



Radioresistant Nasopharyngeal Carcinoma Cells Exhibited Decreased Cisplatin Sensitivity by Inducing SLC1A6 Expression

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Cisplatin-based regimens are commonly used for the treatment of nasopharyngeal carcinoma (NPC) in patients who receive concurrent chemoradiotherapy. The sensitivity of NPC cells to cisplatin is closely associated with the efficacy of radiation therapy. In this study, we established two radioresistant NPC cell lines, HONE1-IR and CNE2-IR, and found that both cell lines showed reduced sensitivity to cisplatin. RNA-sequence analysis showed that SLC1A6 was upregulated in both HONE1-IR and CNE2-IR cell lines. Downregulation of SLC1A6 enhanced cisplatin sensitivity in these two radioresistant NPC cell lines. It was also found that the expression of SLC1A6 was induced during radiation treatment and correlated with poor prognosis of NPC patients. Notably, we observed that upregulation of SLC1A6 led to elevating level of glutamate and the expression of drug-resistant genes, resulted in reduced cisplatin sensitivity. Our findings provide a rationale for developing a novel therapeutic target for NPC patients with cisplatin resistance.

Keywords: nasopharyngeal carcinoma, SLC1A6, cisplatin, radiation-resistance, glutamate

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant tumor in the head and neck with high incidence in southern China and Southeast Asia (Chen et al., 2019; Ji et al., 2019). According to the guidelines of the National Comprehensive Cancer Network (NCCN), radiotherapy and cisplatin-based regimens are the main treatments for NPC patients (Pfister et al., 2020). The cisplatin-based concurrent chemoradiotherapy has been proven to improve the outcome of early and locally advanced NPC (Chen et al., 2011). However, there are a small portion of patients who did not response effectively or became recurrent to these treatments, and their prognosis remains poor (Chen et al., 2011; Karam et al., 2016).

The antitumor mechanism of cisplatin is to form covalent DNA adducts thus interfering with DNA repair (Wang and Lippard, 2005). The combination of cisplatin provides a synergetic effect for

radiotherapy for the reason that cisplatin could enhance the sensitivity to radiation (Boeckman et al., 2005). Interestingly, some studies have revealed that cancer cells could acquire cisplatin resistance after radiation therapy (Eichholtz-Wirth, 1995; Zhuang et al., 2019). Based on our clinical observations, the phenomenon of cisplatin resistance is commonly seen in NPC patients who also resistant to radiotherapy. However, the association of cisplatin and radiation resistance is elusive.

The SLC1A6 (Solute Carrier Family 1 Member 6) is a member of the SLC1A family, which consists of the excitatory amino acid transporters EAAT1–EAAT5 (designated as SLC1A1-3, 6-7) and the alanine serine cysteine transporters ASCT1-ASCT2 (designated as SLC1A4-5) in mammals (Freidman et al., 2020). This transmembrane transporter encoded by SLC1A6 mediates the uptake of L-glutamate and L/D-aspartate (Fairman et al., 1995; Ryan et al., 2009; Vandenberg and Ryan, 2013; Guskov et al., 2016). Although the role of SLC1A6 in cancers was not well documented, other members of the SLC1A family were reported to be overexpressed in multiple tumors and predict poor prognosis. For example, expression of SLC1A1, SLC1A2 or SLC1A3 contributed to promoting tumor progression in solid tumors, such as lung cancer, glioma, and gastric cancer (Ye et al., 1999; de Groot et al., 2005; Tao et al., 2011; Xu et al., 2020; Guo et al., 2021). Besides, the SLC1A members were also reported to be associated with drug resistance. SLC1A1 was upregulated in oxaliplatin-resistant colorectal cancers (Pedraz-Cuesta et al., 2015), and SLC1A3 was associated with L-asparaginase resistance in acute lymphoblastic leukemia (Sun et al., 2019). As mammalian transporters of amino acids, members of the SLC1A family are implicated to impact drug-related metabolic profiles in tumor cells.

In the present study, we established two radioresistant NPC cell lines, HONE1-IR and CNE2-IR. We found that the radioresistant NPC cells acquired the characteristic of reduced cisplatin sensitivity, which was associated with the upregulation of SLC1A6. By inducing SLC1A6, HONE1-IR and CNE2-IR cells increased the cellular glutamate level and drug resistance genes, leading to reduced cisplatin sensitivity.

MATERIALS AND METHODS

Ethical Statement

The approval of this study was obtained from the Ethics Committee of Sun Yat-sen University Cancer Center (SYSUCC, Guangzhou, China). This study met the ethical standard of the Declaration of Helsinki. The nasopharyngeal biopsy was performed in all patients who have submitted their informed consent.

Patients and Specimens

A total of 78 NPC patients treated in Sun Yat-sen University Cancer Center were recruited and completely followed up from February 2011 to August 2014. The NPC biopsy specimens were fixed in 4% paraformaldehyde and paraffin-embedded and used for immunohistochemical (IHC) analysis of SLC1A6 expression.

SLC1A6 expression was scored according to the staining intensity and percentage of positively stained cells. The staining intensity score of SLC1A6 was graded as follows: 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow-brown); 3, intense staining (brown). The staining percentage score of SLC1A6 was graded as follows: 1, percentage of positive cells less than 30%; 2, percentage of positive cells between 30 and 60%; 3, percentage of positive cells more than 60%. The total-score was calculated as the formula: Total score = \sum (Intensity score \times percentage score) = (1 \times percentage score) + (2 \times percentage score) + (3 \times percentage score). Finally, the intensity score, percentage score, and total score were used to verify the prognostic value of SLC1A6 expression for overall survival (OS).

Immunohistochemistry

The 5- μ m paraffin sections were deparaffinized with xylene and rehydrated in graded ethanol. Antigen retrieval was achieved by placing the sections in sodium citrate buffer (pH 6.0) at 95°C for 20 min. The sections were blocked with 5% goat serum in PBS and incubated with primary antibody against SLC1A6 (1:100, Thermo fisher, the United States) overnight at 4°C. The next day, the sections were stained with SP-9000 Detection Kits (Biotin-Streptavidin HRP Detection Systems, ZSGB-Bio, China) and the DAB Kit (ZSGB-Bio, China) was used for color development according to the manufacturer's manual. The sections were counterstained with hematoxylin and observed with a light microscope.

Cells and Cell Culture

Human NPC cell lines CNE2 and HONE1 were gifted from Professor Chaonan Qian (SYSUCC) (Guo et al., 2020). Both the CNE2 and HONE1 cell lines, and their radioresistant cell lines HONE1-IR and CNE2-IR, were cultured in RPMI-1640 medium (Gibco, Thermo fisher, United States), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotics (Penicillin-Streptomycin). Another head and neck cell line SCC9 was cultured in F12-Dulbecco's modified Eagle's medium (Gibco, Thermo fisher, United States), supplemented with 10% FBS and 1% antibiotics (Penicillin-Streptomycin). Customized RPMI-1640 (deprived glutamic acid or aspartic acid) was ordered from Weiga Biotechnology Company (Guangzhou, China), supplemented with 10% dialyzed FBS (Gibco, Thermo fisher, United States). All cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Establishment of Radioresistant Cell Lines

According to our previous study (Guo et al., 2020), HONE1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and reached approximately 50% confluence in 25-cm² flasks. Cells were treated with a dose of 6Gy radiation using an X-rays generator. After radiation, the culture medium was replaced with complete fresh medium and cells were returned to the incubator. Cells were passaged until they reached approximately 90% confluence. The fractionated irradiations were repeated five times and reached a total dose of 30Gy. The interval between each radiation was at least 2-weeks for all cells. Radioresistant cell population were selected and were referred as HONE1-IR cells.

The parental cells without irradiation were used as control cells. The CNE2-IR cells were kindly provided by Professor Yunfei Xia (SYSUCC).

Quantitative Real-Time PCR

The total RNAs were extracted by the TRIzol reagents (RNAiso PLUS, Invitrogen, US) following the manufacturer's manuals. The isolated RNAs were assessed by Bioanalyzer 2,200 (Agilent, United States) to determine their concentrations and quality before conversion to cDNA. Quantitative RT-PCR was carried out using the Real-Time PCR Detection System (Lightcycler 480 II, Roche, United States) based on the manufacturer's manual. Values were expressed as fold changes of the controls using the $2e^{-\Delta\Delta Ct}$ method.

RNA-Seq Transcriptome Analysis

NPC Cells (HONE1 and HONE1-IR, CNE2, and CNE2-IR) were cultured and their total RNAs were extracted as described above and kept at -80°C . Sequencing and bioinformatic analysis were performed by DESeq2 and edgeR platforms with the aid of Novogene Company (Beijing, China). The intensity was used to generate the heatmap by Novomagic platform (Novogene Company). Differentially expressed genes were determined when $p < 0.05$ and the absolute \log_2 fold change of expression was greater than 3.

MTS Assay

HONE1 (HONE1-IR and NC cells) and CNE2 (CNE2-IR and NC cells) were planted into 96-well plates at $1-2 \times 10^3$ cells/200 μL /well, treated with increasing concentration of cisplatin for 24 h. In the last 2 h of incubation, 20 μL of MTS tetrazolium (Promega, United States) was added to each well based on the manufacturer's protocols. Cell viability was examined by assessing the light absorbance at 490–500 nm. The cell survival curves were drawn based on the results obtained.

Colony Formation

HONE1 (HONE1-IR cells and NC cells) and CNE2 (CNE2-IR and NC cells) were planted into 6-well plates at 5×10^2 cells/well. Subsequently, cisplatin was added to the cultured medium at the concentration of 20 μM for 24 h of treatment. After that, the culture medium was replaced by a fresh medium. The cells were cultured for 2 weeks. Crystal violet was used to stain the colonies.

Small Interfering RNA Transfection

HONE1-IR and CNE2-IR cells were planted into 6-well plates one day before transfection to reach about 60–70% confluence. The siRNAs targeting SLC1A6 gene were purchased from Ruibo Company (Guangzhou, China). Transient transfection of HONE1-IR and CNE2-IR cells was performed using Lipofectamine RNAiMax (Invitrogen, United States) according to the manufacturer's protocols. Cells were transfected with a total of 50 pmol siRNA and subjected to Western blot and MTS assays after 24–48 h post-transfection. The siRNA sequences for SLC1A6 were as follows:

SLC1A6-siRNA-1 : 5'-AUGAAAACUGCAAUGACUGUA-3'
SLC1A6-siRNA-2 : 5'-AAGGAAUAAGCCAACGAUGAC-3'

Establishment of SLC1A6 Overexpressed Stable Cell Lines

The cDNA of SLC1A6 was synthesized according to the human full-length open reading frame of SLC1A6 mRNA (NM_001,272,087) and integrated into pcDNA3.1 plasmid. The lentiviral expression plasmid was used to transfect 293T cells for packaging. The culture media of transfected 293T cells was harvested and used to transfect HONE1 or CNE2 cells. Cells expressing SLC1A6 were selected with puromycin (Sigma-Aldrich, United States) in the concentration of 2 $\mu\text{g}/\text{mL}$. SLC1A6-overexpressed stable cells were established after 10 days selection.

Western Blotting Analysis

Following treatments, cells were lysed by RIPA buffer (Beyotime, China) containing protease and phosphatase inhibitors (Life Technologies, United States). Protein concentrations were detected by a BCA kit (Thermo Fisher, United States). Proteins of samples were subjected to 8–10% SDS-PAGE and transferred to PVDF membranes (Biorad, United States). After being blocked with 5% non-fat milk for 1 h, membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies against SLC1A6 (1:1,000, Thermo fisher), γH2AX (1:1,000, Cell Signaling), Beta-actin (1:1,000, Cell Signaling) were used. Then the membranes were washed with TBS containing 0.1% Tween-20, followed by incubation with HRP anti-rabbit (1:1,000, Cell Signaling) secondary antibody. The band intensity values were normalized to that of Beta-actin.

Measurement of Glutamate or Aspartate Level

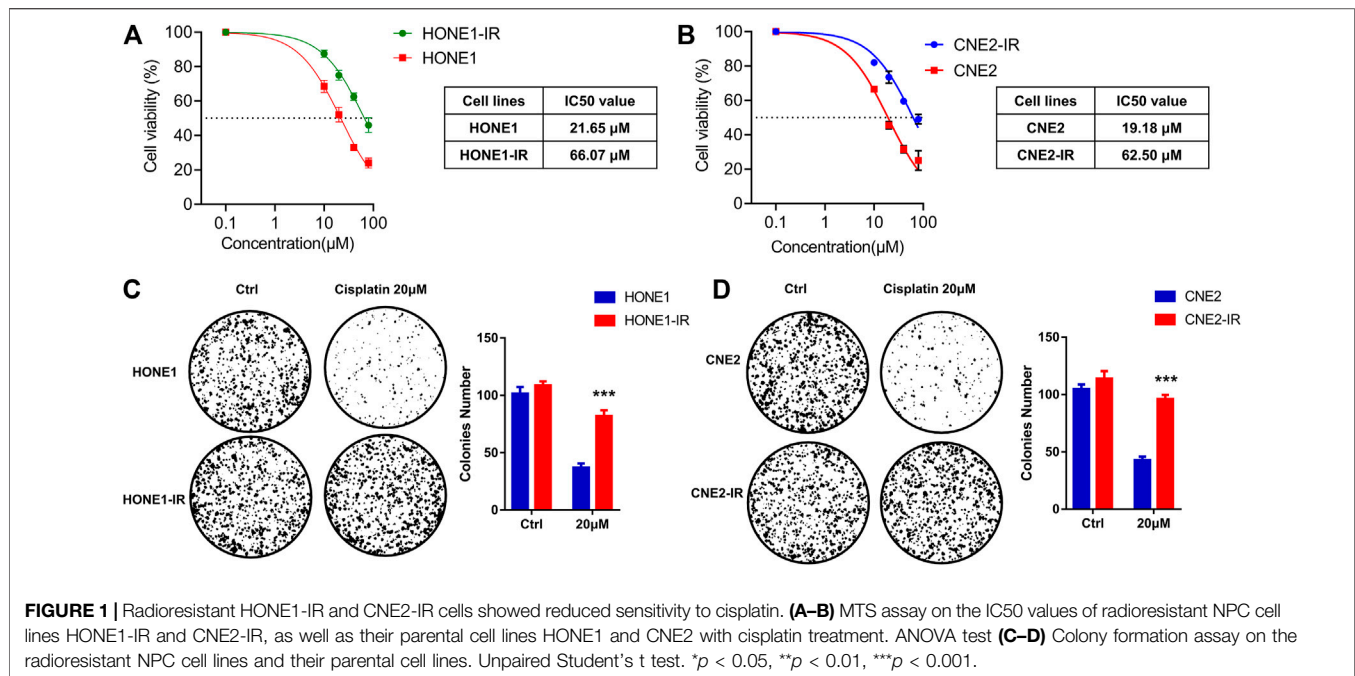
A total of 10×10^6 cells were collected and lysed by ultrasonication (low frequency, 3s, 20 times), glutamate or aspartate content was extracted by glutamate assay kit (Solarbio, China) or aspartate assay kit (Abnova, Taiwan). The level of glutamate or aspartate was detected by comparing the light absorbance value with the standard solution curve. The absorbance value was measured at 340 nm or 570 nm, respectively.

Online and Public Database

The correlation of SLC1A6 expression and survival outcome in patients with head and neck squamous cell carcinoma (HNSCC) in the TCGA database was analyzed on the GEPIA website (<http://gepia.cancer-pku.cn/>).

Statistical Analysis

The data were expressed as the mean \pm SD (the standard deviation). Comparisons between two groups were analyzed by unpaired Student's *t* test. Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Significant *p* value was considered as < 0.05 . In all cases, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.



RESULTS

Radioresistant HONE1-IR and CNE2-IR Cells Showed Reduced Sensitivity to Cisplatin

Two radioresistant human NPC cell lines, HONE1-IR and CNE2-IR, were generated and their resistance to radiation was verified by survival assay (Guo et al., 2020). As the sensitivity to cisplatin is closely associated with radiotherapy efficacy, we compared the sensitivity to cisplatin in radioresistant NPC and their parental cells. MTS assay supported that radioresistant NPC cells exhibited reduced sensitivity to cisplatin compared to their parental cells. Subsequent analysis of chemosensitivity indicated that the cisplatin IC50 of HONE1-IR cells was significantly higher than that of their parental cells (66.07 vs. 21.65 μM). Similarly, CNE2-IR cells also showed higher IC50 than that of their parental cells (62.50 vs. 19.18 μM) (Figures 1A,B). Colony formation assay showed that cisplatin treatment significantly reduced tumor cell proliferation in HONE1 and CNE2 cells, but not in HONE1-IR and CNE2-IR cells (Figures 1C,D). These findings indicated that radioresistant NPC cells reduced cisplatin sensitivity.

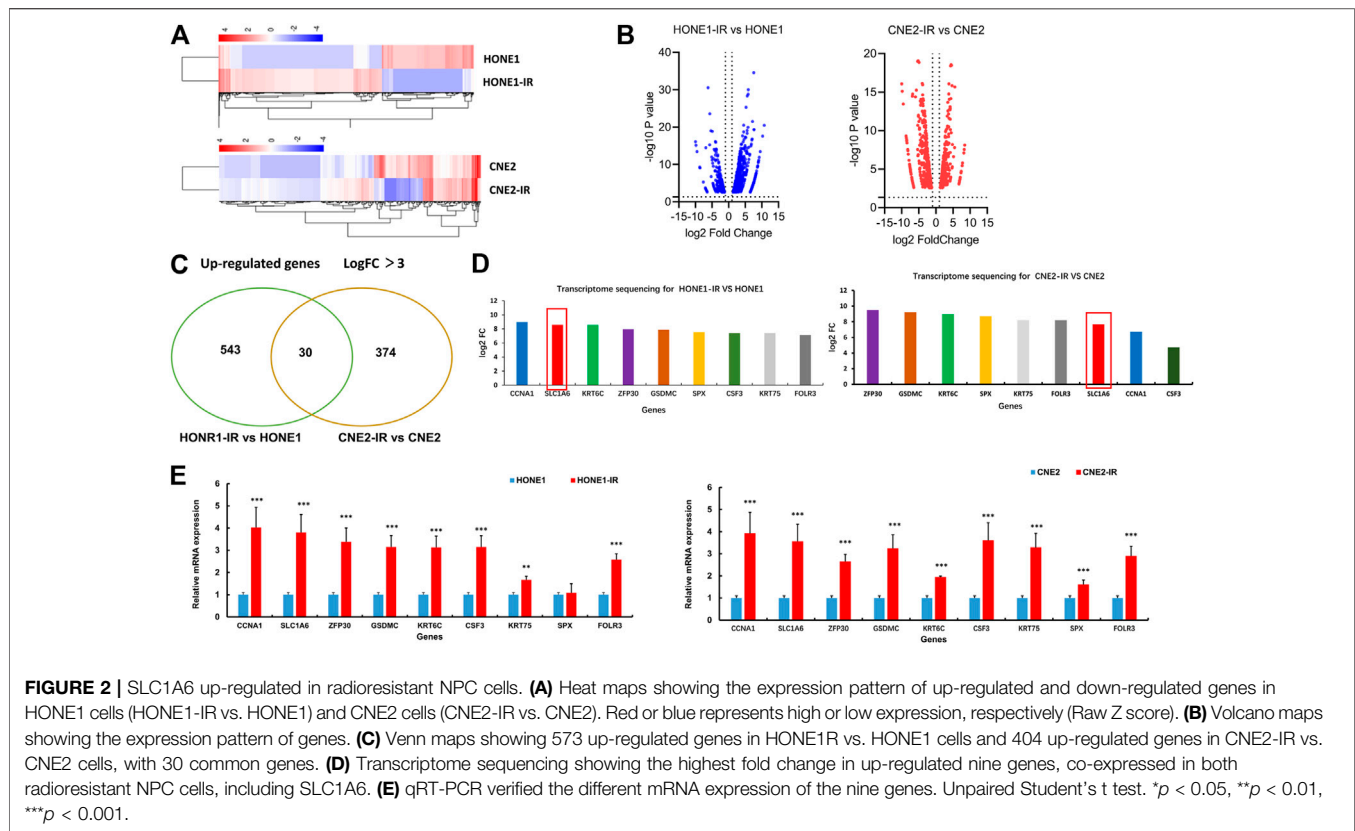
SLC1A6 Gene was Up-Regulated in Both HONE1-IR and CNE2-IR Cells

To explore the potential regulators of cisplatin sensitivity in radioresistant NPC cells, RNA-Sequence was performed to compare the transcriptome profile of HONE1-IR cells and their parental cells. The cutoff criteria were more than 3-fold or less than 3-fold for upregulation or downregulation,

respectively. As for CHE2-IR cells and their parental cells, the same method was performed. Heatmap showed a total of 573 genes in HONE1 cells (HONE1-IR cells vs. their parental cells) and 404 genes in CNE2 cells (CNE2-IR cells vs. their parental cells) were identified to be differentially expressed (Figures 2A,B). There were 30 common genes upregulated in both HONE1-IR and CNE2-IR cells compared to their parental cells (Figure 2C). SLC1A6 gene was particularly noted in the top nine over-expressed genes (Figure 2D) and verified by qRT-PCR (Figure 2E). These results indicated that SLC1A6 might be a crucial gene associated with cisplatin or radiation sensitivity in radioresistant NPC cells.

SLC1A6 Overexpression Conferred Reduced Cisplatin and Radiation Sensitivity in Radioresistant NPC Cells

Furthermore, the up-regulation of SLC1A6 has been verified by Western blots in both HONE1-IR and CNE2-IR cells compared to their parental cells (Figure 3A). To further investigate the role of SLC1A6 in NPC radioresistant cells, siRNA or SLC1A6 overexpression lentivirus was used to knock down or up regulate the expression of SLC1A6 in cells (Figure 3B). Results showed that downregulation of SLC1A6 gene re-sensitized radioresistant NPC cells to cisplatin treatment (Figure 3C). Moreover, overexpression of SLC1A6 decreased the sensitivity to cisplatin in parental cells (Figure 3D). Besides, modulation of the SLC1A6 also impacted the sensitivity to radiation in radioresistant NPC and parental cells (Figures 3E,F). Cisplatin and radiation both cause DNA damage. Increased gamma-H2AX (γH2AX) expression, a biomarker of DNA damage, was noticed in radioresistant NPC cells by



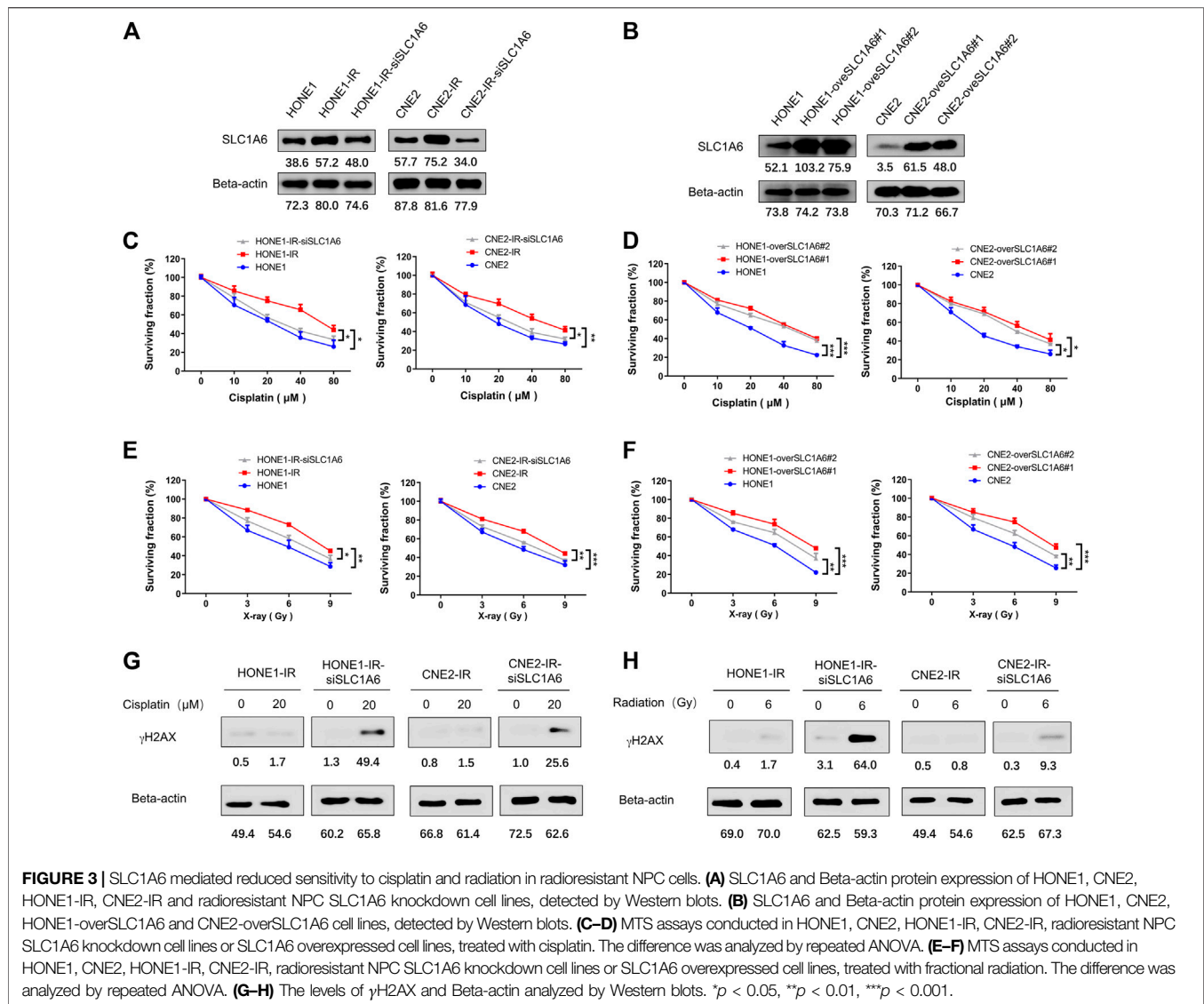
knocking down SLC1A6, followed by cisplatin or radiation treatment (Figures 3G,H). These results demonstrated that SLC1A6 contributed to reducing cisplatin and radiation sensitivity of radioresistant NPC cells.

SLC1A6 Induced by Radiation Treatment and Correlated with Poor Prognosis

As SLC1A6 gene was up-regulated in the radioresistant NPC cells, we next investigated the impact of radiation on SLC1A6. We observed both RNA (Figure 4A) and protein levels (Figure 4B) of SLC1A6 increased during radiation treatment in NPC cells. As NPC belongs to HNSCC, another HNSCC cell line SCC9, was utilized to further verify the role of the SLC1A6 gene on the sensitivity to cisplatin. Similarly, SLC1A6 up-regulation was seen during radiation treatment in SCC9 cells (Figures 4A,B). SLC1A6 over-expressed SCC9 cells exhibited low sensitivity to cisplatin (Figure 4C). Analysis on TCGA database showed that high SLC1A6 expression was correlated with poor prognosis in HNSCC patients (Figure 4D). To further validate the prognostic role of the SLC1A6 in NPC patients, we collected 78 biopsies from NPC patients in Sun Yat-sen University Cancer Center. We demonstrated that high SLC1A6 expression was correlated with poor prognosis in these patients (Figures 4E,F). These results demonstrated that overexpression of SLC1A6 was associated with low therapeutic efficacy and poor survival in NPC patients.

SLC1A6 Up-Regulated Glutamate Level and Drug Resistance Genes

Further experiments were conducted to elucidate the underlying mechanism of SLC1A6 in regulating cisplatin sensitivity in radioresistant NPC cells. SLC1A6 is one of the members of the EAATs family, which transport aspartate, glutamate, and cysteine. These amino acids serve as substrates in several biochemical and metabolic pathways in cancer cells. Previous studies have reported that the EAATs-mediated therapeutic resistance is related to altered tumor metabolic profiles (Ye et al., 1999; de Groot et al., 2005; Tao et al., 2011; Pedraz-Cuesta et al., 2015; Sun et al., 2019; Xu et al., 2020; Guo et al., 2021). It was found that the level of glutamate and aspartate increased in the radioresistant NPC cells compared to their parental cells (Figure 5A). In addition, deprivation of glutamate, not aspartate, in the culture medium re-sensitized radioresistant NPC cells to cisplatin treatment (Figure 5B). Moreover, PCR analysis revealed that the expression of CYP1A1, CYP2C8, CYP2D6, DHFR, GSTP1, and SULT1E1 genes, that are associated with drug catabolism, were significantly higher in radioresistant NPC cells compared to their parental cells (Figure 5C). ABCC1 and ABCC3, that are associated with drug transportation, were also elevated in radioresistant NPC cells (Figure 5C). The expressions of these genes were significantly decreased when SLC1A6 was knocked down in radioresistant NPC cells (Figure 5D). These results collectively supported that SLC1A6 overexpression reduced



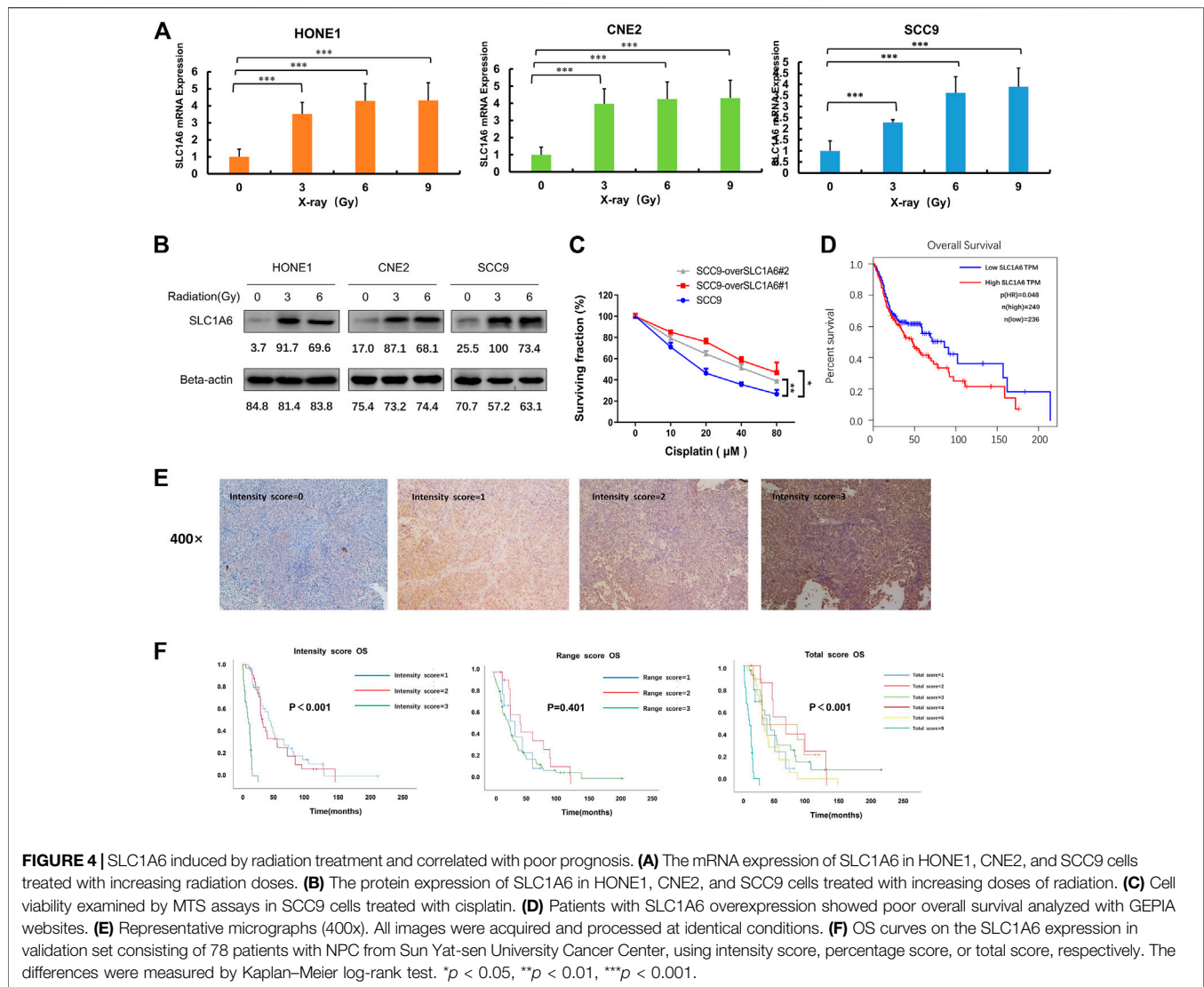
cisplatin sensitivity in radioresistant NPC cells by increasing the level of glutamate and drug resistance genes.

DISCUSSION

Radiation resistance has been a significant obstacle for the local control of NPC. Some patients who are resistant to radiotherapy are also not sensitive to cisplatin treatment. In this study, we observed the decreased sensitivity to cisplatin in radiation resistant NPC cells. Although previous studies have revealed the mechanism of radiation-resistance or cisplatin resistance, the relationship between radiation and cisplatin resistance is complicated and not elucidated. The cause of cisplatin resistance, included aberrant repair of DNA damage, apoptosis pathway defects, activation of drug export system, altered cellular metabolism, reduced oxidative stress, and cancer stem cell induction, etc. (Ikuta et al., 2005; Galluzzi et al., 2012; Zhang et al., 2012; Cruz-Bermúdez et al., 2019) As both radiation and cisplatin

cause DNA damage in tumor cells, they might share the same biological pathway to reverse DNA damage. Cross-resistance mechanisms reported by studies include elevated GSH level, DNA repair enzymes, NF κ B and TNF α , etc. (Chao et al., 1991; Popenborg et al., 1997; Zhu et al., 2019)

Here, the identification of SLC1A6 as the crucial gene conferring reduced cisplatin sensitivity in radiation-resistant NPC cells is novel. In this study, it was found that SLC1A6 expression was upregulated in HONE1-IR and CNE2-IR cells. Down-regulating SLC1A6 expression could significantly rescue the cisplatin sensitivity in HONE1-IR and CNE2-IR cells. The SLC1A6 could be the common factor to reduce DNA damage from radiation or cisplatin treatment, which was confirmed by our study. Our results were in consistent with previous studies that described the radiation-induced cisplatin resistance (Eichholtz-Wirth et al., 1993; Eichholtz-Wirth, 1995; Zhuang et al., 2019). The cisplatin resistance acquired during radiation could explain the phenomenon that some patients with radio-resistance also didn't respond to cisplatin

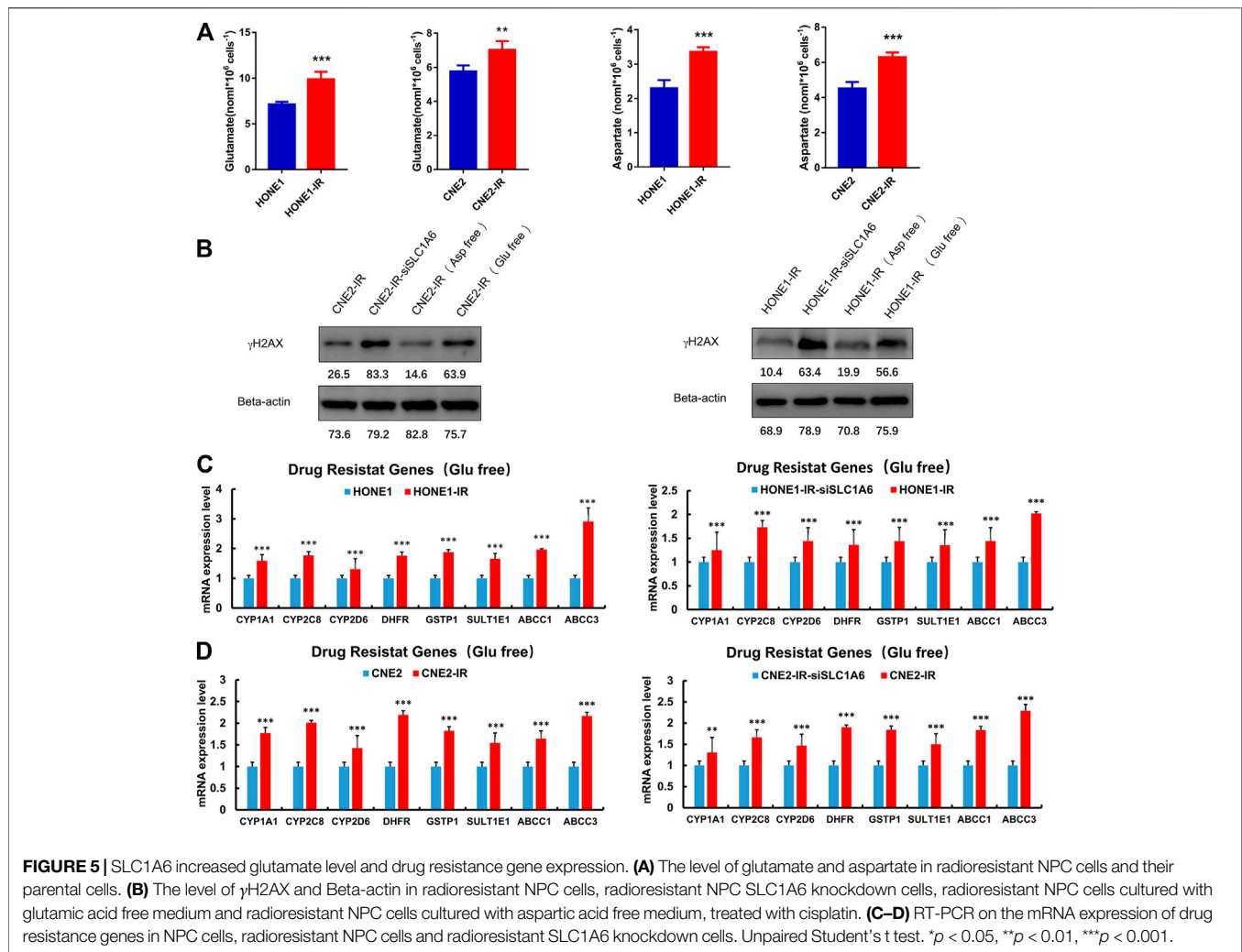


treatment and correlated with the radiation-induced SLC1A6 upregulation. However, the mechanism of radiation induced SLC1A6 overexpression remained far from understood. We hypothesized that genetic reprogramming happened in the process of DNA injury and repair upon radiation.

SLC1A family is thought to contribute to tumor progression by regulating microenvironments and metabolic profiles (Ye et al., 1999; de Groot et al., 2005; Tao et al., 2011; Pedraz-Cuesta et al., 2015; Sun et al., 2019; Freidman et al., 2020; Xu et al., 2020; Guo et al., 2021). SLC1A6 transports aspartate, glutamate, and cysteine, and regulation of these amino acids is essential for numerous biochemical and metabolic pathways such as the TCA cycle or nucleotide synthesis. For example, glutamate could be transformed into glutamine, facilitating nucleotide synthesis and repair DNA damage (Fu et al., 2019). The endocrine resistance breast cancer cells would increase aspartate and glutamate import to sustain DNA, lipid, and protein synthesis (Bacci et al., 2019). We found both glutamate and aspartate were elevated in radioresistant NPC cells. However, only glutamate played a vital role in resisting DNA injury

from cisplatin treatment. These findings suggested that SLC1A6 contributed to metabolic reprogramming in radioresistant NPC cells.

We also uncovered that SLC1A6 promoted the upregulation of drug catabolic genes (CYP1A1, CYP2C8, CYP2D6, DHFR, GSTP1, and SULT1E1) and drug transport genes (ABCC1 and ABCC3). The CYP enzymes have been extensively investigated in drug metabolism, and their inhibitors were proven to be effective in reversing cisplatin sensitivity in cancer cells (Sonawane et al., 2019). Gstp1, a GST family member, is involved in the detoxification of cisplatin via cisplatin-glutathione adducts formation (Li et al., 2019). DHFR and SULT1E1 have been reported to be up-regulated in cisplatin-resistant cells (Marverti et al., 2009; Varamo et al., 2019). The ABC family of transporters is referred to as multidrug resistance proteins that transport substrates across the cellular membranes (Chen et al., 2016). These results implicated that SLC1A6 contributed to cisplatin resistance in radioresistant NPC cells through multiple factors.



In summary, we identified the upregulation of SLC1A6 in radioresistant NPC cells. Overexpression of SLC1A6 is correlated with reduced sensitivity to cisplatin by elevating the level of glutamate and drug resistance genes. Targeting SLC1A6 could be a potential strategy to enhance cisplatin sensitivity in NPC 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 below: NCBI SRA, PRJNA700383.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Sun Yat-sen University Cancer Center, and met the ethical

standard of the Declaration of Helsinki. Informed consent was obtained from each patient before obtaining the nasopharyngeal biopsy.

AUTHOR CONTRIBUTIONS

WH, LW, LC, JY, JW and LN conducted experiments and acquired the data; WH, LW, LC and JY analyzed the data; WH, LW and LC wrote the paper; YC and XL conceived and designed the study. All authors read and agreed on the final manuscript.

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

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