TUMOR-ASSOCIATED ANTIGENS AND THEIR AUTOANTIBODIES: FROM DISCOVERING TO CLINICAL UTILIZATION

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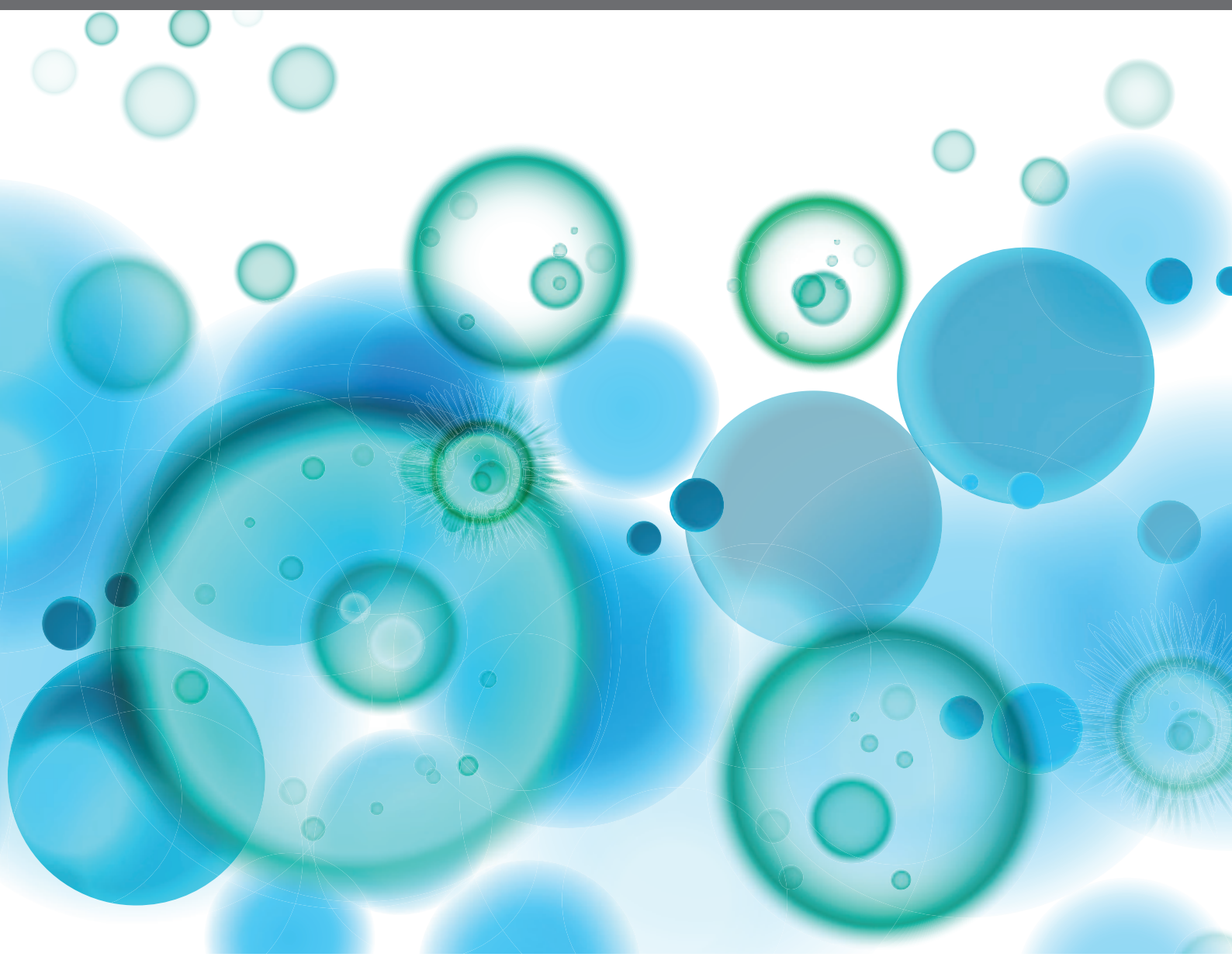

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TUMOR-ASSOCIATED ANTIGENS AND THEIR AUTOANTIBODIES: FROM DISCOVERING TO CLINICAL UTILIZATION

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Editorial: Tumor-associated antigens and their autoantibodies, from discovering to clinical utilization

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cancer, tumor-associated antigen, autoantibody, biomarker, immunodiagnosis

Editorial on the Research Topic
Tumor-Associated Antigens and Their Autoantibodies: From Discovering to Clinical Utilization

The detection of autoantibodies in human sera can be used as an important indicator for the diagnosis of autoimmune diseases and has been widely used in clinical research and clinical practice. Researchers in cancer field have found that various types of autoantibodies against cell self-antigens also exist in the sera from cancer patients (1, 2). These antigens related to tumorigenesis are called tumor-associated antigen (TAA). Through the study of the molecular structure and function of some human autoantigens, it is found that there are a wide variety of cellular autoantigens that induce autoantibody responses. Therefore, these cancer-associated autoantibodies might be used as biomarkers for immunodiagnosis of certain type of cancer, or as a tool to monitor therapy as well as as an indicator to predict disease prognosis (3–9). Different approach and technology, including serological analysis of recombination cDNA expression libraries (SEREX) and proteomics, have been extensively used in the identification of TAAs in cancer (10, 11). A bunch of proteins in cancer and pre-cancer conditions were identified and characterized (12–17). More recently, we have noticed that some of other approaches were also used to identify TAAs and detect anti-TAAs autoantibodies, such as whole genome derived peptide arrays and proteome microarray technology (18–20).

In recent two decades, major developments have been made in the field of research on TAAs and anti-TAAs autoantibodies, and many studies have demonstrated that serum anti-TAAs autoantibodies can be used as effective biomarkers for cancer immunodiagnosis (1–9). The diagnostic value, clinical utility, and pathogenic significance of TAAs or anti-TAAs autoantibodies are the focus of ongoing research.

This special issue mainly focus on the recent studies associated with the idea and possibility that identification of TAAs and their anti-TAAs autoantibodies can be useful for cancer immunodiagnosis and cancer immunotherapies.

In this Research Topic, our guest editors have invited investigators to contribute original research articles as well as review articles which were mainly related to TAAs and anti-TAAs autoantibodies in cancer immunodiagnosis and cancer immunotherapies, and assembled the current Research Topicfor updating the recent advances in this field. In this special issue, we have received 29 submitted manuscripts, and 18 manuscripts with 139 authors have been accepted for publication. For example, a paper of Jiang et al. used a protein array technology and identify a panel of anti-TAAs autoantibodies in the early detection of lung cancer; a paper of Liu et al. used neoantigen reactive T cells combined with tomotherapy to treat a patient with advanced HCC, who reached a long time progress free survival; a paper of Qiu et al. has evaluated the diagnostic value of autoantibody against PDLIM1 for improving the detection of ovarian cancer; a study from Wang et al. has tested and validated anti-14-3-3 zeta autoantibody might be a biomarker for predicting hepatocarcinogenesis; a study from Lu et al. suggests that the combined application of PD-1-based immunotherapy and anti-cancer drugs has become a new expectation for clinical treatment of colorectal cancer. In addition to these original research papers, several review articles were also included in this Research Topic. For example, a review article from Zhang et al. has summarized the latest advances in the classification of immunotherapy and the process of classification, identification and synthesis of tumor-specific neoantigens, as well as their role in current cancer immunotherapy; a review article from Li et al. has provided an overview of the tumor-associated antigens and anti-TAAs autoantibodies as biomarkers in the immunodiagnosis of steosarcoma; a review article from Jin and Wang has proposed a concept of immunogenic cell death (ICD)-based cancer vaccines and summarized sources of ICD-based cancer vaccines and their challenges, which may broaden the understandings of ICD and cancer vaccines in cancer immunotherapy.

In summary, this Research Topic covers many important aspects in cancer immunology, especially relating to TAAs and anti-TAAs autoantibodies. This Research Topicalso includes recent advances in the basic and clinical studies relating to cancer immunodiagnosis and cancer immunotherapy. We hope that this special issue can provide useful and helpful information to investigators in this field.

Author contributions

JZ and XG have written the manuscript and BJ and QZ have revised 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.

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TSPAN1, *TMPRSS4*, *SDR16C5*, and *CTSE* as Novel Panel for Pancreatic Cancer: A Bioinformatics Analysis and Experiments Validation

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Pancreatic cancer is a lethal malignancy with a poor prognosis. This study aims to identify pancreatic cancer-related genes and develop a robust diagnostic model to detect this disease. Weighted gene co-expression network analysis (WGCNA) was used to determine potential hub genes for pancreatic cancer. Their mRNA and protein expression levels were validated through reverse transcription PCR (RT-PCR) and immunohistochemical (IHC). Diagnostic models were developed by eight machine learning algorithms and ten-fold cross-validation. Four hub genes (*TSPAN1*, *TMPRSS4, SDR16C5*, and *CTSE*) were identified based on bioinformatics. RT-PCR showed that the four hub genes were expressed at medium to high levels, IHC revealed that their protein expression levels were higher in pancreatic cancer tissues. For the panel of these four genes, eight models performed with 0.87–0.92 area under the curve value (AUC), 0.91–0.94 sensitivity, and 0.84–0.86 specificity in the validation cohort. In the external validation set, these models also showed good performance (0.86–0.98 AUC, 0.84–1.00 sensitivity, and 0.86–1.00 specificity). In conclusion, this study has identified four hub genes that might be closely related to pancreatic cancer: *TSPAN1*, *TMPRSS4*, *SDR16C5*, and *CTSE*. Four-gene panels might provide a theoretical basis for the diagnosis of pancreatic cancer.

Keywords: pancreatic cancer, WGCNA, diagnostic model, machine learning, bioinformatics, panel

INTRODUCTION

Pancreatic cancer is the seventh leading cause of cancer-related deaths worldwide, and the mortality rate closely parallels the incidence (1). In recent years, deaths associated with pancreatic cancer are gradually increasing and it is predicted to be the second leading cause of cancer-related death by 2030 (2). In the United States, it is estimated that there will be approximately 56,770 new pancreatic cancer cases diagnosed, and 45,750 estimated deaths occurring among these new cases (3). From 2003 to 2015 statistics from China show that the age-standardized 5-year relative survival rate for

pancreatic cancer was only 7.2%(4). Despite advances in pancreatic cancer treatment strategies, the prognosis remains poor, largely due to the lack of early diagnostic approaches (5). Additionally, carbohydrate antigen 19–9 is widely used for the diagnosis of pancreatic cancer, but its sensitivity and specificity are only 0.80 (95% CI: 0.77-0.82) and 0.80 (95% CI: 0.77-0.82), respectively (6, 7). Therefore, the identification of new biomarkers or a panel with high specificity and sensitivity for diagnosing pancreatic cancer are important.

In recent years, with the development of microarray and high-throughput sequencing technologies, gene expression profiles have become an effective source of biomarkers discovery. Weighted gene expression network analysis (WGCNA) has been widely used to reveal the phenotype-related genes by constructing scale-free gene co-expression networks, especially in cancers, including lung (8), bladder (9), breast (10), and pancreatic cancer (11). In developing prediction models, satisfying the sensitivity and specificity requirements are the most interesting and challenging tasks for tumor biomarker screening. Previous studies have shown that machine learning method can improve the accuracy of disease diagnosis or prognosis (12, 13), and cancer models with higher accuracy have been developed by applying those methods (14–17).

Therefore, this study was designed to explore novel biomarkers with high performance using bioinformatics. Potential genes, screened by bioinformatics, will be validated using RT-PCR and IHC experiments. Diagnostic models will

Abbreviations: WGCNA, Weighted gene co-expression network analysis; DEGs, differentially expressed genes; TOM, topological overlap matrix; GS, gene significance; MM, module membership; TCGA, the Cancer Genome Atlas; ICGC, International Cancer Genome Consortium; GTEx, Genotype-Tissue Expression; ROC, receiver operating characteristic; Se, Sensitivity; Sp, Specificity.

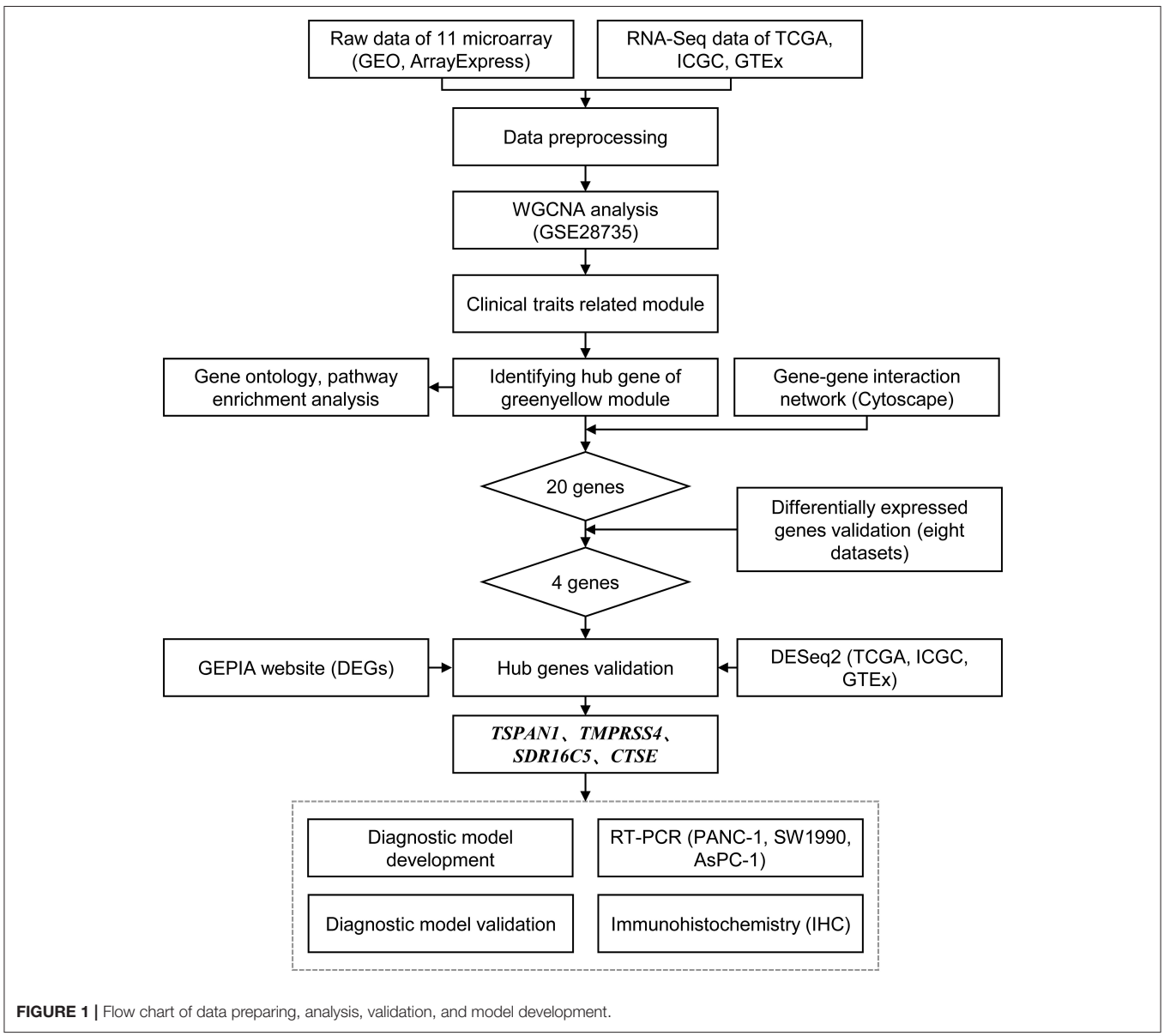

FIGURE 1 | Flow chart of data preparing, analysis, validation, and model development.

be constructed using different machine learning methods and ten-fold cross-validation.

MATERIALS AND METHODS

Data Collection and Preprocessing

The study design is shown in **Figure 1**. A systematic search on two electronic databases (Gene Expression Omnibus and ArrayExpress) was performed for potential datasets before 1 June 2019. Datasets with a sample size >20 were included. Eleven pancreatic cancer microarray datasets from three platforms were downloaded (Affymetrix Human Genome U133 Plus 2.0 Array, Affymetrix Human Gene 1.0 ST Array, and Affymetrix Human Genome U219 Array). The raw data were pre-processed with the "oligo" package and the "affy" packages. The Robust Multichip Average (RMA) function was used for background correction and normalization. In this study, GSE28735 was used to construct a weighted gene co-expression network because it contained the most balanced case and control samples, nine datasets (E-MEXP-2780, GSE15471, GSE16515, GSE32688, GSE71989, GSE106189, GSE62452, E-MTAB-6134, GSE62165) were combined to develop diagnostic models with a total of 818 samples, and the GSE32676 dataset with 32 samples was chosen to externally validate the model's performance. The ComBat algorithm was used to adjust the expression data from nine datasets for batch effects using the "sva" package (18). The characteristics of all microarray datasets are summarized in **Supplementary Table 1**.

Besides, TCGA data on RNA-sequencing (RNA-Seq) was downloaded using the "TCGAbiolinks" package (19), the ICGC data on RNA-Seq was download from Data Portal (https://dcc.icgc.org/releases/current/Projects), normal sample data was download from GTEx Portal (https://gtexportal.org/home/datasets). In total, RNA-Seq data were collected from 598 samples, including 270 cases of carcinoma and 328 cases of normal tissue.

TABLE 1 | Primers sequences of hub genes and internal reference genes.

Gene name	Primers sequences	Amplified fragment size
TSPAN1	Forward 5':TGGGCTGCTATGGTGCTAAG	154 bp
	Reverse 5':GGCACTACCAGCAACGTCAG	
TMPRSS4	Forward 5':GGGAAGTCACCGAGAAGA	107 bp
	Reverse 5':ATGCCACTGGTCAGATTG	
CTSE	Forward 5':CTATACCCTCAGCCCAACTG	169 bp
	Reverse 5':GTTATTCCCACGGTCAAAGAC	
SDR16C5	Forward 5':AATGGGCTGGCAGATTACTG	111 bp
	Reverse 5':CACAATCGTGGTTTTGATCC	
GAPDH	Forward 5':TGACTTCAACAGCGACACCCA	121 bp
	Reverse 5':CACCCTGTTGCTGTAGCCAAA	

Weighted Gene Co-expression Network Analysis

The theoretical framework of the WGCNA algorithm has been described (20). The "WGCNA" package was used to construct the co-expression network (21). Firstly, the quality of samples and genes was checked. Then, outlier samples were removed by cluster analysis using the average linkage method. When constructing a weight co-expression network, the soft threshold power is an important parameter that can emphasize strong and reduce weak correlations between genes. The power of $\beta = 8$ (scale-free $R^2 = 0.86$) was selected to ensure a scale-free network. Then, the adjacency was transformed into a topological overlap matrix (TOM), and the topological overlap dissimilarity (1-TOM) was used as hierarchical clustering input. Next, gene modules were identified using a dynamic hybrid branch cutting method with a minimum size of 30 for the gene

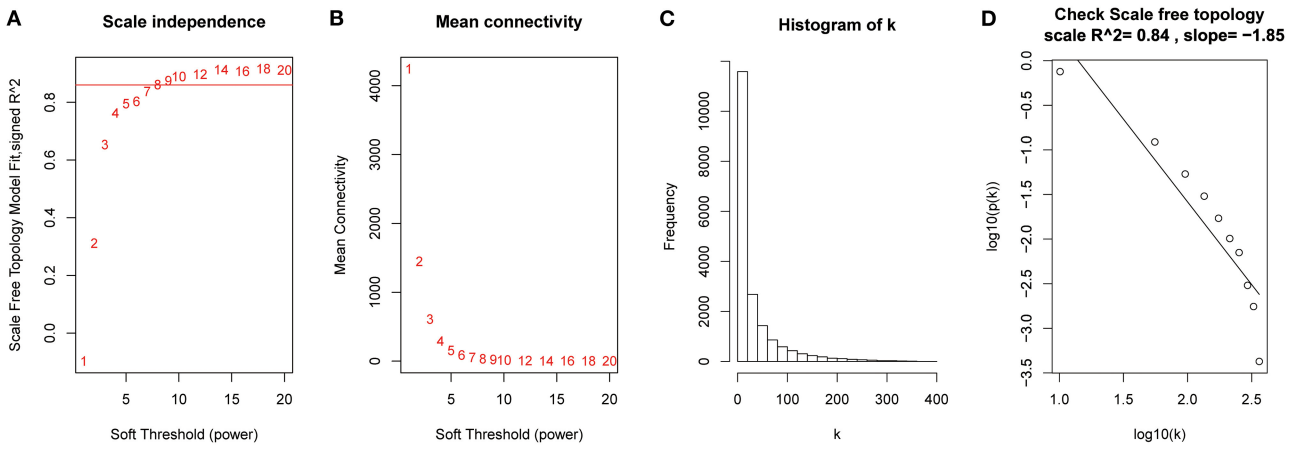

FIGURE 2 | Determination of soft-thresholding power in the weighted gene co-expression network analysis (WGCNA). **(A)** Analysis of the scale-free fit index for various soft-thresholding powers (β). **(B)** Analysis of the mean connectivity for various soft-thresholding powers. **(C)** Histogram of connectivity distribution when $\beta = 8$. **(D)** Checking the scale-free topology when $\beta = 8$.

dendrogram (22), and gene modules with a height of <0.25 were combined. An important goal of WGCNA is to detect the gene module subsets that are closely related to clinical traits. Genes within an identified module may have great biological significance. To this end, gene significance (GS) and module significance (MS) were calculated. Also, module membership (MM) was defined to select highly corrected modules with certain clinical traits.

Identification of the Hub Genes

In gene networks, genes that have many interactions with other genes are defined as hub genes. Hub genes usually play

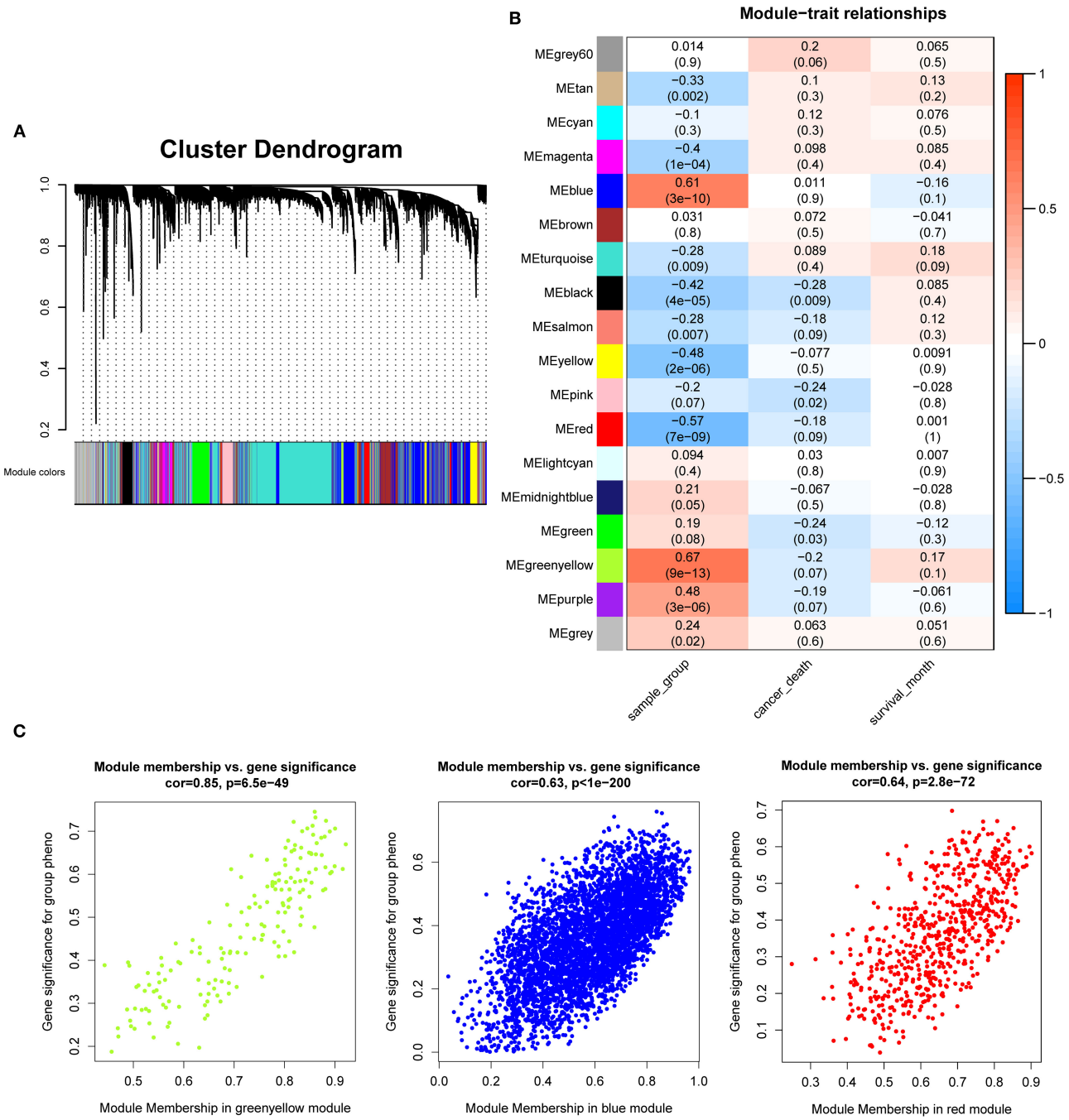

FIGURE 3 | Identification of modules associated with the clinical traits of pancreatic cancer. **(A)** Dendrogram of 18,830 genes clustered based on a dissimilarity measure (1-TOM). **(B)** Heatmap of the correlation between module eigengenes and clinical traits of pancreatic cancer. **(C)** Module membership vs. gene significance in "greenyellow," "blue," and "red" module.

an important role in a biological system (23). All genes in the significant module were included to construct a gene-gene interaction network using the "cytoHubba" Cytoscape plugin (24, 25). The top 20 genes, ranked by degrees of interactions, were selected. These genes may play important roles in the pathogenesis of pancreatic cancer. Then, differentially expressed genes (DEGs) were identified for GSE15471, GSE28735, GSE62165, GSE32688, GSE71989, GSE62452, GSE62165, and GSE32676 datasets, respectively. The "limma" (26) package was used to identify DEGs, false discovery rate (FDR) < 0.05 and $|\log2 \text{ fold change (FC)}| > 1$ were set as the cut-offs. Overall, the hub genes were determined by the intersection of the top 20 genes and the results of the eight DEGs analyses.

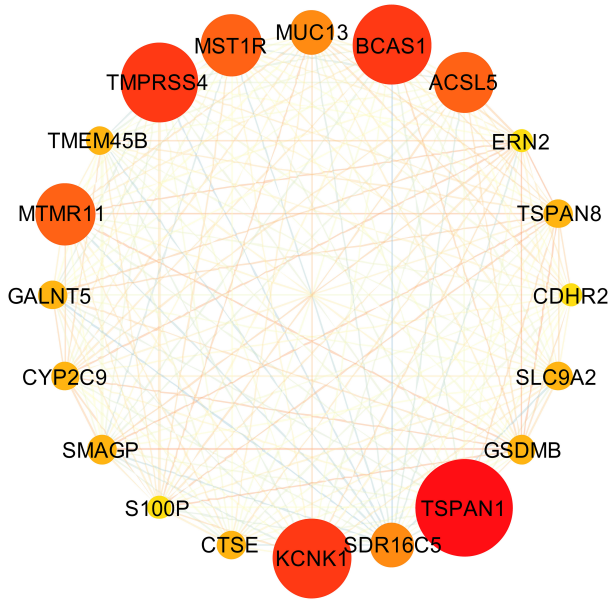

FIGURE 4 | Gene-gene interaction network of the top 20 genes. Through constructing a gene-gene interaction network by using 171 genes obtained from WGCNA analysis, the top 20 genes, ranked by degrees of interactions, were identified.

Validation of the Hub Genes

To validate hub gene expression in pancreatic cancer and normal tissues, the GEPIA tool (http://gepia.cancer-pku.cn/) was firstly applied using the RNA-Seq data (27). It is worth emphasizing that the GEPIA website included the TCGA and GTEx datasets (19, 28). And the transcripts per million (TPM) algorithm was used to measure RNA expression (29). Using the "DESeq2" package, further validation was performed based on the negative binomial distribution model using the raw counts of TCGA, ICGC, and GTEx data (30).

Reverse Transcription PCR (RT-PCR)

cDNA was synthesized using 1 μg of total RNA isolated from three pancreatic cancer cell lines (PANC-1, GCC-PA0001RT; SW1990, GCC-PA0004RT; and AsPC-1, GCC-PA0006RT) and RT-PCR was performed using 400 ng cDNA per 12 μl reactions. The primer sequences used in RT-PCR are described in **Table 1**. Relative expression abundance was determined by ΔCt=Ct (hub gene)—(*GAPDH*). $\Delta Ct \leqq 12$, $12 < \Delta Ct < 16$ and $\Delta Ct \geqq 16$ were considered to be a high expression

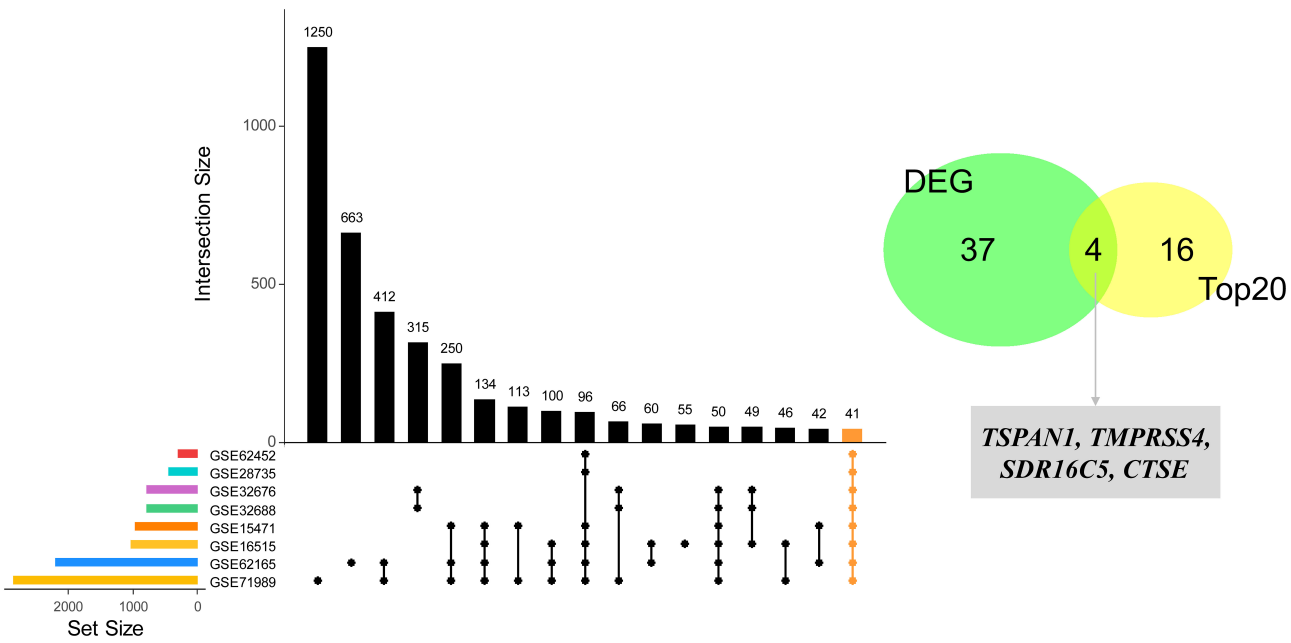

FIGURE 5 | Identification of four hub genes by eight datasets validation. Forty-one DEGs were identified through the intersection of the DEGs of 8 GEO datasets (GSE15471, GSE28735, GSE62165, GSE32688, GSE71989, GSE62452, GSE62165, and GSE32676), and then four hub genes were identified by an intersection with the top 20 genes.

abundance, moderate expression abundance and low expression abundance, respectively.

Immunohistochemistry (IHC)

Specimens of 70 pancreatic cancer tissues and 70 adjacent tissues were deparaffinized and rehydrated. The sections were incubated with polyclonal anti-TSPAN1 antibody (1:1000 dilution) (SANTA CRUZ BIOTECHNOLOGY, sc-376551), anti-TMPRSS4 antibody (1:500 dilution) (proteintech, 11283-1-AP), anti-SDR16C5 antibody (1:300 dilution) (Thermo Fisher, PA5-55229), and anti-CTSE antibody (1:1000 dilution) (SANTA CRUZ BIOTECHNOLOGY, sc-166500). Two independent pathologists evaluated and scored the IHC, and the log2 (H-score) described the semi-quantitative expression of the four proteins.

Diagnostic Model Development and Validation

In this analysis, the merged dataset was used to construct models of pancreatic cancer using four hub genes. A total of 818 samples were randomly assigned into a training cohort and a validation cohort at 7:3 ratios. The GSE32676 dataset was used as the external validation cohort. The support vector machine, random forest, Naive Bayes, neural network, linear

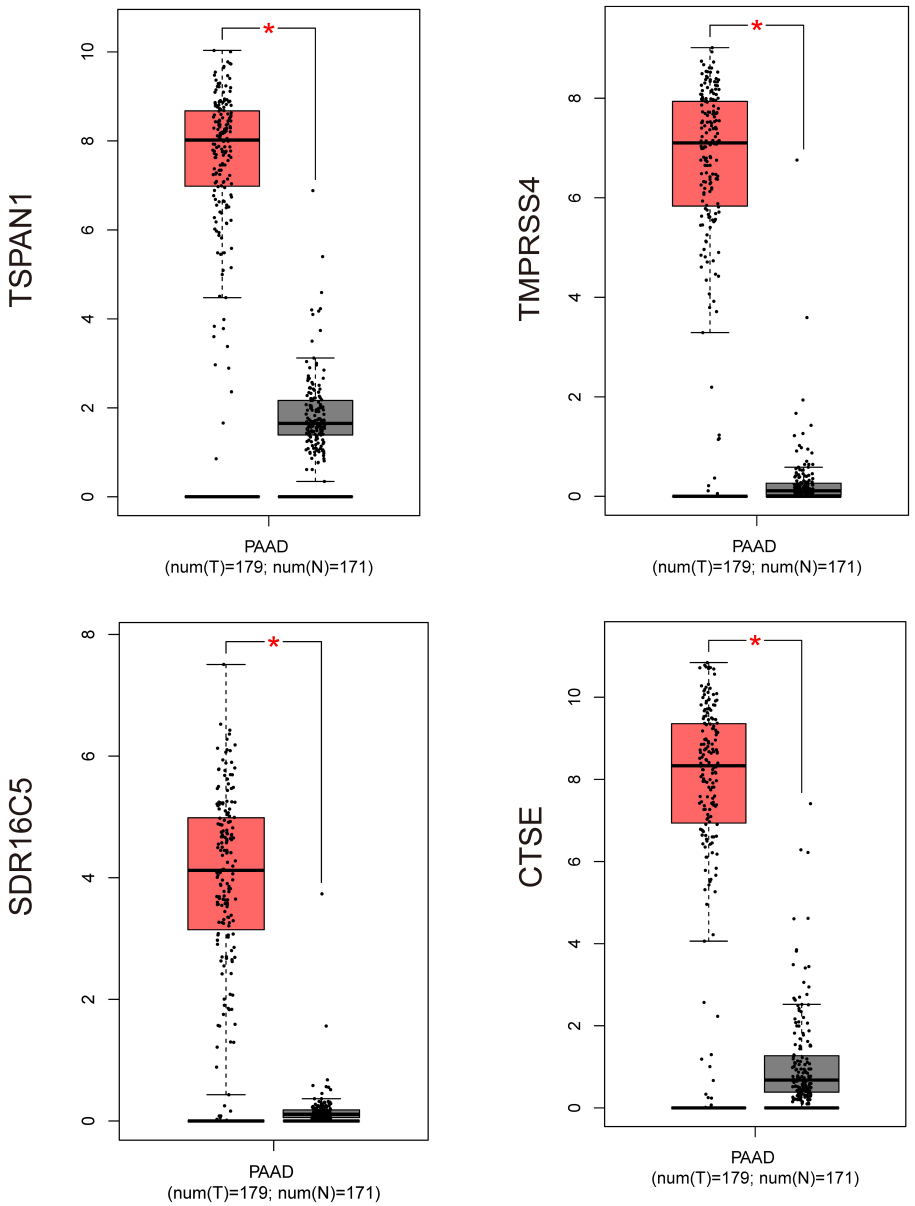

FIGURE 6 | Validation of four hub genes expression by using RNA-Seq data (GEPIA website). *$P \leq 0.05$; PAAD, pancreatic cancer (GEPIA website).

discriminant analysis, mixture discriminant analysis, flexible discriminant analysis, and logistic regression were used to classify pancreatic cancer and normal tissues. To strengthen the robustness of the prediction with these genes, 10-fold cross-validation was also applied reiteratively 100 times. The receiver operating characteristic (ROC) curve was drawn to estimate the diagnostic performance of each model, and the sensitivity and specificity were determined. All statistical analyses were conducted using R 3.5.3.

RESULTS

Gene Co-expression Network Construction and Key Modules Identification

After the quality assessment for the GSE28735 dataset, GSM711915 and GSM711957 samples were removed. Eventually, a total of 18,830 genes and 88 samples were included to construct a gene co-expression network using the "WGCNA" package. In the current study, to ensure a scale-free network, $\beta = 8$ (scale-free $R^2 = 0.86$) was selected (**Figures 2A,B**), and scale-free topology ($R^2 = 0.84$, slope $= -1.85$) was obtained (**Figures 2C,D**). Through the obtained scale-free topology, 18,830 genes were classified as 18 modules (**Figure 3A**). Three modules were acquired that were significantly related to the sample category (greenyellow: $r = 0.67$, $P = 9e\text{-}13$; blue: $r = 0.61$, $P = 3e\text{-}10$; and red: $r = -0.57$, $P = 7e\text{-}9$; **Figure 3B**). The greenyellow module showed the highest correlation with clinical information (cor $= 0.85$, $P = 6.5e{-}49$, **Figure 3C**). Therefore, the 171 genes of the greenyellow module were used for subsequent analyses.

Hub Gene Identification and Validation

Based on the interaction parameters of the 171 genes obtained from WGCNA analysis, the top 20 genes were identified (**Figure 4**). DEGs analysis of eight gene datasets revealed a total of 41 genes, so four hub genes, *TSPAN1*, *TMPRSS4*, *SDR16C5*, and *CTSE* was identified (**Figure 5**). The validation results showed that four hub genes derived from the GEPIA tool were differentially expressed in cancer and normal tissues (**Figure 6**), as was the result of DESeq2 analysis (**Supplemantary Table 2**). The details of the four genes are shown in **Table 2**.

RT-PCR and IHC

The expression of the four hub genes in three cell lines showed that *TSPAN1* and *CTSE* were expressed at high levels, *TMPRSS4* and *SDR16C5* were expressed at medium expression levels (**Figure 7**). IHC staining results are shown in **Figure 8**. The expression levels in pancreatic cancer tissues and adjacent tissues showed as follows: 7.27 ± 0.31 and 6.88 ± 0.14; 7.16 ± 0.24 and 7.02 ± 0.13; 7.15 ± 0.24 and 6.99 ± 0.14; 7.00 ± 0.26 and 6.76 ± 0.09. Higher levels of TSPAN1, TMPRSS4, SDR16C5 and CTSE expression were observed in pancreatic cancer than in normal pancreatic tissue (paired *t*-test, $P < 0.0001$).

TABLE 2 | Summary of four hub genes identified by weighted gene co-expression network analysis.

Gene ID	Official full name	Description	References
TSPAN1	Tetraspanin 1	Cell development, activation, growth, and motility	(31, 32)
TMPRSS4	Transmembrane serine protease 4	Integral component of membrane; regulation of gene expression; scavenger receptor activity	(33, 34)
CTSE	Cathepsin E	Antigen processing and presentation of exogenous peptide antigen via MHC class II; protein autoprocessing; protein catabolic process	(35, 36)
SDR16C5	Short chain dehydrogenase/ reductase family 16C member 5	Activating transcription factor binding; keratinocyte proliferation; oxidation-reduction process	NA

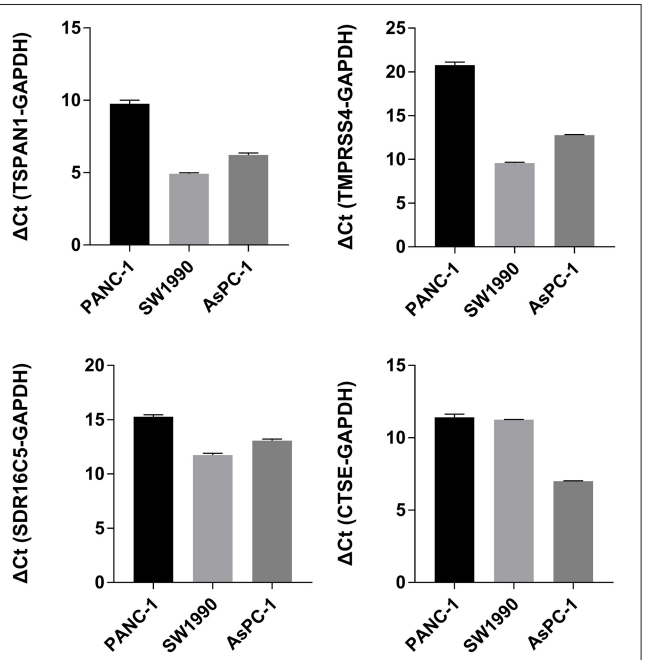

FIGURE 7 | *TSPAN1*, *TMPRSS4*, *SDR16C5* and *CTSE* mRNA expression in three pancreatic cancer cells.

Diagnostic Model Development and Validation

In the validation cohort, the AUC of the eight models constructed by machine learning ranged from 0.87 to 0.92, sensitivity ranged from 0.91 to 0.94, and specificity ranged from 0.84 to 0.86. In the external validation cohort, the AUC of the eight models ranged from 0.86 to 0.98, sensitivity ranged from 0.84 to 1.00, and specificity ranged from 0.86 to 1.00 (**Table 3**).

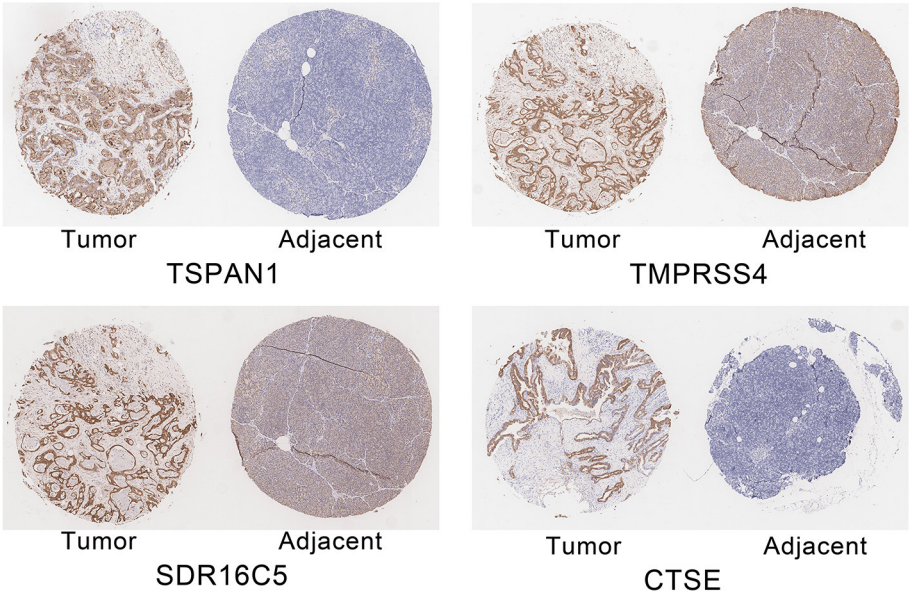

FIGURE 8 | Immunohistochemical staining of TSPAN1, TMPRSS4, SDR16C5 and CTSE.

TABLE 3 | Diagnostic performance of eight machine learning methods for pancreatic cancer.

Methods	Validation (30%)			External validation (GSE32676)		
	AUC	Se	Sp	AUC	Se	Sp
Support vector machine	0.87 (0.79–0.95)	0.92	0.84	0.90 (0.73–1.00)	0.96	0.86
Random forest	0.91 (0.86–0.97)	0.91	0.86	0.94 (0.83–1.00)	0.96	0.86
Naive Bayes	0.91 (0.86–0.96)	0.93	0.84	0.92 (0.77–1.00)	0.96	0.86
Neural network	0.91 (0.86–0.97)	0.94	0.84	0.97 (0.91–1.00)	0.84	1.00
Linear discriminant analysis	0.91 (0.86–0.96)	0.93	0.84	0.95 (0.86–1.00)	1.00	0.86
Mixture discriminant analysis	0.91 (0.87–0.96)	0.91	0.84	0.98 (0.93–1.00)	1.00	0.86
Flexible discriminant analysis	0.91 (0.85–0.96)	0.92	0.84	0.86 (0.71–1.00)	0.84	0.86
Logistic regression	0.92 (0.87–0.97)	0.93	0.84	0.97 (0.90–1.00)	0.96	0.86

AUC, receiver operating characteristic area under the curve value; Se, Sensitivity; Sp, Specificity.

DISCUSSION

There is an urgent need for a relatively reliable, clinically easy to use, cost-effective biomarker panel for the diagnosis of pancreatic cancer. This study identified four hub genes through bioinformatics, DEGs analysis in multiple datasets, experimental verification of mRNA and protein levels. Using machine learning methods, the expression of four hub genes was utilized to construct models with satisfactory diagnostic value.

Pancreatic cancer is a polygenic and highly heterogeneous disease, the diagnosis of which is challenging (37). A single biomarker may not be sufficient for accurate diagnosis, and a panel consisting of multiple biomarkers might be more beneficial and accurate (38). In the study of pancreatic cancer, some diagnostic models have been developed (39–41). However, most models are not cost-effective for patients, because multiple biomarkers are difficult to routinely screen and/or identify clinically. Most importantly, a recent study demonstrated that a three-miRNA panel can be as effective as the panel of 1800 miRNAs (42). It is necessary to weigh the number of biomarkers in clinical application and their predictive abilities. Therefore, the focus of this study is to screen hub genes and explore a diagnostic model with cost-effective performance.

With the development of next-generation sequencing, bioinformatics has been used in many ways of research, such as biomarker screening, molecular mechanism exploration. Currently, WGCNA was widely applied to screen hub genes in various cancers (9). This approach can identify critical cancer driver genes that may be a significant therapeutic target or diagnostic marker (43). In recent years, several biomarkers have been identified in the field of cancer research using WGCNA (44–47). However, most studies only used DEGs or the first 25% variation genes to construct a weighted gene co-expression network, which may result in a loss of genetic

diversity. Moreover, some studies only used the feature selection method to select biomarkers (17, 43, 48). Although this method can reduce the dimensionality of data, these genes that play important roles in the cancer process may be lost.

In this study, transcriptome data related to pancreatic cancer were systematically retrieved and its raw data were preprocessed. During the WGCNA analysis process, all genes were included in the construction of a co-expression network to find diagnostic biomarkers, which enhanced the diversity of genes. After using WGCNA to identify a set of genes highly correlated with pancreatic cancer, hub genes were identified through gene-gene interaction network analysis and DEGs analysis in independent eight datasets. It is important to emphasize the interactions between these genes, it can provide deeper insight into the mechanism of cancer (9, 49–51). To increase the credibility of the selected hub genes. DEGs validation was firstly applied using the RNA-Seq data. And then their gene and protein expression levels were verified through experimental methods, including RT-PCR and IHC methods.

In recent years, many studies have suggested that machine learning can provide promising tools for diagnosis in the cancer domain (13). For example, Pu et al. (52) identified a diagnostic model based on five hyper-methylated CpG sites with 0.82% accuracy using the support vector machine method. It is more practical to explore an optimal panel with few biomarkers and high diagnostic performance. Therefore, this study used the four hub gene expression profiles of 818 samples to construct the diagnostic models through machine learning. After internal verification and external verification, the results showed that panels of the four hub genes had a better diagnostic performance for pancreatic cancer.

Four hub genes were identified by bioinformatics in this study. *TSPAN1* (31, 32), *TMPRSS4* (33, 34), and *CTSE* (35, 36) have previously been studied in pancreatic cancer. Among them, *TMPRSS4* was overexpressed in, and identified as a biomarker of, pancreatic carcinoma (33), *TSPAN1*, *TMPRSS4*, and *CTSE* are potential diagnostic or prognostic markers for pancreatic ductal adenocarcinoma (31, 33, 35), and most of these genes are associated with metastasis and proliferation and in pancreatic cancer. Although *SDR16C5* has not been reported in pancreatic cancer, a study showed that it is involved in the regulation of triple-negative breast cancer (53). Its potential as diagnostic marker warrants further functional investigations on its roles in the development of pancreatic cancer.

Certain important strengths of this study should be emphasized. First, the data used in this study are very comprehensive, and the sample size is the largest in the current study of pancreatic cancer. Second, multiple validations of hub genes expression were executed using eight microarray data sets and RNA-Seq data sets, and the RT-PCR and IHC methods were used to validate their expression at the gene and protein level. Those validations can maximize the reliability of the selected hub genes. Third, logistic regression and several machine learning methods were applied to evaluate the diagnostic ability of our panels. Iterative ten-fold cross-validation repeated 100 times was also used to obtain a robust evaluation of the prediction ability using these genes. There are also some limitations in this study. First, the research samples included in this study were from diverse populations from the USA, France, and Japan. There may exist some differences in gene expression profiles among various ethnic groups. Next, our prediction models will be improved with further validation using independent experimental data.

In conclusion, four hub genes were identified using bioinformatics and experimental verification approaches. More importantly, the four-gene panels can accurately predict pancreatic cancer. Our findings encourage future clinical research to validate the robustness of the diagnostic model and additional functional research.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Life Science Ethics Review Committee of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TL conceived the project. HW, JW, and CY collected the datasets. TL participated in the pre-processing of the datasets and performed the computational analysis. TL and HY drafted the manuscript. JS, PW, CS, LD, GJ, YH, JL, and YY thoroughly revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.649551/full#supplementary-material

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REVIEW
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Neoantigen: A New Breakthrough in Tumor Immunotherapy

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Cancer immunotherapy works by stimulating and strengthening the body's anti-tumor immune response to eliminate cancer cells. Over the past few decades, immunotherapy has shown remarkable efficacy in the treatment of cancer, particularly the success of immune checkpoint blockade targeting CTLA-4, PD-1 and PDL1, which has led to a breakthrough in tumor immunotherapy. Tumor neoantigens, a new approach to tumor immunotherapy, include antigens produced by tumor viruses integrated into the genome and antigens produced by mutant proteins, which are abundantly expressed only in tumor cells and have strong immunogenicity and tumor heterogeneity. A growing number of studies have highlighted the relationship between neoantigens and T cells' recognition of cancer cells. Vaccines developed against neoantigens are now being used in clinical trials in various solid tumors. In this review, we summarized the latest advances in the classification of immunotherapy and the process of classification, identification and synthesis of tumor-specific neoantigens, as well as their role in current cancer immunotherapy. Finally, the application prospects and existing problems of neoantigens were discussed.

Keywords: immunotherapy, neoantigen, tumor-specific antigens, vaccine, personalized cancer immunotherapy

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INTRODUCTION

The human immune system helps us avoid infections and many diseases and protects us from cancer (1, 2). With the ability to recognize its own and non-self substances, the body's immune system can produce natural immune tolerance to its own components and eliminate non-self foreign bodies to maintain the internal environment's stability (3). Cancer occurs when normal cells change and begin to lose control. Since cancer cells are derived from normal cells and are indistinguishable from normal cells, the immune system's ability to recognize cancer cells is minimal (4, 5). Cancer cells can avoid being attacked by the immune system when the immune system mistakenly thinks tumor cells are self-components. The surveillance of the immune system is also progressively weakened by mutations in the tumor. Tumor cells that activate the immune system are gradually screened out until they produce tumor molecules that are not recognized by the immune system. This process is also known as immunoediting of tumor. In this way, tumor cells successfully escape the damage of the immune system and have a chance to develop. What's more, because cancer cells themselves can also release many substances that block the immune system, tumor immune response is often selectively suppressed around the tumor tissue (6, 7), which

explains the ineffectiveness of immunotherapy in many patients: it is the failure to activate the immune response around the tumor tissue rather than the inability to activate the immune response systematically (6–9). In addition, inflammation can promote the development of tumors. Inflammation can release a large number of immunosuppressive cytokines locally in tumor tissue and suppress the immune system through a variety of ways. So cancer still could be caused even with a normal immune system. To overcome this problem, researchers have been looking for ways to help the immune system enhance its antitumor immune responses and improve its capacity to suppress tumor. In recent years, immunotherapy has developed rapidly and become a mature cancer treatment strategy in addition to surgery, chemotherapy and radiotherapy. Immunotherapy has shown a significant therapeutic effect in many human malignant tumors by using the immune system to eliminate cancer cells (10).

With the wide application of high-throughput omics and the development of neoantigen prediction technology, immunotherapy based on neoantigen has become a new research hotspot. Neoantigens are mainly tumor-specific antigens generated by mutations in tumor cells, which are only expressed in tumor cells (11). Neoantigens can also be produced by viral infection, alternative splicing and gene rearrangement (12–14). They are ideal targets for T cells to recognize cancer cells and can stimulate strong anti-tumor immune response. Studies in the past five years have shown that neoantigens play a key role in tumor immunotherapy. The identification, screening and identification of neoantigens accelerate the development of personalized immunotherapy for tumor patients, which will benefit more patients (15). As more scientific and clinical data reveal the remarkable effects of neoantigen-based vaccine therapies in a variety of cancer types, there is ample reason to believe that neoantigen-based therapies will be a promising area of cancer immunotherapy.

INTRODUCTION OF CANCER IMMUNOTHERAPY

Immunotherapy refers to the measures taken to use immunological methods and principles to target the hyper or hypo-immune state of the organism, intervene or adjust the organism's immune function artificially, and strengthen or attenuate the immune response so as to achieve the purpose of treating diseases (16). Immunotherapy enhances the immune system's ability to recognize, target, and eliminate cancer cells in the body, making it a potentially universal cancer solution (17). Immunotherapy, approved as a first-line treatment strategy for multiple cancers in the United States and elsewhere (18), can be used alone or in combination with other cancer treatments (19). Compared with other cancer treatments, immunotherapy has become more precise, personalized, and has fewer side effects (20–22). In recent years, it has gradually become an important development direction of cancer treatment, which is known as the fourth leading cancer treatment technology after surgery, radiotherapy, and chemotherapy.

CLASSIFICATION OF IMMUNOTHERAPIES

Immunotherapy is generally divided into two categories: active immunotherapy and passive immunotherapy (23) (**Figure 1**). Active immunotherapy refers to eliminating cancer cells by stimulating the body's immune system (24). Passive immunotherapy refers to the passive acceptance by an organism of antibodies, cytokines, or transformed immune cells that can directly act on the tumor (25).

Tumor vaccines, one type of active immunotherapy, is an important component in the field of tumor immunotherapy (26). Tumor vaccines can recognize proteins present on specific cancer cells, arrest cancer cell growth, prevent cancer

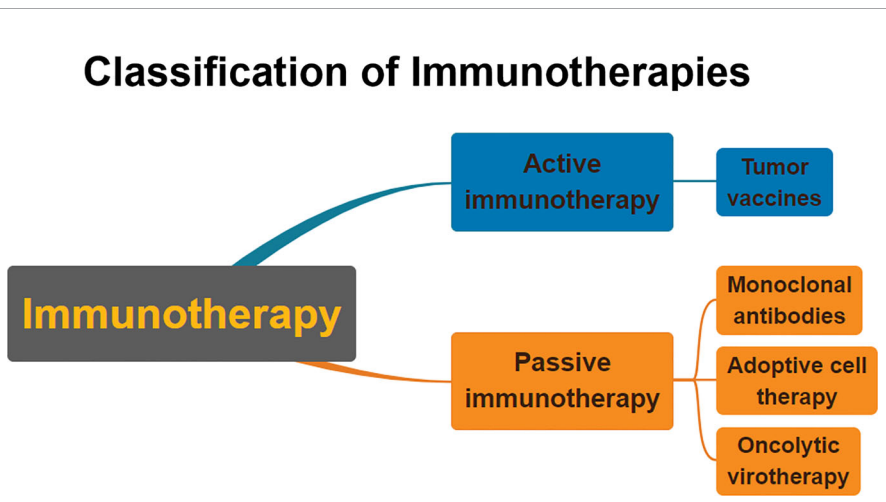

FIGURE 1 | Classification of immunotherapies. Immunotherapy is usually divided into passive immunotherapy and active immunotherapy. Active immunotherapy is mainly cancer vaccines. Passive immunotherapy mainly includes adoptive cell therapy, oncolytic viruses, and monoclonal antibodies.

recurrence, and clear cancer cells that remain after treatment (27–30). Cancer vaccines aim to elicit immune responses against tumor-specific or tumor-associated antigens, thereby facilitating the immune system to attack cancer cells carrying these antigens (30, 31). Tumor vaccines include cell vaccines, DNA vaccines, mRNA vaccines, polypeptide vaccines, dendritic cell vaccines, and others (32, 33). In April 2010, Provenge (sipuleucel-T), the first tumor therapeutic vaccine, was approved by the FDA for the treatment of prostate cancer (34).

Monoclonal antibodies have promising therapeutic effects in the clinic and belong to passive immunotherapy (35). Monoclonal antibody drugs can specifically bind to specific receptors or ligands on the surface of tumor cells or immune cells and block the corresponding signaling pathways, thereby exerting antitumor effects (36). The hottest monoclonal antibodies in the field of oncology at present are immune checkpoint inhibitors (37, 38). Immune checkpoints are immunosuppressive pathways that they can suppress T-cell activity (39, 40), and cancer cells evade the immune response by hijacking this pathway (41–44). The most thoroughly studied immune checkpoints are CTLA-4, PD-1, and PD-L1, which have been approved by FDA for the treatment of a variety of tumors (45, 46). Adoptive cell therapy exerts its tumor-suppressive and killing effects by isolating immunocompetent cells from patients, inducing their differentiation *in vitro*, reconstituting, expanding, and re-infusing them into patients to target antigen-specific tumor cells and exert their tumor-suppressive and tumor-killing effects (47). It belongs to passive immunotherapy (48). The most promising adoptive cell therapies at present are TCR-T(TCR-modified T cell) and CAR-T(Chimeric antigen receptor T cell) (49–51). Oncolytic virotherapy is a form of passive immunotherapy. Oncolytic viruses are a class of tumor-killing type viruses that render them unable to replicate in normal tissues by attenuating or deleting viral pathogenic factors while maintaining replication and killing viability in tumor cells (52, 53). IMLYGIC, the first FDA-approved oncolytic viral drug, was genetically modified from herpes virus type 1 (HSV-1) for the treatment of metastatic melanoma (54).

There are two current successes of immunotherapy, one with PD-1 and PD-L1 mAbs derived through immune checkpoints and the other with adoptive T cell therapy (29, 55). But clinical trials have shown that although having promising potential, immune checkpoint therapies have limited efficacy in many cases, especially in solid tumors with low response rates (56). What's more, adoptive T cells present problems such as poor persistence as well as cytotoxicity *in vivo* and can trigger an inflammatory factor storm (57–59). With the wide application of multi-omics high-throughput technologies and the development of neoantigen prediction technology, neoantigen-based immunotherapy becomes a new research hotspot. In the past five years, studies have shown that neoantigens have a promising outcome in clinical therapy. Technological advances in the identification, screening, and characterization of neoantigens will accelerate the development of individual immunotherapy for cancer patients, thereby benefiting more patients. In the future, neoantigen therapy will become an important therapeutic modality in the field of precision oncology (60).

WHAT IS THE NEOANTIGEN?

Gene mutations caused by genetic instability during carcinogenesis always occur in the non-coding and coding region, and the amino acid sequence changes caused by mutations in the coding region can produce proteins that are not found in normal cells. These proteins can activate the immune system and lead to the immune system's attack on cancer cells (61). Neoantigens can also be produced by viral infection, alternative splicing and gene rearrangement. These aberrant antigens, which can be recognized by immune cells and result from mutations in cancer cell genes, are neoantigen (62). Neoantigens can be presented on the cell surface and subsequently recognized by T cells under the action of major histocompatibility complex (MHC) molecules (63, 64). Tumor antigens are divided into tumor associated antigens (TAA) and tumor specific antigen (TSA) (65). TAA is a protein expressed by unmutated genes and appears to be significantly over-expressed in tumor cells but rarely expressed in normal cells (11). Because TAAs are normal host proteins, they are subject to both central and peripheral tolerance mechanisms (35, 64). Targeting TAAs may also lead to autoimmune toxicity (39); tumor specific antigen (TSA) is a neoantigen resulting from somatic mutations and is expressed only in tumor cells but not in normal cells (66). Because normal cells do not express TSA, they are considered non-self by the immune system, neoantigen specific immune responses are not affected by tolerance. Furthermore, targeting TSAs does not easily induce autoimmunity (39). Thus, neoantigens are ideal targets for therapeutic cancer vaccines and T cell-based cancer immunotherapy. By taking advantage of the immune activity of neoantigens, synthetic neoantigen drugs can be designed according to the situation of tumor cell mutation to achieve the effect of treatment.

CLASSIFICATION OF NEOANTIGENS

Neoantigens can be classified into two categories: shared neoantigens and personalized neoantigens (66, 67) (**Figure 2**). Shared neoantigens refer to mutated antigens that are common across different cancer patients and not present in the normal genome. Shared neoantigens that are highly immunogenic have the potential to be screened for use as broad-spectrum therapeutic cancer vaccines for patients with the same mutated gene (68, 69). Personalized neoantigens refer to mutated antigens that are unique to most neoantigens and completely different from patient to patient. Thus, the personalized neoantigen preparation drug can only be specifically targeted to each patient, that is, personalized therapy (70). Neoantigens, with strong immunogenicity, can reduces the probability of immune escape of tumor cells. However, the different types and quantities of neoantigens in different individuals of the same tumor caused by specificity of mutations showing obvious individual heterogeneity. Therefore, the application of neoantigens in tumor immunotherapy will tend to be personalized (71). Individualized cancer vaccines can work alone or in combination with other therapies to increase the strength and

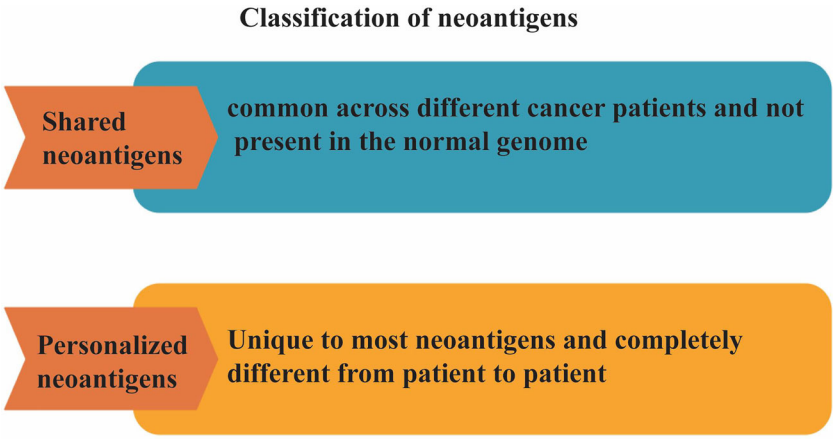

FIGURE 2 | Classification of neoantigens. Neoantigens can be classified into two categories: shared neoantigens and personalized neoantigens. Shared neoantigens refer to mutated antigens that are common across different cancer patients and not present in the normal genome. Personalized neoantigens refer to mutated antigens that are unique to most neoantigens and completely different from patient to patient.

durability of the anti-tumor effect, improve survival and quality of life, and ultimately improve the outcome of cancer treatment for patients (72). The feasibility, safety and immunogenicity of individualized cancer vaccine in the treatment of cancer patients determine that it will be an important development trend in the future (15). It is expected that individualized cancer vaccines will enable most patients to obtain precise treatment in the foreseeable future.

IDENTIFICATION OF NEOANTIGENS

Although neoantigens have made good clinical progress in tumor therapy, the number of neoantigens with immunogenicity is small, and the prediction comparison is difficult (73). Therefore, the neoantigen field needs more optimized algorithms and validated methods for accurate prediction in order to select more reliable neoepitopes of high immunogenicity. At present, the prediction accuracy of tumor neoantigen remains an urgent problem. For tumor neoantigen prediction algorithm, there are many factors to be considered, including HLA typing, expression, mutation analysis, prediction peptide processing, TCR binding force, MHC affinity, PMHC stability, tumor neoantigen source,and so on (74, 75). It also includes T cell recognition, TCR analysis, and immune cell analysis to assess T cell response (74, 76). For neoantigen screening and assessment of T cell responses, in addition to next-generation sequencing, there are high-resolution and tandem mass spectrometry techniques as well as in silico techniques for peptide prediction, but prediction algorithms based on machine learning and AI techniques need to be continuously trained with confirmatory datasets where data type, quality, and quantity can greatly affect algorithm precision (77, 78) (**Figure 3**). Therefore, the continuous accumulation of databases, especially the validated tumor neoantigen data, is extremely critical to improving algorithm accuracy (79, 80). The tumor neoantigen selection Alliance (TESLA) was initiated and formed by the Park Institute for cancer immunotherapy (PICI) and the Cancer Research Institute (CRI) (81). TESLA brings together 36 top biotechnology, pharmaceutical, university, and non-profit research teams, consisting of the National Cancer Center (NCC), PICI, Memorial Sloan-Kettering Cancer Center (MSKCC), MD Anderson Cancer Center, and more than 30 other top neoantigen research institutions. The consortium aims to establish algorithms and standards for global neoantigen testing, make concerted efforts to predict more precise anticancer targets, and advance research and application of personalized tumor vaccines. TESLA scientists discovered algorithmic models and core parameters that can better predict neoantigens and accurately predicted 75% of validated neoantigen targets and filtered to exclude 98% of invalid neoantigen targets, whose findings were published in cell journals (81).

NEOANTIGEN SYNTHESIS PROCESS

The development strategy for tumor neoantigen vaccines is clear. First, obtaining tumor tissue and normal tissue samples from the patient and then identifying the mutant genes of the tumor by comparing the exome sequencing results of the two groups of samples. cDNA microarray or RNA sequence test was performed to select appropriate mutated neoantigens according to gene expression level. Computer analysis is used to predict the adhesion affinity of candidate antigens to HLA, and the gene sequences that are most likely to become neoantigens of tumors are screened. Finally, these mutated genes are designed into vaccines (82, 83). Cancer vaccines can come in many forms, such as peptide vaccines, dendritic cell vaccines, mRNA vaccines, DNA vaccines, and viral vaccines (84) (**Figure 4**). Different forms of vaccines have different advantages and disadvantages (**Table 1**).

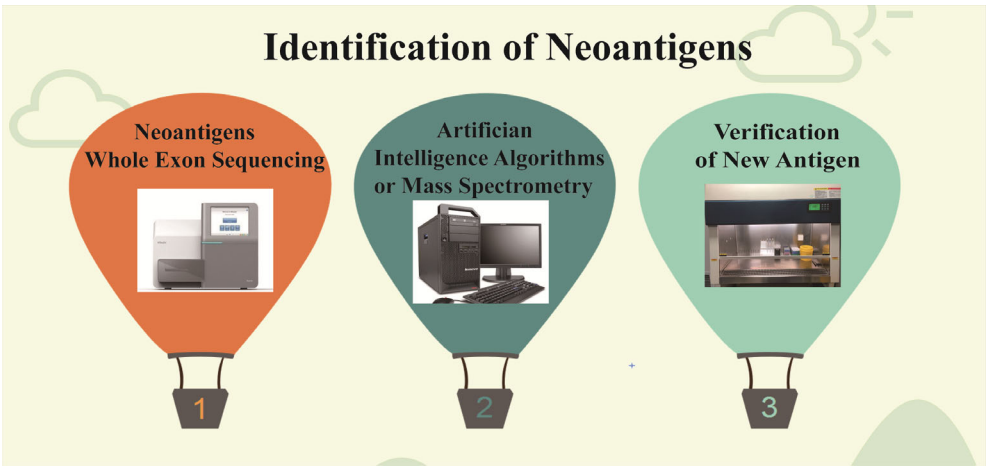

FIGURE 3 | Identification of neoantigens. First, the new antigens were found by high-throughput sequencing, then screened by algorithm and mass spectrometry, and finally verified by experiment.

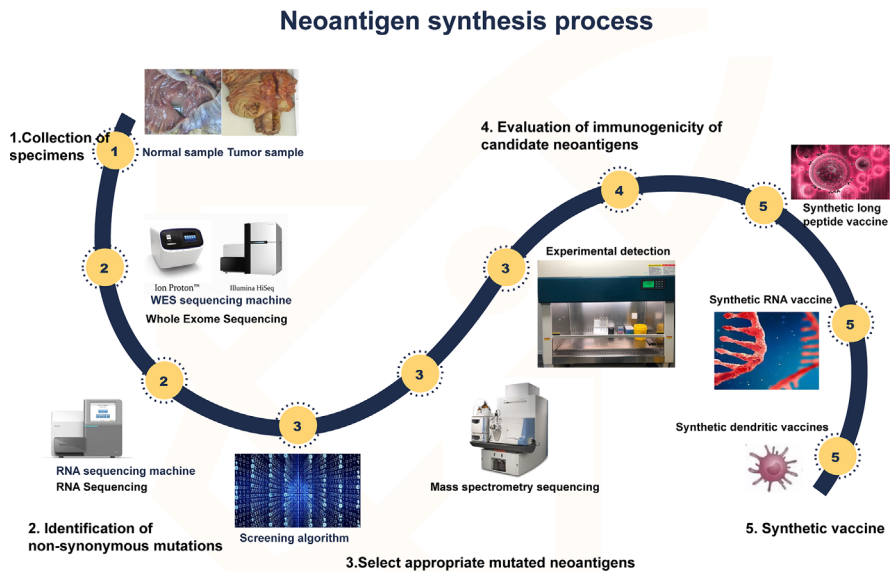

FIGURE 4 | Synthesis of neoantigen. First, tumor and normal tissue samples were obtained. Then, by comparing the sequencing results of the two groups of samples, the mutated gene of the tumor was identified. Using computer, mass spectrometry or experimental methods to screen the gene sequences that are most likely to become tumor neoantigens, and finally these mutated genes can be designed into vaccines, which can take many forms, such as peptide vaccines, dendritic cell vaccines, RNA vaccines, etc.

ADVANCES IN THE STUDY OF NEOANTIGENS

In 2014, Rosenberg reported in Science that he had successfully treated a patient with advanced cholangiocarcinoma, a highly aggressive form of the disease, using lymphocytes that were amplified *in vitro* and specifically recognized cancer cells (85). Whole exome sequencing revealed a total of 26 significant gene mutations in the patient, many of which produced abnormal proteins. By co-culturing these abnormal protein fragments with lymphocytes isolated from the patient's tumor tissue, CD4 positive T cells were found to recognize one abnormal protein, ERBB2IP. Lymphocytes that recognize the aberrant protein produced by the ERBB2IP mutation were expanded, activated, and returned to the patient. After two regurgitation, the lesion shrank significantly throughout the patient's body, leading to complete remission and complete disappearance of the tumor. Cholangiocarcinoma is an extremely difficult disease, and this

TABLE 1 | Advantages and disadvantages of different forms of vaccines.

Vaccines type	Advantages	Disadvantages
mRNA vaccines	1. Cellless production; 2. Delivered to DC efficiently	Easy degradation
DNA vaccines	1. Cellless production; 2. The cost is low; 3. Encode any epitope	There is a risk of mutation
Peptide vaccines	1. Cellless production; 2. Easy to transport; 3. Fully degradable	Degradation produces an unrelated immune response
Dendritic cell vaccines	1. The immune stimulation activity is strong; 2. The clinical effect has been proved	Cost is high

patient achieved complete remission with lymphocyte infusion for several years after the failure of conventional treatments, such as chemotherapy. This report foreshadows the future role of neoantigens in immunotherapy. In 2015, Rosenberg team also reported the discovery of multiple neoantigens in the tissues of patients with digestive tract tumors, including the famous neoantigen derived from KRAS (G12D) mutation (86).

At present, a large number of neoantigens have been discovered, which are unique to cancer cells and recognized by T cells as heterogeneous, and are not affected by immune tolerance mechanism. Therefore, these antigens can be used as effective targets for immune-mediated tumor therapy. In 2017, Ugur Sahin's team reported in Nature that a personalized RNA-based vaccine treatment regimen was used in a trial involving 13 melanoma patients. The therapy predicted the antigen of the mutations detected on each patient's genome and then synthesized a personalized vaccine. Eight of these patients have had no further tumor recurrence over 23 months, five of them with advanced malignant melanoma, two of whom experienced significant tumor shrinkage, and one additional patient treated with the vaccine combined with a PD1 antibody had a complete tumor response. This study demonstrated the clinical feasibility, safety, and antitumor activity of personalized RNA vaccine based on multi-omics (87). In the same year, the vaccine produced by Catherine J. Wu's team using Neoantigen also successfully treated malignant melanoma. Four of the six patients who received the vaccine were free of relapse 25 months after the vaccine, while two patients with recurrent disease were subsequently treated with anti-PD-1 therapy and experienced complete tumor regression (88). Personalized vaccines may be an important way to conquer cancer.

Genocea Biosciences's GEN-009 vaccine trial (NCT03633110) is one of the few Phase 1/2a clinical trials that have shown the best efficacy among the current neoantigen personalization therapies with an estimated completion date of December 2022. The purpose of this study was to evaluate the safety, tolerability, immunogenicity, and antitumor activity of the personalized vaccine GEN-009 for the treatment of patients with solid tumors, which is targeted at a broad range of cancers. The results so far show that 40 doses of the vaccine have been administered, and only a few patients have experienced mild local discomfort caused by the vaccine adjuvant with no dose limited toxicity (DLT) occurred. Ninety-nine percent of the peptides selected for the vaccine produce an immune response, and so far, no patients who have received the vaccine have relapsed (73, 89).

In 2019, Patrick Ott reported in the journal Cell the results of a personalized neoantigen vaccine NEO-PV-01 combined with PD-1 blockade in patients with advanced melanoma, non-small cell lung cancer, or bladder cancer. No treatment-related serious adverse events were observed in 82 patients, and T cell responses were observed in all patients, with no obvious toxic and adverse reactions (90).

A novel bispecific-specific antibody therapy targeting common mutations in TP53 and Ras was reported in Science and Science Immunology in 2021 by Bbert Vogelstein and his team. We all know that TP53 and RAS are important tumor-related genes *in vivo*, which are often mutated. However, p53 and Ras are mainly intracellular, so antibody-based therapy cannot be achieved with conventional methods. However, proteins are degraded by the proteasome into peptides, some of which form complexes with human leukocyte antigen (HLA) proteins and are presented on the cell surface. They have developed a specific bispecific single-chain diabody (SCDB) antibody that targets TP53 and RAS mutations. This bispecific antibody can specifically recognize and activate T cells in vitro and in mice, exerting good anti-tumour effects without cross-reactivity and with a good safety profile (91, 92).

We searched ClinicalTrials.gov (https://clinicaltrials.gov/) and found 77 cases of neoantigen studies, many of them have shown the good application value of neoantigen (Search term is "neoantigen"). However, the study of neoantigen therapy starts relatively late and still in the laboratory stage, and there are no related products on the market at both home and abroad.

PROSPECTS AND CHALLENGES

As the incidence and mortality of cancer continue to increase, people's desire to conquer cancer is more and more urgent. Neoantigen vaccine has shown an obvious effect on tumor treatment in clinical trials and is expected to become an important drug for alleviating the increasing tumor morbidity and mortality in the future. It has attracted the attention of experts in immunotherapy and is a significant development direction in the future. However, there are still some restricting factors in the development of neoantigens, and solving these problems is the key to the widespread popularization of neoantigens.

1. Scarce amount of antigens. Thousands of mutations in non-synonymous genes are typically found in tumor samples, but only a few ultimately meet the antigen criteria. Finding more effective antigens is a problem that needs to be solved. Studies have shown that most specific antigens tend to be distributed in non-coding regions. The development of non-coding research in recent years will also provide assistance for the discovery of new antigens.
2. Screening methods for predicting neoantigens need to be improved. The lack of effective screening methods for

neoantigen is another obstacle to the development of neoantigen therapy. At present, the algorithm of predicting neoantigen is in full bloom. With the development of bioinformatics technology, artificial intelligence and machine learning, we believe that this problem will be solved soon.

3. The development cycle of neoantigen vaccine is too long. The long development cycle of neoantigen vaccine is recognized as a primary obstacle to the application of vaccine. The long development cycle leads to the increase of research and development costs, and the great pressure on laboratories and enterprises is not conducive to the clinical application of vaccines. Patients participating in the trial or treatment have a short survival period and a long development cycle, which may lead to some patients being unable to accept the final treatment due to the long drug development cycle.
4. Preparation and delivery of vaccines remains a challenge. Currently, many methods have been developed for the preparation, formulation and delivery of different cancer vaccines. Technically, however, these vaccines need to be manufactured under GMP conditions. The biggest challenge, especially for small nucleic acid therapies such as mRNA/DNA, is delivery technology.
5. The heterogeneity of the tumor is difficult to resolve. Tumors are heterogeneous in the course of evolution, so each part of the gene that may mutate is different. So it may be a paradox to get local tumor tissue from a patient in the first place to predict the neoantigen of that patient. It is still a difficult problem to use 1-2 specific neoantigens to fully recognize and kill solid tumor tissues.
6. Expensive. Therapies based on neoantigens are mostly personalized, and from initial gene sequencing to validation and production, the cost of treatment can be very high. Cost remains the biggest challenge.

AUTHOR CONTRIBUTIONS

ZZ has written the review. JZ and WS supervised the program. ML, YQ, WG and TL have discussed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Discovering Panel of Autoantibodies for Early Detection of Lung Cancer Based on Focused Protein Array

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Substantial studies indicate that autoantibodies to tumor-associated antigens (TAAbs) arise in early stage of lung cancer (LC). However, since single TAAbs as non-invasive biomarkers reveal low diagnostic performances, a panel approach is needed to provide more clues for early detection of LC. In the present research, potential TAAbs were screened in 150 serum samples by focused protein array based on 154 proteins encoded by cancer driver genes. Indirect enzyme-linked immunosorbent assay (ELISA) was used to verify and validate TAAbs in two independent datasets with 1,054 participants (310 in verification cohort, 744 in validation cohort). In both verification and validation cohorts, eight TAAbs were higher in serum of LC patients compared with normal controls. Moreover, diagnostic models were built and evaluated in the training set and the test set of validation cohort by six data mining methods. In contrast to the other five models, the decision tree (DT) model containing seven TAAbs (TP53, NPM1, FGFR2, PIK3CA, GNA11, HIST1H3B, and TSC1), built in the training set, yielded the highest diagnostic value with the area under the receiver operating characteristic curve (AUC) of 0.897, the sensitivity of 94.4% and the specificity of 84.9%. The model was further assessed in the test set and exhibited an AUC of 0.838 with the sensitivity of 89.4% and the specificity of 78.2%. Interestingly, the accuracies of this model in both early and advanced stage were close to 90%, much more effective than that of single TAAbs. Protein array based on cancer driver genes is effective in screening and discovering potential TAAbs of LC. The TAAbs panel with TP53, NPM1, FGFR2, PIK3CA, GNA11, HIST1H3B, and TSC1 is excellent in early detection of LC, and they might be new target in LC immunotherapy.

Keywords: lung cancer, protein array, tumor-associated antigen, autoantibody, diagnostic model

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INTRODUCTION

Lung cancer (LC) is one of the leading causes of cancer-related deaths worldwide, accounting for 28% of all cancer deaths (1, 2). In China, LC is the first common cause of cancer-related death in men and the second cause in women (3). Due to the lack of effective early diagnosis technology for LC, it remains a challenge to improve the overall survival of patients with LC (4, 5). In the past 50 years, the 5-year survival rate of LC patients at early stage is 60–70%, while it is dreadfully < 5% at late stage (3). Therefore, early diagnosis is a critical factor to reduce the mortality and improve the long-term survival rate of LC patients (6, 7). Low-dose computed tomography (LDCT) emerged as a novel screening method for LC in 1990's, it was reported with 20% reduction of LC-related death in National Lung Cancer Screening Trial (NLST) by LDCT (8). Nevertheless, LDCT has up to 90% false-positive rate, thus it is necessary to confirm the diagnosis by additional invasive surgery or repeated radiation exposure (9), which bring unnecessary burden to the patient's economy and body.

Blood tumor biomarkers are potential for early diagnosis of LC as they have advantages of non-invasion and convenient to access (10, 11). However, multiple tumor biomarkers utilized in clinical practice show low diagnostic accuracy for cancer, such as carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), and cytokeratin-19 fragment (CYFRA 21-1) (12–14). Tumor-associated antigens (TAAs) refer to antigen molecules that exist on tumor cells or normal cells, but they are abnormally expressed in diverse cancers (15). Autoantibodies to TAAs (TAAbs) are produced in early stage of cancers by humoral immune response triggered by abnormal expression of TAAs. In comparison with other types of biomarkers, serum TAAbs appeared earlier and more stable (16). They are a kind of promising biomarkers which could be applied for early diagnosis in cancers (17).

Recently, the protein array technology was commonly applied in identifying new TAAbs, which can simultaneously analyze large number of proteins in parallel and recognize posttranslational modified proteins (18, 19). The mutation of cancer driver genes may be one of the important factors for the occurrence of cancers (20). Based on the 138 cancer driver genes (74 tumor suppressor genes and 64 oncogenes) listed in study of Vogelstein et al. (21), we customized a protein array with 154 human recombinant proteins to explore the autoantibodies against TAAs in LC. The selected TAAbs were further validated by enzyme-linked immunosorbent assay (ELISA). Since single TAAb was limited by low sensitivity and accuracy and combined multiple TAAbs could improve the detection rate of LC effectively (22–24), a series of data mining techniques were performed to establish diagnostic models for LC, such as logistic regression, Fisher discriminate analysis, decision tree (DT), support vector machines (SVM), artificial neural network-multilayer perception (ANN-MLP), and artificial neural network-radial basis function (ANN-RBF). Finally, we evaluated the diagnostic efficacy of these models and chose DT model as the optimal model.

Abbreviations: ANN-MLP, artificial neural network-multilayer perception; ANN-RBF, artificial neural network-radial basis function; AUC, area under the receiver operating characteristic curve; BLD, benign lung disease; CEA, carcinoembryonic antigen; CI, confidence internal; COPD, chronic obstructive pulmonary disease; CYFRA 21-1, cytokeratin-19 fragment; DT, decision tree; ELISA, enzyme-linked immunosorbent assay; LC, lung cancer; LDCT, low-dose computed tomography; NC, normal control; NSE, neuron-specific enolase; ROC, receiver operating characteristic; SEREX, serological analysis of recombination cDNA expression libraries; SERPA, serological proteome analysis; SNR, signal-to-noise ratio; SVM, support vector machines; TAA, tumor-associated antigen; TAAb, autoantibody to TAA.

MATERIALS AND METHODS

Study Populations

In this study, totally 1,204 subjects [555 LCs, 505 normal controls (NCs), and 144 benign lung disease cases (BLDs)] in three independent cohorts (discovery cohort, verification cohort, and validation cohort) were recruited from the First Affiliated Hospital of Zhengzhou University in Henan province, China between November 2016 and April 2019 (**Table 1**). All specimens were collected with patients' written informed consent, and the study protocol was approved by Medical Ethics Committee of Zhengzhou University (Zhengzhou, China). The process of serum specimen preparation and the inclusion criteria of subjects were presented in **Supplementary Texts 1,2**, respectively.

Focused Protein Array

A total of 154 human source recombinant proteins, including 143 proteins encoded by cancer driver genes and 11 proteins (CyclinB1, c-Myc, CIP2A/p90, IMP1, IMP2, IMP3, RalA, RBM39, YWHAZ, and two fragments of Survivin) previously researched in our laboratory, were contained in the focused protein array. The array was customized in CDI Laboratories (Mayaguez, USA). The array screening, data extraction, and analysis were implemented according to the protocol illustrated in **Supplementary Text 3**. Signal-to-noise ratio (SNR) was used to describe the serum level of autoantibodies in the subjects of discovery cohort. Based on the results of array test, we carried out comprehensive analyses to screen candidate TAAbs for LC (**Supplementary Figure 1**).

ELISA

Indirect ELISA was used to detect the level of candidate TAAbs in serum samples of verification cohort and validation cohort. Detailed steps of the indirect ELISA experiment are presented in **Supplementary Text 4**. In this study, the verification cohort was used to test the eligibility of candidate TAAbs, and validation cohort to further validate the diagnostic performance of TAAbs. The positive and negative control sera of the TAAb were set in each plate for quality control. Furthermore, the concentration of autoantibodies in the serum was calculated according to the IgG standard curve of each plate.

The Establishment of Diagnostic Model by Data Mining Methods

All diagnostic models were established by using SPSS Modeler 18.0 software. In order to establish and externally evaluate the diagnostic models, all LCs and NCs in the validation cohort were randomly divided into training ($N = 414$) and test ($N = 186$) sets

TABLE 1 | Characteristics of populations in this study.

	Discovery cohort		Verification Cohort		Validation Cohort		
	LC N (%)	NC N (%)	LC N (%)	NC N (%)	LC N (%)	BLD N (%)	NC N (%)
N	100	50	155	155	300	144	300
Age							
Mean ± SD (years)	61 ± 11	40 ± 13	61 ± 10	60 ± 11	61 ± 11	60 ± 10	57 ± 11
Range (years)	26–85	20–71	30–83	28–81	26–87	29–85	25–89
Gender							
Male	66 (66.0)	23 (46.0)	116 (74.8)	116 (74.8)	185 (61.7)	103 (71.5)	156 (52.0)
Female	34 (34.0)	27 (54.0)	39 (25.2)	39 (25.2)	115 (38.3)	41 (28.5)	144 (48.0)
Smokers							
Yes	45 (45.0)		98 (63.2)		111 (37.0)	78 (54.2)	
No	55 (55.0)		57 (36.8)		178 (59.3)	66 (45.8)	
Unknown	0 (0.0)		0 (0.0)		11 (3.7)	0 (0.0)	
Drinkers							
Yes	26 (26.0)		45 (29.0)		54 (18.0)	36 (25.0)	
No	74 (74.0)		110 (71.0)		233 (77.7)	108 (75.0)	
Unknown	0 (0.0)		0 (0.0)		13 (4.3)	0 (0.0)	
Family history of tumor							
Yes	12 (12.0)		28 (18.1)		22 (7.3)	18 (12.5)	
No	88 (88.0)		127 (81.9)		263 (87.7)	126 (87.5)	
Unknown	0 (0.0)		0 (0.0)		15 (5.0)	0 (0.0)	
Clinical stage							
Stage I	18 (18.0)		11 (7.1)		51 (17.0)		
Stage II	12 (12.0)		11 (7.1)		12 (4.0)		
Stage III	33 (33.0)		58 (37.4)		44 (14.7)		
Stage IV	37 (37.0)		60 (38.7)		81 (27.0)		
Unknown	0 (0.0)		15 (9.7)		112 (37.3)		
Histological type							
SCC	31 (31.0)		42 (27.1)		64 (21.3)		
AD	68 (68.0)		58 (37.4)		177 (59.0)		
SCLC	0 (0.0)		43 (27.7)		32 (10.7)		
Others	1 (1.0)		12 (7.8)		15 (5.0)		
Unknown	0 (0.0)		0 (0.0)		12 (4.0)		
Tumor size							
≤5 cm	60 (60.0)		59 (38.1)		126 (42.0)		
>5 cm	40 (40.0)		80 (51.6)		79 (26.3)		
Unknown	0 (0.0)		16 (10.3)		95 (31.7)		
Lymph node metastasis							
Yes	69 (69.0)		99 (63.9)		124 (41.3)		
No	31 (31.0)		41 (26.4)		72 (24.0)		
Unknown	0 (0.0)		15 (9.7)		104 (34.7)		
Distant metastasis							
Yes	38 (38.0)		61 (39.4)		109 (36.3)		
No	62 (62.0)		79 (50.9)		112 (37.4)		
Unknown	0 (0.0)		15 (9.7)		79 (26.3)		
Benign disease type							
COPD						72 (50.0)	
Chronic bronchitis						72 (50.0)	

AD, adenocarcinoma; BLD, benign lung disease; COPD, chronic obstructive pulmonary disease; LC, lung cancer; NC, normal control; SCC, squamous cell carcinoma; SCLC, small cell lung cancer; SD, standard deviation.

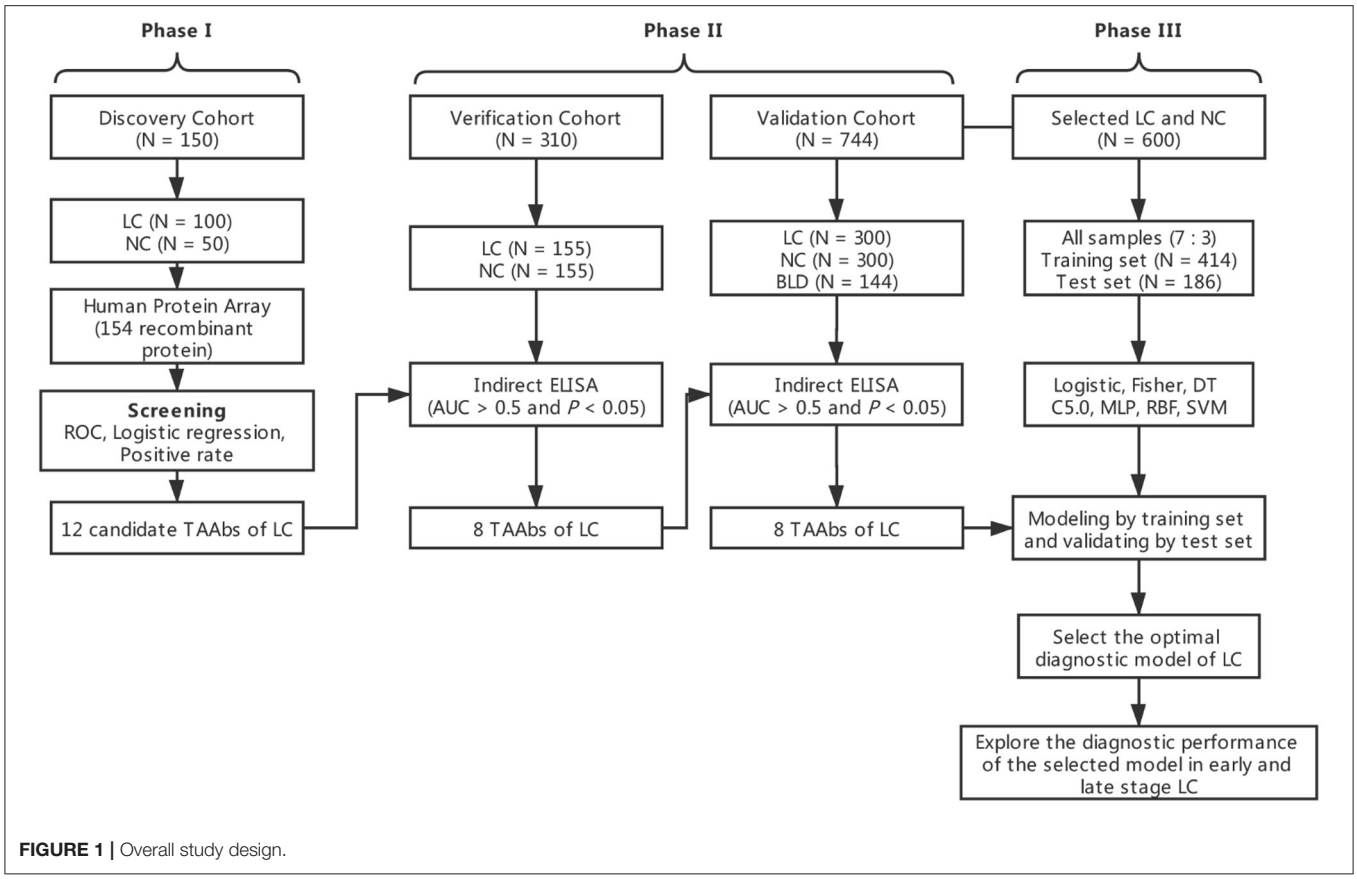

FIGURE 1 | Overall study design.

according to the proportion of 7:3 by SPSS 21.0 software. Logistic regression analysis, Fisher discriminant analysis, DT C5.0, SVM, ANN-MLP, and ANN-RBF were applied to build models based on training set and then the models' performance were validated in test set. Additionally, Logistic regression models were established through forward and backward conditional logistic regression, respectively. The stepwise method and internal cross-validation were used in the Fisher discriminant model. In the construction of DT C5.0 model, decision tree was picked as the model output type with 10-fold cross-validation as internal validation. In order to improve the model, expert and global pruning mode were chosen, meanwhile, pruning severity and the minimum number of record for each sub-branch were set to 80 and 2, respectively. We also constructed models by MLP and RBF methods. MLP had more terminative rules than RBF (using a maximum training time of 1 min) and overfitting prevents the set from being 50.0% when choosing parameters of model. Moreover, we established SVM model in which the expert mode was selected. All methods were applied to distinguish LCs from NC.

Statistical Analysis

SPSS 21.0 software package, GraphPad Prism 5.0, and MedCalc 11 were used to analyze and visualize the data from ELISA in this research. Differences of TAAbs levels among the different groups were analyzed by non-parametric tests and Wilcoxon test with Bonferroni adjustment. The sensitivity, specificity, and AUC with 95% confidence internal (CI) were all calculated by receiver operating characteristic (ROC) curve analysis. The OD value produced at the highest Youden's Index (sensitivity + specificity −1) was set as the cutoff value. The difference was considered statistically significant while $P < 0.05$.

RESULTS

Overall Study Design

The overall study was divided into three phases including the discovery of potential TAAbs, the validation of candidate TAAbs, and the establishment of diagnostic models (**Figure 1**). Briefly, in phase I, the serum samples of discovery cohort containing 100 LCs and 50 NCs were individually profiled on focused protein array. In phase II, 155 LCs and 155 NCs in the verification cohort were matched by age and gender, which was used to verify the screened candidate TAAbs from protein array. In addition, there were 300 LCs, 300 NCs, and 144 BLDs in the validation cohort, which was used to validate the TAAbs from the verification cohort. In phase III, the ELISA results of eight TAAbs of the LCs and NCs in validation cohort were applied to build and test the diagnostic models.

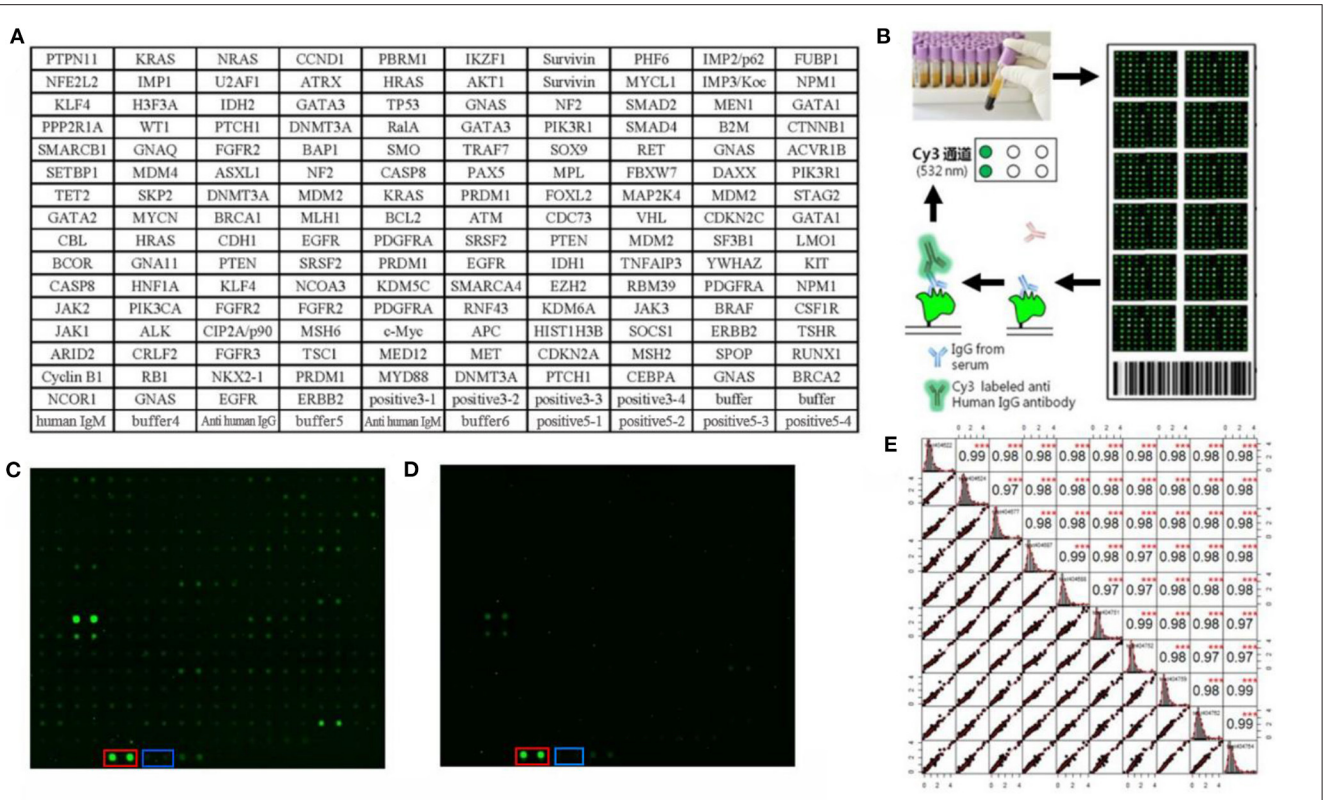

FIGURE 2 | Protein array customization and preliminary results. **(A)** Protein array layout. **(B)** The operation process and principle of the protein array. **(C,D)** Protein fluorescence quantification results of LC and NC, respectively. The red and blue frames highlight the positive control (anti-human IgG) and negative control (buffer). **(E)** The result of repeated experiments by the same serum sample. The lower left showed the distribution of the results after linear fitting, and the upper right showed correlation results between samples after linear fitting (***$P < 0.001$). The middle graph was the cumulative density distribution of a single sample.

Screening 12 Potential TAAbs for LC Based on Focused Protein Array

One hundred serum samples from LCs and 50 sera from NCs were tested by customized protein array. The 154 human recombinant protein, positive control (antihuman IgG) and negative control (buffer) arranged according to the protein array layout that shows in **Figure 2A**. The operation process and principle of the protein array were visualized in **Figure 2B**. As shown in **Figures 2C,D**, the fluorescent scanning signal results of two representative samples illustrated that the IgG response of the LC case was stronger than the NC.

Before the formal experiment, we repeated the tests 30 times in total on the same sample at different times, different arrays, and different locations to evaluate the stability of the array and the operation. From the results, the overall average value of repeatability between different batches of arrays was 0.98, indicating the overall stability was great (**Figure 2E**).

As exhibited in the **Supplementary Figure 1**, based on the criteria of AUC >0.5 and $P < 0.05$ by ROC analysis, the 40 TAAbs were preliminarily screened (**Supplementary Table 1**). Then, totally 15 TAAbs of them were further screened, which included 11 TAAbs selected by regression analysis and four TAAbs studied in our previous research. Whereafter, according to the criteria of the positive rate of LC minus NC was > 10%, we ultimately selected 12 candidate TAAbs which involved in carcinogenesis, such as cell cycle, apoptosis, PI3K pathway, and RAS pathway (**Supplementary Table 2**) for further verification. Higher level of the 12 TAAbs was observed in LCs than NCs ($P < 0.05$) (**Figure 3A**). The AUC of each TAAb was ranged from 0.596 (95% CI: 0.504–0.689) to 0.706 (95% CI: 0.643–0.769) (**Figure 3B**).

Verifying the Candidate TAAbs by ELISA in Verification Cohort

In order to determine the diagnostic validity of 12 TAAbs, we tested these TAAbs in 310 serum samples in the verification cohort (155 LCs and 155 NCs) by ELISA. The results were highly consistent with the discovery phase. According to screening criteria of AUC >0.5 and $P < 0.05$, four TAAbs (P62, Survivin, PBRM1, and JAK2) were excluded. The concentration level of the other eight TAAbs in the serum of LCs was significantly higher than NCs ($P < 0.05$) (**Supplementary Figure 2A**). As displayed in **Supplementary Figure 2B**, GNA11 owned the highest AUCof 0.802 (95% CI: 0.753–0.850).

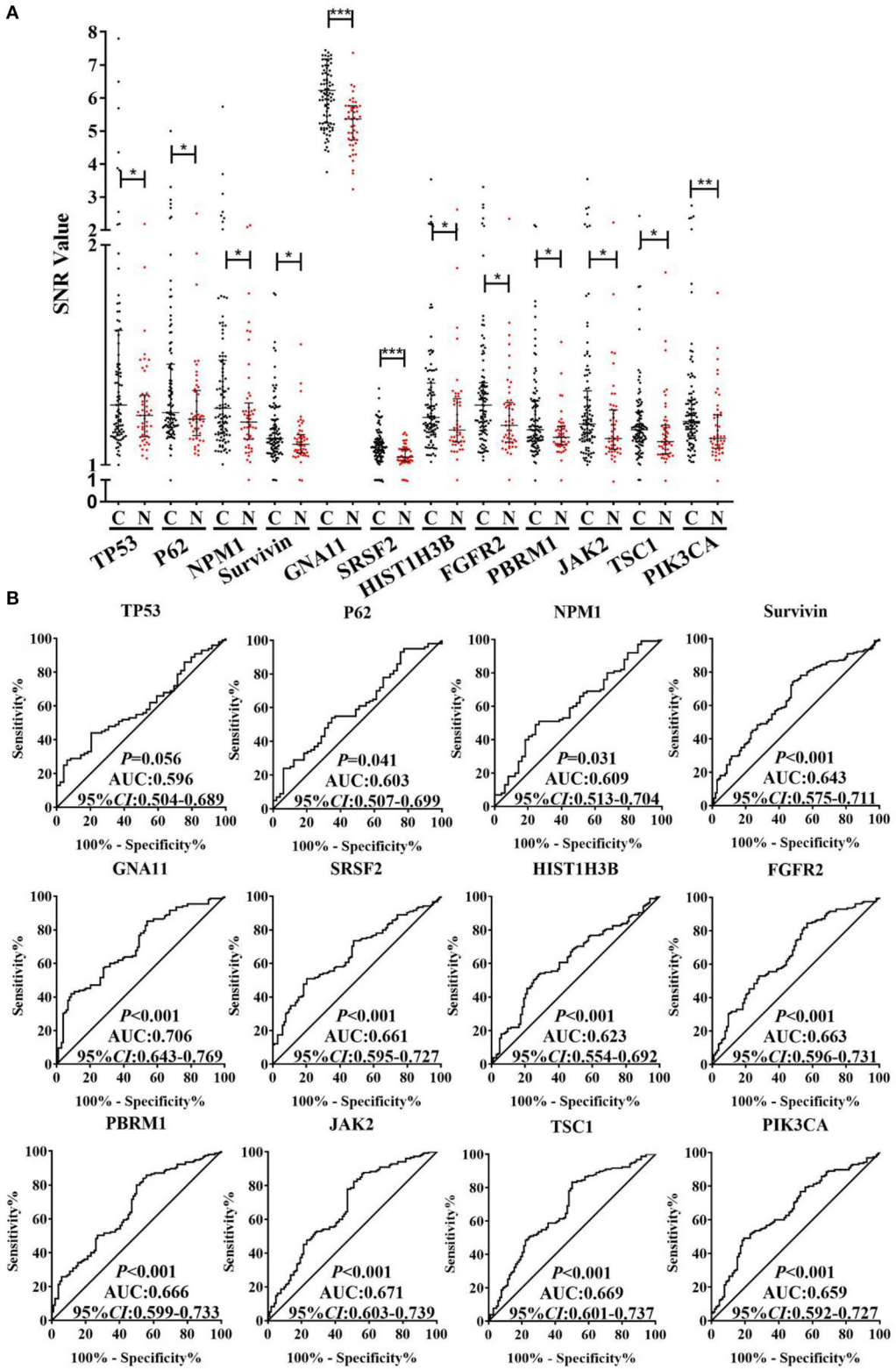

FIGURE 3 | (A) SNR of autoantibodies against 12 TAAs in discovery cohort with 100 LCs and 50 NCs. **(B)** ROC analysis of autoantibodies against 12 TAAs for LC detection in discovery cohort. C, cancer; N, normal; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

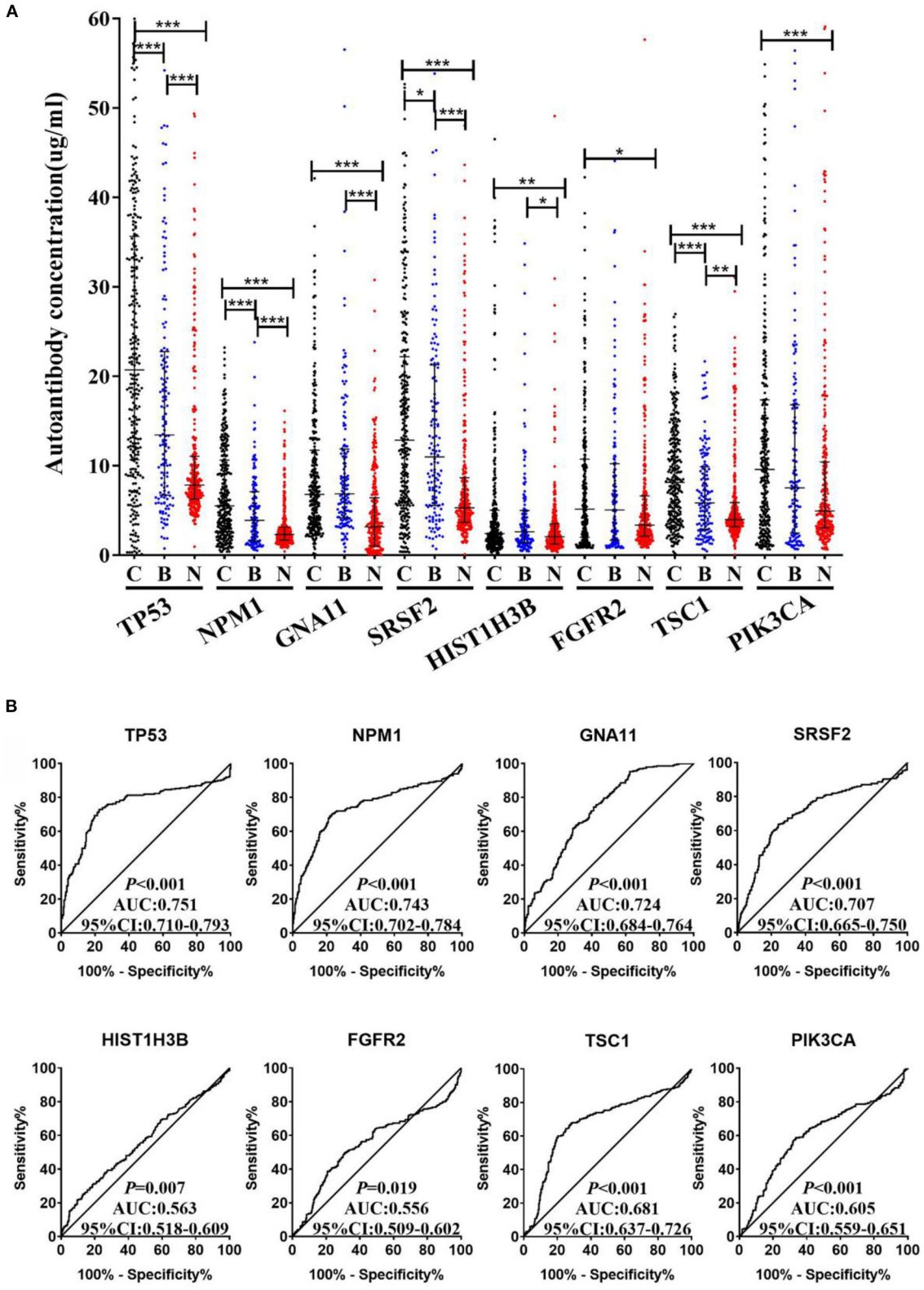

FIGURE 4 | (A) The expression of autoantibodies against eight TAAs in validation cohort with 300 LCs, 144 BLDs, and 300 NCs. **(B)** ROC analysis of autoantibodies against eight TAAs for LC and NC groups in validation cohort. C, cancer; B, benign; N, normal; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

The Performance of the Eight TAAbs in Validation Cohort and Establishment of Diagnostic Model

An independent validation cohort, including 300 LCs, 300 NCs, and 144 BLDs, was then used to validate the above eight TAAbs. As indicated in **Figure 4A**, all eight TAAbs showed significantly higher level in LCs compared with NCs. Interestingly, the serum levels of four TAAbs (TP53, NPM1, SRSF2, and TSC1) in LCs were significantly higher than BLDs. The AUCs of eight TAAbs for distinguishing LCs from NCs were ranged from 0.556 (95% CI: 0.509–0.602) for FGFR2 to 0.751 (95% CI: 0.710–0.793) for TP53 (**Figure 4B**), and the sensitivities were 13.7–43.0% at the specificities ≥90% (**Supplementary Table 3**). Besides, we investigated the correlation of the eight TAAbs and histologies; however, the results revealed that there were no differences among the adenocarcinoma patients, squamous cell carcinoma patients, and small cell lung cancer patients in serum TAAbs ($P > 0.05$) (data not shown).

In order to explore the optimal diagnostic model with higher diagnostic accuracy than single TAAb for LCs, six modeling methods were performed and compared. Clearly, the model established by the DT C5.0 yield the most remarkable diagnostic performance among the six models (**Figure 5**), which contain seven TAAbs (TP53, NPM1, FGFR2, PIK3CA, GNA11, HIST1H3B, and TSC1) and possessed an AUC of 0.897 (95% CI: 0.863–0.924), sensitivity of 94.4%, specificity of 84.9%, and accuracy of 89.9% (**Table 2**). Meanwhile, it also achieved an excellent achievement in the test set, the AUC, sensitivity, specificity, and accuracy were 0.838 (95% CI: 0.777–0.888), 89.4, 78.2, and 83.3% (**Table 2**).

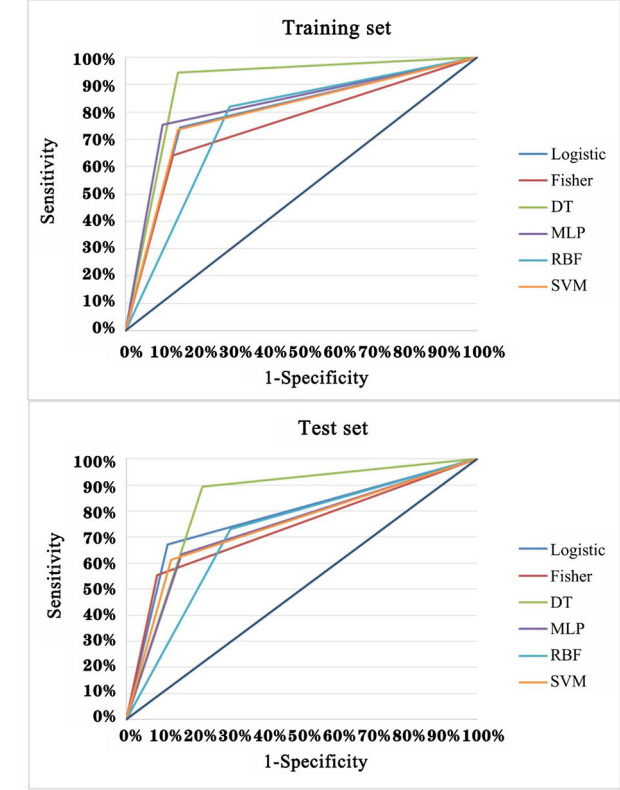

FIGURE 5 | ROC analysis of multiple models for the differential diagnosis of LCs and NCs in training set and test set of validation cohort.

Evaluation of the Performance of the Optimal Model in Different Stages of LC

According to clinical stages I, II, III, and IV (AGCC), stages I and II of LC were defined as early LC ($N = 72$) and stages III and IV as late LC ($N = 141$) (**Table 3**). For the diagnosis of early LC, TP53 owned the highest AUC (95% CI) of 0.840 (0.782–0.898), while the AUC of DT C5.0 model achieved 0.886 (95% CI: 0.845–0.926).The sensitivity of single TAAb in early LC ranged from 13.9 to 48.6%, while it dramatically increased to 94.4% in DT 5.0 model established by seven TAAbs. However, the specificity of the model (82.7%) was slightly reduced compared with the single TAAb (92.0–95.3%). For the late LC, the AUC (95% CI), sensitivity of DT C5.0 model were 0.864 (0.826–0.902) and 90.1%, which were obviously higher than single TAAb. Yet, the specificity of the model was only reduced about 10% in late LC compared with the single TAAb. Moreover, the accuracies of the model in both early and late stages were close to 90%, which highly improved the results of single TAAbs.

DISCUSSION

In recent years, with the rapid development of proteomics methods, the discovery of new serum biomarkers has been greatly promoted by protein array which is a high-throughput method to screen specific antibody targets against protein samples (25). Hence, the protein array technique was selected for high-throughput screening in current research.

Although one study has utilized protein array to identify TAAbs for LC (26), our research design owned several novel features. First, the protein array was customized based on 138 cancer driver genes which were the key carcinogenic factors that could promote the rapid growth of tumors. On this basis, the possibility of screening out meaningful biomarkers was improved to some extent. Second, the candidate TAAbs were verified and validated in the multiple independent cohorts with more than 1,000 samples, so that the diagnostic value of these TAAbs was very reliable on account of the consistency between ELISA and protein array results. Third, we applied multiple data mining methods to establish diagnostic models and then selected the optimal model, which not only yielded further improvements in diagnostic performance but also avoided the insufficiency of using a single modeling approach.

Cancer is a disease that is caused by the DNA sequence in the genomes of cancer cells changing (20). Besides, cancer driver genes were defined as the important genes which related to the occurrence and development of cancer, and the determination of cancer driver genes is key to advancing diagnostics, therapeutics, and treatments (27). Bert Vogelstein et al. (21) summarized 138 cancer driver genes (74 tumor suppressor genes and 64

TABLE 2 | The performance of multiple models in training set and test set for lung cancer detection.

Modeling approach	TAAbs	Training set				Test set				
		P	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Accuracy (%)	*P*	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Accuracy (%)
Fisher	5	<0.0001	0.753 (0.709–0.794)	64.2 (57.4–70.6)	86.4 (80.9–90.9)	74.9	<0.0001	0.732 (0.662–0.794)	55.3 (44.1–66.1)	91.1 (83.8–95.8)	74.7
Logistic	6	<0.0001	0.794 (0.752–0.832)	74.4 (68.0–80.1)	84.4 (78.6–89.2)	79.2	<0.0001	0.776 (0.709–0.834)	67.1 (56.0–76.9)	88.1 (80.2–93.7)	78.5
DT C5.0	7	<0.0001	0.897 (0.863–0.924)	94.4 (90.5–97.1)	84.9 (79.2–89.6)	89.9	<0.0001	0.838 (0.777–0.888)	89.4 (80.8–95.0)	78.2 (68.9–85.8)	83.3
MLP	8	<0.0001	0.824 (0.784–0.859)	75.4 (69.0–81.0)	89.5 (84.3–93.3)	82.1	<0.0001	0.738 (0.669–0.800)	63.5 (52.4–73.7)	84.2 (75.6–90.7)	74.7
RBF	8	<0.0001	0.761 (0.717–0.801)	81.9 (76.0–86.8)	70.4 (63.5–76.6)	76.3	<0.0001	0.716 (0.646–0.780)	72.9 (62.2–82.0)	70.3 (60.4–79.0)	71.5
SVM	8	<0.0001	0.792 (0.750–0.830)	73.5 (67.1–79.3)	84.9 (79.2–89.6)	79.0	<0.0001	0.742 (0.672–0.803)	61.2 (50.0–71.6)	87.1 (79.0–93.0)	75.3

AUC, area under the receiver operating characteristic curve; CI, confidence interval; DT C5.0, Decision Tree C5.0; Fisher, Fisher discriminant analysis; Logistic, Logistic regression analysis; MLP, multilayer perception; RBF, radial basis function; SVM, support vector machines; TAAbs, autoantibodies to tumor-associated antigens; 5 TAAbs, TP53, NPM1, FGFR2, GNA11, and HIST1H3B; 6 TAAbs, TP53, NPM1, FGFR2, PIK3CA, GNA11, and HIST1H3B; 7 TAAbs, TP53, NPM1, FGFR2, PIK3CA, GNA11, HIST1H3B, and TSC1; 8 TAAbs, TP53, NPM1, SRSF2, FGFR2, PIK3CA, GNA11, HIST1H3B, and TSC1.

oncogenes) which can promote or "drive" tumorigenesis when altered by intragenic mutations. We customized a protein array including 154 human recombinant proteins based on the 138 genes to explore the level of autoantibodies to the proteins encoded by these genes, which integrated the merits of cancer driver gene and TAAb.

Applying the protein array technology, we analyzed the level of autoantibodies against 154 proteins in serum from 100 LCs and 50 NCs. According to multiple statistical analyses and screening criteria, 12 TAAb candidates were rapidly identified in the discovery phase. These TAAbs are all involved in some important carcinogenesis functions (**Supplementary Table 2**), and eight of them were first discovered in this research for diagnosis of LC. The remaining four TAAbs have been studied in various cancers, including TP53 (28–30), P62 (31, 32), NPM1 (33, 34), and Survivin (35).

In the verification phase, these 12 TAAbs were tested using indirect ELISA in 155 LCs and 155 matched NCs to assess their performance in distinguishing LCs from NCs. Furthermore, eight TAAbs (TP53, NPM1, GNA11, SRSF2, HIST1H3B, FGFR2, TSC1, and PIK3CA) were further selected on account of their excellent performance in verification cohort and subjected to validation cohort with 300 LCs, 300 NCs, and 144 BLDs. The basically consistent results of multistage and multicohort validation testified the reliability of our study. Remarkably, the level of anti-TP53 was found to be statistically significantly higher in LC than NC, which yielded the highest diagnostic value with the AUC (95% CI) of 0.751 (0.710–0.793). Park et al. (36) also found the significance of anti-TP53 in the diagnosis of LC. Besides, it was regrettably found that the majority single TAAbs had lower diagnostic performance for LC, which was similar to the results shown in previous studies (37). In order to improve the diagnostic value, we combined different TAAbs by using diverse data mining methods.

In recent years, various data mining techniques have been widely used to establish cancer diagnostic models, such as logistic regression analysis (38), Fisher discriminant analysis (39), decision tree (40), support vector machine (41), ANN-MLP, and ANN-RBF (42). However, each method has its own strengths and weaknesses, so the current study aimed to build LC diagnostic models through different modeling methods and validate the diagnostic value of each model for LC in a test set for choosing an optimal model. In result, we selected the decision tree model with a seven-TAAb panel (TP53, NPM1, FGFR2, PIK3CA, GNA11, HIST1H3B, and TSC1) which yield the highest AUCs of 0.897 (95% CI: 0.863–0.924) and 0.838 (95% CI: 0.777–0.888) for distinguishing LCs from NCs in training set and test set. Moreover, the results of the seven TAAbs and the panel of TAAbs in this study showed better discriminatory power for the early-stage LC than the advanced stage (**Table 2**). The above result may imply that autoantibodies to tumor-associated antigens, as a kind of promising biomarkers produced in early stage of tumorigenesis, could own more chances to be applied for early diagnosis in cancers.

However, as to the limitation, the small sample size of early-stage LCs might limit the expansibility of the value of this diagnostic model. Therefore, in our further research, we

TABLE 3 | The diagnostic performance of DT 5.0 model and the seven TAAbs in early and late stage LC.

TAAbs	AUC (95% CI)	*P*	Sensitivity (%)	Specificity (%)	YI	PPV (%)	NPV (%)	Accuracy (%)
Early stage (I + II; *N* = 72)								
TP53	0.840 (0.782–0.898)	0.000	48.6	92.7	0.413	86.89	64.33	70.64
NPM1	0.837 (0.778–0.897)	0.000	48.6	94.0	0.426	89.01	64.65	71.31
GNA11	0.733 (0.672–0.793)	0.000	26.4	95.3	0.217	84.97	56.43	60.86
HIST1H3B	0.567 (0.484–0.650)	0.078	13.9	95.3	0.092	74.85	52.54	54.61
FGFR2	0.639 (0.558–0.719)	0.000	15.3	94.0	0.093	71.80	52.60	54.64
TSC1	0.749 (0.683–0.816)	0.000	18.1	92.0	0.101	69.30	52.89	55.03
PIK3CA	0.668 (0.592–0.744)	0.000	15.3	93.3	0.086	69.62	52.42	54.31
DT C5.0	0.886 (0.845–0.926)	0.000	94.4	82.7	0.771	84.49	93.70	88.56
Late stage (III + IV; *N* = 141)								
TP53	0.710 (0.651–0.769)	0.000	35.5	92.7	0.281	82.86	58.95	64.06
NPM1	0.707 (0.650–0.764)	0.000	27.0	94.0	0.210	81.79	56.27	60.48
GNA11	0.727 (0.679–0.774)	0.000	19.1	95.3	0.145	80.41	54.11	57.24
HIST1H3B	0.565 (0.506–0.624)	0.027	9.2	95.3	0.046	66.39	51.22	52.28
FGFR2	0.509 (0.448–0.571)	0.750	7.8	91.0	−0.012	46.43	49.67	49.40
TSC1	0.641 (0.582–0.701)	0.000	9.2	92.0	0.012	53.54	50.33	50.61
PIK3CA	0.576 (0.516–0.636)	0.010	14.9	90.0	0.049	59.82	51.40	52.45
DT C5.0	0.864 (0.826–0.902)	0.000	90.1	82.7	0.727	83.86	89.28	86.37

AUC, area under the receiver operating characteristic curve; CI, confidence interval; DT C5.0, Decision Tree C5.0; LC, lung cancer; NPV, negative predictive value; PPV, positive predictive value; TAAbs, autoantibodies to tumor-associated antigens; YI, Youden's Index.

will confirm the diagnostic utility of this TAAb panel in a large sample size study to verify our findings, and explore its differential diagnostic performance between benign and malignant pulmonary nodules.

In conclusion, focused protein array based on cancer-driver genes is an effective and fast approach to discovering novel TAAbs. Comprehensive analysis of multiple models established by data mining showed that the DT C5.0 model generated by the combination of seven TAAbs had the highest LC diagnostic value. In consequence, the model may be the auxiliary means for clinicians to diagnose early-stage LC, and it may have a great influence in improving the accuracy of LC diagnosis.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Zhengzhou University (Zhengzhou, China). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LD and JZ: conception and design. LD: administrative support. TW, LP, CS, KW, and XW: provision of study materials or patients. DJ, XZ, ML, YW, PW, HY, and JS: collection and assembly of data. DJ and XZ: data analysis, interpretation, and manuscript writing. All authors: final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.658922/full#supplementary-material

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REVIEW
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Tumor-Associated Antigens (TAAs) for the Serological Diagnosis of Osteosarcoma

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Osteosarcoma (OS) is the most common form of malignant bone tumor found in childhood and adolescence. Although its incidence rate is low among cancers, the prognosis of OS is usually poor. Although some biomarkers, such as p53, have been identified in OS, the association between the biomarkers and clinical outcome is not well understood. Thus, it is necessary to establish a method to identify patients diagnosed with OS at an early stage. It is becoming obvious that anti-tumor-associated antigens (TAAs) autoantibodies (TAAbs) in sera could be used as serological biomarkers in the detection of many different types of cancers. This notion indicates that TAAbs are considered as immunological "sentinels" associated with tumorigenesis underlying molecular events. It provides new insights into the molecular and cellular biology of the differential diagnosis of cancers. What's more, it is reported that a customized TAA array could significantly increase the sensitivity/specificity. TAA arrays also have great application prospects in detecting cancer at an early stage, monitoring cancer progression, discovering new therapeutic targets, and designing personalized treatment. In this review, we provide an overview of the TAAs identified in OS as well as the possibility that TAAs and TAAbs system be used as biomarkers in the immunodiagnosis and prognosis of OS.

Keywords: osteosarcoma, tumor-associated antigen, autoantibodies, biomarker, immunodiagnosis, prognosis

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INTRODUCTION

Osteosarcoma (OS) is characterized by the production of bone-like substances by malignant osteoblasts, which is the most common and highly malignant primary bone tumor that originates from primary osteoblasts (1). It is, like all other sarcomas, rare with an incidence rate of less than 1% of all cancers diagnosed (2). It is estimated by the American Cancer Society that approximately 800 new cases arise in the USA each year, and about 400 of them are children and teens. OS is the most common form of bone cancer in children and adolescents ageing from 10 to 20 years old (3). Although the incidence rate of OS is relatively low among all cancers, OS is highly malignant and can often be neglected on misidentification with benign lesions or trauma since the initial symptoms of the disease are commonly quite nonspecific and subtle. Furthermore, OS progresses aggressively. About 20% of the patients have metastases usually detected in the lung

when initially diagnosed (4–7). OS is one of the most dangerous primary malignant tumors in childhood and adolescence, resulting in a high rate of amputation, disability, and death. At present, therapy for OS is still inadequate. The five-year survival rate is approximately 60% even after pre- and post-operative, neoadjuvant chemotherapy and excision of operable lesions (8). Moreover, tumor size and metastases presented at initial diagnosis always portend a worse outcome (5, 9). Thus, a critical need in the diagnosis and treatment of OS is to select an optimal array of sera biomarkers that can be used in clinic. These biomarkers could assist to detect tumors at an early stage with high specificity/sensitivity and limited invasiveness. This array could help to predict whether patients in need of treatment will develop aggressive tumors.

Over the last few decades, many studies demonstrated that these autologous cells developing into tumors contained tumor-associated-antigens. The abnormal exposure or presentation of these antigens recognized by the human immune system could further trigger autoantibodies, that have been termed tumor-associated autoantibodies (TAAbs), against these cellular antigens. This notion has come from evidence that TAAbs are immunological "sentinels" associated with tumorigenesis underlying molecular events (10–12). The content of TAAbs could increase in the very early stage during carcinogenesis (13). TAAbs are stable with high levels in patients' sera even though the level of the corresponding antigens is low (14) or even after removal of these antigens (15, 16). Such benefits of TAAbs have triggered a growing enthusiasm for applying these TAAbs as serological cancer biomarkers. Moreover, in recent years, increasing attention has been given in humoral immunity to TAAs in particular clinical fields, such as the possible use of TAAs and TAAbs systems as cancer biomarkers not only in detecting cancer at an early stage, but also to monitor cancer progression, discover new therapeutic targets, and design personalized therapeutic interventions (17).

At present, emerging studies engaged at the molecular markers or pathways on OS have revealed the key roles of these molecules (13) in OS tumorigenesis and prognosis. These molecular markers could potentially be used to predict the diagnostic accuracy or micrometastasis when diagnosed and when to treat with chemotherapy. Further, such pathways could also be possible targets for new chemotherapeutic agents in OS. The information of the TAAs of human OS is still inadequate. There are too many genes and corresponding protein products, like melanoma-associated antigen (MAGE) (18), HER2 (19), p53 (20), HSP (21), squamous cell carcinoma antigen recognized by T cells (SART1) (22), SART3 (23), or papillomavirus binding factor (PBF) (24), which were reported to be expressed in OS. Unfortunately, it is generally recognized that they are still insufficient in the application of available clinical information in diagnosing cancer early, designing personalized treatment, and predicting prognosis. Therefore, it is necessary to develop innovative diagnostic and prognostic tools to effectively manage OS by utilizing clinical biomarkers.

This review aims to summarize the established and experimental TAAs and TAAbs tested in OS in recent years. It is useful to summarize the idea and possibility of detecting TAAs and TAAbs in the immunodiagnosis and prognosis of OS.

TAAs IN OS

It is now evident that the sera of cancer patients comprise autoantibodies that can react with TAAs. These TAAs are varied and contain a unique group of autologous cellular antigens (10, 25, 26), including the tumor suppressor p53 (27, 28), oncogene products such as HER-2/neu and ras (29), proteins that protect mRNAs from degradation such as p62 (30) and CRD-BP (31), onconeural antigens (32), differentiation-antigens such as tyrosinase and the cancer/testis antigens (33), and anti-apoptotic proteins such as survivin (34) and LEDGF (35). The factors leading to the production of these autoantibodies are not fully understood. The existing studies suggested that many target antigens were cellular proteins, and their abnormal regulation or overexpression may lead to tumorigenesis. This could take p53 for example (27, 28). In the case of mRNA binding protein p62, a fetal protein missing in adult tissues, immunogenicity seems to be associated with abnormal expression of p62 in cells of tumor (36). In some cancer patients, the immune system seems to have the ability to sense these abnormalities and react by producing autoantibodies (37). Autoantibodies associated with a particular type of cancer are targeted at proteins that are abnormally regulated or activated in the molecular pathways involved in the malignant transformation of that particular type of cancer (38). Taken together, TAAbs may be considered as the reporter of abnormal cellular mechanisms during tumorigenesis (10).

Although the reports on types and functions of TAAs in OS are still limited, these tumors might express some diagnostic and/or therapeutic targets. The numerous TAAs summarized in **Table 1** have been described in previous studies. In human OS, the high expression rate of HER2/erbB-2 was 40-45%, which was related to poor prognosis, early lung metastases, and poor response to preoperative chemotherapy (39, 40). p53 was localized in euchromatic areas of nuclei of OS cells, and was involved in the development of OS, but not correlated with any clinical factors (20, 41). Some previous reports had shown that the expression of P-glycoprotein may have an association with the increasing risk of chemotherapy resistance (42, 43, 76, 77). Afterward, it was reported that hsp27 was overexpressed related to the poor prognosis of OS (44). Sudo et al. suggested that melanoma antigen (GAGE) family members were expressed in substantial numbers of OS as tumor-rejection antigens in the main histocompatibility class-I restriction mode (18). And in sarcoma cell lines, hsp72 was selectively expressed on the cell surface, overcoming the protective effect and acting as a target for natural killer cells (46), which was related to the good response of neoadjuvant chemotherapy (47). The GD2 ganglioside was found overexpressed in OS (48) and later, some groups demonstrated that the therapy anti-GD2 antibody can improve the survival rate of high-risk neuroblastoma (49). Two tumor-rejection antigens, SART1 (22) and SART3 (23), were reported to be expressed in OS, which suggests that these proteins and their

TABLE 1 | Identification of TAAs or TAAbs analyzed in multiple studies.

TAA or TAAb	Description	Observation in OS	Ref.
HER2	Oncogene	Correlates with poor prognosis for patients with OS.	(39, 40)
P53	Tumor suppressor	Fails as a maker in OS because of no significant correlation between p53 expression and the clinical outcome and response to chemotherapy.	(20, 41)
P-glycoprotein	ATP-binding cassette (ABC) transporters	Increased risk for chemotherapy resistance.	(42–43)
Hsp27	Heat shock protein 27, protein chaperone and antioxidant	Correlates with poor prognosis for patients with OS.	(44)
MAGEA	Melanoma antigen family A	Expressed in substantial numbers of OS in a major histocompatibility class-I-restricted manner.	(18, 45)
Hsp72	Heat shock protein 70 family and a chaperone protein	Correlates with a good response to neoadjuvant chemotherapy.	(46, 47)
GD2	Disialoganglioside GD2, a sialic acid-containing glycosphingolipid	Overexpressed in OS.	(48, 49)
SART1, SART3	Squamous cell carcinoma antigen recognized by T cells, tumor-rejection antigens	Potentially used in specific immunotherapies HLA-A24$^+$ patients with OS or malignant fibrous histiocytosis.	(22, 23)
B7-H3	58 kDa glycosylated tumor-associated protein antigen	Potential molecules for use in specific immunotherapies for HLA-A24$^+$ patients with OS or malignant fibrous histiocytosis.	(50)
Melanoma antigen MUC18	MCAM (melanoma cell adhesion molecule) and as CD146 (endothelial antigen)	Correlates directly with tumor progression and metastatic potential.	(51)
CXCR4	Chemokine receptor type 4, an alpha-chemokine receptor	Potentially used as a prognostic factor and as a predictor of potential metastatic development in OS.	(52)
SAA	Serum amyloid A, a family of apolipoproteins associated with high-density lipoprotein (HDL)	Increased SAA levels associated with type of tumor and high-risk OS development.	(53)
CLUAP1	Clustering associated protein 1	Potentially used as a prognostic/diagnostic marker and/or for a target of immunotherapy of OS.	(54)
GAGE 1,2,8	g melanoma antigen (GAGE)	Expressed *GAGE-1, 2, 8* in all 9 OS tissue samples.	(55)
NY-ESO-1	New York esophageal squamous cell carcinoma 1 (NY-ESO-1), a cancer-testis antigen	Expressed NY-ESO-1 in 8 of 9 OS tissue samples.	(55)
Survivin	Inhibitor of apoptosis	Potentially used as an independent predictor of survival for OS patients.	(56)
Midkine	Heparin-binding growth factor	Correlates with the prognosis of patients with OS.	(57)
OSAA-3 and OSAA-5	Unknown	Potentially used as candidates for diagnosis and targets for immunotherapy in OS.	(58)
PBF	Papillomavirus binding factor (PBF)	May contribute to peptide-based vaccination and/or adoptive antigen-specific T-cell therapy of patients with OS and other bone and soft tissue tumors.	(59)
TEM1	Tumor endothelial marker 1 (TEM1), prototypical member of a family of genes expressed in the stroma of tumors.	Potentially used as a target protein for selective therapeutic intervention.	(60)
lL-11Rα	Interleukin-11 receptor alpha-chain	May represent a new therapy for patients with OS pulmonary metastases.	(61)
FAP	Fibroblast activation protein	Might be considered as a novel therapeutic target against this cancer.	(62)
Tim-3	T-cell immunoglobulin and mucin domain-3–containing molecule 3	Potential diagnostic and prognostic biomarker for OS progression.	(63)
PCNA	proliferating cell nuclear antigen	Inhibited OS cell proliferation	(64)
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	A potential therapeutic target for the treatment of metastatic OS	(65)
ANG-lgM	angiogenin	Serve as a biomarker for increased risk of OS	(66)
CDK5	Cyclin-dependent kinase 5	Pro-malignant role	(67)
CEACAM1	Carcinoembryonic antigen related cell adhesion molecule 1	May be a prognostic biomarker for OS	(68)
EZH2	Enhancer of zeste homologue 2	May be tumor-associated antigens of OS	(69)
BMI-1	B cell-specific Moloney murine leukemia virus integration site 1	May be tumor-associated antigens of OS	(69)
p16INK4A	Cyclin-dependent kinase inhibitor 2A	May be a useful biomarker to guide the treatment of OS.	(70, 71)
PRDX 2	peroxiredoxin 2	A candidate for chemotherapy responsiveness marker in OS	(72)
CXCL4, CXCL6	CXC chemokines	Associated with OS patient outcomes	(73)
Anti-hsp60 antibody	Autoantibodies against heat shock protein60	Increases of anti-hsp60 antibodies at the time of first diagnosis of OS.	(74)
Anti-hsp90 antibody	Autoantibodies against heat shock protein90	Correlates with a good response to neoadjuvant chemotherapy and their absence correlates with the occurrence of metastases.	(75)
Anti-MUC18 antibody	Autoantibody against MUC18	Inhibits the development of OS metastases in a preclinical model.	(51)
Anti-midkine	Autoantibody against midline	Inhibits growth of OS cells *in vitro*.	(57)

derived peptides could be used as specific immunotherapy molecules in OS patients with HLA-A24+ or malignant fibrous histiocytosis. It was the uniform expression of B7-H3 (8H9 antigen) on the cell membrane that makes it an attractive candidate for targeted immunotherapy (50). Additionally, a cell adhesion molecule, the human METCAM/MUC18 (melanoma antigen/MUC18), with a high expression level in OS played an important role in the metastasis of OS, suggesting that ABX-MA1 might be a new immunotherapeutic approach for OS (51). Furthermore, the study also concluded that, by using a preclinical model, anti-MUC18 antibodies could inhibit the process of OS metastases (51). In the past few years, a relationship between the expression of CXCR4 and initial metastases was discovered (52). SAA expressed higher in OS than benign bone tumors and normal subjects (53). CLUAP1 as potentially a prognostic/diagnostic marker for OS (54) has been suggested. Jacobs et al. reported that all nine OS tissue samples expressed *GAGE-1, 2*, and *8*, and eight of nine expressed NYESO-1, application of cancer-testis antigens or cancer germline genes expressed in solid tumor research of pediatrics (55). Like the other tumors, survivin was also overexpressed in OS as an anti-apoptotic molecule. Several investigators had revealed that the overexpression of survivin was associated with worse clinical outcome, which may be used as an independent predictor for OS patients in survival field (56). More recently, Maehara et al. showed that the expression level of midkine, a heparin-binding growth factor midkine, has an association with the prognosis of OS patients. Anti-midkine functional antibodies can effectively inhibit the growth of OS cells *in vitro* (57).

In the recent past, several studies have done their best to verify new targeting sites for immunotherapy exploiting neither humoral or cellular immune responses to OS, although their precise role in cell biology remains unclear. Among them, OSAA-3 and OSAA-5, two serological antigens, were identified exclusively in the sera of OS patients, but not in normal individuals, which suggests that these two antigens' immune responses were related to OS (58). Papillomavirus binding factor (PBF) was identified by derived cDNA library screening with autologous tumor-reactive CD8+T cells. PBF was overexpressed in most OS and might be helpful for peptide-based vaccine inoculation and/or adoptive antigen-specific T-cell therapy in OS patients as well as in other bone and soft tissue tumors (59). The report by Rouleau et al. showed that the measured level of endosialin/tumor endothelial marker 1 (TEM1) was low in normal tissues, but in several sarcoma subtypes it was in high levels, suggesting that TEM1 may work as a suitable target protein for selecting therapeutic intervention (60). In more recent years, researchers found that interleukin 11 receptor alpha (IL11Rα) (61) and fibroblast activation protein (FAP) (62) were selectively expressed in OS patients compared with healthy groups, which suggests that they may play roles during the process of OS development and progression.

On the other hand, the recognition that the production of TAAbs was stimulated by human tumors has opened a new chapter in cancer biology. More and more studies began to focus on the possibility that autoantibodies may be used as serological tools to diagnose and manage cancer early (78). It has been reported that the titer of anti-hsp60 autoantibody was increased in OS patients but it was not found to be associated with clinical parameters (74). One year later, the other group reported that such immunoreactivity against hsp90 may have predictive value in OS patients because the presence of anti-hsp90 autoantibodies was associated with a good response to neoadjuvant chemotherapy, and their absence was associated with the occurrence of metastasis (75).

Upon the current limited information, TAAbs in OS seem to target proteins involved in tumorigenesis, with high expression in bone tumors. Unfortunately, many published studies on the identification of TAAs have failed to further detail this association. Additionally, since molecular diagnostic and/or prognostic markers have not been established clinically, risk stratification is mainly based on the initial stage of the illness and the reaction to chemotherapy. It would be essential to develop a new focus to identify diagnostic and prognostic indicators to detect these drug-resistant tumors as soon as possible so that more aggressive treatment can be used to improve the outcomes at the first stage of diseases.

APPROACHES TO TAAs IDENTIFICATION

In the past few decades, several approaches have been used to identify TAAs. The most successful methods are serological screening of cDNA expression libraries and phage display libraries, and the latest methods based on proteomics. Based on these techniques, putative TAAs with high-titer reactivity were identified in sera. Subsequently, TAAbs from these sera were used to isolate the antigen DNA sequence from the cDNA expression libraries. In this way, several new TAAs, including p62 (30) and p90 (79), were discovered in our studies. Afterward, several new and previously defined TAAs (25) were identified with cancer sera using a method called serological analysis of recombinant cDNA expression libraries (SEREX), which was an improvement of the method before (80). In cases of OS, it was the SEREX method that could identify new and previously defined TAAs, including CLUAP1 (54), OSAA-3, and OSAA-5 (58).

With the development of proteomic technology in recent years, the field of bone cancer has entered the era of proteomic research, which aims to identify serum biomarkers using proteomics methods, to carry out early and non-invasive diagnosis of cancer, and monitor the progress of the tumor. By using two-dimensional gel electrophoresis (2-DE) system and mass spectroscopy, one method involved direct analysis of human cancer sera to identify specific protein characteristics of different tumor types (6, 81–84). Li et al. used SELDI-TOF-MS to analyze the patients' sera of OS and osteochondroma, which provided the first example for the identification and verification of the protein biomarker characteristics of OS and found a unique cluster of proteins in the data of patients with bone

tumor, which were discrete SAA subtypes (53). A second approach was used in our research (**Figure 1**) and focused on developing a serum autoantibody library for cancer patients to identify TAAs, in order to better diagnose and manage OS by using customized TAA panels or arrays (78, 85). which was termed as serological proteome analysis (SERPA) (86). Compared with SEREX, the technology of the SERPA allows individuals to screen a large number of sera and to identify a large number of candidate TAAs in a shorter time. The proteome-based approach, commonly known as immunoproteomics (80), can also discriminate antigen subtypes and detect the presence of post-translational modified autoantibodies for specific targets. Moreover, this also sheds light on rapid progress in determining vaccine-associated protein antigens (87), immune-related substances, and biomarkers for disease diagnosis and prognosis (80). In recent years, this approach has been used by our lab to extensively screen sera of certain types of cancer patients such as hepatocellular carcinoma (HCC) (88), esophageal squamous cell carcinoma (ESCC) (89), as well as sera from patients with precancerous lesions such as liver fibrosis (90), to identify and

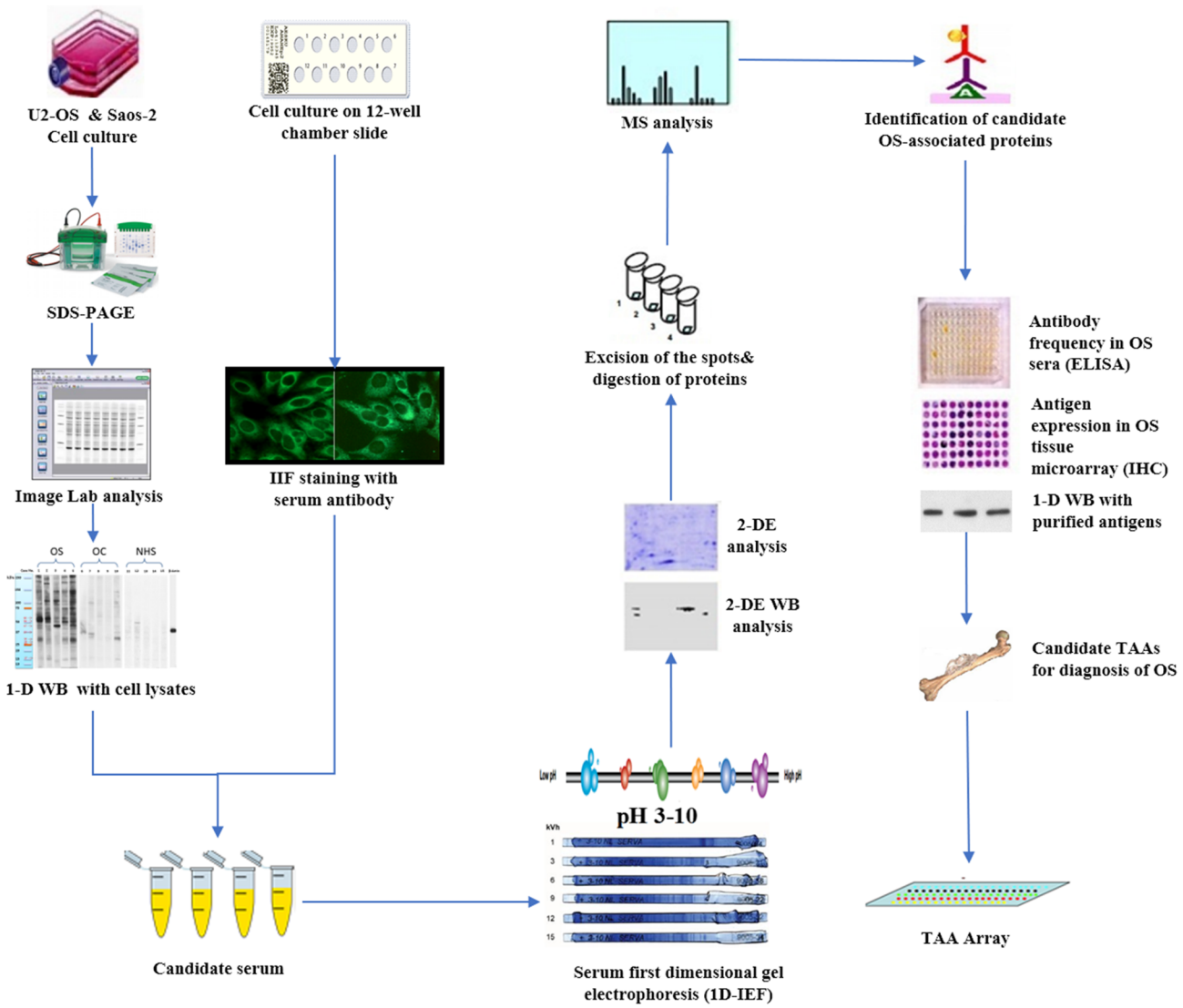

FIGURE 1 | Schematic representation of identification and validation of TAAs using serological proteome analysis (SERPA) approach. In brief, the sera from OS patients and controls were initially examined using extracts of culture cells as a source of antigens in Western blotting and by indirect immunofluorescence (IIF) on whole cells. With these two techniques, we identify sera which have high-titer fluorescent staining or strong signals to cell extracts on Western blotting and narrow the targeting proteins on specific molecular weight bands, and subsequently use the antibodies in these sera as probes in immunoproteomic screening. Cell extracts of cultured human cells was also applied onto the first-dimension gel (isoelectrofocusing gel), and subsequently loaded onto the second-dimension gel (2-DE-SDS-PAGE). The proteins were transferred to the nitrocellulose membrane or visualized by silver staining or Coomassie brilliant blue staining. After immunoblotting with OS sera and control sera, a number of protein spots of interest were excised from the 2-DE gels, digested by trypsin, and subsequently analyzed by mass spectrometry (MS). In subsequent studies, we will characterize the identified cellular proteins that are potential biomarkers in OS.

characterize the latent TAAs. To support the development and characterization of TAAs in our lab, this protocol was developed to screen the immune sera of patients. As shown in **Figure 1**, Western blotting and indirect immunofluorescence (IIF) were used to detect the sera from OS patients and corresponding healthy controls, initially examined by using proteins extracted from tissue culture cells as antigens source. By using these two techniques, the western blotting method identified sera with high-titer fluorescent staining or react with cell extracts on strong signals, then the antibodies were used in these sera as probes for immunoproteomic screening. After cultured human cell extract was applied onto the isoelectrofocusing gel (first-dimensional gel), it was subsequently loaded onto the (2D-SDS-PAGE). Right after the proteins transferred to the nitrocellulose membrane (NC membrane), silver staining or Coomassie brilliant blue staining was used to visualize the spots. Some protein spots were removed from 2D gels and digested with trypsin after immunoblotting with OS sera and control sera, then liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used to do some analysis. In the following studies, a variety of methods, including Enzyme-Linked Immunosorbent Assay (ELISA), 1D Western blotting, and immunohistochemistry (IHC) with tissue arrays, were used to comprehensively characterize and verify the candidate TAAs and TAAbs systems. In addition to the above methods, single molecule array (Simoa) is a new super sensitive detection technology based on the sandwich method of digital ELISA and combined with high-throughput array technology (91, 92). It has a sensitivity 1000 times higher than ordinary ELISA (93). It also has obvious advantages for the detection of low abundance protein markers, and the detection limit can be as low as fg/ml (94). These screening systems have potential application value in cancer immunodiagnosis. Afterwards, the sensitivity and specificity of different antigen-antibody systems as certain types of cancer markers was evaluated for "TAA arrays" systems developed for diagnosing, predicting cancer, and tracking responses of patients treated in the future.

APPLICATION OF TAA-ARRAYS IN OS

Interestingly, TAAbs used as serological markers for the further use of cancer diagnosis stems from the recognition that the expression level of these autoantibodies is usually absent or present in low levels in normal persons and non-cancer patients (85). Comparted with other tumor markers, they present persistence and stability circulation in the sera of cancer patients and may present before the development of cancer, giving them greater early diagnostic and/or prognostic potential (38). Compared with autoimmune diseases, the presence of a particular autoantibody may have diagnostic value; when evaluated individually TAAb has little diagnostic value. This is mainly because of their low frequency, sensitivity, and specificity. Such a limitation has been observed in our previous study and we found it would be optimized by using a properly selected TAA mini-arrays. Furthermore, different TAA arrays were used to diagnose different types of cancer to achieve the required sensitivity and specificity. This then makes immunodiagnosis a feasible auxiliary means to diagnosing tumors and even in predicting prognosis (26).

Our pioneering findings provided evidence that the ability of autoantibodies to detect cancers could be substantially improved by using a series of several TAAs, such as mini-arrays, as target antigens (26, 95, 96). For instance, this mini-array comprised 14 full-length recombinant proteins expressed from cDNAs encoding survivin, CAPERα, RalA, p62, Koc, MDM2, cyclinB1, p53, 14-3-3ζ, p90, IMP1, c-Myc, NPM1, and p16. This mini-array was customized for the detection of hepatocellular carcinoma (HCC). The frequency of autoantibodies to any of these individual TAA was variable, ranging from 5.6% to 21.1% in HCC (97). Nevertheless, with the continuous addition of TAAs in the final 14 TAAs, the positive antibody response of HCC gradually increased to 69.7% in HCC (97). These data indicate that the combined application of multiple TAAs has a higher sensitivity in the serological diagnosis of cancer. More recently, we have evaluated 29 protein antigens identified in OS sera. It was found that only eight protein antigens - DSF70, HMGB1, HCC1, RalA, c-Myc, AnnexinA1, IMP1, and PBP - can induce significantly higher antibody responses in patients with OS with the highest sensitivity and specificity of 0.66 and 0.95, respectively, compared with normal individuals (unpublished data). In a further study, using the panel of these eight TAAs contributes to a 70.7% increase in antibody-positive reactions with an observed AUC of 0.972 (95% CI: 0.867-0.988). These preliminary data extensively supported that not all proteins identified in cancer can be used as be potential TAAs in OS immunodiagnosis or prognosis; only some of them can induce immune responses.

In addition to being potential biomarkers for the immunodiagnosis or prognosis of OS, TAA also has potential as a target for immunotherapy. Vaccinations including peptide-based vaccines are a promising active treatments as more and more TAAs become available for tumor immunotherapy, with the advantage of being easily produced and having minimal toxicity (98–101). For instance, MUC4 could be used as a candidate therapy for the treatment of pancreatic cancer (102). Studies conducted personalized TAA panels to treat patients with glioblastoma multiforme or advanced lung cancer (103). More effective vaccine regimes about TAAs, as well as the mechanism of action of vaccines, should be studied in the future.

CONCLUSIONS

In conclusion, a critical need in the diagnosis and management of OS is to identify a convincing combination of biomarkers used in clinic. These biomarkers could detect tumors at an early stage with limited invasiveness as well as high sensitivity/specificity, which could accurately predict which patients will develop aggressive tumors requiring treatment. Recent research on the TAAs and TAAbs provided a great promise in discovering new

tumor biomarkers. The key to these TAAs or TAAbs is to illustrate a new understanding of the molecular mechanisms associated with the skeletal consequences of malignant tumors. There is growing evidence suggesting these tumors may express multiple diagnostic and/or therapeutic targets, although the information on the repertoire and function of TAA in OS is still limited. Our previous studies have provided strong evidence that enhance cancer detection and treatment; TAA arrays provide promising and powerful tools, even though the clinical application of TAA arrays is still in its infancy. It would be necessary to improve the sensitivity and specificity of TAA by identifying new TAAs and systematically defining the optimal combination of TAAs before TAA arrays could be widely used as tools in screening programs for cancer diagnosis or monitoring cancer progression and guiding therapeutic interventions. The results of these studies are greatly anticipated.

AUTHOR CONTRIBUTIONS

ZG and WL conceived the study. JL and BQ drafted the application sections; they contributed equally to this work. MH, YM, and DL revised and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neoantigen Specific T Cells Derived From T Cell-Derived Induced Pluripotent Stem Cells for the Treatment of Hepatocellular Carcinoma: Potential and Challenges

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Immunotherapy has become an indispensable part of the comprehensive treatment of hepatocellular carcinoma (HCC). Immunotherapy has proven effective in patients with early HCC, advanced HCC, or HCC recurrence after liver transplantation. Clinically, the most commonly used immunotherapy is immune checkpoint inhibition using monoclonal antibodies, such as CTLA-4 and PD-1. However, it cannot fundamentally solve the problems of a weakened immune system and inactivation of immune cells involved in killing tumor cells. T cells can express tumor antigen-recognizing T cell receptors (TCRs) or chimeric antigen receptors (CARs) on the cell surface through gene editing to improve the specificity and responsiveness of immune cells. According to previous studies, TCR-T cell therapy is significantly better than CAR-T cell therapy in the treatment of solid tumors and is one of the most promising immune cell therapies for solid tumors so far. However, its application in the treatment of HCC is still being researched. Technological advancements in induction and redifferentiation of induced pluripotent stem cells (iPSCs) allow us to use T cells to induce T cell-derived iPSCs (T-iPSCs) and then differentiate them into TCR-T cells. This has allowed a convenient strategy to study HCC models and explore optimal treatment strategies. This review gives an overview of the major advances in the development of protocols to generate neoantigen-specific TCR-T cells from T-iPSCs. We will also discuss their potential and challenges in the treatment of HCC.

Keywords: hepatocellular carcinoma, immunotherapy, neoantigen, T cell-derived induced pluripotent stem cells, T cell receptors, T cells

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer worldwide and the third leading cause of cancer-related death, with approximately 50% of HCC cases occurring in China (1). Localized stage HCC has a five-year relative survival rate in near 31% of patients with HCC, in all races (2). About 70% of HCC cases are diagnosed at the advanced stage (3, 4), which requires systemic treatment. Surgical treatment is considered the most comprehensive treatment for HCC. Non-surgical treatments mostly include local treatments such as transcatheter hepatic arterial chemoembolization (TACE), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI). For mid-late stage HCC, molecular targeting drugs such as Sorafenib are currently the most commonly used treatment (**Figure 1**).

While earlier studies were focused on the tumor parenchyma, currently, the research focus has shifted to the tumor stroma and tumor microenvironment. The microenvironment of solid tumor usually contains a large number of stroma and immunosuppressive cells, making it difficult for the immune cells to attack the tumor; the hypoxic environment and nutritional deficiency also hinder the proliferation and survival of T cells (5). Thus, the immune system is unable to clear the tumor. Adoptive cell therapy (ACT) might be able to overcome these challenges. It improves the patient's immune environment, reactivating and proliferating the non-functional immune cells, allowing them to kill the tumor cells in the patient's body.

Tumor immunotherapy has attracted much attention because of its ability to enhance the body's autoimmunity to kill tumors. In addition to adoptive cell therapy, immune-based therapies for HCC have been used in clinical applications or are undergoing clinical trials. Immune-based therapies include methods such as immune checkpoint inhibitors, inhibitory cytokine blockers, oncolytic viruses, and genetic vaccines (6). Studies have shown that after administration of tremelimumab, an antibody targeting CTLA-4, combined with radiofrequency ablation to treat advanced liver cancer, led to a significant increase in CD8+ T cells in the cancer tissue of patients, significantly prolonging the progression-free survival period (7). Monoclonal antibodies that target immune checkpoints have achieved remarkable therapeutic results in different types of tumors, but it also has several challenges, for example, controlling immune-related adverse reactions and applying combination therapy strategies. Moreover, this type of immunotherapy is time-consuming and slow in onset. Further research is needed to design and optimize such immunotherapeutic treatment strategies.

With respect to ACT, chimeric antigen receptor (CAR)-T cell therapy and T cell receptor (TCR)-T cell therapy are most commonly used. T cells express TCRs or CARs that recognize tumor antigens on the cell surface through gene editing technology, rendering T cells the specificity in their functions. Moreover, compared with cumbersome screening to obtain tumor infiltrating lymphocytes (TILs), the acquisition and preparation of genetically engineered T cells is simpler (8). With the leukemia treatment trial of CAR-T cell therapy conducted by the University

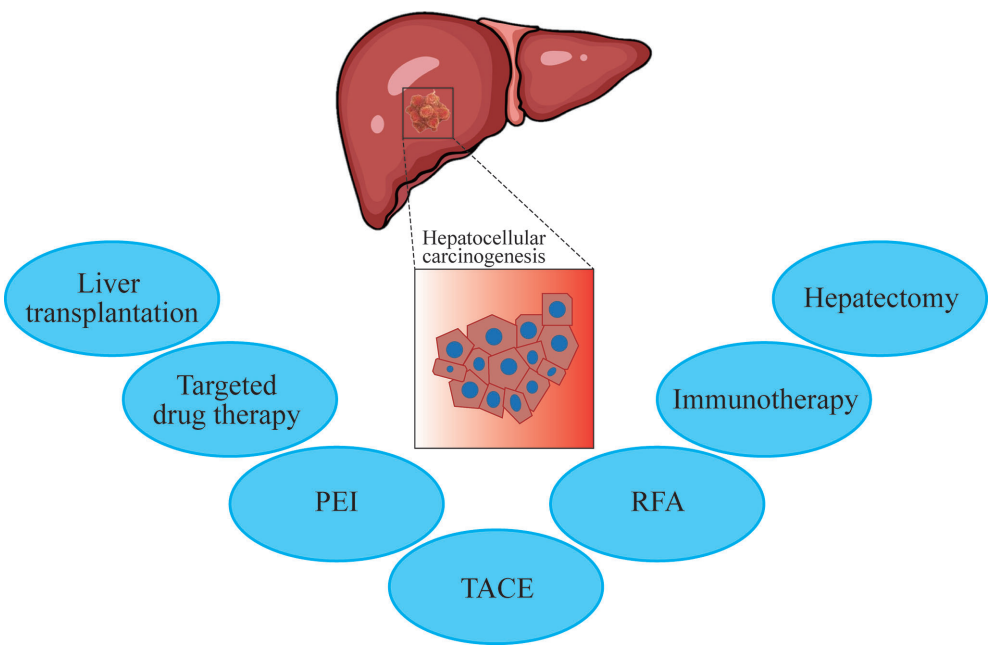

FIGURE 1 | The current treatments for liver cancer include hepatectomy, immunotherapy, transcatheter arterial chemoembolization (TACE), radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), targeted drug therapy (sorafenib/FOLFOX4), and liver transplantation (UCSF standard). According to the patient's condition, such as the degree of liver cirrhosis, liver function (child grade), number of tumors, size of tumors, and metastasis, clinicians select the appropriate treatment plan or perform combination therapy.

of Pennsylvania, it has begun to be widely used in the treatment of tumors (9). The development of CAR-T cell therapy has achieved clinical cures for certain types of tumors in the blood, like B-cell leukemia and lymphoma. Although CAR-T cells have achieved remarkable results in the treatment of certain types of leukemia, their use in treating solid tumors faces several challenges. Therefore, the successful application of TCR-T cell therapy in treating solid tumors makes it one of the most promising immune cell therapy methods for solid tumors, e.g., the use of NY-ESO-1 antigen-specific TCR-T cells against some tumors like melanoma, synovial sarcoma, multiple myeloma and disseminated neuroblastoma (10–13).

TCR-T cell therapy is widely used in tumor therapy because of its wide range of therapeutic targets, high affinity, and high specificity for tumor antigen. However, it also elicits adverse effects, especially off-target effects and severe untargeted toxicity (14–17). Induced pluripotent stem cells (iPSCs) emerged as a viable tool to derive T cells owing to their plasticity, high proliferative capacity, and no ethical restrictions. Therefore, developing a model of neoantigen T cell-derived iPSCs (T-iPSCs) is increasingly being considered in cancer precision medicine, tumor immunotherapy and regenerative medicine, to study their safety and efficacy in treating HCC. Several studies are underway to develop protocols to generate personalized and specific TCR-T cells using T-iPSCs. This review will describe in detail the recent advances made in developing TCR-T cells using T-iPSCs. We will also discuss the potential applications and challenges of using neoantigen T cells derived from T-iPSCs for the treatment of HCC.

IMPORTANCE OF NEOANTIGEN SPECIFIC T CELLS DERIVED FROM T-iPSCs in the Treatment of HCC

Traditional hepatectomy is effective in treating early HCC but not for advanced HCC and metastatic cancer cells. Radiotherapy and chemotherapy have poor selectivity for liver cancer, resulting in damage of normal tissues while killing cancer cells. Although targeted drugs such as sorafenib, donafenib, atezolizumab, and bevacizumab are effective in treating HCC, they cannot suppress the mutation and drug resistance that develops in tumor cells. Extensive research has been carried out to develop drugs to treat tumors. However, molecular-targeted drugs have their own limitations due to drug resistance, side effects, lack of tumor specificity, and unsustained tumor killing activity. Therefore, some patients do not benefit from systemic treatment with molecular-targeted drugs. This challenge can perhaps be overcome by tumor immunotherapy and regenerative medicine.

Immunotherapy does not require surgery; immune cells can be targeted to the tumor through direct injection or through the peripheral. Most tumor patients are in a state of immunosuppression. Therefore, immunotherapy is necessary, to change the overall immune state of the patient or to change the local immune microenvironment of the tumor.

In China, HCC cells usually have integrated HBV-DNA fragments (18) and can therefore synthesize a peptide chain which can bind to major histocompatibility complex (MHC) molecules, enabling it to be recognized by T cells. Hepatitis B surface antigen (HbsAg) may be used as a potential target for TCR-T cell therapy (19). TCR genes can specifically recognize and kill HepG2 tumor cells *in vitro* through CD8+ T cells. They can also eliminate HepG2 tumors in NSG mice (15). Hafezi et al. showed that HBV-specific TCR-T cells have potential applications in organ transplantation patients with recurring HBV-HCC (20). These results indicate that TCR-T cell therapy is efficient in the treatment of solid tumors such as HCC, and adoptive cell transfer therapy of liver cancer might eventually become the optimal solution in its comprehensive treatment.

The selection of TCR targets is a key challenge in TCR-T cell therapy. Generally, there are two criteria for determining target tumor antigens. First, if the antigen is only expressed in tumors; second, if there are enough patients who can benefit from this treatment. Thus, there are three tumor antigens that can be used as suitable targets for TCR-transduced T cell therapy, including cancer-testis antigens, oncogenic virus antigens, and neoantigens. Tumors produce mutated peptides due to accumulated somatic mutations. MHC molecules can bind to such antigen peptides to form new antigens on the cell surface (**Figure 2**). These neoantigens are usually unique to cancer cells and individuals. Therefore, they are ideal targets for designing T cell immunotherapy. Neoantigens may also become potential targets for developing immunotherapy in patients with other cancer types (19).

DEVELOPMENT OF TCR-T CELL TARGETING NEOANTIGENS USING T-iPSCs

The production of TCR-T cell targeting neoantigens requires three steps: screening neoantigens, constructing T-iPSCs, and inducing TCR-T cells.

Screening Neoantigens

According to existing literature, tumor rejection epitopes may be derived from two classes of antigens. The first class is developed from non-mutated proteins to which T cell tolerance is incomplete because of their restricted tissue expression pattern. The second class can be developed from peptides that are entirely absent from the normal human genome, the so called neoantigens (21). Neoantigens are derived from antigens produced by the mutant protein, and antigens produced by the integration of the tumorigenic virus into the human genome. Neoantigens do not cause central immune tolerance and have strong immunogenicity. There are two conditions for screening neoantigens: first, a mutated protein needs to be processed and then presented as a mutant peptide by MHC molecules; second, T cells that can recognize this peptide-MHC complex should be present (21).

All malignant tumors harbor non-synonymous mutations or other genetic alterations (22). This makes it feasible to identify mutant antigens by technical means. Immunogenic antigenic

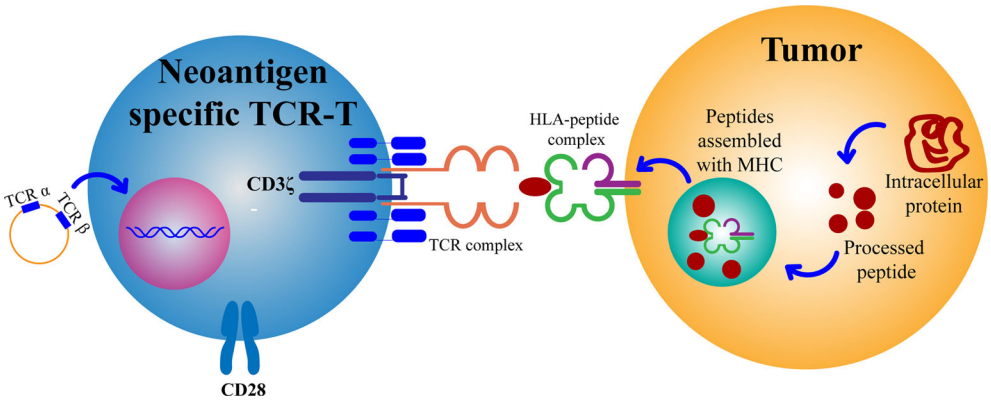

FIGURE 2 | Mature CD8+ T cells with a new antigen-specific TCR gene can recognize antigen targets and thus attack and kill tumor cells. TCR, T cell receptor; HLA, human leukocyte antigen; MHC, major histocompatibility complex.

peptides were identified using next-generation sequencing technology in conjugation with bioinformatics, which predicted epitope peptides binding mutated proteins to human leukocyte antigen (HLA) with high affinity, and stimulated lymphocytes to detect cytokines *in vitro*. Robbins et al. developed a new screening approach involving mining whole-exome sequence data to identify mutated proteins expressed in the tumor of the patient. They then synthesized and evaluated candidate mutated T cell epitopes that were identified using an MHC-binding algorithm for being recognized by TILs. This simplified approach for identifying mutated antigens recognized by T cells avoids the need to laboriously develop and screen cDNA libraries of tumors and may be easier for practical application (23).

Based on whole-exome sequence, using non-synonymous mutations, a tandem minigene (TMG) vector was designed and synthesized, which was transcribed into multi-epitope RNA *in vitro* and transferred to antigen-presenting cells. Subsequently, T cells were stimulated by these antigen-presenting cells. This enables identification of immunogenicity epitopes of RNA and specific mutant epitopes of immunogenicity, as needed. Tran et al. used whole-exome sequence which revealed 26 non-synonymous mutations in a widely metastatic cholangiocarcinoma case. The study used minigene approach wherein multiple minigenes were synthesized in tandem to generate TMG constructs. These constructs were then used as templates for the generation of *in vitro* transcribed (IVT) RNA. Each of these IVT TMG RNAs was then individually transfected into autologous antigen-presenting cells, followed by coculture with TILs. The reactivity predominated in the CD4+ T cell population, as demonstrated by up-regulation of the activation markers OX40 and 4-BB. The study successfully identified HLA-DQB1*0601 restrictive REBB2IP neoantigen sequence NSKEETGHLENGN (24).

Using bioinformatics, multiple antigenic peptide fragments were designed for hotspot mutations of high-frequency mutated genes, and the optimal epitopes were screened *in vitro*, so as to cover more patients with mutations of this gene site. Schumacher et al. demonstrated that isocitrate dehydrogenase 1 (IDH1) (R132H) contains an immunogenic epitope suitable for mutation-specific vaccination. Peptides encompassing the mutated region are presented on MHC class II and they induce mutation-specific CD4+ helper T cell-1 (TH1) responses in gliomas (25). These methods can efficiently screen neoantigens, thus laying the foundation for the subsequent production of neoantigen specific TCR-T cells.

T-iPSCs

Dedifferentiation of adult cells into stem cells was first proposed by Japanese scholar professor Shinya Yamanaka in 2006. Their team proposed that differentiated cells from mouse embryo or adult fibroblasts can be reprogrammed into iPSCs by introducing four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4 (26). In 2007, Takahashi et al. found that iPSCs developed from adult human dermal fibroblasts using Oct3/4, Sox2, c-Myc, and Klf4 (OSKM) were similar to human embryonic stem cells (ESCs) and could differentiate into cell types of the three germ layers (27). In 2008, Park et al. developed a protocol using the four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) to develop iPSCs from reprogramming of human somatic cells. They found that only Oct3/4 and Sox2 were necessary in such a case, and NANOG and LIN28 can replace c-Myc and Klf4, respectively. Adding human telomerase reverse transcriptase (hTERT) and SV40 Large T antigen can improve the efficiency of reprogramming the cells during the cultivation process. They also pointed out that human iPSCs resemble ESCs not only in morphology and gene expression but also in the capacity to form teratomas in immune-deficient mice. The study also established a method to yield patient-specific cells that may be cultured *in vitro* (28). In 2009, Hamilton et al. used a lentiviral transduction system induced by doxycycline and four transcription factors (OSKM) to reprogram mouse embryonic fibroblasts into iPSCs (29). Since then, iPSCs have been widely studied by several researchers, which will pave way for research into treatment methods for various diseases (30).

More than ten years after the advent of iPSCs, a variety of methods for inducing iPSCs *in vitro* have been studied and

improvements have been made to the original method. For example, the use of ROCK inhibitors can improve the efficiency and survival rate of human iPSCs (31). The use of episomes and HDAC inhibitors can lead to efficient production of iPSCs from peripheral blood (32). It was found that reprogramming of adult fibroblasts and mouse embryos into iPSCs using serum-containing media is often not as effective as using serum-free media (33). Some studies have shown that iPSCs can also be successfully developed without c-Myc, but it results in low efficiency and delay in development of iPSCs (34, 35) (**Figure 3**).

The iPSCs induced by T cells are called T-iPSCs, which retain the assembled "endogenous" TCR gene after nuclear reprogramming. Notably, T cells differentiated from T-iPSCs have the same arrangement pattern of TCR genes as original T cells. This makes T-iPSCs an efficient raw material to be used for the propagation of numerous T cells, all of which express antigen-specific TCRs (36, 37).

However, Minagawa et al. discovered that T cells had lost their antigen specificity due to the following factors: (i) there is a rearrangement of the TCR gene at the double positive (DP) stage in the CD8$\alpha\beta$+ T cells generated from human T-iPSCs. The researchers used CRISPR technology to knock out the recombinase gene (RAG2) in T-iPSC to prevent this. (ii) During the treatment stage, they also found that the exogenous and the endogenous TCR chains were paired in some T cells (38). To solve this problem, a single viral vector encoding two TCR chain genes can be used in the viral transfection stage, which will reduce the probability of mismatch between the exogenous TCR chain and the endogenous TCR chain (39). Other studies have used siRNA to down-regulate endogenous CD3, or zinc finger nuclease knockout technology to knock out endogenous TCR to reduce the probability of mismatches (40, 41).

T cells used to induce T-iPSCs are mainly derived by isolating autologous TILs from resected tumors of patients and obtaining peripheral lymphocytes (42). The application of T-iPSC to

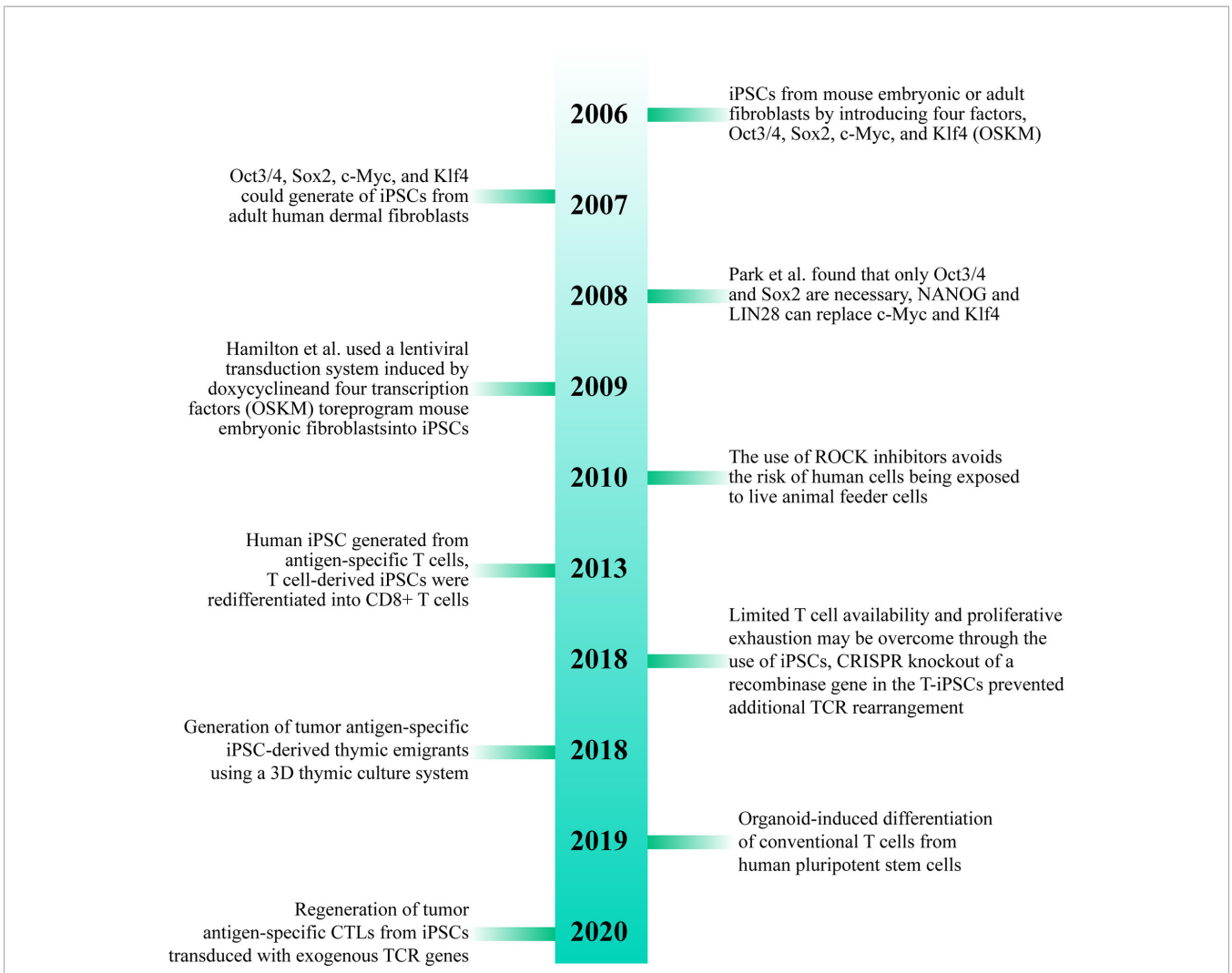

FIGURE 3 | Brief history of T cell-derived induced pluripotent stem cells (T-iPSCs). TCR, T Cell Receptor; iPSCs, induced pluripotent stem cells; CTLs, cytotoxic T lymphocytes.

generate a large number of tumor antigen-specific T cells solves the problem of short lifespan of activated cytotoxic T lymphocytes (CTLs) in traditional tumor immunotherapy. Normal T cells induced by the antigen presenting cells *in vitro* will be inactive in a short period, and therefore, cannot effectively attack tumors (43).

Neoantigen Specific T-iPSCs

The unit of TCR-T executive function is the TCR-CD3 complex, which belongs to the immune protein superfamily, a heterodimer consisting of α- and β-chains. The peptide chain is divided into three parts, the extracellular region, the cellular transmembrane region, and the intracellular region. Moreover, neoantigen specific TCR α- and β-chains are the major components of TCR-T cells. A study identified neoantigen specific TCR α- and β-chains and inserted them into T-iPSCs. After successfully inducing the rearranged TCR gene into iPSC, in the preselection stage, it was able to effectively produce cells which expressed TCR on the surface (43). Compared with the ordinary T-iPSCs, the TCR-iPSCs with the new antigen inserted in them solved the problem of the heterogeneity of the generated T cells. The TCR-iPSC clone produced is simple, fast, and high-quality (44).

A study showed that using gene expression vectors, TCR α- and β-chains transduced into human T lymphocytes could mediate tumor regression. This has been applied by researchers for clinical trials in humans, and no adverse events have been observed so far (11, 17).

According to a previous study, the steps to generate neoantigen specific TCR α chains and β chains are as follows: Tumor reactive T cells derived from TILs and CTLs of peripheral blood from patients and T cells by autologous antigen presenting cells (APCs) are loaded with synthetic tandem minigene (TMG) or peptides encoding mutated antigens. After that, the newly acquired T cells are identified. Reactive T cells are identified based on the expression of active molecules such as 4-1BB on CD8+ T cells and OX-40 on CD4+ T cells, and then purified and amplified using flow cytometry (24). A new method was designed by Cohen at al. for the identification, isolation, and utilization of neoantigen-specific T cells. They used neoepitope MHC/peptide tetramers to combine T cells in the fresh tumor digests and peripheral blood to obtain neoantigen-specific T cells (45, 46). A small number of neoantigen-specific T cells were successfully obtained using peptide-MHC polymerization method, enabling peripheral blood mononuclear cell (PBMC) to become a potential source of neoantigen epitope-specific TCR. Then sequencing of TCR, obtain Neoantigen specific TCR α chains and β chains.

Neoantigen Specific TCR-T Cells

The most critical step is to induce differentiation of neoantigen-specific T-iPSC into mature TCR-T cells. These specific TCR-T cells are monoclonal and highly accurate in cell targeting. Unlike the exogenous TCR transfer technology to induce T cells from hematopoietic stem cells and peripheral blood into tumor antigen-specific T cells, the neoantigen-specific TCR-T cells do not generate mismatched TCR, reducing unnecessary issues in screening and treatment (36). Generally, the induction of lymphoid cells is achieved by constructing a thymic microenvironment in which T cells proliferate. To generate T cells from iPSCs, it needs to be induced into mesoderm, and needs to perform hematopoietic functions (47). Common methods are as follows:

Differentiated and developed CD3+ T cells (3–5) $\times 10^5$ were extracted from PBMC using magnetic beads, and T cells were stimulated in T cell medium with leben-CD3/CD28 magnetic beads and IL-2. The T cell culture medium contained 20 ng/ml IL-2, 10 ng/ml IL-7, and 10 ng/ml IL-15. On the second day after stimulation, oct3/4, SOX2, KLF4, and c-MYC transcription factors packaged with sendai virus were transfected into T cells. Rat embryonic fibroblasts were laid on the 8th day, and the medium containing 5 ng/ml basic fibroblast growth factor (b-FGF) human induced pluripotent stem cells was replaced 12 days after culture. T-iPSCs were collected at 33–40 days. Subsequently, in the presence of bone marrow mesenchymal stem cells (C3H10T1/2), vascular endothelial growth factor (VEGF), stem cell factor (SCF), and Tyrosine Kinase ligand 3 (FLT-3L). iPSCs differentiated into mesodermal CD34+ hematopoietic stem cells and hematopoietic progenitor cells. Cells were transfected with OP9-DL1 FLT-3 and IL-7 culture medium at day 14 to obtain T cell lineage (36).

Vizcard et al. showed that a 3D thymic culture system enables the generation of a homogeneous antigen-specific T cell subset. These T-cells generated a unique product which was named iPSC-derived thymic emigrants (iTEs); they closely mimic naïve T cells and exhibit potent anti-tumor activity. The study has designed the 3D thymic culture method based on the traditional fetal thymic organ culture (FTOC) system (48). Another study proved that by co-cultivation of OP9-DL1/DL4 cells which express the Notch ligand, TCR-iPSC inserted with neoantigens can induce production of tumor-specific CD8+ T cells (49) (**Figure 4**).

POTENTIAL AND CHALLENGES OF TCR-T CELL INDUCED BY T-iPSCs FOR THE TREATMENT OF HCC

Antigen Selection

Among the current TCR-T cell therapies, TCR-T cells with cancer testis antigen (CT) as the target are the most commonly used. CT is expressed in a variety of tumor tissues, but not in normal tissues other than testis, placenta, and fetal ovaries. Because of its unique expression, it has become an ideal target antigen for tumor immunotherapy. The most used antigen target is NY-ESO-1.

NY-ESO-1 is a tumor-sharing antigen screened from esophageal cancer cDNA expression library using serological analysis of recombinant cDNA library (50). The frequency of expression of the antigen NY-ESO-1 in various tumors is different, and so is the frequency of protein expression. The descending order of protein expression in various tumors is: neuroblastoma, synovial sarcoma, and malignant melanoma.

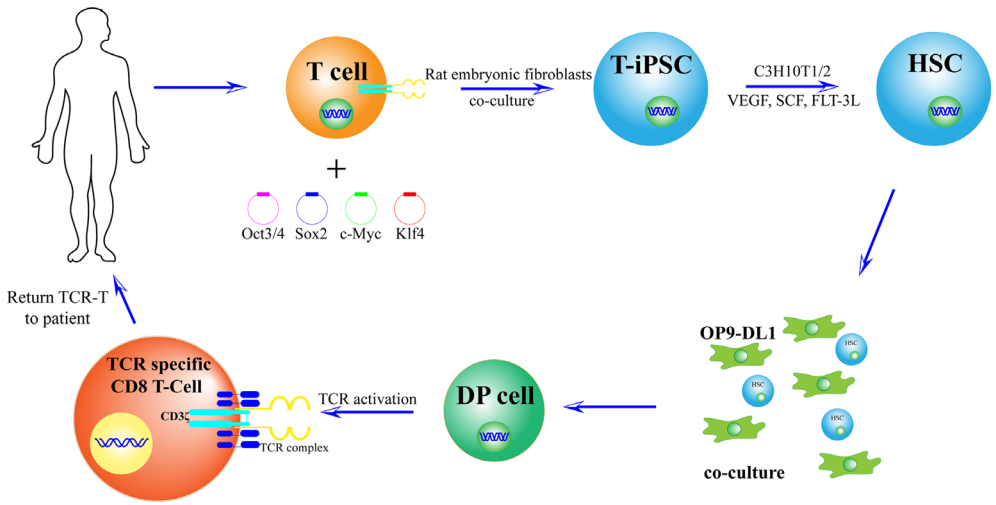

FIGURE 4 | Preparation of new antigen-specific TCR-T cells. First, T cells with new antigen-specific TCR rearrangement gene were isolated from the body. Second, after adding OCT3/4, Sox2, KLF4, and c-MYC, T cells were induced to T-iPSC. Third, in the presence of bone marrow mesenchymal stem cells (C3H10T1/2), vascular endothelial growth factor (VEGF), stem cell factor (SCF), and tyrosine kinase ligand 3 (FLT-3L), T-iPSCs were differentiated into mesodermal CD34+ hematopoietic stem cells (HSCs) and hematopoietic progenitor cells. Cells were transfected to OP9-DL1 FLT-3 and IL-7 culture medium to obtain T cell lineage. Thus, a large number of T cells will be used to recognize and kill tumor cells.

The NY-ESO-1 mRNA is highly expressed in prostate cancer, bladder cancer, breast cancer, multiple myeloma, and hepatocellular carcinoma, and has low expression in oral squamous cell carcinoma and esophageal cancer (51–55). In 2011, it was first reported that TCR-T cell therapy targeting NY-ESO-1 was effective in both melanoma and synovial sarcoma (10). The use of lentivirus-mediated TCR-T cells is also effective in the treatment of hematological malignancies like multiple myeloma (17).

While TCR-T cell therapy has achieved success in other cancers, it is at the clinical trial stage in liver cancer treatment. Currently, there are 6 such clinical trials underway for the treatment of HCC (https://www.clinicaltrials.gov) [Accessed March 15, 2021] (refer to **Table 1** for details).

Although adoptive immunotherapy is relatively new, tumor-specific antigens have been studied for more than 50 years. However, the prolonged research and high investment in this field have brought about very few results. In adoptive immunotherapy there is always a need for suitable immune attack targets (16).

In the past, TCR-T cell therapies targeted tumor-associated antigens (TAAs), which are also expressed in normal cells. Therefore, after treatment with genetically engineered T cells targeting carcinoembryonic antigen (CEA), patients have experienced severe transient colitis (56). Patients receiving MART-1 and gp100 TCR treatment also showed toxicity in the skin, eyes, and ears (57). TCR-T cells targeting MAGE-A12 also produced severe neurotoxicity during the treatment process and caused mortality in patients (58).

Although TAAs have many side effects in targeted therapy, they have other advantages. In recent years, many studies have confirmed that the immune system can detect TAAs, and thus, the serum of patients with cancer contains the respective antibodies (59). Therefore, the anti-TAA/TAA system is of

TABLE 1 | Recent clinical trials related to TCR-T cells for the treatment of HCC.

Clinical Trial	NCT number	Host/Country
Redirected HBV-specific T Cells in patients with HBV-related HCC (SAFE-T-HBV) (SAFE-T-HBV)	NCT04745403	Singapore General Hospital, Singapore
TCR-redirected T cell infusion in subjects with recurrent HBV-related HCC post liver transplantation	NCT02719782	The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China
TCR-redirected T cell treatment in patients with recurrent HBV-related HCC post liver transplantation	NCT04677088	The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China
TCR-redirected T cell infusions to prevent HCC recurrence post Liver transplantation	NCT02686372	The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China
Autologous CAR-T/TCR-T cell immunotherapy for solid malignancies	NCT03941626	Henan Provincial People's Hospital, Zhengzhou, Henan, China
Autologous CAR-T/TCR-T cell immunotherapy for malignancies	NCT03638206	The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

HBV, Hepatitis B Virus; HCC, Hepatocellular Carcinoma; TCR, T Cell Receptor; CAR-T, Chimeric Antigen Receptor T-Cell; TCR-T, T cell receptor T-Cells.

great significance as an early cancer biomarker and for predicting the prognosis of the disease. For example, when anti-TAAs are added as a diagnostic marker combined with alpha-fetoprotein (AFP), the sensitivity and specificity to diagnose HCC are improved, compared with AFP alone (60). Wu et al. used alpha-fetoprotein combined with tumor size and alkaline phosphatase levels to invent a simpler and more efficient prognostic scoring system for predicting overall survival (OS) and disease-free survival (DFS) of patients with spontaneously ruptured liver cancer (60). It was proved that the use of multiple antigen microarrays to detect autoantibodies of TAAs can detect and diagnose cancers (61). Wang et al. proposed that anti-TAA autoantibodies may appear in the early stage of HCC, which can be used as a serological marker for early screening of HCC, and it also provides a new way for HCC detection in AFP-negative patients (62).

There is a need for extensive studies on neoantigens as they can safely target tumors to achieve curative effects, unlike other existing methods. Additionally, it may also become a universal target antigen for immunotherapy in patients with different types of tumors. After adoptive cell therapy, a large number of tumor cells are destroyed in a short time in some patients, which will lead to fatal clinical events (63). In addition, T cells may kill all tumor or infected cells, such as in case of liver cancer caused by HBV infection, leading to serious organ function problems. This can be overcome by transferring suicide genes into T cells or transiently expressing TCR gene through mRNA electroporation (64).

Expression of TCR

There are several challenges that remain, with respect to TCR expression, such as (i) to maximize the expression of TCR on the cell surface; (ii) to reduce the mismatch between the introduced TCR α- or β-chains and the endogenous TCR chains; (iii) to enhance the binding of TCR molecules to CD3 molecules; and (iv) to increase affinity between TCR and tumors (39). Studies have shown that the provision of additional CD3 molecules while giving T cell TCR genes can promote T cell affinity and anti-tumor activity *in vivo* (65). Besides, the liver is a special organ which has many immune suppression mechanisms (66), and may create obstacles in the future to immunotherapy for HCC.

Immune Evasion and Immunosuppression

In addition, tumor immune evasion is also one of the factors that affect TCR-T cell therapy. The loss of autoantigen, loss of HLA molecules, and change of tumor antigen in the process rendered the T cells unable to recognize cancer cells (67, 68). The TCR-T cells derived from iPSCs are monoclonal. It was found that CD8 + T cells express LAG-3 and PD-1 molecules, leading to accelerated T cell depletion (69). Whether this phenomenon will appear in T cells derived from iPSCs, thus reducing its therapeutic effect remains to be seen. Issues with T cell homing is also one of the factors affecting adoptive immunotherapy. The nitration of CCL2 chemokines and the change of glycosylation of T cell surface glycoprotein are the factors that weaken T cell homing (70). Further, in some cases, despite the large number of specific CD8+ T cells, the tumor continues to grow (71). It may be due to inhibitory factors expressed by tumors, such as IL-10 and TGF-β (72) (**Table 2**).

Quality Control

Currently, advancement in the current protocols used to produce neoantigen T cells derived from T-iPSCs holds a great promise for regenerative medicine and therapeutic applications. Researchers have been able to produce functional neoantigen T cells from iPSCs and patient-derived tissues (73, 74). Sometimes, the quality of the patient's T cell affects the production of T-iPSC, which is often not ideal. A study had developed a single-cell droplet microfluidic technology to screen functional TCR-T cells, which can overcome this problem (75). In 2020, HLA was used to transplant donor tissue or cells with the same HLA alleles into HLA haplotype heterozygous patients, which reduced the immune rejection reaction, thereby producing "off-the-shelf" T cells used to treat patients (76). The knock-out of TCRs and/or HLAs can help obtain universal TCR-T cells without MHC restriction. Researchers have been able to expand and scale the production of cells, and developed tools such as 3D thymic culture for mass production of neoantigen T cell. Moreover, development of 3D thymic organoids, coculture of multiple cytokines, and transplantation into 3D thymic organoids have improved the maturity and functionality of T cells. It is crucial to resolve challenges associated with genomic instability and

TABLE 2 | Current challenges, reasons and possible solutions of TCR-T induced by T-iPSCs for the treatment of Hepatocellular Carcinoma.

Challenges	Reasons	Possible solutions
Security	Off-target effects Targeted toxicity	Find neoantigens
TCR mismatch	The introduced TCR chains match the endogenous TCR chains	A single viral vector encoding two TCR chains Gene knock-out
Tumor immune evasion	Loss of autoantigen Loss of HLA molecules Change of tumor antigen Monoclonal TCR-T	Find neoantigens
T cell depletion	LAG-3 and PD-1 overexpression	Gene knock-out Targeted drugs
Problems with T cell homing	Nitration of CCL2 chemokines T cell surface glycoprotein changed	Regulating the expression of chemokines
Liver autoimmune suppression	Regulatory myeloid populations maintain liver immune tolerance	Improve the immune microenvironment

TCR, T Cell Receptor; TCR-T, T cell receptor T-Cells; HLA, human leukocyte antigen; LAG-3, Lymphocyte activation gene-3; PD-1, Programmed Death-1; CCL2, chemokine ligand 2.

formation of teratomas after transplantation of iPSC-derived T cells, in order to achieve its wide-scale clinical use in regenerative medicine and cell therapy.

CONCLUSIONS AND FUTURE PERSPECTIVES

The immune system is an important line of defense for the body to resist external intrusions, supervisors and stabilize the internal environment. Antigen-antibody reactions are the essential means for the immune system to perform its functions. Tumor antigens play important roles in the occurrence and development of tumors and inducing the body's anti-tumor immune responses. Therefore, whether in TCR-T cell therapy or other immunotherapies, finding, identifying and analyzing tumor antigens is the core of tumor immunology research. Among them, neoantigens are considered the most promising tumor antigens. The personalized immunotherapy model based on neoantigens generated by tumor-specific mutations is the main development direction of immunotherapy in solid tumors in the future.

In recent years, the rapid development of immunotherapy has become a new hope for mankind to fight tumors. Academics are of the opinion that the approach of using the immune system to attack tumors will become a turning point in cancer treatment. TCR-T cell therapy can be successfully applied to solid tumors, and has broad scope in the future. However, several challenges associated with the TCR-T cell therapy need to be resolved for its wider application. Improvement in the induction and differentiation of T-iPSCs, TCR-T cell production efficiency and quality, optimized TCR transfection system, transfection efficiency, TCR affinity, and cell expression levels can further improve T cell performance (72). In addition, progress in tumor immunotherapy methods depends on suitable immune targets, and the neoantigens generated by mutations are very ideal specific antigens. In view of the complex immunosuppressive microenvironment of tumors, neoantigen-based immunotherapy with a reasonable combination of immune checkpoint inhibitors and traditional tumor treatment modes such as radiotherapy, chemotherapy, and monoclonal antibodies targeting tumor antigens, can further increase the efficacy of immunotherapy and exert a more synergistic anti-tumor effect.

The induction and redifferentiation technology of iPSCs has gradually advanced, providing great convenience in the establishment of HCC treatment models and application in HCC immunotherapy. The use of T-iPSCs to produce a large number of highly individualized neoantigen specific TCR-T cells that can be used for treatment will also achieve practical application in the near future. Thymic organoids developed from patient-derived tissue have been shown to faithfully recapitulate the disease *in vitro* and could be a useful tool to study disease pathogenesis and screening of novel therapeutic drugs. However, currently, the high cost of preparation and complicated operating procedures of TCR-T cell therapy requires the need for further studies in the future to overcome these challenges.

In conclusion, researchers have been able to generate suitable neoantigen T cells derived from T-iPSCs to apply for clinical application. The optimal combination of TCR-T cell therapy and traditional HCC treatment methods also requires continuous exploration and research, striving to improve the efficacy while making it convenient for clinical application, so that more patients can benefit from it in the near future.

AUTHOR CONTRIBUTIONS

XL contributed significantly to analysis, fund support, and the conception of the review. FL and X-J-NM contributed to manuscript preparation and wrote the manuscript. W-LJ helped analyze and revised the manuscript. YL contributed to manuscript with some constructive suggestions. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immunogenic Cell Death-Based Cancer Vaccines

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Cancer immunotherapy has achieved great advancement in the past decades. Whereas, its response is largely limited in immunologically cold tumors, in an urgent need to be solve. In recent years, an increasing number of studies have shown that inducing immunogenic cell deaths (ICDs) is an attractive approach to activate antitumor immunity. Upon specific stress, cancer cells undergo ICDs and dying cancer cells release danger associated molecular patterns (DAMPs), produce neoantigens and trigger adaptive immunity. ICDs exert a cancer vaccine-like effect and Inducement of ICDs mimics process of cancer vaccination. In this review, we propose a concept of ICD-based cancer vaccines and summarize sources of ICD-based cancer vaccines and their challenges, which may broaden the understandings of ICD and cancer vaccines in cancer immunotherapy.

Keywords: cancer vaccine, danger associated molecular patterns, immunogenic cell deaths, immunotherapy, tumor microenvironment

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INTRODUCTION

Cancer treatment has shifted from tumor cell-centric to tumor microenvironment (TME)-centric with an in-depth understanding of the constitution and function of TME (1). Meanwhile, apart from targeting oncogenic pathways, there is an increasing awareness of the relationship between tumorigenesis and immunity. In the past decades, cancer immunotherapy has revolutionized cancer treatment, with immune checkpoint blockades (ICBs) including programmed cell death protein 1 (PD-1)/programmed cell death protein-ligand 1 (PD-L1)/cytotoxic T lymphocyte associate protein-4 (CTLA-4) being regarded as one of the most promising approaches to treat cancer. However, ICBs do not fit all types of tumor. They encounter unresponsiveness in "cold" tumor (glioblastoma, ovarian cancer, prostate cancer, etc.), characterized by the lack of tumor antigens, T cell priming, activation or infiltration (2). Impaired T cell priming or activation was attributed to defective recruitment of antigen presenting cells (APCs) and lack of co-stimulatory molecules including danger associated molecular patterns (DAMPs) (3). Cancer vaccines are such a strategy which turns "cold" tumor microenvironment to "hot" one assisting to solve the therapeutic resistance of cancer immunotherapy.

Immunogenic cell death (ICD) is a unique cell death featured by activation of dying T cell immune response and release of DAMPs. The most frequent applied DAMPs to evaluate the immunogenicity of cell death including calreticulin (CRT), adenosine triphosphate (ATP) and high

mobility group box 1 (HMGB1). DAMPs induced by tumor cell ICD, as mentioned before, are critical to maturation of dendritic cells (DCs), production of immunosuppressive factors, activation of APCs and T cell co-stimulation (2, 4, 5). The characteristics of ICD provide a theoretical basis for it to be utilized to produce cancer vaccine.

Bonaventura and colleagues have provided an elaborate summary of approaches to turn "cold" tumors into "hot" ones (2). They also highlighted that ICDs induced by chemotherapy or radiotherapy and cancer vaccines are potential approaches to activate and recruit T cells (2). Herein, we will demonstrate the significance of cancer vaccines in cancer immunotherapy, highlight that ICDs can be targets for cancer vaccines and summarize current strategies of inducing ICDs. We believe that this review will provide an updated, deep and comprehensive understanding of ICD-based cancer vaccines and lead the way for cancer immunotherapy.

CURRENT STATUS OF CANCER IMMUNOTHERAPY

Thirty-five years have passed when interferon-α (IFN-α), the first cancer immunotherapy, was approved by FDA for treating hairy cell leukemia (6). The concept of cancer immunotherapy has shifted from enhancement to normalization of antitumor immunity (7). Great progress have been made in ICBs, cancer vaccines, chimeric antigen receptor T (CAR-T) cells therapies, natural killer (NK) Cells therapies, co-stimulatory receptor agonists and cytokines in the past several decades. For example, myeloid cell TREM2 reprogrammed tumor microenvironement and the anti-TREM2 treatment promoted responsiveness of anti-PD-1 immunotherapy (8, 9). Personalized ovarian cancer vaccine produced neoantigen-specific T cells and prolonged survival of OC-bearing mice (10). CAR-NK therapy received great therapeutic responses in 11 enrolled relapsed or refractory CD19-positive cancers, without inducing major immune related adverse effects (irAE) (11). Cancer therapy has gradually entered into the era of immunotherapy. Currently, PD-1/PD-L1 monotherapies or combination therapies have been approved by FDA for first-line therapy of patients with metastatic gastric cancer and esophageal adenocarcinoma cancers, advanced renal cell carcinomas, triple-negative breast cancers, advanced lung cancer or advanced head and neck cancers. Past 5 years have even witnessed appear of neoadjuvant ICBs, which were believed to stimulate a long-lasting immunomodulatory effects on diverse immune cells. Over 100 registered clinical trials on neoadjuvant ICBs were recruiting, undergoing or completed. However, despite the rapid development, cancer immunotherapy do face some challenges need to be solved. Hegde and Chen have summarized top 10 challenges for immunotherapy, including the demand in the advancement in pre-clinical models, further explorations in mechanisms of cancer immunity, assessment of its clinical efficacy, as well as investigation of its combination regimens to improve therapeutic response or reduce irAE (12). Among which, we believe that transforming immunologically cold tumors into hot ones to maximize clinical efficacy of cancer immunotherapy/ICBs is one of the hottest research focuses.

CANCER VACCINES

Cancer vaccines can be categories into genetic vaccines, protein or peptide vaccines and cell vaccines. In the past decade, the recognization of cancer vaccines in immunotherapy gradually increase, especially in its combination with ICBs. Some have already been used in clinical practice. Human papillomavirus, Hepatitis B Virus and Hepatitis C virus vaccines are applied to prevent oncogenic infections, also called prophylactic vaccine. Oncophage, a heat shock protein (HSP) vaccine, was approved in Russia for patients with earlier stage kidney cancer in 2008 (13). Sipuleucel-T (Provenge®) was the first United States (U.S.) Food and Drug Administration (FDA)-approved cancer vaccine (2010), used to treat metastatic prostate cancer. It was a cancer vaccine manufactured with peripheral-blood mononuclear cells and a recombinant protein PA2024 through *ex vivo* incubation. Sipuleucel-T was reported to prolong overall survival of patients with metastatic prostate cancer, but did not delay disease progression (14). Oncophage and Sipuleucel-T are typical examples of therapeutic cancer vaccines, whose clinical realization is quite limited owing to time and cost taken to generate personalized cancer vaccine. Regardless of cancer vaccines formats, cancer vaccines aim at enhancing immunogenicity and promoting antitumor immunity to eliminate cancer cells through the induction of cancer antigens.

Cancer antigens can be classified into tumor associated antigens (TAAs), tumor specific antigens (TSAs) and cancer germline antigens (14). Non-mutant TAAs are suggested to be targets for cancer vaccines. Recently, Sahin and colleagues have tested the effect of FixVac, a liposomal RNA (RNA-LPX) vaccine that targets four non-mutated TAAs in ICB-experienced melanoma. The study showed that the RNA-LPX vaccine alone or combined with ICBs enhanced antitumor immunity, resulted in a better therapeutic response and confirmed that TAAs-based cancer vaccine could be utilized in immunotherapy (15). Whereas, there are still concerns that TAAs-based cancer vaccines encounter therapeutic resistance (central tolerance) as TAAs are generally expressed in normal cells. TSAs, also known as neoantigens, which are derived from tumor specific mutations rather than normal cells. It is believed that neoantigen-based cancer vaccines may be safer, more effective and more likely to spare from resistance, bringing cancer vaccine back to research focus after two decades when cancer vaccine clinical trials encountered a low objective response rate (16). Classified by sources, classical neoantigen-based cancer vaccines include but not limited to synthetic long peptide (SLP) vaccines, DC vaccines, RNA vaccines. In a phase I/Ib surgical resectable methylguanine methyltransferase-unmethylated glioblastoma trial, Keskin and colleagues have shown that SLP vaccination do lead to neoantigen-specific T cell infiltration and response in tumor microenvironment, which sensitizes immunotherapy in tumors

that originally with low tumor burden and immunogenicity (17, 18). Though, unfortunately all the enrolled patients could not spare from death because of tumor progression and recurrence (18), TSA-based cancer vaccines do arouse great attention. There is an urgent need for broaden understandings of vaccine-induced TSAs in stimulating immunity and improvements in manufacturing TSA-based cancer vaccines.

INDUCING IMMUNOGENIC CELL DEATH TO GENERATE CANCER VACCINATION

Immunogenic Cell Death: A Form of Immunogenic Regulated Cell Death

ICDs, contrary to tolerogenic or non-immunogenic cell deaths, are a form of regulated cell deaths that trigger adaptive immunity through production of neoantigens and release of DAMPs and cytokines. Thus, immunogenicity of cancer cell deaths *in vitro* is evaluated by immunostimulatory DAMPs like HMGB1, ATP, CRT, HSP70, HSP90, ANXA1, or cytokines like IFN, CCL2, CXCL1, CXCL10, etc (19). Meanwhile, the vaccination-rechallenge model is applied to detect ICDs *in vivo*. ICDs were initially found to be triggered by chemotherapy or radiation, while accumulative evidence has shown that they can be induced by photodynamic therapy (PDT), targeted therapy, oncolytic viruses, cardiac glycosides, as well as shikonin and capsaicin, extracts of Chinese herbal medicine. The inducement of ICDs and the production of neoantigens reshapes immunosuppressive TME to immunoactivited one, initiate antitumor immunity and improve therapeutic response of immunotherapy. ICD-based therapy is now viewed as attractive candidates for cancer immunotherapy combination regimens.

ICD Induction Realizes Cancer Vaccine-Like Effect

Upon particular stress like chemotherapy, radiotherapy, ICD cascade initiates including generation of tumor specific neoantigens by dying cancer cells, generation of ROS, induction of ER stress, release of DAMPs including exposure of CRT on cell surface (activation of "eat me" signal), secretion of ATP and HMGB1. DAMPs are then recognized by pattern recognition receptors (PRRs), promoting recruitment and maturation of DCs (**Figure 1**). The mature DCs then uptake dying cells, process TSAs to APCs, promote T cells polarizations, engulf CTLs, $CD4^+$ T cells, which leads to production of cytokines and activation of antitumor immunity ultimately (4, 5) (**Figure 1**). During the process, dying cancer cells

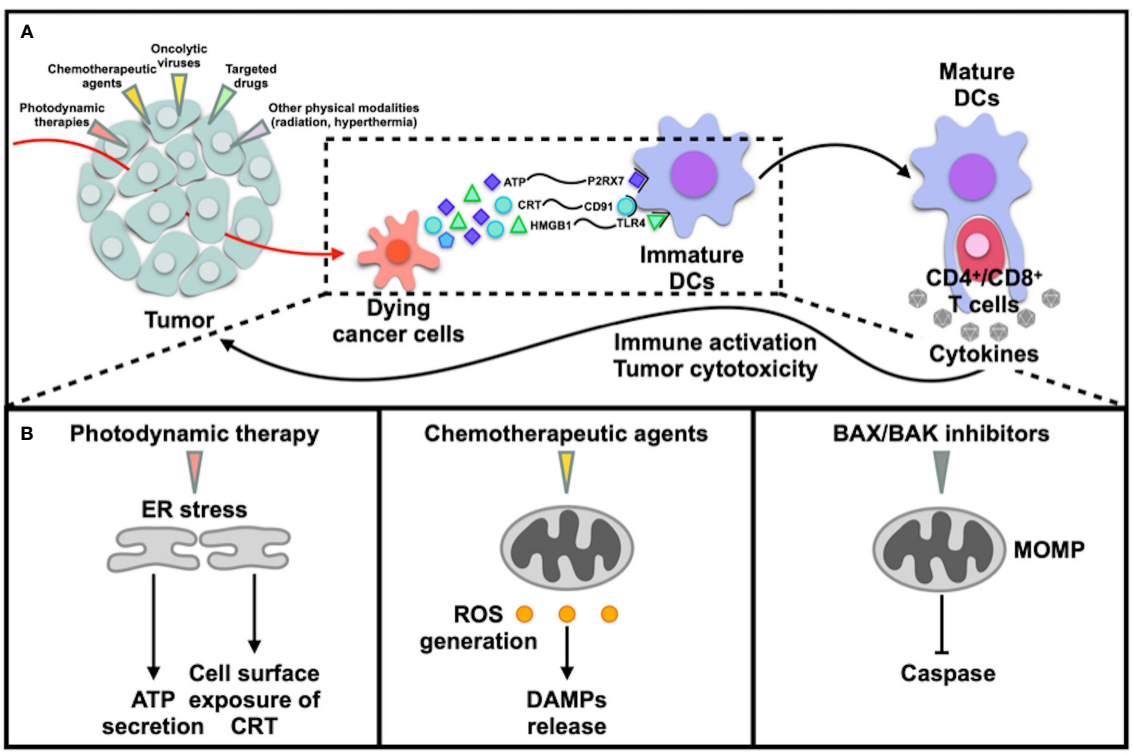

FIGURE 1 | Immunogenic cancer death. **(A)** Various triggers like photodynamic therapies, chemotherapeutic agents, oncolytic viruses, targeted drugs and physical modalities induce immunogenic cell deaths and release of DAMPs, leading to immune activation; **(B)** ICDs were induced via different mechanisms: 1) induction of ER stress directly cause cell deaths; 2) generation of ROS, in which induction of ER stress does not directly cause ICDs; 3) induction of mitochondrial outer membrane permeabilization (MOMP).

generate antigens and enhance immunogenicity to kill tumor cells, in line with the criterion of endogenous cancer vaccines. Recruited DCs may serve as be effective targets for cancer vaccine as well, which is DC vaccine that we are familiar with.

ICD Detection Relies on the Vaccination-Rechallenge Experiment

Immunogenicity of cell deaths induced by therapeutic agents can be assessed *in vivo* and *in vitro* through quantitative analysis of CRT exposure, ATP and HMGB1 secretion, ROS generation or ER stress. At present, there is no specific structure that helps to predict potential ICD inducers. The gold standard experiment to validate ICD or verify an ICD inducer is the vaccination-rechallenge experiment: cancer cells were treated with a potential ICD inducer and cell viability was assessed *via* cytometry after staining with PI and annexin V/DIOC$_6$ (**Figure 2B**). Most of non-dead cancer cells should be at a dying stage. Cell suspension was then injected subcutaneously to immunocompetent mice, which is called vaccination. Living cancer cells were injected a week later, which is called rechallenge. Tumor volumes of mice post-vaccination to post-rechallenge and the ratio of mice spared from tumor formation were recorded (20). The vaccination-rechallenge experiment not only helps to identify an ICD inducer, but also builds a prototype for vaccination with ICD cells.

APPROACHES TO PRODUCE IMMUNOGENIC CELL DEATH-BASED CANCER VACCINES

ICD can be triggered by PDT (21), chemotherapeutic agents (18, 22–31), oncolytic viruses (OVs) (32–34) and targeted drugs (35–38) (**Table 1**), as well as physical modalities such as radiation (52), hyperthermia (53), which won't be discussed herein. In this review, we divide ICD-based cancer vaccines into two categories: ICD inducers (**Figure 2A**) and ICD cells (**Figure 2B**).

Vaccination With ICD Inducers: Photodynamic Therapy

PDT has been proved to have anti-tumor and immune-activation effect in various types of cancers such as cervical cancer, pancreatic cancer, prostate cancer, glioblastoma and clinically approved in treating non-small cell lung cancers and esophageal cancers by the U.S. FDA. PDT requires a light-sensitive dye, also known as a photosensitizer (PS). With the presence of light of specific wavelength (600-900 nm) and oxygen, PDT is able to generate reactive oxygen species (ROS) and cause cell death through inducing endoplasmic reticulum (ER) stress (54, 55).

It is worth noting that PDT's effect on inducing ICDs and enhance tumor immunogenicity is reduced in hypoxic niche (21,

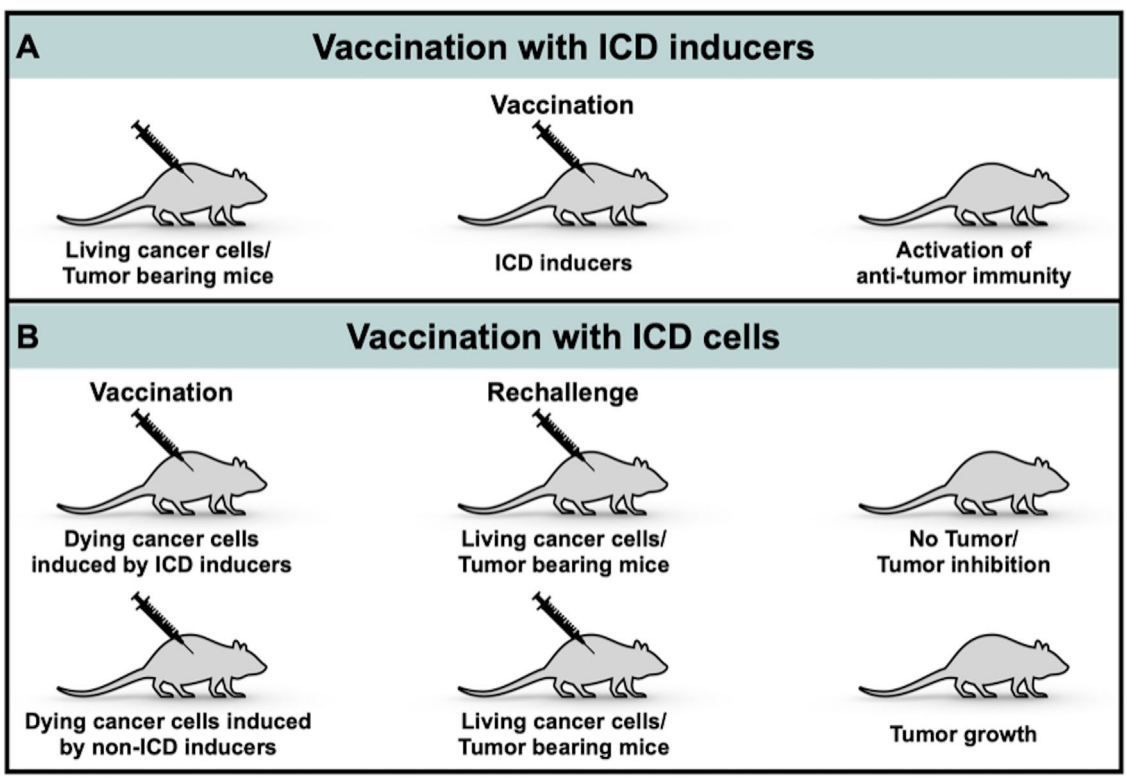

FIGURE 2 | Process of inducing ICD-based cancer vaccines. **(A)** Vaccination with ICD inducers; **(B)** Vaccination with ICD cells.

TABLE 1 | Examples of immunogenic cell death-based cancer vaccine.

Categories	Examples	Cell lines	Vaccination-challenge experiment	ROS generation	ER stress	Biomarkers (DAMPs) for identifying ICDs	Cytokines secretion	Antitumor immunity activation	Other observations	Type of ICDs	Ref
Photodynamic therapies-derived cancer vaccine	PS-PDT; PD-PDT; FAL-ICG-HAuNS; AuNC@MnO2, AM; Ce6/MLT@SAB; CCPS; pRNVs/HPPH/IND; Ds-sP/TCPP-TER NPs)	4T1; B16; CT-26; MC38; GL261; MCA205	+/-	+	+/not mentioned	CRT exposure; ATP release; HMGB1 release	IL-6; IL-12; TNF-α; IFN-γ	Recruitment, maturation and activated antigen presenting functions of DCs; Recruitment, infiltration, proliferation and activation of CTLs; Activation of $CD4^+$ T cells (mostly Tregs) and NK cells activation; Inhibition of MDSCs proliferation	CHOP proteins upregulation; Relieve of hypoxic niche; Caspase-3 activation; PI3K-Akt pathway activation; IDO upregulation	Apoptosis; Ferroptosis; Necrosis	(21, 39–43)
Chemotherapeutic agents-derived cancer vaccine	Doxorubicin; Bortezomib; Melphalan; Paclitaxel	EG7; CT26; PRObe; B16; U266; GL261;	+ (Mostly)	+	Not mentioned in most researches	CRT exposure; ATP release; HMGB1 release; HSP70 exposure; HSP90 exposure; F-actin exposure	IFN-γ; IL-1β; IL-6	Maturation and proliferation of DCs; Proliferation. infiltration of CTLs; Proliferation of NK cells; Proliferation and polarization of macrophages;	Caspase-3 activation; Activation of NF-kB–mediated CCL2 transcription; Activated vesicle exocytosis induced by IkappaB-mediated SNARE;	Apoptosis	(22, 24, 25, 27, 31, 44)
Oncolytic viruses-derived cancer vaccine	Semliki Forest virus; Vaccinia virus; Adenovirus; Measles virus; Coxsackievirus B3; Newcastle disease virus; Seasonal Influenza Vaccine	B16; HOS; A549; CT26; MeWo; PAN02; CMT93; Mel888; Mel624; SkMel28	+	+	Not mentioned in most researches	CRT exposure; ATP release; HMGB1 release; HSP90 exposure;	IFN-α; IFN-β; IFN-γ; IL-12; TNF-α; IL-4; IL-13; IL-6; IL-8; IL-1β; IL-24; CCL3	Activation and maturation of DCs; Recruitment and activation of tumor antigen-specific CTLs; Maintenance of Bregs;	Release of PAMPs; Activation of PI3K/Akt and MAP/ERK/MEK pathway; Release of tumor specific neoantigens	Apoptosis; Necroptosis; Pyroptosis; Autophagy;	(33, 34, 45–50)
Targeted drugs-derived cancer vaccine	7A7 mAb; Cetuximab; Crizotinib	D122; H1650;	+	Not mentioned	+	CRT exposure; ATP release; HMGB1 release; HSP 70 exposure; HSP 90 exposure;	IFN-α; IFN-γ; IL-12; IL-17;	Activation and maturation of DCs; Proliferation, infiltration and activation of CTLs; Promotion of DCs phagocytosis of dying cells; Induction of PD-1 expression on tumor-	Inhibition of XBP1	Apoptosis	(35–37)

(Continued)

TABLE 1 | Continued

Categories	Examples	Cell lines	Vaccination-challenge experiment	ROS generation	ER stress	Biomarkers (DAMPs) for identifying ICDs	Cytokines secretion	Antitumor immunity activation	Other observations	Type of ICDs	Ref
ICD cells-derived cancer vaccine	Necroptotic cancer cells generated by RIPK3 induction systems	CT26	+	Not mentioned	–	ANXA1 exposure; ATP release; LDH release; HMGB1 release;	IFN-γ; CXCL1	infiltrating $CD4^+$ $Foxp3^-$ cells Activation of BMDCs maturation and phagocytosis of dying cells; Cross-priming and proliferation of CTLs	NF-kB activation, which is necessary for immunogenic necroptosis	Necroptosis	(51)

4T1, murine breast cancer cell lines; B16, murine melanoma cell lines; CT-26, MC38, murine colon carcinoma cell lines; GL261, murine glioma cell lines; MCA205, murine fibrosarcoma cell lines; U266, murine myeloma cell lines; ER, endoplasmic reticulum; CRT, calreticulin; DCs, dendritic cells; CTLs, cytotoxicity T lymphocytes; Bregs, regulatory B cells; Tregs, regulatory T cells; NK cells, natural killer cells; MDSCs, myeloid-derived suppressor cells; 7A7 mAb, an anti-murine EGFR Ab; RIPK3, receptor-interacting protein kinase-3; LDH, lactate dehydrogenase; XBP1, X-box binding protein 1; ANXA1, Annexin A1.

56). Meanwhile, PDT exacerbates hypoxia in TME, leading to angiogenesis, tumor progression, metastasis, poor therapeutic response and prognosis (56, 57). Researchers have been trying to solve the problem by developing an oxygen nanocarrier with chlorine e6 encapsulated (C@HPOC) (58). The modified oxygen-boosted PDT displayed infiltration of cytotoxic T lymphocytes (CTLs) in TME and a better induction of ICD in 4T1 murine breast cancer cells by elevating cell surface exposure of CRT, increasing release of HMGB1 and ATP, and afterwards promoting the maturation of DCs (57). Combing metal-organic framework-based nanoparticles to PDT was also of benefit for alleviating PDT-induced hypoxia *via* inhibiting hypoxia inducible factor-1α (59). The combination of nanomaterials or anti-hypoxic treatment with PDT may help it to achieve a better clinical realization.

Vaccination With ICD Inducers: Chemotherapeutic Agents

The initial hypothesis on the relationship between chemotherapy and ICDs could date back to one or two decades ago. It was proposed that the impact of chemotherapy on immune system through induction of ICDs helped it to achieve better clinical efficacy (18). However, not all kinds of chemotherapeutic agents induce ICDs, and some chemotherapeutic agents do have immunogenic effects, but insufficient to cause cell death (28). So far, anthracyclins (22), bleomycin (23), bortezomib (24), cyclophosphamide (25, 26), daunorubicin (22), doxorubicin (22), idarubicin (22), melphalan (27), oxaliplatin (31), paclitaxel (44) and trifluridine/tipiracil (29) have been proved to induce ICDs; while, cisplatin, mitomycin C were proved to induce non-immunogenic apoptosis (22, 28). The reason remains unknown, even though oxaliplatin and cisplatin share similar structures, which raises the bar for seeking out an ICD inducer and confirms the significance of vaccine-rechallenge experiment.

Mechanisms in inducing ICDs for chemotherapeutic agents are different from those for PDT. Instead of inducing ICDs directly through ER stress, chemotherapeutic agents tend to induce generation of ROS. Based on the difference, Wang et al. have divided ICD inducer into two types: type I for those inducing ROS generation (chemotherapeutic agents/targeted therapy), type II for those inducing ER stress (PDT/Oncolytic viruses) (60). Notably, cancer vaccination effects induced by chemotherapeutic agents are weaker than PDT. Take melphalan as an example, hypericin photodynamic therapy (Hyp-PDT) as a positive control, brefeldin A (a tolerogenic cell death inducer) as a negative control, melphalan-induced cancer vaccination effect was in the middle with 40% vaccinated mice protected (62% for Hyp-PDT group, less than 20% for brefeldin A group) (27). However, apart from its cytotoxicity to kill tumor cells directly, chemotherapeutic agents have their advantages compared to ICD inducer in being a cancer vaccine: their extensive role on non-malignant cells in immunosuppressive TME and additional immunogenic effect other than ICDs, which may be utilized to realize better tumor-killing effect (30).

Vaccination With ICD Inducers: Oncolytic Viruses

OVs have become attractive formats of cancer vaccine in immunotherapy considering its direct cytotoxicity mediated by oncolysis, immune activation and anti-angiogenesis (61). Even intratumoral injections of OVs have been proved to activate anti-tumor immunity not only inside tumors and on adjacent TME, but also on distant untreated focuses (62). Previous studies have revealed that oncolytic viruses such as Coxsackievirus B3, Adenovirus, Measles virus, Semliki Forest virus, Newcastle disease virus, influenza A virus elicit ICDs (33, 34, 45–47, 50) and improve immune responses of ICBs (48, 49). The additional release of pathogen-associated molecular patterns (PAMPs) derived from oncolytic viruses further increases immune-activation (63).

Specially, the combination of oncolytic viruses and chemotherapeutic agents have been shown to maximize immunogenicity. For example, the combination of adenovirus and oxaliplatin, both demonstrated to be ICD inducers in colorectal cancer in previous studies (31, 47), leaded to reduced tumor growth and longer median survival CT26 (mouse rectal adenocarcinoma cell line)-bearing mice compared to adenovirus or oxaliplatin alone (64). Instead of systemic administration, researchers chose intratumoral injection *via* interventional radiologic techniques to peruse a high local concentration sufficient to cause ICDs and a relatively low systemic toxicity. Meanwhile, a replicating other than a non-replicating virus was required to overcome transient growth inhibitions and achieve sustained tumor suppressive activity; buffer for oxaliplatin needed to be carefully selected considering its impact on increasing cytotoxicity of oxaliplatin and reducing infectivity of adenovirus (64). These findings confirm synergistic effects of two ICD inducers and provide guidance when one single ICD inducer does not sufficient to provide a strong and long-lasting immune-activation.

Vaccination With ICD Inducers: Targeted Drugs

Epidermal growth factor receptor (EGFR)-targeting mAb 7A7 or cetuximab and tyrosine kinase inhibitor crizotinib were reported to exert ICDs (35–38, 52). EGFR-targeting mAb 7A7 exert an effect on DCs, T cells and NK cells infiltration (65). The combination of targeted drugs and chemotherapy, for example cetuximab plus folinic acid+fluorouracil+irinotecan (FOLFIRI) or crizotinib plus cisplatin), resulted in a better antitumor efficacy (36–38). Cetuximab's ability of inducing ICDs relies on EGFR mutational status and BRAF mutation. It failed to induce ICDs in human colon cancer cells (HT-30 cells lines) ($KRAS^{WT}$) (37). These provide explanations for heterogeneous or limited therapeutic efficacy of some targeted drugs when applied as a single agent to some extent. Liu and colleagues also showed that crizotinib and cisplatin combination upregulated expression of PD-1/PD-L1 and improved therapeutic response to ICBs (37, 38). However, targeted therapy in inducing ICDs is still at a beginning stage calling for more attempts and scientific evidence.

Vaccination With ICD Cells

Expect vaccination with ICD-inducers, vaccination with ICD cells also realizes a cancer vaccine-like effect, which mimics the establishment of vaccination-rechallenge model (**Figure 2B**). Immunocompetent mice were vaccinated with doxycycline-treated necroptotic DD_RIPK3 cells (51). Injected necroptotic cells leaded to the generation of TSAs, proliferation of CTLs, release of HMGB1, production of cytokines like $CXCL_1$, IFN-γ and phenotypic maturation of bone marrow-derived DCs. Remarkably, NF-κB activation but not ER stress was observed in necroptotic cells, indicating immunogenicity of DD_RIPK3 cells was not principally mediated by ER stress, which was a little bit different from up-mentioned classical ICDs (51). Strategies that elevated RIPK3 expression were required since losses of RIPK3 expression exists in many types of cancer and genotypes of ICD cells largely restrict their immunogenicity (6, 51). Compared to ICD inducers, utilizing ICD cells is more able to produce personalized cancer vaccines, but more time-consuming and money-consuming.

DISCUSSION

ICDs generate tumor specific antigens, which serves as endogenous cancer vaccine targets. Recent years, especially last year, have witnessed a growing utilization of nanoparticles in delivering ICD inducers or amplifying cancer cell ICDs. Advantages of ICD-based cancer vaccines are quite obvious. First and foremost, they arouse immunogenicity for "cold" tumor and sensitize immunotherapy. Secondly, their immune stimulation activities reach to distant untreated lesions, which means intratumorally injection with a lower-dose may be chosen to avoid systemic cytotoxicity. Apart from up-mentioned a great variety of inducers, other therapeutic agents such as cardiac glycosides (66), non-steroidal anti-inflammatory drugs (NSAIDs) (67), dinaciclib (an experimental inhibitor of cyclin-dependent kinases) (68) displayed ability to induce ICDs as well.

However, it is still difficult to look for a candidate in a bundle of drugs. Recognitions of universality of ICD inducers, ICD biomarkers and platforms for efficient, time-saving, money-saving, high-throughput drug screening may accelerate drug discoveries. What's more, we have noticed heterogeneous capability of activating anti-tumor immunity. As reviewed previously, TME is made up of various specialized micro environments (1), and there is an intricate crosstalk between each other. Besides fighting against immunosuppressive TME, ICD-based cancer vaccine needs to overcome hypoxia as well to realize a stable and long-lasting effect. Jessup and colleagues have combined two ICD inducers to achieve better immune responses (64). Chen et al. (58) and Cai et al. (59) have worked on modifying the PS with nanoparticles or combining anti-hypoxia treatment to overcome hypoxia in TME as mentioned before. They have set good examples for magnifying clinical effect of ICD-based cancer vaccines. The clinical realization of ICD-based cancer vaccines relies on a deeper investigation of their mechanisms

not merely on tumor immunity or immune microenvironment but also on the whole TME, and more attentions and attempts on combination therapy. It is noteworthy that previous studies haven't take irAE into consideration. Questions like how severe are irAE of ICD-based cancer vaccines or what kinds of people tend to suffer from irAE call for answers. Also, we've noticed that ICDs include apoptosis, ferroptosis, necrosis, autophagy, which may exert a different impact on immunity (69), whereas, few studies take modes of cell deaths into considerations.

In general, the concept that ICD can be competitive cancer vaccine targets provides a solid theoretical basis for application of ICD-based cancer vaccine in "cold" tumor and broadens understandings and approaches for investigation of neoantigen-based cancer vaccine. Owing to immunogenicity induced by ICDs, ICD-based cancer vaccines will be appealing sensitizers for ICBs in the near future.

AUTHOR CONTRIBUTIONS

X-PW and M-ZJ conceived the paper. M-ZJ wrote the paper and developed the figures and tables. X-PW edited the paper. All authors contributed to the article and approved the submitted version.

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The Mechanism of Stimulating and Mobilizing the Immune System Enhancing the Anti-Tumor Immunity

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Cancer immunotherapy is a kind of therapy that can control and eliminate tumors by restarting and maintaining the tumor-immune cycle and restoring the body's normal anti-tumor immune response. Although immunotherapy has great potential, it is currently only applicable to patients with certain types of tumors, such as melanoma, lung cancer, and cancer with high mutation load and microsatellite instability, and even in these types of tumors, immunotherapy is not effective for all patients. In order to enhance the effectiveness of tumor immunotherapy, this article reviews the research progress of tumor microenvironment immunotherapy, and studies the mechanism of stimulating and mobilizing immune system to enhance anti-tumor immunity. In this review, we focused on immunotherapy against tumor microenvironment (TME) and discussed the important research progress. TME is the environment for the survival and development of tumor cells, which is composed of cell components and non-cell components; immunotherapy for TME by stimulating or mobilizing the immune system of the body, enhancing the anti-tumor immunity. The checkpoint inhibitors can effectively block the inhibitory immunoregulation, indirectly strengthen the anti-tumor immune response and improve the effect of immunotherapy. We also found the checkpoint inhibitors have brought great changes to the treatment model of advanced tumors, but the clinical treatment results show great individual differences. Based on the close attention to the future development trend of immunotherapy, this study summarized the latest progress of immunotherapy and pointed out a new direction. To study the mechanism of stimulating and mobilizing the immune system to enhance anti-tumor immunity can provide new opportunities for cancer treatment, expand the clinical application scope and effective population of cancer immunotherapy, and improve the survival rate of cancer patients.

Keywords: anti-tumor immunity, CAR-T, checkpoint inhibitors, immune system, immunotherapy

INTRODUCTION

Tumor microenvironment (TME) is a complex environment in which tumor cells depend for survival and development. The tumor microenvironment is composed of cellular components and non-cellular components (1). The cell components include tumor cells, inflammatory cells, immune cells, mesenchymal stem cells, endothelial cells, and tumor-related fibroblasts. Non-cellular components, including cytokines and chemokines, constitute a complex tumor microenvironment. These cellular and non-cellular components work together to support tumor growth (**Figure 1**). Tumor and its microenvironment interact and promote each other through angiogenesis, immunosuppression and other means (2). Therefore, the influence of tumor microenvironment on treatment must be considered when developing tumor treatment methods, or targeted tumor microenvironment treatment methods should be adopted. In the past, researchers have focused on targeting cancer cells themselves, such as traditional chemoradiotherapy and targeted therapies that target the tumor itself. In recent years, the treatment of tumor against tumor microenvironment has become a hot spot of tumor treatment. Studies have found that this kind of treatment can often bring better results. Anti-angiogenesis therapy and immunotherapy are the mainstream treatment methods for tumor microenvironment at present (**Figure 1**). The aim of tumor immunology therapy is to stimulate or mobilize the body's immune system and enhance tumor microenvironment anti-tumor immunity, so as to control and kill tumor cells (3). There are many methods of tumor immunological therapy (4) (5), including tumor vaccine (6), immune-guided therapy, cell adoptive immunotherapy (7), cytokine therapy, gene therapy (8) and comprehensive therapy. Immunotherapy of tumor, whether a single drug or combination therapy, has shown clinical efficacy in many kinds of cancer, so it has attracted more and more attention (3, 9). Although immunotherapy has great potential, it is currently only applicable to patients with certain types of tumors, such as melanoma, lung cancer, and cancer with high mutation load and microsatellite instability, and even in these types of tumors, immunotherapy is not effective for all patients. In addition, the skin toxicity, gastrointestinal toxicity and other side effects caused by immunotherapy of tumor should not be ignored (10). This article reviews the mechanism of immune resistance, principle of immunotherapy for cancer, the application of new immunotherapy for cancer and immunotherapy in different tumors. It also introduces the new strategies to overcome the resistance of immunotherapy for cancer, the methods to improve the curative effect of immunotherapy and to alleviate side effects, as well as the screening, treatment and effectiveness evaluation of beneficiaries before immunotherapy.

MECHANISMS OF IMMUNE RESISTANCE

Standard immunotherapy has not been as effective as expected in the treatment of aggressive and advanced cancers. This is due to decreased immunogenicity and increased immune tolerance. When the immune "hot" tumor becomes "cold" or immunosuppressed, the tumor recurs (11, 12). Lack of understanding of these mechanisms may hinder the development and clinical application of tumor immunotherapy.

Tumor Cell Heterogeneity

Tumor heterogeneity refers to the presence of cells with many different genotypes or subtypes within the same tumor (13–15).

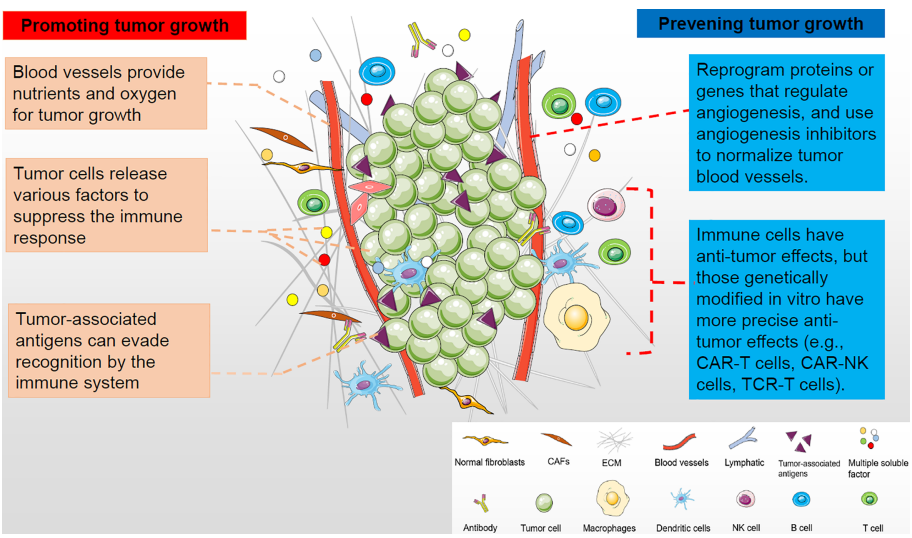

FIGURE 1 | The tumor microenvironment is composed of cellular and non-cellular components that support tumor growth. Tumor and its microenvironment interact and promote each other through angiogenesis and immunosuppression. Therefore, targeting tumor microenvironment in anti-tumor therapy can make greater progress, such as inhibiting tumor angiogenesis and tumor immunity.

Therefore, the same kind of tumor in different individuals can show different therapeutic effects and prognosis, and even the same body tumor cells have different characteristics and differences. Subclones usually survive treatment and lay quiescent. When selective pressure is removed, the cells can produce signaling factors that increase the tumorigenicity and growth ability of tumor cells, a process known as subcloning synergy. The influence of drug-resistant subclones is the basis of the limited efficacy of current immunotherapy (16).

Major Histocompatibility Complex

The loss of MHC Class I and Class II molecules has been shown to be associated with tumor progression. Human MHC or Human Leukocyte Antigen 1 (HLA-I) molecules are encoded by a series of polymorphic genes with multiple alleles or haplotypes. The most common method for these allele alterations is loss of heterozygosity in the coding region of HLA and 2-microglobulin (an essential element of the HLA-I complex), resulting in loss of HLA-I haplotypes, and loss of chromosome 15 (7, 17). Tumor cells also have a tendency to evade NK cell detection by regulating the expression of MHC class I-like NKG2D ligands.

Anti-Apoptotic Mechanisms

Tumor cells use a complex network of hyperactivated signaling pathways to protect cells from apoptosis and ensure their continued survival and proliferation. Common signaling pathways are Ras/Braf/Mek/MAPK pathway and PI3K/Akt pathway. Activation of MAPK pathway can promote the expression of tumor immune editing, immunosuppressive cytokines and checkpoint markers, and reduce the infiltration of CTL (18, 19). The hyperactivated PI3K/Akt pathway has these functions after the loss of its physiological inhibitor, the phosphatase and tensin homolog (PTEN). Abnormal PI3K/Akt signaling pathway inhibits cell apoptosis by up-regulating the expression of Bcl-2 and reducing the level of apoptotic regulators.

Immune Checkpoint Ligands

The coinhibitory checkpoint ligand on the APC interacts with the receptor on the lymphocyte to prevent self-reactivity and maintain peripheral tolerance. When a programmed death receptor of T cell encounters these checkpoint ligands and is unable to fully respond to its target, the T cell experiences impotence or failure (the "off switch") (20, 21). Although a series of features of this ligand are normal tissue, tumor cells are known to have the ability to inhibit tumor-specific t cell function. PD-L1 and CTLA-4 are the most studied checkpoint ligands (6, 22).

NEW APPROACHES TO TUMOR IMMUNITY

Cancer Vaccines

Tumor vaccines amplify tumor-specific T cell responses by generating active immunity by identifying tumor-associated antigens. The active components of cancer vaccines consist of four key components described below: tumor antigens (6), preparations, immune adjuvants, and delivery vectors (23). There are two types of cancer vaccines. One is used to prevent cancer. The other, which is used to treat cancer, is emerging immunotherapy. It stimulates the body's immune system to fight, killing cancer cells and preventing them from spreading and returning.

New York esophageal squamous cell 1 (NY-ESO-1), a kind of cancer/testis antigen (CTA), is a cancer-associated protein found in many invasive tumors (24). It is widely expressed in breast cancer, bladder cancer, prostate cancer, melanoma, NSCLC, hepatocellular carcinoma, ovarian cancer and other cancers, with a range of 20-80%. It is one of the most immunogenic antigens in the body, so it is considered as an ideal target antigen for tumor immunotherapy. NY-ESO-1 cancer vaccine is made with NY-ESO-1 as the target antigen. DC cells ingested cancer-specific antigens composed of NY-ESO-1 derived peptides and presented them to the tumor microenvironment. DC vaccines containing NY-ESO-1 peptide directly stimulate T cells to fight tumors (25). Racotumomab is a murine gamma-type anti-idiotype monoclonal antibody that specifically induces an antibody response against Neu-glycolyl GM3 ganglioside. The best clinically active heat shock protein for advanced NSCLC patients ACTS as an intracellular chaperone, binding and presenting tumor antigens on specialized APC *via* MHCI and II molecules, leading to activation of anti-tumor T cells. Racotumomab has been shown to be a maintenance therapy for advanced non-small cell lung cancer (26). The tumor antigen of the CryoVax vaccine comes from a chaperone released by substances inside the tumor. The vaccine currently targets patients with advanced metastatic colorectal cancer. It can be used as a tumor antigen and adjuvant to regulate the immune response *in vivo*. The aim of the CryoVax vaccine is to create "hot" tumors in these patients and then naturally block the expression of checkpoint molecules. Currently, AlloVax vaccine is mainly used in patients with advanced liver cancer. The chaperones in tumor cells carry autologous tumor-specific peptides (antigens) that confer tumor-specific immunity. AlloVax vaccines contain protein-associated cell lysates (CRCL). After AlloVax injection, CRCL contains a lot of tumor antigens, so it increases the chances of the body producing an effective immune response to all tumors. Ronald Levy and other researchers developed a new cancer vaccine, irradiation of iPSCs as an autogenous anti-tumor vaccine. They found that the new cancer vaccine is suitable for many different types of cancer, including breast cancer and colorectal cancer (27).

CAR-T Cell Immunotherapy

CAR-T cell immunotherapy (chimeric antigen receptor T cell immunotherapy) is a new type of cancer immunotherapy. Car-T therapy extracts some T cells from patients and gene modification them to make T cells express new receptor CAR. After proliferation, they are infused back into the patient's body (**Figure 2**). These T cells can quickly identify and destroy target cells using their CAR receptors (28). Compared with surgery, radiotherapy, chemotherapy, targeted therapy and hematopoietic stem cell transplantation, CAR-T cell immunotherapy is more accurate, flexible, spectral and durable. It has a remarkable curative effect in the treatment of acute leukemia and non-Hodgkin

lymphoma (29). In a recent study, researchers found that CAR-T therapy is effective in treating patients with glioblastoma and can remove 80% of the tumors (30). Moreover, the combination of anti-cancer vaccine and CAR-T therapy can stimulate the immune system to produce memory T cells and prevent the recurrence of tumors (31, 32).

TCR-T Therapy

Although the existing CAR T treatment has shown significant efficacy in clinical trials for acute and chronic lymphoblastic leukemia, the available targets for CAR T treatment are limited, the treatment of solid tumors has not been very effective, and the adverse reactions caused by CAR T treatment are sometimes difficult to control. Compared with CAR T, TCR-T therapy can select more targets and has better efficacy in solid tumors with fewer side effects. TCR-T therapy improves the affinity and combat effectiveness of TCR (T cell antigen receptor) that specifically recognizes tumor-associated antigen by transducing chimeric antigen receptor or TCR α/β heterodimer, enabling T lymphocytes to re-efficiently recognize target cells (33).

In a collaborative trial study, researchers found that preliminary clinical results from patients receiving TCR-T cell therapy showed encouraging positive signs. TCR had better binding affinity after improvement. TCR-T cells showed excellent expression level. The persistence of therapeutic effects has been demonstrated in preliminary studies. In addition, researchers have developed other HLA subtypes to treat more patients with different HLA subtypes in the future. At present, more and more enterprises at home and abroad have carried out research on TCR-T therapy.

Fusion Cell Therapy

Fusion cell therapy is a kind of therapy that uses cancer cells of patients to develop new dendritic cells to attack cancer cells. Through the direct fusion of cancer cells and dendritic cells of cancer patients, new dendritic cells are cultivated. When the new dendritic cells are reinjected near the lymph nodes, they will educate T cells that can remember a lot of cancer antigen features. Even if the cancer cells hide a feature, the T cells will recognize them from other features, leaving the cancer cells nowhere to hide, and finally be killed. A phase II trial of fused cell vaccine + IL-12 in 15 patients with brain tumors (gliomas) showed that the treatment prevented 73 percent of the disease from deteriorating, with a clinical response rate of 40 percent (34). Avigan et al. has investigated the efficacy of the fused cell vaccine in treating kidney cancer (35), showing that the vaccine contains both dendritic cells of the patient and the patient's own cancer antigen, which can induce a wide immune response and make it difficult for cancer cells to escape under the surveillance of the immune system. Avigan et al. found that the combination of the fused cell vaccine and anti-PD-1 antibodies was also applicable to blood cancers such as leukemia and myeloma (36). Because the vaccine is based on the patient's cells, a completely customized cancer vaccine modulation is realized without any side effects.

CAR - NKT Therapy

The researchers found that natural killer T cells (NKT) are a special subset of T cells that have both the T cell receptor TCR and the NK cell receptor on their surface (37). NKT cells destroy cancer cells by releasing cytotoxic particles (38). Car-nkt cell

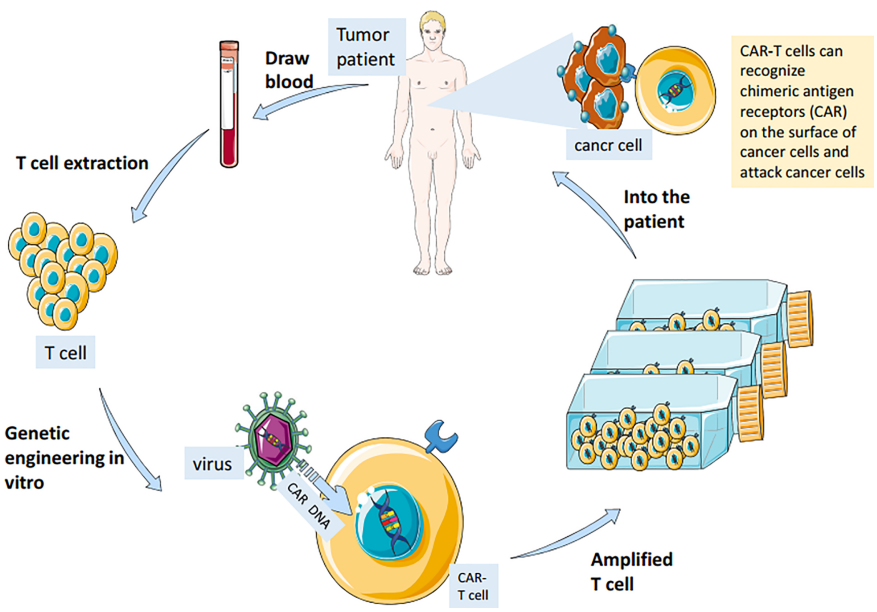

FIGURE 2 | Immune T cells were isolated from patients and genetically engineered *in vitro* to be fitted with chimeric antigen receptors (CAR) that recognize cancer cell surface antigens. The modified cells are amplified in large Numbers *in vitro* and injected back into the patient to achieve the therapeutic effect of accurately identifying and killing cancer cells.

therapy is to separate the NKT cells from the blood of patients or healthy people, and collect them back to the patients after reaching a certain amount of culture *in vitro*. Researchers conducted a phase I clinical trial based on engineered CAR-NKT cells to evaluate CAR-NKT cell therapy in children with recurrent neuroblastoma. At present, two children with neuroblastoma have been treated. One patient was stable at 4 weeks follow-up. The other patient had two bone tumors, one of which disappeared completely (39).

LN - 145 Therapy

LN-145 is a therapy (40) developed by Iovance which amplifies and activates the anti-tumor activity of autologous TILs *in vitro*, and returns them to the patient. TIL is a special lymphocyte mobilized by the immune system in the early stage of cancer. These lymphocytes have the ability to recognize and attack the flow of tumors and penetrate into tumors. LN-145 therapy extracts TIL from patients and then stimulates TIL amplification *in vitro* with IL-2 cytokines. This not only increases the number of TIL, but also activates TIL's anti-tumor ability. Finally, these TILs were injected back into the patient to play a more powerful role in killing cancer cells. LN-145 therapy has shown amazing data in clinical trials (41). FDA approved LN-145 for breach-through treatment of advanced cervical cancer and accelerated its approval for listing.

Checkpoint Inhibitor

After the activation of T cells involved in anti-tumor immune response, the expression of various inhibitory regulatory receptors on the surface of T cells was up-regulated, and combined with the corresponding ligands highly expressed on the surface of tumor cells, which inhibited the immune response and down regulated the intensity of tumor related immune response. In the process of immune response, the sites with inhibitory immunoregulation are called immune checkpoints. Traditional immunotherapy is mainly to induce or strengthen anti-tumor immune response, but due to the existence of immunosuppressive immune regulation, such as immune checkpoints, often cannot produce lasting and effective anti-tumor immune effect. Checkpoint inhibitors can effectively block the inhibitory immunoregulation of PD-1/PD-L1, CTLA-4 and other immune checkpoints, so as to indirectly strengthen the anti-tumor immune response and improve the effect of immunotherapy. The combination of these two checkpoint inhibitors has also been evaluated clinically for improved efficacy but increased toxicity. The anti-CTLA-4 antibody ipilimumab was shown to be effective for advanced melanoma for the first time (42–44). At present, CTLA-4 and PD-1 inhibitors have been used in clinical immunosuppression. Among them, CTLA-4 inhibitors include apilimumab, tremelimumab, etc. PD-1 inhibitors include nivolumab, pembrolizumab, pidilizumab, etc. In recent years, the biomarkers of immunocheckpoint inhibitors have developed rapidly. This is due to the research on the mechanism of the interaction between tumor and host genome, tumor microenvironment and immune function. T cells are activated by two signals (TCR-MHC-peptide recognition antigen and CD28 something CD 80/86 costimulatory signal). Mutations in Tumor Cell Genomes or proteins that are abnormally expressed are processed into peptides that bind (or fail to bind) to MHC class I molecules. Immunogenic peptides (MHC-bond immunogenic peptides) trigger a subsequent immune response. Generally, the higher the mutation load, the higher the possibility of producing MHC-bond peptides. Inhibitory immune cells (such as tumor-associated macrophages) and tumor-causing inflammatory mediators (such as TNF-α、 IL-6、 TGF-β) in tumor microenvironment can stimulate tumor cell proliferation and induce tumor angiogenesis by inhibiting NF-κB and STAT3 signaling pathways, promote the tumor immune escape as well as the tumor invasion and metastasis (45–47).

Regulatory T Cells

Tregs is a subtype of CD4 cells, which can inhibit immune response, maintain immune tolerance and prevent autoimmune. Tregs can enhance immune function and help maintain homeostasis. In cancer, however, Tregs infiltrate the tumor microenvironment (7, 48, 49). This is associated with poor prognosis and poor response to immunotherapy. In animal models, the removal of Tregs has been shown to improve the anti-tumor immune response. CTLA-4 is highly expressed on Tregs. Treatment with anti-CTLA-4 antibody could deplete Tregs in tumor microenvironment in mice. This is achieved through a mechanism that relies on tissue to host macrophages. However, some studies have shown that enhanced tumor therapy *via* anti-CTLA-4 antibody is dependent on interactions with Tregs and T effectors (50, 51).

PD-1/PD-L1 AND TUMOR IMMUNOTHERAPY

In normal and stable state, PD-L1 pathway can maintain immune homeostasis and protect immune system. In canceration, PD-L1 can disrupt the tumor's immune cycle in two ways, thus protecting the tumor from damage by cytotoxic T cells. First, over-expression of PD-L1 on the surface of tumor-infiltrating immune cells in lymph nodes prevents the initiation and activation of new cytotoxic T cells and their recruitment into the tumor (52). Secondly, in the tumor microenvironment, the up-regulation of PD-L1 on the surface of dendritic cells leads to the inactivation of cytotoxic t cells. In both cases, PD-L1 interacts with PD-1, a homologous ligand on the surface of T cells, which can inhibit the function of T cells, alter their phenotypes, induce T cell tolerance, inhibit T cell proliferation, and reduce cytokine production, blocking T cell recognition of tumor cells (53, 54).

Autophagy

Tumors often adapt to resource deprivation through different survival mechanisms, such as autophagy. Autophagy is the process of degradation and recycling of self-nutrients through lysosomal pathway, clearing away damaged organelles and protein aggregates, thereby maintaining cell steady-state catabolism. Autophagy is beneficial to normal cells, but in tumors, it helps malignant cells adjust and adapt to adverse conditions, allowing them to develop and continue to grow. In addition, the PD-1/PD-L1 signaling pathway plays a key role in tumor function and survival (55, 56). Autophagy is influenced by the PD-L1 ligand. The results

of mouse melanoma cells and human ovarian cells showed that cells with high expression of PD-L1 receptor were more sensitive to autophagic inhibitors than cells with low expression of PD-L1 (57). Preclinical studies in mice with human melanoma xenografts have shown that the combination of Anthracycline and autophagy inhibitors enhances the anti-tumor T cell response. The same study also showed that autophagy inhibitors have no significant side effects on the immune system. These results provide new opportunities for cancer therapy, such as drugs targeting the PD-1/PD-L1 axis in combination with autophagy inhibitors. Although autophagy inhibitors in combination with anti-PD-L1 agents offer a new direction for cancer therapy, autophagy inhibitors are only one part of a complex network of pathways that affect the immune system's role in tumor cell death, further research is needed to identify the various scenarios in which autophagy inhibitors are effective against cancer (58).

Tumor-Associated Macrophages

A study has shown that both rats and humans express PD-1 in their Tumor-associated macrophage. As the tumors proliferated, the expression of PD-1 increased significantly in both mice and human tumor-associated macrophage. The higher the expression of PD-1 in tumor-associated macrophage, the lower the phagocytosis of tumor cells. Blocking PD-1/PD-L1 *in Vivo* can increase the phagocytosis of macrophages, slow down the growth of tumor and prolong the survival time of tumor model in mice. The greater the number of megakaryocytes given, the longer the survival time. Thus, PD-1/PD-L1 therapy can also play a role through macrophages, which has substantial significance for the creation of new tumor immunotherapy methods (59, 60).

CD47

CD47 is a widely expressed transmembrane glycoprotein, also known as integrin-associated Protein (IAP), an immunoglobulin superfamily protein that is expressed on the surface of almost every cell in the body. CD47 protects healthy cells from the immune system by binding to the n terminal of the immune cell signal-regulating protein α (SIRPα), signaling "don't eat me" and inhibiting the phagocytosis of macrophages. In the meantime, CD47, as a "don't eat me" signal, protects tumor cells from phagocytosis by macrophages, which has become a new mechanism of tumor development and development, and has also explored a new effective way for tumor immunotherapy (61, 62). CD47 antibodies target a variety of indications, including hematological malignancies such as non-Hodgkin Lymphoma and acute myeloid leukemia (AML), as well as solid tumors such as colorectal cancer, ovarian, and bladder cancers (63).

SCREENING AND EVALUATION OF THE BENEFIT GROUP BEFORE IMMUNOTHERAPY

Due to the remarkable efficacy of immunotherapy in tumor therapy, immunotherapy is regarded as one of the most promising therapeutic strategies in the field of tumor therapy in recent years. Immuno-checkpoint inhibitors have brought great changes to the treatment model of advanced tumors, but the clinical treatment results show great individual differences (64). Only a small number of patients show a lasting response to the drug, most patients do not show a lasting response. It has become an especially urgent problem for doctors and patients how to predict the curative effect and screen the effective immunotherapy population before treatment.

Tumor Microenvironment Scoring System

Zeng et al. found that the tumor microenvironment scoring system is not only related to the prognosis of tumor patients, but also has important value for the screening of immunotherapy beneficiaries. Through PCA algorithm, they scored the tumor microenvironment of gastric cancer patients, and found that the higher the tumor microenvironment score, the stronger the anti-tumor immune response-ability, the greater the potential of benefiting from immunotherapy, and the longer the survival time of gastric cancer patients (65).

TIDE Algorithm

Studies have shown that T-cell function-related tumor immune dysfunction and rejection (TIDE) algorithm can predict the efficacy of immunotherapy. TIDE score was high, the efficacy of immune checkpoint blocking therapy (ICB) was poor, and the survival period after ICB treatment was short. TIDE is better than other markers such as PD-L1, mutation load and IFN-γ in predicting the efficacy of first-line treatment of melanoma with ICB (66).

PD-1/PD-L1

Initial studies focused on the expression of PD-1/PD-L1 in tumor cells and surrounding immune cells. A team found that PD-1/PD-L1 antibodies work by mobilizing macrophages infiltrating tumor tissue to gobble up and destroy tumor cells (58, 67, 68). It is believed that the higher the expression of PD-1/PD-L1, the better the effect of immunotherapy. It does present this trend in clinical practice. However, some patients with negative expression of PD-1/PD-L1 also benefit from the treatment of PD-1/PD-L1 monoclonal antibody (59, 69), so it is not recognized as the only precise biomarker to guide immunotherapy.

Tumor Mutation Load

Through total exon sequencing, Yang et al. found that tumor patients with good therapeutic benefits generally have high somatic mutation load. At the same time, patients with high tumor mutation load are more likely to benefit from anti-CTLA-4 treatment (70). The researchers found a similar pattern in non-small cell lung cancer. In general, the higher the mutation load of the tumor, the stronger the immune response provoked, and the better the effect of immunotherapy. In addition, microsatellite instability (MSI) (21) and DNA mismatch repair functional defect (dMMR) are also potential biomarkers. In the analysis of more than 60,000 tumor samples, Volkov et al. found that MSI-H patients generally had high tumor mutation load (71). On the other hand, it also reflects that the tumor mutation load is correlated with the benefit of immune checkpoint inhibitors (72,

73). Tumor patients with MSI-h characteristics, or tumor patients with mismatched repair gene defect (dMMR), tend to benefit from immune checkpoint inhibitor therapy (74). FDA announces MSI as a molecular diagnostic marker for tumor immune checkpoint inhibitor therapy. This is great progress in using standard biomarkers to guide immunotherapy.

Classical Monocytes With CD14+CD16-HLA-DRhi Phenotype

Researchers selected 20 melanoma patients as study subjects (75) and found that the proportion of classical monocytes with CD14 +CD16-HLA-DRhi phenotype in the peripheral blood of patients can be used as biomarkers for predicting PD-1 drug reactivity. According to the experimental model, the researchers determined 19.38% as the optimal threshold, that is, when the subgroup ratio of CD14+CD16-HLA-DRhi is greater than 19.38%, the treatment regimen with antibody PD-1 is recommended for patients. The results show a bright application prospect of this research.

Invasive T Cells and T Cell Receptor

Tumors with more invasive T cells (CD8+, killer T cells) surrounding cancer cells are known as "hot" tumors. Numerous studies have found that immune checkpoint inhibitors have a very good effect on "hot" tumors (76, 77). Moreover, it is theoretically believed that the more extensive the expression spectrum of T cells after treatment, the more it can reflect that the drug activates the immune function of the body. In fact, some studies have found that immune checkpoint inhibitors have limited effects on "hot" tumors. Scheper et al. argues that rather than categorizing a tumor as "hot" or "cold" simply by the number of T cells it has infiltrated, it should be determined whether the TCR of these t-cells can recognize a tumor (78). They set up a platform to analyze whether infiltrating T cells in tumors can recognize surrounding cancer cells. TCR can be used to accurately analyze whether killer T cells in tumors have the potential to fight cancer. Schumacher et al. (79) suggest that increasing the coverage of TCR on the surface of T cells infiltrating tumors may enhance the therapeutic effect of immune checkpoint inhibitors.

CD39

Simoni et al. found that CD39 may be a predictive biomarker for patients to respond to CD8 + TIL targeted cancer immunotherapy. Whether CD8+ TIL cells express CD39 or can predict the response degree of patients to PD-1 antibody therapy, it is of great help to screen more potential anticancer T cells in TIL and CAR-T cell therapy, and further expand the immunotherapy (80–82).

CONCLUSIONS, PROBLEMS AND PROSPECTIVE

Immunotherapy of tumor is a very popular anti-tumor treatment method in recent years, and is expected to become the main means of anti-tumor treatment in the future. Based on large number of research results, it is not difficult to find that tumor immunotherapy has shown amazing clinical efficacy in many types of cancer, such as melanoma, lung cancer, and cancer with high mutation load of microsatellite instability. Immunotherapeutic drugs have shown excellent results, whether used alone or in combination with other therapies. The rapid progress of PD-1 inhibitors and PD-L1 inhibitors is regarded as a star product to open a new era of cancer immunotherapy. With the discovery of new targets for immunotherapy and the development of new drugs for immunotherapy, people's confidence in overcoming cancer has become more and more firm.

Fortunately, more and more studies have found breakthroughs to improve the efficacy of immunotherapy and reduce side effects, such as thioredoxin, FATP2, bacterial transplantation, etc. Moreover, effective indicators such as tumor mutation load index and tumor microenvironment scoring system can be used to screen the population that can benefit from immunotherapy and evaluate the efficacy. Believe it's only a matter of time before cancer is conquered (83). Through the unremitting efforts of human beings, we can certainly rekindle the hope of life for cancer patients earlier.

AUTHOR CONTRIBUTIONS

XZ conceived and designed the study. ZW and SL wrote this manuscript. ZW, SL, and XZ discussed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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PD-1-siRNA Delivered by Attenuated *Salmonella* Enhances the Antitumor Effect of Chloroquine in Colon Cancer

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The widespread appearance of drug tolerance and the low efficiency of single treatment have severely affected the survival time of the patients with colorectal cancer. Exploring new treatment options and combined treatment strategies have become the key to improving the prognosis. The combination of immunotherapy and chemotherapy have shown good clinical expectations. Here, we studied the cooperative effects of chloroquine, an anti-malarial drug that is now widely used in anti-tumor research, and RNA interference (RNAi) targeting the immune checkpoint molecule Programmed Death-1 (PD-1) delivered with attenuated *Salmonella*. Our results show that chloroquine can not only significantly inhibit the survival of colon cancer cells and induce apoptosis, but also effectively inhibit cell invasion and migration. The results of *in vivo* experiments show that chloroquine can increase the expression of PD-1 in tumor tissues. Combining chloroquine and PD-1 siRNA can further inhibit the growth and metastases of colon cancer and induce apoptosis. The mechanism underlying this phenomenon is the occurrence of chloroquine-induced apoptosis and the effective immune response caused by the attenuated *Salmonella* carrying PD-1 siRNA. This study suggests that the combined application of PD-1-based immunotherapy and anti-cancer drugs has become a new expectation for clinical treatment of colorectal cancer.

Keywords: colon cancer, *Salmonella*, chloroquine, PD-1, siRNA

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignant tumor in men and women worldwide, and it is also one of the leading causes of cancer-related death (1, 2). Although traditional treatment, such as surgery, radiotherapy and chemotherapy, can effectively prolong the survival time of patients with colorectal cancer on early stage, it shows poor efficacy for patients with

distant metastasis and old age (3). More unfortunately, patients with colorectal cancer have developed significant chemoresistance, both primary and secondary drug resistant (4, 5). To explore a more effective and targeted treatment strategy is the key to improving the survival rate of colorectal cancer patients. Studies have shown that tumor immune escape is the key to the rapid development and metastasis of cancer. Based on this, in recent years, cancer immunotherapy has become a hot spot in cancer treatment research, and it is also a more effective and widely applicable treatment method. Among many immunotherapy methods, immune checkpoint therapy is the most broadly studied. Its mechanism is to improve the anti-tumor immune response by inhibiting the immunosuppressive molecules on the surface of thymus dependent lymphocytes (T cells). Among them, cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or programmed cell death 1 (PD-1) are the most widely studied and applied (6–10).

PD-1, also named CD279, is an immunosuppressive receptor, which regulates the activation of T cells by binding to its ligand Programmed death receptor ligand (PD-L1) (11). Studies have shown that PD-1 is mainly expressed on the surface of immune cells, including $CD4^+$, $CD8^+$ T and natural killer (NK) cells, while tumor cells is mainly expressed PD-L1 (12). The combination of PD-L1 molecules on the surface of tumor cells and PD-1 molecules on the surface of immune cells inhibits the anti-tumor immune response of immune cells and mediates the occurrence of immune escape of tumor cells (13). Therefore, PD-1 and PD-L1 have become the key target molecules of immunotherapy. Based on this theory, a variety of monoclonal antibodies targeting PD-1 have been developed worldwide, including opdivo (nivolumab) and keytruda (pembrolizumab), which have been used in clinical practice (14, 15). In addition to monoclonal antibodies, small molecule inhibitors and siRNA based on PD-1 have also entered preclinical research (11, 16). We have also reported attenuated *Salmonella* carrying PD-1 siRNA in the treatment of melanoma (17). At present, PD-1/PD-L1 inhibitors are combined with chemotherapy, radiotherapy, targeted drug therapy and other immunotherapies, which increases the number of $CD8^+$ T cells in the tumor microenvironment of patients, destroys the immune escape of tumors, and enhances the anti-tumor effect of PD-1/PD-L1 inhibitors (18–20).

Chloroquine (CQ) is a wide range of anti-malarial drugs. In addition to malaria, chloroquine is currently the most common drug used to relieve acute and chronic inflammatory diseases, such as rheumatoid arthritis (21), systemic lupus erythematosus (22, 23), and Knot disease (24). Studies have found that chloroquine also can inhibit cell cycle, autophagy and induce apoptosis in lung cancer, liver cancer, and gallbladder cancer, which has good anti-tumor potential (25–28). In addition, it is reported that chloroquine can be used in combination with other chemotherapy to enhance its anti-tumor effect (29). However, the relationship between chloroquine and immunotherapy for tumors is hitherto unclear.

In this study, we first determined the effect of chloroquine on the survival, proliferation and apoptosis of colorectal cancer cells in *in vitro* experiments. To clarify the regulation of immune checkpoint by chloroquine, we established a subcutaneous transplanted-tumor model of colorectal cancer in mice. The results showed that chloroquine could increase the expression of PD-1 in tumor tissues while inducing tumor growth inhibition. Chloroquine combined with PD-1 siRNA delivered with attenuated *Salmonella* could significantly enhance the tumor growth inhibition through upregulation of the number and activity of immune cells in tumor tissues.

MATERIALS AND METHODS

Cell Lines, Mice and Bacteria

Mouse colon cancer cell line, CT26, was obtained from American Type Culture Collection (ATCC, Rockville, MD). Human colon cancer cell lines, RKO and HCT-116 were obtained from Procell (Wuhan, China). BALB/c mice (6-8 weeks, female rat) were purchased from Charles River Laboratories (Beijing, China). RKO, HCT-116 and CT26 cell lines were cultured in high glucose DMEM with 10% FBS, 1% Penicillin streptomycin (all purchased from Gibco, USA). The experimental mice were fed with food and water regularly, Animal experiments were approved by the ethics committee of Xinxiang Medical University (Xinxiang, China). Recombinant attenuated *Salmonella* (containing siRNA-PD1 or siRNA-Scramble plasmids with phP/phQ deletion) has been constructed and stored in our laboratory. The sequence of siPD-1 is as follows: GATCCGGGTTTGAGCCAACCCGTCCAGTTC AAGAG ACTGGACGGGTTGGCTCAAACCTTTTTTGGAAA.

Cell Proliferation and Viability Assay

The RKO, HCT-116 and CT26 cells in the logarithmic growth phase were plated in a 96-well plate (1.2×10^4 cells/well for proliferation assay, 0.4×10^4 cells/well for viability assay), after it adheres to the wall, give Chloroquine (Sigma, USA) of different concentrations (proliferation assay 0-60μM, viability assay 0-480μM). After the effect is over, add 10μl CCK8 reagent (MCE, USA) to incubate for 2 hours, and measure the Optical density (OD) value at 450nm.

Wound Healing Assay

RKO, HCT-116 and CT26 cells are spread in a 6-well plate at 3×10^5 cells/well. After they have adhered to a single layer of cells, use the pipette tip to draw a lane in the 6-well plate. Chloroquine was added to a 6-well plate at different concentrations (0-60μM), and photographed with an inverted phase contrast microscope (Nikon, Japan) at 24 hours and 48 hours for recording and analysis.

Colony Formation Assay

Count the cells, spread 500 cells per well in a 6-well plate, set different concentrations of chloroquine (0-60μM) for treatment, wait for about a week, observe the formation of dot-shaped clones at the bottom of the 6-well plate, fix the cells, stain with crystal violet for 30 minutes and take photos for analysis.

Transwell Assay

Spread the cells in 5×10^4 cells in a transwell, and add chloroquine of different concentrations (0-60μM). Place the transwell in a 37°C constant temperature cell incubator for 24-36 hours, take the transwell out of the incubator, fix cells in the transwell with 4% tissue fixative (Solarbio, Beijing, China) for 30 minutes, stain cells in the transwell with crystal violet staining solution for 30 minutes, wipe the upper chamber cells with a cotton swab, observe cells under a microscope, and take pictures.

Western Blotting

Cells are treated with chloroquine for 12-24 hours, the cell pellet is collected by centrifugation, add lysis buffer (Beyotime Biotechnology, Shanghai, China), and protein is collected. After the animal experiment treatment is over, the subcutaneous tumor is taken out, the tissue is frozen and ground with liquid nitrogen, the lysate is added, and the tissue protein is extracted. Use the BCA protein quantification kit (Beyotime Biotechnology, Shanghai, China) to determine the protein concentration and determine the sample volume. After the electroporation is over, 5% skim milk is blocked at room temperature for 1-2 hours, and the primary antibody is incubated at 4°C overnight. The primary antibodies are as follows: PD-1(1:1000, Abcam, MA), MMP2,Cleaved-caspase-3,PD-L1,CD4,CD8 (1:1000,CST, USA), Bax, Bcl-2, Cytochrome-C(1:1000, Abways, China), and Tubulin (1:3000,Sigma,USA), After incubating for 1 hour with the secondary antibody corresponding to the primary antibody, wash the PVDF membrane (Millpore, USA) and use ECL chemiluminescent solution (Millpore, USA) for chemical imaging. Use Image J software for gray value analysis.

Flow Cytometry

Flow cytometry to detect cell apoptosis: Spread 2.5×10^6 cells/well in a 6-well plate, and after they adhere to the wall, treat them with chloroquine for 12-24 hours. Collect the cell pellets when the cell status changes under the microscope, and use the AnnexinV, FITC Apoptosis Detection Kit (Dojindo, Japan) for detection. Flow cytometry to detect immune cells: After the treatment, the mouse spleen cells were separated and red blood cell lysate (Beyotime Biotechnology, Shanghai, China) was added. Resuspend the cells in PBS and count them. Take about 2×10^6 cells from each experimental group, resuspend them in 200 μl of PBS, and add the corresponding fluorochrome-labeled antibodies (CD3/FITC, CD4/PE and CD8/APC) (all purchased from BioLegend, USA). Incubate for 30 minutes at 4°C in the dark. Flow cytometry analysis of fluorescence intensity.

Animal Modeling and Treatment

CT26 cells were injected subcutaneously into BALB/c mice at 1×10^6 cells to establish a mouse colorectal cancer model. Started the first treatment, 7 days after the model was built. The mice were randomly divided into 5 groups, namely PBS group, Scramble (Scr) group, CQ group, siPD-1 group, CQ+siPD-1 group. Starting from day 7, the PBS group was intraperitoneally injected with 100μl of PBS per day, and the CQ group and CQ+siPD-1 group were intraperitoneally injected with 100μL of CQ (50mg/kg) per day. Scr group, siPD-1 group and CQ+siPD-1 group were given intratumoral injection of *Salmonella* carrying empty and siPD-1 (4×10^5 CFU in 100 μL of PBS/mouse) on the 8th and 14th day. Record tumor volume and mouse body weight and end of treatment on day 21.

Immunohistochemistry (IHC) and HE Staining (HE)

After the treatment, remove the mouse subcutaneous tumor, fix it with 4% tissue fixative for 24 hours, embed the tissue and slice it (thickness 4μM). HE staining with hematoxylin and eosin (Beyotime Biotechnology, Shanghai, China). The sections were processed with an immunohistochemistry kit (Zhongshan Jinqiao, Beijing, China). The slides were incubated with antibodies against CD4, CD8, and PD1 at 4°C overnight. The next day, it was incubated with the secondary antibody for 2 hours, then stained with DAB and counterstained with hematoxylin. Two pathologists judge and count the results.

Terminal-Deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL)

According to the instructions of the TUNEL Apoptosis Detection Kit (Green Fluorescence, Abbkine, Wuhan, China), the tissue sections were fluorescently stained deoxyribonucleotide terminal transferase, based on the notch end labeling method. The tissue sections were deparaffinized and dehydrated, incubated with proteinase K for 15 minutes, and incubated with Tunel staining reagent at 37 °C for 1 hour in the dark, and photographed with a confocal microscope (Nikon, Japan).

Statistics

T-test was used to compare the values between the two groups, and ANOVA was used for three groups and more than three groups. Comparisons were considered to be statistically significant difference when $P < 0.05$.

RESULTS

Chloroquine Inhibits the Survival and Proliferation of Colon Cancer Cells and Induces Apoptosis

In order to clarify the effect of chloroquine on the biological behavior of colon cancer cells, we first examined the survival and proliferation of colon cancer cells treated with chloroquine. The results showed that compared with the control group, different concentrations of chloroquine could significantly reduce the survival rate of three kinds of colon cancer cells (**Figure 1A**). Furthermore, CCK8 proliferation test and plate cloning test showed that chloroquine could significantly inhibit the proliferation of colon cancer cells (**Figures 1B–D**). Furthermore, the effect of chloroquine on apoptosis of colon cancer cells was determined by flow cytometry. The results showed that chloroquine could increase the apoptosis rate of three kinds of colon cancer cells (**Figure 2A**). A variety of pathways can induce

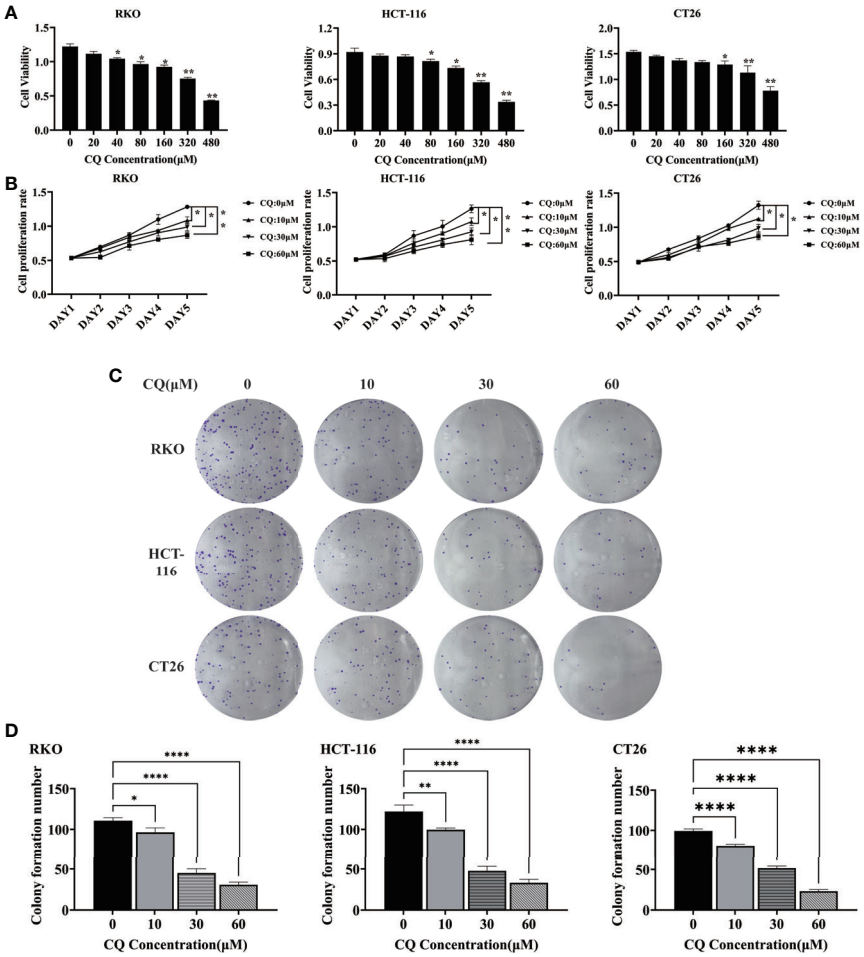

FIGURE 1 | Chloroquine inhibits the survival and proliferation of colorectal cancer cells. **(A)** The cell viability of RKO, HCT-116 and CT26 was detected by CCK8 assay. **(B)** The cell proliferation of RKO, HCT-116 and CT26 was detected by CCK8 assay. **(C, D)** The cell proliferation of RKO, HCT-116 and CT26 was detected by colony-forming assay. (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ *vs* 0μM group).

apoptosis, and mitochondrial apoptosis pathway is the most important one. Our test results showed that chloroquine could significantly affect mitochondrial apoptosis-related molecules, Bax, Bcl-2 and Cytochrome C, and meanwhile induce the expression of apoptosis effector molecule, cleaved caspase-3 (**Figures 2B–D**).

Chloroquine Inhibits the Migration of Colorectal Cancer Cells

We further used the wound healing assay and the transwell assay to detect the effect of chloroquine on the migration ability of colon cancer cells RKO, HCT-116 and CT26. The results showed that as the concentration increased, the migration ability of colon cancer cells decreased (**Figure 3**). Meanwhile, western blotting experiment was used to detect the change of the migration phase protein (MMP2), and the results showed that high concentration of chloroquine significantly reduced the expression of MMP2 (**Figure 3A**). The results above indicate that chloroquine can inhibit the migration ability of colorectal cancer cells. However, we found that chloroquine did not affect the expression of PD-L1 in colon cancer cells (**Figure 3A**).

Chloroquine Up-Regulates the Expression of PD-1 in Tumor Tissues, and the Combination of Chloroquine and PD-1 siRNA Can Further Inhibit Tumor Growth

Based on the fact that chloroquine did not affect the expression of PD-L1 in colon cancer cells, we determined whether chloroquine could affect the expression of PD-1 in tumor tissues. IHC results showed that chloroquine could significantly increase the expression of PD-1 in tumor tissues *in vivo*, mainly on the surface of lymphocytes. Western blotting results also showed that chloroquine up-regulated the expression ofPD-1 in tumor tissues (**Figures 4A, B**). Meanwhile, the results showed that the PD-1 siRNA delivered with attenuated *Salmonella* could significantly reduce the expression of PD-1 in tumor tissues. Tumor tissue weight and size test results showed that chloroquine could inhibit the growth of tumor to a certain

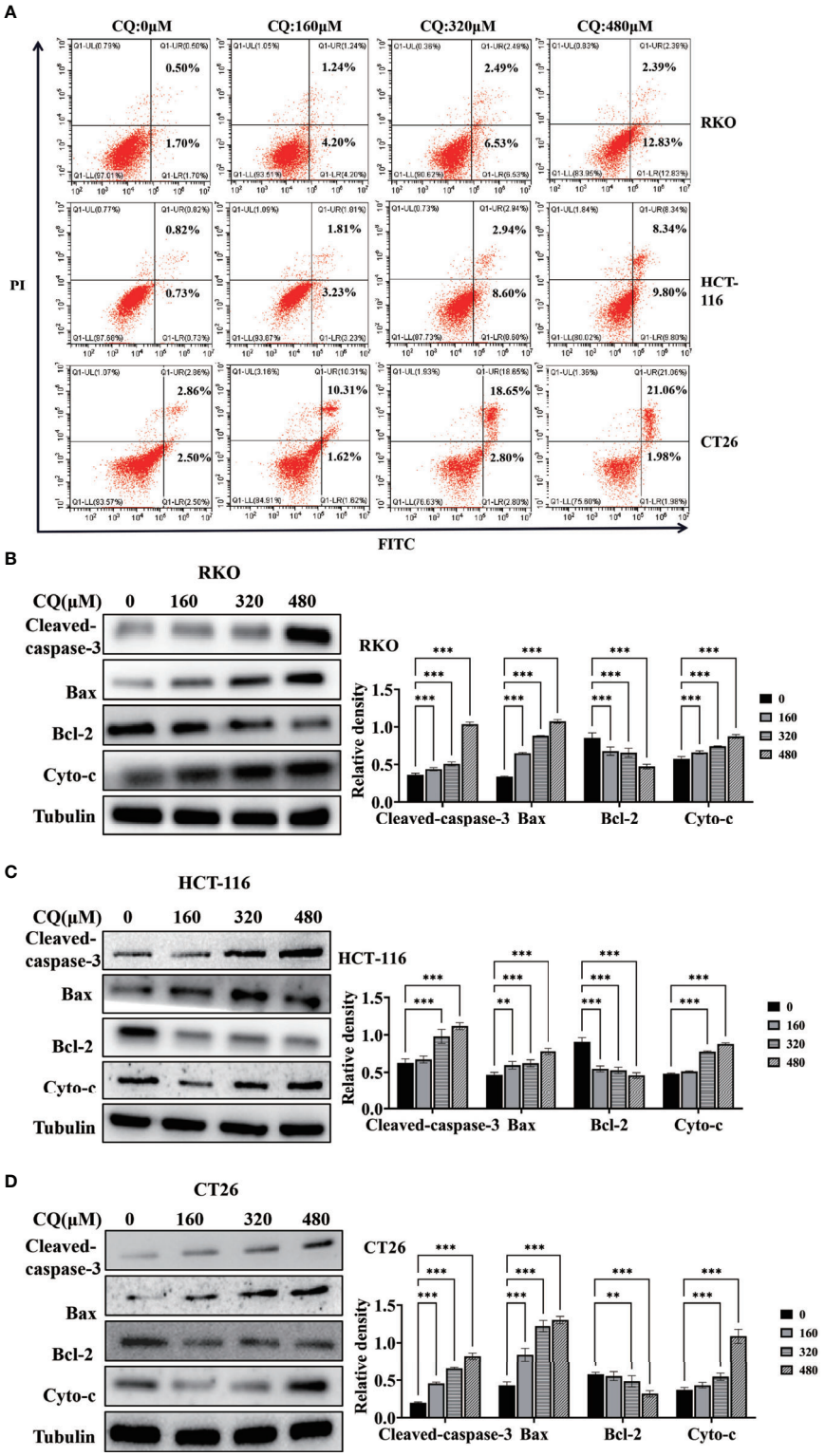

FIGURE 2 | Chloroquine induces apoptosis of colorectal cancer cells and affects the expression of related proteins. **(A)** Flow cytometry detected the apoptotic rate of RKO, HCT-116 and CT26 induced by CQ. **(B–D)** Western blotting analysis for the protein expression of Cleaved caspase 3, Bax, Bcl-2 and Cytochrome c (Cyto-c) after treatment with a chain of concentrations of CQ. (**$p < 0.01$, ***$p < 0.001$ *vs* 0μM group).

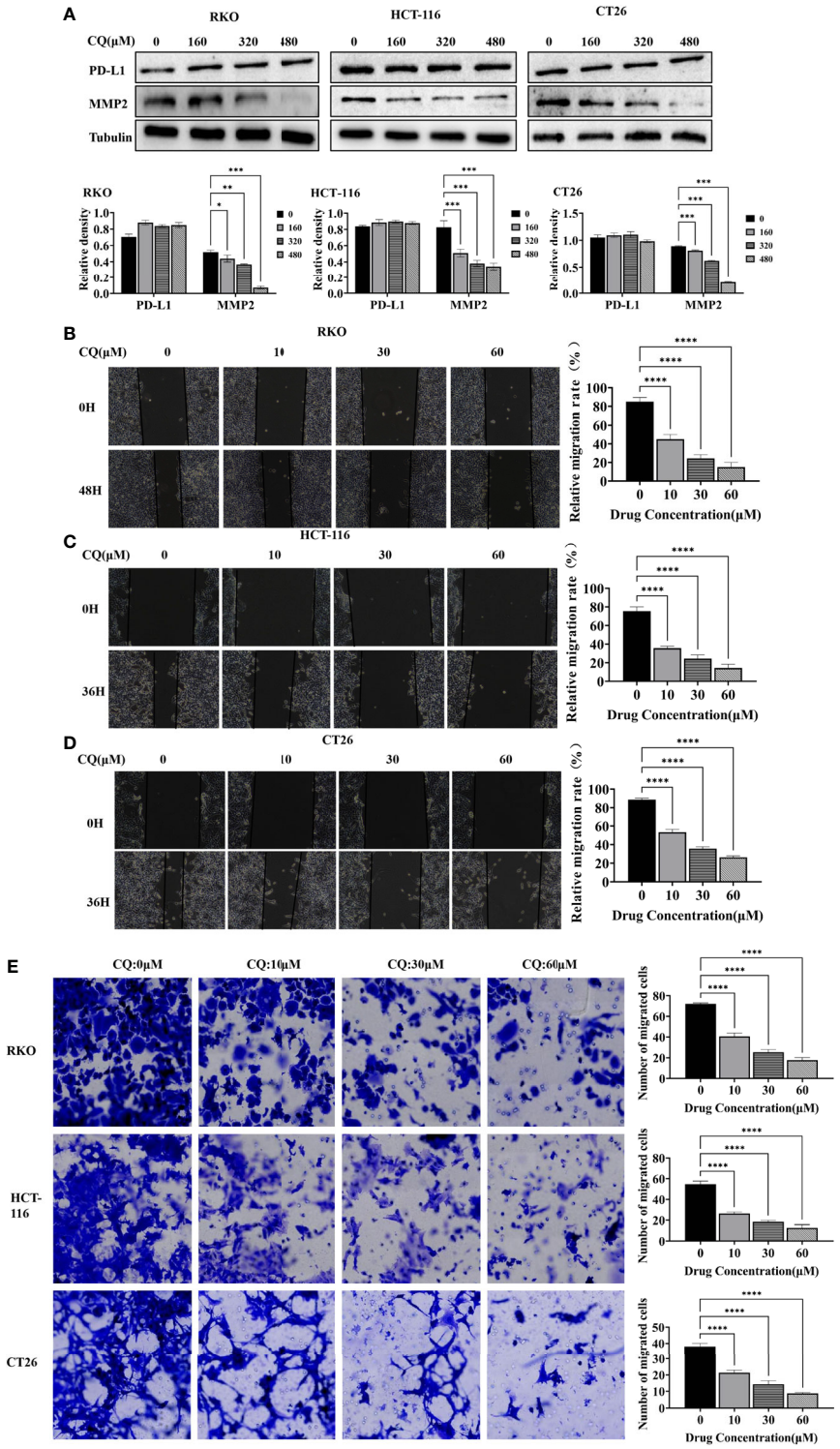

FIGURE 3 | Chloroquine inhibits migration of colorectal cancer cells. **(A)** Western blotting analysis for the protein expression of PD-L1, MMP2 after treatment with a chain of concentrations of CQ. **(B–D)** Wound healing assay detects the effect of different concentrations of CQ on the migration ability of colorectal cancer cells RKO, HCT-116 and CT26. **(E)** Transwell assay detects the effects of different concentrations of CQ on the migration ability of colorectal cancer cells RKO, HCT-116 and CT26. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *vs* 0μM group).

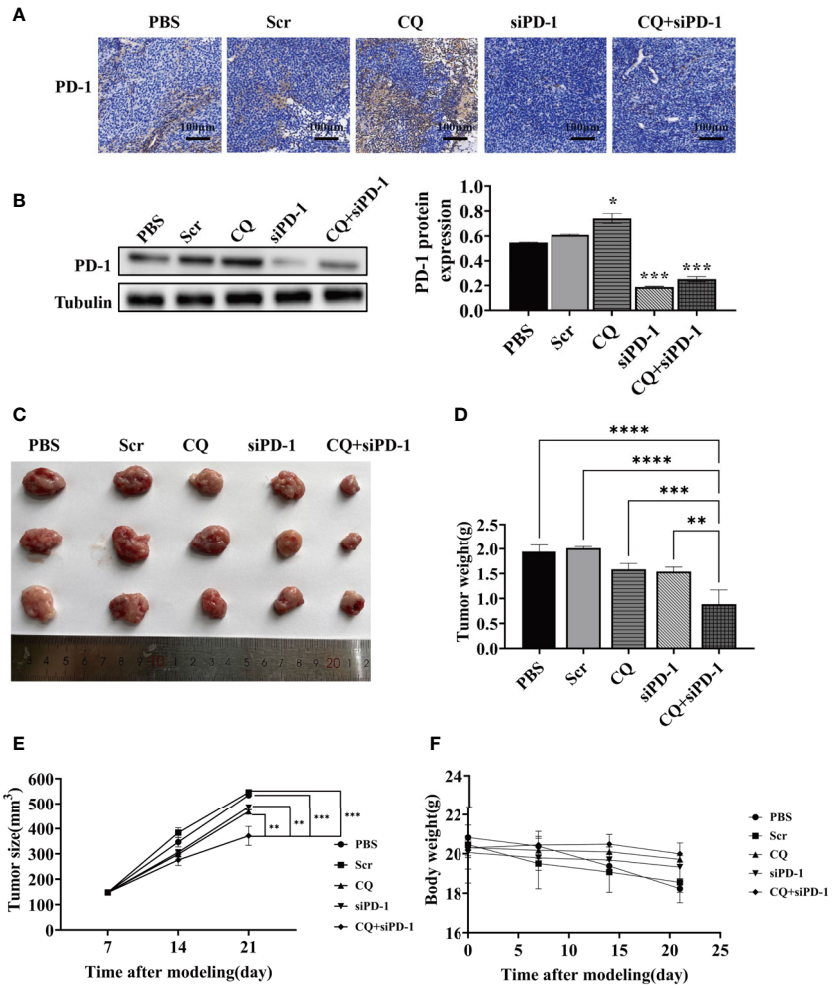

FIGURE 4 | Effect of various treatments on tumor growth in *vivo*. BALB/c mice were subcutaneously inoculated with 1×10^6 CT-26 cells and treated with PBS, attenuated *Salmonella* carrying the scrambled siRNA plasmid, CQ, attenuated *Salmonella* carrying PD-1 siRNA, or CQ plus attenuated Salmonella carrying PD-1 siRNA. **(A)** IHC detects the expression of PD-1 in tumor tissues. **(B)** The protein levels of PD-1 in tumor tissue after the indicated treatments were evaluated by Western blotting analysis. **(C)** Images of the representative tumors in each group on posttreatment day 14. **(D)** Tumor weight analysis. **(E)** Tumor size analysis. **(F)** Body weight analysis. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *vs* CQ+siPD-1 group).

extent, but chloroquine combined with PD-1 siRNA had the most obvious inhibition (**Figures 4C–E**). The weight of the mice was measured, and the results showed western blotting that there was no significant change, indicating that the combination medication did not have significant side effects (**Figure 4F**).The results suggested that chloroquine and PD-1 blockade synergistically elicit anti-tumor effects.

The Combination Treatment of Chloroquine and PD-1 siRNA Can Significantly Induce Apoptosis and Inhibit Migration in Colon Cancer Xenografts

To explore the effect of chloroquine on apoptosis and metastasis of colon cancer cells *in vivo*, the hematoxylin-eosin staining (HE staining), transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL), and western blotting were used to detect apoptosis and protein expression. As shown in HE staining, tissue disorder or necrosis was observed in the CQ, siPD-1 and CQ +siPD-1 groups, with the most severe damage in the combination treatment group (**Figure 5A**). TUNEL results showed that there were more apoptotic cells in tumor tissues in the combination treatment group (**Figures 5B, C**). Western blotting analysis showed that compared with other groups, the combination treatment group could significantly inhibit the expression of MMP2 and induce the expression of apoptotic protein, cleaved caspase-3 (**Figure 5D**). The results above indicate that CQ combined with PD-1 siRNA can significantly promote the apoptosis of colorectal cancer cells and inhibit migration.

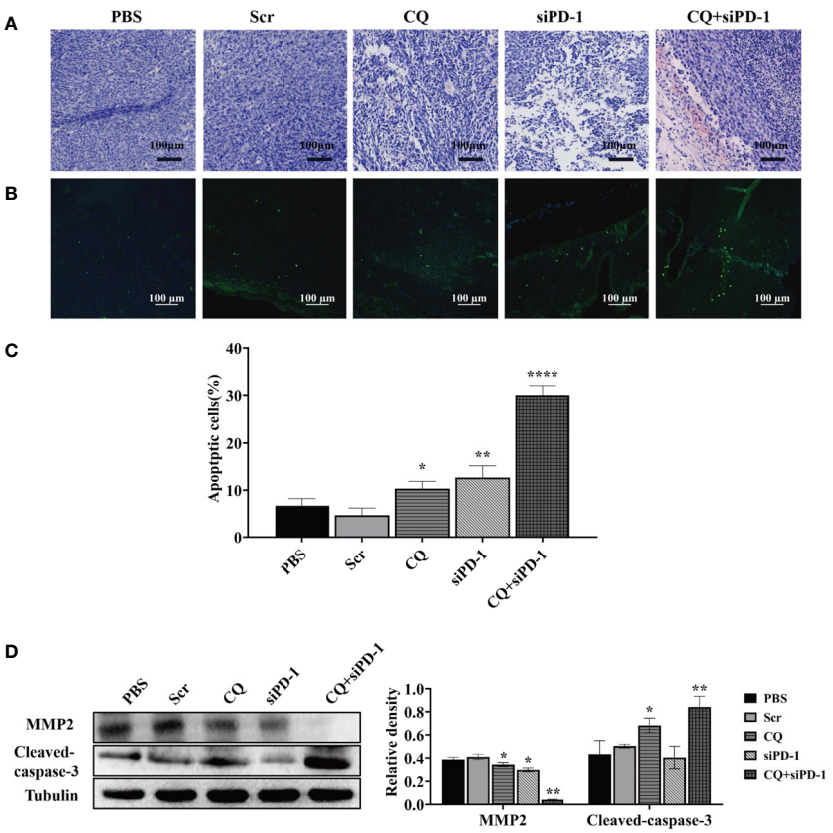

FIGURE 5 | *In vivo* experiments have detected that chloroquine combined with siPD-1 in the treatment of colorectal cancer can significantly inhibit the migration of colorectal cancer and promote cell apoptosis. Tumor tissues were collected on day 14 after the first treatment and were subjected to HE, TUNEL staining, and Western blotting analysis. **(A)** Tissue HE staining. **(B, C)** TUNEL staining and quantitative analysis of tissues. **(D)** The expression of proteins associated with apoptosis and migration.(*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ *vs* PBS or Scr group).

The Combination Treatment of Chloroquine and PD-1 siRNA Significantly Enhance the Infiltration of Effector T Cells Into Tumor Tissues

Furthermore, we used immunohistochemistry (IHC) to detect the expression of CD4, CD8 and PD-1 in colorectal cancer tumor tissues. The results showed that compared with other groups, the expression of CD4 and CD8 in the combination treatment group was higher (**Figures 6A, B**). Western blotting analysis showed that treatment of CQ combined with PD-1 siRNA could increase the expression of CD4 and CD8 (**Figure 6C**). In summary, these results indicate that the combination treatment of CQ and PD-1 siRNA can enhance the infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in colon cancer tissues.

Combination Treatment With Chloroquine and PD-1 siRNA Elicited Systemic and Synergetic Antitumor Immunity

In order to determine whether the anti-tumor effect of chloroquine and PD-1 siRNA combined therapy is due to the activation of systemic tumor-specific cellular immunity, we isolated T lymphocytes from mouse spleen cells of all groups, respectively. The results showed that the combination therapy significantly prolonged the percentage of $CD4^+$ and $CD8^+$ T cells (**Figure 7**). These results indicate that the increase in T cell infiltration may be due to systemic and synergetic anti-tumor immunity exerted by chloroquine and PD-1 siRNA.

DISCUSSION

With the emergence of chemotherapeutic drug resistance and the in-depth research on the mechanism of drug action, many traditional non-chemotherapeutic drugs are used in the treatment of cancer, such as aspirin and metformin, which are called old drug in new use (30–33). Chloroquine, with the molecular formula, C18H26ClN3, is an anti-malarial and anti-inflammatory agent widely used in the treatment of malaria and rheumatoid arthritis (34, 35). In addition, chloroquine is often known as an inhibitor of the end of autophagy, which inhibits the degradation of substances in autophagic vesicles by increasing the potential of hydrogen (pH) of the lysosome, thereby causing cell damage (36). Studies have shown that chloroquine can induce and inhibit tumors, but these studies were mainly focused on proliferation and apoptosis, and the effect of chloroquine on cell migration and invasion is not

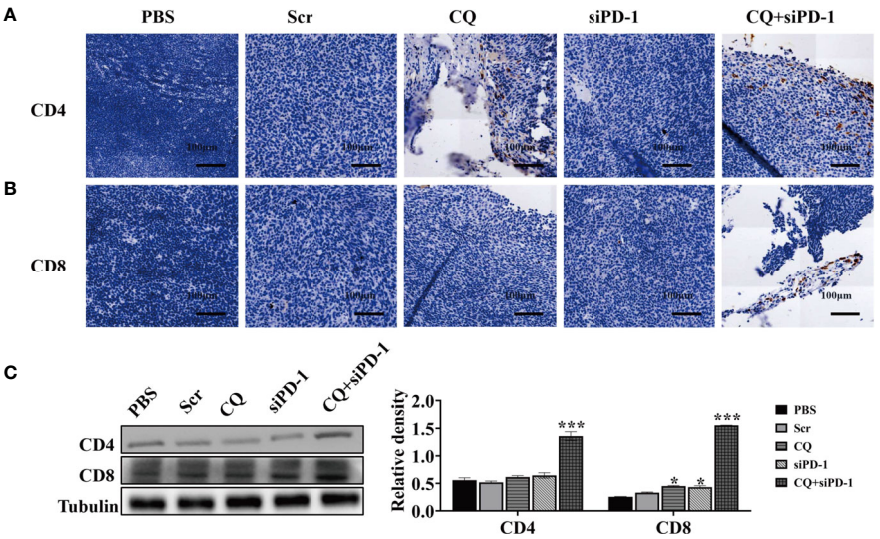

FIGURE 6 | Chloroquine combined with PD-1 siRNA can promote the infiltration of T cells into tumor tissues and inhibit the expression of PD-1. **(A, B)** IHC detects the expression of $CD4^+$ and $CD8^+$ in tumor tissues. **(C)** The protein levels of CD4 and CD8 in tumor tissue after the indicated treatments were evaluated by Western blotting analysis. (*$p < 0.05$, ***$p < 0.001$ *vs* PBS or Scr group).

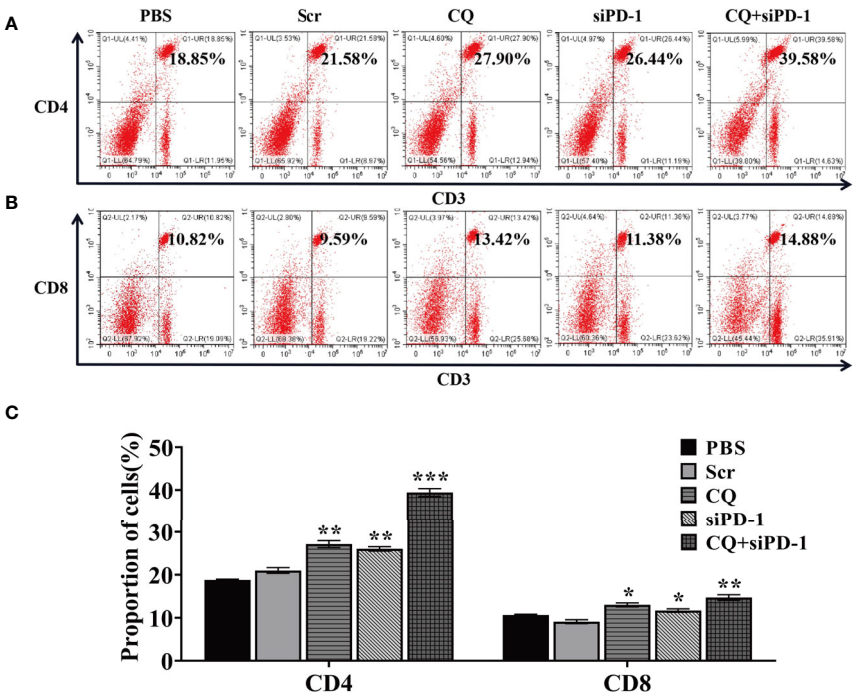

FIGURE 7 | The combination treatment of chloroquine and PD-1 siRNA can regulate the body's anti-tumor immunity. **(A, B)**, Flow cytometry detects the proportion of $CD4^+$ and $CD8^+$ T cells in cells extracted from tumor tissue. **(C)** Data analysis of the ratio of cells positive for each surface marker. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* PBS or Scr group).

very clear (37, 38). Our results show that chloroquine can inhibit the survival, proliferation and induce apoptosis of colon cancer cells, and at the same time can significantly inhibit the invasion and migration of colon cancer cells.

A large number of studies have shown that chemotherapy or radiotherapy alone has little benefit to cancer patients after surgery (39–41). Combination therapies have been widely researched and clinically recognized (42, 43). Among them, the

most extensive research in recent years focused on the combination immunotherapy (44, 45). There are many kinds of immunotherapies, including vaccines, antibodies, cytokines, cell therapies and immunosuppressive agents. Among them, the most widely studied and the most recognized is the treatment based on immune checkpoints (46). At present, the most widely applied is the PD-1/PD-L1 monoclonal antibody, and it shows a significantly better effect than chemotherapy alone (47, 48). This brings new hope to cancer patients. Although there are many studies focused on the anti-tumor effect of chloroquine, there are few studies centered on the relationship between chloroquine and tumor immune response. We first tested the effect of chloroquine on the expression of PD-L1 in tumor cells and found that chloroquine could not significantly affect the expression of PD-L1 in tumor cells. In order to explore this problem, we established a subcutaneous transplanted-tumor model of colon cancer in mice, and further observed its effect on the expression of immune cells PD-1 in tumor tissues. The results showed that the administration of chloroquine could significantly increase the expression of PD-1 in tumor tissues. At the same time, we found that the number of $CD4^+$ and $CD8^+$ T cells in tumor tissues increased significantly. This phenomenon has a dual effect. On one hand, it increases the infiltration of lymphocytes in tumor tissues, which is beneficial to the killing effect of immune cells; on the other hand, the disadvantage is that the increase of PD-1 can inhibit the killing effect of immune cells. Based on this, we used our pre-designed PD-1 siRNA and chloroquine to act on colon cancer tissues. The results found that compared with chloroquine alone, the tumor suppression in the chloroquine combined with PD-1 siRNA group was very obvious, showing a combination effect. On the basis of inhibiting tumor growth, we detected the expression of apoptosis and metastasis-related proteins in tumor tissues. The expression level of apoptosis in the combination treatment group was significantly increased, and at the same time the expression level of the metastasis-related protein,MMP2, was significantly inhibited. To explore the change in the number of immune cells in tumor tissues, we further detected the number of $CD4^+$ and $CD8^+$ T cells in the spleens of mice, and found that the combination treatment group could also significantly increase the number of immune cells in the spleens. These results show that the combination use of chloroquine and PD-1 siRNA can effectively activate the immune response, thereby inhibiting tumors.

Compared with monoclonal antibodies and small molecule inhibitors, the use of siRNA to treat tumors requires an effective delivery system. In addition to viral vectors, recent studies have shown that a variety of bacteria can be used as carriers, among which attenuated *Salmonella* is the most widely studied and applied (49, 50). As a vehicle, attenuated *Salmonella* can carry small hairpin RNA (shRNA) to a variety of tumor sites, including lung cancer, breast cancer, gastric cancer and prostate cancer. The advantages of attenuated *Salmonella* as a carrier are: on one hand, it can target the low-oxygen environment of solid tumors; on the other hand, it can stimulate an effective immune response. In this study, we used phoP/phoQ-deleted *Salmonella Typhimurium* to effectively carry PD-1 siRNA to tumor tissues, and at the same time, activate a robust immune response, exhibiting a good application prospect.

This study clarified that chloroquine could significantly inhibit colon cancer cell proliferation and induce apoptosis while inhibit its migration and invasion capabilities. Combining chloroquine and PD-1 siRNA delivered with attenuated *Salmonella* could effectively inhibit the growth of tumors and had a good synergistic effect. The mechanism was due to the killing effect of chloroquine on tumor cells, the effective immune response and the increases in the number of immune cells. At the same time, siRNA further suppressed the immunosuppressive molecule, PD-1, on the surface of immune cells (**Figure 8**). The research provided a basis and new direction for the clinical application of chloroquine and immunosuppressive agents combined to treat tumors more validly in the future.

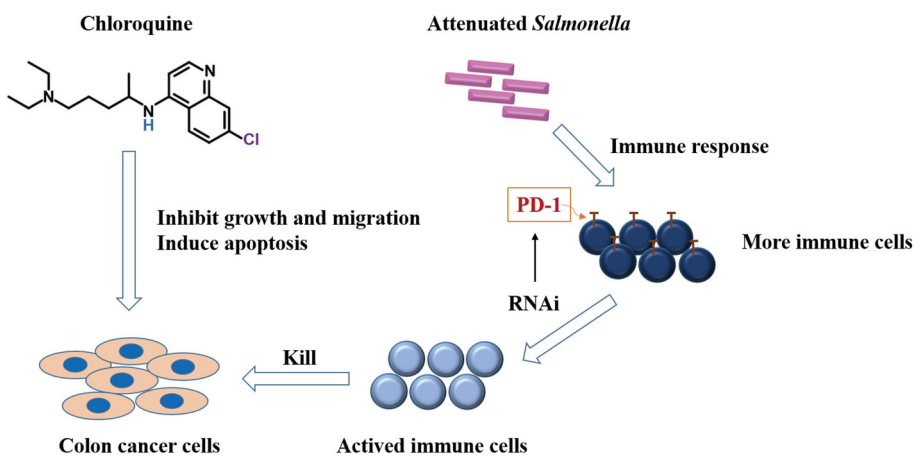

FIGURE 8 | Schematic diagram demonstrating the potential mechanism of chloroquine and PD-1 siRNA in combination.

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 animal study was reviewed and approved by Ethics Committee of Xinxiang Medical University.

AUTHOR CONTRIBUTIONS

JZ, TZ, JG, and MW designed the experiments and wrote the manuscript. SL, JG, HJ, YL, YD, FS, ZL, and SM performed the experiments. SL, JG, and HJ contributed equally to this work. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

REVIEW
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Potential Role of CD47-Directed Bispecific Antibodies in Cancer Immunotherapy

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The prosperity of immunological therapy for cancer has aroused enormous passion for exploiting the novel targets of cancer immunotherapy. After the approval of blinatumomab, a bispecific antibody (bsAb) targeting on CD19 for acute lymphoblastic leukemia, a few of CD47-targeted bsAbs for cancer immunotherapy, are currently in clinical research. In our review of CD47-targeted bsAbs, we described the fundamental of bsAbs. Then, we summarized the information of four undergoing phase I researches, reviewed the main toxicities relevant to CD47-targeted bsAb immunological therapy of on-target cytotoxicity to healthy cells and a remarkable antigen-sink. Finally, we described possible mechanisms of resistance to CD47-targeted bsAb therapy. More clinical researches are supposed to adequately confirm its security and efficacy in clinical practice.

Keywords: cancer immunotherapy, bispecific antibodies (bsAbs), CD47, immune checkpoint, macrophage, phagocytosis

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INTRODUCTION

Cancer is a serious health problem and the second primary cause of death all over the world. Data from the International Agency for Research on Cancer (IARC) displayed that there were about 19.2 million new cancer cases and 9.9 million new cancer deaths in 2020. Over the past few decades, the introduction of cancer immunotherapies, aiming to ameliorate anti-tumor immune responses with less off-target effects, compared with chemotherapies and other agents which straightway kill tumor cells, has significantly improved the survival outcome of patients (1–3). Immunotherapeutic agents are designed to activate or modulate innate or adaptive immune system to assault cancer cells *via* repairing or enhancing natural mechanisms, plenty of which are escaped or damaged in the development of disease, thereby inhibiting tumor growth and metastasis (4–6). Results from the KEYNOTE-024 study on patients with advanced non-small cell lung cancer revealed that the overall survival (OS) rate after five years was twice as high for patients that were treated with Keytruda (31.9%), compared to a chemotherapy regimen (16.3%) (7). Thus, immunotherapy is universally acknowledged to treat, and to heal, several kinds of cancer.

However, monotherapy with these and other drugs based on monoclonal antibodies cannot heal certain types of cancer, especially owing to T lymphocytes not actively participating in destroying tumors, nonetheless monoclonal antibodies merely restrain the combination of growth factors with the corresponding receptors. Monoclonal antibody interdicting the inhibitory signals which defend cancers

from immune cells display fantastic outcomes while treating certain especial kinds of cancers. However, antibodies binding to two or more antigens (bsAbs), together with conjugated agents are research hotspots for chemo and radiotherapies.

THERAPEUTIC BsAbs IN ONCOLOGY

The original concept of bispecific antibody (bsAb) was proposed 50 years ago by Nisonoff and co-workers (8). Subsequently, the utilization of bsAbs to redirect immune cells to kill tumor cells was then certified in the 1980s, which provided a promising immunotherapeutic approach for cancer therapy and diagnosis (9). Blinatumomab (Blincyto®, CD3 × B lymphocyte antigen CD19) was the first bsAb to be ratified by the US Food and Drug Administration (FDA) for the treatment of relapsed or refractory acute lymphoblastic leukemia (AML) in 2014 (10, 11). The remarkable success of blinatumomab has prompted pharmaceutical companies to efficiently generate and produce stable bsAbs. Till date, more than 100 bsAbs with different backbones and over 30 technology platforms have been reported and reviewed (12–16), and approximately, two-thirds of these bsAb therapeutics in the clinical pipeline are designated for the treatment in oncology (17), which either recruit and redirect the immune effector cells to kill cancer cells or interdict diverse signaling pathways *via* restraint of the ligand or the receptor (17–19).

BsAbs are genetically designed recombinant antibodies, which contain two disparate binding domains that allow simultaneous coupling with two diverse antigens or two diverse epitopes of the same antigen (**Figure 1**) (20, 21). Two diverse receptors or ligands on the surface of the same cell might be concurrently targeted by bsAbs, and they will give rise to restraint or stimulation of two diverse signaling pathways. Fusion of anti-tumor binding domain with the fragment crystallizable region (Fc) or the anti-CD3 binding domain is a conventional strategy to generate bsAbs, which has a great potential to recruit immune cells.

The bsAbs can be categorized into two different formats: a non-IgG-like format and an IgG-like format (**Figure 2**), and the structural elements of each format have implications for engagement and mobilization of the immune system. Among the clinically approved bsAbs, Catumaxomab and Hemlibra (emicizumabkxwh) have the IgG-like format, while Blinatumomab has the non-IgG-like format.

The IgG-like bsAbs include the Fc region, which is an important determinant for bsAb in stimulating immune responses. Biochemical properties including affinity, glycosylation and isotype of the Fc region are responsible to trigger different immune effector cells. The Fc region triggers bsAb-mediated functionalities, including antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP) (22).

Unlike the IgG-like bsAbs, the non-IgG-like bsAb format lacks the Fc region and in consequence, the molecular mass (30–50 kDa) is smaller than that of a whole IgG (150 kDa). Because the structure of a non-IgG-like format includes only VH and VL domains or the Fab fragment, such bsAb format depends only on Ag binding capacity for its therapeutic

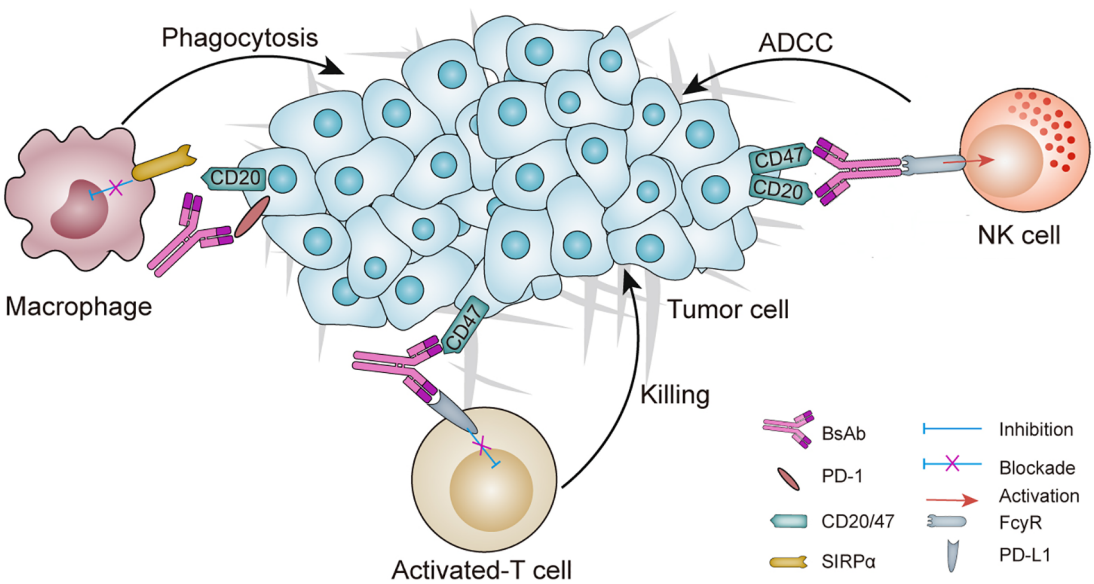

FIGURE 1 | Schematic overview of a BsAb in cancer: mechanisms of action and potential targets. Dual checkpoints blockade is achieved by two-checkpoint blockers integrated into one antibody to inhibit two immune checkpoints simultaneously. Tumor targeted immunomodulators are designed for binding to one tumor-associated antigen (TAA) to inhibit TAA signaling pathway and the other immunomodulating receptor (e.g., PD-L1, CD47), thus regulating the immune system to attack the tumors. ADCC, antibody-dependent cell-mediated cytotoxicity; bsAb, bispecific antibody; NK, natural killer; SIRPα, signal-regulatory protein α.

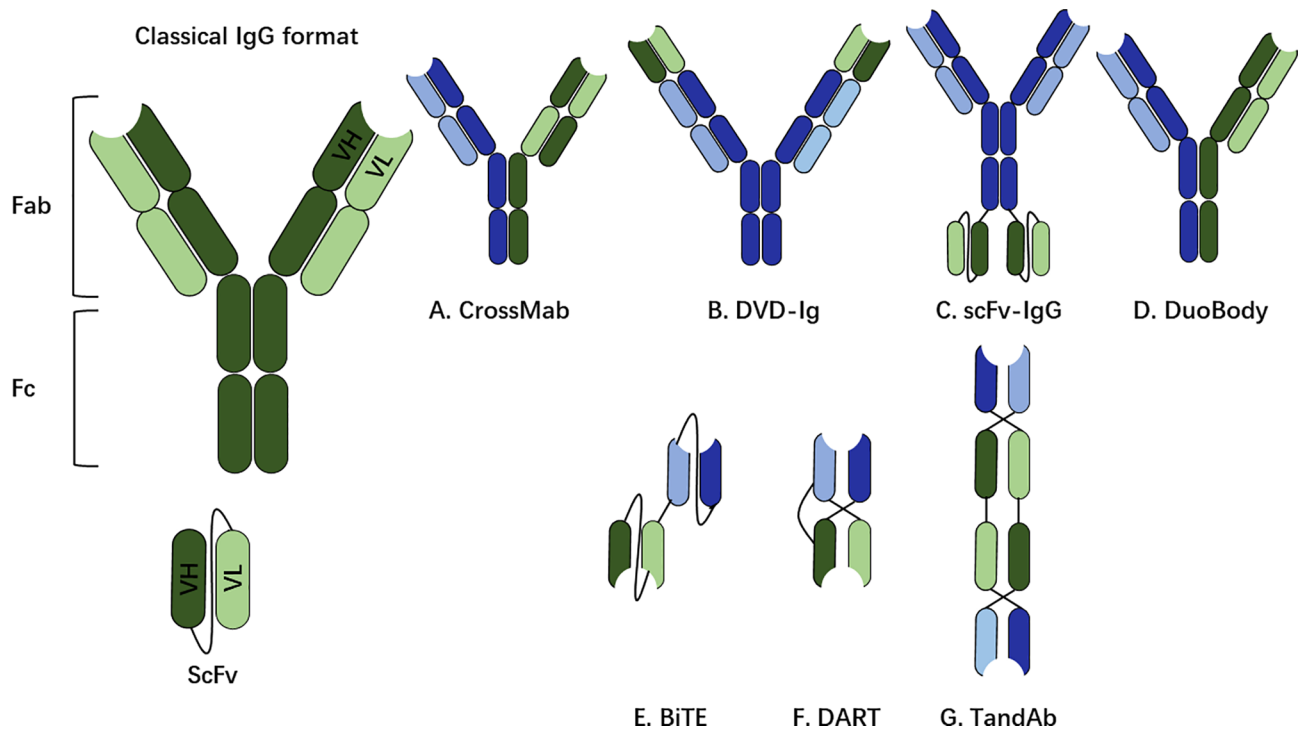

FIGURE 2 | The non-IgG-like format and IgG-like format. The classical structure of a human immunoglobulin G (IgG) for reference, including fragment crystallizable (Fc) region and fragment antigen-binding (Fab). The binding part of the Fab region is called the single chain variable fragment (scFv), which is a synthetic protein composed of the heavy (VH) and light (VL) chains connected by a short peptide linger. **(A–D)** IgG-like bsAb formats (with Fc region). **(E–G)** non-IgG-like bsAb fragments (without Fc region). CrossMab Cross monoclonal antibody, DVD-Ig, dual-variable-domain immunoglobulin; scFv, single-chain variable fragment; BiTE, bispecific T-cell engager; DART, dual-affinity retargeting molecule; TandAb, tandem diabody.

function. A major limitation of the non-IgG like bsAbs is their short-half life.

In contrast to antibody-drug conjugates and naked monoclonal antibodies (mAb), bsAbs can be engineered to recruit diverse effector immune cells through coupling with the membrane-associated antigen expressed from cancer cells, and the second antigen expressed in special immune cells, such as natural killer (NK) cells, effector T cells (23) or immunomodulatory proteins such as cluster of differentiation 47 (CD47) or programmed death receptors 1 (PD-1) (24). The superior effect might be ascribed to the tumor-directing function generated by bsAbs (25). Additionally, bsAbs execute their therapeutic effect by binding to receptors expressed on cell surface, without requiring for receptor internalization, which permits to target a disparate population of tumor antigens. A few of immunotherapies aiming to CD47, such as antibody-drug conjugates, chimeric antigen receptor (CAR) T cells and bsAbs, are now undergoing assessed in clinical trials or preclinical studies. We have recently reported our experience of constructing a novel bsAb fusion protein both targeting EGFR and CD47, which could reduce the "off-target" effects because of CD47 expression on red blood cells (RBCs). Our study demonstrated that bi-SP with modified treatment indices may have a potential to treat CD47+ and EGFR+ cancers in the clinic (26).

CLINICAL EXPERIENCE OF CD47 AS A TARGET FOR CANCER IMMUNOTHERAPY

CD47 [Integrin-associated protein (IAP)] has a molecular weight of 45-55 kDa (**Figure 3**) with five-membrane-spanning segments in the membrane, which coprecipitates with platelet-derived β3 integrin and placental αvβ3 integrin (28). As we know, it is the receptor of thrombospondin family members, and is an extraordinary member of the membrane protein immunoglobulin (Ig) superfamily with a single extracellular IgV-like region (29). CD47 is expressed at the plasma membrane of almost all cell types including mesenchymal stromal cells and blood cells, and is known to mediate vascular smooth cell proliferation and migration (30), platelet activation and spread (31), and recruit granulocytes and T cells to infections positions (32, 33). It was initially introduced as a tumor antigen involved in ovarian cancer, and multiple studies have indicated that CD47 is generally upregulated in various types of malignancies (34), such as myeloma (35), leiomyosarcoma (36), leukemia (37), non-Hodgkin's lymphoma (38), breast cancer (39), osteosarcoma (40), and hepatocellular carcinoma (41). It may be a mechanism of cancer immune evasion, and this mechanism-driving overexpression in tumor cells seems to be a compensatory response to a pro-inflammatory tumor microenvironment with

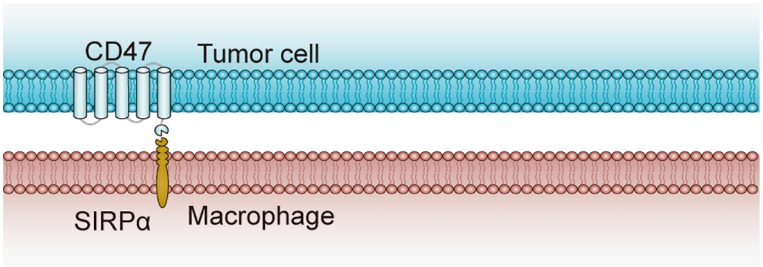

FIGURE 3 | Structure and interaction of CD47 and SIRPα. CD47 contains one N-terminal extracellular IgV-like domain and five-membrane-spanning segments at the membrane. SIRPα, as the CD47 ligand, contains three extracellular IgSF domains, one transmembrane spanning region and an intracellular domain with ITIM motifs. CD47-SIRPα binding prevents the host cell from being targeted for phagocytosis, while anti-CD47 antibodies can block the suppression signal and promote phagocytosis by macrophages (27). CD47, cluster of differentiation 47; ITIM, immunoreceptor tyrosine-based inhibitory motif; SIRPα, signal-regulatory protein α.

high levels of TNF-α (42), which activates NF-κB signaling and thus regulates CD47 expression (43). Therefore, the CD47/SIRPα axis is considered as a novel existing target in immunology (39), and CD47 will have a major role in tumorigenesis, since its enhanced expression in cancer cells enables evasion of phagocytosis (44).

Notably, CD47 sends "don't eat me" signals by inhibiting phagocytosis of tumor cells and triggering an immune evasion and therefore serves as a myeloid-specific immune checkpoint when CD47 binds to signal regulatory protein α (SIRPα) that is an inhibitory receptor on macrophages and dendritic cells. In view of the interaction between CD47 and SIRPα in tumor cells restricts the anti-tumor immune response, treatment with anti-CD47 antibodies that restrain CD47 signaling in tumor cells was able to induce the phagocytosis of tumor cells by macrophages and stimulate anti-tumor immune responses *in vivo* (45). Consequently, a number of CD47-targeting antibodies are in clinical trials of numerous cancers spanning both hematological malignancies and solid tumors.

The most prominent clinically approved CD47-targeted immunotherapeutic is the humanized anti-CD47 antibody (Hu5F9-G4) that targets CD47 to induce phagocytosis, which was developed at Stanford University. Hu5F9-G4 is currently being assessed in phase I clinical research of patients with solid malignancies (www.clinical trials.gov identifier: NCT02216409). Also, HU5F9-G4 can trigger an effective anti-tumor T cell response, which cross-presents cancer cell antigens *via* macrophages, in order to provide protection against tumor recurrence.

A variety of ways have been established to target the CD47 pathway, such as directly interdicting of CD47 or its macrophage receptor SIRPα. Therefore, interdiction of CD47 in preclinical studies utilizing monospecific antibodies has shown promising effects. Nevertheless, the application of anti-CD47 antibodies still faces some safety risk issues. First, CD47 is ubiquitously expressed at the membranes of all cells, including RBCs, under normal and pathological conditions, generating cytotoxicity and antibody depletion. Therefore, comparing to traditional cancer immunotherapeutics, bsAbs targets CD47 along with other tumor antigens as a viable strategy for directing the synergistic benefits of combination therapy specifically toward tumor cells, has a potential to enhance security and efficacy. Secondly, anti-CD47 antibody monotherapy cannot completely get rid of lymphoma. In contrast, combination stratagems which induce adoptive immunity or refer to the utilization of anti-CD20 antibody, macrophage agonists, including IFN-c, interleukin-10, and other agents (e.g., caspase modulators and F-actin regulators), might possess enduring and efficient anti-lymphoma effects. Thirdly, the effects of diverse ways of interdicting CD47, including anti-CD47 antibodies or scFv derived from an antibody, are still uncharted.

BsAb TARGETING CD47 AND OTHER TARGETS THAT ARE CURRENTLY IN CLINICAL TRIALS IN ONCOLOGY

BsAbs co-targeting CD47 and other tumor-specific antigens may improve the binding specificity of CD47-directed antibody, thus enhancing safety and efficacy. Considering these above, bsAbs targeting tumor-specific receptors possessing high binding affinity on one arm and CD47 with lower affinity on the other arm, are nowadays being assessment in clinical trials or are in preclinical study. There are currently four bsAbs constructs targeting CD47 for the treatment of patients with various kinds of cancers, which are undergoing assessed in the clinics at present (**Table 1**).

IBI322

IBI322 is the first anti-CD47/PD-L1 checkpoint bsAb that inhibits both the PD-1/PD-L1 and CD47/SIRP-α pathways, which is used for the treatment of patients with advanced malignancies (25). Preclinical studies have demonstrated that IBI322 can effectively block CD47/SIRP-α interaction and induce macrophages to phagocytose CD47-expressed tumor cells, which is equivalent to anti-CD47 monoclonal antibody.

TABLE 1 | CD47-targeted bsAbs in clinical trials as of March 2021 (44).

bsAb	Sponsor	Format	Targets	Disease area	Development stages	ClinicalTrials.gov Identifier	Status	Preliminary Clinical Data Reported (Ref.)
IBI322	Innovent Biologics (Suzhou) Co. Ltd.	Not available	CD47/ PD-L1	Solid tumors and hematological tumors	Phase I	NCT04338659	Not yet Recruiting	No
					Phase I	NCT04328831	Recruiting	Yes (25, 46)
HX009	Waterstone Hanxbio Pty Ltd	Antibody-receptor fusion	PD-1/ CD47	Malignancies (liver cancer, stomach cancer, and colorectal cancer and so on)	Phase I	NCT04097769	Recruiting	No
IMM0306	ImmuneOnco	Antibody-receptor fusion	CD47/ CD20	B-cell Non-Hodgkin's Lymphoma	Phase I	NCT04746131	Not yet Recruiting	No
TG-1801	NovImmune, TG Therapeutics	Antibody-receptor fusion	CD47/ CD19	Haematologicalmalignancies (B cell lymphoma)	Phase I	NCT03804996	Recruiting	No
SL-172154	Shattuck Labs, Inc	Antibody-receptor fusion	SIRPα-Fc-CD40L	Ovarian Cancer	Phase I	NCT04406623	Recruiting	Yes (47)

And it was suggested that the affinity of IBI322 to PD-L1 was stronger than that to CD47, implying a potential therapy regime in PD-L1-positive cancers (48). IBI322 also effectively blocks the binding of PD-1 to PD-L1 and activates CD4+T lymphocytes, which is comparable to anti-PD-L1 monoclonal antibody. Since PD-L1 is expressed in tumor cells, IBI322 can selectively bind to tumor cells more effectively than anti-CD47 monospecific antibody, thus reducing the possibility of binding to CD47 expressed on RBCs, which could ultimately reduce the toxicity associated with anti-CD47 antibodies. Therefore, IBI322 has stronger anti-tumor activity and higher safety profile.

The first case had been triumphantly administrated in a Phase I clinical trial (CIBI322A101) of the promisingly first-in-class recombinant anti-CD47/PD-L1 bsAb (IBI322) in China. CIBI322A101 is a Phase Ia/Ib clinical study conducted in China to evaluate IBI322 in the treatment of patients with advanced malignancies. Meanwhile, dose selection for first-in-class human (FIH) studies of IBI322, authorized by the National Medical Products Administration (NMPA) for clinical trials (IND No. CXSL1900125) was evaluated, suggesting that the preliminary pharmacodynamics (PD) study with 0.34 mg/kg was rational (46). For scientific confirmation, it still needs more researches at different doses to be conducted in future.

Anti-CD47 antibodies have the tendency to attack normal cells. However, IBI-322 is preferentially distributed in PD-L1-positive tumor cells, thus decreasing the possible adverse effects of this target associated with monospecific anti-CD47 antibodies (25). In addition, bispecific monoclonal antibody enhances cytotoxicity, antibody selectivity and functional affinity by targeting effector cells directly to tumor cells. Preliminary results showed that IBI322 had higher efficacy *in vivo*, tumor-rich distribution and better safety than the mono-specific anti-CD47 antibody (25). Bispecific antibody could offer a lower-cost solution for patients compared with two monoclonal antibody combinatorial therapies. Therefore, the development of the anti-CD47/PD-L1 bsAb will provide patients with a novel, comprehensive, effective, and cost-saving treatment regimen. IBI322 has the potential to benefit more patients in need. Given that IBI322 is currently undergoing a phase I dose escalation trials in China (NCT04328831) and USA (NCT04338659), there is no clinical data reported for it. In China, CIBI322A101 is a Phase 1a/1b clinical study conducted to evaluate IBI322 in the treatment of patients with advanced malignancies. The Phase 1b study will be carried out to evaluate the efficacy of IBI322 in lung, cervical, esophageal, head and neck squamous cell and liver carcinomas. Collectively, more clinical studies are needed still in the clinic.

HX009

As crucial innate and adaptive immune checkpoints on cancer cells, CD47 and PD-L1 coordinate to inhibit immune sensing. Preclinical studies have demonstrated a bsAb co-targeting PD-L1 and CD47 (49) dramatically improved tumor targeting and treatment outcome *vs.* monotherapy *in vitro* and *in vivo*. Anti-PD-1 antibodies restored large amounts of exhausted T cells and anti-CD47 antibodies stimulated phagocytosis of macrophage. Based on this, the anti-PD-1/CD47 bsAb, HX009, was developed by Hangzhou Hanx Biopharmaceutics, Inc. (HanxBio), to treat patients with advanced solid tumors, including gastric cancer, colorectal cancer and liver cancer. Consisting of human IgG4-Fc region of anti-PD-1 mAb and extracellular domain (ECD) of SIRPα, it achieves the synergistic anti-tumor effects by simultaneously activating both innate and acquired immune responses to suppress tumor immune escape and release immune suppression at immune checkpoints.

This antibody-receptor fusion format may have time-cost savings by adapting the natural receptor to the bsAb over the two mAbs-based bsAb for which one more mAb has to be developed. However, due to relatively lower stability of the receptor portion, the antibody-receptor fusion proteins might suffer less stability than the bsAbs based on two mAbs. Currently, HX009 is in early phase I clinical trial (NCT04097769) for patients with advanced solid tumors. So far, no clinical data has been reported for safety and efficacy of HX009.

IMM0306

IMM0306, developed by Shanghai ImmuneOnco Biopharmaceuticals Co. (ImmuneOnco), is a bispecific recombinant antibody-receptor fusion protein. It is designed to target both CD47 and CD20 on B cells but avoid binding to human RBCs, which could simultaneously act on the tumor disease targets and modulate the immune system. By activating the phagocytosis potency of macrophages and triggering antigen-specific T cells *via* tumor antigen presentation, IMM0306 will become a new hot point in the research of cancer immunotherapy in future (50).

Extensive characterization *in vitro* demonstrated that IMM0306 binds to both CD47 and CD20 with affinity 3-8 folds lower than either single-targeted molecule. However, it has greater pro-phagocytosis activity over CD47-positive target cells, and even stronger ADCC activity than Rituximab (an anti-CD20 mAb). Intriguingly, IMM0306 has no binding activity at all toward human RBCs, albeit much lower binding activity toward monkey RBCs (51). Treatment of tumor-implanted SCID mice with IMM0306 significantly inhibited tumor growth and led to eradication of the tumor cells from 5 out of 8 mice, which is much more effective than Rituximab (51). Besides, the *in vivo* studies demonstrated that IMM0306 did not bind to human RBCs and did not induce T cell apoptosis. It can clear lymphoma at a low dose (1.5 mg/kg) (52), showing obvious advantages in safety and clinical development. Preclinical study in non-human primates demonstrated a favorable pharmacokinetic profile with no obvious hemotoxicity following single as well as multiple administrations at different dosage.

All these studies above suggest that antibody-trap like IMM0306 might be an ideal approach for CD47-targeted immunotherapy development since selective avoidance of RBCs mediated antigen-sink as well as anemia could be achieved along with the robust anti-tumor activity. In addition, the preclinical studies confirmed that IMM0306 achieved significant therapeutic effects in various tumor models. IMM0306 is now being assessed in phase I trial (NCT04746131) for the evaluation of safety and effect in patients with B-cell non-Hodgkin's lymphoma. Meanwhile, dose selection of IMM0306, authorized by NMPA for clinical trials (IND No. CTR20192612), is being evaluated for patients with refractory or recurrent CD20 positive B-cell non-Hodgkin's lymphoma. Until now, there is no clinical data reported for IMM0306.

TG-1801

As the first-in-class anti-CD47/CD19 bsAb, TG-1801 is a fully humanized IgG1, targeting the 'don't eat me' self-defense signal which defends cancer cells against the immune system. This bsAb is designed by combining a low-affinity CD47 targeting antibody with a high-affinity antibody against CD19, to make sure that CD47 is employed by the bsAb only on tumor cells that co-expressed both antigens. It has an enhanced Fc-mediated phagocytosis and reserved its activity while the existence of high levels of non-tumor-associated CD47 (53). TG-1801 is designed to selectively target CD47 on CD19+ B cells, sparing RBCs and platelets, with the blockade of CD47-SIRPα macrophage checkpoint on mature B cells. It will avoid off-target toxicity, representing a novel immunological therapeutic strategy with potential for synergistic or complimentary activity with drugs in the current pipeline. In addition, the co-targeting of CD47 and CD19 enhances the expected safety, as well as induces antibody dependent cytotoxicity (ADCC) by retaining the function of its IgG1 Fc region, thereby providing a second mechanism for anti-tumor activity.

To conclude, TG-1801 might play a crucial part in improving the outcome of patients with B cell malignancies. Meanwhile, TG Therapeutics first demonstrated the synergistic effect of TG-1801 in combination with ublituximab (anti-CD20 monoclonal antibody) and umbralisib (PI3K-δ/casein kinase-1ϵ inhibitor) (54), and the synergistic tumor growth inhibition appeared to be mediated by increased infiltration of immune effector cells (55). At present, TG-1801 is undergoing assessment in Phase I trial (NCT03804996) to evaluate its safety and efficacy for treating patients with B cell lymphoma. Thus far, there is no clinical data reported for TG-1801.

SL-172154

SL-172154, developed by Shattuck Labs Inc., is a novel fusion protein consisting of human SIRPα and CD40L (SIRPα-Fc-CD40L) linked *via* a human Fc. It is designed to block the CD47 immune checkpoint while simultaneously activating the CD40 pathway through the dual mechanism, which are both checkpoint blockade and TNF activation. Being one of bifunctional fusion protein candidates, SL-172154 is developed through Shattuck Agonist Redirected Checkpoint (ARC) Platform Technology, which is designed to solve structural limitations. Previous studies found that SL-172154 significantly improved rejection of both primary and secondary tumors, as compared with individual antibodies targeting CD40 and CD47 used alone or in combination, similar to PD-1-Fc-OX40L (56). Notably, the safety and efficacy of the bsAb in nonhuman primates *in vivo* that, the bsAb stimulated dose-dependent elevation in multiple serum cytokines and $CD40^+$ B-cell margination in cynomolgus macaques, without causing hemolysis or thrombocytopenia, provides justification to further explore this strategy in patients with cancers (47).

Currently, SL-172154 is being evaluated in a phase 1 trial (NCT04406623) for patients with ovarian cancer. So far, no clinical data has been reported for safety and efficacy of SL-172154.

BIOSAFETY CONCERNS AND FUTURE DIRECTIONS ASSOCIATED WITH CD47-TARGETED BsAbs

Given the huge potential of anti-CD47 bsAbs, there is ongoing interest in expanding this field in cancer immunotherapy and several bsAbs targeting CD47 have entered into clinical trials. So far in current phase 1 studies, blast reduction and complete

remission rates have been observed, although safe targeting doses to be used in future phase 2 clinical trials still need to be determined for each bsAb targeting CD47 construct. Despite ubiquitously expressed on almost all cell types, on-target cytotoxicity of CD47 to healthy cells and a prominent antigen-sink phenomenon have influenced the development of CD47-targeting agents (57). Therefore, it is important to improve our understanding for potential biosafety problems.

Since CD47 is ubiquitously expressed, potential problems with anti-CD47 antibodies as anticancer agents were possible off-target effects such as anemia (58). CD47 is a crucial regulator of RBCs turnover (59). Buatois et al. (60) indicated that Hu47F9-G4 alone or in combination with other antibodies may cause accidental killing of normal RBCs, potentially resulting in anemia. Hence, there are concerns that CD47-targeting antibodies would expedite RBCs clearance and cause hemolytic anemia since several agents in this class have learn about RBC affinity while others do not. For CD47-targeting bsAbs in preclinical research, various methods are being used to attempt to alleviate this on-target cytotoxicity. For example, the NHP study indicated that a second dose of IBI322 (25) resulted in an extra less drop in the RBCs and hematocrit indices. While RBC counts of both groups began to revert on day 11, IBI322 treatment resulted in a dramatically enhanced RBC count, compared with the Hu5F9 group on day 15. Given that the cross-reaction of IBI322 with cyno PD-L1 and CD47 at approximate affinities to receptors, it is rational to expect that IBI322 could decrease CD47 target-mediated side-effect in patients. CD47 might become an efficient target of this stratagem soon afterward. Comprehensive assessment of IBI322 for the safety and efficacy is necessary in future.

Notably, CD47-targeting agents (i.e., Hu5F9-G4 and TTI-621) could cause acute anemia and thrombocytopenia in people (61–63), which might as well as depend upon the Fc format. The toxicity of anti-CD47 antibodies appears to be Fc-dependent, given that anti-CD47 antibodies, and SIRPα-Fc fusion proteins give rise to this toxicity, while high-affinity SIRPα monomers do not (39, 64, 65). These findings suggest that further studies should aim to optimize the structure of the anti-CD47 bsAbs when attempting to design novel drugs without unwanted side-effects. How to reduce or avoid damage to normal cells while exerting antitumor effects is one of the problems that needs to be considered when designing anti-CD47 therapeutics.

In addition, the expression of CD47 on normal tissues may create an 'antigen sink' that prevents anti-CD47 therapeutic antibodies from reaching tumor cell targets *in vivo*, which also pose a problem in the development of anti-CD47 bsAbs. One strategy to circumvent this issue is by reducing the bsAbs affinity for CD47 but retaining the ability to block the CD47-SIRPα interaction and elevating the affinity to a second tumor antigen. In future studies, more strategies by targeting CD47 and its ligands specifically on tumor cells should be investigated.

Upon macrophage or dendritic cell-mediated phagocytosis of cancer cells by CD47 blockade, these phagocytes may present tumor antigens to T cells to induce anti-tumor T cell responses (66, 67). Therefore, the regimen by combining with T-cell checkpoint inhibitors (with anti-PD-1 or PD-L1 agents) may further augment T cell responses and enhance efficacy. Several pre-clinical studies have demonstrated that CD47 blockade in combination with T cell checkpoint inhibitors can enhance antitumor activity (68–70). Importantly, CD47-targeting agents have the potential to augment existing antibody therapies by adding the benefit of blocking the CD47-mediated inhibitory signal to the established therapeutic effect of pro-phagocytic antitumor antibodies.

We should realize that future improvements in cancer screening and precision medicine will enable the identification and stratification of specific tumor types and/or stages of cancer that would be most amenable to treatment with a certain type or types of anti-CD47 treatment.

CONCLUSION

Recently, we have witnessed much progress with bsAb technologies and therapeutics as reviewed here and elsewhere (13, 71), which have found wide applicability to immunotherapy for cancer treatment. And thus, many bsAb constructs with differing mechanisms of action are being investigated, each with their own limitations and advantages. In this review, we summarized diverse strategies of cancer immunotherapy and characterized the breakthrough in bsAbs development which might be devoted into enhancing the treatment effect and decreasing untoward effects, which mark the beginning of a new era for cancer immunotherapy. Although cancer immunotherapy is rapidly advancing, the effectiveness of bsAbs, especially CD47 bsAbs, can vary between different cancer types, different studies, and even different cancer types, in other words, the therapeutic efficacy of anti-CD47 agents may be tumor specific. Thus, the exploration of novel strategies by targeting macrophage checkpoints is under development. In addition to CD47/SIRPα-targeting antibodies and fusion proteins, the other potential CD47-directed strategies should also be considered.

In summary, two exclusion conditions must be set in the anti-CD47 bsAbs screening process: 1) RBC binding and erythrocyte agglutination induction; 2) Induction of T lymphocyte apoptosis. Without the exclusion of these above, it will be difficult to achieve clinical success. At present, most of the clinical trials targeting CD47 are in phase I clinical trials, which mainly focus on hematoma, thus its value in the solid tumors has yet to be verified. In addition, IgG4 antibodies against CD47 do not respond well to solid tumors, even when used in combination with other antibody drugs. Rigorously, pre-clinical assessments of such treatments are warranted. Based on innovative techniques, we expect that CD47 bsAbs discussed in our review will be more extensively and innovatively designed for immunological therapy, therefore facilitating their effectiveness, as well as decreasing immunologically relevant untoward effects. Future research will be required to raise our comprehension of tumor immunology, which will provide important insights into developing more bsAbs.

AUTHOR CONTRIBUTIONS

YY (1st author) wrote the original draft. ZY and YY (3rd author) revised the manuscript. YY (1st author) prepared tables and figures. YY (3rd author) reviewed and edited. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Advanced HCC Patient Benefit From Neoantigen Reactive T Cells Based Immunotherapy: A Case Report

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Advanced hepatocellular carcinoma (HCC) is a highly lethal disease, mainly due to the late stage at diagnosis and its rapid progression. Although patients with advanced HCC can choose targeted therapy or chemotherapy, overall, the treatment response rate is extremely low and the average survival time is one year more or less. But the application of immunotherapy have led to a paradigm shift in the treatment of HCC, such as TILs (tumor infiltrating lymphocytes),Checkpoint blockade (immune Checkpoint blockade), CAR-T(chimeric antigen receptor T cells) and TCR-T (engineered t-cell receptor T cells). And recent data indicate neoantigens generated when tumors mutate are the main target of tumor-specific TILs, and they are also the main antigens mediating tumor regression in TILs treatment. Moreover, numerous evidences have revealed that radiotherapy lead to massive release of tumor antigens, which may increase the effectiveness of immunotherapy. Based on the above theory, we used neoantigen reactive T cells combined with tomotherapy to treat a patient with advanced HCC (Clinical Trial Study Registration Number: NCT03199807), who reached a long time progress free survival.

Keywords: hepatocellular carcinoma, neoantigen reactive T cells, immunotherapy, tomotherapy, benefit

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INTRODUCTION

In China, hepatocellular carcinoma is among the top five cancer types that have high morbidity and mortality (1). Approximately 80% of the HCC patients were initially diagnosed in an advanced or metastatic stage. Surgery is the first treatment for HCC patients, but not many patients are eligible for surgery, and the 5-year risk of recurrence after hepatectomy is as high as 50%-70% (2, 3). Interventional

therapy and radiotherapy are mostly used as auxiliary treatment methods. In terms of targeted therapy or chemotherapy, the average survival time of patients receiving this treatment is one year more or less, and the remission rate is 2%-18.8% (4–7). Although the use of PD-1 can effectively prolong the survival of patients, the response rate of single-drug use is no more than 16-20%, this suggests that the use of single drugs needs to screen effective populations. In summary, looking for more effective immunotherapy may become a new breakthrough in the field of current HCC treatment.

In a clinical trial of metastatic melanoma, Dr. Rosenberg and his colleagues, who worked at NCI in the United States, confirmed that TILs back-transfusion combined with non-myeloablative chemotherapy or radiotherapy could achieve a clinical remission rate of 40%-72%, and nearly 40% of patients with complete remission (CR) showed no recurrence for more than 7 years (8). The above data indicate that TILs may bring new hope for the treatment of HCC, and the neoantigens that are the main targets of TILs, with its unique advantages, it has become a hot field in today's anti-tumor research.

Neoantigens are a class of antigens derived from normal human genomes. Antigens produced by mutant proteins and oncoviruses are integrated into human genome. They have high affinity with TCR and strong immunogenicity. Compared with the traditional tumor-associated antigen (TAA), neoantigen is not expressed by normal tissues; and thereby, it does not cause central immune tolerance or autoimmune diseases (9). In addition, in 2015, Schumacher TN et al. conducted a tumor mutation spectrum analysis on several common solid tumor types, and showed that the mutation frequency of liver cancer was among the highest (10), and radiotherapy lead to massive release of tumor antigens (11). Based on the above principles, neoantigen reactive T cells combined with tomotherapy have been considered as a promising therapy for advanced cancer. In our previous study, we have combined TCGA data and the NGS sequencing results of the liver cancer samples from our center to screen out the genes with high-frequency mutations (12). Based on the common HLA genotyping (HLA-a*02:01, HLA-a*02:03, HLA-a*02:06, HLA-a*11:01, HLA-a*24:02) in Chinese population, 29 epigenetic peptides were selected and validated the antigenicity and other related parameters of the library (12), and finally we further conducted the clinical study that combined neoantigen reactive T cells with tomotherapy (Clinical Trial Study Registration Number: NCT03199807). Here, we reported a case that had significantly beneficial results from this therapy. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

CASE PRESENTATION

The patient was male, 75-year-old, with a history of chronic hepatitis B. Partial hepatectomy was performed after the diagnosis of primary liver cancer in Apr-2016. In Oct-2016,abdominal Computed Tomography (CT) re-examination revealed multiple enhanced nodules in the liver, and the possibility of metastasis was considered. Therefore, this patient underwent 3 Transarterial Chemoembolization (TACE) operations.

Unfortunately, the abdominal Magnetic Resonance Imaging (MRI) re-examination in Feb-2017 revealed multiple metastatic liver lesions with enlarged hilar lymph nodes.

The patient strongly requested to participate in our clinical trial of tomotherapy combined with Neoantigen Reactive T (NRT) Cells based immunotherapy. In March 2017, this patient received tomotherapy for local lesions in the right anterior lobe of the liver; the cumulative dose was: Planning Target Volume (PTV): 50Gy/10f. After tomotherapy, the patient received personalized NRT immune cells reinfusion combined with IL-2(400WU/d, civ, 5 days in total) and GM-CSF cytokine (150ug/d, ih, 5 days in total) therapy in March 2017, with one month per cycle and a total of four cycles. According to the patient gene expression profile, the neo-antigen was chosen as: (HLA-a*11:01): KRAS-(G12A) p07-16: VVVGAAGVGK; KRAS-(G13D) p07-16: VVVGAGDVGK; PIK3CA-(H1047L) p1046-1054: ALHGGWTTK; IDH1(R132H) p123-142: GWVKPIIIGHHAYGDQYRAT.

This patient was PD-L1(+), and TMB-H. The re-examination after 2 cycles of treatment showed that the lesion on the right anterior lobe of the liver became smaller, and the number of nodules in the more enhanced focus was reduced. The tumor markers were significantly down-regulated, and the changes in HCC specific tumor marker AFP were listed in **Figure 1A**. At the same time, the lymph number gradually increased, and the changes in lymphocytes after per cycle of NRT treatment were shown in **Figure 1B**. After four cycle of NRT, the overall efficacy evaluation was SD, and only regular examination was continued. The amount of T cells reinfusion per cycle and the amount of NRT immune cells detected *in-vivo* were listed in **Table 1**. Except for the fever during the treatment (the highest temperature was 38.3°C, and it was relieved after Paracetamol), there was no other discomfort or obvious liver toxicity.

10 months after the radiotherapy combined with personalized cellular immunotherapy, a review of the abdominal MRI on May 2018 showed that the left outer lobe of the liver appeared, which was considered as disease progression. After full informed consent, the patient was enrolled in the RESCUE project (13) with combined PD-1 antibody and apatinib. From 2018-6, the patient received intravenous injection of PD-1 antibody(SHR-1210,200mg/q2w,iv, two times as a cycle) and apatinib (250mg/d, po). After 2 cycles of PD-1 antibody treatment, the re-examination showed that the intrahepatic mass of porta hepatis and left outer lobe were significantly smaller than before, with the overall efficacy evaluation as partial response (PR). However, due to the hand-foot syndrome side-effect of apatinib, which seriously affected the daily activities of patient, the patient stopped taking apatinib from 2018-7. Therefore, since then, PD-1 antibody single-drug treatment was performed till 2020-6, lasting for a total of 2 years. The patient has been in a fairly good condition. The regular reviews of the tumor showed gradually shrinking of lesion in porta hepatis, and complete response (CR) of intrahepatic lesions in left outer lobe. The patient's imaging changes were shown in **Figure 2**. The target

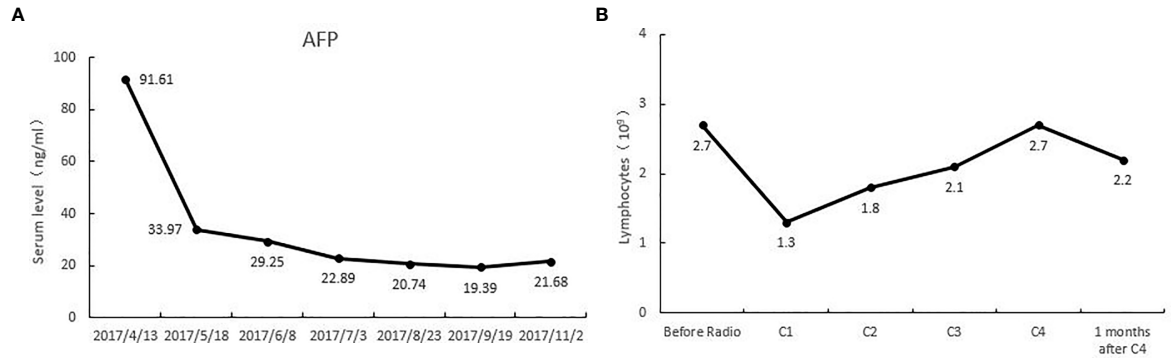

FIGURE 1 | **(A)** alpha fetoprotein (AFP) levels of this patient during the treatment of tomotherapy combined with Neoantigen Reactive T Cells. **(B)** The lymphocyte levels of this patient during the treatment of tomotherapy combined with Neoantigen Reactive T Cells.

TABLE 1 | The amount of T cells reinfusion per cycle and the amount of NRT immune cells detected *in-vivo*.

	Number of NRT (10^{10})	$CD3^+CD4^+CD137^+$ (10^8)	$CD3^+CD8^+CD137^+$ (10^8)
First cycle	1.03	0.62 (0.6%)	0.82 (0.8%)
Second cycle	2.04	1.02 (0.5%)	3.47 (1.7%)
Third cycle	0.43	1.08 (2.5%)	0.52 (1.2%)
Fourth cycle	1.12	0.78 (0.7%)	2.13 (1.9%)

lesion changes at different time points during and after the treatment were listed in **Table 2**.In order to intuitively reflect the patient's prognosis and clinical curative effect, we listed the entire treatment process in **Figure 3**.

DISCUSSION

This is a case of comprehensive treatment with obvious benefits. In the first stage, the patient benefited from tomotherapy combined with Neoantigen Reactive T Cells. The lesion with radiotherapy was significantly reduced, and the remaining lesions were stable. In the second stage, new lesion appeared on the left lobe, and the patient benefited from the treatment of apatinib combined with PD-1 antibody. At the final stage, PD-1 antibody maintenance treatment was going on after the abandonment of apatinib because of the serious side effects, this patient continued to get benefit from treatment. From the genetic test, this patient is positive for TMB-H and PD-L1, which may be a potential predictor of his benefit from immunotherapy. At the same time, this patient has mutations in KRAS and IDH1, which are frequent mutation sites in HCC, providing a genetic basis for neoantigen immunotherapy. The patient's early cell therapy provides a material basis for the subsequent PD-1 antibody to a certain extent.

In this comprehensive treatment, it reflects the synergy of radiotherapy and immunotherapy. In recent years, the synergistic effect of radiation and immunotherapy has been reported widely. Radiation can cause tumor to release tumor antigens, transforming cold tumors into hot tumors. And these changes in immune microenvironment can attract T cells to attack the tumor site, thus synergizing the effects of radiation and immune response (14–16). In addition, radiotherapy can lead to a significant decrease in lymphocyte count, which possibly eliminates the inhibitory or dysfunctional T cells. We also observed this change in our study. The number of lymphocytes in patients declined significantly after radiotherapy. But after we transfused the T cells back into patients, the number of lymphocytes gradually increased and played a synergistic anti-tumor role.

In this comprehensive treatment, it also reflects the synergy of anti-angiogenesis and immunotherapy. Studies have shown that the basic principle of anti-angiogenic drugs combined with immunotherapy is that anti-angiogenic drugs can inhibit the occurrence of angiogenesis and immune escape in the tumor microenvironment, at the same time, this effect can enhance the body's immune response to achieve anti-tumor effects (17, 18). The Keynote524 study also shows that when the two drugs are used in combination, the objective response rate(ORR) of patients with advanced HCC can reach 36%, progression-free survival (PFS) reaches 8.6 months, and overall survival (OS) reaches 22 months (19). We also observed a significant reduction of the lesion in the study. After the patient's left outer lobe of the liver was treated with anti-angiogenesis combined immunotherapy, the lesion was significantly reduced, and the efficacy was evaluated as PR.

Overall, this comprehensive treatment model is safe and well tolerated. Except for hand-foot syndrome caused by apatinib,

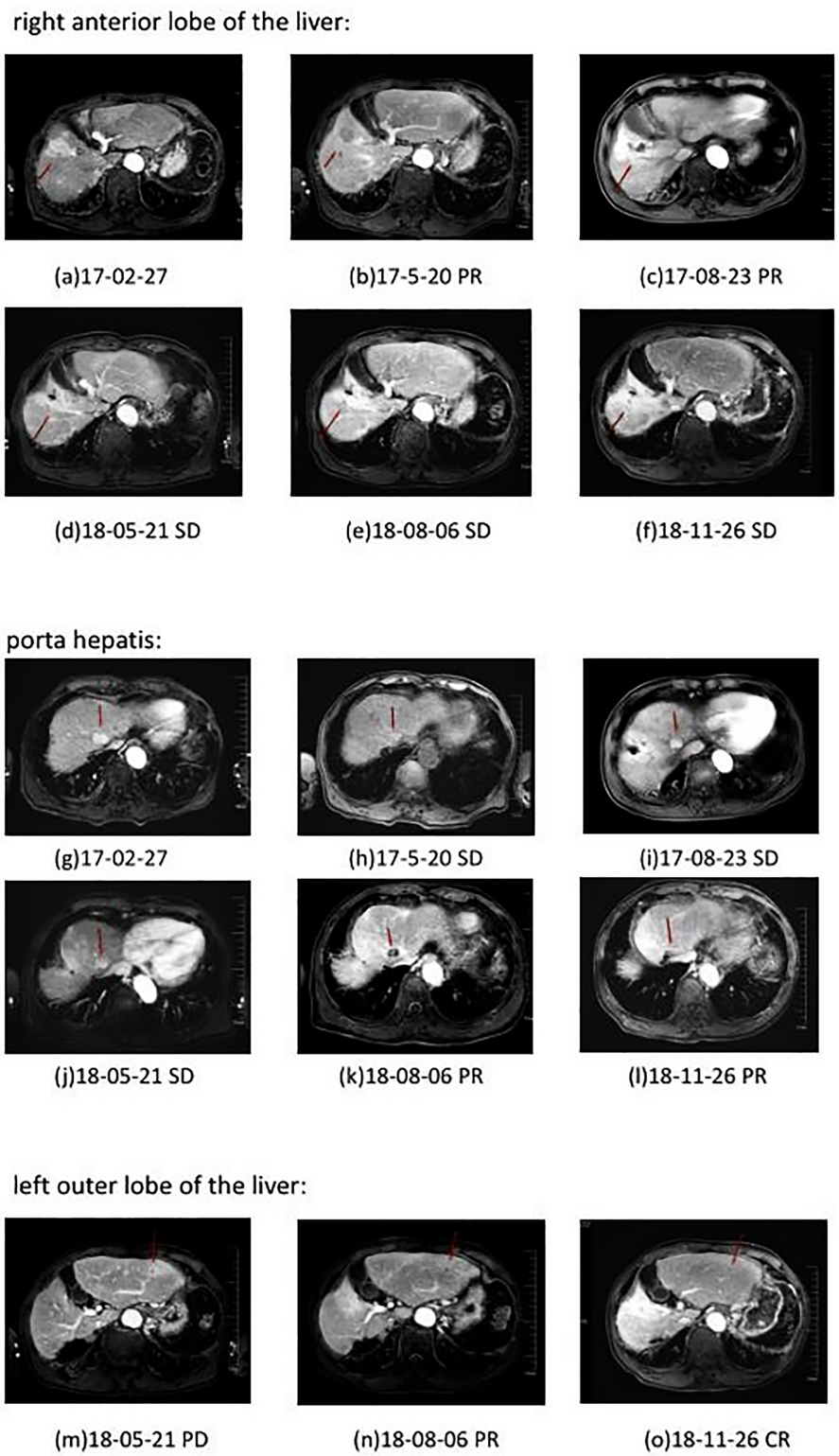

FIGURE 2 | Plain and enhanced MRI of lesions in different parts of patient in different periods. a–f: right anterior lobe of the liver; g–l: porta hepatis; m–o: left outer lobe of the liver. Efficacy evaluation: PD, Disease progression; SD, Stable disease; PR, Partial response; CR, Complete response.

TABLE 2 | The target lesion changes at different time points during and after the treatment.

	2017-2 (mm)	2017-8 (mm)	2018-5 (mm)	2018-8 (mm)	2018-11 (mm)	2020-07 (mm)	2021-3 (mm)
right anterior lobe	33.60	22.93	18.45	18.65	18.60	18.62	18.69
porta hepatis	21.48	18.35	19.08	7.71	9.25	6.21	6.21
left outer lobe	none	none	18.00	7.11	0	0	0

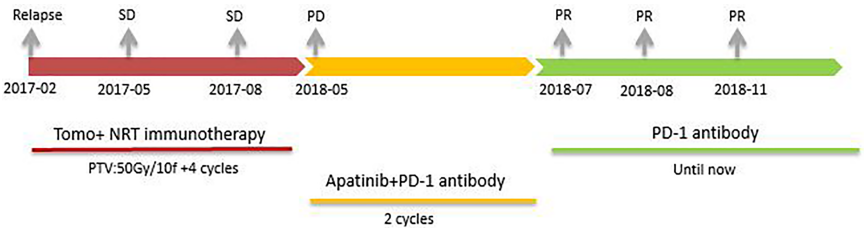

FIGURE 3 | The patient received different treatments at different times. Firstly, tomotherapy combined with Neoantigen Reactive T Cells based immunotherapy (2017-02 to 2017-08, SD). Secondly, PD-1 antibody combined with apatinib(2018-05 to 2018-07, PR). Finally, PD-1 antibody single-drug maintenance treatment (2018-07 until 2020-06, PR in porta hepatis and CR in left outer lobe).

there is no other special discomfort. But it brings significant survival benefits to patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Drum Tower Hospital Affiliated to Nanjing University Medical School. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JShe and BL designed the clinical trial. CL, JShe and QX composed the manuscript and provided figures. CL, JSha, YD, QX, JY, JL, BL and JShe did the work of the acquisition, analysis and interpretation of the data. JShe, YD, ZZ, FC and SL revised the manuscript critically for important intellectual content, and agreement to be accountable for all aspects of the work, in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors ontributed to the article and approved the submitted version.

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Serum Anti-PDLIM1 Autoantibody as Diagnostic Marker in Ovarian Cancer

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Background: Serum autoantibodies (AAbs) against tumor-associated antigens (TAAs) could be useful biomarkers for cancer detection. This study aims to evaluate the diagnostic value of autoantibody against PDLIM1 for improving the detection of ovarian cancer (OC).

Methods: Immunohistochemistry (IHC) test in tissue array containing 280 OC tissues, 20 adjacent tissues, and 8 normal ovarian tissues was performed to analyze the expression of PDLIM1 in tissues. Enzyme-linked immunosorbent assay (ELISA) was employed to measure the autoantibody to PDLIM1 in 545 sera samples from 182 patients with OC, 181 patients with ovarian benign diseases, and 182 healthy controls.

Results: The results of IHC indicated that 84.3% (236/280) OC tissues were positively stained with PDLIM1, while no positive staining was found in adjacent or normal ovarian tissues. The frequency of anti-PDLIM1 autoantibody was significantly higher in OC patients than that in healthy and ovarian benign controls in both training (n=122) and validation (n=423) sets. The area under the curves (AUCs) of anti-PDLIM1 autoantibody for discriminating OC from healthy controls were 0.765 in training set and 0.740 in validation set, and the AUC of anti-PDLIM1 autoantibody for discriminating OC from ovarian benign controls was 0.757 in validation set. Overall, it was able to distinguish 35.7% of OC, 40.6% of patients with early-stage, and 39.5% of patients with late-stage. When combined with CA125, the AUC increased to 0.846, and 79.2% of OC were detected, which is statistically higher than CA125 (61.7%) or anti-PDLIM1(35.7%) alone ($p<0.001$). Also, anti-PDLIM1 autoantibody could identify 15% (18/120) of patients that were negative with CA125 (CA125 <35 U/ml).

Conclusions: The anti-PDLIM1 autoantibody response in OC patients was positively correlated with PDLIM1 high expression in OC tissues, suggesting that the autoantibody against PDLIM1 might have the potential to be a novel serological biomarker of OC, serving as a complementary measure of CA125, which could improve the power of OC detection.

Keywords: autoantibody, tumor-associated antigens, ovarian cancer, PDLIM1, diagnostic marker

INTRODUCTION

Ovarian cancer (OC) is one of the most common cancer among women, with approximately 313,959 new diagnoses and 207,252 deaths worldwide in 2020 (1). Due to the typically asymptomatic early-stage tumors and lack of effective diagnostic methods, most OC patients continue to be diagnosed at advanced stage with high fatality rates and relatively poor long-term survival (2, 3). Although some improvements in diagnosis and treatment have been made, the overall 5-year survival rate still remains as low as 40% (4). The measurement of CA125 level has been embraced by primary care for ovarian cancer, but the measurements with better sensitivity and specificity are especially in need for early disease (5). To address this issue, substantial work needs to be put into the exploration of novel biomarkers that could improve early diagnosis and treatment effect.

The detection of autoantibodies (AAbs) triggered by tumor-associated antigens (TAAs) is showing great potential for the development of blood-based biomarkers (6, 7). Most of TAAs are secreted or shed into the blood from tumor cells, then captured by the immune system and elicit an immune response (8). However, due to the labile features and low titers, TAAs seem not as stable as AAbs. AAbs not only have an immunological amplification effect but also can exist in the peripheral blood for a longer period of time, making them more ideal biomarkers for the detection of cancers than their corresponding TAAs (9). Moreover, AAbs have been identified as the reporters of incipient carcinogenesis, which could occur before any clinical symptoms (10). Therefore, identifying AAbs with good performance will hold favorable clinical applications, especially for the early detection of cancer with a non-invasive method.

The cytoskeleton is a kind of protein fiber network structure and widely exists in eukaryotic cells, which maintains cell morphology and participates in cell movement, cell polarity, cell division, and signal transduction (11). PDLIM1 is a cytoskeletal protein, also known as CLP36, belonging to the PDZ and LIM protein family. Mediated *via* the PDZ domain, PDLIM1 binds to alpha-actinin and is localized to actin stress fibers in the cytoplasm (12). Many studies demonstrated that PDLIM1 plays a role in the regulation of actin cytoskeleton organization in non-muscle tissues (12–14). Studies indicated that the abnormal expression of PDLIM1 is associated with hepatocellular carcinoma, breast cancer, colorectal cancer, and pancreatic cancer (15–18). In Liu's study, PDLIM1 was reported to promote breast cancer cell migration and invasion *in vitro* and metastasis *in vivo* through interaction with α-actinin (17). During metastasis of hepatocellular carcinoma (HCC), PDLIM1 was demonstrated to play an important inhibitory role through activating the Hippo signaling pathway (16). It seems that PDLIM1 plays different regulatory roles in different kinds of cancer cells (16, 17).

In a few studies on autoantibody against PDLIM1, PDLIM1 was identified as a tumor-associated antigen (TAA) due to inducement of autoantibody response in patients with breast cancer and pancreatic cancer (9, 18). No reports have been found about the expression of PDLIM1 in ovarian cancer tissues and whether there is an autoantibody response to PDLIM1 in patients with ovarian cancer. In the current study, we aim to explore the occurrence and presentation level of anti-PDLIM1 autoantibodies in the sera of patients with ovarian cancer and further to evaluate its potential as a biomarker for the detection of OC.

MATERIALS AND METHODS

Immunohistochemistry

OC tissue microarray consisting of tissues from 294 OC patients (14 of 294 were invalid), 20 adjacent tissues, and 8 normal ovarian tissues was obtained from Shanxi Avila Biotechnology Ltd., Co. (Xian, China), and duplicate cores per case of cancer to make sure of a solid result. Immunohistochemistry (IHC) test was performed by following the standard protocols. Anti-PDLIM1 antibody (Santa Cruz Biotechnology, sc-374077, 1:20 dilution) and secondary antibody (MXB Biotechnologies, KIT-9720) were used for IHC testing. Briefly, paraffin-embedded tissue slides were deparaffinized and rehydrated with xylene and graded alcohols. Slides were washed with phosphate-buffered saline (PBS) and subjected to antigen microwave retrieval at 100°C for 15 min and cooled at room temperature for 40 min. Endogenous peroxidase activity was blocked by incubating slides with 3% H_2O_2 for 37°C/30 min. Slides were blocked by 10% blocking reagent (goat serum) for 60 min. After washing with PBS, slides were incubated with PDLIM1 antibody at 4°C overnight. Slides were then incubated with secondary antibody for 60 min at room temperature. To visualize the reaction, slides were incubated with DAB for 2–5 min at room temperature and followed by counterstaining with Gill hematoxylin solution for 1 min and washed for 10 min with running water. Finally, the slides were dehydrated and mounted and were then observed under a microscope (Olympus). The results were read by two independent pathologists. Stain intensity and the percentage of positive cells were scored as follows: (1) for stain intensity, negative, score 0; light brown, score 1; brown, score 2; deep brown, score 3; (2) for percentage of positive cells, scored each criterion on a scale of 0 to 3, ≤5% scored 0; 6–25% scored 1, 26–50% scored 2, and >50% scored 3. For two cores per case of cancer, the result was calculated by the mean value. Final results (defined as IHC-score) were calculated by multiplying the scores of the percentage of positive cells by the stained intensity. The range of IHC-score is 0–9, and if it was greater than 2, the sample was considered as positive result (19). The expression of PDLIM1 in OC and normal ovarian tissues was also explored at the Human Protein Atlas (HPA) (20).

Sera From Patients and Controls

The case-control study including 545 subjects were divided into two cohorts. All female OC cases were age-matched with corresponding healthy controls. Healthy controls had no history of cancer, autoimmune diseases, and ovarian benign diseases. The 182 OC sera and 181 benign controls sera were obtained from two affiliated hospitals of Zhengzhou University in Henan Province, China, and serum collection time spanned July 2017 to December 2018. All healthy control sera (N=182) were from the Biological Specimen Bank of Henan Key Laboratory of Tumor Epidemiology.

The detailed information of the study populations is shown in **Table 1**. All participants in the study have signed the informed consent form. The study was approved by the Ethics Committee of Zhengzhou University.

Recombinant Protein and ELISA Assay

The full-length recombinant protein of PDLIM1 was purchased from Cloud-Clone Corp. (Wuhan, China). PDLIM1 recombinant protein was diluted in carbonate buffer (pH=9.6) to an optimal concentration of 0.25 μg/ml for ELISA testing. The detailed procedure was described in our previous study (21). In brief, the diluted protein was coated onto the bottom of 96-well plates overnight at 4°C, followed by incubation using 2% bovine serum albumin (BSA) for 2 h at 37°C water baths. After washing with phosphate-buffered saline containing 0.05% Tween-20 (PBST), sera with the dilution of 1:100 or the dilution buffers without sera (blank control) were added into corresponding wells for incubation of 1 h at 37°C water baths. In this step, eight sera from four OC patients and four healthy controls were added into every plate for normalization among different plates (CV<15%). Then, plates were washed by PBST followed by incubating with HRP-conjugated goat anti-human IgG at 1:5m000 dilution for 1 h at 37°C water baths. A solution of 3,3',5,5'-tetramethyl benzidine (TMB)-H_2O_2-urea was used as the detecting agent, and 2M sulfuric acid was added into each well as the stopping solution. The optical density (OD) was read at 450 and 620 nm by Multilabel Plate Reader (PerkinElmer).

Statistical Analysis

All data were described by using Median ± IQR (Inter Quartile Range). Kruskal-Wallis H Test, Mann-Whitney U Test, Chi-square test, and Fisher's Exact Test were performed to compare the differences of AAb levels in different groups (if there were more than two groups for comparison, the α value was adjusted by Bonferroni correction). The receiver operating characteristics (ROC) curve analysis was employed to evaluate the diagnostic value of anti-PDLIM1 AAb for OC. In addition, the Youden index (YI), sensitivity, specificity, false positive rate (FPR), false negative rate (FNR), positive predictive value (PPV), and negative predictive value (NPV) were calculated to evaluate the validity and reliability of the anti-PDLIM1 AAb as a diagnostic biomarker. The cut-off value was determined by the maximum of Youden index with specificity of 90%. Statistical analyses were performed by IBM SPSS Statistics 21.0 and GraphPad Prism 8.0. The gene expression of PDLIM1 in OC tissues was investigated in GEPIA (Gene Expression Profiling Interactive Analysis) (22).

RESULTS

PDLIM1 Protein Expression in OC Tissues

The overall study design was shown in **Figure 1**. The expression of PDLIM1 protein was tested and analyzed in OC tissues, adjacent normal tissues, and normal ovarian tissues by IHC

TABLE 1 | Characteristics of study subjects.

Variables	Training set		Validation set		
	OC (%)	Healthy (%)	OC (%)	Benign (%)	Healthy (%)
Number	61	61	121	181	121
Female	61 (100)	61 (100)	121 (100)	181 (100)	121 (100)
Age, years					
Mean ± SD	54 ± 10	51 ± 12	51 ± 12	40 ± 11	52 ± 11
Range	23–74	23–81	20–81	22–66	20–83
Family tumor history				NA	NA
Yes	20 (32.8)		24 (19.8)		
No	41 (67.2)		97 (80.2)		
FIGO				NA	NA
I	6 (9.8)		15 (12.4)		
II	2 (3.2)		9 (7.4)		
III	19 (31.1)		32 (26.4)		
IV	10 (16.4)		20 (16.5)		
Missing	24 (39.3)		45 (37.2)		
Lymph node metastasis				NA	NA
Positive	26 (42.6)		38 (31.4)		
Negative	35 (57.4)		83 (68.6)		
Distant metastasis				NA	NA
Positive	25 (41.0)		34 (28.1)		
Negative	36 (59.0)		87 (71.9)		
Histological type				NA	NA
Serous adenocarcinoma	51 (42.1)		102 (84.3)		
Mucinous adenocarcinoma	1 (0.8)		3 (2.5)		
Clear cell carcinoma	2 (1.7)		3 (2.5)		
Endometrioid adenocarcinoma	7 (5.8)		13 (10.7)		

OC, ovarian cancer.
NA, not applicable.

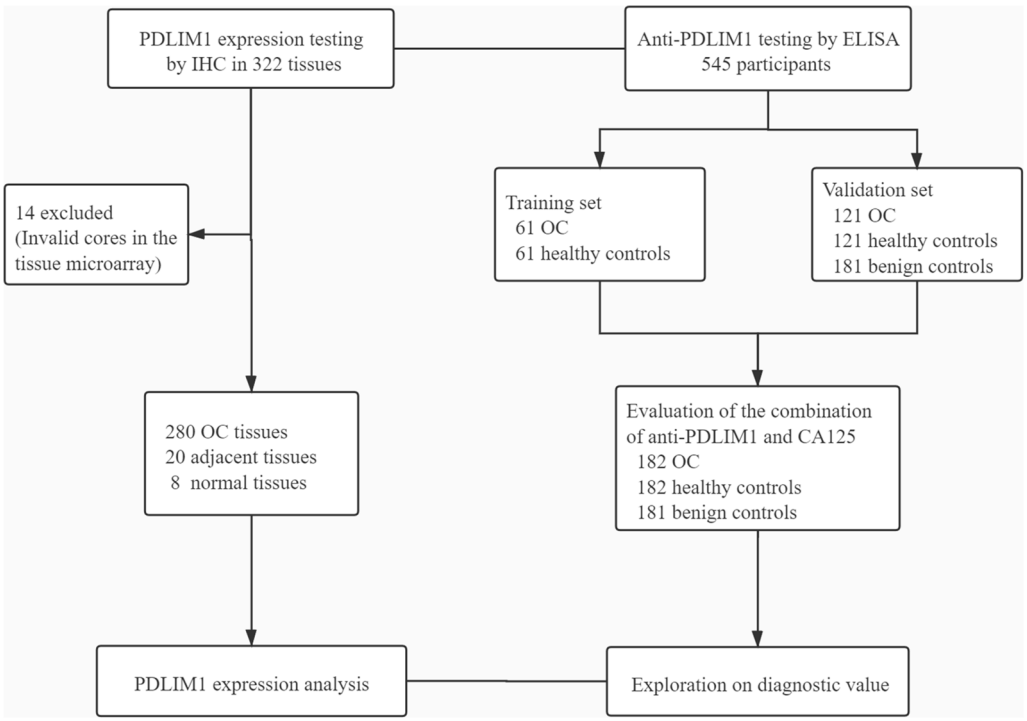

FIGURE 1 | Study design. OC, ovarian cancer; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry.

(**Table 2**). According to the IHC results, PDLIM1 was highly expressed in OC tissues (**Figure 2A**) with cytoplasmic staining pattern, while no cytoplasmic staining was found in both adjacent tissues and normal ovarian tissues (**Figures 2B, C**). There were 84.3% (236/280) of OC tissues (14 of 294 cores were invalid) that were positively stained with PDLIM1 (**Figure 2D**). Based on clinical features such as clinical stage, age, and pathological grade, OC tissues were classified into three subgroups. Across the three subgroups, there were no significant differences in frequency of positive staining (**Figures 2E–G**). By querying the HPA database, it was found that the expression of PDLIM1 had strong or weak staining (8 of 11) in OC tissues, while normal ovarian tissues showed negative staining in ovarian stromal cells. Moreover, we also analyzed the mRNA expression of PDLIM1 in OC and normal ovarian tissues by GEPIA; it was also highly expressed ($p<0.05$) in OC tissues with significant difference (**Figure 2H**).

Detection and Validation of Anti-PDLIM1 Autoantibody by ELISA

To explore the appearance and presentation level of anti-PDLIM1 AAb in the sera from OC patients and healthy controls, the indirect ELISA was performed for the measurement of anti-PDLIM1 autoantibody in two datasets (training and validation). We first tested the autoantibody in 61 OC sera and 61 age-matched healthy control sera in the training dataset. The result showed that anti-PDLIM1 autoantibody not only appeared in the sera from OC patients but also was significantly higher ($p<0.0001$) in OC sera than that in healthy control sera (**Figure 3A**). Then, we

TABLE 2 | Results of IHC analysis.

Tissues	Stages	N	Intensity score		Positive-cell score		IHC-Score	
			Median	IQR	Median	IQR	Median	IQR
OC	I	193	2.0	1.0	3.0	1.0	6.0	6.5
	II	42	2.0	1.0	3.0	0.0	6.0	6.0
	III	33	1.0	1.0	3.0	1.0	3.0	4.0
	IV	12	2.5	1.8	3.0	1.1	7.5	6.4
	Total	280	2.0	1.0	3.0	1.0	6.0	6.4
Adjacent		20	0.0	0.0	0.0	0.0	0.0	0.0
Normal		8	0.0	0.0	0.0	0.0	0.0	0.0

OC, ovarian cancer; IQR, interquartile range; IHC, immunohistochemistry.

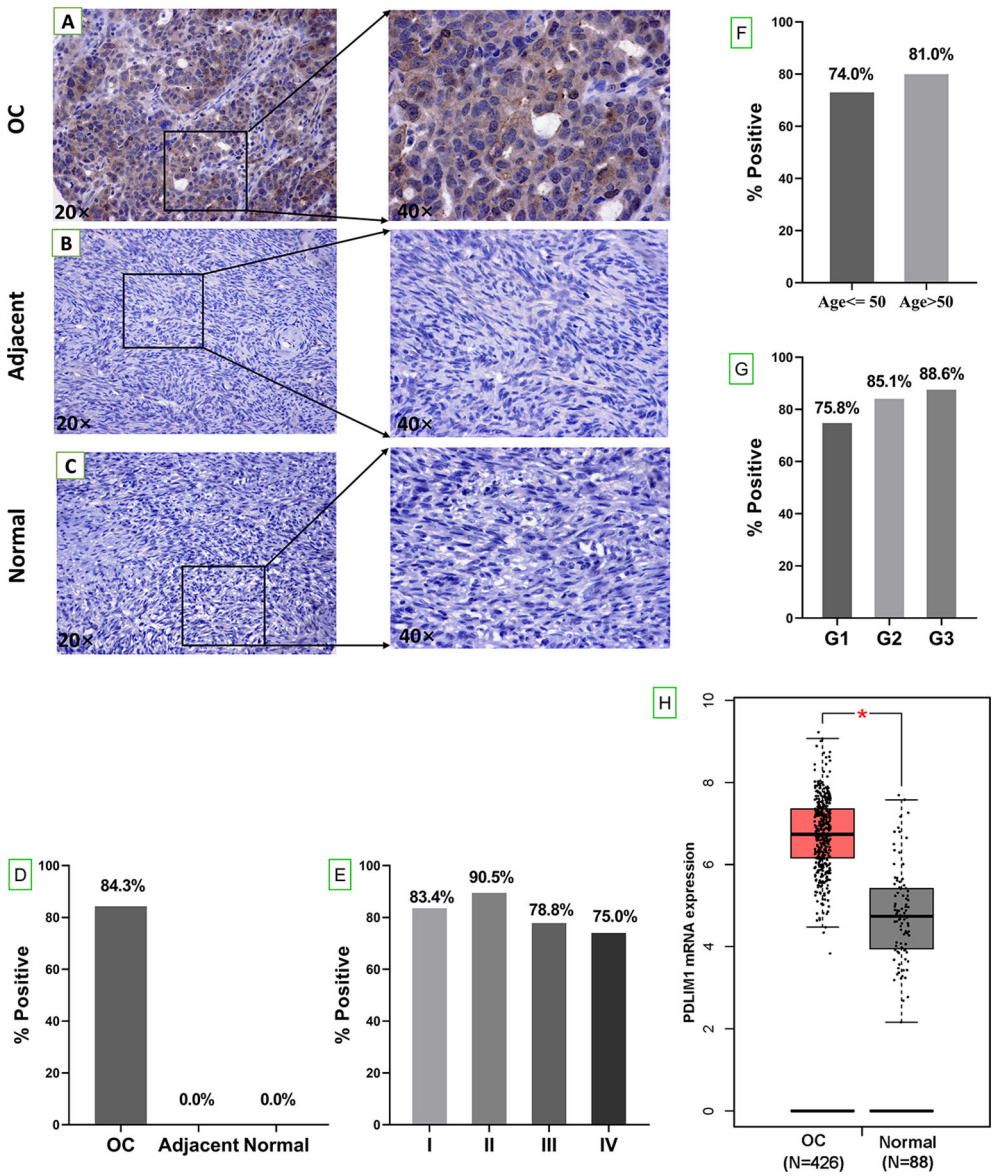

FIGURE 2 | Tissue expression of PDLIM1 by IHC analysis. **(A)** Positive staining of PDLIM1 in a representative ovarian cancer tissue (obtained at 20× and 40× by microscope). **(B)** Negative staining in a representative adjacent tissue (obtained at 20× and 40× by microscope). **(C)** Negative staining in a representative normal ovarian tissue (obtained at 20× and 40× by microscope). **(D)** Positive rates of PDLIM1 in the tissues of 280 OC, 20 adjacent normal tissues, and 8 normal ovarian tissues. **(E)** Positive rates of PDLIM1 in the different stages of 280 OC. **(F)** Positive rates of PDLIM1 in different ages. **(G)** Positive rates of PDLIM1 in different pathological grades. **(H)** The mRNA expression of PDLIM1 in OC and normal tissues from GEPIA database. *$p < 0.05$. The cut-off value was considered as IHC-score >2.

further validated the result from the training dataset with larger sample size in the validation dataset including 121 sera from patients with OC, 181 sera from patients with ovarian benign diseases, and 121 sera from healthy controls. Significant differences in the titer of anti-PDLIM1 AAb were observed between the OC group and each of two control groups (healthy controls and ovarian benign controls) (p<0.0001); the statistical difference still appeared when we combined two control groups into one group, without statistical difference between healthy control group and benign controls group (**Figures 3B, C**). There is no difference detected between early and late stage of OC on the expression of anti-PDLIM1 AAb (**Figures 3D**).

Value of Anti-PDLIM1 Autoantibody in Diagnosis and Differential Diagnosis of OC

The performances of anti-PDLIM1 AAb in training and validation datasets were determined by comparing the parameters that can reflect the diagnostic value of OC. Based on the ROC analysis, the Youden index (YI), sensitivity, specificity, false positive rate (FPR),

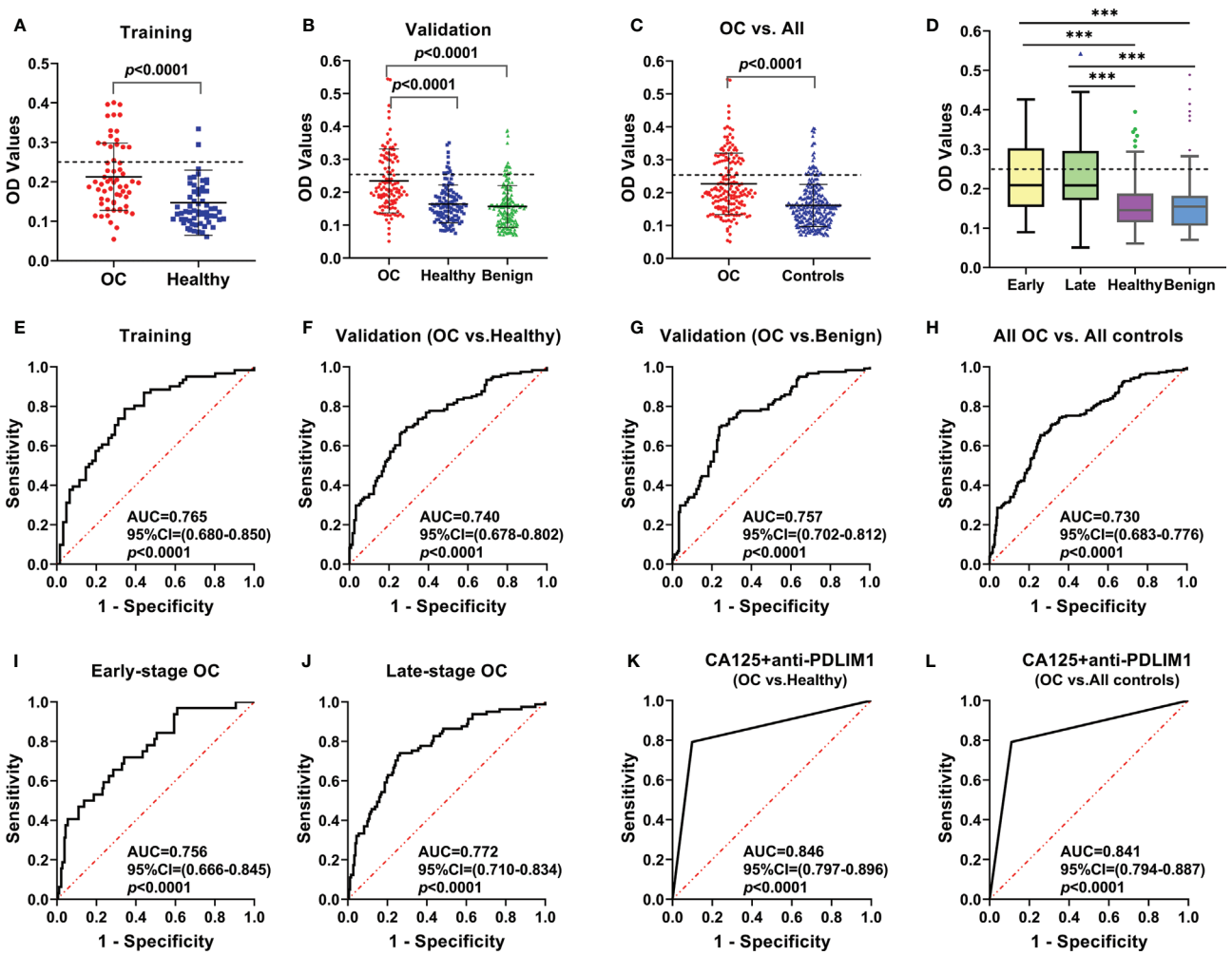

FIGURE 3 | The expression and the diagnostic performance of anti-PDLIM1 AAb in OC. The expression level of anti-PDLIM1 AAb in the training set **(A)**, validation set **(B)**, all OC and all controls (healthy+benign) **(C)**, early-stage and late-stage of OC **(D)**. The cut-off value (dotted line) was determined by the maximum Youden index with the specificity of 90%. The diagnostic performance of anti-PDLIM1 AAb in the training set **(E)**, validation set **(F, G)**, all OC and all controls (healthy+benign) **(H)**, early-stage OC **(I)**, late-stage OC **(J)**. The diagnostic performance of anti-PDLIM1 AAb in combination with CA125 for identifying OC from healthy controls **(K)** or all controls (healthy+benign) **(L)**. ***$p < 0.001$.

false negative rate (FNR), positive predictive value (PPV), and negative predictive value (NPV) were analyzed among different groups (**Table 3**). As shown in **Figures 3E, F**, the areas under the curve (AUC) for identifying OC from healthy controls in training and validation datasets were 0.765 (95% CI: 0.680–0.850) and 0.740 (95% CI: 0.678–0.802), respectively. In the differential diagnosis of OC in the validation dataset (**Figure 3G**), anti-PDLIM1 AAb had the AUC of 0.757 (95% CI: 0.702–0.812). When we combined all

TABLE 3 | Diagnostic value of anti-PDLIM1 AAb to OC.

Subjects	AUC	*p*	Se (%)	Sp (%)	YI	FPR (%)	FNR (%)	PPV (%)	NPV (%)
OC *vs.* Healthy	0.740	<0.0001	35.5	90.1	0.3	9.9	64.5	78.2	58.3
OC *vs.* Benign	0.757	<0.0001	33.9	90.6	0.2	9.4	66.1	78.3	57.8
OC *vs.* (Healthy+Benign)	0.730	<0.0001	31.9	90.1	0.2	9.9	68.1	76.3	57.0
Early *vs.* Healthy	0.756	<0.0001	40.6	90.1	0.3	9.9	59.4	80.4	60.3
Late *vs.* Healthy	0.772	<0.0001	39.5	90.1	0.3	9.9	60.5	80.0	59.8
Early *vs.* Benign	0.748	<0.0001	40.6	90.6	0.3	9.4	59.4	81.2	60.4
Late *vs.* Benign	0.761	<0.0001	33.3	90.6	0.2	9.4	66.7	78.0	57.6
Early *vs.* (Healthy+Benign)	0.739	<0.0001	40.6	90.7	0.3	9.3	59.4	81.4	60.4
Late *vs.* (Healthy+Benign)	0.755	<0.0001	33.3	90.1	0.2	9.9	66.7	77.1	57.5

OC, ovarian cancer; Se, sensitivity; Sp, specificity; YI, Youden Index; FPR, false positive rate; FNR, false negative rate; PPV, positive predictive value; NPV, negative predictive value.

healthy controls and ovarian benign controls from both training and validation datasets, the AAb showed the AUC of 0.730 (95% CI: 0.683–0.776) for distinguishing all OC from all controls (**Figure 3H**). The AUCs for identification of OC with early stage (I+II) and late stage (III+IV) were 0.756 (95% CI: 0.666–0.845) and 0.772 (95% CI: 0.710–0.834), respectively (**Figures 3I, J**), without significant difference.

When a cut-off value was defined as the corresponding point to maximum of Youden index at specificity of 90%, the frequency of anti-PDLIM1 AAb in the different groups were calculated. Among 182 OC sera, 35.7% (65/182) of sera showed positive reaction to PDLIM1 (**Table 4**), which was significantly higher than that in healthy control sera (9.9%, 18/182) and ovarian benign control sera (12.2%, 22/181). Obviously, anti-PDMIL1 AAb had a certain ability to distinguish OC from normal and ovarian benign diseases. In another word, the anti-PDLIM1 AAb seemed more specific to OC across ovarian benign and malignant diseases. To evaluate the performance of anti-PDLIM1 AAb in different subgroups of OC, we divided OC patients into different groups by clinical stage, age, family tumor history, lymph node metastasis, distant metastasis, and histological types, and no significant differences were found across these subgroups (**Figure 4**). Although the positive rate of anti-PDLIM1 AAb in ovarian mucinous adenocarcinoma was as high as 75% (3/4), the sample size was too small to make a difference (p>0.05, Fisher's exact test).

Combination of Anti-PDLIM1 Autoantibody and CA125 for Detecting OC

We used testing data from a total 182 OC sera and total 182 healthy control sera for evaluating the diagnostic value of the combination of anti-PDLIM1 AAb and CA125. As mentioned above, 35.7% (65/182) of OC sera was found to have positive reactivity to PDLIM1. Among 182 OC sera, 120 of OC patients had the test result of CA125; 61.7% (74/120) of OC sera showed positive reaction (>35 U/ml) of CA125 (**Table 4**). When combing anti-PDLIM1 AAb with CA125, the positive rate increased to 79.2% (95/120), which was significantly higher than anti-PDLIM1 AAb or CA125 alone (p<0.001). The combination of the two biomarkers yielded the AUCs of 0.846 (95% CI: 0.797–0.896) and 0.841 (0.794–0.887) for discriminating OC from healthy controls or all controls (healthy + benign), respectively (**Figures 3K, L**). The combination identified 91.7% (11/12) early-stage (I+II) OC and 77.8% (53/63) late-stage (III+IV) OC (**Table 4**). Moreover, anti-PDLIM1 AAb identified 15% (18/120) of OC patients with negative CA125.

DISCUSSION

Due to the heterogeneous attribute, most of ovarian cancer (OC) is insidious and painless in the early stage. Thus, there are only less than 25% of patients with OC detected at the early stage (I+II), and more than 75% of patients with OC are found at the late stage (II+IV) (23). Although the 5-year survival of OC patients has improved owing to advanced treatments, the overall cure rate still remains lower than 30% (24). The transvaginal ultrasonography and cancer antigen (CA) 125 are the most commonly used diagnostic tests for OC; however, they are not specific for OC and could not reflect the evidence of decreased mortality for OC (25, 26). At present, the development of the strategy for early detection of OC is imperative to reduce the mortality of OC. Therefore, with the advantages of stability and convenient detection, a blood-based biomarker such as autoantibodies holds promising interests for identifying individuals and developing strategies of early detection (27, 28). In this study, we tried to explore anti-PDLIM1 AAb as a potential biomarker for the detection of OC. Using different methods (IHC, ELISA, bioinformatics analysis) for validation, the anti-PDLIM1 AAb showed good diagnostic potential as a biomarker for the detection of OC.

To the best of our knowledge, this is the first study to explore the diagnostic value of anti-PDLIM1 AAb for the detection of OC. In a study performed by Hong *et al.*, anti-PDLIM1 AAb was detected in 14 out of the 36 sera (38.9%) from patients with a pancreatic adenocarcinoma, while it was only observed in 4.4% of controls (3 out of 68 subjects including 14 lung adenocarcinoma, 19 colon adenocarcinoma, and 35 healthy subjects) (18). Another study also reported that anti-PDLIM1 AAb identified breast cancer from controls with a sensitivity of 73.4% and specificity of 58.3% (9). However, there is less evidence to show the detection and diagnostic performance of anti-PDLIM1 AAb in OC patients. From our findings, anti-PDLIM1 AAb could distinguish OC from healthy controls with the AUCs of 0.765 and 0.740 in the training and

TABLE 4 | Diagnostic performance of anti-PDLIM1 AAb, CA125, and the combination of them.

Types	Positive, n/N	%	*p*
Anti-PDLIM1+CA125	95/120	79.2	<0.0001[a]
Anti-PDLIM1	65/182	35.7	
CA125	74/120	61.7	0.003[a]
Early (Anti-PDLIM1+CA125)	11/12	91.7	0.080[b]
Late (Anti-PDLIM1+CA125)	53/63	77.8	
Healthy (Anti-PDLIM1)	18/182	9.9	<0.0001[c]
Benign (Anti-PDLIM1)	22/181	12.2	<0.0001[c]

The cut-off was determined by the maximum Youden index with specificity of 90%.

[a]*The frequency of OC patients with positive reaction to both anti-PDLIM1 AAb and CA125 compared with the frequency of only anti-PDLIM1 AAb or CA125 positive.*

[b]*The frequency of OC patients with early stage compared to that with late stage.*

[c]*The frequency of OC patients with positive reaction to anti-PDLIM1 AAb compared with the frequency of healthy or ovarian benign controls with positive reaction to anti-PDLIM1 AAb.*

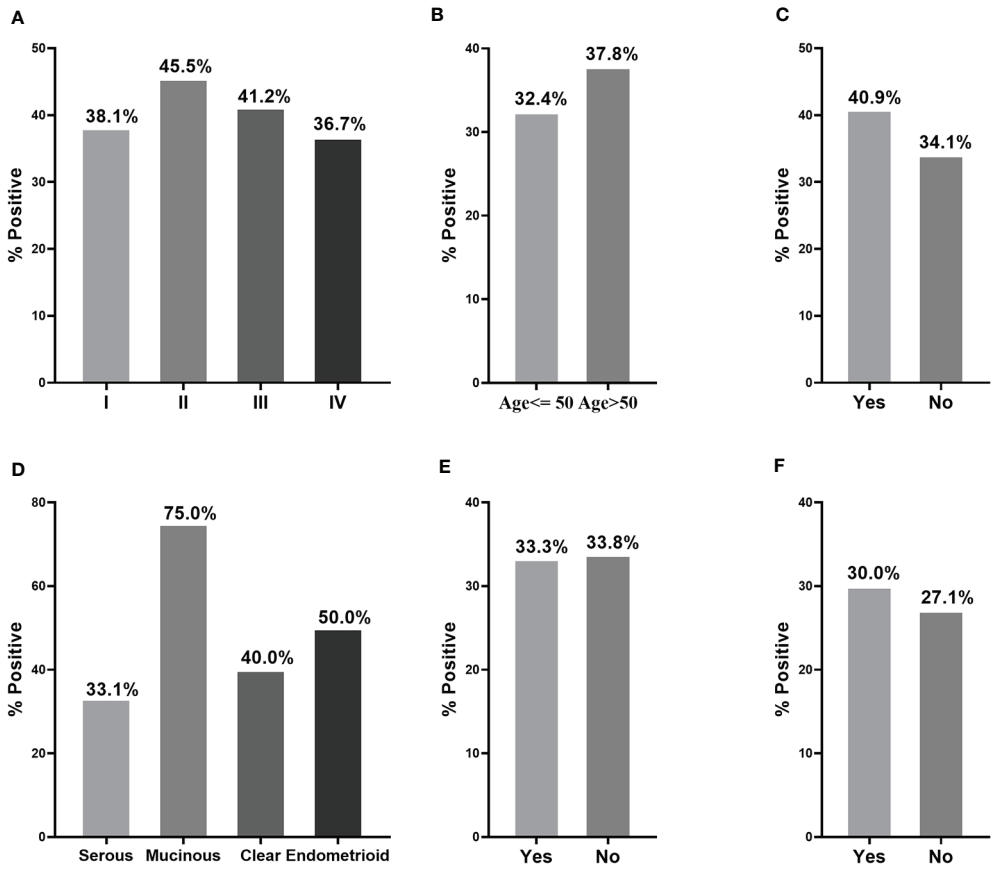

FIGURE 4 | The performance of anti-PDLIM1 AAb in different subgroups of OC patients. The frequency of anti-PDLIM1 AAb among subgroups of OC patients based on stage **(A)**, age **(B)**, family tumor history **(C)**, histological type **(D)**, lymph node metastasis **(E)**, and distant metastasis **(F)**.

validation datasets, and 0.757 for distinguishing OC from benign controls. It could identify 35.7% (65/182) of OC at the specificity of 90.1%. Even though we combined both healthy and benign controls, the AAb still has the AUC of 0.730 with the sensitivity of 31.9% and specificity of 90.1% to discriminate OC. Therefore, this study holds some advantages. First, we measured the expression of anti-PDLIM1 AAb not only in the sera of OC patients and healthy subjects but also in the sera of patients with ovarian benign diseases. Second, the design of two different groups of controls makes our results more dependable. Third, both training and validation datasets in which the sera were from different hospitals could further make a solid conclusion. Last but not least, since the elevated anti-PDLIM1 AAb in OC sera was in line with the high expression of PDLIM1 protein in OC tissue, it was speculated that strong immune response of PDLIM1 AAb in OC patients might be triggered by the high expression of PDLIM1 protein in OC tissues. Consequently, anti-PDLIM1 AAb has great potential as a serological marker of ovarian cancer. Further works are still needed to investigate its potential utility for clinical detection.

Moreover, PDLIM1 plays an important role in the process of tumorigenesis (14, 29, 30). It serves as an important regulator for breast cancer cell migration and metastasis, and the increased expression of PDLIM1 contributes to the progression of breast cancer (17). In addition, a study indicated that PDLIM1 could promote proliferation and suppress apoptosis of chronic myeloid leukemia cells, having an oncogenic role to chronic myeloid leukemia (29). Huang's study showed that PDLIM1 is significantly downregulated in the tissues of metastatic hepatocellular carcinoma (HCC), suggesting that PDLIM1 may be a potential prognostic marker for metastatic hepatocellular carcinoma (16). Ahn et al. reported that PDLIM1 with the interaction to neurotrophin receptor p75 as a mediator of glioma invasion could provide therapeutic strategies for patients with glioblastoma (31). From the IHC analysis in this study, the PDLIM1 protein was highly expressed in OC tissues, while it was not expressed in adjacent or normal ovarian tissues. Based on the aforementioned evidence as well as the results of our current study, we could confirm that PDLIM1 is closely associated with cancers, and thus it may be a tumor-associated antigen in ovarian cancer.

At present, the detection of AAbs attracted much attention to complement CA125 for the screening of women with ovarian cancer (32–34). Since multiplex detection could improve the sensitivity and specificity, a panel with multiple biomarkers was widely reported. A study reported that a panel with four

biomarkers [CA125, macrophage inhibitory factor (MIF), osteopontin (OPN), and anti-IL-8 autoantibodies] could identify 82.0% of OC with early stage compared to 65% with CA125 alone (35). It was demonstrated that the combination of CA125, anti-SBP1, and anti-TP53 greatly improved the sensitivity and specificity of OC identification with the AUC of 0.96 (36). From this study, the combination of anti-PDLIM1 AAb and CA125 could detect 79.2% patients with OC, which is statistically higher than when using CA125 or anti-PDLIM1 AAb alone. In addition, the novel AAb identified in our study could identify 15% (18/120) of patients with negative CA125. Therefore, our findings may provide a novel biomarker for OC detection as a complementary tool to CA125. However, there are limitations in this study. Firstly, the study is a case-control study; further validations in a prospective research work are required to confirm the diagnostic value of anti-PDLIM1 AAb for OC detection based on a large sample size. Secondly, anti-PDLIM1 AAb is not fully specific to OC; it was also detected in breast cancer and pancreatic cancer. Further works are needed to explore the mechanism of PDLIM1 in OC.

In summary, our findings indicated that anti-PDLIM1 AAb was elevated in OC patients compared with healthy controls, which was consistent with the high expression of its corresponding antigen in OC tissues. Anti-PDLIM1 AAb could distinguish OC patients from both healthy subjects and ovarian benign cases, and it showed a good performance especially when combined with CA125. Therefore, anti-PDLIM1 AAb may be used as a potential biomarker for OC detection, and it could improve the sensitivity in identifying OC by the combination with CA125.

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 the Ethics Committee of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XW: Design of the study, conceptualization, writing—review and editing. JZ: Writing—review and editing. CQ: Data collection and analysis, methodology, conducting study, writing—original draft. YD: Conducting study and writing—review and editing. BW: Visualization and writing—review and editing. JS: Statistical analysis. PW: Experiments. HY: Experiments. LD: Data analysis. All authors contributed to the article and approved the submitted version.

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Filamentous Bacteriophage—A Powerful Carrier for Glioma Therapy

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Glioma is a life-threatening malignant tumor. Resistance to traditional treatments and tumor recurrence present major challenges in treating and managing this disease, consequently, new therapeutic strategies must be developed. Crossing the blood-brain barrier (BBB) is another challenge for most drug vectors and therapy medications. Filamentous bacteriophage can enter the brain across the BBB. Compared to traditional drug vectors, phage-based drugs offer thermodynamic stability, biocompatibility, homogeneity, high carrying capacity, self-assembly, scalability, and low toxicity. Tumor-targeting peptides from phage library and phages displaying targeting peptides are ideal drug delivery agents. This review summarized recent studies on phage-based glioma therapy and shed light on the developing therapeutics phage in the personalized treatment of glioma.

Keywords: filamentous bacteriophage, glioma, target peptide, antibody, BBB, BBTB

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INTRODUCTION

Glioma is the most common cerebral malignancy with high morbidity and mortality. Despite the current treatment measures such as surgery, radiation, and chemotherapy, the prognosis and mortality of patients has not improved significantly (1–3). The annual death rate in China is as high as 30,000. Currently, the adverse reactions to glioma drugs are more prominent, and drug resistance is readily developed (1, 4, 5). To overcome the limits of existing therapies, there is a pressing need for a treatment strategy that can selectively target cancer tissues and avoid non-target tissues.

In addition, the blood-brain barrier (BBB) is a formidable obstacle for the transport of most administered therapeutics to the brain (6, 7), and most anti-tumor drugs have difficulty passing the BBB and the blood-brain tumor barrier (BBTB), it is a major hurdle in the development of targeted drugs for glioma (8–10). Therefore, choosing a carrier that can pass through the BBB is very important for glioma treatment.

Filamentous bacteriophages (Ff phage) are nano-scale viruses that infect bacteria and are not harmful to humans (11–13). Ff phage fd, M13, and f1 are stable under harsh conditions and can be manufactured with uniform specifications and low cost (14–17). As well, Ff phage has genetic flexibility. In 1985, Smith et al. reported phage display technology to display a variety of proteins, antibodies, and peptides on the phage coat proteins. Subsequently, phage display libraries were injected intravenously into laboratory animals to screen the targeting peptides (18). Moreover, Ff phage could enter the central nervous system (CNS) without visible toxic effects (19, 20), it can pass through the BBB as a drug carrier, when administered intranasally or through convection-enhanced delivery (CED), and has great research potential for the treatment of brain diseases (21–24).

Furthermore, the phage display library is used quickly and directly to screen peptides targeting tumor and anti-tumor antibodies. To date, there are numerous studies by the phage library screening tumor targeting peptides for target therapy and immunotherapy (25–27), and it has established the method for screening glioma targeting peptides across the BBB, guiding the immunotherapy in patients. These phages and peptides targeting glioma cells could avoid or reduce the toxic effects of anti-cancer drugs (28–31). At the same time, phages, carrying targeted peptides and antibodies, stimulate the immune response and play an immunotherapy role (28, 32–35).

In summary, this review will clarify the strategy for applying Ff phage nanoparticles to glioma treatment. It can be used to direct clinical treatment of tumors and provide new ideas for personalized disease therapy.

BIOPANNING THE TUMOR-TARGETED PEPTIDE

Ff phage is a biological nanomaterial with a length of about 1 um and a diameter of about 7 nm (14, 36). It could specifically infect bacteria and is present in the human body and harmless to humans. Ff phage is made of single-stranded circular DNA and coat proteins. The main coat protein pVIII is located on the phage side and minor coat proteins (pIII, pV I, pVII, and pIX) are located at both tips (**Figure 1**).

Phage display is to insert the DNA sequence of the exogenous peptide into the phage coat protein gene and express the peptide on the surface of the phage along with the expression of the coat protein. The phage displaying peptide still has protein assembly and infection activity (37–39). Based on phage display technology, phage libraries were built and used to select targeting phages, such as tumor-targeted peptides, which improved research efficiency and reduced costs. In addition, these targeted peptides developed functions of cell-targeting, tumor-homing, and cell-penetrating (40–42). Phage libraries were usually screened by using molecules, cells, and tissues *in vitro* or in animals and human patients (38, 43, 44).

Traditional chemotherapeutics have poor accuracy on tumor cells and are prone to adverse reactions. Therefore, the targeted therapy is particularly important for tumor therapy (45–47). Peptides specifically binding to tumor tissues, as carriers to direct drugs to tumor tissues, significantly improved the accuracy of drug targeting (48–51). Although monoclonal antibodies as vectors were successfully applied to anti-tumor, the high molecular weight of antibodies might reduce efficiency (52–55), while the phage peptide library has the benefits of screening for small molecular weight peptides, which can compensate for antibody deficiencies that are widely used in the diagnosis and treatment of glioma.

The screened peptides could combine with markers for imaging. Wang et al. developed an HO-8910 ovarian cancer cell targeting peptide (NPMIRRQ) from the phage library, which demonstrated the ability to selectively bind ovarian cancer cells using immunofluorescence and immunohistochemical assays (56).

The screened peptides could also couple with chemotherapeutic drugs or some gene, and then be used in the tumor-targeted treatment or gene treatment; Du et al. obtained the A54 peptide (AGKGTPSLETTP) by *in vivo* phage display for hepatocarcinoma and conjugated it with doxorubicin for *in vivo* targeted therapy. The study showed the A54-doxorubicin reduced the tumor size and prolongated the long-term survival rate (57).

Furthermore, some specific binding peptides that inhibit tumor growth, invasion, and metastasis, could be used to treat tumors directly. Zhou et al. isolated the peptide, SWQIGGN, from a Ph.D.-C7C phage library with the ovarian cancer cell HO-8910 (58). They found that the peptide controlled cancer cell migration, viability, adhesion capacity, invasion, and tumor growth *in vivo*.

Currently, the screening of tumor-targeted peptides is widely used in targeting the treatment of tumors, such as lung cancer, stomach cancer, liver cancer, colon cancer, and prostate cancer.

FF PHAGE, A TARGETED THERAPY VECTOR

Poor permeability of the cellular plasma membrane to a drug or gene is the main barrier for targeted delivery, while the nature of vectors affects the efficiency of drug delivery in tumors and tumor-affected tissues. Therefore, construction and selection of drug vectors is one of the most important steps of tumor therapy. Ff phage could deliver genes and peptides to mammalian cells, and the structure of Ff phage results in more efficient cellular attachment and ensuing membrane penetration. It has been

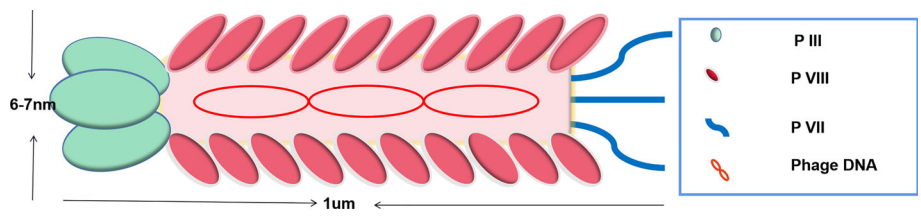

FIGURE 1 | Schematic of Ff phage. Phage consists of a tubular protein coat surrounding a single-stranded circular DNA. Proteins III and VII are the minor coat proteins, present in 3-5 copies. Protein VIII is the major phage coat protein and presents in 2700 copy numbers.

successfully used in treatment under the U.S. Food and Drug Administration (FDA) process (www.fda.gov), and methods for isolating, storing, and producing phages are now becoming more available and better developed under the ATCC (www.atcc.org) and PHE (www.gov.uk/government/organisations/public-health-england) collections.

Ff phage might be an ideal carrier for drug therapy and immunotherapy. First, an exogenous gene could be inserted into the Ff phage genome. Meanwhile, the peptide displayed on the Ff phage presents its natural conformation and the phage has a strong resistance to physical and chemical factors (59–61). Second, the phage displayed exogenous peptides or chemical modifications, which could combine with inorganic nanomaterials/drugs, to form phage-nanocomplexes and drug-loaded phages. It is well utilized in photodynamic cancer therapy (62–64). Some researchers used the Fd phage to display a cancer-targeting peptide on pVIII major coat protein, and then conjugated photosensitizer at the N-terminal end of the targeting peptides, and demonstrated that the complex of phage-photosensitizers was able to selectively target and kill SKBR3 tumor cells *in vitro* (65). Third, the displayed Ff phage triggers every arm of the immune response. Berardinis et al. engineered fd to target mouse dendritic cells (DCs), and activated the innate and adaptive responses without the need of exogenous adjuvants (66). The study has also shown that phage could induce the IL-2 and IFN-γ cytokines, which were useful in tumor immunotherapy (67). Fourth, drug conjugated phage increases the half-life in the blood steam (68), while the toxicity and side effects of hazardous drugs are reduced in combination with the Ff phage.

In a word, Ff phage, as a carrier of therapeutic reagents, has more advantages in targeted therapy, with high specificity, high sensitivity, and reproducibility.

THE APPLICATION OF PHAGE NANOMATERIALS TO GLIOMA THERAPY

Gliomas are aggressive brain tumors and challenging therapeutic cancers that have high mortality (69). The 5-year survival rate of glioma is very low (70), and the prognosis of glioblastoma patients is poor with a median survival of less than 1 year. Recently, cancer research in the U.K. showed that 40% of brain tumor patients survive their cancer for 1 year and more than 10% survive their cancer for 5 years or more.

At present, the clinical therapies for gliomas are surgical therapy, radiation therapy, chemotherapy, gene therapy, and other comprehensives (1, 71–74). However, it is easy to relapse after these treatments, and the patients' survival rates are not significantly improved. Immunotherapy is useful for treating tumors, the mAbs bevacizumab, rituximab, and trastuzumab were already widely used against tumors outside the brain (75–79). But the current immunotherapy for medical glioma is costly and inefficient.

BBB is another significant barrier to the delivery of targeted treatments for brain tumors. Indeed, more than 98% of low-molecular-weight candidate drugs and almost 100% of large therapeutic candidate drugs cannot cross the BBB (80). There is an urgent need for a carrier that carries drugs across the BBB. Phage display technology can be used for the construction of peptide libraries to screen for glioma tumor-targeting peptides and peptides across the BBB. Surface functionalization with these peptides is a sophisticated way to develop drug delivery platforms that cross the BBB and target glioma.

Identification of Targeting Peptide and Antibody

About 30% of all human antibody therapies are derived from phage antibody libraries. In addition, the screening of phage display libraries is an effective tool to obtain peptides that target glioma tumors both *in vitro* and *in vivo* (43, 49, 81, 82).

In Vitro Panning

In vitro panning was used to identify peptides that specifically bind to glioma cells and proteins. Ho et al. isolated GL1 peptide that specifically interacts with primary glioma cells obtained from human biopsy specimens using a phage library and injected the GL1-bearing phages into a mouse (83). They found that the phage targeted the mouse brain tumor and this peptide had the potential to be used for therapeutics to glioma cells.

Glioma stem cells (GSCs) are the major drivers of brain tumors. Beck et al. screened the peptides binding to GSCs from the phage display library, and the administration of GSC-homing peptide into the glioma mice model resulted in penetration into the brain and specific accumulation in glioma. CD133 is a cell surface antigen allowing identification of GBMs. Yoon et al. screened the peptides targeted CD133 from U373 glioma cells, using the phage library, and conjugated the targeting peptide (CBP4) to GNPs. They found that the targeting peptide was effective for passage into the brain extracellular space (84). The protein kinase C (PKC) family plays an important role in glioma, is a potential biomarker to disturb the expression of CD133 on glioma cells, and may have a therapeutic effect on GSCs. Yoon et al. also identified 12-amino-acid peptide-binding toward the PKCd catalytic domain through a phage display library and certificated that the peptide could target and inhibit PKC, provided a novel peptide sequence for a therapeutic strategy to target GSCs. To identify novel peptides targeting malignant gliomas, Wang et al. used a 12-mer peptide phage display library and obtained the peptide (VTWTPQAWFQWV) bound to U87MG cells. In addition, the VTW phage is bound strongly to other human glioma cell lines, including H4, SW1088, and SW1783 (85–87).

The discovery and isolation of antibodies are important for the treatment of glioblastoma (GBM). Insulin-like growth factor binding protein 2 (IGFBP2) is highly upregulated in GBM tissues and plays a crucial role in the invasion of glioma cells. Kondaiah et al. screened scFv phage display libraries using recombinant IGFBP2 and identified that scFv B7J could bind to IGFBP2 and inhibit the migration and invasion of glioma cells (88). Tumor sphere cells more closely resemble the phenotype of primary tumors than do serum-cultured cell lines. Liu et al. derived GBM

tumorspheres from human brain tumor specimens, biopanning the scFvs that bind to CD133 positive GBM tumorsphere cells from scFvs phage library and indicated one scFvs could inhibit the growth of the GBM tumorsphere cells *in vitro*.

Overall, peptides targeting glioma were identified using phage display library *in vitro*. It is useful for further development of novel therapies that target glioma cells and provide novel diagnostic and therapeutic modalities for human brain malignancies.

In Vivo Pannings

In vivo pannings were successful in obtaining organ-specific targeting peptides in the animal model. Peptides and antibodies may be isolated, which recognize subsets of glioma tumors *via in vivo* biopanning of phage display libraries in glioma xenografts.

GBM displays cellular hierarchies with self-renewing glioma-initiating cells (GICs) at the apex. To discover new GIC targets Rich et al. delivered a phage peptide library intravenously to a GBM xenograft *in vivo*, then derived GICs, and then identified the peptides targeting VAV3 and CD97. These peptides could be used for identifying and targeting of GICs (89),

Additional destruction of existing tumor vasculature effectively deprives tumors from blood. With the need to identify novel tumor vascular targeting agents, Lith et al. identified a nanobody C-C7 *in vivo* biopanning of phage display library in an orthotopic mouse model of diffuse glioma, which showed that C-C7 recognized a subpopulation of tumor blood vessels in glioma xenografts and clinical glioma samples (90). Leenders et al. cloned a nanobody phage library from lymphocytes of a llama, which had been immunized with clinical glioma tissue and isolated the nanobodies discriminated incorporated pre-existent vessels in highly infiltrative cerebral E434 xenografts from normal brain vessels *via* biopanning *in vivo* with this library in the orthotopic glioma xenograft models (91).

In vivo biopanning, in appropriate animal models, is a very promising approach for future identifying novel molecular tools for targeting glioma tumors and oncogenic pathways preferentially activated within the tumor hierarchy, which could offer a new strategy for the development of glioma therapy.

Development of Carriers for Targeted Drug Delivery

The BBB and BBTB restrict the entry of drugs given routinely with glioma (92, 93). Thus, effective glioma treatment requires therapeutic agents to penetrate both BBB and BBTB. An emerging solution consists of identifying the peptide vectors that penetrate the BBB/BBTB.

In recent years, numerous studies have focused on modifying the pharmacokinetics of chemotherapeutic drugs by using a delivery vector or by adding targeting properties. Langel et al. developed a tumor-targeted delivery vector gHoPe2 that is based on a cell-penetrating peptide pVEC and a novel glioma-targeting peptide sequence gHo (NHQQQNPHQPPM), which was identified using phage display technology. The vector could be efficiently absorbed into glioma cells and xenograft glioma tumors in a mouse model. In addition, vectored doxorubicin was more effective than free drug in a mouse glioma xenograft model (94). The study demonstrated the general feasibility of the current approach for constructing targeted delivery systems based on the cell-penetrating peptides.

The BBTB is formed by brain tumor capillaries and comprises a barrier that is variably distinct from the BBB, forming an additional hurdle toward treatment. Lin et al. identified a novel BBB/BBTB-penetrating peptide M1 (TFYGGRPKRNNFLRGIR) from the phage displayed peptide library *in vivo* and modified the M1 peptide with a tumor-targeting named M1-RGD (TFYGGRPKRNNFLRGIRRGD), then they conjugated the MI-RGD with drug and applied PDC M1-RGD-PTX to treat glioma and found that it suppressed glioma proliferation and thus extended mouse survival in a glioma xenograft model (95). The study suggested that the peptide M1 could serve as a vector through the BBB and BBTB.

Therefore, the targeting peptides screening from the phage library are effective drug carriers across the BBB and BBTB, and phage display technology has wide applications for treating brain tumors.

FF PHAGE: A POTENTIAL THERAPEUTIC VEHICLE FOR GLIOMAS

The rod-shaped nanoparticles have higher avidity and selectivity for endothelial cells and increase the specificity and vascular targeting for brain endothelium (96). Increasing the length-to-diameter ratio of Ff phage results in more effective cellular attachment and ensuing membrane penetration. The phage maintains the biological activity of the peptide displayed on the phage vector, these properties make Ff phage suitable for use as a vector in the treatment of central nervous diseases (97), and research proved that phages carrying antibodies effectively label Aβ plaques is an efficient and nontoxic delivery vector to the brain and is useful for the treatment of Alzheimer's disease *in vivo* (68).

Ff Phage Could Deliver the Drug to the CNS

The function of BBB is under normal in low-grade glioma (98), Ff phage can pass the BBB and deliver therapeutics directly to the CNS when administered intranasally. It has been applied on protein-based treatments for other drug abuse syndromes. Janda et al. demonstrated that Ff phage displaying cocaine-binding proteins sequester cocaine in the brain and blocked the psychoactive effects of cocaine administered intranasally (23). Additionally, Ff phages have been reported to possess anti-tumorigenic properties. The researchers found that Ff phages could even inhibit the growth of subcutaneous GBM tumors in mice and this activity was mediated in part by lipopolysaccharide molecules attached to virion using the intranasal route.

Convection-enhanced delivery (CED) is a novel approach for administering chemotherapy in patients with brain tumors (99, 100). Additionally, CED is also an effective and safe method for

distributing M13 phage to the brain (97). It reminds us that Ff phage could deliver the medicine to glioma *via* CED.

Normal vascular function is disturbed in high-grade glioma and Ff phage has more capacity to cross the BBB through various routes. Based on Ff phage, a functional dual vector could target and treat glioma intravenously. Hajitou et al. have designed a hybrid AAV/phage with a recombinant adeno-associated virus genome (rAAV) and the capsid of M13 phage as a vector for dual targeting of therapeutic genes to glioblastoma. The phage capsid displayed the RGD4C ligand that binds the αvβ3 integrin receptor and the recombinant rAAV genes expressed from a tumor-activated and temozolomide (TMZ)-induced promoter of the glucose-regulated protein, Grp78 (101). The recombinant vector targeted intracranial tumors in mice following intravenous administration and the gene delivered was expressed in human GBM cells. The construction of a double display Ff phage system was also reported. Sandlie and his team also developed a P III/P VII phage-genome double display system that could simultaneously carry two different exogenous peptides to perform different biological functions (102), and we can infer that the double display phage displayed targeting peptide and antibody could apply for treating glioma.

Taken together, Ff phages have the anti-tumor capability and could be genetically modified to display tumor homing motifs and conjugated to cytotoxic drugs. These phages are harmless when administered intranasally, CED, or intravenously and may present route anti-tumorigenic. Using them as vectors could be useful in the treatment of glioma.

Future Prospects for Personalized Therapy

Glioma is a highly heterogeneous disease with major molecular differences in the expression of tumor cell surface markers in patients with the same grade of cancer (103). Currently, drugs used for glioma are often toxic to normal cells, resulting in serious side effects (104–106), and the broad range of drugs should be improved, as glioma cells are also prone to drug resistance (107, 108) Therefore, personalized therapy is very critical for gliomas.

Extensive research has used the phage display library to identify tumor-specific ligands by panning established tumor cell lines *in vitro* or by panning in an animal model. However, the material derived from the patient has more advantages of clinical relevance. It is tolerable in the human body, several groups have injected Ff phage library into patients without obvious side effects, and it is highly successful to develop a protocol for selecting phage displayed ligands in patients (109, 110). Shukla et al. conducted the toxicity profiles of different doses and phage displayed library formats for cancer patients (111). Then, they obtained and evaluated the tumor-homing phage-antibodies and derived soluble scFv antibodies to patients' tumors and found that these antibodies were cancer-specific (112). Moreover, Ff phages are stable. They retain infectivity after IV injection and circulation in the human body. These studies remind us that phage display technology can be used to identify tumor-specific ligands to develop personalized therapy.

Therefore, in theory, phage display strategies can achieve success when applied to target glioma cells for personalized treatments. Because of the specificity of biomarkers, glioma patients could be administered using phage display libraries and profiled for the presence of cancer targets before treatment. Such cancer-specific peptides can also be obtained from individual cancer patients *in vitro* and then be designed to target cancer treatment for personalized treatments (**Figure 2**).

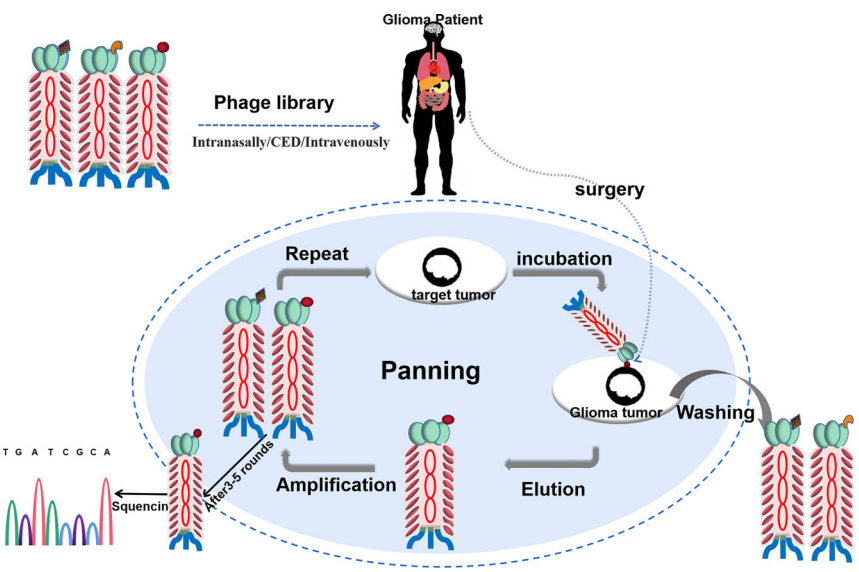

FIGURE 2 | Schematic of affinity-selection of targets-binding phages from a phage library for personalized therapy of glioma. An antibody phage library is administered in glioma patients. After incubation, get the tumor from the patient. The unbound phages are washed and the bound phages are collected and amplified. After 3-5 rounds, the affinitive phages are enriched and sequenced.

CONCLUSION

Gliomas are the most common primary brain tumors. Effective treatment of glioma is hampered by the presence of both BBB and BBTB. In this review, we presented an Ff phage approach to enhance the permeability of drugs through BBB and BBTB.

Although Ff phages have the problem of further optimization and improvement in separation and purification, they also have a number of advantages. Ff phage has a greater level of safety, it is not reproduce naturally in mammalian hosts, and it expresses a wide range of peptides on coat proteins using genetic engineering techniques to attach targeting peptides and antibodies.

Phage display is a high throughput screening strategy to construct peptide libraries that are used to screen glioma targeting peptides. These peptides might cross the BBB/BBTB and target tumors. It can also be used as a drug or drug carrier after being modified. Furthermore, Ff phage is an ideal transport carrier to CNS across the BBB, it has the anti-tumor ability and could be genetically modified to display glioma homing motifs and conjugated to cytotoxic drugs. Moreover, Ff phage displaying targeting peptide has stronger tumor penetrating ability, a higher load of drug delivery ability, and lower toxicity.

Developing carrier-based Ff phage as a drug delivery system can solve the problem of going through the BBB and BBTB. In short, Ff phage display technology is a powerful method of developing highly effective target drug delivery carriers. In addition, it opens the door to the development of personalized therapy agents in the future.

AUTHOR CONTRIBUTIONS

YW: Conceptualization; JS: Methodology and analysis; JC: Data Curation and analysis; CZ: Resources; XL: Visualization; WY: Writing; RC: Supervision; TG: Writing-Reviewing and Editing.

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Identification and Evaluation of Autoantibody to a Novel Tumor-Associated Antigen GNA11 as a Biomarker in Esophageal Squamous Cell Carcinoma

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The study aims to explore the diagnostic value of anti-GNA11 autoantibody in esophageal squamous cell carcinoma (ESCC) from multiple levels. Autoantibody against GNA11 with the highest diagnostic performance was screened out from the customized protein microarray. A total of 486 subjects including ESCC patients and matched normal controls were recruited in the verification and validation phases by using enzyme-linked immunosorbent assay (ELISA). Western blotting analysis was used to verify the ELISA results. Immunohistochemistry (IHC) was used to evaluate GNA11 expression in ESCC tissues and para-tumor tissues. In addition, a bioinformatics approach was adopted to investigate the mRNA expression of GNA11 in ESCC. Results indicated that the level of anti-GNA11 autoantibody in ESCC patients was significantly higher than that in the normal controls, and it can be used to distinguish ESCC patients from normal individuals in clinical subgroups ($p < 0.05$), as revealed by both ELISA and Western blotting. The receiver operating characteristic (ROC) curve analysis showed that anti-GNA11 autoantibody could distinguish ESCC patients from normal controls with an area under the ROC curve (AUC) of 0.653, sensitivity of 10.96%, and specificity of 98.63% in the verification cohort and with an AUC of 0.751, sensitivity of 38.24%, and specificity of 88.82% in the validation cohort. IHC manifested that the expression of GNA11 can differentiate ESCC tissues with para-tumor tissues ($p < 0.05$), but it cannot be used to differentiate different pathological grades and clinical stages ($p > 0.05$). The mRNA expression of GNA11 in ESCC patients and normal controls was different with a bioinformatics mining with The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) data in Gene Expression Profiling

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Interactive Analysis (GEPIA). In summary, anti-GNA11 autoantibody has the potential to be a new serological marker in the diagnosis of ESCC.

Keywords: GNA11, tumor-associated antigen, autoantibody, immunodiagnosis, esophageal squamous cell carcinoma

INTRODUCTION

Esophageal cancer (EC) is one of the common malignant tumors that threaten the health of human beings, with its incidence ranking seventh and cancer-related death ranking sixth worldwide (1). Studies estimated that there were 572,000 new cases and 508,600 deaths of EC around the world in 2018 (2). The incidence of EC in China is among the top 5 in the world (1). It was estimated that there were 246,000 new cases and 188,000 deaths of EC in 2015, which was the fourth leading cause of cancer-related death in China (3). Esophageal squamous cell carcinoma (ESCC) accounts for more than 90% of EC in China, which is a severe healthcare burden (4).

The 5-year overall survival rate of EC is less than 20%, and the prognosis is inferior (5, 6). The poor prognosis of EC is mainly due to the lack of clinical symptoms in patients in the early stage and the paucity of reliable non-invasive testing methods. However, relevant studies pointed out that early diagnosis of EC can significantly improve its poor prognosis, with a 5-year survival rate reaching as high as 80%–90% (7, 8). The standard diagnostic methods for EC screening include endoscopy and pathological biopsy, which are expensive and invasive. Consequently, a critically unmet need in the diagnosis and management of EC is identifying and developing novel non-invasive biomarkers that can complement the traditional diagnostic methods. Researchers have suggested that autoantibodies against tumor-associated antigens (TAAs) could be used as diagnostic biomarkers for the early diagnosis of cancer. These anti-TAA autoantibodies are stable in the circulating blood and can be produced as early as several years before the appearance of clinical symptoms (9–12). Currently, there are a lot of autoantibodies against TAAs that have been used in the early diagnosis of ESCC, such as FOXP3 (13), Fascin (14), Ezrin (15), STIP1 (16), LY6K (17), and MMP7 (18). However, the sensitivity and specificity of these anti-TAA autoantibodies cannot meet the needs of the clinical diagnosis of ESCC as biomarkers. Hence, it is of great importance to identify additional biomarkers with high specificity and sensitivity for the diagnosis of ESCC.

Heterotrimeric G proteins are widely expressed in all eukaryotic cells consisting of three subunits: alpha, beta, and gamma. GNA11 is the alpha subunit of G protein and is involved in a variety of transmembrane signaling systems. Our previous study indicated that GNA11 was involved in the development of hepatocellular carcinoma (HCC) and might be a potential biomarker in HCC detection (19). Our previous study also found that anti-GNA11 autoantibody had the potential to diagnose ESCC when we established a diagnostic model of an autoantibody panel to screen ESCC (20). *GNA11* gene was frequently mutated in the conventional esophageal adenocarcinoma, and the mutation was related to critical cellular pathways including PI3K, RAS, and MAPK, which suggested that GNA11 mutation might be tightly linked to the occurrence of EC (21). In the current study, in order to evaluate the potential of anti-GNA11 autoantibody in the diagnosis of ESCC, the level of anti-GNA11 autoantibody in sera of ESCC patients and matched normal controls was detected by ELISA, and the protein level and mRNA level of GNA11 were further explored by immunohistochemistry (IHC) and bioinformatics. The overall design of the current study is shown in **Figure 1**.

MATERIALS AND METHODS

Serum Samples

Serum samples of 333 ESCC patients and 293 normal controls were used in the study. From July 2017 to October 2018, sera from ESCC patients were collected from a third-level grade A hospital in Henan Province, China. Normal control sera were derived from the biological specimen bank in Henan Key Laboratory of Tumor Epidemiology. All patients underwent pathological examination to confirm that they have not received any treatment prior to collecting blood, and none of the controls had autoimmune diseases or tumor-related diseases. Sera from 90 ESCC patients and 50 normal controls were selected for protein microarray assay, and 486 sera from 243 ESCC patients and 243 normal controls were used for ELISA. **Table 1** shows the detailed clinical information of all participants. The peripheral blood of all subjects under fasting state was collected at 5 ml and placed into vacuum tube without anticoagulants. The collected whole blood samples were centrifuged at 3,000 rpm for 10 min after standing for 1 h at room temperature and then stored at −80°C. This study has been approved by the Ethics Committee of Zhengzhou University, and all the subjects had signed informed consent.

Protein Microarray

The study was authorized by Guangzhou Bochong Biotechnology Co., Ltd., to make focused array protein microarray including 154 recombinant human proteins or protein fragments (CDI lab), which included 143 proteins or protein fragments encoded by 138 cancer driver genes and 11 proteins with high diagnostic value in our previous studies containing IMP1, IMP2, IMP3, CyclinB1, c-Myc, CIP2A/p90, RalA, YWHAZ, RBM39, and two fragments of Survivin. Protein microarray was used to detect the expression levels of corresponding autoantibodies in 140 serums to screen out significant TAAbs associated with ESCC. To eliminate the

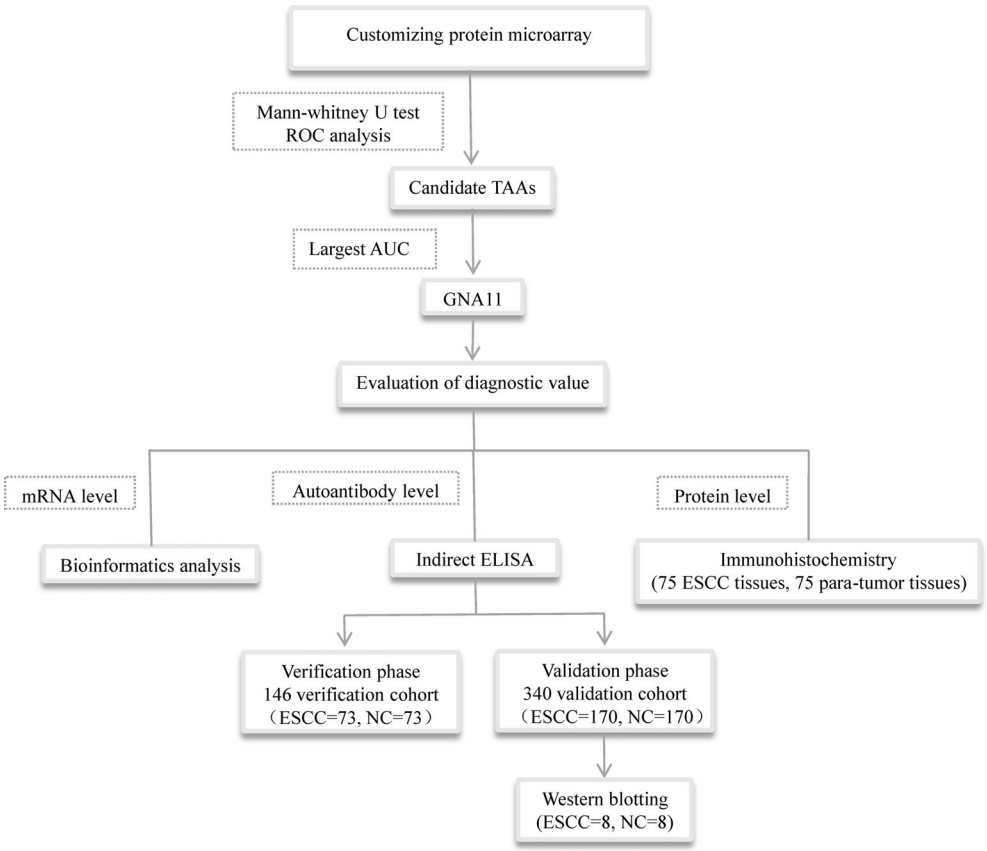

FIGURE 1 | The overall design of the study. TAAs, tumor-associated antigens; ELISA, enzyme-linked immunosorbent assay.

bias brought by the difference of background values, signal-to-noise ratio (SNR) was defined as F median/B median, where F532 Median refers to the median of the foreground value of signal points in the 532-nm channel and B532 Median refers to the median of the background value of signal points in the 532-nm channel. The detailed experimental procedure was performed exactly as the standard protocol described in our previous study (22).

Enzyme-Linked Immunosorbent Assay

The level of anti-GNA11 autoantibody in sera of ESCC patients and normal controls was detected by ELISA. Purified recombinant protein GNA11 was purchased from the LD Biopharma Company (San Diego, USA). Horseradish peroxidase (HRP)-conjugated mouse anti-human IgG (Wuhan Aoko Biotechnology Co. Ltd.) was used as the secondary antibody. Each ELISA plate included eight standard concentrations of 10, 20, 50, 100, 150, 200, 250, and 300 ng/ml of IgG (Solarbio); a positive control; a negative control; and a blank control, which enabled the stability of all the plates. The detailed operation of ELISA was described in our previous study (23). The optical density (OD) of each well was measured at 450 and 620 nm by using a microplate reader (Thermo Fisher Scientific).

Western Blotting

The positive and negative sera of anti-GNA11 autoantibody found by ELISA were randomly selected and detected by Western blotting to verify the immunoreactivity of the sera. The detailed procedure of Western blotting was described in our previous study (24). Briefly, purified recombinant protein GNA11 was electrophoresed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred onto nitrocellulose membrane. Selected serum samples diluted at 1:100 and HRP-conjugated mouse anti-human IgG antibody with a dilution of 1:5,000 were utilized as the primary antibody and the secondary antibody, respectively. The positive reaction signal was obtained by Azure Biosystems with chemiluminescence (C300–C600) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical analysis was performed to detect the expression level of GNA11 protein in 75 ESCC tissues and 75

TABLE 1 | Characteristics of study participants.

Variables	Verification phase (n = 146)		Validation phase (n = 340)	
	ESCC	NC	ESCC	NC
Number	73	73	170	170
Gender				
Male, n (%)	46 (63.01)	46 (63.01)	117 (68.82)	117 (68.82)
Female, n (%)	27 (36.99)	27 (36.99)	53 (31.18)	53 (31.18)
Mean age ± SD (years)	63.8 ± 9.1	63.6 ± 9.0	63.8 ± 8.1	64.8 ± 8.3
Age range (years)	41–82	41–82	42–88	45–88
Tumor site				
Upper thorax	9 (12.33)		25 (14.71)	
Middle thorax	39 (53.42)		51 (30.00)	
Lower thorax	24 (32.88)		37 (21.76)	
Unknown	1 (1.37)		57 (33.53)	
Family tumor history				
Yes	22 (30.14)		27 (15.9)	
No	49 (67.12)		45 (26.5)	
Unknown	2 (2.74)		98 (57.6)	
Histological grade				
High	2 (2.74)		4 (2.35)	
Medium	23 (31.51)		48 (28.24)	
Low	22 (30.14)		35 (20.59)	
Unknown	26 (35.61)		83 (48.82)	
TNM stage				
I	14 (19.18)		60 (35.29)	
II	17 (23.29)		21 (12.35)	
III	28 (38.36)		7 (4.12)	
IV	8 (10.96)		4 (2.35)	
Unknown	6 (8.21)		78 (45.89)	
Lymph node metastasis				
Positive	42 (57.53)		25 (14.71)	
Negative	27 (36.99)		51 (30.00)	
Unknown	4 (5.48)		94 (55.29)	
Distant metastasis				
Yes	8 (10.96)		3 (1.76)	
No	60 (82.19)		62 (36.47)	
Unknown	5 (6.85)		105 (61.77)	

ESCC, esophageal squamous cell carcinoma; NC, normal control.

corresponding para-tumor tissues. Tissue microarray (TMA; CGT No. HEso-Squ150CS-02) and the specific operations were provided by Shanghai Outdo Biotech Co. Ltd. Mouse monoclonal anti-GNA11 antibody was used as the first antibody. Biotin-labeled secondary antibody and diaminobenzidine (DAB) were used as detecting reagents. All the TMA results were evaluated by two independent pathologists without knowing the tumor stage of the TMA sections. Ten fields were randomly selected under the microscope for each microarray, and the score was calculated according to the percentage of positive cells (area score) and staining intensity (color score). There were five scoring conditions for the percentage of stained cells in a cell count, for example, score 0 (less than 10%), 1 (10%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (more than 75%). Staining intensity incorporated four different evaluation criteria: negative, score 0; faint yellow, score 1; brown yellow, score 2; and medium brown, score 3. The final score was attained by multiplying the score of the percentage of positive cells by the staining intensity score, which was negative score 0, weakly positive score 1–4, positive score 5–8, and strongly positive score 9–12.

Bioinformatics Analysis

The expression of GNA11 at the mRNA level was further explored in Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/). The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) normal data were chosen to be compared in esophageal carcinoma (ESCA) patients about the difference of GNA11 expression at the mRNA level. RNA sequencing expression data of ESCC were according to TCGA dataset. And the use of GEPIA was precisely based on the report from literature (25).

Statistical Analysis

IBM statistical software (version 25.0) and GraphPad Prism 8.0 were utilized in the study. All statistical analysis processes were two-tailed tests, and the test level was set as $\alpha = 0.05$. The non-parametric test was applied to compare the difference of anti-GNA11 autoantibody between ESCC patients and normal controls. The frequency of anti-GNA11 autoantibody in different clinical parameters and demographic characteristics in all ESCC patients was analyzed by chi-square test. One-way ANOVA was used to analyze IHC scores in different tissue types. The receiver operating characteristic (ROC) curve was performed to estimate the diagnostic ability of the biomarker in different groups and clinical subgroups. At the same time, Youden's index, positive likelihood ratio (PLR), negative likelihood ratio (NLR), positive predictive value (PPV), negative predictive value (NPV), and accuracy were also calculated to appraise the better ability of anti-GNA11 autoantibody in differentiating ESCC patients from normal controls. The area under the ROC curve (AUC) values of different clinical subgroups were analyzed by DeLong test. The cutoff value was set as mean value plus one SD of the normal control OD values (mean ± SD).

RESULTS

Autoantibody to a Novel Tumor-Associated Antigen Was Found in Sera of Esophageal Squamous Cell Carcinoma Patients by Protein Microarray Technology

In the present study, 86 sera from ESCC patients and 50 control sera were finally selected to evaluate the autoantibody levels with protein microarray. Interestingly, five candidate anti-TAA autoantibodies corresponding to P53, PTEN, GNA11, GNAS, and SRSF2 were identified by the Mann–Whitney U test and ROC analysis (20). In the ESCC group and normal control group, the positive rates of five anti-TAA autoantibodies ranged from 16.28% to 34.88% and 8.16% to 16.33%, respectively. The AUC values of five autoantibodies in diagnosing ESCC ranged from 0.606 to 0.682. The AUC value of anti-GNA11 autoantibody was the highest (0.682, 95% CI: 0.588–0.776) (**Figure 2B**) with sensitivity of 17.44% and specificity of 91.84%. The positive rate of anti-GNA11 autoantibody in the ESCC group and normal control group was 17.44% and 8.16%, respectively. The scatter plot showed that

the level of anti-GNA11 autoantibody in the ESCC group was obviously higher than that in the normal control group ($p < 0.05$) (**Figure 2A**). It indicated that anti-GNA11 autoantibody has the potential to distinguish ESCC patients from normal controls. To verify the hypothesis, the performance of anti-GNA11 autoantibody was further explored in cohorts with a large sample size.

Validating Diagnostic Value of Anti-GNA11 Autoantibody in Sera of Esophageal Squamous Cell Carcinoma Patients and Normal Controls

To further validate the efficacy of anti-GNA11 autoantibody in the immunodiagnosis of ESCC, sera from 243 ESCC patients and 243 normal controls were used in ELISA. All 486 serum samples were divided into two cohorts according to the ratio of 3:7. In the verification cohort (73 ESCC *vs*. 73 NC), a higher expression level of anti-GNA11 autoantibody was observed in ESCC patients (mean ± SD: 0.301 ± 0.054) compared with normal controls (mean ± SD: 0.277 ± 0.049) (**Figure 3A**); and anti-GNA11 autoantibody can distinguish 10.96% of ESCC patients with an AUC of 0.653 at the specificity of 98.63% (**Figure 3B**). A validation cohort of larger sample size (170 ESCC *vs*. 170 NC) was used to further confirm the diagnostic value of anti-GNA11 autoantibody. In this validation cohort, the level of anti-GNA11 autoantibody was distinctly higher in the ESCC group (mean ± SD: 0.345 ± 0.068) than that in the normal control group (mean ± SD: 0.281 ± 0.079) (**Figure 3C**), and the AUC value was as high as 0.751 with sensitivity of 38.24% and specificity of 88.82% (**Figure 3D**). The detailed results are shown in **Table 2**.

Additionally, eight ESCC and eight normal control sera were randomly selected from ESCC patients and normal controls for Western blotting analysis to further confirm the results of ELISA. The molecular weight of GNA11 protein is 45.8 kDa. Results indicated that six of eight ESCC sera showed positive bands with molecular weight near 45 kDa, and none of the eight control sera showed a positive band in the corresponding 45 kDa position. The results of Western blotting are shown in **Figure 4** and were consistent with those of ELISA.

The Effect of Anti-GNA11 Autoantibody in Different Clinicopathologic Characteristics of Esophageal Squamous Cell Carcinoma

The performance of anti-GNA11 autoantibody in clinical variables (including lymphatic metastasis, differentiation, distance metastasis, TNM stage, family tumor history, gender, and age) was further explored. Anti-GNA11 autoantibody could significantly distinguish ESCC patients from normal controls in every subgroup ($p < 0.05$) (**Figures 5A–N**). The AUC values of clinical subgroups ranged from 0.661 to 0.774 ($p < 0.05$). The maximum AUC of 0.774 was observed among female ESCC patients (**Figure 5L**), and the minimum AUC of 0.661 was detected in ESCC patients with TNM stage III and IV (**Figure 5H**). The AUC values in comparison groups regarding lymphatic metastasis, differentiation, distance metastasis, TNM stage, family tumor history, and age was not significantly different ($p > 0.05$), yet it presented a marginal difference in gender ($p = 0.05$). In addition, there were no differences in the positive rates of anti-GNA11 autoantibody in all subgroups ($p > 0.05$) (**Table 3**).

The Expression Level of mRNA and Protein of GNA11

As shown in **Figure 6**, the mRNA level of GNA11 was higher (fold change > 1.4, $p < 0.05$) in ESCC patients compared with normal controls based on TCGA and GTEx data in GEPIA, which was consistent with the difference in serum level of anti-GNA11 autoantibody between ESCC patients and normal controls.

Furthermore, the expression of GNA11 at the protein level was investigated in the Human Protein Altas (HPA; https://www.proteinatlas.org/). However, there was no prior evidence on the

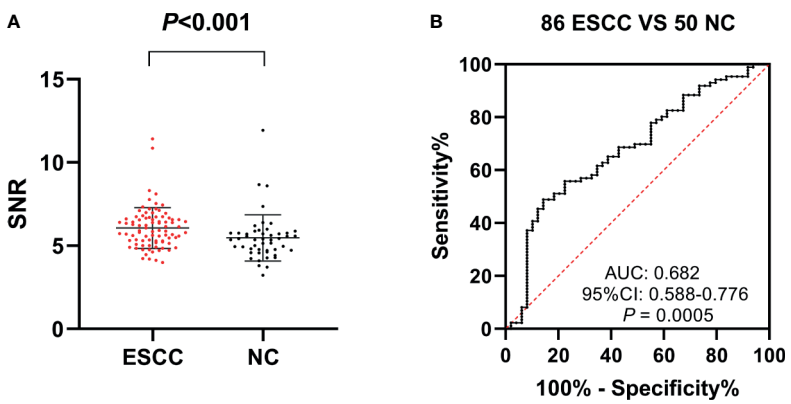

FIGURE 2 | The expression level and diagnostic value of anti-GNA11 autoantibody in ESCC patients and normal controls in screening phase. **(A)** The scatter plot depicts the level of anti-GNA11 autoantibody in ESCC group and normal control group. **(B)** The receiver operating characteristic curve of anti-GNA11 autoantibody in the screening stage. ESCC, esophageal squamous cell carcinoma; NC, normal controls; SNR, signal-to-noise ratio.

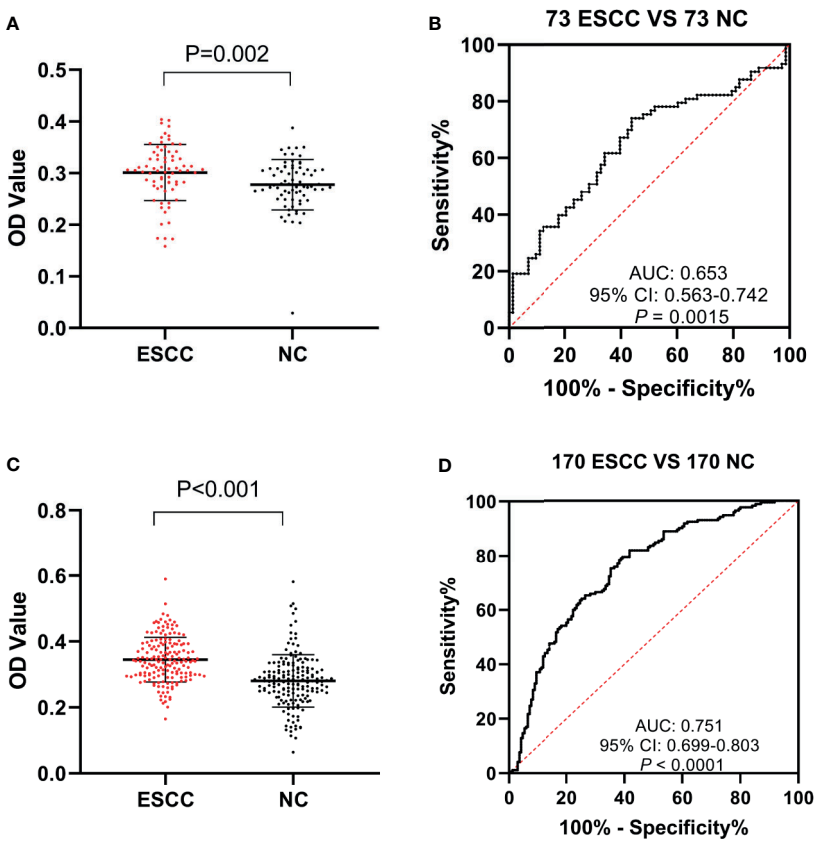

FIGURE 3 | The expression level and diagnostic value of anti-GNA11 autoantibody in the phases of verification and validation. **(A, C)** The expression level of anti-GNA11 autoantibody in ESCC patients and normal controls in verification cohort and validation phase. **(B, D)** ROC analysis of anti-GNA11 autoantibody in the verification and validation phase. ESCC, esophageal squamous cell carcinoma; NC, normal controls; ROC, receiver operating characteristic.

expression of GNA11 at protein in ESCC tissues (**Supplementary Figure 1**). Therefore, IHC was employed to further verify the expression of GNA11 at the protein level in ESCC tissues and para-tumor tissues. TMA slides including 75 ESCC tissue samples and corresponding para-tumor tissues were commercially acquired for this study. The clinical information of 75 ESCC patients including American Joint Committee on Cancer (AJCC) clinical stage and pathological grade was analyzed to explore the association between GNA11 expression and tumor stage. The expression level of GNA11 in tumor tissues and para-tumor tissues was evaluated by staining intensity (color score) and the percentage of positive cells (area score).

Results showed that GNA11 was overexpressed in ESCC tissues compared with para-tumor tissues (final score 1.57 *vs.* final score 0.14, $p < 0.05$) (**Table 4**). The color scores in ESCC tissues were significantly higher than those in para-tumor tissues, whereas the area scores were similar in different groups. In addition, the expression of GNA11 protein was distinctly higher in ESCC patients of pathological grade III compared with pathological grade I and II patients in color score ($p < 0.05$). The area score showed boundary difference in ESCC patients of clinical stage 1–2 compared with clinical stage 3–4 ($p = 0.05$). And the difference in final score was only observed in ESCC patients of pathological grade I compared with pathological grade III ($p < 0.05$). The color score and final score in ESCC patients with clinical stage 3–4 were also higher than those in clinical stage 1–2 patients ($p < 0.05$). **Figure 7** includes representative immunostaining for GNA11 protein in ESCC tissues and corresponding para-tumor tissues.

TABLE 2 | The diagnostic value of anti-GNA11 autoantibody in two stages of validation.

Cohorts	AUC	95% CI	Se (%)	Sp (%)	YI	+LR	–LR	PPV (%)	NPV (%)	Accuracy (%)
Verification	0.653	0.563–0.742	10.96	98.63	9.59	8.00	0.90	88.89	52.55	0.55
Validation	0.751	0.699–0.803	38.24	88.82	27.06	3.42	0.70	77.38	58.98	0.64

AUC, area under the curve; CI, confidence interval; Se, sensitivity; Sp, specificity; YI, Youden's index; +LR, positive likelihood ratio; –LR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

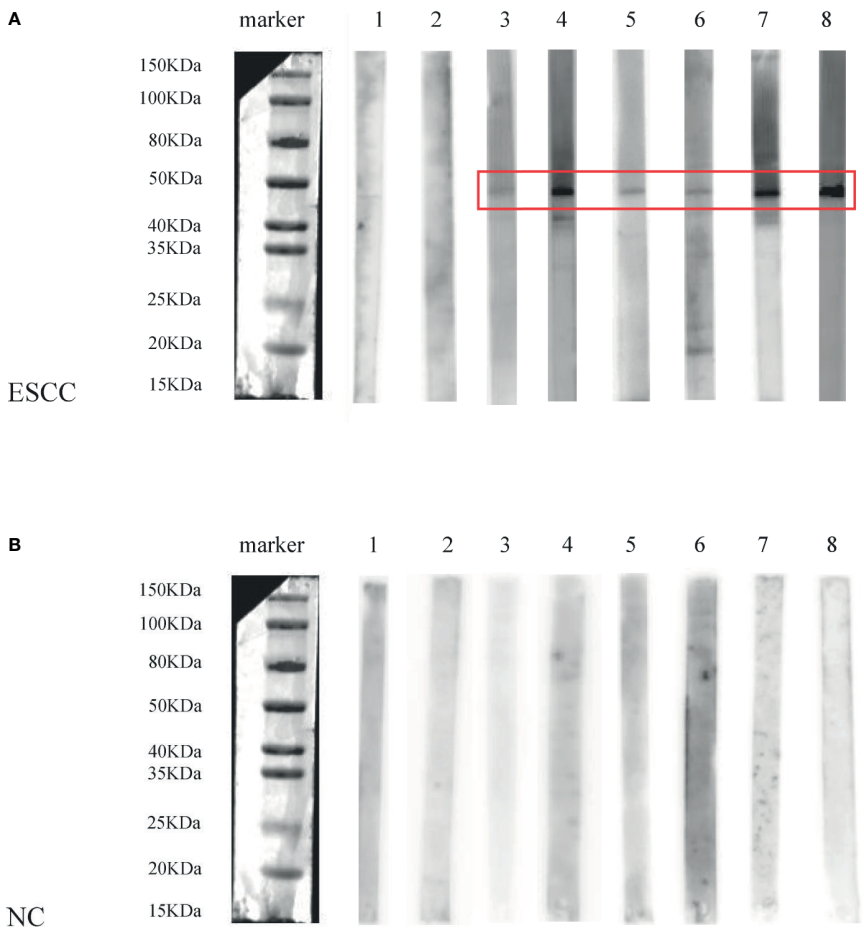

FIGURE 4 | The analysis of anti-GNA11 autoantibody by Western blotting in sera of eight ESCC patients and eight normal controls. ESCC, esophageal squamous cell carcinoma; NC, normal controls. **(A)** The results of Western blotting in sera of 8 ESCC patients, of which No. 1-2 was negative, and No. 3-8 showed a positive band near 45 kDa. **(B)** The results of Western blotting in 8 control sera were negative, and there was no positive band near 45 kDa.

DISCUSSION

Currently, the discovery of reliable biomarkers is still an important goal for the diagnosis of ESCC. However, only a few biomarkers had been used in the clinical detection of ESCC due to the limitation in study population or diagnostic value. So far, only a few tumor-associated proteins have been approved to be used as biomarkers in cancer (26). Therefore, it is necessary to find autoantibodies to TAAs as effective and reliable biomarkers. Protein microarray is an emerging high-throughput technique for protein detection and analysis with the merits of low sample consumption, simultaneous detection of multiple proteins, automation, and high sensitivity (27, 28). It has been widely used to detect protein biomarkers in a variety of cancers such as lung cancer, gastric cancer, and colorectal cancer (29–31). In the current study, the protein microarray containing 154 recombinant proteins was customized to screen out potential TAAs as biomarkers for the diagnosis of ESCC by using ROC analysis and Wilcoxon test. Through an extensive screening and validation, anti-GNA11 autoantibody was finally identified as a potential biomarker for the diagnosis of ESCC. Our study demonstrated that the positive rate of anti-GNA11 autoantibody was 38.24% (65/170), which was significantly higher than that in sera of normal controls 11.18% (19/170) ($p < 0.05$). Besides, our team further explored the performance of anti-GNA11 autoantibody as a biomarker in different subgroups including lymphatic metastasis, differentiation, distance metastasis, family tumor history, TNM stage, gender, and age. However, there was no obvious difference to distinguish these subgroups. Similar results had been seen in other studies about ESCC detection. It was reported that autoantibodies to p53, NY-ESO-1, matrix metalloproteinase-7 (MMP-7), heat shock protein 70 (Hsp70), and peroxiredoxin VI (Prx VI) had no difference in the detection of early-stage and late-stage ESCC patients (32). Chen et al. indicated that tumor-associated autoantibodies against Fascin also showed no difference in histological grade or TNM stage (14). It can be inferred that anti-GNA11 autoantibody may have a relation to the occurrence of ESCC

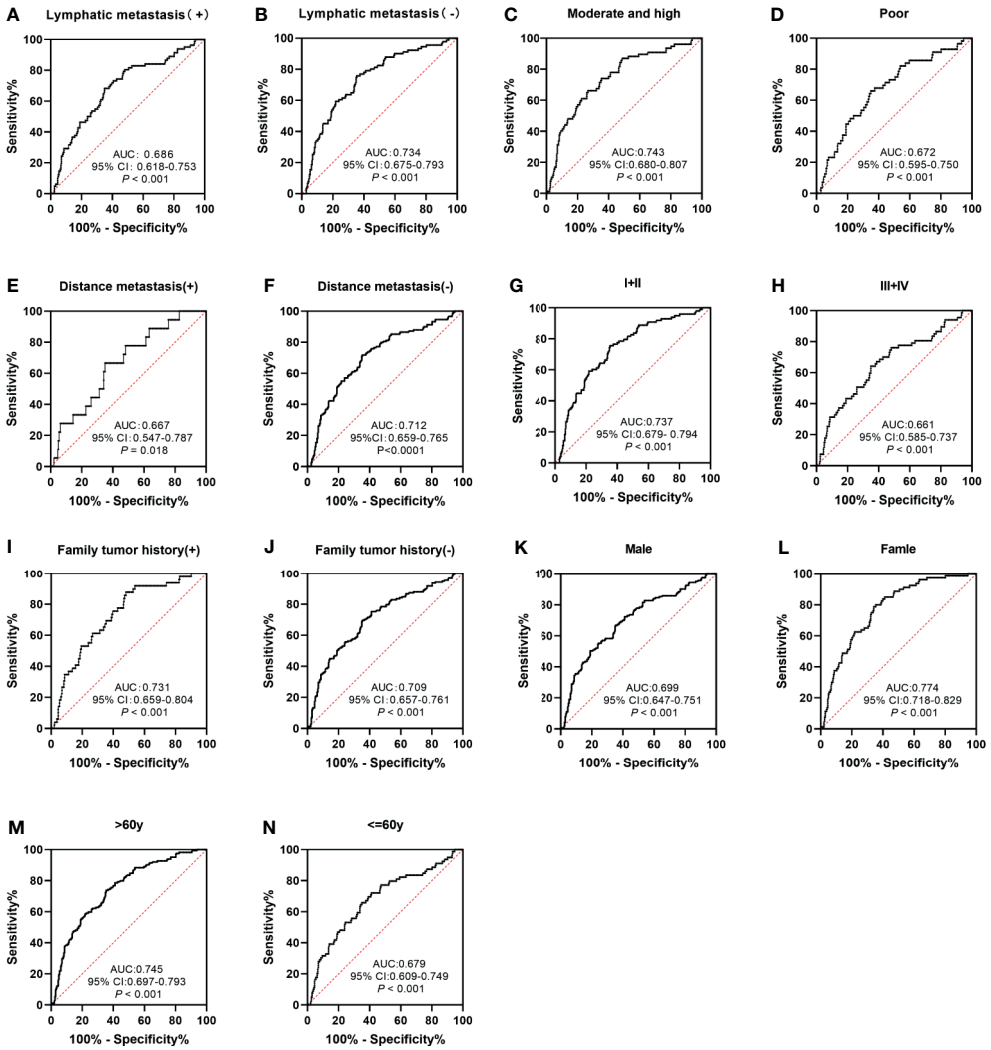

FIGURE 5 | The performance of anti-GNA11 autoantibody according to different clinical variables of ESCC patients. ESCC, esophageal squamous cell carcinoma.

rather than the progression from results above. Through bioinformatics analysis, our team also found that the mRNA level of GNA11 in ESCC is significantly higher than that in the normal control group. IHC analysis further confirmed that GNA11 protein was overall more abundant in ESCC tissues compared with para-tumor tissues. Multiple expression level analyses showed that anti-GNA11 autoantibody is elevated in ESCC patients.

GNA11 involves signaling pathways associated with cell survival including PI3K, RAS, and MAPK; and mutations of the corresponding genes could give the cancer cells a selective growth advantage that develop in a restricted nutritional condition (33). Mutations in *GNA11* will lead to malformations and overgrowth of capillary in capillary malformation patients (34). Some studies have demonstrated that *GNAQ/11* mutation occurs at the early stage of uveal melanoma and clonal expansion is possible only after *GNAQ/11* mutation happens in normal cells (35, 36). Another study indicated that hotspot mutations of Gαq and Gα11 (R183 and Q209) that occurred in uveal melanoma acting as cancer driver genes will destroy the function of GTPase in the MAPK/MEK/ERK pathway, and the implementation of *GNAQ/GNA11* mutation analysis in clinical diagnosis processes might be helpful in making treatment decisions (37). *GNA** (*GNAS*, *GNAQ*, or *GNA11*) aberrations in gastrointestinal excluding appendiceal and colorectal cancer account for 7.3% in all *GNA** mutated tumors, and there is a trend toward lower median overall survival in patients with *GNA** mutated tumors compared with *GNA** wild-type tumors (38). Gα11 is coupled to gonadotropin-releasing hormone receptor (GnRHR) and also involved in negatively regulating the growth of human breast epithelial cells through GnRH–GnRHR system (39). Studies suggested that the methylation of *GNA11* resulted in reduced mRNA expression in breast cancer, which was of great help in the growth of human breast cancer

TABLE 3 | The positive frequencies of anti-GNA11 autoantibody in subgroups.

Categories	Number	Frequency (%)	*p*
Lymphatic metastasis			
Positive	82	26.83	0.468
Negative	91	31.87	
Differentiation			
Moderate and high	77	38.96	0.055
Low	56	23.21	
Distance metastasis			
Positive	18	27.78	0.744
Negative	149	31.54	
Family tumor history			
Positive	49	30.61	0.703
Negative	158	33.54	
Age range (years)			
>60	164	35.37	0.441
≤60	79	30.38	
Gender			
Male	163	32.52	0.563
Female	80	36.25	
TNM stage			
I–II	98	32.65	0.558
III–IV	67	28.36	

cells (40). It was reported that 80% uveal melanomas have *GNAQ* or *GNA11* mutations, which were potential drivers of MAPK activation; and a randomized phase II trial of selumetinib, a selective MEK inhibitor, has shown prospective therapeutic effect for uveal melanomas (41, 42). Consistent with the above studies, we also detected elevated levels of anti-GNA11 autoantibody in sera of ESCC patients, which may indicate changes in *GNA11* gene during the original initiation of ESCC.

The present study reports the potential value of anti-GNA11 autoantibody in the diagnosis of ESCC patients from multiple levels. Anti-GNA11 autoantibody was initially screened out by protein microarray, and the performance of the autoantibody was further verified in two stages to obtain stable and reliable results. From multiple level analyses, we systematically confirmed that anti-GNA11 autoantibody is elevated in ESCC patients and may be a novel marker for the diagnosis of ESCC patients. However, some other studies on *GNA11* mainly focus on the influence of its mutation on melanoma, and there are few studies working on *GNA11* in ESCC. We believe that our subsequent studies on the mechanism of *GNA11* in the occurrence and development of ESCC will fill this gap and provide more data to support our current findings.

CONCLUSIONS

In summary, this study has investigated the expression of GNA11 in ESCC from multiple levels. The results have demonstrated that anti-GNA11 autoantibody can be used as a potential biomarker in the detection of ESCC patients.

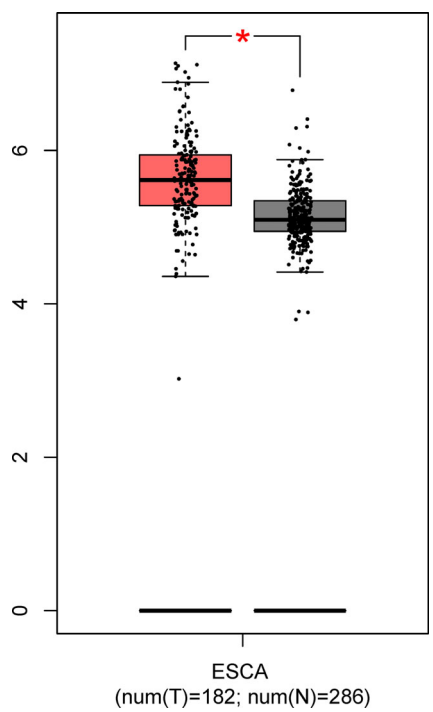

FIGURE 6 | The expression of GNA11 at mRNA level based on TCGA and GTEx data in GEPIA. The red box represents the esophageal carcinoma group, and the gray box chart indicates the normal control group. ESCA, esophageal carcinoma; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; GEPIA, Gene Expression Profiling Interactive Analysis. *$p < 0.05$.

TABLE 4 | The expression of GNA11 between ESCC tissues and para-tumor tissues evaluated by IHC.

Tissue types	N	Color				Area				Final score			
		Score	p^a	p^b		Score	p^a	p^b		Score	p^a	p^b	
Tumor	75	1.99	0.00			0.79	0.71			1.57	0.00		
Pathological grade													
I	17	1.47	0.01	0.01[c]	0.62[d]	0.65	1.00	0.15[c]	0.66[d]	0.96	0.04	0.02[c]	0.45[d]
II	34	1.71	0.00	0.01[c]		0.74	0.98	0.22[c]		1.27	0.00	0.06[c]	
III	24	2.75	0.00			0.96	0.24			2.64	0.00		
Clinical stage													
1–2	34	1.5	0.00	0.02[e]		0.62	0.98	0.05[e]		0.93	0.00	0.01[e]	
3–4	41	2.39	0.00			0.93	0.13			2.22	0.00		
Para-tumor	75	0.21				0.67				0.14			

p^a: compared with para-tumor tissues (one-way ANOVA). p^b: Comparisons between two groups. For pathological grades, c means comparisons of ESCC patients with pathological grade I and II versus pathological grade III; d means comparisons of pathological grade I ESCC patients with pathological grade II. For clinical stages, e represents comparisons of clinical stage 1–2 ESCC patients with clinical stage 3–4. The final score is obtained by multiplying area and color. The data shown in the table are the mean values of the samples. ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry.

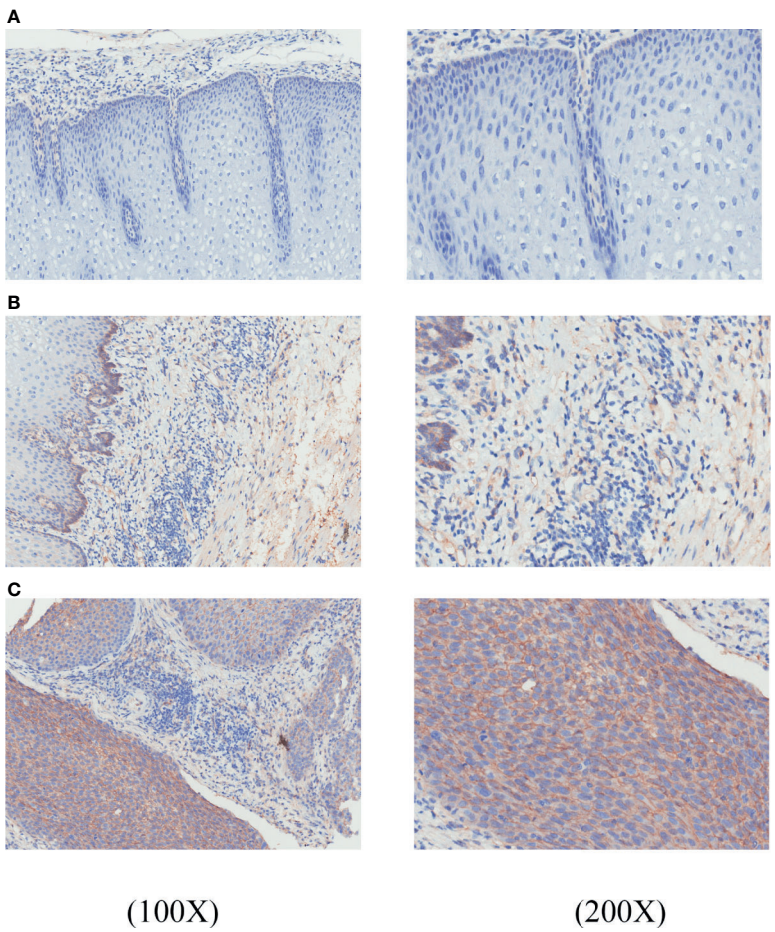

FIGURE 7 | GNA11 is overexpressed in ESCC tissues compared with corresponding para-tumor tissues by immunohistochemistry. The histopathology of these tissue samples was tested by hematoxylin and eosin (H&E) staining. **(A)** A representative negative expression of GNA11 protein in para-tumor tissues at ×100 and ×200 magnification. **(B)** Weak positive expression of GNA11 protein in representative para-tumor tissues at ×100 and ×200 magnification. **(C)** Positive expression of GNA11 protein in representative ESCC tissues at ×100 and ×200 magnification. ESCC, esophageal squamous cell carcinoma.

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 the Institution Review Board of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PW, XY, and JZ conceived the design of the current study. HW and GS conducted experiments and drafted the manuscript. QY and CC participated in the data analysis. HY, XW, JS, and LD helped to draft 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.2021.661043/full#supplementary-material

Supplementary Figure 1 | The expression of GNA11 protein in the tissues of different organs.

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TNF Patterns and Tumor Microenvironment Characterization in Head and Neck Squamous Cell Carcinoma

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Background: HNSCC is a heterogeneous disease, which arises from distinct anatomic subsites, associates with various risk factors and possesses diverse molecular pathological features. Generally, HNSCC is considered as an immunosuppressive disease, characterized by abnormal tumor immune microenvironment. The TNF family plays a crucial role in the survival, proliferation, differentiation, and effector functions in both immune and non-immune cells. However, the expression patterns of TNF in HNSCC remains to be systematically analyzed.

Methods: We downloaded transcriptional profile data of HNSCC from TCGA and GEO datasets. Unsupervised clustering methods were used to identify different TNF patterns and classify patients for further analysis. PCA was conducted to construct a TNF relevant score, which we called risk score.

Results: In this study, we systematically evaluated the patterns of TNF family and tumor immune microenvironment characteristics of HNSCC patients by clustering the expression of 46 members of TNF family. We identified two subtypes with distinct clinical and immune characteristics in HNSCC and constructed a risk scoring system based on the expression profile of TNF family genes.

Conclusion: Risk score serves as a reliable predictor of overall survival, clinical characteristics, and immune cell infiltration, which has the potential to be applied as a valuable biomarker for HNSCC immunotherapy.

Keywords: TNF, head and neck cancer, tumor immune microenvironment, immunotherapy, bioinformatics and biomarkers

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignant tumors worldwide (1, 2). HNSCC is a heterogeneous disease, which arises from distinct anatomic subsites, associates with various risk factors and possesses diverse molecular pathological features (3). Generally, HNSCC is considered as an immunosuppressive disease, characterized by abnormal tumor immune microenvironment (4). Two immune checkpoint inhibitors, pembrolizumab and nivolumab have been approved for the treatment of advanced HNSCC by FDA (5, 6), but only a limited number of patients with HNSCC benefit from immune checkpoint inhibitors. It is therefore urgent to identify reliable molecular biomarkers for risk stratification and therapeutic benefits prediction for immunotherapies in HNSCC (4).

The TNF family, which consists of a 19 TNF ligand superfamily (TNFSF) and a 29 TNF receptor superfamily (TNFRSF), plays a crucial role in the survival, proliferation, differentiation, and effector functions in both immune and non-immune cells (7). A number of TNF family members have been verified to be associated with human diseases including inflammatory disease and cancer (8). Because of the vital role TNF family activities in inflammatory responses regulation, antagonists targeting this signaling to reduce chronic inflammation or promote anti-tumor immunity have been developed, and tested or being tested in clinical trials for inflammatory diseases or cancer (9). In the context of head and neck cancer, TNF signaling was a well-established tumor-promoting pathway *via* either helping tumor cell resist apoptosis or inducing an immune suppressive tumor microenvironment (10–14). For instance, OX40, a member of the TNFRSF, was reported to be highly expressed in the tumor infiltrating lymphocytes of patients with HNSCC, leading to suppressive tumor immune microenvironment (10). Another study demonstrated that TNF-α promotes invasion and metastasis *via* NF-κB pathway in oral squamous cell carcinoma (15). However, the expression patterns and functions of TNF family in HNSCC remains to be systematically analyzed.

In this study, we systematically evaluated the patterns of TNF family and tumor immune microenvironment characteristics of HNSCC patients by clustering the expression of 46 members of TNF family. We identified two subtypes with distinct clinical and immune characteristics in HNSCC and constructed a risk scoring system based on the expression profile of TNF family genes. Risk score serves as a reliable predictor of overall survival, clinical characteristics, and immune cell infiltration, which has the potential to be applied as a valuable biomarker for HNSCC immunotherapy.

Abbreviations: HNSCC, head and neck squamous cell carcinomas; TNFSF, TNF ligands superfamily; TNFRSF, TNF receptor superfamily; GSVA, gene set variation analysis; PCA, Principal component analysis; ssGSEA, single-sample gene set enrichment analysis; PPI, protein-protein interactions; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TME, tumor microenvironment; CAFs, cancer-associated fibroblasts; HPV, human papillomavirus.

MATERIALS AND METHODS

Data and Resources

All data used in this study were obtained from public databases. In total, three HNSCC cohorts were included in our study (TCGA, GSE65858 and GSE41613). For TCGA HNSCC cohort, RNA sequencing data [fragments per kilobase of transcript per million mapped reads (FPKM) values] were downloaded *via* the R package TCGAbiolinks (16). Two GEO datasets were downloaded and processed by R package GEOquery. Then, FPKM values were transformed into transcripts per kilobase million (TPM) values that were more similar to those generated from microarrays. Somatic mutation (SNPs and small INDELs) was downloaded from the University of California Santa Cruz (UCSC) Xena browser (https://xenabrowser.net). All baseline information of HNSCC datasets is summarized in **Supplementary Table S1**.

Unsupervised Clustering for TNF Family Genes

Unsupervised clustering methods were used to identify different TNF patterns and classify patients for further analysis. A total of 46 TNF family genes were used to conduct the unsupervised clustering. A consensus clustering algorithm was performed using the R package ConsensusClusterPlus (17) and was repeated 1,000 times in order to ensure the stability of clustering. The group information after unsupervised clustering of TCGA HNSCC cohort is in **Supplementary Table S2**.

Gene Set Variation Analysis (GSVA) and Single-Sample GSEA (ssGSEA)

The R package GSVA (18) was used to quantify the activity of biological pathways. Immune gene signatures were collected from previously published works (19) (**Supplementary Table S3**). The relative immune signature enrichment scores of each TCGA HNSCC sample are in **Supplementary Table S4**. The ssGSEA algorithm in the R package GSVA was used to estimate the relative abundance of each immune cell in HNSCC. The gene sets defining each immune cell type were downloaded from the study of Charoentong (20) (**Supplementary Table S5**). The relative abundance of each immune cell of each TCGA HNSCC sample was supplemented in **Supplementary Table S6**.

The Protein-Protein Interactions (PPI) Analysis

The protein-protein interactions among TNF family proteins were identified on the STRING according to the instructions (21).

Functional and Pathway Enrichment Analysis

GO analysis was performed to identify enriched GO terms using the R package clusterProfiler (22) with a cutoff p value <0.05 and an adjusted p value <0.2. To identify the most related pathways of TNF family genes, the gseKEGG function of the R package clusterProfiler (22) was used. The DEGs list was estimated between groups with high and low expression of this gene and ranked according to adjusted p value.

DEGs Among TNF Patterns

DEGs among two TNF patterns (**Supplementary Table S7**) were determined using the R package limma (23). The significance criterion for DEGs was set as an adjusted p value < 0.001 and log FC > 1 or < -1.

Generation of the Risk Score

First, the prognostic analysis was performed for each gene in the 693 DEGs using a univariate Cox regression model. A total of 177 genes (**Supplementary Table S8**) with significant prognosis were extracted for further analysis. Then, the expression of these genes was transformed into a Z score. PCA was conducted to construct a TNF relevant score, which we called risk score. Both PC1 and PC2 were selected to serve as signature scores. Risk score = $\Sigma(PC1_i + PC2_i)$, where i is the expression of TNF family pattern related signature genes. Risk score of TCGA and two independent GEO cohorts are in **Supplementary Table S9**.

Statistical Analysis

The normality of the variables was tested using the Shapiro-Wilk normality test (24). For comparisons of two normally distributed groups, statistical analysis was performed by unpaired t tests, and for nonnormally distributed variables, statistical analysis was analyzed by a Wilcoxon rank-sum test. The best cutoff values of each cohort were evaluated using the survcutpoint function in the survminer package. The survival curves for the prognostic analysis were conducted *via* the Kaplan-Meier method, and log-rank tests were utilized to judge differences between groups. Correlation coefficients were computed by Spearman and distance correlation analyses. The univariate Cox regression model was utilized to calculate the hazard ratios (HRs). All statistical p values were two-tailed, with p <0.05 considered as statistically significant. All statistical analyses were conducted using R 4.0.5 (https://www.r-project.org/). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

RESULTS

Multi-Omics Landscape of TNF Family in HNSCC

Firstly, we depicted the multi-omics landscape of 46 TNF family proteins using data from TCGA HNSCC cohort (TNFRSF6B was excluded because of its undetectable expression in TCGA HNSC cohort). We found that the overall mutation rate of all proteins is relatively low in the HNSCC genome (**Figure 1A**). Next, the protein-protein interaction (PPI) network analyzed by STRING showed that TNFSF and TNFRSF had widespread protein interactions, and members inside TNFSF or TNFRSF also interacted extensively (**Figure 1B**). These results demonstrated that members among TNF family formed a complicated network to synergistically mediate TNF pathway in tumor progression. As shown in **Figure 1C**, the expression of most proteins was significantly elevated in tumor tissues compared to normal adjacent tissues.

The Immune Correlation and Prognostic Value of TNF Family in HNSCC

Considering the vital roles of TNF family in immune regulation (7), we speculated that they might be associated with immune cell infiltration in tumor microenvironment in HNSCC. We analyzed the association between expression of 46 TNF family proteins with 23 types of immune cells in TCGA HNSCC cohort respectively, and we found that most of them were significantly positively correlated with immune cell infiltration in tumor tissues (**Figure 2A**). To clarify the prognostic significance of TNF family, we conducted univariate Cox regression of all 46 proteins, and found that 17 proteins were significantly correlated with overall survival of HNSCC patients, of which only TNFRSF12A, EDA, LTBR and EDA2R predicted unfavorable outcomes (**Figure 2B**). These results seemed contradictory to our previous result that most TNF family members were elevated in tumor tissues compared to normal adjacent tissues. However, patients enrolled from TCGA HNSCC cohort have received anti-tumor therapies, and these results indicated that high expression of TNF family members could have better therapeutic responses, thus leading to a better overall survival.

TNF Patterns in the TCGA HNSCC Cohort

TNF family might contribute to the heterogeneity of HNSCC and they were also associated closely with the tumor immune microenvironment. To further identify new probable TNF family patterns, unsupervised clustering was conducted based on the expression of 46 members of TNF family in TCGA HNSCC cohort. As shown in **Supplementary Figures S1A–D**, two clusters could achieve the best clustering efficacy. Accordingly, patients were classified into TNF pattern A and pattern B (**Figure 3A**). TNF pattern A displayed better overall survival, whereas TNF pattern B displayed more advanced pathological stage and grade (**Figures 3B, C**). More elaborate analysis of TNF patterns with clinical characteristics of HNSCC patients showed that TNF pattern A had a low frequency of TP53 mutation and EGFR amplification (**Figures 3D, E**), but a higher rate of HPV infection (**Figures 3F, G**). TP53 mutation and EGFR amplification are unfavorable predictors in HNSCC patients, while HPV infection was a favorable indicator. These results were consistent with TNF pattern A with a better overall survival in HNSCC. Furthermore, almost all TNF family proteins were significantly elevated in TNF pattern A (**Figure 3H**).

Differential Immune Characteristics of TNF Pattern A and B

To further explore the heterogeneity of different TNF patterns, we identified the differentially expressed genes (DEGs) among two groups. A total of 693 TNF pattern-related genes were identified (**Supplementary Table S7**). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that the pathways were enriched in immune-related events (**Figures 4A, B**). Also, we conducted gene set enrichment analysis (GSEA) of differentially expressed genes between TNF pattern A and B, and the result showed that they are enriched in antigen processing and presentation and T cell

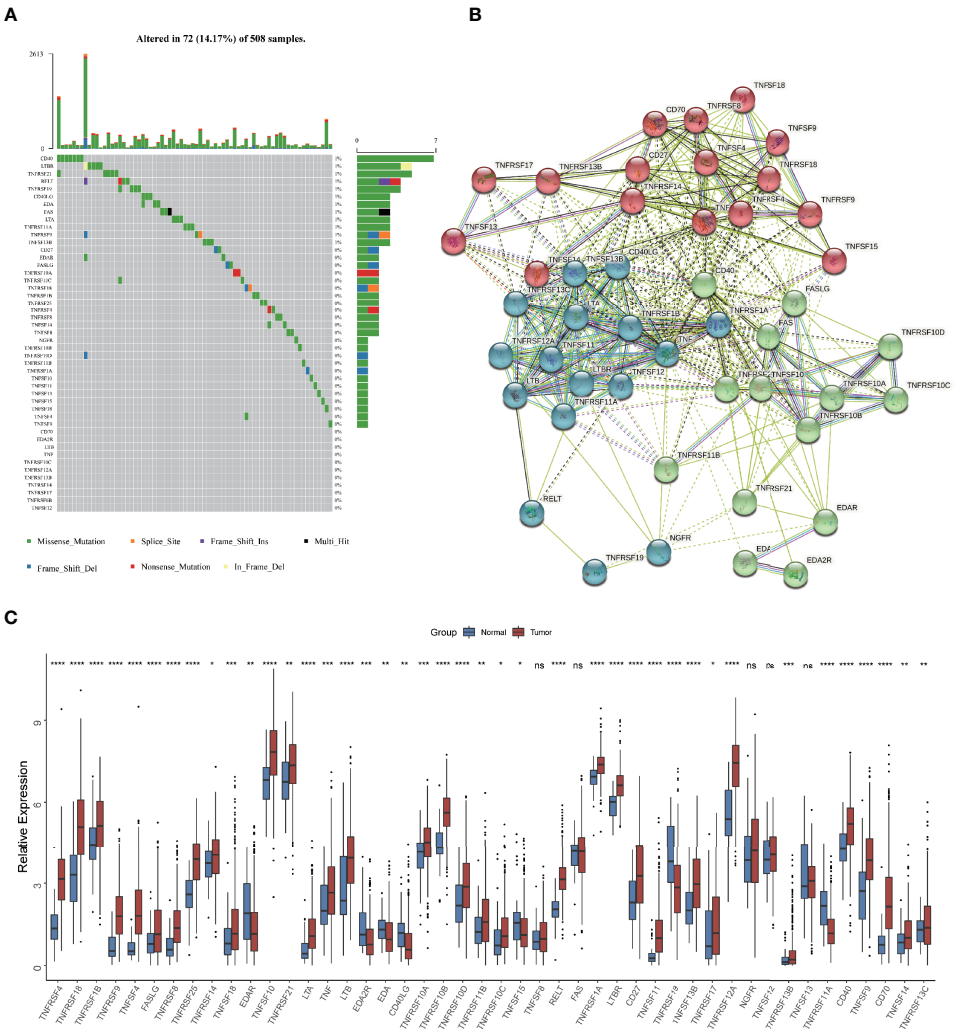

FIGURE 1 | Multi-omics landscape of TNF family in HNSCC. **(A)** The mutation frequency of 46 TNF family proteins in TCGA HNSCC cohort. Each column of the figure represents an individual patient. **(B)** The protein-protein interaction network among TNF family proteins. **(C)** Boxplot shows the expression of 46 TNF family proteins between tumor and normal tissues in TCGA HNSCC cohort. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant)

differentiation (**Supplementary Figure S2**), which was consistent with our GO and KEGG analysis. To further analyze immune cell infiltration and immune-related signatures in two TNF patterns, we estimated the relative abundance of each type of immune cells and relative immune signature enrichment scores in HNSCC by the method of single-sample GSEA (ssGSEA). The results showed that TNF pattern A was significantly associated with immune cell infiltration (especially CD4+ and CD8+ T cell) and relatively hot immune microenvironment signature, such as effector CD8+ T cells and cytolytic activity (**Figures 4C, D**). Considering the vital role of immune checkpoint molecules (PD-1, PD-L1, LAG3 and CTLA-4) in tumor immune microenvironment, we analyzed the expression of these molecules in two TNF patterns. We found that the expression of all four immune checkpoint molecules was significantly elevated in TNF pattern A, which was consistent with our above-mentioned immune-related signatures (**Supplementary Figure S3**).

The Clinical and Transcriptomic Characteristics of TNF-Related Gene Clusters

To further explore the heterogeneity of different TNF patterns, a univariate Cox regression analysis of 693 DEGs certified that 177 genes had prognostic value (**Supplementary Table S8**). Unsupervised clustering analysis based on the expression of these 177 genes also divided HNSCC patients into two clusters, which we called TNF gene clusters (**Supplementary Figure S4**). The clinical analysis showed that different gene cluster had distinct status of TP53 mutation and HPV infection (**Figure 5A**), which was consistent with our previous identified TNF clusters, and TNF gene cluster A also tended to have a

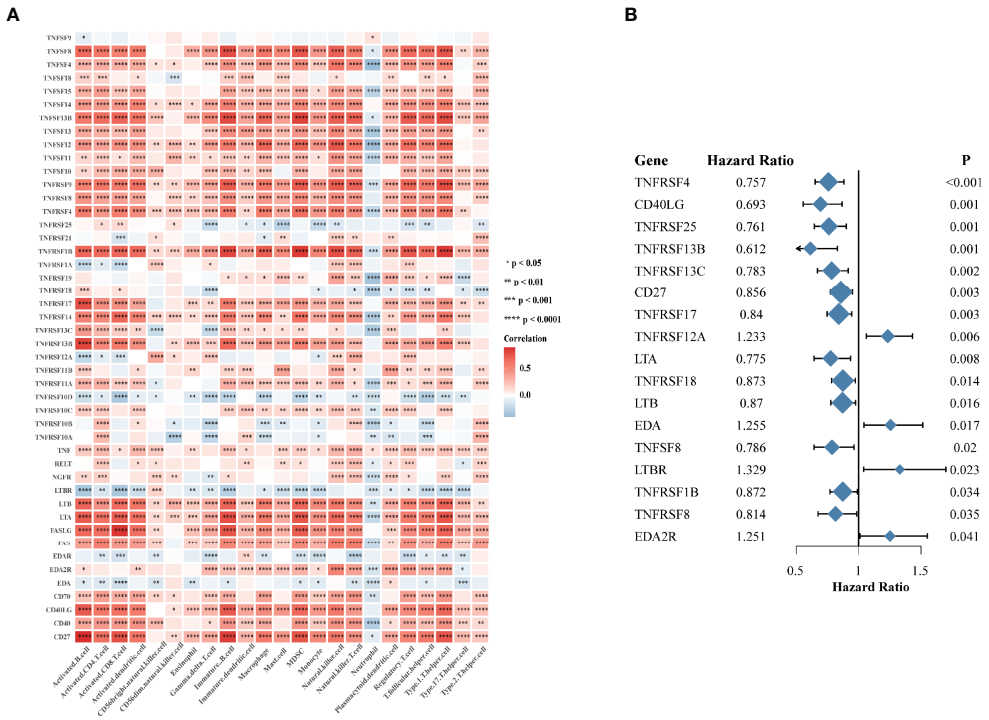

FIGURE 2 | The immune correlation and prognostic value of TNF family in HNSCC. **(A)** Correlation heatmap between 46 TNF family proteins and immune cells in the TCGA HNSCC cohort. Orange indicates positive correlation; blue indicates negative correlation. **(B)** The forest plot of the univariate Cox regression model depicting the 17 statistically significant prognostic factors in TNF family proteins in TCGA HNSCC cohort. Hazard ratio > 1: risk factors for survival. Hazard ratio < 1: protective factors for survival. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

better survival (**Figure 5B**). Further analysis of two TNF gene clusters with immune cell infiltration and immune related signature also showed gene cluster A was enriched in immune cell infiltration and immune signatures (**Figures 5C, D**). Furthermore, we analyzed the expression of four important immune checkpoint molecules (PD-1, PD-L1, LAG3 and CTLA-4) in two TNF gene clusters, and the results showed that TNF gene cluster A showed a significant elevation of all four molecules, which was an indicator of hot immune microenvironment (**Supplementary Figure S5**). Additionally, the expression of most TNF family proteins was significantly elevated in TNF gene cluster A (**Supplementary Figure S6A**).

The Construction of Risk Score and Its Clinical Significance

To evaluate TNF status individually, we further constructed a risk score system based on 177 TNF-related signature genes. High risk score was associated with worse outcome, high frequency of TP53 mutation and EGFR amplification, and low frequency of HPV infection in HNSCC patients (**Figures 6A, B**), whereas TNF pattern A and TNF gene cluster A, which had better overall survival, showed a low risk score (**Figures 6C–E**). Also, high risk score was correlated with less immune cell infiltration and lower immune signature enrichment (**Supplementary Figure S6A, B**). Moreover, risk score was significantly negatively correlated with the expression of immune checkpoint molecules (PD1, PDL1, LAG3 and CTLA4) (**Supplementary Figures S7A–D**). Subsequently, we collected two independent GEO cohorts to further validate prognostic values of our risk score. As shown in **Figures 6F, G**, high risk score was significantly correlated with poor overall survival in two independent cohorts, and TP53 mutation or HPV negative group tended to have high risk scores (**Supplementary Figure S8**).

DISCUSSION

HNSCC is considered as a disease with extraordinary heterogeneity due to its diverse anatomic subsites (1). Risk factors for this disease include tobacco consumption, alcohol abuse and infection with human papillomavirus (HPV), primarily HPV-16 (25–27). With the development of next-generation sequencing, substantial progress has been made over the past decade in the molecular characterization of HNSCC, culminating in The Cancer Genome Atlas (TCGA) (1, 28–30). Based on the multi-omics data from TCGA, a series of studies have identified a number of genes or signatures for risk stratification, prognosis prediction and therapeutic responses prediction. However, novel molecular biomarkers are still urgently to be identified for better management of this disease.

TNF signaling was a well-established tumor-promoting pathway by either helping tumor cells resist apoptosis or

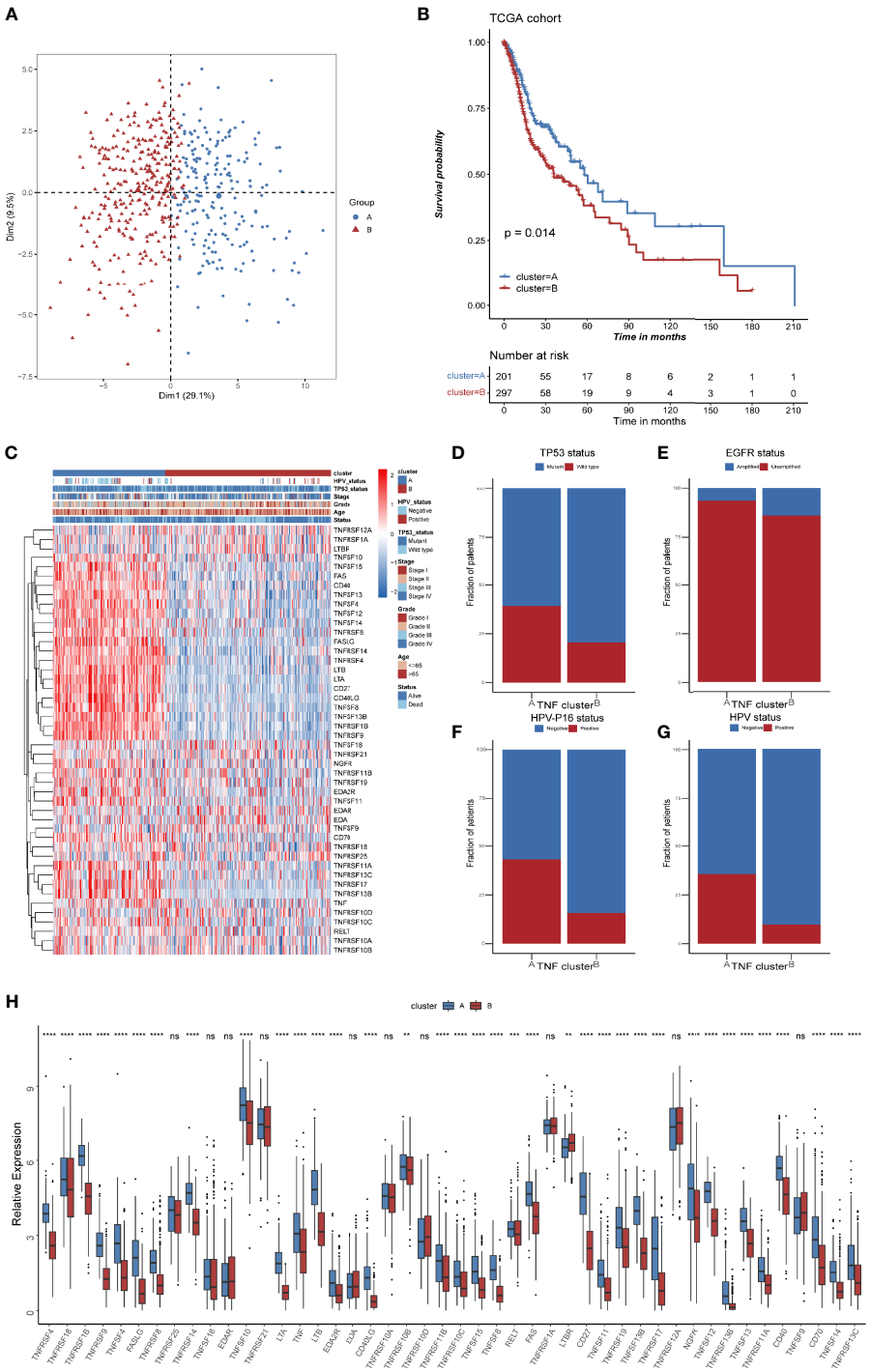

FIGURE 3 | TNF patterns in the TCGA HNSCC cohort. **(A)** Principal component analysis (PCA) for the expression of 46 TNF/TNFR proteins to distinguish two TNF/TNRER patterns in TCGA HSNC cohort. **(B)** Kaplan-Meier survival analysis of two TNF patterns in TCGA HNSCC cohort. **(C)** Unsupervised clustering of 46 TNF family proteins in TCGA HNSCC cohort. Red represents high expression, and blue represents low expression. The TNF patterns, HPV status, TP53 status, stage, pathological grade, age, and survival status were used as sample annotations. **(D–G)** Stacked bar plot of TCGA HNSCC TP53 status, HPV-16 status, HPV status and EGFR status in two TNF patterns, respectively. **(H)** Relative expression of 46 TNF family proteins in TNF cluster A and B in TCGA HNSCC cohort. (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant).

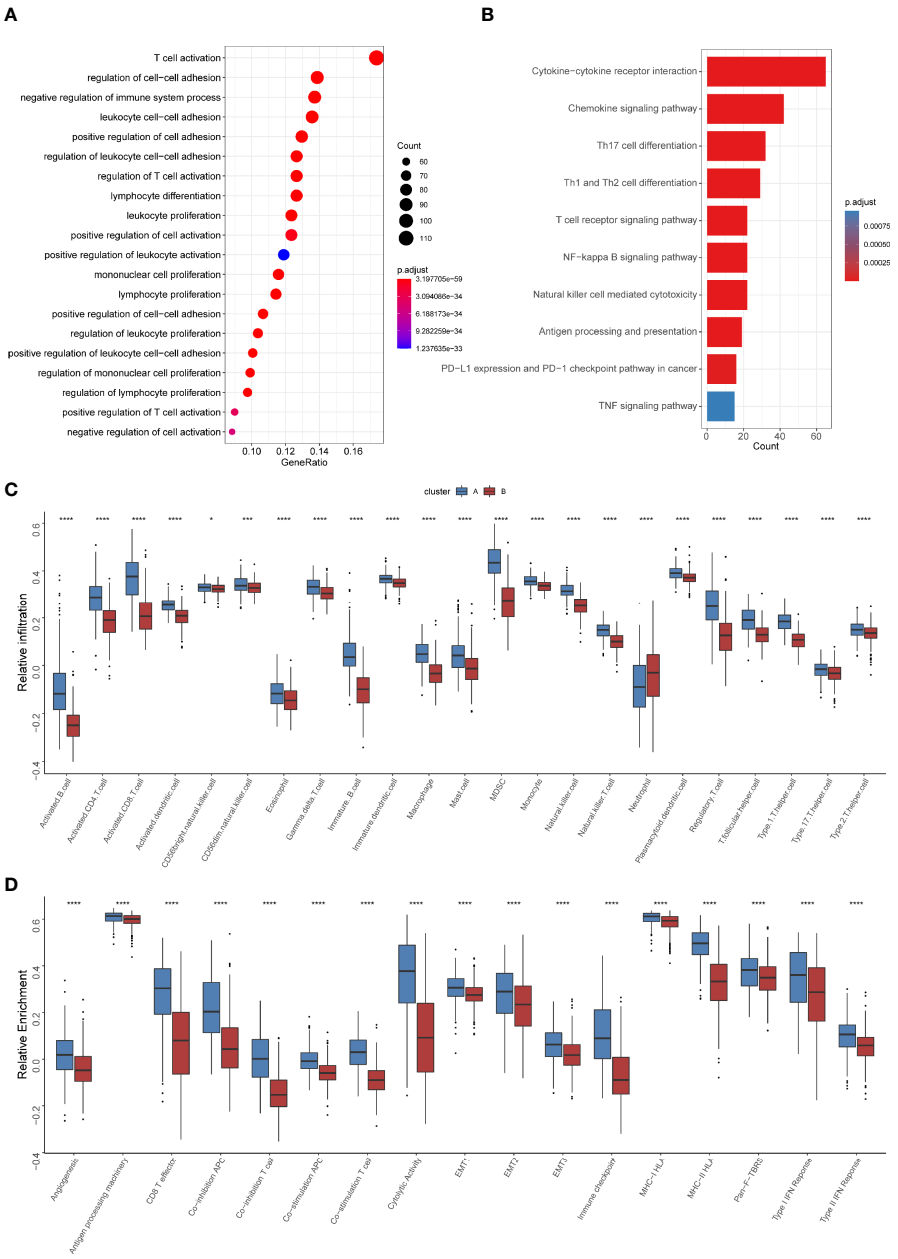

FIGURE 4 | Differential immune characteristics of TNF pattern A and B **(A)** Gene Ontology (GO) analysis depicted the enriched pathways of TNF-related genes. **(B)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of TNF-related genes. **(C)** Relative infiltration of 23 types of immune cells in TNF cluster A and B **(D)** Relative enrichment score of 17 immune related signatures in TNF cluster A and B. (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

inducing an immune suppressive tumor microenvironment in HNSCC (10–14). We firstly systematically analyzed multi-omics data of 46 TNF family genes, and we found that most of these genes were significantly differentially expressed in HNSCC. Further analysis of immune correlation of TNF family genes in HNSCC revealed that most of them were significantly positively correlated with infiltration of various immune cells, including activated $CD4^+$ and $CD8^+$ T cell. Univariate Cox regression of all 46 proteins showed that 17 proteins were significantly correlated with overall survival of HNSCC patients, of which only TNFRSF12A, EDA, LTBR and EDA2R predicted unfavorable outcomes. These results demonstrated that TNF family genes played a crucial role in the development and TME of HNSCC.

Subsequently, we identified two subtypes with distinct clinical and immune characteristics in HNSCC based on the expression profile of TNF family genes. TNF cluster A was characterized by high infiltration of immune cells and enrichment of immune activated signatures along with a better overall survival. TP53

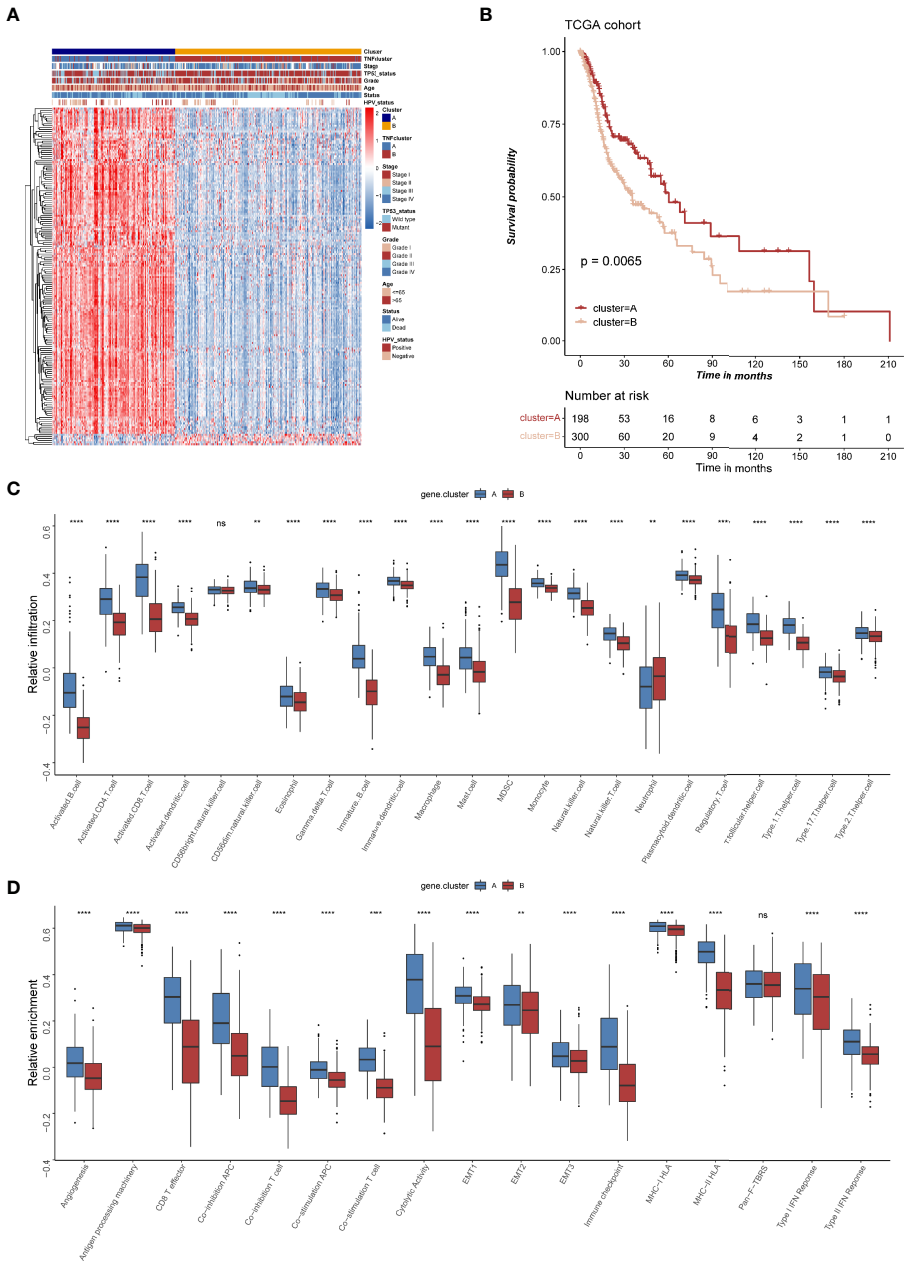

FIGURE 5 | The clinical and transcriptomic characteristics of TNF-related gene clusters. **(A)** Unsupervised clustering of 177 TNF related genes in TCGA HNSCC cohort. Red represents high expression, and blue represents low expression. The TNF gene cluster patterns, TNF patterns, HPV status, TP53 status, stage, pathological grade, age, and survival status were used as sample annotations. **(B)** Kaplan-Meier survival analysis of two TNF gene cluster patterns in TCGA HNSCC cohort. **(C)** Relative infiltration of 23 types of immune cells in TNF gene cluster A and B **(D)** Relative enrichment score of 17 immune related signatures in TNF gene cluster A and B. ($^{**}p < 0.01$, $^{****}p < 0.0001$, ns, not significant).

mutation (72% of tumors) was the most frequent mutation in HNSCC, and it was considered as an early driver genomic alterations (28). Our analysis found that TNF cluster A had lower TP53 mutation frequency compared to TNF cluster B. Another oncogenic driver gene in HNSCC tumor is EGFR, which was overexpressed in 80–90% of HNSCC tumors and was associated with poor overall survival and progression-free survival (31, 32). Our analysis of EGFR amplification in TNF cluster A and B also showed that cluster A had higher fraction of unamplified EGFR compared cluster B. HPV infection is an increasingly common risk factor for HNSCC. HPV infection is associated with most oropharyngeal cancers (>70%) and a small minority of cancers at other head and neck anatomical sites (25, 26), and HPV-positive HNSCC generally has a more favorable prognosis

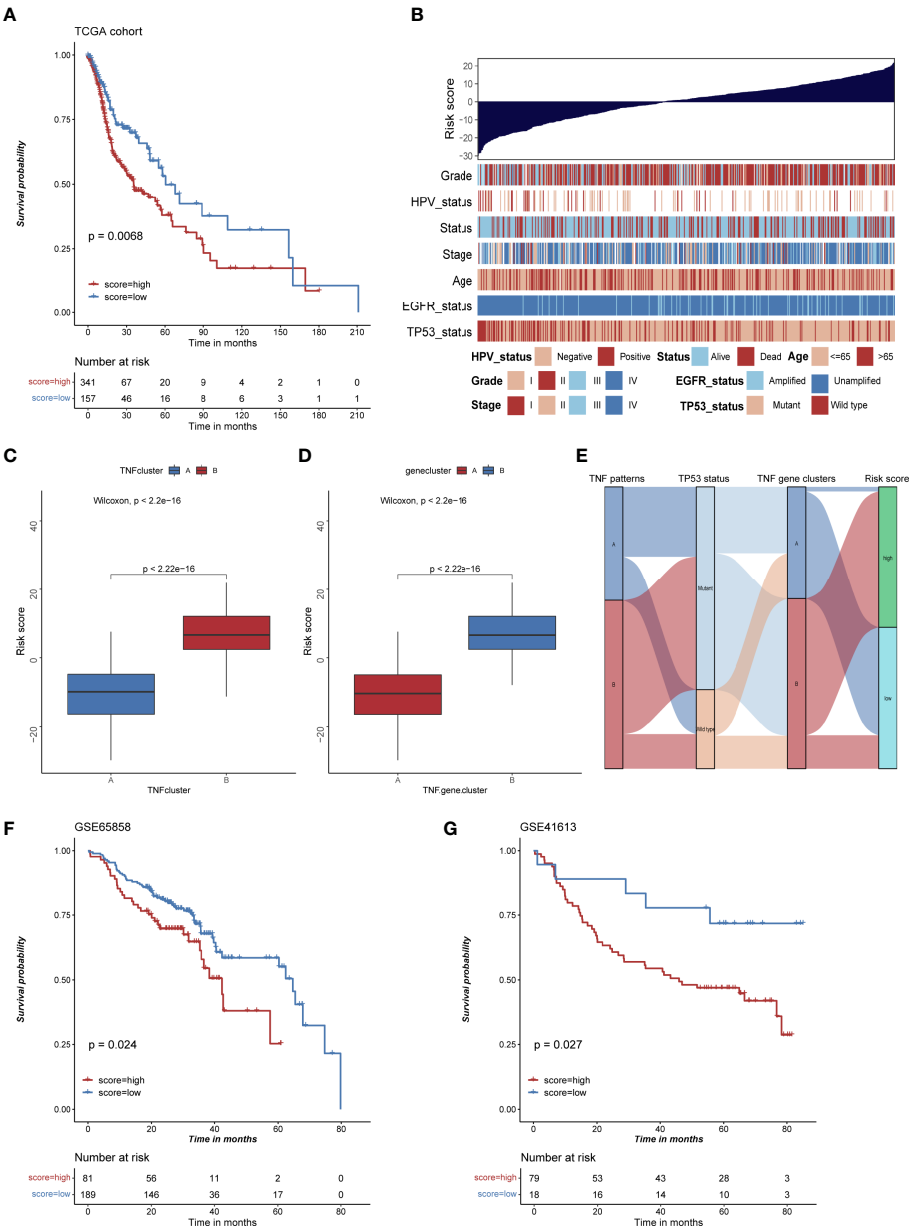

FIGURE 6 | The construction of risk score and its clinical significances. **(A)** Kaplan-Meier survival analysis of high and low risk score group in TCGA HNSCC cohort. **(B)** Bar chart depicting the relationship of risk score and various clinical characteristics in the TCGA HNSCC cohort. HPV status, TP53 status, stage, pathological grade, age, and survival status were used as sample annotations. **(C)** Boxplot of risk score in TNF cluster A and B **(D)** Boxplot of risk score in TNF gene cluster A and B **(E)** Alluvial diagram depicting the relationship of TNF cluster, TNF gene cluster, TP53 status and risk score group, TNF cluster/gene cluster A was associated with low frequency of TP53 mutation and low risk score. **(F)** Kaplan-Meier survival analysis of high and low risk score group in GSE65858 cohort. **(G)** Kaplan-Meier survival analysis of high and low risk score group in GSE41613 cohort.

than HPV-negative HNSCC (1). We also analyzed the relationship between TNF clusters and HPV infection in HNSCC, and the result showed that HPV infection was more frequent in TNF cluster A. All these results were consistent with our previous analysis that TNF cluster A had a better overall survival.

The tumor microenvironment (TME) in HNSCC is a complex and heterogeneous population of tumor cells and stromal cells, which include endothelial cells, cancer-associated fibroblasts (CAFs) and immune cells (1). Generally, HNSCC is considered as an immune cell infiltrating tumor, which are characterized by immunosuppressive TME (33, 34). Two immune checkpoint inhibitors, pembrolizumab and nivolumab have been approved for the treatment of advanced HNSCC by FDA (5, 6), but a limited number of patients with HNSCC derive benefit from

immune checkpoint inhibitors. It is also urgent to identify reliable molecular biomarkers for risk stratification and therapeutic benefit prediction for those therapy strategies in HNSCC (4). In our study, two subtypes identified by us had distinct immune characteristics. TNF cluster A was significantly associated with higher immune cell infiltration, activated immune signatures and expression of immune checkpoint molecules, which indicated an immune "hot" tumor. Based on this result, we speculate that TNF cluster A might derive better responses to immune checkpoint inhibitors.

Considering the distinct characteristics of two TNF clusters, we hypothesized that DEGs between two groups might also have unique characteristics. Accordingly, we conducted unsupervised clustering analysis based on the expression of DEGs and also divided HNSCC patients into two groups, which we called TNF gene cluster A and B. Similar to the results of TNF clusters, two TNF gene clusters also had distinct clinical and immune characteristics. These results demonstrated that there were indeed two immune subtypes based on the expression profile of TNF gene family in HNSCC.

Regarding the clinical significance of our study, we constructed a risk score based on the expression of DEGs that can evaluate risk of HNSCC patients individually. Indeed, the patients with high risk score had worse overall survival, which was validated by two independent GEO cohorts. Also, the patients with TP53 mutation or EGFR amplification had a significantly higher risk score and patients with HPV infection had a lower risk score, which was consistent with our TNF clusters and TNF gene clusters.

However, our study also had some limitations. First, we failed to find expression profile data of HNSCC patients receiving immune checkpoint blockers to validate the predictive value of our risk score to immune checkpoint inhibitors. Second, our analysis was only based on retrospective data from public databases, which need to be further validated by prospective studies in the future.

In conclusion, we identified two subtypes with distinct clinical and immune characteristics in HNSCC and constructed a risk scoring system based on the expression profile of TNF genes. Risk score is capable of serving as a reliable predictor of overall survival, clinical characteristics, and immune cell infiltration, which have the potential to be applied as a valuable biomarker for HNSCC immunotherapy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

QL, YL, MC, and AY conceived and designed the project. QL, CH, QM, JP, WD, XX, DD, XW, XQ, WZ, DS, and MC analyzed and interpreted the data. QL, YL, MC, and AY wrote and revised 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/fimmu.2021.754818/full#supplementary-material

Supplementary Figure 1 | (A–D) Consensus clustering matrices of 46 TNF family proteins in TCGA HNSCC cohort for k = 2-5.

Supplementary Figure 2 | GSEA of DEGs in two TNF patterns in HNSCC.

Supplementary Figure 3 | The relative expression of PD1, PDL1, LAG3 and CTLA4 in two TNF patterns in HNSCC.

Supplementary Figure 4 | (A–D) Consensus clustering matrices of 177 differentially expressed genes in TCGA HNSCC cohort for k = 2-5.

Supplementary Figure 5 | The relative expression of PD1, PDL1, LAG3 and CTLA4 in two TNF gene clusters in HNSCC.

Supplementary Figure 6 | (A) Relative expression of 46 TNF family proteins in TNF cluster A and B in TCGA HNSCC cohort. **(B)** Relative infiltration of 23 types of immune cells in high and low risk group. **(C)** Relative enrichment score of 17 immune related signatures in high and low risk group.

Supplementary Figure 7 | (A–D) The correlation of risk score with the expression of PD1, PDL1, LAG3 and CTLA4 in TCGA HNSCC cohort, respectively.

Supplementary Figure 8 | (A) Risk score in different TP53 status patients in GSE65858 cohort. **(B)** Risk score in different HPV status patients in GSE65858 cohort.

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Serum Anti-14-3-3 Zeta Autoantibody as a Biomarker for Predicting Hepatocarcinogenesis

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Hepatocellular carcinoma (HCC) is a common malignancy worldwide. Alpha-fetoprotein (AFP) is still the only serum biomarker widely used in clinical settings. However, approximately 40% of HCC patients exhibit normal AFP levels, including very early HCC and AFP-negative HCC; for these patients, serum AFP is not applicable as a biomarker of early detection. Thus, there is an urgent need to identify novel biomarkers for patients for whom disease cannot be diagnosed early. In this study, we screened and identified novel proteins in AFP-negative HCC and evaluated the feasibility of using autoantibodies to those protein to predict hepatocarcinogenesis. First, we screened and identified differentially expressed proteins between AFP-negative HCC tissue and adjacent non-tumor liver tissue using SWATH-MS proteome technology. In total, 2,506 proteins were identified with a global false discovery rate of 1%, of which 592 proteins were expressed differentially with 175 upregulated and 417 downregulated (adjusted p-value <0.05, fold-change FC ≥1.5 or ≤0.67) between the tumor and matched benign samples, including 14-3-3 zeta protein. For further serological verification, autoantibodies against 14-3-3 zeta in serum were evaluated using enzyme-linked immunosorbent, Western blotting, and indirect immunofluorescence assays. Five serial serum samples from one patient with AFP-negative HCC showed anti-14-3-3 zeta autoantibody in sera 9 months before the diagnosis of HCC, which gradually increased with an increase in the size of the nodule. Based on these findings, we detected the prevalence of serum anti-14-3-3 zeta autoantibody in liver cirrhosis (LC) patients, which is commonly considered a premalignant liver disease of HCC. We found that the prevalence of autoantibodies against 14-3-3 zeta protein was 16.1% (15/93) in LC patient sera, which was significantly higher than that in patients with chronic hepatitis (0/75, $p = 0.000$) and normal human sera (1/60, 1.7%, $p = 0.01$). Therefore, we suggest that anti-14-3-3 zeta autoantibody might be a biomarker for predicting hepatocarcinogenesis. Further follow-up and research of

patients with positive autoantibodies will be continued to confirm the relationship between anti-14-3-3 zeta autoantibody and hepatocarcinogenesis.

Keywords: SWATH-MS proteome technology, 14-3-3 zeta, biomarker, hepatocarcinogenesis, premalignant liver disease

1 INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, with approximately 840,000 new cases annually. Nearly 800,000 people die of HCC every year (1). Alpha-fetoprotein (AFP) is still the only serum biomarker widely used clinically. However, approximately 40% of cases with normal AFP levels, including those with very early HCC and AFP-negative HCC, cannot be detected early. Thus, there is an urgent need to identify novel biomarkers for patients for whom disease cannot be diagnosed early. Liver cirrhosis (LC) is a common chronic progressive liver disease caused by long-term or repeated diffuse liver damage due to one or more causes (2). It is known that underlying LC and chronic hepatitis (CH) are common precursors of HCC, and 80%–90% of HCCs develop from LC. Thus, LC is considered a premalignant disease of HCC (3). Therefore, autoantibodies detected in premalignant diseases of HCC, such as LC, are of significance for the identification of individuals who will potentially develop HCC.

Many studies have shown that the sera from cancer patients contain autoantibodies that react with a unique group of autologous cellular antigens known as tumor-associated antigens (TAAs) (4). These antigens are usually overexpressed, aberrantly cleaved, posttranslationally modified, mutated, or aberrantly localized in cancerous cells (5, 6). Autoantibodies to TAAs are sometimes detected during the transition to malignancy, such as from chronic liver disease to HCC (7). It has been proposed that these antibody responses could be stimulated by abnormal cellular proteins that are involved in carcinogenesis. Thus, the appearance of autoantibodies to TAAs might predict hepatocarcinogenesis. At present, there are few studies on tumor-associated antigens and autoantibodies to predict hepatocarcinogenesis, and no biomarkers with better sensitivity and specificity have been detected.

In this study, we aimed to screen and identify differentially expressed proteins in very early HCC and AFP-negative HCC and evaluate the feasibility of using autoantibodies against these identified antigens to predict hepatocarcinogenesis with premalignant liver disease, such as LC. We screened and identified novel proteins in AFP-negative HCC tissue that were differentially expressed related to levels in adjacent non-tumor liver tissue using SWATH-MS proteome technology and investigated the prevalence of serum autoantibodies against identified antigens in serial serum samples of AFP-negative HCC patients. Moreover, we sought to detect the prevalence of autoantibodies against identified proteins in LC patient sera and evaluate the feasibility of using serum autoantibodies to predict hepatocarcinogenesis.

2 MATERIALS AND METHODS

2.1 Patients and Serum Samples

HCC tissues and adjacent non-tumorous liver tissue counterparts used for this study were collected from HBV-associated HCC patients who underwent hepatectomy. Sera from 93 patients with LC, 75 patients with CH, and 60 normal human serum (NHS) samples were obtained from outpatients or inpatients. Tissues and sera were collected at Beijing You'an Hospital, Capital Medical University, Beijing, China, between January 2015 and January 2016. HCC tissues and adjacent non-tumorous liver tissues were verified by histopathological examination. The diagnosis of LC and CH was based on clinical, biochemical, and imaging studies, and/or liver histological data, according to the Chronic Hepatitis B Prevention and Treatment Guidelines of 2015. One AFP-negative HCC patient was diagnosed according to the Primary Liver Cancer Treatment Protocols (2011 edition). Patients with LC and CH were followed up for at least 18 months to exclude individuals with primary biliary cirrhosis. This study was approved by the Institutional Review Board of Capital Medical University, Beijing, China (February 28, 2014). Patients/participants provided written informed consent to participate in the study.

2.2 Recombinant Proteins and Antibody

The 14-3-3 zeta construct GST-14-3-3 WT (plasmid ID: 1944), purchased from Addgene (75 Sidney Street, Suite 550A Cambridge, MA 02139, USA), was subcloned into the pET28a vector. The recombinant protein 14-3-3 zeta expressed in ArcticExpress (DE3) RP was purified using nickel column chromatography. Recombinant protein was examined using SDS-PAGE, and the expected molecular size of the expression products was determined using Coomassie blue staining. Western blot analysis was performed to confirm that the bands observed in SDS-PAGE were reactive with the corresponding antibodies.

2.3 Differential Proteomics Between AFP-Negative HCC and Adjacent Non-Tumor Liver Tissue

2.3.1 Sample Preparation

The AFP-negative HCC tissues [AFP (–) -HCC group] and adjacent non-tumorous liver tissues (control group) were cut into pieces and washed with ice-cold phosphate-buffered saline (PBS) at 4°C. The samples were ground into powder in liquid nitrogen and then homogenized on ice in 1 ml of lysis buffer (50 mM HEPES, 6 M urea, 2 M thiourea, and 1× protease inhibitor cocktail). After that, the samples were vortexed for 10 s at 200 rpm 20 times following centrifugation at 20,000×*g* for

40 min at 4°C to collect the supernatant (total protein solution of each sample). The protein concentration of each sample was measured using the BCA protein assay method (8), and each group was subjected to biological triplicate experiments.

2.3.2 Protein Digestion in Solution

Tissue protein solution (100 μg protein) was diluted with an equal volume of 20% 2,4,6-trichloroanisaole (TCA) to purify proteins. The solution was then precipitated for 2 h at −80°C and centrifuged at 20,000×*g* for 30 min at 4°C. The precipitate was suspended in 0.4 ml of acetone (4°C) *via* ultrasonic treatment for 5 min. The resulting mixture was continuously precipitated overnight at −80°C and centrifuged at 20,000×*g* for 30 min at 4°C, the acetone was removed, and the purified proteins were obtained.

Purified proteins were dissolved in 70 μl 0.2% RapiGest SF in 50 mM ABC solution and then reduced and alkylated by incubation with 100 mM tris (2-carboxyethyl) phosphine in 50 mM ABC solution for 30 min at 60°C and 100 mM iodoacetamide in 50 mM ABC solution for 30 min at room temperature in the dark, respectively. Digestion was performed by incubation with trypsin (Promega; enzyme-to-substrate ratio, 1:100) at 37°C with 160 rpm shaking overnight. The enzymatic reaction was stopped by adding 10% trifluoroacetic acid solution at a final concentration of 1% at 37°C for 0.5 h. The digestion was transferred to an ultrafiltration tube and centrifuged at 10,000×*g* for 20 min at 4°C, and the filtrate comprised the sample peptide solution at a final concentration of 1 μg/μl.

2.3.3 Proteomic Data Acquisition

Identified proteomic data were acquired on an AB 5600+ Triple TOF mass spectrometer (AB Sciex) equipped with an Eksigent 400 nano-HPLC system (Eksigent, AB Sciex, USA). A nano-LC MS/MS method was built, and the main steps were as follows: an equal volume of each peptide solution was taken to obtain one pooled peptide solution. The mixed solution was loaded on a nano trap column (350 μm × 0.5 mm, Chrom XP C18-3 μm, 120 A) and washed with acetonitrile–water–formic acid (2:98:0.1, v/v/v) solution for desalting at a flow rate of 2 μl/min for 10 min. After desalting, peptides were separated on an analytical column (75 μm × 20 cm, Sunchrom C18-5 μm, 120, Beijing Happy Science Scientific Co., Ltd.) at a flow rate of 300 nl/min, and gradient elution was performed according to the following method [preparation of phase A (ACN–H_2O–FA (98:2:0.1, v/v/v)] and phase B [ACN–H_2O–FA (0.1:98:2, v/v/v)]: 0 min, 95% and 5%; 1 min, 91% and 9%; 95 min, 75% and 25%; 100 min, 50% and 50%; 101 min, 20% and 80%; 110 min, 20% and 80%; 111 min, 95% and 5%; 120 min, 95% and 5% phase B. The peptide elution was ionized, and then, MS data were acquired using a data-dependent acquisition (DDA) model with an AB 5600+ Triple TOF mass spectrometer.

In the high-resolution model, the first-order spectra were scanned in the range of 300–605 *m*/*z*, 600–805 *m*/*z*, and 800–1,250 *m*/*z*. The TOF MS parameters were set as follows: spray mode, nano-electrospray; GS1, 12; CUR, 30; ISDF, 2,500; IHF, 150; DP, 100; CE, 10; cumulative time, 250 ms; mass deviation, 50 mDa. The dynamic exclusion acquisition time was set to 20 s. The charge-setting range was 2–5. Fragment ion level 2 spectra (MS2) were acquired in high sensitivity mode, with a mass-to-charge ratio (*m*/*z*) ranging from 100 to 1,500 and an acquisition time of 250 ms per cycle.

SWATH label-free was introduced to quantify identified proteins, and the SWATH MS/MS method was established. At first, pooled peptide spectra were acquired with the nano-LC MS/MS method based on the DDA model, but the TOF MS range was set from *m*/*z* 300 to 1,250. The variable window for SWATH-MS was calculated using Variable Window Calculator V 0.2 112513 (AB Sciex). The parameters were set as follows: number of windows, 60; lower *m*/*z*, 300; upper *m*/*z*, 1,250; window overlap (Da), 0.5; rolling collision energy, 15. Product ion spectra were measured in the range of *m*/*z* 100 to 1,500 for 40 ms with the high sensitivity model, and the resulting total cycle time was 1.42 s. SWATH-MS measurements were performed in triplicate for each sample.

2.3.4 Data Processing and Bioinformatics Analysis

DDA data were analyzed with Paragon (ProteinPilot software, AB/Sciex) against the *Homo sapiens* database (https://www.ncbi.nlm.nih.gov/genome/?term=Homo+sapiens). The detected protein threshold [unused Protscope (conf)] of result quality was 0.05 (10%), and false discovery rate (FDR) analysis was chosen. SWATH data were analyzed using PeakView SWATH Processing Micro App (AB Sciex), followed by real-time global correction. The important parameters for processing SWATH data were as follows: peptide confidence threshold, 95%; FDR threshold, 1.0%; 50 ppm *m*/*z* tolerance; 15 min extraction window; shared peptides excluded for SWATH analysis. Protein peak area data were finally obtained.

Protein areas were normalized, and principal component analysis (PCA) and *t*-tests were performed for AFP(−)-HCC *versus* CK. Differentially expressed proteins (DEPs) were considered based on a fold-change of ≥1.5 or ≤0.067 and a *p*-value less than 0.05. Next, DEPs were annotated based on the Gene Ontology Consortium (GO) (9), including cellular component, biological process, and molecular functions, and their systematic information was computed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation (10).

2.4 Enzyme-Linked Immunosorbent Assay

The 14-3-3 zeta recombinant protein was diluted in PBS to a final concentration of 0.5 μg/ml and used to coat a 96-well microtiter plate (Corning 3590, USA), which was incubated at 4°C overnight. The antigen-coated wells were blocked with 10% fetal bovine serum at 37°C for 1 h. Human serum (diluted 1:200) was incubated in the antigen-coated wells for 60 min. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd., China) as a secondary antibody was diluted to 1:10,000 for coating (60 min). Then, it was washed with PBS containing 0.1% Tween 20. A 3,3,5,5-tetramethylbenzidine liquid substrate system (Beijing Solarbio Science & Technology Co., Ltd., China) was used as the detection agent. The optical density (OD) value of all wells was 450 nm. The cutoff value for defining a positive reaction was designated as the mean OD value of the

60 NHS samples plus three standard deviations (mean + 3 SD). Each microtiter plate included 10 NHS samples representing a range of absorbance above and below the mean of 60 NHS samples. The average OD value of 10 NHS samples was used to normalize all OD values to the standard mean of the 60 normal samples. Each sample was tested in triplicate.

2.5 Western Blotting

The purified 14-3-3 zeta recombinant protein was electrophoresed on a 12% SDS-PAGE gel and subsequently transferred to a nitrocellulose membrane. After blocking in TBS with 5% non-fat milk and 0.1% Tween-20 for 1 h at ambient temperature (25°C), the membrane was cut into strips and incubated with patient sera diluted at 1:200 or polyclonal anti-14-3-3 zeta antibody diluted at 1:500. The membranes were finally incubated with HRP-conjugated goat anti-human IgG or HRP-conjugated goat anti-rabbit IgG diluted at 1:10,000 for 1 h. Positive signals were detected using an ECL kit (Thermo Scientific, Waltham, MA, USA) according to the instructions of the manufacturer.

2.6 Indirect Immunofluorescence Assay

An indirect immunofluorescence assay was performed on a Hep-2 cell substrate slide (Medical and Biological Laboratories Co., Ltd.). The sera were diluted to 1:40 with PBS at pH 7.4 and incubated with the slides for 30 min at ambient temperature, followed by extensive washing with PBS. The slides were then incubated with fluorescein isothiocyanate-conjugated goat anti-human IgG secondary antibody for 20 min at ambient temperature and thoroughly washed with PBS. Subsequently, a drop of mounting medium containing 4,6-diamidino-2-phenylindole (Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd., China) was added. The slides were examined under a fluorescence microscope in the dark using a Nikon ECLIPSE Ti at ×200 magnification. Related software was used for image capture and analysis.

Continuous variables are expressed as the mean ± SD or median (25th and 75th percentiles). Count data were described as frequencies. The mean OD value in each group of patient sera was compared using the Mann–Whitney *U* test or one-way analysis of variance. The frequency of anti-14-3-3 zeta autoantibody in each group of patient sera was compared using the chi-squared (χ^2) test with Yate's correction. Two significant levels (0.05, 0.01) were used.

3 RESULTS

3.1 Differentially Expressed Proteomics of *In Situ* AFP-Negative HCC and Adjacent Non-Tumor Liver Tissue

In this project, we identified protein expression *in situ* using AFP-negative HCC tissue [AFP(−)-HCC] and adjacent non-tumor liver tissue (control group, CK). In total, 2,506 proteins were identified with a global FDR of 1% from 17,645 distinct peptides. Then, those identified proteins were quantified by the SWATH label-free method, and quality control was performed. After normalizing the protein areas (**Figure 1A**), *t*-test and clustering analysis results showed good tissue specificity (**Figures 1B, C**). In addition, there were 592 DEPs between AFP(−)-HCC and CK with 175 upregulated and 417 downregulated (adjusted *p*-value <0.05, fold-change ≥1.5 or 0.67; **Figure 1D**).

Cellular component results showed diverse locations of DEPs (**Figure 2A**). Specifically, 510 DEPs primarily located in the cytoplasm, followed by 487 DEPs located in intracellular organelles. Furthermore, 427 DEPs located in the intracellular organelle part. Moreover, 313 DEPs located in vesicles, 288 DEPs located in extracellular exosomes, and 286 DEPs located in extracellular membrane-bounded organelles. A few other DEPs located in intracellular vesicles, bounding membrane of organelles, and plasma membrane bounded cell projections, among others.

Biological processes of DEPs were also analyzed, as shown in **Figure 2B**. Here, 279 DEPs primarily involved in transport, followed by 212 DEPs involved in cellular catabolic processes, 200 DEPs involved in cellular response to chemical stimulus, 195 DEPs involved in organic substance catabolic processes, 193 DEPs involved in response to organic substances, 192 DEPs involved in molecular complex subunit organization, 179 DEPs involved in establishment of localization in cells, 167 DEPs involved in protein localization, and 162 DEPs involved in cellular component assembly. A few other DEPs involved in organic acid metabolic processes, cellular macromolecule localization, and single-organism cellular localization, among others.

In molecular function enrichment results (**Figure 2C**), binding and ligase activity were the predominant pathways. Ligase activity pathways comprised cholate-CoA ligase activity, arachidonate-CoA ligase activity, phytanate-CoA ligase activity, and medium-chain fatty acid-CoA ligase activity, with high rich factors but few DEPs. Binding was the major function of DEPs between AFP(−)-HCC and CK, including uracil binding, pyrimidine nucleobase binding, protein binding, poly (A) RNA binding, binding, RNA binding, and especially protein binding, which was associated with a significant *p*-value. 14-3-3 zeta (H0YB80) was one of those DEPs, and it performs protein domain-specific binding. It was reported that 14-3-3 zeta is a signaling molecule with discrete phosphoserine/threonine-binding modules that regulate tumor progression (11). Our previous studies also demonstrated that the sensitivity and specificity of immunologic diagnosis could be up to 69.7% and 83.0%, respectively, with 14 TAAs based on a microarray that included 14-3-3 zeta (12). Therefore, 14-3-3 zeta was considered a candidate TAA for AFP-negative HCC tests.

KEGG pathways for DEPs were analyzed and enriched (**Figure 3**). Metabolic pathways and ribosome were the most enriched pathways, followed by the spliceosome, pathogenesis, *Escherichia coli* infection, endocytosis, RNA transport, the mTOR signaling pathway, and PPAR signaling, among others. Signaling pathway molecules generally undergo phosphorylation. 14-3-3 proteins, as bridging molecules, can

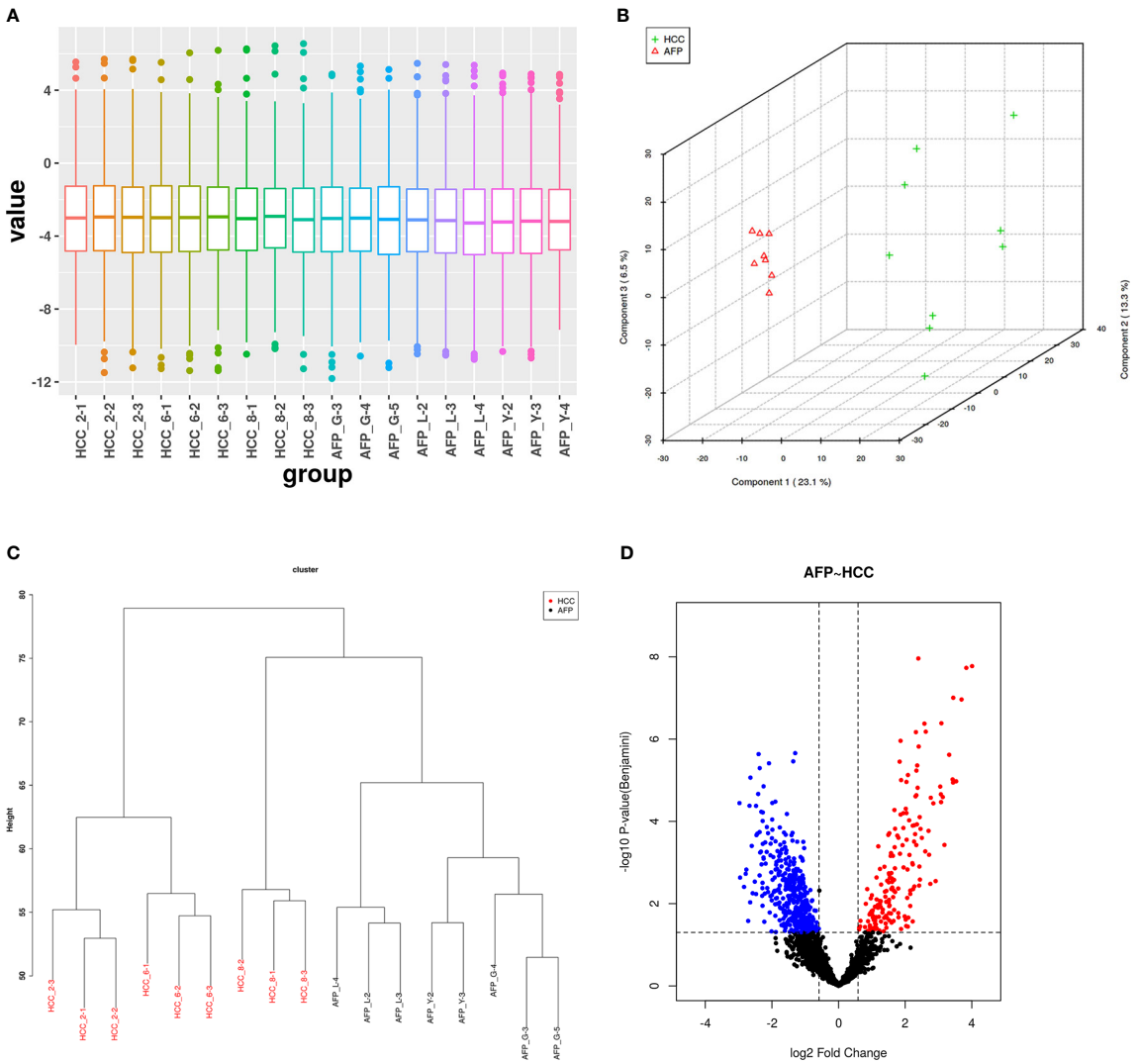

FIGURE 1 | Quality control of SWATH quantification areas between the alpha-fetoprotein (AFP)-negative hepatocellular carcinoma (HCC) tissue and adjacent tissues. **(A)** Statistical results of normalized areas. There was significant specificity between the AFP-negative HCC tissue and adjacent tissues by principal component analysis (PCA) and clustering analysis **(B, C)**. **(D)** Differentially expressed proteins between the HCC tissue and adjacent tissues.

bind to phosphorylated protein molecules to transmit signals (13). If the phosphorylation of pathway molecules is inhibited, the binding fails and signal transmission is blocked. Therefore, 14-3-3 proteins play an important role in signal transduction and could be used as potential TAA-associated molecules.

3.2 Serological Verification in Serial Serum Samples

Autoantibody to 14-3-3 zeta in serial blood samples from the AFP-negative HCC patient was also tested using Western blotting. The results are shown in **Figure 4**. A 61-year-old AFP-negative HCC patient was diagnosed with HCC on January 18, 2016. In this study, the anti-14-3-3 zeta autoantibody appeared in the serum 9 months before the diagnosis of the tumor and gradually increased as the size of the nodule increased from 6 to 14.02 mm, which was subsequently proven to be a tumor nodule. However, there was no significant trend for AFP values, which were less than the upper limit of the normal level.

3.3 Baseline Characteristics of Patients in the LC, CH, and NHS Groups

The baseline characteristics of patients in the LC, CH, and NHS groups are summarized in **Table 1**.

3.4 Frequency and Titer of Autoantibody Against 14-3-3 Zeta in Patients With LC

The 14-3-3 zeta recombinant protein was used as a coating antigen in enzyme-linked immunosorbent assay (ELISA) to screen for an autoantibody against 14-3-3 zeta in sera from

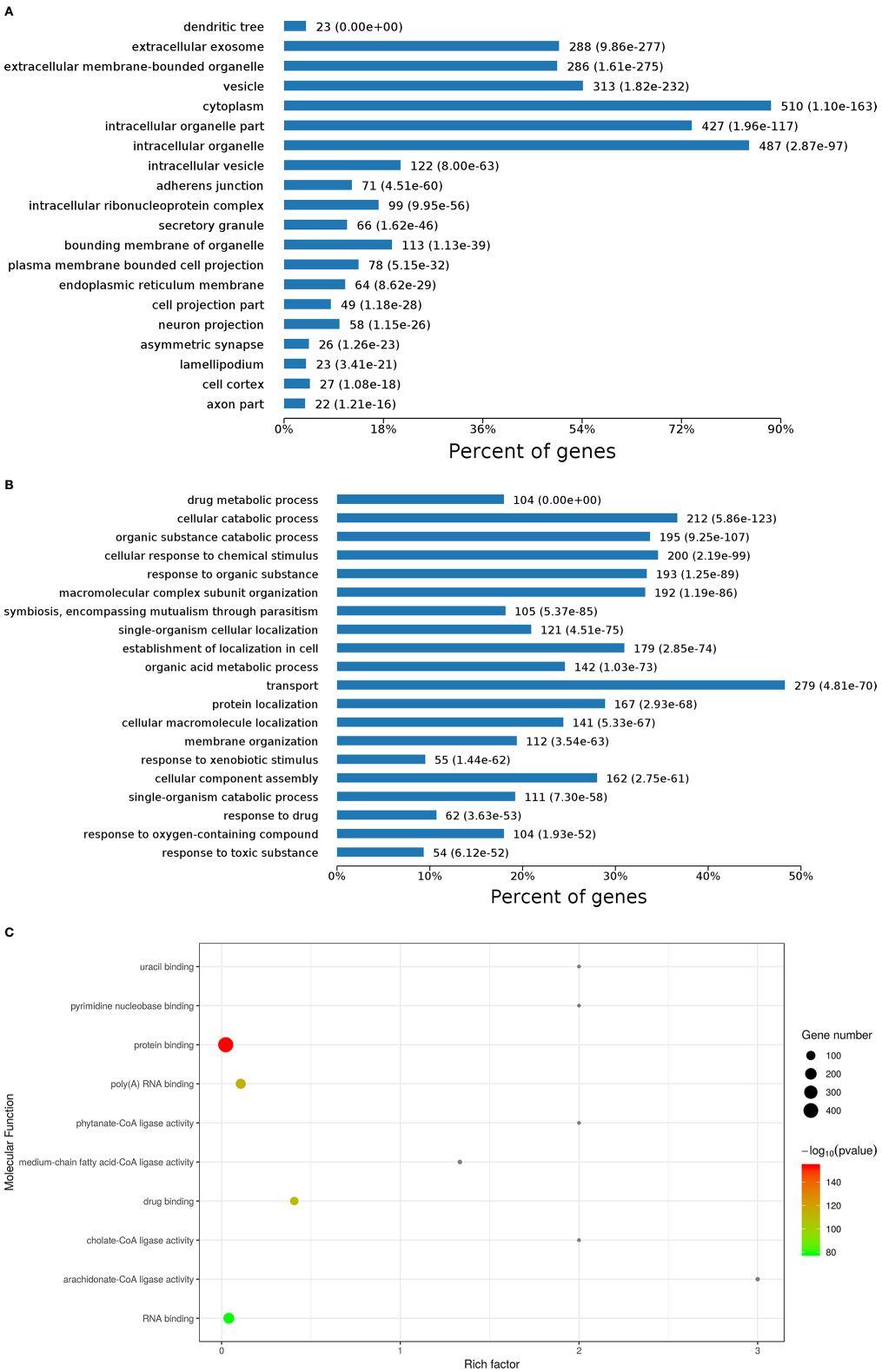

FIGURE 2 | Gene ontology (GO) analysis results of differentially expressed proteins (DEPs) including cellular component **(A)**, biological process **(B)**, and molecular function **(C)** analysis and enrichment.

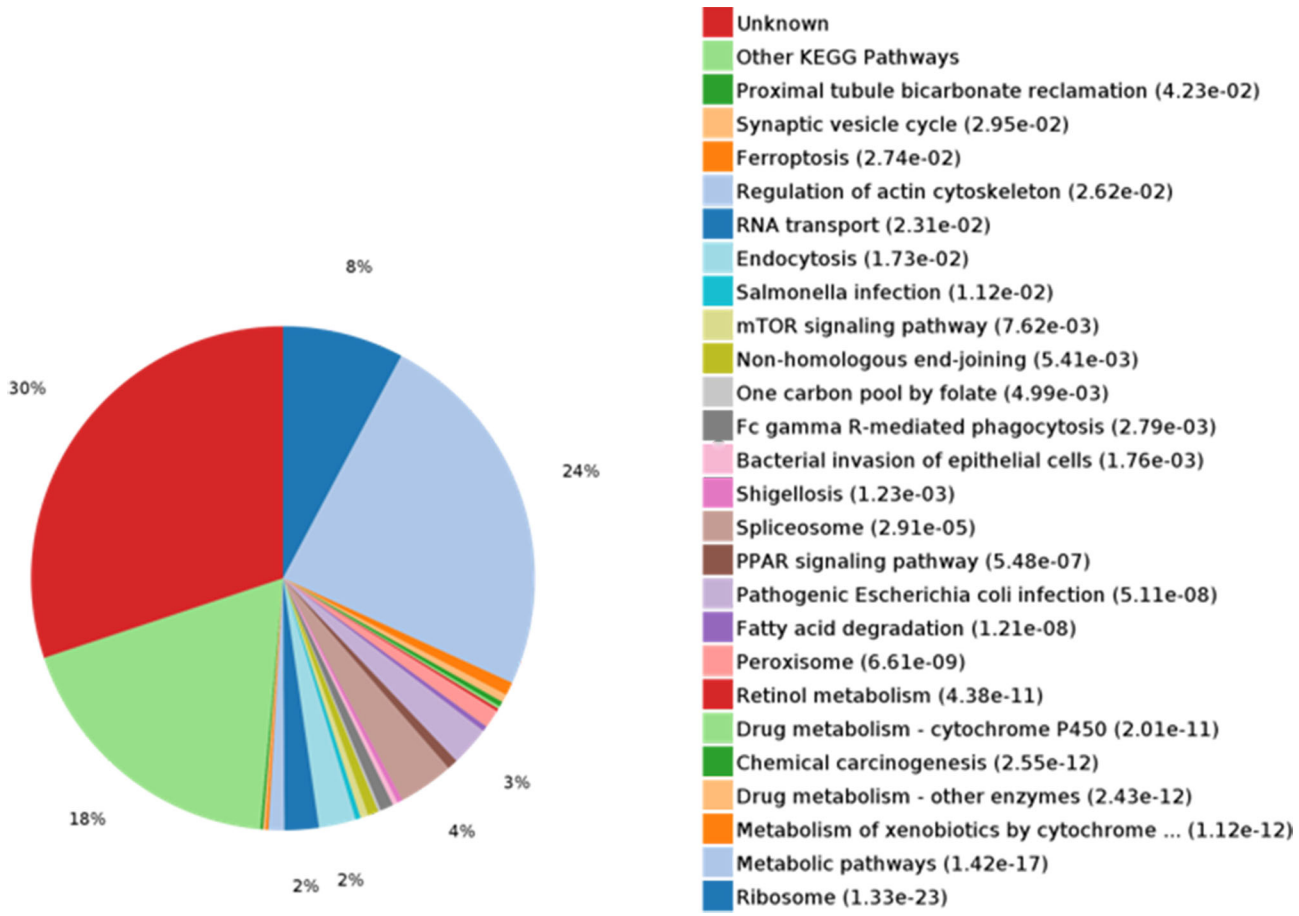

FIGURE 3 | KEGG pathway analysis and enrichment of differentially expressed proteins (DEPs). KEGG metabolic pathway analysis showed that the metabolic pathways were rich in proteins (24%).

patients with LC, CH, and NHS. As shown in **Table 2**, the prevalence of autoantibodies against 14-3-3 zeta was 16.1% (15/93) in LC, which was significantly higher than that in CH and NHS (LC *versus* CH, $p = 0.000$; LC and NHS, $p = 0.01$). The titers of anti-14-3-3 zeta autoantibody in sera from the three groups are shown in **Figure 5**. The average titer of anti-14-3-3 zeta autoantibody in sera from LC patients was much higher than that in CH patients and NHS (LC *versus* CH, $p = 0.000$; LC and NHS, $p = 0.000$). These results were confirmed using Western blot analysis. **Figure 6** shows that representative LC sera with a positive reaction to 14-3-3 zeta based on ELISA showed stronger reactivity in Western blotting than CH and normal sera.

3.5 Detection of Intense Nuclear Staining Pattern in Hep2 Cells Using Indirect Immunofluorescence Assay With Representative Positive LC Sera

To further confirm the reactivity of the 14-3-3 zeta autoantibody in LC sera and the intracellular location of 14-3-3 zeta, commercially purchased Hep2 cell slides were used in an indirect immunofluorescence assay to detect LC sera with a positive anti-14-3-3 zeta autoantibody, as shown by ELISA. As shown in **Figure 7**, representative anti-14-3-3 zeta autoantibody-positive LC sera resulted in an intense cytoplasmic staining pattern, which was similar to the fluorescent staining pattern and cellular location shown with the polyclonal anti-14-3-3 zeta autoantibody.

4 DISCUSSION

Many studies have identified the presence of autoantibodies in human sera, not only in systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (14–16), but also in non-autoimmune diseases such as cancer (17, 18). Although the mechanism underlying the production of such autoantibodies remains poorly understood, they are commonly used as invaluable tools for clinical detection in some diseases (19–22). In recent years, many novel TAAs and autoantibodies, such as p53, p62, and p90, have been isolated and characterized from patients with HCC (23–26). These are potential biomarkers for the early diagnosis of HCC, and a

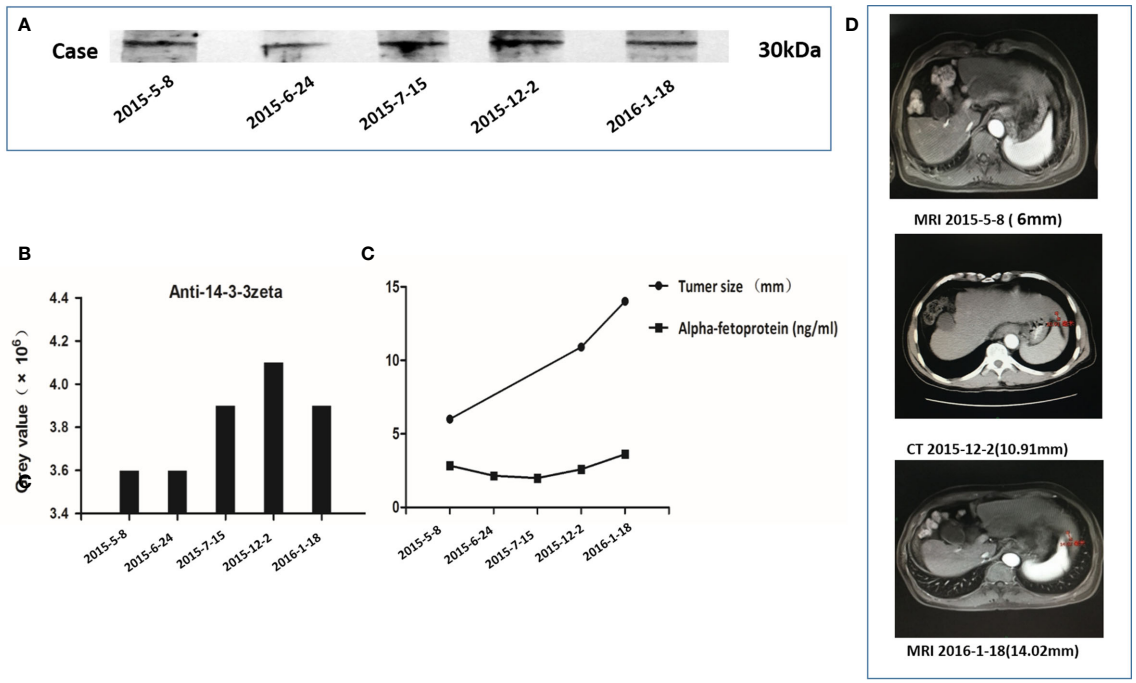

FIGURE 4 | Autoantibody to 14-3-3 zeta in serial serum samples from an alpha-fetoprotein (AFP)-negative hepatocellular carcinoma (HCC) patient. **(A)** The HCC patient was diagnosed on January 18, 2016, with a strong 14-3-3 zeta reactive band. **(B)** During a 9-month period (May 8, 2015, to January 18, 2016), five serum samples obtained from this patient showed a gradual increase in the 14-3-3 zeta reactive band. A stronger reactive band could be observed in the serum 9 months before the diagnosis of HCC. **(C)** The trends in gray values of five serum sample reactive bands were compared with 14-3-3 zeta and AFP values in five serum samples, as well as the tumor nodule size, at three corresponding time points. The gray values and tumor sizes corresponding to the serial serum samples show the same increasing trend. No significant trend in AFP values among the five serum samples was observed, and these values were less than the upper limit of the normal level. **(D)** Imaging data of three corresponding time points of serial serum sampling shows that the size of the nodule gradually increased from 6 to 14.02 mm and was subsequently proven to be a tumor nodule.

TABLE 1 | Baseline characteristics of patients in the LC, CH, and NHS groups.

Variables	LC (*n* = 93)	CH (*n* = 75)	NHS (*n* = 60)
Age (years)	52 ± 13	42 ± 14	39 ± 12
Sex, male/female	70/23	59/16	25/35
HBV/HCV/BC/NBNC	44/23/3/23	56/16/0/3	–
ALT, U/L	37.6 (21.0, 60.7)	76.7 (29.5, 310.4)	–
AST, U/L	48 (31.15, 79.5)	45.6 (25.6, 142.9)	–
TBIL, μmol/L	30.0 (19.95, 51.15)	17.1 (13.1, 38.5)	–
DBIL, μmol/L	7.3 (4.6, 19.75)	4.5 (2.6, 17.2)	–
ALB, g/L	34.2 ± 5.9	41.3 ± 4.7	–
CR, μmol/L	60 (51.3, 70.35)	66.7 (59.5, 73.8)	–
INR	1.2 (1.065, 1.4)	–	–
PT, S	13.5 (11.95, 15.85)	–	–
AFP, ng/ml	4.33 (2.54, 8.935)	–	–
Child–Pugh score	7 (6, 9)	–	–
Child–Pugh grade, A/B/C	61/25/7	–	–
Meld score	11.0 (9, 15)	–	–
Encephalopathy non-/1–2/3–4	85/7/1	–	–
Ascites degree none/low/medium/high	34/49/7/3	–	–

Continuous variables are expressed as the mean ± SD or medians (25th and 75th percentiles); count data are described as frequency.
LC, liver cirrhosis; CH, chronic hepatitis; NHS, normal human sera.

TABLE 2 | Frequency of autoantibody against 14-3-3 zeta in human sera based on ELISA.

Type of sera	No. of tested	Autoantibody to 14-3-3 zeta (%)	*p*-value
LC	93	15 (16.1)	–
CH	75	0 (0)	0.001 (LC *versus* CH)
NHS	60	1 (1.7)	0.01 (LC *versus* NHS)

Cutoff value, mean + 3 SD of NHS.
LC, liver cirrhosis; CH, chronic hepatitis; NHS, normal human sera.

mini-array of multiple TAAs can enhance autoantibody detection for the diagnosis of HCC (27, 28). In addition, studies have shown that some autoantibodies to TAAs can be observed in the serum 6–9 months before the clinical diagnosis of HCC (29, 30), which means that autoantibodies to TAAs might predict hepatocarcinogenesis. Currently, more than 60% of HCC patients cannot obtain timely diagnosis and treatment, which leads to a high mortality rate, especially for very early

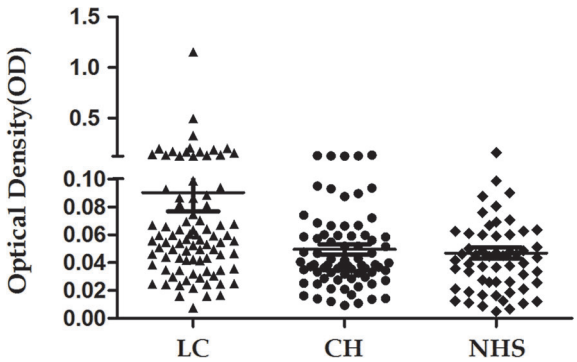

FIGURE 5 | Titer of autoantibody against 14-3-3 zeta in human sera determined by ELISA. The range of antibody titers to 14-3-3 zeta was expressed as that obtained by ELISA. The mean ± 3 SD of normal human serum (NHS) samples is shown in relation to values for all serum samples. The titer of anti-14-3-3 zeta autoantibody in liver cirrhosis (LC) was much higher than that in other types of sera (p = 0.000).

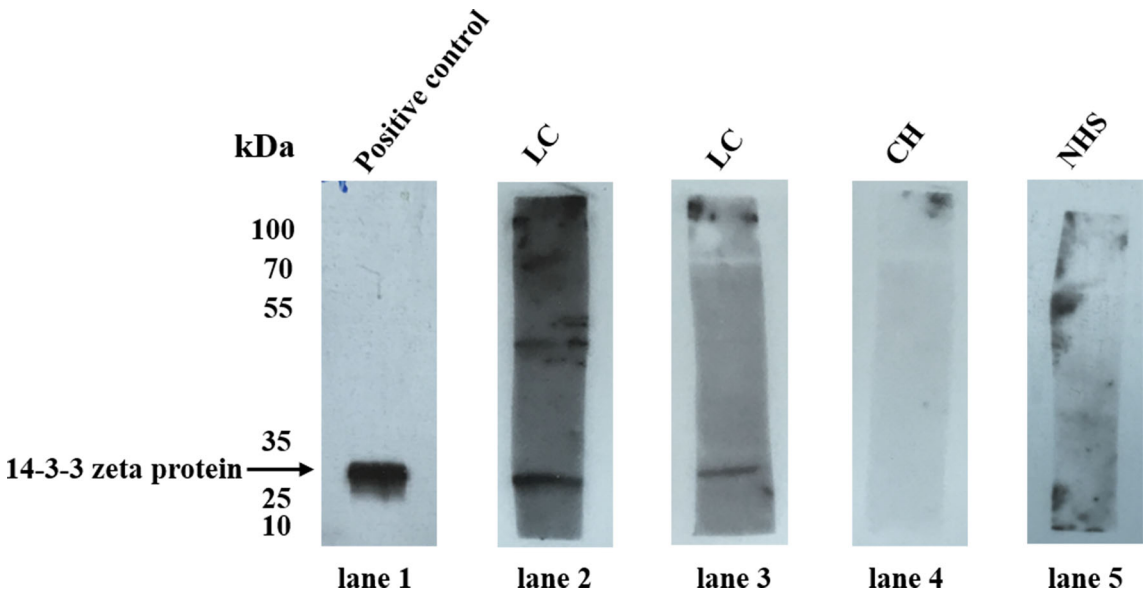

FIGURE 6 | Western blot analysis of representative sera assessed by ELISA. Lane 1, the polyclonal anti-14-3-3 zeta autoantibody was used as a positive control; lanes 2 and 3, two representative liver cirrhosis (LC) serum samples that were positive by ELISA also showed strong reactivity with 14-3-3 zeta recombinant protein by Western blot analysis; lanes 4 and 5, randomly selected chronic hepatitis (CH) sera and normal human serum (NHS) samples, respectively, with negative reactivity to 14-3-3 zeta recombinant protein.

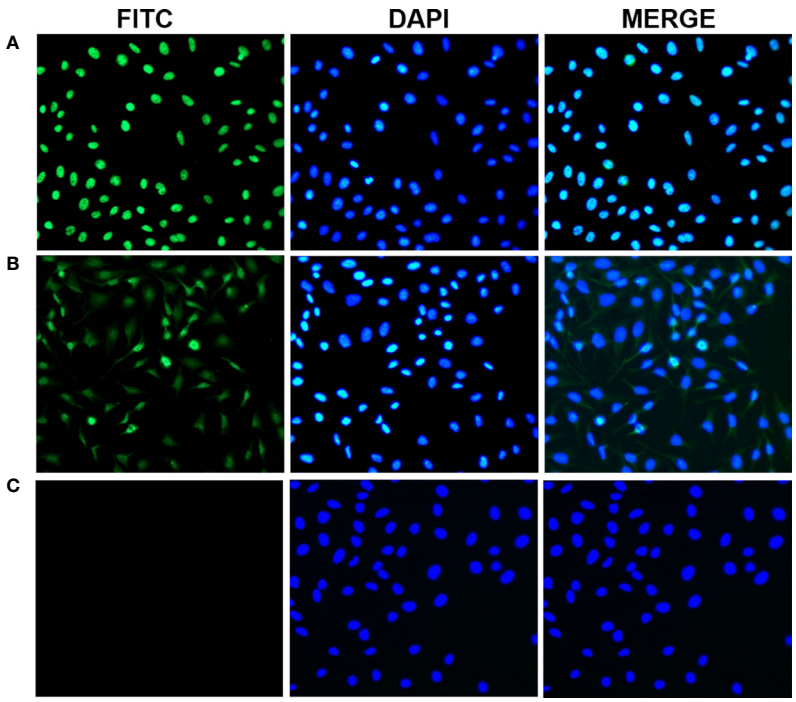

FIGURE 7 | Representative immunofluorescence staining pattern of anti-14-3-3 zeta autoantibody with positive liver cirrhosis (LC) serum. **(A)** A polyclonal anti-14-3-3 zeta antibody that showed a cytoplasmic immunofluorescence staining pattern was used as a positive control. **(B)** A representative anti-14-3-3 zeta autoantibody-positive LC serum sample demonstrated an intense staining pattern. **(C)** normal human serum (NHS) was used as a negative control.

HCC and AFP-negative HCC. Therefore, screening novel autoantibodies to TAAs for AFP-negative HCC and very early HCC is still an important task.

In this study, we aimed to screen and identify novel proteins in AFP-negative HCC and evaluated the feasibility of using the autoantibodies to those proteins to predict hepatocarcinogenesis. Firstly, we screened and identified DEPs between AFP-negative HCC tissue and adjacent non-tumor liver tissue using SWATH-MS proteome technology, which included 14-3-3 zeta protein, a protein-binding protein. The 14-3-3 proteins are a group of highly conserved acidic proteins encoded by different genes. There are seven distinct isoforms, β, γ, ϵ, ζ, η, σ, and τ, in mammals. The 14-3-3 isoforms have been linked to carcinogenesis, as they can be found in conjunction with various target proteins *via* phosphorylation-dependent or non-dependent phosphorylation reactions, and are involved in all human physiological reactions, including cell cycle regulation, signal transduction, apoptosis, proliferation, and differentiation (31). Studies have shown that 14-3-3 zeta, a 14-3-3 protein family member, is overexpressed in various tumor types, including HCC tissue samples (32, 33). In one study, the researchers investigated the association between serum 14-3-3 isoforms and HCC progression and prognosis. They found that serum 14-3-3 β is a potential biomarker for the diagnosis of early-stage HCC and associated with metastasis and poor prognosis. There was no statistical difference in the serum levels of 14-3-3 ϵ, γ, and ζ between HCC and control groups (34). However, our previous studies have shown that the level of anti-14-3-3 zeta autoantibody is significantly higher in the sera of patients with HCC than in those of patients with other chronic liver diseases and NHS. In addition, anti-14-3-3 zeta autoantibody was detected 9 months before the clinical diagnosis of HCC in several patients *via* serial blood sampling (35). These results suggest that anti-14-3-3 zeta autoantibody is a potential biomarker for early-stage HCC screening and diagnosis. However, there are few screening studies on AFP-negative HCC and very early HCC.

By further serological verification, one patient was diagnosed with AFP-negative HCC. The outcome of serial serum sampling showed that the anti-14-3-3 zeta autoantibody appeared in sera 9 months before the diagnosis of HCC and gradually increased as the size of the nodule increased, which was subsequently proven to be a tumor nodule. However, there was no significant trend in AFP values. The levels of AFP in five serial serum samples were less than the upper limit of the normal level. The results suggest that AFP might not be an ideal biomarker for the detection of HCC. Several studies have shown that the sensitivity and specificity of AFP for HCC diagnosis is not optimal, as 40% of HCC cases were not detected by screening for AFP (36, 37). The findings in the present study suggest that positive anti-14-3-3 zeta autoantibody might be a biomarker of carcinogenesis in AFP-negative HCC patients. This confirms our previous findings that indicate that anti-14-3-3 zeta autoantibody could be a potential biomarker for early HCC diagnosis. Concerning AFP-negative HCC patients, the presence of an anti-14-3-3 zeta autoantibody can be detected far earlier than the manifestation of disease based on imaging.

Studies on the level of serum autoantibodies during liver fibrogenesis have shown that this is a potential method for the identification of biomarkers in premalignant liver disease. For example, metallopeptidase inhibitor 1 (TIMP-1), α-2-macroglobulin, and hyaluronic acid (HA) have been identified as serum molecules (38, 39). However, these markers are not liver-specific. To date, few studies have been performed using autoantibodies as biomarkers in premalignant liver disease to identify those who might be at risk of developing HCC. The appearance of anti-14-3-3 zeta autoantibody in the LC stage in patients with HCC indicates that anti-14-3-3 zeta autoantibody might be predictive of hepatocarcinogenesis in premalignant liver disease. Based on these findings, we attempted to detect the prevalence of serum autoantibodies against 14-3-3 zeta in premalignant liver disease. We found that the prevalence of autoantibodies against 14-3-3 zeta protein was significantly higher than that in chronic hepatitis and normal human sera. Therefore, we suggest that anti-14-3-3 zeta autoantibody could be a biomarker to predict hepatocarcinogenesis. Further follow-up of and research on patients with positive autoantibodies will be continued to confirm the relationship between anti-14-3-3 zeta autoantibody and hepatocarcinogenesis.

One limitation of this study is that the 14-3-3 zeta construct, GST-14-3-3 WT (plasmid ID: 1944), in this study was created from mouse origin because a human origin 14-3-3 zeta plasmid could not be obtained. Actually, this should not influence the results because human 14-3-3 zeta and mouse 14-3-3 zeta are highly homologous with only one amino acid difference. In addition, more time is needed to follow up on the outcome of patients with positive autoantibodies, and the serological changes in these patients should be observed to further determine the correlation with carcinogenesis.

CONCLUSION

In conclusion, the comprehensive use of serum biomarkers is a promising method to predict the development of HCC, especially early and AFP-negative liver cancer. Our study found that serum anti-14-3-3 zeta autoantibody is a potential non-invasive serum biomarker for predicting hepatocarcinogenesis, which might significantly improve the diagnosis efficiency for early and AFP-negative liver cancer and reduce the associated fatality rate to a certain extent.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028854.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of Capital Medical University, Beijing, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contributions to the work and approved it for publication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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TC2N: A Novel Vital Oncogene or Tumor Suppressor Gene In Cancers

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Several C2 domain-containing proteins play key roles in tumorigenesis, signal transduction, and mediating protein–protein interactions. Tandem C2 domains nuclear protein (TC2N) is a tandem C2 domain-containing protein that is differentially expressed in several types of cancers and is closely associated with tumorigenesis and tumor progression. Notably, *TC2N* has been identified as an oncogene in lung and gastric cancer but as a tumor suppressor gene in breast cancer. Recently, a large number of tumor-associated antigens (TAAs), such as heat shock proteins, alpha-fetoprotein, and carcinoembryonic antigen, have been identified in a variety of malignant tumors. Differences in the expression levels of TAAs between cancer cells and normal cells have led to these antigens being investigated as diagnostic and prognostic biomarkers and as novel targets in cancer treatment. In this review, we summarize the clinical characteristics of TC2N-positive cancers and potential mechanisms of action of TC2N in the occurrence and development of specific cancers. This article provides an exploration of TC2N as a potential target for the diagnosis and treatment of different types of cancers.

Keywords: TC2N, tumor-associated antigens (TAAs), cancer, signal pathway, molecular mechanism, functional characterization, clinical feature

Abbreviations: TC2N, tandem C2 domains nuclear protein; TAAs, tumor-associated antigens; CDK5, cyclin-dependent kinase 5; P21(CDKN1A) , cyclin dependent kinase inhibitor 1A; P53, tumor protein p53; BAX, a member of the B-cell lymphoma-2(BCL2) gene family; BCL1, B-cell lymphoma-1; IκB, inhibitor of NF-κB; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MMP7, matrix metalloproteinase 7; MMP9, matrix metalloproteinase 9; ALK, anaplastic lymphoma kinase; EBP1, ErbB-3 binding protein 1; AKT, serine threonine kinase; GSK3β, glycogen synthase kinase-3β; PTEN, phosphatase and tensin homolog deleted on Chromosome 10; MMP2, matrix metalloproteinase 2; GALNT3, polypeptide N-acetylgalactosaminyltransferase 3; RBM47, RNA binding motif protein 47.

1 INTRODUCTION

Cancer is an important public health concern worldwide and continues to be of great interest to the scientific community. It is one of the leading causes of death, with approximately 14 million new cases and 8.2 million cancer-related deaths occurring in 2018 (1). This disease is considered the biggest barrier to improving life expectancy in countries in the 21st century (2). Annually, over 4 million new cancer patients and over 2 million cancer-related mortalities are reported in China. Despite the availability of multiple treatment modalities such as surgery, chemotherapy, radiation therapy, and targeted therapy, the 3- and 5-year cancer-specific survival rates remain poor (3–7). While overall cancer related mortalities have decreased (8), it is notable that the reduction in mortality is largely due to early detection and prevention rather than development of better treatment options (9–12).

Most cancers are asymptomatic in the early stages of development (13, 14) largely because of their ability to evade immune surveillance (15, 16). Immune evasion is thought to be driven by two major mechanisms. First, owing to altered antigen presentation or receptor library editing, the immune system is unable to detect tumor populations. Second, the initially effective immune response may become ineffective owing to the presence of an immunosuppressive tumor microenvironment (17–19). Therefore, it is important to explore mechanisms of cancer development to identify new markers for diagnosis and prognosis and to develop effective and novel treatment methods. Developments in both fronts will have substantial implications for improving survival rates of cancer patients.

Numerous studies have shown that certain genes, such as oncogenes and tumor suppressor genes, are risk factors for many types of cancer (20–24). When activated, oncogenes stimulate tumor growth whereas tumor suppressor genes prevent tumor growth and development. In mouse models, where oncogene expression is driven by tissue-specific promoters, tumors appear at high frequency but disappear when the inductive stimulus is turned off (25–27), suggesting that oncogenes are the Achilles' heel of cancer (28). Tumor suppressor genes play a critical role in controlling the cell cycle assuring proper proliferation and differentiation (29). Therefore, identifying these genes is crucial because targeting them may prevent or treat different types of cancers.

The C2 domain was initially thought to be a protein structural domain of calcium-dependent protein kinase C (30–32). Further studies confirmed that the function of the C2 domain was not only calcium-dependent phospholipid binding, but also involved in cellular signal transduction and protein-protein interactions (33). Several proteins that contain a structural domain called the C2 domain have been linked to the regulation of tumorigenesis. For example, a C2 domain-containing protein, DOC2B, plays a tumor-suppressive role in cervical cancer by inhibiting cell proliferation, migration, and invasion (34). Conversely, another C2 domain-containing protein, myoferlin, plays a tumor-enhancing role by promoting metastasis in patients with triple-negative breast cancer (35). Gene encoding tandem C2 domains nuclear protein (TC2N)—a putative C2 domain-containing protein—has recently been shown to function both as an oncogene and a tumor suppressor gene (36–38). TC2N is located on human chromosome 14q32, belongs to the carboxyl-terminal type (C-type) tandem C2 protein family, and contains two C-terminal C2 domains (C2A and C2B) (39). *TC2N* is also an immune system gene similar to *IFI27*, *CASS4*, and *SMARCD3* (40). Given its tumorigenesis properties and its association with the immune system, it has been proposed as a potential target for the detection and treatment of various cancers. In this review, we summarize recent progress in understanding the role and underlying mechanisms of *TC2N* in the occurrence and development of cancer, with a focus on lung cancer, breast cancer, and gastric cancer.

2 TC2N IN CANCERS

TC2N expression is upregulated in different types of cancers, including lung, breast, and gastric cancers. The relevant clinicopathological features and the molecular mechanisms of *TC2N* in these cancers are summarized in **Table 1** and detailed in the rest of this section.

2.1 Lung Cancer

2.1.1 Functional Characteristics and Clinical Features of TC2N in Lung Cancer

TC2N is overexpressed in cancerous lung tissues and cell lines compared with that in normal lung tissues and a human bronchial epithelial cell line, respectively. Hao XL et al. (36) showed that upregulation of TC2N was significantly correlated with advanced TNM stage and high histological grade of disease. Additionally, high TC2N expression levels were associated with poor clinical outcomes and significantly short overall survival. Hence, TC2N expression has been proposed as an independent prognostic factor affecting patient survival. Mechanistically, overexpression of TC2N significantly inhibited apoptosis, promoted cell proliferation, and increased migration and invasion of tumor cells *in vitro*; in contrast, knockdown of TC2N promoted apoptosis and inhibited proliferation of lung cancer cells (41). Furthermore, knockdown of TC2N in tumor tissues resulted in an increase in apoptotic cells, supporting the hypothesis that TC2N overexpression promotes tumorigenesis and growth of lung cancer tumors *in vivo*.

In summary, *TC2N* is a potential novel oncogene in lung cancer, whose expression levels are correlated with cancer progression and patient survival. *TC2N* stimulates cell proliferation, migration, and invasion and reduces apoptosis of lung cancer cells *in vitro* and *in vivo*.

2.1.2 Signaling Pathways Influenced by TC2N in Lung Cancer

2.1.2.1 TC2N Inhibits p53 Signaling Pathway in Lung Cancer

Hao XL et al. (36) proposed that the regulation of cell proliferation, cell cycle, and apoptosis by TC2N is dependent on the p53 signaling pathway. *TP53*, which encodes p53, was initially classified as an oncogene due to its ability to transform cells (43–46). However, the identification of growth-inhibiting and temperature-sensitive mutants of p53 in sporadic cancer samples and familial cancers

TABLE 1 | Functional characteristics and clinical features of *TC2N* in human cancers.

Cancer types	Expression	Role	Functional role	Related genes	Clinical features	References
Lung cancer	Upregulated	Oncogene	Promotes proliferation, migration, and invasion and inhibits apoptosis	*CDK5, P53, P21, BAX, BCL1, IκB, NF-κB, MMP7, MMP9*	Advanced TNM stage, high histological grade, and poor clinical prognosis	(36, 41)
Breast cancer	Upregulated	Anti-oncogene	Inhibits proliferative and colony-forming abilities	*ALK, EBP1, P55γ, AKT, Caspase-3, GSK3β, MYC, BAD, PTEN*	Early clinical stage, small tumor size, low lymph node metastasis, high HER-2 positive rate, and good prognosis	(37)
Gastric cancer	Upregulated	Oncogene	Promotes proliferation, migration, and invasion	*MMP2, MMP9, CATSPERB, GALNT3, RBM47*	Advanced TNM stage, large tumor size, high histological grade, advanced distant metastasis, and poor prognosis	(38, 42)

TC2N, tandem C2 domains nuclear protein; CDK5, cyclin-dependent kinase 5; P21 (CDKN1A), cyclin dependent kinase inhibitor 1A; P53, tumor protein p53; BAX, a member of the B-cell lymphoma-2 (BCL2) gene family; BCL1, B-cell lymphoma-1; IκB, inhibitor of NF-κB; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MMP7, matrix metalloproteinase 7; MMP9, matrix metalloproteinase 9; ALK, anaplastic lymphoma kinase; EBP1, ErbB-3 binding protein 1; AKT, serine threonine kinase; GSK3β, glycogen synthase kinase-3β; BAD, Bcl-2-associated death promoter; PTEN, phosphatase and tensin homolog deleted on chromosome 10; MMP2, matrix metalloproteinase 2; GALNT3, polypeptide N-acetylgalactosaminyltransferase 3; RBM47, RNA binding motif protein 47.

has shown that p53 is in fact a tumor suppressor protein (47–53). p53 functions as the major regulator of central signaling and cell fate decision pathways (54). It is a nuclear transcription factor composed of 393 amino acids with four major functional domains: a transcriptional, a DNA binding, a tetramerization, and a regulatory domain (55). It modifies the expression of multiple genes involved in a variety of biological processes, including cell cycle, apoptosis, senescence, differentiation, and DNA repair (56–66). Moreover, p53 has been associated with diverse biological processes, such as regeneration (67), metabolism (68, 69), interaction with viruses (70), prevention of liver pathologies (71, 72), forming a barrier to stem cell formation (73, 74), endocrinology circuits (75) and serving as the guardian of the tissue hierarchy (76). p53 activity is largely controlled by post-translational modifications, such as phosphorylation (77). CDK5 is a protein kinase that phosphorylates p53 at Ser-15, Ser-33 and Ser-46 (78, 79) and binding of CDK5 to p53 induces activation of the p53 signaling pathway (77). Overexpression of TC2N interferes with CDK5-p53 interaction in the nucleus and induces significant CDK5 degradation by increasing the ubiquitination of CDK5 (**Figure 1**). Therefore, an increase in TC2N levels suppresses CDK5-induced p53 phosphorylation and p53 pathway activation. The expression of other key players in the p53 signaling pathway, such as P21, BAX, and BCL-2, is also downregulated by TCN2 (36). When cells experience stress or undergo uncontrolled division and proliferation, p53 is activated (56, 80). Under these conditions, p53 induces p21 expression, causing cell cycle arrest (81, 82). Furthermore, p53 triggers programmed cell death by triggering apoptosis-related genes, including *bax*, a pro-apoptotic member of the bcl-2 family, when a DNA damage cannot be repaired (83). Hence, reduction of the downstream players in the p53 pathway by TCN2 promotes proliferation and prevents apoptosis.

2.1.2.2 TC2N Promotes NF-κB Signaling Pathway in Lung Cancer

In addition to suppressing the p53 pathway, TCN2 was observed to affect another key signaling pathway in lung cancer cells (41). Over 30 years ago, Sen et al. (84) identified a protein that bound to a specific, conserved DNA sequence in the nucleus of activated B lymphocytes. The protein was named after the identified cell type and the gene it affected: nuclear factor binding near the k light-chain gene in B cells (NF-kB) (85). Since its discovery, NF-κB has been found to be involved in several key processes such as immune regulation, inflammation, cell survival, stress response, embryogenesis, differentiation, proliferation, and cell death (86–96) and it functions primarily by orchestrating the expression of many functionally diverse genes (85, 89, 93, 97, 98). Due to its extensive physiological effects, dysregulation of NF-κB can lead to severe consequences (99, 100), including cancer, neurodegenerative diseases, autoimmune diseases, cardiovascular diseases, and diabetes (85, 99–105).

Most lymphatic or solid tumors, including lung cancer, present with increased NF-κB levels (106). NF-κB in the nucleus is an indicator of active NF-κB signaling, and its levels correlate with the transcription of its target genes (107). Typically, NF-κB levels in the nucleus and its activity are regulated by inhibitor of NF-κB (IκB). IκB acts as a gatekeeper, limiting NF-κB migration into the nucleus by masking its nuclear localization domains (108, 109). Additionally, it prevents activation of NF-κB target genes by masking the DNA-binding domains of NF-κB (108), thereby leading to interruption of the NF-κB signaling pathway. Notably, overexpression of NF-κB—both in the nucleus and cytoplasm of lung cancer cells—correlated with increased expression level of TC2N in these cells (41). Hao XL et al. (41) proposed that this increase in NF-κB expression level is a direct consequence of TC2N overexpression in these cells (**Figure 1**). Overexpression of TC2N enhanced the phosphorylation of IκB but decreased the total IκB protein levels, leading to increased nuclear translocation of NF-κB and subsequent activation of the signaling pathway (41). Additionally, TC2N modulates this process through other downstream proteins in the pathway such as MMP7 and MMP9 (41).

2.2 Breast Cancer

2.2.1 Functional Characteristics and Clinical Features of TC2N in Breast Cancer

Similar to that in lung cancer tissues, the expression of TC2N was markedly upregulated in breast cancer tissues compared with that in adjacent non-cancerous tissues (37). However, unlike that in lung cancer, upregulated TC2N was associated with good

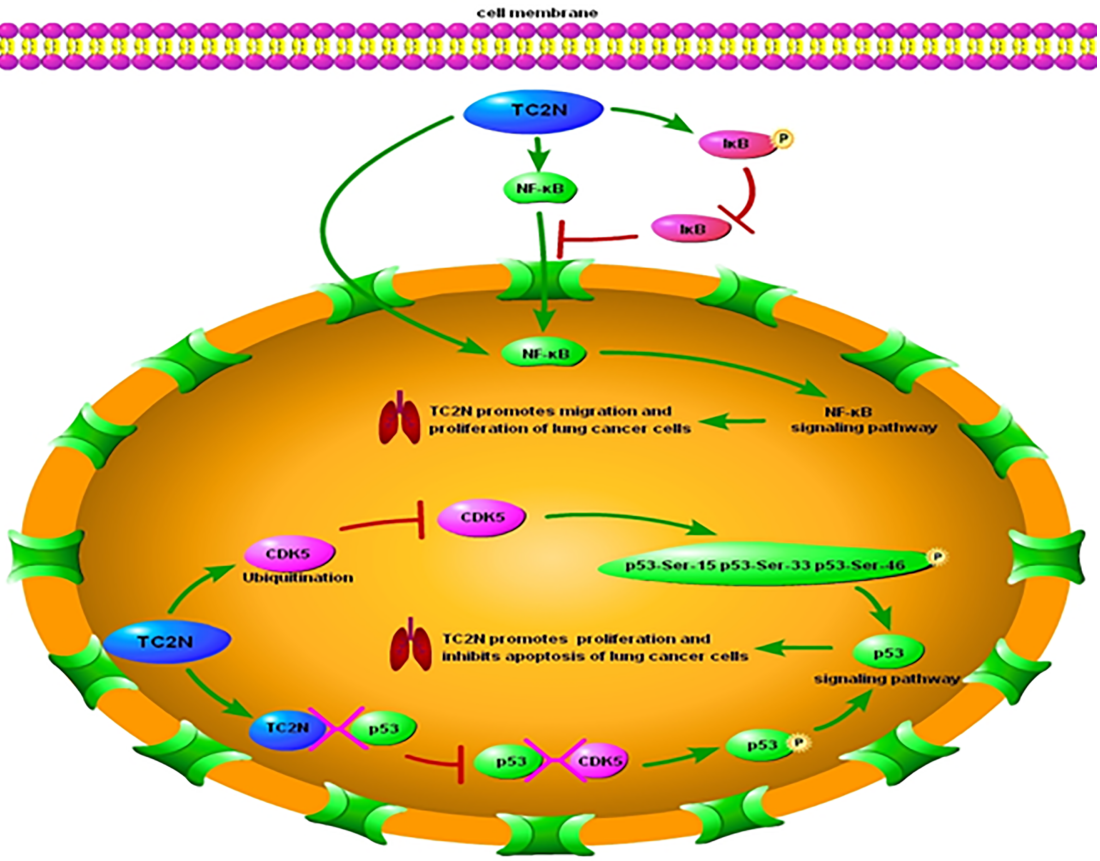

FIGURE 1 | Underlying molecular mechanisms of TC2N in p53 and NF-κB signaling pathways in lung cancer. TC2N, tandem C2 domain nuclear protein; CDK5, cyclin-dependent kinase 5; P53, tumor protein p53; IκB, inhibitor of NF-κB; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

prognosis and overall survival. It positively correlated with the early clinical stage of disease, small tumor size, low lymph node metastasis, and high human epidermal growth factor receptor 2 (HER-2) positivity rate. Additionally, upregulated TC2N inhibited the proliferative and colony-forming abilities of breast cells both *in vitro* and *in vivo*. In summary, in contrast to its role in lung cancer cells, TC2N is a potential tumor suppressor in breast cancer.

2.2.2 Signaling Pathways Influenced by TC2N in Breast Cancer

2.2.2.1 TC2N Inhibits PI3K/AKT Signaling Pathway in Breast Cancer

To explain the tumor suppressive function of TC2N in breast cancer cells, Hao XL et al. (37) proposed that upregulation of TC2N represses the Phosphoinositide 3-kinases/serine-threonine kinase (PI3K/AKT) signaling pathway, which is typically constitutively active in some human cancers (110, 111). PI3K/AKT is a growth-regulating cellular pathway and it is well established that PI3K/AKT signaling enhances tumor cell survival, proliferation, and motility in different tumor types (112–119). PI3Ks form a family of kinases that are expressed in almost all mammalian cells and play essential roles in survival, migration, cell cycle progression, and cell growth (120). PI3K phosphorylates phosphatidylinositol to form inositol lipid, which functions as a second messenger in the human body (121). Similarly, AKT is involved in various physiological processes and is a key regulatory protein for cell growth, survival, metabolism, and proliferation (116, 122–124). The pathogenesis of a variety of human cancers is associated with aberrant regulation of the PI3K/AKT pathway (125–127).

Anaplastic Lymphoma Kinase (ALK) is an activator of the PI3K/AKT signaling pathway, and it induces phosphorylation of the p55γ subunit of PI3K in cancer cells, rather than the usual p85 subunit that is phosphorylated (128, 129). It has been shown that the interaction between ALK and p55γ is crucial for ALK-induced p55γ phosphorylation (128) and subsequent activation of the PI3K/AKT signaling pathway. Another key regulatory step in the activation of PI3K/AKT signaling is phosphorylation of AKT by ErbB-3 binding protein 1 (EBP1). TC2N targets both these key steps to inhibit the PI3K/AKT signaling pathway. TC2N forms a complex with ALK, which prevents the ALK-p55γ interaction and therefore inhibits downstream AKT phosphorylation and consequently the PI3K-AKT signaling pathway (**Figure 2**). Additionally, TC2N inhibits the interaction of EBP1 with AKT, which is necessary for

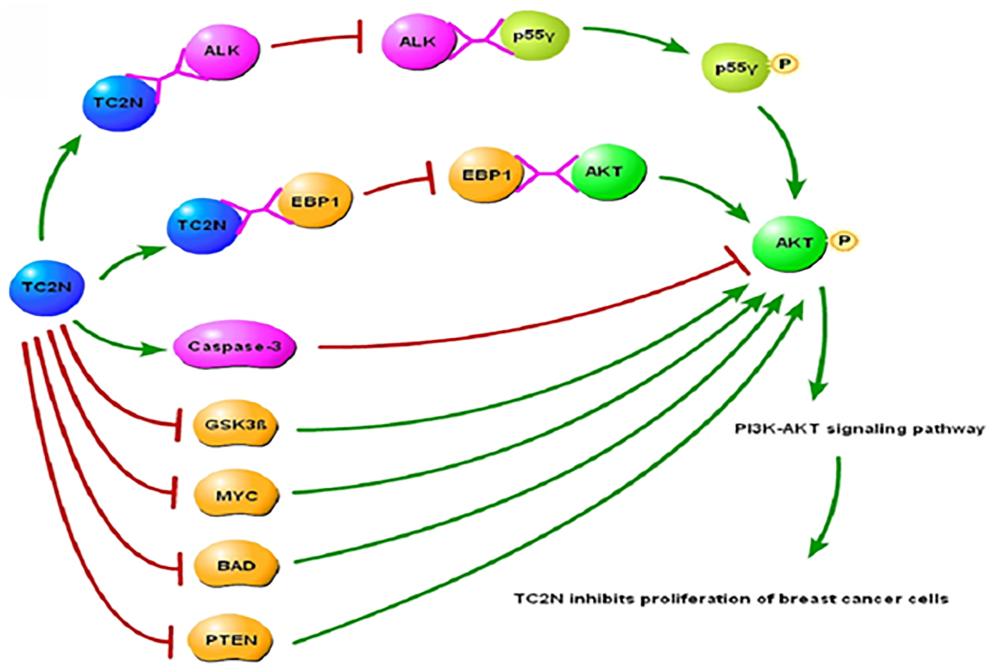

FIGURE 2 | Underlying molecular mechanisms of TC2N in PI3K-AKT signaling pathway in breast cancer. TC2N, tandem C2 domain nuclear protein; ALK, anaplastic lymphoma kinase; EBP1, ErbB-3 binding protein 1; AKT, serine threonine kinase; GSK3β, glycogen synthase kinase-3β; BAD, Bcl-2-associated death promoter; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

phosphorylation of AKT (130, 131) and subsequent PI3K/AKT signaling. Upregulation of TC2N has also been shown to activate AKT inhibitors such as caspase-3 and block AKT inhibitors such as GSK3β, MYC, BAD, and PTEN.

2.3 Gastric Cancer

2.3.1 Functional Characteristics and Clinical Features of TC2N in Gastric Cancer

Similar to lung and breast cancer cells, TC2N is overexpressed in a variety of gastric cancer cell lines and tumor samples compared to normal cells and tissues (38, 42). High TC2N levels were significantly correlated with poorly differentiated histological classification, large tumor size, advanced TNM stage, and advanced distant metastasis. Furthermore, patients with high TC2N expression showed poorer prognosis regardless of TNM stage compared to patients with low TC2N expression. *In vitro*, TC2N knockdown significantly inhibited the proliferation of gastric cancer cells, while TC2N overexpression promoted the growth of these cells. Similar results were observed *in vivo* where downregulation of TC2N inhibited the migration and invasion of gastric cancer cells, whereas overexpression had the opposite effect. Thus, similar to lung cancer, *TC2N* potentially functions as an oncogene in gastric cancer.

2.3.2 Signaling Pathway of TC2N in Gastric Cancer

The mechanism of action of TC2N in gastric cancer remains unclear. Shen L et al. (42) suggested that TC2N might partially affect the migration and invasion ability of gastric cancer by regulating the expression levels of MMP2 and MMP9. MMP2 and MMP9 are known to be involved in cell invasion and tumor metastasis (132). TC2N expression also showed strong positive correlation with the expression of CATSPERB and other cancer related genes such as *GALNT3* and *RBM47* (38). However, the detailed molecular mechanism by which TC2N promotes gastric cancer progression needs further evaluation.

3 CONCLUSION AND FUTURE PERSPECTIVES

High-throughput gene expression profiling facilitates the simultaneous measurement of the expression levels of thousands of genes. A key application of gene expression profiling in cancer is to identify differences in gene expression patterns between tumor and control samples (133). Advances in technology and the declining costs of DNA sequencing have spurred global efforts to discover differentially expressed genes in various cancers. From one such effort, TC2N was found to be widely upregulated in several human cancers, including lung, breast, and gastric cancers. TC2N levels were correlated with multiple clinicopathological features and prognosis, such as TNM stage, histological grade, tumor size, overall survival, lymph node metastasis, and distant metastasis. In support of its involvement in tumorigenesis and tumor progression, *in vitro and in vivo* experiments have shown that TC2N affects proliferation, apoptosis, migration, invasion of tumor cells and

tumor growth in many cancers. The underlying molecular mechanisms of TC2N in several cancers have also been preliminarily explored and suggest that TC2N modulates several key signaling pathways that influence carcinogenesis and cancer progression, including p53, NF-κB, and PI3K/AKT signaling pathways.

Although TC2N is a potential therapeutic target, several questions remain to be addressed. First, the molecular mechanism of TC2N in different types of cancers is not completely understood. For example, while preliminary data suggest that in gastric cancer TC2N modulates the expression of several cancer related genes, the specific pathway affected by TC2N is unclear. Furthermore, while the function of TC2N in lung, breast and gastric cancer have been studied to some extent, its potential role in other cancers, such as cancers associated with the urinary and reproductive systems remain unexplored. Second, while TC2N is upregulated in tumor tissues of some specific cancers, it is not known if TC2N is also upregulated in body fluids such as plasma and urine. The identification of diagnostic biomarkers is a promising avenue for early cancer diagnosis. If TC2N is detectable in plasma or urine, it may facilitate early detection and prognosis assessment using simple and non-invasive tests. Third, it is unknown if TC2N is a tumor-associated antigen and requires further evaluation. Fourth, TC2N is an immune system associated gene, but whether it can serve as a target in personalized immunotherapies remains to be seen. Therefore, more attention should be paid to the clinical value of TC2N in cancer diagnosis and treatment.

In summary, TC2N has been shown to have oncogenic or tumor-suppressive functions in different types of cancers, and could potentially serve as a cancer-specific molecular biomarker for early diagnosis, treatment, and prognosis assessment. While some progress has been made in the mechanistic analysis of TC2N, several questions remain unanswered. Future work needs to focus on understanding the precise molecular mechanism of TC2N in carcinogenesis and tumor progression to explore the potential clinical application of TC2N.

AUTHOR CONTRIBUTIONS

HL and TW contributed to the conception and design of the review. HL wrote the first draft of the manuscript. BL revised the manuscript. All authors contributed to the article and approved the submitted version

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A Diagnostic Model With IgM Autoantibodies and Carcinoembryonic Antigen for Early Detection of Lung Adenocarcinoma

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Immunoglobulin M (IgM) autoantibodies, as the early appearing antibodies in humoral immunity when stimulated by antigens, might be excellent biomarkers for the early detection of lung cancer (LC). We aimed to develop a multi-analyte integrative model combining IgM autoantibodies and a traditional tumor biomarker that could be a valuable and powerful auxiliary diagnostic tool and might improve the accuracy of early detection of lung adenocarcinoma (LUAD). A customized protein array based on cancer driver genes was constructed and applied in the discovery cohort consisting of 68 LUAD patients and 68 normal controls (NCs); 31 differentially expressed IgM autoantibodies were identified. The top 5 candidate IgM autoantibodies [based on the area under the receiver operating characteristic curve (AUC) ranking], namely, TSHR, ERBB2, survivin, PIK3CA, and JAK2, were validated in the validation cohort using enzyme-linked immunosorbent assay (ELISA), which included 147 LUAD samples, 72 lung squamous cell carcinoma (LUSC) samples, 44 small cell lung carcinoma (SCLC) samples, and 147 NCs. These indicators presented diagnostic capacity for LUAD, with AUCs of 0.599, 0.613, 0.579, 0.601, and 0.633, respectively ($p < 0.05$). However, none of them showed a significant difference between the SCLC and NC groups, and only the IgM autoantibody against JAK2 showed a higher expression in LUSC than in NC ($p = 0.046$). Through logistic regression analysis, with the five IgM autoantibodies and carcinoembryonic antigen (CEA), one diagnostic model was constructed for LUAD. The model yielded an AUC of 0.827 (sensitivity = 56.63%, specificity = 93.98%). The diagnostic efficiency was superior to that of either CEA (AUC = 0.692) or IgM autoantibodies alone (AUC = 0.698). Notably, the accuracy of this model in early-stage LUAD reached 83.02%. In conclusion, we discovered and identified five novel IgM indicators and developed a multi-analyte model combining IgM autoantibodies and CEA, which could be a valuable and powerful auxiliary diagnostic tool and might improve the accuracy of early detection of LUAD.

Keywords: lung adenocarcinoma (LUAD), IgM autoantibody, carcinoembryonic antigen (CEA), protein array, cancer driver gene, diagnostic model

INTRODUCTION

With estimates of 2.2 million new cases and 1.8 million deaths, lung cancer (LC) is the second most common cancer and the leading cause of cancer death worldwide, approximately accounting for one-tenth (11.4%) of cancer occurrence and one-fifth (18.0%) of cancer deaths (1). The 5-year survival rate is 57% for patients with localized tumors, while this decreased to 5% for patients at the metastatic stage (2), which indicates that the high mortality rate of LC is closely related to cancer stage. Traditionally, LC is classified into non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). There are two major types of NSCLC: lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) (3). LUAD, as the most common lung malignancy, is frequently found in women and non-smokers (3, 4). A large number of studies have shown that, if LC patients with adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA) could undergo radical surgery, their 5-year disease-free survival rate may approach 100% (5). Therefore, the early diagnosis and treatment of LUAD are essential to reduce the mortality of LC.

Currently, low-dose spiral CT (LDCT) and pathological tissue biopsy are used to screen and detect LC patients clinically, but the former has high false-positive rates and the latter is traumatic, which causes some excessive diagnosis, unnecessary tests, invasive procedures, and, rarely, radiation-induced cancers (6). In recent years, serological biomarkers have received widespread attention because of their advantages, such as being simple, noninvasive, and easily accepted by patients (7, 8). Traditional serum tumor markers had been used in the auxiliary diagnosis of cancers in clinical practice, such as carcinoembryonic antigen (CEA), cancer antigen 125 (CA-125), and cytokeratin-19 fragment (CYFRA 21-1), but their diagnostic ability was limited by their unsatisfactory:: sensitivity and specificity for LC (9, 10).

Tumors are the products of the malignant transformation of normal cells, which are characterized by continuous proliferation and metastasis in the body. The prominent feature of tumor cells in immunology is the appearance of certain tumor-associated antigens that are not visible or have low expressions in normal cells of the same type (11). Due to the presence of tumor-associated antigens, it is bound to be recognized by the body's immune system and thus stimulate adaptive immune responses, including cellular immunity and humoral immunity (12, 13). Immunoglobulin G (IgG) and immunoglobulin M (IgM) autoantibodies are produced as an important part of humoral immunity and are secreted into the blood. Recent researches have provided substantial evidence that patients with cancers could develop humoral immune response and then produce autoantibodies in the early stage even before cancer diagnosis (14, 15). Therefore, as the primary and secondary response products, IgM and IgG autoantibodies have great potential as early diagnostic indicators of LC. Related studies on IgG autoantibodies in the early diagnosis of LC have made admirable progress (16–18). However, studies regarding IgM autoantibodies are limited. Thus, more research is needed to provide evidence for IgM as an earlier indicator for discriminating LC patients and normal individuals.

In the present study, we aimed to screen valuable IgM autoantibody indicators for LUAD by protein array and verify them in another sample cohort with enzyme-linked immunosorbent assay (ELISA). Ultimately, the five candidate IgM autoantibodies and CEA were integrated to construct a diagnostic model to improve the diagnostic efficiency for LUAD. The diagnostic model might be able to improve the treatment status of LC patients and increase their survival rate.

Abbreviations: AIS, adenocarcinoma *in situ*; AUC, area under the receiver operating characteristic curve; BSA, bovine serum albumin; CA-125, cancer antigen 125; CEA, carcinoembryonic antigen; CI, confidence internal; CYFRA 21-1, cytokeratin-19 fragment; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; GO, Gene Ontology; HRP, horseradish peroxidase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC, lung cancer; LDCT, low-dose computed tomography; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MIA, minimally invasive adenocarcinoma; NCs, normal controls; NSCLC, non-small cell lung carcinoma; OD, optical density; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.1% Tween 20; ROC, receiver operating characteristic; SCLC, small cell lung carcinoma; SNR, signal-to-noise ratio.

MATERIALS AND METHODS

Study Population and Serum Collection

All serum samples included in this study were obtained from the Specimen Biobank in Henan Key Medical Laboratory of Tumor Molecular Biomarkers collected from a provincial hospital in Zhengzhou, Henan Province, China, between 2016 and 2019. Two independent sample cohorts (a discovery cohort and a validation cohort) were used in this research. The discovery cohort consisted of 68 LUAD patients (LUADs) and 68 normal controls (NCs) matched by gender and age. In addition, 147 LUADs, 147 matched NCs, 72 LUSC patients, and 44 SCLC patients were included in the validation cohort. The blood samples of all LC patients were drawn upon their first diagnosis without any other cancers, antitumor treatment, and autoimmune diseases. All NCs were individuals who had a health checkup without history of cancer, pulmonary diseases, and autoimmune diseases. The sera were extracted and stored according to standard protocols (19). The study was approved by the Medical Ethics Committee of Zhengzhou University, and all the patients and NCs signed an informed consent before their participation in the study.

The serum CEA test results were provided by the laboratory of the hospital. It was obtained using the MODULARE70 automatic analyzer and supporting kits produced by Roche in Switzerland. The principle was electrochemiluminescence. The experimental operations were carried out by professional and technical personnel. Moreover, the results were released after inspection by experienced laboratory physicians.

Human Protein Array Assay

The human protein array assay was commissioned to BC Biotechnology Co., Ltd. (Foshan, China) based on the conception of our laboratory. The protein chip contained 154 recombinant

proteins or protein fragments, 11 of which (CIP2A/p90, c-Myc, cyclin B1, IMP1, IMP2, IMP3, RalA, RBM39, YWHAZ, and two fragments of survivin), with potential diagnostic value, were reported in our previous research (20, 21); the other 143 were encoded by cancer driver genes. The chips covering the aforementioned proteins were blocked by a blocking buffer [3% albumin from bovine serum (BSA) in a phosphate-buffered saline (PBS) buffer] for 3 h at room temperature. After removing the liquid, 200 μl diluted serum sample (1:50) was added into the corresponding block and incubated on a side swing shaker at 4°C overnight. Then, the chips were washed three times (10 min each time) with 200 μl PBS containing 0.1% Tween 20 (PBST) buffer for each block. The arrays were transferred into an incubation box with 3 ml Cy5-labeled anti-human IgM antibody incubation fluid inside for 1 h in the dark. The wash process was repeated three times with PBST and twice with ddH_2O. After placing the chips in an array dryer for centrifugal drying, scanning and data extraction were performed using LuxScan 10K-A (CapitalBio Corporation, Beijing, China).

Data Analysis for Assays Performed on Protein Array

In order to eliminate the deviation caused by the inconsistency of the background value between different samples, the ratio of the foreground value to the background value of each protein (F median/B median) was calculated, namely, the signal-to-noise ratio (SNR). Statistical analyses of the samples were based on the SNR value. The tests for the test samples were repeated 30 times at different time points, different chips, and different positions to evaluate the stability of the operation.

Selection of Candidate IgM Autoantibodies

Analysis of the receiver operating characteristic (ROC) of each autoantibody in LUADs and NCs was applied based on the SNR value of each samples. Thirty-one IgM autoantibodies were screened and showed higher distinguishing values in LUADs [area under the ROC curve (AUC) > 0.5, $p < 0.05$]. In view of the optimal choice in terms of the number of indicators when constructing the model, we set the top 5 based on AUC rankings (TSHR, ERBB2, survivin, PIK3CA, and JAK2) among the 31 differential autoantibodies as the candidate IgM indicators (**Supplementary Table S1**).

Enzyme-Linked Immunosorbent Assay

ELISA was used to detect the serum levels of candidate autoantibodies discovered by the protein array. The five purified recombinant proteins (TSHR, ERBB2, survivin, PIK3CA, and JAK2) were coated at an optimal concentration of 0.125 μg/ml (50 μl/well) at 4°C overnight. After blocking with 2% BSA buffer (50 μl/well) at 4°C overnight, all serum samples diluted 1:100 were piped into the antigen-coated wells (50 μl/well) and incubated at 37°C for 1 h. Then, the plates were washed three times with PBST. Subsequently, the horseradish peroxidase (HRP)-conjugated anti-human IgM antibody (1:2,000 dilution) or the HRP-conjugated anti-human IgG antibody (1:5,000 dilution) was added into each microplate well (50 μl/well) and incubated at 37°C for 1 h, followed by washing five times with PBST. TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution was used as the detecting reagent (50 μl/well), and the enzyme–substrate reaction was terminated by the addition of a sulfuric acid solution (25 μl/well). Finally, the color change was measured spectrophotometrically at wavelengths of 450 and 620 nm.

The difference of the optical density (OD) values at 450 and 620 nm was applied in further statistical analysis. To ensure the reliability of the serum levels of the IgM autoantibodies, a positive control, a negative control, and two blank controls were used in every plate and the samples of each group were dispersed on the plates.

Statistical Analysis

Data analysis and visualization was performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery), SPSS Statistics 26.0, GraphPad Prism 8.0, and R-4.0.0 software. The χ^2 test and Student's *t*-test were applied to compare the differences in the characteristics between two cohorts. Besides, differences in the levels of autoantibodies in LC patients and NCs were analyzed using the non-parametric Mann–Whitney *U* test. Logistic regression was employed to construct a diagnostic model for LUAD combining autoantibodies and CEA. ROC analysis and the AUC with 95% confidence interval (CI) were used to evaluate the diagnostic performance of the biomarkers and the model. The sensitivity and specificity were determined based on the cutoff value, which was defined as the OD value at the maximum Youden's index, while specificity is more than 90%. In all tests, $p < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

Overall Study Design and Characteristics of the Study Subjects

In the whole study, we employed a three-phase strategy to identify IgM autoantibodies for the early diagnosis of LUAD and to construct a diagnostic model combining potential IgM autoantibodies and CEA (**Figure 1**). In phase I, the serum samples from the discovery cohort composed of 68 LUADs and 68 matched NCs were individually profiled on the protein array for the screening of IgM autoantibodies. A total of 31 IgM autoantibodies were observed to have higher intensity in the sera of LUADs than in NCs. In phase II, the top 5 (AUC ranking) of the 31 overexpressed IgM autoantibodies were tested in a larger cohort comprising sera from 147 LUADs, 147 matched NCs, 72 LUSC patients, and 44 SCLC patients using indirect ELISA. In phase III, we selected 83 LUADs with CEA results and 83 NCs matched by age and sex from the validation cohort. Based on the CEA and IgM autoantibody results of these populations, a diagnostic model was built, which showed a much improved performance using logistic regression.

The clinical characteristics of all the subjects involved in this study are described in **Table 1**. There were no significant differences in sex, smoking history, TNM stage, status of lymph node metastasis, and distant metastasis in LUADs of the two cohorts.

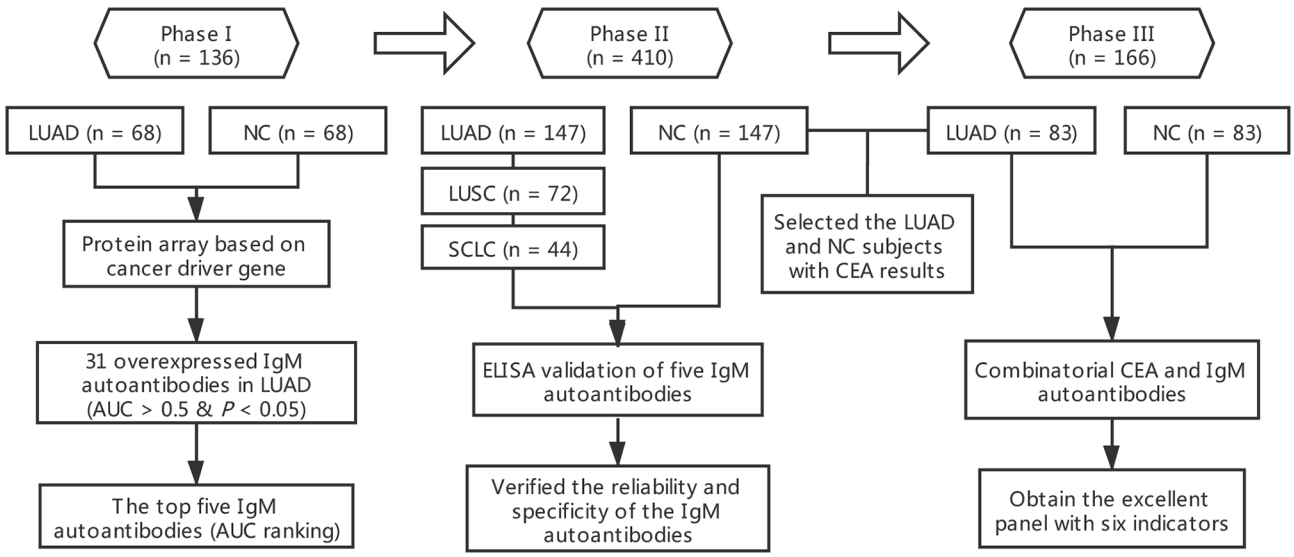


FIGURE 1 | Overall design of this study. AUC, area under the receiver operating characteristic curve; CEA, carcinoembryonic antigen; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NC, normal control; SCLC, small cell lung carcinoma.

TABLE 1 | Descriptive characteristics of the study population.

Variables	Discovery cohort (*n* = 136)		Validation cohort (*n* = 410)			
	LUAD (*n* = 68)	NC (*n* = 68)	LUAD (*n* = 147)	LUSC (*n* = 72)	SCLC (*n* = 44)	NC (*n* = 147)
Age (years)						
Range	35–76	39–78	26–83	26–85	40–78	27–82
Mean ± SD	60.84 ± 8.52	60.58 ± 8.73	57.79 ± 10.79	62.75 ± 10.81	62.82 ± 6.51	57.47 ± 10.71
Sex, *n* (%)						
Male	37 (54.41)	40 (58.82)	75 (51.02)	65 (90.28)	30 (68.18)	75 (51.02)
Female	31 (45.59)	28 (41.18)	72 (48.98)	7 (9.72)	14 (31.82)	72 (48.98)
Smoking, *n* (%)						
Yes	20 (29.41)		51 (34.69)	55 (76.39)	28 (63.64)	
No	48 (70.59)		94 (63.95)	17 (23.61)	16 (36.36)	
Unknown			2 (1.36)			
Stage, *n* (%)						
I and II	26 (38.24)		49 (33.34)	8 (11.11)		
III and IV	42 (61.76)		90 (61.22)	26 (36.11)	17 (38.64)	
Unknown			8 (5.44)	38 (52.78)	27 (61.36)	
Lymph node metastasis, *n* (%)						
Yes	39 (57.35)		79 (53.74)	35 (48.61)	21 (47.73)	
No	20 (29.41)		61 (41.50)	9 (12.50)	2 (4.54)	
Unknown	9 (13.24)		7 (4.76)	28 (38.89)	21 (47.73)	
Distant metastasis, *n* (%)						
Yes	25 (36.77)		54 (36.73)	16 (22.22)	16 (36.36)	
No	41 (60.29)		85 (57.82)	22 (30.56)	3 (6.82)	
Unknown	2 (2.94)		8 (5.44)	34 (47.22)	25 (56.82)	
CEA, *n* (%)						
>5 ng/ml	20 (29.41)		38 (25.85)	7 (9.72)	5 (11.36)	8 (5.45)
≤5 ng/ml	15 (22.06)		45 (30.61)	33 (45.83)	18 (40.91)	128 (87.07)
Unknown	33 (48.53)		64 (43.54)	32 (44.45)	21 (47.73)	11 (7.48)

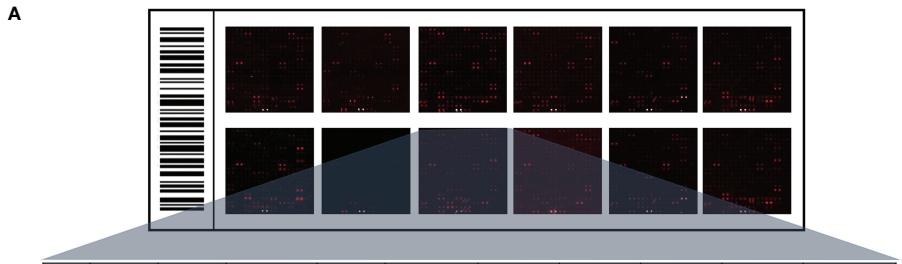
CEA, carcinoembryonic antigen; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NC, normal control; SCLC, small cell lung carcinoma; SD, standard deviation.

Human Protein Array Analysis

In order to identify candidate IgM autoantibodies, we assembled the discovery cohort of 136 serum samples collected from 68 LUADs and 68 matched NCs. The levels of IgM autoantibodies against 154 recombinant proteins were evaluated in the sera using the protein array. As shown in **Figure 2A**, the 154 proteins, the anti-human IgM (positive control), and a buffer (negative control) were arranged in order in the protein chips. The stability test results revealed that the average repeatability between the different batches of chips was 0.98, manifesting that the overall

stability was good (**Figure 2B**). Furthermore, as exhibited in the representative results from LUADs and NCs (**Figures 2C, D**), the IgM response of LUADs was apparently stronger than that of NCs. The positive and negative controls ensured the credibility of the experimental results.

Based on the criteria AUC > 0.5 and $p < 0.05$, 31 of the screened IgM autoantibodies showed higher expressions in LUADs, but much less in NCs (**Figure 3A**). **Supplementary Table S1** illustrates the characteristics and functions of these 31 IgM autoantibodies, which were functionally relevant in tumorigenesis. Subsequently, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analyses of the corresponding genes were performed with DAVID. KEGG pathway enrichment analysis showed that the proteins were mostly involved in pathways in cancer, PI3K–Akt signaling pathway and Ras signaling pathway (**Figure 3B**). GO enrichment analysis revealed that the main biological processes involved cell proliferation, cell migration, regulation of angiogenesis, and adaptive immune response (**Figure 3C**), whereas the molecular functions of these proteins were primarily related to protein binding, ATP binding, and receptor signaling protein tyrosine kinase activity (**Figure 3D**). As for the cellular component terms in the GO analysis, the proteins



	Column-1/2	Column-3/4	Column-5/6	Column-7/8	Column-9/10	Column-11/12	Column-13/14	Column-15/16	Column-17/18	Column-19/20
Row-1	PTPN11	KRAS	NRAS	CCND1	PBRM1(frag)	IKZF1	Survivin	PHF6	IMP2/p62	FUBP1
Row-2	NFE2L2	IMP1	U2AF1	ATRX	HRAS	AKT1	Survivin	MYCL1	IMP3/Koc	NPM1
Row-3	KLF4	H3F3A	IDH2	GATA3	TP53	GNAS	NF2	SMAD2	MEN1	GATA1
Row-4	PPP2R1A	WT1	PTCH1	DNMT3A	RalA	GATA3	PIK3R1	SMAD4	B2M	CTNNB1
Row-5	SMARCB1	GNAQ	FGFR2	BAP1	SMO	TRAF7	SOX9	RET	GNAS	ACVR1B
Row-6	SETBP1	MDM4	ASXL1	NF2	CASP8	PAX5	MPL	FBXW7	DAXX	PIK3R1
Row-7	TET2(frag)	SKP2	DNMT3A	MDM2	KRAS	PRDM1	FOXL2	MAP2K4	MDM2	STAG2
Row-8	GATA2	MYCN	BRCA1	MLH1	BCL2	ATM	CDC73	VHL	CDKN2C	GATA1
Row-9	CBL	HRAS	CDH1	EGFR	PDGFRA	SRSF2	PTEN	MDM2	SF3B1	LMO1
Row-10	BCOR	GNA11	PTEN	SRSF2	PRDM1	EGFR	IDH1	TNFAIP3	YWHAZ	KIT
Row-11	CASP8	HNF1A	KLF4	NCOA3	KDM5C	SMARCA4	EZH2	RBM39	PDGFRA	NPM1
Row-12	JAK2	PIK3CA	FGFR2	FGFR2	PDGFRA	RNF43	KDM6A	JAK3	BRAF	CSF1R
Row-13	JAK1	ALK	CIP2A/p90	MSH6	c-Myc	APC	HIST1H3B	SOCS1	ERBB2	TSHR
Row-14	ARID2	CRLF2	FGFR3	TSC1	MED12	MET	CDKN2A	MSH2	SPOP	RUNX1
Row-15	Cyclin B1	RB1	NKX2-1	PRDM1	MYD88	DNMT3A	PTCH1	CEBPA	GNAS	BRCA2
Row-16	NCOR1	GNAS	EGFR	ERBB2	positive3-1	positive3-2	positive3-3	positive3-4	buffer	buffer
Row-17	human IgM	buffer4	Anti human IgG	buffer5	Anti human IgM	buffer6	positive5-1	positive5-2	positive5-3	positive5-4

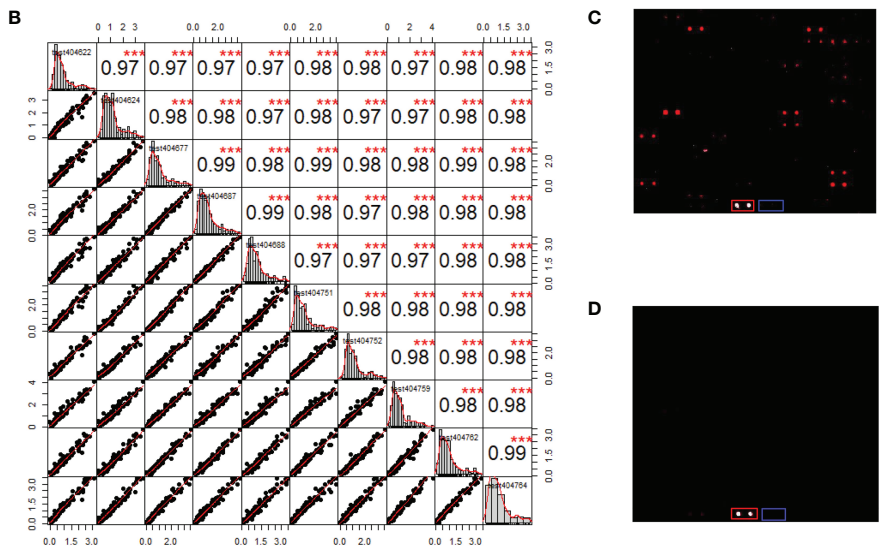


FIGURE 2 | **(A)** Layout of the customized protein array. **(B)** Evaluation of the operational stability of different chips at different time points. The *top right half* represents the correlation results between samples after linear fitting (***$p < 0.001$), while the *bottom left half* depicts the distribution of the results after linear fitting. **(C)** Protein fluorescence quantification results of a LUAD sample. **(D)** Protein fluorescence quantification results of a NC sample [*red* and *blue frames* highlight the positive control (anti-human IgM) and negative control (buffer)]. LUAD, lung adenocarcinoma; NC, normal control.

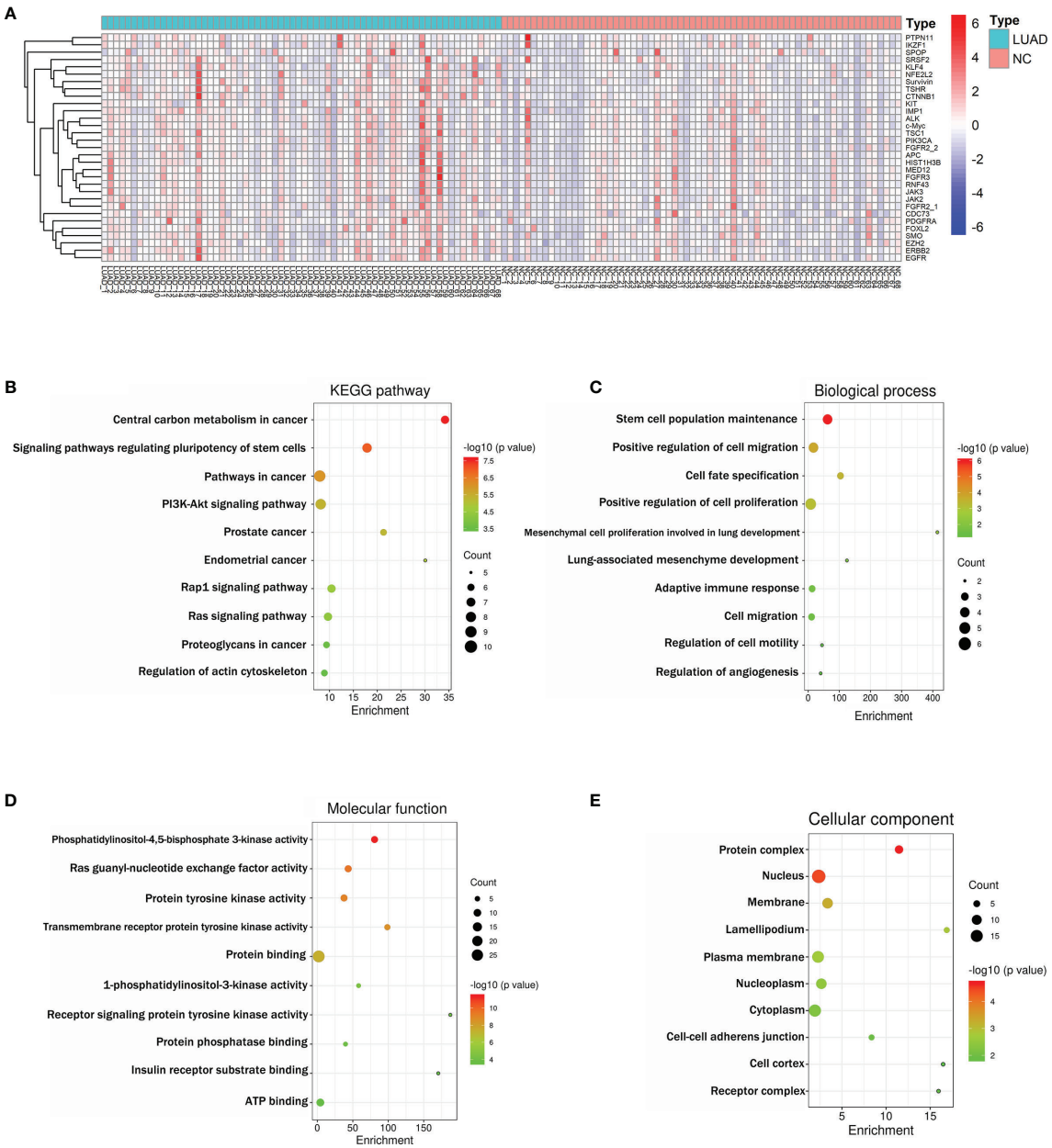


FIGURE 3 | **(A)** Heatmap of the 31 differently expressed IgM autoantibodies between LUAD and NC. **(B)** Schematic representation of the 10 KEGG pathways for the 31 differentially abundant IgM autoantibodies in LUAD (all $p < 0.001$). **(C–E)** Schematic representation of the GO terms for the 31 differentially abundant IgM autoantibodies in LUAD (all $p < 0.05$). GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LUAD, lung adenocarcinoma; NC, normal control. Enrichment (fold enrichment) is defined as the ratio of the two proportions. For example, if 10/22 (i.e., 45.5%) of the input genes involved in "pathways in cancer" and the background information is 393/6,879 genes (i.e., 5.7%) associating with "pathways in cancer", the fold enrichment is roughly 8 (45.5%/5.7%).

were mainly localized in the nucleus, cytoplasm, and plasma membrane (**Figure 3E**).

Performance of Five Candidate IgM Autoantibodies in the Discovery and Validation Cohorts

Of the 31 aforementioned IgM autoantibodies, we selected the top 5 (AUC ranking) as the candidate biomarkers. In the discovery cohort, the levels of IgM autoantibodies against TSHR, ERBB2, survivin, PIK3CA, and JAK2 (the top 5 in AUC ranking) were significantly higher in LUADs than those in NCs (**Figure 4**). The AUCs of the single IgM autoantibodies ranged from 0.665 to 0.758. Similar results were also found in another independent cohort (validation cohort) that included 147 LUADs and 147 matched NCs (**Figure 5**), with AUCs ranging from 0.579 to 0.633. These results indicated the consistency of the results from the protein array

and from ELISA. Moreover, the specificities of the five IgM indicators in LUADs were investigated in 72 LUSC and 44 SCLC patients. Only the titer of IgM autoantibodies against JAK2 in LUSC patients was higher than in NCs ($p = 0.046$) (**Figure 5A**). Moreover, the diagnostic performance of each IgM autoantibody in LUADs of the validation cohort was assessed using a series of evaluation indices in diagnostic experiments (**Table 2**). Regrettably, the individual IgM autoantibodies showed poor diagnostic value, with sensitivities ranging from 9.52% to 17.01% when the specificities were set higher than 90%. The diagnostic accuracies only ranged from 51.70% to 54.76%. Besides, the levels of the five IgM autoantibodies in LUADs with different clinical characteristics (clinical stage, gender, age, smoking history, and condition of lymph node metastasis, and distant metastasis) were stratified and analyzed in **Supplementary Figure S1**.

Since IgG autoantibodies to tumor-associated antigens have been frequently investigated, we also conducted an analysis on the five IgG autoantibodies. As shown in **Supplementary Figure S2**, three IgG autoantibodies (ERBB2, JAK2, and PIK3CA) were found to be significantly higher in LUADs than in NCs (AUC > 0.05, $p < 0.05$).

Construction of a Diagnostic Model Combining IgM Autoantibodies and CEA

Given the insufficiency of a single IgM autoantibody for LUAD detection, we selected and matched the populations (83 LUADs and 83 NCs) with CEA results from the validation cohort to construct a diagnostic model that combines candidate IgM autoantibodies and CEA. Binary logistic regression was adopted to combine the IgM autoantibodies with CEA. It was found that the AUC of CEA alone was only 0.692 and that of the five IgM autoantibodies (combined) was 0.698. However, the AUC of the model that included both five IgM autoantibodies and CEA reached 0.827 (**Figure 6A**). This model effectively improved the diagnostic performance in LUADs. Moreover, the AUCs of this model in early- and advanced-stage LUADs reached 0.774 and 0.860, respectively (**Figures 6B, C**). The formula for the diagnostic model was as follows: $P = 1/[1 + \text{Exp}(-(1.655*\text{ERBB2} + 7.862*\text{JAK2} - 10.285*\text{TSHR} + 17.135*\text{PIK3CA} - 11.294*\text{Survivin} + 0.299*\text{CEA} - 1.899)]$.

Application of the Diagnostic Model Combining IgM Autoantibodies and CEA in Stratified Diagnosis of LUAD

LUADs were stratified according to their clinical characteristics of tumor stage, lymph node metastasis, and distant metastasis. ROC analysis and evaluation of the diagnostic performance were performed for every subgroup and in NCs (**Table 3**). The results revealed that the LUADs in every subgroup could be significantly distinguished from NCs based on this diagnostic model. In addition, the AUCs in LUADs with early-stage disease, lymph node metastasis (−), and distant metastasis (−) were higher than 0.7 (0.744, 0.804, and 0.769, respectively). Moreover, the AUCs in LUADs with advanced-stage disease, lymph node metastasis (+), and distant metastasis (+)

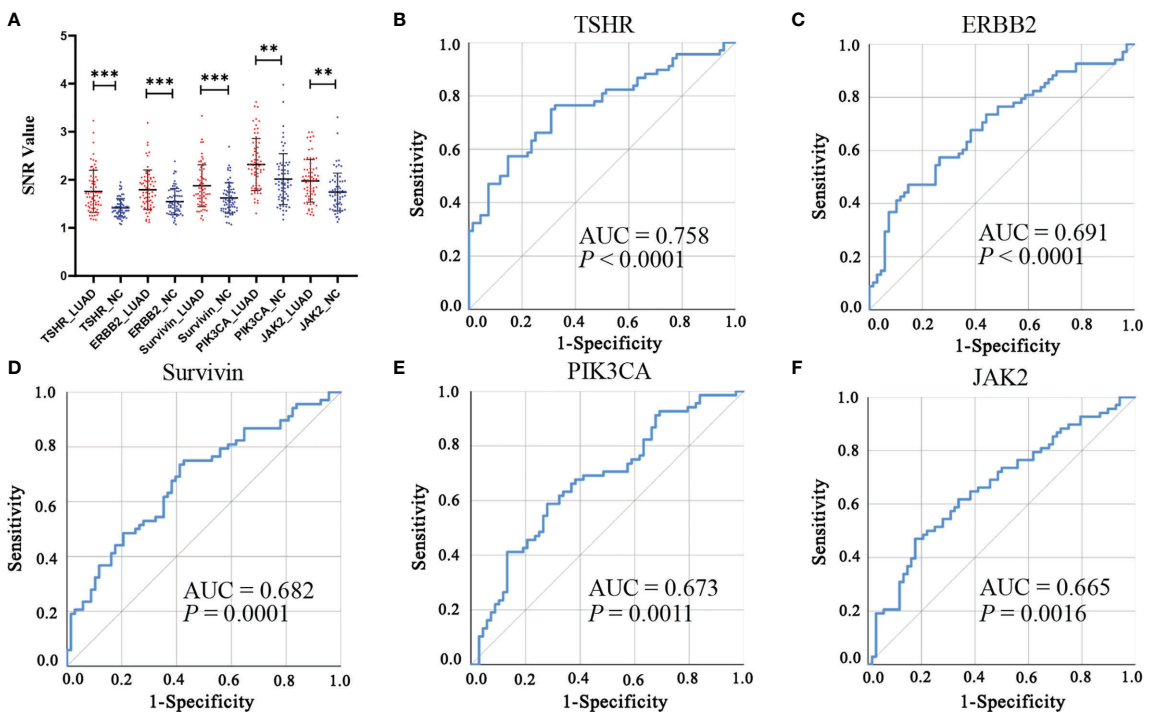


FIGURE 4 | **(A)** Differential expressions of the top 5 (AUC ranking) IgM autoantibodies in the discovery cohort using protein array. ***$p < 0.001$, **$p < 0.01$. **(B–F)** ROC analysis of the top 5 IgM autoantibodies in the discovery cohort. AUC, area under the receiver operating characteristic curve; LUAD, lung adenocarcinoma; NC, normal control; ROC, receiver operating characteristic; SNR, signal-to-noise ratio.

TABLE 2 | Diagnostic value of five IgM autoantibodies in the validation cohort for the detection of lung adenocarcinoma (LUAD).

IgM Autoantibodies	Cutoff	Sen (%)	Spe (%)	AUC (95%CI)	PPV (%)	NPV (%)	+LR	–LR	Accuracy (%)
TSHR	0.432	9.52	93.88	0.599 (0.534–0.664)	60.87	50.92	1.556	0.964	51.70
ERBB2	0.381	17.01	92.52	0.613 (0.548–0.677)	69.44	52.71	2.274	0.897	54.76
Survivin	0.324	14.97	90.48	0.579 (0.514–0.644)	61.11	52.11	1.572	0.940	52.72
PIK3CA	0.363	14.29	93.20	0.601 (0.537–0.666)	67.74	52.09	2.101	0.920	53.74
JAK2	0.341	14.29	93.20	0.633 (0.570–0.697)	67.74	52.09	2.101	0.920	53.74

The optical density (OD) value at the maximum Youden's index while specificity is more than 90% was defined as the cutoff value.
Sen, sensitivity; Spe, specificity; AUC, area under the receiver operating characteristic curve; CI, confidence interval; LUAD, lung adenocarcinoma; OD, optical density; PPV, positive predictive value; NPV, negative predictive value; +LR, positive likelihood ration; –LR, negative likelihood ration.

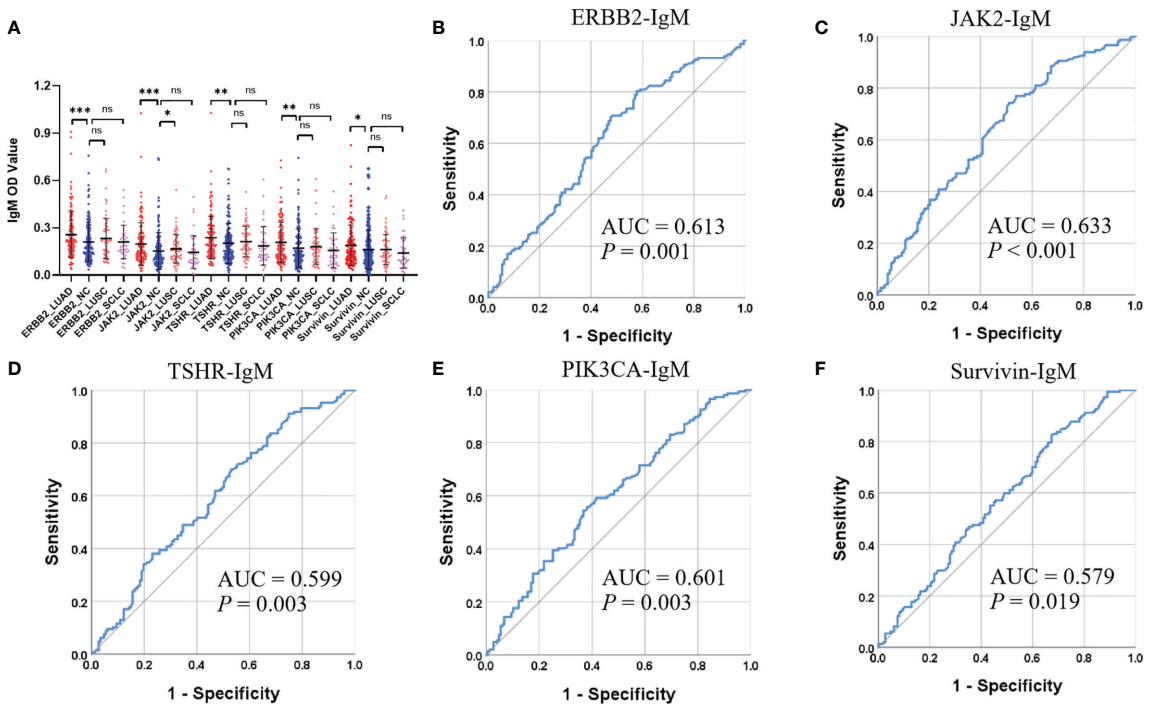


FIGURE 5 | **(A)** Differential expressions of the top 5 (AUC ranking) IgM autoantibodies in the validation cohort using ELISA. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, nsp > 0.05. **(B–F)** ROC analysis (LUAD and NC) of the top 5 IgM autoantibodies in the validation cohort. AUC, area under the receiver operating characteristic curve; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NC, normal control; OD, optical density; ROC, receiver operating characteristic; SCLC, small cell lung carcinoma.

reached 0.85 (0.860, 0.843, and 0.892, respectively). Notably, the accuracy of the model in early-stage LUADs was up to 83.02%. Interestingly, the predictive probability for LUAD derived by the model increased with the higher aggressiveness of the tumor [median: early stage (0.557) *vs.* advanced stage (0.803) and without lymph node metastasis (0.565) *vs.* with lymph node metastasis (0.900) *vs.* without distant metastasis (0.557) *vs.* with distant metastasis (0.905), all $p < 0.05$] (**Table 3**).

The diagnostic value of the model that included three IgG autoantibodies (ERBB2, JAK2, and PIK3CA) and CEA for LUADs is exhibited in **Supplementary Table S2**. The diagnostic performance of the model combining three IgG autoantibodies and CEA (AUC = 0.781, sensitivity = 56.63%, specificity = 90.36%) (**Supplementary Table S2**) was a little lower than that of the model that included five IgM autoantibodies and CEA (AUC = 0.827, sensitivity = 56.63%, specificity = 93.98%) in 83 LUADs *vs.* 83 NCs (**Table 3**). Interestingly, the model that included five IgM autoantibodies and CEA possessed great diagnostic value in early-stage LUAD (AUC = 0.744, $p < 0.05$) (**Table 3**), but the model that included three IgG autoantibodies and CEA had no diagnostic value in early-stage LUAD (AUC = 0.549, $p > 0.05$) (**Supplementary Table S2**).

DISCUSSION

In recent studies, the EarlyCDT-Lung test has achieved remarkable results in distinguishing LC patients from high-risk

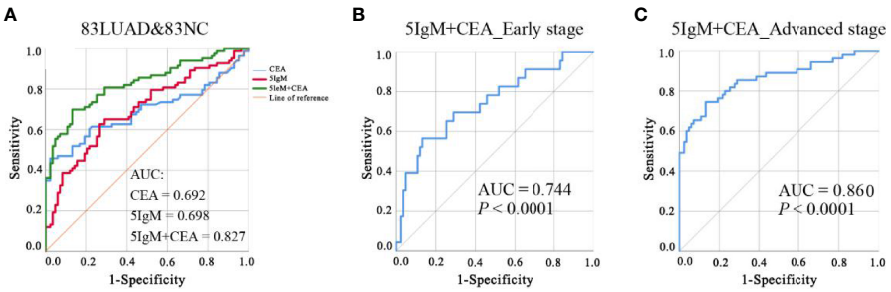


FIGURE 6 | **(A)** ROC analysis of CEA alone, five IgM autoantibodies alone, and the combination of five IgM and CEA for the detection of LUAD from NC. **(B, C)**. ROC analysis of the diagnostic model for LUAD patients at different stages of the disease. AUC, area under the receiver operating characteristic curve; CEA, carcinoembryonic antigen; LUAD, lung adenocarcinoma; NC, normal control; ROC, receiver operating characteristic.

TABLE 3 | Diagnostic value of the model combining five IgM autoantibodies and CEA for LUAD patients with different disease stages.

Group	Median	*p*-value	Sen (%)	Spe (%)	AUC (95%CI)	PPV (%)	NPV (%)	+LR	–LR	Accuracy (%)
All	0.716		56.63	93.98	0.827 (0.765–0.890)	90.38	68.42	9.407	0.462	75.30
Stage										
Early	0.557	0.004	39.13	95.18	0.744 (0.625–0.864)	69.23	84.95	8.118	0.640	83.02
Advanced	0.803		65.45	92.77	0.860 (0.793–0.928)	85.71	80.21	9.053	0.372	81.88
Lymph node metastasis										
No	0.565	0.017	43.75	92.77	0.804 (0.712–0.896)	70.00	81.05	6.051	0.606	79.13
Yes	0.900		63.83	96.39	0.843 (0.764–0.921)	90.91	82.47	17.681	0.375	84.62
Distant metastasis										
No	0.557	0.002	44.19	93.98	0.769 (0.679–0.859)	79.17	76.47	7.341	0.594	76.98
Yes	0.905		67.65	96.39	0.892 (0.816–0.967)	88.46	87.91	18.740	0.334	88.03

Median, median of the predictive probability value; Sen, sensitivity; Spe, specificity; AUC, area under the receiver operating characteristic curve; CI, confidence interval; LUAD, lung adenocarcinoma; PPV, positive predictive value; NPV, negative predictive value; +LR, positive likelihood ration; –LR, negative likelihood ration.

subjects. The panel, which includes seven IgG autoantibodies, demonstrated sensitivity and specificity values for newly diagnosed LC of about 40% and 90%, respectively (22–24). Regrettably, this detection method has not been widely used clinically due to its limitation of low sensitivity. Theoretically, IgM autoantibodies, as the first antibodies produced by the immune response, may be more suitable for screening indicators of early cancer diagnosis. The study by Fitzgerald et al. found that the IgM autoantibodies against CADM1, ICLN, SEC16, and ZNF768 were increased in the serum of colorectal cancer patients and demonstrated that these IgM autoantibodies are potential biomarkers for colorectal cancer (25). However, research works on the screening of autoantibody biomarkers for LC diagnosis and treatment have mainly focused on IgG autoantibodies (26–28). Notably, a study has shown that IgM autoantibodies play a crucial part in the immunosurveillance mechanisms against malignant epithelial cells (29). There is an urgent need to explore the expressions of IgM autoantibodies in LC patients for the screening of indicators that have the potential to identify malignant lesions at an earlier stage.

A fundamental target in cancer research is to understand the mechanisms of cell transformation. Cancer driver genes are involved in tumorigenesis and development across cancer types (30). Hence, IgM autoantibodies against proteins encoded by cancer driver genes might be potential biomarkers for the detection of LUAD. To test this hypothesis, we customized the protein array based on the 138 cancer-driving genes revealed in the genomic landscapes of common forms of human cancer by Vogelstein et al. (31) and endeavored to explore the expressions of IgM autoantibodies in the sera of LUADs and NCs in order to screen for indicators with potential diagnostic value. In phase I of this study, there was a significantly higher IgM autoantibody response to 31 antigens in LUADs than in NCs. In addition, the results indicated that the top 5 IgM autoantibodies (TSHR, ERBB2, survivin, PIK3CA, and JAK2) were capable of identifying patients with LUAD from NC individuals, whether it is the small sample screening data of the protein chip or the large sample verification data of ELISA. Importantly, these indicators showed excellent specificity in LUADs, but without increasing in other subtypes of LC.

ERBB2 is a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases, and its IgG autoantibody has been extensively assessed as a potential diagnostic biomarker for gastric cancer (32) and breast cancer (33). In a recent study, the determination of autoantibodies to TSHR presented helpful in the diagnosis and management of patients with autoimmune thyroid disease (34). In our previous study, potential IgG autoantibodies were screened in 100 serum samples from LC patients and 50 sera from NCs using focused protein array based on cancer driver genes. Interestingly, higher levels of IgG autoantibodies against survivin, PIK3CA, and JAK2 were observed in LC patients than in NCs (19). Therefore, IgM

autoantibodies to TSHR, ERBB2, survivin, PIK3CA, and JAK2 may also be used as potential biomarkers for LUAD.

In phase II of the present study, we found that single-autoantibody indicators always showed an insufficient diagnostic ability, as in previous studies (35–37). Moreover, the AUCs of the five IgM and CEA were only 0.698 and 0.692, respectively. However, combining the five IgM autoantibodies with CEA by logistic regression resulted in a significant improvement in the AUC, sensitivity, and specificity, as well as the positive predictive value (PPV) and negative predictive value (NPV) of the assay. Besides, it is worth noting that the diagnostic accuracy in early-stage LUADs reached 83.02%. The results were better than that of the currently used EarlyCDT-Lung test with an accuracy of about 68% (22, 23, 38). Therefore, integrating two different types of biomarkers tends to have better diagnostic performance than that of a panel of multiple indicators of a single type.

Interestingly, the diagnostic performance of the model with five IgM autoantibodies and CEA (AUC = 0.827) was a little higher than that of the model with three IgG autoantibodies and CEA (AUC = 0.781). Additionally, for early-stage LUAD, the model with five IgM autoantibodies and CEA showed great diagnostic value, but the model with three IgG autoantibodies and CEA had no diagnostic value. This would justify IgM autoantibodies as having the advantage of potentially identifying malignant lesions at an earlier stage than IgG autoantibodies, as reported in the study of Pedchenko et al. (39), where high titers of IgM lung cancer-associated autoantibodies in the serum of patients 2 years before clinical presentation were detected. The diagnostic model that combined IgM and CEA may be a great auxiliary diagnostic tool for LUAD, especially for early-stage LUAD. Meanwhile, the predictive probability value of the diagnostic model increased with the aggressiveness of LUAD, which indicated that the model may have certain predictive power for the condition of patients.

Our study displayed several novel features. Firstly, we adopted a new modality of a customized protein array based on cancer driver genes to improve the possibility of screening potential IgM autoantibodies, which yielded satisfactory results. Secondly, ELISA was applied to validate the candidate IgM autoantibodies in an independent validation cohort with the aim of identifying robust LUAD biomarkers. Thirdly, the model constructed with the combination of IgM autoantibodies and CEA improved the diagnostic value of the existing clinical auxiliary diagnostic method, which implied that multi-analyte tests could ameliorate the problem of the insufficient diagnostic value of biomarkers.

There were certain limitations in this retrospective study. In future work, we will longitudinally explore the levels of these IgM indicators using pre-diagnostic longitudinal sera from LUADs. Moreover, we will verify the performance of the diagnostic model in a larger sample cohort to evaluate its capability in the early stage of LUAD.

In conclusion, we performed a meaningful approach of a protein array assay to identify novel IgM autoantibodies for the detection of LUAD. The diagnostic model that combined five IgM autoantibodies and CEA may be a more accurate method for the early detection of LUAD and possess certain predictive power for the severity of tumor.

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 the Medical Ethics Committee of Zhengzhou University. The patients/participants provided written informed consent to participate in this study. Written informed consent was obtained from individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LD was responsible for study conception and design. LD and JZ provided administrative support. TW, LP, FL, XiZ, and XW contributed to the provision of study materials or patients. XuZ, JL, YW, ML, and DJ collected and organized data. XuZ analyzed and interpreted the data and wrote the manuscript. All authors contributed to the article and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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CD38: A Significant Regulator of Macrophage Function

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Cluster of differentiation 38 (CD38) is a cell surface glycoprotein and multifunctional extracellular enzyme. As a NADase, CD38 produces adenosine through the adenosine energy pathway to cause immunosuppression. As a cell surface receptor, CD38 is necessary for immune cell activation and proliferation. The aggregation and polarization of macrophages are affected by the knockout of *CD38*. Intracellular NAD^+ levels are reduced by nuclear receptor liver X receptor-alpha (LXR) agonists in a CD38-dependent manner, thereby reducing the infection of macrophages. Previous studies suggested that CD38 plays an important role in the regulation of macrophage function. Therefore, as a new marker of macrophages, the effect of CD38 on macrophage proliferation, polarization and function; its possible mechanism; the relationship between the expression level of CD38 on macrophage surfaces and disease diagnosis, treatment, etc; and the role of targeting CD38 in macrophage-related diseases are reviewed in this paper to provide a theoretical basis for a comprehensive understanding of the relationship between CD38 and macrophages.

Keywords: CD38, macrophages, calcium, SASP (senescence-associated secretory phenotype), mechanism

INTRODUCTION

Cluster of differentiation 38 (CD38) is a cell surface glycoprotein and multifunctional extracellular enzyme which is primarily a NAD^+ glycohydrolase and ADPR cyclase (1). As a nicotinamide adenine dinucleotide (NAD^+) hydrolase, most of the NAD^+ catalyzed by CD38 is converted to adenosine diphosphate-ribose (ADPR), and a few molecules are cyclized to form cADPR by ADPR cyclase (2). At an acidic pH, CD38 catalyzes the synthesis of nicotinic acid adenine dinucleotide phosphate (NAADP) from $NADP^+$ (3). cADPR and NAADP are secondary messengers involved in calcium regulation and mobilization, such as calcium signal transduction and release, leading to angiogenesis and tumor progression (4–6). In addition, ADPR is further processed (through CD203a and CD73) to form ADO to cause immunosuppression (2).

As a cell surface receptor, CD38 is necessary for immune cell activation and proliferation. High expression of CD38 in immune cells can affect their functions, such as in macrophages, regulatory T cells

(Tregs), regulatory B cells (Bregs), myeloid-derived suppressor cells (MDSCs) and $CD16^{-}CD56^{+}$ natural killer (NK) cells (7). Macrophages are usually divided into M1 and M2 subsets, which have different functions and transcriptional profiles. The balanced polarization of M1 and M2 macrophages determines the fate of an organ during inflammation or injury (8–10). Pro-inflammatory M1-like macrophages accumulate in metabolic tissues during aging and the acute inflammatory response, including visceral white adipose tissue and the liver. These M1-like macrophages express high levels of the NAD consuming enzyme CD38 and enhance CD38-dependent NADase activity, thereby reducing the tissue NAD^{+} level. A study also found that aging cells gradually accumulated in the visceral white adipose tissue and liver during aging, and the inflammatory cytokines secreted by aging cells (representing the senescence-associated secretory phenotype, SASP) induced macrophage proliferation and the expression of CD38 (11). *In vitro*, it was found that lipopolysaccharide (LPS) in macrophages could upregulate the expression of CD38 in a time-and-dose-dependent manner. Knocking out or blocking CD38 inhibited LPS-induced M1 polarization of macrophages (12). Thus, CD38 is an important regulator of macrophage function. The present review discusses the effects of CD38 expression on the proliferation and polarization of macrophages, the effect of CD38 on macrophage function and its possible mechanisms, the expression level of CD38 on macrophage surfaces related to disease diagnosis, treatment, and prognosis; and the role of targeted CD38 therapy in macrophage-related diseases. Thus, this review aimed to promote our understanding of the relationship between CD38 and macrophages.

CD38 IS A POTENTIAL NEW MARKER OF MACROPHAGES

CD38 was initially considered as a surface activation marker of T cells and was later found to be expressed in other immune and non-immune cells, including macrophages (5). Under appropriate stimulation, macrophages are activated into an inflammatory state, which can be divided into M1 (classical activation) and M2 (alternative activation) subsets. The former are immune effector cells that resist bacterial invasion and phagocytizes and digest necrotic cells, and the latter are mainly responsible for wound healing and tissue repair. The M2b subtype of M2 macrophages has strong immunomodulatory and anti-inflammatory effects (13, 14). Through the comprehensive analysis of the transcriptional characteristics of M1 and M2 macrophages of mice, Jablonski et al. found that CD38 is upregulated by more than 50 times in M1 macrophages, while its expression is downregulated in M2 macrophages compared with that in M0 macrophages, which suggests that CD38 is the exclusive expression pattern of M1 macrophages, while early growth response protein 2 (EGR2) is the exclusive expression pattern of M2 macrophages. Further verification by flow cytometry showed that M1 and M2 macrophages could be distinguished by their relative expression levels of CD38 and EGR2. CD38 labeled most (71%) M1 macrophages *in vitro* (14). However, some studies have shown that bone marrow-derived macrophages (BMDMs) not infected with *Coxiella burnetii*-nine-mile phase II (NMII) strain do not significantly express CD38 and EGR2, the markers of classical activated (M1) and alternative activated (M2) macrophages (15).

M1 macrophages are usually activated by LPS and interferon gama (IFN-γ). After activation, they produce a large amount of interleukin 12 (IL-12) and a small amount of IL-10. Amici et al. found that CD38 is a marker of inflammatory macrophages *in vitro* and in an *in vivo* mouse model. Monocyte analysis in patients with systemic lupus erythematosus showed that although all monocytes expressed CD38, high expression of CD38 in atypical monocyte subsets was related to the disease. These data are consistent with the inflammatory marker role of CD38 in human macrophages and monocytes (9). In addition, Jablonski et al. also found that CD38 was selectively upregulated in BMDMs of inflamed mice (14). *Mycobacterium tuberculosis* cell wall binding factor, trehalose 6,6'-dimethylcholic acid (TDM), is a physiologically useful molecule that can be used to establish macrophage-mediated early events in the pathogenesis of tuberculous granuloma. Seven days after TDM introduction, $CD11b^{+}CD45^{+}$ macrophages with high surface expression of M1 (inflammatory)-like markers CD38 and CD86 were found in the pathological areas of mouse lung tissues (16). In summary, CD38 can be used as a potential new marker of macrophages.

EFFECT OF CD38 ON MACROPHAGE PROLIFERATION AND POLARIZATION

Macrophages exert an effector function against invading microorganisms. CD38 plays an important role in the proliferation and polarization of various immune cells, including macrophages (7). Knockout of *CD38* affected the aggregation and polarization of macrophages. Studies have shown that LPS can induce a series of inflammatory reactions and signal transductions. In cultured macrophages, LPS could upregulate the level of CD38 in a time and dose-dependent manner. It also increased the expression of *CD38* at the mRNA level by activating the Janus kinase signal transducer and transcription activator (JAK-STAT) pathway (17). However, knocking out or blocking CD38 in macrophages might inhibit the M1 polarization of macrophages induced by LPS and reduce nuclear factor kappa B (NF-κB) signal activation. CD38 can regulate the macrophage activation and acute renal injury (AKI) caused by LPS, and can be used as a therapeutic target for AKI caused by sepsis (12).

The recovery of CD38-deficient mice after closed head injury (CHI) was significantly lower than that of wild-type (WT) mice, and the object recognition ability of CD38-deficient mice was significantly lower than that of the WT mice. In addition, we observed that the number of activated microglia/macrophages at the injury site in the CD38-deficient mice was significantly lower than that in the WT mice, while CD38 was expressed in brain microglia. Thus, CD38 plays a beneficial role in the recovery of CHI of mice, and this effect is mediated, at least in part, by an increase in the number of microglia/macrophages, mediated by CD38 (18). It can be seen that CD38 is conducive to macrophage proliferation and polarization.

EFFECT OF CD38 ON MACROPHAGE FUNCTION AND ITS POSSIBLE MECHANISMS

Effect of CD38 on the Intracellular Calcium Concentration of Macrophages

CD38 is a regulator of the intracellular calcium pool and has the characteristics of anti-osteoclastogenesis. Increased expression of CD38 can reduce the number of osteoclasts and bone resorption. Through Ca^{2+}, cAMP, and cytokines (such as tumor necrosis factor alpha (TNF-α), regulating the expression of the NAD^+ sensitive enzyme CD38 might help to couple the strong metabolic activity of osteoclasts and osteoblasts with their respective bone resorption and bone shaping functions (19, 20). In the tumor microenvironment (TME), CD38 regulates the activation of tumor associated microglia/macrophages (TMMs) through the increase of calcium concentration mediated by cADPR. TMMs contribute to an immunosuppressive TME, thus promoting the growth and metastasis of glioma. In addition, the inflammatory process can significantly affect the brain injury resulting from ischemic stroke. The synthesis of calcium by bifunctional receptors and extracellular enzyme CD38 mobilizes the increased calcium concentration mediated by second messengers (e.g., cADPR), which has been proven to be necessary for the activation and migration of myeloid immune cells. The activation, migration, and accumulation of immune cells are the key steps in post-ischemic inflammation. Cu et al. found that CD38 deficiency could reduce the production of chemokines, immune cell infiltration (macrophages and lymphocytes) and brain injury after transient ischemia-reperfusion. Therefore, CD38 might be a therapeutic target for ischemic stroke (21).

Effect of CD38 on the Phagocytosis of Macrophages

Macrophages have a powerful effector function against invading microorganisms. Macrophages internalize pathogens through phagocytosis, then kill them and digest them in phagosomes (22). CD38 can reduce the risk of infection of macrophages. Matalonga identified a molecular mechanism regulated by the nuclear receptor liver X receptor-alpha (LXR), which limits the infection of host macrophages through the transcriptional activation of multifunctional enzyme CD38 (22). LXR agonists reduce intracellular NAD^+ levels in a CD38-dependent manner, resist pathogen-induced changes in macrophage morphology and the F-actin cytoskeleton distribution, and reduce the ability of non-conditioned *Salmonella* to infect macrophages (22). NAD^+ supplementation reversed the morphological transformation of macrophages and the accumulation of dorsal F-actin, and restored the ability of *Salmonella* to infect macrophages in the presence of LXR agonists. Therefore, the mechanism comprises limiting the infection of host macrophages by affecting CD38 and regulating NAD^+ metabolism.

In addition, $CD38^+$ macrophages can limit the growth of intracellular bacteria. Pathogens such as *Haemophilus aegypti*, *Haemophilus influenzae*, *Haemophilus haemolyticus*, *Haemophilus parainfluenzae*, and *Haemophilus parahaemolyticus* lack the ability to synthesize NAD^+; therefore, they rely on the uptake of NAD^+ and NAD^+ precursors [e.g., nicotinamide mononucleotide (NMN) and nicotinamide ribose (NR)] to support metabolism and growth. In fact, NAD^+ and its precursors are necessary for bacterial growth and must be included in the culture medium as factor V. CD38 exists in activated immune cells. As an extracellular enzyme or intracellular enzyme, CD38 promotes the metabolic collapse of pathogens by degrading NAD^+ and its precursors, thus limiting the development or progress of infection. This occurs not only outside the cell, but also inside the cell (23).

CD38 Is Involved in Nerve Injury and Protection of Macrophages

After facial nerve transection in *Cd38* knockout mice, axonal degeneration and demyelination were delayed, and macrophage aggregation decreased. Supplementation with NAD^+ could slow down axonal degeneration and demyelination, but did not change the level of macrophage infiltration after amputation. CD38 deletion and NAD^+ supplementation may have an autonomic protective effect on axonal cells after facial nerve transection (24). Normal or pathological aging is characterized by an increase in the number of aging cells in the brain, mainly astrocytes, which display SASP and are characterized by the release of pro-inflammatory cytokines and chemokines. These pro-inflammatory factors increase the expression of CD38 in astrocytes and microglia (resident brain macrophages), resulting in (i) increased release of pro-inflammatory cytokines and neuroinflammation, and (ii) NAD depletion, accumulation of DNA damage, metabolic dysfunction, and oxidative stress caused by the decreased activity of NAD-dependent enzymes, such as sirtuins and poly(ADP-ribose) polymerase (PARP), resulting from increased CD38 enzyme activity, leading to neuronal damage and eventually, cell death (25).

The Change of CD38 Expression on Macrophage Is Involved in the Regulation of NAD^+ Level

In many tissue types, the expression of CD38 increases with aging. As a NAD^+ hydrolase, CD38 significantly downregulates the level of NAD^+. During aging, the activation of CD38 might increase NF-κB signal transduction. The reason for this is that NF-κB of *Cd38* KO mice was greatly weakened in a collagen-induced arthritis mouse (CIA) model (26). The NF-κB signaling pathway is one of the main signaling pathways involved in the emergence of SASP (27), and most pro-inflammatory genes expressed in aging cells are related to NF-κB (28). The activation of tissue resident macrophages in SASP and the inflammatory environment causes the accumulation of macrophages in the liver, which expresses more CD38, showing increased signs of aging, and tending to promote inflammatory polarization (24). Covarrubias et al. found that aging cells gradually accumulated in the visceral white adipose tissue and liver during aging. Inflammatory cytokines secreted by aging (SASP) cells induced macrophage proliferation and expressed CD38. In addition, pro-inflammatory M1-like macrophages, rather than naive or M2 macrophages, were accumulated in metabolic tissues, including the visceral white adipose tissue and liver, during aging and the acute inflammatory response. These M1-like macrophages

expressed high levels of the NAD-consuming enzyme CD38 and enhanced CD38-dependent NADase activity, thereby reducing the tissue NAD level (11), as described in **Figure 1**. The CD38 expression of macrophages might be the main reason for the decline of NAD^+ in aging tissues.

In the kidney, lung, blood vessels, brain, and other important human tissues, the number of aging cells related to injury or stress secreting pro-inflammatory factors exhibiting SASP increases with age. SASP-conditioned mediators from aging cells can also induce the expression of CD38 in macrophages and endothelial cells. These M1-like macrophages express a high level of CD38 and enhance the CD38 dependent NADase activity, thus reducing the tissue NAD level, while the decrease in NAD^+ levels related to aging might weaken SASP and reduce its pathological effect (29–31).

THE RELATIONSHIP BETWEEN THE EXPRESSION LEVEL OF CD38 ON THE MACROPHAGE SURFACE AND DISEASE DIAGNOSIS, TREATMENT, AND PROGNOSIS

The increase of macrophage CD38 is observed in many diseases, for example, the expression of renal macrophage CD38 in lupus nephritis is significantly increased (32). The cell surface CD38 expressed by small macrophages was significantly increased in lung resection tissues from patients with chronic obstructive pulmonary disease (COPD) patients (33). Among 54 patients infected with HIV with virus inhibition, the level of CD14CD38 (% CD14) increased significantly in 20 patients with significantly reduced memory ability (34). This suggested that the expression level of CD38 on the surface of macrophages might be related to the diagnosis of disease.

In the tumor microenvironment, $CD38^+$ macrophages contribute to the anti-tumor effect. For example, the density of $CD38^+$ $CD68^+$ macrophages is associated with the improvement of postoperative prognosis of liver cancer. The density of $CD68^+$ macrophages is associated with poor prognosis, while CD38 is significantly expressed in the internal environment of human macrophages, which produces high levels of IL-6 and TNF-α, and together with the expression of CD80, it causes more inflammation and helps to inhibit the tumor. Therefore, the density of $CD38^+$ macrophages might correlate positively with the prognosis of patients with hepatocellular carcinoma (HCC) and might be meaningful for routine diagnosis (1, 3, 35). Thus, it can be seen that the expression level of CD38 on the surface of macrophages might be associated with the prognosis of disease.

Tumor associated microglia/macrophages (TMMs) show different or even opposite effects to $CD38^+$ macrophages. TMMs are formed by a small number of $CD38^+$ microglia and infiltrating monocytes in the brain. They secrete IL-1, basic fibroblast growth factor, and VEGF, and regulate Ca^{2+} mobilization through CD38-mediated cADPR, which is conducive to TMM activation, angiogenesis, and immunosuppression (1), thus promoting the growth and metastasis of glioma. Therefore, the expression of CD38 in TMMs might correlate negatively with the prognosis of patients with glioma. Subsequent related studies also supported this view. For example, compared with wild-type mice carrying glioma, CD38-deficient mice showed reduced expansion of glioma and prolonged life. Similar results were obtained by targeting CD38 pharmacologically by administering the CD38 inhibitor, k-rhein (2). The mechanism comprises the activation of immune cells

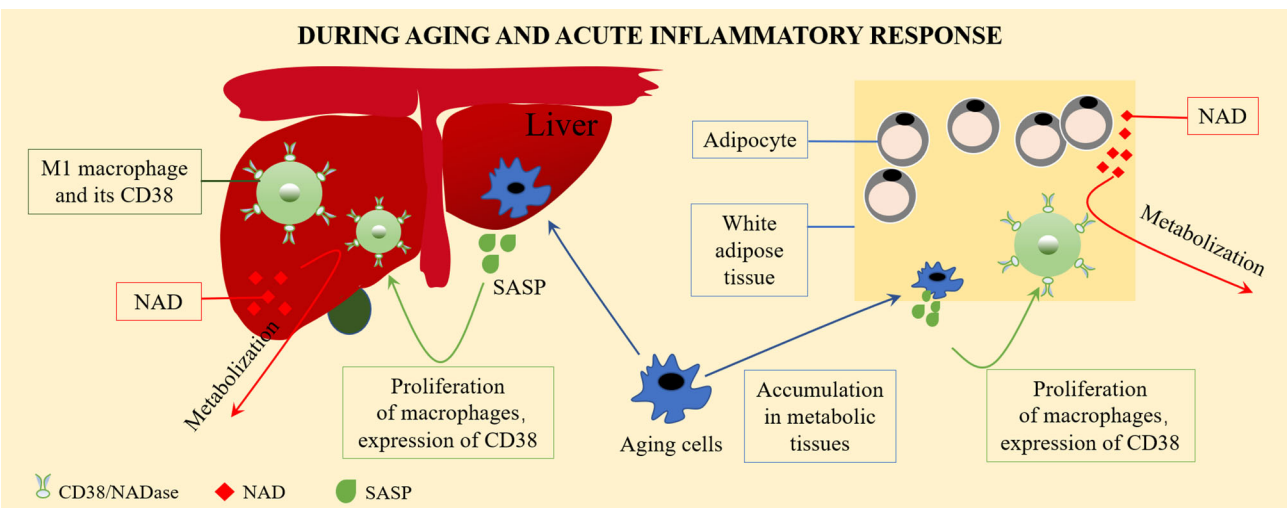

FIGURE 1 | The CD38 expression on macrophage and the regulation of NAD^+ level. Aging cells gradually accumulate in visceral white adipose tissue and liver during aging. Inflammatory cytokines secreted by aging cells (senescence associated secretory phenotype, SASP) induce macrophages to proliferate and to express CD38. Pro-inflammatory M1-like macrophages accumulate in metabolic tissues, including visceral white adipose tissue and the liver, during aging and acute inflammatory responses. These M1-like macrophages express high levels of the NAD-consuming enzyme CD38 and enhanced CD38-dependent NADase activity, thus reduces tissue NAD levels.

(including T cells, natural killer cells, neutrophils, macrophages, and dendritic cells) by inhibiting the formation of adenosine by inhibiting the NAD glycohydrolase activity of CD38. By contrast, through the inhibition of CD38, adenosine further inhibits the antitumor immune response under hypoxia by recruiting bone marrow-derived inhibitory cells and T regulatory cells, which enhance the activity of T effector cells and limit tumor progression (36, 37). This shows that inhibiting CD38 in patients' glioma is conducive to the treatment of glioma and prolong life.

THE ROLE OF TARGETED CD38 (THERAPY) IN DISEASES

The Role of an Anti-CD38 Monoclonal Antibody (mAb)and Its Synergy in the Treatment of Multiple Myeloma

Daratumumab is a human specific IgG1 anti-CD38 antibody, which has been approved as a single drug or combined regimen for the treatment of recurrent/refractory multiple myeloma. Daratumumab triggers $CD38^+$ multiple myeloma cells [via antibody-dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and tumor-associated macrophages (TAMs)] in sensitive and drug-resistant patients, regulates the enzyme activity of CD38, reduces the level of adenosine and reduces adenosine-induced immunosuppression. In addition, Daratumumab reduces the types of inhibitory cells in the TME of multiple myeloma, i.e., it consumes immunosuppressive cells such as $CD38^+$ MDSCs, Tregs, and Bregs, and enhances anti-tumor activity (1). Moreover, in patients with a partial or good response to Daratumumab, cytotoxic T cells increased significantly and enhanced anti-tumor activity. Similarly, Isatuximab can also induce $CD38^+$ Treg depletion and induce the proliferation and functional recovery of effector T cells (**Figure 2**). This suggests that Treg consumption is a key additional mechanism of action of these mAbs (38).

It was found that patients with at least a partial response to Daratumumab showed higher CD38 expression on MM cells (39). In this case, all-transretinoic acid (ATRA) treatment could increase the expression level of CD38 and reduce the expression of complement inhibitory proteins, CD55 and CD59, in MM cells, indicating that ATRA enhances the activity of Daratumumab (40). In addition, the use of immunomodulatory drugs (IMID), such as thalidomide, lenalidomide, and pomalidomide, resulted in the upregulation of CD38 on MM cells, causing them to trigger

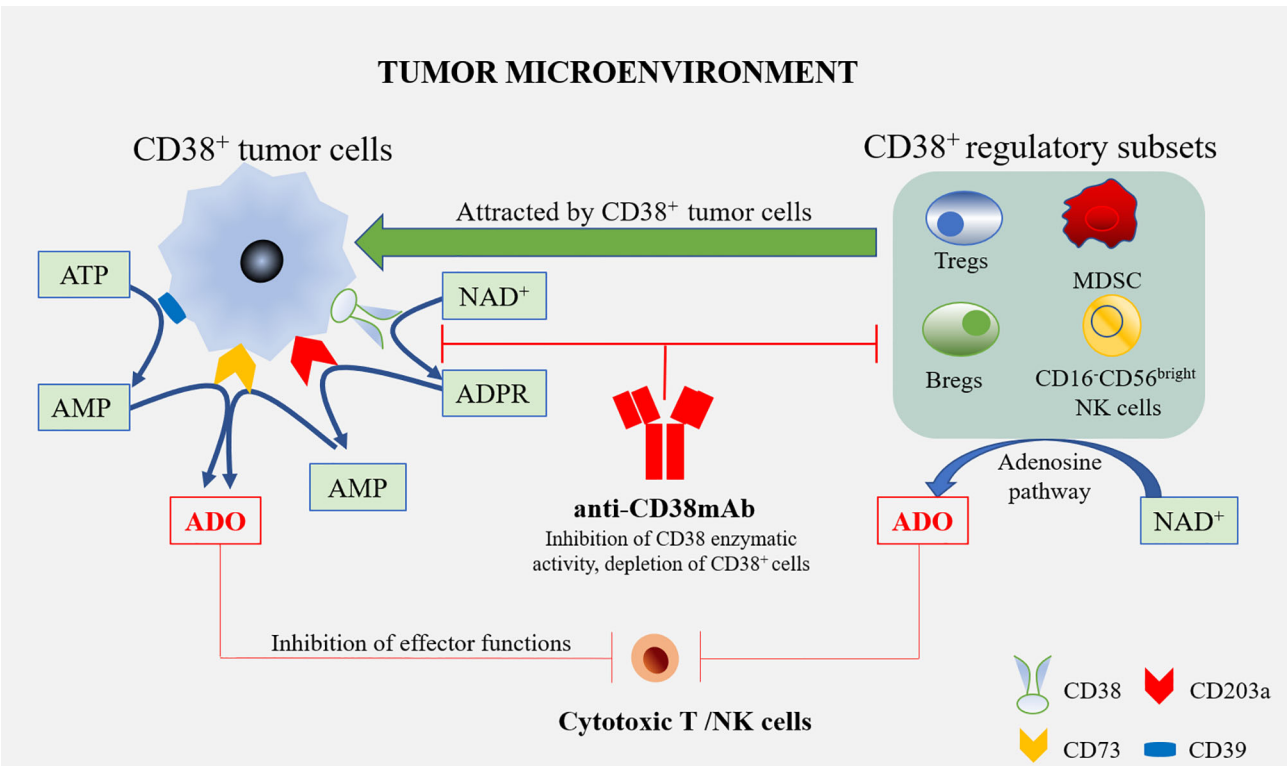

FIGURE 2 | Anti-CD38 mAbs and the treatment of multiple myeloma. Tumor cells are able to attract diverse regulatory subsets, such as Tregs, Bregs, MDSCs, and $CD16^-CD56^{bright}$ NK cells. All these cells may co-operate in the production of ADO, starting from ATP or NAD^+. ADO produced by these cells, as well as tumor cells in the tumor microenvironment, interact with ADO receptors on the cytotoxic T/NK cells to inhibit effector functions, which alleviates the anti-tumor immune response. The treatment of multiple myeloma using Anti-CD38 mAbs might overcome the immune suppression by blocking CD38's enzymatic activity in these cells, and the depletion of $CD38^+$ cells leads to an increased therapeutic response.

Daratumumab-induced NK cell-mediated ADCC (41, 42). Vitamin D can enhance cytotoxic activity *in vitro*; therefore, vitamin D supplementation in an IMID combined test could further improve the therapeutic effect of anti-CD38 antibodies (43). That study also found that the use of DNA methyltransferases (DNMTs) could achieve similar effects. Two DNMTs, azacitidine and decitabine, upregulated *CD38* mRNA levels in MM cells and the amount of CD38 on the cell surface. Therefore, the level of *in vitro* ADCC in cells treated with DNMT was higher than that of untreated cells, supporting the concept that DNMT can be used to improve the therapeutic effect of Daratumumab (44).

Targeted CD38 Therapy for Melanoma

Primary melanoma cell lines can inhibit the proliferation of $CD4^+$ and $CD8^+$ T cells through adenosine-dependent mechanisms; however, it has been found that the use of CD38 inhibitors can reverse this effect and restore T cell proliferation (45). Therefore, blocking the CD38-mediated adenosine pathway seems to reduce the immunosuppression in melanoma (46). A study found that untreated control mice formed well vascularized tumors and developed lung metastasis compared with melanoma-infected mice treated with the NAADP inhibitor Ned-19, suggesting that targeted CD38 inhibition in melanoma is partly caused by reduced NAADP production (47).

Relationship Between Targeted CD38 Therapy and Programmed Cell Death 1 (PD-1/PD-1 Ligand 1 (PD-L1) Efficacy

The use of the PD-1 specific monoclonal antibody nivolumab resulted in tumor volume reduction in one fifth of patients with advanced liver cancer. Nivolumab works by significantly reducing the number of non-reactive T cells and increasing the number of activated T cells expressing CD38 (48). These activated $CD38^+$ tumor infiltrating lymphocytes (TILs) produce cytotoxic compounds and inflammatory cytokines to attack tumors. These cytokines include IFN-γ, which plays a key role in tumor control, upregulates the immune response, and exerts a pro-inflammatory activity on immune infiltration and tumor cells (49). However, CD38 is usually upregulated after a period of anti-PD-1/PD-L1 treatment. The adenosine-promoting activity of CD38 in turn leads to $CD8^+$ T cell inhibition (50). This phenomenon might partly explain the high resistance rate observed in patients treated with PD-1/PD-L1 blockers, thus limiting the therapeutic benefits of anti-PD-1 immunotherapy. By inhibiting the adenosine receptor,

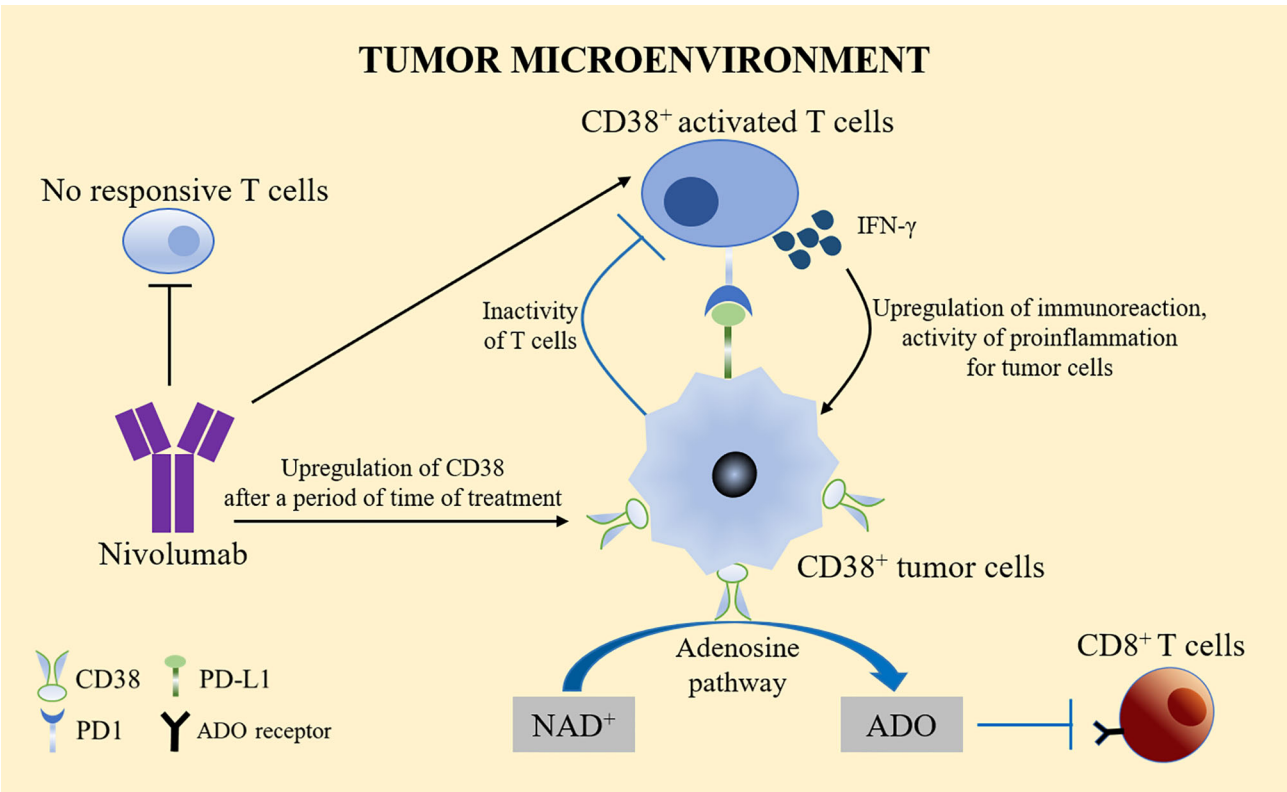

FIGURE 3 | CD38 treatment and PD-1/PD-L1. Nivolumab significantly reduced the number of non-reactive T cells and increased the number of activated T cells expressing CD38. Activated $CD38^+$ TILs produced cytotoxic compounds and inflammatory cytokines, such as IFN-γ to upregulate the immune response and exert pro-inflammatory effects on immune infiltration and tumor cells. However, CD38 is usually upregulated after a period of anti-PD-1/PD-L1 treatment. The adenosine activity of CD38 in turn leads to the inhibition of $CD8^+$ T cells, which might partly explain the high drug resistance rate observed in patients treated with PD-1/PD-L1 blockers. Pharmacological targeting of the adenosine pathway could reverse the immunosuppression caused by the upregulation of CD38.

pharmacological targeting of the adenosine pathway could reverse the immunosuppression caused by the upregulation of CD38 (**Figure 3**).

In another study, it was found that CD38 was the only significantly upregulated gene or protein after anti-PD-L1 treatment in patients with non-small cell carcinoma (NSCLC). Interferon-β and ATRA are important mediators for the upregulation of CD38 in NSCLC cells, which allows the tumor to develop resistance to anti-PD-L1 and anti-PD-1 treatments. This suggests that CD38 might be a potential additional immunotherapeutic target in HCC and NSCLC, and can be used together with PD-1/PD-L1 immunotherapy (51).

Reasons for Choosing CD38 as a Therapeutic Target

The main reasons why CD38 has become a tumor treatment target are as follows: (1) In a hypoxic TME, CD38 acts as an extracellular enzyme to catalyze NAD^+ existing in the TME into ADPR or cADPR. This is the first step in another adenosine production pathway, which typically includes CD39 catalyzing adenosine triphosphate (ATP) to produce adenosine monophosphate (AMP). AMP is then generated from ADPR by CD203. Both pathways rely on CD73 to convert AMP into the final product, namely adenosine (52). Tumor cells can attract different regulatory subsets in the tumor microenvironment, such as Tregs, Bregs, CD16-CD56 bright NK cells, and MDSCs. All these cells may produce adenosine (ADO) from ATP or NAD^+ (7). Under hypoxia, adenosine inhibits the anti-tumor immune response by recruiting bone marrow-derived suppressor cells and Tregs, thereby inhibiting the activity of T effector cells (36, 37). This leads to increased immune resistance of tumor cells and allows faster growth and proliferation rates (53). Therefore, the high expression of CD38 in the TME may lead to poor prognosis. (2) NAADP produced by CD38 is closely related to angiogenesis induced by vascular endothelial growth factor (VEGF) (54). CD38 is an ecto-enzyme highly expressed in endothelial cells (55). Type II CD38 can be internalized into endolysosomes through endocytosis. The acidic environment of endolysosomes is conducive to the production of NAADP (6, 56). NAADP stimulates Ca^{2+} channel of endolysosomes to releases Ca^{2+}, and Ca^{2+} acts as the second messenger to promote angiogenesis, thus promoting tumor growth and metastasis (3). In addition, VEGF is the main angiogenic growth factor, which binds with its receptors VEGFR2 to stimulate the release of Ca^{2+} by VEGFR2/NAADP/TPC2/Ca^{2+} signaling pathway, which also leads to angiogenesis (57) (**Figure 4**).

Macrophages are the key mediators by which monoclonal antibodies to play a therapeutic role, and antibodies targeting CD38 will also affect macrophage function (45). Therefore, the role of CD38 in macrophage diseases, especially in tumor diseases, cannot be ignored. Therefore, targeted CD38 therapy would be a promising treatment for a variety of diseases.

SUMMARY AND PROSPECTS

CD38 has dual functions, acting as an extracellular enzyme and a surface receptor. As a double-edged sword, it ubiquitously expressed in immune cells such as T cells, NK cells, and dendritic cells. CD38 promotes the migration phenotype and

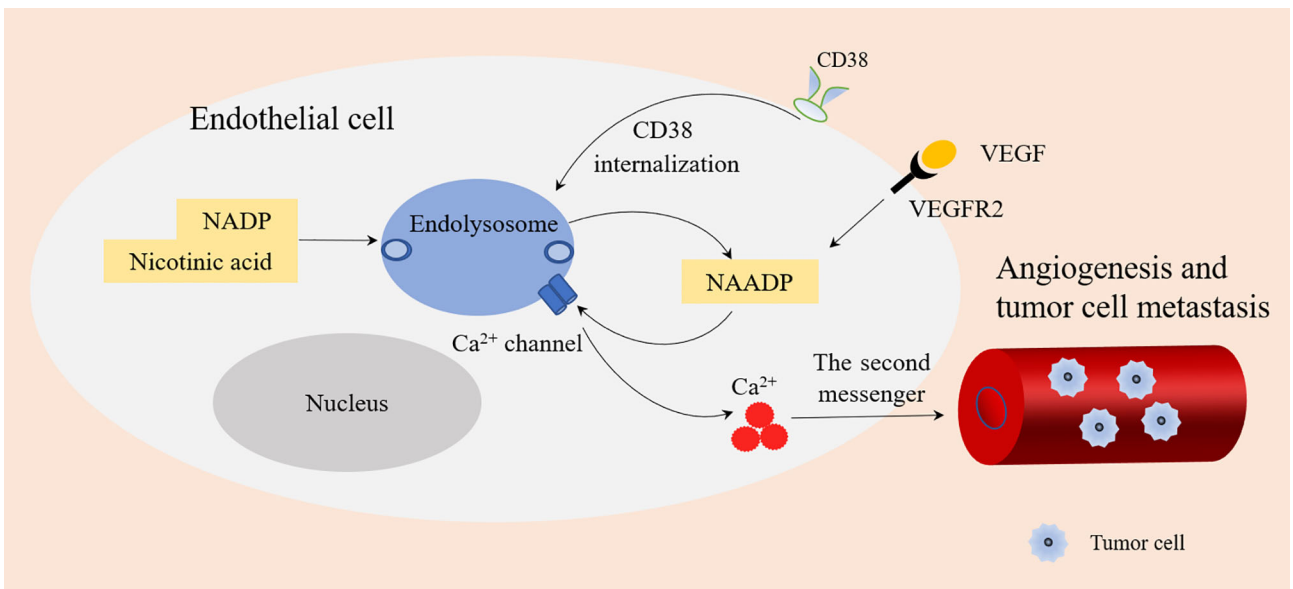

FIGURE 4 | NAADP produced by CD38 and angiogenesis. Type II CD38 is expressed on the cell surface and internalized into endolysosomes through endocytosis. Endolysosomes provide an acidic environment where NADP and nicotinic acid are transformed into NAADP by base exchange reaction. NAADP acts on Ca^{2+} channels of Endolysosomes to promote the release of Ca^{2+} (as a second messenger), which is conducive to angiogenesis and tumor cell metastasis. VEGF binds with its receptors VEGFR2 to stimulate the release of Ca^{2+} by VEGFR2/NAADP/TPC2/Ca^{2+} signaling pathway leading to angiogenesis.

signaling cascade, which is responsible for the activation and proliferation of various immune cells. Pro-inflammatory M1- like macrophages accumulate in metabolic tissues during aging and acute inflammatory reactions. The inflammatory cytokines secreted by aging cells (SASP) induce macrophage proliferation and enhance CD38-dependent NADase activity, thereby reducing the level of NAD in tissues. The decrease of NAD^+ levels related to aging might weaken SASP and reduce its pathological effect. Macrophages might be the main reason for the increased expression of CD38 and decrease of NAD related to aging in aging tissues.

However, more experiments are needed to verify the following aspects: The role and possible mechanism of CD38 in macrophage polarization and proliferation; the exact relationship between CD38, macrophages, and injury to (and protection of) the nervous system; and the real relationship between targeted CD38 therapy and the efficacy of PD-1/PD-L1-targeted therapy.

AUTHOR CONTRIBUTIONS

WL and YL wrote the manuscript and drew the figures. XJ, QL and ZC collected the related papers and helped to revise the manuscript. HP and YZ designed and revised the manuscript. All the authors read and approved the final version of the review.

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