

miR-144-3p serves as a tumor suppressor by targeting FZD7 and predicts the prognosis of human glioblastoma

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Abstract. – OBJECTIVE: Previous research indicated that miR-144-3p was associated with the regulation of the carcinogenic processes, but the role of miR-144-3p in glioblastoma (GBM) remains unclear. In this study, we aimed to analyze the role of miR-144-3p in GBM.

PATIENTS AND METHODS: The expression of miR-144-3p was measured in GBM tissues and adjacent non-malignant tissues using qRT-PCR. The correlation of miR-144-3p with clinicopathological features and prognosis was also analyzed. Cell proliferation, invasions, and migration assay were applied to assess the function of miR-144-3p in vitro. Bioinformatics prediction and luciferase assays were employed to identify the predicted microRNA (miRNA) which regulates Frizzled-7 (FZD7). The levels of FZD7 and FZD7 mRNA were determined by Western blot and RT-PCR.

RESULTS: The results showed that the miR-144-3p expression was significantly downregulated in tumor tissues and GBM cell lines compared with that in normal brain tissues and the normal human astrocytes. The levels of miR-144-3p were negatively correlated with the status of WHO grade and recurrence. Furthermore, patients with low serum levels of miR-144-3p had a significantly shorter median overall survival rate. Multivariate Cox regression analysis confirmed that low level of miR-144-3p expression predicted poor prognosis independently. Further function assays showed that miR-144-3p inhibited proliferation invasion and migration of GBM cells. Finally, miR-144-3p was demonstrated to bind to the wild-type 3' untranslated region of FZD7 but not with its mutant.

CONCLUSIONS: The results of the present study indicate that miR-144-3p suppresses tumor metastasis by targeting FZD7. This work also provides strong evidence that miR-144-3p is an independent prognostic factor for patients with GBM.

Key Words:

miR-144-3p, FZD7, GBM, Prognosis, Progression.

Introduction

Glioblastoma (World Health Organization [WHO] grade IV) is one of the most malignant central nervous system (CNS) cancers in children and adults^{1,2}. Despite aggressive surgical resection and combined temozolomide chemotherapy and radiotherapy (RT), overall survival in GBM is usually less than 12 months and long-term survival is rare^{3,4}. The genetic alterations and epigenetic changes involved in GBM should be explored more intensively to develop a new treatment strategy.

microRNAs (miRNAs) are a group of non-coding nucleic acids, which regulate gene expression that negatively regulate expression of protein-coding genes at the post-transcriptional level⁵. Growing evidence shows that miRNA alteration and dysfunction play important roles during tumorigenesis and metastasis by way of the regulation of cancer cell proliferation, differentiation, apoptosis, and invasion⁶⁻⁸. Previous studies have found that miR-144-3p is downregulated in various types of malignancies, which suggests that miR-144-3p may serve as a tumor suppressor^{9,10}. However, its clinical value and function in GBM remain unclear. FZD7 has been recognized as an oncogene in cancer development, because it activates Wnt signaling¹¹⁻¹³. In the present work, our attention focused on the association between miR-144-3p and FZD7. We further explore the prognostic effect of miR-144-3p in patients with GBM.

Patients and Methods

Patients and Tissue Samples

GBM tissues and adjacent non-tumor tissues used for qRT-PCR and/or Western blot were col-

lected from 111 GBM patients who underwent curative surgery between 2010 and 2012 at the Linyi People's Hospital. All patients' clinical stages and histological grades were evaluated by a group of independent pathologists. No local or systemic treatment had been conducted in these patients before the operation. Tissues were frozen in liquid nitrogen immediately and stored at -8°C after excision. Patient information and tumor pathology are summarized. This study was approved by the Ethical Committee of Linyi People's Hospital.

Cell Culture and Transfection

GBM cell lines U87, T98G, A-172, LN18, LN-229 and the normal human astrocytes (NHAs), were obtained from Cell Bank of Type Culture Collection (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). To obtain stable miR-144-3p-overexpressing GBM lines, T98G and LN18 cells (3×10^5 cells/well) were inoculated and incubated into 6-well plates. Then, miR-144-3p mimic (miR-144-3p) respective negative controls (NC) were purchased from Ambion (Houston, TX, USA), and were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNAs were extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Reaction mixture containing 1-3 μg of total RNA was reverse-transcribed to cDNA by using PrimeScript RT-polymerase (TaKaRa, Dalian, China). The expression levels of miR-144-3p and FZD7 mRNA were quantified using miRNA-specific TaqMan MiRNA Assay Kits (Applied Biosystems, Foster City, CA, USA). The reactions were performed in triplicate by using the ABI PRISM 7000 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). U6 small nuclear RNA was used as an endogenous control. Relative expression levels of miRNAs were analyzed with the comparative cycle threshold (CT). The PCR primers were shown in Table I.

Cell Proliferation Assays

Cell proliferation was measured by CCK-8 assay. Forty-eight hours after transfection,

T98G and LN18 cells per well were seeded into 96-well plates. Cells were incubated with CCK-8 for 2 h at 37°C , and detected the absorbance at the day 3.

Transwell Assays

Tumor cell migration and invasion were carried out using a Transwell insert (8 μm , Corning, Corning, NY, USA). T98G or LN18 cells (1×10^5) were added to the upper compartment of a transwell chamber and allowed to migrate for 24 h at 37°C . Then, a swab was used to wipe out the remaining Matrigel and cells in the upper chamber. The lower chamber contained 10% fetal bovine serum (FBS) for use as a chemoattractant. After cells were cultured at 37°C for 48 h, they migrated to the lower surface and were removed with 70% ethanol for 30 min and stained with 0.2% crystal violet for 10 min. The number of cells migrating or invading through the membrane was counted in five randomly selected fields under a light microscope.

Luciferase Reporter Assay

The 3'-UTR of FZD7 containing the potential binding sites of miR-144-3p or the mutant FZD7 3'-UTR was amplified by PCR and then cloned into XhoI/NotI restriction sites of pRL-TK vector (Promega, WI, USA). T98G cells were seeded in 24-well plates and allowed to settle for 24 h. Then, T98G cells were co-transfected with 0.4 μg of the reporter construct, 0.02 μg of pRL-TK vector, and 5 pmol of miRNA mimic or scramble controls. 48 h after transfection, luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blotting

Cells in monolayer were washed with cold PBS, subsequently lysed in ice-cold mammalian protein extraction reagent lysis buffer. The

Table I. The primers used in the reactions

Gene	Sequence
miR-144-3p	F: 5'TACTGCATCAGGAAGTACTGGA R: 5'GTGCAGG GTCCGAGGT
FZD7	F: 5'CTAGACTAGTGAAAGCGGTTTGGATGAA 3' R: 5'9 CCC AAGCTT CGTCTCCTTGGCCTTATC3'
GADPH	F: 5'-GACTCATGACCACAGTCCATGC-3' R: 5'-AGAGGCAGGATGATGTTCTG-3'
U6	F: 5' CTCGCTTCGGCAGCACA-3' R: 5'AACGCTTCACGAATTTGCGT-3'

total proteins were then electrophoresed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) (Roche, Basel, Switzerland) membranes. Then, these proteins were blocked for 1 h in 5% non-fat powdered milk in tris buffered saline-tween (TBST). Finally, signals were detected by Western Lightning Plus-ECL Kit (PerkinElmer, Boston, MA, USA) according to manufacturer's instructions. The primary antibodies used for immunodetection were mouse monoclonal anti-FZD7 (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-GAPDH antibodies (Santa Cruz, CA, USA), and the secondary antibody was anti-mouse (Abcam, Cambridge, MA, USA).

Statistical Analysis

All computations were carried out using the SPSS software version 17.0 for Windows (IBM Corporation, Armonk, NY, USA). Student's *t*-test, χ^2 -test, and Fisher's exact tests were used to analyze the differences between groups. Probabilities of overall survival were calculated by the Kaplan-Meier method, and compared using the log-rank test. The prognostic variables to predict overall survival were assessed by multivariate Cox proportional hazards regression analysis. $p < 0.05$ was considered statistically significant.

Results

miR-144-3p Expression is Decreased in GBM Cell Lines and Tissues

We firstly determine the expression levels miR-144-3p in GBM tissues by RT-CPR. As shown in Figure 1A, our results indicated that miR-144-3p was significantly down-regulated in GBM tissues compared with the adjacent non cancerous tissues ($p < 0.01$). Then, we detected the expression of miR-144-3p in GBM cell lines and NHAs. As shown in Figure 1B, we found that it showed that miR-144-3p expression was markedly down-regulated in all GBM cell lines compared to that in NHAs (all $p < 0.01$).

The Relationship Between miR-144-3p Expression and Clinical Features

The 111 patients with GBM were classified into two groups according to the median expression level of miR-144-3p. There were 53 patients with low miR-144-3p and 58 patients with high miR-144-3p. The relationship between clinical features and miR-144-3p expression levels in individuals with GBM are summarized Table II. We found that low expression of miR-144-3p was positively associated with WHO grade ($p = 0.003$) and recurrence ($p = 0.001$) in GBM patients. However, no statistically significant correlation was observed between miR-144-3p expression and other clinicopathological factors.

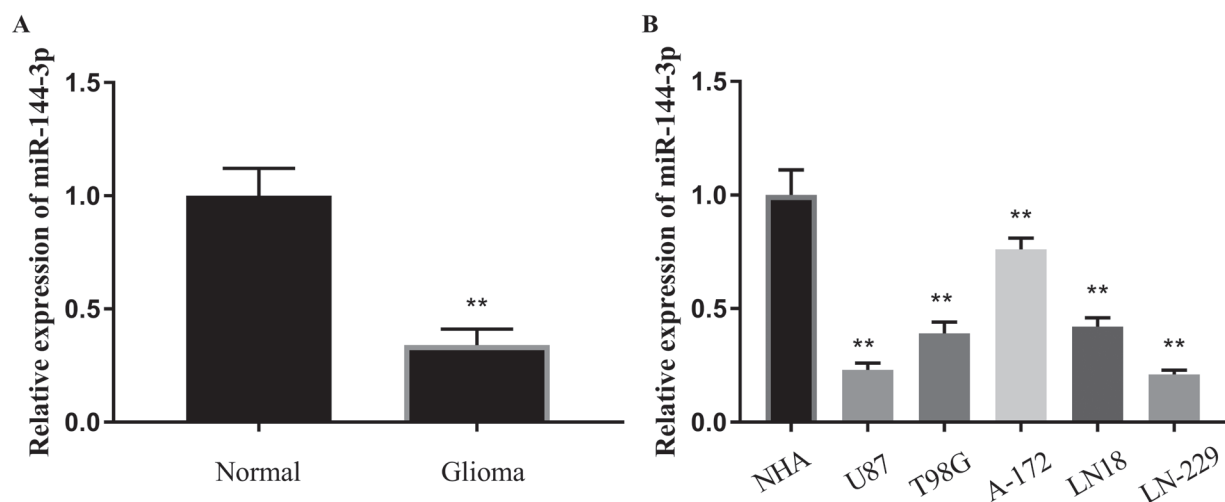


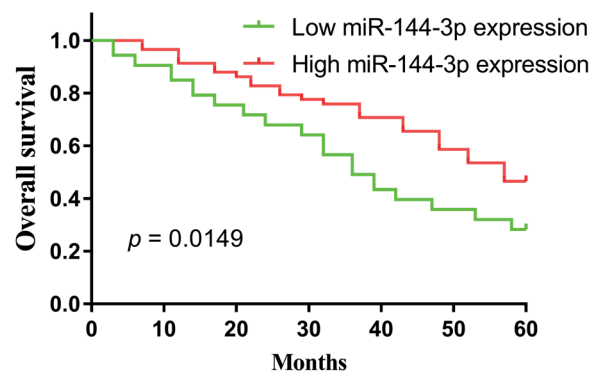
Figure 1. miR-144-3p is frequently decreased in GBM tissues and cell lines. **(A)** Expression level of miR-144-3p is determined in tumor tissues and matched normal tissues by Real-time PCR and normalized to U6. **(B)** Expression level of miR-144-3p is examined by Real-time PCR in NHA and GBM cell lines, including U87, T98G, A-172, LN18 and LN-229. All data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

Table II. The relationship between miR-144-3p expression and clinicopathological characteristics in patients with glioblastoma.

Characteristics	No. of patients	Low expression	High expression	<i>p</i> -value
Age (years)				NS
≤ 45	42	20	22	
> 45	69	33	36	
Gender				NS
Male	78	41	37	
Female	33	12	21	
Tumor size (cm)				NS
≤ 3	35	19	16	
> 3	76	34	42	
Tumor location				NS
Supratentorial	90	44	46	
Infratentorial	21	9	12	
WHO grade				0.003
I-II	54	18	36	
III-IV	57	35	22	
Recurrence				0.001
Yes	51	33	18	
No	60	20	40	

Low-Expression Level of miR-144-3p Predicts Poor Prognosis in GBM Patients

In order to further verify the potential clinical utility of miR-144-3p expression, we explored the prognostic value of miR-144-3p in 111 GBM patients. As shown in Figure 2, it was shown that lower miR-144-3p level was associated with shorter overall survival ($p < 0.0149$). Then, we performed univariate and multivariate analyses to judge whether the miR-144-3p expression level and various clinicopathological features were independent prognostic parameters of GBM patient outcomes. The Cox proportional hazards model of the results revealed that WHO grade ($p = 0.004$), recurrence ($p = 0.001$) and miR-144-3p expression ($p = 0.006$) were associated with overall mortality (Table III). Moreover, results by multivariate analysis low miR-144-3p expression ($p = 0.008$) was a significant and independent indicator of poor prognosis for patients with GBM (Table III).

**Figure 2.** Overall survival rate in patients with low miR-144-3p expression was significantly lower than that in patients with high miR-144-3p expression.

Over-Expression of miR-144-3p Inhibits GBM Progression In Vitro

To further investigate the effect of miR-144-3p overexpression in GBM progression, T98G and LN18 cells were infected with miR-144-3p mimic

Table III. Univariate and multivariate analysis of different prognostic factors for overall survival in patients with glioblastoma.

Prognostic factors	HR	95% CI	<i>p</i> -value	HR	95% CI	<i>p</i> -value
Age	1.213	0.783-1.662	NS	-	-	-
Gender	1.411	0.732-1.522	NS	-	-	-
Tumor size	1.893	0.923-1.933	NS	-	-	-
Tumor location	1.632	0.655-1.691	NS	-	-	-
WHO grade	3.893	1.692-6.522	0.004	3.283	1.442-5.782	0.006
Recurrence	4.213	1.773-7.928	0.001	3.892	1.562-6.892	0.003
MiR-144-3p expression	3.213	1.342-5.669	0.006	2.791	1.129-5.023	0.008

or NC, and the upregulation of miR-144-3p expression was confirmed by RT-PCT (Figure 3A, $p < 0.01$). Then, the effect of NEAT1 on GBM cell growth was determined by CCK-8 assay. As shown in Figure 3B, we found that forced miR-144-3p expression significantly reduced proliferation of both T98G and LN18 ($p < 0.01$, respectively). We further performed to migration and invasion assays to determine whether miR-144-3p could also inhibit migration and invasion in GBM cells. As shown in Figure 3C, when miR-144-3p was overexpressed alone, cell migration ability was enhanced. Moreover, similar findings were also found in invasion assays (Figure 3D). Together, these findings demonstrated that miR-144-3p inhibits GBM cell proliferation, migration and invasion *in vitro*.

miR-144-3p Directly Targeted FZD7

We used online publically available algorithms (microRNA.org and targetscan) to predict miR-144-3p targets. As shown in Figure 4A-B, FZD7 is theoretically a potential target gene of miR-144-3p, and the predicted binding site between miR-144-3p and FZD7 3'-UTR is also illustrated. Dual reporter assays revealed that introduction of miR-144-3p in T98G cells suppressed the activity of a luciferase reporter fused to the wild-type (WT) 3'-UTR of FZD7, but not mutant reporters in the T98G cells (Figure 4C). Subsequently, we investigated whether miR-144-3p regulated the expression of FZD7 mRNA and proteins in the T98G and LN18 cells. As shown in Figure 4D, ectopic expression of miR-144-3p in the both T98G and LN18 cells

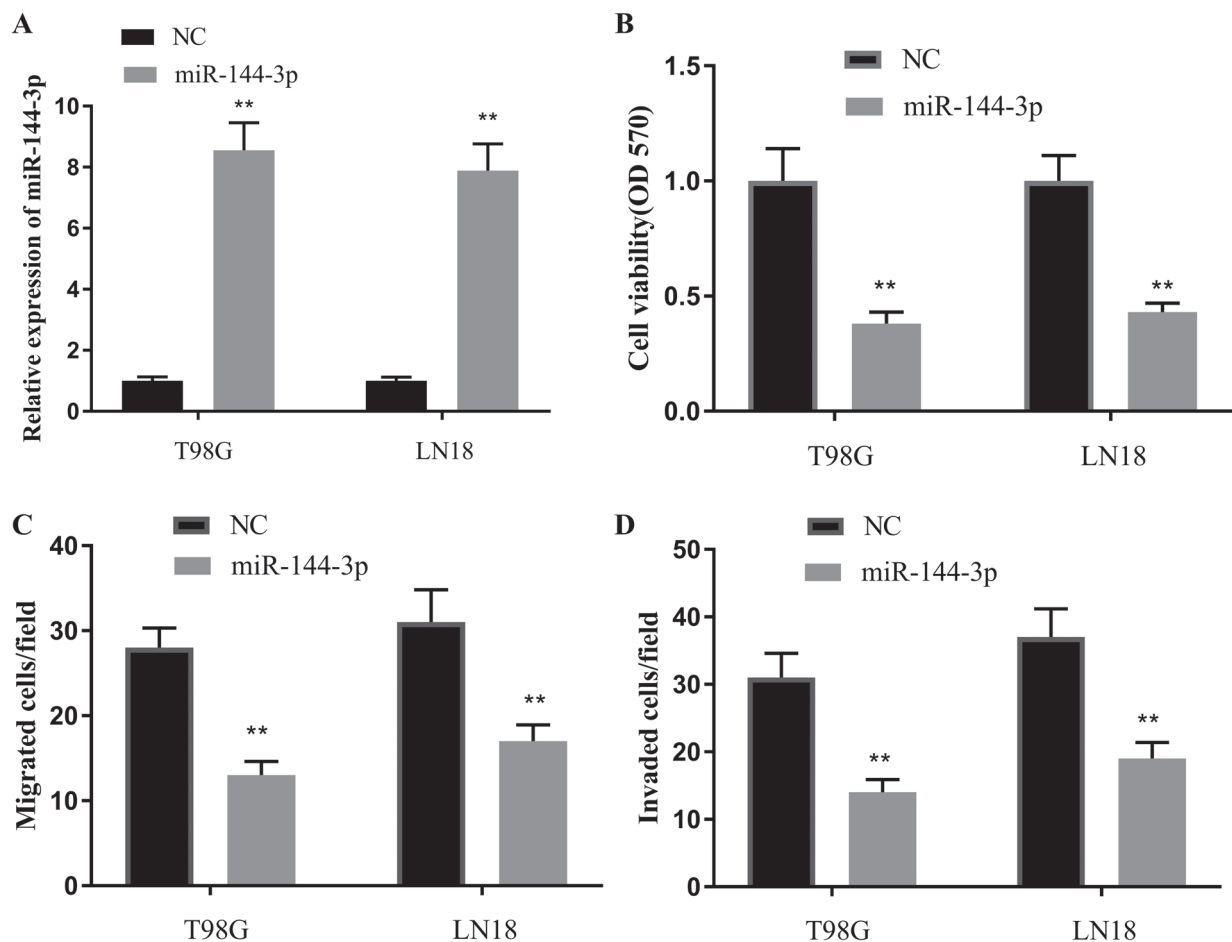


Figure 3. Over-expression of miR-144-3p inhibited GBM cell proliferation, migration and invasion. **(A)** The expression of miR-144-3p in T98G and LN18 cells. After miR-144-3p mimic transfected, the miR-144-3p level increased significantly. **(B)** CCK-8 assay was employed to examine the proliferation ability of T98G and LN18 cells, respectively. Cells were transfected with miR-144-3p mimic or mimics control. **(C-D)** Transwell invasion assay were performed to determined cell migration **(C)** and invasion **(D)** of T98G and LN18 cells. * $p < 0.05$, ** $p < 0.01$.

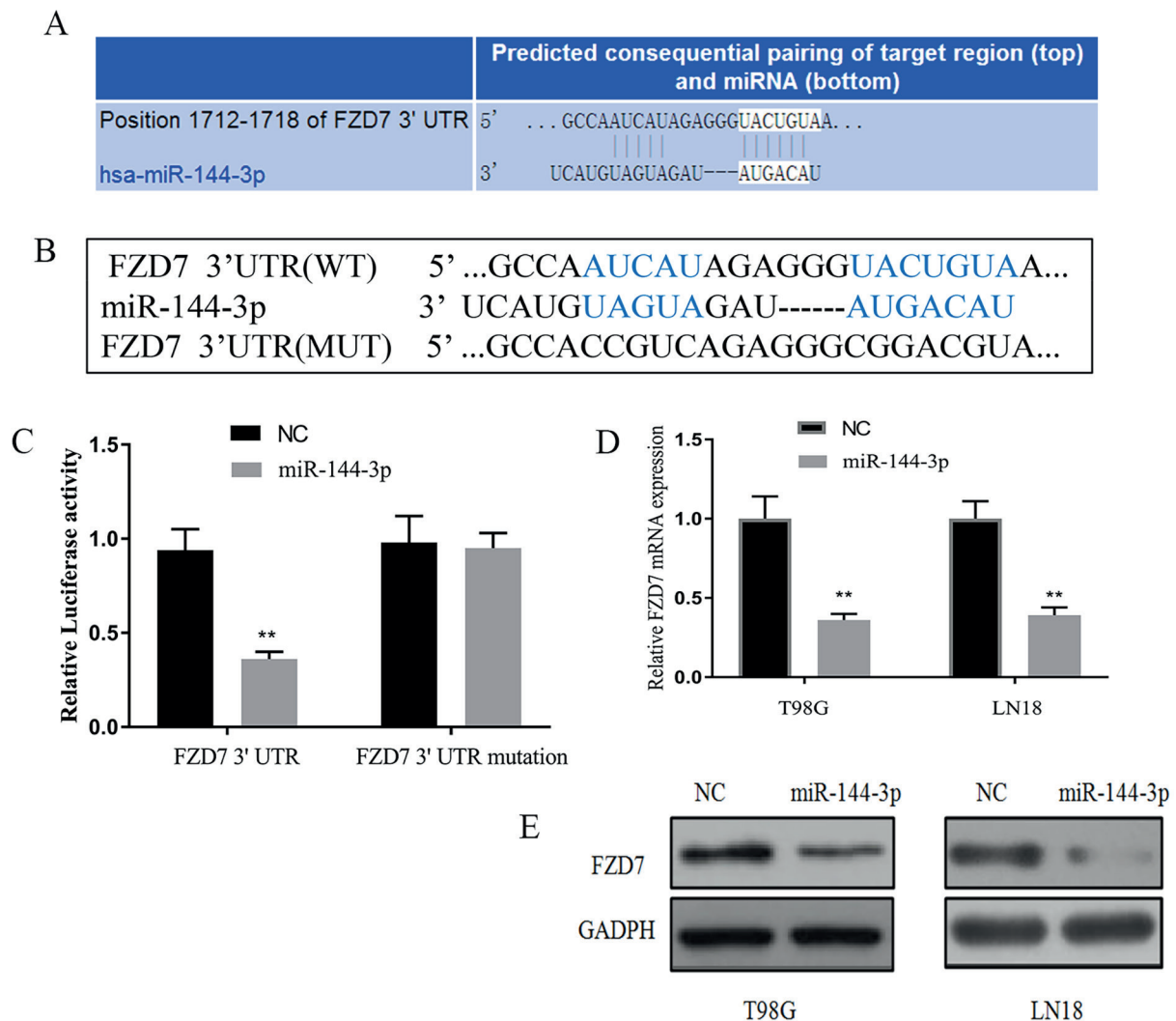


Figure 4. FZD7 is identified as a functional target of miR-144-3p in GBM. **(A)** TargetScan software predicted that FZD7 was a target of miR-144-3p. **(B)** The mutant miR-144-3p binding site was generated in the complementary site for the seed region of miR-144-3p (wt, wild-type; mut, mutant type). **(C)** Luciferase assay showed that reporter activity was inhibited in GBM cells only in the presence of wild type FZD7 and not with a mutated 3'UTR. **(D)** miR-144-3p transfection downregulated FZD7 mRNA as assessed by qRT-PCR. **(E)** miR-144-3p transfection downregulated FZD7 protein levels as assessed by qRT-PCR. * $p < 0.05$, ** $p < 0.01$.

decreased FZD7 mRNA expression. In addition, by Western blot, we also confirmed that ectopic expression of miR-144-3p in the both T98G and LN18 cells decreased FZD7 protein expression (Figure 4E). Taken together, these results suggested that the FZD7-3'-UTR is located at direct binding sites of miR-144-3p.

Discussion

Effective surgical management for GBM was not easy to be done because GBM cells can actively migrate within the brain, often traveling

relatively long distances¹⁴. In order to improve the prognosis of GBM patients, revealing the molecular mechanism for GBM metastasis is pivotal for developing effective therapy. Due to the critical role of miRNAs in progression and development of various tumors, miRNAs have become a research hot point. Previous studies have suggested that a number of miRNAs participate in the pathogenesis of GBM, including miR-200c¹⁵, miR-331-3p¹⁶ and miR-219-5p¹⁷. Therefore, the investigations of miRNAs and their targeting gene are likely to provide not only new marker for predicting the prognosis of GBM patients, but also potential therapeutic strategies.

Previous studies have showed that miR-144-3p plays crucial roles in the development, invasion, and metastasis of some cancers. For instance, Liu et al¹⁸ reported that miR-144-3p was significantly correlated with the metastasis potential in renal cell carcinoma by affecting the epithelial-mesenchymal transition (EMT). Li et al¹⁹ found that miR-144-3p significantly inhibited proliferation, migration, and invasion in gastric cancer cells by inhibiting epithelial-to-mesenchymal transition through targeting PBX3. Zhang et al²⁰ revealed that miR-144-3p inhibited the growth, invasion and migration of laryngeal squamous cell carcinoma cells by targeting ETS-1. Most recently, although Lan et al²¹ reported that miR-144-3p function as a tumor suppressor in GBM by targeting c-Met, more potential mechanism was needed to be further explored.

FZD7 is located on human chromosome 2q33²². It has been confirmed that FZD7 can activate the canonical and/or the non-canonical Wnt signaling pathways in different types of cancers. The suppressive-role of FZD7 has also been reported in various tumors including GBM. For instance, Schiffgens et al²³ reported that the expression levels of FZD7 was significantly up-regulated in GBM patients, and its high levels associated with shorter survival in GBM patients. Qiu et al²⁴ found that forced over-expression of FZD7 promoted proliferation of GBM cells by upregulating TAZ. Previous work indicated that FZD7 could be regulated by miRNAs²⁵. However, whether FZD7 was a direct of miR-144-3p remains unknown.

In the present paper, we found that the expression levels of miR-144-3p were upregulated in both GBM tissues and cell lines, and high miR-144-3p was correlated with the status of WHO grade and recurrence. Kaplan-Meier analysis indicated that low expression of miR-144-3p was associated with poor overall survival in GBM patients. Those results were constant with previous research²¹. In order to explore the specific role of miR-144-3p in development of GBM, we performed *in vitro* experiments. Our results showed that over-expression of miR-144-3p significantly suppressed miR-144-3p inhibited proliferation invasion and migration of GBM cells. Furthermore, we confirmed that FZD7 was a direct downstream target of miR-144-3p by using luciferase reporter assays. These results suggested that miR-144-3p exerted tumor suppressor role in GBM by repressing FZD7 expression.

Conclusions

We highlight a pivotal role for miR-144-3p as a tumor suppressor and provide strong evidence that miR-144-3p might be a potential therapeutic target and a novel prognostic biomarker for GBM.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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