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Original Article

HIF-1α Contributes to Hypoxia-induced Invasion and Metastasis of Esophageal Carcinoma via Inhibiting E-cadherin and Promoting MMP-2 Expression

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Hypoxia-inducible factor-1α (HIF-1α) has been found to enhance tumor invasion and metastasis, but no study has reported its action in esophageal carcinoma. The goal of this study was to explore the probable mechanism of HIF-1α in the invasion and metastasis of esophageal carcinoma Eca109 cells *in vitro* and *in vivo*. mRNA and protein expression of HIF-1α, E-cadherin and matrix metalloproteinase-2 (MMP-2) under hypoxia were detected by RT-PCR and Western blotting. The effects of silencing HIF-1α on E-cadherin, MMP-2 mRNA and protein expression under hypoxia or normoxia were detected by RT-PCR and Western blotting, respectively. The invasive ability of Eca109 cells was tested using a transwell chambers. We established an Eca109-implanted tumor model and observed tumor growth and lymph node metastasis. The expression of HIF-1α, E-cadherin and MMP-2 in xenograft tumors was detected by Western blotting. After exposure to hypoxia, HIF-1α protein was up-regulated, both mRNA and protein levels of E-cadherin were down-regulated and MMP-2 was up-regulated, while HIF-1α mRNA showed no significant change. SiRNA could block HIF-1α effectively, increase E-cadherin expression and inhibit MMP-2 expression. The number of invading cells decreased after HIF-1α was silenced. Meanwhile, the tumor volume was much smaller, and the metastatic rate of lymph nodes and the positive rate were lower *in vivo*. Our observations suggest that HIF-1α inhibition might be an effective strategy to weaken invasion and metastasis in the esophageal carcinoma Eca109 cell line.

Key words: hypoxia-inducible factor-1α, esophageal carcinoma, invasion and metastasis

The hypoxia-inducible factors (HIFs) mediate transcriptional responses to changes in oxygen levels by activating the transcription of hundreds of target genes [1]. HIF-1 is the key transcriptional regulator of the cellular response to a hypoxic environment. It is involved in many cellular processes that help cells and organisms to cope with reduced oxygen and energy supply [2]. HIF-1 is a heterodimer comprised of α and β subunits. While HIF-1β is constitutively expressed, HIF-1α is rapidly degraded under normoxic conditions and can be stabilized by low oxygen tensions, which in turn increases the expression of more than 100 gene products that take part in the regulation of angiogenesis, energy metabolism, invasion and metastasis [3]. Invasion and metastasis is a complicated process involved in attenuated adhesion.

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As a hallmark of epithelial to mesenchymal transition (EMT), E-cadherin is believed to weaken tumor invasion [4], which can be repressed by SNAIL through binding to its E-box elements [5]. In addition, tumor cells have to produce and derive proteases that are used to degrade extracellular matrix and basement membrane. Among these proteases, matrix metalloproteinase-2 (MMP-2) can degrade many kinds of extracellular matrix and play critical roles in invasion [6]. HIF-1α enhances tumor invasion and metastasis through down-regulating E-cadherin [7] and up-regulating MMP-2 [8], but no study has reported its action in esophageal carcinoma. In this study, we used cobalt chloride ($CoCl_2$) to mimic a tumor hypoxic microenvironment [9] and established a xenograft tumor model, in order to detect the effect of HIF-1α on invasion and metastasis *in vitro* and *in vivo*.

Materials and Methods

Cell line and cell culture. Human esophageal cancer line Eca109 was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 media (Sigma Chemical, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 25 U/mL penicillin and 25 ng/mL streptomycin (GIBCO/Invitrogen, Carlsbad, CA, USA) at 37℃ in a humidified atmosphere containing 5% CO_2. The $CoCl_2$ (Sigma Chemical) of 100 μmol/L was used to induce hypoxia-mimicking conditions [9].

Reagents and antibodies. Antibodies against HIF-1α, E-cadherin, MMP-2 and SNAIL were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). GAPDH antibody was acquired from Novus Biologicals (Novus, Littleton, CO, USA). pSuper HIF-1α/siRNA vector and empty pSuper vector were from Shanghai GeneChem Co., Ltd. (Shanghai, China).

Reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Total RNA was reverse-transcribed to make cDNA using the Prime Script RT reagent kit (TaKaRa, Otsu, Japan) and amplified by PCR. The primer sets used for PCR were as follows:

HIF-1α, 5'-ACTCAGGACACAGATTTAGACTTG-3' (sense) and 5'-TGGCATTAGCAGTAGGTTCTTG-3' (anti-sense);
E-cadherin, 5'-ATTCTGATTCTGCTGCTCTTG-3' (sense) and 5'-TGGCATTAGCAGTAGGTTCTTG-3' (anti-sense);
MMP-2, 5'-TGACGGTAAGGACGGACTC-3' (sense) and 5'-ATACTTCACACGGACCACTTG-3' (anti-sense);
GAPDH, 5'-ACCTGACCTGCCGTCTAGAA-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (anti-sense).

The PCR conditions consisted of 10 min at 94℃ followed by 30 cycles of denaturation for 20 s at 95° (HIF-1α: annealing for 20 s at 58°; E-cadherin: 20 s at 56°; MMP-2: 20 s at 58°; GAPDH: 20 s at 57°) and extension for 30 s at 72°, followed by 72° for 10 min. Amplified products were separated by 1.5% agarose gel electrophoresis, photographed under ultraviolet light and analyzed by densitometry. The quantity of each transcript was normalized to that of GAPDH.

Western blotting analysis. Western blotting analysis was performed as described elsewhere [10]. Briefly, proteins were separated on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose membrane. Immunoblots were performed with primary antibodies against HIF-1α (1 : 200), E-cadherin (1 : 200), MMP-2 (1 : 200), SNAIL (1 : 200) and GAPDH (1 : 500), respectively. Membranes were blocked in TBS-T containing 6% nonfat dry milk and then incubated overnight with primary antibody. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma, St. Louis, MO, USA) for 1 h. ECL reagent (GE Healthcare, NJ, USA) was used for protein detection.

Establishment of stable HIF-1α knockdown cells. Eca109 cells were transfected with control siRNA or siRNA against HIF-1α termed 1, 2, 3 or 4. The target sequence of the 1st was GTGATGAAAGAATTACCGAAT, the 2nd was CCGCTGGAGACACAATCATAT, the 3rd was CGGCGAAGTAAAGAATCTGAA and the 4th was CCAGTTATGATTGTGAAGTTA. To generate stably transfected cells, we

transfected Eca109 cells using Tfx-20 (Promega) according to the manufacturer's instructions and selected them with puromycin (at a final concentration of 5 μg/ml) (Sigma) for 14 days. Clones were subsequently selected and screened for diminished HIF-1α expression and expression of E-cadherin and MMP-2 was detected by RT-PCR and Western blotting analysis.

Cell invasion assay. Eca109 cells were cultured in normal medium overnight and then switched to low-serum medium (LSM) with 0.5% FBS for 8 h. Thereafter, cells were harvested and resuspended in LSM. Cell invasion was assayed using a transwell chamber (Corning, Mexico) with a pore size of 8.0 μm. Polycarbonate membrane was coated with matrigel gel. Cells were counted and set to 5×10^5 cells/mL in LSM, and 200 μL of the suspension was added to each insert, while 500 μL of normal medium was added to outside of each insert. After being cultured for 24 h under hypoxia, the cells were scraped from the top surface of the membrane with a cotton swab, and the cells that had invaded the membrane were fixed by paraformaldehyde (40 g/L) and stained with hematoxylin and eosin. Five fields were selected randomly from the central and the surrounding membrane, and then every field of cells was counted.

Establishment of a xenograft tumor model using Eca109 cells. Three-week-old female athymic nude mice (Academy of Medical Sciences, China) were housed in a specific pathogen-free room under controlled temperature and humidity. All animal experiments conformed to the provisions of the Declaration of Helsinki, 1995 (as revised in Edinburgh in 2000). All mice were adapted to the sterile basic supplementary diet and acclimated to laboratory conditions for a week before use. These mice were randomly divided into 3 groups. The first group (n = 10) was an untreated group to be implanted with untreated Eca109 cells, the second (n = 10) was a control siRNA group and the third (n = 10) was the siRNA HIF-1α group. In every group, 2×10^6 viable Eca109 cells were injected into the right lateral thigh. Mice were sacrificed when a tumor formed after 20 days, and then the tumor tissues and bilateral inguinal lymph nodes were removed.

Tumor volume and lymph node metastasis assay. Tumor volume (V) was determined using the following equation: $V = (L \times W^2) \times 0.5$, where L was the length of the long side of the tumor and W was the length of the short side. All lymph nodes were stained with hematoxylin and eosin, embedded in paraffin and cut into pathological sections. The metastatic rate was defined as the number of metastatic nude mice against the total number of mice (10). The positive rate was defined as the number of positive lymph nodes against the total number of nodes in each group.

Expression of E-cadherin, MMP-2 and HIF-1α proteins in xenograft tumors. Tumor tissues were collected from the 3 groups and processed with trypsinization, centrifugation and precipitation. Western blotting analysis was used to detect the expression of E-cadherin, MMP-2 and HIF-1α proteins in xenograft tumors.

Statistical analysis. All results were expressed as the mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls tests for post-hoc comparisons between the means. Statistical significance was determined as $p < 0.05$. All statistical analyses were carried out with SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Hypoxia-induced expression change of E-cadherin, MMP-2 and HIF-1α in human esophageal cancer cells. Hypoxia-dependent expression of E-cadherin and MMP-2 was investigated by RT-PCR. We exposed Eca109 human esophageal cancer cells to normoxia or hypoxia for 6 h, 12 h and 24 h. E-cadherin mRNA was significantly decreased, while MMP-2 increased after exposure to hypoxia compared with normoxia ($p < 0.05$). Moreover, E-cadherin expression in hypoxia at 12 h and 24 h was significantly lower than that at 6 h, while MMP-2 higher than that at 6 h ($p < 0.05$) (Fig. 1A). Western blotting analysis was used to determine E-cadherin and MMP-2 protein expression under hypoxia. Results showed that hypoxia resulted in decreased E-cadherin protein expression and increased MMP-2 expression (Fig. 1B), and these changes were consistent at various mRNA levels. Our results also showed that hypoxia induced up-regulation of HIF-1α protein, whereas its mRNA level remained stable (Fig. 1A and 1B).

The role of HIF-1α on hypoxia-induced expression change of E-cadherin and MMP-2 in Eca109 cells. To determine the effect of HIF-1α

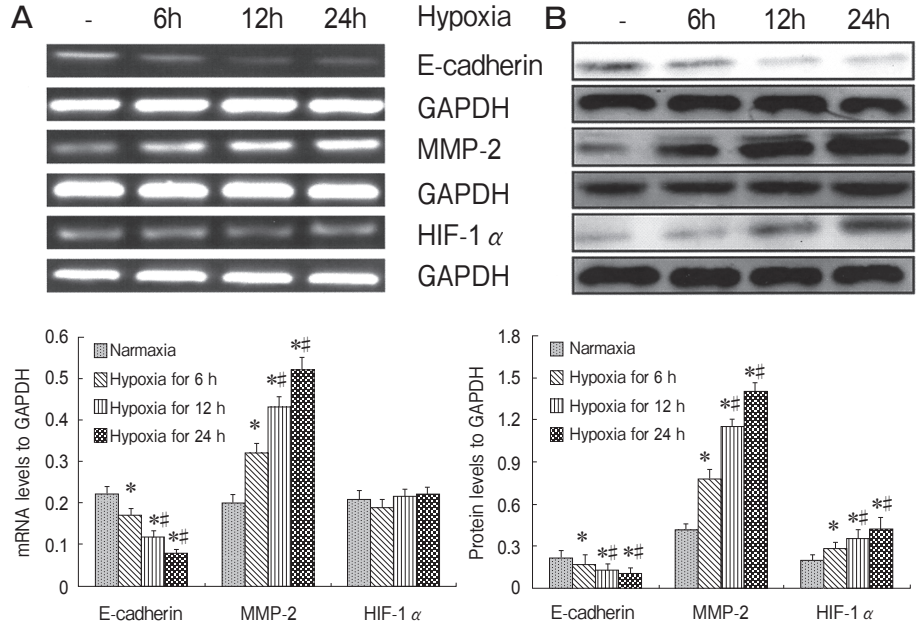

Fig. 1 Hypoxia-induced expression of E-cadherin, MMP-2 and HIF-1α in human esophageal cancer cells. (**A**) RT-PCR was used for analysis of E-cadherin, MMP-2 and HIF-1α mRNA levels in Eca109 cells after exposure to normoxia or hypoxia for the indicated periods. Results were normalized to GAPDH. (**B**) Western blotting analysis was performed to detect E-cadherin, MMP-2 and HIF-1α protein expression after culturing in normoxic or hypoxic conditions. Band intensities were normalized to those of GAPDH. $^{*}p < 0.05$ versus normoxia group. $^{\#}p < 0.05$ versus hypoxia group for 6 h (mean $\pm$ SEM, n = 3).

on hypoxia-induced expression change of E-cadherin and MMP-2, we used siRNA against HIF-1α to down-regulate the expression of HIF-1α in Eca109 cells. Compared with the 1st, 2nd and 4th, the 3rd siRNA against HIF-1α had the best effect (Fig. 2A), so we chose the 3rd siRNA for our study. As shown in Fig. 2B, HIF-1α mRNA and protein were both markedly suppressed in cells transfected with siRNA compared with cells transfected with control siRNA and untreated cells ($p < 0.05$), following exposure to hypoxic conditions for 24 h. In further experiments, treatment with HIF-1α siRNA not only blocked the down-regulation of E-cadherin but also suppressed the up-regulation of MMP-2 mRNA and protein levels under hypoxia. We also detected whether siRNA had an effect on the expression of E-cadherin and MMP-2 under normoxia. Our results showed that compared with those in the control siRNA or untreated group, mice in the siRNA HIF-1α group showed no significant change in either E-cadherin or MMP-2 expression, even though HIF-1α mRNA was significantly inhibited ($p < 0.05$) (Fig. 2C).

SNAIL may have been involved in HIF-1α-mediated suppression of E-cadherin. To further investigate how HIF-1α suppressed E-cadherin, we detected SNAIL expression in Eca109 cells by Western blotting. As shown in Fig. 3, HIF-1α resulted

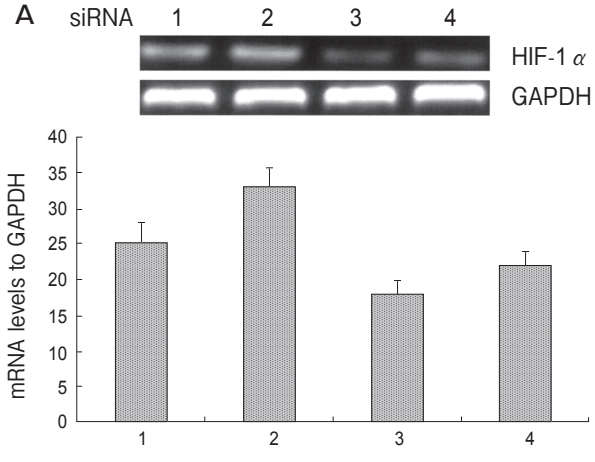

Fig. 2 The role of HIF-1α in hypoxia-induced expression change of E-cadherin and MMP-2 in Eca109 cells. (**A**) Eca109 cells were treated with siRNA against HIF-1αtermed 1, 2, 3 or 4.

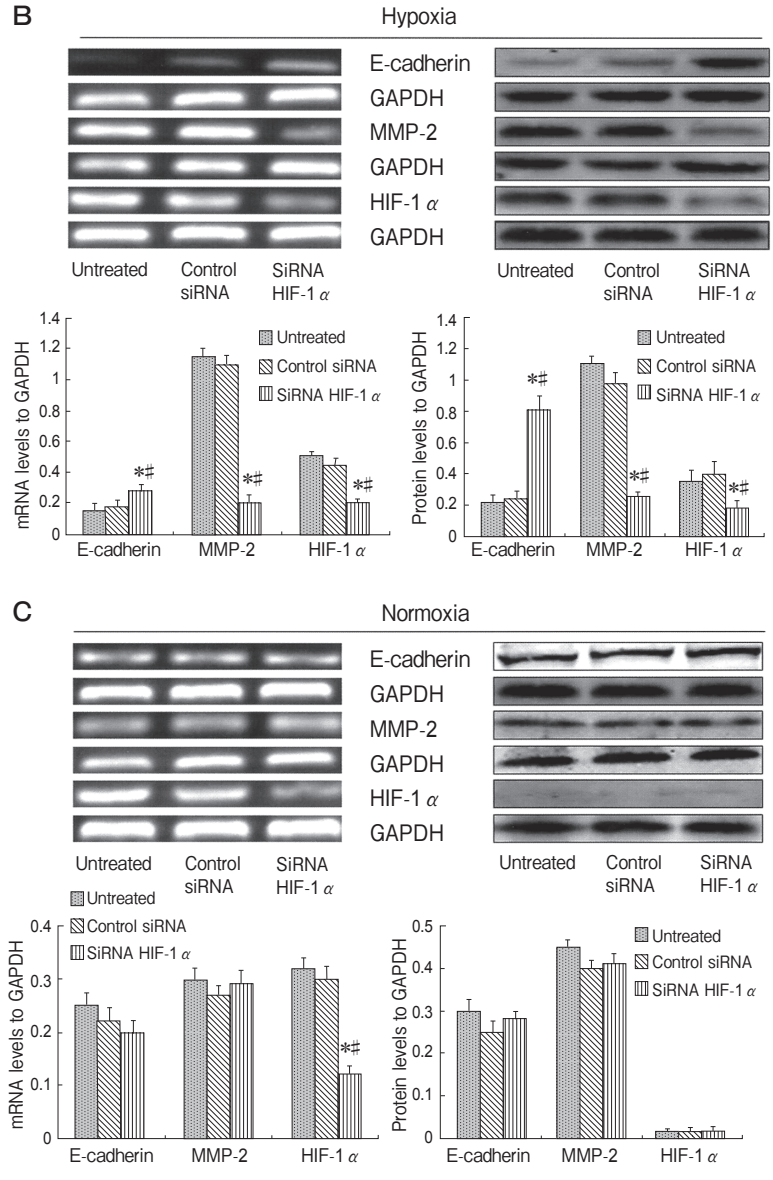

Fig. 2 The role of HIF-1α in hypoxia-induced expression change of E-cadherin and MMP-2 in Eca109 cells. (**B**) Expression of HIF-1α, E-cadherin and MMP-2 under hypoxic conditions for 24 h was examined by RT-PCR and Western blotting. (**C**) Expression of HIF-1α, E-cadherin and MMP-2 under normoxia was examined. Band intensities were normalized to those of GAPDH. $^{*}p < 0.05$ versus untreated group, and $^{\#}p < 0.05$ versus control siRNA group (mean $\pm$ SEM, n = 3). This is a representative experiment out of a total of 3.

in increasing expression of SNAIL in Eca109 cells. Once HIF-1α was blocked, SNAIL expression was inhibited accordingly.

The increase in invasion of Eca109 cells caused by HIF-1α. To detect the effect of HIF-1α on invasion, we assayed cell migration using a transwell chamber. As shown in Fig. 4, compared with that under normoxia, the number of invasive cells was increased significantly under hypoxia, but it decreased following down-regulation of HIF-1α by siRNA ($p < 0.05$). However, the number of invasive cells in the siRNA group was still greater than that under normoxia ($p < 0.05$).

The promotion of tumor growth and metastasis by HIF-1α in xenograft tumor. As shown in Fig. 5A, tumor volume in the siRNA group was

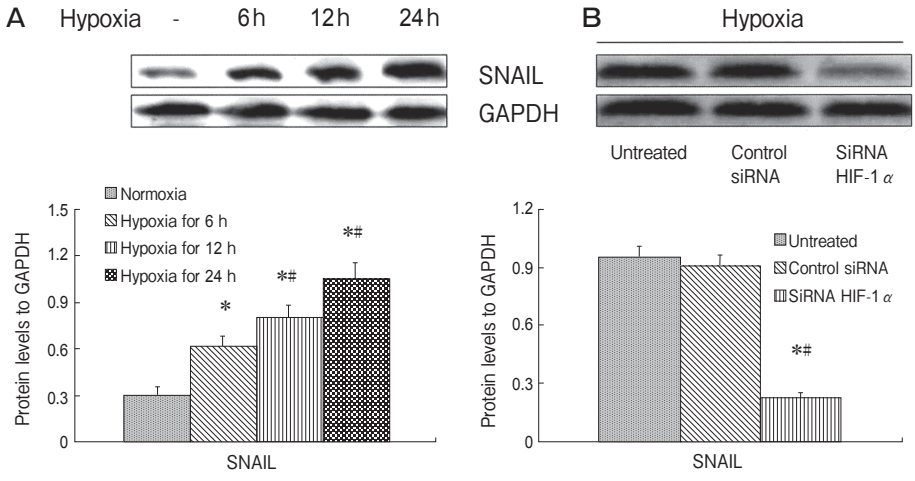

Fig. 3 SNAIL may be involved in HIF-1α-mediated suppression of E-cadherin. (**A**) Western blotting was used for the analysis of SNAIL expression in Eca109 cells after exposure to normoxia or hypoxia for the indicated periods. $^{*}p < 0.05$ versus the normoxia group, $^{\#}p < 0.05$ versus the hypoxia group for 6 h. (**B**) Eca109 cells were treated with siRNA against HIF-1α. Expression of SNAIL protein was examined by Western blotting under hypoxic conditions for 24 h. Band intensities were normalized to those of GAPDH. $^{*}p < 0.05$ versus the untreated group. $^{\#}p < 0.05$ versus the control siRNA group (mean $\pm$ SEM, n = 3).

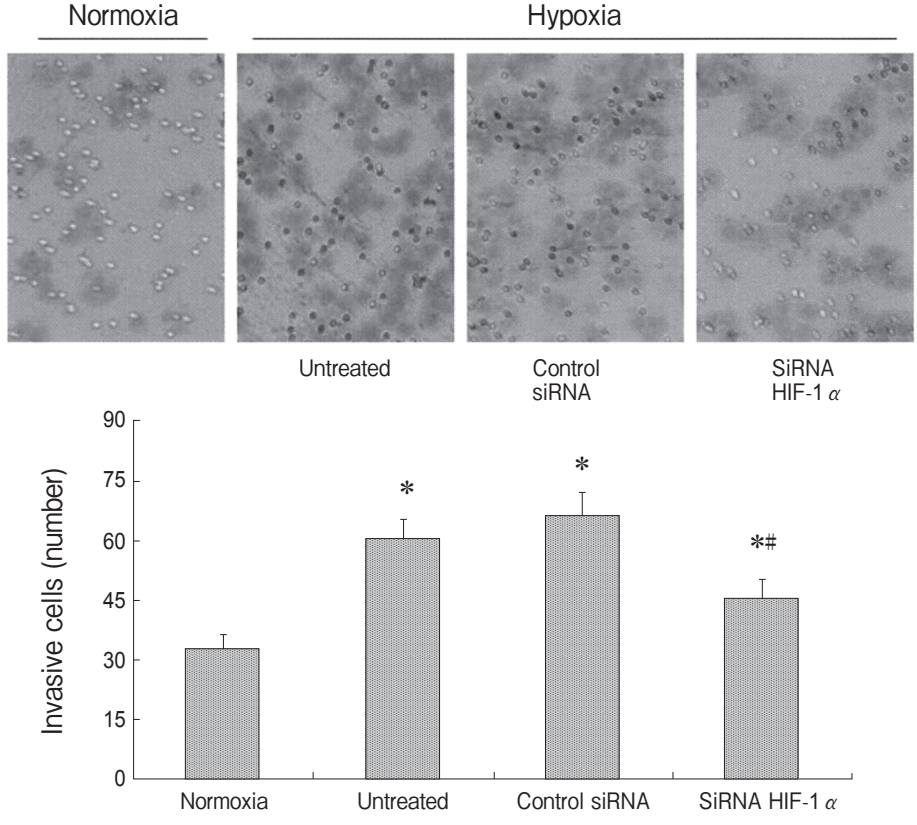

Fig. 4 Hypoxia enhanced Eca109 cell invasion. A transwell chamber was used to evaluate the invasion of Eca109 cells after exposure to hypoxia. $^{*}p < 0.05$ versus the normoxia group. $^{\#}p < 0.05$ versus the untreated group and the control siRNA group (mean $\pm$ SEM, n = 3). Results are expressed as the mean $\pm$ SEM of 3 separate experiments.

$538.0 \pm 45.7\,mm^3$, which was significantly less than the volumes in the untreated group ($1374.2 \pm 95.7\,mm^3$) and the control siRNA group ($1459.6 \pm 116.2\,mm^3$) ($p < 0.05$). The metastatic rate of lymph nodes in the siRNA group was 30% (3/10), which was lower than the rates in the untreated group (100%, 10/10) and the control siRNA group (100%, 10/10) (Fig. 5B); The positive rate of lymph nodes was 29.4% (5/17), which was also much lower than the rates in the untreated group (76%, 19/25) and the control siRNA group (72.4%, 21/29) (Fig. 5C).

Expression of E-cadherin, MMP-2 and HIF-1α in xenograft tumors. E-cadherin, MMP-2 and HIF-1α protein levels were detected by Western blotting. As shown in Fig. 6, both HIF-1α and MMP-2 in the siRNA group were significantly lower than those in the untreated group and control siRNA group ($p < 0.05$), while E-cadherin was higher ($p < 0.05$). HIF-1α, E-cadherin and MMP-2 in the untreated group showed no significant change compared to those in the control siRNA group.

Discussion

The extreme energy demands of the numerous, rapidly dividing tumor cells create a hypoxic environment as the tumor develops and grows, so the majority of locally advanced solid tumors contain regions of reduced oxygen availability [11]. Responses to hypoxia

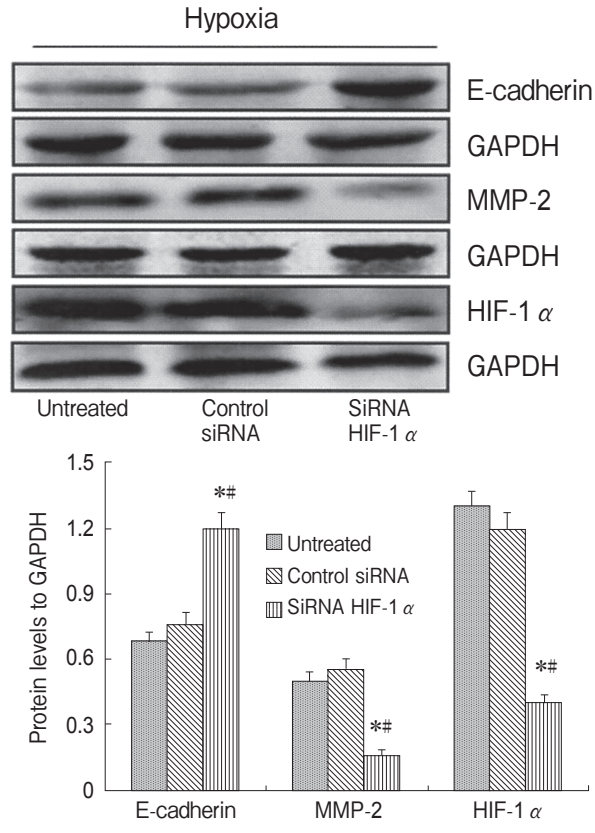

Fig. 6 Expression of E-cadherin, MMP-2 and HIF-1α proteins in xenograft tumors. Band intensities were normalized to those of GAPDH. $^*p < 0.05$ versus the untreated group, and $^\#p < 0.05$ versus the control siRNA group. The results are representative of 3 independent experiments.

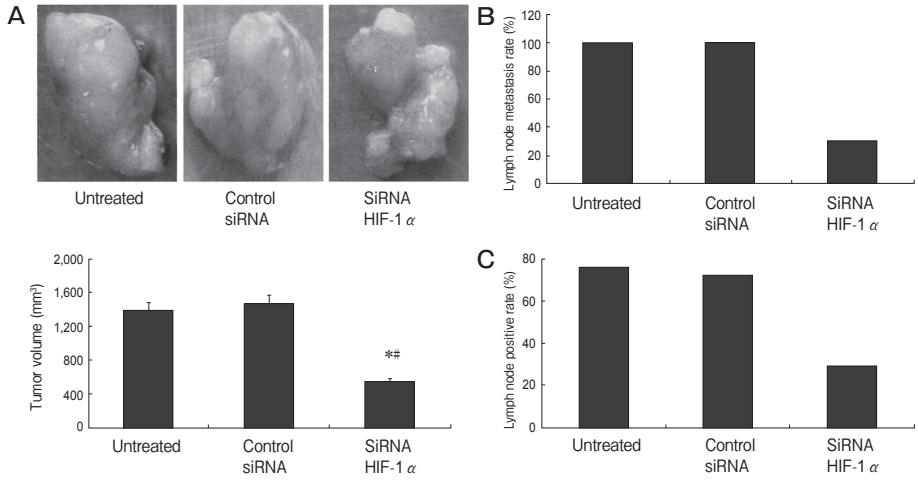

Fig. 5 HIF-1α promoted tumor growth and lymph node metastasis in xenograft tumors. (**A**) The graph showed the tumor volume of representative xenograft tumors in every group, expressed as mm^3. $^*p < 0.05$ versus the untreated group, and $^\#p < 0.05$ versus the control siRNA group. (**B**) Immunohistochemical studies were used to detect the metastatic rate. (**C**) The positive rate of lymph nodes in every group was also assayed by immunohistochemistry.

are mediated mainly through HIF-1α, which is the main mediator of the hypoxia response in the regulation of many hypoxia-inducible genes [12]. To date, HIF-1α expression regulated by hypoxia at the translational or transcriptional level is still controversial. We initially examined the influence of hypoxia on the expression of HIF-1α mRNA and protein levels in an esophageal carcinoma cell line. The expression of HIF-1α mRNA level was stable, regardless of the level of HIF-1α protein expression, in agreement with the results of a previous study [13]. In contrast, HIF-1α protein was increased by hypoxia in the Eca109 cell line.

In normoxia, HIF-1α is hydroxylated by 3 different prolyl hydroxylases, the hydroxylation of which is prevented at low oxygen concentrations. $CoCl_2$ can prevent HIF-1α from binding to the prolyl hydroxylases and thereby interrupt its hydroxylation and subsequent degradation by the proteasome. Thus, $CoCl_2$-mediated stabilization of HIF-1α induces the transcription of HIF-targeted genes in spite of the presence of oxygen [14]. $CoCl_2$ has been widely used as a hypoxia mimic in both *in vitro* and *in vivo* studies [15].

E-cadherin possibly influencing the process of EMT is regulated by HIF-1α through a number of different pathways [16]. However, Gu *et al* recently found that HIF-1α is not correlated with the expression of E-cadherin [17]. Accordingly, we explored whether hypoxia is involved in the regulation of E-cadherin expression. As expected, up-regulation of HIF-1α expression in Eca109 cells corresponded to a decrease in expression of E-cadherin at both the translational and transcriptional levels. These inconsistent observations may be due to the use of different tumor germ lines.

As for MMP-2, our results showed that its expression increased at both the translational and transcriptional levels with HIF-1α protein enhanced. To verify whether HIF-1α affected MMP-2 expression, we used siRNA against HIF-1α to down-regulate the expression of HIF-1α in Eca109 cells. Our results showed that MMP-2 expression of both mRNA and protein levels decreased once Eca109 cells were transfected with HIF-1α siRNA under hypoxia, but the mechanism of this action needs to be explored further.

We also detected the effects of siRNA against HIF-1α on E-cadherin and MMP-2 under normoxia. As shown in Fig. 2C, the expression of HIF-1α protein was very weak, and neither E-cadherin nor MMP-2 expression showed a significant change, even though HIF-1α mRNA was significantly inhibited.

SNAIL was an important breakthrough, providing new insights into the molecular mechanisms of tumor invasion, which can repress E-cadherin through binding to its E-box elements [5]. To further investigate whether SNAIL is involved in the HIF-1α-mediated suppression of E-cadherin, we detected SNAIL expression under hypoxia. SNAIL protein was increased following exposure to hypoxia in Eca109 cells but repressed in the case of HIF-1α knockdown. The results showed that SNAIL may be involved in the suppression of E-cadherin mediated by HIF-1α, which was in agreement with the result of a previous study [18].

Hypoxia-inducible genes regulate several biological processes, including cell proliferation, angiogenesis, metabolism, apoptosis, immortalization and migration [19]. It has been demonstrated that HIF-1α is associated with invasion and distant metastases [20]. Our observation was further confirmed by cell invasion assay. In our study, the number of invading Eca109 cells was enhanced by hypoxia but blocked by siRNA against HIF-1α. However, the number of invasive cells in the siRNA group was still greater than that under normoxia, which may have been due to the fact that the effect of siRNA against HIF-1α was not ideal.

Transwell assay suggested that the regulation of E-cadherin and MMP-2 by hypoxia leads to increased invasion of esophageal cancer cells to facilitate metastasis. This result was also verified *in vivo*. Compared with tumor volumes in the untreated group and control siRNA groups, tumor volumes in the siRNA group were smaller ($p < 0.05$), and the metastatic rate of lymph nodes and the positive rate were lower as well. HIF-1α and MMP-2 protein expression in the siRNA group was significantly decreased, and E-cadherin was significantly increased ($p < 0.05$).

It has been shown that HIF-1α enhances tumor invasion and metastasis through down-regulating E-cadherin [7] and up-regulating MMP-2 [8], but no study has reported its action in esophageal carcinoma. Our observations suggest for the first time that HIF-1α contributes to hypoxia-induced invasion and metastasis of esophageal carcinoma via inhibiting E-cadherin

expression and promoting MMP-2 expression both in vitro and *in vivo*, and HIF-1α inhibition might be an effective strategy to weaken invasion and metastasis in the esophageal carcinoma Eca109 cell line.

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