TITLE

P53 expression in breast cancer discriminates tumours with low probability of non-sentinel nodes infiltration.

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Running title:

Negative nodes and p53 in breast cancer

ABSTRACT

Aim: Several predictive tools of non-sentinel lymph nodes neoplastic involvement when a positive sentinel lymph node is found have been described. However, molecular factors have been rarely evaluated to build these tools. The aim of this study was to establish which factors predicted non-sentinel lymph nodes infiltration in our setting, including some molecular factors.

Methods: We proceeded to a retrospective review of 161 patients with breast cancer, a positive SLN and subjected to ALND, none of which had received neoadjuvant treatment. Features evaluated as predictive factors for non-sentinel node positivity were: menopausal status, tumor size, histological subtype, histological grade, lympho-vascular invasion, extracapsular invasion, Ki67 index, hormonal receptors, CerbB2 and p53 expression, size of sentinel lymph node metastases and number of sentinel lymph nodes affected.

Results: Tumor size (p=0.001), size of sentinel lymph node metastases (p=0.001), lobular invasive carcinoma (p=0.05) and lympho-vascular invasion (p= 0.006) were significantly associated with non-sentinel lymph node positivity. Tumor p53 positive expression was strongly associated with non-sentinel lymph node negativity (p=0.000). In multivariate analysis all these factors but tumor size maintained their significance. The discrimination power of the model calculated by the area under the ROC curve was 0.811 (95% CI 0.741 - 0.880).

Conclusion: p53 expression in breast cancer was highly predictive of non-sentinel lymph node negativity in our study. New studies should evaluate if it would be of use adding p53 expression in other predictive tools previously described.

KEY WORDS:

Sentinel lymph node, Non-sentinel lymph node, Axillary lymph node dissection, p53.

INTRODUCTION

Surgical approach in breast cancer has changed dramatically in recent years. The current practice of axillary lymph node dissection (ALND) in cases with a positive sentinel lymph node (SLN) biopsy has been challenged because of the consequent high morbidity of such dissection. Patients subjected to this procedure have been reported to show severe secondary effects [1,2].

A more conservative approach, as proposed by the Z0011 study of the American College of Surgeons Oncology Group (ACOSOG)[3] and supported by the results of the IBCSG 23-01 trial[4], would circumvent excessive surgery in patients with a low risk of further axillary involvement without impairing survival. Systemic adjuvant treatments and tangential field breast irradiation could be enough to control any axillary residual disease[3].This is also supported by the fact that although the false negative rate in SLN biopsies ranges between 5 and 10% axillary recurrence occurs in less of 2% of SLN negative patients [5].

Even though some concerns about the Z0011 study methodology have been raised [67] the avoidance of ALND in patients with T1 o T2 with only one or two positive SLN has been adopted in many breast units. A further step would be identifying more patients who would benefit from sparing ALND because of their low tumor axillary load. In this setting, predictive tools of non-sentinel node (NSLN) involvement could remain useful. The point is that predictive tools developed in different institutions show lower accuracy when applied to other clinical settings [8].

The aim of our study was to find in our setting which factors predict NSLN infiltration when a positive SLN is found, including clinical factors, pathology and some molecular factors sparsely evaluated in previous reports.

MATERIAL AND METHODS

Patient selection and data collection

The study was a retrospective cohort study. A retrospective review of breast cancer patients treated in the Breast Cancer Unit of the *Hospital del Mar*, Barcelona, was performed from 1 January 2004 to 31 December 2012. Patients included in the study were those with T1 and T2 tumors, whose first treatment was mastectomy or wide local excision (WLE) with a positive SLN biopsy and who had been subjected to ALND (immediate or delayed). The following patients were excluded from the study: those who received neoadjuvant treatment; those with T3, Tis or T1mic following TNM classification; those not undergoing a complete ALND (defined as excision of Berg levels I and II); and those with a negative SLN biopsy. Patients were selected from the Pathology Department database. Clinical data were retrieved from the clinical records. Pathology and molecular data were again retrieved from the Pathology Department database.

Clinical data collected from the patients included age and menopausal status. Pathology data collected from the tumor included size, histology subtype (considering invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC) and others), histological grade (I, II, III) and lympho-vascular invasions (LVI). The SLN information collected included the following: size of SLN metastases (macrometastases, micrometastases or isolated tumor cells (ITCs), as defined in the TNM classification, number of positive SLNs removed and extracapsular invasion. Data collected on ALND pathology included number of NSLN infiltrated.

Data retrieved from the tumor immunohistochemistry study included Ki67 index, estrogen receptor (ER) status, progesterone receptor (PR) status, CerbB2 status and p53 status. Hormone receptors were considered positive if more than 1% of cells showed positive staining by immunohistochemistry, following the American Society of Clinical Oncology/College of American Pathologists guideline[9]. CerbB2 positivity was defined by intense staining in immunohistochemistry or gene amplification by fluorescent in situ hybridization (FISH) following the criteria of the American Society of Clinical Oncology/College of American

Pathologists clinical guideline[9]. P53 was considered positive if 10% or more tumor cells stained by immunohistochemistry, as employed most of the studies about p53 [10].

Pre-surgical Sentinel Node Localization

SLNs were identified following an established protocol in our institution. All patients underwent preoperative lymphoscintigraphy after intratumoral injection of 111 MBq of 99mTc-nanocolloid (GE Healthcare). The volume used depended on whether a tumor was palpable (0.5 mL) or non-palpable (0.3 mL). In non-palpable lesions, radiotracer was injected under ultrasound guidance. In all cases, images were acquired 30-120 min after injection. SLNs were considered to be those first to appear in the dynamic study or in the sequential images in a specific lymph node basin, those directly connected to the injection zone by a lymphatic channel, or those meeting a combination of these criteria. Nodes appearing later in the same lymphatic stations were considered secondary nodes. On the day of surgery, a gamma probe was used to guide dissection to the hot node(s). Counts were recorded per unit of time with the probe in the operative field, before excision (in vivo) and after excision (ex vivo). The wound site was checked for remaining activity.

SLNs and ALND histological examination.

SLNs were routinely examined by serial sectioning. SLNs were cut in two mm slices and for each slice, six sections of 4 μ were obtained, leaving 20 μ of separation between them. Three alternate sections were stained with routine haematoxylin and eosin (H&E) and if negative, the remnant sections were studied immunohistochemically using cytokeratin.

For ALND, three mm slices were obtained for each node and for each slice a single 4 μ section was stained with routine H&E.

Statistical analysis

Data were anonymously collected with Microsoft Access as the patient identity was hidden and patients were identified only with a reference number. Statistical analyses were

performed using PASW version 18 (IBM^{R} SPSS^{R} software). We considered that the minimum number of patients to include in the study was 120. The reason was that using a Chi-square test to compare 80 patients negative for any specified predictor of NSLN positivity with 40 patients positive for that same predictor, significant differences ($p \leq 0.05$) of 25% in NSLN positivity could be detected assuming that the incidence of NSLN positivity in the 80 negative patients was 20%.

Patients were divided into two groups for univariate analyses, namely those with NSLN metastases and those without NSLN metastases. Age between groups was compared using Student's t-test. Univariate analyses with Chi-square or Fisher's exact test were performed to compare categorical variables. Patients with unknown or not applicable data for a specific characteristic were excluded for that specific univariate analysis. A multivariate logistic regression analysis was performed with factors found to be significant in the univariate analyses. The Hosmer and Lemeshow test assessed the goodness-of-fit of the multivariate regression. The discrimination power of the model was quantified by the area under the receiver-operating characteristic (ROC) curve.

All statistical analyses were two sided and p values <0.05 were considered significant.

Ethics

The study was approved by the Ethical Committee for Clinical Research of our research center, the *Institut Mar d'Investigacions Mèdiques* (IMIM)[11].

RESULTS

From 1 January 2004 to 31 December 2012, 189 patients with T1 and T2 tumors initially treated with surgery had a positive SLN biopsy. Of these patients, 161 were subjected to ALND in our center and 54 among them (33.5%) had NSLN involvement. The reasons for not performing ALND in 28 patients were: 12 patients were included in a Spanish multicenter randomized trial (ATM 048/13/2000) and randomized to no ALND, 6 refused any further

surgical intervention, 6 patients had comorbid conditions not advising further interventions and 4 patients were lost of follow-up (no data found in clinical reports).

The mean age of the NSLN-negative group and NSLN-positive group was 58.76 (range 31-83) and 61.66 (range 26-86) years respectively; this difference was not significant (p=0.172).

The description and distribution of the categorized variables and the results of the univariate analysis are shown in Table 1. As a consequence of the low number of patients showing ITCs in the SLN (only 2 patients), univariate analysis for metastasis size in SLN was categorized in two: patients with macrometastases in one group and patients with micrometastases or ITCs in the other group.

As shown, percentage of NSLN infiltration was significantly higher in T2 tumors than in T1 tumors (p=0.001), in patients with SLN macrometastases than in those with micrometastases or ITC (p=0.001), when there was presence of LVI in the tumor (P= 0.006), in ILC compared to other pathology subtypes (p=0.005) and in p53-negative tumors compared to p53-positive ones (p= 0.000). The other factors analyzed were not statistically significant.

Multivariate analysis revealed the same significant predictive factors for positive NSLN, except for tumor size, which lost its significance, although it continued to show a clear trend towards it (Table 2).

Hosner and Lemeshow test confirmed the good of fit of the model based on multivariate logistic regression (p=0.515) and area under the ROC curve was 0.811 (95% CI 0.741 - 0.880), confirming accuracy and good discrimination power (figure 1).

DISCUSSION

Our data regarding the prognostic factors of NSLN involvement are partially consistent with the findings of other studies [12–15] and support the need to redefine indications of ALND in some settings as NSLN involvement was detected in only 33.5% of our patients.

We acknowledge that this is a retrospective evaluation with a limited number of patients included. The real value of the factors herein studied and reported to be predictors of NSLN involvement should be tested in a large prospective study, to validate its clinical usefulness. The use of any predictive nomogram in the clinical setting is highly controversial as all of these predictive tools have been built with retrospective data and a reliable prospective study of their usefulness in taking clinical decisions has not yet been published. Furthermore, developing predictive tools of NSLN involvement in the Z011 trial era could lack of any value, as after obtaining one or two positive SLNs the decision to perform an ALND in many cases would depend on other few well established criteria, as number of SLN infiltrated [16]or extensive extracapsular invasion[17]. In the counterpoint, all those patients not fulfilling the Z0011 trial criteria could benefit also of sparing ALND if it was clear somehow that the remnant tumoral load is minimal. In this scenario, a reliable predictive tool would seem helpful to discuss with the patient the expected benefit of further surgery.

Multiple studies have attempted to define prognostic factors of NSLN involvement. The main significant factors according to some reviews are: tumor size > 20 mm, SLN metastases greater than two mm, evidence of LVI in the tumor, extranodal invasion, tumor histologic grade, and number of positive SLNs[1018,19]. Other factors, such as tumor histology or molecular subtype, number of SLNs removed, proportion of positive SLN/total SLN removed, number and distribution of tumor foci, tumor location, cerbB2 positivity, HR status and age have given controversial results in different institutional studies and meta-analysis [8,18–20].

We consider that the main finding of our research was that a positive p53 expression was a strong negative predictor for NSLN involvement. A limitation of our study was that the number of patients with p53-positive tumors was low. Another limitation of our study was that TP53 gene mutational status was not studied in our set of tumors. As a TP53 mutation not always

translates in p53 overexpression[21,22], our study is unable to conclude if TP53 mutation for itself could account for a high probability of negative NSLNs.

Despite these limitations, the statistical significance was high. Little is described in literature about the loco-regional behavior of tumors overexpressing p53. They are generally considered aggressive cancers[21,22]. It is important to acknowledge that incidence of axillary invasion varies depending on the breast cancer phenotype, triple negative tumors showing less axillary involvement despite their aggressive behaviour[23]. The aggressive behavior of breast cancers showing low axillary involvement would depend mainly in their ability to start hematological dissemination very early during tumor growth. We have started a new study in our department to evaluate local behavior of all breast tumors overexpressing p53 by immunohistochemistry.

In our study ILC was more likely to present NSLN metastasis when compared to IDC and other histologic types (63.2% vs. 29.6%). Some nomograms described in literature include the pathology subtype as a significant factor predicting NSLN involvement[19] but other reviews have not found this feature significant[8]. Sensitivity of axillary ultrasound has been reported to be much lower in ILC compared to other pathology subtypes (36% vs. 76%)[24]. This difference in axillary ultrasound sensibility could be an explanation for our finding: despite performing axillary ultrasound to all our patients previously to surgery, SLN biopsy would have been performed in a high proportion of ILC with a significant axillary neoplastic load as a result of ultrasound inability to detect axillary infiltration in these cases.

Our data confirm the relevance of tumor LVI and SLN macrometastasis as predictive factors of NSLN involvement. Tumor size lost statistical significance in our study in multivariate analyses but its p-value was almost significant. As tumor size is a key clinical factor in breast cancer prognosis and a significant factor in most studies, we decided to keep its value when building a tool to calculate the risk of further axillary involvement.

Extranodal invasion has been described as a strong predictor of NSLN invasion in a meta-analysis [25] but it showed not predictive value in our study. Again, performing preoperative axillary ultrasound systematically on all our breast cancer patients would explain the low incidence of extranodal invasion in our cohort and therefore the absence of significance in the statistical analysis.

We agree with the statement of Koca et al that after comparing 14 predictive nomograms concluded that each breast center should find the most suitable predictive tool [8]. This is supported by Tvedskov et al, who reported a decrease in accuracy when a predictive Finnish model was tested in a Danish cohort[26]. Some predictive factors may be coincident between centers while other maybe not, depending on staging methods and epidemiologic factors.

Other promising tools for predicting NSLN tumor load are being developed, as the OSNA (for One Step Nucleic Acid) technique that detects SLN metastases counting CK 19.9 mRNA copies. In a recent research by Banerjee et al it was shown that mRNA copy numbers <1400 were never associated with histologically proven metastasis in NSLN at ALND[27]. It would be interesting to find out if this cut off for mRNA copy numbers may vary among different breast cancer subtypes or among tumors showing different molecular features as p53 expression.

In conclusion, on the basis of our findings, we propose that some factors predicting NSLN involvement may vary between centers. This could be caused by the differences in breast cancer staging methods when evaluating the axilla. In our clinical setting tumor size, size of SLN metastases, ILC, LVI, and soundly p53 status were predictors of NSLN involvement. Further studies in other centers are needed to confirm p53 expression as a strong predictor of NSLN negativity and if so, its inclusion in predictive nomograms would be strongly recommended.

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Disclosure

All of the authors declare they have no conflict of interest.

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Table I: Description of the cohort and univariate statistical analysis. Not reported or unknown results were excluded for the analysis

Characteristics	Total*	Negative NSLN**	Positive NSLN**	P value
Menopause				
Premenopausal	46 (28.57)	33 (71.7)	13 (28.3)	0.370
Postmenopausal	115 (71.43)	74 (64.3)	41 (35.7)	
Tumour size				
pT1	108 (67.1)	81 (75)	27(25)	0.001
pT2	53 (32.9)	26(41.9)	27(50.9)	
Histological grade				
I	35 (21.7)	23 (65.7)	12 (34.3)	0.767
II	60 (37.3)	43 (71.17)	17 (28.3)	
III	44 (27.3)	32 (72.7)	12 (27.3)	
Not reported	22 (13.6)	9 (41)	13 (59)	
Histology subtype				
IDC and others	142 (88.2)	100 (70.4)	42 (29.6)	0.005
ILC	19 (11.8)	7 (36.8)	12 (63.2)	
Oestrogen receptor				
Positive	145 (90)	93 (64.2)	52 (35.8)	0.06
Negative	16 (10)	14 (87.5)	2 (12.5)	
Progesterone receptor				
Positive	114 (70.8)	74 (65)	40 (35)	0.37
Negative	47 (29.2)	33 (70.3)	13 (29.7)	
c-erbB2				
Positive	17 (10.6)	10 (58.8)	7 (41.2)	0.588
Negative	144 (89.4)	97 (67.4)	47 (32.6)	
Ki 67 index				
<15	40 (24.8)	30 (75)	10 (25)	0.373
$\geq$15	53 (33.0)	35 (66)	18 (34)	
Unknown	68 (42.2)	42 (61.8)	26 (38.2)	
P53				
Positive	29 (18)	28 (96.6)	1 (3.4)	0.000
Negative	130 (80.7)	78 (60)	52 (40)	
Unknown	2 (1.3)	1 (50)	1 (50)	
Lymphovascular invasion				
Yes	36 (22.4)	17 (47.2)	19 (52.8)	0.006
No	125 (77.6)	90 (72)	35 (28)	
Size of SLN metastases				
>2 mm	113 (70.2)	66 (58.4)	47 (41.6)	0.001
$\leq$ 2 mm	48 (29.8)	41 (85.4)	7 (14.6)	
Number of positive SLN				
1	122 (75.7)	84 (68.9)	38 (31.1)	0.255
>1	39 (24.3)	23 (59)	16 (41)	
Nodal extracapsular invasion				
Yes	8 (5)	5 (62.5)	3 (37.5)	1
No	153 (95)	102 (66.7)	51 (33.5)	

* Percentage of the subtype within a specific characteristic in parenthesis

** Percentage of patients with negative or positive NSLN within that subtype in parenthesis

IDC: invasive ductal carcinoma

ILC: invasive lobular carcinoma

TABLE 2
Multivariate analysis for potentially significant pathology and molecular characteristics predicting non-sentinel lymph nodes metastases

Factor	Odds Ratio	95% CI	P-value
Size > 20 mm (T2)	2.171	0.944 - 4.995	0.068
Invasive lobular carcinoma	3.349	1.090 - 10.287	0.035
SLN*metastasis size (< 2 mm)	0.292	0.113 - 0.757	0.011
Lymphovascular invasion	2.746	1.073 - 7.080	0.035
p53 positive	0.048	0.006 - 0.379	0.004

*SLN: Sentinel lymph node

Legend for figure 1:

Receiver-operating characteristic (ROC) curve calculation using the results of our multivariate analysis, area under the curve was 0.811 (95% CI 0.741 - 0.880).

Figure 1: