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Original Research

IRF2-ferroptosis related gene is associated with prognosis and EMT in gliomas ${}^{\bigstar}$

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ARTICLE INFO	A B S T R A C T
Keywords: IRF2 Glioma Prognosis Ferroptosis EMT	Ferroptosis is a new type of programmed cell death that has excellent anti-tumor potential in different tumors. However, the research on ferroptosis in glioma is still incomplete. In this study, we aimed to revealed the relationship between ferroptosis-related genes (FRGs) and glioma. We collected gene expression profiles of glioma patients from the TCGA and CGGA databases. All glioma samples were classified into five subtypes using the R software ConsensusClusterPlus. Subsequently, we performed single sample gene set enrichment analysis (ssGSEA) to explore the correlation between different subtypes and immune status and ferroptosis. Then co- expression modules were constructed via weighted gene co-expression network analysis (WGCNA). A Gene Ontology (GO) analysis was conducted to analyze the potential biological functions of the genes in the modules. Finally, we identified 10 hub genes using the PPI network. The <i>in vitro</i> experiments were used to validate our predictions. We found that the expression level of IRF2 is positively correlated with the grade of glioma. The overexpression of IRF2 could protect glioma cells from ferroptosis and enhance the invasive and migratory abilities. Silence of IRF2 had the opposite effect. In conclusion, we demonstrated a novel ferroptosis-related signature for predicting prognosis, and IRF2 could be a potential biomarker for diagnosis and treatment in glioma.

Introduction

Glioma is the most common primary brain tumor, accounting for more than 80% of all intracranial malignant tumors [1]. The World Health Organization (WHO) divides gliomas into low-grade gliomas (WHO grades I and II) and high-grade gliomas (WHO grades III and IV) according to histopathological characteristics [2]. Although surgery is combined with postoperative radiotherapy and chemotherapy can reduce the chance of tumor recurrence in patients [3], but the overall survival rate of glioma patients, especially WHO grade IV glioblastoma (GBM) patients, is still not ideal. The median survival time of GBM is only 12–15 months [4]. Therefore, it is urgent to explore the biological functions and molecular regulation mechanisms of gliomas to formulate more detailed and effective treatment strategies. Ferroptosis is a new form of programmed cell death characterized by iron accumulation and lipid peroxidation, and its regulation is significantly different from cell death, autophagy and cell necrosis [5]. In terms of cell morphology, the main manifestations are the increase in the inner membrane density of the mitochondria and the decrease of the mitochondrial cristae, but there is no significant change in the nucleus [6]. In terms of mechanism, ferroptosis can be induced by the inhibition of cystine/glutamate system Xc- activity, the decrease of glutathione peroxidase 4 (GPX4) and the increase of reactive oxygen species (ROS) [7]. Studies have shown that ferroptosis has great potential in the treatment of tumors, especially highly aggressive malignant tumors that are resistant to traditional treatments [8]. For gliomas, the research on ferroptosis is far from in-depth. it is necessary to screen and understand the potential biomarkers related to ferroptosis to provide a more

https://doi.org/10.1016/j.tranon.2022.101544

Received 8 June 2022; Received in revised form 20 July 2022; Accepted 11 September 2022

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^{*} We screened out the ferroptosis-related gene IRF2 in gliomas through bioinformation, and verified the high expression of IRF2 in gliomas through the collected clinical samples. Combined with *in vitro* experiments, it is found that IRF2 protects ferroptosis and enhances epithelial mesenchymal transition in glioma. All in all, IRF2 can be used as a potential target for the treatment of glioma.

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complete idea for the treatment of glioma [9].

Interferon regulatory factors (IRFs) are crucial nuclear transcription factors, consisting of 9 members (IRF1–9) in mammals [10]. The IRF family is best known for its role in the regulation of gene expression, which is essential for the induction of IFN responses [11]. A member of the IRF family, IRF2 is involved in a variety of biological processes, including antiviral inflammation and tumorigenesis, cell proliferation and apoptosis, immune cell maturation, and chemotherapy resistance [12]. Currently, only a few studies have demonstrated that IRF2 is associated with tumor immunity in gliomas, with nearly no additional features of the tumor being considered [13]. Therefore, it is vital to improve our understanding of IRF2 even more.

In the current study, we first obtained the mRNA expression profile of FRGs in glioma samples from public databases. Then we evaluated their expression correlations in different samples and investigated the biological roles. Moreover, we chose the hub gene IRF2 for further experiment *in vitro*. Through a combination of bioinformatics analysis and *in vitro* experiments, we proposed the relationship between IRF2 and ferroptosis for the first time, and at the same time, explored the effect of IRF2 on migration and invasion in glioma cells. Our research helps to disclose the underlying mechanism of glioma and supports IRF2 as a potential therapeutic target.

Materials and methods

Data collection

Public RNA-seq data and corresponding clinical information of glioma patients were collected from The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/). The data were sorted out by the R package "Limma", Similarly, we download the related data from the datasets mRNAseq_693 and mRNAseq_325 in the Chinese Glioma Genome Atlas (CGGA) (http://www.cgga.org.cn). All sample data with age less than 18 years old, survival time less than three months and incomplete information were removed. The TCGA-glioma dataset included a total of 459 samples selected. The mRNAseq 693 and mRNAseq 325 dataset in CGGA were merged into one cohort used as validation included a total of 681 samples selected. In addition, the mRNA-seq information of normal brain tissues was obtained from Genotype Tissue Expression project (GTEx, https://www.genome.gov/ Funded-Programs-Projects/Genotype-Tissue-Expression-Project). Ferroptosis related genes were provided by FerrDb database, a professional database collecting ferroptosis-related marker genes [14].

Sample collection

All glioma tissues and non-tumor tissues were obtained from the Department of Neurosurgery, Renmin Hospital of Wuhan University. The patient did not receive radiotherapy and chemotherapy before surgery and signed an informed consent form. Therefore, all studies were conducted under the approval of the Ethics Committee of the Renmin Hospital of Wuhan University (approval number: 2012LKSZ (010)H).

Identification of ferroptosis-related patterns in gliomas

The "ConsensusClusterPlus" package in R software version 4.0.3 was used to identify ferroptosis-related patterns in gliomas based on the expression profile of ferroptosis-related genes in the TCGA database.

Single-sample gene set enrichment analysis (ssGSEA)

In the TCGA dataset, 29 immune signatures representing diverse immune cell types, functions, and pathways were quantified for their enrichment degrees within glioma samples using single-sample gene set enrichment analysis (ssGSEA), respectively. Furthermore, the gene set "WP_Ferroptosis" was obtained from the Molecular Signatures Database (http://www.broad.mit.edu/gsea/msigdb/). ssGSEA performed by R package "GSVA" (23) was applied to calculate the enrichment scores of "29 immune signatures" and "ferroptosis" for each glioma sample.

Weight-gene co-expression network analysis (WGCNA)

We used the "WGCNA" package in the R software to build a scale-free co-expression network [15]. After setting the minimum threshold, the dendrogram was constructed so that the highly correlated ferroptosis-related patterns were in the same module.

Verification of the hub genes in gene module

The online STRING database was used to predict protein-protein interaction (PPI) network of gene module in the WGCNA, and the score was set to 0.4 and visualized by Cytoscape software [16]. The top ten hub genes in specific module were selected according to the MCC value calculated through more than ten calculation methods using Cytoscape plug-in Cytohubba.

Immunohistochemistry (IHC) staining

IHC staining was used to detect the expression of IRF2 in glioma tissues. In short, the paraformaldehyde fixed paraffin tissue chip includes 51 glioma tissue samples. After treatment with 0.3% hydrogen peroxide solution, the sections were incubated with IRF2 antibody for 4 °C overnight and peroxidase–conjugated secondary antibody at 37 °C for 1 h. Finally, Olympus BX40 microscope (Tokyo, Japan) was used to take images, and two observers independently assessed the percentage of IFR2 positive, who were unaware of the specimen data.

Quantitative real-time reverse transcription PCR (qRT-PCR)

The total RNA in the tissue specimen was extracted with TRlzol reagent (Invitrogen). cDNA was obtained by reverse transcription using PrimeScript RT reagent kit (Takara, Japan) and quantitative real-time PCR was performed by using the SYBR Green PCR master mix (Thermo Scientific, Germany). The primers were purchased from Sangon Biotech (Shanghai) and the sequences were listed in: GAPDH primer (forward primer, CCTTCCGTGTCCCCACT; reverse primer, GCCTGCTTCACC- ACCTTC); IRF2 primer (forward primer, CCTCA-GAACGGACGAGATA; reverse primer, GAAGAG-AGGCTGGGGAA).

Cell culture and transfection and reagents

Human U251 and U87 glioblastoma cells were obtained from Shanghai Institute of Biochemistry and Cell Biology. Cells were cultured in Dulbecco's modified Eagle medium at 37°C and 5% CO2, which was supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 ug/ml streptomycin (Servicebio, Wuhan, China). The specific small interfering RNA (siRNA) and Flag plasmids for IRF2 were purchased from MiaoLing Plasmid Sharing Platform. According to the instructions, cells were cultured in a six-well plate and density reaches 90% or more, then lip3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect 2 ug of plasmids. The Culture medium was changed after transfected for 6 h and the cells were harvested after transfected for 24 h for further experiments. RSL3 and Fer-1 (Ferrostatin-1) was purchased from Selleck.

Western blot analysis

The treated cells were lysed on ice for 30 min, then separated by 12% SDS-PAGE and transferred to a PVDF membrane, and blocked with skim milk for 1 h. Incubate the membrane with the corresponding primary antibody overnight at 4 °C: IRF2 antibody, ACSL4 antibody, GPX4

antibody, SLC7A11 antibody, E-cadherin antibody, N-cadherin antibody, Vimentin antibody, Tubulin antibody, GAPDH antibody were purchased from Proteintech. Next, the membranes were probed with horseradish peroxidase conjugated secondary antibody (Antgene, China). Finally, immunoblots were incubated with ECL detection kit (Pierce, Rockford, IL) and visualized by imaging systems, results were quantified and analyzed by Image J.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay: After transfection for 24 h, cells were seeded 5×10^3 per well in 96-well plates. The cell viability was tested at 0, 24, 48, 72 h according to the instructions of the CCK-8 kit (Dojindo, Japan).

Colony formation: 500 cells per well were seeded into 6-well plates and cultured for two weeks. Then, cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.2 crystal violet for 20 min. The colonies were counted and photographed using a microscope.

Edu assays: The cells were inoculated on sterilized slides placed in 6well plates and incubated for 24 h. Next, Edu (C10310-1, RiboBio, Guangzhou, China) was diluted with the culture medium and cultured with these cells for 2 h in dark room. Immediately after staining, the fluorescence was observed under a fluorescence microscope (Olympus BX51, Japan). And the percentage of Edu-positive cells was calculated using the number of Edu-positive cells and the total number of DAPIstained cells.

Reactive oxygen species (ROS) level

The ROS sensitive probe H2DCFDA (MCE, China) was used to detects the total of ROS in the cells. The adherent cells were incubated with 5 μ M H2DCFDA dye at 37 °C for 20-30 min. Then The cells were collected, washed in PBS and measured with a beckman flow cytometer, and finally data were analyzed with FlowJo software. The lipid ROS level in the cells was assessed using C11-BODIPY dye (Thermo Fisher, Waltham, MA, USA). The cells were inoculated on sterilized slides placed in 6-well plates and incubated for 24 h. Then, cells were incubated with 2 μ M C11-BODIPY dye at 37 °C for 30 min. Next, cells were washed in PBS, fixed with 4% paraformaldehyde and images were acquired using inverted microscopy.

Glutathione assay

After cell homogenization, protein concentrations were measured using BCA protein assay kit (Beyotime, China). Next, the cell supernatant was collected by centrifugation for intracellular Glutathione Assay Kit (Beyotime, China). After obtaining the content of GSH, the ratio of GSH to protein concentration was calculated.

Wound healing and transwell assay

Cells were seeded in a 6-well plate and cultured for a certain time to reach a > 90% confluence. A straight vertical line was drawn in the middle of the plate using a sterile pipette tip and cells were cultured in a serum free DMEM for 24 h. Wound healing images were captured using an inverted microscope (Olympus BX51, Japan). ImageJ software was used to analyze the distance of the healed wound. For the transwell assay. We first pretreated the upper well (Corning, USA) with Matrigel (R&D, USA). 5000 cells were seeded into the serum-free medium upper well, and the lower chamber contains 600 ul medium containing 10% FBS, and incubated for 24 h in an incubator (37 °C, 5%CO₂). Finally, the cells were fixed, stained and photographed under an inverted microscope (Olympus BX51, Japan).

Statistical analysis

All data were shown as the mean \pm standard deviation (SD) of at least three experiments. The differential expression of IRF2 in different grades of gliomas was evaluated by Chi square test. Overall survival analysis was evaluated by Kaplan–Meier plots and log-rank tests. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, CA, USA) and R packages. In all analyses, P-values less than 0.05 were considered statistically significant.

Results

Construction of ferroptosis-related patterns

For investigating the expression patterns of ferroptosis-related markers in gliomas, we performed unsupervised clustering through R package "ConsensusClusterPlus". The clustering tended to be stable when consensus matrix k = 5 (Fig. 1A) according to the consensus clustering cumulative distribution function (CDF) (Fig. 1B) and relative changes in the area under the CDF curve (Fig. 1C). Patients in different ferroptosis clusters had distinct clinical outcomes, and the survival prognosis of patients in cluster 3 was significantly worse than that of other clusters (Fig. 1D). In addition, cluster 3 had the highest ferroptosis enrichment score, which was consistent with the results of ssGSEA (Fig. 1E, F). Furthermore, we analyzed the distribution of age group, 1p19q status, IDH status and WHO grade in 5 clusters, the relative proportion of 1p19q-codeletion status and IDH-wildtype status in cluster 3 was higher than other clusters (Fig. 1G–J). These results suggested that ferroptosis-related clustering had clinical predictive effects and could be used for screening and diagnosis of glioma patients.

Identification of hub genes based on ferroptosis feature

In order to screen out the markers related to ferroptosis patterns constructed, we performed differentially expressed analysis among 5 clusters. A total of 3,368 DEGs were identified according to the limiting conditions (the abs of logFC>1 and adj.p value< 0.05). WGCNA was used to construct a co-expression network in these DEGs according to their expression profiles. Based on two evaluation criteria (scale independence and mean connectivity), the soft threshold was set at 5 (Fig. 2A, B) and DEGs were divided into 5 modules (Fig. 2C). The correlation was strongest for brown module and ferroptosis score (r = 0.65, p < 0.05), in addition, brown module was positively correlated to the immune score (r = 0.87, p < 0.05) and stromal score (r = 0.87, p < 0.05) 0.05), as shown in the Fig. 2D. So, this module was selected for further analysis, we found the brown module genes have significant positive module membership values for ferroptosis, immune and stromal scores (Fig. 2E–G). Hub genes played an important role in biological processes and were often identified as potential research targets. We used Cytoscape software to visualize the protein-protein interaction network in the brown module and identified the 10 hub genes with the highest scores, including: IRF1, OAS3, GBP2, IRF2, HLA-B, HLA-C, HLA-A, HLA-E, HLA-F, OAS1 (Fig. 2H). GO analysis was used to identify the genes potential biological functions, found that the genes were enriched in immune and cell adhesion (Fig. 2I). Finally, we chose IRF2 as the research object of this experiment.

The expression of IFR2 was related to the grade and survival prognosis of glioma

To explore the expression level of IFR2 among glioma and non-tumor brain tissues, we found that IRF2 was highly expressed in high-grade gliomas in TCGA datasets (Fig. 3A). In addition, survival data indicated that higher IRF2 expression was correlated with poor Prognosis than lower IRF2 expression (P = 0.0019) (Fig. 3B). To evaluate the expression level of IRF2 in GBM, we selected 78 glioma tissue samples



Fig. 1. Construction of ferroptosis-related patterns. (A) The consensus matrix for k = 5, highly consistent samples were assigned to the same cluster. (B) Cumulative distribution function (CDF) curve for K = 2–9. (C) The delta area plot indicated the relative increase in cluster stability. (D) Survival analysis of the five clusters. (E) Ferroptosis enrichment score. (F) Heatmap of the five FRGs clusters based on ssGSEA scores. (G-J) Expression and distribution in different age, 1p19q status, IDH status, pathological grade.

and 6 normal brain tissues and evaluated IRF2 mRNA levels using qRT-PCR. As shown in Fig. 3C, the mRNA of IRF2 expression was significantly Higher in glioma tissues than matched normal tissues (p < 0.05). Further, we got the same result in IHC analysis (Fig. 3D, E). Altogether, these database and clinical data demonstrate that IRF2 was high-regulated in glioma and the expression of IRF2 was associated with the development of glioma.

IRF2 promoted the proliferation in glioma cells

To investigate IRF2's role in glioma further, we employed plasmid to precisely knockdown and overexpress IRF2 in U251 and U87 cells and western blot was used to verify whether the plasmids were effective (Fig. S1A). The CCk8 assay was performed to assess the viability of U251 and U87 cells, and the results showed that the IRF2 knockdown group dramatically decreased cell proliferation, but the IRF2 overexpression group boosted cell proliferation (Fig. 4A, B). In both cell lines, colony formation experiments revealed that the knockdown group had fewer colonies than the control group, whereas the overexpression group had more colonies (Fig. 4C, D). In addition, Edu assay was used to further evaluate the role of IRF2 in cell proliferation, with Edu positive indicating that cells are in S phase of mitosis. The results showed that the proportion of Edu-positive cells in the IRF2 knockdown group was less than that in the control group, whereas the increase in the IRF2 over-expression group oppositely (Fig. 4E, F). Based on the above results, IRF2 could promote the proliferation of glioma cells.

IRF2 protected ferroptosis in glioma cells

To illustrate the relationship between IRF2 and ferroptosis. After transient transfection of IRF2, relevant ferroptosis measurements will be performed. First, we detected ROS levels in U251 and U87 cells using



Fig. 2. Identification of hub genes based on ferroptosis feature. (A, B) The soft threshold was set at 5 based on scale independence and mean connectivity. (C) Genes were divided into 5 modules. (D–G) The correlation between modules and sample clustering, the brown module was most closely related to ferroptosis. (H) Construction of the protein-protein interaction network in brown module. (I) Gene Ontology enrichment analysis.

H2DCFDA dye. As expected, the ROS content of the IRF2 knockdown group was significantly higher than that of the control group (Fig. 5A, B). We further speculated that ROS occurs as lipid peroxidation. We thus used C11-BODIPY probes to detect oxidized lipids by fluorescence microscopy. The results showed that the level of lipid peroxidation increased in the IRF2 knockdown group, while the opposite result was obtained in the overexpression group (Fig. 5C). GSH, as the main antioxidant in the body, can scavenge free radicals and reduce reactive oxygen species. The GSH assay showed that the GSH content decreased in the IRF2 knockdown group and increased in the overexpression group, which further proved that IRF2 could reduce lipid peroxidation (Fig. 5D). Finally, we used western blot analysis to analyze the expression of several core regulatory proteins of lipid oxidation. The IRF2 knockdown group decreased the expression of GPX4 and SLC7A11 and increased the expression of ACSL4, while the IRF2 overexpression group obtained the opposite results (Fig. 5E, F). All evidence suggested that IRF2 protected glioma cells from ferroptosis.

IRF2 reduced sensitivity to the ferroptosis inducer RSL3 in glioma cells

We further investigated the correlation between IRF2 and ferroptosis by studying the cytotoxicity of the ferroptosis inducer RSL3 in glioma cells. First, CCK8 assay was performed to assess cytotoxic effects. As shown in Fig. 6A and B, the IRF2 knockdown group significantly enhanced the killing effect of RSL3 in U251 cell, while the IRF2 overexpression group partially inhibited the effect of RSL3 in U87 cell. Next, the IRF2 knockdown group increased RSL3-induced lipid peroxidation in U251 cell, while the IRF2 overexpression group obtained the opposite result (Fig. 6C, D). Finally, we found that the ferroptosis inhibitor Fer-1 could significantly reverse the decrease of cell viability and GSH content in the IRF2 knockdown group in U251 and U87 cells (Fig. 6E, F). Therefore, these results further demonstrated that IRF2 was involved in regulating ferroptosis and protected glioma cells from ferroptosis.



Fig. 3. The expression of IFR2 was related to the grade and survival prognosis of glioma. (A) The expression level of IRF2 in high-grade and low-grade gliomas was higher than non-tumor brain tissues. (B) Survival analysis of glioma patients in low-IRF2 and high-IRF2 groups. (C) Clinical samples of 6 non-tumor brain tissues, 24 WHO II and 54 GBM. (D, E) Images of IHC staining for IRF2 in different pathological grade of glioma clinical samples: 5 non-tumor brain tissues, 18 WHO II, 10 WHO III, 18 GBM. Statistical results were shown on the right. * P < 0.05, ** P < 0.01, ns, not significant.

IRF2 enhanced the migration and invasion in glioma cells

Previous results showed that IRF2, as one of the 10 hub genes, was highly associated with glioma cell adhesion and focal adhesion. Therefore, we speculated that IRF2 might be related to the epithelialmesenchymal transition (EMT) of glioma cells. We evaluated the EMT status of U251 and U87 cells based on wound healing and transwell assays. As shown in Fig. 7A and B, the wound healing rate was significantly reduced in the IRF2 knockdown group. Similarly, transwell assay showed that the IRF2 knockdown group inhibited the invasion ability of U251 and U87 cells (Fig. 7C, D). In contrast, the IRF2-overexpressing group promoted cell migration and invasion. The TCGA database indicated that IRF2 was positively correlated with EMT-related proteins (Ncadherin and Vimentin) (Fig. S1B), and IHC analysis also showed increased expression of N-cadherin and Vimentin in clinical samples with high IRF2 expression (Fig. S1C). For further verification, we used Western blot to detect these EMT-related proteins in U251 and U87 cells. The results showed that the expression of N-cadherin and Vimentin was decreased in the IRF2 knockdown group, while the expression of Ecadherin was increased. The results of the IRF2 overexpression group were opposite (Fig. 7E, F). In short, IRF2 could enhance EMT-mediated cell migration and invasion in glioma cells.

Discussion

Ferroptosis is a newly defined form of programmed cell death [6]. Ferroptosis has been studied in many diseases, such as cancer, Alzheimer's disease, ischemia-reperfusion injury and so on [17]. Since the occurrence and progression of cancer cells are accompanied by high metabolism and high oxidative stress levels. In recent years, cancer treatment strategies that target ferroptosis have become a popular direction [18]. Some ferroptosis-inducing medicines, such as erastin and RSL3, block system Xc- to diminish cystine import, while the latter inhibits GPX4 directly, weakening glutathione production and disturbing intracellular redox equilibrium [19]. For example, erastin inhibits cell growth and enhances the cisplatin toxicity of resistant head and neck cancer cells [20]. Inhibition of GPX4 by RSL3 can enhance the anticancer effect of cisplatin *in vitro* and *in vivo* [21]. These experimental results suggest that ferroptosis may be effective in the treatment of tumors, particularly tumors that are resistant to traditional treatments [22].

In recent years, ferroptosis-related genes have been investigated for their prognostic significance in a variety of malignancies. For example, Zhou et al. asserted that the risk score of FRGs, which is based on glioma pathological characteristics, can be used to predict the prognosis of glioma patients in an independent manner [20]. According to Chen et al., the FRGs model was combined with clinical features in order to improve the predictive ability [23]. Our research considerations and analysis methods are roughly the same as them. We used bio-information technology to screen and analyze FRGs in gliomas from the TCGA and CGGA databases. WGCNA was used to build a scale-free gene co-expression network and identify the module that is significantly correlated with ferroptosis. 10 hub genes were successfully screened as a result of the PPI network construction. The GO analysis revealed that hub genes were enriched in immune, cell adhesion, and focal adhesion. This provided us with new ideas for further research into FRGs

The 10 hub genes related to ferroptosis include: IRF1, OAS3, GBP2, IRF2, HLA-B, HLA-C, HLA-A, HLA-E, HLA-F, OAS1. Lei et al. found that the expression profiles of IRF1, IRF2, IRF3, IRF4, IRF5, IRF7, IRF8, and IRF9 were positively correlated with the pathological grade of glioma, and were related to immune cell infiltration [13]. Su et al. constructed a prognostic risk scoring model including OAS3, indicating that OAS3 is a potential prognostic biomarker for low-grade glioma [24]. Liu et al.



Fig. 4. IRF2 promoted the proliferation in glioma cells. (A, B) CCK-8 assay was performed for the detection of the viability of U251 and U87 cells. (C, D) Cell colony formation assay, and the results were shown in the right. (E, F) Edu assay was performed to assess DNA replication. Red represents the Edu positives cells; blue represents the nucleus. Scale bar: 100 μ m. The ratio of Edu-positive cells was calculated by counting red cells/blue cells. * *P* < 0.01, *** *P* < 0.001.

found that GBP2 expression is an independent risk factor for the overall survival rate of pancreatic cancer patients [25]. Fei et al. identified HLA-B as a high-risk immunological gene, and its expression is inversely linked to the prognosis of esophageal squamous cell carcinoma (ESCC) [26]. According to reports, the expression of HLA-F is positively correlated with the malignant phenotype of glioma and negatively correlated with the overall survival rate of patients [27]. The findings of these earlier research suggest that the FRGs has a lot of promise for predicting prognosis and providing possible therapeutic targets for glioma.

Among these hub genes, it is very clear that IRF plays a broad role in

immunity and inflammation by regulating interferon. There are two types of interferon: type I (IFN- α and IFN- β) and type II (IFN- γ). Type I is mostly produced after virus infection, and type II is produced by a limited number of immune cells [28]. In non-immune cells, IFN- γ tends to have an inhibitory effect. Previous studies have shown that IFN- γ is a cytokine that inhibits the proliferation of tumor cells and increases the sensitivity to ferroptosis by reducing SLC3A2 and SLC7A11 [29]. IRF1 and IRF2 have similar structures, but their functions are completely different. IRF1 acts as an activator of IFN- γ , while IRF2 inhibits IFN- γ transcription [30]. Therefore, IRF2 may protect glioma cells from



Fig. 5. IRF2 protected ferroptosis in glioma cells. (A, B) Flow cytometry was performed to measure cellular ROS content, and the results were statistically analyzed. (C) Fluorescence imaging revealed the effect of IRF2 on lipid peroxidation. DAPI was used for nuclear staining. Scale bars: 20μ m. (D) The glutathione assay revealed a regulation of IRF2 on the GSH level of U251 and U87 cells. (E, F) Influence of IRF2 expression on the protein levels of ACSL4, SLC7A11 and GPX4 in U251 and U87 cells. * P < 0.05, ** P < 0.01, *** P < 0.001.

ferroptosis by affecting IFN.

In the experiment, we found that the expression of IRF2 in glioma tissues was significantly higher than that in non-tumor brain tissues, and it increased as the grade of glioma increased. The overall survival of patients with high IRF2 expression in gliomas was poor, which was the same as in the former study [31]. However, the research by Lei et al. had limitations and was only carried out in a limited bioinformation database. In this experiment, we used a lot of clinical data to effectively

prove that IRF2 is highly expressed in gliomas, indicating that IRF2 might be a potential biomarker for glioma. A large number of studies have shown that IRF2 is related to the occurrence and development of tumors [32]. In liver cancer cells, the Knock-down of IRF2 leads to a significant decrease in the invasive ability of cancer cells and a decrease in the expression of STAT3 and MMP9 proteins [33]. In pancreatic cancer, IRF2 is related to the depth of tumor invasion and promotes tumor cell growth [34]. However, in some tumors, overexpression of



Fig. 6. IRF2 reduced sensitivity to the ferroptosis inducer RSL3 in glioma cells. (A) CCK-8 assay was performed for the detection of the viability of U251 and U87 cells after RSL3 treatment. (B) Cell morphology changes were observed by inverted microscopy. Scale bar: 50 μ m. (C, D) Flow cytometry was performed to measure cellular lipid peroxidation, and statistical results were shown on the right. (E) The viability was detected of U251 and U87 cells in the presence of Fer-1. (F) The GSH level was detected of U251 and U87 cells in the presence of Fer-1. *** *P* < 0.001.

IRF2 is found to inhibit proliferation and invasion [35], indicating that the regulatory mechanism of IRF2 in different tumors may be inconsistent, which requires further research to clarify.

Based on previous studies, we found that there is indeed a strong link between ferroptosis and EMT. In the study by Lee J et al., EMT reprogramming increased ferroptosis sensitivity in head and neck cancer [36]. Ferroptosis-related prognostic features reveal EMT status in bladder cancer according to the study by Yan et al [37]. Our study found that IRF2-FRGs can also affect the EMT status of glioma cells, but the mechanism between ferroptosis and EMT needs more in-depth research, which we believe will be a valuable research direction.

Conclusions

In short, we screened out the ferroptosis-related gene IRF2 in gliomas through bioinformation, and verified the high expression of IRF2 in gliomas through the collected clinical samples. Combined with *in vitro* experiments, it is found that IRF2 protected ferroptosis and enhanced epithelial mesenchymal transition in glioma. All in all, IRF2 could be used as a potential target for the treatment of glioma.

Authors contributions

Shiao Tong and Liguo Ye performed the data collection and experimental work. Yang Xu wrote the paper. Qian Sun, Lun Gao, Jiayang Cai



Fig. 7. IRF2 enhanced the migration ability in glioma cells. (A, B) Representative images and statistic graphics of wound healing revealed the migration ability in U251 and U87 cells. (C, D) Representative images and statistic graphics of transwell assay revealed the invasion ability in U251 and U87 cells. (E, F) Influence of IRF2 expression on the protein levels of E-cadherin, N-cadherin and Vimentin in U251 and U87 cells. ** P < 0.01, *** P < 0.001.

and Zhang Ye contributed to the review and revision of the manuscript. Daofeng Tian and Qianxue Chen designed this study. All authors read and approved the submitted version.

Funding

The study was supported by the Hubei Province Health and Family Planning Scientific Research Project (WJ2017M019).

Ethics approval

All study were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and the Ethics Committee of the Renmin Hospital of Wuhan University, Wuhan, China (approval number: 2012LKSZ(010)H).

Declaration of Competing Interest

The authors declared no conflicts of interests.

Acknowledgments

We were very grateful for the support of the department of neurosurgery and central laboratory at Renmin Hospital of Wuhan University.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101544.

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