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MicroRNAs-449a and -449b exhibit tumor suppressive effects in retinoblastoma

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ABSTRACT

Retinoblastoma is the most common pediatric cancer of the eye. Currently, the chemotherapeutic treatments for retinoblastoma are broad-based drugs such as vincristine, carboplatin, or etoposide. However, therapies targeted directly to aberrant signaling pathways may provide more effective therapy for this disease. The purpose of our study is to illustrate the relationship between the expressions of miRs-449a and -449b to retinoblastoma proliferation and apoptosis. We are the first to confirm an inhibitory effect of miR-449a and -449b in retinoblastoma by demonstrating significantly impaired proliferation and increased apoptosis of tumor cells when these miRNAs are overexpressed. This study suggests that these miRNAs could serve as viable therapeutic targets for retinoblastoma treatment.

1. Introduction

Retinoblastoma is the most common pediatric cancer of the eye. Although survival rates in some Western nations are as high as 97%, many children still suffer significant morbidity from loss of an eye [1]. In some countries located within the continents of Africa and Asia, mortality rates are as high as 39–70% due to later detection at advanced stages of the disease [1,2].

Currently, the chemotherapeutic treatments for retinoblastoma are broad-based drugs such as vincristine, carboplatin, or etoposide [1]. However, therapies targeted directly to aberrant signaling pathways may provide more effective therapy for this disease. MicroRNAs (miRNAs) are short, non-coding RNAs that act by regulating target genes. Studies from multiple human cancers have shown that miRNA profiles from tumor tissue are different from normal tissue of the same origin [3,4]. Specific individual miRNAs have now been identified which have confirmed effects in cancer, and some miRNAs can act as either tumor suppressors or as oncogenes by exerting effects on important regulatory cellular pathways. For example, Mu et al. report reduced expression of the tumor suppressor microRNA let-7 in retinoblastoma [5]. In addition, miR-17–92 has been described as an oncogenic microRNA cluster that is overexpressed in several human cancers, including hematopoietic, lung, and colon cancers [6]. Conkrite et al. also showed that overexpression of miR-17–92 is important in the formation of a cohort of retinoblastomas [7]. The miR-34 family of miRNAs is downregulated in many cell lines, including breast, lung and colon cancer [8]. MicroRNA-34a is variably expressed in retinoblastoma, and its overexpression in cell lines can slow proliferation [9].

Similar to the miR-34 family, miRNA-449a and -449b have been consistently reported as having tumor suppressive roles in various human cancers. Transcription of miR-449a and miR-449b are induced by E2F transcription factors. Further, the E2F transcription factors are targets of miR-449a/b, which suggest a feedback loop [10,11]. Kheir et al. have shown that miRNAs-449a and -449b are downregulated in human gastric carcinoma, and that overexpression of these miRNAs can inhibit proliferation and result in cell

Abbreviations: miRNAs, microRNAs; TLDA, taqman low density array; Ct, cycle threshold; ΔCt, delta Ct; RQ, relative quantitation; FFPE, formalin-fixed paraffin-embedded.

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cycle arrest *in vitro* [12]. A decrease in miRNA-449 expression has been reported in testicular cancer cells and lung adenocarcinoma cell lines. Expression of miR-449 in these cell lines affects proliferation, senescence and apoptosis [13]. We have previously reported differential expression of miRs-449a and -449b in human retinoblastomas [14]. In this study, we validate the differential expression of these miRNAs in a cohort of primary human retinoblastomas and retinoblastoma cell lines. Further, this is the first study to link the expression of miRs-449a and -449b to retinoblastoma proliferation and apoptosis. These studies suggest that these miRNAs could serve as viable therapeutic targets for retinoblastoma treatment.

2. Materials and methods

2.1. Human retinoblastoma tumor samples

Use of formalin-fixed, paraffin-embedded tumor samples for this study were approved by the Institutional Review Boards at Ann and Robert H. Lurie Children's Hospital of Chicago (IRB Project No. 2010-14211) and Northwestern University Feinberg School of Medicine (IRB Project No. STU00035305). The retinoblastomas were products of enucleation obtained from our institution. Tumors were excluded if the patient had received any treatment, either chemotherapy or local therapy, prior to enucleation.

2.2. Cell culture

Human retinoblastoma cell lines Y79 and Weri1 were cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin, streptomycin, and glutamine, and incubated at 37 °C with 5% CO_2.

2.3. RNA extraction

For each product of enucleation, we obtained five 20 μm thick formalin-fixed, paraffin-embedded (FFPE) sections. Tumor areas

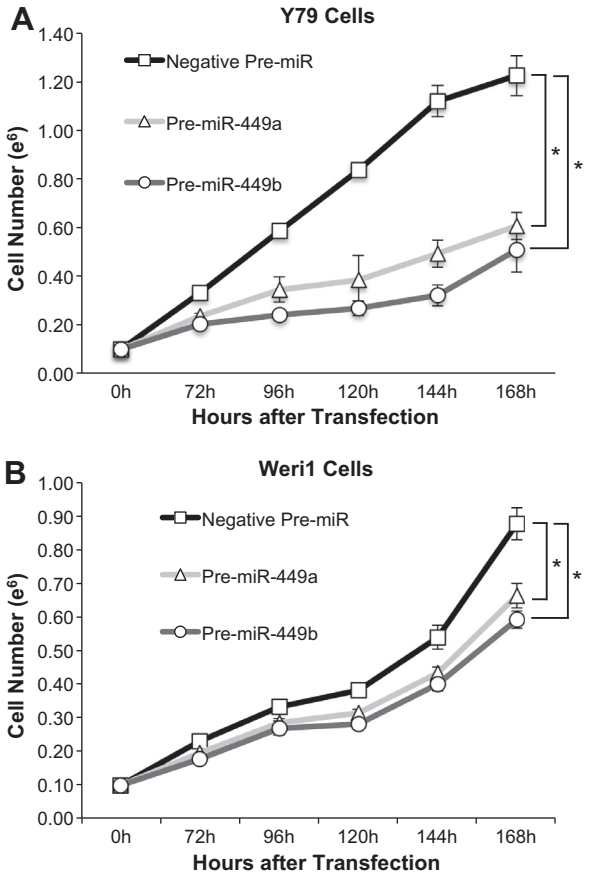

Fig. 2. miR-449a/b overexpression inhibits proliferation of human retinoblastoma cell lines. Human retinoblastoma cell lines Y79 (A) and Weri1 (B) were plated at 10^4/ml and transfected at 50 nm (final concentration) with Pre-miR-449a, Pre-miR-449b, or negative control Pre-miR in a 24 well plate. Cell viability was determined using the Guava Viacount assay for 72–168 h after transfection. Raw data and error bars were derived from three replicate experiments with each sample run in triplicate. Asterisk denotes $p < 0.001$.

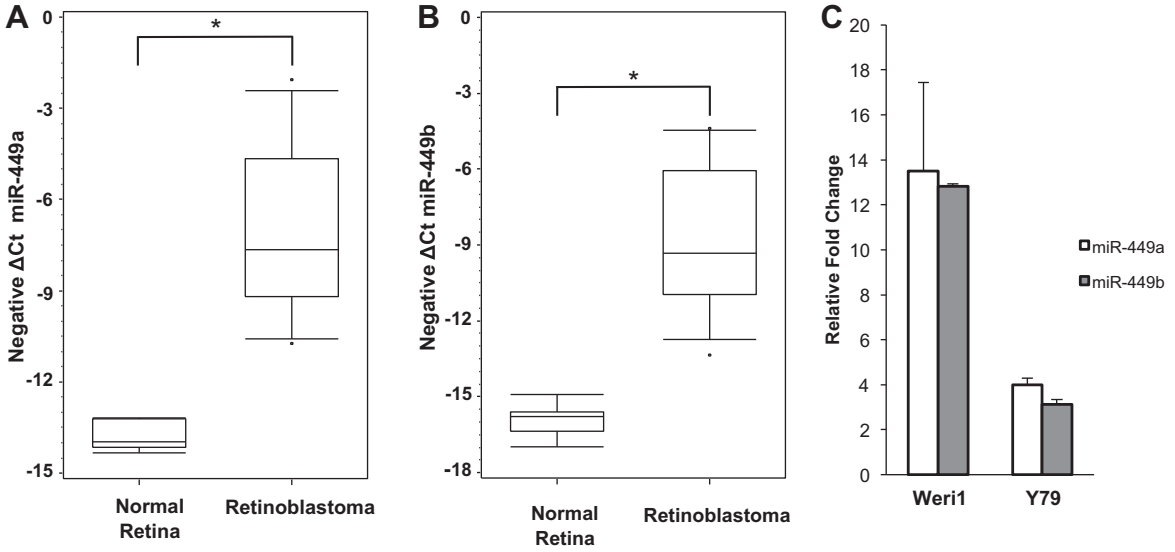

Fig. 1. miR-449a/b is upregulated in human retinoblastoma tumors and cell lines. (A) The expression level of miR-449a was measured in 10 human retinoblastoma tumors as compared to normal pediatric retinas from 3 individuals by real-time PCR. (B) The expression level of miR-449b was measured in 10 human retinoblastoma tumors as compared to normal pediatric retinas from 3 individuals by real-time PCR. (A–B) The centerline represents the median ΔCt and small circles designate outliers. Scale has been inverted to show the inverse-relationship between miRNA abundance and ΔCt value. Raw data and error bars were derived from two independent experiments. U6 was used as an endogenous control. Asterisk denotes $p < 0.0001$. (C) The expression level of miR-449a/b was measured in human retinoblastoma cell lines Weri1 and Y79 as compared to normal pediatric retinas from 3 individuals by real-time PCR. Each bar represents the RQ (relative fold change) value that was calculated using the following equation: $RQ = 2^{-\Delta(\Delta Ct)}$. Raw data and error bars were derived from two independent experiments with triplicate samples. U6 was used as an endogenous control.

were carefully macrodissected from normal tissue and collected in centrifuge tubes. Extraction of total RNA was performed using the Recover All Nucleic Acid Isolation Kit (Applied Biosciences) according to the manufacturer's instructions. For normal controls, RNA was extracted from three pediatric retinas (5, 10, and 10 months) using a standard Trizol protocol (Invitrogen). Normal controls were dissected from whole globes that were obtained from Midwest Eye Banks and National Disease Research Interchange. Use of normal control retinas was approved by the Institutional Review Boards at Ann and Robert H. Lurie Children's Hospital of Chicago (IRB Project Nos. 2010-14211 and 2010-14137) and Northwestern University Feinberg School of Medicine (IRB Project No. STU00035305). Quantity and quality of RNA from all samples and cell lines was determined using NanoDrop ND-1000 spectrophotometer.

2.4. Validation of the differential expression of miR-449 in human retinoblastomas and cell lines

Validations of miRs-449a and -449b were performed using Taqman MicroRNA Individual assays (Applied Biosystems) for real-time PCR. Total RNA from control retinas, retinoblastomas, and retinoblastoma cell lines was reverse transcribed using the MicroRNA Reverse Transcription kit (Applied Biosystems) and analyzed using MicroRNA Individual Assays according to the manufacturer's instructions. Duplicate experiments were used to calculate mean delta Ct (ΔCt), mean RQ (fold change) and standard deviation. MicroRNA expression was normalized to endogenous control MammU6. RQ was calculated using the $2^{-\Delta(\Delta Ct)}$ method where RQ or fold change is equal to $2^{-((\text{Mean } \Delta Ct \text{ Target})-(\text{Mean } \Delta Ct \text{ Calibrator}))}$.

2.5. Transfection and analysis of cell viability and apoptosis in human retinoblastoma cell lines

MicroRNA Pre-miRs (Life Technologies) were combined with RPMI 1640 and transfection reagent Lipofectamine RNAiMAX (Invitrogen). Transfection complex was added to wells of a 24-well plate (50 nm final concentration) and incubated at room temperature for 20 min. Y79 or Weri1 cells were added to each well at a final cell number of 50,000 cells in 500 μL. At each designated time point (72, 96, 120, 144 and 168 h), cell proliferation was measured

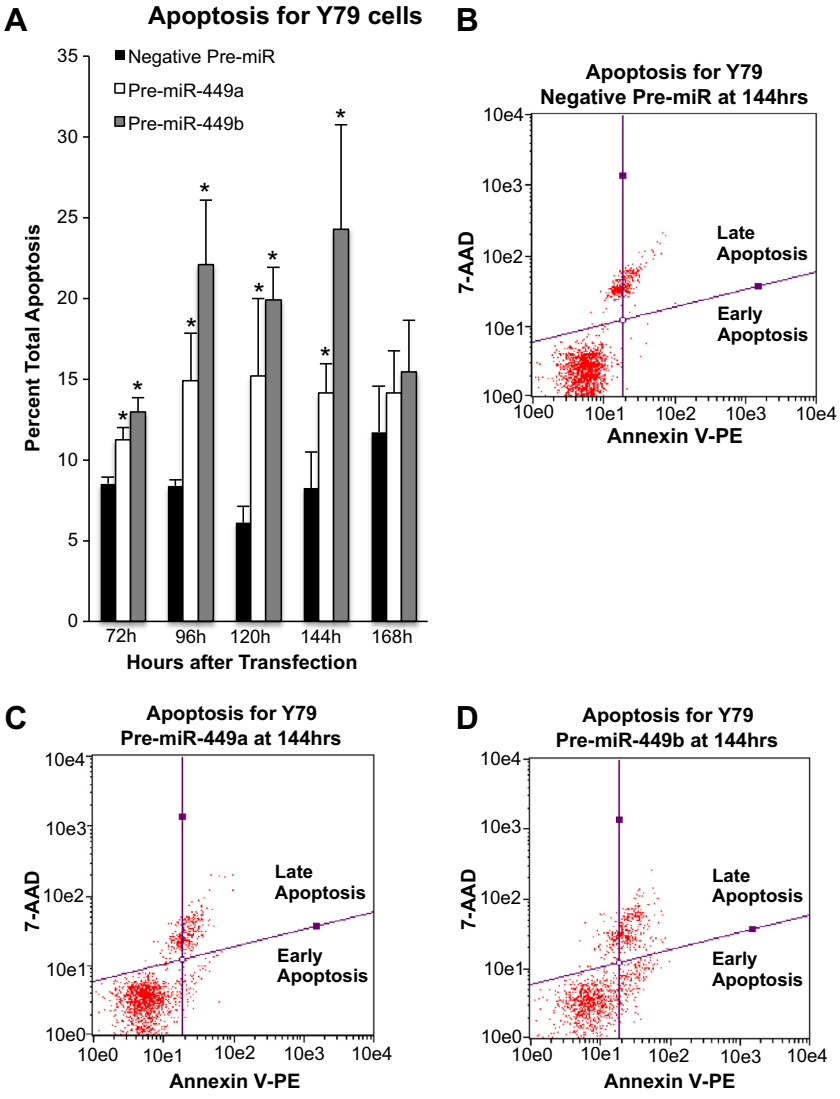

Fig. 3. miR-449a/b overexpression induces apoptosis in the Y79 human retinoblastoma cell line. (A) Total apoptosis (the sum of early and late apoptosis) was determined using the Guava Nexin assay for 72–168 h post-transfection. Raw data and error bars were derived from three replicate experiments. Asterisk denotes $p < 0.05$ (comparing negative Pre-miR to Pre-miR-449a or Pre-miR-449b). (B–D) Representative pictures of early/late apoptosis as visualized using the Guava Nexin assay at the 144 h time point. The lower right quadrant represents early apoptosis (shown), and the upper right quadrant represents late apoptosis (shown).

using the Guava Viacount assay and apoptosis was measured using the Nexin assay on the Guava MiniCyte (Millipore).

2.6. Statistical analysis

The statistical analyses in this manuscript were performed by our co-author, Gang Zhang, a biostatistician in the Biostatistics Research Core at Ann and Robert H. Lurie Children's Hospital of Chicago Research Center. Statistical analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC). Boxplots were generated for the negative delta Ct ($-\Delta Ct$) of three normal retinas in comparison to 10 retinoblastomas with lower and upper whiskers corresponding to 5 and 95 percentiles respectively. For the apoptosis studies, total numbers of cells were compared between negative Pre-miR and Pre-miR-449a or -449b at 72, 96, 122, 144, and 168 h. Proliferation studies were analyzed at 168 h (comparing negative Pre-miR to Pre-miR-449a or -449b). For all studies, comparisons between more than two groups were conducted using the Kruskal–Wallis test, and then followed by the Wilcoxon Rank Sums test between each pair of groups. All tests were two-sided. Tests with p-values less than 0.05 are considered as significant.

3. Results

3.1. Validation of miR-449 differential expression in primary tumors and in cell lines

We validated the differential expression of miR-449a and -449b in a subset of 10 primary human tumors as compared to three normal pediatric retinas. Both miRNAs were confirmed to be upregulated in the tumor cohort (Fig. 1A and B). Next, we evaluated the expression of miR-449a and -449b in two human retinoblastoma cell lines, Weri1 and Y79. In comparison to three normal pediatric retina controls, both miR-449a and -449b were overexpressed in cell lines. The degree of overexpression was higher in Weri1 cells compared with Y79 cells (Fig. 1 C).

3.2. Overexpression of miR-449 in human retinoblastoma cell lines

Reverse transfection was utilized to express miRs-449a and -449b in human retinoblastoma cell lines. At each designated time point after transfection, (72, 96, 120, 144 and 168 h), overexpression of both miR-449a and -449b resulted in decreased

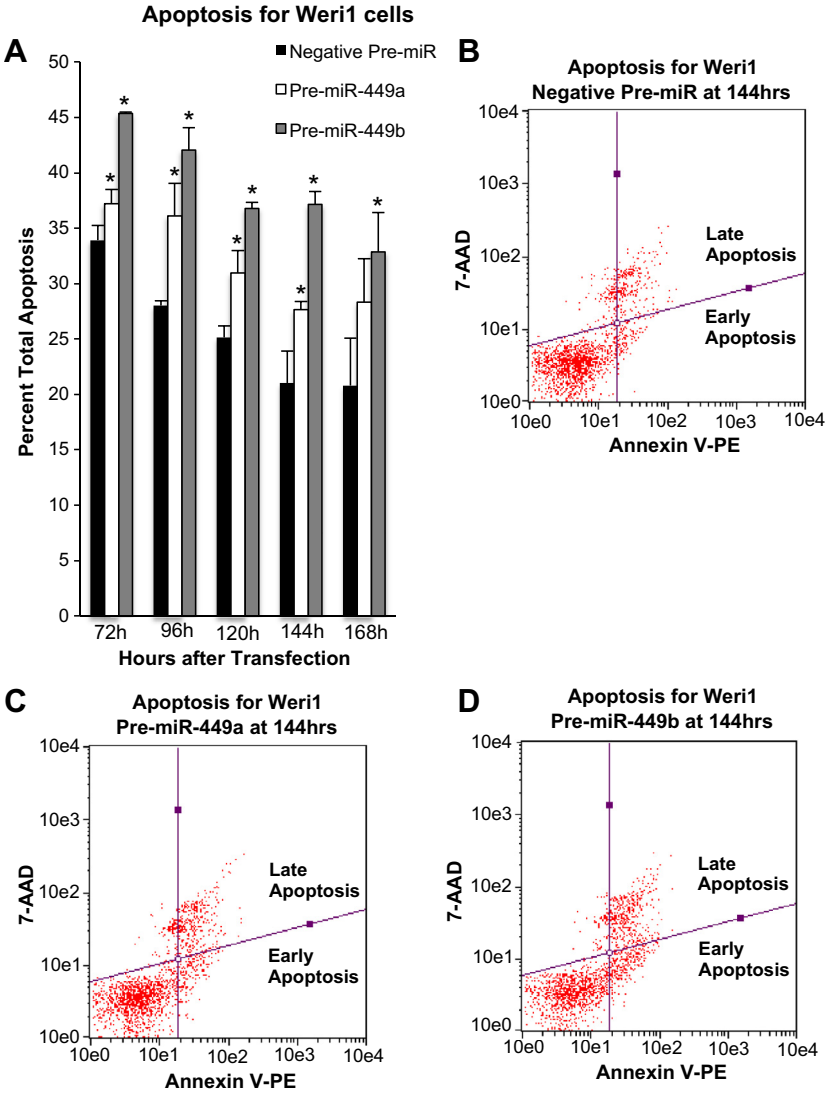

Fig. 4. miR-449a/b overexpression induces apoptosis in the Weri1 human retinoblastoma cell line. (A) Total apoptosis (the sum of early and late apoptosis) was determined using the Guava Nexin assay for 72–168 h post-transfection. Raw data and error bars were derived from three replicate experiments. Asterisk denotes $p < 0.05$ (comparing negative Pre-miR to Pre-miR-449a or Pre-miR-449b). (B–D) Representative pictures of early/late apoptosis as visualized using the Guava Nexin assay at the 144 h time point. The lower right quadrant represents early apoptosis (shown), and the upper right quadrant represents late apoptosis (shown).

proliferation of retinoblastoma cells compared with cells transfected with a negative Pre-miR control. This effect was seen in both Weri1 and Y79 cells, but was more pronounced in Y79 (Fig. 2A and B). Cells transfected with miRs-449 and -449b also showed higher rates of apoptosis at each time point in Y79 cells (Fig. 3). In Weri1 cells, although the absolute percentage of apoptosis decreased each day, apoptosis was lower in cells transfected with a negative Pre-miR than with either miR-449a or -449b (Fig. 4).

4. Discussion

MicroRNA-449a and -449b have been reported in the literature as having tumor suppressive roles and exerting inhibitory effects on the cell cycle [12,13,15]. Transcription of both miRNAs is induced by E2F transcription factors, and in turn, the E2F transcription factors are targets of miR-449a and -449b, suggesting a feedback loop [10,11]. We hypothesize that this may be important in retinoblastoma because the lack of a functional Rb protein allows for uninhibited E2F transcription, and resultant higher proliferative potential in retinoblastoma cells. Another important predicted target of miR-449a and -449b is cyclin D1, which is necessary for progression through the cell cycle [16].

Despite a predicted role as a tumor suppressor that functions to augment both Rb-mediated control over cell proliferation and p53 mediated apoptosis, both miR-449a and -449b were overexpressed in our tumor cohort. A potential explanation for this may be that increased levels of E2F, resulting from failure of normal Rb function, causes a relative increase of these miRNAs, but that the inhibitory effects on proliferation are only seen at higher levels achieved by transfection.

We also find it interesting that though miRs-449a and -449b were significantly upregulated in both Weri1 and Y79 cell lines, the expression was lower in Y79. Y79 cells are considered to be more aggressive than Weri1 cells, and will invade the optic nerve when injected into the vitreous of immunodeficient mice [17,18]. We speculate that the relatively lower expression of miR-449 in Y79 may contribute to or allow this more aggressive behavior.

We have confirmed an inhibitory effect of miR-449a and -449b in retinoblastoma by demonstrating significantly impaired proliferation and increased apoptosis of tumor cells when these miRNAs are overexpressed. These results are exciting because of the potential implications for development of novel therapeutics, which would increase the relative expression of miR-449a and/or -449b, either through introduction of the miRNA, or by use of an agent that will increase expression of this miRNA.

Acknowledgments

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