

Caspase-3 expression in normal oral epithelium, oral submucous fibrosis and oral squamous cell carcinoma

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Abstract

Context: The epithelium atrophy, as the oral submucous fibrosis (OSMF) progresses, is believed to be an after effect of stromal fibrosis, hyalinization, decrease in vascularity and cellularity and is considered as “ischemic atrophy.” Due to hypoxia, caspase-3 get activation and subsequent decrease in viable cell count can occur.

Aims and Objectives: To determine caspase-3 expression in various grades of OSMF and oral squamous cell carcinoma (OSCC) to find out whether upregulation of apoptosis is responsible for the epithelial changes in OSMF.

Subjects and Methods: The control tissue (15 samples from normal oral mucosa) and study group comprising 97 cases of OSMF of different grades and OSCC associated with OSMF were stained with caspase-3 antibody, and the percentage of positive cells was calculated using ImageJ software.

Statistical Analysis: The results obtained were statistically analyzed using ANOVA and Tukey's honest significance difference test and Mann–Whitney U-test.

Results: There was a nuclear expression of caspase-3 in basal and parabasal layers of normal epithelium. There was cytoplasmic expression of caspase-3 in OSMF without dysplasia, total absence of caspase-3 expression in dysplastic epithelium and in majority cases of OSCC. The caspase-3 percentage was increased in OSMF (0%–53%) when compared with OSCC (0%–8%). The statistical comparison of caspase-3 among normal, OSMF and OSCC patients revealed significant correlation ($P < 0.00010$). The comparison within different grades of OSMF and between dysplastic and nondysplastic epithelium OSMF also showed significance ($P < 0.019$).

Conclusions: The decreased expression of caspase-3 in disease progression reflects its role in the malignant transformation.

Key Words: Arecanut, caspase-3, epithelium

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INTRODUCTION

Arecanut chewing is a risk factor for the development of oral submucous fibrosis (OSMF).^[1] The atrophic epithelium in

OSMF is believed to be an after effect of fibrosis, hyalinization and decrease in vascularity.^[2] The tissue homeostasis is regulated by cell proliferation and apoptosis. Activation of caspase-3

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and decrease of viable cell count due to hypoxia have been documented.^[3] There was an increase of apoptotic cells in arecoline-treated human KB (keratin forming tumor cell line HeLa) epithelial cells, and evaluation of caspase-3 expression is a precise method to identify apoptosis.^[4-6] Thus, the study analyzed whether apoptosis contributes to epithelial atrophy by evaluating the expression of caspase-3.

SUBJECTS AND METHODS

The present study was conducted in a total of 112 samples. The normal tissues (15 tissues) were harvested during surgical extraction of the third molar. The OSMF and oral squamous cell carcinoma (OSCC) tissues associated with OSMF were taken as the study group (disease group), which comprised 97 tissues. Both the control and study group tissues were fixed in 10% buffered formalin and embedded in paraffin. The disease group (OSMF) was subdivided into early (29 cases), moderate (34 cases) and advanced (18 cases) following Pindborg grading system^[7] with slight modification and OSMF associated with OSCC (16 cases).

From the control and diseased group, four micron thick sections were prepared and taken on APES coated slides. The sections were stained for caspase-3 antibody to evaluate the apoptotic cells. The sections were dewaxed through two changes of xylene and rehydrated in alcohol in descending order. The antigens were disclosed by heat retrieval method under citrate buffer (pH-6). During the staining procedure, the incubated reagents were washed using phosphate buffer saline (PBS). All the sections were treated with hydrogen peroxidase for 10 min to block endogenous peroxidase and subsequently treated by a protein block for the same time (DAKO REAL EnVision, Denmark). The sections were incubated with caspase-3 monoclonal antibody (clone 3CSPO3, Thermo Fisher Scientific, USA) for 1 h. The excess primary antibody was washed in PBS and followed by poly-horseradish peroxidase secondary antibody (DAKO REAL EnVision, Denmark) incubation for 30 min. Sections were rinsed in PBS; the immunostaining was developed by treating with freshly constituted 3,3'-diaminobenzidine tetrahydrochloride solution for 5 min. The sections were thoroughly rinsed in distilled water to remove unbound chromogen present in nonrepresentative areas. The sections were counterstained with Mayer's Hematoxylin. Further, the sections were dehydrated in alcohol, cleared with xylene and mounted with dibutyl phthalate in xylene. Lymph node tissue as a positive control and a negative control was also stained along with the procedure.

The brown precipitate present in the cytoplasm and nucleus was taken as positivity. From good representative areas, five

photomicrographs were recorded under $\times 40$, both in control and disease group. The number of positive and negative cells for caspase-3 antibody was calculated using "imageJ" software. The percentage of caspase-3 positive cells was calculated by dividing the number of positive cells by a total number of cells present in all the five fields and multiplying by 100. The data were statistically analyzed using ANOVA, exp Tukey's honestly significant difference (HSD) test and Mann-Whitney U-test.

RESULTS

The epithelium in OSMF was variable in thickness. It was hyperplastic, atrophic and dysplastic in nature [Figures 1a-d and 2a-c]. The OSCC cases included in the study were of different grades [Figure 2d]. The caspase-3 positivity was observed as a brown color end reaction, both in the cytoplasm and the nucleus. In normal oral mucosa, the cells present in the basal and spinous layers expressed caspase-3 in the nucleus in a majority of the cases [Figure 3a]. The expression was observed in the upper spinous layer, and the superficial layers also expressed it in the nucleus. The OSMF revealed caspase-3 expression predominantly in the cytoplasm of basal cells. The nuclear expression was also evident in some cases of OSMF [Figures 3b-d and 4a and b]. The caspase-3 expression was negative in 38 cases of OSMF and OSCC which accounted for 39% [Figure 4c] of the cases. In the OSCC case, very few tumor cells expressed caspase-3 in the cytosol [Figure 4d]. The OSMF cases that exhibited dysplasia showed either negative expression or very less percentage of caspase-3 positive cells. The percentage of caspase-3 expressing epithelial cells ranged from 11% to 38% in the normal oral mucosa. The early grade OSMF cases demonstrated caspase-3 mostly in the severely atrophic epithelium. The percentage of caspase-3 immunopositive cells ranged from 0% to 53% in early grade of OSMF. The highest percentage was observed in severe atrophic epithelium of this group. The expression of caspase-3 was negative in 12 cases out of 34 moderate OSMF, and the percentage was 0%–24% in moderate OSMF. The maximum percentage of cells expressing caspase-3 in advanced OSMF cases were similar to normal oral mucosa. With regard to caspase-3 expression in the epithelial dysplasia and nondysplastic lesions, some of the dysplastic lesions expressed caspase-3 in very low percentages when compared with normal mucosa, and a large number of cases revealed total negativity. The OSCC showed a low percentage of caspase-3 positive cells (0%–8%).

On statistical comparison of caspase-3 expressing cells both by ANOVA and Tukey HSD test in OSMF and OSCC revealed a significant correlation at $P < 0.0001$ [Table 1]. The comparison of caspase-3 among the OSMF groups was significant

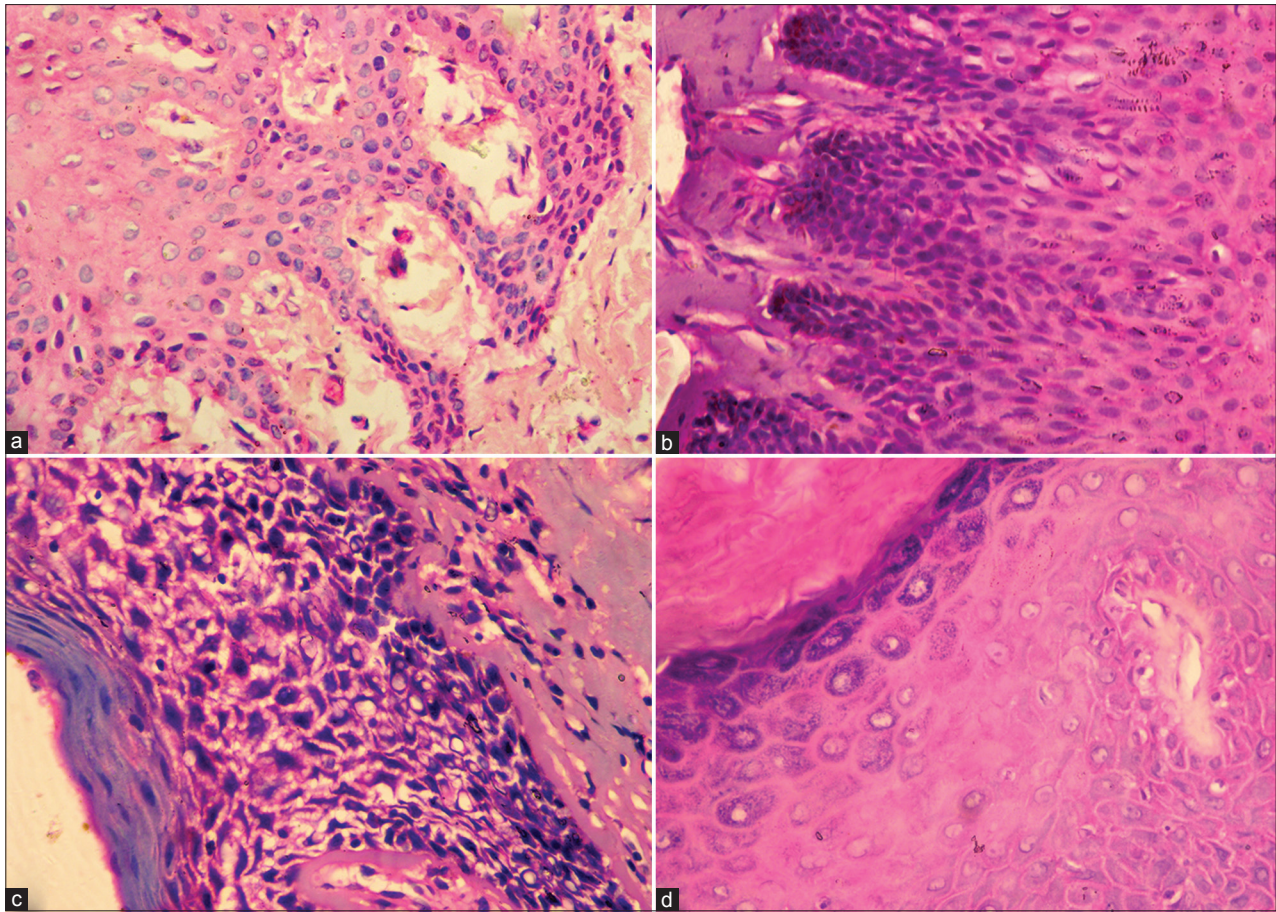


Figure 1: (a) The normal oral mucosa exhibiting stratified squamous epithelium and fibrous connective tissue (H&E stain, X400). (b) The early oral submucous fibrosis demonstrating hyperplastic epithelium (H&E stain, X400). (c and d) The moderate oral submucous fibrosis showing dysplastic and orthokeratinized stratified squamous epithelium (H&E stain, X400)

Table 1: Comparison of caspase-3 expression among normal oral mucosa, different grade of oral submucous fibrosis and oral squamous cell carcinoma using Kruskal-Wallis test

Grades	n	Mean	SD	95% CI for mean		Minimum	Maximum	P
				Lower bound	Upper bound			
Normal	15	21.73	8.884	16.81	26.65	10	40	<0.0001
Early OSMF	29	11.24	14.872	5.58	16.90	0	53	
Moderate OSMF	34	5.38	7.295	2.84	7.93	0	24	
Advanced OSMF	18	10.17	12.557	3.92	16.41	0	38	
Carcinoma	16	2.12	3.575	0.22	4.03	0	10	
Total	112	9.39	11.898	7.17	11.62	0	53	

OSMF: Oral submucous fibrosis, CI: Confidence interval, SD: Standard deviation

($P < 0.019$). The percentage of caspase-3 positive cells in dysplastic and nondysplastic epithelium observed in OSMF showed significance at $P < 0.001$ on statistical comparison under nonparametric Mann–Whitney U-test [Table 2]. On the other hand, statistical comparison among hyperplastic, mild, moderate, severe atrophic epithelium of OSMF and normal oral epithelium showed insignificant correlation. The statistical evaluation of caspase-3 expression between OSCC and dysplastic OSMF epithelium showed significance [Table 2].

DISCUSSION

Apoptosis is a process that is initiated by the stimulation of one of two distinct pathways: The intrinsic death receptor – independent pathway and the extrinsic death receptor – dependent pathway, both of which require sequential functioning of cysteine proteases known as caspases. The activation of initiator caspases such as caspase-8 and caspase-9 leads to proteolytic cleavage at caspase-3 – specific DEVD cleavage motifs of caspase-3.^[8]

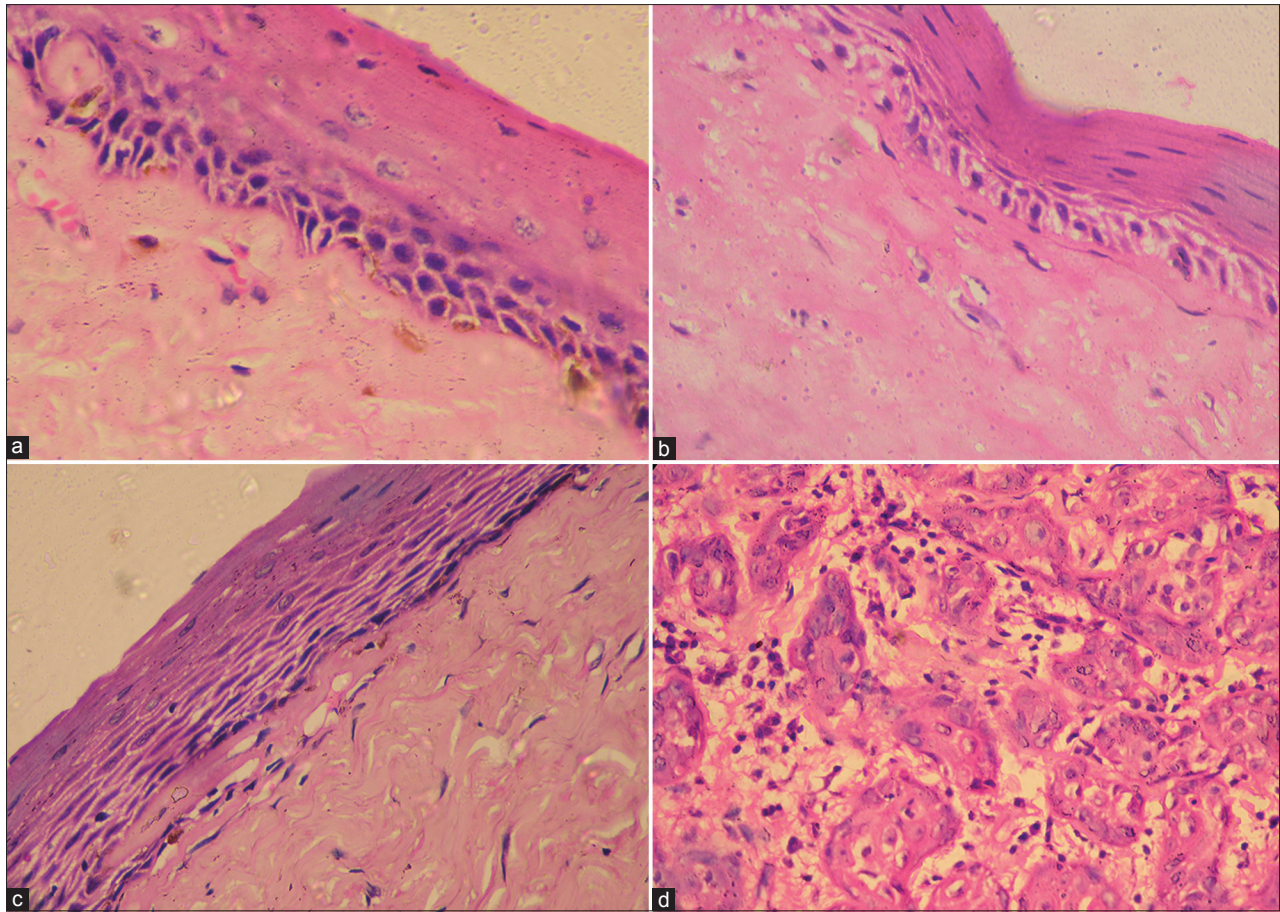


Figure 2: (a-c) Moderate and advanced oral submucous fibrosis showing atrophic epithelium (H&E stain, X400). (d) Oral squamous cell carcinoma exhibiting islands of dysplastic epithelium in the stromal tissue (H&E stain, X400)

Table 2: Comparison of caspase-3 expression among oral submucous fibrosis with dysplasia, without dysplasia and oral squamous cell carcinoma using nonparametric Mann-Whitney U-test used

Parameters	n	Mean	SD	Mean rank	P
OSMF without dysplasia	47	12.30	12.782	50.28	<0.001
OSMF with dysplasia	34	3.35	8.041	28.18	
OSCC	16	2.12	3.575	24.97	0.227
OSMF with dysplasia	34	3.35	8.041	25.75	
OSCC	16	2.12	3.575	17.97	0.003
OSMF without dysplasia	47	12.30	12.782	36.78	

OSMF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, SD: Standard deviation

The activated caspase-3 is the main executor of apoptosis mechanism and results in the cleavage of multiple cytoplasmic and nuclear proteins.^[9] The determination of caspase-3 is a simple, reliable and precise method to recognize apoptosis in the early stage.^[6] In the present study, the nuclear expression was the dominant feature in normal oral mucosa. In addition, some of the OSMF and OSCC cases demonstrated caspase-3 in the same location. The nuclear expression indicates translocation of activated caspase-3 to the nucleus. Caspase-3 specifically activates the endonuclease caspase-activated DNase (CAD).

In proliferating cells, CAD is complexed with its inhibitor, Inhibitor of CAD (ICAD). In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD. CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. In addition to that caspase-3 also induces cytoskeletal reorganization and disintegration of the cell to form apoptotic bodies. Actin binding protein, gelsolin, has been recognized as one of the substrates of activated caspase-3.^[8,10]

In an *in-vitro* experiment, the organotypic epithelium derived from normal keratinocytes and immortalized epithelium stained for caspase-3 revealed nuclear expression of caspase-3 throughout the epithelial layers of immortalized buccal epithelium. However, the normal keratinocytes showed positivity in the basal layer region. This finding was congruent with the finding of the present study although the concepts and results of various studies about apoptosis in oral epithelium were inconclusive and highly controversial.^[11] The surface epithelium of normal subjects, where the cells undergo terminal differentiation demonstrated negative immunoreactivity for cleaved caspase-3 and further, the pro-form of this enzyme is

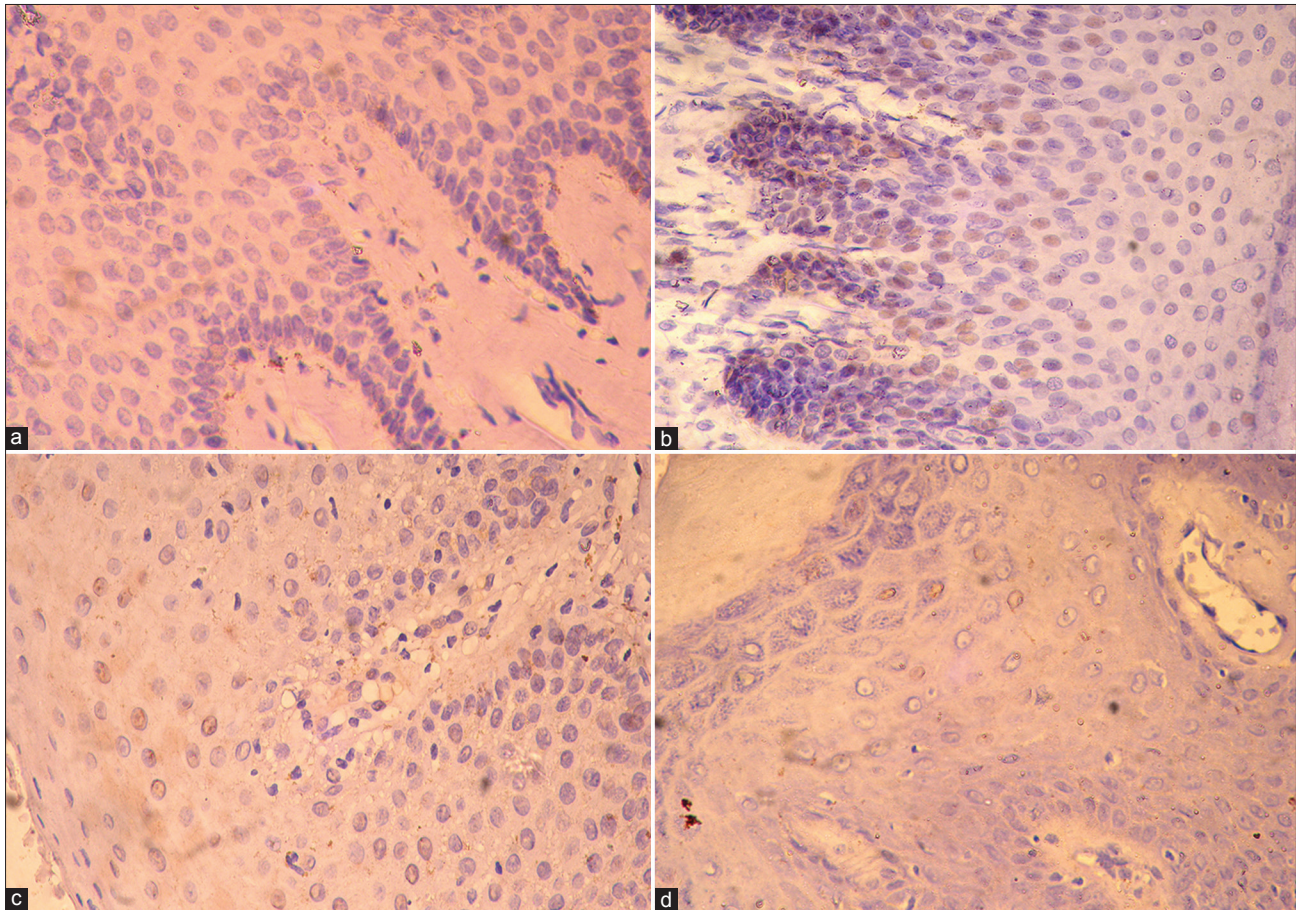


Figure 3: (a) The normal epithelium showing nuclear expression of caspase-3 (IHC, X400). (b and c) The hyperplastic oral submucous fibrosis epithelium exhibiting both nuclear and cytoplasmic expression of caspase-3. (d) A moderate oral submucous fibrosis case demonstrating nuclear expression (IHC stain, X400)

downregulated in differentiated oral epithelial cells.^[12] Perhaps pronounced cytosolic expression of caspase-3 and its location, both in the nucleus and cytosol of keratinocytes, was observed in OSMF and OSCC.

Numerous studies showed this executioner caspase in the cytoplasm of normal tissues and different human tumors. The cytoplasmic anti-caspase-3 immunopositive cells exhibited features consistent with apoptosis. Nevertheless, some of the normal-looking cells also expressed it. The hepatocytes and tumor cells of hepatocellular carcinoma and similarly, the gastric cells and premalignant and malignant lesions of gastric mucosa showed cytoplasmic expression of caspase-3.^[13] The OSCCs showed decreased, as well as cytoplasmic and nuclear distribution of this apoptotic protein in some cases.^[6,12] The predominance of cytoplasmic expression of caspase-3 in various tumors was in accordance with the results of the current study.^[6,9,12]

The dysplastic epithelium in OSMF and OSMF associated OSCC cases showed a less percentage of caspase-3 positive cells. The study on evaluation of apoptotic, necrotic and absolute

cell death indices in OSMF, and normal oral epithelium cases showed no significant changes in these parameters. The authors suggested that there is an increased possibility for decreased cellular proliferation and replenishment in causing epithelial involution, occurring as an end result of fibrosis in OSMF.^[2]

There is some variation in the data between the current study and the results of Rajendran *et al.*^[2] This discrepancy in results might be due to the adaptation of different methods and the sample size of the current study. The common finding of both the studies is that there is no acceleration of apoptosis in OSMF. The reason for significant correlation between the normal and OSMF cases is the absence of caspase-3 expression in 39% of OSMF cases and low percentage of positive cells in the dysplastic epithelium and OSCC cases.

The apoptosis measured by terminal deoxynucleotidyl transferase dUTP nick end-labeling methods showed a different pattern of distribution of apoptotic cells. In the hyperplastic epithelium, the apoptotic cells were distributed in the superficial layers and appeared as a nuclear stain. In the dysplastic epithelium, apoptotic cells were located in the

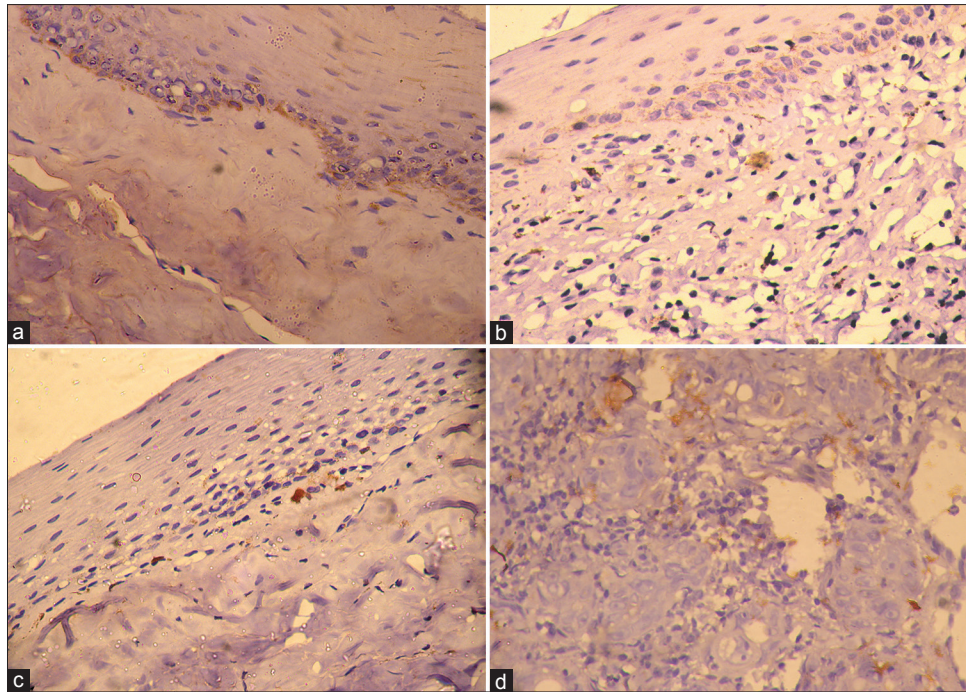


Figure 4: (a and b) Moderate and advanced oral submucous fibrosis with atrophic epithelium revealing cytoplasmic expression of caspase 3 (IHC stain, X400). (c) Advanced oral submucous fibrosis showing negative expression of caspase-3 (IHC stain, X400). (d) Oral squamous cell carcinoma exhibiting cytoplasmic expression of caspase-3 (IHC stain, X400)

middle layers. On the other hand, the severe dysplasia showed apoptotic cells both in the middle and basal layers. When the entire epithelium was replaced by the basaloid cells, there was a decrease in the apoptotic cell population. Based on these findings, the authors suggested that apoptosis of superficial and middle layer keratinocytes promotes the migration of basal cells to the top layer of epithelium which is a feature of carcinoma *in situ*.^[14]

The caspase-3 role in the proliferation of OSCC cells was determined in the presence and absence of caspase-3. It was found that on the inhibition of caspase-3 activity, there was a significant decrease in the proliferation of OSCC cells. The results strongly suggested the notion that caspase-3 signaling facilitates oral cancer progression. The authors suggested from their observation that an increased level of apoptosis is associated with tumor progression with a high turnover of tumor cell.^[15] It is speculated that decrease in caspase-3 expression and its outcome resulting in reduced proliferation of tumor cells may be responsible for less aggressive nature of OSCC developing from OSMF.

The basal cell expression of caspase-3 has a different role that is rather than apoptosis; it contributes to tumor progression. It is also mentioned in the literature that expression of caspase-3 at the lower level may not be sufficient to induce apoptosis in tumor cells. However, it may be sufficient to enhance the proliferation of cancer cells.^[15] There was no difference of

caspase-3 mRNA expression between peritumoral tissues and in normal tissues. Caspase-3 mRNA expression level in tumor tissues was significantly lower than in peritumoral and normal tissues. It has been concluded that survivin can possibly inhibit the synthesis of caspase-3. Hence, it blocks the apoptosis mediated by caspase-3 and finally leads to the development of OSCC.^[16] In an analysis of caspase-3 level estimation in gastric cancer, the results showed the total absence of caspase-3 activity in gastric cancer and in the peritumoral area. On the other hand, the normal mucosa divulged the caspase-3 activity, and it was suggested that lack of caspase-3 activation is involved in the transformation to gastric carcinoma.^[17] Caspase-3 expression was observed in cytoplasm of normal gastric mucosa. Premalignant lesions and gastric malignancy showed decreased expression as the progression of gastroduodenal carcinogenesis and became undetectable in a majority of malignant samples. It is therefore suggested that the apoptosis-resistant property of gastric carcinoma is attributed to the decreased level of caspase-3 in gastric tumors.^[13] The apoptotic cells were identified using DNA *in-situ* end-labeling and showed a gradual rise in apoptotic index from normal oral mucosa to malignant progression. In carcinomas, approximately 25% of tumor cells are involved in cell division, whereas only 0.4% of tumor cells undergo apoptosis.^[18]

The apoptotic cell death rate in basal layer of oral epithelium was lower than the basal layer of well, moderate and poorly differentiated squamous cell carcinomas. In the suprabasal

layer of oral epithelium, the apoptotic index was less than the central cells of well, moderately and poorly differentiated squamous cell carcinomas. Results showed an increase in apoptotic index and decrease in bcl-2/bax ratio of OSCC when compared with the normal oral epithelium.^[19] This finding was in contrast to the result of the current study; caspase-3 is expressed in the cytoplasm and nucleus of the oral carcinomas. Caspase-3 was predominantly expressed in oral carcinomas when compared with normal oral epithelium. Caspase-3 is expressed in proliferative pool of oral epithelium, especially in the condensed chromatin. The surface epithelium of normal subjects, where the cells undergo terminal differentiation demonstrated negative immunoreactivity for cleaved caspase-3, the pro-form of this enzyme is downregulated in differentiated oral epithelial cells. Nevertheless, none of the oral epithelium showed negative expression in the present study. All the oral carcinomas and carcinoma cell lines expressed caspase-3 in the cytoplasm and nucleus of the tumor cells, and cytoplasmic expression was a noticeable finding. Due to activation and subsequent degradation of caspase-3, mostly tumor cells express caspase-3. A positive correlation was observed among caspase staining intensity and tumor staging that is poorly differentiated carcinoma with higher staging showed upregulation of caspase-3. The cytoplasmic expression of caspase-3 in tumor cells reflects the aberrant differentiation in OSCCs and suggested to use as a parameter to assess the prognosis of the tumor.^[12]

The nonneoplastic lesions of breast expressed caspase-3, 6 and 8 as diffuse, weak and inconsistent cytoplasmic staining respectively. The benign lesions expressed it as a slightly stronger staining. The nuclear expression of caspase-3 was observed in a few cases of carcinomas of the breast. The immunoreactivity of all caspases is parallel with the histological progression of the breast lesion. The peritumor epithelium adjacent to the squamous cell carcinoma showed the absence of caspase-3 expression. The epithelial dysplasia showed immunoreactivity for caspase-3 in the parabasal proliferative cells. The carcinoma *in situ* revealed this protein expression in the entire layer of epithelium except the keratin layer in the superficial aspect. The invasive carcinoma demonstrated abundant caspase-3 positive cells in all the cases.^[15] Furthermore, the apoptotic cells in tumor areas, cells without the morphology of apoptosis and the metastatic tumor islands in the lymph nodes were also immunopositive. The apoptotic index was calculated in squamous cell lesions of the oral cavity which showed a gradual increase of this death index from mild dysplasia to severe dysplasia. However, in contrast, there was a gradual decrease in apoptotic index from well-differentiated OSCC to moderately differentiated OSCC which is consequently followed by poorly differentiated OSCC cases.^[20] There was

a slight increase in caspase-3 expression in early OSMF when compared with normal epithelium; however, other groups of OSMF and OSCC showed a decrease in expression. From these observations, it is speculated that the epithelial atrophy in OSMF as the disease progress is not due to an upregulation of apoptosis mechanism. Nevertheless, it may be an outcome of dysregulated epithelial proliferation. In future, an extensive probing on other molecules of apoptosis and proliferative pathway may address this issue in a more authenticated way.

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Conflicts of interest

There are no conflicts of interest.

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