Original Article
The correlation of HIF-1α and transcription factors Snail and E-cadherin with gastric cancer cell proliferation and EMT

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Abstract: Objective: To investigate the correlation of hypoxia inducible factor-1α (HIF-1α), and transcription factors Snail and E-cadherin with gastric cancer cell proliferation and epithelial-mesenchymal transition (EMT). Methods: SGC-7901 gastric cancer cells were cultured under normal both oxygen and hypoxia conditions. MTT assay was used to detect cell proliferation, Transwell assay was used to detect cell migration and invasion, Spearman correlation analysis was used to evaluate the relationship between HIF-1α expression level and EMT-related molecule expression level, qPCR and Western blot were used to quantify HIF-1α and EMT-related proteins Snail, E-cadherin mRNA and protein expression level. Results: Compared with normal gastric mucosal tissue, the expression of HIF-1α and Snail protein in gastric cancer tissue was up-regulated, and the expression of E-cadherin protein was down-regulated. Compared with the normoxia group, the hypoxic group had increased cell migration, invasion and proliferation, significantly up-regulated HIF-1α and Snail mRNA and protein expression levels, and significantly down-regulated E-cadherin mRNA and protein expression levels. Spearman correlation analysis showed that HIF-1α was positively correlated with Snail mRNA and protein expression, while negatively correlated with E-cadherin mRNA and protein expression. Under normal oxygen conditions, down-regulation of HIF-1α led to reduction of cell migration, invasion and proliferation, down-regulation of EMT-related protein Snail and up-regulation of E-cadherin. Down-regulation of HIF-1α under hypoxia could offset cell changes caused by hypoxia. Conclusion: HIF1α promotes gastric cancer cell proliferation and EMT by up-regulating Snail and down-regulating E-cadherin.

Keywords: Gastric cancer, HIF-1α, Snail, E-cadherin, epithelial mesenchymal transition, cell proliferation

Introduction

Since great progress has been made in endoscopic diagnosis technology in recent years, the mortality rate of gastric cancer has shown a significant downward trend. However, the early symptoms have not been clearly identified and defined, and as such the course of the disease often has progressed to the middle and late stages when most patients seek medical treatment, and the 5-year survival rate is usually in the range of 20-25% [1]. Cancer cells in patients with advanced gastric cancer are characterized by proliferation, invasion and metastasis, thus the treatment difficulty is increased, and the prognosis is poor due to high recurrence rate [2]. The process of tumor invasion and migration is complicated, and they are the main cause of tumor metastasis. Tumor cell epithelial-mesenchymal transition (EMT) can induce cellular invasion and migration growth [3]. EMT is a biological process that depolarizes epithelial cells of primary tumors and transforms them into mesenchymal cells to play a role in reducing adhesion [4]. During the EMT period when epithelial cells have the characteristics of mesenchymal cells, decreasing the characteristic expression of epithelial cells such as E-cadherin can reduce the cell adhesion, while increasing the characteristic expression of mesenchymal cells such as Vimentin and N-cadherin can increase the cell invasion and migration. The changes of epithelial and mesenchymal cells are all controlled by the regulation of EMT transcription factor Snail [5, 6]. A large amount of literature suggests that

the progression of gastric cancer is bound up with whether EMT occurs in cancer cells [7, 8]. Recent research supports that there are many factors inducing EMT, including various cytokines such as transforming growth factor β (TGF-β), epidermal growth factor (EGF), or various intracellular signaling pathways such as Hedgehog, RAS, Wnt, NF-κB, Notch and phosphatidylinositol 3-kinase (PI3K), among which hypoxia inducible factor-1α (HIF-1α) is recognized as one of the key inducing factors of EMT [9]. High expression and accumulation of HIF-1α caused by hypoxia have been proved to promote tumor growth and metastasis in various solid tumors.

However, in gastric cancer, the relevant research is generally rare. This study intends to clarify whether HIF-1α and EMT related molecules are activated in gastric cancer by exploring the possible mechanism of HIF-1α and EMT in gastric cancer, so as to provide new theoretical basis for targeted therapy of gastric cancer. Details are as follows.

Materials and methods

Reagents, instruments and tissue cells

Gastric cancer SGC-7901 cell line (Nanjing Cobioer Biosciences Co., Ltd.). RPMI1640 medium (Shanghai Yuchun Biotechnology Co., Ltd.). MTT solution and TBST (Solarbio). DMSO (Nanjing Reagents Co., Ltd.). Enzyme reader (Beijing Potenov Technology Co., Ltd.). Matrigel (Beijing Biodee Biotechnology Co., Ltd.). Reverse transcription kit (Shanghai Yubo Biotechnology Co., Ltd.). Fluorescence quantitative PCR instrument (Beijing Century Kexin Science Co., Ltd.). SDS-PAGE gel (Nanjing Detai Biologics Co., Ltd.) HIF-1α, Snail, E-cadherin, GAPDH primary antibodies and HPR labeled sheep anti-rabbit secondary antibody (AmyJet Scientific Inc.). ECL Luminous color development solution (Beijing Apply Gen Technology Co., Ltd.).

Gastric cancer cells SGC-7901 are cultured in normal oxygen and hypoxia environments

RPMI1640 medium containing 10% fetal bovine serum (FBS) was added to gastric cancer cell SGC-7901 and then cultured in a 5% CO_2 incubator at 37°C. They were divided into the normoxic group, hypoxic group, blank control NC group (without transfection of HIF1α), and HIF1α-shRNA (HIF1α was knocked down and then transfected into SGC-790 cell). Fortyeight hours after transfection, cells in the logarithmic growth phase were taken and inoculated with a concentration of $3×10^3$ cells at a volume of 100 µL per well. Each group was divided into 6 duplicate wells (final concentration = 100 nmol/L). The hypoxic group were given CoCl2 (final concentration = 200 µmol/L) to construct anoxic environment when the cell attachment was completed. The remaining steps were the same as those of the normal oxygen group, all of which were subcultured in a 5% CO_2 incubator at normal temperature for 12 h.

MTT assay is used to detect the proliferation of gastric cancer cells in each group

After culture, 5 g/L MTT (20 µL) was added to each group. The incubation was continued for 4 h, and the supernatant was discarded. DMSO (100 µL) was added to each well, and shaken for 10 min to dissolve the crystals completely. The absorbance [A (490)] value was measured at 490 nm on a microplate reader to indicate cell proliferation activity.

Transwell assay was used to detect the migration and invasion ability of gastric cancer cells in each group

Cell migration experiment: SGC-7901 cells in a logarithmic growth phase were taken, and serum-free DMEM was added to prepare cell suspension. Cell density was adjusted to $2×10^5$/ml. The cell suspension (100 µl) was added to the upper compartment of Transwell chamber (pore diameter of 8 µm). Another 600 µl of DMEM culture solution containing 10% FBS was taken for continuous culture for 24 h in an incubator of 37°C and 5% CO_2. The liquid in the upper compartment was discarded, fixed with anhydrous methanol for 30 min. Cells that failed to penetrate the membrane were wiped off with cotton swabs, dyed with 0.1% crystal violet for 20 min, and the number of migrated cells was recorded under an inverted microscope. Cell invasion experiment: Matrigel matrix was diluted with serum-free DMEM medium in advance (ratio: 1:3). Then, 40 µl of it was taken and spread on the upper compartment surface at the bottom of Transwell chamber (pore diameter of 8 µm), put into a 37°C, 5% CO_2 volume fraction incu-

Table 1. Primer sequence

Primer		Sequence
HIF-1	Upstream	5'-TGCAACATGGAAGGTATTGC-3'
	Downstream	5'-TTCACAAATCAGCACCAAGC-3'
Snail	Upstream	5'-CAATCGGAAGCCTAACTA-3'
	Downstream	5'-CAGATGAGCATTGGCAGCG-3'
E-cadherin	Upstream	5'-CCCTCGACACCCGATTCACCG-3'
	Downstream	5'-CCAGGCGTAGACCAAGAAATG-3'
GAPDH	Upstream	5'-TGTTGCCATCAATGACCCCTT-3'
	Downstream	5'-CTCCACGACGTACTCAGCG-3'

bator to continue culturing for 30 min. The rest of the experimental steps were the same as cell migration experiment.

qPCR is used to detect the mRNA expression level of HIF-1α and EMT related molecules in gastric cancer cells

The total RNA of each group was extracted, and cDNA was synthesized by using a reverse transcription kit. GAPDH was set as the internal reference, and the reaction was performed by real-time fluorescence quantitative PCR. The reaction conditions were 50°C 2 min, pre-denaturation 95°C 2 min, 95°C 15 s, 60°C 15 s, 72°C 1 min, 40 cycles in total. Ct values of each group were measured and standard curves were drawn. The experiments were repeated three times, and the relative expression of the target gene was calculated by 2-ΔΔCt. Primer sequence was shown in **Table 1**.

Western blotting is used to detect the expression levels of HIF-1α and EMT related molecular proteins in gastric cancer cells and normal gastric mucosa tissues

After collecting the cells of each group, the total protein was extracted, and the protein concentration was detected and calculated. Total protein (25 μg) was placed in 10% SDS-PAGE gel for electrophoresis reaction and then transferred to PVDF membrane, sealed with 5% skim milk powder. Primary antibodies (HIF-1α, Snail, E-cadherin diluted at 1:200, GAPDH diluted at 1:1,000) were added and incubated overnight at 4°C. After TBST washing, HPR-labeled goat anti-rabbit secondary antibody (dilution ratio: 1:2,000) was added. After incubation at room temperature for 1 h, the goat anti-rabbit secondary antibody was washed again. ECL luminescence method was adopted for color development. Bio-Rad Chemi DOC MP omnipotent imaging system was applied to analyze and draw the image to measure the gray value of the recorded protein.

Statistical analysis

SPSS 22.0 statistical analysis software package was used for processing. The detailed usage methods were as follows: the data were expressed by mean standard deviation, and the comparison of variable values was tested by T or F. Chi-square test was used for data comparison of classified variables. Spearman method was used to analyze the relationship between HIF-1α and EMT related molecular expression. P < 0.01 was considered statistically significant.

Results

Comparison of the expression of HIF-1α, Snail and E-cadherin proteins in normal gastric mucosa tissue and gastric cancer tissue

Compared with normal gastric mucosa tissue, the expression of HIF-1α and Snail protein in gastric cancer tissue was higher, and the expression of E-cadherin protein was lower (P < 0.05). More details were shown in **Figure 1**.

Comparison of tissue proliferation of gastric cancer with HIF-1α knocked down under different oxygen-containing conditions

Compared with the normoxic group, the proliferation activity of gastric cancer cell SGC-7901 in the hypoxic group increased notably (P < 0.05), knocking down HIF-1α under normal oxygen conditions resulted in cell proliferation reduction, while knocking down HIF-1α under hypoxia conditions could offset cell changes caused by hypoxia (P < 0.05). As shown in **Figure 2**.

Comparison of migration and invasion of gastric cancer tissues knocked down by HIF-1α under different oxygen-containing conditions

Compared with the normoxic group, the number of SGC-7901 cells migrating and invading in the hypoxia group increased remarkably (P < 0.05), knocking down HIF-1α under normal oxygen condition resulted in decreased cell migration and invasion, while knocking down HIF-1α under hypoxia condition could offset cell
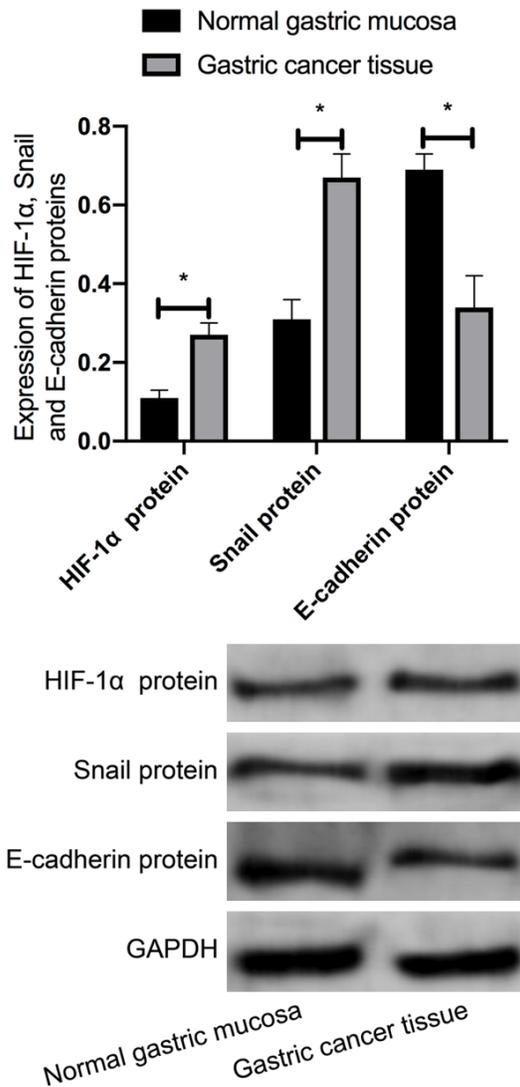
Figure 1. Comparison of the expressions of HIF-1α, Snail and E-cadherin proteins in normal gastric mucosa tissue and gastric cancer tissue. Compared with normal gastric mucosa tissue, the expression of HIF-1α and Snail protein in gastric cancer tissue was up-regulated, and the expression of E-cadherin protein was down-regulated. Note: * represents the comparison between the two groups, P < 0.05.

changes caused by hypoxia (P < 0.05). As shown in **Figure 3**.

Spearman correlation is used to analyze the correlation between the expression of HIF-1α and EMT-related molecules

Spearman correlation analysis showed that the expressions of HIF-1α was positively correlated with Snail mRNA and protein expression (r = 0.740, 0.751, P < 0.001), and negatively correlated with the expressions of E-cadherin
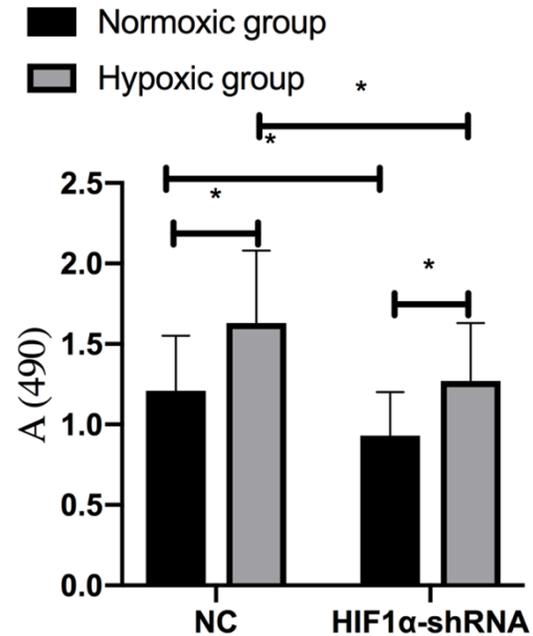
Figure 2. Comparison of proliferation of gastric cancer tissues knocked down by HIF-1α under different oxygen-containing conditions. Compared with the normoxic group, the proliferation activity of gastric cancer cell SGC-7901 in the hypoxic group increased notably. Down-regulating HIF-1α under normal oxygen condition resulted in cell proliferation reduction, while down-regulating HIF-1α under hypoxic conditions offset cell changes caused by hypoxia (P < 0.05). Note: * represents the comparison between the two groups, P < 0.05.

mRNA and protein expression (r = -0.770, -0.766, P < 0.001). More details were shown in **Figure 4**.

Comparison of the expression of HIF-1α, snail and E-cadherin mRNA in gastric cancer cell SGC-7901 under different oxygen-containing conditions

Compared with the normoxic group, HIF-1α and Snail mRNA in the hypoxic group were higher, E-cadherin mRNA was lower (P < 0.05). More details were shown in **Figure 5**.

Comparison of the expression of HIF-1α, snail and E-cadherin in gastric cancer cell SGC-7901 knocked down by HIF-1α under different oxygen-containing conditions

Compared with the normoxic group, HIF-1α and Snail protein in cells of the hypoxic group were higher, and E-cadherin protein was lower (P < 0.05). EMT-related protein Snail was down-regulated and E-cadherin was up-regu-
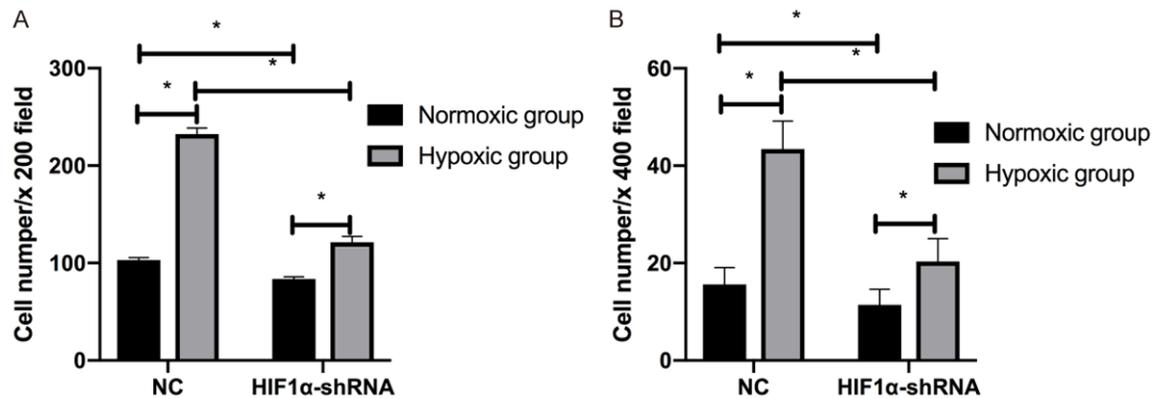
Figure 3. Comparison of the migration and invasion of gastric cancer tissues knocked down by HIF-1α under different oxygen-containing conditions. A. Compared with the normoxic group, the number of migration of gastric cancer cell SGC-7901 in the hypoxic group increased notably. Down-regulating HIF-1α under normal oxygen conditions resulted in cell migration reduction, while down-regulating HIF-1α under hypoxic conditions offset cell changes caused by hypoxia (P < 0.05). B. Compared with the normoxic group, the number of invasion of gastric cancer cell SGC-7901 in the hypoxic group increased notably. Down-regulating HIF-1α under normal oxygen conditions resulted in cell invasion reduction, while down-regulating HIF-1α under hypoxic conditions offset cell changes caused by hypoxia (P < 0.05). Note: * represents the comparison between the two groups, P < 0.05.
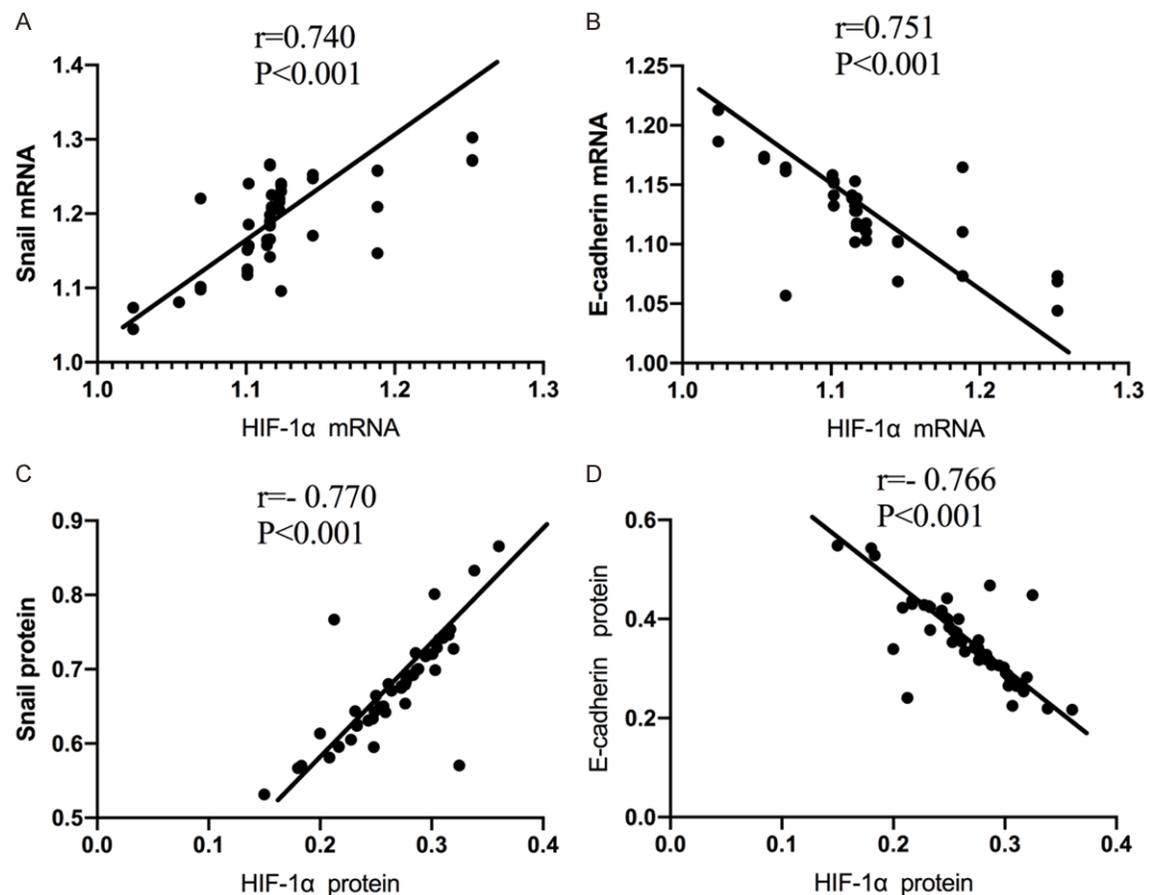
Figure 4. Spearman correlation is used to analyze the correlation between the expression of HIF-1α and EMT-related molecules. A. Spearman correlation analysis showed that HIF-1α mRNA expression was positively correlated with Snail mRNA expression (r = 0.740, P < 0.001). B. Spearman correlation analysis showed that HIF-1α mRNA expression was negatively correlated with E-cadherin mRNA expression (r = -0.770, P < 0.001). C. Spearman correlation

analysis showed that HIF-1α protein expression was positively correlated with Snail protein expression (r = 0.751, P < 0.001). D. Spearman correlation analysis showed that HIF-1α protein expression was positively correlated with E-cadherin protein expression (r = -0.766, P < 0.05).
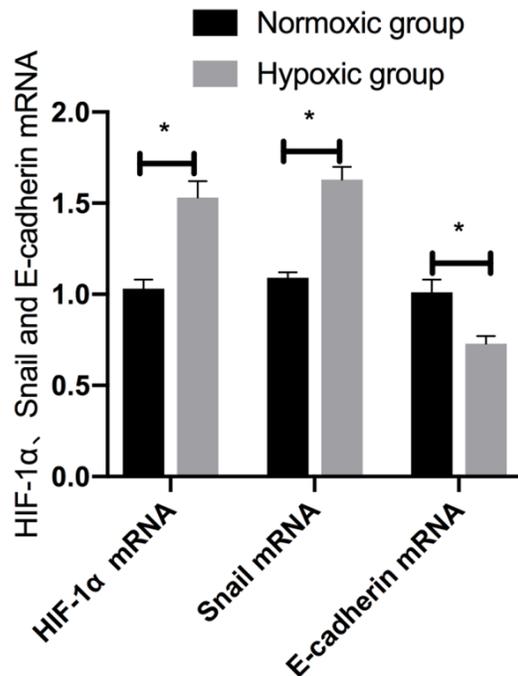
Figure 5. Comparison of the expression of HIF-1α, Snail and E-cadherin mRNA in gastric cancer cell SGC-7901 under different oxygen-containing conditions. Compared with the normoxic group, the hypoxic group had up-regulated HIF-1α and Snail mRNA and down-regulated E-cadherin mRNA. Note: * represents the comparison between the two groups, P < 0.05.

lated after knocking down HIF-1α under normal oxygen conditions. Knocking down HIF-1α under hypoxia conditions could offset cell changes caused by hypoxia (P < 0.05). As shown in **Figure 6**.

Discussion

The continuous and large consumption of oxygen in tumor tissues is mainly attributed to several reasons, such as an imbalance of vascular growth changes, lack of blood perfusion and rapid proliferation rate of tumor cells, thus leading to hypoxia in tissues [10, 11]. In order to maintain a stable blood oxygen state, the tumor cell hypoxia response system is activated, in which hypoxia inducible factor (HIF) participates in tumor proliferation, drug resistance and prognosis by acting on various downstre-

am regulatory factors and promoting tumor cells to undergo anaerobic glycolysis at the same time [12]. The HIF family of proteins are composed of a heterodimer of α and β sub-units, commonly known as HIF-1, 2 and 3, among which HIF-1 is one of the nuclear transcription factors of conservative evolution. In a resting state, only subunits exist in the cytoplasm, and it is continuously degraded by ubiquitination at normal oxygen content. When cells are resistant to hypoxia, HIF-1α accumulation and expression changes easily occur due to inhibition of ubiquitination degradation [13, 14]. HIF-1α over-expression and aggregation occur in glioma, pancreatic cancer, prostate cancer, human tongue squamous cell carcinoma and other solid tumors [15-18]. Excessive accumulation of HIF-1α in tumor cells will affect its phenotypic malignant transformation, accelerate the generation of tumor neovascularization, tumor growth factor and tumor cell metabolic rate, and enhance drug resistance and radiotherapy resistance, thus deteriorating tumor growth and metastasis trajectory [19]. In many studies, tumor invasion and migration can't occur without HIF-1 beam accumulation in cells [20]. However, it is not clear whether the mechanism of cell invasion and migration caused by HIF-1α accumulation in gastric cancer is specific. Therefore, this experiment aimed to explore the possible mechanisms by constructing a hypoxia environment to induce EMT in gastric cancer cells.

According to the summary of data in this study, compared with normal gastric mucosa tissue, HIF-1α and Snail protein expression in gastric cancer tissue are up-regulated and E-cadherin protein expression is down-regulated. Previous studies have recorded high expression data of HIF-1α protein in many solid tumors [21]. The progression of gastric cancer is often accompanied by deletion of E-cadherin protein. The transcription factor Snail protein can restrict the activity of E-cadherin through multiple ways, which can promote EMT-related changes in the process [22]. Other literature reports are similar to the results of this study. Our research found that compared with the normoxic group, cell migration, invasion and prolif-
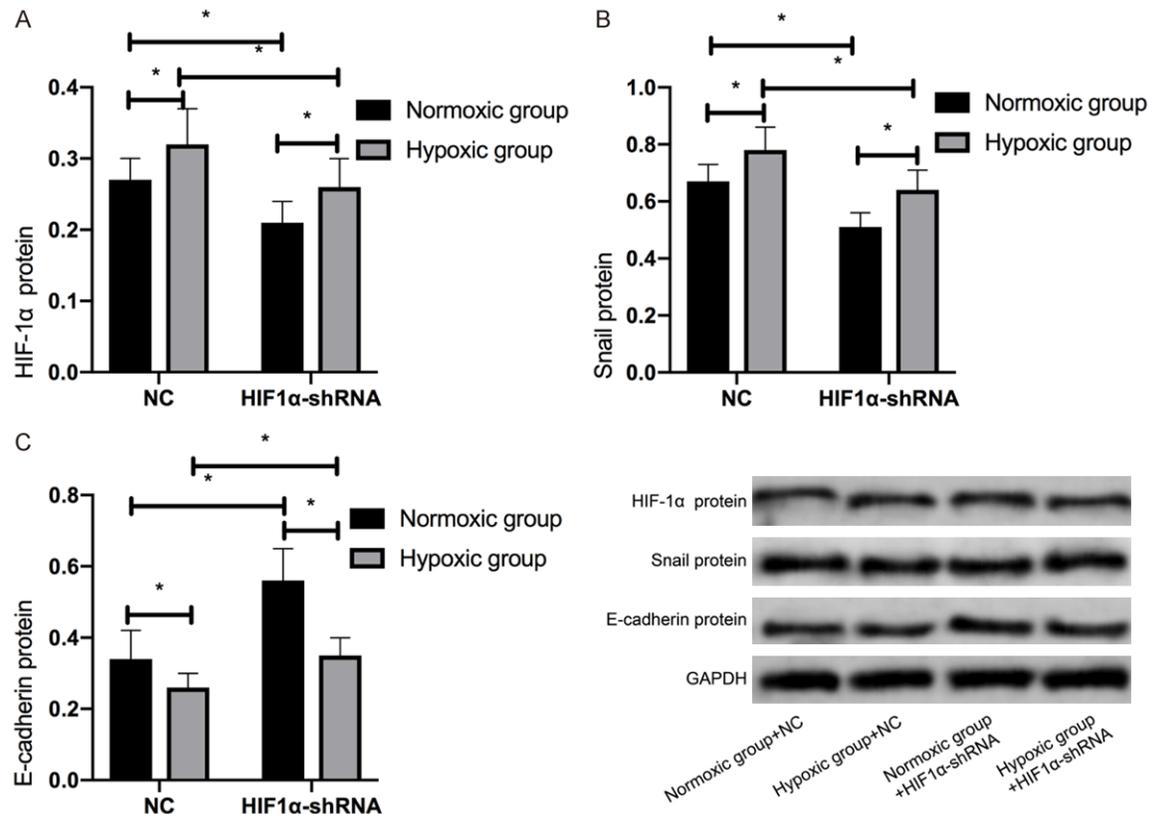
Figure 6. Comparison of the expression of HIF-1α, Snail and E-cadherin in gastric cancer cell SGC-7901 knocked down by HIF-1α under different oxygen-containing conditions. A. Compared with the normoxic group, HIF-1α in the hypoxia group was up-regulated, and HIF-1α was down-regulated after knocking down HIF-1α under normal oxygen and hypoxic conditions. B. Compared with the normoxic group, Snail protein was up-regulated, EMT-related protein Snail was down-regulated after knocking down HIF-1α under normal oxygen conditions, and down-regulation of HIF-1α under hypoxic conditions could offset cell changes caused by hypoxia. C. Compared with the normoxic group, E-cadherin was up-regulated, EMT-related protein E-cadherin was down-regulated after knocking down HIF-1α under normal oxygen conditions, and down-regulation of HIF-1α under hypoxic conditions offset cell changes caused by hypoxia. Note: * represents the comparison between the two groups, $P < 0.05$.

eration in the hypoxic group increased. Under normal oxygen conditions, knocking down HIF-1α led to reduction of cell migration, invasion and proliferation. Under hypoxic conditions, knocking down HIF-1α offset cell changes caused by hypoxia. Hypoxia in a tumor environment is the most typical feature of solid tumors. The hypoxia microenvironment occupies the key regulatory factors in angiogenesis, tumor cell infiltration, distant metastasis and tumor progression [23]. HIF-1α protein accumulation is closely related to biological behavior of tumor cells and angiogenesis in tumors [24-26]. This shows that the deterioration rate of tumor cells can be alleviated by reducing the content of HIF-1α in cells and improving the hypoxic state. On the other hand, we selected EMT related molecules of gastric cancer to

carry out detailed research, and found that compared with the normoxic group, HIF-1α and Snail expression in the hypoxic group were higher and E-cadherin expression was lower. Knocking down HIF-1α under normal oxygen conditions lead to the decrease of EMT related protein Snail, while an increase of E-cadherin. HIF-1α knock-down treatment under hypoxic conditions offset the expression changes caused by hypoxia. Some studies have shown that HIF-1α directly up-regulates the transcription of Snail gene and E-cadherin when hypoxic. HIF-1α is an upstream regulatory factor of Snail and E-cadherin genes. The regulation in the target region mainly depends on the reaction of lysine and lipoxygenase. It can induce EMT in tumor cells by dominant Snail gene expression and can also activate and inhibit

the expression of transcription factors such as E-cadherin [27]. This indicates that inhibition of gastric cancer cells can achieve the goal of killing tumor cells via regulating HIF-1α, Snail and E-cadherin expression. In order to further clarify the expression correlation between HIF-1α, Snail and E-cadherin, Spearman method was adopted to verify it, and we found that the expression of HIF-1α and Snail was positively proportional, while the expression of HIF-1α and E-cadherin was inversely proportional, which again verified the validity of the expression data of the above results

To sum up, HIF-1α induces gastric cancer cell proliferation and EMT by regulating Snail and E-cadherin expression. In this study, the biological behavior of gastric cancer cells and the expression of EMT-related molecules were observed by establishing hypoxic environments and knocking down HIF1α, so as to understand the dominant role of HIF1α in gastric cancer. However, there are still some deficiencies in this article that need to be improved. For example, only one gastric cancer cell line was selected. In order to avoid error data, cells can be appropriately added for research, and other relevant molecules can also be selected for comparative analysis for EMT-related molecules selection. All these are our follow-up ideas with improved research content, in order to provide a better plan for the diagnosis, treatment and prevention of gastric cancer recurrence.

Acknowledgements

Disclosure of conflict of interest

None.

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