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ORIGINAL ARTICLE

Expression of Fas/FasL in patients with oral lichen planus

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Summary

Purpose: *To investigate the malignant potential of oral lichen planus (OLP) on the basis of expression of the Fas/FasL markers in healthy individuals (H), OLP patients and patients with squamous cell carcinoma (SCC).*

Patients and methods: *40 patients with OLP and two control groups were included in this research (H and patients with SCC). Immunohistochemistry for Fas and FasL was carried out using an avidin-biotin peroxidase complex method.*

Results: *Only a low percentage of infiltrating lymphocytes and no keratinocytes were Fas-positive in OLP specimens. The highest percentage of Fas-staining keratinocytes in our survey was identified mostly in H and patients with well-differentiated SCC. In most cases of SCC, OLP and H a high percentage of keratinocytes and lymphocytes were FasL-positive. FasL expression was negatively correlated with the degree of cell differentiation and apoptosis. Taking into consideration that all carcinomas in this survey were highly differentiated, it is not surprising that no statistically significant differences in FasL expression between H, OLP and SCC specimens were detected.*

Conclusion: *Downregulation of Fas expression in keratinocytes and lymphocytes of OLP specimens, together with upregulation of FasL, may serve as initial prognostic biomarker in oral cancer development.*

Key words: Fas, FasL, oral lichen planus, premalignant lesions

Introduction

According to the current definition, precancerous lesion is "a morphologically altered tissue in which cancer is more likely to occur than in apparently normal counterpart" [1]. Cancer develops as a result of the accumulation of genetic errors in the same tissue: the activation of oncogenes and the inactivation of tumor suppressor genes [2]. Oral cancer and, in particular, SCC have been repeatedly linked to apoptotic dysregulations [3]. The presence of genetic changes in precancerous lesions of the oral mucosa underscores the significance of apoptotic deviations during the early steps of malignant transformation [4]. The possible malignant transformation of lichen planus (LP) still remains a subject of contraversional discussions in the literature [5]. The risk of malignant transformation varies between 0.4 and 5% over periods of observation from 0.5 to 20 years, and seems to be independent of the clinical type of OLP or the treatment used [6]. OLP is a T-cell mediated chronic inflammatory oral mucosal disease of unknown etiology. Autoimmunity is considered to be the most probable cause of OLP [7]. Colloid bodies are considered to be among the earliest pathologic changes in OLP, and they, most probably, represent the products of apoptosis [8].

Apoptosis, as a form of cell death, is characterized by morphological as well as biochemical criteria and can be considered as a counterpart of mitosis [9]. At the molecular level, the apoptotic pathway best understood involves that initiated by "death receptors" - Fas/CD95. Fas (CD95/APO-1) is a cell-surface member of the tumor necrosis factor (TNF) receptor superfamily and mediates apoptosis upon engagement by its ligand-FasL [10]. Fas is widely expressed in numerous different cell types throughout the body, whereas FasL expression appears to be more restricted. Following activation,

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different cell types within the immune system express FasL, including T and B cells [11]. There is evidence that FasL precludes inflammatory reactions from sites of immune privilege by triggering Fas-mediated apoptosis of infiltrating pro-inflammatory cells. The ability of FasL to impair immune responses is being pursued as a possible means of protecting tissue transplants from immunological rejection, and therapeutic promise has been reported in some experiments [12].

The immune system appears to recognize some cellular changes during progression from benign to malignant status, and it desperately try to respond [13]. FasL- expressing T cells attempt to engage Fas on the tumor cells, in order to initiate tumor cell apoptosis. As a countermeasure, larger number of tumor cells express FasL, which may enable them to induce apoptosis in tumor infiltrating T cells. The tumor cells also have decreased Fas expression, which would have reduce their susceptibility to the increased number of FasL-expressing T cells. Thus, dynamic changes in T cell killing mechanism occur during the final stage of carcinogenesis: progression of benign lesions into malignancy [14].

We hypothesized that Fas-mediated apoptosis of keratinocytes and lymphocytes could show progression from healthy oral tissue, through premalignant lesions (lichen planus), to oral squamous cell carcinoma (SCC). The aim of this study was to investigate the malignant potential of OLP on the basis of expression of the Fas/FasL markers in keratinocytes and lymphocytes of OLP patients, H, and patients with SCC.

Patients and methods

Patients

Tissue samples were obtained from 40 patients with OLP, 13 H and 12 patients with SCC. All specimens were examined in the Laboratoire Central d' Anatomie Pathologique, Professor J-F. Michiels, Centre Hospitalier Universitaire de Nice, France.

Methods

Immunohistochemistry was carried out using an avidin-biotin peroxidase complex method [15]. For immunostaining with antigen retrieval, citrate buffer (pH 6.0) solution was used. Tissue sections were transferred to a beaker containing each buffer solution and incubated at 95º C in a microwave oven for 17 min to unmask the site of antigen. After taking away from the microwave oven, the tissue sections were left for 20 min in a beaker at room temperature. Then, they were rinsed with PBS and incubated with 0.3% H_2O_2 for 15 min to block the endogenous peroxidase. The tissue sections were then incubated with normal goat serum for Fas and FasL staining. They were treated overnight at 4º C with a mouse monoclonal antibody against Fas (APO-1, DAKO A/S, Denmark) – dilution 1/10 and FasL (KAY-10, Santa Cruz Biotech. Santa Cruz, CA, USA) – dilution 1/20.

Afterwards, the samples were incubated with biotinylated animal-matched secondary antibodies (DAKO A/S, Denmark) at room temperature, and after rinsing with PBS, they were incubated again with avidin-biotin peroxidase for 45 min. Protein expression was visualized using a kit (DAKO) with diaminobenzidine (DAB)-H_2O_2 substrate complex. Each section was left in the DAB solution up to 15 min, and counterstained lightly with Mayer's hematoxylin (Hemalun). PBS was used for all washings between the applications of the staining reagents and also as a diluent buffer for the antibodies.

In OLP patients biopsies were taken from visible oral lesions.

Two control groups were included in this research. In the first one, consisting of 13 H, immunohistochemical examination was carried out in oral mucosa specimens without inflammatory changes. The immunohistochemical procedure was the same as for patients with OLP. In the second control group, immunohistochemical examination was carried out in oral squamous cell carcinoma (SCC) specimens taken from 12 patients with SCC. Immunohistochemistry was also the same as for patients with OLP.

Positive controls for the antibodies mentioned above were: Fas - breast carcinoma (basal membrane, cytoplasmic) and FasL - prostate (basal membrane, cytoplasmic). Staining was considered positive if the nuclear, cytoplasmic or basal membrane staining of the mucosal epithelium cells of OLP was compatible with that of positive control. For negative control, the same procedure was carried out with normal serum instead of each antibody.

Immunohistochemical measurement parameters included total tissue area and total stained area. 500 keratinocytes or lymphocytes were randomly counted in the epithelium (basal and prickle cell layer) and submucosa. Semiquantitative evaluation was performed for each antibody used.

Fas

0 (Negative)
Level 1 (<1% of cells stained with Fas protein)
Level 2 (1-5% of cells stained with Fas protein)

Level 3 (5-25% of cells stained with Fas protein)
Level 4 (25-50% of cells stained with Fas protein)

Fas-L

0 (Negative)
Level 1 (1-5% of cells stained with Fas-L protein)
Level 2 (5-25% of cells stained with Fas-L protein)
Level 3 (25-50% of cells stained with Fas-L protein)
Level 4 (50-100% of cells stained with Fas-L protein)

Each specimen was fixed in formalin for 2-3 days and embedded in paraffin. The sections (4 μm thick) were deparaffinized with xylene and rehydrated with graded ethanol series.

Comparison of differences between the examined groups was carried out using Fisher t-test and Fisher exact test for pairs.

Results

The keratinocytes of OLP (Figure 1C) patients did not show any immunoreactivity for Fas protein, whereas the keratinocytes of H (Figure 1A,B) and patients with SCC (Figure 1E) showed a high percentage of Fas staining reaction (Table 1). Significant differences in the percentage of cells positive for Fas protein were found between the OLP patients vs. H ($p < 0.001$) and vs. SCC patients ($p < 0.001$). No difference was seen between H and SCC patients ($p=0.64$).

In contrast to keratinocytes, positive immunoreactivity for Fas protein was seen in lymphocytes of OLP patients (Figure 1D). Nevertheless, only a low number of the examined OLP specimens exhibited positive Fas staining on the basal membrane and cytoplasm of infiltrating lymphocytes (22.5%) compared with SCC specimens (58.3%; $p < 0.001$; Figure 1F).

FasL protein was detected in the cytoplasm of keratinocytes and lymphocytes in all 3 examined groups. In contrast to Fas-unstained keratinocytes, high percentage of FasL positive keratinocytes (level 3+) was seen in most of the examined OLP (Figure 2C, Table 2) specimens. Besides, the keratinocytes of H (Figure 2A) and SCC (Figure 2E) specimens were also stained in high percentage with FasL protein in most of the examined cases. No statistical difference between those 3 groups was observed ($p=0.34$). In all examined specimens of H, OLP and SCC, FasL was localized in the basal cell layer, although it was occasionally present in the prickle cell layer, too.

In most cases of SCC (83.33%; Figure 2E), OLP (57.5%; Figure 2D) and H (84.62%; Figure 2B), a high percentage of lymphocytes (level 3+) was FasL-positive. This protein was also distributed in the interstitial tissue of the lamina propria mucosae, beneath the epithelium.

Discussion

Our results indicate that Fas expression in keratinocytes was significantly downregulated in OLP patients. Downregulation of Fas expression could be an early event in oral carcinogenesis in an attempt to escape immune surveillance and destruction by activated cytotoxic T cells [16,17].

Fas-positive cells were found mostly in the cellular infiltrate (CI) of OLP, which is in accordance with the results of Dekker et al. [18]. The low number of OLP specimens (n=12) in this study showed Fas-positive reaction throughout the inflammatory CI. In the majority of those cases (10 out of 12) it was noticed low to medium degree of CI. A negative correlation between the percentage of Fas-positive lymphocytes and the degree of CI was proven. According to our results, it seems that apoptotic mechanisms eliminate more lymphocytes in OLP cases with Fas-positive CI than in OLP specimens with Fas-negative CI. On the other hand, our results showed positive correlation between FasL expression in lymphocytes and the degree of CI. Downregulation of Fas and upregulation of FasL-expressing lymphocytes could represent a simultaneous process by which lymphocytes evade Fas-mediated cell death [19,20]. It has been suggested and experimentally supported that aberrations in apoptotic mechanisms may result in failure to eliminate autoimmune lymphocytes, thus driving the appearance of autoimmune diseases [21]. Additionally, mutations affecting genes that code for crucial apoptotic mediators, such as Fas and FasL, have been correlated with autoimmune diseases [22].

Neppelberg et al. showed high expression of Fas and a low rate of apoptosis in the subepithelial CI of OLP [23]. These findings might indicate that, despite enabled Fas-mediated apoptosis, the signal for apoptotic cell death is not transmitted further into the cell. They also believe that escape of Fas-induced apoptosis could be part of the explanation for the formation of massive inflammatory infiltrates in OLP. Besides, elimination of autoreactive lymphocytes through Fas/FasL mediated apoptosis may be blocked by an upregulation of soluble Fas (sFas; increased levels of sFas can be detected in serum) [24]. According to the results of Aliev et al. the mean level of sFas is similar in patients with lichen planus and SCC, but significantly higher in comparison to healthy controls [25].

In this survey all examined oral SCC were Fas-positive and well-differentiated. Muraki et al. detected high level of Fas protein in highly differentiated SCCs, moderate Fas expression in moderately differentiated tumors and no Fas positive cells in the poorly differentiated tumors [26]. That means that the expression of Fas antigen is related to the degree of tumor differentiation.

The results of the present study showed that all examined groups similarly expressed FasL in the cytoplasm of keratinocytes and lymphocytes. FasL ex-

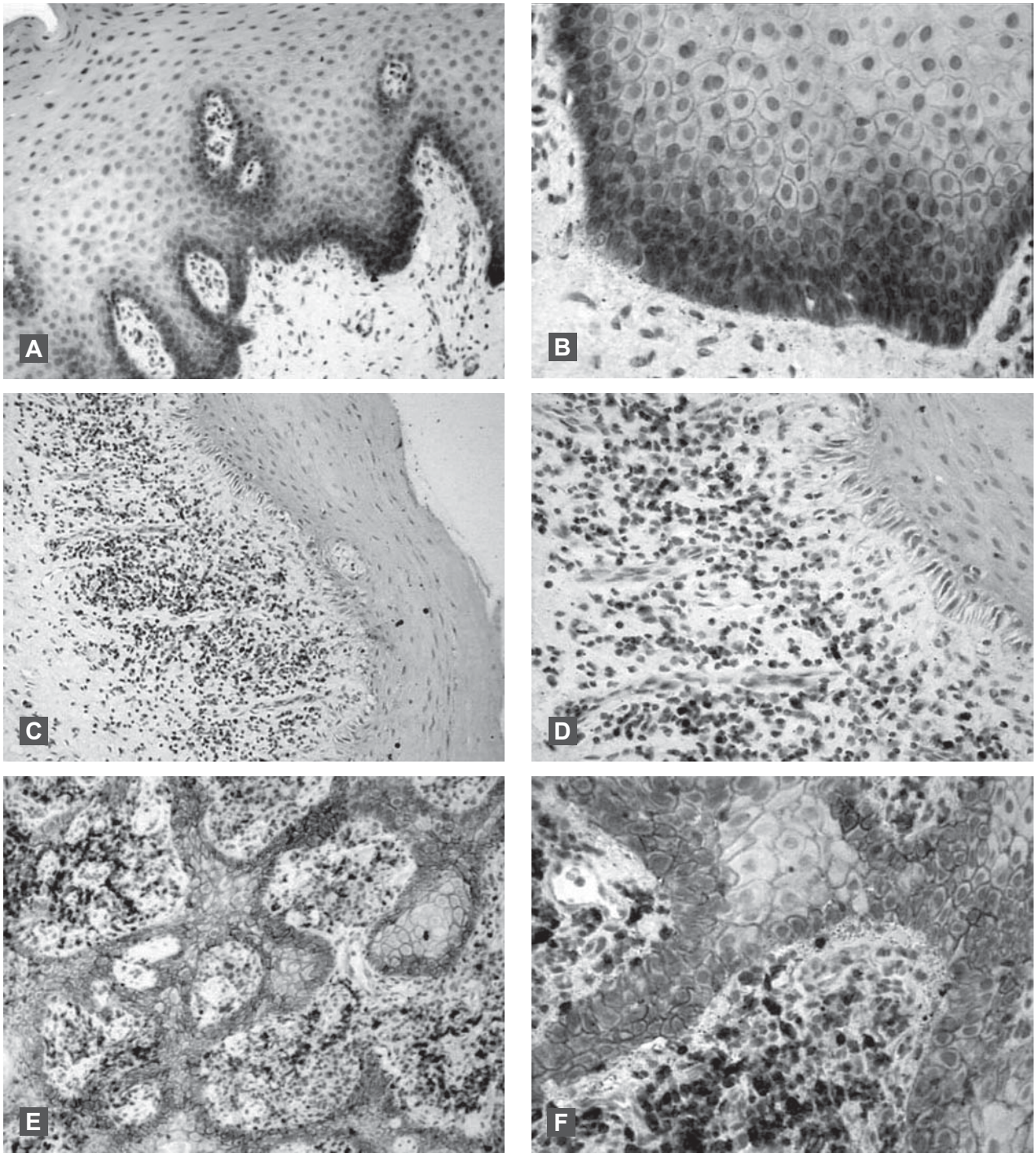

Figure 1. Immunohistochemical localization of Fas antigen. **A:** (healthy control) High percentage of keratinocytes intensively marked with Fas protein on the cell membranes of the basal layer (× 200). **B:** (healthy control) The Fas antigen is stained as granules around the cytoplasm (× 400). **C:** (oral lichen planus) There are no keratinocytes marked with Fas in OLP epithelium. The majority of the mononuclear cells in the subepithelial cell infiltrate express Fas antigen (× 200). **D:** (oral lichen planus) A high percentage of lymphocytes is weakly to moderately stained with Fas marker (× 400). **E:** (oral squamous cell carcinoma) A high percentage of cells are stained with Fas antigen (× 200). **F:** (oral squamous cell carcinoma) Intensive expression of Fas is also seen in the infiltrating lymphocytes of the lamina propria mucosae (× 400).

pression correlates negatively with the degree of cell differentiation and apoptosis [27]. Indeed, Chen and coworkers established that the majority of invasive carcinomas show upregulation of FasL when compared with premalignant lesions, implying that tumor cells may counteract the immune system by killing Fas-expressing cytotoxic T cells [16]. The overexpression of FasL is closely related to the progression of OLP [28]. In general, the degree of FasL expression concurred with the degrees of dysplasia. Taking into consideration that

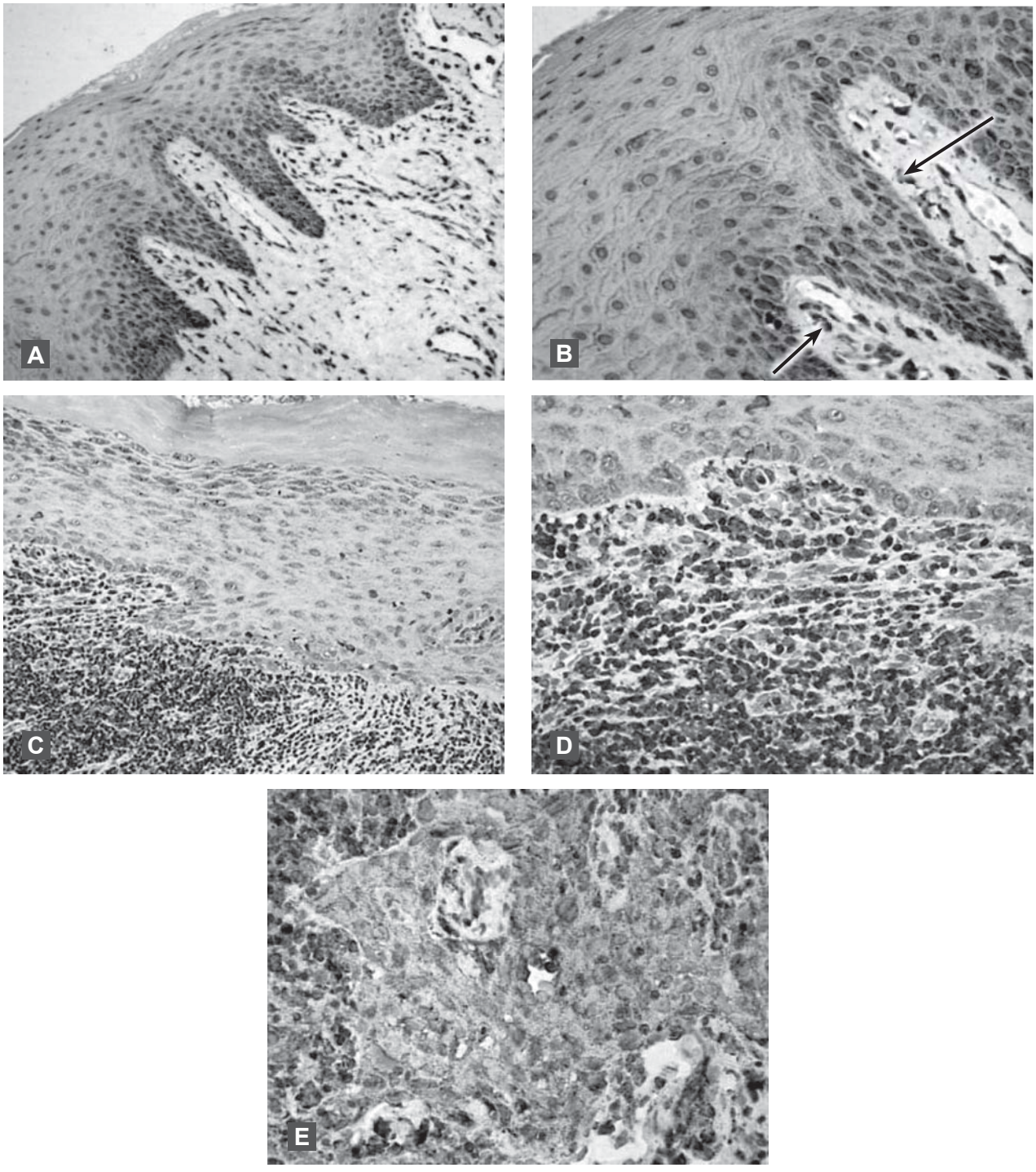

Figure 2. Immunohistochemical localization of FasL antigen. **A:** (healthy control) FasL staining cells are present mainly in the basal cell layer, with a weak reaction in the prickle cell layer (× 200). **B:** (healthy control) FasL is also expressed in the infiltrating lymphocytes of the lamina propria mucosae (arrows; × 400). **C:** (oral lichen planus) FasL staining is observed throughout the epithelium, but mostly in the prickle cell layer (× 200). **D:** (oral lichen planus) A high percentage of infiltrating lymphocytes is intensively stained with FasL protein (× 400). **E:** (oral squamous cell carcinoma) Intensive FasL staining is present in high percentage of cells (× 400).

Table 1. Percentage of keratinocytes stained with Fas protein in patients with squamous cell carcinoma (SCC), oral lichen planus (OLP) and healthy persons (H)

Variable	*SCC*		*OLP*		*H*	
	n	*%*	*n*	*%*	*n*	*%*
0 Fas-negative	4	33.33	40	100	2	15.38
1 Fas (<1 %)	0	0	0	0	0	0
2 Fas (1-5%)	2	16.67	0	0	2	15.38
3+ Fas (5-25%)	6	50	0	0	9	69.24
Total	12	100	40	100	13	100
p* < 0.001						

*Fisher test; Fisher exact test for pairs: SCC vs. OLP: $p < 0.001$, SCC vs. H: $p = 0.67$, OLP vs. H: $p < 0.001$

Table 2. Percentage of keratinocytes stained with FasL protein in patients with squamous cell carcinoma (SCC), oral lichen planus (OLP) and healthy persons (H)

Variable	*SCC*		*OLP*		*H*	
	n	*%*	*n*	*%*	*n*	*%*
0 FasL-negative						
1 FasL (1-5 %)	4	33.33	16	40	4	30.77
2 FasL (5-25%)	3	25	2	5	1	7.69
3+ FasL (25-50%)	5	41.67	22	55	8	61.54
Total	12	100	40	100	13	100
p* = 0.34						

*Fisher test; Fisher exact test for pairs: SCC vs. OLP: $p < 0.13$, SCC vs. H: $p = 0.77$, OLP vs. H: $p < 0.42$

all SCCs in this survey were highly differentiated, it is not surprising that statistically significant difference in FasL expression between H, OLP and SCC specimens was not reached.

Even though we established some quantitative differences in Fas/FasL expression between H, OLP and SCC specimens, we cannot give an exact estimation of the malignant potential of OLP. Nevertheless, downregulation of Fas expression in keratinocytes and lymphocytes of OLP specimens, together with upregulation of FasL may serve as initial prognostic biomarker in oral cancer development.

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