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Absence of myoepithelial cells correlates with invasion and metastasis of Carcinoma ex pleomorphic adenoma

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Abstract. Myoepithelial cells (MECs) are implicated in the development and progression of human salivary gland tumours. Here, we investigate the potential role for MECs in invasion and metastasis of carcinoma ex pleomorphic adenoma (CXPA). Tumour tissues from 40 CXPA patients diagnosed between 1960 and 2014 were obtained. Patient samples were divided into two groups (non-invasive tumours, $n = 10$; and frankly invasive tumours, $n = 30$). Each group was further divided into two subgroups (metastatic tumours and non-metastatic tumours). Immunohistochemistry for MEC markers (α-SMA, CALPONIN, and p63) was performed, and the number and distribution of MECs was quantified. For non-invasive CXPAs, non-metastatic cases ($n = 8$) displayed a significant enrichment in CALPONIN(+) and α-SMA(+) MECs, but not p63(+) MECs, compared with metastatic cases ($n = 2$). Likewise, for frankly invasive CXPAs ($n = 30$), non-metastatic cases showed a significant enrichment for α-SMA(+), CALPONIN(+), and p63(+) MECs compared with metastatic cases ($n = 15$). We demonstrate that non-invasive CXPAs have the potential for metastasis. Furthermore, the tumour capsule may not be the only barrier preventing invasion and metastasis, as a significant reduction in numbers of myoepithelial cells correlates with invasion and metastasis in CXPA patients.

Key words: CXPA; myoepithelial cells; invasion; metastasis.

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Benign pleomorphic adenoma is the most common type of salivary neoplasm, accounting for almost 80% of all salivary tumours. Malignant transformation arising from pleomorphic adenoma often develops in patients with a prolonged history of untreated or recurrent benign pleomorphic adenoma. In the World Health Organization (WHO) classification system, malignant pleomorphic adenoma is subdivided into three categories, in which carcinoma ex pleomorphic adenoma (CXPA) is the most common type. CXPA presents with benign pleomorphic adenoma and epithelial malignancy. Based on the extent of the malignant component, CXPA is categorized into three subtypes[1]: frankly invasive CXPA, if invasion extends >1.5 mm

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beyond the capsule; minimally invasive CXPA, if invasion extends ≤1.5 mm beyond the capsule; and non-invasive CXPA, if the malignancy is confined to the tumour capsule.

Currently, it is thought that non-invasive forms of CXPA rarely metastasize to cervical regions or other distant tissues, and most patients experience remission for many years after treatment[2–4]. Nevertheless, there have been sporadic reports of non-invasive CXPAs leading to cervical or distant metastases[5,6]. Even in our current investigation, we examined one patient with non-invasive CXPA who succumbed to pulmonary metastasis after treatment, and another patient who developed cervical metastasis before treatment.

To investigate myoepithelial cells (MECs) as potential anti-invasive factors, we examined the number and distribution of MECs in different groups of CXPA. As one of the most important structures in the salivary gland, MECs have been implicated in inhibiting the development and progression of salivary gland tumours. Specifically, MECs accelerate the differentiation of epithelial cells, and participate in the formation of basement membrane by means of a paracrine effect. They are also known to suppress tumour invasion by secreting serine protease inhibitors and matrix metalloproteinase inhibitors[7,8].

Since MECs are almost unrecognizable by haematoxylin and eosin (H&E) staining, immunohistochemical staining or electron microscopy is usually employed. To distinguish MECs from myofibroblasts, ductal epithelial cells, or vascular smooth muscle, ideal markers for MECs, must possess high sensitivity and specificity. At present, antibodies against α-smooth muscle actin (α-SMA), CALPONIN, and p63 are commonly used to molecularly identify MECs[9–11].

In this study, we examined the number and distribution of MECs expressing α-SMA, CALPONIN, and p63 in human CXPA patients displaying varying grades of tumour invasion and metastasis. Cumulatively, our data suggest that the enrichment of MECs that ensheath tumour tissues correlates with non-metastatic forms of this cancer. Thus, MECs might play a key role in inhibiting progression of this deadly human disease, warranting future investigation.

Materials and methods

Patient selection and characteristics

This study was approved by the Peking University School of Stomatology Institutional Review Board. The Department of Oral and Maxillofacial Surgery at Peking University School and Hospital of Stomatology provided medical records and pathological specimens from ten patients treated for non-invasive CXPA and 30 patients treated for frankly invasive CXPA between 1960 and 2015. For both groups, patients were further subdivided into non-metastatic or metastatic subgroups. For the non-invasive CXPA cohort, eight out of 10 (80%) were classified as non-metastatic and two out of 10 (20%) were metastatic. For the frankly invasive CXPA cohort, 15 out of 30 (50%) were classified non-metastatic and 15 out of 30 (50%) were metastatic. The histopathological subtype of all 40 patients are identified as adenocarcinoma not otherwise specified (25), mucoepidermoid carcinoma (7), adenoid cystic carcinoma (6), and salivary duct carcinoma (2 cases).

Immunohistochemistry

For each case, paraffin-embedded samples with typical pathological manifestation were chosen to make four consecutive pathological slides of 5 μm thickness. Each slide was deparaffinized and rehydrated with a graded ethanol series. For antigen retrieval, slides were subjected to 0.01 mM citrate buffer (pH 6.0) for 15 min in a microwave oven. A 3% hydrogen peroxide solution was used to block endogenous peroxidase activity for 10 min at room temperature. After rinsing with PBS, slices were incubated for 24 hours at 4 °C with primary antibodies. Primary antibodies and dilutions were as follows: rabbit anti-human-SMA (1:150 dilution, Zhongshan Goldenbridge, Beijing, China), rabbit anti-human-CALPONIN (1:100 dilution, Zhongshan Goldenbridge), rabbit anti-human-p63 (1:100 dilution, Zhongshan Goldenbridge). Sections were washed in PBS three times, and then incubated with HRP conjugate anti-rabbit secondary antibody (Zhongshan Goldenbridge) for 20 min at 37 °C. Colorimetric development with the 3,3′-diaminobenzidine-tetrahydrochloride (DAB) substrate was performed, followed by counterstaining with haematoxylin. Primary antibodies were omitted in negative controls. Tissue from human parotid gland was used as positive controls.

Quantification of immunohistochemical staining

Cells positive for α-SMA or CALPONIN expression displayed yellow or brown particles in cytoplasm, and p63 positive cells contained yellow or brown particles in the nucleus. To determine if a patient's tumour sample was either positive or negative for the MEC markers α-SMA(+), CALPONIN(+), or p63(+), we used an established scoring method[12]. First, we determined the average percentage of cells positive for each marker in at five separate fields of one slide at 400× magnification by manually counting. Second, we numerically ranked the intensity of colorimetric staining in each field as follows: weak, 1; moderate, 2; and intense, 3. For sections that displayed heterogeneous staining, the average staining intensity was used for the intensity score. Finally, the average percentage of cells positive for each marker was multiplied by the staining intensity rank to generate an overall score for each patient's sample. Samples with scores greater than 1 were defined as positive for the presence of MECs. Otherwise, they were classified as negative for the presence of MECs.

Statistical analysis

All data were analysed with SPSS v 20.0 (Chicago, IL, USA). Fisher's exact test and the χ^2 test were used to compare the quantifications of MECs between different patient groups. $P < 0.05$ was considered statistically significant.

Results

Characterization of MEC markers

To confirm that MECs were detectable in patient samples, we used immunohistochemistry to examine the expression of proteins known to be enriched in MECs. MECs are defined by the expression of α-SMA, CALPONIN, and p63. We observed that α-SMA and CALPONIN both localize in the cell membrane and cytoplasm. In contrast, p63 was located in cell nuclei. Furthermore, we observed that signal for all three markers was enriched in cells that ensheath or are closely associated with acinar and ductal epithelial cells, in addition to the epithelial cells of tumours. The presence of stained cells in these anatomical locations was consistent with the established distribution of MECs[9–11]. Thus, we concluded that our staining protocols effectively detected MECs in human tissues.

-SMA staining

First, we examined patient tissues for the presence of α-SMA(+) MECs. Among non-invasive CXPAs ($n = 10$), two out

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