Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

# miR-140-5p suppresses retinoblastoma cell proliferation, migration, and invasion by targeting CEMIP and CADM3

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**Received** February 7, 2018; **Accepted** April 3, 2018; **Published** May 15, 2018

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**Doi:** http://dx.doi.org/10.14715/cmb/2018.64.6.8



**Abstract:** Retinoblastoma (RB) is a childhood intraocular tumor, affecting millions of patients worldwide. MicroRNA-140-5p (miR-140-5p) was demonstrated to be involved in the tumorigenesis of various human cancers; however, its role in RB remains undetermined. In this study, quantitative real-time PCR (qRT-PCR) and Western blot assays were used to determine the expression levels of miR-140-5p, cell migration-inducing protein (CEMIP), and cell adhesion molecule 3 (CADM3) in RB tissues and cell-lines. The proliferation ability was detected by cell-counting kit 8 (CCK-8), Edu staining, and colony formation assay. The cell cycle and migration and invasion abilities were measured by flow cytometry, wound-healing assay and Transwell assays, respectively. The correlation between miR-140-5p and CEMIP/CADM3 were then confirmed by immunofluorescence (IF) and dual-luciferase reporter assays. The results showed that miR-140-5p expression was significantly decreased; however, CEMIP and CADM3 expression was increased in RB tissues and cells. Overexpression of miR-140-5p inhibited proliferation, migration, and invasion of RB cells. We also found that miR-140-5p inhibited CEMIP and CADM3 expressions in RB cells. In addition, we demonstrated that miR-140-5p might negatively regulate the transcriptional activities of CEMIP and CADM3 by targeting their 3'-UTR. Therefore, we suggested that miR-140-5p could be a potential therapeutic target for the treatment of RB through CEMIP and CADM3.

***Key words:*** MicroRNA-140-5p; Retinoblastoma; Cell migration inducing protein; Cell adhesion molecule 3; Proliferation; Migration and invasion.

## Introduction

MicroRNAs (miRNAs), characterized by short sequence in length, are an important subtype of non-coding RNAs, which accounts for over 98% in the human genome (1, 2). Accumulating researches have shown that miRNAs may regulate the gene expression, including oncogenic genes and tumor suppressor genes, by binding to the 3'-untranslated regions (3'-UTR) of target mRNA (3-5). Aberrant expression of miRNAs was frequently found in many different human tumors (6-8). Therefore, miRNAs are considered to be involved in the pathogenesis of various human cancers, such as breast cancer, lung cancer, and oral cancer (9-11). While the exact role of most miRNAs in human cancers remain unclear, increasing evidences have suggested that miRNAs expression exerts potential significance in the diagnosis and prognosis of human cancers (12-14).

Retinoblastoma (RB), occurring mostly in children under five years old, is one of the most common intraocular malignant cancers worldwide (15, 16). Data has showed that RB accounts for over 9,000 new cases per year worldwide, with approximately 200 new cases in the USA (17, 18). Most of the children could survive this malignant tumor; however, the complications may include removal of the affected eye(s) and vision that will affect the whole life of RB patients (19). Therefore, it is essential to elucidate the underlying mechanisms for effective molecular targets for the diagnosis, treatment, and prognosis of RB. Recently, increasing studies have reported that various miRNAs are involved in the tumorigenesis of RB by regulating the expression of tumor suppressors (20, 21). Extensive researches have shown that miR-140-5p is dysregulated and plays as oncogenic or tumor suppressive role in many human cancers, including breast cancer, gastric cancer, and lung cancer (22-24). However, the potential role of miR-140-5p in RB cells remains unclear.

In this study, we observed that mRNA expression level of miR-140-5p was significantly reduced, whereas cell migration inducing hyaluronan binding protein (CEMIP), and cell adhesion molecule 3 (CADM3) mRNA expression were remarkably increased, in both RB tissues and cell-lines. We then assessed the cor-

relation between miR-140-5p and CEMIP/CADM3 and observed that miR-140-5p overexpression could downregulate the expression of CEMIP and CADM3 in RB cells. In the further functional assays, we found that miR-140-5p overexpression significantly inhibited cell proliferation, migration, and invasion in RB cells. Finally, based on the results of bioinformatics analysis, we found that CEMIP and CADM3 may act as two potential targets of miR-140-5p, and dual-luciferase reporter assay was performed to confirm these results. All these results suggested that miR-140-5p may serve as a tumor suppressor in RB by targeting CEMIP and CADM3.

## Materials and Methods

### Patient samples, cell lines and transfection

Thirty pairs of human RB tissues and normal adjacent tissues were obtained from the Zhejiang Provincial People's Hospital, China. This study was performed after obtaining written informed consent from every RB patients, and all methodologies of this study were approved by the Ethics Committee of the Zhejiang Provincial People's Hospital. The human retinal pigment epithelial (HRPE) cells, human RB cell-lines (Y79 and WERI-Rb-1), and 293T cells were obtained from ATCC (Rockville, Md., USA) and cultured in RPMI 1640 medium (Gibco, NY, USA) containing 100 U/L penicillin/streptomycin and 10% fetal bovine serum (FBS) under the conditions of 95% air and 5% $CO_2$ at 37°C.

For the establishment of miR-140-5p overexpressed cell model, Y79 cells were seeded into 24-well plates overnight and followed by the treatment of 50 nM miR-140-5p mimics via Lipofectamine 2000 reagent (Invitrogen, USA) according to the instructions of manufacturer. Negative control (NC) mimics was used as control group.

### RNA extraction and quantitative real-time PCR (qRT-PCR) assay

Total RNA of RB tissues and cell-lines was prepared by TRIzol reagent (#9109, Takara, Japan). Reverse transcription was performed using Bestar qPCR RT kit (#2220, DBI Bioscience, China), and real-time quantitative PCR amplification was performed using BestarTM qPCR MasterMix (#2043, DBI Bioscience, China) following the protocols provided by manufacturer. The sequence of GAPDH primers are: F, 5'-TGTTCGTCATGGGTGTGAAC-3', R, 5'-ATG GCA TGG ACT GTG GTC AT -3'; CEMIP primers are: F, 5'-GCC AAT CCC AAC AACAAC CT-3', R, 5'-CGG TAG TTG GAA TGT GCT CG-3'; CADM3 primers are: F, 5'-ACT ATG CCT GTG CGA ACT G-3', R, 5'-CGG GTA ACC TGG AAT GTC A-3'; U6 primers are: F, 5'-CTC GCT TCG GCA GCA CA-3', R, 5'-AAC GCT TCA CGA ATT TGC GT-3'; miR-140-5p primers are: F, 5'-ACA CTC CAG CTG GGC AGT GGT TTT ACC CTA-3', R, 5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG CTA CCA TAG G-3'. All the primers were designed and purchased from Sangon, China, and the mRNA expression of miR-140-5p was normalized to U6, and the mRNA expression of CEMIP and CADM3 was normalized to GAPDH.

### Immunohistochemical and immunofluorescence analysis

For immunohistochemical analysis, RB and NAT tissues were fixed in 4% formalin and embedded in paraffin. The tissues were cut into 4 μm thick sections. After dewaxation and rehydration, all the sections were incubated with 10 mM citrate buffer at 100°C for 5 mins for antigen retrieval. The non-specific binding sites were blocked by 10% FBS at room temperature for 2 h. Next, the sections were incubated overnight at 4°C with rabbit polyclonal antibodies against CEMIP (1:50, 21129-1-AP, Proteintech Group, Inc.) or rabbit polyclonal antibodies against CADM3 (1:50, MBS2528459, MyBioSource). After washing with PBS for three times, the sections were incubated with anti-rabbit secondary antibody at room temperature for 2 h and detected by streptavidin-horseradish-peroxidase.

For immunofluorescence analysis, treated RB cells were seeded at 80% confluence onto coverslips for 24 h at 37°C. The cells were fixed with 4% paraformaldehyde for 20 mins followed by the treatment of methanol for 2 mins. Next, the cells were incubated with corresponding primary antibodies against CEMIP (1:50, 21129-1-AP, Proteintech Group, Inc.) or CADM3 (1:50, MBS2528459, MyBioSource). The cells were incubated with secondary antibodies that conjugated to Alexa Fluor-488 (1:200, ab150073, Donkey Anti-Rabbit IgG, Abcam) or Alexa Fluor-647 (1:200, ab150075, Donkey Anti-Rabbit IgG, Abcam) at room temperature for 2 h. Finally, the images were taken with a confocal laser-scanning microscope, and quantitative assay has been performed using Image-Pro Plus (version 6.0, Media Cybernetic, Inc, USA).

### Western Blot assay

Proteins from treated HRPE cells and RB cell-lines (Y79, and WERI-Rb-1) were extract using RIPA buffer followed by ultrasonication on ice. Centrifugation (12,000 rpm/mins, 15 mins) was performed to isolate the supernatant, and a BCA kit (Pierce, Rockford) was used to detect the concentration of proteins. After isolation using 10% SDS-PAGE, the proteins were transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA) under 100 V and low temperature. The membranes were incubated with 5% low fat dried milk at room temperature for 2 h to block non-specific sites. The membranes were incubated with primary goat antibodies against CEMIP (1:50, 21129-1-AP, Proteintech Group, Inc.), CADM3 (1:50, MBS2528459, MyBioSource) and GAPDH (1:10000, sc420485, Satna Cruz) at 4°C overnight. After washing with TBST for three times, the membranes were incubated with horseradish peroxidase conjugated donkey-anti-rabbit secondary antibodies at room temperature for 2 h. Finally, the signals were detected with enhanced chemiluminescent reagents.

### Cell proliferation analysis

Cell proliferation of Y79 cells transfected with miR-140-5p or NC mimics was determined by CCK-8 assay, Edu staining, and colony formation assay. For Cell Counting Kit-8 (CCK-8) assay, treated Y79 cells were seeded into 96-well plates at a concentration of 2,000 cells/per well and incubated at 37°C overnight. A CCK-

8 detection kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to determine cell proliferation at every 24 h for three consecutive days following the instructions of manufacturer.

For Edu staining, treated Y79 cells were processed with a Click-iT Plus EdU Alexa Fluor1594 Imaging Kit (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the instructions of manufacturer. DAPI was used to stain cell nucleus.

For colony formation assay, treated Y79 cells were seeded onto 6-well plates at a concentration of 1,000 cells/well for two weeks. Colonies were fixed with methanol for 30 mins followed by staining with Giemsa solution, and the number of colonies was counted.

### Cell cycle analysis

Twenty-four hours after transfection with the miR-140-5p or NC mimics, Y79 cells were collected and centrifuged at 2,000 rpm for 5 mins and washed with PBS for three times. The cells were fixed with ethanol for 30 mins followed by 250 µL 0.05 g/L propidium iodide (PI) treatment for 30 mins at room temperature. Finally, the cells were analyzed using a flow cytometer (FACSCanto™ II, BD Biosciences).

### Migration and invasion analysis

Wound-healing assay and Transwell assay were performed to assess the effects of miR-140-5p on cell migration and invasion, respectively. For wound-healing assay, transfected Y79 cells were seeded onto 6-well plates at a concentration of $2\times10^4$ cells/well and incubated at 37°C. When the cells reached 80% confluence, a straight scratch was made by a plastic pipette tip, and the cells were cultured for an additional 24 h at 37 °C. Images of these scratch were captured at the time of 0 and 24 h after wounding, and the wound width was determined under a microscope.

For Transwell assay, chambers with 8-µm pores (Corning Incorporated, Corning, NY, USA) and coated with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) were used to examine invasion. Treated Y79 cells ($1\times10^4$) were re-suspended in 0.3 mL of culture medium and added to the upper chambers, then 0.5 mL of culture medium supplemented with 20% FBS was added to the lower chambers. After incubation at 37 °C for 24 h, non-invaded cells were removed and the invaded cells were stained 20 mins with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China). Finally, the invaded cells were imaged and counted using a microscope.

### Plasmid construction and dual luciferase activity assay

For plasmid construction, the fragment of CEMIP or CADM3 containing miR-140-5p binding sites was amplified by PCR with specific primers, respectively, and then inserted into a luciferase vector psi-CHECK (Promega, Madison, USA) and named CEMIP-WT or CADM3-WT. The fragment of CEMIP or CADM3 containing mutant miR-140-5p binding sites was also amplified and inserted into vector to produce CEMIP-Mut or CADM3-Mut plasmids.

For dual luciferase activity assay between CEMIP and miR-140-5p, 293T cells were incubated onto 6-well plates for 24 h and then transfected with CEMIP-WT or CEMIP-Mut plasmids and miR-140-5p or NC mimics using Lipofectamine 2000 (Invitrogen). Luciferase activity was detected by the Dual-Luciferase Reporter Assay System (Promega) according to the protocols of manufacturer. Renilla luciferase activity was normalized to Firefly luciferase activity. The dual luciferase activity assay between CADM3 and miR-140-5p was performed same as CEMIP.

### Statistical Analysis

Data was presented as mean ± SEM, and Graghpad (Ver. Prism 7, GraphPad Prism Software, La Jolla, CA, USA) was used for statistical analysis. Data was subjected to *t*-test or one-way analysis of variance (ANOVA) followed by post-hoc to evaluate the difference between means. Differences between groups were considered significant if $P < 0.05$.

## Results

### Expression levels of miR-140-5p, CEMIP, and CADM3 in RB tissues

To determine the underlying roles of miR-140-5p, CEMIP, and CADM3 in the pathogenesis of RB, we firstly assess the mRNA expression by qRT-PCR in 30 paired RB tissues and normal adjacent tissues (NAT). Results have shown that miR-140-5p mRNA expression was remarkably downregulated in RB tissues than NAT ($P < 0.001$, Figure 1A); however, the relative mRNA expression of CEMIP and CADM3 in RB tissues was significantly increased compared to NAT ($P < 0.001$, Figure 1B, and 1C). In addition, we analyzed the protein expression of CEMIP and CADM3 by immunohistochemical analysis and observed that CEMIP and CADM3 expression was significantly higher in RB tissues than NAT (Figure 1D).

### Effect of miR-140-5p overexpression on CEMIP and CADM3 expression in RB cell-lines

In order to assess the correlation between miR-140-

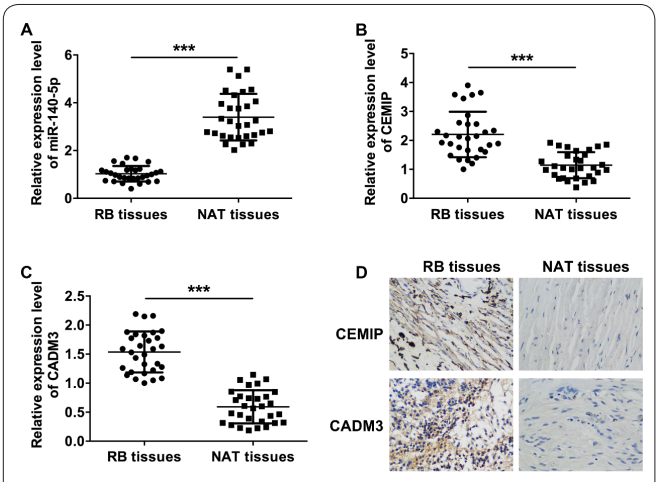


**Figure 1.** Aberrant expression of miR-140-5p, CEMIP, and CADM3 in RB tissues. (A, B and C) The relative mRNA expression of miR-140-5p, CEMIP, and CADM3 was determined by qRT-PCR in 30 paired RB tissues and normal adjacent tissues (NAT) (***$P$<0.001). (D) Immunohistochemical analysis was used to assess the expression of CEMIP and CADM3 in RB tissues and NAT tissues.

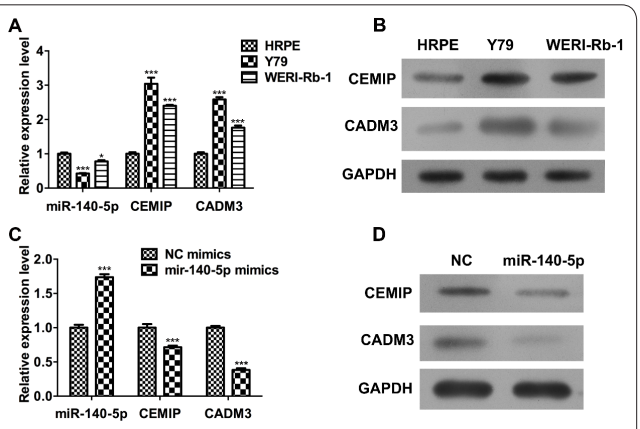


**Figure 2.** Aberrant expression of miR-140-5p, CEMIP, and CADM3 in RB cell lines, and effects of miR-140-5p overexpression on CEMIP, and CADM3 expression. (A) The relative mRNA expression of miR-140-5p, CEMIP, and CADM3 was measured by qRT-PCR in HRPE and RB cell lines: Y79 and WERI-Rb-1(*$P$ < 0.05, ***$P$ < 0.001 *vs*. HRPE cells). (B) The protein expression level of CEMIP and CADM3 was assessed by western blot analysis in HRPE, Y79and WERI-Rb-1 cells. (C) qRT-PCR was performed to evaluated the mRNA expression of miR-140-5p, CEMIP, and CADM3 in miR-140-5p mimics or negative control (NC) mimics treated Y79 cells (***$P$ < 0.001 *vs*. HRPE cells). (D) Western blot analysis was carried out to examined the protein expression of CEMIP and CAD3 in Y79 cells transfected with miR-140-5p or NC mimics.

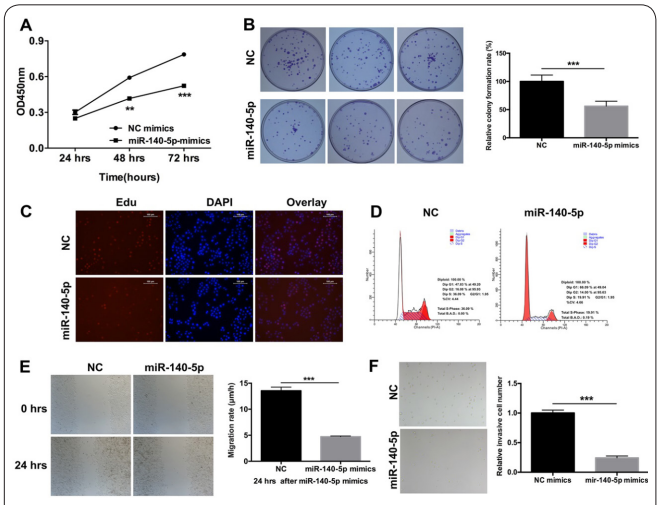


**Figure 3.** Effects of miR-140-5p overexpression on cell proliferation, cycle, migration, and invasion of Y79 cells. (A, B, and C) After transfected with miR-140-5p or NC mimics, RB cells were subjected to proliferation analysis by CCK-8 assay, Edu staining, and colony formation assay (**$P$ < 0.01, ***$P$ < 0.001). (D) The cell cycle of miR-140-5p overexpressed Y79 cells was also determined with flow cytometry analysis. Wound-healing assay (E) and Transwell assay (F) was used to assess the ability of migration and invasion, respectively, in miR-140-5p or NC mimics treated Y79 cells (***$P$ < 0.001).

5p and CEMIP/CADM3, we determined the mRNA and protein expression of CEMIP and CADM3 in the miR-140-5p overexpressed RB cells by qRT-PCR and western blot, respectively. We firstly examined the mRNA expression of miR-140-5p, CEMIP, and CADM3 in the human retinal pigment epithelial (HRPE) cells and RB cell-lines, Y79and WERI-Rb-1. Results from qRT-PCR showed that miR-140-5p mRNA expression was significantly downregulated, whereas CEMIP and CADM3 mRNA expression was remarkably upregulated in Y79 and WERI-Rb-1 cells compared to HRPE cells ($P$ < 0.001, $P$ < 0.05, Figure 2A). Western blot analysis was used to further examine the protein expression of CEMIP and CADM3. The results showed that the protein expression was also significantly increased in RB cell-lines compared to HRPE cells (Figure 2B). We then established a miR-140-5p overexpressed cell model by transfecting Y79 cells with miR-140-5p mimics, and assessed the mRNA and protein expression of CEMIP and CADM3 by qRT-PCR and western blot, respectively, in the miR-140-5p overexpressed Y79 cells. Both the mRNA and protein expression of CEMIP and CADM3 were significantly downregulated in the Y79 cells transfected with miR-140-5p mimics compared to cells transfected with negative control (NC) mimics ($P$ < 0.001, Figure 2C and 2D).

## Effect of miR-140-5p overexpression on cell proliferation, migration, and invasion of RB cells

Since the downregulation of miR-140-5p and upregulation of CEMIP and CADM3 were found in both the RB tissues and cell-lines, we analyzed the underlying functions of miR-140-5p in RB. CCK-8 assay, Edu staining, and colony formation assay were used to detect the effects of miR-140-5p on cell proliferation. The results showed that miR-140-5p overexpression significantly inhibited cell proliferation of Y79 cells ($P$ < 0.01, $P$ < 0.001, Figure 3A, 3B and 3C). Flow cytometry analysis was performed to detect miR-140-5p effects on cell cycle. The results showed a significant decrease in S phase of RB cells transfected with miR-140-5p mimics than cells treated with NC mimics (Figure 3D). In addition, wound-healing and Transwell assays were carried out to evaluate the miR-140-5p roles in migration and invasion of RB cells. The results indicated that the abilities of migration and invasion were significantly attenuated in the miR-140-5p overexpressed RB cells ($P$ < 0.001, Figure 3E and 3F).

## Regulation of CEMIP and CADM3 expression by miR-140-5p

Given that miR-140-5p overexpression regulated CEMIP and CADM3 expression in RB cells, we considered that CEMIP and CADM3 may be two potential targets of miR-140-5p. We firstly confirmed the results that miR-140-5p exerts a regulatory role in the expression of CEMIP and CADM3 in RB cells by immunofluorescence analysis. The results indicated that miR-140-5p overexpression reduced the fluorescence intensity of CEMIP and CADM3 in RB cells (Figure 4A and 4B). CEMIP and CADM3 contained a miR-140-5p binding sites, respectively, according to the bioinformatics analysis via miRDB (Figure 4C and 4D, upper panel). To test whether miR-140-5p could directly target CEMIP and CADM3, dual-luciferase reporter assay was performed in HEK293T cells transfected with either wide-type (WT) or mutated (Mut) CEMIP or CADM3 3'-UTR firefly luciferase constructs and miR-140-5p or NC mimics. HEK293T cells transfected with WT constructs and miR-140-5p mimics significantly attenuated luciferase activity, whereas Mut or NC mimics had no obvious effects ($P$ < 0.05, $P$ < 0.001, Figure 4C

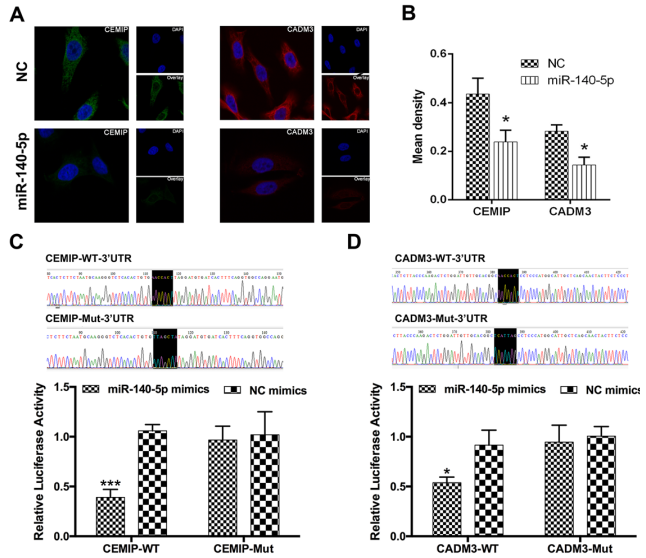


**Figure 4.** CEMIP and CADM3 were the targets of miR-140-5p in Y79 cell line. (A and B) Immunofluorescence analysis was performed to examine the expression of CEMIP and CADM3 in Y79 cells transfected with miR-140-5p or NC mimics, DAPI was used to stain nuclear. Magnification: 120×. (C and D) Luciferase analysis, upper panel showed alignment and base pairing of miR-140-5p with the 3'-UTR of CEMIP and CADM3; Lower panel showed the relative luciferase activity of cells co-transfected CEMIP-WT/CADM3-WT or CEMIP-Mut/CADM3-Mut plasmid with miR-140-3p mimics or NC mimics (*$P < 0.05$, ***$P < 0.001$).

and 4D, lower panel), suggesting that miR-140-5p could decrease CEMIP and CADM3 expression by targeting their 3'-UTR.

## Discussion

It is generally believed that the inactivation of RB gene, which is located on chromosome 13 of humans, is the primary cause of retinoblastoma (25, 26). The RB gene functions as an important tumor suppressor, and aberrant expression of RB gene was found in various human cancers (25). It is well established that miRNAs may participate in the pathogenesis of many human cancers by targeting the tumor suppressive genes. For example, Wang J et al have reported that miR-183 expression was significantly downregulated in both RB tissues and cell-lines, and miR-183 overexpression could inhibit cell growth, migration, and invasion by directly interacting with lipoprotein receptor-related protein 6 (LRP6) (27). Sun Z et al showed that miR-145 expression was also significantly decreased in RB tissues and cell-lines, and overexpression of miR-145 remarkably inhibited cell proliferation, migration, and invasion by targeting ADAM19 in RB cells (28). In addition, miR-17-92 was reported to be upregulated in human RB, and miR-17-92 could promote RB progression by cooperating with RB gene (29). Although miR-140-5p was demonstrated to be involved in the tumorigenesis of various human cancers, the roles of miR-140-5p in RB remains undetermined. In order to evaluate the underlying functions of miR-140-5p in RB, we determined mRNA expression of miR-140-5p by qRT-PCR and found that miR-140-5p mRNA expression was significantly lower in RB tissues and cell-lines. Further, miR-140-5p overexpression inhibited cell proliferation, migration, and invasion, thereby suggesting that miR-140-5p acts as a tumor suppressive agent in RB tumor progression.

Cell migration inducing hyaluronan binding protein (CEMIP), also known as CCSP1 and KIAA1199, is a human protein encoded by CEMIP gene (30, 31). Previous studies have demonstrated that CEMIP overexpression is associated with poor prognosis of patients with various cancers, such as breast cancer, gastric cancer, and colon cancer (32, 33). In addition, knockdown of CEMIP could induce cell apoptosis in various tumor cells, suggesting that CEMIP plays a critical role in cancer progression (34). Cell adhesion molecule 3 (CADM3), a subtype of CAMD family, is a glycoprotein and an important constituent of cell membrane (35). CADM3 plays a critical role in cell-cell and cell-extracellular matrix (ECM) interactions, and the dysfunction of adhesive processes leads to various human diseases, like colorectal cancer, gastric cancer and breast carcinoma (35-37). Unlike miR-140-5p expression, in our study, it was observed that the expression of CEMIP and CADM3 was significantly upregulated in RB tissues and cell-lines. Further, CEMIP and CADM3 may be the two potential targets of miR-140-5p, and miR-140-5p could regulate the expression of CEMIP and CADM3 by directly targeting to them.

In conclusion, downregulated miR-140-5p expression was found in RB, and overexpression of miR-140-5p inhibited RB cell proliferation, migration, and invasion by directly targeting CEMIP and CADM3. Therefore, we suggested that miR-140-5p is involved in the progression and prognosis of RB though inhibition of CEMIP and CADM3. MiR-140-5p could be a potential biomarker for the diagnosis of RB. However, additional studies are required to validate this hypothesis in the prognostic and therapeutic effects of miR-140-5p in RB *in vivo*. Further, studies are required to validate the functions and mechanisms of miR-140-5p in RB.

## Acknowledgments

This work was supported by Wenzhou city technology bureau public welfare projects (Grant No. Y20140619), Natural Science Foundation of Zhejiang Province (Grant No. LQ17H160017), (Grant No. LQ16H070003), Natural Science Foundation of Zhejiang Province (NO:LY15H280003), Traditional Chinese Medicine Scientific Research Foundation of Zhejiang Province (NO:2018ZY001), and Open Foundation from Key Laboratory of Tumor Molecular Diagnosis and Individualized Medicine of Zhejiang Province (NO: ZJZL-SYS003).

## Competing interests

The authors have no commercial or other associations that might pose a conflict of interest.

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