miRNA-133a has anti-tumor effects on glioblastoma

Li Zhao¹, Xian An², Wenhong Yang³, Xu Han²

¹Internal Medicine, Jining No. 1 People's Hospital, Jining, Shangdong Province, China
²Health Care Unit, Jining No. 1 People's Hospital, Jining, Shangdong Province, China
³Nursing Department, Jining No. 1 People's Hospital, Jining, Shangdong Province, China

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Abstract

Introduction: Glioblastomas (GBMs) are primary malignant gliomas and astrocytomas that predominantly threaten patients’ life. The aim of this study was to explain how miRNA-133a plays an important role in GBM patients’ life.

Material and methods: We analyzed the pathology in healthy and GBM tissues by HE staining. We evaluated the MEK protein expression in adjacent and cancer tissues by immunohistochemistry (IHC). By cell experiment, we evaluated the miRNA-133a effects in U87 cells, which are a human primary glioblastoma cell line, biologically by MTT assay, flow cytometer and wound healing assay and measuring the relative protein expressions by WB assay.

Results: GBM patients show poor prognosis of < 1.5-year survival period. Real time polymerase chain reaction (RT-PCR) was used to evaluate miR-133a and MEK levels in glioma tissues and healthy tissues, and the correlation between miR-133a and MEK was analyzed. Cell proliferation was examined by MTT assay. Wound healing assay was performed to detect cell migration. The results show a lower miR-133a level in glioma tissue than healthy tissue, and miR-133a is positively correlated with patients’ overall survival (OS). Fibroblast growth factor 1 (FGF1) agonist rescues miR-133a mimic induced reduction of U87 cell proliferation. FGF1 agonist inhibits miR-133a mimic caused up-regulation of U87 cell apoptosis, U87 cells that were at G1 stage and U87 cell migration. p-MEK/p-PI3K/p-AKT/p-ERK signaling pathways were responsible for the protective role of miR-133a in GBM.

Conclusions: miR-133a plays a protective role in GBM via inhibiting activation of MEK/PI3K/AKT and ERK pathways, and might be an effective therapy for GBM.

Key words: miR-133a, glioblastoma, overall survival, p-MEK/p-PI3K/p-AKT/p-ERK signaling pathways.

Introduction

Glioma is a type of tumor arising from glial cells in the brain or spine, whose most common site is the brain [1]. Glioma constitutes approximately 30% of central nervous system tumors and 80% of malignant brain tumors [2].

While glioblastomas (GBMs) begins from normal brain cells or develops from an existing low-grade astrocytoma [3], it represents 15% of brain tumors [4], thus predominantly threatening patients’ life [5]. About 3/100,000 people develop GBM each year [6]. Glioblastoma is characterized by genetic instability, intra-tumoral histopathological variability and unpredictable survival probability in patients [7, 8]. The clinical hallmarks of GBM are aggressive proliferation and persistent recurrence due...
to invasive infiltration [9, 10]. Despite maximum treatment, GBM frequently recurs [6]. After GBM diagnosis, in patients without treatment overall survival (OS) is typically 3 months [11], while in patients with treatment 5-year OS occurs in less than 3–5% people and 12–15-month OS is most common [6, 12].

Typically treatment of GBM involves surgery after chemotheraphy (temozolomide) and radiation therapy [6]. However, there are no effective methods to anticipate GBM.

PI3K/Akt and Ras-ERK pathways are aberrantly activated pathways in GBM. For instance, the activated Raf-MEK-ERK pathway was reported to enhance activity of GBM cells [13]. PI3K and ERK inhibitors were discovered to effectively treat GBM patients [14]. PI3K/Akt inhibitors were recently reported to be therapeutic options for GBM [15, 16].

microRNAs (miRNAs) lower gene expression through binding to target sites in 3′-UTR of mRNAs, and play critical roles in cancer progression and treatment [17, 18]. miR-133 was first experimentally characterized in mice [19]. In the human genome, miR-133a1, miR-133a2 and miR-133b genes were found on chromosomes 18, 20 and 6, respectively, and a mature sequence was excised from the hairpin 3′ arm. miR-133a down-regulation led to an augmentation of contraction and bronchial smooth muscles (BSM) hyperresponsiveness [20, 21]. Whether miR-133a plays a role in GBM was unknown; the current study aimed to investigate its corresponding effects.

**Material and methods**

**Specimens**

The present study was approved by the Ethics Committee of Jining No. 1 People’s Hospital. A total of 45 cases of glioma tissues as well as 20 cases of normal brain tissues were collected at Jining No. 1 People’s Hospital from 2014 to 2016. All patients signed informed consent. We evaluated the pathology of healthy and glioma tissues by HE staining and estimated the MEK protein expression of healthy and glioma tissues by immunohistochemistry (IHC). IHC determined: 3 points: cells dyed dark brown were strongly positive; 2 points: brown yellow is masculine; 1 point: Light yellow is weak positive and 0 point: No staining is negative.

**Cell culture**

Human glioma cell line U87 was purchased from American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin combination at 37°C in a humidified atmosphere with 5% CO₂.

**Cell transfection**

Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen, USA) following the manufacturer’s instructions. MiR-133a mimic (miR-133a) was transfected into U87 cells with or without FGF1 agonist (GenScript (Nanjing) Co., Ltd., Nanjing, China) treatment at a concentration of 20 μM. MiR-negative control (miR-NC) was used as the negative control (NC). At the same time, U87 cells were treated with FGF1 inhibitor (Z02786-50, GenScript (Nanjing) Co., Ltd., Nanjing, China) alone as the FGF1 inhibitor (K1040, GenScript (Nanjing) Co., Ltd., Nanjing, China) treatment group. Forty-eight h after transfection, the U87 cells were used for the following experiments. The miR-133a was purchased from GenScript (Nanjing) Co., Ltd., Nanjing, China (5′-UUUGGUCCCCUCUACCAG CUG-3′).

**MTT assay**

To examine the cell proliferation, MTT assay was performed. 24 h after transfection, log-phase human glioma U87 cells were seeded into a 96-well plate (5 × 10³) and cultured for 24 h. Then, 20 μl of MTT (5 mg/ml, Sigma, USA) was added. After incubation at 37°C for 4 h, 150 μl of DMSO was added. 10 min after incubation at room temperature, the optical density (OD) at 490 nm was examined using a spectrophotometer. Each experiment was repeated three times.

**Transwell invasion experiment**

The transwell migration assay was conducted with a Corning Inc. transwell chamber. The transwell migration assay, 2 × 104 cells suspended in 100 μl serum-free DMEM were seeded in the upper compartment of the chamber and 800 μl DMEM with 10% FBS were added to the lower compartment of the chamber. The cell of difference groups were treated by difference treatments at the same time and the cells were incubated for another 24 h. After that, the cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet. The non-migrating cells in the upper chamber were removed carefully using a cotton swab. The invasion cells were cells on the lower surface were photographed with an Olympus IX70 inverted microscope in five randomly selected visual fields and the migrated cells were quantified using Image-Pro Plus 6.0 software. Each assay was performed at least three times.

**Wound healing assay**

For cell migratory capacity detection, wound healing assay was performed. 24 h after transfection, U87 cells (10⁶ cells/hole) were seeded in
miRNA-133a has anti-tumor effects on glioblastoma

Arch Med Sci

a 6-well plate and incubated to 100% confluence under normal cell culture conditions with 5% CO₂ at 37°C. Then, wounds were formed by using a sterile pin. Then, cells were cultured in media without serum for 48 h, and observed under an inverted microscope (Olympus, Japan). The width of wounds was measured at 0 h and 48 h.

Apoptosis analysis assay

For detection of cell apoptosis levels, a FITC apoptosis detection kit (Vazyme, US) was used according to the manufacturer's instruction. 24 h after transfection, U87 cells were washed with cold PBS solution. Then, U87 cells were re-suspended in 1× binding buffer solution with Annexin V-FITC and propidium iodide (PI) and incubated for 15 min at room temperature without light. Flow cytometry (FCM) (Becton Dickinson, New Jersey, USA) was used for detection of the apoptotic rate. Tests were performed in triplicate.

Cell cycle analysis

Cells were collected using 0.25% trypsin, then fixed in 70% ethanol at 4°C overnight, and washed with cold PBS solution three times. Subsequently, cells were resuspended with RNase A (Sigma, USA) at 37°C for 30 min, and then cells were incubated with propidium iodide (PI) (0.05 mg/ml) at 4°C for 30 min in the dark. A FACSort flow cytometer (BD Biosciences, USA) was used for accession of the processed cells. CellQuest software (BD Biosciences, USA) was used for data analysis.

RNA isolation, RT-PCR

Total mRNAs and miRNAs were extracted from tissues and cells using Trizol reagent and miRNeasy mini kit (Qiagen, USA) according to the manufacturer's protocol. For miRNA detection, reverse transcription (RT) and quantitative real-time PCR (qPCR) kits were performed. TaqMan microRNA Reverse Transcription Kit was used for RT reactions. Real-time PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen, USA), according to the manufacturer's instructions. The PCR reactions conditions were 95°C for 10 min, followed by 37 cycles at 95°C for 15 s, and 72°C for 30 s. The housekeeping gene GAPDH (for mRNA) or U6 snRNA (for miRNA) was used as a loading control.

Western blot analysis

Cells were harvested by RIPA lysis buffer and protease inhibitor cocktail (Roche). Each sample was electrophoresed on SDS-PAGE, transferred onto PVDF membrane (Millipore). After blocking with 5% BSA for 1 h at room temperature, blots were probed with corresponding primary (MEK, p-MEK, PI3K, p-PI3K, AKT, p-AKT, ERK, p-ERK and GAPDH at 1 : 1000) antibodies overnight at 4°C. HRP labeled secondary antibodies were probed for 1 h at room temperature. GAPDH (Sigma Aldrich, 1 : 10,000) was used as a loading control.

Statistical analysis

Data are displayed as the mean ± SD. All statistical analyses were performed using SPSS 17.0 statistical software (SPSS, Chicago, IL, United States). T-test was used to evaluate the difference between groups. A value of \( p < 0.05 \) was considered statistically significant.

Results

Clinical analysis

Compared with healthy tissues, the cancer cell invasion and migration of cancer tissues were increased by HE staining (Figure 1 A). IHC assay was used to evaluate the MEK protein in healthy and glioma tissues. The results showed the MEK protein in glioma tissues and healthy tissues. The ERK protein expression of glioma tissues was significantly up-regulated compared with that of healthy tissues (\( p < 0.05 \), Figure 1 B). Forty-five cases of glioma tissues and 20 cases of normal brain tissues were collected and used to investigate the expression differences of miR-133a. The results demonstrated that miR-133a in glioma tissues was greatly decreased compared with normal brain tissues (\( p < 0.05 \), Figure 1 C). However, The MEK gene expression of glioma tissues was significantly decreased compared with healthy tissues (\( p < 0.05 \), Figure 1 D). The miR-133a gene expression was negatively correlated with MEK gene expression (\( r = -5.5196 \), Figure 1 E).

miR-133a is positively correlated with patients' OS

Patients were followed up for up to 32.9 months. The relationship between miR-133a level and OS was analyzed. We found that the longer the patients' OS, the higher was the miR-133a level.

Table I. Primer sequences for PCR

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Sequence (5’–3’)</th>
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<tbody>
<tr>
<td>miR-133a:</td>
<td>Forward: 5’UUUGGUCUUCUUCAACACGGUG3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’UAAACAAAGGUAAMUGUGCGA3’</td>
</tr>
<tr>
<td>MEK:</td>
<td>Forward: 5’CTTCTACGGGGCCTTTCTACAGT3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’GCATGATCTGGTGCCTCTCTC3’</td>
</tr>
<tr>
<td>U6:</td>
<td>Forward: 5’GCCATGATCCTGCTTCTCTC3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’GTAGAGGCAGGGATGTCTT3’</td>
</tr>
</tbody>
</table>
Figure 1. Clinical analysis. A – The adjacent and cancer tissues of glioma were evaluated by HE staining (200×). B – The MEK protein expression was analyzed by IHC assay (200×); Healthy tissues were +; Glioma tissues were ++++. C – miR-133a in glioma tissues was strongly down-regulated compared with normal tissues. D – miR-133a in glioma tissues was strongly up-regulated compared with normal tissues. E – miR-133a was negatively correlated with MEK gene expression in glioma (r = –5.5196)
miRNA-133a has anti-tumor effects on glioblastoma

Figure 2. miR-133a is positively correlated with patients’ OS. A – The longer the patients’ OS, the higher the miR-133a level. B – In the high miR-133a group, the medium OS was 27.0 months, while in the low miR-133a group, the medium OS was 8.3 months.

FGF1 agonist rescues miR-133a mimic induced reduction of U87 cell proliferation

Cell proliferation in different groups was assessed by MTT assay. As with the effect of FGF1 inhibitor on U87 cell proliferation, the cell proliferation rate in U87 cells transfected with miR-133a mimic was also significantly decreased compared with the NC group, which was rescued by co-treatment with FGF1 agonist (Figure 3).

FGF1 agonist inhibits miR-133a mimic induced up-regulation of U87 cell apoptosis

FCM was used for evaluation of the apoptotic rate. As with the effect of FGF1 inhibitor on U87 cell apoptosis, the cell apoptosis rate in U87 cells transfected with miR-133a mimic was also significantly higher than that in the NC group, which was inhibited by co-treatment with FGF1 agonist. The corresponding data were analyzed and were consistent with changes of cell apoptosis (Figure 4).

FGF1 agonist inhibits miR-133a mimic induced up-regulation of U87 cell migration

Wound healing assay was performed to display cell migration of U87 cells in different groups. The width of wounds was measured at 0 h and 48 h after different treatments, respectively. There was no significant difference in wound width at 0 h in the 4 groups. Compared with the NC group, miR-133a mimic significantly increased U87 cell migration, which was remarkably decreased by co-treatment of FGF1 agonist, and FGF1 inhibitor showed a similar change with miR-133a mimic (Figure 6).

FGF1 agonist inhibits miR-133a mimic induced up-regulation of U87 cell invasion

Transwell assay was performed to display cell invasion of U87 cells in different groups. Compared with the NC group, miR-133a mimic significantly suppressed U87 cell invasion, which was remarkably decreased by co-treatment of FGF1 agonist, and FGF1 inhibitor showed a similar change with miR-133a mimic (Figure 7).

P-MEK/p-PI3K/p-AKT/p-ERK signaling pathways were responsible for the role of miR-133a in GBM

Compared with the NC group, protein levels of p-MEK/p-PI3K/p-AKT and p-ERK were decreased...
in the miR-133a mimic group, which was reduced by co-treatment of FGF1 agonist, and FGF1 inhibitor showed a similar change with miR-133a mimic (Figure 8).

Discussion

The current study showed that miR-133a played a protective role in GBM. A lower miR-133a level was found in glioma tissue than healthy tissue; moreover, miR-133a was positively correlated with patients’ OS. FGF1 agonist rescued miR-133a mimic induced reduction of U87 cell proliferation. FGF1 agonist inhibited miR-133a mimic caused up-regulation of U87 cell apoptosis, U87 cells that were at G1 stage and U87 cell migration. P-MEK/p-PI3K/p-AKT/p-ERK signaling pathways were regulated by miR-133a in GBM. We provided a potential therapeutic option for GBM.

GBM predominantly threatens patients’ life [5]. Its clinical hallmarks are aggressive proliferation and persistent recurrence due to invasive infiltration [9, 10]. After GBM diagnosis, in patients without treatment, OS is typically 3 months [11]; while in patients with treatment, 5-year OS occurs in less than 3–5% people and 12–15-month OS is the most common [6, 12].

Surgery is the first stage of GBM treatment. Because GBM cells are infiltrated through the brain at diagnosis, despite a “total resection” of tumor, tumors recur in most patients later. Subsequent
miRNA-133a has anti-tumor effects on glioblastoma

Figure 4. FGF1 agonist inhibits miR-133a mimic-induced up-regulation of U87 cell apoptosis. 

A – The cell apoptosis of different groups by flow cytometry. 
B – Similarly to the effect of FGF1 inhibitor, cell apoptosis rate in U87 cells transfected with miR-133a mimic was significantly higher than the NC group, which was inhibited by co-treatment with FGF1 agonist. The corresponding data were analyzed \( *p < 0.05 \).

To surgery, radiotherapy was the mainstay of treatment for GBM, which conducted along with temozolomide (TMZ) [22]. TMZ (chemotherapy) functions by sensitizing tumor cells to radiation [23]. However, GBM contains zones that are highly resistant to radiotherapy. Taken together, there is no effective therapy for GBM.

MicroRNAs function by lowering gene expression through binding to target sites in mRNA 3'-UTR. miR-133a downregulation led to an augmentation of contraction and BSM hyperresponsiveness [20]. Whether miR-133a plays a role in GBM was unknown. We first conducted RT-PCR to examine the miR-133a level in glioma tissues and healthy tissues. The results showed that in glioma tissues, the miR-133a level was much lower than in healthy tissues, which strongly suggested that miR-133a plays a potential role in GBM. However, it is unknown whether there is a correlation between miR-133a and patients' OS.

Based on the new finding, we were inspired to investigate the above question. We found that
with the elongation of patients' OS, the miR-133a level was increased. Meanwhile, the medium OS was 27.0 months (95% CI: 21.1–32.9 months) in the high miR-133a group, while the medium OS was 8.3 months (95% CI: 3.3–10.7 months) in the low miR-133a group, which suggested that miR-133a was positively correlated with patients' OS. We propose the hypothesis that miR-133a might also affect cell activities in vitro.

MTT assay showed that compared with the NC group, U87 cell proliferation was significantly reduced by miR-133a mimics. FCM showed that the cell apoptosis rate in U87 cells treated with miR-133a mimic was significantly higher than in the NC group. Cell cycle analysis demonstrated that U87 cells at G1 stage transfected with miR-133a mimic were significantly more numerous than those in the NC group, which was inhibited by co-administration of FGF1 agonist.

Figure 5. FGF1 agonist inhibits miR-133a mimic induced up-regulation of U87 cells at G1 stage. A – The cell cycle of different groups by flow cytometry. B – Analyzing the statistical data of different groups. Similarly to the effect of FGF1 inhibitor, cells at G1 stage in U87 cells transfected with miR-133a mimic were significantly more numerous than those in the NC group, which was inhibited by co-administration of FGF1 agonist.

*p < 0.05.
miRNA-133a has anti-tumor effects on glioblastoma.

Figure 6. FGF1 agonist inhibits miR-133a mimic induced up-regulation of U87 cell migration. 

A – 0 h and 48 h of cell morphology in different groups by wound healing assay. 

B – Wound width was measured at 0 h and 48 h. Compared with the NC group, miR-133a mimic significantly increased U87 cell migration, which was remarkably decreased by co-treatment of FGF1 agonist. Cells in FGF1 inhibitor treatment showed a similar change with miR-133a mimic. *p < 0.05.
In 1973, FGF was found in pituitary extracts [24]. In humans, 22 members of the FGF family have been identified; all are structurally related signaling molecules [25–27]. FGFs are multifunctional proteins that have regulatory, morphological, and endocrine effects. FGF1 is also known as acidic FGF. FGF1 has been shown clinically to induce angiogenesis in the heart [28]. FGF1 stimulates angiogenesis and the proliferation of fibroblasts, which fills up a wound space early during wound-healing progression.

In conclusion, in the current study, U87 cells were also co-administered with FGF1 agonist and miR-133a mimic. The results demonstrated that FGF1 agonist rescued miR-133a mimic induced reduction of U87 cell proliferation, inhibited miR-133a mimic caused up-regulation of U87 cell apoptosis, U87 cells that were at G1 stage and U87 cell migration. U87 cells treated with FGF1 inhibitor only showed similar effects with miR-133a mimic. Taken together, FGF1 was evidenced to be a potential target for miR-133a. Whereas the downstream molecules were unknown, we further conducted western blot to assess proteins that were related to GBM.

As acknowledged, PI3K/Akt and Ras-ERK pathways are activated in GBM. For instance, the activated Raf-MEK-ERK pathway was reported to enhance activity of GBM cells [13]. PI3K and ERK inhibitors could effectively treat GBM patients.
miRNA-133a has anti-tumor effects on glioblastoma

**Figure 8.** p-MEK/p-PI3K/p-AKT and p-ERK were responsible for the role of miR-133a in GBM. MEK/PI3K/AKT and ERK protein levels showed no significant changes. In the miR-133a group, p-MEK/p-PI3K/p-AKT and p-ERK protein levels were significantly higher than the NC group, which was obviously reduced by FGF1 agonist co-administration, and FGF1 inhibitor showed a similar change with miR-133a mimic.

* $p < 0.05$
PI3K/Akt inhibitors are therapeutic options for GBM [15, 16]. Compared with the NC group, protein levels of p-MEK/p-PI3K/p-AKT and p-ERK were decreased in the miR-133a mimic group, which was remarkably reduced by co-treatment of FGF1 agonist. Also, administration of FGF1 inhibitor only showed similar effects as the miR-133a mimic. These data suggested that miR-133a played a protective role in GBM via inhibiting activation of MEK/PI3K/AKT and ERK pathways, and might be an effective therapy for GBM.

Conflict of interest

The authors declare no conflict of interest.

References