Original Article

Specific silencing of hypoxia inducible factor-1α, hypoxia inducible factor-2α and hypoxia inducible factor-1β by small interference RNA reduces the expression of vascular endothelial growth factor in BGC-823 gastric cancer cells under hypoxia

Yanli Qu1*, Haifeng Wang2*, Ning Zhou1, Yong Tang1

1Department of Digestive System, 2Abdominal Radiotherapy, The Affiliated Tumor Hospital of Xinjiang Medical University, Urumqi, Xinjiang Uygur Autonomous Region, P. R. China. *Equal contributors.

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Abstract: Objectives: The present study investigates the effects of hypoxia-inducible factor (HIF)-1α, HIF-2α and HIF-1β on vascular endothelial growth factor (VEGF) expression in BGC-823 gastric cancer cells under hypoxia. Methods: Hypoxia model was established by CoCl2 method. qRT-PCR and Western blotting were used to determine the expression of VEGF, HIF-1α, HIF-2α, and HIF-1β. Small interference RNA (siRNA) was used to silence HIF-1α, HIF-2α and HIF-1β expression, and then VEGF expression was evaluated using qRT-PCR and Western blotting. Results: Expression of VEGF, HIF-1α, and HIF-2α under hypoxia was significantly increased compared to normoxia. Under hypoxia, HIF-1α expression was first up-regulated and then decreased. HIF-2α expression was slowly up-regulated in hypoxia, and then maintained stable. Expression of HIF-1β mRNA and protein did not change under normoxia or hypoxia. Transfection by siRNA effectively silenced HIF-1α and HIF-2α gene expression, and down-regulated VEGF expression. In addition, the reduction effect of HIF-2α was more prominent. Conclusion: The present study demonstrates that hypoxia results in varied levels of HIF-1α, HIF-2α and HIF-1β expression in gastric carcinoma BGC-823 cells. HIF-1α and HIF-2α have dominant effects in response to hypoxia in gastric cancer. Moreover, interference by RNA inhibits HIF gene expression, and reduces downstream VEGF level. Compared with HIF-1α and HIF-1β pathways, HIF-2α pathway may be more valuable in gastric cancer treatment.

Keywords: Gastric cancer, hypoxia-inducible factor, vascular endothelial growth factor, small interference RNA

Introduction

As one of typical solid tumors, gastric cancer has common characteristics of malignant solid tumors. The characteristics include rapid growth in a short period of time, ischemia and hypoxia in tumor tissues, decreased oxygen consumption rate of mitochondria in tumor cells and reduced production of ATP. In order to meet the needs of growth, tumors have a compensation mechanism for energy metabolism and angiogenesis to adapt to the microenvironment of hypoxia [1]. Hypoxia-inducible factors (HIF) have key effects in gastric cancer compensation mechanism in adaptation to hypoxia, including HIF-1α, HIF-2α and HIF-1β. As a constitutive expression protein, HIF-1β is not sensitive to hypoxia, but can play its regulatory role by binding with hypoxia response factor element (HRE) of its target gene after forming a dimer with HIF-1α or HIF-2α. HIF-1α and HIF-2α have the same β subunit but different α subunit, leading to different functions [2]. Studies show that HIF-1α regulates the angiogenesis, energy metabolism, invasion and metastasis of gastric cancer [3-5], while HIF-2α plays important roles in small-cell lung cancer, colon cancer, cervical carcinoma, liver cancer, and esophageal cancer [6, 7]. Vascular endothelial growth factor (VEGF) gene is closely related with tumor angiogenesis, and encodes VEGF protein that promotes neovascularization and
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vasoperme ability [8, 9]. Studies show that abnormal expression of HIF-1α or HIF-2α increases VEGF transcription [10, 11]. HIF-1α inhibits the activities of IL-8 and c-Myc, and enhances the proliferation of vascular endothelial cells, vascular sprouting and the number and diameter of tumor cells. HIF-2α mainly regulates DII4, ADM1 and Ang2, and increases tumor angiogenesis and microvessel density by inhibiting vascular endothelial cell migration and vascular sprouting [12]. RNA interference is a new technique that specifically shuts down the expression of specific genes, and is widely used in researches on therapies for malignant tumors [11]. In this study, we determine the levels of HIF-1α, HIF-2α, HIF-1β and VEGF in BGC-823 gastric carcinoma cells, and try to understand their regulatory effects on the angiogenesis in gastric cancer.

Materials and methods

Cells

Gastric cancer BGC-823 cells (Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultivated in RPMI-1640 media containing 10% fetal bovine serum (FBS) under 37°C and 5% CO₂. The medium was refreshed every other day. When reaching 80-90% confluency, cells in log-phase growth were used for experiments. In preliminary experiments, 150, 200, 250 and 275 μmol/L CoCl₂ were used to treat BGC-823 cells, and 200 μmol/L was chosen for subsequent experiments due to strongest effect and weakest adverse effect. BGC-823 cells were divided into normoxia group and different hypoxia groups (2, 4, 8, 12, 24, 48 and 72 h), and seeded onto 6-well plates at a density of 4×10⁵ cells/well. After incubation for 24 h, the medium of the seven hypoxia groups of cells was replaced by normal medium supplemented with 200 μmol/L CoCl₂, and the cells were cultured for another 12 h.

To silence HIF-1α, HIF-2α and HIF-1β, small interference RNA (siRNA) of the three genes (SC-35561, SC-353316 and SC-8076, respectively; Santa Cruz Biotechnology, Dallas, TX, USA) were used to transfect BGC-823 cells. In addition, normoxia group, blank (untransfected) group and negative control group (control siRNA) were also included. For the transfection of BGC-823 cells in negative control groups and siRNA groups, the cells were cultivated in 6-well plates (4×10⁵ cells/well) at 37°C and 5% CO₂. When the cells reached 70-80% confluency, the medium was discarded and washed with phosphate-buffered saline (PBS) for three times before incubation for 6 h. The siRNA plasmids (6 μg) were added to 2.5 ml RPMI-1640 medium in vial A before gentle mixing. Lipofectamine 2000 (10 μl) was added to 250 μl RPMI-1640 medium in vial B before incubation at room temperature for 15 minutes. After standing still at room temperature (five minutes), the two were combined, followed by incubation (room temperature; 20 min). Subsequently, the mixture (500 μl) was added into each well. After incubation at 37°C and 5% CO₂ for 48 h, the normoxia group was changed to normal medium, and the medium of the cells in negative control groups and siRNA groups were changed to normal medium supplemented with 200 μmol/L CoCl₂ and cultured for another 12 h.

Quantitative real time polymerase chain reaction (qRT-PCR)

BGC-823 cells (2×10⁵) were thoroughly mixed with 1 ml Trizol for lysis. Then, extraction of total RNA was performed by using phenol chloroform method. To detect the purity, an ultraviolet spectrophotometry (Thermo Scientific, Waltham, MA, USA) was used to measure the ratio of absorbance at 260 nm over 280 nm. Then, cDNA was reverse-transcribed from RNA using Reverse Transcription System (Takara, Dalian, China). The cDNA was stored at -20°C.

SYBR Green quantitative real-time PCR kit (Kapa Biosystems, Wilmington, MA, USA) was used to detect mRNA expression, using GAPDH as internal reference. The reaction system (20 μl) was composed of 4 μl Master Mix, 3.2 μl RNA and 12.8 μl ddH₂O. The primers for HIF-1α were 5’-TGCAACATGGAAGGTATTGC-3' (upstream) and 5’-TTCACAAATCAGCACCAAGC-3' (downstream); the primers for HIF-2α were 5’-TGGTAGCCCTCCTGACCAAGGTATTGC-3’ (upstream) and 5’-TTCAAAATCAGGACCAAGGACAC-3’ (downstream); the primers for HIF-1β were 5’-TGCACTGGAAGGTATTGC-3’ (upstream) and 5’-CTTCCAGTCCAGCAGGGAC-3’ (downstream); the primers for VEGF were 5’-TGTTAGCCCTCCTGACCAAGGTATTGC-3’ (upstream) and 5’-GGACCAAGGACCAAGGACAC-3’ (downstream); the primers for GAPDH were 5’-GAGGACCAAGGACCAAGGACAC-3’ (upstream) and 5’-GGACCAAGGACCAAGGACAC-3’ (downstream).
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5'-GAA GGC TGG GGC TCA TTT-3' (downstream) (Thermo Fisher Scientific, Waltham, MA, USA). PCR condition was: initial denaturation at 95°C for 2 minutes; and then denaturation at 95°C for 15 seconds, annealing at 60°C for 15 s and elongation at 72°C for 1 minutes (40 cycles) (iQ5; Bio-Rad, Hercules, CA, USA). Then, relative expression of target mRNA was determined by 2\(^{-\Delta\Delta C_t}\) method, and expressed as a ratio against GAPDH. All tests were repeated for 3 times.

Western blotting

Cells were cultivated in 6-well plates (1×10\(^6\)/well). Two days after transfection, the cells were collected and mixed with RIPA buffer (100 μl) containing 1 mM phenylmethylsulfonyl fluoride for lysis of 15 min at 4°C. After centrifugation at 12,000 g/min and under 4°C for 5 min, protein concentration in supernatant was determined by BCA kit (RTP7102, Real-Times Biotechnology, Beijing, China). After that, protein (50 μg) was mixed with 2× sodium dodecyl sulfate buffer and denaturized under 100°C for 5 min. Afterwards, 10 μl sample was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel for an electrophoresis at 100 V. Then, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes on ice under 250 mA for 90 min for HIF-1α, HIF-2α or HIF-1β and 45 min for VEGF. After blocking the samples with 5 g/L skimmed milk (25°C; 90 min). Then, rabbit anti-human polyclonal HIF-1α (1:200), HIF-2α (1:100), HIF-1β (1:200) and GAPDH (1:1,000) primary antibodies (Abcam, Cambridge, UK) were used to incubate the membranes at 4°C overnight. After being washed thoroughly with PBST for 3 times (each 15 min), goat anti-rabbit polyclonal horseradish peroxidase-conjugated secondary antibody (1:2,000; Abcam, Cambridge, UK) was used to incubate the membranes for 1 h at room temperature. Then, the membranes were washed with PBST for another three times (15 min). Then, enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used for imaging. To acquire and analyze imaging signals, Image lab v3.0 (Bio-Rad, Hercules,
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CA, USA) was used. The expression of target proteins was calculated relative to the greyscale of GAPDH (n = 3).

Statistical analysis

All data analyses were carried out using SPSS 22.0 statistical software (IBM, Armonk, NY, USA). The results were expressed as means ± standard deviation (SD). The t-test or Wilcoxon rank sum test was used to compare differences between paired groups. In case of homogeneity of variance, analysis of variance was used; in case of heterogeneity of variance, Games-Howell method was used. P < 0.05 indicated statistically significant differences.

Results

Hypoxia for 8 h decreases HIF-1α mRNA expression, but enhances HIF-2α and VEGF mRNA expression in a time-dependent manner

To measure mRNA expression, qRT-PCR was used. The expression of HIF-1α mRNA at 2, 4, and 8 h after hypoxia were not significantly different from that in normoxia group (P > 0.05), but those at 12, 24, 48 and 72 h after hypoxia were significantly lower than that in normoxia group (P < 0.05) (Figure 1A). HIF-2α mRNA levels at 2 and 4 h after hypoxia were not significantly different from that in normoxia group (P > 0.05), while those at 8, 12, 24, 48 and 72 h after hypoxia were significantly higher than that in normoxia group (P < 0.05) (Figure 1B). In addition, HIF-1β mRNA levels at 2, 4, 8, 12, 24, 48 and 72 h were not significantly different from that in normoxia group (P > 0.05) (Figure 1C). Interestingly, the mRNA levels of VEGF at 2, 4 and 8 h after hypoxia were not significantly different from that in normoxia group (P > 0.05), while those at 12, 24, 48 and 72 h after hypoxia were significantly higher than that in normoxia group (P < 0.05) (Figure 1D). The data suggest that HIF-1α mRNA expression is decreased, but HIF-2α and VEGF mRNA expression is increased after hypoxia for 8 h, showing a time-dependent manner.

Expression of HIF-1α, HIF-2α and VEGF proteins are enhanced in hypoxia, but the expression of HIF-1β protein is not

To determine HIF-1α, HIF-2α, HIF-1β and VEGF protein expression, Western blotting was car-
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Figure 3. Expression of HIF-1α, HIF-2α, HIF-1β and VEGF proteins in BGC-823 cells at 12 h after hypoxia. Western blotting was employed to measure protein expression. *, P < 0.05 compared with normoxia group.

ried out. These results showed that HIF-1α protein expression in normoxia group was very low, but it was increased at 2, 4 and 8 h after hypoxia, reaching a peak at 4 h (P < 0.05). However, HIF-1α protein expression at 12, 24, 48 and 72 h was significantly decreased to a level that is not significantly different from that in normoxia group (P > 0.05) (Figure 2A). HIF-2α protein expression in normoxia group was also very low, but it was significantly increased at 4, 8, 12, 24, 48 and 72 h after hypoxia, reaching a plateau after 24 h (P < 0.05 for all) (Figure 2B). HIF-1β protein expression at 2, 4, 8, 12, 24, 48 and 72 h after hypoxia was significantly different from that in normoxia group (P > 0.05) (Figure 2C). Moreover, VEGF protein expression at 2, 4, 8, 12, 24, 48 and 72 h after hypoxia was significantly increased than that in normoxia group in a time-dependent manner (P < 0.05 for all) (Figure 2D). In the following experiments, we chose cells under hypoxia for 12 h for the determination of HIF-1α, HIF-2α, HIF-1β and VEGF expression. The data showed that HIF-1α, HIF-2α and VEGF protein expression at 12 h after hypoxia were significantly higher than that in normoxia group (P < 0.05), but HIF-1β protein expression at 12 h after hypoxia was not significantly different from that in normoxia group (P > 0.05) (Figure 3). The data indicate that expression of HIF-1α, HIF-2α and VEGF proteins is enhanced by hypoxia, but that of HIF-1β protein is not.

Silencing of HIF-1α, HIF-2α or HIF-1β mRNA expression significantly decreases the expression of VEGF mRNA in BGC-823 cells

To test the effect of the siRNA of HIF-1α, HIF-2α and HIF-1β on the mRNA expression of VEGF, qRT-PCR was employed. The results demonstrated that the levels of HIF-1α mRNA in blank group and negative control group were not significantly different from each other (P > 0.05). In addition, the level of HIF-1α mRNA in cells treated with siRNA of HIF-1α was significantly reduced than that in blank group (P < 0.05). Similarly, the levels of VEGF mRNA in blank group and negative control group were not significantly different from each other (P > 0.05). Moreover, the level of VEGF mRNA in cells treated with siRNA of HIF-1α was significantly reduced than that in blank group (P < 0.05) (Figure 4A). Transfection with the siRNA of HIF-2α had similar effect on HIF-2α mRNA and VEGF mRNA expression compared with transfection with siRNA of HIF-1α (Figure 4B). In addition, HIF-1β mRNA levels in blank group and negative control group were not significantly different from each other (P > 0.05). The level of HIF-1β mRNA in cells treated with siRNA of HIF-1β was significantly reduced than that in blank group (P < 0.05). Moreover, the levels of VEGF mRNA in blank group and negative control group were not significantly different from each other (P > 0.05). The level of VEGF mRNA in cells treated with siRNA of HIF-1β was signifi-
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**Figure 4.** Effect of silenced expression of HIF-1α, HIF-2α and HIF-1β mRNA on the expression of VEGF mRNA under hypoxia. A. HIF-1α and VEGF mRNA expression in BGC-823 cells under normoxia condition (A1), cells transfected with siRNA of HIF-1α (B1), untransfected (blank) cells (C1) or cells transfected with negative control (D1). B. HIF-2α and VEGF mRNA expression in BGC-823 cells under normoxia condition (A2), cells transfected with siRNA of HIF-2α (B2), untransfected (blank) cells (C2) or cells transfected with negative control (D2). C. HIF-1β and VEGF mRNA expression in BGC-823 cells under normoxia condition (A3), cells transfected with siRNA of HIF-1β (B3), untransfected (blank) cells (C3) or cells transfected with negative control (D3). D. VEGF mRNA expression in untransfected (blank) BGC-823 cells or cells transfected with siRNA of HIF-1α (B1), HIF-2α (B2) or HIF-1β (B3). qRT-PCR was used to determine mRNA expression. At 48 h after transfection, the medium of the cells in negative control groups and siRNA groups were changed to normal medium supplemented with 200 μmol/L CoCl₂ and cultured for another 12 h. *, P < 0.05.

Significantly reduced than that in blank group (P < 0.05) (Figure 4C). Overall, VEGF mRNA expression in cells transfected with HIF-1α or HIF-1β siRNAs was significantly higher than that in cells transfected with HIF-2α siRNA (P < 0.05) (Figure 4D). These data suggest that silencing of HIF-1α, HIF-2α or HIF-1β mRNA expression significantly decreases the expression of VEGF mRNA in BGC-823 cells.

**Silencing of HIF-1α, HIF-2α or HIF-1β protein expression significantly decreases the expression of VEGF protein in BGC-823 cells**

To test the effect of the siRNA of HIF-1α, HIF-2α and HIF-1β on protein expression of VEGF, Western blotting was carried out. The levels of HIF-1α protein in blank group and negative control group were not significantly different from each other (P > 0.05). The level of HIF-1α protein in cells treated with siRNA of HIF-1α was significantly reduced than that in blank group (P < 0.05). The levels of VEGF protein in blank group and negative control group were not significantly different from each other (P > 0.05). Moreover, the level of VEGF protein in cells treated with siRNA of HIF-1α was significantly reduced than those in blank group (P < 0.05) (Figure 5A). Of note, transfection with the siRNA of HIF-2α had similar effect on the expression of HIF-2α protein and VEGF protein compared with transfection with siRNA of HIF-1α (Figure 5B). In addition, levels of HIF-1β protein in blank group and negative control group were not significantly different from each other (P > 0.05). The level of HIF-1β protein in cells treated with
siRNA of HIF-1β was significantly reduced than those in blank group (P < 0.05). The levels of VEGF protein in blank group and negative control group were not significantly different from...
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each other (P > 0.05). The level of VEGF protein in cells treated with HIF-1β siRNA was significantly reduced than that in blank group (P < 0.05) (Figure 5C). In all, VEGF protein expression in cells transfected with HIF-1α or HIF-1β siRNA was significantly higher than that in cells transfected with HIF-2α siRNA (P < 0.05) (Figure 5D). These results indicate that silencing of HIF-1α, HIF-2α or HIF-1β protein expression significantly reduces the expression of VEGF protein in BGC-823 cells.

Discussion

An important step in the growth of tumor cells is the adaptation to the hypoxic microenvironment, which generates and activates a large number of HIF that is one of the manifestations of adaptation to hypoxia. HIF is a kind of heterodimeric gene transcription factor with regulatory capacities for multiple genes. HIF can be divided into HIF-α and HIF-β according to different subunits. HIF-α includes HIF-1α, HIF-2α and HIF-3α subtypes, among which HIF-1α and HIF-2α are the best studied [13]. HIF-1α and HIF-2α are oxygen-regulated molecules and become accumulated because of inhibited protein degradation under hypoxia; HIF-1β exists in the nucleus and is less affected by oxygen concentration [14]. HIF-1α or HIF-2α needs to form a heterodimer with HIF-1β to bind the hypoxia response factor element (HRE) of target genes. HIF-1α and HIF-2α are located in chromosomes 14q21-q24 and 2p16-p21, respectively, and both contain a basic helix loop helix domain, a PAS domain, an ODD domain and a TAD (C-TAD and N-TAD) domain. Of note, HIF-1α and HIF-2α have 88% basic helix loop helix domain in common, 78% PAS domain in common, 100% C-TAD domain in common, and 0% N-TAD domain in common [15]. Structural similarity and difference between HIF-1α and HIF-2α result in similar and distinct regulatory capabilities between the two [16].

VEGF gene is an important gene involved in many kinds of tumor angiogenesis. Glycoprotein VEGF encoded by VEGF gene promotes angiogenesis in tumor bodies and improves their permeability [17]. It is reported that abnormal expression of HIF-1α and HIF-2α increases the transcription of VEGF. HIF-1α mainly inhibits the activities of IL-8 and c-Myc, enhances proliferation of vascular endothelial cells, promotes neovascularization budding, and increases number and diameter of tumor cells; HIF-2α mainly regulates Dll4, ADM1 and Ang2, inhibits the migration of vascular endothelial cells, suppresses neovascularization budding, promotes intratumoral vascularization, and enhances microvessel density. In contrast to HIF-2α, HIF-1α has more prominent regulatory effects on VEGF under hypoxia [18]. By now, the molecular mechanism for the activation of HIF-1α is still debatable. Most researchers believe that HIF-1α is an acute HIF that is regulated by low oxygen at protein level or transcriptional level. The results of the present study show that the expression of HIF-1α mRNA and protein in BGC-823 cells begins to increase at 4 h after hypoxia, and is decreased afterwards, suggesting that hypoxia regulates the expression of HIF-1α at both transcriptional level and protein level. This is not consistent with most other researchers [19]. By contrast, HIF-2α mRNA and protein expression under hypoxia is elevated with time, being consistent with a previous study in which HIF-2α acts as an inducing factor mediating cell adaptation to chronic hypoxia [20]. In contrast to HIF-1α and HIF-2α, HIF-1β expression is not varied under normoxia or hypoxia. This may be due to the fact that HIF-1β is a structural subunit of HIF and its intracellular expression is independent of oxygen concentration. Of note, α subunit is an activity-regulating subunit of HIF, and its degradation under hypoxia is inhibited, leading to nuclear translocation, as well as a series of responses to hypoxia. After hypoxia induction, VEGF mRNA and protein expression are both elevated, suggesting that hypoxia is a key factor that promotes VEGF production in gastric cancer cells and tumor neovascularity.

The mechanism of action of RNA interference (RNAi) by siRNAs base pairing, and siRNA only degrades mRNA molecules that match the sequence of siRNA. Therefore, siRNA has its unique advantage in specificity compared with treatment with drugs. After using RNA interference technique, HIF-1α, HIF-2α and HIF-1β mRNA and protein expression are inhibited. The inhibition rate is between 85% and 90%. Furthermore, VEGF mRNA and protein expression are also decreased as the inhibition of HIF-1α, HIF-2α and HIF-1β expression, with the effect of HIF-2α silencing being the strongest. HIF-1α, HIF-2α, HIF-1β and VEGF expression in negative control group are not significantly dif-
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different from that in blank group. Therefore, our data suggest that HIF-2α pathway has stronger effect on angiogenesis in gastric cancer under hypoxia than the other two HIF pathways. Angiogenesis in gastric cancer is controlled by a series of factors, and drugs that suppress angiogenesis can only partially inhibit angiogenesis and metastasis in gastric cancer. Therefore, exploring ways to block the targets of multiple vascular growth factors is more practical.

In conclusion, HIF-1α, HIF-2α and HIF-1β are similar and functionally different factors, although they can up-regulate the expression of VEGF in gastric cancer, promote angiogenesis, and create conditions for tumor growth and invasion. Compared with HIF-1α and HIF-1β, inhibition of HIF-2α pathway may be more effective in preventing angiogenesis in gastric cancer, which will provide a novel therapeutic target for the treatment of gastric cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Yong Tang, Department of Digestive System, The Affiliated Tumor Hospital of Xinjiang Medical University, No. 789 East Suzhou Road, Urumqi 830000, Xinjiang Uygur Autonomous Region, P. R. China. Tel: 86-13899880718; E-mail: quyanli73@163.com; ae717ty@163.com

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