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Original Article

Hypoxia-inducible factor-1α and glucose transporter 1 in the malignant transformation of oral lichen planus

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Abstract: Hypoxia-inducible factor-1α (HIF-1α) and glucose transporter 1 (GLUT1) are key factors in numerous physiological and pathological processes. However, studies on their involvement in the pathogenesis of oral lichen planus (OLP) and its progression toward oral squamous cell carcinomas (OSCC) are scarce. In this study, we examined the protein and gene expressions of both HIF-1α and GLUT1 in normal mucosa, nonatrophic OLP (OLPI), atrophic OLP (OLPII), and OSCC resulting from OLP. Tissues were obtained from 60 cases of OLP patients (n=36 for OLPI, n=24 for OLPII), 20 cases of OSCC patients and 30 healthy control individuals. In addition, in order to investigate if the pathological changes are due to hypoxia, we cultured keratinocytes under hypoxia conditions and measured the expression of HIF-1α and GLUT1. The results indicated that the expressions of HIF-1α and GLUT1 were gradually amplified from normal mucosa to OLPI, OLPII, and OSCC. The expression of both HIF-1α and GLUT1 in OLPII was significantly greater than OLPII. Likewise, the HIF-1α and GLUT1 expressions in OSCC were markedly higher compared to both OLPI and OLPII. Similar trends were obtained in real time PCR and Western blot analyses. A progressive increased micro-vessel density (MVD) was also recorded from normal mucosa to OLPI, OLPII, and OSCC. Moreover, the correlation analysis revealed significant positive correlations between HIF-1α and GLUT1 which were both correlated with MVD in the OLP and OSCC groups. Culture of keratinocytes isolated from OLP tissues under hypoxic and normoxic conditions showed a time-dependent inhibition of keratinocyte proliferation and increased expression of HIF-1α and GLUT1 under hypoxia conditions. In summary, we provided new evidence that hypoxia markers HIF-1α and GLUT1 are upregulated in OLP and are potentially involved in pathological changes leading to malignant transformation of OLP. Further characterization of these factors will provide new ideas for the diagnosis and treatment of OLP.

Keywords: Oral lichen planus, oral squamous cell carcinomas, hypoxia, HIF-1α, GLUT1, malignant transformation

Introduction

Oral lichen planus (OLP), a chronic inflammatory dermatologic disorder that touches the oral mucosa, is characterized by a T-cell-mediated immune response against epithelial cells, causing disruption of basement membrane, liquefaction degeneration of the basal cells, and subepithelial band-like infiltration of T lymphocytes [1]. In majority, OLP touches women, especially those older than 40, but the etiology and molecular mechanisms underlying OLP pathogenesis are still illusive [1-3]. Hypoxia, the deprivation of sufficient oxygen supply to a given tissue, elicits numerous cellular processes under physiological and pathological conditions [4, 5], and has recently been incriminated in the pathogenesis of chronic inflammatory diseases such as OLP [6-8]. Nonetheless, studies on the implications of hypoxia in the pathogenesis of OLP are still limited.

Under hypoxia conditions, hypoxia-inducible factor 1a (HIF-1α) is activated and subsequently controls the expression of various transcription factors necessary for mediating adaptive reactions to hypoxia [9]. HIF-1α is equally reported to control the expression of diverse cancer phenotypes including cell proliferation, metastasis and angiogenesis [10-13]. Previous studies indicate that a variety of genes governing cancer biological processes including glucose metabolism and angiogenesis, are regulated by HIF-1α [14]. Especially, HIF-1α was found to control the activity of glucose transporters (GLUTs) which catalyze glucose trans-

Table 1. Primer sequences used in real time qRT-PCR experiments

Gene	Sense (F) and anti-sense (B) primers
GAPDH	F: 5'-GCCTCAAGACCTTGGGCTGGGACTG-3'
	B: 5'-CAGTCCCAGCCCAAGGTCTTGAGGC-3'
HIF-1α	F: 5'-GCTTGCTCATCAGTTGCCAC-3'
	B: 5'-TCGAAATCACCAGCATCCAG-3'
GLUT1	F: 5'-TCCACCATTTTGCTAGAGAAGGCCG-3'
	B: 5'-CGGCCTTCTCTAGCAAAATGGTGGA-3'

port and uptake [14]. Expression of GLUT1 under hypoxemia redirects glucose metabolism towards glycolysis and studies have demonstrated that hypoxia regulates GLUT1 in a HIF-1α dependent manner [15]. However, studies on the expression of GLUT1 in OLP and its correlation with the expression of HIF-1α and pathological changes such as keratinocyte proliferation and angiogenesis on one hand, and with hypoxia conditions on the other hand, have not been reported. Furthermore, OLP can generally develop into malignant transformations and lead to complications such as oral squamous cancer (OSCC) [16]. Nevertheless, the mechanism underlying this process is still ill-defined.

Herein, we aimed to analyze the expression of GLUT1 and HIF-1α in OLP and OSCC and study their correlation and roles in the progression of OLP towards OSCC.

Materials and methods

Study subjects

This study included oral mucosa biopsies obtained from 60 cases of OLP patients (n=36 for non-atrophic OLP (OLPI), n=24 for atrophic OLP (OLPII)), 20 cases of OSCC patients and 30 healthy control individuals. The collected samples were stored at -80°C before use. The patients clinically diagnosed with OLP and OSCC were referred to Huashan Hospital from January 2013 to December 2014. During the diagnosis, we adopted the WHO diagnostic criteria and those of the International Contact Dermatitis Research Group (ICDRG). For the OLP diagnosis, patients with tumor history and those with lichenoid lesions and amalgam fillings were excluded. The study was reviewed and approved by the Ethics Committee of Huashan Hospital affiliated to Fudan University, and each patient gave written informed consent.

Immunohistochemistry analysis

Oral mucosa samples were fixed in 4% paraformaldehyde for 48 hours, embedded in paraffin, and sectioned at 5 μm thickness. Thereafter, tissue sections were deparaffinized in xylene and rehydrated in a series of graded ethanol concentrations. The primary anti-rabbit HIF-1α and GLUT1 antibodies (ABCAM, USA, dilution 1:1000) were added and incubated overnight at 4°C. HRP-labeled goat anti-rabbit IgG (H+L) (Beijing ZSGB-BIO Technology Co., Ltd.) was added as the secondary antibody. The slides were stained with DAB, then washed and counterstained with hematoxylin. Immune complexes were visualized with the Dako REAL™EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse (Dako, USA) according to the manufacturer's procedure. The experiments were repeated for three times. The positive cells were identified by brown color. Normal oral mucosal tissue was used as control. Finally the specimens were observed under microscope (Nikon, Japan) by two pathologists.

Counting microvessel density (MVD)

MVD was measured using a previously reported method [17]. The section was observed at low magnification in order to identify the most vascularized areas. After five hotspot areas with the highest number of capillaries and small venules were identified, the microscopic slides were photographed at high magnification power (200×) and the microvessels were counted using Image J for quantitative immunohistochemical image processing and analysis software (National Institute of Health, Bethesda, MD, USA) and the mean count was calculated.

OLP keratinocytes isolation, culture and proliferation assay

Tissues (0.6 cm×1.0 cm) obtained from OLP patients were immersed in phosphate-buffered saline (PBS) supplemented with 100 mg/ml streptomycin and 100 units/ml penicillin (Gibco, USA) for blood removal. Subsequently, keratinocytes were isolated following a protocol described in detail previously [18]. Isolated keratinocytes were cultured in serum-free medium. Viable third to fourth generation keratinocytes were employed in the present study. Hypoxia was induced using a 5% CO_2, 37°C, 95% N_2 hermetically sealed incubator in which kera-

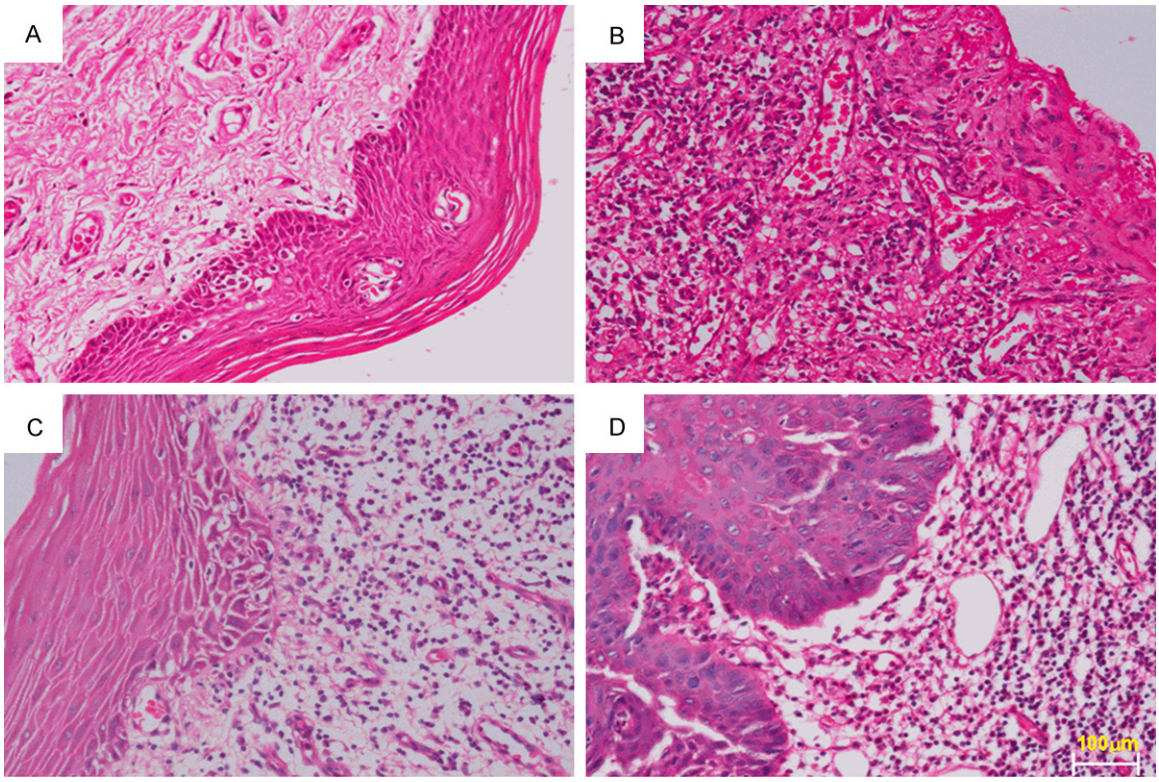

Figure 1. H&E staining of the specimens. (A) Normal control; (B) OLPI; (C) OLPII and (D) OSCC specimens were stained using H&E and the sections observed at a 200× magnification. Only representative images are presented.

tinocytes were cultured for 48 hours. At the same time, keratinocytes cultured in normoxic conditions were used as control in a standard 5% CO_2 incubator maintained at 37°C.

Keratinocyte proliferation assay

The proliferation assay of Keratinocytes from OLP lesion was determined using the 96992 Cell Counting Kit-8 (Sigma-Aldrich) according the instructions provided with the kit manual.

RNA isolation and qRT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, NY, USA) according to the manufacturer's instructions from tissues collected from control group, OLPI, OLPII and OSCC groups. cDNA was prepared from 1 mg total RNA using a cDNA synthesis kit (Promega, Madison, WI). Quantitative reverse transcription-PCR (RT-PCR) was carried out with SYBR Supermix (TAKARA). The primer sequences of HIF-1α, GLUT1 and GAPDH are shown in **Table 1**. The amplification cycle consisted of 2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C, and 1 minute at 60°C. The expression of each target mRNA relative to GAPDH was computed using the ΔΔCT approach.

Western blotting

Total proteins were extracted from tissue specimens and cells using the RIPA lysis buffer (Beyotime, Jiangsu, China). After determination of protein concentrations using the BCA kit (Pierce, Rockford, USA) following the manufacturer's instructions, cell lysates were purified by 10% SDS-PAGE electrophoresis and incubated with primary antibodies (Abcam, Cambridge, UK) against HIF-1α (1:1500 dilution), GLUT1 (1:1000 dilution) and GAPDH (1:1000 dilution). Subsequently appropriate secondary antibodies conjugated with horseradish peroxidase were added for revelation. GAPDH was used as a loading control. Proteins were detected by chemiluminescence (Cell Signaling, Danvers, USA) and densitometry analysis was performed using Image J software.

Data analysis

Each experiment was conducted at least three times. All values were expressed as means ±

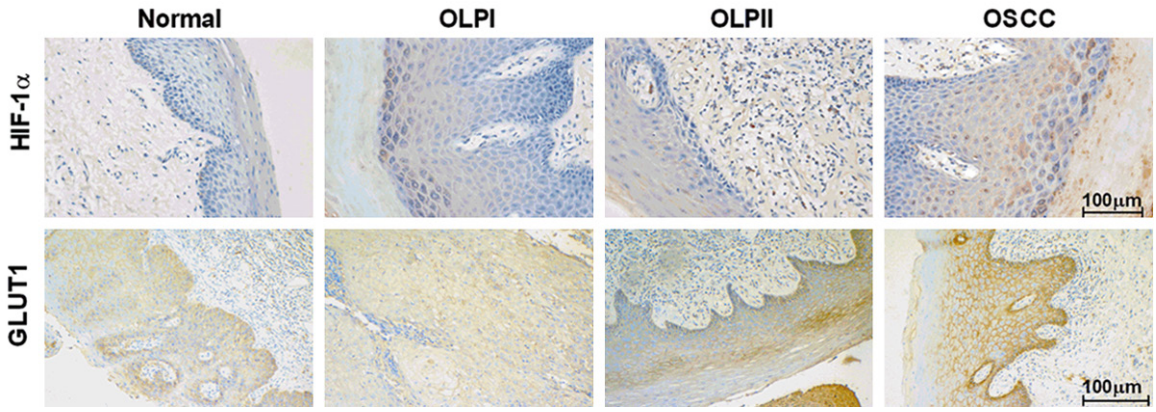

Figure 2. Histochemical staining of specimens. The expression of HIF-1α and GLUT1 in normal, OLPI, OLPII and OSCC tissues was evaluated using the histochemical approach. The expression of both HIF-1α and GLUT1 were gradually increased throughout disease progression. Only representative images (200×) are presented.

SD. Paired and/or unpaired Student's t-tests were used appropriately to evaluate the statistical significance of differences between two group means, and analysis of variance was performed for multiple groups by one-way ANOVA or two-way ANOVA using GraphPad Prism for windows (version 6) at a critical significance level of P<0.05. The correlations between HIF-1α, GLUT1 and MVD were determined by Pearson correlation analysis using GraphPad Prism for windows (version 6).

Results

Pathological characteristics of the samples

Histopathological analysis of samples was performed using H&E staining. As shown in **Figure 1**, normal biopsies exhibited ordinary structure without any sign of pathological changes. Staining of the OLPI tissues showed the presence of keratotic lines and degeneracy of the epithelium, damage of basal cells and subepithelial inflammatory infiltrates. For the OPLII tissues, hyperkeratosis and deterioration of the basal cell layer by liquefaction, and the presence of sub-epithelial lymphoplasmacytic infiltrates could be recorded. The OSCC stained samples displayed multiples comparable characteristics with the OLPII tissues. In addition to that, well differentiated carcinomas could be observed.

HIF-1α and GLUT1 are upregulated in OLP and OSCC tissues

The expression of HIF-1α and GLUT1 in buccal mucosa tissue of OLP patients were significantly higher than that in the normal oral mucosa tissue according to the results obtained from the immunohistochemistry experiments ($P<0.01$). In normal oral mucosa tissue, HIF-1α and GLUT1 were rarely detected. In OLPII oral mucosa tissues the expression of HIF-1α and GLUT1 were higher compared to tissues from OLPI (**Figure 2**). HIF-1α and GLUT1 stained moderately in the cytoplasm of the basal and spinous cell layers of the epithelium and of the sub-epithelial lymphocytic infiltrate cells. In OSCC from OLP, HIF-1α and GLUT1 mainly stained strongly in the cytoplasm of the neoplasm cells.

To further confirm the effective expression of HIF-1α and GLUT1, we performed western blotting and real time PCR analysis. Results showed that the expressions of HIF-1α and GLUT1 at gene and protein levels in normal oral mucosa group were significantly lower than those in OLP I and OLP II groups ($P<0.01$). These expressions were significantly higher in the OLP II group than in the OLP I group ($P<0.01$) (**Figure 3**).

The present results indicated that HIF-1α and GLUT1 are progressively increased from normal mucosa to OSCC with intermediary expression in OLPI and OLPII.

MVD count

As summarized in **Figure 4**, in the OLPI specimens, the mean MVD (55.55) was significantly higher (P<0.01) when compared with the control (37.63). The mean number of MVD was significantly increased in OLPII (90.62) when com-

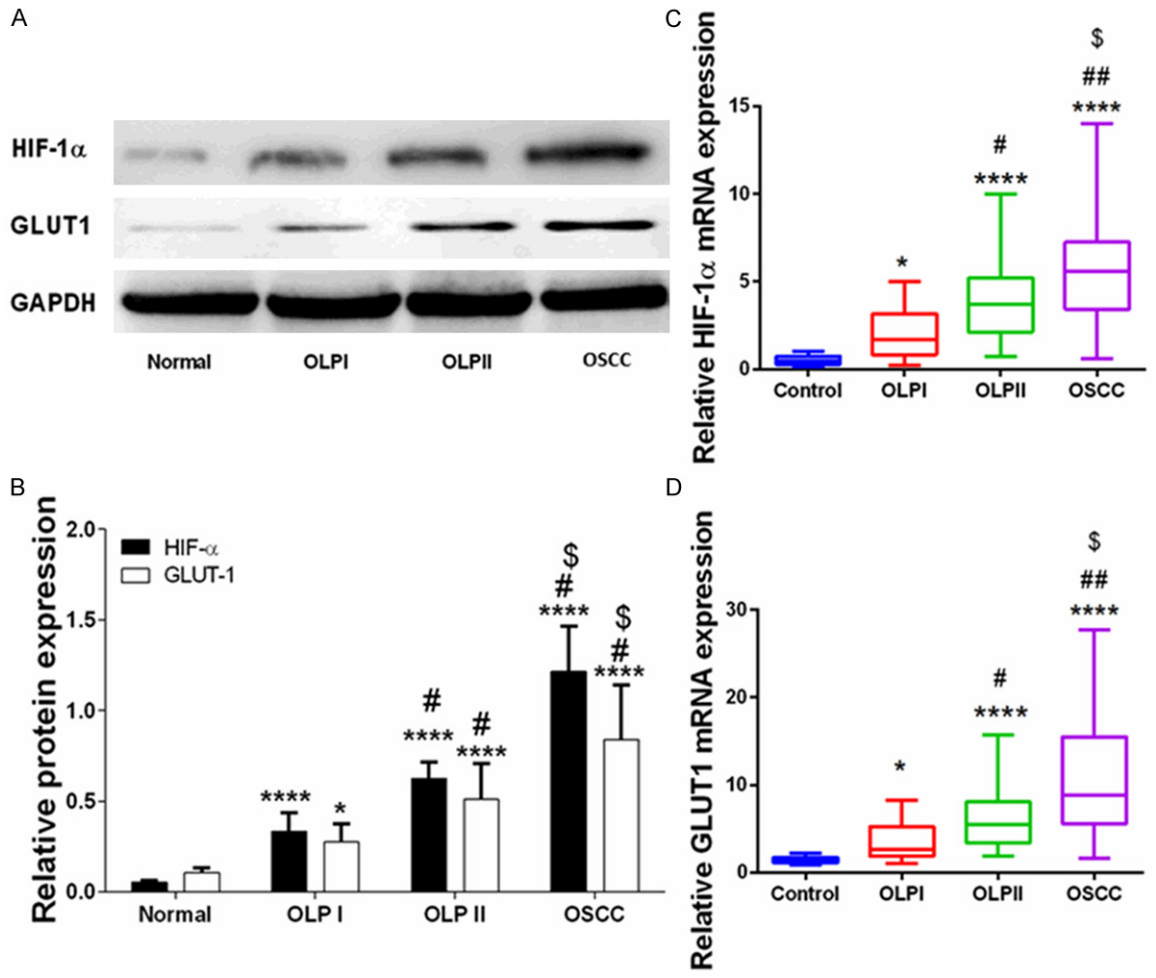

Figure 3. Western blot analysis and real time qRT-PCR analysis of HIF-1α and GLUT1 expression in specimens. A. Representative bands obtained from western blot analysis of specimens. B. Densitometry analysis of bands obtained from western blot analysis of specimens. C. qPCR analysis of HIF-1α mRNA expression. D. qPCR analysis of GLUT1 mRNA expression. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 when compared with the normal group. #P<0.01, ##P<0.0001 when compared with the OLPI group. $P<0.01, when compared with the OLPII group.

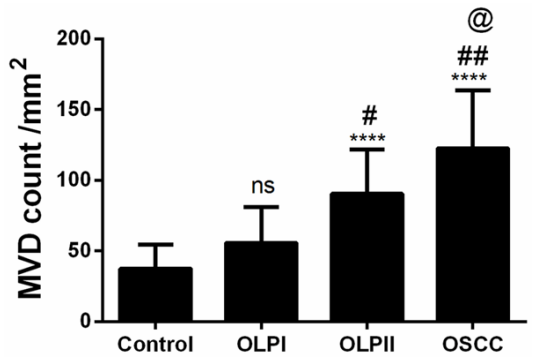

Figure 4. Determination of microvessel density. Specimen were stained using CD34 antibody and the microvessel density (MVD) counted. ****P<0.0001 when compared with the normal group. #P<0.01, ##P<0.0001 when compared with the OLPI group. @ P<0.01, when compared with the OLPII group.

pared with that in OLPI (55.55) and normal mucosa (37.63). The MVD in OSCC was the highest compared with the other specimens.

Correlations between HIF-1α, GLUT1 and MVD

The correlation between HIF-1α and GLUT1 was analyzed by Pearson correlation analysis. A significantly positive correlation was recorded between HIF-1α and GLUT1 protein levels (r= 0.934, P<0.004). Meanwhile, a significantly positive correlation was found between HIF-1α and GLUT1 mRNA expression (r=0.910, P< 0.005). Furthermore, the correlation analysis revealed that there were positive significant correlations between MVD counts and the mRNA expressions of both HIF-1α (r=0.906, P<0.001)

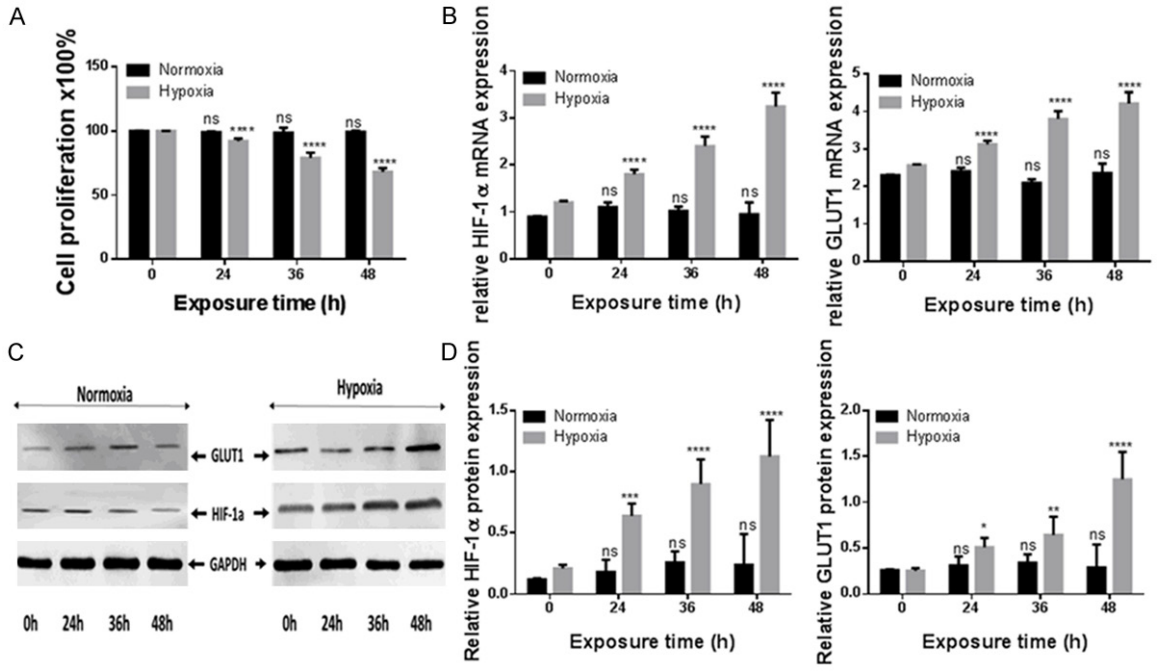

Figure 5. Hypoxia attenuates keratinocyte proliferation and induces the expression of HIF-1α and GLUT1. A. Cell Counting Kit-8 (CCk8) cell proliferation assay showed that hypoxia decreases keratinocyte proliferation. B. qPCR analysis of HIF-1α mRNA expression and GLUT1 mRNA expression. C. Representative bands obtained from western blot analysis of cells cultured under normoxic and hypoxia conditions. D. Densitometry analysis of bands obtained from western blot analysis. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 *vs.* 0 h.

and GLUT1 (r=0.887, P<0.005) within different groups.

Hypoxia attenuates keratinocyte proliferation and increases the expression of HIF-1α and GLUT1

The proliferation of keratinocytes isolated from OLP lesions was determined using CCK-8 assay at 24 h, 36 h and 48 h following hypoxia exposure compared to the normoxic control group (**Figure 5A**). Cell viability was significantly reduced with the exposure (all *P*<0.0001 when compared with the normoxic condition).

On the contrary, we found that the expression of HIF-1α and GLUT1 at gene and protein levels in keratinocytes were significantly and progressively increased with the exposure time while no significant change was recorded for cells cultured under normoxic conditions (**Figure 5B-D**).

Discussion

Oral lichen planus is a disease with the potential ability to undergo malignant transformation. The process from OLP to OSCC is complicated and regulated by many unknown factors [16]. HIF-1α and GLUT1 might play multiple roles in the malignant transformation of OLP. This study showed that the expression of HIF-1α and GLUT1 in OLPII specimens and OSCC from OLP was higher than that of the normal oral mucosa specimens and OLPI specimens. HIF-1α and GLUT1 expression was significantly higher in the OSCC from oral lichen planus specimens than in the OLPII specimens. This suggests that OLPII is more likely to undergo a malignant transformation than OLPI and that HIF-1α and GLUT1 might play an important role in the early stages of malignant transformation.

Hypoxia plays a major role in tumor progression, therapy resistance and for prognosis of oral squamous cell carcinoma (OSCC). The crucial step as a response to hypoxia is the activation and stabilization of the alpha subunit of hypoxia inducible factor 1 (HIF-1α). HIF-1α regulates the expression of different genes to adapt the tumor cells to reduced oxygenation. Main downstream proteins are the glucose transporter 1 (GLUT1), carbonic anhydrase IX (CAIX), and vascular endothelial growth factor (VEGF). Herein, we found that culture of keratinocytes

isolated from OLP tissues under hypoxic conditions caused a time-dependent inhibition of keratinocyte proliferation and increased expression of HIF-1α and GLUT1 under hypoxia conditions. These findings equally incriminate hypoxia into the malignant transformation of OLP.

The significance of GLUT1-specific staining with an oral brush biopsy is more limited than expected but could be used as an additional tool in detecting malignant transformation in the oral cavity [19]. Increased GLUT1 expression in OSCC along with the degree of dysplasia and the histologic grade reflects the expanding glycolytic response to hypoxia [20].

GLUT1 seems to play a decisive role in carcinogenesis [11, 21-24]. Some studies detected an overexpression of GLUT1 in several types of cancer and showed a correlation with a poor prognosis [11, 21, 22]. Considering the negative prognostic value of GLUT1 staining in HNSCC in view of the assessment of the aggressiveness of the tumor, therapy resistance, and overall survival, the question of the importance of early detection of premalignant lesions arises [19].

Along with the increased GLUT1 expression, the expression of HIF-1α also increased in the specimens of oral lichen planus and OSCC from oral lichen planus. Expression of HIF-1α may alter the expression of the GLUT1. Correlation analysis showed that the expression of GLUT1 was correlated with that of HIF-1α. The levels of both proteins were positively correlated with that of the MVD.

Conclusively, these results suggested that GLUT1 and HIF-1α are involved in the angiogenesis and may be partly involved in the progression of OLP towards OSCC.

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Disclosure of conflict of interest

None.

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