Clinico-pathological and molecular subtypes of male breast carcinoma according to immunohistochemistry

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Abstract

Introduction: Male breast carcinoma is a rare condition, but with a trend of increase frequency. In our study, we investigate the clinico-pathological features and overall survival at 35 male cases of primary invasive breast carcinoma correlated with molecular subtypes defined by immunohistochemical profile. Methods: Based on immunohistochemical expression profiles of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor 2 (HER2) and Ki67, EGFR and CK5/6, the male breast cancers were classified into the following molecular subtypes: Luminal A, Luminal B, HER2+, triple negative and unclassified. Results: In our study, we identified 65.7% as Luminal A subtype and 28.6% as Luminal B subtype. The difference was represented by two (5.7%) cases of triple negative subtype, but due to low number of patients, no correlations or prognostic significance could be assessed in these cases. No HER2 or unclassified subtypes were identified. Conclusions: Luminal A tumors are the most frequent subtype in MBC, with a better outcome than Luminal B subtype. We recorded high levels of ER and PR expression, which predict a better response to adjuvant hormonal therapy. At the time of diagnosis, most of the patients were aged and with an advance clinical stage, this requiring implementation of screening programs and increase education of population in order to an early detection.

Keywords: male breast carcinoma, molecular subtype, immunohistochemistry.

Background

Breast cancer is a disease with numerous victims every year, especially within female population worldwide. It is responsible for 14% from all cancer deaths in 2008 [1]. Breast carcinoma among men is an unusually condition, but with a trend of increase frequency. Incidence of male breast carcinoma (MBC) is higher in the last decade than the late 1970s, with 1.2 case per 100 000 versus one per 100 000 [2]. Many cases have a poor prognosis because they are usually diagnosed with an advanced tumor stage [3]. Even if MBC shares many similarities with female breast cancer (BC), there are differences regarding not only the incidence or age distribution, but also prognosis or survival.

Many molecular studies have been performed in the last decade for a better understanding of tumor carcinogenesis. Molecular research in the field of BC have increased and provided new insights on biology of breast cancer with the first studies of Perou CM et al. [4] and Sorlie T et al. [5]. They proposed a new intrinsic molecular classification of BC (Luminal A, Luminal B, ErbB2 overexpression, basal-like and normal-like types), based on gene expression analysis on DNA microarrays. In nowadays, there are a few gene expression models available for describing BC. Some of them are already approved and used in clinical trial, as the 70-gene profile MammaPrint (Agenda, The Netherlands) [6, 7] or Oncotype DX (Genomic Health, USA) [8], and others in course of validation. But, all these microarray techniques are not yet accessible for routine practice for two reasons: the high cost and their inability to easily use formalin-fixed paraffin-embedded sample, most of them requiring fresh samples. For these reasons, immunohistochemistry has been used as a surrogate for gene expression analysis. Definition of molecular subtypes using several biomarkers is still under debate and in a continuum change based on dissemination of the new studies results.

So, at 12th St. Gallen International Breast Cancer Conference 2011, using a panel of five biomarkers (estrogen receptor – ER, progesterone receptor – PR, human epidermal growth factor receptor 2 – HER2/neu and Ki67 index) were accepted four major immunohistochemical subtypes: Luminal A, Luminal B, HER2 positive and triple negative. There intrinsic molecular subtypes correspondent are: Luminal A, Luminal B, HER2 positive and triple negative. The reason for this is that basal-like intrinsic subtype is a very heterogeneous disease. It was able to accurately identify these tumors with 100% specificity and 76% sensitivity by adding two more biomarkers: human epidermal growth factor receptor 1 (EGFR) and cytokeratin 5/6 (CK5/6) [10, 11].

Considering all previous notes, the purpose of our study is to investigate, in a retrospective way, the clinico-pathological features and overall survival at 35 male cases
Materials and Methods

Selection of patients

From the surgical pathology archives of the Department of Pathology, Emergency County Hospital of Constanta, Romania, we identified cases of primary invasive MBC, from January 1, 2001 to December 1, 2012. Clinicopathological records were retrospectively reviewed and we obtained the following data: age at clinical presentation and diagnosis, operative procedure and nodal excision, tumor size, lymph node status and outcome status (overall survival defined as the time, in months, from the date of primary surgery to date of breast cancer-related death). All these information were collected with approval of the Institutional Review Board.

Tissue processing

The Pathology Medical Practice Guidelines of Romanian Health Ministry were used to evaluate tumor samples and all settled for following: histological classification, using the criteria recommended by the World Health Organization [12], grade according to the Scarff-Bloom-Richardson scale modified by Elston CW and Ellis IO [13], TNM pathological stage established by American Joint Committee on Cancer (AJCC) [14].

Immunohistochemistry staining and evaluation

Four μm thick sections of formalin-fixed, paraffin-embedded tissue block of the best representative slide for each case were prepared for immunostains. After epitope retrieval, tissue sections were incubated with five antibodies: estrogen receptor (ER – monoclonal rabbit 1D5 clone), progesterone receptor (PR – monoclonal mouse PR636 clone), HercepTest (rabbit immunoglobulin HercepTest), Ki67 (monoclonal mouse MIB-1 clone), p53 (monoclonal mouse DO-7 clone). In two cases, we also used two more antibodies: CK5/6 (monoclonal mouse D5/16B4 clone) and EGFR (monoclonal mouse EGFR.113 clone). All antibodies are ready to use and were provided from DakoCytomation (Denmark). When the positive internal control was missing, an external one has been included in each immunostaining run. Negative controls were obtained by omission of the primary antibodies from the staining procedure. We used as chromogen 3,3’-diaminobenzidine (DAB), with brown staining of antigen concerned. Sections were finally counterstained with Mayer’s Hematoxylin.

We assessed the antibody distribution pattern, percentage of positive cells and intensity of reaction for all cases. The semiquantitative scoring method was used for assessing immunohistochemical expression of hormonal receptors (ER and PR). A positive result was considered if at least 1% of cells show nuclear immunostain signal [15].

Accurate evaluation of HER2 status (overexpression of the HER2/neu protein or amplification of the HER2 gene) is crucial for a proper treatment and correct prognosis. HER2 protein was scored using the new recommendations of ASCO/CAP Guidelines, and was quantified as followed: 0 if no membrane staining is observed in invasive tumor cells; 1+ if is observed weak, incomplete membrane staining in any proportion of invasive tumor cells, or weak, complete membrane staining in less than 10% of cells; 2+ for complete membrane staining that is non-uniform or weak but with obvious circumferential distribution in at least 10% of cells, or intense complete membrane staining in 30% or less of tumor cells; 3+ if is seen strong and uniform staining of the entire membrane in more than 30% of cells [16]. Cases with HercepTest 2+ score (equivocal) were further analyzed for HER2 gene amplification by CISH (chromogenic in situ hybridization) technique. This method has a high concordance with fluorescence in situ hybridization (FISH), values ranging from 85% [17] to 100% [18].

For Ki67 immunoreexpression, the absolute percentage of nuclear stained cell was recorded and a value of 14% was used as a cut-off value for low or high expression [9, 19]. Immunohistochemical expression of p53 was considered positive if more than 10% of tumor nuclei were stained. EGFR positivity was considered when more than 10% of the membrane of tumor cells were stained [20], and CK5/6 positivity when any cytoplasm and/or membrane staining was seen in the tumor cells [21].

Chromogenic in situ hybridization evaluation

For the detection of human HER2 gene by CISH, was used ZytoDot SPEC HER2 Probe Kit from ZytoVision according to manufacturer’s instructions. In case of high gene amplifications, a large number of dots or large clusters, comprising an area greater than five dots, were visible in the nuclei [18].

Definition of molecular subtypes of breast carcinoma using biomarkers

Based on immunostochemical expression profiles of ER, PR, HER2, Ki67, EGFR and CK5/6, the male breast cancers were classified into following molecular subtypes: Luminal A (ER+ and/or PR+, HER2-, low Ki67), Luminal B (ER+ and/or PR+, HER2+, any Ki67 or ER+ and/or PR+, HER2-, high Ki67), HER2+/ER-(ER-, PR-, HER2+), triple negative (ER-, PR-, HER2-, CK5/6+ and/or EGFR+) and unclassified (all six biomarkers are negative).

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software program, version 20.0. Descriptive statistics were calculated, providing mean and standard deviation for continuous variables and frequency (percentage) for categorical variables. The association between continuum variable was done using independent Student’s t-test and for categorical variable was established by Fisher’s exact test. Kaplan–Meier analysis was performed to calculate survival curves and log-rank test was used to assess the statistical significance of the differences between IHC subtypes. A two-tailed p-value <0.05 was considered significant.

Results

In our department, 158 surgical interventions of male patients were recorded during 2001 and 2012, from which 96 cases were diagnosed with benign lesions, 25 cases with
in situ carcinoma and 37 cases with invasive carcinoma. From all invasive carcinoma, only 35 cases proved to be primary invasive carcinoma, the other two were metastatic carcinoma. The age of the patients range from 40 to 81-year-old (mean 64.14±9.4 years) for all cases, five (14.3%) cases being under the age of 50 years and 30 (85.7%) cases older than 50 years. The most frequent symptom was a nodular mass (26 cases) and few cases presented with homolateral axillary adenopathy (three cases) or nipple abnormalities (two cases – retraction; four cases – ulceration). Left breast was more affected (57.1%) than right breast (42.9%). Most of the tumors were found in the retroareolar region (45.7%), the second position being occupied by upper left quadrant (31.4%). In three cases, tumor was large enough to extend all quadrants of the breast. Mean tumor diameter was 2.9±1.9 cm (range from 0.5 cm to 8 cm).

According to the histological classification, invasive ductal carcinoma – no special type (CDI–NOS) was the most frequent form (82.9%). Few cases were represented by invasive cribriform carcinoma (3/35), invasive papillary carcinoma (1/35), invasive lobular carcinoma (1/35) and one case of mix carcinoma (CDI–NOS and CLI). About half of the tumors were poorly differentiated (45.7%), closely followed by moderate differentiated tumors (40%). Few cases were well-differentiated tumors (5/35). Axillary lymph node involvement was identified in 68.6% cases and vascular invasion was observed in 37.1% of cases. Few cases were well-differentiated tumors (5/35). Axillary lymph node involvement was identified in 68.6% cases and vascular invasion was observed in 37.1% of cases. According to pTNM stage, the most frequent class was Stage 3B (40%), followed by Stage 1 (22.9%) and Stage 2A (20%).

Concerning immunohistochemical results, for all the cases we got 88.6% (31/35) cases for ER positive and 77.1% (27/35) cases for PR positive. HER2/neu immunohistochemical expression with 3+ score was identified in eight cases and with 2+ score was observed in three cases. For these cases, with an equivocal immunohistochemical expression, we performed CISH to assess the true amplification of HER2/neu gene. Only one case proved to have a true gene amplification and so HER2 positive was identified in 25.7% (9/35) cases. We also observed that 80% cases were Ki67 positive (18 – low expression, 10 – high expression) and 25.7% (9/35) cases were p53 positive. We assess the immunohistochemical expression of CK5/6 and EGFR in two cases and, we got a positive immunostain only for CK5/6.

**Molecular subtypes using immunohistochemical criteria**

Based on immunohistochemical expression of biomarkers, 65.7% (23/35) were identified as Luminal A subtype, 28.6% (10/35) as Luminal B subtype. The difference was represented by two (5.7%) cases of triple negative subtype, both of them being poorly differentiated CDI–NOS, with Stage 4 and high percentage of axillary lymph node metastases. Due to low number of patients with triple negative subtype, no correlations or prognostic significance could be assessed in these cases. For this reason, this subtype of tumors was excluded from further statistical analysis. In Table 1 are the main clinico-pathological features of invasive male breast carcinoma correlated with intrinsic luminal molecular subtypes.

### Table 1 – Correlation between the main clinico-pathological features of invasive MBC and molecular luminal subtypes according to IHC

<table>
<thead>
<tr>
<th></th>
<th>Total (n=33)</th>
<th>Luminal A (n=23)</th>
<th>Luminal B (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean age [years]</strong></td>
<td>64.1±9.7</td>
<td>63.1±9.5</td>
<td>66.1±10.3</td>
<td>0.442*</td>
</tr>
<tr>
<td>(range)</td>
<td>(40–81)</td>
<td>(40–81)</td>
<td>(48–80)</td>
<td></td>
</tr>
<tr>
<td><strong>Age [years]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>5 (15.2%)</td>
<td>4 (17.4%)</td>
<td>1 (10%)</td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>28 (84.8%)</td>
<td>19 (82.6%)</td>
<td>9 (90%)</td>
<td></td>
</tr>
<tr>
<td><strong>Mean tumor diameter [cm]</strong></td>
<td>2.6±1.6</td>
<td>2.3±1.5</td>
<td>3.4±1.8</td>
<td>0.078*</td>
</tr>
<tr>
<td>(range)</td>
<td>(0.5–6.5)</td>
<td>(0.5–6)</td>
<td>(1–6.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td>Yes</td>
<td>22 (66.7%)</td>
<td>12 (52.2%)</td>
<td>0.013*</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>11 (33.3%)</td>
<td>11 (47.8%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Mean number of positive lymph node examined [range]</strong></td>
<td>2.4 (0–10)</td>
<td>1.4 (0–5)</td>
<td>4.8 (2–10)</td>
<td>0.002*</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>19 (57.6%)</td>
<td>18 (78.3%)</td>
<td>1 (10%)</td>
<td>0.000*</td>
</tr>
<tr>
<td>III</td>
<td>14 (42.4%)</td>
<td>5 (21.7%)</td>
<td>9 (90%)</td>
<td></td>
</tr>
<tr>
<td><strong>AJCC staging</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I–II</td>
<td>18 (54.5%)</td>
<td>18 (78.3%)</td>
<td>0</td>
<td>0.000*</td>
</tr>
<tr>
<td>Stage III–IV</td>
<td>15 (45.5%)</td>
<td>5 (21.7%)</td>
<td>10 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>ER status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>31 (93.9%)</td>
<td>23 (100%)</td>
<td>8 (80%)</td>
<td>0.085*</td>
</tr>
<tr>
<td>Negative</td>
<td>2 (6.1%)</td>
<td>0</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td><strong>PR status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>27 (81.8%)</td>
<td>20 (87%)</td>
<td>7 (70%)</td>
<td>0.336*</td>
</tr>
<tr>
<td>Negative</td>
<td>6 (18.2%)</td>
<td>3 (13%)</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td><strong>HER2+</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8 (24.2%)</td>
<td>0</td>
<td>8 (80%)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Negative</td>
<td>25 (75.8%)</td>
<td>23 (100%)</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td><strong>Ki67 index</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative/low</td>
<td>24 (72.7%)</td>
<td>23 (100%)</td>
<td>1 (10%)</td>
<td>0.000*</td>
</tr>
<tr>
<td>High</td>
<td>9 (27.3%)</td>
<td>0</td>
<td>9 (90%)</td>
<td></td>
</tr>
<tr>
<td><strong>P53</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7 (21.2%)</td>
<td>1 (4.3%)</td>
<td>6 (60%)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Negative</td>
<td>26 (78.8%)</td>
<td>22 (95.7%)</td>
<td>4 (40%)</td>
<td></td>
</tr>
</tbody>
</table>

*Independent t-test; §Fisher’s exact test.

The mean age of Luminal A subtype was 63-year-old with a mean period of follow-up 75.3±33.4 months (range from 9 to 126 months). Regarding histopathological types, we found that 82.6% were CDI–NOS, 8.7% were invasive cribriform carcinoma (2/23) and the difference was...
represented evenly by invasive lobular carcinoma (1/23), invasive papillary carcinoma (1/23). Most of Luminal A cases were moderate differentiated (56.5%), but all the five well-differentiated tumors of our study were met only in this molecular subtype (21.7%). Regarding the stage, the most frequent was Stage 1 (34.8%), closely followed by Stage 2A (30.4%). The difference was represented by Stage 2B (13%), and Stage 3B (21.7%). Axillary lymph node metastasis was found in 52.2% (12/23) and lympho-vascular invasion was present in 17.4% of cases (4/23).

In Luminal B subtypes (Figure 1), the mean age was almost similar to that of Luminal A (66-year-old) with a mean period of followed up 53.6±28.6 months.

The mean tumor diameter was greater than Luminal A (3.4 cm versus 2.3 cm). The most frequent histopathological type was also CDI–NOS (80.0%), the other two cases being represented by one case of mix carcinoma (CDI–NOS associated with ILC) and one case with invasive cribriform carcinoma. Almost all cases were poorly differentiated carcinoma (90%) and one case was moderate differentiated carcinoma. Luminal B subtype was characterized in our study by a high percentage of Stage 3B tumors (90%) and only one case with Stage 3C. Lympho-vascular invasion was present in 70% of cases.

We found a significant correlation between luminal molecular subtype and tumor grade, lymph node status, AJCC stage, Ki67 index and p53 expression, but no significant correlation with age, tumor diameter or hormonal status (Table 1).

Using Kaplan–Meier method we evaluated the patients survival curve, which showed a statistical significant differences between patients with Luminal B tumors subtypes and those with Luminal A subtypes (p=0.007). The median survival for Luminal A cases was 120 months and 94.3 months for Luminal B group. The five-year relative survival rate for Luminal B was lowest than for Luminal A, 22% versus 81% (Figure 2). Also, stratifying patients regarding p53 immunoexpression, we observed an important and significant difference (p=0.015), p53 positive group having a worse survival than those with p53 negative (Figure 3).

Figure 1- Luminal B subtype: (a) Macroscopic aspect of MBC – a nodular, solid tumor with hard consistency; (b) ER immunoexpression – nuclear stain in 85% of tumor cell, ×40; (c) HER2+ immunostain with 2+ score (in case CISH – low level of HER2 gene amplification), ×100; (d) Ki67 immunostain – nuclear stain in more than 14% of tumor cells, ×40.
In the last decades, immunohistochemical markers have been used successfully as a surrogate in determining molecular subtypes (initially described by gene expression analysis), and based on the last guidelines of IHC-subtypes definitions [9] we observed that Luminal A subtype represent the highest proportion (65.7%) in our study, the second position being occupied by Luminal B (28.6%). Our results are similar to other studies who also emphasized the presence of a high frequency of Luminal subtypes in male patients, these proportions being higher than female counterpart, and low proportion or even absence of HER2 and basal-like subtype [22–24]. If proportion of Luminal A subtype in women BC range from 56% to 61% [25–27], in MBC were recorded values between 75% to 83% [22–24]. For our group, we obtained a value which is smaller than the records from literature regarding MBC, but still higher than female proportions. On the other hand, proportion for Luminal B subtypes is higher than those reported in different studies (6.2% to 21%) and also from female BC, were frequency range from 9% to 16% [27]. If HER2 and triple negative subtypes are frequent in female BC, in MBC these subtypes are less described or even absent [23, 24]. Basal-like subtype was represented by a very small number of cases (5.7%) in our group, which it made impossible for statistical analysis, and no HER2 or unclassified subtypes were identified.

Age is considered to be the single biggest risk for MBC [28]. Average age at diagnosis for our group was 64-year-old (range 40 to 81 years), consistent with other studies [23, 29], 86% cases were more than 50-year-old (higher than average age of diagnosis for female BC), but with no statistical significance between molecular subtypes. Tumor stage and axillary nodal status are already well-established prognostic factors, and for both of them we proved to be a statistically significant difference between molecular luminal subtypes. For our group we observed that positive lymph node average was nearly two times higher in Luminal B tumors than in Luminal A and all the stage I tumors were met only in Luminal A. In Luminal A subtype we identified a high proportions of well to moderate tumors with low clinical stages, in contrast with Luminal B subtype were poorly differentiated tumors with high clinical stage predominate.

Immunohistochemical expression of hormone receptors was represented by high values (ER with 88.7% and PR with 77.1%), in agreement with previous reports [24, 30], suggesting a beneficial role of neoadjuvant endocrine therapy for these patients [9]. HER2 protein (the product of HER2/neu oncogene, located on chromosome 17q21) is a member of the epidermal growth factor receptor family (EGFR) and plays an important role for cell differentiation and adhesion [31]. Overexpression or amplification of HER2/neu is encountered in 10–34% of invasive female BC [32]. It is associated with poor prognosis, earlier relapse and short survival time in both node-negative and -positive patients in some studies [33] whereas others did not [30]. In MBC the rate of HER2 immunopositivity widely range from one study to another, but in general has been reported highly proportions (30–56%) comparing with those from female BC [34–37]. In our study, HER2 was positive in 25.7% cases, result that do not differ considerably from female counterpart. The HER2 status is also an important predictive factor for HER2-targeted antibody Trastuzumab (HercepTest), which combined with chemotherapy increases response rates and survival [38].

P53 oncogene play an essential role in cell apoptosis and its alterations are the most frequently gene modification encountered in malignant tumors, inhibiting cell death through apoptosis [39, 40]. The prognostic role of p53 is still under debate. There are reports that proved that p53 overexpression was correlated with poor survival [41] but other failed [34, 42]. In our study, p53 immunoexpression was positive in 25.7% of cases, in agreement with literature where it has been reported proportions of p53 immunostain range from 18% to 58% [30, 43]. In our group of patients,
positivity of p53 predicts a worse outcome, with a statistical difference between the two-Luminal subtypes.

Conclusions

Our study demonstrated that the most frequent molecular subtypes are Luminal A and Luminal B with a worse outcome for the latter. Triple negative subtype was identified in a few patients and no case for HER2 subtype. We recorded high levels of ER and PR expression, which predict a better response to adjuvant hormonal therapy. At the time of diagnosis, most of the patients were aged and with an advance clinical stage, this requiring implementation of screening programs and increase education of population in order to an early detection. Even if our study has a low statistical power, because of the small number of patients, still our results can be useful for future multicenter clinical trials.

Acknowledgments

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References


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