Impact of CD44+CD24- cells on non-sentinel axillary lymph node metastases in sentinel node-positive breast cancer

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Abstract. Although complete axillary lymph node dissection (ALND) is the standard for evaluating axillary status after the identification of a positive sentinel lymph node (SLN) in breast cancer; approximately 40-60% of SLN-positive patients have negative non-SLN. In this study, to explore putative breast cancer stem cells with CD44+CD24- in the SLN, we retrospectively analyzed the expression of CD44+CD24- on metastatic tumor cells within SLNs as a predictive factor for positive non-SLNs (NSLNs). We tested 271 patients for SLNs using serial sectioning with cytokeratin immunohistochemistry (IHC) and hematoxylin-eosin staining and identified 67 patients who had a positive SLN biopsy and complete ALND. CD44 and CD24 expression was detected using double-staining IHC. Twenty-eight (41.8%) out of 67 patients had positive NSLN metastases. Seven positive SLNs with micrometastases were not available for the evaluation of CD24 and CD44 expression. Out of the remaining 60 patients, 19 (31.7%), 44 (73.83%) and 37 (61.7%) patients had CD24+, CD44+ and CD44+CD24- metastatic tumor cells in SLNs, respectively. Positive NSLN metastasis was significantly associated with the primary tumor size (P=0.004), CD24- expression (P=0.04), CD44+ expression (P=0.01) and CD44+CD24- expression (P=0.02). This report provides the first evidence of the existence of a putative stem-like phenotype within the SLN, which is significantly associated with positive NSLN in early breast cancer patients.

Introduction

Lymphatic dissemination is a major first route for breast cancer metastasis (1,2). Thus, lymph node involvement is the most significant predictor of outcome in breast cancer (3),

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and the lymph node status may be a pivotal factor in decisions regarding patients' treatment plans. Whether lymphatic and hematogenous spreading occurs in a synchronous or metachronous fashion remains controversial (4), experimental evidence suggests that intranodal tumor deposits can and do act to seed downstream sites within the lymph node chain and systemically (5,6). The sentinel lymph node concept is based on the principle that a primary tumor is drained by an afferent lymphatic channel that courses to the first, or 'sentinel', lymph node in that specific regional lymphatic basin (7). Although recent sentinel lymph node biopsy technique have shown that metastases start out as single cells that detach from the primary tumor and travel to the lymph nodes (8-11), few studies have examined what factors control the ability of tumor cells to survive, establish and show progressive growth in a lymph node environment (12-14).

Although complete axillary lymph node dissection (ALND) is the standard for evaluating the axillary status after the identification of a positive sentinel lymph node, approximately 40-60% of SLN-positive patients have negative non-sentinel lymph nodes (NSLN) (15). Several studies have analyzed the association of various clinicopathological features in SLN-positive breast cancers to additional NSLN metastases. Tumor size, nuclear grade, presence of lymphovascular infiltration and size of the SLN metastatic tumor were predictive factors of NSLN positivity (16-19); however, tumor cell biology remains poorly understood.

According to the cancer stem cell hypothesis, cancer stem cells defined as a subset of tumor cells with stem cell-like features, have the capacity to self renew and to differentiate (20-22). In breast cancer, cells with positive CD44 expression and low or negative CD24 expression (CD44+CD24-) have been identified as candidate breast cancer stem cells based on xenotransplant assays in non-obese/severe combined immunodeficient mice (23). The inherent properties of stem cells may impart their transformed counterparts with the ability to evade traditional antitumor therapies and to establish metastases (24-26).

Systemic dissemination is another route for breast cancer metastases, and recent data have shown a subpopulation (CD44+CD24-) of breast cancer cells in the bone marrow in early breast cancer patients (27); however, the clinical presence and implications of this subpopulation (CD44+CD24-)

of breast cancer cells in sentinel lymph nodes has not been reported.

The aim of the present study was to demonstrate the existence of a subpopulation (CD44$^+$CD24$^-$) of breast cancer cells in sentinel lymph nodes and to investigate whether these cells have an impact on non-sentinel lymph node metastases.

Materials and methods

Patients. Between September 2005 and December 2008, 271 consecutive cases of SLN biopsy at Jikei University Hospital were enrolled prospectively into the Jikei Lymph Node Database. Our study population involved 67 cases that fulfilled the following criteria: primary invasive breast carcinoma with clinically negative axilla and no prior systemic treatment; successful SLN biopsy in which metastatic disease was identified; and complete ALND with at least 10 nodes examined.

Technique for sentinel node biopsy. All the patients received a subdermal injection of ^{99m}Tc phytate colloid on the day of surgery (0.25 ml, 15 MBq) or the day before surgery (0.5 ml, 30 MBq) and a lymphoscintigraphy was performed. On the day of surgery, with the patients under general anesthesia, 5 ml of isosulfan blue dye (Lymphazurin; Covidien, Mansfield, MA) was injected peritumorally and the breast was massaged for 5 min. In patients who underwent phytate colloid injection, a handheld γ detection probe (NeoProbe; Covidien, Mansfield, MA) was used to scan the axilla transcutaneously and to identify the most radioactive area. Through an axillary incision over this hot spot, the SLNs were identified as those with blue dye uptake, radiotracer uptake or both.

Identification of sentinel lymph node metastases. The SLN was dissected and sectioned at 2- to 3-mm intervals. The nodal tissue was quickly frozen in liquid nitrogen, and a single 5-μm-thick section stained with hematoxylin and eosin (H&E) was examined intraoperatively (frozen-section analysis). If the section was positive, a complete ALND was performed immediately. After frozen-section analysis, the remaining frozen tissue was fixed in formalin and embedded in paraffin. Another 5-μm-thick H&E stained section was evaluated as a frozen section control. If this section showed no evidence of metastasis, IHC-stained sections were prepared from the paraffin block using the cytokeratin antibody CAM 5.2 (Becton-Dickinson Immunocytometry Systems, San Jose, CA). NSLNs were examined using routine single-section H&E staining. Each primary tumor was evaluated for the size of the invasive component, the histological type, the nuclear grade, the estrogen and progesterone receptor status, the HER2/neu status and the presence of lymphovascular invasion. The hormonal status was regarded as positive if >10% of the cells were stained using IHC.

Control using single-staining immunohistochemistry. Following the confirmation of lymph node metastases using H&E staining and cytokeratin IHC, immunohistochemistry procedures were performed on 3-μm tissue sections using monoclonal antibodies for the adhesion molecules CD44 (clone 156-3C11, diluted 1:300; Lab Vision Corp., Fremont,

Table I. Patients, tumor, SLNs and NSLNs characteristics.

Variable	N	(%)	Median	Range
Total no. of patients		271		
Age (years)			51.6	25-82
No. of patients with positive SLNs	67	24.7		
No. of patients who underwent complete ALND	67	100		
Size of primary tumor (mm)			24.6	0.2-70
Histological subtype				
Invasive ductal carcinoma	57	85.1		
Invasive lobular carcinoma	7	10.4		
Mucinous carcinoma	3	4.5		
Estrogen receptor positive	53	79.1		
Progesterone receptor positive	47	70.1		
HER2/neu expression positive (3+)	3	4.5		
Lymphatic infiltration positive	34	50.7		
Vessel infiltration positive	7	10.4		
No. of SLNs identified			2.2	1-4
No. of positive SLNs			1.4	1-4
Size of largest SLN metastases (mm)			5.3	2-10
No. of LNs removed			10.6	1-33
No. of positive NSLNs			1.4	0-20

SLN, sentinel lymph node; NSLN, non-sentinel lymph node; ALND, axillary lymph node dissection.

CA) and CD24 (clone SN3b, diluted 1:1000; Lab Vision Corp.). To control the reliability of the CD44 and CD24 double staining, single staining with CD44 and CD24 was also performed for consecutive tissue sections.

Immunohistochemistry using a double-staining technique. Staining for CD44 and CD24 was performed using the iView™ DAB Detection Kit and UltraView™ Universal Alkaline Phosphatase Red Detection Kit (Ventana, Tucson, AZ, USA) according to the manufacturer's instructions for Ventana automated slide stainers. The first kit uses a horseradish peroxidase conjugate and DAB substrate system that enables the visualization of CD24 protein as a brown stain. The second kit uses an alkaline-phosphatase conjugate and Fast Red substrate that stains CD44 an intense red.

Evaluation. Cells that stained red without much interference from the brown stain were identified as CD44$^+$CD24$^-$ or CD44$^+$CD24^{low}. The percentages of CD44$^+$ cells, CD24$^+$ cells and CD44$^+$CD24$^-$ or CD44$^+$CD24^{low} cells were estimated for the entire tumor area. Cell surface staining ≥10% was considered a positive result.

Statistical analyses. Descriptive statistics were used to assess the frequency distribution among the study population.
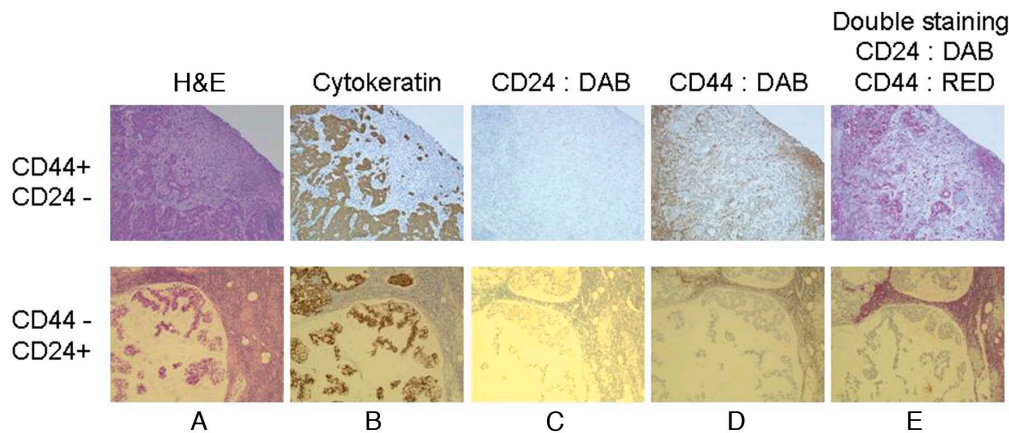
Figure 1. Immunohistochemical analyses of metastatic tumor cells in sentinel lymph node. (A) Hematoxylin and eosin staining. (B) Immunohistochemical staining for cytokeratin (brown). (C) Single staining for CD24 (brown). (D) Single staining for CD44 (brown). (E) Double staining for CD44 (red) and CD24 (brown).

Associations between the presence of positive NSLNs and various characteristics including age, primary tumor size, histological type, estrogen and progesterone receptor status, HER2/neu overexpression, nuclear grade, lymphovascular infiltration in the primary tumor, number of tumor positive SLNs, size of the SLN metastases and the expression of CD44 and CD24 were analyzed using the χ^2, Fisher exact test, and Wilcoxon rank-sum tests, with StatView® software (version 5, 1998; SAS Institute, Inc., Cary, NC). P-values of <0.05 were considered statistically significant.

Results

Descriptive characteristics of patients, tumors, SLNs and NSLNs. We evaluated 271 consecutive patients who underwent SLN biopsy for breast cancer. Of these patients, 67 (24.7%) had a positive SLN. All the patients with a positive SLN underwent a complete ALND. The descriptive characteristics of the patients, tumors, SLNs and NSLNs are listed in Table I. The median patient age was 51.6 years (range, 25-82 years). None of the patients had received prior systemic chemotherapy. The predominant primary tumor histological subtype was invasive ductal carcinoma (57 patients; 85.1%). The median tumor size was 24.6 mm (range, 0.2-70 mm). Overall, 79.1% of the tumors were positive for estrogen receptor, 70.1% were positive for progesterone receptor and 95.5% were HER2/neu negative. Lymphatic infiltration was identified in 50.7% of the tumors, and vessel infiltration was identified in 10.4% of the tumors.

The median number of SLNs identified was 2.2 (range, 1-4). The median number of positive SLNs was 1.4 (range, 1-4). The median size of the SLN metastases was 5.3 mm (range, 2-10 mm). The number of patients with additional positive NSLNs was 28 patients (41.8%). The median number of positive NSLNs was 1.4 (range, 0-20). The median number of removed lymph nodes was 10.6 (range, 1-33).

Correlation between clinicopathologic features and positive NSLNs. Twenty-eight of 67 the patients (41.8%) had positive NSLN metastases. Table II summarizes the results of statistical analyses to determine the relationship between the clinicopathologic variables and positive NSLNs. A univariate analysis revealed that the primary tumor size (P=0.004), tumor histological type (P=0.02) and number of SLNs with metastasis (P=0.03) were significantly associated with positive NSLNs. The size of the SLN metastases (P=0.08) was associated with positive NSLNs.

Identification of CD24⁺ and CD44⁺ metastatic tumor cells in SLNs and correlation between positive NSLN metastases and CD44⁺CD24⁻ cells. Seven positive SLNs with micrometastases were not available for the evaluation of CD24 and CD44 expression. Out of the remaining 60 patients, 19 (31.7%) and 44 (73.3%) exhibited CD24⁺ and CD44⁺ metastatic tumor cell in SLNs, respectively.

To investigate the combined expression of CD24 and CD44 within tumor cells, we performed a double immunohistochemical analysis (Fig. 1C). Out of the 60 patients, 37 (61.7%) patients had CD44⁺CD24⁻ metastatic tumor cells in SLNs (Fig. 2).

Table III summarizes the associations between the positive NSLNs metastases and their expressions. CD44⁺ tumor cells were detected in 61% of the negative NSLNs and 89% of the positive NSLNs. CD24⁺ tumor cells were detected in 42% of the negative NSLNs and 19% of the positive NSLNs. CD44⁺CD24⁻ stem cell-like tumor cells were detected in 48% of the negative NSLNs and 78% of the positive NSLNs. CD44⁺CD24⁻ cells were detected in 30% of the negative NSLNs and 7% of the positive NSLNs. Both CD44⁺ and CD24⁺ cells were detected in 12% of the negative NSLNs and 11% of the positive NSLNs. Both CD44⁻ and CD24⁻ cells were detected in 9% of the negative NSLNs and 37% of the positive NSLNs. Positive NSLN metastases were significantly associated with CD24⁻ expression (P=0.04), CD44⁺ expression (P=0.01) and CD44⁺CD24⁻ expression (P=0.02).

Discussion

We investigated the impact of the stem/progenitor phenotype defined by CD44 positivity and CD24 negativity in sentinel lymph nodes (SLN) on non-sentinel lymph node (NSLN) metastases.

Table II. Characteristic differences between negative NSLNs and positive NSLNs.

	Negative NSLNs			Positive NSLNs			
	No. of patiens (n=39)	(%)	Median (range)	No. of patiens (n=28)	(%)	Median (range)	P-value
Patient and tumor characteristics							
Patient age (years)			51.7 (27-78)			51.3 (25-82)	0.9[a]
Tumor size (mm)			20.3 (0.1-45)			30.6 (12-70)	0.004[a]
Tumor size (AJCC)							0.09
T1							
Tmic (≤1 mm)	1	3		0	0		
T1a (2-5 mm)	1	3		0	0		
T1b (6-10 mm)	3	8		0	0		
T1c (11-20 mm)	16	41		8	29		
T2	17	44		17	61		
T3	0	0		3	11		
Tumor type							0.02
Invasive ductal carcinoma	35	61		22	39		
Invasive lobular carcinoma	1	14		6	86		
Mucinous carcinoma	3	100		0	0		
Nuclear grade							0.4
G1	28	72		24	86		
G2	5	13		2	7		
G3	6	15		2	7		
Estrogen receptor status							0.9
Negative	8	21		6	15		
Positive	31	79		22	85		
Progesterone receptor status							0.5
Negative	13	33		7	25		
Positive	26	67		21	75		
HER2/neu expression							0.8
Score 0+, 1+, 2+	37	95		27	96		
Score 3+	2	5		1	4		
Lymphatic infiltration							0.17
Negative	22	56		11	39		
Positive	17	44		17	61		
Vessel infiltration							0.95
Negative	35	90		25	89		
Positive	4	10		3	11		
Sentinel lymph node characteristics							
No. SLNs removed			2.1 (1-4)			2.5 (1-4)	0.1[a]
No. positive SLNs			1.2 (1-3)			1.7 (1-4)	0.03[a]
1	32	82		15	54		0.04
2	6	15		9	32		
3	1	3		1	4		
4	0	0		3	11		
No. positive non-SLNs						3.3 (1-20)	
Size of SLN metastases			2.6 (0.15-8)			3.7 (0.2-10)	0.08[a]
Isolated tumor cells or clusters >0.2 mm	9	23		3	11		0.4
Micrometastases (>0.2-2 mm)	11	28		8	29		
Macrometastases (>2 mm)	19	49		17	59		

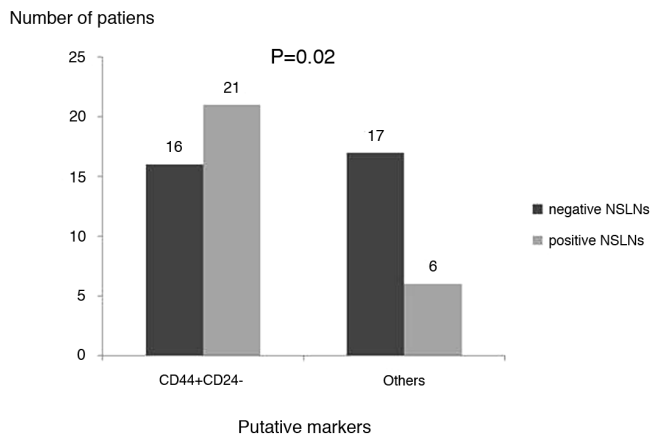SLN, sentinel; NSLN, non-sentinel lymph node. [a]Wilcoxon rank-sum tests.

Figure 2. The differences of CD44+CD24- and other phenotypes between negative NSLNs and positive NSLNs.

Table III. CD24 and CD44 expression differences between negative NSLNs and positive NSLNs.

	Negative NSLNs No. of patiens (n=33)	Positive NSLNs No. of patiens (n=27)	P-value
CD24 expression			0.04
Negative	19 (58)	22 (81)	
Positive	14 (42)	5 (19)	
CD44 expression			0.01
Negative	13 (39)	3 (11)	
Positive	20 (61)	24 (89)	
Combined			
CD44+CD24-	16 (48)	21 (78)	0.02
CD44+CD24+	4 (12)	3 (11)	
CD44-CD24+	10 (30)	2 (7)	
CD44-CD24-	3 (9)	1 (37)	

NSLN, non-sentinel lymph node.

Recently, SLN biopsy has been described as an accurate means of assessing regional lymph node involvement (28-30). If an SLN biopsy specimen is histopathologically negative, the risk of missed axillary disease is extremely low. SLN biopsy alone without complete axillary lymph node dissection (ALND) has been adopted as an accurate method of staging the axilla while avoiding much of the morbidity associated with a complete ALND. Nevertheless, a complete ALND is the standard treatment for breast cancer patients with SLN metastases, because the total number of involved nodes provides important prognostic information, as a larger number of positive nodes portends a poorer survival, and ALND can influence survival via local-regional control of the axilla. However, whether ALND should be performed in every patient with detectable SLN metastases, particular in those in whom the perceived risk of additional disease is low, remains debatable (31,32). Approximately 40-60% of patients with positive SLNs are found to have no other nodal metastases (15), and the therapeutic benefit of complete ALND after a positive SLN biopsy is minimal because patients with SLN metastases will generally receive systemic adjuvant therapy, regardless of the presence of any additional nodal metastases, and any residual disease may be eradicated by systemic therapy. Several studies have analyzed various clinicopathologic features in case with SLN-positive breast cancers to determine factors that might help predict the involvement of non-sentinel axillary lymph nodes (15-19). The size of the primary tumor and the size of the SLN metastasis as well as the presence of lymphovascular invasion in the primary tumor were the most common predictive factors.

Our data showed that the tumor size, tumor histological type and number of positive SLNs were associated with NSLN metastases. Using these clinicopathological features, a nomogram for predicting the likelihood of additional nodal metastases in breast cancer patients with a positive sentinel node biopsy has been recently developed (31). However, little is known about molecular events that regulate the ability of breast cancer cells to survive and grow in sentinel lymph nodes.

CD44 is a cell adhesion molecule known to be expressed in most cell types (32) and has been associated with stem cells in normal breast tissue (33). CD24 is expressed during the early stages of B-cell development and is highly expressed on neutrophils (34). Whereas CD24 is not present in adult human tissues, its expression has been observed in human carcinomas (35-37). In normal breast tissue, CD44 is localized to the cell membranes of basal/myoepithelial cells and a subset of luminal epithelial cells. Meanwhile, CD24 expression is occasionally found on the apical membranes of luminal cells (38).

Recent data have suggested that the decreased expression or loss of CD24 seems to be characteristic of the stemness of a tumor cell (34,39). CD44 and CD24 have been shown to regulate the invasion and metastasis of breast cancer cells either positively or negatively (40-44). Another report using genome-wide gene expression profiles showed that CD44+ cells showed a more mesenchymal stem cell-like profile that was enriched with genes involved in cell motility, proliferation and angiogenesis, whereas CD24+ cells highly expressed genes implicated in carbohydrate metabolism and RNA splicing (45).

We found that 73.3% of the metastatic tumor cells in SLNs were CD44+ and 31.7% were CD24+. CD44+CD24- metastatic cells in the SLN were significantly associated with NSLN metastases.

Although the concept of cancer stem cells has been controversial, previous reports have suggested that a subpopulation of CD44+CD24- breast cancer cells is responsible for the self-renewing properties and malignant behavior of human breast tumors (23). These putative cancer stem cells have been reported to constitute 12-60% of the tumor cells in clinical breast cancer specimens (39,45). The cell line MDA-MB-468 LN derived from MDA-MB-468, which has a high lymphatic metastatic ability was, reported to have a higher proportion (96.4%) of cells with a CD44+CD24- stem cell-like phenotype and to be associated with a high clonogenic potential, having a

great ability to survive and grow in foreign microenvironments such as lymph nodes (46).

We found that a high proportion (61.7%) of SLN metastatic cells were CD44+CD24-. Furthermore, the presence of CD44+CD24- tumor cells in SLNs was significantly associated with a high frequency of non-SLNs metastases. Clinically, these data may support the ability of stem cell-like cells to survive and exhibit autonomous growth in a foreign microenvironment.

The present study is the first to analyze the presence of cancer stem cell-like tumor cells in sentinel lymph nodes, and our study revealed that the presence of CD44+CD24- tumor cells in SLNs was associated with a high frequency of positive NSLNs.

The status of CD44+CD24- tumor cells in SLNs appears to be a predictor of NSLN metastases, and this information may be useful for determining the necessity of subsequent ALND. This finding also has implications for future therapeutic strategies aimed at the selective targeting of this stem cell-like phenotype to block this early stage of the metastatic process.

Acknowledgements

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