

Hypoxia promotes epithelial-mesenchymal transition of hepatocellular carcinoma cells via inducing Twist1 expression

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Abstract. – OBJECTIVE: To investigate whether hypoxia microenvironment induced hepatocellular carcinoma cells SMMC-7721 epithelial-mesenchymal transition (EMT) and to explore the underlying molecular mechanism.

MATERIALS AND METHODS: In this study, SMMC-7721 cells were cultured under normoxia and hypoxia conditions, respectively. RT-PCR and Western blot were used to monitor the expression level of EMT-related markers, E-cadherin, and vimentin, as well as hypoxia inducible factor-1 α (HIF-1 α) and Twist1. Then we performed the transwell invasion assays to detect the ability of cell invasion.

RESULTS: The results demonstrated that hypoxia micro-environment could induce hepatocellular carcinoma cells SMMC-7721 EMT and enhance the cell invasion ability. Furthermore, knockdown of Twist1 by using specific siRNA could reverse hypoxia-induced EMT process.

CONCLUSIONS: Hypoxia promotes hepatocellular carcinoma cells SMMC-7721 EMT by up-regulating the expression of Twist1.

Key Words:

Hepatocellular carcinoma, Hypoxia, Epithelial-mesenchymal transition, Invasion.

ment is an indispensable factor for occurrence and development of tumors, while hypoxia is one of the features of the microenvironment in solid tumors². Despite the regulatory role of hypoxic environment in the distant invasion and metastasis of tumors, the specific regulation mechanism remains unclear yet. Epithelial-mesenchymal transition (EMT) refers to a complicated process, in which epithelial cells in malignant tumors, under the effects of various factors, can be transformed into the cells with the mesenchymal phenotype through specific procedures^{3,4}. Usually initiating from the invasion and metastasis of malignant tumors, EMT can ultimately lead to an increment in the invasive capability of malignant tumor cells. Research has shown that multiple transcriptional factors are involved in EMT via regulation, in which Twist, as one of the key regulatory factors to initiate the EMT, also participates in the invasion and metastasis of multiple malignant tumors^{5,6}. In this study, we aimed to investigate how hypoxic microenvironment regulated the EMT in HCC SMMC-7721 cells and the potential molecular mechanism to further disclose the action mechanism of invasion and metastasis of HCC cells, which can provide new evidence and therapeutic targets for clinical diagnosis and treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. HCC, for its hidden onset and susceptibility to distant invasion and metastasis, usually leads an extremely high recurrence rate and metastasis rate in patients after operation¹. Thus, research into the invasion and metastasis mechanisms of HCC is of great significance. The specific microenviron-

Materials and Methods

Material

Human hepatic carcinoma SMMC-7721 cell strain was purchased from Institute of Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences; Trizol RNA

extraction reagent was purchased from TaKaRa Bio (Shiga, Otsu, Japan); PCR primers were synthesized by Tianyi Huiyuan Biology (Shanghai, China) Co., Ltd; polyclonal rabbit anti-human HIF-1 α , E-cadherin, Vimentin, Twist1 and β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); horseradish peroxidase-labelled goat anti-mouse or anti-rabbit IgG was purchased from Boster Biological Technology Co., Ltd (Wuhan, Hubei, China); Enhanced chemiluminescence (ECL) kit was purchased from Millipore (Billerica, MA, USA); fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA); Dulbecco's Modified Eagle Medium (DMEM-H) culture medium was purchased from Gibco (Grand Island, NY, USA); radioimmunoprecipitation assay (RIPA) for proteins in cells and tissues and bicinchoninic acid (BCA) kit were purchased from Beyotime Biotechnology Institute (Atlanta, GA, USA); 0.45 mm polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Billerica, MA, USA); 6-well plate, 25 cm² plastic culture bottle, and 24-well transwell chambers (pore size: 8 μ m) were purchased from BeaverBio Biotechnology Co., Ltd (Atlanta, GA, USA); Matrigel for invasion experiment was purchased from BD (Hercules, CA, USA); Co170R-230-0200 tri-gas cell incubator for simulating the hypoxic environment for cell culture was purchased from Eppendorf (Hamburg, Germany); inverted phase contrast microscope was purchased from Olympus (Tokyo, Japan); horizontal, vertical, and transferring electrophoresis tanks were purchased from Liuyi Biotechnology Co., Ltd. (Beijing, China).

Cell Culture

In a cell incubator (37°C, 5% CO₂, and saturated humidity), human hepatic carcinoma SMMC-7721 cell strain was cultured using the Dulbecco's Modified Eagle Medium (DMEM-H) culture medium containing 10% inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL gentamycin. Cells were then digested using 0.25% trypsin for passaging, and those 3rd and 4th cells in logarithmic phase were adopted for consecutive experiments. After 70 to 80% of cells had been fused, cells were starved overnight using serum-free medium, and then placed under the normal oxygen environment and the hypoxic environment for a certain period of culture. Cells were then divided into groups according to the experiment requirements.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Primer sequence of β -actin: upstream sequence 5'-TCAGGCGTCTGTAGAGGCTT-3', downstream sequence 5'-ATGCACATCCTTCGATAAGACTG-3'. Primer sequence of HIF-1 α : upstream sequence 5'-AGCTTCCAGACACGCTATCAT-3', downstream sequence 5'-CGGTACAACGAGCTGTTTCTAC-3'. Primer sequence of E-cadherin: upstream sequence 5'-ATTTTTCCTCGACACCCGAT-3', downstream sequence 5'-TCC-CAGGCGTAGACCAAGA-3'. Primer sequence of Vimentin: upstream sequence 5'-AGTCCACTGAGTACCGGAGAC-3', downstream sequence 5'-CATTTCACGCATCTGGCGTTC-3'. Reaction conditions of PCR: initial denaturation at 94°C for 2 min; denaturation at 94°C for 20 s, annealing at 56°C for 1 min, extension at 72°C for 30 s, for a total of 32 cycles; extension at 72°C for 5 min. Amplified products were preserved at 4°C. Statistical analysis was performed for the results of Real-time quantitative PCR.

Western Blotting Detection

SMMC-7721 cells were cultured for 24 h respectively under the normal oxygen environment and hypoxic environment, and cells adhering to the wall in the 6-well plate were washed using pre-heated phosphate buffer saline (PBS) for three times. Thereafter, cells were transferred into the radioimmunoprecipitation assay (RIPA) for lysis. With the cell scraper, we removed the cells gently and placed into the EP tubes, which were then put on the ice for 15 min of lysis. Eppendorf (EP) tubes were placed into a centrifuge for centrifugation at 12000 rpm for 5 min with the sediment being discarded. The supernatant was then preserved at -20°C. Bicinchoninic acid (BCA) method was employed to detect the concentration of protein followed by sample loading. At 80 V, proteins were coagulated, and then separated and transferred onto the membrane at 120 V. After the membrane was blocked using 5% skimmed milk for 1 h, polyclonal rabbit anti-human HIF-1 α , E-cadherin, Vimentin, Twist1 and β -actin antibodies were added onto the membrane for incubation at 4°C overnight, and the membrane was washed using tris buffered saline-tween 20 (TBS-T) on a decoloring shaker for three times (5 min/time). Horseradish peroxidase-labelled goat anti-rabbit IgG was added onto the membrane for 1 h of incubation at room temperature, and washed using TBST for three times (5 min/

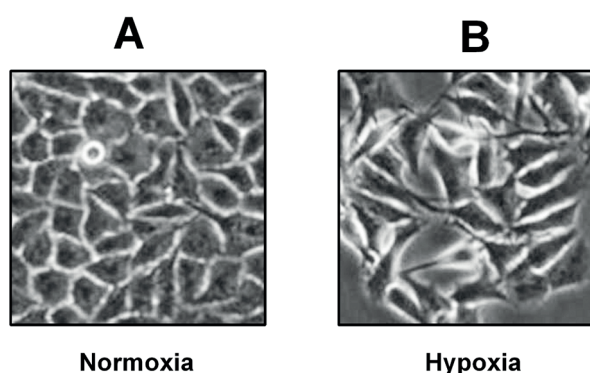


Figure 1. Morphological changes of the SMMC-7721 cells under light microscope ($\times 400$). A, SMMC-7721 cells under normoxic condition; B, SMMC-7721 cells under hypoxic condition.

time). Thereafter, ECL reagent was added onto the membrane for exposure followed by image scanning, digitalization for specific stripes on the images using Quality One. The relative value of each experiment was calculated with the internal reference gray value.

Invasion Experiment of Tumor Cells

Cell invasion experiment: before the experiment, Matrigel was melted at 4°C . $40\ \mu\text{L}$ diluted Matrigel was spread on the surface of polycarbonate microporous membrane, and the volume ratio of Matrigel to the serum-free medium was 1:3. Then, the medium was placed into an incubator for coagulation at 4 h for later use. SMMC-7721 cells in logarithmic phase were starved in serum-free DMEM-H culture solution for 24 h, and digested using 0.25% ethylene diamine tetraacetic acid (EDTA). Thereafter, a single-cell suspension was prepared using serum-free DMEM-H medium, in which cell density should be adjusted to $3 \times 10^5/\text{mL}$. In each upper transwell chamber, $200\ \mu\text{L}$ serum-free cell suspension was added, and cells were divided into groups with 3 replicative wells in each group according to the requirement of the experiment. In each lower transwell chamber, we added the DMEM-H culture medium containing 10% FBS with $600\ \mu\text{L}$ in each well followed by 36 h of culture in an incubator. After culture, transwell was taken out and the medium was removed by washing using PBS for 3 times. In upper transwell chambers, cells that failed to pass through the membrane were scraped gently using wet cotton swabs, and remaining cells were fixed in 4% paraformaldehyde for 20 min.

Thereafter, transwell chambers were dried at room temperature followed by further staining using crystal violet for 20 min. Then cells that passed through the membrane in 5 microscopic fields (central, left, right, upper and lower fields) were counted under the inverted microscope ($400\times$), and the average of cell count was calculated. (2) Remaining procedures were the same as those in the migration experiment.

Statistical Analysis

All values were expressed by mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) was carried out on SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) for statistical analysis. $p < 0.05$ suggested that the difference had statistical significance. Tukey's HSD (honestly significant difference) test was used in conjunction with the ANOVA to find means that are significantly different from each other.

Results

Morphological Changes of SMMC-7721 Cells Induced by Hypoxic Microenvironment

Under the normal oxygen environment, cells were turned into the typical epithelial polygon-shape, and tight conjunctions among cells could be identified. After 24 h of culture under hypoxic environment, cell polarity among the hepatic carcinoma cells disappeared, and the flat epithelial shaped cells in tight conjunction were replaced by the loosely connected and spindle-shaped cells, which were in disorganized arrangement and free state (Figure 1).

Hypoxic Microenvironment Induces the EMT of SMMC-7721 Cells

After 24 h of culture respectively under the normal oxygen environment and hypoxic environment, the RT-PCR and Western blotting results showed that compared with the cells cultured under the normal oxygen environment, significant decreases were seen in the mRNA and protein expressions of E-cadherin in the SMMC-7721 cells with the epithelial phenotype under the hypoxic environment, while in those cells with the mesenchymal phenotype, the mRNA and protein expressions were significantly elevated with the dramatic upregulation in the expressions of HIF-1 α (Figure 2).

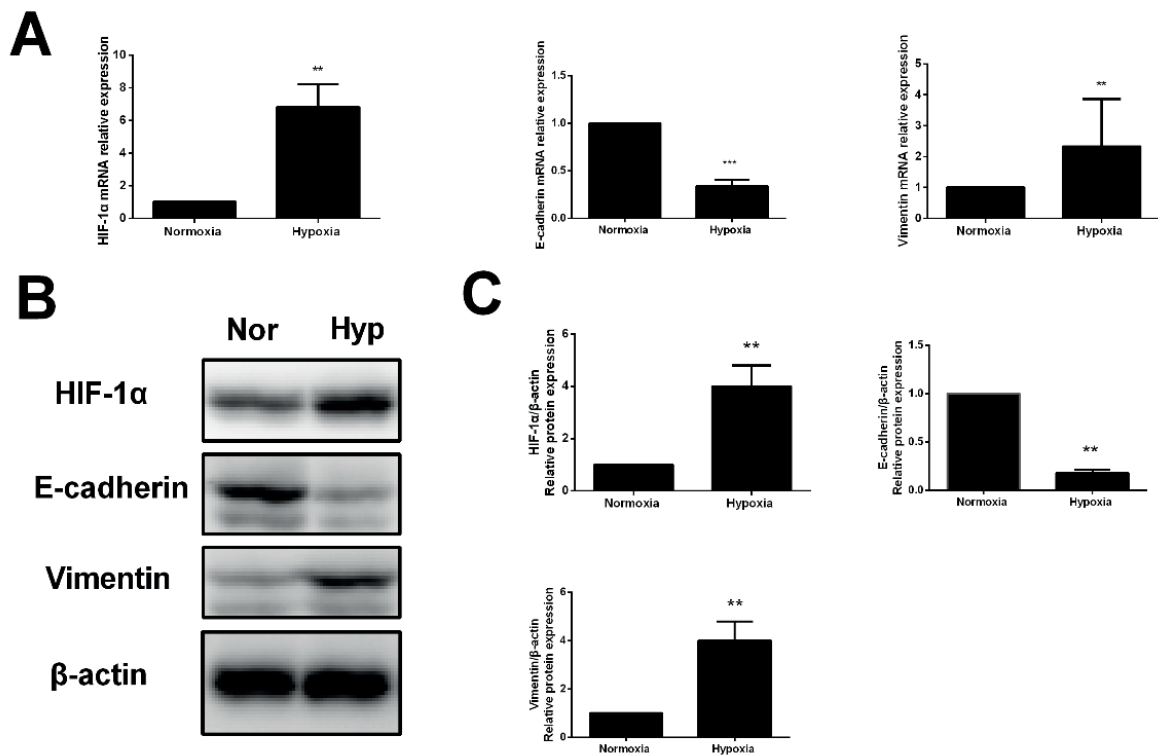


Figure 2. EMT of SMMC-7721 cell induced by hypoxia microenvironment. **A**, RT-PCR analysis of HIF-1α, E-cadherin, Vimentin mRNA; **B**, Western blot analysis of HIF-1α, E-cadherin, Vimentin protein; **C**, Quantification of Western blot results.

Hypoxic Microenvironment Enhances the in-vitro Invasion Capability of SMMC-7721 cells

After 24 h of culture respectively under the normal oxygen environment and hypoxic environment, the invasion capability of SMMC-7721 cells in the hypoxic environment was significantly augmented in comparison with the cells cultured under the normal oxygen environment (Figure 3).

Silencing the Expression of Twist1 Gene Inhibits the EMT Induced by Hypoxic Environment

Through applying the siRNA for targeted silencing of Twist1 expression, we further investigated the role of Twist1 in hypoxia-induced EMT. RT-PCR and Western blotting results showed that after the expression of Twist was silenced, EMT induced by hypoxic environment was significant-

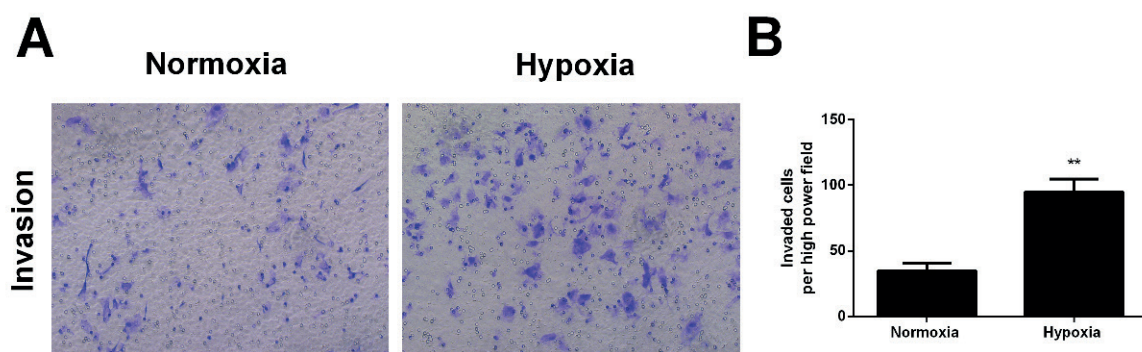


Figure 3. The invasion potentiality of SMMC-7721 enhanced by hypoxia microenvironment (crystal violet staining $\times 200$). **A** and **B**, transwell assay detect the invasion abilities of SMMC-7721 cells and quantification analysis of the results.

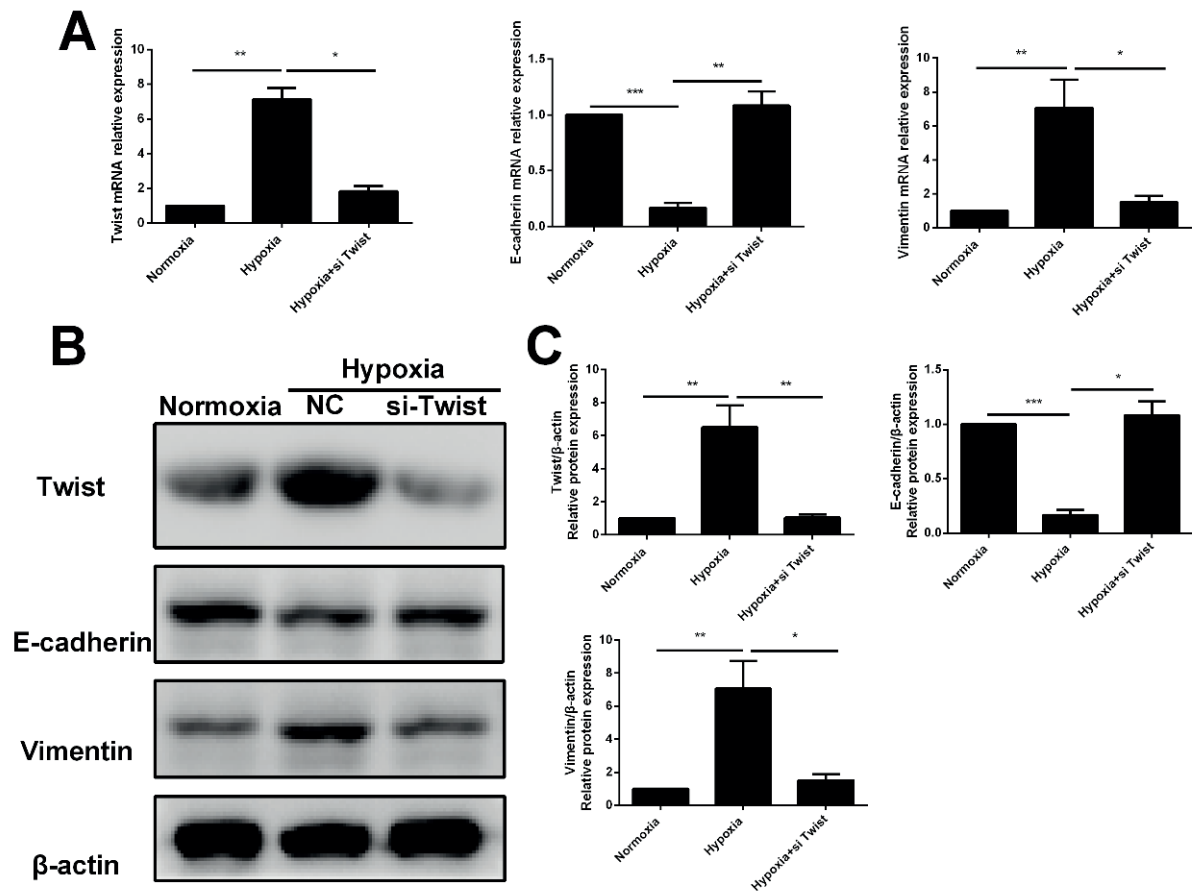


Figure 4. Knockdown of Twist inhibits hypoxia microenvironment induced EMT. *A*, RT-PCR analysis of Twist, E-cadherin, Vimentin mRNA; *B*, Western blot analysis of Twist, E-cadherin, Vimentin protein; *C*, Quantification analysis of Western blot results.

ly reversed with an increase in the expression of E-cadherin and decrease in the expression of Vimentin (Figure 4).

Silencing the Expression of Twist1 Weakens the in-vitro Invasion Capability Induced by Hypoxia

Furthermore, the effect of Twist1 on cell invasion induced by hypoxic microenvironment was verified. In the transwell invasion experiment, we found that after the expression of Twist1 was blocked using siRNA, a significant decrease was identified in the invasion capability of SMMC-7721 cells cultured under the hypoxic environment (Figure 5).

Discussion

In China, cases of hepatic carcinoma have occupied over 50% of the quantity in the world,

and its mortality rate is only secondary to the gastric carcinoma and esophagus cancer. The occurrence and development of hepatocellular carcinoma are sequentially evolutionary processes with the involvement of multiple factors and procedures, and patients usually experience three stages, i.e. hepatitis, liver cirrhosis, and hepatic carcinoma. Hepatocellular carcinoma, with a high malignancy, rapid progression and susceptibility to the intrahepatic and distant metastasis, greatly threatens the health and life of human beings⁷. During the growth of a solid tumor, rapid proliferation in tumor cells and delayed angiogenesis in the local tumor tissues contribute to the insufficiency of blood supply, which generate the tumor cells into a hypoxic and ischemic microenvironment^{8,9}. Hypoxia, one of the basic features of the microenvironment in tumors, is also involved in the invasion, metastasis, proliferation, and apoptosis of malignant tumors¹⁰⁻¹². Hepatocellular carcinoma is the most

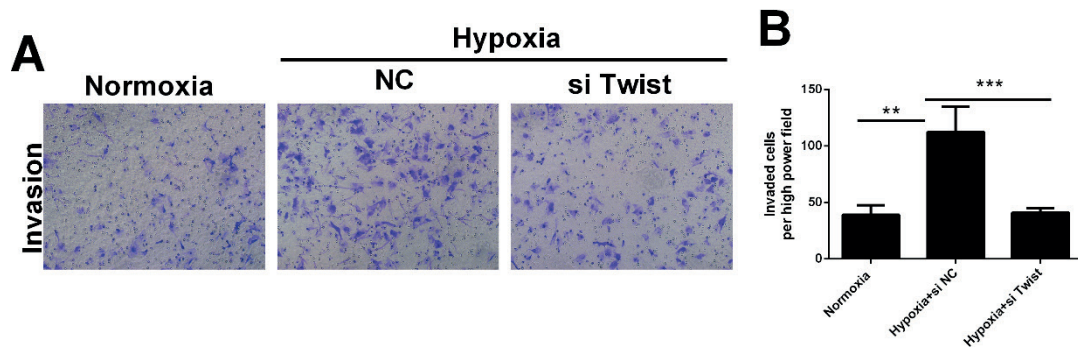


Figure 5. Twist specific siRNA attenuates hypoxia environment induced SMMC-7721 cell invasion (crystal violet staining $\times$ 200). A and B, transwell assay detect the invasion abilities of SMMC-7721 cells and quantification of the results.

common solid tumor, and its growth is quite rapid, which renders the cells in local tumors into the hypoxic microenvironment, especially common in massive hepatocellular carcinoma. Previous studies^{13,14} have indicated that the level of HIF-1 α is significantly elevated in the HCC tissues, and negatively correlated with the clinical prognosis. Additionally, in the HCC tissues, tumor cells under hypoxic environment are usually superior in the invasion and metastasis capability, in which the initiation of EMT may play an essential role^{15,16}. EMT is a highly conservative process, and, through the specific procedure, can eliminate the polarity of epithelial cells in adhesive form; thereby, through destroying the tight conjunction among cells, and breaking through the basal membrane, those epithelial cells transform into the mesenchymal cells with the capabilities of migration and invasion. Not only the EMT is involved in the embryonic formation and the development and chronically degenerative fibrosis of multiple tissues and organs, but also it plays an important role in the invasion and metastasis of malignant tumors¹⁷. The occurrence of EMT is usually accompanied with the decrease in E-cadherin, phenotypic marker of epithelial cells, and the increase in Vimentin, phenotypic marker of mesenchymal cells¹⁸. Research has confirmed that various factors could induce the occurrence of EMT, while hypoxic microenvironment is one of the key factors inducing the EMT¹⁹. Local hypoxia and ischemia microenvironment can cause the EMT-characteristic transition of many tumor cells, like ovarian cancer²⁰ and prostate cancer²¹, to enhance the capability of invasion and metastasis. The Twist is a newly discovered oncogene that is mainly involved in the cell differentiation and embryonic development; also, it is one of the key regulatory

factors in EMT^{22,23}. Research has indicated the close association between the Twist and occurrence and development of multiple malignant tumors in clinical practice, including breast cancer [Chen, 2015 #17503], prostate cancer [Raatikainen, 2015 #17504], gastric cancer, and hepatic carcinoma. All these researches revealed the high expressions of Twist in those tumor tissues. Also, some studies showed that the abnormally activated Twist gene under the hypoxic microenvironment in tumors, through regulating the expression of downstream targeting genes, can intervene a series of biological events, like cell proliferation, differentiation, apoptosis, and invasion, thereby affecting the occurrence and development of a variety of tumors^{24,25}. However, few investigations have reported the roles and regulation mechanism of Twist gene in EMT of hepatocellular carcinoma under the hypoxic microenvironment. In this work we firstly cultured the SMMC-7721 cell strains, respectively under the normal oxygen environment and hypoxic environment, and verified whether *in-vitro* hypoxic microenvironment could induce the EMT in SMMC-7721 cells through RT-PCR and Western blotting. The results showed that compared with the cells cultured in the normal oxygen environment, cells after 24 h of culture in the hypoxic environment experienced the transition from the initial polygon shape to the fusiform, cam-biform and fibroblast-like shape with the decrease in protein expression of epithelial phenotypic E-cadherin and increase in protein expression of epithelial phenotypic Vimentin. Transwell invasion experiment showed that after culturing in a hypoxic environment, SMMC-7721 cells that experienced EMT gained stronger invasion capability. To further investigate the mechanism of EMT in the hypoxic microenvironment, we

detected the activity of Twist gene, the key regulatory gene in EMT. The results indicated that compared with the cells cultured in a normal oxygen environment, the Twist gene in the SMMC-7721 cells was significantly activated after 24 h of culture in the hypoxic microenvironment, suggesting its potential involvement in the EMT in SMMC-7721 cells under the hypoxic environment. To clarify the action mechanism of Twist gene in EMT, the expression of Twist was silenced using the specific Twist siRNA to further investigate the effect on the initiation of EMT and invasion capability of cells. This study showed that after the Twist gene was specifically silenced, hypoxia-mediated EMT and invasion in SMMC-7721 cells were reversed, suggesting the essential role of Twist gene in the process of EMT. Based on the results of this study, we inferred that hypoxic microenvironment, through activating the Twist gene, can regulate the EMT in SMMC-7721 cells, enhancing the invasion capability of SMMC-7721 cells, thus facilitating the distant invasion and metastasis of HCC.

Conclusions

Preliminarily we showed that hypoxic microenvironment in the SMMC-7721 cells can activate the Twist gene to induce the occurrence of EMT and invasion and metastasis of tumor cells, which can deepen the understandings on the mechanisms of invasion and metastasis of HCC. Thus, in-depth studies on the roles and regulation mechanism of Twist gene in the EMT of HCC through targeted interruption of Twist gene expression to reverse the EMT process and inhibit the invasion and metastasis of tumor cells will suggest new evidence for the prophylaxis of invasion and metastasis and specific treatment of HCC. Hence, it has a great clinical significance and promising application prospect.

Conflict of interest

The authors declare no conflicts of interest.

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