MicroRNA-361-3p regulates retinoblastoma cell proliferation and stemness by targeting hedgehog signaling

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Abstract. Retinoblastoma (RB) is the most common type of intraocular malignancy in children. During RB oncogenesis, sonic hedgehog (SHH) is commonly differentially expressed. Additionally, microRNAs (miRs) are known to serve crucial roles as oncogenes or tumor suppressors. Specifically, miR-361-3p has been revealed to serve a vital role in cutaneous squamous cell carcinoma, cervical cancer, prostate cancer, colorectal cancer, gastric cancer, hepatocellular carcinoma, breast cancer and lung cancer. However, the role of miR-361-3p in RB and the potential molecular mechanisms involved remain unknown. Therefore, the current study aimed to determine the involvement of miR-361-3p in the development of RB by targeting SHH signaling. In the present study, miR-361-3p expression levels in RB tissue and serum samples obtained from 10 patients with RB, normal retinal tissue and serum samples obtained from 10 healthy controls, and two human RB cell lines (Y79 and Weri-Rb-1) were determined using reverse transcription-quantitative polymerase chain reaction. In addition, a cell counting kit-8 assay, a cell transfection assay, a MTT assay, western blotting, a tumor sphere formation assay and a luciferase assay were used to assess the expression, function and molecular mechanism of miR-361-3p in human RB. It was demonstrated that miR-361-3p was significantly downregulated in RB tissues, RB serum and RB cell lines compared with normal retinal tissues and normal serum. The ectopic expression of miR-361-3p decreased RB cell proliferation and stemness. Furthermore, GLI1 and GLI3 were verified as potential direct targets of miR-361-3p. miR-361-3p was also revealed to exhibit a negative correlation with GLI1/3 expression in RB samples. Taken together, the results indicate that miR-361-3p functions as a tumor suppressor in the carcinogenesis and progression of RB by targeting SHH signaling. Thus, miR-361-3p should be further assessed as a potential therapeutic target for RB treatment.

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Introduction

Retinoblastoma (RB) is the most prevalent type of primary malignant intraocular cancer of infancy and childhood, particularly in children <5 years old (1). It represents ~4% of all pediatric malignancies (2). It is estimated that 5,000 cases of RB are diagnosed each year, worldwide, with 250-300 cases in the United States alone (2). The clinical manifestations of RB vary greatly with disease stage (3). In its earliest clinical stage, RB presents as a small transparent or slightly translucent lesion in the sensory retina (2). Larger tumors exhibit retinal blood vessel dilation or foci of chalk-like calcification (3). Often, RB of any size presents as leukocoria, also known as the white pupillary reflex, at more advanced stages (2). The survival rate of RB is >95% in the United States, but is 50% worldwide as RB is often detected when it has invaded the orbit or brain (2). Current disease management includes chemotherapy, enucleation, laser therapy or cryotherapy (4). However, the most common treatment protocol includes chemotherapy combined with immunotherapy, which is cytotoxic to RB cells (5). However, this treatment produces various adverse effects, including the acceleration of RB progression, meaning that the development of other therapeutic methods, particularly those that target specific signaling pathways, is required (4,6).

MicroRNAs (miRs) are small (20-25 nucleotides), noncoding RNAs that regulate the expression of certain target genes in various physiological and pathological processes (7). They serve key roles as oncogenes or tumor suppressors and thus regulate tumor progression and metastasis (8,9). Several critical miRs have been identified in RB progression. Hsa-miR-373 may be involved in RB invasion and metastasis, while hsa-miR-125b and hsa-let-7b may function as tumor suppressors by co-regulating cell division protein kinase 6, cell division cycle 25A and lin-28 homolog A (10,11). Furthermore. hsa-miR-181a may serve a key role in the cyclin-dependent kinase inhibitor 1B-regulated cell cycle pathway (10). Certain miRs, including hsa-miR-25, hsa-miR-18a and hsa-miR-20a may affect the progression of RB by coregulating BCL2-like

1 (10). Furthermore, miR-497 functions as a tumor suppressor in RB carcinogenesis and progression (12). He *et al* (13), demonstrated the suppressive effect of miR-143 in RB by targeting CDK6. In addition, miR-31 and miR-200a expression was revealed to be significantly reduced in human RBs and was determined to mediate the regulation of RB proliferation (4). miR-125b is also significantly upregulated in RB, promoting tumor growth and suppressing apoptosis (11). Castro-Magdonel *et al* (14), has recently revealed a miR landscape that exhibits a core-cluster of 30 miRs in RB. miR-3613 was also identified as being a potential and critical downregulatory marker in RB (14). However, the regulatory mechanisms involving miR in RB are yet to be fully elucidated.

Accumulating evidence has indicated that miR-361 serves important roles in various types of human tumor, including cutaneous squamous cell carcinoma (15), cervical cancer (16), prostate cancer (17), colorectal cancer (17), gastric cancer (18), hepatocellular carcinoma (19), breast cancer (20) and lung cancer (21). Additionally, the positive expression of miR-361-5p indicates a better prognosis for patients with breast cancer (20). Furthermore, Chen *et al* (21) revealed that miR-361-3p suppresses tumor cell proliferation and metastasis by directly targeting Src homology 2 B adapter protein 1 in non-small cell lung cancer (NSCLC).

According to Chen *et al* (21), sonic hedgehog (SHH) signaling proteins are frequently expressed in RB and are associated with aggressive clinicopathological features, including optic nerve invasion, choroidal invasion, extraocular invasion and metastasis. The activation of SHH signaling is regulated by the glioma-associated oncogene homologue (GLI) transcription factors (GLI1, 2 and 3), which induce several downstream pathways, including ATP-binding cassette G2 (22). The SHH pathway has been demonstrated to be dysregulated in various different types of cancer, including basal cell carcinoma and medulloblastoma (23,24). However, few studies have assessed the role of SHH signaling in RB (25).

As miRs are involved in neoplastic progression, the current study hypothesized that the SHH pathway may serve a key role in the development or progression of RB by regulating miR expression as it has been reported that miRs are involved in many types of invasive tumor by targeting SHH proteins (25). To the best of our knowledge, this is the first study to determine the involvement of miR-361-3p in RB by targeting SHH signaling.

Materials and methods

Clinical samples. The present study was performed under the approval of the Clinical Research Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University (Qiqihar, China), and written informed consent was obtained from each patient prior to surgical excision. RB tissues, RB serums and matched adjacent, non-tumorous normal tissues were collected from 10 patients with RB who did not receive any preoperative therapies, including radiotherapy and chemotherapy. All patients (aged from 1 to 30 months) underwent ophthalmectomy at The Third Affiliated Hospital of Qiqihar Medical University between 2014 and 2017. The clinical characteristics of the patients with RB included in the current study are presented in Table I. Non-tumorous normal tissues were obtained separately from an opposite quadrant of the tumor site (>5 mm from the margin of the tumor) to avoid RB contamination. Control serums were obtained for 10 healthy participants. Following surgery, fresh RB and serum specimens were immediately snap-frozen in liquid nitrogen and stored at -80˚C for further examination.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from RB cells or tissues using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The experiment was performed as previously described (26). The Taqman miR Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used. RT-qPCR analysis of miR was performed using the TaqMan miR assay system (Applied Biosystems, Foster City, CA, USA). Small nuclear RNA U6 was utilized as an endogenous control. The relative expression of mRNA was determined using a SYBR Green RT-qPCR assay (Invitrogen; Thermo Fisher Scientific, Inc.), with β-actin as an endogenous control. All RT-qPCR procedures were performed using an ABI 7500 thermocycler (Invitrogen; Thermo Fisher Scientific, Inc.). Relative expression was calculated using the $2^{-\Delta\Delta cq}$ method (27). The thermocycling conditions were as follows: 95˚C for 2 min, followed by 30 cycles of amplification at 94˚C for 40 sec, annealing at 55˚C for 40 sec and extension at 72˚C for 1 min, with a final elongation step at 72˚C for 10 min. The primers utilized were as follows: GLI1 forward, 5'-TTC CTACCAGAGTCCCAAGT-3' and reverse, 5'-CCCTAT GTGAAGCCCTATTT-3'; GLI3 forward, 5'-GAAGTGCTC CACTCGAACAGA-3' and reverse, 5'-GTGGCTGCATAG TGATTGCG-3'; cluster of differentiation (CD)133 forward, 5'-GAAAAGTTGCTCTGCGAACC-3' and reverse, 5'-CTC GACCTCTTTTGCAATCC-3'; SRY box-2 (SOX-2) forward, 5'-GGGAAATGGAGGGGTGCAAAAGAGG-3' and reverse, 5'-TTGCGTGAGTGTGGATGGGATTGGTG-3'; Nanog forward, 5'-TCCTCCTCTTCCTCTATACTAAC-3' and reverse, 5'-CCCACAATCACAGGCATAG-3'; β-actin forward 5'-AAGGGACTTCCTGTAACAATGCA-3' and reverse, 5'-CTGGAACGGTGAAGGTGACA-3'; miR-361-3p forward, 5'-ACACTCCAGCTGGGTCCCCCAGGTGTGATTC-3' and reverse, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCA GTTGAGAAATCAGA-3'.

RB cells and cell transfection. Two human RB cell lines (Y79 and Weri-Rb-1) were obtained from The American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were then left to grow in a humidified incubator containing 5% CO_2 at 37˚C. miR-361-3p, control miR or miR-361-3p inhibitor (all miRs, 100 μM; all miRs, Shanghai GenePharma Co., Ltd., Shanghai, China) were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following 48 h, subsequent experiments were performed. The sequences of miR-361-3p and control miR were as follows: miR-361-3p, 5'-UCCCCCAGG UGUGAUUCUGAUUU-3'; control miR, 5'-UUCUCCGAA CGUGUCACGUTT-3'.

Table I. Clinical characteristics of patients with RB.

Case	Gender	Age (months)	Optic nerve invasion	Choroidal invasion	Extraocular invasion	Differentiation
1	Male	23	Yes	Yes	No	Poor
2	Male	25	No	No	No	Moderate
3	Female	13	Yes	Yes	No	Moderate
4	Male	4	Yes	Yes	No	Poor
5	Male	16	Yes	Yes	No	Poor
6	Male	22	Yes	Yes	No	Poor
7	Female	7	Yes	No	No	Moderate
8	Male	19	Yes	No	No	Poor
9	Female	8	Yes	Yes	No	Moderate
10	Female	10	Yes	Yes	No	Poor

MTT assay. An MTT assay was utilized to assess RB cell proliferation. Y79 and Weri-Rb-1 cells ($5x10^3$ cells per well) were seeded in 96-well plates. At 24, 48, 72 and 96 h following transfection, MTT (Beyotime Institute of Biotechnology, Haimen, China) at a final concentration of 0.5 mg/ml was added to each well and cultured at 37˚C for 4 h. The solution was removed and the precipitates were dissolved in dimethyl sulfoxide. Absorbance was measured at 570 nm using a microplate reader.

Cell counting kit-8 (CCK-8) assay. RB cell proliferation was assessed using A CCK-8 assay (Beyotime Institute of Biotechnology) was performed to confirm the results of proliferation, according to the manufacturer's protocol. Y79 and Weri-Rb-1 cells were seeded into 96-well plates ($5x10^3$ cells per well). Following 48 and 96 h of transfection, the CCK-8 reagent was added to each well (dilution, 1:10) and cells were incubated for 2 h at 37˚C. Absorbance was measured at a 450 nm using a microplate reader.

Western blotting. Cells were harvested using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was determined using the BCA method (Beyotime Institute of Biotechnology). Total protein (20 μg) was separated using SDS-PAGE (10% gels) and electrophoretically transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, membranes were blocked with 5% non-fat milk solution for 1 h at room temperature, and then incubated with the following primary and secondary antibodies (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight: GLI1 (cat. no. sc-20687; 1:500), GLI3 (cat. no. sc-20688; 1:500), CD133 (cat. no. sc-19365; 1:500), SOX-2 (cat. no. sc-365823; 1:500), Nanog (cat. no. sc-374103; 1:500) and β-actin (cat. no. sc-47778; 1:1,000). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated immunoglobulin G secondary antibodies (horseradish peroxidase conjugated goat anti-rabbit Immunoglobulin G, cat. no. sc-2004 and horseradish peroxidase conjugated goat anti-mouse Immunoglobulin G, cat. no. sc-2005; each, 1:10,000; each, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Membranes were then washed and proteins were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and exposed to X-ray film (Eastman Kodak, Rochester NY, USA). ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA) was used for densitometry.

Tumor sphere formation assay. Y79 and Weri-Rb-1 cells ($5x10^3$ cells per well) were seeded into ultra-low attachment 24-well plates (Corning Incorporated, Corning, NY, USA). All cells were maintained in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), containing 20 ng/ml epidermal growth factor (Invitrogen; Thermo Fisher Scientific, Inc.) and 10 ng/ml basic fibroblast growth factor (Invitrogen; Thermo Fisher Scientific, Inc.). Following 7 days of culture in a humidified incubator containing 5% CO_2 at 37˚C, tumor spheres were counted and images were captured at a magnification of x200 using a light microscope (Nikon Corporation, Tokyo, Japan).

TargetScan online tool analysis. The TargetScan online tool (http://www.targetscan.org/vert_72/) was utilized to predict the targets of the miRNAs being assessed.

Luciferase assay. The 3' untranslated (UTR) regions of GLI1 and GLI3 containing putative miR-361-3p binding sites were constructed based on the psiCHECK-2 plasmid (Promega Corporation, Madison, WI, USA). Each plasmid was co-transfected with miR-361-3p or control miR (miR without targets) in RB cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase and Renilla signals were measured 48 h following co-transfection using a Dual-Luciferase Reporter Assay kit (Promega Corporation) according to the manufacturer's protocol and normalized against the activity of *Renilla* luciferase activity.

Statistical analysis. Each experiment was performed in triplicate and repeated at least three times. Data are expressed as the mean ± standard deviation and were analyzed using a Student's t-test or one-way analysis of variance followed by a Tukey's multiple comparison test. A Pearson's correlation analysis performed to assess the correlation between miR-361-3p and Gli1 expression. Statistically significant differences were

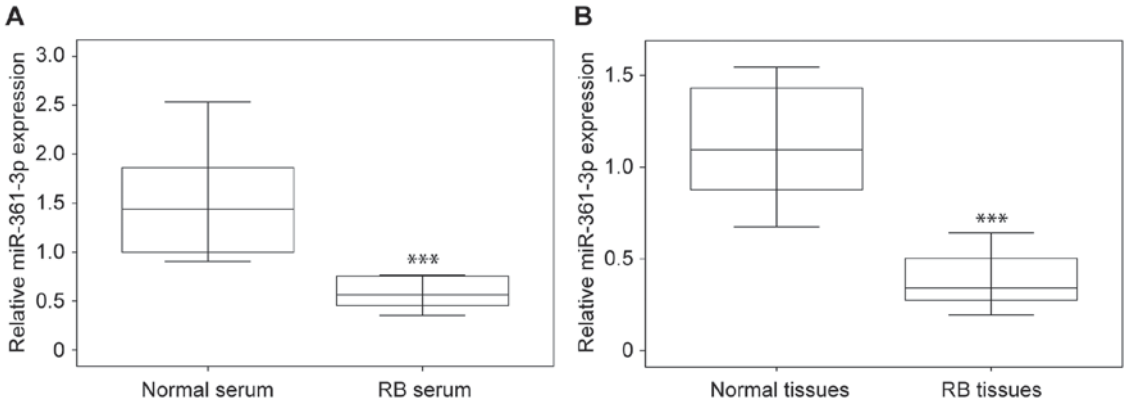

Figure 1. Expression of miR-361-3p is downregulated in the serum and tissue of patients with RB. (A) Reverse transcription-quantitative polymerase chain reaction analysis of miR-361-3p expression in the serum of 10 patients with RB and 10 healthy participants. (B) miR-361-3p expression in RB samples and normal tissue obtained from patients with RB. ***P<0.001 vs. normal serum or normal tissues. miR, microRNA; RB, retinoblastoma.

determined using SPSS version 16 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant result.

Results

Expression of miR-361-3p is downregulated in the serum and tumor tissues of patients with RB. serum and tumor samples were obtained from 10 patients with RB and 10 healthy participants to assess the expression of miR-361-3p. The results revealed that the expression of miR-361-3p was significantly downregulated in RB serum (Fig. 1A). Furthermore, compared with normal adjacent tissues, miR-361-3p levels were significantly decreased in RB samples (Fig. 1B). These results indicate that miR-361-3p levels are decreased miR-361-3p RB patient serum and tumor samples.

miR-361-3p inhibits RB cell proliferation. To study the function of miR-361-3p in RB, functional assays were performed in Y79 and Weri-Rb-1 cell lines. The results demonstrated that the expression of miR-361-3p was increased in RB cell lines transfected with miR-361-3p when compared with control miR (Fig. 2A). Cell malignancies between the miR-361-3p and control miR groups were also compared. An MTT cell proliferation assay was performed following Y79 and Weri-Rb-1 cell transfection with miR-361-3p or control miR. The results revealed that miR-361-3p significantly reduced RB cell proliferation (Fig. 2B). Cell proliferation was further analyzed using a CCK-8 assay. The results revealed that miR-361-3p inhibited Y79 and Weri-Rb-1 cell proliferation (Fig. 2C). Furthermore, miR-361-3p was inhibited using a miR-361-3p inhibitor (Fig. 2D). The results of the proliferation assays revealed that the inhibition of miR-361-3p significantly promoted RB cell proliferation (Fig. 2E and F). These results indicate that miR-361-3p reduces RB cell proliferation.

miR-361-3p restrains RB cell stemness. In order to investigate the effect of miR-361-3p on RB cell stemness, the expression of stem cell markers including CD133, SOX-2 and Nanog were assessed using RT-qPCR. The expression levels of the aforementioned genes were significantly decreased in miR-361-3p overexpressed RB cells (Fig. 3A). Western blotting further confirmed the reduction of stem cell markers in miR-361-3p-overexpressed RB cells (Fig. 3B). A tumor sphere assay was subsequently performed to detect the ability of RB stem cells to self-renew. The results revealed that miR-361-3p markedly reduced RB cell self-renewal (Fig. 3C). In addition, a miR-361-3p inhibitor was transfected into RB cells and cells markers were identified using RT-qPCR and western blotting. The results demonstrated that the inhibition of miR-361-3p significantly increased the expression of these markers (Fig. 4A and B). A tumor sphere assay was then performed and it was determined that the miR-361-3p inhibitor promoted RB cell self-renewal (Fig. 4C). The results indicated that miR-361-3p inhibited Y79 and Weri-Rb-1 cell stemness.

miR-361-3p directly targets GLI1 and GLI3. To assess the molecular mechanism underlying the effect of miR-361-3p on RB cells, potential miR-361-3p targets were predicted using TargetScan online tool which identified two conserved binding sites of miR-361-3p in the 3'UTR of GLI1 and GLI3 mRNA (Fig. 5A). The current study hypothesized that GLI1 and GLI3 were potential targets of miR-361-3p due to their function in RB development. To confirm this, a luciferase reporter assay was performed using a psiCheck-2 reporter with wild-type of GLI1 or GLI3 3'UTR. The results revealed that miR-361-3p significantly inhibited luciferase activity (Fig. 5B). The protein expression levels of GLI1 and GLI3 were assessed in Y79 and Weri-Rb-1 cells following miR-361-3p transfection. As presented in Fig. 4C, GLI1 and GLI3 expression levels in Y79 and Weri-Rb-1 cells were reduced following miR-361-3p transfection. Together, these results demonstrated that GLI1 and GLI3 are miR-361-3p targets.

Negative correlation between miR-361-3p and GLI1/3 in RB samples. A Pearson's correlation analysis was performed to assess miR-361-3p and its targets, GLI1 and GLI3. As presented in Fig. 6, a negative correlation was observed between miR-361-3p and GLI1/3 in RB tissues.

Discussion

According to a previous study, miR-361 has been implicated in the development and progression of various types of

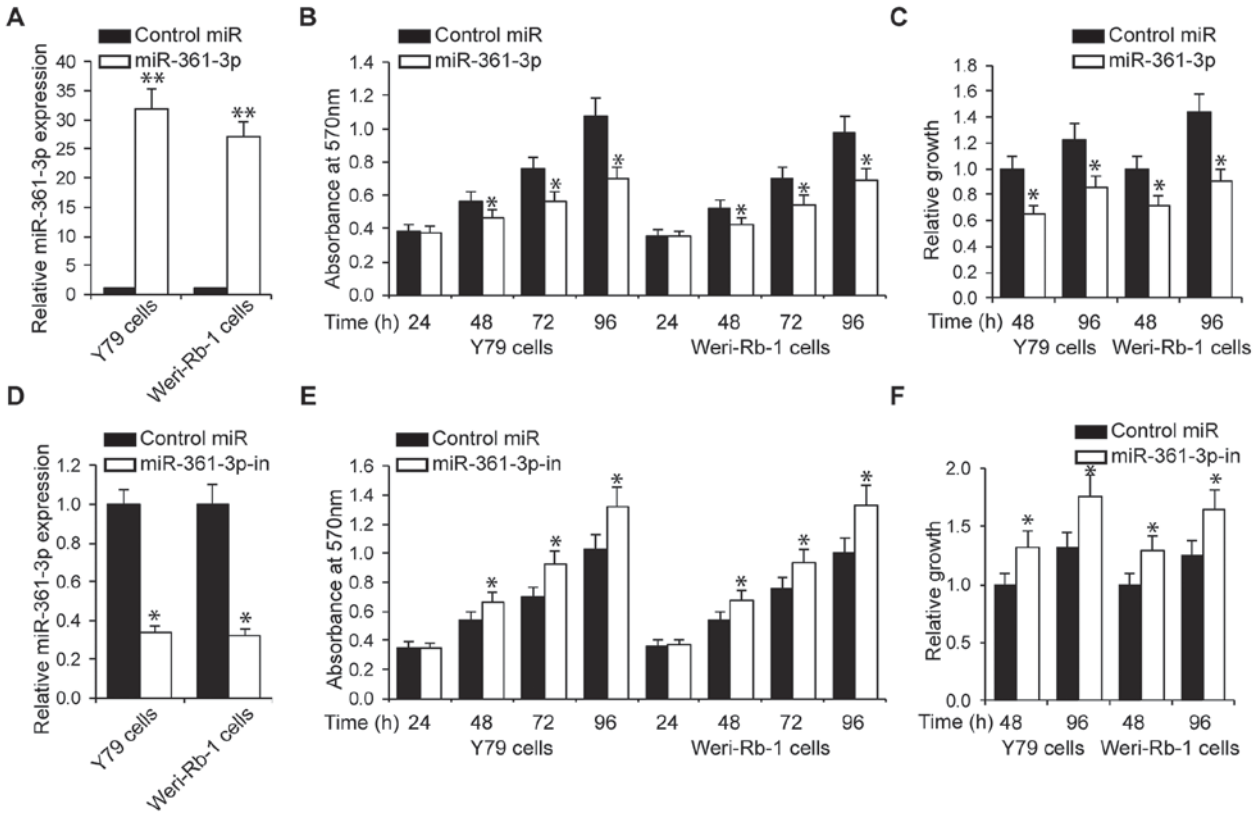

Figure 2. miR-361-3p inhibits RB cell proliferation. (A) RT-qPCR analysis of miR-361-3p expression in Y79 and Weri-Rb-1 RB cells following transfection with miR-361-3p or control miR for 48 h. (B) An MTT assay was performed in Y79 and Weri-Rb-1 RB cells that exhibited miR-361-3p overexpression. (C) Relative cell proliferation was determined using a CCK-8 assay. (D) RT-qPCR analysis of miR-361-3p inhibitor expression in Y79 and Weri-Rb-1 RB cells following transfection with a miR-361-3p inhibitor or control miR for 48 h. (E) An MTT assay was performed following miR-361-3p inhibition. (F) Relative cell growth was assessed using a CCK-8 assay following miR-361-3p inhibition. *P<0.05 and **P<0.01 vs. control miR. miR, microRNA; RB, retinoblastoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, cell counting kit-8; in, inhibitor.

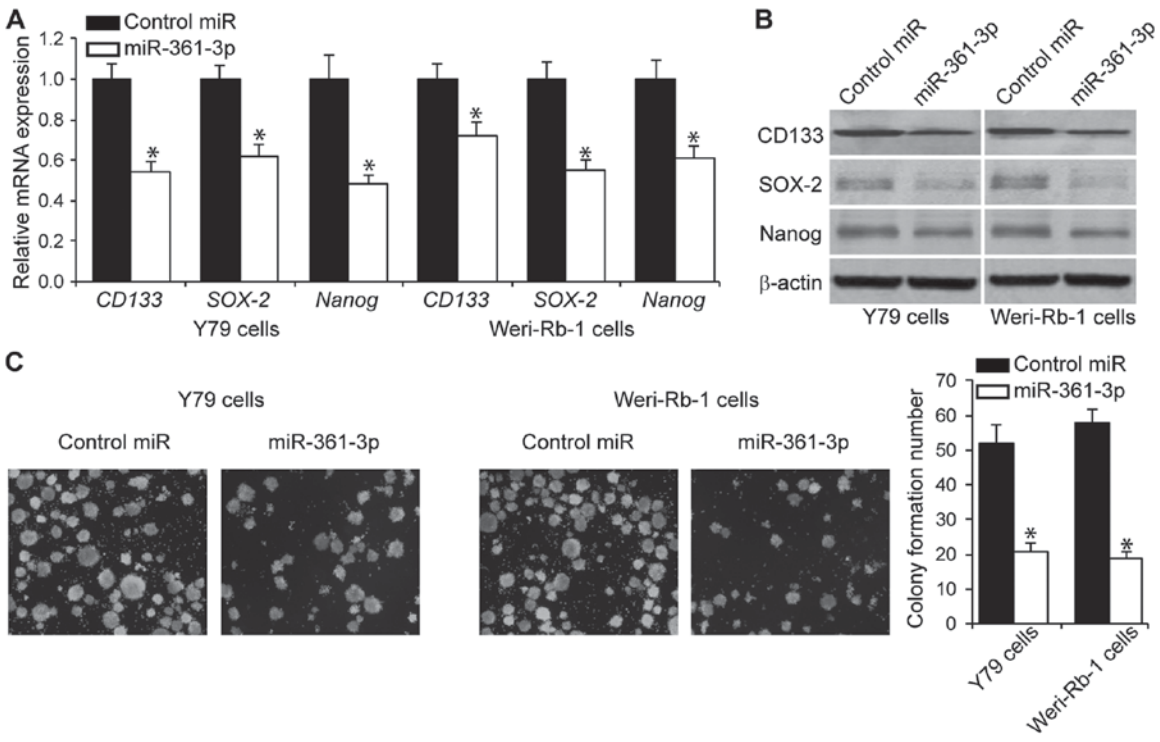

Figure 3. miR-361-3p overexpression reduces RB cell stemness. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blotting of the expression level of stem cell markers (CD133, SOX-2 and Nanog) in Y79 and Weri-Rb-1 RB cells transfected with miR-361-3p. (C) A tumor sphere assay was performed in Y79 and Weri-Rb-1 RB cells transfected with miR-361-3p (magnification, x200). *P<0.05 vs. control miR. miR, microRNA; RB, retinoblastoma; CD, cluster of differentiation; SOX-2, SRY box-2.

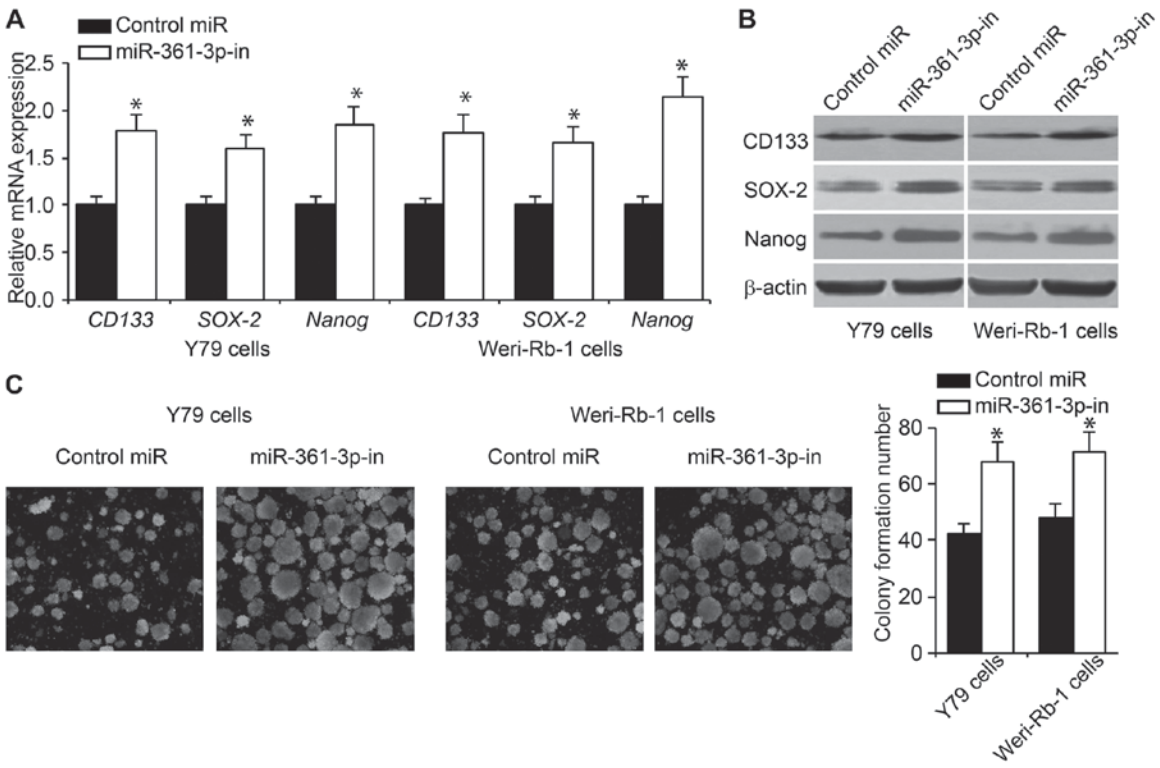

Figure 4. miR-361-3p inhibition promotes RB cell stemness. (A) Reverse transcription-quantitative polymerase chain reaction analysis and (B) western blotting of stem cell marker (CD133, SOX-2 and Nanog) expression in Y79 and Weri-Rb-1 RB cells transfected with a miR-361-3p inhibitor. (C) A tumor sphere assay was performed in Y79 and Weri-Rb-1 RB cells transfected with a miR-361-3p inhibitor (magnification, x200). *P<0.05 vs. control miR. miR, microRNA; RB, retinoblastoma; CD, cluster of differentiation; SOX-2, SRY box-2; in, inhibition.

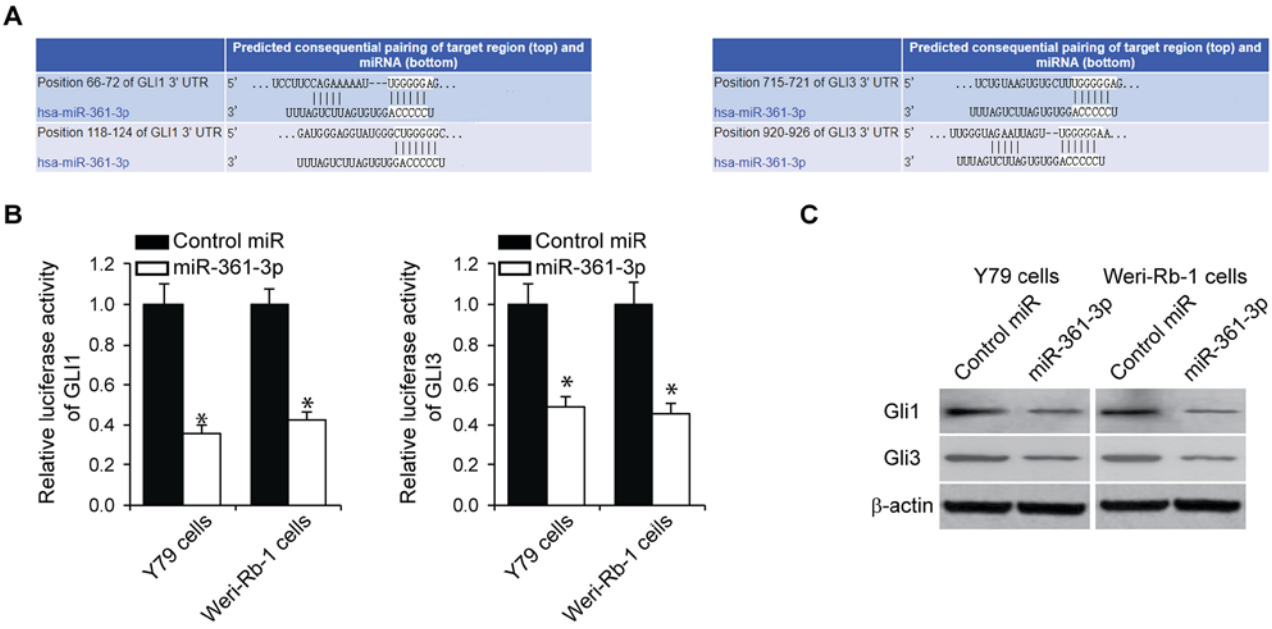

Figure 5. miR-361-3p directly targets GLI1 and GLI3. (A) A graphical representation of miR-361-3p binding sites in wild-type GLI1 and GLI3 3'-UTRs. (B) A luciferase assay was performed in Y79 and Weri-Rb-1 RB cells transfected with miR-361-3p and GLI1 or GLI3 3'UTR luciferase reporter plasmids. (C) Western blotting was performed to assess GLI1 and GLI3 expression in Y79 and Weri-Rb-1 RB cells transfected with miR-361-3p. *P<0.05 vs. control miR. miR, microRNA; RB, retinoblastoma; GLI, glioma-associated oncogene homologue; UTR, untranslated region.

cancer (21). In the present study, it was determined that the expression of miR-361-3p was 0.5-fold lower in the serum of patients with RB compared with normal serum and 0.33-fold lower in RB tumor tissues in comparison with healthy control samples. These results are consistent with a previous study by Chen *et al* (21), who also reported a decreased miR-361 expression profile in NSCLC tissues compared with normal lung tissue, and an inverse association with advanced stage and

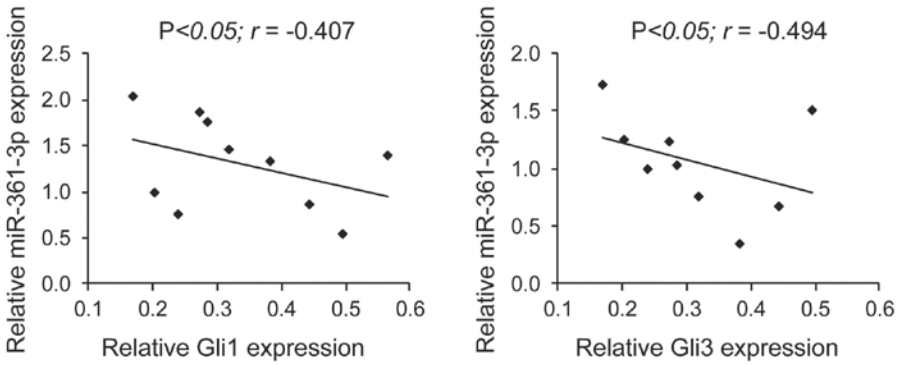

Figure 6. Negative correlation between miR-361-3p and GLI1/3 in RB samples. A Pearson's correlation analysis was performed between miR-361-3p and GLI1/3 in 10 RB tissues GLI1, P<0.05, *r*=-0.407; GLI1, P<0.05, *r*=-0.494. miR, microRNA; GLI, glioma-associated oncogene homologue; RB, retinoblastoma.

lymph node metastasis. Furthermore, it has been demonstrated that miR-361 is downregulated in colorectal carcinoma, gastric cancer (18) and castration-resistant prostate cancer (17), indicating that miR-361-5p may serve a tumor-suppressive miR role. However, miR-361-5p has been revealed to serve as an oncogene in cervical cancer progression by enhancing cell proliferation and invasion (16).

In the current study, Y79 and Weri-Rb-1 cells were utilized. However, these cell lines are in suspension and are thus difficult to use for the assessment of cell migration and invasion (6,28). For this reason, the current study only assessed cell proliferation and stemness, which serves as a limitation. Despite this, the results demonstrated that miR-361-3p inhibits RB cell proliferation in two human RB cell lines (Y79 and Weri-Rb-1). Similarly, the CCK-8 assay results revealed that miR-361-3p inhibited the proliferation of Y79 and Weri-Rb-1 cells. Montoya *et al* (4) also investigated the miR-mediated regulation of RB proliferation. It was demonstrated that miR-31 and/or miR-200a overexpression restricted the expansion of a highly proliferative cell line (Y79), but did not affect the growth rate of a less aggressive cell line (Weri-Rb-1) (4).

Cancer cell stemness is a key feature of cancer progression and often determines patient survival (29,30). Common stemness characteristics of stem and cancer stem cells (CSCs) include self-renewal, differentiation potential and stemness involves the organization of a microenvironment that protects stem cell niche or stem cells (31,32). Specifically, CSCs are a group of cells within the tumor mass, which exhibit stemness properties that promote cancer progression, including involvement in self-renewal cloning, growing, metastasizing, homing and re-proliferating (30). In addition, CSCs may influence the nutrient supply delivery of neighboring cells and cooperate in immune system elusion (33). They may also increase heterogeneous cell populations, which exhibit a high plasticity potential (33) and a high resistance to unfavorable factors within the tumor microenvironment, and may induce cell death (34) and quiescence (21).

miRs regulate diverse basic cellular functions, including proliferation, migration and invasion (35). Therefore, in the current study, RB cell lines were transfected with miR-361-3p, following which cell stemness ability was assessed via the detection of stem cell marker expression. The results demonstrated that miR-361-3p overexpression downregulated the expression of CD133, SOX-2 and Nanog. In addition, miR-361-3p significantly decreased RB cell self-renewal. Therefore, it was concluded that miR-361-3p inhibits the stemness ability of Y79 and Weri-Rb-1 cells. The key pathogenic mechanism in the development of RB is the inactivation of two RB gene alleles; a regulator of the G1 and S phase of the cell-cycle (36). Despite previous studies, there is currently no clinically acceptable biomarker that identifies the risk of RB metastasis (25). Different signaling pathways may be of great relevance to the oncogenesis and development of RB. Li *et al* (12) demonstrated that miR-497 overexpression decreases the proliferation, migration and invasion of human RB cells by targeting vascular endothelial growth factor A. Furthermore, Bai *et al* (11) revealed that miR-125b promotes tumor growth and suppresses apoptosis by targeting DNA Damage Regulated Autophagy Modulator 2 in RB. SHH signaling is another major pathway that is dysregulated in RB (6,25). SHH, a vertebrate homologue of the Drosophila segment polarity gene product, hedgehog, is associated with retina cell proliferation and differentiation during the process of embryonic development (37,38). SHH-associated pathways may proceed directly or indirectly by activating a network of cascades (39,40), which affect the apoptosis and metastasis of tumor cells (41) in various types of malignancy, including in pancreatic cancer (42) or ovarian cancer (43). Additionally, Song *et al* (6) demonstrated that the blockade of SHH signaling decreases RB cell viability and induces apoptosis by activating the phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) pathway. Additionally, previous studies have implied that the potential effect of the PI3K/Akt pathway on SHH signaling is regulated by GLI-dependent transcriptional activity (44). This is concordant with the current study as miR-361-3p was revealed to significantly reduce the expression of GLI1 and GLI3 protein in Y79 and Weri-Rb-1 cells. Thus, the results demonstrated that GLI1 and GLI3 are targets of miR-361-3p and that a negative correlation exists between miR-361-3p and GLI1/3 in RB samples. However, the absence of a non-cancerous cell line as a control is a limitation of the present study. Further assessment is therefore required to confirm these results.

In conclusion, the results of the present study indicate that miR-361-3p regulates the proliferation and metastasis of RB cells by targeting SHH signaling. The current study also revealed that SHH signaling serves an important role in the development of RB and that miR-361-3p should be assessed as a potential therapeutic target for RB treatment. The results of the present study may also enhance the development of

targeted therapeutic approaches for RB and improve the efficacy of current RB management. However, further preclinical studies are necessary to determine the role of the miR-361-3p in different malignant tumors and to reveal SHH-associated signaling transduction.

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Availability of data and materials

All data and materials supported the results of the present study are available in the published article.

Authors' contributions

DZ and ZC performed experiments, and collected and analyzed the data. DZ wrote the manuscript and ZC designed the experiments. Each author read the manuscript and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Clinical Research Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University.

Patient consent for publication

Written informed consent obtained from all patients prior to publication.

Competing interests

The authors declare that they have no conflicts of interests.

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