MicroRNA-194 represses glioma cell epithelial-to-mesenchymal transition by targeting Bmi1

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Abstract. MicroRNA-194 (miR-194) is frequently dysregulated in many types of cancer. However, the function of miR-194 in glioma remains unknown. In the present study, we aimed to investigate the biological functions of miR-194 in glioma and the potential molecular mechanism of miR-194 involved in glioma progression. We found that miR-194 expression was significantly reduced in glioma specimens and cell lines, as detected by real-time quantitative polymerase chain reaction (RT-qPCR) analysis. The overexpression of miR-194 inhibited while the suppression of miR-194 promoted cell migration, invasion and epithelial mesenchymal transition (EMT) in glioma cells. Bioinformatics analysis showed that the B cell-specific moloney murine leukemia virus insertion site 1 (Bmi1) was a direct target of miR-194, which was validated by Dual-Luciferase reporter assay, RT-qPCR and western blot analysis. The restoration of Bmi1 expression significantly abrogated the suppressive effect of miR-194 on glioma cell EMT. Taken together, the present study suggests that miR-194 inhibits glioma cell EMT by targeting Bmi1 providing novel insights into understanding the pathogenesis of glioma. The restoration of miR-194 may be a potential therapeutic strategy for glioma treatment.

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Abbreviations: Bmi1, B cell-specific moloney murine leukemia virus insertion site; EMT, epithelial-to-mesenchymal transition; FBS, fetal bovine serum; miRNAs, microRNAs; RT-qPCR, real-time quantitative polymerase chain reaction; UTR, 3'-untranslated region

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Key words: glioma, miR-194, Bmi1, epithelial-to-mesenchymal transition

Introduction

Glioma is one of the most prevalent brain tumors, with a high yearly mortality rate worldwide (1,2). In spite of the current advances in cancer treatments, glioma is still an intractable disease due to the rapid growth, angiogenesis and metastasis of glioma cells (3,4). The overall survival has not been significantly improved during the past decades (4,5). Therefore, there is an urgent need to develop new therapies for glioma. However, the mechanism of glioma tumorigenesis is still not fully understood that hampers the development of new treatments for glioma.

In recent years, microRNAs (miRNAs), a group of small and non-coding RNA, have emerged as potential and promising tools for cancer diagnosis and treatments (6,7). miRNAs can regulate target gene expression by interacting with the 3'-untranslated region (UTR) of the mRNA that inhibits translation or induces mRNA degradation (8,9). Thus, miRNAs function as native regulators of gene expression. miRNAs are capable of modulating cancer cell proliferation, apoptosis, differentiation, migration and invasion by targeting oncogenes or tumor suppressors (10). There is increasing evidence suggesting that various miRNAs are dysregulated in glioma, which function as oncogenes or tumor suppressors (11,12), highlighting their potential as novel agents for the treatment of glioma. However, the precise role of miRNAs in glioma remains largely unknown.

The B cell-specific moloney murine leukemia virus insertion site 1 (Bmi1) is a self-renewal gene that is overexpressed in multiple human cancers (13). Bmi1 is a key component of the polycomb regulatory complex-1 regulating the transcription of a variety of important genes (14,15). The inhibition of Bmi1 induces tumor cell apoptosis and increases susceptibility to chemotherapy and radiation therapy, indicating an oncogenic role of Bmi1 (16,17). Bmi1 can induce cell transformation and promote cancer proliferation *in vivo* and *in vitro* through transcriptional repression of tumor suppressor genes including p16^{INK4A} and p14^{ARF} (18-20). The epithelial-to-mesenchymal transition (EMT) is an essential process for cancer invasion and metastasis (21). Notably, a recent study has shown that Bmi1 plays an important role in inducing EMT (22-24). Bmi1 plays an important role in glioma tumorigenesis through promoting the invasion, migration, angiogenesis and proliferation of glioma cells (25-28). Therefore, targeted therapy on

Bmi1 may have promising and potential therapeutic values on glioma treatment.

Emerging evidence has suggested that miR-194 functions as a tumor suppressor in many types of human cancers including lung cancer (29), hepatocellular carcinoma (30) and colorectal cancer (31). However, the role of miR-194 in glioma remains unclear. In the present study, we aimed to explore the biological role of miR-194 and the underlying molecular mechanism in regulating glioma procession. We found that miR-194 expression was significantly decreased in glioma tissues and cell lines. Overexpression of miR-194 inhibited while the suppression of miR-194 promoted cell migration, invasion and EMT of glioma cells. Bioinformatic analysis indicated that Bmi1 was the direct target of miR-194 in glioma cells by which miR-194 regulated the glioma EMT process. Our findings provide a better understanding of the mechanism of glioma development and suggest that targeting Bmi1 by miR-194 may serve as an attractive treatment method for glioma.

Materials and methods

Tissue specimens and cell line. A total of 20 glioma specimens were histopathologically diagnosed at the Department of Neurosurgery, the Second Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). The specimens were resected prior to any therapeutic treatment. Normal brain tissues obtained from internal decompression patients undergoing surgical operation were used as a control. Prior written informed consent of the patients and approval were obtained from the Institutional Human Experiment and Ethic Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. This study was performed in accordance with the Helsinki Declaration. U87, U251, SHG44 and A172 glioma cell lines and normal human astrocytes (NHA) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin mix (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. For the analysis of Bmi1 expression, RNA was reverse-transcribed into cDNA by Moloney murine leukemia virus (M-MLV) reverse transcriptase (Takara Bio, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. For the analysis of miR-194 expression, RNA was reverse-transcribed into cDNA by TaqMan MicroRNA reverse transcription kit (Applied Biosystems, Inc., Carlsbad, CA, USA). Small nuclear RNA U6 was used for normalization. The PCR amplification was performed by using the SYBR-Green Master Mix kit (Bio-Rad Laboratories, Hercules, CA, USA) on an Applied Biosystems AB 7500 real-time PCR system (Applied Biosystems). The primers used were as follows: miR-194, forward, 5'-atggacctggggccacgaag-3' and reverse, 5'-tctggcct

gggagcgtcg-3'; U6, forward, 5'-cgcttcggcagcacatatactaa-3' and reverse, 5'-tatggaacgcttcacgaatttgc-3'; Bmi1, forward, 5'-atgca tcgaacaacgagaatcaagatcact-3' and reverse, 5'-tcaaccagaagaa gttgctgatgaccc-3'; and GAPDH, forward, 5'-ccatgttcgtcatggtg tg-3' and reverse, 5'-ggtgctaagcagttggtggtg-3'. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized against U6 or GAPDH and then compared with the control group.

Oligonucleotides, plasmids and transfection. miR-194 mimics, miR-194 inhibitor and their negative controls were purchased from Shanghai GenePharma, Co., Ltd. (Shanghai, China). The open reading frame of Bmi1 was inserted into the pcDNA3.0 vector (Invitrogen). All the transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Migration and invasion assay. For cell migration, $1x10^5$ cells transfected with miR-194 mimics or miR-194 inhibitors were placed in the upper chambers of a Transwell (Costar, Cambridge, MA, USA) with serum-free medium and the lower chambers were filled with medium containing 10% FBS. For cell invasion, the upper chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA). After being cultured for 24 h at 37˚C, the non-migrated or non-invaded cells on the top of the well were gently removed and the cells on the lower surface of the membrane were fixed with 70% ethanol and stained with 0.1% violet (Sigma-Aldrich). The cells were observed and counted under a microscope (Olympus Corp., Tokyo, Japan).

Western blot analysis. Proteins were extracted by cell lysis buffer and the protein concentration was detected by a BCA kit (Beyotime Institute of Biotechnology, Haimen, China). A total of 50 μg protein was separated by SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). The membrane was blocked by 5% non-fat milk and then incubated with primary antibodies at 4˚C overnight. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (Bosis, Beijing, China) for 1 h at 37˚C. Ultimately, the membrane was detected by an ECL western blotting kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The image was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The values were normalized against GAPDH and then were compared with the control group. Primary antibodies used in this experiment including anti-E-cadherin, anti-vimentin, anti-Bmi1 and anti-GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Dual-Luciferase reporter assay. The Bmi1 3'-UTR harboring the seed-matched sequences of miR-194 with or without mutation was inserted into pmirGLO Dual-Luciferase vector (Promega Corp., Madison, WI, USA). Glioma cells were seeded into 24-well plates and co-transfected with miR-194 mimics or miR-194 inhibitor and the constructed wild-type or mutant pmirGLO-Bmi1 3'-UTR into glioma cells using Lipofectamine 2000. After 48 h, the luciferase activity was analyzed by a Dual-luciferase reporter assay system kit (Promega).
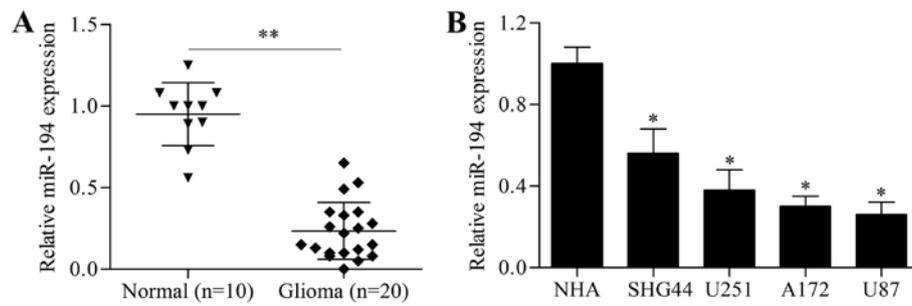
Figure 1. The expression of miR-194 in glioma tissues and cell lines. (A) Relative miR-194 expression in glioma tissues and normal brain tissues was examined by RT-PCR. **P<0.01 vs. normal. (B) RT-qPCR analysis of miR-194 in glioma cell lines (U251, SHG44, A172 and U87) and normal human astrocytes (NHA). *P<0.05 vs. NHA.
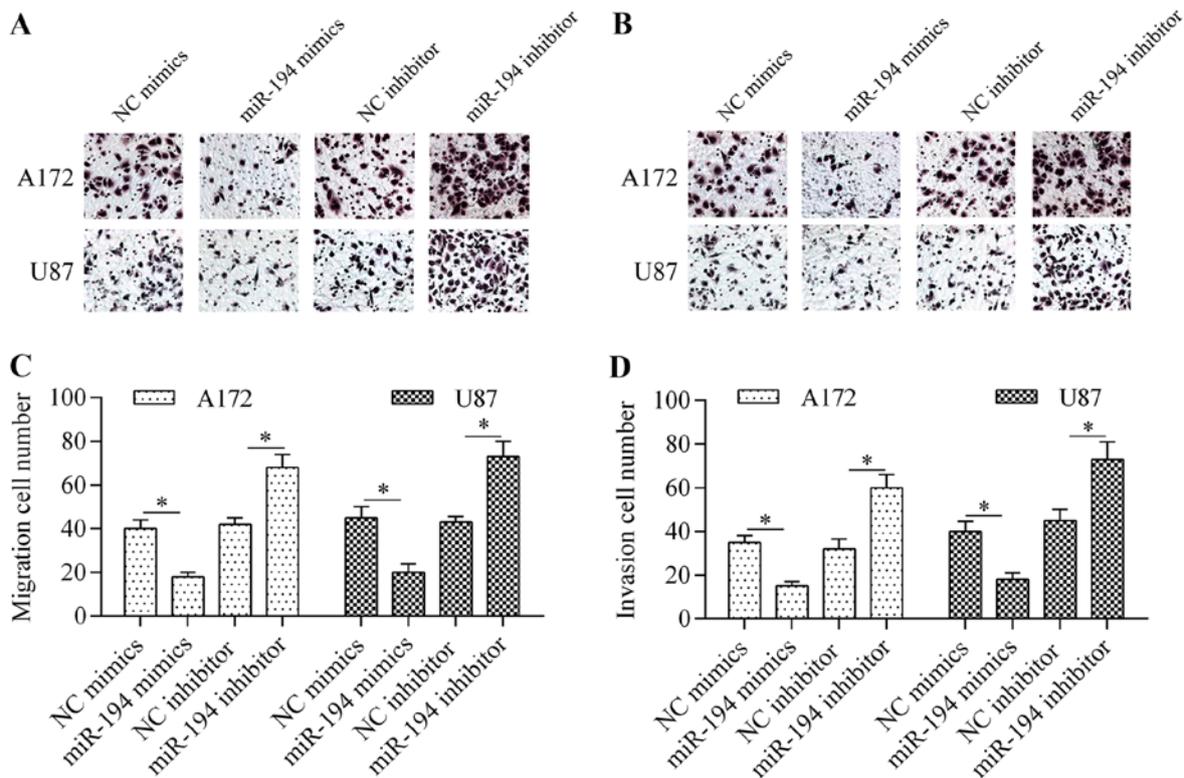

Figure 2. miR-194 represses glioma cell migration and invasion. A172 and U87 cells were transiently transfected with miR-194 mimics or miR-194 inhibitors. (A) Glioma cell migration and (B) invasion were detected by Transwell assay. (C) Quantitative cell number of migrated cells. (D) Quantitative cell number of invaded cells. *P<0.05.

Data analysis. Data are reported as means ± standard deviation (SD). Statistical analyses were performed using SPSS version 11.5 software (SPSS, Inc., Chicago, IL, USA). Differences were calculated by the Student's t-test or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc. When the P-value was <0.05, the difference was considered to be statistically significant.

Results

miR-194 is frequently lower in glioma tissues and cell lines. To determine whether miR-194 is involved in glioma development, we first analyzed the expression patterns of miR-194 in glioma specimens by RT-qPCR. The results showed that miR-194 was frequently downregulated in glioma tissues compared with normal brain tissue (Fig. 1A). Moreover, the expression levels of miR-194 were also decreased in the four glioma cell lines examined, particularly in A172 and U87, when compared with normal human astrocytes (Fig. 1B). These results indicate that miR-194 may play an important role in glioma.

miR-194 suppresses glioma cell migration and invasion. To investigate the biological role of miR-194 in glioma, we performed gain- and loss-of-function experiments by transiently transfecting miR-194 mimics or miR-194 inhibitors in A172 and U87 cells. We then detected the functional effect of miR-194 on glioma cell migration and invasion by Transwell assay. The results showed that miR-194 overexpression significantly inhibited glioma cell migration (Fig. 2A and C) and invasion (Fig. 2B and D) in A172 and U87 cells. Conversely,
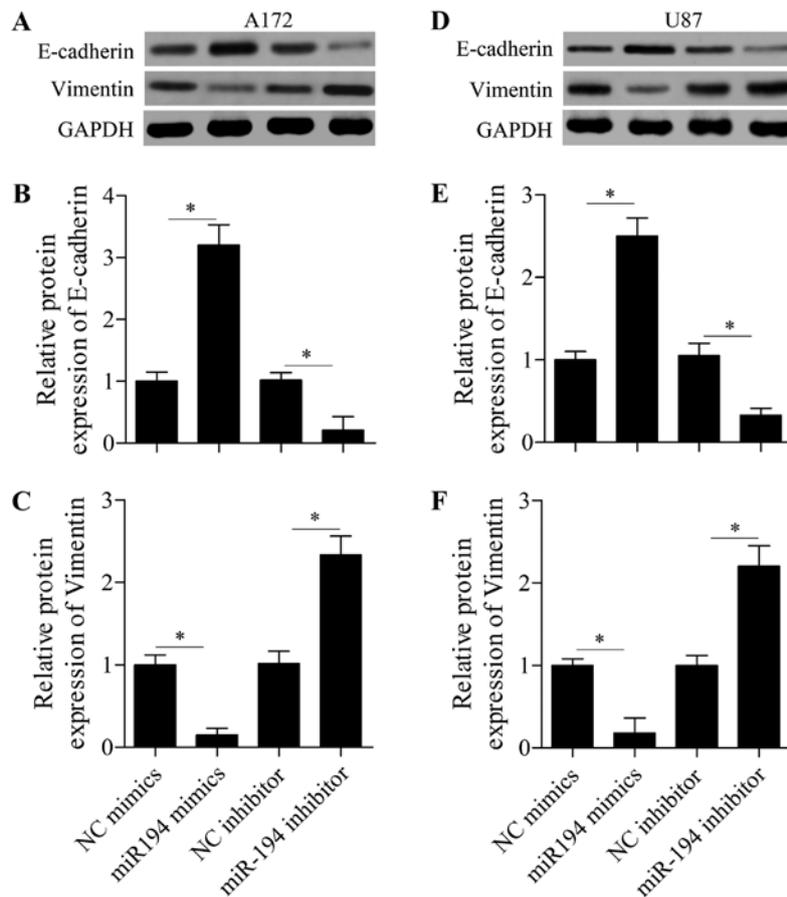
Figure 3. miR-194 inhibits glioma cell EMT. The effect of miR-194 overexpression or suppression on E-cadherin and vimentin was detected by western blot analysis in A172 (A-C) and U87 (D-F) cells. The relative expression of proteins was quantified by Image-Pro Plus 6.0 software. *P<0.05.
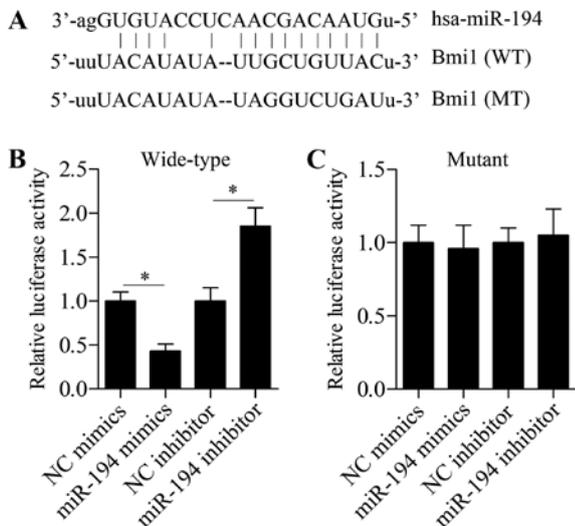

Figure 4. miR-194 targets the 3'-UTR of Bmi1. (A) Schematic representation of the seed region between miR-194 and Bmi1 3'-UTR. (B and C) Dual-Luciferase report assay. U87 cells were co-transfected with the miR-194 mimics or miR-194 inhibitors and report vectors containing wild-type (WT) or mutant (MT) Bmi1 3'-UTR. After 48 h, the luciferase activity was analyzed by a dual-luciferase reporter assay system kit. *P<0.05.

miR-194 suppression markedly increased glioma cell migration (Fig. 2A and C) and invasion (Fig. 2B and D). These data imply a suppressive role of miR-194 on glioma metastasis.

miR-194 suppresses EMT of glioma cells. To further investigate the contribution of miR-194 on glioma metastasis, we examined the biological role of miR-194 in regulating glioma cell EMT. Western blot analysis showed that the expression of epithelial marker E-cadherin was significantly increased, whereas expression of the mesenchymal marker vimentin was markedly decreased by miR-194 overexpression in A172 cells (Fig. 3A-C). In contrast, miR-194 suppression showed the opposite effects (Fig. 3A-C). Similar results were also observed in U87 cells (Fig. 3D-F), suggesting a suppressive role of miR-194 in glioma cell EMT.

miR-194 directly targets Bmi1. To investigate the underlying molecular mechanism by which miR-194 regulates glioma cell EMT, we used bioinformatic analysis to identify the potential target of miR-194. Among these predicted target genes, Bmi1, an important regulator for cancer metastasis (22-24), caught our attention. The predicted binding site between miR-194 and Bmi1 3'-UTR is illustrated in Fig. 4A. To verify that Bim1 is a direct target gene of miR-194, we performed a Dual-Luciferase reporter assay. The 3'-UTR fragments of Bmi1, which contain a wild-type or mutant binding site of miR-194, were cloned into the luciferase reporter vector. Overexpression of miR-194 significantly restrained the luciferase activity in U87 cells transfected with luciferase reporter vector containing wild-type Bmi1 3'-UTR, whereas miR-194 suppression promoted the luciferase activity (Fig. 4B). However, neither miR-194
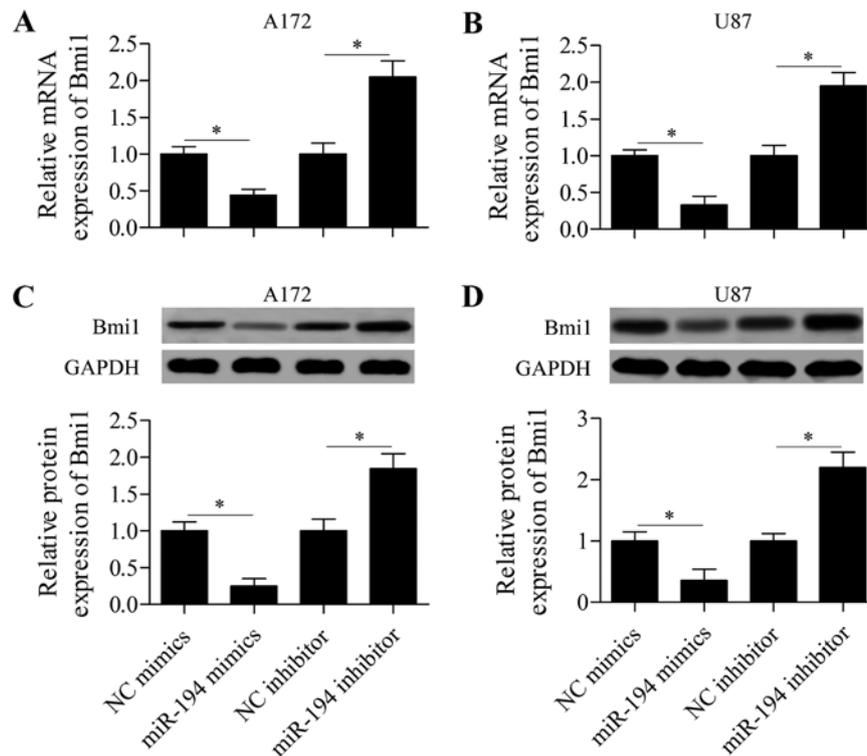
Figure 5. miR-194 regulates Bmi1 expression. A172 and U87 cells were transfected with miR-194 mimics or miR-194 inhibitors for 48 h. The mRNA expression of Bmi1 in (A) A172 and (B) U87 cells was detected by RT-qPCR analysis. The protein expression of Bmi1 in (C) A172 and (D) U87 cells was detected by western blot analysis. The relative expression of proteins was quantified by Image-Pro Plus 6.0 software. $^{*}P<0.05$.

overexpression nor suppression showed an obvious effect on luciferase activity of the luciferase reporter vector containing a mutant 3'-UTR (Fig. 4C). Next, we further determined the direct effect of miR-194 on regulating Bmi1 expression. RT-qPCR analysis showed that the mRNA expression of Bmi1 was significantly decreased or increased by miR-194 overexpression or suppression, respectively (Fig. 5A and B). Consistently, western blot analysis identified that Bmi1 protein expression was regulated by miR-194 (Fig. 5C and D). Taken together, these results suggest that miR-194 regulates Bmi1 expression by directly targeting the 3'-UTR of Bmi1.

miR-194 inhibits glioma EMT through downregulating Bmi1. To verify whether the tumor suppressor role of miR-194 in glioma is mediated by suppressing the expression of Bmi1, we performed a rescue assay. Glioma cells were co-transfected with miR-194 mimics and Bmi1-overexpressing vector. The decreased protein expression of Bmi1 induced by miR-194 overexpression was significantly restored by transfection with a Bmi1-overexpressing vector (Fig. 6A and E). As expected, the inhibitory role of miR-194 on glioma EMT was rescued by Bmi1 overexpression (Fig. 6A-H). These results suggest that the suppressive role of miR-194 on glioma EMT is mediated by inhibiting Bmi1.

Discussion

It has now been clearly established that miRNAs play an important role in regulating the tumorigenesis and metastasis of glioma (32-34). Therefore, the identification of glioma-associated miRNAs as biomarkers for glioma diagnosis, prognosis

and treatment is of great importance. In the present study, we found that miR-194 was a novel glioma-associated miRNA that was decreased in glioma specimens and cell lines. The functional experiments indicated that miR-194 inhibited glioma cell migration, invasion and EMT, possibly through targeting and regulating Bmi1. Collectively, our data suggest that miR-194 plays an important role in glioma procession, indicating a novel therapeutic target for glioma.

Growing evidence has demonstrated that miR-194 is a tumor-associated miRNA that is frequently dysregulated in many types of human cancers. miR-194 expression was markedly decreased in hepatocellular carcinoma and miR-194 overexpression inhibited cell proliferation by targeting mitogen-activated protein kinase 4 (30). Other studies reported that miR-194 was frequently downregulated in colorectal cancer (35,36). miR-194 can inhibit colorectal carcinogenesis by targeting mitogen-activated protein kinase 4 (37) or Akt2 (31). Several studies have reported that miR-194 represses the progression and metastasis of non-small lung cancer through targeting bone morphogenetic protein 1 and p27[kip1] (38), forkhead box A1 protein (39) and human nuclear distribution protein C (29). miR-194 can suppress gastric cancer cell proliferation and EMT through targeting RING box protein 1 (40) and forkhead box protein M1 (41). The tumor suppressive role of miR-194 is also observed in oral squamous cell carcinoma (42), bladder cancer (43) and clear cell renal cell carcinoma (44). However, the role of miR-194 in glioma remains unknown. In line with these findings, this study demonstrated that miR-194 functioned as a tumor suppressor in glioma. We found that miR-194 was decreased in glioma tissues and cell lines. Overexpression of miR-194
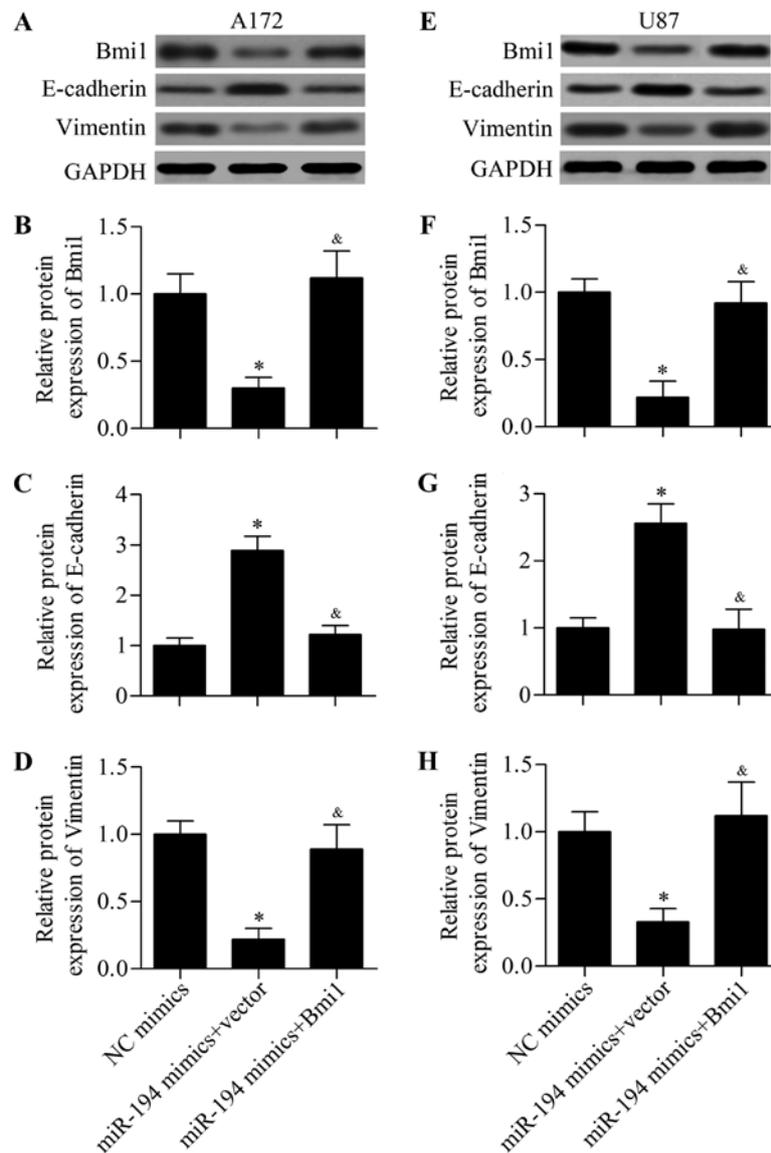
Figure 6. miR-194 functions a tumor suppressor through Bmi1. Cells were co-transfected with miR-194 mimics and pcDNA-Bmi1 vector encoding the full-length coding sequence without the 3'-UTR region. The expression levels of Bmi1, E-cadherin and vimentin in (A-D) A172 and (E-H) U87 cells were detected by western blot analysis. The relative expression of proteins was quantified by Image-Pro Plus 6.0 software. *P<0.05 vs. NC mimics; &P<0.05 vs. miR-194 mimics+vector.

inhibited glioma cell migration, invasion and EMT through inhibiting Bmi1. However, an oncogenic role of miR-194 is observed in pancreatic ductal adenocarcinoma that promotes tumor growth and progression through inhibiting the tumor suppressor DACH1 (45). Therefore, the precise role of miR-194 in cancer progression requires further investigation.

Bmi1 has been suggested as an oncogene that is frequently elevated in various cancer types and is correlated with a worse prognosis (13). Bmi1 has been reported to inhibit tumor suppressor genes including p14^{ARF}, p16^{INK4a}, KIF1Bβ and TSLC1 (46). Bmi1 is a critical regulator for EMT and cancer metastasis (47). Knockdown of Bmi1 abolishes the ionizing irradiation-induced EMT of breast cancer cells (48). Twist1 and Bmi-1 can act cooperatively to inhibit E-cadherin and p16^{INK4a}, leading to EMT induction (49). Bmi1 plays an important role in development of the central nervous system; however, the dysregulation of Bmi1 contributes to the development of brain tumors (50,51). Overexpression of Bmi1 was

found in human medulloblastomas, correlated with activation of the sonic hedgehog pathway (50). Bmi1 is highly elevated in glioma tissues and patients with high levels of Bmi1 show a poor survival rate (52). Bmi1 promotes glioma cell migration and invasion via nuclear factor kappa B and p16 (25,28,53). Consistently, our results showed that the inhibition of Bmi1 by miR-194 significantly suppressed the glioma cell migration, invasion and EMT, further implying that Bmi1 can serve as a therapeutic target for preventing glioma metastasis.

In the present study, we demonstrated that miR-194 functions as an EMT regulator in glioma via targeting Bmi1. We showed that miR-194 could directly target the 3'-UTR of Bmi1 and inhibit Bmi1 expression. Our findings are consistent with a previous study which revealed that miR-194 suppresses the EMT of endometrial cancer cells by targeting Bmi1 (54). The present study further confirms that Bmi1 is a functional target of miR-194. A recent study (55) suggested that targeting Bmi1 by miRNAs is a promising strategy for cancer prevention.

Deng *et al* (55) reported that miR-376c suppresses cervical cancer cell proliferation and invasion by targeting Bmi1. miR-494-3p enhances the radiosensitivity of oral squamous carcinoma cells through targeting Bmi1 (56). miR-452 and miR-403 can inhibit the metastasis of non-small cell lung cancer through inhibiting Bmi1 (57,58). miR-218, functioning as a negative regulator of Bmi1, is also found in bladder cancer (59) and esophageal squamous cell carcinoma (60). Besides these miRNAs, miR-429, miR-200c, mIR-221 and miR-320a have been demonstrated to directly target Bmi1 in various cancer cells (22,61-63). These studies indicate that Bim1 undergoes epigenetic regulation by various miRNAs that may contribute to the development of cancer. A better understanding of Bmi1 by miRNAs will provide a new insight into understanding glioma pathogenesis.

EMT is considered as major modulator of metastasis formation in epithelial solid tumors, and the EMT of glioma has also been widely studied (64). Twist1, ZEB1/ZEB2 and SNAI1/SNAI2 have been suggested as the major regulators for EMT in glioma (65-67). Various miRNAs such as miR-200b, miR-590-3p and miR-181c are reported to be involved in regulating EMT of glioma (68-70). Our present study showed for the first time that miR-194 was decreased in glioma which could inhibit glioma cell migration, invasion and EMT. Importantly, we found that Bmi1 was a direct target of miR-194 in glioma cells by which miR-194 inhibited glioma cell EMT. miR-194 may serve as a novel biomarker as well as a therapeutic target in glioma. However, further *in vivo* studies are required to fully elucidate the exact role and molecular mechanism of miR-194 in the regulation of glioma.

Acknowledgements

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