CELL CYCLE
2021, VOL. 20, NO. 19, 1935–1952
https://doi.org/10.1080/15384101.2021.1966962

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Up-regulation of miR-663a inhibits the cancer stem cell-like properties of glioma via repressing the KDM2A-mediated TGF-β/SMAD signaling pathway

Lei Wang[a,*], Bojuan Lang[a,*], Youdong Zhou[a,*], Jinyang Ma[a,b], and Keqi Hu[d]

[a]The First College of Clinical Medical Science, China Three Gorges University, Yichang, Hubei, China; [b]Department of Neurosurgery, Yichang Central People's Hospital, Yichang, Hubei, China; [c]Department of Pathology, Yichang Central People's Hospital, Yichang, Hubei, China; [d]Department of Neurosurgery, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangyang, Hubei, China

ABSTRACT

Emerging reports have shown that microRNAs (miRNAs) function as vital regulators in tumor development via modulating gene expression at the posttranscriptional level. Here, we explored the role and underlying mechanism of miR-663a in the proliferation, migration, invasion, and cancer stem cell-like (CSC) properties of glioma cells. Quantitative reverse transcription PCR (qRT-PCR) was implemented to detect miR-663a expression in glioblastoma tissues and the adjacent normal tissues. Additionally, gain- and loss-of-function assays of miR-633a were performed on U-251 MG cells or human primary glioblastoma cancer cells (pGBMC1). Cell proliferation, migration, invasion, CSC properties, and profiles of stem cell markers (including CD133, CD44) were examined by the MTT assay, Transwell assay, tumorsphere experiment, and Western blotting, respectively. The dual-luciferase reporter gene assay was performed to testify the targeted relationship between miR-663a and lysine demethylase 2A (KDM2A). The results showed that miR-663a was down-regulated in glioblastoma tissues and cells. Overexpressing miR-663a repressed the proliferation, migration, invasion, CSC properties of U-251 MG cells and pGBMC1, while miR-663a knockdown had the opposite effects. The *in-vivo* experiment confirmed that miR-663a repressed the growth of U-251 MG cells in nude mice. When cocultured with THP1 cells, U-251 MG cells gained enhanced proliferation, migration, invasion, and CSC properties. MiR-633a overexpression reversed THP1-mediated effects on U-251 MG cells, and reduced the "M2" polarization of THP1 cells. What's more, Mechanistically, KDM2A was targeted by miR-663a. KDM2A knockdown suppressed the progression and CSC properties of U-251 MG cells *in vitro*, and dampened TGF-β. Overall, those data revealed that up-regulating miR-663a reduced glioma progression by inhibiting the KDM2A-mediated TGF-β/Smad pathway.

ARTICLE HISTORY
Received 28 May 2021
Revised 1 August 2021
Accepted 7 August 2021

KEYWORDS
Glioma; KDM2A; macrophages; miR-663a; stemness; TGF-β

1. Introduction

Glioma is the most frequent primary intracranial tumor, making up 81% of all malignant cerebral tumors, with high mortality and morbidity [1]. Surgery, radiotherapy, and chemotherapy are the main methods in treating glioma. However, the recurrence rate of glioma remains high due to invasive growth, drug resistance, and extensive heterogeneity [2]. As the crucial part of the brain tumor microenvironment (TME), tumor-associated macrophages (TAMs) play an emerging role in tumor progression and the control of anti-tumor immune responses [3]. TAMs make up to one-third of the tumor mass, and they widely participate in tumor growth, invasion, angiogenesis, and chemoresistance [4,5,6]. In addition, glioma stem cell-like cells (GSCs) are highly invasive. The maintenance of GSCs requires specific changes in cells and a larger TME regarding signal transduction and metabolism [7]. However, the function of cerebral TME in GSC proliferation and invasion remains elusive.

MicroRNAs (miRNAs), a group of small non-coding RNAs which have less than 24 nucleotides, are involved in the occurrence, angiogenesis, invasion and apoptosis of glioma [8]. For example, miR-451 dampens the proliferation, and invasion of glioma cells *in vivo* and

CONTACT Keqi Hu keqidoc@163.com Department of Neurosurgery, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, No.136 Jingzhou Street, Xiangcheng Disreict, Xiangyang, Hubei 441021, China

*These authors contributed equally to this work

Supplemental data for this article can be accessed here

in vitro by targeting alcium binding protein 39 (CAB39), thereby inhibits the mTOR (mechanistic target of rapamycin kinase)/HIF-1α (hypoxia-inducible factor 1 subunit alpha)/ VEGF (vascular endothelial growth factor) signaling pathway [9]. In addition, miR-320a suppresses tumor invasion by targeting Aquaporin-4 (AQP4) [10]. MiR-663a, a miRNA, is dysregulated in various cancers and involved in cancer progression as a tumor suppressor. Zhang C et al. showed that miR-663a abates cell proliferation and invasion of hepatocellular carcinoma (HCC) *in vitro* and *in vivo* by regulating transforming growth factor beta 1 (TGF-β1) [11]. Besides, miR-663a impedes HCC cell proliferation by targeting high mobility group AT-hook 2 (HMGA2) [12]. However, little is known about the specific mechanism and effect of miR-663a on glioma cells.

Lysine demethylase 2A (KDM2A), also known as JHDM1A, FBXL11 or Ndy2, is the first demethylase identified to contain the JmjC domain and plays an essential role in regulating cell proliferation [13,14]. Previous studies have shown that KDM2A is abnormally expressed in a variety of cancers and affects tumor progression. For example, the expression of KDM2A is elevated in gastric cancer (GC) tissues. Overexpressed KDM2A promotes GC cell growth and movement by down-regulating programmed cell death 4 (PDCD4) [15]. In addition, KDM2A is upregulated in non-small cell lung cancer (NSCLC), and knocking down KDM2A represses cell proliferation and invasion [16]. Besides, KDM2A overexpression is related to poor survival in ovarian cancer patients, and knocking down KDM2A can promote tumor cell apoptosis and hamper proliferation by inhibiting the phosphatidylinositol 3-kinase, putative (PI3K)/ AKT serine/threonine kinase (AKT)/mTOR signaling pathway [17]. Furthermore, Kurt IC and his colleagues revealed that silence of KDM2B (also known as JHDM1B, FBXL10, or Ndy1), an analog of KDM2A, significantly enhances the sensitivity of tumor necrosis factor-associated apoptosis-inducing ligand (TRAIL) and accelerates glioma cell apoptosis [18]. Nevertheless, the role of KDM2A in glioma needs to be further probed.

In this study, we aimed to investigate the role of miR-663a in the development of glioma cells by targeting the KDM2A/TGF-β pathway. As the results showed, miR-663a was downregulated in glioblastoma tissues. Up-regulating miR-663a or down-regulating KDM2A could reduce the stemness and malignant behaviors of glioma cells both *in vivo* and *in vitro*. Further experiments indicated that the KDM2A-mediated TGF-β signaling pathway is involved in miR-663a-meidated anti-tumor effects, which is hopefully a new therapeutic target for glioma.

2. Materials and methods

2.1. Ethics statement

A total of 50 glioblastoma tissues and the paired adjacent normal tissues were collected from surgical specimens of patients from June 2016 to May 2018 in Xiangyang Central Hospital. All of those patients received surgical treatment in Xiangyang Central Hospital. The histopathological diagnosis, stage, and grade of glioblastoma were based on the 2016 WHO Classification of Tumors of the Central Nervous System. Overall survival (OS) was defined as the time from the initiation of surgery to death of any cause or the most recent follow-up. This study was approved by the Ethics Committee of the Xiangyang Central Hospital. Patients' data and samples were handled according to the ethical and legal standards adopted by the Declaration of Helsinki (2013). All patients agreed to get involved in this study and signed a letter of authorization.

2.2. Cell culture

Human monocyte cell line THP1 cells, human glial cell line HEB cells, human glioblastoma cell lines including U-251 MG, A172, LN18 cells were commercially provided by the Cell Center of the Chinese Academy of Sciences (Shanghai, China). They were cultured in the Dulbecco modified Eagle medium (DMEM)-F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (Invitrogen, CA, USA) in an incubator at 37 °C with 5% CO_2. DMEM-F12 and FBS were obtained from Thermo Fisher Scientific (MA,

USA). During the logarithmic growth phase, 0.25% trypsin (Thermo Fisher HyClone, Utah, USA) was used for trypsinization and passage.

2.3. Human primary glioblastoma isolation

Primary glioblastoma cell isolation was referred in a previous study [19]. Briefly, three fresh tumor samples (all diagnosed as astrocytoma, grade IV based on WHO Classification) were obtained from patients undergoing surgical treatment at the Xiangyang Central Hospital. The tissues were quickly washed by PBS, and got trypsinization at 37°C for 1 h. Centrifugation (at room temperature, 1000 rpm for 5 min) was used for removing the undigested tissues. Then the digested cells were cultured in the DMEM-F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, CA, USA). Next, the cells were cultured maintained at 37°C in a 5% CO2 incubator. After three times of passage, the primary glioblastoma cells (named as pGBMC1, pGBMC2, pGBMC3) was used for further experiments.

2.4. The co-culture model of THP1-glioma cell

The interaction model was established using a Transwell chamber (0.4 μm in diameter) composed of 6 wells. 2×10^5 U-251 MG cells were inoculated in the lower layer, and 6×10^5 mononuclear macrophages THP-1 were inoculated in the upper layer (ratio 1: 3). The co-culture model was then incubated at 37°C, and the culture medium was altered 24 hours later. After incubation for 72 hours, the upper inserts containing TAMs were discarded. The collected TAMs were cocultured with U-251 MG cells again according to the above method, and the lower chamber cells were harvested.

2.5. Cell transfection

Small inference RNA targeting KDM2A (Si-KDM2A), Si-RNA negative control (si-NC), miR-663a mimics, miR-663a inhibitors, and the negative control (NC) were obtained from GenePharma Co., Ltd. (Shanghai, China). THP1, U-251 MG, and pGBMC1 were inoculated into 24-well plates (3×10^5 cells/well) and cultured at 37°C with 5% CO_2 for 24 hours before cell transfection. Next, 10 μl Si-KDM2A or si-NC, miR-663a mimics, miR-663a inhibitors or the negative control (NC) (20 μmol/L) was mixed with 150 μl Opti-Mem medium (Gibco; Thermo Fisher Scientific, Inc.), then 5 μl Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added and mixed. After a 5-min incubation at room temperature, the mixture was subsequently added into the above cells in 24-well plates. Then the plates were incubated at 37°C for 24 h. Followed by that, the medium was replaced with DMEM-F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After another 24-hour culturing, the cells were collected for subsequent experiments. The transfection validity was detected by quantitative reverse transcription PCR (qRT-PCR).

2.6. Quantitative reverse transcription PCR (qRT-PCR)

The total RNA was isolated from tissues and cells with the TRIzol reagent (Invitrogen, Waltham, MA, USA) as requested. The RNA content and purity were determined by Nanodrop-spectrophotometer. The complementary DNA (cDNA) of mRNA was synthesized from 1 μg of total RNA using the PrimeScript-RT Kit (Madison, WI, USA). The cDNA of miRNAs was performed using All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, Maryland, USA). Then we employed SYBR®Premix-Ex-Taq™ (Takara, TX, USA) and the ABI7300 system to conduct qRT-PCR of KDM2A, IL-4, IL-10, CD206, CD133, and CD44. The miRNA Q-PCR Detection Kit was used for the qRT-PCR of miR-663a. The total volume of the PCR system was 30 μL, and each sample contained 300 ng cDNA. The amplification was initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 85°C for 20 seconds. All fluorescence data were converted into relative quantification, with U6 as the internal reference of miR-663a and GAPDH is the internal control of KDM2A, IL-4, IL-10, CD206, CD133, and CD44. qRT-PCR was done three times. The primer sequences are as Table 1.

Table 1. The primers in qRT-PCR.

Genes	Forward primers (5′-3′)	Reverse primers (5′-3′)
miR-663a	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
KDM2A	CCTCCTGGTAGCACTTTGGA	TGCCTCACTCAGTCACATGT
IL-4	TGCCTCCAAGAACACAACTG	CTCTGGTTGGCTTCCTTCAC
IL-10	AGCTGTGGCCAGCTTGTTAT	GTAGAGACGGGGTTTCACCA
CD206	GGCGGTGACCTCACAAGTAT	TTTTCATGGCTTGGTTCTCC
CD133	TTGTGGCAAATCACCAGGTA	TCAGATCTGTGAACGCCTTG
CD44	ACCATTTCAACCACACCACG	AGTTGCCTGGATTGTGCTTG
TGF-β1	CTTTCCTGCTTCTCATGGCC	TCCAGGCTCCAAATGTAGGG
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT
GAPDH	CAGGAGGCATTGCTGATGAT	GAAGGCTGGGGCTCATTT

2.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cells were seeded into 96-well plates (2000 cells per well), and cultured in DMEM-F12 medium for 24 hours. They were then treated with temozolomide (0–25 μg/mL, dissolved in dimethyl sulfoxide and diluted in DMEM-F12 medium) for 48 hours. Subsequently, 10 μL MTT solution (5 mg/mL) was added to each well and incubated with the cells at 37°C for 4 hours. The absorbance value was measured at 450 nm using a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). Each experiment was conducted three times.

2.8. Cell sphere culture

U-251 MG or pGBMC1 cell was cultured in DMEM-F12 added with of N2 and B27 (0.5×, Invitrogen, Carlsbad, CA, USA), bFGF and EGF (50 ng/mL, Biovision), glutamine (1×, Invitrogen, Carlsbad, CA, USA), and 1% penicillin/streptomycin for 2–3 days, and the first-generation cell spheres could be seen. After 4–5 days, the cell spheres grew well and were in the logarithmic growth stage. The cell spheres were collected, mechanically dissociated into single cells with a pipettor for 2 min. The cells were then screened with a 400-mesh filter, centrifuged at 1000 rpm for 3 min, harvested, and further cultured in non-serum DMEM-F12 medium.

2.9. Transwell assay

THP1 and U-251 MG cells/pGBMC1 were co-cultured for 1–3 days. Then, 2×10^5 U-251 MG or pGBMC1 cells were resuspended in 200 μL serum-free DMEM-F12 and added to corresponding upper inserts. DMEM-F12 (600 μL) containing 10% FBS was added to the lower chamber. Unmigrated cells were wiped off the membranes, and the cells migrated and attached to the lower chamber were fixed with 4% paraformaldehyde for crystal violet staining. Five high-powered representative fields of view were randomly selected to calculate the number of transmembrane cells, and the mean value of three multiple wells was taken to represent the migrative and invasive ability of tumor cells.

2.10. The dual-luciferase reporter assay

The potential targets of miR-663a were predicted through ENCORI (http://starbase.sysu.edu.cn/index.php). According to the predicted binding sites, the luciferase reporting vectors (KDM2A-WT, KDM2A-MUT, JUNB-WT, JUNB-MUT, TGF-β-WT, and TGF-β-MUT) were cloned and inserted into the 3′UTR of psiCHECK-2 plasmid (Promega, Madison, WI, USA). Among them, KDM2A-WT and TGF-β-WT contained the binding sites with miR-663a, while KDM2A-MUT and TGF-β-MUT were mutant of binding sites with miR-663a. pGBMC1 and U-251 MG (4.5×10^4) were inoculated into 48-well plates and cultured to 70% confluence. Then, they were co-transfected with KDM2A-WT, KDM2A-MUT, TGF-β-WT, TGF-β-MUT, and miR-663a mimics or negative controls using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). 48 hours after the transfection, the luciferase activity was measured 48 hours after the transfection. All experiments were repeated three times.

2.11. Western blotting

Cells were lysed with RIPA lysis buffer (Beyotime Biotech, China), and total proteins in tumor tissues, U-251 MG cells, and TAMs were isolated. Then, 50 μg total protein was running in 12% polyacrylamide gel for 100 V electrophoresis for 2 hours and transferred to polyvinylidene fluoride (PVDF) membranes. After 1 hour of blocking with 5% skimmed milk at room temperature, the membranes were rinsed in TBST three times (10 min

each time). Subsequently, the membranes were incubated with anti-CD133 (1: 1000, ab216323, Abcam, MA, USA), anti-CD44 (1: 1000, ab51037, Abcam), anti-iNOS (1: 1000, ab178945, Abcam), anti-CD86 (1: 1000, ab239075, Abcam), anti-KDM2A (1:1000, ab191387, Abcam), anti-TGF-β1 (1:1000, ab215715, Abcam), anti-CD80 (1:1000, ab134120, Abcam), anti-Arg1 (1:1000, ab203490, Abcam), anti-CD163 (1:1000, ab182422, Abcam), anti-Smad2 (1:1000, ab40855, Abcam), anti-p-Smad2 (1:1000, ab188334, Abcam), anti-Smad3 (1:1000, ab40854, Abcam), anti-p-Smad3 (1:1000, ab52903, Abcam), and anti-β-actin (1:1000, ab8227, Abcam) at 4°C overnight. After the membranes were washed with TBST, they were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody (concentration: 1:300) at room temperature for 1 hour. Nest, TBST was utilized to wash the membranes three times (10 min each). Finally, the Western Blot reagent (Invitrogen, US) was used for chromogenic imaging, using ImageJ 1.44 software for density determination.

2.12. Flow cytometry

The glioma cells including U-251 MG and pGBMC1 in logarithmic phase were digested with 0.25% trypsin, centrifuged, and re-suspended in cold PBS. Then the apoptosis of the cells was determined using the Annexin-V FITC Apoptosis Detection Kit (Cat.No. AB_2869082, BD Pharmingen™, CA, USA) according to the manufacturer's instruction. Then the cells were analyzed by flow cytometry (FACScan, BD Biosciences) and apoptotic fractions were investigated by CELL Quest 3.0 software.

2.13. Animal experiment

Sixty 6-week-old male BALB/c Nude mice weighing 18–20 g were purchased from the Animal Experimental Center of Wuhan University. Mice were caged and fed with food and water during a 12-hour light-dark cycle at 20 ~ 25 °C with 50 ~ 52% humidity. The Ethics Committee of Xiangyang Central Hospital approved this experiment, which was in line with the Guidelines of the National Institutes of Health on animal care and use.

2.14. Tumor xenograft model

Mice were anesthetized using 1% sodium pentobarbital solution (50 mg/kg) via intraperitoneal injection. U-251 MG or pGBMC1 cells were transfected with negative control (NC), miR-663a mimics (miR-663a) or miR-663a inhibitors (miR-663a-in). Then 0.1 mL U-251 MG (2×10^7 cells/mL) or pGBMC1 (2×10^7 cells/mL) cell suspension was subcutaneously injected into the armpit of nude mice (6-week-old male mice, n = 5 in each group). From the 3rd day of injection, the tumor size was measured by a Vernier calliper (tumor volume = length × width^2/2). After 4 weeks, the tumor was removed and weighted. The tumor tissues were fixed with 10% neutral-buffered formalin. Then histopathological examinations including hematoxylin and eosin (H&E) staining, IHC staining and immunofluorescence staining were performed. The KI67 Cell Proliferation Kit (IHC,cat #E607235-0100, Sangon Biotech (Shanghai) Co., Ltd.) was used for evaluating cell proliferation in the tumor tissues.

2.15. Immunofluorescence staining

For evaluating KDM2A expression in U-251 MG or pGBMC1 cells transfected with miR-663a mimics (miR-663a) or miR-663a inhibitors (miR-663a-in), the cells were fixed by 4% paraformaldehyde (at room temperature for 30 min) and washed with PBS for 3 times (5 min each time). Next, 0.5% Triton X-100 was used for permeabilizing the cells for 10 min at room temperature. The cells were blocked with 1% BSA at room temperature for 30 min, and then incubated with the primary antibody anti-KDM2A (Abcam, UK, #ab238945, 1:100), anti-CD11b (Abcam, UK, #ab1211, 1:100), and anti-CD206 (Abcam, UK, #ab125028, 1:100) at 4°C overnight. The next day, the cells were washed with PBS for 3 times (5 min each time) and then incubated with the fluorescent-labeled Cy3 secondary antibody (Beyotime, China, #A0516, 1:100) and fluorescent-labeled FITC secondary antibody (Beyotime, China, #A0568, 1:100) for 1 h at 37°C in the dark. Finally, 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) solution (Beyotime, Shanghai, China) was used for staining the nuclei.

The fluorescence imaging was observed on a fluorescence microscope (Olympus, 200× magnification).

For detecting microglia/macrophage polarization in the tumor tissues, tissue immunofluorescence staining was performed. The fresh glioblastoma tissues and the paired adjacent normal tissues were fixed by 4% paraformaldehyde and embedded in paraffin wax. Then 4-μm thick sections were prepared, dewaxed, permeabilized with 0.2% Triton, and incubated with 5% goat serum. Then the sections were incubated with the primary antibodies including anti-CD11b (Abcam, UK, #ab1211, 1:100) and anti-CD206 (Abcam, UK, #ab125028, 1:100) at 4°C overnight. The next day, the sections were washed with PBS for 3 times (5 min each time) and then incubated with the fluorescent-labeled Cy3 secondary antibody (Beyotime, China, #A0516, 1:100) and fluorescent-labeled FITC secondary antibody (Beyotime, China, #A0568, 1:100) for 1 h at 37°C in the dark. Finally, 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) solution (Beyotime, Shanghai, China) was used for staining the nuclei. The fluorescence imaging was observed on a fluorescence microscope (Olympus, 200× magnification).

2.16. Statistical analysis

SPSS17.0 (SPSS Inc., Chicago, Illinois, USA) was adopted for analysis. The data were presented as mean ± standard deviation (x ± s). One-way analysis of variance followed by Turkey's test was used for multi-factor comparison, and paired comparison was made by *t* test. The overall survival of glioblastoma patients with different level of miR-663a was evaluated using Kaplan-Meier survival curve. $P < 0.05$ was statistically significant.

3. Results

3.1. miR-663a was downregulated in glioblastoma tissues

A total of 50 glioblastoma tissues and the paired adjacent normal tissues were collected, and the miR-663a level was detected. As the data showed, miR-663a had a lower level in the glioblastoma tissues compared with that in the adjacent normal tissues (Figure 1(a)). Then the Kaplan-Meier survival curve suggested that lower level of miR-663a predicted poorer overall survival of glioblastoma patients (P = 0.041, Figure 1(b)). Interestingly, the miR-663a level was also downregulated in human glioblastoma cell lines including U-251 MG, A172, LN18 cells, primary glioblastoma cell (named as pGBMC1, pGBMC2, pGBMC3) compared with that in the human glial cell line HEB cells (Figure 1(c)). Next, we detected IL-4, IL-10, CD206, CD133 mRNA alterations in the above clinical samples. As the data showed, all of the four mRNAs were enhanced in the glioblastoma tissues compared with that in the adjacent normal tissues (Figure 1(d–g)). Next, tissue immunofluorescence staining was used for detecting microglia/macrophage polarization in the tumor tissues. It was found that $CD11b^{+}CD206^{+}$-labeled M2 polarized microglia/macrophages were significantly accumulated in the glioblastoma tissues compared with that in the adjacent normal tissues (Figure 1(h,i)). Consistent with our results, the expression of IL-4, IL-10, CD206, CD133 and ABCG2 was enhanced in the tumor tissues of Glioblastoma multiforme (GBM), and Brain Lower Grade Glioma (LGG) compared with those in the normal tissues (sup Figure (a–e)). Therefore, it's supposed that miR-663a and TAMs have potential effects on glioma development.

3.2. Overexpressing miR-663a inhibited the proliferation, migration and invasion of glioblastoma cells

miR-663a mimics, or miR-663a inhibitors were transfected into U-251-MG and pGBMC1 cells. qRT-PCR result illustrated that miR-663a was upregulated in both U-251 MG and pGBMC1 cells transfected with miR-663a mimics, but downregulated when miR-663a inhibitors were transfected (Figure 2(a,b)). We evaluated the proliferation, apoptosis, migration and invasion of the two cells via MTT assay, flow cytometry, and transwell assay, respectively. The data indicated that compared with NC group, miR-663a inhibited the proliferation, migration, invasion, and aggravated apoptosis of the two cells, while miR-663a-in exerted the opposite effects (Figure 2(c–g)). To

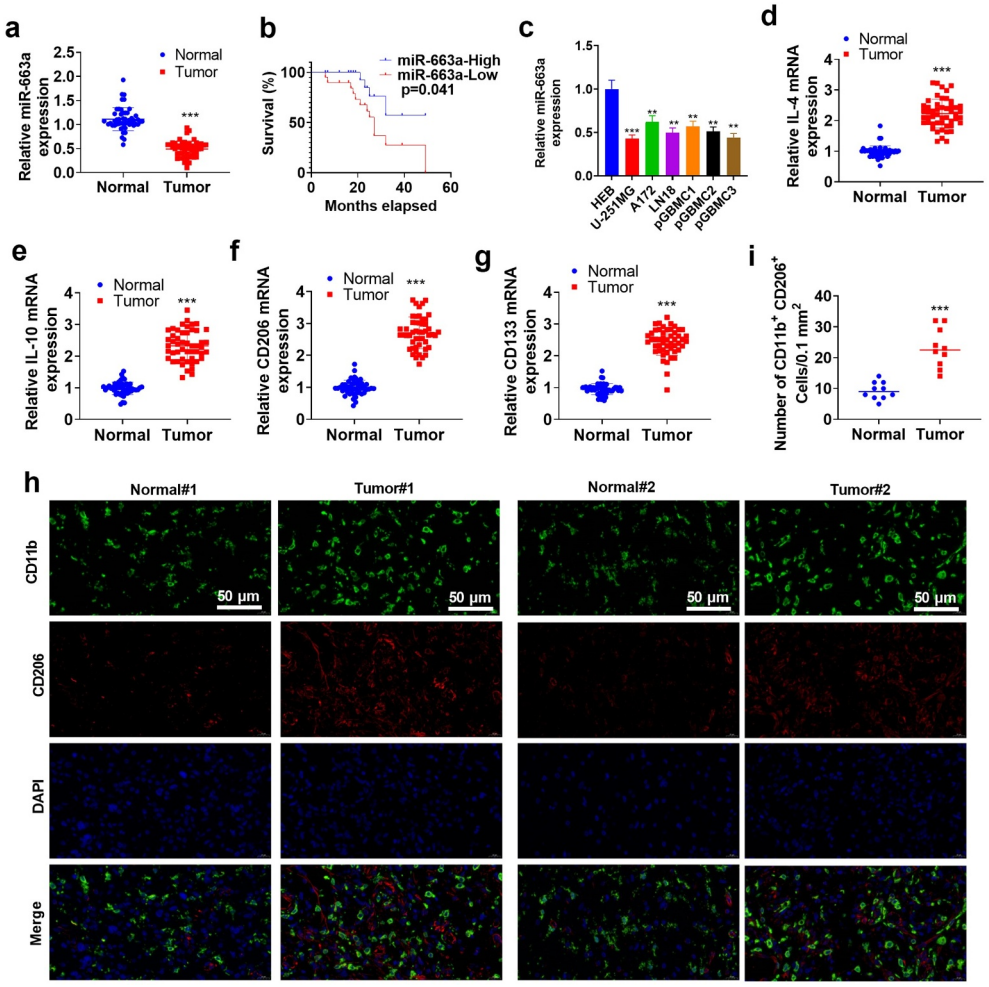

Figure 1. miR-663a was down-regulated in glioblastoma tissues and glioblastoma cells.

(a) Determination of miR-663a expression was made by qRT-PCR in 50 cases of glioblastoma tissues and adjacent normal tissues. (b) Kaplan-Meier curves were applied to analyze the relationship between the high and low expression levels of miR-663a and the overall survival (OS) rate of the glioblastoma patients. (c) The expression of miR-663a in human glioblastoma cell lines including U-251 MG, A172, LN18 cells, primary glioblastoma cell (named as pGBMC1, pGBMC2, pGBMC3) and human glial cell line HEB cells lines was analyzed by qRT-PCR. (d–g) The mRNA expression of IL-4 (d), IL-10 (e), CD206 (f) and CD133 (g) in glioblastoma tissues and adjacent normal tissues were monitored by qRT-PCR. (h–i) The number of $CD11b^{+}CD206^{+}$ cells in 10 pairs of glioblastoma tissues and adjacent normal tissues was determined Immunofluorescence staining (scale bar = 50 μM). The values shown represent the mean ± standard deviation (SD). All experiment were repeated for three times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs. HEB group).

verify the role of miR-663a *in vivo*, miR-663a mimics or miR-663a inhibitors were transfected into U-251-MG and pGBMC1 cells, which were then used for in vivo experiment. We found that up-regulation of miR-663a abated tumor volume and weight, while downregulation of miR-663a promoted tumor growth (compared with NC group, Figure 2(h–j)). Next, the results of Ki-67 staining showed that the miR-663a group had less Ki67-positive cell rate, while the miR-663a-in group had higher Ki67-positive cell rate (compared with NC group, Figure 2(k)). The above results confirmed that overexpressing miR-663a has inhibitive effects in glioblastoma cells.

3.3. miR-663a overexpression mitigated the CSC properties of glioblastoma cells

miR-663a mimics or miR-663a inhibitors were transfected into U-251-MG and pGBMC1 cells.

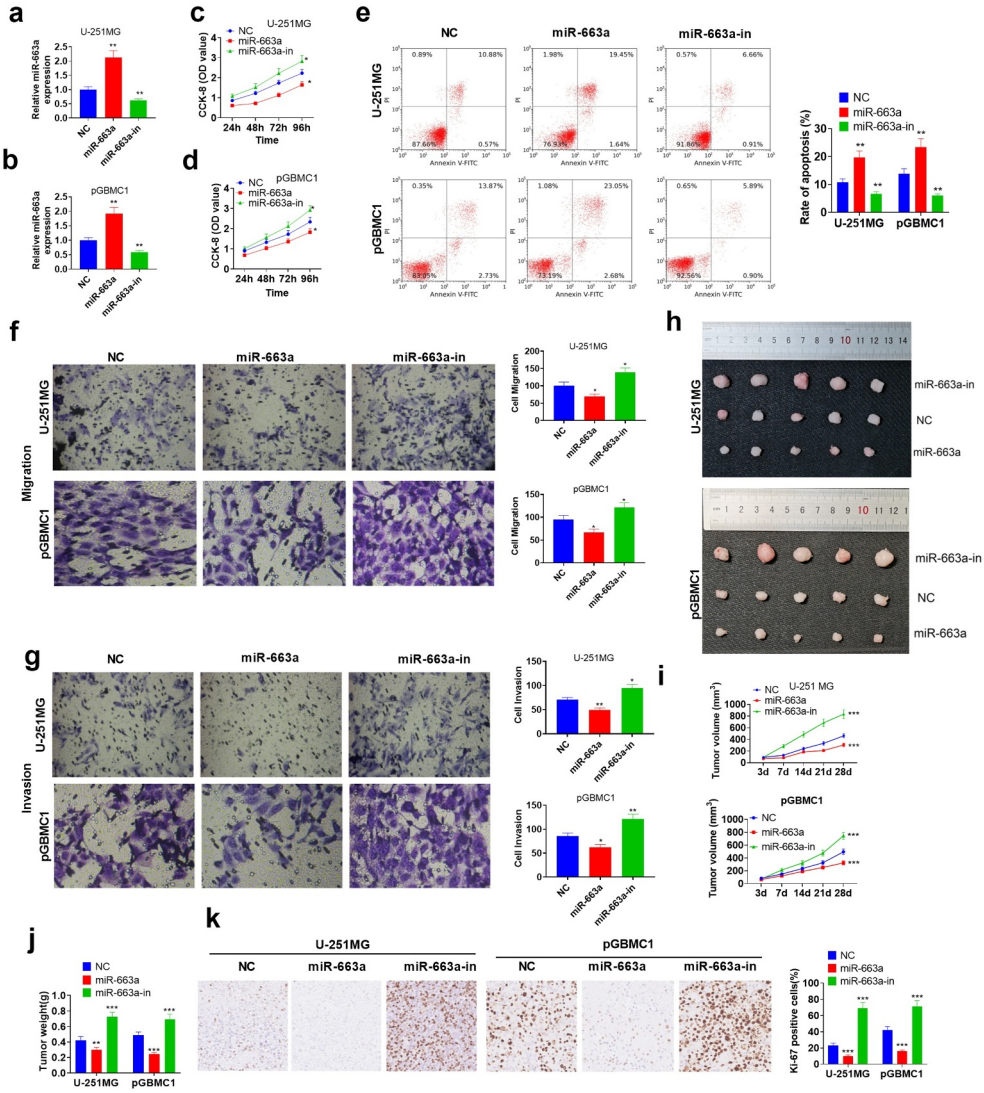

Figure 2. miR-663a suppressed the proliferation, migration and invasion of glioblastoma cells.

(a and b) The expression of miR-663a in U-251 MG and pGBMC1 cells after the transfection of miR-663a mimics or miR-663a inhibitors or negative control (NC) was compared by qRT-PCR. (c and d) MTT assay detected the proliferation of U-251 MG and pGBMC1 cells after transfection with miR-663a mimics or miR-663a inhibitors. (e) Flow cytometry was performed to evaluated the apoptosis of U-251 MG and pGBMC1 cells. (f–g) The migration and invasion of U-251 MG and PGBMC1 cells transfected with miR-663a mimics and miR-663a inhibitors were measured by Transwell assay. U-251 MG and pGBMC1 cells were transfected with miR-663a mimics or miR-663a inhibitors or negative control (NC) and used for *in-vivo* experiment. (h) Tumor images obtained 28 days after subcutaneous injection of tumor cells. (i) Tumor volumes at 3, 7, 14, 21, and 28 days. (j) The mice were sacrificed at the 28th day and the tumor weight was calculated. (k) Immunohistochemical staining of Ki-67 in the xenograft tumor nodules (magnification: ×200. Scale = 50 μM). the Ki-67 positive cell rate was calculated. The values shown represent the mean ± standard deviation (SD). All experiment were repeated for three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (vs. NC group).

The spheres of the two cells were evaluated by the cell spheres assay, and the results revealed that the sphere ability of the cells in the miR-663a group was reduced compared with that in the NC group, while that in the miR-663a-in group was promoted (Figure 3(a,b)). qRT-PCR and Western blotting were employed to compare the expression of stem cell markers (CD133, and CD44) in U-251 MG and pGBMC1 cells. The results illustrated that the expression of the above markers was down-regulated in the miR-663a group compared with that in the NC group. On the contrary, miR-663a-in enhanced CD133 and CD44 mRNA levels (Figure 3(c,d)). The protein levels of CD133 and

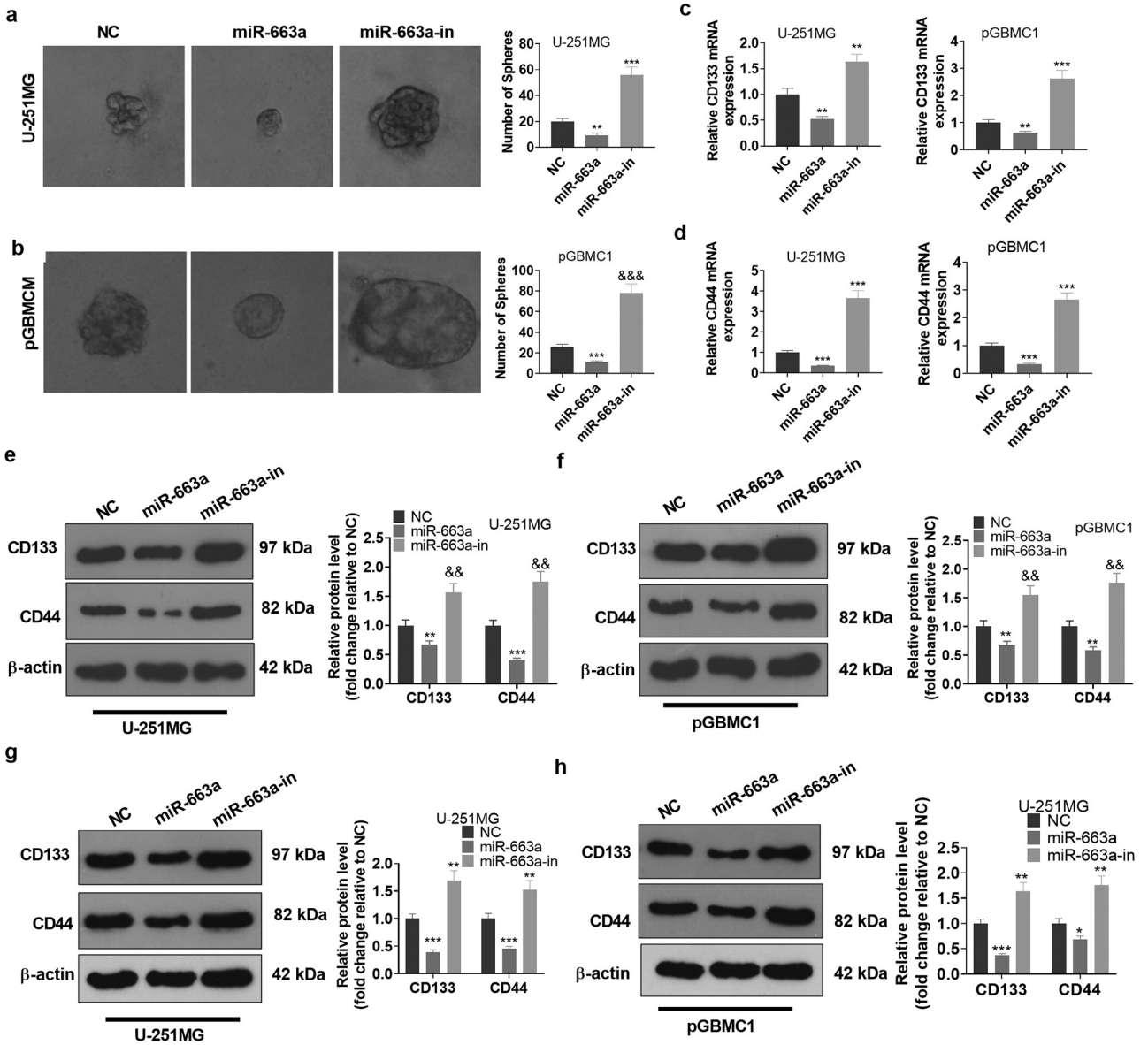

Figure 3. miR-663a attenuated the stem cell properties of glioblastoma.

U-251 MG and pGBMC1 cells were transfected with miR-663a mimics or miR-663a inhibitors or negative control (NC). U-251 MG and pGBMC1 cells were cultured in DMEM-F12 medium added with of N2 and B27 (0.5×), bFGF and EGF (50 ng/mL), glutamine (1×) for 3 days and them culture in non-serum DMEM-F12 medium. (a and b) Tumor sphere formation assay was used to determine the self-renewal ability of U-251 MG and pGBMC1 cells transfected with miR-663a mimics and miR-663a inhibitors. The number of spheres was counted. (c and d) The mRNA expression of CD133, and CD44 in U-251 MG and pGBMC1 cells after transfection of miR-663a mimics and miR-663a inhibitors was determined by qRT-PCR. E-H: The profiles of CD133, and CD44 in U-251 MG and pGBMC1 cells (e,f), as well as the xenograft tumor nodules (g,h) were tested by Western blot. The values shown represent the mean ± standard deviation (SD). All experiment were repeated for three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (vs.NC group).

CD44 in the cells and formed tumor tissues were detected by western blot. It was found that miR-663a overexpression attenuated CD133 and CD44 protein levels, and miR-663a inhibitors promoted those protein levels in U-251 MG and pGBMC1 cells (Figure 3(e–h)). The above results indicated that miR-663a suppressed the CSC properties of glioblastoma cells.

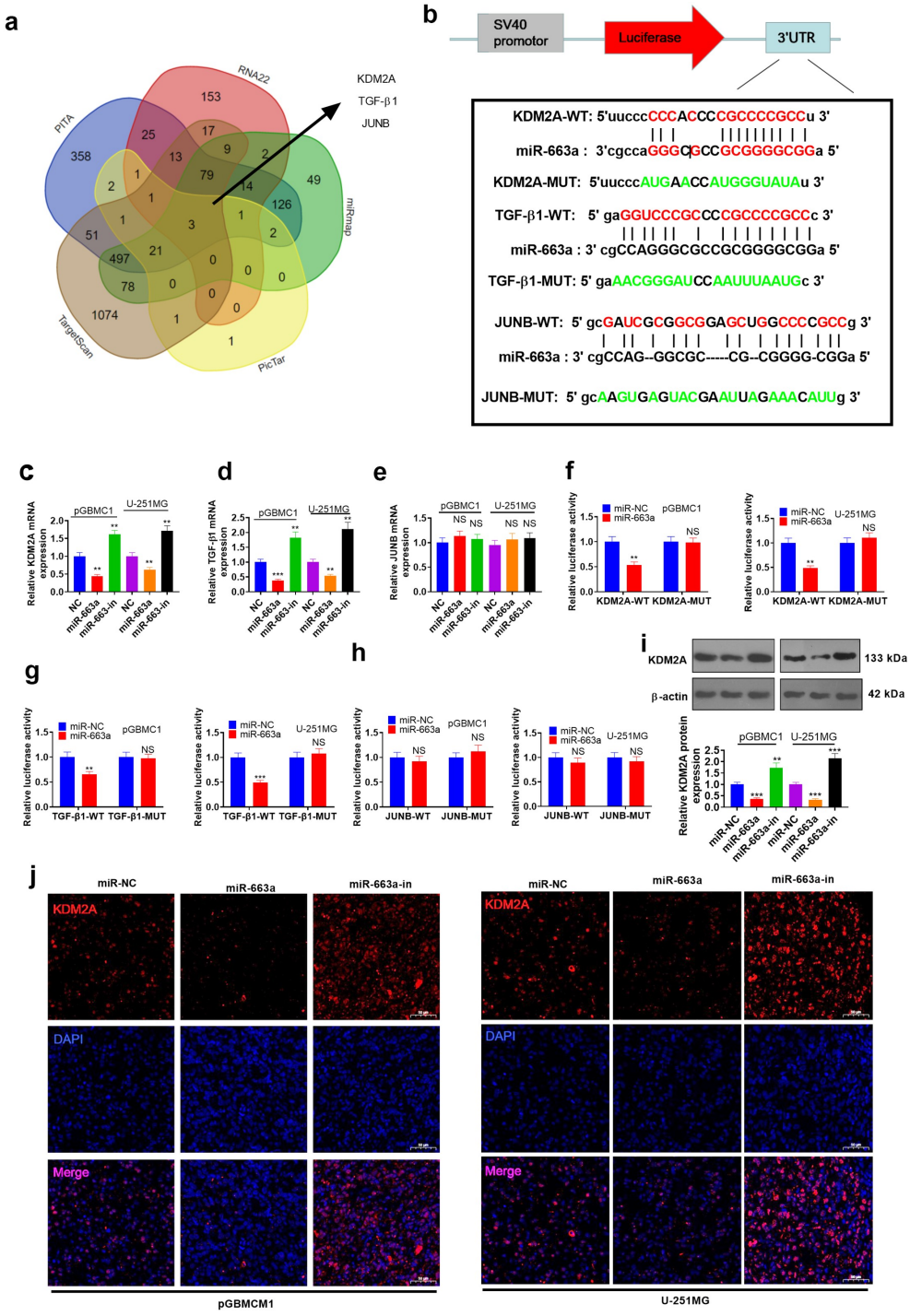

Figure 4. miR-663a targeted and inhibited the expression of KDM2A.

(a and b) The ENCORI (http://starbase.sysu.edu.cn/index.php) online database was employed for bioinformatics analysis for searching the downstream targets of miR-663a. Venn diagram analysis showed that miR-663a targeted KDM2A, TGF-β1, and JUNB. The luciferase reporting vectors (KDM2A-WT, KDM2A-MUT, JUNB-WT, JUNB-MUT, TGF-β-WT, and TGF-β-MUT) were cloned and inserted into the 3'UTR of psiCHECK-2 plasmid. (c–e) The KDM2A, TGF-β1, and JUNB mRNA profiles in PGBMC1 and U-251 MG cells transfected with miR-663a mimics or miR-663a inhibitors or negative control (NC) were assessed by qRT-PCR. NS indicated $P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0{,}001$ (vs.NC group). N = 3. (f–h) pGBMC1 and U-251 MG cells were transfected with the luciferase reporting vectors (KDM2A-WT, KDM2A-MUT, JUNB-WT, JUNB-MUT, TGF-β-WT, and TGF-β-MUT), and miR-663a or miR-NC. The dual-luciferase reporter assay was performed to confirm the targeting relationship between miR-663a and KDM2A, miR-663a and TGF-β1, miR-663a and JUNB. (i) After transfection of miR-663a mimics and miR-663a inhibitors into pGBMC1 and U-251 MG cells, the protein expression of KDM2A was verified by western blot. (j) The expression of KDM2A in PGBMC1 and U-251 MG cells was detected by cellular immunofluorescence (red for KDM2A and blue for nucleus). The values shown represent mean ± standard deviation (SD). N = 3 for each group. NS indicates $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs. miR-NC group).

3.4. MiR-663a targeted KDM2A

It was testified that miR-663a had a tumor-suppressive effect in glioma, and we were curious about the downstream regulatory pathway of miR-663a. By using ENCORI (http://starbase.sysu.edu.cn/index.php), we found that miR-663a targeted KDM2A, TGF-β1, and JUNB (Figure 4(a,b)). Next, we examined the expression of KDM2A, TGF-β1, and JUNB in miR-663a mimics or miR-663a inhibitors-transfected U-251 MG and pGBMC1 cells. As a result, the expression of KDM2 and TGF-β1 was down-regulated in U-251 MG and pGBMC1 cells after miR-663a overexpression, while JUNB showed no significant changes (Figure 4(c–e)). Therefore, we used the dual-luciferase reporter assay to verify the targeted relationship between miR-663a and KDM2A, TGF-β1, and JUNB. Our results showed that miR-663a mimics significantly inhibited the luciferase activity in U-251-MG and pGBMC1 cells transfected with KDM2A-WT or TGF-β1-WT, but had no effect on U-251-MG and pGBMC1 cells transfected with KDM2A-MUT, TGF-β1-MUT, JUNB-MUT or JUNB-WT (Figure 4(f,h)). Hence, we suspected that miR-663a played a role in glioma through KDM2A. Then, we detected KDM2A

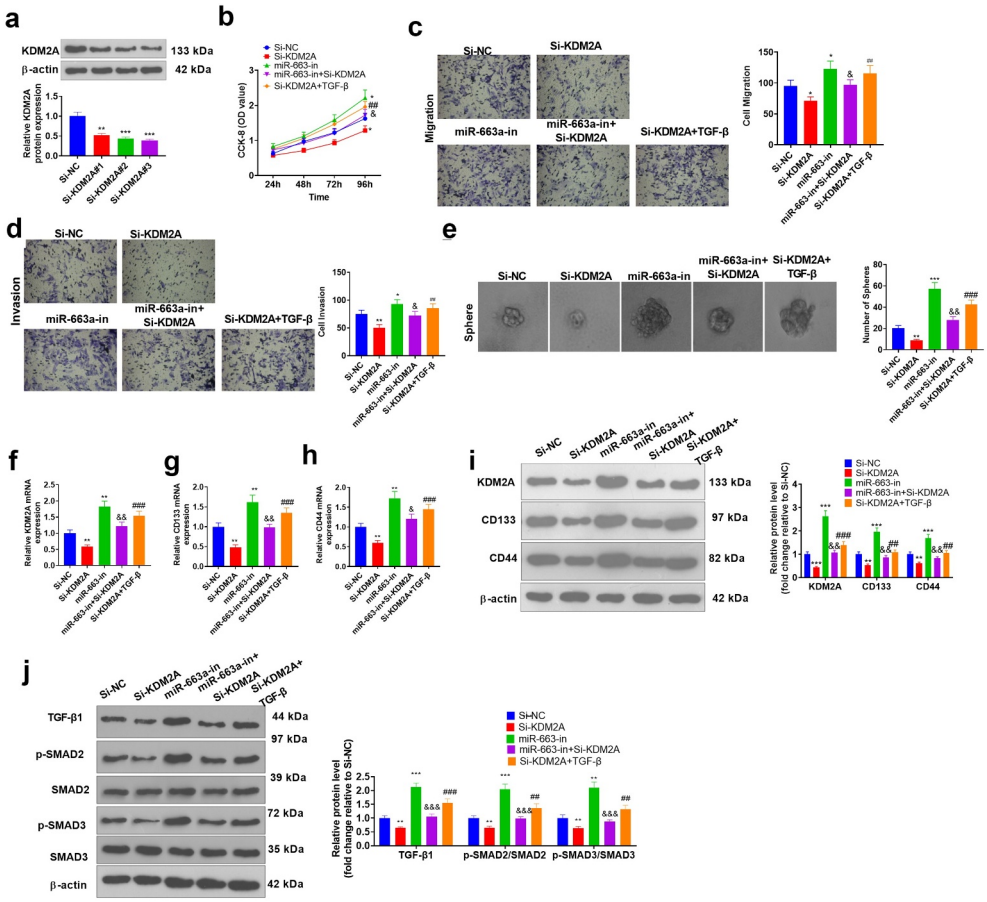

Figure 5. Knockdown of KDM2A abated the miR-663a-in-mediated carcinogenic effects.

(a) A KDM2A knockdown model was constructed in U-251-MG cells using si-KDM2A. The protein level of KDM2A was tested by western blot. The third sequence of si-KDM2A showed the most significant inhibition of KDM2A and was used for the further experiment. Si-KDM2A and/or miR-663a inhibitors were transfected into U-251 MG cells, and TGF-β (10 ng/ml) was used for activating TGF-β pathway. (b) U-251 MG cell proliferation was tested by MTT assay. (c and d) Transwell assay was implemented to detect the migration and invasion of U-251 MG cells. (e) Tumor sphere formation was adopted to determine the self-renewal ability of U-251 MG cells. (f–i) The mRNA and protein expression of KDM2A, CD133, and CD44 in U-251 MG cells was tested by qRT-PCR and western blot, respectively. J. The protein levels of TGF-β1, SMAD2, SMAD3 were determined by western blot. The displayed value represents the mean ± standard deviation (SD). n = 3. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (vs.Si-NC group); &$P < 0.05$, &&$P < 0.01$, &&&$P < 0.001$ (vs. miR-663a-in group); ##$P < 0.01$, ###$P < 0.001$ (vs. si-KDM2A group).

expression in U-251-MG and pGBMC1 cells by western blot, and immunofluorescence. The data suggested that overexpressing miR-663a notably repressed protein expression of KDM2A, while miR-663a knockdown promoted KDM2A expression (compared with NC group, Figure 4(i,j)). These results indicated that miR-663a was the upstream regulator of KDM2A and negatively regulated the KDM2A expression.

3.5. Knocking down KDM2A attenuated the stimulative effect of miR-663a knockdown on U-251-MG cells

A KDM2A knockdown model was constructed on U-251 MG cells (Figure 5(a)). Next, the U-251 MG cells were transfected with miR-663a-in, or treated with TGF-β (10 ng/ml). U-251 MG cells with low expression of KDM2A had less proliferative, migration and invasive abilities (vs. Si-NC group or miR-663a-in group), while TGF-β enhanced the proliferative, migration and invasive abilities of U-251 MG cells with KDM2A knockdown (compared with Si-KDM2A group, Figure 5(b–d)). The sphere ability of U-251-MG cells was evaluated by the cell sphere assay. The results showed that the U-251-MG cell sphere was dampened after the down-regulation of KDM2A (vs. Si-NC group or miR-663a-in). The addition of TGF-β enhanced tumorsphere formation compared with the Si-KDM2A group (Figure 5(e)). KDM2A, and the stem cell markers in U-251 MG cells were determined by qRT-PCR and Western blotting. As the results showed, the expression of KDM2A, CD133, and CD44 was down-regulated in the Si-KDM2A group (vs. the Si-NC group) and miR-663a-in+Si-KDM2A group (vs. the miR-663a-in group, Figure 5(f–i)). Additionally, the protein levels of TGF-β1, SMAD2, SMAD3 were determined by western blot. As the data showed, Si-KDM2A inhibited TGF-β1 and the phosphorylation of SMAD2 and SMAD3 (compared with si-NC group or miR-663a-in group, Figure 5(j)), and the treatment of TGF-β enhanced TGF-β1, the phosphorylation of SMAD2 and SMAD3 (vs.Si-KDM2A group, Figure 5(j)). The above results suggested that KDM2A knockdown attenuated the stimulative effect of miR-663a-in-mediated U-251-MG cells via repressing TGF-β/Smad signaling pathway.

3.6. Overexpressing miR-663a attenuated the stimulative effect of THP1-mediated U-251-MG cells

We co-cultured THP1 with U-251 MG cells for three days. The collected TAMs were then co-cultured with U-251 MG cells for 1 or 3 days. Western blot results showed that THP1 cells stimulated upregulation of KDM2A, CD133, and CD44 in U-251 MG cells (sup Figure 2(a)), and promoted the sphere formation ability, proliferation, migration, invasion and reduced temozolomide sensitivity of U-251 MG cells (sup Figure 2(b–e)). qRT-PCR illustrated that miR-633a was down-regulated in both THP1 and U-251 MG cells when THP1 and U-251 MG cells were cocultured (Figure 6(a,b)). Secondly, THP1 cells were transfected with miR-663a mimics, and then co-cultured with U-251-MG for three days. Subsequently, the miR-663a expression in U-251 MG cells was monitored by qRT-PCR. It turned out that miR-663a was overexpressed in the THP1-3d+miR-663a group compared with that in the THP1-3d+miR-NC group (Figure 6(c)). Next, Western blot result showed that KDM2A, CD133, and CD44 were downregulated in the THP1-3d +miR-663a group (vs. the THP1-3d+miR-NC) (Figure 6(d)). Meanwhile, the cell sphere assay demonstrated that U-251-MG cell sphere formation ability was reduced after up-regulating miR-663a (Figure 6(e)). Transwell assay illustrated U-251-MG cells showed reduced migration and invasion ability after miR-663a up-regulation (Figure 6(f–g)). MTT assay revealed that compared with the THP1-3d+miR-NC group, the sensitivity of U-251 MG cells to temozolomide was significantly increased in the THP1-3d+miR-663a group (Figure 6(h)). The above results confirmed that overexpressing miR-663a attenuated the stimulative effect of THP1-mediated U-251-MG cells.

3.7. Overexpressing miR-663a attenuated the "M2" polarization of THP1 cells

qRT-PCR was performed to detect the IL-4, IL-10 and CD206 mRNA expression in THP1 cells cocultured with U-251-MG cells. As the data showed, all of IL-4, IL-10 and CD206 mRNA levels

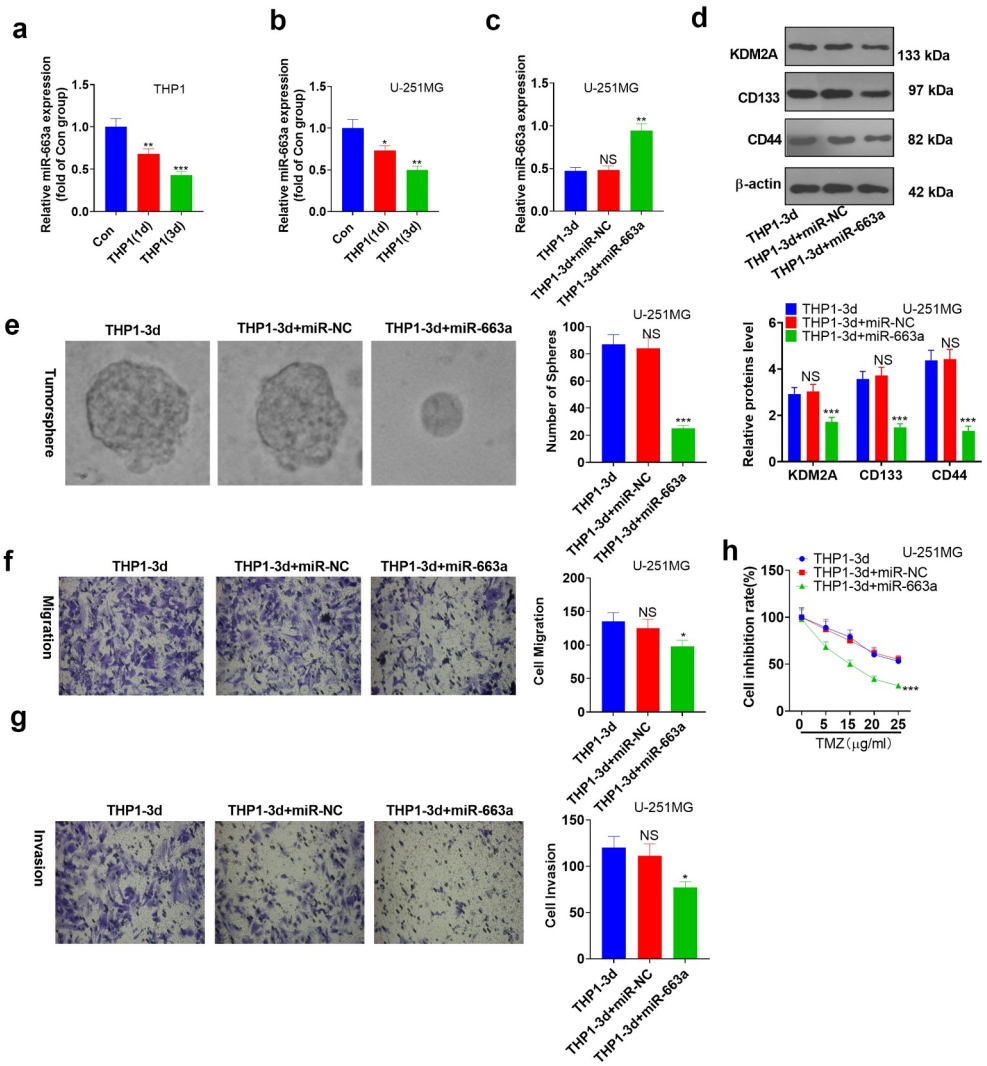

Figure 6. Overexpressing miR-663a weakened the THP1-mediated promotive effects of U-251 MG cells.

(a and b) THP1 cells were co-cultured with U-251 MG cells for 1–3 days, and the mRNA expression of miR-633a in TAMs and U-251 MG cells was detected by qRT-PCR. THP1 cells were transfected with miR-663a mimics or miR-NC, and then cocultured with U-251 MG cells for 3 days. (c) The miR-663a profile in THP1 cells was examined by qRT-PCR. (d) Western blot was utilized for detecting the expression of KDM2A, CD133, and CD44 in U-251 MG cells cocultured with TAMs transfected with miR-663a mimics. € The number of tumorspheres was counted and the morphology of tumorspheres was observed under a light microscope. (f and g) Transwell assay was applied to monitor migration and invasion in U-251 MG cells after being cocultured with TAMs transfected with miR-663a mimics. (h) U-251 MG cells cocultured with TAMs transfected with miR-663a mimics were treated with 0–25 μg/mL temozolomide for 24 h, the proliferation of U-251 MG cells was determined by MTT assay. The values shown represent mean ± standard deviation (SD). The values were shown as mean ± standard deviation (SD), n = 3 for each group. NS indicates $P > 0.05$ (vs. Con group or THP1-3d group). *$P < 0.05$, **$P < 0.01$, ***$P <$ 0,001 (vs. Con group or THP1-3d+miR-NC group).

were enhanced in THP1 cells co-cultured with U-251-MG cells (compared with Con group, Figure 7(a–c)). However, the transfection of miR-663a in THP1 cells reduced IL-4, IL-10 and CD206 expressions (compared with the THP1-3d+miR-NC group, Figure 7(d–f)). Double immunofluorescence of CD11b and CD206 in THP1 cells was performed, and western blot to test the polarization of TAMs. The result indicated that $CD11b^{+}CD206^{+}$ cell rate was significantly increased when THP1 cells were cocultured with U-251-MG cells (compared with Con group), while miR-663a upregulation led to attenuated $CD11b^{+}CD206^{+}$ cell rate (compared with THP1 (3d)+miR-NC group, Figure 7(g–i)). Moreover, the protein levels of iNOS, CD86, CD80 were

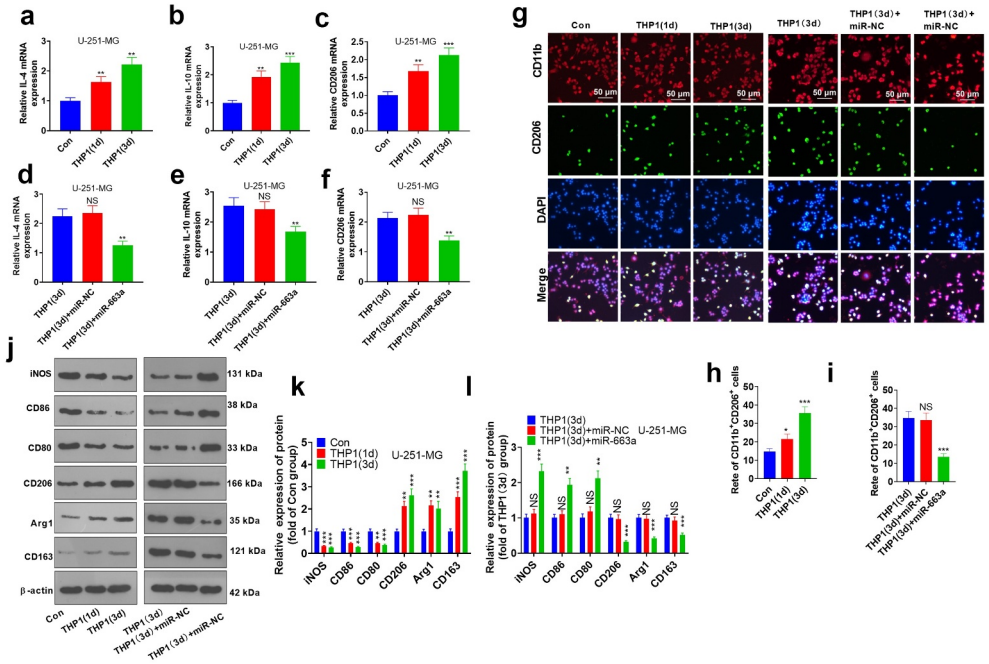

Figure 7. Overexpressing miR-663a attenuated the IL-4, IL-10 and CD206 expressions in THP1 cells.

THP1 cells were co-cultured with U-251 MG cells for 1–3 days. (a–c) qRT-PCR was performed to detect the IL-4, IL-10 and CD206 mRNA expression in THP1 cells cocultured with U-251-MG cells. THP1 cells received the transfection of miR-663a mimics and then were co-cultured with U-251-MG cells. (d–f) qRT-PCR was performed to detect the IL-4, IL-10 and CD206 expression in THP1 cells. (g–i) Double immunofluorescence of CD11b (red) and CD206 (green) in THP1 cells was performed. The rate of $CD11b^{+}CD206^{+}$ cell was counted. (j–l)Western blot was conducted to test the protein level of "M1" markers (including inos, CD86, and CD80), and "M2" markers (including CD206, Arg1, and CD163) of THP1 cells. The values were shown as mean ± standard deviation (SD), n = 3 for each group. NS indicates $P > 0.05$ (vs. THP1-3d group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (vs.Con group or THP1-3d+miR-NC group).

downregulated, and CD206, Arg1, CD163 were overexpressed in THP1 cells cocultured with U-251-MG cells. forced overexpression of miR-663a in THP1 cells reversed this tendency, as iNOS, CD86, CD80 were enhanced, and CD206, Arg1, CD163 were repressed in THP1 cells with upregulated miR-663a (Figure 7(j–l)). The above data suggested that THP1 cells are more polarized into M2 phenotype when they were co-cultured with glioma cells, and upregulation of miR-663a inhibited M2 polarization of THP1 cells.

4. Discussion

Glioma is the most frequent intracranial malignancy in humans. Due to its proliferation and invasion, it remains a therapeutic challenge [20]. In this study, we found that miR-663a was downregulated in glioblastoma tissues and cells. Forced overexpression of miR-663a repressed the proliferation, invasion, growth, and the CSC properties of glioblastoma cells.

Increasing studies have confirmed that the detection of miRNAs in cerebrospinal fluid and brain tissues has significant value in the diagnosis of glioma, and miRNAs are involved in the development of glioma stem cells and the TME [21,22]. For example, miR-21 heightens glioma cell migration and invasion by activating the SRY-box transcription factor 2 (SOX2)/β-catenin pathway [23]. MiR-218-5p was downregulated in glioma tissues and reduced glioma cell invasion by targeting Lipoma HMGIC fusion partner-like 3 (LHFPL3) [24]. MiR-663a, a miRNA, acts as a tumor suppressor gene to inhibit cell proliferation and invasion of gallbladder cancer [25] and lung small-cell cancer [26] via targeting different genes. In addition, photodynamic therapy (PDT) induces apoptosis of glioma cells by up-regulating miR-663a [27], suggesting that miR-663a is a potential therapeutic target in glioma. Here, we revealed that miR-663a was downregulated in glioblastoma tissues and cells. The lower level of miR-663a was associated with poorer survival of glioblastoma

patients. Additionally, the functional assays confirmed that miR-663a is an antitumor gene in glioblastoma via repressing the proliferation, migration, invasion, and cancer stem cell-like properties of glioblastoma cells both *in vivo* and *in vitro*.

As a vital part of the brain TME, TAMs play an emerging role in regulating tumor progression and controlling anti-tumor immune responses. TAMs are recruited to the glioma environment and release various growth factors and cytokines in response to those factors produced by cancer cells, thus accelerating tumor cell proliferation and invasion [3,28]. For instance, the inhibitor of the CSF-1 receptor (CSF-1 R) to target TAMs attenuates intracranial growth of patient-derived glioma xenografts in a mouse proneural GBM model. Mechanistically, CSF-1 R inhibitor restrains the releasing of granulocyte-macrophage CSF (GM-CSF) and interferon-γ (IFN-γ) from glioma cells, and mitigates the "M2" polarization of TAMs [29]. In another study, it is revealed that M2-polarized TAMs are more recruited and induced in the hypoxic areas of glioma. The HIF inhibitor acriflavine (ACF) markedly suppresses the M2 polarization of macrophages by inhibiting macrophage colony-stimulating factor receptor (M-CSFR) in macrophages and TGF-β in glioma cells under hypoxic conditions [30]. In addition, the alteration of miRNAs in TAMs affects the antitumor immune response [31]. Taking miR-181b as an example, its lower level is correlated with high-grade glioma patients. MiR-181b inhibits EGFR-dependent Vascular cell adhesion molecule-1 (VCAM-1), which induces monocyte adhesion and leads to promotive tumor growth and invasion activity [32]. Here, we found that miR-663a was downregulated in both THP1 and U-251-MG cells when they were cocultured. THP1 cells induced accelerated proliferation, migration, invasion and CSC properties of U-251-MG cells, and those effects were reversed by miR-663a upregulation.

The modulation of TAMs polarization has been identified as an effective method against TAMs-mediated glioma progression [33]. For instance, Georgieva et al. showed that M2 phenotype macrophages can promote transplanted tumors survival and growth [4]. In this study, we found that that $CD11b^{+}CD206^{+}$ positive M2 microglia/macrophages are more accumulated in the glioblastoma tissues, and the GBM/LGG tumor tissues have more expression of M2 phenotype markers, such as IL-4, IL-10 and CD206. Actually, IL-4, IL-10 and CD206 have been regarded as therapeutic targets in cancers [34,35,36]. Presently, we constructed a co-cultured model of THP1-U-251-MG cells. We found that U-251-MG cells enhanced the "M2" polarization of THP1 cells. Upregulation of miR-663a promoted the transformation of "M2" phenotype into "M1" phenotype. Therefore, miR-663a is suggested to affect the TAMs-glioblastoma interaction.

As a member of the KDMs family, KDM2A modulates cell proliferation, differentiation, apoptosis, and senescence by regulating the H3K36 methylation level and expression of related signaling pathways, thus affecting tumor development and formation. Previous studies have shown that KDM2A is carcinogenic in many types of cancer [37,38,39]. TGF-β is a multifunctional cytokine that regulates the proliferation, differentiation, and survival or apoptosis of many cells and is the core molecule that maintains the malignant phenotypes of glioblastoma [40,41]. TGF-β functions by activating specific receptors in multiple intracellular pathways, leading to receptor-regulated phosphorylation of Smad2/3 protein and promotive effect of glioma invasion [42]. For example, knocking down retinol dehydrogenase 10 (RDH10) inhibits glioma cell metastasis by inhibiting the TGF-β/SMAD signaling pathway [43]. Inhibition of dioxin/aryl hydrocarbon receptor (AhR) reduces the survival rate and invasiveness of glioma cells by inhibiting the TGF-β/Smads signaling pathway [44]. Thus, inhibiting the TGF-β/Smads signaling pathway can abate glioma progression. Additionally, Xu WH et al. indicated that the TGF-β pathway might be a downstream target of KDM2A in regulating cell proliferation. Knocking down KDM2A represses HEK293T cell proliferation by inhibiting the TGF-β signaling pathway [14]. On the other hand, many studies have shown that miRNAs affect tumor progression by targeting KDM2A [37,45]. miR-3666 overexpression significantly reduces the KDM2A level in glioma cells, thus inhibiting the proliferation, migration and invasion of glioma cells [46]. Based on the above

findings, we probed the role of miR-663a and KDM2A/TGF-β/Smads in glioma. Analysis of online websites showed a binding site between miR-663a and KDM2A, and the dual-luciferase reporter assay confirmed that their expression is negatively correlated. Overexpressing miR-663a significantly suppressed the expression of KDM2A in glioma cells. Moreover, we found that knocking down KDM2A attenuates the TGF-β/Smad pathway. It has been found that overexpression of miR-663a attenuates the KDM2A/TGF-β/Smad signaling pathway and alleviates the CSC properties of glioma mediated by THP1 cells. Our study is the first to investigate the relationship between miR-663a and KDM2A and their functions in glioma.

In conclusion, this study revealed that miR-663a is downregulated in glioblastoma and serves as a favorable factor in predicting the overall survival of glioblastoma patients. Overexpression of miR-663a represses the KDM2A/TGF-β/Smad signaling pathway and attenuates the CSC properties of glioblastoma cells. However, further experiments are needed to investigate the functions of miR-663a on TAMs-(isolated from glioblastoma tissues) mediated malignant progression both in vitro and *in vivo*. Overall, our study may provide new insights into the pathogenesis of TAMs in glioma and bring novel prevention and treatment options for glioma.

Authors' contribution

Conceived and designed the experiments: Keqi Hu;

Performed the experiments: Lei Wang, Bojuan Lang, Youdong Zhou, Jinyang Ma, Keqi Hu;

Statistical analysis: Youdong Zhou, Jinyang Ma, Bojuan Lang, Keqi Hu;

Wrote the paper: Lei Wang, Bojuan Lang, Keqi Hu.

All authors read and approved the final manuscript.

Data availability statement

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request. http://www.xfszxyy.cn/publish/cbnews/201510/16/cb5942_1.shtml.

Disclosure statement

The authors 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.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not- for- profit sectors.

Ethics statement

Our study was approved by the Ethics Review Board of Xiangyang Central Hospital, Affiliated Hospital of Arts and Science.

References

[1] Ostrom QT, Bauchet L, Davis FG, et al. The epidemiology of glioma in adults: a "state of the science" review. Neuro Oncol. 2014;16(7):896–913.

[2] Han W, Shi J, Cao J, et al. Emerging roles and therapeutic interventions of aerobic glycolysis in glioma. Onco Targets Ther. 2020 [Published 2020 Jul 16];13:6937–6955.

[3] Roesch S, Rapp C, Dettling S, et al. When Immune cells turn bad-tumor-associated microglia/macrophages in glioma. Int J Mol Sci. 2018 [Published 2018 Feb 1];19 (2):436.

[4] Georgieva PB, Mathivet T, Alt S, et al. Long-lived tumor-associated macrophages in glioma. Neurooncol Adv. 2020 [Published 2020 Sept 25];2(1):vdaa127.

[5] Wu J, Yang H, Cheng J, et al. Knockdown of milk-fat globule EGF factor-8 suppresses glioma progression in GL261 glioma cells by repressing microglial M2 polarization. J Cell Physiol. 2020;235(11):8679–8690.

[6] Glass R, Synowitz M. CNS macrophages and peripheral myeloid cells in brain tumours. Acta Neuropathol. 2014;128(3):347–362.

[7] Thomas TM, Yu JS. Metabolic regulation of glioma stem-like cells in the tumor micro-environment. Cancer Lett. 2017;408:174–181.

[8] Rolle K. miRNA multiplayers in glioma. From bench to bedside. Acta Biochim Pol. 2015;62(3):353–365.

[9] Nan Y, Guo H, Guo L, et al. MiRNA-451 inhibits glioma cell proliferation and invasion through the mTOR/HIF-1α/VEGF signaling pathway by targeting CAB39. Hum Gene Ther Clin Dev. 2018;29 (3):156–166.

[10] Xiong W, Ran J, Jiang R, et al. miRNA-320a inhibits glioma cell invasion and migration by directly tar-

geting aquaporin 4. Oncol Rep. 2018;39 (4):1939–1947.
[11] Zhang C, Chen B, Jiao A, et al. miR-663a inhibits tumor growth and invasion by regulating TGF-β1 in hepatocellular carcinoma. BMC Cancer. 2018 [Published 2018 Nov 28];18(1):1179.
[12] Huang W, Li J, Guo X, et al. miR-663a inhibits hepatocellular carcinoma cell proliferation and invasion by targeting HMGA2. Biomed Pharmacother. 2016;81:431–438.
[13] Tsukada Y, Fang J, Erdjument-Bromage H, et al. Histone demethylation by a family of JmjC domain-containing proteins. Nature. 2006 Feb 16;439(7078):811–816. Epub 2005 Dec 18. PMID: 16362057.
[14] Xu WH, Liang DY, Wang Q, et al. Knockdown of KDM2A inhibits proliferation associated with TGF-β expression in HEK293T cell. Mol Cell Biochem. 2019 June;456(1–2):95–104. Epub 2019 Jan 2. PMID: 30604066.
[15] Huang Y, Liu Y, Yu L, et al. Histone demethylase KDM2A promotes tumor cell growth and migration in gastric cancer. Tumour Biol. 2015 Jan;36 (1):271–278. Epub 2014 Sep 23. PMID: 25245333.
[16] Wagner KW, Alam H, Dhar SS, et al. KDM2A promotes lung tumorigenesis by epigenetically enhancing ERK1/2 signaling. J Clin Invest. 2013 Dec;123 (12):5231–5246. Epub 2013 Nov 8. PMID: 24200691; PMCID: PMC3859406.
[17] Lu DH, Yang J, Gao LK, et al. Lysine demethylase 2A promotes the progression of ovarian cancer by regulating the PI3K pathway and reversing epithelial-mesenchymal transition. Oncol Rep. 2019 Feb;41(2):917–927. Epub 2018 Nov 27. PMID: 30483796; PMCID: PMC6313075.
[18] Xiong J, Guo G, Guo L, et al. Amlexanox enhances temozolomide-induced antitumor effects in human glioblastoma cells by inhibiting IKBKE and the Akt-mTOR signaling pathway. ACS Omega. 2021 [Published 2021 Feb 5];6(6):4289–4299.
[19] Kurt IC, Sur I, Kaya E, et al. KDM2B, an H3K36-specific demethylase, regulates apoptotic response of GBM cells to TRAIL. Cell Death Dis. 2017 June 29;8(6):e2897. PMID: 28661478; PMCID: PMC5520939.
[20] Ware ML, Berger MS, Binder DK. Molecular biology of glioma tumorigenesis. Histol Histopathol. 2003;18 (1):207–216.
[21] Zhou Q, Liu J, Quan J, et al. MicroRNAs as potential biomarkers for the diagnosis of glioma: a systematic review and meta-analysis. Cancer Sci. 2018;109 (9):2651–2659.
[22] Liu Y, Li X, Zhang Y, et al. An miR-340-5p-macrophage feedback loop modulates the progression and tumor microenvironment of glioblastoma multiforme. Oncogene. 2019;38(49):7399–7415.
[23] Luo G, Luo W, Sun X, et al. MicroRNA-21 promotes migration and invasion of glioma cells via activation of Sox2 and β-catenin signaling. Mol Med Rep. 2017;15 (1):187–193.
[24] Li Z, Qian R, Zhang J, et al. MiR-218-5p targets LHFPL3 to regulate proliferation, migration, and epithelial-mesenchymal transitions of human glioma cells. Biosci Rep. 2019 [Published 2019 Mar 1];39(3): BSR20180879.
[25] Ma Q, Zhang Y, Liang H, et al. EMP3, which is regulated by miR-663a, suppresses gallbladder cancer progression via interference with the MAPK/ERK pathway. Cancer Lett. 2018;430:97–108.
[26] Zhang Y, Xu X, Zhang M, et al. MicroRNA-663a is downregulated in non-small cell lung cancer and inhibits proliferation and invasion by targeting JunD. BMC Cancer. 2016 [Published 2016 May 16];16:315.
[27] Yuan SX, Li JL, Xu XK, et al. Underlying mechanism of the photodynamic activity of hematoporphyrin-induced apoptosis in U87 glioma cells. Int J Mol Med. 2018;41(4):2288–2296.
[28] Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. Nat Neurosci. 2016;19 (1):20–27.
[29] Pyonteck SM, Akkari L, Schuhmacher AJ, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. Nat Med. 2013;19(10):1264–1272.
[30] Guo X, Xue H, Shao Q, et al. Hypoxia promotes glioma-associated macrophage infiltration via periostin and subsequent M2 polarization by upregulating TGF-beta and M-CSFR. Oncotarget. 2016;7 (49):80521–80542.
[31] Sahraei M, Chaube B, Liu Y, et al. Suppressing miR-21 activity in tumor-associated macrophages promotes an antitumor immune response. J Clin Invest. 2019;129 (12):5518–5536.
[32] Liu YS, Lin HY, Lai SW, et al. MiR-181b modulates EGFR-dependent VCAM-1 expression and monocyte adhesion in glioblastoma. Oncogene. 2017;36 (35):5006–5022.
[33] Zhu C, Kros JM, Cheng C, et al. The contribution of tumor-associated macrophages in glioma neo-angiogenesis and implications for anti-angiogenic strategies. Neuro Oncol. 2017;19(11):1435–1446.
[34] Ito SE, Shirota H, Kasahara Y, et al. IL-4 blockade alters the tumor microenvironment and augments the response to cancer immunotherapy in a mouse model. Cancer Immunol Immunother. 2017;66 (11):1485–1496.
[35] Mocellin S, Marincola FM, Young HA. Interleukin-10 and the immune response against cancer: a counterpoint. J Leukoc Biol. 2005;78(5):1043–1051.
[36] Jaynes JM, Sable R, Ronzetti M, et al. Mannose receptor (CD206) activation in tumor-associated macrophages enhances adaptive and innate antitumor

immune responses. Sci Transl Med. 2020;12(530): eaax6337.

[37] Ou R, Zhu L, Zhao L, et al. HPV16 E7-induced upregulation of KDM2A promotes cervical cancer progression by regulating miR-132-radixin pathway. J Cell Physiol. 2019 Mar;234(3):2659–2671. Epub 2018 Aug 21. PMID: 30132864.

[38] Wang F, Liang S, Liu X, et al. LINC00460 modulates KDM2A to promote cell proliferation and migration by targeting miR-342-3p in gastric cancer. Onco Targets Ther. 2018 Oct 2;11:6383–6394. PMID: 30323616; PMCID: PMC6174301.

[39] Xi C, Ye NY, Wang YB. LncRNA LINC01278 accelerates colorectal cancer progression via miR-134-5p/KDM2A axis. Eur Rev Med Pharmacol Sci. 2020 Oct;24(20):10526–10534. PMID: 33155208.

[40] Kaminska B, Wesolowska A, Danilkiewicz M. TGF beta signalling and its role in tumour pathogenesis. Acta Biochim Pol. 2005;52(2):329–337.

[41] Frei K, Gramatzki D, Tritschler I, et al. Transforming growth factor-β pathway activity in glioblastoma. Oncotarget. 2015;6(8):5963–5977.

[42] Kaminska B, Cyranowski S. Recent advances in understanding mechanisms of TGF beta signaling and its role in glioma pathogenesis. Adv Exp Med Biol. 2020;1202:179–201.

[43] Guan F, Kang Z, Wang L, et al. Retinol dehydrogenase 10 promotes metastasis of glioma cells via the transforming growth factor-β/SMAD signaling pathway. Chin Med J (Engl). 2019;132(20):2430–2437.

[44] Gramatzki D, Pantazis G, Schittenhelm J, et al. Aryl hydrocarbon receptor inhibition downregulates the TGF-beta/Smad pathway in human glioblastoma cells. Oncogene. 2009;28(28):2593–2605.

[45] Kong Y, Zou S, Yang F, et al. RUNX3-mediated up-regulation of miR-29b suppresses the proliferation and migration of gastric cancer cells by targeting KDM2A. Cancer Lett. 2016 Oct 10;381(1):138–148. Epub 2016 Aug 3. PMID: 27497248.

[46] Shou T, Yang H, Lv J, et al. MicroRNA-3666 suppresses the growth and migration of glioblastoma cells by targeting KDM2A. Mol Med Rep. 2019 Feb;19(2):1049–1055. Epub 2018 Nov 27. PMID: 30483744; PMCID: PMC6323202.