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ORIGINAL ARTICLE

Cys–X–Cys ligand 9 might be an immunological factor in the pathogenesis of oral submucous fibrosis and its concomitant oral lichenoid lesion

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Abstract
Objectives Oral submucous fibrosis (OSF) is a chronic oral precancerous disease primarily caused by betel quid chewing. Some OSF patients are concomitant with oral lichenoid lesion (OLL), a white-streak lesion with a higher risk for cancerization, in OSF mucosa. Immunological reaction has been considered as one of their common pathogenic mechanisms. Cys–X–Cys ligand 9 (CXCL9) is an important factor to recruit effector neutrophils and lymphocytes in immunological reactions. However, the expression levels of CXCL9 in OSF and OLL remain unclear.
Materials and methods We investigated the expression levels of CXCL9 in 10 normal buccal mucosa (NBM) samples and 56 OSF concomitant with OLL patients, and evaluated the possible mechanism of CXCL9 on their pathogenesis.
Results Our results showed NBM demonstrated negative CXCL9 expression. OSF stained positive CXCL9 mainly in the cytoplasm of inflammatory cells and endothelial cells throughout the superficial layer of connective tissue, while its concomitant OLL showed much stronger CXCL9 in all mononuclear cells of subepithelial inflammatory infiltration (p=0.0006). There was an upregulated trend of CXCL9 expression from NBM to OSF to OLL. However, no significant association between CXCL9 expression and clinicopathologic parameters of patients was found.
Conclusions In conclusion, CXCL9 was found for the first time to contribute to the immunological pathogenesis for both OSF and its concomitant OLL, indicating a continuously enhanced intensity of immunoreactivity in their pathogenic process.
Clinical relevance CXCL9 might be a useful tool to monitor the phase and disease severity of OSF and OLL, and a potential target for further clinical therapy for both lesions.

Keywords Immunological reaction · CXCL9 · Oral submucous fibrosis · Oral lichenoid lesion · Betel quid

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Introduction

Oral submucous fibrosis (OSF) is a chronic, debilitating, and potentially cancerous oral mucosal disease caused primarily by chewing betel quid, which is the fourth most common psychoactive substance in the world [1, 2]. There are approximately 600 million betel quid chewers worldwide, amounting to 10–20 % of the world's population [3]. OSF is considered as an early indicator of damage to the oral mucosa and carries a significantly high transformation rate of 3–19 % to oral squamous cell carcinoma [4].

In OSF patients, the major clinical presentation of the mucosa is distinctively pearly white with loss of elasticity. The lesion on the buccal mucosa has a more mottled and fibrous consistency. Histopathologically, OSF is characterized by inflammatory cell infiltration followed by progressive accumulation of collagen fibers within the lamina propria and the underlying submucosal layer and associated epithelial atrophy [5, 6]. Some OSF patients have

concomitant oral lichenoid lesions (OLL) in the fibrous mucosa [6, 7]. Their clinical features are usually characterized by white streaks in a lace-like pattern on the mucosa with atrophy and erosions [6]. The main microscopic features include a band-like zone of lymphocyte infiltration confined to the lamina propria, liquefaction degeneration of basal keratinocytes, and thickening of the spinous layer [8, 9].

The full etiology and pathogenesis of both OSF and OLL remain poorly understood, but both of these chronic inflammatory diseases seem to share common immunopathological features involving T cell-mediated allergic responses [10, 11]. Thus, there should be some common factors triggering the immunological process in the development of both OSF and OLL in one patient.

Chronic inflammation has been found to play a key role in immunological disorders, while it is now well established that chemokines act as important signaling molecules in determining tissue-specific trafficking and positioning of leukocyte subsets within both normal and inflamed tissues [12]. Chemokines are a family of small and structurally related proinflammatory peptides with high homology. They exhibit a peculiar function of attraction and recruitment of different cell types during physiological processes of maturation and trafficking of immune cells to induce, maintain, and amplify the inflammatory reactions [13, 14]. According to NH_2-terminal cysteine motifs, chemokines are classified as CXC, CC, CX3C, and C subfamilies. Cys–X–Cys ligand 9 (CXCL9), so-called inflammatory/inducible chemokine induced by IFN-γ, is an important member of CXC subfamily regulated by proinflammatory stimuli, and can orchestrate innate and adaptive immune responses to control the recruitment of effector neutrophils and lymphocytes in infection and inflammation sites [15–17]. It has been documented that increased expression of CXCL9 can be shown in both basal keratinocytes and dermal mononuclear cells of oral lichen planus [18] and skin lichen planus [19]. Meanwhile, in our previous study, the enhanced expression of CXCL9 was also found in the inflammatory cells of lamina propria of OSF [20]. However, the differential expression of CXCL9 protein between OSF and its concomitant OLL lesion has not been previously explored.

To assess the potential differential expression of CXCL9 protein between OSF and its concomitant OLL, we studied the expression trend of CXCL9 protein from normal mucosa to OSF to its concomitant OLL by immunohistochemistry and Western blot. We found that there was an upregulated trend of CXCL9 expression from normal buccal mucosa (NBM) to OSF to OLL, indicating a continuously enhanced intensity of immunoreactivity in their pathogenic process. With the results of this study, we considered that CXCL9 expression may be a useful tool for monitoring the phase and disease severity of OSF and OLL.

Material and methods

Patients and tissue samples collection

Under an ethical guideline of the Central South University Ethics Committee, 68 patients with both clinically defined OSF and concomitant unilateral buccal OLL were recruited from the Department of Oral and Maxillofacial Surgery, Xiangya Hospital of Changsha in China between March 2007 and May 2011. Among them, five patients had previous local or general drug treatments for oral mucosal lesions, three had general history of systemic autoimmune diseases (hepatitis B, diabetes, and autoimmune thyroid diseases), and four patients had received amalgam or other metal restoration for the teeth close to OLL lesion. Twelve patients were excluded in our present study. All patients had the habit of betel quid chewing. Eventually, we obtained 56 pairs of buccal OSF and concomitant OLL samples. Ten unmatched NBM tissues were obtained from ten healthy volunteers, who underwent surgery for either third molar impactions or trauma. Informed consent was obtained from all donors. Immediately after surgical removal, each pair of OSF and concomitant OLL sample was divided into two parts: one part was placed in 10 % neutral-buffered formalin for 24–48 h and embedded in paraffin for pathological analysis; the residual sample was snap-frozen with liquid nitrogen and stored at −80 °C for Western blot. After final pathological analysis, all 56 pairs (53 males and 3 females) of samples were diagnosed as buccal OSF and concomitant OLL. Patient's age, gender, duration of betel quid chewing, duration of OSF disease, and OSF histopathological grade were used as parameters as shown in Table 1. All control samples were assessed as NBM. In the present study, the clinical and pathological diagnostic and grading criteria employed to classify OSF were based on the concept of Pindborg and Sirsat [1]. The diagnosis of OLL was made by clinical and histopathological features based on the revised WHO diagnostic criteria of OLL [21]. All samples were collected from buccal lesions because the buccal mucosa is the commonly affected site of OSF and OLL [1, 21].

Immunohistochemistry analysis

Immunohistochemical studies were done using the avidin–biotin–peroxidase method. Briefly, 3-μm-thick sections were mounted on silanized slides. After being deparaffinized and rehydrated, the sections were subjected to 2 min heat-induced antigen retrieval. After treatment with 3 % hydrogen peroxide, the sections were incubated with

Table 1 Clinicopathological parameters of OSF-OLL patients

Parameter	Value
Man (*n*)	53
Woman (*n*)	3
Age range (years)	20–56
Median age (years)	30.7
Duration of disease (years)	0.02–5
Median duration of disease (years)	1.4
Duration of betel quid chewing (years)	0.17–10
Median duration of betel quid chewing (years)	3.8
OSF histological grade (*n*)	
Early stage	16
Moderately advanced stage	30
Advanced stage	10
Site of OLL (*n*)	
Left buccal	19
Right buccal	37

10 μg/ml monoclonal anti-CXCL9 (R&D, Minneapolis, MN, USA). Slides were then incubated for 30 min with the biotinylated IgG (Santa Cruz Biotechnology, CA, USA). Antigen–antibody complexes were visualized with diaminobenzidine. Subsequently, the slides were counterstained with Mayer's Hematoxylin, differentiated, dehydrated, and mounted. Based on our previous study [20], the immunostaining intensity of CXCL9 was graded as follows: negative (≤10 % of cells stained), positive (>10 %, but ≤70 % of cells stained), and strongly positive (>70 % of cells stained). All immunohistochemical slides were scored independently by two investigators who were blinded to the patient's clinical data. Occasional disagreements were discussed to reach a consensus. In cases of persistent differences between them, the sections were studied by a third independent observer and the majority decision was then considered.

Western blot analysis

Thirty micrograms of proteins from OSF, OLL samples, and normal controls was separated by 12 % SDS–PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking, filters were incubated with mouse monoclonal anti-CXCL9 (R&D, Minneapolis, MN, USA; 2 μg/ml dilution) followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, California, CA, USA) diluted at 1:2,000. As an internal control, samples were probed with mouse monoclonal anti-β-actin antibody (BD Biosciences, San Jose, CA, USA). Bands were visualized using the ECL system (Amersham, Buckinghamshire, UK) and signal intensity was analyzed by the Bandscan software (Glyko, Novato, CA, USA).

Statistical analysis

The positive rates of CXCL9 expression among NBM, OSF, and OLL groups or among early stage, moderately advanced stage, and advanced stage of OSF were analyzed by chi-square test. Meanwhile, the association between clinical parameters of patients (age, gender, duration of betel quid chewing, duration of disease, and histopathologic grade) and immunohistochemical results was analyzed with the chi-square or Fisher's exact test (if $N<5$). A p value <0.05 was considered significant. The statistical analysis was carried out by using the SPSS for Windows 15.0 program (SPSS Inc., Chicago, WI, USA).

Results

CXCL9 expression in NBM, OSF, and OLL samples detected by immunohistochemistry

We examined CXCL9 expression in 10 NBM samples and 56 pairs of OSF and its concomitant OLL samples by immunohistochemistry. The results showed that all NBM samples demonstrated negative CXCL9 expression (Fig. 1a, b). However, 73.2 % (41/56) OSF samples showed positive CXCL9 expression mainly located in the cytoplasm of inflammatory cells and endothelial cells throughout the superficial layer of connective tissue (Fig. 1c, d). Fifty-four out of 56 OLL samples (96.5 %) were stained strongly positive CXCL9 in the cytoplasm of the majority of mononuclear cells of subepithelial inflammatory infiltration (Fig. 1e, f). The expression of CXCL9 in NBM, OSF, and OLL was compared using the chi-square test. The positive rate of CXCL9 in OSF samples was obviously higher than the NBM samples ($\chi^2=19.3$, $p=1.1\times10^{-5}$) while significantly lower compared with its concomitant OLL samples ($\chi^2=11.7$, $p=0.0006$). The data indicated that there was a gradually increased trend of CXCL9 expression levels with statistical significance from NBM to OSF to OLL. Moreover, of OSF samples, positive expression of CXCL9 was found in 62.5 % (10/16) early-stage OSF samples, 80.0 % (24/30) moderately-advanced-stage samples, and 70 % (7/10) advanced-stage samples. Overexpression of CXCL9 was observed in moderately-advanced-stage OSF samples (24/30) compared with early-stage (10/16) and advanced-stage samples (7/10), but this difference was not statistically significant ($\chi^2=1.7$, $p=0.20$; $\chi^2=0.4$, $p=0.51$, respectively; shown in Table 2).

Associations between the expression of CXCL9 and clinicopathological parameters in OSF–OLL samples

Associations between CXCL9 expression in OSF samples and clinicopathological features of patients were analyzed

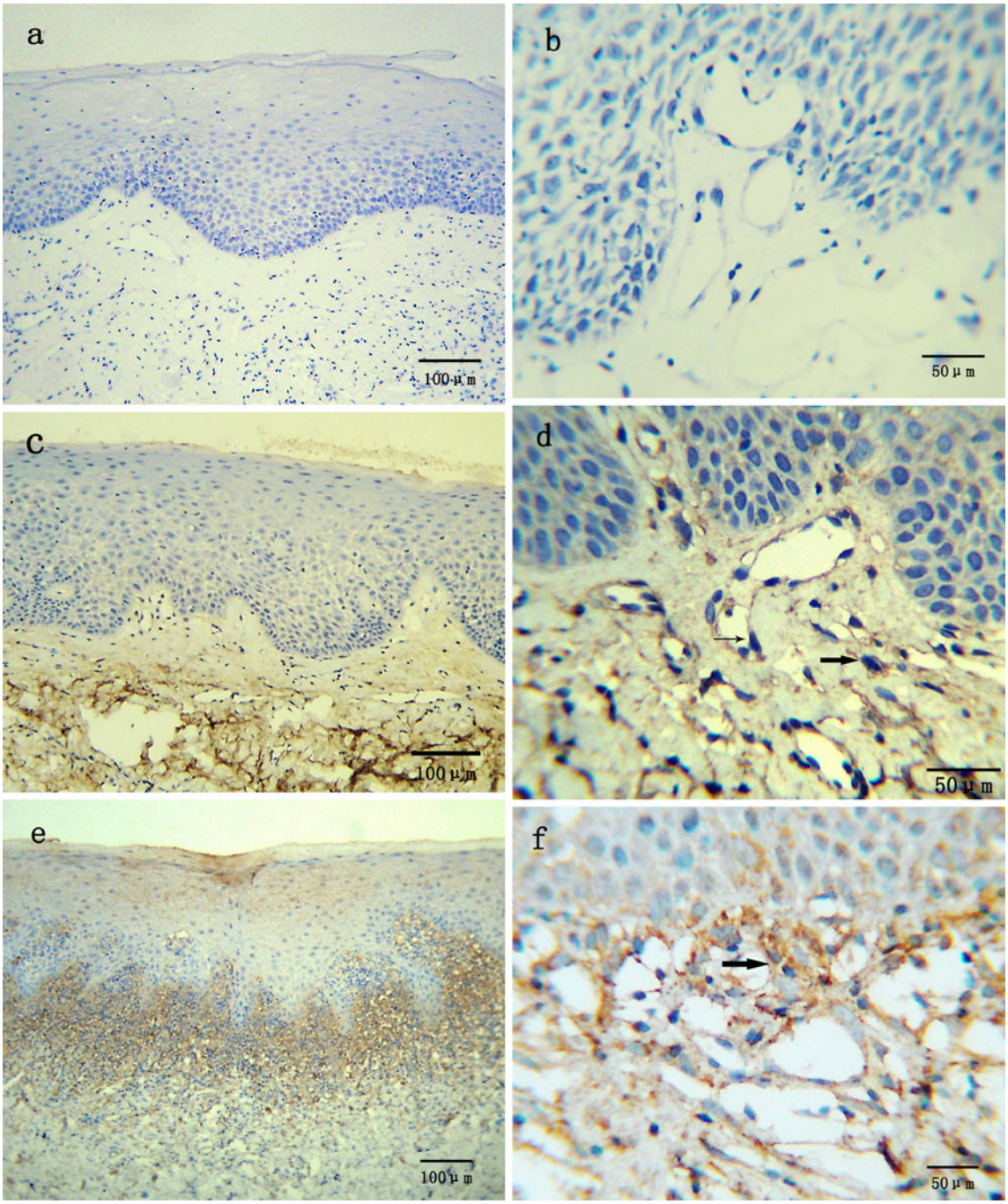

Fig. 1 Comparison of immunohistochemical analysis on CXCL9 among NBM, OSF, and OLL. **a** Negative CXCL9 staining in NBM (original magnification, ×200). **b** Negative CXCL9 staining in NBM (original magnification, ×400). **c** Immunohistochemical image of CXCL9 from OSF mucosa showed diffuse CXCL9 positivity throughout the superficial layer of connective tissue (original magnification, ×200). **d** Immunohistochemical image of CXCL9 from OSF mucosa showed CXCL9 positivity in the cytoplasm of inflammatory cells (*thick arrow*) and endothelial cells (*thin arrow*; original magnification, ×400). **e** Immunohistochemical image of CXCL9 from OLL mucosa showed intensive and continuous CXCL9 positivity in the juxtaepithelial lamina propria (original magnification, ×200). **f** Immunohistochemical image of CXCL9 from OLL mucosa showed intensive CXCL9 positivity in the cytoplasm of all mononuclear cells (*thick arrow*) of subepithelial inflammatory infiltration (original magnification, ×400). There is a gradually increased level of CXCL9 with the statistically significance from NBM to OSF and from OSF to OLL ($p < 0.0001$)

by chi-square test, but no significant association was found in all parameter items including age, gender, duration of betel quid chewing, duration of disease, and OSF histological grade (shown in Tables 3 and 4). Meanwhile, no significant association was found between CXCL9 expression in OLL samples and clinicopathological features including age, gender,

Table 2 Expression of CXCL9 in NBM, OSF, and OLL (cases)

Group	*N*	CXCL9	
		(+)	(−)
NBM	10	0 (0 %)	10 (100 %)
OSF	56	41(73.2 %)	15 (26.8 %)
E	16	10 (62.5 %)	6 (37.5 %)
M	30	24 (80.0 %)	6 (20.0 %)
A	10	7 (70.0 %)	3 (30.0 %)
OLL	56	54 (96.5 %)	2 (3.5 %)

E early stage, *M* moderately advanced stage, *A* advanced stage

duration of betel quid chewing, and duration of disease. Limited sample size may be the main reason (shown in Table 4).

CXCL9 expression in NBM, OSF, and OLL samples detected by Western blot

We detected CXCL9 protein expression in six NBM samples, six OSF, and six OLL samples. The expression levels of CXCL9 protein were remarkably increased in OSF samples compared with NBM controls ($p<0.05$), and the expression levels remained gradually elevated in OLL samples ($p<0.05$). A representative Western blot result was presented in Fig. 2.

Discussion

OLL is well recognized as a chronic inflammatory disease related to a T lymphocyte-mediated process involving cytotoxic activity against basal keratinocytes, and commonly caused by allergies to metal restoration in mouth and some specific drugs [22]. Although OLL may occur in every region of the oral mucosa, the buccal mucosa is the most common site [21, 23]. In the present study, we found that not all OLL lesions in OSF patients can be found closed to some metal restoration. The persistent mechanical and chemical stress to the oral mucosa caused by betel quid chewing may be the key etiology for concomitant OLL in OSF buccal mucosa. Lots of evidences also support the immunological basis for OSF, including increased levels of immune complexes, raised serum levels of immunoglobulin, the detection of various autoantibodies, and various HLA types of patients, as well as the dispersed distribution of $CD4^+$ T lymphocytes and macrophage in the juxta-epithelial connective tissues [11, 24, 25]. Meanwhile, OLL could be found in both non-OSF betel quid chewers and OSF patients, while the prevalence of OLL in cases of OSF was higher than the prevalence of OLL in non-OSF cases [26]. These evidences suggest that immunoreactivity could be an important factor triggering the development of both OSF and OLL in one patient, though the full pathogenesis of both lesions is believed to be multifactorial [25, 27].

The role of CXC chemokines in several types of inflammatory and immunological disorders has been investigated

Table 3 Correlation between the expression of CXCL9 and clinicopathological parameters in OSF samples ($n=56$)

	Cases	CXCL9 (+)	CXCL9 (−)	*p* value
Age (years)				
<30	33	25 (75.8 %)	8 (24.2 %)	0.6 (<30 vs ≥30)
≥30	23	16 (69.6 %)	7 (30.4 %)	
Gender				
Female	3	1 (33.3 %)	2 (66.7 %)	0.1 (female vs male)
Male	53	40 (75.5 %)	13 (24.5 %)	
Duration of betel quid chewing (years)				
<4	30	24 (80 %)	6 (20 %)	0.2 (<4 vs ≥4)
≥4	26	17 (65.4 %)	9 (34.6 %)	
Duration of disease (years)				
<1	20	15 (75 %)	5 (25 %)	0.8 (<1 vs ≥1)
≥1	36	26 (72.2 %)	10 (27.8 %)	
Histological grade				
E	16	10 (62.5 %)	6 (37.5 %)	0.2 (E vs M)
M	30	24 (80 %)	6 (20 %)	
M	30	24 (80 %)	6 (20 %)	0.5 (M vs A)
A	10	7 (70 %)	3 (30 %)	

E early stage, *M* moderately advanced stage, *A* advanced stage

Table 4 Correlation between the expression of CXCL9 protein and clinicopathological parameters in OLL samples (n=56)

	Cases	CXCL9 (+)	CXCL9 (−)	p value
Age (years)				
<30	33	32 (97 %)	1 (3 %)	0.8 (<30 vs ≥30)
≥30	23	22 (95.7 %)	1 (4.3 %)	
Gender				
Female	3	3 (100 %)	0 (0 %)	0.7 (female vs male)
Male	53	51 (96.2 %)	2 (3.8 %)	
Duration of betel quid chewing (years)				
<4	30	30 (100 %)	0 (0 %)	0.1 (<4 vs ≥4)
≥4	26	24 (92.3 %)	2 (7.7)	
Duration of disease (years)				
<1	20	20 (100 %)	0 (0 %)	0.3 (<1 vs ≥1)
≥1	36	34 (94.4 %)	2 (5.6 %)	

widely and was recently reviewed [28]. CXCL9, an important marker of immunogenicity, is secreted primarily by various kinds of inflammatory cells upon stimulation with interferon-gamma during inflammatory and immune processes. CXCL9 has been strongly implicated in the recruitment of activated $CD4^+$ and $CD8^+$ T cells, NK cells, and plasmacytoid and dendritic cells in several immunological diseases including Graves' disease, type I diabetes mellitus, Addison's disease, psoriasis, skin lichen planus, and vascular gland nephritis. CXCL9 signals through a G-protein-coupled receptor and can be mediated via JAK–STAT signaling pathway [29–32].

In the present study, we showed for the first time that there was progressive expression of CXCL9 protein in inflammatory cells of the superficial layer in connective tissue from normal mucosa to OSF to concomitant OLL. One of the hallmarks of OSF histopathological features is a juxta-epithelial and diffuse mononuclear cell infiltration in the lamina propria. Accordingly, in present study, the enhanced expression of CXCL9 was found in the inflammatory cells of lamina propria of OSF, indicating that CXCL9 might contribute to an ascending chemotactic gradient in the sub-epithelial connective tissue and the pronounced recruitment of inflammatory cells in OSF. Furthermore, our data showed

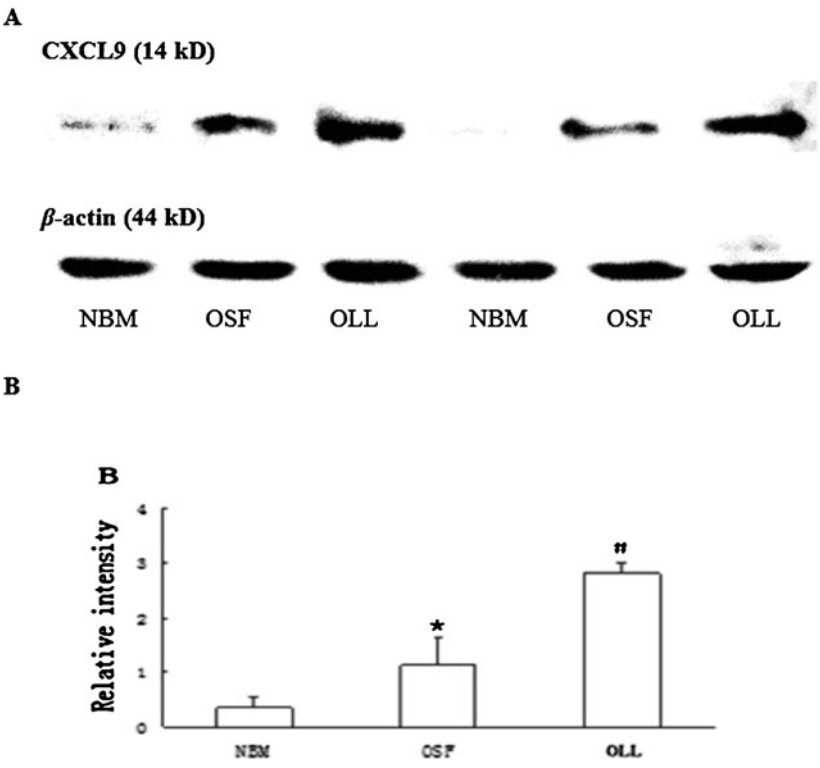

Fig. 2 Western blotting analysis of CXCL9 expression among NBM, OSF, and OLL. **a** Representative blots of CXCL9 among NBM, OSF, and OLL were presented. **b** Quantitative analysis of CXCL9 protein using data obtained from all Western blotting results was shown. Staining for CXCL9 protein revealed gradually increasing levels from NBM to OSF to OLL. *p<0.05 vs. NBM , $^{\#}p$<0.05 vs. OSF

that CXCL9 protein was present in endothelial cells of OSF microvessels, leading us to hypothesize that the expression of CXCL9 in endothelial cells promotes infiltration of inflammatory cells and their adherence to endothelial cells. This might have a detrimental effect in the repair process of the vasculature and result in the eventual atresia of OSF microvessels. Meanwhile, no relationship was found between CXCL9 expression and the pathological grades of OSF, indicating that the CXCL9 expression might be persistent in the whole process of OSF development. In addition, persistent inflammatory reaction in the juxta-epithelial tissue of OSF, which is maintained and amplified by CXCL9, could result in the gradual fibrosis of subepithelial connective tissue [33–35]. In our Western blot analysis, some NBM samples can detect weak CXCL9 expression, while no positive expression of CXCL9 protein was found in NBM by immunohistochemical analysis. The sensitivity of anti-CXCL9 used in the present study might be higher in Western blot analysis than in immunohistochemical analysis. However, the gradually upregulated trend of CXCL9 expression from NBM to OSF to OLL shown in Western blot was consistent with the results in immunohistochemistry.

The concomitant and localized OLL in OSF cases, in our present study, demonstrated an upsurging CXCL9 staining in mononuclear cells of subepithelial inflammatory infiltration, suggesting that this area of mucosa could be a more sensitive "trigger point" subjected to an extremely strong immunoreactivity during the chronic development of OSF lesion. So far, there is still few reference related to OLL and CXCL9. Imanguli [36] observed that increased expression of CXCL9 in submucosal infiltrating cells and keratinocytes of OLL among chronic graft-versus-host disease patients supported CD8 immigration, proliferation, and cytotoxic differentiation. In the present study, we did not find clearly stained CXCL9 in the epithelial cell layers of concomitant OLL in OSF which would indicate some special characteristics for this type of OLL compared with simple OLL lesions. In addition, only 3 of the 56 paired samples were from females of median age just about 30 years old. This differs from the classic clinical characteristics of simple OLL which occurs frequently in the fifth decade of life and is more common in females [23]. The main reason may lie in the fact that the vast majority of betel quid chewers are young males. Moreover, we consider that the intensive recruitment of inflammatory cells to subepithelial tissue of OLL lesion by CXCL9 protein could initiate the onset of the particularly pathological features of OLL, including the band-like zone of lymphocyte infiltration and the ultimate liquefaction of the basal layer. In the present study, we excluded OLL lesions likely from allergies to metal restoration and use the OLL related to OSF as positive control just in order to assess whether the betel quid stress could cause inhomogeneous damages for different areas of the oral mucosa through some immune-related mechanisms. We found OLL lesion in OSF mucosa was an obviously intensive area of immunological reaction, which made a possible foundation for our further research on whether the cancerization of OSF mucosa might come from the malignant change of OLL in OSF. In fact, as a premalignant lesion, OLL has been considered to have a more increased risk of development of oral cancer than other precancerous lesions [9, 21]. Whether the intensive CXCL9 expression in concomitant OLL lesion on OSF mucosa could serve as an essential switch to trigger other immune-related mechanisms for malignant change is still an unanswered question needing further research.

Conclusions

In summary, we for the first time found that there was an upregulated trend of CXCL9 expression from NBM to OSF to OLL, indicating that there was a continuously enhanced intensity of immunoreactivity in their common immunological pathogenic process, which was possibly caused by migrating lymphocytes continuously recruited by CXCL9 protein. Therefore, CXCL9 might be a novel immunological factor in the pathogenesis of OSF and its concomitant OLL.

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Conflict of interest The authors declare that they have no conflict of interest.

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