

MicroRNA-133a inhibits the malignant behavior of glioma via downregulation of matrix metalloproteinase 9

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Abstract. MicroRNA (miR)-133a expression has been reported to be downregulated in numerous human malignancies. However, the expression levels and function of miR-133a have not yet been investigated in human glioma. In the present study, the expression of miR-133a was analyzed by reverse transcription-quantitative polymerase chain reaction. Following transfection of miR-133a, cell proliferation, cell migration, cell invasion and luciferase assays, and western blot analysis were conducted in glioma cell lines. The present study demonstrated that miR-133a was downregulated in human glioma tissues compared with in normal adjacent tissues. In addition, the results indicated that miR-133a was likely to directly target matrix metalloproteinase 9 in glioma. These results suggest that miR-133a may be considered as a target for the treatment of human glioma.

Introduction

Gliomas are solid tumors of the central nervous system, which represent >30% of central nervous system tumors and account for 70% of malignant primary brain tumors (1,2). It is estimated that there are 20,000 newly diagnosed cases each year in the USA (2). Histologically, gliomas are divided into oligodendrogliomas, astrocytomas, anaplastic astrocytomas, glioblastomas, and numerous other subtypes (3). Malignant gliomas, including anaplastic astrocytomas and glioblastoma multiforme are the most common types of primary brain

tumor, accounting for ~60% of gliomas (4,5). The standard therapeutic strategies for glioma treatment, including surgical resection, and adjuvant radiotherapy and chemotherapy, have improved the 5-year survival rates from 2 to 10%; however, the prognosis for patients diagnosed at an advanced stage remains poor (6,7). It has previously been demonstrated that dysregulated tumor suppressor genes or oncogenes are closely associated with the development and progression of glioma (8). Therefore, more effective therapeutic strategies and targets for the treatment of glioma should be developed, alongside an increased understanding of the molecular pathogenesis of glioma.

Previous studies have indicated that microRNAs (miRNAs) are important in numerous aspects of human cancer (9-12). miRNAs are a class of non-coding RNA molecules (length, ~18-25 nucleotides), which induce mRNA degradation and suppress protein translation, predominantly by binding to the seed sequences in the 3'-untranslated region of mRNAs (13,14). miRNAs influence various physiological and pathological processes, including proliferation, differentiation, apoptosis, metabolism, migration and invasion (11,15).

It has previously been estimated that miRNAs are involved in almost every biological process by targeting ~30% of protein-coding genes in the human genome (16). Since the first miRNA expression profiles were obtained from a range of cancer types using bead-based flow cytometry, hundreds of evolutionarily conserved miRNAs have been identified in different species, including plants, animals and viruses (10,17). There is also an increasing amount of evidence suggesting that miRNAs may function as tumor suppressors and oncogenes (18). Downregulated miRNAs in cancer may normally function as tumor suppressor genes and inhibit cancer by regulating oncogenes. Conversely, upregulated miRNAs in cancer may function as oncogenes by negatively regulating tumor suppressors (19). Therefore, the identification of miRNA target genes is critical to understand the function of miRNAs in cancer development and progression. It has also been suggested that miRNAs may be a target for cancer therapy.

The expression levels of miR-133a have been reported to be downregulated in numerous human malignancies (20-28); however, to the best of our knowledge, the role of miR-133a

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in human glioma has not yet been investigated. In the present study, the expression levels and effects of miRNA-133a on human glioma were examined. The results of the present study demonstrated that miR-133a was downregulated in human glioma tumor tissues compared with their normal adjacent tissues (NATs), and miR-133a was able to suppress cell proliferation, migration and invasion by directly targeting matrix metalloproteinase 9 (MMP9). These findings may have therapeutic implications, and may be useful for the development of novel treatments for human glioma.

Materials and methods

Clinical specimens. The present study was approved by the ethics committee of The First Affiliated Hospital of Chongqing Medical University (Chongqing, China) and informed consent was obtained from all patients. The glioma and NAT specimens for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were obtained from 50 patients (32 male and 28 female; age range, 37-84 years) who had undergone surgery at Daping Hospital (Chongqing, China) between January 2010 and December 2014. Of the 50 gliomas, 22 were classified as low-grade [World Health Organization (WHO) I and WHO II] gliomas and 28 were classified as high-grade (WHO III and WHO IV) gliomas (29). None of the patients had received chemotherapy, immunotherapy or radiotherapy prior to specimen collection. Tissues were snap-frozen in liquid nitrogen and stored at -80°C.

Cell culture and transfection. The U251 and U87 human glioma cell lines were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). U251 and U87 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a 5% CO₂ cell incubator.

Mature miR-133a mimics, negative control (NC) miRNA mimics and the luciferase reporter plasmid were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence of miR-133a mimics was 5'-UUUGGU CCCCUUCAACCAGCUG-3'. The sequence of NC mimics was 5'-UUCUCCGAACGUGUCACGUTT-3'. Transient transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

RNA isolation and RT-qPCR analysis. The tissues were homogenized and the total RNA was extracted from tissues with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentration and purity of all RNA samples were measured using an ND-2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RT-qPCR analysis was performed using One Step SYBR® PrimeScript™ miRNA RT-PCR kit (Takara Bio, Inc., Otsu, Japan) in a CFX96™ Real-Time system and a C1000™ Thermal Cycler (both Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturers' protocols. The primers were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China) Each reaction was performed in a final

volume of 25 µl. The cycling conditions were as follows: 42°C for 5 min; 95°C for 10 sec; and 40 cycles of 95°C for 5 sec, 55°C for 30 sec and 70°C for 30 sec. Each sample was analyzed in triplicate and the data were normalized using the endogenous U6 small nuclear RNA. The relative expression of miR-133a was analyzed by use of the 2^{-ΔΔCq} method (30).

Cell proliferation assay. Cell proliferation was determined using Cell Counting kit-8 (CCK-8) detection kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The transfected cells (with miR-133a mimics or NC) were seeded at a density of 3x10³ cells/well into 96-well plates. Cell proliferation was assayed every 24 h for 5 days. Briefly, 10 µl CCK-8 solution was added to each well and incubated at 37°C for 2 h. Absorbance was measured at 450 nm in an ELISA reader (Elx800; Bio-Rad Laboratories, Inc.). The suppression rate of proliferation was calculated using the following formula: Suppression rate = (1 - OD_{miR-133a}/OD_{miR-NC}) x 100%. OD indicates optical density. All the experiments were performed in triplicate.

Cell migration and invasion assays. Migration and invasion of glioma cell lines were assessed using Transwell chambers with an 8-µm pore polycarbonate membrane (Costar; Corning Incorporated, Corning, NY, USA). For the migration assay, 5x10⁴ transfected cells (with miR-133a mimics and NC) were harvested and suspended in 200 µl DMEM with 0.1% FBS. These cells were placed into the upper chamber. A volume of 0.5 ml DMEM with 20% FBS was then added to the lower chamber as a chemoattractant. For the invasion assay, 5x10⁴ transfected cells were placed into the upper chamber, which was coated with Matrigel (BD Biosciences, San Jose, CA, USA). A volume of 0.5 ml DMEM with 20% FBS was then added to the lower chamber as a chemoattractant. Cells were incubated for a further 12 h for the migration assay and 24 h for the invasion assay. In the two assays, the invaded cells on the lower surface were fixed with 100% methanol (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China), stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China), and were then counted under an inverted microscope (CKX41; Olympus Corporation, Tokyo, Japan) to calculate the relative numbers (magnification, x100). Each experiment was repeated at least three times.

Western blotting. Mouse anti-human monoclonal primary antibodies against MMP9 (dilution, 1:1,000; sc-21733) and β-actin (dilution, 1:1,000; sc-47778), which were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), were used in the present study. Following 72 h of transfection with miR-133a or NC, the cells were washed twice with ice-cold phosphate-buffered saline and lysed with radioimmunoprecipitation assay lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM Na₃VO₄ and 1 mM NaF]. The protein concentration was measured using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Equal quantities of protein (20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology). For western blotting, the

membranes were blocked with 5% skimmed milk and incubated with the primary antibodies at dilutions specified by the manufacturer's protocols at 4°C overnight. The membranes were washed with Tris-buffered saline with 0.5% Tween 20 (TBST; Beyotime Institute of Biotechnology) and then incubated with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000; Santa Cruz Biotechnology, Inc.; sc-2005) in TBST. The protein bands were developed with enhanced chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) and imaged with a FluorChem imaging system (version 4.1.0; Alpha Innotech, San Leandro, CA, USA).

Luciferase assay. The U251 and U87 cells were transfected with 0.5 µg MMP9-3'-UTR-Wild type or MMP9-3'-UTR-Mutant, and 40 nmol miR-133a mimics or NC in a 12-well plate using Lipofectamine® 2000, according to the manufacturer's protocol. The activities of the firefly and *Renilla* luciferases in cell lysates were determined using the Dual-Luciferase Reporter assay system (Promega Corporation, Madison, WI, USA) 48 h post-transfection. The firefly luciferase activity was normalized to the *Renilla* luciferase activity for each transfected well. Each reporter plasmid was transfected at least three times (on different days) and each sample was assayed in triplicate.

Statistical analysis. Data are presented as the mean ± standard deviation. Data were analyzed using Student's t-test and analysis of variance in Stata 10.0 (StataCorp LP, College Station, TX, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-133a is downregulated in glioma tumor tissues compared with NATs. miR-133a expression levels were detected in all glioma tissues and NATs using RT-qPCR. As presented in Fig. 1A, miR-133a was significantly decreased in glioma tissues compared with NATs ($P < 0.01$). These results indicate that miR-133a may have an important role in glioma.

Furthermore, the present study investigated whether the expression levels of miR-133a were associated with tumor grade and stage. The statistical analysis demonstrated that the expression levels of miR-133a were significantly lower in high-grade glioma (WHO III and WHO IV) compared with in low-grade glioma (WHO I and WHO II; Fig. 1B; $P < 0.01$).

miR-133a suppresses cell proliferation in U251 and U87 glioma cells. To investigate the effects of miR-133a on glioma cell proliferation, the CCK-8 assay kit was used. Upregulation of miR-133a markedly inhibited cell proliferation in glioma U251 and U87 cells (Fig. 2; $P < 0.01$). These results indicate that miR-133a may function as a tumor suppressor in human glioma.

miR-133a suppresses cell migration and invasion in U251 and U87 glioma cells. To determine the influence of miR-133a on tumor cell migration and invasion, the Transwell assay was used. As shown in Fig. 3, the migratory and invasive ability of U251 and U87 glioma cells transfected with miR-133a was markedly decreased compared with those transfected with NC ($P < 0.01$). These results indicate that miR-133a may decrease the migration and invasion of glioma cells.

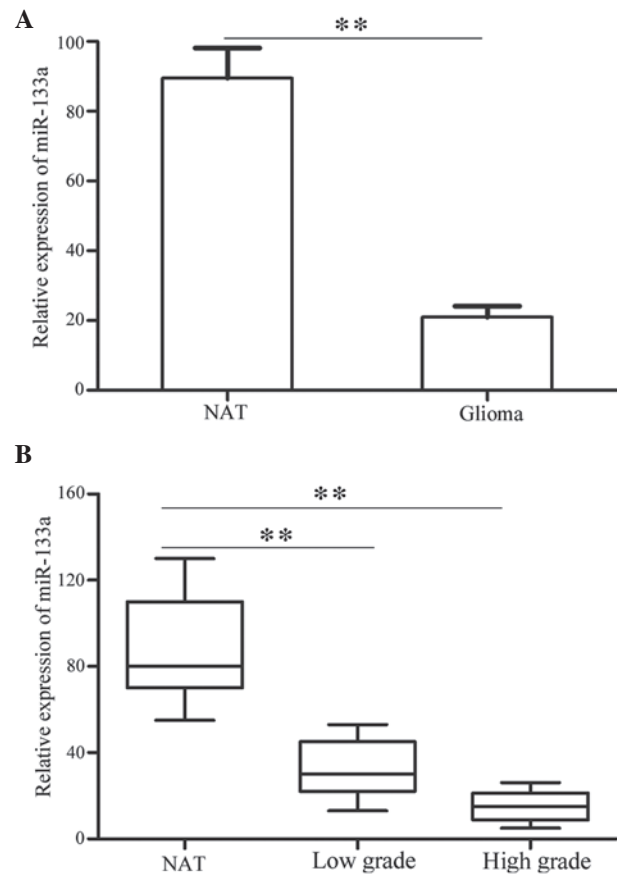


Figure 1. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-133a expression in glioma tissues and NATs. miR-133a expression is decreased in glioma tissues. Data are presented as the mean ± standard deviation. (B) Expression levels of miR-133a were significantly lower in high-grade glioma (WHO III and IV) compared with in low-grade glioma (WHO I and II). Data is presented in the box plot as mean and range. ** $P < 0.01$. NAT, normal adjacent tissue; WHO, World Health Organization; miR, microRNA.

MMP9 is a direct target gene of miR-133a in glioma cells. To identify the target of miR-133a in glioma cells, TargetScan (<http://www.targetscan.org>) was used and MMP9 was predicted to be a target of miR-133a (Fig. 4A). To verify whether miR-133a directly targets MMP9, luciferase reporter assays were performed. As presented in Fig. 4B, miR-133a significantly inhibited the luciferase activity of wild-type MMP9 but not that of mutant MMP9 in U251 and U87 glioma cell lines ($P < 0.01$).

Furthermore, western blotting was performed to investigate whether MMP9 was decreased following transfection of U251 and U87 glioma cells with miR-133a. As presented in Fig. 4C, MMP9 was significantly downregulated in U251 and U87 glioma cells post-transfection with miR-133a ($P < 0.01$). These results suggest that MMP9 may be a direct target gene of miR-133a in the U251 and U87 glioma cell lines.

Discussion

Previous studies have indicated that miRNAs are aberrantly expressed in several types of human cancer, and are widely involved in the regulation of cancer development (30,31). However, the underlying molecular mechanisms by which miRNAs regulate carcinogenesis and cancer progression remain

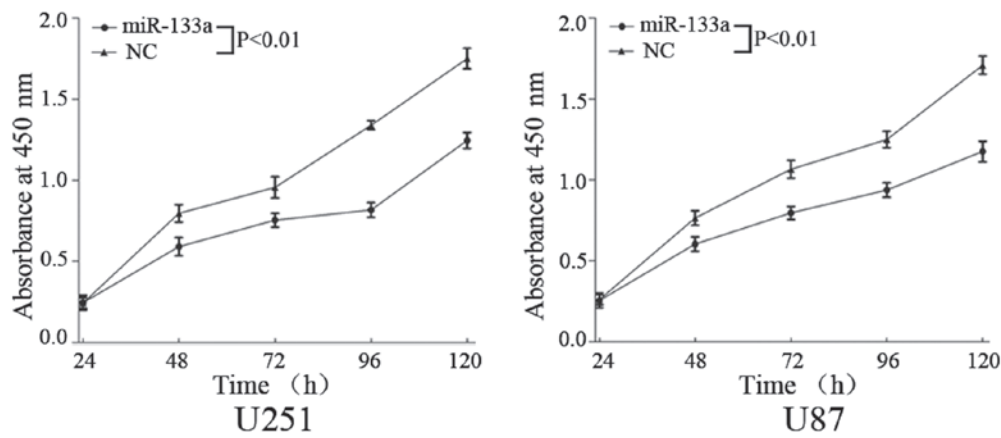


Figure 2. Cell Counting Kit-8 assay was performed to determine cell viability. Upregulation of miR-133a markedly suppressed cell proliferation in U251 and U87 glioma cell lines. Data are presented as the mean \pm standard deviation. miR, microRNA; NC, negative control.

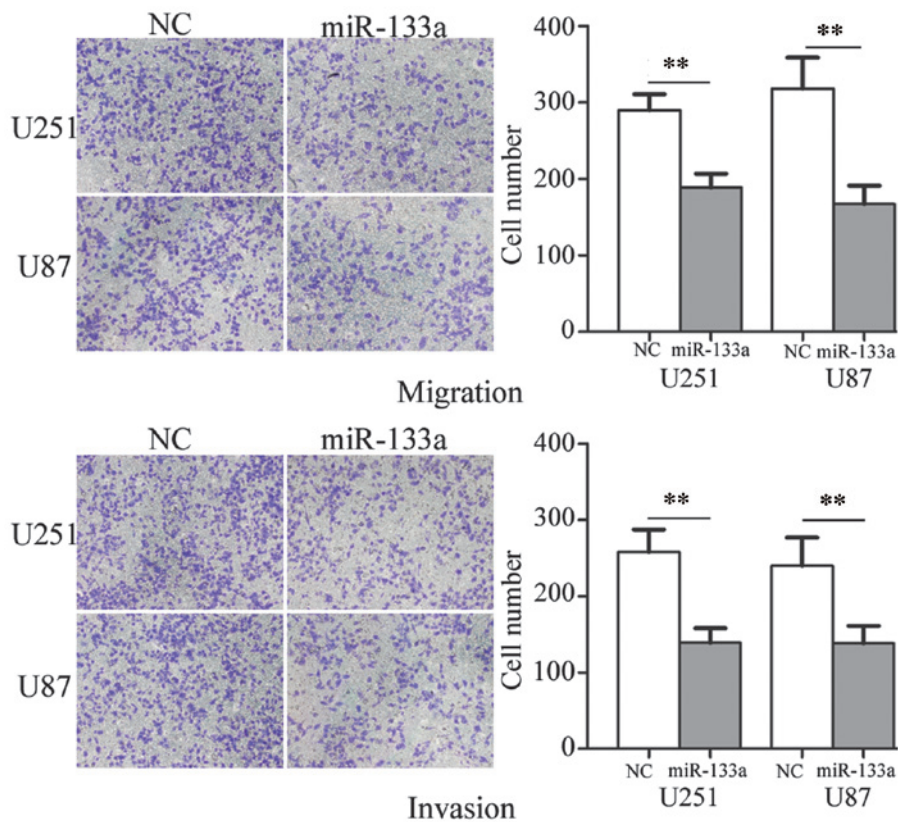


Figure 3. miR-133a inhibited cell migration and invasion in the U251 and U87 glioma cell lines. Subsequent to a 12 h incubation, the number of U251 and U87 cells that traversed the Transwell membrane was significantly decreased following transfection with miR-133a. Subsequent to a 24 h incubation, the number of U251 and U87 cells that traversed the Matrigel-precoated Transwell membrane was significantly decreased following transfection with miR-133a. Data are presented as the mean \pm standard deviation. ** $P < 0.01$. NC, negative control; miR, microRNA.

to be elucidated. Investigation into differentially expressed miRNAs in cancer tissue samples has provided important information to aid understanding of carcinogenesis (32). The present study is, to the best of our knowledge, the first to demonstrate that miR-133a is downregulated in human glioma, which led to the hypothesis that miR-133a may exert a tumor-suppressive effect in human glioma development and progression. By analyzing overexpression of miR-133a in human glioma cell lines, the present study verified that miR-133a reduced cell proliferation, migration and invasion, also suggesting a tumor

suppressive role of miR-133a. Therefore, these results may have clinical implications in the future.

miR-133 is one of the most studied and best characterized miRNAs. miR-133a and miR-133b are on chromosome 18 in the same bicistronic unit (20). miR-133a has been verified as a muscle-specific miRNA, which may modulate myoblast differentiation and participate in heart and myogenic diseases (33,34). It has been identified as downregulated in numerous human malignancies, including prostate cancer (21), bladder cancer (20), renal cell carcinoma (22), pancreatic

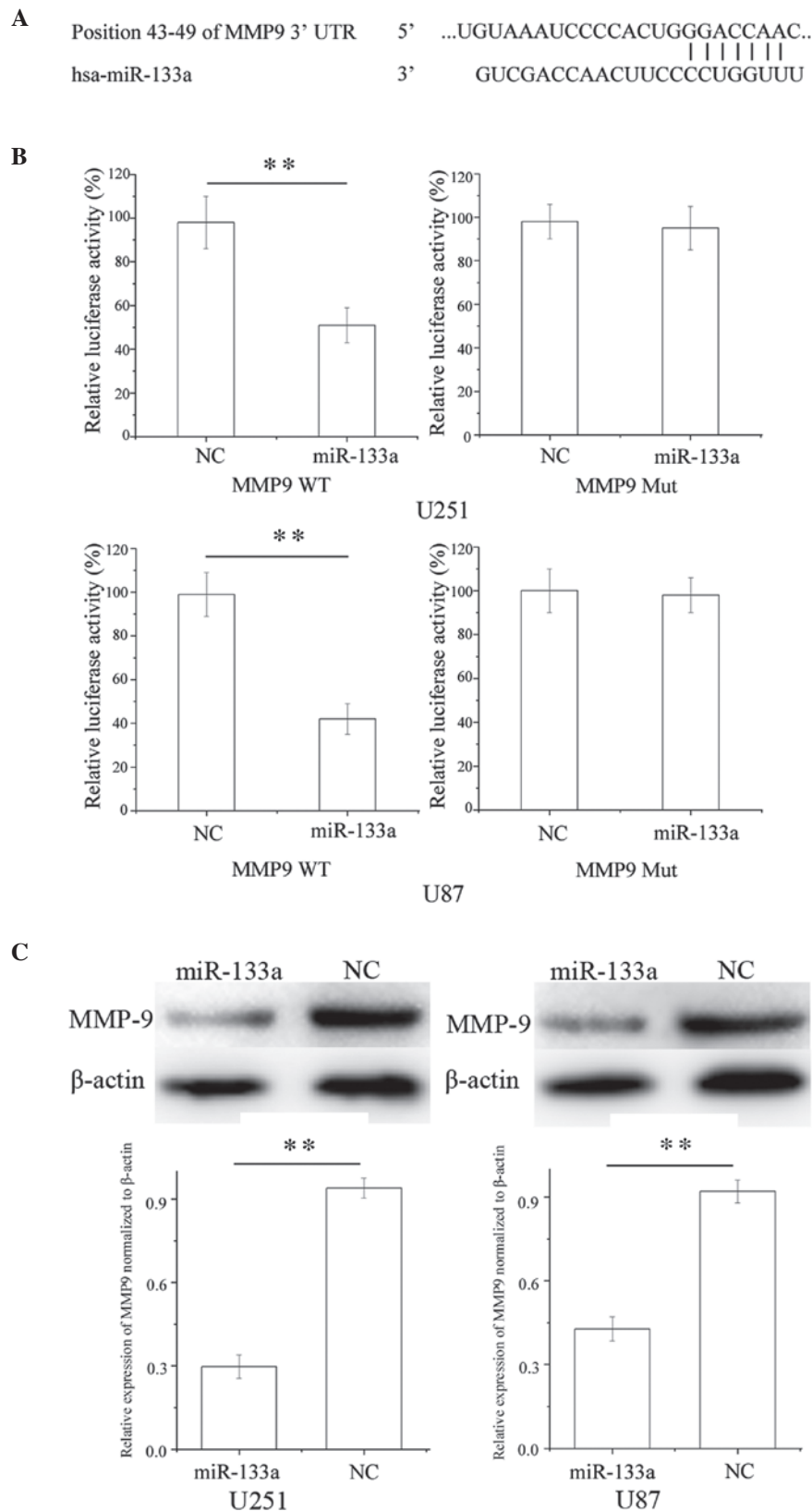


Figure 4. (A) TargetScan determined that MMP9 mRNA contains a seven-nucleotide seed match for miR-133a at position 43-49 of the MMP9 3'-UTR. (B) MMP9 may be a direct target of miR-133a *in vitro*. Overexpression of miR-133a significantly inhibited the WT but not the Mut luciferase activity of MMP9 in U251 and U87 glioma cell lines. (C) MMP9 was significantly reduced in U251 and U87 glioma cells following transfection with miR-133a. Data are presented as the mean \pm standard deviation. **P<0.01. UTR, untranslated region; MMP9, matrix metalloproteinase 9; miR, microRNA: WT, wild-type; Mut, mutant.

ductal adenocarcinoma (23), esophageal squamous cell carcinoma (24), ileal carcinoid tumors (25), rhabdomyosarcoma (26),

and hepatocellular and lung carcinomas (27,28). Recent studies have suggested that miR-133a inhibits cell proliferation,

migration and invasion in bladder and prostate cancer by targeting epidermal growth factor receptor (EGFR) and its downstream effector proteins (20,21). Previous studies have reported the effect of ectopic miR-133a on inhibition of cancer cell growth in lung squamous cell carcinoma, maxillary sinus squamous cell carcinoma, tongue squamous cell carcinoma, esophageal squamous cell carcinoma, renal cell carcinoma and rhabdomyosarcoma (22,26,32,35-37). It has been suggested that miR-133a may be important in these types of cancer and may serve as a potential therapeutic target for their treatment.

Identification of miR-133a target genes is critical for understanding its role in tumorigenesis. Studies have demonstrated that it may regulate oncogenic transcripts in human cells, including EGFR (21), moesin (38), Sp1 transcription factor (39), fascin actin-bundling protein 1 (40), insulin like growth factor-1 (41), glutathione S-transferase pi 1 (42), LIM and SH3 protein 1 (43) and transgelin 2 (44). The present study hypothesized that miR-133a may function as a tumor suppressor via downregulation of MMP9 in glioma. miR-133a transfection resulted in decreased cell viability, and reduced migration and invasion in human glioma cells. These results suggested that it may be useful in the development of novel molecular markers and therapeutic strategies to inhibit metastasis in glioma.

MMP9, which is a member of the zinc-dependent endopeptidases family, is a 92 kDa type IV collagenase and a key component in the basement membrane (45-47). Due to its extracellular matrix degrading characteristics, it has been demonstrated that upregulation of MMP9 facilitates metastatic spread of numerous human malignancies, and it appears to be one of the most important molecules to directly promote cancer metastasis (48). MMP9 has been shown to be enhanced or activated by oncogenic proteins in several types of human cancer, including prostate, breast, pancreatic and bladder cancer (47,49-52). MMP9 expression has also been detected in human glioma tissues, where it may facilitate multiple biological events required for glioma progression, including invasion, migration and dissemination of glioma cells (53). Furthermore, Yang *et al* (48) demonstrated that MMP9 expression is associated with higher WHO grade, which is a key prognostic factor for patients with glioma. Recent evidence has also suggested that MMP9 has a distinct role in tumor angiogenesis, predominantly by regulating the bioactivity of vascular endothelial growth factor, which is considered the most promising factor in interfering with tumor angiogenesis and, thus, a novel therapeutic target (54). In the present study, the results suggested that miR-133a suppressed glioma cell migration and invasion via downregulation of MMP9. Therefore, miR-133a may be useful as a predictive value for early detection of tumor metastasis, and as a therapeutic target for suppression of human glioma invasion.

In conclusion, the present study is the first, to the best of our knowledge, to demonstrate that miR-133a is downregulated in human glioma, and contributes to cell proliferation, migration and invasion by directly targeting MMP9 in human glioma. The identification of candidate target genes of miR-133a may provide further understanding regarding the potential carcinogenic mechanisms in human glioma. The findings of the present study have therapeutic implications and may be exploited for further research into the treatment of human glioma. Future investigations are required to address whether

miR-133a may be useful in cancer treatment, and may be a potential therapeutic target for the treatment of glioma.

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