

Original Article

MicroRNA-664 regulates cell invasion and migration and epithelial-mesenchymal transition by targeting TGF- β signal in glioblastoma

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Abstract: This study aimed to investigate the effects of miR-664 on glioblastoma cell invasion and migration and to reveal its possible molecular mechanisms. The expression of miR-664 was measured by quantitative real-time PCR (qRT-PCR) in glioblastoma tissue and the normal brain tissue as well as in two human glioblastoma cell lines. Additionally, the expression of transforming growth factor beta receptor II (TGFBR2) was detected by qRT-PCR and western blot. The effect of miR-664 on glioblastoma cell invasion and migration were studied by transwell assay. The epithelial-mesenchymal transition (EMT)-related protein (N-cadherin, E-cadherin and vimentin) expression was detected by Western blot. Furthermore, the miR-664 target was searched and the underlying mechanism was clarified by reporter assay. The result showed that miR-664 expression was inhibited while TGFBR2 expression was up-regulated in glioblastoma tissue and cell lines. Overexpression of miR-664 suppressed glioblastoma cell invasion and migration, promoted E-cadherin expression, and inhibited expressions of N-cadherin and vimentin. Further study showed that TGFBR2 was the direct target of miR-664 and miR-664 overexpression regulated cell invasion and migration via TGF- β signal. The data indicated that miR-664 may suppress tumor invasion and migration by targeting TGF- β signal and regulate EMT progress of glioblastoma cells.

Keywords: Glioblastoma, miR-664, TGF- β signal, cell migration, cell invasion

Introduction

Glioblastomas is the most common and aggressive cancer that begins within the brain, accounting for 15% of brain tumors [1]. It can start from normal brain cells or develop from an existing low-grade astrocytoma [2]. It is reported that about 3 per 100,000 people develop this disease annually [3]. Most patients with glioblastomas survive approximately 12 to 15 months following diagnosis, and only 3 to 5% of people surviving greater than five years [3]. Therefore, understanding the molecular mechanisms of glioblastomas tumorigenesis to discover novel therapeutic targets is badly needed for patients with glioblastomas.

Presently, transforming growth factor- β (TGF- β) is considered to be an important factors responsible for glioblastoma tumorigenesis [4,

5]. TGF- β is a family of polypeptides, regulating numerous biologic functions including cell proliferation, migration, angiogenesis and differentiation [6]. The overexpression of TGF- β has been reported in various malignant entities [7, 8]. In glioblastomas patients, elevated level of TGF- β is associated with advanced tumor stages, and poor outcome [9].

microRNAs (miRNAs) are a class of small non-coding RNAs, which play an important role in regulating gene function [10]. It has been found that miRNAs play a key role in tumor progression, serving as oncogenes or tumor suppressor genes [11]. Specially, TGF- β can be regulated by miRNAs to play roles in glioblastomas. A recent study revealed that miR-663 inhibits the proliferation, migration and invasion of glioblastoma cells via targeting TGF- β [12]. He et al. [13] have reported that miR-181c can inhibit

Table 1. Primers used for targets amplification

Name	Forward primer	Reverse primer
TGFBR2	GGAATGTCTTGGGCAAATCT	ACCTGAATGCTTGTGCTTTTATT
E-cadherin	AACGCATTGCCACATACAC	AACGCATTGCCACATACAC
N-cadherin	AACTCCAGGGGACCTTTTC	CAAATGAAACCGGGCTATC
Vimentin	TCCAAGTTGCTGACCTCTC	TCAACGGCAAAGTTCTCTTC
GAPDH	GGGAGCCAAAAGGGTCAT	GAGTCTCCACGATACCAA

glioblastoma cell invasion, migration and mesenchymal transition by targeting TGF- β pathway.

Recently, miR-664 has been suggested to be implicated in several cancers, such as osteosarcoma, breast cancer and cervical cancer [14-16]. However, its role in glioblastoma as well as its relationship with TGF- β in glioblastoma development remains largely unknown. Therefore, the present study investigated the expression of miR-664 in glioblastoma tissues and cells and investigated the effects of miR-664 on glioblastoma cell migration and invasion and mesenchymal transition. Moreover, the target of miR-664 was investigated in order to determine the underlying mechanism of miR-664 in glioblastoma.

Materials and methods

Patients and samples

A total of 13 patients who were diagnosed with glioblastoma from January 2013 to January 2016, and received surgery in our hospital were enrolled in this study. The diagnosis of glioblastoma was pathologically defined according to World Health Organization (WHO) classification and each case was confirmed by two pathologists. Glioblastoma tissue and the normal brain tissue samples were obtained from the enrolled cases. Then these samples were frozen immediately with liquid nitrogen, and stored at -80°C till RNA extraction.

All the patients were informed with the consent and this experiment was approved by the hospital's protection of human ethics committee, the First Affiliated Hospital of Nanchang University.

Cell culture and transfection

Human glioblastoma cell lines U87-MG and T98G, and the normal human astrocytes cell line HEB were obtained from European Collection of Cell Cultures (Wiltshire, UK). These cells were cultured in the Dulbecco's Modified

Eagle Medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and streptomycin (Gibco) in an atmosphere of 5% CO₂ at 37°C.

miR-664 mimic, miR-664 scramble and TGFBR2-siRNA were synthesized by Sangon Biotech (Shanghai, China). Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from tissues and cells using TRIzol Reagent (Invitrogen, CA, USA). The isolated RNA was treated with RNase-free Dnase I (Promega Biotech, USA). The concentration and purity of the isolated RNA were detected with SMA 400 UV-VIS (Merinton, Shanghai, China). Then purified RNA (at density of 0.5 μ g/ μ L) with nuclease-free water was reverse-transcribed using the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen, CA, USA). The expressions of targets were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY, USA) with the SYBR ExScript RT-qPCR Kit (Takara, China). To confirm that only one product was amplified and detected, melting curve analysis of amplification products was conducted at the end of each PCR. Phosphoglycer-aldehyde dehydrogenase (GAPDH) was used as the internal controls. Primers used for targets amplification were shown in **Table 1**.

Western blot analysis

Cells were lapped with RIPA assay (Sangon Biotech, Shanghai, China) lysate containing phenylmethanesulfonyl fluoride (PMSF; Sigma, USA). After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was collected for the measurement of protein concentrations using BCA Protein Assay Kit (Pierce, Rochford, IL). Then protein samples (50 μ g per lane) were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes. After the membranes were blocked in PBST (0.1 % triton in 19 PBS), they were probed with primary antibodies (1:1,000 for transforming growth factor beta receptor II (TGFBR2), E-cadherin, N-cadherin, and vimentin, and 1:5,000 for GAPDH) at 4°C overnight. Then the membranes were incubated with appropriate horse-

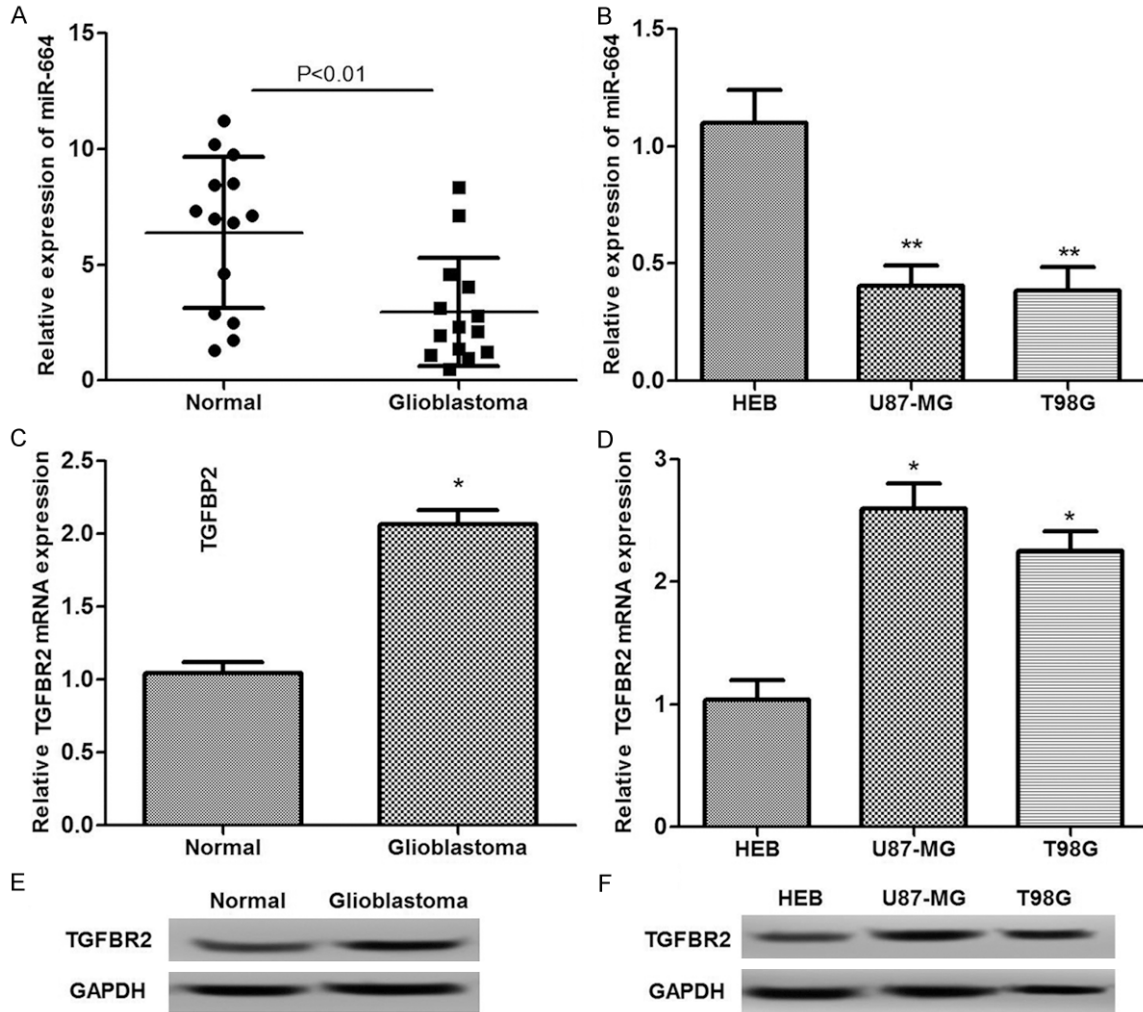


Figure 1. The relative expression levels of miR-664 and TGFBR2 in glioblastoma tissues and cell lines assayed by qRT-PCR (A-D) and western blot (E and F). (A, C and E) Represent in glioblastoma tissues sample, and (B, D and F) represent in cell lines. *: $P < 0.05$, **: $P < 0.01$.

radish peroxidase-conjugated secondary antibodies (1:1,000). Finally, the immunoreactive protein bands were developed by enhanced chemiluminescence and analyzed by a densitometer.

Invasion and migration assay

Transwell was used for cell invasion and migration assay. For invasion assay, cells in each group were incubated in serum-free RPMI-1640 medium containing 0.01% bovine serum albumin (BSA; Sigma, USA) for 24 h. The upper layer of Transwell chamber was enveloped with serum-free DMEM medium supplemented with 50 mg/L Matrigel and then air-dried at 4°C. Then the medium was sucked out, and 50 µL fresh serum-free medium containing 10 g/L BSA was added and cultured for 30 min at

37°C. After that, Transwell was put into the 24-well plates and cultured with DMEM medium mixed with 10% FBS. Cells in Transwell were suspended with serum-free DMEM medium for 48 h. The upper cells on microporous membrane were removed by washing with PBS buffer. After being fixed in ice-cold alcohol, Transwell from each group was stained with 0.1% crystal violet for 30 min, and then decolorated with 33% acetic acid. Each experiment was performed in triplicate. For migration assay, the process was similar with the invasion assay except the upper layer of Transwell chamber without Matrigel.

Luciferase reporter analysis

Vectors of TGFBR2-3'-UTR was synthesized by Sangon Biotech (Shanghai, China). The dual

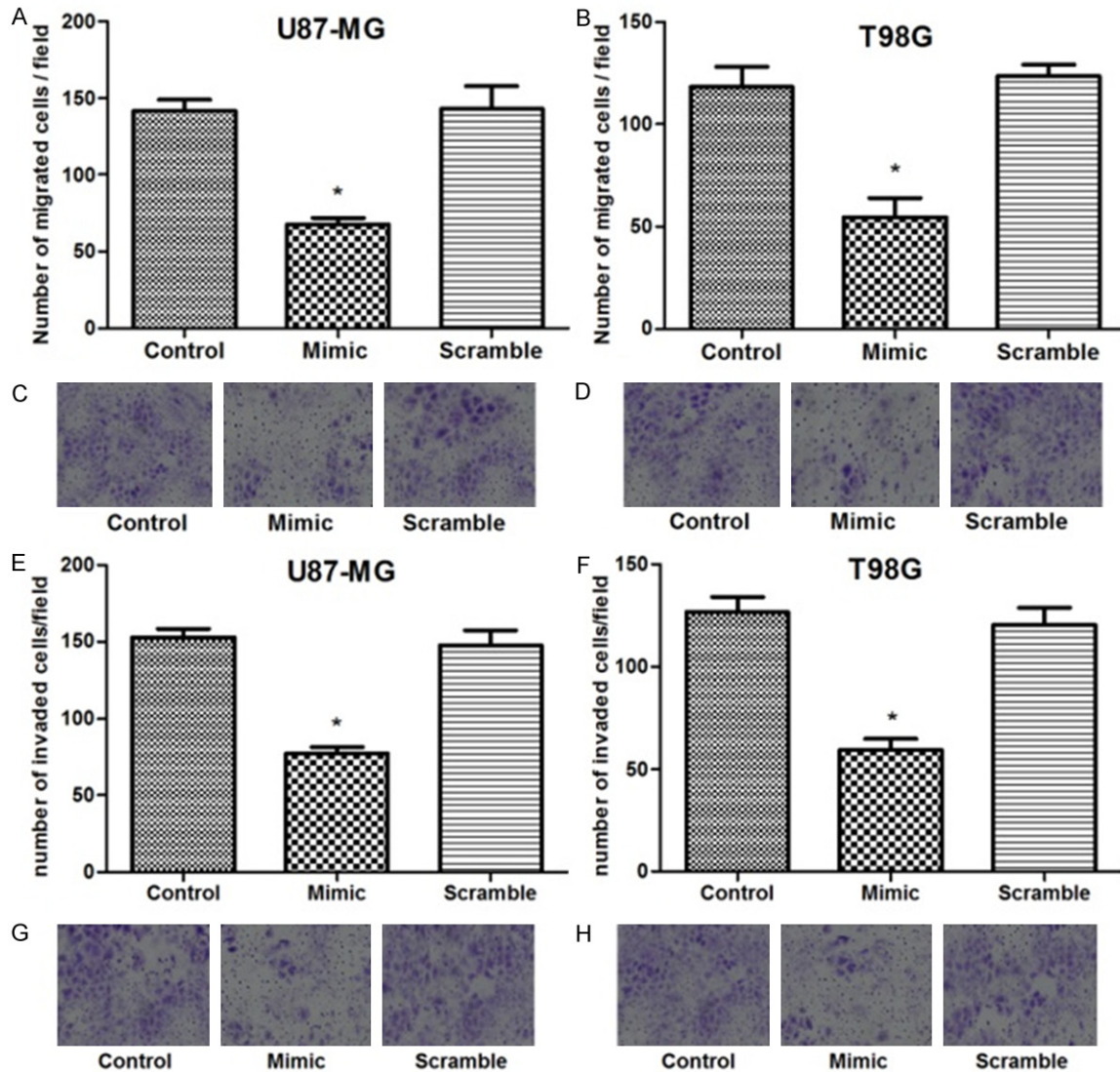


Figure 2. The effects of miR-664 on glioblastoma cells migration (A-D) and invasion (E-H) assayed by Transwell. miR-664 overexpression significantly decreased the number of migrated and invaded cells. *: $P < 0.05$.

luciferase reporter plasmids TGFBR2-WT (containing the wild-type TGFBR2 putative 3'-UTR-binding site) and TGFBR2-Mut (containing the mutant TGFBR2 3'-UTR) were constructed. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega) at 48 h after cell transfection. The relative reporter activity was normalized by r-luc activity.

Statistical analysis

All data in this study were expressed as mean \pm standard error of mean (SEM). Independent sample t-test was used to calculate the differ-

ence between two groups using the graph prism 5.0 software (GraphPad Prism, San Diego, CA). Analysis of variance (ANOVA) was used to calculate the difference for more than three groups. $P < 0.05$ was defined as statistically significant.

Results

Expression of miR-664 in glioblastoma tissue and cell lines

The relative expression levels of miR-664 and TGFBR2 in glioblastoma and the normal brain tissue tissues, as well as in glioblastoma cells

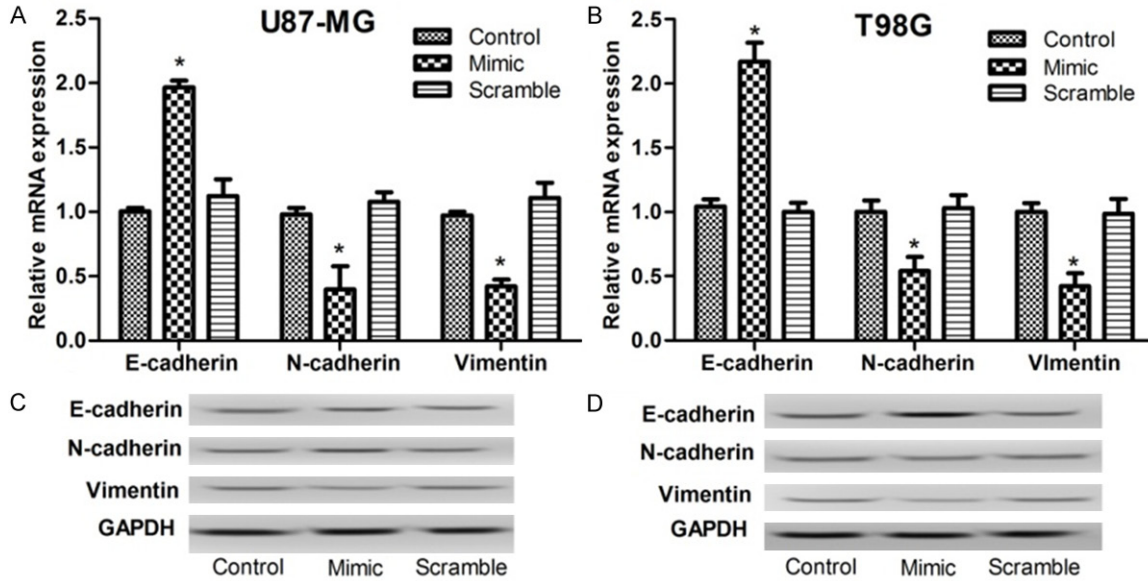


Figure 3. The relative expression levels of epithelial-mesenchymal transition-related proteins (N-cadherin, E-cadherin and vimentin) assayed by qRT-PCR (A and B) and western blot (C and D). *: $P < 0.05$.

and normal human astrocytes were determined using qRT-PCR and western blot analyses. As presented in **Figure 1A** and **1B**, the relative mRNA levels of miR-664 were significantly decreased in glioblastoma tissues and cells in comparison with the normal tissues and cells ($P < 0.01$). Additionally, mRNA and protein expression levels of TGFBR2 in glioblastoma tissues and cells were significantly higher than that in normal tissues and cells ($P < 0.05$) (**Figure 1C-F**).

miR-664 overexpression inhibited cell migration and invasion

The role of miR-664 in invasion and migration of glioblastoma cells was assayed by Transwell. The results showed that the number of migrated glioblastoma cells decreased significantly in miR-664 mimic group compared with that in miR-664 scramble and control groups ($P < 0.05$) (**Figure 2A-D**). Similarly, the number of invaded glioblastoma cells in miR-664 mimic group was significantly less than that in miR-664 scramble and control groups ($P < 0.05$) (**Figure 2E-H**).

miR-664 overexpression regulated the EMT-related protein expression

In order to further explore the mechanisms of miR-664 inhibiting glioblastoma cell invasion

and migration, the expressions of EMT-related proteins, N-cadherin, E-cadherin and vimentin were detected. As shown in **Figure 3A-D**, when miR-664 was overexpressed, the expressions of E-cadherin increased significantly in both U87-MG and T98G cells ($P < 0.05$). On the contrary, the expressions of N-cadherin and vimentin decreased significantly in two cell lines in miR-664 mimic group than in the other two groups ($P < 0.05$).

TGFBR2 was the target for miR-664

The gene sequences of TGFBR2 regulated by miR-664 in the two kinds of cells were shown in **Figure 4A**. The relative luciferase activities were shown in **Figure 4B1** and **4B2**. The relative luciferase activity of the reporter that contained the wild-type 3'-UTR of TGFBR2 was significantly decreased in miR-664-mimic-transfected cells compared to control cells in both U87-MG and T98G cells ($P < 0.05$). However, mutation of the predicted binding site of miR-664 on the TGFBR2 3'-UTR rescued the luciferase activity. Moreover, the relative TGFBR2 expression was significantly decreased in miR-664 mimic group compared with in miR-664 scramble and control groups ($P < 0.05$) (**Figure 4C1-D2**). In addition, the relative mRNA and protein expression of TGFBR2 was down-regulated in cells transfected with silenced TGFBR2

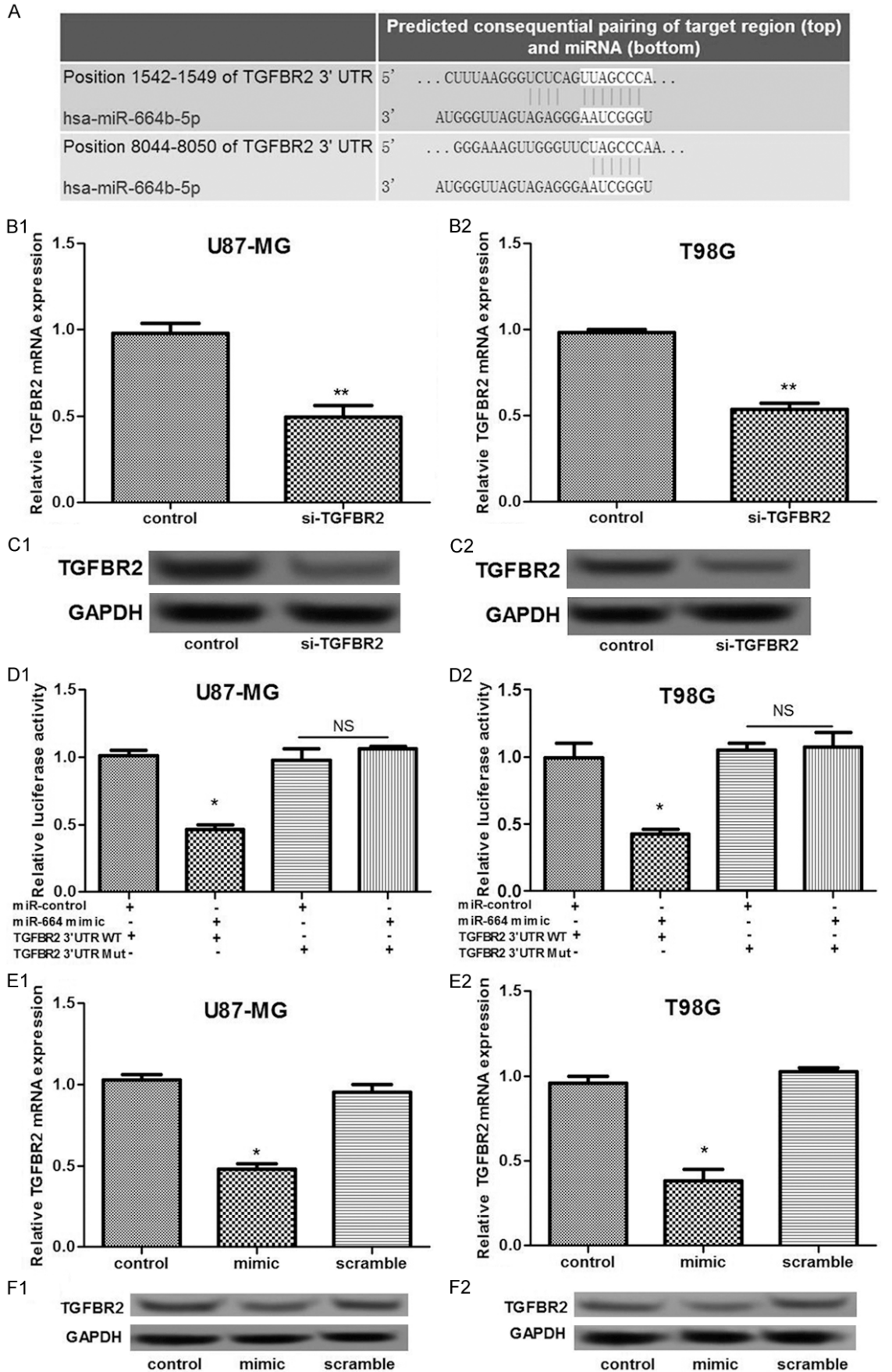


Figure 4. (A) The gene sequences of TGFBR2 regulated by miR-664. (B1 and B2) The relative luciferase activities in wild-type 3'-UTR of TGFBR2 and mutant TGFBR2 3'-UTR in transfected cells. (C1 and C2) The relative mRNA level of TGFBR2 in the two kinds of cells. (D1 and D2) The protein level of TGFBR2 in the two kinds of cells. (E1-F2) The relative expression levels of TGFBR2 in silenced TGFBR2 transfected cells assayed by qRT-PCR (E1 and E2) and western blot (F1 and F2). *: P < 0.05.

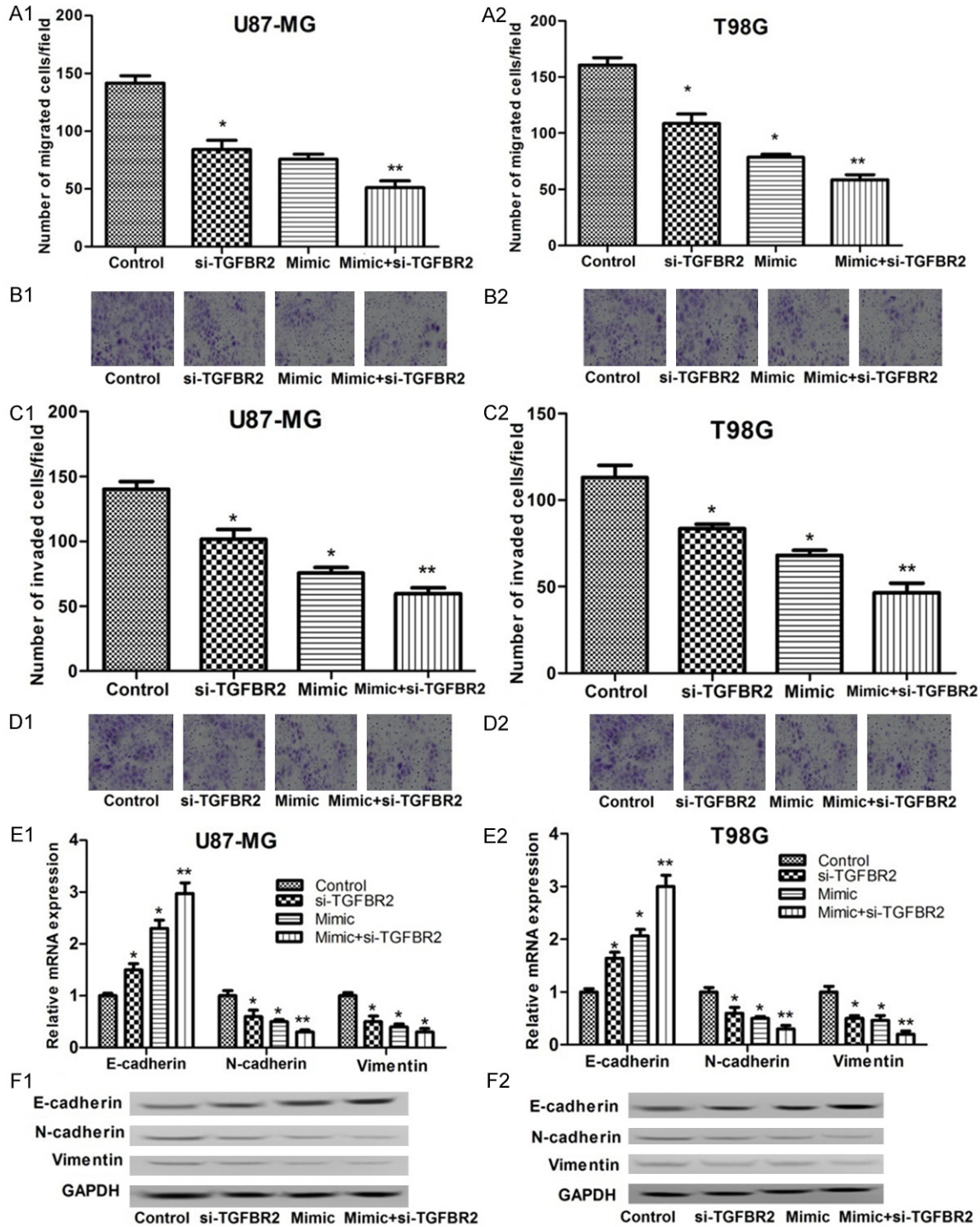


Figure 5. The effects of miR-664 overexpression and TGFBR2 suppression on glioblastoma cells migration (A1-B2) and invasion (C1-D2) assayed by Transwell. The relative expression levels of N-cadherin, E-cadherin and vimentin after miR-664 overexpression and TGFBR2 suppression assayed by qRT-PCR (E1 and E2) and western blot (F1 and F2). *: P < 0.05, **: P < 0.01.

(Figure 4E1-F2). These results suggested that TGFBR2 was negatively regulated by miR-664.

miR-664 overexpression regulated cell invasion and migration via TGF- β signal

Further investigation of the relationships between miR-664 inhibiting glioblastoma cell invasion and migration and TGF- β signal revealed that both TGFBR2 inhibition and miR-664 overexpression could significantly inhibit the invasion and migration of glioblastoma cells ($P < 0.05$). Additionally, when miR-664 mimic combined with si-TGFBR2, the inhibitory effect was optimal ($P < 0.01$) (Figure 5A-D). Furthermore, both miR-664 mimic and si-TGFBR2 could increase the expression of E-cadherin, and decrease the expressions of N-cadherin and vimentin significantly ($P < 0.05$), especially when miR-664 mimic combined with si-TGFBR2 ($P < 0.01$) (Figure 5E, 5F). The results indicated that miR-664 overexpression inhibiting glioblastoma cell invasion and migration and EMT may be achieved by regulating TGF- β signal.

Discussion

Recently, abnormal miRNA expression has been revealed in tumorigenesis and in various malignancies [17]. A series of miRNAs have been experimentally verified to be associated with cellular proliferation, invasion and migration, such as miR-34a [18], miR-191 [19], and miR-214 [20]. Therefore, identification of the functions of differentially expressed miRNAs may provide new insights into the molecular mechanisms underlying disease development.

In the present study, we demonstrated that miR-664 was down-regulated in glioblastoma tissues and cells. Interestingly, miR-664 has been found down-regulated in several other cancers. For instance, Ding et al. [21] have found that the expression of miR-664 is significantly down-regulated in cutaneous malignant melanoma tissues and compared with normal controls. In addition, Yang et al. [22] firstly demonstrated that miR-664 expression was overall down-regulated in both primary cervical carcinoma tissues and cervical cancer cell lines. Therefore, we speculate that miR-664 may be a candidate tumor suppressor of glioblastoma. To our best knowledge, our study is the first to report such a role for miR-664 in glioblastoma.

Also in the present study, by up-regulating the expression level of miR-664 in U87-MG and T98G cells, we found that cell invasion and migration were significantly suppressed, suggesting that miR-664 may play a vital role in inhibiting invasion and migration of glioblastoma cells. Recent studies have revealed that EMT plays an important role in tumor migration and invasion [23, 24]. Developing glioblastoma cells displayed EMT-like changes, including increased expression of vimentin and N-cadherin and decreased levels of E-cadherin [25]. Therefore, we investigated the expressions of EMT-related proteins, N-cadherin, E-cadherin and vimentin to further explore the mechanisms of miR-664 inhibiting glioblastoma cell invasion and migration. The results showed that the expressions of E-cadherin increased significantly, while the expressions of N-cadherin and vimentin decreased significantly in two cell lines when miR-664 overexpressed. These findings are consistent with previous reports showing that protein expression of E-cadherin was significantly up-regulated by miR-664 overexpression in cervical cancer cells [22]. As a result, EMT may be a potentially mechanism of miR-664 regulated invasion and migration in glioblastoma.

Recent studies have reported that TGF- β signaling plays an critical role in the regulation of proliferation, migration and invasion in glioblastoma [26, 27]. Importantly, TGF- β signaling has also been found to promote EMT in several cancers including glioblastoma [28-30]. Specially, TGF- β exerts its effects by binding to TGFBR2. TGFBR2 is a key receptor in regulating the TGF- β signaling pathway, mutations in which may inactivate or activate the TGF- β signaling pathway [31, 32]. Narushima et al. [33] have reported that TGFBR2 plays an important role in regulation of glioblastoma stem cell properties. In the present study, we found that TGFBR2 was up-regulated in glioblastoma tissues and cells; besides, luciferase reporter analysis showed that TGFBR2 was a target of miR-664. Furthermore, further study revealed that miR-664 suppression or TGFBR2 overexpression could inhibit cell invasion and migration as well as regulate the expressions of EMT-related proteins, suggesting that miR-664 could regulate the migration and invasion of glioblastoma cells by TGF- β signaling.

In conclusion, the miR-664 expression was negatively correlated with the expression of TGFBR2 and in glioblastoma. miR-664 overexpression inhibited the migration and invasion of glioblastoma cells as well as EMT via TGF- β signaling. Therefore, more clinical studies are needed to confirm the role miR-664 as a target of gene therapy in glioblastoma.

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Disclosure of conflict of interest

None.

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