·Basic Research·

Suppressive effect of microRNA-143 in retinoblastoma

Li-Lun Wang[1], Hai-Feng Hu[2], Yan-Qin Feng[1]

[1]Department of Ophthalmology, Affiliated Hospital of Yan'an University, Yan'an 716000, Shaanxi Province, China
[2]Department of Oncology, Affiliated Hospital of Yan'an University, Yan'an 716000, Shaanxi Province, China
Correspondence to: Yan-Qin Feng. Department of Ophthalmology, Affiliated Hospital of Yan'an University, Yan'an 716000, Shaanxi Province, China. fengyanqin2016@163.com
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Abstract

• AIM: To investigate microRNA-143 expression and effect on suppression of retinoblastoma (RB) cells.

• METHODS: The expression of microRNA-143 was investigated and compared in normal human retina tissue samples and in RB cell lines of Y79 and Weri1. The microRNA-143 mimics were transfected into the RB cell lines separately, and its effect on RB cell lines was detected using reverse-transcription quantitative polymerase chain reaction and Western blotting methods.

• RESULTS: The microRNA-143 expression was significantly suppressed in RB cell lines. Overexpression of microRNA-143 significantly lowered cell viability and invasion of the RB cell lines, and increased the number of apoptotic cells. Meanwhile, the Bax expression was up-regulated and much higher in the microRNA-143 mimics transfected group than that in the negative control and the microRNA-143 inhibitor groups.

• CONCLUSION: MicroRNA-143 exhibits suppressive effects in RB. The current study provides the perspective of a potential therapeutic treatment for RB.

• KEYWORDS: retinoblastoma; microRNA-143; proliferation; invasion; apoptosis

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INTRODUCTION

Although the incidences of retinoblastoma (RB) are rare, it is the most common intraocular malignant tumor that normally affects infants and young children, resulting in serious risk of visual dysfunction [1]. As clinical treatment continues to improve, the treatment has also shifted from life protection of children to life quality improvement of children, *i.e.* eye preservation and maintaining visual function. Early detection and diagnosis of RB is critical to serve that purpose, given the fact that the age of children with RB are usually less than 5 years old. However, due to the poor cooperation of children undergoing fundus examination, and the lack of clear and intuitive imaging approaches, early detection of RB proved quite difficult. In certain African and Asian countries, mortality rates of RB reach as high as 39%-70%, due to failure of early detection and diagnosis [2]. Currently, the main therapeutic methods include chemotherapy, radiotherapy, and eye removal (enucleation surgery). However, therapies targeted directly to aberrant signaling pathways might be more promising than conventional treatment strategies. Thus achieving a deeper understanding of the molecular and cellular mechanism of RB is necessary, and so is developing more effective treatment.

Previous studies [3-4] have reported that microRNAs, as novel molecules, play vital roles in cancer development by functioning as tumor suppressors or oncogene. MicroRNAs are a class of endogenous, short (22-25 nucleotides in length), non-coding RNA molecules, which serve as key regulators of gene expression through the post-transcriptional silencing of target messenger RNAs by compete binding. The expression patterns of microRNAs in certain tumor tissues are different from those in normal tissues of the same origin [5]. Approximately 60% of the protein-coding genes were regulated by microRNAs and the roles of target genes revealed that microRNA functions as either oncogene or a tumor suppressor[6]. MicroRNA-143 has been reported to act as a tumor suppressor for colorectal cancer, bladder cancer and gastric cancer, as well as a promising non-invasive biomarker for clinical diagnosis[7-10]. The expression levels of microRNAs were fluctuated enormously in different tumor tissues, tumor node metastasis (TNM) stages and physiological processes, showing that microRNAs were deeply associated with cellular proliferation and differentiation, carcinogenesis and apoptosis. Alteration of microRNA-143 expression is closely correlated with carcinogenesis, therefore microRNA-143 could be used as a novel method for cancer diagnosis and treatment[9,11].

In the present study, the effect of microRNA-143 on the biological behaviors of the human RB tissues, and RB cell lines of Y79 and Weri1 were assessed, in an attempt to present theoretic basis for immunotherapy of RB.

SUBJECTS AND METHODS

Tissue Sample and Cell Lines Human retinoblastoma tumor samples (n=44) and normal retina tissue samples (n=20) were obtained from the Department of Ophthalmology (Affiliated Hospital of Yan'an University, Shaanxi Province, China). The present study was approved by the Ethnic Committee of Yan'an University, and written informed consent was obtained from patients. Tumor samples were excluded from experiment if the RB patients had undergone chemotherapy or other treatment earlier than enucleation.

Human malignant retinoblastoma cell lines Y79 and Weri1 were obtained from the American Type Culture Collection (ATCC). Tissue samples were collected at surgery, immediately frozen in liquid nitrogen, and then stored until RNAs and proteins extraction were performed. Cell lines were cultured in DMEM with high glucose and sodium pyruvate, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 mg/mL streptomycin, at 5% CO_2 and 37℃.

Transient MicroRNA Transfection For functional analysis, the control microRNA, microRNA-143 mimics, and microRNA-143 inhibitor were all obtained from GenePharma (Shanghai, China). Double-stranded scrambled RNA was used as negative control (NC). The microRNA was transfected into the cells using Lipofectamine 2000 (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions. The sequences used in this study are as follows: miRNA control, 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-143 mimics, 5'-GCGCAGCGCCCTGTCTCCCAGCCT-3'; miR-143 inhibitor, 5'-GAGCUACAGUGC UUCAUCUCA-3'.

RNA Extraction, Reverse Transcription, and Real-time Polymerase Chain Reaction Analysis Total RNAs from normal retina tissues, RB tissues, and RB cell lines were isolated from harvested cells and human RB tissues with Trizol (Invitrogen Life Technologies, CA, USA) according to the standard procedure. RNAs were reverse transcribed using the PrimeScriptTM real-time polymerase chain reaction (RT-PCR) Kit (Takara). RT-PCR reactions were performed using SYBR Premix DimerEraser (Takara). PCR cycles were carried out by initial denaturation at 95℃ for 5min, then running 40 cycles at 95℃ for 10s, prior to running the cycle at 60℃ for 1min. Duplicate experiments were conducted to calculate the mean delta Ct, mean fold change (RQ) and standard deviation (SD). MicroRNA expression level was normalized to endogenous control U6.

Western Blotting Analysis of Cell Apoptosis Primary antibodies against Bax (catalog number ab32503, diluted 1:1000) and Bcl-2 (catalog number ab32124, diluted 1:1000) were purchased from Abcam (Cambridge, UK). Tumor tissues from human were grinded into powder in liquid nitrogen with radio immunoprecipitation assay (RIPA) buffer, and the total tissue proteins were extracted. The supernatants were collected, and protein concentration was evaluated using BCA assay kit (Thermo Fisher, USA). Forty microgram of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then was transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked for 30min at room temperature with 5% non-fat dry milk and incubated for 2h with primary antibodies against Bax, Bcl-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a humidified container at 37℃. After that, membranes were washed three times with phosphate buffer saline-Tween-20 (PBST), prior to incubating with HRP-conjugated secondary antibody for 2h. Lastly, the membranes were washed with PBST three times and the proteins of interest were detected using electrochemical luminescence (ECL) (Millipore, Billerica, MA, USA). Quantity One Software (Bio-Rad, USA) was used to quantify the intensities of Bax, Bcl-2 and GAPDH bands.

Luciferase Reporter Assay Luciferase activity was measured 24h after transfection using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The firefly and Renilla reniformis luciferase activities were measured 30s later. Experiments were conducted in triplicate independently.

Cell Viability Assay Cell viability was measured by conducting the cell counting Kit-8 (CCK8) assay. The transfected cells were seeded into 96-well plates at the density of 1×10^4 cells per well. After that, cell absorption was measured using a CCK8 kit (R&D Systems, M.N., USA) according to the manufacturer's instruction at different time points. The data were collected from independent experiments performed in triplicate.

Flow Cytometry Analysis of Cell Cycle Transfected cells were trypsinized and collected. After being centrifuged and washed three times, cell concentration was adjusted to 1×10^6 cells/mL, and fixed in 75% ethanol at -20℃ for 16h. After fixation, cells were washed with cold phosphate buffer saline (PBS) and then stained with 0.5 mL of propidium iodide (PI) staining buffer, which contains 200 mg/mL RNase A, 50 μg/mL PI, at 37℃ for 30min in the dark. Cell cycle analysis was conducted using Flow Cytometer (BD Biosciences, San Jose, CA, USA), and the wavelength was set at 488 nm. Cell cycle and apoptosis analysis kit was purchased from Beyotime Institute of Biotechnology (China). For each group, 10 000 events were acquired. The DNA of the cells can be stained by DNA binding dye. Cells in S phase have more DNA than those in G1 phase. They will bind proportionally more dye, fluorescing more brightly until their DNA content was doubled. The cells in G2 phase will be approximately twice as bright as cells in G1 phase. The percentage of cells in each cell phases (G0/G1, S and G2/M)

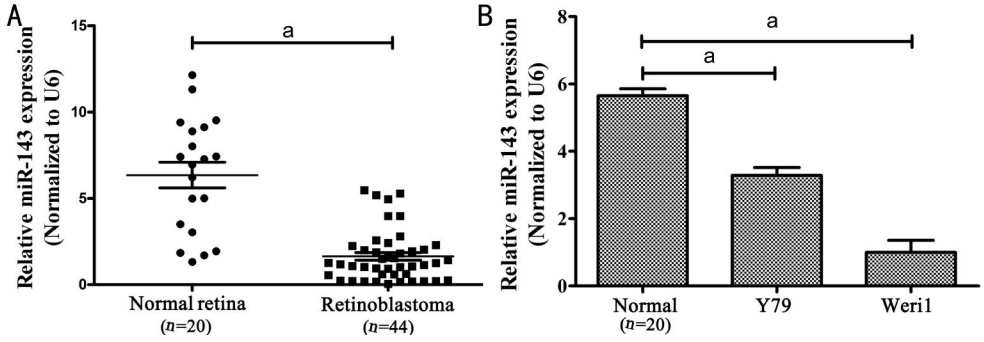

Figure 1 MicroRNA-143 expression was decreased in human RB samples and cell lines compared with normal retina tissues (normalized to U6) A: Quantitative RT-PCR analysis showed that microRNA-143 is significantly down-regulated in RB tissues (n=44) compared with normal retina tissues (n=20) (P<0.001); B: MicroRNA-143 expression is significantly higher in normal retina tissues (n=20) compared with that of Y79 and weri1 cell lines (P<0.001). Data were presented as the mean±SD of three replicates. [a]P<0.001.

was calculated and compared. Each test was conducted in triplicate.

Cell Invasion Assay The invasive potential of RB cells were examined using 24-well Transwell plates (Corning Life Sciences, MA, USA) pre-coated with Matrigel basement membrane matrix, at a concentration of 1 mg/mL, according to the manufacturer's instructions. A total number of 2×10^4 cells were seeded per well in the upper chamber in DMEM with 0.1% serum, the lower chamber contained 0.6 mL of medium supplemented with 10% FBS to stimulate cell invasion. Transwell plates were incubated in a 5% CO_2 atmosphere at 37℃ for 24h. The non-invading cells were removed from top well, while the bottom cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and photographed for each well. Data were collected from three separate experiments each time.

Statistical Analysis Inter-group comparison of the variables were conducted using the Student's t-test or one-way ANOVA, with the SPSS 17.0 (Chicago, Illinois, USA). A P-value <0.05 was regarded to indicate a statistically significant difference.

RESULTS

Expression of MicroRNA-143 in Human Retinoblastoma Samples and Y79, Weri1 Cell Lines To analyze the expression pattern of microRNA-143 in human RB samples and RB cell lines of Y79 and Weri1, we performed a comparison of microRNA-143 expression between RB samples (n=44) and normal retina tissues (n=20) using the quantitative RT-PCR technique. Our analysis showed that the expression level of microRNA-143 was significantly reduced in the RB samples, compared with that in the normal retina tissues (Figure 1A). Additionally, we examined the expression pattern of microRNA-143 in RB cell lines of Y79 and Weri1, and made comparison with that in normal retina tissues, respectively. Our findings showed that microRNA-143 was significantly decreased in Y79 and Weri1 cell lines compared with that in normal retina tissues (Figure 1B).

Overexpression of MicroRNA -143 Inhibited the Proliferation and Growth of Retinoblastoma Y79 Cells To investigate the biological role of microRNA-143 on RB development and progression, we up-regulated (Figure 2A) and down-regulated (Figure 2B) microRNA-143 by transfecting RB Y79 cells with microRNA-143 mimics and microRNA-143 inhibitor to measure the effects, respectively. The effect of microRNA-143 on the proliferation of RB Y79 cells was measured using the CCK8 assay (Figure 2C, 2D). As indicated in Figure 2C, the overexpression of microRNA-143 by transfecting RB Y79 cells with microRNA-143 mimics was found to significantly suppress the growth rate of RB cancer cells at 24h post transfection (P<0.001) compared with that in non-targeting microRNA mimics-transfected group of RB Y79 cells (microRNA-NC). Reversely, in the group of microRNA-143 inhibitor, microRNA-143 was silenced in RB Y79 cells, cell proliferation was observed to significantly increase at 24h post-transfection compared with that in NC group (Figure 2D). Additionally, the altered cell cycle distribution is closely associated with the up-regulation and down-regulation of microRNA-143. Overexpressed microRNA-143 led to cell cycle arrest in G1 phase. As shown in Figure 2E and 2F, overexpression of microRNA-143 by microRNA-143 mimics transfection decreased considerably the cells percentages in the S-phase, while increased the cells percentage in the G0/G1-phase, compared with that in the NC transfected cells. On the contrary, the silence of microRNA-143 exhibited the counter effects. The results provided evidence that overexpression of microRNA-143 was interfered with cell cycle, inducing G1-phase arrest.

MicroRNA -143 Repressed the Invasion of Retinoblastoma Y79 Cells To evaluate the role of microRNA-143 in the invasion of RB Y79 cell lines, groups of RB Y79 cells transfected with microRNA-143 mimics, microRNA-143 inhibitor and microRNA-NC were observed by performing Transwell assays. The overexpression of microRNA-143 (microRNA-143 mimics group) decreased

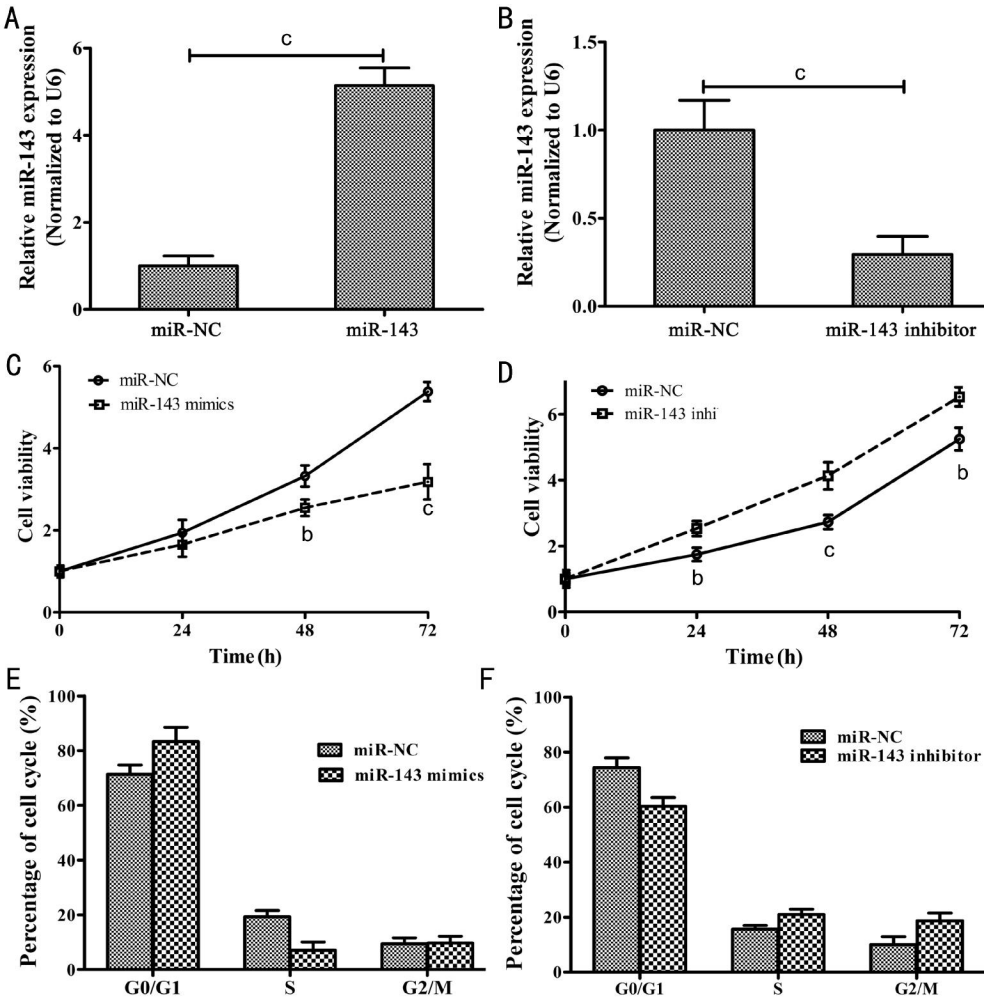

Figure 2 MicroRNA-143 inhibits RB Y79 cells viability and interferes with RB Y79 cell cycle progression A: MicroRNA-143 expression level was measured in RB Y79 cells transfected with microRNA-143 mimics by RT-PCR; B: MicroRNA-143 expression level was measured in RB Y79 cells transfected with microRNA-143 inhibitor by RT- PCR; The U6 snRNA was used as a normalizer (endogenous control); C: CCK8 assay showed the viability of RB Y79 cells was suppressed when transfected with microRNA-143 mimics; D: CCK8 assay showed the viability of RB Y79 cells was enhanced when transfected with microRNA-143 inhibitor; E: Flow cytometry analysis of cell cycles suggested that the overexpression of microRNA-143 increased the percentage of cells in G1 phase, and decreased the cells percentage in S phase; F: When microRNA-143 was silenced in RB Y79 cells, the cells percentage in G1 phase was decreased, while the cells percentage in S phase was increased. The data were collected from the experiment conducted three times independently. $^{b}P<0.01$, $^{c}P<0.001$.

the invasion of RB Y79 cells, while the down-regulation of microRNA-143 in the microRNA-143 inhibitor group, tumor cell invasion was promoted significantly (Figure 3B).

Effects of the MicroRNA -143 on Cell Apoptosis of Human Retinoblastoma Y79 Cell Lines To further investigate the mechanism behind the association between microRNA-143 and RB Y79 cell growth, the percentage change in the cell apoptosis was measured using Flow Cytometry and Western blotting analysis. As shown in Figure 4A, microRNA-143 overexpression significantly increased the percentage of apoptotic cells in RB Y79 cell lines, while the silence of microRNA-143 significantly decreased the percentage of apoptotic cells.

In the Western blotting analysis, Bax and Bcl-2 antibodies were included to measure the apoptosis. As a pro-apoptosis regulator, Bax can accelerate programmed cell death by binding to, and antagonizing the apoptosis repressor Bcl-2. Under stress conditions, Bax undergoes a conformation change that results in the translocation to the mitochondrion membrane, which lead to the release of Cytochrome C and triggers apoptosis. Bcl-2 plays a role of apoptosis suppressor in a vast variety of cell systems, regulating cell death by controlling the mitochondrial membrane permeability. Bcl-2 inhibits Caspase activity by preventing the release of Cytochrome C or by binding to the apoptosis-activating factor. Therefore, the change of expression pattern of Bax and Bcl-2 can be used to indicate and measure cell apoptosis. As show in Figure 4B, in the group of microRNA-143 mimics (microRNA-143 overexpression), the expression of Bax was increased and Bcl-2 was decreased, compared with that in the microRNA-NC group. In the group of microRNA-143 inhibitor (microRNA-143

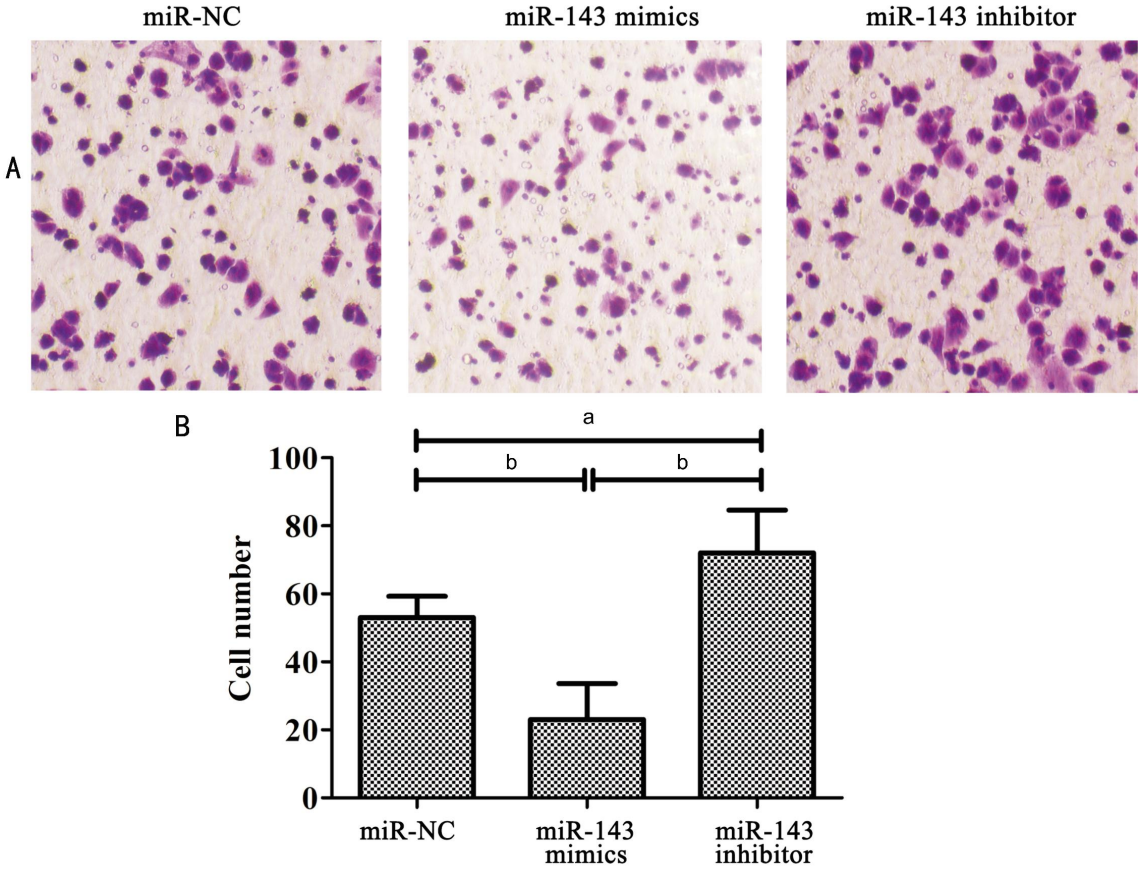

Figure 3 MicroRNA-143 repressed the invasion of RB Y79 cells A: Transwell invasion assay showed that invasion of RB Y79 cells was inhibited significantly when transfected with microRNA-143; B: Transwell invasion assay suggested that RB Y79 cells transfected with microRNA-143 mimics significantly reduced the invasive potential compared with that in RB Y79 cells with microRNA-NC and microRNA-143 inhibitor (^{b}P<0.001); All the experiments were performed in triplicate independently. The data represent the mean±SD of 3 replicates. ^{a}P<0.05, ^{b}P<0.001.

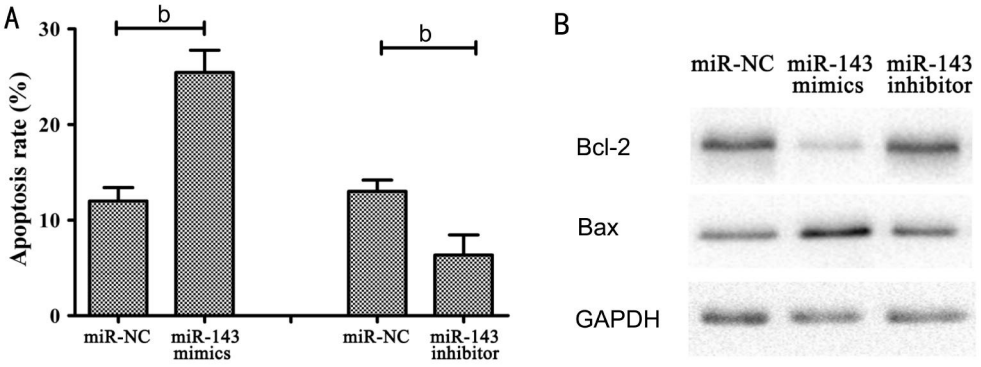

Figure 4 MicroRNA-143 induced the apoptosis of retinoblastoma cells A: The apoptosis rate of cells in the microRNA-NC group was significantly lower than that in the microRNA-143 mimics group (^{b}P<0.001); but was significantly higher than that in the microRNA-143 inhibitor group (^{b}P<0.001); B: Western blotting analysis of cell apoptosis. For the RB Y79 cells transfected with microRNA-143 mimics, Bax (pro-apoptosis regulator) expression was increased and Bcl-2 (anti-apoptosis regulator) expression was decreased, compared with microRNA-NC group and microRNA-143 inhibitor group. For the RB Y79 cells transfected with microRNA-143 inhibitors, Bax and Bcl-2 expressions exhibited the reversed effect, *i.e.* Bax expression was suppressed and Bcl-2 expression was enhanced. GAPDH was used as an internal control. The data were based on the experiment conducted three times independently.

silence), the expression of Bax was down-regulated, while the expression of Bcl-2 was up-regulated compared with that in the microRNA-NC group. Our data showed that overexpression of microRNA-143 can significantly increase the percentage of apoptotic cells. The result is a solid evidence that microRNA-143 is able to promote the apoptosis of RB Y79 cells.

DISCUSSION

RB is one of the several heritable childhood cancers, resulting in reduced vision and/or impaired hearing.

Increasing evidence has showed that genetic or epigenetic alterations in a vast variety of oncogenes and tumor suppressor genes are associated with RB progression [12-13], therefore RB exhibits both hereditary and sporadic incidences. The malignant tumor RB normally arises in children's eyes, and was found to be the result of two distinct genetic changes, each leading to the dysfunction of one of the two homologous copies at a single genetic locus. Mutations of the genes may be inherited from one parent, and might rise during gametogenesis or might occur in the somatic cells[13-14].

Numerous studies have confirmed that microRNAs were involved in the regulation of signaling molecules through translational repression and/or gene splicing, modulating around one thirds of human gene by functioning as oncogene or tumor suppressor gene [1-5,15]. MicroRNAs are also associated with cell proliferation, differentiation and apoptosis, and the altered expression of microRNAs could enhance or reduce the incidences of cancer. Most microRNAs are highly conserved, and exhibits varied expression level and pattern with the change of timing, tissues and cells [11,16-17]. A better understanding of the carcinogenic mechanisms might help identify the biomarker of RB and develop new therapeutic methods.

In the present manuscript, we focused on the study of microRNA-143 expression pattern in the human RB Y79 cells. The microRNA-143 expression vector, which expresses and regulates microRNA-143 in RB Y79 cells was constructed accordingly to examine its effect on human RB Y79 cells. Moreover, it was observed that the over-expression of microRNA-143 can induce the apoptotic effect in RB Y79 cells and suppress the proliferation of RB Y79 cells following transfection with microRNA-143 mimics. The comparison of expression level between normal retina tissues and RB tissues were performed using quantitative RT-PCR and Western blotting. Our data suggested that microRNA-143 was significantly down-regulated in human RB samples and RB Y79 cell line, compared with that in normal retina tissues. As the major characteristics of tumor cells, cell viability and invasion were examined by CCK8 and Transwell assay, respectively. Our results showed that overexpression of microRNA-143 reduced viability and invasion in RB Y79 cells (Figure 2C, 2D), while in the microRNA-143 inhibitor group, RB Y79 cell viability and invasion were significantly higher than that in the NC group and in the microRNA-143 mimics group. Moreover, the current study identified that microRNA-143 was involved in the cell cycle of tumor cells. The cell cycle progression includes an orderly set of events, culminating in cell growth and leading to the division and duplication of its DNA, producing two daughter cells consequently. In cancer, regulation of the cell cycle goes awry, and derangement of normal cell growth and behavior occurs [18-20]. In the present study, cells lines transfected with microRNA-143 mimics resulted in the augmentation of cells percentage in G1 phase, while caused decrease of cells percentage in S phase. In contrast, in the group of RB Y79 cells transfected with microRNA-143 inhibitor, the percentage of cells in S phase grows and that in G0/G1 phase were reduced. Our data confirmed that the up-regulation of microRNA-143 could interfere with cell cycle progression, promoting G1 phase arrest. Li *et al* [18] investigated association between microRNA-143 and Syn-1 regulation, indicating that microRNA-143 expression was significantly lower in melanoma tissues than in normal tissues. Further experiments showed that the overexpression of microRNA-143 reduced cell proliferation, promoted G1 phase arrest and induced apoptosis in melanoma [21-22]. This observation concurs with our findings that microRNA-143 exerts inhibitory on the progression of cell cycle.

Apoptosis, also known as, programmed cell death, involves an ordered and orchestrated cellular process under certain physiological and pathological conditions. Understanding of the mechanism of apoptosis is essential, given it plays a pivotal role in the pathogenesis of a variety of diseases. In some cases, the problem is too much apoptosis, such as in degenerative diseases; while in others, too little apoptosis occurred. Caner is known for its capability of immortal regeneration, in which the incidences of apoptosis remain slim [11,19,21-22]. Bax belongs to the Bcl-2 protein family. Bax protein forms a heterodimer with Bcl-2, and functions as an apoptotic activator [20,23-25]. The expression of this gene is regulated by the tumor suppressor P53 and was involved in P53-mediated apoptosis. Bcl-2 is an anti-apoptotic and the founding member of the Bcl-2 family of proteins, which paly either a pro- or anti-apoptotic role in cell death signaling. Bcl-2 family members form heterodimers or homodimers, and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. The pro-survival members contain four Bcl-2 homology regions (BH1-BH4), whereas the BH3-only members contain only the BH3 amphipathic helix, which mediates their interaction with the groove of multi-domain Bcl-2 family members. Human Bcl-2 gene mutations are known causes of many cancers, including melanoma, breast, prostate, chronic lymphocytic leukemia, and lung cancer [23-25]. In the present study, the apoptosis of RB Y79 cells were measured by comparing the apoptosis rate using flow cytometry, as well as the expressions of pro-apoptotic regulator Bax and anti-apoptotic regulator Bcl-2 using Western blotting analysis. Our findings suggested that the apoptosis rate in the group of microRNA-143 overexpression was significantly higher than that in the NC group (P<0.001), and the apoptosis rate in the microRNA-143 down-regulation group was significantly lower than that in the NC group.

The outcome of Western blotting analysis on apoptosis is in agreement with that of flow cytometry. In the group of RB Y79 cells transfected with microRNA-143 mimics, the expression of pro-apoptotic gene Bax was enhanced and anti-apoptotic gene Bcl-2 was reduced. While in the group of RB Y79 cells transfected with microRNA-143 inhibitors, the results suggested otherwise, Bax expression was reduced and Bcl-2 expression was enhanced. These findings are the concrete evidence that overexpression of microRNA-143 is capable of inducing apoptosis of human RB Y79 cells.

In conclusion, the present study demonstrated the suppressive effect of microRNA-143 on human retinoblastoma cells, causing proliferation and invasion reduction, inducing apoptosis of RB Y79 cells. MicroRNA-143 also interferes with cell cycle progression, inducing arrest in G1-phase. These results revealed the potential therapeutic capabilities of microRNA-143 for RB treatment.

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Conflicts of Interest: Wang LL, None; **Hu HF,** None; **Feng YQ,** None.

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