

Hypoxia-induced miR-181b enhances angiogenesis of retinoblastoma cells by targeting PDCD10 and GATA6

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Abstract. Previous findings showed that miR-181b is upregulated under hypoxic conditions in retinoblastoma cells. Since hypoxia is a common feature of retinoblastoma that affects tumor progression as well as tumor therapy, in the present study, we investigated the regulatory mechanism of miR-181b under hypoxic conditions, and examined the role of miR-181b in retinoblastoma responses to hypoxia (chemoresistance and angiogenesis) and possible downstream genes. The level of hypoxia-inducible factor-1 α (HIF-1 α) and miR-181b was detected to examine the link between them. Tube formation and cell cytotoxicity assays were used to clarify the effects of miR-181b on hypoxic responses of retinoblastoma cells. Bioinformatics analysis was performed to predict potential targets of miR-181b and western blotting was used to verify these targets. The results showed a significantly increased expression of HIF-1 α in hypoxia-treated retinoblastoma cells. Downregulation of HIF-1 α using a small-interfering RNA (siRNA) knockdown technology did not decrease the expression of miR-181b. Through gain- and loss-of-function studies, miR-181b was demonstrated to significantly stimulate the ability of capillary tube formation of endothelial cells. Programmed cell death-10 (*PDCD10*) and GATA binding protein 6 (*GATA6*) were identified as the target genes of miR-181b. To the best of our knowledge, results of the present study provide the first evidence that miR-181b was upregulated by hypoxia in retinoblastoma in an HIF-1 α -independent manner. miR-181b increased tumor angiogenesis of retinoblastoma cells. Additionally, miR-181b exerts its angiogenic function, at least in part, by inhibiting *PDCD10* and *GATA6*. Thus, it is a new potentially useful therapeutic target for retinoblastoma.

Introduction

Retinoblastoma is the most common and malignant intra-ocular tumor in children (1). Chemotherapy combined with local therapy has become the first-line treatment for early retinoblastoma, and over 95% of patients with retinoblastoma survive their malignancy in developed countries (2). However, once extraocular dissemination occurred, the rate of ocular salvage and patient survival were low. The leading cause of treatment failure and cancer-associated mortalities in retinoblastoma are chemoresistance and metastasis to distant organs. This is particularly prominent in developing countries due to the ignorance of the parents, and delay in referral which is associated with delayed diagnosis and treatment (3,4).

Hypoxia is a common feature of solid tumor including retinoblastoma and responses of tumor cells to hypoxia are important for tumor progression as well as tumor therapy. Previous studies demonstrated that hypoxic responses of tumor cells were involved in various biological processes including cellular proliferation, apoptosis, chemotherapy resistance, angiogenesis and migration (5-7). Thus, the presence of a hypoxic region in retinoblastoma may be a common pathway mediating chemoresistance and metastasis, rendering hypoxia a new therapeutic target. Hypoxic responses are an outcome of the interplay between the tumor cells and microenvironment. However, the exact mechanism was a complicated process involving the coordination of different factors that have not been adequately understood yet.

MicroRNAs (miRNAs) are a family of small non-coding RNA oligonucleotides that regulate gene expression by binding to sites in the 3'-untranslated region (3'-UTR) of the corresponding mRNA. Dysregulation of miRNAs is a key process involved in each stage of many cancer types from initiation to metastasis. Studies have shown there is a functional link between hypoxia and miRNA dysregulation (also termed hypoxia-regulated miRNAs, HRMs) which plays an important role in cell survival in low oxygen environment in cancer (8-10).

We identified miR-181b as an HRM in retinoblastoma cells that stimulated proliferation of retinoblastoma cells in our previous study (11). However, the function of miR-181b in retinoblastoma has yet to be elucidated. In the present study, we aim to investigate the regulatory mechanism of miR-181b

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under hypoxic conditions and evaluate the possible roles of miR-181b in hypoxic responses of retinoblastoma cells.

Materials and methods

Cell and culture. HXO-RB44 cells (human retinoblastoma cells) were provided by Professor Heping Xu (Zhongnan University, China) and human umbilical vein endothelial cells (HUVECs) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HXO-RB44 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (both from Gibco-BRL, Grand Island, NY, USA) in an incubator (37°C, 5% CO₂). HUVECs were grown in M-199 medium (Gibco-BRL) with 20% FBS, 100 U/ml penicillin (Biological Industries, Bet Haemek, Israel) and 1 U/ml fibroblast growth factor 2 (FGF2; Upstate Biotechnology, Lake Placid, NY, USA) in a humidified incubator (37°C, 5% CO₂). To induce hypoxic conditions, the cells were maintained in an InVivo200 hypoxia workstation (Biotrace International, Ruskinn Life Sciences, UK) with a steady flow of gas mixture (1% O₂, 5% CO₂ and 94% N₂).

Small-interfering RNA (siRNA)-mediated hypoxia-inducible factor-1 α (HIF-1 α) silencing. siRNAs targeting HIF-1 α and a scrambled sequence (GenePharma, Shanghai, China) were transfected into HXO-RB44 cells according to the manufacturer's instructions. Briefly, the cells were plated into 24-well plates (5x10⁵ cells/well) with FBS-free medium overnight. The cells were transfected with siHIF-1 α and scrambled sequence using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA USA). After 6 h, the cells were re-fed with fresh medium containing 10% FBS and experiments were performed 48 h later. Sequences for the siRNA of HIF-1 α used in this study were: siHIF-1 α -816 forward, GCUCAAUUUAUGAAU AUUATT and reverse, UAAUAUUCAUAAAUUGAGCGG; siHIF-1 α -1171 forward, GAAGGAACCUGAUGCUUU ATT and reverse, UAAAGCAUCAGGUUCCUUCTT; siHIF-1 α -1504 forward, CGAUGGAAGCACUAGACAATT and reverse, UUGUCUAGUGCUUCCAUCGGA.

Detection of the expression of HIF-1 α . Total RNA was isolated from HXO-RB44 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity was assessed using NanoDrop (Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA (2 μ g) was reverse transcribed into single-strand DNA using the First Strand Synthesis kit (Invitrogen). The resulting cDNA was used to perform quantitative PCR with Rotor-Gene 3000 Real-Time PCR System (Corbett Robotics, Australia) using SYBR-Green reporter dye (Invitrogen) and β -actin was used for normalization. The PCR amplification protocol was 95°C for 1 min followed by 40 cycles of 95°C for 15 sec, 62°C for 15 sec, 72°C for 30 sec, then followed by 95°C for 15 sec, 60°C for 1 min, 95°C for 30 sec and a 30-sec final extension at 37°C. Specific primers (Sangon Biotech Inc., Shanghai, China) used for HIF-1 α were: forward, CGTTCCTTCGATCAGTTGTC and reverse, TCAGTGGTGGCAGTGGTAGT. The primer sequences for β -actin were: forward, CTCATCCTGGCCTCGCTGT and reverse, GCTGTCACCTTCACCGTTCC. The relative

expression was analyzed using the 2^{- $\Delta\Delta$ Ct} method and all the experiments were performed in triplicate.

Transfection of miRNAs. HXO-RB44 cells in the exponential growth phase were seeded in 24-well plates (4x10⁴ cells/well). miR-181b precursor and inhibitor and the negative control (Ambion, Foster City, CA, USA) were transfected into the cells at a final oligonucleotide concentration of 30 pmol/well using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were incubated in a hypoxia workstation for 48 h prior to experiments.

Detection of miR-181b by TaqMan RT-PCR. Total RNA was extracted from cells using TRIzol (Invitrogen). The RT and PCR reactions were performed using the Hairpin-itTM miRNAs Real-Time PCR Quantitation kit (GenePharma, Shanghai, China). For reverse transcription (RT), the reaction was carried out as follows: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then kept at 4°C. The primer used for RT was GTCGTA TCCAGTGCGTGTCTGGAGTCGGCAATTGCACTGGA TACGACACCCAC. The PCR amplification was conducted as follows: 95°C for 1 min followed by 40 cycles of 95°C for 15 sec, 62°C for 15 sec, 72°C for 30 sec, and a 30-sec final extension at 37°C. The relative amounts of miRNAs were normalized against the U6 RNA using the 2^{- $\Delta\Delta$ Ct} method, all experiments were performed in triplicate for each sample. The primers used for PCR amplification were: miR-181b forward, GGGAACATTCATTGCTG and reverse, CAGTGCGTGTC GTGGAGT; U6 forward, GCTTCGGCAGCACATATACTA AAAT and reverse, CGCTTCACGAATTTGCGTGTTCAT.

Tube formation assay. Matrigel-coated (BD Pharmingen, San Jose, CA, USA) 24-well plates were incubated at 37°C for 2 h prior to the experiment. HUVECs were seeded onto the plates (4x10⁴ cells/well) and cultured at 37°C for 6 h with i) M-199 medium in normal conditions, ii) M-199 medium in hypoxic conditions and iii) culture medium of pre-transfected retinoblastoma cells in hypoxic conditions. The number of the capillary-like structures was scanned and quantified under a light microscope (Zeiss, Aoboe Cohen, Germany) in five lower-power fields (magnification, x100).

Cell cytotoxicity assay. Vincristine (VCR), etoposide VP-16 (VP-16) and carboplatin were purchased from the Pharmaceutical Corporation of Qilu, China. HXO-RB44 cells were seeded into 96-well plates (6x10⁵ cells/well) and treated with VCR (0.1, 1, 5, and 10 μ g/ml), VP-16 (2, 20, 100, and 200 μ g/ml) and carboplatin (2, 20, 100, and 200 μ g/ml) at different concentrations, respectively. Wells with medium only were set as blank and wells with cells but no drugs as control. After being cultured under normal and hypoxic conditions for 72 h, cell viability was determined by a CCK-8 Kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The 50% inhibitory concentration (IC₅₀) for each drug was calculated according to a dose-response curve by non-linear regression analysis. Pre-transfected HXO-RB44 cells (miR-181b precursor, and inhibitor and negative control) were treated with drugs as mentioned above and the IC₅₀ value was calculated. The experiments were performed three times and three parallel samples were measured each time.

Bioinformatics analysis of target genes of miR-181b. Target genes of miR-181b were predicted by retrieving the miRBase (<http://microrna.sanger.ac.uk/>), TargetScan (<http://www.targetscan.org>) and Pictar (<http://pictar.mdcberlin.de>) databases. Target prediction was performed by applying the three algorithms and two experimentally validated databases: TarBase and miRecords (<http://mirsystem.cgm.ntu.edu.tw/>) provided by the miRSystem. Targets receiving more than three positive votes were selected as miR-181b targets for subsequent analysis to reduce the quantity of false-positive results.

Reverse transcription and PCR amplification. Extraction and assessment of RNA were performed as described above. RNA was reverse transcribed with reverse transcriptase to synthesize the cDNA using a random primer. Two micrograms of total RNA in a 25- μ l reaction was carried out at 42°C for 60 min, then the reaction mixture was incubated at 95°C to inactivate the reverse transcriptase and denature the template. The PCR reaction was conducted in a final reaction volume of 25 μ l containing 2 μ l of cDNA, 1 μ l of gene-specific primers (10 μ M), 0.2 μ l of dNTPs (25 mM), 1 μ l Taq DNA polymerase (1 U/ μ l) and 2.5 μ l of 10X buffer. The cycling conditions for the PCR reaction were: 95°C for 10 min, followed by 35 cycles of 95°C for 20 sec, 60°C for 20 sec, 72°C for 30 sec, then followed by 72°C for 7 min and then kept at 4°C. The amplified products were visualized using 2% agarose gel and ethidium bromide under UV transillumination. Expression was considered positive if the expected band of gene was observed. The gene-specific primers used for PCR were: visinin-like 1 (VSNL1) forward, ATGGGGAAACAGAATAGCAA and reverse, TCATTTCTGAATGTCACACTG; pleiomorphic adenoma gene 1 (PLAG1) forward, CAAGATTCTCAAGCATCGTCA and reverse, TCCAAGGCTCCCCACTG; glutamate receptor 1 (GRI1) forward, AAATCTACAGCAATGCTGCGCA and reverse, CTTGATGACTTCTCTGTC; protease-activated receptors 4 (PAR-4) forward, GGGACCTCGGAACCAAC and reverse, TGTATCTGCCTGGGACTG; programmed cell death-10 (PDCD10) forward, CCTAAACGAAAGGCACGAG and reverse, GCCCTGCGGTTCTGGTA; T-cell leukemia/lymphoma 1 (TCL1) forward, ATCATCGAGCTCCAGGCTGGAGCTGGTTTCCATG and reverse, ATCATCAGATCTCGTCCAAATACACGAACCTTCTCCC; E2F transcription factor 5 (E2F5) forward, ATCCAGCATGGCAACTCAA and reverse, TCATCTGCCGGGGTAGGAG; GATA binding protein 6 (GATA6) forward, CTCCAACCTCCACCTCTTCTAAC and reverse, GCCCATCTTGACCCGAAT.

Western blotting. The cells were incubated with buffer containing protease inhibitor cocktail (0.01 mg/ml of aprotinin, pepstatin A and leupeptin) and PMSF (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 5 min. The cells were lysed by sonicator (Episonic, Santa Ana, CA, USA) with 25% power for 1 min, then centrifuged at 4°C (50,000 x g for 20 min) and the supernatant was transferred to a new sterile 1.5 ml EP tube. Protein concentrations were measured using the BCA Protein Assay kit (Bio-Rad, Hercules, CA, USA). The western blot analysis was subsequently performed. Briefly, 50 μ g of total protein was separated by SDS-PAGE on 10% gel and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) at 200 mA for 1.5 h. The membranes were incubated

with primary antibodies (1:50 in 1% BSA/TBST) overnight at 4°C. The primary antibodies (mouse monoclonal) against PDCD10, GATA6, PLAG1 and E2F5 were all purchased from Bioworld (St. Louis, MN, USA). The membranes were incubated with secondary antibodies [1:5,000 in 1% BSA/TBST, goat anti-mouse IRDye 800CW, LI-COR Biosciences (Lincoln, NE, USA)] for 1 h at room temperature. The immunoreactivity of proteins was detected using an ECL reagent (Millipore). The mean density of each band was quantified using Image J software with tubulin (Sigma-Aldrich; 1:1,000 in 1% BSA/TBST), used as an internal control.

Statistical analysis. Data were presented as means \pm SD if not specified otherwise. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The difference between groups was analyzed using the unpaired Student's t-test (only two groups) or one-way analysis of variance (three or more groups) with significance accepted as $P < 0.05$.

Results

Upregulation of miR-181b in retinoblastoma cells under hypoxia is HIF-1 α independent. Our previous findings (11) confirmed that hypoxia upregulated the expression of miR-181b in retinoblastoma cells. Since HIF-1 α is the master regulator of transcription factors under hypoxic conditions, we aimed to determine whether the expression of miR-181b is regulated by HIF-1 α . We detected the expression of HIF-1 α in retinoblastoma cells under normal and hypoxic conditions, respectively, and found a 2.4-fold increased expression of HIF-1 α under hypoxia as compared to normal conditions (Fig. 1A). To better understand the connection between HIF-1 α and miR-181b, we reduced the level of HIF-1 α using siRNA technology. In order to silence the target gene more effectively, we designed three pairs of different sequences of siRNA (HIF-1 α -816 targeted 816-836 sites, HIF-1 α -1171 targeted 1171-1191 sites and HIF-1 α -1504 targeted 1504-1524 sites). Quantitative PCR results showed HIF-1 α -1504 reduced the level of HIF-1 α >50% (Fig. 1B). However, the expression of HIF-1 α was largely blocked by siHIF-1 α -1504, and no significant change of the expression of miR-181b (Fig. 1C) was identified, indicating that the increased expression of miR-181b under hypoxia was not regulated by HIF-1 α .

Hypoxia enhances the capacity of tube formation of HUVECs. To examine the effect of hypoxia on the ability of tube formation of HUVECs, HUVECs were exposed to normoxia or hypoxia for 6 h, and the capillary-like structures were calculated. The results showed HUVECs formed more capillary-like structures under hypoxia than under normoxia (Fig. 2).

miR-181b significantly promotes capillary tube formation of HUVECs under hypoxia. To examine the biological mechanism of miR-181b in tumor-hypoxic responses, we further detected the effect of miR-181b on the ability of capillary tube formation of HUVECs *in vitro*. Retinoblastoma cells were transfected with miR-181b precursor and inhibitor to upregulate or downregulate their miR-181b levels. Their culture medium was added

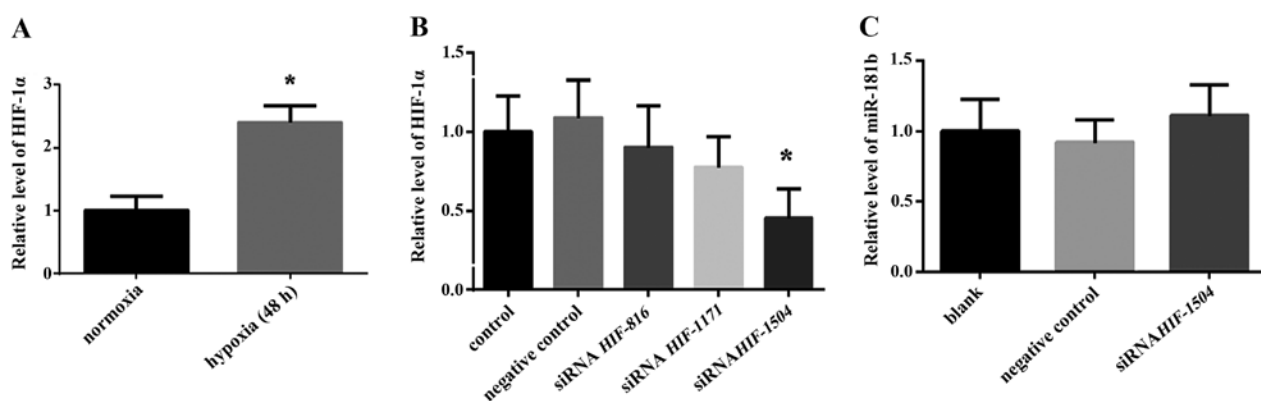


Figure 1. Hypoxia-induced miR-181b overexpression in retinoblastoma cells (HXO-RB44) was not regulated by hypoxia-inducible factor-1 α (HIF-1 α). (A) When exposed to hypoxia for 48 h, the expression of HIF-1 α in HXO-RB44 cells was upregulated compared to normal conditions. (B) HXO-RB44 cells were transfected with small-interfering RNAs (siRNAs) against HIF-1 α or scrambled sequence as control under hypoxic conditions, while the sequence siHIF-1 α -1504 decreased the expression of HIF-1 α effectively. (C) The level of miR-181b was not suppressed by siHIF-1 α -1504 simultaneously. The data are presented as means \pm SD (* P <0.01).

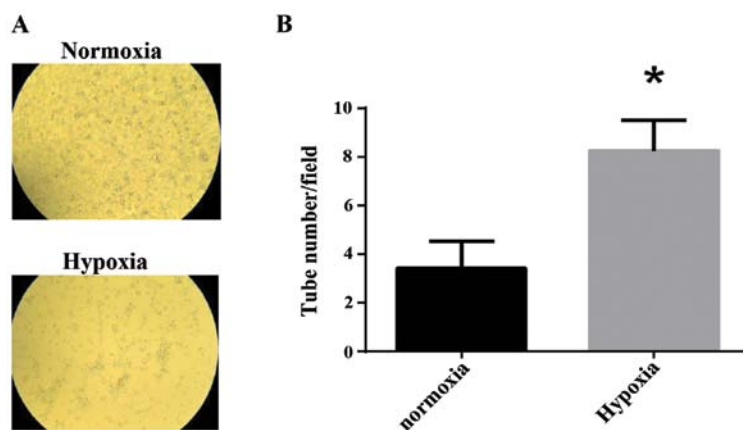


Figure 2. Human umbilical vein endothelial cells (HUVECs) formed more capillary-like structures when exposed to hypoxic conditions. After being cultured under low-oxygen conditions for 6 h, the ability of capillary tube formation of HUVECs was significantly increased compared to under normoxia (* P <0.01).

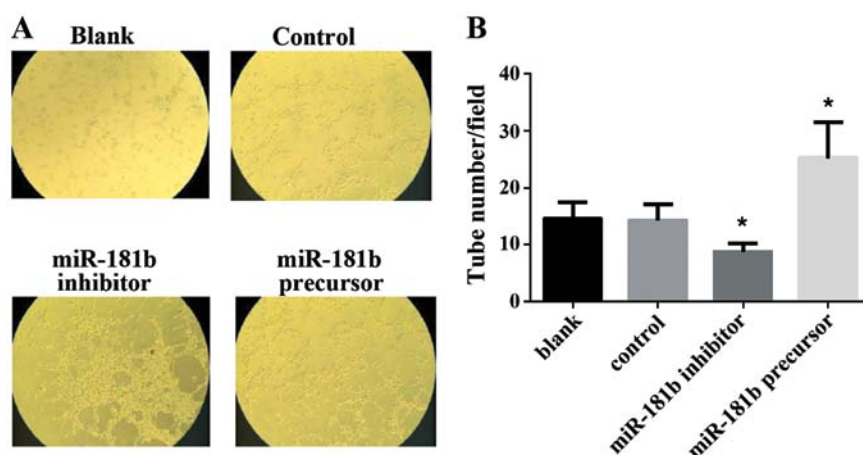


Figure 3. miR-181b enhanced capillary tube formation of human umbilical vein endothelial cells (HUVECs) *in vitro*. HUVECs were cultured with culture medium of retinoblastoma cells (pre-transfected with miR-181b precursor, and inhibitor and the negative control, respectively) or ordinary M-199 medium as blank for 6 h under hypoxic conditions. Medium of miR-181b precursor increased the ability of tube formation of HUVECs while the medium of miR-181b inhibitor reduced the ability of tube formation compared with the control (* P <0.01).

to HUVECs. The culture medium of miR-181b precursor-transfected retinoblastoma cells significantly increased the capillary tube formation of HUVECs. By contrast, the medium

of miR-181b inhibitor led to the suppression of tube formation of HUVECs. The results suggested that miR-181b enhanced the angiogenesis of HUVECs *in vitro* (Fig. 3).

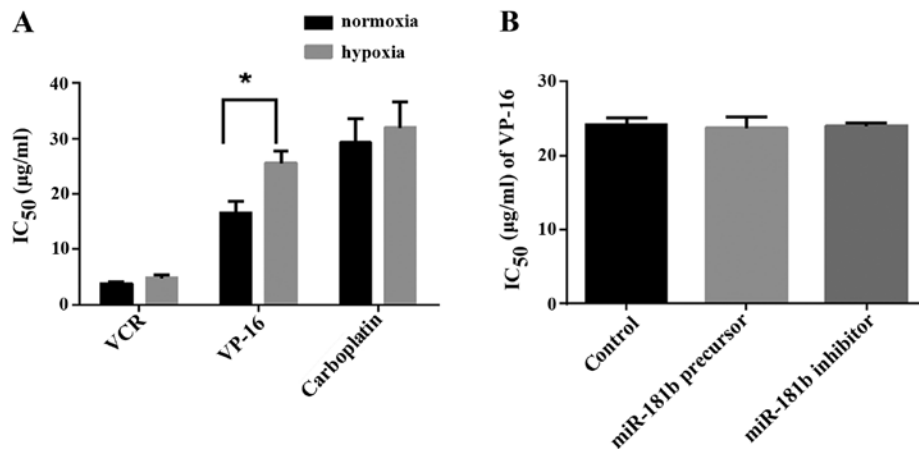


Figure 4. Induced chemoresistance to VP-16 by hypoxia is independent of miR-181b in retinoblastoma cells. IC₅₀ of drugs was assessed to determine the chemoresistance of drugs. (A) After being cultured under hypoxia for 72 h, only IC₅₀ of VP-16 was increased significantly (*P<0.01). (B) Pre-transfected (miR-181b precursor, and inhibitor and control) cells were treated with VP-16 under hypoxia for 72 h. No significant change of IC₅₀ compared to the control was identified, indicating hypoxia-induced chemoresistance of VP-16 is independent of miR-181b (P>0.05).

Gene	Putative binding sites	
Pleiomorphic adenomagen 1 (PLAG1)	5' ...UUAUUAAGCUAAUAAUGAAUGUU...	3501-3507
	3' UGGGUGGCUGUCGUUACUUAACAA	miR-181b
	5' ...ACAGAUGAACAGAAUGAAUGUU...	4389-4395
GATA binding protein 6 (GATA6)		
	3' UGGGUGGCUGUCGUUACUUAACAA	miR-181b
	5' ...CAGCAUUUUUUAUAAUGAAUGUA...	720-726
E2F transcription factor 5 (E2F5)	3' UGGGUGGCUGUCGUUACUUAACAA	miR-181b
	5' ...AAAUUUUUUUUUAAUAAUGAAUGUA...	83-89
	3' UGGGUGGCUGUCGUUACUUAACAA	miR-181b
protease activated receptors 4 (PAR4)	5' ...UUUUAAACUACUGAUUGAAUGUU...	44-50
	3' UGGGUGGCUGUCGUUACUUAACAA	miR-181b
	5' ...CAU-GUUAGUUGCCAGCUACC	348-370
programmed cell death-10 (PDCD10)		
	3' GTATTAA C AA TAT T TGGTGG	miR-181b
	5' ...CCAGGCCGCGAGCCCGUGGAUG	375-381
T-cell leukemia/lymphoma 1 (TCL1)	3' GGUGGCUGUCGUUACUUAACAA	miR-181b
	5' ...GCCUACAGCACAU--UGAAUGUU...	232-238
	3' GGGUGGCUGUCGUUACUUAACAA	miR-181b
Glutamate receptor 1 (GRIA1)	5' ...GGGUGGCUGU--CGUUAUUAACAA	313-336
	3' C C C G G C G C C A T C C G T G A A T G T T	miR-181b
	5' ...GGGUGGCUGU--CGUUAUUAACAA	

Figure 5. Bioinformatics analysis predicted target genes of miR-181b. There were eight predicted target genes that contained putative binding sites for miR-181b at the 3'-untranslated region (3'-UTR).

Induced chemoresistance to VP-16 by hypoxia is independent of miR-181b in retinoblastoma cells. To elucidate the possible role of miR-181b as a hypoxic responses regulator, we examined the effect of miR-181b on chemoresistance in retinoblastoma cells. VCR, VP-16 and carboplatin are the most widely used chemotherapeutic agents in retinoblastoma. The regimen containing these three drugs has become the first-line therapy for retinoblastoma in recent years. We used IC₅₀ which describes the concentration of the drugs that lead to inhibition

of cell growth in 50% of the treated cells to represent the chemoresistance. If a drug has a higher IC₅₀, stronger cell resistance to drugs should be considered. When exposed to hypoxic conditions for 72 h, the IC₅₀ values of three drugs were higher than those of the normal conditions, with only the difference of VP-16 (25.66 vs. 16.57 µg/ml) being statistically significant (Fig. 4A). It meant that hypoxia was one of the incentives of acquired resistance of VP-16. We further characterized the role of miR-181b in regulating hypoxia-induced chemoresistance of VP-16 by modulating its levels in retinoblastoma cells. But transient transfection of miR-181b precursor and inhibitor did not show any effect on the IC₅₀ of VP-16 compared to control (23.76, 24.03 and 24.17 µg/ml, respectively, Fig. 4B). The data indicated hypoxia-induced chemoresistance of VP-16 in retinoblastoma cells was miR-181b-independent.

Prediction of target genes of miR181b. The conservation of the miRNA binding sites in the 3'-UTR of the orthologous genes is a significant feature in predicting miRNA targets. It is conceivable that the use of multiple prediction tools lead to a higher confidence in predicting miRNA target gene pairs. We adopted a consensus target prediction approach of using multiple miRNA target prediction databases: miRBase, TargetScan and PicTar to identify target genes of miR-181b. Eight genes contained predicted binding site for miR-181b at 3'-UTR (Fig. 5). Further investigation was performed to exclude the false-positive. We detected the mRNA level of these genes and found *GATA6*, *PDCD10*, *PLAG1* and *E2F5* were steadily expressed in retinoblastoma cells (Fig. 6). The four genes were selected as candidates of miR-181b.

miR-181b inhibits PDCD10 and GATA6 production in retinoblastoma cells. We examined the regulation of miR-181b on the expression of the four genes mentioned above by western blotting. The results showed that pretreatment with miR-181b precursor decreased PDCD10 and GATA6 expression while pretreated miR-181b inhibitor increased PDCD10 and GATA6 expression (Fig. 7). These results suggested that miR-181b acted as a negative regulator of PDCD10 and GATA6 under hypoxia in retinoblastoma cells.

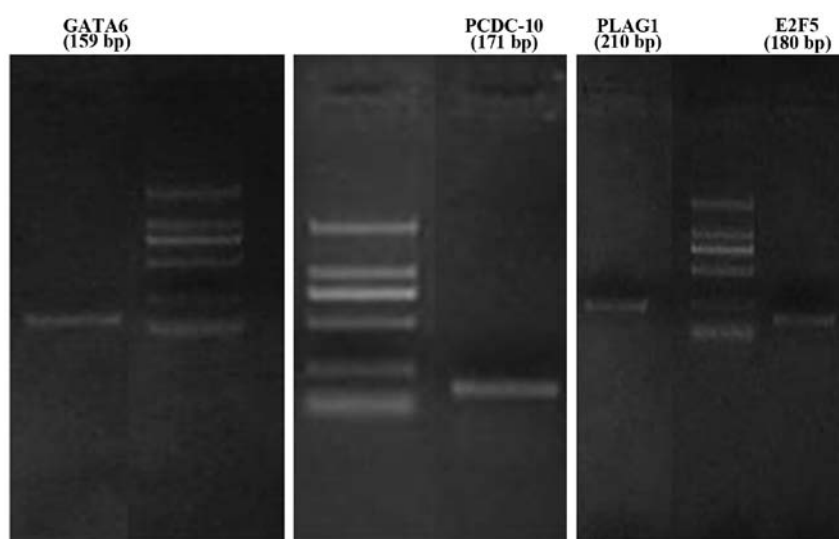


Figure 6. RT-PCR detected the expression of potential target genes in retinoblastoma cells. The *GATA6*, *PDCD10*, *PLAG1* and *E2F5* genes were steadily expressed in retinoblastoma cells. *GATA6*, GATA binding protein 6; *PDCD10*, programmed cell death-10; *PLAG1*, pleiomorphic adenoma gene 1; *E2F5*, E2F transcription factor 5.

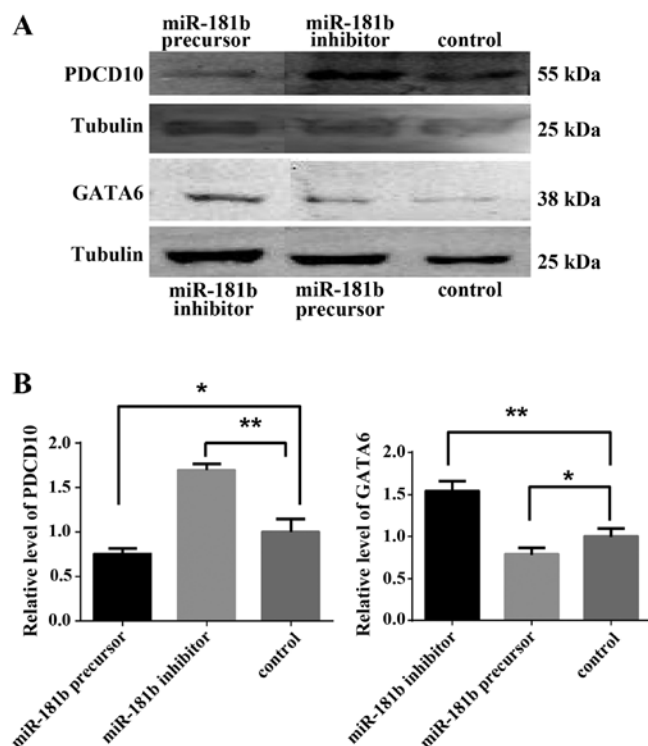


Figure 7. Regulation of miR-181b on the expression of *PDCD10* and *GATA6*. The levels of *PDCD10* and *GATA6* expression were obviously inhibited by transfection with miR-181b precursor to upregulate miR-181b level in cells, while knockdown of miR-181b with miR-181b inhibitor, showed a significantly increased expression of *PDCD10* and *GATA6* (* $P < 0.05$, ** $P < 0.01$). *GATA6*, GATA binding protein 6; *PDCD10*, programmed cell death-10.

Discussion

Hypoxia is an important pathological process of solid tumors, and hypoxic regions are present in retinoblastoma (12). The findings of our previous study showed that miR-181b was upregulated by hypoxia in retinoblastoma cells, although the molecular mechanisms of responses to hypoxia remained

unknown. *HIF-1* is the most studied gene that plays a central role in cancer hypoxic responses (13). HIF-1 is composed of HIF1 α and HIF1 β subunits. HIF-1 α is considered as the most important regulator under hypoxic conditions in various solid tumors and a high expression of HIF-1 α was confirmed in the progression of retinoblastoma (14-16). Many miRNAs, such as miR-21, -210, -103 and -195 were reported to be induced and upregulated by HIF-1 α in cancer cells under hypoxic conditions (17,18). Thus, whether miR-181b is also a controlled candidate of HIF-1 α remains to be determined. Results of this study showed that the expression of HIF-1 α in retinoblastoma cells was elevated substantially following exposure to hypoxia for 48 h. When using HIF-1 α siRNA to reduce the level of HIF-1 α , the repression of miR-181b was not observed synchronously. These results suggested the expression of miR-181b under hypoxia was not regulated by HIF-1 α . A possible explanation for this finding is that HIF-1 α may be crucial under hypoxic conditions but it is not the single regulatory factor. Mounting evidence has indicated HIF alone is insufficient to implement the full program of adaptive changes of cancer under hypoxic stress. Recently, transcription factors responding to hypoxia, such as p53 and NF- κ B, have been shown to affect the expression of selected miRNAs (19-21). Instead, mTOR, endoplasmic reticulum (ER) stress were also reported to be involved in hypoxic responses via HIF-independent pathways (22,23). It seems that miRNAs interface with both HIF-driven and -independent pathways to form an interconnected regulatory network under hypoxic stress. We have demonstrated that miR-181b transcriptional upregulation in hypoxia in retinoblastoma is HIF-1 α -independent. However, additional studies are needed to identify the network between miR-181b and hypoxia in retinoblastoma.

miR181-b is a type of miRNA endogenously expressed in cells of the inner and middle retina. Dysregulated expression of miR-181b has been demonstrated in different tumors (24,25). Retinoblastoma is a malignancy originating from the retina. However, to the best of our knowledge, there are no reports available concerning the possible roles of miR-181b in the devel-

opment of retinoblastoma. We have identified miR-181b as an HRM of retinoblastoma in a previous study (11). Since chemoresistance and angiogenesis were main responses to hypoxia in many tumors including retinoblastoma, we examined the functions of miR-181b in hypoxic responses of retinoblastoma. Firstly, we determined the influence of hypoxia on the ability of tube formation of HUVECs and found HUVECs formed more capillary tubes under hypoxic as compared to normal conditions. Subsequently, through up- and downregulation of miR-181b expression in retinoblastoma cells, we demonstrated that miR-181b enhanced the ability of capillary tube formation of HUVECs *in vitro*. Additionally, we identified that retinoblastoma cells showed a stronger resistance to VP-16 in hypoxic conditions, although the same chemoresistance was not observed in VCR and carboplatin (these three were the most popularly used chemotherapy drugs in retinoblastoma). Further investigation suggested the chemoresistance induced by hypoxia was miR-181b-independent. These results, however, were not consistent with those of previous studies and there is controversy regarding the roles of miR-181b in human malignancies. For instance, Sun *et al* reported overexpression of miR-181b increased the sensitivity of glioma cells to teniposide, while Takiuchi *et al* found miR-181b led to the resistance of pancreatic cancer cells to gemcitabine by activating NF- κ B (26,27). These discordant findings suggested that miR-181b may play organ-specific roles in part due to the different cell contexts of tumors and pharmacological characteristics. To the best of our knowledge, the present study provides the first evidence that hypoxia-induced miR-181b may play a critical role in angiogenesis and metastasis in retinoblastoma.

To gain insight into the molecular mechanism involved in the function of miR-181b, we predicted eight target genes of miR-181b using bioinformatics analysis. This prediction was further confirmed by evaluating the effects of up- and downregulation of miR-181b on specific target gene expression in retinoblastoma cells. We also determined *PDCD10* and *GATA6* as downstream genes of miR-181b. *PDCD10* (also known as cerebral cavernous malformation 3, CCM3) was expressed in endothelial cells and was essential for vascular development (28). Previous results showed silencing CCM3 or loss of CCM3 (decreased expression of CCM3) stimulated sprouting and tube branching or activated endothelial angiogenesis (29-31). These data indicated the roles of *PDCD10* as an anti-angiogenic transcription factor in vascular development/maturation. *GATA6* is a transcription factor belonging to the GATA family which controls the development and differentiation of a wide spectrum of cell lineages especially in HUVECs. Considerable evidence suggests the importance of *GATA6* in the regulation of vascular endothelial cells, which enables angiogenic function and endothelial cell survival (32). *GATA* gene families may be involved in regulating homeostasis of the eye vasculature (33). Evidence also suggested *GATA6* as a target for repression by miR-181s (34). In agreement with the abovementioned results, we found that upregulation of miR-181b in retinoblastoma cells inhibited the expression of *PDCD10* and *GATA6*, weakened the anti-angiogenic functions of *PDCD10* and *GATA6*, and promoted the angiogenesis of retinoblastoma cells.

In conclusion, there were three important results in the present study: hypoxia-induced overexpression of miR-181b

in retinoblastoma cells is HIF-1 α independent; miR-181b significantly increased the ability of capillary tube formation of HUVECs; and the enhanced effects of miR-181b on angiogenesis may be due to the decreased expression of *PDCD10* and *GATA6*. Our results suggest that miR-181b acts as an oncogenic miRNA in retinoblastoma cells to promote metastasis under a hypoxic microenvironment. These results add to the mounting evidence that miR-181b is crucial in promoting cancer development and may assist in the development of new therapeutic regimens against hypoxic tumors.

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