MicroRNA-543 inhibits proliferation, invasion and induces apoptosis of glioblastoma cells by directly targeting ADAM9

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Received February 22, 2017; Accepted July 20, 2017

DOI: 10.3892/mmr.2017.7332

Abstract. Glioma is the most common type of malignant brain tumor in humans and accounts for 81% of all malignant brain tumor cases in adults. The abnormal expression of microRNAs (miRs) has been reported to be important in the formation and progression of various types of human cancer, including glioblastoma (GBM). Therefore, studies into the expression, and roles of microRNAs as diagnostic and prognostic markers, as well as their therapeutic value for patients with GBM are warranted. The expression and roles of miR‑543 have been reported in several types of human cancer. However, the role of miR‑543 in GBM remains unclear. In the current study, the expression pattern of miR‑543 in GBM, the effects of miR‑543 on GBM cells and the underlying molecular mechanism was determined. The results of the present study demonstrated that miR‑543 was significantly downregulated in GBM tissue samples and cell lines. Furthermore, the upregulation of miR‑543 inhibited GBM cell proliferation and invasion, as well as promoted cell apoptosis. In addition, a disintegrin and metalloproteinase 9 (ADAM9) was identified to be a direct target gene of miR‑543. Furthermore, ADAM9 was significantly upregulated in GBM tissue samples and its expression was inversely correlated with miR‑543 expression in GBM tissue, suggesting that miR‑543 downregulation may contribute to ADAM9 upregulation in GBM. Finally, the results of the rescue experiment indicated that ADAM9 overexpression significantly reversed the effects of miR‑543 on the proliferation, invasion and apoptosis of GBM cells, suggesting that miR‑543 serves as a tumor suppressor in GBM through ADAM9 regulation. Overall, these findings indicate that the miR‑543/ADAM9 signaling pathway may provide as a potential therapeutic strategy for GBM.

Introduction

Glioma is the most common type of malignant tumors in the brain; it originates from neural stromal cells and accounts for 81% of all malignant brain tumors in adults (1,2). The formation and progression of glioma is extremely complex and involves aberrant activation of proto-oncogenes and inactivation of tumor suppressor genes (3-5). Glioma could be graded from grade I to grade IV, based on the degree of malignancy according to the World Health Organization (WHO) classification (6). Glioblastoma (GBM), the highest-grade glioma, is characterized by a heterogeneous population of cells that are genetically unstable, highly infiltrative, angiogenic, and resistant to chemotherapy (7). Despite recent advances in multimodal therapies, the prognosis of GBM patients remains poor, with an average five-year survival rate of only 4 to 5% (8,9). The average survival time of GBM remains only 14.6 months following surgical treatment combined with radiation and chemotherapy (10). The high mortality of GBM is partially due to rapid growth, angiogenesis and metastasis of GBM cells (11,12). Therefore, improving understanding regarding the underlying mechanisms involved in GBM occurrence and progression, which would be essential to identify novel therapeutic methods for patients with this disease, is clinically significant.

MicroRNAs (miRNAs) are a group of endogenous, single-stranded and non-protein-coding short RNAs with approximately 19-24 nucleotides in length (13). miRNAs negatively regulate the gene expression through imperfect base pairing with the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs), resulting in translation repression or mRNA degradation (14). Each miRNA could directly modulate hundreds of target genes, and more than one-third of genes may be regulated by miRNAs (15). miRNAs play important roles in diverse biological processes, such as cell proliferation, cycle, death, differentiation, metastasis and drug resistance (16-18). The deregulation of miRNAs has been detected in a wide variety of human cancers, such as glioma (19), prostate cancer (20), gastric cancer (21), breast cancer (22) and lung cancer (23). Increasing evidence suggested that the abnormal expression of some specific miRNAs are closely related to tumorigenesis and tumor development, and some miRNAs also act as tumor suppressors or oncogenes depending on the characteristic of their target genes (24-26).
Therefore, further investigation of the expression and roles of miRNAs in GBM is important to develop novel and efficient therapeutic targets for patients with GBM.

The expression and roles of miR-543 have been reported in numerous types of human cancers (27-29). However, miR-543 had not been well studied in GBM. In our present study, we investigated the miR-543 expression pattern in GBM, the effects of miR-543 on GBM cells and the involved molecular mechanisms.

Materials and methods

Tissue sample and cell lines. This study was approved by the Ethical Committee of Shenzhen Second People's Hospital. All patients enrolled in this study gave written informed consent. A total of 19 cases of GBM tissues (age range, 31-69 years; median age, 45; twelve males and seven females) and 8 cases of normal brain tissues were obtained from Department of Neurosurgery, Shenzhen Second People's Hospital between February 2013 and May 2015. Normal brain tissues were collected from internal decompression patients treated with surgical operation. None of these patients had any radio-therapy or chemotherapy before surgical resection. All tissue samples were immediately snap-frozen and stored at -80°C until further use.

Cell lines and transfection. Four GBM cell lines U87, U251, LN229, and T98 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (both from Invitrogen, Carlsbad, CA, USA). Human astrocytes (NHAs) purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) were cultured in astrocyte medium (ScienCell Research Laboratories). All cells were grown at 37°C in a fully humidified atmosphere containing 5% CO₂ in air.

MiR-543 mimics and miRNA mimics negative control (NC) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). A disintegrin and metalloproteinase 9 (ADAM9) overexpressed plasmid (pcDNA3.1-ADAM9) and blank plasmid (pcDNA3.1) were synthesized by Chinese Academy of Sciences (Changchun, China). Cell transfection was performed by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or cells using TRIzol (Invitrogen) according to the manufacturer's protocol. To examine miR-543 expression levels, total RNA was transcribed by TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). qPCR was performed using TaqMan MicroRNA PCR kit on an ABI Prism 7500 Sequence Detection system (both from Applied Biosystems). To quantify miRNA expression of ADAM9, cDNA was synthesized using a First-Strand cDNA Synthesis kit (Invitrogen) and followed by qPCR with SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China). U6 and GAPDH was used as internal standard to normalize the expression of miR-543 and ADAM9 mRNA, respectively. The primers were designed as follows: miR-543 forward, 5'-CCAGCCTACATGGCCAGCAATTCA TGGTT-3' and reverse, 5'-CTCAACTTGTGGTGCTGGA-3'; U6 forward, 5'-CTCGGTTCCGCACGACATATACT-3' and reverse, 5'-AGCTTCAAGTTGCGGTCT-3'; ADAM9 forward, 5'-GCTAGTGGACTGAGATTTGG-3' and reverse, 5'-TTATACCCAGGGGAGGACAC-3'; GAPDH forward, 5'-CATCACCATTTCAAGGACGC-3' and reverse, 5'-TGACCTTGCCCCACAGCTTTG-3'. Relative expression was calculated using the 2^ΔΔCt method (30).

Cell Counting Kit-8 (CCK-8) assay. CCK-8 assays were carried out to assess the cell proliferation. Briefly, transfected cells were trypsinized, collected and seeded into 96-well plate at a density of 3,000 cells/well. Cells were then incubated at 37°C with 5% CO₂, and CCK-8 assay was performed on days 0, 1, 2, and 3. Ten microliters of CCK-8 were added to each well. The plates were incubated at 37°C for another 4 h. The absorbance was determined with a microplate reader (Bio-Rad, Richmond, CA, USA) at the wavelength of 450 nm. Each sample was performed in triplicate and repeated three times.

Transwell invasion assay. The invasion assay was performed using Transwell chambers (8 mm pores; Costar, Corning, NY, USA) pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). Following transfection for 48 h, 5×10⁴ cells were added into the top of the Transwell chambers in FBS-free DMEM, and DMEM containing 20% FBS was added to the lower chambers. The cells were incubated at 37°C with 5% CO₂ for 24 h. Cells that did not invade through the pores were removed from the top chambers with a cotton swab while the invasive cells were fixed with 100% methanol and stained with 0.1% crystal violet. The number of invasive cells was counted in five random visual fields under an inverted microscope (Olympus Corporation, Tokyo, Japan) and images were captured under x200 magnification.

Flow cytometry analysis. Transfected cells were collected at 48 h post-transfection, washed with ice-cold PBS and then fixed in 80% ice-cold ethanol. Subsequently, cells were re-suspended in cold PBS to a concentration of 1x10⁶ cells/ml. Annexin V-FITC apoptosis detection kit (Invitrogen) was used to examine cell apoptosis, as described by the manufacturer. The cell apoptotic rates of the transfected cells were determined using a flow cytometry (Beckman Coulter Inc., Brea, CA, USA).

Bioinformatics analysis. The candidate targets of miR-543 predicted by computer-aided algorithms were obtained from TargetScan (www.targetscan.org/) and miRanda (www.microrna.org).

 Luciferase reporter assay. Luciferase reporter plasmids, psiCHECK2-ADAM9-3'-UTR-wild type (Wt) and psiCHECK2-ADAM9-3'-UTR-mutant (Mut), were synthesized and purchased from GenePharma. For luciferase reporter assay, cells were co-transfected with the psiCHECK2-ADAM9-3'-UTR-Wt.
or psiCHECK2-ADAM9-3'-UTR-Mut, and miR-543 mimics or NC using Lipofectamine 2000, following to the manufacturer's instructions. After co-transfection for 48 h, the Dual-Luciferase Reporter Assay system (Promega, Manheim, Germany) was used to measure the activities of Renilla luciferase and firefly luciferase, according to the manufacturer's protocol. Renilla luciferase activity was normalized to firefly luciferase activity.

Western blot analysis. Total protein was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA), and the protein concentration was measured using the bicinchoninic acid protocol (Pierce, Rockford, IL, USA). Equal amounts of protein was loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Subsequently, the membrane was blocked with 5% skimmed milk in TBS/0.1% Tween (TBST) at room temperature for 1 h, and incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study were mouse anti-human monoclonal ADAM9 antibody (1:1,000 dilution; sc-377233; Santa Cruz Biotechnology, Santa Cruz CA, USA) and mouse anti-human monoclonal GAPDH antibody (1:1,000 dilution; ab125247; Abcam, Cambridge, UK), at 4°C overnight. Following a 2 h incubation with a goat-anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; Santa Cruz Biotechnology), the protein bands were visualized using the enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). GAPDH was used as a loading control.

Statistical analysis. Data were expressed as mean ± SD. Statistical analyses were performed using Mann-Whitney’s U test or one-way ANOVA with SPSS 16.0 software (SPSS, Chicago, IL, USA). P<0.05 was considered to be statistically significant.

Results

Reduced miR-543 expression levels in GBM tissues and cell lines. The expression of miR-543 in 19 cases of GBM tissues and 8 cases of normal brain tissues was investigated using RT-qPCR. Data were expressed as mean ± SD. Statistical analyses were performed using Mann-Whitney’s U test or one-way ANOVA with SPSS 16.0 software (SPSS, Chicago, IL, USA). P<0.05 was considered to be statistically significant.

Reduced miR-543 expression levels in GBM tissues and cell lines. The expression of miR-543 in 19 cases of GBM tissues and 8 cases of normal brain tissues was investigated using RT-qPCR to explore the potential roles of miR-543 in GBM. As shown in Fig. 1A, miR-543 expression level was significantly decreased in GBM tissue compared with that in normal brain tissues (P<0.05). The expression of miR-543 in four GBM cell lines (U87, U251, LN229, T98) and NHAs was determined to confirm the reduced miR-543 expression level in GBM tissues. miR-543 expression level was lower in GBM cell lines than that in NHAs (Fig. 1B, P<0.05). These results suggested that miR-543 was downregulated in GBM and may act as a tumor suppressor.

ADAM9 is a direct target of miR-543 in GBM. Bioinformatics analysis was conducted to identify the candidate target genes of miR-543 and to investigate the underlying mechanism by which miR-543 regulates GBM cell proliferation, invasion and apoptosis. Among these predicted targets, ADAM9, which is an important regulator for cancer formation and progression (31-33), attracted attention and was selected for...
Figure 2. Restoration expression of miR-543 inhibited tumour progression in GBM cells. (A and B) Expression levels of miR-543 in U87 and U251 cells transfected with miR-543 mimics or NC were assessed using RT-qPCR. *P<0.05 compared with NC. (C and D) Relative cell proliferation in U87 and U251 cells was examined after the cells transfected with miR-543 mimics or NC using CCK-8 assays. *P<0.05 compared with NC. (E and F) Effect of miR-543 on cell invasion was evaluated by using Transwell invasion assay. *P<0.05 compared with NC. (G and H) Apoptotic rate of U87 and U251 cells transfected with miR-543 mimics or NC were measured using flow cytometry analysis and compared with that of the cells involving NC. *P<0.05 compared with NC. GBM, glioblastoma; CCK-8, Cell Counting Kit-8.
Luciferase reporter assay was performed on U87 and U251 cells to validate interaction between miR-543 and the predicted binding site of ADAM9. The overexpression of miR-543 decreased wild-type (Fig. 3B, P<0.05) but not mutant ADAM9 reporter activity, suggesting that miR-543 specifically targeted the 3’UTR of ADAM9. To confirm whether ADAM9 are regulated by miR-543, the mRNA and protein expression of ADAM9 were examined in U87 and U251 cells transfected with miR-543 mimics or NC. Results showed that restoration expression of miR-543 reduced the ADAM9 expression levels in U87 and U251 cells at both mRNA and protein levels (Fig. 3C and D, P<0.05). These results suggested that ADAM9 is a direct target of miR-543 in GBM.

Further confirmation (Fig. 3A). Luciferase reporter assay was performed on U87 and U251 cells to validate interaction between miR-543 and the predicted binding site of ADAM9. The overexpression of miR-543 decreased wild-type (Fig. 3B, P<0.05) but not mutant ADAM9 reporter activity, suggesting that miR-543 specifically targeted the 3’UTR of ADAM9. To confirm whether ADAM9 are regulated by miR-543, the mRNA and protein expression of ADAM9 were examined in U87 and U251 cells transfected with miR-543 mimics or NC. Results showed that restoration expression of miR-543 reduced the ADAM9 expression levels in U87 and U251 cells at both mRNA and protein levels (Fig. 3C and D, P<0.05). These results suggested that ADAM9 is a direct target of miR-543 in GBM.

ADAM9 is upregulated in GBM tissues and inversely correlates with miR-543 levels. Further experiments were utilized to detect the ADAM9 expression in 19 cases of GBM tissues and eight cases of normal brain tissues. RT-qPCR and Western blot results revealed that ADAM9 mRNA and protein expression levels were higher in GBM tissues relative to that in normal brain tissues (Fig. 4A and B, P<0.05). Spearman’s correlation analysis was then performed to explore the relationship between miR-543 and ADAM9 mRNA expression in GBM tissues. As shown in Fig. 4C, ADAM9 mRNA was inversely correlated with miR-543 levels in GBM tissues (Fig. 4C, r=-0.6685, P=0.0018), suggesting that the downregulation of miR-543 may be an important cause for the upregulation of ADAM9 in GBM.

Upregulation of ADAM9 reverses the effects of miR-543 on GBM cell proliferation, invasion and apoptosis. Rescue experiments were performed in U87 and U251 cells to further explore the role of ADAM9 on the biological roles induced by miR-543 in GBM. Then, western blot analysis was used to measure the ADAM9 expression in U87 and U251 cells transfected with pcDNA3.1-ADAM9 or pcDNA3.1. Our data showed that ADAM9 protein was significantly upregulated in U87 and U251 cells after transfection with pcDNA3.1-ADAM9 (Fig. 5A, P<0.05). Functional rescue results showed that the reintroduction of ADAM9 reversed the effects of miR-543 overexpression on GBM cell proliferation (Fig. 5B and C, P<0.05), invasion (Fig. 5D and E, P<0.05) and apoptosis (Fig. 5F and G, P<0.05). These results indicated that miR-543 inhibits GBM cell proliferation and invasion, as well as promotes apoptosis by targeting ADAM9.

Discussion

Recently, accumulated studies demonstrated that abnormally expressed miRNAs may act as oncogenes or tumor suppressor genes and is important on the formation and progression of various human cancers, including GBM (34-36). Therefore, investigation on the expression and roles of miRNAs may have potential tumor diagnostic and prognostic values, as well as therapeutic values for patients with GBM (37). In our current study, miR-543 was lowly expressed in GBM tissues and cell lines. The resumption expression of miR-543 inhibited the proliferation and invasion, as well as enhanced the apoptosis of GBM cells. In addition, ADAM9 was identified as a novel target of miR-543. Therefore, miR-543 played tumor suppressing roles through the downregulation of ADAM9 in GBM.

miR-543 was aberrantly expressed in numerous types of human cancers. For example, in colorectal cancer, miR-543 was downregulated in tumor tissues and cell lines. The low miR-543 expression level was inversely correlated with the metastatic status of patients with colorectal cancer and the metastatic.
potential of colorectal cancer cell lines (27). miR-543 was
lowly expressed in endometrial cancer (28). However, contrary
to these results, miR-543 was upregulated in gefitinib-resistant
non-small cell lung cancer (29), hepatocellular carcinoma (38),
gastric cancer (39) and osteosarcoma (40). These conflicting
findings indicated that the expression pattern of miR-543 in
human cancer has tissue specificity.

miR-543 has been demonstrated to be a tumor suppressor.
Fan et al reported that the enforced expression of miR-543
suppressed the proliferation and metastasis of colorectal
cancer cells both in vitro and in vivo (27). Bing et al revealed
that miR-543 re-expression inhibited cell monolayer prolifera-
tion, anchorage-independent growth, migration and invasion
of endometrial cancer (28). However, in non-small cell lung
cancer, Bi et al found that miR-543 acts as an oncogene by
attenuating tumor cell proliferation and invasion (29). Yu et al
demonstrated that the upregulation of miR-543 enhanced the
cell growth and motility of hepatocellular carcinoma (38).
Li et al showed that the ectopic expression of miR-543
promoted the cell proliferation and cell cycle progression
of gastric cancer (39). Besides, the restoration expression
of miR-543 promoted the osteosarcoma cell growth in vitro
and in vivo (40).

These studies may appear contradictory, because miR-543
acted as an oncogene in certain types of cancer and a tumor
suppressor in others. These contradictory results may be
explained by the imperfect complementarity of the interac-
tions between miRNAs and their target genes (41).

Identification of cancer-specific miRNAs and their target
genes is pivotal for understanding their roles in tumorigen-
esis and tumor development (42,43). Several target genes
of miR-543 have been identified, such as KRAS, MTA1 and
HMGA2 in colorectal cancer (27), FAK and TWIST1 in
endometrial cancer (28), PTEN in lung cancer (29), PAQR3
in hepatocellular carcinoma (38), SIRT1 in gastric cancer (39)
and PRMT9 in osteosarcoma (40). Herein, we focused on
the mechanisms of miR-543 in regulating cell proliferation,
invasion and apoptosis of GBM cells. Bioinformatics analysis
was performed to search candidate targets of miR-543,
among which ADAM9 was predicated as a potential target
of miR-543. Luciferase reporter assay indicated that miR-543
directly targeted the 3'-UTR of ADAM9. Following, we
confirmed that ADAM9 mRNA and protein levels were
negatively regulated by miR-543 in GBM cells. Additionally,
ADAM9 was upregulated in GBM tissues and inversely
correlated with miR-543 expression level. Besides, rescue
experiments demonstrated that upregulation of ADAM9
rescued the effects induced by miR-543 overexpression on
GBM cells. These results suggest that miR-543 can directly
and negatively regulate ADAM9 expression by binding to the
3'-UTR of ADAM9.

ADAMs are members of the metzincin superfamily of
matrix metalloproteinases (MMP) (44). To date, 21 functional
ADAMs have been described in humans and 40 in different
organisms (45). ADAMs are involved in many different
biological functions, including fertilization, adhesion, migra-
tion and proteolysis (44,46). ADAM9 is a membrane-anchored
metalloproteinase and is one of the first ADAM proteins to
be identified and characterized. It consists of an N-terminal
prodomain followed by a metalloprotease domain, a disinte-
grin domain and cysteine-rich region, an epidermal growth
factor repeat, a transmembrane domain and a cytoplasmic tail
with potential SH3 ligand domains (47,48). Previous studies
reported that ADAM9 was upregulated in several types
of human cancers, such as renal cell cancer (31), non-small
cell lung cancer (49), prostate cancer (50), hepatocellular
carcinoma (32), colon cancer (33) and pancreatic cancer (51).
In glioma, ADAM9 was highly expressed in tumor tissues.
In addition, high expression levels of ADAM9 were signifi-
cantly correlated with poor prognosis in lower-grade glioma.
Furthermore, multivariate analysis indicated ADAM9 expres-
sion as an independent marker of poor survival (52). Functional
assays revealed that ADAM9 plays important roles in glioma
growth and metastasis (19,53). These studies all indicated that
ADAM9 may be a novel and promising target for therapeutic
intervention in patients with glioma.

Figure 4. ADAM9 expression was upregulated and negatively correlated with
miR-543 expression in GBM tissues. (A and B) RT-qPCR and western blot
analysis were performed to detect ADAM9 mRNA and protein expression in
GBM tissues and normal brain tissues. *P<0.05 compared with normal brain
tissues. (C) Negative relationship between ADAM9 mRNA and miR -543
expression was analysed by Spearman's correlation analysis in GBM tissues.
ADAM9, a disintegrin and metalloproteinase 9; GBM, glioblastoma.
Figure 5. Overexpression of ADAM9 reversed miR-543-induced tumour suppressive effects on GBM cell behaviours. (A) Western blot analysis showed that ADAM9 protein expression level was increased in U87 and U251 cells transfected with pcDNA3.1-ADAM9. *P<0.05 compared with pcDNA3.1. (B and C) CCK-8 assay was used to detect proliferation in U87 and U251 cells treated with NC, miR-543 mimics or miR-543 mimics+pcDNA3.1-ADAM9. *P<0.05 compared with NC and miR-543 mimics+pcDNA3.1-ADAM9. (D and E) Transwell invasion assay of U87 and U251 cells transfected with NC, miR-543 mimics or miR-543 mimics+pcDNA3.1-ADAM9. *P<0.05 compared with NC and miR-543 mimics+pcDNA3.1-ADAM9. (F and G) Flow cytometry analysis of U87 and U251 cells transfected with NC, miR-543 mimics or miR-543 mimics+pcDNA3.1-ADAM9. *P<0.05 compared with NC and miR-543 mimics+pcDNA3.1-ADAM9. ADAM9, a disintegrin and metalloproteinase 9; GBM, glioblastoma; CCK-8, Cell Counting Kit-8.
In conclusion, miR-543 was downregulated in GBM and played an important role in regulating GBM cell proliferation, invasion and apoptosis. Moreover, ADAM9 is the target gene of miR-543. These results suggested that miR-543/ADAM9 pathway may be investigated as an important strategy for the treatment of patients with GBM.

References