

CLINICAL SCIENCE

Evaluation of prognostic factors in stage IIA breast tumors and their correlation with mortality risk

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ABSTRACT: Breast tumors exhibit extensive molecular and clinical heterogeneity. One of the most utilized breast carcinoma classifications is based on its molecular aspects and subdivides breast cancer into five major groups based on the expression of certain genes. In this study, we evaluated which factors are important in determining a prognosis after 5 years of follow-up for patients with clinical stage IIA breast tumors. We took into consideration the different phenotypes (luminal A luminal B HER-2 overexpression, basal and triple-negative), various epithelial-mesenchymal (EMT) molecular markers and adhesion molecules (E-cadherin, P-cadherin, N-cadherin, vimentin, twist snail and slug) and NOS-2, in addition to clinical and demographic data, tumor characteristics and treatment types.

METHODS: The study population consisted of 82 patients with breast cancer. We analyzed eight molecular markers by immunohistochemistry on tissue microarrays containing breast tumor specimens from patients with ten years of follow-up, and we classified each tumor according to its estrogen receptor, progesterone receptor and HER-2 expression. We then placed the tumor into one of the above categories.

RESULTS: The presence of several clinical and demographic factors, various histopathologies, treatment forms and several immunohistochemical markers were not associated with a worse prognosis for group IIA patients. The factors that were associated with a mortality risk were the triple-negative (odds ratio (OR) = 11.8, 95% confident interval (CI) = 2.0-70.3, $P=0.007$) and basal (OR = 18.4, 95% CI = 1.8-184.7, $P=0.013$) phenotypic patterns.

CONCLUSIONS: The EMT markers and NOS-2 were not mortality risk factors. Basal and triple-negative phenotypic patterns were related to a higher mortality risk in patients with stage IIA tumors.

KEYWORDS: Breast cancer; molecular markers; staging; tissue microarray; immunohistochemistry.

Carvalho SMT, Stiepcich MA, Fregnani JH, Nonogaki S, Rocha RM, and Soares FA. Evaluation of prognostic factors in stage IIA breast tumors and their correlation with mortality risk. Clinics. 2011;66(4):607-612.

Received for publication on November 11, 2010; First review completed on December 2, 2010; Accepted for publication on January 12, 2011

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INTRODUCTION

Breast cancer is the most frequent malignant neoplasm among women worldwide, and in Brazil, and it is one of the leading causes of cancer-related death.^{1,2} Despite the increased incidence in Brazil and other countries, large international studies show that higher-efficiency screening programs, better diagnostic image definition and the appropriate use of adjuvant drug therapies have led to improved patient survival in the last few decades.^{3,4} A long-term survival patient is defined as one who is still living 5 years after first being diagnosed with cancer.⁵ For breast cancer, 5- and 10-year survival rates are 88% and 77%, respectively, both of which are quite good as compared with

the 5-year survival rate of all other types of cancer combined.^{5,6} Breast tumors classified as stage IIA are tumors presenting with local or disseminated disease in the homolateral axillary lymph nodes. At this stage, around 80% of patients survive disease-free after 5 years;^{7,8} the data also show that 20% will die within the same time interval despite the good prognosis associated with these cases.⁸

With the evolution of genomic techniques and, consequently, the use of molecular markers, prognostic information must be continuously refined. However, these data are still not sufficient for breast cancer specialists and clinical oncologists when compared with the standard anatomical-pathological reports used to determine the best course of action in some specific cases.

At the beginning of this century, some groups studied the molecular signature of invasive ductal carcinomas (IDCs)^{9,10} and showed that the phenotypic diversity in breast tumors was associated with diversity in gene expression. These gene expression profiles were called luminal (A and B), HER-2 overexpression, basal and similar to normal breast.

Importantly, this classification showed prognostic value and validated well-known clinical behaviors demonstrated in the literature.

Some molecular markers of epithelial-mesenchymal transition (EMT) (e.g., vimentin, snail, slug and twist) are correlated with survival, and their expression may be associated with a more aggressive phenotype, more mesenchymal differentiation, and thus, decreased survival.¹¹⁻¹³ The IHC markers that have been studied include adhesion molecules (E-cadherin, N-cadherin and P-cadherin) and nitric oxide synthase 2 (NOS-2).

On the basis of these observations and with the intention of improving the immunohistochemical signature of stage IIA breast tumors, we designed our study to evaluate which factors affect the mortality rate in 10 years of follow-up, taking into account the different phenotypes (luminal A, luminal B, HER-2 overexpression, basal and triple-negative), the different EMT molecular markers and adhesion molecules (E-cadherin, P-cadherin, N-cadherin, vimentin, twist, snail and slug) and NOS-2, in addition to clinical and demographic data, tumor characteristics and treatment types.

MATERIALS AND METHODS

Study design, patients' characteristics and tissue samples

This study design followed the main criteria defined by REMARK.¹⁴ The study population consisted of 82 patients with IDC who were operated on at A. C. Camargo Hospital from 1980 to 1999; they were previously identified from surveys on file in the hospital's Department of Pathology. The study protocol was approved by the Research Ethics Committee at the Center for Treatment and Research, Cancer Hospital, A. C. Camargo. The patients selected for this study were diagnosed as having clinical stage IIA breast cancer (T0N1M0, T1N1M0 and T2N0M0)¹⁵ and having a sufficient amount of representative material from the tumor for the study. In this study, we analyzed 82 cases of female patients diagnosed with stage IIA IDC.

We selected patients with a 5-year minimum follow-up period (60 months). All of the patients were alive, and those patients with less than 60 months of follow-up were excluded from the analysis. The maximum follow-up period was 10 years (120 months), and we counted mortality at any time during the follow-up. The date of the first consultation at the Department of Mastology, Cancer Hospital, A. C. Camargo, was considered as the start date for clinical follow-up, and the date of the last consultation or death was considered as the end date. We considered those cases in which the patients

did not return for consultation and did not respond to telephone calls or telegraphs as loss to follow-up.

We reviewed the clinical records, noting the relevant clinical-pathological information (clinical and demographic data, anatomopathological staging, therapeutic adjuvant, follow-up, relapse and survival) in an Excel spreadsheet.

For all of the selected cases, we recovered the original hematoxylin/eosin (H&E)-stained slides and the corresponding paraffin-embedded tissue blocks, from which we made new histological sections. Using conventional slides, we stained the representative areas of the invasive component to orient the puncturing and removal of small core biopsies, which were used to construct a tissue microarray (TMA). A core was removed from each original tumor block using a specially designed precision instrument for constructing TMAs (Beecher Instruments®, Silver Spring, Maryland), as described previously.¹⁶ The diameter of each block core used for the TMA was 1.0 mm. The histological sections from the TMA blocks, with an average thickness of 4 µm, were mounted on special electrically charged slides (Instrumedics Inc.®, Richmond, USA) to facilitate their orientation after the microtomy and for immunohistochemistry (IHC) analysis.

Immunohistochemistry of TMAs

The immunohistochemistry (IHC) study was performed with antibodies that recognized the different markers used. For the IHC reactions, we followed the specific standardized protocols from the manufacturers and previous reports for each marker. All of the reactions included positive and negative controls that were stained in parallel with the slides from the study. Table 1 shows the complete list of the nine antibodies used with their respective clones, dilutions and manufacturers.

Evaluation of IHC

Immunostaining was evaluated by light microscopy. When evaluating the IHC reactions, we took into account only the percentage of stained cells and classified them according to a specific expression pattern for each antibody and the number of positive cells.¹⁷ For the nuclear markers, the cells were considered positive when the percent of stained nuclei was greater than 1% and negative when this staining was less than 1%. For cytoplasmic and membranous markers, we considered as positive those cases with more than 10% stained cells. Examples of positive and negative cases are shown in Figures 1 through 3.

Table 1 - Antibodies and protocols used in the reactions.

Antibody	Clone	Dilution	Manufacturer
Estrogen receptor	Rabbit monoclonal antibody SP1	1:400	Neomarkers, CA, USA
Progesterone receptor	PgR636	1:300	Dako, Carpinteria, CA, USA
HER-2	Rabbit polyclonal	RTU ^a	Dako
NOS-2	16	1:100	BD Transduction San Jose, CA, USA
Twist 2	3C8	1:250	Novus Biologicals, Littleton, USA
Slug	Polyclonal	1:100	Abcam
Snail	E18	1:500	Santa Cruz Biotechnology, (Santa Cruz, CA, USA)
Vimentin	Vim3B4	1:400	Dako, Carpinteria, CA, USA
E-Cadherin	36	1:600	BD Transduction San Jose, CA, USA
N-Cadherin	6G-11	1:50	Dako, Carpinteria, CA, USA
P-Cadherin	56C1	1:100	Neomarkers, CA, USA

^aReady to use.

Table 2 - Case distribution according to clinical and demographic variables, histopathologic variables, treatment forms and mortality within 120 months of follow-up^a among women with stage IIA tumors (n = 82).

VARIABLES	CATEGORIES	Mortality within 120 months				P **
		No		Yes		
		n	%	n	%	
Clinical and Demographic ^a Data						
Age	Up to 50 years	24	72.7	9	27.3	0.807
	>50 years	33	70.2	14	29.8	
Skin color	White	44	68.8	20	31.3	0.264
	Non-white	9	90.0	1	10.0	
Number of pregnancies	None	10	71.4	4	28.6	1.000
	One or more	41	70.7	17	29.3	
Hormonal status	Pre-menopause	26	72.2	10	27.8	0.744
	Post-menopause	31	68.9	14	31.1	
Family history	No	42	71.2	17	28.8	1.000
	Yes	10	71.4	4	28.6	
Histopathology ^a						
Tumor size	Up to 2.0 cm	14	87.5	2	12.5	0.127
	2.1 - 5.0 cm	41	65.1	22	34.9	
Scarff-Bloom-Richardson	Degrees 1 and 2	50	73.5	18	26.5	0.332
	Degree 3	8	57.1	6	42.9	
Nuclear grade	Degrees 1 and 2	28	87.5	4	12.5	0.008
	Degree 3	30	60.0	20	40.0	
Mitotic index	0 to 9	37	78.7	10	21.3	0.106
	10 or more	9	56.3	7	43.8	
Lymph node metastasis	No	45	68.2	21	31.8	0.329
	Yes	12	86.7	2	14.3	
Treatment ^a						
Neoadjuvant chemotherapy	No	49	73.1	18	26.9	0.345
	Yes	3	50.0	3	50.0	
Neoadjuvant radiotherapy	No	50	72.5	19	27.5	0.574
	Yes	2	50.0	2	50.0	
Adjuvant chemotherapy	No	21	70.0	9	30.0	0.846
	Yes	31	72.1	12	27.9	
Adjuvant radiotherapy	No	15	68.2	7	31.8	0.705
	Yes	37	72.5	14	27.5	
Hormone therapy	No	27	79.4	7	20.6	0.149
	Yes	45	64.1	14	35.9	

^aLiving patients with less than 60 months of follow-up were excluded from the analysis.

**Chi-square test or Fisher's exact test.

The IHC slide readings were performed on a shared optic microscope. ER (estrogen receptor) and PR (progesterone receptor) status was determined on the basis of IHC staining. Tumors were considered HER-2-positive only if they were scored as 3+ by IHC or if they were *HER-2* amplified (ratio > or = 2.0) on the basis of fluorescence *in situ* hybridization (FISH). In the absence of positive FISH data, tumors with indeterminate IHC scores (2+) were considered negative.

Immunophenotypical classification

From the results of the IHC reactions, we identified five sub-groups based on the immunohistochemical expression of ER, PR, HER-2 and CK5, 6, as follows: luminal A (ER positive, PR positive and HER-2 negative), luminal B (ER positive and/or PR positive and HER-2 positive), HER-2 over-expression (ER negative, PR negative and HER-2 positive), triple-negative (ER negative, PR negative and HER-2 negative) and basal pattern (ER negative, PR negative, HER-2 negative and/or CK5 and 6 positive).

Statistical methods

The results were analyzed using SPSS v.13 (SPSS Inc., USA, 2004) and GraphPad Prism v.4.02 (GraphPad Software

Inc., USA, 2000). We characterized the sample using descriptive statistics. We performed association analysis between categorical variables using the chi-square or Fisher's exact test, depending on the values we observed in the contingency tables. We used logistic regression to identify independent risk factors associated with mortality. The final models were adjusted for chemotherapy completion, hormone therapy and age (as a continuous variable). The adjustments for age, chemotherapy completion and hormone therapy were based on a greater potential for interference with the outcome (death). In all of the models, the control variables were not significant. For the mortality analysis, we truncated the follow-up period to 120 months, and we used a cross-sectional evaluation of the data. A 5% significance level was used for all of the tests.

RESULTS

When analyzing the stage IIA patients, we observed that there were no significant associations between mortality and any of the clinical, demographic or treatment-related variables (Table 2). When we analyzed the histopathologic variables, we observed that none of them was significantly

Table 3 - Case distribution according to biomolecular variables and mortality within 120 months of follow-up^a among women with stage IIA tumors (n = 82).

VARIABLE	CATEGORIES	Mortality within 120 months				P **
		No		Yes		
		n	%	n	%	
ER	Negative	6	42.9	8	57.1	0.021
	Positive	52	76.5	16	23.5	
PR	Negative	19	59.4	13	40.6	0.071
	Positive	39	78.0	11	22.0	
HER-2	Negative	53	72.6	20	27.4	0.437
	Positive	5	55.6	4	44.4	
CK 5/6	Negative	49	72.1	19	27.9	0.736
	Positive	8	66.7	4	33.3	
NOS-2	Negative	18	64.3	10	35.7	0.320
	Positive	36	75.0	12	25.0	
E-Cadherin	Negative	22	62.9	13	37.1	0.146
	Positive	32	78.0	9	22.0	
N-Cadherin	Negative	53	73.6	19	26.4	0.059
	Positive	2	33.3	4	66.7	
P-Cadherin	Negative	30	68.2	14	31.8	0.518
	Positive	24	75.0	8	25.0	
Vimentin	Negative	53	72.6	20	27.4	0.199
	Positive	1	33.3	2	66.7	
Twist	Negative	22	64.7	12	35.3	0.370
	Positive	29	74.4	10	25.6	
Snail	Negative	0	-	0	-	NA
	Positive	55	71.4	22	28.6	
Slug	Negative	8	66.7	4	33.3	0.745
	Positive	43	70.5	18	29.5	

^aLiving patients with less than 60 months of follow-up were excluded from the analysis.

^bNot available because of the reduced number of cases in one of the categories.

**Chi-square test or Fisher's exact test.

associated with mortality, except for the nuclear grade variable; the majority of patients who died had grade 3 tumors ($P = 0.008$) (Table 2). For the biomolecular variables and mortality, we observed that ER expression was positive in significantly more patients who did not die than those that did die ($P = 0.021$) (Table 3).

When we analyzed the distribution of the cases according to their phenotypic profile, we observed a larger proportion of luminal A patients among the patients who did not die than those that did die ($P = 0.006$) and a larger proportion of patients who died in the triple-negative ($P = 0.016$) and basal sub-groups ($P = 0.018$) (Table 4).

Using logistic regression, we individually tested the luminal A, triple-negative and basal phenotype variables (models) adjusted for age, chemotherapy completion and hormone therapy, in clinical stage IIA tumors. When evaluating the mortality risk within 120 months, we observed that the patients with a phenotypic pattern other than luminal A had an increased risk of dying (Model A, OR = 6.3, 95% CI = 1.7-22.6, $P = 0.005$). We also observed that patients with the triple-negative and basal patterns had an increased risk of dying, respectively (Model B, OR = 11.8, 95% CI = 2.0-70.3, $P = 0.007$) and (Model C, OR = 18.4, 95% CI = 1.8-184.7, $P = 0.013$), independent of the effect of hormone therapy, adjuvant chemotherapy or age (Table 5).

When analyzing the mortality risk within 120 months according to luminal B and HER-2 overexpression patterns in multivariate analysis, (model adjusted for age, hormone therapy and adjuvant chemotherapy completion), we

Table 4 - Case distribution according to the phenotypic biomolecular profile and mortality within 120 months of follow-up^a among women with stage IIA tumors (n = 82).

VARIABLE	CATEGORIES	Mortality within 120 months				P **
		No		Yes		
		n	%	n	%	
Luminal A ^b	No	8	44.4	10	55.6	0.006
	Yes	50	78.1	14	21.9	
Luminal B ^b	No	56	71.8	22	28.2	0.577
	Yes	2	50.0	2	50.0	
HER-2 over-expression ^b	No	55	71.4	22	28.6	0.627
	Yes	3	60.0	2	40.0	
Triple-negative ^b	No	55	75.3	18	24.7	0.016
	Yes	3	33.3	6	66.7	
Basal ^b	No	56	75.7	18	24.3	0.018
	Yes	2	28.6	5	71.4	

^aLiving patients with less than 60 months of follow-up were excluded from the analysis.

^bGroup determination:

Luminal A: (ER⁺ or PR⁺) and HER-2⁻.

Luminal B: (ER⁺ or PR⁺) and HER-2⁺.

HER-2 overexpression: ER⁻ and PR⁻ and HER-2⁺.

Triple-negative: ER⁻ and PR⁻ and HER-2⁻.

Basal: Triple-negative and (EGFG⁺ or CK5/6⁺ or p63n⁺ or P-cadherin⁺ or CK14⁺).

**Chi-square test or Fisher's exact test.

observed that these patterns were not associated with a risk factor of mortality (luminal B, OR = 0.9, 95% CI = 0.1-12.3, $P = 0.921$) and (HER-2, OR = 0.4, 95% CI = 0.1-2.8, $P = 0.342$).

DISCUSSION

Breast cancer is one of the most common tumors in women. According to data from the Instituto Nacional de Câncer INCA (Brazilian National Cancer Institute), there will be an estimated 49,240 cases of breast cancer in Brazil in 2010.¹

Only a few published studies have analyzed which prognostic factors influence long-term patient survival with clinical stage IIA breast cancer. This deficit was the main impetus for conducting our study.

In this study, we analyzed stage IIA breast tumors, taking into account the variables of age, race, number of

Table 5 - Evaluation of the mortality risk within 120 months^a according to the luminal A, triple-negative and basal phenotypic patterns using multivariate analysis (logistic regression). Models were individually adjusted by age, hormone therapy and adjuvant chemotherapy (number of deaths considered in the model = 21).

VARIABLE	CATEGORY	n	OR	95% CI	P
Luminal A	No	16	6.3	1.7-22.6	0.005
	Yes	57	1.0	Reference	
Triple-negative	No	30	1.0	Reference	0.007
	Yes	43	11.8	2.0-70.3	
Basal	No	66	1.0	Reference	0.013
	Yes	6	18.4	1.8-184.7	

^aLiving patients with less than 60 months of follow-up were excluded from the analysis.

pregnancies, hormonal status and family history. We did not find any statistically significant correlation with mortality risk within the 120-month follow-up period.

According to the literature, one important factor in determining long-term survival is age.^{18,19} Younger patients (30-35 years) have a worse survival rate as compared with patients aged 70 or older. In our analysis, we did not find any statistically significant correlation between age and survival during the 120 months.

Considering the histopathologic variables, we observed that the nuclear grade variable was the only one associated with mortality. For variables related to treatment and mortality within 120 months, none had a statistically significant association. Several studies have shown an association between high histological grade and reduced disease-free survival.^{18,20-22} The histological grade also has a significant impact on the selection of patients who will undergo systemic adjuvant treatment. The histological grade takes into consideration tubular formation, nuclear grade and mitotic index.²³ However, the individual prognostic value of each one of its components—especially the nuclear grade—has not received much attention in the literature.

The patients who were ER-positive had a reduced mortality rate within the 120-month follow-up period ($P=0.021$). Previous findings have demonstrated that ER positivity confers a better prognosis for the patients, independent of sub-group, which allows for the use of specific anti-hormonal medication.^{3,6}

Luminal A tumors are characterized by their positive ER expression and/or positive PR expression and negative HER-2 expression profile.²⁴ According to Zaha et al.,²⁵ tumors in the luminal A sub-group are more often associated with ER positivity, a larger percentage of patients in stages I and II and moderately differentiated tumors. Fernandes et al.²⁶ observed that the presence of hormone receptors defines a sub-group with more favorable morphological characteristics.

When analyzing the stage IIA patients in relation to the mortality risk within 120 months, the multivariate analysis showed that the patients with a phenotypic pattern other than luminal A had an increased risk of dying (6.3 times) within 120 months, independent of the effects of hormone therapy, chemotherapy and age. We also observed an increased risk in the triple-negative pattern (11.8 times) and the basal pattern (18.4 times). Even with statistical significance, it is important to emphasize that the confidence interval is very wide because there were few cases with a positive basal pattern (only six cases in the model). Although this result should be interpreted with caution, the basal pattern may nonetheless represent a risk factor for death.

In general, the literature indicates that patients with a basal or triple-negative breast tumor pattern have a worse prognosis.²⁷⁻²⁹ Haupt et al.³⁰ observed that when a tumor belonged to the basal sub-group, it was associated with a worse prognosis in both early and advanced stages in addition to being more frequently associated with visceral dissemination. Recently, De Brot et al.³¹ demonstrated that patients with basal sub-group tumors had an average disease-free survival of 28 months and an overall survival of 36 months, of which only 50% were disease-free. Basal sub-group tumors also appear to be more common among

young women. In our experience, more than 50% of the cases in this population have a basal phenotype.³²

This association between decreased survival and non-luminal A tumors can also be analyzed in relation to the expression of EMT markers. The progression of breast cancer is a result, among others, of a process involving a loss of epithelial characteristics and the acquisition of mesenchymal properties, which results in a more aggressive tumor phenotype.³³ Sarrío et al.³⁴ observed a correlation between EMT and the basal pattern. Makdissi et al.³⁵ observed that in invasive breast carcinoma, tumor E-cadherin protein expression may be related to overall breast cancer survival. In our study, we did not observe a relationship between the phenotypic profiles and the expression of EMT markers in stage IIA tumors by IHC. We assume that the expression of these EMT markers in breast tumors depends on the degree of transformation of epithelial cells into mesenchymal cells and that the entire process occurs via specific and complex regulation, in which each one of these factors can act in a distinct way during tumor progression.

In conclusion, none of the EMT markers or NOS-2 was related with mortality risk for stage IIA breast cancer. The mortality risk was associated with the tumor not belonging to the luminal A sub-group but instead belonging to the basal or triple-negative subgroup.

There is still much to learn about the biology of these tumors, and certainly the variable expression of the different markers that define basal- and triple-negative-type tumors should help clarify the distinctions between subpopulations. Even so, in our study, we observed no correlations between the studied IHC markers and survival. Thus, robust population-based studies with appropriate patient populations are important to define these prognostic factors.

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