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Downregulation of α-L-fucosidase 1 suppresses glioma progression by enhancing autophagy and inhibiting macrophage infiltration

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Abstract

α-L-Fucosidase 1 (FUCA1), a lysosomal enzyme that catalyses the hydrolytic cleavage of the terminal fucose residue, has been reported to be involved in tumorigenesis. However, the clinical significance and biological roles of FUCA1 in glioma remain largely unknown. We analyzed FUCA1 expression according to data in Oncomine, The Cancer Genome Atlas, and Chinese Glioma Genome Atlas databases and further verified FUCA1 expression with immunohistochemistry and real-time PCR analysis in glioma tissues. The results showed that FUCA1 overexpression was significantly associated with high-grade glioma as well as high mortality rates in the survival analysis. Data analyzed in cBio-Portal showed that alterations in FUCA1 (1.4%) were correlated with worse survival in glioblastoma multiforme patients. Functional experiments showed that downregulation of FUCA1 suppressed glioma growth in vitro and in vivo. Conversely, overexpression of FUCA1 had the opposite effects on glioma. Mechanistically, transient inhibition of FUCA1 promoted the formation of large acidic vacuoles, as revealed by staining with acridine orange, increased the ratio of LC3-B/LC3-A, and modified the expression of Beclin-1 and Atg12, which are autophagic markers. Upregulation of FUCA1 attenuated starvation-induced autophagy in glioma. In addition, lower levels of tumor-infiltrating macrophages, including CD68$^+$ (−30%), F4/80$^+$ (−50%), and CD11c$^+$ macrophages (−50%), were identified in FUCA1-downregulated glioma tissues, and CCL2/CCL5 neutralizing Abs blocked this effect. These results show that FUCA1 could serve as a potential therapeutic target for the treatment of patients with glioma by enhancing autophagy and inhibiting macrophage infiltration.

KEYWORDS
autophagy, glioma, macrophage, prognosis, α-L-fucosidase 1

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1 | INTRODUCTION

Glioma is the most common and malignant CNS cancer. The most common forms are AA (WHO grade III) and GBM (WHO grade IV), with the latter accounting for approximately 65% of all gliomas.[1] Moreover, the 5-year survival rate of AA is <30%; that of GBM is even worse (<10%), and most GBM patients die within 2 years after diagnosis.[1,2] Based on this status quo, it is vital to clarify all the potential genes associated with the pathogenesis of glioma.

α-L-Fucosidase-1 is a lysosomal enzyme that encodes a fucosidase that removes terminal fucose residues from glycoproteins.[3] Mutation of *FUCA1* causes a malignant genetic disease called fucosidosis.[4,5] Recent studies have shown that FUCA1 is associated with some types of carcinoma, such as thyroid cancer,[6] breast cancer,[7,8] and colon cancer.[9] Cheng et al reported that there was a greater than 139-fold increase in FUCA1 mRNA expression in breast tumor samples compared with normal breast tissue, and lower FUCA1 levels predicted a significantly inferior overall survival rate.[7] Otero-Estévez et al reported that the expression of FUCA1 can be regulated at the transcriptional level in colorectal cancers.[9] Ezawa et al[10] showed that *FUCA1* acts as a p53 target gene (its overexpression suppresses the growth of cancer cells and induces cell death by removing fucose from EGFR) and contributes to the repression of EGFR signaling. Baudot et al[11] reported that p53 directly regulates the glycosidase FUCA1 to promote chemotherapy-induced cell death. However, the effects of FUCA1 in different cancers are different. In addition, the role of FUCA1 and the more detailed mechanisms of its involvement in glioma are unclear.

The purpose of this study was to investigate the expression and molecular mechanism of FUCA1 in glioma. Consistent with the high expression of FUCA1 in breast cancers, FUCA1 expression was higher in glioma tissues than in normal tissues and was associated with WHO grade, as confirmed by bioinformatics analysis and IHC. Additionally, in vitro and in vivo functional experiments showed that FUCA1 silencing suppresses glioma growth by enhancing autophagy and inhibiting macrophage infiltration.

2 | MATERIALS AND METHODS

2.1 | Glioma specimens

Human samples were obtained from patients of Tianjin Huanhu Hospital, and the study was approved by the Ethics Committee of Tianjin Huanhu Hospital. The human glioma tissue samples used in this study were from 6 patients with grade I (6 for PCR), 16 patients with grade II (8 for IHC, 8 for PCR), 16 patients with grade III (8 for IHC, 8 for PCR), and 22 patients with grade IV (10 for IHC, 12 for PCR); these patients were graded according to the WHO classification. Normal brain tissues (5 cases) were obtained from patients with brain trauma or cerebral hemorrhage, for PCR. All participants signed informed consent forms and were aware of the study details.

2.2 | Cells and reagents

The human glioma cell lines U-87 MG (U87) and U-251 MG (U251) were obtained from iCell Bioscience, and cultivated in DMEM (Thermo Fisher Scientific) with 10% FBS (Thermo Fisher Scientific) in a humidified incubator with 5% CO_2 at 37°C. To induce autophagy, the cells were starved in Earle's balanced salt solution (Thermo Fisher Scientific).

The HRP-conjugated secondary Abs and Abs against Atg12 (4180), Beclin (3495), LC3-A/B (12741), F4/80 (70076S), CD11c (97585S), and GAPDH (5174) were from Cell Signaling Technology. Antibodies against FUCA1 (ab197285) and CD68 (ab125212) were from Abcam. Acridine orange was purchased from Sigma-Aldrich.

2.3 | Oncomine database analysis

The Oncomine database (https://www.oncomine.org/resource/login.html) was used to determine the expression level of the *FUCA1* gene in various types of cancers.

2.4 | Expression and prognostic significance analysis in GEPIA

The online database GEPIA (http://gepia.cancer-pku.cn/index.html)[12] was used to determine the expression and prognostic significance of FUCA1 in the LGG cohort, GBM cohort, and normal controls.

2.5 | UALCAN analysis

UALCAN (http://ualcan.path.uab.edu)[13] is an interactive web portal that facilitates in-depth analysis of TCGA gene expression data. UALCAN was used to clarify the expression of FUCA1 in glioma patients and normal controls.

2.6 | Mutation analysis of *FUCA1* in glioma in cBioPortal

The cBioPortal for Cancer Genomics (http://cbioportal.org) provides a web resource for exploring, visualizing, and analyzing multidimensional cancer genomics data.[14] cBioPortal was used to evaluate the mutation rate of *FUCA1* in GBM.

2.7 | Transient silencing and overexpression of FUCA1 in glioma cell lines

The sequences of siRNAs targeting the FUCA1 and pcDNA3.1-FUCA1 overexpression plasmids were synthesized by GenePharma. The siRNA sequences were as follows: siScr, UUCUCCGAACGUGUCACGUTT; siFUCA1-I, GGUCCACAGAUCCAGAUAATT; and siFUCA1-ii, GCAGAGUUUGCUUGGACUATT. U87 and U251 cells were transfected with specific siRNAs or plasmids using the Lipofectamine 2000 reagent (Thermo Fisher Scientific) in antibiotic-free Opti-MEM medium (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.8 | Generation of stable FUCA1 knockdown U87 cell lines

The knockdown lentivirus vectors of FUCA1 (LV16-shFUCA1, GGUCCACAGAUCCAGAUAATT) and LV16 were purchased from GenePharma. U87 cells were infected with lentivirus vector (LV16-shFUCA1 or LV16) and screened by puromycin (5 μg/mL) to successfully construct stable FUCA1 knockdown U87 cell lines. The knockdown efficiency was evaluated by qRT-PCR and western blotting.

2.9 | Cell viability assay

U87 or U251 cells transfected with siFUCA1 or pcDNA3.1-FUCA1 plasmid were seeded in 96-well plates at 5000 cells/well and were cultured for 24, 48, 72, and 96 hours in the presence or absence of 3-MA (1 mmol/L, S2767; Selleck), respectively. Then CCK-8 solution (K009-500; ZETA) was added to each well for 1-4 hours at 37°C, and the absorbance was measured at 450 nm using a microplate reader (Molecular Devices).

2.10 | Quantitative real-time PCR

Samples were collected and homogenized in TRIzol (Thermo Fisher Scientific). RNA extraction and reverse transcription were carried out following standard procedures. RNA was reverse transcribed using the SureFireRT kit (06-104; Abgen). Real-time PCR was undertaken using GreenHOToGo Taq Mix (05-110121; Abgen). The PCR primers were designed as follows: FUCA1 forward, 5′-GTTTTGACGACAAAGCATCACG-3′ and reverse, 5′-CAATTCACCAACCAAATCCCGA-3′; CCL2 forward, 5′-GAGAGGCTGAGACTAACCCAGA-3′ and reverse 5′-ATCACAGCTTCTTTGGGACACT-3′; CCL5 forward, 5′-ACCAGTGGCAAGTGCTCC A-3′ and reverse, 5′-ACCCATTTCTTCTCTGAGTTGGCA-3′; and GAPDH forward, 5′-CAATGACCCCTTCATTGACC-3′ and reverse, 5′-GACAAGCTTCCCGTTCTCAG-3′. The PCR cycles were carried out by initial denaturation at 95°C for 5 minutes and then running 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. Duplicate experiments were carried out to calculate the mean ΔΔCq, mean RQ (fold change), and SD on a LightCycler 480 II (Roche).

2.11 | Cell staining with acridine orange to detect autophagy

A final concentration of 1 mg/mL acridine orange was added to the cells for 20 minutes. Photographs were obtained with a fluorescence microscope (LSM880; Zeiss), a 488-nm band-pass blue excitation filter and a 515-nm long-pass barrier filter.

2.12 | Western blot analysis

Proteins were extracted from cells using RIPA lysis buffer and PMSF (Solarbio) following the manufacturer's protocols. Then the proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Merck Millipore). After being blocked with BSA, the blots were incubated with Abs against FUCA1, Atg12, LC3-A/B, Beclin, or GAPDH. The blots were then incubated with goat anti-mouse IgG HRP or goat anti-rabbit IgG HRP for 90 minutes at room temperature, treated with enhanced chemiluminescence reagent, and exposed to chemiluminescent film.

2.13 | Immunohistochemistry

The paraffin-embedded brain tissues were sectioned into slides, and IHC was carried out on a YN-05MY automatic immunohistochemical staining system (Shenzhen Yongnian Technology) with Abs against FUCA1, CD68, F4/80, and CD11c. Immunostained sections were imaged with a positive fluorescence microscope (Carl Zeiss). A minimum of 10 fields per section were analyzed.

2.14 | Correlation analysis of FUCA1 expression and immune infiltration level in glioma in TIMER and TISIDB

The TIMER (https://cistrome.shinyapps.io/timer/) is devoted to comprehensively investigate the molecular characterization of tumor-immune interactions.[15] As a user-friendly web portal, TISIDB integrates multiple types of data resources in oncoimmunology, allowing users to facilitate a comprehensive investigation of tumor-immune interactions.[16] Here, we analyzed the correlation between the expression of FUCA1 and the infiltration of immune cells, including B cells, CD8+ T cells, DCs, monocytes, neutrophils, natural killer cells, T cells, and regulatory T cells.

2.15 | Xenograft model with nude mice

All animal studies were approved by the Ethics Committee of Tianjin Huanhu Hospital. In total, 5×10^6 U87 cells stably transfected with LV16 or LV16-shFUCA1 were injected s.c. into the axillary fossa of male athymic BALB/c nude mice. After injection, the

tumor volume was measured with a caliper and was calculated as a × b2/2 (a, long axes; b, short axes). Forty-two days after inoculation, the mice were killed, and the tumors were removed for the next IHC assay.

To block CCL2/CCL5 in vivo, mice were injected i.p. with hamster anti-mouse CCL2 Ab (200 µg/mouse, BE0185; BioXCell), human CCL5/RANTES Ab (20 µg/mouse, MAB278; R&D Systems), or control solution twice a week.
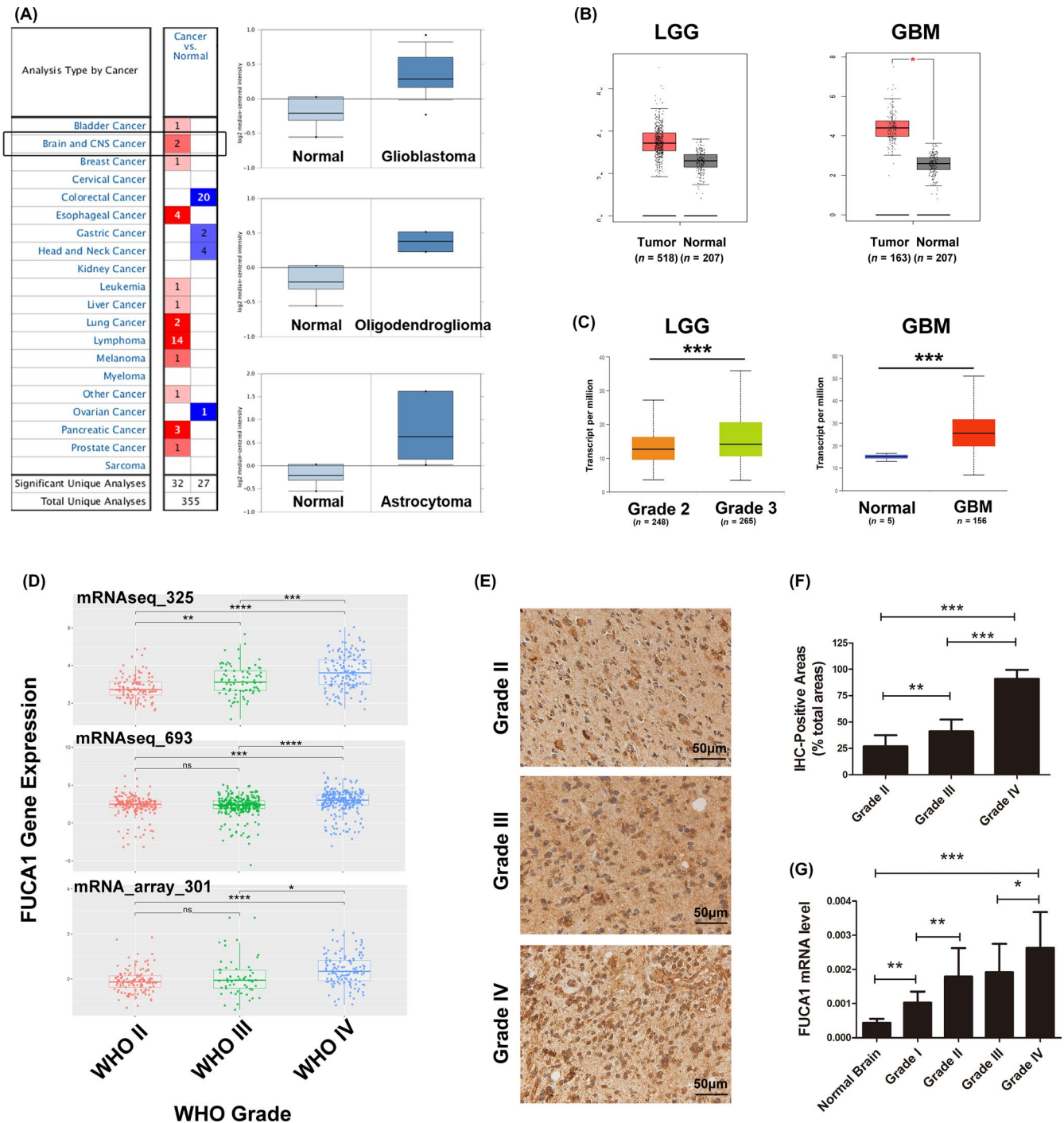

FIGURE 1 Expression of α-l-fucosidase 1 (FUCA1) in glioma. A, Expression of FUCA1 mRNA levels in different cancer types in the Oncomine database. B, Gene Expression Profiling Interactive Analysis of FUCA1 expression in low grade glioma (LGG) and glioblastoma multiforme (GBM) tissues compared with normal brain tissues. C, UALCAN analysis of FUCA1 expression in LGG and GBM. D, Expression of FUCA1 in 3 Gene Expression Profiling Interactive Analysis microarray datasets. E, F, Immunohistochemistry analysis of FUCA1 expression in different WHO grade glioma tissues from Tianjin Huanhu Hospital (grade II, n = 8; grade III, n = 8; grade IV, n = 10). Scale bar = 50 µm. G, Quantitative RT-PCR analysis of FUCA1 mRNA expression in glioma tissues of different WHO grades compared with normal brain tissues from Tianjin Huanhu Hospital (normal brain, n = 5; grade I, n = 6; grade II, n = 8; grade III, n = 8; grade IV, n = 12). Data are mean ± SD. *$P < .05$, **$P < .01$, ***$P < .001$, one-way ANOVA

2.16 | Statistical analysis

The results generated in Oncomine are displayed with fold change and *P* value. The results obtained from UALCAN and GEPIA are displayed as the mean ± SEM and *P* value. Survival curves were generated by the Kaplan-Meier method. The correlation between the expression of different genes was evaluated by Spearman's correlation and statistical significance, and $P < .05$ was considered statistically significant.
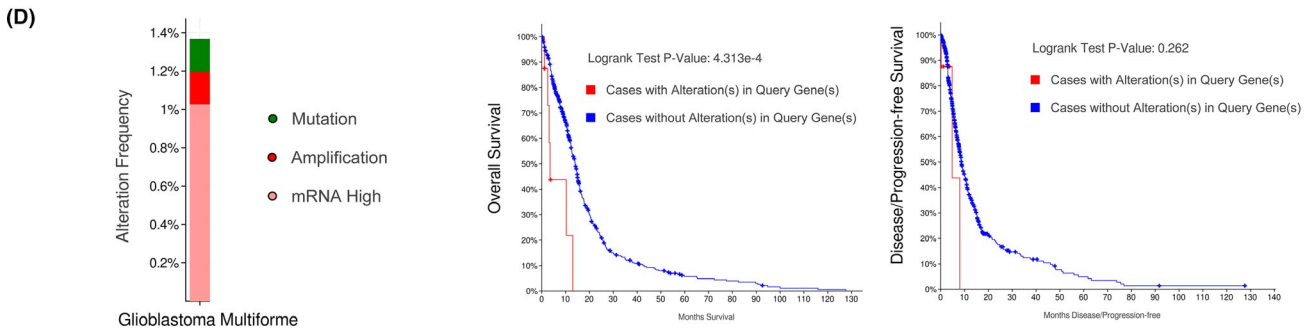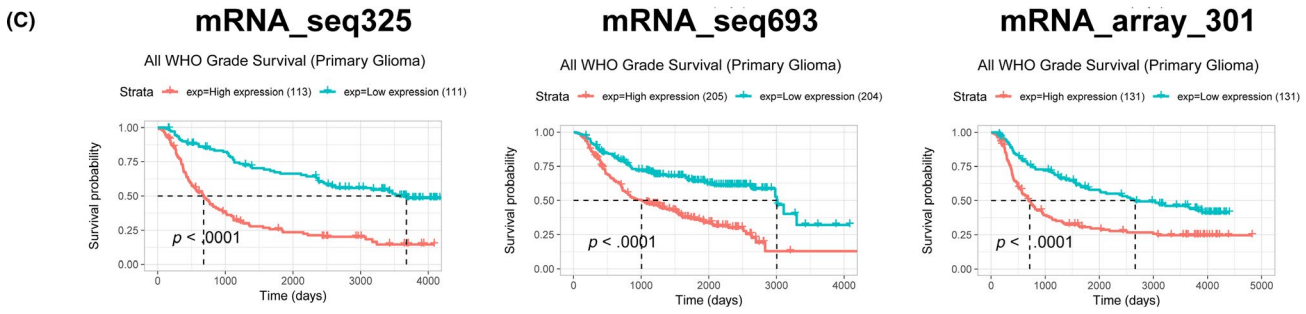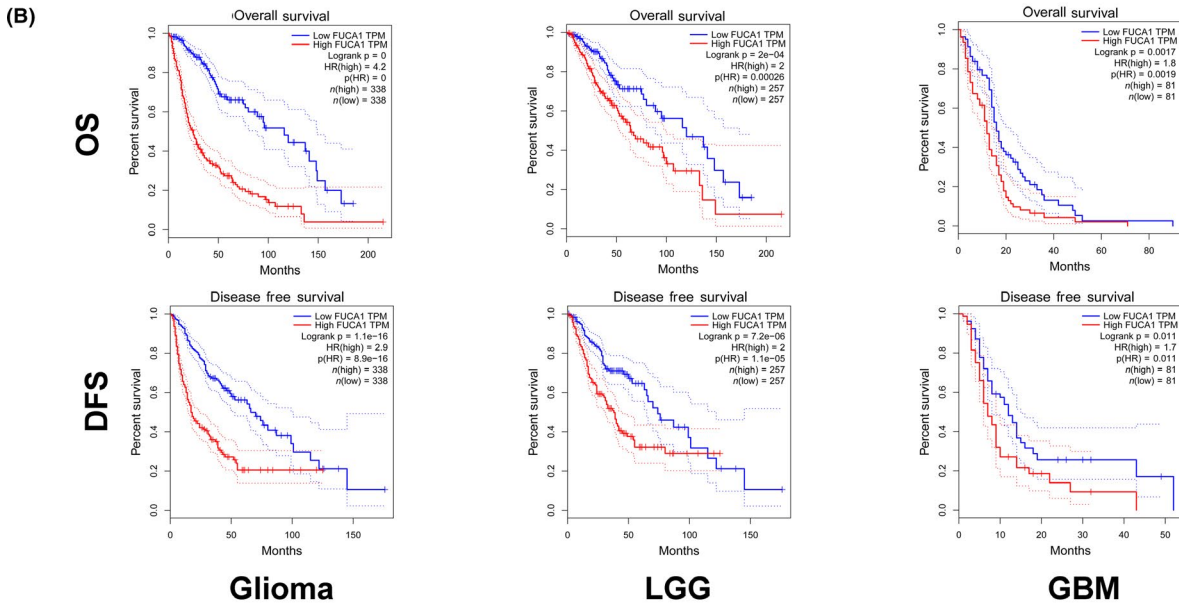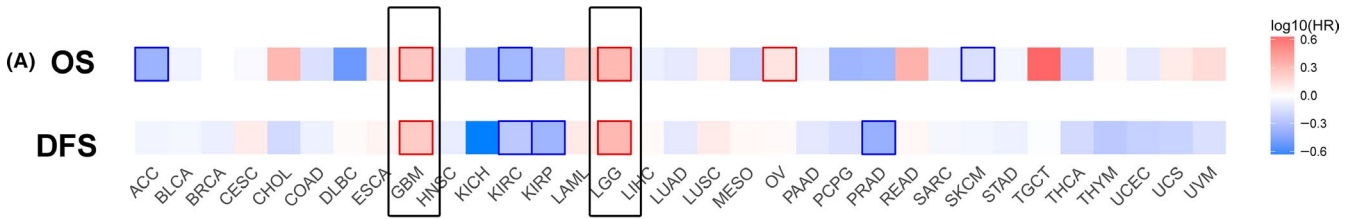
FIGURE 2 Prognostic significance of α-l-fucosidase 1 (FUCA1) expression in glioma patients. A, Survival map of FUCA1 in different cancers obtained from Gene Expression Profiling Interactive Analysis (GEPIA). ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO,mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma. B, GEPIA analysis of the prognostic significance of FUCA1 expression in glioma patients. Relationships between FUCA1 expression and overall survival (OS)/disease-free survival (DFS) in patients with glioma (left panels), LGG (middle panels), and GBM (right panels). C, Chinese Glioma Genome Atlas analysis of the prognostic significance of FUCA1 expression in glioma patients in three datasets. D, cBioPortal analysis of the correlation between FUCA1 alteration rate and survival in GBM
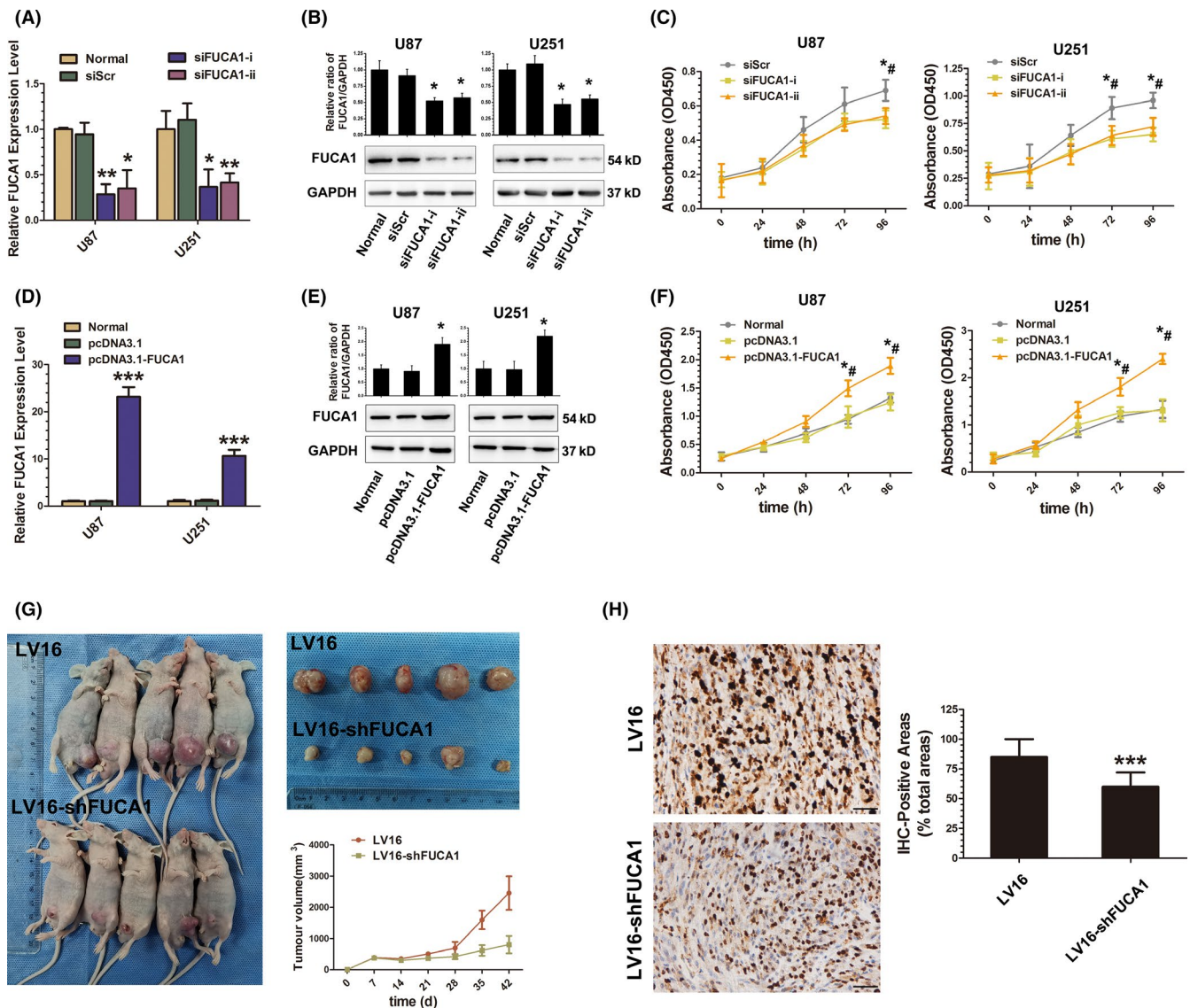
FIGURE 3 Effects of α-l-fucosidase 1 (FUCA1) on glioma growth in vitro and in vivo. A, B, FUCA1 siRNA caused an obvious downregulation of FUCA1 mRNA (A) and protein (B) levels in U87 and U251 cells. Data are mean ± SD. *P < .05, **P < .01, vs scrambled siRNA (siScr) group, one-way ANOVA. C, Viability of U87 and U251 cells was determined by CCK-8 assay after transfection with siScr or siFUCA1. Data are mean ± SD. *P < .05 siFUCA1-i vs siScr group, #P < .05 siFUCA1-ii vs siScr group, one-way ANOVA. D-F, Overexpression of FUCA1 caused by pcDNA3.1-FUCA1 has opposite effects on glioma. G, U87 cells stably transfected with LV16-shFUCA1 or LV16 were injected s.c. into nude mice (n = 5 mice per group). Subcutaneous tumor volumes are shown. H, Ki-67 immunohistochemistry staining of tumors derived from (D). Representative images are shown (scale bar = 20 μm). Data are mean ± SD. **P < .01, ***P < .001, one-way ANOVA. OD450, optical density at 450 nm

FIGURE 4 Inhibition of α-l-fucosidase 1 (FUCA1) induces autophagic cell death in U87 and U251 cells. A, Transient FUCA1-knockdown cells were subjected to acridine orange staining to detect the formation of acidic vesicular organelles (AVOs). Autophagic cell death values were calculated as the percentage of acridine orange-positive cells relative to the total number of cells in each random field. B, Western blot analyses for LC3-A/B, Beclin 1, and Atg12 expression in U87 and U251 cells transfected with siFUCA1 for 48 h. Data are mean ± SD. *$P < .05$, **$P < .01$ vs scrambled siRNA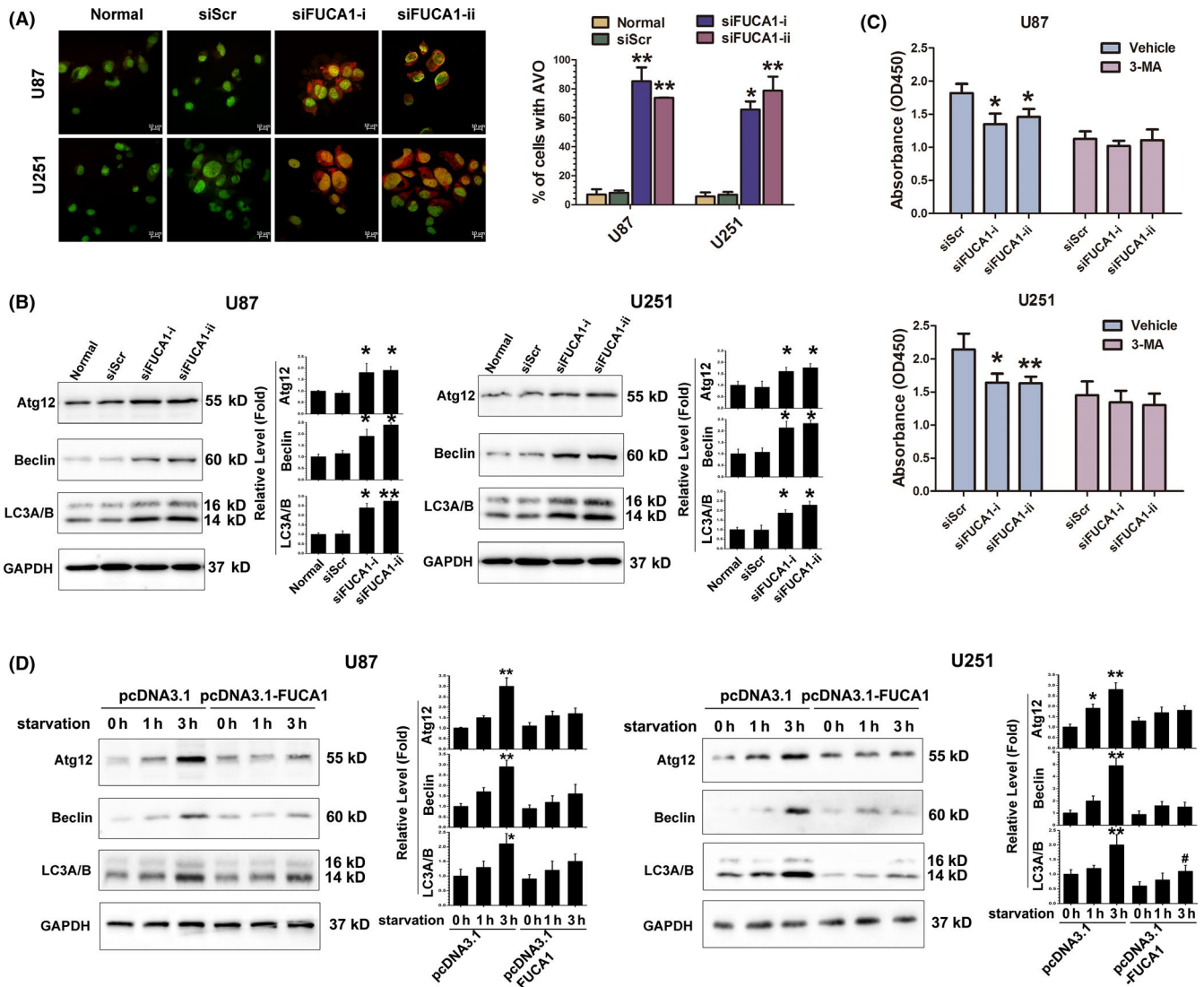 (siScr) group, one-way ANOVA. C, CCK-8 analysis revealed that 3-MA blocked FUCA1 knockdown-induced cell death (72 h). Data are mean ± SD. *$P < .05$, **$P < .01$, vs siScr group. D, Western blot analysis of LC3-A/B, Beclin 1, and Atg12 expression in starved U87 and U251 cells transfected with pcDNA3.1 or pcDNA3.1-FUCA1. Data are mean ± SD. *$P < .05$ 3 h vs 0 h in the pcDNA3.1 group; #$P < .05$ 3 h vs 0 h in the pcDNA3.1-FUCA1 group, one-way ANOVA

3 | RESULTS

3.1 | Expression level of FUCA1 in glioma

First, the Oncomine database was used to determine the FUCA1 mRNA levels in cancer and normal tissues (Figure 1A). Two brain and CNS datasets met the inclusion criteria, and the Shai Brain dataset was selected for analysis. Compared with that in normal tissues, the mRNA expression of FUCA1 was higher in different glioma types, including glioblastoma (fold change = 1.506, $t = 6.545$, $P = 2.42E-6$), oligodendroglioma (fold change = 1.529, $t = 5.655$, $P = .002$), and astrocytoma (fold change = 2.004, $t = 3.034$, $P = .017$). To further evaluate FUCA1 mRNA

expression, we used established computational approaches (GEPIA and UALCAN) to analyze the RNA sequencing data from TCGA and the CGGA database. Both GEPIA (Figure 1B) and UALCAN (Figure 1C) results revealed that FUCA1 expression was markedly higher in the GBM group than in the normal group and was positively correlated with WHO grade, as determined with 3 CGGA microarray datasets (Figure 1D). Finally, immunohistochemistry (Figure 1E-F) and qRT-PCR (Figure 1G) analyses were used to examine the expression of FUCA1 in glioma tissues. We found that the expression levels of FUCA1 in the high-grade samples (grade III and grade IV) were significantly higher than those in the grade II tumor samples (Figure 1). These results further confirmed that FUCA1 was highly expressed in glioma.
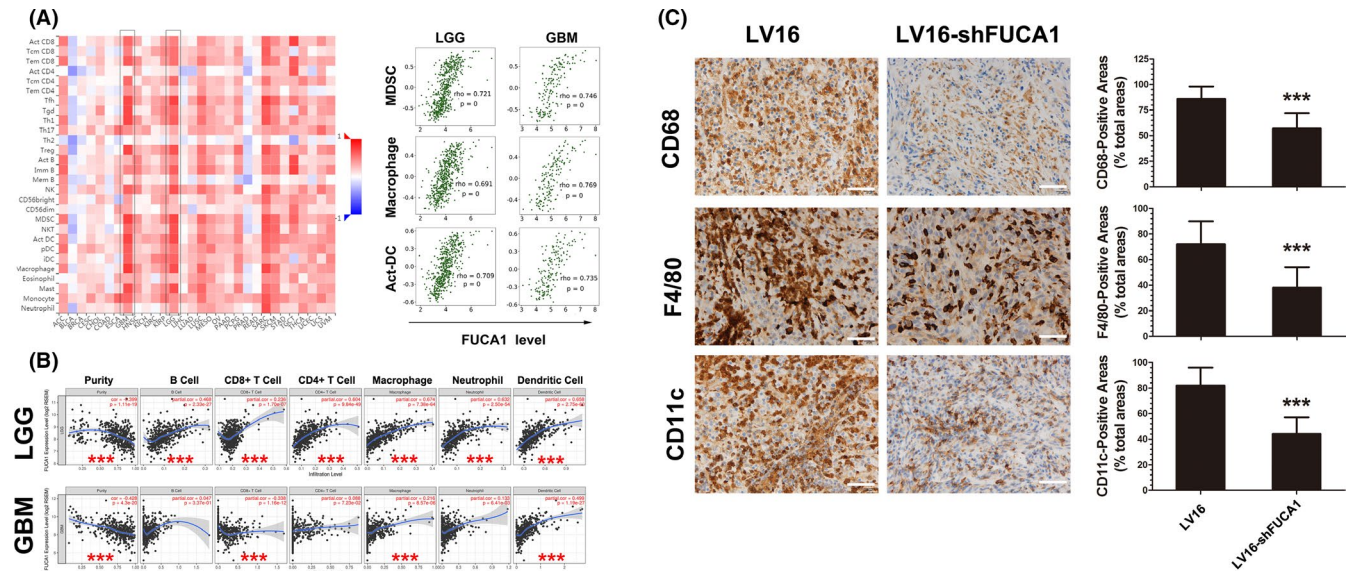
FIGURE 5 Silencing α-l-fucosidase 1 (FUCA1) inhibits macrophage infiltration into glioma. A, Immune infiltration levels in low grade glioma (LGG) and glioblastoma multiforme (GBM) from TISIDB. B, Immune infiltration levels in LGG and GBM from Tumor Immune Estimation Resource. C, Immunohistochemistry staining of CD68, F4/80, and CD11c from LV16- or LV16-shFUCA1-derived tumors (scale bar = 50 μm). Data are mean ± SD. ***$P < .001$, one-way ANOVA

3.2 | Prognostic significance and mutations of *FUCA1* in glioma

To investigate whether FUCA1 expression was correlated with prognosis in LGG and GBM, we evaluated the impact of FUCA1 expression on survival rates using GEPIA. Using the "Survival Map" module of GEPIA, we obtained the OS and DFS significance map for FUCA1, which showed significant results in GBM, LGG, and kidney renal clear cell carcinoma (Figure 2A). Moreover, the results obtained from the "Survival Analyses" module showed that high expression of FUCA1 was marginally associated with worse OS and DFS compared with low expression of FUCA1 in patients with LGG (n = 514) and GBM (n = 162) (Figure 2B). Moreover, CGGA analysis showed that FUCA1 expression was negatively associated with survival in primary glioma patients in 3 CGGA microarray datasets (n = 895) (Figure 2C). Therefore, it is conceivable that high FUCA1 expression leads to poor prognosis in glioma patients.

Furthermore, the data analyzed in cBioPortal showed that FUCA1 was altered in 7 (1.4%) of 585 sequenced patients in TCGA dataset (glioblastoma, TCGA Cell 2013, n = 585), which implied that mutation or DNA copy-number alteration of FUCA1 occurs at a low rate in GBM (Figure 2D). The alterations in FUCA1 were correlated with worse OS (P = 4.313E-4) in GBM patients; however, there was no significant difference in DFS between patients with and without FUCA1 alterations (P = .262) (Figure 2E).

3.3 | Effects of FUCA1 on glioma growth in vitro and in vivo

To further verify the function of FUCA1 on glioma growth, we separately transfected U87 and U251 cells with 2 siRNA molecules for

FUCA1, namely, siFUCA1-i and siFUCA1-ii, with siScr as a control, to silence the *FUCA1* gene. The FUCA1 siRNA treatments resulted in a decline of more than 60% in FUCA1 mRNA levels (Figure 3A) and a decline in protein levels of more than 50% (Figure 3B), rendering a low growth rate, as verified by CCK-8 assay (Figure 3C). We then evaluated the functional role of FUCA1 overexpression in glioma cells. As shown in Figure 3D-F, overexpression of FUCA1 also significantly enhanced the proliferation of glioma cell lines. Furthermore, to evaluate the proof-of-principle influence of FUCA1 on glioma growth in vivo, the U87[LV16-shFUCA1] cell line, which stably knocked down FUCA1, or the control cell line U87[LV16] was implanted into the flanks of nude mice to establish a mouse s.c. model of human cancer. As shown in Figure 3G, the mean tumor volume of the mice in the LV16-shFUCA1 groups was dramatically reduced at 28, 35, and 42 days compared to that in the LV16 group. At the end of the experiment, a significant decrease in tumor volume was observed in the LV16-shFUCA1-treated group (Figure 3G). In addition, IHC staining revealed downregulation of Ki-67 in the LV16-shFUCA1 group compared to the control group (Figure 3H). This result is consistent with the effects of siFUCA1 on cultured human glioma cells in vitro, indicating that FUCA1 depletion inhibits glioma growth in vitro and in vivo.

3.4 | Downregulation of FUCA1 induces autophagic death in glioma cells

α-l-Fucosidase 1 is an enzyme in lysosomes, the main structure mediating autophagic death, leading us to wonder whether FUCA1 is involved in the regulation of autophagic cell death. As shown in Figure 4A. A significant number of autophagic cells was observed by detecting the formation of AVOs, a morphological characteristic

TABLE 1 Correlation analysis between α-l-fucosidase 1 (FUCA1) and related genes and markers of immune cells in Tumor Immune Estimation Resource

Description	Gene markers	LGG None Cor	LGG None P value	LGG Purity Cor	LGG Purity P value	GBM None Cor	GBM None P value	GBM Purity Cor	GBM Purity P value
B cells	CD19	0.405	***	0.337	***	0.219	***	0.160	.0623
	CD79	0.307	***	0.319	***	0.354	***	0.342	***
CD8+ T cells	CD8A	0.339	***	0.217	***	0.320	***	0.210	.0138
	CD8B	0.330	***	0.225	***	0.454	***	0.372	***
DC cells	CD1C	0.399	***	0.400	***	0.415	***	0.238	*
	HLA-DPA1	0.671	***	0.560	***	0.620	***	0.524	***
	HLA-DPB1	0.689	***	0.667	***	0.676	***	0.584	***
	ITGAX	0.543	***	0.458	***	0.273	***	0.028	.748
M1	IRF5	0.716	***	0.665	***	0.577	***	0.349	***
	NOS2	0.075	*	−0.102	*	0.010	.899	0.055	.526
	PTGS2	0.149	***	0.065	.15	0.321	***	0.157	.0664
M2	CD163	0.571	***	0.583	***	0.685	***	0.525	***
	MS4A4A	0.679	***	0.693	***	0.797	***	0.685	***
	VSIG4	0.642	***	0.609	***	0.73	***	0.565	***
Monocytes	CD86	0.732	***	0.690	***	0.721	***	0.587	***
	CSF1R	0.610	***	0.531	***	0.597	***	0.390	***
Neutrophils	CCR7	0.362	***	0.338	***	0.506	***	0.389	***
	CEACAM8	0.007	.877	0.016	.7	−0.057	.483	−0.148	.0839
	ITGAM	0.665	***	0.594	***	0.571	***	0.333	***
NK	KIR2DL1	0.008	.858	0.028	.535	0.144	.076	0.097	.259
	KIR2DL3	0.150	***	0.145	**	0.024	.768	−0.036	.677
	KIR3DL1	0.034	.436	0.024	.602	0.022	.784	0.016	.851
	KIR3DL2	0.187	***	0.195	***	0.109	.178	0.093	.282
T cell exhaustion	CTLA4	0.426	***	0.362	***	0.493	***	0.372	***
	GZMB	0.234	***	0.247	***	0.517	***	0.383	***
	HAVCR2	0.715	***	0.669	***	0.642	***	0.446	***
	LAG3	0.178	***	0.214	***	0.024	.767	0.062	.473
	PDCD1	0.460	***	0.438	***	0.377	***	0.302	***
T cell general	CD2	0.562	***	0.540	***	0.599	***	0.478	***
	CD3D	0.523	***	0.470	***	0.617	***	0.501	***
	CD3E	0.555	***	0.528	***	0.587	***	0.488	***
TAM	CD68	0.751	***	0.725	***	0.732	***	0.559	***
	CCL2	0.463	***	0.431	***	0.566	***	0.410	***
	IL10	0.574	***	0.537	***	0.735	***	0.594	***
Tfh	BCL6	0.009	.836	0.039	.392	−0.044	.558	−0.116	.176
	IL21	0.067	.129	0.056	.22	0.079	.329	0.067	.439
Th1	IFNG	0.243	***	0.195	***	0.121	.138	0.098	.253
	STAT1	0.350	***	0.320	***	0.143	.079	0.184	.0313
	TNF	0.246	***	0.196	***	0.319	***	0.147	.0874
Th2	GATA3	0.439	***	0.393	***	0.248	*	0.276	*
	IL13	−0.044	.32	−0.038	.412	−0.152	.061	−0.102	.234
	STAT5A	0.683	***	0.622	***	0.531	***	0.397	***

(Continues)

TABLE 1 (Continued)

| Description | Gene markers | LGG | | | | GBM | | | |
| | | None | | Purity | | None | | Purity | |
		Cor	P value	Cor	P value	Cor	P value	Cor	P value
Th17	IL17A	−0.009	.833	−0.020	.66	0.041	.614	−0.034	.696
	STAT3	0.490	***	0.500	***	0.086	.289	0.037	.665
Treg	FOXP3	−0.112	.012	−0.093	.042	0.339	***	−0.584	***
	TGFB1	0.614	***	0.555	***	0.419	***	0.239	*

Abbreviations: Cor, Correlation; DC, dendritic cell; GBM, glioblastoma multiforme; LGG, brain lower grade glioma; NK, natural killer; TAM, tumor-associated macrophage; Tfh, T follicular helper cell; Th, T helper cell; Treg, regulatory T cell.

*$P< .01$; ***$P< .001$.

TABLE 2 Correlation analysis between α-l-fucosidase 1 and related genes and markers of macrophages in Gene Expression Profiling Interactive Analysis

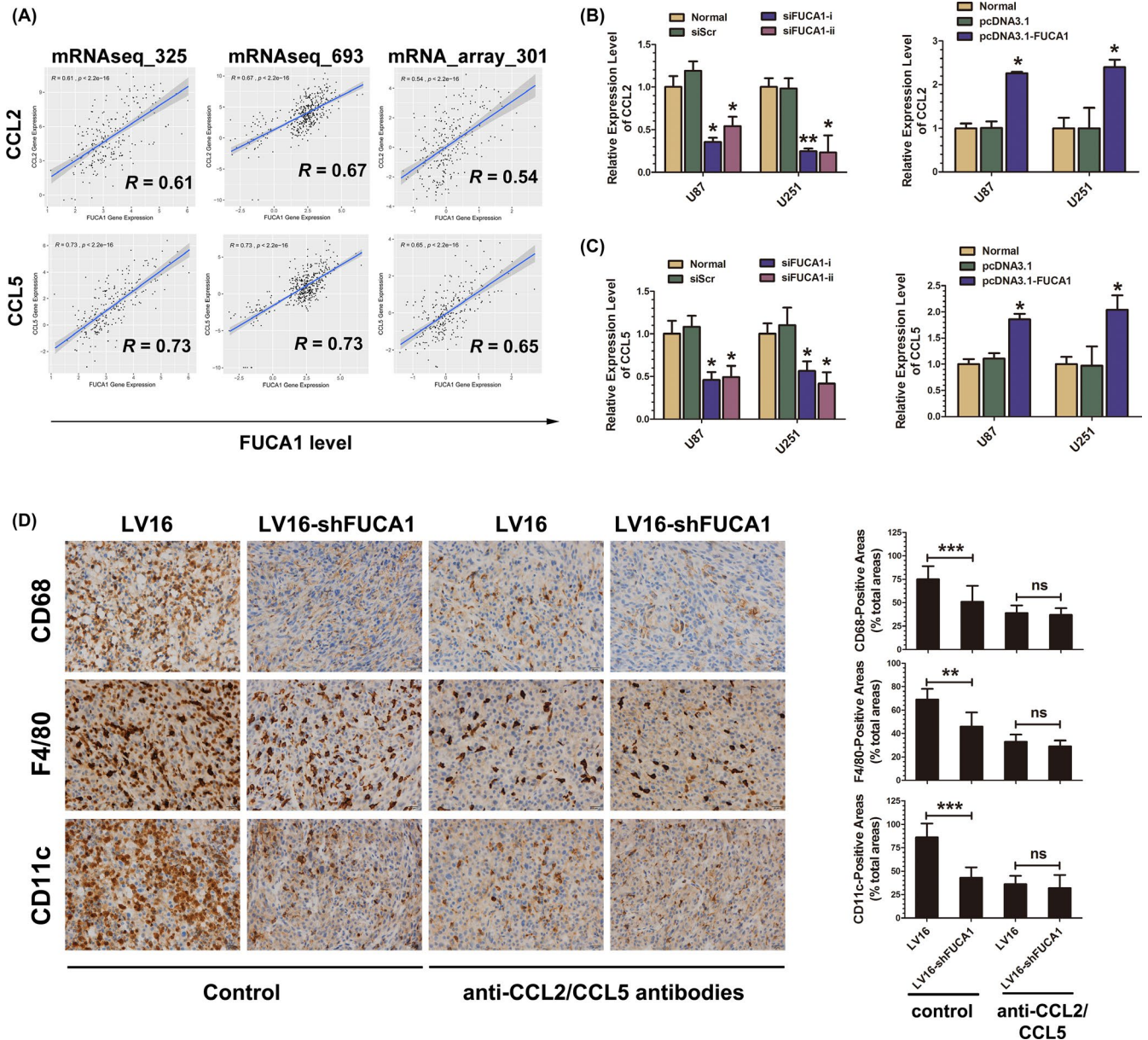| Description | Gene marker | Glioma | | LGG | | GBM | |
		R	P value	R	P value	R	P value
Monocytes	CD86	0.640	***	0.620	***	0.590	***
	CD115 (CSF1R)	0.400	***	0.450	***	0.540	***
TAM	CCL2	0.410	***	0.340	***	0.340	***
	CD68	0.750	***	0.730	***	0.750	***
	IL10	0.670	***	0.710	***	0.580	***
M1 Macrophage	INOS (NOS2)	0.044	.25	0.063	.15	−0.086	.28
	IRF5	0.490	***	0.540	***	0.500	***
	COX2 (PTGS2)	0.240	***	0.088	*	0.250	***
M2	CD163	0.690	***	0.680	***	0.620	***
	MS4A4A	0.730	***	0.700	***	0.680	***
	VSIG4	0.650	***	0.660	***	0.560	***

Abbreviations: GBM, glioblastoma multiforme; LGG, brain lower grade glioma; TAM, tumor-associated macrophage.

*$P<0.01$; ***$P<0.001$.

of autophagy, by acridine orange staining. More than 70% of the cells that were transiently transfected with the FUCA1 siRNA were positive for AVO staining (Figure 4A). Furthermore, we detected changes in the autophagic markers LC3-A/B, Beclin, and Atg12 in FUCA1 siRNA-treated glioma cells by western blot (Figure 4B). The results showed that the LC3-B to LC3-A conversion and the expression of Beclin and Atg12 was significantly increased in both U87 and U251 cells that were transfected with siFUCA1 compared to the expression in cells transfected with siScr. We then used 3-MA, an autophagy inhibitor, to confirm that FUCA1 knockdown-mediated cell death is autophagic cell death. The CCK-8 results showed that 3-MA blocked FUCA1 knockdown-induced cell death (Figure 4C). Moreover, as shown in Figure 4D, overexpression of FUCA1 significantly impaired LC3-B accumulation induced by starvation. The upregulation of Atg12 and Beclin caused by starvation was also attenuated (Figure 4D). Taken together, these results showed that silencing FUCA1 induces autophagic cell death in U87 and U251 glioma cells.

3.5 | Silencing FUCA1 inhibits macrophage infiltration into glioma by downregulating CCL2/CCL5 expression

Tumor-infiltrating lymphocytes can correlate with either better or worse prognosis in cancer. Therefore, we investigated whether FUCA1 expression was correlated with immune infiltration levels in LGG and GBM data from TISIDB (Figure 5A) and TIMER (Figure 5B). The data obtained from TISIDB in the LGG and GBM cohorts showed that FUCA1 expression had significant correlations ($r > .65$) with infiltrating levels of T follicular helper cells (LGG, $r = .736$; GBM, $r = .712$), regulatory T cells (LGG, $r = .678$; GBM, $r = .724$), myeloid-derived suppressor cells (LGG, $r = .721$; GBM, $r = .746$), activated DCs (LGG, $r = 0.709$; GBM, $r = 0.735$), macrophages (LGG, $r = .691$; GBM, $r = .769$), and mast cells (LGG, $r = .696$; GBM, $r = .697$) (Figure 5A). The association between FUCA1 and immune marker genes of different immune cells was analyzed with the "gene" and "correlation" modules of TIMER, and the results showed that FUCA1

(A)

(B)

(C)

(D)

FIGURE 6 Silencing α-l-fucosidase 1 (FUCA1) inhibits macrophage infiltration by downregulating CCL2/CCL5 expression. A, Correlation between FUCA1 and CCL2/CCL5 in 3 Chinese Glioma Genome Atlas datasets. B, C, Quantitative RT-PCR analyses for CCL2 (B) and CCL5 (C) expression in U87 and U251 cells transfected with siFUCA1 or pcDNA3.1-FUCA1 for 24 h. Data are mean ± SD. *$P < .05$, **$P < .01$, one-way ANOVA. D, Immunohistochemistry staining of CD68, F4/80, and CD11c from LV16- or LV16-shFUCA1-derived tumors in the presence of CCL2/CCL5 neutralizing Abs. Data are mean ± SD. **$P < .01$, ***$P < .001$, one-way ANOVA. ns, not significant

mRNA expression had a significant negative correlation with tumor purity (LGG, $r = -.399$, $P = 1.11E-19$; GBM, $r = -.428$, $P = 4.3E-19$) and, moreover, that FUCA1 mRNA expression positively correlated with the infiltration level of macrophages (LGG, $r = .674$, $P = 7.38E-64$; GBM, $r = .216$, $P = 8.75E-06$), neutrophils (LGG, $r = 0.632$, $P = 2.50E-54$; GBM, $r = .133$, $P = 6.41E-03$), and dendritic cells (LGG, $r = .658$, $P = 2.75E-60$; GBM, $r = .499$, $P = 1.19E-27$) in LGG and GBM (Figure 5B, Table 1). The results from both TISIDB and TIMER confirmed that FUCA1 was positively correlated with macrophage infiltration (Figure 5A,B), which was shown to be the main infiltrating immune cell type in glioma. Furthermore, we evaluated the

correlation between the FUCA1 expression level and macrophage-related markers with GEPIA (Table 2). The results revealed that the FUCA1 expression level was significantly correlated with most immune marker sets of macrophages in LGG and GBM, such as CD68, MS4A4A, IL10 and CD163 (Table 2). According to the above results, we predicted that FUCA1 expression is correlated with the immune infiltration level in LGG and GBM, especially with the infiltration of macrophages. To further confirm the role of FUCA1 in macrophage infiltration, we detected the CD68^{+}, F4/80^{+}, and CD11c^{+} macrophage populations using IHC. As shown in Figure 5C, FUCA1 silencing decreased the frequency of CD68^{+} macrophages (−30%,

FIGURE 7 Schematic representation of the processes involved in the effects of α-l-fucosidase 1 (FUCA1) on glioma development. Effects of FUCA1 silencing on tumor development are the result of the induction of autophagic cell death, and the reduction in macrophage recruitment from circulation through promotion of CCL2/CCL5 expression
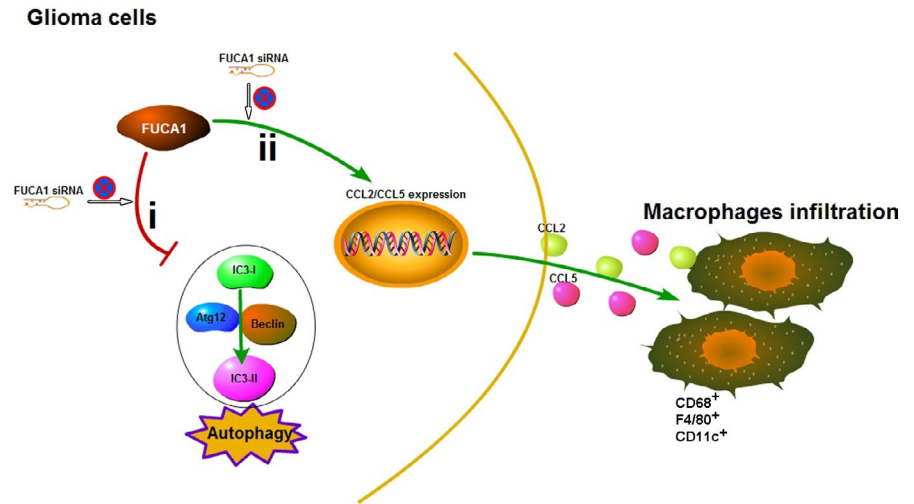
$P < .001$), F4/80^{+} macrophages (-50%, $P < .001$), and CD11c^{+} macrophages (-50%, $P < .001$) in glioma, which was consistent with the results obtained from TISIDB and TIMER.

As chemotactic factors, CCL2/CCL5 play an important role in the recruitment of macrophages, and we determined the correlation between FUCA1 and CCL2/CCL5 in CGGA data (Figure 6A). The results were confirmed by qRT-PCR analysis of mRNA levels, which showed that CCL2 (Figure 6B) and CCL5 (Figure 6C) mRNA levels were reduced in FUCA1-depleted cells and increased in FUCA1-overexpressing cells. Our subsequent in vivo studies indicated that blocking CCL2/CCL5 using neutralizing Abs greatly inhibited the role of FUCA1 on macrophage accumulation in glioma (Figure 6D). Taken together, these results confirmed that silencing FUCA1 inhibits macrophage infiltration into glioma through downregulation of CCL2/CCL5 expression.

4 | DISCUSSION

α-l-Fucosidase 1 is a lysosomal enzyme involved in the degradation of fucose-containing glycoproteins and glycolipids. It is reported to be associated with some types of carcinoma, and it has decreased expression and functions as a tumor suppressor in colorectal cancer[9] and hepatocellular carcinoma[17]; however, it is overexpressed and acts as an oncogene in breast[7] and thyroid cancers.[9] This difference could be due to different molecular mechanisms. In this study, we aimed to discover the clinical significance and exact biological function of FUCA1 in glioma tumorigenesis.

For the expression of FUCA1 in glioma patients, data mining (Oncomine, GEPIA, UALCAN, and CGGA), IHC staining, and qRT-PCR analysis confirmed that FUCA1 expression was increased in LGG/GBM tissues compared with normal tissues and was associated with high WHO grade. Moreover, we found that the survival time of patients with higher FUCA1 expression was significantly shorter than that of patients with lower expression, and the cBioPortal results showed that alterations in FUCA1 were correlated with worse survival in GBM patients.

Our results and the results of previous studies suggest that the expression and prognostic role of FUCA1 in human cancers is complicated. In glioma, FUCA1 overexpression was significantly associated with high-grade glioma as well as high mortality rates, which prompted us to directly assess the role of FUCA1 in glioma development through functional cellular experiments. In the functional cellular experiments, we synthesized FUCA1 siRNA and FUCA1 overexpression plasmids and transfected them into the glioma cell lines U87 and U251 and found that silencing FUCA1 led to glioma cell growth inhibition. Overexpression of FUCA1 had opposite effects. This result is consistent with the effects of FUCA1 siRNA on the U87 xenograft tumor model, indicating that FUCA1 silencing inhibits glioma growth in vitro and in vivo.

What mechanisms are involved? α-l-Fucosidase 1 is an enzyme in lysosomes, the main structure mediating autophagic death, leading us to speculate that FUCA1 is involved in the regulation of autophagic cell death. We found that FUCA1 depletion led to the formation of large acidic vacuoles, induced an increased LC3-B/LC3-A ratio, and modified the expression of Beclin-1 and Atg12, which indicated that autophagic cell death occurred.

Another important highlight of our study is the correlation of FUCA1 expression and macrophage infiltration levels in glioma. Our data indicated that FUCA1 silencing decreased CD68^{+} macrophages (-30%, $P < .001$), F4/80^{+} macrophages (-50%, $P < .001$), and CD11c^{+} macrophages (-50%, $P < .001$) in glioma, which was consistent with the results obtained from TISIDB and TIMER. Additionally, CCL2/CCL5 neutralizing Abs inhibited the effect of FUCA1 on macrophage accumulation in glioma. It is known that intense infiltration of TAMs facilitates malignant growth of glioma, correlates with glioma progression and tumor grade, and predicts poor survival of glioma patients.[18,19] Our results confirmed that FUCA1 inhibits glioma development not only by inducing autophagy but also by regulating macrophage recruitment. Recent studies have revealed the mechanism of macrophage infiltration in glioma. Ge et al[20] reported that CD70 affects tumor migration and growth and macrophage infiltration by regulating the expression of the *CD44* and *SOX2* genes.

Fang et al[21] reported that extracellular ATP regulated glioma-associated microglia/macrophage infiltration by influencing macrophage inflammatory protein-1α and monocyte chemoattractant protein-1 expression, which was consistent with our speculation that silencing FUCA1 inhibits macrophage infiltration into glioma through downregulation of CCL2/CCL5 expression. Further work is needed to clarify the exact mechanisms by which FUCA1 silencing induces CCL2/CCL5 reduction.

Collectively, we hypothesize that the effect of FUCA1 silencing on tumor development can be explained by the induction of autophagic cell death, and the reduction in macrophage recruitment from circulation (Figure 7). We anticipate that FUCA1 can be used as a valuable target for glioma treatment and as a prognostic biomarker.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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