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ORIGINAL ARTICLE

Altered expression of mRNA for *HIF-1α* and its target genes *RTP801* and *VEGF* in patients with oral lichen planus

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OBJECTIVE: To explore a potential causal contribution of the transcription factor *HIF-1α* and its target gene, *RTP801* and *VEGF*, to the development of oral lichen planus (OLP).

DESIGN RELEVANT: Twenty-two adult OLP patients were enrolled in this study. All OLP diagnoses were verified by histopathological characteristics. Normal mucous specimens were collected from 12 controls after various oral surgeries.

MATERIAL AND METHOD: RNA was isolated from OLP and control specimens. Microarray was performed using BiostarH-40s gene chip. Expression of *HIF-1α*, *VEGF* and *RTP801* was evaluated using quantitative real-time polymerase chain reaction (qPCR). Unpaired *t*-test and one-way ANOVA was used for statistical analysis.

RESULTS: Microarray results showed that *RTP801* expression was lower in OLP than in controls (779 *vs* 3090). qPCR further confirmed that expression of *RTP801* was similarly lower in OLP than in controls (0.363 *vs* 1.473, $P < 0.001$); expression of *VEGF* was also lower in OLP (0.448 *vs* 1.74, $P = 0.012$). In contrast, expression of *HIF-1α* was higher in OLP than in controls (11.12 *vs* 1.628, $P < 0.001$).

CONCLUSION: The oral mucosa of OLP is hypoxic. Genes that are activated by hypoxia, such as *RTP801* and *VEGF*, and their signal cascades may be novel potential therapeutic targets for OLP.

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Keywords: HIF-1α; RTP801; VEGF; oral lichen planus

Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease that causes bilateral white striations, papules, or plaques on the buccal mucosa, tongue, and gingivae. Erythema, erosions, and blisters may or may not be present (Vincent *et al*, 1990; Silverman *et al*, 1991; Lodi *et al*, 2005). OLP is a common non-infectious oral mucosal disorder among adult patients that attend oral pathology and oral medicine clinics (Sugerman *et al*, 2000). Approximately 1–2% of the general adult population is affected by OLP in the world, although the prevalence of the disease is unknown in many areas (Axell and Rundquist, 1987). Patients with OLP may have a slightly increased risk of oral cancer, such as oral squamous cell carcinoma (SCC), though it is unlikely that OLP is inherently premalignant (Holmstrup *et al*, 1988; Eisenberg, 2000; Silverman, 2000). However, the etiology of OLP remains undetermined. One theory is that OLP is a T-cell-mediated autoimmune disease in which autocytotoxic $CD8^+$ T-cells trigger the apoptosis of oral epithelial cells (Sugerman *et al*, 2000). Others include virus infection and psychological stress and anxiety (Vallejo *et al*, 2001; Lodi *et al*, 2005; Lundqvist *et al*, 2006).

Genes linked to apoptosis and malignancy have been investigated in OLP, including transformation growth factor (TGF) beta 1, Smad7 and telomerase RNA component (hTR) (O'Flatharta *et al*, 2002; Sugerman *et al*, 2002; Karatsaidis *et al*, 2003). Hypoxia-inducible factor (HIF) 1 is a heterodimeric transcription factor that plays a general role in activating homeostatic responses to hypoxia. HIF-1 controls numerous genes of pivotal importance for cellular metabolism, angiogenesis, cell cycle regulation and inhibition of apoptosis (Galanis *et al*, 2008). HIF-1 is composed of HIF-1α and HIF-1β. HIF-1α is an important mediator for hypoxia-induced cell death; it also plays an equally important role in mediating hypoxia-induced ischemia tolerance as well (Wang *et al*, 1995; Carmeliet *et al*, 1998; Bergeron *et al*, 2000). It has been suggested that HIF-1α plays a role in oral SCC (Fillies *et al*, 2005; Sasabe *et al*, 2007; Liang *et al*, 2008).

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An HIF-1 dependent gene, RTP801, has been identified recently. It is ubiquitously expressed in multiple human tissues. Its transcription is regulated by hypoxia (Shoshani *et al*, 2002). Schwarzer *et al* showed evidence for anti-apoptotic role of RTP801 in two different cell lines, PC-3 prostate cancer cells and HaCaT keratinocytes. Their data revealed that down-regulated RTP801 expression induced apoptosis in cancer cells as well (Schwarzer *et al*, 2005). Another target gene of HIF-1 is vascular epidermal growth factor (VEGF). Its transcription under hypoxia condition depends on HIF-1α induction. VEGF regulates angiogenesis and vascular permeability in hypoxia (Galanis *et al*, 2008). To date, there have not been extensive studies of HIF-1α, VEGF and RTP801 in OLP.

In our study, we investigated the gene expressions of *HIF-1α*, *RTP801* and *VEGF* in OLP and the relationship between *HIF-1α* and *RTP801*. We report here that *HIF-1α* expression is significantly increased in OLP specimens as compared to normal specimens, whereas expression of *RTP801* and *VEGF* are markedly decreased. These results suggest that *HIF-1α* and its target genes, *RTP801* and *VEGF*, may play important roles in the pathogenesis of OLP. These genes may provide to be potential targets of OLP therapy.

Materials and methods

Patients and normal mucous specimens

All patients and normal oral mucous specimens were provided by Jiangsu Province Stomatological Hospital, Nanjing, China. This study was approved by Ethical Committee of Nanjing Medical University, Nanjing, China. All OLP diagnoses were evaluated by the authors and histopathological confirmation was verified by an oral pathologist according to the clinical and histopathological definition of OLP by the World Health Organization in 1978 and the criteria proposed by van der Meij *et al* in 2003 (van der Meij and van der Waal, 2003). Briefly, the clinical criteria are presence of bilateral (mostly symmetrical) lesions, including a lace-like network of slightly raised gray-white lines (reticular); erosive lesions; atrophic lesions; bulbous and plaque type lesions. When reticular lesions are present elsewhere in the oral mucosa, other types of lesions are considered to be a subtype of the reticular lesions. Histopathological criteria are the presence of a well-defined band-like zone of cellular infiltration (mostly of lymphocytes) confined to the superficial part of the connective tissue, and signs of liquefaction degeneration in the basal cell layer.

Twenty-two adult OLP patients (7 male, 15 female, age range 20–73) were enrolled in this study. Clinical appearances of oral mucosa in this study cohort included: (i) white reticular, (ii) atrophic and erythematous, (iii) erosion, (iv) sensitivity and pain occurred in buccal mucosa, dorsum and lateral of the tongue, gingival and alveolar mucosa. Histopathological diagnosis criteria for OLP were (i) variable degree of parakeratosis, (ii) liquefaction of the basement layer, (iii) marked layered lymphocytic infiltrate immediately underlying the epithelium observed in lesion area. Sixteen specimens were taken from buccal mucosa; six specimens were taken from margin of tongue. All specimens were taken from the lesion area. Half of specimen was immerged in 10% formalin for histological examination and half of specimen was immersed in liquid nitrogen immediately following the biopsy. The mucosa sections embedded in paraffin were cut (5 μm) and stained with hematoxylin and eosin. OLP was determined histologically by our Department of Pathology. Patients with systemic diseases were excluded from the study. Twelve normal mucous specimens (five males, seven females, age range 20–62) were collected during oral surgery from patients with oral mucous cyst, wisdom tooth that needed removal, or abnormal bone fibrogenesis. All normal mucous specimens were without evidence of OLP. There is no sign of inflammation when surgery was processed. Fresh specimens were taken and frozen in liquid nitrogen immediately.

RNA isolation

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) from fresh frozen specimens according to the manufacturer's instructions. The RNA precipitate was completely dissolved in diethylpyrocarbonate treated water (Ambion Inc., Austin, TX, USA). The RNA was then cleaned using RNeasy Mini columns (QIAGEN Sciences, Germantown, MD, USA) according to the manufacturer's instructions. Then it was stored at −80°C for later cDNA microarray and quantitative real-time polymerase chain reaction (qPCR) assays.

Microarray analysis

Complementary DNA microarray analysis was carried out using BiostarH-40s double dot human whole gene chip (Boxing Corp., Shanghai, China). The fluorescence-labeled cDNA probe was prepared by reverse transcription using a T7-promoter linked Oligo-dT primer. The probes from normal oral mucosa were labeled with Cy3-dCTP, while those from OLP were labeled with Cy5-dCTP. The cDNA probes were mixed and precipitated with ethanol, and then resolved in 5 × sodium chloride/sodium citrate (SSC)/0.2% sodium dodecyl sulfate (SDS) hybridization solution. Probes and chips were denatured in a 95°C water bath for 2 min. The probes were then applied to a BiostarH-40s gene chip. They were hybridized in a sealed chamber at 42°C for 16–18 h. Following hybridization, probe arrays were washed for three times with a solution of 2 × SSC/0.2% SDS, 0.1 × SSC/0.2% SDS and 0.1 × SSC for 10 min each. The arrays were then dried at room temperature. The chips were read by a Scan Array 4000 Scanner (General Scanning Inc., Bedford, MA, USA). The overall intensities of Cy3 and Cy5 were normalized and adjusted using a coefficient according to the ratios of the 40 located housekeeping genes. The acquired image was further analyzed by GenePix Pro 3.0 software (Axon Instrument, Sunnyvale, CA, USA) with a computer to obtain the intensities of fluorescent

signals and the Cy3/Cy5 ratio. The data were calculated as an average of the two repeated spots. Quality assessment was based on the number of spots in each channel (Cy3, Cy5) with adequate signal-noise (S/N) ratio. The S/N ratio was >3. The threshold value of *Z*-score normalization was between 0.4 and 2.5. If the absolute value of the natural logarithm of Cy5/Cy3 was >0.69 (meaning that the variation of gene expression was more than twofold, i.e., Cy5/Cy3 >2 or Cy5/Cy3 <0.5), the difference between two groups was defined as significant.

qPCR analysis

Total RNA was digested with DNase I (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Expression of *HIF-1α* and *RTP801* genes were estimated by qPCR using a primer pair designed as follows: For *HIF-1α*, the forward primer was 5′-GGCGCGAACGACAAGAAAAAG-3′ and the reverse primer was 5′-CCTTATCAAGATGCGAACTCACA-3′. For *RTP801*, the forward primer was 5′-AGCCAGTTGGTAAGCCAGG-3′ and the reverse primer was 5′-GCCAGAGTCGTGAGTCCAG-3′. For *VEGF*, the forward primer was 5′-CAACATCACCATGCAGATTATGC-3′ and the reverse primer was 5′-CCACAGGGACGGGATTCTTG-3′. Human Beta-actin (*β-Actin*) was used as reference or 'housekeeping' gene; the forward primer was 5′-CATGTACGTTGCTATCCAGGC-3′, and the reverse primer was 5′-CTCCTTAATGTCACGCACGAT-3′.

qPCR was performed using an ABI 7000 Real-Time instrument (Applied Biosystems, Foster, CA, USA) and SYBR green real-time PCR master mix (Toyobo Co., Osaka, Japan). The PCR mixture (25 μl) consisted of 12.5 μl master mixture, 0.5 μl PCR forward primer, 0.5 μl PCR reverse primer, 0.5 μl ROX reference dye, 2.0 μl template cDNA and 9 μl DNase and RNase free water. qPCR was done using 40 cycles of 30 s at 60°C and 45 s at 72°C. Gene expression was analyzed using Rotor-Gene 5.0 software (Bosch Institute, Camperdown, NSW, Australia). Detected target transcripts were normalized to the endogenous housekeeping gene *β-actin*.

Statistical analysis

Values are presented as means ± s.e.m. One-way ANOVA was used to assess the overall level of significance across experimental groups. Unpaired *t*-tests were then done to compare mean differences between two different groups. $P < 0.05$ was considered to be statistically significant. Statistical package for social science (SPSS, version 16.0, Chicago, IL, USA) was used for statistical analysis.

Results

Microarray analysis

Specimens from OLP patients and normal controls were used for microarray analysis. Of the three clinical forms of OLP (reticular, atrophic and erythematous and erosive; Eisenberg, 2000; Silverman, 2000), 15 of our patients had the erosive with erythematous form. Normal mucosa was labeled with Cy3-dCTP while OLP mucous was labeled with Cy5 dCTP. In total, expression of 4000 genes was analyzed using GenePix Pro 3.0 software. The data showed lower expression level of *RTP801* in OLP patients compared to normal controls. The mean value of *RTP801* was 3090 in the controls (Cy3) while it was 779 in OLP specimens (Cy5). The ratio of Cy5:Cy3 for *RTP801* was 0.252 (Table 1).

Detection of RTP801, HIF-1α and VEGF mRNA expression by qPCR

To confirm the microarray results, qPCR was done to quantify differences of mRNA expression between OLP patients and normal controls. When normalized for *β-Actin*, *RTP801* mRNA expression was lower in OLP patients (Figure 1a). Overall, *RTP801* mRNA expression was clearly decreased in OLP compared to normal controls (Control *vs* OLP: 1.473 ± 0.349 *vs* 0.363 ± 0.100, $t = 3.85$, df = 32, $P = 0.0002$) (Figure 1b). ANOVA for between groups variables has $F = 14.825$ with $P = 0.001$.

Because *RTP801* is one of the target genes of *HIF-1α* (Shoshani *et al*, 2002), mRNA expression of *HIF-1α* was measured by qPCR. After normalization, *HIF-1α* mRNA expression was found to be elevated in most OLP patients compared to normal controls (Figure 2a). Overall, *HIF-1α* mRNA expression was significantly

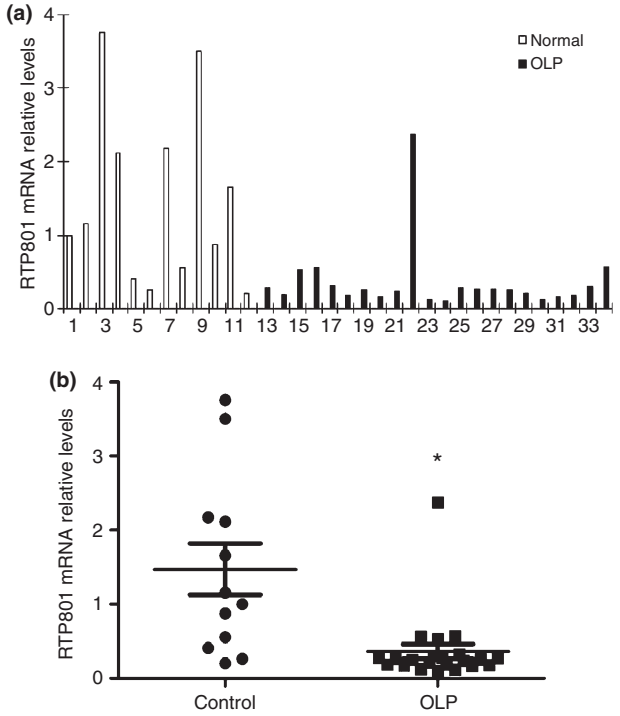

Figure 1 *RTP801* mRNA expression in OLP specimens and normal controls. **(a)** Bars denote *RTP801* expression in individual samples. Open bars denote normal controls; filled bars denote OLP specimens. **(b)** Bars denote the mean ± s.e.m. for *RTP801* expression. Filled circles denote normal controls; filled squares denote OLP specimens. *, $P < 0.001$ compared to normal controls

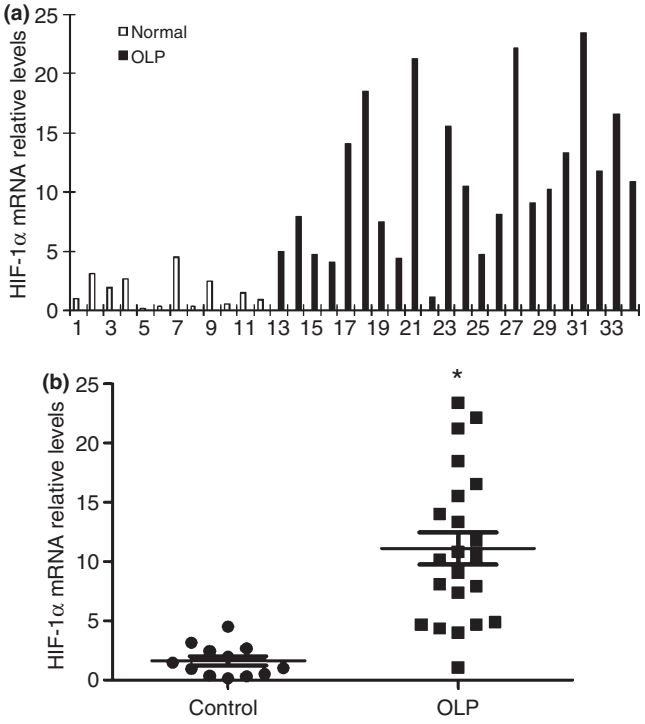

Figure 2 *HIF-1α* mRNA expression in OLP specimens and normal controls. **(a)** Bars denote *HIF-1α* expression in individual samples. Open bars denote normal controls; filled bars denote OLP specimens. **(b)** Bars denote the mean ± s.e.m. for *HIF-1α* expression. Filled circles denote normal controls; filled squares denote OLP specimens. *, $P < 0.001$ compared to normal controls

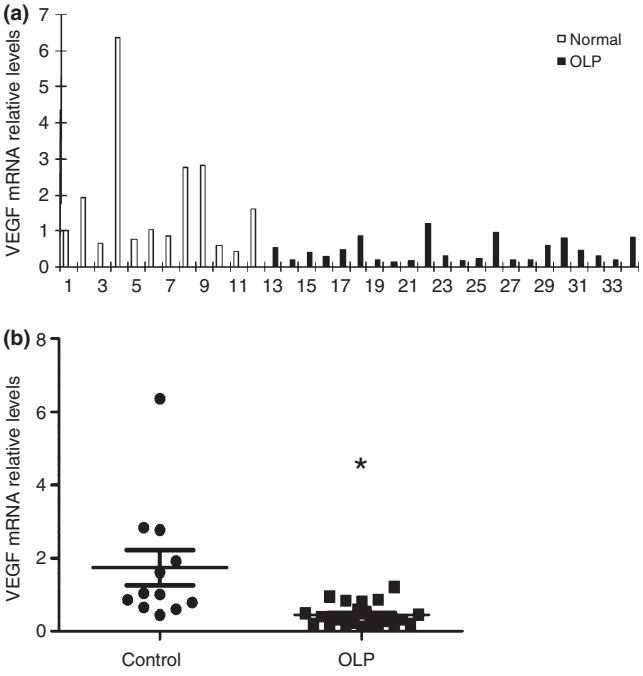

Figure 3 VEGF mRNA expression in OLP specimens and normal controls. **(a)** Bars denote *VEGF* expression in individual samples. Open bars denote normal controls; filled bars denote OLP specimens. **(b)** Bars denote the mean ± s.e.m. for *VEGF* expression. Filled circles denote normal controls; filled squares denote OLP specimens. *, $P < 0.01$ compared to normal controls

increased in OLP (Control *vs* OLP: 1.628 ± 0.390 *vs* 11.12 ± 1.356, $t = 5.073$, df = 32, $P < 0.0001$) (Figure 2b). ANOVA for between groups variables has $F = 21.48$ with $P < 0.001$.

Expression of another *HIF-1α* target gene, *VEGF*, was also evaluated by qPCR. Similar to *RTP801* expression, *VEGF* mRNA expression was lower levels in OLP patients than in normal controls (Figure 3a). Overall, *VEGF* mRNA expression was markedly decreased in OLP compared to normal controls (Control *vs* OLP: 1.740 ± 0.481 *vs* 0.448 ± 0.066, $t = 3.57$, df = 32, $P = 0.0012$) (Figure 3b). ANOVA for between groups variables has $F = 12.733$ with $P = 0.001$.

Correlation of HIF-1α and RTP801 mRNA expression in OLP

Correlation analysis was carried out using SPSS statistical software. Pearson's correlation analysis exhibited a negative correlation between *HIF-1α* and *RTP801*expression in OLP patients with *r* (Pearson correlation coefficient) = −0.427 and $P = 0.047$ (Table 2). No significant correlation of *HIF-1α* and *VEGF* was detected in OLP patients (data not shown).

Table 1 Ratio of *RTP801* mRNA levels in OLP specimens compared to normal controls

Gene name	*Cy3 (normal oral mucosa)*	*Cy5 (OLP mucosa)*	*Ratio (Cy5/Cy3)*
RTP801	3090	779	0.252

Normal oral mucosa were labeled with Cy3-dCTP; OLP specimens were labeled with Cy5-dCTP. Ratios are based on the mean values of gene expression from microarray data analysis.

Discussion

While OLP is one of the most common dermatological diseases presenting in the oral cavity, the mechanisms by which the disease develops remain undefined. In the present work, we examined gene expressions of *HIF-1α* and its target genes, *RTP801* and *VEGF* in mucosa specimens from OLP patients and normal controls. Down-regulated *RTP801* expression was detected by microarray analysis. qPCR confirmed the results of microarray. Expression of the target genes was significantly down-regulated while expression of HIF-1 was significantly up-regulated in OLP (Figures 1–3).

HIF-1 is a well-studied transcription factor complex, although the mechanism by which HIF-1 interacts with its target genes remains obscure. HIF-1 is activated in hypoxic condition in mammalian cells, and it then regulates transcription of genes in angiogenesis, erythropoiesis, glycolysis, iron metabolism and cell survival (Semenza, 2001). The HIF pathway directly regulates the transcription of at least 70 different effector genes. Perhaps 1–5% of all human genes are HIF-1 target genes induced by hypoxia (Semenza, 2003; Wenger *et al*, 2005). HIF-1 is a heterodimer composed of a constitutively

Table 2 Correlation of *HIF-1α* and *RTP801* expression in OLP specimens

	Correlations	*HIF-1α*	*RTP801*
HIF1a	Pearson correlation	1.000	−0.427*
	Sig. (two-tailed)		0.047
	N	22	22
RTP801	Pearson correlation	−0.427*	1.000
	Sig. (two-tailed)	0.047	
	N	22	22

*Correlation is significant at the 0.05 level (two-tailed).
Pearson's correlation analysis was carried out in OLP specimens using SPSS statistical software. $*P < 0.05$ denotes a significant negative correlation between *HIF-1α* and *RTP801*.

expressed HIF-1β subunit and an inducible HIF-1α, the expression of which is highly regulated by cellular oxygen concentration. HIF-1α is an important transcription factor in nucleus during hypoxia. HIF-1α translocates into the nucleus and heterodimerizes with HIF-1β. HIF-1 complex then binds to hypoxia response elements (HREs), which are present in the promoter or enhancer regions of HIF-1 target genes (Wenger *et al*, 2005). After HIF-1 interacts with transcriptional coactivators, such as (cAMP response element binding) - binding protein (CBP)/p300, the target gene expression is induced (Semenza, 2003). HIF-1α has dual effect on pro- and anti-apoptosis, the balance between the relative contributions of proliferative and cell death signals will determine survival (Bacon and Harris, 2004). Overexpression of HIF-1α in alveolar epithelial cells results in enhanced apoptosis because of the activation of tumor suppressor p53 in the hypoxia condition. The interaction of HIF-1α and p53 activate gene, such as cyclin-dependent kinase inhibitor 1A (p21), which, in turn, causes cell death (Krick *et al*, 2005). HIF-1α overexpression is an indicator of prognosis in patients with SCC of the oral floor (Fillies *et al*, 2005).

RTP801 is a target gene of HIF-1α involving in the hypoxia-induced apoptosis pathway (Shoshani *et al*, 2002; Semenza, 2003). HIF-1α regulates transcription of *RTP801* by directly binding HRE in the promoter region of *RTP801* (Shoshani *et al*, 2002). RTP801, like HIF-1α, has dual effects of pro- and anti-apoptosis on the target cells. For instance, overexpression of *RTP801* in MCF-7 and PC12 cell lines prevented apoptosis under hypoxia, while liposome delivery of *RTP801* to mouse lungs induced a prominent apoptotic response on the target cells (Shoshani *et al*, 2002). Study in prostate cancer cell line, PC-3, suggested that reduced RTP801 levels by blocking phosphoinositide 3-kinase (PI3K) pathway sensitized cells toward apoptosis, whereas elevated levels of RTP801 induced by hypoxia or overexpression desensitized cell to apoptotic stimuli (Schwarzer *et al*, 2005). It was shown that reduced RTP80 may contribute to apoptosis and suppress cancer cell growth.

HIF-1α promotes angiogenesis through regulating transcription activation of VEGF. VEGF is a major modulator of angiogenesis that promotes endothelial cell migration toward a hypoxia area (Vaupel, 2004; Dery *et al*, 2005). VEGF and its receptors activate a PI3KAkt pathway involved in wound healing and endothelial cell survival (Abid *et al*, 2004; Santos *et al*, 2007). Study in acute gastric injury suggested that blocking endogenous VEGF effects with anti-VEGF antibodies exacerbated mucosal injury, while administration of recombinant VEGF reduced the severity of mucosal injury (Matsui *et al*, 2002). Moreover, blocking VEGF signaling by an inhibitor of VEGF receptor 2 (VEGFR-2) reduced epithelial proliferation in mouse model of wound healing (Ko *et al*, 2005). When PI3K pathway was blocked by its inhibitors, LY294002 or Rapamycin, both RTP801 and VEGF expression were reduced in PC-3 cell line and thus promoted apoptosis (Schwarzer *et al*, 2005).

While the histopathological features of OLP are defined as dense subepithelial lympho-histiocytic infiltrate, increased numbers of intra-epithelial lymphocytes, and apoptotic basal keratinocytes (Sugerman *et al*, 2002), the trigger for keratinocyte apoptosis in OLP is unknown. When hypoxia occurs locally, HIF-1α is stabilized and promotes the transcription of its target genes including anti-proliferative and pro-apoptotic genes, such as p53, BNIP3 and NIX (Bacon and Harris, 2004; Krick *et al*, 2005). Overexpression of *HIF-1α* in OLP patient demonstrates that oral mucous is under hypoxia condition, and implies oral mucous epithelial cells undergo apoptosis in the lesion area of OLP. Moreover, it is possible that reduced RTP801 expression might evoke apoptosis and anti-proliferation in mucosa epithelial cells of OLP, as down-regulated *RTP801* enhanced apoptosis in prostate cancer cells (Schwarzer *et al*, 2005). Further, the lower *VEGF* expression under hypoxia may impair epithelial repair and induction of apoptosis via PI-3K pathway in OLP patients. Normally, increased *VEGF* expression under hypoxia promotes angiogenesis, endothelial cell migration, and survival (Vaupel, 2004; Santos *et al*, 2007). Our previous data showed that Bcl2-associated X (Bax) protein level was increased in OLP (Fan *et al*, 2004). Furthermore, p53 was positively detected in OLP biopsies (Dekker *et al*, 1997). Together, these results suggest that *HIF-1α* and its target genes, *RTP801* and *VEGF*, may play important roles in the chronicity of oral mucosa lesions of OLP patients. Further research will investigate the molecular pathway of apoptosis and inflammatory response under hypoxia in OLP, in particular, the factors influencing the PI-3K pathway. HIF-1α and its signal cascade may be novel potential therapeutic targets for OLP.

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