

Original Article

ZNF395 facilitates macrophage polarization and impacts the prognosis of glioma

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Abstract: The immune microenvironment of glioma attributes to the initiation and development of glioma; however, the underlying mechanisms of tumor microenvironment formation have not been fully understood. In this study, we revealed that Zinc Finger Protein 395 (ZNF395), a member of the Kruppel C2H2-type zinc-finger protein family and also known as a common transcription factor, was aberrantly overexpressed in glioma and positively associated with the poor clinicopathological features and the prognosis of patients with glioma based on the analyses of TCGA, CGGA and other datasets. Further in vitro experimental data demonstrated that the upregulation of ZNF395 promoted the proliferation of glioma cells. In addition, functional enrichment analysis showed that ZNF395 was involved in immune processes and correlated with macrophage infiltration and polarization. Moreover, C-C Motif Chemokine Ligand 20 (CCL20), one of the ZNF395 co-expressed genes, was validated as the downstream factor under the transcriptional regulation of ZNF395. Importantly, cell co-culture experiments confirmed that ZNF395 upregulated both the intracellular and secreted CCL20 level of glioma cells and induced M2 macrophage polarization which is known to promote the malignant progression of glioma. Taken together, our findings suggested that ZNF395 might play an essential role in glioma development, and inhibition of ZNF395 might be a plausible strategy for glioma therapy.

Keywords: ZNF395, glioblastoma, macrophage, M2-polarization, CCL20

Introduction

Central nervous system tumors are a group of highly heterogeneous malignant tumors with increasing morbidity and mortality every year worldwide. In 2020, 308,000 new cases and 251,000 deaths were reported globally [1]. Glioma is the most common primary intracranial tumor accounting for 81% of cases, and glioblastoma (GBM), the most common glioma histology, has a very poor prognosis [2]. The survival time of patients is usually less than two years, with 1-year survival rate of 37.2% and 5-year survival rate of less than 5% [3]. Due to the high invasive ability, tumor heterogeneity, and the characteristics of tumor-initiating cell dormancy, the rapid progression and high recurrence rate of glioma attribute to the poor prognosis of glioma patients [4]. Chemotherapy, radiation therapy, and neurosurgical resection are the common standard treatments for glioma,

while immunotherapy, mainly immune checkpoint inhibitor, has been increasingly used in the last decade [5, 6]. In addition, Tumor-infiltrating macrophages have been reported to participate in the progression of glioma and influence the outcome of tumor therapy [7, 8], which highlights the importance of identifying novel molecules for targeting the macrophages in glioma immunotherapy.

As a member of C2H2-type zinc finger protein family, ZNF395 is a newly discovered nuclear-cytoplasmic shuttling transcription factor of 613 amino acids long [9]. ZNF395 transcriptionally regulates the downstream genes, including CXCL10 and CXCL11, and functions in immune response and tumor development [10, 11]. In addition, as a target gene of the hypoxia inducible factor HIF-1 α , ZNF395 facilitates the hypoxic induction of IL-1 β , IL-6, IL-8, and LIF in U87-MG cells and contributes to the hypoxia

associated inflammation [12]. However, its association with the progression and immune microenvironment regulation in glioma is still unclear, which is the focus of this study.

In this study, RNA-seq data from The Cancer Genome Atlas (TCGA), the Chinese Glioma Genome Atlas (CGGA) datasets and GEO datasets were analyzed to explore the aberrant expression of ZNF395 and the correlation between ZNF395 expression and clinicopathological features, prognosis, and infiltrating immune cell subsets in gliomas. More importantly, we experimentally validated the findings from the bioinformatics analysis that macrophage infiltration and M2 macrophage polarization were associated with high ZNF395-expression of glioma. Our findings may shed light on the role and the functional mechanism of ZNF395 in glioma.

Methods

Data collection

The RNA sequencing fragments per kilobase million (FPKM) data and the corresponding clinical information were downloaded from TCGA database for further analysis. First, the mRNA levels of ZNF395 in pan-cancers were accessed using the Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn/index.html>). Next, the dataset of mRNAs-seq_325 containing the mRNA and clinical data of 325 glioma samples was acquired from the CGGA database (<http://www.cgga.org.cn/>). In addition, the transcriptome data of glioma tissues from GSE16011 dataset (117 tumors and 8 normal tissues) and GSE datasets were utilized to verify the aberrant expression of ZNF395. Finally, the clinical information extracted from TCGA cohort and CGGA database was utilized to investigate the correlations between ZNF395 expression and clinical features (age, WHO grade, histology, etc.).

Analysis of ZNF395 as a prognostic marker

Based on the median expression of ZNF395, patients in TCGA and CGGA cohorts were divided into two groups: ZNF395-low and ZNF395-high groups. These two subgroups were distinguished with different IDH1 status and 1p/19q co-deletion. Kaplan Meier (KM) survival curves were then plotted with orders in R software.

Gene enrichment analysis of ZNF395-coexpressed genes

The differentially expressed genes between the ZNF395-low and the ZNF395-high groups were screened by Limma package and ggplots in R software when $\log_2[\text{fold change}] > 1.5$ and adjusted $P < 0.05$ were defined as the thresholds. Furthermore, the ZNF395-coexpressed genes were analyzed with the GO and KEGG pathway enrichment tools by R clusterProfiler.

Correlation analysis between ZNF395 and immune signature

Tumor Immune Estimation Resource (TIMER) (<https://cistrome.shinyapps.io/timer/>) was applied to evaluate the levels of immune cell infiltration and to analyze the association between the immune cell infiltration and the prognosis. The ESTIMATE immune score of each sample was calculated by applying Estimate package of R software. In addition, CIBERSORT algorithm was used to characterize each immune cell subtype, and deconvolution algorithm was used to accurately quantify the levels of different immune cell subtypes.

Cell culture and transfection

The human glioma cell line SHG-44, U251 and the human monocytic cell line THP-1 were obtained from the Chinese Academy of Sciences. The glioma cells were cultured in DMEM medium (Gibco, BRL, Grand Island, NY, USA), and THP-1 cells were cultured in RPMI1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Yuchun Biotech Co., Ltd., Shanghai, China) in a humidified 5% CO₂ atmosphere at 37°C.

ZNF395 overexpressing plasmid and control pcDNA3.1 plasmid were purchased from Genomeditech Co., Ltd. (Shanghai, China) and stably transfected to the glioma cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), respectively, following the manufacturer's instructions. Briefly, about 80-90% confluent glioma cells were transfected with indicated plasmids. The transfection mix was removed 8 h after transfection, and the cells were harvested 48 h after transfection for further analysis.

Table 1. The sequences of primers used for RT-qPCR

Gene	Primer Sequence (5'-3')
ZNF395 Forward	CGAAAAAAGAAAGAACTCTGTG
ZNF395 Reverse	CTGTGTCCCCAGATGGAG
CCL20 Forward	TGCTGTACCAAGAGTTTGCTC
CCL20 Reverse	CGCACACAGACAACCTTTTCTTT
Arg-1 Forward	CTCCAAGCCAAAGCCCATAGAG
Arg-1 Reverse	AGGGGCTGTCATTGGGGACATC
CCD206 Forward	CTGTGTCCCCAGATGGAG
CCD206 Reverse	CACCCATTCGAAGGCACTC
IL-10 Forward	GCCTTATCGGAAATGATCCA
IL-10 Reverse	AGGGGAGAAATCGATGACAG
GAPDH Forward	TCATTTCTGGTATGACAACGA
GAPDH Reverse	GTCTTACTCCTTGGAGGCC

RT-qPCR

The total RNA from transfected cells was extracted using TRIzol[®] reagent (Beyotime Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol. ZNF395 or CCL20-specific complementary DNA was generated from 20 ng total RNA using Reverse Transcription System (cat. no. A3500; Promega Corporation, Madison, WI, USA). The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the primer sequences were presented in **Table 1**. The PCR conditions were as follows: 94°C for 2 min, 40 cycles of 94°C for 15 sec/60°C for 20 sec/72°C for 20 sec, and 72°C for 30 min. The relative mRNA levels of ZNF395 or CCL20 from three replicates were calculated using the standard 2- $\Delta\Delta C_q$ method.

Western blot analysis

Standard western blot analysis protocol was followed. Briefly, cells were lysed with RIPA buffer (Beyotime), and the cell lysates were quantified by the BCA method. A total of 20 μ g proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with anti-ZNF395 (A-AP14567c; Abgent, San Diego, USA), anti-CCL20 (A1756, ABclonal, Wuhan, China), or anti-GAPDH (A19-056, ABclonal) antibodies. An enhanced chemiluminescence system (ECL Detection System; Amersham Pharmacia Biotech, Piscataway, NJ) was utilized to detect the signals.

Cell proliferation assays

At 24 h after transfection, cells were harvested and reseeded into a 96-well plate at 5000 cells/well. Cell growth was measured at different time points (24 h, 2, 3 and 4 days) by using the Cell Counting Kit-8 (Beyotime) according to the manufacturer's protocol.

ELISA

The supernatants of indicated cells were collected and centrifuged at 22,000 $\times$ g at 4°C for 15 min. The levels of secreted CCL20 or IL-10 were determined by ELISA using Human CCL20 Elisa kit (EK0453; Boster Biological Technology, Ltd., Wuhan, China) or IL-10 Elisa kit (EK0416, Boster), respectively, according to the manufacturer's protocol.

Dual-luciferase reporter assay

A dual-luciferase reporter plasmid under the control of CCL20 promoter sequence was constructed by Genomeditech Co., Ltd. This reporter gene plasmid and pRL-TK (internal control plasmid for transfection normalization) were co-transfected with ZNF395 overexpressing plasmid or vector control into HEK293T cells. Dual-Luciferase Reporter Assay System (Yeasen, China) was used to detect the luciferase activity at 48 h after transfection. The relative luciferase activity of different groups was calculated.

Statistical analysis

Data were presented as the mean $\pm$ standard deviation from at least three independent experimental replicates. Differences between two groups were analyzed using the Student's t-test. Correlation analyses were performed by utilizing the Spearman correlation test. The associations between ZNF395 expression and the clinicopathological parameters of the glioma patients were analyzed using the chi-squared test. Survival analysis was performed with the Kaplan-Meier method using two-sided log-rank test. R software (v4.3.2) was used for statistical analysis and the graphing of bioinformatics analysis. Graphpad Prism 6 was utilized for processing the data from cell-based experiments.

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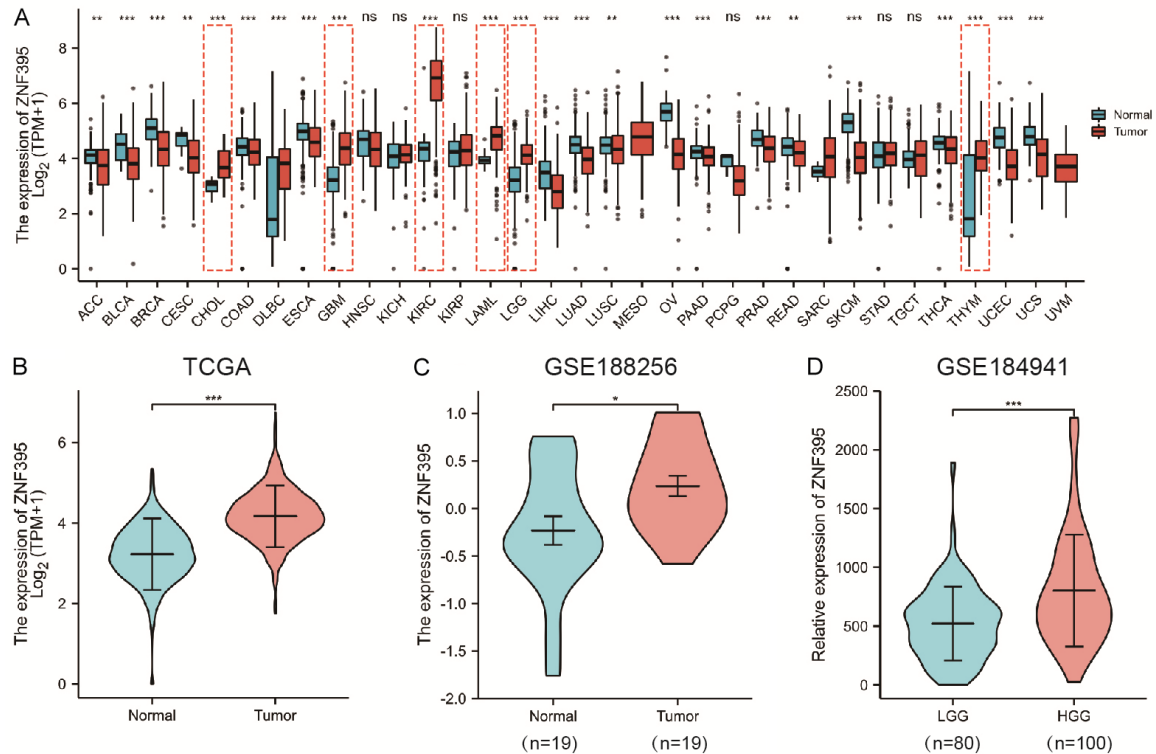


Figure 1. ZNF395 mRNA level in glioma tissues extracted from different datasets. (A) Differential ZNF395 mRNA expression between tumor and normal tissues according to TCGA database. TPM: Transcripts Per Kilobase of exon model per Million mapped reads. (B, C) Differential ZNF395 mRNA expression between glioma and normal tissues in TCGA dataset (B) and in GSE188256 cohort (C). (D) Differential ZNF395 mRNA expression between low-grade glioma and high-grade glioma in the GSE184941 cohort. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

ZNF395 was upregulated in GBM and correlated with clinical parameters

The differential expression of ZNF395 between tumor and normal tissues in 33 cancer types from TCGA database was analyzed and plotted, and the upregulation of ZNF395 was found in Cholangiocarcinoma (CHOL), GBM, KIRC, LAML, LGG and THYM (**Figure 1A**). The expression pattern of ZNF395 in glioma was separately displayed in **Figure 1B**. The aberrantly high expression of ZNF395 in glioma tissues compared to normal tissues was also validated using samples from GSE188256 dataset (**Figure 1C**, $P < 0.05$). Furthermore, according to GSE188256 dataset, the ZNF395 level was significantly higher in high grade glioma (HGG) than in low grade glioma (LGG) (**Figure 1D**, $P < 0.001$).

The clinicopathological characteristics of glioma patients from TCGA cohorts were collected,

and the correlation between ZNF395 and those parameters was analyzed as shown in **Table 2**. ZNF395 level was strongly associated with WHO grade ($P < 0.001$), 1p/19q co-deletion ($P < 0.001$) and histological type ($P < 0.001$); however, no association between ZNF395 expression and IDH status, gender, age and race was observed ($P > 0.05$).

Furthermore, the prognostic value of ZNF395 expression in glioma was evaluated, and the KM curves were plotted. ZNF395 was found as a prognostic factor for overall survival (OS) and disease-free survival (DFS) in glioma (**Figure 2A** and **2B**). The prognosis of glioma patients with high ZNF395 expression was worse than that of patients with low ZNF395 expression. However, the prognostic value of ZNF395 was dependent to the status of IDH status and 1p/19q deletion, as ZNF395 expression was only associated with the prognosis of patients harboring wild type IDH and no1p/19q codeletion (**Figure 2C-F**).

Table 2. Correlation between ZNF395 expression and the clinico-pathologic characteristics of glioma

Characteristic	Low expression of ZNF395	High expression of ZNF395	P
n	348	348	
WHO grade, n (%)			< 0.001
G2	131 (20.6%)	93 (14.6%)	
G3	132 (20.8%)	111 (17.5%)	
G4	52 (8.2%)	116 (18.3%)	
IDH status, n (%)			0.052
WT	111 (16.2%)	135 (19.7%)	
Mut	234 (34.1%)	206 (30%)	
1p/19q codeletion, n (%)			< 0.001
codel	120 (17.4%)	51 (7.4%)	
non-codel	228 (33.1%)	290 (42.1%)	
Gender, n (%)			0.251
Female	157 (22.6%)	141 (20.3%)	
Male	191 (27.4%)	207 (29.7%)	
Age, n (%)			0.189
≤ 60	284 (40.8%)	269 (38.6%)	
> 60	64 (9.2%)	79 (11.4%)	
Race, n (%)			0.339
Asian	5 (0.7%)	8 (1.2%)	
Black or African American	20 (2.9%)	13 (1.9%)	
White	318 (46.6%)	319 (46.7%)	
Histological type, n (%)			< 0.001
Astrocytoma	101 (14.5%)	94 (13.5%)	
Glioblastoma	52 (7.5%)	116 (16.7%)	
Oligoastrocytoma	70 (10.1%)	64 (9.2%)	
Oligodendroglioma	125 (18%)	74 (10.6%)	
Age, median (IQR)	45 (34, 57)	47 (35, 60)	0.133

According to CGGA datasets, ZNF395 expression significantly correlated with WHO grade (**Figure 3A**) and non-codeletion of 1p/19q (**Figure 3B**). In all WHO grade primary glioma, higher ZNF395 expression predicted a poor prognosis (**Figure 3C**), while it couldn't predict the prognosis of patients with recurrent glioma (**Figure 3D**).

ZNF395 promoted the proliferation of glioma cells

To investigate the function of ZNF395 in glioma progression, we manipulated the expression of ZNF395 in glioma SHG-44 and U251 cells via transfection of ZNF395 overexpressing plasmid or control plasmid. The overexpression of ZNF395 in SHG-44 was confirmed by RT-qPCR and western blot (**Figure 4A** and **4B**), and the cell proliferation was measured by CCK8 assay.

We found that overexpression of ZNF395 promoted the proliferation of SHG-44 cells (**Figure 4C**, $P < 0.05$). Similar results were obtained with U251 cells (**Figure 4D-F**).

Functional annotation and pathway enrichment of ZNF395-associated genes

To understand the molecular mechanisms underlying the biological function of ZNF395 in glioma, differential expression genes (DEGs) analysis was performed between ZNF395-low and ZNF395-high groups by Limma package of R software. The top 15 gene sets that were significantly positively (up) or negatively (down) correlated with ZNF395 were shown in the heatmap (**Figure 5A**). The Spearman correlation heatmap was shown in **Figure 5B**. The results showed that the expression of CCL20 was most closely associated with ZNF395 level (**Figure 5C**).

We further employed GO enrichment analysis of the upregulated ZNF-associated genes and found the enrichment of these genes in the processes including

the activation of receptor ligand activity, extracellular structure organization, extracellular matrix organization, cell chemotaxis and myeloid leukocyte migration (**Figure 6A**). Although KEGG enrichment analysis showed that these up-regulated genes were mainly involved in PI3K-Akt signaling pathway and cytokine-cytokine receptor interaction (**Figure 6B**), both enrichment analyses suggested the influence of ZNF395 on the tumor microenvironment of glioma, raising the possibility that ZNF395 might affect the infiltrating of immune cells.

Relationship between ZNF395 expression and tumor-infiltrating immune cells

Due to the enrichment in several immune-associated signaling pathways in ZNF395-high

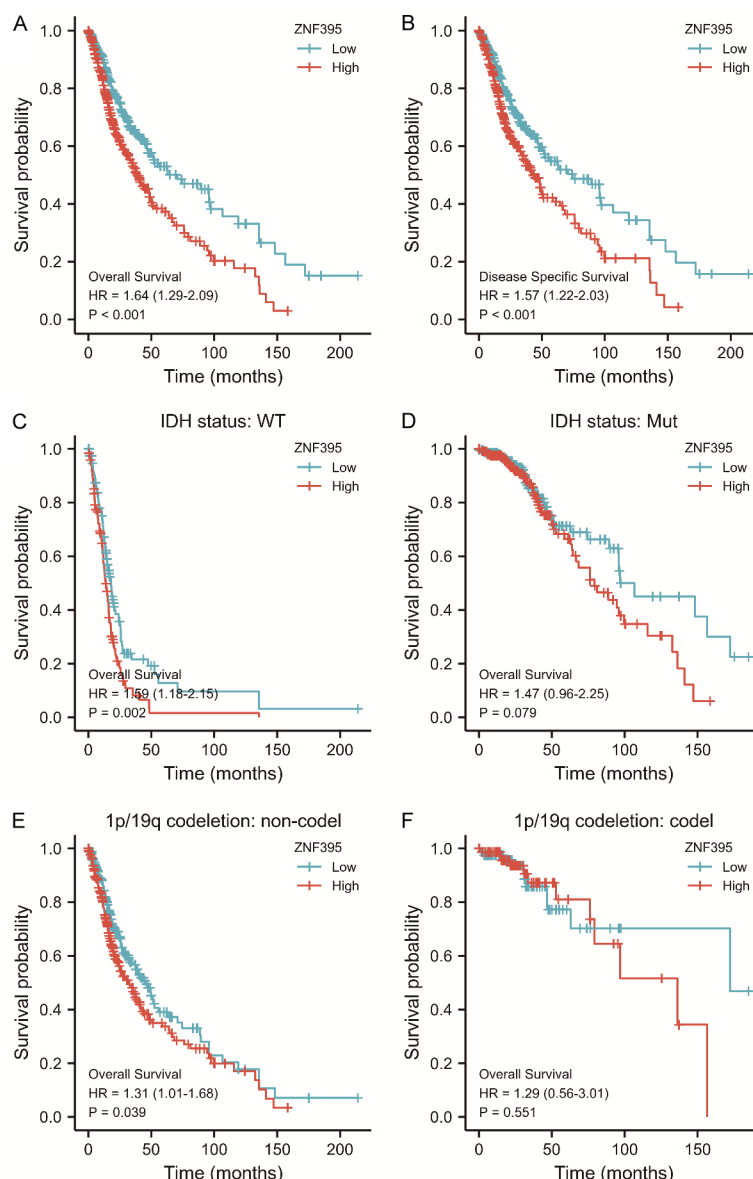


Figure 2. The prognostic role of ZNF395 expression in glioma from TCGA datasets. Overall survival analysis (A) and disease-free survival analysis (B) of patients with ZNF395-low or ZNF395-high mRNA expression in all glioma patients. (C, D) Survival analysis of ZNF395 mRNA expression in different IDH mutant status. (E, F) Survival analysis of ZNF395 mRNA expression in different 1p/19q codeletion status.

group, we investigated the association between ZNF395 and tumor immune microenvironment. TIMER was utilized to analyze the potential associations between ZNF395 expression and the degree and the composition of immune cell infiltration in glioma. As shown in **Figure 7A**, ZNF395 expression was positively associated with the levels of macrophages, neutrophils and T helper cells and was negatively correlated with CD56^{bright} NK cells, mast cells and B cells. The specific association between the infil-

tration levels of all six immune cell types and ZNF395 level was shown in **Figure 7B**. Furthermore, glioma samples from TCGA cohort were divided into ZNF395-low and ZNF395-high groups based on the ZNF395 level, and the composition of 22 subtypes of immune cells in these two groups of was evaluated by CIBERSORT method. Higher percentage of M2 macrophage and resting memory CD4⁺ T cell were found in ZNF395-high group (**Figure 7C**), consistent with the results from KEGG enrichment and TIMER analyses.

ZNF395 promoted macrophage M2 polarization via transcriptionally upregulating CCL20

According to ZNF395 co-expressing analysis above, CCL20 was upregulated in ZNF395-high glioma tissues, and JASPAR datasets predicted the transcriptional regulation between CCL20 and ZNF395. Thus, we investigated this regulation by using luciferase reporter assay. The promoter region of CCL20 was cloned into a luciferase reporter plasmid, and dual-luciferase reporter assay was performed in HEK-293T cells. The over-expression of ZNF395 significantly enhanced the relative luciferase activity driven by CCL20 promoter ($P < 0.05$) compared to the luciferase

activity in vector control cells (**Figure 8A**). Consistently, both CCL20 mRNA and protein levels were upregulated in ZNF395-overexpressed glioma cells (**Figure 8B-D**), indicating that ZNF395 enhanced CCL20 transcription.

More importantly, we investigated the effect of ZNF395 on M2 microphage polarization by co-culture experiments. The culture media were collected from the culture dishes of cells over-expressing ZNF395 or control vector and were

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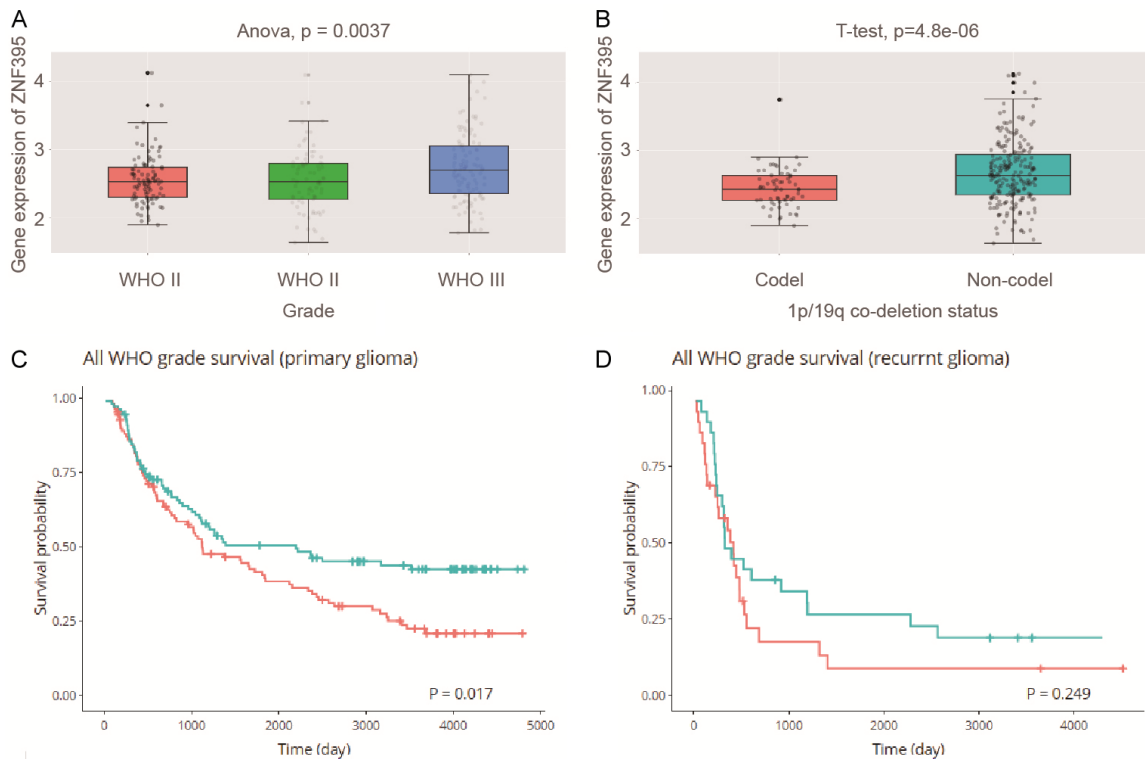
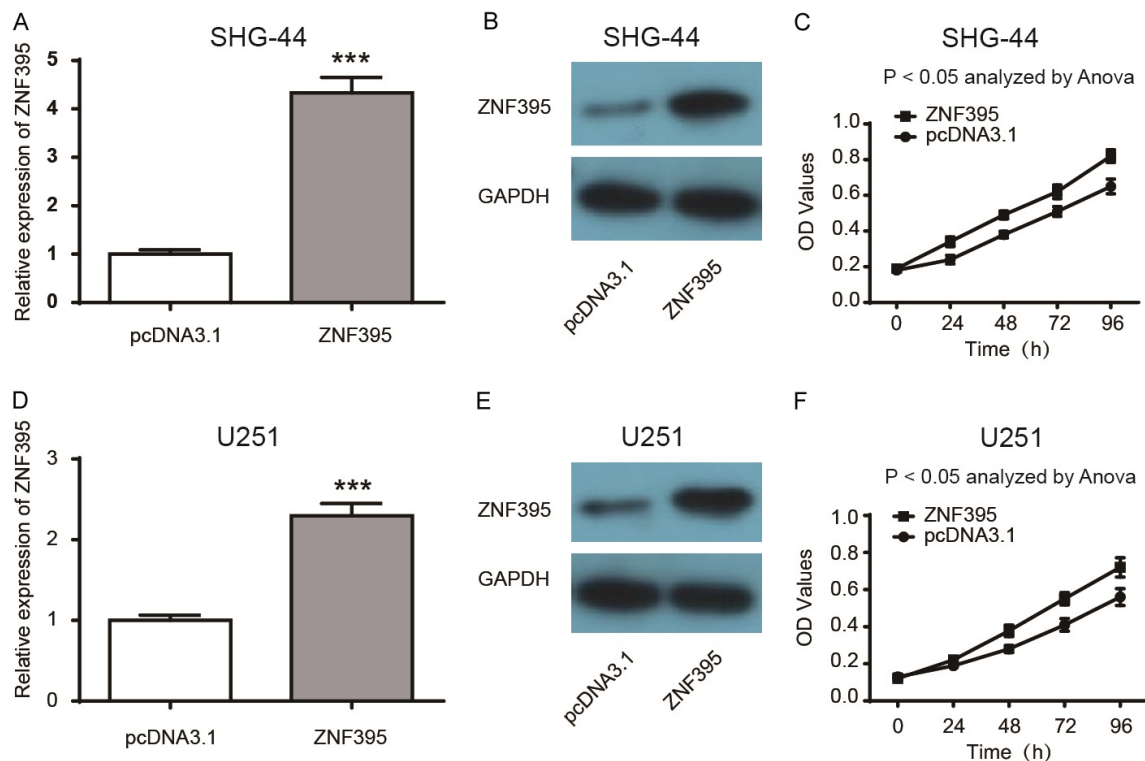


Figure 3. ZNF395 mRNA expression in glioma tissues from CGGA datasets. A. ZNF395 expression in different histological types of glioma. B. ZNF395 expression in patients with different 1p/19q status. C. Overall survival analysis of ZNF395 mRNA expression in all WHO grade patients with primary glioma. D. Overall survival analysis of ZNF395 mRNA expression in all WHO grade patients with recurrent glioma.



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Figure 4. ZNF395 promoted the proliferation of glioma cells. (A, B) SHG-44 cells were transfected with ZNF395-expressing plasmid, and the overexpression of ZNF395 was confirmed by RT-qPCR (A) and by Western blot (B). (C) The proliferation of ZNF395- or vector-transfected SHG-44 cells was measured by CCK8 assay. (D, E) U251 cells were transfected with ZNF395-expressing plasmid, and the overexpression of ZNF395 was confirmed by RT-qPCR (D) and by Western blot (E). (F) The proliferation of ZNF395- or vector-transfected U251 cells was measured by CCK8 assay. ***P < 0.001, n = 3.

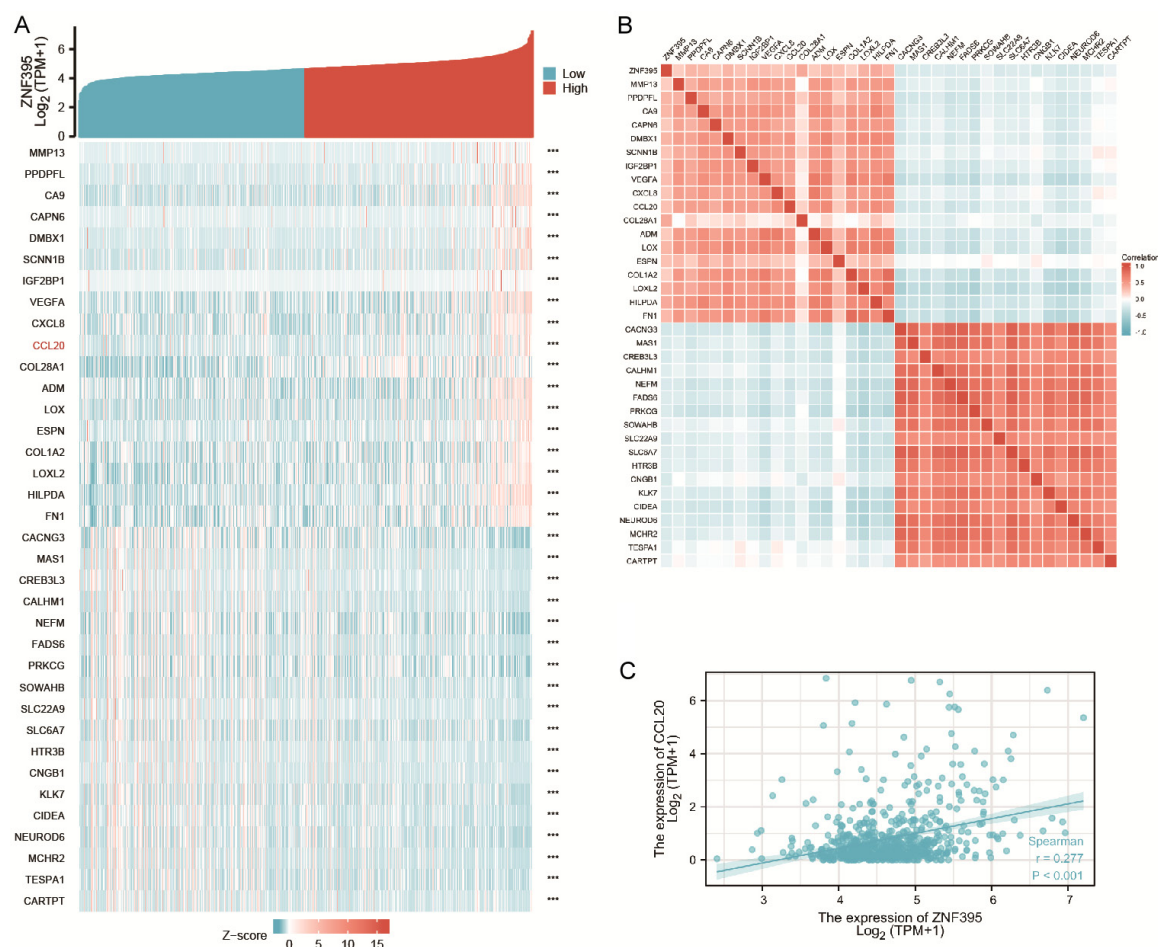


Figure 5. Screening of ZNF395-coexpressing genes in TCGA datasets. A. Heat map showing the top 18 genes positively or negatively associated with ZNF395 expression in glioma. B. The correlation among ZNF395-coexpressing genes was presented in Spearman Heatmap. C. CCL20 mRNA level was positively correlated with ZNF395 expression in glioma tissues.

used as condition media to treat M0-induced THP-1 cells. We found that, after 24 h of co-culture, the expression levels of ARG-1, CD206 and IL-10 were significantly upregulated in THP-1 cells treated with ZNF395-overexpressing glioma cell culture medium (Figure 8E). The secreted level of IL-10 in THP-1 cell culture medium was also increased after being treated with ZNF395-overexpressing glioma cell culture medium (Figure 8F), suggesting that ZNF395-overexpressing glioma cells facilitated the M2 polarization of infiltrating macrophages.

Discussion

The latest cancer statistics released in 2021 indicated the poor outcomes of patients with glioma [1]; hence, it is imperative to further understand the molecular mechanisms underlying the tumorigenesis and development of glioma in order to improve the clinical outcomes. It has been known that the initiation and progression of glioma is closely correlated with the components of tumor microenvironment, especially the tumor-infiltrating immune cells [13-15]. Tumor-associated macrophages

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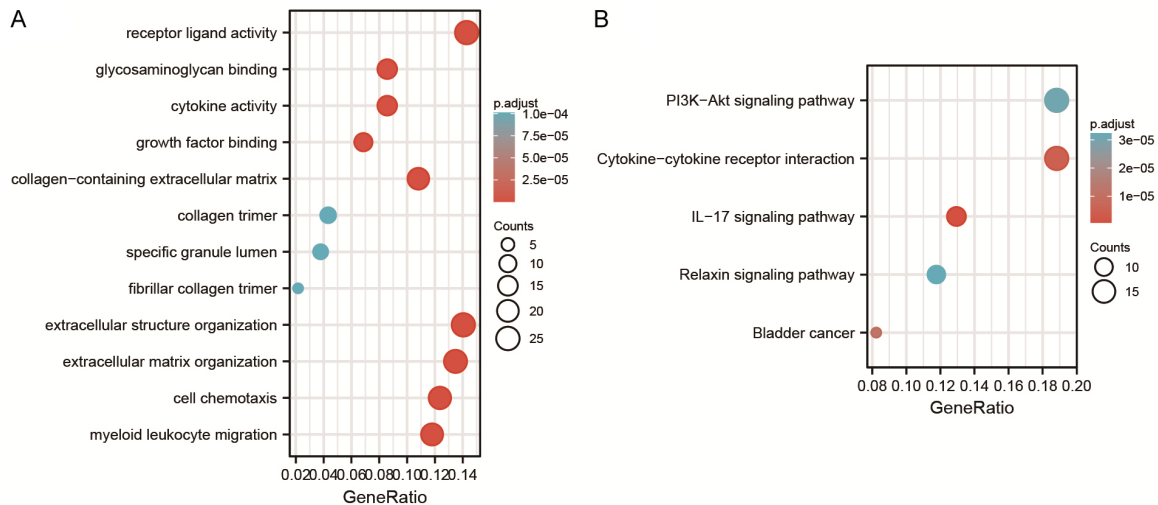


Figure 6. Gene enrichment analysis of ZNF395 in TCGA datasets. A. GO analysis of ZNF395-associated genes. B. KEGG analysis for the pathway enrichment of ZNF395-associated genes.

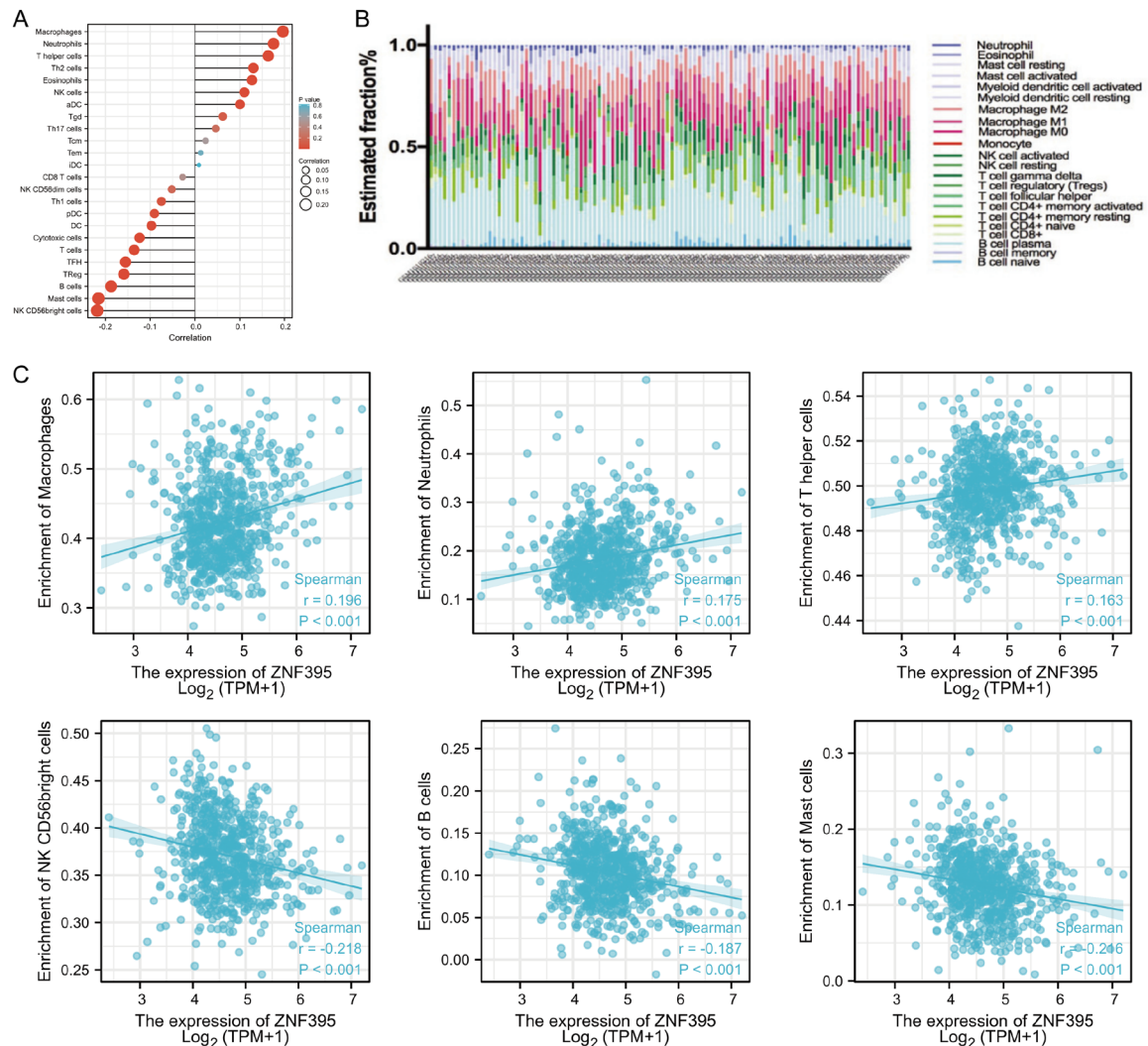


Figure 7. Correlation of ZNF395 with infiltrating immune cells in glioma. A. Correlation between ZNF395 expression and immune cell infiltration in glioma analyzed by R Software. B. All 22 subtypes of infiltrating immune cells were

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plotted according to the ZNF395 expression level in GEPIA. C. The correlation between ZNF395 expression and infiltrating immune cells, including macrophages, neutrophils, T helper cells, CD56bright NK cells, mast cells and B cells.

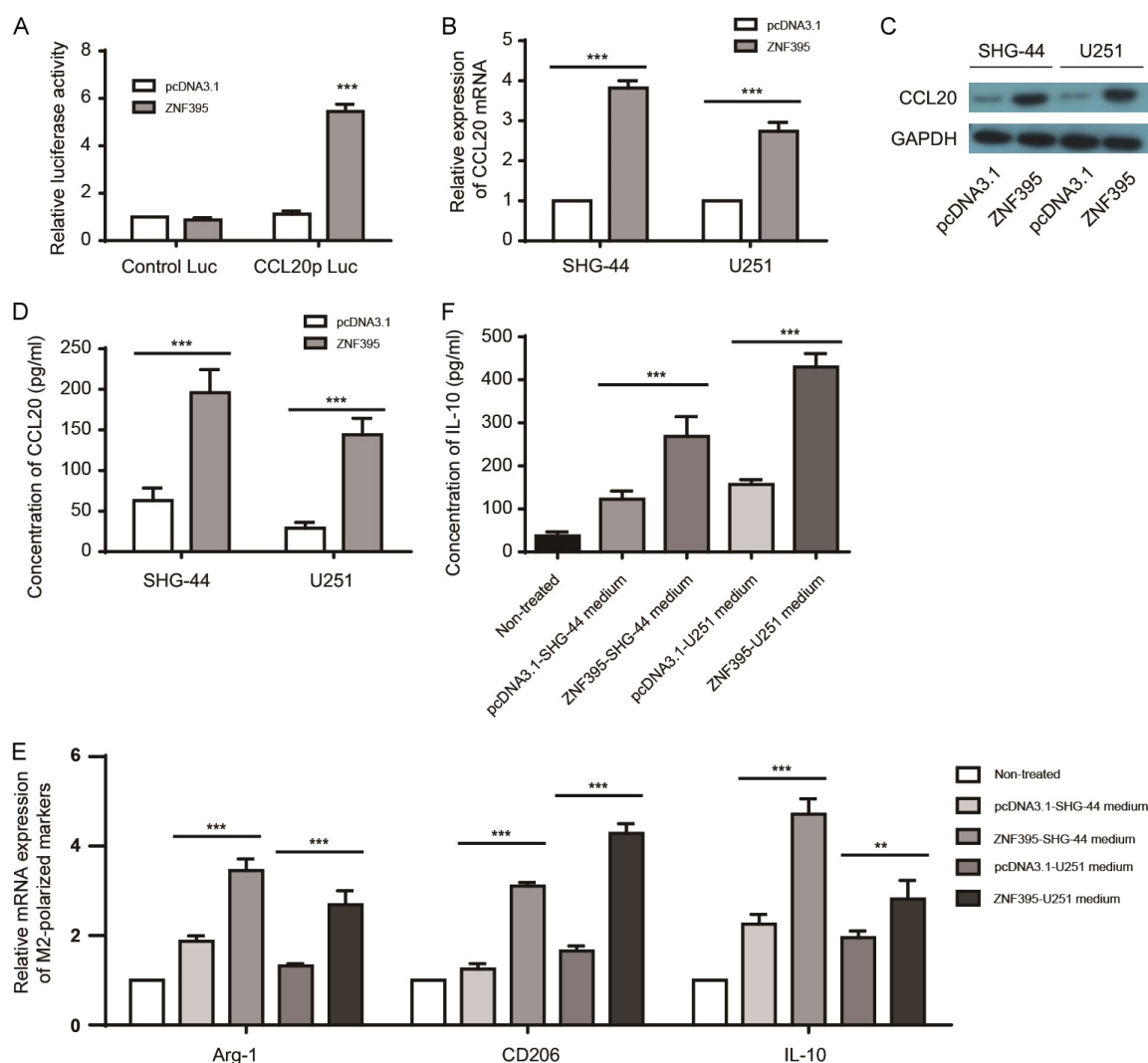


Figure 8. ZNF395 increased the expression of CCL20 and promoted M2 polarization of THP-1 cells. (A) Dual luciferase report assay confirmed the transcriptional regulation of CCL20 by ZNF395. (B-D) Overexpression of ZNF395 in glioma cells increased the expression of CCL20 mRNA as determined by RT-qPCR (B), the intracellular CCL20 protein as determined by Western blot (C), and the secreted CCL20 protein as determined by ELISA (D). (E) The mRNA levels of Arg-1, CD206 and IL-10 in THP-1 cells treated with indicated culture medium were determined by RT-qPCR. (F) Secreted IL-10 protein level in THP-1 cells treated with indicated culture medium was determined by ELISA. **P < 0.01, ***P < 0.001, n = 3.

(TAMs), as an important subset of immune cells, were extensively involved in the progression of tumors including glioma [16, 17]. In this study, we demonstrated that ZNF395 was an important regulator of glioma, not only regulating glioma cell proliferation, but also directly modulating the transcription of CCL20 which, in turn, enhanced self-renewal ability and the tumorigenic activation of M2-like TAM polarization.

Previous studies have reported that hypoxia-induced ZNF395 can transcriptionally up-regulate cancer-related genes and interferon-stimulated genes, such as IFIT1/ISG56, IFI44 and IFI16, in an IKK signaling-dependent manner, indicating ZNF395 as a novel transcription factor that promotes inflammation and cancer progression [11, 12, 18]. Furthermore, elevated expression of ZNF395 has been frequently observed in human cancers, including kidney

cancer, breast cancer, chondrosarcoma, and glioma, where ZNF395 exhibits an oncogenic role in promoting carcinogenesis [19-22]. However, the tumor-suppressing effects of ZNF395 in liver cancer, lung cancer and pancreatic cancer were also reported [23-25]. In addition, although the transcriptional regulation of ZNF395 on its target genes has been well studied, the functional correlation between ZNF395 and tumor immune response, particularly the macrophage polarization in glioma immune micro-environment, has not been determined.

In this study, we were the first to demonstrate that overexpression of ZNF395 enhanced the proliferation of glioma cells in normoxia condition through a series of *in vitro* experiments. Furthermore, using various bioinformatics analysis tools, we revealed that ZNF395 was closely correlated with cytokine-cytokine receptor interaction, which is related to tumor micro-environment, suggesting a potential link between ZNF395 expression and the level of immune cell infiltration. Moreover, we also confirmed that the expression of ZNF395 was correlated with the level of macrophages infiltration in the tumor microenvironment. Glioma-associated macrophages, the TAMs in glioma, were divided into either M1 or M2 polarized macrophages producing multiple cytokines and angiogenic factors. Based on the correlation between survival and the TAM expression in glioma patients, previous studies classified TAM into M1 and M2 subtypes according to their characteristics and specific markers. Although this is not an absolute classification, these two subtypes typically exhibit opposite phenotypes: the M1-like subtype is associated with the inhibition of angiogenesis and tumor growth, which may offer promising therapeutic advantages, while the M2-like subtype is associated with high-risk glioma patients and tumor invasion [26-28]. The classic features of M2-polarized macrophages include higher expression of genes including CD163, Arg-1 and CD206 and the secretion of high levels of anti-inflammatory cytokines such as IL-10 and TGF- β [29, 30]. IL-10 has been reported to activate JAK/STAT3 pathway, leading to tumor growth [31], and TGF- β promotes the migration of cancer cells via upregulating integrin and MMP2 [32, 33]. Furthermore, the extracellular vesicles produced by M2-polarized macrophages transfer oncogenic molecules and facilitate the development of glioma [34, 35]. Thus,

through these multiple mechanisms, M2 polarization of macrophages induced by ZNF395 overexpression promotes the progression of glioma, suggesting that suppressing ZNF395 could be a potential therapeutic approach for inhibiting M2 polarization and inhibiting glioma development.

Another interesting finding in this study was the association of ZNF395 with CCL20 and TAMs. ZNF395 overexpression increased the expression of CCL20, and the level of M2 polarization marker was increased after M0-induced THP-1 cells were treated with the condition medium from ZNF395-overexpressing cells. CCL20, a member of the CC family and the only chemokine ligand for C-C motif chemokine ligand-receptor 6 (CCR6), was found overexpressed in ZNF395-high group in TCGA cohorts. Upregulation of CCL20 was also detected in ZNF395-overexpressing glioma cells *in vitro*. Dual luciferase reporter assay confirmed that CCL20 was transcriptionally regulated by ZNF395. CCL20 chemokine, also known as macrophage inflammatory protein (MIP)-3 α , Exodus 1 or liver and activation regulatory chemokine (LARC) [36], is mainly involved in cell immune regulation and structural homeostasis [37-39]. Recently, high expression of CCL20 and its receptors was observed in several types of tumors, demonstrating the role of CCL20 signaling in their development. In addition, high serum CCL20 was also recognized as a potential diagnostic and prognostic biomarker for multiple tumors [40-43]. And CCL20 could promote tumor progression via both intracellular pathways and regulating the tumor microenvironment [44-47]. Recent studies reported that CCL20 promoted M2 polarization of macrophages [48, 49], which strongly supports the functional axis among ZNF395, CCL20 and M2 macrophages in this study.

In summary, our findings indicate that ZNF395 expression is correlated with poor prognosis of glioma patients and, importantly, ZNF395 promotes the tumorigenicity of glioma through direct transcriptional induction of CCL-20 and the regulation of macrophage polarization in the brain. These findings provide new insights into our understanding of the function and the underlying mechanism of ZNF395 in tumorigenesis, and the results of this study will aid in establishing tailored targeting strategies.

Disclosure of conflict of interest

We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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