Translational Oncology 14 (2021) 101196

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

Loss of FAM60A attenuates cell proliferation in glioma via suppression of PI3K/Akt/mTOR signaling pathways

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ARTICLE INFO

Keywords:
FAM60A
Cell proliferation
PI3K/Akt/mTOR
Glioma
Biomarker

ABSTRACT

Background: Glioma is a common malignant tumor of the central nervous system with a high incidence and mortality. Family with sequence similarity 60 member A (FAM60A) is a new subunit of the Sin3 deacetylase complex. The clinical significance and biologic role of FAM60A in glioma remain unclear.
Methods: The expression of FAM60A in normal glial cells, glioma cells, and five-paired gliomas, and adjacent noncancerous tissues was quantified using real-time polymerase chain reaction (PCR) and western blotting. FAM60A protein expression in 179 archived, paraffin-embedded glioma samples was analyzed using immunohistochemistry. The roles of FAM60A in glioma cell proliferation and tumorigenicity were explored *in vitro* and *in vivo*. The underlying molecular mechanisms were elucidated using Western blot assay. Serum exosomal FAM60A levels of glioma patients were detected using electron microscopy, western blot, and real-time PCR.
Results: FAM60A expression was significantly up-regulated in glioma tissues and cell lines and positively associated with a worse outcome in glioma. Knockdown of FAM60A could inhibit glioma cell proliferation and tumorigenicity *in vitro and in vivo*. Besides, FAM60A expression was detectable in extracted serum exosomes with a higher expression in the glioma cancer group than in the normal group.
Conclusions: Loss of FAM60A attenuates cell proliferation in glioma by suppressing PI3K/Akt/mTOR signaling pathways. Therefore, FAM60A may act as a prognostic biomarker and therapeutic target for glioma.

Introduction

Glioma is the most frequent brain tumor disease in adults, accounting for 40.49% of intracranial tumors [1]. The average life span of glioma patients is not more than five years from the beginning of diagnosis [2]. Glioma is a malignant disease with high mortality and disability. The pathogenesis of glioma should be explored at the level of molecular biology to diagnose and treat glioma [3]. At present, the diagnosis and treatment of glioma are in the stage of continuous improvement. However, the survival rate of glioma patients has not been significantly improved [4,5]. Once diagnosed, most of the patients are in the interim and end-stage, and the survival rate after an operation is not optimistic. Therefore, it is urgent to search for prognostic markers of glioma to analyze the prognosis of patients, improve the postoperative quality of life of glioma patients, and select reasonable follow-up treatment to improve the survival rate of patients [6].

Family with sequence similarity 60 member A (FAM60A) is a new subunit of the Sin3 deacetylase complex [7]. The mRNA level of

Abbreviations: FAM60A, Family with sequence similarity 60 member A; PI3K, Phosphoinositide 3-kinase; PtdIns, Phosphatidylinositol; PKB, protein kinase B; WHO, the World Health Organization; HEB, human normal glial cell; FBS, fetal bovine serum; qRT-PCR, quantitative real time-polymerase chain reaction; NC, nitrocellulose; IHC, immunohistochemical; TCGA, the Cancer Genome Atlas; OS, overall survival; DFS, disease-free survival; HDAC, histone deacetylase complex; MAPK, mitogen-activated protein kinase.

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https://doi.org/10.1016/j.tranon.2021.101196
Received 22 June 2021; Received in revised form 2 August 2021; Accepted 5 August 2021

FAM60A increases in rapidly proliferating stem cells and decreases during differentiation as proliferation slows down, indicating that FAM60A is crucial in the proliferation of poorly differentiated cells [8]. FAM60A silenced can inhibit the growth, migration, and invasion of esophageal cancer tumor cells *in vitro*, suggesting that FAM60A may be a therapeutic target and biomarker for esophageal cancer [9]. However, there is no information on whether FAM60A is implicated in glioma cell proliferation. Therefore, it is meaningful to explore the role of FAM60A in the proliferation potential of glioma cells and its specific mechanism.

PI3K-Akt-mTOR is important in cancer among three major signaling pathways [10]. Phosphoinositide 3-kinase (PI3K) is a family of related intracellular signal transducer enzymes which can phosphorylate the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) [11]. Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes. mTOR is a serine/threonine-protein kinase downstream of PI3K/Akt, regulating cellular processes, and protein synthesis and transcription [12,13]. PI3K-Akt-mTOR inhibits the activity of pro-apoptotic members through phosphorylation while activating anti-apoptotic members [14]. The PI3K-Akt-mTOR signaling pathway is crucial in glioma stem cell self-renewal and resistance to chemotherapy or radiotherapy, which is believed to be the root of treatment failure, glioma recurrence, and metastasis [15]. Cancer cells can enhance endogenous repair and regenerative processes by releasing paracrine factors, including secretome, miRNAs, and exosomes [16]. Exosomes are a class of membrane-bound vesicles with diameters from 30 to 100 nm, deriving from endosomes and containing various proteins, lipids, and RNAs from origin cells [17].

In this study, FAM60A expression was significantly up-regulated in glioma tissues and cell lines and positively associated with a worse outcome in glioma. A series of experiments were performed to explore the function of FAM60A in glioma progression. Loss of FAM60A could inhibit glioma cell proliferation and tumorigenicity *in vitro* and *in vivo*. Then the potential regulatory mechanisms of FAM60A in glioma were explored. Loss of FAM60A regulated glioma cell proliferation with suppression of PI3K/Akt/mTOR signaling pathways. Besides, FAM60A expression was detectable in extracted serum exosomes with a higher expression in the glioma cancer patients group than in the normal group. Therefore, FAM60A is a prognostic biomarker and therapeutic target for glioma.

Methods

The current study has been submitted to and approved by the Ethic Committee for Clinical Research of the First Affiliated Hospital of Wannan Medical College (IRB file No. 2018-03-07). All participants were given written informed consent. The experimental research on xenografted tumor model has been approved by the Ethic Committee for Clinical Research of the First Affiliated Hospital of Wannan Medical College (IRB file No. LLSC-2020-095) and reporting of these experiments complied with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. These studies were conducted in accordance with the Declaration of Helsinki (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/).

Human tissue samples

Fifty pairs of adjacent non-tumor tissues and glioma samples were collected from the Department of Neurosurgery of The First Affiliated Hospital, Wannan Medical College (Wuhu, Anhui, PR China) from March 2018 to February 2020. Two pathologists evaluated all specimens according to the World Health Organization (WHO) guidelines. All tissues were received at surgery and directly preserved in liquid nitrogen. No local or systemic treatments were administered to these patients before surgery. The survey and experiments have obtained patients' consent and been approved by the Ethic Committee for Clinical Research of the First Affiliated Hospital of Wannan Medical College. The study was conducted in accordance with the Declaration of Helsinki (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/).

Cell culture

Human normal glial cell (HEB) and glioma cell lines (LN382, U251, U87MG and A172) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The cell lines were characterized by DNA fingerprinting, cell vitality detection, isozyme detection, and mycoplasma detection. The last cell characterization was performed on September 2020. The five cell lines were cultured in high-glucose Dulbecco's Modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco, Bethesda, MD, USA) and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin). All the cells were incubated in 5% CO_2 at 37°C.

Cell transfection

The lentivirus constructing of FAM60A knockdown was obtained from Genepharma (Shanghai, China). Glioma cell lines were plated in 6 wells dishes at 50% confluence and infected with FAM60A knockdown lentivirus (termed as shFAM60A), or a scramble control (termed as shCtrl) in U251 and LN382 cells, respectively. Pools of stable transductions were generated by selection using puromycin (4μg/ml) for 2 weeks. The above protocol of cell transfection according to the manufacturer's instructions. The cells were cultured after transfection and harvested for the following experiments.

Quantitative real time-polymerase chain reaction (qRT-PCR)

Quantitative real time-polymerase chain reaction was performed as previously described [18]. In brief, the RNAs were extracted from cells or tissues with TRIzol reagent (Invitrogen, Burlington, ON, Canada), 0.4 μg RNA was adopted to synthesize cDNA using a first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The RNA concentration was examined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR analysis was performed using the CFX-96 (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. PCR amplification was conducted as follows: initial denaturation at 95°C for 30s, 40 cycles of 95°C for 22s 60°C for 30s. GAPDH were used as controls. The following primer sequences were used: primers for FAM60A, forward primer 5′-CTCCAGTTCTCGATTCACTGAC-3′ and reverse primer 5′-CGAGTCTCATGCAATCCAAAACA-3′; primers for GAPDH forward primer 5′-CCAGGTGGTCTCCTCTGA-3′ and reverse primer 5′-GCTGTAGCCAAATCGTTGT-3′. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method. Each sample was repeated in triplicate and analyzed using the Relative Quantification Software.

Western blot assay

Western blot assay was performed as previously reported [18]. In brief, the proteins from cells or tissues were exacted with RIPA lysis buffer (Sigma-Aldrich, St Louis, MO). The concentration of proteins was measured with a BCA assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The 10% SDS-PAGE isolated protein was transferred to a 0.22μm nitrocellulose (NC) membrane (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked at room temperature with 5% non-fat milk for 2h. Then, the membranes were incubated in specific primary antibodies including FAM60A (1:1000, ab167180, Abcam, Cambridge, UK), PI3K (1:1000, #4249, Cell Signaling Technology, Danvers, MA, USA), Akt(1:1000, #2920, Cell Signaling Technology, Danvers, MA, USA), mTOR(1:1000, #2983, Cell Signaling Technology, Danvers, MA, USA), p-Akt(1:1000, #4060, Cell Signaling Technology, Danvers, MA,

USA), p-mTOR(1:1000, #5536, Cell Signaling Technology, Danvers, MA, USA), HDAC1(1:1000, #2062, Cell Signaling Technology, Danvers, MA, USA), Alix(1:1000, #2171, Cell Signaling Technology, Danvers, MA, USA), HSP70(1:1000, #4876, Cell Signaling Technology, Danvers, MA, USA), GM130(1:1000, #553612480, Cell Signaling Technology, Danvers, MA, USA), Flotillin-1(1:1000, #18634, Cell Signaling Technology, Danvers, MA, USA), EpCAM(1:1000, #5532626, Cell Signaling Technology, Danvers, MA, USA), p-PI3K (1:1000, ab182651, Abcam, Cambridge, UK) and β-Actin (1:3000, A1978, Sigma, Victoria, BC, Canada) at 4°C overnight. The membranes were washed with 0.1% TBST three times for 5min, and then incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 2h, and washed with 0.1% TBST three times for 5min each. Chemiluminescent ECL Plus reagents (Pierce, USA) were added to visualize the reaction products. The membranes were scanned with Tanon 5200 (Tanon, Shanghai, PR China). The band intensity was measured by densitometry using the Quantity One Software (Tanon, Shanghai, PR China). The protein levels were normalized with that of β-actin. All experiments were repeated in triplicate, and the representative results were shown.

Immunohistochemical (IHC) staining

Paraffin-embedded adjacent non-cancerous tissues or tumor tissues were cut into 5μm-thick slides for immunohistochemical analysis of FAM60A protein. After baking for 2h at 60°C, the tissues were immersed in xylene for deparaffinization and ethanol of decreased concentrations for rehydration. Then, the slides were incubated with the FAM60A primary antibodies (1:100, ab247130, Abcam, Cambridge, UK) for 12h at 4°C. The next day, the slides were washed with PBS and incubated with biotinylated secondary antibody for 2h at room temperature. Finally, slides were treated with ImmunoPure Metal enhanced DAB substrate kit (Pierce, Rockford IL) according to the instructions of manufacturers.

MTT assay

U251 and LN382 cells from each group were collected and inoculated into 96-well plates, respectively. After 48h of incubation, 50μL MTT solution (Sigma, 5mg/mL) was added to each well. Following incubation in the incubator for 3h and subsequent absorption of the supernatant, 150μL DMSO was added and shook evenly in a platform bed. At 490nM wavelength, the absorbance value of each well was measured by Microplate Reader. Six replicates were set up in each group, and the mean value was taken.

Colony formation assay

U251 and LN382 cells after transfection were added into 6-well plates and maintained in a medium under standard conditions. Plates were then rinsed via ice-cold phosphate buffer saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) after incubation at 37°C for two weeks. Subsequently, 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) was used to fix colony cells for 15 min, followed by 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) staining at room temperature. The number of colony cells was imaged and counted under an optical microscope (Nikon, Japan).

Transwell assay

Cell migration and invasion were performed with Transwell chambers (BD Biosciences, USA). After 48h of corresponding treatment, U251 and LN382 cells in each group were inoculated into the upper chamber of the Transwell chamber at the density of 4×10^4 cells/well (100μL culture medium containing 5% fetal bovine serum). Besides, 500μL culture medium containing 10% fetal bovine serum was added to the 24-well culture plate of the lower chamber. After 24h of routine culture, the chamber was removed, and cells from the upper layer of microporous membranes were wiped with cotton swabs. After that, cells were immobilized in 4% paraformaldehyde solution for 10min at room temperature and stained with 0.5% crystal violet solution (Sigma-Aldrich; Merck KGaA) for 15min. In the last step, 5 visual fields were randomly selected and observed under an optical microscope (Nikon, Japan) to count the number of cells invading the sub-layer of the microporous membranes of the chamber.

Wound healing assay

Cell migration was observed using a wound-healing assay. When the transfected U251 and LN382 cells were maintained in a 6-well plate, achieving 90–95% confluence, scratches were generated using the micropipette tips. The wound state was observed at 0 and 72 h after scratching with an X71 inverted microscope (Olympus, Tokyo, Japan). Wound widths in the images were measured and percentage of wound size was calculated as follows: [wound width at 72h/wound width at 0h] × 100%.

Xenografted tumor model

Four to six weeks old (average weight: 15 g) BALB/c nude mice (male/female ratio 1:1) were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science, and maintained under specific pathogen-free conditions. No statistical method was applied for the sample size estimation for the animal study. Each experimental group had enrolled 5 nude mice in an unrandomized manner to ensure the precision of the results. The experimental protocol was approved by the Wannan Medical College Animal Experimental Ethics Committee and reporting of these experiments complied with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. The cells (2×10^6 U251-shFAM60A cells and U251-shCtrl cells) were injected subcutaneously into the right dorsal flank. The tumor sizes were measured using a Vernier caliper every day when the tumors were readily visualized. Tumor formation in nude mice was observed by measuring the tumor volume calculated by the following formula: volume = (length × widthˆ2)/2. On day 28, animals were euthanized, and tumors were excised and weighed. The exclusion criteria of animal experiments are that the bodyweight of the mouse was statistically significantly changed compared to the others. The xenograft tumor was festered seriously and influenced the measurement of tumor volume.

Rescue experiments

The rescue experiment of histone acetylase inhibitor CPTH2 (cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl)hydrazine, #S1242, Selleck Chemicals, Houston, TX, USA) for silenced FAM60A in U251 and LN382 cells was carried out. U251 and LN382 cells were first stably transfected with negative control shRNA or FAM60A shRNA, and then used CPTH2 (50 nM) to inhibit the expression of acetylase in the FAM60A-silenced U251 and LN382 cells, which were divided into 3 groups, including negative control shRNA, FAM60A shRNA and FAM60A shRNA/ CPTH2 groups. The following assay were performed by western blot.

Exosome isolation

Exosomes were obtained from the serum by using ExoQuick precipitation solution (System Biosciences, Mountain View, CA, USA) according to manufacturer's instructions. Briefly, 250μL of serum were mixed with 63μL of ExoQuick solution and incubated overnight at 4°C. After centrifugation at 1500 × g for 30 min, the pellets were suspended in 50μL PBS and filtrated through a 0.22μm filter (EMD Millipore, Billerica,MA, USA). The isolated exosomes were stored at −80°C until use.

Transmission electron microscopy

Twenty microliters of the prepared exosomes were pipetted onto formvar carbon-coated copper grids and allowed to adsorb for 10 min before excess fluid was drained. The adsorbed exosomes were then negatively stained with 2% (w/v) phospphotungstic acid (pH 6.8)for 5min and was air-dried under an electric incandescent lamp, and analyzed with a transmission electron microscope (FEI Tecnai 12, Philips), bar = 200 nm.

Size analyses of exosomes

The isolated exosomes were diluted in PBS and were analyzed using a NanoSight LM 10-HSBFT 14 Instrument (NanoSight, Malvern, UK) according to the manufacturer's protocol. The size of exosomes were then analyzed by using the Nanoparticle Tracking Analysis 2.0 (NTA 2.0) software.

Bioinformatics analysis

The expression of FAM60A and glioma clinical data were downloaded from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga/) and Chinese Glioma Genome Atlas (CGGA) database (http://www.cgga.org.cn/index.jsp). Cut-off values were determined using mean expression level of FAM60A. The expression of FAM60A and glioma clinical data is summarized in Supplemental Tables 1, 2.

Statistical analysis

All data from three independent experiments were expressed as mean ± SD. Statistical analyses using SPSS 19.0 software (SPSS Inc., USA). Student's t-test was used to examine the differences between two groups. And one-way analysis of variance (ANOVA) were used to examine the differences multiple groups. Overall survival and disease-free survival were analyzed using Kaplan-Meier estimator and tested via the log-rank. Statistics were regarded significant when $P < 0.05$.

Results

FAM60A expression is highly up-regulated in human glioma cells and tissues

The Cancer Genome Atlas (TCGA) database shows that the FAM60A levels are significantly up-regulated in 156 glioma patients than 5 normal brain tissues ($P < 0.05$) (Fig. 1A). The FAM60A expression in a series of glioma cell lines and the normal glial cell HEB was measured by real-time PCR and western blot analyses. FAM60A is strikingly up-regulated in cell lines (LN382, U251, U87MG, and A172) compared to normal glial cell HEB ($P < 0.05$) (Fig. 1B, C). Furthermore, IHC and western blot analyses reveal that the expression levels of FAM60A in five pairs of human glioma tissues have higher FAM60A expressions than matched adjacent noncancerous tissues (Fig. 1D, E). The results indicate that FAM60A expression is up-regulated in glioma.

FAM60A upregulation is positively correlated with poor clinical outcomes of glioma

The low-grade (WHO I/II) and high-grade (WHO III /Ⅳ) glioma groups were analyzed using real-time PCR, IHC, and western blot analyses to investigate the FAM60A mRNA and protein expression levels in different-grade glioma tissues. The results demonstrate higher FAM60A expression in high-grade (WHO III /Ⅳ) gliomas compared to low-grade (WHO I/II) gliomas (Fig. 2A–C). Subsequently, Kaplan–Meier survival analysis of cohort of glioma patients from our laboratory with the log-rank test was performed. Log-rank and Kaplan–Meier analysis tests reveal that different WHO grade glioma patients group with higher FAM60A expression have poorer overall survival (OS) and disease-free survival (DFS) rates than groups with lower FAM60A expression ($P < 0.05$; Fig. 2D–G). Similar OS results were obtained in all WHO grade glioma patients from CGGA and TCGA database ($P < 0.05$; Supplemental Fig. 1A–lH). Collectively, FAM60A expression is a valuable prognostic marker generally for glioma patients for patients with major pathologic types of glioma specifically.

Knockdown of FAM60A inhibits the proliferation of glioma cells in vitro

To evaluate the function role of FAM60A in glioma cells, we stably knocked down the expression of FAM60A by two CPVL-specific

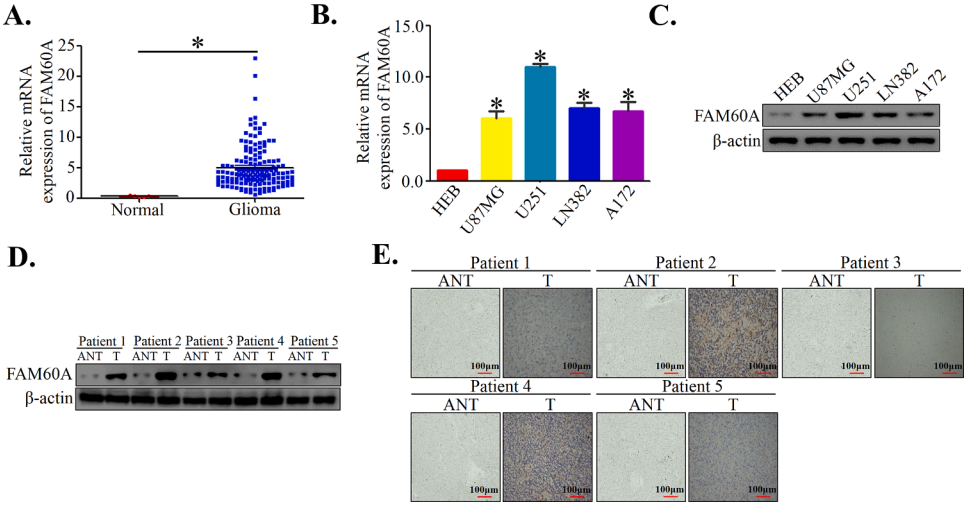

Fig. 1. FAM60A expression is highly up-regulated in human glioma cells and tissues. (A) Relative FAM60A mRNA expression in normal brain specimens (n=5) and glioma specimens (n=156) from TCGA. Bar graph data are presented as mean ± SEM; *, $P < 0.05$ (t-test). (B) Relative FAM60A mRNA expression in normal brain cell (HEB) and glioma cell lines (LN382, U87MG, U251, and A172) determined by real-time PCR. Experiments are conducted in triplicate. Bar graph data are presented as mean ± SEM; *, $P < 0.05$ (*t*-test). (C) Western blot analysis of FAM60A expression in normal brain cell (HEB) and glioma cell lines (LN382, U87MG, U251, and A172). β-actin is adopted as a loading control. (D) Western blot analysis of FAM60A expression in matched primary glioma tissues (T) and adjacent noncancerous tissues (ANT). The patients were clinically grade characterized (patients#1: GradeII; patients#2: Grade Ⅳ; patients#3: Grade I; patients#4: Grade Ⅳ; patients#5: Grade III). β-actin is adopted as a loading control. (E) IHC staining analysis of FAM60A protein expression in matched primary glioma tissues (T) and adjacent noncancerous tissues (ANT). The patients were clinically grade characterized (patients#1: GradeII; patients#2: Grade Ⅳ; patients#3: Grade I; patients#4: Grade Ⅳ; patients#5: Grade III). Scale bars, 100μm (200 × magnification).

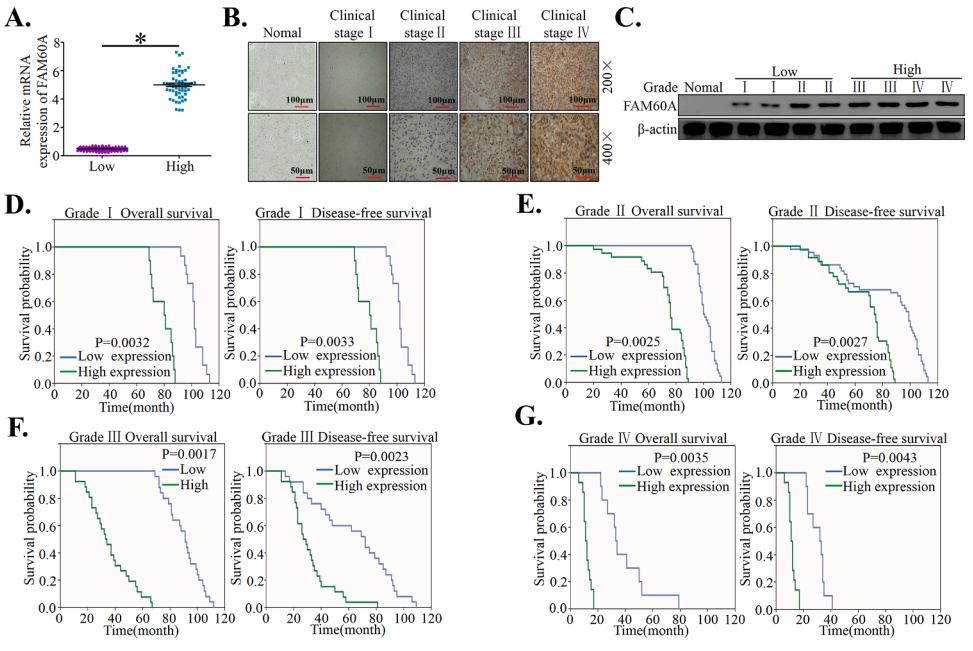

Fig. 2. FAM60A expression is positively correlated with poor clinical outcomes of glioma. (A) Relative FAM60A mRNA expression in glioma specimens of low clinical grades and high clinical grades from our laboratory determined by real-time PCR. Experiments are conducted in triplicate. Bar graph data are presented as mean ± SEM; *, P < 0.05 (*t*-test). (B) IHC staining analysis of FAM60A protein expression in normal brain tissues and glioma tissues of different clinical grades. Scale bars, 100 μm (200 × magnification) for upper panels and 50 μm for lower panels (400 × magnification). (C) Western blot analysis of FAM60A expression in glioma specimens of low clinical grades and high clinical grades. β-actin is adopted as a loading control. (D–G) Kaplan-Meier survival curves of overall survival (OS) and disease-free survival (DFS) with univariate analyses (log-rank) for different WHO grade glioma patients group (Grade I(D); GradeII(E); Grade III(F); Grade Ⅳ(G))with low FAM60A-expressing versus high FAM60A-expressing tumors from our laboratory. The log-rank test is adopted to compare differences between the two groups. P < 0.05 (*t*-test and ANOVA).

lentiviral shRNAs (shFAM60A #1 and shFAM60A #2) in U251 and LN382 cells. As shown in Fig. 3A–D, shFAM60A#2 exhibited the most evident knockdown efficiency and was chosen for the subsequent in *vivo* and mechanism experiment (Fig. 3A–D). FAM60A knockdown reduces the colony formation ability of the U251 and LN382 cells (Fig. 3E). Transwell assay shows that U251 and LN382 cell invasion is robustly reduced after the downregulation of FAM60A (P < 0.05) (Fig. 3F). FAM60A knockdown significantly inhibits U251 and LN382 cell proliferation using the MTT assay (P < 0.05) (Fig. 3G, H; Supplemental Fig. 2A, B). Moreover, the wound healing assay reveals that the knockdown of FAM60A remarkably suppresses U251 and LN382 cell migration capability (P < 0.05) (Fig. 3I, J). Therefore, the

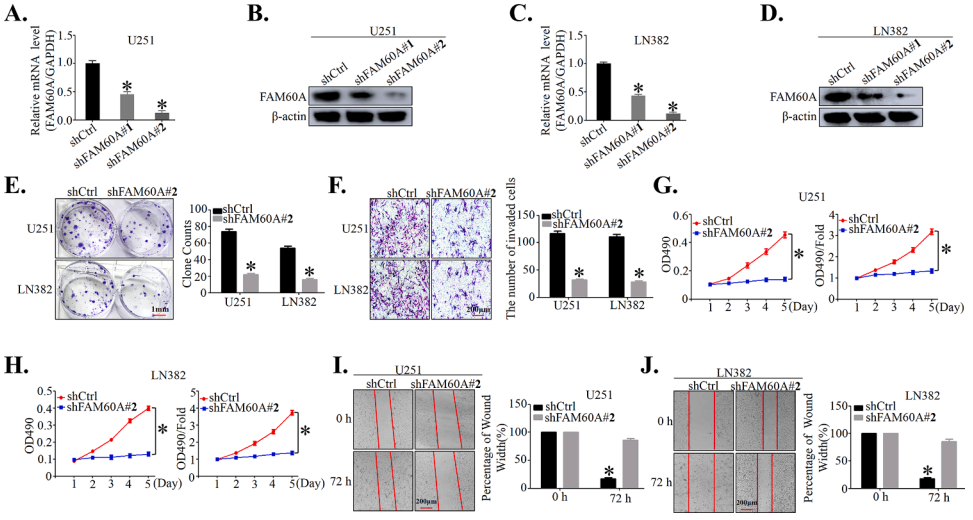

Fig. 3. Knockdown of FAM60A inhibits the proliferation of glioma cells *in vitro*. (A) Relative FAM60A mRNA expression in U251 cells expressing two FAM60A shRNA (shFAM60A#1, shFAM60A#2) determined by real-time PCR. Experiments are conducted in triplicate. Bar graph data are presented as the mean ± SEM; *, P < 0.05 (*t*-test). (B)Western blot analysis of FAM60A expression in U251 cells expressing two FAM60A shRNA (shFAM60A#1, shFAM60A#2). β-actin is adopted as a loading control. Experiments are conducted in triplicate. (C) Relative FAM60A mRNA expression in LN382 cells expressing two FAM60A shRNA (shFAM60A#1, shFAM60A#2) determined by real-time PCR. Experiments are conducted in triplicate. Bar graph data are presented as the mean ± SEM; *, P < 0.05 (*t*-test). (D) Western blot analysis of FAM60A expression in LN382 cells expressing two FAM60A shRNA (shFAM60A#1, shFAM60A#2). β-actin is adopted as a loading control. Experiments are conducted in triplicate. (E) Colony formation assay was used to investigate the proliferation capacity of the FAM60A-silenced U251 and FAM60A-silenced LN382 cells. Representative pictures are shown on the left, and the number of colonies was counted on the right. Experiments are performed in triplicate. Bar graph data are presented as mean ± SEM; *, P < 0.05 (*t*-test). Scale bars, 1mm (20 × magnification). (F) Transwell assay was used to investigate the invasion of the FAM60A-silenced U251 and FAM60A-silenced LN382 cells. Representative pictures are shown on the left, and the number of invaded cells was counted on the right. Experiments are performed in triplicate. Bar graph data are presented as mean ± SEM; *, P < 0.05 (*t*-test). Scale bars, 200 μm (100 × magnification). (G) MTT assays were used to investigate the proliferation rates in FAM60A-silenced U251 cells. Experiments are conducted in triplicate. Bar graph data are presented as the mean ± SEM; *, P < 0.05 (*t*-test). (H) MTT assays were used to investigate the proliferation rates in FAM60A-silenced LN382 cells. Experiments are conducted in triplicate. Bar graph data are presented as the mean ± SEM; *, P < 0.05 (*t*-test). (I) Wound healing assay is adopted to investigate the migration capacity of the FAM60A-silenced U251 cells. Representative images of the wound healing assay are shown on the left, and the percentage of wound width (%) on the right. Experiments are performed in triplicate. Bar graph data are presented as mean ± SEM; *, P < 0.05 (*t*-test). Scale bars, 200 μm (100 × magnification). Wound healing assay is adopted to investigate the migration capacity of the FAM60A-silenced LN382 cells. Representative images of the wound healing assay are shown on the left, and the percentage of wound width (%) on the right. Experiments are performed in triplicate. Bar graph data are presented as mean ± SEM; *, P < 0.05 (*t*-test). Scale bars, 200 μm (100 × magnification).

abovementioned results suggest that knockdown of FAM60A can inhibit the proliferation of glioma cells *in vitro*.

Knockdown of FAM60A inhibits the tumorigenicity of glioma cells in vivo

Mouse xenograft models were established to determine whether FAM60A could affect tumorigenesis *in vivo*. The shRNA or FAM60A shRNA U251 cells were injected at flank into nude mice. The tumor volume of the FAM60A knockdown group decreases to approximately one-fourth of the control group, and the tumor weight of the FAM60A knockdown group decreases to approximately one-fifth of the control group (Fig. 4A–C). Therefore, FAM60A is crucial in promoting glioma tumorigenesis.

FAM60A regulates glioma cell proliferation via the PI3K/Akt/mTOR signaling pathways

U251 and LN382 cells were transfected with FAM60A shRNA. Signaling pathways involved in tumor proliferation that FAM60A might activate were analyzed by examining the phosphorylated forms of PI3K, Akt, and mTOR using western blot assay to uncover the molecular mechanism mediating the cell proliferation effect of FAM60A. FAM60A knockdown significantly decreases the expression of HDAC1 and the phosphorylation of PI3K, Akt, and mTOR, whereas the total expression of PI3K, Akt, and mTOR does not change (Fig. 5A, B). The histone acetylase inhibitor (CPTH2) could rescue the diminished expression of HDAC1 and phosphorylation of PI3K, Akt, and mTOR (Fig. 5C, D). Therefore, loss of FAM60A attenuates glioma cell proliferation through suppression of PI3K/Akt/mTOR signaling pathways.

Serum exosomal FAM60A level is up-regulated in glioma cancer patients

Exosomes were extracted from 50 serum samples from glioma cancer patients and 50 serum samples from normal persons. Vesicles are characterized by several methods, such as electron microscopy (Fig. 6A) and western blot (Fig. 6B). The high presence of the exosomal markers Alix, HSP70, GM130, Flotillin-1, and EpCAM confirmed the purity of isolated glioma-secreted exosomes in the serum. The results show that FAM60A expression is detectable in extracted serum exosomes with higher expression in the glioma cancer group than in the normal group (Fig. 6C). Exosomal FAM60A in serum is stable and can serve as a promising biomarker for glioma cancer patients.

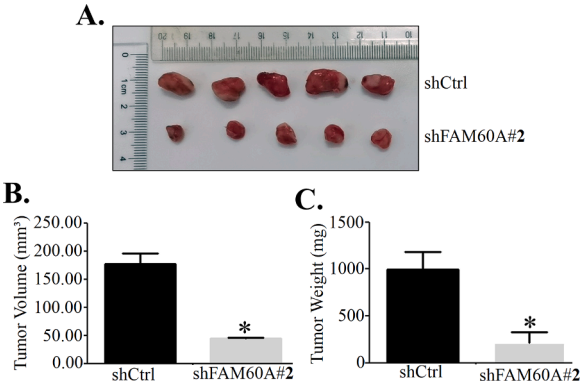

Fig. 4. Knockdown of FAM60A inhibits the tumorigenicity of glioma cells *in vivo*. (A) Macroscopic view of tumor harvested of two indicates treatment groups (n=5). (B) Comparison of the tumor volume of two indicated treatment groups at the end-point (n=5). Bar graph data are presented as mean ± SEM; *, $P < 0.05$ (*t*-test). (C) Comparison of the tumor weight of two indicates treatment groups at the end-point (n=5). Bar graph data are presented as mean ± SEM; *, $P < 0.05$ (*t*-test).

Discussion

As a vital element of the neuroma, glioma is a common malignant tumor in the central nervous system over the world [19]. However, it is still a great challenge for neurosurgery because the average survival time of glioma patients is less than one and a half years, and there is no valid treatment for this cancer [20]. Much progress has been made in the treatment of glioma. However, glioma has a strong invasion and resistance to chemotherapy and radiotherapy, so clinical treatment for glioma is difficult. Hence, the inner molecular mechanism should be explored further for the treatment of glioma.

This study provides new insights and strong evidence that FAM60A is crucial in the tumorigenicity and progression of glioma. FAM60A knockdown decreased the proliferation and tumorigenicity of glioma cells. The loss of FAM60A attenuates glioma cell proliferation by suppressing PI3K/Akt/mTOR signaling pathways.

FAM60A has been described as a conserved metazoan-specific protein found from flies to humans and has a unique sequence that does not exhibit any sequence homology to other known proteins in the human proteome [7]. FAM60A is an essential core subunit of a variant Sin3a complex in ES cells required to promote rapid proliferation and prevent unscheduled differentiation [8,21]. FAM60A is required for proper embryogenesis due to its regulation of DNA methylation at specific gene promoters [13]. FAM60A is crucial in the production stabilities of recombinant CHO cell lines [22]. However, FAM60A has an N-terminal conserved GATA-like zinc finger motif, showing that it may bind to DNA in transcriptional regulation. Human FAM60A binds to promoters of TGF-1R and SMAD2 genes in cancer cells and functions as a repressor of genes in the TGF-beta signaling pathway [7]. FAM60A can bind to the promoter of cyclin D1. Upon depletion of FAM60A, elevated levels of cyclin D1 mRNA and protein were observed. Human FAM60A can act as a sub-unit of the SIN3-histone deacetylase complex (HDAC), crucial in transcriptional repression [7].

In this study, FAM60A expression levels and the clinical significance in glioma were evaluated with large numbers of clinical tissue samples. FAM60A mRNA and protein expression levels in 50 glioma tissue samples were detected by real-time PCR, western blot, and IHC, respectively. The results showed that aberrantly high expression levels of FAM60A were likely associated with adverse prognoses in glioma. FAM60A expression in 4 glioma cell lines was detected and a FAM60A lentiviral vector was constructed to stably knock down FAM60A to clarify the biological behavior of FAM60A in glioma with which stable transduction of U251 and LN382 cell lines was conducted. Results demonstrated that FAM60A knockdown could suppress the proliferation of U251 and LN382 *in vitro* and *in vivo*. However, the mechanisms of FAM60A in cell proliferation in glioma cells have not been studied until now. In this study, FAM60A knockdown could inhibit the expression of HDAC1 and the phosphorylation of PI3K, Akt, and mTOR, whereas the total of PI3K, Akt, and mTOR was not changed. We used CPTH2, a histone acetylase inhibitor to inhibit the expression of acetylation in the CPVL-silenced U215 and LN382cells. The results revealed that CPTH2 could rescue the diminished expression of HDAC1 and phosphorylation of PI3K, Akt, and mTOR.

Aberrations in various cellular signaling pathways can regulate cellular metabolism, tumor development, growth, proliferation, metastasis, and cytoskeletal reorganization. The fundamental cellular signaling cascade involved in processes, including the PI3K/Akt/mTOR signaling pathways, are closely related to the mitogen-activated protein kinase (MAPK) pathway. It is a crucial and intensively explored intracellular signaling pathway in tumorigenesis. Various activating mutations in oncogenes and the inactivation of tumor suppressor genes are found in diverse malignancies across almost all pathway members [23]. The PI3K/Akt/mTOR signaling pathways is serving as a target for cancer therapy [14,15]. Exosomes are emerging as local and systemic cell-to-cell mediators of oncogenic information and are crucial in cancer progression through horizontal transfer of various bioactive molecules,

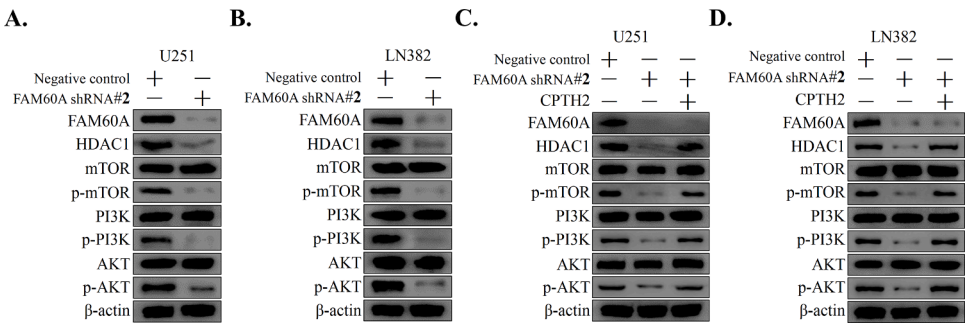

Fig. 5. FAM60A regulates glioma cell proliferation through the PI3K/Akt/mTOR signaling pathways. (A) Western blot analysis of the PI3K/Akt/mTOR signaling pathway downstream response genes and HDAC1 protein expression in FAM60A-silenced U251 cells. β-actin is adopted as a loading control. (B) Western blot analysis of the PI3K/Akt/mTOR signaling pathway downstream response genes and HDAC1 protein expression in FAM60A-silenced LN382 cells. β-actin is adopted as a loading control. (C) Western blot analysis of the PI3K/Akt/mTOR signaling pathway downstream response genes and HDAC1 protein expression in CPTH2 treatment on FAM60A-silenced U251 cells. β-actin is adopted as a loading control. (D) Western blot analysis of the PI3K/Akt/mTOR signaling pathway downstream response genes and HDAC1 protein expression in CPTH2 treatment on FAM60A-silenced LN382 cells. β-actin is adopted as a loading control.

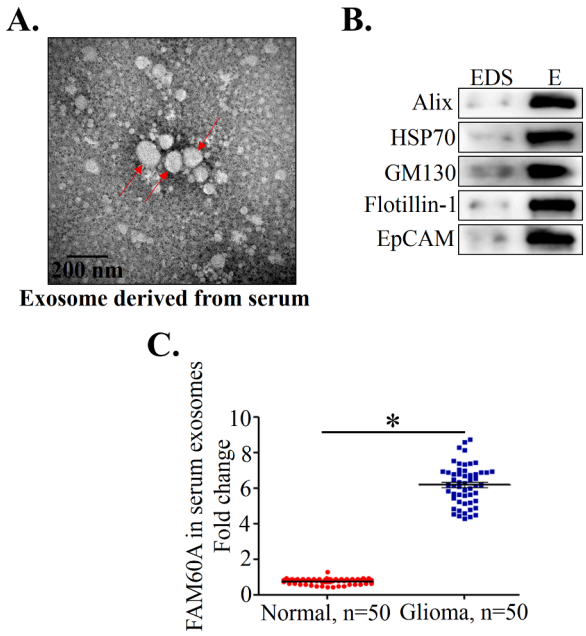

Fig. 6. Serum exosomal FAM60A level is upregulated in glioma cancer patients. (A) Representative images of the exosome (indicated by red arrows) are derived from the sera of glioma cancer patients, detected from an electron microscope. (B) The exosome markers from purified serum exosomes are analyzed by western blotting in exosomes (Es) and exosome-depleted supernatant (EDS). (C) qRT-PCR for the abundance of FAM60A in serum exosomes. The levels of FAM60A in extracted serum exosomes from the glioma cancer patients group are significantly higher than in the normal group. Experiments are conducted in triplicate. Bar graph data are presented as mean ± SEM; *, $P < 0.05$ (*t*-test) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

such as proteins and mRNAs [24]. Exosomes are secreted from multiple cells and participate in intercellular communication by transmitting intracellular cargoes [25]. Proteins can be protected from degradation in the circulation by exosomes and transferred between cancer cells, transmitting signals and phenotypes through exosomes [26,27]. However, the functions of exosomal proteins derived from glioma cells are still unknown. FAM60A expression is detectable in extracted serum exosomes with a higher expression in the glioma cancer patients group than in the normal group.

In conclusion, the current study demonstrated that FAM60A is highly elevated in glioma patient samples and cell lines, and FAM60A expression is positively correlated with poor clinical outcomes of glioma patients. Loss of FAM60A attenuates glioma cell proliferation through PI3K/Akt/mTOR signaling pathways. FAM60A is detected in glioma tissues and exosomes, suggesting its potential use as a biomarker and promising therapeutic target of glioma. Full understanding of the exact role of FAM60A in human glioma may provide an opportunity to develop a novel therapeutic strategy by inhibiting its expression in glioma cells. Translational researches on the clinical application of FAM60A are required to produce a methodology and evaluate the molecular diagnostic application of FAM60A in glioma.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

CRediT authorship contribution statement

Xiaocen Liu: Investigation, Formal analysis, Writing – review & editing. **Mengying Zhang:** Investigation, Formal analysis, Writing – review & editing. **Xiaolong Zhu:** Investigation, Formal analysis, Writing – review & editing. **Yingying Wang:** Data curation, Formal analysis, Writing – review & editing. **Kun Lv:** Conceptualization, Visualization, Writing – review & editing. **Hui Yang:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Grant Nos. 81802503 to Hui Yang and 82072370 to Kun Lv), the Key Projects of Natural Science Research of Universities in Anhui Province (Grant No. KJ2020A0607 to Xiaocen Liu), and Peak Training Program for Scientific Research of Yijishan Hospital, Wannan Medical College (Grant No. GF2019G15 to Hui Yang).

Acknowledgments

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101196.

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