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ORIGINAL RESEARCH

CASC8 lncRNA Promotes the Proliferation of Retinoblastoma Cells Through Downregulating miR34a Methylation

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Background: *CASC8* lncRNA has been proven to be oncogenic in a variety of cancers, but its role in other types of cancer remains unclear. This study was to investigate the role of *CASC8* in retinoblastoma (Rb).

Methods: RT-qPCR was performed to determine the expression of *CASC8* and miR34a in paired Rb and nontumor tissue. Overexpression of *CASC8* and miR34a in Rb cells was achieved to evaluate the interaction between them. Methylation-specific PCR was used to analyze the effect of *CASC8* overexpression on *MIR34A* gene methylation. CCK8 assays were used to analyze cell proliferation.

Results: The results showed that *CASC8* expression was upregulated and miR34a expression downregulated in Rb tissue. Moreover, miR34a expression was negatively correlated with the of *CASC8* expression in Rb tissue. Overexpression of *CASC8* decreased expression of miR34a and increased methylation of *MIR34A* in Rb cells. In addition, overexpression of *CASC8* reduced the inhibitory effects of miR34a on Rb-cell proliferation.

Conclusion: *CASC8* may promote Rb cell proliferation by downregulating miR34a methylation.

Keywords: retinoblastoma, *CASC8*, miR34a, methylation, proliferation

Introduction

Retinoblastoma (Rb) is a rare malignant tumor that originates from the photosensitive tissue of the eye, known as the retina.[1] Rb is caused mainly by genetic mutation in retinal nerve cells. Cells with certain mutations will grow uncontrollably, leading to the formation of tumors.[2] Rb affects mainly patients <5 years old.[3] With proper treatment, 95% of Rb patients can be cured, and both eyes can be saved in 70%–80% cases.[4,5] However, Rb in some cases can also spread to other parts of the body, such as the spine and brain, leading to poor prognosis.[6] Therefore, novel therapeutic approaches are needed to further improve the survival of Rb patients.

Molecular factors play crucial roles in the pathogenesis of Rb.[7,8] Functional characterization of molecular pathways, such as p53, RB1, and NFκB, provides novel insights to the development of novel anticancer therapies, such as targeted therapy.[9,10] It has been well established that ncRNAs, such as lncRNAs), andmiRNAs are not involved in protein synthesis, but regulate the expression of cancer-related genes to suppress or promote cancer development.[11] Therefore, ncRNAs are potential targets for targeted cancer therapy.[12] However, the function of most ncRNAs is barely known. lncRNA *CASC8* has been proven to be

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significantly correlated with several types of cancer, such as lung cancer,[13] while its involvement in Rb is unknown. Our preliminary RNA-seq analysis revealed altered expression of *CASC8* in Rb, and expression of *CASC8* in Rb was negatively correlated with miR34a (data not shown), which is a well-established tumor-suppressive miRNA.[14] This study was thus carried out to analyze interactions between *CASC8* and miR34a in Rb.

Methods

Rb Patients

The Ethics Committee of Shenzhen Hospital of Integrated Chinese and Western Medicine approved this study. A total of 62 Rb patients (39 males and 23 females, 9 months to 3 years, 4 months, 2.0±0.4 years) were enrolled at this hospital between March 2016 and January 2019. All patients were newly diagnosed Rb patients. No recurrent Rb patients were enrolled. Patients with a family history of malignancy were excluded. Before this study, no therapy had been performed on these patients. The International Classification for Intraocular Retinoblastoma was used to stage the 62 Rb patients. Based on these criteria, patients were divided into group A (n=11), B (n=12), C (n=10), D (n=15), and E (n=14). In addition, patients included 28 cases with low-grade Rb and 34 high-grade Rb. All patients provided signed informed consent.

Specimen Collection and Rb Cells

During histopathological biopsy for the diagnosis of Rb, paired Rb and nontumor tissue samples were collected from each patient. A liquid-nitrogen tank was used to store all fresh tissue samples before subsequent experiments. Y79 and C33A (ATCC) human Rb cell lines were used. Cells were cultivated in a 5% CO_2 and 95% humidity incubator at 37°C. The cell-culture medium was composed of RPMI 1640 (80%) and FBS (20%).

Cell Transfection

A *CASC8*-expression vector was constructed using a pcDNA3.1 vector (Invitrogen) as a backbone. Vector construction was provided by Invitrogen. Mimics of miR34a and negative control (NC) miRNA were from Invitrogen. Y79 and C33A cells were cotransfected with 40 nM miRNA mimic or 10 nM vector using Lipofectamine 2000 (Sangon). Control cells were untransfected. NC cells were miRNA- or empty vector–transfected cells. The following experiments were carried out at 48 hours after all transfections.

RNA Preparations

A Trizol Plus RNA-purification kit (Thermo Fisher Scientific) was used to extract total RNA from both paired nontumor and Rb tissue samples, as well as Y79 and C33A cell lines. Genomic DNA removal was performed using DNase I (Invitrogen) at 37°C for 2 hours. RNA integrity was checked using a urea–PAGE gel (6%). Only RNA samples of satisfactory quality were subjected to the following experiments.

RT-qPCR

An SSRT III reverse transcription (RT) system (Thermo Fisher Scientific) was used to perform RT to synthesize cDNA samples with RNA samples as a template. Samples of cDNA were used as a template to perform all qPCR using a Kapa SYBR Fast qPCR kit (Roche). The endogenous control of *CASC8* was 18S rRNA. To measure expression levels of mature miR34a, addition of poly(A), miRNA RT,

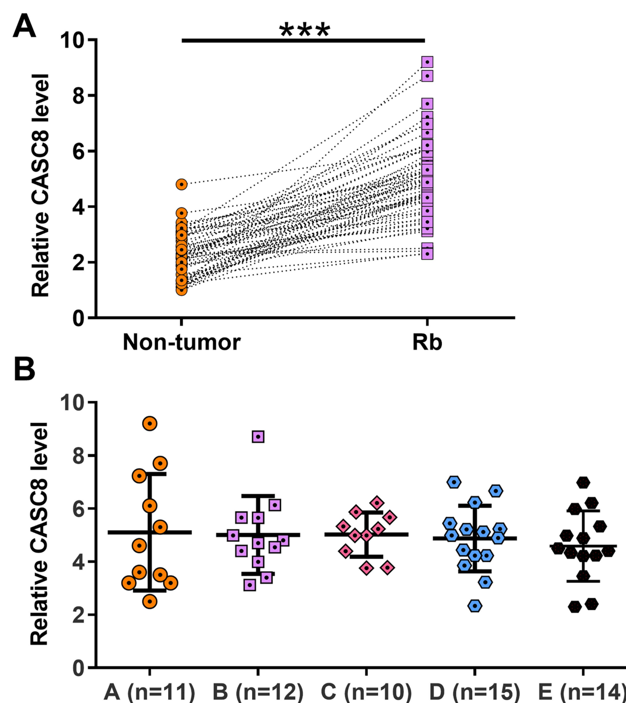

Figure 1 *CASC8* expression was upregulated in Rb tissue. Paired Rb and nontumor tissue were collected from the 62 Rb patients enrolled in this study. Tissue samples were subjected to RNA preparations and RT-qPCR used to determine the expression of *CASC8* in EC. Three replicate PCRs were included in each experiment. Ct values of *CASC8* were normalized to endogenous control 18S rRNA using the $2^{-\Delta\Delta CT}$ method. The sample with the biggest ΔCT value was set to value "1". All other samples were normalized to this sample. Mean values of data are presented (**A**). Expression of *CASC8* in Rb tissue among patients at different clinical stages (**B**). ***p<0.001.

and qPCRwere performed using a GeneCopoeia All-in-One miRNA qRT-PCR detection kit. The internal control of miR34a was U6. Three replicates were included in each experiment. The $2^{-\Delta\Delta CT}$ method was used to normalize gene-expression levels.

Methylation-Specific PCR

Isolation of genomic DNA from Y79 and C33A cells was performed using a Monarch genomic DNA-purification kit (NEB), with all steps performed following the manufacturer's instructions. DNA samples were converted using a Methylation-Gold kit (Zymo Research). To analyze methylation of the *MIR34A* gene, methylation-specific PCR and routine PCR were performed using Taq 2× master mix (NEB). PCR products were sequenced to make sure that the correct PCR products were obtained.

CCK8 Assays

Y79 and C33A cells were collected at 48 hours posttransfection. Cells were digested with 0.25% trypsin, washed with cold PBS, and counted. Cells were then cultivated in a 96-well plate at 37°C with 3,000 cells in 0.1 mL medium per well. To determine cell proliferatione, OD values at 450 nm were measured every 24 hours for 96 hours. At 4 hours before measurement, CCK8 solution (Sigma-Aldrich) was added to each well to reach 10%.

BrdU Assays

After transfection, Y79 and C33A cells were transferred to a 96-well cell-culture plate (3,000 cells in 0.1 mL medium per well). Cell culture was performed for a further 48 hours, and peroxidase-coupled anti-BrdU antibody (Sigma–Aldrich) was used to incubate cells after fixation. Peroxidase substrate was then used to incubate cells for 2 hours after washing, and OD values at 450 nm were measured later.

Statistical Analysis

Data of three biological replicates of each experiment are expressed as means ± SD. Paired *t*-tests were used to compare paired tissue samples. ANOVA with Tukey's test was used to compare multiple groups. Correlations were analyzed by linear regression. $P<0.05$ was statistically significant.

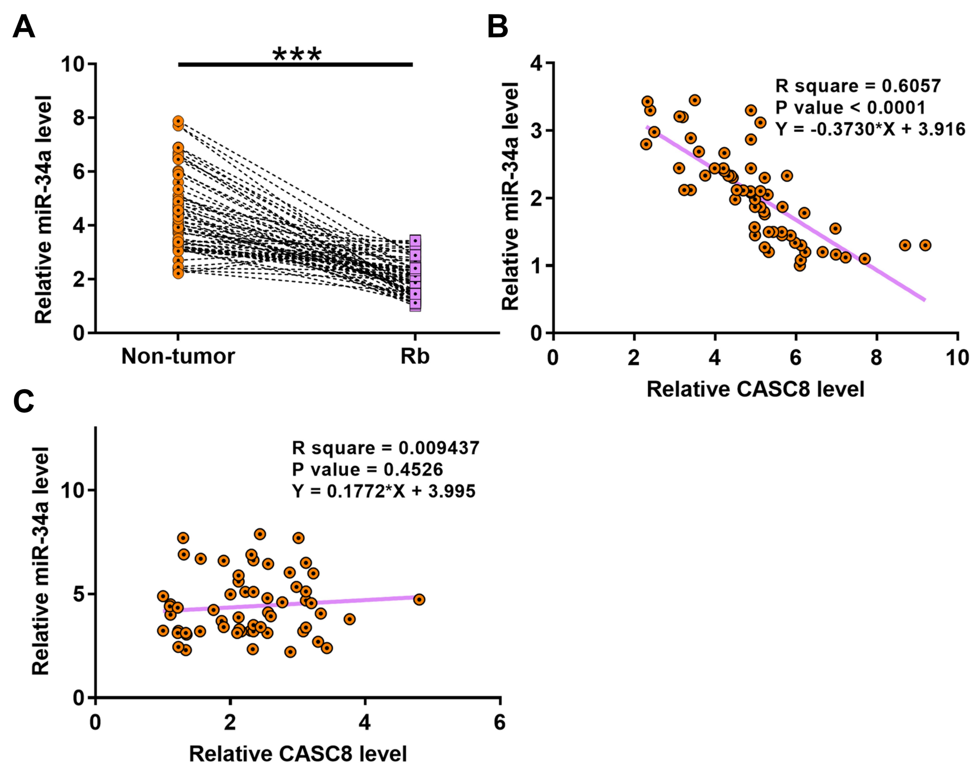

Figure 2 Expression of miR34a was downregulated in Rb and inversely correlated with *CASC8* expression. Paired Rb and nontumor tissue from 62 Rb patients were also subjected to RNA preparations and RT-qPCR used to analyze differential expression of miR34a in Rb. Ct values of miR34a were normalized to the endogenous control, U6, using the $2^{-\Delta\Delta CT}$ method. The sample with the biggest ΔCT value was set to value "1". All other samples were normalized to this sample. Three replicate PCRs were included in each experiment. Mean values of data are presented (**A**). ***$p<0.001$. Linear regression was carried out to analyze correlations between expression levels of miR34a (**B**) and *CASC8* (**C**) across Rb tissue and nontumor tissue.

Results

Expression of *CASC8* Upregulated in Rb tissue

Expression of CASC8 in paired Rb and nontumor tissue samples was determined by RT-qPCR. Compared with non-tumor tissue, expression of *CASC8* was significantly higher in Rb tissue (Figure 1A, $p<0.001$), indicating the possible involvement of *CASC8* in Rb. Expression of *CASC8* in Rb tissue was compared among patients at different clinical stages. It was observed that *CASC8* expression was not significantly different among groups A–E (Figure 1B).

miR34a Downregulated in Rb and Inversely Correlated with *CASC8*

Expression of miR34a in paired Rb and nontumor tissue samples from 62 Rb patients was determined by RT-qPCR. It was observed that expression of miR34a was significantly decreased in Rb tissue compared with nontumor tissue (Figure 2A, $p<0.001$). Linear regression was carried out to analyze the correlation between the expression of miR34a and *CASC8* across Rb tissue and nontumor tissue. It was found that the expression of miR34a and *CASC8* were inversely and significantly correlated in Rb tissue (Figure 2B). However, the correlation between them was not significant in nontumor tissue (Figure 2C).

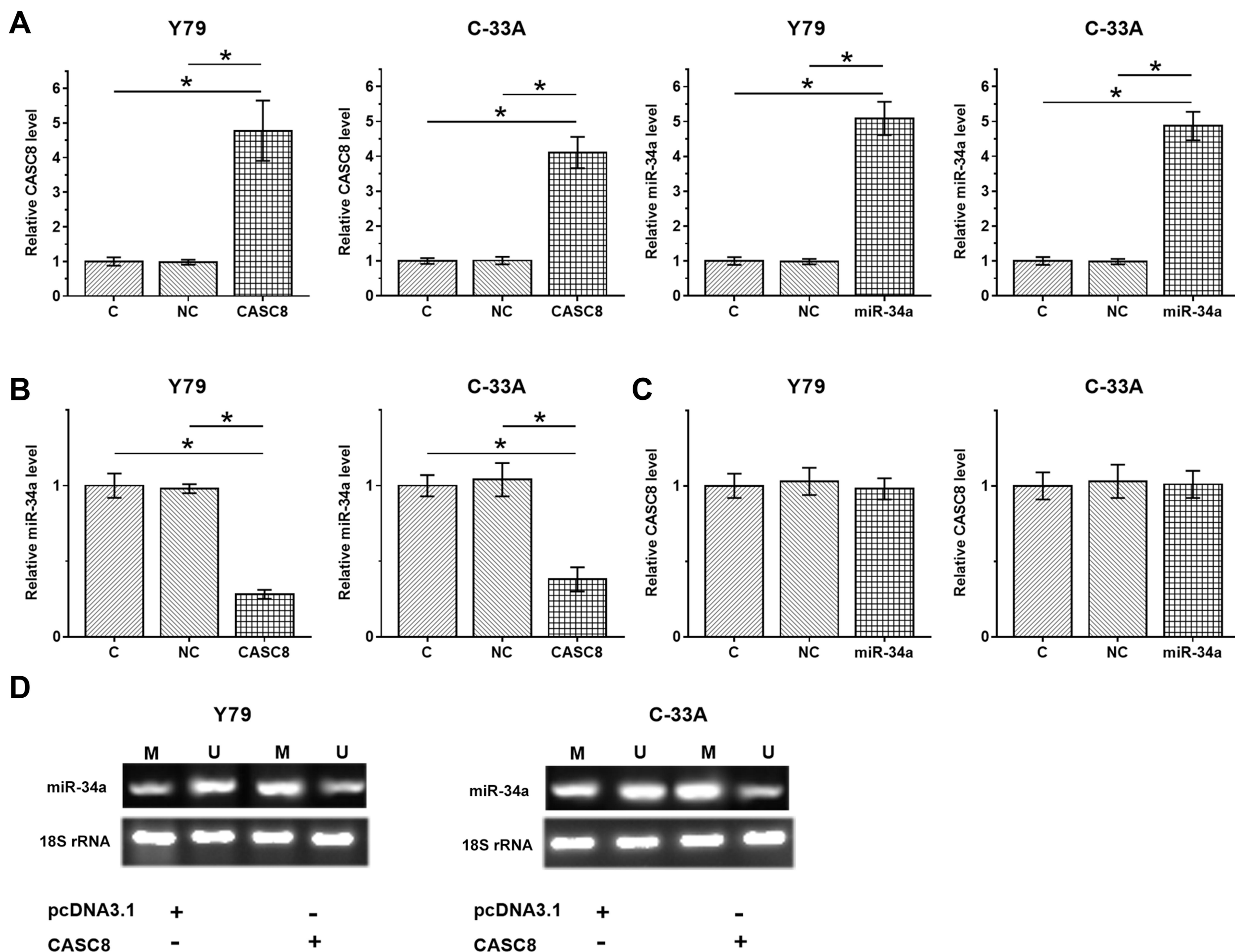

Figure 3 *CASC8* overexpression downregulated miR34a in Rb cells through methylation. To analyze possible interactions between *CASC8* and miR34a, a *CASC8* expression vector or miR34a mimic was transfected into Y79 and C33A cells. RT-qPCR was performed at 48 hours posttransfection to confirm overexpression of *CASC8* and miR34a (**A**). Expression of miR34a in cells with *CASC8* overexpression (**B**) and expression of *CASC8* in cells with miR34a expression (**C**) were also analyzed by RT-qPCR (**C**). Values of the control group were set to "1", and other groups were normalized to the control group. MSP was performed on cells transfected with either empty pcDNA3.1 vector or cells transfected with a *CASC8* -expression vector to analyze the effects of *CASC8* overexpression on methylation of miR34a (**D**). Three independent replicates were included in each experiment, and data are expressed as means ± SD. *$p<0.05$.

Abbreviations: M, methylation; U, unmethylaed; C, control (no transfection); NC, negative control (cells transfected with empty vector or NC miRNA).

CASC8 Overexpression Downregulated miR34a in Rb Cells Through Methylation

To analyze possible interactions between *CASC8* and miR34a, the CASC8-expression vector or miR34a mimic was transfected into Y79 and C33A cells. RT-qPCR was performed at 48 hours posttransfection to confirm overexpression of *CASC8* and miR34a (Figure 3A, $p<0.05$). Compared with controls, downregulated miR34a was observed in cells transfected with the *CASC8*-expression vector (Figure 3B, $p<0.05$), while no significant effect on *CASC8* expression was observed after miR34a-mimic transfection (Figure 3C, $p>0.05$). Methylation-specific PCR was performed to analyze the effects of *CASC8* overexpression on methylation of miR34a. Compared with cells transfected with the empty vector, cells transfected with *CASC8*-expression vector showed significantly increased methylation of miR34a (Figure 3D).

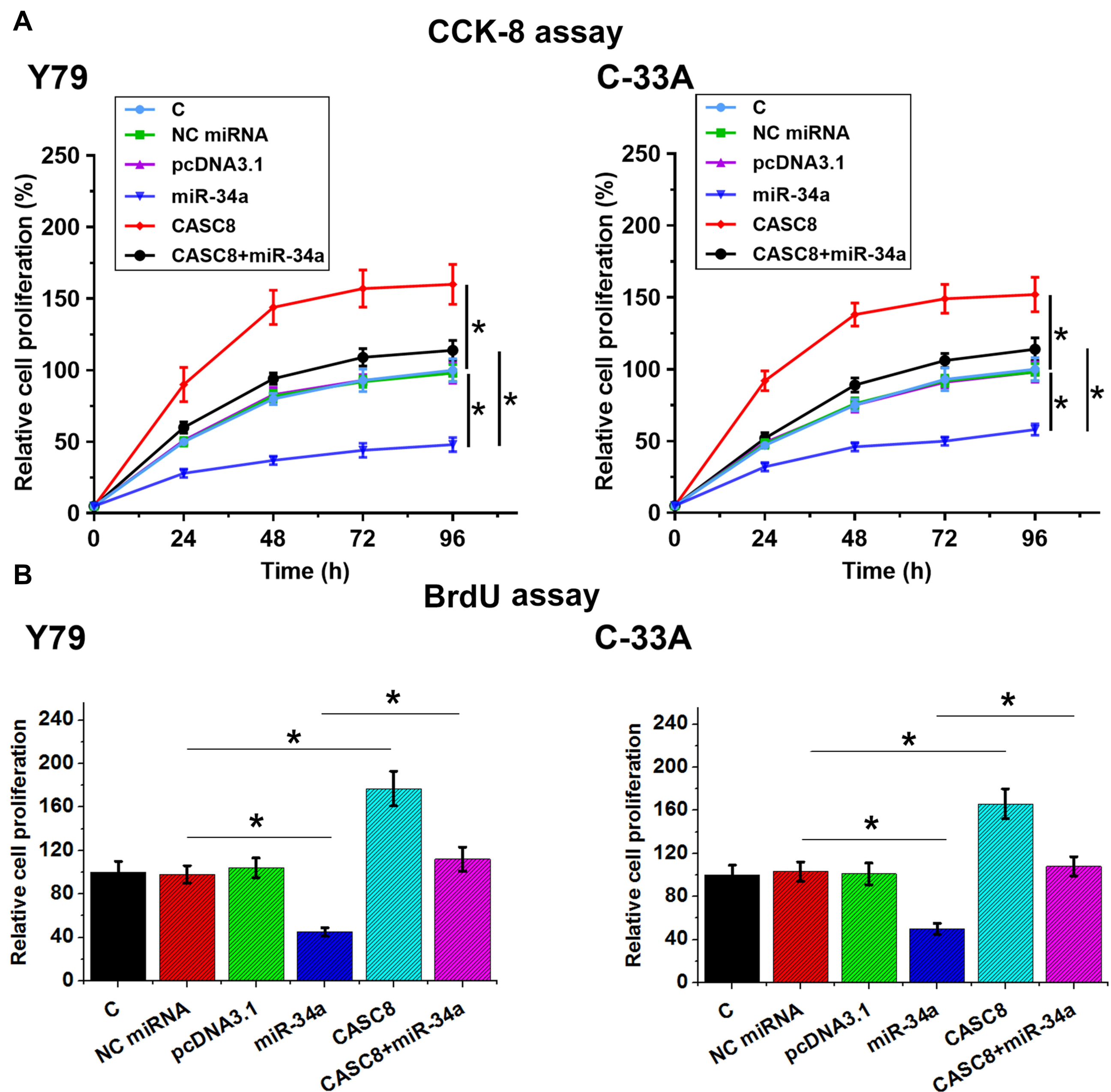

Figure 4 *CASC8* overexpression promoted the proliferation of Rb cells through miR34a. Possible involvement of *CASC8* in regulation of proliferation of Rb cells was analyzed by both CCK8 (**A**) and BrdU assays (**B**). Cell proliferation was reflected by OD values measured at 450 nm. For CCK8 assays, measurement of OD values was performed every 24 hours until 96 hours. For BrdU assays, cells were fixed after cell culture for a further 48 hours and OD values measured later. Three independent replicates were included in each experiment, and data are expressed as means ± SD. *$p<0.05$.

Abbreviations: C, control (no transfection); NC, negative control (cells transfected with empty vector or NC miRNA).

CASC8 Overexpression Promoted Proliferation of Rb Cells Through miR34a

Possible involvement of *CASC8* in regulation of the proliferation of Rb cells was analyzed by CCK8 (Figure 4A) and BrdU assays (Figure 4B). Compared with the control group, *CASC* overexpression significantly increased the proliferation rate of Rb cells, while miR34a overexpression significantly decreased the proliferation rate of Rb cells. In addition, *CASC8* overexpression reduced the inhibitory effects of miR34a on Rb-cell proliferation (Figure 4, $p<0.05$).

Discussion

In this study, we analyzed the interaction between *CASC8* and miR34a in Rb, and found that *CASC8* expression was upregulated in Rb. Moreover, *CASC8* downregulated expression of miR34a by methylation of the *MIR34A* gene, thus promoting the proliferation of Rb cells.

Studies have investigated correlations between genetic polymorphisms of *CASC8* and the risk of cancer.[15] It has been observed that compared with the population with the rs10505477 TT genotype, those with TC or CC genotype were at significantly decreased risk of many types of cancer.[15] Correlations between *CASC8* polymorphisms and risk of cancer have been reported in colorectal cancer,[16] hepatocellular carcinoma,[17] lung cancer,[13] and acute lymphoblastic leukemia.[18] However, the expression pattern and function of *CASC8* in cancer biology remains unclear. This study is the first to report on the upregulation of *CASC8* in Rb. In addition, we found that upregulation of *CASC8* increased the proliferation rate of Rb cells. Therefore, *CASC8* may play oncogenic roles in Rb by promoting cancer-cell proliferation.

miR34a plays tumor-suppressive roles in many types of cancer, including Rb.[19–22] In cancer biology, miR34a targets multiple oncogenic genes, such as *CD44* and *CMET*, to suppress the growth and metastasis of tumors. In Rb, miR34a not only regulates the chemosensitivity of cancer cells to chemotherapy[19] but also suppresses cancer development by inhibiting cancer-cell proliferation.[20] Therefore, overexpression of miR34a is considered a promising target for the treatment of Rb. However, based on our knowledge, the upstream regulator of miR34a in many cancers, including Rb, is unknown. This study confirmed the inhibitory effects of miR34a on Rb-cell proliferation. miR34a is involved in cancer biology mainly through interaction with cancer-related pathways, such as MAGEA–p53 signaling.[19] Our results showed that *CASC8* might be an upstream inhibitor of miR34a, and could downregulate the expression of miR34a through a methylation pathway. However, the methylation factors involved in this process remain unclear.

Interestingly, we found that *CASC8* and miR34a were inversely correlated in Rb tissue, but not nontumor tissue. Therefore, the interaction between *CASC8* and miR34a might be mediated by certain pathological factors. Future studies are needed to identify these factors.

In conclusion, the expression of *CASC8* is upregulated in Rb, and *CASC8* may upregulate miR34a expression in Rb cells through methylation of the *MIR34A* gene, thus promoting the proliferation of Rb cells.

Ethics Approval and Consent to Participate

The Ethics Committee of Shenzhen Hospital of Integrated Chinese and Western Medicine approved this study, and informed consent was obtained from all participants. Data collected from participants were kept confidential and accessible only by the researchers.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, took part in drafting the article or revising it critically for important intellectual content, agreed to submit to the current journal, gave final approval to the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that there are no competing interests associated with the manuscript.

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