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.

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.

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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. 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. 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) 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 telegrams 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. 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 (Intrumedics 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. 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 | PgR635          | 1:300    | Dako, Carpinteria, CA, USA                                                   |
| HER-2             | Rabbit polyclonal  | RTU*     | Dako                                                                         |
| NOS-2             | 16                 | 1:100    | BD Transduction San Jose, CA, USA                                            |
| Twist 2           | 3CB                | 1:250    | Novus Biologicals, Littleton, USA                                           |
| Slug              | Polyclonal         | 1:100    | Abcam                                                                       |
| Snail             | E18                | 1:500    | Santa Cruz Biotechnology, (Santa Cruz, CA, USA)                              |
| Vimentin          | Vimi84            | 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                                                           |

*Ready to use.
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 $\geq 2.0$) 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
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 II A 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, we 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 Cancer 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 3 - Case distribution according to biomolecular variables and mortality within 120 months of follow-upa among women with stage IIA tumors (n = 82). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| VARIABLE        | CATEGORIES      | n   | %    | No  | %    | Yes | %    | P   | **  |
| 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        | 55  | 71.4 | 22   | 28.6 |      |
| Positive        | 8              | 66.7 | 4    | 33.3 | 0.745 |
| Slug            | Negative        | 43  | 70.5 | 18   | 29.5 |      |
| 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.

| Table 4 - Case distribution according to the phenotypic biomolecular profile and mortality within 120 months of follow-upa among women with stage IIA tumors (n = 82). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| VARIABLE CATEGORY | n   | OR     | 95% CI  | P   | **  |
| Luminal A        | No  | 8     | 44.4   | 10   | 55.6 | 0.006 |
| Luminal B        | Yes | 50    | 78.1   | 14   | 21.9 |      |
| Luminal C        | No  | 56    | 71.8   | 22   | 28.2 | 0.577 |
| HER-2 overexpressionb | No  | 55    | 71.4   | 22   | 28.6 | 0.627 |
| Triple-negativeb | Yes | 3     | 60.0   | 2    | 40.0 |      |
| Basalb           | 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 (EGF+ or CK5/6+ or p63+ or P-cadherin+ or CK14+). **Chi-square test or Fisher's exact test.

**DISCUSSION**

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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 monthsa 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.10,11 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.12,20-22 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.2,6

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 morphologic 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.7-29 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 a median 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 Sarrio et al.34 observed a correlation between EMT and the basal pattern. Makdisi 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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