

ORIGINAL ARTICLE

Evaluation of myofibroblasts in oral squamous cell carcinoma using H1 calponin: An immunohistochemical study

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral mucosa. Stromal myofibroblasts play an important role in tumor invasion and metastasis, due to its ability to modify the extracellular matrix. The purpose of this study was to evaluate and compare the presence of myofibroblasts in normal mucosa, early invasive carcinoma and different grades of OSCC. **Materials and Methods:** The study included the archival tissues of 18 OSCC of well, moderate and poorly differentiated grades, three early invasive carcinomas and five normal mucosa. Myofibroblasts were identified by immunohistochemical detection of h1 calponin. **Results:** The percentage and intensity of h1 calponin were examined and positive immunostaining was observed in the myofibroblasts of all SCCs and early invasive carcinomas; however, these cells did not stain in the normal epithelium specimens. The presence of myofibroblasts was significantly higher in invasive pattern of OSCCs compared to normal mucosa cases ($P < 0.070$). A significant difference was not observed between the different grades of OSCC ($P \leq 0.812$). **Conclusion:** These findings show the presence of myofibroblasts in OSCC but not in normal mucosa, suggesting that the genetically altered epithelium (carcinomatous epithelium) may have an inductive effect on the adjacent stroma to produce myofibroblasts. Also transdifferentiation of myofibroblasts is induced somewhere in the invasive stage of SCC irrespective of the epithelial cell differentiation.

Key words: Bryne's invasive tumor front grading system, calponin, myofibroblast, Oral squamous cell carcinoma

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy worldwide.^[1] Transformation of normal oral mucosa (NOM) to SCC represents a complicated process involving numerous etiologic factors.^[2] During progression to invasive carcinoma, neoplastic cells activate the underlying connective tissue and generate a phenotypically altered and specific tumor stroma, which may influence the cancer cells.^[3] Tumor stroma comprises of immunocompetent and

inflammatory cells, endothelial cells, fibroblasts and a subtype specific of fibroblasts called myofibroblasts. Myofibroblasts exhibit a phenotype between fibroblasts and smooth muscle cells.^[4] Myofibroblasts are derived mainly from fibroblasts and also from smooth-muscle cells, pericytes, macrophages, hepatic stellate cells, epithelium and bone marrow.^[5] The tumor-promoting effect of myofibroblast is based on the direct cytokine stimulation of cancer cells, maintenance of vascularity and on their capacity to produce enzymes which degrade molecules like lysyl oxidase which enhance the structural integrity of matrix. Myofibroblasts create a physical barrier between carcinoma cells and immune system of the: body against cancer.^[5] An increase in myofibroblasts may be useful to predict the prognosis of OSCC patients, since cervical node metastasis is one of the major prognostic factors in patients with OSCC.^[6]

Earlier studies using alpha-smooth muscle actin (α -SMA) have been done to compare the distribution of myofibroblasts

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in NOM and histological grades of OSCC.^[7] The present study uses h1 calponin to compare the distribution of myofibroblasts in NOM, early invasive carcinoma and histological grades of OSCC. Calponins are components of the smooth muscle thin filament that are suggested to regulate interactions between actin and myosin II.^[8]

MATERIALS AND METHODS

The study included the archival tissues of 18 OSCC cases of well, moderate and poorly differentiated grades, three early invasive carcinomas and five normal mucosa selected from the Department of Oral and Maxillofacial Pathology, Rajah Muthiah Dental College and Hospital, Annamalai University. All specimens were fixed in 10% buffered formalin and embedded in paraffin wax. Two sections of 3–5 μm thickness were sliced from each tissue block for routine hematoxylin and eosin staining and immunohistochemical staining. The hematoxylin and eosin stained sections of OSCC were examined to confirm the diagnosis of OSCC histopathologically. The other sections were mounted on glass slides coated by aminopropyl triethoxy silane (APES; Sigma Chemical Co., USA) and processed for subsequent immunohistochemical study.

The sections were deparaffinized at 60°C for 1 h. The sections were dewaxed in xylene and rehydrated in descending grades of alcohol. The slides were placed in a coplin jar, with citrate buffer solution. Antigen retrieval was performed under stream pressure using pressure cooker. The sections were covered with 3% hydrogen peroxide for 10 min followed by treatment with protein block for 10 min. The sections were covered completely with optimally diluted mouse monoclonal primary antibody to h1 calponin (Biogenix) for 30 min. Then the slides were kept in phosphate-buffered saline (PBS) buffer bath for 5 min. Then the slides were treated with poly horseradish peroxidase (HRP) enzyme (Dako REAL EnVision, Denmark) for 30 min. The slides were then washed with PBS and immunostaining was carried out by staining with DAB (3,3'-diaminobenzidine tetrahydrochloride) for 5 min. The slides were immersed in Mayer's hematoxylin for 7 min and blueing was done for 10 min. The sections were dehydrated in ascending grades of alcohol and air dried thoroughly and mounted using DPX.

INTERPRETATION OF STAINING

Presence of brown-colored end product at the site of target antigen was considered as positive immunoreactivity. Cytoplasmic staining of stromal spindle cells observed in the experimental slides of well, moderate and poorly differentiated SCC was considered as positive immunoreactivity.

Immunostaining was assessed by the evaluation of the staining intensity and percentage of positive-staining stromal cells, according to the method proposed by Tuxhorn *et al.*^[9] The

percentage of immunopositive cells in the non-inflammatory and non-endothelial stromal cells immediately adjacent to the carcinomatous islands and normal was recorded as: 0% = no positive cells, 1 = 1–33% positive cells, 2 = 34–66% positive cells and 3 = 67–100% positive cells. Staining intensity was considered 0 when there was no staining; 1, in parts where positivity was observed only at a magnification of $\times 40$; 2, in cases where staining was obvious at $\times 10$, but not $\times 4$; and 3, in fields where immunopositive cells were seen even at $\times 4$. Multiplication of the percentage and intensity scores comprised the 'staining index' of each specimen. This index was classified as zero (0), low (1–2), moderate (3–4) and high (6–9).

Representative areas were selected from each slide. Dark brown stained stromal spindle cells of each representative area were selected and cell counting was performed using the "cell counter" in Image J software. Differences in the presence of myofibroblasts between groups were analyzed using Kruskal–Wallis and Mann–Whitney tests.

RESULTS

Histopathological observations of the study samples were done and grading was confirmed. According to the "Invasive Tumor Front Grading System" by Bryne *et al.*, (1969), the study samples were grouped into three grades. The sample comprised of 10 cases of OSCC of grade I, seven cases of OSCC of grade II and four cases of OSCC of grade III.

The study samples were evaluated immunohistochemically for myofibroblast expression by h1 calponin. The staining index in histological grades of OSCC and invasive patterns of OSCC are calculated.

Expression of calponin in myofibroblast was compared in 21 OSCC and five NOM using Kruskal–Wallis test. Statistical analysis showed a significant difference in the expression of calponin between normal and OSCC ($P < 0.001$) [Table 1].

There was no statistically significant difference in the expression of calponin among well, moderate and poorly differentiated and early invasive OSCC ($P < 0.812$). Statistical analysis showed a nearing significance ($P < 0.070$) [Table 2] in the expression of calponin among the invasive patterns of OSCC. Between grades I and III, there was significant difference ($P < 0.02$) [Table 3] in the expression of calponin.

DISCUSSION

With approximately 500,000 new cases annually, SCC of the head and neck represents one of the sixth most common cancers in the world.^[10] According to the 'tissue organization field theory', cells are normally in a proliferative state and do not tend to be quiescent. Thus, mutated epithelial or stromal cells and disturbed stromal–epithelial interactions may be equally responsible for the induction of carcinogenesis.^[11] During

progression to invasive carcinoma, neoplastic cells activate the underlying connective tissue and generate a phenotypically altered and specific tumor stroma, which may influence the cancer cells.^[3] Carcinomas induce a modified stroma through expression of growth factors that promotes angiogenesis, altered ECM expression, accelerated fibroblast proliferation and increased inflammatory cell recruitment.^[12] Some stromal events such as fibroblast's activities, myofibroblast's differentiation and presence of some specific stromal proteins like proteolytic enzymes, fibronectins and laminin-5 have been reported as the main features of stromal tumor.^[2] Fibroblasts play a major role in regulating and maintaining extracellular homeostasis and when activated after tissue injury, are responsible for wound contraction, fibrosis, scarring and regulation of inflammatory reactions.^[4] In addition they differentiate into contractile and secretory fibroblasts with abundant endoplasmic reticulum, pronounced Golgi apparatus and α -SMA fibers. These α -SMA-positive fibroblasts, termed myofibroblasts, synthesize extracellular matrix components and several proteinases, growth factors and cytokines.^[13] Derek *et al.*, 2007^[14] suggested that exposure of epithelial cells to matrix metalloproteinases (MMPs) can lead to increased levels of cellular reactive oxygen species (ROS) that stimulate transdifferentiation to myofibroblast-like cells. Myofibroblasts were originally identified in granulation tissue as modified fibroblasts with prominent rough endoplasmic reticulum. Later, myofibroblast were most often defined as fibroblasts positive for α -SMA and containing actin microfilaments and vimentin as identified by immunohistochemistry.^[15] Tumor growth factor (TGF)- β induces expression of α -SMA and is considered the major growth factor promoting myofibroblast development. Squamous carcinoma cells may directly induce a myofibroblast phenotype in primary fibroblasts through the

secretion of TGF- β 1. Oral fibroblasts undergo TGF- β -induced myofibroblast differentiation and this effect is enhanced by IL-1 β .^[16] Myofibroblasts are present in the stroma of most human OSCC and the two dominant patterns, 'spindle' and 'network', have been described by several authors (Vered *et al.*, 2009 and Kellermann *et al.*, 2007).^[17,3] Myofibroblast appears to be a key player in the carcinogenesis and progression of OSCCs.

In the present study, the role of myofibroblast in progression of OSCC was evaluated. The expression of myofibroblast: of OSCC was evaluated. The expression of myofibroblast between five normal mucosal specimen [Figure 1] and 21 OSCC specimens [Figures 2 to 4] were analyzed using calponin. The study showed a consistently increased calponin expression levels ($P < 0.001$) in almost all OSCC tissues compared to normal mucosa which was devoid of myofibroblasts [Figures 3 and 5]. The finding suggests that the increase in myofibroblasts in the stroma of OSCCs may be an important event in the invasion of epithelial cells. This finding is in agreement with those reported by Etemad *et al.*, in 2009,^[7] Eliene *et al.*, in 2011^[18] who found that the presence of myofibroblasts was significantly higher in OSCCs compared to normal mucosa and dysplastic epithelium which were devoid of it. Barth *et al.*, in 2004^[19] investigated tumor-free mucosa and SCCs of the oral cavity, the pharynx and larynx and showed the presence of α -SMA positive myofibroblasts in SCCs and their absence in tumor-free mucosa. Kellermann *et al.*, 2007^[20] in a correspondence article reported myofibroblasts at the invasive front of the OSCCs and no myofibroblast were found in the stroma of normal mucosa and epithelial dysplasia. Zidar *et al.*, in 2002^[21] found increased presence of myofibroblast in squamous carcinoma of larynx and lack of myofibroblast in the stroma of normal laryngeal mucosa and laryngeal epithelial hyperplastic lesions. Chaudhary *et al.*, in 2012^[22] compared the presence of myofibroblasts in OSCC, verrucous carcinoma (VC), high-risk epithelial dysplasia (HRED), low-risk epithelial dysplasia (LRED) and NOM. The α -SMA positive myofibroblast was expressed in 97.29% of OSCC, 86.66% of VC and 46.66% of HRED but not in NOM and LRED. In the present study, the association between expression of calponin in myofibroblast and the parameters such as tumor grade and invasive patterns of OSCC were also evaluated. The expression of calponin among early invasive SCC and well, moderate and poorly differentiated OSCCs was compared. All the grades showed significant expression of calponin compared to NOM but there was no significant difference in calponin expression

Table 1: Expression of myofibroblast in normal and OSCC

	Nil	Low	Moderate	High	P value*
Normal epithelium (5)	5	0	0	0	0.001 (significant)
OSCC (21)	0	9 (42.85%)	9 (42.85%)	3 (14.28%)	

*Kruskal-Wallis test. OSCC=Oral squamous cell carcinoma

Table 2: Expression of myofibroblast in invasive patterns of OSCC

	Nil	Low (%)	Moderate (%)	High (%)	P value*
Grade I (10)	0	4 (40)	6 (60)	0	0.070 (nearing significance)
Grade II (7)	0	3 (42.85)	3 (42.85)	1 (14.28)	
Grade III (4)	0	1 (25)	1 (25)	2 (50)	

*Kruskal-Wallis test. OSCC=Oral squamous cell carcinoma

Table 3: Mann-Whitney test for comparison between grade I and grade III

Groups	N	Mean rank	Mann-Whitney U	Wilcoxon W	z	P value*
Grade I	10	5.75	2.500	57.500	2.261	0.028 (significant)
Grade III	3	11.17				

*Mann-Whitney test

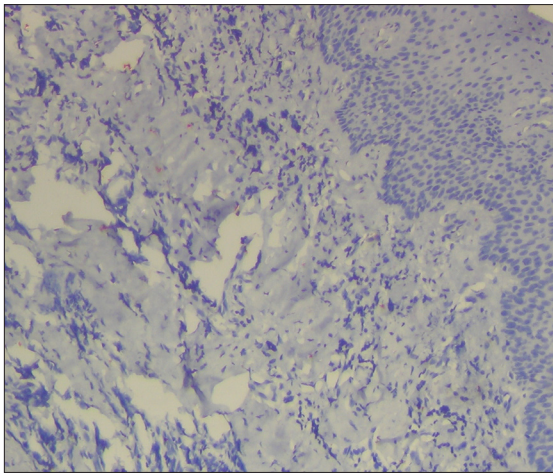


Figure 1: Low power magnification showing normal mucosa with negative immunoeexpression of calponin (IHC stain, x100)

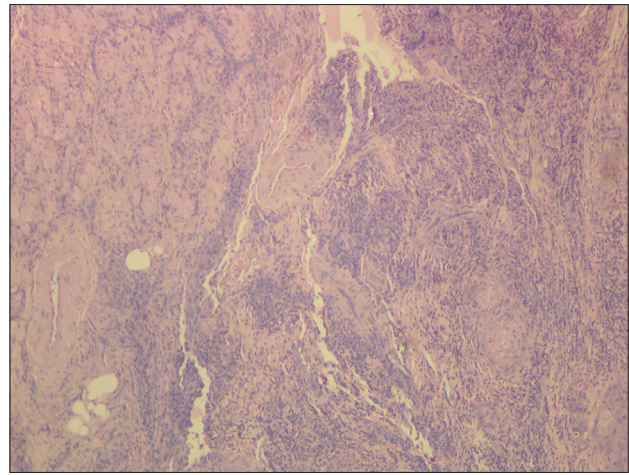


Figure 2: Low power magnification showing poorly differentiated squamous cell carcinoma (H&E stain, x40). H&E = Hematoxylin and eosin

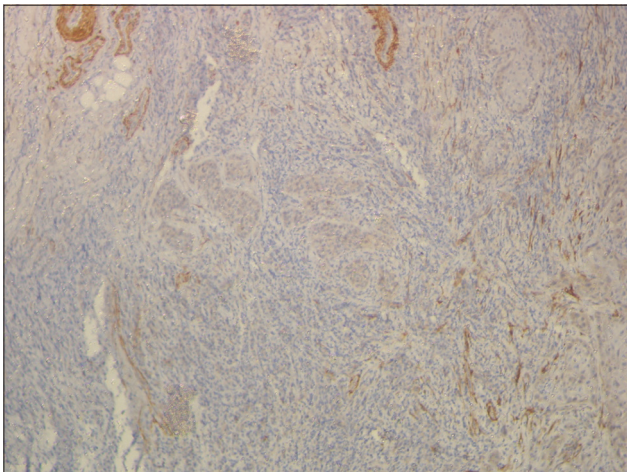


Figure 3: Low power magnification showing poorly differentiated squamous cell carcinoma with calponin expression in stromal spindle cells (IHC stain, x40)

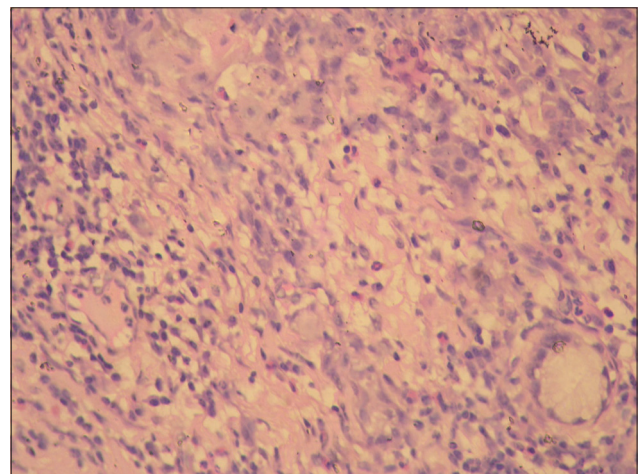


Figure 4: High power magnification showing moderately differentiated squamous cell carcinoma (H&E stain, x400)

among the grades of OSCC. This is in accordance with the results obtained by Etemad *et al.*, in 2009^[7] who were unable to find significant difference between the three histologic grades of OSCC. Also study by Kellermann *et al.*, in 2008^[3] showed no correlation between SCC differentiation and the observation of myofibroblasts. These findings may suggest that the transdifferentiation of myofibroblasts is induced somewhere in the invasive stage of OSCC irrespective of the tumor cell differentiation. The histological features of OSCC may differ widely from area to area within the same tumor and it is believed that the most useful prognostic information can be deduced from the invasive front of the tumors, where the deepest and presumably most aggressive cells reside. The invasive front, defined as the band of tissue between the tumor front and adjacent normal tissue, may better reflect tumor prognosis than other parts of the tumor.^[23] According to Bryne's Invasive Tumor Front Grading System, a remarkable difference was observed between the grades of invasion when the OSCC samples were classified by their

invasive patterns, ($P < 0.070$) in the presence of stromal myofibroblasts. The calponin expression was significantly higher in grade III invasive pattern when compared to grade I. In a similar study by Eliene *et al.*, in 2011,^[18] presence of myofibroblast was higher in tumors with a more diffuse histological pattern of invasion. These findings suggest that myofibroblasts are associated with the creation of a permissive environment for tumor invasion in OSCC and play an active role in OSCC invasion and metastasis. Kellermann *et al.*, 2007^[20] demonstrated that an abundance of myofibroblasts leads to more aggressive behavior of the SCCs, including an elevated proliferative potential. The abundant presence of myofibroblasts, particularly at the invasive tumor front, was significantly associated with shorter overall survival. Vered *et al.*, in 2005^[17] and Mashhadiabbas *et al.*, in 2010^[24] concluded that the expression of myofibroblasts was an index of invasive behavior of odontogenic lesions and they suggested that target therapy can be beneficial as an auxiliary method for treatment of more invasive lesions.

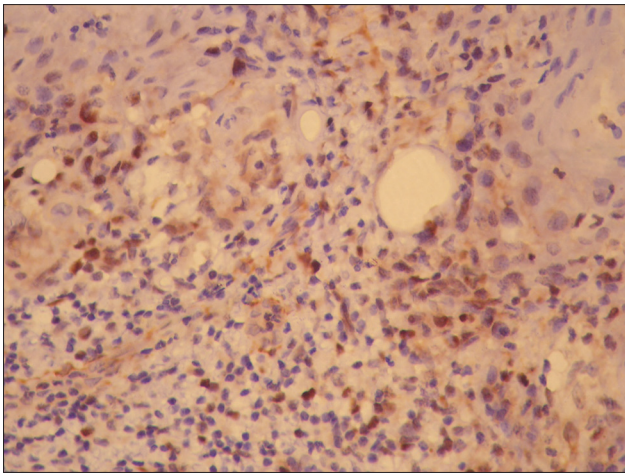


Figure 5: High power magnification showing moderately differentiated squamous cell carcinoma expressing calponin (IHC stain, x400)

CONCLUSION

In view of the observance of the presence of myofibroblasts in SCC and their absence in normal, it seems that the genetically altered epithelium (carcinomatous epithelium) may have an inductive effect on the adjacent stroma to produce myofibroblasts. Also lack of myofibroblasts in normal epithelium and their appearance in early invasive carcinoma indicates that transdifferentiation of myofibroblasts is induced somewhere in the invasive stage of SCC irrespective of the epithelial cell differentiation.

REFERENCES

- Shah JP, Johnson NW, Batsakis JG, editors. Text book of Oral Cancer. 1st ed. United Kingdom: Martin Dunitz Publisher; 2003. p. 4.
- Kuffer R, Lombardi T. Premalignant lesions of the oral mucosa. A discussion about the place of oral intraepithelial neoplasia (OIN). *Oral Oncol* 2002;38:125-30.
- Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: Induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation. *Oral Oncol* 2008;44:509-17.
- Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? *Histol Histopathol* 2002;17:599-621.
- Brian E. The myofibroblast in health and disease. *Rev Esp Patol* 2008;41:3-10.
- Greenberg JS, Naggar AK, Mo V, Roberts D, Myers JN. Disparity in pathologic and clinical lymph node staging in oral tongue carcinoma. Implication for therapeutic decision making. *Cancer* 2003;98:508-15.
- Etamad-Moghadam S, Khalili M, Tirgary F, Alaeddini M. Evaluation of myofibroblasts in oral epithelial dysplasia and squamous cell carcinoma. *J Oral Pathol Med* 2009;38:639-43.
- Ferjani I, Fattoum A, Maciver SK, Manai M, Benyamin Y, Roustan C. Calponin binds G-actin and F-actin with similar affinity. *FEBS Lett* 2006;580:4801-6.
- Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: Induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res* 2002 8: 2912-23.
- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;55:74-108.
- Beacham DA, Cukierman E. Stromagenesis: The changing face of fibroblastic microenvironments during tumor progression. *Semin Cancer Biol* 2005;15:329-41.
- Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature* 2004;432:332-7.
- Desmoulière A, Guyot C, Gabbiani G. The stroma reaction myofibroblast: A key player in the control of tumor cell behaviour. *Int J Dev Biol* 2004;48:509-17.
- Radisky DC, Kenny PA, Bissell MJ. Fibrosis and cancer: Do myofibroblasts come also from epithelial cells via EMT? *J Cell Biochem* 2007;101:830-9.
- Eyden B. The myofibroblast: An assessment of controversial issues and a definition useful in diagnosis and research. *Ultrastruct Pathol* 2001;25:39-50.
- Thode C, Jørgensen TG, Dabelsteen E, Mackenzie I, Dabelsteen S. Significance of myofibroblasts in oral squamous cell Carcinoma. *J Oral Pathol Med* 2011;40:201-7.
- Vered M, Shohat I, Buchner A, Dayan D. Myofibroblasts in stroma of odontogenic cysts and tumors can contribute to variations in the biological behavior of lesions. *Oral Oncol* 2005;41:1028-33.
- de-Assis EM, Pimenta LG, Costa-e-Silva E, Souza PE, Horta MC. Stromal myofibroblasts in oral leukoplakia and oral squamous cell carcinoma. *Med Oral Patol Oral Cir Bucal* 2012;17:e733-8.
- Barth PJ, Schenck zu Schweinsberg T, Ramaswamy A, Moll R. CD34+ fibrocytes, alpha-smooth muscle antigen-positive myofibroblasts, and CD117 expression in the stroma of invasive squamous cell carcinomas of the oral cavity, pharynx, and larynx. *Virchows Arch* 2004;444:231-4.
- Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. *Histopathology* 2007;51:849-53.
- Zidar N, Gale N, Kambic V, Fischinger J. Proliferation of myofibroblasts in the stroma of epithelial hyperplastic lesions and squamous carcinoma of the larynx. *Oncology* 2002;62:381-5.
- Chaudhary M, Gadabail AR, Vidhale G, Mankar Gadabail MP, Gondivkar SM, Gawande M, et al. Comparison of myofibroblasts expression in oral squamous cell carcinoma, verrucous carcinoma, high risk epithelial dysplasia, low risk epithelial dysplasia and normal oral mucosa. *Head Neck Pathol* 2012;6:305-13.
- Bryne M. Is the invasive front of an oral carcinoma is the most important area for prognostication? *Oral Dis* 1998;4:70-7.
- Mashhadiabbas F, Starbashi M, Moshref M, Elahi M. Immunohistochemical detection and ultrastructure of myofibroblasts in the stroma of odontogenic cysts and ameloblastoma. *IRCMJ* 2010;12:453-7.

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