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Effect of hypoxia on the Twist1 in EMT of cervical cancer cells

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Abstract. – OBJECTIVE: To explore the effect of hypoxia on the Twist1 expression in epithelial-mesenchymal transition of the cervical cancer cells.

MATERIALS AND METHODS: In this study, we simulated the normoxia and hypoxia environment, where HeLa cells were cultured, respectively. Cell invasion ability was measured by the transwell assay, while the GLI-1 protein and mRNA expressions were measured by Real-time polymerase chain reaction (RT-PCR) and Western blot assays. After that, HeLa cells were transfected with the GLI-1-specific siRNA, followed by the measurement of mRNA and protein expressions using RT-PCR and Western blot assays, as well as the cell invasion ability by the transwell assay.

RESULTS: We found that in hypoxic environment, GLI-1 was up-regulated in HeLa cells, with enhanced invasion ability. However, silencing the expression of GLI-1 could reverse the up-regulation of GLI-1 compromising the invasion ability of HeLa cells.

CONCLUSIONS: Hypoxia may account for the increased invasion of HeLa cells, which is realized by the up-regulated GLI-1.

Key Words:
Cervical cancer, Hypoxia, GLI-1, Invasion.

Introduction

Cervical cancer, as the most frequent gynecological tumor in clinical practice, has taken up nearly half of the incidences of all tumors in female reproductive system, with highly ranked incidence rate and mortality rate in developing countries [1,2]. Distant infiltrative invasion and metastasis of cervical cancer have been deemed as the major causes accounting for the death of patients [3]; strategies aiming to suppress the invasion ability of cervical cancer cells have been regarded as the key link for effective treatment. Nevertheless, current development against these strategies has been stagnated due to the elusive mechanism relating to the cell metastasis and invasion. Thus, research focusing on this aspect has been endowed a great clinical significance. During the rapid growth of solid malignant tumors, excessive proliferation of tumor cells bounds to result in the severe ischemia and imbalance between the oxygen supply and energy consumption in local tissues[4]. Localized hypoxic microenvironment, as one of the major characteristics of solid tumors, is critical to maintaining the energy metabolism of tumor cells, angiogenesis and enhancing the invasion ability of tumor cells[5-7]. Through activating a series of downstream signaling pathways, hypoxia is involved in the regulation of tumor development and progression in each stage[8]. Scholars have shown that Hedgehog (Hh) signaling pathway is activated in hypoxic microenvironment[8], and the abnormally high expressions in a variety of malignant tumor tissues, e.g. the colorectal cancer[9], breast cancer[10] and liver cancer[11], are closely correlated with the proliferation, invasion and metastasis of malignant tumor cells. Latest researches[12] also indicate that GLI-1, as the transcription factor of Hh signaling pathway, and mediating the transcription of the most of the target genes of Hh signaling pathway, is potentially involved in the distant metastasis of cervical cancer. In light of the scarce information regarding to the role of GLI-1 gene in the distant invasion of cervical cancer, we conducted this study to investigate the effect of simulated hypoxic microenvironment on the invasion ability of HeLa cells and the potential molecular mechanisms, aiming to uncover the action mechanism of GLI-1 in the distant metastasis of cervical cancer cells, providing evidence and therapeutic target for the clinical diagnosis and treatment of cervical cancer.

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Materials and Methods

Materials

HeLa cell strain (China Center for Type Culture Collection, Wuhan, China); Silencer TM Select GLI-1 siRNA (Shanghai GenePharma Co., Ltd); TRIzol RNA extract kit (TaKaRa, Otsu, Shiga, Japan); PCR primers of β-actin, GLI-1, MMP2 and MMP9 (Shanghai Tianyi Huiyuan Biotechnology Co., Ltd.); rabbit anti-human GLI-1, HIF-1α and β-actin polyclonal antibodies (Abcam, Cambridge, MA, USA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Boster, Wuhan); protein marker (Thermo Fisher Scientific, Waltham, MA, USA); enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA); fetal bovine serum (FBS) (Zhejiang Tianhang Biotechnology Co., Ltd., China); Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA); radio-immunoprecipitation assay (RIPA) kit (Beyotime, China); Pierce BCA kit (Thermo Fisher Scientific, Waltham, MA, USA); 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA); 6-well plate and 25 cm^2 culture flask (Beavorbio, Suzhou, China); 24-well transwell chamber (pore size: 8 μm) (Corning, Corning, NY, USA); Matrigel (BD Biosciences, Franklin Lakes, NJ, USA); Co170R-230-0200 tri-gas incubator EP (Eppendorf, Hamburg, Germany); invert phase-contrast microscope (Olympus, Tokyo, Japan); horizontal electrophoresis tank, vertical electrophoresis tank and transfer tank (Liuyi, Beijing, China).

Cell Culture

HeLa cell strains were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL gentamycin in an incubator (37°C, 5% CO_2 and saturated humidity). After culture, cells were digested and passaged using 0.25% trypsin, and those in the 3rd or 4th generation and logarithmic phase were harvested for following experiments. When cell confluence reached 70% to 80%, cells were starved using serum-free medium, and then placed in normoxia and hypoxia environment for indicated time and grouped according to the experiment requirement.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Primers for β-actin: upstream, 5′-AGCGGGAAATCGTGCGTG-3′, downstream, 5′-CAGGGTACATGGTGGTGCC-3′; GLI-1: upstream, 5′- CCGTCATCTCCGACTTCATCT-3′, downstream, 5′-GTGTCTCCTCCCTGTTGTTCTG-3′; HIF-1α: upstream, 5′-TCAACACGACACCGGATAAA-3′, downstream, 5′-CCGCGAGCTATCTTTCTTCA-3′. Temperatures and cycles in PCR were set as follows: pre-denaturation at 94°C for 2 min; 32 cycles of denaturation at 94°C for 20 s, annealing at 56°C for 1 min and extension at 72°C for 30 s; extension at 72°C for 5 min. Amplified product was preserved at 4°C, and statistical analysis was also performed for the real-time quantitative PCR results.

Western Blot Assay

HeLa cells, following 24 hours of culture in normoxia and hypoxia environment in 6-well plate, were washed three times using pre-heated phosphate buffered saline (PBS) and placed in radioimmunoprecipitation assay (RIPA) reagent for lysis. Then, cells adhering to the wall were collected using a cell scraper and transferred into Eppendorf (EP) tubes on ice for lysis for 15 min. Lysate was then centrifuged for 5 min at 12000 rpm, and the supernatant was collected and preserved at -20°C. Prior to the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), we measured the protein concentration using BCA method. Then, proteins on gel were subjected to the polyvinylidene difluoride (PVDF) membrane which was later blocked using 5% non-fat milk for 1 h. Thereafter, proteins were probed using the rabbit anti-human polyclonal antibodies of anti-GLI-1, -HIF-1α and -β-actin at 4°C overnight, followed by three washes in TBST (5 min for each). HRP-conjugated goat anti-rabbit IgG was then used for incubation for 1 h at room temperature, and the membrane was then washed using Tris-buffered saline and Tween 20 (TBST) three times (5 min for each). Immunoblots were then developed using the ECL reagent and analyzed using Quality One software in terms of grey value. The relative expression of targeted proteins was corrected with the expression of β-actin.

siRNA Transfection

Matrigel was melted at 4°C in advance, and diluted with the serum-free medium in ratio of 1:3. Next, 40 μL diluted Matrigel were smeared on the surface of polycarbonate well which was preserved in the incubator. Cells in logarithmic phase were inoculated on the 6-well plate at density of 3×10^5/well; on the following 24 hours of culture, cell confluence reached 50% to 60%. At

that time, the original medium was substituted by the serum-free medium, and transfection of GLI-1 siRNA or control siRNA was performed using Lipofectamine™ RNAi MAX in accordance with the instructions. Transfection was performed in replicate wells; in each well, the concentrations of GLI-1 siRNA, and control siRNA were set as 100 nmol/l. Following 6 hours of transfection, the medium was replaced with fresh serum-free medium; 48 hours later, mRNA and proteins were extracted for following experiments. This experiment was conducted in triplicate.

Cell Invasion Experiment

Cell invasion experiment: Matrigel was melted at 4°C in advance, and diluted with the serum-free medium in ratio of 1:3. Then, 40 μL diluted Matrigel were smeared on the surface of polycarbonate well which was coagulated in the incubator for 4 h. HeLa cells in logarithmic phase were starved in serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) medium for 24 h and digested with 0.25% EDTA-trypsin to prepare the single cell suspension with serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) medium. Next, 200 μL HeLa cell suspension were inoculated into the transwell upper chamber at density of 1×10^8/L, while in the lower chamber, 600 μL Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) were added. In each group, there were triplicate wells. Cells in this experiment were grouped as follows: normoxia control, hypoxia, normoxia + GLI-1-siRNA, and hypoxia + GLI-1 siRNA. After 48 hours of culture in an incubator, transwell chamber was taken out and cells were fixed in methanol without any medium. The chamber was dried at room temperature for 5 min, and cells were stained using 0.1% crystal violet for 20 min, followed by three washes in phosphate-buffered saline (PBS). Cells that failed to get into the lower chamber were removed, and under the microscope, 5 vision randomly were selected for cell count in lower chamber. This experiment was carried out in triplicate and the average was used for statistical analysis.

Statistical Analysis

All the values were presented in $\chi \pm s$. One-way ANOVA was performed using SPSS 19.0 (IBM, Armonk, NY, USA), and $p<0.05$ suggested that the difference had statistical significance. Tukey's HSD (honestly significant difference) test was used in conjunction with an ANOVA to find means that were significantly different from each other.

Results

Hypoxic Microenvironment Enhances the In Vitro Invasion Ability of HeLa Cells

Under the different concentration of oxygen, HeLa cells were cultured for 48 hours with the cells cultured in normal environment as control. The results showed that HeLa cells in the hypoxia group manifested enhanced invasion ability in comparison with the control group ($p<0.05$; Figure 1).

Hypoxic microenvironment up-regulates the GLI-1 in HeLa cells

Through 24 hours of culture in normoxia and hypoxia, RT-PCR and Western blot assay showed that compared to the normoxia group, HeLa cells in hypoxia group exhibited significant up-regula-

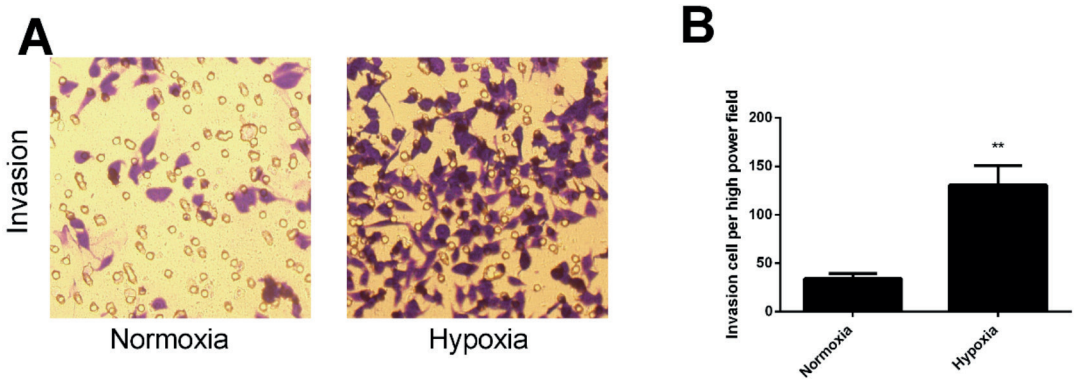

Figure 1. Hypoxia promoted the invasion of HeLa cells (crystal violet staining× 200). ***A,*** Transwell assay detect the invasion abilities of HeLa cells; ***B,*** Quantification analysis of the number of invaded cells.

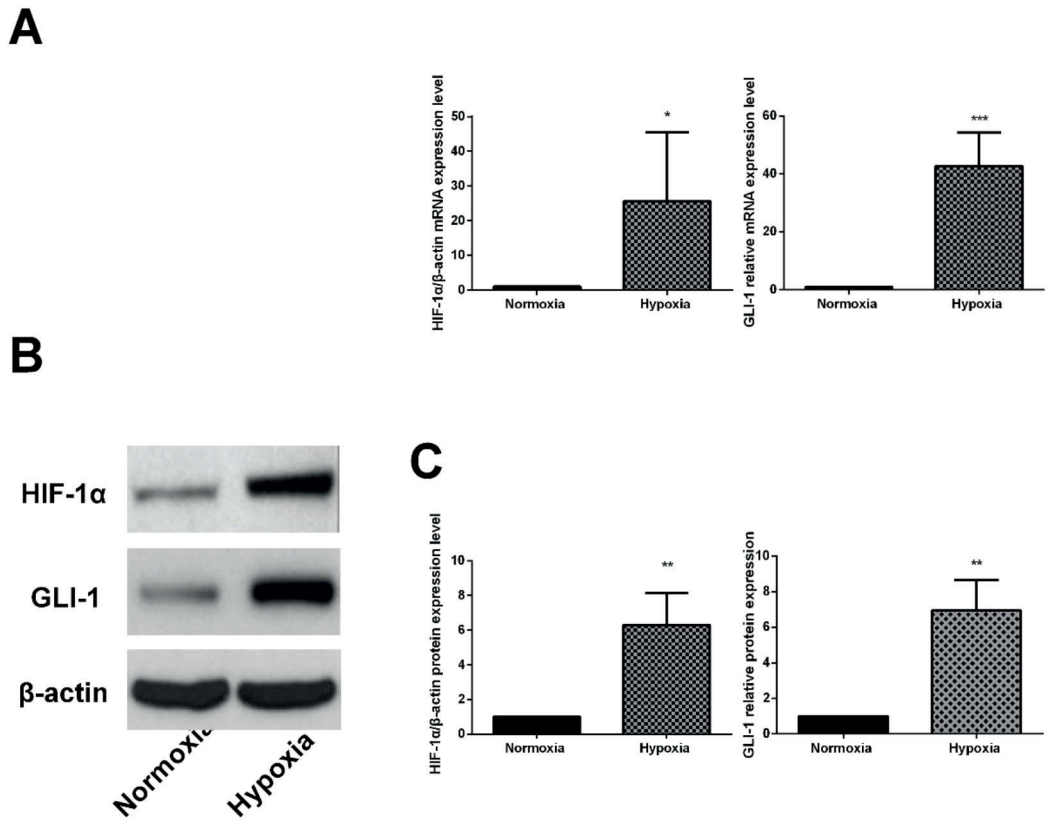

Figure 2. Hypoxia Microenvironment Up-Regulated the Expression of GLI-1 in HeLa Cells. ***A,*** RT-PCR analysis of mRNA expression of GLI-1; ***B,*** Western blot analysis of protein expression of GLI-1; ***C,*** Quantification analysis of Western blot results.

tion in mRNA and protein expressions of GLI-1 ($p<0.05$; Figure 2).

Targeted Inhibition of GLI-1 Using GLI-1 siRNA Suppresses the mRNA and Protein Expressions in HeLa Cells

Following 48 hours of transfection using GLI-1 siRNA or control siRNA for HeLa cells, RT-PCR and Western blotting assays both showed evident down-regulation in GLI-1 expressions in GLI-1 siRNA group when compared with the GLI-1 control group and normal control group with statistically significant differences ($p<0.05$; Figure 3).

Silencing of GLI-1 Curbs the Hypoxia-Induced In Vitro Invasion Ability

We further verified whether GLI-1 affects the hypoxia-induced invasion of HeLa cells. In the transwell cell invasion assay, we found that after GLI-1 was silenced using the specific siRNA, the invasion ability of HeLa cells cultured in hypoxia was significantly deprived in comparison with those in hypoxia group and hypoxia + control siRNA group (Figure 4).

Discussion

Cervical cancer, one of the most frequent female malignant tumors, has developed into the infiltrative cancer at the time of diagnosis in almost 60% of patients, concomitant with distant invasion and metastasis in varying degrees, severely impeding the treatment and prognosis[13]. In clinical practice, any local infiltration could result in a significant decrease in the survival rate of patients, while distant invasion has been considered as the major cause for poor prognosis[14]. Thus, searching for the specific molecules that can predict the early-stage invasion and metastasis of cervical cancer show a great significance for the implementation of individualized treatment and amelioration of prognosis. For

the majority of solid tumors, they always appear with hypoxic microenvironment, and during the development and progression of tumors, the adaptive ability of tumor cells to the hypoxia has been regarded as a key link[15,16]. In response to the stress in hypoxic metabolism, a series of signal pathways are initiated in tumor cells to enhance the abnormal proliferation and metastasis and generation of drug tolerance through promoting the peripheral angiogenesis, enhancing the cellular metabolism and migration, etc. Hypoxic microenvironment can regulate the expression of multiple genes, so as to facilitate the infiltration and metastasis of malignant tumors, in which Hh signal pathway exerts a key effect[17-19]. In mammals, Hh signaling pathway is crucial not only to the embryonic development, but also to the growth and differentiation of cells after embryonic development, and the abnormal activation almost participates in the development of malignant tumors in all systems[20,21]. GLI-1, as the downstream molecule of Hh signaling pathway, acts as the nuclear transcription factor to bind to the promoter to activate the transcription of target genes. It has been observed that in a variety of gastric cancer, breast cancer, ovarian cancer, hepatic cancer and colorectal cancer, SHH, SMO, PTCH and GLI in this pathway are abnormally activated, suggesting that the activation of such a pathway is associated with the development and progression of malignant tumors[22-26]. The ligand SHH can bind to its receptor PTCH to eradicate the inhibitory effect of PTCH on SMO, and the resultant activated SMO can further activate GLI-1. Thereby, GLI-1 nuclear translocation can initiate the transcription of downstream genes to enhance the infiltration and invasion of tumor cells [27]. Therefore, not only does GLI-1 serve as a key role in Hh signaling pathway, but also the up-regulation of GLI-1 is one of the indicators suggesting the activation of this pathway. The research has shown the abnormal overexpression of GLI-1 in cervical squamous cell carcinoma tissues, suggestive of the potential involvement of GLI-1 in the development of cervical cancer[28]. In this experiment, we firstly verified the effect of *in vitro* hypoxic microenvironment on the invasion ability of HeLa cells. Results showed that in comparison with the normoxia control, HeLa cells, following 48 hours of hypoxia microen-

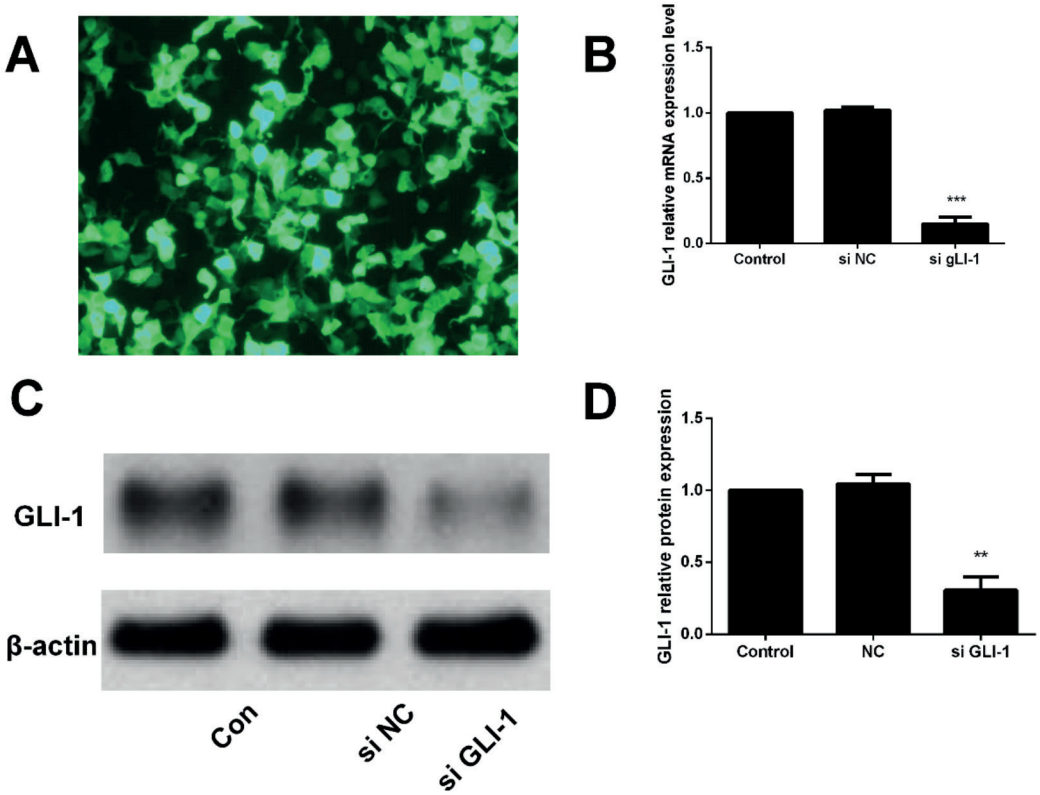

Figure 3. The effective expression of exogenous gene silence of GLI-1. ***A-B***, RT-PCR analysis of GLI-1 mRNA; ***C***, Western blot analysis of GLI-1 protein; ***D,*** Quantification of Western blot results.

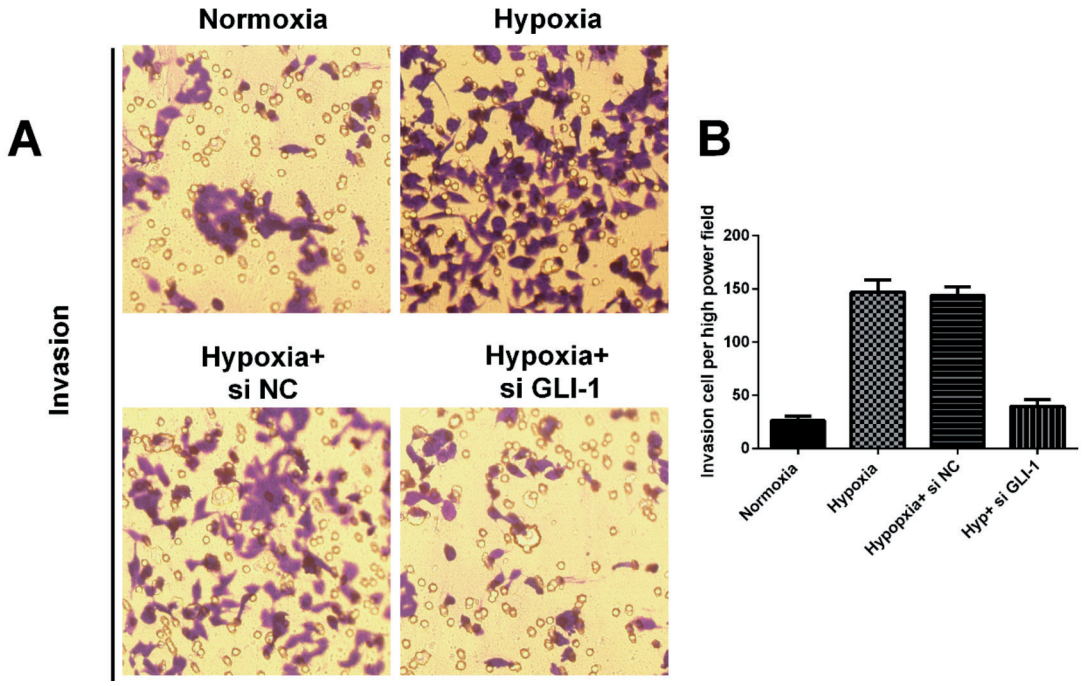

Figure 4. GLI-1 specific siRNA attenuates hypoxia induced HeLa cells invasion (crystal violet staining× 200). ***A*** and ***B:*** transwell assay detect the invasion abilities of HeLa cells and quantification of the results.

vironment, gained a substantially enhanced invasion ability. Sequentially, we probed the potential molecular mechanism under the hypoxic microenvironment. It was indicated that in comparison with normoxia control, HeLa cells after 48 hours of hypoxic culture manifested the abnormally activated GLI-1, which suggested that it may be involved in the invasion in hypoxic microenvironment. To further clarify the action mechanism of GLI-1 in the invasion ability of HeLa cells in hypoxic microenvironment, we adopted the GLI-1 siRNA to silence the expression. Consequently, we found that after the GLI-1 was silenced specifically, hypoxia-induced cell invasion was suppressed, suggestive of the key role of GLI-1 in this process. In light of the study above, we suggest that hypoxia can modulate the transcription of downstream genes via targeting GLI-1 gene, so as to promote the distant invasion and facilitate the development and progression of cervical cancer.

Conclusions

We preliminarily showed that, for HeLa cells, the hypoxic microenvironment can activate the GLI-1 in Hh signaling pathway to promote the distant metastasis of tumor cells, which is conducive to the understanding of the pathogenesis of cervical cancer. Thus, in-depth study on the role of GLI-1 in the development and progression of cervical cancer and the regulation mechanism is helpful to develop strategy to block the expression of GLI-1 to achieve the goal of suppressing the distant metastasis of tumor cells. This is expected to provide new ideas and evidence for the specific treatment of cervical cancer, showing a great clinical significance and promising application prospect.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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