Original Article

Comparison of expression of myofibroblasts in normal oral mucosa, oral epithelial dysplasia, and oral squamous cell carcinoma using α-SMA and vimentin: An immunohistochemical study

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Abstract

Background: Myofibroblasts are thought to play critical roles in inflammation, growth, repair, premalignancy, and malignancy. This study was done to evaluate, compare and co- relate the progressive increase in the immunohistochemical expression of myofibroblasts in normal oral mucosa, epithelial dysplasia, and oral squamous cell carcinoma (OSCC). To compare and co-relate the expression of myofibroblasts in normal oral mucosa, epithelial dysplasia, and oral squamous cell carcinoma. To co-relate the progressive increase in myofibroblasts expression in normal oral mucosa, epithelial dysplasia, and oral squamous cell carcinoma. **Materials and Method:** Forty-nine paraffin-embedded tissue blocks with 7 cases of normal oral mucosa, 21 cases of epithelial dysplasia, and 21 diagnosed cases of OSCCs were studied. The samples were subjected to heat-induced antigen retrieval methods followed by staining using primary mouse monoclonal antibodies against α-smooth muscle actin (SMA) and vimentin. Staining index of all the sections was calculated. Statistical analysis was performed using the Kruskal–Wallis test, Mann–Whitney U test, and Chi-square test. Values of *P* less than or equal to 0.05 ($P \leq 0.05$) were considered statistically significant. **Results:** Statistically significant staining index was obtained by α-SMA and vimentin between normal oral mucosa, epithelial dysplasia, and OSCC. **Conclusion:** Myofibroblast may play a role only during initial tumorigenesis that is the conversion of severe dysplasia into OSCC.

Keywords: α-SMA, immunohistochemistry, myofibroblasts, vimentin

Introduction

It has been very much perceived that the cancers of the oral cavity and the pharynx are a general medical issue and therefore, there is an extraordinary number of fatality and individuals experiencing sicknesses or inability in numerous countries.[1]

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The occurrence of oral cancer is getting higher in most countries, particularly in developing countries. In India, it positions number one among men and third among women.[2]

Despite therapeutic advances, survival rates for patients with oral squamous cell carcinoma (OSCC) remain at approximately 50% and have not improved over several decades. Improving detection, diagnosis, and treatment of precancerous changes and early asymptomatic cancers is imperative to increase survival and improve functional outcomes for persons at risk to develop oral cancer. Oral carcinogenesis proceeds through a stepwise accumulation of genetic damage over time. Genetic mutations often produce early phenotypic changes that may present as clinically apparent, recognizable lesions. Oral lesions that have been identified clinically as having potential for malignant conversion include leukoplakia, erythroplakia, lichen planus, and submucous fibrosis.[3]

As there is a transformation of normal epithelium to precancerous and to squamous cell carcinoma, the stroma likewise changes from normal to primed to activated or tumor-associated known as the stromal reaction.[4] In the past, the stroma was regarded as a support tissue for cancer cells, the outcomes obtained in various scientific studies indicated that it can control the processes of tumor invasion and metastasis.[5]

One of the stromal responses is the presence of specific fibroblasts called myofibroblasts.[4] Myofibroblasts have smooth muscle-like features due to the presence of contractile apparatus and were first seen by "Gibbiani" in granulation tissue during wound healing under an electron microscope.[6,7]

These cells play a key role in physiologic procedures like wound healing and pathologic conditions such as reactive lesions, benign tumors, locally aggressive tumors, and malignancies affecting the oral cavity. Myofibroblasts, that are found in normal skin tissues, pulmonary septa, and periodontal ligaments, are distinctive and attributable to their location, they are known as juxtaparenchymal cells.[5,8,9] Some authors also named myofibroblasts as cancer-associated fibroblasts.[10]

Because of their contractile features and capability to produce extracellular matrix components, cytokines, proteases, and proangiogenic factors, myofibroblasts have been involved in the progression of many tumors, including OSCC. Therefore, myofibroblasts might be utilized as a stromal marker for the progression of oral premalignancy into malignancy.[4]

In this study, we intend to evaluate the immunohistochemical expression of myofibroblasts using alpha-smooth muscle actin (α-SMA) and vimentin in formalin-fixed paraffin-embedded sections of normal oral mucosa, epithelial dysplasia, and OSCC and to find out the correlation between the expression of myofibroblasts within the three groups.

Materials and Methods

After obtaining permission from the institutional ethics committee, the study sample included 49 archival tissue specimens embedded in paraffin wax blocks of reported cases, which were previously diagnosed in the Department of Oral Pathology and Microbiology, Himachal Institute of Dental Sciences, Paonta Sahib.

Epithelial dysplasia was recorded as mild, moderate, and severe and the OSCC cases were histologically graded as well, moderately, and poorly differentiated. Inflammation was minimal in normal tissues and all were devoid of pathologic conditions. Samples were categorized as:

- Group I: consisted of 7 cases of normal oral mucosa.
- Group II: consisted of 21 cases of epithelial dysplasia.
- Group III: consisted of 21 cases of OSCC.

Endothelial cell lined blood vessels were used as an internal positive control for α-SMA and human tonsil were used as a positive control for vimentin.

Sections of 4-micron and 3-micron thickness were prepared from each paraffin block using semi-automatic microtome. Figures 1-3 shows the Immunohistochemical staining of myofibroblasts for α SMA and vimentin in various grades of dysplasia and OSCC. The 4-micron sections were floated onto albumin- coated slides and stained according to routine H and E protocol. The 3-micron sections were floated on poly-L-lysine coated slides for immunohistochemical staining with α-SMA and vimentin.

Immunohistochemical staining procedure

Immunostaining for demonstration of Myofibroblasts was performed according to the manufacturer's instructions, using

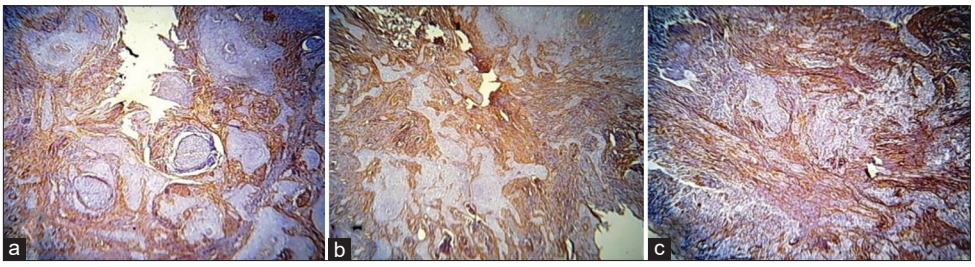


Figure 1: Immunohistochemical staining for α–SMA, (a) well differentiated squamous cell carcinoma (4x); (b) moderately differentiated squamous cell carcinoma (4x); (c) poorly differentiated squamous cell carcinoma (4x)

primary mouse monoclonal antibodies against α-SMA (clone 1A4; Biogenex) and vimentin (clone V9; Biogenex). According to datasheet provided, both the antibodies stain cytoplasm only.

Procedure

The staining protocol was followed according to manufacturer's instructions. Sections were allowed to dry at room temperature overnight and heated at 60°C on a slide warming table for 1 hour before staining. Sections were deparaffinized in two changes of xylene for 10 min each. Rehydration was done in 100% alcohol for 5 min, 85% alcohol for 5 min, and 70% alcohol for 5 min. Slides were rinsed in running tap water for 5 min. Antigen retrieval was performed by heat-induced epitope retrieval (HIER) technique using microwave (EZ retriever system, Biogenex). For this section were immersed in 10 mM sodium citrate buffer (pH 6.0) in the containers supplied with the retrieval system and two cycles were run: first cycle at 90° c for 10 minutes and second cycle at 95°c for 15 minutes. Slides were cooled to room temperature for 30 minutes and washed with phosphate-buffered saline (PBS) in three changes for 3 minutes each. Slides were then incubated with peroxide block (3% hydrogen peroxide) for 10 minutes in the humidifier chamber to block endogenous peroxidase activity. Slides were washed with three changes of PBS for 3 min each. Incubation with power block (for nonspecific background staining) was done for 15 minutes in the humidifier chamber. Sections were then incubated with mouse monoclonal antibody to α-SMA and vimentin, respectively for 1 hr at room temperature. Slides were washed with three changes of PBS for 3 min each. Slides were then incubated with a super enhancer for 25 minutes. Incubation with secondary antibody labeled with horseradish peroxidase was done for 30 min. Slides were again washed with three changes of PBS for 3 min each. Incubation with 3,3'- diaminobenzidine tetrahydrochloride (DAB) chromogen for 5-10 minutes. After this, slides were washed in running tap water for 2 minutes. All slides were then counterstained with Harris haematoxylin for 30 seconds. Slides were washed in running tap water for 5 minutes and dehydrated in 70% alcohol for 2 min, 80% alcohol for 2 min, and 100% alcohol for 2 min. For clearing, slides were placed in two changes of xylene for 2 min each. All slides were mounted in dibutyl phthalate polystyrene xylene (DPX). With every batch of immunohistochemistry (IHC) staining, positive control was used to standardize the procedure.

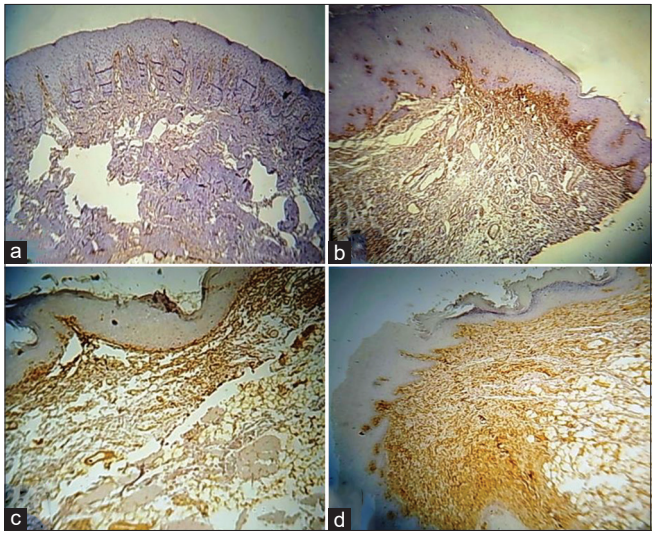


Figure 2: Immunohistochemical staining for vimentin, (a) normal oral mucosa (4x) (b) mild dysplasia (4x); (c) moderate dysplasia (4x); (d) severe dysplasia (4x)

Cell counting and scoring

Histopathological pictures were captured using Labovision Freedom 5000 5 MP Wi-Fi Camera. To evaluate the level of α-SMA and vimentin expression the percentage of positive stained stromal spindle cells (at 400x in five microscopic fields) and the staining intensity were graded on a scale of 0–3 by three observers according to the method proposed by Tuxhorn JA *et al.*[11]

Percentage of myofibroblasts

0: 0% positive cells.
1: 1–33% positive cells.
2: 34–66% positive cells.
3: 67–100% positive cells.

Staining intensity

0: no staining.
1: staining obvious only at 400x.
2: staining obvious at x100 but not at 40x.
3: staining obvious at 40x.

For each sample, the staining percentage and staining intensity scores were multiplied to give the staining index. Staining index was classified as:

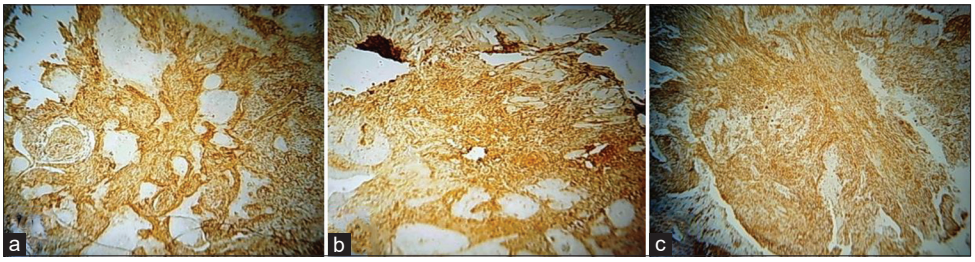


Figure 3: Immunohistochemical staining for vimentin, (a) well differentiated squamous cell carcinoma (4x); (b) moderately differentiated squamous cell carcinoma (4x); (c) poorly differentiated squamous cell carcinoma (4x);

Staining index

0: Zero.
1–2: Low.
3–4: Moderate.
6–9: High.

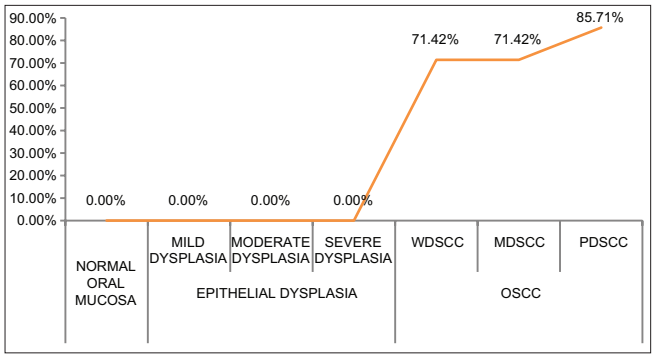
Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis test, Mann–Whitney U test, and Chi-square test. Values of *P* less than or equal to 0.05 ($P \leq 0.05$) were considered statistically significant.

Discussion

In our study, α-SMA positive myofibroblasts were not seen in normal oral mucosa and epithelial dysplasia [Graph 1]. The staining index was zero in all of these cases. These findings are in accordance with the previous studies done by Kellerman MG *et al.* (2008),[12] Rodrgues PC *et al.* (2015),[13] and Smitha A *et al.* (2019).[14] Similar results have also been reported by various other authors.[14-19] But in contrast to our study, small amount of myofibroblasts were reported in high-risk epithelial dysplasia by Chaudhary M *et al.* (2012),[20] Kapse SC *et al.* (2013),[21] Gupta K *et al.* (2015),[22] and Khalid A *et al.* (2019).[23]

The literature[12-17] along with our study findings indicates that stromal myofibroblasts cannot be used as a marker to predict malignant transformation potential of epithelial dysplasia. This relative absence of myofibroblasts in the stroma of epithelial dysplasia may also reflect its low malignant transformation potential despite its high prevalence.



Graph 1: Line diagram showing percentages of positive cases (α-SMA) α SMA: alpha-smooth muscle actin; WDSCC: well-differentiated squamous cell carcinoma; MDSCC: moderately differentiated squamous cell carcinoma; PDSCC: poorly differentiated squamous cell carcinoma; OSCC: oral squamous cell carcinoma

Five out of 7 cases of both well-differentiated squamous cell carcinoma (WDSCC) as well as moderately differentiated squamous cell carcinoma (MDSCC) showed α-SMA positive myofibroblasts and 6 out of 7 cases of poorly differentiated squamous cell carcinoma (PDSCC) showed α-SMA positive myofibroblasts [Graph 1]. Overall, out of total 21 cases of OSCC, 16 cases showed α-SMA positive myofibroblasts that means 71.9% cases are positive. Similar findings have also been reported by Rodrigues PC *et al.* (2015)[13] (59.8%), Jayaraj G *et al.* (2015)[15] (45.3%), Chaudhary M *et al.* (2012)[20] (97.29%), Kapse SC *et al.* (2013)[21] (70%), in OSCC though the percentage of positive cases differs. The staining index was zero in 5 cases, low in 4 cases, moderate in 8 cases, and high in 4 cases of OSCC [Table 1].

A statistically significant difference was observed between normal oral mucosa, epithelial dysplasia, and OSCC ($P < 0.05$) but the distribution of myofibroblasts was not significantly different between three histological grades of Squamous Cell Carcinoma ($P = 0.145$). Studies done by Kellerman MG *et al.* (2008)[12] and Moghadam ES *et al.* (2009)[17] also revealed no statistically significant difference in the mean number of myofibroblasts between well, moderately, and poorly differentiated OSCC and suggested that transdifferentiation of myofibroblasts is induced during the invasive stage of carcinomatous epithelium and further loss of tumoral differentiation does not affect the number of cells. In contrast to our study, Rodrigues PC *et al.* (2015)[13] observed significantly higher expression of myofibroblasts in MDSCC and PDSCC as compared to WDSCC. The difference in positive findings can be due to tumor stroma because tumors lacking fibrous stroma were devoid of myofibroblasts. This has been postulated by Jayaraj J *et al.* (2015).[15] Our findings along with previous studies indicated that myofibroblasts are not associated with the transformation of epithelial dysplasia into OSCC. Dysplasia represents an intermediate step, wherein the stroma is losing its control over epithelial morphogenesis. Once the dysplastic epithelium accumulates further mutations resulting in the

Table 1: Mean immunohistochemical staining index of myofibroblasts for α-SMA in normal oral mucosa and different grades of epithelial dysplasia and OSCC

Groups		Number of Cases (*n*)	α-SMA Staining Index			
			Zero	Low	Moderate	High
Normal Oral Mucosa		7	7	0	0	0
	Mild	7	7	0	0	0
Epithelial Dysplasia	Moderate	7	7	0	0	0
	Severe	7	7	0	0	0
	WDSCC	7	2	2	3	0
OSCC	MDSCC	7	2	2	2	1
	PDSCC	7	1	0	3	3

WDSCC: well-differentiated squamous cell carcinoma; MDSCC: moderately differentiated squamous cell carcinoma; PDSCC: poorly differentiated squamous cell carcinoma; OSCC: oral squamous cell carcinoma

invasion, the stroma responds by acting as a co-conspirer . Hence, myofibroblasts differentiation is an event that occurs in the carcinogenesis process.[15]

A major issue in our study is that there is no way of knowing whether the dysplasias would transform into OSCC or remain as dysplastic entities. But there seems to be a genetic alteration in the carcinomatous epithelium, which produces an inductive effect on the adjacent stroma to produce myofibroblasts. These mutated epithelial-stromal interactions may be responsible for the induction of initial carcinogenesis only. Additional investigations on these myofibroblasts and the related factors will help us to clarify how and to what extent these cells contribute to carcinogenesis.

In this study, myofibroblasts were found surrounding the tumor islands and cords in the stroma, and often in the deep invasive front of the tumors. Some tumors expressed only a few myofibroblasts in delicate rows surrounding and abutting the tumor islands, while others showed an abundance of myofibroblasts in the stroma, which were organized in a syncytium. In our study, we also found that some epithelial cells at the periphery of tumor islands have also been stained by α-SMA. This has not been reported by any of the studies till date. As we know that epithelial cells can undergo epithelial-mesenchymal transitions and transform into myofibroblasts.[8] So this may be the reason for the positive staining of these cells.

All the cases of normal oral mucosa, dysplasia, and OSCC showed a positive reaction of α-SMA around blood vessels, which was used as an internal positive control.

The other marker used in our study to observe myofibroblasts was vimentin. In our study, normal oral mucosa (n = 3/7, 42.85%), mild dysplasia (n = 4/7, 57.4%), moderate dysplasia (n = 3/7, 42.85%), severe dysplasia (n = 5/7, 71.42%), WDSCC (n = 6/7, 85.7%), MDSCC (n = 7/7, 100%), PDSCC (n = 7/7, 100%) showed positive spindle cell staining with vimentin [Graph 2]. Similar results were obtained by Kapse SC *et al.* (2013)[21] though the percentage of positive cases differs due to variation in fibrous component as stated above. Some of the inflammatory cells also took the stain that may be due to background staining.

Out of 21 cases, OSCC staining index was zero in one case, moderate in 3 cases, and high in 17 cases. In the case of epithelial dysplasia, staining index was zero in 11 cases, low in 9 cases, and zero in 1 case. Four cases of normal oral mucosa showed zero staining index and three cases showed a low staining index [Table 2].

Vimentin expression was detected in the cytoplasm of connective tissue cells only but not in the epithelial cells which were in `accordance with the studies done by Kapse SC *et al.* (2013),[21] Sawant SS *et al.* (2014),[24] and Liu LK *et al.* (2010).[25] Vimentin, a general connective tissue marker, stained all the connective tissue components, which was in accordance with the study done by Smitha GP *et al.* (2016).[26] In our study, we concluded that vimentin cannot be used alone as a marker to observemyofibroblasts as it stains a lot of other connective tissue cells including fibroblasts adipocytes, muscle cells, and endothelial cells. Till date only Moghadam ES *et al.* (2009)[17] and Kapse SC *et al.* (2013)[21] have compared the staining performance of vimentin and α-SMA to stain myofibroblasts. They also concluded that α-SMA stains myofibroblasts better than vimentin.

In our study, staining index of vimentin was higher in OSCC than epithelial dysplasia and normal oral mucosa. Statistically significant difference was found in staining index between normal oral mucosa, epithelial dysplasia, and OSCC (p = <0.05) but when staining index of WDSCC, MDSCC, and PDSCC was compared, no statistically significant results were obtained (P = 0.145); also there was no significant difference in the staining index of epithelial dysplasia and normal oral mucosa (P = 0.614).

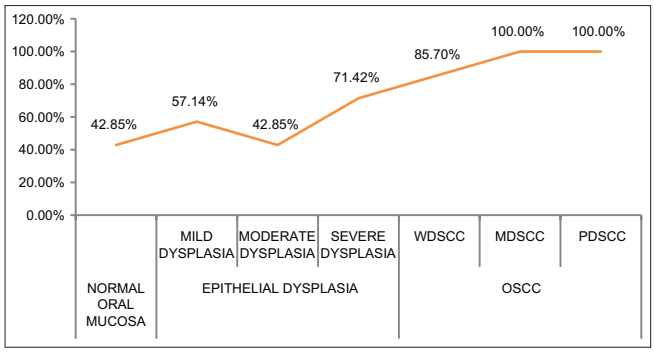
Since statistically significant staining index was obtained by α-SMA and vimentin between normal oral mucosa, epithelial dysplasia, and OSCC, we can conclude that myofibroblast may play a role only during initial tumorigenesis that is the conversion of severe dysplasia into OSCC.

Liu LK *et al.* (2010)[25] studied the pattern of tumor invasion at normal tissue tumor interface using vimentin. They concluded that increased expression of vimentin was associated with decreased expression of e-cadherins and hence higher chances of metastasis and recurrences. Hence, myofibroblasts do play a key

Table 2: Mean immunohistochemical staining index of myofibroblasts for vimentin in normal oral mucosa and different grades of epithelial dysplasia and OSCC

Groups		Number of Cases (*n*)	Vimentin Staining Index			
			Zero	Low	Moderate	High
Normal Oral Mucosa		7	4	3	0	0
	Mild	7	3	4	0	0
Epithelial Dysplasia	Moderate	7	5	1	1	0
	Severe	7	3	4	0	0
	WDSCC	7	1	0	0	6
OSCC	MDSCC	7	0	0	3	4
	PDSCC	7	0	0	0	7

WDSCC: well-differentiated squamous cell carcinoma; MDSCC: moderately differentiated squamous cell carcinoma; PDSCC: poorly differentiated squamous cell carcinoma; OSCC: oral squamous cell carcinoma



Graph 2: Line diagram showing percentages of positive cases (vimentin) WDSCC: well-differentiated squamous cell carcinoma; MDSCC: moderately differentiated squamous cell carcinoma; PDSCC: poorly differentiated squamous cell carcinoma; OSCC: oral squamous cell carcinoma

role in tumor invasion and metastasis and epithelial-mesenchymal interactions at the tumor invasion front but their identification is challenging.

In our study, we observed that vimentin has high sensitivity and poor specificity as stated by Painter JT *et al.* (2010).[27] It can be used to differentiate epithelial cells from stromal cells but cannot be used to differentiate fibroblasts from myofibroblasts because vimentin stains a number of stromal cells as explained above but α-SMA is a better marker than vimentin as it stains myofibroblasts and smooth muscle fibers only. Tomasek JJ *et al.* (2002)[6] also stated that α-SMA is the most reliable marker for differentiated myofibroblasts because of higher specificity.

The network pattern of myofibroblasts in OSCC represents higher invasive features and weaker prognosis. We can also say that due to more amount of myofibroblasts in network arrangement, neoplastic lesions show more rigorous invasive behavior in contrast to spindle pattern.[23]

In future, if our findings are established by further examinations, therapeutic targeting of myofibroblasts, their byproducts, or factors responsible for their transdifferentiation from fibroblasts may be beneficial to OSCC patients and can probably be considered as a new auxiliary method that will cause fewer complications. However, further studies are recommended for more reliable achievements to be reached.

Conclusions

Benefitting from the advantage of myofibroblasts in physiologic processes and blocking the processes leading to the causation and progression of OSCC is the need of the hour. Additional knowledge and clinical studies involving this unique cell may provide us with an effective target for cancer therapy. In the future, it will be worth exploring the autocrine and paracrine effects of transformed myofibroblasts and stromal epithelium interaction as a potential therapeutic approach against cancer progression via drug interaction.

Clinical significance

Different surgical methods, radiotherapy, and chemotherapy, which are currently used in the treatment of squamous cell carcinoma cause some complications that have not improved the life expectancy of the patients in 25 years. If our findings are confirmed by future investigations, therapeutic targeting of myofibroblasts, their byproducts, or factors responsible for their transdifferentiation from fibroblasts may be beneficial to OSCC patients and can probably be considered as a new auxiliary method that will cause fewer complications.

Limitations

The limitations of this study take account of the fact that there was no way to determine whether the cases of oral epithelial dysplasia would have remained as dysplastic entities or whether they would have transformed into OSCC. However, further studies with an increased sample size are recommended for more reliable achievements to be reached.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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