Evaluation of molecular classification by immunohistochemistry in advanced breast cancer treated with epirubicin and docetaxel: clinical, pathological, therapeutic and prognostic differences

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Abstract

Objective: To describe clinicopathological differences in molecular subtypes classified by immunohistochemistry (IHC) in patients with locally advanced breast cancer and to study the survival in each subtype. **Patients and Methods:** Immuno-histoquímica staining of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) was performed in order to classify the carcinoma into five molecular subtypes. Clinical, pathological and biological factors were analyzed among subtypes using Kaplan-Meier curves and Cox proportional hazards. **Results:** Luminal A (LA: 40.4%) and triple negative (TN: 24.6%) tumors were the most frequent and had the best rate of disease free survival (DFS) at 5-year (80% and 67%, respectively; $P = 0.016$). Luminal B/HER2 (LB/HER2) negative tumors showed more aggressive characteristics: large and undifferentiated tumors with nodal involvement N2, and reduced DFS (48%); they share tumoral similarities and poor prognosis with the LB/HER-positive. HER2 tumors are frequent in postmenopausal women, they are undifferentiated, with high percentage nodal N2 associated to Stage IIIB and poor DFS (58%). In the multivariate analysis, the subtype LA had the best prognosis. The luminals B (both) and TN had the worst prognosis in the death risk with a hazard ratio of 3.3 ($P = 0.004$) and 3.4 ($P = 0.003$), respectively. **Conclusion:** Molecular classification by IHC is accessible, simplified and allows us to establish clinical, pathological, treatment and prognosis differences in the different subtypes.

Key words: Neoadjuvant chemotherapy. Immunohistochemistry. Molecular subtype. Advanced breast cancer. Docetaxel. Epirubicin.

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Introduction

Breast cancer histological classification is mainly based on morphology and ductal carcinoma is the most common variant (70-86%)¹, which entails higher morbidity and mortality. Even when tumors have clinical, histopathologic or stage-wise similar profiles, they can have marked differences in response (carcinoma clinical and prognostic behavior) that are thought to be due to molecular factors²⁻⁵.

Breast cancer is a heterogeneous condition, and the histologic diagnosis obtained from biopsy-extracted tissue requires further support of multigene testing to identify the gene expression profile and its correlation with the different phenotypes, which enables changing the way cancer is classified²⁻⁶.

Advances in molecular biologic techniques have enabled breast cancer simplified molecular classification with a panel of immunohistochemistry (IHC) biomarkers, since obtaining information from a genomic relationship matrix is not always feasible. The most widely known markers are estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2) and Ki-67, which define the treatment and prognosis of the disease. This allows for carcinomas to be categorized in subtypes that are equivalent, but not identical to the intrinsic subtypes²⁻¹⁴, and is a valid alternative to the costly genomic microarrays^{7-11,14}.

There are specific biomarkers that enable sub-classification, such as basal cytokeratins (Ck) (Ck5, Ck6, Ck14, Ck17), which can identify basal cell-like (BCL) tumors^{2,4,12-14}. Ki-67 enables discriminating luminal A and B tumors^{13,14}. Other biomarkers are also helpful in carcinoma sub-classification (EGFR, P63, BCL-2, p53, MUC1 and COX-2)^{11,14}.

This report describes the prevalence of the main clinical, pathologic and IHC characteristics, as well as the clinical course of each molecular subtype; the therapeutic implications and survival pattern of each subtype are also identified in patients with locally advanced breast cancer (LABC) who underwent neoadjuvant chemotherapy (NC) with anthracyclines and taxanes.

Patients and methods

Patient selection

Longitudinal, retrospective study assessing patients with malignant breast tumors who received medical care at the High Specialty Medical Unit of No.3

Obstetrics and Gynecology Hospital of La Raza National Medical Center, IMSS, during the period from January 5, 2009, to December 31, 2015. A total of 126 patients with ages ranging from 18 to 75 years, with clinical diagnosis of LABC (stage III) and inflammatory carcinoma (cT4d) without distant metastasis were included. Breast cancer was staged according to the American Joint Committee on Cancer Criteria (6th edition)¹⁵.

The breast was assessed by physical examination and imaging techniques (mammography and/or ultrasound); each patient underwent imaging studies to document distant disease: bone scan, liver ultrasound and chest X-ray.

Tissues were obtained by tru-cut or open incisional biopsy for the primary invasive carcinoma diagnosis; molecular diagnosis was obtained by ER, PgR and HER2 IHC staining. Hormone receptor (HRe: ER and PgR) cell staining higher than 1% was regarded as positive (+). HER2 amplification by IHC was considered to be positive with 3+ staining or 2+ with positive chromogenic in situ hybridization technique. HER2 1+ or 0 staining was regarded as negative (-).

Multimodal treatment plan

Details on the chemotherapy regimen and surgery are described in a previous study¹⁶. Patients received a NC regimen of 6 to 8 cycles based on sequential epirubicin and docetaxel (E-D) followed by surgery and radiotherapy. Patients with positive HRe by IHC received adjuvant tamoxifen 20 mg/day for at least 5 years; no patient with HER2+ report received neoadjuvant trastuzumab.

Pathologic response definition

Pathologic response to NC was assessed when the breast and lymph nodes (LN) were extirpated. Pathologic complete response (pCR) was defined as complete tumor remission of every invasive cell in the breast and axillary LN; residual disease in the breast, such as carcinoma in situ (CIS), was included in the pCR.

Infiltrating ductal carcinoma (IDC) and infiltrating lobular carcinoma (ILC) comprise almost the totality of breast carcinomas; for statistical purposes, we established 3 histologic groups: IDC, ILC and other carcinomas (e.g. metaplastic, mixed, and inflammatory, among others less common) characterized by the worst prognosis.

Classification of molecular subtypes

According to St. Gallen 2013 recommendations¹⁷, we used the molecular classification by dividing breast cancer into 5 subtypes. The novelty was the inclusion of LB/HER2- carcinoma by defining it as a tumor with ER+ and PgR < 20%, with Ki-67 > 14% and HER2-. Tumor histologic grade was assessed using the modified Bloom-Richardson system¹⁸.

Since biomarkers such as Ki-67, e-cadherin, epidermal growth factor receptor, CK5/6 and CK14 were not available, we used molecular alterations of the 3 IHC-assessed biomarkers that are associated with tumor grade. We used the biological classification proposed in the study by von Minckwitz⁸: LA, LB/HER2-, LB/HER2+, HER2+ and TN. For the latter subtype, we preferred to use the simple classification based on ER, PgR and HER2 negative expression.

Statistical analysis

The main purpose was to describe the most important clinicopathological features according to the IHC-defined molecular subtype. The second purpose was to examine the prognostic relevance of each one of the subtypes on 5-year disease-free survival (DFS) and overall survival (OS).

Age (years) and tumor size (cm) were regarded as continuous variables and were assessed by means of single-factor variance (ANOVA). Pair-wise differences between molecular subtypes were assessed with Tukey's correction by means of adjusted P-values. Normality assumptions were corroborated by means of bias, kurtosis, bar chart and Shapiro-Wilk hypothesis test calculation. Age and tumor size were also assessed as ordinal variables.

Clinical, pathologic and IHC categorical variables (ER, PgR and HER2) in the study population were described as frequencies and percentages according to the primary tumor molecular subtype and were assessed using χ^2 tests.

Kruskal-Wallis test was used for ordinal variables when the 5 molecular subtypes were compared: age (< 40 years, 41 to 50 years, > 51 years), tumor size (T2, T3, T4), clinical lymph node (N1, N2, N3) and clinical stage (IIIA, IIIB, IIIC).

DFS and OS were calculated with the Kaplan-Meier method¹⁹ and log-rank tests were carried out to compare the groups. The DFS interval was calculated from the date of surgery to the date of the first event (local, regional or distant relapse) or to the date of death for any reason.

The OS interval was recorded from the date of NC initiation to the date of death for any reason or last contact. Lost and uneventful cases were censored. DFS and OS intervals were recorded in months.

Cox²⁰ univariate regression analysis was used to identify the prognostic relevance of the crude hazard ratio (HR) of molecular factors (LA, LB/HER2-, LB/HER2+, HER2, TN) related to clinical and pathologic variables that influence on survival. Subsequently, those variables that yielded a univariate result of P.10 were included in the multivariate model.

The Cox²⁰ multivariate regression analysis was carried out using the stepwise forward elimination method with the model established at P.10. The molecular factors (subtype) were adjusted to the primary clinical (age, tumor size, lymph node) and pathologic variables (histologic type, histologic grade, lymphovascular invasion [LVI]). Dummy variables were categorized and models were constructed with all predictors regardless of statistical significance.

Log rank-tests were used in the univariate and multivariate models to compare differences in the time-to-event outcome. The results were expressed in HRs with their corresponding 95% confidence intervals (CI).

All tests were two-tailed. The level of statistical significance was established at $p < 0.05$ or 95% CIs were not to include the 1 value. The database and statistical analyses were carried out using the statistical program SPSS, version 15 (SPSS Inc., Chicago, IL).

Results

Clinicopathological variables correlation with molecular subtypes

Clinical and pathological characteristics of all cases associated with each molecular subtype are described in tables 1 and 2. Most cases were LA (40.4%), followed by TN tumors (24.6%).

No significant differences were observed at diagnosis when patient mean age was compared between the molecular subtypes (ANOVA, $F = 0.696$; $p = 0.596$) (Table 1). When the differences were pair-wise assessed, the highest difference, without being significant, was observed when LB/HER2- subtype patients' mean age was compared versus the LB/HER2+ ($p = 0.732$) and TN ($p = 0.529$) subtypes.

When stratified by age group, LB/HER2- (57.9%), LA (51%) and HER2 tumors (50%) tend to occur in older women (> 50 years). LB/HER2+ cases were more common in the 41 to 50 years' age group. The occurrence

Table 1. Molecular subtypes baseline clinicopathological characteristics

Molecular subtype: Characteristic	LA (HRe+, HER2-)	LB (HRe+, HER2-)	LB/HER2 (HRe+/-, HER2+)	HER2 (HRe-, HER2+)	TN (HRe-, HER2-)	p
	n = 51 (40.4%)	n = 19 (15.0%)	n = 13 (10.3%)	n = 12 (9.5%)	n = 31 (24.6%)	
Age (years) Average $\pm$ SD	51.2 $\pm$ 8.6	53.6 $\pm$ 9.0	49.5 $\pm$ 11.1	51.0 $\pm$ 7.6	49.4 $\pm$ 10.3	0.596
Age (stratum)						
< 40 years	4 (7.8)	2 (10.5)	1 (7.6)	1 (8.3)	7 (22.6)	0.212
41 to 49 years	21 (41.2)	6 (31.6)	6 (46.2)	5 (41.7)	12 (38.7)	
> 50 years	26 (51.0)	11 (57.9)	6 (46.2)	6 (50.0)	12 (38.7)	
Tumor (cm) Average $\pm$ SD	7.0 $\pm$ 1.6	8.7 $\pm$ 2.3	9.0 $\pm$ 2.9	8.8 $\pm$ 3.3	8.0 $\pm$ 2.2	0.004
Clinical tumor (cT)						
cT2	3 (5.9)	3 (15.8)	0 (0.0)	0 (0.0)	0 (0.0)	0.037
cT3	33 (64.7)	3 (15.8)	7 (53.8)	5 (41.7)	13 (41.9)	
cT4	15 (29.4)	13 (68.4)	6 (46.2)	7 (58.3)	18 (58.1)	
Clinical lymph node (cN)						
cN1	30 (58.8)	5 (26.3)	4 (30.8)	3 (25.0)	14 (45.2)	0.032
cN2	21 (41.2)	13 (68.4)	9 (69.2)	8 (66.7)	16 (51.6)	
cN3	0 (0.0)	1 (5.3)	0 (0.0)	1 (8.3)	1 (3.2)	
Clinical stage (cIII)						
cIIIA	36 (70.6)	6 (31.6)	7 (53.8)	5 (41.7)	13 (41.9)	0.012
cIIIB	15 (29.4)	12 (63.2)	6 (46.2)	6 (50.0)	17 (54.8)	
cIIIC	0 (0.0)	1 (5.3)	0 (0.0)	1 (8.3)	1 (3.3)	
ER by IHC						
Positive	51 (100)	19 (100)	13 (100)	0 (0.0)	0 (0.0)	0.000
Negative	0 (0.0)	0 (0.0)	0 (0.0)	12 (100)	31 (100)	
PgR by IHC						
Positive	42 (82.4)	14 (73.7)	8 (61.5)	0 (0.0)	0 (0.0)	0.000
Negative	9 (17.6)	5 (26.3)	5 (38.5)	12 (100)	31 (100)	
HER2 by IHC						
Negative	51 (100)	19 (100)	0 (0.0)	0 (0.0)	31 (100)	0.000
Positive	0 (0.0)	0 (0.0)	13 (100)	12 (100)	0 (0.0)	

LA: luminal A; LB: luminal B/HER2-; LB/HER2: luminal B/HER2+; pure HER2: human epidermal growth factor receptor 2; TN: triple-negative; c: clinical; SD: standard deviation; ER: estrogen receptor; PgR: progesterone receptor; IHC: immunohistochemistry.

of TN tumors is common in the premenopausal woman (61.3%) (Table 1).

With regard to primary tumor size at diagnosis, the largest average diameter of initial tumor occurred in LB/HER2+ tumors (9 $\pm$ 2.9 cm), followed by LB/HER2- (8.7 $\pm$ 2.3 cm) and HER2 tumors (8.8 $\pm$ 3.3 cm) (Table 1). Significant differences were observed in tumor size (ANOVA, $F = 4.063$; $p = 0.004$). When pair-wise assessed, the greatest significant difference was observed in LA versus LB/HER2- ($p = 0.037$) and LB/HER2+ tumors ($p = 0.036$).

As regards clinical tumor (cT), there was a higher trend towards cT4 (skin lesion: ulcer, edema or erythema) in high-risk tumors: LB/HER2- (68.4%), HER2 (58.3%) and TN (58.1%); conversely, the trend towards cT3 or cT2 presentation often occurs in LA (70.6%) and LB/HER2+ tumors (53.8%) with moderate grade.

With regard to clinical axillary lymph node disease (cN), the analysis of HER2 tumors with overexpression detected a higher prevalence of cN2 lymph nodes in the LB/HER2+ (69.2%) and HER2 subtypes (66.7%), and in the absence of HER2 expression, cN2 lymph nodes were more prevalent in the LB/HER2- (68.4%) and TN (51.6%) subtypes. A trend towards cN1 axillary lymph node disease was detected in LA tumors (58.8%) (Table 1).

Advanced stage disease (stage III) showed significant differences ($p = 0.012$). The LB/HER2- (63.2%), HER2 (50%) and TN (54.8%) subtypes showed a higher percentage of stage IIIB cases; conversely, the trend towards stage IIIA tumors was more common in the LA (70.6%) and LB/HER2+ subtypes (53.8%). Stage IIIC was the subgroup with the lowest frequency (3 cases) (Table 1).

Table 2. Molecular subtypes baseline clinicopathological characteristics

Molecular subtype:	LA (HRe+, HER2-)	LB (HRe+, HER2-)	LB/HER2 (HRe+/-, HER2+)	HER2 (HRe-, HER2+)	TN (HRe-, HER2-)	p
Characteristic	n = 51 (40.4%)	n = 19 (15.0%)	n = 13 (10.3%)	n = 12 (9.5%)	n = 31 (24.6%)	
Histologic grade						
Grade 2	51 (100.)	0 (0.0)	8 (61.5)	2 (16.7)	17 (54.8)	0.000
Grade 3	0 (0.0)	19 (100.)	5 (38.5)	10 (83.3)	14 (45.2)	
LV invasion						
No	39 (76.5)	8 (42.1)	9 (69.2)	10 (83.3)	23 (74.2)	0.067
Yes	12 (23.5)	11 (57.9)	4 (30.8)	2 (16.7)	8 (25.8)	
Affected side						
Right breast	27 (52.9)	6 (31.6)	7 (53.8)	5 (41.7)	15 (48.4)	0.562
Left breast	24 (47.1)	13 (68.4)	6 (46.2)	4 (58.3)	16 (51.6)	
Quadrant						
UEQ	27 (52.9)	14 (73.7)	8 (61.5)	10 (83.4)	20 (64.5)	0.400
LEQ	3 (5.9)	1 (5.3)	1 (7.7)	1 (8.3)	2 (6.5)	
UIQ	6 (11.8)	1 (5.3)	2 (15.4)	1 (8.3)	5 (16.1)	
LIQ	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.5)	
Central	14 (27.5)	3 (15.7)	2 (15.4)	0 (0.0)	2 (6.5)	
Menopausal status						
Pre-menopause	21 (41.2)	7 (36.8)	7 (53.8)	4 (33.3)	15 (48.4)	0.770
Post-menopause	30 (58.8)	12 (63.2)	6 (46.2)	8 (66.7)	16 (51.6)	
Inflammatory carcinoma						
No	50 (98.0)	16 (84.2)	12 (92.3)	11 (91.7)	25 (80.6)	0.099
Yes	1 (2.0)	3 (15.8)	1 (7.7)	1 (8.3)	6 (19.4)	

LA: luminal A; LB: luminal B/HER2-; LB/HER2: luminal B/HER2+; pure HER2: human epidermal growth factor receptor 2; TN: triple-negative; LV: lymphovascular; ILC: infiltrating lobular carcinoma; IDC: infiltrating ductal carcinoma.

According to the histologic grade, grade 2 (G2) carcinomas were especially predominant in the LA (100%) and LB/HER2+ subtypes (61.5%). Grade 3 (G3) was common in subtypes LB/HER2- and HER2 (100% and 83.3%, respectively), which are related to larger tumors (Table 2).

IDC histogenesis was predominant in 4 subtypes, particularly in LB/HER2- (73.7%) and TN (74.2%). In contrast, ILC histogenesis was mainly observed in patients with LA subtype (60.8%) (Fig. 1). A higher percentage of pCR was observed in groups phenotypically with HER2 overexpression regardless of HR status: HER2+/HR- (41.7%) and HER2+/HR+ (38.5%). The LB/HER2- subtype had the lowest pCR rate (21.1%) (Fig. 2).

For the entire group, a slight predominance of left breast tumors (52.4%) was observed at diagnosis. When assessed by subtype, tumors were located mainly at upper left external quadrant (52.9 to 83.4%) in all molecular subtypes. All luminal tumors: LA (27.5%), LB/HER2- (15.7%) and LB/HER2+ (15.4%) tended to develop towards the central region of the breast (Table 2).

HRe+ disease expression was dominant in all luminal subtypes, with all tumors positively staining for ER and PgR in 65.8% and 50.7%, respectively. HER2

overexpression occurred in 19.8% of cases. Postmenopausal women accounted for a slightly higher number of cases (n = 72). LB/HER2- (57.9%) and LB/HER2+ tumors (30.8%) were more affected by LVI owing to their aggressive histology. Inflammatory carcinoma is often associated with tumors with the poorest prognosis: LB/HER2- (15.8%) and TN (19.4%).

Molecular subtypes survival

Over a mean 57.7 ± 2.4 -month follow-up (95% CI: 52.8 to 62.6 months) there was a period of 6,535 person-years recorded, with 51 relapses (40.4%) and 35 deaths (31.7%).

The univariate survival analysis by means of the Kaplan-Meier method detected clear differences between the molecular subgroups: the cases with HER2 (58%) and LB/HER2- (48%) showed a reduced 5-year DFS in comparison with the LA, TN and LB/HER2+ cases, which showed a better DFS rate regardless of pCR (80%, 67% and 63%, respectively). The main DFS difference occurred in LA versus LB/HER2- and HER2 tumors (p = 0.016) (Fig. 3A).

Figure 1. Carcinoma distribution according to molecular subtypes histogenesis. Lobular carcinoma was predominant in the LA subtype (60.8%), while ductal carcinoma was it in 4 subtypes, particularly in LB (73.7%) and TN (74.2%). Ca: carcinoma; LA: luminal A; LB: luminal B/HER2-; LB/HER2: luminal B/HER2+; pure HER2: human epidermal growth factor receptor 2; TN: triple-negative.

Figure 2. pCR distribution according to molecular subtypes. The LB/HER2 (38.5%) and HER2 subtypes (41.7%) showed the best pCR rates, whereas the LB subtype had the lowest pCR rate (21.1%) LA: luminal A; LB: luminal B/HER2-; LB/HER2: luminal B/HER2+; pure HER2: human epidermal growth factor receptor 2; TN: triple-negative; pCR: pathologic complete response.

After receiving NC, only two subtypes showed a superior effect on 5-year OS: LA (84%) and TN (67%). The subtypes with the poorest survival were those of patients with LB/HER2-, LB/HER2+ and HER2 tumors

(53%, 48% and 51%, respectively). The largest significant OS difference occurred in LA tumors in comparison with the group with the HER2-positive phenotype (HER2+ and LB/HER2+; $p = 0.022$) (Fig. 3B).

Figura 3. Kaplan-Meier curves analysis. Patients with advanced-stage breast cancer were stratified by molecular subtypes; there are significant differences in DFS (A) and OS (B).

Molecular subtypes prognostic significance

Cox univariate and multivariate analysis was carried out in order to examine between-subtype differences with regard to clinical and pathologic factors. ER, PgR and HER2 parameters have been used to define each one of the molecular subtypes, and these parameters were therefore not used to model their data.

The Cox univariate analysis results show that age, tumor size, histology and lymph node status were independent DFS predictors in some subtypes; however, in the multivariate analysis, only tumor size and grade had significant prognostic relevance.

In the multivariate analysis, tumor size was an independent DFS clinical predictor in all subtypes. The mortality risk in patients with cT4 TN tumors is 3.4-fold higher ($p = 0.003$, such as in LB/HER2- (HR = 3.3; $p = 0.003$), LB/HER2+ (HR = 3.3; $p = 0.004$) and HER2 tumors (HR = 3.1; $p = 0.005$) (Table 3).

The pathologic predictor that significantly impacted on prognosis was G3, with a similar mortality risk being observed in LB/HER2- and LB/HER+ tumors (hormone receptor-positive group), as well as in HER2 and TN tumors (hormone receptor-negative group), with HRs of 2.4 ($p = 0.040$), 2.5 ($p = 0.019$), 2.2 ($p = 0.046$) and 2.5 ($p = 0.019$), respectively. The other pathological factors were also assessed without any significant prognostic relevance being found.

DFS average for LA was 66.1 ± 2.8 months, for LB/HER2-, 42.5 ± 6.9 months, for LB/HER2+, $47.9 \pm$

5.8 months, for HER2, 42.8 ± 8.6 months, and for TN, 48.5 ± 3.8 months. This way, DFS was better for LA in comparison with the other 4 subtypes, which are characterized for a poor prognosis.

Discussion

Hormone receptor and HER2 testing with IHC staining, which can be used as an approach to breast cancer category or molecular class, has demonstrated its usefulness by classifying tumors according to clinical results in similar strata, just as defined in the gene expression profile, which suggests this classification is robust^{2,4,6,9-11,14}.

Several studies suggest using 4 to 5 biomarkers (ER, PgR, HER2, HER1 and CK5/6), and others suggest additionally including the Ki-67 biomarker to specifically classify carcinomas, observing that there is good correlation between the gene profile and the profile by IHC^{4,6,9-14}.

Molecular subtypes clinicopathological profile

LUMINAL A SUBTYPE

It is the most commonly recorded tumor in various studies (39% to 67%)^{6,8-11,21-24}. Tumors with the HR+ and HER2- phenotype with moderate histologic grade (G2) or grade 1 (42% to 89%)^{6,10,21} are more likely to

Table 3. Clinicopathological characteristics prognostic impact on DFS by molecular subtype by means of Cox univariate and multivariate analysis (n = 126)

Univariate analysis	LA		LB		LB/HER2+		HER2		TN	
	Ref.		HR	p	HR	p	HR	p	HR	p
Age: < 50 vs. > 50	1		3.8	0.006	2.2	0.178	4.1	0.013	2.0	0.128
Tumor: < T3 vs T4	1		2.4	0.079	1.9	0.283	3.4	0.032	1.4	0.416
LN: N1 vs. N2-3	1		3.3	0.014	2.0	0.252	3.7	0.025	1.9	0.167
Grade: G2 vs. G3	1		1.8	0.386	1.6	0.467	1.9	0.356	1.3	0.586
Carcinoma: L vs. D	1		4.7	0.005	2.6	0.133	5.0	0.009	2.4	0.099
Univariate analysis	HR*	P	HR*	p	HR*	p	HR*	p	HR*	p
Tumor: < T3 vs T4	3.2	0.004	3.3	0.003	3.3	0.004	3.1	0.005	3.4	0.003
Grade: G2 vs. G3	1.8	0.219	2.4	0.040	2.5	0.019	2.2	0.046	2.5	0.019

DFS: disease-free survival; HR: crude hazard ratio; LA: luminal A; LB: luminal B/HER2-; LB/HER2: luminal B/HER2+; HER2: human epidermal growth factor receptor 2; TN: triple-negative; Ref: reference; L: lobular; D: ductal; LN: lymph node.

*HR adjusted by age, tumor, lymph node and histology.

be LA. They exhibit favorable clinical characteristics, and it is the subtype that includes higher ILC percentage (15% to 23%)²². At diagnosis, these tumors are relatively small (< 2 cm) or stage I-II (44% to 47%)^{6,11,21}.

High expression of HR+ disease (ER alpha and/or PgR) is common, especially ILC shows ER positive status (60% to 90%) and genes related to ER activation (e.g., LIV1, cyclin D1)^{3,25}, as well as high levels of BCL-2 and GATA3²⁶ and low Ki-67 indices (7% to 33.9%)^{5,10,11,22,24}. These tumors express low mitotic index (69%)⁶ and less frequent p53 mutation (15%)^{5,6,11}, they do not express HER2/neu and are characterized for losing e-cadherin expression, which is present in ductal carcinomas⁴.

Treatment response

These phenotypically HRe+ and HER2- tumors generally show low response or resistance to chemotherapy^{8,11,14}. Other studies conclude the opposite, and recommend prolonged chemotherapy treatment (for longer than 16 weeks)²⁷. These tumors are often highly sensitive to endocrine therapy^{11,14,27}.

Prognosis

Most cases evolve with a good prognosis, by showing higher survival and low rates of recurrence^{2,3,9-11}. The pCR rate ranges from 3% to 8.9%, indicating that pCR would not be a marker of survival for this subtype^{8,11,22,25,27,28}.

From the study

T3 tumors (64.7%) and cN1 lymph node involvement (58.8%) were common. In contrast with other studies^{9-11,22,24,27}, our data showed a higher percentage of pCR (29.4%); one possible explanation for this is the higher number of ILC cases with G2 LA tumors and less ILC with pCR recorded in the other subtypes. We observed the best DFS rate in favor of LA tumors (80%) and a mortality rate of 22.9%. In the multivariate analysis, the mortality risk is 2.2-fold higher in cT4 tumors (stage IIIB) in comparison with < T3 tumors.

LB/HER2- (OR LB) SUBTYPE

Its frequency is lower than that of the LA subtype (8.5% to 39.8%)^{8,22,24}. The St. Gallen consensus¹⁷ divides the luminal B subtype in two groups: LB with HER2- (ER+/PgR- or low with HER2- and high Ki-67 indices or G3) and LB with positive HER2 (ER+/any PgR and Ki-67 with HER2+). Not the entire LB group is HER2- (approximately 30% is HER2+)^{6,13}.

It is not stable in the classification, since it is excluded in many studies, and its molecular pattern and clinical evolution are therefore unclear. In view of the lack of consistent data on this subtype, we were based on data of our study. These tumors are characterized for showing more aggressive characteristics, and they often occur in postmenopausal women with an average age of 53.6 ± 9 years; these tumors are mainly IDC (73.7%) and include aggressive histologies (e.g. mixed

carcinomas); stage IIIB is common (63.2%) and are associated with undifferentiated tumors (100%) with cN2 lymph node conglomerates (68.4%). HRe+ disease expression is common (ER: 100%; PgR: 73.7%), with low or absent HER2 expression.

In addition, these tumors express high BCL2 and Ki-67 indices^{11,13,24} and show p53 mutation^{3,11}. The grade establishes differences in luminal A and B tumors^{13,14,22}, they are mutually excluding (an LB/HER2- tumor cannot be LA and vice versa).

Treatment response

It is the most challenging subtype in terms of treatment²². Some reports indicate that HRe+/HER2- disease has poor response to standard chemotherapy, with low pCR rates (8.2% to 16%)^{8,22,27,28}. Different studies have shown a beneficial effect when chemotherapy is added to endocrine therapy in LB/HER2- phenotype carcinomas with high Ki-67 proliferative index^{13,27,28}, and therefore anthracyclines with higher cumulative doses have an effect on the tumor, and drugs that damage the DNA or microtubules are active through non-mitotic mechanisms^{22,29}. The condition of being less hormone-sensitive doesn't preclude their treatment with endocrine therapy.

Prognosis

These tumors show less favorable results in comparison with LA tumors^{13,22,28}. Although they share tumor similarities, LB/HER2- and LB/HER+ tumors have prognostic differences. There is still controversy on whether there are any characteristics to discriminate between those tumors that benefit from chemotherapy addition to endocrine therapy (e.g., LB/HER2- with few positive LN < 3)^{14,28}. Differences in grades and some histologic variants are probably due to inter-observer variance in different studies^{6,8-11,21-24,28,30}.

From the study

It showed the lowest pCR rate (21.1%) and the highest residual tumor rate (78.9%). In spite of a lower pCR rate in LB versus LA ($p = 0.484$) and LB/HER2+ ($p = 0.282$), there were no significant differences. This subtype showed the poorest DFS (48%) and the highest percentage of deaths (25.7%). In the multivariate analysis, cT4 tumors (stage IIIB) had a 3.3-fold mortality risk, similar to the other subtypes. In our cohort, pCR does not appear to be an OS marker.

LB/HER2+ SUBTYPE

It accounts for 6% to 18%^{8,10,11,21-24,28,30}. Its definition corresponds to a mixture of HRe+ disease with HER2+. Some studies define it as luminal B (ER+ and/or PgR+, HER2+)^{9-11,21}. It has similar clinical and pathologic characteristics to LB. Stage I-II is common (39% to 54%)⁶.

Our data showed an average age of 49.5 ± 11.1 years. These tumors are characterized for being IDC (69.2%) that include histologic types with poor prognosis, and considerably large size (> 5 cm). Stage IIIA was slightly predominant (53.8%), with cN2 lymph node disease (69.2%) and G2 (61.5%) being notorious.

HRe+ disease is common (ER: 100%; PgR: 61.5%), with HER2, HER1 and cyclin E1 high expression⁵. They also express Ki-67 (32% to 44.1%)^{10,24}, low mitotic index (68%) and low p53 mutation (23%)⁶. They are mutually excluding with the HER2+ subtype (since HER2+ cannot be LB/HER2+).

Treatment response

The use of anthracyclines and taxanes is the treatment of choice^{8-10,14}; however, these tumors are less chemosensitive than those only overexpressing HER2. They are highly sensitive to anti-HER2 targeted therapy. Although sensitivity to endocrine therapy is low, they can receive hormone therapy with tamoxifen or aromatase inhibitors¹⁴.

Prognosis

Generally, these tumors have a poor prognosis, just as LB tumors. Owing to their mixed phenotype, they can have a heterogeneous pattern in the ER and PgR status with variability in the results. The cPR rate ranges from 17.2% to 40%^{8,22,27,28}.

From the study

This subtype showed the second best pCR rate (38.5%) and a favorable 5-year DFS (63%). For this cohort, as in the LB subtype, pCR does not appear to be an OS marker. It showed a low mortality rate (11.4%) in spite of its inherent aggressive biology. According to the multivariate analysis, the mortality risk for cT4 tumors (stage IIIB) is 2.3-fold higher than for cT3 tumors (stage IIIA).

HER2 SUBTYPE

Its frequency is low (5.6% to 29.9%)^{8-11,21-24,30}. It is also known as C-erbB2. Tumors with *HER2/neu* gene

amplification and ER and PgR null expression are more likely to be phenotypically pure HER2 tumors^{2,3,6,14}.

These tumors have clinical and pathologic similarities with TN tumors, and are common in postmenopausal women (77% to 86.8%)^{10,21}; most carcinomas are large tumors > 2 cm or at advanced stage (IIB/III) (53% to 68%)^{6,10,11,21}, with high prevalence of undifferentiated cases (35.1% to 70%)^{6,21,30}; lymph node conglomerates are present, and are associated with G3 as in TN⁶; they are accompanied by CIS in a high percentage (55.9%)¹⁰.

They can express other poor prognosis markers such as GATA4, angiogenesis genes, high levels of Ki-67 (50% to 63.2%)^{6,10,11,24}, p53 mutation (43% to 89%)^{5,6,11,23} and high mitotic index (69%)⁶.

Treatment response

These tumors are sensitive to trastuzumab and/or lapatinib³¹ simultaneous with neoadjuvant anthracyclines and taxanes; a limited number of anti-HER2 therapy preoperative cycles is recommended.

The beneficial effect of adding an anti-HER2 monoclonal therapy to NC increases the opportunity to obtain a pCR (32% to 58%)^{8,14,15,22,27,31}. In one meta-analysis, anti-HER2 therapy with NC had a higher percentage of pCR in all HER2+ cases, with up to 46.4% vs. 25.4% without trastuzumab. When the HER2+ groups were compared, the HER2+/HRe- group obtained a better pCR (38.5%) than the HER2+/HRe+ group (18.4%).

Prognosis

High levels of tumor-infiltrating lymphocytes (TIL) are associated with good results in HER2+ disease treated with carboplatin³²; further studies validating this are necessary. Aggressiveness of the disease is due to its high potential of recurrence and distant metastasis. Prognosis is poor when compared with the luminal subtypes^{6,8-11, 21-24}, and DFS and OS intervals are shorter.

From the study

Although it had the best pCR rate (42.7%), this subtype showed a short DFS interval (58.3%). The mortality rate was 14.3%. In the multivariate analysis, cT4 tumors (stage IIIB) had a 3.1-fold increase in the mortality rate.

TRIPLE-NEGATIVE SUBTYPE (TN)

It accounts for 7.4% to 30%^{6,8-11,21-24,30}. Its immunophenotypical essential characteristic is HER2, ER

and PgR lack of expression^{2-6,14,33-35}. It was initially named BCL or basal-like by Perou². Most TN tumors fall into the basal-like spectrum and are commonly HER2- (70% to 90%)^{11,33-36}. Most claudin-low tumors are clinically TN as well. Apparently, 10% of basal-like tumors and 15% to 25% of claudin-low tumors are HRe+^{6,9}.

Although there is no absolute classification, this subtype has been sub-categorized into 5 or 6 specific groups and an unknown group^{2,5,25,36}, all of them with different responses to NC: the BCL-1 subgroup showed a higher pCR rate (52%) than the BCL-2 (0%) and luminal AR+ (10%)³⁶, which indicates that these are not homogeneous carcinomas^{14,25,36}.

Although the TN and basal-like concepts are not synonyms, they tend to be exchangeable, since they share more than 70% of molecular characteristics (e.g. lack of HER2, ER and PgR biomarkers expression)^{14,33,34}.

Most basal-like cases are sporadic. It is usual finding them in young premenopausal women (26% to 39%)^{6,10,21}, with early menarche, multiparous, with increased body mass index^{4-6,21}. By ethnicity, Afro-American women (24.3% to 39%) are at higher risk of TN disease^{6,35}.

These tumors are characterized for being IDC (80%) with aggressive histology (e.g., metaplastic, atypical medullary carcinoma)⁶, > 2 cm in size (42% to 62%)^{10,21,22,34,35} or at stage II or III (62% to 81.8%)^{6,11,34}, and are poorly differentiated (57.5% to 82%)^{6,10,11,21,30,34}. Lymph node involvement is evident (54% to 59%)^{6,10,11,21} and they are associated with CIS (14.4%)¹⁰.

Most BCRA1 mutation carriers younger than 50 years are TN^{2,5,34,35}; these tumors intensely express HER1 (60%) and cytokeratines (Ck5, Ck6, Ck14, Ck17) in 50-80%, which are characteristic of the basal epithelial layer^{4,6,33,34}, in addition to expressing high Ki-67 proliferative index (68% to 75.3%)^{4,6,11,24,25}, p-cadherin^{4,34} and high mitotic counts (87%)⁶. They are associated with the p53 mutation (44% to 82%)^{4,6,11,24,34}.

Treatment response

Although not susceptible to standard targeted therapy, these tumors are sensitive to anthracyclines plus taxanes^{8,9,14} and cyclophosphamide¹⁴.

Carriers of the *BCRA1* suppressor gene dysfunctional mutation benefit from the use of platinum^{14,37,38}. There is a need to find targeted treatments, such as poly (ADP ribose) polymerase (PARP) and/or anti-CSC, to define their biological complexity and the most adequate treatment³⁸. Genes associated with pCR are different in the HER2 and TN subtypes, and

chemosensitivity can therefore vary^{9,33}. The lack of HRs hinders the use of usual endocrine therapies^{2-5,14,27}.

Prognosis

Although these tumors show high pCR rates (20% to 43%)^{8,9,22,27}, high grade and high sensitivity to NC have no prognostic relationship with lymph node metastatic capability^{6,10,11,34}. Patients with high levels of TIL are associated with good pCR outcomes^{32,39}, but have shorter DFS and OS intervals, especially those patients with post-chemotherapy residual tumor, either due to the lack of therapeutic options or owing to the aggressive biological nature of the disease.

When compared with the luminal subtypes after adjusting for lymph node disease and other variables, these patients have the poorest prognosis (although not uniformly), since this subtype shows higher aggressiveness owing to its potential for distant metastasis (e.g., visceral organs)^{2-6,21,34}. Therefore, this subgroup of carcinomas with HRe- and HER2- phenotype can constitute a mixture of heterogeneous tumors with biological and prognostic differences³³⁻³⁶.

From the study

The pCR rate (29%) was similar to that for the LA subtype. A small sub-population was aged < 40 years (22.6%). In contrast with other studies^{6,9-11,21,34}, grade 3 was less common in TN tumors (45.2%), and the second best DFS rate was observed (67%). In spite of the good prognosis, this tumor was first-place in terms of deaths (25.7%) similar to LB. In the multivariate analysis, the mortality risk is 2.4-fold higher in cT4 (stage IIIB) than in cT3 tumors (stage IIIA).

There is variability in the subtype results in different trials, which can be due to the type of patient selection, inter-observer assessment variability (histology and grade) and inter-laboratory IHC assessment (e.g. several commercially-available anti-ER antibodies), as well as the type of molecular classification (4 or 5 groups)^{6-11,21-26,30}; there is also diversity in the results with regard to luminal tumors defined by IHC (4 to 5 biomarkers) or by molecular classification (4 or 5 subtypes)^{2-6,9-11,22-25,28,30}, and establishing comparisons is therefore not easy.

The limitations of this study are worth mentioning. Molecular tumor classification by IHC might be less robust due to the lack of specific biomarkers to define luminal and TN tumors, to the fact that the LB/

HER2- subtype is not used by all international groups and to a limited number of cases for some subtypes (LB, LB/HER2+, HER2), which might overestimate or underestimate the proportion of survivors or the mortality risk. Our study investigated mainly the clinical, pathological and immunophenotypical differences of LABC heterogeneous behavior aided by surrogate molecular markers using IHC.

The advantage of this series include a standardized oncological treatment where 80% of patients had access to the recommended 8-cycle full course with sequential E-D at standard doses in a single hospital, and the IHC study of ER, PgR and HER2 carried out by a single laboratory, which enabled finding differences with higher statistical strength.

pCR depends on the molecular subtype and establishes significant survival differences. Patients with the LA and TN subtypes had a beneficial DFS effect after receiving sequential E-D with a survival increase. The log-rank test (1.21; $p = 0.016$) for DFS showed that LB, LB/HER2+ and HER2 carcinomas had the worst 5-year DFS. TN and LB tumors showed the highest percentage of deaths.

The multivariate analysis confirmed, in all subtypes, that patients with T4 tumors (stage IIIB) have a mortality risk 2.1 to 2.4-fold higher than those with T3 tumors (stage IIIA), adjusted for age, tumor, lymph node, grade and histology; the difference was significant when considered as a whole ($p = 0.010$). Our data have similarities with other studies^{2-13,21-30}, confirming that molecular classification by IHC enables establishing differences in the response to NC and assessing the prognostic value in each subtype.

Conclusion

This investigation provides clinical, pathologic and biological information that is additional to that established by research on this complex disease. Not all carcinomas respond equally to chemotherapy, and the molecular classification used in this study enabled us to identify significant differences in the different subtypes by determining their prevalence, their relationship with clinical, tumor and treatment characteristics, as well as the survival pattern in this study cohort.

Carcinoma prognostic and predictive factors clinical value has been strengthened with the addition of molecular classification to daily clinical practice, which is highly useful to understand tumor biology. Prognosis is the reflection of the different results, which suggests the need for the development of new therapeutic approaches for the molecular subtypes with poorer response.

Conflict of interests

The authors declare there is no conflict of interests.

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