Bivariate RNA and DNA Content Analysis in Breast Carcinoma: Biological Significance of RNA Content

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ABSTRACT
Flow cytometric studies of mammary carcinoma have been limited to DNA content analysis. Simultaneous analysis of DNA and RNA has been applied to hematological and certain solid neoplasms and has been shown to provide valuable information in the clinical assessment of these tumors. To determine whether measuring RNA content during flow cytometric analysis provides additional information in the clinical assessment of breast carcinoma, dual-parameter analysis of DNA and RNA content on freshly disaggregated breast carcinoma specimens was performed. RNA content, divided along the mean (≤1.6 and >1.6), correlated with tumor grade, histological type, hormonal status, and patient survival. DNA aneuploidy was noted in 247 (69.2%) neoplasms and correlated significantly with tumor grade and survival. RNA content, proliferative fraction, and tumor stage were independent prognostic indicators. Our results indicate that measurement of cellular RNA content provides additional biological information that may be useful in the clinical assessment of breast carcinoma.

INTRODUCTION
Previous flow cytometric studies of mammary carcinomas have been restricted to the analysis of DNA content. These investigations provided evidence for the clinical utility of such analysis in the evaluation of these neoplasms. However, no consensus has been reached regarding the role of DNA content information in the management of individual patients or on its independence from known prognostic factors in breast carcinomas (1).

Cellular RNA content reflects the translational activity (2–4) of neoplastic cells and may add further biological information to the flow cytometric evaluation of breast carcinoma. Simultaneous analysis of DNA and RNA content by flow cytometry was achieved by Darzynkiewicz et al. (2) in 1975 using acridine orange. Subsequent investigations have shown that analysis of RNA content allows discrimination between cycling and noncycling cells, classification of various leukemias and lymphomas, and assessment of the biological behavior of certain tumors (5–24). Similar studies of solid neoplasms, exclusive of mammary carcinoma, have also demonstrated a diagnostic and prognostic merit for such analysis (25–36).

This is the first study to apply this technique in the determination of concomitant RNA and DNA content in the biological evaluation of breast carcinoma.

MATERIALS AND METHODS
We retrospectively reviewed histograms of 1170 fresh tumor tissue and fine-needle aspiration specimens from patients with breast carcinoma that were submitted to the flow cytometry laboratory of the Department of Pathology at the University of Texas M. D. Anderson Cancer Center from November 1982 to October 1990. Criteria for inclusion were based on: (a) the analysis of at least 5000 cells in any given sample; (b) the availability of Giemsa-stained cytospin preparations for each specimen; and (c) the presence of >80% neoplastic elements with at least 50% intact cells in each sample. Three hundred fifty-seven specimens from an equal number of patients met these criteria and comprised the materials for this study.

Flow cytometric information, including the DNA index, proliferative fraction, and RNA index, was recorded from the histograms of these tumors. The hematoxylin and eosin-stained slides of all cases were reviewed, and tumors were graded according to Black’s nuclear grading system. Demographic clinicopathological and patient follow-up information were obtained from surgical pathology reports and by reviewing patients’ medical records.

Staging and Therapeutic Protocols
After multidisciplinary breast evaluation, patients were staged according to the American Joint Committee on Cancer staging system into: stage I, invasive tumors ≤2 cm with a negative axilla; stage II, invasive tumors >2 cm but <5 cm or nonfixed axillary adenopathy; and stage III, invasive tumors 5 cm or greater, or skin involvement, or fixed (matted) axillary adenopathy. No systemic adjuvant therapy was given to stage I patients after mastectomy or breast conservation surgery during the time period of this study. Patients with stage II disease and patients who refused preoperative chemotherapy for stage III disease were offered a clinical trial to evaluate the role of additional chemotherapy. Four cycles of vinblastine and methotrexate following standard postoperative treatment and six cy-

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cles of 5-fluorouracil, doxorubicin, and cyclophosphamide were given to patients <50 years old regardless of estrogen receptor status and in patients older than 50 years with estrogen receptor-negative assays. For patients >50 years old with estrogen receptor-positive tumors, a randomized comparison of the efficacy of tamoxifen for 5 years to combination chemotherapy (six cycles of 5-fluorouracil, doxorubicin, and cyclophosphamide followed by four cycles of vinblastine and methotrexate) was offered.

To determine whether the extent of residual disease in the mastectomy specimen after preoperative chemotherapy for stage III disease could be used as a guide to plan postoperative adjuvant treatment, the clinical trial during the time period of this study (1982-1990) consisted of three cycles of VACP\(^2\) administered at 21-day intervals followed by a modified radical mastectomy (complete removal of all breast tissue and an axillary lymph node dissection). Patients with histologically confirmed complete response and those with >1 cm\(^3\) of residual tumor received five additional cycles of VACP; those with no response to preoperative chemotherapy were crossed over to receive five cycles of methotrexate, 5-fluorouracil, and vinblastine. Patients with partial responses were assigned randomly to receive five additional cycles of VACP or methotrexate, 5-fluorouracil, and vinblastine. All patients received radiation to the chest wall and regional lymph nodes.

**Flow Cytometry**

Single-cell suspension from neoplastic tissues were prepared by mechanically mincing fresh tissue in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA), forcing the minced tissue through a stainless steel wire mesh, and passing the filtrate through a syringe and into a Petri dish. Samples were washed twice with PBS (Irvine Scientific) containing 2 mM/liter MgCl\(_2\). Cells were resuspended in PBS and filtered through a 37-μm nylon mesh. The cell count was then adjusted to 1.0 × 10\(^6\) cells/ml. A cytospin preparation was obtained and evaluated for cellular integrity and clumping.

**DNA and RNA Staining by Acridine Orange.** Simultaneous staining of DNA and RNA was performed by a two-step method using acridine orange (37). An aliquot of the fresh cell suspension was subjected to detergent treatment (0.1% Triton X-100) at a low pH to render the cells permeable to the dye while allowing cells to retain their integrity. Cells were subsequently stained with a solution containing acridine orange in the presence of EDTA to denature double-strand RNA. Acridine orange intercalates into double-stranded DNA and upon excitation by a 488-nm wavelength emits green fluorescence with a maximum intensity at 530 nm. The dye also simultaneously stacks electrostatically on single-strand RNA and emits red fluorescence with a maximum intensity at 640 nm. To ensure the specificity of RNA staining, cells were incubated with RNase A for 20 min. RNase A-treated cells showed a >90% decrease in fluorescence intensity at >610 nm compared with untreated samples.

For dual-parameter analysis of the DNA and RNA content, an EPICS Profile II cytometer (Epics Division, Coulter Corp., Hialeah, FL) with an argon-ion laser emitting 488 nm (15 mW) was used. A 525 band-pass for green fluorescence (DNA) and a 610 long-pass filter for red fluorescence (RNA) were used. Peak versus integral signals were used to discriminate doublets. Analyses of cell cycle distribution was computed according to the model described by Johnston et al. (38).

**DNA and RNA Analysis.** Ploidy was determined by the DNA index, which is the ratio of the relative Go/G1 of a tumor sample to that of healthy lymphocytes. Diploid DNA content was designated by a DNA index of 1.00, and DNA aneuploidy was indicated by the presence of a second distinct peak. DNA hyperdiploidy was defined by a DNA index of >1.00, and DNA hypodiploidy was determined when the DNA index was <1.00 after mixing the test sample with standard biological lymphocytes from healthy donors and occasionally the patients’ own normal tissue. Samples were also mixed with normal lymphocytes when overlapping near-diploid aneuploid peaks were present. The coefficient of variation of the diploid peak for all samples ranged from 2.3 to 6.5% (mean, 3.9% ± 1.4%). The coefficient of variation for the aneuploid stem line ranged from 3.2 to 7.8% (mean, 5.0%). The proliferative index (S + G2-M) was dichotomized into groups of >10% and <10% based on the mean value of the samples analyzed.

Relative RNA indexes for individual samples were expressed as the ratio of the mean RNA level of the Go/G1 peak of the test sample divided by the mean RNA level of normal lymphocytes. The RNA value of the aneuploid peak was used for DNA aneuploid tumors in this study. RNA indexes of ≤1.6 and >1.6 were used as a cutoff between samples on the basis of the best discriminatory value in the statistical analysis.

**Statistical Methods**

The prognostic effect of a variable was assessed using the log rank test and the Kaplan-Meier method. Fishers’ exact test was used to determine the correlations between flow cytometry data and tumor characteristics. The stepwise multivariate proportional hazard model of Cox was applied in the analysis of factors predictive of survival.

**RESULTS**

All 357 patients included in this cohort had undergone mastectomy. The follow-up period ranged from 52 to 95 months, with a mean of 78 months. The study population was comprised of 256 (71.7%) white, 47 (13.2%) black, 39 (10.9%) Hispanic, 4 (1.1%) Asian, and 11 (3.1%) ethnically unknown patients. Patients’ ages ranged from 26.1 to 86.4 (mean, 54.0 ± 13.4) years. Tumor size ranged from 0.5 to 13 cm (mean, 2.94 ± 1.99 SD). Three hundred thirty (92.4%) tumors were invasive ductal (including atypical medullary carcinoma) and 27 (7.6%) were lobular carcinomas. One hundred forty-six (40.8%) tumors were Black’s nuclear grade I, 191 (53.5%) were nuclear grade II, and 14 (3.9%) were nuclear grade III; this information was missing for 6 (1.7%) of the tumors. Clinical stage of disease was stage I in 62 (17.4%) patients, stage II in 255 (71.4%) patients, and stage III in 40 (11.2%) patients. Estrogen receptor-negative (<10 fmol) tumors constituted 28.3% (101 cases) of all

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\(^2\)The abbreviation used is: VACP, vincristine-doxorubicin-cyclophosphamide-prednisone.
samples, and hormone receptor-positive (>10 fmol) tumors comprised 67.5% (241 cases); hormone receptor status was not available for 4% (15 cases). Axillary lymph nodes were negative for disease in 166 (46.5%) cases and were positive in 191 samples, and hormone receptor-positive (>10 fmol) tumors.

**Table 1** Univariate analysis of demographic and tumor characteristics and survival of patients with mammary carcinoma

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Alive</th>
<th>Dead</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>276</td>
<td>81</td>
<td>0.07</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>205</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>31</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>27</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55</td>
<td>142</td>
<td>41</td>
<td>0.92</td>
</tr>
<tr>
<td>≥55</td>
<td>134</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3.5</td>
<td>174</td>
<td>27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt;3.5</td>
<td>102</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Black’s nuclear grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>162</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>58</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>II</td>
<td>196</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>22</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>DNA ploidy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneuploid</td>
<td>185</td>
<td>61</td>
<td>0.25</td>
</tr>
<tr>
<td>Diploid</td>
<td>90</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>RNA Content (index)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≤1.6</td>
<td>163</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>&gt;1.6</td>
<td>112</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Proliferative index</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≤10</td>
<td>163</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>&gt;10</td>
<td>112</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Hormone status</td>
<td></td>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td>Negative</td>
<td>71</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>197</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>142</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>134</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The profound cellular and biological heterogeneity of mammary carcinomas complicates attempts to better predict their clinical behavior and treatment response. Recently, several new biomarkers with putative clinical relevance in assessing their behavior have been investigated (39–42). Of these, flow cytometry has emerged as a versatile, expedient, and objective technique for clinical and investigative studies (43–53). Previous flow cytometric analysis of mammary carcinomas has been restricted to DNA content measurement because of the archival nature of retrospective studies and the convenience of
using the less cumbersome DNA fluorochromes. Even though studies have shown a strong overall correlation between the flow cytometric analysis of the proliferative index and clinical outcome, a definitive role for flow cytometric DNA analysis in the management of individual patients with breast cancer remains controversial (1, 39, 45, 53, 54).

Using dual-parameter analysis, several studies have shown that the RNA index correlates with the DNA content as well as
other biological factors in several hematoreticular and solid neoplasms (6–36). Moreover, cellular heterogeneity can be minimized in RNA content-based analysis (9). Despite these advantages, the application of this technique, especially in solid tumors, has been limited. This is largely because of technical difficulties in retrieving intact cells from solid neoplasms, critical optimization of acridine orange staining, and the tendency of this fluorochrome to stain plastic tubing of flow cytometers (3, 4).

Acridine orange staining of tumor cells requires preservation of cellular integrity and full denaturation of double-stranded RNA without altering the double strands of DNA. This can be achieved by a careful dissociation of tumor tissues and adjustment of the dye and ionic concentrations in solutions (38). To minimize the effect of marked cell disruption on the RNA analysis in this study, only specimens with more than 50% intact neoplastic cells were analyzed. It has to be realized, however, that inevitably variable proportions of bare nuclei and partially disrupted cells will be present and this may reduce the overall RNA values. However, tumor cells contain nucleoli, the site of RNA synthesis, and this may partially reflect their RNA content (7, 26).

Our univariate analysis showed that the RNA level correlates significantly with the clinical outcome. Patients with a high RNA content have lower proliferation indices than patients with a low RNA content.

**Table 2** Correlation between flow cytometric data and forms of therapy in patients with mammary carcinomas

<table>
<thead>
<tr>
<th>Therapy</th>
<th>DNA ploidy A</th>
<th>RNA content &gt;1.6</th>
<th>RNA content ≤1.6</th>
<th>PI &gt;10</th>
<th>PI ≤10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29</td>
<td>78</td>
<td>0.38</td>
<td>51</td>
<td>55</td>
</tr>
<tr>
<td>No</td>
<td>79</td>
<td>167</td>
<td></td>
<td>130</td>
<td>115</td>
</tr>
<tr>
<td>Postoperative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>190</td>
<td>78</td>
<td>0.28</td>
<td>130</td>
<td>136</td>
</tr>
<tr>
<td>No</td>
<td>55</td>
<td>30</td>
<td></td>
<td>50</td>
<td>35</td>
</tr>
</tbody>
</table>

* a, diploid; A, aneuploid; PI, proliferative index.

**Table 3** Correlation between different tumor characteristics and flow cytometric information in patients with mammary carcinomas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Ploidy (D vs. A)</th>
<th>RNA content &gt;1.6</th>
<th>RNA content ≤1.6</th>
<th>PI ≤10</th>
<th>PI ≥10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type</td>
<td>=0.27</td>
<td>=0.01</td>
<td></td>
<td></td>
<td>=0.55</td>
</tr>
<tr>
<td>Size</td>
<td>=0.35</td>
<td>=0.13</td>
<td></td>
<td></td>
<td>=0.03</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>&lt;0.001</td>
<td>=0.12</td>
<td></td>
<td></td>
<td>=0.003</td>
</tr>
<tr>
<td>Hormone status</td>
<td>=0.07</td>
<td>=0.05</td>
<td></td>
<td></td>
<td>=0.003</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>=0.73</td>
<td>=0.52</td>
<td></td>
<td></td>
<td>=0.02</td>
</tr>
</tbody>
</table>

* a, diploid; A, aneuploid; PI, proliferative index.
RNA content had a worse survival outcome than those with a low RNA content. RNA analysis, therefore allowed for further discrimination of the clinical behavior of patients within the DNA diploid and aneuploid tumors. Similar association between RNA content and tumor biological behavior has been reported in different solid neoplasms (25, 26, 32, 34). RNA content also correlated significantly with tumor grade, histological subtype (ductal versus lobular), and hormonal status. No significant association between RNA content and tumor size, tumor stage, or lymph node status was found. These results underscore the independence of RNA content from the traditional prognostic indicators. Our data may, therefore, lend further support to the putative association between ribosomal RNA levels and the oncogenic activity in neoplastic cells.

In agreement with the majority of previous flow cytometric studies of mammary carcinomas, our results show a significant correlation between the proliferative fraction and survival in both univariate and multivariate statistical analyses (39, 45, 50–52, 54–58). The proliferative fraction was also significantly associated with tumor grade, size, hormone receptor, and lymph node status, as previously shown by others (53, 54, 57, 59).

Although our results on the incidence of DNA ploidy is in concordance with previous investigations (51, 54, 60–65), no significant correlation between this feature and survival was found. DNA ploidy, as previously reported (66–71), was significantly correlated with tumor grade and stage in the present study. This association suggests an interdependence between DNA ploidy pattern, and these parameters and may explain the lack of statistical significance in multivariate analysis. Our data also show that traditional clinicopathological factors such as nuclear grade, lymph node status, tumor stage, and hormonal status maintained their association with the pathobiological characteristics of these tumors. In our study, a significant difference in survival between treated and nontreated patients was observed. Patients who underwent pre- and/or postsurgical treatment were associated with poor outcomes. This can be attributed to the selection of patients with high-risk factors for adjuvant therapy.

Overall, our results indicate that the RNA content, proliferative fraction, and tumor stage are independent predictors of the clinical course in this cohort and that RNA analysis provides additional biological information that may subserve other prognostic factors. Prospective clinical trials applying this technique may accurately define the role of RNA in the management of individual patients with this disease.

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