MicroRNA-101-3p inhibits proliferation in retinoblastoma cells by targeting EZH2 and HDAC9

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Abstract. Retinoblastoma is the most frequent intraocular malignant tumor type to occur in childhood. MicroRNA (miR)-101-3p has been reported to function as a tumor suppressor in various types of cancer. However, the biological function and underlying mechanisms of miR-101-3p in retinoblastoma are largely unknown. In the present study, it was identified that miR-101-3p was downregulated in retinoblastoma. MTT and flow cytometry assays demonstrated that ectopic overexpression of miR-101-3p significantly inhibited cell viability and cell cycle progression in WERI-Rb-1 and Y79 cells. *In vivo* mouse experiments further confirmed the anti-proliferative role of miR-101-3p in retinoblastoma. Additionally, predictions with TargetScan software indicated that the 3'-untranslated regions of enhancer of zeste homolog 2 (EZH2) and histone deacetylase (HDAC9) mRNAs are targeted by miR-101-3p. Accordingly, a dual luciferase reporter gene assay demonstrated that miR-101-3p directly targeted EZH2 and HDAC9 to suppress the proliferation of retinoblastoma cells. Meanwhile, the restoration of EZH2 or HDAC9 expression countered the anti-proliferative effect of miR-101-3p on WERI-Rb-1 and Y79 cells. Collectively, these data highlight the role of miR-101-3p in the tumorigenesis of retinoblastoma, and indicate its suitability as a novel therapeutic target.

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Introduction

Retinoblastoma is the most frequent intraocular malignant tumor type to occur in childhood (1). Retinoblastoma is a heritable cancer caused by mutations or deletions of the tumor suppressor gene, retinoblastoma 1 (Rb1) (2). Although the allelic inactivation of the Rb1 gene serves an important role in the tumorigenesis of retinoblastoma, other oncogenes or tumor suppressors may also be important in the development and progression of the disease (3,4). Further delineating the molecular events involved in the oncogenesis of retinoblastoma should provide novel insights into the etiology of this tumor.

MicroRNAs (miRNAs/miRs) are highly conserved, endogenous, small non-coding RNAs of ~22 nucleotides in length (5). miRNAs pair imperfectly with the 3'-untranslated region (3'UTR) of specific mRNAs to regulate the transcriptional and post-transcriptional expression of target genes (6). Mounting evidence has demonstrated that miRNAs are involved in numerous physiological and pathological processes (7,8). Of note, the dysregulation of miRNAs serves a vital role in almost every aspect of tumor biology (9,10). miRNAs may act as oncogenes or tumor suppressors by regulating different target genes (11,12). Martin *et al* (13) investigated differentially expressed miRNAs in 12 retinoblastomas as compared with three normal human retina samples using the Taqman Low Density Array and identified that miR-129-3p, miR-382, miR-504, miR-22 and miR-129-5p were significantly downregulated in retinoblastoma. Furthermore, Huang *et al* (14) used microarrays to profile three retinoblastoma samples and one healthy retina sample and the results indicated that the expression levels of let-7b, let-7c, miR-24, miR-125b, miR-191, miR-181a and miR-423 were downregulated in retinoblastoma. Additionally, microarray data provided by Zhao *et al* (15) reported that miR-494, let-7e, miR-513-1, miR-513-2, miR-518c-5p, miR-129-1, miR-129-2, miR-198, miR-492, miR-498, miR-320, miR-503 and miR-373-5p were upregulated in retinoblastoma. Therefore, it is imperative to further elucidate the roles of miRNAs in the tumorigenesis of retinoblastoma.

miR-101-3p has been reported to be downregulated in various types of cancer, and functions as a tumor suppressor via targeting multiple oncogenes. For instance, it has been demonstrated that miR-101-3p loss in prostate cancer increases the expression of SUB1, activating genes associated with an

aggressive tumor phenotype (16). Zhang *et al* (17) reported that miR-101-3p suppresses cholangiocarcinoma angiogenesis via targeting vascular endothelial growth factor. Furthermore, in human colon cancer cells, the downregulation of miR-101-3p was observed, which was associated with the upregulation of cyclooxygenase-2 (18). In addition, the expression of miR-101-3p in non-small cell lung cancer was reported to be significantly decreased (19). However, the biological function and underlying mechanisms of miR-101-3p in retinoblastoma are largely unknown.

The aim of the present study was to determine the role of miR-101-3p in retinoblastoma, and investigate whether miR-101-3p targeted enhancer of zeste homolog (EZH2) and histone deacetylase 9 (HDAC9) to exert its role in retinoblastoma.

Materials and methods

Cell culture. The human retinoblastoma cell lines WERI-Rb-1 and Y79 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmids. pEnter (cat. no. P100001), pEnter-EZH2 (cat. no. CH882009) and pEnter-HDAC9 (cat. no. CH804907) were purchased from Vigene Biosciences, Inc. (Rockville, MD, USA). Wild-type and mutant 3'UTRs of EZH2 and HDAC9 were synthesized by Genewiz (Suzhou, Jiangsu, China) and were inserted into a pMIR-REPORT™ luciferase plasmid (cat. no. AM5795; Thermo Fisher Scientific, Inc.).

Patients and tissues. A total of 12 human retinoblastoma tissue samples (7 males and 5 females; median age, 31 months), and 3 normal retinal tissues (2 males and 1 female; median age, 56 months) from individuals who succumbed to conditions other than ophthalmologic diseases, were obtained from the Second Affiliated Hospital of Nanchang University (Nanchang, China) between January 2015 and December 2016. All patients with retinoblastoma were diagnosed and treated for the first time, and had not received adjuvant treatments prior to this study in order to avoid treatment-induced expression changes. All specimens were histopathologically diagnosed by two pathologists. The current study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University, and written informed consent was obtained from the parents or guardians of patients.

Transfection. MicrON® miR-101-3p agomir (cat. no. miR40000099-1-10) and an miR-control (cat. no. miR04201-1-10) were purchased from Guangzhou Ribobio Co., Ltd. (Guangzhou, Guangdong, China). WERI-Rb-1 and Y79 cells were seeded in in 6-well plates ($5x10^4$ cells per well) and transfected with 50 nM miR-101-3p agomir or miR-control using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h post-transfection, the cells were subjected to subsequent analysis. To determine the roles of target genes on the effect of miR-101-3p, co-transfection of 50 nM

miR-101-3p agomir+1 µg control plasmid (pEnter) or 50 nM miR-101-3p agomir+1 µg pEnter-EZH2/pEnter-HDAC9 plasmid in WERI-Rb-1 and Y79 cells was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.). At 5 days post co-transfection an MTT assay was performed.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from prepared tissue or cultured cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). For miRNA a specific Bulge-Loop™ miR-101-3p RT primer (cat. no. ssD809230011) and a U6 RT primer (cat. no. ssD0904071008; both Guangzhou Ribobio Co., Ltd.) were used for the RT reaction. For mRNA, an Oligo(dT)$_{15}$ primer was used to reverse transcribe the mRNA into cDNA. The RT reaction was performed using an RT system (cat. no. A3500; Promega Corporation, Madison, WI, USA). The temperature protocol for RT was as follows: 42°C for 30 min, 70°C for 10 min and hold at 4°C. qPCR was performed using an SYBR-Green PCR Master mix (Takara Biotechnology, Co., Ltd., Dalian, China) on an ABI7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Bulge-Loop™ miRNA qPCR primer sets for miR-101-3p (cat. no. miRQ0000099-1-1) and RNU6B (cat. no. MQP-0202) were purchased from Guangzhou Ribobio Co., Ltd. The primers used for detecting EZH2, HDAC9 and GAPDH were as follows: EZH2 forward, 5'-GGAACAACG CGAGTCGG-3' and reverse, 5'-CTGATTTTACACGCTTCC GC-3'; HDAC9 forward, 5'-GAACTCTAAGCCAGATGG GG-3' and reverse, 5'-GCCCACAGGAACTTCTGACT-3'; GAPDH forward, 5'-ATGGTGAAGGTCGGTGTGAA-3' and reverse, 5'-GAGTGGAGTCATACTGGAAC-3'. RNU6B or GAPDH served as internal controls. The PCR protocol for detection of miR-101-3p and RNU6B was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 2 sec, 60°C for 20 sec and 70°C for 10 sec. The PCR protocol for detection of EZH2, HDAC9 and GAPDH was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 15 sec. Cq values were calculated, and fold change was determined using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. The cells were lysed with radioimmunoprecipitation assay lysis buffer [50 mM TrisHCl (pH 7.6), 150 mM NaCl, 1 mM EGTA, 1% Triton NP-40, 1% sodium deoxycholate, 0.1% SDS and 50 mM NaF]. The protein concentration in the extract was measured by the Bradford assay (Bio-Rad, Richmond, CA, USA). Equal amounts (30 µg) of protein were separated by 10% SDS-PAGE. The proteins were then transferred electrophoretically onto polyvinylidene fluoride membranes. Subsequently, membranes were incubated with primary antibody at 4°C overnight. The primary antibodies used included rabbit monoclonal anti-EZH2 (cat. no. ab150433; 1:1,000), anti-HDAC9 (cat. no. ab109446; 1:1,000) and anti-α-tubulin (cat. no. ab4074; 1:2,000; all Abcam, Cambridge, UK). Membranes were then incubated at room temperature for 1 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. ab205718; 1:2,000; Abcam). An enhanced chemiluminescence detection system (Amersham; GE Healthcare, Chicago, IL, USA) was used for developing bands, and the membrane was exposed on X-ray film (Kodak, Tokyo, Japan). α-tubulin was used as the loading control.
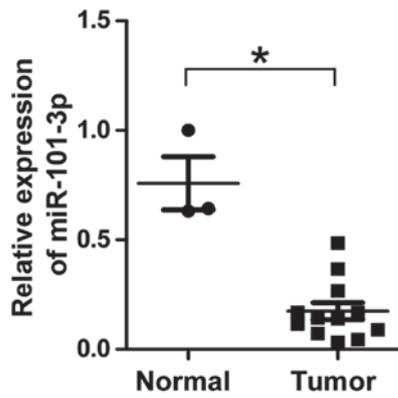
Figure 1. miR-101-3p is downregulated in retinoblastoma. Reverse transcription-quantitative polymerase chain reaction assays were performed in 12 retinoblastoma and three normal retina specimens. The expression level of miR-101-3p in retinoblastoma samples was significantly downregulated compared with normal retinas. *P<0.05. miR, microRNA.

MTT assay. Cell viability was assessed with an MTT assay. Cells (10^3 cells/well) were cultured in 96-well plates. At the same time each day for 5 days, 20 μl MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well and incubated for a further 4 h. Following removal of the culture medium, 200 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added and the optical density at 490 nm was measured using an Elx808 enzyme immunoassay analyzer (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Flow cytometry. Flow cytometry was performed to analyze cell cycle distribution. Cells were harvested, washed with cold PBS and fixed in cold 70% ethanol overnight at 4°C, followed by treatment with 0.25 mg/ml RNase A for 30 min at room temperature. After staining with propidium iodide (PI; 0.05 mg/ml; Sigma-Aldrich; Merck KGaA) for 15 min at room temperature, the samples were analyzed using a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and was analyzed using ModFit LT 4.1 (Verity Software House, Topsham, ME, USA).

Animal experiments. The animal studies were approved by the Ethical Committee of the Second Affiliated Hospital of Nanchang University. A total of 10 male BALB/cA nude mice (age, 5 weeks; weight, 15.2-17.8 g) were purchased from Charles River Laboratories, Inc. (Beijing, China). All animals were maintained at 22°C and 80% relative humidity with a 12-h day/night cycle and were provided food and water *ad libitum*. Y79 cells (10^6) were subcutaneously (s.c.) transplanted into the flank of each nude mouse. Once tumors reached ~40 mm³, nude mice were assigned to two groups (n=5 per group). Agomir and miR-control (30 μl PBS with 10 nmol/l) were injected into the tumor tissue in each group by multipoint intratumoral injection every 2 days. The tumor sizes were monitored, and the tumor volume was calculated using the following formula: Tumor volume=0.5x long radius x short radius². At day 16 following the first miR injection, mice were sacrificed and the weight of the tumors was measured.

Luciferase reporter assay. Cells were co-transfected with the reporter constructs (pMIR-REPORT-EZH2-3'UTR-WT,

pMIR-REPORT-EZH2-3'UTR-Mut, pMIR-REPORT-HDAC9- 3'UTR-WT or pMIR-REPORT- HDAC9-3'UTR-Mut) and miR-101-3p agomir or miR-control, using Lipofectamine 3000 according to the manufacturer's protocol. pRL-TK *Renilla* (Promega Corporation) was used to normalize the efficiency of the transfection. At 48 h, cells were harvested and the luciferase activity was analyzed using the Dual Luciferase Assay kit (Promega Corporation) according to the manufacturer's protocol. *Renilla* luciferase activity was used as an internal control.

Bioinformatics analysis. Targetscan software (version 7.1; targetscan.org/vert_71/) was used to predict the putative targets of miR-101-3p.

Statistical analysis. The results were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). All experiments were performed three times. Data are presented as the mean ± standard deviation. Differences between two groups were compared using Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-101-3p is downregulated in retinoblastoma and suppresses proliferation in retinoblastoma cells. Previous studies have demonstrated that miR-101-3p is downregulated in several types of cancer, prompting the investigation of the expression of miR-101-3p in retinoblastoma in the present study. RT-qPCR assays were performed for 12 retinoblastoma and 3 normal retina specimens. As demonstrated in Fig. 1, the expression of miR-101-3p was significantly downregulated in retinoblastoma samples compared with in the normal retina samples (P<0.05), suggesting that miR-101-3p may serve a functional role in retinoblastoma development. Next, the effects of miR-101-3p on retinoblastoma cells were investigated. Transfection with an miR-101-3p agomir (Fig. 2A) significantly attenuated the viability of WERI-RB-1 and Y79 cells (P<0.05; Fig. 2B). PI staining and flow cytometry were subsequently performed to analyze the effect of miR-101-3p on cell cycle progression. As demonstrated in Fig. 2C and D, miR-101-3p overexpression significantly increased the proportion of WERI-RB-1 and Y79 cells in G_1 phase (P<0.05). Collectively, these data suggest that miR-101-3p serves an anti-proliferative role in retinoblastoma cells.

miR-101-3p inhibits tumor growth in nude mice. To further explore the function of an miR-101-3p agomir in *in vivo* tumor growth, a tumor model was established by transplanting (s.c.) Y79 cells into the flanks of nude mice. Once tumors reached ~40 mm³, 30 μl PBS with 10 nmol/l agomir or miR-control was injected into the tumor tissue every 2 days, and the tumor sizes were monitored. As indicated in Fig. 3A and B, treatment with miR-101-3p agomir significantly inhibited tumor growth in nude mice compared with in the controls (P<0.05). Consistently, the mean tumor weight in the Y79/miR-101-3p agomir mice was significantly decreased in comparison with the control (P<0.05; Fig. 3C). These *in vivo* findings further indicate that miR-101-3p markedly suppresses tumor growth in retinoblastoma.
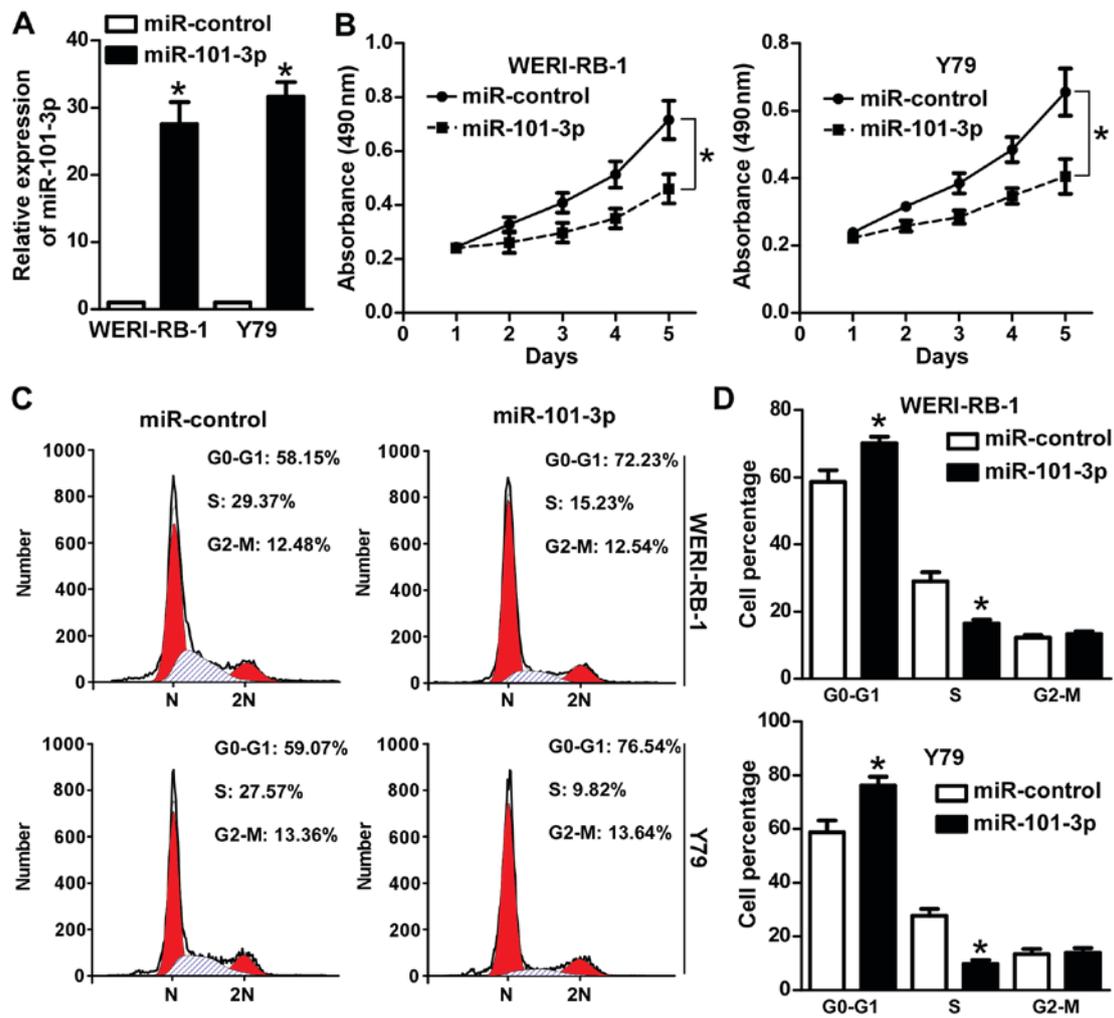
Figure 2. miR-101-3p suppresses proliferation in retinoblastoma cells. (A) miR-101-3p agomir transfection significantly upregulated the expression of miR-101-3p in WERI-RB-1 and Y79 cells. (B) WERI-RB-1 and Y79 cells were transfected with an miR-control and miR-101-3p agomir, and cell viability at the indicated times was assessed by MTT assays. (C and D) Effects of miR-101-3p on WERI-RB-1 and Y79 cell cycle were analyzed by flow cytometry. Overexpression of miR-101-3p resulted in G_1 arrest in WERI-RB-1 and Y79 cells. *P<0.05. miR, microRNA.
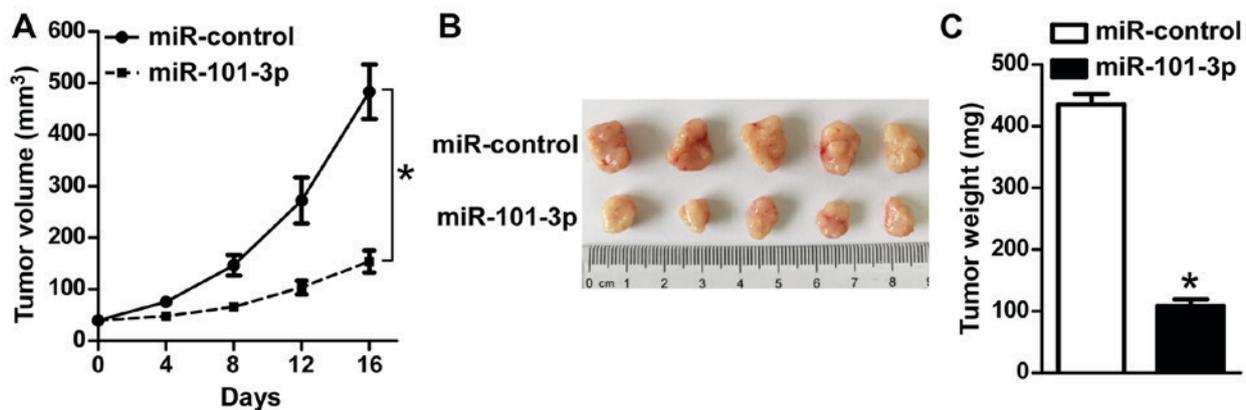
Figure 3. miR-101-3p inhibits tumor growth in nude mice. (A) Tumor volume was assessed every 4 days. The results demonstrated that the growth of tumors in the miR-101-3p agomir group was significantly slower compared with the miR-control group. (B) Images of tumors extracted from mice inoculated with Y79 cells and treated with miR-101-3p agomir or miR-control. (C) Mean tumor weight of the indicated groups. *P<0.05. miR, microRNA.

miR-101-3p directly targets EZH2 and HDAC9 in retinoblastoma cells. Next, the role of miR-101-3p in regulating HDAC9 in retinoblastoma cells was explored. Initially, bioinformatics analysis with TargetScan was performed to predict the potential

targets of miR-101-3p. Among these targets, the key roles of EZH2 and HDAC9 in retinoblastoma have been well demonstrated (21-23). As indicated in Fig. 4A, potential miR-101-3p binding sites were identified in the 3'UTRs of EZH2 and
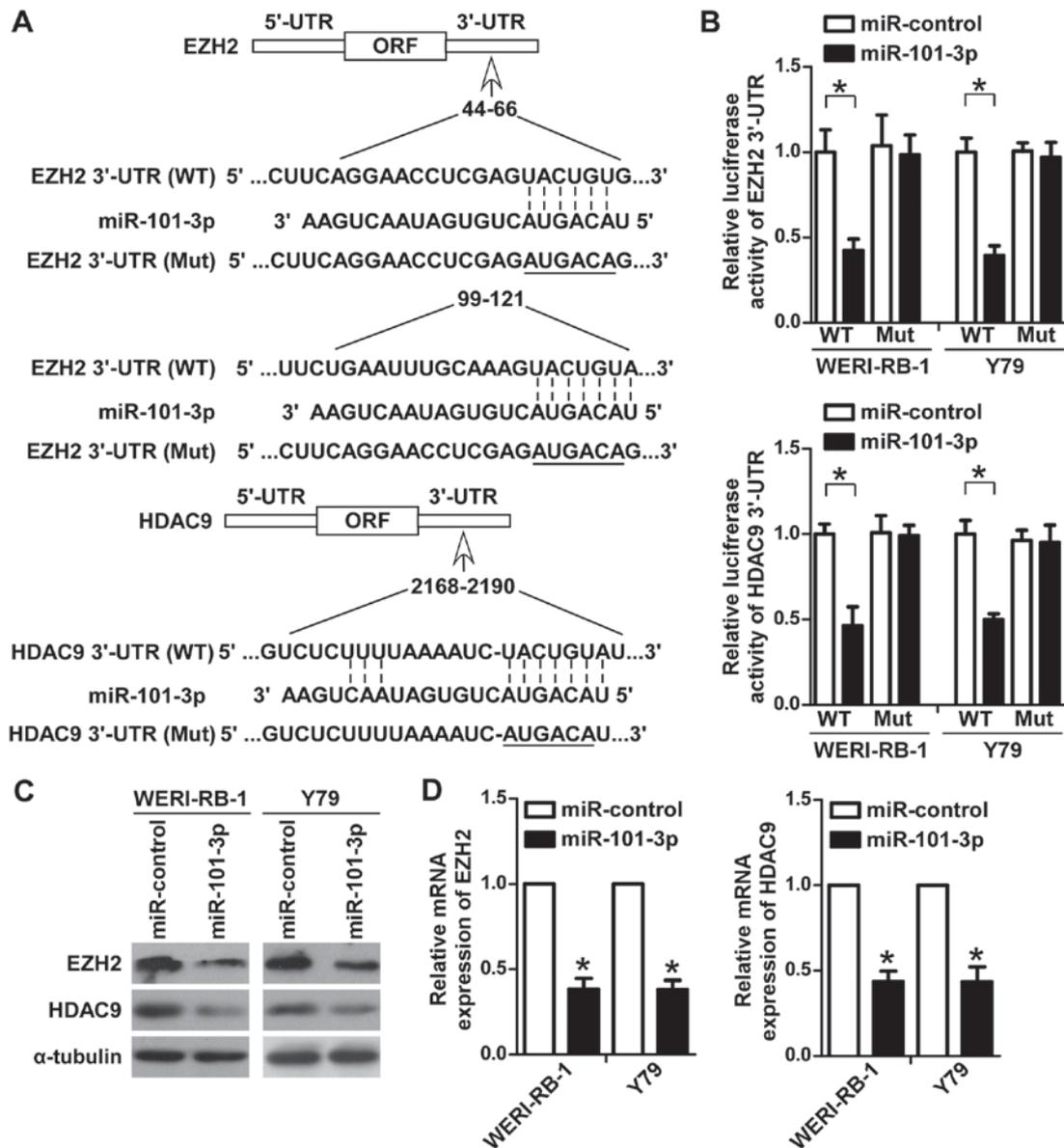
Figure 4. miR-101-3p directly targets EZH2 and HDAC9. (A) EZH2 and HDAC9 were identified as candidate targets for miR-101-3p. The wild-type or mutant putative miR-101-3p binding sites in the 3'UTRs of EZH2 and HDAC9 mRNAs are shown. (B) Luciferase activity assays demonstrated that miR-101-3p overexpression significantly decreased the luciferase activity in WERI-RB-1 and Y79 cells transfected with the wild-type 3'UTR of EZH2 or HDAC9. No effects on the mutant 3'UTRs for EZH2 or HDAC9 were observed. $^*P<0.05$. (C) Overexpression of miR-101-3p suppressed the levels of EZH2 and HDAC9 protein. (D) Overexpression of miR-101-3p reduced the mRNA levels of EZH2 and HDAC9. $^*P<0.05$ vs. relative control. miR, microRNA; EZH2, enhancer of zeste homolog 2; HDAC9, histone deacetylase 9; 3'UTR, 3'-untranslated region; WT, wild-type; Mut, mutant; ORF, open reading frame.

HDAC9. To determine whether miR-101-3p directly targets EZH2 and HDAC9 in retinoblastoma cells, luciferase reporters containing wild-type or mutant miR-101-3p binding sites in the 3'UTRs of EZH2 and HDAC9 were constructed (Fig. 4A). The reporter vectors were co-transfected with miR-101-3p or the control miRNA into WERI-RB-1 and Y79 cells. As demonstrated in Fig. 4B, miR-101-3p significantly inhibited the luciferase activity of the reporters containing EZH2 and HDAC9 wild-type 3'UTRs, but not of those containing respective mutant 3'UTRs (P<0.05). To further confirm the regulatory role of miR-101-3p on EZH2 and HDAC9 in WERI-RB-1 and Y79 cells, western blotting and RT-qPCR assays were performed. As indicated in Fig. 4C, the protein levels of EZH2 and HDAC9 were markedly decreased in WERI-RB-1 and Y79 cells transfected with the miR-101-3p agomir compared

with the control group. Consistently, the mRNA expression levels of EZH2 and HDAC9 were significantly decreased in WERI-RB-1 and Y79 cells transfected with the miR-101-3p agomir compared with the control group (P<0.05; Fig. 4D). Collectively, these data suggested that miR-101-3p directly targeted EZH2 and HDAC9 in retinoblastoma cells.

Restoration of EZH2 or HDAC9 hinders the anti-proliferative role of miR-101-3p in WERI-RB-1 and Y79 cells. To determine whether EZH2 and HDAC9 were involved in the anti-proliferative role of miR-101-3p in retinoblastoma cells, a rescue experiment was performed by transfecting WERI-RB-1 and Y79 cells with miR-101-3p or the control miRNA along with pEnter, pEnter-EZH2 or pEnter-HDAC9 (Fig. 5A). As illustrated in Fig. 5B and C, MTT assays demonstrated that the
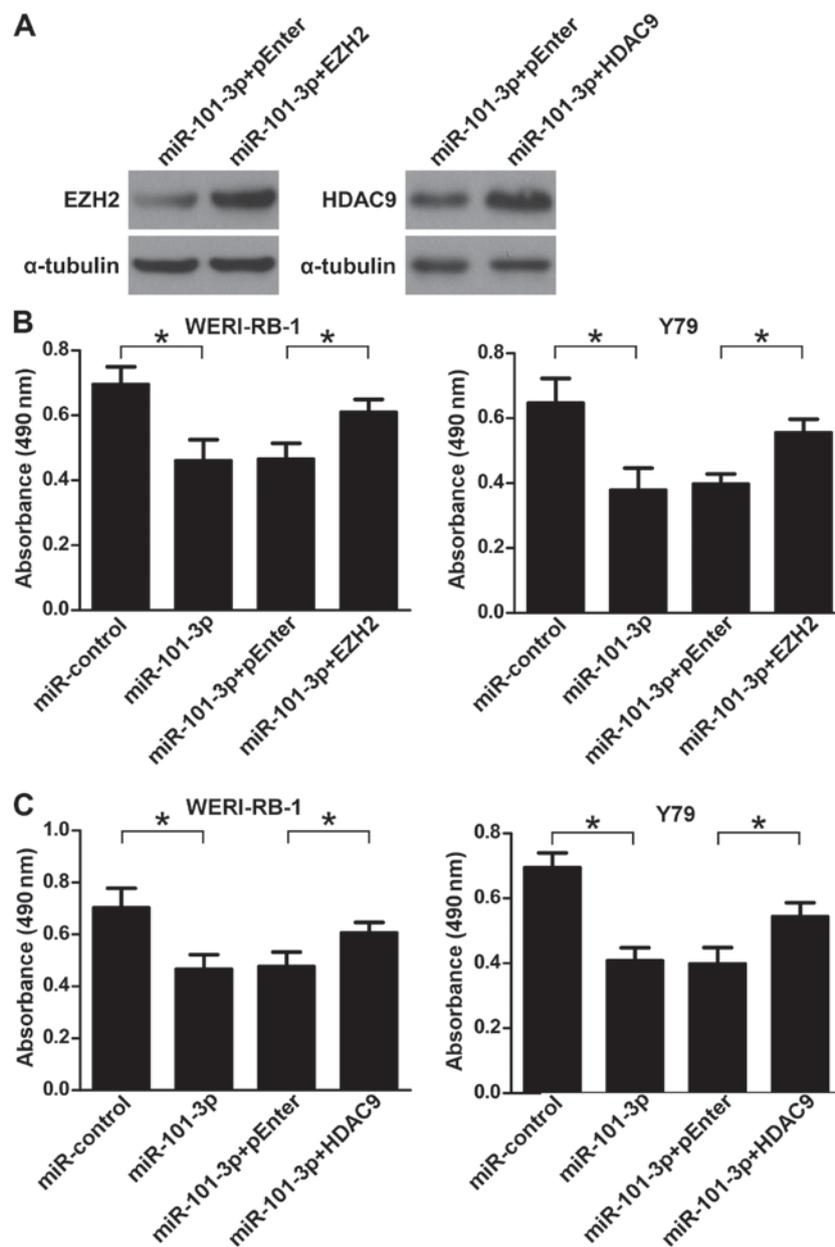
Figure 5. Restoration of EZH2 or HDAC9 expression reverses the effect of miR-101-3p on WERI-RB-1 and Y79 cells. (A) Overexpression of EZH2 or HDAC9 in indicated cells was confirmed by performing western blotting. (B) WERI-RB-1 and Y79 cells were transfected with miR-control or miR-101-3p, and pEnter or pEnter-EZH2. Cell viability was determined by an MTT assay. (C) WERI-RB-1 and Y79 cells were transfected with miR-control or miR-101-3p, and pEnter or pEnter-HDAC9. Cell viability was determined by an MTT assay. *P<0.05. EZH2, enhancer of zeste homolog 2; HDAC9, histone deacetylase 9; miR, microRNA.

ectopic overexpression of EZH2 or HDAC9 partially reversed the miR-101-3p-induced suppression of retinoblastoma cell viability (P<0.05). These data indicated that downregulation of EZH2 and HDAC9 was involved in mediating the anti-proliferative role of miR-101-3p in retinoblastoma cells.

Discussion

Accumulating evidence has revealed that the aberrant expression of miRNAs may be involved in the development and progression of retinoblastoma (24,25). Thus, a greater understanding of the specific miRNAs associated with retinoblastoma tumorigenesis may be crucial for exploring novel therapeutic strategies for this malignancy. In the present study, it was identified that the expression level of miR-101-3p was significantly downregulated in retinoblastoma. The overexpression of miR-101-3p inhibited retinoblastoma cell proliferation *in vitro* and *in vivo*. Furthermore, data based on two retinoblastoma cell lines indicated that miR-101-3p directly targets EZH2 and HDAC9 to modulate retinoblastoma cell proliferation. These implicate miR-101-3p as a tumor suppressor in retinoblastoma.

Several lines of evidence have demonstrated that miR-101-3p is downregulated in various types of cancer, including hepatocellular carcinoma, non-small cell lung cancer and gastric cancer (19,26,27). In line with these studies, the

downregulation of miR-101-3p was also identified in retinoblastoma, suggesting that miR-101-3p may serve an important role in the development and progression of this disease. In the present study, it was identified that the ectopic overexpression of miR-101-3p significantly inhibited the viability of WERI-RB-1 and Y79 retinoblastoma cells. Accordingly, flow cytometry analyses demonstrated that miR-101-3p suppressed cell cycle progression. In addition, *in vivo* mouse experiments further confirmed the anti-proliferative role of miR-101-3p on retinoblastoma cells. These results strongly indicate that miR-101-3p is involved in the proliferation of retinoblastoma cells.

It is well documented that miRNAs exert their functions by regulating multiple target genes. A study by Lin *et al* (28) demonstrated that miR-135b promoted lung cancer metastasis by regulating multiple key components in the Hippo pathway, including LATS2, β-TrCP and NDR2. Le *et al* (29) demonstrated that a subset of miR-125b target genes affect neuronal differentiation. In the present study, it was identified that miR-101-3p inhibited retinoblastoma cell proliferation by directly targeting EZH2 and HDAC9. EZH2, the catalytic core protein of polycomb repressor complex 2, is a highly conserved histone methyltransferase (30). EZH2 suppresses the expression of target genes by catalyzing the trimethylation of K27 of histone H3 (31). The upregulation of EZH2 has been identified in various types of malignancy, including colorectal, breast and prostate cancer (32-34). Khan *et al* (21) observed that EZH2 was overexpressed in retinoblastoma specimens. The pro-proliferative role of EZH2 in cancer cells is also well established. Lian *et al* (35) demonstrated that EZH2 promotes laryngeal cancer cell proliferation by regulating Runt-related transcription factor 3 expression. Additionally, the inhibition of EZH2 by a small-molecule inhibitor suppresses tumor cell growth via induction of the tumor-suppressor protein p16^{INK4A} (36). Previous studies have also reported the expression and functional roles of HDAC9 in various types of cancer. Upregulated expression of HDAC9 was identified to promote oral squamous cell carcinoma growth and cell cycle progression through targeting of the transcription factors myocyte enhancer factor 2D and nuclear receptor subfamily 4 group A member 1 (37). Additionally, a study by Zhao *et al* (38) demonstrated that HDAC9 epigenetically repressed the transcription of p53 via binding to its proximal promoter region, thus enhancing the proliferation of osteosarcoma cells.

In the present study, it was revealed that miR-101-3p negatively regulated the expression of EZH2 and HDAC9 in retinoblastoma cells. Furthermore, the results of luciferase reporter assays further validated that miR-101-3p inhibited the expression of EZH2 and HDAC9 by directly binding to their 3'UTRs. The restoration of EZH2 or HDAC9 expression partially reversed the anti-proliferative effect of miR-101-3p in retinoblastoma cells. This strongly indicates that the inhibition of cell proliferation induced by miR-101-3p is mediated by regulating the expression of EZH2 and HDAC9.

In conclusion, it was demonstrated that miR-101-3p is downregulated in retinoblastoma. In addition, the overexpression of miR-101-3p suppresses the proliferation of retinoblastoma cells via directly targeting EZH2 and HDAC9. These data highlight an important role for miR-101-3p in the tumorigenesis of retinoblastoma, and may inform the development of novel therapeutic methods.

Availability of data and materials

All data generated or analyzed in this study are available within the published article.

Authors' contributions

QJ, WH and LC performed the *in vitro* experiments and analyzed the data. YY performed the animal experiments. KS collected the clinical tissue samples. QJ and ZY designed the study, wrote the manuscript and revised the manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University and written informed consent was obtained from the parents or guardians of all patients. The animal studies were approved by the Ethical Committee of the Second Affiliated Hospital of Nanchang University.

Patient consent for publication

Written informed consent for the publication of their data was obtained from the parents or guardians of patients involved in the present study.

Competing interests

The authors declare that they have no competing interests.

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