

Original Article

Analysis of the correlation of the expression level of hypoxia-inducible factor-1 α with the glycosylation of oral squamous cell carcinoma

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Abstract: Objective: To investigate the correlation of the expression level of hypoxia-inducible factor-1 α (HIF-1 α) with the glycosylation of oral squamous cell carcinoma (OSCC). Methods: We conducted an immunohistochemical SP method to detect the expression levels of HIF-1 α and O-glycosylation-related proteins (O-linked N-acetylglucosamine [O-GlcNAc], O-GlcNAcase [OGA], and O-GlcNAc transferase [OGT]) in 30 cases of OSCC tissues that were surgically removed and confirmed by pathology in our hospital from January 2018 to July 2020. Meanwhile, the expression levels of O-GlcNAc, OGA, and OGT under the action of the HIF-1 α inhibitor PX-478 were detected by Western blotting in the human OSCC cell line (Tca8113 line). Results: ① The expression of HIF-1 α and O-glycosylation-related proteins in OSCC was reported at an increased level. ② The positive expression of HIF-1 α was associated with the age and tumor size of OSCC patients ($P < 0.05$); the positive expression of O-GlcNAc and OGT was related to the tumor size of OSCC patients ($P < 0.05$). ③ Expression of HIF-1 α , O-GlcNAc and OGT in OSCC tissues was positively correlated (ρ correlation coefficient = 0.550). ④ Under HIF-1 α inhibition, a statistically significant decrease occurred in the expression levels of O-GlcNAc and OGT at a dose of 25 μ M PX-478 ($P < 0.05$), but a statistically significant increase occurred in OGA ($P < 0.05$). ⑤ Under the action of PX-478, there was a statistically significant and gradual decrease in the OGT content over time ($P < 0.05$). Conclusions: The expression of HIF-1 α and O-glycosylation-related proteins increases in OSCC, and the expression level increases proportionally with tumor volume. Expression of HIF-1 α and O-GlcNAc and OGT was positively correlated. HIF-1 α inhibition by PX-478 led to decreased expression levels of O-GlcNAc and OGT but the increased expression level of OGA. PX-478 can affect Tca8113 glycosylation by reducing the expression level of OGT.

Keywords: Hypoxia-inducible factor-1 α , expression level, oral squamous cell carcinoma, O-glycosylation, correlation

Introduction

Oral squamous cell carcinoma (OSCC), a clinically common oral malignancy, has up to 260,000 new cases per year, including 120,000 deaths. The overall survival rate within five years is less than 50% [1]. Surgical treatment is currently still the first choice for OSCC. The main challenge is that a single surgical treatment or chemoradiotherapy is usually not ideal due to advanced or locally moderate-advanced disease. Thus, the search for an effective new target for treatment is the focus of current clinical studies.

Glycosylation is essential for post-translational modification forms of proteins, which involves many biological processes such as protein degradation and cellular immunity and is often involved in the development of tumors [2]. O-glycosylation modification refers to the connection of a single O-linked N-acetylglucosamine (O-GlcNAc) with O-glycosidic bonds on the oxygen atoms of the hydroxyl groups of protein threonine or serine. It has been shown that O-glycosylation modification participates in a series of complex cellular activities such as cellular response, gene transcription, and protein degradation in a mechanism relating to phos-

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Table 1. Statistics of main experimental reagents

| Experimental reagents | Manufacturer |
|-------------------------------------------|---------------------------------------------|
| SP immunohistochemical staining kit | Active Motif (USA) |
| DAB stain | Shanghai Beinuo Biology |
| Mouse O-GlcNAc monoclonal antibody | Thermo (USA) |
| Rabbit OGT polyclonal antibody | Abcam (USA) |
| Rabbit OGA monoclonal antibody | Abcam (USA) |
| Rabbit HIF-1 α monoclonal antibody | Thermo (USA) |
| Fetal bovine serum | Corning (USA) |
| DMEM medium | Beijing Jiehuibogao Biotechnology Co., Ltd. |
| Double-stranded siRNA | Invitrogen (USA) |
| PX-478 | Tuscon (USA) |

phorylation modification [3]. The addition and removal of O-GlcNAc modification groups are mainly accomplished by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) synergistically. Thanks to its importance in O-glycosylation function, it has become a hot topic in current researches about cancer metabolism [4]. However, there are few studies that have been conducted on the association between hypoxia-inducible factor-1 α (HIF-1 α) expression and OSCC glycosylation.

The literature on HIF-1 α , a transcription factor mainly involved in regulation of cellular hypoxia response [5], has highlighted that it can induce cells to undergo survival responses such as autophagy in a low-oxygen environment. Down-regulation of HIF-1 α has been found to promote A549 apoptosis in OSCC cells and inhibit cancer cell growth [6]. This study aimed to evaluate the correlation of the expression level of HIF-1 α with glycosylation of OSCC. The expression levels of HIF-1 α and O-glycosylation-related protein in SCC cancer tissues and O-glycosylation-related protein under HIF-1 α inhibition were detected by immunohistochemical SP method and Western blotting, respectively.

Materials and methods

Source of specimens

OSCC tissues were collected from 30 OSCC patients (23 males and 7 females aged 38-75 years with a median age of 58 years) who underwent surgical resection and were confirmed pathologically in our hospital between January 2018 and July 2020. The human OSCC cell line (Tca8113 line) was obtained from the Cell Resource Center of Shanghai Institutes for

Biological Sciences of Chinese Academy of Sciences. The collection of samples fully met the ethical requirements of medical research. Obtained informed consent from the patient and donated tissues voluntarily.

Main reagents

Statistics of main experimental reagents were shown in **Table 1**.

Methods

Tissue processing. Paraffin-embedded OSCC tissue samples were taken for serial sectioning (thickness: 4 μ m); the sections were placed on cation-resistant glass slides and sent to the oven for baking for 2 h at a temperature of 60°C and then removed and stored for future use [7].

Immunohistochemical SP method. The sections were removed and subjected to high-temperature and high-pressure antigen retrieval with citrate hydrochloride buffer at pH 6.0. After cooling to room temperature, rinse twice with double-distilled water every 1 minute, and rinse three times with phosphate-buffered saline (PBS) buffer every 2 minutes. After swinging off the PBS solution, the primary antibodies were added on the sections respectively, as follows: mouse O-GlcNAc monoclonal antibody, rabbit OGT polyclonal antibody, rabbit OGA monoclonal antibody, and rabbit HIF-1 α monoclonal antibody (except for the negative control, PBS solution was used instead of the primary antibody), and incubated for 2 h at 37°C followed by rinsing four times with PBS buffer every 5 minutes [8]. After swinging off the PBS solution, biotin-labeled secondary antibodies were added and incubated at 37°C for

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30 min and then rinsed three times with PBS buffer every 5 minutes. After swinging off the PBS solution, DAB color development, counter-staining, and differentiation were performed. Then dehydration, mounting, and microscopic examination were performed.

Cell culture. Tca8113 cell line was inoculated in DMEM medium containing 10% fetal bovine serum and cultured at a constant temperature of 37°C with 5% CO₂ and saturated humidity, and the culture medium was renewed every 1-2 days. Cells in the logarithmic phase were selected for testing, with three times each group [9].

siRNA transfection. Double-stranded siRNAs targeting human ATG1 and ATG12 genes were encapsulated within the liposome reagent RNAiMAX and injected into Tca8113 cells at 30 nM each [10]. T8113 cells were analyzed 48 hours after transfection, in which siRNA with scrambled sequences in the experiment was used as a control.

Western blotting. Transfected Tca8113 cells were collected, lysed with cell lysate at 4°C, and extracted for total protein, followed by incubation on ice for 2 h. Later, the cells were centrifuged at 1400 r/min for 10 min to collect their supernatant [11]. Protein quantification was performed with a BCA protein concentration assay kit, and 40 μ g of total protein samples were subjected to electrophoresis, separated with equal proteins, and transferred to membranes, blocked in 5% skimmed milk powder for 2 h, and then incubated overnight at 4°C with primary antibodies (Mouse O-GlcNAc monoclonal antibody, 1:500, Thermo, USA; Rabbit OGT polyclonal antibody, 1:500, Abcam, USA; Rabbit OGA monoclonal antibody, 1:500, Abcam, USA) [12]. After washing the membrane three times with PBS, it was incubated at room temperature for 2 h with secondary antibodies (HRP labeled goat anti-rabbit IgG, 1:3000, Abcam, USA). The second washing was followed by color development with ECL luminescence solution. The gray values of the target bands were analyzed with a gel image processing system. Gray value = target protein/ β -actin [13].

Result determination

By referring to the American ASCO/CAP scoring system (2007), the immunohistochemical

results were analyzed using Image-proplus6.0 software. The presence of brownish-yellow granules in the nucleus and cytoplasm of cells stained with immunohistochemical antibodies against HIF-1 α , O-GlcNAc, OGA, and OGT was used as a positive marker. When positive cell counts were \geq 10% judged as positive; when positive cell counts were $<$ 10% judged as negative.

Statistical analyses

For statistical analysis, GraphPad Prism (GraphPad Software, version 7.0) and SPSS (IBM SPSS Statistics 22, version 7.0) were used. The measurement data were represented as ($\bar{x} \pm s$), and t-test was performed. The enumeration data were represented as [n (%)], and χ^2 test was performed. The correlation was analyzed by the exact probability method of paired design fourfold table data. $P < 0.05$ was deemed as a statistically significant difference.

Results

Expression of HIF-1 α and O-glycosylation related proteins in OSCC tissue

Among the 30 OSCC tissues, 23 (77%) were positive in HIF-1 α , and 27 (90%) in O-GlcNAc, OGT, and OGA. HIF- α was mainly expressed in the cytoplasm, nucleus of the tumor, and was enhanced around the necrotic tissue of the tumor and at the edge of tumor invasion. O-GlcNAc, OGT, and OGA were observed in the tumor epithelial parenchyma, and there was also a low positive expression in the tumor stroma and inflammatory cells (**Figure 1**).

Factors affecting the positive expression of HIF-1 α and O-glycosylation related proteins in OSCC tissues

HIF-1 α in OSCC tissues was associated with age and tumor size ($P < 0.05$); O-GlcNAc and OGT was related to tumor size ($P < 0.05$), and no significant correlation between OGA positivity and clinicopathological parameters was observed ($P > 0.05$) (**Table 2**).

Correlation of the positive expression of HIF-1 α and O-GlcNAc and OGT in OSCC tissues

22 of 30 OSCC tissues had consistent positive expression of HIF-1 α and O-GlcNAc and OGT.

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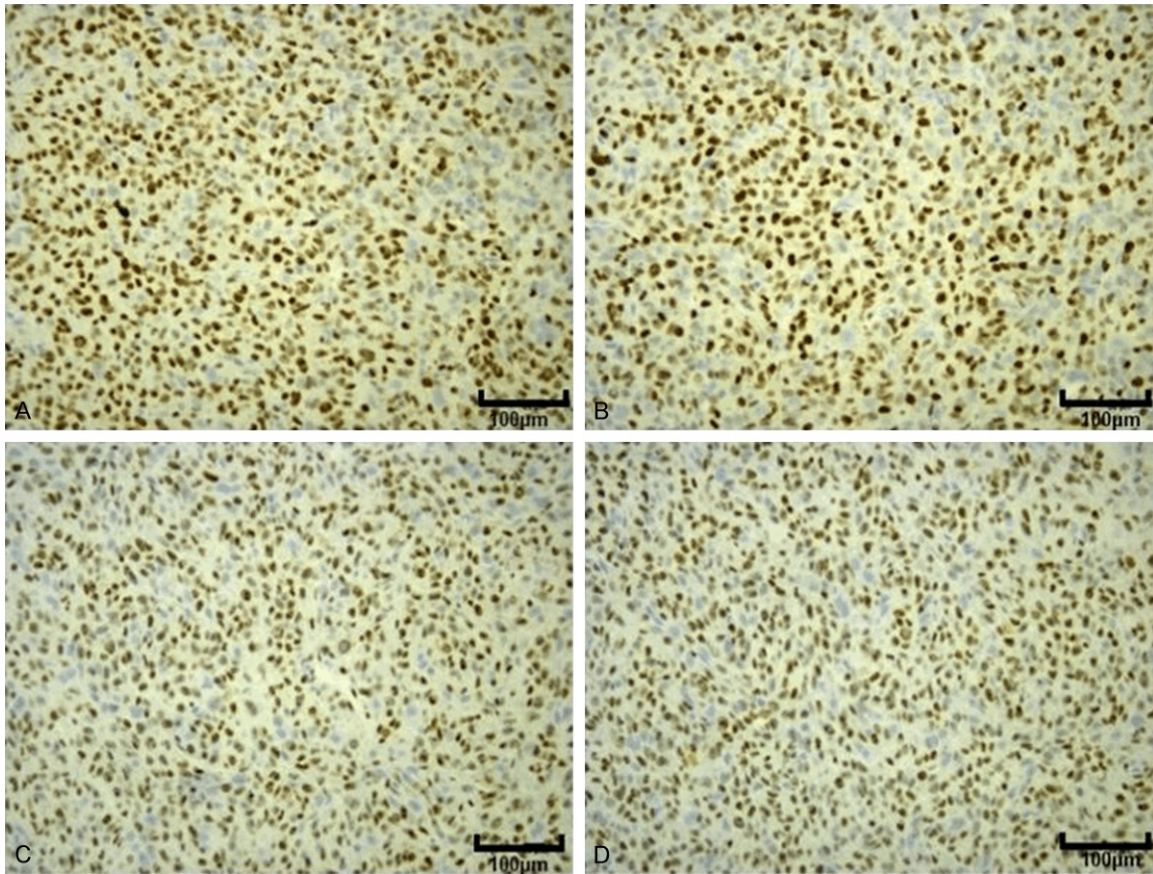


Figure 1. SP staining images of HIF-1 α and O-GlcNAc, OGT and OGA in OSCC tissues. Notes: (A) Positive expression of HIF-1 α in OSCC tissues (IHC 200 \times); (B) Positive expression of O-GlcNAc in OSCC tissues (IHC 200 \times); (C) Positive expression of OGT in OSCC tissues (IHC 200 \times); (D) Positive expression of OGA in OSCC tissues (IHC 200 \times).

Table 2. Correlation of the expression of HIF-1 α and O-GlcNAc, OGT and OGA with clinicopathological parameters

| | HIF-1 α expression | | | O-GlcNAc expression | | | OGT expression | | | OGA expression | | | |
|---------------|---------------------------|----|---|---------------------|----|---|----------------|----|---|----------------|----|---|-------|
| | + | - | P | + | - | P | + | - | P | + | - | P | |
| Sex/n | - | - | - | - | - | - | - | - | - | - | - | - | |
| Male | 23 | 18 | 5 | 0.708 | 22 | 1 | 0.061 | 22 | 1 | 0.061 | 20 | 3 | 0.314 |
| Female | 7 | 5 | 2 | | 5 | 2 | | 5 | 2 | | 7 | 0 | |
| Age | - | - | - | - | - | - | - | - | - | - | - | - | |
| ≥ 55 y | 18 | 17 | 1 | 0.005 | 16 | 2 | 0.804 | 16 | 2 | 0.804 | 17 | 1 | 0.320 |
| < 55 y | 12 | 6 | 6 | | 11 | 1 | | 11 | 1 | | 10 | 2 | |
| Tumor size/n | - | - | - | - | - | - | - | - | - | - | - | - | |
| ≥ 4 cm | 17 | 16 | 1 | 0.010 | 17 | 0 | 0.037 | 17 | 0 | 0.037 | 16 | 1 | 0.390 |
| < 4 cm | 13 | 7 | 6 | | 10 | 3 | | 10 | 3 | | 11 | 2 | |
| TNM staging/n | - | - | - | - | - | - | - | - | - | - | - | - | |
| Stage I-II | 14 | 10 | 4 | 0.526 | 11 | 3 | 0.051 | 11 | 3 | 0.051 | 12 | 2 | 0.464 |
| Stage III-IV | 16 | 13 | 3 | | 16 | 0 | | 16 | 0 | | 15 | 1 | |

Contingency correlation analysis revealed that the positive expression of HIF-1 α and O-GlcNAc,

and OGT was positively correlated (ϕ correlation coefficient = 0.550) (Table 3).

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Table 3. Correlation of the positive expression of HIF-1 α and O-GlcNAc and OGT in OSCC tissues

| O-GlcNAc, OGT | HIF-1 α | | χ^2 | P | ϕ Correlation coefficient |
|---------------|----------------|---|----------|-------|--------------------------------|
| | + | - | | | |
| + | 22 | 1 | 11.468 | 0.001 | 0.550 |
| - | 5 | 6 | | | |

Effect of inhibiting HIF-1 α expression on O-glycosylation related proteins in Tca8113 cells

When Tca8113 cells were treated with PX-478 at 0 μ M, 5 μ M, and 25 μ M, respectively, it displayed that with the increase of the dose, the expression of O-GlcNAc and OGT tended to decrease, and there was statistically significant at a dose of 25 μ M ($P < 0.05$). However, the expression of OGA showed a statistically significant increase with the increase in inhibitor dose ($P < 0.05$) (Figure 2).

Changes in OGT expression in Tca8113 cells at different time points under the inhibitory effect of HIF-1 α

The treatment of Tca8113 cells with 25 μ M PX-478 for 0 h, 4 h, 8 h, and 16 h showed that the OGT content tended to exhibit a statistically significant decrease with increased time ($P < 0.05$) (Figure 3).

Discussion

As a common malignancy in the oral and maxillofacial region, OSCC is characterized by local invasiveness and multiregional neoplastic necrosis. There is evidence that the necrotic areas are mostly characterized by high hypoxia and high acidity, which will lead to low sensitivity to chemotherapy and resistance to anti-cancer drugs [14]. Hypoxia-inducible factor (HIF) is one of the most critical factors that trigger neoplastic hypoxia. HIF-1 α is not merely a subunit that determines HIF activity, but also the only transcription factor that can exert its activity in a hypoxic environment. It is now well established that overexpression of HIF-1 α is interrelated with tumor growth in the head, stomach, and breast and increases tumor mortality [15].

In addition, glucose metabolism also exerts a crucial role in differentiation and cell survival in head and neck squamous cell carcinoma cells.

The efficient glycolysis has a high potential to increase uridine-N-acetylglucosamine diphosphate levels [16]. On the other hand, increased glucose levels may induce Wnt and protein glycosylation, corresponding to the glycosylation of EGFR (epidermal growth factor receptor). Both HIF1 and P53 have potential binding sites on the DPAGT1 promoter, indicating that HIF1 could remain stable under hypoxic conditions while guiding the up-regulation of genes (PDK1, GLUT1, etc.) that advance glycolysis. However, effective substances activated by P53 can serve as an inhibitor of glycolytic enzymes and have an inhibitory effect on the glycosylation reaction of tumor cells in normal homeostasis [17].

Glycosylation, the process of adding sugars to lipids or proteins under enzymatic regulation, can further improve the function and structure of proteins and make the regulatory function more accurate. Most proteins with essential biological functions such as tumor-inhibiting factor and transcription factors are glycosylated in vivo. Data from several studies suggest that the malignant transformation of many malignant tumor cell lines in vitro and the occurrence and development in vivo are related to the excessive activation of glycosylation [18].

Expression of O-glycosylation-related proteins was observed primarily in the tumor epithelial parenchyma, and a low expression in the tumor stroma and inflammatory cells. In this study, we found that the expression of O-glycosylation-related proteins in OSCC increased, and the expression levels of O-GlcNAc and OGT increased proportionally with tumor volume. This finding is consistent with that of Wang [19] et al. who found that 17 (89.5%) of 19 adjacent samples of esophageal squamous cell carcinoma had elevated expression level of O-glycosylation-related proteins as detected by immunohistochemistry. This suggested that the expression of O-glycosylation-related proteins in OSCC increased, and the expression level was positively correlated with the tumor size. The expression of HIF-1 α was observed mainly in the cytoplasm and nucleus and enhanced around the necrotic tissue of the tumor and at the edge of tumor invasion. The expression of HIF-1 α was found to be elevated in OSCC, and the expression level increased proportionally with age and tumor volume. This

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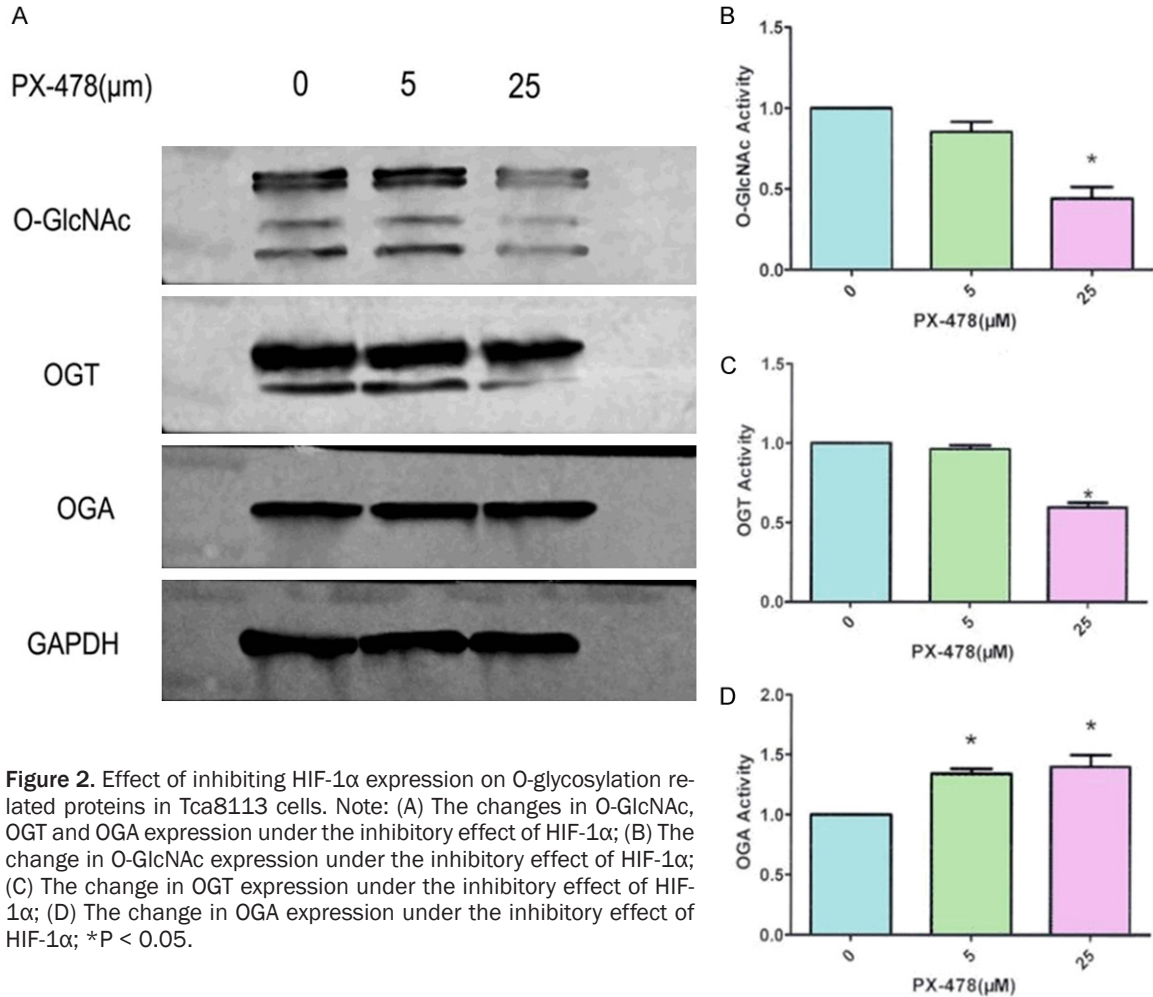


Figure 2. Effect of inhibiting HIF-1 α expression on O-glycosylation related proteins in Tca8113 cells. Note: (A) The changes in O-GlcNAc, OGT and OGA expression under the inhibitory effect of HIF-1 α ; (B) The change in O-GlcNAc expression under the inhibitory effect of HIF-1 α ; (C) The change in OGT expression under the inhibitory effect of HIF-1 α ; (D) The change in OGA expression under the inhibitory effect of HIF-1 α ; *P < 0.05.

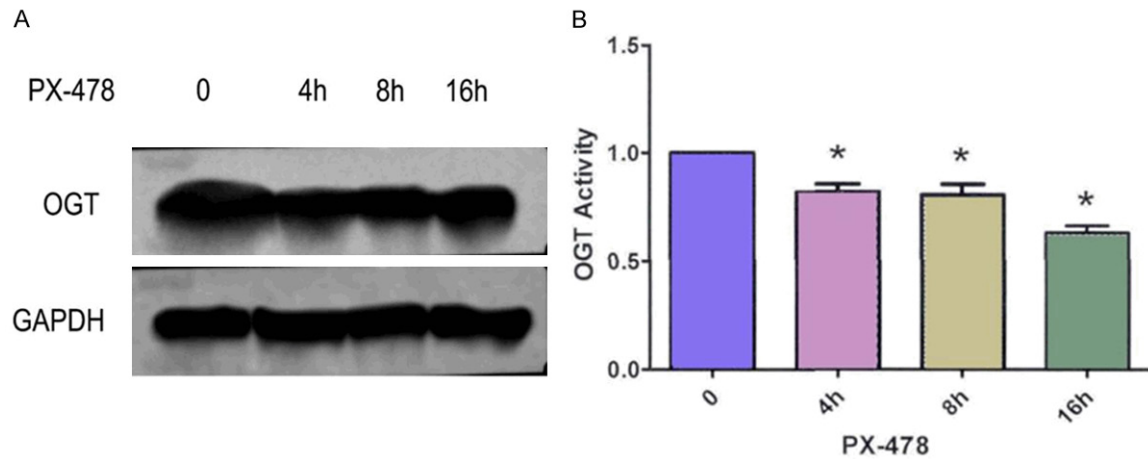


Figure 3. Changes in OGT expression in Tca8113 cells at different time points under the inhibitory effect of HIF-1 α . Notes: (A) Histogram of OGT expression at different time points under the inhibitory effect of HIF-1 α ; (B) The changes in OGT expression at different time points under the inhibitory effect of HIF-1 α ; P < 0.05.

was similar to the findings of Agaet al., whose study detected elevated HIF-1 α expression in

tissue samples from 55 patients with OSCC by immunohistochemistry [20].

Simultaneously, this study also found that the expression of HIF-1 α and O-GlcNAc and OGT in OSCC tissues was positively correlated (ρ -correlation coefficient = 0.550). It suggested that there may be a mechanism of mutual regulation between HIF-1 α and O-GlcNAc, OGT. This is also consistent with previous observations, which showed that OGT and O-GlcNAc glycosylation would improve HIF-1 α stability, facilitate aerobic glycolysis, and promote ER stress to regulate apoptosis [21].

To further investigate the correlation of HIF-1 α with the expression of O-glycosylation-related proteins, we treated Tca8113 cells with PX-478, an inhibitor of HIF-1 α . After inhibition of HIF-1 α , O-GlcNAc and OGT decreased while the expression of OGA increased, indicating that PX-478 could affect Tca8113 glycosylation by regulation of the expression of OGT. This finding broadly supports the work of other studies in this area linking the expression of O-GlcNAc modification with the HIF-1 α inhibition [22], suggesting that decreased expression of O-GlcNAc and OGT, as well as increased expression of OGA, were noted in Tca8113 cells by inhibition of HIF-1 α .

OSCC progression is a multifactorial synergistic mechanism, and how to suppress tumors through gene regulation still requires further research. At present, patients with moderate-advanced OSCC who seek medical treatment in clinical practice have the poor effect of single surgical therapy. Postoperative chemotherapy complications will also lead to a reduced survival rate. Therefore, how to effectively improve the healing of postoperative wounds and reduce complications is still a challenge in current OSCC therapy.

In conclusion, the expression of HIF-1 α and O-glycosylation-related proteins increases in OSCC, and the expression level increases proportionally with tumor volume. Expression of HIF-1 α and O-GlcNAc and OGT is positively correlated. Inhibition of HIF-1 α by PX-478 resulted in decreased expression of O-GlcNAc and OGT, while the expression level of OGA increased. Moreover, PX-478 can affect Tca8113 glycosylation by decreasing OGT expression.

Disclosure of conflict of interest

None.

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