Short Communication

Induction of lymphangiogenesis in and around axillary lymph node metastases of patients with breast cancer

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We studied the presence of lymphangiogenesis in lymph node (LN) metastases of breast cancer. Lymph vessels were present in 52 of 61 (85.2%) metastatically involved LNs vs 26 of 104 (25.0%) uninvolved LNs (P<0.001). Furthermore, median intra- and perinodal lymphatic endothelial cell proliferation fractions were higher in metastatically involved LNs (P<0.001). This is the first report demonstrating lymphangiogenesis in LN metastases of cancer in general and breast cancer in particular.

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Lymph node (LN) status is the most important prognostic factor for patients with breast cancer. The presence and the extent of axillary LN metastases reflect the probability that the cancerous process has spread through the body and both are strongly correlated with the development of distant metastases and with shortened disease-free and overall survival. Lymph node metastases are more than passive tumour deposits. Metastatic tumour sites are capable of inducing a vascular stroma and can actively contribute to tumour progression and to further metastatic spread. To what extent processes involved in progression of primary tumours, such as angiogenesis and lymphangiogenesis, contribute to progression of secondary sites is largely unknown. Reports have suggested differences between primary tumours and secondary sites and between different secondary sites. Whereas primary breast tumours grow angiogenesis dependently, we demonstrated that 90% of breast cancer liver metastases grow according to an angiogenesis-independent replacement pattern (Stessel et al, 2004). The growth of breast cancer LN metastases, on the contrary, was angiogenesis dependent and lymphangiogenesis and hypoxia in the metastases were correlated with angiogenesis and hypoxia in the primary tumours (Van den Eynden et al, 2005). Guidi et al (2000) demonstrated that the presence of vascular hot spots in LN metastases, but not in the primary breast tumours was associated with decreased survival.

In the present study, we compared the expression of the lymphatic endothelium-specific markers Prox-1, LYVE-1 and podoplanin in metastatically involved and uninvolved LNs of patients with breast cancer. Prox-1 and LYVE-1 are, respectively, a transcription factor and a hyaluronan receptor that show specificity for lymphatic endothelial cells. D2-40 was originally described as a selective monoclonal antibody to a M₄ 40 000 O-linked sialoglycoprotein that reacts with a fixation-resistant epitope in lymphatic endothelium (Kahn and Marks, 2002). Recently, the D2-40 antibody has been shown to specifically recognise podoplanin, a glomerular podocyte membrane protein (Schacht et al, 2005) and has been shown to be a very sensitive and specific marker for lymphatic endothelium in most tissues (Evangelou et al, 2005) and especially in breast cancer (Van der Auwera et al, 2005). We investigated the presence and extent of lymphangiogenesis in LN metastases of breast cancer using the podoplanin antibody.

MATERIALS AND METHODS

Patients and samples

One hundred and ten patients with operable breast cancer were included in this study, 49 patients with LN-negative and 61 patients with LN-positive breast cancer. Clinico-pathological features are compared between both study groups in Table 1, using the UICC TNM system. Formalin-fixed paraffin-embedded tissue blocks of one metastatically involved and – if available – one uninvolved LN of patients with LN-positive breast cancer and one metastatically uninvolved LN of patients with LN-negative breast cancer were selected for immunohistochemical examination. Of six LN-positive patients, no uninvolved LN was available.

Expression of lymphatic endothelium-specific and vascular markers

Immunohistochemical stainings for the lymphatic endothelium-specific markers podoplanin (clone D2-40, Dako, Glostrup, Denmark), Prox-1 (polyclonal, Reliatech, Braunschweig, Germany) and LYVE-1 (polyclonal, Reliatech) and for the panendothelial
Presence of lymph vessels and lymphangiogenesis

An immunohistochemical staining for podoplanin (clone D2-40, Dako) and an immunohistochemical double staining with anti-podoplanin (clone D2-40, Dako) and anti-Ki-67 antibodies (clone MIB-1, Dako) were performed on sections of a subset (n = 20) of metastatically involved and uninvolved LNs. A detailed description of the protocol of these stainings has been published before (Van der Auwera et al, 2005) (Figure 1).

Statistical analysis

Statistical analyses were performed with the SPSS 13.0 software package. A P-value < 0.05 was considered statistically significant. Correlations between categorical variables (e.g. presence of LVs, metastatic involvement) were analysed using a χ² test. LEC% in different groups was compared with a Kruskal–Wallis nonparametric test.
microscopy at different focal planes further reduces this bias. The methodology adopted in this study to measure ECP% is widely accepted in the angiogenesis field (Vermeulen et al., 2002) and has recently been introduced in the lymphangiogenesis field (Dadras et al., 2003; Beasley et al., 2002; Straume et al., 2003; Koukourakis et al., 2005; Van der Auwera et al., 2005). The median intranodal LECP% of 6.0% suggests that lymphangiogenesis in metastatically involved LNs is higher than in primary breast tumours: we previously demonstrated that LECP% in primary breast tumours was 1.83% (Van der Auwera et al., 2005).

Our results corroborate the concept of LN lymphangiogenesis, which was recently introduced and shown to be involved in the recruitment of dendritic cells to inflamed LNs (Angeli et al., 2006). The authors demonstrated that the lymphangiogenic response in the LNs was particularly localised in the subcapsular space and around B-cell follicles from where LVs penetrated into the cortex. Whether the outgrowth of new LVs in metastatically involved axillary LNs originates from the subcapsular marginal sinus or from LVs in areas of lipomatosis or in fibrous septa remains to be elucidated.

Different hypotheses could be raised about the role of LN lymphangiogenesis in metastatically involved LNs. On the one hand, LN lymphangiogenesis might be part of the immunological reaction against the tumour cells and on the other hand be involved in tumour progression and metastases, as in primary tumours. Therefore, LN lymphangiogenesis, as LN angiogenesis, might contribute to the metastatic spread of breast cancer. In a transgenic mouse model of skin cancer, Hirakawa et al. (2005) demonstrated that the induction of lymphangiogenesis by vascular endothelial growth factor A is involved in tumour progression. They furthermore showed that VEGF-A also induces lymphangiogenesis in the sentinel LN and that lymphangiogenesis is induced
before metastasising (Hirakawa et al, 2005). Based on our data, it is difficult to investigate the mutual contribution of local and remote mechanisms to the induction of lymphangiogenesis in LNs of patient with breast cancer. However, the difference in LECP% in uninvolved LNs of patients with N0 vs N+ breast cancer is intriguing in this context.

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Table 2 Expression of different vascular markers in metastatically uninvolved LNs of patients with breast cancer

| CD31 | CD34 | Podoplanin | Prox-1 | LYVE-1* |
|------|------|------------|--------|--------|
| High endothelial venules | + | + | – | – |
| Littoral cells (lining subcapsular and trabecular sinuses) | – | – | Very focally and faintly | – |

Fibrous capsule and trabecular fibrous septa

| BV | LV |
|----|----|
| +b | +b |

Fibrous and lipomatous areas

| BV | LV |
|----|----|
| +b | +b |

Perinodal fat

| BV | LV |
|----|----|
| +b | +b |

BV = blood vessel; LV = lymph vessel. *LYVE-1 positivity of macrophages and of intravascular proteins often hampers interpretation. CD31 and CD34 expression of lymphatic endothelial cells is mostly fainter than expression of blood vessel endothelial cells.

Table 3 Comparison of the presence of LVs between metastatically involved and uninvolved LNs

| Presence of LVs |
|-----------------|
| No | Yes |

Uninvolved LN

| No breast cancer | 37 | 12 |
| N+ breast cancer | 41 | 14 |
| Metastatically involved LN | 9 | 52 |

Uninvolved LN is divided in LN from N0 and N+ patients with breast cancer. P-value (metastatically involved vs uninvolved LNs) <0.001. P-value (uninvolved LNs N0 vs N+)= 0.91.

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