ABSTRACT

Primary tumors from breast cancer patients were evaluated for the biochemical presence of three steroid cytosolic receptors and by DNA histogram analysis using flow cytometry. These parameters were compared with the histological and staging diagnoses and the patients' survival over a 36-month period. A total of 74 patients with primary breast tumors were evaluated. The breast samples invariably demonstrated a peak population of diploid G_0 phase cells which contained 2C amounts of DNA, as determined by mixing experiments using normal human breast tissues or trout erythrocytes as fixed standards. The tumors were classified into five DNA histogram types based on their DNA index distributions established by flow cytometry. These results showed that 21% of the tumors were diploid and indistinguishable from the diploid population of normal breast cells, 8% were hypodiploid, 11% were hypertetraploid, 8% were multiploid, and the remaining 52% were hyperdiploid. The DNA index values varied from 0.78 (hypodiploid) to 2.60 (hypertetraploid). The percentages of S-phase cells were lowest in the diploid and hypertetraploid tumors and highest in the hypodiploid tumors. Among the 24 patients who died during the 36-month follow-up, 92% (22 of 24) were classified in one of the aneuploid groups. Three high-risk groups identified on the basis of survival after 36 months were distinguished: hypodiploid (50% survival); multiploid (43% survival); and hyperdiploid (50% survival). Rates of survival in the diploid and hyperdiploid groups were 87 and 71%, respectively. The hypodiploid group was distinguished by having the lowest mean estrogen cytosolic receptor value [26 ± 13 (S.D.) fmol/mg], progesterone cytosolic receptor value (13 ± 15 fmol/mg), and androgen cytosolic receptor value (<1 ± 1 fmol/mg). In contrast, the diploid tumors had some of the highest receptor values, with mean estrogen cytosolic receptor value equal to 102 ± 114 fmol/mg, progesterone cytosolic receptor value equal to 74 ± 110 fmol/mg, and androgen cytosolic receptor value equal to 65 ± 80 fmol/mg.

The lowest survival rates (17% after 36 months) occurred in patients over 67 years of age who had aneuploid tumors, compared to 100% survival in patients over 67 years of age with diploid tumors.

Our results demonstrate the value of using flow cytometry and steroid receptor values as supplements to histopathology for the characterization of subgroups of mammary cancer patients. The ability to identify patients with a good prognosis compared to those at high risk of recurrence and death will be valuable in the design of future prospective treatment studies.

INTRODUCTION

The primary goal in tumor pathology is to develop anatomical, histological, and cytological grading systems which will determine if a tissue is benign or malignant and, if it is malignant, the type of malignancy. However, morphological techniques require a high degree of skill and experience and are subjective in their interpretation. Therefore, objective, quantitative, and precise parameters for differentiating cancer cells from normal cells are urgently needed.

During the last few years, the number of papers which show the importance of using DNA as a marker for predicting neoplastic progression and as a differential descriptor of benign versus malignant tumor tissues has increased significantly (5, 6, 12, 60). New instrumentation and sample preparation methods have enabled us to utilize flow cytometry in a simple, quantitative, and objective manner for determining the DNA distribution in tissues.

The evaluation of estrogen and progesterone receptors in breast cancer tissue is important when attempting to predict response to hormone therapy and for selecting the clinical treatment best suited for the patient (1–3, 20, 29). The concentrations of various steroid receptors are low in normal mammary cells but are frequently quite high in tumor tissues from breast cancer patients. Tumors with ER+ and PR+ values are the ones most likely to respond to endocrine treatment (30–33, 58) and are usually slower growing. Tumors with ER+/PR− or ER−/PR− values are thought to contain cells which are generally less differentiated (37) and less responsive to endocrine therapy and which tend to be more invasive (15, 16, 19). The treatment of breast cancer patients with estrogen (44), androgens (47), progestins, or antagonists of these steroids (18, 47) can produce tumor regression in some of the patients treated (47). However, our understanding of and our ability to identify those tumors which will metastasize quickly and spread in a virulent manner have been limited to hormone receptor and histological evaluations (22, 23).

Tumors from 74 primary breast cancer patients were evaluated in the present study. Analyses were performed on these tumors to quantitate estrogen, progesterone, and androgen cytosolic receptor levels. The tumors were further evaluated by flow cytometry for cellular DNA content (DI) relative to normal nondiploid mammary cells. These parameters were then re-

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1 To whom requests for reprints should be addressed, at University of Tennessee Center for the Health Sciences, Dept. of Obstetrics and Gynecology, 1924 Alcoa Highway, Knoxville, TN 37920.
3 The abbreviations used are: ER+, estrogen receptor-positive; PR+, progesterone receptor-positive; AR+, androgen receptor-positive; AR−, androgen receptor-negative; NIM, nuclear isolation medium; DAP, 4′,6-diamidino-2-phenylindole dihydrochloride; CV, coefficient of variation; PI, proliferation index of the aneuploid cells; DCC, dextran-coated charcoal; ERL, estrogen cytosolic receptors; PRL, progesterone cytosolic receptors; ARL, androgen cytosolic receptors; DI, DNA index.
related to surgical staging and to a patient followup after 36 months in order to correlate these with patient survival.

**MATERIALS AND METHODS**

**Patients.** Follow-up studies to relate surgical staging (42), patient survival, and recurrence histories with steroid receptor and DNA analyses were made possible through use of the Cancer Registry at the Johnson City Medical Center Hospital. The tumor registry is authorized by the American Cancer Society, the NIH, and the American College of Surgeons as a regional tumor registry. It is operated in accordance with the rules outlined in the NIH Guide to Protection of Human Subjects, Federal Registry (46 FR 8366) as defined in 45 CFR 46.101(b). The names of all patients were recorded by the investigators in a coded manner so that subjects would not be identified by name in the data summary. The mean age of the 74 patients was 61 ± 14 (S.D.) years. The limiting consideration for our choice of patient samples was the availability of this follow-up data. We obtained 36 months of follow-up information on 74 breast tumor patients whose tumor samples were received during 1979 and 1980 for estrogen receptor analysis. Neoplastic lesions of the breast were classified in accord with the criteria of F. W. Stewart (53). These patients were evaluated histologically at the time of surgery and identified as containing tumor cells by the pathologist on duty. Surgical stages were defined as: 1, local involvement of breast; 2, breast tumor plus at least 1 positive lymph node; 3, breast tumor plus 2 or more nodes or distant sites; and 3+, breast tumor plus multiple metastatic sites or remote involvement (brain, bone, liver). The individual histological slides were reevaluated by a single pathologist (D. S.) to confirm the presence of malignant cells.

**Sample Preparation.** The breast tissues were stored at −80°, and a small tissue segment, 3 to 5 cu mm, was cut from the frozen sample for flow cytometric analysis. After thawing in Dulbecco's phosphate-buffered saline with calcium [0.01 mM phosphate, NaCl (0.85 g/liter), calcium chloride (0.1 g/liter); Grand Island Biological Co., Grand Island, NY], at room temperature for 5 min to rinse off any blood contamination, each tissue cube was placed in a 60-mm Petri dish, and 1 to 3 ml of NIM (pH 7.2) containing DAPI (DAPI = a DNA-specific stain) was added (56). The tissue was minced in the NIM-DAPI solution (2 min. filtered through a 70-μm mesh filter, syringed 3 times slowly through a 23-gauge needle attached to a 3-ml disposable plastic syringe, and placed in the refrigerator until analysis (56). Normal diploid breast tissues (2C = 7.8 pg of DNA/cell) from 5 mastectomy patients and 2 trout erythrocytes (2C = 5.2 pg of DNA/cell) were prepared by the same method (26) and were used to establish the 2C portion of the DNA tumor data (Chart 1, A and B). The nondiploid tumors were further analyzed subsequent to their initial sample run by mixing with normal 2C breast cells in order to accurately determine the diploid channel and the presence of 2 peaks in the mixture. Furthermore, multiple samples of selected breast tumors, especially all of the hypodiploid tumors, were reanalyzed to estimate the number of aneuploid nuclei (Chart 1, C, D, and E).

**Flow Cytometry.** DNA per nuclei values were determined by utilizing a new fluorescence-electronic cell volume flow cytometer. Measurements were acquired using DAPI excitation-emission dichroic mirror-filter sets which isolated the 365-nm mercury emission line to excite the fluorochrome and a 400-nm dichroic mirror with a 450-nm interference filter to optimize the emission signal.

**DNA Data Analysis.** A data acquisition, storage, and analysis system as described previously (54, 56) was used in these studies to acquire the 1000-channel single-parameter DNA histograms. The CV (average CV, 2 to 3%) was determined by a user-interactive computer analysis program using the following formula:

\[
\text{CV} = \frac{\text{Width at } \frac{1}{2} \text{ ht}}{\text{Peak channel no.} \times 2.3} \times 100
\]

The number of cells in G0/G1, S, and G2 + M were obtained utilizing an assembler program in which the vertical cursor control line of the Tektronix 4013 terminal empirically sets the limits of integration. The number of G0/G1 cells were calculated by integrating the minimum cell number values immediately to the left and to the right of the G0/G1 curve, respectively. A similar integration was performed to obtain the cell number in the G2 + M curve. The remaining cells comprised the S phase population. This simple integration procedure, using a high-resolution flow system with CVs in the range of 1 to 3%, is sufficient for obtaining precise data (56).

**Proliferation Index.** The PI for each of the aneuploid cell tumors is an estimate of how many aneuploid nuclei were in the S + G2 + M portions of the aneuploid DNA histogram and were computed as described previously (54). This computation is an estimate of how fast the aneuploid tumor may be dividing. The measurements of the aneuploid population would be little influenced by the small number of diploid S and G2 + M cells which may overlap with the greater number of aneuploid G0/G1 cells.

**Steroid Receptor Analysis.** Mammary tissue (approximately 0.5 g, wet weight) was homogenized and sonicated with a Polytron (PT-10, Brinkman) for 1 min in Tris:EDTA:sodium molybdate:monothioglycerol:glycerol buffer [Tris (10 mM):EDTA (1.5 mM):sodium molybdate, (10 mM):monothioglycerol (15 mM):20% glycerol, pH 7.5]. The homogenate was centrifuged at 100,000 × g for 1 hr at 0°C, and the supernatant was retained as the cytosolic preparation. The cytosol was treated for 5 min with Norit DCC (0.05% Norit in 0.1% dextran) and centrifuged to remove endogenous steroids + DCC; the "stripped" supernatant was then assayed for estrogen, progesterone, and androgen receptors.

A multiple-dose DCC assay was used to determine saturation for tumor receptor values. [17-3H]Estradiol (New England Nuclear 517; specific activity, 143 Ci/mmol), [3H]-R520 (promegestrone = progesterone ligand; New England Nuclear 555; specific activity, 87 Ci/mmol), and [3H]-R1881 (an androgen ligand; New England Nuclear 590; specific activity, 86 Ci/mmol) were used as the isotopic probes (concentration range, 0.02 to 2.0 nmol/ml) and competitively displaced with 200-fold greater levels of unlabeled diethylstilbestrol, R520, or dihydrotestosterone, one, respectively.

The remaining cytosolic fraction was analyzed for total protein using the Lowry assay (28) after pretreatment, following the procedure of Ross and Schatz (45), to remove interference by glycerol and monothioglyco...
Chart 1. DNA flow cytometer histograms of mammary tissues. Samples were prepared as described in "Materials and Methods" utilizing NIM-DAPI to isolate single nuclei from the tissues and simultaneously stain with the DNA-specific fluorochrome, DAPI. Each section represents the number of nuclei measured (Y-axis) versus the relative intensity of fluorescence (DNA content) per nucleus (X-axis). The CV of the peaks ranged between 2 and 3%. A, normal breast; DI = G0, tumor/G0, normal = 1.0. B, diploid tumor; DI = 1.0. C, hyperdiploid tumor; DI >1.0 and <2.0. D, hypertetraploid tumor; DI ≥2.0. E, multiploid tumor; DI = multiple aneuploid peaks. F, hypodiploid tumor; DI <1.0.

cerol. Protein determinations were run on the cytosolic fraction, which contains less protein than does the total homogenate. The lower protein values, the added protective agents, and the "stripping" procedure all contributed to yield slightly higher receptor cut-off values than have been reported by some groups (27).

Samples were incubated overnight at 0°, and the reaction was stopped by adding 0.25% (final concentration) of DCC. Aliquots (50 μl) of the supernatant containing the bound 3H-steroids were prepared in a toluene Omnifluor (New England Nuclear) cocktail (4 g/liter) containing 30% Triton X-100 and counted in a Beckman LS-9000 scintillation counter. The unquenched counting efficiency was approximately 80%, and the results were converted to dpm by the Compton edge method using a cesium external standard. Results were normalized to fmol of steroid bound per mg of cytosolic protein (fmol/mg). The nonspecifically bound [3H]estradiol (in the presence of 200 times excess unlabeled estradiol) was subtracted from the total steroid bound to give specifically bound steroid (17). For purposes of predicting endocrine responsiveness, the mammary tumors were grouped as: ER+, >50 fmol/mg; and PR+ or AR+, >20 fmol/mg (Table 2). Using this criterion, 43% of the primary breast cancer patients were ER+, 44% were PR+, and 51% were AR+.
**Statistical Analysis.** For simple comparisons, $x^2$ analyses or Student's $t$ tests were performed. Application of bivariate linear correlation (Pearson) and Brown-Forsythe (10) analysis of variance (robust to violations of the assumption of equal variances) and log-linear regressions were performed. Computations were performed manually and with the Biomedical Department program, which is a statistical software package. Multiple comparisons were performed using the Tukey-Kramer pairwise method (13, 39, 51), and the life table analysis was performed using the Kaplan-Meier product limit estimate of survivorship (21).

## RESULTS

As shown in Chart 1, human breast cancer tumors analyzed by flow cytometry show a wide variety of DNA histogram types. Normal human mammary tissue (Chart 1A) and diploid breast cancer tumor cells (Chart 1B) reveal similar patterns, with no aneuploid G$_{0}$/G$_{1}$ peaks. Therefore, the PI$_{a}$ equals zero for these DNA type populations. The first peak on each of the normal and diploid curves represents the G$_{0}$/G$_{1}$ nuclei containing the 2C complement of DNA, and the smaller peak (right side) represents the G$_{2}$ + M nuclei containing 4C amounts of DNA, with the population between these peaks being S-phase nuclei. Peaks which are greater or less than the diploid G$_{0}$/G$_{1}$ and G$_{2}$ + M peaks or apparently aneuploid G$_{0}$/G$_{1}$ peaks in the diploid regions are considered to contain aneuploid nuclei.

The normalized ploidy shift on the X-axis is expressed by the DI, where DI is the ratio of aneuploid G$_{0}$/G$_{1}$ modal channel number to the normal breast cell 2C or diploid G$_{0}$/G$_{1}$ modal channel number. Therefore, both the normal breast tissues and the diploid tumors have a DI = 1.0 value. The 2C DNA value was not normalized to a set channel number, because the variance in DNA content from hypodiploid to hypertetraploid made it difficult to fit all of the curves to a normalized channel. Instead, normal breast tissues and tumor erythrocytes were utilized to establish the 2C value for the samples.

Table 1 shows the distribution of these tumor cells based on 5 DNA histogram types (illustrated in Chart 1): diploid, hyperdiploid, hypodiploid, multiploid, and hypertetraploid. All of the tumors contained normal diploid cells, and 79% had distinct aneuploid tumor populations. The tumor samples with only diploid G$_{0}$/G$_{1}$ populations comprised 21% of the tumors and could not be distinguished from normal nuclei based on DNA content. The largest group with a DI >1.0 but <2.0 was the hypodiploid type (52%). Some of the tumors had nuclei with DNA amounts greater than 4C (hypertetraploid = 11%), and some had populations less than 2C (hypodiploid = 8%), while other tumors had more than one DNA type present (multiploid = 8%).

**Patient Analysis.** Data from the flow analysis of tumor DNA, the fmol/mg cytosolic steroid receptor levels (ER$_{a}$, PR$_{a}$, and AR$_{a}$), the patient follow-up or survival, the surgical information on tumor staging, the lymph node involvement at the time of surgery, and the ages for all patients were compiled and statistically analyzed. These tumors represent 74 primary breast cancer patients with a mean age of 61 years ± 14.

**Cytosolic Steroid Receptors.** The distribution frequency of positive steroid receptor values was 43% for ER$_{a}$, 44% for PR$_{a}$, and 51% for AR$_{a}$ (Table 2). These values are in good agreement with several other literature reports (1, 27, 40) on the distribution of receptors found in primary breast cancers. The levels of steroid receptors measured in these DNA histogram types (Table 3) ranged from 1 to 502 fmol/mg for the ER$_{a}$, from 1 to 431 fmol/mg for the PR$_{a}$, and from 1 to 247 fmol/mg for the R5020 or PR$_{a}$, and from 1 to 247 fmol/mg for the R1881 or AR$_{a}$. Receptor values for the mammary tumors were grouped as negative or positive (ER+, >50 fmol/mg; PR+ or AR+, >20 fmol/mg) as shown in Table 2.

<table>
<thead>
<tr>
<th>Receptors (fmol/mg of cytosolic receptor)</th>
<th>% of ER$_{a}$</th>
<th>% of PR$_{a}$</th>
<th>% of AR$_{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>9</td>
<td>33</td>
<td>26</td>
</tr>
<tr>
<td>4-20</td>
<td>20</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>21-50</td>
<td>28</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>51-100</td>
<td>16</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>101-150</td>
<td>9</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>151-600</td>
<td>18</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Percentage of positive tumors</td>
<td>43$^a$</td>
<td>44$^a$</td>
<td>51$^a$</td>
</tr>
</tbody>
</table>

**Total population (N)** 74

$^a$ Greater than 50 fmol/mg of cytosolic protein = ER+.

$^b$ Greater than 20 fmol/mg of cytosolic protein = PR+ or AR+.

$^c$ Insufficient tissue to allow androgen receptor determination on 16 tumor samples.

| Percentage of Aneuploid Nuclei. The DNA histograms were evaluated for the percentage of aneuploid nuclei and expressed as a percentage of the total nuclei observed (diploid and aneuploid). The total population showed a mean of 28% aneuploid cells. The diploid DNA histogram type was considered to have zero aneuploid nuclei but with a PI$_{a}$ of 4%. The high-risk group

**Aneuploid Proliferation Index.** The PI$_{a}$ were calculated for each of the tumors analyzed by flow cytometry. Because no distinct population of cells outside of the diploid peak position could be discerned in the diploid tumors, their PI$_{a}$ values are listed as zero. The PI$_{a}$ was 4 ± 5 (n = 15), which is similar to the normal nonpregnant mammary control tissues run in this study (PI$_{a}$ = 3 to 8). Of the aneuploid groups, the hypertetraploid

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was obtained (Table 3). Patients with the lowest 3-year survival were the hypodiploid tumors. This is similar to data reported recently by several investigators. Hypodiploid tumors (Table 3, Group 5) gave the lowest Pla, which could indicate a slower rate of growth, although 36-month survival was only 50% for this group. These tumors (hypertetraploid) also showed a distinct increase with increasing age, while percentage of aneuploid nuclei and survival both decreased with increasing age categories (Table 3, Groups 9 to 11), all 3 receptor values were over 67 years of age (n = 7) who also were in one of the high-risk groups. Those patients over age 67 with diploid tumors had 100% (n = 4) 3-year survival.

Patient Survival. Follow-up information on patient survival was obtained (Table 3). Patients with the lowest 3-year survival were grouped together as a high-risk group (Table 3, Group 6). The high-risk group and the deceased groups (Table 3, Groups 6 to 8) showed significantly higher aneuploid percentage compared to the diploid group. When the subjects were divided into 3 age categories (Table 3, Groups 9 to 11), all 3 receptor values showed a distinct increase with increasing age, while percentage of aneuploid nuclei and survival both decreased with increasing age. The lowest survival rates (17% after 36 months) occurred in those patients over age 67 years (n = 7) who also were in one of the high-risk groups. Those patients over age 67 with diploid tumors had 100% (n = 4) 3-year survival.

The data from Table 3 were evaluated statistically using Pearson's correlation coefficient as shown in Table 4. The percentages of aneuploid nuclei were correlated directly (p ≤ 0.05) to Pla, and both were inversely related to survival.

**Table 3**

<table>
<thead>
<tr>
<th>Group comparisons (n)</th>
<th>Survival (days)</th>
<th>Age (yr)</th>
<th>ERa (fmol/mg)</th>
<th>PRa (fmol/mg)</th>
<th>ARa (fmol/mg)</th>
<th>% of aneuploid nuclei</th>
<th>Pla</th>
<th>% of survival (≥36 mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0. Total population (n = 74)</td>
<td>985 ± 404*</td>
<td>61 ± 14</td>
<td>86 ± 99</td>
<td>47 ± 78</td>
<td>38 ± 50</td>
<td>28 ± 23</td>
<td>18 ± 13</td>
<td>50/74 = 68%</td>
</tr>
<tr>
<td>1. Diploid (n = 15)</td>
<td>978* (60-1554)*</td>
<td>62 (31-89)</td>
<td>38 (1-502)</td>
<td>16 (1-247)</td>
<td>18 (1-270)</td>
<td>23 (7-270)</td>
<td>15 (0.1-83)</td>
<td></td>
</tr>
<tr>
<td>2. Hyperdiploid (n = 38)</td>
<td>1038 ± 306</td>
<td>64 ± 13</td>
<td>102 ± 114</td>
<td>74 ± 110</td>
<td>65 ± 80</td>
<td>4 ± 5°</td>
<td>0°</td>
<td>13/15 = 87%</td>
</tr>
<tr>
<td>3. Hypodiploid (n = 6)</td>
<td>1057 ± 385</td>
<td>60 ± 15</td>
<td>73 ± 92</td>
<td>39 ± 112</td>
<td>38 ± 93</td>
<td>33 ± 27°</td>
<td>19 ± 34</td>
<td></td>
</tr>
<tr>
<td>4. Multiploid (n = 7)</td>
<td>1065 (284-1546)</td>
<td>59 (31-89)</td>
<td>35 (1-502)</td>
<td>20 (1-310)</td>
<td>30 (1-113)</td>
<td>32 (5-75°)</td>
<td>16 (7-46)</td>
<td></td>
</tr>
<tr>
<td>5. Hypertetraploid (n = 8)</td>
<td>619 ± 470</td>
<td>48 ± 11°</td>
<td>26 ± 13</td>
<td>13 ± 15</td>
<td>&lt;1.0</td>
<td>47 ± 2°</td>
<td>27 ± 26</td>
<td>3/6 = 50%</td>
</tr>
<tr>
<td>6. High-risk (3, 4, 5) (n = 21)</td>
<td>654 (81-1087)</td>
<td>34 (34-64)</td>
<td>33 (9-40)</td>
<td>5 (1-38)</td>
<td>&lt;1.0 (0-1)</td>
<td>37 (33-83)</td>
<td>17 (3-70)</td>
<td></td>
</tr>
<tr>
<td>7. Deceased within 3 yr (n = 24)</td>
<td>727 (469-1546)</td>
<td>65 (59-75)</td>
<td>66 (4-335)</td>
<td>15 (1-255)</td>
<td>13 (4-70)</td>
<td>57 (16-73)</td>
<td>17 (8-52)</td>
<td></td>
</tr>
<tr>
<td>8. Deceased aneuploid (n = 24)</td>
<td>720 (343-777)</td>
<td>65 (34-79)</td>
<td>41 (4-335)</td>
<td>15 (1-255)</td>
<td>5 (1-102)</td>
<td>35 (3-83)</td>
<td>15 (3-70)</td>
<td></td>
</tr>
<tr>
<td>9. Patients &gt;67 yr (n = 22)</td>
<td>531 ± 316</td>
<td>63 ± 12</td>
<td>69 ± 72</td>
<td>34 ± 56</td>
<td>32 ± 30</td>
<td>33 ± 26°</td>
<td>19 ± 14</td>
<td>0/24 = 0.0%</td>
</tr>
<tr>
<td>10. Patients 50-67 yr (n = 34)</td>
<td>496 ± 287</td>
<td>64 ± 12</td>
<td>62 ± 70</td>
<td>40 ± 60</td>
<td>36 ± 31</td>
<td>38 ± 25°</td>
<td>20 ± 15</td>
<td>0/20 = 0.0%</td>
</tr>
<tr>
<td>11. Patients &lt; 50 yr (n = 18)</td>
<td>486 ± 1186</td>
<td>64 ± 39</td>
<td>46 ± 289</td>
<td>24 ± 237</td>
<td>30 ± 102</td>
<td>36 ± 83</td>
<td>16 (3-70)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4**

<table>
<thead>
<tr>
<th>Survival</th>
<th>% of aneuploid</th>
<th>Pla</th>
<th>Age</th>
<th>ERa</th>
<th>PRa</th>
<th>ARa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival</td>
<td>% of aneuploid</td>
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<td>Pla</td>
<td>Age</td>
<td>ERa</td>
<td>PRa</td>
<td>ARa</td>
</tr>
</tbody>
</table>

Mean ± S.D.
Median.
Numbers in parentheses, range.
Zero percentage of aneuploid cells for Group 2, and the percentage of diploid is S + G2 only.
Since no aneuploid nuclei could be distinguished in the diploid group, P1 = 0.
*p < 0.01. Significantly different from diploid group by Student's t test.

DNA Histogram and Survival in Breast Cancer

**Table 5**

<table>
<thead>
<tr>
<th>DNA Histogram and Survival in Breast Cancer</th>
</tr>
</thead>
</table>
| Patient Survival. Follow-up information on patient survival was obtained (Table 3). Patients with the lowest 3-year survival were grouped together as a high-risk group (Table 3, Group 6). The high-risk group and the deceased groups (Table 3, Groups 6 to 8) showed significantly higher aneuploid percentage compared to the diploid group. When the subjects were divided into 3 age categories (Table 3, Groups 9 to 11), all 3 receptor values showed a distinct increase with increasing age, while percentage of aneuploid nuclei and survival both decreased with increasing age. The lowest survival rates (17% after 36 months) occurred in those patients over age 67 years (n = 7) who also were in one of the high-risk groups. Those patients over age 67 with diploid tumors had 100% (n = 4) 3-year survival.

The data from Table 3 were evaluated statistically using Pearson's correlation coefficient as shown in Table 4. The percentages of aneuploid nuclei were correlated directly (p ≤ 0.05) to Pla, and both were inversely related to survival.

**Steroid Receptor Ratios.** Table 5 shows the 5 DNA histogram types related to estrogen and progesterone receptor status. Note that all hypodiploid tumors with their poor prognosis (50% 3-year survival) fell into the ER- group. There was a 77% 3-year survival for ER+/PR+ tumor patients. The high-risk groups showed a greater proportion of tumors with either ER- or PR-values and only a 48% 3-year survival. Patients with AR+ tumors (Table 6) unexplainably had an even poorer prognosis. In the high-risk group, there were 2 patients with ER+/AR+ tumors, and all died within the 3-year follow-up period (3 of these patients were over 67 years of age).

**Surgical Stage.** When the samples were divided on the basis of surgical staging (Table 7), all stages were observed in both the diploid and the high-risk groups. No significant differences were observed in staging and DNA histogram types according to Brown-Forsythe analysis of variance statistics (10). The diploid and the hypertetraploid groups had the least number of Stage 3 tumors, although the difference was not statistically significant, and no single DNA type was unique to a particular surgical stage. Survival in patients with Stage 1 and 2 cancers was 69%, compared to the 44% survival in Stage 3 patients at the 3-year follow-up.
Collectively, the diploid and the hypertetraploid DNA types represented 33% of the tumors studied (Table 1). On the average, these 2 types contained the highest ER, PR, and AR levels, the lowest PI of the DNA histogram types (Table 3), and the lowest percentage of tumors classified as Stage 3 (Table 7). However, they did demonstrate different survival rates in which 87% of the diploid tumor patients and only 50% of the hypertetraploid tumor patients were surviving at 36 months (Table 7).

Hypodiploid Group. A summary of the 6 patients in the hypodiploid group (Table 8) illustrates how low the receptor values were in all of the patients and that the percentage of aneuploid nuclei values were all greater than 32%. All 3 surgical stages were represented in the hypodiploid group. The mean age of the hypodiploid patients was 48 ± 11 years, while the diploid group showed a mean age of 64 ± 14 years.

Patients Surviving for Less Than 3 Years. Within the total group of 74 patients, 24 individuals died within the 36-month follow-up period (Table 9). In this group of patients, the mean ER, PR, AR values were lower than the diploid values, but they overlapped, and the differences were not statistically significant. The percentages of aneuploid nuclei were significantly higher (p < 0.01) in the patients surviving <36 months compared to levels for the diploid group.

Life Table Analysis. Graphs were derived using life table analysis by the product limit method (Kaplan and Meier, 1958) for estimating the survivorship function for censored data, with some patients still alive at the conclusion of the study (21). Survival was compared among patients in the diploid, hypertetraploid, and high-risk groups (Chart 2). The curves were analyzed by the K-sample extension of the Gehan's Generalized Wilcoxon test for censored data (26). A trend is evident in the life table survival compared to the DNA histogram type at the end point measurements (Chart 2A) in which the high-risk group (48%), the hypertetraploid group (71%), and the diploid group (87%) begin to show differences in survival. In the life table survival compared to surgical staging (Chart 2B), there is less distinction between groups.

DISCUSSION

The relationship between nuclear differentiation and cancer prognosis has been studied for many years (53). Despite development of a number of histological classifications, a definitive system, which is broadly applicable and accepted by most pathologists, has not been developed. The intrinsic subjective nature of histopathological nuclear grading certainly has contributed to the difficulties in application of the many thoroughly described staging or grading systems. On the other hand, nuclear differentiation in cancer prognosis remains an extremely important parameter to be further developed.

In recent years, substantial interest has arisen for developing additional parameters to supplement the morphological criteria for diagnosing and grading tissues in general as benign or as malignant with a propensity for metastasis. Such new parameters are especially needed for breast cancers to overcome the current problems met in differentiating malignant tumors from the benign lumps of fibrocystic disease which quite frequently develop in women over 45 years of age. Furthermore, ultrasound and self-breast examinations often detect very small lumps which are too little for estrogen receptor studies. The small sample size required for flow cytometry study (≤3 to 5 cu mm) would allow DNA ploidy determinations to be made on the smallest of these.
The most important nonhistological parameter for differentiating benign and malignant cells is their DNA content. Recently, one DNA flow cytometric study with 92 human breast cancers showed aneuploidy in 92% of the cases (38), while benign breast tumors were diploid (24, 37). Mörk and Laerum (36), in a study of 56 human brain tumors, showed that benign tumors were diploid or near diploid, while most malignant tumors were significantly aneuploid. In a study of aspirated biopsy material from 56 human brain tumors, showed that benign tumors were diploid or near diploid, while most malignant tumors were significantly aneuploid. In a study of aspirated biopsy material from 56 human brain tumors, showed that benign tumors were diploid or near diploid, while most malignant tumors were significantly aneuploid. In a study of aspirated biopsy material from 56 human brain tumors, showed that benign tumors were diploid or near diploid, while most malignant tumors were significantly aneuploid.

The distributions of diploid (21 and 24%) and aneuploid (79 and 76%) primary breast tumors were in close agreement (Table 1) for the 2 separate studies reported. They are also in agreement with several other studies (8, 41, 55).

Supportive evidence for the importance of nuclear DNA characteristics by the Feulgen static microspectrophotometric method in cancer prognosis has been presented. For example, Zetterberg and Esposti (60) in 1980 studied 43 patients with prostatic carcinoma in which the 21 disease-free patients (greater than 5 years) were characterized by a diploid DNA type, while the 22 patients who died within 3 years had aneuploid carcinomas. Kreichbergs et al. (23) characterized the DNA types in 48 chondrosarcoma patients with subsequent accumulation of survival data for 10 years. Twenty-two chondrosarcomas were characterized as diploid, and 26 were found to be aneuploid. The diploid tumor patients had a highly significant survival ad-

to be analyzed by flow cytometry methods and reported for neuroblastoma (52), testicular seminomas and malignant teratomas (61), murine tumors (11, 52, 56), and human tumors (6, 7, 55). The distributions of diploid (21 and 24%) and aneuploid (79 and 76%) primary breast tumors were in close agreement (Table 1) for the 2 separate studies reported. They are also in agreement with several other studies (8, 41, 55).
Flow cytometry has been reviewed recently (8). Divided into DNA histogram types (n = 74). The 36-month survival for diploid tumor patients was 87%; for hyperdiploid tumor patients, it was 71%; and for the high-risk tumor patients (hypodiploid, multiploid, and hypertetraploid tumors), it was 48%. Reference: Kaplan-Meier product-limit estimate of survivorship (21, 25).

Survival = days from receptor assay (breast surgery) to death or May 1, 1983. B, 55% survival. Reference: Woolley (59), in 1982, prospectively studied a subpopulation of patients with tumors which are hormonally nonresponsive and potentially very aggressive. Further research is needed to verify the present results.

Histological studies of breast cancer tumors have shown that medullary and scirrhus carcinomas have a higher percentage of S + G₂ + M cells than do lobular, mucous, ductal, colloid, papillary, or tubular carcinomas (29, 37, 38). The present study had 4 patients with breast carcinomas identified as scirrhus. They were all hyperdiploid or hypertetraploid tumors, surgical Stage 1 or 2, and all were alive at the 3-year follow-up period. Squamous cell carcinoma, one of the most aggressive histological breast carcinomas, is usually ER− (50), and might be predicted to show high P₄ values in patients with squamous tumors. Although patient numbers were small in this study, such receptor and P₄ values appear to identify a subpopulation of patients with tumors which are hormonally responsive. Further research is needed to verify the present results.

The evaluation of benign breast tumors, especially atypical lobular, medullary, and ductal hyperplasia of benign tumors (thought to have from 2 to 6 times greater risk of subsequent mammary carcinoma) would be of particular importance in future studies (43). The evaluation of breast cancer patients using life table analysis of progesterone receptors and interval to metastasis, showed greater rates of metastasis in PR− tumors (46). Simultaneous evaluation of ER and PR have shown a better correlation for predicting endocrine response. Patients with ER+/PR− tumors show a 75 to 85% response to endocrine therapy, while ER−/PR− and ER+/PR− tumor-bearing patients show less than 10% response to endocrine therapy (31, 40, 48). These results agree with our findings of increased survival in the ER+/PR− total group (77% surviving) and the ER+/PR− high-risk group (50% surviving) compared to the ER+/PR− total group (54% surviving) or the ER+/PR− high-risk group (25% surviving). Our investigation showed that the highest P₄ values occurred in the ER+/PR− tumors. Although patient numbers were small in this study, such receptor and P₄ values appear to identify a subpopulation of patients with tumors which are hormonally responsive and potentially very aggressive. Further research is needed to verify the present results.

Histological studies of breast cancer tumors have shown that medullary and scirrhus carcinomas have a higher percentage of S + G₂ + M cells than do lobular, mucous, ductal, colloid, papillary, or tubular carcinomas (29, 37, 38). The present study had 4 patients with breast carcinomas identified as scirrhus. They were all hyperdiploid or hypertetraploid tumors, surgical Stage 1 or 2, and all were alive at the 3-year follow-up period. Squamous cell carcinoma, one of the most aggressive histological breast carcinomas, is usually ER− (50), and might be predicted to show high P₄ values in patients with squamous tumors. Although patient numbers were small in this study, such receptor and P₄ values appear to identify a subpopulation of patients with tumors which are hormonally responsive. Further research is needed to verify the present results.
closely overlap with the normal diploid population and cannot always be differentiated. We have been able to develop instrumentation and preparation methods (56) for analyzing the DNA content of fresh tissue nuclei with CVS in the range of 1 to 2%. The resolution in this study using frozen tissue was 2 to 3% CV. The advantage of using fresh tissue is the convenience it allows for sample transportation and batch processing. A method for comparing the percentage CVSs of the tumors to normal tissue percentage of CV has been described (56) and would aid in distinguishing suspected overlapped peaks in future studies.

Those tumors with the highest Pla values frequently (but not always) contained the lowest values for steroid receptors, indicating that these tumors were more cytokinetically active than those with ER+PR+ values. This finding agrees with studies by Kute et al. (24), Olszewski et al. (38), and Raber et al. (41), who used flow cytometry, by Silverstrini et al. (49) and Auer et al. (5), who used autoradiography to measure S-phase fractions in breast cancer. Among these studies, it is particularly important to note which ones evaluated the proliferation index for the entire population of cells (i.e., the in vitro [3H]thymidine incorporation approach) and which studies were able to evaluate Pla for tumor cells only. The latter technique would give a more accurate measurement of the aggressiveness of the tumor population. To date, no method has been devised which can simultaneously measure Pla and steroid receptor values for the aneuploid population exclusively. Such a method would be highly desirable.

The age relationship to receptor content, DNA histogram type, and survival was interesting. All of the receptor values in this study increased with increasing age. Patients over 67 years old who were in one of the high-risk groups had the poorest (17%) survival rate even though their steroid receptor values were high. In contrast, the patients over 67 years old with diploid tumors had 100% survival at 3 years.

In conclusion, based on a simple, 5-min preparation technique (56) requiring only a tiny portion of tissue (0.1 mg wet weight), frozen breast tumor samples were analyzed by flow cytometry in order to supplement the morphological and receptor data with DI analysis and percentage of aneuploid nuclei. Three subpopulations of tumor patients, those with hypertetraploid, multiploid, or hypotetraploid nuclei, demonstrated the lowest 36-month survival rates. Two of these subpopulations, the hypertetraploid and the multiploid groups, contained some of the highest tumor loads (percentage of aneuploid nuclei) and the highest Pla values measured.

Work is in progress to investigate quantitative descriptors other than DNA which may be useful in differentiating benign from malignant tissues and for predicting the propensity for recurrence. Some of these descriptors include electronic nuclear volume, tumor-specific monoclonal antibodies, and certain enzymes which are important for DNA synthesis, transport of amino acids across the cell membrane, and proteolytic activity. A multiparameter flow cytometric study of this nature will only be of value if the resolution of the DNA measurement is not compromised. In any event, a larger number of patients with long-term survival data will be an important supplement to the results presented in this paper. It is hoped that these parameters will accurately predict the biological behavior of tumors in general and add to our diagnostic, prognostic, and therapeutic knowledge of cancer.

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Prognostic Indicators Including DNA Histogram Type, Receptor Content, and Staging Related to Human Breast Cancer Patient Survival

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