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Stem cell-related markers in primary breast cancers and associated metastatic lesions

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Abstract

It has been reported previously that: 1) normal breast epithelial cells that are CD24-/44+ express higher levels of stem/progenitor cell associated genes; 2) cancer cells that have undergone epithelial to mesenchymal transition display CD24-/44+ cell surface expression, a marker for breast cancer stem cells; 3) loss of E-cadherin is a preliminary step in epithelial to mesenchymal transition; and 4) vimentin is a marker of mesenchymal phenotype.

We hypothesized that stem cell subpopulations would be more frequent in metastatic than in primary tumors. Therefore we assessed by immunohistochemical analysis, tissue microarrays containing tissue from primary and associated metastatic breast cancers for expression of CD24, CD44, E-cadherin and vimentin to evaluate candidate cancer-initiating cell populations in breast cancer subtypes and metastatic lesions. The occurrence of CD24-/44+ and CD24+/44- cells did not differ in primary vs matched lymph node or distant and locoregional metastatic lesions; E-cadherin expression was decreased in primary vs lymph node metastases (P=0.018) but not decreased in distant and locoregional metastases relative to primary tumor, while vimentin, was more frequently expressed in lymph node and distant and locoregional metastases (P=0.013, P=0.004) than in matched primary cancers. Thus, the frequency of CD24-/44+ cells does not differ in metastases relative to the primary breast cancer but differs by tumor stage and subtype.

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Keywords

Breast cancer subtypes; CD44; CD24; Vimentin; E-cadherin; Metastases

Introduction

CD24-/44+ cell surface expression has been proposed as a marker for breast cancer stem cells [1-4]. Normal breast epithelial cells that are CD24-/44+ express higher levels of stem/progenitor cell associated genes and cancer cells that have undergone epithelial to mesenchymal transition reportedly display the CD24-/44+ phenotype [5]. Epithelial to mesenchymal transition is a morphogenetic program essential for embryonic development and wound healing, but can adversely cause fibrosis and metastatic cancer progression when deregulated [2]; Vimentin is a marker of mesenchymal phenotype [6]. The dissolution of the E-cadherin mediated adherens junction is a key preliminary step in epithelial to mesenchymal transition and may occur early or late in the growing epithelial tumor as a first step toward stromal invasion, intravasation, extravasation and distant metastasis [7].

Human breast tumors are genetically heterogeneous, consisting of phenotypically diverse cells. Breast cancer cells with a CD24-/44+ cell population have been suggested to have tumor-initiating properties with stem cell-like and invasive features, though it is unclear whether their presence within a tumor has clinical implications [3,4]. Honeth *et al* [8] assessed the prevalence of cells with different CD24/44 phenotypes within breast cancer subtypes by double-stain immunohistochemistry to quantify CD44 and CD24 expression in 240 human breast tumors. The CD24-/44+ cell population was most common in the basal-like subgroup, and particularly common in BRCA1-mutated familial tumors, of which 94% included CD24-/44+ cells. The CD24-/44+ cells were rare in HER2 overexpressing (HER2+) tumors, which had a predominantly CD24+ status. The fact that not all basal-like tumors and very few HER2+ tumors contain CD24-/44+ cells, suggests that tumor-initiating properties are not wholly confined to CD24-/44+ cells and other markers remain to be identified [8].

Park *et al* [9] evaluated expression of stem cell-related markers in breast cancers of all subtypes and histological stages by immunohistochemical analyses of 12 proteins, including CD44, CD24 and vimentin. CD44 expression was lower in invasive compared to *in situ* tumors, especially in luminal A subtype cancers. CD24-/44+ cells were detected in 69% of all tumors, with 100% of the basal-like and 52% of HER2+ tumors containing these cells, results quite different from those reported by Honeth *et al* [8].

Horiguchi *et al* [10] investigated the significance of CD24 and CD44 expression for predicting responses to chemotherapy and prognosis in primary breast cancer patients receiving neoadjuvant chemotherapy. These authors reported a significant correlation between CD24 expression and response to chemotherapy, whereas CD44 expression was correlated with prognosis. Thus, these authors suggested that CD24 and CD44 expressing cells may serve as predictive and prognostic factors, respectively.

To examine the significance of CD44, CD24, vimentin and E-cadherin expression and correlations among these markers, in light of differing reported results, we evaluated the expression of these markers in two well-characterized, separate cohorts of primary breast cancers and their associated axillary lymph node metastases and distant and locoregional metastases on tissue microarrays with linked immunohistochemical expression data obtained previously, clinical features and patient outcome. We hypothesized that the frequency of CD24-/44+ cells and of vimentin positive cells would be higher in the lymph node and distant and locoregional metastases relative to the primary breast cancer from the same

patient. We also expected that we would find more frequent expression of CD24-/44+ and CD24+/44+ in triple negative cancers, based on a previous report [8].

Materials and Methods

Breast cancer tissue microarrays

The studies of human subjects were approved by the Ohio State University Institutional Review Board and the Hacettepe University Ethics Committee. All tissues were anonymized by removal of identifiers and assignment of random numbers before supplying slides, blocks and linked clinical information to investigators, as described previously [11]. Selection criteria for the first tissue microarray included invasive breast cancers treated prior to 1998, for which clinical information, including stage, grade, histological type, treatment, site of first recurrence and disease-free and overall survival were available; for 303 primary breast cancers there was tissue for primary tumor only, 226 with tumor and axillary lymph nodes, and 35 with only lymph node tissue with metastatic lesion. Cores (0.6 mm) from each tumor and metastasis were placed in quadruplicate blocks, with 50 assorted control tissues, including normal breast tissue. The second tissue microarray was prepared from breast cancer cases that were diagnosed between 1984 and 2008, at Hacettepe University; 69 primary breast cancers and their associated isolated chest wall recurrences after mastectomy (n=28, 41%) or distant and locoregional metastases (n=41, 59%) available in the pathology archive were selected. Tissue cores (1 mm) from both primary blocks and blocks with metastatic lesions were punched from each donor paraffin block using an advanced tissue arrayer (Chemicon – Advanced Tissue Arrayer-ATA100). Each lesion was represented in duplicate, one core from central and one from the peripheral area of the tumor, on the tissue microarray.

Immunohistochemical analysis

Tissues were sectioned at 4 μm, sections placed on positively charged slides, and deparaffinized and rehydrated through xylenes and graded alcohols. Prior to antigen retrieval, slides were blocked for endogenous peroxidase in 3% H_2O_2 for 5 min. Antigen retrieval was performed in a vegetable steamer with Dako citrate buffer for 25 min, followed by cooling for 15 min. Slides were then placed on a Dako Autostainer immunostaining system. Primary antisera were diluted as in Table 1 and placed on tissue sections for 30 min at room temperature. Detection systems used are also listed in Table 1. Slides were counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions and coverslipped.

CD24 and CD44 expression was determined by double staining of tumor cells, as described by Honeth *et al* [8]; the first primary antibody, to CD24, was incubated for 30 min at room temperature. DAB chromogen was used to develop CD24, producing a brown precipitate. Before the second primary antibody was applied, serum-free protein block was added (Dako code X0909) to minimize background and crossover between primary antibodies. CD44 antibody was then incubated on slides for 60 min at room temperature. Vulcan Fast Red was used to develop CD44, producing a bright fuchsia precipitate so that the two primary antibodies could be easily differentiated. Also according to Honeth *et al* [8], the proportion of CD24-/CD44+ tumor cells was determined as the percentage of cells negative for DAB staining but positive for Vulcan Fast Red staining. The frequencies of CD24+/CD44- cells and of CD24+/CD44+ cells were determined similarly. These proportions were defined as presence or absence of CD24-/CD44+, CD24+/CD44-, CD24+/CD44+ phenotypes for statistical purposes. In this situation for example CD24-/44+ variable includes a range from only a few cells to almost all tumor cells showing no staining with DAB (for CD24) but

positive staining with permanent red (for CD44). CD44 staining was membranous, CD24 cytoplasmic.

Expression of vimentin and E-cadherin was scored as follows: sections were scored from 0-3 where "0" corresponds to lack of positive staining and "3" represents the most intense staining. Scores were calculated as follows: average intensity of the stain (1-3) × average percentage of positive cells. Therefore, a section with intensity 1 and 50% positive cells would have a score of 1 × 50 = 50. Cases were then divided into four scores as follows: 0: negative, 1-100: low expressors, 101-200: moderate expressors, 201-300: high expressors. These scores were converted into a two-scale as follows: 0: negative; 1: 1-300, positive; though tumors were grouped as non-expressors *vs* expressors for some statistical modeling. Final scores represent averages of two scores for both tissue microarrays. E-cadherin staining was membranous and vimentin cytosolic and often very strongly perinuclear. For CD24/44 stains, we used only percentage of cells stained in scoring and grouped them as negative or positive.

Slides were scored independently by two pathologists (SC, CI) blinded to breast cancer subtype; two pathologists (GG, SB) converted scores to numbers, selected cut off values for each marker and entered data into Excel and SPSS files. The 423 breast cancers that could be scored were divided into subtypes of breast cancer as defined by their protein expression profiles using designated stains, as described previously [11].

Statistical Analysis

Not all marker or clinical data were available on all subjects, and percentages refer to cases for which data for a specific variable were available. Associations between categorical variables (eg. marker score data, menopausal status, ER status) were evaluated using chi-square or Fisher exact tests. McNemar tests were used to analyze concordance in marker expression in tumor tissue and matching metastatic tissue. Mann Whitney U test and Kruskal Wallis test were used to compare scores for expression of proteins involved in DNA-damage response [11]. The results of DNA damage response scores are given as Median ± Interquartile Range. Relationships of marker expression and clinical features were evaluated in relation to disease subtype using univariate and multivariate logistic regression models.

Disease-free survival was assessed from the time patients were disease-free to date of recurrence and/or death. Patients with metastases at diagnosis were excluded from disease-free survival analyses and only subjects with stage I-III tumors at diagnosis were included. Kaplan-Meier and Cox regression models were used to evaluate disease-free survival, where differences in distributions were evaluated based on clinical characteristics and marker expression. The p-values reported in relation to disease-free survival correspond to log rank tests unless otherwise noted.

Results

Breast cancer characteristics

The characteristics of cases included in the first tissue microarray were previously described (see Table 2 of [11]). Due to missing HER2 status, subtype could be determined in only 302 cases. Most of these cases were classified as to subtype (also referred to as gene class or gen-class). These include luminal A (ER and/or PR positive, ErbB2 negative); luminal B (ER and/or PR positive, ErbB2 positive); ErbB2+++ (ER and PR negative, ErbB2 IHC/FISH +++); basal-like TN (ER, PR, ErbB2 negative and CK5/6 and/or EGFR positive); and TN non-basal (ER, PR, ErbB2, CK5/6, EGFR negative). According to this classification, the ratios were as follows: luminal A (60%, 179/302); 15% (47/302) were luminal B; 10%

(29/302) HER2+; and 15% (47/302) triple negative. Among the 47 triple negative cases, CK5/6 and EGFR expression scores were available in all but 4. Of the remaining 43, 33 (77%) showed a basal-like phenotype as determined by EGFR and CK5/6 staining. The tissue microarray analyses included 109 subjects with both tumor tissue and metastatic tissue; 96% of metastatic tissues were from lymph nodes. The second tissue microarray was prepared from 69 cases that were diagnosed between 1984 and 2008, at Hacettepe University, which had samples of both primary and distant and locoregional metastatic tumor in pathology archive. All patients were treated with mastectomy; of the 28 locoregional recurrences (41%), most (22, 78%) were chest wall recurrences, 4 were supraclavicular, 2 were in ipsilateral axillary lymph nodes. Almost all patients with isolated chest recurrence after mastectomy will develop distant metastases [12]. The sites of distant metastases were as follows: 9 (13%) bone, 4 (6%) liver, 4 (6%) lung, 1 (1%) brain, 6 (9%) serous membranes, 9 (13%) soft tissue, 3 (4%) bone marrow, 1 (1%) stomach, 3 (4%) ovary, 1 (1%) colon. Patients were between 30-90 years of age (mean 46.6). Primary tumors were diagnosed as: 52 (75%) invasive ductal carcinoma; 9 (13%) invasive lobular carcinoma; 6 (9%) mixed (ductal+lobular) carcinoma; 1 (1%) mucinous; 1 (1%) tubular carcinoma. The data for the two tissue microarrays were analyzed separately.

Correlation of CD24/44 subpopulations with breast cancer subtype, clinical features and patient outcome

The proteins assessed in this study, E-cadherin, vimentin, CD44 and CD24, were examined for correlations of expression with proteins previously assessed [11], as well as for correlations with breast cancer subtypes and other clinical features (see Table 2 and supplementary Table 1**).** As expected [13-15], E-cadherin was expressed significantly less frequently in invasive lobular cancers, and more frequently in PR negative cases. When the lobular and mixed invasive tumors with a lobular component were excluded from the analysis, all the significant associations remained the same. Negative/very low E-cadherin expression was observed in only 4% and 9% of triple negative non-basal and basal-like tumors (see Supplementary Table 1). Vimentin expression was observed significantly more frequently in ER-, PR-, and basal-like tumors (P=0.002, P=0.004, P=0.009, respectively) (Supplementary Table 1). Vimentin expression was related to breast cancer subtype (P=0.004,) as follows: high vimentin expression is observed in 25% of luminal A, 16% of luminal B, 45% of HER2+, 50% of basal and 33% of non basal triple negative (P=0.004).

Overall the CD24+/44- subpopulation was observed in 29% of breast cancers, CD24-/44+ in 18%, CD24+/44+ in 16% of cases; 45% of cases showed none of the subpopulations. 13% showed two subpopulations, 2% three subpopulations. CD24-/44+ cells were present in 15% of luminal A, 19% of luminal B, 38% of HER2+, 45% of basal and 25% of non-basal triple negative (P=0.001, see Table 2), whereas CD24+/44- cells were found in 30% of luminal A, 48% of luminal B, 41% of HER2+, 27% of basal-like and 25% of non-basal triple negative cancers. The distribution of CD24+/44- did not show significant differences among the various breast cancer subtypes. In multivariate analysis the presence of CD24-/44+ cells was an independent factor for the basal phenotype.

Grade (P<0.001), stage (P<0.001), ER (P<0.001), PR (P<0.001), HER2 overexpression (P=0.002), triple negative (P<0.001) and basal status (P=0.007), breast cancer subtype (P<0.001), EGFR (P=0.007), CD24+/44+ (P=0.05) expressing cells were associated with disease-free survival in univariate analysis. In multivariate analysis of clinical features, only stage was independently related with disease-free survival, as shown previously [11], whereas the presence of CD24-/44+ and CD24+/44+ tumors was not independently associated with disease-free survival. In logistic regression, the presence of CD24-/44+ cells was independently related with basal phenotype (not shown).

Associations of protein expression with lymph node and distant and locoregional metastasis

The presence of subpopulations of CD24+/44- and CD24-/44+ cells did not differ in the primary vs lymph node metastases (P=1.000 and P=0.845 for the CD24+/44- and CD24-/44+, respectively) and primary vs distant and locoregional metastases (P=0.180 and P=0.625, for the CD24+/44- and CD24-/44+, respectively) (Table 3). After excluding the locoregional recurrences after mastectomy, the results remained the same. Vimentin was expressed more frequently in lymph node metastatic lesions (P=0.013) and distant and locoregional metastases (P=0.004) vs the primary site. E-cadherin expression was lower in lymph node metastases (P=0.018), but did not differ in distant and locoregional metastases relative to primary tumor. Even after excluding locoregional recurrences, vimentin remained higher in distant and locoregional metastases and E-cadherin and CD24/44 status was not statistically significantly different (see Table 3 for summary of these results and Figure 1 for examples of immunohistochemical detection of protein expression).

Discussion

The purpose of this study was to determine if stem cell-like and mesenchymal cell properties, as determined by the phenotypic expression of CD24-/44+ cells, E-cadherin and vimentin differed in the primary tumor and the metastatic lesions from the same patients. Contrary to our primary hypothesis, the expression of CD24-/44+ cells did not differ in these sites. However, E-cadherin and vimentin expression differed in these sites with increased expression of the mesenchymal marker, vimentin, found in lymph node and distant and locoregional metastases relative to the primary tumor. Vimentin expression was previously reported to be similar in primary and distant metastases [16], suggesting the need for further comparative studies of this marker. It will be interesting in the future to examine the expression of other proteins, more recently defined as stem cell markers or epithelial to mesenchymal transition markers, in primary and metastatic cancers to determine if they might be enriched in metastatic lesions or in primary cancers associated with metastatic lesions. Cimino-Matthews et al [17] have recently shown that the epithelial cell adhesion molecule, EpCAM is over-expressed in breast cancer metastases. We did find that CD24-/CD44+ subpopulations were increased in basal-like, triple negative cancers in accord with the previous study [8]. The lack of greater expression of cancer stem cell markers in metastatic sites does not necessarily contradict the importance of cancer stem cells as the tumor initiating cells; stem cells at metastatic sites can also give rise to differentiated progeny. Chemotherapy treatment may selectively affect numbers of stem cells and most cases in our study groups were treated with multiple chemotherapeutic agents. However, the data, particularly for cases with distant metastases, is old and chemotherapy regiments were very heterogeneous. Also the time between chemotherapy treatment and recurrences were not uniform. Thus, we cannot estimate the effect of chemotherapy stress on the numbers of cancer stem cells found in our cases.

Epithelial to mesenchymal transition is necessary in many physiological events such as embryogenesis, wound healing and cell migration. It is a rapid and often reversible cell phenotype change, in which epithelial cells are detached from each other by loss of their cell-to-cell adhesion structures and by adjusting cell polarity and then by changing the intermediate filaments of cytoskeleton, mainly from keratins to vimentin. The loss of E-cadherin and subsequent alteration of the adherens junction is a key preliminary step in epithelial to mesenchymal transition [18]. E-cadherin expression is lost in the lobular breast cancer phenotype [14,15]. In addition, prior studies show that E-cadherin expression is lost in other breast cancer subtypes and is associated with poor prognosis [19].

E-cadherin was present in only 9% of basal-like triple negative tumors in the current study, suggesting a possible role for E-cadherin loss in the development of these tumors. We found that E-cadherin expression loss, relative to the matched primary tumor, was significantly more frequent in lymph node but not in distant and locoregional metastases. As E-cadherin loss is an early event during epithelial to mesenchymal transition, but is reversible, the genetic control mechanisms that keep E-cadherin repressed may operate differently in lymph node and distant metastases. Vimentin is a marker of mesenchymal differentiation and its expression has been observed previously in triple negative and basal breast tumors [20]. We confirmed high vimentin expression in ER-, PR- and basal tumors and found that vimentin expression is significantly more frequent in matched lymph node and distant and locoregional metastases compared to primary tumors. This result suggests that up-regulation of vimentin expression is a nonreversible change associated with epithelial to mesenchymal transition, accompanying progression of breast cancer.

CD24-/44+ tumor cells or aldehyde dehydrogenase 1 positive tumor cells are considered cancer stem cells that possess the properties of self-renewal and tumorigenicity. The occurrence of the CD24-/44+ phenotype was reportedly high in basal-like tumors and lower in tumors of luminal subtype and particularly low in the HER2+ tumors [21]. As expected, the results of this study confirm that CD24-/44+ expressing cells were more frequent in basal tumors and along with age, the presence of CD24-/44+ cells was an independent factor associated with basal phenotype. However, there was not an increased frequency of CD24-/44+ expressing cells in metastatic lesions relative to the primary cancers, nor was presence of these cells an independent predictor of disease-free survival. It has been suggested that hematogenous spread is more likely in triple negative tumors, as visceral metastases are seen more commonly [22]. However the incidence of lymph node metastasis is reported to be not less frequent than for other breast cancer subtypes in current studies [23]. Our study shows that lymph node and distant and locoregional metastases are not enriched for breast cancer stem cells. These results suggest that stem cell pools in individual primary breast tumors may be related to the biology of the breast cancer subtypes and remain unaltered in metastatic lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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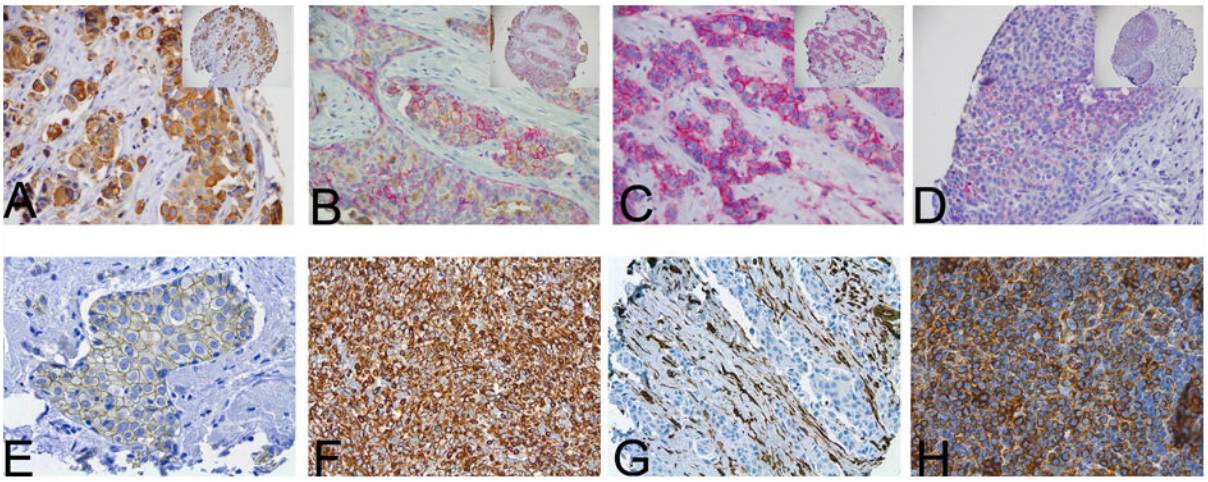

Figure 1. Examples of immunohistochemical detection of protein expression

(**A**) Primary tumor, cytoplasmic CD24 positive; (**B**) Primary tumor, dual positivity of cytoplasmic CD24 and membranous CD44; (**C**) Primary tumor, strong membranous CD44 positivity; (**D**) Primary tumor, weak membranous CD44 positivity; (**E**) Primary tumor, membranous E-cadherin positivity; (**F**) Primary tumor, strong vimentin positivity; (**G**) Primary tumor, weak vimentin positivity; (**H**) Metastatic lymph node, strong vimentin positivity (corresponding to primary tumor in G)

Table 1
Primary antisera and detection kits used in immunohistochemical studies

Antiserum	Description	Dilution	Detection Kit
Vimentin	Dako M0275	1/250	Labeled Streptavidin-Biotin Complex
E-cadherin	Dako M3612, clone NCH-38	1/100	Labeled Streptavidin-Biotin Complex
CD44	Dako mAb DF1485	1/600	Mach 3 mouse, Biocare Med
CD24	Neomarkers mAb SN3b	1/50	Mach 4 alk phosp, Biocare Med

Table 2
Characteristics of CD24-/44+ tumors

	All tumors	No CD24-/44+ cell present	At least 1 cell CD24-/44+	*P*
Grade				
I	25 (7.4%)	22 (8.4%)	3 (4%)	0.005
II	152 (45%)	128 (48.7%)	24 (32%)	
III	161 (47.6%)	113 (43%)	48 (64%)	
Recurrence				
No, unknown	312 (84.8%)	254 (87%)	58 (76.3%)	0.021
Yes	56 (15.2%)	38 (13%)	18 (23.7%)	
ER				
Negative	94 (26.3%)	61 (21.5%)	33 (44.6%)	<0.001
Positive	264 (73.7%)	223 (78.5%)	41 (55.4%)	
PR				
Negative	119 (33.7%)	83 (29.9%)	36 (48%)	0.003
Positive	234 (66.3%)	195 (70.1%)	39 (52.0%)	
Triple Negative				
No	293 (86.7%)	241 (90.3%)	52 (73.2%)	<0.001
Yes	45 (13.3%)	26 (9.7%)	19 (26.8%)	
Gene Class				
Luminal A	152 (57.6%)	129 (62.9%)	23 (39%)	0.001
Luminal B	42 (15.9%)	34 (16.6%)	8 (13.6%)	
HER2+	29 (11%)	18 (8.8%)	11 (18.6%)	
Basal	33 (12.5%)	18 (8.8%)	15 (25.4%)	
Triple negative other	8 (3%)	6 (2.9%)	2 (0.8%)	
Basal-like				
No	302 (90.1%)	248 (93.2%)	54 (78.3%)	<0.001
Yes	33 (9.9%)	18 (6.8%)	15 (21.7%)	
Vimentin				
Low	242 (68%)	200 (70.9%)	42 (56.8%)	0.020
High	114 (32%)	82 (29.1%)	32 (43.2%)	
E-cadherin				
Low	265 (74.6%)	212 (75.7%)	53 (70.7%)	0.372
High	90 (25.4%)	68 (24.3%)	22 (29.3%)	

Not associated with age, race, menopause, histotype, stage, HER2 status. In multivariate analyses of associations of markers, clinical features with basal phenotype, CD24-/44+ was significantly associated (*P*=0.026, odds ratio 2.87; 95% confidence interval for odds ratio 1.13 to 7.25).

Table 3

Expression in primary tumor vs lymph node metastases distant/locoregional metastases

Primary tumor	Expression in primary breast cancers vs lymph node metastases			Expression in primary breast cancers vs distant metastases		
	Negative in lymph node metastases	Positive in lymph node metastases	*P*	Negative in distant metastases	Positive in distant metastases	*P*
CD24-/44+						
All cells negative	58 (82.9%)	14 (63.6%)	0.845	30 (96.8%)	3 (30.0%)	0.625
At least one cell positive	12 (17.1%)	8 (36.4%)		1 (3.2%)	7 (70.0%)	
Total	70 (100.0%)	22 (100.0%)		31 (100.0%)	10 (100.0%)	
CD24+/44-						
All cells negative	35 (63.6%)	21 (56.8%)	1.000	2 (22.2%)	2 (6.3%)	0.180
At least one cell positive	20 (36.4%)	16 (43.2%)		7 (77.8%)	30 (93.8%)	
Total	55 (100.0%)	37 (100.0%)		9 (100.0%)	32 (100.0%)	
Vimentin						
Negative	4 (57.1%)	14 (14.9%)	**0.013**	16 (88.9%)	14 (51.9%)	**0.004**
Positive	3 (42.9%)	80 (85.1%)		2 (11.1%)	13 (48.1%)	
Total	7 (100.0%)	94 (100.0%)		18 (100.0%)	27 (100.0%)	
E-cadherin						
Negative	13 (34.2%)	10 (15.6%)	**0.018**	6 (66.7%)	2 (5.6%)	1.000
Positive	25 (65.8%)	54 (84.4%)		3 (33.3%)	34 (94.4%)	
Total	38 (100.0%)	64 (100.0%)		9 (100.0%)	36 (100.0%)	