Long non-coding RNA SNHG20 promotes cell proliferation, migration and invasion in retinoblastoma via the miR-335-5p/E2F3 axis

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Abstract. Current therapies for retinoblastoma (RB) are unsatisfactory and there is an urgent need for the development of new treatment modalities. Small nucleolar RNA host gene 20 (SNHG20) has been reported to serve a key oncogenic role in the development of various types of cancer, but its role in RB tumorigenesis remains to be fully determined. The present study aimed to investigate the expression patterns and biological roles of SNHG20 in RB. The expression levels of SNHG20 were measured via reverse transcription-quantitative PCR in RB tissues and cell lines. The impact of SNHG20 status on cell proliferation, survival, migration and invasion was determined using small interfering RNA and a range of established experimental assays. The SNHG20/microRNA (miR)-335-5p/E2F transcription factor 3 (E2F3) signaling axis was further investigated using a dual-luciferase activity reporter system and an RNA pull-down assay combined with bioinformatics analyses. SNHG20 expression was significantly increased in RB tissues and cell lines. Silencing of SNHG20 in RB cells was shown to inhibit cell proliferation, clonogenic survival, migration and invasion. Moreover, mechanistic investigations demonstrated that SNHG20 could enhance the expression of E2F3 by sponging of miR-335-5p. These data suggested that the long non-coding RNA SNHG20 may promote cell proliferation, migration and invasion in RB via the miR-335-5p/E2F3 axis.

Introduction

Retinoblastoma (RB) is a common type of primary intraocular malignancy in children (1). RB is caused by mutations in the RB gene and can be life-threatening without treatment when detected in its advanced form (2,3). Treatments for RB mainly include enucleation and/or combinations with chemotherapy, as well as laser treatment or cryotherapy (4,5). Current treatment modalities have associated limitations, such as the systemic side effects of intravenous chemotherapy, and there is an urgent need for the development of novel treatment options for patients with RB.

Long non-coding RNAs (lncRNAs) are a large and diverse class of transcribed RNA molecules that are >200 nucleotides in length and are not translated into proteins (6). The specific expression patterns of certain lncRNAs have been associated with various types of cancer; for example, H19 has been reported to be upregulated in hepatocellular carcinoma (7,8); however, the role of most lncRNAs in oncogenesis remains to be fully determined.

Small nucleolar RNA host gene 20 (SNHG20) is a lncRNA that is considered to have a major role in tumorigenesis (9). SNHG20 is involved in the pathogenesis of various types of cancer, including lung, prostate, gastric and cervical cancer, and glioma (10-14). A recent meta-analysis of 1,149 patients from 15 studies concluded that elevated SNHG20 expression levels are associated with poor prognosis, including overall survival and metastasis, in various types of cancer (15). These data strongly support the hypothesis that SNHG20 acts as an oncogene in most cancer types, such as colorectal cancer and non-small cell lung cancer. However, the role of SNHG20 in the pathogenesis of RB remains unknown.

Interactions between lncRNAs and microRNAs (miRNAs/miRs) that collaborate to form regulatory networks and control gene expression have been observed in numerous studies, and are important factors in understanding the complex molecular mechanisms of various diseases (16,17). miR-335-5p is a miRNA that has decreased expression patterns in various cancer types, including non-small cell lung, colorectal and thyroid cancer (18-20). E2F transcription factor 3 (E2F3) is a transcription factor that is known to promote the progression of various cancer types (21,22) and has recently been found to be highly expressed in RB (23). However, to the best of our knowledge, it is not known whether SNHG20, miR-335-5p and E2F3 can form a lncRNA-miRNA-mRNA regulatory network that drives the pathogenesis of RB. Therefore, the present study aimed to examine the expression patterns of SNHG20 in RB and to identify its potential role in the miR-335-5p/E2F3 signaling axis.

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Materials and methods

Participants and tissue samples. To study the expression pattern of SNHG20 in RB at the tissue level, tissue samples were obtained from the specimen bank of The First People's Hospital of Lianyungang (Lianyungang, China), which collects and stores tissue samples removed from patients who have received surgical therapy at The First People's Hospital of Lianyungang dating back to January 2010. Written informed consent was obtained from all the patients (or their legal guardians), whose surgically removed tissue samples were collected with the agreement that the collected samples could be used in future scientific research. In the present study, RB tissues were obtained from 24 patients (average age, 32±11 months; 10 male and 14 female patients) with RB, and after matching by age and sex, retinal tissues removed for non-RB reasons (mainly ocular trauma) were obtained from eight patients to serve as a control. The tissues were collected between January 2011 and February 2020. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki, and was approved by the Ethics Committee of The First People's Hospital of Lianyungang (approval no. LW-20190620001).

Cell culture. The human RB cell lines, Y79, SO-RB50 and WERI-RB1, and the human retinal pigment epithelial cell line (ARPE-19) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO_2/95% air at 37˚C. Cell culture medium was replaced every 3 days.

Reverse transcription-quantitative (RT-q)PCR. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissues or cells. A Transcriptor First Stand cDNA Synthesis kit (Roche Diagnostics; for mRNA; reaction condition: 55˚C for 30 min) or a miScript II RT kit (Qiagen, Inc.; for miRNA; reaction conditions: 37˚C for 60 min and 95˚C for 5 min) was used to synthesize the cDNA via RT of RNA, according to the manufacturers' instructions. qPCR was then performed using a LightCycler 480 Real-time PCR system (Roche Diagnostics) according to the manufacturer's instructions with a LightCycler® 480 SYBR-Green I Master (Roche Diagnostics). U6 snRNA was chosen as the housekeeping gene for normalization when evaluating the relative expression levels of lncRNA or miRNA, while GAPDH was chosen as the housekeeping gene for normalization when evaluating the relative expression levels of other target genes. The following primers were used for RT-qPCR: SNHG20, forward (F) 5'-ATGGCTATAAATAGATACACGC-3' and reverse (R) 5'-GGTACAAACAGGGAGGGA-3'; miR-335-5p, F 5'-UGUUUUGAGCGGGGGUCAAGAGC-3' and R 5'-CUCUCAUUUGCUAUAUUCA-3'; U6, F 5'-CTCGCTTCGGCAGCACATATACTA-3' and R 5'-ACGAATTTGCGTGTCATCCTTGC-3'; E2F3, F 5'-GATGGGGTCAGATGGAGAGA-3' and R 5'-GAGACACCCTGGCATTGTTT-3'; GAPDH, F 5'-GCACCGTCAAGGCTGAGAAC-3' and R 5'-TGGTGAAGACGCCAGTGGA-3'. The thermocycling conditions for qPCR were as follows: Initial denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 10 sec and annealing at 65˚C for 1 min. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (24).

Small interfering RNA (siRNA) transfection. Cells were assigned to four groups and transfected with either a negative control (NC) siRNA (si-NC, 5'-GGATACGGAGTACTATAGC-3') or with 100 nM si-SNHG20 (#1, 5'-GCCUAGGAUCAUCCAGGUUTT-3'; #2 5'-UAUAGCCAUACACAACAGGTT-3'; #3, 5'-GCCACUCACAAGAGUGUAUTT-3'; Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, exponentially growing cells were seeded in a 24-well plate ($2x10^5$ cells/well in 500 μl RPMI-1640 medium) the day before transfection, in order to reach 30-50% confluence at the time of transfection. Subsequently, 20 pmol siRNA oligomer was diluted in 50 μl Opti-MEM® I Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.) and 1 μl Lipofectamine 2000 was diluted in 50 μl Opti-MEM® I Reduced Serum Medium. After 5-min incubation at room temperature, the diluted oligomer was combined with the diluted Lipofectamine 2000, was mixed gently and incubated for 20 min at room temperature. Subsequently, the oligomer-Lipofectamine 2000 complexes were added to each well containing cells and medium, and the cells were incubated at 37˚C in a CO_2 incubator for 24 h. The efficacy of siRNA transfection was validated via RT-qPCR 24 h after transfection, and the siRNA that had the highest knockdown efficiency was used in further experiments.

miRNA transfection. miR-335-5p mimic (5'-UCAAGAGCAAUAACGAAAAAUGU-3'), miR-335-5p inhibitor (5'-ACAUUUUUCGUUAUUGCUCUUGA-3') and their corresponding negative controls (miRNA-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; miRNA inhibitor-NC, 5'-CAGUACUUUUGUGUAGUACAA-3') were purchased from Shanghai GenePharma Co., Ltd. Similar to the aforementioned siRNA transfection protocol, miRNA transfection was performed using Lipofectamine 2000 with a final concentration of 0.2 pmol/μl, according to the manufacturer's instructions.

Cell-Counting Kit-8 (CCK-8) assay. Cell proliferation was measured using the CCK-8 assay kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. Cells were seeded in 96-well plates ($1x10^3$ cells/well) and cultured in growth medium with CCK-8 solution (10 μl/well) for 0-72 h at 37˚C. Subsequently, the absorbance was measured at 450 nm using a Model 680 microplate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. Colony formation assays were used to assess the clonogenic survival of RB cells following the modulation of SNHG20 expression. After siRNA transfection, cells ($8x10^2$) were cultured in 60-mm dishes for 10 days, fixed with 4% formaldehyde solution at room temperature for 30 min, and stained with 0.2% crystal violet at room temperature for 30 min. Colony numbers were counted according to a 50-cell exclusion rule (i.e., only colonies containing >50 individual cells were counted).

Cell migration assay. The impact of SNHG20 status on cell migration was determined using a cell migration assay. Equal numbers of cells (10^5 cells) were seeded in the upper Transwell (pore size, 8 μm; Costar; Corning, Inc.) chamber (without Matrigel coating) with serum-free medium. The lower chamber contained culture medium supplemented with 10% FBS. After incubating for 18 h in a humidified atmosphere of 5% CO_2/95% air at 37˚C, the cells on the upper surface of the membrane were removed. The membranes were then fixed with 4% paraformaldehyde at room temperature for 15 min, and the cells on the undersurface were stained with 0.1% crystal violet at room temperature for 15 min. Cells were counted under a light microscope with a total magnification of x100).

Cell invasion assay. A Transwell (Costar; Corning, Inc.) with a pore size of 0.8 μm was used to evaluate cell invasion. Cells were seeded in the upper Matrigel-coated (pre-coated at 37˚C for 30 min) chamber with serum-free culture medium. The lower chamber contained culture medium supplemented with 10% FBS. After incubating for 8 h in a humidified atmosphere of 5% CO_2/95% air at 37˚C, cells in the upper chamber were removed carefully with a cotton swab. Cells that had traversed to the reverse side of the membrane were fixed in 4% methanol at room temperature for 15 min, stained with 10% Giemsa at room temperature for 15 min and counted under a light microscope with a total magnification of x100).

miRNA target prediction. The starBase database v2.0 (http://starbase.sysu.edu.cn/) was used to predict the targeting associations between miRNA (miR-335-5p) and lncRNA (SNHG20) or mRNA (E2F3).

Luciferase reporter assay. Based on the results of the miRNA target prediction, the binding sites of miR-335-5p on SNHG20 and E2F3 were validated using a luciferase reporter assay. A pMIR-REPORT vector (Invitrogen; Thermo Fisher Scientific, Inc.) with the 3'-untranslated region fragments of SNHG20 and E2F3 was constructed [SNHG20: Wild-type (WT), 5'-GCUCUUG-3' and mutant (MUT), 5'-CGAGAAC-3'; E2F3: WT, 5'-GCUCUUG-3' and MUT, 5'-GCGAGAAC-3']. Cells were co-transfected with the miR-335-5p mimic or miR-NC and the reporter constructs using Lipofectamine 2000 with a final concentration of 0.2 pmol/μl according to the manufacturer's instructions and as aforementioned. Luciferase activity was measured using a Dual Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's instructions. The luciferase activity of *Renilla* was used to normalize the firefly luciferase activity.

RNA pull-down assay. To determine interactions between the lncRNA SNHG20 and miR-335-5p, an RNA pull-down assay was performed. Synthesized SNHG20 and Bio-NC probes were purchased from Shanghai GenePharma Co., Ltd and were biotinylated using a Biotinylation Kit (Abcam) according to the manufacturer's instructions. Cells were transfected with the biotinylated lncRNA using Lipofectamine 2000 with a final concentration of 0.2 pmol/μl according to the manufacturer's instructions and as aforementioned. A total of 48 h post-transfection, the cells were collected and lysed in lysis buffer [50 mM Tris-HCl (pH 7.0), 10 mM EDTA, 1% SDS supplemented with 200 U/ml RNAse inhibitor solution, and 5 μl/ml protease inhibitor cocktail (EMD Millipore)]. Using the Magnetic RNA-Protein Pull-Down kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, M-280 streptavidin magnetic beads (Invitrogen; Thermo Fisher Scientific, Inc.) were incubated with cell lysates at room temperature for 15 min, and then the miR-335-5p expression levels were determined via RT-qPCR, which was conducted as aforementioned.

Western blotting. Cells were lysed in RIPA buffer (Abcam) and the protein concentrations were determined using a BCA protein assay kit. The supernatants (20 μg total protein/lane) were resolved via SDS-PAGE on 10% gels and transferred to a PVDF membrane (EMD Millipore). The membrane was then blocked with nonfat dried milk diluted in TBS-0.1% Tween at room temperature for 1 h and incubated with the following primary antibodies overnight at 4˚C: Anti-E2F3 (1:1,000; cat. no. ab152126; Abcam) and anti-GAPDH (1:1,000; cat. no. ab181602; Abcam). Following incubation, the membrane was washed and incubated with an appropriate secondary antibody [goat anti-rabbit IgG H&L (HRP); 1:5,000; cat. no. ab205718; Abcam) for 1 h at room temperature. The relative expression of target proteins was visualized using a FluorChem™ E imager (ProteinSimple) with Digital Darkroom software (version 4.1.4; ProteinSimple). Protein expression levels were normalized to the expression of the GAPDH housekeeping gene.

Statistical analysis. Data are presented as the mean ± SD and each experiment was repeated three times. An unpaired Student's t-test was used for statistical comparisons between two groups. For comparisons between >2 groups, one-way ANOVA was used for statistical comparisons and the Bonferroni test was employed for post hoc test. All statistical analyses were performed using SPSS, version 22.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Elevated expression levels of SNHG20 in RB tissues and cell lines. To evaluate the expression patterns of SNHG20 in RB, SNHG20 expression was examined in RB tissues and RB cell lines (WERI-RB1, SO-RB50 and Y79) via RT-qPCR. Compared with normal tissues, the expression levels of SNHG20 were significantly elevated in RB tissues (Fig. 1A). Similar expression patterns were found in all three RB cell lines compared with those in the ARPE-19 cell line (Fig. 1B). Y79 and SO-RB50 cells showed the highest expression levels of SNHG20, and were selected for subsequent experiments.

SNHG20 silencing inhibits the proliferation, migration and invasion of RB cells. To further investigate the role of SNHG20 in RB, three siRNAs were designed to target SNHG20 (si-SNHG20#1, #2 and #3) and were transfected into Y79 and SO-RB50 cells. The expression levels of SNHG20 were determined via RT-qPCR and it was demonstrated that the expression levels of SNHG20 were significantly decreased (by ≥50%) after transfection with SNHG20 siRNA compared

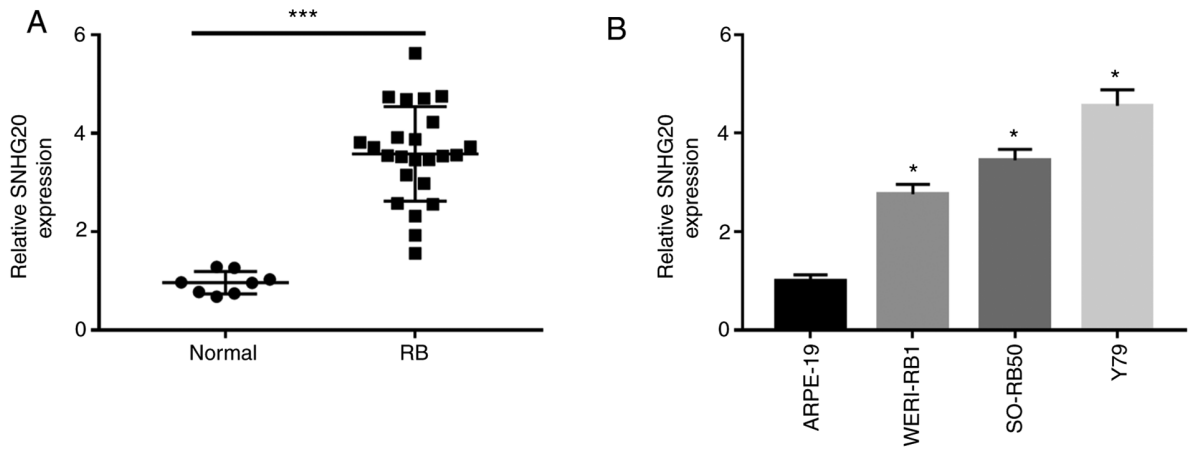

Figure 1. Expression levels of SNHG20 are elevated in RB tissues and cell lines. SNHG20 expression in (A) RB tissues (n=8 normal tissue; n=24 RB tissue) and (B) cell lines as examined via reverse transcription-quantitative PCR. Each experiment was performed in triplicate and repeated three times with similar results unless otherwise specified. *P<0.05 vs. ARPE-19; ***P<0.001. SNHG20, small nucleolar RNA host gene 20; RB, retinoblastoma.

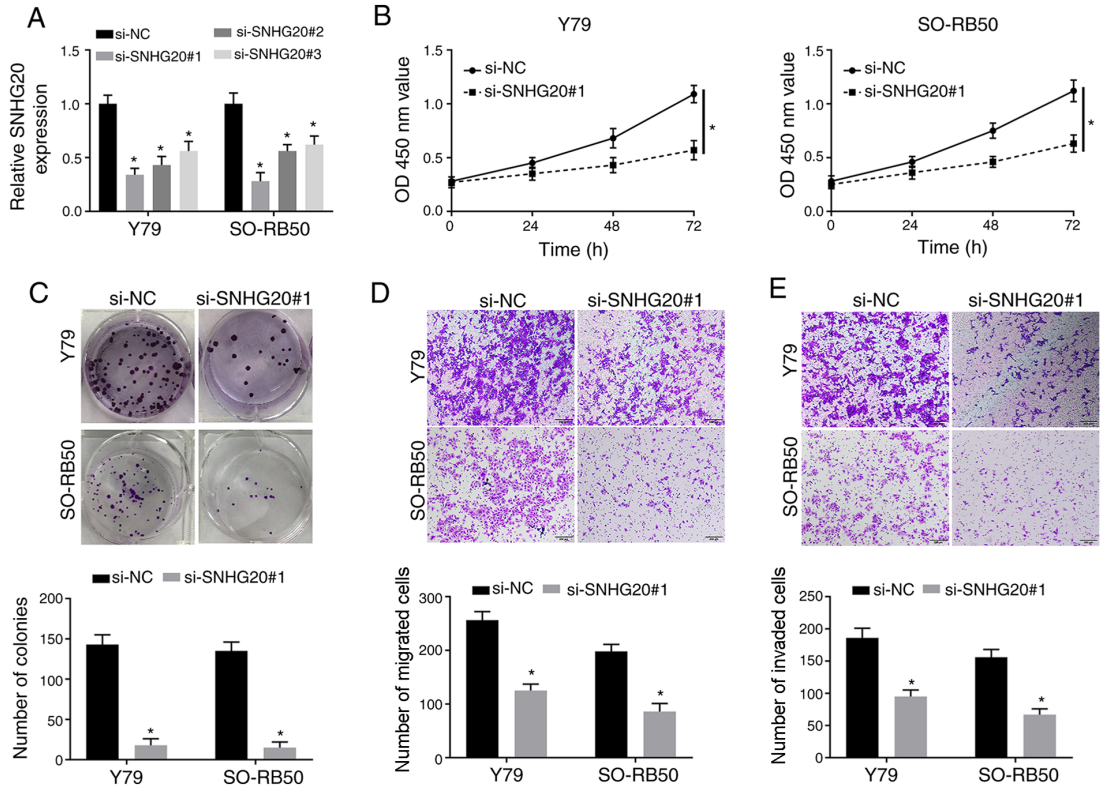

Figure 2. Knockdown of SNHG20 inhibits RB cell proliferation, migration and invasion. Analyses of (A) SNHG20 expression, (B) cell proliferation, (C) clonogenic capacities, (D) cell migration (magnification, x100) and (E) invasion (magnification, x100) in RB cells transfected with si-SNHG20#1 or si-NC. Each experiment was performed in triplicate and repeated three times with similar results. *P<0.05 vs. si-NC. NC, negative control; si, small interfering; SNHG20, small nucleolar RNA host gene 20; OD, optical density; RB, retinoblastoma.

with si-NC. Moreover, the si-SNHG20#1 construct showed the highest level of SNHG20 knockdown (Fig. 2A) and was therefore selected for subsequent experiments.

The results from the functional assays demonstrated that knockdown of SNHG20 significantly inhibited the proliferation (Fig. 2B), clonogenic survival (Fig. 2C), migration (Fig. 2D) and invasion (Fig. 2E) in Y79 and SO-RB50RB cells.

The bioinformatics analysis identified that SNHG20 possessed a potential binding site for miR-335-5p (Fig. 3A). The transfection efficiency of the miR-335-5p mimic is shown in Fig. 3B. Results from the luciferase activity reporter assay demonstrated that luciferase activity was significantly inhibited by the miR-335-5p mimic in cells transfected with SNHG20-WT (Fig. 3C and D). A direct combination of SNHG20 and miR-335-5p was confirmed based on the results of the RNA pull-down assay (Fig. 3E). Moreover, the RT-qPCR results indicated that the expression levels of miR-335-5p were increased after SNHG20 knockdown (Fig. 3F).

Based on the results of the bioinformatics analyses (Fig. 4A), E2F3 was identified to be a potential target for

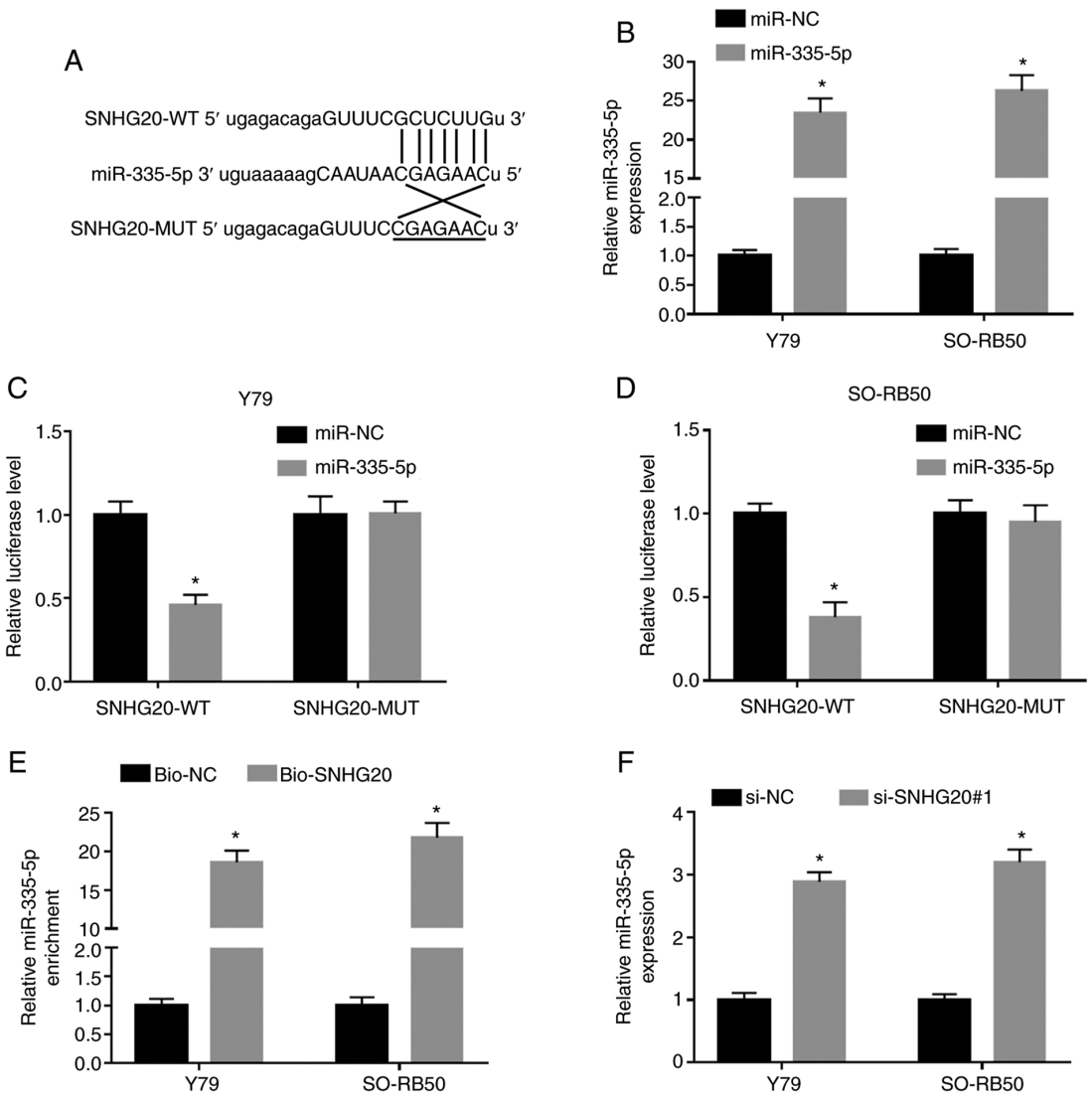

Figure 3. SNHG20 directly targets miR-335-5p in RB. (A) A putative binding site of SNHG20 was located in miR-335-5p. (B) RT-qPCR results identified that, compared with miR-NC, miR-335-5p increased miR-335-5p expression in RB cells. Transfection with miR-335-5p mimic decreased the luciferase activity of SNHG20-WT but not SNHG20-MUT in (C) Y79 and (D) SO-RB50 cells. *P<0.05 vs. miR-NC. (E) A significant enrichment of miR-335-5p was obtained using the SNHG20 pull down pellets (Bio-SNHG20) compared with Bio-NC. *P<0.05 vs. Bio-NC. (F) RT-qPCR results indicated that SNHG20 negatively regulated miR-335-5p expression in RB cells. Each experiment was performed in triplicate and repeated three times with similar results. *P<0.05 vs. si-NC. NC, negative control; si, small interfering; SNHG20, small nucleolar RNA host gene 20; miR, microRNA; RB, retinoblastoma; WT, wild-type; MUT, mutant; Bio, biotinylated; RT-qPCR, reverse transcription-quantitative PCR.

miR-335-5p. These findings were validated via the luciferase activity reporter assay (Fig. 4B), which demonstrated that luciferase activity was significantly inhibited by the miR-335-5p mimic in cells transfected with E2F3-WT. Compared with the miR-NC, overexpression of miR-335-5p significantly inhibited the expression of E2F3 at the mRNA and protein levels (Fig. 4C and D).

SNHG20 promotes RB progression via the regulation of the miR-335-5p/E2F3 axis. To validate the associations between SNHG20, miR-335-5p and E2F3, the Y79 and SO-RB50 RB cell lines were transfected with si-NC, si-SNHG20#1 or SNHG20#1 + miR-335-5p inhibitor. The transfection efficiency of the miR-335-5p inhibitor is shown in Fig. 5A. Knockdown of SNHG20 significantly increased the expression of miR-335-5p, which was reversed by inhibition of miR-335-5p (Fig. 5B). Western blotting results demonstrated that knockdown of SNHG20 significantly inhibited the expression of E2F3, which was reversed by inhibition of miR-335-5p (Fig. 5C). Similar regulatory patterns were found in the other functional studies, which demonstrated that knockdown of SNHG20 significantly inhibited cell proliferation (Fig. 5D), clonogenic survival (Fig. 5E), migration (Fig. 5F) and invasion (Fig. 5G), which were reversed by inhibition of miR-335-5p.

Discussion

The current study investigated the expression patterns of lncRNA SNHG20 in RB and examined its role in the pathogenesis of RB. The present results demonstrated that the expression of SNHG20 was elevated in RB and served a role in oncogenesis by promoting cell proliferation, migration and invasion via the miR-335-5p/E2F3 axis. To the best of our knowledge, this was the first study to evaluate the

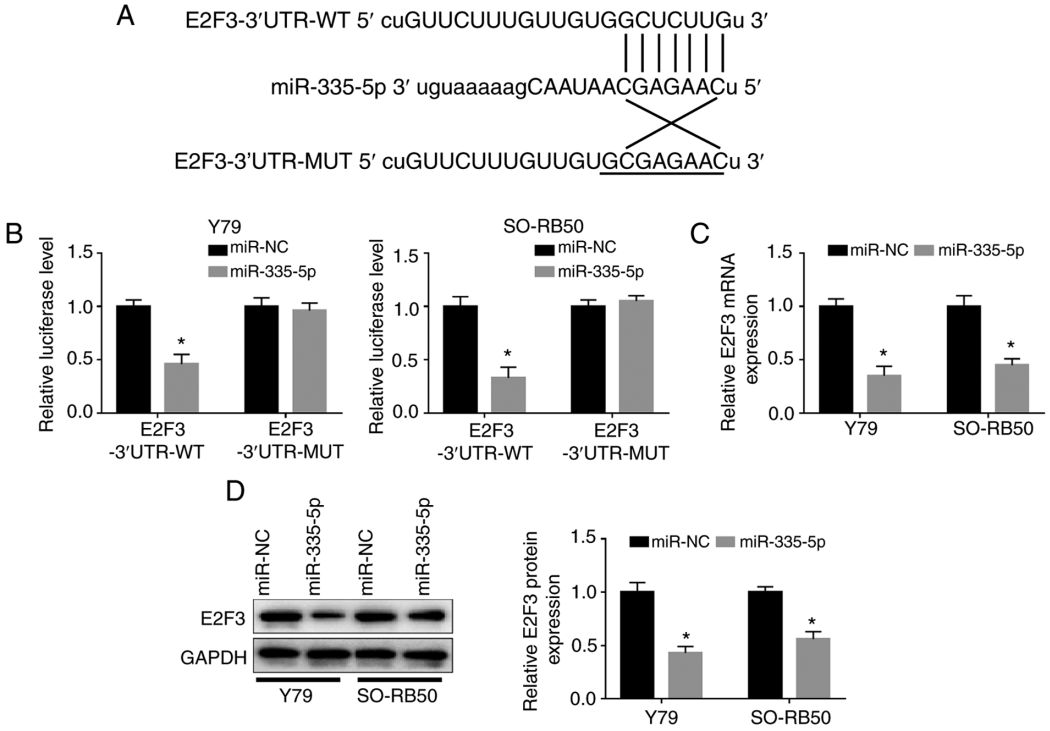

Figure 4. miR-335-5p directly targets E2F3 in RB. (A) A putative binding site of E2F3 was located in miR-335-5p. (B) Transfection with miR-335-5p mimic decreased the luciferase activity of E2F3-WT but not E2F3-MUT. (C) Reverse transcription-quantitative PCR and (D) western blotting demonstrated that miR-335-5p negatively regulated E2F3 expression in RB cells. Each experiment was performed in triplicate and repeated three times with similar results. *P<0.05 vs. miR-NC. NC, negative control; miR, microRNA; RB, retinoblastoma; WT, wild-type; MUT, mutant; E2F3, E2F transcription factor 3.

expression patterns and role of SNHG20 in RB. Furthermore, for the first time, a lncRNA-miRNA-mRNA regulatory network (SNHG20/miR-335-5p/E2F3) in RB was identified that may provide further opportunities in RB research.

The present data suggested SNHG20 as a potential novel target in RB. Moreover, it was observed that dysregulation of the SNHG20/miR-335-5p/E2F3 axis was associated with cell proliferation, migration and invasion of RB. These data suggested that this axis could be involved in various cancer phenotypes and may contribute to the pathogenesis of RB. In addition, SNHG20 may serve as a biomarker in RB, for early diagnosis, prognosis and prediction of response to treatment.

Accumulating evidence supports a central role for lncRNAs in tumorigenesis, with numerous lncRNAs being dysregulated in various cancer types, such as NEAT1 in prostate cancer (25). In RB, several lncRNAs have been reported to be responsible for different cellular functions and may have roles as prognostic and predictive biomarkers (26). While SNHG20 has been studied in numerous other types of cancer, there is a lack of evidence for its role in RB.

The present study evaluated the expression patterns of SNHG20 in RB tissues and several RB cell lines, identifying a ~4-fold increase in SNHG20 expression levels in RB tissues compared with in normal tissues. To further investigate the role of SNHG20 in RB and based on the bioinformatics predictions, two other regulators, miR-335-5p and E2F3, that are known to be involved in multiple cancer types were examined.

Although the role of miR-335-5p has not yet been reported in RB, its role as a tumor suppressor is well established in other cancer types, such as gastric cancer (27-29). However, the targets of miR-335-5p involved in tumorigenesis vary across different studies. Tang *et al* (30) reported copine 1 to be a target of miR-335-5p that may be involved in the pathogenesis of non-small cell lung cancer. Moreover, Yao *et al* (31) revealed that miR-335-5p negatively regulated granulosa cell proliferation via serum/glucocorticoid regulated kinase family member 3. These data indicated that dysregulated miR-335-5p may contribute to tumorigenesis via different cancer-specific mechanisms.

In the present study, E2F3 was selected as a potential target of miR-335-5p, which was validated via the luciferase activity reporter assay. The current study reported the role of the SNHG20/miR-335-5p/E2F3 axis in RB cell lines. As E2F3 has been shown to be involved in several types of cancer (22), the lncRNA-miRNA-mRNA regulatory network (the SNHG20/miR-335-5p/E2F3 axis) identified in the current study may also exist in other types of cancer.

Although the present study identified and initially validated the SNHG20/miR-335-5p/E2F3 axis in RB, it should be noted that other underlying mechanisms may control the abnormal expression pattern of SNHG20 in RB. For instance, Cui *et al* (32) reported that SNHG20 could promote cell proliferation and invasion in gastric cancer by upregulating miR-495-3p, which may act to inhibit the expression of the zinc finger protein X-linked gene. Furthermore, Lingling *et al* (33) revealed that SNHG20 promoted the proliferation, migration and invasion of non-small cell lung cancer cells by sponging miR-154. There may also be numerous other genes targeted by miR-335-5p that could contribute to tumorigenesis. A previous network enrichment analysis indicated that several genes involved in metastasis and tumor invasion, such as plasminogen activator, urokinase receptor, CDH11, collagen

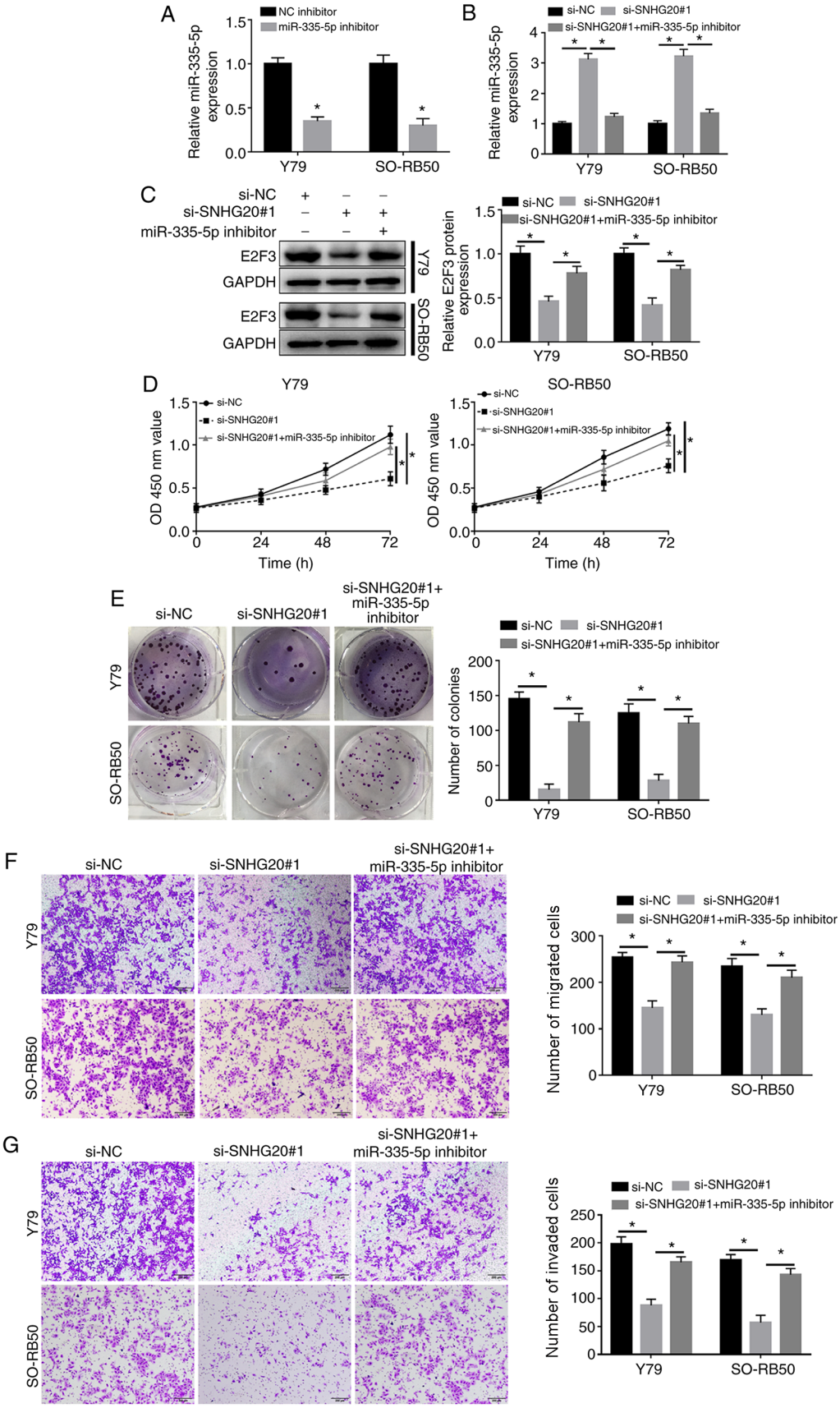

Figure 5. SNHG20 promotes RB progression via regulation of the miR-335-5p/E2F3 axis. (A) RT-qPCR results demonstrated that, compared with NC inhibitor, the miR-335-5p inhibitor decreased miR-335-5p expression in RB cells. (B) RT-qPCR results revealed that, compared with si-NC, transfection of si-SNHG20#1 increased miR-335-5p expression in RB cells, which was reversed by miR-335-5p inhibitor treatment. Knockdown of SNHG20 significantly inhibited the (C) expression of E2F3, (D) cell proliferation, (E) clonogenic capacities, (F) cell migration (magnification, x100) and (G) invasion (magnification, x100) in RB cells, which could be reversed by the miR-335-5p inhibitor. Each experiment was performed in triplicate and repeated three times with similar results. *P<0.05 vs. NC inhibitor or as indicated. NC, negative control; miR, microRNA; RB, retinoblastoma; E2F3, E2F transcription factor 3; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; SNHG20, small nucleolar RNA host gene 20; OD, optical density.

type IV α 2 chain, cellular communication network factor 2, cathepsin K, MMP7, platelet derived growth factor subunit A, TIMP metallopeptidase inhibitor (TIMP) 1 and TIMP2, were regulated by miR-335-5p (28). These studies suggested that the role of SNHG20 in tumorigenesis may be significantly complex and requires further investigation.

The present study observed the expression pattern of SNHG20 at both the cell and tissue levels in RB. Several methods were used to investigate and validate the SNHG20/miR-335-5p/E2F3 regulatory network, including luciferase reporter and RNA pull-down assays. Despite robust and convincing experimental observations, the current study had some limitations. Firstly, the SNHG20/miR-335-5p/E2F3 regulatory network was only validated at the cellular level and requires further validation in animal models. Secondly, as aforementioned, the SNHG20/miR-335-5p/E2F3 axis could be only one of several potential mechanisms accounting for the abnormal expression patterns of SNHG20 in RB. Finally, although the present study used several methods to initially validate the SNHG20/miR-335-5p/E2F3 axis, other advanced methods such as RNA sequencing may be used to further examine the underlying mechanisms. In addition, it should be noted that the lack of protein validation tests for the migration and invasion assays is also a limitation, and further assays are required in the future.

In conclusion, the present study identified the mechanism of SNHG20 in RB tumorigenesis. It was found that SNHG20 was highly expressed, and may promote cell proliferation, migration and invasion via the miR-335-5p/E2F3 axis. However, additional in-depth studies are required to further elucidate the underlying molecular mechanisms.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JS and ZZ designed and conducted the study, including data analysis, and are responsible for confirming the authenticity of all the raw data. JS created the draft of the manuscript and ZZ revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the First People's Hospital of Lianyungang (approval no. LW-20190620001). Written informed consent was obtained from the parents or legal guardians of the patients; surgically removed tissue samples were collected from the patients with the agreement that the collected samples could be used in future scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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