell maintenance responses (46). TNF-α/NF-κB signaling is closely associated with glioma proliferation (47). Also, CD86 is an unfavorable prognostic biomarker in lower-grade glioma (48). It is worth noting that PTPRC, TLR8, TLR4, and TNF all exhibit functions related to IL-8 regulation. Interleukin-8 (IL-8) has been revealed as a critical regulator of central nervous system (CNS) function and development with participation in many CNS disorders including gliomas (49, 50). These are the key genes of immunity and stemness from different ferroptosis regulation patterns. This suggests that among the multiple FRGs, some genes regulate GBM stem cells and the immune microenvironment. The connection between them is also the direction of our next research.

Based on five FRGs, a prognostic model was established and validated in TCGA-GBM, GSE13041, and CGGA-325. These databases have authoritative gene expression and clinical information for GBM. The prognostic model contained five genes (NCOA4, TFRC, STEAP3, AKR1C1, and AKR1C3). The mRNA expression of genes was verified using qRT-PCR. Based on the HPA database, genes were verified at the protein levels. These FRGs affect many of the key processes involved in the tumorigenesis and progression of cancer, especially glioma. NCOA4 is a selective cargo receptor for the autophagic degradation of ferritin in glioma which is known as ferritinophagy (16, 51). The TFRC expression was higher in glioma (52), and the progression and oncogenicity of glioma were regulated by hsa-miR-144-3p/TFRC signaling (53). STEAP3 emerged as an important protein that induces mesenchymal transition and stem-like traits in glioma (54). AKR1C1 and AKR1C3 are members of the AKR superfamily which has been previously shown to be associated with oncogenic potential and proliferation capacity (55), and selective targeting of AKR1C proteins in GBM could delay the acquisition of resistance to TMZ of astroglioma cells (56). This prognostic model could predict tumor prognosis, and targeting these prognostic model genes may provide new ideas for the development of targeted treatment tools.

Our results demonstrated that a high-risk score was associated with a worse prognosis. Three-year and 5-year ROC curves indicated the 5-gene signature as a potential diagnostic factor in GBM patients. Moreover, outcome of the nomogram showed that risk score and age were associated with GBM prognosis, and it was consistent with the actual clinical situation. We investigated the correlation between high- and low-risk groups and immune cells with the CIBERSORT, CIBERSORT-ABS, QUANTISEQ, XCELL, MCPCOUNTER, and EPIC algorithms. Tregs were elevated in the high-risk group. The findings of this study are in line with those presented in previous studies, Tregs play important known roles in suppressing the immune response and maintaining immune homeostasis (57). Innovatively supporting that the abundance of nonpolarized M0 macrophages rather than M1 or M2 macrophages assembly in glioblastoma that contributed to the malignancy of tumor was proposed recently (58). Also, a recent mice study showed that increasing glioma-associated monocytes in intracranial murine GL261 leads to an increase in intratumoral and systemic myeloid-derived suppressor cells (59). In summary, regulation of ferroptosis in GBM patients may be important in controlling the inflammatory and immune responses. Research on immune checkpoints has now become a new hotspot. In this study, significant differences in the expression of immune checkpoints between high- and low-risk groups suggested that the sensitivity to immunotherapies is associated with a risk score. Moreover, our risk score may screen out potential ICB responders. This provides a new idea for *in-vivo* experiments of immunotherapy. However, GBM patients with low OS exhibit higher expression of markers characterizing immune response activity and T cell infiltration (60). Besides, the presence of the blood–brain barrier cannot be ignored for the nature of immunotherapy. In fact, considering that targeting these FRGs indirectly improves immunotherapy, many questions need answering.

This study still has some limitations. First, all the data used to construct and validate the prognostic model were obtained from publicly available datasets. These three GBM databases inevitably lead to the neglect of intra-tumor heterogeneity in different databases. As confirmed in the study, tumor heterogeneity has an important impact on diagnosis and treatment (61). A prospective study is needed to assess the potential application of the signature. Second, for the five key genes related to GBM stemness, which FRGs or pathways regulate them remains to be further elucidated. Third, although the survival benefits and immune-related biological processes with ferroptosis-related gene signature have been revealed through functional analysis, *in-vivo* and *in-vitro* experiments are needed to further elucidate the specific mechanism, preferably at the single-cell level in humans. Finally, we expect that this work will provide clues on immunity, stemness, and prognosis characteristics for future studies.

CONCLUSIONS

In summary, by analyzing the expression of ferroptosis-related genes in GBM, we identified three ferroptosis regulation patterns of GBM patients. Comparison of the DEG of three patterns and unveiled five key genes involved in immunity and stemness. A prognostic model based on five FRGs was built. The risk score can be a good predictor of prognosis and also predicts the degree of immune infiltration and ICB responsiveness.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: The Cancer Genome Atlas (TCGA) database, https://portal.gdc.cancer.gov/, TCGA-GBM, Chinese Glioma Genome Atlas (CGGA), http://www.cgga.org.cn/, CGGA-325, and National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO), https://www.ncbi.nlm.nih.gov/geo, GSE13041.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH, JD, and HZ conceived and designed the study and revised the manuscript. JD, FW, JJ, HJ, and XY provided analytical technical support. JD, NW, and JZ participated in the production of charts and pictures. SH supervised the study. JD designed and completed qRT-PCR experiments. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Supplementary Figure 1 | Independent prognostic value of the risk score in the train set and construction of the predictive nomogram. **(A)** Univariate Cox regression analysis of the signature. **(B)** multivariate Cox regression analysis. **(C)** A nomogram of the risk score for predicting 1-, 3-, and 5-year survival.

Supplementary Figure 2 | Sankey diagram shows the results of our overall study. Different ferroptosis regulation patterns, three clusters of DEGs, risk scores, and survival status were linked together.

Supplementary Table 1 | FRGs and classification.

Supplementary Table 2 | Primer sequences for qRT-PCR.

Supplementary Table 3 | Clinical characteristics of patient cohort.

Supplementary Table 4 | Eligible genes in the modules.

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Glioblastoma Relapses Show Increased Markers of Vulnerability to Ferroptosis

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Background: Despite the availability of various therapy options and being a widely focused research area, the prognosis of glioblastoma (GBM) still remains very poor due to therapy resistance, genetic heterogeneity and a diffuse infiltration pattern. The recently described non-apoptotic form of cell death ferroptosis may, however, offer novel opportunities for targeted therapies. Hence, the aim of this study was to investigate the potential role of ferroptosis in GBM, including the impact of treatment on the expression of the two ferroptosis-associated players glutathione-peroxidase 4 (GPX4) and acyl-CoA-synthetase long-chain family number 4 (ACSL4). Furthermore, the change in expression of the recently identified ferroptosis suppressor protein 1 (FSP1) and aldehyde dehydrogenase (ALDH) 1A3 was investigated.

Methods: Immunohistochemistry was performed on sample pairs of primary and relapse GBM of 24 patients who had received standard adjuvant treatment with radiochemotherapy. To identify cell types generally prone to undergo ferroptosis, co-stainings of ferroptosis susceptibility genes in combination with cell-type specific markers including glial fibrillary acidic protein (GFAP) for tumor cells and astrocytes, as well as the ionized calcium-binding adapter molecule 1 (Iba1) for microglial cells were performed, supplemented by double stains combining GPX4 and ACSL4.

Results: While the expression of GPX4 decreased significantly during tumor relapse, ACSL4 showed a significant increase. These results were confirmed by analyses of data sets of the Cancer Genome Atlas. These profound changes indicate an increased susceptibility of relapsed tumors towards oxidative stress and associated ferroptosis, a cell death modality characterized by unrestrained lipid peroxidation. Moreover, ALDH1A3 and FSP1 expression also increased in the relapses with significant results for ALDH1A3, whereas for FSP1, statistical significance was not reached. Results

obtained from double staining imply that ferroptosis occurs more likely in GBM tumor cells than in microglial cells.

Conclusion: Our study implies that ferroptosis takes place in GBM tumor cells. Moreover, we show that recurrent tumors have a higher vulnerability to ferroptosis. These results affirm that utilizing ferroptosis processes might be a possible novel therapy option, especially in the situation of recurrent GBM.

Keywords: ferroptosis, glioblastoma, glioma, immunohistochemistry, protein expression, cell death, therapy resistance, relapse

INTRODUCTION

The poor overall survival (OS) of glioblastoma (GBM) patients, even after extensive therapy including neurosurgical resection followed by combined adjuvant radiation therapy and chemotherapy with temozolomide (TMZ), is attributed to its genetic heterogeneity, diffusely infiltrating growth pattern, high proliferation rate and therapy resistance (1–3). Whilst the exact mechanisms underlying the resistance to treatment remain unknown, it seems to involve radiation-resistant tumor stem cells (4–6). Until today, the methylation status of the O-6-methylguanin-DNA methyltransferase (MGMT) promoter remains one of the most significant prognostic markers (7). Current research aims at finding new therapeutic targets to improve the patients' prognosis. In the course of this, it was shown that the activation of the iron-dependent cell death ferroptosis can drive cancer therapy by inducing cell death, which led to the hypothesis that ferroptosis may offer new therapeutic targets for difficult-to-treat entities, including GBM (8).

The induction of ferroptosis might amplify the effect of certain chemotherapeutics (9). For instance, diffuse large B cell lymphomas and renal cell carcinomas show a high susceptibility to the ferroptosis inducer erastin (10, 11). Similar promising effects were seen for the combination of erastin with TMZ in glioma and GBM cells (12, 13).

The process of ferroptosis defines an iron-dependent oxidative destruction of lipid bilayers leading to rupturing of cellular membranes and cell death (14, 15). In 2018, ferroptosis was classified as a regulated cell death modality sensitive to lipophilic antioxidant agents (16). One way to achieve this is to perturb lipid hydroperoxide detoxification systems and to trigger iron-dependent reactive oxygen species (ROS) generation (17).

The *three hallmarks of ferroptosis* include the loss of the lipid peroxide regeneration system, an increase of polyunsaturated fatty acids (PUFAs) and an increase of redox active iron (17). The key enzyme in ferroptosis is glutathione-peroxidase 4 (GPX4). When GPX4 is either inhibited or has lost its function, ROS accumulate and cause cell death (17). Especially tumor cells rely on enzymes like GPX4 to prevent oxidative stress and assure their survival by therapy resistance. A loss of GPX4 might offer a way to selectively kill therapy-resistant tumor cells and prevent relapse (18). Because of its importance in the process, GPX4 was chosen as one marker of ferroptosis in this study.

PUFAs define the second hallmark of ferroptosis because they are more susceptible for radicals or oxidation and hence, for generating ROS. They get activated and oxidized by enzymes like acyl-CoA-synthetase 4 (ACSL4) and lipid-oxygenase. Because of its important regulatory role in in the ferroptotic cell death process (19) and its sensitizing effects on (tumor) cells towards ferroptosis (20), ACSL4 was also chosen as marker for ferroptosis in this study. Moreover, an ACSL4 depletion showed inhibiting effects on GBM tumor cell growth (20). The ferroptosis suppressor protein 1 (FSP1) was recently identified to be the second mainstay in ferroptosis control and therefore included (21).

In addition to these canonical ferroptosis players, the enzyme aldehyde dehydrogenase 1A3 (ALDH1A3) was analyzed, which has been discussed as a stem cell marker in GBM (22–25). The isoform ALDH1A3 appears to be the most active one in GBM (23). Interestingly, an *in-vitro* study showed that ALDH knock-out cells were more sensitive to therapy with TMZ compared to the wildtype cells (26).

The mechanisms through which TMZ induces ROS production (13, 27) and some type of autophagic cell death – possibly ferroptosis - are still unknown (28, 29). We hypothesize that they might involve accumulating aldehydes and complex interactions between aldehyde dehydrogenase (ALDH) (26, 30), key ferroptosis player GPX4 (13, 31), and cystine/cysteine (32). A combination of TMZ and ferroptosis inducing agents thus might be a promising approach in GBM patients (12).

The aim of the present study was to analyze the expression of ferroptosis-associated proteins in GBM. To address the expression evolvement, we compared the change the change of GPX4, ACSL4, FSP1 and ALDH1A3 expression in corresponding pairs of primary and recurrent GBM. Our study

Abbreviations: $^{+}$ = positive; ACSL4, acyl-CoA-synthetase long chain family member 4; ALDH, aldehyde dehydrogenase; DAB, 3,3'-diaminobenzidine; DAPI, 4',6-Diamidino-2-phenylindol; FITC, fluorescein isothiocyanate; FPKM, fragments per kilobase of transcript per million mapped reads; FSP1, ferroptosis suppressor protein 1; GBM: glioblastoma; GFAP, glial fibrillary acidic protein; GPX4, glutathione peroxidase 4; HE, hematoxylin and eosin; Iba1, ionized calcium-binding adapter molecule 1; IDH, isocitrate dehydrogenase; IHC, immunohistochemistry; IF, immunofluorescence; IRS, immunoreactive score; OS, overall survival; PFS, progression free survival; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; TCGA, the Cancer Genome Atlas; TMZ, temozolomide; y, years.

thus reveals new insights into ferroptosis in context of GBM, particularly during the course of patient treatment.

MATERIAL AND METHODS

Material

24 pairs of primary and recurrent GBM (all isocitrate dehydrogenase (IDH) wildtype; median age 58 years, range 27-78 years; 17 male, 7 female) were retrieved from the archive of the Institute of Pathology of the Technical University Munich (**Table 1**). All patients had received surgery at the Department of Neurosurgery of the Klinikum rechts der Isar between 2003 and 2017. Diagnoses were confirmed and reevaluated for this study by experienced neuropathologists according to the classification of brain tumors by the World Health Organization, 2016 (33). Clinical information was gathered by searching the hospital information system. Specification of the MGMT promotor status was obtained by searching the information system of the Institute of Pathology. The MGMT promotor status had been determined by the methylation quantification of endonuclease-resistant DNA (MethyQUESD) method (34).

All patients had been treated following the standard Stupp scheme (6 weeks concomitant radiochemotherapy with TMZ, followed by up to six cycles of TMZ alone (2)), and availability of tissue samples of primary and recurrent tumor was given. For GPX4, ACSL4 and ALDH1A3, 24 pairs of primary and recurrent tumors were included for immunohistochemistry (IHC) and double immunofluorescence (IF) with cell-type specific markers. The expression of FSP1 was analyzed in 13 pairs. Double immunofluorescence for GPX4 plus ACSL4 was performed for 5 pairs of primary and corresponding recurrent tumors.

A data analysis with transcriptome profiling datasets from the Cancer Genome Atlas (TCGA) regarding a change of ACSL4 and GPX4 gene expression in pairs of primary and recurrent GBM was performed to verify our results. The dataset and associated clinical information were acquired from the TCGA official website (https://portal.gdc.cancer.gov). Six corresponding pairs of primary and recurrent GBM were available (TCGA-06-0210, TCGA-06-0190, TCGA-19-4065, TCGA-14-1034, TCGA-06-0125, TCGA-06-0211). For each patient, six to nine transcriptome profiling datasets including the gene quantification expression were accessible. The ACSL4 and GPX4 gene expression from each primary GBM was compared to the corresponding recurrent GBM using the natural logarithm of the gene quantification expression (ln(expression value)).

This retrospective study was approved by the local Ethics Committee of the Technical University Munich (vote number 164/19 S-SR) and conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were cut in 2 µm-thin sections and deparaffinized followed by epitope unmasking in pH 6.0 citrate buffer at 95°C for 30 minutes. After incubating with endogenous peroxidase, the slides were quenched with 1.5% H_2O_2 and blocked in a mixture of blocking buffer (1x phosphate buffered saline (Thermo Fisher Scientific, USA), 1% bovine serum albumin (Biochrom AG, Germany), 0.2% gelatin of cold-water fish skin (SIGMA-ALDRICH®, St. Louis), 0.1% triton X 100 (Carl Roth GmbH+Co. KG, Germany)) with 2.5% normal horse serum (Vector Laboratories, UK) before avidin (Vector Laboratories, USA) was added. Afterwards, incubation was performed with primary antibodies against ACSL4, GPX4, ALDH1A3 and FSP1 overnight at 4°C. The used antibodies with corresponding dilution are listed in **Table 2**. The antibody diluent consisted of blocking buffer and biotin. On the next day, biotinylated secondary anti-rabbit IgG, anti-rat IgG or anti-mouse IgG antibodies, were diluted at the rate of 1:400 and incubated for 30 minutes. Afterwards, the ABC-reagent (Vector Laboratories, USA) was applied and incubated for 30 minutes. Antibody complexes were detected with 3,3'-diaminobenzidine (DAB) reagent (Vector Laboratories, USA). Finally, counterstaining with haematoxylin was performed. Positive controls (human liver tissue for ACSL4, ALDH1A3 and FSP1; human kidney tissue for GPX4) served as quality assurance.

The cytoplasmatic staining was analyzed using the immunoreactive score (IRS) established by Remmele and

TABLE 1 | Patient data.

Sex	**Male** **Female**	**17** **7**	**n = 24**
Age at first diagnosis	median	58 y	n = 24
	range	27-78 y	
PFS/time between primary tumor and relapse	median	9 mth	n = 19
	range	3-53 mth	
OS/time between first diagnosis and death/today	median	18 mth	n = 19
	range	9-71 mth	
Time between relapse diagnosis and death/today	median	11 mth	n = 19
	range	3-56 mth	
MGMT promoter status	methylated	5	n = 22
	unmethylated	17	

24 patients have been included, of which 5 were still alive at the time of this study. y, years; mth, months. PFS, progression free survival; OS, overall survival; MGMT, O-6-methylguanine-DNA methyltransferase. The MGMT promotor status of 22 patients was available. Survival data was accessible for 19 patients.

TABLE 2 | Used antibodies.

Antibody	Company	Clone	Host species	Dilution
Anti-ACSL4	Santa Cruz, USA	Monoclonal, clone IgG_{2b}	mouse	IHC: 1/100 IF: 1/20
Anti-GPX4	Abcam, UK	Monoclonal, clone EPNCIR144	rabbit	IHC: 1/3000 IF: 1/1000
Anti-ALDH1A3	Thermo Fisher Scientific, USA	polyclonal	rabbit	IHC: 1/1000
Anti-FSP1	developed in house	IgG2 monoclonal antibody raised against a N-terminal peptide of hXCT, clone 3A12-1-1	rat	IHC: undiluted
2nd antibody, anti-rabbit	Vector Laboratories, USA	IgG	rabbit	IHC: 1/400
2nd antibody, anti-mouse	Vector Laboratories, USA	IgG	mouse	IHC: 1/400
2nd antibody, anti-rat	Vector Laboratories, USA	IgG	rat	IHC: 1/400
anti-GFAP	Dako, USA	monoclonal	mouse	IF: 1/50
anti-GFAP	Dako, USA	polyclonal	rabbit	IF: 1/500
anti-Iba1	Abcam, UK	monoclonal	mouse	IF: 1/500
anti-Iba1	Wako, USA	polyclonal	rabbit	IF: 1/500
2nd antibody	Invitrogen/Thermo Fisher Scientific, USA	Polyclonal, (Alexa Fluor 568/488)	mouse	IF: 1/2000
2nd antibody	Invitrogen/Thermo Fisher Scientific, USA	Polyclonal, (Alexa Fluor 568/488)	rabbit	IF: 1/2000

Stegner (35). The score is a product of the percentage of positive cells (0 = 0%, 1 = <10%, 2 = 10-50%, 3 = 51-80%, 4 = >80%) and staining intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = strong positivity) allowing total values from 0 to 12. Three randomly chosen high power fields (600-fold magnification; ocular 10-fold, objective 60-fold) containing tumor core were examined and the mean was calculated.

Immunofluorescence

To further investigate in which cells ferroptosis in principle may occur, double immunofluorescence staining with cell markers including glial fibrillary acidic protein (GFAP), which is expressed in tumor cells and astrocytes, along with ionized calcium-binding adapter molecule 1 (Iba1) for detecting microglia cells was performed (36, 37). Like for IHC, the samples were deparaffinized, unmasked, quenched and blocked. Afterwards, anti-ACSL4 and anti-GPX4, respectively, antibodies each in combination with anti-GFAP or Iba1 antibody (all diluted in blocking buffer (1x phosphate buffered saline, 2.5% donkey serum, 1% bovine serum albumin, 0.2% gelatin of cold-water fish skin, 0.1% triton X 100) were incubated overnight at 4˚C, followed by the second antibody, which incubated for 45 minutes. The used antibodies with corresponding dilution are listed in **Table 2**. Autofluorescence Quenching Kit including 4',6-Diamidino-2-phenylindol (DAPI) (Vector Laboratories, USA) was used to reduce autofluorescence and to counterstain the nuclei. While the ferroptosis-related enzymes were stained in a green-fluorescent dye with excitation at 488 nm detectable with the fluorescein isothiocyanate (FITC) filter, the cell type-specific markers were colored in a red-fluorescent dye with absorption at 568 nm and detected with the rhodon filter. The blue-fluorescent nuclei were detected with the DAPI filter.

The same procedure was applied to the ACSL4 and GPX4 double immunofluorescence. Only the incubation time of the first antibodies was shortened to two hours to reduce background straining.

For quantification, the amount of nuclei was counted. Afterwards, a percentage of GPX4-positive ($^+$) and $ACSL4^+$ cells, as well as $GFAP^+$ and $Iba1^+$ cells was estimated. Furthermore, the number of co-expressing cells (GPX4 or ACSL4 plus GFAP or Iba1 and ACSL4 plus GPX4) was counted. Again, three high power fields (630-fold magnification; ocular 10-fold, objective 63-fold) containing tumor core were examined and the mean calculated. Following amounts were calculated: the ACSL4- and GFAP-co-stained cells divided by the amount of GFAP-positive cells ($ACSL4^+/GFAP^+$), $ACSL4^+/Iba1^+$, $GPX4^+/GFAP^+$ and $GPX4^+/Iba1^+$, as wells as $GFAP^+/ACSL4^+$, $Iba1^+/ACSL4^+$, $GFAP^+/GPX4^+$ and $Iba1^+/GPX4^+$.

Statistical Analysis

Statistical analyses were performed with R Version 3.6.1. Since pairs of samples from the same patient had to be compared and a normal distribution was not always given, all significance was tested using the Wilcoxon signed-rank tests were used for comparisons of primary and recurrent tumors and Spearman's rank correlation coefficients with corresponding tests for assessment of associations between quantitative data. Since the TCGA-dataset was also not coherently normally distributed, the Wilcoxon signed-rank test was applied once more. For correlating the IHC results with clinical outcome, a test on association was performed and a cut-off score was estimated using the 'coin' package which calculated the best threshold to discriminate patients with regard to OS (38). Kaplan-Meier survival curves are shown for the corresponding groups. Moreover, the p-value was also estimated by the "maxstat"-function from the "coin"-package. For all tests, statistical significance was defined as $p<0.05$.

RESULTS

Dynamic Changes in Ferroptosis-Related Enzymes in Primary and Recurrent GBM

By immunohistochemistry, a significant increase of ASCL4 and ALDH1A3 expression and a significant decrease of GPX4 expression was observed when comparing primary and corresponding recurrent tumor. FSP1 expression increased slightly, not significantly, though.

Following, the results are demonstrated in detail. The IRS allows values from 0 to 12. Inconsistencies between absolute values and difference (Δ) are due to rounding.

The average of ACSL4 expression increased from IRS 2.40 in the primary tumors to IRS 4.99 in the recurrent tumors (**Figures 1B, C**, **2A**). This change of 4.58 IRS points was highly significant ($p<0.001$).

Expression of GPX4 decreased from IRS 6.53 in the primary to IRS 2.17 in the recurrent tumors (Δ 4.36 IRS points, **Figures 1F, G**, **2B**). The decrease could be detected in 23 out of 24 patients and was highly significant ($p<0.001$).

FSP1 expression increased slightly from IRS 1.46 to 2.08 (Δ 0.62 IRS points). This change was not significant ($p=0.174$, **Figures 1J, K**, **2C**).

ALDH1A3 expression increased in 22 out of 24 patients (**Figures 1N, O**, **2D**). The increase from IRS 2.24 in the primary to IRS 6.18 in the recurrent tumors (Δ 3.94 IRS points) was highly significant ($p<0.001$).

The complete results of immunohistochemistry are summarized in **Table 3**.

A TCGA data analysis was performed to verify our results. The gene expression level was normalized using fragments per kilobase of transcript per million mapped reads (FPKM). Although insignificant ($p=0.094$), the ACSL4 gene expression increased in five out of six patients (TCGA-06-0210, TCGA-06-0190, TCGA-19-4065, TCGA-06-0125, TCGA-06-0211) by an average of 0.36 (**Figure 2E**). Moreover, the GPX4 gene expression decreased in four out of six patients (TCGA-06-0210, TCGA-06-0190, TCGA-19-4065, TCGA-14-1034) by an average of 0.06 (**Figure 2F**), again, not significantly, though ($p=0.844$). The insignificant results may be allegeable by the small sample size.

The results of the TCGA data analysis are summarized in **Table 4**. **Figure 1** also includes hematoxylin and eosin stain, as well as ki67 immunohistochemistry as proliferation marker for all examples of primary and recurrent GBM.

Co-Expression Analysis With Immunofluorescence

Co-expression of ferroptosis-related proteins ACSL4 and GPX4 with the cell type specific markers GFAP and Iba1 was analyzed

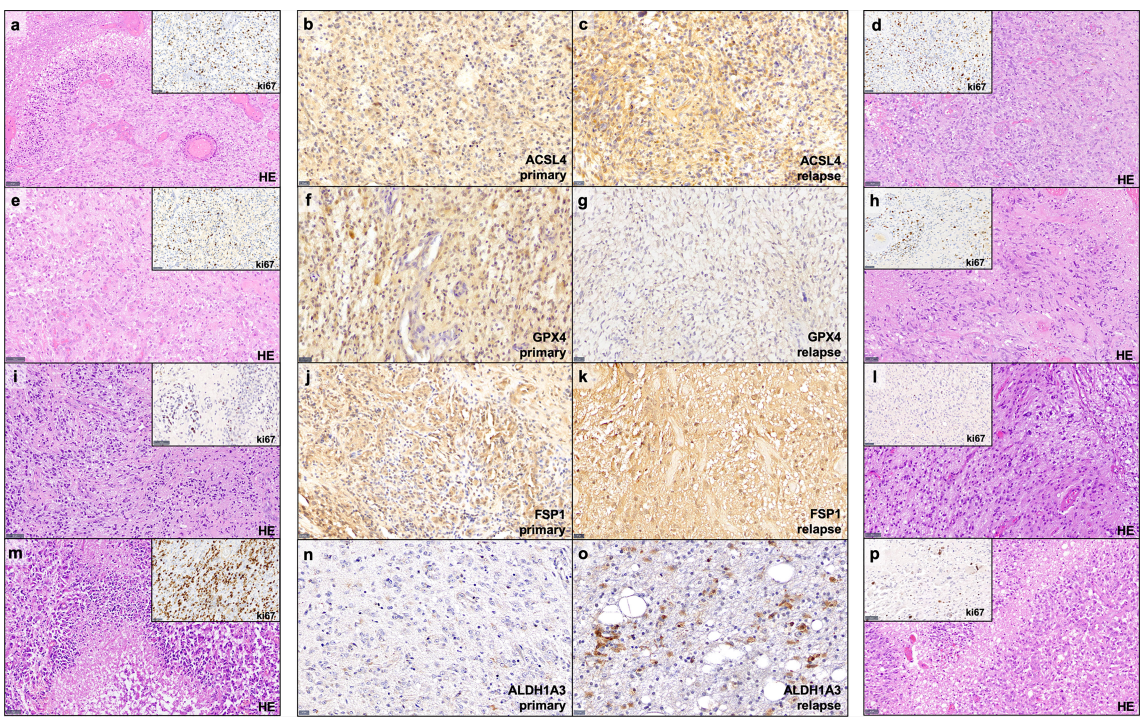

FIGURE 1 | Examples of immunohistochemistry staining. Shown are paired GBM primary and relapse with stronger ACSL4 and ALDH1A3 expression in the relapse **(C, O)** compared to the primary tumor **(B, N)**. In contrast, GPX4 and FSP1 display a stronger expression in the primary tumor **(F, J)** compared to their relapse **(G, K)**. Furthermore, for each example of primary and recurrent GBM, a hematoxylin and eosin (HE) stains as well as a ki67 immunohistochemistry of representative areas are provided **(A, D, E, H, I, L, M, P)**. Scale bars of ACSL4, GPX4, FSP and ALDH1A3 immunohistochemistry: all 20 µm; Scale bars of HE and ki67 immunohistochemistry: all 50 µm.

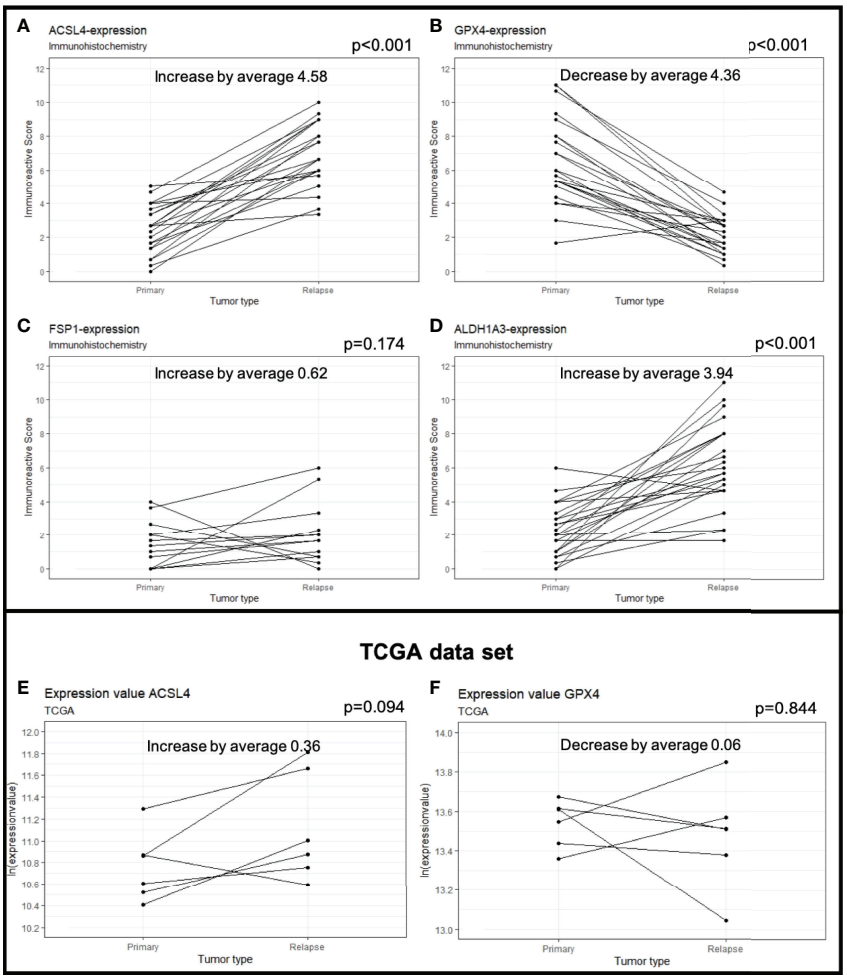

FIGURE 2 | Protein expression in primary and recurrent GBM. The dots mark the level of IRS in the primary and relapse tumor. Each line represents the change in expression of one patient, showing increase of expression for ACSL4 **(A)**, FSP1 **(C)** and ALDH1A3 **(D)**. GPX4 expression decreases significantly in 23 out of 24 patients **(B)**. The results were verified by analyses of the TCGA data set showing an increase in ACSL4 expression **(E)** and a decrease in GPX4 expression **(F)**.

to further investigate which cell types become vulnerable to ferroptosis. Many GFAP-positive ($^+$) cells expressed the ferroptosis-associated markers labelled by a yellow signal (**Figures 3A–F**). In combination with Iba1, however, only a few cells showed a clear yellow signal indicating co-expression with ACSL4 and GPX4 (**Figures 3G–L**). To anticipate: the co-expression analysis shows that 70% to 80% of the cells expressing the ferroptosis-associated marker genes ACSL4 or GPX4 do also express GFAP.

In detail and illustrated in **Figures 4A, B**, the overall amount of ACSL4$^+$ cells in both combinations increased significantly from primary to recurrent tumors. Moreover, a higher amount of GFAP$^+$ cells also expressed ACSL4 compared to Iba1$^+$ cells. The amount of ACSL4$^+$ cells of the GFAP$^+$ cells increased

TABLE 3 | Summary of results of immunohistochemistry.

Protein	IRS total		IRS primary		IRS recurrent		Δ primary-recurrent				
	Mean	Range	Mean	Median	Mean	Median	Mean	Median	SD	p-value	Z-score
ACSL4	4.69	0.00-10.00	2.40	2.50	6.99	6.67	+4.58	+5.00	2.12	<0.001	-7.181
GPX4	4.35	0.33-11.00	6.53	6.00	2.17	2.17	-4.36	-4.33	2.25	<0.001	-7.047
FSP1	1.77	0.00-6.00	1.46	1.33	2.08	1.67	+0.62	+0.67	2.28	0.174	-1.360
ALDH1A3	4.21	0.00-11.00	2.24	2.00	6.18	5.83	+3.94	4.17	2.91	<0.001	-6.672

TABLE 4 | Summary of the TCGA analysis.

Protein	Ln(expression-value) total		Ln(expression-value) primary		Ln(expression-value) recurrent		Δ primary-recurrent				
	Mean	Range	Mean	Median	Mean	Median	Mean	Median	SD	p-value	Z-score
ACSL4	7.22	1.42-12.90	7.07	7.47	7.40	7.70	+0.36	+0.36	0.42	0.094	-1.676
GPX4	9.45	4.06-14.95	9.51	9.25	9.38	9.07	-0.06	-0.08	0.31	0.844	-0.197

TABLE 5 | Summary of results of co-expression analysis by immunofluorescence.

Proteins		primary		recurrent		Δ primary-recurrent				
		Mean	Median	Mean	Median	Mean	Median	SD	p-value	Z-score
$ACSL4^+$ in	$GFAP^+$	47.1%	44.4%	76.5%	79.3%	+29.3%	+30.8%	18.6%	<0.001	-6.647
	$Iba1^+$	24.6%	19.1%	28.7%	28.6%	+4.2%	10.4%	19.3%	0.027	-2.209
$GPX4^+$ in	$GFAP^+$	78.4%	79.6%	40.3%	39.4%	-38.0%	-40.4%	18.1%	<0.001	-7.124
	$Iba1^+$	34.5%	32.5%	25.3%	23.4%	-9.2%	-9.7%	20.0%	0.001	-3.192
$GFAP^+$ in	$ACSL4^+$	76.1%	76.9%	70.3%	72.6%	-5.8%	-4.4%	14.7%	0.026	-2.223
$Iba1^+$ in		22.2%	20.0%	16.1%	17.4%	-6.1%	-0.1%	16.0%	0.026	-2.232
$GFAP^+$ in	$GPX4^+$	79.1%	82.9%	72.1%	76.3%	-6.9%	-6.6%	17.1%	0.006	-2.747
$Iba1^+$ in		13.6%	12.5%	20.2%	15.5%	+6.6%	+4.9	15.1%	0.024	-2.255
$ACSL4^+$ with	$GPX4^+$	18.9%	15.7%	16.2%	17.1%	-2.7%	-3.4%	7.5%	0.625	-7.181

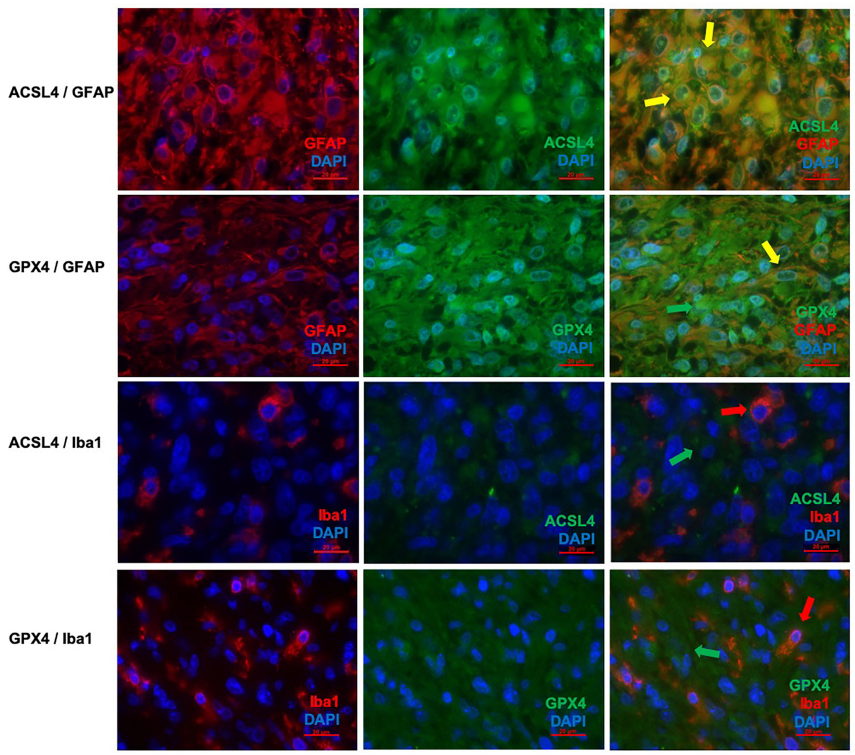

FIGURE 3 | Co-staining of ferroptosis-related proteins with cell type specific markers. **(A–C)** Double immunofluorescence with ACSL4 and GFAP in a primary GBM shows several cells with co-expression (yellow arrow). **(D–F)** Double immunofluorescence with GPX4 and GFAP shows co-expression in many cells in a primary GBM (yellow arrow). Additionally, one cell expressing only GPX4 (green arrow) is marked. **(G–I)** Double immunofluorescence with ACSL4 and Iba1 in a primary GBM shows several $Iba1^+$ cells which mostly do not express ACSL4 (red arrow: Iba1-positive cell lacking ACSL4 expression; green arrow: ACSL4-positive non-microglial cell). **(J–L)** Double immunofluorescence with GPX4 and Iba1 in a relapse GBM. Most cells express either GPX4 (green arrow) or Iba1 (red arrow). Scale bars all 20 μm.

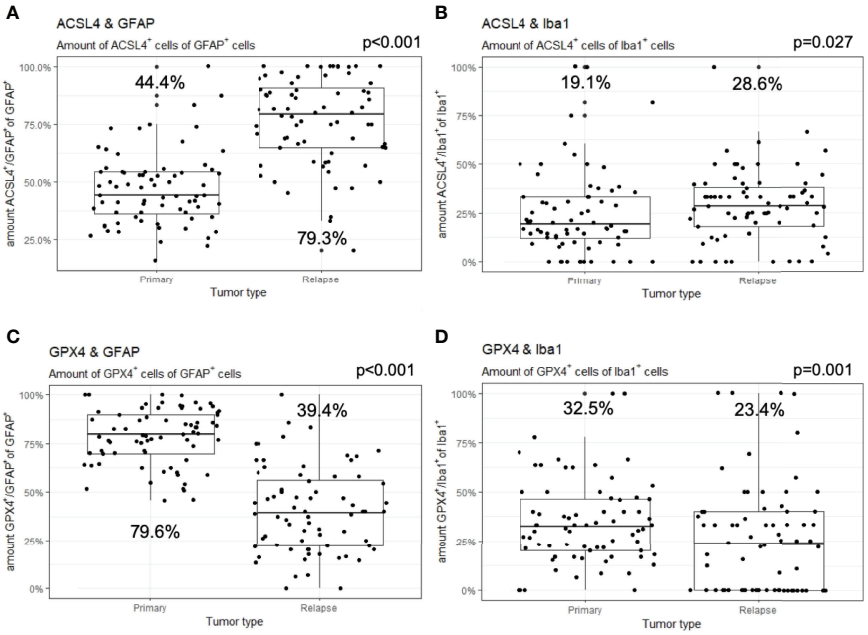

FIGURE 4 | Co-expression of ferroptosis-associated markers with GFAP and Iba1. Given are the numbers of $ACSL4^+$ or $GPX4^+$ cells of $GFAP^+$ or $Iba1^+$ cells. The dots indicate the amounts of each patient in the primary and relapse tumor. **(A)** The number of $ACSL4^+/GFAP^+$ cells increases significantly in the relapse. **(B)** The amount of $ACSL4^+/Iba1^+$ cells increases significantly in the relapse. **(C)** The number of $GPX4^+/GFAP^+$ cells decreases significantly in the relapse. **(D)** The amount of $GPX4^+/Iba1^+$ cells decreases significantly in the relapse.

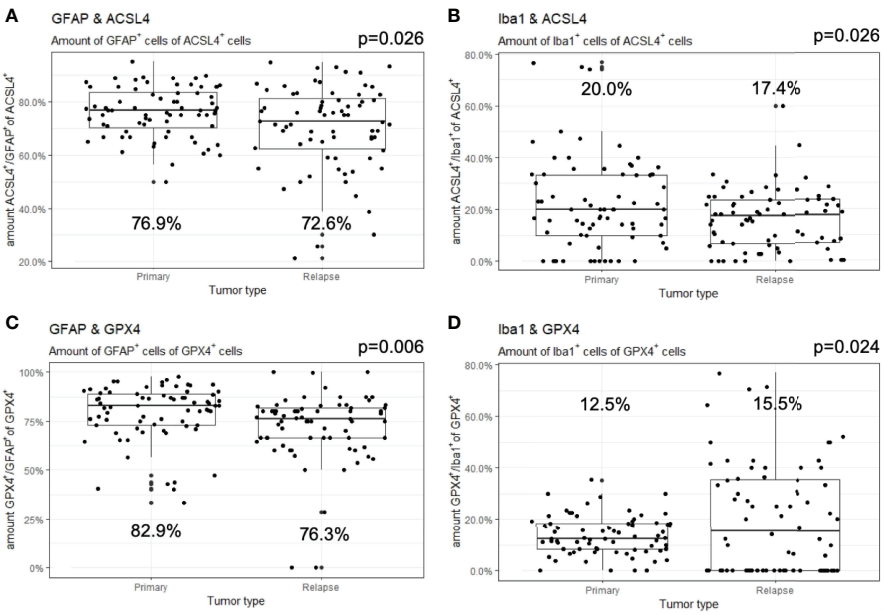

FIGURE 5 | Co-expression of GFAP and Iba1 with ACSL4 and GPX4. Given are the numbers of $GFAP^+$ or $Iba1^+$ cells of $ACSL4^+$ or $GPX4^+$ cells. The dots indicate the amounts of each patient in the primary and relapse tumor. **(A)** The number of $GFAP^+/ACSL4^+$ cells decreases insignificantly in the relapse. **(B)** The amount of $Iba1^+/ACSL4^+$ cells significantly decreases in the relapse. **(C)** The number of $GFAP^+/GPX4^+$ cells decreases significantly in the relapse. **(D)** The amount of $Iba1^+/GPX4^+$cells increased significantly in the relapse.

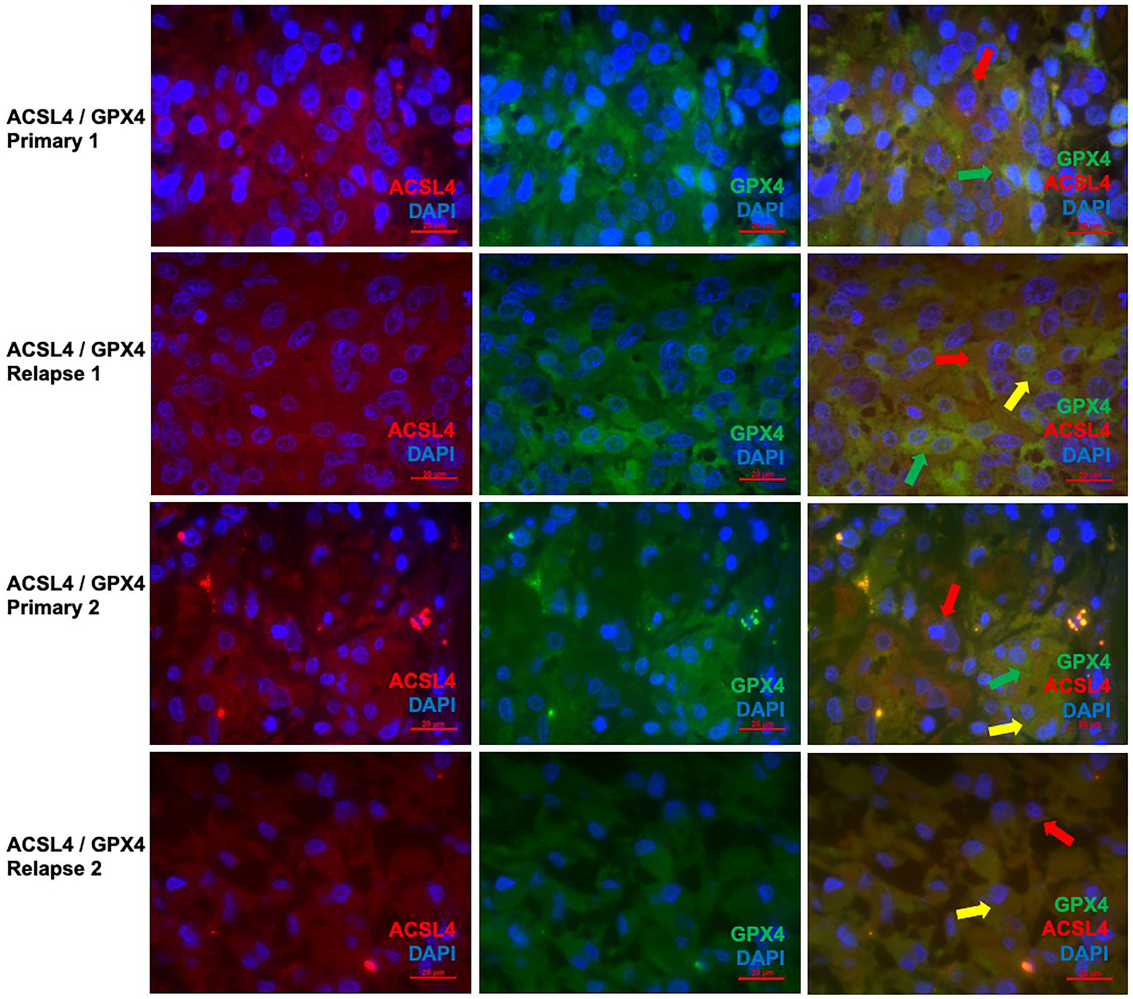

FIGURE 6 | Co-staining of both ferroptosis-related proteins in pairs of primary and recurrent GBM. **(A–C)** Double immunofluorescence with ACSL4 and GPX4 in a primary GBM (patient A) shows cells expressing either ACSL4 (red arrow) or GPX4 (green arrow). **(D–F)** Double immunofluorescence with ACSL4 and GPX4 in a relapse GBM (patient A) shows several cells with co-expression (yellow arrow), as well as only ACSL4$^+$ cells (red arrow) and GPX4$^+$ cells (green arrow). **(G–I)** Double immunofluorescence with ACSL4 and GPX4 in a primary GBM (patient B) shows ACSL4$^+$ cells (red arrows) and GPX4$^+$ cells (green arrow). Some cells express both ACSL4 and GPX4 (yellow arrow). **(J–L)** Double immunofluorescence with ACSL4 and GPX4 in a relapse GBM (patient B). Whilst many cells show co-expression (yellow arrow), one cell only expressing ACSL4 is marked (red arrow). Scale bars all 20 µm.

significantly by an average of 29.3% ($p<0.001$) between primary and relapsed tumor. The number of ACSL4$^+$ cells in the Iba1$^+$ cell population increased significantly by an average of 4.2% ($p=0.027$).

The count of GPX4$^+$ cells of GFAP$^+$ cells decreased significantly by an average of 38.0% between primary and recurrent tumors ($p<0.001$), while the amount of GPX4$^+$ cells of Iba1$^+$ cells decreased significantly by an average of 9.2% ($p=0.001$) in the relapse (**Figures 4C, D**).

The number of GFAP$^+$ of ACSL4$^+$ cells decreased insignificantly by an average of 5.8% in the recurrent tumors ($p=0.026$, **Figure 5A**). Moreover, the amount of Iba1$^+$ cells of ACSL4$^+$ cells was three to four times lower in the primary tumor and decreased significantly by an average of 6.1% in the recurrent tumor ($p=0.026$; **Figure 5B**).

A similar tendency can be observed in **Figure 5C** with the amount of GFAP$^+$ cells of GPX4$^+$ cells. This number decreased significantly by an average of 6.9% ($p=0.006$). On the other hand, the amount of Iba1$^+$ cells of the GPX4$^+$ cell population was three to four times lower (**Figure 5D**). It increased significantly by an average of 6.6% ($p=0.024$). The quantitative results of the described double immunofluorescences are summarized in **Table 5**.

Since the changes in expression of ACSL4 and GPX4 from primary to relapse GBM were already analyzed quantitively in the IHC, double immunofluorescence of these two proteins was

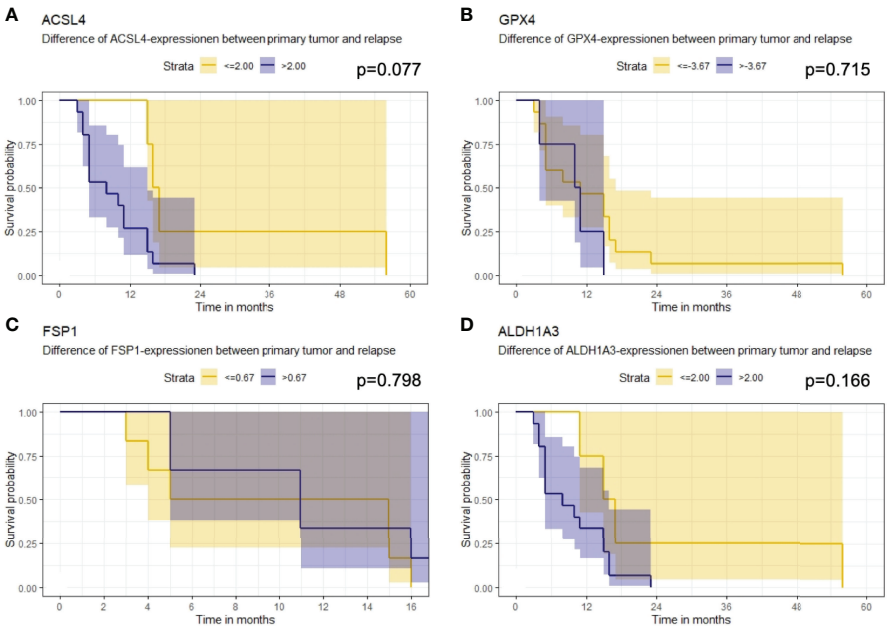

FIGURE 7 | Kaplan-Meier survival curves. Starting point for measurement of survival time was the time at recurrent confirmation. The p-value describes a possible association between enzyme expression and OS. **(A)** Patients whose ACSL4 expression increased by more than 2.00 IRS points had a worse outcome, although. **(B)** GPX4 cutpoint is at -3.67. Patients with a higher pronounced decrease in GPX4 expression had a slightly better outcome. **(C)** FSP1 cutpoint is at 0.67. There is no relevant association. **(D)** ALDH1A3 cutpoint is at 2.00. Patients with a higher increase had a worse outcome.

performed to show parallel expression and possible interactions in a qualitatively way. With a mean of 18.9% in primary and a mean of 16.2% in relapse GBM, several cells show co-expression, although there is no significant difference (p=0.625). Whilst GPX4 remained prominent in the primary tumors (**Figures 6A–C, G–I**), ACSL4 was expressed more in relapse GBM (**Figures 6D–F, J–L**), affirming the results of IHC.

Association of Ferroptosis-Associated Markers With Overall Survival

Furthermore, the association of the expression dynamic of ACSL4, GPX4, FSP1 and ALDH1A3 with OS was evaluated. The IRS differences between primary and recurrent tumor were calculated for each marker and a threshold for every enzyme was estimated. Patients with a larger increase than 2.00 in their ACSL4 expression (**Figure 7A**) showed a poorer overall survival with a median of 8 months compared to patients with an increase of up to 2.00 and with a median OS of 16.5 months. However, no significant association between ACSL4 expression an OS was observed (p=0.077). Given that the GPX4 expression decreased in almost all patients, the calculated cutpoint amounts to -3.67 (**Figure 7B**). Patients with an even stronger decrease (≤ -3.67) displayed a slightly better overall survival with a median of 11 months compared to patients with a smaller decrease with a median of 10.5 months, yet again no statistically significant association between expression and OS was found (p=0.715).

The threshold for FSP1 was 0.67 (**Figure 7C**). Patients with a higher increase in expression had a median OS of 11.0 months, those with a lower change had a median OS of 10.0 months. There was no significant association between FSP1 expression

TABLE 6 | Summary of results of survival analysis.

Protein	Cutpoint	Time between relapse diagnosis and death		p- value
		Mean	Median	
ACSL4	≤2.00	26.0	16.5	0.077
	>2.00	9.3	8.0	
GPX4	≤-3.67	13.6	11.0	0.715
	>-3.67	10.0	10.5	
FSP1	≤0.67	9.7	10.0	0.798
	>0.67	10.8	11.0	
ALDH1A3	≤2.00	24.8	16.0	0.166

and OS (p=0.798). For ALDH1A3 expression, a cut-off value of 2.00 was identified (**Figure 7D**). Patients with a stronger increase trended to have a poorer overall survival with a median of 8.0 months compared to the patients with less increase than 2.00 IRS points with a median OS of 16 months. Again, no significant association was observed (p=0.166).

In addition, the change in expression of each enzyme was correlated with the methylation status of the MGMT promoter. No significant correlations between the difference of expression of ACSL4 (rho=-0.146, p=0.518), GPX4 (rho=0.205, p=0.359), ALDH1A3 (rho=0.146, p=0.518) or FSP1 (rho=0.336, p=0.336) and status of methylated MGMT promoter were detected.

The results of the survival analysis are summarized in **Table 6**.

DISCUSSION

Despite extensive therapy, the prognosis of GBM remains very poor. Therefore, new therapeutic approaches are a key topic of many studies. Ferroptosis has been discussed as possible novel therapeutic target in cancer, particularly in light of a number of recent reports that suggested that therapy-resistant cancer cells and those undergoing epithelial-mesenchymal transition display a high vulnerability towards ferroptosis (8, 39). Thus, the objective of this study was to analyze whether ferroptosis is in principle activated in GBM and moreover, if there is a difference in vulnerability between primary and relapsed tumors. Various therapy options examined the effect of ferroptosis induction *in vitro* and ex vivo (17, 19, 40). All of these studies could in fact confirm an increased therapy response after the induction. Therefore, the following enzymes implicated in the process of ferroptosis were chosen (17): ACSL4, GPX4 and FSP1.

Our study shows that ACSL4 expression increases in GBM relapses compared to their primaries, whilst the GPX4 expression decreases. The results of the TCGA data analysis verified these results. An increased expression of ACSL4 is ultimately linked to an increased generation of activated PUFAs, which are used to produce oxidative stress and activate even more PUFAs by forming radicals in the presence of iron. Upon esterification into membranes they may become peroxidized thus rendering cells more sensitive to ferroptosis (19, 41, 42). Moreover, the decrease of GPX4 expression implies that the detoxifying capacity might be diminished. Both changes increase the propensity of cells to undergo lipid peroxidation (19, 43). This can also be caused by chemotherapeutics, radiation or simply burning energy (44–46).

Since FSP1 was recently shown to efficiently protect against ferroptosis caused by GPX4 deletion or inhibition (21, 47), it was hypothesized that its expression may increase in response to a loss of GPX4 expression. Accordingly, a slight, although not significant increase in expression could be detected. Unlike the glutathione/GPX4 axis that directly reduces lipid hydroperoxides in the membranes to its corresponding alcohols, the oxidoreductase FSP1 regenerates extra-mitochondrial ubiquinone to ubiquinol, that in turn either directly or indirectly *via* vitamin E prevents the lipid peroxidation chain reaction by reducing peroxyl radicals in phospholipid acyl chains (8). Furthermore, FSP1 was described as a protein prohibiting ferroptosis through the suppression of lipid peroxidation. Although two of the defined hallmarks can be detected in GBM relapse, additional studies are warranted to show that there is indeed increased lipid peroxidation in respective tissues as this would ultimately tell us that an imbalance in PUFA enrichment of membranes and a compromised protecting system sensitizes tumors to ferroptosis (17). The antibody against human FSP1 was reported in 2019 (21). Thus, its analysis was complemented retroactively with sufficient material from only 13 patients left.

This is the first study that investigated a potential relationship between ALDH1A3 and ferroptosis susceptibility. IHC demonstrated a significant increase of ALDH1A3 between GBM primary and relapse. This is in accordance with the mesenchymal transformation taking place during occurrence of recurrent tumors or a selection of GBM tumor stem cells surviving. ALDH1A3 has been associated with mesenchymal differentiation in GBM by keeping cells in an undifferentiated, stem-cell-like state which might also lead to therapy resistance (22–24). Since ALDH1A3 is involved in the detoxification of aldehydes generated as secondary products by lipid peroxidation, an increase in ALDH1A3 expression could present a cellular response towards more lipid peroxidation in GBM relapse.

Furthermore, since the quantitative changes of ACSL4 and GPX4 were already analyzed in the IHC results, the double immunofluorescence with ACSL4 and GPX4 was performed on only 5 pairs of primary and relapse GBM to demonstrate possible interactions. The detected co-expression, with GPX4 dominating in the primary and ACSL4 in the relapse GBM, indicates a complex equilibrium-like relation between the two ferroptosis-markers. Regarding this, Sha et al. examined the combined status of ACSL4 and GPX4 expression in breast cancer patients. They discovered that the combined status could predict pathological complete response to chemotherapy due to their balance-like interactions. Moreover, patients with a high ACSL4 and low GPX4 status showed higher sensitivity to chemotherapy leading to the assumption that a combination of ACSL4 inducer and GPX4 inhibitor could be beneficial for treatment efficacy (48).

In combination with the results of the double immunofluorescence with GFAP and Iba1, we furthermore provide intriguing evidence that ferroptosis is more likely to take place in GBM tumor cells and not in the surrounding microglia cells.

There was no significant association between the change of expression of ferroptosis-associated proteins and OS. Nevertheless, patients with a high increase of ACSL4 expression had a poorer OS than those with a low increase. This suggests that patients with a higher content of PUFAs in membranes have a poorer overall outcome. Liu et al. identified 19 ferroptosis-related genes in glioma using data from genome atlases including TCGA, upon which they evaluated a risk score (49). The risk score of those genes positively correlated with glioma malignancy, as well as migration and invasion.

While higher risk scores regarding the ferroptosis-related genes were associated with worse prognosis, the receiver operating characteristic curve generated by the risk score could predict patient OS. Since six signature genes of the 19 ferroptosis-related genes were involved in the GPX4 regulation, GPX4 and its role in ferroptosis might play a crucial role regarding survival of glioma patients. Furthermore, mesenchymal cancer cells associated with drug resistance proofed to be selectively dependent on GPX4 (18). Therefore, GPX4 inhibitors were selectively lethal to these cells, offering yet another therapeutic option. Moreover, patients with a higher ALDH1A3 increase showed poorer OS. This might lead to the assumption that the detoxifying systems including GPX4 and ALDH1A3 can sense the oxidative stress level in the cell and therefore coordinate up- and downregulation accordingly. When there is more ROS accumulating due to the downregulation of one of the systems, the other one possibly increases.

The lack of significance may be attributable to the small sample size and, therefore, to the wider confidence intervals. Nevertheless, these results support the hypothesis that an increased generation of lipid hydroperoxides and increased vulnerability towards ferroptosis may occur during primary and relapse diagnosis. It remains, however, to be explored, whether TMZ in fact contributes to the sensitization of GBM towards the ferroptotic process. Sehm et al. combined several ferroptosis inducers like erastin and sorafenib with TMZ and showed that TMZ works in an xc-system expression dependent manner (12). Furthermore, Buccarelli *et al.* reported increased glioblastoma stem-like cells susceptibility to TMZ after induction of ferroptosis (40). TMZ treatment thus may act as a possible ferroptosis inducer but further experiments including a control group without the treatment remain necessary. Moreover, the amount of TMZ may have an effect on this process. If more TMZ, maybe in form of more TMZ cycles, influences, or even amplifies the ferroptosis induction, has yet to be investigated.

A shortcoming of this study is the small sample size, which can be explained by the poor prognosis of GBM, where death occurs often before relapse and in case of relapse situation, only a small portion of GBM patients receive re-resection. The high value of our cohort is demonstrated by considering the small number of 6 pairs of primary and recurrent GBM available at the TCGA data base. The heterogeneity of GBM was not fully respected in this study. By calculating a mean of three randomly chosen tumor containing areas, we tried to incorporate the heterogeneity, though. As the heterogeneity plays a key role for therapy resistance, following studies should address differences in intratumoral expression. Another limitation consists of a missing control group.

To conclude, this is the first study analyzing ferroptotic processes in GBM between the primary and relapse tumor. Based on our results, ferroptosis likely takes place in GBM tumor cells. Moreover, we showed that there is a dynamic in the expression of ferroptosis-associated between primary and recurrent GBM with a higher vulnerability to ferroptosis in the relapses. These results affirm that utilizing ferroptosis processes might be a possible novel therapy option especially in the situation of recurrent GBM. Particularly relevant for GBM is the role of TMZ, although it remains to be determined whether it acts as a true ferroptosis trigger or a sensitizer. Nonetheless, prospective trials should be geared to examine a possible link between TMZ and ferroptosis and to validate its true clinical value.

CONCLUSION

Our study implies that ferroptosis may take place in GBM tumor cells due to the profound changes in the expression of ACSL4 and GPX4. Moreover, we show that recurrent tumors have a higher vulnerability to ferroptosis. These results affirm that utilizing ferroptosis processes might be a possible novel therapy option especially in the situation of recurrent GBM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Technical University Munich. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HK, MC, JS, YW, and FL-S contributed to conception and design of the study. HK, JG, GP, and FS-G organized the database. HK and BH performed the statistical analysis. GP performed the TCGA analyses. HK wrote the first draft of the manuscript. MC, JS, and FL-S wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Iron Transporters and Ferroptosis in Malignant Brain Tumors

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Malignant brain tumors represent approximately 1.5% of all malignant tumors. The survival rate among patients is relatively low and the mortality rate of pediatric brain tumors ranks first among all childhood malignant tumors. At present malignant brain tumors remain incurable. Although some tumors can be treated with surgery and chemotherapy, new treatment strategies are urgent owing to the poor clinical prognosis. Iron is an essential trace element in many biological processes of the human body. Iron transporters play a crucial role in iron absorption and transport. Ferroptosis, an iron-dependent form of nonapoptotic cell death, is characterized by the accumulation of lipid peroxidation products and lethal reactive oxygen species (ROS) derived from iron metabolism. Recently, compelling evidence has shown that inducing ferroptosis of tumor cells is a potential therapeutic strategy. In this review, we will briefly describe the significant regulatory factors of ferroptosis, iron, its absorption and transport under physiological conditions, especially the function of iron transporters. Then we will summarize the relevant mechanisms of ferroptosis and its role in malignant brain tumors, wherein the role of transporters is not to be ignored. Finally, we will introduce the current research progress in the treatment of malignant brain tumors by inducing ferroptosis in order to explain the current biological principles of potential treatment targets and treatment strategies for malignant brain tumors.

Keywords: iron transport, transporters, ferroptosis, malignant brain tumors, therapeutic strategy

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1 INTRODUCTION

Brain tumors can be categorized as primary malignant types and secondary forms from metastasis (1). Of these, roughly 40% will be malignant and the incidence rate of malignant brain tumors is higher in males (2, 3). Primary brain tumors are the first common tumor and the first cause of tumor death in children (3). Brain tumors can be classified based on origin, such as glioblastoma (GBM), neuroblastoma and meningioma (4). GBM is the most common and aggressive malignant primary brain tumor, with a limited response to the current standard of treatment. Most GBM patients can only live up to 15-20 months (5).

Malignant brain tumors are commonly intratumoral heterogenic, which likely explains their poor clinical prognosis of malignant brain tumors poor and easy to relapse (6). Despite current

multimodality treatment efforts, combining in surgical resection when feasible, with radiotherapy, chemotherapy and symptomatic treatment, the median survival remains short (7).

Iron is necessary for life (8). Iron plays an extremely significant role in brain development and function, and is involve in many biological processes such as embryonic neuronal development, myelin formation, neurotransmitter synthesis and oxidative phosphorylation (9, 10). Iron deficiency impairs the function of iron-requiring enzymes in all tissues, however, excessive iron accumulation leads to toxicity through oxidative stress activation of cell death signaling pathways (11). To maintain adequate and safe amounts of iron levels, cells express a the coordination of a wide variety of proteins, which tightly control both intracellular and systemic iron metabolism (12). Iron transporters participate in the regulation of iron uptake, storage and distribution, wherein help maintain iron homeostasis (13).

Ferroptosis is an iron-dependent form of regulated cell death (14). The intracellular iron homeostasis and balance between the oxidation and reduction of phospholipids is tightly associated with ferroptosis. Ferroptosis occurs when iron overload induces lipid peroxidation (11). Recent studies showed that ferroptosis is involved in the death of pathological cells in malignant brain tumors, which may have a therapeutic potential towards malignant brain tumors (15, 16). The specific way of ferroptosis inhibiting cancer may be to induce oxidative stress and resist treatment antagonism of cancer cells, in which iron transporters may has a stronger role. Although great progress has been made in the study of the biological function and disease correlation of ferroptosis, its biological signal pathway and underlying mechanism remain to be elucidated.

Starting from the iron transport in the body under physiological conditions, we further summarize the specific mechanism of iron metabolism disorder and ferroptosis in the pathological condition of malignant brain tumors, in particular, the crucial role of transporters. Finally, we summarized the specific mechanisms and targets for inducing ferroptosis in the treatment of malignant brain tumors and introduced potentially related drugs.

2 IRON PHYSIOLOGY

2.1 Iron and Iron Transporters

2.1.1 Iron Function

Iron is a vital micronutrient for nearly all living organisms due to its significant role in many biological processes such as catalyzing redox reactions and transporting oxygen. In addition, iron is essential for the functions of many enzymes and prosthetic groups (17, 18).

2.1.2 Dietary Sources of Iron

Iron is required across all human life stages, from embryological development, to infancy or old age. Estimated daily average iron requirements are the highest in pregnancy 3rd trimester (19). Despite having an efficient iron recycling mechanism, humans need to absorb about 10% of our total iron needs from regular dietary to maintain normal health. Dietary iron exists as either heme iron or non-heme iron. Heme iron is derived from hemoglobin, myoglobin and neuroglobin found in animal foods, and its absorption is not affected by diet; meanwhile non-heme iron is found mainly in plant foods, and its absorption is influenced by inhibitors and enhancers found in the diet. Nonetheless both are affected by iron storage levels in the body (19, 20).

2.1.3 Iron Absorption

The absorption site of iron is mainly in the mucosa of duodenum and upper jejunum. In a nutshell, iron absorption can be divided into two steps; first iron in food enters intestinal mucosal cells, second iron in intestinal mucosal cells crosses the cell membrane into capillaries and is transported systemically to the whole body in bloodstream (19).

2.1.4 Iron Transport

In humans, a number of proteins have evolved which tightly regulate iron homeostasis since we cannot rapidly excrete iron in the urine and iron must be transported and stored intracellular on a protein carrier due to extremely low free iron levels both systemically and intracellular (21). These includes the proteins that are involved in iron transport, both in the circulation and intracellularly, the reductases and oxidases that facilitate the movement of iron across cell membranes, and other proteins that regulate these processes (22). Iron transporters are vital role to maintain iron homeostasis in the body, and a total of 22 iron transporters have been identified (**Table 1**). The functions of several iron transporters are introduced below.

Transferrin (TF) is regulator of free iron levels in body fluids, binding, sequestering, and transporting Fe^{3+} ions. This iron carrier protein helps maintain iron availability systemically and prevents tissue oxidative damage caused by excessive free radical accumulation (23).

SLC25A37 (Mitoferrin 1, Mfrn1) is a solute carrier localized in the mitochondrial inner membrane. When iron enters cells, Mfrn1 transport iron into mitochondria, which is used to synthesize mitochondrial heme and iron sulfur clusters. Mitoferrin-1 is necessary for neuronal energy metabolism and influences brain function (24).

SLC11A2 (Divalent metal cation transporter 1, DMT1), is a proton-dependent iron importer of Fe^{2+}, is involved in systemic iron recycling and cellular iron absorption. DMT1 is located on the parietal membrane of duodenal intestinal epithelial cells, where it brings dietary free iron into cells and promotes iron absorption (25). DMT1 is also involved in transferrin/transferrin receptor 1 (TF/TFR1) pathway, wherein transports iron absorbed by this pathway from the endosome into the cytosol (26).

SLC40A1 (Ferroportin 1, Fpn1), is a major iron export protein, is expressed in many cells, such as placental syncytiotrophoblasts, wherein plays a role in transferring maternal iron to the fetus and releasing iron from tissue into the blood. It should be noted that inactivating the murine Fpn1 gene globally is embryonic lethal (27).

TABLE 1 | Iron Transporters. For detailed information about the gene tables, please visit: http://www.bioparadigms and http://www.org.genecards.org.

Human gene name	Protein name	Aliases	Substrates	Tissue and cellular expression	Sequence accession ID	Mouse KO model
SLC11A1	NRAMP1	NRAMP1 NRAMP LSH	Mn^{2+}, Fe^{2+}, other divalent metal ions	Phagolysosomes of phagocytes (macrophages, neutrophils)	NM_000578.4	No
SLC11A2	DMT1	NRAMP2 DCT1	Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{1+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}	Widespread, including intestine (duodenum), erythroid cells, kidney, lung, brain, testis (Sertoli cells), thymus	NM_001174125.2	Yes
SLC22A17	BOIT	BOCT, NGALR	1-methyl-4-phenyl-pyridinium (MPP (+)), Fe	Brain	NM_020372.4	No
SLC25A28	Mitoferrin 2 (Mfrn2)	MRS3/4, MRS4L	Fe^{2+}	Ubiquitous (heart, liver, kidney)	NM_031212.4	No
SLC25A37	Mitoferrin 1 (Mfrn1)	HT015, MSC, MSCP	Fe^{2+}	Fetal liver, bone marrow, spleen, placenta, liver, brain	NM_016612.4	No
SLC39A14	ZIP14, LZT-Hs4	ZIP14 KIAA0062 NET34	Zn, Fe, Mn, Cd	Widespread, liver	NM_001128431.4	Yes
SLC40A1	MTP1, IREG1	Ferroportin 1 (FPN1)	Fe^{2+}	Duodenum, macrophages, liver Kupffer cells, placenta, kidney	NM_014585.6	Yes
SLC41A1	MgtE	MgtE, NPHPL2	Mg^{2+} (Sr^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ba^{2+}, Cd^{2+})	Kidney, heart, testis, skeletal muscle, prostate, adrenal gland, thyroid	NM_173854.6	Yes
SLC41A2	SLC41A1-L1, SLC41A1-like 1	SLC41A1-L1	Mg^{2+} (Ba^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+})	Highest expression in cerebellum, lymph nodes, stomach, lungs, testis, skin	NM_032148.6	No
SLC46A1	PCFT	HCP1	Reduced folates, folic acid, antifolates, heme	Small intestine, choroid plexus, kidney (proximal tubule), liver (sinusoidal), placenta	NM_080669.6	No
SLC48A1	HRG-1	HHRG-1 HRG1, HRG-1	Heme	Liver, heart, CNS, kidney, skeletal muscle, small intestine	NM_017842.3	No
SLC49A1	FLVCR1	FLVCR, MFSD7B, AXPC1, PCARP	Heme	Ubiquitous, high expression in intestine, liver, kidney, brain, bone marrow	NM_014053.4	No
SLC49A2	FLVCR2	MFSD7C, CCT, EPV, PVHH, FLVCRL14q	Heme	Liver, kidney, brain, lung, placenta, fetal liver, bone marrow	NM_017791.3	No
SLC57A1	NIPA1	NIPA1, SPG6, FSP3	Mg^{2+}, Sr^{2+}, Fe^{2+}, Co^{2+}	Constitutively express at low levels, significant enrichment in the brain (human); widely expressed, including heart, kidney, liver, colon, less in the brain, not in the small intestine (mouse)	NM_144599.5	No
SLC57A3	NIPAL1	NIPA3	Mg^{2+}, Sr^{2+}, Ba^{2+}, Fe^{2+}, Cu^{2+}	Biased expression in esophagus, skin and 13 other tissues	NM_207330.3	No
SLC58A2	TUSC3	N33	Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}	Placenta, pancreas, testis, ovary, heart, prostate	NM_006765.4	No
TF	TF	Transferrin HEL-S-71p, PRO1557, PRO2086, TFQTL1	Fe^{2+}	Liver	NM_001063.4	No
ABCB6	ABCB6	ABC, LAN, MTABC3, PRP, umat	Iron	Ubiquitous expression in testis, ovary and 25 other tissues	NM_005689.4	No
ABCB7	ABCB7	ABC7, ASAT, Atm1p, EST140535	Iron	Ubiquitous expression in duodenum, heart and 25 other tissues	NM_004299.6	No
ABCB8	ABCB8	MITOSUR M-ABC1 MABC1 EST328128	Organic and inorganic molecules	Mitochondria, cardiac	NM_001282291.2	No

(Continued)

TABLE 1 | Continued

Human gene name	Protein name	Aliases	Substrates	Tissue and cellular expression	Sequence accession ID	Mouse KO model
ABCG2	ABCG2	BCRP ABCP MXR EST157481 CD338	Protoporphyrin IX (PPIX), heme, sphingosine-1-P	Biased expression in small intestine, duodenum and 12 other tissues	NM_004827.3	Yes

2.2 Brain Iron Transport

2.2.1 Brain Iron Function

Iron in the brain plays a crucial role in maintaining normal physiological function through its participation in many cellular activities such as mitochondrial respiration, myelin synthesis, neurotransmitter synthesis and metabolism (10). Iron is also essential in enzymes involved in the production of monoamines (dopamine, epinephrine, norepinephrine and serotonin), which are involved in social emotional development, executive function and memory processes. Therefore, maintaining iron homeostasis is essential for normal physiological activity of the brain (28).

Blood-brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB) are of great significance to maintain the relative stability of physical and chemical factors in the internal environment of brain tissue and prevent harmful substances in blood from entering brain tissue (29). The BBB and BCSFB also controls iron transport from the bloodstream to the brain parenchyma, allowing for some independence of brain iron levels from the total body iron and providing some resistance to systemic iron toxicity (30, 31). Different cells types in the brain acquire iron through different pathways, which involving a myriad iron transporters (**Table 2**) (29).

Herein we provide a summary of recent literature unveiling the mechanism of iron transport and regulation across the BBB and BCSFB, as well as the characteristics of iron transport and metabolism in different cell types of the central nervous system (CNS) such as neurons, microglia, astrocytes, and oligodendrocytes.

2.2.2 Iron and Iron Transporters in BBB and BCSFB

CNS is tightly sealed from the changeable milieu of blood by the BBB and the BCSFB (31). BBB is an heterogenous multicellular complex system. This system includes tightly connected endothelial cells and a unique basement membrane. In addition to the parenchymal basement membrane, the basement membrane also contains an ensheathment of astrocytic end-feet, pericytes and perivascular antigen-presenting cells (32). BCSFB lies at the choroid plexuses in the lateral, third and fourth ventricles of the brain where the choroid plexus epithelial cells of the nonporous capillary wall contain a special carrier system for transporting various substances. This system is responsible for the exchange of substances between cerebrospinal fluid (CSF) and blood, and transport across BBB and BCSFB is important for the entry of iron into brain (33, 34).

TF/TFR1 pathway may be the main route of iron transporter across the luminal (apical) membrane of the BBB. Additionally, non-transferrin-bound iron (NTBI) uptake from the blood through luminal DMT1 and H-ferritin uptake may be partly responsible for iron transport across the BBB. Iron transport across the abluminal (basal) membrane is a Fpn1/hephaestin (Fpn1/Heph) and/or Fpn1/ceruloplasmin (CP)-mediated process (35, 36).

TF/TFR1/DMT1 pathway is an important pathway for iron transport across the BCSFB. Furthermore, iron export from the choroid epithelium to the CSF is mediated by the Fpn1/CP or Fpn1/Heph pathways. Beyond restriction of the access of

TABLE 2 | Proteins Involved in Brain Iron Transport.

Gene name	Fe species bound	Presence in					Function
		BBBBCSFB	Neurons	Microglia	Astrocytes	Oligodend-rocytes	
TF (Transferrin)	Fe^{3+}	+	+	+	+	+	Transport iron to cells
DMT1 (SLC11A2)	Fe^{2+}	+	+	+	+	+	Involved in iron absorption
Zip14 (SLC39A14)	Fe^{2+}				+		Transporter of NTBI
FPN1 (SLC40A1)	Fe^{2+}	+	+	+	+	+	Iron export from cells
CP (Ceruloplasmin)	Fe^{2+}	+	+		+		Peroxidation of Fe^{2+} to Fe^{3+}
HEPH (Hephaestin)	Fe^{2+}	+	+	+		+	Peroxidation of Fe^{2+} to Fe^{3+}
Ferr (Ferritin)	Fe^{3+}	+	+	+		+	Intracellular iron storage protein

"+" *refers to the existence of corresponding genes.*

substances from the blood to the CSF, it is possible that the BCSFB has a bigger impact on iron removal from the brain than iron uptake into the brain (35–37).

2.2.3 Iron and Iron Transporters in Neurons

Iron is essential for neuron development and function (38). First iron is an essential cofactor for enzymes involved in energy metabolism and amino acid biosynthesis. Iron also plays a significant role for division of embryonic neurons as it is a cofactor for the enzyme ribonucleotide reductase. In addition, during early embryonic development, the dysfunction of yolk sac cells caused by excessive iron uptake leads to the necrotic degeneration of neuroectodermal cells (39, 40).

The neuronal expression levels of the TFR1 reflects their need for iron (41). DMT1 is also expressed in neurons, suggesting that after transferrin binding, iron is transported to the cytoplasm through DMT1 (42). DMT1 is involved in hippocampal neuronal iron uptake during development and memory formation (43). The presence of NTBI in brain extracellular fluids suggests that neurons can also take up iron as transferrin-free iron (44). Fpn1 and Heph are involved in the output of iron from the neuron (45, 46).

2.2.4 Iron and Iron Transporters in Microglia

Microglia have vital roles in brain development and CNS homeostasis, including programmed cell death, clearance of apoptotic newborn neurons, as well as pruning developing axons and synapses (47, 48). Microglia are immune cells of the CNS, which are implicated in brain inflammation and can modulate the transport and metabolism of essential metal iron according to the anti-inflammatory and pro-inflammatory environment (49).

The mechanism of iron transport in microglia has been addressed in cell culture. The different sources of cells include primary adult mouse microglia (49), primary 2-day-old Sprague-Dawley microglia, primary newborn Wistar rat microglia (50), primary C57BL/6 mice microglial (51) and BV-2 microglial cells (52). Microglial cells interact with both TF bound-iron (TBI) and NTBI. TBI is taken up *via* the TFR1/DMT1 pathway, and after the release of iron in the acidic milieu of the endosome, this is translocated into the cytosol by DMT1 or other transporters (53). For NTBI uptake, an endogenous cell surface ferrireductase reduces Fe^{3+} to Fe^{2+} for uptake by DMT1 in a pH-dependent manner at the cell surface (54).

2.2.5 Iron and Iron Transporters in Astrocytes

Astrocytes are the most abundant glial cells in the brain (55). In healthy CNS tissue, astrocytes maintain homeostasis of extracellular fluids, provide energy substrates to neurons, modulate local blood flow, and play essential roles in synapse development and plasticity (56). In addition, astrocytic end-feet form intimate contacts with the abluminal side of brain capillary endothelial cells (BCECs) in all brain regions. This close relationship makes it denotes an important role in nutrient capture from the circulating blood such as iron (57). Astrocytes theoretically can transport iron directly from BCECs to neurons and oligodendrocytes through intracellular transport (58).

The TF cycle is probably not the main process by which astrocytes obtain iron from endothelial cells (59). It is more likely that DMT1 mediates some of this uptake, since this transporter is strongly expressed in the astrocyte end-feet contacting with BCECs directly. This suggests that astrocytes can potentially uptake NTBI directly from BCECs (57).

In addition, the zinc transporter Zip14 and resident transient receptor potential channels have been suggested to be involved in the uptake of NTBI by astrocytes (60). Fpn1 and CP are highly expressed on astrocytic cell membranes, and both proteins may be essential in iron mobilization from these cells into the extracellular brain space (61, 62).

2.2.6 Iron and Iron Transporters in Oligodendrocytes

Oligodendrocytes create myelin sheaths for CNS axons, assist in the jumping and efficient transmission of bioelectric signals, maintain and protect the normal function of neurons (63, 64). Oligodendrocytes are the cells with the highest iron levels in the brain. Oligodendroglia cells require iron as a cofactor for several enzymes involved in the proliferation and differentiation of oligodendrocyte precursor cells (OPCs), as well as enzymes required for the production of cholesterol and phospholipids, which are essential myelin components (65, 66).

In oligodendrocytes, TF/TFR1/DMT1 pathway plays a significant role in iron transport in immature oligodendrocytes, however the proportion of iron transported by this pathway may decrease with the beginning of myelination (36). DMT1 is essential for OPC maturation and normal myelination in mouse brain, which is considered to be a crucial pathway for many cells to uptake NTBI (67). Extensive literature suggests that H-ferritin is the main source of iron in oligodendrocytes, conferring high buffering capacity for iron (68). Heph is expressed by mature oligodendrocytes and plays a role in iron efflux from these cells, but white and gray matter oligodendrocytes can regulate iron efflux differently; while white matter oligodendrocytes upregulate the expression of Cp in the absence of Heph, likely as a fail-safe mechanism, gray matter oligodendrocytes lacks such compensatory pathway (69).

3 FERROPTOSIS AND TRANSPORTERS IN MALIGNANT BRAIN TUMORS

3.1 The Transport Mechanisms in Ferroptosis

Ferroptosis is a form of iron-dependent regulatory cell death distinguished from necrosis, apoptosis and autophagy (70), which can be triggered by the small-molecule compound erastin and RSL3 (71, 72). Iron and polyunsaturated fatty acids (PUFAs) act as raw materials for lipid peroxidation to promote the occurrence of ferroptosis (73, 74). While glutathione peroxidase 4 (GPX4) using glutathione (GSH) as the substrate effectively removes excess ROS through antioxidant mechanism and inhibits ferroptosis (75). The increase of intracellular iron content, the accumulation of ROS and excessive lipid peroxidation are crucial to induce ferroptosis (76). Ferroptosis

is closely related to iron metabolism, amino acid metabolism and lipid metabolism in cells. Therefore, iron transporters and amino acid transporters involved in metabolism have a marked effect on the cell sensitivity to ferroptosis (70, 77).

3.1.1 Iron Transporters in Ferroptosis

DMT1 and TfR1 are involved in the absorption of intracellular iron (78, 79), while Fpn1 transports iron from the cell to the blood (27). They are both ubiquitous and crucial proteins that regulate the iron content in cells and are essential for the maintenance of iron homeostasis (**Table 3**). Iron is essential for cell growth, but it can promote the formation of toxic ROS during ferroptosis. In the case of excessive iron in cells, Fe^{2+} and H_2O_2 can generate hydroxyl radicals (OH^-) through Fenton reaction, promoting the oxidation of PUFAs on the cell membrane, greatly accelerating lipid peroxidation and ultimately causing cell damage or death (80). Therefore, increasing the expression of TFR1 or decreasing the expression of Fpn1 will increase the accumulation of iron in the cell and result in ferroptosis. DMT1 located on the lysosomal membrane mediates iron transfer and the inhibitors of DMT1 can kill cells by accelerating lysosomal iron overload and an increase of ROS production (81).

Recently identified ferroptosis-related iron transporters ZIP14 (SLC39A14) can transport manganese, iron and zinc (**Table 3**). However, its main function is to transport manganese ions, while iron ions are not the main transport substrate of ZIP14 under normal physiological conditions (82, 83). Only in the state of iron overload, ZIP14 exhibits the function of transporting iron ions and mediating ferroptosis (84).

3.1.2 Amino Acid Transporters in Ferroptosis

The amino acid transporter system Xc^- on the cell membrane is composed of two core components, SLC7A11 (Solute Carrier Family 7 Member 11, xCT) and SLC3A2 (Solute Carrier Family 3 Member 2, 4F2hc), involved in the exchange of extracellular cystine (Cys2) by transporting intracellular glutamate (Glu) (**Table 3**) (70). Intracellularly, Cys2 will be reduced to cysteine (Cys), thereby promoting the synthesis of GSH, the cofactor of GPX4. As a central regulatory protein for ferroptosis, GPX4 can convert GSH to oxidized glutathione (GSSG) whilst also reducing lipid hydroperoxides (L-OOH) to lipid alcohols (L-OH), which is the main mechanism to prevent lipid peroxidation and inhibit ferroptosis (85). In fact, knockout and inactivation of GPX4 both contribute to ferroptosis (86). Ferroptosis inducer erastin can result in GSH depletion and GPX4 inactivation by inhibiting system Xc^- transport of cystine (71), while RSL3 directly induces ferroptosis by inhibiting the activity of GPX4 (72). Cys is the crucial limiting amino acid for intracellular GSH synthesis and GSH depletion directly affects the function of GPX4. Therefore, system Xc^- that participates in the uptake of Cys2 is considered to be one of the most critical regulators of ferroptosis. Recent studies suggest that regulation of TP53 (87), Nrf2 (15), ATF4 (88), BECN1 (89) or interferon γ(IFNγ) released by $CD8^+$ T cells (90) significantly inhibits the system Xc^-, leading to a decrease in GSH synthesis and ferroptosis.

The transmembrane transport of glutamine (Gln) is dependent on SLC1A5 (Solute Carrier Family 1 Member 5) and SLC38A1 (Solute Carrier Family 38 Member 1) (**Table 3**). After entering the cell, Gln is catalyzed by glutaminase (GLS) and broken down into Glu and ammonia in the mitochondria (91). Subsequently, Glu can be converted to α-ketoglutarate (α-KG) that is involved in the oxidative energy supply as an important intermediate for the tricarboxylic acid (TCA) cycle (92). Glu is an indispensable molecule for generating GSH, which can effectively scavenge intracellular ROS. In cancer cells, inhibition of ferroptosis has been shown to be associated with high levels of Gln (93). Although glutaminolysis promotes cancer cell growth, this metabolic process can also induce ferroptosis toward cell death (94). The pivotal role of dihydrolipoamide dehydrogenase (DLD) in prompting ferroptosis induced by cystine deprivation or cystine import inhibition has been recently confirmed. Apart from stimulating DLD to produce hydrogen peroxides, α-KG can be further converted into acetyl-CoA, facilitating fatty acid synthesis and lipid peroxidation-dependent ferroptosis (95). MIR137 (microRNA137) has also been recently identified as a negative regulator of erastin or RSL3-induced ferroptosis through down-regulation of SLC1A5 in melanoma cells (96).

3.2 Ferroptosis and Malignant Brain Tumors

In 2021, the World Health Organization (WHO) released the fifth edition of the Classification of Tumors of the Central

TABLE 3 | The characteristics of ferroptosis-related transport protein associated with malignant brain tumors.

Gene symbol	Alias	Protein name	Subcellular	Substrates	Related Brain Cancer
SLC7A11	xCT	Cystine/glutamate transporter	Plasma membrane	Cystine, Glutamate	Glioblastoma, Neuroblastoma
SLC3A2	4F2hc	4F2 cell-surface antigen heavy chain	Lysosome, Plasma membrane	L-type amino	Glioblastoma, Neuroblastoma
SLC1A5	ASCT2	Neutral amino acid transporter B (0)	Plasma membrane	Glutamine	Glioblastoma
SLC38A1	SNAT1	Sodium-coupled neutral amino acid transporter 1	Plasma membrane	Glutamine	Glioblastoma
SLC11A2	DMT1	Natural resistance associated macrophage protein 2	Plasma membrane, Endosome, Mitochondrion	Fe^{2+}	Glioblastoma
SLC40A1	Fpn1	Solute carrier family 40-member 1	Plasma membrane	Fe^{2+}	Glioblastoma, Neuroblastoma
SLC39A14	ZIP14	Metal cation symporter ZIP14	Plasma membrane	Mn^{2+}, Fe^{2+}, Zn^{2+}	
TFR1	TFRC	Transferrin receptor protein 1	Plasma membrane	Fe^{3+}	Glioblastoma, Neuroblastoma

Nervous System (CNS) (WHO CNS5). Among various brain tumors, childhood brain tumors, adult gliomas and meningiomas are currently the most common brain neoplasia. Neuroglioma is one of the common primary central nervous system tumors that originate from glial cells. GBM is the most malignant and deadliest type of neuroglioma (97). Neuroblastoma is the most common extracranial tumor in children and nearly half of neuroblastoma occurs in infants and young children under 2 years of age (98). Meningiomas are tumors originating from arachnoid cap cells, most of which are benign. However, about 3% meningiomas are malignant, including invasive meningiomas (99). The current treatment methods for malignant brain tumors mainly include surgical resection, radiotherapy and chemotherapy.

Recently increasing numbers of studies have shown that ferroptosis is associated with the pathological process of a variety of neurological diseases, including neurodegenerative diseases, neurotrauma and brain tumors (100). Nevertheless, there has been less research on brain tumors compared to the other types of tumors so far. It is undeniable that ferroptosis, a new form of non-apoptotic cell death, will open up new therapeutic avenues for eliminating brain tumor cells (101).

Soon after ferroptosis was defined, researchers injected iron-containing water into the rats transplanted with glioma-35 cells and then focused on treating the tumor site with radiotherapy (102). They found that the tumor volume in the experimental group was significantly smaller than that in the control group. Mechanistically, in a separate report, it is suggested that iron-containing water treatment before radiation induces glioma cell death through the combination of apoptosis and ferroptosis (103). Furthermore, ferroptosis is proved to be involved in the GBM cell death which can be induced by neutrophils. It appears that this process requires activation signals given by the tumor microenvironment. When mature neutrophils infiltrating into the tumors are activated, they will trigger lipid peroxidation by transferring myeloperoxidase into GBM cells and increase cellular ROS, finally causing tumor cell ferroptosis (104).

Although most ferroptosis-related studies have concentrated on gliomas, neuroblastoma, another malignant brain tumor, is gradually coming into focus. Research suggests that overexpression of Mitochondrial ferritin (FtMt) in dopaminergic neuroblastoma cell line SH-SY5Y cells can significantly inhibit erastin-induced ferroptosis (105). This is mainly due to FtMt-mediated inhibition of cellular labile iron pool (LIP) and the accumulation of cytoplasmic ROS which protects against effects of ferroptosis. In another study with SH-SY5Y, the ferroptosis inhibitor Ferrostatin-1 (Fer-1) was found to have a neuroprotective effect under Rotenone-induced oxidative stress conditions (106).

In a recently published study, researchers evaluated the expression of Merlin/Neurofibromin2 (NF2) and the ferroptosis regulator GPX4 in patients with primary meningioma and found a positive correlation between them. They speculated that the inactivation of NF2 in meningiomas may be more likely to cause ferroptosis. Furthermore, it has been determined that inhibition of NF2 and E-Cadherin can promote ferroptosis-related cytotoxicity and lipid peroxidation in meningioma cell lines. The transcription factor MEF2C has been shown to regulate the transcription of NF2 and E-cadherin genes. Silencing MEF2C, the expression levels of NF2 and E-cadherin in meningiomas decreased, which inhibited the growth of meningiomas mediated by ferroptosis (**Figure 1**). Therefore, MEF2C can be used as a potential molecular target for the treatment of aggressive meningiomas through modulating ferroptosis (107).

3.3 The Role of Transporters Associated With Ferroptosis in Malignant Brain Tumors

Ferroptosis plays a key role in the development of malignant brain tumors. As an important part of ferroptosis, relevant transporters can regulate amino acid metabolism and iron metabolism and are essential for the maintenance of iron homeostasis. Disorders of iron homeostasis in the brain will increase the risk of tumors, which may be one of the factors leading to the increased incidence of brain tumors (108). In addition, a group of ferroptosis-related genes have been discovered that may predict the prognosis of glioma patients based on clinical databases (109). In terms of iron metabolism, CDGSH iron-sulfur domain-containing protein 1 (CISD1) (110), poly r(C) binding protein 1 (PCBP1) (111) and transferrin (TF) (94) have a marked impact on ferroptosis by regulating the cellular content of iron. Here we compared the survival curve of brain tumor patients with the expression of ferroptosis-related genes and the results showed that the decrease in survival rate was related to the high-level expression of the protein required for iron intake (**Figure 2**). These data indicate that a better understanding of the role of ferroptosis-related transporters in malignant brain tumors may help provide more options for the treatment and prevention of brain tumors.

The obvious increase of lipid and cytoplasmic ROS is an important feature of ferroptosis and part of its regulatory factors have been used as small molecule drug targets to induce the death of cancer cells. Fpn1 can inhibit ferroptosis by reducing the accumulation of iron-dependent lipid ROS. Studies have found that in neuroblastoma cells, erastin induces the accumulation of iron and the low expression of Fpn1 involved in iron outflow (112). Furthermore, hepcidin, an amino acid peptide hormone (113) that binds with Fpn1 and stimulates Fpn1 degradation, increases antitumor activity of Erastin. This suggests that Fpn1 can be used as a potential therapeutic target for neuroblastoma in the future and Fpn1 inhibitors may provide a new approach for the treatment of neuroblastoma.

In neuroblastoma, gene amplification of the oncogenic transcription factor MYCN makes tumor cells more malignant and difficult to eliminate. Increased TFR1 expression and decreased Fpn1 expression in MYCN-amplified neuroblastoma cells results in high intracellular iron content. Overexpression of MYCN activates Xc^{-}/GPX4 pathway, resulting in increased intracellular cystine and enhanced antioxidant protection (114). Therefore, the use of system Xc^{-} selective inhibitors or TFR1 agonists to treat MYCN-amplified neuroblastoma will

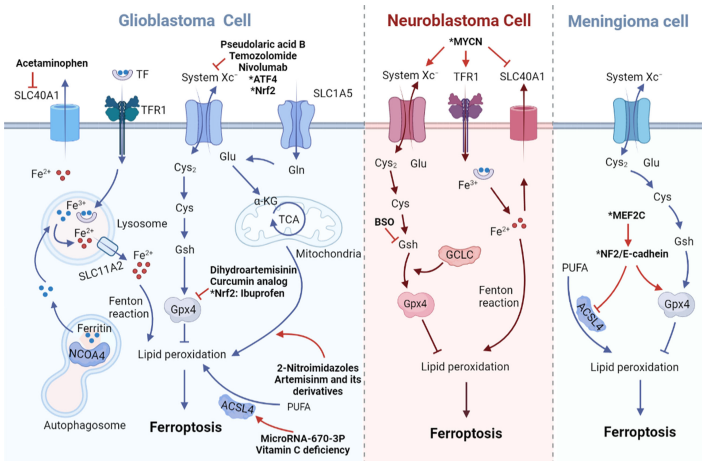

FIGURE 1 | Impacts of ferroptosis-related transport proteins in three malignant brain tumor cells. In GBM cell, iron transport-related proteins DMT1 (SLC11A2), Fpn1 (SLC40A1), TFR1 and amino acid transporters system Xc^- (SLC7A11/SLC3A2), ASCT2 (SLC1A5) regulate the occurrence of ferroptosis all together. In MYCN-amplified neuroblastoma cell, lipid peroxidation and cell death are promoted due to increased expression of TFR1 and System Xc^- and lower expression of Fpn1. In meningioma cell, MEF2C mediated upregulation of NF2 and E-cadherin inhibits Erastin-induced ferroptosis. Arrows indicate promotion and blunt-ended lines indicate inhibition. Cys, cysteine; Cys2, cystine; GSH, glutathione; GPX4, glutathione peroxidase 4; Glu, glutamate; Gln, glutamine; TF, transferrin; TFR1, transferrin receptor 1; PUFA, polyunsaturated fatty acid; ACSL4, acyl-CoA synthetase long-chain family member 4; TCA, tricarboxylic acid cycle; α-KG, α-ketoglutarate; NCOA4, nuclear receptor coactivator 4; ATF4,activating transcription factor 4; Nrf2, nuclear factor erythroid-2-related factor; MYCN, BHLH Transcription Factor; MEF2C, Myocyte Enhancer Factor 2C; NF2, neurofibromatosis type 2; BSO, buthionine sulphoximine.

increase the level of lipid peroxidation and eventually lead to ferroptosis of tumor cells (**Figure 1**).

In addition to neuroblastoma, GSH depletion caused by system Xc^- inhibition is associated with other malignant brain tumors (115). Nuclear factor (erythoid-derived)-like 2(Nrf2) overexpression or Kelch-like ECH associated protein 1(Keap1) knockdown can accelerate the growth of glioblastoma and promote the development of glioma cells (15). Similarly, xCT is positively regulated by Nrf2 and plays a crucial role in the inhibition of ROS accumulation during the ferroptosis process of glioma cells. Drug inhibitors targeting system Xc^- can rescue ROS generation, thereby increasing the sensitivity of glioma cells to ferroptosis and achieving the goal of treating malignant gliomas (**Figure 1**) (15).

The first-line treatment anti-tumor drug Temozolomide can inhibit the growth of glioblastoma. In order to explore the role of ferroptosis in this process, researchers treated human glioblastoma cell line TG905 cells with siRNA and found that knockdown of DMT1 reduced the level of ROS and iron production induced by Temozolomide (116). In addition, down-regulation of DMT1 also increased the expression of GPX4, Nrf2 and HO-1, thereby preventing the occurrence of ferroptosis. Temozolomide induces ferroptosis of some glioblastoma cells by increasing the expression of DMT1, so the divalent metal transporter DMT1 can be used as a drug target in glioblastoma.

4 THERAPEUTIC STRATEGY

Mounting evidence suggests ferroptosis plays a beneficial role in tumors treatment. With the need for new treatments for malignant brain tumors, increased attention has been paid to drugs inducing ferroptosis that designed based on the regulatory pathways of ferroptosis. The main types of malignant brain tumors targeted by the novel Ferroptosis-based include GBM (117), fibrosarcoma (118), head and neck carcinoma (119).

Ferroptosis can be induced by increasing intracellular iron or ROS level (11). Inhibition of the glutathione peroxidase GPx4 or glutamate/cystine antiporter system Xc^- through the drugs is beneficial, promoting ferroptosis though increased ROS accumulation. Nrf2-Keap1 pathway promotes cell proliferation and diminishes ferroptosis (15). Although some studies have reported that inhibiting ferroptosis by activating Nrf2 pathway can play a neuroprotective role, for example, astrocytes protect neurons from ferroptosis by activating the Nrf2 pathway to supply neurons with GSTM2 and other antioxidants, inhibiting Nrf2 pathway in tumor cells to promote ferroptosis plays a therapeutic effect (120). ATF4 and Pseudolaric acid B promotes ferroptosis in a xCT-dependent manner (89, 121). Dihydroartemisinin initiates ferroptosis through GPx4 inhibition (122). Ibuprofen induces ferroptosis *via* downregulation of Nrf2-Keap1 signaling pathway (123).

Other mechanisms of promoting ferroptosis have also been reported, including activating the transcription factor BACH1 (BTB domain and CNC homology 1) (124) or Nox4 (121) to promote oxidative stress, inhibition of autophagy (125), vitamin C deficiency to reduce proliferation (126) and targeting ACSL4 which suppresses proliferation (127). Based on these mechanisms, related drugs have been found, such as 2-Nitroimidazoles, temozolomide, artemisinin and its derivatives.

Ferroptosis inducers may expand our arsenal of frontline therapeutic agents for combinatory approaches. Temozolomide toxicity operates is boost by ferroptosis (128). Androgen receptor

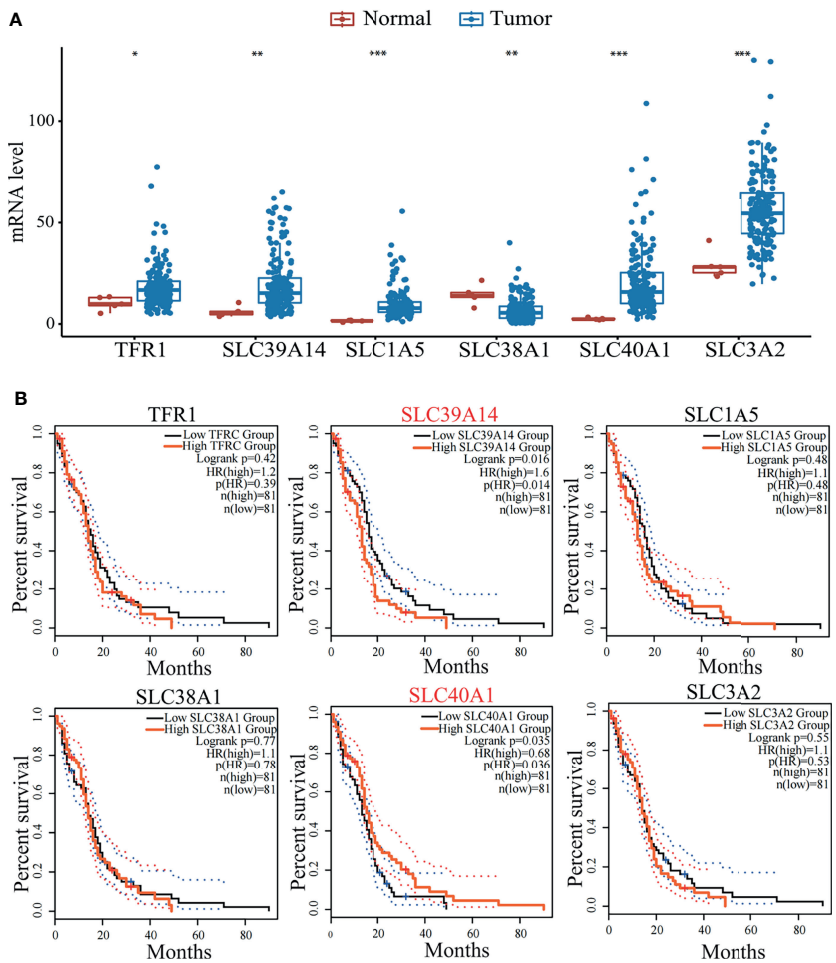

FIGURE 2 | **(A)** Expression level of transporters (TFR1, SLC39A14, SLC1A5, SLC38A1, SLC40A1, SLC3A2) in tumor patients and normal people. Data mined from TCGA (https://cancergenome.nih.gov/). *p < 0.05, **p < 0.01, ***p < 0.001, compared with tumor patient group. **(B)** Survival curves of GBM patients mined from GEPIA2 (http://gepia2.cancer-pku.cn/). GBM patients were stratified into high or low expression groups based on the expression level of transporters (TFR1, SLC39A14, SLC1A5, SLC38A1, SLC40A1, SLC3A2) of patients. p<0.05 in Log-rank test. OS, overall survival in months.

(AR) ubiquitination is induced by the curcumin analog which suppresses growth of temozolomide-resistant GBM through disruption of GPX4-mediated redox homeostasis (129). Furthermore, T cell-promoted tumor ferroptosis is an anti-tumor mechanism, and targeting this pathway in combination with immunotherapy is another potential therapeutic approach (91, 130, 131). Nivolumab therapy revealed that clinical benefits correlate with reduced expression of SLC3A2 and increased IFNγ and CD8 (91).

Although many anticancer compounds that promote ferroptosis have been found, there are still many treasures to be discovered. Drugs targeting other mechanisms of ferroptosis need to be explored, such as targeted iron accumulation. A systematic assessment of the relationship between ferroptosis related genes (FRGs) expression profiles and the occurrence and development of tumors based on the Cancer Genome Atlas (TCGA), Chinese Glioma Genome Atlas (CGGA) datasets and FerrDb datasets may unveil new targets (77, 132). In fact, the potential impact of Acetaminophen in ferroptosis through interaction with CD44, HSPB1, and SLC40A1 was found this way (132).

To find out the potential correlation of GBM with transporters involved in ferroptosis. Here we compared the expression of several ferroptosis related transporters (SLC7A11, SLC3A2, SLC1A5, SLC38A1, SLC11A2, SLC40A1, SLC39A14, TFR1, TF) in normal people and GBM patients based on TCGA data. It is worth mentioning that the differentially expressed genes (DEGs) covered the majority of the transporters that we screened related to ferroptosis. Box-plot shows the expressions of SLC3A2, SLC1A5, SLC40A1, SLC39A14 and TFR1 increased significantly, the expression of SLC38A1 decreased significantly (**Figure 2A**). The effect of DEGs on the survival curve of GBM patients was further explored based on TCGA data (**Figure 2B**). As shown in the Kaplan-Meier survival curve, median survival of GBM patients changed significantly according to the expression of SLC39A14 (p = 0.016) and

SLC40A1 ($p = 0.035$), but the mechanism behind it remains to be explored. The above analysis further proves the potential of targeting these transporters and ferroptosis in the treatment of GBM.

Ferroptosis induction may prove as an effective therapeutic strategy against malignant brain tumors, yet a wide range of ferroptosis inducers are prone to off-target effects and may cause significant damage to normal cells. Therefore, it is urgent to develop tumor targeting delivery strategies of ferroptosis inducers. At present, many research are focusing on this aspect. Class I histone deacetylase (HDAC) inhibitors can selectively inhibit ferroptosis in neurons, but promote ferroptosis in tumor cells, which may be due to its different epigenetic regulation on the two cells. The combination of HDAC inhibitors and ferroptosis inducers can not only reduce the dosage of ferroptosis inducers to reduce toxicity, but also protect neurons (133, 134). Nano-targeting of WA allows systemic application and suppressed tumor growth due to an enhanced accumulation at the tumor site (135, 136).

At present, the treatment strategy targeting ferroptosis has been widely studied in various tumors, among which the advanced treatment strategy can potentially use for malignant brain tumors as well. Some new therapeutic mechanisms are worth learning. For example, gene interference by transferring genes with adeno-associated virus and iron nanoparticles enhance ferroptosis and inhibit tumor growth (137); ferroptosis inducer erastin or rsl3 is used independently or in combination with standard-of-care second-generation for the treatment of advanced prostate cancer (138); and activating ferroptosis by sequestering iron in lysosomes kills cancer stem cells (139). Studies have showed that targeted ferroptosis can used to overcome drug resistance of tumors. For example, Vorinostat promotes ferroptosis to overcome the resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) (140); and artesunate inhibits growth of therapy-resistant renal cell carcinoma through induction of ferroptosis (141). Some advanced strategies are for targeted therapy. For example, Photodynamic therapy site-specifically produces reactive oxygen species for the Fenton reaction, which promotes ferroptosis and suppresses tumors (142); and catalytic nanomedicine that contains natural glucose oxidase and ultrasmall Fe_3O_4 nanoparticles selectively and effectively strengthens ferroptosis of tumor cells (143). In short, the essence can be drawn from the treatment of other tumors and used in the treatment of malignant brain tumors.

Inducing ferroptosis of tumor cells is a newly discovered strategy for the treatment of malignant brain tumors, but many problems remain to be solved, including elucidating the mechanism of ferroptosis in different malignant brain tumors, discovering new therapeutic targets for inducing ferroptosis of tumor cells, and increasing the tumor cell targeting of ferroptosis inducers. It is worth noting that the regulation of iron transport in tumor cells and the expression of transporters related to ferroptosis may have good therapeutic potential. Many transporters have become drug targets in recent years (144, 145). At the same time, clarifying iron transport under physiological conditions also provides an important research basis for targeted therapy of tumor cells, crucial to avoid the damage of normal tissues through off target effects.

AUTHOR CONTRIBUTIONS

LC proposed the research. JZ and YW both reviewed the literature and collected references. JZ, YW, and LT wrote the manuscript and finalized the paper. All authors contributed to the article and approved the submitted manuscript.

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LncRNA *PELATON*, a Ferroptosis Suppressor and Prognositic Signature for GBM

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PELATON is a long noncoding RNA also known as long intergenic nonprotein coding RNA 1272 (*LINC01272*). The known reports showed that *PELATON* functions as an onco-lncRNA or a suppressor lncRNA by suppressing miRNA in colorectal cancer, gastric cancer and lung cancer. In this study, we first found that *PELATON*, as an onco-lncRNA, alleviates the ferroptosis driven by mutant *p53* and promotes mutant *p53*-mediated GBM proliferation. We also first confirmed that *PELATON* is a new ferroptosis suppressor lncRNA that functions as a ferroptosis inhibitor mainly by mutant *P53* mediating the ROS ferroptosis pathway, which inhibits the production of ROS, reduces the levels of divalent iron ions, promotes the expression of *SLC7A11*, and inhibits the expression of *ACSL4* and *COX2*.*PELATON* can inhibit the expression of *p53* in *p53* wild-type GBM cells and regulate the expression of *BACH1* and *CD44*, but it has no effect on *p53*, *BACH1* and *CD44* in *p53* mutant GBM cells. *PELATON* and *p53* can form a complex through the RNA binding protein EIF4A3. Knockdown of *PELATON* resulted in smaller mitochondria, increased mitochondrial membrane density, and enhanced sensitivity to ferroptosis inducers to inhibit GBM cell proliferation and invasion. In addition, we established a favourite prognostic model with *NCOA4* and *PELATON*. *PELATON* is a promising target for the prognosis and treatment of GBM.

Keywords: lncRNA, ferroptosis, *PELATON*, *LINC01272*, ROS

Abbreviations: FPI: ferroptosis potential index; GBM: Glioblastoma; PCD: programmed cell death; GPX4: glutathione peroxidase; ROS: reactive oxygen species; NCOA4: Nuclear Receptor Coactivator 4; TMZ: temozolomide; lncRNAs: Long non-coding RNAs; ACSL4: Acyl-CoA Synthetase Long Chain Family Member 4; COX2: Prostaglandin-Endoperoxide Synthase 2; SLC7A11: Solute Carrier Family 7 Member 11; MM: module membership; GS: gene significance; KEGG: Kyoto Encyclopedia of Genes and Genomes; OS: overall survival; IDH: Isocitrate Dehydrogenase; FPS: free-progression survival; GO: Gene Ontology; GSEA: Gene Set Enrichment Analysis; TCGA: The Cancer Genome Atlas; ES: enrichment score; ssGSEA: single-sample gene set enrichment analysis; TOM: topological overlap matrix.

INTRODUCTION

Glioblastoma (GBM) is the most common malignant brain tumour of the central nervous system, accounting for approximately 45% of central nervous system tumours, with an annual incidence of 3.19 cases/100000 people (1–3). Recent studies have shown that the main factors affecting the prognosis of glioblastoma patients include the degree of surgical resection of the tumour tissue and the molecular classification of the tumour. With improvements in surgical accuracy and progress in tumour imaging, it is easier to distinguish glioblastoma from normal brain tissue and maximize the removal of tumour tissue. However, due to the invasive growth of glioblastoma, tumour cells often infiltrate normal brain tissue, resulting in treatment failure and recurrence (4). At present, the conventional treatment of glioblastoma patients mainly includes optimal and safe surgical resection of tumour tissue, followed by adjuvant radiotherapy and chemotherapy (5, 6). An increasing number of studies are exploring targeted and personalized therapies for glioblastoma, such as targeting DNA repair, tumour growth, apoptosis, invasion, and angiogenesis and overcoming resistance to chemotherapeutic drugs, including temozolomide (7–10). Despite this, recurrence and drug resistance of glioblastoma are still common, and recurrent tumour cells grow faster and more aggressively. In the past decade, the poor prognosis of patients with glioblastoma has not improved significantly, and the overall median survival time remains at 16-18 months (11). Therefore, according to the pathogenesis of glioblastoma, identifying new therapeutic targets and developing effective alternative clinical therapies are still urgent problems to be solved.

Ferroptosis was first proposed by Dr. Brent R. Stockwell in 2012 as an iron-dependent programmed cell death (PCD), which is different from autophagy, apoptosis, and necrosis (12–14). The process involves high levels of iron ions, accumulation of reactive oxygen species, changes in mitochondrial morphology and lipid peroxide metabolism genes (15–17). Ferroptosis is characterized by the depletion of glutathione and a decrease in glutathione peroxidase (GPX4) activity. As a result, lipid oxides cannot be metabolized by the GPX4-catalyzed glutathione reductase reaction, and bivalent iron ions oxidize lipids to produce reactive oxygen species (ROS) (13, 18, 19). Related studies have shown that, as a new mechanism of cell death, ferroptosis may be involved in the development of disorders such as cancer, neurodegenerative diseases, inflammatory diseases, cardiovascular diseases, and T cell immunity (14, 20, 21). One of the reasons for the high degree of malignancy and drug resistance of glioblastoma is that these tumours can effectively escape ferroptosis (22). The induction of glioblastoma ferroptosis molecules or the synthesis of small molecule drugs and nanomaterials provides new ideas for the treatment of glioblastoma (23–28). For example, loss of COPI coat complex subunit zeta 1 induces nuclear receptor coactivator 4 (NCOA4)-mediated autophagy and ferroptosis in glioblastoma cells (29). The curcumin analogues ALZ003 and quinkalim can lead to ferroptosis in glioma cells, thus opening new avenues for the treatment of temozolomide (TMZ)-resistant glioblastoma (30, 31). Iron oxide nanoparticles are safe and effective ferroptosis and apoptosis inducers and can be used as a combination therapy for glioblastoma (32, 33).

Long noncoding RNAs (lncRNAs) may promote or suppress the occurrence and development of tumours (34). They are involved in tumour invasion and metastasis, apoptosis, proliferation, drug resistance, and angiogenesis and regulate the expression of target genes at the transcriptional and posttranscriptional levels. An increasing number of studies have shown the important role of lncRNAs in the regulation of ferroptosis in cancer, but only a few have focused on GBM (23). At present, many reports have established the prognosis model of ferroptosis related genes in cancer including GBM by screening the differentially expressed ferroptosis related genes in the database and other bioinformatics analysis, so as to evaluate the tumour immune microenvironment and immune cell infiltration, which has good predictive value for the survival and immunotherapy of tumour patients (35, 36).Therefore, it is still urgent to further explore and study new molecules in GBM ferroptosis, so as to provide guidance for the clinical treatment of GBM.

In this study, we obtained 13 known ferroptosis mRNAs and 12 unreported ferroptosis lncRNAs, found that LncRNA *PELATON* and NCOA4 were prognostic ferroptosis genes, and constructed a favourite ferroptosis risk model for GBM. We also found that *PELATON* was mainly involved in the ROS ferroptosis pathway by mutant *p53*, and in *p53* mutant-type GBM cells, it suppressed the expression of ferroptosis driver genes and promoted the expression of ferroptosis suppressor genes. *PELATON* is a novel ferroptosis suppressor. Knockdown of *PELATON* promoted the production of ROS and the levels of divalent iron ions, the mitochondria decreased, the cell membrane density increased, and GBM cells displayed proliferation inhibition.

MATERIALS AND METHODS

Collection of GBM Datasets

Based on The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/), we performed transcriptome profiling by next-generation sequencing and obtained the corresponding clinical information of the GBM set. The GSE43378 (GPL570) dataset was obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), which contains gene expression and clinical data of GBM.

WGCNA Analysis

A cluster dendrogram of the genes was constructed to check for outliers using the hclust function. After removing the outlier genes, the R package "weighted gene co-expression network analysis" ("WGCNA") was used to establish the co-expression network of highly expressed genes (37). In our study, we used the pick Soft Threshold function to determine the soft-thresholding powers β over R2. Using the value of β for which the value of R2 is maximum with the transformed gene expression matrix, we constructed the adjacency matrix and topological overlap matrix (TOM). For the construction of the module, a dendrogram of

genes was constructed with a dissTOM matrix using the hclust function with different colours. Based on the TOM dissimilarity measurements, we established an average hierarchical linkage clustering. Module dendrograms were built by setting the minimum genome to 30, and highly similar modules were merged by setting a cutoff of < 0.25. The dissimilarity of the module eigengenes was calculated using the module eigengenes function. The association between eigenvalues and FPI was assessed using Pearson's correlation.

Ferroptosis Potential Index (FPI)

The FPI was calculated according to the method of Liu Z et al. (38). We assessed the ferroptosis level, which was established based on the expression data for genes positively or negatively regulating ferroptosis. The enrichment score (ES) for a gene set that positively or negatively regulated ferroptosis was calculated using single-sample gene set enrichment analysis (ssGSEA) in the R package 'GSVA' (39), and the normalized differences between the ES of the positive components and negative components were defined as the FPI to computationally dissect the ferroptosis levels/trends in the tissue samples.

GEPIA Analysis

Differentially expressed genes, OS, and FPS were integrated using Gene Expression Profiling Interactive Analysis 2 (GEPIA2, http://gepia2.cancer-pku.cn/) (40). We identified the differentially expressed genes with |log2FC| values > 1 and q values < 0.05 using LIMMA. OS and DFS were evaluated using the Kaplan–Meier method with the median cutoff and compared using the log-rank test.

Enrichment Analysis

We utilized the "clusterprofiler" package to conduct Gene Set Enrichment Analysis (GSEA) analysis for GO enrichment and KEGG (41). KEGG pathway analysis was performed on ferroptosis genes using the R package "clusterprofiler" (41). Meanwhile, adjusted p<0.05 was regarded as statistically significant.

Antibodies and Reagents

The reagents, chemicals, and antibodies used in this study were as follows: SLC7A11 (Abcam, ab175186, Massachusetts, US), COX2 (Abcam, ab179800, Massachusetts, US), GPX4 (Abcam, ab125066, US),ACSL4 (Abcam, ab155282, Massachusetts, US), BACH1 (Abcam, ab180853, Massachusetts, US), CD44 (Abcam, ab243894,Massachusetts,US), P53(Proteintech, CatNo.60283-2-Ig, China), GAPDH (Proteintech, 60004-1-Ig, Wuhan, China), DMSO (MP Biomedicals, 19605580, California, USA), and erastin (MedChemExpress, HY-15763, Shanghai, China).

Tissue Collection, Glioblastoma Cell Lines and Primary Cell Culture

These procedures were performed as previously described in detail in our previous study (42, 43). Human clinical sample and data were collected from the Department of Neurosurgery, Central South University. All human experiments were performed in accordance with the Declaration of Helsinki and approved by the Joint Ethics Committee of the Central South University Health Authority. All subjects provided informed written consent. Primary tumour samples were minced about lmm3 with a GentleMACS Dissociator (Miltenyi Biotec). The cells were digested with trypsin and incubated at 37° for 10 minutes, then tissue suspension was filtered through the filter screen(Jet Biofil) to remove the undigested tissue residue, and centrifuged at 800 rpm for 5 ~ 8 minutes, Cells were cultured in DMEM/F12 containing 10% FBS, 5% CO2 and 37°C. Primary tumour cells were tested by GFAP, nestin, and CD133 staining and subcutaneous implantation in nude mice.

Cell Transfection Assay

Cells with approximately 80% confluence were transiently transfected with 3.1- or 3.1-*PELATON* Plasmid, *PELATON*-siRNA ([226] 5′-GCAGCACAGUCACAUCCUATT-3′, [342] 5′-GCGCCUGUCCAGGACAAGUTT-3′, and [478] 5′-GCACAGAAGUCUCUUCCCUTT-3′). siRNAs were synthesized by RiboBio (Guangzhou, China). Cell transfection was performed using Lipofectamine 3000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

RT–qPCR

Total RNA was extracted from cells using TRI Reagent (Molecular Research Center, TR118, Cincinnati, OH 45212, USA), and its concentration and purity were determined using a Nanodrop2000 microultraviolet spectrophotometer. The extracted RNA was reverse transcribed into cDNA using the RevertAid RT Reverse Transcription Kit (Thermo Scientific, K1691, USA) according to the instructions of the manufacturer, and qPCR was carried out on a real-time fluorescence quantitative instrument (Bio–Rad, 788BR06968, USA). The gene-specific primers used are as follows:

PELATON Forward:5 ′ACAAAGATGAGACGCAGGCT 3′;
PELATON Reverse:5 ′GTTAAGGGCCCGGGAATCTG 3′;
SLC7A11 Forward: 5′GGACAAGAAACCCAGGTGGT 3′;
SLC7A11 Reverse: 5′GCAGATTGCCAAGATCTCAAGT 3′;
COX2 Forward:5 ′CTATCCTGCCCGCCATCATC 3′;
COX2 Reverse: 5 ′GGGATCGTTGACCTCGTCTG 3′;
GPX4 Forward: 5′AGATCCAACCCAAGGGCAAG 3′;
GPX4 Reverse: 5′GGAGAGACGGTGTCCAAACT 3′;
ACSL4 Forward: 5′GCCCCTCCGATTGAAATCAC 3′;
ACSL4 Reverse:5 ′AGCCGACAATAAAGTACGCAA 3′;
BACH1 Forward: 5′ CGCCTCAGCTCTGGTTGAT 3′;
BACH1 Reverse: 5′ ATCAGCCTGGCCTACGATTC 3′;
CD44 Forward: 5′ AGTCACAGACCTGCCCAATG3′;
CD44 Reverse: 5′ TTGCCTCTTGGTTGCTGTCT3′;
GAPDH Forward: 5 ′GAATGGGCAGCCGTTAGGAA 3′;
GAPDH Reverse: 5′AAAAGCATCACCCGGAGGAG 3′;

GAPDH was used as an internal control. The relative transcriptional levels of the target genes were calculated using the $2^{-\triangle\triangle CT}$ method. Datas were mean ± SEM for three independent experiments.

Western Blot Analysis

Cells were lysed in RIPA buffer (Beyotime, Shanghai, China) for 30 min and centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were collected. The protein concentration was determined using the BCA method (Thermo Scientific, 23222, USA). The proteins were separated by SDS–PAGE and transferred to a polyvinylidene fluoride membrane (Merck Millipore, ISEQ00010, USA). The PVDF membrane was incubated for 1 h in 5% skim milk powder at room temperature and then incubated with the corresponding anti-antibody overnight at 4°C. After washing thrice for 10 min with PBST, the membrane was incubated with the secondary antibody at 37°C for 1 h. The protein bands were visualized using enhanced chemiluminescence reagents (Abbkine, Wuhan, China, BMU102-CN). The ChemiDoc imaging system (Bio–Rad, USA) was used to capture the images and quantify the intensity of the protein fragments.

Coimmunoprecipitation and RNA-Binding Protein Immunoprecipitation Assay

Cells were extracted with lysis buffer, and the supernatants were incubated with the indicated antibodies for 1 h at 4°C. Then, the samples were precipitated with agarose beads for 1 h at 4°C. The immunocomplexes were washed from agarose beads with Poly FLAG Peptide and then subjected to the second co-IP with the indicated antibodies and agarose beads. The final retrieved protein was detected by Western blotting. The coprecipitated RNAs were detected by RT–qPCR.

Transmission Electron Microscopy

These procedures have been previously described in detail (42).

Transwell Assay

The glioma cell suspension (1×10^6 cell/ml, 100 μL) was added to the transwell chamber covered with Matrigel (Corning, 256234, USA), and 600 μl medium containing 15% FBS was added to the 24-well subplate chamber. The transwell chamber was removed after 48 h of culture and fixed with 4% formaldehyde for 30 min. The cells were stained with 0.1% crystal violet and washed thrice with PBS. Five microscope fields were photographed for each group, and the cell numbers were counted using ImageJ software. The experiment was repeated three times.

Wound-Healing Assay

The glioma cells were inoculated into a 6-well plate and transfected for 48 h. A 2 mm width scratch was made in the middle of the tissue culture plate and cultured for another 48h. Photographs were taken at certain time points, and the scratch healing rate was calculated using ImageJ software. Datas were mean ± SEM for three independent experiments.

Detection of Intracellular ROS Levels

To calculate the production of intracellular ROS, a reactive oxygen species detection kit (Biosharp, Shanghai, China) was used. First, the ROS probe H2DCFH-DA was diluted to 10 μM in serum-free culture medium, and 1 ml H2DCFH-DA working solution was added to each well at 37°C in the dark for 30 min. Then, the cells were washed with serum-free medium 3 times to fully remove H2DCFH-DA that did not enter the cells. Finally, the cells were observed under a fluorescence microscope and photographed.

Iron Ion Detection

After protein extraction, the protein concentration was determined using the BCA method (Thermo Scientific, 23222, USA). Iron levels in the samples were determined using an iron ion detection kit (Leagene, Beijing, China) according to the manufacturer's instructions. The corresponding reagents were added in turn and mixed gently at 37°C for 10 min, and the absorbance of the detection well was measured at 562 nm. Finally, the plasma and serum Fe (μM/L) were measured as follows: [Fe]= [A determination-(A serum blank × 0.970)]/A standard × 35.8.

Fluorescence *In Situ* Hybridization (FISH)

Paraffin sections of glioma and normal brain tissues were baked at 42°C for 2 h, dewaxed with xylene, dehydrated in graded ethanol solutions (100%-95%-80%-50%-30%) for 5 min, treated with DEPC water for 2×5 min, and washed with PBS (pH 7.4) for 2 × 5 min. Afterwards, the sections were treated with 0.3% Triton X-100 for 15 min to permeabilize the membranes and washed with PBS for 2×5 min. Subsequently, the sections were digested with RNase-free protease K (20 g/ml) at 37°C for 20 min and washed with 100 mM Gly/PBS and PBS. Then, 4% paraformaldehyde (4°C) was added for 5 min to fix the samples. Triethanolamine buffer (100 mM, pH 8.0) containing 0.25% (w/v) acetic anhydride was discharged for 15 min and washed with PBS. Then, the following steps were performed using an *in situ* hybridization detection kit from RiboBio (Guangzhou, China) according to the instructions of the manufacturer to avoid light in the whole process, and glioma cells were used as in the RiboBio FISH kit. The sections were analysed using a confocal microscope.

Statistical Analysis

The most significant ferroptosis gene signatures associated with the OS of patients with GBM were identified using the Lasso-penalized Cox regression model (44). We set 10-fold cross-validation as the criterion to prevent overfitting with the penalty parameter lambda. Then, we used the time-dependent receiver operating characteristic (ROC) curve and the area under the curve (AUC) to identify the prognostic accuracy of the two-gene signature model in the discovery set and internal set with the package "survival ROC" (45). To separate patients into high-risk and low-risk score groups, we set the median risk score as the cutoff value and then used Kaplan–Meier survival analysis and the log-rank test to evaluate differences in OS between the two

groups. The nomogram was established based on the "regplot" package.

SPSS 21.0 (IBM Corp., Armonk, NY, USA) and Prism 7.0 were used for statistical analysis. Statistical analysis was performed using the t test and analysis of variance. Statistical significance was set at $p < 0.05$. The measured data were expressed as "mean ± SEM". A single factor analysis of variance (ANOVA) was used for comparison among the groups. Datas were mean ± SEM for three independent experiments.

RESULTS

Identification of Ferroptosis LncRNAs in GBM

To identify the ferroptosis genes of GBM, WGCNA analysis was used to identify the key module correlated with ferroptosis in GBM. Based on mRNAs found to be expressed at high levels (average expression of FPKM >0.5) in the TCGA cohort, which includes 18293 genes, 25 co-expression modules were constructed (**Supplementary Figures 1A-D**) (37), in which the red module containing 1049 genes showed the highest correlation with ferroptosis (**Figures 1A, B**). There was a highly significant correlation between the module membership (MM) of the red module and FPI gene significance (GS) (**Figure 1C**) (38). In addition, we used a two-sided hypergeometric test to find 12 ferroptosis driver sets (FDR=7.32eE-06) and 8 suppressor sets (FDR=0.000345) in the red module (**Supplementary Figures 1E, F**) (46). Furthermore, the differentially expressed gene (DEG) analysis indicated that 58% (610/1049) of the ferroptosis genes of the red module were differentially expressed in GBM (**Supplementary Figure 1G**). We obtained 13 known ferroptosis mRNAs and 12 unreported ferroptosis lncRNAs from 610 DEGs (**Figure 1 D** and **Table 1**).

The Favourite Ferroptosis Risk Model for GBM

To determine whether the above 25 ferroptosis genes are associated with the clinical prognosis of patients with GBM, we used survival coxph function to perform univariable Cox proportional hazard regression on the TCGA cohort. Then, by a single factor test followed by Lasso regression analysis, two prognostic ferroptosis

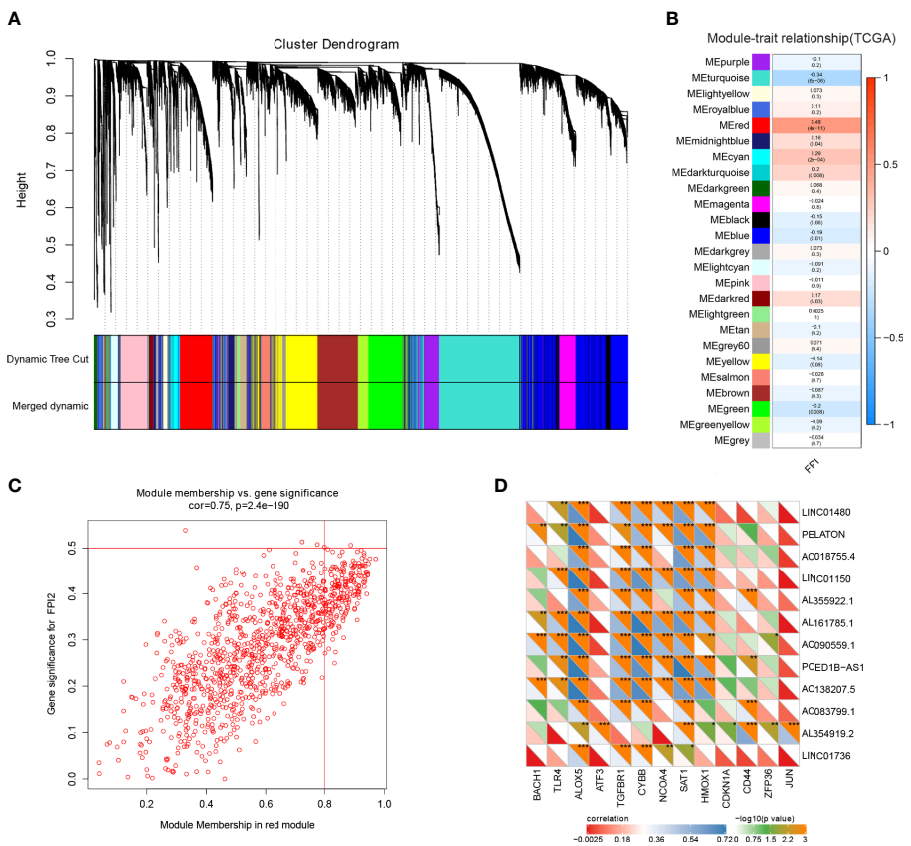

FIGURE 1 | Ferroptosis gene identification in GBM. **(A)** Dendrogram of all highly expressed genes clustered based on a dissimilarity measure (1-TOM) together with assigned module colours in the GBM cohort of TCGA. **(B)** Heatmap of the correlation between module genes and FPI in GBM. Each range contains the Pearson correlation coefficient and P value. **(C)** Significant correlation between the module membership of the red module and FPI gene significance. **(D)** Identification and correlation of 13 known ferroptosis-associated mRNAs and 12 unreported ferroptosis lncRNAs by Pearson correlation analysis from 610 DEGs (*$p < 0.05$; **$p < 0.001$; ***$p < 0.001$).

TABLE 1 | Screening of 12 LncRNAs and 13 mRNAs associated with Ferroptosis.

RNA	Gene abbreviation	Full name	NCBI Entrez Gene	Ensembl
LncRNA	LINC01480	Long Intergenic Non-Protein Coding RNA 1480	101927931	ENSG00000270164
	PELATON	Plaque Enriched LncRNA In Atherosclerotic And Inflammatory Bowel Macrophage Regulation	100506115	ENSG00000224397
	AC018755.4	NA	NA	ENSG00000273837
	LINC01150	Long Intergenic Non-Protein Coding RNA 1150	101927624	ENSG00000229671
	AL355922.1	NA	NA	ENSG00000136315
	AL161785.1	NA	NA	ENSG00000224307
	AC090559.1	NA	NA	ENSG00000255197
	PCED1B-AS1	PCED1B Antisense RNA 1	100233209	ENSG00000247774
	AC138207.5	NA	NA	ENSG00000265743
	AC083799.1	NA	NA	ENSG00000203644
	AL354919.2	NA	NA	ENSG00000254545
	LINC01736	Long Intergenic Non-Protein Coding RNA 1736	101927532	ENSG00000228058
mRNA	BACH1	BTB Domain And CNC Homolog 1	571	ENSG00000156273
	TLR4	Toll Like Receptor 4	7099	ENSG00000136869
	ALOX5	Arachidonate 5-Lipoxygenase	240	ENSG00000012779
	ATF3	Activating Transcription Factor 3	467	ENSG00000162772
	TGFBR1	Transforming Growth Factor Beta Receptor 1	7046	ENSG00000106799
	CYBB	Cytochrome B-245 Beta Chain	1536	ENSG00000165168
	NCOA4	Nuclear Receptor Coactivator 4	8031	ENSG00000266412
	SAT1	Spermidine/Spermine N1-Acetyltransferase 1	6303	ENSG00000130066
	HMOX1	Heme Oxygenase 1	3162	ENSG00000100292
	CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	1026	ENSG00000124762
	CD44	CD44 Molecule (Indian Blood Group)	960	ENSG00000026508
	ZFP36	ZFP36 Ring Finger Protein	738	ENSG00000128016
	JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	3725	ENSG00000177606

genes were identified: LncRNA *PELATON* and *NCOA4* (**Supplementary Figures 2A–C**). Combining the regression coefficients with gene expression values, a risk score formula was created as follows: risk score = -0.69641**NCOA4*+ 0.35167**PELATON*.

To evaluate the predictive ability of the ferroptosis risk model with *NCOA4* and LncRNA *PELATON* for patients with GBM, we performed Kaplan–Meier survival and time-dependent ROC analysis in the discovery set of TCGA (n=161) and the internal set of GSE43378 (n=50). In the discovery set, the higher the risk score (**Figure 2A**), the greater the number of deaths (**Figure 2B**), and the lower the survival rate of patients with GBM (**Figure 2C**), the predictive accuracy of the signature was 0.70, 0.74 and 0.75 at 1, 3, and 5 years, respectively (**Figure 2D**). We obtained consistent results in the internal set (**Supplementary Figures 2D–G**).

To develop a clinically applicable tool that can easily assess the prognosis of patients with GBM, we established a graphical nomogram. The nomogram was based on the discovery set for predicting overall survival (OS). The independent prognostic factors were age, sex, original subtype, isocitrate dehydrogenase (IDH) status, and ferroptosis risk score. A nomogram capable of predicting the OS probabilities of GBM at 1, 2 and 3 years was constructed (**Figure 2E**). The calibration curves at 1, 2 and 3 years showed good consistency between actual observation and prediction by the nomogram (**Figure 2F**).

PELATON in the ROS-Mediated Ferroptosis Pathway by Mutant *p53*

To reveal the effects of *PELATON* on GBM progression, we performed Gene Ontology (GO) and KEGG analyses by Gene Set Enrichment Analysis (GSEA) on RNA-seq data from the TCGA cohort in GBM. Both GO and KEGG analyses suggested that reactive oxygen species (ROS) biosynthesis was mainly in response to the ferroptosis pathway involved by *PELATON* (**Figure 3A** and **Supplementary Figures 3A–C**). To identify which is likely the most important molecule of ROS biosynthesis involved in the ferroptosis suppressor *PELATON*, we analysed the top 20 genes that are differentially expressed between GBM and normal brain tissue (**Figure 3B**) and then wanted to determine which of these is most commonly mutated or overexpressed in human GBM, which revealed *P53*, *RYR2* and *IDH1* at the top of this analysis, with a mutation rate of *p53* up to 30% (**Figure 3C**).

Since wild-type *p53* is a tumour suppressor gene that mainly acts as a transcription factor and prevents oncogenesis, its coding gene *p53* is highly mutated, and its activity is almost abrogated in ~50% of human cancers (47). Combining **Figure 5C**, next, we mainly focused on the *p53* mutant-type GBM cells to explore the function of *PELATON*. By hTFtarget database analysis, we determined that *P53* regulates ferroptosis-related target genes, such as the ferroptosis suppressor genes *SLC7A11*, *GPX4*, and *CD44* and the ferroptosis driver genes *ACSL4* and *BACH1*. Then, pcDNA3.1-*PELATON* was transfected into GBM U251 cells, which is a *p53* mutant-type GBM cell line with lower levels of *PELATON* expression (**Supplementary Figure 3D**), and *PELATON* was knocked down in PG-3 cells, which are primary cultured *p53* mutant-type GBM cells with high levels of *PELATON* expression (**Supplementary Figure 3E**). We found that the overexpression of *PELATON* inhibited *ACSL4* expression and promoted *SLC7A11* expression in U251 cells

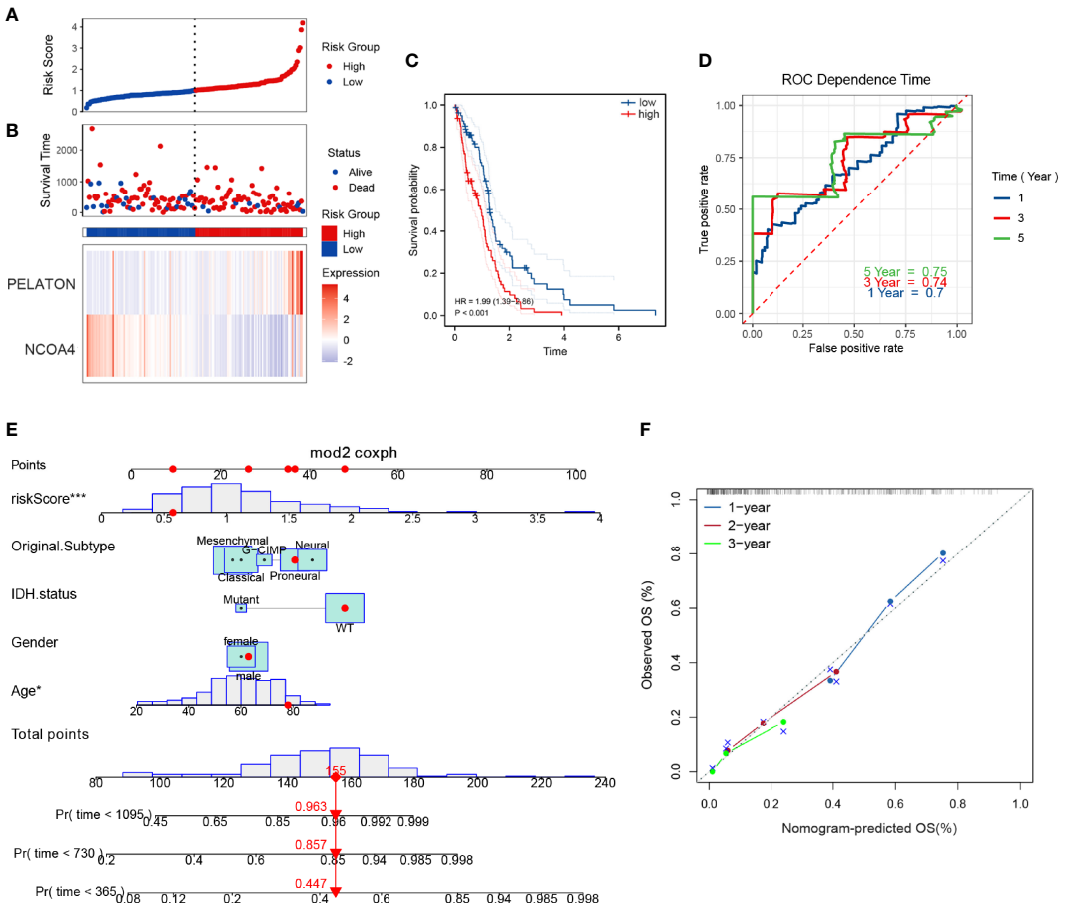

FIGURE 2 | Ferroptosis risk prognosis model with *NCOA4* and *PELATON*. The distribution of risk factors **(A, B)**, Kaplan–Meier survival analysis **(C)**, and time-dependent ROC curves at 1, 3, and 5 years **(D)** between patients at high and low risk based on the *NCOA4* and *PELATON* prognostic models in the TCGA discovery set. **(E)** Nomogram integrating the ferroptosis risk score, age, sex, original subtype and IDH status. **(F)** Calibration curve for predicting OS at 1, 2 and 3 years.

(**Figures 3D, F**), whereas knockdown of *PELATON* promoted *ACSL4* expression and inhibited *SLC7A11* expression in PG-3 cells (**Figures 3E, G**). In wild-type *p53* primary cultured GBM PG-1 cells, *PELATON* inhibited the expression of *BACH1* and *CD44* (**Supplementary Figures 3F, H**), but *PELATON* had no effect on the expression of *GPX4*, *BACH1* and *CD44* in the *p53* mutant GBM cells, such as PG-3 and PG-2 (**Figures 3D–G** and **Supplementary Figures 3G, I**), suggesting that the mutant site of *P53* may affect the binding of *P53* and target genes. In addition, in mutant *p53* GBM cells, we also found that the overexpression of *PELATON* inhibited the expression of the ferroptosis-driven gene *COX2* (**Figures 3D, F**), and knockdown of *PELATON* promoted the expression of *COX2* (**Figures 3E, G**).

Bioinformatics correlation analysis showed that *PELATON* was negatively correlated with *p53* (**Supplementary Figure 4A**), and wild-type or mutant *P53* in GBM patients did not affect *PELATON* expression (**Supplementary Figure 4B**). Further results showed that *PELATON* inhibited the expression of wild-type *p53* in GBM PG-1 cells but had no effect on mutant *p53* in GBM PG-2 and PG-3 cells (**Supplementary Figures 3H, I**). In wild-type *p53* GBM PG-1 cells, simultaneous overexpression of *PELATON* and *P53* inhibited *PELATON*'s regulation of *BACH1* and *CD44* (**Supplementary Figure 4C**). Further research and bioinformatics prediction found that *PELATON* and *P53* can form a complex through the RNA-binding protein EIF4A3, which suggests a possible mechanism by which *PELATON* mediates ferroptosis in *p53* wild-type or mutant GBM cells (**Figure 3H** and **Supplementary Tables 2, 3**) (48). The above data suggested that *PELATON* suppressed the expression of ferroptosis driver genes and promoted the expression of ferroptosis suppressor genes, suggesting that *PELATON* may be a ferroptosis suppressor.

PELATON Is a Novel Ferroptosis Suppressor in GBM

Transmission electron microscopy observation showed that mitochondria decreased, the cell membrane density increased, and cristae decreased or even disappeared after *PELATON* was knocked down in PG-3 primary GBM cells, whereas pcDNA3.1-*PELATON* U251 cells had a relatively normal mitochondrial

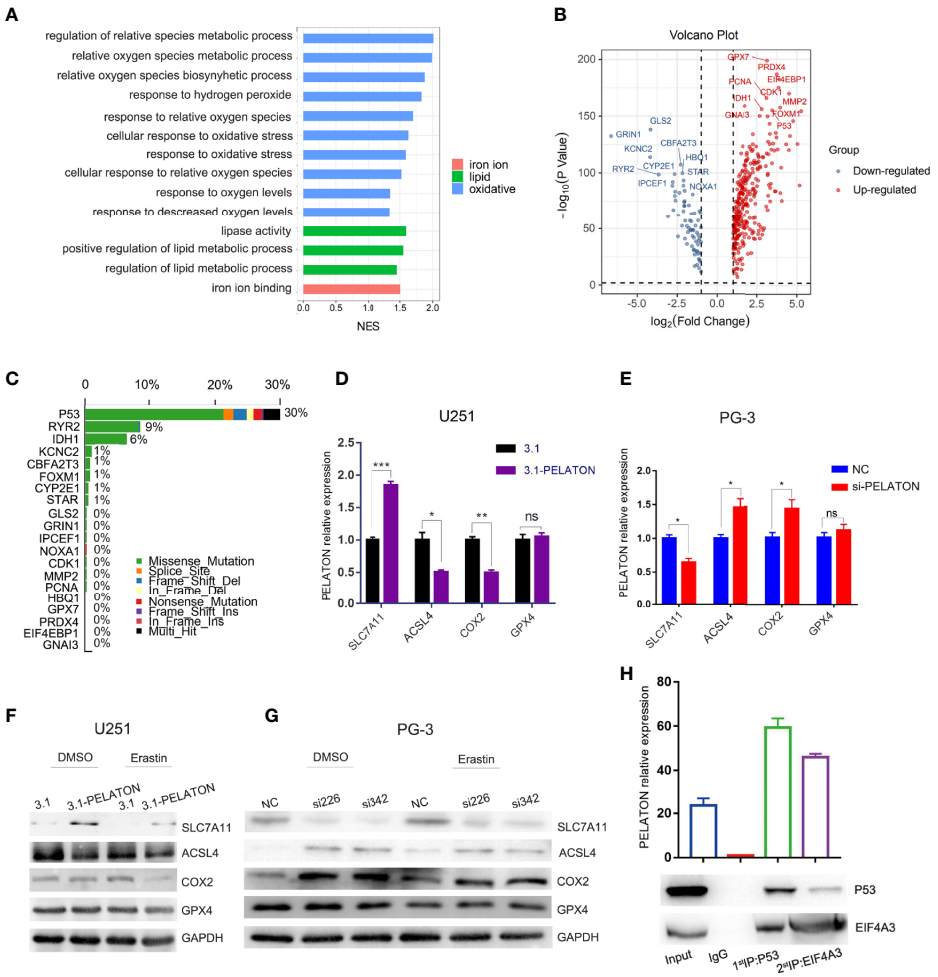

FIGURE 3 | *PELATON* in the ROS-mediated ferroptosis pathway by mutant *p53*. **(A)** GO analysis of biological processes related to *PELATON* in GBM. **(B)** Top 20 genes involved in ROS biosynthesis that are differentially expressed between GBM and normal brain tissue. **(C)** Gene mutation or overexpression in human GBM. RT-qPCR analysis of *ALSL4*, *COX2*, *SLC7A11* and *GPX4* when *PELATON* was overexpressed **(D)** or knocked down **(E)** in glioblastoma cells (*p< 0.05, **p < 0.01; ***p < 0.001; ns, not significant). Datas were mean ± SEM for three independent experiments. western blot analysis of ALSL4, *COX2*, *SLC7A11* and *GPX4* when *PELATON* was overexpressed **(F)** or knocked down **(G)** in glioblastoma cells. independent experiment was repeated for three times. **(H)**. The *PELATON-EIF4A3-P53* complex was detected by two-step immunoprecipitation and RT–PCR, Datas were mean ± SEM for three independent experiments.

morphology (**Figures 4A, B**). The increase in reactive oxygen species and divalent iron ions is a sign of ferroptosis. By determining the levels of ROS and divalent iron ions, we found that knockdown of *PELATON* PG-3 in primary GBM cells promoted the production of ROS and induced the levels of divalent iron ions (**Figures 4C, E**), even after treatment of GBM cells with the ferroptosis inducer erastin (10 μM) for 4 h, and the opposite effect was observed in pcDNA3.1-*PELATON* U251 cells (**Figures 4D, F**).

PELATON Promotes GBM Cell Phenotypes

Although there is a known relationship between *NCOA4* and ferroptosis (29, 49, 50), there is no information about *PELATON* in ferroptosis. *PELATON* is a long intergenic nonprotein coding RNA1272 (also known as *LINC01272*). Few studies have indicated that it promotes cancer cell migration and invasion, such as gastric cancer (51, 52), colorectal cancer (53), and non-small-cell lung cancer (54), but there is no report in GBM. *PELATON* showed significantly higher expression in GBM tissues and primary GBM cells, which were named PG-2, PG-3, PA-2, and PA-3 (42), and was mainly located in the plasma membrane of GBM cells (**Supplementary Figure 5A** and **Figures 5A–C**). Patients with the highest 20% *PELATON* expression had significantly shorter overall survival and free-progression survival (FPS) than the remaining GBM patients (**Supplementary Figures 5B, C**).

The CCK8 assay showed that pcDNA3.1-*PELATON* increased U251 cell proliferation, comparable with that of pcDNA3.1 U251 cells, which have relatively low expression *PELATON*. Knockdown of *PELATON* inhibited the

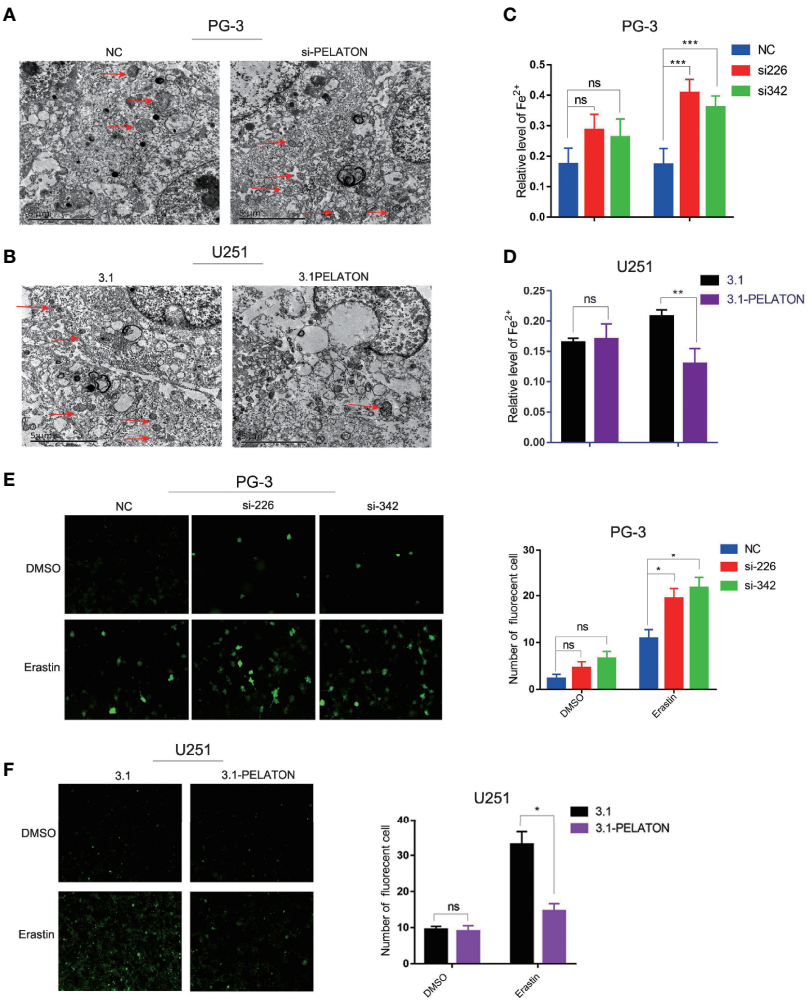

FIGURE 4 | *PELATON* is a novel ferroptosis suppressor in GBM. The effect of downregulation **(A)** or upregulation **(B)** of *PELATON* on the morphology of mitochondria in glioblastoma cells assayed by transmission electron microscopy, independent experiment was repeated for three times. The effect of downregulation **(C)** or upregulation **(D)** of *PELATON* on the levels of iron in glioblastoma cells (**p < 0.01; ***p < 0.001; ns: not significant), Datas were mean ± SEM for three independent experiments. The effect of downregulation **(E)** or upregulation **(F)** of *PELATON* on the levels of reactive oxygen species in glioblastoma cells (*p < 0.05; ns, not significant), Datas were mean ± SEM for three independent experiments. Fluorescence intensity of the active oxygen probe photographed by laser confocal microscopy (left) and quantification of the fluorescence intensity of the reactive oxygen species probe (right).

proliferation of PG-3 primary GBM cells, which have relatively high *PELATON* expression (**Figure 5D**). Wound-healing and transwell assays showed that pcDNA3.1-*PELATON* promoted active migration and invasion in U251 cells and vice versa (**Figures 5E, F** and **Supplementary Figures 5D, E**). We also assessed the effect of *PELATON* on GBM cell proliferation in the presence of the ferroptosis inducer erastin, in which ROS- and iron-dependent signalling is required for erastin-induced ferroptosis. pcDNA3.1-*PELATON* U251 cells and PG-3 primary GBM cells with high *PELATON* expression resisted ferroptosis induced by erastin in a concentration- and time-dependent manner, whereas knockdown or low *PELATON* expression promoted ferroptosis induced by erastin to inhibit PG-3 cell proliferation (**Figure 5G**).

DISCUSSION

PELATON is a long noncoding RNA also known as long intergenic nonprotein coding RNA 1272 (*LINC01272*), small integral membrane protein 25 (*SMIM25*), or GC-related lncRNA 1 (*GCRL1*). A handful of reports indicated that *PELATON* has dual functions as an oncogene or a suppressor gene by acting as a miRNA sponge (53, 55, 56). *PELATON* promotes metastasis of colorectal cancer or gastric cancer by targeting the *miR-876*/ITGB2 axis (53) or *miR-885*-3p/CDK4 (52). *PELATON* also inhibits lung cancer and non-small cell lung cancer by targeting the *miR-7-5p*/CRLS1 axis or by inhibiting *miR-1303* (52, 57). Our research first showed that *PELATON* is highly expressed in gliomas and functions as an oncogene to promote

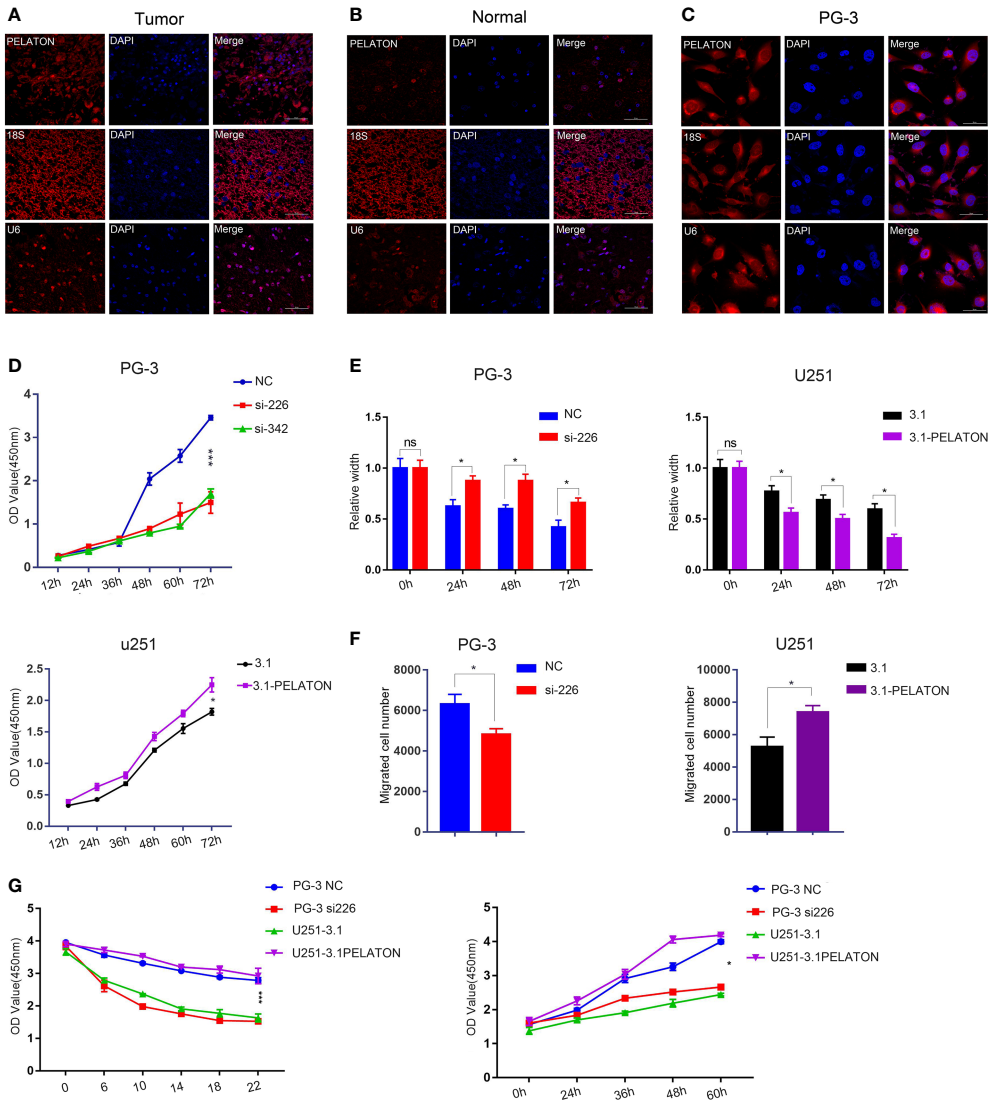

FIGURE 5 | *PELATON* promotes GBM cell phenotypes. Fluorescence *in situ* hybridization of *PELATON* in GBM tissues **(A)**, normal brain tissues **(B)**, and GBM cells **(C)**. **(D)** CCK8 analysis of *PELATON* knockdown or overexpression on glioblastoma cell proliferation (*p < 0.05; ***p < 0.001), independent experiment was repeated for three times. **(E)** Quantification of the migration ability of PG-3 (left) and U251 (right) cells after interference or overexpression with *PELATON* (*p < 0.05; ns, not significant), Datas were mean ± SEM for three independent experiments. **(F)** Quantification of the number of invasive cells after knockdown (left) or overexpression (right) of *PELATON* in glioblastoma cells (*p< 0.05), Datas were mean ± SEM for three independent experiments. **(G)** CCK8 analysis of *PELATON* overexpression or knockdown on proliferation and sensitivity to the ferroptosis inducer erastin (*p< 0.05, ***p < 0.001), Datas were mean ± SEM for three independent experiments.

the proliferation and invasion of *P53* mutant-type GBM cells by inhibiting ferroptosis.

Ferroptosis is an iron-dependent PCD in which cells die because of the toxic accumulation of lipid ROS (58). In cancer, the goal of treatment is to activate ferroptosis and cause the death of tumour cells that are resistant to other PCDs. An increasing number of studies have identified several drivers and suppressors of ferroptosis. Zhou et al. annotated the genes in 784 articles on the ferroptosis FerrDb website and found 253 regulatory factors, including 108 drivers, 69 suppressors, 35 inducers, and 41 inhibitors (46). It is expected that interfering with ferroptosis-related drivers and suppressors, inducers and inhibitors will provide new approaches for the treatment of cancer and metabolic diseases (23–25, 30). The common ferroptosis drivers are *PTGS2*/*COX2* (59), *ACSL4* (1), *NCOA4*, *BECN1* (60), *BACH1* and *P53* (58, 61). *P53* promotes ferroptosis by inhibiting the expression of *SLC7A11* or increasing the expression of SAT1, GLS2, and PTGS2. *P53* also inhibits ferroptosis by directly inhibiting the activity of dipeptidylpeptidase-4 or by inducing the expression of cyclin-dependent kinase inhibitor 1A (61). Ferroptosis suppressors have also achieved good research results, such as nuclear factor, erythroid 2-like 2 (*NRF2*) (20, 62–66),

SLC7A11 (15), *CD44* and *GPX4* (13, 18, 58, 61). The cystine/glutamate antiporter *SLC7A11* (also known as *xCT*) is used to uptake cysteine for glutathione biosynthesis and antioxidant defence. *SLC7A11* is a ferroptosis suppressor gene that is overexpressed in many human cancers (16). Drugs that target *SLC7A11* and block cystine uptake can cause ferroptosis. *SLC7A11* is regulated by the transcription factors NRF2, ATF4, and *P53* (61). *GPX4*, a type of glutathione peroxidase (GPX), is a key inhibitor of ferroptosis. Overexpression of *GPX4* endows tumour cells with resistance to ROS-induced cell death, while silencing *GPX4* sensitizes tumour cells (16, 64, 67). In our study, we first confirmed that *PELATON* is a novel ferroptosis suppressor that functions as a ferroptosis inhibitor mainly by mutant *p53* mediating the ROS ferroptosis pathway. In *p53* mutant-type GBM cells, *PELATON* inhibits the production of ROS, reduces the levels of divalent iron ions, promotes the expression of *SLC7A11*, and inhibits the expression of *ACSL4* and *COX2*. GBM cells with *PELATON* knockdown showed smaller mitochondria, increased mitochondrial membrane density, and decreased mitochondrial cristae. To explore the possible mechanism between *PELATON* and P53, we found that *PELATON* and *P53* can form a complex through the RNA binding protein EIF4A3 (*PELATON*- EIF4A3- P53). *EIF4A3* is reported to be a new anticancer target whose consumption or inhibition will activate *p53* and inhibit the growth of cancer cells. *PELATON* may inhibit the RNA and protein expression of P53 through the *PELATON*-EIF4A3-P53 complex to inhibit GBM ferroptosis, which suggests a possible mechanism by which *PELATON* mediates ferroptosis in *p53* wild-type or mutant GBM cells.

It is well known that the resistance of cancer cells to chemotherapy is a major obstacle in cancer treatment. Activation of the ferroptosis pathway can induce cancer cell death, especially in the case of drug resistance, and enhance the sensitivity of tumours to chemotherapeutic drugs (68). Studies have shown that TMZ combined with erastin can significantly improve antitumor activity, which reflects the importance of ferroptosis in the treatment of gliomas (31, 69, 70). Our experiments confirmed that knockdown of *PELATON* enhanced the sensitivity of GBM cells to erastin and inhibited the proliferation of tumour cells. Overexpression of *PELATON* inhibited the effect of erastin on glioma cells. It is suggested that interference with *PELATON* may provide a new target for treating glioma patients.

Nowadays, many reports screened differentially expressed genes of ferroptosis in the database, and then conduct enrichment analysis, interactive network analysis, univariate and multivariate Cox regression analysis to establish the prognosis model to predict the overall survival time, tumour immune microenvironment and immune cell infiltration (71–73). However, the study of ferroptosis in GBM needs to be further deepened. We not only screened 12 lncRNAs which closely related to ferroptosis, but also proposed a ferroptosis prognostic model with *NCOA4* and *PELATON* for patients with GBM, risk score = -0.69641**NCOA4*+ 0.35167**PELATON*. The higher the risk score is, the greater the death rate among patients with GBM. The survival rate of patients with GBM in the high-risk group was significantly lower than that in the low-risk group. Compared with other methods that require multiple genes for risk scoring to determine the survival of patients (74), we only use two genes to predict the effect, which is relatively accurate, predictive accuracy of the signature was 0.70, 0.74 and 0.75 at 1, 3, and 5 years.

In conclusion, we confirmed that *PELATON* is a new ferroptosis suppressor and an oncogene and established a prognostic model and diagram of ferroptosis in GBM patients with *NCOA4* and *PELATON*, provided that *PELATON* alleviates ferroptosis driven by wild-type or mutant *p53* and suppresses wild-type or mutant *p53*-mediated GBM proliferation. Knockdown of *PELATON* enhances the sensitivity to ferroptosis inducers to inhibit GBM cell proliferation and invasion. *PELATON* is an important target for the prognosis and treatment of GBM.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://cancergenome.nih.gov/, https://www.ncbi.nlm.nih.gov/geo/ (GSE43378).

AUTHOR CONTRIBUTIONS

HJF mainly performed the experiments, and ZYZ mainly performed the information analysis. DYL, QQL, SMC and ZPZ helped with the experiments, and MHW proofread the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.817737/full#supplementary-material

Supplementary Figure 1 | Ferroptosis-related gene clustering and identification in GBM. Sample gene clustering to detect seven outliers **(A)**. The FPI of gene clustering was calculated **(B)**. Set the soft-thresholding value to 4 (scale free R2 = 0.9, mean connectivity=115.71) **(C)** and cut height to 0.25 **(D)**. Intersection of ferroptosis driver and suppressor gene sets in FerrDb. The enrichment results of the constructing module in ferroptosis driver **(E)** and suppressor **(F)** gene sets (FDR<0.05). **(F)** Intersecting genes between DEGs (left, 6093) of GBM and the red module (right, 1050); 610 overlapping genes were selected (| Log2 (fold change) | ≥ 1, and $p < 0.05$).

Supplementary Figure 2 | Ferroptosis-related gene identification of *NCOA4* and *PELATON*. **(A)** Results of the univariate Cox regression analyses of OS in the TCGA cohort. Seven genes (*PELATON, NCOA4, AL354919.2, HMOX1, AL355922.1, CD44, and ALOX5*) were identified. **(B)** LASSO coefficient plot of 25 genes (13 mRNAs and 12 lncRNAs) correlated with ferroptosis. **(C)** The optimal parameter (λ) was chosen by cross validation. The distribution of risk factors **(D–E)**., Kaplan–Meier survival analysis **(F)**, and time-dependent ROC curves at 1, 3, and 5 years **(G)** between patients at high and low risk based on the *NCOA4* and *PELATON* prognostic models in the internal set GSE43378.

Supplementary Figure 3 | *PELATON* regulates *BACH1* and *CD44* in *p53*-mediated ferroptosis. **(A–C)** KEGG analysis of the ferroptosis signalling pathway related to *PELATON* in GBM. **(D)** The expression of *PELATON* in U251 cells after treatment with 3.1 or 3.1-*PELATON* (***$p < 0.001$), Datas were mean ± SEM for three independent experiments. **(E)** The expression of *PELATON* in PG-3 glioma primary cells after treatment with siRNAs (si226, si342, and si478), Datas were mean ± SEM for three independent experiments. (**$p < 0.01$). **(F–G)** RNA level changes of *BACH1* and *CD44* when *PELATON* was knocked down or overexpressed in glioblastoma cells, Datas were mean ± SEM for three independent experiments. (*$p < 0.05$, ***$p < 0.001$, ns: not significant). **(H–I)** Protein level changes of *BACH1*, *CD44* and *P53* when *PELATON* was knocked down or overexpressed in glioblastoma cells, independent experiment was repeated for three times.

Supplementary Figure 4 | Regulatory Relationship between *PELATON* and *P53*. **(A)** Bioinformatics correlation between *PELATON* and *P53*. **(B)** The difference in *PELATON* expression between wild-type and mutant *P53* in GBM patients. **(C)** western blot analysis of *BACH1*, *CD44* and *P53* when *PELATON* and *P53* were overexpressed in glioblastoma cells, independent experiment was repeated for three times.

Supplementary Figure 5 | Association of *PELATON* expression between patient prognosis and GBM cell phenotype. **(A)** RT-qPCR analysis of *PELATON* in glioma primary cells (PG-1, PG-2, PG-3, PA-1, PA-2, PA-3) and glioma cell lines (U118, U251). PG-1, PG-2, and PG-3 are primary cells from patients with glioblastoma, and PA-1, PA-2, and PA-3 are primary cells from patients with astrocytoma. Datas were mean ± SEM for three independent experiments. Kaplan–Meier curves showing overall survival **(B)** and disease-free survival **(C)** of patients with GBM stratified based on *PELATON* expression levels ($p < 0.05$ p.adjust<0.25). **(D)** The effect of *PELATON* overexpression or knockdown on the invasion ability of glioblastoma cells (left), independent experiment was repeated for three times. **(E)** Migration ability of PG-3 and U251 cells after interference or overexpression with *PELATON*. Photos were taken at 0, 24, 48 and 72 hours, independent experiment was repeated for three times.

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REVIEW
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Pharmacological targets for the induction of ferroptosis: Focus on Neuroblastoma and Glioblastoma

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Neuroblastomas are the main extracranial tumors that affect children, while glioblastomas are the most lethal brain tumors, with a median survival time of less than 12 months, and the prognosis of these tumors is poor due to multidrug resistance. Thus, the development of new therapies for the treatment of these types of tumors is urgently needed. In this context, a new type of cell death with strong antitumor potential, called ferroptosis, has recently been described. Ferroptosis is molecularly, morphologically and biochemically different from the other types of cell death described to date because it continues in the absence of classical effectors of apoptosis and does not require the necroptotic machinery. In contrast, ferroptosis has been defined as an iron-dependent form of cell death that is inhibited by glutathione peroxidase 4 (GPX4) activity. Interestingly, ferroptosis can be induced pharmacologically, with potential antitumor activity *in vivo* and eventual application prospects in translational medicine. Here, we summarize the main pathways of pharmacological ferroptosis induction in tumor cells known to date, along with the limitations of, perspectives on and possible applications of this in the treatment of these tumors.

Keywords: ferroptosis, cancer cell, brain tumors, GPX4, system x_c^-, lipid ROS, iron

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INTRODUCTION

Cancer is one of the most frequent pathologies worldwide; according to the World Health Organization (WHO) statistics, there were 18.1 million new cases and 9.6 million deaths related to this disease in 2018 (https://www.who.int/news-room/fact-sheets/detail/cancer). Cancers are difficult to treat because they employ multiple molecular mechanisms to evade different types of cell death, such as apoptosis, due to their overexpression of antiapoptotic proteins such as Bcl-2 and Bcl-xL and low expression of proapoptotic factors such as Bax, Bim and Puma (**Figures 1A, B**) (1). At the same time, it is known that the low efficacy of apoptosis induction with conventional therapies is due to the robust antioxidative defenses of tumor cells (2). Among the main antioxidants that confer apoptosis resistance on tumor cells is glutathione (GSH) (3, 4).

Due to the high resistance of tumors to apoptosis, the induction of necroptosis was postulated to be a potential therapeutic approach (5, 6). In contrast to apoptosis, which does not generate an

inflammatory response, necroptosis induces death by cellular explosion, which generates a microenvironment of proinflammatory signals that could favor tumor death (5, 6). Thus, necroptosis, a form of regulated necrosis dependent on RIPK1, RIPK3 and MLKL, was postulated as a potential therapy for cancer (**Figures 1A, B**) (7–9). Unfortunately, several tumor cells evade necroptosis efficiently by inhibiting the expression of RIPK3 *via* epigenetic control mechanisms (10–12).

In line with this idea, new and emerging forms of regulated cell death with characteristics of necrotic disintegration have

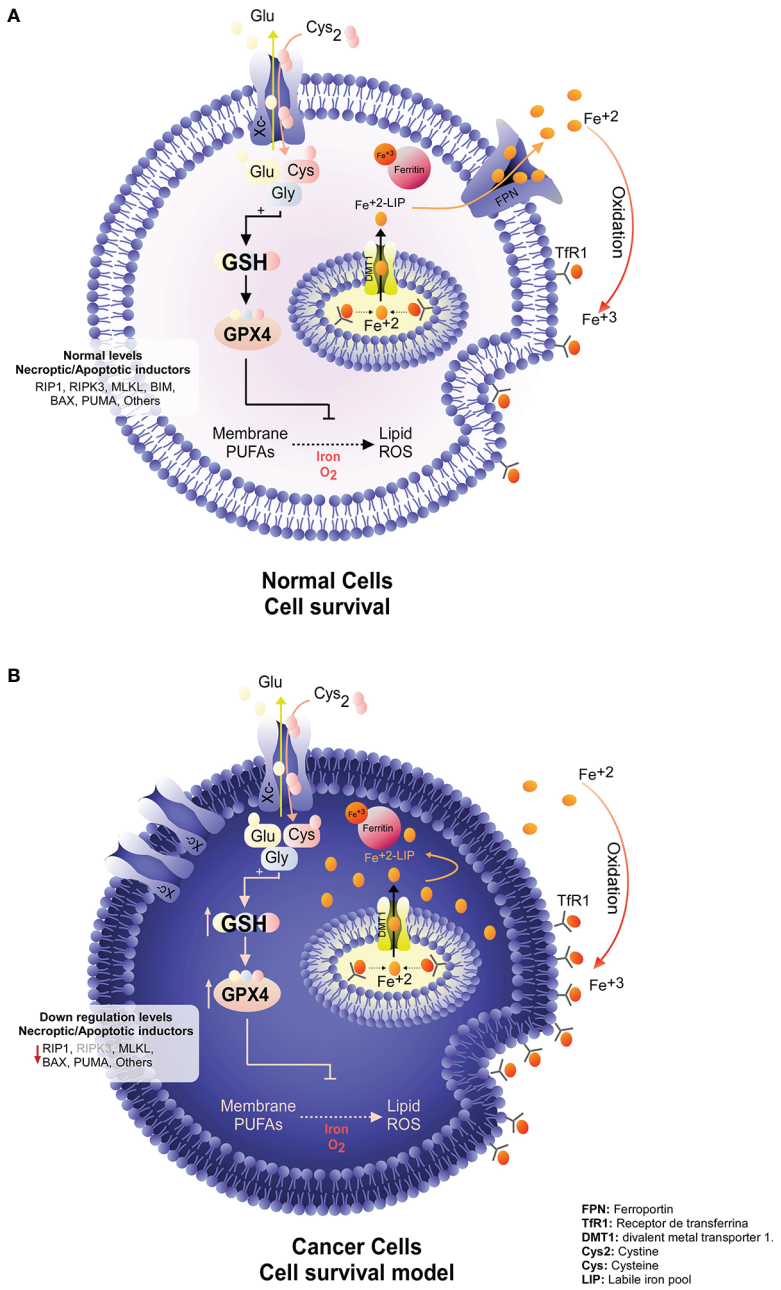

FIGURE 1 | Survival programs in normal and tumor cells. **(A)** Under physiological conditions, normal cells maintain stable levels of death-executing proteins while maintaining a constant balance of nutrients and trace elements, promoting cell survival. **(B)** To avoid death, tumor cells activate various mechanisms, such as decreasing the expression of proapoptotic and necroptotic genes while increasing antioxidant defense by increasing GSH synthesis and GPX4 levels. In this way, ROS are efficiently eliminated, avoiding the damage produced by the accumulation of iron due to low FPN levels. This death evasion program makes many types of cancer highly difficult to treat, as classical apoptosis induction therapies fail because the machinery for the execution of this pathway is not available. FPN, Ferroportin; TfR1, Transferrin Receptor 1; DMT1, Divalent Metal Transporter 1; Cys2, Cystine; Cys, Cysteine; LIP, Labile Iron Pool.

been described and postulated as treatments for cancer; among these, ferroptosis is highlighted (13–19). Here, we describe the main pharmacological targets for the induction of ferroptosis with emphasis on the treatment of brain tumors.

OVERVIEW OF THE INDUCTION OF FERROPTOSIS IN CANCER CELLS: TARGETING SYSTEM x_c^-

System x_c^- is an antiporter that imports cystine and exports glutamate from the cell in a 1:1 ratio (**Figures 1A, B**). System x_c^- is composed of 2 subunits: the SLC7A11 subunit (also called xCT), with a transport function and solute carrier family 3 member 2 (SLC3A2; also called CD98hc or 4F2hc), a chaperone with a plasma membrane anchoring function (20–23). For the purposes of this review, we refer only to the SLC7A11 subunit, given the importance of cystine transport to the cell (**Figures 1A, B**). In this context, the uptake of cystine into the cell is essential to maintain the redox state, since the reduced form of this amino acid (nonessential) is necessary for the biosynthesis of the main intracellular antioxidant, glutathione (GSH) (**Figure 1A**). Interestingly, most cancer cells overexpress SLC7A11 (22), suggesting a strong dependence on GSH to maintain the levels of controlled reactive oxygen species (ROS) (**Figure 1B**); thus, SLC7A11 is an potential therapeutic target. Interestingly, in 2012, it was determined that the small molecule erastin (13, 24) targeted SLC7A11 for inhibition, which led to depletion of GSH, inducing a type of death dependent on iron and lipid ROS, called ferroptosis (14). This type of cell death was inhibited by radical trapping antioxidants (RTAs) such as Ferrostatin-1 (Fer-1), lipophilic antioxidants such as vitamin E or iron chelators such as Desferoxamine (DFO) (18, 25), with potential application in the treatment of cancer and other pathologies (**Figure 2B**) (26).

Thus, when tumor cells are incubated with erastin, cell death is induced independent of caspases (13) or mitochondrial oxidative stress but in a manner dependent on iron, ROS and lipid ROS (14). Even though there is evidence that mitochondria could be involved, regulating the "avidity" for ferroptosis induction (27–29), they are not necessary for activation of this pathway (30). Inhibition of system x_c^- results in intracellular depletion of cysteine because extracellular cystine (Cys_2) is imported through SLC7A11 and reduced intracellularly to cysteine (**Figure 2B**) (16, 31). Intracellular cysteine is necessary for the biosynthesis of GSH (16, 32). In turn, GSH is a cofactor for the selenoprotein GPX4, a hydroperoxidase responsible for detoxifying toxic hydroperoxides to alcohols (15). Therefore, erastin triggers indirect inhibition of GPX4 activity mediated by GSH depletion (**Figure 2B**).

Despite this apparent dependence of cells on system x_c^-, animals with knockout of the *slc7a11* gene are fertile and develop completely normally (33), which prompted the consideration of SLC7A11 inhibition as an eventual cancer therapy with few adverse effects (**Figure 2A**).

Thus, although many tumor cells can evade apoptosis and necroptosis due to their low expression of key genes for the activation of these pathways (**Figure 1B**) (1, 10, 11), RNA-seq data show that most cancer cells have high expression levels of SLC7A11 and GPX4 (https://portals.broadinstitute.org/ccle). Similarly, tumor cells are "addicted" to iron because they have decreased expression of ferroportin (FPN), the iron efflux pump, and overexpress the transferrin receptor (TfR1), the iron importer (**Figure 1B**) (34–37). Indeed, excess iron contributes to both tumor initiation and tumor growth (34). These observations indicate that SLC7A11, GPX4, iron and ferroptosis are potential therapeutic targets for cancer (**Figures 2B**, **3**). However, there are cancer cells that do not express FPN (MCF-7 cells, among others) and therefore accumulate excess intracellular iron but are still resistant to ferroptosis (38, 39). An explanation for this phenomenon is the recent finding that in addition to GPX4 and iron, acyl-CoA synthetase long-chain family member 4 (ACSL4) is another component that dictates sensitivity to ferroptosis (39). Reinforcing this concept, ACSL4 is a key protein because it incorporates long polyunsaturated fatty acids (PUFAs) into membranes, which allows lipid peroxidation to proceed and ferroptosis to be carried out (39–41). In another context, the erastin analog imidazole ketone erastin (IKE) has been shown to be metabolically stable and a potent inducer of ferroptosis in tumor cells *in vivo* (19, 42). Thus, induction of ferroptosis in tumor cells through inhibition of SLC7A11 may be a promising treatment for use in patients.

Interestingly, high doses of glutamate can inhibit system x_c^-, emulating the effects induced by erastin (14, 43, 44). However, it is known that the responses to glutamate treatment are diverse and can induce cell death by apoptosis or necroptosis (45, 46) and eventually by other pathways of regulated necrosis. Thus, although high doses of glutamate can inhibit system x_c^-, they are not necessarily a specific inducer of ferroptosis in tumor cells but could induce ferroptosis in normal tissues under pathophysiological conditions (44, 47–49).

Ferroptosis Beyond the Inhibition of System x_c^-

Although the concept of ferroptosis was initially described in response to treatment with erastin, various ferroptosis inducers (FINs) have been developed to act independently of cystine uptake and GSH levels. FINs are currently classified into four classes (I-IV) (40, 50): class I FINs induce GSH depletion (**Figure 2B**), class II FINs inhibit GPX4 (**Figure 3**), class III FINs deplete GPX4 (**Figure 4**), and class IV FINs act through iron oxidation/iron overload (**Figure 5**) (summarized in **Table 1**). Interestingly, two research groups recently described a new player in the regulation of ferroptosis in parallel: ferroptosis suppressor protein 1 (FSP1) (**Figure 4**) (54, 55). Previously called apoptosis-inducing mitochondria-associated factor 2 (AIFM2), FSP1 is a flavoprotein with extramitochondrial oxidoreductase activity that can be recruited into the plasma membrane due to myristoylation. FSP1 in the plasma membrane catalyzes the conversion of ubiquinone (coenzyme Q10, CoQ10) to ubiquinol at the expense of NADPH (56).

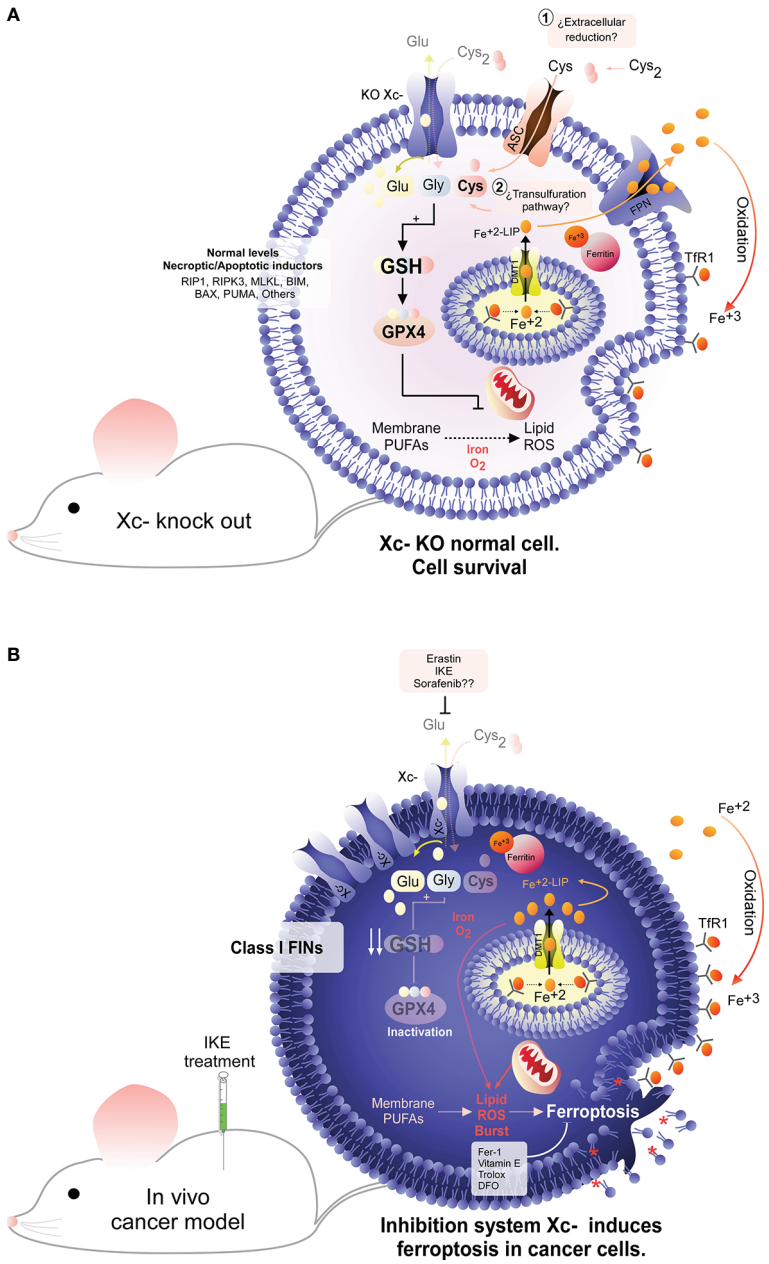

FIGURE 2 | System x_c^- dependence in cancer cells. Under physiological conditions, the nonessential amino acid cysteine is present as cystine due to the extracellular oxidative environment. To maintain a stable intracellular cysteine level, the presence of the cystine/glutamate antiporter (system x_c^-) is necessary. Interestingly, genetic deletion of system x_c^- does not produce any damage in animals, suggesting that normal cells do not depend on this antiporter to maintain the intracellular cysteine level. In line with this idea, compensatory mechanisms, such as the transsulfuration pathway, may exist for the recovery of the intracellular cysteine level **(A)**. Conversely, it has been widely described that tumor cells have a high dependence on system x_c^- for the cellular uptake of cysteine **(B)**. Pharmacological inhibition of this antiporter results in the depletion of intracellular cysteine, inducing an abrupt decrease in the GSH level, which ultimately triggers inactivation of GPX4, the main hydroperoxidase in the cell. Inactivation of GPX4 due to inhibition of system x_c^- results in an overwhelming overload of lipid ROS that ultimately induces tumor death by ferroptosis **(B)**. Interestingly, it has been determined that inhibition of system x_c^- can induce tumor death both *in vitro* and *in vivo*, identifying this antiporter as a potential therapeutic target for cancer.

Thus, the FSP1-ubiquinone-ubiquinol axis inhibits lipid peroxidation and ferroptosis in parallel to GPX4 and independent of GSH levels (54, 55). An inhibitor of FSP1 (iFSP1) (54) that may stimulate the induction of ferroptosis was identified by drug screening. In this context, the iFSP does not fit within any class of FINs (I-IV) because it does not target GPX4 or iron metabolism. Thus, we suggest that FINs that do not target GPX4 or iron metabolism but, as their mechanism

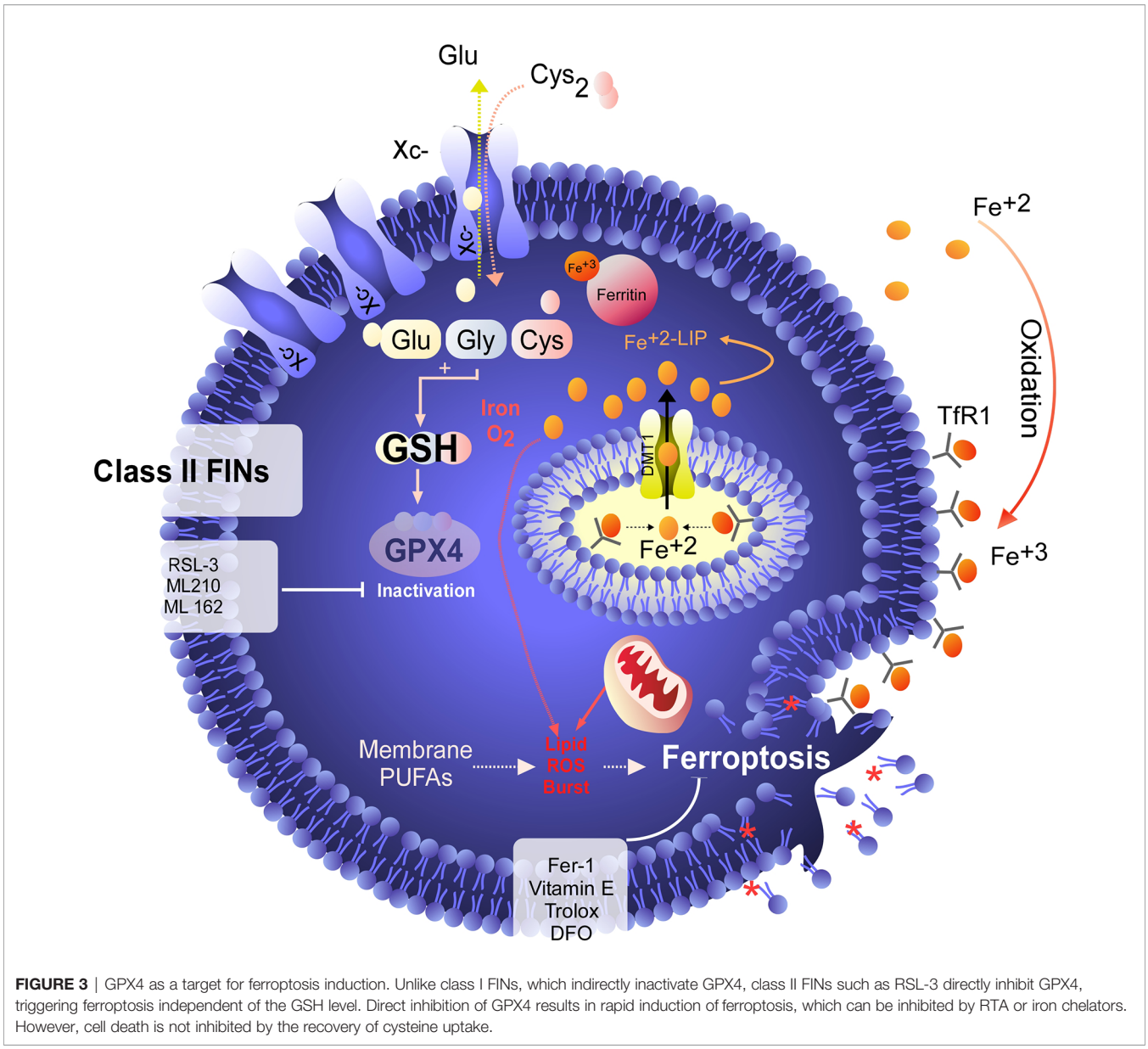

FIGURE 3 | GPX4 as a target for ferroptosis induction. Unlike class I FINs, which indirectly inactivate GPX4, class II FINs such as RSL-3 directly inhibit GPX4, triggering ferroptosis independent of the GSH level. Direct inhibition of GPX4 results in rapid induction of ferroptosis, which can be inhibited by RTA or iron chelators. However, cell death is not inhibited by the recovery of cysteine uptake.

involves CoQ10, can be classified into class V (**Figure 4**). Because FIN56 depletes GPX4 and CoQ10 (52), this compound has a dual classification and should also be reclassified into class V. Despite the existence of various ferroptosis inducers, not all of them have therapeutic potential *in vivo* (40). However, it has been shown that the use of class IV inducers could have potential therapeutic effects *in vivo* to treat high-risk neuroblastomas (17).

INDUCTION OF CELL DEATH IN NERVOUS TISSUE

Normal adult neurons are equipped to survive because they express low levels of proapoptotic proteins and high levels of antiapoptotic proteins (57, 58). Furthermore, it has been shown that as neurons mature, they lose chemosensitivity to staurosporine and doxorubicin (58). This evidence suggests that brain tumors would be highly resistant to conventional antineoplastic agents, given the preconditioning of this type of cell to efficiently evade apoptosis. At the same time, it has been shown that tumor cells of astroglial origin (T98G, U251 and A172) efficiently evade necroptosis induced by chemotherapeutic agents because they do not express RIPK3 due to epigenetic modifications (10, 11).

Thus, the development of new therapies for the treatment of brain tumors that do not involve the induction of apoptosis or necroptosis as the main strategy is urgently needed. In this sense, in recent years, the induction of ferroptosis has gained great relevance as a possible therapeutic approach to induce cell death in brain tumors (17, 50, 59, 60). Considering this concept in the

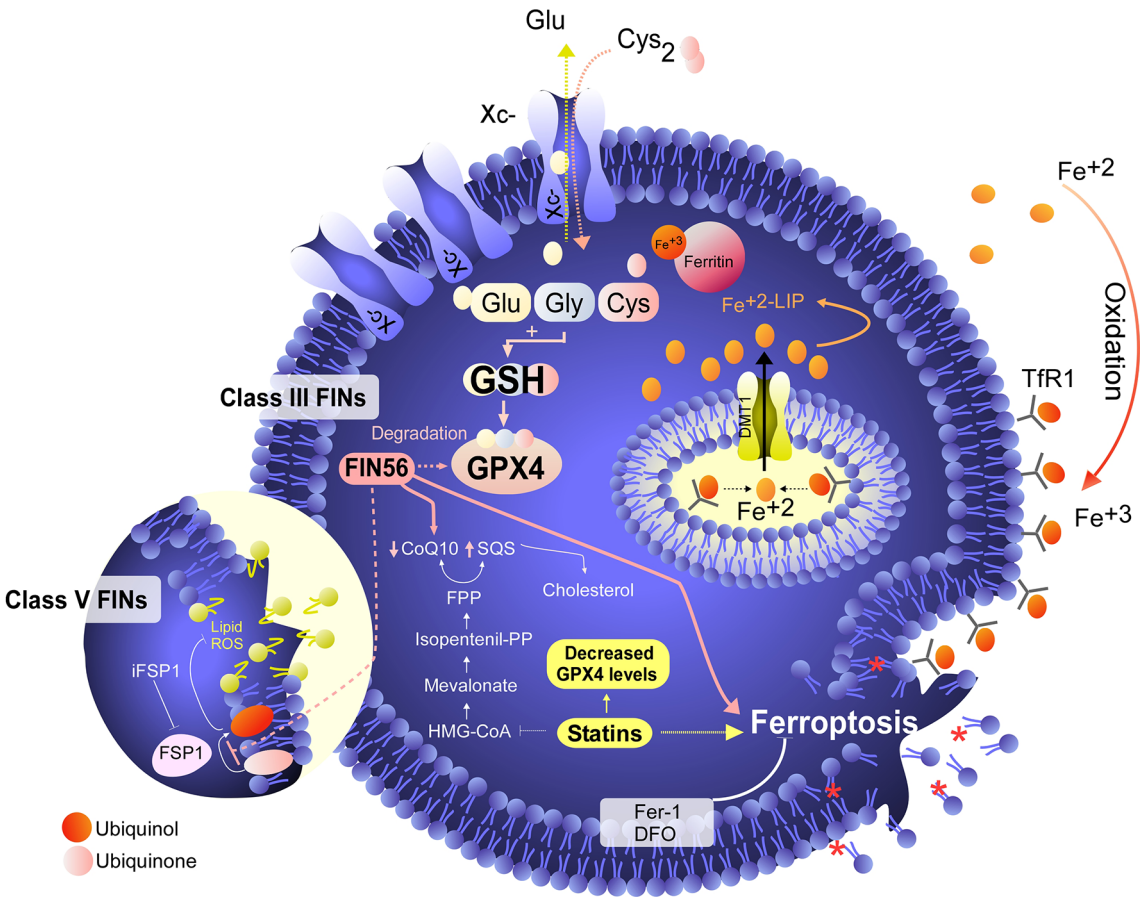

FIGURE 4 | Degradation of GPX4/CoQ10 or inhibition of FSP1 induces ferroptosis in cancer cells. Class III FINs are molecules that act independently of system x_c^- activity, the GSH level and direct inhibition of GPX4. These molecules, including FIN56, induce degradation of GPX4, which leads to ferroptosis induction. In addition to degrading GPX4, FIN56 also induces degradation of coenzyme Q10 (ubiquinone) by altering the mevalonate pathway. The importance of coenzyme Q10 degradation in the execution of ferroptosis is assumed because the function of a protein called FSP1 (a class V FIN) was recently described (33, 34). In this scenario, FSP1 converts extramitochondrial ubiquinone (the oxidized form of coenzyme Q10) to extramitochondrial ubiquinol (the reduced form of coenzyme Q10), and ubiquinol acts as an endogenous RTA that inhibits ferroptosis independent of the presence of GPX4. In this context, by inducing coenzyme Q10 degradation, FIN56 can inhibit the effects of FSP1 to confer resistance to ferroptosis. On the other hand, the inhibitor of FSP1 (iFSP1) controls ferroptosis without degrading CoQ10.

following sections, we focus on the induction of ferroptosis in neuroblastoma (NB) and glioblastoma multiforme (GBM).

Neuroblastoma

NB is the most common pediatric extracranial tumor, accounting for more than 15% of all cancer deaths in children (61). NB is classified as low-, intermediate- and high-risk (62). While low-risk and intermediate-risk NBs generally have a good prognosis given that they develop into benign ganglioneuromas or enter remission due to surgical or pharmacological treatment, high-risk NBs have few treatment options (17, 62, 63). The main diagnostic characteristics of high-risk NB are that it appears after 18 months of age, has MYCN amplification, or exhibits activation of telomere maintenance mechanisms (62, 63). In line with this observation, current therapies against the NB include treatment with cycles of cisplatin, etoposide, vincristine, doxorubicin, and cyclophosphamide (64), which are preferential inducers of apoptosis. However, this type of pharmacological treatment generates multidrug-resistant clones, which greatly hinders the eradication of this type of tumor and favors its relapse (64).

Classical Pharmacological Induction of Ferroptosis in Neuroblastoma

Considering that classical NB eradication therapies generally fail, it has been proposed that the induction of ferroptosis could be a feasible therapeutic approach. In this context, when the sensitivity of NB cell lines to classic ferroptosis inducers such as erastin or RSL-3 was studied (**Figures 2B**, **3**), it was determined that most of the models (SHSY-5Y, SK-N-SH, NB69, SK-N -DZ, NLF, and CHP-134 cells, among others) are highly insensitive to SLC7A11 or GPX4 inhibition (17, 65, 66). At the same time, there is very little information on the potential use of iFSP1 as a possible strategy against NB, since this

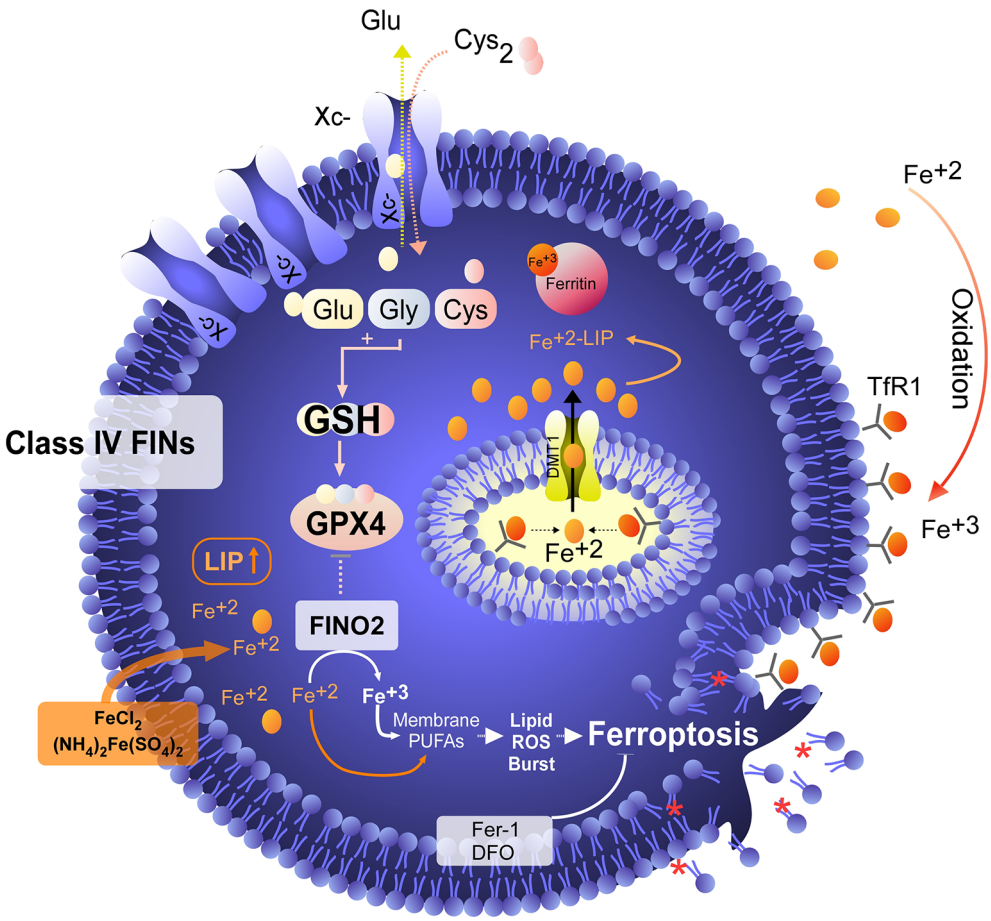

FIGURE 5 | Iron overload or peroxidation induces ferroptosis in tumor cells. Class IV FINs are ferroptosis inducers that directly involve metabolism and iron levels in the cell. On the one hand, we have found synthetic molecules, such as $FINO_2$, that alter the metabolism of iron, favoring its intracellular oxidation. In addition to promoting the oxidation of iron, $FINO_2$ indirectly inhibits the activity of GPX4. On the other hand, when the labile iron pool (LIP) is increased by exogenous treatment with iron or iron nanoparticles, an overload of this metal is generated, which induces lipid peroxidation without the need for GPX4 inhibition. Class IV FINs are fairly attractive agents for the induction of ferroptosis because tumor cells are addicted to iron due to their low ferroportin (FPN) expression and high levels of transferrin receptor (TfR) expression, which favors an increase in the LIP. In this context, treatment with exogenous iron (e.g., $FeCl_2$) in combination with $FINO_2$ would eventually be a potent inducer of ferroptosis in tumor cells. Unfortunately, the development of $FINO_2$ analogs for *in vivo* use is necessary to test whether the increases LIP and iron peroxidation are synergistic to specifically kill tumor cells.

TABLE 1 | Principal Ferroptosis Inducers.

FIN Class	Target	Example
I	System x_c^-	Erastin, Sulfasalazine, Glutamate (14); IKE (19);
II	Inhibition of GPX4	RSL-3 (15); ML210, ML162 (51)
III	Depletion of GPX4	FIN56 (52), Statins (51), withaferin A (17)
IV	Oxidation/Overload of Iron	$FINO_2$ (53); $(NH4)_2Fe(SO4)_2$ (17); $FeCl_2$ (48)
V	Inhibition FSP1/Depletion CoQ10	iFSP1 (54); FIN56 (52)

compound has only been tested in the IMR-5/75 cell line without major effects on viability (54). Based on this background, the scientific community has focused on the search for new strategies for ferroptosis induction in NB, not through the classical targets but instead through the use of combined therapies or noncanonical inducers of ferroptosis, as potential treatments for high-risk NB (17, 67).

TYPICAL AND ATYPICAL PATHWAYS TO INDUCE FERROPTOSIS IN NEUROBLASTOMA

Because NB generally presents resistance to Erastin and RSL-3, it is necessary to search for new ferroptosis inducers. To this end, it was recently determined that treatment with the natural

compound withaferin A (WA) can eradicate high-risk NB (17) by inducing ferroptosis through the canonical pathway, this means with GPX4 as a direct target. On the other hand, *via* a noncanonical pathway, where keap1 is the target, thus favoring an increase in labile iron pool (LIP) (17). This dual behavior of WA, similar to that of a mixture of FIN56 and FINO2 (**Figures 4**, **5**) (52, 53) (compounds that have not been tested in NB models), seems to render it a promising drug therapy for NB, since to date, it is unknown whether *in vivo* application of $FINO_2$ is possible (40). Fortunately, WA has been shown to be effective in promoting the eradication of NB *in vivo* (17, 50). Interestingly, even though WA has been shown to induce iron-dependent lipid peroxidation and GPX4 depletion, Fer-1 treatment does not completely rescue NB cells from cell death (17). This suggests two alternatives; the first is that WA induces other types of ferroptosis-independent death in NB. However, WA induces lipid peroxidation, which is completely inhibited by treatment with DFO and partially inhibited by Fer-1, suggesting a strong iron dependence (17).

In this context, as a second alternative, the authors suggest that WA could eventually favor an overload of lipid ROS of various origins that may not necessarily be inhibited by Fer-1, such as lipid ROS generated by H_2O_2 (17). It is important to note that Fer-1 does not inhibit death induced by H_2O_2 treatment (14) or by extracellular H_2O_2 production mediated by pharmacological doses of ascorbic acid (68), because these treatments preferentially induce conventional necrosis. However, it has recently been determined that NADPH-cytochrome P450 reductase (POR) favors the induction of ferroptosis due to the cytoplasmic production of H_2O_2 (69), which is inhibited by Fer-1 or by the intracellular expression of catalase (69) but thus far, this finding is limited to cervical cancer cells (HeLa).

In this context, and considering the particularities of NB cells, it is likely that it is possible to classify the cell death induced in this type of tumor (or others) as ferroptosis, even when it is not inhibited by Fer-1, if it has other hallmarks, such as lipid peroxidation and iron dependence. Indeed, it has recently been determined that inhibition of lipid peroxidation mediated by liproxstatin-1 treatment is not sufficient to rescue SLC7A11 KO melanoma cells from death (70). Furthermore, it has also been shown that there is strong induction of lipid peroxidation during the activation of noncanonical pyroptosis that is not necessarily related to the direct execution of this death pathway (71). This evidence could limit lipid peroxidation as an exclusive hallmark of ferroptosis, driving the definition of ferroptosis toward a type of death *dependent on lipid peroxidation* (72).

DOES TARGETING SLC7A11-GSH AXIS IN NEUROBLASTOMA INDUCE FERROPTOSIS?

In another context and emphasizing that MYCN is a protein overexpressed in NB, recent advances have been achieved to determine that MYCN favors an increase in intracellular iron *per se*, which could favor the pharmacological sensitization of NB to ferroptosis induction (66, 67). Thus, the authors determined that inhibition of SLC7A11 with sulfasalazine (14) or inhibition of GSH synthesis with Buthionine sulfoximine (BSO) favors the induction of ferroptosis in models of NB with MYCN amplification (67). This *in vitro* evidence from patient samples is closely related to an eventual clinical application, since the toxicity of BSO has been evaluated in a phase I clinical trial, as a possible treatment for NB in conjunction with other drugs (73). Despite being relatively well tolerated, the treated patients presented vomiting/nausea as adverse effects (73). However, there is also evidence indicating that the administration of BSO can trigger kidney failure in animal models (74) and patients (75). Thus, special precautions must be taken when trying to directly extrapolate *in vitro* findings to *in vivo* models or patients.

Curiously, some of the NB cell lines used in this study show partial resistance to death induced by the SLC7A11 inhibitor and GSH depletor erastin (17), which leads to an intriguing question: why are some NB cell lines resistant to erastin but sensitive to inhibition of GSH synthesis or inhibition of SLC7A11 mediated by sulfasalazine? In this scenario, it is important to highlight that in lung adenocarcinoma cells, it was recently determined that the SLC7A11 inhibitor HG106 preferentially induces GSH depletion and cell death by apoptosis, which is inhibited by the recovery of cysteine uptake, but without eventual induction of ferroptosis, since DFO treatment does not prevent cell death (76). This evidence suggests that although HG106 has the same target as erastin (SLC7A11), there are other off-targets that favor the induction of one type of death over another (apoptosis or ferroptosis) or the production of particular ROS that trigger differential cellular responses (22). Despite these pharmacological dichotomies, which induce different types of death even when the target is the same, or which have differential action mechanisms in response to treatment with SAS, erastin (IKE) or HG106, the message that remains the same: SLC7A11 is a potent therapeutic target for cancer (**Figure 2B**).

IRON OVERLOAD AS A POSSIBLE TREATMENT FOR NEUROBLASTOMA

It was shown that NB cells with MYCN amplification are particularly sensitive to the induction of death mediated by treatment with auranofin (a rheumatoid arthritis drug) (67). Although the authors attribute the effect of auranofin to the induction of ferroptosis, treatment with Fer-1 only partially rescues cells from cell death, even when there is an increase in lipid peroxidation, and treatment with DFO effectively prevents cell death and ROS production (67). Again, this finding leads us to conclude that apparently in NBs, the lipid ROS generated are specific to this tumor type or there are parallel mechanisms of cell death, since Fer-1 is not capable of completely inhibiting cell death, even when the evidence points to iron and lipid ROS dependency. Accumulating evidence, the literature indicates that iron accumulation and increased LIP are strong candidates for exploiting the pharmacological sensitivity of high-risk NB to

ferroptosis induction (**Figure 5**) (17, 50, 66, 67, 77). Thus, the use of compounds that promote the mobilization or uptake of iron in this type of tumor, in combination with ferroptosis inducers, could exploit the vulnerabilities of this tumor to favor its eradication. However, further studies are still needed to determine the potential lethal effects on nervous tissue and to assess whether these types of therapeutic agents can penetrate the blood–brain barrier.

GLIOBLASTOMA

Overview of Glioblastoma Treatment

Malignant gliomas are one of the most devastating and frequently diagnosed brain tumors in adults and are associated with a short life expectancy of only 12 to 15 months (78). The WHO classifies this type of tumor as grade I to IV, the latter being called glioblastoma multiforme (GBM), which corresponds to the most advanced stage and has a shorter life expectancy (78–80). The current incidence of GBM in the USA is approximately 7 per 100,000 inhabitants (79). Currently, therapy for GBM is based on surgery accompanied by radiation therapy and chemotherapy, since GBM cannot be completely removed surgically due to its infiltrative nature (78). Although radiotherapy increases the life expectancy of patients, 90% of GBMs exhibit recurrence at the original tumor site after therapy (81). Thus, all hopes for the treatment of this type of tumor are placed on the development of new agents or pharmacological strategies for successful chemotherapy. To date, the main pharmacological approaches for the treatment of GBM include the use of antiangiogenic therapies (bevacizumab, sunitinib, vandetanib), immunotherapy (anti-PD-1/PD-L1 antibodies) and various other molecular approaches, such as inhibitors of mTOR, EGFR, HSP90, and PI3K (78, 82). Unfortunately, GBMs acquire resistance to these types of treatment (78, 82). In this scenario, as a therapeutic strategy, one of the most commonly used compounds is temozolomide (TMZ), an oral alkylating agent (80, 83, 84) that targets the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT), which has been shown to prolong the life expectancy of patients when used in conjunction with radiotherapy (84–86). Unfortunately, most GBMs recur after 2 years with cell populations resistant to this type of therapy due to stem cell properties (87–89). Based on accumulating evidence and the strong resistance of GBM to multiple therapies, the development of new drugs for the treatment of these devastating tumors is urgently needed.

Pharmacological Ferroptosis Induction: A Therapy Against Glioblastoma?

Based on the above premise, pharmacological induction of ferroptosis could exploit the vulnerabilities of GBM cells and sensitize them to death when used in combination with other antineoplastic compounds. In line with this idea, the evidence suggests that combined treatment with ferroptosis inducers plus other antineoplastic therapies (e.g., TMZ or radiation) could lead to sensitization to this type of death in GBM cells (60, 90). This is because most GBM cells are resistant to either SLC7A11 inhibition (erastin treatment) or GPX4 inhibition (RSL-3 treatment) (54, 91, 92), although they express ACSL4 (93).

In line with this observation, high levels of SLC7A11 expression are considered to predict poor survival in patients with malignant glioma (94). At the same time, high expression of SLC7A11 is associated with epileptic seizures, stem cell properties, increased migration and invasion, neurosphere formation and increased expression of Nanog, Sox-2 and Nestin, among other proteins (95, 96). Thus, the expression of this transporter is considered a possible biomarker for the diagnosis of GBM. In this scenario, it is tempting to speculate that SLC7A11 blockade could be an excellent therapy for GBM, since its high expression level indicates a strong dependence on its function.

However, current evidence has shown that GBM cells, such as U87, U251, and U373 cells, are highly insensitive to treatment with SAS or erastin (72, 97), a phenotype that could be related to resistance mechanisms mediated by ATF4 and Nrf-2 that favor overexpression of SLC7A11 (21, 97, 98). Furthermore, the use of SAS in a clinical trial against glioma did not show a response, and various adverse effects were observed (99), which greatly complicates its future use as a ferroptosis-inducing drug in patients. It is important to note that various studies have suggested that GBM cells (and cells of other lineages) have unique sensitivity to death (theoretically ferroptotic) mediated by sorafenib treatment (97, 100). However, recently, it was shown that sorafenib failed to induce ferroptosis in a wide panel of tumor cell lines (including GBM cell lines) (72), which leads us to take special care in the interpretation and specificity of sorafenib in triggering ferroptosis.

Molecular Pathways That Confer Resistance to Ferroptosis in Glioblastoma

In this scenario, where GBM cells show great resistance to inhibition of system x_c^-, it is possible to speculate that they obtain cysteine intracellularly from another source that implies mechanisms independent of the function of SLC7A11, which would explain the resistance to treatment with erastin or SAS. The main metabolic pathway that supplies cysteine intracellularly in tumor cells independent of the transport activity of SLC7A11 is the transsulfuration pathway (101). The transsulfuration pathway allows methionine to be used as a substrate for cysteine biosynthesis through various enzymatic reactions (101). At the same time, it has been shown that inhibition of this pathway in tumor cells makes it possible to recover sensitivity to erastin in certain cell lines other than GBM cell lines (102). Unfortunately, inhibition of the expression of cystathionine β-synthase (CBS), a key protein in the transsulfuration pathway, has been shown to promote GBM progression (103), while in other tumor models, CBS inhibition effectively causes cell death (104), which suggests that GBM cells could be resistant to ferroptosis induction, including that mediated through inhibition of system x_c^- and the transsulfuration pathway.

On the other hand, one possible explanation for the strong resistance of GBM cells to the induction of ferroptosis is the protective effect exhibited by FSP1 in this type of tumor (**Figure 4**), since most GBM cells express high levels of this protein, and cotreatment with iFSP1 and RSL-3 strongly sensitizes them to ferroptosis (54, 72). However, cotreatment with erastin or SAS + iFSP1 fails to induce death in GBM cells (72). This evidence corroborates the findings that FSP1 acts independently of the GSH level (54, 55) and that it apparently can only have synergistic effects with direct GPX4 inhibitors such as RSL-3 or ML162.

Interestingly, the GPX4 depletor FIN56 (**Figure 4**) was recently shown to induce ferroptosis in *in vitro* and *in vivo* GBM models (105); this was the first study to use this compound *in vivo*. However, the trial was not carried out with tumors in nervous tissue but rather in nude mice with subcutaneous tumors, which makes it difficult to extrapolate the possible eventual effects of FIN56 on the brain, and it is not known whether this compound can cross the blood–brain barrier to be considered a potential therapy in the future.

Based on accumulating evidence and given the limitations of the use of direct GPX4 inhibitors for the treatment of tumors *in vivo*, the best therapeutic approach seems to be inhibition of SLC7A11. Along these lines, it has been demonstrated that cotreatment with IKE and radiation favors ROS production and induces cell death in GBM models (60). Concurrently, cotreatment with erastin and TMZ has been found to sensitize GBM cells to death (90). This eventual therapeutic strategy offered by treatment with SLC7A11 inhibitors should be exploited in the future in the search for compounds with synergistic activity that exploit the vulnerabilities of GBM cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

Although there are several inducers of ferroptosis, the potential use of these drugs as cancer treatments is limited because they have little bioavailability for action *in vivo*. However, with the development of IKE, an avenue was opened for ferroptosis induction as an *in vivo* treatment by targeting system x_c^- (19). To date, evidence suggests that inhibition of system x_c^- could be a safe therapeutic approach as a tumor suppressor. Unfortunately, several types of tumors, including NB and GBM, are resistant to system x_c^- inhibition for ferroptosis induction (13, 17, 92). Thus, combination therapies of other antineoplastic drugs with IKE may represent an option for the treatment of cancers highly resistant to cell death. However, ferroptosis dogma dictates that GPX4 is the key protein (15, 106); thus, all efforts have been focused on the development of new drugs for its inhibition. Although there are direct GPX4 inhibitors, such as RSL3 (15), they have little application *in vivo* (40), and GPX4 deletion in some types of cancer is not lethal (51), suggesting that there may be other mechanisms in addition to GPX4 inhibition to suppress lethal lipid peroxidation. FSP1, GCH1 and BH4/BH2 are proteins with the ability to inhibit ferroptosis independently of GPX4 and GSH levels (54, 55, 65), and FSP1 is a *druggable* protein (54). In line with this idea, a new avenue has been opened for the development of drugs that include SLC7A11, GPX4 and FSP1 inhibitors with potential *in vivo* application as a cancer treatment.

AUTHOR CONTRIBUTIONS

LF, MB, KS, AG and FN conceived the ideas and concepts. LF wrote the article. AG, KS, MV and FN critically revised the manuscript. MB generated the scientific illustrations. All authors approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Ferroptosis in Glioma Immune Microenvironment: Opportunity and Challenge

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Glioma is the most common intracranial malignant tumor in adults and the 5-year survival rate of glioma patients is extremely poor, even in patients who received Stupp treatment after diagnosis and this forces us to explore more efficient clinical strategies. At this time, immunotherapy shows great potential in a variety of tumor clinical treatments, however, its clinical effect in glioma is limited because of tumor immune privilege which was induced by the glioma immunosuppressive microenvironment, so remodeling the immunosuppressive microenvironment is a practical way to eliminate glioma immunotherapy resistance. Recently, increasing studies have confirmed that ferroptosis, a new form of cell death, plays an important role in tumor progression and immune microenvironment and the crosstalk between ferroptosis and tumor immune microenvironment attracts much attention. This work summarizes the progress studies of ferroptosis in the glioma immune microenvironment.

Keywords: glioma, ferroptosis, immune microenvironment, immunotherapy, GPX4

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INTRODUCTION

Glioma is a threatening primary malignancy tumor in the central nervous system (1, 2), which is divided into grades I-IV according to WHO standard with glioblastoma (WHO grade IV) as the most malignant and common subtype (3). The standard therapy for glioma patients is the Stupp protocol, which consists of maximal safe surgical resection or a diagnostic biopsy, followed by concurrent chemoradiotherapy and then maintenance chemotherapy, where chemotherapy is comprised of temozolomide (4). Although glioblastoma (WHO IV) patients receive the most effective treatment/surgery with radiotherapy and chemotherapy after diagnosis (5, 6), the median survival time is only about 18 months (7), and that is mainly the result of a glioma infiltration boundary and/or the resistance of chemotherapy. Consequently, new therapeutic approaches for glioma are urgently needed (8).

Recently, immunotherapy represented by PD-1/PD-L1 and CTLA-4 has shown excellent clinical effects on numerous tumors such as melanoma and non-small cell lung cancer (9, 10), which have rekindled researchers' faith in glioma treatment. Unfortunately, its effect is extremely limited in glioma and relevant clinical data show that it works on less than 10% of glioblastoma patients (11) An increasing number of studies have confirmed that it is a result of the glioma immunosuppressive

microenvironment (12), therefore, the distruption of the immunosuppressive microenvironment and revision of the glioma from a 'cold tumor' to a 'hot tumor' is practical to relieve the glioma immunotherapy resistance (8).

The glioma immune microenvironment is composed of glioma cells, immune cells, cytokines and so on (12). Glioma cells can recruit numerous kinds of cell including immune cells that move to the niche by secreting cytokines (like TGF-β, GM-CSF) (13, 14) and then revise these cells into 'tumor-friendly' phenotypes (15). In this case, the recruited cells may serve as a physical barrier to prevent later immune cells from approaching and attacking the tumor cells Also, the recruited immune cells can also secrete cytokines (such as IL1-β, TGF-β) that continue to assimilate later recruited immune cells as 'tumor-friendly' phenotypes (16, 17). Under this "snowball" interaction, coupled with the unique central nervous system microenvironment, such as the blood-brain barrier (18) and hypoxia (19, 20), acidic tumor microenvironment (2, 21), tumor cells can escape immune surveillance (22) and eventually set the glioma immunosuppressive microenvironment (23, 24).

Ferroptosis is a form of regulated cell death driven by lipid peroxidation, a consequence of imbalance between cell metabolism and redox homeostasis (25). It is different from other cell death such as apoptosis, pyroptosis in morphology, biochemistry and gene (26). Its key process is phospholipids with polyunsaturated fatty acyl tails (PUFAs) are oxidized in an iron- or oxidoreductase- dependant way and ultimately induce cell death (27). Recently, researchers found that activating ferroptosis could improve temozolomide treatment effectiveness in GBM-bearing mice (28), and lonizing radiation could induce cell ferroptosis. The above means that ferroptosis is vital for glioma chemotherapy and radiotherapy (29).

OVERVIEW OF FERROPTOSIS AND POTENTIAL SINGLING PATHWAY IN GLIOMA

The main characteristics of ferroptosis include: cell morphology (mitochondria crista, volume reduction, and increase of membrane density); cellular composition [cellular ROS is elevated and lipid peroxidation is significantly increased (27)]. Meanwhile, the intracellular pool of antioxidant executor (GSH or/and glutathione peroxidase 4) was shrunk, and phospholipid peroxide (PLOOH) is the executive driver of ferroptosis (26, 27). With step by step studies, researchers found that ferroptosis could be regulated by a variety of ways including redox homeostasis (30), iron metabolism (31), mitochondrial activity (32), metabolism of amino acids, lipids, and glucose (33). Ferroptosis pathways can be broadly divided into glutathione peroxidase 4 (GPX4) -dependent and -independent pathways (25, 26) (**Figure 1**).

GPX4 Dependent Ferroptosis Pathway

Glutathione peroxidase 4 (GPX4), also known as phospholipid hydrogen peroxide glutathione peroxidase (PHGPx), is a selenoprotein required for peroxidized phospholipids (34). Cystine/glutamic acid reverse transporter (system x_c^-) is an upstream regulator (25, 35) and its dysfunction can increase glutamic acid levels and reduce cystine levels (36), which in turn leads to the exhaustion of the intracellular pool of glutathione (GSH), the main reducing substance of human body (37). Subsequently, this causes GPX4 reduction (27), then induces more PUFAs to turn to PLOOH and eventually induces ferroptosis (25). Besides, lonizing radiation also could regulate GPX4 activity directly and then shape ferroptosis (38).

System x_c^- plays an important role in GPX4 relative pathway, whether the system x_c^- dysfunction could result in the pool of GSH and GPX4 shrinking (35), and then gives birth to intracellular PLOOH explode and ultimately induces ferroptosis (26, 33). The monitors regulating system x_c^- are SLC7A11 (39), SLC3A2 (40), NRF2 (41) and so on (42). Stephanie demonstrated that SLC7A11 expression is associated with seizures and predicts poor survival in patients with malignant glioma (43) Ju et al. proved that NRF2 is a potential prognostic biomarker and is correlated with immune infiltration in the brain's lower grade glioma (44). Long et al. found that dysregulation of system x_c^- enhances Treg function that promotes VEGF blockade resistance in glioblastoma (45). The above indicates that system x_c^- should be a key hub between ferroptosis and the glioma immune-microenvironment.

Cystine metabolism is a vital segment in the GPX4-dependent ferroptosis pathway and the main factors affecting cystine metabolism include the transsulfuration pathway and/or the methionine cycle (46). As a vital brick for GSH synthesis, cystine plays a key role in glioma progression, Liu et al. confirmed that methionine and cystine double deprivation stress suppresses glioma proliferation by inducing reactive oxygen species (ROS) and autophagy (47), Wang et al. demonstrated that methionine deprivation can reset numerous immune pathways such as macrophages, T cell activation pathways in glioma (48), as cystine and methionine are all in methionine cycle (49), and there should be cystine/methionine-ferroptosis-immunity related pathways. Simultaneously, glioma cells can selectively uptake methionine, cysteine, and serine (47, 50, 51), so other cells will uptake or store less of these amino acids than glioma cells, which limits the production of cysteine and GSH. It remains to be determined whether it would induce other cells to include immune cells more sensitive to ferroptosis than glioma cells and whether DNA/RNA methylation is vital for glioma escape ferroptosis, as methionine is the major methyl donor (52, 53). Unfortunately, the researchers did not conduct this corresponding work.

In addition, the mevalonate pathway also participated in GPX4 activity regulation and isopentenyl pyrophosphate was the core factor regulating the transcription efficiency of GPX4 (54). E. Cimini et al. has confirmed that zoledronic acid, an aminobisphosphonate drug, can inhibit glioma cell proliferation by interfering with mevalonate pathway of Vγ2 T-cells (55). Deven found that LXRβ knockdown decreased cell cycle progression, cell survival, and decreased feedback repression of the mevalonate pathway in densely-plated glioma cells. LXRβ regulates the expression of immune response gene sets and lipids known to be involved in immune modulation (56) and these

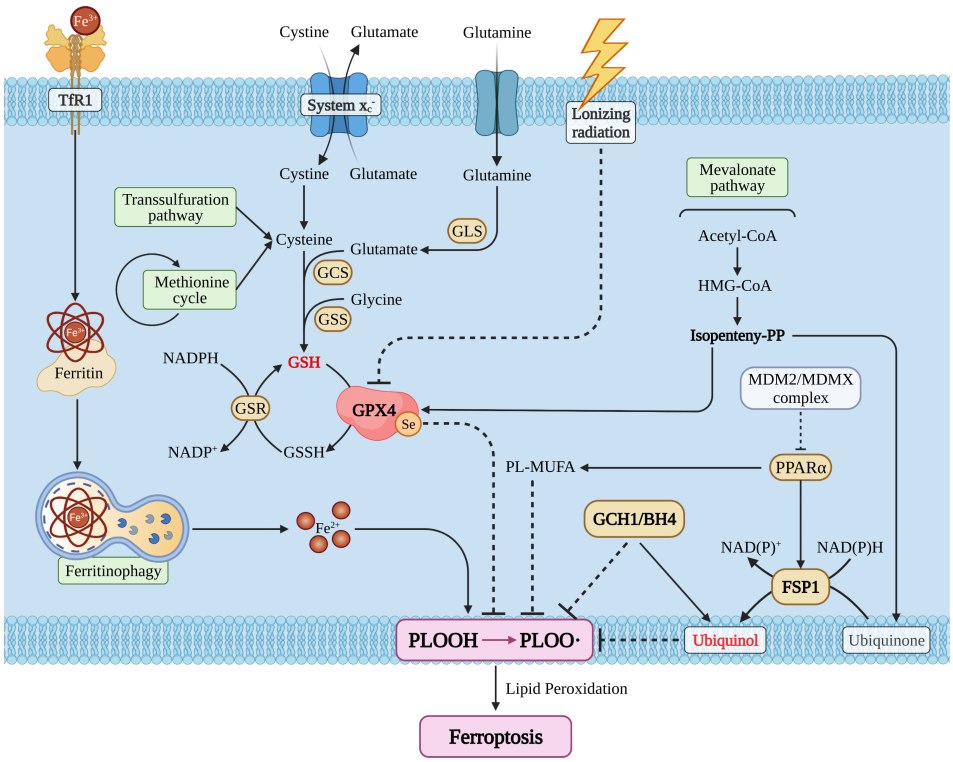

FIGURE 1 | The snapshot of ferroptosis pathways. TfR1, transferrin receptor 1; GLS, glutaminase; GCS, glutamylcysteine synthetase; GSS, glutathione synthetase; GSH/GSSH, glutathione; GSR, glutathione S-reductase; GPX4, glutathione peroxidase 4; MDM2, mouse double minute 2; MDMX, mouse double minute 4; PPARα, peroxisome proliferator activated receptor alpha; FSP1, ferroptosis suppressor protein 1; GCH1, GTP cyclohydrolase 1; BH4, tetrahydrobiopterin; PL, phospholipid; MUFA, monounsaturated fatty acid.

works imply that targeting the mevalonate pathway could disturb ferroptosis and immunity in glioma.

Currently, researchers have demonstrated that lonizing radiation could consume GSH, inhibit GPX4 activity, and induce ferroptosis (25, 54), and they denote that ferroptosis should be essential for glioma treatment because radiotherapy is an important part of Stupp strategy (8). Zhang et al. revealed that inhibition of TAZ contributes to radiation-induced senescence and growth arrest in glioma, and immune-related genes are specifically affected as the long-term effect (57). However, we did not know whether ferroptosis cells would act as or release cytokines that induce glioma cells to adapt to radiotherapy resistance.

GPX4 Independent Ferroptosis Pathway

Although GPX4 is the core molecule of ferroptosis, we have now found other pathways that influence PLOOH synthesis and ferroptosis (25, 26).

The first is the ferroptosis inhibition protein 1(FSP1) (58–60), which can reduce the mevalonate pathway produced ubiquinone translate to ubiquinol, suppress production of PLOOH, and eventually inhibit ferroptosis (58). Furthermore, FSP1 could also be activated by the MDM2/MDMX-PPARα axis (25, 61), and in addition to activating FSP1 functions, PPARα also regulates the conversion of PL-MUFA to PLOOH by ACSL3-mediated MUFA way. It has been reported that FSP1 can protect cells from ferroptosis which is induced by GPX4 inhibition/knockout (26). Zou et al. demonstrated that TGF−β1 increases FSP1 expression in human bronchial epithelial cells (62), as TGF−β1 is an important cytokine that can be secreted by glioma or/and immune cell (17, 63, 64), and it means that FSP1 could be a nexus between glioma or/and immune cell ferroptosis.

A critical factor in inducing ferroptosis is the imbalance of intracellular iron metabolism which could cause iron overload. Through the specific receptor TFR1 (transferrin receptor 1), circulating iron (Fe^{3+}) can be imported into the cell and stored mostly within ferritin (Fe^{3+}), changing to cytoplasmic iron (65). A small pool of cytoplasmic free Fe^{2+} could directly catalyze the formation of free radical formation *via* the Fenton Reaction where changes of ferritin expression levels affect the homeostasis of iron metabolism by altering the intracellular free and redox active iron pool. Researchers have reported that the overexpression of NCOA4 reinforces the degradation of ferritin, which releases excessive cytoplasmic free Fe^{2+} and subsequently, promotes ferroptosis (66).

As a "double-edged sword", autophagy is crucial in glioma progress (67, 68) due to the unbridled proliferation tumor cells that require a large amount of nutrients. Also, an appropriate

level of autophagy is conducive to ensure necessary cellular function, as their own bricks, could be reused but excessive levels of autophagy will induce cell 'self-digestion' and eventually induce glioma death (69). Recent studies have proven that autophagy can participate in ferroptosis and the main progress is called ferritinophagy (25), and the key interaction hub of these two pathways is NCOA4 and FTH1. Zhang et al. confirmed that COPZ1 is the key molecule that mediates autophagy-dependent ferroptosis in glioma (70). Meanwhile, autophagy is essential for immune cell proliferation and function, and Enyong confirmed that autophagy-dependent ferroptosis drives tumor-associated macrophage polarization *via* the release and uptake of the oncogenic KRAS protein (71). Sun et al. confirmed that autophagy-dependent ferroptosis-related signature is closely associated with the prognosis and tumor immune escape of patients with glioma (72). Therefore, we recognize autophagy should be one of the hubs between ferroptosis and immunity in glioma.

Moreover, researchers have also confirmed that GTP cyclohydrase 1 (GCH1) inhibits the production of PLOOH through its metabolite BH2/4. Meanwhile, BH4 could also reduce PLOOH pool by regulating the production of Ubiquinol (26). Anh proved that the GCH1 knockdown with short hairpin RNA led to GBM cell growth inhibition and reduced self-renewal in association with decreased CD44 expression (73). Yan et al. showed that blocking CD44 inhibited glioma cell proliferation by regulating autophagy (67) and this means GCH1 could induce glioma cell ferroptosis and influence immunity by autophagy.

Furthermore, AMPK associated energy stress and Hippo pathways are all associated with ferroptosis by regulating the PLOOH pool (25, 26), and these factors are also vital for the glioma immunosuppressive microenvironment (GIME) and glioma proliferation (24).

FERROPTOSIS AND IMMUNE MICROENVIRONMENT

Inducing tumor cell death is one of the effective methods to treat cancer, so inducing cancer cell ferroptosis is a feasible way for glioma treatment (34). Dead cells can release a series of "find me" and "eat me" signals for immune cells to locate, migrate, and clean dead cells which is confirmed by the phenomenon that ferroptosis tumor cells can be effectively engulfed by macrophages *in vitro* (74). The calreticulin (CRT), a soluble ER-associated chaperone, is one of the ferroptosis-mediated proteins which regulate the tumor microenvironment. Ferroptosis facilitates the translocation of CRT to expose it on the surface of tumor cells, where CRT could serve as a potent "eat-me" signal and induce a robust antitumor immune response (75). However, the signal communication between ferroptosis glioma cells and surrounding immune cells is not clear (76) (**Figure 2**).

The potential signal is the arachidonic acid (AA) oxidation product released by ferroptosis cells therefore, it has been hypothesized that lipoxygenases (LOXs) can not only induce the PUFAs production but also promote ferroptosis cells to release immune signals and regulate tumor immunity (26). A study has shown that ferroptosis cells can release eicosanoids (5-HETE, 11-HETE, 15-HETE, etc.) when GPX4 was suppressed. Contrarily, ferroptosis cells reduce the production of pro-inflammatory lipids when GPX4 activity was increasing, afterwards inhibiting the production of TNF and IL-1 β by the NF-kB pathway (77). A liposome analysis offerroptosis cells found that the accumulation of oxygenated AA-containing phosphatidylethanolamine species was associated with ALOX15 (78), which can shape adaptive immune response by inhibiting dendritic cell maturation and T cell helper cell 17 (TH17) differentiation *via* activating transcription factor NRF2 (79).

Prostaglandin E2 (PGE2) is considered to be one of the important immunosuppressive factors and it can be released after most death cells (26, 80) and then disturb immune cells mainly in the following ways: 1. directly inhibit NK, cytotoxic T cell clean function (81, 82), 2. inhibit the infiltration of Conventional Type 1 dendritic cell (cDC1) into tumor niche *via* inhibiting the secretion of CCL5 and XCL1 by NK cells (83), and 3. inhibit cDC1-dependent $CD8^+$ T cell-mediated immune response (84). Yoshiteru proved that inhibition of macrophagic PGE2 synthesis is an effective treatment for the induction of anti-glioma immune response (85).

Recent studies have confirmed that GPX4 activity is associated with chronic inflammation (26), and current studies have confirmed that glioma progression is related to chronic inflammation (86). Moreover, Xu et al. demonstrated that GPX4 is crucial for protecting activated Treg cells from lipid peroxidation and ferroptosis and offered a potential therapeutic strategy to improve cancer treatment (87). All of the above has to remind us that GPX4 may be a hub to connect ferroptosis and inflammation/immune in glioma.

In addition to releasing lipid mediators, ferroptosis cells can also release HMGB1 in an autophagy-dependent manner (88). HMGB1 belongs to DAMPs and is one of the key elements for tumor cell immunogenicity, as it will bind to its receptor and activate the immune system once it is released outside of cell (89). Wen et al. confirmed that RAGE is essential for HMGB1 mediated TNF releasing in macrophage when they respond to ferroptosis cells (90). Lowenstein et al. considered that HMGB1-activated dendritic cells, loaded with glioma antigens, migrate to cervical lymph nodes to stimulate a systemic $CD8^+$ T cells cytotoxic immune response against glioma and induce immunological memory (91).

In addition to the above cytokines, there are other cytokines worth exploring (76). Although researchers believe that the cytokines are critical for the "crosstalk" between ferroptosis cells and immune cells, the mechanism remains unclear. Additionally, attention should also be paid to off-target effects of ferroptosis induction (92).

CHALLENGES OF FERROPTOSIS IN GIME

The glioma immunosuppressive microenvironment (GIME) is the main reason for poor efficacy of immunotherapy in glioma (8, 22, 23). The rapid proliferation of glioma causes an arduous microenvironment such as acidity, limited of nutrients, and

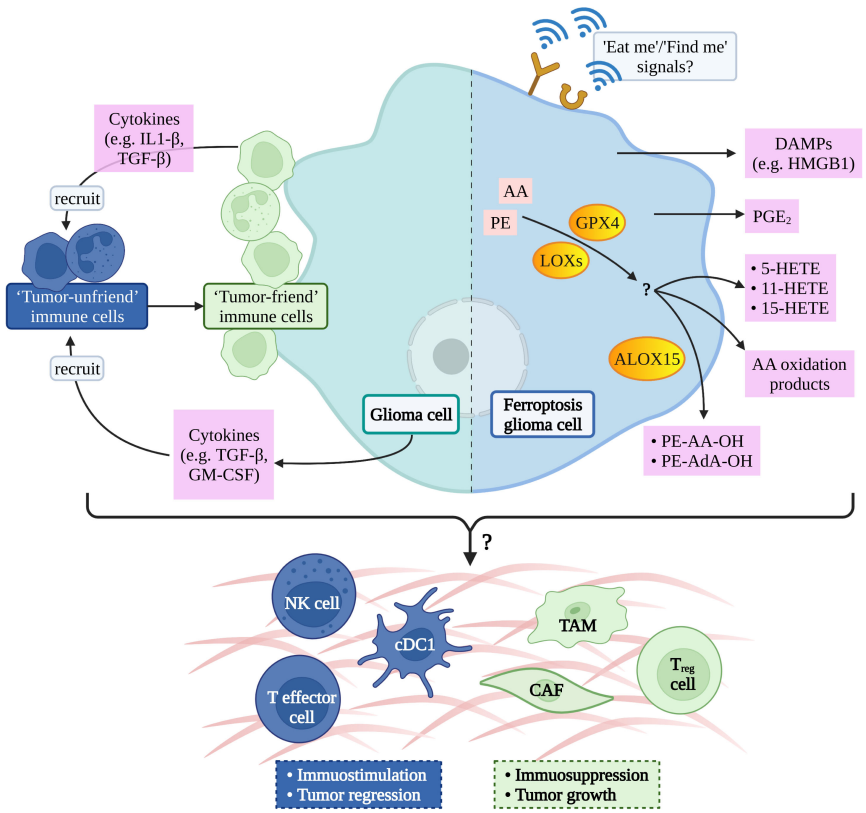

FIGURE 2 | Possible ferroptotic signals in glioma immune-microenvironment. AA, arachidonic acid; PE, phosphatidylethanolamine; GPX4, glutathione peroxidase 4; LOXs, lipoxygenases; ALOX15, arachidonate lipoxygenase 15; DAMPs, damage-associated molecular patterns; HMGB1, high mobility group box-1; PGE2, prostaglandin E2; HETE, hydroxy eicosatetraenoic acid; AdA, adrenic acid; IL1, interleukin-1; TGF, transforming growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; NK, natural killer; cDC1, conventional type 1 dendritic cell; TAM, tumor- associated macrophage; CAF, cancer-associated fibroblast; T reg cell, regulatory T cell.

oxygen (47, 93, 94). In this circumstance, immune cells will betray, retreat, or die as they cannot adapt (95, 96) but glioma can adapt to this harsh microenvironment due to their own tremendous plasticity (97, 98). The blood-brain barrier can also hinder immune cells from migrating to the tumor (99, 100). Also, many of the inhibitory cytokines secreted by glioma (101) and inhibitory immune cells suppress the antitumor effect of immune cells (21, 102). In addition, glioma cells can also secrete numerous cytokines to trap immune cells as they present 'non-tumor cells' markers (95, 103) and then these "tricked" cells secrete cytokines and continue to later recruit immune cells (104). In this circumstance, glioma cells escape immune surveillance (22, 105)and we should take the above into account when considering glioma immunotherapy (106). Meanwhile, immunotherapy combination regimens (22), administration mode, and timing (107) can also influence the therapeutic efficacy. Currently, accruing studies demonstrate that ferroptosis is crucial for tumor progression and targeting ferroptosis maybe a latent way to remodel the tumor immune microenvironment (26, 27, 34). While we have already done a brief description above, we should also recognize the challenges.

Although ferroptosis does play a crucial role various tumor immune microenvironments, its own mechanism is still unclear (25, 26), which is reflected on the following aspects: 1. The exactly mechanism of PLOOH in ferroptosis is unclear. At present, although it is clear that PLOOH is the ultimate executor of ferroptosis, the exact mechanism of PLOOH inducing ferroptosis is unknown (26); 2. Ferroptosis studies lack a 'gold standard'. Although we have made great progress in ferroptosis study (26), we have not yet found a relative "gold standard" like LC3, and P62 in autophagy (108) and researchers usually select one or more targets such as GPX4, P53, FTH1 (109–111) in a paper, even worse the targets just like scraped together, which has troubled the following researchers. 3. Ferroptosis shows a 'double-edged sword' role in diseases. It is easy to understand that ferroptosis plays different roles in different diseases such as the beneficial outcome of inducing ferroptosis in tumor cells is for disease (112), but inhibiting ferroptosis in stroke is beneficial to the prognosis (113). We hypothesize that ferroptosis may also play different roles in one disease, for example, and there may be tumor cells that choose to sacrifice themselves. Then, the secreted cytokines can make the surrounding tumor cells in a stress state and finally avoid ferroptosis (26). 4. What and how ferroptosis cells release signals after death and what are the functions of these signals

(33, 71). 5. The crosstalk between ferroptosis and other forms of death is not clear (26, 27, 33). For cells that may suffer from different kinds of death at the same time (114), it is unknown how can they communicate with each other or whether ferroptosis works more or less in cell death. This is because we found cells suffer ferroptosis but cells still died after we used ferroptosis inhibitors in a proper dose. This means that ferroptosis can induce other kinds of cell death, and/or it plays a minor effect in cell death, or we use inhibitors after the 'reversible point' and once this threshold is exceeded, ferroptosis will be irreversible

Recently, we also found that ferroptosis is vital for tumor immunity such as macrophage phagocytosis (71) and T cell killing (115) but the "crosstalk" between ferroptosis and the glioma immunosuppressive microenvironment is not clear. Additional issues to be addressed are: 1. The signal interaction between ferroptosis cells and surrounding immune cells is not clear which is mainly manifested in the specific cytokines of 'find me' and 'eat me' released by ferroptosis cells (26). 2. Will the cytokines released by ferroptosis cells help other glioma cells escape immune surveillance by seducing or misleading immune cells (34, 88, 103)? 3. Whether GPX4-induced chronic inflammation engaged in glioma progression or outcome (116). 4. What is the role of ferroptosis in glioma immunotherapy tolerance? (95, 103).

CONCLUSION

The clinicians and researchers are always trying to find new treatments for tumors and it is comforting that treatment methods such as immunotherapy and oncolytic virus have been found. Unfortunately, immunotherapy, which has shed light on numerous tumor treatments, does not always work regarding glioma. Increasing research demonstrates that this is result of the glioma immunosuppressive microenvironment, so researchers are searching for an antidote for remodeling GIME. Ferroptosis, a new form of cell death, plays an important role in glioma cell and immune cell. The exactl mechanism is unclear and multipley works demonstrate that it is deserved to explore its role in GIME and how to regulate ferroptosis for glioma therapy. Although there are still many obstacles in the cognition of crosstalk between ferroptosis and GIME, we believe we will address this with further studies and new technologies, such single cell sequencing and spatial transcriptomics. This will not only improve our understanding of ferroptosis and GIME but also provide a new solution for glioma immunotherapy resistance, a new breakthrough point for glioma treatment.

AUTHOR CONTRIBUTIONS

KW proposed the research. KW, JW, and JHZ wrote the manuscript and finalized the paper. AZ, YL, and JYZ both reviewed the literature and collected references. XW and JMZ reviewed the literature. All authors contributed to the article and approved the submitted manuscript.

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Mechanisms of long non-coding RNAs in biological phenotypes and ferroptosis of glioma

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Glioma, one of the most common malignant tumors in the nervous system, is characterized by limited treatment, high mortality and poor prognosis. Numerous studies have shown that lncRNAs play an important role in the onset and progression of glioma by acting on various classical signaling pathways of tumors through signaling, trapping, guiding, scaffolding and other functions. LncRNAs contribute to the malignant progression of glioma *via* proliferation, apoptosis, epithelial-mesenchymal transformation, chemotherapy resistance, ferroptosis and other biological traits. In this paper, relevant lncRNA signaling pathways involved in glioma progression were systematically evaluated, with emphasis placed on the specific molecular mechanism of lncRNAs in the process of ferroptosis, in order to provide a theoretical basis for the application of lncRNAs in the anticancer treatment of glioma.

KEYWORDS

lncRNAs, glioma, phenotypes, ferroptosis, mechanism

Introduction

Human glioma, histologically originates from the neuroectoderm and is recognized as the most prevalent and lethal intracranial tumor, accounting for more than 50% of cerebral tumors (1, 2). Based on the malignancy characteristics, glioma can be classified as WHO grade I-IV. In addition, the latest version of the WHO classification divides

glioma into more biologically and molecularly defined pathological subtypes (3). Currently, glioma is still difficult to treat. Although the current therapeutic schemes have advanced in operation, radiotherapy and chemotherapy, the prognosis of glioma patients still remains pessimistic due to the high rates of relapse and inevitable metastasis (4). Thus, it is critical to determine the exact molecular mechanisms leading to glioma onset and progression.

Long non-coding RNAs (lncRNAs) are the molecules that are more than 200 nucleotides in length and have no/little protein-coding functions/potentials and/or lack open reading frames. LncRNAs can modulate gene expression at the transcriptional or post-transcriptional levels (5), and several lines of evidence have shown that lncRNAs have been associated with the occurence and development of many human tumors, including glioma. More specifically, lncRNAs are involved in modulating the development of malignant glioma cells by altering cellular proliferation, apoptosis, drug resistance and ferroptosis (6). LncRNAs have been associated with the onset and development of many human malignant tumors, including glioma.

Ferroptosis is a cell death pathway characterized by iron dependency and excessive lipid peroxidation, making it unique in comparison to other cell death process such as apoptosis, necrosis and pyroptosis. According to the recent 2018 consensus derived from the nomenclature committee on cell death, ferroptotic cell death is a type of regulated cell death (RCD), in contrast to accidental cell death (ACD) which is caused by physical, chemical or other factors (7). Ferroptosis is a double-edged sword that plays a dual role in tumors *via* damage-associated molecular patterns (DAMPs) (8, 9). In this review, we summarized the functions and mechanisms of lncRNAs in the genesis and malignant development of glioma. We conclude that lncRNAs play a crucial role in glioma ferroptosis, contributing to deeply understand glioma pathogenesis and provide future directions for others.

Classification and molecular mechanism of lncRNAs

According to their position in the reference genome, lncRNAs can be classified into the following five categories: sense, antisense, bidirectional, intronic and intergenic (10). According to their mechanism of action, lncRNAs can be divided into five functional types, and each lncRNA can coexist with multiple functions (**Figure 1**). 1) Molecular guide. LncRNAs act as molecular guides of ribonucleoprotein complexes to specific sites on chromatin (11). 2) Scaffolds. LncRNAs bind different effector molecules as binding scaffolders of protein complexes, and combine with these effector proteins to jointly regulate gene transcription in time and space (12). 3) Signals. LncRNAs stimulate a variety of signaling molecules to regulate their downstream signaling pathways during cell transcription (13). 4) Competitive endogenous RNAs (ceRNAs).

LncRNAs can be used as miRNA sponges that contain miRNA binding sites where miRNAs are sequestered, inhibiting miRNA target genes (14). 5) Molecular decoys. LncRNAs can act as molecular decoys *via* allosteric binding to specific proteins to inhibiting the function of downstream proteins (15).

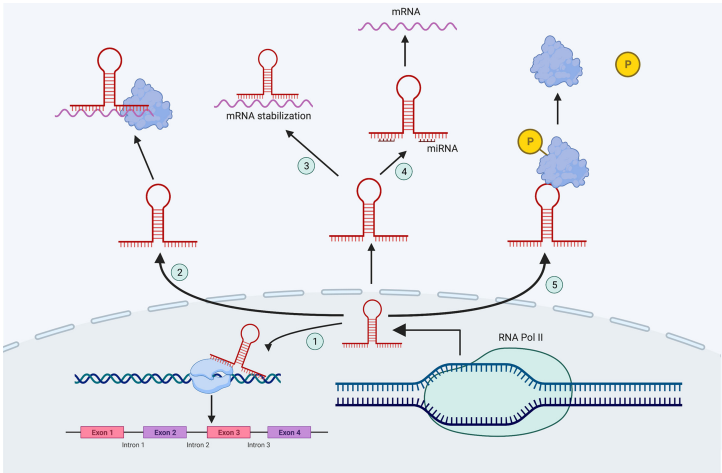

FIGURE 1
The mechanisms of lncRNAs. ① Molecular guide; ② Scaffolds; ③ Signals; ④ CeRNAs; ⑤ Molecular decoys.

LncRNAs regulate the malignant progression of glioma

Abnormal expression of lncRNAs in glioma has been revealed to be interrelated tightly with the prognosis of glioma patients including overall survival (OS) and survival quality. For instance, lncRNA ROR1-AS1 was up-regulated in glioma and indicated a poor clinical outcome. Kaplan–Meier curves indicated that the 5-year survival rate of glioma patients was obviously higher in patients with lower ROR1-AS1 expression (16). Moreover, LINC01494 was over-expressed in glioma and was associated with a poor prognosis in glioma patients (17).Chen et al. analyzed the relationship between the expression of lncRNA CPS1-IT1 and the pathological characteristics of glioma, and found that low expression of lncRNA CPS1-IT1 led to elevated WHO grade and poor prognosis (18). In addition, the expression of lncRNA CASC7 was related to glioma progression and WHO stage, and was positively correlated with patient prognosis (19).

LncRNAs and the proliferation of glioma

As one of the most important biological characteristics of tumor cells, cellular proliferation determines the occurrence and development of tumors. Previous studies have shown that lncRNAs ultimately affect the proliferation of glioma cells through a series of downstream pathways (**Table 1**).

P21, as a cyclin-dependent kinase inhibitor encoded by the CDKN1A gene, inhibits the formation of the CDK2-CDK1 complex and mediates the G1 phase arrest of p53- dependent cell cycle. Numerous studies have shown that lncRNAs regulated the cell cycle by affecting the expression of p21 protein, leading to the proliferation of glioma cells. For example, a study (20) comparing 108 glioma to control tissue samples found that lncRNA SNHG20 was highly expressed in glioma tissue and negatively correlated with patient prognosis. Further study of the specific mechanism showed that lncRNA SNHG20 accelerated the G0/G1 cycle by reducing p21 transcription, ultimately leading to the glioma cell proliferation. LncRNA SNHG3 was also highly expressed in glioma tissues and promoted the proliferation of glioma cells. It recruited EZH2 to the promoters of KLF2 and p21, and epigenetically inhibited KLF2 and p21 (21).

Moreover, lncRNA SNHG6 and SNHG16 promoted the proliferation of glioma cells *via* reducing p21 mRNA levels (22, 23). In addition to small nucleolar RNA host gene (SNHG), lncRNA RP11-732M18.3 induced the degradation of p21 and increased the proliferation of glioma cells. It recruited 14-3-3β/α to UBE2E1, and the binding of 14-3-3β/α to UBE2E1 enhanced the degradation activity of UBE2E1 on p21 *via* ubiquitination (24).

Another important molecular pathway activated during the development of human cancers, including glioma, is the Wnt/β-catenin signaling pathway (31, 32). LncRNA ADAMTS9-AS1 was confirmed to be involved in the positive regulation of Wnt/β-catenin signaling pathway in glioma, leading to glioma cell proliferation (25). Zhou et al. showed that lncRNA H19, as a ceRNA, directly bound to miR-342 and inhibited its expression. Knockdown of miR-342 in turn promoted Wnt5a and β-catenin expression to positively regulated the Wnt5a/β-catenin signaling axis and glioma cell proliferation (26). Similarly, lncRNA CTBP1-AS2 also functioned as a ceRNA and specifically bound miR-370-3p to inhibit its expression. Sequestration of miR-370-3p by CTBP1-AS2 prevented miR-370-3p 3'UTR binding and disinhibition of Wnt7a, and miR-370-3p knockdown activated Wnt7a/β-catenin signaling. Both actions accelerated the proliferation of glioma cells (27).

Several investigations have demonstrated that inhibition of the PI3K/AKT signaling pathway blocked cellular proliferation and played an anti-tumor role by inhibiting the cell cycle and inducing apoptosis (33, 34). For instance, lncRNA SNHG20

TABLE 1 Representative lncRNAs and related signaling pathways in glioma proliferation.

LncRNA	Expression	Downstream Targets	Proliferation	References
SNHG20	upregulated	P21, CCNA1	promote	(20)
SNHG3	upregulated	EZH2, KLF2, P21	promote	(21)
SNHG6	upregulated	P21	promote	(22)
SNHG16	upregulated	P21, caspase 3/9, cyclinD1/B1	promote	(23)
RP11-732M18.3	upregulated	14-3-3β/α, UBE2E, P21	promote	(24)
ADAMTS9-AS1	upregulated	Wnt/β-catenin pathway	promote	(25)
H19	upregulated	miR-342, Wnt5a/β-catenin pathway	promote	(26)
CTBP1-AS2	upregulated	miR-370-3p, Wnt7a/β-catenin pathway	promote	(27)
SNHG20	upregulated	PTEN/PI3K/AKT pathway	promote	(28)
XIST	upregulated	miR-126, IRS1/PI3K/Akt pathway	promote	(29)
LBX2-AS1	upregulated	PI3K-Akt-GSK3β pathway	promote	(30)

promoted the activation of the PI3K/AKT signaling pathway and accelerated the proliferation of glioma cells by inhibiting PTEN (28). LncRNA XIST, a molecular sponge of miR-126, promoted glucose metabolism and led to the glioma cell proliferation through regulation of the IRS1/PI3K/Akt pathway (29). LncRNA LBX2-AS1 knockdown caused a significant decrease in both GSK3β and Akt phosphorylation, suggesting that it promoted cell proliferation by activating the PI3K-Akt-GSK3β pathway (30).

LncRNAs and apoptosis in glioma

Apoptosis, or programmed cell death, is strictly regulated at the genetic level, resulting in the orderly and efficient elimination of damaged cells (35). As an important biological process of cell metabolism, apoptosis is affected by many factors and is involved in the activation, expression and regulation of a series of genes. Dysfunctional apoptosis is closely related to tumorigenesis. At present, lncRNAs have been confirmed to activate or inhibit the apoptosis of glioma cells through downstream molecules (**Table 2**).

Traditionally, p53-induced apoptosis was considered a main mechanism that inhibited tumor development by regulating downstream target genes. At present, numerous P53 target genes are involved in apoptosis regulation, which can be mainly divided into two categories: death receptor family and the bcl-2 family. Numerous studies have shown that lncRNAs regulated the expression and degradation of p53 through a variety of downstream molecules, thus affecting the glioma cell apoptosis. LncRNA FOXD2-AS1 was significantly upregulated in glioma tissues and mainly distributed in the nucleus. By binding to EZH2, FOXD2-AS1 weakened the recruitment ability of p53, thus inhibiting glioma cell apoptosis and promoting malignant progression of glioma (36). LncRNA SNHG20 increased MDM2 level by binding miR-4486, which enhanced the degradation of P53 protein and ultimately inhibited the apoptosis of glioma cells (37). In addition, studies have confirmed that p53 binds to the lncRNA ST7-AS1 promoter to increase its transcription. Subsequently, lncRNA ST7-AS1 regulated p53 expression by binding to PTBP1, and forming a positive feedback loop to inhibit the progression of invasive glioma (38).

Apoptosis is regulated by many genes, among which the bcl-2 and caspase families are the most important. Bcl-2 and bax genes are important regulatory apoptotic genes that act antagonistically to each other in apoptosis regulation, and caspase-3 is a critical apoptotic execution protease. Numerous studies have proved that lncRNAs regulate the apoptosis of glioma cells by acting on them. Specifically, LncRNA ANCR regulated PTEN expression *via* binding and interacting with EZH2, thus inhibiting the apoptosis of glioma cells. Moreover, high expression of lncRNA ANCR reduced bax expression and promoted bcl-2 expression to produce an anti-apoptotic effect (39). Similarly, lncRNA LOC101928963 inhibited PMAIP1 expression, which also induced bcl-2 and reduced bax expression, and ultimately inhibited the apoptosis of glioma (40). Furthermore, lncRNA GAS5 increased Caspase-3/7 activity and promoted apoptosis *via* regulating GSTM3 (41). LncRNA PCED1B-AS1, on the other hand, inhibited caspase-3 activity *via* miR-19-5p/PCED1B axis, thereby activating glioma proliferation and limiting apoptosis (42).

A large amount of evidence has confirmed the strong correlation between P53 and lncRNAs, and these lncRNAs regulate tumor apoptosis as regulatory factors or effectors of P53. In addition, Liu et al. reported multiple lncRNAs expression levels under various antitumor drugs. By detecting the expression changes of lncRNAs in doxorubicin and resveratrol treated glioma cells, MIR155HG was up-regulated in response to resveratrol-induced apoptosis, GAS5 was up-regulated during doxorubicin-induced apoptosis, and MEG3 and ST7OT1 were up-regulated under apoptosis induced by both agents (43). These results indicate that lncRNAs can be used as targets of multiple chemotherapy drugs to promote glioma cell apoptosis, and a more complete lncRNAs action network is conducive to the development of more therapeutic targets and new chemotherapy drugs.

TABLE 2 Representative lncRNAs and related signaling pathways in glioma apoptosis.

LncRNA	Expression	Downstream Targets	Apoptosis	References
FOXD2-AS1	upregulated	EZH2, P53 pathway	inhibit	(36)
SNHG20	upregulated	miR-4486, MDM2-P53 pathway	inhibit	(37)
ST7-AS1	downregulated	PTBP1, Wnt/β-catenin pathway	promote	(38)
ANCR	upregulated	PTEN, EZH2, Bax, Bcl-2	inhibit	(39)
LOC101928963	upregulated	PMAIP1	inhibit	(40)
GAS5	downregulated	GSTM3, Caspase 3/7	promote	(41)
PCED1B-AS1	upregulated	miR-19-5p, PCED1B	inhibit	(42)

LncRNAs and the EMT process in glioma

Epithelial-mesenchymal transformation (EMT) refers to the transformation of epithelial-to-mesenchymal cells and is recognized as an integral part of glioma invasion and migration. EMT is characterized by the loss of cell adhesion, changes in cytoskeletal components and the acquisition of migration and invasion characteristics (44). In addition to the invasion process, apoptosis, chemotherapy and immunotherapy resistance during glioma progression are also involved in EMT (45). EMT regulation is a complex network that includes multiple signaling pathways involving the TGFβ family, Wnts, Notch, EGF, HGF, FGF and HIF. Numerous studies have confirmed that lncRNAs regulated the EMT process of glioma cells through downstream pathways (**Table 3**).

Zinc-finger E-box-binding homeobox 1 (ZEB1) is an important regulator of EMT. LncRNA was known to function as a ceRNA to regulate ZEB1 *via* multiple pathways in regulation of EMT process of glioma cells (46). LncRNA linc00645, for instance, played a key role in TGF-β-triggered glioma cell EMT through competing with miR-205-3p and promoting the expression of downstream molecule ZEB1 (47). LncRNA UCA1 partially rescued the inhibitory effect of miR-204-5p on ZEB1 *via* binding and inhibiting miR-204-5p, which promoted the EMT process of glioma cells (48). Hypoxia-induced glioma cells upregulated lncRNA HOTTIP and sponge inhaled endogenous miR-101, resulting in increased ZEB1 expression and promoting EMT process (49). LncRNA HOXC-AS2 formed a positive feedback loop with ZEB1 through miR-876-5p to regulate the EMT in glioma, providing a potential therapeutic target for glioma prevention (50).

The Wnt signaling pathway also plays an important biological role in EMT in glioma. In this sense, lncRNA CTBP1-AS2 regulated the Wnt7a-mediated EMT by binding miR-370-3p (27), whereas lncRNA H19 inhibited EMT Wnt/β -catenin pathway (51).

In conclusion, a variety of lncRNAs can regulate the EMT process of glioma through ZEB1, which is closely related to tumor metastasis and drug resistance. ZEB1, a zinc-finger transcription factor induces EMT by regulating E-cadherin and vimentin. In-depth understanding of the molecular mechanism of lncRNAs control of EMT can not only reveal the process of metastatic drug resistance of tumor cells, but also provide new therapeutic targets and treatment options for effective cancer treatment.

LncRNAs and TMZ resistance in glioma

Chemotherapy is a common postoperative treatment strategy for glioma treatment (52). Temozolomide (TMZ) is a second-generation oral alkylating agent that can easily cross the blood-brain barrier, therefore it is the standard first-line chemotherapy agent in the clinical treatment of glioma (53, 54). TMZ exerts its antitumor effects mainly through inducing base mismatch, DNA repair aberration, DNA chain break and cell death (55). However, TMZ can only slightly improve the survival of patients with glioma, because many patients develop resistance to TMZ, resulting in poor or no response to it (56).

At present, a string of studies have described the mechanism of glioma drug resistance to chemotherapy, and these mechanisms may involve lncRNAs (**Table 4**) and the β-catenin signaling pathway. LncRNA RMRP modulated TMZ resistance in glioma by regulating ZNRF3 levels and the Wnt/β-catenin signaling pathway to form a positive feedback loop (57). LncRNA MIR155HG was highly expressed in glioma tissues and promoted glioma resistance to TMZ by binding PTBP1 to regulate the Wnt/β-catenin pathway (58). It was found that lncRNA SOX2OT reduced the methylation level of SOX2 by interacting with ALKBH5, thus improving the SOX2 expression and activating the Wnt5a/β-catenin signaling pathway to promote TMZ resistance in glioma cells (59). In addition, lncRNA SNHG15 also activated the β-catenin signaling pathway by promoting SOX2 expression (60).

TMZ resistance in glioma cells may be epigenetically regulated by lncRNAs. For example, one report showed that lncRNA SNHG12 was activated by DNA methylation in the promoter region CpG island, and lncRNA SNHG12 regulated the MAPK/ERK signaling pathway and G1/S cell cycle transition through competitive binding of miR-129-5p. Thus, DNA methylation of lncRNA SNHG12 ultimately regulated TMZ resistance in glioma cells (61).

TABLE 3 Representative lncRNAs and related signaling pathways of EMT process in glioma.

LncRNA	Expression	Downstream Targets	EMT	References
linc00645	upregulated	miR-205-3p, ZEB1	promote	(46)
UCA1	upregulated	miR-204-5p, ZEB1	promote	(47)
HOTTIP	upregulated	miR-101, ZEB1	promote	(48)
HOXC-AS2	upregulated	miR-876-5p, ZEB1	promote	(49)
CTBP1-AS2	upregulated	miR-370-3p, Wnt7a/β-catenin pathway	promote	(27)
H19	upregulated	Vimentin, ZEB1, Wnt/β-Catenin pathway	promote	(50)

TABLE 4 Representative lncRNAs and related signaling pathways of TMZ resistance.

LncRNA	Expression	Downstream Targets	TMZ resistance	References
RMRP	upregulated	ZNRF3, Wnt/β-catenin pathway	promote	(56)
MIR155HG	upregulated	PTBP1, Wnt/β-catenin pathway	promote	(57)
SOX2OT	upregulated	SOX2, Wnt5a/β-catenin pathway	promote	(58)
SNHG15	upregulated	miR-627-5p, CDK6, SOX-2	promote	(59)
SNHG12	upregulated	miR-129-5p, MAPK1, E2F7	promote	(60)

Postoperative temozolomide chemotherapy has become the standard treatment for glioma. However, acquired TMZ resistance limits the treatment of patients with glioma, especially relapsing glioma. As mentioned above, some lncRNAs are associated with glioma drug resistance, which involves not only intracellular processes but also factors in the glioma microenvironment. Elucidate the molecular mechanism of TMZ resistance, which is helpful to rationally design the combined treatment plan to block TMZ chemotherapy resistance.

LncRNAs and ferroptosis in glioma

As a matter of fact, iron is an essential nutrient and microelement for cell growth, no exception for cancer cells. Moreover, iron-dependent ferroptosis induces inflammation reaction to promote the initiation and advancement of cancers in early stages. On the other hand, cancer can be restrained by anti-cancer immune response triggered by ferroptosis and the release of damage-associated molecular pattern (DAMPs). Up to now, lncRNAs owing to diversities and complex functions is thought to be closely related to ferroptosis of various diseases based on explosive growing studies. Zhang et al. found that (62) curcumenol could hinder the progression of lung cancer by slowing down the multiplication and accelerating cell death as an effectual component of Wenyujin. Finally, they verified that lncRNA H19 could enhance the transcription activity of ferritin heavy chain1 (FTH1), a biomarker of ferroptosis, by interacting with miR-19b-3p as a competent endogenous RNA. Shi et al. found that (63) lncRNA AAB expressed highly and increased Fe^{2+} level to exert antitumor effect in cardiac microvascular endothelial cells (CMECs). Furthermore, they demonstrated that lncRNA AAB caused the disturbance between MMP9 and TIMP1 balance by sponging miR-30b-5p in CMECs. They even constructed a nanocomplex delivering si-lncRNA AAB into CMECs to provide a potential treatment method for cardiac hypertrophy patients. Besides, Luo et al. found that (64) lncRNA RP11-89 heightened the migration and expansion of bladder cancer *via* the miR-129-5p/PROM2 axis. It is acknowledged that prominin2 (PROM2) is the key molecule to inhibit ferroptosis. Evidence showed PROM2 executed a crucial role in the traffic of iron mediated by transferrin and altered the sensitive of cancer cells to ferroptosis.

The metabolism of iron

Iron is one of the most indispensable metals for humans. Biological iron participates in various metabolic processes, including cellular proliferation and death, especially ferroptotic cell death. Intracellular iron exists in two oxidative states, Fe^{2+} and Fe^{3+}, which can be randomly converted into different forms. Iron can be transported by binding to serum transferrin (TF) or lactotransferrin as Fe^{3+}. Endocytosis occurs when serum TF binds to transferrin receptor (TFRC), allowing Fe^{3+} to be released into the cell. In contrast, lactotransferrin can directly shift iron into the cytoplasm (65). TFRC is an important component for iron uptake in the membrane, which can govern the labile iron pool (LIP) by conveying Fe^{3+} into the cytoplasm to promote various biological activities. Fe^{3+} is reduced into Fe^{2+} by STEAP3 once it enters the cytoplasm, and Fe^{2+} is stored in the LIP. Fe^{2+} is important for metabolic and biochemical processes, such as energy metabolism in the mitochondria. TFRC actions alter intracellular iron content, Fe^{2+} levels and reactive oxygen species (ROS) levels. Ye et al. found that (66) TFRC rescued the reduction in iron, Fe^{2+} and ROS concentrations caused by YTHDF1 knockdown in hypopharyngeal squamous cell carcinoma (HPSCC) cells. The study also showed that (67) TFRC might also intervene in glutathione peroxidase 4 (GPX4)-dependent ferroptosis. GPX4 is a key molecule that modulates ferroptosis. In 2021, Ma et al. found that (68) lncRNA RP1-86C11.7 could interact with hsa-miR-144-3p to increase the expression level of TFRC. RP1-86C11.7 enhanced proliferation, migration and progression in glioma. Consequently, accumulation of unstable LIP leads to the overproduction of lipid peroxidation, which is another vital process of ferroptosis in addition to iron metabolism.

Lipid peroxidation

Lipid peroxidation is a characteristic of ferroptosis that is driven by free radicals, including ROS and reactive nitron species (RNS) (69). During lipid peroxidation, oxidants attack lipids, such as polyunsaturated fatty acids (PUFAs), to produce lipid hydroperoxides (LOOHs) and reactive aldehydes that rely on the catalysis of the ALOX family (70). ROS consist of superoxide

anion (O_2·-), hydroxyl radicals (HO·), hydrogen peroxide (H_2O_2) and singlet oxygen (O^2), which are generated by insufficient reduction of oxygen during hypoxia or in response to other physical and chemical reactions. There are two pathways that produce ROS. One is the NADPH oxidase (NOX) pathway. Another is the Fenton reaction, in which Fe^{2+} interacts with H_2O_2 to produce Fe^{3+}, HO·and OH-. In return, O_2·- interacts with Fe^{3+} to produce Fe^{2+}. The entire process is called the Haber-Weiss cycle (70). These free radicals contribute to oxidative stress and damage proteins and nucleic acids, a process closely related to the carcinogenic potential in malignant diseases. Bountali et al. demonstrated that (70) lncRNA MIAT knockdown promoted the accumulation of ROS and enhanced cell apoptosis to further influence other cancer-related genes in glioma. It was highly possible that MIAT exerted its effectiveness on ferroptosis by changing ROS levels in glioma. Ahmadov et al. found that (71) N-acetyl cysteine (NAC), a ROS scavenger, could reverse the phenotype caused by the decline of ROS level due to lncRNA HOTAIRM1 knockdown in glioma cells. What's more, they elucidated that intracellular ROS decrease mediated by HOTAIRM1 contributed to the radiation resistance in glioma. Lulli et al. found that (72) miR-370-3P weakened the proliferation and invasion by directly inhibiting lncRNA NEAT1 in glioma. NEAT1 encouraged the activation of HIF1-α and HMGA1, which were both connected to oxidative stress in glioma. Currently, lncRNA NEAT1 is commonly an oncogene in cancers. Zhen et al. found that (73) lncRNA NEAT1 could be a tumor-enhancer by regulating miR-449b-5p/c-Met axis in glioma. Collectively, these data suggest that the lncRNA NEAT1 may affect ferroptosis by controlling molecules related to oxidative stress in glioma. However, the specific mechanisms by which these lncRNAs affect ferroptosis remain unexplored. In fact, there are many regulators or pathways that modulate intracellular ROS content, such as lipophagy, ferritinophagy, GPX4 and NOXs, and that may be altered by lncRNAs.

Other potential molecular mechanisms in ferroptosis

The mechanisms of ferroptosis are complicated and obscure. Many molecules, in addition to those mentioned above, are involved in this important biological process (8, 70, 74).. As we all known, glutathione peroxidase 4 (GPX4) is considered as the gatekeeper and hub molecule in ferroptosis. GPX4 belongs to Glutathione peroxidases (GPXs) family, which currently contains GPX1-GPX8. In general, GPXs are involved in the reduction reactions of H_2O_2 and small hydroperoxides *via* glutathione (GSH) as reductant. Besides, only GPX4 can catalyze the reduction of hydroperoxides in the complicate lipids, even located in the biomembranes or lipoproteins. Rich evidences (75), have demonstrated GPX4 plays a crucial role in the process of ferroptosis. GSH, comprised of cystenie, glutamate and glycine, is required for GPx4 to execute its functions. Cystine is transported into cells through the protein complex systmeXc- in plasma membrane which consists of SLC7A11/xCT (solute carrier family 7 member 11) and SLC3A2 (solute carrier family 3 member 2). Cystine will be transformed into cysteine to form GSH once it enters into cells. On the other hand, GPX4 can catalyze phospholipid hydroperoxides (PLOOH) to produce phospholipid alcohols (PLOH) and decrease the stock of lipid peroxidants (69, 70). However, to inhibit any one step above will suppress the function of GPX4 directly or indirectly to cause the accumulation of PLOOH to promote ferroptosis. In addition, the stability of SLC7A11 can also influence the occurrence of ferroptosis. Zhao et al. found that (76) OTUB1, an ovarian tumor (OTU) family member deubiquitinase positively regulated the stability of SLC7A11 to support ferroptosis in glioma. Moreover, Liu et al. found that (77) CD44, the biomarker of cancer stem cells directed the process of ferroptosis by promoting the interaction between OUTB1 and SLC7A11, which suggested that CD44 might be involved in the progression of ferroptosis. Chen et al. (78) found that differential expression of lncRNA TMEM161B-AS1 regulated the two ferroptosis-related genes (FANCD2 and CD44) separately by sponging hsa-miR-27a-3p. They also confirmed that depletion of FANCD2 and CD44 caused the accumulation of iron and lipid ROS, suggesting that low expression of lncRNA TMEM161B-AS1 could promote cell apoptosis and ferroptosis in glioma. What's more, Zhang et al. found that (79) lncRNA OIP5-AS1 inhibited ferroptosis in prostate cancer with long-term cadmium exposure through miR-128-3p/SLC7A11 signaling. Obviously, SLC7A11 played a crucial role in ferroptosis by regulating the transportation of cystine. As mentioned before, oxidative stress involved molecules could cause irreversible or lethal damage to cells. Particularly, NOXs, controlled positively by DPP4/CD26 and other kinases, constitute part of the membrane-bound enzyme complexes that transport electrons necessary for the production of free radicals, including ROS, that promote lipid peroxidation in ferroptosis (69). Another study also reported that (70) DPP4 was involved in the reduction reaction of O_2 to O_2·- in a NOXs-dependent manner.

It is clear that numerous molecules are involved in the complex process of ferroptosis, yet only a few molecules have been declared to influence ferroptosis by interacting with lncRNAs in glioma (**Figure 2**). Ferroptosis is a newly defined process of cell death that plays an important role in the progression of many diseases, especially tumors. Therefore, it is worth exploring the specific and profound mechanisms whereby lncRNAs contribute to glioma to provide new potential targets for therapy.

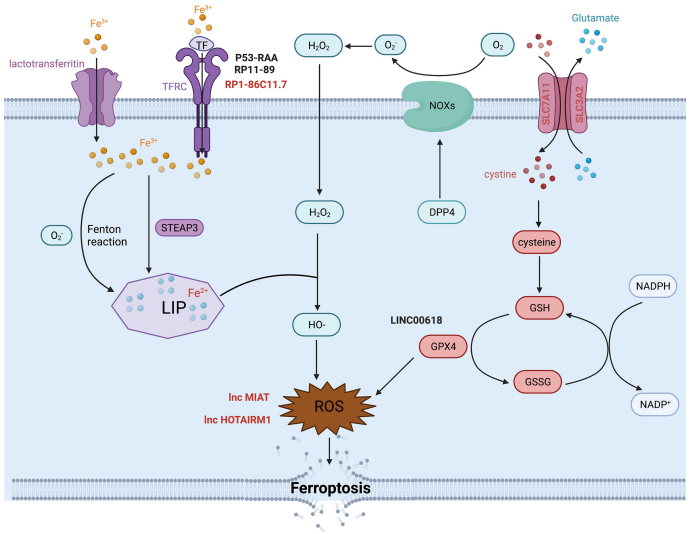

FIGURE 2
The molecular mechanism and some lncRNAs regulation of ferroptosis.

Conclusion and prospectives

Glioma is the most prevalent and dangerous CNS tumor with a very poor prognosis. It is noteworthy that GBM patients still remain hopeless prognosis even though they were performed complex treatment scheme combining operation with radio- and chemo- therapy. More depressingly, chemotherapy drugs can't be easily targeted to GBM due to special location comparing to other non-intra tumors. Therefore, to find some specific targeted molecules for GBM is of vital and urgent. To clarify the pathological molecular mechanisms of GBM is of vital and helps other scientists further explore corresponding target therapy to some extent. And we noticed noncoding RNAs, including miRNAs, circRNAs and lncRNAs play critical and significant roles in suppressing or provoking the initiation and progression of glioma. In particular, lncRNAs which is under the spotlight, impact various aspects of glioma, such as proliferation, invasion, migration, EMT, cell death, stemness of glioma stem cells and resistance to radiotherapy and chemotherapy, by interacting with proteins, mRNAs, enzymes and other noncoding RNAs and interfering countless signal pathways. Thus, it can be seen lncRNAs indeed involves in the development and progression of glioma. However, the specific and precise mechanisms of lncRNAs still need to be further probed in future. For example, whether lncRNAs involves in the ferroptosis of glioma or not? And can lncRNAs put an effect on the ferroptosis progress by some molecules or similar pathways involved in the proliferation, EMT, apoptosis and TMZ of glioma?

Recently, ferroptosis, a novel class of cell death, has attracted much attention in various diseases. However, few articles have discussed the mutual interaction between lncRNAs and ferroptosis in glioma compared with other diseases. Similar relationships between lncRNAs and ferroptosis found in other diseases may also be present in glioma. For instance, Mao et al. (80) reported that lncRNA P53RAA promotes ferroptosis by accumulating iron and lipid ROS and displacing p53 from the G3BP1-p53 complex. Simultaneously, P53RAA can decrease the expression of the metabolic molecule SLC7A11, a regulator of iron concentration. It is well-known that p53 is a classical tumor suppressor. Therefore, the following question arises: can p53-related lncRNA induce ferroptosis in glioma (81)? Besides, He et al. (82) and Shi et al. (83) built a novel ferroptosis-related lncRNAs panel which provides some assertive evidence for delving into the relationship between lncRNAs and ferroptosis in glioma. Meantime, they released some implications and values for the potential therapy plan related to immunotherapy for glioma patients.

Glioma is different from other tumors due to its heterogenity and has a special tumor microenvironment (TME) consisted of cancer cells and immune cells, including macrophages, nature killing cells, dendritic cells and et al. The growth of glioma cells is dependent on the iron element comparing with other non-malignant cells (84). Moreover, glioma mostly occurred in the brain and iron is usually transported mediated by TFR into the brain, which indicated that we can induce the ferroptosis to consume the iron to prevent the growth of malignant cells. However, how to achieve it? As mentioned above, lncRNAs can act various role in the progress of ferroptosis in glioma. If lncRNA

X (which means any suitable lncRNA), high tissue-specificity, were found to induce ferroptosis by increasing the iron concentration or TFR in brain, we can focus on to synthesize a kind of drug to change the expression of lncRNA X to induce ferroptosis to obstruct the proliferation of glioma cells. Besides, we can monitor the change of lncRNA X level in tissue to hint us to take precautions against the glioma. Furthermore, human brain, enriched in lipid, is the most susceptible to the progress of oxidative stress reaction which also are very meaningful to ferroptosis (85). It is possible for others to develop some related and helpful therapy plans targeting lncRNAs involved in ferroptosis followed the same mind above accordingly. Several works have showed that the links between immune cells within TME and ferroptosis implicated that some immune therapy targeting ferroptosis might can be explored (86, 87). More promisingly, it has been verified that CD4, CD8 and CD36 T cell within TME can induce the ferroptosis by accumulating the lipid ROS (88), which suggest we can mainly concentrate on some immune-related lncRNAs involved in the ferroptosis to scout the links and the potential therapy. In addition, the induction of ferroptosis can prevent the formation of acquired drug-resistance which is significant and meaningful clinically.

Ferroptosis is a complex process and a newly discovered modality of cell death. The interactions between ferroptosis and other processes of cell death have been explored, as the mechanisms of ferroptosis have become increasingly clearer. Wang et al. (89) found that LINC00618 knockdown reduced early apoptosis. In addition, LINC00618 can inhibit GPX4, a key regulator of ferroptosis, and increase the concentration of intracellular iron and lipid ROS. Ultimately, they suggested that LINC00618 can increase ferroptosis in a manner dependent on cell apoptosis. Therefore, we suggest that lncRNAs might act as bridge molecules between ferroptosis and

apoptosis, including cellular and necrotic apoptosis. Moreover, this evidence highlights the potential crosstalk or interrelationship amongst cell apoptosis, necrosis, autophagy and ferroptosis that may occur in or to contribute to many diseases and should be the focus of future studies.

In conclusion, lncRNAs play a crucial role in the occurrence and development of glioma. Targeting these lncRNAs may help glioma patients to obtain potential treatment benefits. In addition, the identifications of lncRNAs may contribute to the early detection and diagnosis of glioma. However, in order to fully understand the function of lncRNAs in the neoplastic process of glioma, several key issues must be solved. For example, since lncRNAs have various functions and can regulate a variety of cellular processes, it is necessary to analyze the specific molecular mechanisms of it. In addition, whether the participation of lncRNAs in clinical application has sufficient reliability and sensitivity or not remains to be verified. We believe that the use of robust sequencing techniques can shed light on the roles of lncRNAs in glioma development and could accelerate the clinical application of lncRNAs in diagnosis, treatment, and prognostic evaluation of glioma.

Author Contributions

TX, QL, XY, and JG conceived the concept and wrote the manuscript. ZL, MH, XJ, ZW, YL, DH, and FZ finished the table and figure. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The molecular mechanisms of ferroptosis and its role in glioma progression and treatment

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Ferroptosis is one of the programmed modes of cell death that has attracted widespread attention recently and is capable of influencing the developmental course and prognosis of many tumors. Glioma is one of the most common primary tumors of the central nervous system, but effective treatment options are very limited. Ferroptosis plays a critical role in the glioma progression, affecting tumor cell proliferation, angiogenesis, tumor necrosis, and shaping the immune-resistant tumor microenvironment. Inducing ferroptosis has emerged as an attractive strategy for glioma. In this paper, we review ferroptosis-related researches on glioma progression and treatment.

KEYWORDS

ferroptosis, molecular mechanism, role, glioma progression, combination therapy

Introduction

Ferroptosis is an iron-dependent form of programmed cell death, is more immunogenic than apoptosis. During ferroptosis, the level of reactive oxygen species (ROS) increases and induces lipid peroxidation (LPO) (1, 2). Ferroptosis is widely present in the development of many cancers, such as liver cancer, gastric cancer, lung cancer, colorectal cancer, ovarian cancer, breast cancer, glioma, and hematologic tumors (3). Ferroptosis has attracted increasing attention since its naming in 2012 (4).

The process of ferroptosis involves multiple signaling pathways and regulatory mechanisms that interact with other cell death modalities in the development of glioma (3, 5–7). It has been shown that increased ROS during ferroptosis can initiate LPO by interacting with polyunsaturated fatty acids in lipid membranes, thereby mediating chemoresistance in gliomas (8). A deeper understanding of the mechanism

of ferroptosis in glioma progression is of great significance for the research and improving the existing therapies for glioma.

Taking into account the histopathological manifestations and alterations in genes, molecules and signaling pathways, in 2021, WHO proposed the fifth edition of the CNS tumor classification, which comprehensively introduced the latest classification criteria for gliomas, using the terms "diffuse" and "restrictive" to define different types of gliomas, replacing the original Roman numeral grading method of grade I-IV. The latest published classification shows that diffuse gliomas occurring mainly in adults and mainly in children have some molecular differences and should be classified as adult and pediatric types; moreover, adult diffuse gliomas that occur as angiodysplasia and necrosis should be diagnosed as glioblastoma (9).

The damage-associated molecular pattern (DAMP) of ferroptosis is more specific than the other forms of cell death (Figure 1, Universal mechanisms of DAMP release) (10). On the one hand, ferroptosis can recruit and activate numerous immune cells at the tumor site and drive dendritic cell maturation *in vitro* (11, 12), and ferroptosis inducers can function as sensitizers for anti-tumor immunotherapy (13–15). Studies have shown that ferroptosis combined with radiotherapy and chemotherapy can partially overcome drug resistance, limit glioma growth and prolong survival (14, 16, 17). Alternatively, ferroptosis is a unique form of autophagy (18), and results in iron accumulation, which is not only associated with iron uptake and new blood vessels formation during tumor growth (19), but also serves as an important factor involved in the construction of an immunosuppressive glioma microenvironment, such as the regulation of proliferation of B cells, T cells and immunophenotypic differentiation of tumor-associated macrophages (20). In conclusion, ferroptosis is involved in multiple aspects of the glioma progression, thus, targeting ferroptosis may be a potential strategy for glioma therapy.

Molecular mechanism of ferroptosis

In 2012, a new type of iron-dependent programmed cell death has been described and named ferroptosis by Professor Stockwell et al. (4). The process of ferroptosis can be briefly described as the activation of lipoxygenase by free ferrous ions through the Fenton reaction, leading to peroxidation of polyunsaturated fatty acids (PUFAs) on cell membranes, and the increased level of LPO causes loss of cell permeability and eventually cellular ferroptosis (21). Ferroptosis is regulated by a combination of iron metabolism, LPO and antioxidant systems, impairing the homeostasis of any of these processes may trigger ferroptosis (22). There are diverse cellular defense systems in response to LPO in cells, including the classical pathway mediated by GPX4, the non-classical pathway mediated by FSP1 independently of GPX4, as well as a third pathway in which dihydroorotate dehydrogenase (DHODH) interacts with GPX4 to block ferroptosis in the inner mitochondrial membrane by reducing ubiquinone to form ubiquinol (23). The ferroptosis related mechanism will be discussed in detail below (Figure 2, Molecular mechanism of ferroptosis) (24).

The involvement of important molecules in the process of ferroptosis

Glutathione peroxidase 4(GPX4), is a crucial regulator of endogenous ferroptosis. GPX4 can convert glutathione (GSH) to

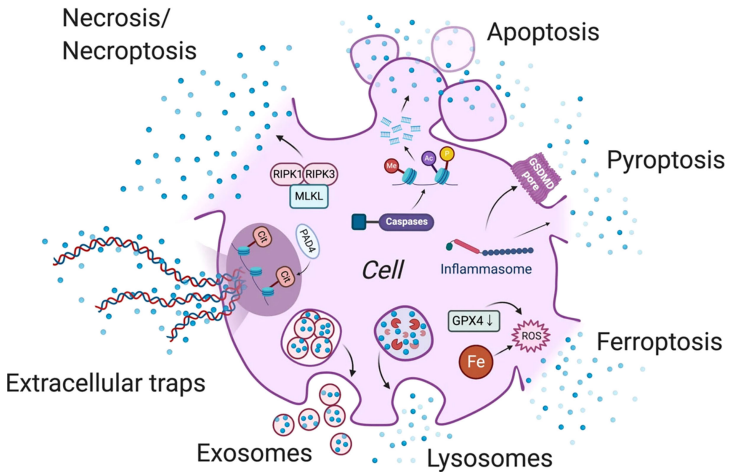

FIGURE 1
Universal mechanisms of DAMP release.

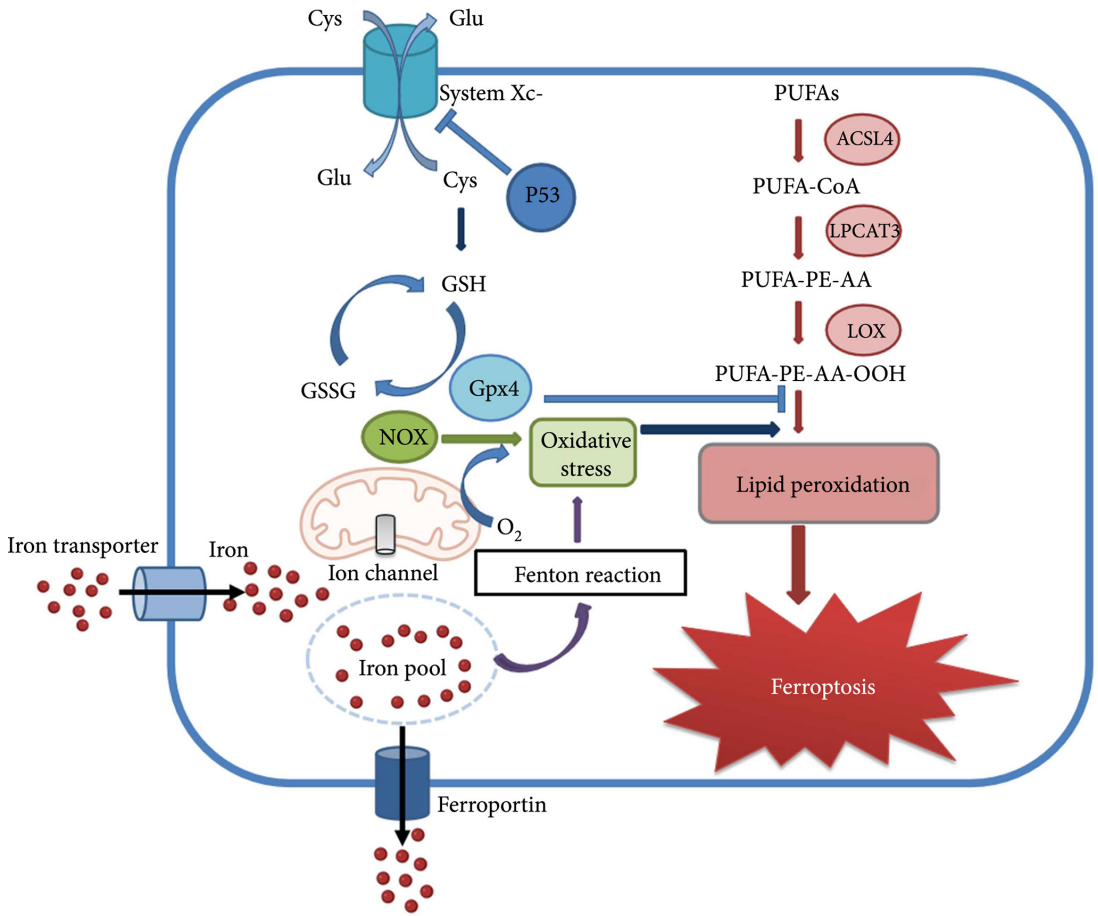

FIGURE 2
Molecular mechanism of ferroptosis.

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oxidized glutathione and reduce cytotoxic lipid peroxides to the corresponding alcohols. By inhibiting the formation of lipid peroxides, GPX4 prevents the production of LPO reaction products, reduces cell membrane damage, and thus alleviates cellular ferroptosis (25). Down-regulation of GPX4 expression can make cells more sensitive to ferroptosis, and knockdown of GPX4 induces ferroptosis. Whereas studies show that GPX4 expression was significantly increased in human glioma patients compared to brain tissue of healthy patients (26).

The X^{c-}system, a heterodimer composed of subunits SLC7A11 and SLC3A2, is an important component of the cellular antioxidant system and is closely associated with exogenous ferroptosis processes. It is widely distributed in the phospholipid bilayer and is involved in intracellular cystine uptake and GSH synthesis (27). The inhibition activity of the X^{c-}system will result in reduced GPX4 activity, ROS accumulation, and lipid oxidative stress, causing cellular ferroptosis. SLC7A11 was reported to be expressed at higher levels in GBM patient biopsies or glioma cell lines than in normal brain tissue (28).

ACSL4, acyl-CoA long-chain synthase 4, expressed in the endoplasmic reticulum and mitochondrial outer membrane, is an essential molecule in lipid metabolism. This molecule is mainly responsible for catalyzing the formation of acetyl coenzyme A from lipids. It is closely related to the production of ROS and the process of ferroptosis, and thus has potential to be an indicator of ferroptosis sensitivity (29). ACSL4 down-regulates glioma cell proliferation and mediates up-regulation of ferroptosis levels in gliomas. Studies have shown that ACSL4 expression is down-regulated after glioma occurs (30). Additionally, it was reported that GPX4 knockdown leads to ferroptosis, while double knockdown of GPX4 and ACSL4 genes can reverse GPX4 knockdown-induced ferroptosis (31).

FSP1 (ferroptosis-suppressor-protein1), is a ubiquinone oxidoreductase, it was initially described as a pro-apoptotic gene called apoptosis-inducing factor 2(AIFM2) in mitochondria and is now considered as a glutathione-independent ferroptosis resistance molecule. FSP1 can act in parallel with the GPX4 pathway, thus preventing glutathione deficiency-induced ferroptosis (32).

Moreover, FSP1 can use NADPH to catalyze the reduction of the lipophilic radical scavenger ubiquinone (CoQ10), and the FSP1-CoQ10-NADPH pathway can synergistically inhibit ROS elevation with the GPX4 system to prevent ferroptosis caused by oxidative damage (33).

DHODH, an iron-containing flavin-dependent enzyme, is an important molecule in nucleotide metabolism, which inhibits mitochondrial ferroptosis *via* regulating the production of the antioxidant ubiquinol(CoQH2) in the inner mitochondrial membrane (34). It was shown that inhibition of DHODH promotes ferroptosis as it increases LPO in mitochondria, and DHODH can act synergistically with GPX4 to inhibit ferroptosis in the mitochondrial inner membrane (35).

TP53, a widely studied oncogene, can repress the expression of SLC7A11, a component of the X^{c-}system, at the transcriptional level, in turn targeting the diamine acetyltransferase SAT1 and the mitochondrial glutaminase GLS2, which are involved in the regulation of glutamine metabolism, to enhance cellular ferroptosis (36).

Furthermore, p53 can inhibit ferroptosis by directly inhibiting dipeptidyl peptidase 4 (DPP4) activity or by inducing cell cycle protein-dependent kinase inhibitor 1A (CDKN1A/p21) expression (36). Recent studies have shown that the regulation of ferroptosis by p53 contributes to its tumor suppressive function (37). In addition, p53 plays a dual role in mediating ferroptosis in glioma (38).

Nrf2, a transcription factor that regulates redox metabolism, contains a basic leucine zipper DNA-binding domain at the C-terminus and plays a key role in the cellular response to oxidative stress. Many enzymes and proteins involved in LPO are target genes of NRF2, such as glutamate-cysteine ligase and glutathione peroxidase (GPX). Through interactions with p53, GPX4, X^{c-}system, etc., Nrf2 affects ferroptosis (39).

The role of ferroptosis in glioma progression

The relevance of ferroptosis and glioma has been widely recognized for influencing various vital processes in the development of glioma. It is not only involved in the construction of an acidic, hypoxic, immunosuppressive glioma microenvironment, but is also closely related to glioma cell proliferation, angiogenesis, tumor necrosis, and invasive growth.

Induced ferroptosis can mediate altered oxidative metabolism in glioma cells, trigger changes toward of macrophage polarization in the glioma microenvironment, and interfere with the proliferation and function of immune cells.

Ferroptosis influence glioma cell proliferation

Ferroptosis has been proposed to play an important role in glioma cell proliferation (40, 41). Inhibition of ferroptosis accelerates glioma proliferation and metastasis and promotes angiogenesis and malignant transformation of gliomas. Ferroptosis attenuates the viability of glioma cells, and activation of ferroptosis inhibits glioma cell proliferation. It was revealed that reduced ferroptosis in human glioma tissue and glioma cells might be associated with ACSL4, an important molecule in the emergence of ferroptosis (30). This study found ACSL4 expression was decreased in glioma cells and reduced expression of ACSL4 compared to the normal human brain. Furthermore, their speculates confirmed that ACSL4 plays an essential role in regulating ferroptosis and proliferation in glioma cells and knocking down the gene significantly improved the viability of glioma cells (30). Another investigation on non-coding RNA

MicroRNA-670-3p again demonstrated that targeting the inhibition of ACSL4 and thus ferroptosis in human glioblastoma cells has a pro-tumor effect (42).

It was noted in studies on drugs that dihydrotanshinone I, a natural antitumor drug commonly used in clinical practice, can significantly proliferate human glioma cells and promote human glioma cell death (43). After treatment of human glioma cells with dihydrotanshinone I, GPX4 expression decreased while ACSL4 expression increased, inducing ferroptosis in human glioma cells. Then, the inhibitory effect of dihydrotanshinone I on the proliferation of glioma cells was blocked after the application of ferroptosis inhibitors (43). It can be concluded that the effect of dihydrotanshinone I on the proliferation of glioma cells is derived from ferroptosis. Additionally, elevated expression levels of Nrf2, a ferroptosis-related molecule, in glioma patient samples exhibited pro-tumor proliferative utility by regulating the X^{c-}system-mediated reduction in ferroptosis (44).

Ferroptosis promotes the progression of glioma necrosis

Tumor necrosis is a prevalent phenomenon in gliomas, especially high-grade glioblastomas, which is strongly associated with the highly aggressive growth of the tumor. Necrosis progresses alongside tumor progression, causing inflammation and cytokine storms resulting in multi-organ disorders, poor prognosis and death; the degree of tumor necrosis is negatively correlated with survival in glioma patients (45). There is a general consensus that tumor necrosis is caused by a hypoxic tumor microenvironment and rapidly proliferating tumor cells that exceed the capacity of the vascular supply (46). Moreover, it is proposed that necrosis is caused by iron-dependent oxidative stress and may partially follow the ferroptosis pattern (47). Glioma tissue that undergoes necrosis recruits immune cells by releasing its corresponding DAMP. Findings show that neutrophils extensively infiltrate the tumor necrosis area and increase with tumor progression, and that the degree of infiltration of glioma tumor-associated neutrophils positively correlates with the degree of tumor necrosis (47).

Furthermore, studies suggest that neutrophils are participating in the process of promoting tumor necrosis and that process is achieved by triggering ferroptosis in tumor cells. Besides, elevated glutamate levels in areas of glioma necrosis can cause increased tumor necrosis by inhibiting the X^{c-}system in ferroptosis, thereby inducing ferroptosis (47).

Ferroptosis has an impact on glioma angiogenesis

Aberrant vascular network formation with high permeability is another important feature and critical event in the glioma process. The formation of new blood vessels effectively promotes the infiltrative spread of gliomas, highly aggressive growth and leads to a therapeutic resistance. Augmented microvessels are mostly observed in areas of tumor infiltration and necrosis, and the density correlates positively with the malignancy of the glioma. It is believed that glioblastoma is one of the most vascularized human tumors (48). Glioma-associated macrophages, which play an invaluable role in iron metabolism and ferroptosis, are the most abundant immune cells in the glioma microenvironment and are engaged in all phases of angiogenesis, ranging from angiogenesis early sprouting to late neovascularization and the stabilization of neovascularization (49). It was reported that the number of macrophages around proliferating micro-vessels in glioblastoma was significantly increased. The release of angiogenic factors was promoted by stimulation of macrophages, while macrophages in gliomas promoted neovascularization through cyclooxygenase 2-mediated secretion of cyclooxygenase 2(COX2) and IL-6. Studies revealed that ferroptosis recruits glioma-associated macrophages and indirectly acts on macrophage-mediated neointima formation (50). The other evidence that ferroptosis influences glioma angiogenesis has been derived from studies of the transcriptional activator ATF4. It is an integral molecule in cellular oxidative metabolism and is highly expressed in gliomas, promoting cell migration and anchorage-independent cell growth, allowing tumor cells to adapt to the glioma microenvironment, thus, ATF4 plays a role in promoting proliferation and angiogenesis in gliomas (19). Furthermore, ATF4 acts in an X^{c-}system-dependent manner, mediated by the SCL7A11 molecule. Findings show that the ferroptosis inducers erastin and RSL3 reduce ATF4-induced tumor angiogenesis (19).

Ferroptosis raises immune resistance to glioma

Resistance to treatment in glioma is mainly due to the immunosuppressed glioma microenvironment, which is a major obstacle to glioma treatment. Ferroptosis has dual aspects in the glioma progression. On the one hand, ferroptosis is the main form of programmed cell death in the glioma process, causing tumor cell death; On the other hand, ferroptosis is engaged in shaping the immunosuppressive glioma microenvironment, contributing to a decrease in the host's anti-tumor immunity and promoting tumor propagation (51). Bioinformatics data analysis revealed that the expression of ferroptosis-related genes was associated with immunosuppression in gliomas, and studies showed that the severity of ferroptosis was significantly associated with the clinical prognosis of gliomas (41). There is a large number of immune cells infiltrating the ferroptosis-enriched glioma. However, most of these immune cells are immunomodulatory cells, such as Treg, neutrophils, and glioma-associated macrophages. A subset of glioma-associated macrophages can be divided into two subtypes, M1 and M2, representing two different forms of effects that inhibit and promote tumor progression (52, 53). A study of 1750 patients

showed that a higher proportion of tumor-promoting M2-type macrophages was demonstrated in the immunosuppressed glioma microenvironment. Furthermore, ferroptosis in gliomas promotes macrophage infiltration and induces M2-type polarization of macrophages (46). Being present in the vascular niches in close contact with brain endothelial cells, glioma stem cells have tumor initiation properties and self-renewal ability that contribute to the immunosuppressive microenvironment of glioma. These findings show that ferroptosis is involved in the stemness regulation of glioma stem cells. As an example, OTUB1, a deubiquitinating enzyme overexpressed in gliomas, regulates SLC7A11, a critical inhibitory molecule in the ferroptosis X^{c-} system, directly through the ubiquitinase-proteasome degradation system, forming the OTUB1/SLC7A11 axis and thus promoting the stemness of glioma cells (54).

Ferroptosis involvement in glioma treatment

A standard therapy for glioma treatment is a combination of surgery, radiotherapy, and chemotherapy, but the effectiveness of these therapies is limited due to the inherent treatment resistance of glioma. Being one of the important forms of cell death, ferroptosis can suppress the development of glioma. Studies have shown that molecules causing ferroptosis can play an aggressive role in the treatment of glioma (6, 55), and as such, ferroptosis can be used as a combination therapy in the treatment of glioma, improving the sensitivity of radiotherapy and chemotherapy. In conclusion, targeting ferroptosis-related genes might have potential value in the treatment of glioma.

Inducing ferroptosis promotes sensitivity to glioma therapy

Radiotherapy (RT), an important component of standard therapy for glioma, uses X-rays to destroy tumor tissue and can directly trigger multiple types of DNA damage, such as base damage, single-strand breaks (SSB), double-strand breaks (DSB), thereby inducing cycle arrest, senescence, and multiple forms of death in highly proliferative tumor cells with some therapeutic effect. However, due to the heterogeneity of glioma, radiation therapy's effect is inadequate (56). Studies suggest that the combination of ferroptosis inducer sorafenib and radiotherapy play a collaborative role in killing glioma cells (17). Alternatively, ferroptosis inducers 2-nitroimidazole, doranidazole and misonidazole can mediate altered oxidative stress metabolism in glioma stem cells, such as elevated levels of metal reductase steap3 and NADH by doranidazole, which can act as a sensitizer to counteract resistance to radiotherapy and produce cytotoxicity to limit glioma growth and significantly prolong survival (13) (more details see Table 1).

Oral alkylating agent TMZ is the first-line chemotherapeutic agent in the treatment of glioma. With the advantages of easy penetration of the blood-brain barrier, stable acidic environment and no superimposed toxicity with other drugs, it can prolong the survival time of glioma patients to some extent, but only partial patients can benefit from TMZ chemotherapy due to drug resistance (57). Several studies have found that the use of ferroptosis-inducing agents can increase TMZ sensitivity. The combined use of the ferroptosis inducer erastin and TMZ was reported to enhance TMZ sensitivity through multiple pathways (14); *in vitro* use of hydroxychloroquine (HCQ) and its derivative quinacrine(QN), which traverses the blood-brain barrier and impairs TMZ-induced autophagy, can induce ferroptosis and thus increase TMZ sensitivity (6) (more details see Table 1).

Ferroptosis-related drugs in the treatment of glioma

Many drugs can work in glioma treatment by affecting the process of ferroptosis. Dihydroartemisinin (DHA) has been shown to exert anticancer activity by enhancing ferroptosis through the production of ROS and inhibition of GPX4 initiation (58). Amentoflavone (AF), a polyphenol widely found in cypress, has anti-inflammatory and anti-tumor effects. Findings show that AF can trigger glioma ferroptosis in an autophagy-dependent manner to exert anti-tumor effects (59). The accumulation of reactive oxygen species and LPO can be observed in glioma cells treated with the curcumin analogue ALZ003.*In vitro* and animal studies have shown that ALZ003 can inhibit the growth of TMZ-resistant gliomas by acting on GPX4, a crucial molecule in the ferroptosis pathway, while having no cytotoxic effect on normal astrocytes (15) (more details see Table 1).

Furthermore, due to the tight relationship between ferroptosis and lipid metabolism, many glioma therapeutic agents can exert therapeutic effects by mediating cellular ferroptosis through LPO. Brucine, an indole alkaloid extracted from the seeds of strychnine, promotes LPO, causing ferroptosis in glioma cells eventually inhibiting glioma cell growth *in vitro* and *in vivo* (60). The non-steroidal anti-inflammatory drug (NSAID) ibuprofen induces ferroptosis of glioma cells, and its effects are coupled with an abnormal increase in intracellular LPO (61).

Ferroptosis-based combination therapy in glioma

It was shown that NFKB activating protein(NKAP), an important regulator of mitosis, can positively regulate SLC7A11, a key molecule of ferroptosis, and knockdown of

TABLE 1 This table lists compounds currently known to induce or promote ferroptosis as sensitizers in the treatment of glioma.

Classification	Compound	Mechanism
Class I ferroptosis inducers	Erastin	Inhibit SLC7A11 activity
	PE	Inhibit SLC7A11 activity
	IKE	Inhibit SLC7A11 activity
	SAS	Inhibit SLC7A11 activity
	Sorafenib	Inhibit SLC7A11 activity
	Glutamate	Inhibit SLC7A11 activity
	BSO	GSH depletion
	DPI2	GSH depletion
	Cisplatin	GSH depletion
Class II ferroptosis inducers	1S,3R-RSL3	Inhibit GPX4 activity
	ML162	Inhibit GPX4 activity
	ML210	Inhibit GPX4 activity
	Altretamine	Inhibit GPX4 activity
	Withaferin A	Inactivate/deplete GPX4
Class III ferroptosis inducers	FIN56	Degrade GPX4, activate SQS and deplete CoQ10
	Statins (fluvastatin, simvas-tatin, lovastatin acid)	Inhibit HMG-CoA reductase (inhibit CoQ10 synthesis, reduce GPX4 expression)
Class IV ferroptosis inducers	Ferric ammonium citrate/sulfate	Iron loading
	FeCl2	Iron loading
	Hemoglobin	Iron loading
	Hemin	Iron loading
	Nonthermal plasma	Promote the release of Fe2+ from ferritin
	Lapatinib + siramesine	Upregulate TfR1 and downregulate FPN1
	Salinomycin	Inhibit iron translocation and deplete ferritin
	Artesunate, DHA	Endogenous Fe2+ causes the cleavage of endoperoxide bridge
	FINO2	Inhibit GPX4 activity, Oxidize ferrous iron and lipidome
Other ferroptosis inducers and promoters	BAY 87–2243	Inhibit mitochondrial complex I
	BAY 11–7085	Upregulate HMOX1
	Auranofin/Ferroptocide	Inhibit thioredoxin
	iFSP1	Inhibit FSP1
	4-CBA	CoQ10 depletion
	DAHP	Inhibit GCH1
	Methotrexate	Inhibit DHFR
	MF-438/CAY10566	Inhibit SCD1
	JQ-1	Promote ferritinophagy

NKAP gene can increase the level of LPO and cause oxidative damage, which in turn induces glioma ferroptosis and suppresses glioma progression (62). Furthermore, the knockdown of NKAP gene resulted in glioma cell lines that were more sensitive to ferroptosis inducers. Based on this, we think that NKAP knockdown combined with ferroptosis induction therapy has the potential to be used in the treatment of glioma.

A homologous protein mouse double minute (MDM2) and murine double minute X (MDMX) form a complex that promotes ferroptosis sensitivity in glioma cells (63). The study revealed this complex inhibits cellular antioxidant defense by modulating the activity of the major lipid regulator PPARα, which influences cellular lipid metabolism and promotes oxidative damage, leading to LPO-mediated cellular ferroptosis (63). There is a potential to design combined ferroptosis-inducing therapy with MDM2 and MDMX agonists for application in the treatment of glioma.

It is believed that the increase in intracellular iron ions, which contributes to the increase in the unstable iron pool, promotes the Fenton reaction and the generation of toxic phospholipid hydrides can induce glioma ferroptosis. Study reveals that gallic acid (GA) can effectively reduce Fe^{3+} to Fe^{2+} and is able to induce ferroptosis in GBM cells as a substrate for the sustained Fenton reaction (64, 65). Zhang et al. designed a GA-based targeted nanomedicine that combines ferroptosis and photothermal therapy for glioma treatment (65) (more details see Table 2).

TABLE 2 This table lists nanoparticles currently known to induce or promote ferroptosis in the treatment of glioma.

Classification	Compound	Mechanism
Nanoparticles	AMSNs	GSH depletion
	LDL-DHA	Loading natural omega 3 fatty acid
	ZVI NPs	Iron loadin
	FeGd-HN@Pt@LF/	Increase intracellular Fe2+ and H2O2 levels
	RGD2	delivery system releasing Fe3+ and doxorubici
	DGU : Fe/Dox	Consists of Fe3+ ion, tannic acid and sorafenib
	SRF@FeIIITA	Increase intracellular
	PSAF NCs	Fe2+ levels
	MON-p53	Iron loading, inhibit SLC7A11

Tables 1 and 2, references from the article(Ferroptosis, radiotherapy, and combination therapeutic strategies).

Conclusion and outlook

Glioma is the most common primary brain tumor in the central nervous system. The current standard treatment for glioma improves slightly the survival of patients, and all the treatments have shown some limitations and drug resistance. Ferroptosis is a newly defined form of cell death that plays an important role in the progression of glioma, affecting glioma cell proliferation, invasion, tumor necrosis, angiogenesis, and participating in the construction of an immunosuppressive glioma microenvironment. Moreover, ferroptosis can interfere with other modes of cell death. Thus, the induction of ferroptosis in gliomas has the potential to be a new option beyond standard therapies. Existing clinical reports and drug studies have shown that ferroptosis inducers used in combination with radiotherapy or TMZ can improve glioma treatment resistance, many drugs based on ferroptosis can play an aggressive role in glioma treatment, and targeting ferroptosis can contribute to the improvement of glioma treatment outcome. In order to apply ferroptosis in glioma treatment further, we need to perform a more in-depth study of the mechanisms involved in ferroptosis, to identify the population for which ferroptosis therapy is suitable; the toxicity of ferroptosis-inducing drugs, and drug delivery issues, we need to answer how to cross the blood-brain barrier effectively while avoiding off-target effects.

Author contributions

ML wrote and revised the review article; YZ established the article outline and revised it; LS provided some of the material; SS and NA have adjusted some expressions of the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ferroptosis: A novel therapeutic strategy and mechanism of action in glioma

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Glioma is the most common malignant tumor of the central nervous system and resistance is easily developed to chemotherapy drugs during the treatment process, resulting in high mortality and short survival in glioma patients. Novel therapeutic approaches are urgently needed to improve the therapeutic efficacy of chemotherapeutic drugs and to improve the prognosis of patients with glioma. Ferroptosis is a novel regulatory cell death mechanism that plays a key role in cancer, neurodegenerative diseases, and other diseases. Studies have found that ferroptosis-related regulators are closely related to the survival of patients with glioma, and induction of ferroptosis can improve glioma resistance to chemotherapy drugs. Therefore, induction of tumor cell ferroptosis may be an effective therapeutic strategy for glioma. This review summarizes the relevant mechanisms of ferroptosis, systematically summarizes the key role of ferroptosis in the treatment of glioma and outlines the relationship between ferroptosis-related ncRNAs and the progression of glioma.

KEYWORDS

ferroptosis, glioma, GPx4, system xc-, ncRNA

Introduction

Malignant tumors of the central nervous system are one of the most common types of cancer in humans and the incidence in the population is increasing year by year (1). Glioma is the most common primary malignant tumor of the adult central nervous system (2), accounting for 80% of all tumors (1). According to the latest WHO classification criteria (3), the pathological types of gliomas are divided into low-grade gliomas (LGG, grades 1–2) and high-grade gliomas (HGG, grades 3–4). WHO grade 4 glioblastoma (GBM) has the worst prognosis (4), with a median overall survival (OS) of only 12–17 months (5). At present, the treatment of glioma involves surgery, supplemented by radiotherapy, or a combination of radiotherapy and chemotherapy. LGG uses adjuvant chemotherapy with procarbazine, lomustine, and vincristine (6), and

the survival time is significantly prolonged (7). Although high-grade gliomas are usually treated with temozolomide (TMZ) after surgery, the STUPP radiotherapy and chemotherapy regimen has prolonged the survival of patients to some extent (8), but the prognosis of some patients is still poor (9). This may be due to the limitations of glioma surgery methods, which do not allow complete separation of lesions from normal brain tissue, and insufficient tumor vascularization due to the existence of the blood-brain barrier and rapid proliferation of tumor cells (10). It is difficult to reach the tumor through blood circulation and achieve sufficient concentration to achieve localized function. Furthermore, HGG cells, especially GBM cells, exhibit extreme invasiveness (11) and heterogeneity (12). Therefore, treatment with a single chemotherapeutic agent can make glioma cells resistant (13), further complicating their heterogeneity and leading to glioma recurrence (14).

Ferroptosis is a novel iron-dependent regulatory cell death (RCD) method proposed by Dixon et al. (15). A large number of studies have shown that the role of ferroptosis in tumor therapy is particularly important (16–20). By inducing ferroptosis, tumor cell growth, migration, and invasion can be inhibited, achieving the purpose of tumor therapy (17). Drug resistance of tumor cells during chemotherapy can be reversed by inducing ferroptosis (18). After induction of ferroptosis, it can spread among surrounding cells, increasing the antitumor effect of chemotherapeutic drugs (19) and the sensitivity of tumor cells to chemoradiotherapy drugs (20). These findings provide new insight into the treatment of drug-resistant tumors. Recent studies have found that noncoding RNAs (ncRNAs) also play a key role in the ferroptosis of tumor cells (21, 22). ncRNAs such as microRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs) are widely involved in iron metabolism, ROS metabolism, and ferroptosis-related amino acid metabolism in tumor cells (22). Glioma cells have extensive heterogeneity and are prone to drug resistance to chemotherapeutic drugs, resulting in an unsatisfactory patient prognosis (23). A study has found that the ferroptosis inducer erastin can enhance the sensitivity of GBM cells to TMZ (24). Therefore, this new strategy to induce ferroptosis in tumor cells may have great potential in the treatment of glioma. Current research focuses on how to reduce drug resistance in glioma chemotherapy and deepens the mechanism of classical ferroptosis-inducing pathways such as GPX4 in glioma. Ferroptosis-related ncRNAs and nanoparticle therapy targeting ferroptosis in gliomas have received increasing attention. This review summarizes the mechanism of ferroptosis in tumors and the research progress of ferroptosis in the treatment of glioma, as well as the key role of ferroptosis-related ncRNAs, and ultimately the potential clinical value of ferroptosis in personalized treatment regimens for glioma.

Mechanisms of ferroptosis in tumors

Ferroptosis is fundamentally different from apoptosis, pyroptosis, autophagy, and other cell death methods, and shares no similarities in terms of morphology, biochemistry, or genetics (25). Ferroptosis is characterized by the availability of redox-active iron (26) the loss of lipid peroxide repair capacity by the phospholipid hydroperoxidase GPX4 (27), and oxidation of polyunsaturated fatty acid (PUFA)-containing phospholipids (28). Ferroptosis is triggered in tumor cells by cysteine (Cys) depletion or by inhibition of glutathione peroxidase 4 (GPX4) (29). Subcellular structural changes are manifested by the reduction or disappearance of mitochondrial cristae in tumor cells and the destruction of the inner and outer mitochondrial membranes (30). Below we provide an overview of its mechanism around important features of ferroptosis in tumor cells.

Disruption of iron homeostasis

Iron is the basis of tumor cell proliferation, metabolism, invasion, and disruption of the intracellular environment and cytoplasmic iron homeostasis is a key regulator that induces ferroptosis (31). Tumor cells are more proliferative than normal cells and have a greater need for iron (32). The excess free iron produced by the Fenton reaction (33) or iron-containing lipoxygenase (34) oxidizes PUFA on the cell membrane to increase the formation of lipid ROS. Hydroxyl free radicals are the most active substances in ROS, which can trigger the production of PUFAs in membrane lipids. peroxidation, leading to ferroptosis in cells (35). Therefore, an increase in Fe^{2+} content in cells increases the sensitivity of cells to ferroptosis, and iron chelators such as deferoxamine can inhibit ferroptosis by chelating Fe^{2+} in cells to interfere with the production of oxidized lipids (27). Transferrin (Tf) usually binds to the transferrin receptor (TfR1) to transport iron from the intracellular environment into cells in the form of iron-transferrin complexes, a process that is important in ovarian cancer (36), sarcoma (37), and other tumors were significantly up-regulated. TfR1-mediated downregulation of the iron-transferrin complex reduces cellular iron uptake, thus inhibiting ferroptosis (38). Furthermore, heat shock protein HSPB1 and phosphorylated HSPB1 also reduce iron uptake in cells from the internal environment (39), reducing the sensitivity of cells to ferroptosis. Endogenous iron is released into the cytoplasm by lysosomes under the influence of acidic conditions, and this process depends on the phagocytosis of ferritin by nuclear receptor coactivator 4 (NCOA4) (40). Knockdown of

NCOA4 inhibits ferritin degradation, reduces cytoplasmic iron, and up-regulates ferritin heavy chain (FTH1) expression, thus inhibiting erastin-induced ferroptosis (41). Furthermore, the pentaspanin membrane glycoprotein prominin-2 promotes the formation of exosomes and transports cytoplasmic iron out of the cell (42). CDGSH iron-sulfur cluster domain 1 (CISD1) (43) and the iron-sulfur cluster biosynthesis enzyme (NFS1) (44) reduce cell susceptibility to ferroptosis by uptake of cytoplasmic iron.

Lipid peroxidation

Human cell membranes are rich in PUFA-acylated glycerophospholipids and have a wide variability (45) and the scavenging of peroxidized PUFAs can inhibit ferroptosis (46). PUFAs are very sensitive to free radical or enzyme-mediated oxidation (47), and peroxidized PUFAs bind to the glycerophospholipids of the cell membrane and participate in tumor cell ferroptosis and cause cell membrane destruction (48). Excess GSH accumulation due to down-regulation of GPX4 is a molecular mechanism leading to lipid peroxidation (49). GPX4 can reduce lipid hydroperoxides to lipid alcohols to avoid lipid peroxidation (50). Rapamycin complex 1 (mTORC1) upregulates GPX4 expression and inhibits membrane lipid peroxidation (51). The ferroptosis activator RSL3 can induce ferroptosis in tumor cells by silencing or inhibiting GPX4 expression (26). System Xc^- inhibition is another molecular mechanism leading to lipid peroxidation (49). System Xc^- is a glutamate (Glu)/cystine antiporter composed of the solute carrier family 3 member 2 (SLC3A2) and the solute carrier family 7 member 11 (SLC7A11). The ferroptosis activator erastin inhibits system Xc^- blocking the entry of cystine into cells, and the lack of intracellular Cys leads to a decrease in GSH and a decrease in GPX4 activity (52). The tumor suppressor protein BRCA1-associated protein 1 (BAP1) inhibits SLC7A11 expression in a deubiquitinating-dependent manner and induces lipid peroxidation to promote ferroptosis (53). Membrane-associated progesterone receptor component 1 (PGRMC1) inhibits SLC7A11 through autophagic degradation of lipids and induces ferroptosis in paclitaxel-resistant tumor cells (54). NADPH oxidase (NOX) and the tumor suppressor gene p53 (especially the acetylation-deficient mutant p53-3KR) also inhibit SLC7A11 (55). A recent study found that the homology of m6A reader YT521-B containing 2 (YTHDC2) can induce ferroptosis in lung adenocarcinoma cells by inhibiting SLC7A11 (56). Moreover, YTHDC2 suppressed SLC3A2 by inhibiting Homeobox A13 (HOXA13) indirectly (57) and was also found to affect system Xc^- function in lung adenocarcinoma cells.

Accumulation of reactive oxygen species

Reactive oxygen species (ROS) are closely related to the proliferation and death of tumor cells. A certain amount of ROS can promote tumor signal transduction and promote tumor cell proliferation, growth, and adaptation to hypoxia. However, excessive ROS accumulation promotes antitumor signaling, triggers oxidative stress, and induces cell death (58). The content of ROS in tumor cells is higher than in normal cells, and the neuronal redox sensing channel TRPA1 can improve the defense ability of tumor cells against ROS (59). The continuous accumulation of ROS in tumor cells eventually leads to the disappearance of mitochondrial ridges and the destruction of mitochondrial membranes, leading to ferroptosis (60). GPX4 (61), vitamin E (α-tocopherol), and coenzyme Q 10 (CoQ 10) can reduce membrane lipid ROS (62). The Fenton reaction, NADPH-dependent lipid peroxidation, GSH depletion, and decreased GPX4 activity can all promote ROS accumulation in tumor cells (63), which induces ferroptosis in tumor cells.

These studies revealed the complex regulatory mechanism of ferroptosis in tumor cells, which can promote or inhibit ferroptosis by regulating key regulators of ferroptosis. We list the key regulators related to ferroptosis in glioma and their regulatory mechanisms and further explore the application of ferroptosis in the treatment of glioma.

Mechanisms of ferroptosis in glioma

Glioma cells undergo marked metabolic reprogramming (such as the Warburg effect) and GBM cell membrane lipid species are highly cell-type specific, with lipid metabolism involved in the occurrence and progression of GBM (64). The reprogramming of lipid metabolism is affected by the efficiency of acetyl-CoA (65) and isocitrate dehydrogenase (IDH) (66). In particular, the IDH mutation is particularly important for the prognosis of glioma. The IDH mutation inhibits the function of the wild-type IDH product α-ketoglutarate by abnormally producing 2-hydroxyglutarate (2-HG), thus affecting the lipid metabolism of glioma cells (67). Ivanov et al. (68) fed glioma-transplanted rats with a prolonged diet including iron-containing water and found that it promoted the growth of gliomas in rats and improved the effects of radiotherapy, which disappeared after the injection of deferoxamine. Another study (69) suggested that iron and iron metabolism could affect the prognosis of patients with glioma, and the study also found that key regulators of ferroptosis were also important in neuronal function. Alim (70) found that selenium could inhibit GPX4-dependent ferroptosis in neuronal cells. GPX4 depletion leads to

neurodegeneration *in vivo* and *in vitro* (71). Below, we focus on the key regulators of ferroptosis and elaborate on the mechanisms of ferroptosis in glioma (Figure 1).

GPX4

GPX4 is a selenoprotein that belongs to the glutathione peroxidase family (GPX1-8) and is a key regulator in ferroptosis (72). Normally active GPX4 can reduce lipid peroxide (LPO) to alcohol or reduce intracellular H_2O_2 to water to avoid or reduce cell membrane lipid peroxidation. Reduced or inactivated GPX4 activity leads to an excessive accumulation of ROS on membrane lipids that leads to ferroptosis (73). Fragile X-related protein-1 RNA binding protein (FXR1) in glioma cells can bind to GPX4 mRNA and up-regulate GPX4 expression (74). Nrf2 can also inhibit ferroptosis by up-regulating GPX4 expression in glioma cells (75). Conversely, activation of the p38 and ERK pathways in GBM decreased the levels of GPX4 protein (76). Helena Kram et al. (77) performed immunohistochemistry on sample pairs of primary and relapse GBM of 24 patients who had received standard adjuvant treatment with radiochemotherapy. They found that the expression of GPX4 decreased significantly during tumor relapse. this study shows that recurrent tumors have a higher vulnerability to ferroptosis.

System Xc^-

System Xc^- consists of two parts, SLC7A11 and SLC3A2, and its main function is to transport extracellular cystine into cells and reverse glutamate (Glu) transport. A study has found that SLC7A11 is up regulated in a variety of tumor cells, promotes glutathione (GSH) synthesis to inhibit damage from oxidative stress to tumor cells, and is negatively correlated with the median OS of patients (78). System Xc^- also plays a significant role in ferroptosis in gliomas. The proper functioning of System Xc^- function is critical for neuronal signaling (79). Activating transcription factor 4 (ATF4) can increase neovascularization within gliomas and shape neovascularization in a SLC7A11-dependent manner (80). The expression of p53 is deregulated in GBM, and studies have found that the expression of p53 and SLC7A11 is negatively correlated in glioma cells (81) and that p53 inhibits the expression of the SLC7A11 gene (82). Furthermore, p62 binds to p53 and inhibits p53 ubiquitination in GBM. The canonical p62-mediated Nrf2 activation pathway plays an important role in the regulation of ferroptosis in wild-type GBM p53 and inhibits ferroptosis by upregulating the expression of SLC7A11.

In GBM p53 mutants, the strong interaction of p62 with mutant p53/Nrf2 enhances the inhibitory effect of mutant p53 on Nrf2, thus reversing the classical p62-mediated Nrf2 activation pathway (83). However, one study found that the tumor stem cell marker CD44 inhibited ferroptosis in tumor cells in a manner dependent on the deubiquitinase OTUB1, and overexpression of CD44 improved the stability of the SLC7A11 protein by promoting the interaction between SLC7A11 and OTUB1 (84). High expression of OTUB1 was also found in clinical samples of glioma and was positively correlated with SLC7A11 expression (85). Furthermore, the NF-κB pathway activator protein of the NF-B pathway promotes the splicing and maturation of SLC7A11 mRNA by binding to m6A, thus

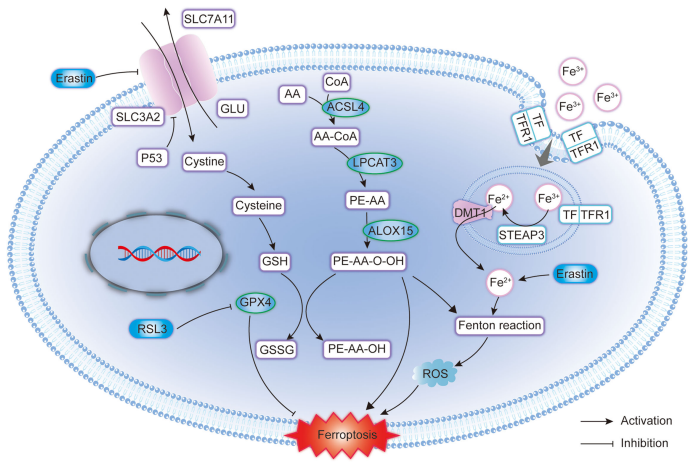

FIGURE 1
Molecular pattern diagram of ferroptosis in glioma. GPX4, Glutathione Peroxidase 4; SLC7A11, Solute carrier family 7 membrane 11; SLC3A2, Solute carrier family 3 membrane 2; GSH, Glutathione; GSSG, Glutathione disulphide; ACSL4, Acyl-CoA synthetase long-chain family member 4; ROS, Reactive oxygen species; DMT1, Divalent metal transporter 1; LPCAT3, Lysophosphatidylcholine acyltransferase 3; STEAP3, Six-transmembrane epithelial antigen of the prostate 3; TFR1, Transferrin receptor 1; TF, Transferrin; ALOX15, Arachidonate 15-lipoxygenase.

upregulating the expression of SLC7A11 and inhibiting ferroptosis in GBM cells (86).

Other regulators

MDM2 and MDMX are negative regulators of p53, and inhibition of MDM2 or MDMX increases ferroptosis suppressing protein 1 (FSP1) expression in glioma cells. The MDM2-MDMX complex regulates lipid peroxidation and promotes ferroptosis in glioma cells by altering the activity of PPARα (87). Knockdown of COPZ1 in GBM cells leads to increased expression of nuclear receptor coactivator 4 (NCOA4), and inhibition of FTH1 leads to ferritin degradation, resulting in excessive accumulation of intracellular Fe^{2+}, leading to ferroptosis (88). Inhibition of the expression of the matrix remodeling-related protein MXRA8 can also up-regulate NCOA4 and down-regulate FTH1 expression, and MXRA8 is positively correlated with the macrophage marker CSF1R. One study co-cultured glioma cell with M2 macrophages and found that MXRA8 knockdown in glioma cells attenuated the infiltration of M2 macrophages, while the addition of Fer-1 restored the infiltration of M2 macrophages (89). The deletion of NCOA4 can inhibit the reduction in the level of the FTH1 protein caused by cystine deprivation, and cystine deprivation simultaneously induces the accumulation of light chain 3 (LC3)-II protein associated with microtubules, enhances ferritin phagocytosis, and then promotes ferroptosis in GBM cells (90). The study (91) has also found that phosphorylation of heat shock protein 27 (HSP27) in GBM cells can resist erastin-induced ferroptosis, while down-regulation of HSP27 promotes erastin-induced ferroptosis and can function as a negative regulator of ferroptosis.

The above studies confirmed the feasibility of treating glioma with classical ferroptosis regulators and mechanisms such as GPX4, SLC7A11, and FSP1. Below, we focus on the key regulators of ferroptosis in the above-mentioned gliomas and describe current ferroptosis-related glioma treatment strategies.

Ferroptosis in glioma treatment

Regulation of GPX4

Studies have found that ibuprofen can induce ferroptosis in glioma cells by down-regulating GPX4 expression in Nrf2-regulated cells (75). GPX4 is a key regulator for dual artemisinin (DHA)-induced ferroptosis in GBM cells (92). DHA induces endoplasmic reticulum (ER) stress in glioma cells, leading to upregulation of ATF4 and protein kinase R-like ER kinase (PERK). ATF4 induces the overexpression of heat shock protein family A (Hsp70) member 5 (HSPA5) and increases GPX4 expression and activity. GPX4 neutralized DHA induces lipid peroxidation, thus protecting glioma cells from ferroptosis (93). Curcumin analogs induce androgen receptor (AR) ubiquitination to inhibit GPX4 activity, thus promoting ferroptosis and reducing resistance to TMZ in GBM cells (94). Studies have found that FXR1 expression is increased in TMZ-resistant glioma cells, and targeted inhibition of FXR1-GPX4 can reduce the drug resistance of TMZ-resistant glioma cells (74). Dihydrotanshinone I (DHI) increases ACSL4 expression in glioma cells and down-regulates GPX4 to inhibit glioma cell proliferation (95). The anti-malaria drug artesunate (ART) induces ferroptosis in GBM cells by regulating the p38 MAPK and ERK signaling pathways and reducing the level of GPX4 protein (76). Although gastrodin reduces the level of malondialdehyde (MDA) in rat glioma cells, which in turn increases GPX4 activity and inhibited ferroptosis in rat glioma cells (96), plumbagin induces GPX4 degradation in glioma cells *via* the lysosomal pathway and leads to GPX4-dependent cell death (97).

RSL3, a small molecule compound that can target GPX4, induces glycolytic dysfunction and autophagy-dependent ferroptosis in glioma cells (98). While down regulating GPX4, RSL3 also activates the nuclear factor kappa-B (NF-κB) pathway to induce ferroptosis in GBM cells. However, the study found that knockdown of GPX4 alone did not effectively induce ferroptosis in glioma cells. NF-κB pathway activation combined with GPX4 silencing induces ferroptosis and inhibits glioma growth and recurrence (99). Ferroptosis activators can inhibit GPX4 expression and synergize with radiotherapy, inducing ferroptosis in glioma cells without increasing DNA damage (100). Local chemotherapy is also a new direction in the treatment of glioma, increasing local chemotherapy drugs while minimizing the impact on normal cells, to inhibit tumor growth and recurrence. The study has reported on the use of gene therapy-based iron oxide nanoparticles (IONP) to deliver GPX4 small interfering RNA (siRNA) and cisplatin (Pt). Nanoparticles activate NADPH oxidase (NOX) to increase H_2O_2 levels while releasing si-GPX4 to inhibit GPX4 expression, causing excessive ROS accumulation and triggering ferroptosis in glioma cells (101). In one study, paclitaxel-loaded iron oxide nanoparticles (IONP@PTX) enhanced the expression of autophagy-related proteins Beclin1 and LC3II, inhibited the expression of p62 protein, and GPX4, and induced ferroptosis in GBM cells (102). Tumor immunity-related studies have found that certain tumor lesions that occur during early tumor progression (i.e., ischemia) recruit neutrophils to sites of tissue damage. However, neutrophils can induce ferroptosis in GBM cells by regulating

GPX4, creating a positive feedback loop that exacerbates the development of internal GBM necrosis (103).

Regulation of SLC7A11

Studies have found that ibuprofen affects GPX4 expression in glioma cells by downregulating Nrf2 and at the same time inhibits the activity of SLC7A11 (75). Activation of the Nrf2-Keap1 pathway up-regulates SLC7A11 to release a large amount of Glu out of glioma cells, thereby affecting the tumor microenvironment, which may be related to the decreased survival rate of patients with glioma with high expression of SLC7A11 (104). Like ibuprofen, in addition to downregulating GPX4, plumbagin can significantly down-regulate SLC7A11 mRNA and protein levels in glioma cells and induce ferroptosis in glioma cells (97). As a first-line drug for chemotherapy with GBM in clinical practice, TMZ can affect GPX4 and reduces the activity of SLC7A11. Studies have found that gliomas with high expression of SLC7A11 are more sensitive to erastin-TMZ combination therapy and have better therapeutic effects (105). ATF4 is a key regulator in cellular metabolism and maintenance of oxidative homeostasis, and upregulation of SLC7A11 expression by ATF4 improves the resistance of gliomas to chemotherapeutic drugs such as TMZ (106). However, the latest study found that down-regulation of ATF4 in GBM cells inhibited CHAC1 expression and blocked sevoflurane (Sev)-induced ferroptosis (107). Activating transcription factor 3 (ATF3) in glioma can promote ferroptosis of glioma cells by upregulating NOX4 and SOD1 to produce H_2O_2 and promote the strychnine-induced accumulation of H_2O_2, and by downregulating SLC7A11 to prevent degradation of H_2O_2 (108). Pseudolaric acid B (PAB) increases the intracellular iron content in the glioma by upregulating the transferrin receptor, activating NOX4, and producing excess H_2O_2 and LPO. PAB blocks cystine supply through the p53-mediated SLC7A11 pathway, depleting intracellular GSH and further exacerbating H_2O_2 and LPO accumulation (109). RSL3 is a GPX4 inhibitor, and recent studies have found that RSL3 can down-regulate SLC7A11 expression by activating the NF-κB pathway (99).

Regulation of other key regulators of ferroptosis

Differences in transferrin receptor (TfR) in normal human astrocytes (NHA) and GBM cell lines may be the key to DHA selective killing of tumor cells to induce ferroptosis (92). A team designed cRGD/Pt + DOX@GFNPs (RPDGs) nanoparticles to promote the simultaneous occurrence of apoptosis and ferroptosis by disrupting redox homeostasis in mouse GBM-resistant cells. Using the Fenton reaction of gallic acid (GA)/Fe^{2+} to catalyze nanoparticles in the intracellular environment, Pt (IV) depletes intracellular GSH and increases the accumulation of reactive oxygen species (ROS), thus inducing ferroptosis in GBM-resistant cells (110). Fe_3O_4-siPD-L1@M-$_{BV2}$ increased Fe^{2+} accumulation in mouse GBM-resistant cells and significantly decreases the expression of programmed death-ligand 1 (PD-L1). Fe_3O_4-siPD-L1@M-$_{BV2}$ also increases the ratio of effector T cells to regulatory T cells in drug-resistant GBM (111). Amentoflavone (AF) can not only induce autophagy in glioma cells by regulating the AMPK/mTOR pathway but is also associated with ferroptosis in gliomas. Knockdown of autophagy-related protein 7 (ATG7) was found to increase ferritin heavy chain 1 (FTH1) expression and inhibits AF-induced ferroptosis. It demonstrates that AF triggered ferroptosis in an autophagy-dependent manner, thereby suppressing glioma growth and recurrence (112). Another study found that siramesin combined with lapatinib mediates ferroptosis in glioma cells through iron release in lysosomes and protease degradation of HO-1 (113). Doranidazole and misonidazole can induce ferroptosis by blocking metabolic alterations in mitochondrial complex I and II of hypoxic glioma stem cells (GSC) that trigger responses to oxidative stress (114).

The above studies demonstrate the mechanism by which different drugs treat glioma by modulating key regulators of ferroptosis (Table 1). In addition, ferroptosis-related ncRNAs also have a certain influence on the treatment of glioma. Next, we will elaborate on ferroptosis-related ncRNAs.

Ferroptosis-related ncRNAs in glioma treatment

Ferroptosis and ncRNAs are closely related to tumors (115). Among ncRNAs, miRNAs, lncRNAs, and circRNAs are all involved in the potential regulatory mechanisms of tumor ferroptosis (116). ncRNAs can regulate the protein levels of ferroptosis-related genes (117), influence the expression of mRNA of ferroptosis-related genes (118), lead to modification of m6A (117), and control epigenetic activity (119). ncRNAs induce ferroptosis by regulating cellular iron metabolism, ROS metabolism, and lipid metabolism. Recent studies have found that ncRNAs also play a key role in glioma ferroptosis (Figure 2). GPX7 is a member of the glutathione peroxidase

TABLE 1 Summary of ferroptosis-associated agents.

Ferroptosis-associated agents	Mechanism	Function	Study
Ibuprofen	down-regulates GPX4 expression and inhibits the activity of SLC7A11	Induces ferroptosis	(75)
Dual artemisinin	up-regulates ATF4 induces the overexpression of HSPA5 and increases GPX4 expression and activity	Inhibits ferroptosis	(93)
Curcumin analog	induces AR ubiquitination to inhibit GPX4 activity	Induces ferroptosis	(94)
Dihydrotanshinone I	increases ACSL4 expression and down-regulates GPX4	Induces ferroptosis	(95)
Artesunate	down-regulates GPX4	Induces ferroptosis	(76)
Plumbagin	induces GPX4 degradation *via* the lysosomal pathway and down-regulates SLC7A11 mRNA and protein expression	Induces ferroptosis	(97)
RSL3	inhibits GPX4 and down-regulates SLC7A11 expression by activating the NF-κB pathway	Induces ferroptosis	(99)
IONP	activates NOX to increase H_2O_2 levels while releasing si-GPX4 to inhibit GPX4 expression	Induces ferroptosis	(101)
IONP@PTX	up-regulates the expression of autophagy-related proteins Beclin1 and LC3II, and inhibits the expression of p62 and GPX4	Induces ferroptosis	(102)
Temozolomide	down-regulates GPX4 and reduces the activity of SLC7A11	Induces ferroptosis	(105)
Amentoflavone	induces autophagy by regulating the AMPK/mTOR pathway, and down-regulates FTH1 expression	Induces ferroptosis	(112)
Siramesin and lapatinib	*Via* iron release in lysosomes and protease degradation of HO-1	Induces ferroptosis	(113)

family (GPX), and miR-29b can inhibit GPX7 expression, thus improving the sensitivity of glioma cells to erastin-induced ferroptosis (120). miR-670-3p inhibited GBM cell ferroptosis by downregulating ACSL4 expression, while the miR-670-3p inhibitor increased the antitumor effect of TMZ (121). miR-18a inhibited ferroptosis related to p53-SLC7A11 in GBM cells by down-regulating the expression of ALOXE3 (122). The lncRNA TMEM161B-AS1 increases FANCD2 and

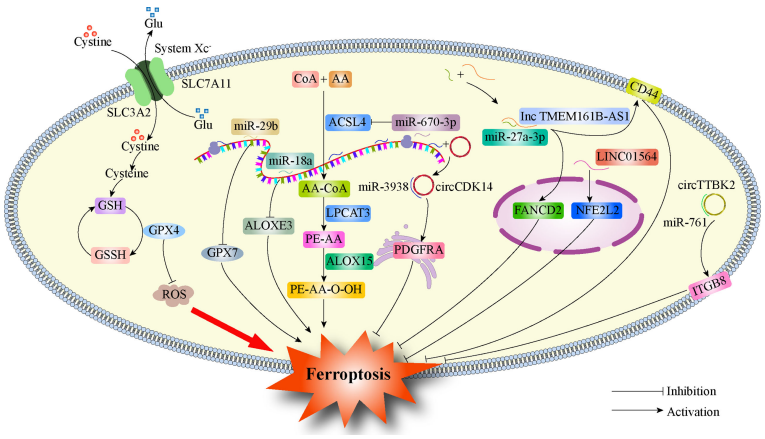

FIGURE 2
Regulation of ferroptosis by ncRNAs in glioma. GPX4, Glutathione Peroxidase 4; SLC7A11, Solute carrier family 7 membrane 11; SLC3A2, Solute carrier family 3 membrane 2; GPX7, Glutathione Peroxidase 7; ACSL4, Acyl-CoA synthetase long-chain family member 4; ALOXE3, Arachidonate lipoxygenase 3; PDGFRA, Platelet-derived growth factor receptor; FANCD2, FA Complementation Group D2; CD44, Cluster of differentiation 44; NFE2L2, Nuclear factor erythroid 2-like 2; ITGB8, Integrin subunit beta 8.

TABLE 2 The regulatory role of ferroptosis-related ncRNAs in glioma progression.

NcRNA	Cell Lines	Mechanism	Function	Study
miR-29b	U87 T98G LN229 A172	Target GPX7	Induces ferroptosis and enhances glioma cell sensitivity to erastin-induced ferroptosis	(120)
miR-670-3p	U87MG A172	Target ACSL4	Inhibits ferroptosis	(121)
miR-18a	U87MG U251	Target ALOXE3	Inhibits ferroptosis and promote migration	(122)
lncRNA TMEM161B-AS1	U87MG U251	Sponge with mir-27a-3p and upregulate the expression of FANCD2 and CD44	Inhibits ferroptosis	(123)
LINC01564	LN229/TMZ U251/TMZ	Upregulate the expression of NFE2L2	Inhibits ferroptosis and promote TMZ resistance	(124)
circ CDK14	HBE SF126 U251 U87	Sponge with mir-3938 and upregulate the expression of PDGFRA	Inhibits ferroptosis	(125)
circ TTBK2	LN229 U251 NHA	Sponge with mir-761 and upregulate the expression of ITGB8	Inhibits ferroptosis	(126)

CD44 expression by adsorbing hsa-miR-27a-3p, inhibits apoptosis and ferroptosis, but reduces the resistance to TMZ in GBM cells (123). LINC01564 has the opposite effect, inhibiting ferroptosis by upregulating NFE2L2 expression and enhancing glioma cell resistance to TMZ (124). Circular RNA CDK14 upregulates the expression of the PDGFRA oncogene in GBM cells by adsorbing miR-3938 and reducing the sensitivity of GBM to erastin-induced ferroptosis (125). The circRNAs TTBK2 and ITGB8 are highly expressed in glioma tissues and cells, and TTBK2 can inhibit ferroptosis in glioma cells by up-regulating ITGB8 by adsorbing miR-761 (126). Table 2 summarizes the findings from recent studies as these results suggest that ncRNAs play a key role in ferroptosis in gliomas and may become new therapeutic targets for gliomas.

Conclusions and future prospects

The heterogeneity of glioma cells alters the sensitivity of gliomas to different chemotherapeutic drugs. The use of a certain chemotherapeutic drug alone in the treatment process cannot achieve the expected desired effect, and new treatment methods are needed to supplement it. The induction of ferroptosis in tumor cells has attracted increasing attention as a new strategy for the treatment of glioma. Previous studies have preliminarily explored the mechanism of ferroptosis in glioma and found that improved iron metabolism and resistance to lipid peroxidation are prevalent in glioma cells. However, recurrent GBM showed high sensitivity to ferroptosis. These studies demonstrate ferroptosis as a new option for glioma treatment in the face of tumor resistance and recurrence. Protein molecules such as GPX4 and System Xc^- and ferroptosis-related ncRNAs have become important targets for glioma therapy. Current studies have demonstrated the value and potential of treating glioma through the canonical pathway and factors of ferroptosis. However, GPX4 is an essential gene in mammals, and whether drugs that inhibit GPX4 to treat tumors will bring unbearable side effects to glioma patients remains to be further studied. Therefore, it is particularly important to explore non-canonical pathways of ferroptosis in the treatment of glioma, such as the treatment of glioma through the HO-1 pathway. In addition, the current study does not involve the treatment of recurrent glioma, and ferroptosis-related ncRNA research and molecular therapy are also in their infancy. Therefore, it is particularly important to study ferroptosis-related mechanisms in greater detail in glioma and to explore ferroptosis-related ncRNAs, nanoparticles, and exosomes. Currently, the clinical trials investigating ferroptosis applied to the treatment of glioma are still incomplete. Inducing ferroptosis to destroy tumor cells and reducing damage to normal cells of the central nervous system is the key to promoting the clinical

translation of research findings. In conclusion, as a complement to current therapeutic approaches, ferroptosis has immense potential in the treatment and prognosis of glioma.

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GZ and YF participated in the conception and designed this review. ZZ provided administrative support. GZ wrote the manuscript. YF and XL revised the manuscript. All authors contributed to this article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ferroptosis in glioma treatment: Current situation, prospects and drug applications

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Ferroptosis is a regulatory form of iron-dependent cell death caused by the accumulation of lipid-based reactive oxygen species (ROS) and differs from apoptosis, pyroptosis, and necrosis. Especially in neoplastic diseases, the susceptibility of tumor cells to ferroptosis affects prognosis and is associated with complex effects. Gliomas are the most common primary intracranial tumors, accounting for disease in 81% of patients with malignant brain tumors. An increasing number of studies have revealed the particular characteristics of iron metabolism in glioma cells. Therefore, agents that target a wide range of molecules involved in ferroptosis may regulate this process and enhance glioma treatment. Here, we review the underlying mechanisms of ferroptosis and summarize the potential therapeutic options for targeting ferroptosis in glioma.

KEYWORDS

glioma, ferroptosis, targeting treatment, reactive oxygen species, iron metabolism

Introduction

Glioma is the most common malignancy of the central nervous system (CNS) and manifests with highly invasive growth, neovascularization, and resistance to various combination therapies (1). Despite advanced therapeutic strategies, including aggressive surgery, radiotherapy, and chemotherapy, glioblastoma (GBM) patients still show poor prognosis and a median overall survival of less than 16 months (2). Despite aggressive treatment measures, including maximal safe surgical resection followed by external irradiation therapy accompanied with adjuvant temozolomide (TMZ) treatment, approximately 90% of grade WHO IV gliomas recur locally within 2 years (3). Gross

total resection (GTR), defined as complete radiectomy of the contrast-enhanced region of high-grade glioma (HGG) and T2-weighted/fluid attenuated inversion recovery (T2/FLAIR) MRI-indicated hyperintensive nonenhancing lesions, almost always fails to completely remove all microscopic residual tumor cells (4). Similar to other malignancies, GBM exhibits a distinct anti-DNA-damage phenotype, which leads to chemoresistance (5).

Hence, therapies targeted to gliomas have not been considered sufficiently effective (6). However, ferroptosis has recently attracted considerable interest, especially because the mechanism involves downregulation and silencing of genes involved in the initiation and execution of cancer necroptosis (7). Ferroptosis is a unique iron-dependent form of nonapoptotic cell death in which the affected cells are morphologically, biochemically, and genetically distinct from apoptotic, necrotic, and autophagic cells (8). Ferroptosis is driven by the lost lipid repair enzymatic activity of glutathione peroxidase 4 (GPX4) and subsequent accumulation of lipid-based reactive oxygen species (ROS), particularly lipid hydroperoxides (9). As a common recognition feature, ferroptotic cells appear as clear and transparent round cells under the microscope, mainly composed of empty cytosol, which is called the "ballooning phenotype". In addition, ferroptotic cells also have ultrastructural changes in mitochondria such as volume decreased, bilayer membrane density increased, outer mitochondrial membrane (OMM) destroyed, and mitochondrial cristae disappeared.

To promote tumor growth, cancer cells exhibit a higher iron demand than normal cells. This iron dependence makes cancer cells more susceptible to ferroptosis (10). Therefore, induced ferroptosis induction may offer the unique possibility of effectively eradicating certain tumor cells, especially those in a highly mesenchymal state (11) and those that evade drug treatment (12). Furthermore, ferroptosis plays a pivotal role in suppressing tumorigenesis by eliminating cells in environments that lack key nutrients or produce cellular stress or that are infected with pathogens (13). The ferroptotic sensitivity of cancer cells may be related to the activation of Ras-mitogen-activated protein kinase (MEK) (14), which contributes to the upregulation of transferrin receptor 1 and increased intracellular iron levels, as well as to the additional formation of ROS *via* inhibited cystine-based reactions (15). Many other molecules in different pathways have been found to be involved in ferroptosis in glioma (16), and the related content is summarized in this review.

Focused overview of ferroptosis pathways

An overview of ferroptosis pathways is shown in Figure 1.

Iron metabolism in ferroptosis

The regulatory mechanism that coordinates intracellular iron homeostasis is centered on iron regulatory proteins (IRPs), which exerts effects by binding to iron-responsive elements (IREs) (17, 18). Under physiological conditions, cellular iron absorption is controlled mainly by the plasma membrane protein transferrin receptor 1 (TFR1), and therefore, knocking down TFR1 expression can block transferrin-bound iron entry into a cell (19, 20), preventing ferroptosis caused by erastin or cystine deprivation (21). Diminishing ferritin expression (22) or FPN1 or ceruloplasmin depletion increases the cell sensitivity to ferroptosis (23, 24). In addition, reduced IRP2 activity, increased expression of transferrin (Tf) and the transferrin receptor (TFR) (19), and recognition of FTH1 by a specific cargo receptor (nuclear coactivator 4, NCOA4), which leads to formation of a complex that fuses with lysosomes (25), cause an abnormal increase in unstable intracellular iron stores, a critical factor in ferroptosis. Other iron metabolism-related proteins also affect cell sensitivity to ferroptosis (26), and certain genes exert the same effects. Recently, the critical role played by STEAP3 in cancer has been extensively investigated, and STEAP3 has thus been found to be a key regulator of ferroptosis by mediating iron metabolism (27, 28). Overexpression of STEAP3 contributes to iron uptake and maintains iron stores (29), supporting the proliferation of multiple types of cancer cells (30–32). Hence, dysregulation of iron metabolism is an important contributing factor to ferroptosis.

Lipid peroxidation in ferroptosis

Lipids are critical for maintaining the membrane integrity of a cell, and extensive peroxidation of lipids changes the assembly, composition, structure, and dynamics of lipid membranes (33). Polyunsaturated fatty acids (PUFAs) containing phospholipids (PLs; PUFA PLs) are substrates for lipid peroxidation (34). ROS are free radicals and/or oxygen derivatives, including superoxide anions, hydrogen peroxide, hydroxyl radicals, lipid hydroperoxides, peroxy radicals, and peroxynitrite (35). Membranes containing high levels of PUFAs are extremely sensitive to ROS effects and highly vulnerable to lipid peroxidation (36, 37). Lipid undergo peroxidation through two routes: nonenzymatic autoxidation and enzymatic PL peroxidation; the former pathway is known as "nonenzymatic PL autoxidation".

Nonenzymatic peroxidation of lipids is mediated by carbon- and oxygen-based radicals and can be divided into three discrete stages: initiation, proliferation, and termination (33). The initial phase involves a series of reactions collectively known as "Fenton

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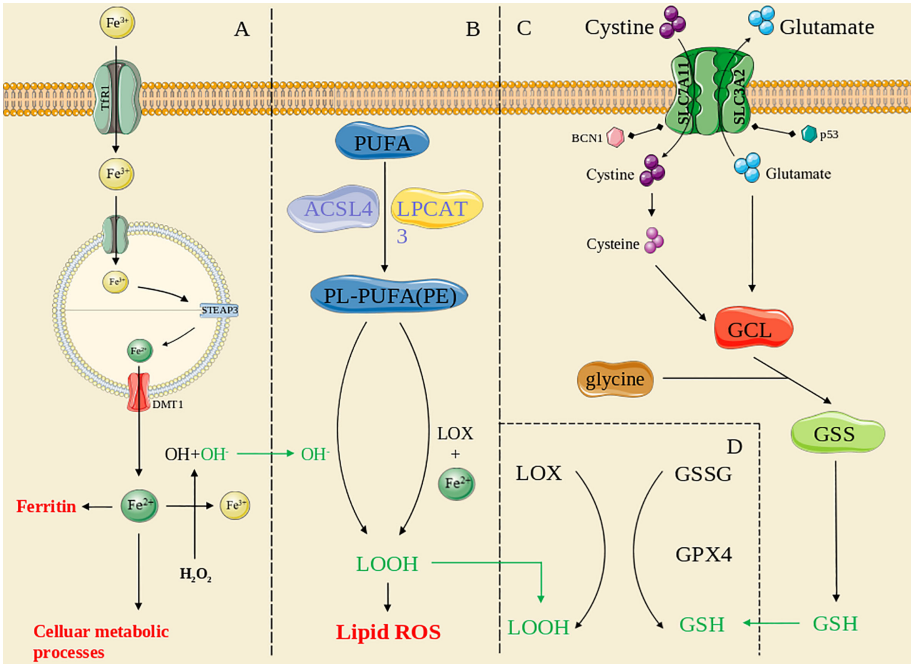

FIGURE 1
The overview of ferroptosis pathways. **(A)**: the iron metabolism pathway; **(B)**: the lipid peroxidation pathway; **(C)**: the antioxidant systems pathway; **(D)**: the GPX4-mediated pathway. The green line means the substance acts across pathways.

chemistry" in which labile iron reacts with endogenous hydrogen peroxide or superoxide to form oxygen-based radicals (38). Radical compounds produce new radicals, which are markers of the proliferative phase. The hydroxyl and peroxide radicals produced through a Fenton reaction can form a resonant stable carbon-based radical by extracting hydrogen from the bis allylic methylene of a membrane PUFA, which can react with molecular oxygen in solution to form the lipid peroxide radical ROO^{-}, which can remove a hydrogen from a different bis allylic methylene to generate peroxidized lipid (ROOH) and another carbon-based radical that can react with oxygen (33, 39, 40). Finally, antioxidants terminate radical propagation (41).

Enzymatic PL peroxidation is mainly mediated by cyclooxygenases (COXs), cytochrome p450 species (CYPs), NADPH oxidase (NOX), and, especially, lipoxygenases (LOXs) (42). Arachidonic acid (C20:4) and linoleic acid (LA; C18:2) are substrates for LOX (43), and ferric iron is a cofactor of LOX (44, 45). In contrast to 5-lipoxygenases, 12- and 15-lipoxygenases exhibit incomplete regional selectivity in producing lipid peroxides (46) and are thought to respond to intact phospholipids and do not promote hydrolysis for peroxidation (47, 48). Lipid hydroperoxides (LOOHs) and the autoxidation products of PUFAs are currently markers of ferroptosis (49, 50).

Antioxidant systems in ferroptosis

In addition to lipid peroxidation, the cellular antioxidant system contributes to ferroptosis by decomposing ROS. GPX4 is a central factor in anti-ferroptosis reactions (51). This protein is expressed as several isoenzymes with different subcellular locations and distinct tissue-specific expression patterns (52, 53). GSH is a cofactor of GPX4, and GSH synthesis is maintained by the amino acid antiporter SLC7A11/xCT/system (54). Some small-molecule compounds can regulate the activity of glutamate-cysteine ligase (GCL) and xCT (8) and thus affect GSH synthesis, eventually leading to ferroptosis. Several other small-molecule compounds can directly inhibit GPX4 activity or cause GPX4 protein degradation (55, 56). Nonoxidized dopamine and activated heat shock protein family A member 5 (HSPA5) prevent GPX4 degradation (57, 58), whereas heat shock protein 90 (HSP90)-dependent chaperone-mediated autophagy promotes erastin-induced GPX4 degradation (59). Furthermore, GPX4-independent ferroptosis pathways have been identified. Ferroptosis inhibitory protein (FSP1) and CoQ10 facilitate a shuttle of reducing equivalents derived from NAD(P)H to the lipid bilayer (60). In addition, POR is involved in ML210-induced ferroptosis (61), and P53 can affect ferroptosis without GPX4 inhibition (62). The main regulatory factors are described in detail in the next section.

Critical factors of ferroptosis in glioma

Ferroptosis follows multiple pathways and involves pivotal factors that are regulated by many different regulators. Certain regulators exert valuable regulatory effects and metabolic changes in glioma cells. In this section, the regulators best characterized to date are described, and additional regulators are presented in Table 1.

GPX4

GPX4, a core factor in the antioxidant system, regulates certain LOX activities by controlling cellular peroxide formation (82). LOX binds to molecular oxygen when iron is oxidized into trivalent iron and adds this molecular oxygen to a PUFA after proton extraction from the bis-allylic positions of the PUFA, leading to the enzymatic peroxidation of the PUFA (43). The GPX4-mediated antioxidant system can reduce the peroxide concentration, which may affect LOX activity, reducing the peroxidation rate of PUFAs and ultimately inhibiting ferroptosis (63). Studies have pointed out that 15-LOX and its linoleic acid (LA)-derived metabolites exerted protumorigenic effects on GBM cells *in vitro* (83). This report may imply that GPX4 affects ferroptosis by regulating LOX activity and can be exploited for glioma treatment.

GSH is a reducing substrate for GPX4, and its interaction with SCL7A11 plays a crucial regulatory role in ferroptosis. However, both GSH and SCLA11 activities are intricately regulated by p53 and NRF2, among other proteins., as described in detail in a subsequent section (64).

Western blot and immunohistochemistry (IHC) analyses showed relatively high expression levels of Gpx4 in glioma tissues and cell lines, and its expression was found to be augmented as the glioma grade increased. In addition, experiments showed that knocking down GPX4 expression inhibited the proliferation and migration of glioma cells (84). Previously, inhibition of GPX4 activity was thought to induce apoptosis (85), and combined with the aforementioned findings, it can be concluded that GPX4 inhibition can also induce ferroptosis, which may become a new research target.

Nrf2

Under normoxic conditions, Nrf2, a transcription factor, binds to Kelch-like ECH-associated protein 1 (Keap1) and is inactivated by proteasome degradation after ubiquitination (86). After cells contact a large number of electrophiles or cytotoxic agents or enter into an oxidative stress state, Nrf2 dissociates from Keap1 and rapidly transfers to the nucleus where interacts with antioxidant response elements (AREs) to ultimately maintain intracellular redox homeostasis (65). Nrf2 regulates

TABLE 1 Critical factors of ferroptosis in glioma.

Factors	Targets	Mechanism	Reference
GPX4	peroxide↓	affect LOX activity, reducing to peroxidation of PUFAs, inhibit ferroptosis	Seibt et al. (63)
	GSH	reduce LOOH, inhibit LPO, inhibit ferroptosis	Ursini et al. (64)
Nrf2	Keap1	dissociates from Keap1, interacts with ARE, maintain intracellular redox homeostasis	Zhang et al. (65)
	MRP1↑	prevents GSH efflux from the cells, strongly restrains ferroptosis	Cao et al. (66)
	xCT↑	reduced ROS formation, prevents ferroptosis	Fan et al. (67)
P53	xCT↓	combination with response elements in the xCT promoter region, inhibit its expression	Jiang et al. (68)
	USP7	promotes nuclear translocation of USP7, removes H2Bub1, reduces the expression of xCT	Wang et al. (69)
	SAT1	induces elevated ALOX15 levels, causes ferroptosis *via* oxidation of PUFA	Ou et al. (70)
BAP1	xCT↓	decrease H2Aub occupancy on the promoter and gene body of xCT	Zhang et al. (71)
OTUB1	p53	regulate the p53 pathway by regulating the activities of Mdm2 and Mdmx	Sun et al. (72)
			Chen et al. (73)
	xCT	Inactivation of OTUB1 lead to a substantial reduction in xCT levels	Liu et al. (74)
ATF4	xCT	ATF4 knockout will reduced xCT transporter activity	Dixon et al. (75)
			Chen et al. (76)
	ROS	ATF4 deficiency increases ROS levels	Angeli et al. (77)
NCOA4	iron homeostasis	iron-bound NCOA4 interacts with the ubiquitin E3 ligase HERC2, reduce the ferritinophagy	Mancias et al. (78)
	FTH1↓	decreased FTH1 levels would cause cells to respond to several ferroptosis-inducing agents	Hayashima et al. (79)
YAP/TAZ	Nuclear translocation	YAP/TAZ be phosphorylated by MOB1	Masliantsev et al. (80)
	autophagy↑	activated YAP/TAZ promotes autophagy, affects ferroptosis	Sun et al. (81)

The symbol ↓ means target factor level reduced, the symbol ↑ means target factor level rises.

ferroptosis by regulating the expression of genes related to GSH regulation (genes that encode proteins involved in GSH synthesis and, supply cysteine mediated by xCT, GSH reductase, GPX4), iron regulation (including export and storage of iron, heme synthesis, and catabolism), and NADPH regeneration (87–89). Considering recent research, we speculated that Nrf2 partially targets xCT to regulate GPX4 synthesis and function, thus regulating ferroptosis. When Keap1 activity is inhibited, Nrf2 activity increases, leading to the upregulated expression of the ATP-binding cassette (ABC)-family transporter multidrug resistance protein 1 (MRP1), which prevents GSH efflux from the cells and profoundly inhibits ferroptosis (66). The expression of Nrf2 was increased 3-fold in human GBM compared to that in normal brain tissue (67). Both the low expression of Keap1 and the overexpression of Nrf2 led to a significant increase in xCT mRNA levels (up to a 5-fold increase), which subsequently reduced ROS formation. In contrast, both the overexpression of Keap1 and the low expression of Nrf2 eventually led to a substantial increase in ROS levels (67). Thus, the levels of NRF2 are directly related to ferroptosis sensitivity, as increased NRF2 expression prevents ferroptosis, and decreased NRF2 expression enhances the sensitivity of cancer cells to ferroptosis (67, 90).

P53

The tumor suppressor p53 is a transcription factor that regulates various cellular responses through selective transcriptional regulation of various target genes or interaction with other proteins. Studies have shown that xCT is a target of p53 and that p53 sensitizes cells to ferroptosis through transcriptional inhibition of xCT expression (68). The combination of p53 with response elements in the xCT promoter region inhibited xCT expression and increased the sensitivity of cancer cells to ferroptosis inducers such as erastin; however, $p53^{3RK}$ failed to induce cell cycle arrest, senescence, or modulation and inhibited xCT expression, ultimately promoting the response to stress induced by ROS (68). However, another acetylation-defective mutant of p53, p534KR98 (with a lysine K98 substitution), showed no ability to reduce xCT expression (91). As recently reported, p53 sensitized cells to erastin-induced ferroptosis through a comprehensive pathway. P53 promotes nuclear translocation of USP7 (a deubiquitinase) that removes the H2Bub1 mark (monoubiquitinated histone H2B on lysine 120) from the regulatory region of the xCT gene. Loss of the H2Bub1 mark inhibited the expression of xCT, leading to ferroptosis (69).

Low-molecular-weight polyamines such as putrescine, spermidine, and spermine are amino acid-derived polycationic alkylamines involved in the regulation of cell growth, proliferation, and differentiation (92). Spermidine/spermine N1-acetyltransferase 1 (SAT1) is a rate-limiting enzyme that controls polyamine catabolism in cells by acetylating spermidine and spermine mediated through acetyl-coenzyme A (93). Overexpression of SAT1 causes a rapid depletion of spermidine and spermidine levels and an increase in putrescine abundance, which causes significant cellular growth inhibition and mitochondrial pathway apoptosis (94). SAT1 has been confirmed to be a transcriptional target of p53, and only the ferroptosis inhibitor ferrostatin-1 was able to inhibit ROS-induced cell death in SAT1-overexpressing cells. In contrast to its effect on conventional pathways, SAT1 exerted no effect on xCT or GPX4 expression or activity but induced an increase in ALOX15 level, which in turn led to ferroptosis mediated *via* the oxidation of PUFAs (70).

Glutamine metabolism affects ferroptosis and exerts a particularly high effect on serum-dependent pathways after amino acid deficiency (19). GSL2 (glutaminase 2) in mitochondria is a transcriptional target of p53 and is the core glutaminase in the glutamine-to-glutamate metabolic pathway (95). the GSL2 is transcribed by p53 and mediates the generation of GSH in LN-2024 cells (a human glioblastoma cell line) to enhance their antioxidant capacity (96).

In addition to the aforementioned effects, p53 inhibited ferroptosis in some tumor cells. For example, studies showed that binding of p53 to dipeptidyl peptidase-4 (DPP4) inhibited ferroptosis in colorectal cancer cells, and certain DPP4 inhibitors completely blocked erastin-induced cell death in p53-deficient colorectal cancer cells (97). These studies suggest that the inhibition of p53 activity is specific to ferroptosis inducers (98). The tumor suppressor CDKN1A/p21 induces cell cycle arrest and senescence (99, 100). Although the cell cycle arrest mediated by CDKN1A is insufficient to inhibit ferroptosis (101), the induction of p53 increases GSH synthesis and thus inhibits ferroptosis (102).

According to The Cancer Genome Atlas (TCGA) data, 78% of GBM cases present with mutations in the p53 pathway (103), including direct mutations in the p53 gene (in secondary GBM) and a loss of the INK4A/ARF (CDKN2A) gene locus, PTEN mutations and EGFR amplification/loss (in primary GBM) (104). Since p53 is involved in various cellular responses involving the cell cycle or leading to apoptosis, differentiation and DNA damage, the regulatory effect of p53 on ferroptosis needs to be assessed on the basis of the situation, and further research is required (105).

BAP1

BRCA1-associated protein 1 (BAP1) is a tumor suppressor with functions such as tumor suppression, cell cycle control, DNA damage repair, and differentiation (106–109) that is widely recognized as a deubiquitinating enzyme (DUB) (110). Study results have suggested that wild-type (WT) BAP1 significantly decreased H2Aub occupancy on the promoter and gene body of

xCT, but the C91A mutant did not exert this effect (71). Because WT BAP1 exhibited DUB activity and BAP1 C91A did not in this experiment, WT BAP1 was the clear cause of inhibited xCT expression (71). Therefore, BAP1 may be recruited by other proteins in the PR-DUB complex, such as ASXL1, which also strongly bind to the xCT promoter (111). BAP1 has been frequently shown to inactivate the expression of genes with mutations or deletions in tumor cells (77), but its behavior in glioma is abnormal. For example, although BAP1 is generally considered to be a chromatin-associated protein and thus to reside within the nucleus (112), recent studies have found it in both the nucleus and cytoplasm of glioma cells, suggesting BAP1 protein is differentially distributed in glioma cells (113, 114). Notably, high cytoplasmic abundance of BAP1 was significantly associated with low overall survival, and nuclear abundance of BAP1 cells was not correlated with overall survival (114). Since BAP1 shows aberrant cytosolic abundance in glioma and because the BAP1-related pathway inhibiting ferroptosis is located in the nucleus, the abnormal distribution of BAP1 in glioma cells, compared to that in other cancer cells, and the BAP1 regulatory pathway in the nucleus can be new research targets.

OTUB1

The ubiquitin hydrolase OTUB1 was previously thought to regulate the p53 pathway by regulating the activities of Mdm2 and Mdmx (72, 73), but OTUB1 has been found to interact directly with xCT to regulate xCT independent of p53 (74). The expression of OTUB1 in glioma compared to adjacent tissues and its expression level was correlated with the low survival of glioma patients (115). Coimmunoprecipitation assays showed that the endogenous OTUB1 protein was coprecipitated with an anti-xCT-specific antibody, and endogenous xCT was coprecipitated with an anti-OTUB1-specific antibody. *In vitro* GST pull-down assays confirmed that OTUB1 is a binding partner of xCT (74). Inactivation of OTUB1 directly led to a substantial reduction in the xCT level, and this effect was confirmed to sensitize cells to erastin and the ferroptosis inhibitor ferrostatin-1 (8, 74). However, the sensitization effect caused by OTUB1 knockdown, which affected both cysteine and glutathione levels in glioma, was rescued by the overexpression of xCT (115). Notably, the ectopic overexpression of xCT is evident occurs in many cancers (68, 116–118). Hence, xCT levels may be stabilized by the absence of OTUB1, promoting ferroptosis and ultimately inhibiting tumor growth (74).

ATF4

Activating transcription factor 4 (ATF4) is another key transcriptional regulator and mediator of metabolism and oxidative homeostasis (76, 119) that can be activated by several stress signals, such as those triggered by anoxia, hypoxia, endoplasmic reticulum (ER) stress, oxidative stress and amino acid deprivation (120). ATF4 expression is significantly higher in malignant gliomas than in untransformed human brain tissue; moreover, ATF4 can promote the proliferation and migration of glioma cells, and patients with high ATF4 expression exhibit a relatively short overall survival time (76). ATF4 expression resulted in a significant increase in xCT mRNA levels in human glioma specimens compared to that in normal brain tissue (a 5-fold increase in gliomas with a WHO° II classification and 19-fold in gliomas with a WHO° IV classification), and xCT protein levels were increased with AFT4 levels. xCT antiporter activity is determined on the basis of extracellular glutamate levels, and ATF4 knockout significantly reduced glutamate release and cystine uptake, which in turn significantly reduced xCT transporter activity (75, 76). These data suggest that ATF4 deficiency increases ROS levels in cells, but the accumulation of ROS has been shown to prevented by chelation of the iron internalized by cells, and the effects produced by ATF4 overexpression can be inhibited by sorafenib and erastin (76, 77). In addition, the growth-promoting effect of ATF4 on cells is mediated by xCT.

Pathological vessels constitute a the specific microenvironmental niche in primary brain tumors (121, 122). The expression level of ATF4 affected the growth of tumor vessels; specifically, ATF4 overexpression increased the number and length of tumor vessels, and ATF4 knockdown led to the opposite effect (76). The effects of ATF4 activity on tumor vessels were regulated by ferroptosis; moreover, erastin and RSL3 inhibited angiogenesis in glioma, and this inhibitory effect was attenuated with increased expression of ATF4 expression, although the outcome was not notable (76). ATF4 is thought to interact with components associated with ER stress (123) and to prevent cellular resistance to partial ferroptosis inducers, such as TMZ and dihydroartemisinin (124). Therefore, ATF4 is involved in multiple pathways and thus presents possibilities for ferroptosis regulation, which may lead to new research prospects.

NCOA4

Nuclear receptor coactivator 4 (NCOA4) is a selective cargo receptor for autophagic turnover that binds to ferritin to mediate its delivery to autophagosomes and subsequently to the lysosome for ferritin degradation and concomitant iron release (78, 125, 126). When the cellular iron content is high, iron-bound NCOA4 interacts with the ubiquitin E3 ligase HERC2 to target NCOA4 for proteasomal degradation, which subsequently reduces ferritinophagy. However, when the cellular iron content is low, this interaction is inhibited, stabilizing NCOA4, which in

turn increases ferritinophagic flux and iron release in lysosomes (78). This mechanism enables NCOA4 to regulate cellular iron homeostasis, determine the ferritin energy flux, and affect the sensitivity of ferroptosis-inducing agents (127–129).

Previous studies reported that NCOA4 activity led to inhibited FTH1 activity levels and that decreased FTH1 levels caused cells to respond to several ferroptosis-inducing agents, such as erastin (79, 130). Cystine deprivation led to ferroptosis, which decreased FTH1 protein levels in control glioblastoma cells (carrying NCOA4 T98G). In NCOA4-deficient GBM cells (NCOA4-knockout [KO] cells), cystine deprivation exerted little effect on the FTH1 level, and therefore, cystine removal did not cause cell death (79). Furthermore, cystine deprivation caused increases in the amount of microtubule-associated protein light chain 3 (LC3)-II (which is related to autophagosome formation) in NCOA4 T98G-mutant cells (79, 131, 132). This finding suggests that cystine deprivation induces NCOA4-mediated ferritin iron release, which in turn leads to the ferroptosis of GBM cells (79).

YAP/TAZ

Yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are two dominant effectors of the Hippo pathway. The Hippo pathway is a potent tumor suppression pathway, and its core kinases include mammalian STE20-like protein kinase 1/2 (MST1/2) and large tumor suppressor ½ (LATS1/2), which inhibit proliferation by inhibiting YAP and TAZ (133, 134). After receiving an activation signal, MST1/2 associates with Salvador 1 (SAV1) to activate the Hippo pathway and to phosphorylate LATS1/2 and its coenzyme factor MOB1. The latter then phosphorylates the transcription cofactor YAP/TAZ, and phosphorylated YAP/TAZ is isolated in the cytoplasm and not translocated to the nucleus (80). Moreover, cell density and cellular communications can influence the regulation of ferroptosis induced by YAP/TAZ (81). For example, Yang et al. showed that TAZ, but not YAP, was abundantly expressed in several cancer cell lines and underwent density-dependent nuclear translocation (135, 136). TAZ depletion led to cell resistance to various ferroptosis inducers, while overexpression of the constitutively active form of TAZ, TAZS89A, sensitized cells to ferroptosis (137).

Additionally, YAP/TAZ regulates autophagy, and overexpression of MST1/2 or contact inhibition caused by high cell density inactivates YAP/TAZ activity, suppressing the transport of autophagosome components mediated by actin-myosin complexes and reducing LC3 levels (134). In contrast, knocking down LATS1/2 activities promotes YAP/TAZ activity and autophagy, which in turn induces ferroptosis (81). Compared to that of TAZ knockdown, the inhibitory effect of YAP knockdown on ferroptosis inducers (erastin, etc.) was more significant, and the knockdown of both YAP and TAZ induced the most significant inhibitory effect (138). The expression of both YAP and TAZ was elevated in multiple tumor types, including glioma cells, and was associated with the grade of malignancy, which was highest in GBM patients (139). YAP is also regarded as an independent prognostic factor for low-grade gliomas, and studies have shown that YAP/TAZ can control GBM cell plasticity (140), which may indicate a high value for YAP and TAZ in glioma and ferroptosis research.

Therapeutic drugs for glioma based on targeting ferroptosis

Compared with widely used ferroptosis drugs, particularly the few drugs used to treat glioma, many drugs are used to treat other malignancies, but these drugs induce drug resistance and fail to cross the blood–brain barrier, making them ineffective glioma treatments (150). TMZ is a widely used chemotherapeutic drug, but the resistance it causes is a very serious problem. Recently, research has been focused on weakening the resistance of malignant tumor cells to TMZ, and to this end, combinations of drugs and molecular hybridization are being tested (151). In addition, photodynamic therapies for ferroptosis may be used to overcome the blood–brain barrier in glioma treatment (152). Some newly tested drugs, such as dihydroartemisinin (DHA) and sulfasalazine (SAS), have shown obvious ferroptosis-inducing effects on glioma cells, and most of these drugs have been previously used to treat other malignancies. In this section, we provide an overview of the dominant therapeutic drugs used for glioma treatment that target ferroptosis. A list of these drugs is also provided in Table 2.

Dihydroartemisinin (DHA)

Artemisinin (ART) is the active component extracted from *Artemisia annua*, and DHA, its main active derivative, has been shown to exert desired cytotoxic effects on various human malignancies (153–156).

Studies showed that the DHA-activiated pathway consumed the reduced form of glutathione (GSH) and that the oxidized form (GSSG) accumulated in glioma cells, leading to increasing levels of lipid ROS and malondialdehyde (MDA, the end product of lipid peroxidation) in glioma cells (124). In addition, transmission electron microscopy showed that the size of mitochondria was decreased, the number of mitochondrial ridges was decreased, and the bilayer membrane density was increased in DHA-treated cells, which was consistent with the ultramorphological features of cells undergoing ferroptosis (63, 157, 158). These observations also prove that DHA induced

TABLE 2 Therapeutic Drugs towards Glioma Treatment by targeting Ferroptosis.

Drugs	Targets	Mechanism	Reference
DHA	GSH↓	consumes the reduced form GSH, oxidized GSSG accumulates, increases lipid ROS and MDA, inactivates GPX4 indirectly	Chen et al. (124)
TMZ	xCT↑	significantly reduced G1 phase and prolonged G2 phase	Sehm et al. (141)
	DMT1↑	broke iron homeostasis	Xue et al. (142)
		synergistically mediate the inhibition of cell activity with GPX4, Nrf2, and HO-1	Song et al. (143)
SAS	ROS↓	scavenge ROS	Aruoma et al. (144)
	ATF4↑	increase ATF4 expression, induce ER stress, decreased cell viability	Sehm et al. (145)
	xCT↓	inhibited the xCT antiporter activity hallmarked	Sehm et al. (145)
Pseudolaric acid B (PAB)	NOX4↑	activated Nox4 contributed to intracellular H2O2 and lipid peroxide and glioma cell death	Wang et al. (146)
	p53	induce GSH depletion, result in xCT inhibition	Wang et al. (146)
Ibuprofen	Nrf2↓	inhibit system xCT, inactivate GPX4 indirectly	Gao et al. (147)
Amentoflavone (AF)	FTH↓	block intracellular iron trafficking and storage to break iron homeostasis *via* modulating FTH	Chen et al. (148)
ALZ003(a curcumin analog)	AR(Androgen receptor)	induces FBXL2-mediated AR ubiquitination, leading to AR degradation then degrade GPX4	Chen et al. (149)

ferroptosis in glioma cells (159). To determine the targets of regulated by DHA in ferroptosis, the expression of GPX4, xCT and ACSL-4 was determined. GPX4 expression was downregulated and decreased with increasing DHA concentrations in DHA-treated groups compared to controls, while the levels of xCT and ACSL-4 were unchanged (159).

The effect of DHA on the induction of ferroptosis depended on multiple factors. Inhibition of the PERK/ATF4 signaling pathway enhanced the ferroptosis rate in DHA-induced glioma cells, and ATF4-induced HSPA5 expression was induced by increasing the GPX4 level in glioma cells undergoing DHA-induced ferroptosis (124). Thus, HSPA5 inhibitors synergistically enhanced the antitumor effects of DHA. Both the iron chelator deferoxamine (DFO) and lipid peroxidation were shown to inhibit ferrostatin-1 (Fer-1) activity, and liproxstatin-1 (Lip-1) inhibited the DHA-induced production of ROS, lipid ROS and MDA (159). Thus, both Fer-1 and Lip-1 reversed DHA-induced ferroptosis. Because DHA affects many high-impact targets and since these effects are regulated by multiple factors, studies into its selective killing effect on glioma cells are promising research directions.

Temozolomide (TMZ)

TMZ is widely used as the first-line treatment of malignant gliomas, but its antitumor effects have not been clearly identified. Ferroptosis is considered one of the pathways targeted by TMZ, and TMZ affects ferroptosis in glioma cells in several ways. The efficacy of TMZ in human glioma depends on xCT expression, and xCT expression in cells is increased after TMZ treatment (141). TMZ induced toxicity in both xCT-silenced and xCT-overexpressing glioma cells, and the toxicity increased with increasing TMZ concentration. Significantly fewer TMZ-treated cells were found to be in the G1 or prolonged G2 phase, and xCT-silenced cells were more sensitive to TMZ than xCT-overexpressing cells (141). Astrocytes and neurons were less susceptible than glioma cells to TMZ, suggesting special implications for TMZ treatment of glioma. Moreover, the effect of TMZ was enhanced when it was combined with erastin or sorafenib (141).

TMZ induces ferroptosis through the divalent metal transporter DMT1, which regulates iron levels and maintains iron homeostasis (8, 142). Both DMT1 mRNA and protein expression levels were significantly increased in glioma cells treated with TMZ (143). When DMT1 activity was inhibited, GPX4, Nrf2, and HO-1 activity was also inhibited, and the ability of TMZ to reduce cell viability was diminished (143). These results suggest that TMZ induces the ferroptosis of glioma cells and that this effect was associated with xCT and DMT1 expression.

Sulfasalazine (SAS)

SAS has been shown to scavenge ROS (144), induce cancer apoptosis (160), and attenuate glioma-induced epilepsy (161, 162). Recent studies showed that SAS significantly increased

ATF4 expression in glioma cells and induced ER stress, decreasing cell viability (145). Cell death was prevented by treatment with iron chelators and ferroptosis inhibitors, and high concentrations of SAS specifically inhibited the expression of an xCT antiporter activity marker (145), confirming that high concentrations of SAS inhibited xCT activity and induced ferroptosis in glioma cells. In experiments with a rat model, SAS significantly reduced glioma cell proliferation, exerted no significant toxic effects on normal neurons (163) and mild toxicity on astrocytes, and did not affect brain cell viability (145). However, due to low brain penetration, SAS showed poor efficacy in newly diagnosed and recurrent malignant glioma (150, 164). This problem is expected to be improved by convection-enhanced delivery (CED) (163).

In addition, SAS is likely to be used in several drug combinations. For example, molecular hybridization product of SAS and DHA, called AC254, showed significantly higher effects on glioma cells than either drug administered separately or in other drug combinations (165). AC254 led to changes in glioma cell shape and activity and terminated cell division, which were significantly better outcomes than those induced by the parent drugs and their mixture with other drugs (165). SAS enhanced the ability of TMZ to reduce human GBM cell activity (151), which may solve the problem of TMZ resistance.

Conclusions and perspectives

As a recently discovered form of cell death, ferroptosis shows many potential applications to glioma treatment. Recent studies have revealed three major pathways of ferroptosis, namely, iron metabolism, lipid peroxidation, and antioxidant system pathways (26). Ferroptosis is primarily regulated by the inhibition of xCT, accumulation of ROS, inhibition of GPX and GSH, which are mediators of many secondary regulatory pathways. In addition to these findings, increasing evidence links ferroptosis with autophagy, which has led to multiple research directions (166). The regulatory pathways of ferroptosis and the relationship of these pathways between ferroptosis and other forms of cell death remain to be further investigated.

Glioma cells show sensitivity to multiple types of specific ferroptosis inducers. Several critical factors inducing ferroptosis show different degrees of abnormal manifestation in glioma cells; for example, GPX4, Nrf2 and ATF4 show high expression compared with normal cells, and p53 shows complex regulatory effects. These findings provide therapeutic targets for glioma. However, few studies have focused on the specific activities of ferroptosis-related factors in glioma, and to identify more factors and their complex roles, more experiments need to be conducted.

Ferroptosis provides potential targets for further glioma treatment. Due to the complex regulatory mechanism of ferroptosis, many drugs show completely different effects *in vivo* than *in vitro* or show varying degrees of antagonistic effects in different pathways. In summary, the specific mechanism of ferroptosis remains unclear, and the indicators of ferroptosis are not obvious. Therefore, research on ferroptosis-related drugs needs to be conducted based on information obtained through additional detailed studies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

YZ, CF, and HX designed the review and wrote the manuscript. YL, XW, and LY conceived the artwork and performed the bibliographical research. AZ, AS, and DZ supervised the writing. All the authors revised and approved the final version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Temporal radiographic and histological study of necrosis development in a mouse glioblastoma model

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Tumor necrosis is a poor prognostic marker in glioblastoma (GBM) and a variety of other solid cancers. Accumulating evidence supports that necrosis could facilitate tumor progression and resistance to therapeutics. GBM necrosis is typically first detected by magnetic resonance imaging (MRI), after prominent necrosis has already formed. Therefore, radiological appearances of early necrosis formation and the temporal-spatial development of necrosis alongside tumor progression remain poorly understood. This knowledge gap leads to a lack of reliable radiographic diagnostic/prognostic markers in early GBM progression to detect necrosis. Recently, we reported an orthotopic xenograft GBM murine model driven by hyperactivation of the Hippo pathway transcriptional coactivator with PDZ-binding motif (TAZ) which recapitulates the extent of GBM necrosis seen among patients. In this study, we utilized this model to perform a temporal radiographic and histological study of necrosis development. We observed tumor tissue actively undergoing necrosis first appears more brightly enhancing in the early stages of progression in comparison to the rest of the tumor tissue. Later stages of tumor progression lead to loss of enhancement and unenhancing signals in the necrotic central portion of tumors on T1-weighted post-contrast MRI. This central unenhancing portion coincides with the radiographic and clinical definition of necrosis among GBM patients. Moreover, as necrosis evolves, two relatively more contrast-enhancing rims are observed in relationship to the solid enhancing tumor surrounding the central necrosis in the later stages. The outer more prominently enhancing rim at the tumor border probably represents the infiltrating tumor edge, and the inner enhancing rim at the peri-necrotic region may represent locally infiltrating immune cells. The associated inflammation at the peri-necrotic region was further confirmed by immunohistochemical study of the temporal development of tumor necrosis.

Neutrophils appear to be the predominant immune cell population in this region as necrosis evolves. This study shows central, brightly enhancing areas associated with inflammation in the tumor microenvironment may represent an early indication of necrosis development in GBM.

KEYWORDS

Glioblastoma, tumor necrosis, magnetic resonance imaging, MRI, mouse model, TAZ

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults. GBM is almost always first captured on brain CT and/or MRI, with MRI as the current gold-standard radiologic diagnostic modality that assists with pre-operative planning. Earlier studies reported that MRI findings closely correlated with histological grade of diffuse astrocytic tumors, including high grade gliomas (1, 2). Moreover, certain MRI features, such as contrast enhancement, necrosis, edema, mass effect, and intra-tumoral hemorrhage, have been shown to correlate with poor prognosis and clinical outcomes (3–5). Yet, unless identified incidentally on brain imaging obtained for other purposes, most GBMs remain undetected and undiagnosed until the tumors have progressed to the extent that they cause symptoms, edema, and brain compression as demonstrated by mass effect (6). By that time, rapid tumor expansion may have irreversibly damaged the surrounding normal brain parenchyma. Microscopic infiltration is usually so extensive at the time of diagnosis that tumors are incompletely resectable, so even maximal surgical resection is non-curative (6). Furthermore, genotoxic stress exerted by the hypoxic/ischemic tumor microenvironment promotes tumor evolution and molecular heterogeneity, rendering therapeutics ineffective. It has been well-established that early surgical resection results in improved overall survival among patients with both low-grade and malignant gliomas (7, 8). Under the assumption that early tumor detection can lead to early surgical resection, which in turn improves the overall survival, it is imperative to identify diagnostic markers to detect malignant tumor progression in the early and asymptomatic stages (4, 6). One histopathological feature associated with GBM progression is the formation of a necrotic core, caused by large-scale cell/tissue death. Tumor necrosis is a poor prognostic marker in GBM and a variety of other solid cancers (9–11). Accumulating evidence suggests that a necrotic core may facilitate tumor progression and evolution, thus promoting acquisition of resistance and negatively impacting patients' responses to therapeutics (12).

Clinically, GBM necrosis has been long used as a radiographic diagnostic criterion to differentiate glioblastoma from other, lower-grade gliomas (13), and it is typically first detected by MRI. However, since most patients do not undergo brain imaging until the later, symptomatic stages of tumor progression, prominent necrosis has already formed by the time tumors are detected on imaging and confirmed by histopathology (2, 13). Therefore, radiological appearances of early necrosis formation and the temporal-spatial development of necrosis alongside tumor progression remain poorly understood. This knowledge gap leads to a lack of reliable radiographic diagnostic/prognostic markers in early GBM progression to detect necrosis.

It is commonly thought that necrosis is due to chronic ischemia-linked oxygen and nutrient deprivation in cancers and that the resulting metabolic stress is the cause of cell death. At the cellular level, although necrosis was previously thought to be a catastrophic and disordered cell death process (14), studies in a variety of pathological situations have found that necrosis can occur in a regulated fashion and includes several cell death mechanisms (15, 16). Whether necrosis in cancers is regulated through similar mechanisms remains unclear. Recent studies in glioblastoma suggested that immune components, such as neutrophils, can lead to oxidative stress-induced tumor cell death, such as ferroptosis, thereby amplifying tumor necrosis (17). Increased expression of ferroptosis-promoting genes was detected in the GBM necrotic area (17). Ferroptosis-related genes were also linked to immunosuppressive microenvironment and poor prognosis of GBM (18–22). These studies suggested that ferroptosis is involved in the development of a necrotic core in GBM.

Recently, we reported a xenograft GBM murine model in which ectopic expression of an active Hippo pathway transcriptional coactivator with PDZ-binding motif (TAZ) mutant (TAZ^{4SA}) in the LN229 human GBM cell line can lead to orthotopic tumors which recapitulate the extent of GBM necrosis seen among patients (17). In this study, we utilized this model to perform a temporal radiographic and histological study of necrosis development. Our study indicated that more prominently enhancing areas associated with inflammation in the tumor microenvironment may represent an early indication of necrosis development in GBM.

Materials and methods

Real-time brain MRI imaging on GBM tumor-bearing mice

MRI was conducted on a 7T MRI scanner (Bruker BIOSPEC 70/20 USR) with a 4-channel mouse brain surface array coil. Under anesthesia with 1.5-4% isoflurane, each animal was positioned prone on a 37°C heating pad with body temperature and respiratory rate monitored. The animal's whole brain was imaged coronally in a spatial resolution of 133 μm × 133 μm × 500 μm using a T_1-weighted spin-echo sequence (repetition time (TR)/echo time (TE)/flip angle (FA) = 500 ms/9.5 ms/90°), a T_2-weighted rapid acquisition with relaxation enhancement sequence (TR/TE/FA = 2066 ms/36 ms/180°), and an 8-echo gradient-echo sequence for T_2* mapping (TR/TE/FA = 1733 ms/4.5 ms/50°, echo spacing 5.5 ms). T_1-weighted MRI was repeated about 15 minutes after a bolus injection of 0.2 mmol/kg gadolinium (Gadavist, Bayer Schering Pharma) through the lateral tail vein. The thickness for the mouse MRI images was 0.5 mm/slice. Heatmaps of MRI signal intensities at each timepoint indicated in Figures 1B, 2B, 3B were generated *via* ImageJ using the "Interactive 3D Surface Plot" function after manually outlining the brain area.

Radiographic analysis of GBM necrosis *via* MRI

Subjects were retrospectively selected from a cohort of patients seen in Penn State Hershey Neuro-Oncology clinic between December 2018 and March 2019, and only patients with histopathologically confirmed WHO grade 4 malignant gliomas (i.e., GBMs) were included in this study (n=75). Pre-surgical, post-contrast axial T1-weighted fat saturated (T1 FS) MRI images with a slice thickness of 5 mm from patients with histologically-confirmed GBMs were retrospectively analyzed. MRI images were acquired *via* standard multi-contrast sequences including postcontrast fat saturated T1 TSE sequence using either 1.5T or 3.0T magnet (Siemens Healthcare) after injection of 0.1mmol/kg of gadolinium (Gadavist, Bayer Schering Pharma). Central necrosis was defined as non-enhancing areas within enhancing tumor with irregular inner margins on post-contrast T1-weighted images. Only pre-existing data were obtained *via* review of electronic medical records (EMR) and imaging studies (MRI), and therefore no further data collection or subject recruitment were conducted for this study. The study procedures and data collection were approved by the Institutional Review Board (IRB) of Penn State Hershey Medical Center. Per the Penn State IRB, human subject research presented in Figure 4 was exempt from informed consent requirements. Heatmaps of signal intensities as in Figure 4B were generated *via* ImageJ using the "Interactive 3D Surface Plot" function after manually outlining the brain area.

Mice and orthotopic xenograft tumor models

Six-to-eight-week-old female athymic nude mice (Nu(NCr)-Foxn1nu Strain Code: 490, Charles River) were used for the GBM orthotopic xenograft mouse models. For tumorigenesis experiments, human GBM cells were first transduced with a retroviral vector expressing firefly luciferase. These cells were then transduced with retroviral vectors expressing the indicated cDNAs. For each mouse, 3×10^5 cells were injected into the right hemisphere at coordinates (+1, +2, -3). For tumor sample preparation and histology, whole brain tissue from tumor-bearing animals was fixed with 4% neutral-buffered formalin, embedded in paraffin, and submitted to the Penn State College of Medicine comparative medicine histology core, cut into sections 5 μm thick, and stained with hematoxylin and eosin (H&E). Areas of tumor and central necrosis were manually traced. All experiments described in this study were carried out with the approval of the Penn State University Institutional Animal Care and Use Committee and in accordance with its guidelines.

Cells

Human GBM cell line, LN229 (CRL-2611), purchased from ATCC, was cultured in Dulbecco's modified Eagle's medium (DMEM; 10-013-CV, Corning) supplemented with 10% fetal bovine serum (FBS; Gibco, 10437028) and 1% Antibiotic–Antimycotic Solution (30-004-CI, Corning) at 37 °C with 5% CO_2. The cell line was not authenticated in this study. The cell line was confirmed as *Mycoplasma* negative before experiments. Unless otherwise indicated, cells were grown to 50% confluence.

Time course radiographic and histologic quantification of areas of interest (e.g., necrotic, peri-necrotic, cellular tumor, and total tumor)

For quantification as in Figures 1, 2, T1 post-gadolinium images were acquired as above. Quantification was performed using images containing tumors with the largest cross-sections. Each specific area of interest (e.g., yellow arrow and green arrowhead as in Figure 1) was manually traced using the freehand tool and measured using the "Analyze" function in ImageJ. Data for tumor areas were normalized to corresponding whole brain area, whereas other areas of interest (i.e., necrotic,

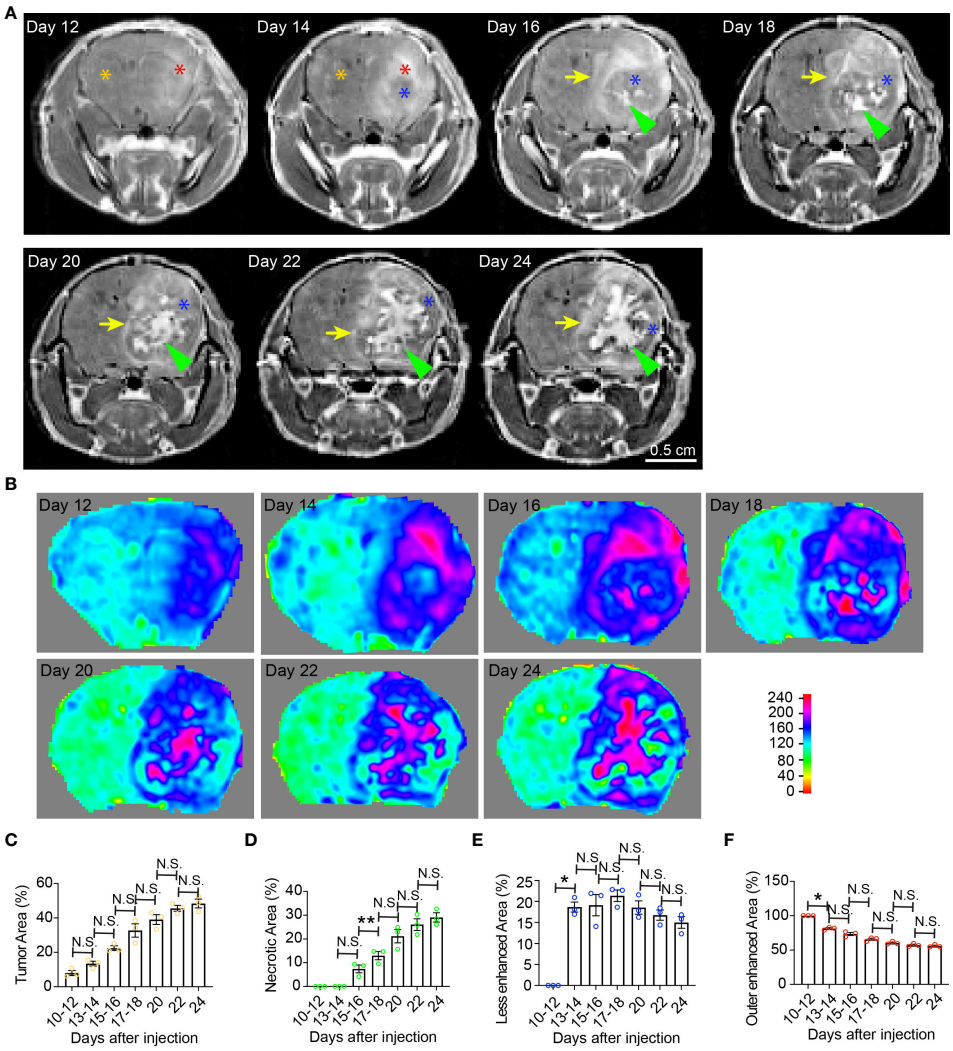

FIGURE 1
Temporal radiographic characterization of tumor necrosis development during early tumor progression *via* MR imaging. **(A)** Temporal development of GBM tumor necrosis during early, asymptomatic stages of tumor progression *via* representative serial T1-weighted post-gadolinium MRI scans of one LN229TAZ(4SA) tumor-bearing mouse. Orange asterisk: Normal brain parenchyma in the non-tumor containing hemisphere; red asterisk: Enhancing areas in the tumor-containing hemisphere; blue asterisk: The center of the tumor becomes less enhancing from day 14 onward, likely representing densely packed tumor cells; green arrowheads: Prominently enhancing foci within the less enhancing tumor stroma, likely representing active development of necrosis, which starts appearing from day 16. Yellow arrows: Peripheral enhancing interface between the less enhancing tumor tissue, as marked by blue asterisks, and the normal brain parenchyma. **(B)** Heatmaps of signal intensities generated using T1 post-contrast MRI images as in panel **(A)**. **(C)** Quantification of tumor area (outlined by the peripheral enhancing interface indicated by yellow arrows) normalized to whole brain area at each timepoint indicated above in panel **(A)**. p_{ANOVA}=0.003. Results of post hoc test for each continuous time point are indicated. **(D)** Quantification of necrotic areas—labeled by green arrowheads in panel **(A)**—normalized to corresponding tumor at each timepoint indicated above. p_{ANOVA}=0.0009. Results of post hoc test for each continuous time point were indicated. **(E)** Quantification of less-enhancing areas—labeled by blue asterisks in panel **(A)**—normalized to corresponding tumor at each timepoint indicated above. p_{ANOVA}=0.012. Results of post hoc test for each continuous time point are indicated. **(F)** Quantification of the outer enhanced areas—labeled by red asterisks in panel **(A)**—normalized to corresponding tumor at each timepoint indicated above. p_{ANOVA}=0.0011. Results of post hoc test for each continuous time point are indicated. Three mice were imaged as replicates with consistent observations; each datapoint shown in the bar graphs represents an animal (n=3). RM one-way ANOVA. Sidak's multiple comparisons test was used in the post hoc test. All center values shown are mean values, and all error bars represent standard errors of the means (s.e.m). N.S.,$p > 0.05$. *,$p < 0.05$; **,$p < 0.01$.

enhanced, and less enhanced areas) were normalized to corresponding tumor size. For quantification shown in Figure 5, paraffin-embedded, H&E-stained sections collected at each indicated timepoint prepared as above were used for quantification. Necrosis (N) is defined as acellular regions (appearing pale pink) within tumors as identified by H&E stain, and a cellular tumor (CT) region is defined as a hypercellular region. Quantification was performed using

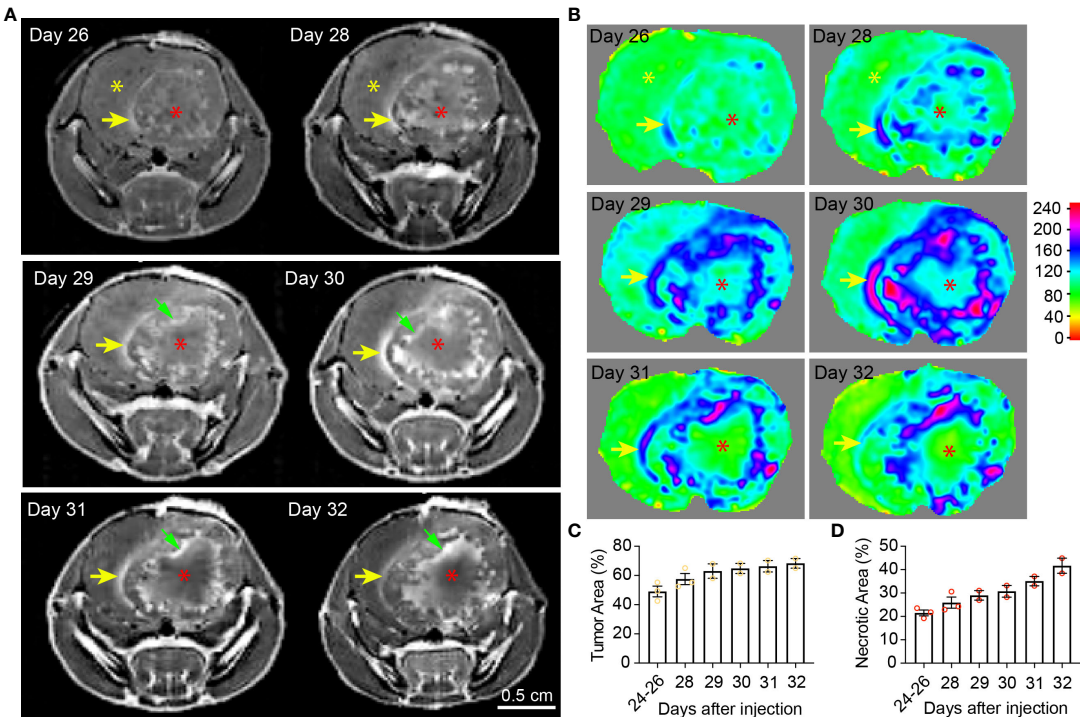

FIGURE 2
Temporal radiographic characterization of tumor necrosis development during late tumor progression *via* MR imaging. **(A)** Temporal development of GBM tumor necrosis during late tumor progression after onset of overt tumor-associated symptoms in tumor-bearing mice *via* representative serial T1-weighted post-gadolinium MRI scans of one LN229TAZ(4SA) tumor-bearing mouse (different from the animal used in Figure 1). Yellow asterisks: Brain parenchyma in the normal hemisphere; yellow arrows: More prominently enhancing rim at the outer edge of the tumor boundary, representing the tumor-infiltrating front; red asterisks: More prominently enhancing (but later unenhancing from day 29 onward) foci within the tumor stroma, representing active development of necrosis; green arrows: Prominently enhancing rim at the necrosis-cellular tumor (N-CT) interface, representing areas of active inflammation. **(B)** Heatmaps of signal intensities generated using T1 post-contrast MRI images as in panel **(A)**. **(C)** Quantification of tumor area (outlined by the peripheral enhancing interface indicated by yellow arrows) normalized to whole brain area at each timepoint indicated above. p=0.3684. **(D)** Quantification of necrotic areas—labeled by red asterisks as in panel **(A)**—normalized to corresponding tumor area at each indicated timepoint as above. p=0.1164. Three mice were imaged as replicates until reached the terminal stage (one at day 28, the other two at day 32; n=2-3); each datapoint shown in the bar graphs represents an animal. All center values shown are mean values, and all error bars represent standard errors of the means (s.e.m). Mixed-effects analysis.

sections with the largest cross-sections. Regions of interest were manually traced using the freehand tool and measured using the "Analyze" function in ImageJ. All statistical calculations and plotting were performed using GraphPad Prism 9.

Immunofluorescent staining and analyses

Immunofluorescent staining was performed as previously described (23). For histological samples, paraffin-embedded 5-µm sections were deparaffinized and rehydrated in successive baths of xylene and ethanol (100%, 95%, 70%, and 50%), followed by heat-induced (95 °C) epitope retrieval in 10 mM sodium citrate buffer (pH = 6.0). After one-hour block with 5% BSA/PBS at room temperature, samples were incubated overnight at 4°C with primary antibodies diluted in 2.5% BSA/0.05% Triton X-100/PBS. The next day, sections were washed three times with 0.1% Triton X100/PBS prior to incubation with secondary antibody diluted in 2.5% BSA/0.05% Triton X-100/PBS for 60-90 minutes at room temperature. Then, sections were again washed three times with 0.1% Triton X-100/PBS, labeled with 4,6-diamidino-2-phenylindole (DAPI) for nuclear visualization, rinsed with PBS, and mounted in ProLong Gold Antifade Mountant (P10144, Invitrogen). Primary antibodies, ultra-LEAF purified rat anti-mouse Ly-6G (1A8, 127620, Biolegend) and rabbit anti-mouse/human CD11b (ab133357, Abcam), were diluted at a 1-to-100 concentration. Secondary antibodies, Alexa Fluor 488 donkey anti-rat IgG (712-545-150, Jackson ImmunoResearch) and Alexa Fluor 594 donkey anti-rabbit IgG (711-585-152, Jackson ImmunoResearch), were diluted at 1-to-200 concentration. For analysis of percentage of CD11b+ and Ly6G+ doubly-positive cells in tumors collected at various time points as in Figure 6, images were acquired within 1–3 days following immunofluorescent staining as above using

an Olympus CX41 microscope PLCN 40x objective. All images were taken within the tumor adjacent to the central necrosis, in so-called peri-necrotic regions, where infiltrating immune cells were most abundant. All images were first converted to 8-bit grayscale images, followed by background subtraction, thresholding, and quantification using the Analyze Particles function in ImageJ. Quantifications of double-positive and triple-positive cells were performed by using the same approach as above on images generated by Image Calculator using the "AND" function in ImageJ. Corresponding DAPI images were obtained for visualization of cellular nuclei and for normalization of percentage of positive signals per cell. Data were plotted as percentage of CD11b+ (singly-positive) normalized to all DAPI+ cells within one high-power 40X field, or percentage of CD11b+–Ly6G+ (doubly-positive) cells normalized to all CD11b+ cells within one high-power 40X field. For analysis of CD11b+ and Ly6G+ cells in tumors or tumor borders as in Figure 6D, images were acquired within 1–3 days following immunofluorescent staining as above using a Leica SP8 inverted confocal laser scanning microscope with 63x objective. Images were then stacked with maximal intensity using the "Z project" function and merged using ImageJ.

Results

Temporal radiographic characterization of tumor necrosis development *via* MR imaging

Clinically, GBM tumor necrosis is typically first identified radiographically *via* T1-weighted MRI with contrast prior to surgery, as histopathological examination cannot be performed until the tumor specimen has been removed. To study the development of tumor necrosis in GBM, we carried out a longitudinal radiographic imaging study on LN229$^{TAZ(4SA)}$ tumor-bearing mice, in which tumor necrosis progressively forms during tumor development (17). LN229$^{TAZ(4SA)}$ tumor-bearing mice were imaged every other day in the early tumor progression stages (starting 10-12 days after tumor implantation) before the onset of any overt tumor-associated symptoms (around 24 days after tumor implantation). During early tumor progression, neoplastic tissue can be readily distinguished from the normal brain tissue by its enhanced contrast signals (Figures 1A, B, day- 12 and 14 scans, red asterisks). The contrast enhancement is likely the result of leaky vasculature and the lack of a blood-brain barrier. In addition to neoplastic tissues with leaky vessels, tissues undergoing active inflammation can demonstrate enhancement due to infiltration of immune cells with resultant edema. We noticed the centers of tumors started showing less enhancement on day 14 (Figure 1A, blue asterisk; and Figure 1B). This likely represents proliferating tumor cells becoming more compact in the tumor center compared to the periphery. H&E staining of tissue sections at this stage confirmed this notion (Figure 5, day-10 scan). The decreased enhancement of solid tumor components between the contrast-enhancing rims may be related to the dense packing of tumor cells in that region, leaving little extracellular space and therefore resulting in reduced contrast enhancement. Changes in MRI appearance for necrosis were first detected on day-16 scan, in which the center of the tumor contained heterogeneous and brighter-enhancing signals on post-contrast T1 spin echo sequence (Figure 1A, day-16 scan, green arrowhead; and Figure 1B, day-16 scan), likely representing actively developing central necrosis, and a contrast-enhancing rim surrounding the tumor, likely representing tumor-infiltrating fronts (Figure 1A, day-16 scan, yellow arrow; and Figure 1B, day-16 scan). The relatively less-enhancing core (Figure 1A, day 16-24 scans, blue asterisks) of the tumor surrounding the brightly enhancing central necrosis (Figure 1A, day 16-24 scans, green arrowheads) was in turn surrounded by a relatively more enhancing peripheral rim (Figure 1A, day 16-24 scans, yellow arrows). Quantification of these radiological features showed that tumor and necrotic areas, which are the areas enclosed by the outer contrast-enhancing rim (Figure 1A, day 16-24 scans, yellow arrows) and the intratumoral contrast-enhancing foci (Figure 1A, day 16-24 scans, green arrowheads), respectively, both gradually enlarged with tumor growth (Figures 1C, D). In contrast, the relatively less-enhancing areas (Figure 1A, day 14-24 scans, blue asterisks) did not expand along with tumors after they appeared around day 14 (Figure 1E), while the peripherally-enhancing rims (Figure 1A, day- 12 and 14 scans, red asterisks) became thinner (Figure 1F).

In the second imaging study, MRI scans were acquired more frequently after the LN229$^{TAZ(4SA)}$ tumor-bearing mice developed overt tumor-associated symptoms (i.e., starting 24 days after tumor implantation). At this time, the relatively more prominent contrast-enhancing rim on the outer edge of the tumor boundaries remained visible throughout the course of imaging (Figures 2A, B, yellow arrows). Using this contrast-enhancing rim as the border of the expanding tumor front, we found that tumors continue expanding (Figure 1C), although the expansion is slower when compared to earlier stages, between days 11-24 (comparing Figure 2C to 1C). Notably, the regions representing central tumor necrosis, which initially demonstrated more prominent enhancement than other solid tumor areas (Figures 2A, B, day- 26, 28 and 29 scans, red asterisks vs. yellow asterisks), became gradually less contrast-enhancing from day 31 onward (Figures 2A, B, red asterisk on day-31 scan vs. red asterisks on day- 28 and 29 scans). The loss of contrast signal in the central areas reflects the occurrence of extensive tumor tissue death (Figure 5A, day-30 scan). We also observed another more brightly contrast-enhancing rim at the interface of solid tumor and central tumor necrosis (i.e., N-CT interfaces) (Figure 2A, green arrows) from day 29 onward after

the implantation. This second rim of enhancement likely represented the active area undergoing inflammation flooded with infiltrating immune cells (e.g., tumor-associated neutrophils, TANs, and tumor-associated macrophages and microglia, TAMs) and interstitial fluid. Using this second contrast-enhancing rim as the outline of the necrotic area, we found that necrotic cores continue expanding at this stage (Figure 1D), although more slowly when compared to stages between days 11-24 (comparing Figure 2D to 1D). Together, these temporal studies suggested hyperactivated TAZ-driven GBM tumor necrosis positively correlated with tumor and symptomatic progression, consistent with what has been reported in clinical human GBM studies. The solid portions of tumors between these two prominently enhancing rims were relatively less-enhancing (Figures 2A, B, day 26-32 scans) as observed in Figure 1 (day 14-24 scans).

Comparison of different MRI sequences for the temporal visualization of tumors and tumor necrosis

Tumor necrosis in GBM patients is typically first identified radiographically *via* T1-weighted MRI performed with a gadolinium contrast-enhancing agent. T1-weighted pre- and post-gadolinium images are especially useful in investigating breakdown of the blood-brain barrier (e.g., tumors, abscesses, brain inflammation, or viral encephalitis.) (24). In the imaging of GBM, T2-weighted sequences are almost always obtained concurrently given their capacity to detect tumor infiltration with edema. (T2*) or susceptibility weighted images are commonly utilized when attempting to detect structural changes related to intracranial hemorrhage (e.g., arteriovenous malformation, cavernoma, hemorrhage within a tumor, punctate hemorrhages in diffuse axonal injury, thrombosed aneurysm, or some forms of calcification.) (24, 25). To compare and contrast these different MRI modalities for the visualization of tumors and tumor necrosis in our GBM mouse model, we conducted a temporal radiographic imaging study on $LN229^{TAZ(4SA)}$ tumor-bearing mice using different MRI modalities. When T1-weighted post-gadolinium imaging was utilized, temporal tumor growth and formation of the prominent necrotic core was visualized (Figures 3A, B, T1-post, arrows). Contrarily, in non-contrast T1 imaging, tumors were barely detectable (Figures 3A, B, T1-pre). While early-stage tumors were readily visible with enhanced signals in the T2 setting (day 13-17), they gradually lost their enhanced T2 signals and became almost isointense to the surrounding parenchyma in later stages (Figures 3A, B, T2, arrows). On T2* gradient echo images—specifically used for the detection of blood products or microhemorrhages in tumors (24, 25)– the tumors in the mouse model did not show enhanced signals (Figures 3A, B, T2*). To ensure the clinical translatability of observations from our GBM mouse model, we also obtained images acquired with different MRI sequences from GBM patients in the clinical setting. Similar to the above observation in our mouse model, tumor necrosis was best visualized in T1-weighted post-contrast images as less-enhancing areas with irregular inner margins near the enhancing edges (Figures 4A, B, T1-post, arrows). These results indicated that T1-weighted post-contrast MRI represents the best imaging modality for visualization and characterization of tumor necrosis both in pre-clinical and clinical GBM models, especially in advanced stages.

Histological characterization of the temporal development of GBM tumor necrosis

While MRI studies provided real-time information about necrosis development with high clinical translatability, the resolution of biological/physiological occurrence provided by MRI scans is somewhat limited to the large-scale tissue level. To allow finer examination of necrosis development at the cellular level in the hyperactivated TAZ-driven mouse GBM model, we performed temporal histological studies by collecting brain tissue from the $LN229^{TAZ(4SA)}$ tumor-bearing mice at different stages throughout tumor progression, from asymptomatic stages (i.e., on days 10, 16, and 20 after tumor implantation), to the symptomatic stage (i.e., from day 24 following implantation and onward), and eventually at the endpoint (i.e., day 30 after implantation). We saw that on day 10 after tumor implantation, tumor tissue was readily visible and could be distinguished from the normal brain parenchyma by its purple stain on H&E sections. At this time, tumors appeared relatively small and homogeneous, although tumor cells are more compact in the tumor center compared to the periphery (Figure 5A, day-10 sections, red-outlined vs. green-outlined areas). Consistent with above MRI studies, tumor sections collected from day 16 and onward after tumor implantation appeared more heterogeneous, with a small central eosinophilic, pale-pink appearing, acellular region of necrosis (Figure 5A, day-16 sections). At this stage, the tumor can be subdivided into three areas, including periphery (less dense, Figure 5A, day-16, green-outlined area), cellular tumor zone (dense, Figure 5A, day-16 sections, blue-outlined area), and necrotic area (Figure 5, day-16 sections, red-outlined area). These three areas correspond to the relatively more enhancing peripheral rim (Figure 1A, day 16-24 sections, yellow arrows), the relatively less enhancing core (Figure 1A, day 16-24 sections, blue asterisks) of the tumor, and the brightly enhancing central necrosis (Figure 1A, day 16-24 sections, green arrowheads), respectively, that were observed in the MRI scans. The histologically-distinct areas, including cellular tumor zone and necrotic area, can be similarly observed in the H&E sections obtained from a GBM patient (Figure 5B). Furthermore, in concordance with results from the above MRI

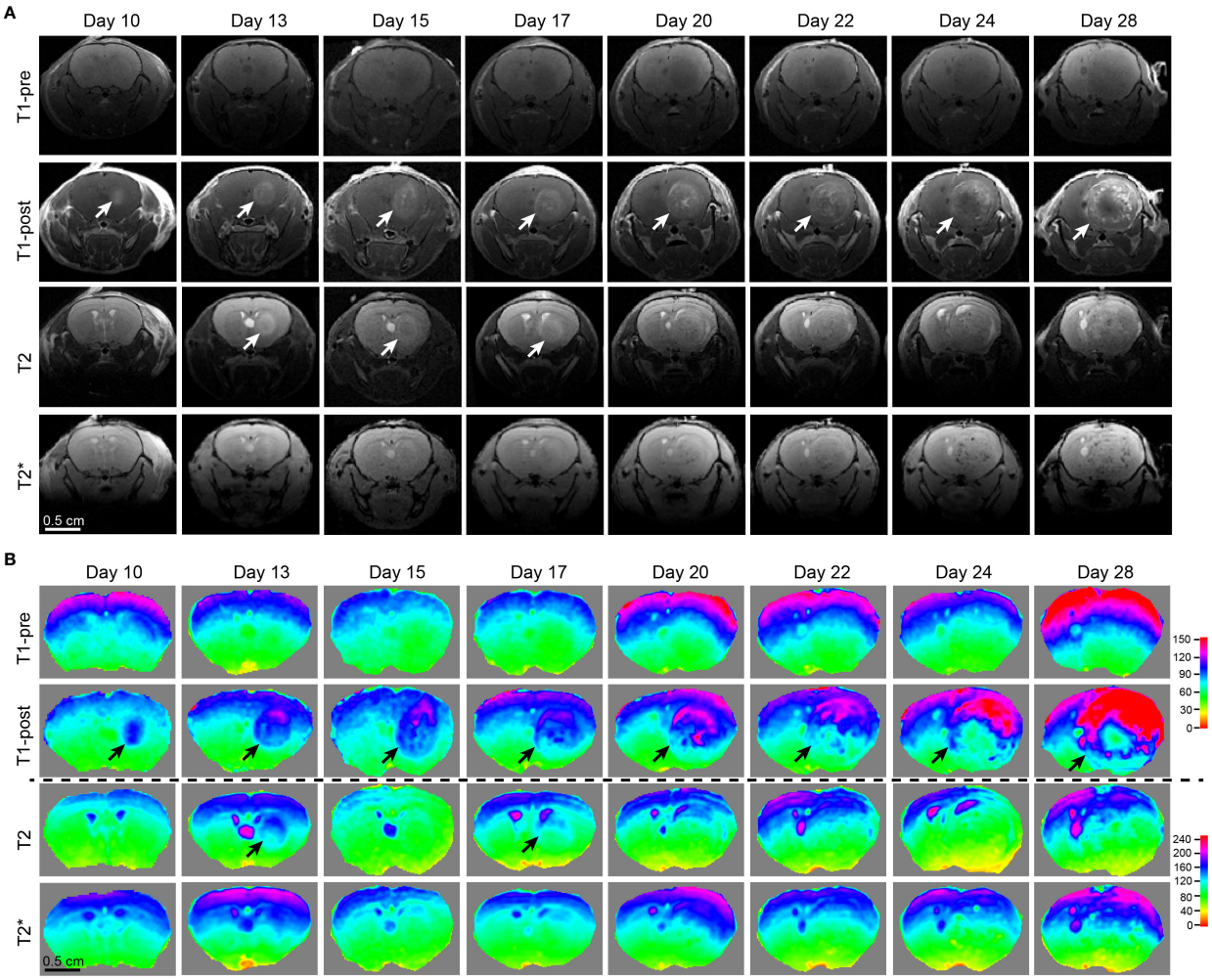

FIGURE 3
Comparison of different MRI modalities for temporal visualization of tumors and tumor necrosis using a murine GBM model. **(A)** Images acquired from various frequently utilized MR imaging modality for diagnostics and pre-operative planning in the clinical setting on the GBM tumor-bearing mouse devised by our lab. Starting from 10 days post tumor cell implantation, the mouse underwent serial brain MRI. Time points were pre-determined based on our previous histological studies on days 10, 13, 15, 17, 20, 22, 24, and 28, which is the end point of this GBM tumor-bearing mouse. **(B)** Heatmaps of signal intensities generated using MRI images as in panel **(A)**. Arrows: Enhancing areas likely representing tumors. One mouse was imaged.

studies, the size of tumor necrosis positively correlated with the size of LN229$^{TAZ(4SA)}$ tumors and symptomatic progression in mice (Figures 5C, D). Interestingly, we consistently observed small round cells with dense, dark purple nuclei (yellow arrowheads), characteristic of mouse immune cells and morphologically distinct from human tumor cells with diffuse, light-purple nuclei (red arrowheads) in the peri-necrotic regions starting on day 16 after tumor implantation (Figure 5A), indicating that prominent infiltration of mouse immune cells began around this time. Morphologically-similar cells were also seen in the peri-necrotic zone (PNZ) and necrotic area of the samples from GBM patients (Figure 5B, arrowheads).

Immunohistochemical characterization of the temporal development of GBM tumor necrosis

To validate our MRI results and to further examine the correlation between immune cells and tumor necrosis at various stages of tumor development, we performed immunohistochemistry using a commonly used myeloid cell marker, CD11b. Because neutrophils (i.e., tumor-associated neutrophils, TANs) have been reported to be enriched in the necrotic region (17), we also used Ly6G, a murine neutrophil marker, to monitor TANs. Brain tumors were visible on

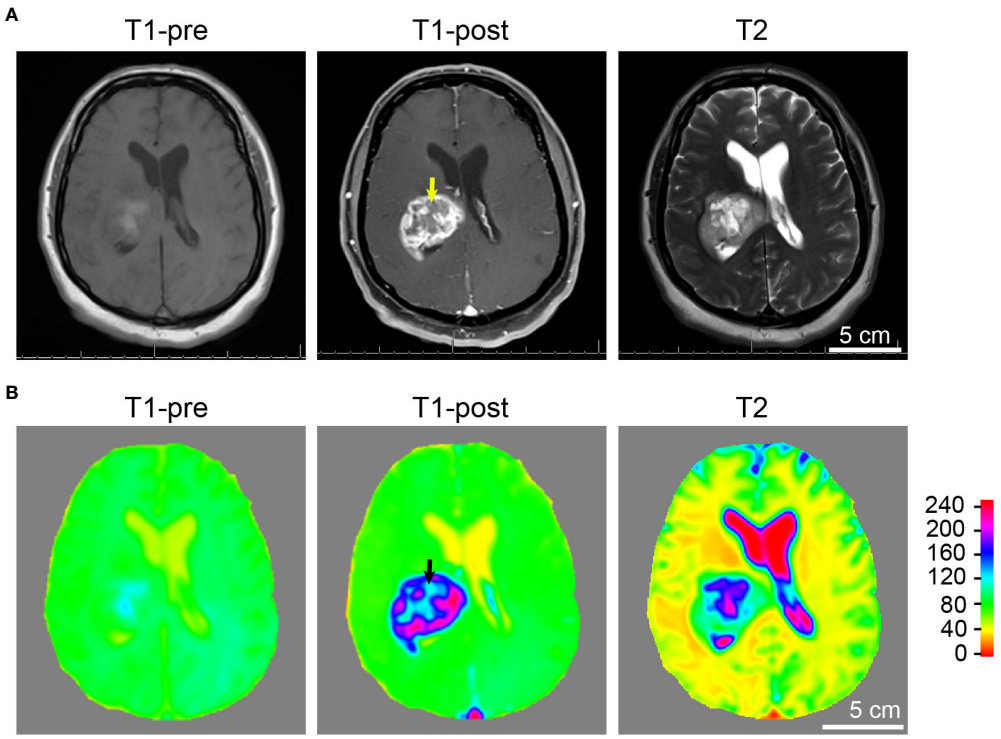

FIGURE 4
Tumor and necrosis visualized in a glioblastoma patient using standard MRI sequences. **(A)** A panel of representative images comparing T1 weighted pre-contrast (T1-pre), T1 weighted post-contrast (T1-post), and T2 sequences, which are routinely utilized in diagnostics and pre-operative planning for glioblastoma patient, are comparable to the images acquired using the murine GBM model which we devised. It is evident that T1-weighted post-gadolinium image remains by far the best sequence for the visualization of GBM tumor necrosis. **(B)** Heatmaps of signal intensities generated using MRI images as in panel **(A)**. Arrows: Less enhancing foci representing the central necrotic areas with irregular inner margins near the enhancing edges.

hematoxylin and eosin (H&E) staining at day 10 after tumor cell implantation, but there was no detectable tumor necrosis (Figure 5A, day-10 sections). In these tumors, $CD11b^+$ cells could be detected, whereas few $Ly6G^+$ cells were seen (Figure 6A, day-10 sections; Figures 6B, C). At day 16 after tumor cell implantation, the derived tumors contained a few necrotic foci that were infiltrated with cells labeled by both CD11b and Ly6G (Figure 6A, day-16 sections; Figures 6B, C). As tumors further progressed and the necrotic areas further expanded, $CD11b^+$ cells were more frequently observed in the PNZ than in the tumor border, where few $CD11b^+$ cells were found in the brain parenchyma (Figure 6D). As the $CD11b^+$ cell population increased in the PNZ, the abundance of $CD11b^+Ly6G^+$ double-positive cells also increased (Figures 6B, C). These results indicated that $CD11b^+Ly6G^+$ cells spatially and temporally coincided with tumor necrosis and tumor progression in $LN229^{TAZ(4SA)}$ tumors, consistent with what has been previously described (17). Overall, these results confirmed the above MRI observations that immune cells and tumor necrosis are temporally and spatially correlated.

Discussion

While necrosis is common in solid malignancies at advanced stages, especially GBMs, the nature and mechanisms driving its development and evolution remain obscure, particularly in the early stages of tumor progression. Clinically, brain MRI plays a pivotal role and remains the primary follow-up modality in assessing therapeutic response and prognosis in GBM patients once the histopathological diagnosis has been confirmed. GBM patients typically undergo brain MRI once every 2-3 months following the initial tumor resection. While tumor necrosis is typically first detected *via* brain MRI among GBM patients, the radiological appearance and temporal evolution of early necrosis formation alongside tumor progression remains poorly understood, resulting in the lack of reliable radiographic diagnostic and prognostic markers in early GBM progression. This is unsurprising given that unless the lesion is identified incidentally, most GBM patients do not undergo brain imaging until they progress to the later, symptomatic stages when symptoms result from extensive tumor expansion and perilesional edema which compress normal brain parenchyma,

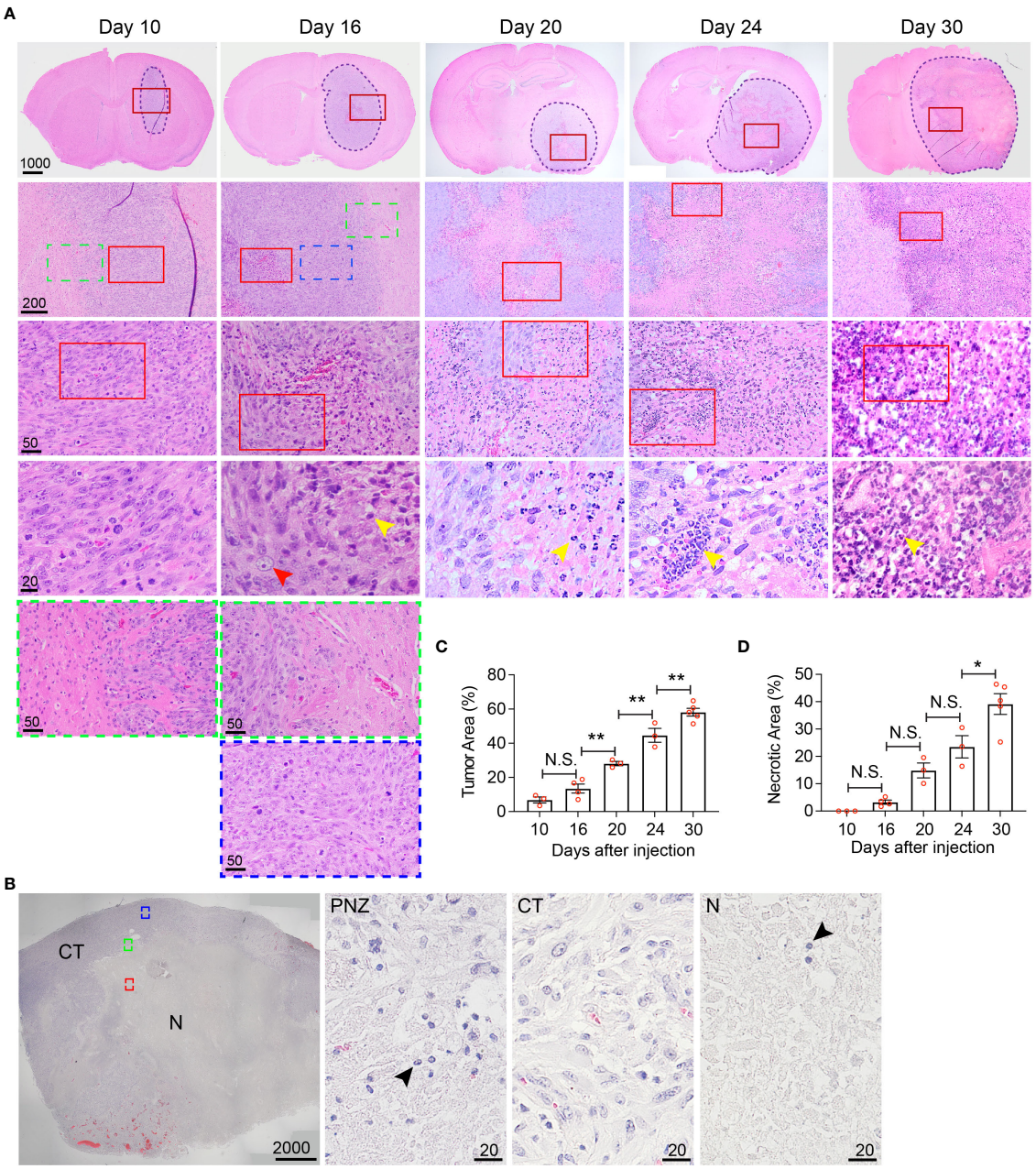

FIGURE 5
Histological characterization of the temporal development of GBM tumor necrosis. **(A)** Temporal development of GBM tumor necrosis from early to late tumor progression using representative time-course H&E-stained formaldehyde-fixed paraffin embedded sections of LN229TAZ(4SA) brain tumor-bearing mice. Each column represents images acquired from the same animal at a certain tumor progression stage. In each section, the tumor stroma has been traced out using a dashed line. Areas marked by red rectangles were further magnified in the images in the bottom of each column. For day 10 and 16 tumors, the areas marked by green (tumor border) or blue (cellular tumor) rectangles were also magnified and shown in the bottom of the correspondent columns. Yellow arrowheads: small, round, dark purple nuclei, likely representing mouse immune cells; red arrowheads: large, light purple nuclei, representing LN229 human tumor cells. **(B)** Representative image of a H&E-stained formaldehyde-fixed paraffin-embedded human GBM brain section showing cellular and necrotic tumor areas. The areas marked by green (peri-necrotic zone, PNZ), blue (cellular tumor, CT), or red (necrotic area, N) rectangles were magnified and shown on right. Arrowheads: small, round, dark purple nuclei, likely representing immune cells. Specimens from three different patients (n=3) were examined independently with similar observations. **(C)** Quantification of tumor area normalized to whole brain area at each timepoint indicated in panel **(A)**. $p_{ANOVA} < 0.0001$. Results of post hoc test for each continuous time point are indicated. **(D)** Quantification of necrotic areas, appearing as pale pink, acellular regions, normalized to corresponding tumor area at each timepoint indicated above $p_{ANOVA} < 0.0001$. Results of post hoc test for each continuous time point are indicated. Tumors from three to five tumor-bearing mice were sectioned and imaged in parallel as replicates with consistent observations (n=3-5); each datapoint shown in the bar graphs represents an animal. Scale bar is in μm. Ordinary one-way ANOVA. Sidak's multiple comparisons test was used in the post hoc test. All center values shown are mean values, and all error bars represent standard errors of the mean (s.e.m). N.S., $p > 0.05$. *,$p < 0.05$; **,$p < 0.01$.

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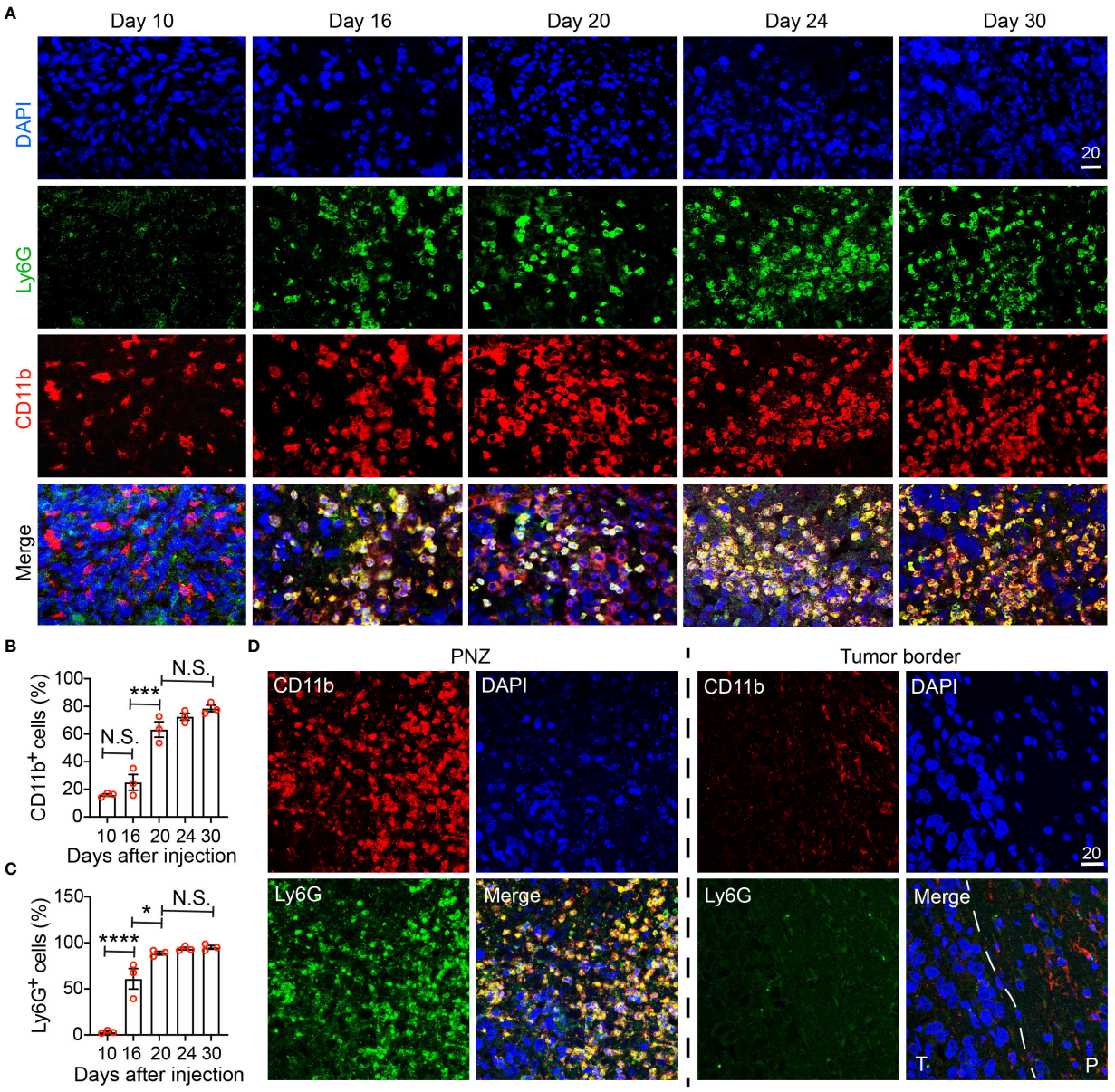

FIGURE 6
Immunohistochemical characterization of the temporal development of GBM tumor necrosis. **(A)** Temporal development of GBM tumor necrosis from the early to the late tumor progression using representative time-course CD11b, Ly6G, and DAPI immunofluorescence staining on brain sections collected from LN229$^{TAZ(4SA)}$ brain tumor-bearing mice. (same cohort of animals as in Figure 5). For day 10, an area of cellular tumor was imaged. For day 16-30, an area of peri-necrotic zone at the interface between cellular and necrotic tumor was imaged for each sample. Images in each column were acquired from the same animal. **(B)** Percentage of CD11b$^+$ cells normalized to all DAPI$^+$ cells in one high-power 40X field collected at each timepoint indicated in panel **(A)**. p_{ANOVA}<0.0001. Results of post hoc test for each continuous time point are indicated. **(C)** Percentage of Ly6G$^+$ cells normalized to all CD11b$^+$ cells in one high-power 40X field collected at each timepoint indicated in panel **(A)**. p_{ANOVA}<0.0001. Results of post hoc test for each continuous time point are indicated. Each datapoint shown in the bar graphs represents an animal. Ordinary one-way ANOVA. Sidak's multiple comparisons test was used in the post hoc test. All center values shown are mean values, and all error bars represent standard errors of the means (s.e.m). N.S., $p > 0.05$. *,$p < 0.05$; ***,$p < 0.001$; ****,$p < 0.0001$. **(D)** Comparison of immunofluorescent images as above acquired from a terminal-stage (i.e., day 30) section between the peri-necrotic zone, denoted by PNZ, and from the tumor border; dashed line outlines the tumor (T) and normal parenchyma (P) border. Tumors from three tumor-bearing mice were sectioned and imaged in parallel as replicates with consistent observations (n=3). Scale bar is in μm.

distort vascular supply, and reconfigure the neurotransmitter environment. By such a time, prominent central tumor necrosis is already well established and easily detected on imaging.

In this study, we utilized an orthotopic xenograft GBM murine model which recapitulates the extent of GBM necrosis seen among patients (17) to fill the above gap in understanding *via* temporal radiographic and histological characterization of necrosis evolution, hoping to identify possible radiographic diagnostic/prognostic markers in early GBM progression. Overall, images acquired *via* T1- and T2- weighted (including T2*) MRIs on our murine model were comparable to those from GBM patients. Nevertheless, unlike intra-lesional blood products including microhemorrhages commonly reported in MRI images of GBM, we did not observe much susceptibility signals in our murine GBM tumor-bearing animals on T2* MRI, suggesting lack of intra-lesional blood products or microhemorrhages in our GBM murine model. This was unsurprising, given that we did not observe extensive intra-tumoral microvascular proliferation or subsequent hemorrhaging on histological studies with our murine GBM model. Additionally, the development of radiographically visualized necrosis positively correlates with the tumor size as well as symptomatic progression. Necrosis at later stages of tumor progression appears as a non-enhancing central-tumoral region on post-contrast T1-weighted images, probably due to extensive tumoral tissue death resulting in the loss of contrast enhancement. Such lack of central enhancement coincides with the radiographic and clinical definition of necrosis among GBM patients. Interestingly, while necrosis eventually loses enhancement and appears unenhancing on T1-weighted post-contrast MRI when compared to normal parenchyma, tissue actively undergoing necrosis first appears more prominently enhancing in the early stages of tumor progression. This feature probably coincides with active inflammation with resulting loss of the blood-brain barrier and extravasation of contrast. Moreover, as necrosis evolves, two contrast-enhancing rims are observed at later stages. The outer rim at the tumor border likely represents the infiltrating tumor edge, and the inner rim at the peri-necrotic region may represent locally infiltrating immune cells which facilitate the development of tumor necrosis, as reported in previous studies (17). These radiographic findings associated with early stages of GBM tumor progression and necrosis evolution, to our knowledge, have not yet been reported in literature.

There are several limitations to this study. First, findings reported in this study are limited to one type of murine xenograft model; whether these findings can be reproduced using other types of GBM murine xenograft models—in particular, GBM patient-derived xenograft murine models—and other non-xenograft models awaits future study. Second, the resolution of biological and physiological processes provided by MRI imaging is somewhat limited to the large-scale tissue level, and it would be clinically inappropriate to generate diagnoses or prognostic information regarding malignancy based solely on radiographic changes without verification from histological or molecular studies. This may be addressed in future murine studies by incorporating MRI compatible cellular contrast dye specific for tumor tissue as well as other tumor-associated immune cells (e.g., TANs or TAMs) as technology and tools in cellular and cell-tracking MRI evolve and become available (26–28).

It is well-established that both chemotherapy and radiation result in tumor tissue death, and therefore necrosis may be a useful predictor of tumor response to treatments. If we have a better understanding of the temporal evolution of intratumoral necrosis, and if we are able to accurately measure it, this may serve as a potential biomarker for monitoring treatment responses of individual tumors and allow tailoring of treatment in real time. Radiographically, the combination of chemotherapy and radiation often provokes contrast enhancement and seeming enlargement of the residual tumor, mimicking tumor progression in so-called pseudoprogression (29). It has long been a challenge to distinguish treatment-related necrosis and its resultant radiographic changes from true disease progression, especially among patients with treatment-resistant tumors. This poses a major hurdle in the follow-up and surveillance of patients with high-grade gliomas, including GBM, as additional surgical biopsy or multi-modal imaging studies, such as perfusion MRI or MR spectroscopy, are necessary for a conclusive diagnosis, which risks delaying treatment of true disease progression. Conventional grading of gliomas does not predict therapeutic response of individual tumors even with same histological grade, and as a result, contrast-enhanced MRI has been the most widely utilized clinical tool to guide diagnosis, surgical navigation, and radiation treatment planning. Additionally, it is the most common objective assessment with which to monitor treatment responses to standard adjuvant chemotherapy and radiation. Moreover, for patients with gliomas that are non-resectable due to being in eloquent locations, MRI remains the gold standard for routine surveillance of these patients and monitoring of potential malignant transformation from low- to high-grade tumors. In some circumstances, MRI may be the only assessment used for diagnosis and for differentiation of low-grade tumors from high-grade ones.

Therefore, knowledge gained from our study may provide neuroradiologists with new insights to more accurately interpret post-treatment radiographic changes among GBM patients, allowing recognition of radiographic changes of necrosis and low-to-high grade malignant transformation at earlier stages, which could in turn facilitate and guide treatment planning among a multi-disciplinary neuro-oncology team to minimize delays and improve success. These potential implications remain to be further explored in future studies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Penn State University Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Penn State University Institutional Animal Care and Use Committee.

Author contributions

PY and WL conceived the project and designed the experiments. PY and WL performed stereotaxic intracranial surgeries in mice. PY performed histological studies of mouse brain tumor specimens with assistance from SC and WL. JW performed mouse MRI. PY performed mouse and human radiographical analyses under the supervision of KT and WL. DA, MG, BZ, and KT provided MRI results from GBM patients seen at the Neurooncology clinic at Penn State Hershey Medical Center. PY and WL wrote an original manuscript. All authors provided intellectual input and edited the manuscript. WL supervised all aspects of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Autophagy-dependent ferroptosis as a potential treatment for glioblastoma

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Glioblastoma (GBM) is the most common malignant primary brain tumor with a poor 5-year survival rate. Autophagy is a conserved intracellular degradation system that plays a dual role in GBM pathogenesis and therapy. On one hand, stress can lead to unlimited autophagy to promote GBM cell death. On the other hand, elevated autophagy promotes the survival of glioblastoma stem cells against chemotherapy and radiation therapy. Ferroptosis is a type of lipid peroxidation-mediated regulated necrosis that initially differs from autophagy and other types of cell death in terms of cell morphology, biochemical characteristics, and the gene regulators involved. However, recent studies have challenged this view and demonstrated that the occurrence of ferroptosis is dependent on autophagy, and that many regulators of ferroptosis are involved in the control of autophagy machinery. Functionally, autophagy-dependent ferroptosis plays a unique role in tumorigenesis and therapeutic sensitivity. This mini-review will focus on the mechanisms and principles of autophagy-dependent ferroptosis and its emerging implications in GBM.

KEYWORDS

autophagy, ferroptosis, glioblasoma, glioblastom stem cells, therapeutics

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults, with an annual incidence of about 3.23 cases per 100,000 people and a median survival (regardless of treatment) of approximately 8 months, with a one-, five- and ten-year survival rates of 42.8, 7.2 and 4.7%, respectively, based on recent statistical analysis of the Central Brain Tumor Registry of the United States (CBTRUS) (1, 2). Temozolomide is one of the first-line chemotherapeutics for the treatment of GBM due to its DNA alkylating activity and its ability to cross the blood-brain barrier (3). However, GMB patients often develop temozolomide resistance after one year of treatment. One reason for this clinical challenge is that glioblastoma stem cells (GSC) can survive after surgical resection and are highly resistant to chemotherapy and radiotherapy (4). Specifically, autophagy is a cellular recycling mechanism that confers robust chemoresistance and radiation resistance to GSC, resulting in

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GBM regeneration and the inability to kill them by standard therapies (5). Therefore, understanding the process and function of autophagy is important for developing effective anticancer approaches in GBM.

Autophagy is a catabolic process that promotes the recycling of cellular components under stress conditions (such as nutrient deficiency or microbial infection), thereby restoring cell homeostasis (6). It can be divided into macroautophagy, microautophagy and chaperon-mediated autophagy (CMA) (7). Macroautophagy involves the formation of autophagosomes that encapsulate senescent proteins or damaged organelles into lysosomes for degradation and recycling (8). The macroautophagy process is dynamically mediated by autophagy-related (ATG) family proteins through the formation of distinct protein complexes under the control of post-translational modifications (9). Microautophagy is driven by direct engulfment of cytoplasmic cargo to lysosomes under infectious conditions (10). In CMA, heat-shocked homologous 70 kDa (HSC70) proteins recognize KFERQ motifs in target proteins and facilitate their transfer to lysosomes through the lysosome-associated membrane protein 2A (LAMP2A) receptor (11). This review will focus on macroautophagy, simply referred to as autophagy from now on.

Autophagy is involved in the regulation of various cell death modalities, thereby determining cell fate (12). In addition to promoting cell survival, excessive autophagy can also trigger cell death, especially the iron-dependent form of nonapoptotic ferroptosis. Notably, ferroptosis was originally described as an autophagy-dependent cell death (13). Growing evidence from independent groups highlights that autophagy promotes iron accumulation and lipid peroxidation, key metabolic hallmarks of ferroptosis (14, 15). Consequently, genetic or pharmacological inhibition of the autophagy machinery can suppress ferroptosis sensitivity in various disease models. Moreover, pharmacological induction of autophagy-dependent ferroptosis may be a game-changing antitumor strategy compared to traditional inhibition of autophagy to limit tumor growth (16).

In this review, we summarize the current understanding of the process and basis of autophagy-dependent ferroptosis. We also discuss the implications of induction of autophagy-dependent ferroptosis for the treatment of GBM.

Molecular mechanism of autophagy-dependent ferroptosis

The activation of autophagy machinery is significantly increased within cells treated with classical ferroptosis inducers, such as small-molecule compounds erastin and RSL3. Compared to wide-type cells, autophagy deficient cells (e.g., ATG5$^{-/-}$ and ATG7$^{-/-}$) exhibit higher survival rate during ferroptosis (17). *In vitro* studies further show that ferroptosis is dependent on autophagy machinery (18, 19). Indeed, excessive formation of autophagosomes or abnormal increase of lysosomal activity will cause the accumulation of intracellular iron and lipid peroxides by selectively degrading proteins regulating iron and redox homeostasis (e.g., ferritin, GPX4, ARNTL, and lipid droplets), promoting the occurrence of ferroptosis (Figure 1). The selective role of autophagy in promoting ferroptosis is discussed from the following six aspects.

Degradation of iron regulatory protein

Excessive ferrous iron can promote the generation of reactive oxygen species (ROS) through Fenton reaction, thus causing toxic effects on cells (20). Under normal physiological conditions,

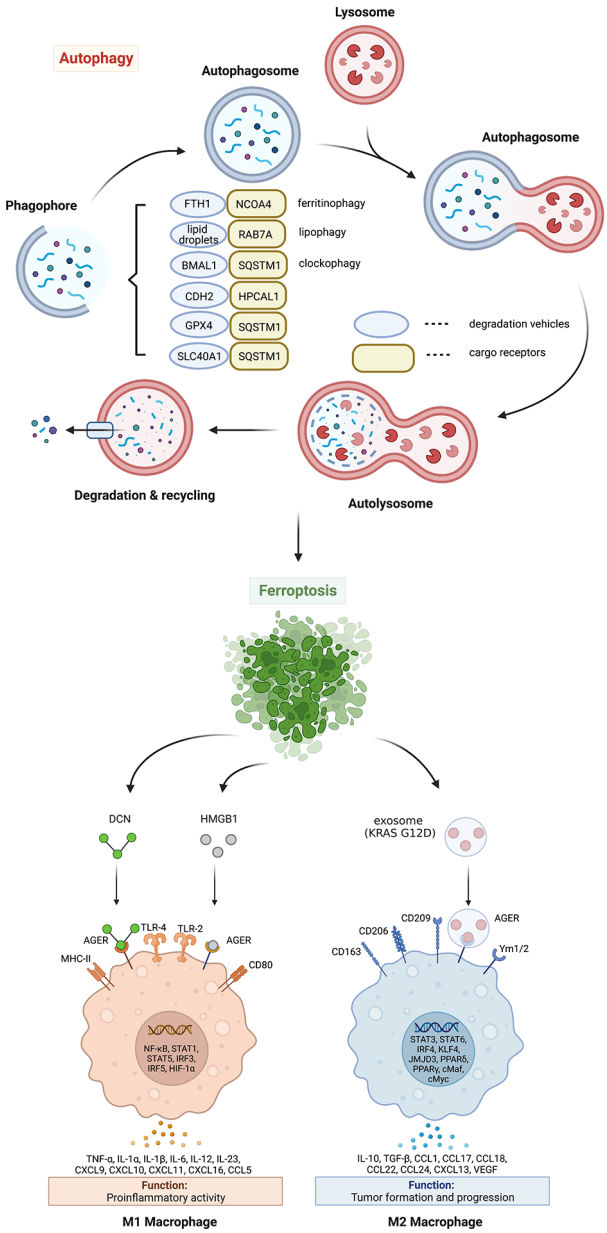

FIGURE 1
Mechanism and significance of autophagy-dependent ferroptosis. Autophagy promotes ferroptosis by selectively degrading anti-ferroptosis proteins or organelles through multiple autophagy receptors. DAMP release from ferroptotic cells can trigger inflammatory and immune responses in macrophages by activating the AGER pathway. AGER, advanced glycosylation end-product specific receptor; BMAL1, brain and muscle ARNT-like 1; CDH2, cadherin 2; DCN, proteoglycan; FTH1, ferritin heavy chain 1; GPX4, glutathione peroxidase 4; HPCAL1, hippocalcin like 1; HMGB1, high mobility group box 1; IL, interleukin; MHC, major histocompatibility complex; NCOA4, nuclear receptor coactivator 4; RAB7A, member RAS oncogene family; SLC40A1, solute carrier family 40 member 1; SQSTM1, sequestosome 1; TLR, toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

intracellular ferrous iron is absorbed by the ferritin heavy chain 1 (FTH1, also known as FTH). Ferrous iron is then oxidized to ferric iron and stored in ferritin. In addition, excess ferrous iron is transported out of the cell *via* iron exporter solute carrier family 40 member 1 (SLC40A1, also known as ferroportin-1 or FPN1) on the cell membrane (21). Ferroptosis can be induced by increasing iron absorption and decreasing iron storage or preventing iron release. At least two mechanisms mediate iron accumulation and subsequent ferroptosis by promoting autophagic degradation of ferritin or SLC40A1 (Figure 1). The degradation of ferritin is mediated by nuclear receptor coactivator 4 (NCOA4)-dependent ferritinophagy in erastin-treated mouse embryonic fibroblasts and human pancreatic cancer cells (17). In contrast, the autophagy receptor sequestosome 1 (SQSTM1, best known as p62) is required for the elimination of SLC40A1 to promote iron-dependent ferroptosis in cancer cells *in vitro* and *in vivo* (22). Although these studies highlight that autophagy increases toxic iron accumulation to induce ferroptosis, whether autophagy selectively affects iron accumulation in different subcellular organelles remains unknown.

Degradation of lipid droplet

Lipid droplets are highly dynamic organelles that not only store lipids but also release them under stressful conditions. The process of lipid droplet degradation through autophagy is called lipophagy (23). The free fatty acids generated by lipophagy promote adenosine 5′-triphosphate generation through β oxidation in mitochondria. Unlike lipid droplets known to play a role in preventing lipotoxicity by storing fatty acids, lipid droplet degradation mediated by RAB7A lipophagy (Figure 1) can promote RSL3-induced lipid peroxidation and ferroptosis in human liver cancer cells (24). In contrast, the overexpression of tumor protein D52 (TPD52) effectively inhibits RSL3-induced lipid peroxidation and ferroptosis by promoting lipid storage or inhibiting lipophagy (24). These findings suggest that lipophagy provides a lipid supply for subsequent lipid peroxidation during ferroptosis. In addition to RAB7A, further identification of lipid droplet-specific autophagy receptors is important for the development of inhibitors targeting lipophagy-dependent ferroptosis.

Degradation of circadian regulator

The circadian clock is endogenous and controls numerous cellular physiological processes, including iron metabolism, oxidative stress, and cell death, by regulating circadian switches (25). Clockophagy is a type of selective autophagy that degrades circadian rhythm-regulating proteins during ferroptotic cancer cell death (26). The clockophagic degradation of basic helix-loop-helix ARNT like 1 (BMAL1, also known as ARNTL1), the core protein of circadian clock, promotes lipid peroxidation and ferroptosis by increasing lipid storage in droplets through the Egl-9 family hypoxia inducible factor 2 (EGLN2, also known as PHD1)-mediated hypoxia inducible factor 1 subunit alpha (HIF1A) degradation (27). Moreover, SQSTM1 is required for clockophagy-mediated BMAL1 degradation (27) (Figure 1), supporting that SQSTM1 is a multisubstrate autophagy receptor for ferroptosis.

Degradation of GPX4

Glutathione peroxidase 4 (GPX4), formerly known as phospholipid hydrogen peroxide glutathione peroxidase (PHGPx), is one of the core regulators and targets of ferroptosis (28). GPX4 is the fourth member of the selenium-containing GPX family with a unique ability to scavenge membrane lipid hydroperoxide products to alcohols (29). In 2014, a targeted metabolomics study showed that the overexpression or knockdown of GPX4 can regulate the cytotoxicity of 12 ferroptosis inducers (28). Mechanistically, GPX4 uses its catalytic activity to weaken lipid peroxide toxicity and maintain membrane lipid bilayer homeostasis. RSL3, an inhibitor of GPX4, covalently binds to GPX4 and inactivates GPX4, leading to the accumulation of intracellular peroxides and triggering ferroptosis (28). As a cofactor of GPX4, glutathione (GSH) deficiency inactivates GPX4 and triggers ferroptosis. Therefore, the inhibition of GPX4 activity and the decrease of GPX4 expression can destroy the balance of cellular redox system, causing the accumulation of lipid ROS and ferroptosis. Both erastin and RSL3 induce autophagy flux and affect GPX4 levels through SQSTM1-mediated GPX4 protein degradation in multiple cancer cells (22) (Figure 1). Moreover, pharmacological inhibition of mammalian target of rapamycin complex 1 (mTORC1) by rapamycin also reduces GPX4 protein levels, while *vice versa* RSL3 inhibits mTORC1, supporting a relationship between autophagy and ferroptosis (30). FIN56, another ferroptosis inducer, also promotes GPX4 protein degradation and lipid peroxidation in an autophagy-dependent manner (31). Since CMA also mediates ferroptosis machinery protein degradation, such as GPX4 and acyl-CoA synthetase long-chain family member 4 (ACSL4) (32, 33), the receptors of which are heat shock proteins, it is necessary to further elucidate the roles of different types of autophagy in promoting ferroptosis.

Degradation of CDH2

Historically, hippocalcin like 1 (HPCAL1) is a neuron-specific Ca2+-binding protein that control central nervous system responses (34). In terms of tumor formation and development, HPCAL1 exhibits tumor-promoting activity in GBM by the activation of the embryonic developmental signals, especially the WNT-CTNNB1/β-catenin pathway (35). Recently, HPCAL1 was identified by quantitative proteomic approach as a novel autophagy receptor that triggers autophagy-dependent ferroptosis by selectively degrading cadherin 2 (CDH2) (Figure 1) (36). Mechanistically, the degradation of CDH2 is initiated by protein kinase C theta (PRKCQ)-mediated HPCAL1 phosphorylation on Ter149. Notably, starvation-induced autophagy does not require HPCAL1, which establishes the first autophagy receptor to induce ferroptosis. Furthermore, transmembrane protein 164 (TMEM164) acts as a specific promoter of ferroptosis-related autophagosome formation, but not ATG9A-dependent and starvation-induced autophagosome

formation (37). These studies provide important insights into the upstream signaling and downstream mediators of autophagy-dependent ferroptosis.

Organelle-specific initiation of autophagy-dependent ferroptosis

In addition to lipid droplets, other organelles play context-dependent roles in mediating autophagy-dependent ferroptosis (38). For example, the lysosomal cysteine protease cathepsin B (CTSB) promotes autophagy-dependent ferroptosis *via* translocation from lysosome into nucleus to cause DNA damage signals and to activate stimulator of interferon response cGAMP interactor 1 (STING1, also known as STING or TMEM173)-dependent DNA sensor pathways (39) (Figure 2). In addition, the MAPK-STAT3-CTSB pathway is required for erastin-induced ferroptosis in pancreatic cancer cells (40). The inhibition of STAT3 through small molecules (e.g., cryptotanshinone and S31-201) or siRNA as well as blockade of CTSB activity (using CA-074Me) or vacuolar type H+-ATPase (using bafilomycin A1) limits ferroptosis (40). These findings suggest that there is organelle communication between the lysosome and the nucleus to initiate autophagy-dependent ferroptosis.

Mitochondria play an important role in the process of ferroptosis, including participating in mitochondrial DNA biosynthesis, ROS metabolism and mitochondrial iron storage and transport (41, 42). The DNA sensor hub STING1 links mitochondrial DNA damage, autophagy, and ferroptosis. Specifically, anti-HIV drug zalcitabine-induced mtDNA depletion and oxidative DNA damage activates the cGAS-STING1 pathway, thereby triggering STING1-dependent autophagy and subsequent autophagy-mediated ferroptosis (43) (Figure 2). Mitochondria associating with lipid droplets in fat-oxidizing tissues are recently identified as peidroplets mitochondria, which have unique ATP synthesis and pyruvate oxidation capacities (44), potentially suggesting a functional role in autophagy-dependent ferroptosis through lipophagy. Furthermore, the iron-binding nuclear protein pirin (PIR) can hijack HMGB1 in the nucleus, thereby inhibiting the translocation of HMGB1 to the cytoplasm and subsequent activation of beclin 1 (BECN1)-dependent autophagy and ferroptosis in pancreatic cancer cells (45, 46). These findings explain the persistent activation of DNA damage, DAMP release, and autophagy flux during ferroptotic death.

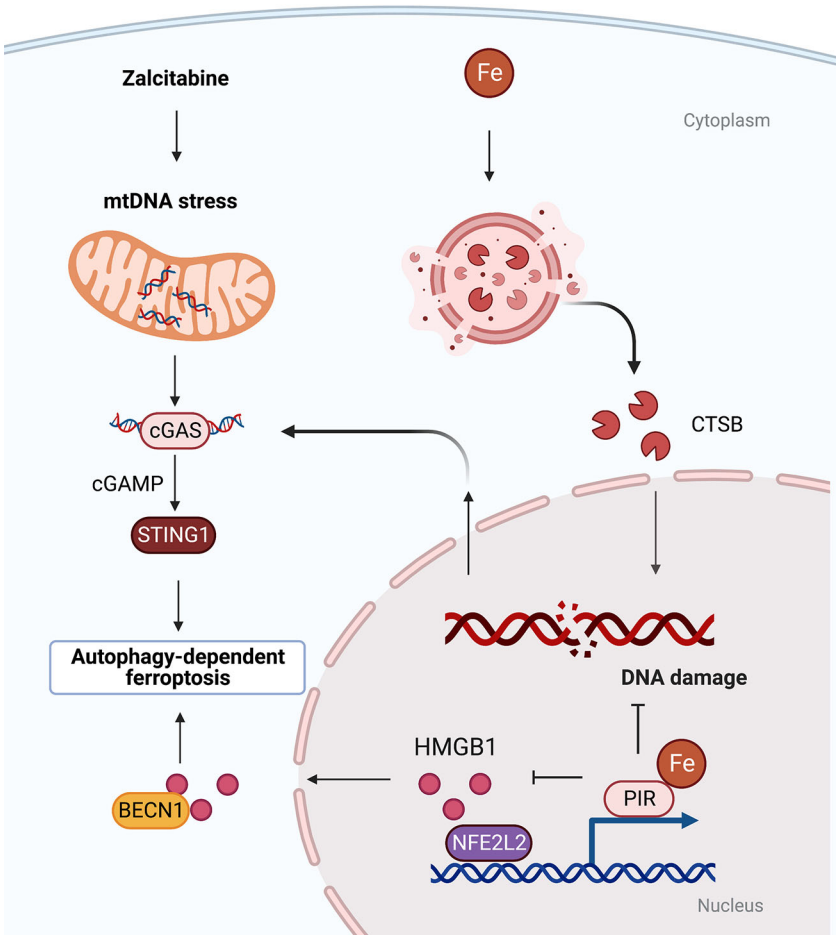

FIGURE 2
Organelle-specific initiation of autophagy-dependent ferroptosis. There is crosstalk between lysosomes, nucleus and mitochondria. Oxidative damage to nuclear or mitochondrial DNA triggered by iron-dependent CTSB translocation or anti-HIV drug zalcitabine can activate STING-dependent autophagy and ferroptosis. BECN1, beclin 1; cGAS, cyclic GMP-AMP synthase; CTSB, cathepsin B; HMGB1, high mobility group box 1; PIR, pirin; STING1, stimulator of interferon response cGAMP interactor 1.

Targeting autophagy-dependent ferroptosis signaling network in GBM

GBM cells promote autophagy under adverse conditions (e.g., nutrient deficiency, oxidative or hypoxic stress) to maintain their survival and evade responses to cancer therapy (47–49). The progression of GBM is associated with decreased autophagy capacity (50). In a *KRAS*-driven mouse model of GBM, inhibition of ATG significantly reduced tumor growth and oncogenic progression, suggesting that autophagy is critical for GBM initiation and growth (51). While temozolomide induces autophagy to kill GBM, GSCs exert self-protection by activating autophagy (52). The current study aims to find new therapeutic targets to improve patient outcomes. Recent studies have shown that ferroptosis exists in GBM tumor cells, and recurrent tumors are more prone to ferroptosis treatment (53). These results confirm that exploiting the ferroptosis process may be a possible new therapeutic strategy, especially in the setting of recurrent GBM.

There is also evidence that GBMs have significantly increased iron requirements compared to normal tissues, and GSCs uptake twice as much iron as non-stem tumor cells (54). Thus, GBM cells have a strong iron reliance. Targeting iron-related proteins or increasing intracellular iron levels are considered as feasible methods for GBM treatment (Table 1). Amentoflavone induces ferroptosis in glioma cells though ATG7-mediated autophagy to break iron homeostasis (55). Interestingly, both coatomer protein complex subunit zeta 1 (COPZ1) and tripartite motif containing 7 (TRIM7) are associated with ferroptosis by regulating intracellular iron metabolism in GBM (57, 59). Genetic inhibition of COPZ1 or TRIM7 suppresses tumor growth *in vitro* and *in vivo*, mechanistically by inducing NCOA4 expression and promoting ferritinophagy, followed by increased intracellular levels of ferrous iron and ultimately ferroptosis (57, 59). More recently, multifunctional nanomaterials (including ultrasmall iron oxide nanoparticles and iron oxide nanoparticles loaded with paclitaxel) have the effects of increasing the intracellular iron level, catalyzing fenton reaction, generating ROS and lipid peroxidation, ultimately inducing ferroptosis *via* a BECN1-dependent autophagy pathway (56, 58).

In addition to iron addiction, GBM has a strong capacity of lipid synthesis, which is related to its malignant degree. Breaking lipid metabolism balance in GBM can induce ferroptosis to inhibit tumor growth. Therefore, exploiting this metabolic alteration in GBM to induce ferroptosis may be another effective therapeutic direction.

The damage-associated molecular patterns (DAMPs) released by dead, dying, or stressed cells act as alarm signals to trigger innate and adaptive immune responses (60). The early release of high mobility group box 1 (HMGB1), proteoglycan core proteoglycan (DCN), or mutated KRAS-G12D protein during ferroptosis is an active process involving secretory autophagy, lysosomal exocytosis, and exosome secretion (61–63) (Figures 1, 2). Once released by ferroptotic cells, these extracellular DAMPs bind to the receptor advanced glycosylation end-product specific receptor (AGER) on macrophages and trigger either proinflammatory cytokine production in a nuclear factor-κB (NF-κB)-dependent manner or macrophage polarization-associated tumor progression (Figure1). Hypoxic glioma-derived exosomes promote M2-like macrophage polarization by enhancing autophagy induction (64). Taken together, pharmacological or genetic inhibition of the DAMP-AGER axis can limit the ability of ferroptotic cancer cells to induce tumor-protective immune responses.

Conclusion and outlook

Autophagy is a degradation process controlled by a cascade of ATG protein complexes, each of which regulates different stages of initiation and formation of autophagic membrane structures. Compared with autophagy to promote cell survival, the molecular mechanism by which autophagy promotes cell death is poorly understood (65, 66). The discovery of ferroptosis as an autophagy-dependent cell death provides an opportunity to suppress cancers with excessive autophagy (67). Selective autophagic degradation of anti-ferroptosis proteins or organelles promotes iron-dependent oxidative damage and cell death. For GBMs, induction of autophagy-dependent ferroptosis facilitates clearance of drug-resistant cancer stem cells. Although several experimental ferroptosis activators are available, there is still a shortage of related drugs that can be used in clinical trials. In the future, we need more in-depth work to determine the specific mechanism of autophagy-dependent ferroptosis (68), identify circulating biomarkers to monitor the activity of this pathway (69), and design the next generation of ferroptosis-related drugs (70).

TABLE 1 *In vivo* or *in vitro* studies targeting autophagy-dependent ferroptosis in GBM.

Compounds/methods	Targets of autophagy-dependent ferroptosis	*In vitro* models	*In vivo* models	Refs.
Amentoflavone	ATG7-dependent autophagy	Human GBM cell lines U251 and U373	BALB/c nude mice bearing subcutaneous xenograft	(55)
Ultrasmall iron oxide nanoparticles (USIONPs)	Beclin1/ATG5-dependent autophagy	Human GBM cell line U251	NA.	(56)
Silencing COPZ1	NCOA4 mediated autophagy	Human GBM cell line U87MG and U251	Nude mice bearing intracranial xenograft tumors	(57)
Iron oxide nanoparticles loaded with paclitaxel (IONP@PTX)	Degradation of GPX4	Human GBM cell line U251	BALB/c-nu mice bearing GBM xenografts	(58)
Silencing TRIM7	NCOA4-mediated ferritinophagy	Human GBM cell line A172 and U87MG	Non-obese diabetic-severe combined immunodeficient NOD-SCID mice bearing subcutaneous and intracranial tumor xenograft	(59)

Author contributions

YX proposed the research. YX and TH both reviewed the literature and collected references. YX, RK, and DT wrote the manuscript and finalized the paper. JL provided some of the material. HZ and XL have adjusted some expressions of the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Ferroptosis-based drug delivery system as a new therapeutic opportunity for brain tumors

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The brain tumor is a kind of malignant tumor with brutal treatment, high recurrence rate, and poor prognosis, and the incidence and death rate is increasing yearly. Surgery is often used to remove the primary tumor, supplemented by radiotherapy and chemotherapy, which have highly toxic side effects. Therefore, there is an urgent need to explore new strategies, methods, and technologies that can genuinely improve the treatment of brain tumors. Ferroptosis differs from traditional apoptosis's morphological and biochemical characteristics, and ferroptosis possesses its unique characteristics and mechanisms, opening up a new field of ferroptosis treatment for cancer. It has been found that there is a close relationship between ferroptosis and brain tumors, and a novel nano-drug delivery system based on ferroptosis has been used for the ferroptosis treatment of brain tumors with remarkable effects. This review firstly analyzes the characteristics of ferroptosis, summarizes the mechanism of its occurrence and some factors that can be involved in the regulation of ferroptosis, introduces the potential link between ferroptosis and brain tumors, and clarifies the feasibility of ferroptosis in the treatment of brain tumors. It then presents the ferroptosis nano drug delivery systems developed under different metabolic pathways for ferroptosis treatment of brain tumors. Finally, it summarizes the current problems and solutions of ferroptosis nano drugs for brain tumor treatment, aiming to provide a reference for developing ferroptosis nano drugs against brain tumors.

KEYWORDS

ferroptosis, brain tumors, nano drug delivery systems, glioblastoma, neuroblastoma

1 Introduction

Brain tumors are different types in the brain's central nervous system (CNS) and are mainly divided into primary and secondary (1). Due to the specificity of the site of occurrence, brain tumors have become one of the essential tumors that endanger human life and health, and the disease burden is increasing year by year. In 2018, the estimated number of new cases of brain tumors worldwide was close to 300,000, accounting for about 1.6% of all new cases of malignant tumors and ranking 17th in the incidence of malignant tumors (2). Therefore, the treatment of brain tumors has been a global problem. The factors causing brain tumors are partly exogenous factors, including bad habits in life, environmental pollution, radioactive elements, etc. In contrast, endogenous factors involve hereditary genetic factors, congenital or acquired immune defects, etc (3). After the brain tumor grows unrestrainedly in the skull to a certain extent, it will compress the local tissues or nerves and increase the intracranial pressure, which will cause paralysis and mental disorder and seriously endanger the life and health of the patient. Surgery is the best treatment option for patients with advanced brain tumors, which can be supplemented with radiotherapy or chemotherapy to achieve the best treatment effect (4). However, for some advanced brain tumors, due to the extensive infiltrative growth of malignant tumor cells into the skull, it is difficult to distinguish them from normal cells, which increases the difficulty of tumor cell resection, and the higher the grade of malignant brain tumor, the higher the malignancy and the worse the prognosis (5).

The traditional cell death modalities are apoptosis, autophagy, and necrosis (6). Ferroptosis is a newly discovered mode of cell death in recent years, which differs from traditional cell death in terms of morphology, mechanism of onset, and biochemical characteristics (7). Ferroptosis is an iron-dependent, concomitant lipid peroxide accumulation mode of death (8). Many studies at this stage have shown that many malignancies, including brain tumors, exhibit sensitivity to ferroptosis (9, 10). Iron is an essential element in living organisms, especially for malignant tumor cells that require it more to maintain their vital activities such as proliferation, differentiation, and migration (11). Ferroptosis has attracted much attention in the anti-tumor field, and many ferroptosis-based drugs have been gradually applied in clinical treatment with specific effects (12, 13). However, due to the high complexity of the human brain, the high invasiveness of tumor cells, tumor heterogeneity, and the existence of the blood-brain barrier (BBB), many factors hinder the effective delivery of therapeutic drugs to tumors, resulting in insufficient drug accumulation or even acquired tumor resistance. These limitations significantly reduce the effectiveness of ferroptosis therapy (14, 15). Therefore, there is an urgent need to develop ferroptosis drug delivery systems that can efficiently cross the BBB and target tumor cells at brain lesion sites.

Booming nanomaterials offer a promising platform for the safe and efficient treatment of tumors (16). Nanomaterials can deliver drugs directly to the focal area to improve efficacy and can be transported by the bloodstream (17). Its effect is exerted in the focal area rather than the whole body, enhancing anti-tumor efficiency while reducing damage to normal body tissues (18). Nanomedicine refers to the use of nanotechnology and formulation science to carry an API (or active molecule) in a nano-sized (1-1000 nm) drug carrier, also known as a nanocapsule (19). In recent years, the successful development of nanomaterials and technologies has provided a promising platform for the safe and efficient treatment of brain tumor nano drugs (20). The properties of nanomedicines are manifested as 1) effective increase in drug solubility, 2) adequate protection of unstable drugs against premature degradation, 3) tumor targeting through passive or active targeting mechanisms, and 4) multiple drugs can be delivered by one nanocarrier to exert synergistic effects (21, 22). Ferroptosis nano drugs designed for the unique environment of brain tumors can protect the loading components, target brain tumors, cross the blood-brain barrier more efficiently, reduce the damage to normal cells, improve drug accumulation and intratumoral penetration in brain tumor tissues, reduce toxic side effects on normal tissues, etc., and show excellent application value and development prospects in brain tumor therapy (23, 24). This review systematically reviewed the ferroptosis nano drug delivery systems designed by different metabolic pathways, the current status of clinical applications, challenges, and opportunities based on the metabolic pathways of ferroptosis.

2 Overview of ferroptosis

2.1 Basic concepts and characteristics

The definition of ferroptosis was first described in 2012 when Dixon et al. studying human fibrosarcoma cells, found a significant increase in intracellular lipid reactive oxygen species (ROS) after treatment with the anti-tumor drug elastin and the concomitant appearance of cell separation and death (25). However, the number of cell deaths was reversely reduced with an iron chelator, so they speculated that the concentration of iron ions and lipid ROS influenced this mode of cell death. They formally named this mode of death ferroptosis.

Ferroptosis is a newly discovered form of programmed cell death (PCD) that distinguishes itself from traditional cell death modalities such as apoptosis, cell necrosis, and cell autophagy (26). Ferroptosis is mainly caused by the imbalance between the production and degradation of intracellular lipid reactive oxygen species. When the cellular antioxidant capacity is reduced and lipid reactive oxygen species accumulate, it can cause cellular ferroptosis (27). Ferroptosis is iron-dependent and is characterized by a lipid peroxide-aggregated cell death pattern that differs significantly from traditional cell death modalities such as apoptosis, autophagy, and necrosis at the cell morphology, biochemical characteristics, and genetic level (28, 29). Morphological aspects of ferroptosis are manifested by smaller mitochondria, increased density of mitochondrial membranes, progressive contraction of mitochondria and reduction or disappearance of cristae, breakage of the outer mitochondrial membrane, and an increase in lipid reactive oxygen radicals, which maintain the cell membrane without rupture while chromosomes are not condensed (30). The biochemical features of ferroptosis include the aggregation of iron

ions, glutathione (GSH) depletion, and lipid peroxide aggregation (31). Gene-level changes in ferroptosis are manifested by the tight regulation of ferroptosis by intracellular signaling pathways, including the regulatory pathway of iron homeostasis, the RAS/Raf/MAPK pathway, and the cystine transport pathway. The immunological profile of ferroptosis is characterized by the release of proinflammatory mediators (e.g., HMGB1) by damage-associated molecular patterns (DAMPs). Susceptibility to ferroptosis is closely linked to many biological processes, including amino acid, polyunsaturated fatty acid metabolism, and the biosynthesis of GSH, phospholipids, NADPH, and coenzyme Q10 (32).

2.2 Mechanism of ferroptosis occurrence

2.2.1 Disorders of iron metabolism

Iron, an essential trace element for the human body, is also a critical factor in the occurrence of ferroptosis (33). The main component ingested by the human body through food is Fe^{3+}, which is reduced to Fe^{2+} by the action of intestinal epithelial cell reductase. After binding to transferrin, part of it is smoothly released into the cell into the unstable iron pool (LIP) with the assistance of transferrin receptors 1 and 2. In contrast, the remaining part will form ferritin. Ferritin is composed of ferritin heavy chain (FTH1) and light chain (FTL) together, which stores and regulates ferric ions (34). Iron autophagy is a process in which ferritin undergoes autophagic degradation guided by Nuclear receptor coactivator 4 (NCOA4), producing Fe^{2+} (35). Due to the high reactivity and instability of Fe^{2+}, the body's iron homeostasis is imbalanced once the Fe^{2+} in the body is overloaded. Metabolism is disturbed, making it prone to Fenton's reaction, in which the overloaded Fe^{2+} reacts to form hydroxyl radicals, promoting the formation of lipid ROS and causing ferroptosis in cells (Figure 1A).

2.2.2 Imbalance of amino acid metabolism

GSH, a tripeptide amino acid, is central to the metabolism of ferroptosis amino acids. It is synthesized in two steps by cysteine, glycine, and glutamate catalyzed by GSH synthase and glutamate-cysteine ligase (36). GSH has a significant effect as an antioxidant in reducing lipid peroxidation reactions and antioxidant stress by scavenging peroxides present in cells, and its regulators include glutathione peroxidase 4 (GPX4) and cystine/glutamate reverse transporter (system Xc^-) (Figure 1B). GPX4 is a defensive antioxidant enzyme belonging to a selenoprotein essential for mammalian development. It can specifically catalyze the conversion of GSH to oxidized glutathione (GSSG), which reduces toxic lipid peroxides in the membrane environment to non-toxic lipid alcohols, thereby mitigating the damage caused by oxidative stress (37). Inactivating GPX4 leads to the unavailability of GSH, imbalance of amino acid metabolism *in vivo*, lipid peroxide pooling, increased damage caused by oxidation, and induction of ferroptosis.

The cystine/glutamate reverse transporter (system Xc^-) serves as an amino acid-specific shipping protein that controls the entry and exit of amino acids into and out of cells. It is essentially a membrane Na^+-dependent cystine/glutamate reverse transporter, mainly found in the phospholipid bilayer of biological cell membranes, formed by the glycosylated light chain subunit SLC3A2 and the non-glycosylated heavy chain subunit SLC7A11 linked by disulfide bonds, a heterodimer (38). The system Xc^- can transfer intracellular glutamate out of the cell while transferring extracellular cystine into

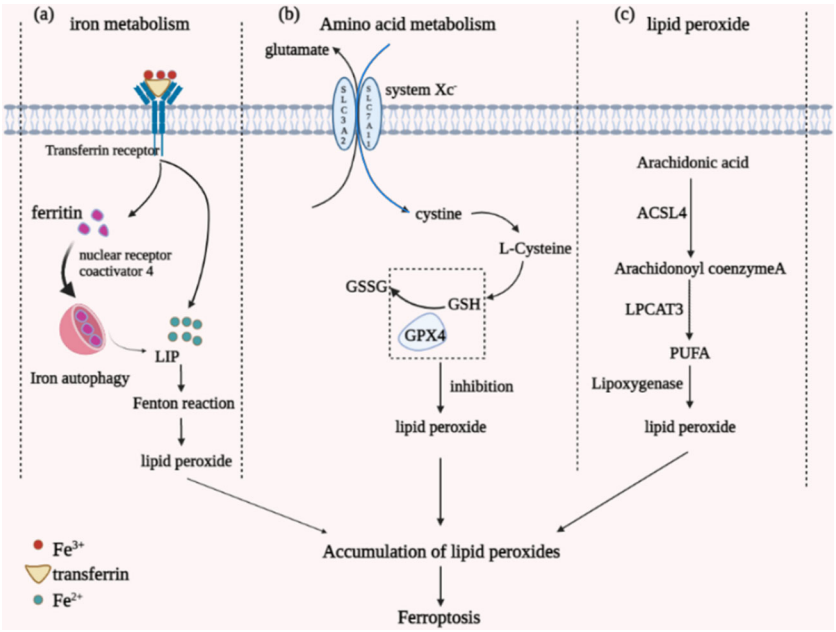

FIGURE 1
Diagram of the different mechanisms by which ferroptosis occurs. **(A)** iron metabolism; **(B)** amino acid metabolism; **(C)** lipid peroxide aggregation.

the cell, which promotes the formation of GSH. By inhibiting the formation of system Xc^-, sufficient GSH cannot be synthesized, leading to an imbalance in amino acid metabolism and ferroptosis.

In addition to the above inhibition of GSH by interfering with GPX4 and system Xc^-, some specific compounds can be used to inhibit GSH. e.g., buthionine imine (BSO), a small molecule inhibitor that targets and inhibits GSH during GSH synthesis. APR-246 (PRIMA-1) is a toxic compound that binds GSH and depletes intracellular GSH content (39). Thus, by interfering with GPX4 expression, inhibiting system Xc^-, or directly acting on GSH to deplete or under-synthesize it, it can imbalance amino acid metabolism *in vivo*, contributing to the accumulation of lipid ROS *in vivo* and causing ferroptosis.

2.2.3 Lipid peroxide aggregation

The central aspect of ferroptosis is the iron-dependent dysregulation of lipid oxidation metabolism, and polyunsaturated fatty acids (PUFAs) are vital substances in the accumulation of lipid peroxides in ferroptosis (40). Under normal conditions, PUFAs are essential substrates for lipid peroxidation reactions and contain diallyl hydrogen atoms. PUFAs are the most fragile of lipids, and their structurally unsaturated double bonds, ester bonds between glycerol and fatty acids, are highly susceptible to lipid peroxidation by enzymes and free radicals (ROS). The process of accumulation of peroxides through the action of PUFAs consists of two main parts: the Fenton reaction and the enzymatic reaction pathway. The Fenton reaction, which is the formation of an unstable iron pool by the high activity of Fe^{2+} in the cell, generates a large amount of free radical material that can separate the hydrogen atoms in the diallyl carbon through the Fenton reaction (41), allowing the accumulation of large amounts of peroxides (Figure 1C).

The enzymatic process involved in the reaction is mainly lipoxygenase but also requires the participation of acetyl coenzyme A synthase long-chain family 4 (ACSL4) and lysophosphatidyl choline acyltransferase 3 (LPCAT3), which is associated with lipid remodelling (42). The reaction uses free PUFAs arachidonic acid as the primary phospholipid substrate, which is finally oxidized by lipoxygenase to form lipid peroxides after two-step esterification by ACSL4 and LPCAT3 (43). When the synthesis of the above three enzymes is inhibited, the oxidation of PUFAs to form lipid peroxides is also affected. On the contrary, excessive activation of the three enzymes or exogenous supplementation of PUFAs leads to increased oxidation, accumulation of lipid peroxides, and massive catabolism to produce toxic aldehydes such as malondialdehyde (Malondialdehyde) or 4-hydroxy-2-nonenal (4-Hydroxynonenah'yl, 4-HNE) combined with a continuous intracellular oxidation reaction that renders the organism essential proteins inactive, which triggers ferroptosis (44).

2.3 Mechanisms regulating ferroptosis

According to the above preliminary summary and analysis of the mechanism of ferroptosis, the trigger of ferroptosis mainly involves the disorder of iron metabolism due to the imbalance of iron ion concentration, the disorder of amino acid metabolism due to the inactivation and depletion of GSH, and the lipid peroxidation aggregation driven by polyunsaturated fatty acids. Therefore, various compounds, genes, and pathways associated with iron metabolism, amino acid metabolism, and lipid metabolism can be involved in the regulation of ferroptosis, and active regulation can trigger ferroptosis and destroy tumor cells (Table 1). Some of the potential regulatory mechanisms are described below.

2.3.1 Iron metabolism

Dixon et al. found a reversal in the number of cell deaths after adding iron chelators during ferroptosis studies, suggesting the possibility of iron metabolism on the regulation of ferroptosis. Later, Gao et al. found the effect of ferritin carrier transferrin (TF) on ferroptosis, further confirming the critical role of iron metabolic processes on ferroptosis (61). The iron metabolic process involves iron ions' storage, transport, export, and degradation processes and contains various proteins and genes. Transferrin and transferrin receptor (TFRC) are essential regulators of iron ion transport. Cysteine desulfurase (NFS1), an iron-sulfur cluster biosynthetic enzyme, decreases ferritin expression and stimulates transferrin expression, thereby increasing the risk of ferroptosis in tumor cells (45). Heme is a Fe^{2+}-containing protein catalyzed by heme oxygenase-1 to produce Fe^{2+}. The Fe^{2+} generated from the degradation of large amounts of heme increases the level of iron ions in the intracellular unstable iron pool, inducing ferroptosis.

TABLE 1 Major regulatory mechanisms of ferroptosis.

Types of regulatory pathways	Regulatory factors	References
Iron metabolism pathway	NFS1	(45)
	Heme oxygenase-1	(46)
	SLC40A1	(46)
	PCBPs	(47)
GPX4 pathway	SMG9	(48)
	CREB	(49)
	ZEB1	(50)
	RSL3	(51)
system Xc^- pathway	ATF3	(52)
	INFγ	(53)
	Erastin	(54)
	p53	(55)
Lipid peroxide pathway	Vitamin E	(56)
	Carbon-deuterium bond	(56)
	MOFs	(57)
Other pathways	VDAC	(58)
	CL	(59)
	FtMt	(60)

Iron ions are transported into the cell by transferrin and stored in the unstable iron pool or ferritin. SLC40A1 is an iron ion transport carrier mainly responsible for the export of Fe^{2+}. SLC40A1 can be regulated by heparin antimicrobial peptide, which can degrade SLC40A1 in the lysosome to reduce iron export. The accumulation of large amounts of iron ions in tumor cells causes ion metabolism to be disturbed, triggering ferroptosis (46). Polycomb binding proteins (PCBPs), an iron molecular chaperone, can participate in iron metabolic processes, on the one hand interacting with ferritin to promote the oxidation of Fe^{2+} to Fe^{3+} for storage, and on the other hand, being essential regulators of the unstable iron pool, regulating the storage and export utilization of iron ions within the iron pool (47). For example, Fe^{2+} was used to regulate the activity of cofactor iron-containing enzymes. The occurrence of ferroptosis can be controlled by regulating polycomb binding protein expression.

2.3.2 GPX4

GPX4 reduces toxic lipid peroxides and is an essential effector of ferroptosis. By regulating the expression of GPX4, the accumulation of peroxides in tumor cells can be controlled, and ferroptosis can be regulated. Han et al. demonstrated the interaction between SMG9 and GPX4 after a small-scale screening of RNAi, i.e., SMG9 is a GPX4 binding protein that promotes the degradation of GPX4 protein, thereby inhibiting GPX4 activity (48). Knockdown or depletion of SMG9 content *in vivo* significantly increased GPX4 protein content and enhanced activity. CREB is a ubiquitous transcription factor that inhibits lipid peroxidation and prevents ferroptosis by binding to the promoter region of GPX4 and stimulating cell viability. Moreover, binding the protein P300 (EP300) to CREB exerts even stronger facilitation (49). ZEB1, a transcription factor, acts oppositely to CREB by repressing GPX4. ZEB1 inhibits GPX4 promoter transcriptional activity by binding to the GPX4 promoter region motif and decreases GPX4 expression (50). The advent of nano-drug vectors has also opened up more possibilities for GPX4 regulation. For example, RSL3 was originally a ferroptosis inducer that targets GPX4 and reduces GPX4 activity. Amphiphilic polymeric micelles linked to nitroimidazole-coupled peptides *via* an azobenzene linker were used to load RSL3, enabling the rapid and precise release of RSL3, which was able to significantly reduce GPX4 expression, with a twofold increase in anti-tumor efficiency compared to RSL3 (51).

2.3.3 System Xc^-

GSH prevents the accumulation of lipid peroxides and resists oxidative stress, while system Xc^- plays a vital role in synthesizing GSH, which becomes an essential regulator of ferroptosis. Therefore, the regulation of ferroptosis can be achieved by regulating system Xc^- levels. ATF3 is a transcription factor, and SLC7A11 is a critical component of system Xc^-. ATF3 binds tightly with the promoter of SLC7A11 to regulate the transcription of SLC7A11 to achieve the repression of system Xc^- and reduce the synthesis of GSH. In addition, ATF3 has a facilitative effect on the reduction of GPX4 activity-induced ferroptosis by RSL because ATF3 enhances the sensitivity of RSL3 to act on GPX4, and the activity is more easily reduced (52). The glycosylation protein INFγ can target system Xc^- and reduce the expression levels of mRNA and protein of both SLC7A11 and SLC3A2 subunits, resulting in reduced system Xc^- activity and inhibition of GSH synthesis. Also, INFγ can be used with ferroptosis inducers such as RSL3 to reduce system Xc^- activity even more effectively, cells are blocked from G1 to S cycle, and cells die (53). Erastin is a typical ferroptosis inducer. It also achieves its effect mainly by inhibiting system Xc^-. Still, it was also found to be counterproductive if activation of system Xc^- subunit SLC7A11 expression could attenuate the effect of Erastin (54). Co-loading Erastin and rapamycin constructed the nano-programmed drug delivery system through the nano-emulsification method; Erastin acts on system Xc^- and rapamycin acts on GPX4, which can synergistically reduce GSH synthesis and utilization and accumulation of lipid peroxides, resulting in ferroptosis of tumor cells (54). p53 is a tumor suppressor in cell proliferation, apoptosis, and other metabolic processes (62). It was also found to decrease the expression of SLC7A11 and thus inhibit system Xc^-, increasing the susceptibility of tumor cells to ferroptosis. In addition, Erastin can upregulate the expression of p53, which further enhances the inhibitory effect and reduces the synthesis of GSH, improving the induction of ferroptosis (55).

2.3.4 Lipid peroxide regulation

The accumulation of lipid peroxides is a significant cause and driver of ferroptosis. Inhibition of lipid peroxide formation can effectively stop ferroptosis from occurring, and common ways of inhibition include preventing peroxide formation and scavenging already-formed peroxides. Vitamin E is a relatively ideal antioxidant to provide electrons to peroxyl radicals, scavenging the free radicals attacking PUFAs and terminating the formation of peroxides. Another method is to label the part of PUFAs susceptible to oxidation on the diallyl with carbon-deuterium bonds because deuterium atoms are not easily replaced by oxidation. This method applies to both Fenton and enzymatic reaction pathways and has a wide range of applications to inhibit lipid peroxide formation effectively (56). Metal-organic backbones (MOFs) are crystalline materials with a periodic network structure formed by molecular self-assembly of metal ions or ionic clusters with organic ligands. Hao et al. devised a strategy for peroxisome accumulation based on catalytic MOFs for ferroptosis therapy. Bimetallic MOFs were synthesized using iron porphyrins as linkers and copper ions as metal nodes. In the tumor microenvironment, the exfoliated MOFs acted as inducers of the Fenton reaction, generating a large number of hydroxyl radicals to accumulate lipid peroxide, effectively inhibiting tumor growth in living mice and providing a new opportunity to treat tumors insensitive to apoptosis (57).

2.3.5 Other regulation methods

The study of ferroptosis found that some mitochondria-related pathways also have an essential role in regulating ferroptosis. Voltage-dependent anion channel (VDAC), a mitochondrial pore protein, has a role in promoting ferroptosis. Under normal conditions, mitochondria exchange standard ions and molecules with the cytoplasmic matrix through the VDAC. When a globin

microtubulin inhibits this process on the VDAC, mitochondrial metabolism is affected, leading to hyperpolarization and massive production of lipid peroxides (58). In addition, some ferroptosis inducers also affect VDAC, such as the increased permeability of the outer mitochondrial membrane, the opening of membrane ion channels, and the imbalance of intracellular homeostasis after the action of Erastin on VDAC, leading to dysfunctional mitochondrial metabolism and oxidation, increased ROS production, and enhanced lipid peroxidation, which in turn cause the development of cellular iron necrosis (63, 64). Cardiolipin (CL) is part of phospholipids in mitochondria, which has a promotional effect on ferroptosis, and it can form a complex with cytochrome C (59). Cytochrome C plays a role in the mitochondrial respiratory chain to transfer electrons, which will be destroyed when CL is peroxidized, and cytochrome C will be released to participate in the respiratory chain as an electron carrier, producing large amounts of lipid peroxides and contributing to ferroptosis in tumor cells. Mitochondrial ferritin (FtMt) is an iron storage protein found in mitochondria and can regulate iron metabolism. Wang et al. found that when FtMt was overexpressed, it had an inhibitory effect on Erastin-induced lipid peroxide production and iron ion level in the iron pool, and reasonable regulation of FtMt expression level could promote ferroptosis in tumor cells (60).

3 Brain tumors and ferroptosis

The two central elements that induce ferroptosis in tumor cells have been identified through the study of ferroptosis are the accumulation of iron levels or lipid peroxides within the tumor cells (65). Ferroptosis plays a vital role in anticancer research. The ferroptosis mechanism has been applied to kill relevant tumor cells (e.g., breast, gastric, and lung cancer). Recent studies have also highlighted the importance of ferroptosis in brain tumors. This article describes the progress that has been made in the means of ferroptosis in brain tumors.

3.1 Glioma

Glioma is an intracranial tumor that originates from glial cells of the nervous system and has a high incidence among brain tumors. It is a malignant tumor that is aggressive, drug-resistant, and has a poor prognosis (66). Traditional cancer treatment strategies (such as radiotherapy and chemotherapy) mainly target relevant genes and proteins that can induce apoptosis, e.g. Caspase-3 is one of the targets of cancer therapy, and activation of Caspase-3 plays a role in inhibiting tumor cells, but cancer cells gradually become resistant to apoptosis, which makes it difficult for traditional treatment regimens to be efficient. Ferroptosis may become an excellent alternative to solve this bottleneck. Ferroptosis as an ideal strategy for glioma treatment has the following main features: (1) glioma exhibits cell necrosis, and mesangial cells play an important role in this process. Mesangial cells accumulate on tumor cells in the early stage of glioma and activated mature mesangial cells release characteristic particles to induce lipid peroxidation in tumor cells and promote ROS production, leading to ferroptosis of tumor cells (67). It was found that gliomas have a strong capacity for lipid synthesis, and the high content of PUFAs in gliomas compared to normal cells contributes to the induction of ferroptosis in gliomas through positive regulatory mechanisms, such as increased expression of lipoxygenase or ACSL4 (68). (2) Glioma can alter the expression capacity of enzymes and proteins related to iron metabolism and accumulate iron content in tumor cells to maintain normal proliferation and metastasis processes (69). Glioma stem cells in gliomas can proliferate indefinitely and require large amounts of nutrients and metal elements for indefinite proliferation, so they also exhibit a high demand for iron. Transferrin receptors are overexpressed on glioma stem cells, and extracellular transferrin carrying large amounts of iron, which is generally mediated into cells through transferrin receptors, can overcome the standard blood-brain barrier mechanism, causing glioma stem cells to take up more iron from extracellular sources and disrupting iron metabolism in the brain. Therefore, reducing intracellular iron content by inhibiting iron uptake by glioma stem cells is a potential anticancer strategy (70). Zhang et al. found that coatomer protein complex subunit zeta 1 (COPZ1) was not only associated with increased tumor grade and poor prognosis in glioma patients but was also strongly associated with ferroptosis. Inhibition of COPZ1 expression induces ferritin phagocytosis and activates ferroptosis. elevated Fe^{2+} levels trigger the Fenton reaction, which promotes ROS production and leads to ferroptosis (71). (3) It was found that the expression of GPX4 was significantly higher in glioma tissues than in normal brain tissues. The expression of GPX4 was progressively enhanced with increasing WHO glioma grading (72). Induction of GPX4 inactivation increases intracellular lipid peroxidation leading to ferroptosis. Therefore, an effective treatment strategy is an induction of GPX4 inactivation by GSH depletion or GPX4 inhibitors (e.g., RSL3). It was also found that system Xc^- could assist in transporting glutamate and cystine inside and outside the cell, maintaining amino acid homeostasis, promoting GSH formation, and reducing the risk of ferroptosis. In glioma cells, when the availability of intracellular glucose decreases, glioma cells exhibit a high dependence on glutamine, and by inhibiting the formation of system Xc^-, GSH cannot be adequately synthesized and amino acid metabolism is imbalanced, leading to ferroptosis can be achieved in the treatment of glioma. Lyuzosulfapyridine is an oral anti-inflammatory drug with an inhibitory effect on system Xc^-, and it has been clinically used in treating glioma patients. However, there is a lack of safety, and is prone to potential neurological risks in patients with malignant glioma. Temozolomide is an alkylating anti-tumor agent that can cross the blood-cerebrospinal fluid barrier and is clinically used in the first-line treatment of brain tumors. However, some patients with glioma have a natural resistance to temozolomide and are also susceptible to temozolomide resistance during chemotherapy. Glioma cells become resistant to temozolomide by enhancing the expression of system Xc^- (73). Combining radiotherapy, immunotherapy, and chemotherapy with temozolomide in postoperative patients with high-grade glioma can improve efficacy, reduce drug resistance, reduce immunosuppression,

improve patients' quality of life, and prolong overall survival time. System Xc^- in ferroptosis will continue to be a major potential target for current and future regimens such as radiotherapy and chemotherapy. Through system Xc^-, ferroptosis plays an important role in modulating the response of Glioma to radiotherapy, immunotherapy, and TMZ, addressing the problem of poor safety and low utilization of traditional therapeutic agents that are not available, Ferroptosis offers additional advantages. Both artemisinin and its derivatives (e.g., dihydroartemisinin and artesunate), the active ingredients extracted from the Chinese medicine Artemisia annua, can induce ferroptosis in tumor cells by enhancing heme oxygenase-1 expression and intracellular pools of unstable iron (74). Dihydroartemisinin can act as an inhibitor of GPX4 and system Xc-, increasing the accumulation of lipid peroxides in glioblastoma cells and inducing ferroptosis (75). However, its clinical application is limited because of its deficiencies, such as poor stability and poor solubility in water. The emergence of nano drug delivery carriers provides direction for anti-glioblastoma of dihydroartemisinin, such as polymeric nanoparticles and inorganic nanoparticles can be loaded with dihydroartemisinin, which improves its solubility, biocompatibility, and targeting in water and provides a new technology for ferroptosis treatment of brain tumors (74).

3.2 Neuroblastoma

Neuroblastoma (NB) is the most common extracranial tumor in children and the most common tumor in infants and children and is also known as the king of childhood cancers (76). Neuroblastoma is clinically widely heterogeneous, mainly exhibiting malignant tumor features such as high metastasis and susceptibility to recurrence. At the same time, a few can regress to benign tumors without treatment or even disappear entirely (77). It has been found that neural tumor cells evade ferroptosis through dopamine produced in the brain. Dopamine drives overexpression of ferritin, which redistributes iron ions that should otherwise enter the unstable iron pool of the cell into the mitochondria, resulting in a reduction of intracellular lipid ROS produced by the Fenton reaction and inhibiting ferroptosis (60). The neuroblastoma oncogenic transcription factor MYCN can also escape the risk of ferroptosis by promoting the expression of system Xc^- and some system Xc^- inhibitors (ATF3, INFγ) might increase the chance of ferroptosis in tumor cells by reducing the effect of MYCN (78). Neuroblastoma is a typical MYC-driven cancer, and patients with neuroblastoma usually present with massive amplification of the N-MYC gene (MYCN), which leads to uncontrolled cancer cells. MYCN-amplified neuroblastoma is highly cysteine-dependent and sensitive to ferroptosis. Hamed et al. performed single amino acid deprivation assays on MYCN-high-expressing neuroblastoma cells and MYCN-low-expressing neuroblastoma cells. They found that MYCN-high-expressing neuroblastoma cells were strongly dependent on cysteine, an amino acid, and that deprivation of cysteine resulted in massive death of MYCN-high-expressing cancer cells (79). This study demonstrates that when cysteine intake is restricted, cysteine is heavily used for protein synthesis, which triggers ferroptosis and can significantly inhibit terminal neuroblastoma, suggesting that the high dependence of MYCN-driven brain tumor cells on cysteine is a novel therapeutic avenue that can be exploited to induce ferroptosis in cancer cells.

3.3 Meningioma

Meningioma is the most common central nervous system tumor, accounting for approximately one-third of all primary brain tumors. It mainly affects the elderly, with an increased incidence over 65 years of age, more in women than men, and less frequently in children. It usually follows a benign course with a pretty good outcome, and surgery and/or radiation therapy remain the standard of care (80, 81). According to the 2016 World Health Organization classification (4th edition), meningiomas are classified into three histological grades. The prognosis remains excellent for grade I meningiomas, with an overall 10-year survival rate greater than 90%. However, while most meningiomas, especially grade I meningiomas, can be cured by surgery alone, they become clinically challenging for grade II and III recurrent meningiomas because there are no clear standard treatment options after re-excision or re-radiation. Grade III meningiomas have an inferior prognosis, with an overall 10-year survival rate of 33%. Many chemotherapeutic agents and hormonal therapies have been tried with only modest benefits (82). NF2 is a mutated gene in brain tumors, and deletion of NF2 predisposes meningiomas to ferroptosis, and E-cadherin is negatively associated with ferroptosis. The transcription factor MEF2C positively regulates the transcription of all their genes. Activation of MEF2C promotes the expression of NF2 and E-cadherin in meningiomas and causes ferroptosis in tumor cells.MEF2C may be a potential therapeutic target for ferroptosis in meningiomas (83).

4 Nanodelivery system mediating ferroptosis in brain tumors

Some protective mechanisms exist within the brain cells to inhibit the occurrence of ferroptosis, Nrf2 is a stress-induced transcription factor that can be involved in iron metabolic processes, and GSH synthesis and metabolism-related enzymes are also under its control (84). Nrf2 can also bind to Kelch-like ECH-associated protein 1 (Keap1) and be present in the cell in an inactivated state; keap1 is the control Nrf2 produces a switch for its action. When cells encounter oxidative stress or cytotoxic drugs, the two become separated, and Nrf2 is activated. Its activation promotes iron storage, limiting ROS production and increasing the antioxidant capacity of tumor cells, making them less susceptible to ferroptosis (Figure 2). In addition, the blood-brain barrier (BBB) formed by the tight junctions between capillary endothelial cells in the brain, surrounded by a layer of stellate cells outside the basement membrane, prevents almost all drugs from entering the brain lesion site to exerting therapeutic effects, which is the main obstacle facing the development of drugs for the treatment of brain tumors (85). Therefore, there is an urgent need

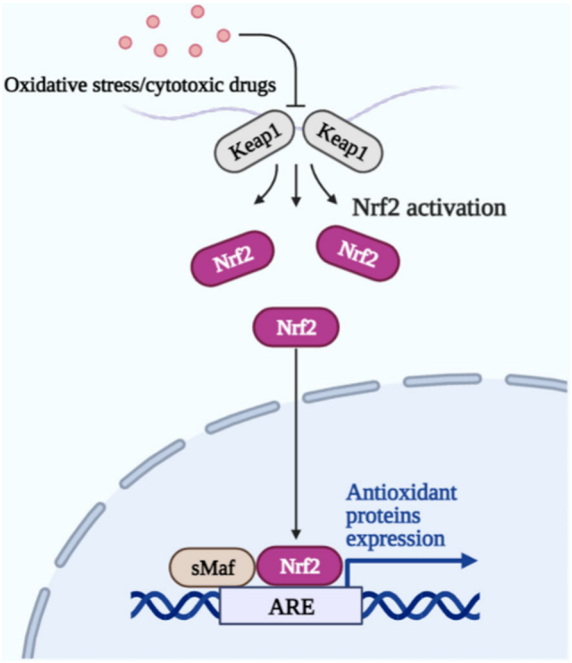

FIGURE 2
Upon oxidative stress or cytotoxic drug stimulation, Keap1 is separated from Nrf2, and Nrf2 is activated. Together with the sMaf protein in the nucleus, it binds to the antioxidant response element (ARE) and drives the expression of antioxidant protein genes to protect cells.

to develop drug delivery systems that can efficiently cross the BBB and target tumor cells at the focal site.

Most of the clinically used chemotherapeutic agents are apoptosis-inducing drugs, such as DOX, cisplatin, and paclitaxel. Due to their prolonged use, problems such as drug resistance, metastasis, relapse, and adverse effects have emerged (86, 87). In recent years, researchers around brain tumors have worked to develop antitumor drugs with low toxicity, high efficiency, low side effects, and high bioavailability. For example, Kar et al. found that Concanavalin A (Con A), a carbohydrate-binding protein of the cohesin family, is an ideal therapeutic agent for gliomas, and high doses of Con A inhibit the growth of glioma by disrupting the thiol/disulfide balance of tumor cells and causing oxidative stress, as well as inducing inflammatory factors and programmed apoptosis (88). In addition, Hacioglu et al. found an inhibitory effect of capsaicin on glioma (89). Kar et al. found an inhibitory effect of the trace element boron on glioma (90). Capsaicin and boron can interfere with the signaling pathways of the regulatory factors ACSL4 and GPX4 and induce ferroptosis. Although all three newly discovered chemotherapeutic agents mentioned above have the potential to treat glioma, they are all dose-dependent and require a certain precise range of dose concentrations to achieve a better inhibitory effect, such as high doses of Con A (250 and 500 μg/ml) to achieve the effect; the inhibitory effect of boron on glioma tumor cells is proportional to its dose concentration. The inhibitory effect of capsaicin on glioma started only when the concentration exceeded 50 μM. It is not difficult to find that the search for the optimal dose will inevitably prolong the drug development process, and dose dependence may become an important factor limiting the better anti-cancer effect of these chemotherapeutic drugs. In addition, the potentially toxic effects of these chemotherapeutic drugs need to be further studied, for example, the toxicity of capsaicin to normal cells is unknown, and the toxicity to organs also needs to be further studied. Therefore, there is still a long way to go before the actual clinical application.

Compared with ordinary chemotherapeutic drugs, high-end formulation nanomedicines have greater clinical application value and advantages. Specifically, (1) nanomedicines modified by targeting groups can achieve targeted drug delivery, which can reduce the dose of drugs and does not require high doses to bring the desired therapeutic effect and reduce its side effects as some chemotherapeutic drugs (e.g. Con A) do. (2) Chemotherapeutic drugs have short *in vivo* half-life and low bioavailability, which require frequent dosing and long treatment cycles for patients. Nanomedicine can solve this problem, and nanocarriers can extend the elimination half-life of drugs, increase the effective blood concentration-time, reduce the frequency of drug administration, and reduce the pain caused by treatment. (3) Nanomedicines can efficiently cross the BBB and specifically target the drug delivery system of tumor cells at the focal site, dramatically increasing their efficacy (85). (4) Nanodrugs can enter capillaries through blood circulation and also cross the endothelial cell gap to achieve targeted drug delivery and improve the bioavailability of drugs. Chemotherapeutic drugs kill tumor cells and normal tissue cells, especially the cells of blood and lymphatic tissues, which are growing vigorously in the human body and have strong killing power, which is harmful and the bioavailability of drugs is low.

Nanotherapies in the context of ferroptosis have gradually become a hot research topic. As the research on ferroptosis modulators and nanomedicine technology has steadily advanced, people have started to combine some ferroptosis modulating inducers with nanomedicine technology to construct novel ferroptosis nanomedicine delivery systems for brain tumor treatment, which not only substantially improves the targeting of the drug and makes it easier to cross the blood-brain barrier but also reduces the adverse effects and improves the bioavailability of the drug *in vivo*. Compared to other types of anticancer nano drugs, ferroptosis-based nano drugs have excellent physicochemical properties such as superparamagnetic properties, good biocompatibility, and low-cost advantages. The combination of multiple therapies is more advantageous than single therapeutic strategies in clinical cancer treatment, such as efficient synergistic therapeutic effects and reduction of toxic side effects by a single dose. Iron-dead nano drugs also have more combination therapeutic modalities than other types of nano drugs, such as magnetic iron-based nanotherapeutics that simultaneously enable the precise diagnosis of tumors through magnetic resonance imaging (MRI) (91). It can characterize different tissues of the same density and chemical structures of the same tissue by imaging display. This facilitates the differentiation of gray matter from white matter in the brain and has great superiority in the early diagnostic effect of brain tumors. The application of ferroptosis nano delivery lines based on different metabolic pathways in brain tumors is summarized in the following section (Table 2).

4.1 Iron metabolism strategy

Ferroptosis is iron-dependent cell death, and iron metabolism is pivotal in the overall ferroptosis process. Iron metabolism involves numerous reactive processes of iron ions, during which many regulatory proteins and genes (ferritin, transferrin) are involved. Its highly complex process, mediated by any pathway, affects the intracellular iron ion content and impacts iron metabolism. Many ferroptosis nano drug delivery systems are designed based on iron metabolism pathways. They are primarily iron-based nanomaterials, which act on specific reaction sites of tumor cells through their high iron content and participate in the Fenton reaction process, increasing the formation of lipid ROS and causing ferroptosis of tumor cells.

Shen et al. designed a FeGd-HN@LF/RGD_2 nanoparticle, a hybridized nanoparticle formed by coupling lactoferrin, RGD dimer, and cisplatin loaded with Fe_3O_4/Gd_2O_3. The nanoparticles can release Fe^{3+} and Fe^{2+} to participate in the Fenton reaction and promote lipid ROS production in brain tumor cells. At the same time, the cisplatin fraction can stimulate the production of hydrogen peroxide (H_2O_2), another substrate of the Fenton reaction, jointly accelerating the Fenton reaction and inducing ferroptosis in brain tumor cells (92). GOD-Fe_3O_4@DMSNs are ferroptosis nanocatalysts with excellent biodegradability and compatibility made from natural glucose oxidase (GOD) and ultra-small Fe_3O_4 nanoparticles integrated into large pore size and degradable dendritic silica nanoparticles. This ferroptosis nanocatalyst was found to release natural glucose oxidase *in vivo*, consume glucose from brain tumor cells, and produce hydrogen peroxide to promote the Fenton reaction, which can trigger ferroptosis and effectively inhibit the activity of glioma cells, providing a promising ferroptosis treatment strategy for glioma patients (93). Zhang et al. extracted gallic acid from gallus compounded it with Fe^{2+} into a nanocarrier (GFNP), and co-loaded the precursor drug inert Pt and chemotherapeutic drug adriamycin to construct cRGD/Pt + DOX@GFNPs nanoformulations. *In vivo* evaluation showed that GFNPs could induce ferroptosis by generating Fe^{2+} to promote the Fenton reaction and produce lipid ROS. In addition, Pt and adriamycin could induce apoptosis in brain tumor cells, ultimately obtaining ferroptosis synergistic with apoptosis to inhibit glioma efficiently (94). Alexandra et al. prepared a hybrid nano preparation (MIONzyme-GOx) based on nano preparation by coupling a natural enzyme (GOx) with an iron oxide-based nano preparation (MIONzyme) and wrapping it with a biocompatible carboxymethylcellulose as a shell was prepared (95). The results of *in vitro* experiments on brain tumor cells showed that the GOx released from this preparation could generate H_2O_2 with glucose, which was then catalyzed by iron oxide nanoparticles to promote the Fenton reaction and generate lipid ROS, which further induced ferroptosis and successfully inhibited brain tumor cell proliferation.

4.2 Amino acid metabolic strategy

Amino acid metabolism involves the antioxidant GSH, which effectively avoids intracellular peroxide production. GPX4 and system Xc^-, which are involved in the utilization and synthesis of GSH, respectively, and also play essential roles in the stabilization of amino acid metabolism, have also been described previously, both belonging to the critical regulators of ferroptosis. This paragraph reviews nano drugs that inhibit system Xc^- or the GPX4 pathway to interfere with normal amino acid metabolism and thus induce ferroptosis.

TABLE 2 Ferroptosis nano-delivery system based on different metabolic pathways.

Metabolic pathways	Nanomedicine	Type of study	Model used	Applications	Reference
Iron metabolism	FeGd-HN@LF/RGD_2	*In vivo* *In vitro*	U-87MG	GBM	(92)
	GOD-Fe_3O_4@DMSNs	*In vivo* *In vitro*	U-87MG	GBM	(93)
	cRGD/Pt + DOX@GFNPs	*In vivo* *In vitro*	U-87MG	GBM	(94)
	MIONzyme-GOx	*In vitro*	U-87MG,U-118MG	GBM	(95)
Amino acid metabolism	FA/Pt-si-GPX4@IONPs	*In vivo* *In vitro*	U-87MG,P3#GBM and NHAs	GBM	(96)
	WA	*In vivo* *In vitro*	IMR-32 and SK-N-SH	NB	(97)
	35GB	*In vitro*	U-87MG	GBM	(98)
	MNP@BQR@ANG-EXO-siGPX4	*In vivo* *In vitro*	Ln229	GBM	(66)
Other	IO-LAHP NPs	*In vivo* *In vitro*	U-87MG	GBM	(99)
	IONP@PTX	*In vivo* *In vitro*	U251 and HMC3	GBM	(100)

Zhang et al. constructed a nano drug (FA/Pt-si-GPX4@IONPs) based on the specific pathophysiological characteristics of glioblastoma (GBM), which is based on porous iron oxide nanoparticles (IONPs) that carry cisplatin (Pt) and small interfering RNA (si-GPX4) inside, and the surface of the drug-carrying IONPs is modified with Folic acid, which can bind to the highly expressed folate receptor on the surface of glioma (96). *In vitro*, cytological experiments showed that FA/Pt-si-GPX4@IONPs produced significant killing effects on GBM cells but not on normal human astrocytes (NHA). During the intracellular degradation of nano drugs, IONPs significantly increased iron and ferrous ions (Fe^{2+} and Fe^{3+}) levels in GBM cells (96). They generated strongly cytotoxic hydroxyl radicals by the Fenton reaction between them and H_2O_2, which oxidized unsaturated fatty acids and triggered ferroptosis. At the same time, the piggybacked si-GPX4 inhibited the expression of GPX4, synergistically improving the effect of induced ferroptosis. The therapeutic approach achieved significant results both *in vitro* and *in vivo*, and FA/Pt-si-GPX4@IONPs nano drugs are expected to be applied in treating GBM. Hassannia et al. identified withaferin A (WA), a natural ferritin inducer in neuroblastoma, to inhibit neuroblastoma cell appreciation as well as inhibit the growth and recurrence of murine neuroblastoma heterogeneous tumors by inhibiting GPX4 or targeting Keap1 to increase the unstable iron pool, which in turn suggested a new therapeutic strategy by iron induction to kill cancer cells effectively. The use of multifunctional nanocarriers with targeting, degradability, and pH sensitivity to wrap WA for the preparation of nanomedicines can solve the drawbacks of poor solubility and many side effects of systemic administration of WA, improve the targeted accumulation at tumor sites, and enhance the inhibitory effect on neuroblastoma (97). As a natural biological vesicle has become an essential vehicle for treating many diseases, Li et al. designed and developed an engineered exosome with endogenously modified brain tumor targeting peptide and bound to magnetic nanoparticles by antibody complexation. A multifunctional nano drug (MNP@BQR@ANG-EXO-siGPX4) was subsequently constructed by loading small interfering RNA (siGPX4), a vital protein of the ferroptosis pathway GPX4, and Brequinar (BQR), an inhibitor of DHODH, onto the surface of exosomes and mesoporous silica, respectively (66). The nano drug can be enriched in the brain under local magnetic localization. The engineered exosomes modified with angiopep-2 (Ang) peptide can trigger transcytosis, allowing the particles to cross the BBB and target GBM cells by recognizing the LRP-1 receptor. The synergistic ferroptosis treatment of GBM is achieved by the triple action of catabolism of dihydrolactate dehydrogenase and glutathione peroxidase four ferritin defense axis, combined with Fe_3O_4 nanoparticle-mediated Fe^{2+} release. The results of this study suggest that this nano drug provides a new idea for enhanced ferroptosis for the synergistic treatment of GBM (66). Protein disulfide isomerase (PDI) has the hazard of interfering with nascent proteins to worsen glioma disease. PID is generally overexpressed in glioma cells, maintaining redox stability in tumor cells. Glioma cells also show a significant dependence on PID. Kyani et al. described a PDI nanomolecular inhibitor, 35GB, and showed that 35GB was able to upregulate the expression level of system Xc^- subunit SLC7A11 and inhibit the exchange function of system Xc^-, resulting in decreased GSH synthesis and lipid peroxide pooling. 35GB also affects the expression of gene HMOX1, and overexpression of heme oxygenase-1, a source of supplied iron, disrupts iron metabolism in the organism and induces ferroptosis. 35GB can cross the blood-brain barrier and is expected to be a novel nano-inducer of ferroptosis in glioblastoma (98).

4.3 Other

Lipid peroxidation pooling is a significant feature of ferroptosis, and to induce ferroptosis in brain tumor cells *via* lipid metabolic pathways, free PUFAs must be activated (101). PUFAs are susceptible to oxidative free radical attack and lipid peroxidation. Supplementing PUFAs levels by exogenous or increasing intracellular levels of oxidized free radicals is extremely important for the induction of ferroptosis. There is a strong link between the level of PUFAs and ferroptosis in cancer cells, with the most vital ability to induce ferroptosis effect by linolenic acid in PUFAs (102). Zhou et al. developed an iron oxide particle (IO-LAHP) that replenishes PUFAs in the body by modifying linoleic acid hydroperoxides (LAHP) and hydrophilic oligomers on the surface of iron oxide nanoparticles (IO NPs) (99). Under acidic conditions, a Fenton-like reaction between Fe^{2+} ions released from iron oxide particles (IO-LAHP NPs) and linoleic acid hydroperoxides (LAHP) on the nanoparticle surface resulted in the formation of specific single-threaded oxygen ($^1O_2$) enabling tumor-specific therapy based on ROS-mediated mechanisms. *In vitro* cellular experiments demonstrated that IO-LAHP NPs could effectively increase intracellular ROS levels in glioma cells (U87MG), which induced ferroptosis in cancer cells. *In vivo* mouse tumor model experiments further confirmed their significant inhibitory effect on tumor growth. This also suggests that exogenous supplementation of *in vivo* PUFAs levels is feasible. The potential exists for this novel nanoparticle for the treatment of brain tumors. The relationship between lipid metabolism and ferroptosis is complex. There is not only an inducing relationship but also a possible inhibiting relationship, e.g., exogenous monounsaturated fatty acids (MUFAs), which can reduce the oxidative sensitivity of cells and inhibit the occurrence of ferroptosis (103). Therefore, the correct use of the relationship of lipid metabolism can have the most significant effect on eradicating brain tumor cells. Chen et al. used iron oxide nanoparticles loaded with paclitaxel to construct a nano drug (IONP@PTX). Using U251 and HMC3 as cell models, *in vitro* studies revealed that IONP@PTX inhibited cell migration and invasion ability, increased the levels of iron ions, ROS, and lipid peroxidation, enhanced the expression of autophagy-related proteins Beclin1 and LC3II, and inhibited the expression of p62 and ferroptosis-related protein GPX4 *in vitro* (104). *In vivo*, pharmacodynamic studies revealed that IONP@PTX significantly inhibited tumor volume in GBM xenografts and decreased the expression level of GPX4 protein in tumor tissues. Thus, IONP@PTX may inhibit GBM growth by enhancing the autophagy-dependent ferroptosis pathway and may be a potential ferroptosis inducer for ferroptosis-based tumor therapy (100).

5 Prospect and conclusion

Currently, the treatment and late convalescence of malignant brain tumors are clinically tricky, both to reduce the disruption of normal brain tissue cells during treatment and to protect essential central nervous functions of the brain. Ferroptosis, as a specific form of programmed cell death, is characterized by the accumulation of lipid peroxides. Its main mechanisms of occurrence and regulatory signals are still complex, involving the Fenton reaction, GPX4, and system Xc^-, among others. Ferroptosis is essential in tumorigenesis and progression and is expected to be developed as a new cancer treatment strategy. The study of nano drug delivery systems has become a quality option in brain tumor treatment, overcoming some of the drawbacks of direct drug administration, prolonging the duration of drug action, and having great potential in improving drug efficacy. Intracellular iron is the basis of ferroptosis, and with the development of nanotechnology, various iron-based nanomaterials, such as iron oxide nanoparticles, amorphous iron nanoparticles, and organic iron frameworks, have shown attractive therapeutic advantages due to their ability to deliver exogenous iron to activate tumors (105). However, reliance on hydrogen peroxide for peroxide production is inefficient when brain tumor cells are under weakly acidic conditions, and excessive use of exogenous metals may cause potential adverse effects on human health, including acute and chronic damage (105, 106). Therefore, there is an urgent need to develop some non-iron-based nano drugs to induce ferroptosis.

Many studies have shown that combining multiple therapies is more advantageous than a single treatment strategy in clinical cancer treatment, such as efficient synergistic effects and reduced adverse effects with a single dose. Photodynamic therapy has been developed in the last century and licensed by the relevant regulatory authorities for cancer treatment. Similar to ferroptosis, they both produce ROS in cells. Photodynamic therapy has certain advantages in treating brain tumors. It can treat minimally invasive areas and protect other areas of the brain as much as possible, reducing the risk of treatment. By combining ferroptosis with photodynamic therapy, the anti-tumor effect is significantly enhanced. The tumor suppression rate is increased dramatically while reducing the possible adverse toxicity associated with ferroptosis treatment. The combination of ferroptosis with photodynamic therapy for combined anti-tumor therapy has been extensively studied, e.g., breast cancer, liver cancer, and lung cancer (106–108). However, there are fewer studies on brain tumors, which is a crucial direction for future research.

Tumor immunotherapy recognizes and kills tumor cells by stimulating the intrinsic immune system with minor damage to normal tissues. However, immunotherapy still has some problems in tumor treatment, such as immune response being only effective for a small proportion of patients and low efficiency due to insufficient immunogenicity. Immunotherapy combined with ferroptosis has emerged as a promising and effective combination of cancer treatment. In terms of the combination mechanism, there is a potential relationship between immunotherapy itself and ferroptosis, as interferon γ released from immunotherapy-activated $CD8^+$ T cells down-regulates the expression levels of mRNA and protein of two subunits of system Xc^-, SLC7A11 and SLC3A2, inhibiting tumor cells of cystine uptake, thus promoting lipid peroxidation and ferroptosis of tumor cells; ferroptosis of tumor cells occurs along with the release of immunogenic antigens that induce immunogenic cell death of tumor cells, thus contributing to the anti-tumor efficacy of immunotherapy (109–111). Zhang et al. designed a bionic magnetic vesicle with leukocyte membranes containing the transforming factor-β inhibitor Ti, membranes wrapped with Fe_3O_4 magnetic nanoparticles, and programmed cell death antibody 1 (Pa) immobilized on the surface. In tumor cells, Ti and Pa can create an immunogenic microenvironment that increases intracellular hydrogen peroxide levels and promotes the Fenton reaction causing ferroptosis (112). Nanotherapy combining ferroptosis with immunotherapy is still a promising therapeutic option for tumors. It is expected to be applied to the treatment of brain tumors through further studies in the future.

The ferroptosis-based nano drug delivery system has provided many opportunities to treat brain tumors. However, it is still not entirely in the mature stage, and the biological safety of nano drugs is still a concern. Most of the research experiments are conducted on animals, and the practical application in the clinic is yet to be developed. Future research is expected to break through the bottlenecks and grow truly efficient and non-toxic anti-tumor nanomedicines.

Author contributions

Conceptualization: YY, PJ, and ZY. Writing—original draft preparation: PJ, HC, JG, YX, PW, and LX. Writing—review and editing: YY, PJ, HC, and ZY. Supervision: YY and ZY. Project administration: PJ. Funding acquisition: YY and ZY. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Opportunities and challenges related to ferroptosis in glioma and neuroblastoma

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A newly identified form of cell death known as ferroptosis is characterized by the peroxidation of lipids in response to iron. Rapid progress in research on ferroptosis in glioma and neuroblastoma has promoted the exploitation of ferroptosis in related therapy. This manuscript provides a review of the findings on ferroptosis-related therapy in glioblastoma and neuroblastoma and outlines the mechanisms involved in ferroptosis in glioma and neuroblastoma. We summarize some recent data on traditional drugs, natural compounds and nanomedicines used as ferroptosis inducers in glioma and neuroblastoma, as well as some bioinformatic analyses of genes involved in ferroptosis. Moreover, we summarize some data on the associations of ferroptosis with the tumor immunotherapy and TMZ drug resistance. Finally, we discuss future directions for ferroptosis research in glioma and neuroblastoma and currently unresolved issues.

KEYWORDS

ferroptosis, neuroblastoma, glioblastoma, GPX4, immune

Introduction

Despite their small percentage (approximately 1%) among all invasive cancer cases, malignant central nervous system (CNS) tumors are representative tumor types in children and adolescents as well as the major cause of death related to cancer in males younger than 40 and females younger than 20. As a result, malignant CNS tumors are the third and fourth leading cause of cancer-related death among individuals in the age ranges of 0-14 and over 40 years old, respectively (1, 2). Common malignant CNS tumors include glioma and neuroblastoma (NB). Gliomas account for 24.5% of all primary CNS tumors, while malignant tumors account for 80.9%. Gliomas usually have a poor prognosis. Glioblastoma (GBM) is a representative malignant CNS tumor (49.1% of all malignancies) with the shortest observed median patient survival. Although advanced therapeutic methods, including temozolomide (TMZ) therapy and tumor-treating fields (TTFields), are applied in the clinic, treated patients have a median survival time of only approximately

15 months. GBM has a poor prognosis, and only 5.8% of patients survive for five years (1–3). NB is another malignant tumor with a sympathetic nervous system origin and accounts for approximately 7-8% of childhood malignant tumor cases and approximately 15% of cancer-related deaths. Patients suffering from high-risk NB have a 5-year survival rate of less than 50% (4).

Ferroptosis is associated with iron and reactive oxygen species (ROS) and primarily results in cytological changes, including oxidative stress. As a result of strong membrane lipid peroxidation and oxidative stress, mitochondrial cristae are reduced or absent, the outer mitochondrial membrane is ruptured, and the mitochondrial membranes are condensed, resulting in weaker plasma membrane selective permeability and increased oxidative stress. At least three cytoprotective systems against ferroptosis with distinct subcellular localizations have been identified in recent studies: glutathione peroxidase 4 (GPX4) located in the cytoplasm and mitochondria; ferroptosis suppressor protein 1 (FSP1) located at the plasma membrane, which promotes ubiquinone regeneration; and dihydroorotate dehydrogenase (DHODH) located in the mitochondria. GPX4 can remarkably prevent ferroptosis by decreasing the levels of phospholipid hydroperoxides and thereby inhibiting lipid peroxidation mediated by lipoxygenase. FSP1, which promotes ubiquinone regeneration at the plasma membrane, uses NAD(P)H to catalyze the regeneration of nonmitochondrial coenzyme Q10 (CoQ10), which blocks ferroptosis by inhibiting lipid peroxide propagation. In parallel with mitochondrial GPX4, DHODH reduces ubiquinone (CoQ) to ubiquinol (CoQH2), an antioxidant capable of resisting ferroptotic activity, which inhibits ferroptosis within the inner mitochondrial membrane (independent of cytosolic GPX4 or FSP1) (5–7).

The prognosis of glioma and neuroblastoma is not particularly satisfactory. Currently, it is necessary to develop effective therapeutic approaches for glioma and neuroblastoma. A valid way to circumvent therapeutic resistance in cancer cells is targeting the ferroptotic pathway because of the high level of iron accumulation and the accompanying increase in ROS production. However, ferroptosis-related therapy application in glioma and neuroblastoma is still challenging because several aspects of the mechanisms of ferroptosis are still unclear. In this article, we present the progress in ferroptosis research in glioma and neuroblastoma and relevant future perspectives.

Ferroptosis

Ferroptosis is a form of nonapoptotic cell death that results from the accumulation of intracellular iron and increased toxic lipid peroxide reactive oxygen species. In the prevention of ferroptosis, antioxidant systems can help decrease oxidative stress. Inhibition of an antioxidant system can contribute to the induction of ferroptosis in tumor cells. As a result, antioxidant systems are capable of remarkably regulating ferroptosis in cells and are also one of the major areas of research on ferroptosis at present.

The Xc system is also referred to as the cystine/glutamate reverse transporter protein. GPX4 essentially constitutes the selenoprotein family and mainly mediates the reduction of peroxides to the corresponding alcohol. This antioxidant system prevents ferroptosis by transporting cysteine through the Xc system for the synthesis of glutathione (GSH), which in turn helps GPX4 reduce peroxides. As a major antioxidant component, GSH participates in a wide range of redox reactions in the body to maintain physiological homeostasis. GPX4 can critically regulate ferroptosis and is known to determine cell fate. Upregulating or inhibiting these antioxidant systems to regulate ferroptosis can impact the development of various diseases. Moreover, studies have identified various drugs and molecules as inducers of ferroptosis that act by restricting Xc system activity (8, 9).

There is increasing evidence that inhibiting GPX4 activity does not necessarily lead to ferroptosis in cells. FSP1 on the plasma membrane reduces ubiquinone with NADPH as a cofactor, thereby preventing the peroxidation of lipids. GSH is not required as a cofactor for this process, nor does this process depend on GPX4. As a result, in contrast to GPX4, FSP1 may be regarded as a ferroptosis inhibitor, and the expression of FSP1 confirms the sensitivity of cells to ferroptosis (8, 10, 11).

DHODH, located in the inner mitochondrial membrane, is the enzyme involved in the 4th rate-limiting step in pyrimidine biosynthesis and is capable of catalyzing dihydroorotic acid (DHO) to be oxidized to orotate (OA) and CoQ (ubiquitin) for further reduction to CoQH2 (ubiquinone), which is associated with the respiratory complex and affects electron transfer in the oxidative respiratory chain. Further studies have shown that inhibition of DHODH results in ferroptosis in cells with low GPX4 expression and increases the sensitivity of cells with high GPX4 expression to ferroptosis. DHODH can act synergistically with GPX4 to inhibit mitochondria-related ferroptosis without dependence on FSP1 (5, 12).

As one of the most important mechanisms regulating ferroptosis, antioxidant systems have always been important. With in-depth research, an increasing number of relevant molecules have been discovered, creating directions for further research and application of ferroptosis in glioma and neuroblastoma.

Ferroptosis in glioma

Glioma is a representative malignant CNS tumor. Currently, surgery, radiotherapy, chemotherapy, and tumor treatment fields (TTFields) are the most common treatments for clinical glioma, but they have a poor prognosis in patients, particularly those who suffer from high-grade gliomas, including GBM. The exploration of new therapeutic methods and therapeutic targets for glioma remains a hot spot. Targeting the ferroptotic pathway can serve as an effective treatment for glioma (Figure 1).

Ferroptosis-related gene network in glioma

The Xc-GSH-GPX4 network serves as the primary antioxidant barrier against ferroptosis. As a direct target gene, recombinant solute carrier family 7, member 11 (SLC7A11) is repressed by p53.

It is a key component of the cystine-glutamate antagonist system (xCT system), which mediates the uptake of extracellular cystine in exchange for glutamate within the cell. The direct interaction between ubiquitin hydrolase ovarian tumor domain protease domain, ubiquitin aldehyde binding protein 1 (OTUB1) and SLC7A11 stabilizes the SLC7A11 protein, and OTUB1 knockdown triggers SLC7A11 expression-dependent ferroptosis (13). Moreover, exogenous overexpression of NF-κB activating protein (NKAP) positively regulates SLC7A11 to promote cellular resistance to ferroptosis inducers (14).

Current research indicates that glutathione peroxidase 4 plays a critical role in ferroptosis. It has been demonstrated that a number of molecules affect the expression of GPX4 in gliomas to regulate ferroptosis. RSL3 (a GPX4 inhibitor) inactivates GPX4 and induces glycolytic dysfunction in glioma cells with reduced ATP and pyruvate content as well as HKII, PFKP, and PKM2 protein levels, which in turn induces ferroptosis (15). Knockdown of RNA-binding fragile X mental retardation syndrome-related protein 1 (FXR1) promotes TMZ-induced ferroptosis, thereby overcoming TMZ resistance. FXR1 has been proven to bind to the GPX4 mRNA transcript and exert a positive regulatory effect on GPX4 expression (16). γ-Glutamyltransferase 1 (GGT1) is an enzyme that cleaves extracellular glutathione. In GBM cells with GGT1 expression, drug inhibition or GGT1 deletion was shown to inhibit the increase in the intracellular glutathione levels induced by the cellular density and the cell viability affected by cystine deprivation. In addition, cystine deprivation led to glutathione depletion and ferroptosis in GBM cells deficient in GGT1 independent of a high cellular density. Exogenous expression of GGT1 in GBM cells deficient in GGT1 suppressed glutathione depletion and ferroptosis induced by cystine deprivation at a high density (17). Even more exciting, GPX4 expression is obviously reduced during tumor recurrence, whereas acyl-CoA synthetase long chain family member 4 (ACSL4) expression exhibits an obvious increase. Moreover, aldehyde dehydrogenase family 1, subfamily A3 (ALDH1A3) and FSP1 expression levels are also increased during recurrence, with the increase in ALDH1A3 expression being significant. It appears that exploiting the ferroptotic process may be a new therapeutic option, especially in patients with recurrent GBM (18). These findings provide new insights into the treatment of recurrent GBM and may contribute to the development of a basis for treating gliomas by targeting ferroptosis in an effective manner.

TP53 encodes p53 promoting cell cycle arrest, senescence, and apoptosis, which are three canonical functions of p53 involved in tumor suppression. This gene is the most frequently mutated tumor suppressor gene in all human cancers. The TP53 gene has been found to be activated under various conditions and to play an important role in the control of ferritin by regulating lipid, energy, and iron metabolism (19, 20). SLC7A11 is a key inhibitor of ferroptosis enhanced by p53. P62 (a stress-induced adaptor protein) inhibits ubiquitination, promotes ferroylation, and suppresses the expression of SLC7A11 in p53-mutant (MT) GBM, whereas it weakens ferroylation and increases SLC7A11 expression in p53-wild-type (WT) GBM (21). There is evidence that Rho family GTPase 1 (RND1) interacts with p53, leading to the deubiquitination of p53. In addition, overexpression of RND1 promotes the activity of the p53-SLC7A11 signaling pathway and triggers lipid peroxidation and siderosis in GBM cells (22). Reduced cystine uptake inhibits downstream GSH biosynthesis, impairing the ability of GPX4 to inhibit siderosis. In addition to downregulating SLC7A11 and impairing GSH biogenesis, p53 promotes ferroptosis through the regulation of other metabolic pathways. The rate-limiting enzyme in polyamine breakdown is arginine/arginine N1-acetyltransferase 1 (SAT1). In recent studies, we found that p53 could induce SAT1 expression, slowing the growth of xenograft tumors. As a result of SAT1 induction, arachidonate 15-lipoxygenase (ALOX15) was upregulated. The p53/SAT1/ALOX15 axis is therefore partially responsible for p53-mediated ferroptosis and tumor suppression (19, 23, 24). In addition, arachidonate 12-lipoxygenase (ALOX12) plays an important role in these functions. p53 promotes the activity of ALOX12. ALOX12 is bound by SLC7A11 and thus sequestered from its substrate, polyunsaturated fatty acids (PUFAs), including those esterified in membranes. ALOX12 is released when p53 downregulates SLC7A11, oxidizing membrane PUFAs and initiating ferroptosis (25, 26). Therefore, the p53/SLC7A11/ALOX12 axis is independent of the decrease in GSH biogenesis and GPX4 activity and is therefore a separate pathway from the p53/SLC7A11/GPX4 pathway. p53 inhibits the expression of SLC7A11 in the antiferroptosis system, and it can also inhibit the serine synthesis pathway as well as the transsulfuration pathway by inhibiting phosphoglycerate dehydrogenase and cystine synthase (CBS), respectively, thus limiting the expression of GSH (19, 27). Mouse double minute 2 homolog (MDM2) is the major E3 ubiquitin-protein ligase that degrades p53, but it is also a p53 target gene. MDM2 and its homolog MDMX can negatively regulate the tumor suppressor p53. Inhibition of MDM2 and MDMX leads to an increased FSP1 protein level, which in turn increases the coenzyme Q10 level. In addition, the MDM2-MDMX complex can alter peroxisome proliferator-activated receptor α (PPARα) activity to regulate lipid metabolism (28). In summary, several studies have been conducted on p53 and ferroptosis to date, and most support a role for p53 in ferroptosis (19, 29). Ferroptosis is promoted by the multiple roles of p53 in regulating cellular metabolism, particularly lipid, iron, ROS, and amino acid metabolism. It remains to be seen whether other metabolic target genes of p53 or metabolic processes modulated by p53 (including autophagy) contribute to p53's ferroptosis-regulating role.

In recent years, ACSL4 was found to partially activate long-chain fatty acid metabolism and immune signal transduction, indicating that it might be a regulator of ferroptosis (30). ACSL4 overexpression was found to decrease GPX4 overexpression and increase ferroptosis marker levels, such as 5-hydroxyeicosatetraene (5-HETE), 12-HETE and 15-HETE, in glioma cells (31). miR-670-3p inhibits ferroptosis in glioblastoma cells by inhibiting ACSL4. As a result, inhibition of miR-670-3p could be an alternative strategy for the treatment of glioblastoma (32). Heat shock protein 90 (Hsp90) and dynamin-related protein 1 (Drp1) actively regulate and stabilize ACSL4 expression during ferroptosis in glioma triggered by erastin. Hsp90 overexpression and Drp1 dephosphorylation change the mitochondrial morphology and

increase lipid peroxidation mediated by ACSL4 to promote ferroptosis (33).

GPX7 is another member of the glutathione peroxidase family (GPX) and participates in oxidative stress and tumorigenesis. GPX7 silencing enhances oxidative stress associated with ferroptosis in glioma cells, while GXP7 deletion sensitizes gliomas to ferroptosis induced by erastin. In addition, miR-29b was found to repress GPX7 expression directly after transcription (34).

Recent studies have confirmed that tetrahydrobiopterin (BH4), a significant cofactor for multiple enzymes, can remarkably inhibit ferroptosis. The GTP cyclohydrolase 1 (GCH1)-BH4 axis controls BH4 synthesis and reduces intracellular CoQ and ROS accumulation, thereby leading to ferroptosis inhibition. In addition, GCH1/BH4 exerts a selective inhibitory impact on nuclear receptor coactivator 4 (NCOA4)-mediated ferritin autophagy and affects iron metabolism (8, 35). This provides a new direction for ferroptosis research in glioma. Coatomer protein complex subunit zeta 1 (COPZ1) negatively regulates NCOA4 activity, and COPZ1 knockdown induces NCOA4-mediated ferritin phagocytosis (36). Downregulation of matrix-remodeling-associated protein 8 (MXRA8) increases the intracellular levels of lipid peroxidation in glioma cells, leads to NCOA4 upregulation and inhibits ferritin heavy chain 1 (FTH1). MXRA8 is significantly associated with various infiltrating immune cells, such as NK cells, macrophages, and neutrophils. MXRA8 knockdown in glioma cells attenuates M2 macrophage infiltration. Accordingly, MXRA8 facilitates glioma progression and critically affects glioma ferroptosis and the immune microenvironment (37).

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) controls the expression of genes associated with oxidative stress and can reliably maintain redox stability and resistance to oxidative stress. High levels of NRF2 lead to sensitivity in glioblastoma dependent on the expression of its proferroptotic target ATP binding cassette subfamily C member 1 (ABCC1), resulting in GSH depletion upon blockade of the Xc system by erastin (38).

With ongoing research progress, the mechanisms regulating ferroptosis are becoming increasingly clear. Further research on the ferroptosis-related gene network will provide new ideas and broad opportunities for the treatment of glioma, not only primary high-grade gliomas such as GBM but also recurrent gliomas. However, there are a few issues that require further exploration. For example, we must determine how to more effectively and precisely induce ferroptosis in glioma cells and improve the efficacy and safety of this treatment.

Ferroptosis-related compounds in glioma

Chemotherapy is one of the basic therapeutic strategies for glioma. TMZ is currently one of the first-line chemotherapeutic drugs for glioma, especially high-grade glioma. However, with the widespread use of TMZ, the median survival time of GBM patients has improved by only approximately 2.6 months. Frustratingly, as GBM patients receive long-term TMZ therapy, resistance inevitably develops, resulting in treatment efficacy dropping significantly or even disappearing. New replacement drug regimens remain to be developed (39). In the Table 1, we list the recent advances in drug-induced glioma ferroptosis for the treatment of glioma (40–51).

From the Table 1, we can see that many drugs used in the past also have a good effect on ferroptosis and that they inhibit the growth of glioma cells by targeting different ferroptotic pathways and target genes. This suggests that it is possible to find new uses for these drugs related to treatment targeting ferroptosis.

Ferroptosis-inducing nanoparticles

The use of rationally designed nanomaterials for the treatment of cancer is an emerging field that has led to tremendous medical success. The administration of ferroptosis-inducing nanoformulations with accurately tuned physicochemical properties is as an extended and feasible therapeutic strategy for tumors. We compiled recent research advances related to the induction of ferroptosis in glioma cells by nanomaterials. (Table 2) (52–56).

These different nanodrugs offer a new direction for ferroptosis-based therapy for gliomas. The different designs are very interesting. It is beneficial to generate nanoparticles encapsulated with Fe3O4 and Ce6 acoustic sensitizers, and external loading of C6 cell membranes is performed to achieve tumor cell enrichment of the material. Transient opening of the blood–brain barrier can be achieved with focused ultrasound (US). This sonodynamic therapy (SDT) combines targeting of ferroptosis in glioma cells with SDT (53). However, noninvasive destruction of the blood–brain barrier (BBB) by focused ultrasound may lead to the entry and/or exit of some harmful substances at the same time. In addition, the combination of ferroptosis-targeting therapy and immunotherapy is also a good treatment strategy (54). A membrane-modified drug delivery system was constructed by loading small interfering RNA targeting programmed cell death 1 ligand 1 (PD-L1) on Fe3O4 and externally on the BV2 cell membrane. This system promoted synergy between ferroptosis induction and immunotherapy by reducing the expression of PD-L1 *in situ* in drug-resistant GBM tissues, which was combined with the effect of ferroptosis induction by Fe2+ in Fe3O4. Some studies have also been conducted on the combination of chemotherapeutic drugs with nanomaterials (55). Gallic iron nanoparticles combined with the chemotherapeutic agent cisplatin produce a dual killing effect. The material's photothermal responsiveness and ability to be imaged by MRI provide a new way to treat GBM. Recent studies have also combined exosomes with nanomaterials to create a composite ferroptosis platform (56). A study engineered exosomes by modifying the ANG-targeting peptide on the surface of the exosomes, giving them a greater ability to cross the blood–brain barrier. Next, they constructed a nanomaterial with an Fe3O4 core, a mesoporous silicon shell and a modified anti-CD63 antibody on the surface of the mesoporous silicon shell for branching exosomes. Ultimately, the ferroptosis-related therapeutic effect of the system was achieved by encapsulating a drug or small interfering RNA targeting a critical ferroptotic pathway in the mesoporous silicon shell and exosomes.

TABLE 1 Ferroptosis-inducing drugs in GBM.

Drug name	Target	Cell line and Animals	Pathway	Impact on Ferroptosis	Ref.
Dihydroartemisinin (DHA)	GPX4	U251, U373 and HT22	PERK/ATF4/HSPA5 pathway	• Increase GPX4 expression and activity • Upregulate ATF4	(40)
Brucine	ATF3	U118, U87, U251 and A172	Trigger ATF3 upregulation and translocation into the nucleus through activation of ER stress	• Promote H2O2 accumulation through upregulation of NOX4 and SOD1 • Downregulate catalase and xCT	(41)
Pseudolaric acid B (PAB)	Transferrin receptor	Rat C6 and human SHG-44, U87 and U251 glioma cells	Upregulate transferrin receptor; p53-mediated xCT pathway	• Upregulate transferrin receptor • Promote H2O2 and lipid peroxide generation • Deplete intracellular GSH *via* the xCT pathway mediated by p53	(42)
Amentoflavone (AF)	Autophagy-dependent ferroptosis	U251 and U373 glioma cells	AMPK/mTOR pathway	• Decrease the GSH level in tumor tissue • Increase the expression of LC3B, Beclin1, ATG5, and ATG7	(43)
RSL3	GPX4	U87 and U251	NF-κB pathway	• Increase the concentration of lipid ROS and downregulate proteins related to ferroptosis (GPX4, ATF4, and SLC7A11) • Activate the NF-κB pathway	(44)
Dihydrotanshinone I	GPX4 and ACSL4	U87 and U251	GPX4 and ACSL4 pathway	• Decrease the GPX4 level and increase the ACSL4 level • Reduce the GSH/GSSG ratio	(45)
Apatinib	Nrf2	U87 and U251	VEGFR2/Nrf2/Keap1 pathway	• Decrease Nrf2 and p-VEGFR2 expression	(46)
Sevoflurane	GPX4 and ATF4	U87 and U251	ATF4-CHAC1 pathway	• Increase ROS levels and the Fe^{2+} concentration • Downregulate GPX4, upregulate transferrin and activate ATF4	(47)
Plumbagin	xCT and GPX4	U87, U251, C6 and GL261	NQO1/GPX4 pathway	• Downregulate xCT and GPX4 • Increase NQO1 activity	(48)
Curcumin analog (ALZ003)	FBXL2	U87 and A172	GPX4 pathway	• Decrease GPX4 expression • Induce lipid peroxidation and ROS accumulation	(49)
Capsaicin	ACSL4 and GPX4	U87 and U251	GPX4 and ACSL4 pathway	• Increase ACSL4, 5-HETE, MDA and TOS levels and decrease GPX4, GSH and TAS levels	(50)
Boric acid (BA)	ACSL4 and GPX4	GBM C6 cells	ACSL4/GPx4SEMA3F/NP2 pathways	• Increase ACSL4 levels and decrease GPX4 levels • Upregulate SEMA3F/NP2	(51)

TABLE 2 Ferroptosis-inducing nanoparticles in GBM.

Nanoparticle name	Target	Cell line and Animals	Impact on Ferroptosis	Ref.
(FA)/Pt-si-GPX4@IONPs	GPX4	U87MG, P3#GBM and NHA	• Increase iron (Fe^{2+} and Fe^{3+}) levels; increase H2O2 levels through the activation of lower NOX • Inhibit GPX4 expression	(52)
PIOC@CM NPs	GPX4	C6	• Increase the ROS level and deplete GSH upon ultrasonic irradiation • Inhibit GPX4 expression	(53)
Fe3O4-siPD-L1@M-BV2	GPX4 and PD-L1	GL261, HT-22 and BV2	• Induce the maturation of DCs and decrease the protein expression of PD-L1 • Inhibit GPX4 expression	(54)
cRGD/Pt + DOX@GFNPs (RPDGs)	N/A	U87 and NHA	• Deplete GSH and elevate the ROS level	(55)
Fe3O4@mSiO2 NPs	DHODH and GPX4	LN229 and A172	• Inhibit GPX4 and DHODH expression • Deplete GSH and elevate the ROS level	(56)

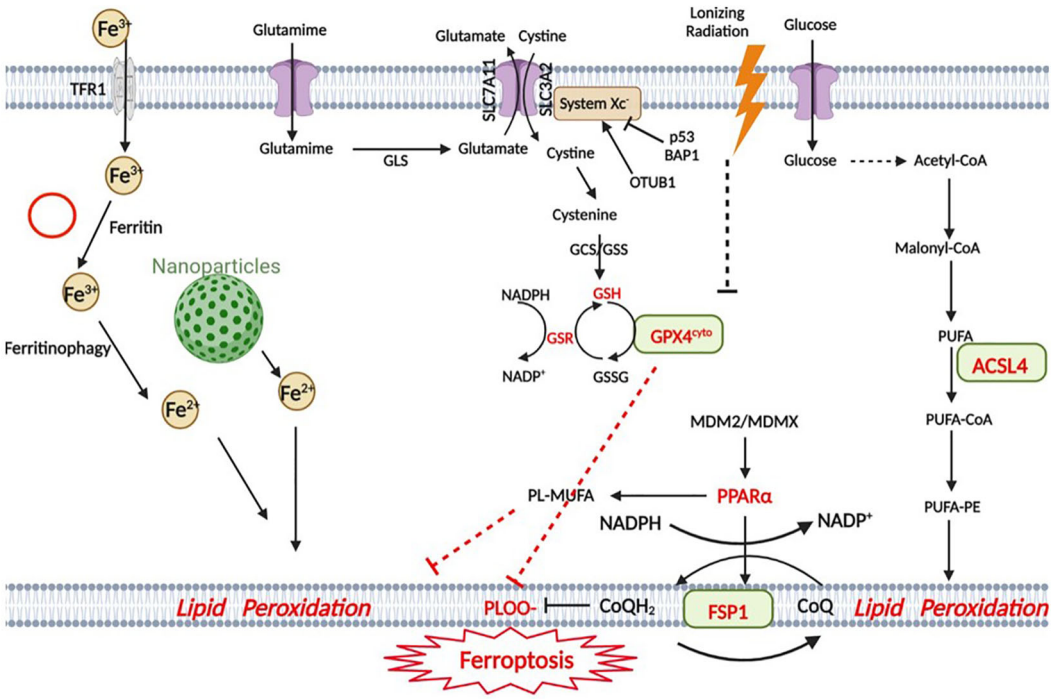

FIGURE 1
Snapshot of ferroptotic pathways. Ferroptosis in GBM is triggered by four main regulatory pathways: iron metabolism, the GPX4 pathway, the FSP1 pathway and lipid metabolism. In iron metabolism, Fe3+ is transported into the cell by TfR1 (transferrin receptor) and subsequently reduced to Fe2+, and some nanoparticles are involved in iron metabolism. The GPX4 pathway is the classic ferroptotic pathway, and the Xc- system plays an important regulatory role in this pathway. p53 is closely related to this pathway. The MDM2-MDMX complex regulates lipid metabolism by altering PPARα activity and ultimately interacts with the FSP1 protein. In lipid metabolism, AA (as well as other PUFAs) is metabolized by ACSL4 and eventually participates in lipid peroxidation. (Created with BioRender).

In general, the different designs are interesting and well designed. In conclusion, to achieve ferroptosis-targeted therapy with nanomaterials, the following steps must be achieved: blood–brain barrier penetration, tumor targeting, and ferroptosis induction. Nanomaterials with properties that enable these events may be new agents for glioma therapy in the future. However, the design of different nanomaterials is relatively complicated, such as the camouflage achieved with different cell membranes and the encapsulation of different drugs, and further improvements and validation in industrial production and human experimental safety are still needed. However, we believe that with the continuous progress of medical-industrial crossover technology, an increasing number of nanoagents will start to capture attention and provide new insights for the treatment of glioma in combination with ferroptosis-inducing agents.

Ferroptosis and TMZ resistance

TMZ is still a most effective drugs for glioma chemotherapy. Ferroptosis can considerably affect TMZ resistance in glioma, and ferroptosis resistance may serve as a mechanism of TMZ resistance in glioma. TMZ increases LDH, MDA and iron levels and decreases GSH levels in glioma cells to induce ferroptosis. In addition, ROS levels and DMT1 expression are elevated, and GPX4 expression is decreased in cells treated with temozolomide; these events are under the regulation of the Nrf2/HO-1 pathway (57).

In addition to ferroptosis inducers and xCT inhibitors, quinacrine (a compound capable of crossing the blood–brain barrier) has been found to impair autophagy but increase the sensitivity of glioblastoma stem cells (GSCs) to TMZ and trigger ferroptosis in GSCs (58). A long non-coding RNA LINC01564 promotes glioma cell resistance to TMZ by upregulating Nrf2 expression, which counteracts the effects of MAPK8 ablation on glioma cell apoptosis and ferroptosis to inhibit ferroptosis (59).

Further study of the ferroptosis mechanism in glioma TMZ resistance will contribute to new insights into the clinical reversal of glioma TMZ chemoresistance. However, details are still needed for clinical application.

Ferroptosis and immunotherapy

One of the most effective ways to treat cancer is to induce tumor cell death. Immunotherapy is considered a milestone in precision medicine. It elicits significant therapeutic responses in patients who have developed resistance to other conventional therapies (60). However, immunotherapy is not particularly effective in glioma, especially in GBM. A growing body of research suggests that the glioma immunosuppressive microenvironment (GIME) contributes to the poor efficacy of glioma immunotherapy (61–63). The rapid proliferation of gliomas creates a harsh microenvironment that is acidic with nutrient scarcity and hypoxia (64–66). As a result,

immune cells become immunosuppressive or inactive or die (67, 68), whereas glioma cells may be able to adapt to this harsh microenvironment due to their substantial plasticity (69, 70). Additionally, the blood–brain barrier prevents immune cells from migrating to tumors (71, 72). Furthermore, many suppressive cytokines secreted by gliomas (73) and suppressive immune cells suppress the antitumor activity of immune cells (74, 75). Furthermore, glioma cells can secrete a large number of cytokines to capture immune cells. Glioma cells are able to escape immune surveillance in this case (62, 76). To treat glioma successfully, it is therefore essential to remodel the immune microenvironment.

This is of great importance for improving traditional drug resistance, as ferroptosis is closely related to antitumor immunity and the immune microenvironment. Calreticulin (CRT), a soluble chaperone associated with the endoplasmic reticulum (ER), is one of the proteins that regulates the tumor microenvironment. As a result of ferroptosis, CRT is translocated onto tumor cells, where it can induce a robust immune response against the tumor (77). Neutrophils have been reported participate in apoptosis by accumulating iron-dependent lipid peroxide, which results in iron atrophy in GBMs. Intratumoral depletion of ACSL4 or overexpression of GPX4 reduces tumor necrosis and aggressiveness (78). By harnessing the cytotoxic potential of the immune system, notably that of tumor-specific cytotoxic T cells, immunotherapy is a promising strategy to treat malignancies. As a result of their antitumor effects, CD8+ T cells are a crucial component of the tumor microenvironment; they also play a key role during every stage of tumor development. Ferroptosis is a metabolic vulnerability of tumor-specific CD8+ T cells, whereas GPX4-deficient T cells display a high sensitivity to ferroptosis and are thus incapable of exerting antitumor effects. Overexpression of GPX4 inhibits ferroptosis in CD8+ T cells and simultaneously restores the production of cytotoxic cytokines *in vitro* or increases the number of tumor-infiltrating CD8+ T cells *in vivo*, thereby enhancing tumor control (79–81). In contrast, increased ferroptosis facilitates immune cell activation and infiltration but attenuates the killing of tumor cells through cytotoxic activity (82). Moreover, enhanced ferroptosis contributes to the recruitment of tumor-associated macrophages (TAMs) and M2 polarization (83). These factors contribute to the creation of an immunosuppressive immune microenvironment, which may lead to immune escape. Further studies are needed to balance the dual effects in the future.

Interestingly, ferroptosis exhibits immunogenicity *in vitro* and *in vivo*, triggering a vaccination-like effect in immunocompetent mice, in which ATP and high mobility group box 1 (HMGB1), the most typical injury-related molecular patterns associated with immunogenic cell death, can be passively released and act as immunogenic signals that affect the immunogenicity of early ferroptotic cancer cells (84). Thus, this novel discovery provides a new direction for vaccine therapy.

Clinical trials of immune checkpoint inhibitors (ICIs) have demonstrated a broad clinical impact and early success. Some but not all cases of ICI response have been associated with the expression of immune checkpoint molecules, including PD-1 ligand (PD-L1) (85). Some patients with PD-L1-positive tumors do not respond to treatment, while some patients with PD-L1-negative tumors may benefit from ICI therapy due to tumor heterogeneity (86, 87). TYRO3 inhibits anti-PD-1/PD-L1-induced ferrogenesis in tumor cells by suppressing the AKT/NRF2 axis and amplifies a favorable tumor microenvironment by reducing the ratio of M1/M2 macrophages, thus contributing to the efficacy of anti-PD-1/PD-L1 therapy (88). More effective immune checkpoints or more valid regulatory pathways need to be explored to overcome resistance in glioma patients.

Although ICI immunotherapy has been shown to have significant positive effects in some cancer patients, there is still evidence of drug resistance in many tumors, including GBM, due to tumor heterogeneity, low tumor-infiltrating T-cell (TIL) levels, loss of target antigens and off-target toxicity (89, 90). Chimeric antigen receptor T (CAR-T) cell immunotherapy targeting neoantigens that are derived from somatic mutations and expressed on only tumor cells has led to a new approach in cancer immunotherapy. CAR-T cell therapy has achieved certain success in both basic research and small-scale clinical research (91). B7-H3 (CD276) is expressed on CNS tumors, and B7-H3-specific CAR-T cells were designed for therapy in diffuse intrinsic pontine glioma (DIPG), producing exciting results (92). Frustratingly, there are no cases of relevant CAR-T cells designed to induce ferroptosis in gliomas. In addition, taking advantage of CAR-T cells to transform the immune microenvironment and enhance ferroptosis in tumor cells is a novel direction to be explored.

In conclusion, with increasing research, immunotherapy is becoming more specific and individualized, which provides opportunities for therapy in glioma. The effects of ferroptosis and immunotherapy are bidirectional, i.e., ferroptosis can further influence the effect of immunotherapy by affecting the immune microenvironment, and the effect of immunotherapy can be further enhanced by enhancing ferroptosis. However, there are still some details and limits that need to be further researched for glioma therapy.

Potential Biomarkers of Ferroptosis

With the development of sequencing technology and the creation of databases, bioinformatic analysis now plays an important role in identifying potential targets and drug effects and predicting prognosis. We compiled the recently published literature on biogenic analysis to provide potential new ideas for future research (Supplement 1) (93–105). As shown in the table, different studies identified different targets, and some of the studies explored several targets.

Although a large number of bioinformatic analysis studies currently provide us with ferroptosis-related targets in low-grade glioma (LGG) and GBM, they still have many limitations and points of controversy due to the lack of rigorous experimental support. Many of the studies relied on only computer technology. Bioinformatic analysis may be a future direction, but the validity and clinical significance of the molecules identified with this approach need to be further explored.

Ferroptosis in neuroblastoma

Neuroblastoma is one of the most prevalent extracranial tumors in children, accounting for the majority of childhood cancer-related deaths, especially in high-risk cases. High-risk NB is characterized by the appearance of this disease after the age of 18 months, the amplification of MYCN (MYCN Proto-Oncogene, BHLH Transcription Factor), or the activation of mechanisms for telomere maintenance (106, 107). The scientific community is committed to finding new strategies related to ferroptosis based on the characteristics of high-risk NB as a potential therapy for high-risk NB (108, 109).

The ferroptosis-related gene network in neuroblastoma

The characteristics of NB are significantly different from those of glioma, and the focus is also different. Genomic amplification of the oncogene MYCN acts as an essential oncogenic event in high-risk NB, occurring in approximately 50% of high-risk cases, and MYCN amplification is strongly related to a poor NB prognosis (OS < 50%). MYCN-amplified NB shows a system-dependent increase in the level of the Xc-cystine/glutamate reverse transporter protein for ROS detoxification mediated by increased transcription of this receptor (108). As a result, MYCN amplification may be a potent target in NB, and much research has focused on this aspect. MYCN induces massive lipid peroxidation when consuming cysteine, the rate-limiting amino acid in the biosynthesis of GSH, which sensitizes cells to ferroptosis. When the uptake of cysteine in MYCN-amplified pediatric NB is restricted, the use of cysteine in protein synthesis can inevitably cause GSH-induced ferroptosis and spontaneous tumor regression of low-risk NB (110). In addition, NB cells with amplified MYCN can easily undergo ferroptosis due to the upregulation of TFRC-encoded transferrin receptor 1, which reprograms cellular iron metabolism through the upregulation of TFRC (Transferrin Receptor) expression. TFRC-encoded transferrin receptor 1 is a pivotal iron transporter protein on the cell membrane, and elevated iron uptake facilitates the accumulation of unstable iron pools, resulting in elevated lipid peroxide production. TFRC overexpression in NB cells is also capable of inducing selective sensitivity to ferroptosis inhibition by GPX4 (111, 112).

Ferroportin (Fpn) is the only iron export protein that partially regulates the intracellular iron concentration. Fpn knockdown has been shown to increase the accumulation of iron-dependent lipid ROS to accelerate erastin-induced ferroptosis, and Fpn may be an appropriate target for NB treatment (113). Mitochondrial ferritin (FtMt), a kind of iron storage protein in the mitochondria, also exerts a protective effect during erastin-induced ferroptosis (114). Recent mechanistic studies have shown that downregulation of CDC27 results in obviously reduced expression of ornithine decarboxylase 1 (ODC1), a recognized direct target of MYCN. ODC1 inhibition markedly undermines the promotive effects of CDC27 on NB cells in terms of proliferation, metastasis and the sphere-forming capacity (115).

Ferroptosis-related compounds in neuroblastoma

There are currently several drugs for NB treatment, including cisplatin, etoposide, vincristine, doxorubicin, and cyclophosphamide. These drugs are the most effective inducers of apoptosis. However, this type of drug therapy creates multidrug-resistant clones, which makes eradicating this type of tumor much harder and favors tumor recurrence (116). The induction of ferroptosis through the use of drugs and agents in NB can be used to achieve better therapeutic outcomes, and this is also another hot topic in current research. Inducing ferroptosis has great potential as an anticancer therapeutic strategy in various NB tumor types, particularly in tumors with RAS mutations. The ferroptosis inducers erastin and RSL3 reduce RAS mutation-rich N2A cell (mouse neuroblastoma N2A cells) viability by increasing ROS levels and inducing cell death. In contrast, ferroptosis inhibitors lower the high ROS levels and reduce viability defects in erastin- or RSL3-treated cells. Ferritin (Fth) heavy chain 1, a ferrous oxidase that converts redox-active Fe2+ into redox-inactive Fe3+, may control the N2A-induced hypersensitivity response to ferroptosis. Overexpression of Fth reduces ROS levels and cell death and induces GPX4 expression. Additionally, NB cell lines present remarkably lower Fth expression than other cancer cell lines (117).

In addition, withaferin A (WA), a natural ferroptosis inducer in NB, activates Kelch-like ECH-associated protein 1 to activate the nuclear factor-like 2 pathway and produces increased intracellular unstable Fe(II) levels after heme oxygenase-1 is excessively activated, inducing ferroptosis or inactivating GPX4 (109). Chlorido[N,N'-disalicylidene-1,2-phenylenediamine]iron(III) complexes in NB cell lines produce lipid-based ROS and induce ferroptosis with greater efficacy than the therapeutic drug cisplatin (118).

Conclusion

Despite advances in multimodal treatment, midbody treatment and the prognosis of gliomas and neuroblastoma are discouraging. Ferroptosis is a newly identified form of programmed cell death (PCD) dependent on iron that differs from apoptosis, cell necrosis, and autophagy. It plays a very important role in GBM and NB. This article summarizes the mechanisms involved in the roles of ferroptosis in GBM and NB. To summarize, we report that (1) the GPX4 pathway remarkably affects GBM and NB and that direct or indirect inhibition of GPX4 disrupts lipid peroxidation. (2) MYCN amplification may be a potent target in NB. (3) Nanodrugs may be new therapeutic agents for treating glioma and neuroblastoma. (4) The complexity of the tumor immune microenvironment and regulatory mechanisms need to be further explored.

Therefore, future research directions should include an in-depth study of ferroptosis, identification of key targets in the ferroptotic pathway and validation of their relationships in glioma and neuroblastoma, application of ferroptosis biomarkers in clinical

prevention and monitoring, exploration of a new generation of ferroptosis-targeting systems, and finally, validation of the relationship between immunity and ferroptosis in glioma and neuroblastoma.

Author contributions

HC and BL made equal contributions to the work. HX and GL took charge of project conception and design. HC and BL proposed the research and finalized the paper. QW, ZG and BF searched and summarized the literature. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1065994/full#supplementary-material

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Glossary

CNS	Central nervous system
NB	Neuroblastoma
GBM	Glioblastoma
TMZ	Temozolomide
TTFields	Tumor-treating fields
ROS	Reactive oxygen species
GPX4	Glutathione peroxidase 4
FSP1	Ferroptosis suppressor protein 1
DHODH	Dihydroorotate dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate
GSH	Glutathione
SLC7A11	Recombinant solute carrier family 7, member 11
OTUB1	OTU domain, ubiquitin aldehyde binding protein 1
OTU	Ovarian tumor domain protease
NKAP	NF-κB activating protein
FXR1	Fragile X mental retardation syndrome-related protein 1
NKAP	NF-κB activating protein
RND1	Rho family GTPase 1
HD	, High cell density
ALDH1A3 Aldehyde dehydrogenase family 1	subfamily A3
ACSL4	Acyl-CoA synthetase long chain family 4
ALOX15	Arachidonate 15-lipoxygenase
PUFAs	Polyunsaturated fatty acids
PPARα	Peroxisome proliferator-activated receptor α
Hsp90	Heat shock protein 90
Drp1	Dynamin-related protein 1
PPAR	Peroxisome proliferator-activated receptor
BH4	Tetrahydrobiopterin
GCH1	GTP Cyclohydrolase1
NCOA4	Nuclear receptor coactivator 4
COPZ1	Coatomer protein complex subunit zeta 1
MXRA8	Matrix remodeling-associated protein 8
FTH1	Ferritin Heavy Chain 1
Nrf2	Nuclear factor erythroid 2-related factor 2
ATF4	Activating transcription factor 4
NOX4	NADPH oxidase 4
SOD1	Superoxide dismutase 1
PAB	Pseudolaric acid B
SOD1	Superoxide dismutase 1
ATG5	Autophagy related 5
VEGFR	Vascular endothelial growth factor receptor
NQO1	NAD(P)H quinone dehydrogenase 1
MDA	malondialdehyde
SDT	Sonodynamic therapy
BBB	Blood-brain barrier
GIME	Glioma immunosuppressive microenvironment
TAM	Tumor-associated macrophages
ICIs	Immune checkpoint inhibitors
TILs	Tumor-infiltrating T cells
CAR-T	Chimeric antigen receptor T
DIPG	Diffuse intrinsic pontine glioma
AF	Amentoflavone
NOX	NADPH oxidase
PFI	Progression-free interval
LGG	Low-grade glioma
OS	Overall survival
Fpn	Ferroportin
FtMt	Mitochondrial ferritin
WA	Withaferin A
PCD	Programmed cell death

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Ferroptosis and PPAR-gamma in the limelight of brain tumors and edema

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Human malignant brain tumors such as gliomas are devastating due to the induction of cerebral edema and neurodegeneration. A major contributor to glioma-induced neurodegeneration has been identified as glutamate. Glutamate promotes cell growth and proliferation in variety of tumor types. Intriguently, glutamate is also an excitatory neurotransmitter and evokes neuronal cell death at high concentrations. Even though glutamate signaling at the receptor and its downstream effectors has been extensively investigated at the molecular level, there has been little insight into how glutamate enters the tumor microenvironment and impacts on metabolic equilibration until recently. Surprisingly, the 12 transmembrane spanning tranporter xCT (SLC7A11) appeared to be a major player in this process, mediating glutamate secretion and ferroptosis. Also, PPARγ is associated with ferroptosis in neurodegeneration, thereby destroying neurons and causing brain swelling. Although these data are intriguing, tumor-associated edema has so far been quoted as of vasogenic origin. Hence, glutamate and PPARγ biology in the process of glioma-induced brain swelling is conceptually challenging. By inhibiting xCT transporter or AMPA receptors in vivo, brain swelling and peritumoral alterations can be mitigated. This review sheds light on the role of glutamate in brain tumors presenting the conceptual challenge that xCT disruption causes ferroptosis activation in malignant brain tumors. Thus, interfering with glutamate takes center stage in forming the basis of a metabolic equilibration approach.

KEYWORDS

glioblastoma, glutamate, peritumoral edema, SLC7A11, ferroptosis

Introduction

Malignant primary brain tumors account for approximately 30% of all primary brain tumors diagnosed annually in the United States (1). Gliomas are the most common of these and represent one of the leading causes of morbidity and mortality in neurological practice (2). Glioblastomas (also referred to CNS WHO grade 4 glioma) with their median survival time of less than 15 months are considered to be the most malignant brain tumor entity (3). To date, conventional treatment includes surgical resection of the bulk tumor mass, followed by radiotherapy and alkylating agent-based chemotherapy. Even with these advanced therapies developed over the last two decades, survival times have only been extended a few months, and a cure remains elusive. In addition, certain biological properties of glioma make complete surgical resection nearly impossible and radiochemotherapy less effective or ineffective in treating residual glioma cells (4–6). At the cellular level, treatment resistance can be explained by the intra- and intertumoral heterogeneity observed in glioblastomas. Based on genomic and transcriptomic analyses of bulk tumors, glioblastomas can be categorized into four molecular subtypes, namely proneural, neural, classical, and mesenchymal (7, 8). However, a follow-up study revealed that all molecular subtypes coexist within a brain tumor heterogeneously (5). The clinical prognosis remained unaffected except for individuals belonging to the proneural subtype. In glioblastomas with high proportions of alternative subtypes, patients with dominant proneural subtype had poorer survival outcomes. In addition, the existence of glioblastoma stem cells (GSC) also contributes to resistance to adjuvant therapy and promote tumor recurrence (9).

Untreated cases of glioblastoma are commonly associated with perifocal edema resulting from blood-brain barrier disruption. These events can lead to devastating neurological sequelae, such as hemiparesis or cognitive decline (10). Whether the tumor-related edema zone should be resected presents a controversial issue until now, but according to a recent study, surgical resection of the peritumoral edema zone has been found not to carry a greater risk of postoperative complications. It even delayed tumor recurrence than simply removing the contrast-enhancing tumor alone (11).

There has been an association between glioblastoma-induced edema and alterations of tumor-associated genes inside the edema region, in terms of upregulations of e.g. c-myc, ERK, or AKT, and downregulation of tumor-suppressors such as p53 (11). Additional bioinformatic analysis of 'The Cancer Genome Atlas' (TCGA) data revealed that occurrence of tumor-related brain edema affects inflammatory gene expression, e.g. by increasing IL-10 levels (12), which in turn was shown to promote glioma cell invasion (13, 14). As a result, the threshold for T cell activation can be raised, and their antitumor activity can be directly suppressed (15). Aside from that, HMOX1-positive myeloid cells are also capable of secreting IL-10, causing T cell dysfunction and immune evasion (16). These recent data strongly suggest that these edema-enriched genes are crucial for gliomagenesis and tumor angiogenesis. The presence of these gene alterations in the edema region is in line with the observation that this area represents the biologically active part of the glioma microenvironment (6).

Brain tissues in the peritumoral area show evidence of oxidative stress, manifested through increased production of reactive oxygen species (ROS) and decreased antioxidant-enzyme defenses such as catalase, glutathione peroxidase, and superoxide dismutase (17, 18). The oxidative stress in the glioma microenvironment is closely related to iron homeostasis, since the balanced amount of intracellular iron governs the oxidation state of phospolipids (19, 20) (Figure 1). An iron overload induces lipid peroxidation and subsequent cell death (21). In a recent study, it was found that GSC can absorb iron from the glioma microenvironment more effectively by upregulating their expression levels of ferritin and transferrin receptor (TfR) 1 (22). The proliferation of glioma cells is facilitated by such TfR overexpression-mediated oxidant accumulation, which inactivates cell cycle regulators and promotes S-phase entry (23).

In addition to the direct iron-related mechanism of tumor progression, another relevant upstream mechanism for the development of edema or glutamate-induced excitotoxicity is represented by the glutamate-cystine antiporter system x_c^- (24, 25) (Figure 1). In glioblastoma cells, increasing cystine import through system x_c^- drives the production of antioxidant glutathione, whereas the inhibition of cystine import, e.g. by application of system x_c^- inhibitors, decreases the antioxidative capabilities of (brain) tumor cells (26). Several molecular aspects modulate these antioxidative properties, including the expression of mitoferrin-1, Nrf2, and catalase, among others (27–29). Thus, the tumor itself expresses a variety of molecules that serve to decrease the oxidative stress in its tissue, promoting growth. Interestingly, blocking system x_c^- allows oxidative agents to accumulate intracellularly, which leads to cell death in the form of ferroptosis (30) (Figure 1). Ferroptosis inhibits malignant brain tumors and tumor-related edema by inducing oxidative stress in tumor cells and through antagonism of the treatment resistance that is strongly displayed by malignant gliomas (31).

In recent years, different pathways have been identified that promote an understanding of the biological actions of malignant tumors and their related cerebral edema. The benefits of current treatment options are still modest, so the investigation of newly identified, tumor- and edema-specific targets could translate quickly into clinical applications, ultimately improving survival rates and quality of life for patients. In this review, we outline recent advances in the treatment of tumor-associated cerebral edema and discuss the overlap between ferroptosis induction in the tumor and the role of ferroptosis adjacent to the edema site.

Origins and relevance of tumor-induced cerebral edema

Aside from rapid growth and diffuse brain infiltration, peritumoral cerebral edema represents a feared hallmark of high-grade gliomas (HGGs, CNS WHO grades 3 and 4) (6, 32). This

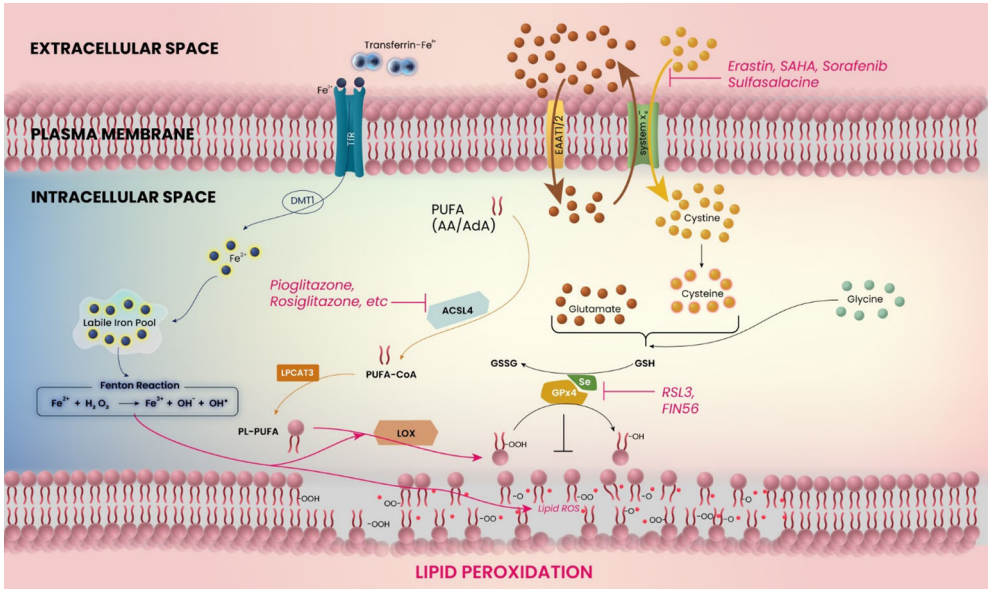

FIGURE 1
Schematic model for the mechanism of ferroptosis in glioblastoma. The figure shows the related molecules and pathways of ferroptosis. Ferroptosis is induced by inhibition of system x_c^- or glutathione peroxidase 4 (GPX4), or accumulation of iron (Fe^{2+}) ions. The catabolic enzyme acyl-CoA synthetase long-chain family member 4 (ACSL4) must be expressed.

process involves an increase in fluid content in the surrounding parenchyma. As a result, the volume and, correspondingly, the clinical reflection of the mass-effect of the space-occupying intracranial process rises significantly (33, 34). According to their cause, there are four known types of cerebral edema, namely vasogenic, cytotoxic, interstitial, and osmotic edema (6, 32, 35–37). It cannot be denied that the vasogenic component is the major player in the progression of HGGs. In most cases, disruption of the blood-brain barrier and increased vascular permeability are responsible for the described fluid accumulation (38). This results in impaired oxygen transport, which increases the symptoms elicited by the edema (39). The disturbed fluid discharge increases intracranial pressure (40). This can be compensated in the first line by reduction of the intracerebral volume of blood and liquor (41), but soon this reserve is exhausted and the intracranial pressure rapidly rises. In the final stage, the swelling brain compresses essential brain areas as well as the venous outflow from the brain and thus results in an episode of ischemia that consequently leads to brain death. In fact, the malignancy of primary brain tumors correlates highly with the development of peritumoral edema (6, 17). The modified tumor microenvironment, known as perifocal or perilesional brain swelling, has been traditionally believed to originate primarily from vasogenic mechanisms and is also associated with the region of tumor-related angiogenesis (6, 42). Morphologically, tumor vessels show characteristic features such as altered capillary endothelial with fenestration of hyperplasia (glomeruloid tufts), irregular basal membranes and extravascular spaces, and also convoluted and sinusoidal abnormalities (43). As a consequence of this altered vascular architecture, gliomas accumulate extracellular water in the peritumoral zone while losing blood-brain barrier integrity and permeability selectivity (44). A crucial cellular component found within the tumor microenvironment is the astrocytic glial cell. Astroglial changes such as altered cytoskeletal arrangements, cytoplasmatic processes and filopodia, and altered expression of water channels (i.e. aquaporin-4) were indentified in perifocal areas (45, 46). These astroglial transformations may reflect a desperate attempt to restore the extracellular balance of fluids. In addition to astrocytes, microglia are also present in the tumor microenvironment and may influence the survival of patients (47). Recently, tumor-associated microglia/macrophages have been found to hold an important role in shaping the tumor microenvironment in mice (48). These data are particularly interesting because up to 50% of microglia/macrophages are estimated to constitute the tumor (49). Furthermore, beyond the bulk tumor mass, an analysis of the peritumoral zone has shown that activated microglia accumulate at the tumor and therby constitute a major component of the perifocal area that contributes to tumor-related edema (45). In total, the mechanisms of this perilesional edema and the cellular and molecular composition of the microenvironment are only partially understood, and further studies are required to assess how a cell-type specific intervention may elevate patients' symptoms.

Based on the mostly vasogenic nature of peritumoral edema (44), it is relevant to identify the various angiogenic factors secreted by HGGs. The most prominent candidates associated with tumor angiogenesis are vascular endothelial growth factor (VEGF) and angiopoietin, as both can stimulate endothelial and perivascular progenitor cell growth as well as tube formation (50). Several mechanisms in which VEGF is involved, lead to an increase in the membrane permeability and thus are responsible for edema formation. Secretion of nitrogen oxide and phosphorylation of occludin are mainly driven by VEGF and result in relaxation of

tight junctions (51). Another factor is the hydrostatic edema. This type of edema induces a shift in the liquor drainage due to the bulk tumor mass, creating a subsequent hydrostatic pressure gradient between the ventricle and brain parenchyma. As a result, fluid is forced into the brain tissue (52–54).

Possibilities to broaden the therapeutic toolbox to treat perifocal edema

In clinical settings, the first choice remains the administration of synthetic glucocorticoids (i. e. dexamethasone) and, in rare cases, osmotically active compounds like mannitol (55). These clinical procedures usually reduce the edema rapidly. The underlying mechanisms consist of blocking nitrogen oxide synthase (NOS), accelerating the depletion of bradykinin, and reducing expression of VEGF by tumor cells (56, 57). These procedures stabilize the tight junctions and therefore reduce the efflux of capillary fluid into the brain parenchyma. However, there is also evidence that glucocorticoids may even accelerate the process of tumor progression (58). For instance, glucocorticoids have strong glycolytic effects and enhance fructose 2,6-bisphosphate production - the most potent stimulator of phosphofructokinase 1 - as well as lactate secretion, which may counteract the further action of anticancer drugs (59). Furthermore, frequent adverse reactions become increasingly relevant in long-term treatment with glucocorticoids, and can cause immuno-suppression, reduction in quality of life, and limiting treatment modalities (60).

With this wide variety of undesirable side effects, alternative treatment targets need to be indentified and utilized. Since it has been found that glutamate influx may contribute to cell-swelling (61, 62), therapeutic targeting of glutamate homeostastis-related proteins as system x_c^- and EAAT1/2 may be potentially beneficial in treatment of tumor-related cerebral edema. In glioma, the decrease in EAAT2 (also known as solute carrier family 1 member 2 (SLC1A2) or glutamate transporter 1 (GLT-1)) correlates with tumor malignancy (63), making the potential involvement of EAAT in tumor-associated diseases much more relevant. For breast and colon cancers beneficial effects of EAAT2 upregulation have already been reported, and the antineoplastic effects have already been well studied (64, 65). Contrary to the system x_c^-, EAAT2 is poorly expressed by glioblastoma cells (66, 67). It regulates the entry of glutamate into these cells (Figure 1), ultimately decreasing extracellular clearance. Under normal conditions, EAAT2 is predominantly expressed in astrocytes, although detection is also possible in oligodendrocytes and neurons (68, 69). EAATs, in general, are membrane-bound pumps. Up to 90% of extracellular glutamate uptake can be accounted for by these transporters (70, 71), making them the single most important mechanism for a glutamate equilibrium. Upregulation of these receptors, in turn, leads to a substantial shift of extracellular to intracellular glutamate. Their expression can be modified by multiple substances and levels, including signaling pathways through PI3K and NF-κB, as well as EGF, PPARγ and pituitary adenylate cyclase-activating polypeptide (67, 71, 72).

When EAATs get activated, the sodium ion-driven uptake of glutamate also leads to the uptake of water, which may contribute to cytotoxic edema (54). However, observations from liver failure-induced cerebral edema suggest a decrease of EAAT2 accompanied by a concurrent increase in AQP4 expression (73, 74). These actions can elevate extracellular glutamate levels, until reaching neurotoxic amounts of glutamate leading to the activation of NMDA receptors associated with neurotoxicity (75, 76). Mechanistically, in terms of tumor-related edema, its formation can result from a rise in glutamate, as well as leukotrienes and vascular endothelial growth factors, which increase the permeability of the brain vessels surrounding the tumor, that leads to the influx of protein-rich influx in the brains' white matter (77). In cases of the other forms of edema such as cellular or interstitial edema, the pathophysiological mechanism differs and may involve other consequences, e.g. increased sodium influx.

When AMPA receptor involvement in cerebral edema, tumor unrelated and evoked e.g. by traumatic brain injury, was assessed, it was found that blocking AMPA receptor activity attenuates edema (78–80). Interestingly, in studies using rodent ischemic models, AMPA-R and NMDA-R antagonistic actions have demonstrated promising results. However, despite these positive preclinical findings, clinical trials using AMPA receptor and NMDA receptor antagonists have been unsuccessful (81, 82). Nonetheless, the neuronal microenvironment near the tumor plays an important role for the tumor progression. In breast and prostate carcinoma cells, it was shown that tumor behavior is responsive to modulation of neurotransmitter activity (83), indicating the importance of chemical released by neuronal tissue. In the specific case of brain cancer, it is important to understand how malignant tumor cells and the neuronal cells in the brain communicate with each other in a reciprocal manner. Previous efforts have been made to address these questions. A soluble form of neuroligin-3, a synaptic protein, was able to activate PI3K-mTOR in high-grade glioma, and increased neuroligin-3 expression was negatively associated with patient survival (84). In addition to the dependency on such molecules, it was shown that glioma membrane depolarization drove tumor proliferation (85). In the same study, it was found that glioma provide electrical feedback to neurons in the circuit, thereby regulating their own, activity-driven growth. These data strongly illustrate how the neuronal compartment in the brain in the near vicinity of the tumor is involved in the tumor's progression.

xCT, microenvironment and tumor-associated cytotoxicity

As another glutamate level-regulating protein, the amino acid transporter xCT (system x_c^-, SLC7A11) is expressed in various cancers including high-grade gliomas (HGGs). Its specific modulation of tumor microenvironment is revealed to be a hallmark of primary malignant brain tumors. In particular, this

modulation influences the tumor-induced neurotoxicity and perifocal edema (32). De- and methylation processes as well as imbalances between the histone deacetylases and acetylases play a critical role in tumor development, whereas the link between these epigenetic regulatory mechanisms and the malignant glioma progression is the transporter system (86).

The inhibition of the cystine/glutamate antiporter xCT leads to a decrease in neurodegeneration, perifocal edema and prolonged survival *in vivo*. Furthermore, this supports the hypothesis that the formation of edema is, to some extent, influenced by the death of peritumoral cells (24) (Figure 1). Inhibition of xCT primarily disrupts its neurodegenerative and microenvironment-toxifying activity (87). A decrease in glioma cell proliferation was associated with higher concentrations of xCT inhibitors in an L-cystine-dependent manner (24, 88, 89). Glioblastoma cells derived from human patients have been shown to be susceptible to the cytotoxic effects of xCT-inhibitors (90). Additionally, cytotoxic effects of substances such as temozolomide are augmented during sulfasalazine-mediated xCT inhibition (91), and in another study it was found that the glioma-toxic effect of sulfasalazine alone was detectable at concentrations above 200 μM (89). HGGs use xCT to increase glutamate levels and manipulate neuronal glutamate signaling for their own growth advantage, leading to chemotherapeutic resistance and a toxic tumor microenvironment for neurons. Reactive oxygen species (ROS) activate transient receptor potential (TRP) channels with the result of a potentiated glutamate release *via* the TRP-channels. The system x_c^- modulates the tumor microenvironment with impact on host cells and the cancer stem cells (92).

Glioma-associated microglia/macrophages (GAMs) are an important cell population component of glioblastoma microenvironment. The increasing glutamate levels cause transcriptional changes in GAMs. These cells respond to extracellular glutamate excess in the glioblastoma microenvironment with increasing expressions of genes related to glutamate transport and metabolism such as GRIA2 (GluA2 or AMPA receptor 2), SLC1A2 (EAAT2), SLC1A3 (EAAT1), decreasing expression of xCT and increasing expression of GLUL (glutamine synthetase) (93).

Regarding the modulation of chemotherapeutic therapy of HGGs, many promising inhibitors and activators of xCT have been detected so far. The key problem of a specific modulation of xCT in gliomas is the ubiquitous expression of xCT also in vital tissue cells which makes it difficult to specifically target expression of xCT in tumor cells, especially because of its essential role in physiology of the CNS (94, 95).

The main cytotoxic tool in countering HGGs is the autophagy-inducing standard chemotherapeutic agent temozolomide. Interestingly, silencing xCT expression in human glioma cells is associated with a higher vulnerability towards temozolomide. However, gliomas with a high xCT expression are more vulnerable towards combinatorial treatment with temozolomide and erastin, a ferroptosis inducing agent (87).

The HDAC-inhibitor SAHA achieves equilibrium with the xCT transporter and is specific to malignant brain tumors, while leaving physiological xCT levels in healthy brain parenchyma unaffected. Consequently, the reduction of extracellular glutamate levels leads to a decrease in neuronal cell death and normalization of the tumor microenvironment. Reducing neurodegeneration results in less damage to the surrounding healthy brain parenchyma (94).

Activating transcription factor 4 (ATF4) is a critical oxido-metabolic regulator that contributes to the malignancy of HGGs by promoting cell proliferation, migration and tumor angiogenesis through the modification of the microenvironment in a potentially harmful way. ATF4 activation is associated with an elevation of xCT levels. The ATF4-induced proliferation is extenuated by xCT inhibition and ferroptosis inducers such as sorafenib and erastin. Moreover, erastin is able to reduce the ATF4-induced angiogenesis. ATF4 and xCT are tightly connected *via* a xCT-dependent configuring of the vascular architecture (31). Interestingly, ATF4 suppression comes along with an increased temozolomide susceptibility and autophagy in HGGs leading to a migratory stop after temozolomide application. ATF4 activation comes along with a xCT elevation resulting in an elevated temozolomide resistance. Thus, ATF4 can be regarded as a chemo-resistance gene in gliomas being determined by its transcriptional target xCT. Inactivation of ATF4 might be a key strategy to eliminate chemo-resistance in human gliomas (96). These findings open the door to new strategies of pharmacological interventions on tumor-associated genes by epigenetic priming (86).

Involvment of ferroptosis in brain tumor treatment and the implications for associated edema

Ferroptosis is a recently discovered mechanism for cell death, characterized by the accumulation of iron ions and lipid peroxidation during cell death (30, 97) (Figure 1). This can ultimately be caused by ROS accumulation through inhibition of glutathione (GSH) or GSH-dependent selenoprotein glutathione peroxidase 4 (GPx4) (6, 30), with the latter being expressed most abundantly in testes and brains (98). This accumulation of iron ions can then lead to a continuous cycle of lipid oxidation and further iron accumulation. Interestingly, inhibitors of apoptosis, necrosis and autophagy cannot reverse this type of cell death (30).

Blocking system x_c^-, which displays a strong expression in malignant tumors such as glioblastoma, induces ferroptosis in the tumor cells. As this poses the question if system x_c^- may be a potential target during chemotherapeutic intervention, it also of particular interest to examine whether ferroptosis is involved in the tumor-associated edema. Recently, it was found that the standard treatment for edema, dexamethasone, sensitizes cells to ferroptosis (99), which would allow to target the tumor and its edema simulatenously by potential medication regimens. *In vivo* treatment with the ferroptosis inducer sulfasalazine in mice with glioma also reduces the tumor-associated edema (89). In line with this finding, system x_c^- inhibition by RNA-mediated silencing improves tumor associated-edema in glioma (24). In contrast to this improvement, rats after subarachnoid hemorrhage developed edema that improved after the inhibition of ferroptosis (100),

instead of its induction. Thus, the underlying pathology that leads to edema seems to be important for the interventions to be taken in edema, and it may be possible that tumor-related edema appear to be more reliant on the ferroptosis status at the tumor site.

Therefore, it would be valuable to assess other ferroptosis-inducing drugs regarding their ability to modify tumor-related edema. At the moment, four distinct classes of ferroptosis-inducing drugs play a major role. Most important in treatment are class 1 and class 2 inducers (95, 101). However, class 3 (GSH depletion compounds, e. g. acetaminophen) and class 4 (lipid peroxidation inducers, e. g. $FINO_2$) also play significant roles.

Class 1 inducers, such as erastin, primarily target the aforementioned transporter system x_c^-, ultimately depleting the cells of cystine and glutathione. Interestingly, it has been reported, that especially some glioma cells, that were therapy-resistent to current treatments, are characterized by increased synthesis of polyunsaturated fats (102) – a dependency that can be readily exploited by GPx4 inhibitors (103).

Class 2 inducers, such as Ras-selective lethal 3 (RSL3) and ferroptosis inducer 56 (FIN56), directly target and inhibit GPx4 through/via downstream process (101). This presents another therapeutic angle, as some trials with knockout human cancer cells have shown a partial resistance to erastin, but not RSL3 (104).

However, serious side effects have been reported involving the induction of ferroptosis in cardiomyocytes (105, 106). A probable explanation for this could be the counteraction of the vital, protective, and antioxidant role that GPx4 plays in many cell types (17, 107). Therefore, therapeutic use should be exercised with caution.

As a side note, a number of compounds can protect against ferroptosis-induced tissue damage. These include thiazolidinediones (TZDs), which are a class of PPARγ agonists (108, 109), as well as LOX-inhibitors, DPP4-mediated lipid peroxidation suppression and iron chelators (97).

Ultimately, ferroptosis-inducing agents such as erastin and RSL3 could potentially serve as extension to standard treatments, especially in cancers that seem resistant to current drug regiments. Potential side effects, however, should be taken into consideration.

Role of PPARγ in brain tumors and cerebral edema

Glioma cells are represented by various cellular and molecular alterations that may contribute to their pathological effects and may also represent therapeutical targets. Amongst those altered signaling pathways, glioma cells express lower endogenous levels of peroxisome proliferator-activated receptor gamma (PPARγ) compared to healthy brain tissue (67, 110). PPARγ is a ligand-activated transcription factor that plays an important role in differentiation at a cellular level, as well as glucose and lipid homeostasis. It has been shown to inhibit cellular proliferation and angiogenesis, while promoting differentiation and inducing apoptosis through multiple pathways (111). One of the main obstacles for drugs designated for intracranial effect is the crossing of the blood-brain barrier. This can be easily overcome by PPARγ agonists, as demonstrated for pioglitazone in human glioma xenograft model (112). As shown in Table 1, a wide variety of antineoplastic efficacy could be seen with PPARγ agonists.

While some effects in cerebral neoplasms and edema have been reported, the exact relation is not yet fully understood. To illustrate, rosiglitazone has been shown to cause G2/M arrest and apoptosis in certain glioblastoma cell lines (113), furthermore a delay in the age of onset of seizures has been demonstrated in genetically susceptible mice, when utilizing pioglitazone (114). In a clinical trial, an extended median survival of 19 months has also been reported for diabetic patients with glioblastoma who received additional treatment with PPARγ agonists, compared to 6 months of extended survival for patients receiving the standard treatment (115). However, it should be noted that the observed result indicating longer survival for the PPARγ agonist group was not considered statistically significant due to the small sample size used in the study. Currently, classic PPARγ agonists such as pioglitazone are FDA approved primarily as oral antidiabetics but the possibility of generating tissue-specific drugs has been validated (116). At the moment, treatment with first-generation TZDs poses many obstacles, as they hold a wide variety of side effects, limiting their use.

TABLE 1 Assessment of PPAR gamma agonists for their oncological value.

Author	Year	Agonist	Cells	Mechanism induced	Mechanism hindered
Grommes et al.	2013	Pioglitazone	LN-229		Tumor volume
Pestereva et al.	2012	Ciglitazone	T98G neurospheres, primary GSC	NANOG	SOX2
Wang et al.	2012	Rosiglitazone	U87MG, U251 MG		TGF-ß, P-SMAD3, SMAD3/SMAD4 complex
Wan et al.	2011	Pioglitazone	U87MG, U251 MG, T98G		ß-catenin
Lee et al.	2011	Pioglitazone	T98G		AKT, MMP
Charawe et al.	2008	Ciglitazone	U87, T98G neurospheres		EGF, Tyk2-STAT3
Coras et al.	2007	Troglitazone	SMA-560, U87MG, F98		TGF-ß, migration (organotopic model)
Spagnolo et al.	2007	Pioglitazone	GL261	Superoxide in glioma cells	
Grommes et al.	2006	Pioglitazone	C6, A172, U87MG		Ki67, tumour volume
Grommes et al.	2005	GW7845	C6, A172, U87MG		Ki67, tumour migration

Recent studies have linked PPARγ to ferroptosis. For instance, dendritic cells in the immune system were shown to require PPARγ to undergo ferroptosis in response to RSL3, a finding that has an impact on antitumor immunity (117). The impact of PPARγ is not limited to cancer, since also other pathologies such as diabetic retinopathy were shown to be influenced in their ferroptotic behavior by PPARγ (118). In neuronal tissue, PPARγ-mediated ferroptosis was found to be relevant in the context of traumatic brain injury (119) and in intracerebral hemorrhage (120).

In addition to PPARγ's role in tumor tissue and ferroptosis that ocurrs within, its importance also expands to edema. For example, in the context of traumatic brain injury PPARγ-modulating substances like pioglitazone and rosiglitazone led to decreases in edema in rodent models (121). Their use is, however, associated with peripheral edema (122). Though rosiglitazone, for example, has been shown to decrease edema following a hemorrhagic event (123), further studies are required to investigate PPARγ specifically in the context of cerebral edema as a result of an adjacent glioma.

According to studies in glioma cell lines and glioma stem cells, PPARγ agonist pioglitazone enhances the functional expression of EAAT2 (124). It suggests that glioblastoma cells at the peritumoral zone may be able to improve glutamate transport, which may lead to alleviation of tumor-related edema. PPARγ agonists have additional effects associated with lipid metabolism and ferroptosis (Figure 1). Polyunsaturated fatty acids (PUFAs) play a crucial role in the process of ferroptosis. To induce lipid peroxidation, the PUFA catabolic enzyme acyl-CoA synthetase long-chain family member 4 (ACSL4) must be expressed (109). This enzyme is essential for ferroptosis and responsible for esterifying CoA into PUFAs such as arachidonic acid (AA) and adrenic acid (AdA). By forming Acyl-CoA, PUFAs are activated for fatty acid oxidation. Utilizing pharmaceutical inhibition of ACSL4 with thiazolidinedione ligands, a class of PPARγ agonists such as pioglitazone and rosiglitazone, has demonstrated that PPARγ agonists can suppress ferroptosis sensitivity (109). Based on our current understanding, there is a notable absence of comprehensive and in-depth studies exploring the molecular mechanisms underlying the regulation of PPARγ and ferroptosis in glioblastoma. Further investigations on the role of PPARγ in glioma microenvironment and ferroptosis are required.

Conclusion

In this review, we propose a fresh view on metabolic homeostasis in context of glioma-induced neurodegeneration and peritumoral edema. We discussed the glutamate signaling cascade and glutamate-EAAT-xCT axis with a special focus on ferroptosis. Brain tumors display a deranged microenvironment with metabolic changes. We discussed xCT and PPARγ as therapeutic targets addressing brain swelling and metabolic homeostasis. We provide supporting evidence for the conceptual challenge that xCT disruption causes ferroptosis activation in malignant brain tumors. We raised the potential involvement of PPARγ agonists in the context of glioblastoma and tumor-related edema.

Author contributions

EY and MD designed the concept, structure and content of the review. EY and MD wrote the manuscript with input from SS, AH, DC, JKD, IM, LZ, H-HS, and NS. EY, SS and MD prepared all tables and figures. All authors listed provided critical revisions to the article. All authors contributed to the article and agreed to submit the manuscript in its current state.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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