MicroRNA-34a inhibits the proliferation and promotes the chemosensitivity of retinoblastoma cells by downregulating Notch1 expression

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Received May 21, 2019; Accepted February 11, 2020

DOI: 10.3892/mmr.2020.11238

Abstract. MicroRNAs (miRs) are potential therapeutic targets for tumors. The aims of the present study were to investigate the regulatory effects of miR-34a on the proliferation, apoptosis and chemosensitivity of retinoblastoma (RB) cells, and to identify the possible underlying mechanism involving Notch1. It was found that miR-34a was downregulated, and Notch1 was upregulated in HXO-RB44 and Y79 cells. In addition, Notch1 was identified to be a target gene of miR-34a, which could be downregulated by the increased expression of miR-34a. It was demonstrated that miR-34a upregulation and Notch1 downregulation significantly inhibited proliferation, promoted apoptosis and enhanced the carboplatin sensitivity of HXO-RB44 and Y79 cells. The transfection of miR-34a mimics + Notch1 siRNA further enhanced the above anti-tumor responses in HXO-RB44 and Y79 cells. Collectively, the present results suggested that miR-34a may negatively regulate Notch1 expression and may be a potential therapeutic target for RB.

Introduction

Retinoblastoma (RB) is the most common intraocular malignant tumor in pediatric patients <5 years old (1,2). Furthermore, RB has an incidence rate of 1 in 15,000-20,000 live births (3). Currently, the primary approaches for the clinical treatment of RB include laser photo-coagulation, chemotherapy and radiotherapy (4-6). Despite substantial improvements in the treatment of RB, the survival of patients remains poor (7). Thus, it is important to investigate novel therapeutic targets to facilitate the development of treatments for RB.

Previous studies have shown that microRNAs (miRNAs/miRs) are potential therapeutic targets for tumors (8-11). miRNAs are small endogenous non-coding RNAs, 18-22 nucleotides in length, that can modulate mRNA translation via binding to the 3'-untranslated region (3'-UTR) of their target genes (12). A variety of miRNAs have been reported to serve oncogenic or anti-tumor roles in RB cells by regulating cell proliferation, apoptosis, migration, invasion and the cell cycle (9-11). miR-106b promotes the proliferation, migration and invasion of RB cells by inhibiting Zinc Finger and BTB Domain Containing 4 (13). In addition, miR-101-3p suppresses the proliferation of RB cells by targeting Enhancer of zeste homolog 2 and Histone deacetylase 9 (14). Exogenous miR-34a inhibits cell proliferation and increases the apoptotic activity of RB cells (15). However, the specific regulatory role of miR-34a on the chemosensitivity of RB cells is not fully understood.

The anti-tumor potential of miR-34a is closely associated with the regulation of various signaling pathways, such as the Wnt/β-Catenin (16), PI3K/AKT/survivin (17) and Notch signaling pathways (18). The Notch signaling pathway is a highly conserved pathway that is involved in the regulation of several fundamental cellular processes, such as cell proliferation, stem cell maintenance and differentiation (19). Furthermore, the Notch family of proteins, which consists of Notch1, Notch2 and Notch3, serve a key regulatory role in RB (20). Li *et al* (21) indicated that miR-433 suppresses cell proliferation and metastasis in RB by inhibiting Notch1. However, whether the regulatory effect of miR-34a on RB is related with the Notch signaling pathway is unknown.

The present study investigated the regulatory effects of miR-34a on the proliferation and chemosensitivity of RB cells, as well as the related regulatory mechanism involving the Notch signaling pathway. It was found that miR-34a may inhibit the proliferation, and promote the apoptosis and chemosensitivity of RB cells by downregulating Notch1. Therefore, the present study may provide a novel theoretical basis for the treatment of RB.

Materials and methods

Cell culture. The immortalized human normal retinal vascular endothelial cell line ACBRI-181, and the RB cell lines HXO-RB44 and Y79 were obtained from the American Type Culture Collection. Cells were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (BD Biosciences) and 1% penicillin/streptomycin (Gibco;

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Key words: retinoblastoma, microRNA-34a, Notch1, proliferation, chemosensitivity

Thermo Fisher Scientific, Inc.). All cells were maintained in a humidified 5% CO_2 atmosphere at 37˚C.

Dual-luciferase reporter gene assay. TargetScan software (22) was used to predict the targeting relationship between miR-34a and Notch1. The 3'-UTR containing the miR-34a binding site of Notch1 was amplified and cloned into Psi-CHECK2 reporter vector (Promega Corporation) to construct wild-type (WT) Psi-CHECK2-WT-Notch1-3'-UTR (Notch1-WT) and mutant (MUT) Psi-CHECK2-MUT-Notch1-3'-UTR (Notch1-MUT). For the luciferase assay, miR-34a mimics or miR-34a negative control (NC) mimics (20 nmol/l) were co-transfected with reporter plasmids (20 nmol/l) into HXO-RB44 and Y79 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). HXO-RB44 and Y79 cells were grouped as follows: i) MUT + mimics group, transfected with Notch1-MUT and miR-34a mimics; ii) MUT + NC group, transfected with Notch1-MUT and miR-34a NC mimics; iii) WT + mimics group, transfected with Notch1-WT and miR-34a mimics; and iv) WT + NC group, transfected with Notch1-WT and miR-34a NC mimics. Luciferase activity was assessed using a dual-luciferase kit (Promega Corporation) after 48 h of transfection, and was normalized to *Renilla* luciferase activity.

Cell transfection and experimental grouping. miR-34a mimics, Notch1 small interfering RNAs (Notch1 siRNA-1 and -2) and their corresponding NC (miR-34a NC and Notch1 siRNA NC) were supplied by Shanghai GenePharma Co., Ltd. The sequences were: miR-34a mimics forward, 5'-UGGCAG UGUCUUAGCUGGUUGU-3' and reverse, 5'-ACAACCAGC UAAGACACUGCCA-3'; miR-34a NC forward, 5'-UCACAA CCUCCUAGAAAGAGUAGA-3' and reverse, 5'-UCUACU CUUUCUAGGAGGUUGUGA-3'; Notch 1 siRNA-1 forward, 5'-CACCAGUUUGAAUGGUCAAdTdT-3' and reverse, 5'-UUGACCAUUCAAACUGGUGTdTd-3'; Notch 1 siRNA-2 forward, 5'-UGGCGGGAAGUGUGAAGCGdTdT-3' and reverse, 5'-CGCUUCACACUUCCCGCCATdTd-3'; Notch 1 siRNA NC forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATt-3'. These substances (20 nmol/l) were transfected into HXO-RB44 and Y79 cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). HXO-RB44 and Y79 cells were randomly divided into five groups: i) BLANK, cells without transfection; ii) miR-NC + si-NC, transfected with miR-34a NC and Notch1 siRNA NC; iii) miR-34a mimics + si-NC, transfected with miR-34a mimics and Notch1 siRNA NC; iv) miR-NC + Notch1 siRNA, transfected with miR-34a NC and Notch1 siRNA-1; and v) miR-34a mimics + Notch1 siRNA, transfected with miR-34a mimics and Notch1 siRNA-1. All cells were cultured for 48 h at 37˚C with 5% CO_2. Some HXO-RB44 and Y79 cells ($1x10^5$ cells/well) were further treated with 40 μM Z-VAD (Selleckhchem) for 48 at 37˚C with 5% CO^2. Cells were randomly divided into four groups: i) Z-VAD; ii) Z-VAD + miR-34a mimics; iii) Z-VAD + Notch1 siRNA; and iv) Z-VAD + miR-34a mimics + Notch1 siRNA.

Cell Counting Kit-8 (CCK-8) assay. The viability of HXO-RB44 and Y79 cells was detected using a CCK-8 assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were seeded into 96-well plates ($2x10^3$ cells/well), and cultured for 24, 48, 72 or 96 h at 37˚C with 5% CO^2. CCK-8 solution (10 μl) was then added to each well and the cells were incubated at 37˚C for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader.

5-Bromo-2-deoxyuridine (BrdU) assay. The proliferation of HXO-RB44 and Y79 cells was measured using a BrdU kit (6813; Cell Signaling Technology, Inc.). Cells were seeded into 96-well plates ($1x10^4$ cells/well) and incubated overnight at 37˚C. Subsequently, cells were labeled with 10 μM BrdU solution for 3 h at 25˚C, denatured with 100 μl FixDenta solution for 30 min at 25˚C and incubated with peroxidase-conjugated anti-BrdU antibody (6813; 1:1,000; Cell Signaling Technology, Inc.) for 1.5 h at 25˚C. The absorbance of each well was measured at 450 nm using a microplate reader.

Flow cytometry assay. The apoptotic rate of HXO-RB44 and Y79 cells was examined using an Annexin V-FITC apoptosis detection kit (KGA108-1, Nanjing KeyGen Biotech Co., Ltd.). Cells were washed three times with PBS, suspended in 500 μl binding buffer, and incubated with 5 μl Annexin V-FITC and 5 μl PI for 15 min in the dark at room temperature. Cell apoptosis was detected using a Cytomics FC500 flow cytometer (Beckman Coulter, Inc.), and the data were analyzed by CytoDiff CXP software (version 2.0, https://www.beckmancoulter.com, Beckman Coulter, Inc.) (23).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from ACBRI-181, HXO-RB44 and Y79 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 500 ng RNA was then RT into cDNA with a Revert Aid First Strand cDNA Synthesis kit at 42˚C for 45 min (Thermo Fisher Scientific, Inc.). RT-qPCR was performed on a RT-qPCR instrument (Bio-Rad Laboratories, Inc.) with SYBR green qPCR Master mix (Thermo Fisher Scientific, Inc.). The program included 94˚C for 3 min, 40 cycles of 94˚C for 15 sec and 60˚C for 30 sec. Primers used for RT-qPCR were as follows: miR-34a forward, 5'-GCCACTATGTAGCGGGTT TC-3' and reverse, 5'-ACCTGCGCTAAGAACTGAGG-3'; Notch1 forward, 5'-TCAACGCCGTAGATGACCT-3' and reverse, 5'-TCTCCTCCCTGTTGTTCTGC-3'; Notch1 siRNA-1 forward, 5'-CAUGGUAGUCACUAACAUATT-3' and reverse, 5'-UAUGUUAGUGACUACCAUGTT-3'; Notch1 siRNA-2 forward, 5'-GCACGCGGAUUAAUUUGCCA-3' and reverse, 5'-UGCAAAUUAAUCCGCGUGC-3'; si-NC forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'; U6 forward, 5'-CTC GCTTCGGCAGCACA-3' and reverse, 5'-ACGCTTCAC GAATTTGCGT-3'; and GADPH forward, 5'-AGCCACATC GCTCAGACA-3' and reverse, 5'-TGGACTCCACGACGT ACT-3'. Relative expression level was calculated by the $2^{-\Delta\Delta Cq}$ method (24).

Western blot analysis. Total proteins were extracted from HXO-RB44 and Y79 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was measured with a bicinchoninic acid kit (Yeasen Biotechnology Co., Ltd.).

Protein samples (30 μg) were subjected to 10% SDS-PAGE and then transferred to a nitrocellulose membrane. After being blocked with 5% skim milk for 30 min at 37°C, the membrane was incubated with the following primary antibodies: Notch1 (14-5785-81; 1:1,000; Chemicon International; Thermo Fisher Scientific, Inc.), p16 (101169-T38; 1:1,000; Sino Biological, Inc.), proliferating cell nuclear antigen (PCNA, 101118-T46; 1:1,000, Sino Biological, Inc.), Bcl-2 (ab185002; 1:1,000; Abcam), Bax (2774s; 1:1,000; Cell Signaling Technology, Inc.), matrix metalloproteinase (MMP)-9 (3852s; 1:1,000; Cell Signaling Technology, Inc.), cleaved caspase-3 (9661s; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (ab8245; 1:1,000; Abcam) at 4˚C overnight. The membrane was subsequently incubated with peroxidase-labeled secondary antibody (anti-rabbit IgG; ab6721; 1:5,000; Abcam) at room temperature for 1 h. The protein blots were visualized using an enhanced chemiluminescence kit (Invitrogen; Thermo Fisher Scientific, Inc.). The density of western blot bands was analyzed using Quantity One 1-D Analysis software (version 4.6.9, Bio-Rad Laboratories, Inc.).

Evaluation of drug sensitivity. HXO-RB44 and Y79 cells were seeded into 96-well plates at a density of $4x10^3$/ml. When the cells had adhered, carboplatin (CBP; Sigma-Aldrich; Merck KGaA) was added into each well at a concentration of 0, 3, 6, 9 or 12 μg/ml. Subsequently, the cells were cultured for 48 h at 37˚C. Then, CCK-8 reaction solution (10 μl) was added into each well and the cells were incubated at 37˚C for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader. The inhibition rate (IR; %) was calculated according to the following formula: IR (%) = $(1-A_{Medicine}/A_{Control})$ x100%. The CBP concentration that caused the death of half of HXO-RB44 or Y79 cells was calculated, and considered the IC_{50}.

Statistical analysis. All statistical analyses were performed using SPSS version 22.0 (IBM Corp.). All experiments were repeated three times. Data are presented as the mean ± standard deviation. A one-way ANOVA followed by Tukey's post hoc test was used to analyze the differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-34a is downregulated and Notch1 is upregulated in RB cells. The expression levels of miR-34a and Notch1 in ACBRI-181, HXO-RB44 and Y79 cells were detected using RT-qPCR. It was found that the mRNA expression level of miR-34a was significantly lower in HXO-RB44 and Y79 cells compared with ACBRI-181 cells (P<0.05; Fig. 1A). However, the mRNA expression level of Notch1 in HXO-RB44 and Y79 cells was significantly higher compared with ACBRI-181 cells (P<0.05; Fig. 1B).

miR-34a downregulates Notch1 in RB cells. Notch1 was silenced in HXO-RB44 and Y79 cells via the transfection of si-Notch1-1 and si-Notch1-2. The RT-qPCR results indicated that that the expression levels of Notch1 was significantly decreased by si-Notch1-1 and si-Notch1-2 (P<0.001; Fig. 1C). In addition, miR-34a was overexpressed in HXO-RB44 and Y79 cells via the transfection of miR-34a mimics. The RT-qPCR results showed that the mRNA expression levels of miR-34a were significantly increased by miR-34a mimics in HXO-RB44 and Y79 cells (P<0.001; Fig. 1D). Furthermore, the effect of miR-34a mimics on the expression levels of Notch1 was analyzed using si-Notch1-1. Compared with the BLANK group, the mRNA and protein expression levels of Notch1 in HXO-RB44 and Y79 cells were significantly decreased by miR-34a mimics and Notch1 siRNA transfections (P<0.05). In addition, it was found that Notch1 was further downregulated in the miR-34a mimics + Notch1 siRNA group compared with the miR-NC + Notch 1 siRNA group (P<0.05; Fig. 1E and F).

Notch1 is the target gene of miR-34a. TargetScan analysis predicted that the binding site of Notch1 to miR-34a was at the 3'-UTR region (Fig. 2A). Based on the result that the Notch1 promoter contains the putative miR-34a binding site, the target relationship between Notch1 and miR-34a was further analyzed by dual-luciferase reporter gene assay. It was demonstrated that the luciferase activity of the WT + mimics group was significantly decreased compared with the WT + NC group (P<0.05; Fig. 2B).

miR-34a upregulation and Notch1 downregulation inhibit the proliferation of RB cells. The viability of HXO-RB44 and Y79 cells was detected with a CCK-8 assay. The viability of HXO-RB44 and Y79 cells was significantly decreased by the transfection of miR-34a mimics and Notch1 siRNA compared with the BLANK group (P<0.05; Fig. 3A). Furthermore, the expression levels of the proliferation-related proteins p16 and PCNA were measured using western blotting. Compared with the BLANK group, PCNA expression levels were significantly decreased, and p16 expression levels were significantly increased by the transfection of miR-34a mimics and Notch1 siRNA in HXO-RB44 and Y79 cells (P<0.05; Fig. 3B). The proliferation of RB cells was further analyzed using a BrdU assay. Compared with the BLANK group, the optical density (OD) 450 values of HXO-RB44 and Y79 cells were significantly decreased by miR-34a mimics and Notch1 siRNA (P<0.05; Fig. 3C). Furthermore, it was found that the transfection of miR-34a mimics + Notch1 siRNA decreased cell viability, upregulated p16 expression, downregulated PCNA expression and decreased OD450 values of HXO-RB44 and Y79 cells, compared with the transfection of miR-34a mimics and Notch1 siRNA alone (P<0.05; Fig. 3A-C).

miR-34a upregulation and Notch1 downregulation promotes the apoptosis of RB cells. The apoptotic rate of HXO-RB44 and Y79 cells was detected using Annexin/PI double staining. It was demonstrated that the transfection of miR-34a mimics and Notch1 siRNA alone significantly increased the percentage of apoptotic cells compared with the BLANK group (P<0.05). Furthermore, the transfection of miR-34a mimics + Notch1 siRNA further increased the percentage of apoptotic cells (P<0.05; Fig. 4A). The expression levels of the apoptosis-related proteins Bax, Bcl-2, MMP-9 and cleaved caspase-3 were measured by western blotting. Compared with the BLANK group, the transfection of miR-34a mimics and Notch1 siRNA alone significantly upregulated Bax and

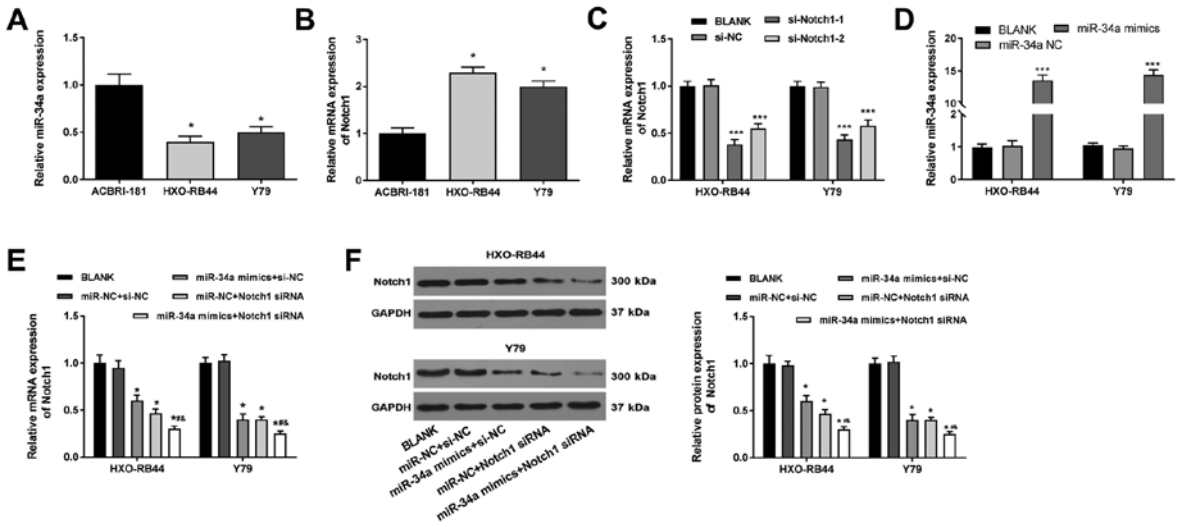

Figure 1. Expression levels of miR-34a and Notch1 in RB cells. (A) mRNA expression levels of miR-34a was detected by RT-qPCR in ACBRI-181, HXO-RB44 and Y79 cells. (B) mRNA expression levels of Notch1 were detected by RT-qPCR in ACBRI-181, HXO-RB44 and Y79 cells. *P<0.05 vs. ACBRI-181 group. (C) mRNA expression of Notch1 was detected by RT-qPCR in si-Notch1 transfected HXO-RB44 and Y79 cells. ***P<0.001 vs. BLANK and si-NC group. (D) mRNA expression levels of miR-34a in HXO-RB44 and Y79 cells 48 h after transfection. ***P<0.001 vs. BLANK and miR-34a NC group. (E) mRNA expression levels of Notch1 in HXO-RB44 and Y79 cells 48 h post-transfection. (F) Protein expression levels of Notch1 were detected by western blotting in HXO-RB44 and Y79 cells at 48 h post-transfection. *P<0.05 vs. BLANK and miR-NC + si-NC group. $^{\#}$P<0.05 vs. miR-34a mimics + si-NC group. $^{\&}$P<0.05 vs. miR-NC + Notch1 siRNA group. Data are presented as the mean ± standard deviation, with three replicates. siRNA, small interfering RNA; NC, negative control; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 187-193 of NOTCH1 3' UTR	5' ...UAUUUUCCACAGAAACACUGCCU...	7mer-m8	-0.51	98	-0.51	6.934	0.79
mmu-miR-34a-5p	3' UGUUGGUCGAUUCUGUGACGGU						

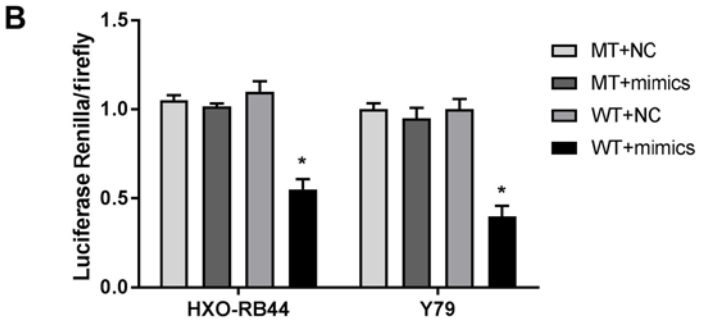

Figure 2. Notch1 is the target gene of miR-34a. (A) Binding target of Notch1 and miR-34a was predicted by TargetScan software. (B) Luciferase activity was measured using a dual-luciferase reporter gene assay. Data are presented as the mean ± standard deviation, with three replicates. *P<0.05 vs. WT + NC group. WT, wild-type; MUT, mutant; NC, negative control; miR, microRNA; 3'-UTR, 3' untranslated region.

cleaved caspase-3 expression, and downregulated Bcl-2 and MMP-9 expression (P<0.05). Furthermore, it was found that the changes in expression of these apoptosis-related proteins were more significant in cells transfected with miR-34a mimics + Notch1 siRNA, compared with those transfected with miR-34a mimics and Notch1 siRNA alone (P<0.05; Fig. 4B and C). In addition, the inhibiting effect of Z-VAD, a caspase inhibitor, on cleaved caspase-3 expression level was significantly reversed by the transfection of miR-34a mimics and Notch1 siRNA alone, and particularly by the transfection of miR-34a mimics + Notch1 siRNA (P<0.05; Fig. 4D).

miR-34a upregulation and Notch1 downregulation increases the sensitivity of RB cells to CBP. The sensitivity of HXO-RB44 and Y79 cells to CBP was evaluated by CKK-8 assay. Compared with the BLANK group, the transfection of miR-34a mimics and Notch1 siRNA alone significantly increased the IR of cells treated with different concentrations of CBP. Furthermore, it was demonstrated that the transfection of miR-34a mimics + Notch1 siRNA further increased the IR (P<0.05; Fig. 5A). These results suggest that the CBP IC_{50} was significantly lower in cells transfected with miR-34a mimics and Notch1 siRNA alone compared with the BLANK group. The CBP IC_{50} was lowest in cells transfected with miR-34a mimics + Notch1 siRNA compared with the different groups (P<0.05; Fig. 5B).

Discussion

miRNAs serve key regulatory roles in the occurrence and development of RB (25,26). It has been previously reported

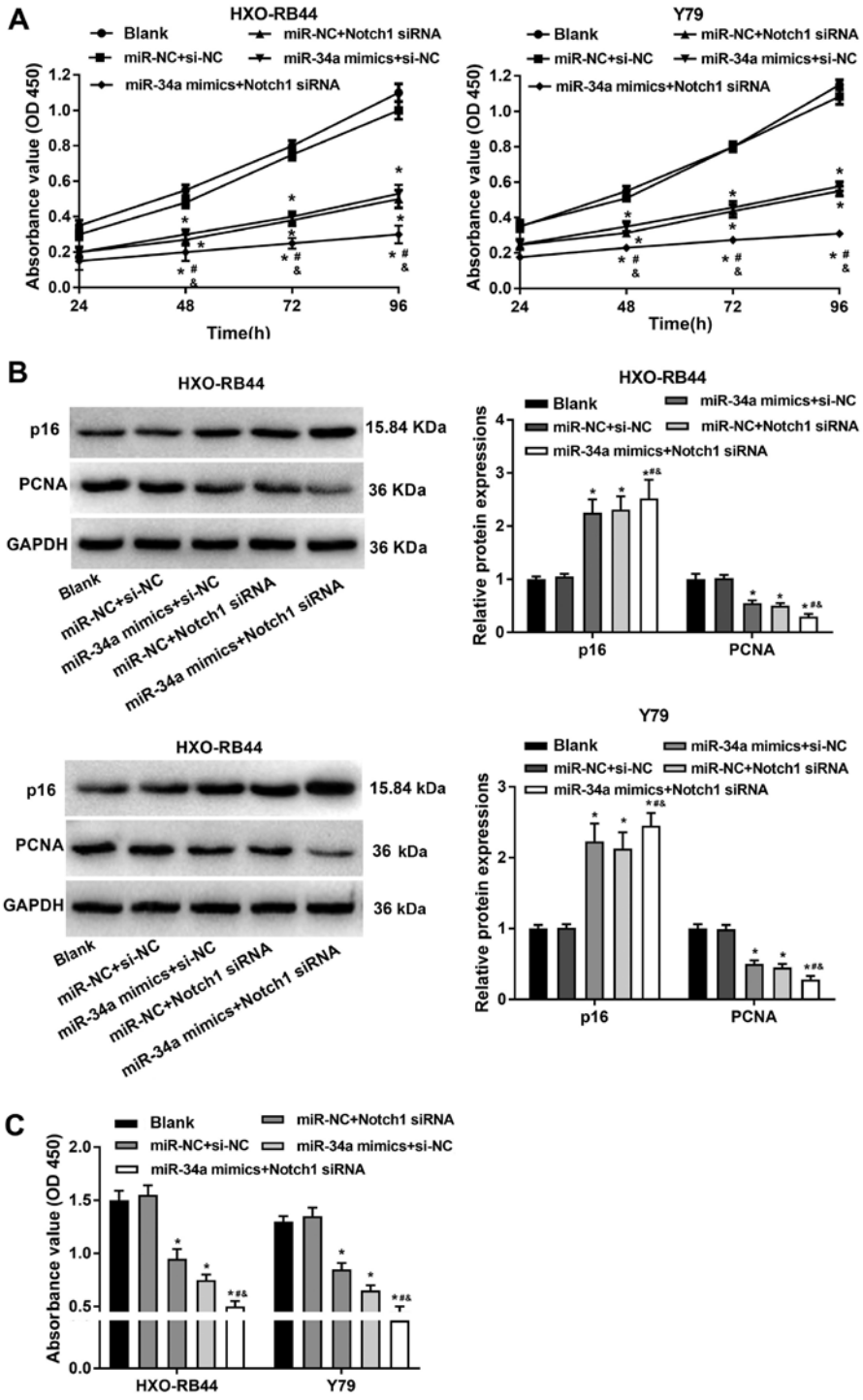

Figure 3. miR-34a inhibits the proliferation of HXO-RB44 and Y79 cells. (A) Cell viability of transfected HXO-RB44 and Y79 cells was measured using a Cell Counting Kit-8 assay. (B) Protein expression levels of p16 and PCNA were measured by western blotting 48 h post-transfection. (C) OD450 values of cells were measured using a 5-Bromo-2-deoxyuridine assay 48 h post-transfection. Data are presented as the mean ± standard deviation, with three replicates. *P<0.05 vs. BLANK and miR-NC + si-NC group. #P<0.05 vs. miR-34a mimics + si-NC group. &P<0.05 vs. miR-NC + Notch1 siRNA group. OD, optical density; siRNA, small interfering RNA; miR, microRNA; NC, negative control; PCNA, proliferating cell nuclear antigen.

that miR-34a expression levels are decreased in breast cancer, cervical cancer, pancreatic cancer, cholangiocarcinoma and RB (15,27-29). In relation to this, the results of the present study showed that the expression levels of miR-34a were significantly lower in RB cell lines compared with human normal retinal vascular endothelial cells. Therefore, the present results suggested that miR-34a is downregulated in RB, which is consistent with the aforementioned previous studies. In addition, previous studies suggested that miR-34a acts as a potential tumor suppressor by modulating cell proliferation, apoptosis, migration and invasion (15). miR-34a suppresses colorectal cancer metastasis by attenuating cell migration and invasion (30). In addition, miR-34a significantly decreases the proliferation rate and increases the apoptotic rate of gastric cancer cells (31). Exogenous miR-34a also inhibits the proliferation and promotes the

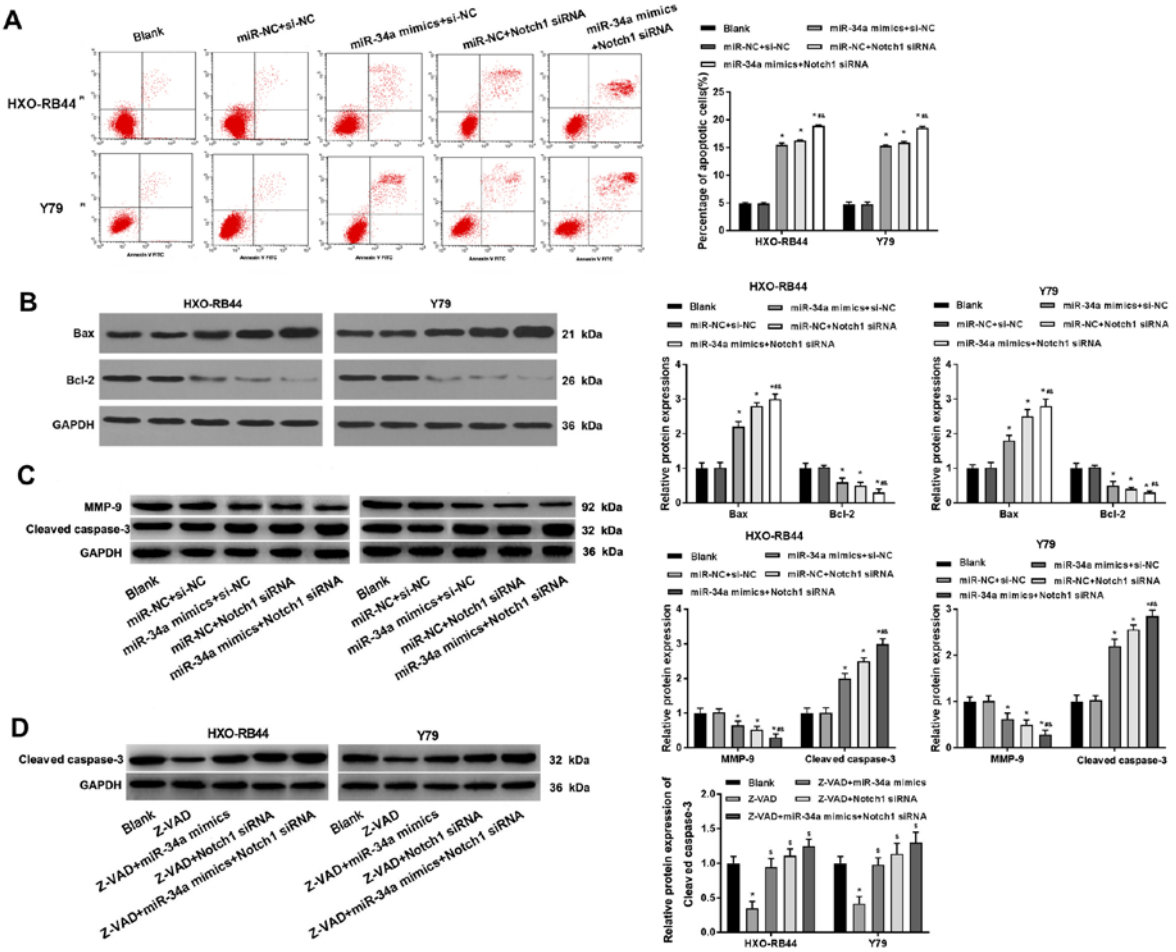

Figure 4. miR-34a promotes the apoptosis of HXO-RB44 and Y79 cells. (A) Cell apoptosis of HXO-RB44 and Y79 cells was detected by flow cytometry 48 h post-transfection. (B) Protein expression levels of Bax and Bcl-2 were measured by western blotting. (C) Protein expression levels of MMP-9 and cleaved caspase-3. *P<0.05 vs. BLANK and miR-NC + si-NC group. (D) Protein expression levels of cleaved caspase-3 were measured by western blot in Z-VAD-treated HXO-RB44 and Y79 cells at 48 h post-treatment. *P<0.05 vs. BLANK. Data are presented as the mean ± standard deviation, with three replicates. #P<0.05 vs. miR-34a mimics + si-NC group. &P<0.05 vs. miR-NC + Notch1 siRNA group. $P<0.05 vs. Z-VAD group. siRNA, small interfering RNA; miR, microRNA; NC, negative control; MMP, matrix metalloproteinase.

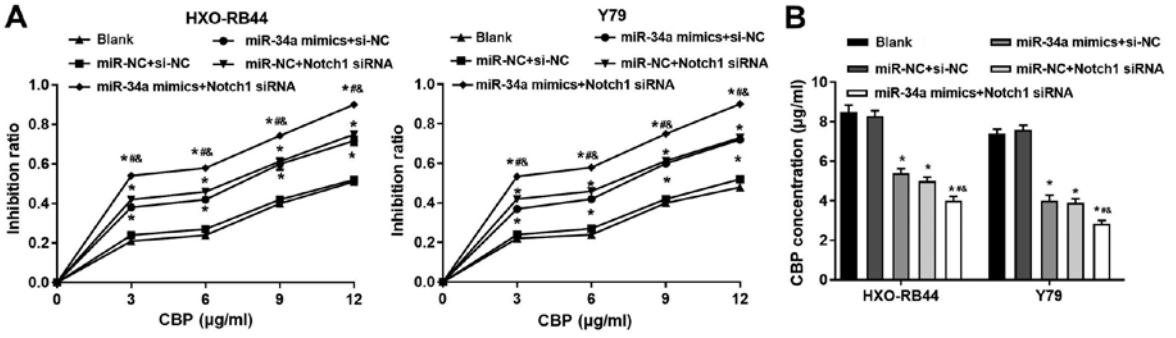

Figure 5. miR-34a promotes CBP sensitivity of HXO-RB44 and Y79 cells. (A) Inhibition ratios of CBP-treated HXO-RB44 and Y79 cells were detected using a Cell Counting Kit-8 assay 48 h post-treatment. (B) CBP IC_{50}. Data are presented as the mean ± standard deviation, with three replicates. *P<0.05 vs. BLANK group and miR-NC + si-NC group. #P<0.05 vs. miR-34a mimics + si-NC group. &P<0.05 vs. miR-NC + Notch1 siRNA group. miR, microRNA; siRNA, small interfering RNA; CBP, carboplatin; NC, negative control.

apoptosis of RB cells (15). In the present study, it was found that miR-34a inhibited the proliferation and promoted the apoptosis of HXO-RB44 and Y79 cells. Therefore, the present results are consistent with previous studies, and demonstrated that miR-34a may be a potential therapeutic target for RB.

The Notch signaling pathway has been identified as a potential therapeutic target for a variety of cancer types, including pancreatic cancer (32), hepatocellular carcinoma (33) and RB (20). In addition, the Notch family of proteins serve vital roles in retinal development (34). During early retinal development in mammals, Notch1 and Notch3 are primarily

present in the central portion of the retina, and Notch2 is predominantly expressed in the retinal periphery and pigment epithelial cells (35,36). A previous study showed that Notch1 expression levels were significantly higher compared with Notch2 and Notch3 expression levels in Y79 cells (37). The present results suggested that Notch1 was upregulated in RB cells compared with human normal retinal vascular endothelial cells, consistent with the results from a previous study, which identified that Notch1 is highly expressed in human RB cells (20). Furthermore, previous studies showed that Notch1 is a target of miR-34a (38-40). miR-34a inhibits cell invasion via the downregulation of Notch1 in cervical carcinoma and choriocarcinoma (38). In addition, miR-34a inhibits the proliferation, and induces the apoptosis of glioblastoma cells by targeting Notch1 (39). miR-34a inhibits the proliferation and invasion of endometrial cancer cells by downregulating Notch1 (40). In line with previous studies, the present results indicated that Notch1 may be a target gene of miR-34a, and that Notch1 may be downregulated by miR-34a upregulation. In addition, downregulation of Notch1 inhibited the proliferation and promoted apoptosis of HXO-RB44 and Y79 cells. Thus, Notch1 may act as an oncogene in RB (37). In addition, miR-34a-induced downregulation of Notch1 may contribute to the anti-tumor response. Thus, miR-34a may inhibit the proliferation and promote the apoptosis of RB cells by downregulating Notch1.

Pediatric patients with RB are primarily treated with chemotherapy using drugs such as CBP, vincristine and etoposide (ETO) (41). However, drug resistance greatly limits the prognosis of patients with RB (42,43). Previous studies have revealed that miR-34a can, to a certain extent, increase the chemosensitivity of RB cells (44,45). miR-34a downregulation elevates the survival rate and viability of RB cells following carboplatin, adriamycin and vincristine treatment (44). Furthermore, miR-34a restores ETO and CBP chemosensitivity, and increases the apoptosis of RB cells (45). The present results suggested that miR-34a increased the IR and CBP IC_{50} of HXO-RB44 and Y79 cells. This result is consistent with previous studies, and indicates that miR-34a promotes the chemosensitivity of RB cells to CBP treatment. In addition, miR-34a increases the chemosensitivity of breast cancer stem cells to Paclitaxel by downregulating Notch1 (46). miR-34a sensitizes the chemosensitivity of breast cancer cells to adriamycin by targeting Notch1 (47). As Notch1 is a target gene of miR-34a, the present results suggested that miR-34a may improve the chemosensitivity of RB cells by downregulating Notch1, and thus aid in the treatment of RB.

In conclusion, it was found that miR-34a was downregulated and Notch1 was upregulated in HXO-RB44 and Y79 cells. In addition, miR-34a upregulation inhibited the proliferation, promoted the apoptosis and enhanced the CBP chemosensitivity of RB cells by downregulating Notch1. The present results provide a novel regulatory mechanism of miR-34a in RB cells, and may facilitate the treatment of RB as overexpression of miR-34a may be a potential therapeutic strategy.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to research related to future studies, but are available from the corresponding author on reasonable request.

Authors' contributions

SZ was responsible for the conception and design of the study, and development of the manuscript. FG and WY performed the experiments, and were involved in the collection and supervision of data. WY participated in drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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