# MicroRNA-504 targets AEG-1 and inhibits cell proliferation and invasion in retinoblastoma

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**Abstract.** The dysregulation of microRNAs (miRNAs/miRs) has become increasingly recognized as a primary feature of retinoblastoma (RB). Furthermore, miRNAs have been demonstrated to be involved in the occurrence and development of RB. Therefore, it is crucial to investigate the expression profile and roles of miRNAs in RB in order to identify potential therapeutic targets to treat patients with RB. The expression profile and biological roles of miRNA-504 (miR-504) have been reported in numerous types of human cancer; however, the roles of miR-504 in RB remain unknown. In the present study, it was demonstrated that miR-504 expression was significantly decreased in RB tissues and cell lines. Functional analysis identified that resumption of miR-504 expression suppressed cell proliferation and invasion in RB. Furthermore, astrocyte elevated gene-1 (AEG-1) was determined to be a direct target of miR-504 in RB, and a negative correlation between miR-504 and AEG-1 mRNA expression levels was observed in RB tissues. Additionally, the tumor-suppressing effects of miR-504 overexpression in RB cells could be rescued by AEG-1 upregulation. In conclusion, these results indicated a significant role of the miR-504/AEG-1 pathway in inhibiting the aggressiveness of RB, suggesting that this miRNA may be employed as a therapeutic target for the treatment of patients with this disease.

## Introduction

Retinoblastoma (RB) is the most common type of intraocular malignant tumor in children and accounts for ~3% of all childhood cancer types (1). The morbidity of RB is relatively stable, with one case for every 15,000-20,000 live births, or ~9,000 novel cases globally every year (2). Untreated RB progresses rapidly, causing blindness and may spread to the brain via the optic nerve (3). Patients with RB are typically diagnosed at more advanced stages in developing countries, with these patients exhibiting poorer prognosis compared with those in developed countries (4). Notable advancements have been made recently in the therapeutic approaches used to treat patients with RB, including enucleation, laser therapy, cryotherapy, thermotherapy and chemotherapy (5). On the contrary, the therapeutic outcomes of these techniques are poor, primarily due to delays in diagnosis and treatment, in addition to metastasis to distant organs and chemoresistance (6-8). Therefore, understanding the mechanisms underlying the formation and progression of RB is crucial for the identification of novel therapeutic strategies for patients with this malignant disease.

MicroRNAs (miRNAs/miRs) refer to a group of endogenous, non-coding short RNAs comprising of 17-24 nucleotides (9). miRNAs negatively modulate gene expression via translational suppression or induction of mRNA degradation by directly interacting with complementary sequences in the 3'-untranslated regions (3'-UTRs) of their target genes (10). An increasing number of previous studies have demonstrated that miRNAs are dysregulated in approximately all human cancer types, and are involved in the regulation of critical cellular behaviors, including cell proliferation, cycle progression, apoptosis, differentiation, invasion, metastasis, angiogenesis and metabolism (11-13). Numerous miRNAs, including miR-21 (14), miR-143 (15), miR-498 (16) and miR-613 (17), have been identified to be aberrantly expressed in RB. The dysregulation of miRNAs may serve tumor-suppressing or oncogenic roles in the genesis and development of RB, which may be primarily ascribed to the biological functions of their target genes (18,19). With regards to the roles of miRNAs in RB, an improved understanding of the association between miRNAs and RB may lead to the identification of valuable therapeutic targets, improving clinical outcomes.

The expression profile and biological roles of miR-504 have been reported in various types of human cancer (20-22); however, the functions of miR-504 in RB remain unknown. The aim of the present study was to detect miR-504 expression in RB, analyze the roles of miR-504 in the development of RB, and to determine the possible underlying mechanisms.

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## Materials and methods

*Clinical specimens and cell lines*. RB tissues were obtained from 23 patients (age range, 15-34 years; average age, 23 years; 14 males and 9 females) who were diagnosed with RB and treated via surgical resection at the China-Japan Union Hospital of Jilin University (Changchun, China). Normal retinal tissues were collected from seven patients (five males and two females) suffering from globe rupture. Their ages ranged between 28 and 63 years, and the average age was 41 years. All tissues were obtained between March 2015 and January 2017. Patients that had been treated with laser therapy, cryotherapy, thermotherapy, chemotherapy or radiotherapy were excluded from the present study. All tissues were immediately frozen in liquid nitrogen and subsequently stored at -80˚C until further extraction of protein or RNA. The present study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University. Written informed consent was provided by all enrolled patients.

A total of three RB cell lines (Y79, SO-RB50 and Weri-RB1) and the normal retinal pigmented epithelial cell line ARPE-19, used as the control in the present study, were purchased from The American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and grown at 37˚C in a humidified incubator containing 5% $CO_2$.

*Cell transfection*. The miR-504 mimics and negative control miRNA (miR-NC) were chemically synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The miR-504 mimics sequence was 5'-AGACCCUGGUCUGCACUCUAUC-3' and the miR-negative control (NC) sequence was 5'-UUC UCCGAACGUGUCACGUTT-3'. An astrocyte elevated gene-1 (AEG-1)-overexpressing plasmid was constructed using a pcDNA3.1 (+) basic plasmid supplied by Shanghai GenePharma Co., Ltd. (Shanghai, China), and was defined as pcDNA3.1-AEG-1. The restriction sites were *Nhe*I and *Hind*III. An empty pcDNA3.1 vector was used as the control for pcDNA3.1-AEG-1 transfection. Y79 and Weri-RB1 cells were transfected with miRNA mimics (100 pmol) or plasmid (4 $\mu$g) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Co-transfection of miR-504 mimics and pcDNA3.1-AEG-1 was additionally performed using Lipofectamine® 2000. After 8 h, the culture medium was replaced with fresh DMEM containing 10% FBS. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were conducted after 48 and 72 h incubations, respectively. Cell Counting Kit-8 (CCK-8) and cell invasion assays were conducted at 24 and 48 h post-transfection, respectively.

*RT-qPCR*. Total RNA was extracted from tissues or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. For the detection of miR-504 expression, cDNA was produced using the TaqMan® MicroRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The RT temperature protocol was as follows: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. qPCR was subsequently performed using the TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The thermocycling conditions were as follows: 50˚C for 2 min, 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 sec; and annealing/extension at 60˚C for 60 sec. For the quantification of AEG-1 mRNA expression, total RNA was reverse transcribed with the RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The RT temperature protocol was as follows: 37˚C for 5 min, 42˚C for 60 min, 70˚C for 10 min and left on ice. The synthesized cDNA was subjected to qPCR using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.). The thermocycling conditions for qPCR were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. U6 small nuclear RNA and GAPDH were utilized as endogenous controls for miR-504 and AEG-1 mRNA, respectively. All reactions were performed on the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific, Inc.). Each assay was performed in triplicate and repeated three times. The $2^{-\Delta\Delta Cq}$ method was used to analyze gene expression (23). The primers were designed as follows: miR-504, 5'-AGACCCTGGTCTGCACTCTAT-3' (forward) and 5'-GCGAGCACAGAATTAATACGAC-3' (reverse); U6, 5'-CAAGGATGACACGCAAAT-3' (forward) and 5'-GCG AGCACAGAATTAATACGAC-3' (reverse); AEG-1, 5'-TGT TGAAGTGGCTGAGGG-3' (forward) and 5'-CAGGAAATG ATGCGGTTG-3' (reverse); and GAPDH, 5'-CGGAGTCAA CGGATTTGGTCGTAT-3' (forward) and 5'-AGCCTTCTC CATGGTGGTGAAGAC-3' (reverse).

*CCK-8 assay*. Transfected Y79 and Weri-RB1 cells were collected following 24 h incubation, and were inoculated into 96-well plates at a density of 3,000 cells/well. A CCK-8 assay was conducted at four time points: 0, 24, 48 and 72 h following inoculation. At each time point, 10 $\mu$l CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added into each well, and the cells were incubated for an additional 2 h at 37˚C. The absorbance value of each well was detected at a wavelength of 450 nm by a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

*In vitro cell invasion assay*. *In vitro* cell invasion assays were performed in order to measure the invasive ability of RB cells using Transwell chambers (8-$\mu$m pore size) precoated with Matrigel (both from BD Biosciences, San Jose, CA, USA). Transfected Y79 and Weri-RB1 cells were collected following 48 h of incubation at 37˚C, resuspended in FBS-free DMEM and plated into the upper chambers with an initial density of $5x10^4$ cells/chamber. The lower chambers were coated with 500 $\mu$l DMEM containing 20% FBS. After 24 h incubation at 37˚C, cells that had not invaded via the pores were gently removed using a cotton swab, whereas the invasive cells were fixed with 4% paraformaldehyde at 37˚C for 30 min and stained with 0.1% crystal violet at 37˚C for 30 min. Images of the invasive cells were captured and the number of invasive cells was counted in at least five randomly selected visual fields under a light microscope (magnification, x200; Olympus IX83; Olympus Corporation, Tokyo, Japan).

*Target prediction*. TargetScan (Release 7.2; http://www.targetscan.org/vert_71/) and miRanda (Release Last Update: 2010-11-01; http://34.236.212.39/microrna/home.do) were used to predict the putative targets of miR-504.

*Luciferase reporter assay*. For the reporter assay, the plasmids pmirGLO-AEG-1-3'-UTR wild-type (wt) and pmirGLO-AEG-1-3'-UTR mutant (mut), respectively containing the wt and mut miR-504 binding site in the 3'-UTR of AEG-1, were chemically synthesized by Shanghai GenePharma Co., Ltd. Y79 and Weri-RB1 cells were seeded into 24-well plates at a density of $1.0x10^5$ cells/well one night prior to transfection. pmirGLO-AEG-1-3'-UTR wt or pmirGLO-AEG-1-3'-UTR mut was transfected into cells using Lipofectamine® 2000 in the presence of miR-504 mimics or miR-NC. Transfected cells were harvested at 48 h following incubation, and the luciferase activity was measured using the Dual-Luciferase Reporter System (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The firefly luciferase activity of each sample was normalized to that of the *Renilla* luciferase activity.

*Western blot analysis*. The expression of AEG-1 protein was detected by western blot analysis. Radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) was applied to isolate total protein from tissues or cells. Protein expression was quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Equal amounts of total protein (30 $\mu$g) were loaded, separated by 10% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% skimmed milk diluted in Tris-buffered saline containing 0.1% Tween 20 buffer (TBST) at room temperature for 1 h, and further incubated overnight at 4˚C with the following antibodies: Mouse anti-human AEG-1 monoclonal antibody (1:1,000 dilution; cat. no. sc-517220; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-human GAPDH monoclonal antibody (1:1,000 dilution; cat. no. sc-166574; Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were washed three times with TBST and probed with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h. The protein signals were observed using an enhanced chemiluminescence kit (EMD Millipore) according to the manufacturer's protocol. Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was utilized for quantification of densitometry.

*Statistical analysis*. All statistical analysis was performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation from at least three independent experiments. A Student's two-tailed t-test and one-way analysis of variance (ANOVA) was employed to compare the differences between groups. A Dunnett's test was used as a post hoc test following ANOVA. Spearman's correlation analysis was utilized to analyze the association between miR-504 and AEG-1 in RB tissues. P<0.05 was considered to indicate a statistically significant difference.

## Results

*miR-504 expression is reduced in RB tissues and cell lines*. To evaluate the expression status of miR-504 in RB, the expression levels of miR-504 in 23 RB and seven normal retinal tissues were analyzed. The results of RT-qPCR demonstrated that miR-504 expression levels were significantly lower in RB tissues compared with the normal retinal tissues (P<0.05; Fig. 1A). Subsequently, miR-504 expression in three RB cell lines was measured using RT-qPCR. miR-504 expression was significantly downregulated in all three RB cell lines (Y79, SO-RB50 and Weri-RB1) compared with in the normal retinal pigmented epithelial cell line ARPE-19 (P<0.05; Fig. 1B). The results indicated that the dysregulation of miR-504 may be involved in the development and progression of RB.

*miR-504 suppresses cell proliferation and invasion in RB*. To investigate the specific roles of miR-504 in RB, miR-504 mimics or miR-NC were transfected into Y79 and Weri-RB1 cells, which exhibited notably lower miR-504 expression levels compared with SO-RB50 cells. Following transfection, RT-qPCR was utilized to assess the transfection efficiency, which revealed that transfection of miR-504 mimics significantly increased the levels of miR-504 in Y79 and Weri-RB1 cells (P<0.05; Fig. 2A). The results of the CCK-8 assay demonstrated that exogenous miR-504 expression significantly decreased the proliferation of Y79 and Weri-RB1 cells compared with the control (P<0.05; Fig. 2B). *In vitro* cell invasion assays were subsequently employed to determine the effect of miR-504 upregulation on the invasive ability of RB cells. It was demonstrated that miR-504 restoration was able to significantly suppress the invasion of Y79 and Weri-RB1 cells compared with the control (P<0.05; Fig. 2C). From the CCK-8 assay, it was observed that the inhibitory effects of miR-504 on RB cell proliferation had no statistical significance at 24 h following incubation. *In vitro* cell invasion assays were performed in Y79 and Weri-RB1 cells following 24 h incubation. Thus, it was proposed that the reduced invasive potential may be due to anti-invasive mechanisms rather than reduced cell number. The results demonstrated that miR-504 may exert tumor-suppressor activity in RB.

*AEG-1 is a direct target of miR-504 in RB cells*. To understand the mechanisms underlying the proliferation and invasion suppression by miR-504 in RB cells, bioinformatics analysis was applied to determine potential miR-504 targets. As presented in Fig. 3A, it was identified that the 3'-UTR of AEG-1 contains a binding site for miR-504. AEG-1 was selected for further experimental verification as the gene has been reported to be closely associated with the genesis and development of RB (24). A luciferase reporter assay was adopted to confirm whether miR-504 could directly target the 3'-UTR of AEG-1. It was observed that miR-504 upregulation significantly attenuated the luciferase activity of the plasmid containing wt AEG-1 3'-UTR in Y79 and Weri-RB1 cells compared with the control (P<0.05); however, the luciferase activity of the plasmid harboring the mutated binding site was markedly unaffected (Fig. 3B). Via RT-qPCR and western blot analysis, AEG-1 mRNA and protein levels were examined in Y79 and Weri-RB1 cells transfected with miR-504 mimics or miR-NC;

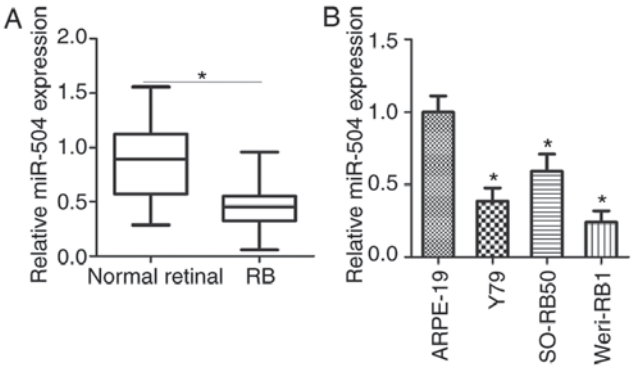


Figure 1. miR-504 is downregulated in RB tissues and cell lines. (A) Relative miR-504 expression was analyzed by RT-qPCR in 23 RB and seven normal retinal tissues. *P<0.05 vs. normal retinal tissues. (B) Expression levels of miR-504 in RB cell lines and the normal retinal pigmented epithelial cell line ARPE-19 were determined using RT-qPCR. *P<0.05 vs. ARPE-19. Results are presented as the mean ± standard deviation. miR, microRNA; RB, retinoblastoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

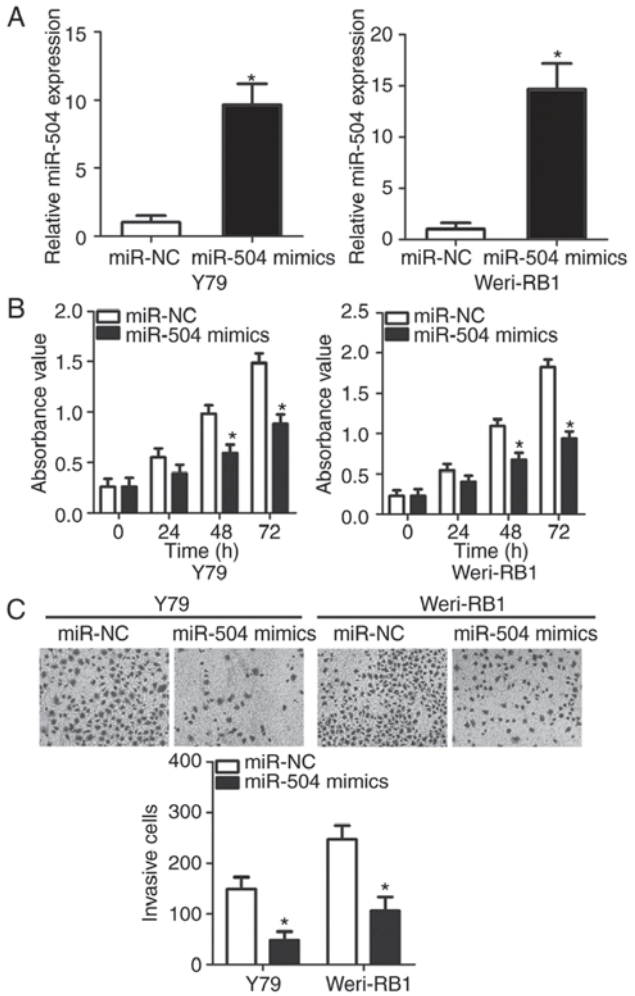


Figure 2. Overexpression of miR-504 inhibits the proliferation and invasion of RB cells. (A) Y79 and Weri-RB1 cells were transfected with miR-504 mimics or miR-NC. Reverse transcription-quantitative polymerase chain reaction analysis was performed to quantify the efficiency of the miR-504 mimics transfection in Y79 and Weri-RB1 cells. *P<0.05 vs. miR-NC. (B) Cell Counting Kit-8 and (C) *in vitro* cell invasion assays were utilized to detect the proliferation and invasion of cells (magnification, x200). *P<0.05 vs. respective miR-NC. Results are presented as the mean ± standard deviation. miR, microRNA; NC, negative control.

whether miR-504 affects endogenous AEG-1 expression was also investigated. Overexpression of miR-504 significantly suppressed AEG-1 mRNA (P<0.05; Fig. 3C) and protein (P<0.05; Fig. 3D) expression levels in Y79 and Weri-RB1 cells compared with the control. The reported results indicated that miR-504 directly targeted the 3'-UTR of AEG-1 and inhibited its expression in RB cells.

*Upregulation of AEG-1 in RB tissues and its inverse correlation with miR-504*. AEG-1 was predicted as a direct target gene of miR-504 in RB. Thus, AEG-1 expression was detected in RB tissues, and a potential association between miR-504 and AEG-1 expression was evaluated. The data from RT-qPCR analysis revealed that the mRNA expression levels of AEG-1 were significantly higher in RB tissues compared with normal retinal tissues (P<0.05; Fig. 4A). Spearman's correlation analysis was performed to further demonstrate the association between miR-504 and AEG-1 mRNA expression in RB tissues. The analysis revealed a negative correlation between miR-504 and AEG-1 mRNA levels in RB tissues (r=-0.5463; P=0.0070; Fig. 4B), suggesting that the upregulation of AEG-1 in RB tissues may be partly attributed to miR-504 downregulation.

*AEG-1 restoration rescues the suppressive effects induced by miR-504 overexpression in RB cells*. In order to demonstrate that miR-504 inhibits RB cell proliferation and invasion by suppressing AEG-1 expression, whether the reported effects could be rescued by upregulating AEG-1 was investigated. Y79 and Weri-RB1 cells were co-transfected with miR-504 mimics and AEG-1 overexpression plasmid (pcDNA3.1-AEG-1) or empty pcDNA3.1, and the transfected cells were subsequently subjected to a series of functional assays. Western blot analysis confirmed that co-transfection with pcDNA3.1-AEG-1 successfully eliminated the miR-504 overexpression-induced inhibition of AEG-1 protein expression in Y79 and Weri-RB1 cells (P<0.05; Fig. 5A). Subsequently, CCK-8 and *in vitro* cell invasion assays demonstrated that the tumor-suppressing roles of miR-504 in Y79 and Weri-RB1 cell proliferation (P<0.05; Fig. 5B) and invasion (P<0.05; Fig. 5C) were mitigated by AEG-1 overexpression. Collectively, the results of the present study suggest that miR-504 exerts its antitumor effects in RB, at least partly, via the negative regulation of AEG-1.

## Discussion

The dysregulation of miRNAs has been increasingly reported as a primary feature of RB (18,19). Notably, miRNAs have been demonstrated to be involved in the occurrence and development of RB, and thus have been proposed as potential therapeutic targets to treat patients with RB (16,25,26). Therefore, it is crucial to investigate the expression profile and detailed roles of miRNAs in RB to identify potential therapeutic targets. In the present study, miR-504 expression was significantly downregulated in RB tissues and cell lines. Additionally, functional experiments revealed that overexpression of miR-504 led to a significant decrease in the proliferation and invasion of RB cells *in vitro*. Furthermore, AEG-1 was demonstrated to be a direct target gene of miR-504 in RB. Its expression was significantly upregulated in RB tissues, and negatively correlated

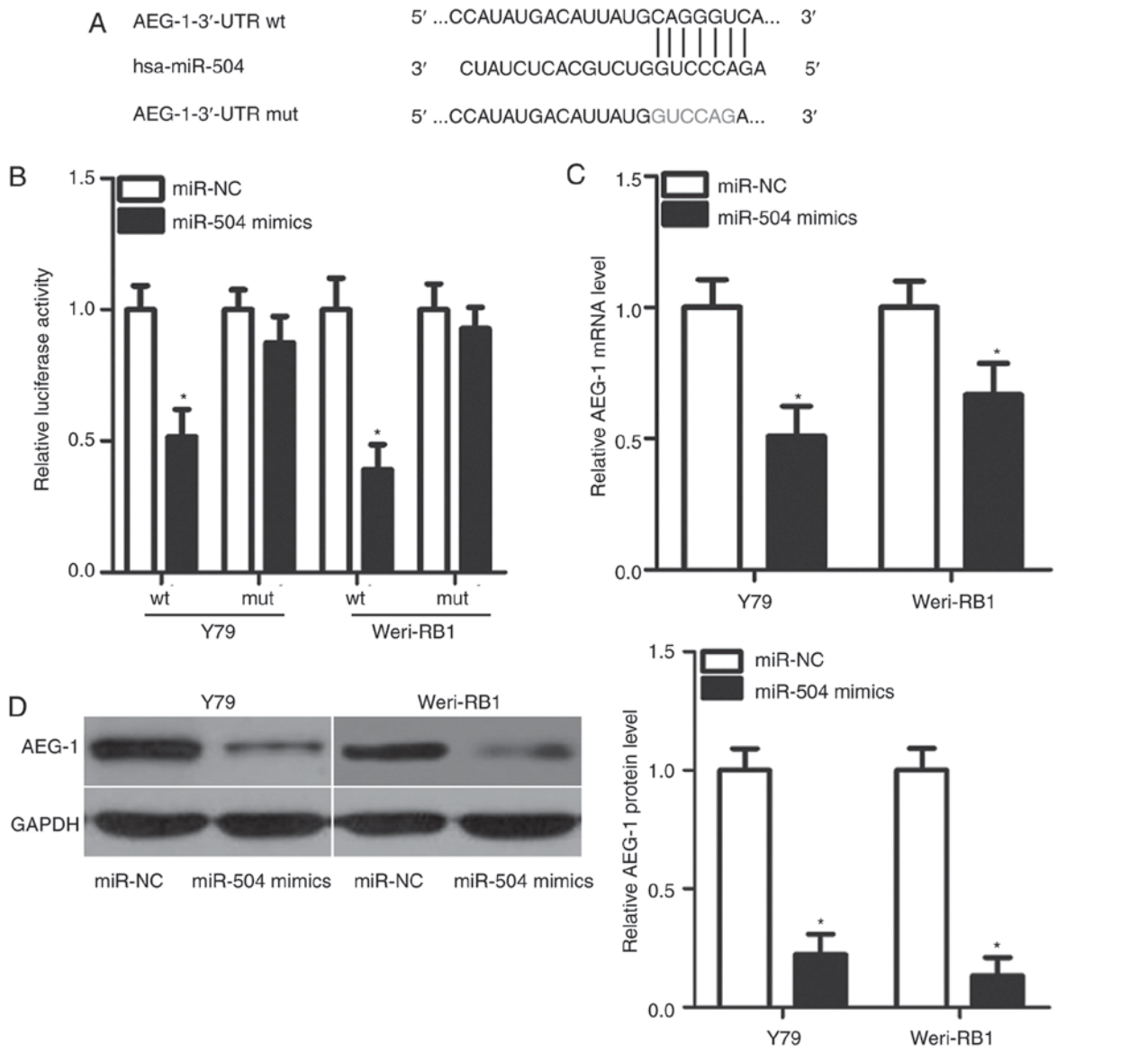


Figure 3. miR-504 decreases AEG-1 expression in RB cells by directly targeting its 3'-UTR. (A) Potential wt and mut miR-504 binding sites in the 3'-UTR of AEG-1. (B) Y79 and Weri-RB1 cells were co-transfected with miR-504 mimics or miR-NC and wt or mut reporter plasmids. The luciferase reporter assay was conducted to determine whether miR-504 was able to directly target the 3'-UTR of AEG-1. *P<0.05 vs. miR-NC. (C) mRNA and (D) protein expression of AEG-1 in Y79 and Weri-RB1 cells transfected with miR-504 mimics or miR-NC was measured by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. *P<0.05 vs. respective miR-NC. Results are presented as the mean ± standard deviation. AEG-1, astrocyte elevated gene-1; miR, micro RNA; mut, mutant; NC, negative control; UTR, untranslated region; wt, wild-type.

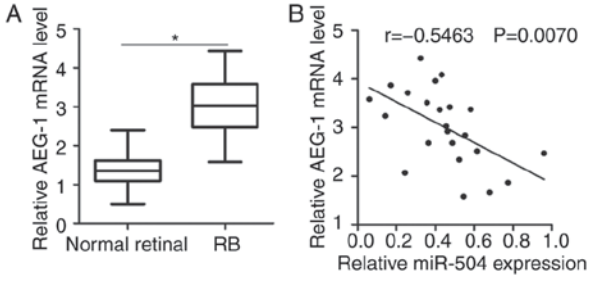


Figure 4. Negative correlation between miR-504 and AEG-1 mRNA expression levels in RB tissues. (A) mRNA expression levels of AEG-1 in 23 RB and seven normal retinal tissues were measured by reverse transcription-quantification polymerase chain reaction. *P<0.05. Results are presented as the mean ± standard deviation. (B) Spearman's correlation analysis was used to determine the association between miR-504 and AEG-1 mRNA expression levels in RB tissues. r=-0.5463; P=0.0070. AEG-1, astrocyte elevated gene-1; miR, microRNA; RB, retinoblastoma.

with miR-504 expression levels. Additionally, rescue experiments revealed that the tumor-suppressing roles of miR-504 in RB cells could be successfully mitigated by upregulating AEG-1. These results suggest that miR-504 inhibited the proliferation and invasion of RB cells by directly targeting AEG-1. Therefore, miR-504 may be considered as a potential therapeutic target for the treatment of RB.

miR-504 has been identified as dysregulated in numerous types of human cancer. For example, miR-504 was significantly downregulated in glioma tissues and cell lines (20,27). Low miR-504 expression was observed to be correlated with aggressive clinicopathological factors (20,27). In addition, Kaplan-Meier survival analysis demonstrated that patients with glioma and low expression levels of miR-504 demonstrated significantly lower survival rates compared with patients with high miR-504 expression levels (20). Additionally, Cox

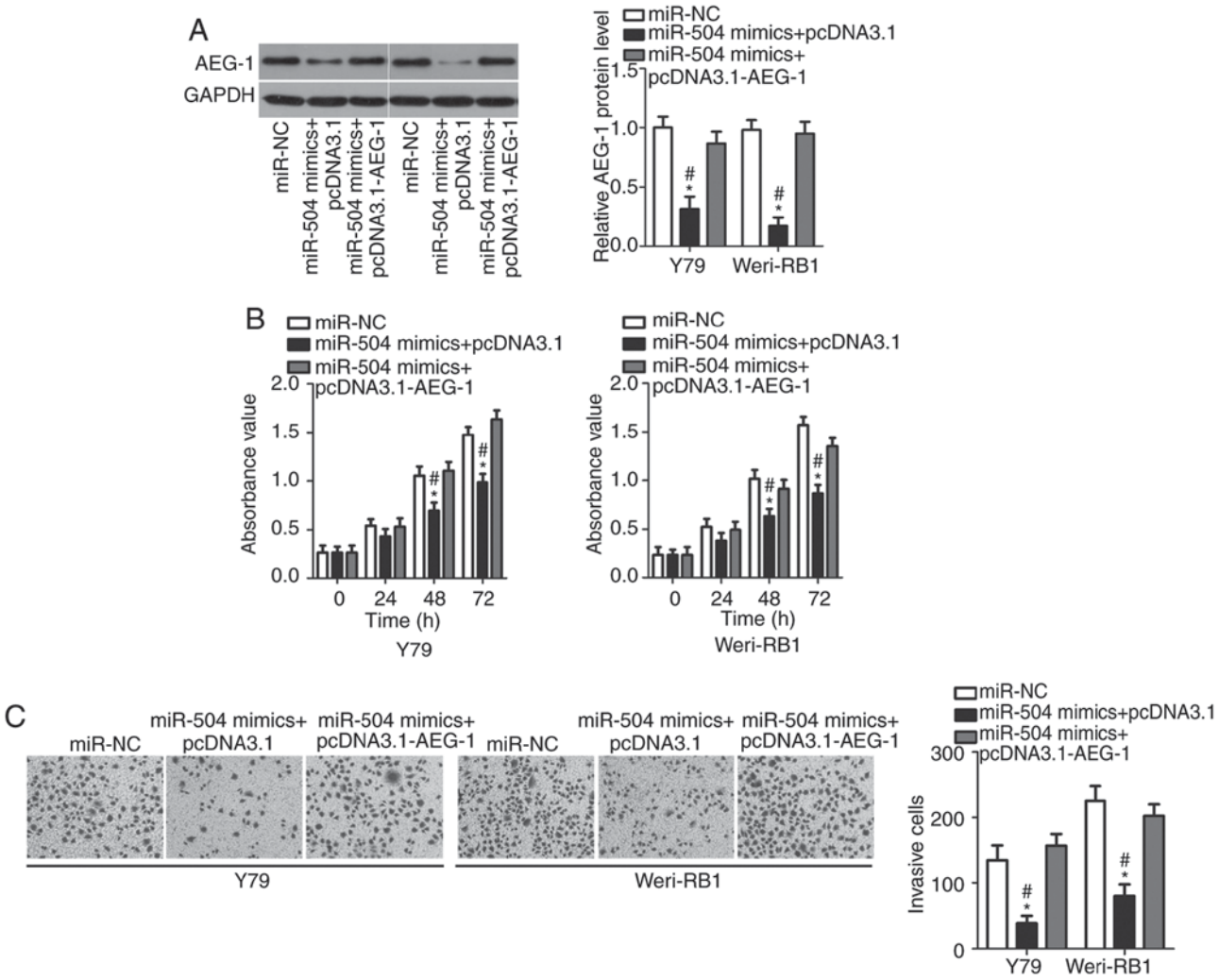


Figure 5. Restoring AEG-1 expression eliminates the inhibitory effects induced by miR-504 overexpression in RB cells. (A) Y79 and Weri-RB1 cells were transfected with miR-504 mimics, and co-transfected with AEG-1 overexpression plasmid (pcDNA3.1-AEG-1) or empty pcDNA3.1. After 72 h, western blot analysis was conducted to determine the protein expression levels of AEG-1. *P<0.05 vs. miR-NC; #P<0.05 vs. miR-504 mimics + pcDNA3.1-AEG-1. (B) Proliferative and (C) invasive abilities of the aforementioned cells were analyzed by Cell Counting Kit-8 and *in vitro* cell invasion assays (magnification, x200), respectively. *P<0.05 vs. miR-NC; #P<0.05 vs. miR-504 mimics + pcDNA3.1-AEG-1. Results are presented as the mean ± standard deviation. AEG-1, astrocyte elevated gene-1; miR, microRNA; NC, negative control.

regression analysis identified miR-504 expression as an independent prognosis-predicting factor for patients with malignant glioma (20). miR-504 downregulation was additionally detected in hypopharyngeal squamous cell carcinoma (21) and oral squamous cell carcinoma (22); however, miR-504 was highly expressed in nasopharyngeal carcinoma-radioresistant cell lines (28). The serum levels of miR-504 in patients with nasopharyngeal carcinoma were upregulated during different weeks of radiotherapy, and correlated with tumor, lymph node and metastasis stages, and total tumor volume (28). In addition, patients with nasopharyngeal carcinoma and high miR-504 expression levels exhibited a relatively lower therapeutic effect ratio of complete response but a higher ratio of partial response compared with patients with low miR-504 expression (28). These findings suggest that the expression profile of miR-504 may exhibit tissue specificity and may be an effective diagnostic and prognostic marker in these types of cancer.

The abnormal expression of miR-504 contributes to the malignant phenotype of numerous types of human cancer. For instance, Cui *et al* (27) identified that miR-504 upregulation suppressed glioma cell proliferation, induced cell cycle arrest and promoted apoptosis. Kikkawa *et al* (21) demonstrated that miR-504 overexpression inhibited cell proliferation and induced cell cycle arrest in hypopharyngeal squamous cell carcinoma. Soutto *et al* (29) observed that trefoil factor 1 attenuated gastric cancer cell proliferation, increased apoptosis *in vitro* and decreased growth *in vivo* via miR-504-mediated p53 activation. Yang *et al* (22) reported that the miR-504/forkhead box protein P1 (FOXP1) axis contributed to the tumor-suppressing roles in oral squamous cell carcinoma induced by connective tissue growth factor. These findings suggest that miR-504 may be investigated as a potential therapeutic target in antitumor treatment.

Numerous targets of miR-504 have been identified, including FOXP1 (27) in glioma, cell division protein kinase 6 (21) in hypopharyngeal squamous cell carcinoma and neuropilin 1 (28) in nasopharyngeal carcinoma. AEG-1, also known as metadherin, was revealed as a direct and functional target of miR-504 in RB. It is located on chromosome 8q22 and is highly expressed in various types of human malignancy, including thyroid cancer (30), glioma (31), colorectal

cancer (32), hepatocellular carcinoma (33) and cervical cancer (34). AEG-1 expression is upregulated in RB tissues and cell lines, and is strongly correlated with the tumor stage of patients with RB (24). AEG-1 promotes oncogene activity in the genesis and progression of RB via deactivation of the extracellular signal-related kinase signaling pathway (24). These findings suggest that suppression of AEG-1 may be a potential therapeutic method to treat patients with RB.

In conclusion, the results of the present study provided novel evidence to support the tumor-suppressive roles of miR-504 in RB. Additionally, AEG-1 was reported as a novel direct target of miR-504 associated with the pathophysiology of RB. On the basis of these results, it was proposed that the miR-504/AEG-1 pathway may be an innovative therapeutic target in the treatment of RB; however, the effects of miR-504 on RB tumor growth and metastasis were not investigated *in vivo*. Therefore, this is a limitation of the present study and requires investigation in future studies.

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**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

**Authors' contributions**

LingW and LinaW designed the present study. LinaW and XL performed the reverse transcription-quantitative polymerase chain reaction assays, western blot analysis and the Cell Counting Kit-8 assay. YM and FW conducted the *in vitro* cell invasion and luciferase reporter assays. LingW analyzed the data of the present study. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, China), and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of China-Japan Union Hospital of Jilin University. Written informed consent was obtained from all patients for the use of their clinical tissues.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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