MicroRNA-382 inhibits cell proliferation and invasion of retinoblastoma by targeting BDNF-mediated PI3K/AKT signalling pathway

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Abstract. It has previously been demonstrated that multiple microRNAs (miRNAs or miRs) are aberrantly expressed in retinoblastoma (RB) and contribute to RB initiation and progression. miR-382 has been revealed to be aberrantly expressed and therefore exhibits a key role in the progression of various types of cancer. However, the expression pattern, functional roles and underlying molecular mechanism of miR-382 in RB remain unknown. The present study investigated the expression levels of miR-382 and its effects on RB cells and the underlying regulatory mechanism of its action. It was demonstrated that miR-382 was downregulated in RB tissues and cell lines. Upregulation of miR-382 inhibited RB cell proliferation and invasion in vitro. Additionally, brain-derived neurotrophic factor (BDNF) was identified as a novel target of miR-382 in RB. BDNF was upregulated in RB tissues and negatively associated with miR-382 expression levels. Furthermore, BDNF overexpression rescued the tumour-suppressing effects on RB cells induced by miR-382. miR-382 inactivated the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signalling pathway in RB. These findings suggested that miR-382 serves as a tumour suppressor in RB, in part, by targeting the BDNF-mediated PI3K/AKT signalling pathway. The results of the present study suggest a potential therapeutic strategy for treating RB patients in the future.

Introduction

Retinoblastoma (RB) is the most common intraocular malignant tumour that normally affects infants and young children, with a prevalence ranging from 1:15,000 to 1:20,000 in children under the age of 5 years in the USA (1,2). RB can be classified into two groups: Hereditary and nonhereditary. Hereditary RB patients account for 30-40% cases and is usually associated with a positive family history; those patients with no family history are generally classified as nonhereditary (3-5). Previous studies demonstrated that high oncogene expression level, loss of tumour suppressors and epigenetic changes of oncogenic methylation may be involved in RB formation and progression (6-8). Currently, the primary treatments for RB are enucleation, chemotherapy and focal therapy, such as laser or cryotherapy (9). Despite improvements in therapeutic treatments, the prognosis for patients with RB remains poor, with a mortality rate of 50-70% among children in underdeveloped countries (10). This poor outcome is largely due to diagnosis and treatment delay (11). Therefore, further exploration of the biology and molecular mechanisms of RB that cause RB procession and metastasis and investigation of novel therapeutic methods to improve the prognosis of patients with RB are necessary.

MicroRNAs (miRNAs or miRs) are a group of endogenous, single-strand, non-coding 19-25 nucleotide RNAs that modulate more than half of the genes in human cells (12). Over 1,000 miRNAs have been identified within the human genome (13), and they may regulate gene expression by interacting with complementary sites in the 3'-untranslated regions (3'-UTRs) of their target mRNA molecules, thereby leading to mRNA degradation or post-transcriptional translational repression (14). miRNAs act as key regulators in a wide variety of physiological and pathological processes, such as cell proliferation, cycle, differentiation, apoptosis, metabolism and death (15-17). An increasing number of studies reported miRNA dysregulation in various kinds of human cancers, such as gastric cancer (18), glioma (19), lung cancer (20), bladder cancer (21) and RB (22). The abnormally expressed miRNAs are involved in progression and development of various cancers and serve as oncogenic and tumour suppressors by directly targeting the oncogenes and tumour-suppressor genes, respectively (23-25). Several miRNAs were correlated with RB tumorigenesis and development and might serve as
independent prognostic markers or therapy targets for patients with this malignancy (26-28). Therefore, aberrant miRNAs and their functions in RB need to be explored to benefit the discovery of novel diagnosis biomarkers and therapeutic strategies.

miR-382 is aberrantly expressed and play important roles in several types of cancer (29-31). However, the expression pattern, functional roles and underlying molecular mechanism of miR-382 in RB remains unknown. This study aimed to investigate the miR-382 expression levels in RB tissues and cell lines. Furthermore, the effects of miR-382 on RB cells and the underlying regulatory mechanism of its action were also examined.

Materials and methods

Tissue samples. This study was approved by the Human Subjects Committee of Beijing Tsinghua Chang Gung Hospital. Informed consent was also obtained from all patients. A total of 26 RB tissues were obtained from patients who underwent enucleation at Beijing Tsinghua Chang Gung Hospital (Beijing, China). Nine normal retina samples were collected from paediatric ruptured globe. RB tissues were excluded from experiment if the RB patients had been treated with chemotherapy or other treatments prior to enucleation. None of the patients received thermotherapy, cryotherapy, chemotherapy and radiotherapy before surgery. Tissues were immediately snap frozen in liquid nitrogen and transferred at -80°C in the refrigerator for storage.

Cell lines, culture conditions and transfection. Three human RB cell lines (Y79, WERI-RB-1 and SO-RB50) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were grown in a humidified atmosphere at 37°C with 5% CO₂. Further, cells were incubated at 37°C in a 5% CO₂ atmosphere for 24 h. Subsequently, the cells were re-suspended with FBS-free RPMI-1640 medium as a single-cell solution and seeded into the upper chambers at a density of 5x10⁴ cells/chamber. RPMI-1640 medium supplemented with 20% FBS was added into the lower chamber as a chemoattractant. Transwell chambers were then incubated at 37°C in a 5% CO₂ atmosphere for 24 h. Subsequently, the non-invasive cells were carefully removed with cotton swab, whereas the invasive cells were fixed with 100% methanol at 37°C for an additional 4 h. Finally, the absorbance was examined at a wavelength of 450 nm using a multifunction microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each assay was performed in 5-well replication and repeated three times.

Matrigel invasion assay. The invasive capacity of RB cells was examined using 24-well Transwell chambers (8 µm; Corning Costar, Cambridge, MA, USA) pre-coated with Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA). After incubation for 48 h, the transected cells were collected, re-suspended with FBS-free RPMI-1640 medium as a single-cell solution and seeded into the upper chambers at a density of 5x10⁴ cells/chamber. RPMI-1640 medium supplemented with 20% FBS was added into the lower chamber as a chemoattractant. Transwell chambers were then incubated at 37°C in a 5% CO₂ atmosphere for 24 h. Subsequently, the non-invasive cells were carefully removed with cotton swab, whereas the invasive cells were fixed with 100% methanol for 5 min, stained with 0.1% crystal violet for 10 min and washed in PBS. The number of invasive cell was counted in five randomly selected fields under an inverted microscope (CKX41; Olympus, Tokyo, Japan).

miR-382 target prediction. The potential targets of miR-382 were analyzed with miRNA target prediction algorithms; PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/).
Western blot analysis. The total proteins of the cell lines and tissues were extracted using a radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) in the presence of a proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA). The total protein concentration was examined using the bicinchoninic acid assay protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), the membranes were incubated at 4°C overnight with primary antibodies: rabbit anti-human polyclonal BDNF (sc-20981; 1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human monoclonal phosphoinositide 3-kinase (PI3K) antibody (ab86714; 1:1,000 dilution; Abcam, Cambridge, MA, USA), rabbit anti-human polyclonal p-PI3K antibody (cat. no. 4228; 1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA), mouse anti-human monoclonal p-protein kinase B (AKT) (sc-271966; 1:1,000 dilution; Santa Cruz Biotechnology), mouse anti-human monoclonal AKT (sc-56878; 1:1,000 dilution; Santa Cruz Biotechnology), and mouse anti-human monoclonal GAPDH (sc-32233; 1:1,000 dilution; Santa Cruz Biotechnology). The membranes were subsequently washed in TBST thrice and probed with corresponding horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology) for 2 h at room temperature. Finally, the protein signals were detected using Pierce™ ECL Western Blotting Substrate (Pierce Biotechnology, Inc.) and analysed with Quantity One® software (version 4.62; Bio-Rad Laboratories, Inc.). GAPDH was used as a loading control.

Statistical analysis. All data are expressed as mean ± standard deviation, and the differences between groups were analysed with Student's t-test or one-way analysis of variance using SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA). Spearman's correlation analysis was utilised to analyse the correlation between miR-382 and BDNF mRNA expression levels in RB tissues. *P<0.05 was considered statistically significant.

Results

miR-382 expression is downregulated in RB tissues and cell lines. Firstly, we measured miR-382 expression in 26 RB tissues and 9 normal retina tissues using RT-qPCR. The miR-382 expression levels were significantly downregulated in RB tissues compared with that in normal retina tissues (Fig. 1A, P<0.05). Moreover, the miR-382 expression levels in three RB cell lines (Y79, WERI-RB-1 and SO-RB50) were examined. As shown in Fig. 1B, miR-382 expression decreased in RB cell lines compared with that in normal retina tissues (P<0.05). These results suggested that miR-382 may play crucial roles in RB occurrence and development.

miR-382 inhibits RB cell proliferation and invasion in vitro. To determine the potential roles of miR-382 in RB, Y79 and WERI-RB-1 cells were transfected with miR-382 mimics or miR-NC. RT-qPCR confirmed that miR-382 was markedly upregulated in Y79 and WERI-RB-1 cells after transfection with miR-382 mimics (P<0.05; Fig. 2A and B). CCK-8 assay was performed to investigate the effect of miR-382 on RB cell proliferation. The miR-382 upregulation significantly inhibited the Y79 and WERI-RB-1 cell proliferation (P<0.05; Fig. 2C and D). We further performed Matrigel invasion assay to determine the effect of miR-382 on RB cell invasion. The invasive capacities of Y79 and WERI-RB-1 cells were obviously suppressed in cells overexpressing miR-382 compared with those in the miR-NC group (P<0.05; Fig. 2E and F). These results suggested that miR-382 may play tumour-suppressing roles in RB.

BDNF is a direct target gene of miR-382 in RB. To elucidate the underlying molecular mechanisms of miR-382 in RB, bioinformatics analysis was conducted to predict the putative targets of miR-382. Among these potential targets, BDNF (Fig. 3A) was selected for further validation owing to its potential roles in RB malignancy regulation (33,34). To confirm this prediction, luciferase reporter assay was used to determine whether miR-382 could directly target the 3'-UTR of BDNF. The restoration expression of miR-382 decreased the luciferase activity of reporter plasmid that contained the wide-type BDNF 3'-UTR (P<0.05; Fig. 3B). In contrast, no significant inhibition was observed for the Mut reporter after miR-382 mimic transfection. RT-qPCR and western blot analysis were utilised to detect the BDNF expression in Y79 and WERI-RB-1 cells transfected with miR-382 mimics or miR-NC. As shown in Fig. 3C and D, miR-382 overexpression decreased BDNF expression at both
the mRNA and protein levels in Y79 and WERI-RB-1 cells (P<0.05). Taken together, these data indicate that BDNF is a direct target of miR-382 in RB.

**BDNF is upregulated and inversely correlated with miR-382 expression in RB tissues.** To further explore the relationship between miR-382 and BDNF, we detected the BDNF expression at the mRNA and protein levels in 26 RB tissues and nine normal retina tissues using RT-qPCR and western blot analysis, respectively. RB tissues had increased BDNF expression at both the mRNA (P<0.05; Fig. 4A) and protein levels (P<0.05; Fig. 4B) compared with the normal retina tissues. Additionally, Spearman's correlation analysis indicated a statistically significant inverse correlation between miR-382 and BDNF mRNA level in RB tissues (r=-0.7284, P<0.001; Fig. 4C).

**BDNF reverses the tumour-suppressing effects of miR-382 on RB cell proliferation and invasion.** Rescue experiments were performed to evaluate whether BDNF is responsible for the tumour-suppressing effects of miR-382 in RB cells. Y79 and WERI-RB-1 cells were transfected with miR-382 mimics or miR-NC and co-transfected with pcDNA3.1-BDNF or pcDNA3.1. Western blot analysis revealed that the restoration expression of BDNF abolished the inhibition caused by the miR-382 mimics in Y79 and WERI-RB-1 cells (P<0.05; Fig. 5A). Subsequently, CCK-8 and Matrigel invasion assays were performed in the above mentioned cells. Notably, the resumption expression of BDNF rescued the suppressive effects of miR-382 on the proliferation (P<0.05; Fig. 5B and C) and invasion (P<0.05; Fig. 5D and E) of Y79 and WERI-RB-1 cells. These results suggested that miR-382 exerted its
tumour-suppressing roles in RB, at least in part by regulating BDNF.

miR-382 inhibits PI3K/AKT signalling in RB. BDNF is involved in PI3K/AKT signalling pathway regulation (35,36).
Therefore, we analysed the effects of miR-382 on PI3K, p-PI3K, AKT and p-AKT protein expression in Y79 and WERI-RB-1 cells transfected with miR-382 mimics or miR-NC. The miR-382 upregulation was found to reduce the p-PI3K and p-AKT expression but did not affect the total PI3K and AKT expression in Y79 and WERI-RB-1 cells (Fig. 6). These results suggested that miR-382 exerts suppressive roles in RB cells by directly regulating BDNF and indirectly regulating the PI3K/AKT signalling pathway.

Discussion

miRNAs serve as post-transcriptional gene regulators that potentially play critical roles in the proliferation, cycle,
BDNF was also demonstrated to be a direct downstream target of miR-382 in RB. In this study, miR-382 was significantly downregulated in RB tissues and cell lines. Furthermore, miR-382 upregulation suppressed cell proliferation and invasion of RB in vitro. BDNF was also demonstrated to be a direct target of miR-382 in RB. Importantly, miR-382 inactivated PI3K/AKT signalling pathway in RB. To the best of our knowledge, this is the first study to investigate the expression and role of miR-382 in RB.

miR-382 was frequently found to be abnormally expressed in multiple human cancers. For instance, miR-382 was significantly downregulated in non-small cell lung cancer (29). Additionally, low miR-382 expression level was correlated with the tumour stage and metastasis of non-small cell lung cancer (29). The miR-382 expression level was decreased in highly metastatic osteosarcoma cell lines and relapsed osteosarcoma tissues. Low miR-382 expression level was inversely associated with relapse and positively associated with metastasis-free survival in osteosarcoma patients (42). The miR-382 expression was relatively low in oesophageal squamous cell carcinoma for patients with poor outcome. Kaplan-Meier analysis indicated a significant reverse correlation between miR-382 expression and survival time of oesophageal squamous cell carcinoma patients (43). The downregulation of miR-382 was also observed in prostate (44), ovarian (45) and colorectal (30) cancers. However, miR-382 was highly expressed in tumour tissues and associated with pathological grade and clinical stage in breast cancer (31). These findings indicated that miR-382 expression has tissue specificity and may be a prognostic target for cancers.

Aberrant miR-382 expression was reportedly associated with various human cancers. Chen et al found that ectopic miR-382 expression repressed cell proliferation, migration and invasion in non-small cell lung cancer (29). Xu et al revealed that miR-382 overexpression attenuated osteosarcoma cell growth, metastasis, epithelial-mesenchymal transition and chemoresistance (42,46). Zhang et al reported that restoration expression of miR-382 impeded cell proliferation and motility in prostate cancer (44). Tan et al demonstrated that resumption expression of miR-382 decreased cell proliferation, metastasis and epithelial-mesenchymal transition in ovarian cancer (45). Zhou et al revealed that enforced miR-382 expression inhibited colorectal cancer cell growth, migration and invasion in vitro (30). These studies indicate that miR-382 is a tumour suppressor in certain types of cancer. However, miR-382 serve as an oncogene in breast cancer by promoting cell viability, clonogenicity, survival, migration and invasion in vitro and tumourigenesis or metastasis in vivo (31). This contradiction may be explained by the ‘imperfect complementarity’ of the interactions between miRNAs and their target genes (47).

Several targets of miR-382 have been identified, such as SETD8 (29) in lung cancer, KLF12 (46), HIPK3 (46), YB-1 (42) in osteosarcoma, COUP-TFII (44) in prostate cancer, ROR1 (45) in ovarian cancer, NR2F2 in colorectal cancer (30), and RERG (31) in breast cancer. In this study, BDNF was demonstrated to be a direct downstream target gene of miR-382 in RB. To explore the mechanism underlying the tumor-suppressing roles of miR-382 in RB, bioinformatics analysis was performed to predict the candidate targets of miR-382. BDNF was predicted as a potential target of miR-382. Luciferase reporter assay was conducted to confirm this prediction, and found that miR-382 could directly targeted the 3'-UTR of BDNF. Additionally, RT-qPCR and western blot analysis demonstrated that miR-382 negatively regulated endogenous BDNF expression at both mRNA and protein level in RB cells. Furthermore, BDNF was upregulated in RB tissues and inverse correlated with miR-382 expression level. Besides, rescue experiments indicated that BDNF overexpression reversed the suppressive effects of miR-382 on RB cells. Taken together, it is reasonable to suggest that alterations in miR-382 expression regulates the proliferation and invasion of RB cells via directly targeting BDNF.

BDNF, a member of the neurotrophin family, plays an essential role in neural maturation and differentiation (48). BDNF was upregulated in several human malignancies, such as bladder cancer (49), glioma, gastric cancer (50), colorectal cancer (51) and breast cancer (52). Moreover, aberrant BDNF expression was involved in tumourigenesis and tumour development. In colorectal cancer, BDNF knockdown inhibited cell proliferation, migration and invasion and enhanced apoptosis (51,53). In lung cancer, BDNF downregulation suppressed cell proliferation and invasion and increased apoptosis (54). In RB, BDNF was upregulated in tumour tissues and cell lines (33) and contribute in RB occurrence and progression regulation (34). All these findings suggested that BDNF is worthy of investigation as a potential target for the treatment of patients with RB.

In conclusion, miR-382 was significantly downregulated in RB tissues and cells. Additionally, we demonstrated that miR-382 acts as a tumour suppressor in RB through direct targeting of BDNF and indirect regulation of the PI3K/AKT signalling pathway. These results contribute evidence that miR-382 plays an important role in tumour malignant progress, and miR-382 may represent a potential gene-targeting approach for RB treatments.
References


