DNA Content Analysis of Breast Lobular Invasive Carcinoma

J da Costa Silva Neto, V Arias, N Shirata, A Filho, A Filho

Citation

Abstract
Breast cancer is highly prevalent worldwide. In Brazil, mortality consequent to the breast cancer is one of the most frequent in women. Lobular carcinoma present high risk of bilateral development and recurrence; it is related the hormonal expression, have rapid progression, and does not show reactivity for E-cadherin protein. The DNA pattern of lobular carcinoma is disputable; some studies have reported it as predominantly diploid and others, in contrast, predominantly aneuploid. The objective of this study was to analyze the pattern of the DNA-ploidy in infiltrating lobular breast carcinoma, and correlate ploidy characteristics with well-documented prognostic factors: expression of the protein p53, C-erb-B2, estrogen receptor, size of the tumors, invaded lymph node, distant and post-surgery metastasis. DNA content revealed predominant aneuploidy (63.16% of the cases) and showed a nearly significant correlation with lymph node status (p=0.07); the other parameters did not show significant association. DNA content is not a reliable parameter to evaluate the aggressiveness of lobular carcinoma.

INTRODUCTION
Breast cancer is one of the most prevalent cancers and affects millions of women worldwide. Around one million new cases are reported annually ('). Lobular invasive carcinoma (LIC) represents 10% of all breast cancer variants. LICs occur in both breasts and generally have a multicentric development in the same breast. Invasive LIC have diffuse pattern that importantly difficult its identification by image methods ('). This is an important concern because the clinical approach is thought to be different between ductal and lobular carcinomas ('). Among many ancillary techniques available presently, the evaluation of DNA content is considered relevant due to its remarkable prognostic impact for many tumours ('). The evaluation of aneuploid DNA content in breast tumors is believed to be a valuable parameter to define malignancy in spite of a number of malignant neoplastic lesions present in the samples ('). Increased incidence of aneuploidy is related to markers of poor prognosis such as estrogen and progesterone receptor negative tumors, tumors from patients with positive axillary's lymph nodes, tumors greater than 2 cm in diameter, and patients younger than 35 years of age ('). DNA ploidy, measured by image analysis, is predominantly associated with markers of cell differentiation and is adjudicate as ancillary option for prognosis ('). Despite of controversies, nuclear DNA content is believed to provide an objective marker of tumour aggressiveness ('). Specifically to lobular carcinoma of the breast, there is modest information about the value of ploidy evaluation. This is consequence, in part, of the low number of cases of lobular carcinoma in contrast to the ductal carcinoma. But the existing results are controversial and the value of ploidy to LIC prognosis is disputable ('). The prognostic significance of DNA ploidy has been widely studied in breast cancer as a whole, but their clinical utility remains contentious. The type of tumour material can significantly influence the DNA measurements mainly if flow cytometry is used ('). This is important because based on DNA aneuploidy pattern, e.g., patients with node negative breast cancer can be stratified into low-risk and high-risk subgroups and also. Identify high-risk patients with lymph node negative breast cancer who might benefit from additional adjuvant therapy ('). Part of this assumption was corroborated by studies with rigorous follow-up that showed that recurrence rate in patients with in situ carcinoma of the breast was significantly related to nuclear size of the primary lesion. As nuclear changes might be related to DNA content, DNA ploidy analysis showed that more than 80% of these lesions were DNA aneuploid, with a distribution similar to that found in invasive carcinomas. Interestingly, this finding raised the hypothesis that the DNA pattern of an invasive...
carcinoma was already established at the pre-invasive stage of DCIS (\(10\)).

The goal of this study was to analyze, by static cytometry, the DNA content pattern of well-documented series of lobular carcinoma and establish the importance of ploidy feature on lobular breast carcinoma and correlates these findings with immunohistochemical evaluation classically used to assess lobular carcinoma aggressiveness.

**MATERIAL AND METHODS**

One hundred cases of LBC were retrospectively identified and selected for this study from the files of the Department of Pathological Anatomy of the A.C. Camargo Cancer Hospital of São Paulo, between 1983 and 2002. The cases selected were processed by the Division of Pathology of the Adolfo Lutz Institute (ALI) /SP for the study of the DNA-ploidy content and immunohistochemistry reactions. The cases were revised and categorized according to WHO (World Health Organization) classification (\(12\)). Mixed cases of lobular carcinoma and invasive ductal carcinomas were excluded.

Previously silanized slides were used for Feulgen-thionine staining and immunohistochemical reactions, prepared with sections of 5µm and 4µm, respectively.

**STATIC CYTOMETRY**

Ploidy measurement was performed with Becton & Dickinson CAS 200 system (Becton & Dickinson Cellular Imaging Systems - San Jose, CA, USA). The samples were deparaffined for 20 minutes in xylene in a oven at 60°C; keeping home temperature for 20 minutes and hydration at xylene, following a decreasing sequence of the concentration until arriving to distilled water where they were washed many times to remove the excess of alcohol. Afterwards, the sections were stained with Feulgen-Thionine CAS Quantitative DNA kit (Becton & Dickinson Cellular Imaging Systems - San Jose, CA, USA). This kit is based on the method which uses chloride acid to carry out the DNA hydrolysis, and consequently promoting the formation of aldehyde groups in the desoxirribosic fraction of the DNA. At a second stage, these aldehyde groups are processed through the use of the Schiff reagent. The color intensity is directly proportional to the DNA content (mass) in accordance with the Lambert-Beer’s absorption law.

**ANAlysis of the Images**

DNA ploidy was assessed by CAS 200 Becton & Dickinson System which is supplied of a program to quantitatively evaluate the DNA ploidy (version 3.0, 804860 66 Mhz PC EISA 32 bits).

The microscope used was a Leica Diastar, especially developed for this system. It is supplied of a pair of cameras of two different wave lengths to catch the images: 500nm and 620nm. The entire system is interconnected to two video monitors with the proposed wave lengths.

**CALIBRATION OF THE IMAGE ANALYZER**

The calibration proceeding (external reference) is made through histological transverse sections of rat hepatocytes (external diploid control) that are submitted to the Feulgen-thionine’s simultaneously with the LBC’s. The variation coefficient of the reference must not be greater than 5%. In the CAS 200 system it is necessary to count more than 20 cells so as to perform the calibration. Any value smaller than 20 is not accepted by the system which impede subsequent readings. For this reason, the calibration was made by counting 20 cells of rat hepatocytes. To increase the reading rigor and to obtain data which served for comparison with the findings of the LBC sample, endothelial cells were used as an internal diploid reference control.

**THE PLOIDY INTERPRETATION**

Approximately 150 to 200 atypical cells of each LBC case were evaluated. After the definition of the cross threshold, integral cells were selected one by one, excluding those with fragmented and added nucleus.

The DNA analyses of the cells were distributed in histograms similar to the Gauss’ curves, for further interpretation. At the same time, the cells were distributed in a scatter type of plot which compared Area (µ3) versus Mass in the different cases of LBC.

**HISTOGRAMS INTERPRETATION**

The evaluation of the indexes was based on the following classification (\(13\)): DNA index (ID) of 0.80 to 1.20 classified as diploid, from 1.20 to 1.30 as peridiploid and from 1.80 to 2.20 as tetraploids. Aneuploid cases are all those which do not belong to the above intervals, including those named as “more probably triploid” = 1.40 – 1.60. For dichotomic comparison (aneuploid versus peridiploid), all categories, except aneuploid, were generically categorized as peridiploid.

**IMMUNOHISTOCHEMICAL REACTION**

Avidin-biotin-peroxidase complex assay for immunohistochemical reaction was used with the
monoclonal antibody E-Cadherin antibody (DAKO, clone NCH38, DAKO Corporation, Carpinteria, CA, USA). In brief, deparaffinized and re-hydrated sections were immersed in EDTA/TRIS pH 9.0, preheated at 96°C for 40 minutes. The slides were incubated with 3.0% hydrogen peroxide in methanol for 30 minutes, followed by incubation with a protein blocker at home temperature for 20 minutes (DAKO Corporation, Carpinteria, CA, USA) before incubating with the primary antibody diluted 1:300, overnight at 4 °C. Sections were sequentially washed in 3 baths of PBS and incubated with biotin secondary antibody (Kit StreptABComplex/HRP Duet, DAKO, K0492, DAKO Corporation, Carpinteria, CA, USA), for 20 minutes at room temperature. Finally, samples were incubated with avidin-biotin-peroxidase complex (Kit StreptABComplex/HRP Duet, DAKO Carpenteria, CA, USA) in the pre-established title of 1/200, for 20 minutes in water/humid chamber at home temperature and developed with 3,3'-diamino-benzidine for 15 minutes (DAB System Chromogenic Substrate, DAKO, DAKO Carpenteria, CA, USA).

ETHIC COMMITTEE

The present study was approved by both Ethic Committees from Adolfo Lutz Institute and the A.C. Camargo Cancer Hospital of São Paulo/Brazil. Information about the procedure and follow up were obtained from data of the patient files, by direct medical supervision of them.

RESULTS

Three hundred and sixty-eight cases of breast invasive lobular carcinoma (LIC) were assessed, diagnosed and registered at the Pathology Department of the A.C. Camargo Cancer Hospital. From these, 100 cases were randomly selected and revised for histopathological study, resulting in 77 cases which were suitable for the study.

Cases with strong or weakly E-cadherin positive reactions were excluded. From 77 selected LIC cases 51 were E-cadherin negative, 15 were positive and 11 were weakly-positive. Only the negative reactions were selected for the sequence of the work.

Table 01 show a summary of the principal data obtained per group: group of patients that were alive until the time of the survey (simply identified as “alive”), group of patients that died until the date of the survey (denominated “dead”) and every case of negative E-cadherin. Lymph node invasion was observed in 50% (16/32) of the cases. The metastases were present in 48.28% (n=29).

The following results attempted to correlate ploidy with clinical and laboratorial parameters of breast cancer characteristics. No significant information was observed correlating age (p=0.179), menarche (p=0.787), menopause (p=0.437), and age at first pregnancy (p= 0.461).

Table 02 depicted the correlation between DNA content patterns and the classic surgical pathological parameters. Importantly, no significant result was proved to correlate with DNA content pattern.

The correlation of DNA content pattern with TNM clinical pathological parameters is showed in Table 3. The size of primary tumor was not informative (p=0,343) but it was observed that in the group of dead patients the average was 8 cm and in contrast to the group of living patients where the average was of 3.31 cm. The status of lymph node (LN) only showed a nearly statistically significance (p=0.07) and, interestingly, the number of invaded LN wasn't (p=0.884). Table 4 show the not significant correlation of DNA content pattern with tumoral vascular embolus (p=0.184), metastasis prior treatment (p=0.571) and metastasis after treatment (p=0.648). Additionally, localization of the LIC was correlated but no significant result was observed (p=0.254).

Finally, Table 5 exhibit the correlation of DNA content pattern with three of the most widely used immunohistochemical markers in breast cancer routine. Estrogen receptor status was not significant (p=0,363) in comparison to the DNA ploidy as well as the C-erb-B2 (p=0.648). It was not possible to calculate the p53 expression significance because all cases were negative.

The survival curve for aneuploid patients was not possible to be figured. The diploid patients however have showed a survival average of 8.6 years old (Figure 1).
**DNA Content Analysis of Breast Lobular Invasive Carcinoma**

**Figure 1**
Table 1: Clinical data from the patients with immunohistochemical-proven negative E-cadherin.

<table>
<thead>
<tr>
<th></th>
<th>Patients alive (n=10)</th>
<th>Patients died (n=10)</th>
<th>Negative E-cadherin (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age average</strong></td>
<td>55</td>
<td>45</td>
<td>11</td>
</tr>
<tr>
<td><strong>Left breast affected</strong></td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td><strong>Histologic grade</strong></td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 2**
Table 2: Correlation of DNA content pattern with surgical pathological parameters.

<table>
<thead>
<tr>
<th>Vascular tumor emboli</th>
<th>PD</th>
<th>AP</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>p = 0.018</td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
<td>7</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3**
Table 3: Correlation of DNA content pattern with TNM clinical pathological parameters.

<table>
<thead>
<tr>
<th>Size of the Tumor (cm)</th>
<th>PD</th>
<th>AP</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (&lt; 2 cm)</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>p = 0.034</td>
</tr>
<tr>
<td>T1c (2-5 cm)</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4**
Table 4: Correlation of DNA content pattern with clinical pathological parameters.

<table>
<thead>
<tr>
<th>Vascular tumor emboli</th>
<th>PD</th>
<th>AP</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>p = 0.002</td>
</tr>
<tr>
<td>Positive</td>
<td>3</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td>12</td>
<td>15</td>
<td>27</td>
<td>p = 0.001</td>
</tr>
</tbody>
</table>

**Histological grade**

<table>
<thead>
<tr>
<th>Grade</th>
<th>PD</th>
<th>AP</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>p = 0.027</td>
</tr>
<tr>
<td>II</td>
<td>12</td>
<td>15</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nuclear grade</th>
<th>PD</th>
<th>AP</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>p = 0.024</td>
</tr>
<tr>
<td>Grade II</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

| PD= diploid AP= aneuploid |
DNA Content Analysis of Breast Lobular Invasive Carcinoma

**DISCUSSION**

The importance of DNA-ploidy analysis to precisely identify cases of lobular carcinoma with aggressive phenotype was not achieved in our study. All the correlations we have used to test the value of ploidy in relation to clinical and laboratorial parameters did not show significance, except by a modest result in relation of aneuploid versus lymph node status. The assessment of informative data regarding ploidy evaluation in this series showed several limitations, most of them related to the restrictive study design we have opted. First, well-documented files from patients with lobular carcinoma were very difficult to be found, in part due to the small number of cases when compared with invasive ductal carcinoma. The lack of more accurate information related to the follow up of some patients limited the analysis of survival curve. Secondly, we were very rigorous to classify LIC, not only by histological parameters but also by the inclusion of E-cadherin negative result as necessary condition to ratify the diagnosis of breast LIC. This is, indeed, a disputable option because under this circumstance, true LIC could be excluded if they presented focal E-cadherin positive reaction in residual ductal structures. Notwithstanding, the cases analyzed showed that the majority of classic variant of LIC showed aneuploid associated with important characteristics of aggressive clinical behavior such as size of the tumor greater than 5 cm and histological grade II classification (16). Aneuploid cases corresponded to 57.1% of cases and, as previously mentioned, correlated with lymph node status but not with distant metastasis. The predominance of aneuploid in LIC was previously reported by Zandona and colleagues (14) that also observed that aneuploid is more frequently found in LICs than in medullary, papillary or colloid variants of breast carcinoma. Our results are similar to Spiethoff and co-workers which found 68% of aneuploid among the lobular carcinomas they studied (15). Most important, aneuploid was correlated to high grade histological pattern which support its use as a prognostic marker (16).

Ploidy content analysis did not correlate with the expression of c-erb-B2 and p53, and neither with estrogen receptors, but the majority of LIC positive for estrogen receptors were aneuploid. Clinically, LICs positive for estrogen receptors are thought to be more responsive for hormonal therapy are related to better prognosis (17). Conversely, the absence, or faint expressions of p53 and c-erb-B2 are associated with worse therapeutic results and poor survival rates (18).

The results obtained from ploidy analysis could be questionable because there are a number of inherent biases involved in the process of DNA content evaluation. Errors of extracting appropriate area of the tumor and processing the tissues samples can directly influence the analysis (19).

Additionally, conflicting results can also occur depending on the methodology chosen to evaluate ploidy. Flow cytometry (FC) is generally preferred due to its great sensitivity to analyze a tissue sample. The most important advantage of FC is the multiparameter evaluation of single cells and the ability to work with very small samples (20). However, flow cytometry do not discriminate the type of cellular population
studied. Accordingly, DNA content of neoplastic cells is evaluated in combination with a plethora of non neoplastic cells, which can lead to discrepancies between flow and static cytometry analyzes (\(c_{0}\)). Static cytometry is very specific for samples with few target cells. The association between flow and static cytometry demonstrated that diploid DNA status associated with no lymph node infiltration correlated with good prognosis; and, aneuploid DNA status are related with survival curve significantly shorter than those observed for cases with diploid pattern (\(c_{0}\)).

Ten from 50 patients (20.0%) died in this series, with a survival rate of 50 months (± 27.6); until the conclusion of the work 15 patients were alive (30.0%) and the follow up of 25 (50.0%) was interrupted. The survival curve for peridiploid patients showed that the patients had a total survival rate of 8.6 years. This value is encouraging and future study with improved casuistic of lobular carcinoma, can show most significant results related to DNA content evaluation.

ACKNOWLEDGMENTS

The authors are indebted with Prof. Dr. Fernando Soares for allowing us to study the material from AC Camargo Cancer Hospital (São Paulo-Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES – Budget No. 0001070000.0382005.

CORRESPONDENCE TO

Adhemar Longatto Filho, M.Sc., PhD, PMIAC
Life and Health Sciences Research Institute
School of Health Sciences
University of Minho
Campus de Gualtar
4710-057 Braga, Portugal

References

Author Information

Professor of Cytopathology, FEJAL/CESMAC/FCBS

Victor Arias, M.D., Ph.D.
Department of Pathology, Federal University of São Paulo

Neuza Kasumi Shirata, M.Sc.
Division of Pathology, Adolfo Lutz Institute

Adauto Castelo Filho, M.D., Ph.D.
Division of Infectious Disease, Federal University of São Paulo (UNIFESP)

Adhemar Longatto Filho, M.Sc., PhD, P.M.I.A.C
Life and Health Sciences Research Institute, School of Health Sciences, University of Minho