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Original Article

miR-145 suppressed human retinoblastoma cell proliferation and invasion by targeting ADAM19

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Abstract: MicroRNAs (miRNAs) play critical roles in retinoblastoma (RB) initiation and progression, aberrant expression of miR-145 had been frequently reported in cancer studies. However, the role and mechanism of its function in RB is still unclear. In this study, our data showed that miR-145 was downregulated in RB tissues and cell lines. Overexpression of miR-145 suppressed RB cell proliferation, migration and invasion in vitro. ADAM19 was identified as a direct target of miR-145. Silencing of ADAM19 significantly inhibited RB cell proliferation, migration and invasion. In addition, a reverse correlation between miR-145 and ADAM19 expression was noted in RB tissues. Taken together, these findings suggested that miR-145 functions as a tumor suppressor in RB by directly targeting ADAM19. miR-145 could be an anticancer therapeutic target for RB patients.

Keywords: Retinoblastoma, miR-145, ADAM19

Introduction

Retinoblastoma (RB) is the most common primary intraocular malignancy usually occurring in childhood [1]. The mortality rate among children diagnosed with RB is 50-70% in the underdeveloped countries [2]. Children with RB are at risk for three life-threatening problems, including metastasis of RB, intracranial neuroblastic malignancy, and second primary tumors [3]. Therefore, it is of great significance to elucidate the molecular mechanisms of RB and look for new therapeutic strategies for this malignancy.

MicroRNAs (miRNAs) are small, non-coding, 19-25 nucleotide RNAs that regulate gene expression by binding to the 3'-UTR of their target mRNA molecules to repress their transcription or induce mRNA degradation [4]. MiRNAs play important roles in a range of biological processes, such as differentiation, development, proliferation and metabolism [5, 6]. In addition, accumulating evidence showed that miRNAs can function as oncogenes or tumor suppressors and contribute to cancer development [7]. For example, Cui et al showed that miR-186 suppressed the growth and metastasis of non-small cell lung cancer cells through targeting ROCK1 [8]. Li et al reported that miR-720 inhibited breast cancer cell invasion and migration by targeting TWIST1 [9]. Xu et al found that miR-374a promoted gastric cancer cell proliferation, migration and invasion by targeting SRCIN1 [10]. These findings suggested that targeting the miRNAs may potentially lead to a novel strategy of diagnose and therapy to the malignancy.

In the present study, we investigate the potential role of miR-145 in the progression of RB. Our results showed that miR-145 was significantly downregulated in RB tissues and cell lines, up-regulation of miR-145 could inhibit RB cell proliferation, migration and invasion in vitro. We experimentally confirmed that miR-145 targets the 3'-UTR of ADAM19. And silencing of ADAM19 significantly inhibited RB cell proliferation, migration and invasion. In addition, a reverse correlation between miR-145 and ADAM19 expression was noted in RB tissues. Thus, our data suggested that miR-145 may represent a therapeutic target for RB treatment.

Materials and methods

Cell lines and patient samples

The human RB cell lines (Y79, Weri-Rb1, and SO-RB50) were purchased from the Institute of

Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. 18 freshly frozen retinoblastoma samples and 6 normal retina samples were collected from Shanghai Tenth People's Hospital. (Normal retina sample was obtained from a pediatric ruptured globe). This study was approved by the institute research ethics committee of Shanghai Tenth People's Hospital.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissue samples and cell lines using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The concentration and quality of total RNA were assessed via spectrophotometric and electrophoretic assay. First-strand cDNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen). Real-time PCR was carried out in the ABI PRISM 7300 system (Applied Biosystems) using the SYBR Premix Ex Taq™ (TaKaRa). Relative expression levels of miR-145 and ADAM19 were calculated by the $2^{-\Delta\Delta CT}$ method after normalization with reference to expression of U6 and GAPDH.

Transfection

Human RB cell lines (Y79) were transfected with miR-145 mimic, miR-NC, si-ADAM19 and si-NC (RiboBio) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 48 h after transfection, cells were harvested for Western blot or qRT-PCR analyses.

Luciferase reporter assay

HEK-293T cells were cotransfected with 20 μM of either miR-145 mimics or the miR-NC and 500 ng of psiCHECK2-ADAM19 3'-UTR-Wt or psiCHECK-2-ADAM19 3'-UTR-Mut. Cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was detected using a GloMax fluorescence reader (Promega). A psiCHECK-2 vector that provides constitutive expression of Renilla luciferase was cotransfected as an internal control.

Cell proliferation assays

To evaluate cell proliferation, 2000 RB cells per well were plated into wells of 96-well plates and transfected with miR-145 mimics or miR-NC. At 24, 48, 72 and 96 h after transfection, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, -diphenyl-tetrazolium bromide (MTT) assay following the manufacturer's protocol.

Cell migration and invasion assays

Migration and invasion were examined using a transwell chamber (Millipore). For the migration assay, $1x10^5$ transfected cells were plated into the upper chamber, and cultured in RPMI 1640, while RPMI 1640 with 10% FBS was added to the lower chamber. After 24 h incubation at 37°C, cells remaining on the upper surface of membrane was removed, and membrane was stained with 20% methanol and 0.5% crystal violet. Cell images were obtained using an inverted microscope (Olympus). For invasion assays, the upper chamber was precoated with Matrigel (BD).

Western blot

Cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and phenylmethylsulfonylfluoride (Roche). Fifty micrograms of protein extracts were separated by 10% SDS-PAGE, transferred to 0.22 mm nitrocellulose membranes (Sigma) and incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). The ADAM9 and GAPDH antibody was purchased from Abcam.

Statistical analysis

All data were presented as mean ± SD and analyzed by using SPSS 18.0. Two-tail Student's t test and ANOVA were performed to determine the differences. P Values < 0.05 was considered significant.

Results

Expression of miR-145 is downregulated in human RB tissues and cell lines

qRT-PCR was performed to examine the expression levels of miR-145 in a subset of 18 human

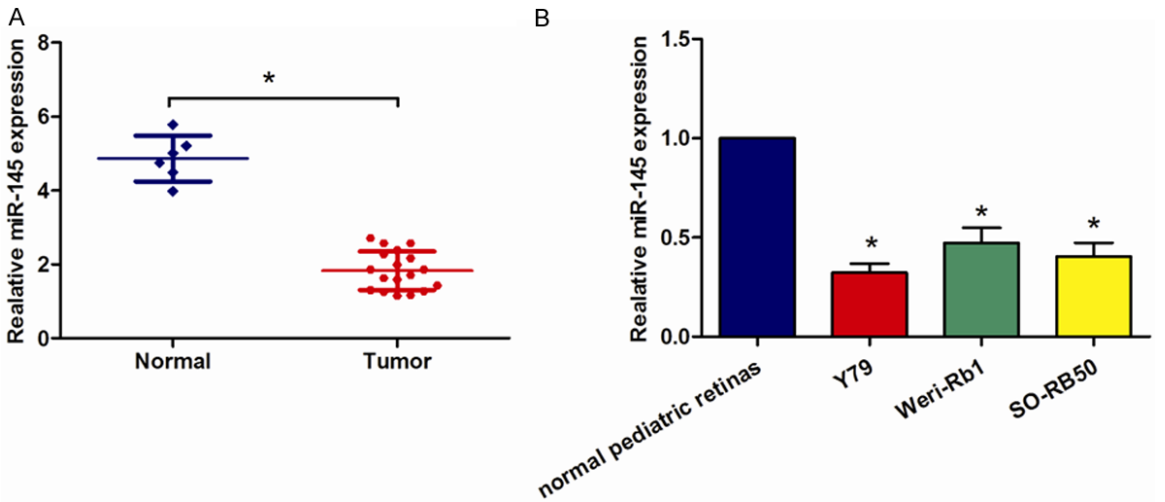

Figure 1. miR-145 is down-regulated in human retinoblastoma. A. miR-145 expression was significantly down-regulated in RB tissues as compared with normal pediatric retinas. B. miR-145 expression was higher in human normal pediatric retinas than in RB cell lines. *P < 0.05.

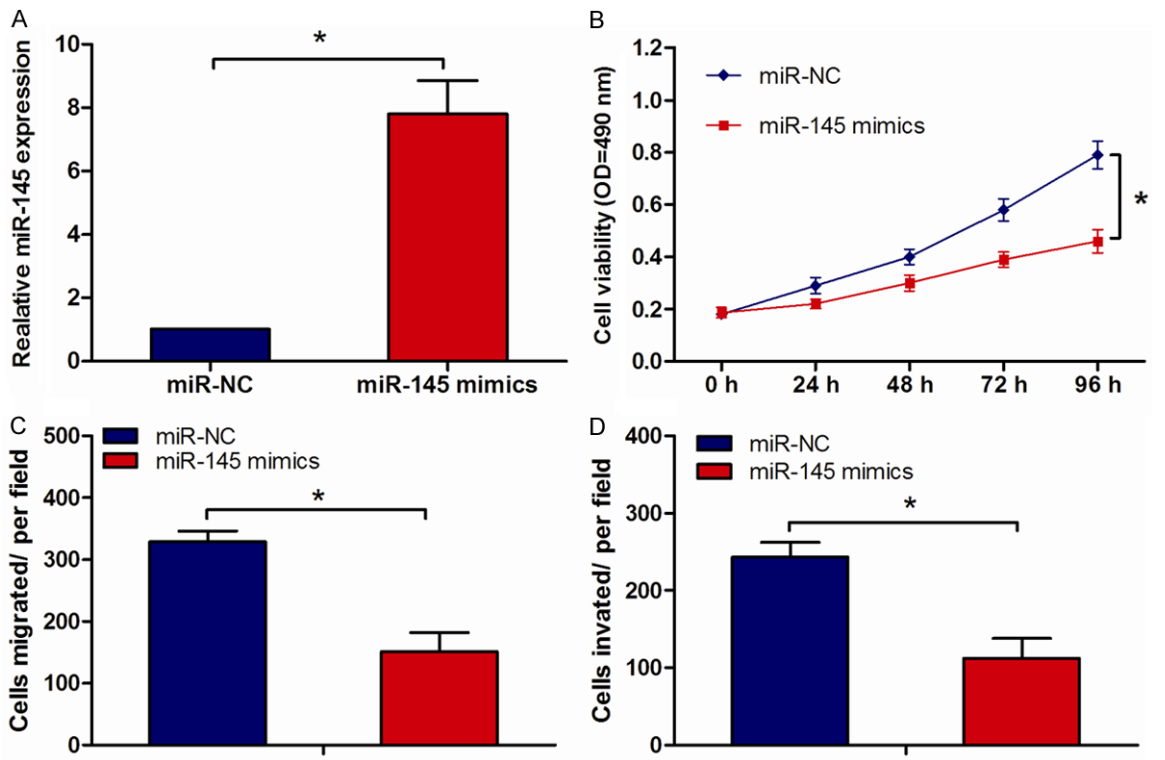

Figure 2. miR-145 suppresses cell proliferation, migration and invasion in vitro. A. The relative expression level of miR-145 in Y79 cells transfected with miR-145 mimics or miR-NC was determined by qRT-PCR. B. miR-145 inhibited Y79 cell proliferation, cell proliferation were determined by MTT assay. C. miR-145 decreased migration ability of Y79 cells, cell migration ability were determined by transwell migration assay. D. miR-145 suppressed invasion ability of Y79 cells, cell invasion ability were determined by transwell invasion assay. *P < 0.05.

RB tissues as compared to 6 normal pediatric retinas. Our results showed that the expression levels of miR-145 were significantly downregulated in RB tissues compared to normal pediatric retinas (**Figure 1A**, P < 0.05). Moreover, we tested the expression of miR-145 in three RB

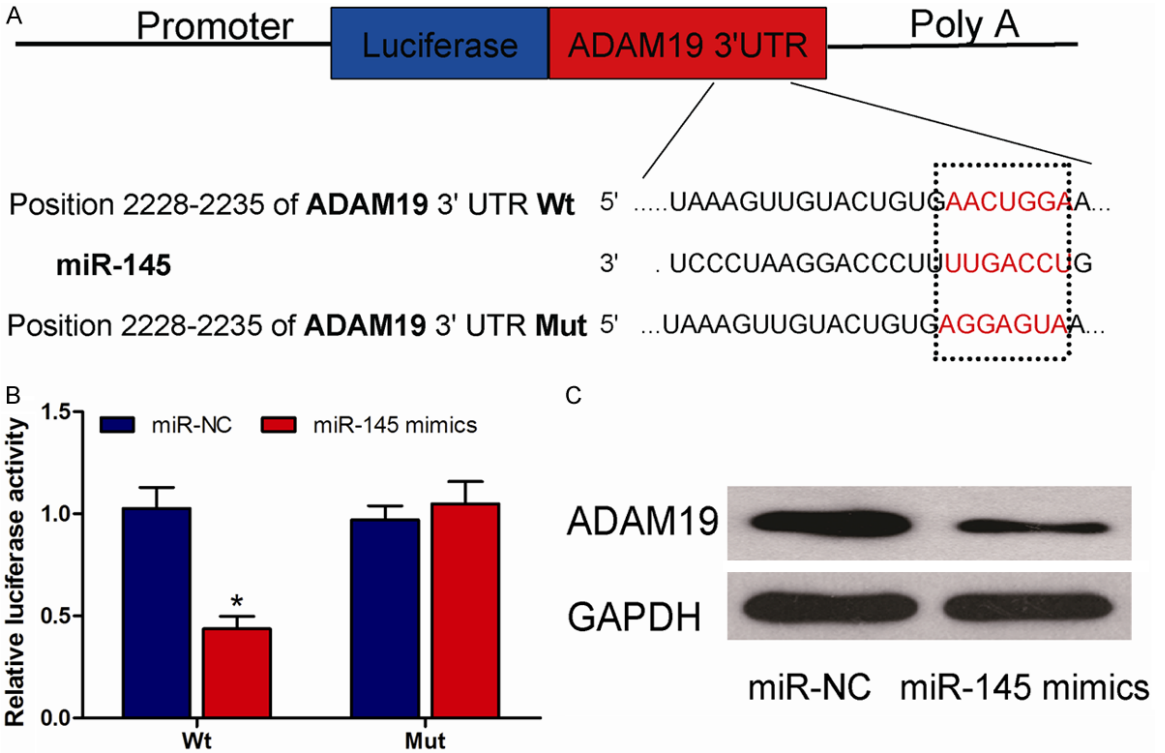

Figure 3. miR-145 directly targets ADAM19. A. Wild-type (Wt) and mutant (Mut) sequences of the putative miR-145 target sequences of ADAM19 3'-UTR. B. miR-145 significantly inhibited the luciferase activity of the ADAM19 Wt 3'-UTR but not that of the Mut in HEK-293T cells. C. Overexpression of miR-145 reduced the ADAM19 protein levels. *P < 0.05.

cell lines (Y79, WERI-RB-1, and SO-RB50). In comparison to 6 normal pediatric retina controls, the expression of miR-145 was decreassed in all these cell lines (**Figure 1B**, P < 0.05).

miR-145 suppresses cell proliferation, migration, and invasion in vitro

To determine the potential role of miR-145 in RB, cell proliferation, migration and invasion assays were performed. Y79 cells were transfected with a miR-145 mimics, and the expression of miR-145 was confirmed by qRT–PCR (**Figure 2A**, P < 0.05). The MTT assay was performed to explore the effect of miR-145 in RB cell proliferation. Our data revealed that overexpression of miR-145 significantly suppressed the proliferation of Y79 cells (**Figure 2B**, P < 0.05). We further investigated the effect of miR-145 on RB cell migration and invasion. Transwell migration assay showed that overexpression of miR-145 dramatically inhibited the migration ability of Y79 cells (**Figure 2C**, P < 0.05). Similarly, transwell invasion assay suggested that miR-145 markedly inhibited the invasive capacity of Y79 cells (**Figure 2D**, P < 0.05).

ADAM19 is a direct target of miR-145 in RB cells

To further explore the mechanism by which miR-145 suppresses RB cell proliferation, migration and invasion, we conducted a bioinformatics screen to identify potential downstream target genes. ADAM19 was predicted to be a potential target of miR-145 (**Figure 3A**). Luciferase activity assay showed that miR-145 significantly inhibited the luciferase activity of the ADAM19 Wt 3'-UTR but not that of the mutant in HEK293 cells (**Figure 3B**, P < 0.05). Moreover, overexpression of miR-145 significantly suppressed ADAM19 protein expression (**Figure 3C**, P < 0.05). Taken together, these data indicated that ADAM19 was a direct target of miR-145 in RB cells.

ADAM19 inhibition results in similar effects as miR-145 overexpression

We further investigated whether ADAM19 silencing by siRNA could induce effects on RB

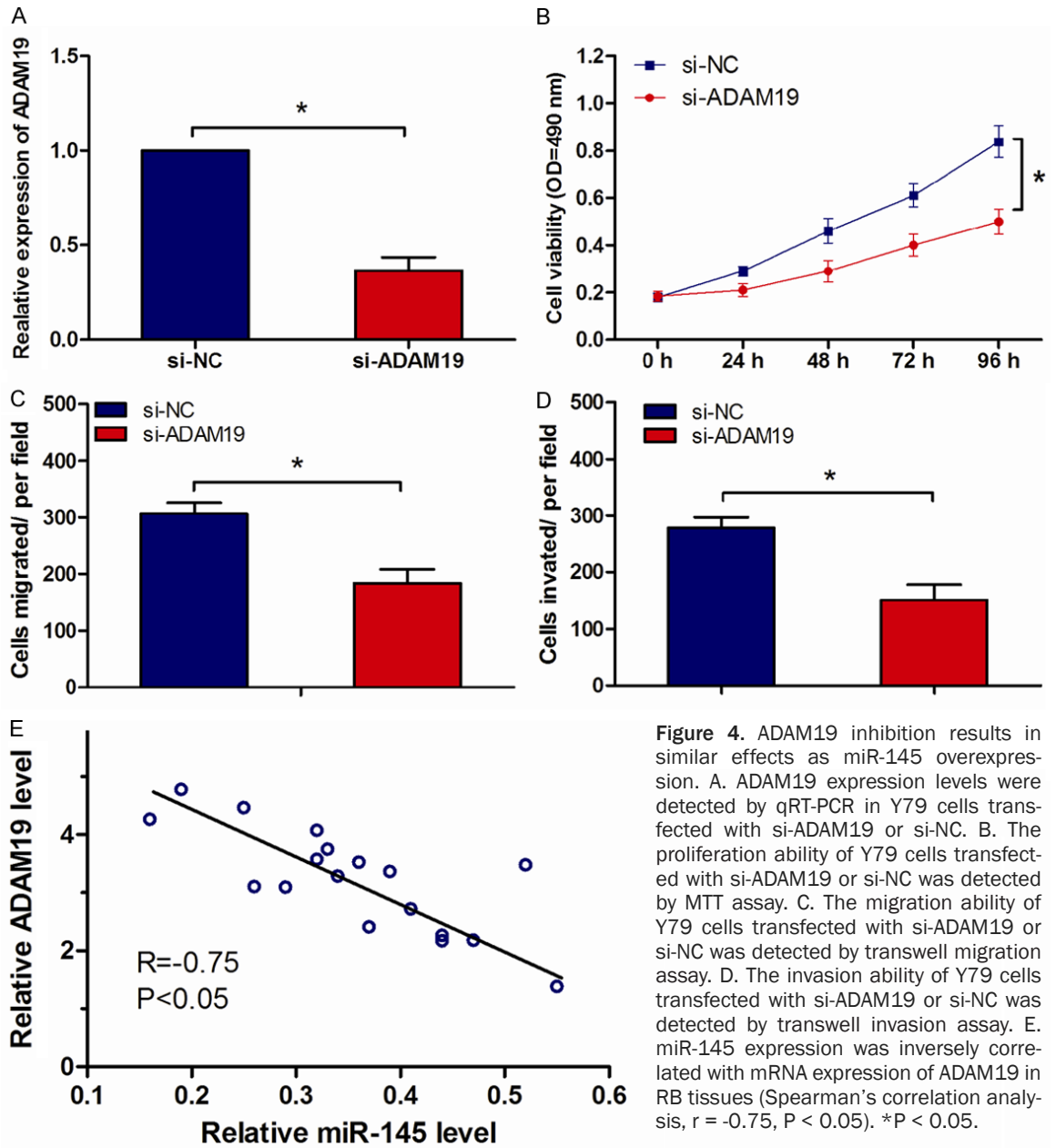

Figure 4. ADAM19 inhibition results in similar effects as miR-145 overexpression. A. ADAM19 expression levels were detected by qRT-PCR in Y79 cells transfected with si-ADAM19 or si-NC. B. The proliferation ability of Y79 cells transfected with si-ADAM19 or si-NC was detected by MTT assay. C. The migration ability of Y79 cells transfected with si-ADAM19 or si-NC was detected by transwell migration assay. D. The invasion ability of Y79 cells transfected with si-ADAM19 or si-NC was detected by transwell invasion assay. E. miR-145 expression was inversely correlated with mRNA expression of ADAM19 in RB tissues (Spearman's correlation analysis, r = -0.75, P < 0.05). *P < 0.05.

cells similar to those caused by the overexpression of miR-145. si-ADAM19 or si-NC was transiently transfected into Y79 cells, qRT-PCR was used to confirm the expression of ADAM19 (**Figure 4A**, $P < 0.05$). The inhibition of ADAM19 by siRNA significantly suppressed the proliferation, migration and invasion of Y79 cells (**Figure 4B-D**, $P < 0.05$). Furthermore, we explored the mRNA expression of ADAM19 in the 18 cases of RB tissues used before. As shown in **Figure 4E**, when ADAM19 mRNA levels were plotted against miR-145 expression, a significant inverse correlation was observed ($P < 0.05$).

Discussion

In this study, we identified a tumor suppressive role of miR-145 in RB cells. Our findings showed that miR-145 was downregulated in RB tissues and cell lines. Overexpression of miR-145 inhibited RB cell proliferation, migration and invasion. ADAM19 was identified as a target of miR-145 in RB cells. Moreover, our data showed that ADAM19 inhibition result in similar effects as miR-145 overexpression in RB cells. And a reverse correlation between miR-145 and ADAM19 expression was noted in RB tissues.

Accumulating evidence revealed the important function of miRNAs in RB progression [11]. For example, Lei et al showed that miR-101 could function as a tumor suppressor in human RB cells by targeting EZH2 [12]. Wang et al suggested miR-183 suppressed RB cell growth, invasion and migration by targeting LRP6 [13]. Chen et al revealed that miR-338-3p could suppress RB cell proliferation, invasion and migration through targeting PREX2a [14]. Recent studies showed that miR-145 could act as a tumor suppressor in some cancers. For example, Xiong et al reported that miR-145 could functions as a tumor suppressor by targeting NUAK1 in human intrahepatic cholangiocarcinoma [15]. Zhang et al found that miR-145 suppressed migration and invasion of lung cancer cells via down-regulating FSCN1 expression [16]. Ozen et al showed that miR-145-5p could inhibit proliferation of prostate cancer cells via down-regulating SOX2 expression [17]. Our study further expanded the tumor suppressive role of miR-145 in RB.

ADAM19 is a member of a disintegrin and metalloproteinases (ADAMs), which are involved in various biological functions, such as fertilization, embryonic development, cell adhesion, cell migration, cell signaling, proteolytic shedding and proteolysis [18]. Dysregulation of many ADAM19 has been observed in the regulation of growth factor activities and integrin functions, leading to promotion of cell growth and invasion in human tumors [19]. For example, Shan et al found that decreased expression of ADAM19 could inhibit migration and invasion of non-small cell lung cancer cells [20]. Zhang et al showed that miR-30c suppressed cancer cell growth, migration and invasion by directly targeting ADAM19 which promoted the malignance of colon cancer cells [21]. In our study, we found that ADAM19 could also be regulated by miR-145 in RB, supporting its oncogenic role in RB.

In summary, our study provided experimental evidence that miR-145 suppressed cell proliferation, migration and invasion in RB by directly targeting ADAM19. These findings suggested that miR-145 may provide a potential therapeutic target for RB treatment.

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Disclosure of conflict of interest

None.

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