

Identification of SLC1A5 as a Novel Candidate Oncogene for Glioma and Its Involvement in the LGG Immune Microenvironment

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Research Article

Keywords: SLC1A5, immune infiltration, immunomodulators, prognostic signature, proliferation, migration, glioma

Posted Date: April 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-355956/v1>

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Abstract

Background: Solute Carrier Family 1 Member 5 (SLC1A5), a member of the amino acid carrier system, is considered an oncogene in various tumors. Several targeted drugs targeting SLC1A5 are being developed. However, the molecular mechanisms of SLC1A5 in gliomas are not fully known.

Methods: In this study, multiple datasets from Chinese Glioma Genome Atlas (CGGA) database and The Cancer Genome Atlas (TCGA) database were analyzed. Gene Set Enrichment Analysis (GSEA) was performed to identify biological functions and signaling pathways of SLC1A5. The correlation between SLC1A5 expression and immune infiltrates or gene signatures of immune cells was then determined using CIBERSORT and online analysis tools (TIMER, TSIDB). Finally, a reliable prognostic signature based on SLC1A5-related immunomodulators prognostic signature (SLC1A5-IPS) was established using the lasso-cox method. The model divided patients into low- and high-risk groups. The prognostic value of the risk scores was evaluated with univariate, multivariate, nomogram and receiver operating characteristic (ROC) curves.

Results: It was found that high expression of SLC1A5 was associated with poor prognosis of glioma. Enrichment analysis indicated that SLC1A5 was regulated tumor immune response. Moreover, SLC1A5 was closely associated with immune cells infiltration and gene signatures of CD8 cells were positively correlated with SLC1A5 expression. Moreover, the developed SLC1A5-IPS risk model showed that the high-risk scores of SLC1A5-IPS accurately predicted poor prognosis of LGG. The constructed nomogram exhibited good prediction of the overall survival (OS) of LGG patients in all datasets. Finally, *in vitro* assays verified that SLC1A5 knockdown led to a loss of migration and proliferation ability of U87 and U251 cells.

Conclusions: SLC1A5 regulates immune cells infiltration in glioma and promotes the proliferation and migration ability of glioma cells, thereby affecting the prognosis of patients. It therefore holds huge potential as a treatment target in glioma and provide a new direction for drug development.

Background

Glioma is among the most common intracranial malignant tumors. According to classification by the World Health Organization (WHO), the disease ranges from WHO grade II to WHO grade IV in adults [1]. Notably, Glioblastoma (GBM, WHO IV) accounts for a majority of the glioma cases (56.6 %). In addition, only 5% of patients with GBM survive more than 5 years after receiving active treatment. Currently, surgery, chemotherapy and radiotherapy are the most commonly used methods of treating patients with glioma. However, cancer immunotherapy, which was shown to have a significant therapeutic effect in a variety of cancers, has spurred great research interest for possible use in the treatment of gliomas. By manipulating the immune system, immunotherapy can inhibit tumors from suppressing the immune system hence leading a lasting anti-tumor activity with fewer side effects [2]. Moreover, numerous studies

have demonstrated the anti-tumor effect of immunotherapy in the brain, providing insights for the development of new treatment strategies for malignant glioma.

Over the past few decades, increasing research on immune metabolism fundamentally revolutionized the field of immunology, with promising results. Notably, decades of research effort has largely focused on metabolite transporters whose main functions include nutrient transport and providing energy for cells. In immune cells, the transport of extracellular metabolites into intracellular affects the immune response and cellular interactions. For example, the importation of glucose and Amino Acids (AAs) is largely involved in pro-inflammatory responses while the uptake of Fatty Acids (FA) was shown to drive pro-resolving and anti-inflammatory phenotypes [3]. Additionally, many metabolites cannot easily diffuse into cells, so they need to be actively transported through the cell membrane [4]. For instance, it was previously shown that upregulating glucose transporter type 1 (GLUT1, a glucose transporter) led to an increase in T cell activity while a decrease in glucose uptake was reported to impair T cell activation [5]. Therefore, the precise regulation of transporter expression is essential to satisfy the diverse metabolic needs of immune cells and initiate alternative pathways to adapt to changes in the cellular environment.

In mammals, most metabolites are transported through transmembrane proteins in the solute carrier (SLC) family, most of which are expressed in immune and tumor cells. For instance, the Solute Carrier Family 1 Member 5 (SLC1A5) is an important member of the amino acid carrier system and is mainly responsible for the transmembrane transport of glutamine and some neutral amino acids, in a Na^+ dependent manner [6, 7]. In addition, SLC1A5 is among the most widely studied transporters and is involved in the progression of tumors by playing a role in proliferation, apoptosis and the cell cycle. Previous studies showed that SLC1A5 was up-regulated in many tumors. For example, SLC1A5 was reported to be an important oncogene that was involved in the proliferation of cancer cells in breast cancer, endometrial cancer, prostate cancer, melanoma, acute granulocytic leukemia and hepatocellular carcinoma [8–12]. Moreover, the occurrence of tumors is not only related to the acceleration of tumor cell proliferation but is also closely associated with the inhibition of tumor cell death. Notably, SLC1A5 was also reported to induce tumor cell apoptosis in non-small cell lung cancer and colorectal cancer [13, 14]. Previous research using prostate cancer mice xenograft models also showed that SLC1A5 influenced the E2F-regulated cell cycle genes to induce the growth of tumor cells [9]. Furthermore, *in vitro* and *in vivo* experiments demonstrated that SLC1A5 inhibits the metastatic ability of gastric cancer cells by regulating the mTOR signaling pathway [15].

Over the past few decades, attention has been paid to the effect of SLC1A5 on tumor cells and only few studies exist on its effect on immune cells in the tumor microenvironment. In 2014, Nakaya et al. revealed that deficiency of SLC1A5 impaired the activation of Th1 and Th17 and attenuated inflammatory T-cell responses in mouse models of immunity and autoimmunity [16]. In addition, SLC1A5 was shown to be required for T Cell Receptor (TCR)-stimulated activation of the metabolic kinase mTORC1. Nonetheless, the effect of SLC1A5 on the immune microenvironment is still unclear. Therefore, the present study revealed that SLC1A5 was a prognostic marker in glioma and its high expression was associated with poor prognosis. Moreover, enrichment analysis showed that SLC1A5 plays an important role in immunity

and tumor progression. Further studies also uncovered that Low-grade Glioma (LGG) patients with high expression of SLC1A5 had higher levels of immune cell infiltration. Additionally, the study summarized 12 SLC1A5-related immunomodulators then built a reliable prognostic signature model. Finally, the effect of knocking down SLC1A5 in glioma cell lines was explored and this not only further revealed the importance of SLC1A5 in glioma but also shed light on the association between the glioma microenvironment and glioma.

Methods

Acquisition of expression data and clinical information from the TCGA and CGGA databases

The study obtained the expression profiles and corresponding clinical data on glioma patients from The Cancer Genome Atlas (TCGA), (<http://cancergenome.nih.gov/>) and Chinese Glioma Genome Atlas (CGGA), (<http://www.cgga.org.cn/>). The RNA expression profiles were processed through normalization and the corresponding clinical data was merged through the R package. Additionally, WHO grade II and III were defined as LGG according to the WHO classification standards for glioma. Finally, the study obtained complete data on 613 glioma patients from the TCGA database and 782 glioma patients from the CGGA database.

Bioinformatics Analysis

In order to evaluate the relationship between the expression levels of SLC1A5 and patient's survival time, a survival curve was created by using the "survminer" and "survival" packages in R. Thereafter, differences in the expression of SLC1A5 were analyzed with regard to gender, age, grade, IDH status and 1p19q status using the "beeswarm" R package, in the TCGA and CGGA databases. Additionally, univariate and multivariate Cox analyses were performed to determine the independent risk factors for glioma. Finally, the Receiver Operating Characteristic (ROC) curve was generated for 1, 3 and 5 years after which the Area Under the Curve (AUC) was calculated.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed using the GSEA 4.0.0 software where the expression levels of SLC1A5 served as a phenotype label. Results from the Gene Ontology (GO) enrichment analysis included three parts, namely; Cellular Component (CC), Molecular Function (MF) and Biological Process (BP). Thereafter, the results were imported into the cytoscape 3.8.2 software to build an interactive network diagram and the auto annotate package was used for classification and annotation. Moreover, functional pathway analysis was conducted through the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

Analysis of Immune Infiltration

First, the “CIBERSORT R script v1.03” and “limma” packages in R were used to analyze the infiltration levels of different immune cells in LGG. The levels of immune cell infiltration between the high and low SLC1A5 expression groups were then analyzed based on the screening results from CIBERSORT and the expression levels of SLC1A5. Notably, the TIMER web server (<https://cistrome.shinyapps.io/timer/>) is a comprehensive resource for the systematic analysis of immune infiltrates across different types of cancer. Therefore, the study used the somatic copy number variation (SCNA) module of TIMER to explore the correlation between SCNA and abundance of immune infiltrates in LGG. Additionally, TISIDB (<http://cis.hku.hk/TISIDB/>) is a web portal for tumor and immune system interaction and integrates multiple heterogeneous data types. Therefore, the study used TISIDB to search for immune infiltrates related to SLC1A5. Finally, associations between the abundance of Tumor-infiltrating Lymphocytes (TILs) and the expression levels of SLC1A5 were analyzed using the Lymphocyte module.

The Lasso Regression Model for SLC1A5 related immunomodulators and Risk Survival Analysis

In order to examine which immunomodulator might be regulated by SLC1A5, the TISIDB database was used to analyze the relationship between immunomodulators and the expression of SLC1A5. Therefore, the study selected 43 genes for immunoinhibitors and immunostimulators in immunomodulators that were significantly related to the expression of SLC1A5 (Spearman correlation test, $p < 0.05$). Thereafter, 27 genes that were significantly associated with the prognosis of clinical survival were screened using univariate Cox regression analysis ($p < 0.05$). Moreover, the genes were subjected to the Least Absolute Shrinkage and Selection Operator (LASSO) analysis using the “glmnet” package (with the perfect Penalty parameter λ related to the minimum 10-fold crossover verification). Furthermore, the study developed an SLC1A5-related immunomodulators prognostic signature (SLC1A5-IPS) involving 6 genes related to immunoinhibitors and 6 genes associated with immunostimulators. The risk score was calculated using the following formula:

$$\text{Risk score} = \sum_{i=0}^n \text{Coef}_i * x_i$$

where Coef_i is the coefficient and x_i is the FPKM value of each selected gene. This was applied to each LGG patient in both the TCGA and CGGA datasets.

Additionally, patients were divided into the low-risk and high risk groups according the median risk score. Thereafter, Kaplan-Meier (KM) survival curves and the Wilcoxon test were used to evaluate correlations between the two risk groups and the clinical characteristics. Notably, the “pheatmap” package showed the expression levels of 12 SLC1A5 related immune genes and the risk score as well as various clinical and molecular pathological features, including the risk scores, age, gender, IDH status and 1p19q status. Moreover, univariate and multivariate Cox regression analyses were performed to determine the prognostic value of the risk score, IDH status, 1p19q status, grade and age. Furthermore, a nomogram

was constructed using the “rms” package in order to evaluate whether the risk scores could improve the predictive performance. The nomogram was constructed by including prognostic factors such as risk scores, age, grade, 1p19q and IDH. Following this, the calibration curve of the nomogram (1/3/5-years) was generated by plotting the nomogram prediction probabilities against the observed rates. The discriminative ability of the nomogram was then assessed using the C-index. In addition, time-dependent ROC curves were employed to determine the 1-, 3- and 5-year prognostic accuracy of the risk score, age and grade, using the “survival ROC” package.

Cell culture and *in vitro* functional assays

The human glioma cell lines U87, U251, H4, HS683, TJ905 and LN229 as well as the immortalized normal astrocytoma cells line, SVG, were obtained free of charge from the Cancer Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). The cells were cultured in DMEM (Gibco, USA), supplemented with 10% fetal bovine serum (PAN; Adenbach, Germany) then incubated at 37°C in an environment saturated with 5% CO₂. On the other hand, the lentiviruses included Lv-SLC1A5-RNAi (SLC1A5-RNA interference lentivirus) and Lv-shNC (black lentivirus), which were designed and generated by the GenePharma Co.,Ltd (shanghai). Transfection was conducted following instructions on the kit of lentiviruses. Moreover, total cellular protein was extracted following instructions on the RIPA extraction kit (Beyotime Biotechnology, China) after which western blot analysis was performed. Furthermore, the colony formation and MTT assays, previously described by Yao et al. were used to determine the clonogenic potential [17]. Finally, the migratory ability of cells was determined through the transwell chamber system (BD Biosciences, U.S.A.).

Statistics analysis

Kaplan-Meier curves were plotted to estimate the 5-year survival rates and compared using the log-rank test. In addition, the Wilcoxon or Kruskal test was used to evaluate whether there were significant differences in the expression levels of SLC1A5 with regard to age, grade, IDH status, 1p19q status and histology. Moreover, univariate and multivariate analyses were performed to examine the risk factors, such as the expression levels of SLC1A5, risk scores, age, grade, IDH status and 1p19q status. The prognostic effect of the SLC1A5-related genes signature was estimated using Cox and Lasso regression analyses. Furthermore, a nomogram chart was generated through multivariate cox regression analysis in the two databases, after which the C-index was calculated. All the R packages were run through R Studio version 1.3.959 while all the statistical analyses were conducted using R v3.5.1 (<https://www.r-project.org/>) and Prism 8 (GraphPad Software Inc., La Jolla, CA).

Results

Differences in the expression of SLC1A5 are associated with poor prognosis in glioma

The results showed that the expression levels of SLC1A5 were higher in GBM than in LGG (Fig. 1a). In addition, the high SLC1A5 expression group had a shorter Overall Survival (OS) than the low expression category in both the TCGA ($p < 0.001$) and CGGA ($p < 0.001$) databases (Fig. 1b, c). The study then investigated the expression pattern of SLC1A5 based on different clinical characteristics, including grade, age, IDH status and 1p19q status. The findings revealed significant differences ($p < 0.001$) in the expression levels of SLC1A5 between grade, IDH status and 1p19q status (Fig. 1d-k) in both the TCGA and CGGA databases. Additionally, univariate and multivariate survival analyses showed that SLC1A5 was an independent risk factor for OS. Other independent risk factors included grade and age, in both databases (Fig. 1l, m). ROC curve analysis in the TCGA database also showed that SLC1A5 had a significant prognostic value (one-year AUC = 0.712, three-year AUC = 0.695, five-year AUC = 0.695) and this was verified in the CGGA database (Fig. 1n, o). These results therefore indicated that SLC1A5 can be used as an independent risk factor to predict the survival time of patients with glioma.

SLC1A5 plays a role in immune responses and regulates tumor related signaling pathways

In order to assess the function of SLC1A5 and the signaling pathways involved, the study used the median expression of SLC1A5 to group patients into the high and low expression categories. GSEA was then applied to determine whether the two groups had significant enrichment in KEGG signaling pathways and molecular functions. Thereafter, the GSEA results were imported into the cytoscape software to construct a network and perform cluster analysis. The results showed that SLC1A5 was enriched in immune responses and the top three clustering results involved activation of alpha beta T cells, mediated immunoglobulin immunity cytotoxicity and migration of macrophage chemotaxis extravasation leukocytes (Fig. 2a). Additionally, KEGG pathway analysis revealed that several immune-related and tumor signaling pathways were enriched in the high-risk group. Such included the p53 signaling pathway, JAK-STAT signaling pathway, pancreatic cancer, pathways in cancer, B cell receptor signaling pathway, T cell receptor signaling pathways, natural killer cell mediated cytotoxicity and the intestinal immune network for IgA production (Fig. 2b). These results therefore suggested that SLC1A5 might be involved in tumor immune responses to regulate tumor progression.

Association between SLC1A5 and immune cells in LGG

The study then examined the relationship between the expression levels of SLC1A5 and infiltration of immune cells in glioma. Therefore, the levels of immune cell infiltration between the high and low SLC1A5 expression groups were analyzed. The results showed significant differences in the levels of T cells CD8, T cells regulatory (Tregs) between the high and low SLC1A5 expression groups in LGG ($p < 0.05$) as shown in (Fig. 3a). However, only the levels of activated dendritic cells were different between the high and low SLC1A5 expression groups in GBM ($p = 0.038$). Moreover, the study used the SCNA module of the TIMER database to verify the association between the copy numbers of the SLC1A5 gene and the immune infiltration levels. The findings revealed that the levels of B cell, CD8 + T cell, CD4 + T cell, macrophage, neutrophil and dendritic cell infiltration were associated with the copy numbers of SLC1A5 in LGG (Fig. 3b). Furthermore, a heatmap of the relationships between the levels of tumor-infiltrating

lymphocytes and expression of SLC1A5 showed that LGG had the most significant results among all the cancers (Fig. 3c). Based on the correlation between SLC1A5 and each phenotype in the differentiation process of CD8 + T cells, the study further examined the association between SLC1A5 and various CD8 + signatures. The results showed that SLC1A5 was positively correlated with all the T cell clusters in the TCGA cohort (Fig. 3d). In addition, the correlation between SLC1A5 expression and the gene signatures of CD8 + T cells in LGG was analyzed. The results showed that HLA-DMB, C3AR1, FCER1g and CD37 had a significant correlation with SLC1A5 (Fig. 3e-g). These results suggested that SLC1A5 was involved in the infiltration of immune cells in LGG.

The prognostic value of SLC1A5-IPS in LGG

The TISIDB database was used to assess the correlation between SLC1A5 expression and immunomodulators in LGG through spearman's correlation analysis (Additional file 1: Tab. S1). 27 immunostimulators were identified, including C10orf54, CD276, CD40, CD48, CD86, CXCR4, ICOSLG, IL6, IL6R, MICB, TMEM173, TMIGD2, TNFRSF14, TNFRSF8, TNFSF13, TNFSF13B, ENTPD1, CXCL12, TNFRSF13C, IL2RA, TNFSF18, CD28, ULBP1, TNFRSF4, TNFRSF25, TNFSF9 and NT5E ($p < 0.05$) (Fig. 4a). Notably, 16 immunoinhibitors were significantly associated with SLC1A5 ($p < 0.05$) in LGG. These included CD96, CSF1R, HAVCR2, IDO1, IL10, IL10RB, LGALS9, PDCD1, PDCD1LG2, PVRL2, TGFB1, TGFB1, CD274, LAG3, CD244 and CD160 (Fig. 4a). Moreover, univariate cox analysis was used to examine the prognostic value of the SLC1A5-related immunomodulators. The findings revealed that 27 immunomodulators were significantly associated with the OS of LGG patients in the TCGA cohort (Fig. 4b). Additionally, the study conducted a LASSO Cox analysis based on the 27 SLC1A5-related immunomodulators in the TCGA cohort (Fig. 4c, d) in order to build the prognostic signature of the SLC1A5-related immunomodulators, for predicting the OS of LGG patients. The analysis generated the prognostic signature containing 12 SLC1A5-IPS then calculated the coefficient of each (Fig. 4e). Based on the coefficients of the 12 immunomodulators, the risk score of each LGG patient in TCGA was calculated after which the patients were divided into the high and low risk groups based on the median risk score. The KM survival curve showed that the low-risk group had a longer OS than the high-risk category ($p < 0.0001$) and this was verified by the CGGA test cohort ($p < 0.0001$) as shown in (Fig. 4f, g). Distribution of the risk scores and patients' survival status in the TCGA and CGGA test cohorts are highlighted in (Fig. 4h, i). Moreover, a ROC curve of the model was plotted and the AUC value of SLC1A5-IPS showed a remarkable predictive ability for OS in the TCGA cohort (1-year AUC = 0.887, 3-year AUC = 0.808, 5-year AUC = 0.764) as shown in (Fig. 4j). The CGGA cohort also demonstrated that SLC1A5-IPS had a similar predictive ability (1-year AUC = 0.689, 3-year AUC = 0.698, 5-year AUC = 0.707) as shown in (Fig. 4k).

Analysis of the prognostic value of the twelve SLC1A5-IPS genes

A heatmap of the SLC1A5 expression profiles was plotted to describe the relationship between differences in the expression of SLC1A5 and risk scores, in the TCGA and CGGA databases. The results showed that there was an increase in the expression levels of CD274, ULBP1, TNFRSF14, CD96, PDCD1, PDCD1LG2, MICB, IL10RB and CD276 with an increase in the risk score. However, there was a decrease in

the expression levels of CD160, TNFRSF13C and CXCL12 with an increase in the risk score (Fig. 5a, b). Additionally, the study examined the correlation between the expression levels of the SLC1A5-related immunomodulators and the OS of LGG patients, in the CGGA database. The findings revealed that LGG patients with higher expression levels of CD160, CD274, CD276, IL10RB, MICB, ULBP1, CD96, PDCD1, TNFRSF14 and PDCD1LG2 had a shorter survival time than those in the low expression group ($p < 0.05$) (Fig. 5c-n). The result was validated in the TCGA database (Additional file 1: Fig. S1a). These results therefore indicated that the SLC1A5-IPS genes were associated with poor prognosis in LGG patients.

Stratification analysis of the SLC1A5-IPS

The study further examined whether there was an association between clinical characteristics and the risk scores. The results showed that LGG patients in the TCGA cohort with WHO grade III, 1p19q non-codel and IDH wildtype had higher risk scores. However the risk scores had no association with age and gender (Fig. 6a-e). We also validated these findings in the CGGA cohort (Additional file 1: Fig. S1b). In order to further evaluate the reliability of SLC1A5-IPS in predicting the prognosis of LGG patients with different clinical characteristics, the study analyzed the difference in survival time between the high and low risk score groups. The results showed that the high-risk group was associated with worse prognosis in LGG patients with WHO grade II, WHO grade III, 1p19q Codel and 1p19q non-Codel (Fig. 6f-g, j-k). Similar results were obtained in LGG patients aged ≤ 41 or > 41 (Fig. 6l, m). In addition, there was no significant difference in the OS of LGG patients with IDH wildtype or IDH mutant between the high and low risk score groups (Fig. 6h, i). In addition, this model was validated in the CGGA dataset (Additional file 1: Fig. S1c). These results therefore indicated that the risk score can be a prognosis predictor of in LGG patients with different clinical characteristics.

SLC1A5-IPS is an independent prognostic factor and was validated using a nomogram

Univariate and multivariate analysis of different prognostic factors in LGG patients were performed. These factors included age, gender, grade, 1p19q, IDH and risk scores. Results from multivariate analysis in the TCGA cohort showed that the risk score could be an independent prognostic factor (HR = 2.223, 95% CI = 1.648-3.000, $p < 0.001$) (Fig. 7a). Similar results were obtained from multivariate analysis in the CGGA validation cohort (HR = 1.467, 95% CI = 1.168–1.844, $p < 0.001$) as shown in (Fig. 7b). The results therefore showed that SLC1A5-IPS, as an independent prognostic factor, might be useful for the clinical diagnosis of LGG. Moreover, a nomogram was established using the TCGA and CGGA database to explore the effect of age, grade, IDH, 1p19q and risk scores on the OS of LGG patients (Fig. 7c; Additional file 2: Fig. S2a). Calibration plots of 1-, 3- and 5- year OS showed that the nomogram performed well in the TCGA database (Fig. 7d-f) and CGGA database (Additional file 2: Fig. S2b-d). Additionally, time-dependent ROC curves were plotted to verify the prognostic efficiency of age, grade and risk scores in predicting survival. The AUC of age, grade and risk scores was 0.667, 0.683 and 0.764, respectively (5-year OS) (Fig. 7g-i) and validated in CGGA database (Additional file 2: Fig. S2e-g). Furthermore, the C-index was calculated to evaluate the prediction accuracy of the nomogram in the TCGA database and a

stable C-index of 0.868 was obtained. These results indicated that the nomogram was a stable predictive model for OS in LGG patients.

SLC1A5 regulates the proliferative and migratory ability of glioma cells

In order to ascertain the expression levels of SLC1A5 in tumor and normal cell lines, western blot analyses were conducted. The results showed that the expression levels of SLC1A5 were higher in tumor cell lines than in normal cells (Fig. 8a). Based on these results, the study further examined the functions of SLC1A5 in the U87 and U251 cell lines (Fig. 8b, c). Therefore, a loss-of-function experiment was conducted where SLC1A5 was knocked-down using shRNA. The colony formation assay indicated that suppressing the expression of SLC1A5 inhibited the proliferative ability of the U87 and U251 cells (Fig. 8d). Moreover, the MTT assay showed that cell growth was inhibited in the SLC1A5-knockdown cell lines, compared to the control group (Fig. 8e, f). Furthermore, the transwell migration assay indicated that knockdown of SLC1A5 inhibited the migratory ability of the U87 and U251 cells (Fig. 8g-i). These results therefore suggested that SLC1A5 promotes the proliferation and migratory ability of glioma cells.

Discussion

Tumor immunotherapy is a new method of cancer treatment that includes the use of monoclonal antibody immune checkpoint inhibitors, therapeutic antibodies, cancer vaccines, cell therapy and small molecule inhibitors. Immunotherapy mainly acts by restoring the body's normal anti-tumor immune response by restarting and maintaining the tumor-immune cycle, thus controlling and eliminating tumors [18, 19]. Up to date, immunotherapy has been shown to have a strong anti-tumor effect against a number of solid tumors such as melanoma, non-small cell lung cancer, kidney cancer and prostate cancer [20–24]. However, immunotherapy of glioma is still a challenge due to lack of immune organs in the central nervous system and the protective effect of the blood-cerebrospinal fluid barrier on tumor cells. Therefore, finding more effective immunotherapy drugs and selecting appropriate patients for treatment remains a huge challenge. In glioma, biomarkers that can accurately indicate the patient's immune status and effect of treatment, are very valuable in guiding clinical treatment decisions. The present study showed that SLC1A5 was closely related to the prognosis of survival in patients and the immune responses in glioma. Additionally, expression of the SLC1A5 gene was associated with the levels of immune cell infiltration and immunomodulators. Furthermore, Cox and KM survival analyses were used to examine the reliability of the prognostic model. Finally, the prognostic value of the risk score was verified using the nomogram.

According to existing literature, high expression of SLC1A5 is associated with poor prognosis in lung, liver, breast and rectal cancers. However, no study exists on the link between SLC1A5 and glioma. The present study first evaluated differences in the expression levels of SLC1A5 in different grades of glioma. The study then analyzed the survival time in the high and low expression groups in order to ascertain whether SLC1A5 can be used as a prognostic marker in glioma. KM survival curve analysis showed that low expression of SLC1A5 was associated with good prognosis in glioma patients. Results from univariate

and multivariate Cox analyses also showed that SLC1A5 was a high-risk factor and could be used as a prognostic marker for glioma patients. Additionally, ROC curve analysis showed that the AUC value for SLC1A5 was > 0.7 , indicating that SLC1A5 was a good prognostic factor. Moreover, *in vitro* experiments revealed that knock down of SLC1A5 inhibited the proliferative and migratory ability of glioma cells. These results further confirmed that SLC1A5 played a role in the development and progression of glioma.

Recent studies showed that SLC1A5 plays an important role in the immune system. For instance, SLC1A5 was shown to be involved in coupling the TCR and CD28 signals to regulate the activation of CD4 + T cells and T cell mediated immunity. In addition, Wu et al revealed that inhibition of the asparagine (Asn) transporter, SLC1A5, impaired the activity and response of CD8 + T cell [25]. Moreover, the T cell response was shown to be the most important host response that controlled tumor growth and development. Notably, CD8 + and CD4+, which are important subgroups involved in anti-tumor immunity, were both regulated by the expression of SLC1A5. Consequently, functional and pathway enrichment analyses were conducted and the results indicated that the enrichment of SLC1A5 was involved in the activation of CD4+/CD8 + T cells and immune pathways. Interestingly, the results showed that SLC1A5, a transporter, was involved in immune infiltration in gliomas. To the best of our knowledge, no study has investigated the association between SLC1A5 and immune infiltration. The present study showed that SLC1A5 was closely associated with the levels of infiltration of activated CD8 T cells (Act CD8), central memory CD8 T cells (Tcm CD8), effector memory CD8 T cells (Tem CD8) and regulatory T cells (Treg) in LGG. These results therefore indicated that SLC1A5 not only directly participates in the proliferation of tumor cells in gliomas but is also involved in the tumor immune microenvironment thus affecting the immune responses.

The current open-access high-throughput gene expression data is useful in the discovery of potentially more reliable and powerful biomarkers for glioma. Notably, several studies have tried to establish a prognostic prediction model for glioma based on gene expression [25–27]. In addition, prognostic immune signatures in glioma have previously been described [28–30]. For instance, Luo et al. selected eight immune related genes that were differently expressed in glioma to build a prognosis prediction model that achieved a moderate prognostic accuracy (C-index = 0.827) [31]. The same study also constructed a robust immune-related signature consisting of 6 genes. The signature was able to predict overall survival in primary LGG [28]. Similarly, the present study used SLC1A5-associated immunomodulators to establish immune gene signatures for LGG. The prognostic risk score model developed in this study included 12 SLC1A5-IPS. The results revealed that the high-risk group was closely associated with poor prognosis of LGG in the TCGA and CGGA datasets. Moreover, the relationship between the risk score and clinical subtypes was explored. The findings revealed that the prognosis of the low-risk group was better and this was equally applicable to the grade, IDH status, 1p19q status and age group. Furthermore, the study established a predictive nomogram that included the risk scores and other clinical characteristics in the TCGA database. The nomogram was validated and had a C-index of 0.868. The results further showed that the risk score of SLC1A5-IPS had excellent prognostic value and might be useful in the development of LGG-related prognostic markers.

The prognostic value of individual genes within SLC1A5-IPS was also investigated. The results showed that the differential expression of all genes was related to clinical characteristics and the risk score of LGG. Based on these results, KM survival analysis was conducted. The findings revealed that apart from TNFRSF13C and CXCL12, low expression of the SLC1A5-IPS genes was significantly associated with a longer survival time in LGG. Previous studies partly highlighted the molecular mechanisms involved in regulating glioma by these genes. For instance, Orin et al. showed that the immunosuppressive phenotype in glioma could be modulated by up-regulating the expression of CD274 in circulating monocytes and tumor-infiltrative macrophages [32]. Additionally, Lemke et al. demonstrated that overexpression of CD276 was correlated with malignancy and poor survival [33]. Moreover, ULBP1 was shown to regulate NK-cell-mediated lysis and enhanced the NK-cell-mediated immune surveillance ability in IDH mutant glioma patients [34]. Over the recent years, the discovery of PDCD1 (programmed cell death 1, PD1) as an important target for immunotherapy and the appearance of monoclonal antibodies (mAbs) against PD-1 (nivolumab and pembrolizumab) have spurred great research interest. However, clinical trials showed that the treatment efficacy of PD-1 mAbs in glioma was not satisfactory [35–39]. Furthermore, it was shown that lack of biomarkers guiding the Immune Checkpoint Blockade (ICB) in some patients might be related to the failure of treatment approaches [40]. Therefore, it is possible that SLC1A5 can be used as a biomarker for immunotherapy and can be combined with PD-1 mAb to kill tumor cells and induce systemic antitumor immunity.

Despite the contributions of this study to literature, it had a number of limitations. First the study was mainly based on public databases and therefore lacks verification from *in vivo* and *in vitro* studies. Second, the specific regulatory mechanism of SLC1A5 in glioma and its effect on immune cells are still unclear. Third, the value of SLC1A5-IPS in guiding prognosis and treatment needs to be explored further in future studies.

Conclusions

This was the first study to explore the expression patterns, prognostic value and potential functions of SLC1A5 in primary glioma. The results demonstrated that SLC1A5 is a key player in immune responses and causes T cell infiltration in LGG. The study also demonstrated that SLC1A5-IPS, based on the risk score, may serve as a diagnostic and prognostic marker for LGG. Therefore, this study provides new insights on how SLC1A5 affects prognosis and the immune microenvironment in glioma. This might be useful in the development of SLC1A5-targeted drugs for cancer therapy in future.

Abbreviations

SLC1A5: Solute Carrier Family 1 Member 5; CGGA: Chinese Glioma Genome Atlas; TCGA: The Cancer Genome Atlas; GSEA: Gene Set Enrichment Analysis; SLC1A5-IPS: SLC1A5-related immunomodulators prognostic signature; WHO: World Health Organization; GBM: Glioblastoma; AAs: Amino Acids; FA: Fatty Acids; GLUT1: Glucose transporter type 1; SLC: Solute carrier; TCR: T Cell Receptor; LGG: Low grade glioma; ROC: Receiver Operating Characteristic; AUC: Area Under the Curve; GO: Gene Ontology; CC:

Cellular Component; MF: Molecular Function; BP: Biological Process; KEGG: Kyoto Encyclopedia of Genes and Genomes; SCNA: Somatic copy number variation; TILs: Tumor-infiltrating Lymphocytes; LASSO: Least Absolute Shrinkage and Selection Operator; KM: Kaplan Meier; OS: Overall Survival; Asn: Asparagine; Tregs: regulatory T cells ; Act CD8: Activated CD8 T cells; Tcm CD8: Central memory CD8 T cells; Tem CD8: Effector memory CD8 T cells; PDCD1: Programmed cell death 1; mAbs: Monoclonal antibodies; ICB: Immune Checkpoint Blockade.

Declarations

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

We would like to thank Zujue Cheng, Department of Neurosurgery, The Second Affiliated Hospital of Nanchang University, for his valuable comments on the revision of the manuscript.

Funding

This study was supported by Science and Technology Research Project of Jiangxi Provincial Department of Education (Grant numbers GJJ200133).

Authors' contributions

JX and ZC conceived and designed the idea to this paper; PC, and YG participated in its design and coordination and supervised the study. PC and RH collected and analyzed the data and drafted the paper; PC, RH and YG analyzed the data and revised the final paper. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Consent to publish was obtained from all authors.

Competing interests

The authors declare that they have no competing interests.

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Figures

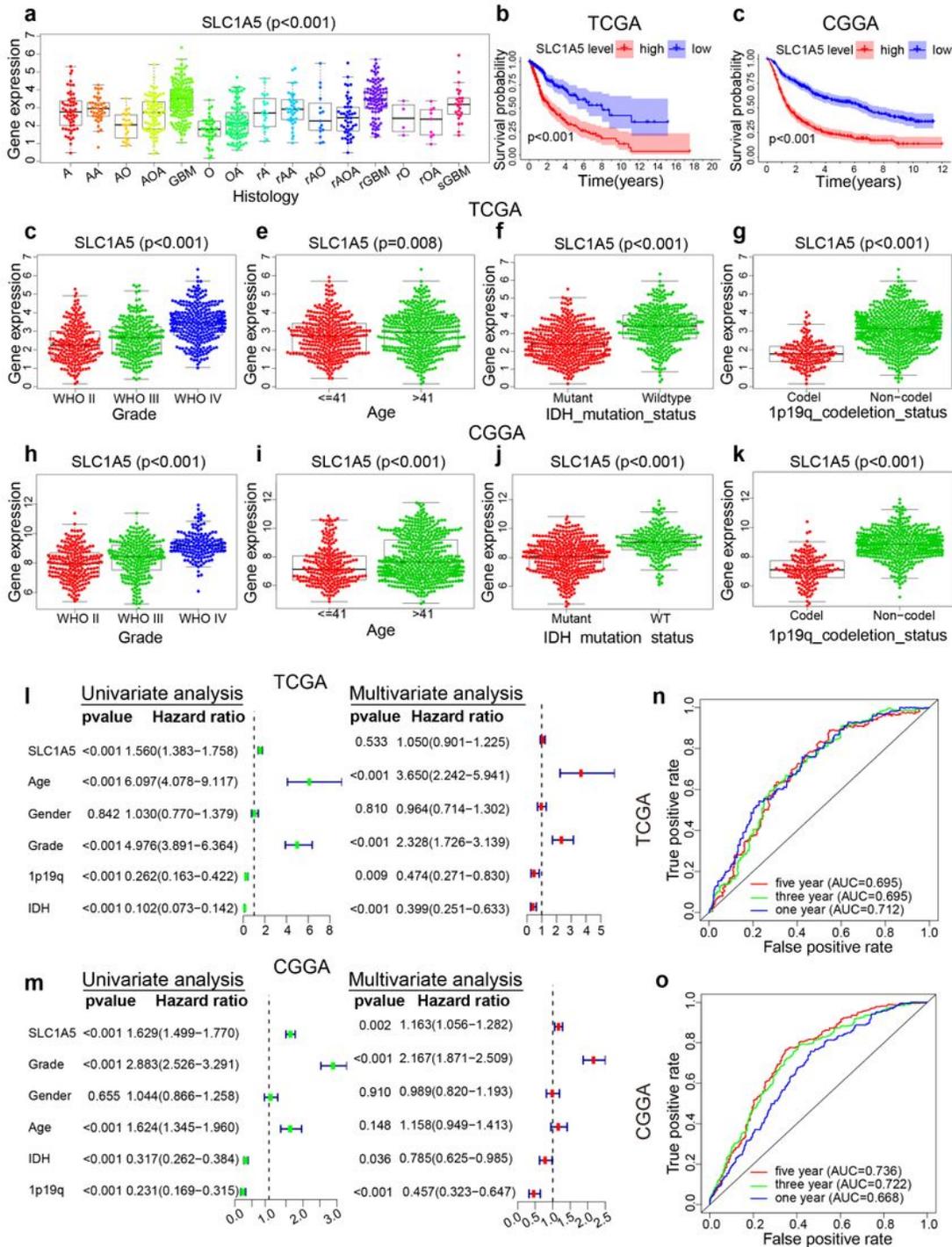


Figure 1

Bioinformatics analysis of SLC1A5 in TCGA and CGGA database. a Expression levels of SLC1A5 are significantly different between tumor subtypes in CGGA ($p < 0.001$). b, c A high expression level of SLC1A5 is correlated with poor prognosis in TCGA and CGGA databases. d-k The mRNA expression level of SLC1A5 in glioma patients with different grade status, age, IDH status, and 1p19q status in TCGA and CGGA databases. l, m Pooled HRs based on univariate analysis and multivariate analyses performed to

assess the effect of SLC1A5 expression on OS in patients with glioma in TCGA and CGGA databases. n, Receiver operating characteristic (ROC) curves showing the ability of SLC1A5 to predict the 1/3/5-year survival in patients from TCGA and CGGA databases.

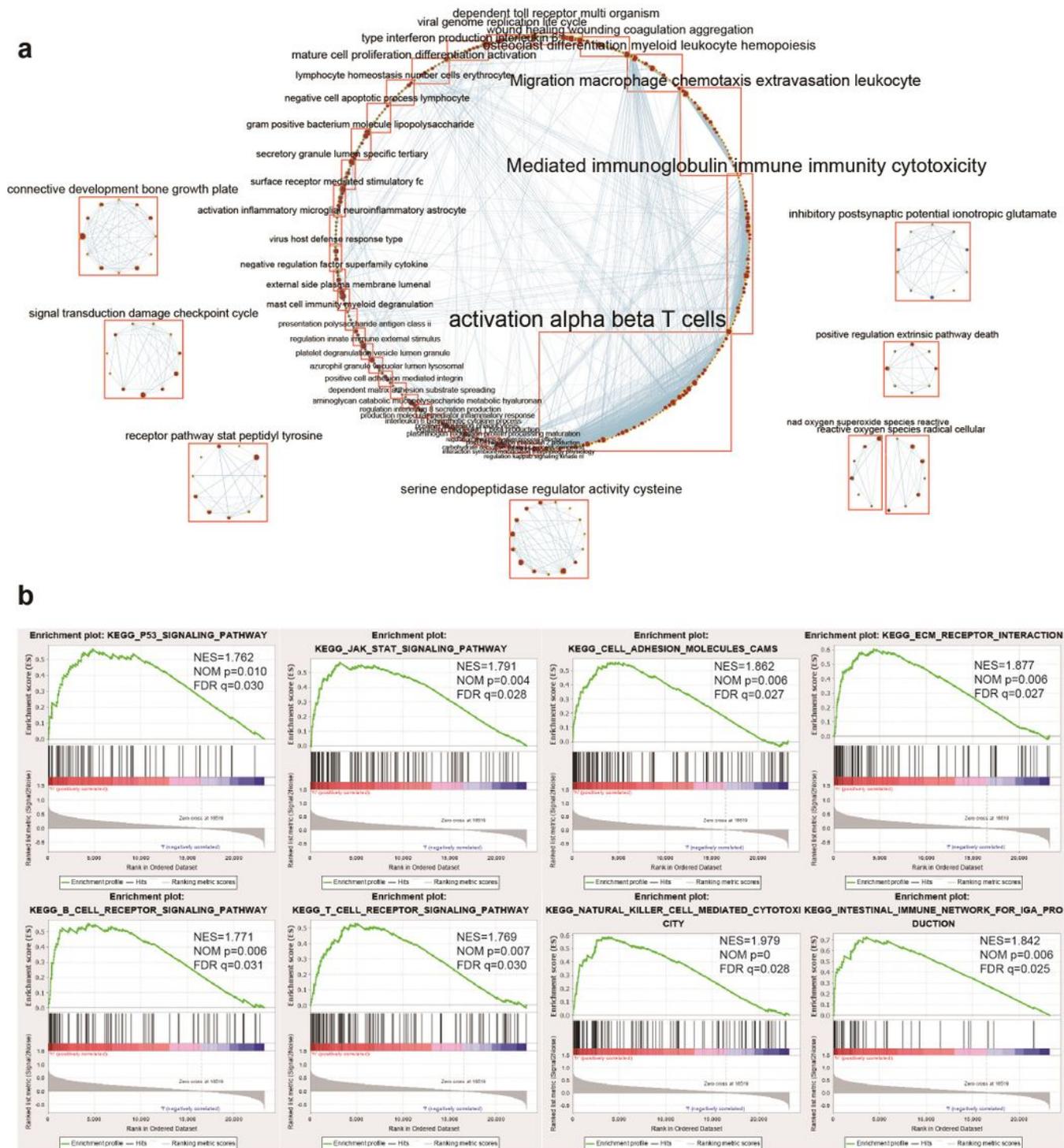


Figure 2

GSEA enrichment analysis of SLC1A5 in the TCGA database. a GO functional enrichment analyses. b KEGG pathway analysis identified ten significantly enriched pathways.

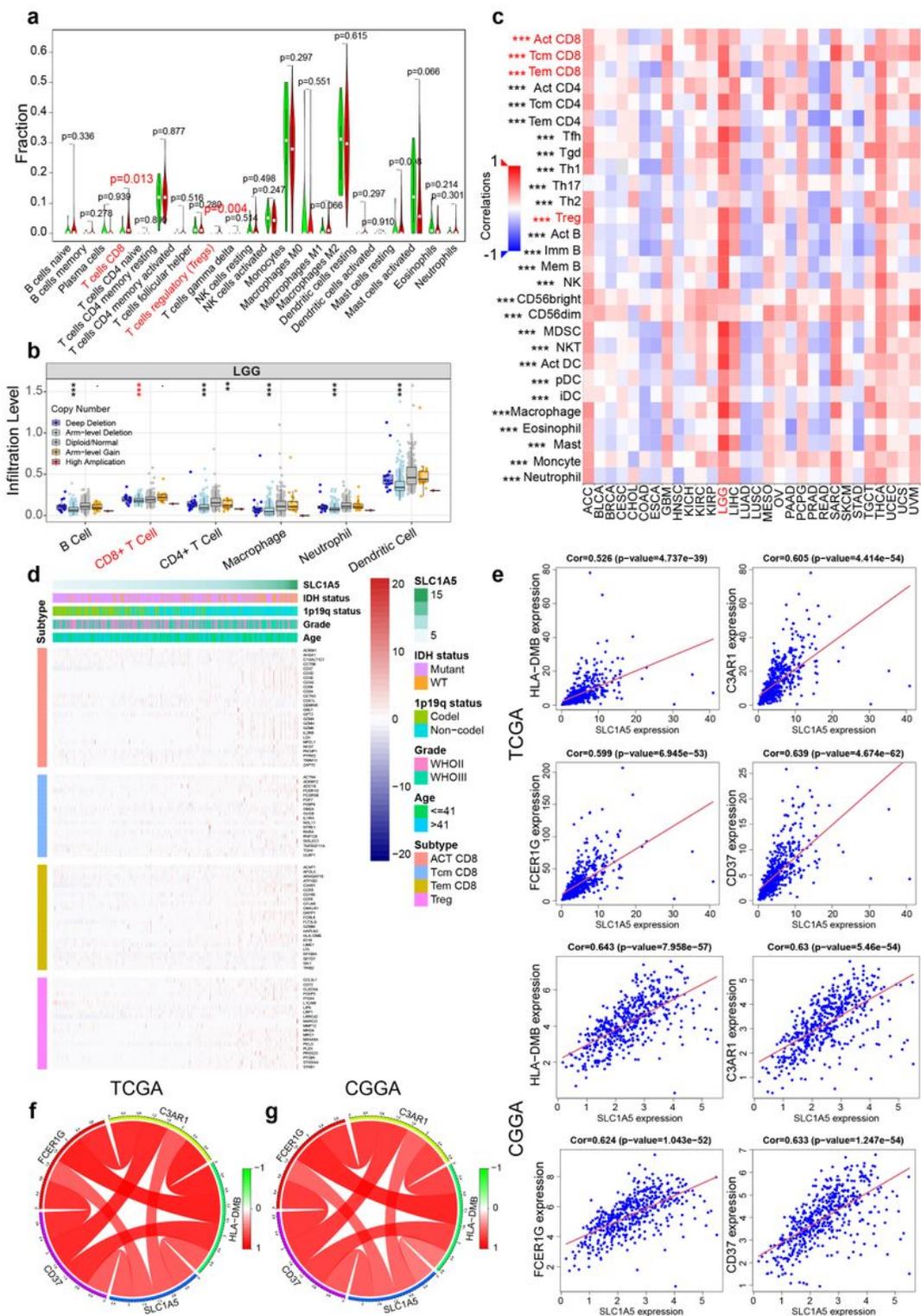


Figure 3

Characterization of immune cell infiltrate between low-, and high SLC1A5 expression group. a The infiltration level of 22 types of immune cells between high and low SLC1A5 expression groups as determined by CIBERSORT. b Association between SLC1A5 copy numbers and immune cells infiltration levels in LGG cohorts. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Correlation between SLC1A5 expression levels and immune cell subsets. c A correlation heatmap showing immune cell types significantly associated

performance of the SLC1A5-related immunomodulators. c-e The least absolute shrinkage and selection operator (LASSO) regression was performed, the minimum criteria and coefficients were calculated. f, g Kaplan–Meier curves show that the high-risk subgroup had worse overall survival than the low-risk subgroup in TCGA and CGGA databases. h, i Distributions of risk scores [based on SLC1A5-related immunomodulators prognostic signature (SLC1A5-IPS)] and survival status of LGG patients in the TCGA and CGGA databases. j, k Receiver operating characteristic (ROC) curves showing the ability of SLC1A5-IPS to predict the 1/3/5-year survival rate of patients in the TCGA and CGGA databases.

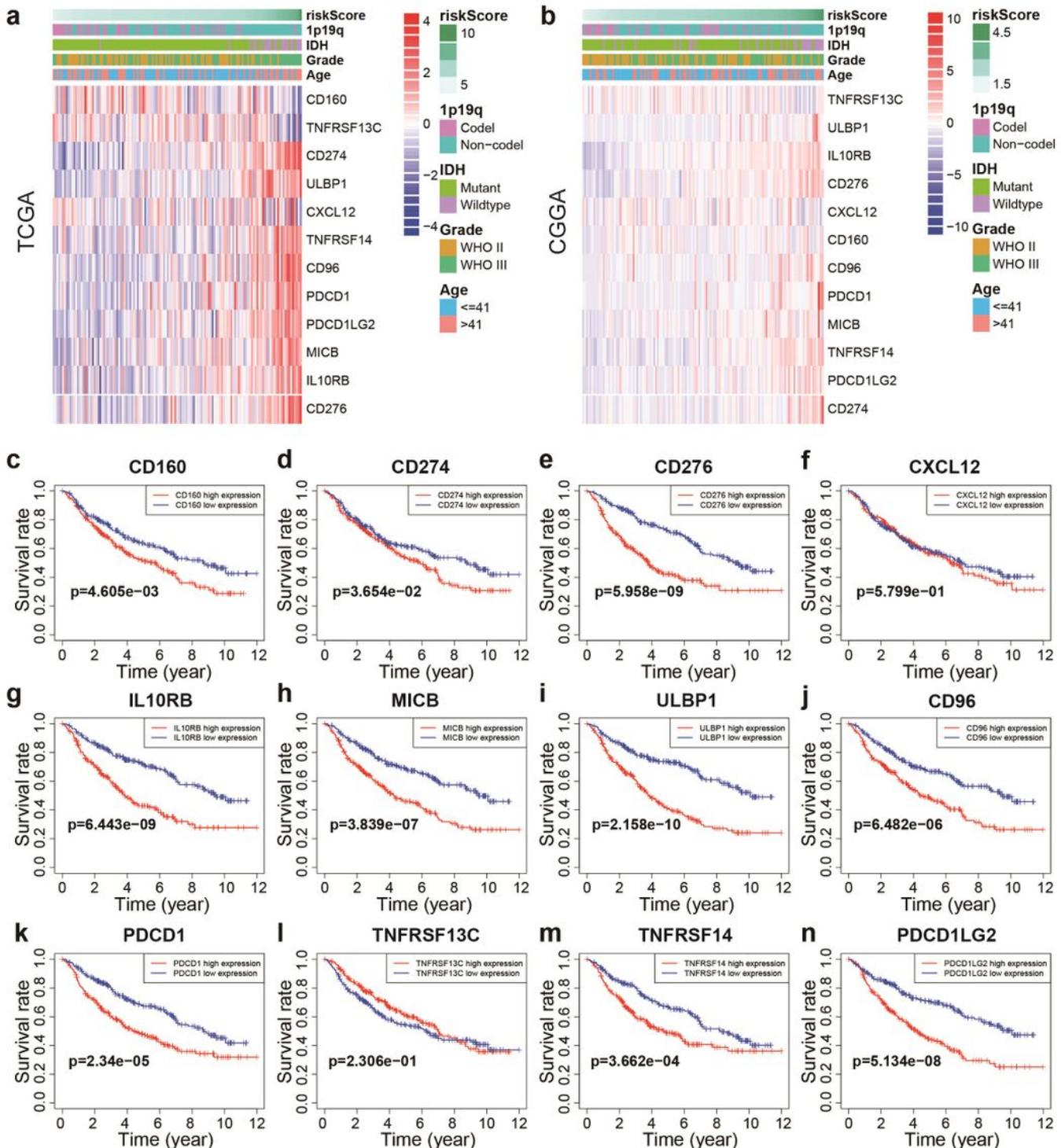


Figure 5

SLC1A5-related immunomodulators expression and survival analysis of patients with LGG. a, b Heatmap showing the association between the expression levels of twelve SLC1A5-related immunomodulators and clinicopathological features in TCGA and CGGA datasets. c–n Kaplan–Meier curves showing that patients with different expression levels of the twelve SLC1A5-related immunomodulators had different overall survival rates.

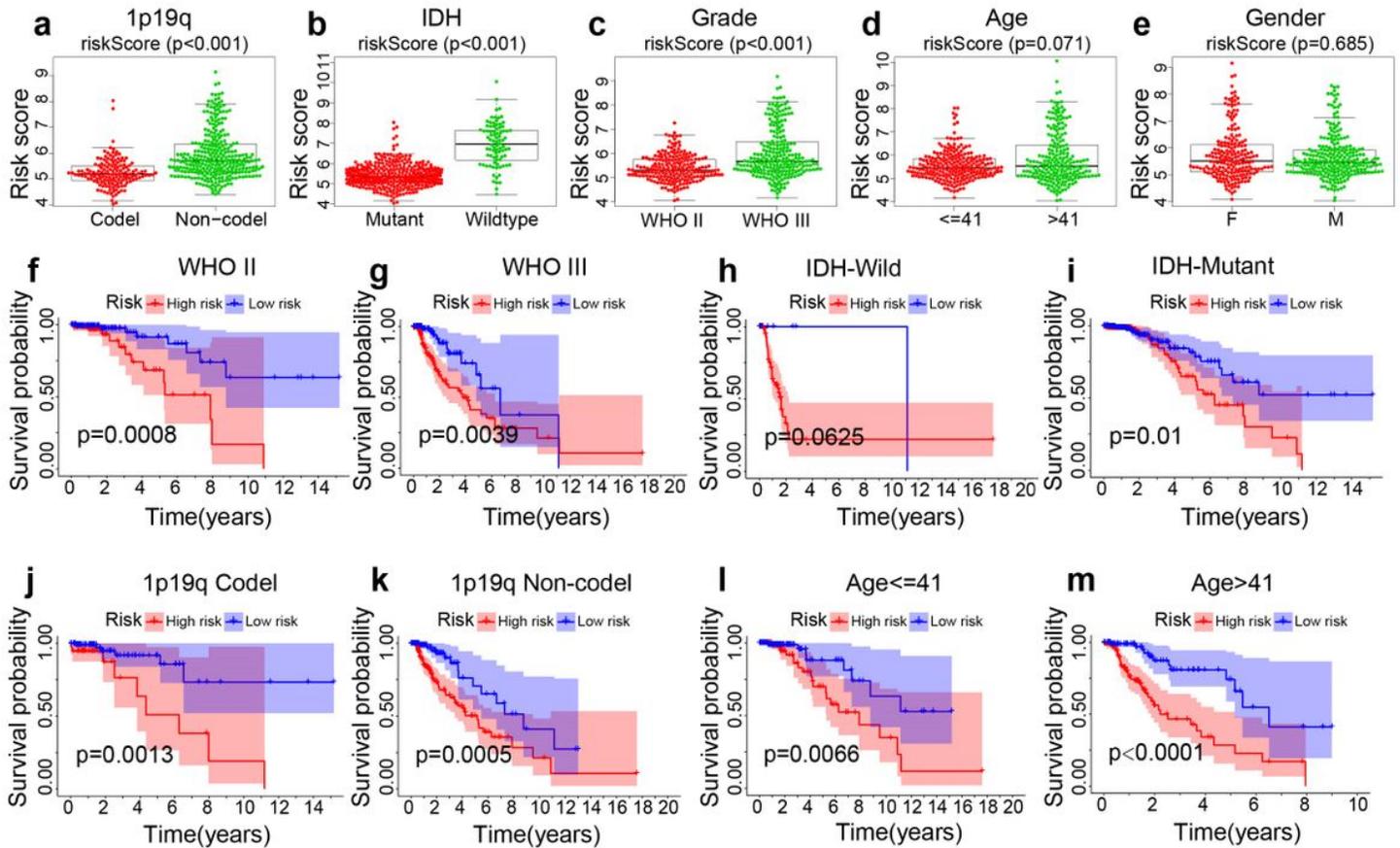


Figure 6

Stratification analysis of the SLC1A5-IPS. a-e Patients with different clinicopathological features (including IDH mutation status, 1p/19q co-deletion status, age, gender, and WHO grade) had different levels of risk scores, calculated based on the SLC1A5-related immunomodulators (SLC1A5-IPS). f–m The SLC1A5-IPS retained its prognostic value in multiple subgroups of LGG patients (including patients with WHO grade II or III, patients with mutant or wildtype IDH, patients with 1p19q codel or non-codel, and patients aged ≤ 41 or > 41 years).

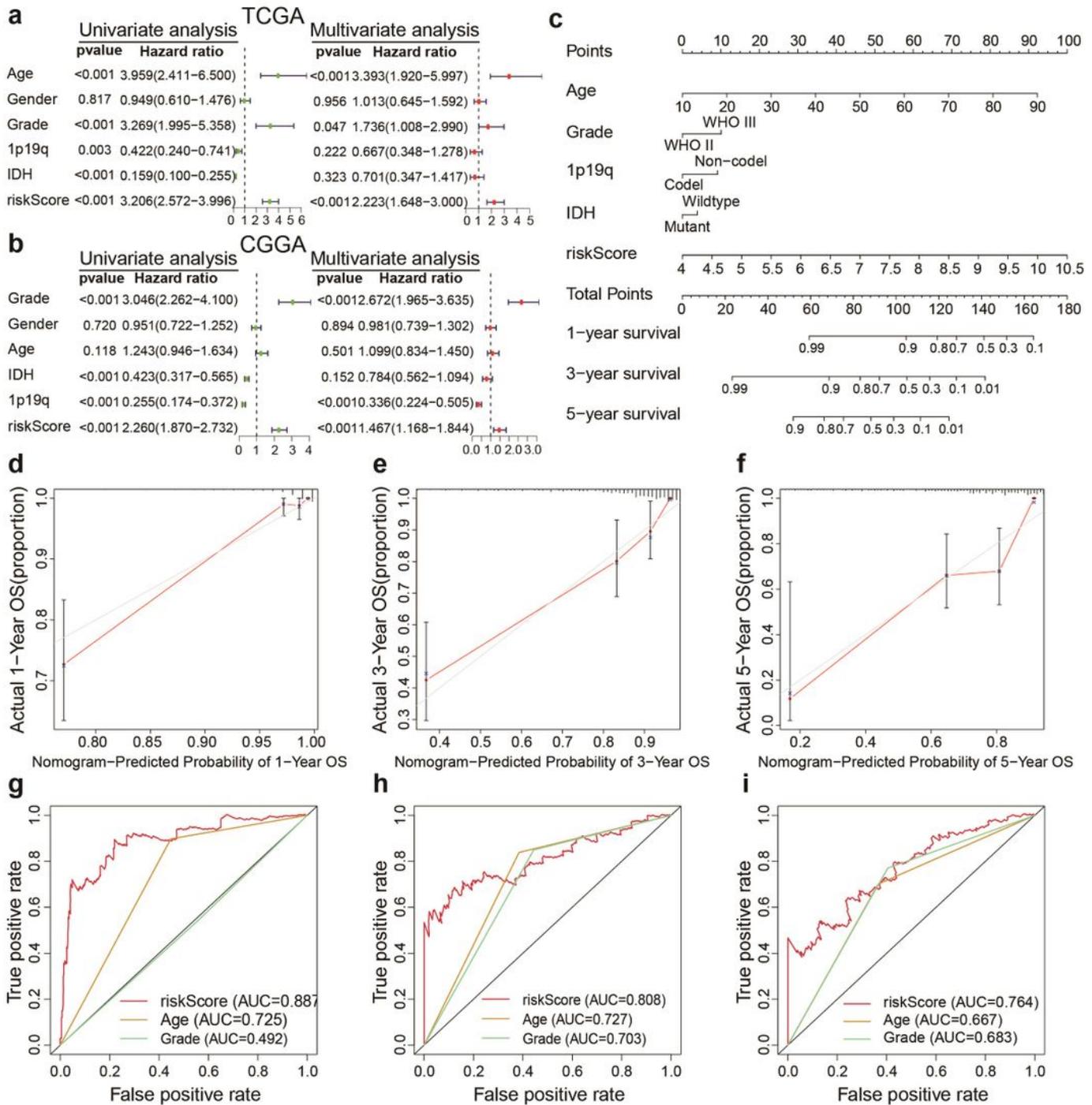


Figure 7

Construction of the nomogram and nomogram calibration. a, b Univariate and multivariate analyses revealed that the risk score [based on the SLC1A5-related immunomodulators prognostic signature (SLC1A5-IPS)] was an independent prognostic predictor in the TCGA and CGGA databases. c Nomogram based on risk score, age, IDH status, 1p19q status and WHO grade. d–f Calibration plots showing the ability of the nomogram to predict the 1-, 3-, and 5-years OS in the TCGA dataset. g–i Time-dependent

receiver operating characteristic (ROC) curves for the nomogram, risk score, age and grade in the TCGA dataset (for predicting 1, 3, and 5-year OS).

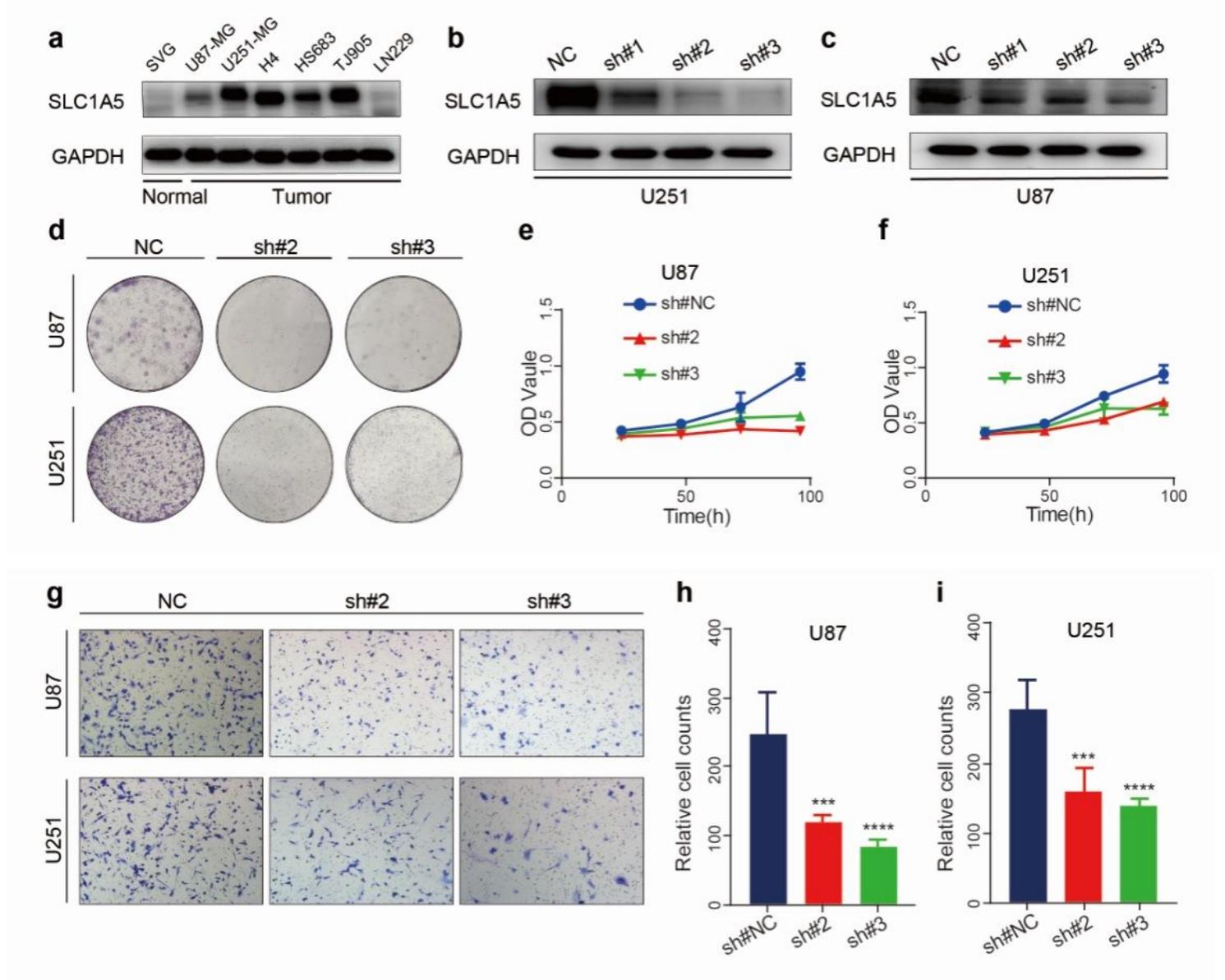


Figure 8

SLC1A5 knockdown inhibits glioma cell proliferation and migration in vitro. a The expression profile of SLC1A5 between different glioma cell lines and normal cell lines. b, c SLC1A5-knockdown in U251 and U87 cell lines was confirmed at the protein level. d Clone formation capacity by U87 and U251 cells was assessed using the clone formation assay. e, f Cell proliferation of U87 and U251 cells was determined using the MTT assay. g-i Knockdown of SLC1A5 inhibited the migration of U87 and U251 cells, the results are displayed in graphs.

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