miR-29a inhibits human retinoblastoma progression by targeting STAT3

SHU LIU¹, XIAOMENG ZHANG¹, CHUNMEI HU², YINGXUE WANG³ and CHUNLING XU¹

Departments of ¹Ophthalmology, ²Tumor and Hematology and ³Electrical Diagnosis, The Second Hospital of Jilin University, Changchun, Jilin 130041, P.R. China

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Abstract. Retinoblastoma (RB) is the most common malignancy that occurs during childhood. Growing evidence supports a crucial role for microRNAs (miRNAs) in regulating the initiation and progression of RB. Aberrant expression of microRNA-29a (miR-29a) has been found in many types of cancers, but not including RB. Therefore, the aims of the present study were to evaluate the regulatory role and underlying mechanism of miR-29a in human RB. In the present study, we found that miR-29a expression was significantly downregulated in RB tissues and cell lines. Overexpression of miR-29a in RB cells significantly inhibited cell proliferation, migration, and invasion and promoted cell apoptosis in vitro. Additionally, signal transducer and activator of transcription 3 (STAT3) was identified as a direct target of miR-29a in RB cells. miR-29a overexpression in RB cells not only inhibited STAT3 expression but also altered expression of its downstream genes, including Bcl2, cyclin D1 and matrix metalloproteinase 2 (MMP-2). STAT3 mRNA expression was upregulated in RB tissues and negatively correlated with miR-29a expression. Reintroduction of STAT3 without 3’-untranslated region (3’UTR) reversed the inhibitory effects of miR-29a on cell proliferation, migration and invasion. In vivo study confirmed that overexpression of miR-29a also inhibited tumor formation of RB in a nude mouse model by repressing STAT3. Collectively, these data suggest that miR-29a exerts a tumor suppressor effect on RB by repressing STAT3, supporting the targeting of miR-29a as a potentially effective therapeutic method for RB.

Introduction

Retinoblastoma (RB) is the most common intraocular malignancy in childhood with a high mortality rate, especially in developing countries (1,2). Although great efforts have been made in the treatment of RB in recent years, the survival rate remains poor mainly due to limitations in the early diagnosis of the disease and the development of metastasis (3). Therefore, it is crucial to explore the key molecular mechanisms involved in RB initiation and development to identify new diagnostic markers and therapeutic targets.

MicroRNAs (miRNAs) are small (18-25 nucleotide long), non-coding RNAs that regulate gene expression via binding to the 3’-untranslated region (3’UTR) of target mRNAs, leading to mRNA degradation or translational inhibition (4,5). It has been demonstrated that miRNAs are involved in diverse biological processes, including metabolic homeostasis, cell proliferation and cell apoptosis (6,7). Accumulating evidence shows that the altered expression of miRNAs are involved in the initiation and progression of cancer (8), suggested that miRNAs can serve as diagnostic markers and therapeutic targets in human cancers. miRNAs have now been identified to serve as either tumor suppressors or as oncogenes in RB by exerting effects on important regulatory cellular pathways (9,10).

An accumulating body of evidence shows that microRNA-29a (miR-29a) expression is dysregulated and plays crucial roles in progression and development of multiple cancers (11-16). Yet, the role and underlying mechanism of miR-29a in RB cells remain unclear. The aims of the present study were to investigate miR-29a expression and clinical significance, and determine the biological function of miR-29a in RB and explore the possible regulating mechanisms in RB cells.

Materials and methods

Human tissue samples and cell lines. Twenty human RB specimens and 5 retina tissues were obtained from patients with RB and ruptured globe at the Department of Ophthalmology, the Second Hospital of Jilin University, respectively. All patients had not received preoperative radiotherapy and/or chemotherapy prior to enucleation. All tissues were stored in liquid nitrogen until RNA isolation. This study was approved by the Ethics Committee of Jilin University. Written informed consent was obtained from each patient.

Two human RB cell lines (Y79 and SO-RB50) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China).

Correspondence to: Dr Chunling Xu, Department of Ophthalmology, The Second Hospital of Jilin University, 218 Zhiqiang Street, Nanguan, Changchun, Jilin 130041, P.R. China
E-mail: xuc@jlu.edu.cn

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All cells were maintained at 37°C in a humidified 5% CO₂ atmosphere in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin.

**Plasmid, microRNA mimic, and transfection.** Full-length STAT3 (without the 3'UTR) was amplified by PCR and inserted into the eukaryotic expression vector pCNA3.1 (+) (Invitrogen). The STAT3 3'UTR target site for miR-29a was amplified by PCR, and subcloned into the pGL3-control vector (Ambion, Austin, TX, USA) and named WT-STAT3-3'UTR. Quick-Change Mutagenesis kit (Stratagene, Heidelberg, Germany) was used for mutagenesis of the miR-29a target-site in the STAT3 3'UTR, and was referred to as MT-STAT3-3'UTR. The miR-29a mimic or corresponding negative control (miR-NC) were chemically synthesized by GenePharma Co. (Shanghai, China). For the transfection experiments, 2x10⁵ RB cells were seeded in a 6-cm dish, and cultured in RPMI-1640 with 10% FBS up to 70-80% confluence. Then transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**RNA extraction and quantitative real-time PCR (qRT-PCR).** Total RNA was isolated from tissue samples and cell lines by the TRIzol reagent (Invitrogen) following the manufacturer's protocol. For miR-29a expression analysis, total RNA was reverse transcribed to cDNA using the microRNA reverse transcription kit (Takara, Dalian, China), and then were quantified with SYBR miRNA detection assays (Takara) under the ΔΔCT method. The U6 and GAPDH were used as control for miR-29A and STAT3 mRNA, respectively using the 2⁻ΔΔCT method.

**Cell proliferation, cell cycle distribution, apoptosis, migration and invasion analyses.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed with 100 U/ml penicillin, and 100 mg/ml streptomycin. Briefly, 5x10⁵ RB cells were seeded in a 6-cm dish, and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin.

**Western blotting.** RB cells and tissues were harvested and lysed with RIPA buffer (Beyotime, Jiangsu, China), following by quantification with the BCA protein assay kit (Pierce, Bonn, Germany). The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, Germany). The membranes were blocked with 5% nonfat milk and then incubated with the following primary antibodies overnight at 4°C: anti-STAT3 (1:1,000, cat. no. sc-293151), anti-Bcl-2 (1:1,000, cat. no. sc-56015), anti-cyclin D1 (1:1,000, cat. no. sc-70899; all from Santa Cruz Biotechnology, Inc.), anti-cyclin E1 (1:1,000, cat. no. sc-2931), anti-Bcl-2 (1:1,000, cat. no. sc-2931), anti-cyclin D1 (1:1,000, cat. no. sc-70899; all from Santa Cruz Biotechnology, Inc.). The blots were washed with PBST and incubated with horseradish peroxidase (HRP)-conjugated corresponding secondary antibody for 2 h at room temperature. Protein bands were observed using an enhanced chemiluminescence system (Thermo Fisher Scientific, Inc.) and exposed to X-ray film. GAPDH was used as a control.

**Xenograft tumor model.** BALB/c-nu mice (5-6 weeks of age and weighing 20-25 g) were purchased from the Experimental
miR-29a is downregulated in RB tissues and RB cell lines. To investigate the expression status of miR-29a in RB and RB cell lines, real-time RT-PCR (qRT-PCR) was conducted. Expression of miR-29a was lower in the 20 RB tissues and 2 RB cell lines than that observed in the 5 normal retinal tissues (P<0.05; Fig. 1), suggesting that the downregulation of miR-29a may be involved in human RB tumorigenesis.

Overexpression of miR-29a inhibits cell proliferation and promotes apoptosis in RB cells. To assess the biological effects of miR-29a on RB cells, Y79 and SO-RB50 cells were transiently transfected with miR-29a mimic and miR-NC, and then cell proliferation, cell cycle distribution and apoptosis were determined. qRT-PCR confirmed miR-29a overexpression in Y79 and SO-RB50 cells (Fig. 2A). The MTT assay showed that restoration of miR-29a expression in Y79 and SO-RB50 cells significantly inhibited cell proliferation (Fig. 2B). Since cell proliferation is closely associated with cell cycle arrest, we investigated the effect of miR-29a on the cell cycle. FACS analysis showed that transfection of the Y79 and SO-RB50 cells with the miR-29a mimic significantly increased the proportion of cells in the G0/G1 phase and reduced the proportion of S phase cells compared to the cells transfected with miR-NC (Fig. 2C). In addition, we found that restoration of miR-29a in Y79 and SO-RB50 cells significantly increased the cell apoptosis rate (Fig. 2D).

miR-29a inhibits the migration and invasion of RB cells. To study the effect of miR-29a on migration and invasion abilities, wound healing and invasion chamber assays were performed in Y79 and SO-RB50 cells transfected with miR-29a or miR-NC, respectively. It was found that restoration of miR-29a in RB cells significantly decreased migration (Fig. 3A) and invasion (Fig. 3B) capacity.

STAT3 is a direct target gene of miR-29a in RB cells. Three miRNA databases (Targetscan, Pictar and Miranda) were used to identify a putative miR-29a-binding site. Among the candidate targets, the 3'UTR of human STAT3 contains a putative region that matches to the seed sequence of miR-29a (Fig. 4A). To confirm whether miR-29a directly binds to STAT3, luciferase activity assay was performed. It was found that miR-29a overexpression markedly reduced the luciferase activity of the WT-STAT3-3'UTR, but not the MT-STAT3-3'UTR in Y79 and SO-RB50 cells (Fig. 4B). To further confirm the effect of miR-29a on STAT3 expression, we analyzed the STAT3 expression at the mRNA and protein levels in Y79 and SO-RB50 cells transfected with miR-29a mimic or miR-NC by qRT-PCR and western blot analysis, respectively. We found that the STAT3 expression at the mRNA and protein levels was decreased in the Y79 and SO-RB50 cells transfected with miR-29a mimic compared with cells transfected with miR-NC (Fig. 4C and D). We also found that miR-29a overexpression also significantly inhibited cyclin D1, Bcl-2 and MMP-9 expression, several downstream proteins of STAT3, in the Y79 and SO-RB50 cells (Fig. 4D). These results indicate that STAT3 is a direct target of miR-29a in RB cells.

STAT3 expression is upregulated, and inversely correlated with miR-29a expression in RB tissues. Next, we detected the STAT3 mRNA expression in the 20 RB tissues and 5 normal...
Figure 2. miR-29a inhibits the proliferation and induces the apoptosis of RB cells. (A) miR-29a expression was confirmed to be increased in the miR-29a mimic-transfected cells, compared with those transfected with miR-NC cells, as confirmed by qRT-PCR. (B-D) Cell proliferation, cell cycle distribution and apoptosis were determined in Y79 and SO-RB50 cells transfected with miR-29a or miR-NC. *P<0.05, **P<0.01.

Figure 3. miR-29a inhibits cell migration and invasion of RB cells. (A) miR-29a overexpression inhibited RB cell migration, as determined by wound healing assay. (B) miR-29a overexpression inhibited RB cell invasion, as determined by Transwell invasion assay. *P<0.05, **P<0.01.
We found that RB tissues had a higher expression of STAT3 mRNA relative to the normal retinal samples (Fig. 5A). In addition, a statistically significant inverse correlation was found between the miR-29a level and STAT3 mRNA level in RB tissues by Spearman's correlation analysis (r = -0.731; P<0.001, Fig. 5B).

To further determine whether STAT3 is a functional target of miR-29a in RB cells, we performed a rescue experiment involving transfection of STAT3 plasmids (lack of 3’UTR) into miR-29a-expressing RB cells. RB cells transfected with the STAT3-overexpressing plasmid showed restoration of STAT3 expression, which was reduced via miR-29a overexpression (Fig. 6A). Moreover, STAT3 overexpression reversed the effect of miR-29a on cell proliferation, cell cycle, apoptosis, migration and invasion (Fig. 6B-F). Taken together, these results indicated that miR-29a exerts its biological effect in RB by suppression of STAT3.

miR-29a inhibits tumor growth in a mouse model. We assessed the in vivo therapeutic efficacy of miR-29a in BALB mice. Y79 cells transfected with miR-29a mimic or miR-NC were subcutaneously injected into the flank regions of nude mice. We found that tumor growth was slower in the miR-29a group compared to the miR-NC group (Fig. 7A). On day 35, the mice were sacrificed, tumors were removed and weighed. The tumor size and weight in the miR-29a group were significantly decreased compared to these parameters in the miR-NC group (Fig. 7B and C). We also detected STAT3 protein expression in tumor tissues of nude mice, and found that STAT3 expression was decreased in the miR-29a group (Fig. 7D). Taken together, these data showed that miR-29a inhibits tumorigenicity in vivo.

Discussion

It is well documented that various microRNAs are involved in the initiation and development of RB by acting as oncogenes or tumor-suppressor genes (9,10). For example, Liu et al. reported...
that ectopic expression of miR-124 significantly suppressed cell proliferation, colony formation, migration and invasion, induced cell apoptosis in the RB cells by repressing STAT3 (18). Wu et al found that enforced expression of miR-204 in RB cells inhibited proliferation and invasion in vitro and suppressed tumor growth in vivo by targeting cyclin D2 and MMP-9 (19). Martin et al demonstrated that overexpression of miR-449a and miR-449b in RB cells significantly impaired proliferation...
and increased apoptosis of tumor cells (20). Gui et al showed that miR-21 functions as an oncogene in RB via regulation of the PTEN/PI3K/AKT pathway (21). In the present study, we found that RB tissues and cell lines had higher expression of miR-29a compared with that noted in normal retinal tissues. Function assays demonstrated that restoration of miR-29a in RB cells impaired cell proliferation, migration and invasion, and induced cell apoptosis in vivo, as well as impaired tumor growth in vivo. These results support the conclusion that miR-29a plays a crucial role in RB progression.

miR-29a, located on chromosome 7q32 (22), has been reported in the literature as playing a tumor suppressive role and exerting inhibitory effects on cell proliferation, migration, and invasion in a subset of cancer (12-16). However, the biological function and underlying mechanism of miR-29a in RB have not been explored. Here, our results showed that miR-29a expression in RB specimens was significantly lower than that in normal retinal tissues. Interestingly, the expression of miR-29a in human RB cell lines was also downregulated compared to normal retinal tissues. Subsequently, we found that overexpression of miR-29a in RB cells inhibited cell proliferation and promoted cell apoptosis, as well as decreased the migration and invasion capacity of RB cells in vitro. Furthermore, we observed that miR-29a overexpression decreased tumor growth in vivo. These results suggested that miR-29a functions as a tumor suppressor in RB.

It is well documented that miRNAs exert their biological function in cancer by regulating expression of their downstream target genes (23). Through three target prediction programs (TargetScan7.1, PicTar and miRDB) putative miR-29a targets were predicted. Our analysis suggests that STAT3 is a potential target of miR-29a. Subsequently, STAT3 was identified as a potential functional target of miR-29a in RB by luciferase assay, qRT-PCR and western blot analysis. STAT3 has been reported to be involved in the development and progression of cancer by regulating cell proliferation, migration, invasion, cell apoptosis and cell cycle arrest (24,25). Importantly, STAT3 exerts its biological role by regulating multiple downstream gene, such as cyclin D1 (cell cycle related gene), survivin, Bcl-xL, Bcl-2 (cell apoptosis-related gene), VEGF, and MMP-2 and MMP-9 (cell invasion-related gene) (24-28). Recently, a study showed that STAT3 expression was upregulated in RB tissues, and that knockdown of STAT3 inhibited cell proliferation in vitro, and suppressed tumor growth in vivo (28,29). A recently study revealed that STAT3 was regulated by miR-124 in RB (18). In this study, we found that miR-29a overexpression significantly inhibited STAT3 expression and expression of its downstream genes cyclin D1, Bcl-2 and MMP-9. In addition, we found that miR-29a was inversely correlated with STAT3 mRNA in human RB tissues. Overexpression of STAT3 partially reversed the effects of miR-29a on RB cell proliferation, cell cycle, apoptosis, migration and invasion. Our in vivo study also confirmed that miR-29a suppressed tumor growth in nude mice by repressing STAT3. These observations provide the first line of evidence, to the best of our knowledge, that miR-29a exerts its tumor suppressor role in RB via the regulation of STAT3.

In summary, our findings confirmed an inhibitory effect of miR-29a in RB by demonstrating significantly impaired proliferation, migration, invasion and promotion of apoptosis in tumor cells by suppressing STAT3, suggesting that miR-29a could be a potential therapeutic target for RB in the future.

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References


