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SLC7A11 negatively associates with mismatch repair gene expression and endows glioblastoma cells sensitive to radiation under low glucose conditions

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The cystine/glutamate antiporter xCT (SLC7A11) is frequently upregulated in many cancers, including glioblastoma (GBM). SLC7A11-mediated cystine taken up is reduced to cysteine, a precursor amino acid for glutathione synthesis and antioxidant cellular defense. However, little is known about the biological functions of SLC7A11 and its effect on therapeutic response in GBM. Here, we report that the expression of SLC7A11 is higher in GBM compared with normal brain tissue, but is negatively associated with tumor grades and positively impacts survival in the bioinformatic analysis of TCGA and CGGA database. Additionally, a negative association between SLC7A11 and mismatch repair (MMR) gene expression was identified by Pearson correlation analysis. In the GBM cells with glucose-limited culture conditions, overexpression of SLC7A11 significantly decreased MMR gene expression, including MLH1, MSH6, and EXO1. SLC7A11-overexpressed GBM cells demonstrated elevated double-strand break (DSB) levels and increased sensitivity to radiation treatment. Taken together, our work indicates that SLC7A11 might be a potential biomarker for predicting a better response to radiotherapy in GBM.

Key words: SLC7A11, glioblastoma, mismatch repair, radiotherapy

Glioblastoma multiforme (GBM) is the most common malignant adult brain tumor and newly diagnosed patients have a life expectancy of 13–15 months [1, 2]. GBM patients inevitably progress or relapse after treatment, despite the advances in understanding tumor biology, improved surgical techniques, and chemoradiotherapies [3]. Therefore, there is a dire need to identify novel potential therapeutic targets for effective therapeutic options of GBM.

As the key component of the amino acid transporter system Xc-, a twelve-pass transmembrane protein SLC7A11 is involved in the efflux of glutamate along with the influx of extracellular cystine [4, 5]. The imported cystine is reduced to cysteine to serve as the precursor for glutathione (GSH) synthesis, maintaining intracellular redox balance, and reducing hydrogen peroxide [5]. GSH is required for optimal activity of GSH peroxidase 4 (GPX4), a key inhibitory enzyme of an iron-dependent form of non-apoptotic cell death called ferroptosis [6, 7]. Thus, intracellular transport of cystine by SLC7A11 is important to avoid oxidative stress and cell death in cancer cells. In line with the potential oncogenic role of SLC7A11, the expression of SLC7A11 is often upregulated in cancer cells, including GBM cells and patient samples, as well as correlates with tumor growth and poor survival [8, 9]. However, it was recently found that overexpression of SLC7A11 in GBM cells promotes cell death under glucose deprivation [10–12]. Given that normal astrocytes are not sensitive to glucose deprivation, the difference in glucose sensitivity may provide an effective therapeutic approach, such as radiation, for GBM.

Mismatch repair (MMR) is an essential pathway responsible for the repair of base mismatches arising during DNA replication or otherwise caused by DNA damage. MMR deficiency resulting from inactivating MMR gene mutations or silenced expression predisposes cells to tumorigenesis [13, 14]. The MMR system depends on several key genes, including mutS homolog 2 (MSH2), mutS homolog 6 (MSH6), mutL homolog 1 (MLH1), as well as Exonuclease 1 (EXO1), DNA Polymerase Delta 1 (POLD1), and replication factor C (RFC) family members [15, 16]. GBM with MMR deficiency is resistant to temozolomide treatment due to escaping the futile cycle of mismatch repair [17, 18], but double-strand break (DSB) repair is reduced in MMR-deficient tumors, triggering the sensitivity of MMR-deficient tumor cultures to radiation and other DSB inducers [19, 20].

In this study, we found that SLC7A11 is overexpressed in glioma and negatively associated with MMR gene expression

in the TCGA and CGGA databases. SLC7A11 overexpression in GBM cells with a low glucose supplement decreased some key MMR genes' expression and sensitized GBM cells to radiation.

Materials and methods

Bioinformatic analysis. The 695 samples in the glioma RNA-seq data set downloaded from The Cancer Genome Atlas (TCGA) comprised 257 patients with Grade II, 266 patients with Grade III, 167 patients with Grade IV, and 5 normal samples. Among the 690 glioma patients, 614 patients were provided with clinical information of 'radiation therapy', including 420 patients who received radiation therapy and 194 patients who did not. To improve the analysis of the differences in expression of *SLC7A11* between glioma and normal tissues, the Genotype-Tissue Expression (GTEx) database was introduced and an RNA-seq data set was extracted from 1,152 normal brain tissues. The R package "limma" was used to merge and de-batch TCGA and GTEx data. All of these data were downloaded from UCSC Xena (http://xena.ucsc.edu/). In addition, two RNA-seq data sets (n=325 and n=693) and one microarray data set (n=301) were downloaded from the Chinese Glioma Genome Atlas (CGGA) (http://www.cgga.org.cn/). R software was used to analyze the obtained data, to yield the expression levels of *SLC7A11* in normal brain tissue and gliomas, the correlation between the expression levels of *SLC7A11* and MMR genes, and the prognostic information for *SLC7A11* in gliomas. In terms of functional enrichment analysis, this study used GSEA software for KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses of *SLC7A11* against the TCGA glioma database.

Cell culture. U87 and T98G cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (4,500 mg/l; 25 mM) or low glucose (900 mg/l; 5 mM) containing L-glutamine, sodium pyruvate, and sodium bicarbonate (Cat. #D6429), supplemented with 10% FBS (Life Technologies), 2 mmol/l L-glutamine, penicillin, and streptomycin (Sigma).

Immunoblotting. Whole-cell lysates or tumor protein extracts were prepared in lysis buffer supplemented with PhosSTOP phosphatase inhibitor and complete protease inhibitor tablets (Roche). The primary antibodies are anti-MLH1 (#4256, Cell Signaling Technology), anti-MSH6 (#5424, Cell Signaling Technology), anti-EXO1 (ab95068, Abcam), anti-SLC7A11 (#12691, Cell Signaling Technology), and β-Actin (#3700, Cell Signaling Technology).

qPCR. Real-time quantitative PCR was performed using Fast SYBR Green Master Mix on a Quant Studio 6 FlexPCR system (Applied Biosystems). PCR primers used in this study are as follows: *MLH1*, F: 5'-GTTCTCCGGGAGATGTTGCATA-3', R: 5'-TGGTGGTGTTGAGAAGGTATAACTTTG-3'; *MSH6*, F: 5'-GGGATACAGCCTTTGACC-3', R: 5'- GTTTACAGCCCTTCTTGG-3'; *EXO1*, F: 5'-TCTGAGAGGTAGTTAATTTGG-3', R: 5'-TACATCCATCAAATACGAGA-3'; *GAPDH*, F: 5'-GTCTCCTCTGACTTCAACAGCG-3', R: 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Clonogenic survival. U87 and T98G cells expressing SLC7A11 or vector control were treated with radiation and then replated at cloning densities. Cells were grown for 11 to 14 days and then fixed/stained with methanol-acetic acid and crystal violet, respectively, and scored for colonies of 50 cells or more. The cytotoxicity of SLC7A11 overexpression in the absence of radiation treatment was calculated as the ratio of surviving treated cells relative to surviving vector control cells. Radiation survival data from treated cells were corrected for plating efficiency by normalizing to non-irradiated control cells. Cell survival curves were fitted using the linear-quadratic equation, and the mean inactivation dose was calculated and used to determine the radiation enhancement ratio. Radiosensitization is indicated by a radiation enhancement ratio of significantly greater than 1.

Irradiation. Irradiations were carried out using a Philips RT250 (Kimtron Medical) at a dose rate of approximately 2 Gy/min.

γH2AX staining. For γH2AX immunofluorescence experiments, cells were grown and treated on coverslips in 12-well dishes. Following treatment, cells were fixed and stained with antibodies recognizing γH2AX antibody (clone JBW301; Millipore) and DAPI. Samples were imaged with an Olympus IX71 FluoView confocal microscope (Olympus America) with a 60× oil objective.

Statistical methods. In terms of bioinformatics, differences in the expression levels of *SLC7A11* between normal subjects and glioma patients were analyzed using one-way ANOVA. The Pearson method was used to analyze the correlation between *SLC7A11* and MMR genes. Kaplan-Meier (K-M) survival-curve analysis was used to analyze the survival. The screening criteria for the GSEA results were $|NES| > 1$, FDR <0.25, and $p<0.05$. All statistical analyses were performed using R software (version 3.6.3, https://www.r-project.org/) and GSEA software (version 4.1.0, https://www.gsea-msigdb.org/gsea/downloads.jsp). A p-value <0.05 was considered significant in two-sided statistical tests.

Results

***SLC7A11* is overexpressed in gliomas but predicts a better prognosis.** To gain the insight of the potential role of SLC7A11 in glioma, we first analyzed the expression of *SLC7A11* in glioma samples in the TCGA and GTEx database, which revealed significantly higher *SLC7A11* expression in gliomas (n=690) than in normal brain tissue (n=1,157), and the expression of *SLC7A11* was the highest in the WHO grade II glioma compared with WHO grade III and WHO grade IV glioma (Figure 1A, upper-left). Next, we did further analysis in gliomas with all grades based on RNA-seq and microarray datasets in the CGGA database, and the results

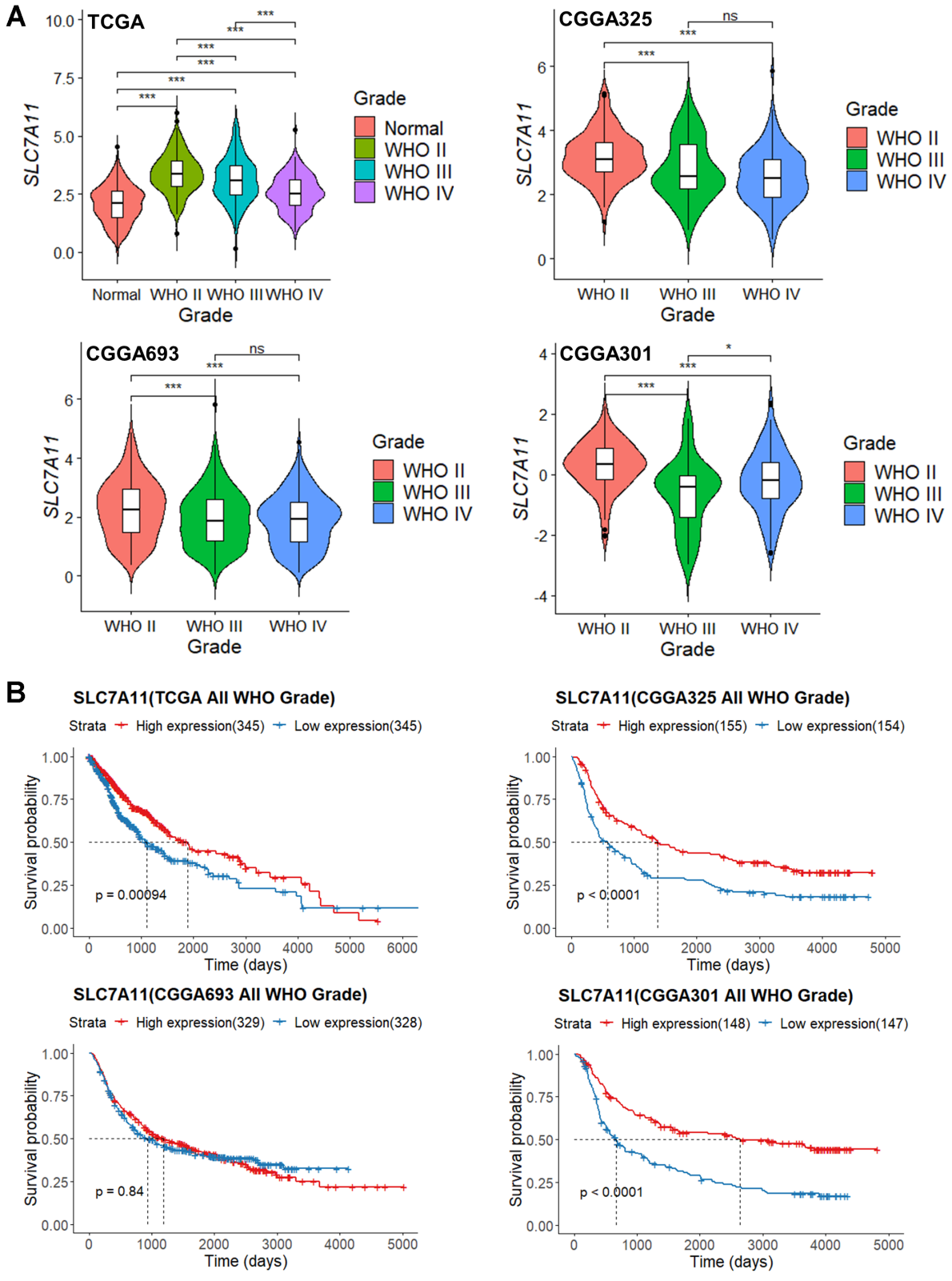

Figure 1. SLC7A11 is overexpressed in gliomas but predicts a better prognosis. A) Violin plots showing the expression level of SLC7A11 in gliomas and normal brain tissue from TCGA, GTEx, and CGGA. ns, *, and * indicate no statistical difference, p<0.05, and p<0.001, respectively. B) K-M survival curves in the four data sets from TCGA and CGGA showing overall survival in glioma patients with high or low *SLC7A11* expression levels.**

clearly showed that the expression levels of *SLC7A11* were also the highest in the WHO grade II glioma compared with WHO grade III and WHO grade IV glioma (Figure 1A, p<0.001). Our analyses suggest that higher expression of *SLC7A11* may be associated with glioma tumorigenesis, but not progression.

We further explored the prognostic value of *SLC7A11* expression levels by analyzing the survival information of glioma patients in the TCGA and CGGA databases. Glioma patients were divided into the high- and the low-expression groups (with a cut-off of 50%) to draw the Kaplan-Meier survival curve (Figure 1B). The results demonstrated that the overall survival was significantly prolonged for glioma patients with a high *SLC7A11* expression in TCGA (p=0.00094), CGGA seq325, and CGGA array301 (p<0.0001), whereas there was no significant difference in survival for glioma patients in CGGA seq693 (p=0.84). Therefore, our bioinformatics analysis validated the upregulation of *SLC7A11* in gliomas, but the *SLC7A11* expression is reversely correlated with tumor grades, as well as predicts a better prognosis

Negative association between the expression of *SLC7A11* and mismatch repair genes (MMR) in gliomas. In order to further explore the biological functions of SLC7A11 in glioma, we again divided high- and low-expression groups of *SLC7A11* from the RNA sequence in the TCGA database. GSEA software was used for the single-gene KEGG enrichment analysis of *SLC7A11* (Figure 2A). Interestingly, we found that the lower *SLC7A11* was enriched in gene sets related to the mismatch repair and nucleotide excision repair (NER). Given that the MMR pathway closely reflects the efficacy of chemoradiotherapy in glioma, we next focused on the association of *SLC7A11* expression with MMR gene transcriptional levels by the Pearson correlation analysis. The analysis results in Figure 2B showed that the expression of most MMR genes, including, *MLH1, MSH6, POLD1, EXO1, PCNA, RFC2, RFC3,* and *RFC4*, exhibits a negative correlation with *SLC7A11* expression in TCGA glioma samples. These findings suggest the regulatory function of SLC7A11 in MMR genes' expression, which might render the SLC7A11-overexpressing gliomas vulnerable to genotoxic agents or radiation treatment.

SLC7A11 overexpression inhibits the MMR genes' expression in GBM cells and exhibits reduced DSB repair. To determine whether SLC7A11 regulates MMR genes' expression in glioma cells, the effects of overexpression of SLC7A11 on some key MMR proteins were measured in U87 and T98G GBM cells. Given that higher SLC7A11 expression promotes GBM cell death under glucose deprivation, and it is not an ideal model for cell culturing without glucose supplement, we performed our cell-based investigations in a low glucose medium (900 mg/l, 5 mM glucose). We found that the overexpression of SLC7A11 significantly reduced the protein levels of MLH1, MSH6, and EXO1 in both cell lines, an observation consistent with the negative correlation of *SLC7A11* and these MMR genes' expression in human glioma samples (Figure 3A). To further examine whether the regulation of MMR genes' expression by SLC7A11 is at the transcriptional level, we detected the *MLH1, MSH6,* and *EXO1* mRNAs and found their transcript levels significantly decreased upon the SLC7A11 overexpression (Figure 3B).

It has been established that MMR-deficient tumors and cells exhibit reduced DNA DSB repair ability [19]. Since the phosphorylated (S139) histone 2AX (γH2AX) serves as a well-established biomarker of DSBs [21], we sought to determine the capability of DSB repair in GBM cells with SLC7A11 overexpression. As shown in Figures 3C and 3D, higher endogenous levels of residual γH2AX foci in the SLC7A11 overexpressed U87 and T98G cells were observed relative to vector control cells. Therefore, upregulation of SLC7A11 leads to dysregulation of MMR genes' expression and a lower DSB repair capacity.

Overexpression of SLC7A11 sensitizes GBM cells to DSB inducers under low glucose stress. The abrogated DSB repair capability upon SLC7A11 overexpression prompted us to detect whether it can cause GBM cells' sensitivity to genotoxic treatment. First, we analyzed the difference of clonogenic formation upon radiation treatment of both U87 and T98G cells in the absence or presence of SLC7A11 overexpression cultured in low glucose media. As shown in Figures 4A and 4B, higher SLC7A11 levels render both cell lines sensitive to radiation, and the radiation enhancement ratio (RER) in SLC7A11 overexpressed cells was significantly higher compared with control cells (U87 cells, RER = 1.6±0.1; T98G cells, RER = 2.1±0.1). To extend our *in vitro* observations to clinical samples, we extracted a total of 614 patients with radiation-related clinical data, and divided the patients into two groups: radiotherapy group (n=420) and non-radiotherapy group (n=194) in the TCGA database, and analyzed their survival in the above two groups respectively. Intriguingly, the results showed that there was no significant difference in the patient survival between high and low *SLC7A11* expression in the non-radiotherapy group (p=0.35), while the patients with higher *SLC7A11* expression had a significantly better prognosis than lower *SLC7A11* patients in the radiotherapy group (p=0.0021) (Figure 4C). To further test the effect of SLC7A11 on the sensitivity of GBM cells to DSB inducer, we used DSB-inducing compound mitomycin C and determined the IC50 of mitomycin C in control and SLC7A11-overexpressed cells by CCK-8 assay. As expected, treatment of mitomycin C proved more detrimental for U87 and T98G cells with SLC7A11 overexpression than vector control (Figures 4D, 4E). Taken together, these data thus indicate that higher expression of SLC7A11 in GBM cells sensitizes to DSB inducers under low glucose conditions.

Discussion

In GBM, GSH is an important antioxidant preventing the accumulation of ROS and promoting GBM cell survival and growth [22, 23]. SLC7A11 indirectly activates GPX4

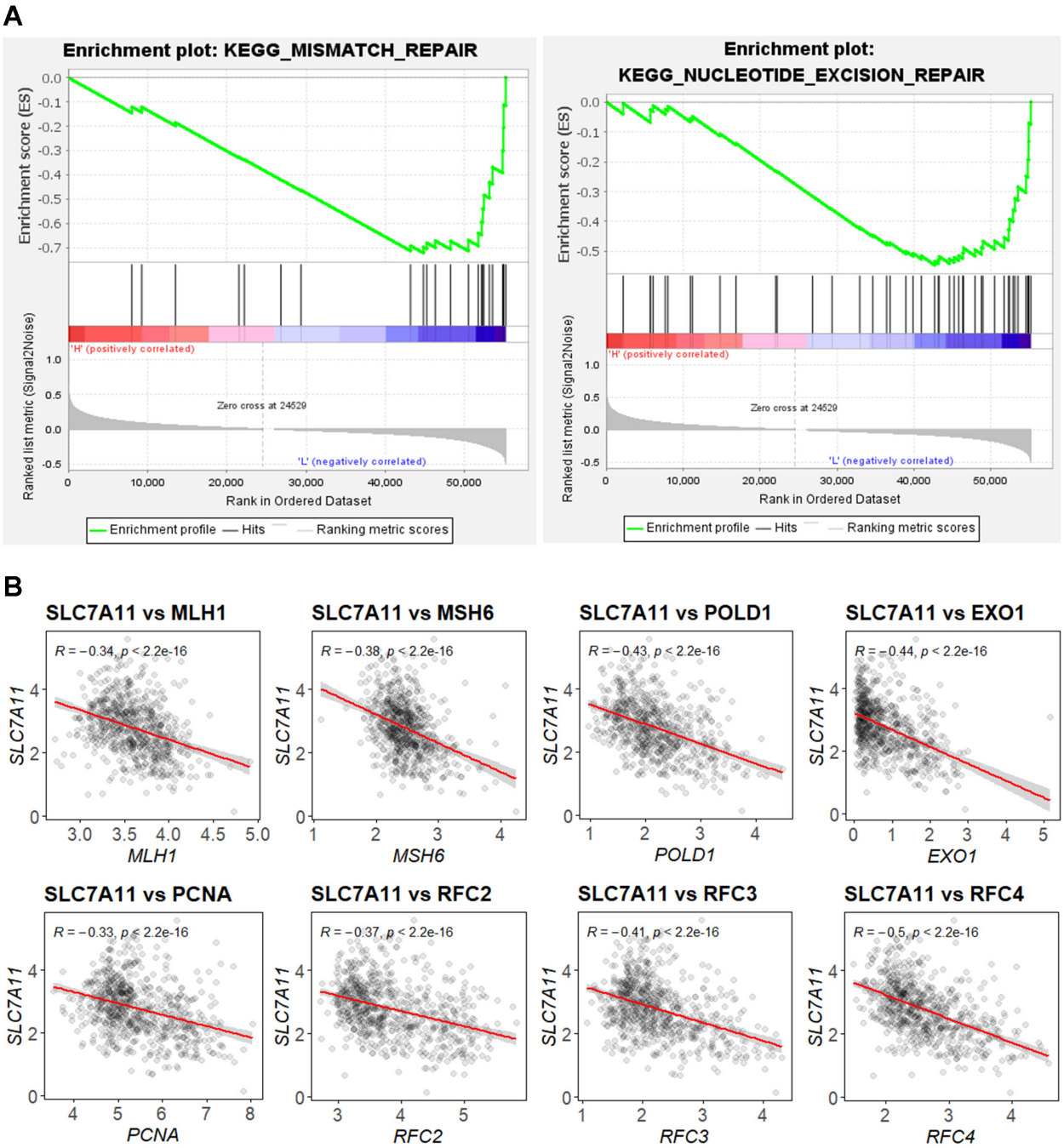

Figure 2. Negative correlation between the expression of SLC7A11 and mismatch repair genes in gliomas. A) GSEA enrichment analysis of SLC7A11 using the TCGA database (n=695). Mismatch repair and nucleotide excision repair were enriched in the low SLC7A11 phenotype. |NES| >1, FDR <25%, p<0.01. B) Expression of SLC7A11 in glioma is negatively associated with mRNA levels of major MMR genes (*MLH1*, *MSH6*, *POLD1*, *EXO1*, *PCNA*, *RFC2*, *RFC3*, and *RFC4*) in the TCGA database using Pearson correlation analysis (p<0.0001).

and decreases toxic lipid ROS by facilitating cystine import and GSH synthesis [11]. Therefore, SLC7A11 is frequently overexpressed in glioblastoma patients and increased expression of SLC7A11 is correlated with tumor growth, seizure, and poor prognosis [9, 24]. Our bioinformatics analysis confirmed the upregulation of *SLC7A11* in gliomas relative to normal brain tissues, validating the oncogenic function of SLC7A11. Rober et al. found that higher *SLC7A11* expression predicted shorter patient survival based on the genomic data in the REMBRANDT (National Institutes of Health Reposi-

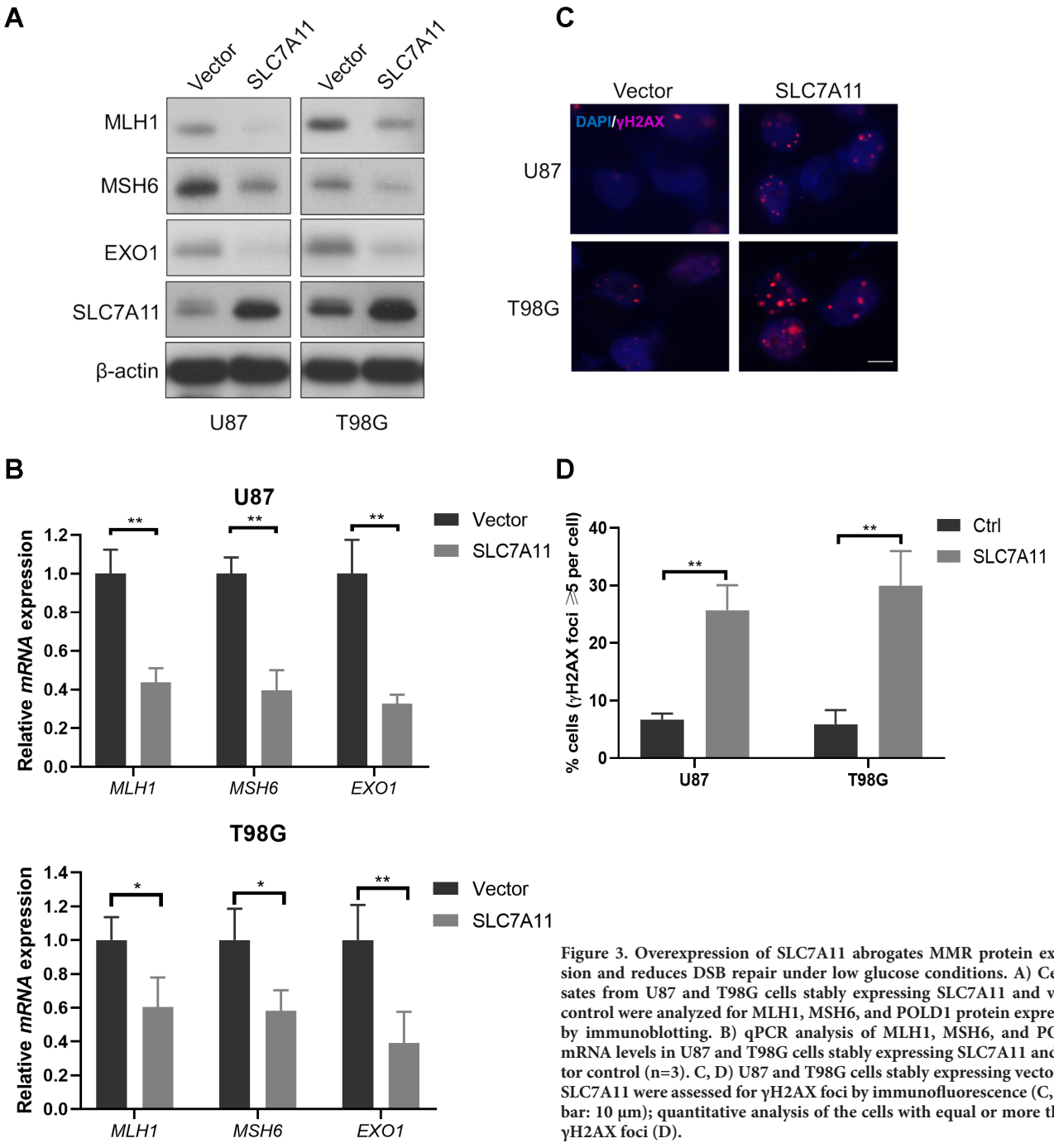

Figure 3. Overexpression of SLC7A11 abrogates MMR protein expression and reduces DSB repair under low glucose conditions. A) Cell lysates from U87 and T98G cells stably expressing SLC7A11 and vector control were analyzed for MLH1, MSH6, and POLD1 protein expression by immunoblotting. B) qPCR analysis of MLH1, MSH6, and POLD1 mRNA levels in U87 and T98G cells stably expressing SLC7A11 and vector control (n=3). C, D) U87 and T98G cells stably expressing vector and SLC7A11 were assessed for γH2AX foci by immunofluorescence (C, scale bar: 10 μm); quantitative analysis of the cells with equal or more than 5 γH2AX foci (D).

tory for Molecular Brain Neoplasia Data) database [9]. However, we here show that increased *SLC7A11* expression correlates with a better prognosis in patient samples from the TCGA and CGGA databases. Further investigations will help to elucidate the effect of SLC7A11 on GBM patient survival.

GBM cells usually grow in a hypoxic and low glucose microenvironment in the brain, which causes cells to adapt to uptake glucose to maintain their survival [25, 26]. This tumor microenvironmental stress leads glioma cells to change their metabolism style and gene expression [27]. However, the well-established *in vitro* GBM cell culture conditions contain high glucose, and may not be able to recapitulate the above alterations. In our study, we cultured GBM cells in a low glucose medium, mimicking the more physiological conditions. We found that the overexpression of SLC7A11 in GBM cells lacking enough glucose decreased the mRNA

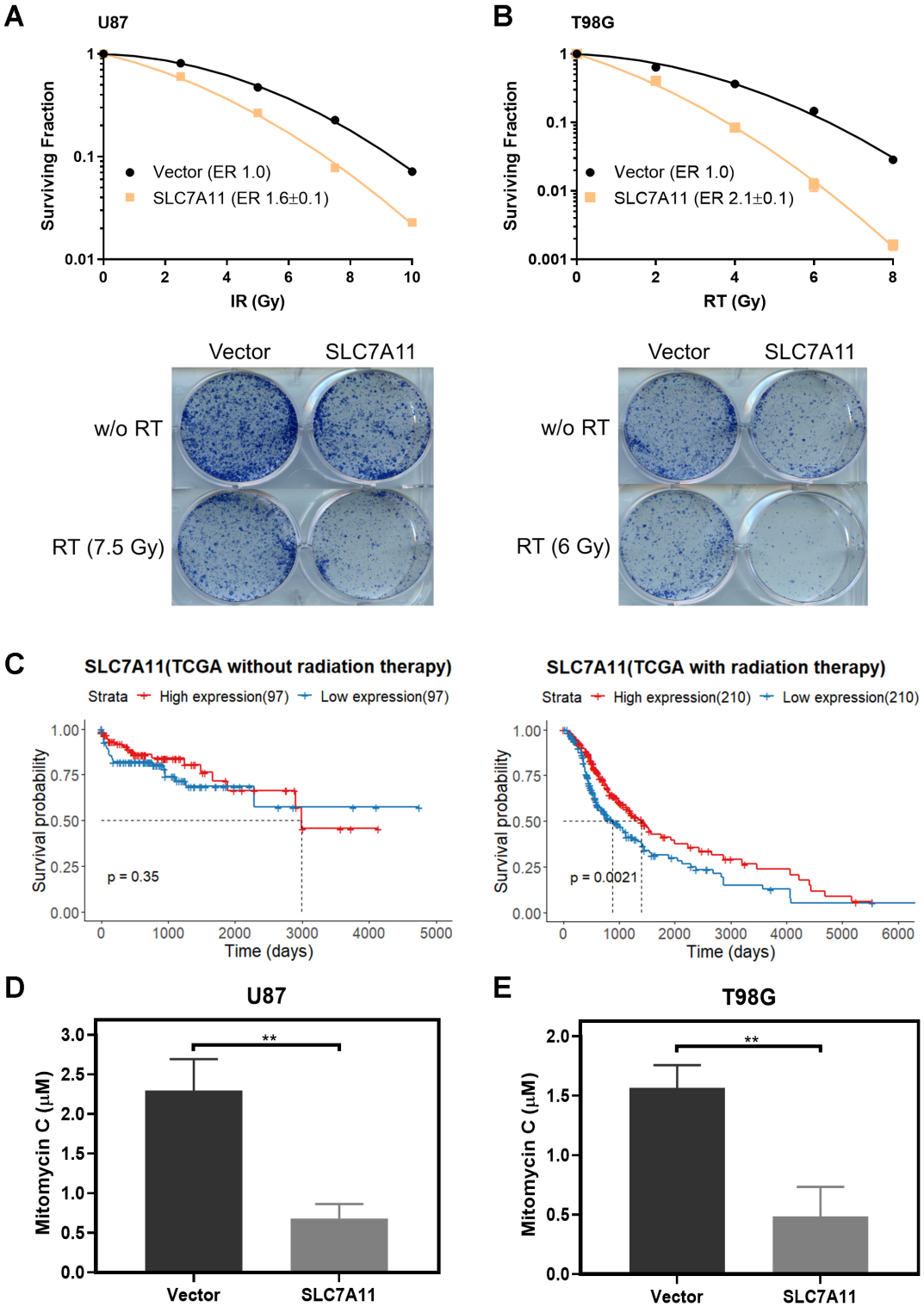

Figure 4. Overexpression of SLC7A11 sensitizes glioma cells to DSB inducers. A, B) U87 cells (A) and T98G cells (B) stably expressing SLC7A11 and vector control were treated with radiation (0, 2.5, 5, 7.5, 10 Gy) and processed for clonogenic survival 24 h post-radiation. Data are from a single representative experiment (plots) or are the mean radiation enhancement ratio ± SE for n=2 independent experiments. Images of clonogenic plates are shown (lower panel). C) K-M survival curves of non-radiotherapy and radiotherapy groups in glioma patients in the TCGA database with high or low *SLC7A11* expression levels. D, E) Cytotoxicity of mitomycin C was measured by CCK-8 assays. Displayed are the average concentrations (μM) that inhibit 50% of the normal growth.

and protein levels of key MMR genes. This phenomenon was not observed in GBM cells when cultured in a high glucose medium (data not shown). In support of this observation, a negative association between *SLC7A11* and MMR genes' expression was also identified in the glioma samples of the TCGA database. Given the low glucose microenvironment of GBM cells *in vivo*, our results raise the possibility that the SLC7A11 upregulation in GBM tumors induces MMR deficiency through downregulating the gene expression.

Functional loss of the MMR system due to the downregulation of MMR genes has been reported in tumors under hypoxic conditions [28–30]. Specifically, the major regulators of this process including hypoxia-inducible factor-1 alpha (HIF-1α) directly downregulate the expression of MMR genes in cancer cells [30]. The combination of SLC7A11 overexpression and low glucose supplement dramatically leads to NADPH depletion (with increased NADP+/NADPH ratio) and a marked increase in cellular reactive oxygen species (ROS) levels [10, 12, 31]. Notably, ROS activates HIF-1α through both transcriptionally promoting *HIF-1α* gene expression and enhancing protein stability [32–34]. Therefore, it is conceivable that SLC7A11-mediated ROS induction under glucose starvation suppresses MMR genes' expression via activating HIF-1α. On the other hand, epigenetic modulations of MMR genes' expression, such as hypoacetylated and hypermethylated histone H3 within *MLH1* and *MSH6* adjacent promoter regions, provides another layer of suppressive regulation [35–37]. However, epigenetic reprogramming in glucose-starved glioma cells with high SLC7A11 expression is still unknown. Thus, it will be of great interest to determine whether SLC7A11 plus limited glucose-mediated MMR gene downregulation is through increased ROS levels, HIF-1α activity, and/or epigenetic mechanisms.

MMR deficiency in GBM causes drug resistance to temozolomide that mediates the formation of O^6 methylguanine-containing mismatches [17, 38]. Mechanistically, in the absence of a functional MMR response, O^6meG:T is not targeted for the "futile MMR repair" cycle. Therefore, MMR deficient GBM cells avoid programmed cell death and survive, albeit with the accumulation of a massive number of DNA mispairs [18, 39]. In this line, higher *SLC7A11* expression levels have been connected to chemoresistance in glioma patients [24]. However, MMR-deficient cells have impaired DSB repair ability and sensitize to DSB inducers [19]. Consistent with these findings, our study shows that SLC7A11-overexpressing GBM cells cultured in low glucose medium demonstrated elevated DSB levels, and GBM cells with SLC7A11 overexpression sensitize to radiation treatment and mitomycin C. Importantly, in our bioinformatics analysis of the TCGA database, prolonged survival was observed in GBM patients with higher *SLC7A11* expression upon radiation treatment. Therefore, our data clearly warrant further studies assessing the therapeutic efficacy of radiotherapy in GBM tumors with high levels of SLC7A11 expression.

In summary, we demonstrate a potential link between SLC7A11 expression and MMR deficiency in GBM cells and patients. Under glucose-limited conditions, the overexpression of SLC7A11 results in downregulation of *MLH1, MSH6,* and *EXO1* gene expression, leading to higher DSB levels and radiosensitization. Based on this study, we propose the potential patient stratification based on the SLC7A11 expression to predict the response of radiotherapy in GBM.

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