Breast Cancer Metastasis to the Axillary Lymph Nodes: Are Changes to the Lymph Node "Soil" Localized or Systemic?

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Heather L Blackburn[1], Darrell L Ellsworth[1], Craig D Shriver[2] and Rachel E Ellsworth[3]

[1]Chan Soon-Shiong Institute of Molecular Medicine at Windber, Windber, PA, USA. [2]Clinical Breast Care Project, Murtha Cancer Center, Walter Reed National Military Medical Center and Uniformed Services University, Bethesda, MD, USA. [3]Clinical Breast Care Project, Murtha Cancer Center, Windber, PA, USA.

ABSTRACT: Metastasis is a multistep process that is not well understood. Colonization of a secondary organ requires specific molecular alterations of the host microenvironment. To determine the temporal and spatial changes associated with metastatic dissemination to the axillary lymph nodes, gene expression profiles were compared between histologically normal lymph nodes from node-positive patients and tumor-free nodes from node-negative patients. Using a stringent false discovery rate correction (<0.05) for multiple hypothesis testing, we did not detect any differentially expressed genes between the lymph node groups. Thus, the presence of metastatic cells within the lymphatic system does not elicit widespread changes in gene expression through the axillary basin; rather, lymph nodes independently respond to disseminated tumor cells.

KEYWORDS: Breast cancer, microenvironment, lymph node metastasis

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CORRESPONDING AUTHOR: Rachel E Ellsworth, 620 Seventh Street, Windber, PA 15963, USA. Email: r.ellsworth@wriwindber.org

Introduction

Breast cancer has become the most common cancer diagnosed in women. In 2015, nearly 232 000 patients were diagnosed with invasive breast cancer in the United States. Although breast cancer mortality decreased by 36% between 1989 and 2013, more than 40 000 women were predicted to die of breast cancer last year.[1] Because most of the breast cancer deaths are caused by metastases, improved understanding of metastatic processes is critical to decreasing breast cancer mortality.

Metastatic colonization is dependent on molecular and phenotypic changes in disseminated tumor cells and the secondary microenvironment. This idea was first proposed by Paget[2] in 1889 when he described his seed and soil model of metastasis in which many cells with metastatic potential (the "seed") may be shed from the primary tumor; however, only cells that find a new microenvironment (the "soil") supportive of tumor growth will form successful metastases. Of all the steps in metastasis, tumor growth at a secondary site is the most challenging[3] and may be facilitated by increased expression of genes involved in transcription, immune response, and signal transduction in metastatic cancer cells.[4] Within the secondary microenvironment, processes including establishment of a premetastatic niche, suppression of the immune response, increased angiogenesis, reversing metastatic dormancy, and response to signals for cellular growth have been associated with establishment of metastatic deposits.[5,6]

To improve our understanding of molecular changes that occur in the secondary microenvironment of the axillary lymph nodes, we previously performed microarray analysis using RNA isolated from both metastatic (involved) and nonmetastatic (noninvolved) lymph node tissues available for research. From the metastatic lymph nodes, the residual, histologically normal lymph node tissue was microdissected and gene expression profiles were compared with those from the tumor-free lymph nodes. Twenty-two differentially expressed genes were identified that were involved in processes such as immune response, cellular proliferation, and epithelial-mesenchymal transition (EMT), demonstrating that the presence of metastatic breast cells is associated with molecular alterations in the host lymph node tissue.[7]

Patterns of gene expression in axillary lymph nodes with well-established metastatic deposits may represent late-stage changes in the microenvironment needed to support micro- or macrometastatic tumor growth. To determine whether similar changes in immune response, proliferation, and EMT or other pathways are altered before the establishment of detectable metastases, we compared gene expression profiles between histologically normal (metastasis free) lymph nodes from patients who had other clinically detectable metastases (node-positive patients) and histologically normal lymph nodes from patients with no evidence of metastatic colonization in the axillary chain (node-negative patients). These data will improve our

understanding of (1) if and how the lymph node microenvironment changes during the earliest stages of metastatic colonization and (2) whether the presence of metastatic deposits within the lymph nodes leads to systemic changes throughout the axillary chain.

Methods

Patients

Tissue samples were obtained under research protocols approved by the Human Use Committee and Institutional Review Board at Walter Reed National Military Medical Center. All subjects enrolled in the Clinical Breast Care Project voluntarily agreed to participate and signed a written statement of informed consent. To be eligible to participate in this study, a patient was required to be (1) at least 18 years of age, (2) mentally competent and willing to sign the informed consent documents, and (3) a patient at one of the breast centers with evidence of breast disease.

Specimen collection and characterization

Women with invasive breast cancer who were diagnosed with positive lymph nodes (n = 24), defined as metastatic deposits that were greater than 0.2 mm in diameter, or who were diagnosed with negative lymph nodes (n = 40) with no evidence of metastases and had frozen negative lymph node tissues were selected for this study. Eight patients with positive lymph nodes and 15 patients with negative lymph node status had multiple negative nodes available, resulting in a total of 34 metastasis-free lymph nodes from node-positive patients and 56 negative lymph nodes from node-negative patients. To determine whether isolated tumor cells were present within the negative lymph nodes, serial sections were analyzed by immunohistochemistry analysis (MDR Global, Windber, PA, USA), with at least 3 sections analyzed per lymph node. Within 15 minutes of surgical excision, lymph nodes were subjected to a comprehensive pathologic evaluation and then frozen in optimal cutting temperature medium. Tissues were stored in liquid nitrogen until used in this study.

Gene expression analysis

For each patient, individual lymph nodes were subjected to laser microdissection using an ASLMD microdissection system (Leica Microsystems, Wetzlar, Germany). To preserve RNA integrity, all microdissections were conducted within 15 minutes for each node. All RNA isolation, amplification, and hybridization procedures followed previously published methods.[8] Briefly, RNA was isolated with the RNAqueous-Micro Kit (Thermo Fisher Scientific, Foster City, CA, USA) and treated with deoxyribonuclease I to remove contaminating genomic DNA. Following isolation, the RNA integrity was determined with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was then labeled with biotin and converted to amplified RNA (aRNA) using 2 rounds of amplification with a MessageAmp II aRNA Amplification Kit (Thermo Fisher Scientific). The concentration and quality of all samples were determined with a NanoDrop 1000 (NanoDrop Products, Wilmington, DE, USA) and a 2100 Bioanalyzer. Aliquots of aRNA were purified, fragmented, and hybridized to GeneChip Human Genome U133A 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) and scanned on a GeneChip Scanner 3000.

Statistical analysis

Pathologic characteristics of all samples were analyzed using a χ^2 test with significance set at $\alpha = 0.05$. The gene expression data were analyzed with Partek Genomics Suite v6.6 (Partek Inc., Saint Louis, MO, USA). Intensities for each probe set were derived from robust multiarray average background correction, quantile normalization, median polish summarization, and $\log_2$ transformation. Standard quality control parameters recommended by the manufacturer were then used to determine the integrity of the data. To investigate whether the gene expression patterns could be effectively separated based on the lymph node status of each patient, we used the principal component analysis (PCA) function in Partek. Differentially expressed genes were identified by analysis of variance (ANOVA) using a stringent False Discovery Rate (FDR) correction (<0.05) to correct for multiple hypothesis testing.

Results

Pathologic characteristics

The age at which breast cancer was diagnosed did not differ significantly ($P = .65$) between women with positive lymph nodes (average age = 53.3 years) and those with negative nodes (average age = 54.8 years). Most patients in both groups self-described their ancestry as European American (71% and 60%) followed by African American (21% and 30%) in node-positive and node-negative patients, respectively. Sentinel lymph node biopsy was more common in the node-negative (82%) compared with node-positive (67%) patients, but this difference was not significant ($P = .15$). Within the node-positive patients, 12 (50%) had extranodal extension (ENE), growth, or spread of cancer cells outside the lymph node capsule, which has been associated with poor prognosis. The number of positive lymph nodes was significantly higher ($P = .009$) in patients with ENE (mean: 5.08, range: 1-12) compared with patients without (mean: 1.91, range: 1-4). None of the pathologic characteristics differed significantly between the 2 groups of patients (Table 1).

Gene expression

An average of 13 769 (average call rate: 61.81%) probes was expressed across all of the lymph node samples (range: 12 457-15 204). Using a stringent FDR multiple comparison correction, ANOVA did not detect any significant differences in gene

expression in negative lymph nodes between the 2 groups of patients. Similarly, PCA was unable to segregate patients based on patterns of gene expression (Figure 1).

This study had 80% power to detect a >1.5-fold difference in gene expression between groups (http://bioinformatics.mdanderson.org/MicroarraySampleSize/). Using an unadjusted $P < .05$, 14 genes were identified with >1.5-fold differential expression between lymph node groups (Table 2); however, none of these genes were significant using a Bonferroni correction for multiple comparisons. Pathway analysis also failed to detect pathways that differed significantly between groups.

Table 1. Pathologic characteristics of patients included in this study.

	NODE-POSITIVE PATIENTS (N=24)	NODE-NEGATIVE PATIENTS (N=40)	*P* VALUE
Tumor size			.126
T1	0.52	0.62	
T2	0.26	0.33	
T3	0.22	0.05	
Tumor grade[a]			.928
1	0.22	0.26	
2	0.30	0.31	
3	0.48	0.43	
ER/HER2 status			.522
ER+/HER2–	0.62	0.58	
ER+/HER2+	0.04	0.16	
ER–/HER2+	0.17	0.10	
ER–/HER2–	0.17	0.16	
Ki67			.713
<20%	0.44	0.50	
>20%	0.56	0.50	

[a]Grade was assigned using the Nottingham histologic score.[9,10]

Discussion

Previous research in our laboratory found that the lymph node microenvironment within a single patient differs depending on whether or not the lymph node harbors a metastatic deposit.[7] Changes in gene expression in histologically normal lymph node tissue associated with metastatic colonization include heightened immunotolerance, reversal of the EMT, and cellular growth and proliferation, each of which may support successful metastatic growth in a distant microenvironment. In that study, tissue from lymph nodes harboring established metastases was evaluated; thus, differences in gene expression may support growth of mature metastases and may not reflect the earliest changes within the lymph node microenvironment that allow disseminated tumor cells to survive in a secondary organ and escape dormancy.

In this study, all lymph nodes evaluated were free of clinically detectable metastatic deposits. One group of negative lymph nodes was collected from female patients who had other positive lymph nodes within the axilla, whereas the other group of lymph nodes was from women with negative lymph node status. Our data provide information about the temporal and spatial mechanisms of metastatic spread. Because the lymph nodes evaluated here were free of metastatic breast cells, these nodes represent one of the earliest stages of metastasis, sharing a milieu within the axillary basin with other metastatic lymph nodes but not harboring breast tumor cells. The observation that patterns of gene expression in these "at-risk" lymph nodes do not differ from expression profiles in lymph nodes from nonmetastatic patients suggests that (1) the presence of foreign cells may be required to initiate changes to the lymph node microenvironment and (2) these changes may be localized and not systemic throughout the axilla. Our data also suggest that the lymph nodes do not promote dissemination of tumor cells to other lymph nodes in the axilla.

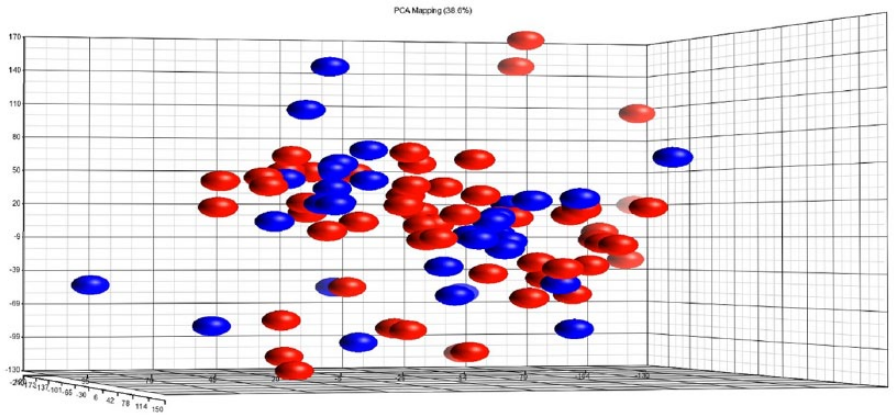

Figure 1. Principal component analysis (PCA) for gene expression profiles in negative lymph nodes. Red spheres = negative lymph nodes from patients without metastasis (n = 56); blue spheres = negative lymph nodes from patients with metastatic deposits in other lymph nodes within the axilla (n = 34).

Table 2. Fourteen genes differentially expressed in metastasis-free lymph nodes with an unadjusted $P < .05$, >1.5-fold difference.

GENE SYMBOL	ACCESSION NUMBER	GENE NAME	PROBE ID	*P* VALUE	FOLD CHANGE
Genes downregulated in lymph node tissue from node-positive patients					
C4BPA	NM_000715	Complement component 4–binding protein alpha	205654_at	.0021	0.664
C6orf62	NM_030939	Chromosome 6 open reading frame 62	213872_at	.0173	0.659
LAIR2	NM_002288	Leukocyte-associated immunoglobulin-like receptor 2	207509_s_at	.0016	0.653
MMP12	NM_002426	Matrix metallopeptidase 12	204580_at	.0249	0.637
FOLR1	NM_016725	Folate receptor 1	211074_at	.0155	0.631
TFPI2	NM_006528	Tissue factor pathway inhibitor 2	209278_s_at	.0081	0.627
Genes upregulated in colonized lymph node tissues from node-positive patients					
APOLD1	NM_001130415	Apolipoprotein L domain containing 1	221031_s_at	.0039	1.846
AREG	NM_001657	Amphiregulin	205239_at	.0058	1.800
CTGF	NM_001901	Connective tissue growth factor	209101_at	.0030	1.622
CXCR4	NM_001008540	Chemokine (C-X-C motif) receptor 4	209201_x_at	.0058	1.516
			211919_s_at	.0047	1.510
CYR61	NM_001554	Cysteine-rich angiogenic inducer 61	201289_at	.0085	1.560
DUSP1	NM_004417	Dual specificity phosphatase 1	201041_s_at	.0063	1.583
JUN	NM_002228	Jun proto-oncogene	201466_s_at	.0380	1.500
SRSF6	NM_006275	Serine/arginine-rich splicing factor 6	206108_s_at	.0002	1.600

Extranodal extension is the spread of metastatic tumor cells through the nodal capsule of the lymph node into the surrounding adipose and connective tissue. The presence of ENE in sentinel lymph nodes has been associated with metastasis in nonsentinel lymph nodes[11] and increased risk of recurrence and overall mortality.[12] In our data set, patients with ENE had significantly higher numbers of metastatic lymph nodes. Thus, migration of tumor cells out of the involved lymph nodes and into the perinodal space allows metastatic cells to disseminate between individual lymph nodes and, once inside a new lymph node, may illicit gene expression changes that promote metastatic growth.

The metastatic process remains poorly understood; however, one recent hypothesis suggests that metastasis may be influenced by genetic predisposition. When a transgenic mouse strain showing high metastatic propensity was outcrossed with various inbred strains of mice, significant variability in the propensity for metastasis was observed. Because each animal inherited the transgene influencing metastasis, any differences among the offspring in metastatic capacity may be attributable to their genetic background.[13] A number of genes have been identified in mice and humans as candidate genes for metastatic predisposition, including *bromodomain*-containing protein 4 (BRD4), breast cancer metastasis suppressor 1 (BRMS1), checkpoint kinase 2 (CHEK2), glutathione peroxidase 4 (GPX4), ligase IV, DNA, ATP-dependent (LIG4), NAD(P)H dehydrogenase, quinone 1 (NQO1), ribosomal RNA–processing protein 1 (RRP1B), signal-induced proliferation-associated gene 1 (SIPA1), and tumor protein p73 (TP73).[14] In this study, evaluation of probes corresponding to these genes did not reveal any statistical differences in expression levels, and differences in expression between groups were <1.1-fold. DNA variants within these purported metastasis susceptibility genes should lead to systemic effects; however, lack of differential expression within the metastatic microenvironment suggests that if these genes contribute to metastasis, it is not through altered transcription levels.

One limitation of this study was the use of RNA isolated from whole lymph node specimens. The lymph node is a heterogeneous organ that includes structures such as vessels and sinuses. Using intravital microscopy, Pereira et al[15] traced the movement of breast cancer cells into the lymph node. Tumor cells entered through the afferent lymphatic vessels and proliferated in the subcapsular sinus and later the parenchyma. In establishing the premetastatic niche, vascular endothelial growth factor receptor 1–positive hematopoietic bone marrow progenitor cells were first detected in the lung parenchyma.[16] These data suggest that the earliest changes in the secondary organ are spatially organized; thus, molecular changes that may be present in the afferent lymphovascular vessels or parenchyma may be masked when analyzing RNA from whole sections of a lymph node. In addition,

this study evaluated the earliest stages of metastasis when uninvolved lymph nodes were present within a metastatic axilla, but were metastasis free. Future studies should investigate the relationship between the first foreign cells entering the lymph node and changes in the lymph node microenvironment. These nodes may harbor either single or small clusters of breast tumor cells or those cells derived from bone marrow that establish a premetastatic niche. Recently developed approaches such as single-cell sequencing may allow improved resolution of these early alterations in both tumor and lymph node tissue that create the microenvironment supportive of metastatic growth, including mechanisms associated with escape from dormancy, angiogenesis, proliferation, and decreased immunosurveillance.[17–19]

Conclusions

Gene expression profiles do not differ significantly in metastasis-free lymph nodes, regardless of the presence or absence of breast cancer metastases within the axilla. These findings suggest that changes in the microenvironment associated with metastases reflect late-stage alterations associated with tumor growth and progression, rather than supporting the earliest stages of colonization. In conjunction, these data demonstrate that the presence of metastatic cells does not elicit widespread changes through the axillary basin, but rather lymph nodes respond to disseminated tumor cells independently.

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Author Contributions

REE and DLE conceived and designed the experiments. REE analyzed the data. REE wrote the first draft of the manuscript. HLB, DLE, and REE contributed to writing of the manuscript. HLB, DLE, CDS, and REE agreed with manuscript results and conclusions. DLE and REE jointly developed the structure and arguments for the paper. HLB, DLE, CDS, and REE made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

REFERENCES

1. American Cancer Society. *Breast Cancer: Facts and Figures 2015-2016*. Atlanta, GA: American Cancer Society; 2015.
2. Paget S. The distribution of secondary growths in cancer of the breast. *Lancet*. 1889;133:571–573.
3. Mathot L, Stenninger J. Behavior of seeds and soil in the mechanism of metastasis: a deeper understanding. *Cancer Sci*. 2012;103:626–631.
4. Ellsworth RE, Seebach J, Field LA, et al. A gene expression signature that defines breast cancer metastases. *Clin Exp Metastasis*. 2009;26:205–213.
5. Lorusso G, Ruegg C. New insights into the mechanisms of organ-specific breast cancer metastasis. *Semin Cancer Biol*. 2012;22:226–233.
6. Kaplan RN, Rafii S, Lyden D. Preparing the "Soil": the premetastatic niche. *Cancer Res*. 2006;66:11089–11093.
7. Valente AL, Kane JL, Ellsworth DL, Shriver CD, Ellsworth RE. Molecular response of the axillary lymph node microenvironment to metastatic colonization. *Clin Exp Metastasis*. 2014;31:565–572.
8. Field LA, Deyarmin B, Shriver CD, Ellsworth DL, Ellsworth RE. Laser microdissection for gene expression profiling. *Methods Mol Biol*. 2011;755:17–45.
9. Bloom HJ, Richardson WW. Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. *Br J Cancer*. 1957;11:359–377.
10. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*. 1991;19:403–410.
11. van la Parra RF, Peer PG, Ernst MF, Bosscha K. Meta-analysis of predictive factors for non-sentinel lymph node metastases in breast cancer patients with a positive SLN. *Eur J Surg Oncol*. 2011;37:290–299.
12. Nottegar A, Veronese N, Senthil M, et al. Extra-nodal extension of sentinel lymph node metastasis is a marker of poor prognosis in breast cancer patients: a systematic review and an exploratory meta-analysis. *Eur J Surg Oncol*. 2016;42:919–925.
13. Hunter KW. The intersection of inheritance and metastasis: the role and implications of germline polymorphism in tumor dissemination. *Cell Cycle*. 2005;4:1719–1721.
14. Ribelles N, Santonja A, Pajares B, Llacer C, Alba E. The seed and soil hypothesis revisited: current state of knowledge of inherited genes on prognosis in breast cancer. *Cancer Treat Rev*. 2014;40:293–299.
15. Pereira ER, Kedrin D, Jones D, Beech E, Taghian A, Padera TP. Abstract P3-01-22: Sentinel lymph node metastases in breast cancer: a contributor to distant metastases? In: Proceedings of the Thirty-Eighth Annual CTRC-AACR San Antonio Breast Cancer Symposium; December 8-12, 2015; San Antonio, TX.
16. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 2005;438:820–827.
17. Hurst RE, Bastian A, Bailey-Downs L, Ihnat MA. Targeting dormant micrometastases: rationale, evidence to date and clinical implications. *Ther Adv Med Oncol*. 2016;8:126–137.
18. Barkan D, Green JE. An in vitro system to study tumor dormancy and the switch to metastatic growth [published online ahead of print August 11, 2011]. *J Vis Exp*.
19. Barkan D, Green JE, Chambers AF. Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. *Eur J Cancer*. 2010;46:1181–1188.