RESEARCH COMMUNICATION

Myofibroblast Stromal Presence and Distribution in Squamous Epithelial Carcinomas, Oral Dysplasia and Hyperkeratosis

Safora Seifi*, Shahryar Shafaei, Ensieh Shafigh, Seid Mehdi Sahabi, Hamidreza Ghasemi

Abstract

**Purpose:** Stromal elements play a key role in growth and development of different neoplasms. Myofibroblasts are the major components and occur in stromal tissue during carcinogenesis processes. The purpose of this study was to review the frequency and the distribution pattern of myofibroblasts (αSMA-positive) in the stroma of squamous epithelial carcinoma and to compare values with those for oral dysplasia and hyperkeratosis.

**Methods:** We evaluated αSMA protein frequency in hyperkeratosis (N =18), oral epithelial dysplasia (N=18) and oral squamous cell carcinoma (N=18) using immunohistochemistry.

**Results:** αSMA-positive expression was observed in 67% of OSCC tissue samples with network and spindle patterns, whereas it was seen in 22% with a focal pattern in dysplasia and in 6% with a scanty pattern in hyperkeratosis cases. **Conclusion:** These findings suggest that an increase in number of myofibroblasts and change in their distribution pattern occurs during carcinogenesis which can be an expression of their role in tumor invasive characteristics.

**Keywords:** Squamous cell carcinoma - dysplasia - hyperkeratosis - αSMA - immunohistochemistry

Introductions

Myofibroblasts are involved in stromal reactions (Adegbuyega et al., 2002) and they are key players in enhancing the connective tissue in wound repair and fibrous tissues (Desmouliere et al., 2004). A unique group of smooth-muscle-like fibroblasts, although they can secrete some cytokines, chemokines, prostaglandins, growth factors and matrix components, they play a key role in inflammatory, growth, and wound repair processes and also are a cause of cancer (Muchaneta-Kubara et al., 1997). Myofibroblasts occur in a variety of organs. (Varayoud et al., 2001) in human and animal normal tissues (Tomasek et al., 2002), in pathological conditions in benign and malignant lesions (Fletcher, 1998), in reactive lesions such as giant cell fibroma (Weather and Campbell, 1974), peripheral giant cell granuloma (Dayan et al., 1989) and in cyclosporine A induced hyperplasia (Yamasaki et al., 2006).

Histologically, normal oral mucosa is composed of epithelium and connective tissue. The connective tissue around the epithelium has protective and nutritional roles for the epithelium. The presence of cancer is followed by some changes that happen in epithelium and the normal stroma and normal stroma becomes a reactive one. The formation of reactive stroma is associated with the secretion of cytokines such as TGFβ-1 from cancerous cells that promotes differentiation of fibroblasts into myofibroblasts, increases the number of blood vessels, increases the inflammatory cells, causes desmoplasia, decreases the expression of epithelial markers (cadherins), and increases the expression of mesenchymal markers such as vimentin. Myofibroblasts in turn secret cytokines and matrix metalloproteases which in turn contribute to the destruction of extracellular matrix and cause tumor growth. (Tuxhorn et al., 2002; De Werer and Mareel, 2003).

The role of inflammatory and endothelial cells in tumor protection and angiogenesis has already been described (Chen et al., 2005), but there is little known about the role of myofibroblasts in carcinogenesis of oral cancer. The way in which cells are arranged in a tissue is defined as cell distribution (Neville et al., 2009). There has been always a question concerning whether the stromal cells distribution in carcinogenesis is random or their distribution is involved in invasive tumor behavior and prognosis. Few studies have been done on cell distribution of the stromal elements and the importance of them in the multistage process of oral carcinogenesis. Some previous studies have suggested a role of myofibroblasts presence and distribution in facilitation of tumor progression (Vered et al., 2009).

However, some other studies only described the myofibroblasts cell distribution and did not study
its relationship with invasive tumor characteristics (Kellermann et al., 2007). Because the expression of αSMA (α-smooth muscle actin) represents the presence of myofibroblasts in different stromal tissues, and also considering the fact that few studies were done on the mentioned subject, these both led is to investigated the presence and distribution of αSMA-positive myofibroblasts in the stroma of squamous epithelial carcinoma and compare it with oral dysplasia and hyperkeratosis using immunohistochemical methods.

**Materials and Methods**

In this cross-sectional descriptive-analytic study, archive samples between 2003 and 2009 from department of oral and maxillofacial pathology in faculty of dentistry at Babol (North of Iran) University of Medical Sciences were studied and those with dysplasia, oral squamous cell carcinoma and oral hyperkeratosis were included. To complete the number of samples, archive samples between 1991 and 2009 from the Laboratory of pathology at Babol’s Shahid Beheshti Hospital were also included in the study. Totally, 54 paraffin blocks were selected including 18 samples of each lesion. Mild, moderate, and severe dysplasia each was observed in 6 samples. Based on the Neville et al., epithelial dysplasia is classified into 3 groups: (Neville et al., 2009)

a. Mild epithelial dysplasia, shows relatively few cytological aberrations (There is mitosis, pleomorphism, hyperchromatism, etc) involving only basal and parabasal layers of the epithelium.

b. Moderate epithelial dysplasia is when the cytological aberrations extend from basal and parabasal layers to the half of the epithelium.

c. Severe epithelial dysplasia is when the cytological aberrations extend from basal and parabasal layers to more than the half of the epithelium.

Squamous cell carcinoma fitted graded into well-differentiated and moderately differentiated (grade 1 and 2, respectively). Grade 3 (poorly differentiated) was not seen.

Criteria was for classification of OSCC into grades 1-3 according to Neville et al., 2009

a. well differentiated (low grade)(grade1): A tumor with low cellular and nuclear pleomorphism and with high keratin production and with enlarges slowly, metastasizes later in its course, such a tumor is labeled well differentiated.

b. Poorly differentiated (High grade)(grade3): A Tumor with high cellular and nuclear pleomorphism and with no or little keratin production and with enlarges rapidly, metastasizes early in its course, and is called poorly differentiated.

c. Moderate differentiated (grade2): A Tumor with a microscopic appearance these two extremes is termed moderately differentiated carcinoma.

Since some squamous cell carcinoma samples were incisional biopsies, determination of the exact differentiation grade and their comparison were not possible.

Clinical information including age, sex, and the location of the lesion were extracted from patients’ files. Then 4-micron sections were prepared from each paraffin block and were stained using hematoxylin-eosin staining protocol and were restudied. The appropriate blocks from each lesion were selected and 3-micron sections were prepared and again were analyzed by an oral pathologist. Immunohistochemical staining was done using standard Avidin Biotin Peroxidase method.

**Staining method:**

The sectioned tissues were incubated at 37°C for 18 hours, and after that they were incubated at 80°C for 20 minutes. They were deparaffinized in xylene and dehydrated in graded alcohol series followed by antigen retrieval by boiling in citrate buffer at 70-80°C for 30 minutes in an autoclave. The samples were taken out of autoclave after turning it off and were inserted into the citrate buffer for 5 minutes. After drying, under and around the tissues were marked with a pen. The samples were then inserted into the following solutions in this order: Dual Endogen Enzyme Block (to eliminate non-specific affinity) for 5 to 10 minutes, one to two drops of primary antibody (clone 1A4, DAKO, A / S Glostrup, Denmark) αSMA solution with high concentration anti-human rabbit polyclonal antibody (at a dilution of 1/100) for 30 minutes, Streptavidin, DAB chromogen (in order to see the resulted products), and Mayer’s hematoxylin counterstain. The samples were washed in distilled water for 5 minutes between each stage. Dehydration was done with graded alcohol and xylene and samples were mounted using entelan. The stained samples were analyzed at 40-time-magnification under the light microscope (Olympus, BX51). To evaluate the accuracy of the study, positive control of ductal carcinoma of breast (positive αSMA) and negative control (mouse non-immunise serum with eliminated primary antibody) were used. Staining of endothelial cells of the blood vessels with αSMA was used as internal positive control. Five cases of normal oral mucosa with αSMA served as control group. The cytoplasm of those αSMA-stained myofibroblasts in the squamous cell carcinoma of adjacent islands and tumor layers and in dysplasia and hyperkeratosis located under mucosa were counted in 100 cells at 40-time-magnification and the calculated average number was considered as the percentage of stained cells. The αSMA-stained endothelial cells of blood vessel were not included in the calculation. Each section was counted twice and the counting was controlled by another pathologist afterward. The results were scored as follows: (Kellermann et al., 2007)

Score 1 (-) (Negative): If myofibroblasts were not stained with αSMA, or if less than 1% of myofibroblasts were stained with αSMA.

Score 2 (+) (scanty): If more than 1% and less than 50% of myofibroblasts were stained with αSMA.

Score 3 (++) (Abundant): If more than 50% of myofibroblasts were stained with αSMA.

Considering the distribution pattern of myofibroblasts, the arrangement of positive-stained cells was classified into 3 groups:

1. Focal: If myofibroblasts had a focal arrangement or
myofibroblast stromal presence and distribution in squamous lesions had no special arrangement in different areas of connective tissue and stroma.

2 - Network: Myofibroblasts with vesicular nucleus and abundant cytoplasm arranged in multiple rows with interwoven network of cytoplasmic extensions forming a network in the stroma of the connective tissue.

3- Spindle: Myofibroblasts arrange in one to three rows in a regular order in the periphery of the neoplastic islands or in the connective tissues with distinctive cell margins around myofibroblasts and malignant tissue. (Vered et al., 2009)

The results of the study on each lesion was recorded in SPSS(17) and analyzed with Anova, Chi-Square test, Kruskal - Wallis tests. The statistical significance level set at 0.05.

Results

The clinical data of age, sex, the location of the lesion are summarized in Tables 1 and 2. Positive staining was immunohistochemical brown cytoplasmic color using αSMA marker in myofibroblasts of the positive-stroma. Score 3(++) was observed in 8 cases of oral squamous cell carcinoma, score 2(+) in 4 cases, and score 1 (-) in 6 cases (Figure 1- 4). Score 3(++) was observed in 1 case of epithelial dysplasia, score 2(+) in 3 cases, and score 1(-) in 14 cases (Figure 5-8). Score 2(+) was observed only in 1 of the hyperkeratosis cases and score 1 (-) in 17 cases (Figure 9,10) (Tables 3 and 4). Endothelial cells of blood vessel stained with brown αSMA occurred in all lesions. The samples of oral squamous cell carcinoma were made up of large amounts of haphazardly arranged αSMA-positive cells. The αSMA expression was less in samples that showed increased secretion from inflammatory cells as a stromal reaction. Tumors without tumor islands in some areas did not have αSMA expression. The expression of αSMA was mostly observed between and around of neoplastic islands. The relationship between αSMA expression in hyperkeratosis,

Table 1. Frequency of Age and Sex in OSCC, Epithelial Dysplasia and Hyperkeratosis

<table>
<thead>
<tr>
<th></th>
<th>Hyperkeratosis</th>
<th>Epithelial Dysplasia</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (SD ± Mean)</td>
<td>3/10 ± 06/36</td>
<td>1/11 ± 49/47</td>
<td>3/16 ± 06/45</td>
</tr>
<tr>
<td>Sex Male</td>
<td>6 (3/33%)</td>
<td>11 (1/61%)</td>
<td>12 (7/66%)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (7/66%)</td>
<td>7 (9/38%)</td>
<td>6 (3/33%)</td>
</tr>
</tbody>
</table>

P=0.000

Table 3. Results of IHC staining with αSMA in OSCC, Epithelial Dysplasia and Hyperkeratosis

<table>
<thead>
<tr>
<th>Type of Lesion</th>
<th>Score1(-)</th>
<th>Score2(+)</th>
<th>Score3(++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperkeratosis</td>
<td>17 (4/94%)</td>
<td>1 (6/5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Epithelial dysplasia</td>
<td>14 (8/77%)</td>
<td>3 (7/16%)</td>
<td>1 (5/5%)</td>
</tr>
<tr>
<td>SCC</td>
<td>6 (3/33%)</td>
<td>4 (2/22%)</td>
<td>8 (5/44%)</td>
</tr>
</tbody>
</table>

Total 18 (100%) 18 (100%) 18 (100%) 54 (100%)

Table 4. Frequency of Stage of αSMA Positive Myofibroblasts in OSCC, Epithelial Dysplasia and Hyperkeratosis

<table>
<thead>
<tr>
<th>Stage</th>
<th>Hyperkeratosis</th>
<th>Epithelial Dysplasia</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>17 (4/94%)</td>
<td>14 (8/77%)</td>
<td>6 (3/33%)</td>
</tr>
<tr>
<td>+</td>
<td>1 (6/5%)</td>
<td>3 (7/16%)</td>
<td>4 (2/22%)</td>
</tr>
<tr>
<td>++</td>
<td>0 (0%)</td>
<td>1 (5/5%)</td>
<td>8 (5/44%)</td>
</tr>
</tbody>
</table>

Total 18 (100%) 18 (100%) 18 (100%) 54 (100%)
The results of this study showed that αSMA expression in the stroma of squamous epithelial carcinoma was greater than its expression in epithelial dysplasia and oral hyperkeratosis which was in accordance with αSMA-positive myofibroblasts role in invasive behavior of oral squamous cell carcinoma. Various studies reported the presence of stromal myofibroblasts in invasive breast, throat and larynx cancers. (Barth et al., 2004; Yazhou et al., 2004; Cimpean et al., 2005)

Stroma plays a key role in the development of oral cancer. Some stromal events such as fibroblasts’ activities, myofibroblasts' differentiation and presence of some specific stromal proteins like proteolytic enzymes, fibronectins, and 5 laminin have been reported as the main features of stromal tumor by some reports. However, there are few studies on biophysics importance of stromal tumors (Nielsen et al., 2008) and most of them have studied the role of epithelium and epithelial factors (Torres-Rendon et al., 2009). In 2008, Kellerman et al. studied the presence of myofibroblasts using immunohistochemical staining with αSMA protein in 83 cases of tongue squamous cell carcinoma and 34 samples as the control group (8 cases of normal oral mucosa and 16 cases of dysplasia) and reported that the stroma of the oral mucus and epithelial dysplasia did not occur in any αSMA cells except for the endothelial cells of blood vessels. However, many myofibroblasts were observed in 60% of cases of the oral squamous cell carcinoma. Kellerman's study results are, in a form, in accordance with the results of our study. However, in our study, αSMA-positive myofibroblasts were seen in 67% and 22% of oral squamous cell carcinoma and epithelial dysplasia, respectively. Regarding hyperkeratosis, except for one case, positive staining was seen only in epithelial cells of the blood vessels. In order to identify stromal myofibroblasts, Etemad Moghadam et al. in 2009 used αSMA, vimentin, and desmin markers on 40 samples of squamous cell carcinoma, 15 cases of dysplasia , and 15 samples of normal oral epithelium. Stained cells with all three markers were seen in oral cancer, but negative staining presented in dysplasia and normal epithelium. They concluded that the presence of myofibroblasts in the stroma of oral cancer is an expression of their key role in carcinogenesis process. (Etemad Moghadam et al., 2009)

Cimpean et al. used immunohistochemical methods to study the importance of αSMA and CD34 markers in benign and malignant breast tumors in 112 women patients with a mass in their breasts and reported that the expression of αSMA was negative in normal breast tissues but CD34 was positive and concluded that the mentioned markers can be useful in distinction of benign and malignant breast tumors in some severe cases. (Cimpean et al., 2005) Stromal activity of the host tissue is the main stage in the growth and development of cancer invasion. However, specific mechanisms of stromal activity by tumor cells have not been fully understood. Presence of myofibroblasts is a main phenomenon of stromal activity (Adegboyega et al., 2002). Neoplastic changes that happen in the epithelium are followed by some changes in stroma that are caused by factors like PDGF and TGFβ1 from stromal surrounding tumor cells which in turn promote the differentiation of fibroblasts into myofibroblasts (Varayoud et al., 2001). Some researchers believe that perhaps old fibroblasts in the connective tissue undergo mutations and change into myofibroblasts. Some others, however, believe that this happens as a result of induction by a variety of cytokine from cancerous cells (Tuxhorn., 2002; Desmouliere et al., 2004). No αSMA-positive myofibroblasts were found in the non-tumor stromal cells of oral squamous cell carcinoma. Myofibroblasts arranged in network and spindle arrangements were seen around tumor islands which demonstrated the role of stromal myofibroblasts in tumor’s invasion. An increase in the number of myofibroblasts during carcinogenesis was also observed.

Adegboyega et al. (2002) used αSMA immunohistochemical staining on myofibroblasts, positive vimentin for normal colon mucosa, hyperplastic polyps and colorectal adenomatous in their research. αSMA-negative fibroblasts and positive vimentin ones were observed in colon mucosa, whereas αSMA-positive and vimentin fibroblasts were observed in hyperplastic and neoplastic polyps. They concluded that in neoplastic cases intercellular fibroblasts differentiate into myofibroblasts in the stroma of squamous cell carcinoma. They also studied its relationship with the tumor stage and reported that there was a relationship between the expression of αSMA and tumor stage.

The presence of myofibroblasts in stromal tumor brings up the possibility that secreted factors from tumor cells may pass through the basal lamina and reach the connective tissue beneath it and play a role in differentiation of fibroblasts into myofibroblasts. (Torres-Rendon., 2009) Considering the design and cell distribution, hyperkeratosis was seen only in 1 case and dysplasia in 4 cases in which myofibroblasts staining was positive, focal and scattered cell arrangements were also seen and most myofibroblasts were separately and focally arranged and some others had scattered and scanty arrangement around blood vessels. Spindle arrangement occurred in 8 cases and network arrangement was occurred in 4 cases. In network configuration, myofibroblasts were arranged in several abundant layers around the neoplastic islands. Increased number of myofibroblasts and their epithelial arrangement was seen that in some areas they were interwoven with neoplastic islands forming a network appearance. In spindle arrangement the myofibroblasts were arranged in rows with fewer numbers around neoplastic islands. It seemed that the
higher the number of myofibroblasts, the more invasive the tumor behavior was. This is due to the fact that secreted matrix metalloproteinase from myofibroblasts plays a role in tumor invasiveness and its weak prognosis. Matrix metalloproteinase has a role in destruction of extracellular matrix, tumor formation, migration, invasion, metastasis, angiogenesis, and induction of apoptotic clones (Lynch et al., 2002). The results of this study showed that there seem to be a relationship between cell arrangement pattern and tumor invasive behavior. Scattered focal arrangement was observed in pre-cancerous lesions while network and spindle ones were observed in neoplastic lesions. It can be said that because of higher number of myofibroblasts in network arrangements they show more severe invasive behavior in comparison to spindle arrangement. To date, just few studies were done on the design and arrangement of cells and their role in invasive behavior of tumors. It seems an area of interest which needs hard work.

Shimasaki et al (2006) confirmed the role of the distribution and arrangement of myofibroblasts in bladder carcinoma and tumor invasive behavior. Fascicular and reticular arrangements were seen in invasive and non-invasive bladder carcinoma, respectively. They concluded that the myofibroblasts distribution pattern can give some information on carcinoma invasion characteristics. A relationship between myofibroblast distribution pattern and invasive tumor characteristics has also been reported (Tuxhorn et al., 2002). However, other studies only mentioned an increase in number (percentage) of myofibroblasts in tumor invasion and did not study their cellular distribution pattern (Etemad Moghadam et al., 2009; Torres-Rendon et al., 2009).

Using immunohistochemical methods, Vered et al. (2009) studied the design and distribution pattern of myofibroblasts. Scanty arrangement was observed in hyperplasia and dysplasia. However, network arrangement and spindle arrangement of squamous cell carcinoma was observed in 23% and 77% of cases, respectively. They too, confirmed the role of network arrangement in invasive tumor behavior and weak prognosis of oral cancer. They also studied the diffuse and focal patterns of TGFβ1 cells and explained the relationship between positive TGFβ1 cell arrangement and tumor invasive behavior. Kellermann et al. (2007) used αSMA immunohistochemical staining to study cell distribution and pattern of dysplasia and squamous cell carcinoma. Negative staining presented in all dysplastic lesions. However, myofibroblasts had fascicular and reticular arrangements. They reported that patients with larger number of myofibroblasts in invasive front had shorter lives.

Different surgical methods, radiotherapy and chemotherapy methods are currently used in the treatment of squamous cell carcinoma and each of them cause some complications that has not improved the life expectancy of the patients in 25 years (Khan et al., 2003; Riviere et al., 2006). Because of observed increase in αSMA expression in the stroma of oral squamous cell carcinoma, target therapy and production of anti-αSMA can probably be considered as a new auxiliary method that will cause fewer complications. However, more studies are recommended for more reliable achievements to be reached.

In Vered et al study, they reported the presence of myofibroblasts in stromal surrounding of cysts and odontogenic tumors using immunohistochemical staining. They reported that αSMA expression in the stroma of solid ameloblastoma and odontogenic keratocyst (parakeratinized type) was higher than dentigerous cyst and unicystic ameloblastoma and ameloblastic fibro odontoma. They concluded that αSMA expression of myofibroblasts was an index of invasive behavior of odontogenic lesions and it seems that target therapy can be beneficial as an auxiliary method for treatment of more invasive lesions (Vered et al., 2005). Differentiation of fibroblasts into myofibroblasts under the influence of TGFβ1 cytokine secreted from cancerous cells can cause cancer progression through parakrin effects stimulating angiogenesis. At the same time, autokrin effects cause Ras gene mutation and produces pre-invasive signals. (Varayoud et al., 2001)

The role of immunological and inflammatory factors and endothelial cells, and angiogenesis had already been known, but few studies had been done on myofibroblasts and their role in squamous cell carcinoma. Hence, this study expressed the role and importance of myofibroblasts considering their presence and distribution in oral squamous cell cancer.

In conclusion, results of this study showed an increase in the number of αSMA-positive myofibroblasts and change in distribution pattern during oral carcinogenesis process which can be an expression of their role in tumor invasive characteristics. It is suggest which network arrangement of myofibroblasts in squamous cell carcinoma represented higher invasive characteristics and weaker prognosis.

It seems that the main limitations of the study were small sample volume of SCC, the fact that some oral carcinomas were incisional, estimated grades and stages, and small sample volume of epithelial dysplasia. To achieve better results we suggest an enlarged sample size of oral SCCs and dysplasia and evaluation of the connection between αSMA and different SCC grades and dysplasia.

Acknowledgments

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References


