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ORIGINAL ARTICLE

Expression of ADAM10, Fas, FasL and Soluble FasL in Patients with Oral Squamous Cell Carcinoma (OSCC) and their Association with Clinical-Pathological Parameters

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Abstract ADAM10 has been implicated in the progression of various solid tumors. ADAM10 regulates the cleavage of the FasL ectodomain from the plasma membrane of different cell types, generating the soluble FasL fragment (sFasL). Currently, there are few studies in oral squamous cell carcinoma (OSCC) that correlate levels of ADAM10 and FasL in the tumor microenvironment with clinical parameters of the disease. To determine the expression of ADAM10, Fas, FasL and sFasL in patients with OSCC and its association with TNM stage. Twenty-five patients with OSCC and 25 healthy controls were included. Biopsies of tumor tissue from patients with OSCC and buccal mucosa in controls were obtained. ADAM10, Fas, and FasL were analyzed by Western blotting. sFasL was quantified by ELISA. ADAM10 and Fas decreased significantly in OSCC compared with controls. Relatedly, within the OSCC group, Fas and ADAM10 decreased in accordance with tumor disease stage; in stages I/II, as well as in tumors of smaller diameter (T1-T2), ADAM10 showed higher levels when compared to patients with T3-T4 tumors and in stage III-IV. FasL in the tumor microenvironment and serum FasL showed no significant differences between both groups. Levels of complete FasL and cleaved FasL were positively correlated in controls; this correlation is preserved in patients with tumors in early stages (I-II), but is lost in later stage (III-IV). The dysregulation of ADAM10, Fas and FasL could be useful indicators of the progression and severity of OSCC.

Keywords Oral cancer · ADAM10 · sFasL · Fas · FasL

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Introduction

Oral squamous cell carcinoma (OSCC) is an important health problem, and represents the most common malignancy in the head and neck region. In 2012, the estimated annual incidence of this disease was approximately 299,000 cases [1]. Despite recent advances in the field of oncology, the prognosis for OSCC remains poor [2]. Greater understanding of the biology of OSCC has the potential to facilitate the diagnosis, staging and monitoring of this disease.

It is well known that ADAM enzymes (A Disintegrin And Metaloproteinase), a family of 21 proteins, which belong to the family of the metzincins, contain a metalloproteinase domain. ADAM enzymes have been implicated in various physiological events such as adhesion, cell migration and fusion, and are also involved in proteolysis and ectodomain cleavage of different

cell surface receptors [3]. In turn, ADAM10 can be processed by proteolytic action of ADAM15 and ADAM9, generating a 55 kDa soluble form of ADAM10 [4]; however the functional role of this cleaved form of ADAM10 is not clear. ADAMs have been found to be involved in the development of several pathologies; in cancer they favor tumor promotion, growth and invasion by regulating the activity of growth factors, adhesion molecules, cytokines and angiogenesis [5].

There is evidence that some ADAMs, including ADAM10, are overexpressed in various malignancies, including OSCC [6, 7]. ADAM10 is an important regulator of the proteolytic processing of at least 40 substrates, one of which is Fas ligand [8]. Fas ligand (FasL) belongs to the TNF family. FasL triggers extrinsic apoptosis through its receptor (Fas) [9]. It has been shown that modulation of apoptosis by ADAM10 is dependent on Fas/FasL interactions, as ADAM10 is principally responsible for the cleavage of the FasL ectodomain from the plasma membrane of some cell types, inhibiting FasL-mediated apoptosis and generating a portion of soluble FasL (sFasL) [10, 11]. Several studies indicate that there is an association between levels of sFasL and progression of several types of malignant tumors [12]. To date, there have been few studies in oral squamous cell carcinoma (OSCC) that correlate levels of ADAM10, FasL, and sFasL in the tumor microenvironment with clinical and pathological parameters of the disease.

Material and Methods

Ethical Considerations The study was approved by the Ethics Committees of the Jalisco Cancer Institute (Instituto Jalisciense de Cancerología) and the University of Guadalajara (Universidad de Guadalajara). The experiments were carried out in accordance with the principles of the Declaration of Helsinki. All participants signed a document signifying informed consent in order to participate in the study.

Patient Information The present study included 25 OSCC patients recruited from the Oncological Surgery Clinic of the Jalisco Cancer Institute. Tumor tissues were collected before the patients received chemotherapy or radiotherapy. The 25 healthy subjects were recruited with the assistance of the Oral Surgery Department at the University of Guadalajara; a biopsy of buccal mucosa was obtained from these controls. Both groups were recruited from 2012 to 2015. In the group of patients with OSCC, 18 men and 7 women, with an average age of 61.2 years (range 31–87 years) were included. Patients were diagnosed according to histological grade as well-differentiated (n = 5), moderately differentiated (n = 12) and poorly differentiated (n = 4), and according to the TNM stage I (n = 2), stage II (n = 4), stage III (n = 6) and stage IV (n = 13). Histological grade and staging were performed in accordance with the TNM system and the WHO histological differentiation guidelines.

OSCC Specimens and Control Samples In both study groups, tissue samples were obtained by biopsy punch of 5 mm. for T3 and T4 tumors, two or more biopsies were obtained. The fragments were stored at −80 °C until use for extraction and analysis of proteins.

Serum Collection The blood samples of all participants in the study were collected in Vacutainer tubes (BD, USA) without additives. The tubes were kept at room temperature for 10 min and then centrifuged at 1500 rpm for 15 min. The serum was placed in 2 ml tubes (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until use.

Western Blotting Detection of ADAM10, FasL and Fas was performed by Western blotting. Proteins were extracted from oral mucosa homogenates using RIPA buffer (SIGMA R 0278; Sigma, NJ, USA). Proteases inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and clarified by centrifugation at 4 °C, 12,000 rpm for 15 min. Protein concentration was determined by the Bradford method (Coomassie brilliant blue G-250, 95 % ethanol, concentrated phosphoric acid and water). The total protein (25 μg) was mixed with loading buffer under reducing conditions, electrophoresed on 7.5–12 % SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad, CA, USA). Non-specific binding was blocked with 3 % non-fat dry milk. Subsequently, membranes were incubated with primary antibodies overnight: anti-ADAM10 (1:500) and anti-FasL (1:200), both from Santa Cruz Biotechnology and anti-Fas (1:500) (Thermo Fisher Scientific Waltham, MA, USA). HRP-conjugated anti-mouse or anti-rabbit secondary antibodies were used to reveal the blots: anti-mouse IgG (1:1000) and anti-rabbit IgG (1:1000) were from Santa Cruz Biotechnology. Immune detection was performed using a chemiluminescence imaging system Microchemi 4.2 (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). Densitometric analysis was performed with the Gelquant v12.2 (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). As an internal control to confirm that similar amounts of protein were loaded into each lane, β-actin levels were determined using a monoclonal anti-actin IgG (1:200) (Santa Cruz Biotechnology) and were revealed with anti-mouse IgG peroxidase (also from Santa Cruz Biotechnology).

Enzyme-Linked ImmunoSorbent Assay The quantitative determination of soluble Fas ligand in the serum of patients and controls was performed in triplicate using an ELISA kit (DFL00 R&D Systems, MN, USA) in accordance with the manufacture's instructions. Signal was detected using a Microplate Reader WHY101 (Poweam Medical Systems Co., Nanjing, Jiangsu, China).

Statistical Methods Statistical analysis was performed with SPSS Statistics software version 20.0 (SPSS, Inc., Chicago, IL, USA). Normally distributed variables were analyzed using one-way analysis of variance (ANOVA) or the Student t test. For the analysis of correlations of continuous variables, the Pearson coefficient were calculated. Data are shown as the mean ± Standard deviation (SD) of the mean. Only p values ≤0.05 were considered significant.

Results

ADAM10 Protein Expression ADAM10 was present at significantly higher levels in controls with respect to patients with OSCC. This pattern was observed both with the soluble or cleaved form (55 kDa) and the complete form (65 kDa) (Fig. 1a, b, c).

Although no significant differences were found when analyzing ADAM10 levels between each of the TNM stages, when the OSCC patients were sub-grouped according to disease progression into early stages (I and II) or advanced stages (III-IV), an important distinction was found. The expression levels of ADAM10 were similar between control subjects and patients in early stages of the disease, while we found a statistically significant decrease when we compared the advanced stages group to the controls (Fig. 2a, b).

One of the most important clinical indicators of prognosis in OSCC is tumor diameter. As observed with TNM stage, the values of ADAM10 in patients with smaller tumors (T1-T2), were similar to controls, whereas tumors of larger diameter (T3-T4) exhibited significantly lower levels of protein compared to the controls and (T1-T2) (Fig. 2c, d).

When the other clinical and demographic characteristics of OSCC patients were evaluated with respect to the expression of ADAM10, the only significant difference was found to be that of gender. Levels of ADAM10 were found to be significantly decreased in men as compared to women. This is consistent with the observation that the majority of the patients

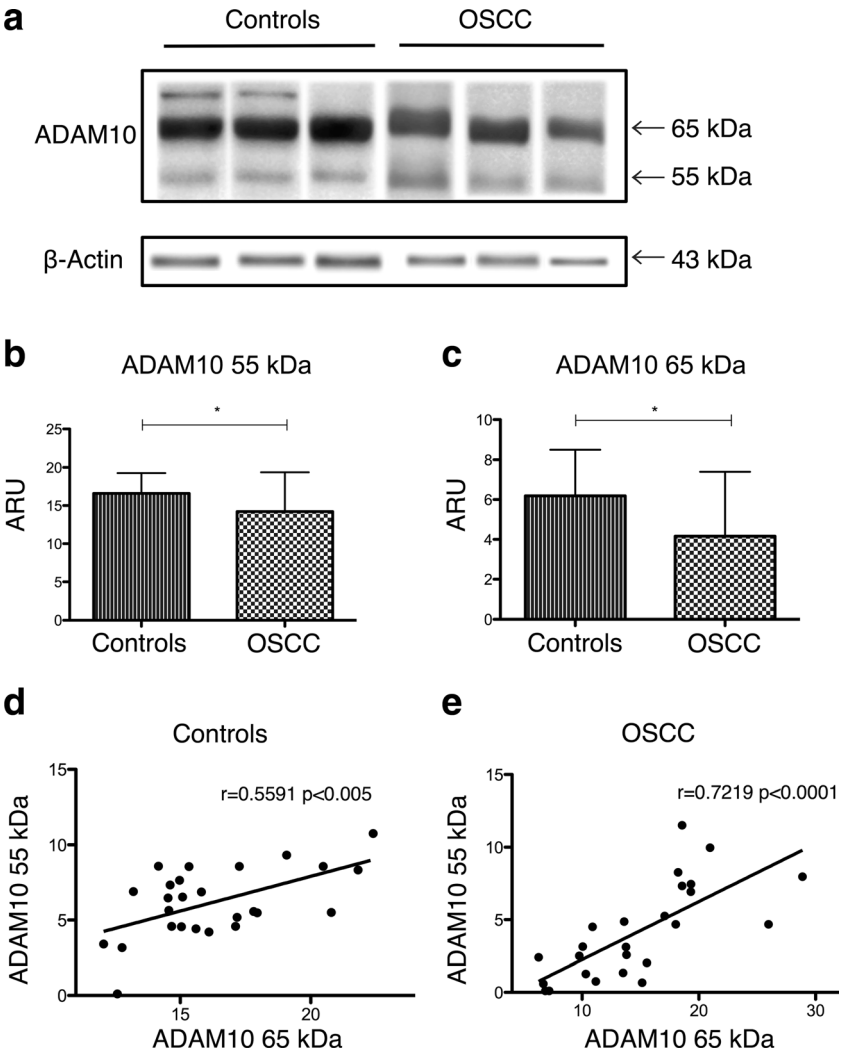

Fig. 1 ADAM10 protein determination (65 kDa and 55 kDa) in homogenized tissue by Western blot. (**a**) Representative blot of controls and OSCC samples. Observed full bands of ADAM10 (65 kDa) and cleaved ADAM10 (55 kDa). Actin was used as an internal control; (**b**) ADAM10, (55 kDa) protein quantification, $*p = 0.013$ controls vs. OSCC patients; (**c**) ADAM10 (65 kDa) protein quantification $*p = 0.048$ controls vs. OSCC patients. Each bar represents the mean value ± Standard deviation (SD). Results are expressed as Area Relative Units (ARU); (**d**) Correlation analysis betwen ADAM10 (55 kDa) and ADAM10 (65 KDa), in controls, $p = 0.0030$, $r = 0.5591$ and (**e**) Correlation analysis between ADAM10 55 kDa and ADAM10 65 KDa, in OSCC patients, $p < 0.0001$, $r = 0.7219$. OSCC (oral squamous cell carcinoma)

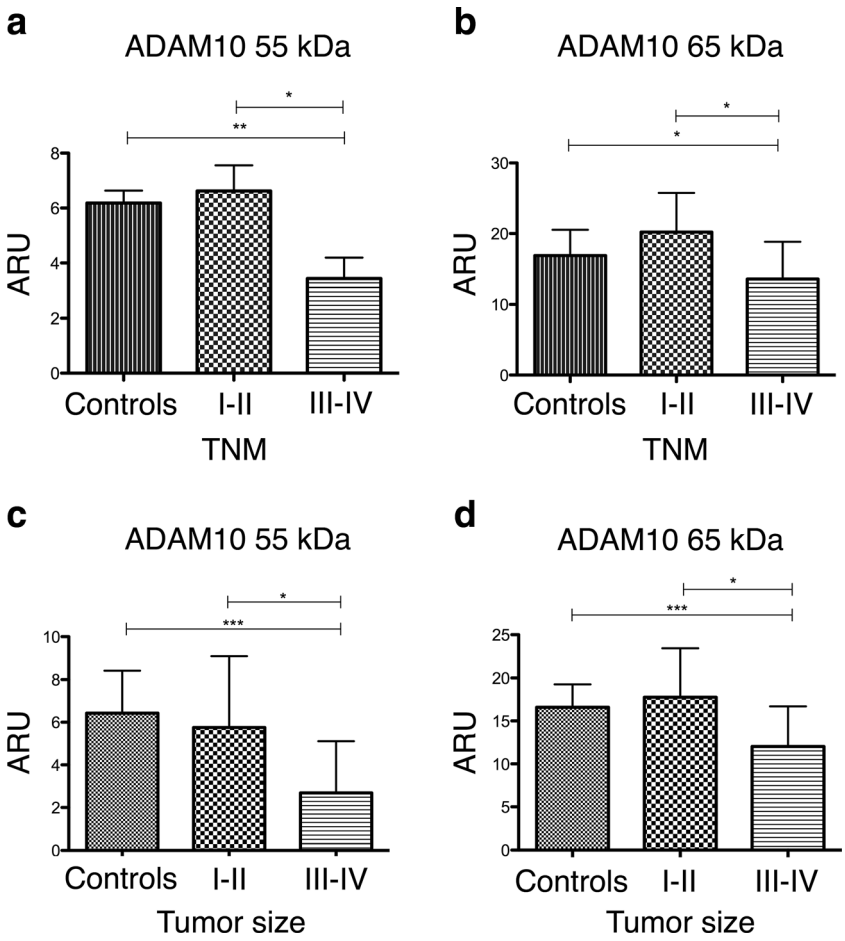

Fig. 2 ADAM10 tissue protein levels (55 and 65 kDa) by Western blot according to TNM stage (TNM: I-II, III-IV) and tumor size (T: T1-T2, T3-T4). (A) ADAM10, protein determination and grouped according to TNM stage. ADAM10, (55 kDa), $^{**}p = 0.0020$ controls vs. III-IV; $^{*}p = 0.0289$ I-II vs. III-IV; (B) ADAM10, (65 kDa), $^{*}p = 0.0158$ controls vs. III-IV; $^{*}p = 0.0215$ I-II vs. III-IV; (C) ADAM10 protein levels and grouped by tumor size, ADAM10, (55 kDa), $^{***}p < 0.0001$ controls vs. T3-T4; $^{*}p = 0.0144$ T1-T2 vs. T3-T4; (D) ADAM10, (65 kDa), $^{***}p < 0.0005$ controls vs. T3-T4; $^{*}p = 0.0144$; $p = 0.0106$ T1-T2 vs. T3-T4. Each bar represents the mean value ± Standard deviation (SD). Results are expressed as Area Relative Units (ARU). OSCC (oral squamous cell carcinoma)

with more advanced tumors are men (Table 1). Additionally, we found a significant positive correlation between the 55 kDa and 65 kDa forms of ADAM10 (Fig. 1d, e). This correlation was found to be tighter in the group of patients with OSCC.

Soluble Fas Ligand Levels in Serum ADAM10 is the main sheddase responsible for the generation of the cleaved form of FasL. In various malignant neoplasms and inflammatory based diseases it has been observed that there is an association between levels of sFasL and damage or disease progression. In this study, no significant differences were found between the levels of sFasL in controls (mean: 58.39 pg/mL) compared to patients with OSCC (mean: 62.04 pg/mL) (Fig. 4). When sFasL levels were compared to other clinical and histopathological variables in the group of patients with OSCC, significant differences were not found (data not shown).

Protein Expression of Fas and FasL In addition to determining the levels of sFasL in serum, we quantified the complete form (40 kDa) and the cleaved form (26 kDa) of FasL in tissue homogenates, but we found no significant differences between controls and OSCC patients (Fig. 3c, d). However, we note that the 26 kDa form of FasL was found to be significantly elevated in patients with stage I-II TNM compared to those with stage III-IV disease (Fig. 3c).

With respect to levels of Fas, we note that there are significantly decreased levels in OSCC patients compared to controls (Fig. 3f). When analyzing the correlation between Fas and FasL in healthy tissue, we observed a significant positive correlation between Fas and both the complete form (40 kDa) and cleaved (26 kDa) form of FasL; this was in contrast to the OSCC patients where we did not find significant correlations (Fig. 3a, b, c, d).

Interestingly, we found that, as in the control subjects, the group of patients with early stage OSCC displayed a strong correlation between Fas and complete FasL (40 kDa); however, in patients with tumors in stages III and IV, this correlation is lost (Fig. 3e, f).

Discussion

The tumor microenvironment plays a complex and dynamic role in solid tumors of epithelial origin. In this context, there is evidence pointing to extra-cellular matrix (ECM) proteinases as central players in modulating the tumor microenvironment [13]. Overexpression of the ADAM10

Table 1 Clinical and pathological characteristics of patients with oral squamous cell carcinoma (OSCC)

Variables		Patients	%
Age	<50	7	(72)
	>50	18	(28)
Gender	Male	18	(28)
	Female	7	(72)
Smoking history	Smoker	10	(40)
	Non-smoker	7	(28)
	Ex-smoker	8	(32)
Alcohol use	Yes	8	(72)
	No	17	(28)
Localization	Tongue	10	(40)
	Tonsil	3	(12)
	Retromolar zone	2	(8)
	Lip	4	(16)
	Floor of the mouth	2	(8)
	Alveolar mucosa	2	(8)
	Palate	2	(8)
Tumor size	T1	2	(8)
	T2	4	(16)
	T3	6	(24)
	T4	13	(52)
Lymph nodes	Yes	15	(60)
	No	4	(16)
	Non-evaluable	6	(24)
Total TNM	I	2	(8)
	II	4	(16)
	III	6	(24)
	IV	13	(52)
Diferenciation*	Well	5	(20)
	Moderate	12	(48)
	Poor	4	(16)
Total		25	100

* Incomplete/not evaluables

metalloproteinase has been observed in oral squamous cell carcinoma (OSCC) [6, 7] and in other malignancies such as pancreatic carcinoma [14], nasopharyngeal carcinoma [15], lung cancer [16], and cervical cancer [17], among others [18]. However, in the previous studies on tumor tissues from patients with OSCC, ADAM10 was quantified only in epithelial cells, using immunohistochemistry, and without regard for the global context of the cells of the ECM, within which the entire tumor grows.

There are few studies that have evaluated the levels and the role of ADAM10 in adult tissues under physiological conditions. Weber et al. analyzed the direct implication of ADAM10 in morphogenesis and stratification of adult keratinocytes through regulation of the Notch signal. That work suggested that ADAM10 is a central regulator in the development and maintenance of epithelial growth and it was also suggested that ADAM10 acts as a tumor suppressor in the skin of adult mice [19].

ADAM10 is not only expressed in epithelial cells, but is also present on the cell surface of lymphocytes [20], endothelium [21], and neurons [22], among other cell types. Actually, some of the major studies about the functionality of ADAM10 and its substrates have been developed using experimentally mouse fibroblast cell lines and dermal fibroblasts [10, 23, 24]. Due to this, it is not surprising that in our work we have observed higher levels of ADAM10 in total tissue homogenates in healthy controls compared to tumor tissues. This sheddase, in tumor cells, promotes degradation of tissue and the ECM. Interestingly, we found that tumors in advanced stages (III-IV) and larger sizes (T3-T4), significantly decreased ADAM10 (Fig. 2c, d). This suggests that the role of ADAM10 in regulating tumor progression and growth is important not only in the ECM, but in other cells that are also involved in the tumor microenvironment.

The positive correlation found between the 65 kDa active form of ADAM10 and the 55 kDa soluble or cleaved form, which was found to be stronger in patients with OSCC (Fig. 1e) indicates that, in this neoplasia, there might exist a tight regulation of the expression and probable enzymatic activity of ADAM10. This suggests the need to further investigate the concept of ADAM9, ADAM15 and gamma secretase as being responsible for the proteolytic processing of ADAM10 in the context of cancer [4].

The study of apoptosis and its role in cancer has been an important subject of many research groups. There is evidence that points to the existence and interaction of Fas/FasL signaling as being responsible for the promotion of tumor activity [12]. Our results showed no significant difference between the complete form (40 kDa), nor soluble form (26 kDa) of FasL, from patients with OSCC with respect to the homogenates of tissues from healthy controls (Fig. 2). However, we did observe increased cleaved FasL (26 kDa) in the tumor microenvironment of patients with TNM stages I-II, with respect to the samples from patients in stage III-IV. These data contrast with those reported in other studies regarding the levels of Fas ligand in malignant tumors. However, it is important to note that we utilized the Western blot as our detection method to analyze the levels of FasL and to date there are no other studies that use this methodology, so that, similarly to ADAM10, we do not have a reference against which to contrast our results. It is known that, under certain conditions, the 26 kDa fragment of FasL blocks extrinsic apoptosis [25]. Relatedly, it also been reported that the Fas/FasL interaction is involved in cell survival [26]. The majority of studies indicate that sFasL is overexpressed in the serum of various malignancies [12]. In this study, we found that although the levels of sFasL were slightly higher in OSCC patients compared with controls, the difference was not statistically significant (Fig. 4) which is consistent with previous

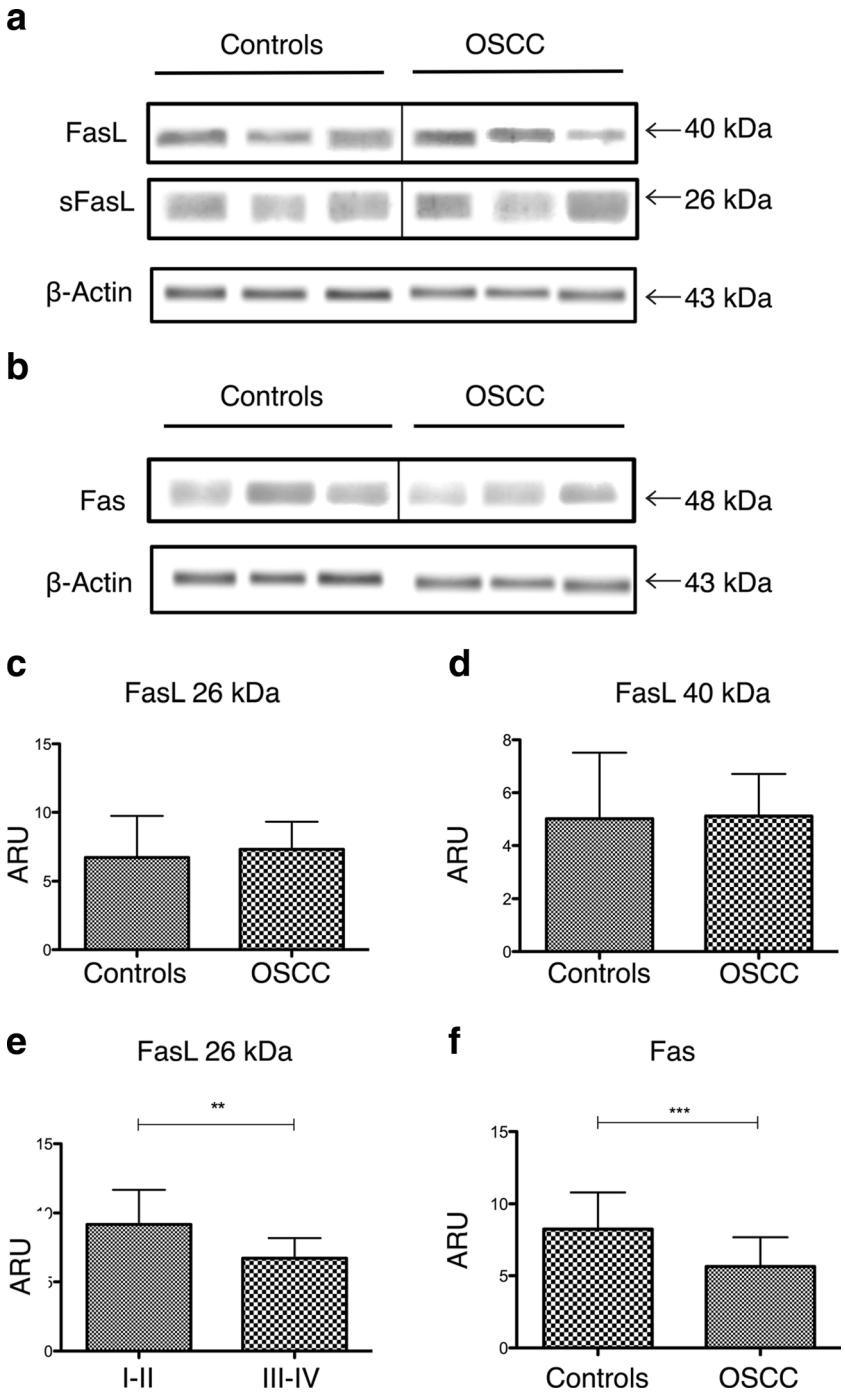

Fig. 3 Fas and FasL proteins in homogenized tissue determined by Western blot. (**a**) Representative Blot of controls and OSCC samples. Observed full bands of FasL (40 kDa) and cleaved sFasL (26 kDa). Actin was used as an internal control; (**b**) Representative Blot of controls and OSCC samples. Observed bands of Fas (48 kDa). Actin was used as an internal control; (**c**) sFasL 26 kDa protein quantification, $p = 0.4340$ controls vs. OSCC patients; (**d**) FasL (40 kDa) protein levels, $p = 0.5655$ controls vs. OSCC patients; (**e**) sFasL (26 kDa) and TNM stage, $^{**}p = 0.0066$ I-II vs. III-IV; (**f**) Fas protein levels, $^{***}p = 0.0002$ controls vs. OSCC patients. Each bar represents the mean value ± Standard deviation (SD). Results are expressed as Area Relaltive Units (ARU). OSCC (oral squamous cell carcinoma)

studies of sFasL in patients with head and neck carcinoma [27] and chronic lymphocytic leukemia [28].

In contrast with the Western blot detection of FasL, which allows the identification of isoforms or incomplete forms of the protein, the ELISA technique has certain limitations. It is not possible to establish whether serum soluble FasL corresponds to the proteolytically cleaved form of 26 kDa, or exosomes containing the complete form of the 40 kDa protein [29]. It has been shown that recognition of the integral or membrane anchored form of FasL by a sensitized cell expressing Fas, triggers the extrinsic pathway of apoptosis. This process is responsible for immunosuppression, the maintenance of immune privilege, or that controversial tumor counter-attack [12]. Although the mechanisms are not completely elucidated, it has been suggested that the proteolytically cleaved form of FasL, does not have apoptotic activity and, to the contrary, leads to blockage of the apoptotic pathway [30]. The 40 kDa form could play an important role in regulating cell death in healthy tissue, and, when expressed on tumor cells, may be a likely strategy to escape anti-tumor

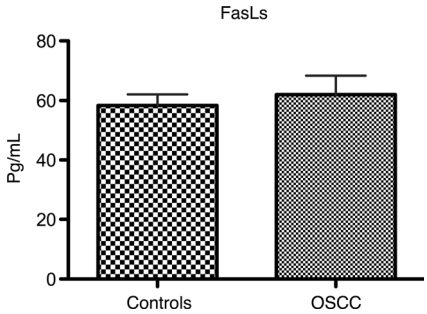

Fig. 4 Serum levels of soluble Fas ligand by ELISA. The quantification of sFasL showed no significant differences, $p = 0.8090$ controls vs. OSCC patients. Controls (mean: 58.39 ± 3.70 pg/mL) OSCC patients (mean: 62.05 ± 6.24 pg/mL). Each bar represents the mean value ± Standard deviation (SD). Results are expressed as picograms/mL. OSCC (oral squamous cell carcinoma)

immune responses. We believe that based on these findings, further studies that will allow us to identify the different forms of the FasL soluble protein would be of great importance.

With respect to the expression of Fas, our results are consistent with previous studies in OSCC [31, 32] and other carcinomas using immunohistochemistry [12]. The behavior of Fas in control subjects suggests that, unlike tumor cells, healthy tissue cells retain the ability to activate the extrinsic apoptotic signal. However, it is not possible to determine whether this apoptotic signal is constitutively active or if the levels of Fas on healthy tissue can change depending on the sensitivity of the cells for the induction of apoptosis. It has been shown that, in healthy epithelia, there exists the simultaneous coexpression of Fas and FasL, principally at the level of the basement membrane, which can be both a physiological regulator of healthy skin, and simultaneously play an important role in the pathogenesis of cancer [33].

Our results showed that only in healthy tissue, there is a statistically significant positive correlation between both forms of FasL (40 kDa and 26 kDa) and Fas (Fig. 5). However, this correlation is lost in patients with OSCC, which

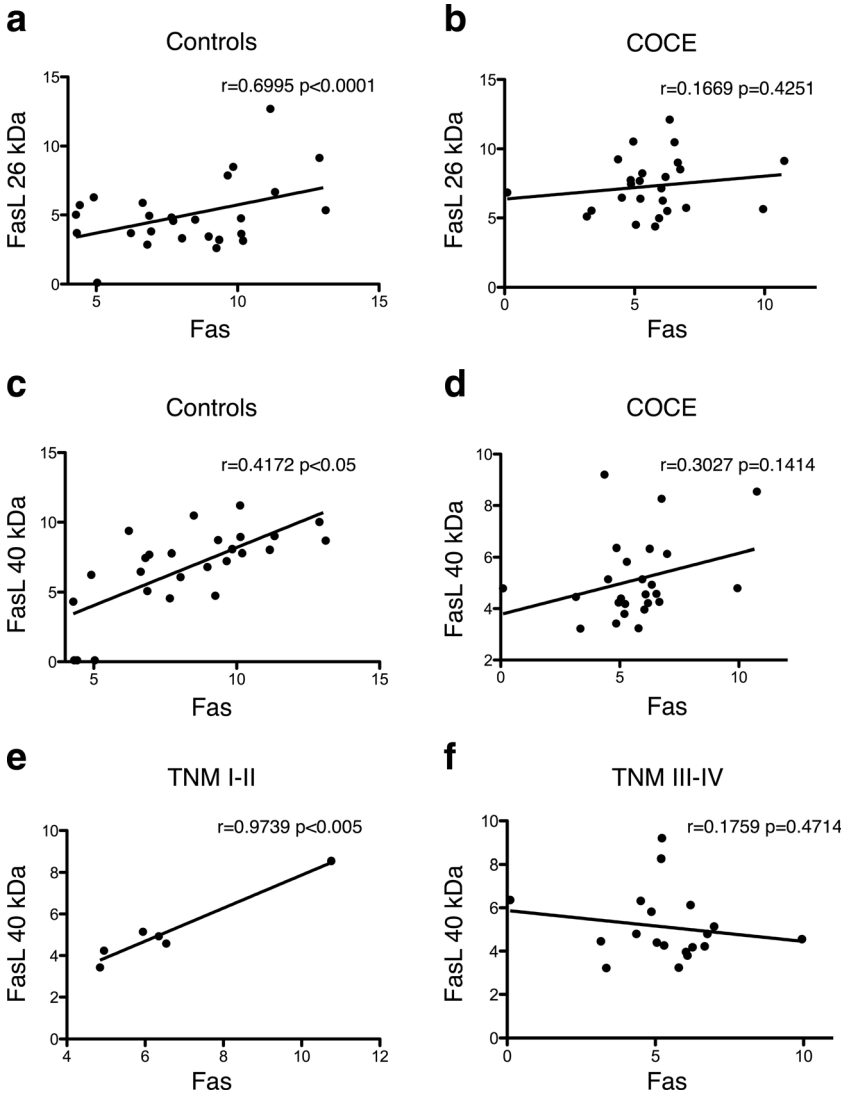

Fig. 5 Pearson's correlation coefficient analysis of Fas and FasL. (**a**) Correlation between: FasL (26 kDa) and Fas in controls, $***p < 0.0001$, $r = 0.6995$; (**b**) FasL (26 kDa) and Fas in OSCC patients, $p = 0.4251$, $p = 0.1669$; (**c**) FasL (40 kDa) and Fas in controls, $*p = 0.0340$, $r = 0.4172$; (**d**) FasL (40 kDa) and Fas in OSCC patients, $p = 0.1414$, $r = 0.3027$; (**e**) FasL (40 kDa) and Fas in TNM I-II patients, $**p = 0.0010$, $r = 0.9739$; (**f**) FasL (40 kDa) and Fas in TNM III-IV patients, $p = 0.4714$, $r = 0.1759$. OSCC (oral squamous cell carcinoma)

suggests that globally, and in the cells of the healthy oral mucosa, there could be a balance between both proteins, principally between the soluble form of FasL (26 kDa) and Fas. These findings allow us to speculate that although high levels of Fas are found in healthy tissue, at the same time cleaved FasL may provide a signal that blocks apoptosis in cells expressing Fas. However, in cancer patients, this relationship appears to be altered, which would result in the loss of the balance between both proteins. This would then imply a possible mechanism involving the activation of apoptosis to the detriment of a group of sensitive Fas-expressing cells, which may be leukocytes, fibroblasts, endothelial or tumor cells themselves.

In conclusion, the findings of our project allow us to speculate that ADAM10 and the Fas system are major players in the pathophysiology of OSCC.

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