Original Investigation | PACIFIC COAST SURGICAL ASSOCIATION

Correlation of Breast Cancer Axillary Lymph Node Metastases With Stem Cell Mutations

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IMPORTANCE Mutations in oncogenes *AKT1*, *HRAS*, and *PIK3CA* in breast cancers result in abnormal PI3K/Akt signaling and tumor proliferation. They occur in ductal carcinoma in situ, in breast cancers, and in breast cancer stem and progenitor cells (BCSCs).

OBJECTIVES To determine if variability in clinical presentation at diagnosis correlates with PI3K/Akt mutations in BCSCs and provides an early prognostic indicator of increased progression and metastatic potential.

DESIGN, SETTING, AND PARTICIPANTS Malignant (BCSCs) and benign stem cells were collected from fresh surgical specimens via cell sorting and tested for oncogene mutations in a university hospital surgical oncology research laboratory from 30 invasive ductal breast cancers (stages IA through IIIB).

MAIN OUTCOMES AND MEASURES Presence of *AKT1*, *HRAS*, and *PIK3CA* mutations in BCSCs and their correlation with tumor mutations, pathologic tumor stage, tumor histologic grade, tumor hormone receptor status, lymph node metastases, and patient age and condition at the last follow-up contact.

RESULTS Ten tumors had mutations in their BCSCs. In total, 9 tumors with BCSC mutations and 4 tumors with BCSCs without mutations had associated tumor present in the lymph nodes (P = .001).

CONCLUSIONS AND RELEVANCE Tumors in which BCSCs have defects in PI3K/Akt signaling are significantly more likely to manifest nodal metastases. These oncogenic defects may be missed by gross molecular testing of the tumor and are markers of more aggressive breast cancer. Molecular profiling of BCSCs may identify patients who would likely benefit from PI3K/Akt inhibitors, which are being tested in clinical trials.

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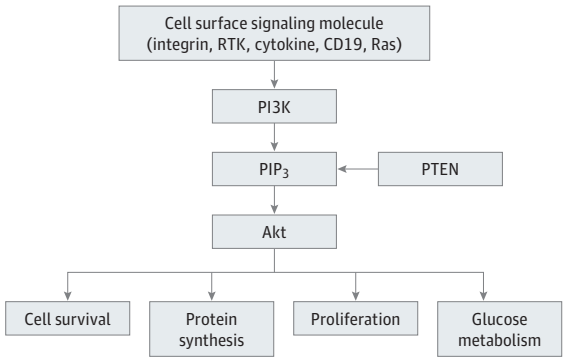
In stem cell theory, breast cancer stem and progenitor cells (BCSCs) are central to cancer proliferation, metastatic potential, and outcome measures. Breast cancer stem and progenitor cells have the capacity to initiate tumors, produce multiple tumor cell lineages, and maintain a continuous population of malignant stem cells through self-renewal.[1,2] Although it is unknown exactly how malignant stem cells arise, the function of benign stem cells is to maintain normal tissues and organs. Following malignant transformation, they likely retain their stem cell qualities and acquire malignant abilities through the acquisition of cancer-associated genetic mutations.

Breast cancer stem and progenitor cells constitute a small percentage of the total number of cells within a tumor. However, experiments in murine models have shown that 200 cancer stem cells can form tumors when transplanted into the cleared mammary fat pad of transgenic mice.[3,4] In contrast, more than 10 000 nonspecific tumors cells are required to create the same result.[5-7] These qualities of self-renewal, tumor cell lineage propagation, and remodeling of surrounding tissues are required to sustain primary and metastatic disease.[8]

Findings from previous murine and human mammary tissue studies[4,7] suggest that benign breast stem and progenitor cells can be distinguished from malignant stems cells based on CD44, CD49f (or CD29), and CD24 cell surface markers. Neither benign nor malignant stem cells express endothelial or leukocyte markers CD31 and CD45. However, it has been shown that benign and malignant breast tissues contain positively and negatively expressing CD44, CD24, and CD49f cells.[9] An-

Figure 1. PI3K/Akt Signaling Pathway, With Downstream Results



Extracellular signaling molecules interact with surface g-proteins, resulting in activation of PI3K, which phosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which in turn activates Akt. Activation of Akt initiates several intracellular processes related to cell survival, protein synthesis, proliferation, and glucose metabolism. PTEN acts as a negative feedback mechanism on PIP_3.

other way to distinguish benign from malignant stem cells is genetic-based analysis to detect cancer-associated changes. Previous research found no oncologic abnormalities in stem and progenitor cells in benign breast tissue; however, oncogenes in the PI3K/Akt signaling pathway were identified in stem and progenitor cell populations in breast cancer.[9]

The PI3K/Akt pathway is implicated in cell proliferation, signaling, and metastatic potential and is a target for chemotherapeutic agents now in clinical trials.[10-12] PI3K is a transmembrane protein that when activated by cellular growth signaling molecules results in initiation of cellular proliferation through phosphatidylinositol 3,4,5-trisphosphate and Akt[13,14] (**Figure 1**). Mutations in proteins along the PI3K/Akt signaling pathway have been found in colon, pancreatic, and lung cancer, and it is the most commonly mutated pathway in breast cancer.[10,15,16] The *PIK3CA* gene encodes the catalytic subunit of the PI3K enzyme. Activating mutations result in increased initiation of the downstream cascade.[17-19] Previous findings indicate that *PIK3CA* mutations are present in 30% to 40% of breast tumors.[15] Mutations have been associated with *AKT1* (OMIM 164730) (4%-8% of tumors) and less frequently with *HRAS* (OMIM 190020) and *PTEN* (OMIM 601728).[10,20,21] Mutations of *PIK3CA* (OMIM 171834) cluster in "hot spots," with the 3 most common mutations at *E5542K*, *E545K*, and *H1047R*, although additional oncogenic mutations have been identified.[10,17] Mutations in the pathway have been found in BCSCs, but the clinical implications have not been explored.[9]

We propose that differences in tumor behavior and clinical outcomes may be due to the genetic differences in BCSCs that are present in breast cancers.[22,23] We hypothesized that breast cancers with stem and progenitor cell mutations in genes of the PI3K/Akt signaling pathway would be associated with more aggressive breast cancers. To test this hypothesis, we correlated the mutation status of genes in the PI3K/Akt signaling pathway in malignant BCSCs with tumor pathologic features and early clinical outcomes among patients with breast cancer.

Methods

This study was approved by the Oregon Health & Science University institutional review board. Women with invasive ductal carcinoma of the breast exceeding 1.0 cm were identified at tumor board meetings and enrolled in the study. Solid-tissue breast specimens were collected at the time of mastectomy or lumpectomy before any adjuvant treatment. Specimens were obtained directly from the operating room and evaluated by a pathologist, and approximately 1 g of tumor tissue was immediately transferred to the laboratory for processing in mammary epithelial cell-specific medium (Epicult; StemCell Technologies). Samples were minced and placed in a solution containing 50% mammary epithelial cell-specific medium, 50% fetal bovine serum, and 6% dimethyl sulfoxide (ATCC) and cryopreserved at −80°C for a variable period ranging from several weeks to months. Samples were thawed, the cryopreserving fluid was removed by centrifuge, and minced tissue was digested in mammary epithelial cell-specific medium containing collagenase and hyaluronidase overnight. Cells were tested for viability with trypan blue, counted, and were labeled with fluorochrome-conjugated monoclonal antibodies against human CD45 and CD31 (fluorescein isothiocyanate conjugated), CD24 (phycoerythrin), CD49f (phycoerythrin-cyanine 5, and CD44 (phycoerythrin-cyanine 7). Isotype control testing indicated no nonspecific binding. Subpopulations were separated based on surface antibody labeling and collected by discriminatory gating. The $CD31^+$ and $CD45^+$ endothelial cells and leukocytes were removed leaving lineage negative cells. Cells were sorted into the following 4 lineage-negative populations: $CD49f^+CD24^+$, $CD49f^+CD24^-$, $CD49f^-CD24^+$, and $CD49f^-CD24^-$. The frequency of CD44 expression was evaluated for these cell populations in most patients. Sorted cell populations then underwent whole genomic amplification (REPLI-g Mini; Qiagen). Following amplification, samples of 10 ng of whole genomic DNA were screened for 410 mutations in 30 human oncogenes using an array system (MassARRAY; Sequenom Inc). As previously described,[24] this protocol involves polymerase chain reaction amplification of sequences of interest, followed by primer extension and mass spectrometry (matrix-assisted laser desorption ionization-time-of-flight mass spectrometry).

Following tissue analysis, medical records were reviewed, and additional data were compiled retrospectively for each patient. Data collected included patient age at diagnosis, race/ethnicity, tumor hormone receptor status, *ERBB2* (formerly HER2/Neu) status, tumor histologic grade, pathologic tumor stage, and condition at the last follow-up contact. χ^2 Test and Fisher exact test were used for statistical analyses. *t* Test was used to compare parametric data between groups.

Results

Thirty invasive ductal breast carcinomas were obtained. The characteristics of breast tumors, including patient age at diagnosis, tumor size, and tumor hormone receptor status, are

Table 1. Tumor Characteristics by Patient

Patient No.	Tumor Mutation	Tumor Size, cm	Tumor Histologic Grade	ER	PR	*ERBB2* Status	TNM at Diagnosis	Pathologic Tumor Stage	No. of Positive Nodes/Total No. of Nodes Evaluated
1	*AKT1* exon 2 E17K	2.3	2	–	–	–	T2bN1aM0	IIB	2/19
2	*PIK3CA* exon 20 H1047R	4.5	3	+	+	+	T2N2aM0	IIIA	4/30
3	*HRAS* exon 2 Q161R	3.2	2	+	–	–	T2N1(mi)M0	IIB	1mi/16
4	*PIK3CA* exon 20 H1047R	3.5	1	+	+	–	T2N1M0	IIB	3/13
5	*PIK3CA* exon 9 E545K	1.7	2	+	+	–	T1CN1(mi)M0	IB	1mi/4
6	*PIK3CA* exon 9 E545K	3.2	1	+	–	–	T2N0M0	IIA	0
7	*PIK3CA* exon 9 E545K	3.3	3	+	+	+	T2N1(mi)M0	IIB	1mi/4
8	*PIK3CA* exon 20 H1047R	2.5	3	–	–	–	T2N1M0	IIB	2/19
9	*PIK3CA* exon 9 E545K	2.2	1	+	+	–	T2N1(mi)M0	IIB	1mi/14
10	*PIK3CA* exon 4 N345K	5.0	1	+	+	–	T4bN1M0	IIIB	2/4
11	*PIK3CA* exon 4 G1049R[a]	1.2	2	+	+	–	T1cN0M0	IA	0
12	NA	2.8	2	+	+	–	T2N0M0	IIA	0
13	NA	3.0	3	–	–	–	T2N0M0	IIA	0
14	NA	2.4	3	–	–	+	T2N0M0	IIA	0
15	NA	1.7	2	+	+	–	T3N0M0	IIB	0
16	NA	1.3	3	+	+	–	T1cN0M0	IA	0
17	NA	3.0	2	+	+	–	T2N0M0	IIA	0
18	NA	2.4	1	+	+	–	T2N1(mi)M0	IIA	1mi/2
19	NA	2.2	3	–	–	–	T2N0M0	IIA	0
20	NA	1.7	3	–	–	–	T1cN0M0	IA	0
21	NA	4.2	2	+	+	+	T2N1M0	IIB	3
22	NA	2.9	2	+	–	–	T2N0M0	IIA	0
23	NA	1.5	1	+	–	–	T1N1(mi)M0	IB	1mi/3
24	NA	1.8	2	+	+	–	T1N0M0	IA	0
25	NA	3.1	2	+	+	–	T2N1M0	IIB	2/18
26	NA	4.2	2	–	+	–	T2N0M0	IIB	0
27	NA	4.8	3	+	+	–	T2N1aM0	IIB	0
28	NA	5.7	3	+	+	–	T3N0M0	IIB	0
29	NA	2.2	2	+	+	–	T2N0M0	IIA	0
30	NA	1.7	3	+	+	–	T1cN0M0	IA	0

Abbreviations: ER, estrogen receptor expression; mi, micrometastatic disease; NA, not applicable; PR, progesterone receptor expression.

[a] Normal human variant.

summarized in **Table 1**. The mean follow-up time was 22 months after diagnosis. One patient was lost to follow-up contact. The most common tumor histologic grade was 2. Six patients (20%) had positive lymph nodes, and an additional 6 patients had micrometastatic deposits (<0.2-cm metastatic focus of tumor) identified in lymph nodes. The correlation between tumor size and macroscopic lymph node metastases did not achieve statistical significance ($P > .05$). All patients with macroscopic lymph node metastases underwent completion axillary lymph node dissection. Patients with only micrometastatic disease did not.

Among 30 tumors, 10 tumors (33%) had BCSCs with *AKT1*, *HRAS*, or *PIK3CA* mutations, 8 of which have been previously reported.[9] Three different mutations (E545K, N345K, and H1047R) were detected in *PIK3CA*, a single mutation was detected in *AKT1*, and a single mutation was detected in *HRAS* (Table 1). *PIK3CA* G1049R (rs1219132) is considered a normal variant and was found in the lineage-negative $CD49f^+CD24^+$ and lineage-negative $CD49f^-CD24^+$ cells of one tumor. A subset of the tumors was specifically assessed for CD44 positivity, which varied based on BCSC population. In the $CD49f^+CD24^+$ sorted cell population of 20 of 26 patients, greater than 85% of the cells were also $CD44^+$. In $CD49f^+CD24^-$, $CD49f^-CD24^+$, and $CD49f^-CD24^-$ populations, expression was variable among patients, with a mean of 60% (range, 20% [4 of 20 patients] to 90% [9 of 20 patients]) of cells being $CD44^+$. Breast cancer stem and progenitor cells with and without mutations were assessed for CD44 positivity, and no significant difference was observed between the 2 groups.

When the presence of any BCSC mutation correlated with patient and breast cancer characteristics, no statistically significant correlations were found with patient age at diagnosis, tumor size, tumor histologic grade, estrogen receptor expression, progesterone receptor expression, or *ERBB2* status (**Table 2**). However, a statistically significant correlation was observed between the presence of BCSC mutations and axil-

Table 2. Tumor and Patient Characteristics

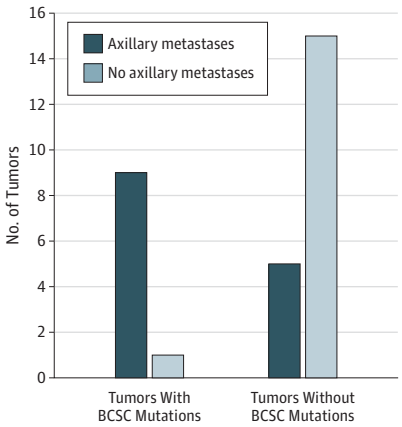
Characteristic	Tumors With BCSC Mutations (n = 11)	Tumors Without BCSC Mutations (n = 15)	*P* Value[a]
Age at diagnosis, mean, y	59	54	.34[b]
ER positive, % (No./total No.)	80 (8/10)	75 (15/20)	>.99
PR positive, % (No./total No.)	60 (6/10)	70 (14/20)	.97
ERBB2 overexpression, % (No./total No.)	20 (2/10)	10 (2/20)	>.58
Tumor size, mean (range), cm	3.1 (1.7-5.0)	2.7 (1.2-5.7)	.45[b]
Disease progression, % (No./total No.)	30 (3/10)	0	.03
Positive nodes, including micrometastases, % (No./total No.)	90 (9/10)	20 (4/20)	.001

Abbreviations: BCSC, breast cancer stem and progenitor cell; ER, estrogen receptor; PR, progesterone receptor.

[a] Fisher exact test unless otherwise indicated.

[b] Unpaired 2-tailed *t* test.

Figure 2. Axillary Lymph Node Metastases and Breast Cancer Stem Cell Mutations



Lymph node metastases are significantly increased in patients having breast cancer stem and progenitor cell (BCSC) mutations compared with patients not having BCSC mutations (*P* = .001).

lary lymph node metastases (*P* = .02). This significance was more pronounced when micrometastatic disease was included (*P* = .001) (**Figure 2**).

Three of 10 patients with BCSC mutations experienced disease progression after diagnosis following indicated chemotherapy, hormone therapy, and a trastuzumab regimen. Two patients died of disease, and 1 patient has brain metastases. No patients with BCSCs without mutations have evidence of disease. At the time of writing, patients with BCSC mutations had been followed up for a mean of 29 months, while patients without BCSC mutations had been followed up for a mean of 19 months. The difference in the mean follow-up periods was statistically significant (*P* = .001).

Discussion

Patients with tumors in which BCSCs have a genetic abnormality of the PI3K/Akt signaling pathway are significantly more likely to have lymph node metastases. While axillary lymph node metastases are known to correlate with tumor size, BCSC mutation in this study was an independent predictor of lymph node metastasis. Because 4 of 20 patients (20%) without BCSC mutations had axillary lymph node metastases, a PI3K/Akt mutation in BCSCs may not be a requirement for axillary lymph node metastases. However, a significant correlation was found between the 2 factors, with 9 of 10 patients (90%) with BCSC mutations having nodal metastases. Five of 10 patients (50%) with BCSC mutations had axillary lymph node macrometastatic disease, and an additional 4 of 10 patients (40%) had micrometastatic disease. Micrometastatic disease in lymph nodes is of uncertain prognostic significance.[25,26] Given the link between PI3K and metastatic potential, it could be that micrometastases harboring PI3K/Akt mutations may carry a different risk for distant metastatic disease. Longer patient follow-up periods and a larger sample size will determine if this subset of patients demonstrates an increased risk and may benefit from specifically designed use of adjuvant chemotherapy.

In the present study, we showed that tumors in which BCSCs have PI3K/Akt mutations exhibit variable hormone receptor expression, tumor histologic grade, and other tumor characteristics, suggesting that these early BCSC mutations do not restrict the type of breast cancer that develops. This finding has also been observed in murine investigations in which *PIK3CA* mutations in luminal cells produced heterogeneous tumors.[27] In additional support of this theory, similar *PIK3CA* and *AKT1* mutations are found in ductal carcinoma in situ.[20,21] Their detection in both ductal carcinoma in situ and BCSCs suggests that they are present in the early development of breast cancer and in some cases may be responsible for promoting early breast cancer development.[21,28-30] The retention of BCSCs along with PI3K/Akt mutations in tumors years after tumor initiation indicates that these cells continue to have an important role in tumor architecture and function. With regard to *ERBB2*, evidence suggests that *PIK3CA* mutations contribute to resistance to trastuzumab in *ERBB2*-positive breast cancers.[31] In this study, an insufficient number of *ERBB2*-amplified tumors were examined to discern if there is a correlation between BCSC abnormalities and axillary lymph nodal metastases in this specific group of tumors.

PI3K/Akt signaling abnormalities also did not correlate with patient age at diagnosis. Unlike *BRCA1* and *BRCA2* mutations, which are associated with early-onset breast cancer, PI3K/Akt abnormalities are not germline mutations but rather somatic mutations. Accordingly, it is not unexpected that they have a later onset than the breast cancers associated with *BRCA* mutations.

The prognostic significance of specific PI3K/Akt signaling pathway mutations in breast cancers remains controversial.[32-34] It is likely that the variability of these mutations, the heterogeneity of the tumors, and the complexity of the pathway contribute to this conflicting evidence. Our findings in BCSCs are consistent with studies[10,15,31-34] showing that *PIK3CA* and *AKT* mutations in breast cancers are associated with factors that may indicate poor prognosis and decreased survival rates. However, other studies[32,35,36] have shown improved disease-free survival rates, lower tumor histologic grades, and increased rates of estrogen receptor positivity in patients with tumors bearing *PIK3CA* mutations. Our study findings indicate that the answer to this controversy may lie in identifying mutations in BCSCs, as well as mutations in the tumor as a whole.

The collection of BCSCs from fresh surgical specimens, performance of molecular analyses, and subsequent correlation with clinical outcomes support embarking on a new way of approaching breast cancer diagnosis and treatment planning. The results of this study support concomitant evaluation of BCSCs along with assessment of the breast cancer overall. The analysis of BCSCs can generate specific information about tumor growth and metastatic potential that may not be obtained from analysis of the tumor progeny cells alone. Simultaneous molecular analyses of both the tumor and BCSCs may better identify patients who are likely to benefit from specific therapeutic regimens. Similarly, simultaneous BCSC and tumor analysis may increase the number of patients who might benefit from treatment but be missed by tumor analysis alone. For example, PI3K/Akt signaling pathway inhibitors now being tested in clinical trials may prove beneficial to patients with BCSC mutations even if genetic analysis of the accompanying tumors demonstrates no PIK/Akt mutation. The use of BCSC-specific and tumor-targeted chemotherapeutic agents may prove to be synergistic with each other, providing a novel therapeutic approach. Cancer stem cell therapeutics is an area of rapidly expanding knowledge. Future studies with larger cohorts, more outcomes data, and longer follow-up periods will allow us to more critically evaluate the significant early results reported in this study.

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Invited Commentary

Breast Cancer Stem Cells—Ready for Their Close-up?

Kristine E. Calhoun, MD

A growing body of evidence suggests that malignant stem cells have a role in the growth, spread, and clinical outcomes of breast cancers. In "Correlation of Breast Cancer Axillary Lymph Node Metastases With Stem Cell Mutations," Donovan et al[1] have built on prior notable works devoted to understanding the behavior of breast cancers. This group of authors previously demonstrated that breast cancer stem cells are distinctively different compared with benign and progenitor stem cells found in normal breast tissue, especially with regard to mutations in genes critical for maintaining normal cellular metabolism and proliferation.[2]

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In their present investigation, Donovan et al[1] propose that differences in malignant behavior, and ultimately patient clinical outcomes, may be due to mutations identified in breast cancer stem cells, specifically alterations in the PIK3CA/AKT1 pathway. Among 30 analyzed tumors, 11 contained mutations in *PIK3CA*, *HRAS*, or *AKT1*. Clinical correlation revealed that individuals whose tumors contained stem cell mutations had significantly higher rates of axillary metastases (90%) and were the only patients to have disease progression. Based on these results, the authors postulated that testing for such genetic mutations among patients with breast cancer will likely have a role in the future and may be useful in therapeutic decision making and treatments.

This is an elegant study contributing to the growing evidence that implicates breast cancer stem cells in the complex clinical behavior of breast cancer. That said, some questions still need to be answered before widespread application of this testing into clinical practice. First, it will be important to see if these preliminary findings hold when more than 30 tumors are tested because this is admittedly a small sample size. Second, identifying whether these findings are consistent among all tumor types, and not just invasive ductal carcinomas, will be of interest as well. Third, revisiting the cohort to examine if follow-up times longer than 2 years result in equalization of the outcome curves is encouraged, as is a determination of how easily such testing can be adopted by pathology departments of variable size and means.

Most critically, in an era when medical fiscal responsibility is necessary and costs are much more visible, it will be mandatory to determine if such testing is economically feasible. It is not uncommon for a patient with newly diagnosed breast cancer to undergo advanced testing with magnetic resonance imaging, as well as tumor profiling with gene assays, which can individually cost multiple thousands of dollars. If breast cancer stem cell testing can be performed in a cost-effective manner, and if the results ultimately yield information that influences therapeutic interventions as suggested by the authors, then additional costs will be justified, and such testing can avoid becoming just another shiny new toy.

ARTICLE INFORMATION

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