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Lymph Node Involvement in Immunohistochemistry-Based Molecular Classifications of Breast Cancer

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Abstract

Background—Prognosis and treatment options differ for each molecular subtype of breast cancer, but risk of regional lymph node (LN) metastasis for each subtype has not been well-studied. Since LN status is the most important predictor for prognosis, the aim of this study is to investigate the propensity for LN metastasis in each of the five breast cancer molecular subtypes.

Methods—Under an IRB-approved protocol, we retrospectively reviewed the charts of all pathologically confirmed breast cancer cases from 1/2004 to 6/2012. Five subtypes were defined as luminal A (hormone receptor +, Ki67 low), luminal B (hormone receptor+, Ki67 high), luminal-human epidermal growth factor receptor 2 (HER-2), HER-2-enriched (hormone receptor negative), and triple negative (TN).

Results—A total of 375 patients with complete data were classified by subtype: 95 (25.3%) luminal A, 120 (32%) luminal B, 69 (18.4%) luminal-HER-2, 26 (6.9%) HER-2-enriched, and 65 (17.3%) TN. On univariate analysis, age (<50), higher tumor grade, HER-2 + status, tumor size, and molecular subtype were significant for LN positivity. Molecular subtype correlated strongly with tumors size (X^2 ; $p=0.0004$); therefore, multivariable logistic regression did not identify molecular subtype as an independent variable to predict LN positivity.

Conclusions—Luminal A tumors have the lowest risk of LN metastasis, while luminal HER-2 subtype has the highest risk of LN metastasis. Immunohistochemical-based molecular classification can be readily performed and knowledge of the factors that affect LN status may help with treatment decisions.

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Keywords

molecular subtypes of breast cancer; immunohistochemistry

INTRODUCTION

Gene expression profiling has shown that breast cancer is a heterogeneous disease that can be classified into molecularly distinct subtypes [1]. Perou and colleagues [1] identified breast cancer cells that shared gene expression patterns resembling luminal epithelial cells (luminal), myoepithelial (or basal) cells, and/or cells with over-expression of the human epidermal growth factor receptor 2 (c-Erb-2 or HER-2) gene. High cost and special processing of tumor samples have limited the use of genomic profiling in routine clinical practice. Immunohistochemical (IHC) evaluation for the presence of several proteins: estrogen receptor (ER), progesterone receptor (PR), HER-2, and Ki-67 [2, 3] correlate closely, although not exactly, with tumor subtypes identified by DNA microarray expression profiles. The IHC-based subtype classification is a convenient surrogate, and multiple studies have shown reproducible associations between specific IHC-defined subtypes with their characteristic clinical and biologic behaviors/prognostic outcomes [4], especially for the triple negative phenotype [5-8]. Risk factors [9, 10], prognosis [11, 12], and treatment response rates [13, 14] are well-known to differ between the subtypes. Thus, these IHC-derived molecular characteristics can inform us of the potential biological behavior of each subtype. However, the risk of regional nodal metastasis for each subtype has not been well-studied. Because lymph node (LN) status is the most important predictor for prognosis and treatment, the aim of this study is to investigate the relationship between the breast cancer molecular subtype and LN status. We hypothesized that there will be a significant difference in propensity for LN metastasis among the different breast cancer subtypes.

In this study, the five subtypes are defined as luminal A (Estrogen Receptor [ER] and/or Progesterone Receptor [PR] positive, proliferation marker Ki-67 low, HER-2 negative), luminal B (ER/PR positive, Ki-67 high, HER-2 negative), luminal HER-2 (ER/PR positive, HER-2 over-expressed or amplified), HER-2-enriched (ER/PR negative, HER-2 over-expressed or amplified), and triple negative (TN, ER/PR negative, HER-2 negative). The TN can be further subdivided into basal (cytokeratin [CK] 5/6-positive, and/or epidermal growth factor receptor 1 [EGFR]) and non-basal.

MATERIALS AND METHODS

Under an IRB-approved protocol, we retrospectively reviewed the charts of all pathologically confirmed breast cancer cases at the University of Texas Medical Branch in Galveston, TX from 1/2004 to 6/2012. A total of 749 breast cancer cases were identified. Patients were excluded if their diagnosis was *in-situ* cancer only, if definitive LN staging was not performed prior to neoadjuvant therapy, or if there was insufficient clinicopathologic data available. After applying exclusion criteria, only 375 patients with invasive breast cancer and complete clinicopathological data were analyzed. These cases were classified according to molecular subtype and LN status. Data gathered included demographic factors (age at diagnosis, race/ethnicity, type of medical insurance), tumor characteristics (breast cancer histological type, tumor grade, tumor size) and LN status (positive LN is >0.2 mm tumor deposit detected by routine histology), and immunohistochemical (IHC) biomarkers for subtyping (ER, PR, Ki-67, HER-2 neu, EGFR, and CK 5/6). IHC staining was performed at the time of breast cancer diagnosis, except for 25 TN cases, where some information was missing but tissue blocks were available. These cases underwent retrospective biomarker staining for CK5/6 and EGFR. A total of 5 cases

underwent retrospective fluorescence *in-situ* hybridization (FISH) analysis to evaluate for HER-2 amplification.

Tumor grade was determined by the Bloom-Richardson scoring system (tubules, pleomorphism, and mitotic rate). ER or PR was considered a positive result with 1% tumor cells with strong nuclear staining [15]. ER or PR was considered negative if 0% of the tumor cells demonstrated nuclear staining. Ki-67 antigen, a marker of mitoses, was considered “low” if the number of positively stained nuclei represented <14% of the total nuclei evaluated, and “high” if 14% [3]. Her-2-neu was determined using a two-step process. The initial IHC-based evaluation was performed using a polyclonal rabbit anti-human c-erbB-2/neu antibody and reported semiquantitatively as either negative (0, no staining or 1+ staining defined as <10% of tumor cells with weak, incomplete, membranous staining) or positive (3+ staining, defined as at least 30% of the tumor cells with uniform, complete, intense membranous staining). Equivocal results (2+ staining, membrane staining that was non-uniform or weak but with obvious circumferential distribution in at least 10% of cells or intense complete membrane staining in 30% or less of tumor cells) were reflexively studied with a FISH assay using the Pat Vysion HER-2 neu DNA Probe Kit on formalin-fixed, paraffin-embedded tissue. Positive controls (high-level protein expression, low-level protein expression and internal control) and negative control were routinely used in the evaluation of the assay. Fixation of the examined tissue as well as the scoring technique [16] were performed in accordance with the College of American Pathologists and the American Society of Clinical Oncology joint guideline on testing for HER-2 status in invasive breast cancer. Specimens were scored and categorized as HER-2 negative if the result is “HER-2 not amplified” and positive if “amplified”.

IHC Antibodies

Slides were stained on an automated immunostainer (Dako autostainer, Carpinteria, CA, USA). Primary antibodies were all purchased from Dako and included the ER/PR PharmDx kit (# SK310 ER clone1D5, and PgR1294), c-erbB-2/neu antibody (# A0485, 1:400), Ki67 (clone MIB-1, # IR626), EGFR (# M3563, clone H11, 1:200), CK5/6 (# M723, clone D5/16B4, 1:100), and negative controls mouse IgG antibodies (# IR750). Antigen retrieval, secondary antibodies, and detection were performed using the Envision FLEX kit (Dako, # K8002).

Molecular classification by IHC

Tumors were classified into five major molecular subtypes using IHC and/or FISH for HER-2, where appropriate: luminal A (ER+ and/or PR+, HER-2–, Ki67 14%), luminal B (ER+ and/or PR+, HER-2–, Ki67 >14%), luminal HER-2 (ER+ and/or PR+, HER-2+), HER-2-enriched (ER–, PR–, HER-2+), and TN (ER–, PR–, HER-2–). The TN subtype can be further subdivided into basal-like (TN, EGFR+ and/or CK5/6+) and non-basal-like (TN, EGFR–, CK5/6–) [6, 17].

Statistical Methods—Frequencies and percentages were reported for categorical variables (such as histology, grade, and LN status). Chi-squared test and Fisher's exact test were used to evaluate the association between LN status and patient's demographic and tumor characteristics. Uni-covariate and multi-covariate logistic regression models estimated the odds ratios for LN metastasis. Since HER-2 expression was strongly correlated with molecular subtype, we excluded HER-2 from the multivariate logistic regression in order to assess the impact of molecular subtype. Otherwise, all other variables in the uni-covariate model were included. P-values were two-sided and were considered statistically significant when less than 0.05. All analyses examining the association between LN status, tumor

characteristics, and demographic data were conducted using SAS (version 9.1.3, Cary, NC) statistical software.

RESULTS

A total of 749 breast cancer cases were identified during the study period. Patients with ductal carcinoma *in situ* only, without pathologically determined LN metastasis prior to neoadjuvant chemotherapy, or incomplete clinical data were excluded from the study. The demographic and clinicopathological characteristics of the 375 eligible patients are shown in Table 1. Approximately one-third of the patients were diagnosed with breast cancer before age 50. The most common breast cancer subtype was luminal B (32%), followed by luminal A (25.3%), luminal HER-2 (18.4%), TN (17.3%), and HER-2 enriched (6.9%). For patients <50 years, luminal B (28.9%) and luminal HER-2 (24.8%) were the most prevalent subtypes, whereas luminal B (33.5%) and luminal A (27.6%) characterized the most common tumors for patients ≥50 years. There were differences in subtypes by ethnicity (Table 1): among the White and Hispanic populations, the most prevalent subtypes were luminal A and B, representing equivalent distributions (28 and 29%, respectively for White patients; 30.8 and 37%, respectively for Hispanic patients). In contrast, among African-American patients, luminal B was most prevalent at 37%, with luminal A representing the least prevalent subtype (11.9%); HER-2-enriched was 13%, representing the greatest number of patients compared to White (5.6%) or Hispanic (3.8%).

The incidence of LN-positive tumors for all patients was 50.1%, and was highest in the younger age group (<50 yrs), and among the Hispanic patients (62.8% and 71.2%, respectively, Table 2). Additionally, LN-positivity correlated with increasing tumor size (Table 2, $p<0.001$). Among the IHC-based individual biomarkers (ER, PR, Ki67, HER-2), only HER-2 status was significantly predictive of LN status ($p=0.005$, Table 2). Both HER-2-enriched and luminal HER-2 subtypes had the highest incidence of LN-positive tumors (58% and 65.2%, respectively; $p=0.0104$, Chi-Sq, Table 2, Figure 2).

On univariate logistic regression analysis, the association of LN positivity correlated with age (<50), higher tumor grade, HER-2-positive status and molecular subtype, and tumor size (Table 3). African American and Hispanic patients were 1.9 and 3.2 times more likely than White patients to have positive lymph nodes. Patients with Medicaid versus privately insured patients were 1.5 times more likely to have positive nodes at presentation (Table 3). A multivariate logistic regression analysis determined that specific independent predictors of LN positivity were age <50, ethnicity (African American and Hispanic vs. White), tumor grade, and tumor size (Table 3). Since tumor size was strongly associated with molecular subtype ($p=0.0004$, Figure 1), subtype was not significant on multi-covariate analysis. The most prevalent molecular subtype of T1 tumors was luminal A (56.8%, Figure 1), which was also the subtype that had the lowest propensity for LN metastasis (38.9%, Table 2), whereas luminal HER-2 was the most common subtype for T4 tumors (30.8%, Figure 1), and also the subtype with the highest percentage of LN metastasis (65.2%, Table 3). After adjusting for age, race, grade and T stage, Financial Class was no longer significant.

Finally, to examine whether there were differences in LN positivity between the TN basal versus the non-basal tumors, only 40/65 (61%) patients were evaluable with available IHC staining data (Table 4). There were no differences in the incidence of LN positivity between basal and non-basal TN breast cancers (Table 4, $p=0.2$).

DISCUSSION

Perou et al [1] initially identified four “intrinsic” breast cancer subtypes: basal-like, HER-2-enriched, luminal and normal breast-like, based on unsupervised hierarchical clustering in a genome-wide analysis. Subsequent studies have further stratified the luminal into less proliferative A tumors and more proliferative B tumors, and the HER-2 phenotype into triple positive versus hormone receptor-negative, HER-2-positive breast cancer. The normal breast-like phenotype was not reproducible in other studies; this subtype is now considered an artifact and is not used [8]. The 2011 St. Gallen International Expert Consensus Group has endorsed the use of IHC-based molecular subtypes as a surrogate for the intrinsic subtypes of breast cancer [19].

Although IHC-based molecular classification of breast cancer can be performed easily in clinical practice, currently, no consensus or standard definition stratifying breast cancer into IHC-based molecular subtypes exists between published studies. For example, the threshold for ER/PR-positive in some studies is at 10% of the cells stained positive [14, 20]. Also, some studies do not stain for the Ki67 antigen [21], while other studies employ tumor grade [14] as a surrogate for cellular proliferation. The number of subclassifications is also varied [11, 14, 22]. We subdivided the HER-2 positive tumors because HER-2-enriched breast cancers had an odds ratio of 2.1-fold risk for positive LN compared to luminal A, whereas luminal HER-2 cancers showed a higher (2.9-fold) risk (Table 3). Our data is in agreement with Van Calster et al [23], who have shown that triple-positive tumors are most likely to be LN positive. Luminal A and TN breast cancers are the least likely to present with positive LN. Among the TN breast cancers, we did not find any differences in the incidence of positive LN between the basal versus the non-basal breast cancers (Table 4). However, it is interesting to note that the basal-like breast cancers have markedly higher recurrence rates, compared to non-basal TN cancers [12], with a higher incidence of visceral metastasis [12, 24, 25], suggesting that the poor prognosis of basal-like breast cancers may be due to a higher propensity for distant (rather than regional) spread, as well as a paucity of adequate systemic therapies [17].

We found that IHC-based molecular subtypes correlated with LN status only by univariate analysis (Table 2, $p=.0104$; Table 3). Because tumor size was strongly associated with molecular subtype ($p=0.0004$, Figure 1), molecular subtype was not significant after adjusting for tumor size. Our data suggests that the molecular subtype reflects the biological behavior of the breast cancer, including the primary tumor's ability to achieve a particular size prior to clinical detection (either by imaging or physical examination), and its relative ability to metastasize to regional LN at the time of diagnosis.

There are several limitations to our study. The majority of patients were not included in this study because they had Stage 0 (*in-situ*) disease and no need for LN evaluation. We also excluded patients with incomplete clinicopathologic data, which introduces inherent selection bias in this study. This is a retrospective, descriptive study spanning eight-and-a-half-year period of time. Our most recent institutional standard for IHC evaluation of TN tumors (the addition of CK5/6 and EGFR staining) was not applied on the majority of TN breast cancers (diagnosed prior to application of the current practice). We subjected 25 cases to CK5/6 and EGFR IHC staining, and 5 cases for FISH analysis of HER-2 amplification for this study since this was not performed at the time of original patient diagnosis. Another limitation is that our study is from a single institution with relatively small sample sizes within each molecular subtype. However, similar to our findings, a study by Voduc et al [12] ($N=2,985$), independently verified that the highest rates of LN positivity occurred in the luminal HER-2 and HER-2-enriched tumors (54% and 55% of patients), whereas the lowest rates of positive LN were found in luminal A and TN tumors (41%, 36%, respectively).

Contemporary surgical management of axillary LN in breast cancer emphasizes a minimally invasive approach, decreasing the morbidity of axillary interventions while maximizing diagnostic information gained through increased pathologic scrutiny of only the sentinel LN, without compromising patient survival [26]. However, an area of continued controversy exists in the accuracy of sentinel LN biopsy after neoadjuvant chemotherapy. Many surgeons advocate forgoing sentinel LN staging prior to neoadjuvant chemotherapy for select patients who are clinically node-negative (by palpation, ultrasound evaluation and/or negative needle biopsy) [27]. The benefit is that the patient would undergo a single operation upon completion of preoperative chemotherapy. Concurrent with the surgical treatment of their primary breast cancer, the patient would undergo sentinel LN biopsy after chemotherapy [27]. Limitations of this practice include: 1) needle biopsy of a radiographically suspicious axillary LN can have a false-negative result of over 30% [28], and 2) the false-negative rate of sentinel LN biopsies after neoadjuvant chemotherapy is higher at 12% [29] compared to the desirable rate of <5% [30]. False-negative LN results may alter adjuvant systemic treatment recommendations and/or additional recommendations for locoregional control (subsequent axillary lymph node dissection/radiation therapy), potentially impacting care for some patients. Knowledge of molecular subtype and risk of LN metastasis may be useful for surgeons and patients deciding to forgo sentinel LN biopsy prior to neoadjuvant chemotherapy.

Conclusions

The prevalence of LN metastasis among the five breast cancer molecular subtypes is significantly different on univariate analysis. In particular, luminal A tumors have the lowest risk of LN metastasis and the greatest number of T1 tumors, while the luminal HER-2 subtype has the highest risk of LN metastasis and the greatest number of T4 tumors. After adjusting for tumor size, molecular subtype was not significantly correlated with LN status on multivariable logistic regression. IHC-based molecular classification can be readily performed and knowledge of the factors that affect LN status may help with treatment decisions.

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Figure 1.
Correlation between tumor size and molecular subtype (Chi-square analysis, $p=0.0004$, see Table 2).

Figure 2.
Correlation between lymph node status and molecular subtype (Chi-square analysis.
 $p=0.0104$, see Table 2).

Table 1

Demographic and tumor characteristics

Variable		N	%
Age	<50	120	32.0
	50	255	68.0
Financial Class	Insured, other	144	38.4
	Uninsured	14	3.7
	Medicare	120	32.0
	Medicaid	97	25.9
Ethnicity	White	231	61.6
	African-American	84	22.4
	Hispanic	52	13.9
	Other	8	2.1
Histology	Ductal	346	92.3
	Lobular	24	6.4
	Other	5	1.3
Grade	Well-differentiated	65	17.3
	Moderately-differentiated	149	39.7
	Poorly differentiated	150	40.0
	N/A	11	2.9
ER Status	Positive (1%)	279	74.4
	Negative (<1%)	96	25.6
PR Status	Positive (1%)	253	67.5
	Negative (<1%)	122	32.5
Ki-67	N.A.	1	
	< 14%	112	29.9
	14%	262	70.1
HER-2 Status	Positive *	96	25.6
	Negative	279	74.4
LN Status	Positive #	188	50.1
	Negative †	187	49.9
Molecular Subtype	Her-2 Enriched	26	6.9
	Luminal/Her-2	69	18.4
	Luminal A	95	25.3
	Luminal B	120	32.0
	Triple Negative	65	17.3
Tumor Size	N.A.	2	
	T1	142	38.1
	T2	147	39.4
	T3	45	12.1
	T4	39	10.5

N.A. data not available.

* HER-2 is positive if IHC shows 3+ staining or FISH shows amplification.

Eight patients (9/188 or 4.7%) had pN1_{mi} (micrometastases defined as >0.2 mm but <2 mm tumor deposit).

† Four patients (4/187 or 2.1%) had pN0i+ (tumor deposit <0.2 mm detected by H&E).

Table 2

Demographic and tumor characteristics by lymph node status

Variable		LN positive	LN negative	P value *
Age	<50	76 (62.8%)	45 (37.2%)	.0007
	50	112 (44.1%)	142 (55.9%)	
Financial Class	Insured	74 (51.4%)	70 (48.6%)	.0019
	Uninsured	8 (57.1%)	6 (42.9%)	
	Medicare	44 (36.7%)	76 (63.3%)	
	Medicaid	62 (63.9%)	35 (36.1%)	
Ethnicity	White	99 (42.9%)	132 (57.1%)	.0002
	African-American	50 (59.5%)	34 (40.5%)	
	Hispanic	37 (71.2%)	15 (28.8%)	
	Other	2 (25%)	6 (75%)	
Histology	Ductal	177 (51.2%)	169 (48.8%)	.2557
	Lobular	10 (41.7%)	14 (58.3%)	
	Other	1 (20.0%)	4 (80.0%)	
Grade	Well-differentiated	20 (30.8%)	45 (69.2%)	.0028
	Moderately-differentiated	85 (57%)	64 (43%)	
	Poorly-differentiated	79 (52.7%)	71 (47.3%)	
	N.A.	4 (36.4%)	7 (63.6%)	
ER Status	Positive	143 (51.3%)	136 (48.7%)	.4592
	Negative	45 (46.9%)	51 (53.1%)	
PR Status	Positive	128 (50.6%)	125 (49.4%)	.7977
	Negative	60 (49.2%)	62 (50.8%)	
Ki-67	<14%	49 (43.8%)	63 (56.3%)	.1140
	14%	138 (52.7%)	124 (47.3%)	
HER-2	Positive	60 (62.5%)	36 (37.5%)	.0050
	Negative	128 (45.9%)	151 (54.1%)	
Molecular Subtype	Her-2 Enriched	15 (57.7%)	11 (42.3%)	.0104
	Luminal-HER-2	45 (65.2%)	24 (34.8%)	
	Luminal A	37 (38.9%)	58 (61.1%)	
	Luminal B	63 (52.5%)	57 (47.5%)	
	Triple Negative	28 (43.1%)	37 (56.9%)	
Tumor Size	T1	44 (31%)	98 (69%)	<.0001
	T2	73 (49.7%)	74 (50.3%)	
	T3	37 (82.2%)	24 (17.8%)	
	T4	34 (87.2%)	5 (12.8%)	

N.A. = data not available

* P value was based on Chi-squared test or Fisher's exact test.

Table 3

Uni- and multi-covariate logistic regression models for lymph node positive breast cancer

Covariate	Uni-covariate		Multi-covariate	
	Odds Ratio (95% CI)	p-value	Odds Ratio (95% CI)	p-value
Age (50 vs. <50)	0.467 (0.300-0.728)	0.001	0.562 (0.337-0.935)	0.0265
Financial Class (Uninsured vs. insured)	1.178 (0.384-3.614)	0.775		
Financial Class (Medicare vs. insured)	0.511 (0.303-0.863)	0.012		
Financial Class (Medicaid vs. insured)	1.565 (0.898-2.726)	0.114		
Ethnicity (African-American vs. White)	1.961 (1.180-3.258)	0.009		
Ethnicity (Hispanic vs. White)	3.289 (1.710-6.326)	<0.001	3.229 (1.533-6.803)	0.002
Ethnicity (Other vs. White)	0.444 (0.088-2.549)	0.327		
Histology (Lobular vs. Ductal)	0.682 (0.295-1.577)	0.371		
Histology (Other vs. Ductal)	0.239 (0.026-2.157)	0.202		
Grade (moderate vs. well-differentiated)	2.987 (1.609-5.545)	0.001	2.692 (1.287-5.630)	0.0085
Grade (poor vs. well-differentiated)	2.503 (1.351-4.636)	0.004	1.879 (0.822-4.297)	0.135
ER Status(negative vs. positive)	0.839 (0.527-1.336)	0.460		
PR Status (negative vs. positive)	0.945 (0.613-1.456)	0.798		
Ki-67 (14% vs. <14%)	1.431 (0.917-2.233)	0.115		
HER-2 Status (negative vs. positive)	0.509 (0.316-0.818)	0.005	NA	
Subtype (HER-2 -Enriched vs. Luminal A)	2.138 (0.886-5.156)	0.091		
Subtype (Luminal-HER-2 vs. Luminal A)	2.939 (1.543-5.599)	0.001		
Subtype (Luminal B vs. Luminal A)	1.733 (1.003-2.992)	0.049		
Subtype (Triple Negative vs. Luminal A)	1.186 (0.625-2.254)	0.602		
Tumor Size (T2 vs. T1)	2.197 (1.359-3.553)	0.001	2188 (1.291-3.710)	0.0036
Tumor Size (T3 vs. T1)	10.301 (4.434-23.931)	<0.001	9.669 (3.940-23.730)	<0.0001
Tumor Size (T4 vs. T1)	15.145 (5.550-41.330)	<0.001	12.522 (4.402-35.620)	<0.0001

CI: confidence interval.

NA: not applicable, HER-2 not included in the model

Table 4

Triple negative breast cancers

Type	N	LN negative	LN positive	p-value Fisher Exact Test
Basal	27	13	14	
Non-Basal	13	8	5	
Total	40	21	19	p=0.2