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Hypoxia promotes migration and invasion of gastric cancer cells by activating HIF-1α and inhibiting NDRG2 associated signaling pathway

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Abstract. – OBJECTIVE: Gastric cancer has been become the fourth most prevalent cancer in whole world and the third most common cancer in Asian countries. This study aimed to discuss the invasive and migration mechanisms of gastric cells.

MATERIALS AND METHODS: Human gastric cancer line, BGC-823 cell, was treated with hypoxia and divided into Hypoxia-12 h, Hypoxia-24 h, Hypoxia-36 h, Hypoxia-48 h and Hypoxia-72 group. Meanwhile, blank BGC-823 cells were assigned as Normal group. mRNA and protein expression of N-myc downstream-regulated gene 2 (NDRG2), Twist, E-cadherin and hypoxia-inducible factor 1α (HIF-1α) were evaluated by using quantitative Real-time PCR (qRT-PCR) and Western blot assay, respectively. Invasion and migration of BGC-823 cells were also examined in this study.

RESULTS: Hypoxia treatment significantly enhanced invasion and migration ability of BGC-823 cells compared to that of Normal group ($p<0.05$). Hypoxia treatment significantly reduced E-cadherin and NDRG2 expression compared to that of Normal group ($p<0.05$). Hypoxia treatment significantly increased Twist and HIF-1α expression compared to that of Normal group ($p<0.05$). HIF-1α inhibitor, YC-1, significantly suppressed the effects of hypoxia treatment on E-cadherin and Twist expression ($p<0.05$). Meanwhile, YC-1 treatment also significantly suppressed the effects of hypoxia treatment on NDRG2 and HIF-1α expression.

CONCLUSIONS: Hypoxia promoted the migration and invasion of gastric cancer cell BGC-823 by activating HIF-1α and inhibiting NDRG2 associated signaling pathway.

Key Words
Gastric cancer, Hypoxia, HIF-1α, NDRG2, Invasion.

Introduction

Gastric cancer has become the fourth most prevalent cancer worldwide and the third most common cancer in Asian countries since 70% of gastric cancer occurs in the developing countries[1,2]. In China, gastric cancer is the third leading reason for cancer-associated death, closely following the lung cancer and liver cancer[3,4]. Although the complete resection and chemo- or radio-therapy have been extensively applied in clinical, due to the drug resistance, cancer recurrence and metastasis, the 1-year, 2-year and 3-year survival rate remains very low with 89%, 74% and 63%, respectively[5,6]. Hypoxia is a well-known characteristic for the malignant cancers and plays a critical role in chemotherapeutic drug resistance and tumor metastasis[7,8]. Scholars[9,10] reported that hypoxia is considered to be the biomarker for the metastases of solid tumors, poor prognosis and chemotherapeutic drug resistance. Hypoxia always induces the production of a transcriptional factor, hypoxia-inducible factor 1 (HIF-l), playing important roles in chemotherapeutic drug resistance[11]. HIF-1 is mainly composed of two subunits, including HIF-1α and HIF-1β, both of which conduct the activity of HIF-1[12]. HIF-1α has been proven to participate in many aspects of tumorigenesis, including metastasis, proliferation, chemo-resistance and tumor angiogenesis[13,14]. N-myc downstream-regulated gene 2 (NDRG2) contributes to the cellular differentiation, tumor suppression, and plays critical roles in cellular stress[15]. Liu et al[16] showed that NDRG2 is over-expressed in the tumor cells suffering from the hypoxic stress and contributes to the hypoxia-induced radio-therapeutic resistance in HeLa cells. Moreover, hypoxia modulates the biological functions of tumor cells by inducing epithelial-to-mesenchymal transition (EMT), which is a critical process for malignant tumor transformation and metastasis[17]. However, the correlation among EMT, NDRG2, and HIF-1α, have not been investigated and the effects of HIF-1α on invasion and migration of tumor cells have not been fully clarified. Therefore, we investigated the invasive and migration mechanisms of gastric cells, by

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evaluating the expression of HIF-1α, NDRG2 and EMT associated molecules in BGC-823 cells, such as E-cadherin, Twist.

Materials and Methods

Cell Culture

The human gastric cancer cell line, BGC-823, was purchased from American Type Culture Collection (ATCC) Cell Bank (Manassas, VA, USA). BGC-823 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco BRL. Co. Ltd., Grand Island, NY, USA) supplementing with 100 microg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. This study has been approved by the Ethics Committee of Affiliated Hospital of Guilin Medical College (Guilin, China).

Cell Treatment and Trial Grouping

For the hypoxia treatment conditions, GBC-823 cells were treated with 1% O_2 and 5% CO_2 balancing with the N_2 gas, and cultured for 12 h (Hypoxia-12 h group), 24 h (Hypoxia-24 h group), 36 h (Hypoxia-36 h group), 48 h (Hypoxia-48 h group) and 72 h (Hypoxia-72 h group) at 37°C. Meanwhile, GBC-823 cells treating with 5% CO_2 and normal O_2 were assigned as the Normal group.

Quantitative Real-Time PCR (qRT-PCR) Assay

The total RNAs were extracted by using TRIzol method according to manufacturer's instructions (Tiangen Biotech Co. Ltd., Beijing, China). Complementary DNA (cDNA) templates were gained by using reverse transcription kit according to the manufacturers' instruction (Invitrogen, Carlsbad, CA, USA). The SybrGreen PCR master Mix (TaKaRa, Otsu, Shiga, Japan) was used to amplify the target genes. The primers for Real-time PCR were listed in Table I. Thermal cycling parameters for PCR were listed as follows: denaturation step at 95°C for 2 min, followed by 35 cycles of amplification of 94°C for 30 s, 60°C for 20 s and 72°C for 20 s. The relative expression levels of target genes were calculated by the Real-time PCR Detection System (Mx3000P, Agilent Technologies, Santa Clara, CA, USA) using the formula of $2^{-\Delta\Delta ct}$.

Invasion and Migration Assay

The 24-well plates (Corning, NY, USA) were used to evaluate the invasion and migration abilities of BGC-823 cells. Firstly, BGC-823 cells were starved for 24 h before suspending and incubating with Roswell Park Memorial Institute-1640 (RPMI-1640) without fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). For the migration assay, a total of 1×10^5 BGC-823 cells were seeded and cultured in the upper chamber (Corning, Corning, NY, USA). For the invasion assay, a total of 2×10^5 BGC-823 cells were seeded and cultured in the upper chamber carrying the basement-membrane matrix (Mode: 356234, BD Biosciences, Franklin Lakes, NJ, USA) coated inserts. The lower chamber was filled with the RPMI-1640 supplementing with FBS (at 20% concentration). BGC-823 cells were incubated for 1 day at 37°C, then the BGC-823 cells on inferior surface of inserts were removed by using the cotton swabs (Corning, Corning NY, USA). Finally, the invasive and migrated BGC-823 cells were treated with 4% paraformaldehyde (Sigma-Al-

Table I. Primers and sequences for the PCR assay.

Name	Primer	Sequence	Size
Homo GAPDH	Forward Reverse	5'- TCAAGAAGGTGGTGAAGCAGG -3' 5'- TCAAAGGTGGAGGAGTGGGT -3'	115 bp
Homo E-cad	Forward Reverse	5'-CGTAGCAGTGACGAATGTGG -3' 5'- CTGGGCAGTGTAGGATGTGA-3'	175 bp
Homo Twist	Forward Reverse	5'- GCGGCCAGGTACATCGACTTCCTCT-3' 5'- CATGGACCAGGCCCCCTCCATCCTC-3'	141 bp
Homo NDRG2	Forward Reverse	5'-AACCCAAACGCCCAGCGATCCTTAC -3' 5'-GGCATCCACATGAACCCGCACAAAG -3'	131 bp
Homo HIF-1a	Forward Reverse	5'-TCCAAGAAGCCCTAACGTGT -3' 5'-TGATCGTCTGGCTGCTGTAA -3'	180 bp

drich, St. Louis, MO, USA) and were counted by using the microscope (Mode: BX51, Olympus, Tokyo, Japan). All of the assays were repeated for at least for six times and at least 10 random fields in each well were analyzed in this study.

Western Blot Assay

Total proteins in BGC-823 cells were extracted with lysis buffer (Beyotime Biotech., Shanghai, China). A total of 0.2 μg-extracted lysates were separated by using 15% sodium dodecyl sulfate ploy acrylamide gel electrophoresis (SDS-PAGE, Amresco Inc., Solon, OH, USA). Then, the proteins were electrotransferred onto the commercial polyvinylidene difluoride (PVDF, Amresco Inc., Solon, OH, USA). Polyvinylidene difluoride (PVDF) membranes were incubated and blocked by using 10% FBS (Gibco BRL. Co. Ltd., Grand Island, NJ, USA) in phosphate-buffered saline (PBS, Beyotime Biotech. Shanghai, China) containing 0.05% Tween-20 solution (Beyotime Biotech. Shanghai, China). Polyvinylidene difluoride (PVDF) membranes were treated with rabbit anti-human epithelial cadherin (E-cadherin) monoclonal antibody (1:4000; Cat. No. ab40722, Abcam Biotech., Cambridge, MA, USA), rabbit anti-human Twist polyclonal antibody (1:2000; Cat. No. ab50581, Abcam Biotech.), rabbit anti-human NDRG2 polyclonal antibody (Cat. No. ab11910, Abcam Biotech.), rabbit anti-human HIF-1α monoclonal antibody (Cat. No. ab16897, Abcam, Biotech.) and rabbit anti-human GAPDH polyclonal antibody (1:2000; Cat. No. ab9485, Abcam Biotech.) for 2 h at room temperature. PVDF membranes were then incubated with 1:2000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cat. No. ab6721, Abcam Biotech., Cambridge, MA, USA). The band signals were visualized with commercially enhanced chemiluminescence kit (ECL, Amresco Inc., Solon, OH, USA). Finally, the band signals analyzed by employing Glyko Bandscan gel scanning system (version: 5.0, Glyko, Novato, CA, USA).

Statistical Analysis

The measurements or data were represented as mean ± standard deviation (SD). The data were analyzed with SPSS software (version: 19.0, (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). Student's *t*-test was used to analyze the data between two groups. Tukey's post-hoc test was used to validate the analysis of variance (ANOVA) for comparing measurement data among groups. A statistical significance was defined when $p<0.05$. Data were obtained from at least six independent experiments.

Results

Hypoxia Treatment Enhanced Invasion and Migration Ability of BGC-823 Cells

The invasion and the migration ability reflect the metastasis and proliferation of tumor cells [18]. Therefore, we firstly investigated the effects of hypoxia treatment on the invasion and migration ability of BGC-823 cells. The results showed that hypoxia treatment significantly increased the migration ability of BGC-823 cells at 12 h, 24 h and 36 h, compared to the that of Normal group (Figure 1A, $p<0.05$). Meanwhile, hypoxia treatment also significantly enhanced the invasion ability of BGC-823 cells at 12 h, 24 h and 36 h, compared to that of Normal group (Figure 1B, $p<0.05$). Interestingly, the effects of hypoxia treatment on migration or invasion were reduced at 48 h and 72 h, however, which were also higher compared to that of Normal group (Figure 1, $p<0.05$).

Hypoxia Treatment Reduced E-Cadherin Expression

The levels of critical EMT biomarker, E-cadherin [19], were evaluated by using both of RT-PCR assay and Western blot assay, respectively (Figure 2). The RT-PCR results showed that the E-cadherin levels in Hypoxia treatment group were significantly reduced compared to that in Normal group (Figure 2A, $p<0.05$). Meanwhile, the Western blot assay results also indicated that E-cadherin levels in Hypoxia treatment group were significantly decreased compared to that in Normal group (Figure 2B, $p<0.05$). Moreover, the E-cadherin levels were also decreased following with the treatment time of Hypoxia (from 12 h to 72 h) (Figure 2).

Hypoxia Treatment Increased Twist Expression

Twist is considered to be a regulator for EMT and could promote the tumor migration and invasion[20]. Therefore, in this study, we also examined the expression of Twist in BGC-823 cells. Both of the RT-PCR (Figure 3A) and Western blot assay (Figure 3B) results indicated that the hypoxia treatment significantly increased the Twist expressions compared to that of Normal group ($p<0.05$). Moreover, the Twist levels were increased following with the increased treatment time of hypoxia (Figure 3).

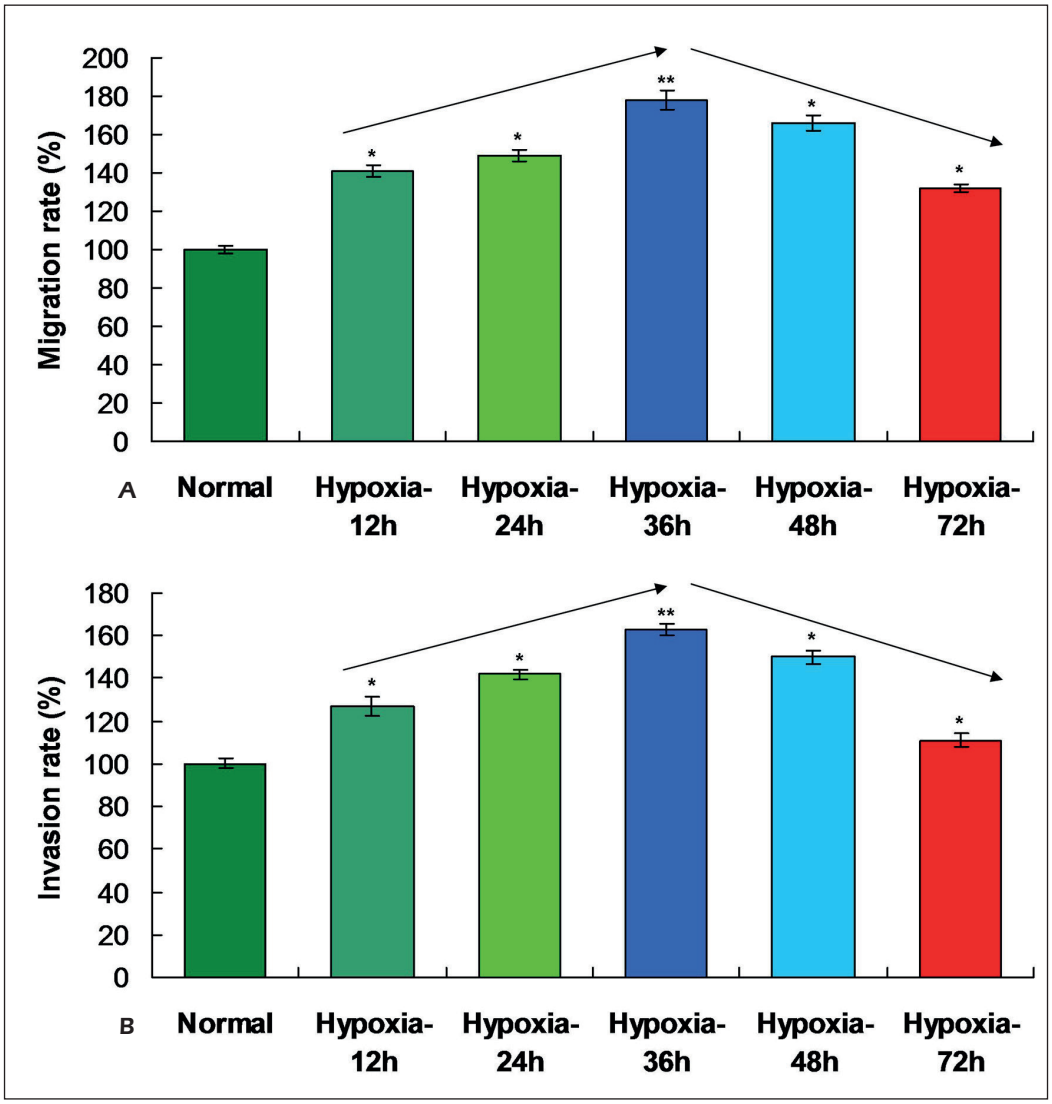

Figure 1. Evaluation for the migration and invasion ability of BGC-823 cells in different hypoxic microenvironment. **A**, Migration ability for the BGC-823 cells. **B**, Invasion ability for the BGC-823 cells. *p<0.05, **p<0.01 vs. Normal group. The black arrows represent the change tendency of migration or invasion.

Hypoxia Treatment Reduced NDRG2 Expression

The previous study[21] reported that NDRG2 plays critical roles in the cellular stress, such as hypoxia stress. Therefore, we evaluated expression of NDRG2 in BGC-823 cells. The results showed that the NDRG2 mRNA levels (Figure 4A) and NDRG2 protein levels (Figure 4B) in hypoxia treatment were significantly higher compared to that in Normal group (p<0.05). Furthermore, both of NDRG2 mRNA and protein levels were reduced following with the decreased treatment time of hypoxia (Figure 4).

Hypoxia Treatment Induced Up-Regulation of HIF-1α

HIF-1 participates in the chemotherapeutic drug resistance of tumor cells[22], therefore, this study also clarified levels of HIF-1α in BGC-823 cells. The results illustrated that the HIF-1α mRNA levels in hypoxia treatment groups were significantly higher compared to that in Normal group (Figure 5A, p<0.05). Meanwhile, the HIF-1α protein levels were also significantly higher in hypoxia treatment groups compared to that in Normal group (Figure 5B, p<0.05). Moreover, both of NDRG2 mRNA and protein levels were increased following with the enhanced treatment time of hypoxia (Figure 5).

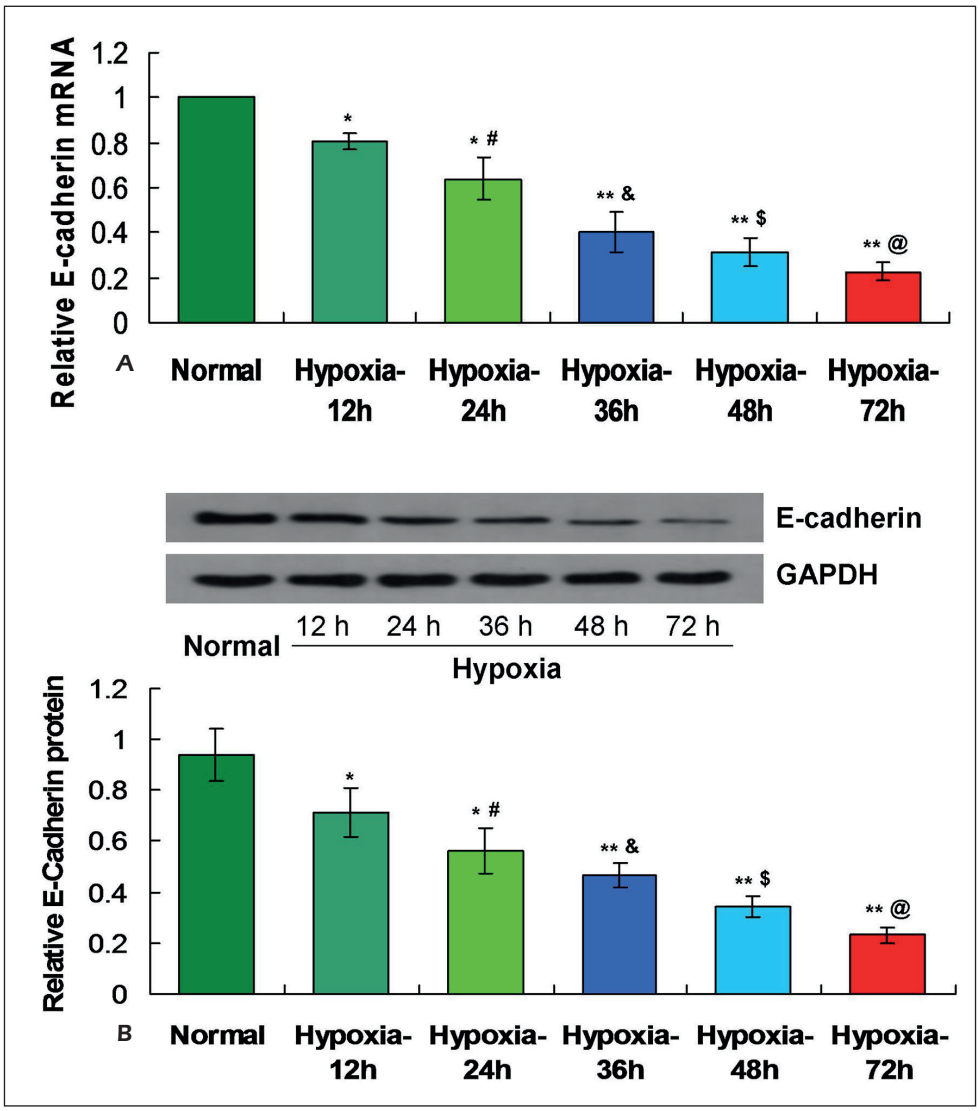

Figure 2. Examination for the mRNA and protein expression of E-cadherin by using qRT-PCR and Western blot assay. **A**, qRT-PCR analysis for E-cadherin mRNA expression. **B**, Western blot assay for E-cadherin protein expression. *$p<0.05$, **$p<0.01$ vs. Normal group. #$p<0.05$ vs. Hypoxia-12 h group. &$p<0.05$ vs. Hypoxia-24 h group. $$p<0.05$ vs. Hypoxia-36 h group. @$p<0.05$ vs. Hypoxia-48 h group.

YC-1 Treatment Suppressed Effects of Hypoxia Treatment on E-cadherin and Twist Expression

YC-1 (3-(5-hydroxymethyl-2-furyl)-1-benzylindazole) is a specific HIF-1 inhibitor that could block the HIF-1α activity[23]. In this study, we applied the YC-1 to treat the BGC-823 cells and observed the effects of YC-1 on E-cadherin and Twist expression. The results indicated that the YC-1 treatment significantly increased the E-cadherin expression compared to that in un-treated cells suffering from hypoxia stimuli at 12 h, 24 h, 36 h, 48 h and 72 h (Figure 6A, $p<0.05$). Meanwhile, the YC-1 treatment also significantly decreased the Twist expression compared to that in un-treated cells suffering from hypoxia stimuli at 24 h, 36 h, 48 h and 72 h (Figure 6B, $p<0.05$).

YC-1 Treatment Suppressed Effects of Hypoxia Treatment on NDRG2 and HIF-1α Expression

In this study, the effects of YC-1 on NDRG2 and HIF-1α expression were also examined by using Western blot assay. The results showed that the YC-1 treatment significantly increased the NDRG2 expression (Figure 7A), significantly de-

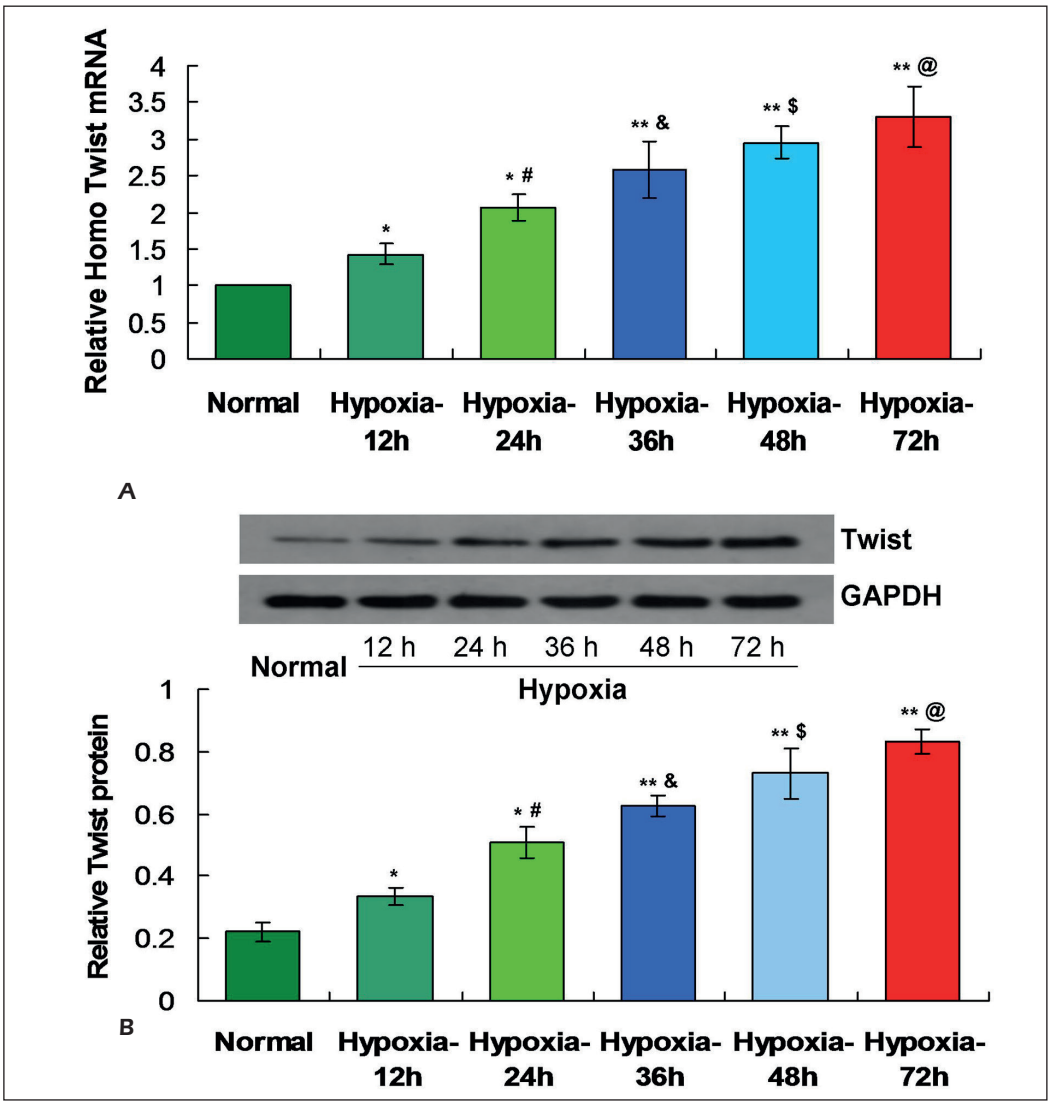

Figure 3. Observation for the mRNA and protein expression of Twist with qRT-PCR and Western blot assay. **A**, qRT-PCR analysis for Twist mRNA expression. **B**, Western blot assay for Twist protein expression. *$p<0.05$, **$p<0.01$ vs. Normal group. $^{\#}p<0.05$ vs. Hypoxia-12 h group. $^{\&}p<0.05$ vs. Hypoxia-24 h group. $^{\$}p<0.05$ vs. Hypoxia-36 h group. $^{@}p<0.05$ vs. Hypoxia-48 h group.

creased HIF-1α expression (Figure 7B) compared to that in un-treated cells suffering from hypoxia stimuli ($p<0.05$).

Discussion

Gastric cancer is one of the most frequently occurred cancers and also the second-reason for cancer-associated mortality[24]. The hypoxia is a critical risk factor for inducing the tumor migration, invasion, and could drive the tumor progression by triggering a series of transcriptional processes and responses[25,26]. Therefore, clarifying the specific mechanism for the hypoxia-caused migration and invasion is extremely critical. In the present study, we firstly investigated the effects of hypoxia treatment on the migration and invasion of BGC-823 cells. The results showed that the hypoxia significantly induced the tumor cell migration and invasion. Actually, this finding consistent with the previously works[27-29] that proved the effects of hypoxia on tumor cell metastasis, migration and invasion. However, the above studies have not clarified the potential mechanisms for the hypoxia caused tumor cell

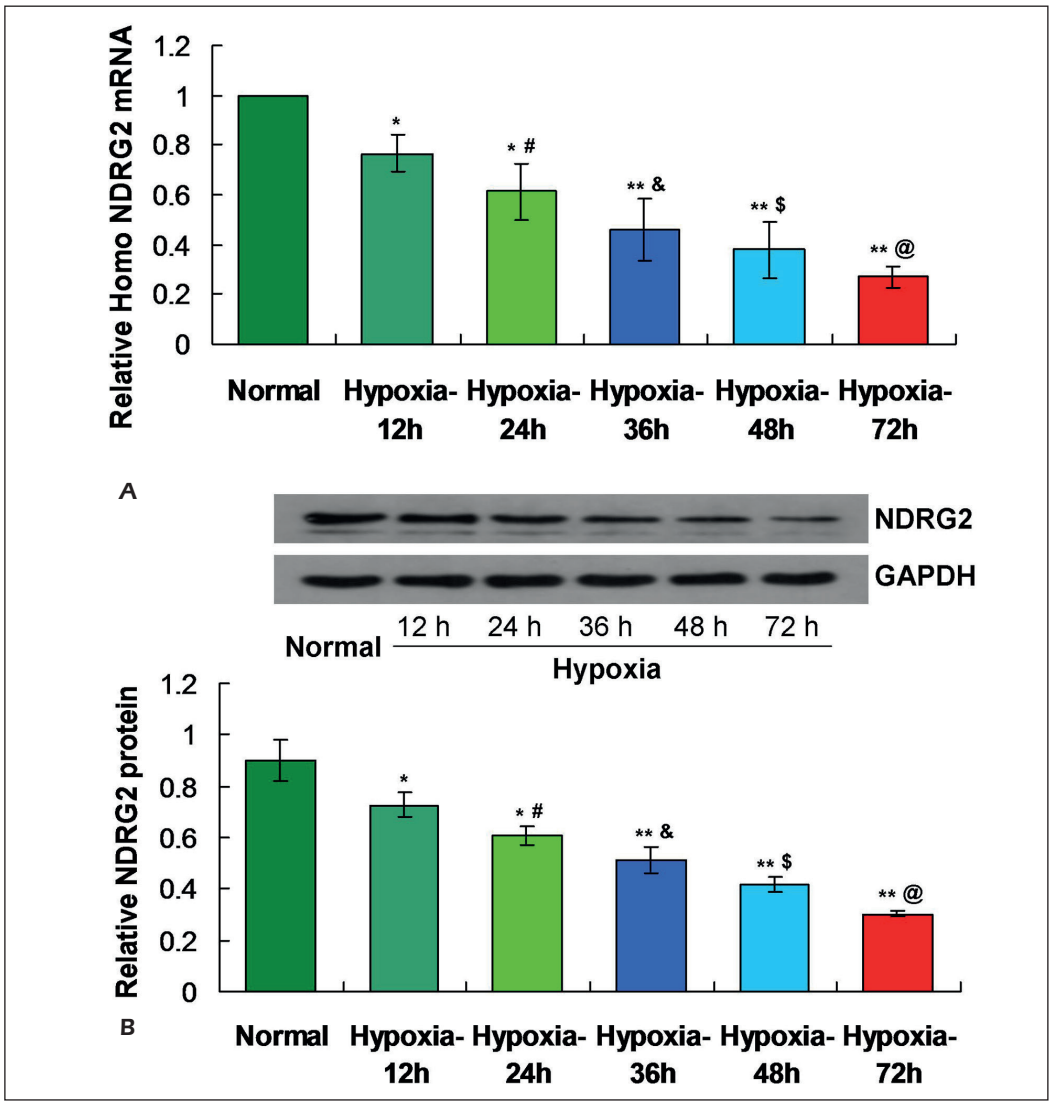

Figure 4. Evaluation of mRNA and protein expression of NDRG2 by using qRT-PCR and Western blot assay. **A**, qRT-PCR analysis for NDRG2 mRNA expression. **B**, Western blot assay for NDRG2 protein expression. *$p<0.05$, **$p<0.01$ vs. Normal group. $^{\#}p<0.05$ vs. Hypoxia-12 h group. $^{\&}p<0.05$ vs. Hypoxia-24 h group. $^{\$}p<0.05$ vs. Hypoxia-36 h group. $^{@}p<0.05$ vs. Hypoxia-48 h group.

metastasis, migration and invasion. Therefore, the hypoxia-associated effects on tumor migration and invasion were investigated in the following experiments. The EMT characterizes by the expression of cell adhesion molecule, E-cadherin, and which is closely associated with the tumor cell migration and invasion[30]. Twist could independently inhibit the expression of E-cadherin and un-regulate the expression of fibronectin and N-cadherin. This research discovered that under the different hypoxic microenvironment, the expression of HIF-1α, NDRG2, E-cadherin and Twist is different. Our results showed that the E-cadherin expression was significantly decreased and Twist expression was significantly increased in the BGC-823 cells undergoing hypoxia treatments. Meanwhile, the NDRG2 expression was significantly decreased and HIF-1α expression was significantly increased in the BGC-823 cells undergoing hypoxia treatments. Moreover, the effects of hypoxia on the E-cadherin, NDRG2, HIF-1α and Twist were enhanced following with the increased hypoxia treatment time. Therefore, the above data suggest that the hypoxia induced

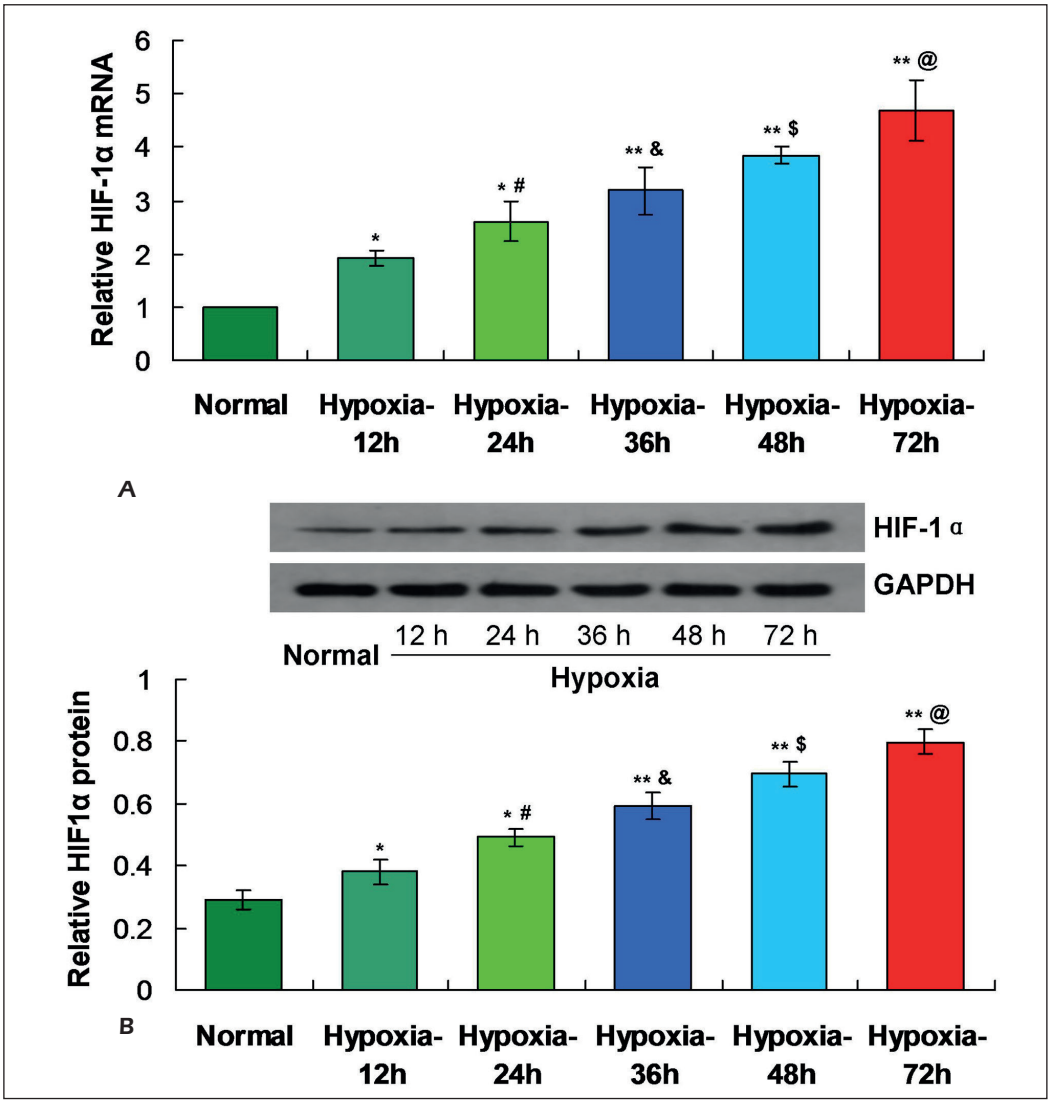

Figure 5. Detection for HIF-1α expression by using qRT-PCR and Western blot assay. **A**, qRT-PCR analysis for HIF-1α mRNA expression. **B**, Western blot assay for HIF-1α protein expression. *$p<0.05$, **$p<0.01$ *vs.* Normal group. $^{\#}p<0.05$ *vs.* Hypoxia-12 h group. $^{\&}p<0.05$ vs. Hypoxia-24 h group. $^{\$}p<0.05$ vs. Hypoxia-36 h group. $^{@}p<0.05$ *vs.* Hypoxia-48 h group.

the BGC-823 cells migration and invasion by promoting EMT, activating the HIF-1α and inhibiting the NDRG2 associated signaling pathway. Researches[31,32] reported that HIF-1α expresses in liver cancer, breast cancer, prostate cancer, and modulates the biological functions. In order to confirm the HIF-1α pathway mediated effects of hypoxia on the cell migration and invasion, the HIF-1α specific inhibitor, YC-1[33], was added to the BGC-823 cells. The results showed that YC-1 treatment significantly increased the E-cadherin and NDRG2 expression, significantly decreased Twist and HIF-1α expression compared to that in un-treated cells suffering from hypoxia stimuli. These data also suggest that the hypoxia causes the EMT-mediated BGC-823 cells migration and invasion by triggering the HIF-1α signaling pathway.

Conclusions

Hypoxia treatment significantly enhanced invasion and migration ability of BGC-823 cells. We found that hypoxia treatment significantly reduced E-cadherin and NDRG2 expression and sig-

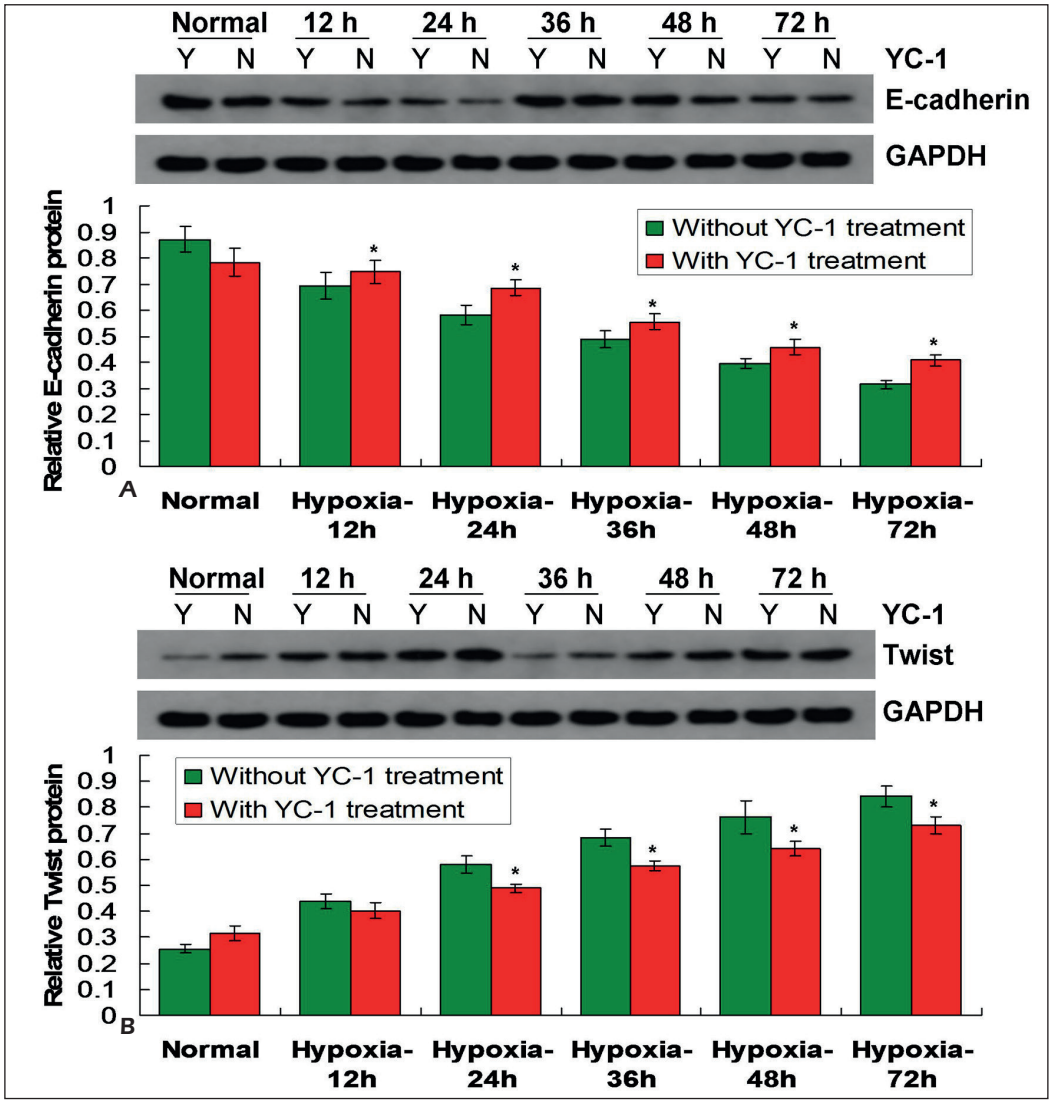

Figure 6. Investigation for the E-cadherin and Twist expression in BGC-823 cells treating with YC-1 treatment by using Western blot assay. **A**, Western blot assay and statistical analysis for E-cadherin expression. **B**, Western blot assay and statistical analysis for Twist expression. *$p<0.05$ *vs.* Normal group. The black arrows represent the change tendency of migration or invasion.

nificantly increased Twist and HIF-1α expression compared to that of Normal group. The HIF-1α inhibitor, YC-1, significantly suppressed the effects of hypoxia. Therefore, the hypoxia promotes the migration and invasion of gastric cancer cell BGC-823 by activating the HIF-1α and inhibiting the NDRG2 associated signaling pathway.

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Conflict of Interests:

The Authors declare that they have no conflict of interests.

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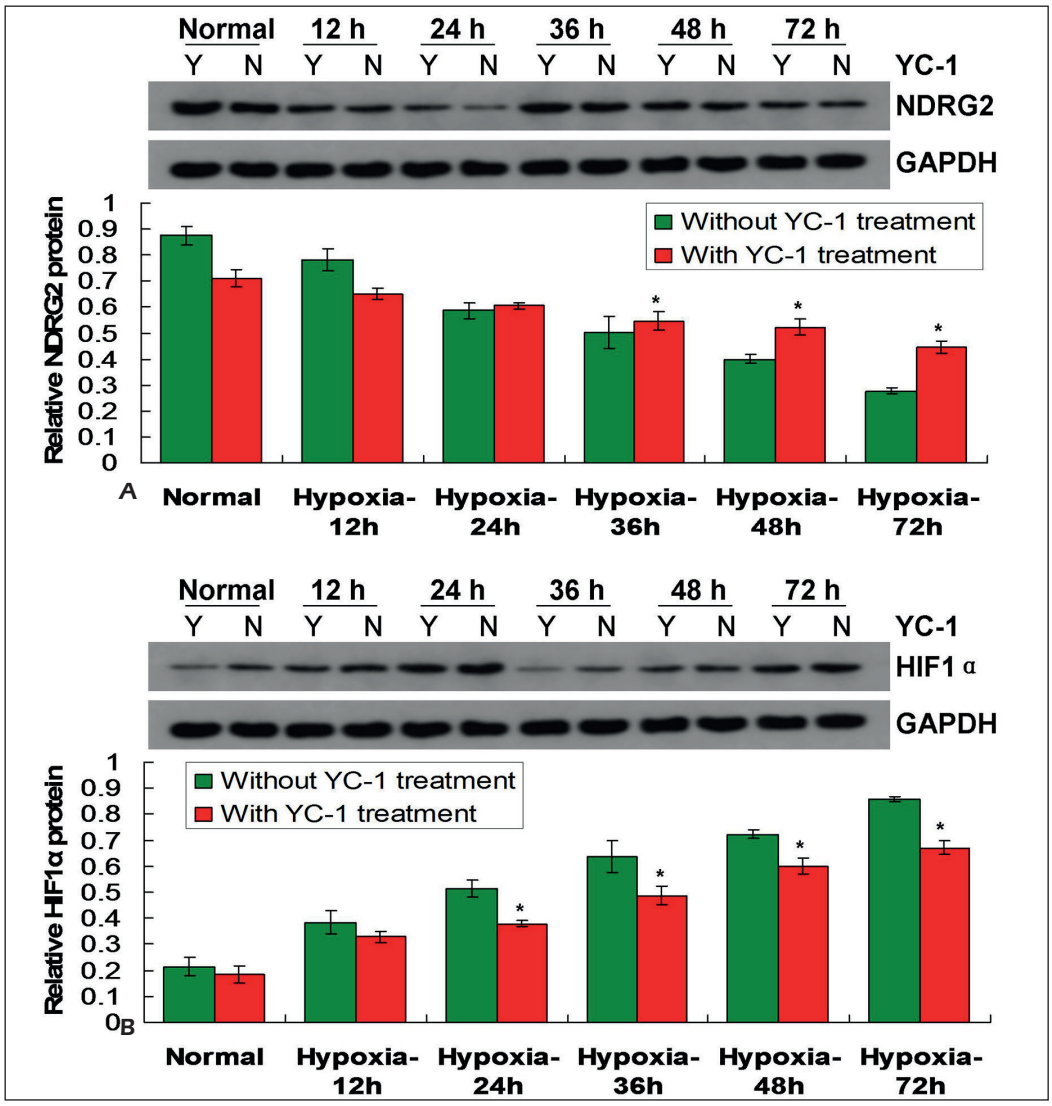

Figure 7. Examination for NDRG2 and HIF-1α expression in BGC-823 cells treating with YC-1 treatment by using Western blot assay. **A**, Western blot assay and statistical analysis for NDRG2 expression. **B**, Western blot assay and statistical analysis for HIF-1α expression. *p<0.05 vs. Normal group. The black arrows represent the change tendency of migration or invasion.

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