Biochemical and Biophysical Research Communications 458 (2015) 307–312

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNAs let-7b/i suppress human glioma cell invasion and migration by targeting IKBKE directly

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ARTICLE INFO

Article history:
Received 20 January 2015
Available online 3 February 2015

Keywords:
Glioma
Let-7b/i
IKBKE
E-cadherin

ABSTRACT

We demonstrated that IKBKE is overexpressed in human gliomas and that the downregulation of IKBKE markedly inhibits the proliferative and invasive abilities of glioma cells, which is consistent with the results reported by several different research groups. Therefore, IKBKE represents a promising therapeutic target for the treatment of glioma. In the present study, we verified that the microRNAs let-7b and let-7i target IKBKE through luciferase assays and found that let-7b/i mimics can knock down IKBKE and upregulate E-cadherin through western blot analysis. Moreover, the expression levels of let-7b/i were significantly lower in glioma cell lines than that in normal brain tissues, as determined by quantitative real-time PCR. Furthermore, let-7b/i inhibit the invasion and migration of glioma cells, as determined through wound healing and Transwell assays. The above-mentioned data suggest that let-7b/i inhibit the invasive ability of glioma cells by directly downregulating IKBKE and indirectly upregulating E-cadherin.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and formidable primary brain tumour [1]. Despite modern treatments with the current standard of care, the outcome of GBM patients remains poor with a median life expectancy of 15–17 months [2]. Fewer than 16% of patients survive more than three years [3]. The tumours exhibit highly invasive and neurologically destructive ability, similarly to the capacity of diffusively infiltrate normal brain tissue [4], which promotes glioma recurrence. These features of gliomas hinder conventional effective therapy [5]. However, recent advances in genetics research are ongoing to delineate the underlying molecular mechanisms. Gene therapy has become an attractive treatment strategy.

IKBKE (inhibitor of nuclear factor kappa-B kinase subunit epsilon), which is also called IKKε and IKKi, is a member of the IκB kinase (IKK) family [6,7]. In prior studies, IKBKE was found to be overexpressed and activated in breast cancer [8,9], ovarian cancer [10] and prostate cancer [11]. These studies also showed that IKBKE can induce cell survival, growth and chemoresistance [10,12,13] and that the overexpression of IKBKE results in malignant transformation [9,14]. Previous studies in our laboratory have demonstrated that IKBKE is overexpressed in human gliomas and that the silencing of IKBKE by siRNA can reduce the invasive capability and proliferation of glioma cells [15].

The miRNAs of the let-7 family have been demonstrated to reduce GBM cell growth and migration via Ras inhibition [16–18]. At present, there is no data on the expression of let-7 in human gliomas, but some indirect data indicate a correlation between low let-7 expression and poor prognosis of human glioma [19,20]. Furthermore, a bioinformatics analysis has revealed that IKBKE is a preferential target gene of the microRNAs let-7b and let-7i. Therefore, the use of let-7b and let-7i may be a novel therapeutic strategy for human glioma.

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http://dx.doi.org/10.1016/j.bbrc.2015.01.105
0006-291X/

In this study, we investigated that the effects of miR- let-7b and miR-let-7i on the invasion and migration of the glioma cell lines U251 and U87 occur through the direct targeting to IKBKE and explored the possible mechanisms responsible for this action.

2. Materials and methods

2.1. Cell lines and tissue samples

The human GBM cell lines U251 and U87 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C with 5% CO_2.

Tissue specimens and clinical information were obtained, and the study was approved by the Institutional Review Board of Tianjin Medical University, China. Three normal brain tissues were obtained from patients with traumatic brain injury and brain tumours for internal decompression. Immediately after surgery, the samples were snap-frozen and stored in liquid nitrogen.

2.2. Analysis of microRNA with candidate target genes

Previous studies performed in our laboratory have shown a positive correlation between IKBKE expression and the invasion and migration of human glioma cell. To identify which type of miRNAs can directly regulate the expression of IKBKE, we further analysed our data using several databases. First, we used TargetScan 6.2 (http://www.targetscan.org) to search for the miRNAs that target IKBKE. We then used microRNA.org - Targets and Expression (http://www.microrna.org).

2.3. RNA extraction and quantitative real-time PCR analysis (qRT-PCR)

The total RNA from the GBM cells (U251 and U87) and tissues was extracted using the TRIzol reagent (Invitrogen) following the manufacturer's protocol. The concentration and purity of RNA were determined using a NanoDrop® ND-1000 spectrophotometer. The GoScript™ Reverse Transcriptase system (Promega, USA) was used for cDNA synthesis according to the manufacturer's protocol.

The expression levels of mature let-7b/i were quantified by qRT PCR using the Hairpin-it™ miRNA qPCR Quantitation Kit (GenePharma Co., Ltd.). All of the PCR reactions were performed using SYBR Green PCR reagents (Applied Biosystems) according to the manufacturer's instructions, and the PCR cycling conditions were the following: 95 °C for 3 min and 40 cycles of 95 °C for 12 s and 62 °C for 40 s. The melting curve was obtained from 62 °C to 95 °C with readings performed every 0.2 °C for 2 s. U6 was used as the internal control. The data are shown as fold changes and analysed initially using the Bio-Rad CFX Manager 3.0 software (Bio-Rad, USA).

2.4. miRNA and IKBKE siRNA transfection

The oligonucleotide sequence of the let-7b mimics was 5′-UGA GGU AGU AGG UUG UGU GGU U-3′, and that of the let-7i mimics was 5′-UGA GGU AGU AGU UUG UGC UGU U-3′. A scrambled siRNA sequence (5′-UUC UCC GAA CGU GUC ACG UTT-3′) was used as the negative control. The sequence of the IKBKE siRNA was 5′-GCA CCA CAT CTA TAT CCA TGC -3′. All of these siRNAs were generated by GenePharma (Co. Ltd., Shanghai, China). The cell transfections were conducted according to the recommendations provided by GenePharma.

2.5. Luciferase activity assay

The pEZX-IKBKE-3′UTR-Subcloning and pEZX-IKBKE-3′UTR-Mut plasmids were purchased from GenScript (Nanjing, China). The putative let-7b/i binding site was cloned into the AsiSI/XboI site of the pEZX-MT01 vector downstream of the luciferase gene to generate pEZX-IKBKE vectors (Fig. 2B).

For the luciferase reporter assay, U251 and U87 cells were cultured in 96-well plates (2000 cells per well) and then transfected with 5 pmol of the let-7b/i mimics oligonucleotide using X-tremeGENE siRNA Transfection Reagent. Twenty-four hours after transfection, the cells were transfected with 0.1 μg of either the pEZX-IKBKE-3-UTR-Subcloning plasmid or the pEZX-IKBKE-3′UTR-Mut plasmid using 0.2 μl of the X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland). Forty-eight hours after the second transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA).

2.6. Wound healing cell migration assays

U251 and U87 cells were seeded in six-well dishes (2×10^5 cells/well) and then transfected with let-7b and let-7i mimics (100 pmol/5 μl). Forty-eight hours after transfection (~90% confluence), an incision was made by scraping the cell monolayer with a sterile pipette tip in the central area of the confluent culture to create an artificial wound. To remove detached cells, the cells were then washed with PBS, and fresh growth media was added. The cells were then incubated at 37 °C, and images of the wound area at 48 h after injury were captured using an inverted microscope. The normalized wound area was calculated using TScratch software [21].

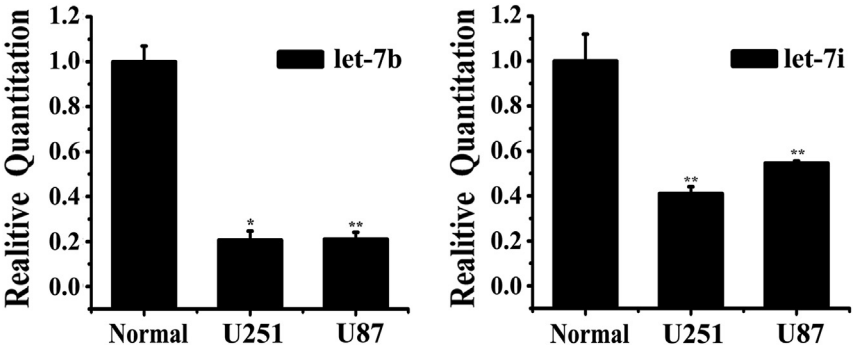

Fig. 1. The expression levels of the microRNAs let-7b/i in normal brain tissues and two different glioblastoma cell lines (U251 and U87) were compared (*$p < 0.05$; **$p < 0.01$).

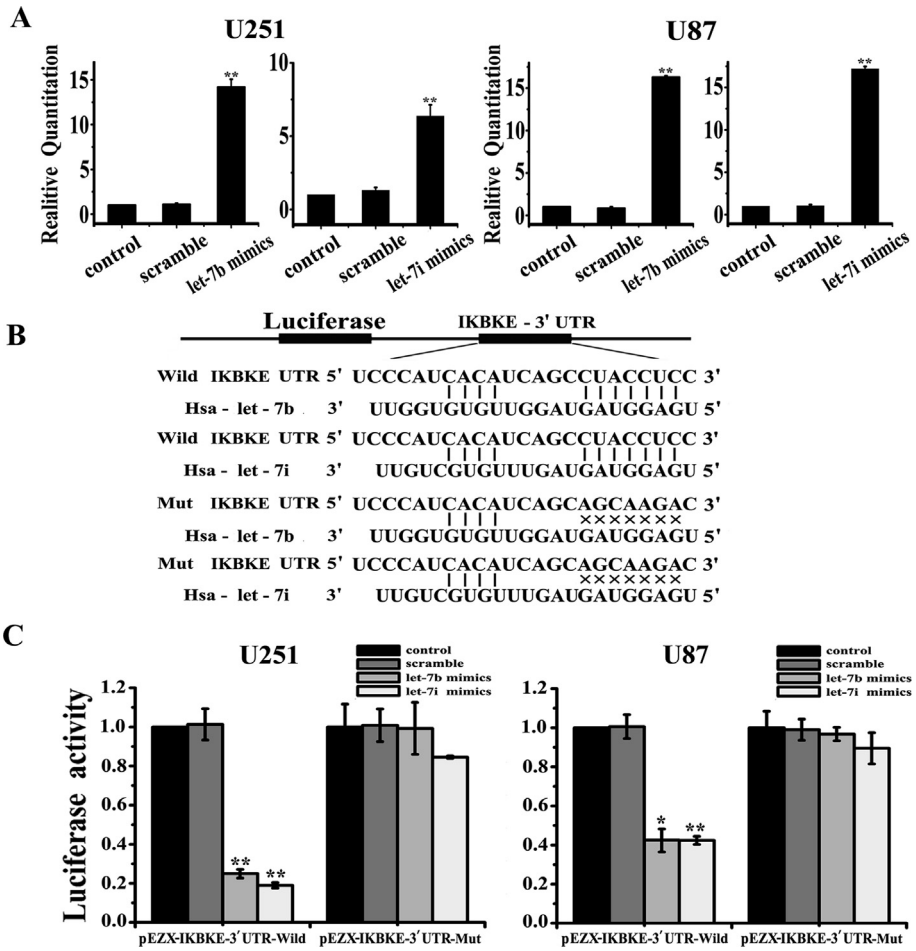

Fig. 2. Analysis of IKBKE as a target gene of let-7b/i. A Relative quantification of let-7b and let-7i in U251 and U87 cells following transfection with the let-7b and let-7i mimics. The quantification was performed through quantitative real-time PCR assays. B Schematic representation of the pEZX-IKBKE 3′UTR-containing reporter constructs. C Luciferase activity in the human glioma cells co-transfected with the let-7b/i mimics and the pEZX-IKBKE 3′UTR plasmids.

2.7. Transwell cell invasion assay

The ability of U251 and U87 cells to migrate was assessed using Transwell filters (8-μm pore size, Millipore, USA). The Transwell filters were place into 24-well plates coated with Matrigel on the upper surface of chamber. Forty-eight hours after transfection, a total of 5 x 10^4 cells were resuspended in 100 μl of serum-free DMEM and plated on the upper chamber of the Transwells. After incubation for 48 h at 37 °C in a 5% CO_2 humidified incubator, the cells in the inner chamber were removed from the upper side with a cotton swab, and the cells attached to the bottom side of the membrane were fixed with 100% methanol, stained with crystal violet, counted and imaged under an inverted microscope at x200 magnification (Olympus Corp., Tokyo, Japan) over ten random fields in each well. Each experiment was performed in triplicate.

2.8. Protein extraction and western blot analysis

The cells were harvested at 48 h after transfection, and the total protein from the glioma cell lines was extracted using the RIPA lysis buffer with proteinase inhibitor. The homogenates were clarified by centrifugation at 20,000 × g and 4 °C for 15 min, and the protein concentration was measured by the bicinchoninic acid method. Then, 50 μg of protein mixed with 5 × SDS loading buffer was loaded into each lane and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA), and the membranes were incubated with primary antibodies against IKBKE and E-cadherin (1:1000 dilution, Abcam UK) and then with an HRP-conjugated secondary antibody (1:5000 dilution, Zhongshan Bio Corp., Beijing, China). The specific protein was detected using a Super Signal protein detection kit (Pierce, USA). After washing with stripping buffer, the membrane was re-probed with antibody against GAPDH (1:5000 dilution, Santa Cruz, USA).

2.9. Statistical analysis

The SPSS software (version 16.0; SPSS Inc.) was used for the statistical analyses. Statistical significance was considered at p values of less than 0.05.

3. Results

3.1. Let-7b/i expression is downregulated in glioma cell lines

To further study the biological role of let-7b/i in human glioma tissues, the expression levels of let-7b/i in normal brain tissues and glioma cell lines were measured by miRNA quantitative RT-PCR. As shown (U251:let-7b, $P < 0.05$; let-7i, $P < 0.01$; and U87:let-7b/i, $P < 0.01$; Fig. 1), the expression of let-7b/i was markedly down-regulated in glioma cell lines compared with normal brain tissues.

3.2. Let-7b/i directly target IKBKE

3.2.1. The bioinformatics analysis identified IKBKE as a preferential candidate target gene of the microRNAs let-7b and let-7i

Using TargetScan 6.2, 99 miRNAs were shown to have a direct association with the IKBKE gene, and as the analysis using microRNA.org - Targets and Expression identified 25 miRNAs. As a result, 10 of them are the same. TargetScan 6.2 showed that let-7b and let-7i exhibit a stronger binding force with the target gene IKBKE.

The quantitative real-time PCR results showed that let-7b/i expression is upregulated in U251 and U87 cells by 14.19 and 6.37 and by 16.30 and 17.21-fold ($P < 0.01$), respectively, after transfection with the let-7b/i mimics compared with the expression levels observed in the control and scramble-treated cells (Fig. 2A).

The IKBKE protein was found to be significantly downregulated in glioma cells with ectopic let-7b/i expression, as determined by western blotting (Fig. 3A), suggesting that let-7b/i induce a silencing effect on endogenous IKBKE expression.

In addition, the relative luciferase activity was significantly decreased in the cells co-transfected with let-7b/i and the IKBKE-3′UTR-Wild plasmid compared with the negative control cells and the scramble plasmid-treated cells. Moreover, let-7b/i did not suppress the luciferase activity in the IKBKE-3′UTR-Mut plasmids-treated cells (U251:let-7b/i, $P < 0.01$; U87:let-7b, $P < 0.05$; let-7i, $P < 0.01$; Fig. 2C). Taken together, these results reveal that let-7b/i specifically recognize binding sites in the IKBKE 3′UTR and directly repress IKBKE expression.

3.3. Let-7b/i inhibit the migration and invasion of human glioma cells in vitro

The migration of cells to the wound area was analysed at 48 h after injury, and the results revealed that the overexpression of let-7b/i led to a marked inhibition of wound healing compared with the negative control and cells transfected with the scrambled mimics, indicating impaired migration ($P < 0.05$, Fig. 3A). As shown in Fig. 3B, the overexpression of let-7b/i significantly impaired the invasion of both U251 and U87 cells across a Transwell chamber compared with the negative control and scramble group ($P < 0.05$). Collectively, these data indicate that let-7b/i play a role in glioma cell migration and invasion *in vitro*.

3.4. Let-7b/i inhibit the E-cadherin-mediated invasive ability of human glioma cells by directly regulating IKBKE

Previous studies conducted in our laboratory have shown that IKBKE exerts a significant impact on the invasion and migration of human glioblastoma cell lines [15], possibly by regulating E-cadherin expression. E-cadherin plays a critical role in the control of cell invasion and metastasis [22]. We therefore investigated IKBKE-related pathways. As shown in Fig. 4, an obvious activation of E-cadherin was observed in U251 and U87 cells after transfection with IKBKE siRNA. Consistently, the overexpression of let-7b/i led to a marked downregulation of IKBKE and a marked upregulation of E-cadherin. These data suggest that the anti-invasion activity of let-7b/i in glioblastoma cells likely acts

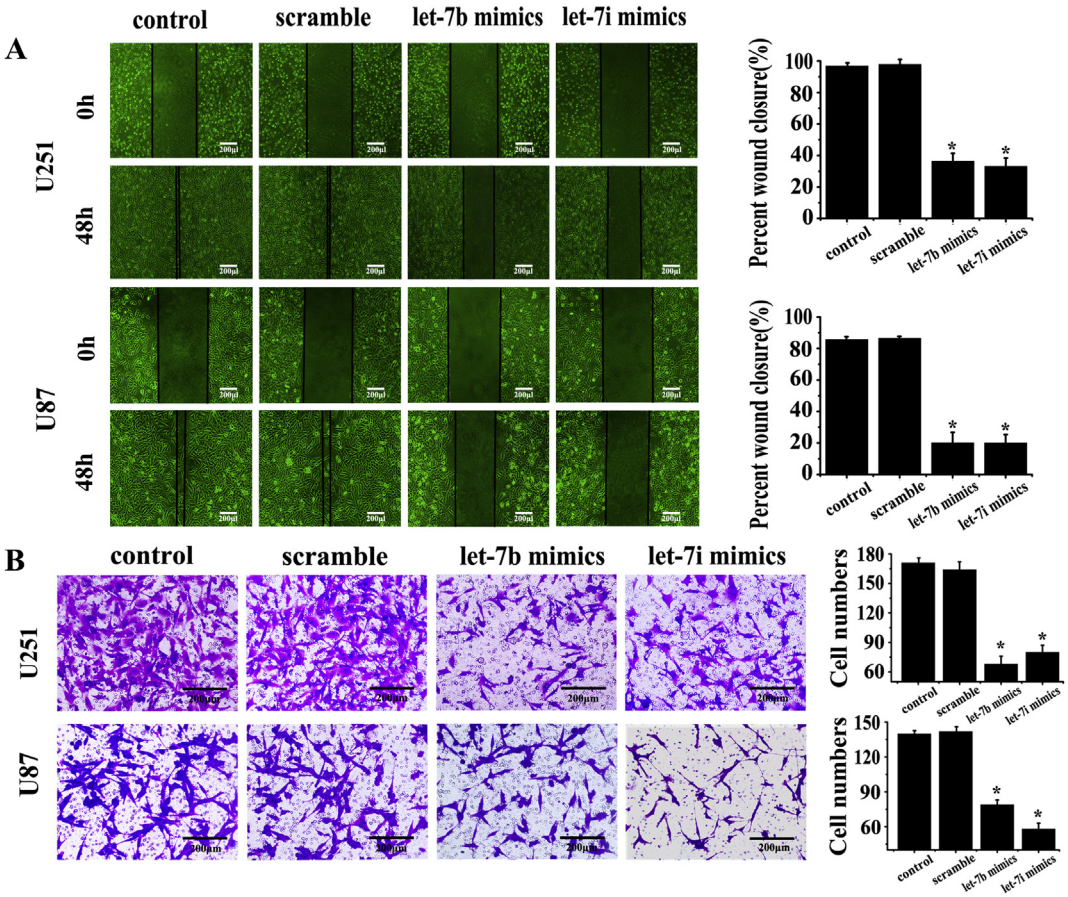

Fig. 3. Effects of the upregulation of let-7b/i on the invasion and migration of glioma cells. A Classic wound healing assay for assessing the migration of the let-7b/i mimics-treated cells. B Transwell assays for assessing the invasion of the let-7b/i mimics-treated cells.

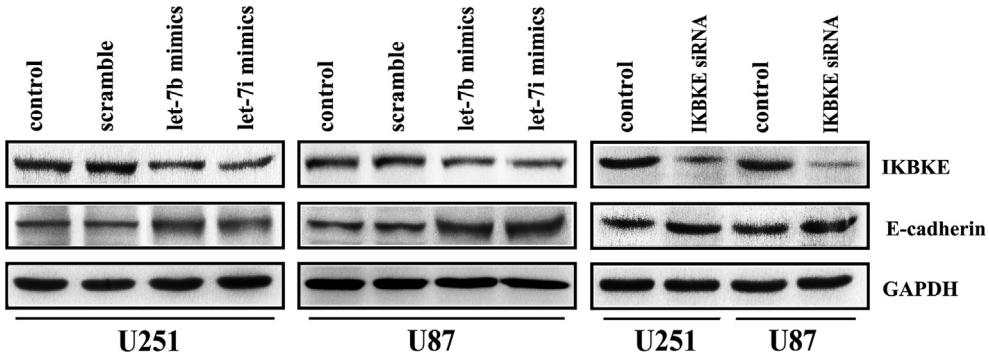

Fig. 4. Let-7b/i upregulate E-cadherin by downregulating IKBKE IKBKE and E-cadherin expression in U251 and U87 cells treated with the let-7b/i mimics and IKBKE siRNA was analysed by immunoblotting.

through the direct downregulation of IKBKE and the indirect upregulation of E-cadherin.

4. Discussion

IKBKE has been identified as an oncogenic protein and found to be upregulated in many tumour specimens. Previous studies conducted in our laboratory have demonstrated that IKBKE downregulation can markedly inhibit the proliferative and invasive abilities of human glioma cells [15]. Elevated expression of IKBKE is not a consequence of gene amplification, which indicates that several mechanisms can upregulate IKBKE expression [23]. Therefore, exploring the mechanisms that upregulate IKBKE may be useful for the development of therapeutic strategies against human glioma.

MicroRNAs (miRNAs) are an abundant class of small (~22 nucleotides) non-coding RNA molecules that control gene expression through mRNA degradation or translational repression at the posttranscriptional level by binding to target messenger RNAs (mRNAs), particularly the 3′-untranslated regions (3′-UTRs) of specific mRNAs [24]. Previous studies have shown that miRNAs play an important role in various biological processes, such as cell differentiation, proliferation, apoptosis, and tumour metastasis [25]. The miRNAs belonging to the let-7 family are highly conserved in both sequence and function. The let-7 family has been demonstrated to act as tumour inhibitors by suppressing oncogenes and pivotal regulators of some signal pathways, including HMGA2, MYC, and RAS [26]. For instance, let-7a exhibits a lower expression level in high-grade glioma and inhibits glioma malignancy by suppressing its target transcript K-ras [27], whereas let7-b inhibits the growth and facilitates the differentiation of benign breast disease by inhibiting the expression of BSG [28]. In addition, reduced let-7g expression can promote mammary carcinoma cell migration and invasion *in vitro* [29], and the overexpression of let-7i can decrease the resistance of ovarian and breast cancer cells [30]. This study showed a markedly low expression of the microRNAs let-7b and let-7i in glioma cell lines compared with normal brain tissues by quantitative real-time PCR, and we confirmed that IKBKE is a target gene of let-7b/i. Therefore, the use of let-7b and let-7i may be a novel therapeutic strategy for human glioma.

Lee et al. [17] demonstrated that let-7 miRNA reduces the migration of glioblastoma cells. Mao et al. [20] showed that LIN28A expression enhances the invasiveness of GBM cells by downregulating let-7b and let-7g. These results are consistent with the findings of our studies. The overexpression of let-7b/i led to a marked inhibition of cell migration and significantly impaired the invasion of both U251 and U87 cells. Although it is well known that the miRNA let-7 family play important roles in the proliferation and invasion of human glioma by targeting K-ras, we hypothesize that let-7b/i suppress the potential invasion and migration of human glioma by targeting the IKBKE gene. Liang et al. [31] demonstrated that ILK can promote cell migration and invasion by downregulating E-cadherin in glioma cells. In our study, IKBKE knock down by siRNA markedly suppressed E-cadherin in human glioma cells. Epithelial cadherin (E-cadherin), a typical tumour inhibitor that belongs to the calcium-dependent adhesion protein family, regulates the cell and cell-matrix adhesion [32] and has been identified as a tumour suppressor in many cancers, including oesophageal, gastric, breast [33], lung cancers [34] and gliomas [35]. Correlative studies have shown that E-cadherin plays an important role in maintaining the epithelial cell morphology and structural integrity [36], as well as in maintaining the cell shape and regulating cell adhesion [37]. The low expression or absence of E-cadherin can cause tumour cells to lose contact inhibition, resulting in unrestricted cell proliferation and loss of differentiation [32]. Moreover, the connections between the tumour cells can be released, resulting in their easy migration from the primary tumour to the adjacent tissues or lymphatic vessels [38,39]. The loss of its expression is a hallmark of the epithelial–mesenchymal transition (EMT). The invasion of the gliomas is closely related to the recurrence and development of the tumour, whereas the epithelial–mesenchymal transition (EMT) is related to the invasion and migration of the gliomas [35]. Wound healing and Transwell assays have shown that the overexpression of let-7b and let-7i can inhibit the invasion and migration of glioma cells and upregulate E-cadherin, as determined by western blot.

In conclusion, all of these data suggest that let-7b/i inhibit the invasive ability of glioma cells by directly downregulating its target IKBKE and indirectly upregulating E-cadherin. However, IKBKE was found to act as a serine/threonine protein kinase, the mechanism through which is can regulate E-cadherin remains to be elucidated. Enhancing the expression of let-7b/i may be a novel and useful therapeutic strategy for the treatment of glioblastoma.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the China National Natural Scientific Fund (Grant No. 81172405) and the Tianjin Science and Technology Committee (Grant No. 13JCZDJC31000 and 13JCQNJC10800).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.105.

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