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ORIGINAL ARTICLE

In stage II/III lymph node-positive breast cancer patients less than 55 years of age, keratin 8 expression in lymph node metastases but not in the primary tumour is an indicator of better survival

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Abstract Axillary lymph node status is one of the most important prognostic variables for breast cancer (BC). To investigate and understand the clinical, histopathological and biological factors that affect prognosis in node-positive young breast cancer patients, we compared the phenotype of 100 primary tumours with their corresponding loco-regional lymph node (LN) metastases using conventional immunohistochemistry (IHC) markers currently in use for molecular classification of breast cancer. By comparing the expression of ER, PR, HER-2, Ki67, K8, K5/6 and vimentin, we found that expression of HER-2, Ki67, K8 and vimentin is frequently lost in lymph node metastases. Between the primary tumour and corresponding lymph node metastases, expression of keratins K8 and K5/6 significantly changed. Expression of K8 in lymph node metastases, but not in primary tumours, segregates patients in two sub-groups with different outcomes. Survival of patients with K8-positive LN metastases at 5 years in comparison with patients with K8-negative LN metastases was 75 vs 48 %, at 10 years 62 vs 22 % and at 20 years 53 vs 14 % ($p<0.001$). K8 immunostaining of tissue from the lymph node metastasis allows defining a sub-group of lymph node-positive BC patients with a highly unfavourable outcome, for whom therapeutic options might have to be reconsidered.

Keywords Breast cancer · Lymph node metastasis · Keratin 8 · Immunohistochemistry · Survival

Introduction

Axillary lymph node status is one of the most relevant prognostic factors in primary breast cancer (BC). Patients with metastases to axillary lymph nodes (LN) are at higher risk of recurrence [1, 2]. It has been estimated that almost 50 % of patients with positive lymph node involvement will develop distant metastases within 5 years, while women without lymph node involvement have a 30 % risk [1]. Many clinical and pathological parameters such as tumour size, tumour grade, age at diagnosis [2–4], lympho-vascular invasion [5], hormone receptor status and HER2 amplification [6, 7] are generally considered as predictors of recurrence. Although these clinical and biological factors are significantly associated with the presence of lymph node involvement, they fail to predict the development of later recurrences [1]. Today, we are unable to distinguish among lymph node-positive BC patients those with high and low risk of recurrence. Comparing pathological and molecular characteristics between primary tumours and corresponding metastatic sites might help not only to understand heterogeneity and biology of the metastatic process but also to predict patient outcome [8], especially in those young patients who already present with axillary node metastases at initial diagnosis.

Molecular classification of BC is associated with clinical behaviour and outcome [9, 10]. BC can be molecularly classified according to expression of oestrogen receptor (ER),

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progesterone receptor (PR) and HER2, and the Ki67 expression-based index of proliferation [11] as well as type of expressed keratin (K) [12–14]. Ks are the largest family of intermediate filaments [15]. As part of their diversity in number, structure and tissue-specific expression, Ks serve as important immunohistochemical markers, also because during tumour progression, the pattern of Ks expression in primary tumour tissue is often maintained in metastases [16]. The epithelium of breast duct and lobules contains luminal and basal/myoepithelial cells [17]. Luminal cells express low molecular weight Ks, such as K7, K8, K18 and K19, while basal/myoepithelial cells [18] express high molecular weight Ks, such as K5 and K14 [17].

A wide range of biomarkers has been studied in primary BC and matched LN metastatic tissue, but to date, biomarker is not used for prognostic purposes [19–25]. Our aim in this study was to compare the expression of biomarkers in daily use for BC classification (ER, PR, HER2, Ki67) and BC phenotypic differentiation (K8 for luminal cells, K5/6 for basal/myoepithelial and vimentin as EMT marker) between primary and matched nodal tumour tissue and to investigate the relationships between primary tumour and metastatic LN as well as potential prognostic relevance in axillary LN metastases.

Material and methods

Patients

One hundred lymph node-positive (LN+) patients with locally advanced BC at diagnosis were enrolled in this retrospective study. Inclusion criteria were women with stages II–III invasive BC, age at diagnosis 55 years or younger, long follow-up if still alive and absence of second primary BC or other malignancies. All women were resident in North-Eastern Italy and received a diagnosis of primary BC at least 20 years before the censoring date of the study (31 December 2013) as reported previously [14]. According to those criteria, 154 women were selected, 54 of whom were excluded due to the impossibility to perform all IHC analyses, because of the minimal amount of metastatic node tissue. Formalin-fixed and paraffin-embedded (FFPE) tissues from the initial surgical intervention were used. Clinical information on follow-up was obtained from medical records.

H&E slides of primary tumours and metastatic lymph nodes were revised in double-blind fashion by two pathologists (R.B., G.S.). Histologic classification and tumour grading were done according to the World Health Organization (WHO 2003) [26] and Elston and Ellis grading system [27] respectively. The cohort of patients was followed through the general population-based Friuli Venezia Giulia Cancer Registry from the date of BC diagnosis to death or until 31 December 2013, whichever came first. The Ethics Committee of the University of Trieste had given its approval for the study. The patients enrolled in the present study had also been part of a previous study [14].

Tissue microarrays

Representative areas of both primary tumour and corresponding metastatic regional nodes were selected by two pathologists (G.S. and R.B.) for TMA construction. Tissue cores were of 1.0 mm in diameter taken at the border of the primary tumour in the donor paraffin block and placed into a recipient paraffin block using a tissue-arrayer (Galileo TMA CK3500; Integrated Systems Engineering, Milano, Italy), as previously described [28]. Multiple samples were taken to represent heterogeneous histological areas. Of each TMA block, 4-μm thick sections were cut, mounted on Superfrost® Plus (Thermo Scientific) microscope slides and heated at 37 °C overnight for IHC analysis.

Immunohistochemical staining

IHC staining was performed following standard procedures [29], according to the manufacturer's instructions for each MAb as reported previously [14]. Immunostaining was performed with the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for ER, PR, K8, K5/6 and vimentin. Prediluted antibodies for ER (CONFIRM™ anti-Estrogen Receptor clone SP1; Ventana Medical System, Tucson, AZ), PR (CONFIRM™ anti-Progesterone Receptor clone 1E2; Ventana Medical System, Tucson, AZ), vimentin (CONFIRM™ anti-Vimentin clone V9; Ventana Medical System, Tucson, AZ) and 1:100 dilution for K5/6 (clone D5&16B4; Aczon Biotech, Monte San Pietro, Bologna, Italy) were applied after antigen retrieval in 0.1 M pH 8 Tris-Borate, 1 mM EDTA at high temperature. Anti-K8 antibody (clone M20; Abcam, Cambridge, UK) was used at 1:250 dilution after antigen retrieval in 10 mM citrate buffer pH 6 at high temperature in a water bath. For visualisation, DAB Substrate kit for Peroxidase (Vector Laboratories, Burlingame, CA, USA) was used. Immunostaining for Ki67 (clone MIB-1; DakoDenmark A/S, Glostrup, Denmark), 1:200 dilution, and HER-2 (clone CB11; Thermo Scientific, Astmoor Runcorn, Cheshire, UK), 1:300 dilution, was performed in Lab Vision Autostainer 480S (Thermo Scientific) with the UltraVision LP Large Volume Detection System HRP Polymer (Lab Vision Corporation, Thermo Scientific) according to the manufacturer's recommended protocol. Briefly, after deparaffinisation and rehydration, tissue sections were washed twice in 0.05 M TBS + Tween 20 (Bio-Optica). Tissue slides were treated with high pH antigen retrieval for 20 min in heated water bath and washed four times. To reduce non-specific background staining, endogenous peroxidase was blocked with hydrogen peroxide for 10 min. Primary

antibodies were applied and incubated according to the manufacturer's instructions. Subsequently, the sections were incubated with primary antibody Enhancer (LabVision, Thermo Scientific) for 20 min at room temperature. After washing, HRP Polymer was applied for 30 min at room temperature. For visualisation, chromogen 3,3′-diaminobenzidine (DAB) (LabVision, Thermo Scientific) was used. Positive and negative control slides were used in each IHC assay.

Evaluation of immunohistochemistry

Immunostaining was quantitatively evaluated by two different observers in a blinded fashion (DP, RB), using light microscopy and counting positive cells across three high power fields (HPF at × 40 magnification). Tumours were considered positive for ER and PR if more than 10 % of tumour nuclei were stained [30, 31, 21], because this was the cut-off used for giving hormone therapy at the time of diagnosis. HER-2 expression was scored according to ASCO guidelines [32]. HER-2 gene amplification in the case of equivocal staining (2+) was assessed by SISH assays using the ultraView SISH DNP Detection Kit (Ventana) in Benchmark XT automated slide strainer instrument, as previously described [33]. Primary tumours and lymph node metastases positive in more than 10 % of cells were defined as positive for K8 and K5/6 as reported previously for keratins in BC [34, 35]. Nevertheless, also a 1 % cut-off was recorded and independently estimated for K8 (data not shown) with minimal variation in the results as shown in Supplementary Fig. 2. Any positive staining in tumour cells for vimentin was considered as positive expression [36]. Tumours were stratified into two groups according to their proliferative activity: Ki67 threshold of 14 % was used to discriminate low proliferation index (<14 %) and high proliferation index (≥14 %) [37, 11]. Tumours were classified into four main molecular classes according to ER, PR, HER-2, Ki67, K8 and K5/6 staining profile [37, 38, 11, 39]. Cases ER-positive and/or PR-positive, HER-2-negative, low Ki67 and K8-positive were classified as Luminal A, cases either (i) ER-positive and/or PR-positive, HER-2-negative, high Ki67 and K8-positive, or (ii) ER-positive and/or PR-positive, and HER-2-positive were classified as Luminal B, tumours ER-negative, PR-negative and HER-2-positive were defined HER-2+ type, and cases ER-negative, PR-negative, HER-2-negative and K5/6-positive were classified as triple negative (TN), which is considered a surrogate for basal-like [40]. If primary tumour or lymph node metastatic tissue staining pattern did not meet one of the above-mentioned panel criteria, the case was defined as 'unclassified'.

Statistical analysis

Staining for each marker was dichotomised into negative and positive expression, according to the previously described parameters. Marker expression was compared between primary tumour and lymph node metastasis using the Wilcoxon matched-pairs signed-ranks test. Associations between clinical-pathological factors and categories of markers were tested for significance using the chi-square test (or Fisher's exact test depending on sample size) for categorical variables. Overall survival (OS), defined as the time lapse between the date of diagnosis and the date of BC-specific death or the end of follow-up (FU), was the end point evaluated in this study. The log-rank test and Kaplan-Meyer curves were used to assess dependence of patient survival on single variables. To estimate the joint effects of the analysed covariates on patient survival and confirm the results of the log-rank test, data were analysed by fitting Cox proportional hazard regression model. Cox analysis included pathological variables (histologic type of tumour, tumour grade, tumour size, number of positive lymph nodes and age at diagnosis), K8 expression status in lymph node metastases and molecular subtype of BC. As post-estimation for the proportional hazards assumption the Grambsch and Therneau test for Schoenfeld residuals was run. All p values are two-sided with values <0.05 regarded as statistically significant. The p values between 0.05 and 0.07 were considered as 'borderline'. Statistical analyses were performed with the Stata/SE 12 package (Stata, College Station, TX).

Results

Patients

Mean age of patients at diagnosis was 47.6 years (range 28–55). During the period of observation, nine patients were lost at follow-up because of emigration. All details are reported in Table 1. As expected for histologic grade, most cases were of grade 2 (42 %) and grade 3 (56 %). The number of metastatic lymph nodes was up to three for 49 women and more than three for 51.

Patients underwent radical mastectomy and breast conservation surgery accompanied by axillary dissection. All patients submitted to conserving surgery (10) were treated with radiotherapy. In detail, in patients with three or fewer lymph nodes involved, only the breast was irradiated; in patients with more than three metastatic lymph nodes with capsule invasion, both breast and axilla were irradiated. Patients were treated with adjuvant chemotherapy with cyclophosphamide, methotrexate and fluorouracil (CMF), or epirubicin, cyclophosphamide and fluorouracil (EC/ECF) regimens according to standard protocols. ER-positive patients were submitted to hormone therapy with tamoxifen. No specific treatment with trastuzumab was performed in HER2+ patients, because it was not available at the time of diagnosis.

Table 1 Main pathological features

Variable	Number of cases
Histologic type	
Ductal	88
Lobular	10
Mucinous	2
Histologic grade	
1	2
2	42
3	56
Tumour size (cm)	
≤2	42
2–5	49
≥5	7
Missing	2
Stage	
II	43
III	57

The mean period of follow-up was 11 years (range 0–28 years). In this period, 67 patients developed recurrences, while 24 did not.

Comparison of markers expression between primary tumour and correspondent axillary LN metastasis

The expression of five out of seven markers (HER-2, Ki67, K8, K5/6, vimentin) was significantly different between primary tumour and corresponding loco-regional metastases as reported in Table 2 and in Supplementary Data. Most markers were more often positive in the primary tumour than in the corresponding lymph node metastases, but K5/6 was more often positive in lymph node metastases (46 %) than in the corresponding primary tumour (19 %) ($p<0.001$).

Vimentin was concordant in primary and metastatic tumour tissues in 53 cases, positive only in primary BC in 30 cases and only in LN in 16 cases. Vimentin expression in LN metastases was related to early BC death (<5 years) ($p=0.02$).

Table 2 Immunohistochemistry-positive results for BC primary tumours and corresponding loco regional metastasis ($N=100$ cases)

Markers	Primary tumour	Node metastases	*p* value
ER	73 %	77 %	0.1
PR	61 %	70 %	0.3
HER-2	33 %	24 %	0.04
Ki67≥14 %	66 %	39 %	0.0003
K8	89 %	59 %	<0.001
K5/6	19 %	46 %	<0.001
Vimentin	44 %	30 %	0.04

Relationship of markers with clinical and pathological features

The relationship between marker expression and clinical pathological features was evaluated for both primary tumours and corresponding metastatic nodes. Only significant associations are reported here.

ER- and PR-negative primary tumours were of higher histological tumour grade ($p=0.004$ for ER, $p=0.06$ for PR). This result was also confirmed for lymph node metastases ($p=0.002$ and $p=0.05$ for ER and PR, respectively). Less differentiated primary tumours were mainly HER-2-positive and Ki67 high ($p=0.04$ and $p=0.02$ respectively). HER-2-positive metastases were also more often less differentiated ($p=0.001$). Patients with higher tumour grade had frequently K5/6-positive lymph node metastases ($p=0.009$), but not primary tumours ($p=0.2$). Absence of K8 expression in lymph node metastases was significantly associated with the development of recurrence ($p=0.04$).

Molecular classification

Based on IHC results, BC primary tumours were classified as follows: 28 luminal A, 45 luminal B, 14 HER-2+ and 13 TN subtypes. There were no significant differences in age at diagnosis between molecular subtypes ($p=0.2$), even for women of less than 40 years ($p=0.6$). Molecular subtypes were concordant between primary BC and LN in 54 patients (54 %). In non-concordant cases (46 cases), about 60 % (28 cases) LN metastases were of a less aggressive subtype, as shown in Table 3. IHC staining for each marker in primary tumours and corresponding lymph node metastases is reported in detail in Supplementary File 1 and in Supplementary Results.

Table 3 Schematic representation of molecular classification in primary tumour and metastatic lymph nodes

Primary tumour → Metastatic LN ↓	Luminal A (28)	Luminal B (45)	TN/basal (13)	Her2+ non-luminal (14)
Luminal A	18	21	0	0
Luminal B	8	24	2	4
TN/Basal	1	0	8[b]	1
Her2+ non-luminal	0	0	1	8
Unclassified	1[a]	0	2[c]	1[d]

In the first row, classification refers to primary BC; in brackets, the number of cases is shown. In the columns, the number of cases related to the specific molecular subtype detected at the LN level is reported

[a] The case was positive for PR only

[b] Six LN were classified as basal and two as TN

[c] One cases was PR+, ER−, HER-2+, K5/6+ and K8− and the other one was PR+, ER−, HER-2−, K5/6+ and K8−

[d] This case was unclassified because it is not evaluable

Survival analysis

At the end of follow-up, 21 women were alive, 9 died of any cause different from BC and 61 patients died from breast carcinoma. Nine patients were lost to follow-up.

K8 expression in loco-regional metastasis was significantly associated with BC-specific survival in the entire cohort of patients. K8 expression in lymph node metastasis was associated with longer survival ($p<0.001$), as shown in Fig. 1. Conversely, expression of K8 in the primary tumour did not significantly affect BC-specific survival ($p=0.5$), (Supplementary Fig. 1). Of note, 33 patients lost expression of K8 in loco-regional metastases, while the primary tumour was positive, and 27 of those died of BC ($p<0.001$) (Fig. 2). Survival analysis according to K8 expression in LN metastases stratified per molecular subtype showed that K8-positive LN metastasis is significantly associated with BC-specific survival only in patients with luminal subtype ($p=0.03$ for luminal A and $p=0.0005$ for luminal B, $p<0.001$ for luminal A and B together), but not in HER-2+ and TN subtypes ($p=0.2$ and $p=0.9$, respectively) as shown in Fig. 2. The hazard ratio for BC-specific death in patients with K8-negative loco-regional metastasis overall was 4.4 times as high as in patients with K8-positive LN metastases ($p=0.001$), and in patients with luminal primary BC, this was even 5.2 (95 % confidence interval 1.52–20.0; $p=0.003$). Survival for patients with K8-positive LN metastases compared to those negative was 75 vs 48 % at 5 years, 62 vs 22 % at 10 years and 53 vs 14 % at 20 years. A representative image of K8 staining in primary BC and synchronous lymph node metastasis is shown in Fig. 3. Loss of expression in lymph node metastases of keratin 18, functionally related to K8, was also related to worse prognosis and cancer-specific death (data not shown).

Cox regression analysis confirmed the result on K8 expression obtained by Log-rank test. Multivariate survival analysis showed a significantly worse prognosis only for the number of positive lymph nodes (more than three positive) ($p=0.03$) with a protective effect for LN metastases expressing K8 ($p<0.001$), as shown in Table 4. Tumour grade, size and histological type did not influence patient survival because of the population selected for our study. Post-estimation test for Cox regression revealed that the proportional-hazard assumption has not been violated. No significant differences in overall survival were detected for the other biomarkers.

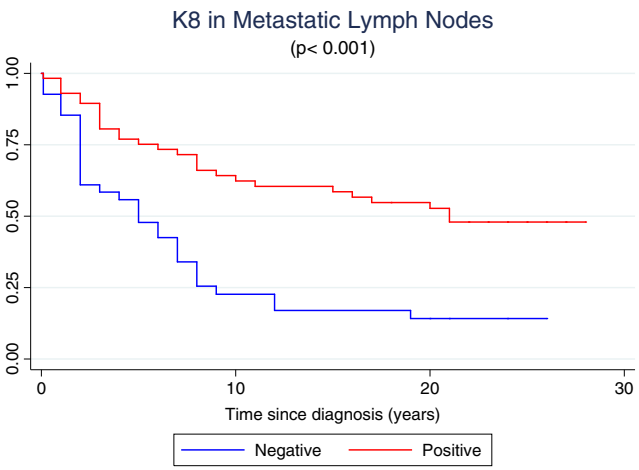

Fig. 1 Kaplan-Meyer overall survival curves according to K8 expression in axillary metastatic LN

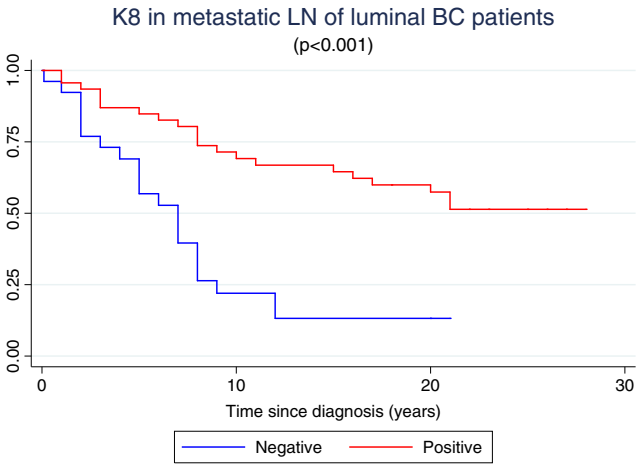

Fig. 2 Kaplan-Meyer overall survival curves according to K8 in axillary metastatic LN in patients with primary luminal breast cancers

Discussion

Axillary lymph node status (LN) and ER status are the most important prognostic variables for breast cancer patients. In this study, we analysed 100 BC patients aged 55 years or less with involvement of loco-regional axillary lymph nodes at initial diagnosis.

Concordance of BC subtypes based on molecular classification was found in most cases (54 %), in agreement with others [21]. Incongruity between primary BC and synchronous metastatic LN cannot be ascribed to pre-analytical conditions because both specimens were collected during the same surgical intervention, prior to any therapy and processed simultaneously with the same workflow and methodology. All specimens were conventionally fixed in 10 % neutral-buffered formalin for a maximum fixation time of 48 h including pre-fixation time in formalin.

In non-concordant cases (46 %), the molecular subtype of the LN metastasis was mostly less aggressive, highlighting heterogeneity in tumour progression. In very few cases (as shown in supplementary results), acquisition of ER or PR in LN metastases might be due to the use of a 10 % cut-off at the time of diagnosis. Discordance in ER, PR and HER2 status between primary tumour and metastatic lesions was reported before, including acquisition of the biomarker in

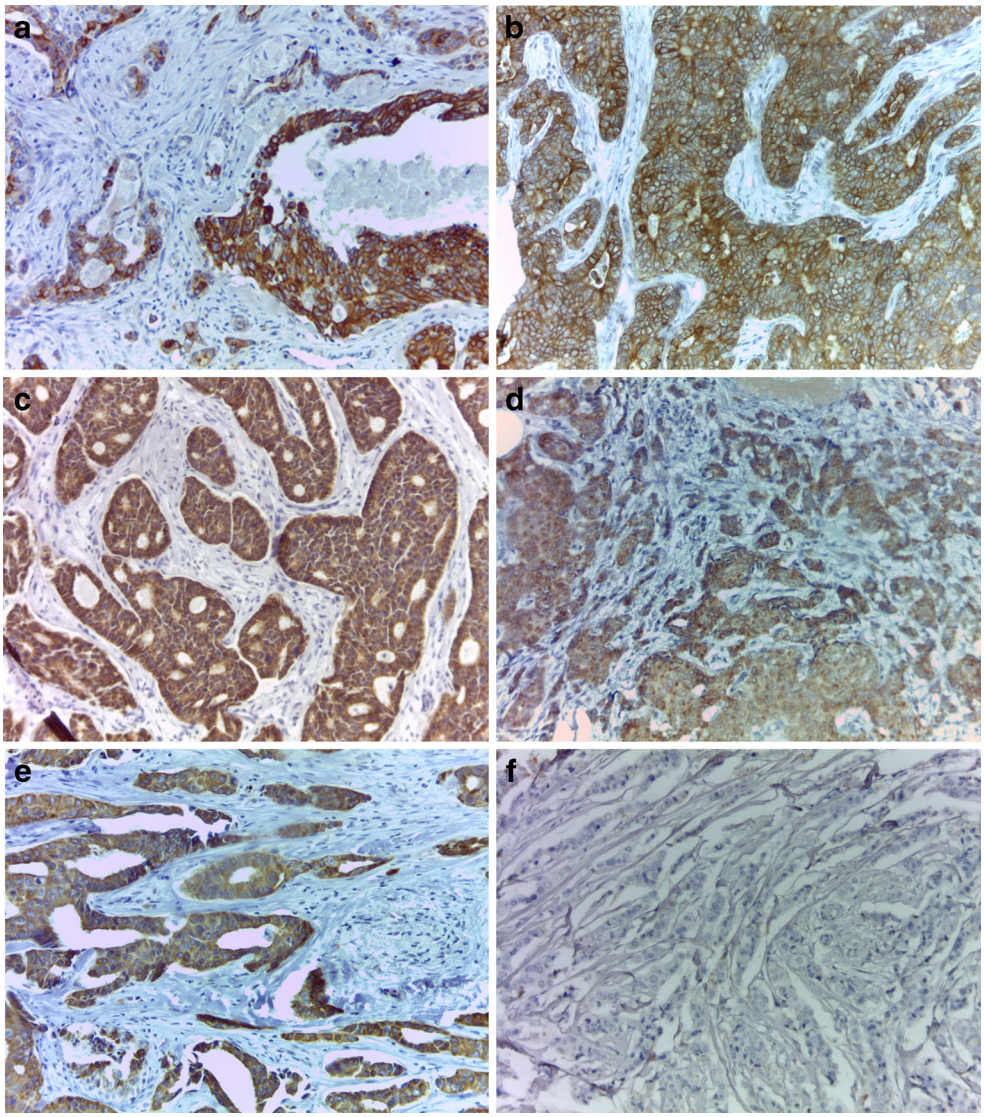

Fig. 3 Immunohistochemical stain for K8 in primary breast cancers (**a**, **c**, **e**) and paired synchronous metastatic lymph nodes (**b**, **d**, **f**). Magnification × 20. **a**, **b** and **c**, **d** refer to K8 positivity in both primary BCs and metastatic LN, while **e**–**f** refer to loss of K8 at the LN level

LN metastases [41, 42] as a consequence of tumour heterogeneity. Synchronous axillary lymph node metastases, even though local, may contain potentially distant metastatic breast cancer cells. Hence, determination of hormone receptor and HER2 status in synchronous axillary nodal metastasis, along with that of the primary tumour, may help to guide therapy and to evaluate the risk of recurrence of lymph node metastatic primary invasive breast cancer [42].

Other authors reported a lower frequency of subtype discordance mostly with a shift towards a more aggressive subtype [22], especially in luminal A patients. Variation between primary tumour and metastasis might be explained by a variety of factors ranging from clonal selection during the metastatic process [43] to phenotypic plasticity related to a new microenvironment in lymphatic tissue [44]. Concordant expression of IHC markers in primary and node metastatic sites might reflect metastatic cells utilizing gene sets similar to those in the primary tumour. Discordant expression might reflect metastatic cells using a distinctly different set of genes [45]. Breast cancer tissue contains molecularly heterogeneous cell populations [46]. Consequently, it is not surprising that the IHC profile of the primary tumour and its lymph node metastases is not always identical. Metastatic tumour deposits are not exact morphological or a molecular replicas of the primary neoplasms from which they arose; as a consequence, metastatic tumours at different sites may display widely disparate features [47].

In our study, the expression of five out of seven markers was significantly different (HER-2, Ki67, K8, K5/6, vimentin). Ki67, HER2 and vimentin were less expressed in node metastases than in primary tumours, suggesting less aggressive metastatic clones. Loss of HER2 amplification in synchronous lymph node metastases with prognostic implications has been reported previously [41]. The Ki67 labelling index in LN metastases was significantly lower than in matched primary tumours (data not shown), in agreement with

Table 4 Cox multivariate regression analysis for hazard ratio in breast cancer survival

Variable	HR	95 % confidence interval	*p* value
Histologic type			
Lobular vs ductal	1.34	0.58–3.1	0.5
Histological grade[a]	1.07	0.64–1.78	0.8
Tumour size[b]	0.89	0.56–1.43	0.6
Number of positive nodes[c]	1.83	1.04–3.23	0.04
Age at diagnosis[d]	1.02	0.98–1.08	0.2
K8 in metastatic LN[e]	0.40	0.23–0.72	0.002
Molecular subtypes			
Luminal B vs luminal A	1.04	0.59–1.98	0.9
HER2+ vs luminal A	2.55	1.02–6.32	0.04
TN vs luminal A	1.87	0.74–4.75	0.2

[a] Grade of the tumour was a continuous variable

[b] Tumour size was included as categorical variable as follows: 1 and ≤2 cm, 2 and >2 cm, or ≤5 cm, and 3 and >5 cm

[c] Number of positive lymph nodes: ≤3 or >3

[d] Age at diagnosis was a continuous variable

[e] Dichotomized in negative or positive

Cabibi et al. [20]. Other authors reported significant increase in Ki67 labelling index in metastases as compared with primary tumours [48]. However, case study selection and cut-off criteria could account for these discrepancies.

K8 was less frequently expressed in metastases, while only of K5/6, the expression rate in loco-regional metastatic lymph nodes increased, but without any impact on survival. We hypothesise that as a result of clonal selection, tumour cells with basal/myoepithelial characteristics more easily colonise lymph nodes. K5/6 expression in primary BC has been associated with a higher number of metastatic lymph nodes [49]. K5/6-positive BC has a poor prognosis, also in TN patients [49], but to our knowledge, there are no reports on K8 and/or K5/6 expression in LN metastases of BC. A single study [50] reports decreased expression only of K5 in metastatic LN.

Vimentin, a marker of mesenchymal phenotype, is more frequently expressed in lymph node metastases than in matched primary BC [23]. We found vimentin expression more often in primary BC than in corresponding LN metastases. This disagreement might be ascribed to differences in scoring, as in the earlier study, low expressing and negative cases were put together in a single group [23]. Biomarker expression in loco-regional lymph nodes might not have the same prognostic value as their expression in the primary tumour. Our findings are in agreement with those reported by Cummings et al. [47] who proposed that treatment decisions should not be based only on morphological features of the primary tumour, as key biological attributes of metastases may significantly affect disease outcome [47].

Although in our cohort, K8 expression in primary tumours did not have any impact on prognosis (Supplementary Fig. 1), K8 expression in lymph node metastasis was predictive for better survival. Conversely, most patients (27 out of 33) with a primary tumour expressing K8 but with negative loco-regional LN metastases died of BC. K8 expression status affected survival only in luminal BC patients in our cohort. This cannot be explained as an effect of therapy, because as a rule, patients with luminal BC derive less benefit from chemotherapy [51]. All our ER+ patients received tamoxifen and adjuvant chemotherapy, and those with loss of K8 expression in lymph node metastases died of BC within 10 years. This finding might have implications for treatment of this sub-group of BC patients, for whom conventional chemotherapy does not prevent late recurrence [52].

IHC staining for K8 LN metastases allows to identify a sub-group of lymphnode-positive luminal BC patients with highly unfavourable outcome: The risk of BC-specific death among patients with K8 negative loco-regional metastases was 4.6 times as high as in patients with K8-positive LN metastases. Log-rank and multivariate analysis showed that only the number of positive lymph nodes and K8 expression in LN metastases remains as independent variables affecting survival (Table 3). This was corroborated by IHC staining of K18, functionally related to K8, of which loss of expression in lymph node metastases was associated with poor prognosis and cancer-specific death (data not shown). Loss of K8 expression in LN metastases might identify less differentiated and more aggressive tumour cells. While responsible mechanisms are unclear, a possibility is K8 function as plasminogen receptor on the cell surface of BC cells, which has been associated with increased invasiveness in vitro and in vivo [53]. Other experiments showed reduced expression of K8 along progression of human breast cancer into an invasive phenotype [54]. Furthermore, Iyer et al. reported that K8 expression is correlation with a less invasive phenotype, while loss of K8 characterises highly invasive dedifferentiated cancers [18].

A limitation of our study is that it was performed on TMAs, which might not reflect tumour heterogeneity in full. To overcome this limitation, multiple areas were selected and included in the TMA. An additional limitation might be our case selection criteria: patient age, stage of disease and LN-positive status. Our conclusions indeed apply only to LN-positive (excluding micro- or very small LN metastases) stage II or III young (55 years or less at diagnosis) breast cancer patients.

Our data strongly support the hypothesis that biomarkers can have a different biological meaning in metastases: Markers useful for prognosis in primary tumours such as Ki67 might be less or not at all informative in lymph node metastases.

Conclusions

Loss or absence of expression of keratin 8 in lymph node metastases appears to define a sub-group of young (less than 55 years) stage II or III patients with a luminal type of breast cancer, with a more aggressive clinical course. Biomarker expression patterns in primary tumours might not have the same meaning as those in metastatic lesions, which emphasises the need to include metastatic tissue in IHC marker studies. Larger validation studies are needed to confirm our findings, but if confirmed, K8 expression might be used for prognosis and therapy in lymph node-positive luminal breast cancer patients.

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Conflict of interests None declared

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