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Relationship between major and minor salivary gland mucoepidermoid carcinoma malignancy grading and presence of stromal myofibroblasts: immunohistochemical study

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OBJECTIVE: Mucoepidermoid carcinoma (MEC) is the most common malignant salivary tumour, classified as low, intermediate and high grade. Myofibroblasts are the main stromal component and are included as prognostic factor in some tumours. The aim of this study was to evaluate the myofibroblasts in the stroma of MEC with possible relationship to malignancy grading.

METHODS: Twenty-five cases of MEC (six low grade, 11 intermediate grade, four high grade and four metastasis) were stained for vimentin, desmin and smooth muscle actin (SMA) for the identification of myofibroblasts. Transforming growth factors (TGFβ1 and TGFβRII) were also assessed in our study.

RESULTS: Myofibroblasts were present in all cases, in amounts varying according to histological grading. TGFβ1 was positive in squamous cells of intermediate grade tumours, and in the stroma of only four cases. TGFβRII was positive in most squamous and intermediate cells, regardless of malignancy grading.

CONCLUSIONS: Our study showed that the analysis of neoplastic stroma must be added to the studies of neoplastic cells to draw a better picture leading to tumour diagnosis and prognosis.

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Mucoepidermoid carcinoma (MEC) is the most common salivary gland malignant neoplasm, histologically characterised by the presence of various cell types resembling the excretory duct of salivary glands (1–5). This tumour has been classified as low, intermediate and high grades of malignancy based on histological grading criteria. Suggested grading criteria have included, either alone or in combination, the relative proportion of cell types, degree of invasiveness, pattern of invasion, degree of maturation of cellular components and proportion of tumour composed of cystic space relative to solid growth. Although most studies have shown that MEC histological grading has prognostic significance, there is still wide discussion about this theme.

Recently, studies have been focusing on stromal features of various tumours, aiming to add more information in the diagnostic and prognostic prediction of neoplastic lesions. In special, desmoplasia, a process characterised by important deposition of collagen by myofibroblasts, has been pointed out as a valuable prognostic factor (6, 7). These cells are regarded as reparative cells, and the fibroblasts are thought to be their precursor. Differentiation of fibroblasts into myofibroblasts is due to growth factors and cytokines activities, amongst which, transforming growth factor β1 (TGFβ1) is the main agent (8).

The present study investigated the presence of stromal desmoplasia by identification of myofibroblasts in MEC of salivary glands, aiming to correlate the later with the different malignancy grades. Additionally, the presence of TGFβ1 and its receptor TGFβRII was analysed with the perspective of understanding its role in the desmoplasia.

Materials and methods

Paraffin-embedded specimens of 25 cases diagnosed as MEC of major and minor salivary glands were retrieved from the archives of the Surgical Pathology Service of the Oral Pathology Department of the Dental School of the University of São Paulo and from the Pathology Department of A.C. Camargo Hospital. Hematoxylin- and eosin-stained sections were re-analysed and

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Table 1 Antibodies used

Types	*Clone*	*Dilution*	*Incubation time*
Vimentin[a]	V9	1 : 50	120 min
SMA[b]	1A4	1 : 100	60 min
Desmin[a]	D33	1 : 150	60 min
TGFβ1[c]	Sc146	1 : 1000	Overnight
TGFβRII[c]		1 : 1000	Overnight

[a]DAKO; [b]Bio Genese Laboratories; [c]Santa Cruz Biotechnology Inc.

histological grading criteria based on scores established by Auclair et al. (9) and Goode et al. (10) were applied. Therefore, tumours were classified as low, intermediate and high grades of malignancy.

Sections of 3 μm were then submitted to streptavidin–biotin peroxidase immunohistochemistry method against vimentin, smooth muscle actin (SMA), desmin, TGFβ1 and TGFβRII. Positive controls were included in the study, and negative controls were performed by omission of the primary serum. Antibodies, clones and sources as well as protocol details are described in Table 1. The results were analysed qualitatively.

Results

From the 25 cases of MEC included in the study, six were classified as low-grade, 11 as intermediate-grade, four as high-grade and four as metastatic tumours.

Tumour stromal cells were positive for vimentin and SMA. The latter denoted the presence of myofibroblasts, the expression of which varied according to tumour grading. Low-grade MEC presented intense positivity in spindle cells located in the periphery of neoplastic cell blocks and cystic spaces. Scattered cells positive for SMA were also seen in other areas of the stroma (Fig. 1A). Intermediate-grade MEC stroma showed spindle cells positive for SMA only in focal areas at the periphery of neoplastic cell nests. This positivity was less intense and in fewer cells than in cases of low-grade MEC. In areas where inflammatory component was present, collagen deposition and myofibroblasts were not observed. Yet, some cases showed complete absence of SMA-positive cells (Fig. 1B). High-grade MEC stroma showed only a scarce number of spindle cells positive for SMA. They were located at the periphery of neoplastic cell blocks and intermingling the stroma (Fig. 1C). Only one case of metastatic MEC

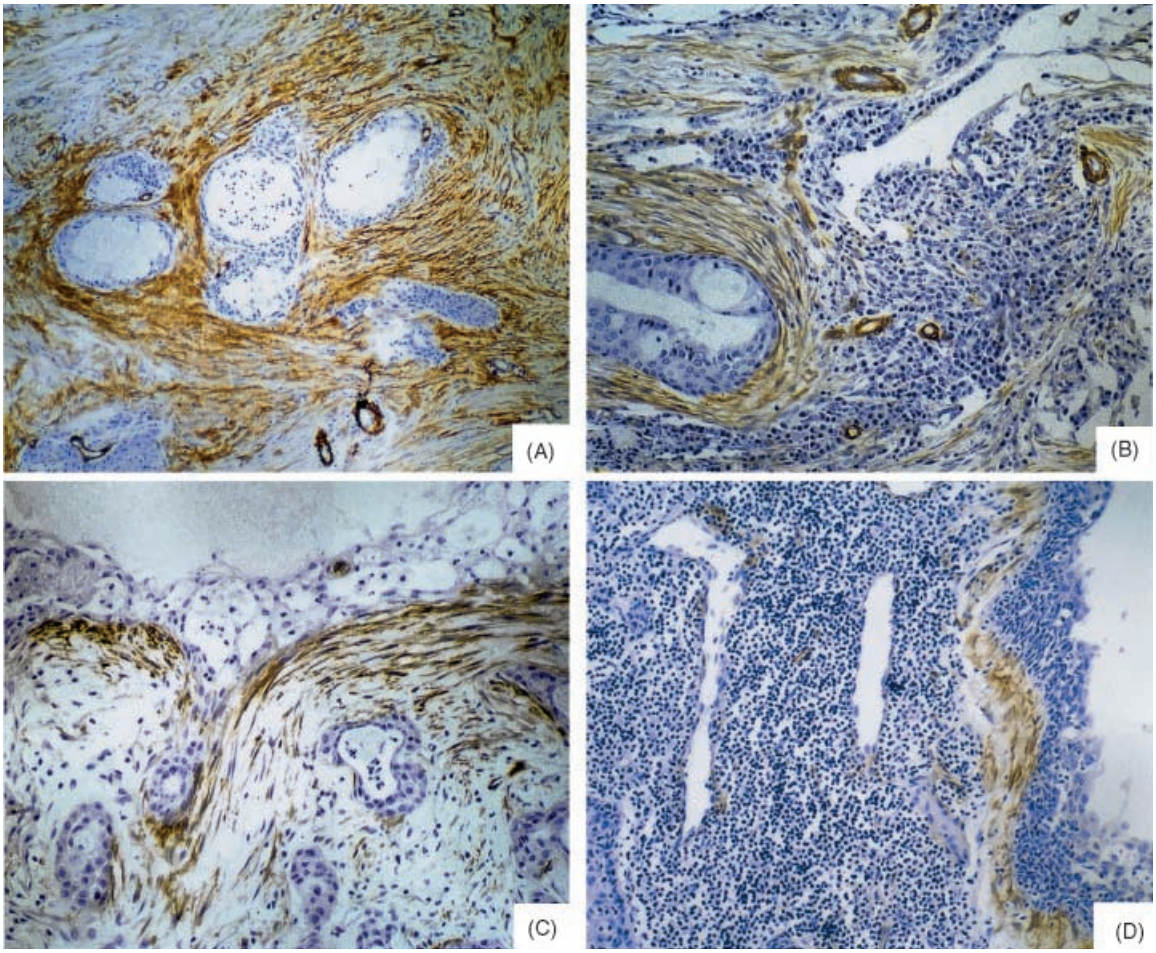

Figure 1 Immunostaining for SMA: (A) in MEC of low grade of malignancy (100×); (B) in MEC of intermediate grade of malignancy (200×); (C) in MEC of high grade of malignancy; (200×); (D) in metastasis (100×).

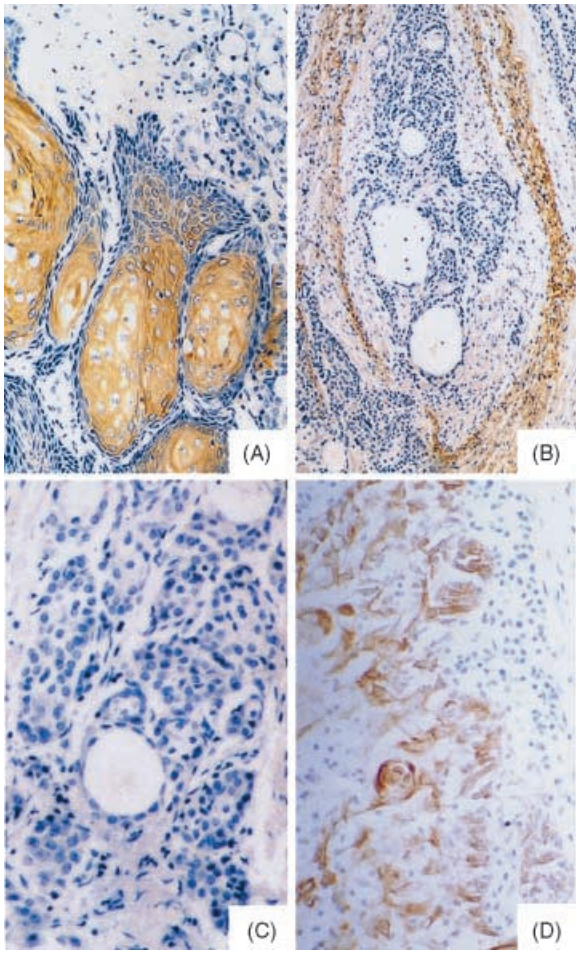

Figure 2 Immunostaining for TGFβ1 in MEC. (A) Squamous cells morphology, present in blocks and/or cystic spaces of intermediate grade MEC (200×); (B) stromal spindle cells were positive (100×); (C) intermediate cells were negative for this antibody in all high-grade tumours (200×); (D) the metastatic tumour was positive (200×).

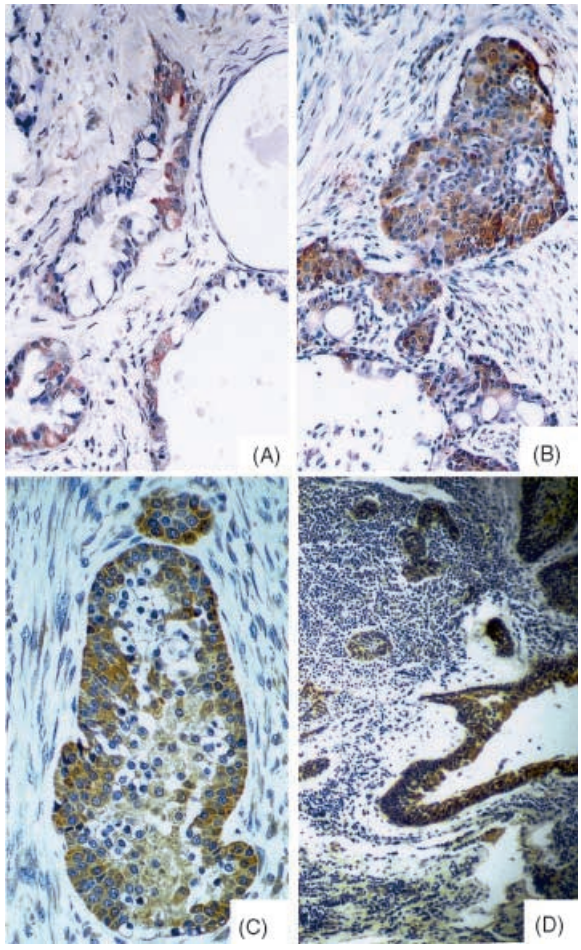

Figure 3 Immunostaining for TGFβRII in MEC. Squamous and intermediate cells were positive for TGFβRII, regardless of malignancy grading. (A) MEC of low grade (200×); (B) MEC of intermediate grade (200×); (C) MEC of high grade (200×); (D) metastasis (100×).

presented spindle cells positive for SMA in the stroma, which were at the periphery of a cystic area (Fig. 1D). Neoplastic MEC cells were negative for these antibodies. Neoplastic MEC cells as well as stromal cells were negative for desmin.

TGFβ1 was expressed by tumour MEC cells bearing squamous morphology, present in blocks and/or cystic spaces of intermediate-grade MEC (Fig. 2A). Stromal spindle cells were positive for this factor in only four cases (Fig. 2B). Mucous, clear, cuboidal, columnar and intermediate cells were negative for this antibody as well as all high-grade tumours (2C). Only one metastatic tumour was positive for TGFβ1 (Fig. 2D).

Most neoplastic squamous and intermediate cells were positive for TGFβRII, independent of malignancy grade (Fig. 3A–D). Cuboidal cells lining cystic spaces as well as mucous and clear cells were negative for this receptor. Only one case classified as low-grade MEC was negative for TGFβRII, as it was composed by cystic structures lined by cuboidal and columnar neoplastic cells. Spindle cells of tumour stroma were as a role negative for this antibody.

Discussion

Our results revealed six cases of low-grade MEC, 11 cases of intermediate-grade and four cases of high-grade tumours using the classification system proposed by Auclair et al. (9) and Goode et al. (10). Additionally, four metastatic tumours were included in the study.

Amongst all MEC, low-grade tumours were the easiest to identify due to their similarity with the salivary gland excretory duct. High-grade tumours were difficult to diagnose, especially when squamous cell carcinoma was the differential diagnosis. Staining for mucous is the diagnostic choice, when differentiating between high-grade MEC and squamous cell carcinoma. However, the high-grade MEC included in our study were mainly composed of intermediate cells instead of squamous cells as described by Evans (11). These tumours deserve a careful examination by all pathologists, and histochemical as well as immunohistochemical techniques may be good diagnostic tools (12).

Histological grading of tumours aims a prognostic prediction of the patient's life, as it is well known that

high-grade tumours present high rate of local recurrence (13, 14). In spite of molecular biology advances, histological grading is an important predictor factor, especially when MEC is compared with other salivary gland tumours (5).

Stromal myofibroblasts are considered an inhibitory factor in tumour progression. In our study, presence of myofibroblasts in MEC was revealed by the expression of vimentin and SMA. This cell phenotype is commonly found in either normal or pathological conditions and according to several studies, association of these proteins characterises mesenchymal cell cytoskeleton (15–18). Contrarily, Roche (19) stated that presence of desmin is essential for myofibroblast characterisation; however, other studies show that presence of desmin and/or myosin associated with vimentin and SMA only reveals different myofibroblast phenotypes (15, 18).

In our results, great desmoplasia was observed in low-grade MEC when compared with high-grade tumours. This data is comparable to those of Vasuedo & Harris (20) and Schürch and co-workers (21, 22) in their study of breast adenocarcinoma. The presence of these cells may be an attempt to block tumour progression and could probably be considered as a prognostic factor.

Several factors related to tumorigenesis may influence myofibroblast differentiation. Amongst these, TGFβ1 is pointed out as one of the main factors of SMA expression induction (8). TGFβ1 is also related to SMA transcription levels, translocation and turnover (7). The expression of this factor in MEC may represent an effort for cell differentiation as the presence of TGFβ1 was only seen in squamous areas and luminal structures, which could be compared with epidermal and dermal cells (23, 24).

The majority of MEC cells were negative for TGFβ1. This result disagrees with those found by Maiorano et al. (25), who described a wider presence of TGFβ1 in neoplastic cells of thyroid carcinomas than in adenomas, suggesting that this factor is related to tumorigenesis. In this sense, salivary gland carcinomas and thyroid carcinomas may follow distinct tumorigenesis pathways.

Only four MEC included in our series presented TGFβ1-positive stromal cells. However, it was not possible to confirm whether positive cells were myofibroblasts or fibroblasts under myofibroblastic differentiation process and further investigation is necessary to clarify this matter. In spite of this, Desmouliére et al. (8) and Massagué (26) state that TGFβ1 is a powerful extracellular matrix modulator, being also related to myofibroblastic modulation.

TGFβ1 is yet directly accountable for fibroblast attraction, placing it in an intimate contact with tumour cell, and then inducing SMA expression and homing of tumour cells in their position (7). This fact might explain the better prognosis of low-grade MEC, which presented the greatest expression of myofibroblasts. On the contrary, in inflammatory areas of intermediate-grade MEC, myofibroblasts were absent. The presence of inflammatory component in malignant breast neoplasms has been pointed out as an inhibitory factor for TGFβ1 synthesis. This may occur by liberation of interferon (INF), which promotes reduction in SMA mRNA synthesis, interfering with fibroblastic differentiation into myofibroblasts (27). INF action may be diminished by granulocyte-macrophage colony-stimulating factors (GM-CSF) action, which acts as myofibroblastic differentiation stimulator (7). Another factor to be considered during inflammatory process is the inflammatory infiltrate. Macrophagic infiltrate has been related to declined TGFβ1 synthesis and to basement membrane destruction, benefiting tumour invasion (28). These data justify our results that showed no myofibroblasts in inflammatory areas of intermediate-grade MEC. It could then be suggested that inflammatory infiltrate in MEC stroma stops myofibroblast differentiation, being indicative of a worse prognosis, as it facilitates progression of neoplastic cells. A busy environment is therefore described in inflammatory situations; however, signalling pathways leading to different cell phenotypes need investigation.

One metastatic tumour included in our study presented neoplastic cells positive for TGFβ1. This fact was not surprising as TGFβ1 expression was demonstrated in infiltrative breast cancer nodal metastasis (28, 29). These authors suggest that overexpression of TGFβ1 stimulates tenascin synthesis, an extracellular matrix protein that aids tumour invasion and metastasis. The dual role of TGFβ1 in tumorigenesis – stimulation and inhibition of tumour progression – is a consequence of different concentrations of this factor (7). Actually TGF-β primarily inhibits the proliferation of many cell types, but in many tumours, cells start secreting non-physiological levels of TGF-β, which may lead to progression of the tumour and metastasis (30). The roles of TGFβ in tumour development and metastasis is not yet fully defined and studies involving specific tumours and/or *in vitro* and *in vivo* models should be better evaluated (31).

It is well established that TGFβ1 controls cell differentiation and expression of collagen and fibronectin in rhabdomyoblasts, which express TGFβRII (26). In addition, absence of TGFβRII in metastasis is related to salivary gland tumour progression (32). Our results showed a massive expression of TGFβRII in all neoplastic cells associated with lack of TGFβ1, which deserves further investigation. Other studies have demonstrated that TGFα, would, in specific situations, compete with TGFβ1-binding sites (33, 34).

Our study showed that analysis of neoplastic stroma must be added to the studies of neoplastic cells to draw a better picture leading to tumour diagnosis and prognosis.

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