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Research Paper: A Comparative, semi-quantitative Evaluation of Myofibroblasts Between Mucoepidermoid Carcinoma and Pleomorphic Adenoma Using α -Smooth Muscle Actin Marker

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ABSTRACT

Introduction: Myofibroblasts are the main stromal components that constitute the desmoplastic reaction of host cells to inductive stimuli exerted by tumor cells. The purpose of this study was to evaluate the score of myofibroblasts using α -smooth muscle actin marker (α–SMA) in mucoepidermoid carcinoma (MEC) in comparison with pleomorphic adenoma (PA)and study the amount presence of these cells in those tumors.

Materials and Methods: The study included 20 cases of mucoepidermoid carcinoma and 20 cases of pleomorphic adenoma, using paraffin-embedded blocks that are appropriate for immunohistochemistry staining. 5 cases of mucocele and 5 cases of oral squamous cell carcinoma served as the negative and positive control. The expression of α -smooth muscle actin marker was determined by the immunohistochemically stained section. Myofibroblasts presence was assessed by a semiquantitative scale based on the score of immunopositive staining(0,1+,2+and 3+). The data were analyzed statistically with SPSS (ver.24) statistical software and using Mann-Whitney, Chi-Square, and Spearman`s correlation tests.

Results: There are no significant differences in the mean score of positive cells for mucoepidermoid carcinoma and pleomorphic adenoma with mean and standard deviation (2.45±0.89) and (2.10±0.91) (Pvalue=0.182). Also, there are no significant differences between low and high grades of mucoepidermoid carcinoma (Pvalue=0.4).

Conclusion: In conclusion, despite the presence of α-SMA-positive myofibroblast cells in the connective stroma, PA capsule septa, and the stroma of MEC cases with different grades of malignancy, no significant difference was detected in the frequency of myofibroblast cells between the two groups.

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Introduction

Salivary gland tumors are an important part of oral and maxillofacial lesions. Although uncommon, these tumors are not rare.(1) Pleomorphic Adenoma (PA) is the most common benign salivary gland tumor that has the following three components: an epithelial cell component, a myoepithelial cell component, and a stromal component.(2)

Mucoepidermoid carcinoma (MEC) is a malignant epithelial neoplasm, which is composed of different components such as mucosal, epidermoid, intermediate, columnar, and clear cells. MEC is usually graded into low, intermediate, and high based on its histological characteristics; each of these grades indicates a different grade of aggressiveness.(3)

Coordinated activity of epithelial cells with their supporting stroma plays a key role in the control of growth and the distinction of normal from pathological conditions. Fibroblasts and myofibroblasts form the major component of Tumor-associated Stroma Cells (TASC).(4)

Myofibroblasts are activated fibroblasts containing the contractile protein of smooth muscle cells, especially vascular smooth muscles such as α-Smooth Muscle Actin (α-SMA). The presence of α-SMA is the most reliable marker for myofibroblast phenotype.(5)

Myofibroblasts play an important role in connective tissue remodeling. These cells can also affect epithelial cells and other connective tissue cells, leading to tumor invasion or angiogenesis.(6)

Nearly 30 years ago, myofibroblasts were observed in the stroma of various invasive and metastatic malignant tumors. At that time, it was thought that the presence of these cells was part of the host response to the invasion of malignant cells. However, over the past ten years, it has been shown that the presence of myofibroblasts at the invasion site is not part of the host defense mechanism against tumor invasion, but a mechanism promoting the invasion process.(7)

The present study was conducted to determine the level of myofibroblast cell staining with the smooth muscle actin-α marker as a marker of tumor invasiveness in mucoepidermoid carcinoma in comparison with benign pleomorphic adenoma tumor and to study the role and possibility of the presence of these cells in different grades of malignancy.

Material and methods

In this descriptive cross-sectional study, all pathology reports archived in the pathology departments of Shahid Sadoughi University of Medical Sciences (Yazd, Iran), and Al-Zahra Hospital (Isfahan, Iran) were reviewed, and 20 samples of the MEC (11 low grade MEC and 9 high grade MEC) and 20 samples of the PA tumors were selected Based on the pathological reports in the archive.

The sample selection criteria were:

1. Having sufficient tissue section of at least 10 High Power Field (HPF)
2. Having a suitable paraffin-embedded tissue section for cutting and staining

Moreover, five cases of normal salivary gland tissue (Mucocele) (as negative control) and five cases of squamous-cell carcinoma tissue (as positive control) were considered. (figure 1) After separating the suitable paraffin blocks, a 3µm thick section was isolated from each block and sent to the immunohistochemistry laboratory for immunohistochemistry staining.

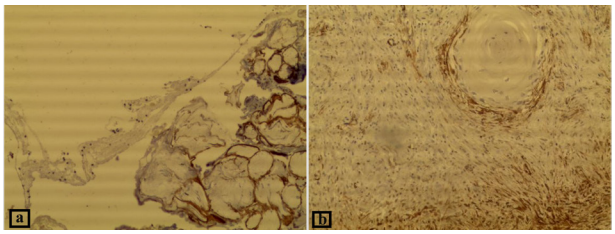

Figure 1. (a)The negative reaction of normal salivary gland tissue (Mucocele) to α-SMA marker (IHC*10), (b) The intense stainability of α-SMA in one case on Squamous cell carcinoma (positive control) (IHC*10)

The prepared samples were mounted on microscopic slides; after the removal of wax in xylene, they were immersed in ethanol, washed in distilled water, placed in 3% hydrogen peroxide, and finally washed in distilled water for 15 minutes.

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For antigen modification, the slides were placed in citrate buffer solution (PH= 6.0) and then put in a microwave at 92°C for 10 minutes. After cooling down to room temperature for 20 minutes, the slides were placed in mouse anti-human α-SMA antibody (Dako A/S, Denmark, clone 1A4)

with a dilution of 0.01 for 60 minutes at room temperature. For antibody detection, a Histofine Peroxidase Polymer kit (rabbit anti-mouse) (multi, Nichirei, Tokyo, Japan) was used. The tissue sections were washed in PBS for 10 minutes, and an AEC Substrate Chromogen kit (Zymed, San Francisco, CA, USA) was added to them; then, they were washed in PBS for two minutes, counterstained with Mayer solution (Pioneer Research Chemical, Colchester, UK), and covered by intermediate mounting material. The sections stained with monoclonal anti-α-SMA antibody were blindly examined by two experts via Olympus light microscope. Then, the distributions of stained myofibroblast cells were scored semi-quantitatively according to Gunhan (8) and Whitaker (9)method as follows:

Score 0: no reaction to α-SMA marker
Score 1: weak and sparse reaction
Score 2: medium stainability as a low-rate, diffuse or sparse reaction
Score 3: intense stainability in a diffuse and clear manner

After scoring, the stainability scores of MEC slides were compared with their malignancy grades. Using the SPSS version 24 (Chicago, IL, USA), the coded data were analyzed via Mann–Whitney U, Chi-square, and Spearman's correlation tests. $p<0.05$ was considered to be statistically significant.

Ethical Considerations

All samples were related to patients referring to the hospital for maxillectomy; the operation had surgical indications for the patients. This research was approved by the Committee of Ethics in Human Research at Shahid Sadoughi University of Medical Sciences, Yazd,Iran (record No. 213)

Results

The immunohistochemical test was performed on 20 samples of each of the MEC and PA tumors using α-SMA marker. The results were collected and recorded in special tables. In the table 1, the frequencies of different α-SMA-positive cell scores in the two groups are presented.

Table 1. Frequencies of myofibroblast cells' stainability scores in MEC and PA groups

Stainability score / Type of lesion	0		+1		+2		+3		total	
	N	%	N	%	N	%	N	%	N	%
Mucoepidermoid carcinoma	1	5	2	10	4	20	13	65	20	100
Pleomorphic adenoma	0	0	7	35	4	20	9	45	20	100
Total	1	2.5	9	22.5	8	20	22	55	40	100

According to these results, only one of the MEC cases did not show an immunohistochemical reaction to the α-SMA marker, while the majority of PA and MEC cases showed intermediate -to-high positive reactions with the mentioned marker. (figure 2,3)

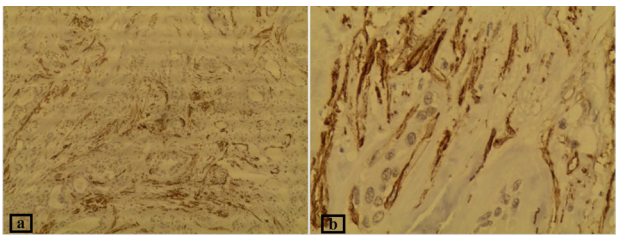

Figure 2. (a and b) medium stainability of α-SMA in one case of PA (IHC*10 and IHC*40)

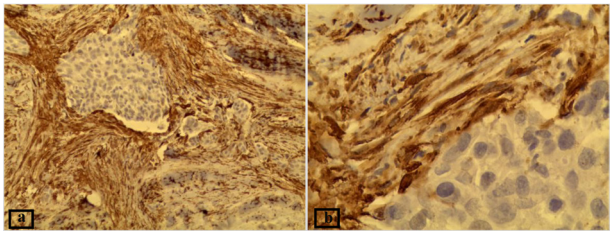

Figure 3. (a and b) The intense stainability of α-SMA in one case of MEC (IHC*10 and IHC*40)

The means and standard deviations in MEC (2.45 ± 0.89) and PA (2.10 ± 0.91) tumors are shown. The results of the Mann–Whitney U test showed no significant difference concerning the average α-SMA-positive cells' stainability score between MEC and PA tumors (p=0.182).

In the table 2, the frequencies of different α-SMA-positive cell scores in different grades of MEC malignancy. The results of the Chi-square test indicated no significant difference between the average α-SMA-positive cells' stainability scores and different grades of malignancy (p=0.4). it should be noted that, among the examined samples, there was no case of MEC with an intermediate grade of malignancy.

Table 2. Frequencies of myofibroblast cells' stainability scores in different malignancy grades of MEC (Chi-square test, p=0.4)

Stainability score / Grade of malignancy	0		+1		+2		+3		total	
	N	%	N	%	N	%	N	%	N	%
Low	1	5	2	10	2	10	6	30	11	55
High	0	0	0	0	2	10	7	35	9	45
Total	1	5	2	10	4	20	13	65	20	100

Discussion

Evidence suggests that the secretion of soluble factors by stromal myofibroblast cells is involved in the process of tumor invasion and progression. Stroma cells with myofibroblastic differentiation features are the predominant cell type in primary and metastatic epithelial tumors. The presence of α-SMA-positive cells in mesenchymal cells of epithelial proliferation indicates that a mutual connection between epithelium and mesenchyme is necessary before the onset of the invasion.(13) Myofibroblast cells have been identified on the invasive edge of many malignant tumors originating in the colon, breast, liver, lung, prostate, pancreas, and oral carcinoma.(10)

Zidar et al. stated that invasion to the basement membrane is necessary for inducing stromal myofibroblast response.(11) Kojc et al. showed that the absence of CD34-positive stromal cells and the presence of α-SMA-positive stromal myofibroblasts are associated with the conversion of laryngeal squamous intraepithelial lesions to Squamous Cell Cancer (SCC). It has also been reported that the proliferation of myofibroblasts is significantly associated with the grade of malignancy, disease grade, regional recurrence, and growth rate of tumor cells in oral SCC. (12)

In this study, myofibroblast cells were studied in MEC and PA tumors based on Gunhan (8) and Whitaker (9) method. the results showed no statistically significant difference in the frequency of myofibroblast cells between the two types of tumors, strengthening the inferences that myofibroblast cells are an integral part of the growing tumors' stroma and that the density of myofibroblast cells cannot differentiate benign from malignant lesions. This finding is consistent with what Vered et al. found when examining myofibroblast cells in invasive and non-invasive CGCGs (Central Giant Cell Granuloma). Vered et al. believed that the biological behavior of CGCG lesions is more associated with the origin of myofibroblasts rather than their density. (14)

Soma et al. examined benign and malignant salivary gland tumors and showed that the frequencies of myofibroblasts in benign tumors were significantly higher than malignant ones. Their findings suggested that the absence of myofibroblasts was involved in the spread of malignant tumors, indicating their role in tumor diffusion restriction. They also believed that myofibroblasts play different roles via the expression of different proteins during different biological processes and the expression of different phenotypes in different organs.(15)

Mashhadi Abbas et al. conducted a study on

myofibroblasts of odontogenic cysts and ameloblastoma and showed that the presence of myofibroblasts might be effective in odontogenic keratocyst's (OKC) aggressive behavior toward dentigerous cyst (DC), but the hypothesis was not confirmed when the same comparison was made between DC, OKC, and ameloblastoma. (5) Syamala et al. interpreted the presence of myofibroblasts in the wall of odontogenic cysts as a homeostatic response helping cystic dilatation.(16) Their findings were in line with the results of the present study concerning the presence of myofibroblast cells in the stroma of growing tumors.

In this study, the diffusion pattern of α-SMA-positive cells in different grades of MEC was not significant, and no significant difference was detected between different MEC grades in terms of marker expression.

Sorbal et al. considered stromal myofibroblasts as a barrier to tumor progression. They believed that the proliferation of these cells could be an attempt to prevent tumor progression and also a possible prognostic factor. Their study also pointed to the dual role of TGFβ1 in tumor inhibition and progression; they stated that high expression of TGFβ1 contributes to tumor invasion and metastasis by stimulating the synthesis of tenascin and extracellular matrix proteins. They also showed that α-SMA-positive spindle cells were mostly observed in the peripheral parts of low-grade MEC cases, and regionally in the environment of neoplastic cell nests in intermediate-grade MEC cases. They reported a small number of α-SMA-positive cells in highly malignant tumors. They only observed one instance of α-SMA-positive cells in the stroma and at the periphery of cystic regions among the MEC cases.(17)

Vered et al. formulated the hypothesis that the higher the frequency of myofibroblast cells in the stroma, the more aggressive behavior is expected from cysts and odontogenic tumors.(18) This hypothesis was not confirmed in the Mashhadi Abbas et al. study, but it was confirmed in Anusai et al.'s study on two odontogenic cysts. (13)

The results of in vitro studies have shown that the mechanical properties of tumor-activated myofibroblasts prevent the infiltration of T-lymphocyte and macrophages into the nodule. The effect of activated T-cells on the differentiation of α-SMA-positive stromal cells in gastric carcinoma has been reported. it has also been suggested that tumor-related myofibroblast cells may be involved in leukocyte migration through cytokine secretion. Therefore, considering the different results obtained in the previous studies, it can be inferred that the initial presence of inflammatory cells can be important in myofibroblast cell differentiation; but, with increasing the number of myofibroblast cells in later stages, the frequency of inflammatory cells decrease.(13) Nonetheless, this inference requires further investigation.

Conclusion

According to the results of this study, despite the presence of α-SMA-positive myofibroblast cells in the connective stroma, PA capsule septa, and the stroma of MEC cases with different grades of malignancy, no significant difference was detected in the frequency of myofibroblast cells between the two groups. The results suggest further studies to evaluate the exact role of stromal myofibroblasts in salivary gland tumors and their possible involvement in the pathogenesis of these lesions.

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