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Hypoxia promotes invasion of retinoblastoma cells *in vitro* by upregulating HIF-1α/MMP9 signaling pathway

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Abstract. – OBJECTIVE: The aim of this study is to investigate the effect of hypoxia-inducible transcription factors-1α (HIF-1α)/matrix metalloproteinase 9 (MMP9) signaling pathway on hypoxia triggered invasion in retinoblastoma cell line HXO-RB44.

MATERIALS AND METHODS: HXO-RB44 cells were cultured under hypoxia conditions for 24 h. The effect of hypoxia on invasion ability of HXO-RB44 cells was monitored with transwell invasion assay; the mRNA and protein expression levels of HIF-1α and MMP9 were detected by Real-time polymerase chain reaction (RT-PCR) and Western blot; luciferase assay was performed to assess the MMP9 regulation by HIF-1α, and HIF-1α regulation by hypoxia. Furthermore, HIF-1α and MMP9 siRNA were used to investigate the effect of HIF-1α/MMP9 signaling on hypoxia-induced cell invasion.

RESULTS: The results demonstrated that hypoxia could promote HXO-RB44 cells invasion. The mRNA and protein level of HIF-1α and MMP9 were upregulated by hypoxia treatment, whereas HIF-1α and MMP9 siRNA could reverse these processes.

CONCLUSIONS: Hypoxia promotes retinoblastoma cell line HXO-RB44 invasion by activating HIF-1α/MMP9 signaling pathway.

Key Words:
Retinoblastoma, Hypoxia, HIF-1α, MMP9, Invasion.

Introduction

As one of the most common ocular primary malignant tumors in children below 3 years old, retinoblastoma with the familial hereditary inclination is severely threatening the life and health of affected children[1,2]. Retinoblastoma is characterized by the features of hidden onset, rapid growth, susceptibility to intracranial and distant invasion and metastasis[3,4]. In recent years, various new surgical techniques and individualized comprehensive therapy have been developed, which have gained significant efficacy in the clinical treatment of retinoblastoma. However, distant metastasis and invasion of tumors remain the major fatal cause of affected patients. Thus, in-depth research into the molecular mechanism of invasion and metastasis of retinoblastoma is of great clinical significance for controlling the growth of tumor and improving the prognosis of patients. During the rapid growth of tumors, contradictions lying between the rapid proliferation of tumor cells and blood supply in the tumor contribute to the formation of hypoxia-ischemia microenvironment in the local tissues of tumor, further affecting the growth, angiogenesis, invasion and metastasis[5-7]. Research has shown that hypoxia inducible factor-1 (HIF-1) is the key factor in regulation of intracellular oxygen concentration[8,9]. Besides, through regulating the transcription of downstream target genes, such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9), it is also involved in a variety of biological events, including the angiogenesis and distant invasion of tumor cells[5,10,11]. In this study, we aimed to investigate the effect of *in-vitro* hypoxic microenvironment on the invasive activity of the human retinoblastoma HXO-RB44 cells, and explore the potential mechanism of HIF-1α/MMP9 signaling pathway in this process.

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Materials and Methods

Materials

Human retinoblastoma HXO-RB44 cell lines were purchased from China Center for Type Culture Collection (College of Life Sciences, Wuhan University); TRIzol RNA extraction kit was purchased from Invitrogen (Carlsbad, CA, USA); primer synthesis was performed by Tianyi Huiyuan Biotech (Beijing, China); monoclonal rabbit anti-human HIF-1α and MMP9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); horseradish peroxidase (HRP)-labeled goat anti-mouse or -rabbit immunoglobulin G (IgG) was purchased from Cell Signaling Technology (Danvers, MA, USA); medium-molecular protein marker was purchased from Thermo Fisher (Waltham, MA, USA); enhanced chemiluminescence (ECL) kit was purchased from Millipore (Billerica, MA, USA); fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd.; Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium was purchased from Hyclone (Logan, UT, USA); radio-immunoprecipitation assay kit (RIPA) and bicinchoninic acid (BCA) quantification kit were purchased from Beyotime Biotechnology Institute (Wuhan, China); 6-well plate and T25 plastic culture bottle were purchased from Beaver (Ware, UK); 24-well Transwell chamber (8 μm) was purchased from Corning (Corning, NY, USA); Matrigel used for invasion experiment was purchased from BD (Franklin Lakes, NJ, USA); MCO-5M tri-gas incubator for simulating the ischemic environment for cell culture was purchased from Sanyo (Tokyo, Japan); TS100 binocular inverted microscope was purchased form Nikon (Tokyo, Japan); horizontal, vertical and transfer electrophoresis systems were purchased from Beijing Liuyi Biotechnology Co., Ltd. (Beijing, China).

Methods

Cell Culture

In a cell incubator (37°C, 5% CO_2 and saturated humidity), human retinoblastoma HXO-RB44 cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution and 2 mmol/L glutamines. After cells were digested using 0.25% trypsin and passaged, culture medium was replaced every 1 or 2 days, and cells in the logarithmic phase were used for experimental research. When 70 to 80% of cells were infused, they were starved using serum-free culture medium and placed in a hypoxic incubator for culture. In different time point (8, 16, and 24 h), we detected the relevant indexes of HXO-RB44 cells. Regular culture conditions were set as follows: 37°C, 5% CO_2 and 20% O_2; the oxygen concentration in hypoxic culture was set as 1%.

RNA Extraction and Real-time PCR Detection

HIF-1α and MMP9 expressions were detected via RT-PCR. Pre-cooled phosphate buffer saline (PBS) was used to wash the cells in each group for 3 times, and 1 mL TRIzol RNA extraction reagent was added to each well. Procedures were carried out to extract the total RNA according to the instructions, and RNA concentration was calculated using the A260/A280 ratio detected by ultraviolet spectrometry, which was repeated for 3 times. cDNA was prepared by reverse transcription of RNA that was extracted using reverse transcription kit for PCR. Primers used in PCR were synthesized by Invitrogen (Carlsbad, CA, USA). Sequences of primers were as follows: β-actin upstream primer 5'-ATGGGGAAGGTGAAGGTCG-3', downstream primer 5'-GGGTCATTGATGGCAACAATATC-3'; HIF-1α upstream primer 5'- AAGTCCCGCCGTGAAGTG-3', downstream primer 5'- ACGCCAAGGTCTGAAGGTC-3'; MMP9 upstream primer 5'- ATCCCCCAACCTTTACCA-3', downstream primer 5'- TCAGAACCGACCCTACAA-3'. Reaction conditions were set as follows: initial denaturation at 95°C for 3 min; 94°C for 30 s, 48°C for 30 s and 72°C for 1 min for 35 cycles; extension at 72°C for 10 min. 1.5% agarose gel electrophoresis was carried out for PCR products, and gray scan was performed using the images obtained by gel imaging system, which was repeated for 3 times.

Western Blotting

Human retinoblastoma HXO-RB44 cells were respectively cultured in regular environment and hypoxic environment, and the adhesive cells in 6-well plate were washed using pre-cooled PBS and treated using RIPA. Then, cell lysate was collected using a cell scraper and transferred into a 1.5 mL Eppendorf (EP; Hamburg, Germany) tube for lysis on ice for 30 min. EP tube was then centrifuged at 12000 rpm for 10 min with the sediment being discarded. Supernatant was preserved

at -20°C. After samples were all loaded, proteins were aggregated through electrophoresis at 80 V, and separated and transferred onto a membrane at 100 V. Membrane was then blocked using 5% skimmed milk. Rabbit anti-human HIF-1α (1:1000) and MMP9 polyclonal antibodies and the mouse anti-human β-actin monoclonal antibody were added onto the membrane for incubation at 4°*C* followed by washing membrane on a decoloring shaker using Tris-buffered saline + Tween 20 (TBST) for 3 times (5 min/time). Next, horseradish peroxidase (HRP)-labeled goat anti-mouse or rabbit IgG was added on the membrane for incubation for 1 h at room temperature followed by washing membrane using TBST for 3 times (5 min/time). Then, electro chemiluminescence (ECL) reagent was added for 1 min of reaction at room temperature followed by X-ray exposure, fixation, and color development.

Cell Invasion Capability Via Transwell Cell Invasion Experiment

Matrigel was prior melted at 4°C, and 40 μL diluted Matrigel (volume ratio between Matrigel and serum-free culture medium was 1:3) were spread on the surface of each polycarbonate microporous membrane. Then, the polycarbonate microporous membrane was placed into an incubator for 4 h for later use. On the next day, the retinoblastoma HXO-RB44 cells in logarithmic phase were starved in a serum-free DMEM culture for 24 h followed by digestion using 0.25% ethylene diamine tetra acetic acid (EDTA) trypsin and preparation of single-cell suspension in serum-free DMEM medium. After the cell density was adjusted to 4×10^5/mL, we detected cell viability using trypan blue staining, and cells with viability above 90% were used for following experiments. In the upper chamber of each transwell, 200 μL serum-free cell suspension was added; in the lower chamber of each transwell, 600 μL DMEM medium containing 20% fetal bovine serum (FBS) was added to each well. The plate was delivered into the incubators for 24 h of incubation in regular and hypoxic environment. Chambers were taken out after the completion of culture, and washed using PBS for 3 times to remove the medium. Wet cotton swabs were used to gently scrape the cells in the upper chamber that failed to pass through the membrane followed by 20 min of fixation in 4% paraformaldehyde. Thereafter, chambers were dried at room temperature followed by further 20 min of staining using crystal violet. After staining, chambers were placed under inverted microscope to count the number of cells that passed through the membrane, and the cell counts in 5 visions (central, upper, lower, right and left visions) were recorded to calculate the average.

Statistical Analysis

All values were presented as χ±s, and statistical analysis was carried out using SPSS 20 (SPSS Inc., Armonk, NY, USA). One-way analysis of variance (ANOVA) was carried out in comparisons among groups, and Student' *t*-test was performed for intergroup comparison. $p<0.05$ suggested that the difference had statistical significance.

Results

Effect of Hypoxic Environment on Invasion Capability of HXO-RB44 Cells

Detections of invasion capability were carried out for retinoblastoma HXO-RB44 cells cultured in regular and hypoxic environment, respectively. Results of transwell invasion experiment showed that in comparison with the cells cultured in regular environment, a significant increase in invasion capability was identified in HXO-RB44 cells cultured in the hypoxic environment (Figure 1).

Hypoxic Environment Activated the HIF-1α/MMP9 Signaling Pathway

After the retinoblastoma HXO-RB44 cells were respectively cultured in regular and hypoxic environment, those cells were used for detecting the mRNA and protein expressions of HIF-1α and MMP9 via RT-PCR and Western blot. The results showed that compared with the cells cultured in regular environment, the mRNA and protein expressions of HIF-1α and MMP9 in HXO-RB44 cells cultured in hypoxic environment were significantly increased, suggesting that hypoxic microenvironment could activate the HIF-1α/MMP9 signaling pathway (Figure 2).

HIF-1α Upregulated the Activity of MMP9 Promotor

We adopted the luciferase reporter genes in this study, and transfected the retinoblastoma HXO-RB44 cells using overexpression plasmid of pCGN-HAM-HIF-1α and p GL33-MMP9-promoter plasmid. At the same time, in each well, cells were also transfected using p RL-TK for internal reference. After 24 h of culture in hypoxic

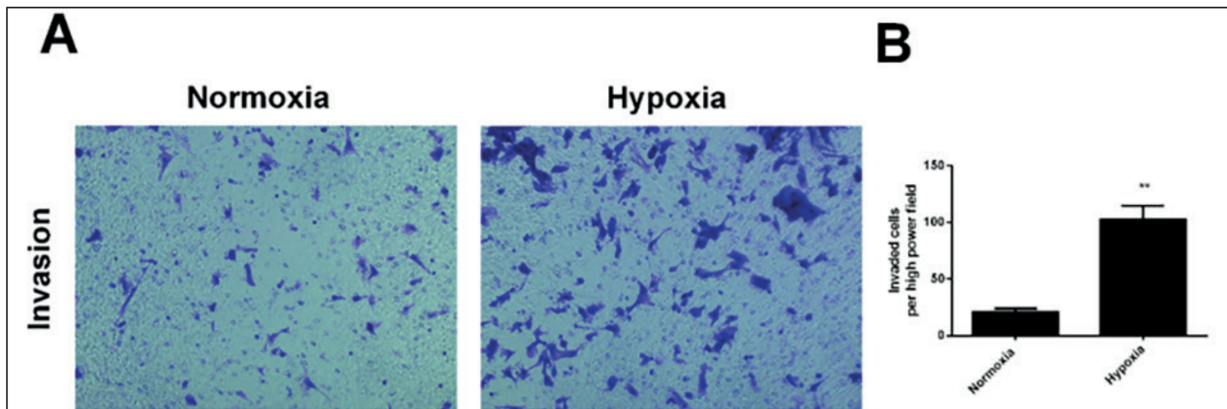

Figure 1. Hypoxia enhances the invasion ability of HXO-RB44 cells. ***A,*** The transwell invasion assay in HXO-RB44 cells treated with hypoxia. ***B,*** The quantitative result of transwell invasion assay in HXO-RB44 cells.

environment, cells were harvested for detecting the activity of luciferase. Results showed that compared with the cells transfected using empty plasmid, the activity of MMP9 plasmid in cells that were transfected using overexpression plasmid of HIF-1α was significantly elevated and the difference had statistical significance ($p < 0.05$; Figure 3).

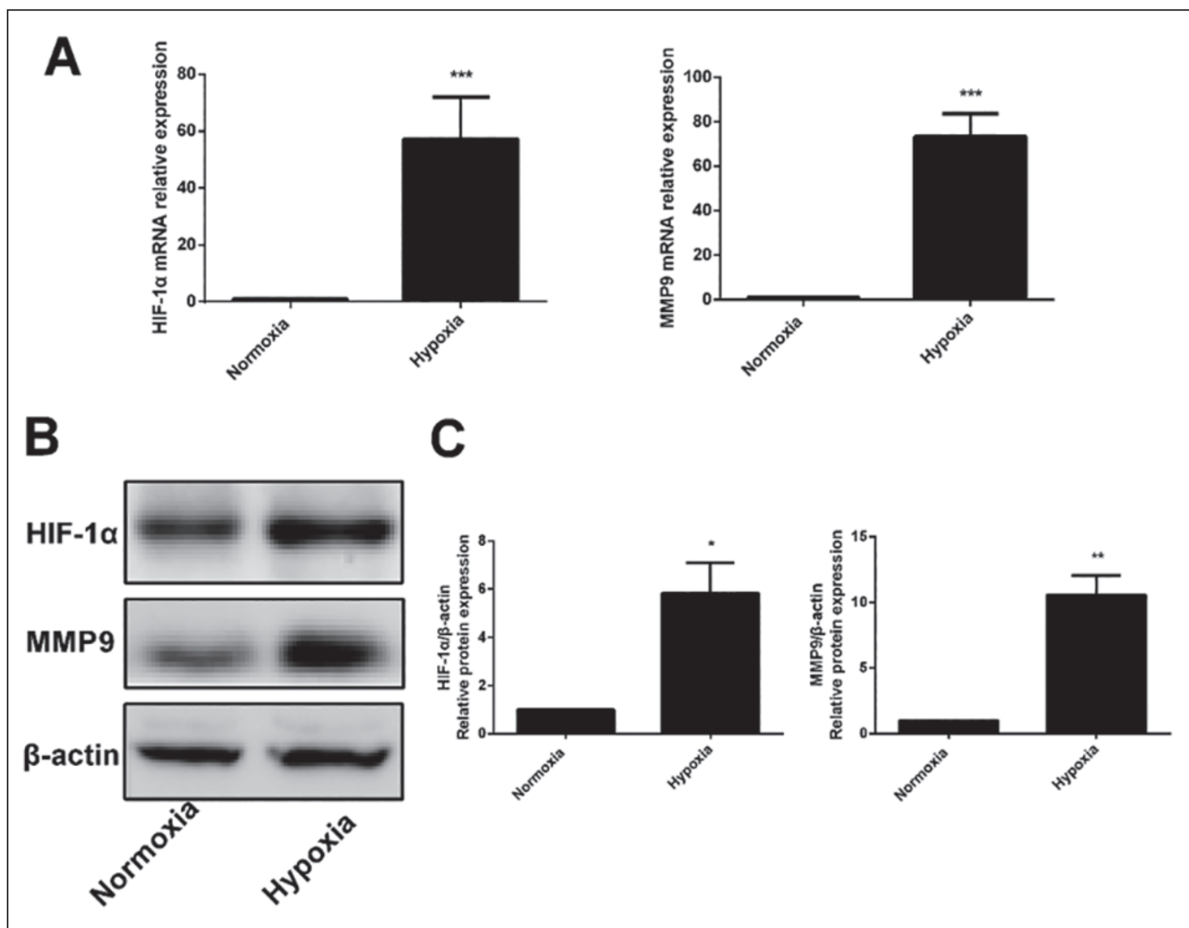

Figure 2. HIF-1α/MMP9 signaling was activated by hypoxia treatment. ***A,*** RT-PCR analysis of HIF-1α and MMP9 mRNA. ***B,*** Western blot analysis of HIF-1α and MMP9 protein. ***C,*** Quantification of Western blot results.

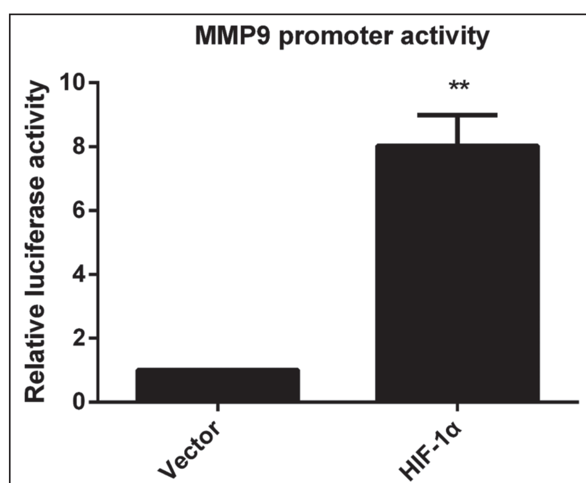

Figure 3. Relative luciferase activity of MMP-9 promoter after transfection with HIF-1α overexpression plasmid.

Hypoxic Environment Upregulated the Activity of HIF-1α promotor

Results of luciferase reporter gene detection showed that after HXO-RB44 cells were cultured in hypoxic environment, the activity of HIF-1α promotor in cells was remarkably enhanced, and the difference had statistical significance ($p < 0.05$; Figure 4).

Effects of HIF-1α and MMP9-Specific siRNAs on HIF-1α/MMP9 Signaling Pathway

Being transfected using HIF-1α and MMP9-specific siRNAs and relevant NC siRNA for 24 h, retinoblastoma HXO-RB44 cells were treated in hypoxic environment for other 24 h followed by detection of mRNA and protein expressions of HIF-1α and MMP9 through RT-PCR and Western blot. Results revealed that compared with the NC group, the mRNA and protein expressions of HIF-1α and MMP9 in cells transfected by HIF-1α siRNA, were significantly decreased, and the difference had statistical significance ($p < 0.05$); there were no remarkable changes in mRNA and protein expressions of HIF-1α in cells transfected by MMP9-specific siRNA, but the mRNA and protein expressions of MMP9 were significantly reduced, and the difference had statistical significance ($p < 0.05$; Figure 5).

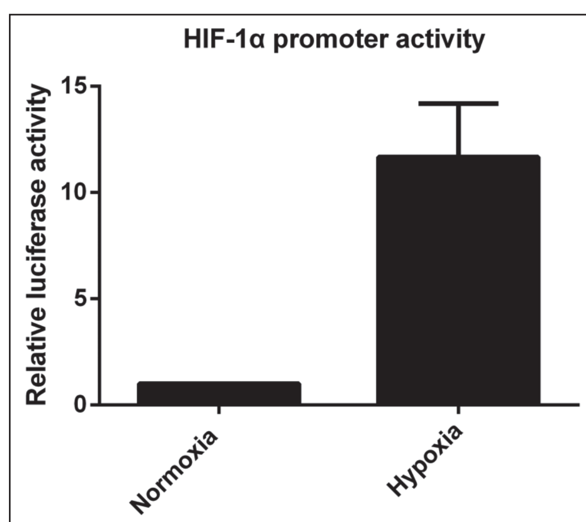

Figure 4. Relative luciferase activity of HIF-1α promoter after treated by hypoxia for 24 hours.

Correlation Between HIF-1α/MMP9 Signaling Pathway and Hypoxia-Promoted HXO-RB44 Cell Invasion Capability

In the Transwell migration experiment, we found that after 24 h of culture in hypoxic environment, no significant differences were observed in comparison of invasion capability of HXO-RB44 cells between hypoxia control group and hypoxia si NC group, but the invasion capability of cells respectively transfected by HIF-1α siRNA and MMP9 siRNA was remarkably inhibited, and the difference had statistical significance ($p < 0.05$; Figure 6).

Discussion

Retinoblastoma, frequently seen in children aged below 3 years old in clinical practice, is a kind of tumor with high malignancy that originates from photoreceptor precursor; it is characterized by the biological features, such as high malignancy, rapid growth in local tissues and susceptibility to the intracranial and distant metastasis and recurrence. The occurrence and development of retinoblastoma are commonly regulated by multi-genes, factors and steps[12,13]. Conventional therapeutic methods include ophthalmectomy, photodynamic therapy, radiotherapy and chemotherapy specifically for metastatic tumors. In recent years, the application of new diagnostic and therapeutic methods in clinical practice of ophthalmology play an important role in the treatment of retinoblastoma, but poor efficacy has been gained in metastasis or invasion of retinoblastoma; therefore, the prognosis of this treatment remains poor[14,15]. The essence of this problem is that the onset of retinoblastoma is at

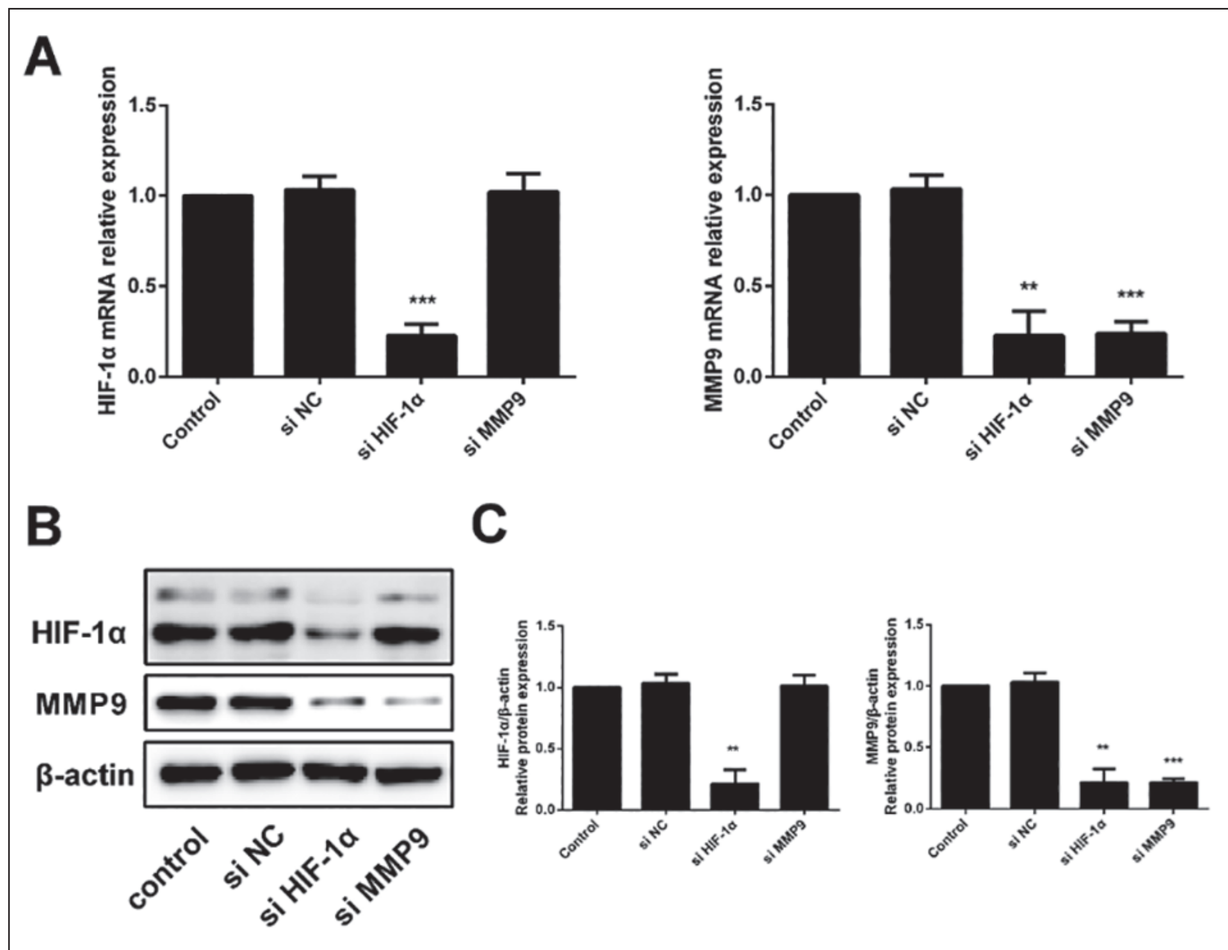

Figure 5. The effect of HIF-1α and MMP9 siRNA on HIF-1α/MMP9 signaling under hypoxia condition. ***A,*** RT-PCR analysis of HIF-1α and MMP9 mRNA. ***B,*** Western blot analysis of HIF-1α and MMP9 protein. ***C,*** Quantification of Western blot results.

a relatively young age, and most patients usually have suffered the distant metastasis or invasion at the time of diagnosis, which is also one of the major causes for the death of pediatric patients with retinoblastoma. In addition, during the formation and growth of tumors, due to the infinite proliferation of tumor cells, when the tumor volume reaches 3 or 4 mm^3, blood supply of tumor tissues can hardly satisfy the need of tumor cells for rapid growth, finally resulting in the insufficiency of blood supply in local tumor tissues, and formation of ischemic and hypoxic microenvironment inside the tumor tissues[16,17]. Literature[18,19] reported that hypoxic microenvironment can induce the expressions of a variety of genes to promote the invasion and metastasis of tumor cells, thus regulating the occurrence and development of tumors. Hypoxia inducible factor-1 (HIF-1), as the most important transcriptional regulatory factor in hypoxic microenvironment for cells in mammal animals, consists of α and β subunits, in which HIF-1α is the sole transcriptional factor with the activity to regulate the concentration of oxygen[20]. Due to the high instability in the normal concentration of oxygen, HIF-1α is easily degraded by hydrolase, but when it comes to the hypoxic environment, HIF-1α can enter the nucleus to bind with the β subunit to form the stable dimer, thus regulating the transcription of downstream target genes[21]. Currently, it has been proved that there are more than 30 kinds of downstream target genes of HIF-1α that are widely involved in various biological processes, including the energy metabolism, angiogenesis, distant metastasis and invasion of tumor cells, thus contributing to the occurrence and development of tumor[22,23]. MMP9 is the downstream target gene of HIF-1α, which can induce the expression of MMP9 to promote

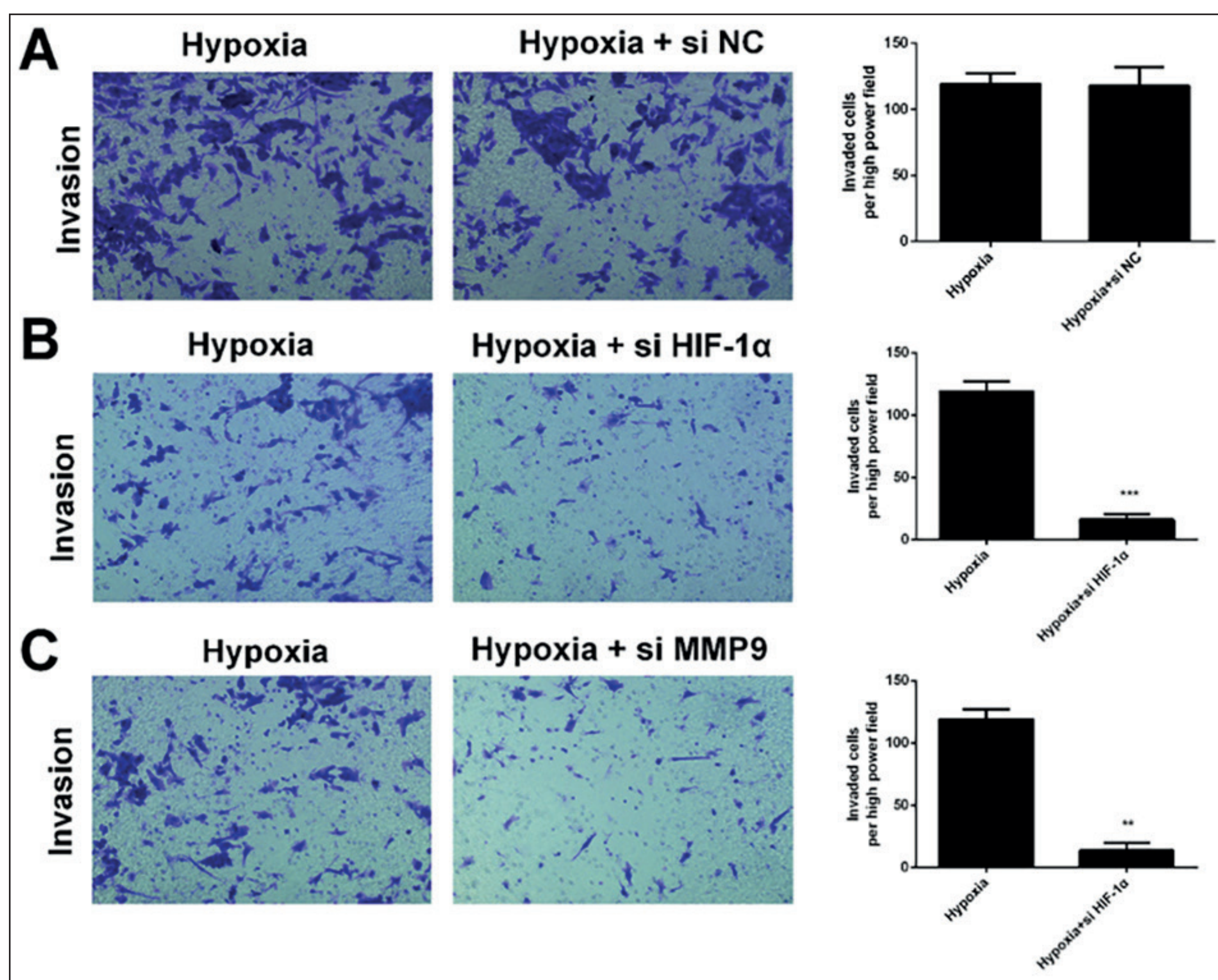

Figure 6. HIF-1α siRNA and MMP9 siRNA attenuates hypoxia triggered HXO- RB44 invasion (crystal violet staining ×200). ***A, B,*** and ***C,*** Transwell assay detect the invasion abilities of HXO-RB44 cells and quantification of the results.

the accelerated degradation of extracellular composition and vascular basement membrane; thus, invasive tumor cells are more apt to pass through the extracellular matrix and basement membrane of primary lesion to infiltrate into the peripheral vessels and lymph-vessel for infiltration and metastasis, thereby providing conditions for distant invasion and metastasis of tumor cells[24]. Research has shown that HIF-1α is correlated with onset of multiple tumors, including colorectal cancer[25], liver cancer[21], and retinoblastoma[26]. All these studies have shown that HIF-1α is highly expressed in these tumor tissues. In addition, some literature[21] reported that many genes that belong to the MMP family are highly expressed in the tumor tissues of retinoblastoma, suggesting that MMP9 might be crucial to the occurrence and development of retinoblastoma. However, there remain few studies reporting the effect of HIF-1α/MMP9 signaling pathway in the onset of retinoblastoma and the relevant regulation mechanism under the hypoxic microenvironment. In this study, we firstly adopted the transwell cell invasion experiment to verify whether *in-vitro* hypoxic microenvironment can induce the invasion of retinoblastoma HXO-RB44 cells. The results showed that compared with the cells cultured under the regular environment, a significant increase was found in the invasion capability of HXO-RB44 cells that had been cultured for 24 h under the hypoxic environment. Furthermore, we investigated the relevant molecular mechanism how the invasion capability of cells was enhanced under the hypoxic microenvironment. RT-PCR and Western blotting experiments revealed that compared with the cells cultured under the regular environment, HIF-1α/MMP9 signaling pathway in the HXO-RB44 cells that

had been cultured under the hypoxic environment was significantly activated, suggesting that HIF-1α/MMP9 signaling pathway might be involved in the invasion of retinoblastoma tumor cells under the hypoxic environment. Then, we detected the role of HIF-1α in regulating the activity of the promotor of the target gene of MMP9 using the dual-luciferase. The results showed that under the hypoxic environment, the activity of MMP9 promotor was remarkably enhanced. Additionally, in comparison with the control group, the activity of MMP9 promotor was upregulated after transfection of HIF-1α. To clarify the regulatory role of HIF-1α/MMP9 signaling pathway in the invasion of HXO-RB44 cells, we adopted the specific siRNAs of HIF-1α and MMP9 to block the relative gene expression, and further determined the effect on cell invasion. Studies have shown that after the HIF-1α/MMP9 signaling pathway was specifically silenced, hypoxia-induced cell invasion was significantly inhibited, indicating that HIF-1α/MMP9 signaling pathway plays a crucial role in this process. Based on the studies above, we inferred that hypoxia could promote the distant invasion and metastasis of HXO-RB44 cells through activating the HIF-1α/MMP9 signaling pathway, thus promoting the occurrence and development of retinoblastoma.

Conclusions

We preliminarily observed that HXO-RB44 cell strains of retinoblastoma in the hypoxic microenvironment can promote the distant metastasis and invasion of tumor cells through activating the HIF-1α/MMP9 signaling pathway, which might be conducive to enhancing the understandings on pathogenesis of osteosarcoma. Therefore, in-depth studies are required to figure out the role of HIF-1α/MMP9 signaling pathway in the occurrence, development of osteosarcoma and the relevant regulation mechanism. Moreover, the invasion and metastasis of tumor cells can be inhibited through specifically blocking the HIF-1α/MMP9 signaling pathway, which may provide new ideas and reference for specific treatment of retinoblastoma. Thus, this treatment method has a great clinical significance and a promising application prospect.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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