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*Original Article*

# miR-410 promotes the proliferation and migration in retinoblastoma cells via CETN3

Xiaohui Fan[1], Chao Yang[1], Shuxia Fan[2]

*Departments of [1]Ophthalmology, [2]Nursing, Binzhou People's Hospital, Binzhou 256610, Shandong, China*

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**Abstract:** miR-410 is recently recognized as a key player in various cancers. In the present study, we sought to emphasize the mechanisms governing the regulation of CETN3 expression by miR-410 in retinoblastoma. MTT and Colony formation assay were used to detect cell proliferation and the colony formation ability of retinoblastoma cells. Wound healing, Migration, and invasion assay were respectively used to explore the migration, and invasion in retinoblastoma cell line. Real-time polymerase chain reaction (RT-PCR) was performed to determine the expression level of miR-410. Western Blot was used to determine the expression of related proteins. We found that miR-410 is significantly upregulated in retinoblastoma. Over-expression of miR-410 apparently promotes retinoblastoma cell proliferation, migration and invasion. In addition, we identified that CETN3 was the direct target of miR-410. Over-expression miR-410 inhibited the expression of CETN3. And CETN3 showed the reverse trend. MiR-410 could activate the Wnt signaling pathway. Our study found that miR-410 functioned as a tumor oncogene by downregulating CETN3 expression, providing a potential diagnostic and therapeutic target for the treatment of retinoblastoma.

**Keywords:** miR-410, CETN3, proliferation, migration, retinoblastoma

## Introduction

Retinoblastoma (Rb) is the most common primary infancy intraocular malignant tumors, which is initiated by the allelic loss of RB gene, and has seriously threatened the life and visual function of children [1, 2]. The morbidity rate of RB is about 1/15000 [2], and has a high death rate approximately 5%. Because ages of children generally are less than 3 years old, the early detection of tumors is quite difficult. So if without timely treatment, RB grows and spreads easily in the eye, and finally leads to retinal detachment and necrosis, intracranial invasion along the optic nerve, threatening the child's eyesight and life [3]. Among all the treatments for retinoblastoma enucleation of eyeball is the most common and useful treatment. In order to elevate the quality of life, the conservative treatments were given more and more attention by oculists. At present, the conservative treatments mainly consist of photocoagulation, cryocoagulation, radiotherapy and chemoradiotherapy [4, 5], but most of the therapies have side effects. Further study on the pathomechanism of RB and seeking more effective therapy is of great significance for clinical treatment of RB.

MicroRNAs (miRNAs), the endogenous non-protein-coding RNA about 22 nucleotides, are widely expressed in various tissues and organs of eukaryotes, that regulate the degradation and translation of mRNAs by base pairing with the 3'-untranslated region of target mRNAs [6, 7]. At present, a large number of researches demonstrate that mRNA plays important roles in the development and progression of malignant tumors and heart diseases. Many miRNAs showed unique tissue-specific and developmental stage-specific expression patterns, suggesting potential unique functions in the retina and other ocular tissues. It is reported that a cluster of miRNAs was identified as highly expressed in retinoblastoma, including miR-373, miR-503, miR-320, and so on [8]. Dalgard found that exogenous miR-34a suppressed retinoblastoma cells growth [9]. Moreover, miR-183 was confirmed to inhibit the proliferation, invasion and migration of retinoblastoma cell.

Low-expression miR-17-92 in coordination with RB1 gene inactivation was reported to induce tumorigenesis [10].

A body of evidence demonstrates that miR-410 was involved in several types of human cancer. Chen et al showed that over-expression of miR-410 led to proliferation inhibition and reduced invasion capability in glioma cells [11]. Chien WW et al found that miR-410 could inhibit the cell proliferation of breast carcinoma by regulating pRb/E2F pathway [12]. Wang et al showed that miR-410 was up-regulated in liver and colorectal tumors [13]. Several studies have shown that miR-410 could significantly inhibit the expression of VEGF in prostate cancer [14-17]. Over-expression of miR-410 efficiently suppresses VEGFA expression, thus preventing retinal angiogenesis [18]. However, the role and function of miR-410 in retinoblastoma development as well as the mechanisms involved are still unknown. In the present study, we sought to emphasize the mechanisms governing the regulation of CETN3 expression by miR-410, providing a potential diagnostic and therapeutic target for the treatment of retinoblastoma.

## Materials and methods

### *Human tissue samples*

Human RB tissues and two normal retina tissues were provided by the Binzhou People's Hospital. All subjects were diagnosed and confirmed by a pathologist. All of the experiments were obtained with the understanding and written consent of each subject. The present study methodologies conformed to the standards set by the Declaration of Helsinki, and the study methodologies were approved by the ethics committee of Binzhou People's Hospital.

### *Cell lines and cell culture*

The human retinoblastoma cell lines, including Y79 and SO-RB50, and the normal human retinal pigment epithelial cell line ARPE-19 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All the cancer cells were cultured in RPMI-1640 medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine, and 10% heat-inactivated fetal bovine serum (FBS). The normal human retinal pigment epithelial cell line ARPE-19 was grown in DMEM medium with 10% FBS, 10 ng/ml cholera toxin, 5 μg/ml transferring, 5 μg/ml insulin, 100 ng/ml hydrocortisone, and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES). All cells were cultured in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C.

### *miRNA transfection experiments*

For the transfection experiments, $3 \times 10^5$ cells were seeded in a 6-cm dish in antibiotic-free RPMI 1640 with 10% fetal bovine serum. After 24 hours, the specific miRNA mimics and control oligos were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. A specific miRNA mimic is a duplex RNA, the sense sequence of which is equal to the sequence of the miRNA. A non-targeting sequence (control mimic) was used as the negative control. All of the above-mentioned RNAs were synthesized by RiboBio (Guangzhou, China).

### *RNA extraction and RT-PCR*

Total RNA was isolated from cells or frozen tissues using Trizol reagent (Invitrogen Life Technologies). The concentrations of RNA were determined using a NanoDrop ND-1000 instrument (ThermoFisher, Waltham, MA, USA) and aliquots of the samples were stored at -80°C. For the reverse transcription (RT) reaction of the miRNA, specific miRNA RT primers were used. U6 small nuclear noncoding RNA served as an internal control. To measure CETN3 mRNA, cDNA was synthesized using DNA polymerase I and RNaseH, and then purified using QiaQuick PCR extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Aliquots of the reaction mixture were used for quantitative PCR with TaqMan 2 × Universal PCRMaster Mix (Applied Biosystems, Foster City, CA, USA), using the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and a final step of 72°C for 45 s. All PCR experiments were performed in triplicate.

### *Cell proliferation, colony formation and cell cycle assay*

Cells were cultured in 96-well plates at $6 \times 10^3$ cells/well, and the surviving fractions were determined at 1-5 days using the MTT assay, as previously described [19]. The absorbance was measured with a spectrophotometer (Bio-Rad

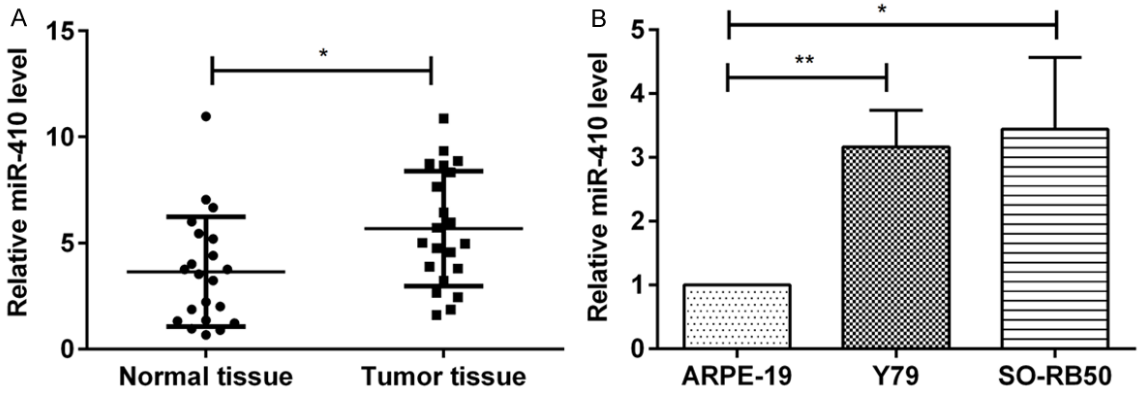


**Figure 1.** miR-410 expression is upregulated in retinoblastoma tissues and cell lines. A. The expression of miR-410 in retinoblastoma tissues and their matched adjacent noncancerous tissues were determined by RT-PCR. B. The expression of miR-410 in retinoblastoma cell lines and normal retinal pigment epithelial cell line ARPE-19 were determined by RT-PCR. *, $P<0.05$; **, $P<0.01$.

Laboratories, Hercules, CA, USA) at 570 nm. Each experiment was performed in triplicate.

For colony formation assay, cells were seeded in a 6-well plate and seeded for 10 days. Colonies were fixed with paraformaldehyde and stained with 1% crystal violet (Sigma-Aldrich) and the numbers of colonies were counted. The fixed cells were incubated with propidium iodide (PI) and ribonuclease a for 30 min, and then detected by flow cytometric analysis using FL2 histogram of a flow cytometer (FACSort; Becton-Dickinson, SanJose, CA, USA).

*Western blot assay*

Total protein was separated by 12% SDS polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% nonfatmilk and incubated with a rabbit anti-DRAM2 polyclonal antibody at a dilution of 1:200 (Sigma Aldrich), or a mouse anti-β-actin monoclonal antibody at a dilution of 1:2000 (Sigma, San Francisco, CA, USA). The membranes were subsequently incubated with a goat anti-mouse or anti-rabbit horseradish peroxidase secondary antibody (Sigma). The protein complexes were detected using the enhanced chemiluminescencere agents (Pierce, Rockford, IL, USA) and β-actin was used as an internal control.

*Cell migration and invasion assays*

HXO-Rb44 cells were grown to confluence in 12-well plastic dishes, and treated with the miR-410 mimic and its negative control (miR-NC), or the miR-410 inhibitor and its negative control (anti-miR). Then, 24 h after transfection, linear scratch wounds (in triplicate) were created on the confluent cell monolayers using a 200 μlpipette tip. To remove cells from the cell cycle prior to wounding, cells were maintained in serum-free media. To visualize migrated cells and wound healing, images were taken at different time points. A total of 10 areas were selected randomly from each well, and the cells in three wells from each group were quantified. For the invasion assays, 24 h after transfection, $1 \times 10^5$ cells in serum-free media were seeded in Transwell migration chambers (8 μm pore size; Millipore, Corning Inc., Corning, NY, USA). The upper chamber of the Transwell inserts was coated with Matrigel (Sigma Aldrich, St Louis, MO, USA). Medium containing 20% FBS was added to the lower chamber. After 24 h, the noninvading cells were removed with cotton wool. Invasive cells located on the lower surface of the chamber were stained with May-Grunwald-Giemsa stain (Sigma Aldrich) and counted using a microscope (Olympus, Tokyo, Japan). Experiments were independently repeated three times.

*DNA constructs and luciferase reporter assay*

The 3'-UTR sequence of CETN3 predicted to interact with miR-410 or a mutated sequence within the predicted target sites, and inserted into the XbaI/FseIsites of the pGL3 control vector (Promega, Madison, WI, USA). The mutant 3'UTR of CETN3 (various nucleotides within the

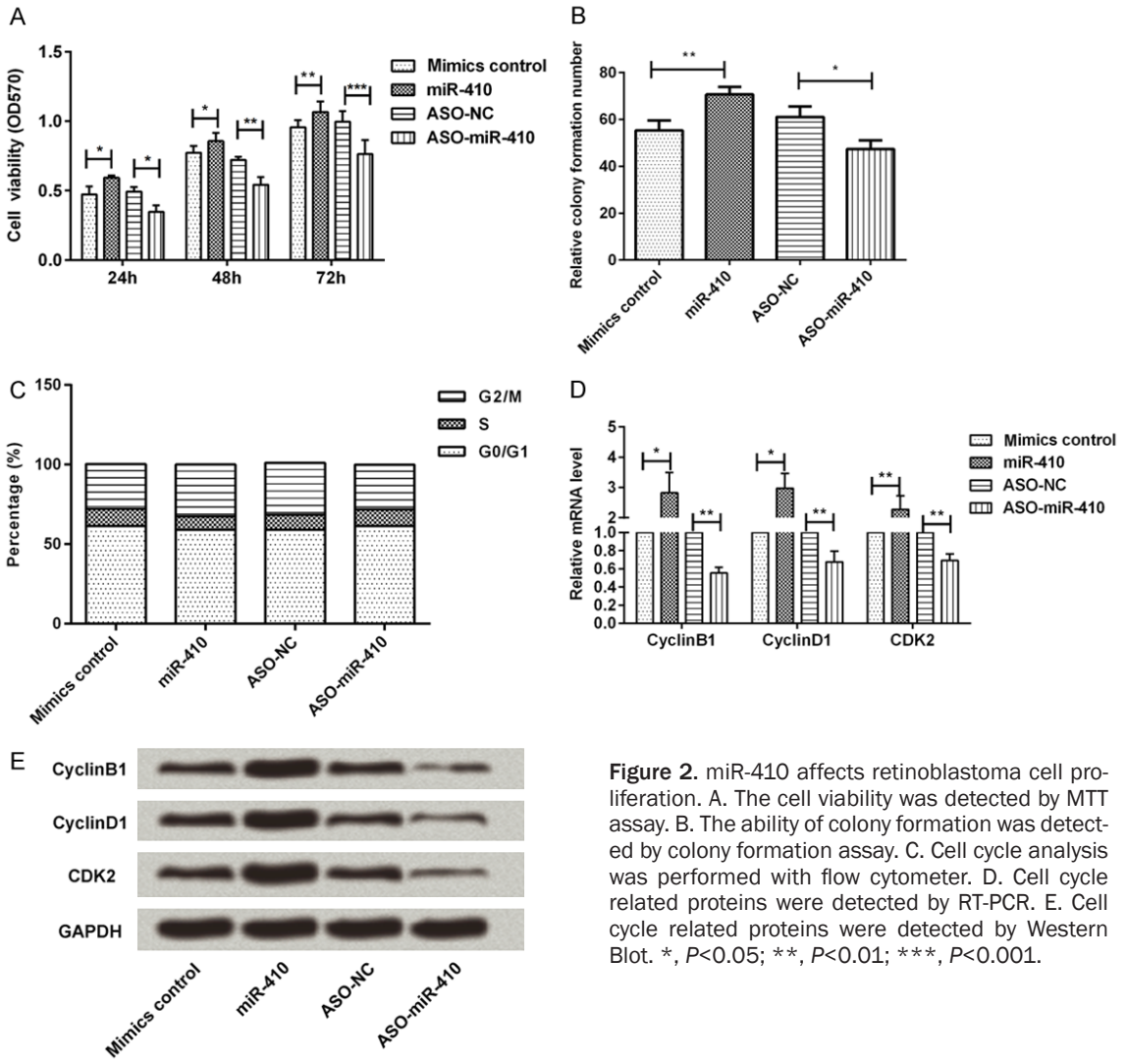


**Figure 2.** miR-410 affects retinoblastoma cell proliferation. A. The cell viability was detected by MTT assay. B. The ability of colony formation was detected by colony formation assay. C. Cell cycle analysis was performed with flow cytometer. D. Cell cycle related proteins were detected by RT-PCR. E. Cell cycle related proteins were detected by Western Blot. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

binding sites were mutated) was amplified using CETN3 3'UTR as the template. For the luciferase activity analysis, cells were co-transfected with the miR-410 mimic and CETN3 3'UTR, or mutant 3'UTR, together with the controls. The Dharma FECT Duotransfection reagent (Thermo Fisher Scientific, Waltham, A, USA) was used for 48 h, and the luciferase assays were performed with the Dual-Glo Luciferase assay (Promega, Beijing, China) according to the manufacturer's instructions.

*Statistical analysis*

The statistical significance of the differences was examined using Student's t test. The data are expressed as the mean ± SD. A *P* value of <0.05 was considered to indicate statistically significant difference. All the experiments were repeated at least three times, and each experiment included triplicate wells. One representative experiment was selected for drawing the diagrams and for the data analysis.

## Results

*miR-410 is upregulated in retinoblastoma cells*

RT-PCR was used to determine the expression level of miR-410. As shown in **Figure 1**, the expression of miR-410 in retinoblastoma tissues was significantly increased compared with the normal retina tissues. Furthermore, the expression of miR-410 in retinoblastoma cells, including Y79 and SO-RB50, was significantly increased compared with that in ARPE-19 (P< 0.05).

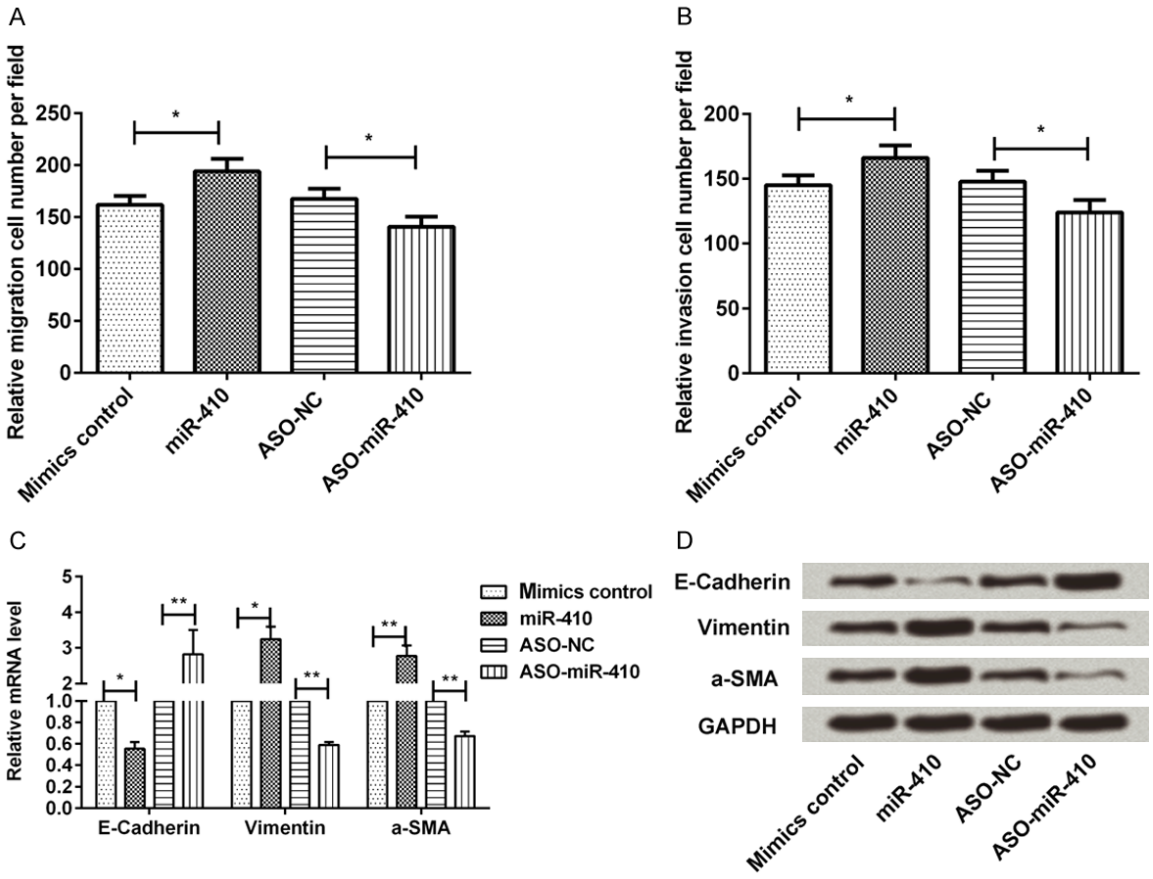


**Figure 3.** miR-410 affects retinoblastoma cell migration and invasion. A. Wound-healing assays were used to analyze the effect of miR-410 on cell migration. B. Transwell assays were used to analyze the effect of miR-410 on cell invasion. C. Epithelial-mesenehymal transition related proteins were detected by RT-PCR. D. Epithelial-mesenehymal transition related proteins were detected by Western Blot. *, $P<0.05$; **, $P<0.01$.

*miR-410 promote the proliferation of retinoblastoma cells*

MTT assay was used to determine the cell viability of retinoblastoma cells in each group (**Figure 2A**). The results showed that over-expression miR-410 could increase the viability of retinoblastoma cells compared with control group significantly (P<0.05). The viability of retinoblastoma cells in ASO-miR-410 treated group was significantly decreased compared with that in control group (P<0.05). And the differences were increased with time prolonged. Colony formation assay was used to detect the colony formation ability of retinoblastoma cells (**Figure 2B**). The results showed that over-expression miR-410 could increase the ability of colony formation, while suppressed the expression of miR-410 significantly decreased the ability of colony formation in retinoblastoma cells (P< 0.05). Then we found that over-expression miR-410 could accelerate the cell cycle of retinoblastoma cells, while suppressed the expression of miR-410 slowed down the cell cycle of retinoblastoma cells (**Figure 2C**). RT-PCR and Western blot were used to examine cell cycle related proteins, including cyclinB1, cyclinD1, and CDK2, respectively (**Figure 2D** and **2E**). Over-expression of miR-410 significantly increased the expression of cyclinB1, cyclinD1, and CDK2 (P<0.05). However, knock-out miR-410 significantly decreased the expression of cyclinB1, cyclinD1, and CDK2 (P<0.05). These results indicated that miR410 played an important role in regulating the proliferation of retinoblastoma cells.

*miR-410 promote the migration and invasion of retinoblastoma cells*

To analyze the role of miR-410 in cell migration and invasion, wound-healing and Transwell assays were performed. As shown in **Figure 3A** and **3B**, over-expression of miR-410 significant-

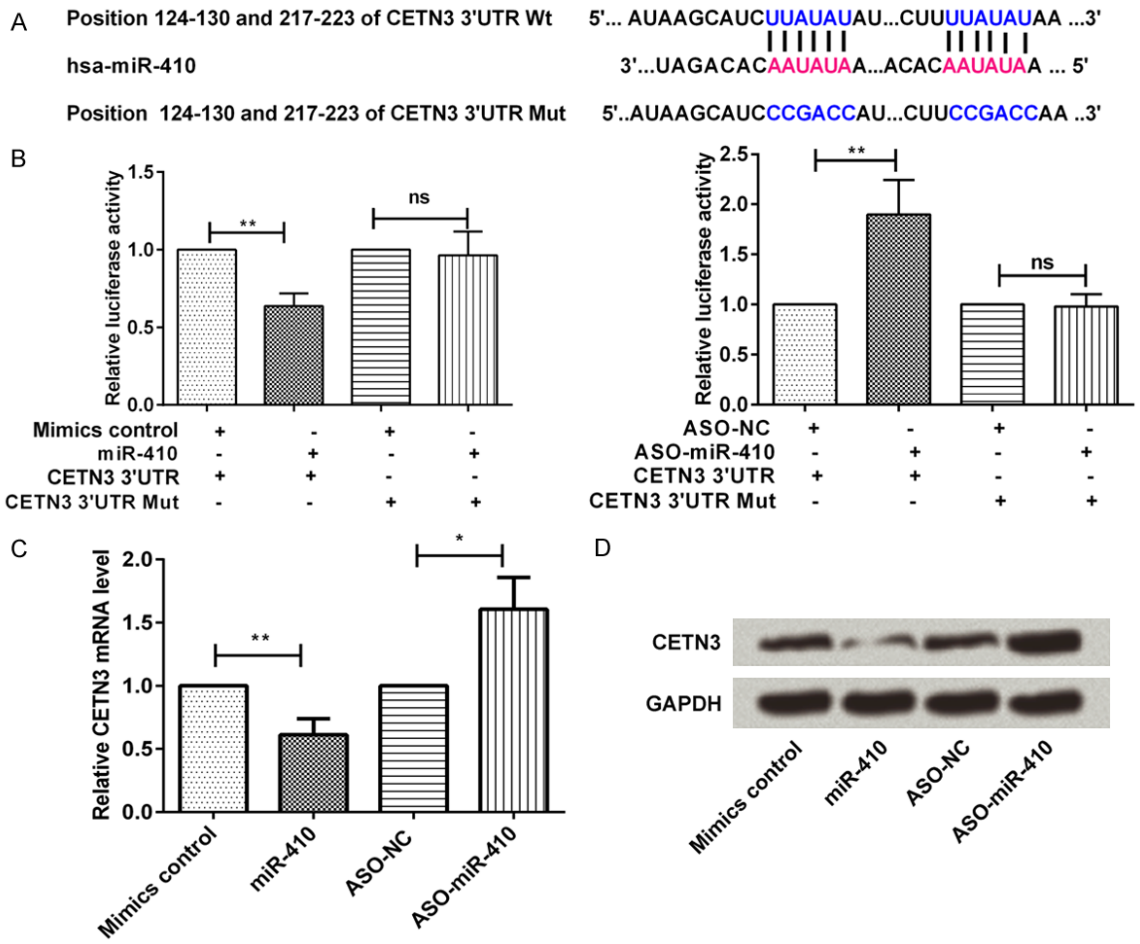


**Figure 4.** miR-410 can lead to downregulated CETN3 expression by directly targeting the CETN3 3'UTR. A, B. Relative luciferase activity of HXO-Rb44 cells co-transfected with increasing amounts of miR-410 mimic and CETN3 3'UTR-luciferase reporter vector that contained a wild-type sequence (CETN3 3'UTR-wt). C, D. The expression of CETN3 was detected by RT-PCR and Western Blot analysis, respectively. *, *P*<0.05; **, *P*<0.01.

ly increased the migration and invasion of retinoblastoma cells (P<0.05), while knock-out miR-410 significantly decreased the migration and invasion of retinoblastoma cells (P<0.05). Furthermore, we determine the expression of epithelial-mesenehymal transition (EMT) related protein. As shown in **Figure 3C** and **3D**, over-expression of miR410 significantly decreased the expression of E-Cadherin and increased the expression of Vimentin and a-SMA significantly (P<0.05). Knock-out miR-410 showed the reverse trend. The results indicated that miR-410 could promote epithelial-mesenehymal transition of retinoblastoma cells.

*CETN3 is direct target genes of miR-410*

miR-410 decreased the luciferase activity of wildtype CETN3 3'UTR compared with the normal cells, while knock-out miR-410 had the reverse trend. However, there was no difference in luciferase activity in CETN3 3'UTR-mut groups, indicating that CETN3 is a direct target of miR-410 (**Figure 4A** and **4B**). RT-PCR and Western blotting were used to examine the expression of CETN3 at mRNA level and protein level, respectively (**Figure 4C** and **4D**). Over-expression of miR-410 could significantly decrease the expression of CETN3, while knock-out miR-410 significantly increased the expression of CETN3 (P<0.05).

*CETN3 inhibit the proliferation, migration, and invasion of retinoblastoma cells*

MTT assay and colony formation assay were used to determine the proliferation of retinoblastoma cells (**Figure 5A** and **5B**). CETN3 sig-

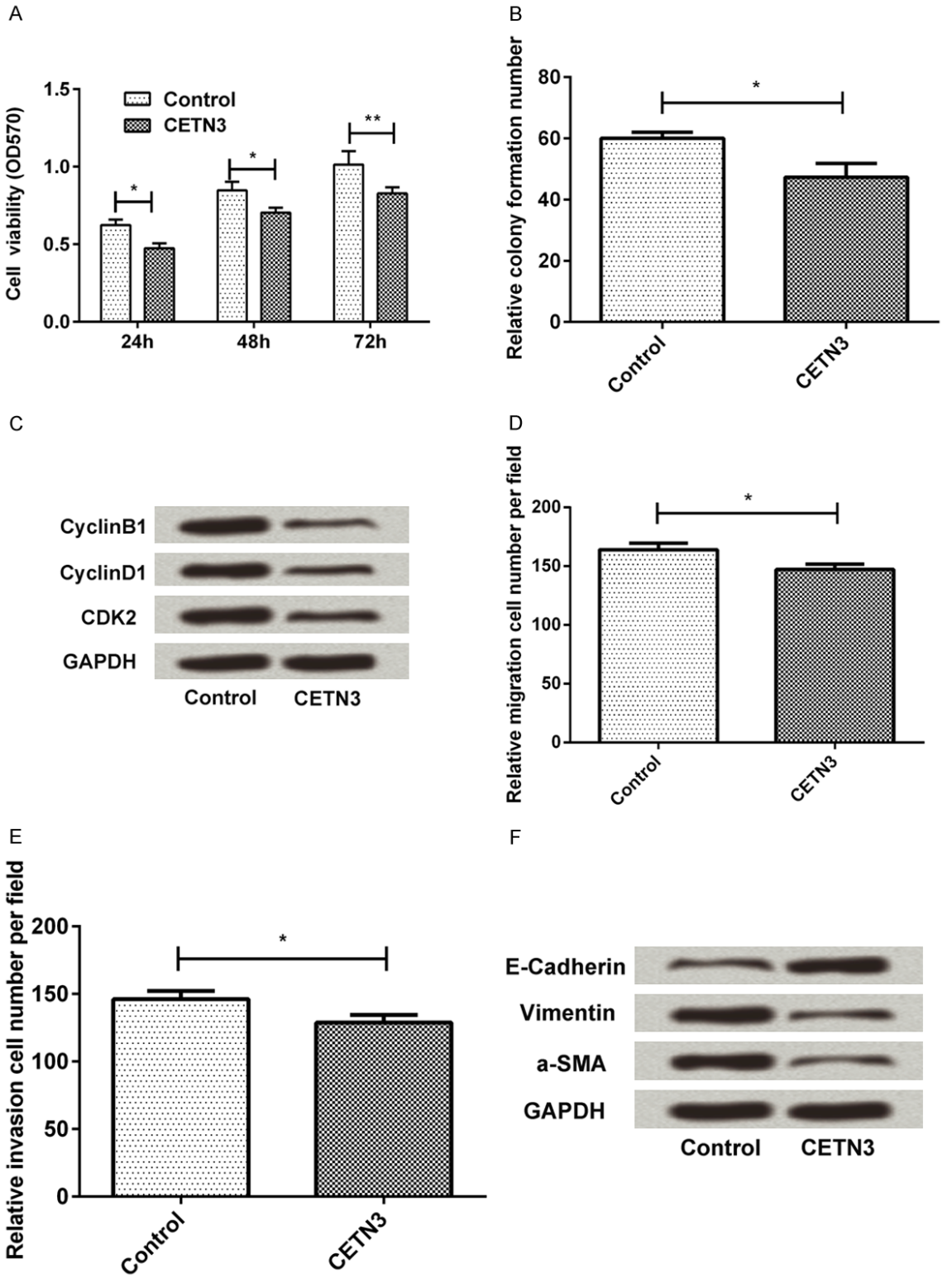


**Figure 5.** CETN3 inhibit the proliferation, migration, and invasion of retinoblastoma cells. A. The cell viability was detected by MTT assay. B. The ability of colony formation was detected by colony formation assay. C. Cell cycle analysis was performed with flow cytometer. D. Wound-healing assays were used to analyze the effect of miR-410 on cell migration. E. Transwell assays were used to analyze the effect of miR-410 on cell invasion. F. Epithelial-mesenehymal transition related proteins were detected by Western Blot. *, $P<0.05$; **, $P<0.01$.

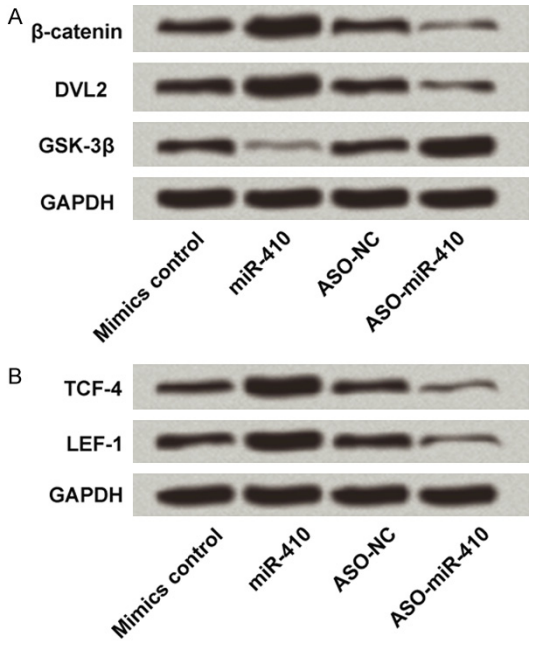


**Figure 6.** miR-410 induced the activation of Wnt/β-catenin. A. Wnt/β-catenin pathway related proteins were detected by Western Blot. B. Wnt/β-catenin signal pathway downstream transcription factors were detected by Western Blot. *, *P*<0.05; **, *P*<0.01.

nificantly decreased the cell viability (P<0.05), and the difference was increased with time prolonged. Western blotting was used to examine the cell cycle related protein level after the over-expression of CETN3, which showed that over-expression of CETN3 decreased the level of cell cycle related proteins, including cyclinB1, cyclinD1, and CDK2, indicating that CETN3 slowed down the cell cycle of retinoblastoma cells (**Figure 5C**). As shown in **Figure 5D** and **5E**, CETN3 could significantly inhibit the migration and invasion of retinoblastoma cells (P<0.05). Western blotting was used to determine the expression of epithelial-mesenehymal transition related protein, which showed that CETN3 decreased the expression of Vimentin and a-SMA, while increased the expression of E-Cadherin (**Figure 5E**).

*miR-410 induced the activation of Wnt/β-catenin*

As shown in **Figure 6A**, over-expression of miR-410 increased the expression of β-catenin and DVL2, and decreased the expression of GSK-3β. Moreover, we found that over-expression of miR-410 increased the expression of TCF-4 and LEF-1 (**Figure 6B**). These results indicated that miR-410 could activate the Wnt/β-catenin signal pathway and increase the expression of its downstream transcription factor.

## Discussion

Recent findings indicate that abnormal expression of miRNAs is an important factor in the development of cancers [20, 21]. It is reported that a cluster of miRNAs was identified as highly expressed in retinoblastoma [8]. In this study, we focused on the role of miR-410 in retinoblastoma cells. We found that the expression of miR-410 were much higher in retinoblastoma cell lines, Y79 and SO-BR50, compared with adjacent controls. The result allowed us to speculate that miR-410 may provide a survival change to retinoblastoma patients. We found that miR-410 could promote cell proliferation by inhibiting the cell apoptosis of retinoblastoma. Moreover, up-regulated miR-410 could promote the ability of migration and invasion of retinoblastoma cells. Further studies proved that miR-410 promoted cell migration and invasion via EMT. These data suggest that miR-410 may act as a tumor promoter in retinoblastoma.

Up to now, a large number of miRNAs have been demonstrated to participate in tumor development through regulation their target genes. Each miRNA regulates about 200-800 target genes, and 60% of the protein-encoding genes are predicted to be regulated by miRNAs [22]. For further study to elucidate the role of miRNA/target gene axis in retinoblastoma tumourigenesis, we analyzed the role of CETN3 in retinoblastoma.

It is reported that centrosome plays an important role in mitosis/meiosis and has shown to be amplified in cancer [23, 24]. As critical constituents of the centriole, centrins play an important role in cytokinesis and cell cycle progression [25]. Centrins, members of the EF-hand superfamily, are localized to the centrosome, which are found in eukaryotes and participate in many cellular processes, including centriole and basal bady duplication [26-29]. There are three members of centrin family of calcium binding proteins (CETN) in mammals, with variable sequence, tissue expression, and functional properties [30]. Centrin 3 (CETN3) is the first centriole-associated protein and par-

ticipates in the initiation of centrosome duplication in animals [31]. It has been shown that Tbcentrin3 plays an essential role in cell motility [32]. In the present study, we found that CETN3 could significantly inhibit the cell migration and invasion of retinoblastoma. According to previous reports, injection of CETN3 protein was able to prevent centrosome duplication [31, 33, 34]. CETN3 could effectively inhibit the cell proliferation and migration in retinoblastoma. Furthermore, we proved that CETN3 is a direct target gene of miR-410, and is markedly downregulated by miR-410 at the posttranscriptional level in retinoblastoma. These data indicated that miR-410 functions as a tumor oncogene by down-regulating CETN3 expression, providing a potential diagnostic and therapeutic target for the treatment of retinoblastoma.

EMT is a key early event induced tumor invasion and metastasis, which provides the basis for the invasion and metastasis of tumor cells of epithelial origin [35]. Many signal transduction pathways involved in the EMT of tumor cells, such as MAPK, Notch, TGF-β, and Wnt pathway [36, 37]. Non-canonical Wnt signaling cascade was considered to be responsible for the development of many malignancies [38]. Wnt5a is a typical representative Wnt protein which signals via non-canonical Wnt pathway. There are some evidences that over-expression of Wnt5a is involved in the aggressiveness and initiation of EMT by activating transcription factors in tumor cells, such as breast cancer, prostate cancer, colon cancer, and gastric cancer [39-41]. When the Wnt/β-catenin pathway is abnormally activated, the adhesion between the tumor cells is weakened, including the EMT process of tumor cells [42], enhancing the ability of tumor cell invasion and metastasis. In the present study, we found miR-410 could activate Wnt/β-catenin signal pathway and increase the expression of its transcription factors.

In conclusion, we found that miR-410 function as a tumor promoter for retinoblastoma by inhibiting the expression of CETN3. Furthermore, over-expression of miR-410 activates Wnt/β-catenin signal pathway, which may promote EMT, thus promoting the migration and invasion of retinoblastoma cells. These results will provide the theory basis for the clinical treatment.

## Disclosure of conflict of interest

None.

**Addres correspondence to:** Xiaohui Fan, Department of Ophthalmology, Binzhou People's Hospital, No. 515, Huangheqi Road, Binzhou 256610, Shandong, China. E-mail: fanxiaohui1122@126.com

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