

## RESEARCH ARTICLE

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# Proliferative Activity of Myoepithelial Cells in Normal Salivary Glands and Adenoid Cystic Carcinomas Based on Double Immunohistochemical Labeling

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## Abstract

**Objective:** To investigate the proliferative activity of myoepithelial cells (MEC) in normal salivary glands (NSG) and adenoid cystic carcinomas (ACC) Study design. Twenty -three salivary gland specimens (13 ACC, 10 NSG) were studied using double immunohistochemical labeling for  $\alpha$  smooth muscle actin ( $\alpha$ -SMA) and proliferative cell nuclear antigen (PCNA). **Results:** There was a significant difference in PCNA reactivity in normal samples between myoepithelial cells of the parotid glands and of the submandibular glands, rates being higher in the latter. Neoplastic myoepithelial cells exhibited higher expression than neoplastic epithelial cells. In addition, myoepithelial cells of the cribriform type of ACC showed PCNA reactivity lower than those of the tubular type, whereas there was no statistically significant difference in epithelial cell rates. We could not identify myoepithelial cells in solid pattern due to  $\alpha$ -SMA negativity; although high PCNA reactivity was evident. **Conclusion:** These data suggest that the myoepithelial cell has a key role in ACC oncogenesis, more so than its epithelial cell counterparts. Moreover, the data provide a histopathological interpretation for aggressive clinical features of submandibular ACC, as the myoepithelial cells were less differentiated as compared to those of parotid glands.

**Keywords:** Myoepithelial cells- adenoid cystic carcinomas- PCNA-  $\alpha$ -SMA- double immunohistochemical labeling

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## Introduction

Myoepithelial cells associate with the acini and intercalated ducts of the salivary gland, providing support for end pieces during active secretion of saliva (Togarrati et al., 2017; Carraro and Stripp, 2017; Chen et al., 2012) Other researchers have demonstrated that myoepithelial cells have crucial properties, such as promoting differentiation of epithelial cells by synthesizing and accumulating extracellular matrix and basement membranes, secreting high amounts of tumor suppressors and proteinase inhibitors, and inhibiting angiogenesis (Costa et al., 2008) and invasion (Ye et al., 2017; Aguiar et al., 2015; Xiao et al., 1999). The myoepithelial cell is for these reasons called the natural tumor suppressor (Duivenvoorden et al., 2017; Barsky, 2003). In addition, the role of myoepithelial cells has been demonstrated in the pathogenesis and biologic behavior of different types of salivary gland tumors (Avci et al., 2012) and non-neoplastic conditions (Ihrler et al., 2010; Nashida et al., 2013).

However, most researchers have studied either proliferation of all tumor cells without distinguishing

between them (Kawasaki et al., 2011; Shousha, 2011), or proved involvement of myoepithelial cells in the pathogenesis of tumors (Ihrler et al., 2010; Barsky and Karlin, 2006). Few studies have characterized the proliferation of myoepithelial cells in salivary gland tumors compared with other types of cells in the same tumor (Norberg et al., 1997; Anderson et al., 2014). On the other hand, some studies did not find any proliferative capacity of normal myoepithelial cells (Daniele et al., 1996), and most studies that showed proliferation using double immunohistochemical labeling were done on rats (Uzeda et al., 2017; Bartsch et al., 2000). Ihrler was the first to use this technique in parotid glands and demonstrated increased proliferation, but only during inflammation (Ihrler et al., 2002).

In this study, we evaluated the proliferative activity of myoepithelial and epithelial cells in ACC, which has predominantly myoepithelial differentiation. In addition, we evaluated the proliferative activity of the myoepithelial cells of normal salivary glands (submandibular and parotid).

We believe that more research is needed to better understand the role of myoepithelial cells in tumors and

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to benefit from them in regenerative medicine approaches and in therapeutic ways of malignancy, such as with antiangiogenesis therapy and protease inhibitors.

## Materials and Methods

### Tissue specimens

Twenty-three salivary gland specimens (48% from males, 35% from females, 17% unknown; age range 24-60 years) were retrieved from the archives of University Mouasa Hospital, Damascus University. Five-micron serial sections were cut. The diagnoses comprised normal salivary gland (n=10) and adenoid cystic carcinoma (n=13). The normal salivary glands were divided into four cases of parotid gland obtained from pleomorphic adenoma from outside the capsule (Ihrler et al., 2004) and six cases of submandibular gland eradicated in the context of excision of squamous cell carcinoma arising in the oral cavity and larynx. Adenoid cystic carcinoma was classified according to the dominant pattern (five cribriform, five tubular, and three solid). It was also divided according to gland into six cases of parotid gland ACC and seven cases of submandibular gland ACC.

### Immunohistochemical Double Staining for Actin and PCNA

Deparaffinized slides were subjected to microwave pre-treatment with target retrieval solution (citrate buffer, pH 6, 15 minutes). Endogenous alkaline phosphatase and peroxidase activity was blocked with dual endogenous enzyme block containing hydrogen peroxide (0.05%). For staining for PCNA and  $\alpha$ -SMA, the EnVision G|2 Doublestain system was applied (Table 1). The first antibody (monoclonal anti-PCNA, ready-to-use, Dako) was applied at room temperature for 30 minutes and was visualized using HRP/DAB+. The second antibody (monoclonal anti- $\alpha$ -SMA, ready-to-use, Dako) was applied at room temperature for 30 minutes and was visualized using AP/Permanent Red. Mayer's hematoxylin was used as a counterstain. The vessels within the stroma were used as internal positive controls for  $\alpha$ -SMA (Ihrler et al., 2002). The positive controls for PCNA was the follicular tissues of the tonsil (Birajdar et al., 2014).

To determine the labeling index, the percentage of PCNA-positive cells within a total of 400 cells was calculated (Madan et al., 2015, Ihrler et al., 2004). The mean percentage of cellular proliferation and the standard deviation (SD) were calculated. Statistical analysis of data was assessed using Chi-square test.

## Results

### Normal salivary glands

$\alpha$ -SMA-positive myoepithelial cells with red color in cytoplasm were identified at the periphery of the acini and intercalated ducts. In the stroma, blood vessels showed positivity to anti-actin antibodies. An interaction to PCNA was observed in nuclei of cells with brown color, where the proliferating myoepithelial cells showed double stain: actin and PCNA (Figure 1). However, they were few (1.6%). The PCNA reactivity of the submandibular

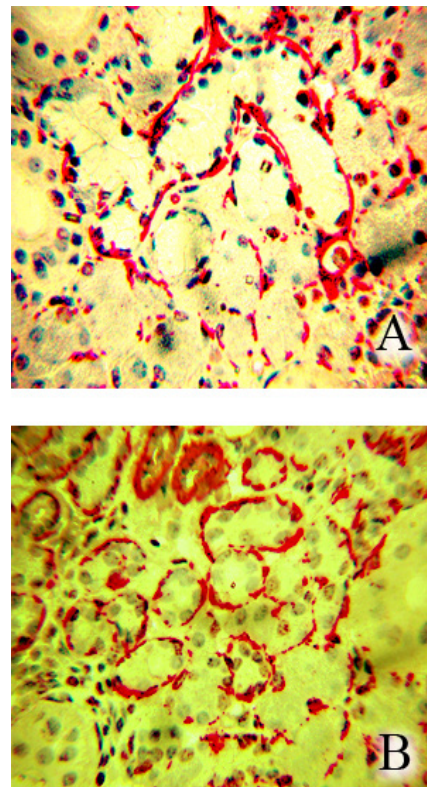


Figure 1. Double Immunohistochemistry for PCNA (brown) and Actin (red) in Normal Salivary Glands Showing Myoepithelial Cells Proliferation in Submandibular Gland (A) and Parotid (B).

gland was significantly higher than of the parotid gland ( $p=0.008$ ).

### Adenoid Cystic Carcinoma

In general,  $\alpha$ -SMA-positive myoepithelial cells were located close to the pseudocystic lumens of the cribriform type, whereas they were at the periphery of epithelial cells in the tubular type. PCNA-positive nuclei were scattered throughout the neoplastic myoepithelial and epithelial components of cribriform and tubular types. On the other hand, the solid pattern showed a negative reaction to  $\alpha$ -SMA and high expression of PCNA (Figure 2).

PCNA reactivity of the myoepithelial cells of ACC was higher compared to the normal sample ( $p<0.001$ ) and lower in the cribriform pattern than in the tubular pattern ( $p<0.001$ ). According to site, PCNA-positive myoepithelial cells of submandibular tumors were higher than those of the parotid ( $p<0.001$ ). There were also statistical differences between epithelial and myoepithelial cells in cribriform and tubular patterns ( $p=0.019$  and  $p=0.002$ , respectively) (Figures 2 and 3). The mean number of PCNA-positive nuclei and standard deviation

Table 1. Details of the Antibodies Used for Immunohistochemistry

Specificity	Clone	Dilution	Source	Buffer (AR)
$\alpha$ -SMA	1A4	Ready-to-use	Dako	citrate
PCNA	PC10	Ready-to-use	Dako	citrate

$\alpha$ -SMA, alpha-smooth muscle actin; PCNA, Proliferative cell nuclear antigen.

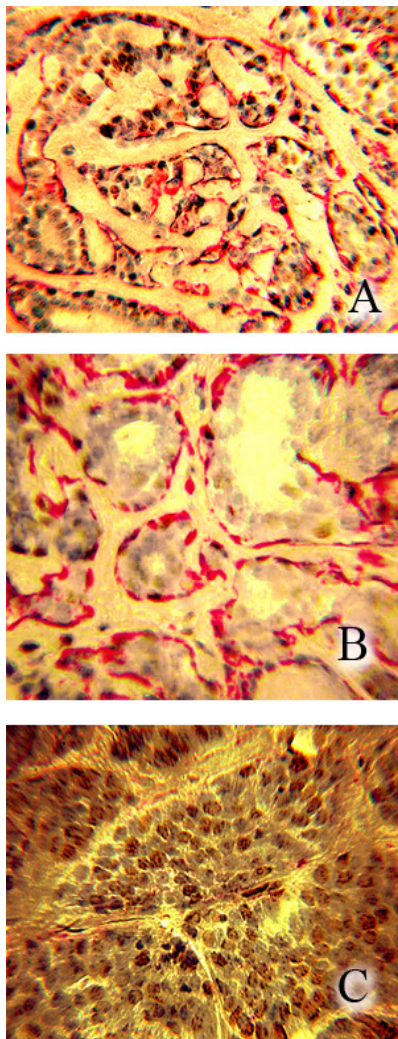


Figure 2. Double Immunohistochemistry for PCNA in ACC

Table 2. Percentage of PCNA- Positive MECs (Mean%  $\pm$  SD) in NSGs and ACCs

	NSGs	ACCs*
Parotid (4)	0.9 $\pm$ 1.2	8.7 $\pm$ 3.56
SM (6)	2 $\pm$ 1.74	19.9 $\pm$ 13.47
Total (10)	1.6 $\pm$ 1.57	15.4 $\pm$ 11.77

PCNA, Proliferative cell nuclear antigen; MEC, Myoepithelial cell; SD, standard deviation; ACCs, adenoid cystic carcinomas; NSGs, normal salivary glands; SM, submandibular. without the solid pattern (3 cases)\*

(SD) are summarized in Table 2 and Table 3.

## Discussion

The size of the study sample might be considered as a limitation for the study but it isn't exceptional! there are many published studies have the same size, especially the subgroups of the sample in every study (Zhu et al., 1997; Ihrler et al., 2002; Ihrler et al., 2004).

the sample size in the current study was determined using G Power software after conducting a pilot study.

Table 3. Percentage of PCNA- Positive MECs and ECs\* (Mean%  $\pm$  SD) in ACC

	EC+MEC	EC	MEC
Cribriform (5)	13.6 $\pm$ 10.10	14.9 $\pm$ 12	12.4 $\pm$ 8.46
Tubular (5)	16.6 $\pm$ 12.03	14.8 $\pm$ 9.38	18.5 $\pm$ 14.72
Solid (3)	28 $\pm$ 12.58	-	-

EC\*, Epithelial cells

### Normal salivary glands

We found in our study that the proliferative activity of myoepithelial cells in normal salivary glands is 1.6%. This result is supported by the multicellular theory of salivary gland tumors, which says that all of the cells of the salivary glands are able to proliferate (Aure et al., 2015). In contrast to the bicellular theory, which is not supported by direct evidence (Dardick et al., 1990). In addition, many studies have shown how myoepithelial cells proliferate in normal salivary glands of rats and during the pathological condition of atrophy (Shah et al., 2016; Takahashi et al., 2005). Moreover, in humans, our results agree with the Ihrler study, who proved myoepithelial cell proliferation in normal parotid glands (Ihrler et al., 2002), which multiplied twenty-fold during inflammation of the gland (Ihrler et al., 2004). However, our results disagreed with Ihrler's in terms of percentage of myoepithelial cell proliferation in the parotid, where it accounted for 0.9%, while with Ihrler, it was 0.2%. This may be due to the difference in the methods and materials used; Ihrler used an LSAB Kit system and Ki67, while we used an EnVision system and PCNA.

Myoepithelial cell proliferation has been explained by the cellular replacement required to maintain the gland in a steady state (Maria et al., 2014) or by the physiological regeneration of the acini and intercalated duct (Holmberg and Hoffman, 2014). We endorse these explanations because the myoepithelial cells, as stated previously, play an important role in differentiation and polarization of epithelial cells, remodeling the basal lamina and producing many of the proteins that are important to enhance these functions, such as laminin and collagen (Shah et al., 2016).

However, what is striking in our findings is the difference in cell proliferation between the parotid gland (0.9%) and the submandibular gland (2%). It is known that one of the myoepithelial cell's functions is to support the acini during secretion, so these differences may be due to differences in effort and activity of each gland; the parotid gland produces 25% of the saliva, while the submandibular gland produces 60% (Rzymska-Grala et al., 2010). In addition, the submandibular gland secretes saliva during rest, the longest period (Proctor, 2016). Therefore, the submandibular gland may require a lot of the regeneration provided by myoepithelial cell proliferation. On the other hand, in rats, Takahashi et al., (2001) explained the high percentage of myoepithelial cell proliferation in parotid during atrophy: the myoepithelial cells of the rat submandibular gland are more numerous than those of parotid gland because they associate with both the acini and the intercalated ducts whereas, in parotid gland, they only associate with intercalated ducts (Ohtomo et al., 2013, Shah et al., 2016). Thus, they need

to proliferate more than those of submandibular gland. Similarly, the same situation exists in the parotid and submandibular glands of humans, but reserved. The intercalated ducts associated with myoepithelial cells of the parotid gland are greater in number and longer than those of the submandibular gland (Shah et al., 2016). Thus, there are more myoepithelial cells in parotid glands than in submandibular glands. Therefore, it is unnecessary for myoepithelial cells in parotid glands to proliferate as actively as in submandibular glands during regeneration.

#### *Adenoid Cystic Carcinoma*

**The Cells and Patterns.** We found statistical differences between epithelial and myoepithelial cell proliferation in cribriform and tubular patterns. In addition, the epithelial cell proliferation in the cribriform pattern was similar to its proliferation in the tubular pattern (14.9% and 14.8%, respectively). However, there is considerable variation in the proportion of myoepithelial cell proliferation between the cribriform pattern (12.4%) and tubular pattern (18.5%), with statistical differences. Thus, ACC progress is only associated with an increase in myoepithelial cell proliferation. This gives us an indication that the myoepithelial cell has a more fundamental role in ACC than the epithelial cell does because cell proliferation is one of the most important biological mechanisms in oncogenesis (Huang et al., 2017) and PCNA is the marker that increases whenever tumor malignancy increases (Anggorowati et al., 2017). Myoepithelial cells are more differentiated in the cribriform pattern and less differentiated in the tubular pattern, with a peak in the solid pattern where they lose their ability to produce actin. This is explained by the abundance of extracellular matrix and basal lamina in the cribriform pattern produced by myoepithelial cells (Du et al., 2016). These materials are the same as those produced by this cell in pleomorphic adenoma, which is evidence of differentiation and integrity (Furuse et al., 2005), while they disappear in the tubular and solid types. Moreover, these abundant materials are responsible for the formation of pseudocystic structures in the cribriform pattern, which are filled with such secretions (Dwivedi et al., 2013). Eneroth explained the good prognosis of cribriform pattern compared to the solid pattern by the existence of these materials (Eneroth et al., 1968). Santucci and Bondi (1989) found a positive correlation between the number of gland-like spaces per square millimeter of tumor and the survival of the patient. da Cruz Perez et al., (2006) also found that the rate of survival was the highest with the cribriform pattern. Similarly, Nascimento et al., (1986) found that cribriform is the best pattern, followed by tubular, then solid. In myoepithelial carcinomas, their nodular architecture and limited infiltration of the tumor cells to the stroma were explained by secreting an abundant extracellular matrices in addition to large amounts of proteinase inhibitors and angiogenic inhibitors. However, these properties might be modified by carcinogenesis (Kong et al., 2015). And maybe for the same modification some ACCs, which demonstrated positive CD105 vessels, had a high tendency to metastasize, where CD105 is a neoangiogenesis marker (Tadbir et al., 2012). And maybe for the same properties

of myoepithelial cells, adenoid cystic carcinoma is characterized for its long clinical behavior and delayed metastases (Sung et al., 2003) Where myoepithelial cells had a role in the control of new vessel formation of ACC (Cardoso et al., 2009). It is known that paracrine interactions between myoepithelial and luminal epithelial cells are necessary to establish epithelial cell polarity, and inhibit cell migration and invasion. However, myoepithelial cells lose their properties as tumor progresses (Lo et al., 2017). However, our study suggest that transforming events started from myoepithelial cells.

**The Glands.** We found that the proliferation of myoepithelial cells in ACC arising in the submandibular gland was higher than in the parotid. We can explain this result through our first result, in which PCNA-positive myoepithelial cells in normal submandibular glands were significantly higher than in the parotid gland, and differentiation of normal tissues is inversely correlated with the proliferating activity of their cells (Blau and Baltimore, 1991). Therefore, the neoplastic myoepithelial cells in the submandibular gland logically have the highest proliferative capacity compared to their counterparts in the parotid gland.

This result is supported by the clinical features of ACC arising in the submandibular gland, where it is noted that the submandibular gland tended to be involved with grade III neoplasms-the tumors with a predominantly solid pattern, whereas grade I tumors predominate in the palate and parotid gland (Szanto et al., 1984). In addition, Politi et al., (2014) found that metastases were more numerous in the submandibular gland compared with the parotid and palate region, and this is explained by drainage of lymph from the primary tumor location and the preoperative duration of the symptoms. However, our result can add a histopathological explanation to these clinical findings.

In conclusion, Myoepithelial cells play a key role in ACC oncogenesis, more than their epithelial cell counterparts, because their differentiation gradually decreases with the progress of the tumor, starting from cribriform, passing to tubular and then to solid. Moreover, this study provides a histopathological interpretation for the aggressive clinical features of submandibular ACC, where myoepithelial cells were less differentiated in submandibular gland ACC compared to parotid gland ACC. This finding was also demonstrated between normal submandibular and parotid glands.

#### *Conflict of interest*

The authors declare that they have no conflict of interest.

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