Hypoxia facilitates epithelial-mesenchymal transition-mediated rectal cancer progress

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Received July 1, 2016
Accepted September 1, 2016
Published December 2, 2016
DOI http://dx.doi.org/10.4238/gmr15048936

ABSTRACT. Rectal cancer is a commonly observed tumor in clinics, and epithelial-mesenchymal transition (EMT) is very important for tumor invasion and metastasis. We established a rectal cancer HCT-116 cell hypoxia model and detected cell proliferation, invasion, and EMT-related protein expression in this model, aiming to analyze the effect of hypoxia on rectal cancer cell EMT. Rectal cancer cell line HCT-116 was cultured in normoxic, hypoxic, or anaerobic environment, and hypoxia-inducible factor-1α (HIF-1α) mRNA expression was detected in the cells by real-time PCR. Cell proliferation was tested by MTT assay; cell invasion was determined by transwell assay, and HIF-1α, epithelial-cadherin, and Snail protein levels were evaluated by western blot analysis. HIF-1α mRNA level significantly increased in the anaerobic group compared to that in the normoxic and hypoxic groups (P < 0.05). HCT-116 cell proliferation in the anaerobic group was obviously higher than that in the other two groups, with the hypoxic group showing stronger proliferative ability than the normoxic group (P < 0.05). Compared to the normoxic group, the HCT-116 cells demonstrated
enhanced cell invasion and migration in hypoxic and anaerobic groups. HIF-1α and Snail expressions were upregulated, whereas epithelial-cadherin expression had declined in the hypoxic and anaerobic groups, compared to those in the normal control (P < 0.05). Therefore, hypoxia promoted rectal cancer cell progress by increasing HIF-1α to induce EMT.

**Key words:** Hypoxia; HCT-116; HIF-1α; E-cadherin; Snail

**INTRODUCTION**

Rectal cancer is a common digestive malignant tumor observed in clinics, with a high mortality rate. Its poor prognosis is closely related to tumor invasion and metastasis, whereas invasion and metastasis are based on epithelial-mesenchymal transition (EMT) (Roy and Majumdar, 2012; Wang et al., 2013). EMT refers to the transformation of epithelium-derived cells to mesenchymal cells (Zhao et al., 2011). Epithelial-cadherin (E-cadherin) and Snail are directly or indirectly involved in EMT (Li et al., 2012).

Tumor microenvironment, mainly composed of different types of cells, extracellular matrix, signaling molecules, acid, hypoxia, and other factors, has been extensively studied in recent years (Fan et al., 2014; Xiong et al., 2015). It was pointed out that in the tumor microenvironment, cytokines, hypoxia, and signaling pathways may regulate the process of rectal cancer EMT (Thiery et al., 2009; Hongo et al., 2013).

Hypoxia-inducible factor-1α (HIF-1α) is overexpressed in the anoxic environment. It can bind to the hypoxia response element and activate the downstream target genes to promote tumor invasion and metastasis. This process is completed by regulating hypoxia signal and mediating hypoxia effects (Pez et al., 2011; Woo et al., 2011). Therefore, this study established a rectal cancer HCT-116 cell hypoxia model to analyze the effect of hypoxia on rectal cancer cell EMT.

**MATERIAL AND METHODS**

**Materials**

**Cells and reagents**

Rectal cancer cell line HCT-116 was obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, China. Mouse anti-human HIF-1α, E-cadherin, and Snail polyclonal antibodies and alkaline phosphatase-labeled goat anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). PCR primers were acquired from Boya Biotechnology Engineering Company (Shanghai, China). Transwell chamber was obtained from BD Company (San Jose, CA, USA).

**Instruments and equipment**

Cell incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Inverted microscope was obtained from Nikon (Minato-ku, Tokyo, Japan). PCR thermocycler was acquired from Biometra (Göttingen, Germany). Centrifuge was obtained from Beckman (Danvers, MA, USA). Refrigerator was purchased from SANYO (Moriguchi, Osaka, Japan).
Experimental methods

Cell culture

Rectal cancer HCT-116 cells were maintained in RPMI-1640 medium and were incubated at 37°C in a 5% CO₂ environment.

Hypoxia cultivation

The cells were maintained in RPMI-1640 medium at 1% O₂, 37°C, and 5% CO₂ for 6 h.

Anaerobic cultivation

CoCl₂ was added to the medium to induce anaerobic environment. Cells were cultured in RPMI-1640 containing 600 µM CoCl₂ for 6 h.

Real-time PCR

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Germantown, MD, USA), following manufacturer protocols. D₂₆₀ nm/D₂₈₀ nm ratio was calculated to evaluate the purity of extracted RNA. Total 200 ng RNA was used to obtain cDNA, after synthesizing the poly(A) tail. The primer sequences used in the experiments are listed in Table 1. PCR procedure included denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase was selected as the internal reference. The relative expression level was calculated by the 2⁻ΔΔCt method.

Table 1. Primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Annealing temperature (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>5'-CACCATGAGGCTACACTGTTCC-3'</td>
<td>60</td>
<td>561</td>
</tr>
<tr>
<td></td>
<td>5'-TTAACCATTGGCGACAGCGG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GACAGGTCATCACTGACACTTGGG-3'</td>
<td>60</td>
<td>314</td>
</tr>
<tr>
<td></td>
<td>5'-GACTGCTTCAACACTTGTGATTC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HIF-1α, hypoxia-inducible factor-1α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

MTT assay

HCT-116 cells in logarithmic phase were treated with 20 µL 5 mg/mL 3'-(4',5'-dimethylthiazol-2-yl)-2',5'-diphenyltetrazolium bromide. After 4-h incubation, the cells were treated with 150 µL dimethyl sulfoxide, and the plate was read at 570 nm wavelength on a Microplate Reader (BioTek, Winooski, VT, USA).

Transwell assays

Invasion test: Matrigel was added to the transwell chamber at 4°C overnight. After the membrane of the transwell chamber was treated with serum-free medium at 37°C for 1 h, HCT-116 cells were seeded in the upper chamber, whereas RPMI-1640 medium was added to the lower chamber. Following this, the membrane was stained with Giemsa and was observed under the microscope.

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Migration test: The cells were seeded in the upper chamber of the transwell chamber, with treated membrane, without Matrigel. RPMI-1640 medium was added to the lower chamber. Following this, the membrane was stained with Giemsa and was observed under the microscope.

**Western blot analysis**

Total protein was extracted and separated by 8% SDS-PAGE. After transferring and blocking at room temperature for 1 h, the PVDF membrane was treated with 1:1200 HIF-1α, E-cadherin, and Snail polyclonal antibodies at 4°C overnight. Next, the goat anti-rabbit secondary antibody was added to the membrane at room temperature for 1 h. Finally, the membrane was developed by enhanced chemiluminescence.

**Statistical analysis**

All data analysis was performed using the SPSS17.0 software (IBM Corporation, Armonk, NY, USA). All experiments were performed in triplicates. The data are reported as means ± standard deviation. The chi-square test and the *t*-test were used for comparison between groups. P < 0.05 was considered statistically significance.

**RESULTS**

**HIF-1α mRNA expression in HCT-116 cells after hypoxia or anaerobic treatment**

Real-time PCR was performed to test HIF-1α mRNA expression in HCT-116 cells cultured under normoxic, hypoxic, or anaerobic condition. HIF-1α mRNA level significantly increased in the anaerobic group, compared to that in the normoxic and hypoxic groups (P < 0.05; Figure 1).

![Figure 1. Hypoxia-inducible factor-1α (HIF-1α) mRNA expression in HCT-116 cells after hypoxia or anaerobic treatment. *P < 0.05, compared to the normoxic group. # P < 0.05, compared to the hypoxic group.](image)

**HCT-116 cell proliferation after hypoxia or anaerobic treatment**

HCT-116 cell viability after hypoxia or anaerobic treatment was detected. HCT-116 cells proliferated with time. HCT-116 cell proliferation in the anaerobic group was obviously
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higher than that in the other two groups, with hypoxic group showing stronger proliferative ability than the normoxic group (P < 0.05; Figure 2).

**Figure 2.** HCT-116 cell proliferation after hypoxia or anaerobic treatment.

**HCT-116 cell invasion and migration after hypoxia or anaerobic treatment**

Transwell assay was performed to determine HCT-116 cell invasion and migration after hypoxia or anaerobic treatment. Compared to the normoxic group, HCT-116 cells demonstrated enhanced cell invasion and migration in the hypoxic and anaerobic groups (P < 0.05). Anaerobic group presented obviously higher cell invasion and migration than the hypoxic group (P < 0.05; Figure 3).

**Figure 3.** HCT-116 cell invasion and migration after hypoxia or anaerobic treatment (400X). A. Normoxic group invasion; B. normoxic group migration; C. hypoxic group invasion; D. hypoxic group migration; E. anaerobic group invasion; F. anaerobic group migration.

**HIF-1α, E-cadherin, and Snail protein expressions after hypoxia or anaerobic treatment**

HIF-1α, E-cadherin, and Snail protein expressions were evaluated after hypoxia or anaerobic treatment. HIF-1α and Snail expressions were upregulated, whereas E-cadherin level had declined in hypoxic and anaerobic groups compared to those in the normal control (P < 0.05; Figures 4 and 5).
DISCUSSION

Recurrence and metastasis are the main causes of death in rectal cancer patients, leading to excess oxygen consumption and oxygen undersupply (Trisciuglio et al., 2010). It has been shown that hypoxic microenvironment may induce tumor cell apoptosis and suppress tumor growth and proliferation through a series of signaling pathways (Spivak-Kroizman et al., 2013). Tissue hypoxia can also elevate the malignancy of tumor cells, especially by inducing EMT (Savagner, 2010). In this study, we cultured rectal cancer HCT-116 cells under anaerobic, normoxic, and hypoxic conditions and tested cell proliferation, invasion, and EMT-related protein expressions, to analyze the effect of hypoxia on rectal cancer cell EMT and its related mechanisms.

Real-time PCR was performed to detect HIF-1α mRNA expression in HCT-116 cells, cultured under normoxic, hypoxic, or anaerobic condition. It was found that HIF-1α
mRNA level significantly increased in anaerobic and hypoxic groups, compared to that in the normoxic group. Research has demonstrated that HIF-1α expression level is closely related to the oxygen level. Sufficient oxygen supply maintains HIF-1α at low levels, whereas severe hypoxia may induce HIF-1α overexpression, promoting tumor cell response to hypoxic state (Copple, 2010; Wu et al., 2014).

While studying the effect of hypoxia on rectal cancer HCT-116 cell behavior, we found that HCT-116 cells proliferated with time. HCT-116 cell proliferation in the anaerobic group was obviously higher than that in the other two groups, with the hypoxic group presenting stronger proliferative ability than the normoxic group. This suggested that hypoxia could increase the proliferation of HCT-116 cells.

This study further investigated the influence of hypoxia on HCT-116 cell invasion and migration. Compared to the normoxic group, HCT-116 cells demonstrated enhanced cell invasion and migration in the hypoxic and anaerobic groups. Anaerobic group presented obviously higher cell invasion and migration than the hypoxic group. This indicated that hypoxic conditions might enhance HCT-116 cell invasion and migration, as their invasion and migration obviously increased following aggravation of hypoxia.

It has been observed that most malignant tumor cells undergo EMT, which is the basic requirement for tumor cell invasion of surrounding tissues and for metastasis (Gulhati et al., 2011; Makrodouli et al., 2011). In addition, EMT is regulated by E-cadherin and Snail (Larriba et al., 2010). This study observed that HIF-1α and Snail expressions were upregulated, whereas E-cadherin level had declined in hypoxic and anaerobic groups compared to those in the normal control. E-cadherin is one the most important regulatory factors involved in the process of EMT (Qin et al., 2016). Tumor cells with lower E-cadherin expression present stronger migration and invasion abilities, suggesting that E-cadherin is correlated to tumor metastasis (Hwang et al., 2011). Snail can inhibit E-cadherin expression to induce EMT, thus promoting tumor invasion and metastasis (Galván et al., 2010; Zheng et al., 2015).

Under anaerobic conditions, rectal cancer HCT-116 cells showed HIF-1α level elevation, enhanced cell proliferation, downregulated E-cadherin expression, and increased Snail level, thus promoting EMT. However, more in-depth investigations are required to clarify the specific mechanism of EMT in rectal cancer hypoxia. Regulation of the hypoxic microenvironment may become the new research direction for clinical treatment of rectal cancer.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

REFERENCES


