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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

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

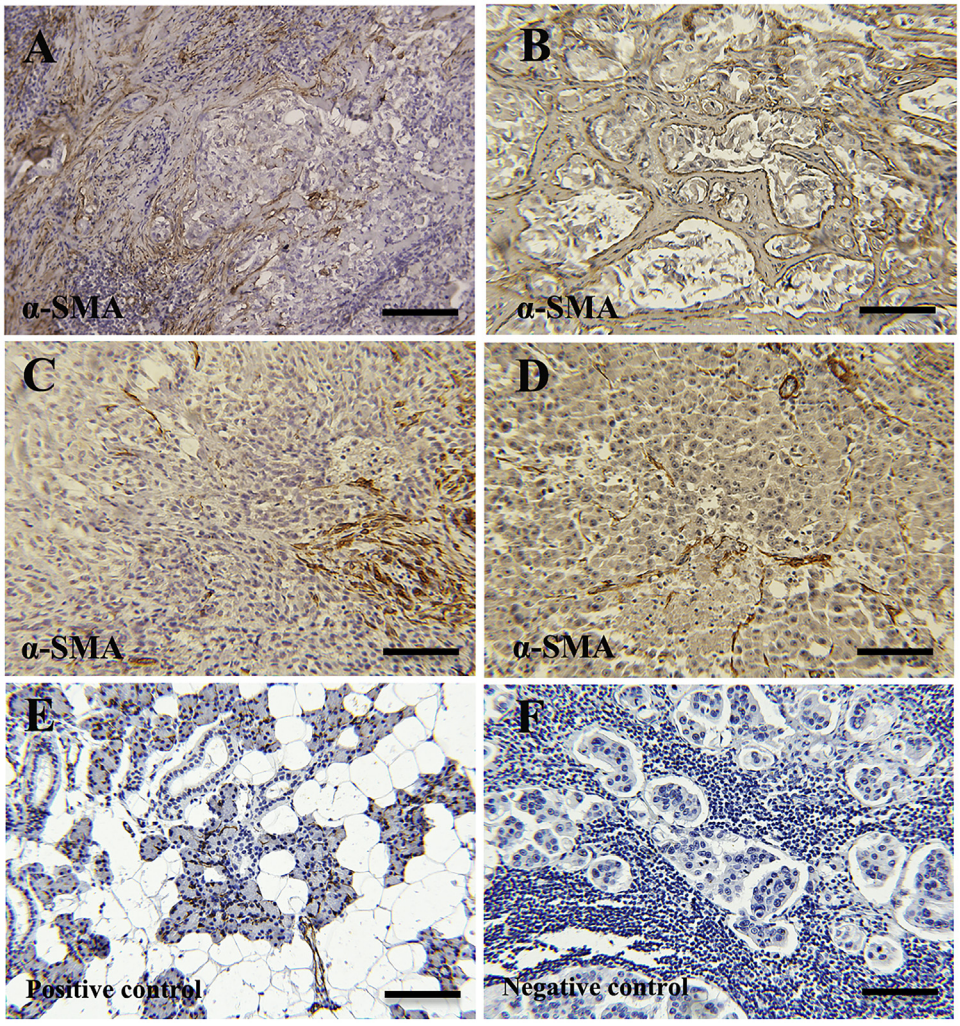

Fig. 1. Immunohistochemical staining ofα-SMA is shown. (A) Non-invasive CXPA with metastasis: MECs (brown staining) decrease in number and form a discontinuous sheath surrounding tumour cells. (B) Non-invasive CXPA without metastasis: positive MECs form a consecutive line-structure surrounding tumour cells. (C) Frankly invasive CXPA without metastasis: MECs are reduced in number compared to non-invasive CXPA. Remaining MECs are disorganized. (D) Frankly invasive CXPA with metastasis: MECs are rare, and complete MEC layers are absent. (E) Tissue from human parotid gland was used as positive controls. Nuclei were counterstained with haematoxylin (blue). (F) Primary antibodies were omitted in negative controls (magnification 10×).

of 10 patients (20%) were classified as metastatic (metastasis subgroup). We found that an average of 41% of tumour cells expressed α-SMA, and the mean staining intensity score was 2 (overall score: 0.82). Therefore none of these tumours were classified as α-SMA(+). Additionally, complete MEC layers did not surround the majority of the tumour nests in these metastatic patients. In contrast, eight out of patients (80%) did not display metastasis (non-metastasis subgroup). We found that an average of 74.5% of tumour cells expressed α-SMA in these patients, and the mean staining intensity score was 2.3 (overall score 1.71). All of these tumours were therefore classified as α-SMA(+). Additionally, we observed complete MEC layers enclosing the majority of tumour nests in these non-metastatic patients (Fig. 1, Table 1).

Among invasive CXPAs ($n = 30$), one out of 15 patients (6.7%) with metastasis (metastasis subgroup) was considered α-SMA(+), displaying 55% of tumour cells positive for α-SMA, and a staining intensity score of 2 (overall score 1.1). Meanwhile, seven out of 15 patients (46.7%) without metastasis (non-metastasis subgroup) were considered α-SMA(+), displaying an average of 58.3% of tumour cells positive for α-SMA, and a mean staining intensity score of 2.1 (overall score 1.22). Furthermore, the number of complete MECs layers was larger in the non-metastasis subgroup compared to the metastasis subgroup (Fig. 1 and Table 1).

Cumulatively, for both non-invasive and invasive CXPAs, the non-metastatic subgroups displayed a statistically higher incidence and enrichment for α-SMA(+) MECs compared to the metastatic subgroups ($P = 0.022$ for non-invasive CXPAs and $P = 0.035$ for invasive CXPAs). Furthermore, the α-SMA(+) MECs observed in non-metastatic subgroups mainly ensheathed tumour nests (Table 1).

CALPONIN staining

Next, we examined patient tissues for the presence of CALPONIN(+) MECs. In non-invasive CXPAs, two out of 10 patients (20%) were classified as metastatic (metastasis subgroup). We found that an

Table 1. The expression of α-SMA, CALPONIN, and p63 in MECs between different groups.

	n	α-SMA		Calponin		p63	
		Positive cases (%)	*P*	Positive cases (%)	*P*	Positive cases (%)	*P*
Non-invasive CXPA							
Metastasis	2	0 (0%)		0 (0%)		0 (0%)	
Non-metastasis	8	8 (100%)	**0.022**	8 (100%)	**0.022**	7 (75%)	0.133
Frankly invasive CXPA							
Metastasis	15	1 (6.7%)		2 (13.3%)		1 (6.7%)	
Non-metastasis	15	7 (46.7%)	**0.035**	8 (53.3%)	**0.02**	9 (60%)	**0.008**

Significant *P* values are shown in boldface.

average of 24% of tumour cells expressed CALPONIN, and the mean staining intensity score was 1.5 (overall score: 0.36). Therefore, none of these tumours were classified as CALPONIN(+). Moreover, complete MEC layers did not surround the majority of the tumour nests in these metastatic patients. In contrast, 8/10 patients (80%) did not display metastasis (non-metastasis subgroup). We found that an average of 69.2% of tumour cells expressed CALPONIN in these patients, and the mean staining intensity score was 2.6 (overall score: 1.80). All of these tumours were therefore classified as CALPONIN(+) (Fig. 2 and Table 1).

Among invasive CXPAs ($n = 30$), two out of 15 patients (13.3%) with metastasis (metastasis subgroup) were considered CALPONIN(+), displaying an average of 52% of tumour cells positive for CAL-

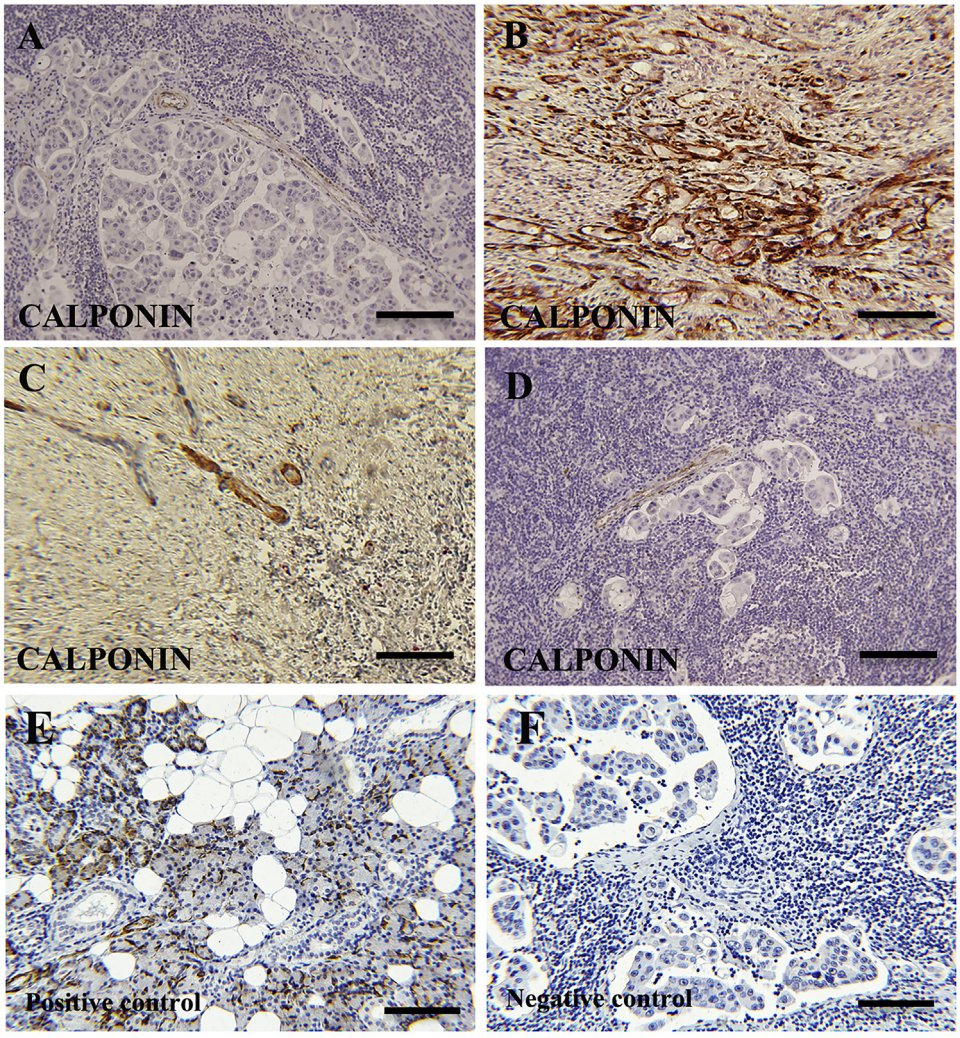

Fig. 2. Immunohistochemical staining of CALPONIN is shown. (A) Non-invasive CXPA with metastasis: MECs (brown staining) are rare, except for some blood smooth muscle that stain positive. (B) Non-invasive CXPA without metastasis: MECs form a continuous sheath surrounding tumour cells. (C) Frankly invasive CXPA without metastasis: MECs are reduced in number compared to non-invasive CXPA. Remaining MECs are disorganized. (D) Frankly invasive CXPA with metastasis. MECs are rare, except for some blood smooth muscle cells that stain positive. (E) Tissue from human parotid gland was used as positive controls. Nuclei were counterstained with haematoxylin (blue). (F) Primary antibodies were omitted in negative controls (magnification 10×).

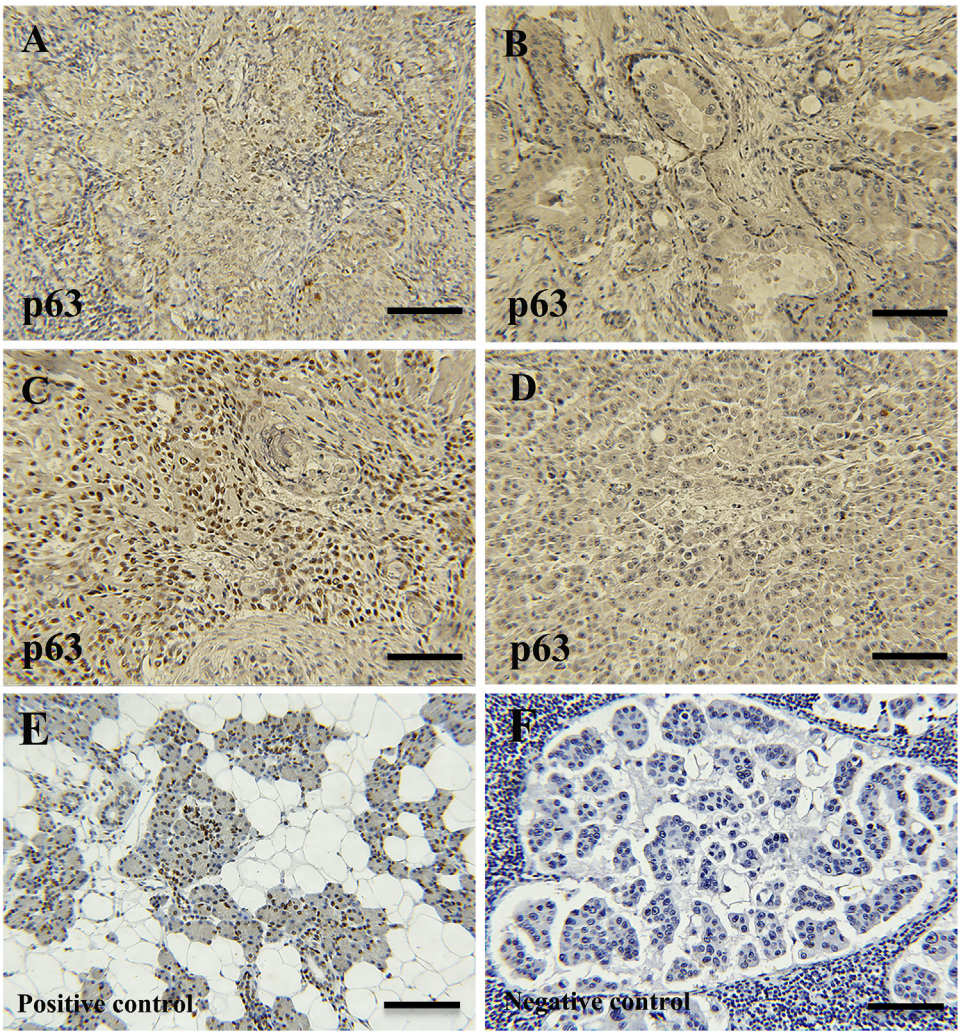

Fig. 3. Immunohistochemical staining of p63 is shown. (A) Non-invasive CXPA with metastasis: MECs (brown staining) are rare, and some MECs form a discontinuous sheath surrounding tumour cells. (B) Non-invasive CXPA without metastasis: MECs are abundant, and form a discontinuous sheath surrounding tumour cells. (C) Frankly invasive CXPA without metastasis: MECs are disorganized. (D) Frankly invasive CXPA with metastasis: MECs are rare. (E) Tissue from human parotid gland was used as positive controls. Nuclei were counterstained with haematoxylin (blue). (F) Primary antibodies were omitted in negative controls (magnification 10×).

PONIN, and a mean staining intensity score of 2 (overall score 1.04). Meanwhile, eight out of 15 patients (53.3%) without metastasis (non-metastasis subgroup) were considered CALPONIN(+), displaying an average of 57.5% of tumour cells positive for CALPONIN, and a mean staining intensity score of 1.9 (overall score 1.10). Furthermore, the number of complete MECs layers was larger in the non-metastasis subgroup compared to the metastasis subgroup (Fig. 2 and Table 1).

Cumulatively, for both non-invasive and invasive CXPAs, the non-metastatic subgroups displayed a statistically higher incidence and enrichment for CALPONIN (+) MECs compared to the metastatic subgroups ($P = 0.022$ for non-invasive CXPAs and $P = 0.02$ for invasive CXPAs). Furthermore, the CALPONIN (+) MECs observed in non-metastatic subgroups mainly ensheathed tumour nests (Table 1).

p63 staining

Next, we examined patient tissues for the presence of p63(+) MECs. In non-invasive CXPAs, two out of 10 patients (20%) were classified as metastatic (metastasis subgroup). We found that an average of 31% of tumour cells expressed CALPONIN, and the mean staining intensity score was 2 (overall score 0.62). Therefore, none of these tumours was classified as p63(+). Moreover, complete MEC layers did not surround the majority of the tumour nests in these metastatic patients. Meanwhile, seven out of eight patients (75%) without metastasis (non-metastasis subgroup) were considered p63(+), displaying an average of 71.5% of tumour cells positive for p63, and a mean staining intensity score of 1.8 (overall score: 1.29) (Fig. 3 and Table 1).

Among invasive CXPAs ($n = 30$), one out of 15 patients (6.7%) with metastasis (metastasis subgroup) were considered p63(+), displaying an average of 51% of tumour cells positive for p63, and a mean staining intensity score of 2 (overall score 1.02). Meanwhile, nine out of 15 patients (60%) without metastasis (non-metastasis subgroup) were considered p63(+), displaying an average of 52.8% of tumour cells positive for p63(+), and a mean staining intensity score of 2.1 (overall

score 1.11). Furthermore, the number of complete MECs layers was larger in the non-metastasis subgroup compared to the metastasis subgroup (Fig. 3 and Table 1).

Cumulatively, for invasive CXPAs, the non-metastatic subgroups displayed a statistically higher incidence and enrichment for p63(+) MECs compared to the metastatic subgroups ($P = 0.008$). Moreover, the p63(+) MECs observed in non-metastatic subgroups mainly ensheathed tumour nests (Table 1).

Discussion

MECs are located in exocrine glands, such as salivary glands, mammary glands, lacrimal glands, and sweat glands. They have many functions. With rapid advances in molecular biotechnology, physiopathological studies on MECs have experienced dramatic developments in recent years.

MECs differ in distribution in different salivary glands. In minor salivary glands, the cell body of MECs cover acinar epithelial cells, and flat processes of MECs extend to the intercalated duct. MECs are absent around the secretory duct and interlobular duct. In the submandibular gland, MECs mainly surround acinar cells and the intercalated duct. They seldom associate with the secretory duct or intercalated duct. Nevertheless, in the parotid gland and sublingual gland, MECs ensheath the secretory duct, intercalated duct, and interlobular duct[13]. Resembling smooth muscle cells, a microfilament is found in the cell body of MECs, which is likely to facilitate the most important function of MECs: assisting saliva secretion through contractions.

MECs lie between acinar cells, ductal cells, and basement membrane. They participate in the formation of basement membrane by excreting type IV collagen. As malignant transformation of acinar and ductal cells occurs, MECs and basement membrane hamper tumour invasion because they serve as a natural barrier. So far, a number of studies verified that MECs secrete serine protease inhibitors and certain matrix metalloproteinase inhibitors (MMP-2 and MMP-9) in a paracrine fashion. This enables MECs to regulate proliferation of tumour cells and impede their ability to degrade extracellular matrix, all of which is required for invasion and metastasis.

In the mammary gland, Man et al.[14] found that as tumours develop from hyperplasia to carcinoma in situ, and ultimately to invasive carcinoma, MECs dramatically decrease in number. MEC layers are gradually destroyed, as well. Likewise, Jones et al.[15] and Polyak and Hu[16] reported that as mammary tumours invade, the number of MECs drop, and their normal physiology is disrupted.

MECs—especially those associated with tumours—are not easily distinguished from myofibroblasts or vascular smooth muscle cells in H&E stained histological sections. Therefore, investigators must resort to electron microscopy or immunohistochemical staining to examine MECs. For immunohistochemistry, the ideal markers for MECs should have high sensitivity and specificity, and allow them to be easily differentiated from other cells types that have similar morphology.

In the mammary gland, Liang et al. immunohistochemically labelled MECs using antibodies against α-SMA, CALPONIN and p63. This methodology was used to examine MECs residing in normal breast tissue and in diseased conditions, including usual hyperplasia, atypical ductal hyperplasia, carcinoma in situ, and invasive carcinoma. As a result, these researchers concluded that these three molecular markers define the cell identity of MECs[17].

In the salivary gland, Tang and Zhou[18] used p63, CALPONIN, and D2-40 to identify MECs in benign pleomorphic adenomas, malignant salivary gland tumours, and tissues near malignancy. They found that MECs are less abundant in malignant tissues than tissues outside of the malignancy or in benign pleomorphic adenomas. They suggested that the decreased abundance and dysfunction of MECs might increase the severity of malignancy for salivary gland tumours. Additionally, irregularly differentiated MECs might accelerate the development of salivary gland tumours[18].

Moreover, a number of studies found that low PLAG1 expression is associated with an invasive type of CXPA and high PLAG1 expression is associated with low-grade histopathological subtypes of CXPA including myoepithelial carcinoma and epithelial–myoepithelial carcinoma[19,20]. Taken together, PLAG1 is a potential biomarker to indicate the histological status of CXPA and shows prognostic value in this disease.

Even though PALG1 shows a high prevalence in MECs, it is not a satisfactory specific biomarker for MECs, since PLAG1 can be detected in the epithelial and myoepithelial components of PA and CXPA[21–23].

For the salivary gland, the role of MECs in tumour invasion and metastasis is unknown. To study the relationship between MECs and CXPA invasion and metastasis, we examined MECs in various cohorts of CXPA patients using three antibodies that detect proteins enriched in this cell type (α-SMA, CALPONIN, and p63). It's well established that in CXPA there exist a variety of histopathological subtypes, such as epithelial carcinoma, adenocarcinoma not otherwise specified, mucoepidermoid carcinoma, adenoid cystic carcinoma, salivary duct carcinoma, and so on. Since epithelial carcinoma is almost composed of neoplastic epithelial cells, in order to avoid a biased result we excluded those cases histopathologically identified as epithelial carcinoma. And the identified subtypes of all included patients are as follows: adenocarcinoma not otherwise specified (25), mucoepidermoid carcinoma (7), adenoid cystic carcinoma (6), salivary duct carcinoma (2 cases).

In non-invasive CXPAs, non-metastatic cases displayed a statistically significant enrichment for CALPONIN(+) and α-SMA(+) MECs compared with the metastatic group. Additionally, the number of complete MEC layers in cases without metastasis was much higher than in metastatic samples. This suggests that most non-invasive CXPAs possess abundant MECs that form complete layers surrounding tumour cells. Yet, in a minority of non-invasive CXPAs, the overall quantity of MECs is much smaller, and they do not ensheath tumour tissue. We propose that one mechanism underpinning the invasiveness and metastasis of CXPA involves the gradual loss of functional MECs. This can account for the clinical observation that most non-invasive CXPAs progress in a benign manner without cervical or distant metastasis, and a few cases develop metastasis before or after treatment. Our data suggest that in non-invasive CXPAs, both the tumour capsule and MECs may serve as a barrier preventing tumour cells from metastasizing. In frankly invasive CXPAs, we obtained similar results. In general, we found fewer numbers of MECs in frankly invasive CXPAs than in non-invasive CXPAs. Furthermore, we observed fewer MECs in the metastatic subgroup than in the non-metastatic subgroup. Cumulatively, these results suggest that a decreased number of MECs may promote tumour invasion and metastasis of CXPA.

Nevertheless, we should point out that incomplete MEC layers do not correlate with tumour invasion and metastasis directly, since in non-invasive CXPAs without metastasis we found some MEC layers surrounding a malignancy that were incomplete or even absent. Similarly, Jing et al.[24] reported eight patients with mam-

mary ductal carcinoma in situ. In these individuals, 130 randomized carcinomatous ducts were examined, and only 31 ducts were found to have a complete MEC layer. Therefore, incompleteness of the MEC layer does not appear to serve as an independent predictor of tumour invasion and metastasis.

In conclusion, a small number of non-invasive CXPAs have the potential for metastasis. The tumour capsule may not be the only barrier preventing invasion and metastasis of CXPA. We propose that reduction in the number of myoepithelial cells promotes the invasion and metastasis of CXPA, and these findings warrant future investigation.

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Competing interests

None.

Ethical approval

Ethical approval obtained from Peking University School and Hospital of Stomatology Biomedical Institutional Review Board PKUSSIRB-201626008.

Patient consent

Not required.

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