ORIGINAL ARTICLE

Lymphatic vascular density and lymphangiogenesis during tumour progression of carcinoma ex pleomorphic adenoma

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Aims: To assess lymphatic vascular density (LVD) and lymph vessel endothelial proliferation in a series of carcinoma ex pleomorphic adenoma (CXPA) that represents the tumour in the different carcinogenesis phases and tumour progression.

Methods: In 8 cases of early CXPA (intracapsular and minimally invasive tumours), 8 of advanced CXPA (widely invasive tumours) and 10 of pleomorphic adenoma (PA) without malignant transformation, lymphatic vessels and proliferating cells were detected using the antibodies D2-40 and Ki-67 respectively.

Results: Comparing early tumours with advanced ones, LVD was not significantly different at the tumour margin. In contrast, regarding intratumoural lymphatics, PA without malignant transformation and early CXPA contained rare, if any, lymph vessels, whereas in widely invasive carcinomas they were more numerous. However, neither intratumoural nor peritumoural LVD were increased in comparison to adjacent normal salivary gland tissue. In no case did dual immunohistochemistry using D2-40 and the cell proliferation marker Ki-67 reveal the existence of proliferating lymphatics. Carcinomatous emboli were found in peritumoural as well as in intratumoural lymphatics only in advanced CXPA without myoepithelial differentiation.

Conclusion: In CXPA, the lymphatic network is mainly composed of pre-existing lymphatics which are rare in tumours that have not infiltrated outside the confines of the original PA. In the widely invasive CXPA, intratumoural as well as peritumoural lymphatics are a conduit for carcinoma cells, but in carcinomas with myoepithelial differentiation, the neoplastic cells seem to have a lower invasion capacity.

Pleomorphic adenoma (PA) is the most common benign tumour arising in salivary glands.¹ Carcinoma ex pleomorphic adenoma (CXPA), an epithelial malignancy that arises in or from a PA,¹ has been considered to belong to the salivary carcinoma group which has moderate risk for neck metastasis.² This tumour accounts for between 4.5% and 15% of all cancers of these glands.^{3–5}

In human cancers, lymphatic vascular density (LVD) has been analysed within the main neoplastic mass (intratumoural lymphatics) as well as at the tumour margin (peritumoural lymphatics). Pre-existing peritumoural lymphatics have been considered functional, accessible and sufficient for lymphatic metastasis.^{6–8} Intratumoural lymphatics, in spite of being proposed as non-functional in tumour models, are associated with an adverse clinical outcome and nodal metastasis in certain types of human tumours, such as cutaneous melanoma⁹ and squamous cell carcinoma of head and neck, and uterine cervix.^{10–11} However, in other neoplasms, such as breast, ovarian, endometrial and lung cancers,^{7–12–14} no intratumoural lymphatic network has been found.

Furthermore, there is great debate whether tumours promote newly formed vessels (lymphangiogenesis) or whether pre-existing lymphatics provide the main avenue for nodal metastasis.^{15–16} Evidence for lymphangiogenesis has been found in melanoma and head and neck squamous carcinoma,^{10–17–18} but not in other malignancies such as breast cancer.^{7–16–19–21} Consequently, it has been hypothesised that the reported discrepancies may reflect genuine differences between the malignant behaviour of various human neoplasms.¹⁰

To our knowledge, this is the first study in which LVD and lymph vessel endothelial proliferation have been assessed in CXPA. Lymphatic vessels were detected using the monoclonal antibody D2-40 that is considered an excellent lymphatic endothelium marker.^{22–23} However, as D2-40 reactivity has also

been described in non-endothelial normal cells and up-regulated in certain kinds of neoplastic cells,^{23–24} we also aimed to verify its immunoreactivity in the cellular components of CXPA.

MATERIALS AND METHODS

The present study was approved by the Committee of Ethics of the University of Campinas, Brazil and was performed in 16 cases of CXPA and 10 cases of PA without malignant transformation which were retrieved from the files of the Department of Pathology of the University of Campinas. CXPA was defined as a malignant epithelial neoplasm arising in association with a primary or recurrent PA. These tumours were classified according to extent of invasion beyond the capsule of the previous PA¹ as: intracapsular (without invasion), 4 cases; minimally invasive (≤ 1.5 mm of invasion), 4 cases; and widely invasive, 8 cases. Demographic and clinical information was obtained from the patients' medical records.

Immunohistochemistry

The following antibodies were used (table 1): D2-40 (for detection of lymphatic vessels); CD34 (for blood vessels); Ki-67 (for proliferating endothelial and epithelial cells); and α -smooth-muscle actin (α -SMA), vimentin, and cytokeratins (CK7 and CK14) (for classifying the carcinomas according to the presence of epithelial and/or myoepithelial cells.)

Single-staining for D2-40, α -SMA, vimentin, CK7 and CK14 was carried out using the EnVision System (Dako Corporation, Glostrup, Denmark).²⁵ Briefly, 5 μ m sections from each paraffin block were deparaffinised, hydrated and endogenous peroxidase activity was quenched by immersion of the slides in 3%

Abbreviations: CXPA, carcinoma ex pleomorphic adenoma; LVD, lymphatic vascular density; PA, pleomorphic adenoma

Table 1 Details of the antibodies used for immunohistochemistry					
Specificity	Clone	Isotype	Dilution	Source	Buffer (AR)
CD34	QBEnd 10	IgG ₁	1:50	Dako*	Citrate
CK7	OV-TL 12/30	IgG ₁	1:100	Dako*	Citrate
CK14	LL002	IgG ₃	1:1000	Neomarkers†	Citrate
Vimentin	V9	IgG ₁	1:100	Dako*	Citrate
α-SMA	1A4	IgG _{2a}	1:200	Dako*	None
D2-40	D2-40	IgG ₁	1:200‡	Dako*	Tris-EDTA

AR, antigen retrieval.
*Dako Corporation, Glostrup, Denmark.
†LabVision Neomarkers, Fremont, CA, USA.
‡Except for double-staining when the dilution was 1:100.

hydrogen peroxide. The antigen retrieval (AR) was achieved by boiling them in a steamer immersed in citrate buffer (pH 6.0) except for alpha-SMA, which did not undergo any AR (table 1). After cooling, the sections were incubated at 4°C, with the primary antibody, overnight and then with the EnVision polymer HRP (code K1491; Dako Corporation, Glostrup, Denmark) for 1 h at 37°C. Subsequently, sections were stained for 5 min at 37°C with 3.3'-diaminobenzidine tetrahydrochloride (DAB) and counter-stained with haematoxylin.

Double-labelling immunohistochemical staining (EnVision doublestain, code K1395, Dakopatts, Denmark) was performed for D2-40/CK14, D2-40/CD34 and Ki67/D2-40. Briefly, a monoclonal antibody anti-D2-40 or anti-Ki67 (table 1) was applied after antigen retrieval using Tris-EDTA buffer (pH 8.9) and incubated overnight at 4°C; detection was achieved using the EnVision polymer HRP and DAB to visualise the binding of the first antibody. The sections were then incubated with a second antibody against CK14, CD34 or D2-40 (table 1) at 4°C overnight. EnVision polymer linked to alkaline phosphatase

and fast red as a substrate chromogen system were used to complete the second immunostaining.

The isotype-matched negative controls did not show coloured precipitate on the tissue, which indicates that artifactual staining was minimised.

Evaluation of staining

Immunohistochemical reactions for D2-40, D2-40/CK14, D2-40/CD34, and D2-40/Ki-67 were interpreted by two authors (AA and AS) using a double-headed microscope. The initial evaluation was performed to verify the expression pattern of the antibodies in both single- and double-stained sections.

Quantitative analysis of the intratumoural and peritumoural lymphatic vessel density was carried out in sections that were single-stained for D2-40, as previously described.²⁶ Because this antibody is also expressed by myoepithelial cells, double stains for D2-40/CK14, and D2-40/CD34 on serial sections were used to confirm the identity of lymphatic vessels. Three distinct sets of measurements were performed in each tumour section, in

Figure 1 Immunostaining of lymphatic vessels in pleomorphic adenoma (PA) without malignant transformation and in intracapsular and minimally carcinoma ex pleomorphic adenoma (CXPA). In PA without malignant transformation (A) and in intracapsular CXPA (B), rare and small intratumoural D2-40 positive lymph vessels (arrows) are seen. In minimally invasive CXPA, the carcinoma cells invade the tissues beyond the PA capsule (C) and at the tumour margin (D); regularly-shaped D2-40 positive lymph vessels (brown, arrows) are seen close to a small salivary duct with CK 14-positive basal cells (red) and at some distance from neoplastic cells (arrows) (double immunostaining with D2-40 and CK 14 antibodies).

which single lymphatic vessels were manually counted at $200\times$ (0.7386 mm^2 field): three fields with the most vascularised areas (hotspots) were identified (a) within the tumour mass (intratumoural lymphatic density), (b) within an area $500\text{ }\mu\text{m}$ from the tumour border (peritumoural lymphatic density) and (c) within the normal tissue adjacent to the lesion. We also evaluated: (a) whether there was invasion of the D2-40 intratumoural or peritumoural lymphatic vessels by carcinoma cells (tumour emboli), and (b) whether benign and malignant myoepithelial as well as epithelial cells expressed D2-40. Finally, lymphatic endothelial proliferation was assessed by counting the number of Ki-67 positive lymphatic endothelial cells per lymphatic vessel.

Statistical analysis

For comparison between different tumour groups we used the Kruskal–Wallis test. Comparisons between intratumoural, peritumoural and normal tissue in the same tumour group were done with the help of the Wilcoxon test. All calculations were performed with the help of the SAS V.8.0 and SPSS V.10.0 programs. Significance level was always 0.05.

RESULTS

The CXPA group included 11 women and 5 men; tumours were located in the parotid glands in 9 cases, in the submandibular glands in 5, and in the minor salivary glands in 2. The average age of the intracapsular CXPA group was 47, in the minimally invasive 67 and in the frankly invasive 65. The CXPA were classified into two main groups according to the extent of invasion beyond the capsule of the previous PA: early tumours, 8 cases (4 intracapsular and 4 minimally invasive carcinomas); and advanced tumours, 8 cases (8 widely invasive carcinomas). In early tumours, nodal metastasis at the time of primary surgery was not present in any cases, whereas in advanced tumours it was detected in 2/7 cases.

The PA group without malignant transformation included 5 women and 5 men (average age 41); tumours were located in the parotid glands in 8 cases and in the submandibular glands in 2.

Lymphatic vessel density and endothelial proliferation

Normal salivary gland and pleomorphic adenoma without malignant transformation

In the normal salivary glands, LVD was 8.75 ± 1.16 . Lymphatic vessels were dispersed in adipose tissue and adjacent to the interlobular salivary ducts and blood vessels but were not seen within the intralobular stroma. In pleomorphic adenoma without malignant transformation, rare lymph vessels were detected within the tumour in 1/10 cases (fig 1A).

Early tumours (intracapsular and minimally invasive CXPA); 8 cases

In this group, according to cellular composition, all cases were composed of carcinoma without myoepithelial differentiation; in only two of them, rare and small intratumoural lymph vessels were observed (fig 1B). In contrast, at the tumour margin, LVD was 6.63 ± 3.89 and the distribution as well as the morphological appearance of the lymphatic vessels was similar to that seen in normal salivary glands (fig 1C,D). Comparing the density of lymphatics in normal salivary glands with that in the peritumoural as well as intratumoural region (fig 2A), only the latter was significantly lower ($p = 0.34$, $p = 0.04$ respectively).

Advanced tumours (widely invasive CXPA); 8 cases

In widely invasive CXPA, intratumoural LVD was 6.25 ± 7.09 and peritumoural LVD 7.14 ± 4.53 (fig 2B). Comparing normal salivary glands with the intratumoural as well as the peritumoural region, no significant difference was found in terms of LVD ($p = 0.59$ and $p = 0.32$ respectively). According to cellular composition, these tumours were classified as: carcinomas with myoepithelial differentiation, four cases; and without myoepithelial differentiation, four cases. In the former group, intratumoural lymphatics were detected in two cases; they were morphologically similar to those in normal tissues (fig 3C). In CXPA without myoepithelial differentiation, intratumoural lymphatic vessels were observed in three cases; in two of them, dilated lymphatics containing carcinoma emboli were seen within the tumour mass as well as at the tumour margin (fig 3A,B).

Comparing early with advanced CXPA groups, the peritumoural region of advanced tumours showed a slight increase in LVD but the difference was not significant ($p = 0.79$). In contrast, intratumoural LVD was significantly lower in early tumours than in advanced ones ($p = 0.04$).

All lymphatic endothelial cell nuclei were negative for proliferation marker Ki-67, whereas strong positivity was seen in adjacent tumour cells and blood endothelial cells, which served as a positive internal control (fig 3D).

D2-40 expression in tumour cells

In PA without malignant transformation and in CXPA, variable proportions of benign as well as malignant myoepithelial cells showed D2-40 expression in the cytoplasm. Among the myoepithelial cells, D2-40 positivity was found in those from tubulo-ductal structures (fig 4A), and in solid cellular masses. Regarding epithelial cells, D2-40 expression was detected in only two cases (one intracapsular and one widely invasive CXPA) and in these, only in malignant cells. In the widely

Figure 2 Box and whisker plots of the lymphatic vascular density (LVD). (A) In intracapsular and minimally invasive carcinoma ex pleomorphic adenoma (CXPA) (early CXPA): (1) intratumoural LVD; (2) peritumoural LVD; (3) LVD in normal tissue adjacent to the lesion. (B) In widely invasive CXPA (advanced CXPA): (1) intratumoural LVD; (2) peritumoural LVD; (3) LVD in normal tissue adjacent to the lesion. The limits of the box represent the 25th and 75th percentiles, and the centre bar the median. The whiskers are equivalent to the 5th and 95th percentiles.

Figure 3 Immunostaining of lymph vessels in widely invasive carcinoma ex pleomorphic adenoma (CXPA). (A) In CXPA without myoepithelial differentiation (ie, CXPA composed of epithelial cells only), peritumoural D2-40 positive lymph vessels containing tumour emboli (arrow) are seen. The inset shows the carcinoma emboli. (B) Within the tumour mass, dilated intratumoural D2-40 positive (brown) lymph vessels containing malignant epithelial cells within the lumen and CD34 positive (red) blood capillaries are seen (double immunostaining with D2-40 and CD34 antibodies). (C) CXPA composed of myoepithelial cells only; note the regularly-shaped intratumoural D2-40 positive lymph vessel without tumour embolus. The inset shows CXPA composed of malignant myoepithelial cells of hyaline type. (D) At the tumour margin, regularly-shaped, non-proliferating lymphatic vessels (red) are found in CXPA with myoepithelial differentiation. Note the D2-40 negative blood vessel (arrow) and the tumour cells with positive nuclear Ki-67 staining (double immunostaining with D2-40 and Ki-67 antibodies).

invasive CXPA, the carcinomatous cells showed squamous differentiation and D2-40 was expressed predominantly within the basal tumour cell layer, with enhanced membrane-staining pattern (fig 4B).

In normal salivary glands, D2-40 stained myoepithelial cells were located in the acinar and ductal structures.

DISCUSSION

Based on the extent of carcinomatous invasion beyond the PA capsule, CXPA may be classified as intracapsular, minimally invasive and frankly invasive.²⁷ Intracapsular and minimally invasive carcinomas have been considered low-grade neoplasias, in which metastasis rarely occurs.^{28–30} In contrast, widely

invasive CXPA are typically high-grade carcinomas with frequent metastases and disease-related deaths.^{27 28}

In the current study, a series of CXPAs that represent the tumour in the different carcinogenesis phases was analysed, thus allowing the characterisation of tumour lymphatics and the assessment of lymphangiogenesis during tumour progression.

In our series, peritumoural LVD was not significantly different in intracapsular and minimally invasive CXPA (early tumours) compared with widely invasive ones (advanced tumours). In contrast, regarding intratumoural lymphatics, early CXPA contained rare, if any, lymph vessels, whereas in widely invasive carcinomas they were more numerous.

Figure 4 D2-40 expression in non-endothelial cells. (A) In adenoma areas of carcinoma ex pleomorphic adenoma (CXPA), benign myoepithelial cells of tubulo-ductal structures are stained by D2-40 (brown) and CK14 (red) (double immunostaining with D2-40 and CK14 antibodies). (B) In the widely invasive CXPA with squamous differentiation, D2-40 (brown) is expressed in the basal tumour cell layer and in lymph vessels (arrows). Note the CD34 (red) positive blood vessels (double immunostaining with D2-40 and CD 34 antibodies).

However, neither intratumoural nor peritumoural LVD increased in comparison to adjacent normal salivary gland tissue. In addition, in no case did dual immunohistochemistry using D2-40 and the cell proliferation marker Ki-67 reveal the existence of proliferating lymphatics, contrasting with the strong expression of Ki-67 in adjacent tumour cells and blood endothelial cells. These results suggest that lymphangiogenesis did not occur, or was minimal, during the process of malignant transformation of PA into carcinoma and, thus, peritumoural as well as intratumoural lymphatic vessels were pre-existing. Therefore, carcinoma arising in PA is similar to breast cancer,^{7 19 31} where lymphangiogenesis was not detected and intratumoural lymphatics were considered “entrapped vessels” adjacent to pre-existing normal lobules and ducts.¹⁹ Nevertheless, CXPA differs markedly from head and neck squamous cell carcinoma^{10 17} and malignant melanoma,¹⁸ where proliferating intratumoural lymphatics have been seen. These findings lend support to the hypothesis that tissue specific tumour features might be responsible for the presence or absence of a “lymphangiogenic switch”.³¹ However, the mechanisms involved in the promotion of newly formed lymph vessels and the role of this phenomenon in tumour progression still need to be clarified. In breast carcinogenesis, as lack of lymphangiogenesis cannot be explained by the absence of proteins associated with lymphangiogenic growth (VEGF-C and VEGF-D) or of their receptors (VEGFR-3),^{32 33} Vleugel *et al*³¹ suggested that factors that contribute to lymphangiogenic growth inhibition should be investigated.

Regarding neoplastic lesions representing the early phases of tumour progression, rare studies have assessed LVD and lymphangiogenesis. In these, comparing premalignant with pre-invasive intraductal proliferations of the breast and nevi with malignant melanoma *in situ*, no significant increase was found in terms of LVD.^{19 31 34} In our series, intracapsular (in situ) and minimally invasive CXPAs as well as PA without malignant transformation showed rare, if any, intratumoural lymph vessels. However, it should be noted that intracapsular CXPA shows significant differences in relation to other pre-invasive epithelial proliferations. These refer to neoplasias restricted to the epithelium without stromal invasion, whereas intracapsular CXPA defines a tumour that has not infiltrated outside the confines of the original PA,¹ which is composed of an intimate admixture of ductal epithelium, myoepithelium and stroma.¹ The stroma is produced by adenoma cells and consists of an admixture of mucoid, myxoid and chondroid tissues. Non-neoplastic stroma (pre-existing stroma), in which the lymph vessels are situated, was only found beyond the PA capsule, which could explain the presence of intratumoural lymphatics only in widely invasive CXPA. This idea is in agreement with the findings reported by Vleugel *et al*³¹ in breast ductal carcinoma *in situ*. These authors suggested that the inverse correlation between lymph vessel density and lesion size could be due to replacement of stroma by tumour epithelium, rather than the collapse of lymph vessels.

Dilated peritumoural lymph vessels, often containing tumour emboli, have been reported in different types of human cancer, suggesting that these lymphatics are sufficient for metastatic dissemination.^{7 19} In contrast, intratumoural lymphatics have been considered non-functional due to the results of fluorescent dye uptake measurements.²⁰ However, in head and neck squamous cell carcinomas it has been shown that intratumoural lymphatics are involved in nodal metastasis.^{10 17 35} In the current study, in widely invasive CXPA, carcinomatous emboli were found in peritumoural as well as in intratumoural lymphatics. These findings suggest that in the advanced phase of tumour progression, intratumoural lymphatics should be regarded as an additional pathway that might increase the

Take-home messages

- Intracapsular (in situ) and minimally invasive carcinoma ex pleomorphic adenoma (CXPA) as well as pleomorphic adenoma without malignant transformation showed rare, if any, intratumoural lymph vessels.
- In widely invasive CXPA, carcinomatous emboli were found in peritumoural as well as in intratumoural lymphatics.
- In no case did dual immunohistochemistry using D2-40 and the cell proliferation marker Ki-67 reveal the existence of proliferating lymphatics, suggesting that the lymphatic network is mainly composed of pre-existing vessels.
- In CXPA with myoepithelial differentiation, the inherent properties of the myoepithelial cells are likely to contribute to its low tendency to invade lymph vessels.

propensity of the CXPA to metastasise. Nevertheless, at the early phase, where the malignant cells are still contained within the parent PA, the scarcity of intratumoural lymph vessels probably contributes to the very low metastatic risk of these tumours.

Besides the presence of peritumoural as well as intratumoural lymph vessels, other factors related to tumour biology may contribute to increase the risk of metastases.^{8 19} Carcinomas arising in PA encompass a wide spectrum of histological patterns in which malignancies with epithelial and/or myoepithelial differentiation³⁶ are included. In the present investigation, lymphatic emboli and cervical metastases were present only in widely invasive carcinomas without myoepithelial differentiation, suggesting that the frequency of these events may also be influenced by tumour cellular composition. In CXPA with myoepithelial differentiation, the inherent properties of the myoepithelial cells (including secretion of low levels of matrix-degrading proteinases and of high levels of various anti-invasive proteinase inhibitors)³⁷ are likely to contribute to its low tendency to invade lymph vessels and, thus, to metastasise.

In relation to D2-40 reactivity with other non-endothelial cells, this antibody recognises an epitope on the podoplanin molecule that is expressed in myoepithelial cells of the normal salivary glands.²³ Podoplanin seems to play an important role in mediating cellular contractile properties and cytoskeletal reorganisation.²³ In our series, benign and malignant myoepithelial cells were D2-40 positive, suggesting that this antibody might be used as an additional tool to identify neoplastic myoepithelial cells in salivary gland tumours.

Regarding carcinomatous cells, Schacht *et al*,²³ Dumoff *et al*²⁴ and Ordóñez³⁸ reported expression of D2-40 in skin, uterine cervix and lung squamous cell carcinomas respectively. Antibody D2-40 was generated against an oncofetal M2A antigen (40-kd O-linked sialoglycoprotein), which is expressed in the fetal testis and in germ cell neoplasia.³⁹ It has been suggested that this antigen could have a role in epithelial tumour progression by enhancing tumour cell spread via lymphatic vessels. However, in our series only a few D2-40 positive carcinoma cells were observed in two cases (intracapsular and widely invasive CXPA respectively), but in both, nodal metastases were not detected. Interestingly, in one of them (a widely invasive CXPA), the carcinoma cells showed squamous differentiation and D2-40 was expressed predominantly within the basal tumour cell layer. Based on these

findings we speculate whether D2-40 expression is implicated in squamous cell differentiation since podoplanin expression has been found in normal basal keratinocytes and predominantly in poorly differentiated squamous cell carcinoma.²³

In conclusion, in CXPA, the lymphatic network is mainly composed of pre-existing lymphatics which are rare in tumours that have not infiltrated outside the confines of the original PA. In the widely invasive CXPA, intratumoural as well as peritumoural lymphatics are a conduit for carcinoma cells, but in carcinomas with myoepithelial differentiation, the neoplastic cells seem to have a lower invasion capacity.

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