The Prognostic Significance of Ploidy Analysis in Operable Breast Cancer

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The nuclear DNA content of 98 operable breast cancers was determined by flow cytometric analysis using paraffin-embedded tissue. All patients were on follow-up and failure of treatment or recurrences were identified. DNA ploidy data in the form of ploidy status and DNA index (DI) has been correlated with various clinical and histopathologic factors. The only significant correlation using univariate analysis exists between the histologic grade and DI ($P < 0.025$), recurrence of the disease and ploidy status ($P < 0.005$), and recurrence of the disease and DI ($P < 0.005$). The absence of correlation of ploidy status with other tumor derived factors indicates the independent nature of ploidy as a prognostic factor. Multivariate analysis showed that in the whole-group ploidy ($P < 0.01$), tumor margin ($P < 0.01$), and menopausal status ($P < 0.01$) were significant factors in the order mentioned. DI with a cut of at 1.29 is not found to be a significant factor in the multivariate analysis. The maximum prognostic value of ploidy status was observed in the postmenopausal group ($P < 0.0005$). In the node-negative group ploidy status ($P < 0.05$) is the only independent significant factor predicting for early relapse. It is concluded that ploidy status is an independent prognostic factor predicting for recurrence of the disease. In the node-negative subgroup this could be used to identify the subset of patients who may benefit from adjuvant treatment.
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BREAST CANCER is a heterogeneous disease. Even in the same clinical stage, different patients behave differently. There is thus a need to determine significant prognostic factors, for deciding appropriate therapy in a given case.

Axillary lymph node status is one of the important prognostic factor in patients with breast cancer.[1] Patients with node-negative tumors have better survival compared with patients where lymph nodes are involved. However, it has been reported that approximately 30% of node-negative patients recur and die within 10 years. A prognostic marker that could identify this high-risk subgroup among node-negative patients would be of great value in individualizing therapy.

The nuclear DNA content has recently emerged as a new prognostic indicator in breast cancer.[2–6] Using flow cytometric study, thousands of nuclei can be evaluated for their DNA content with a good resolution. Presence or absence of an aneuploid peak and the S-phase fraction are of prognostic value.[7,8] Breast carcinomas with a non-diploid or aneuploid nuclear DNA content are associated with less favorable prognosis compared with tumors with a diploid DNA content. It is this determination of biologic aggressiveness and cell proliferation kinetics that makes ploidy analysis an important parameter affecting tumors of all sizes and stages.

The use of paraffin-embedded tissues for DNA analysis is a new technique.[9] The advantage of using archival blocks for DNA analysis is that a long follow-up is available and the DNA ploidy pattern can be analyzed in that context. There is, however, no consensus about how the DNA histograms should be analyzed. Various groups have used different classification to analyze the DNA histograms, keeping in view the presence or absence of aneuploid peaks and percent S-phase fraction. Due to inter-

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observer variation in interpretation and different computer programs used to find S-phase, comparison between different studies where percent S-phase has been calculated is not possible. DNA index (DI), which is the ratio of the mean fluorescence channel of the aneuploid peak to that of the G1/G0 peak, allows better assessment of the histograms.[10]

The current study analyzes the DNA ploidy patterns from paraffin-embedded tissues of patients with breast cancer. The ploidy data has been correlated with various other histopathologic and clinical parameters. The prognostic significance of DNA ploidy in various subgroups using multivariate analysis has been studied.

Patients and Methods

Patients with breast cancer, who had been treated with radical or modified radical mastectomy and were being observed at the Breast Cancer Clinic, were taken up for the study. Detailed clinical data, recorded at the time of surgery, was obtained from their records. Patients with axillary lymph node involvement received six cycles of adjuvant chemotherapy[11] in the form of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF). Node-negative patients were observed with no adjuvant therapy. All patients were managed with the above-mentioned protocol.

Of 152 operable patients with breast cancer registered at the breast cancer clinic during the period 1980 to 1987, complete data with follow-up was available for 108 patients. Paraffin-embedded blocks were available for 104 patients. In 6 cases the DNA histogram was uninterpretable; the data presented here concern 98 patients.

For the first 2 years after definitive surgery the patients were observed at 3-month intervals, after which the patients were examined at 6-month intervals. Chest radiographs and serum alkaline phosphatase tests were done at each follow-up visit. Computed tomography (CT) and radionuclide scans were done in selected cases, depending upon the degree of suspicion. Chest wall and regional lymph node recurrences were confirmed histologically using fine-needle aspiration.

A detailed histopathologic examination of mastectomy specimen was carried out in all cases. Histologic grading was done by the criteria proposed by Bloom and Richardson.[12] The various histologic parameters analyzed were histologic grade, histologic type, nuclear grade, tumor margin, tumor necrosis, lymphatic and blood vessel invasion. It was assessed whether there was a tumor infiltration for the lymph nodes, and also for the presence or absence of sinus histiocytosis and germ cell hyperplasia.

Measurement of Nuclear DNA Content

Fifty-micron-thick sections were taken from the paraffin block, which had a substantial tumor area. The sections were dewaxed, rehydrated, and then treated with 1% pepsin solution (pH 1.5) using the technique of Hedley *et al.*[13] With repeated vortexing a cell suspension was obtained that was passed through a mesh and then washed with saline. Cells were stained using a hypotonic solution of propidium iodide (0.05 mg/ml in 1.12% sodium citrate) that was added to the cell pellet and kept on ice for 15 minutes. For controls, paraffin blocks were made from normal breast tissue and processed as for tumor samples. The histograms obtained from ten different normal breast tissues showed the diploid pattern.

DNA content was measured using FACScan cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). A 488-nm argon laser line run at 15 mW was used for fluorescence excitation. A 585 ± 22 nm band pass filter was used in front of red photomultiplier to block the laser light. For each histogram 5000 nuclei were examined. The first peak from the left, which corresponds to G0/G1 of the cell cycle, was by convention set at channel 50 for all tumors.

DNA Index Determination

The degree of DNA content aberration was expressed by the DNA index. With this method we have assumed that the G0/G1 peak with the smallest DNA content represents normal diploid cells in all samples. Tumors with a DI of less than 1.0 have been erroneously labeled as DI = 1.0.

Coefficient of variation (CV) of the G0/G1 peak varied from 3.2 to 7.2 with a mean of 4.4. Tumors with CV over 8% and those with excessive debris were not included in the study (six). Tumors were labeled as diploid when the DI was 1. Tumors with a shoulder close to G0/G1 peak were also labeled as diploid. The histogram was classified as nondiploid when there were more than one peak or multiple peaks. The histograms were analyzed without the knowledge of histopathologic findings and clinical data.

Results

The clinical characteristics of patients studied is shown in Table 1. Thirty-six patients had recurrences: 11 recurrences were in the chest wall and supraclavicular area, and 25 were distant metastasis. Recurrences appeared within a mean time interval of 28 months from the date of surgery (range, 8 to 60 months).

DNA Ploidy and Recurrence

The DNA data has been correlated with recurrence in two ways. First, tumors with a single peak have been labeled as diploid (this also includes peaks with a shoulder effect); all other tumors with more than one peak have been labeled as nondiploid. Second, DI have been cal-

TABLE 1. Clinical Characteristics of 98 Patients With Breast Cancer

Mean age (yr)	46.93
Mean age at menarche (yr)	14.07
Age at 1st child birth (yr)	21.93
Average parity	3.80
Menopausal status	
Premenopausal	50 (51.0%)
Postmenopausal	48 (49.0%)
Tumor size	
T1	6 (6.0%)
T2	57 (58.1%)
T3	29 (29.5%)
T4	6 (6.0%)
Lymph node involvement	
0	28 (28.5%)
1–3	30 (30.6%)
> 4	40 (40.8%)
Failure of treatment	36 (36.7%)
Locoregional recurrence	11 (30.5%)
Distant metastasis	25 (69.4%)

culated and a DI of 1.29 taken as the cutoff value. This was calculated after correlating distribution analysis of DI with recurrences at various levels of DI.

Twenty-three of the cancers were diploid (23.4%) and 75 (76.5%) were nondiploid. The distribution of DNA indices with recurrence is shown in Figure 1. In diploid tumors the prognosis was favorable ($P < 0.01$) compared with the nondiploid group. Cancers with a DI of less than 1.29 (52) had a favorable prognosis compared with those with a DI of more than 1.30 (46) ($P < 0.01$).

Correlation With Other Prognostic Factors

Correlation of ploidy and DI with several clinical and histopathologic factors using chi-square analysis is shown in Table 2. The only significant factors correlating are recurrence and histologic grade of the tumor. The age at diagnosis also had no impact on the DNA content of the

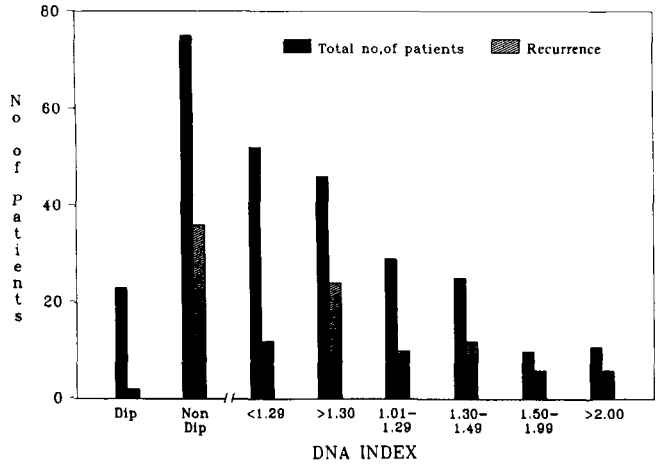

FIG. 1. Bar chart showing total number of patients and recurrence in various subgroups classified according to diploid or nondiploid status and DNA index.

TABLE 2. Clinicopathologic Features of 98 Patients With Breast Cancer and Their Relation to Ploidy and DNA Index

	Nondiploid			DNA index		
Feature	Percent	Percent	*P*	> 1.30	Percent	*P*
Age (yr)						
< 49	59	80	NS	27	46.5	NS
> 50	41	70		19	47.5	
Menopausal status						
Premenopausal	51	82	NS	22	44	NS
Postmenopausal	49	71		24	50	
Tumor size						
T1	6	66		3	50	
T2	58	74	NS	23	40	NS
T3–4	36	83		20	57	
Histologic grade						
1	5	100		5	100	
2	68	75	NS	27	40	< 0.025
3	26	77		14	53	
Tumor necrosis						
None	62	79	NS	28	49	NS
Present	38	69		11	30.5	
Tumor margin						
Circumscribed	9	62.5	NS	2	25	NS
Infiltrative	91	75		34	42.5	
Nodal status						
Node negative	29	71	NS	11	39	NS
Node positive	71	78.5		35	50	
Recurrence						
Absent	63	65	< 0.001	22	35.5	< 0.005
Present	37	94		24	66.6	

NS: not significant.

tumor: mean age at diagnosis of patients with a diploid tumor was 48.13 years (SD ± 8.75) and patients with nondiploid tumors, 46.61 years (SD ± 10.66). The ages of the patients in the node-negative (45.38 years, SD ± 9.16) and node-positive group (47.03 years, SD ± 10.62) also were not significantly different. Similarly, there was no correlation between menopausal status and ploidy.

Multivariate Analysis Findings

Multivariate analysis was done to find out the relative importance and the independent nature of the various variables. The results of multivariate analysis are shown in Table 3. For the whole group the most important independent prognostic factors were ploidy ($P < 0.001$), type of tumor margin ($P < 0.01$), and menopausal status ($P < 0.05$). The traditionally established factors like lymph node status and histologic grade did not figure as independent factors. Because of the possibility that the prognostic significance of DNA analysis might be obscured by adjuvant therapy, the patients were also stratified on the basis of lymph node involvement and menopausal status, and then analyzed.

Discussion

Indian patients with breast cancer differ from the western group of patients because of various clinical charac-

TABLE 3. Results of Multivariate Analysis

Patient group	Significant factors (*P*)		R^2 (%)	
All patients*	Ploidy	(< 0.001)	10.0	
	Tm margin	(< 0.01)	7.0	
	Menopausal status	(< 0.05)	5.0	
			Total (10 Var)	(26.0)
Node negative*	Ploidy	(< 0.05)	14.4	
			Total (9 Var)	(36.6)
Node positive*	Ploidy	(< 0.005)	8.3	
	Tm margin	(< 0.05)	7.0	
	Menopausal status	(< 0.05)	5.0	
			Total (9 Var)	(27.5)
Premenopausal*	No variables meet the criteria			
Postmenopausal*	Ploidy	(< 0.001)	24.8	
	Tm margin	(< 0.05)	8.0	
			Total (9 Var)	(40.7)

Var: variable.
* Dependent variable recurrence.

teristics. Indian patients have a high parity, early age at first childbirth, and are younger[14,15] (Table 1). Also, because patients are younger at the time of presentation, the premenopausal and postmenopausal patients are nearly equally represented in the group (51% *versus* 49%, respectively). The prognostic factors involved may also be different.

Various clinical and histopathologic factors have been correlated in the past with an early recurrence of disease in breast cancer. As mentioned earlier, of a number of histologic parameters studied, lymph node status ($P < 0.05$) and histologic grade ($P < 0.05$) correlate significantly with recurrence in univariate analysis (data not shown for other parameters).

S-phase analysis, although reported as a prognostic factor, is subject to a lot of variation depending on whether fresh tissue or paraffin-embedded tissue is used, with the fixed tissue giving rise to a lot of debris and high background. A defined aneuploid peak is firm and reproducible evidence. The use of DI allows a more objective assessment of the histogram removing thereby the interobserver variation. The DI is easy to calculate and is a reliable index. It has been seen that the recurrence rate is higher in tumors with higher DI. When the tumors were grouped into two groups, DI 1.30 to 1.49 and DI more than 1.50, it was found that the recurrence rate was higher in the more than 1.50 group (57% *versus* 48%). The exact significance of this observation is not known.

Ploidy Analysis as a Unique Prognostic Indicator and Its Relationship With Other Factors

Most studies[3,4,6,16] have shown that DNA aneuploidy is associated with unfavorable prognosis in breast cancer. The association with survival is, however, weak and there are some groups that do not show a correlation.[17,18] Ploidy as an independent prognostic factor has been shown by only one group,[6] whereas some have found ploidy significant in a univariate analysis only.[2] In this study using multivariate analysis we report the ploidy status to be the most important independent prognostic factor in the whole group, node-negative, node-positive, and postmenopausal group of patients (Table 3).

It is an established fact that nuclear DNA content correlates with the histologic grading of the tumor, with poorly differentiated tumors more likely to be aneuploid.[2,4,19,20] This is expected as both factors give information regarding the biological aggressiveness and differentiation of the tumor. In this study the histologic grade is the only tumor-derived factor that correlates with the DNA content of the tumor. The association of other clinical and tumor-derived factors with DNA content is controversial. Whether tumor size should correlate with DNA content is also not clear; some groups have shown a correlation whereas some have not.[17,21,22] The absence of any correlation between DNA content and clinical and tumor-derived factors in this series points toward an independent nature of DNA content as a prognostic factor and its capability to affect prognosis in all groups of patients and all sizes of primary lesions.

Beside histologic grade other factors like tumor margin, tumor necrosis, and lymphatic invasion did not correlate with the ploidy analysis. Previous studies have, however, shown conflicting results regarding the relationship of axillary nodal status and ploidy analysis.[17,21,22] In this study no correlation was found between DNA content and lymph node status; independently, however, they are significant factors in predicting recurrence.

Tumor margin as a prognostic factor has not been given its due importance. Lately, however, some studies[10] have reported this to be a significant factor in multivariate analysis. The tumor margin in this study has been described as infiltrative or circumscribed and it is a significant, independent predictor of recurrence in the multivariate analysis of the whole group ($P < 0.01$), node-positive group ($P < 0.05$), and postmenopausal group ($P < 0.05$) of patients.

Ploidy and the Node-Negative Group

Axillary lymph node-negative group is one such area where the treatment protocols are being actively discussed and the line of management is changing. Recent trials[14,15] have shown a significant increase in disease-free survival in the node-negative group when these patients received adjuvant therapy. Both of the trials, however, selected a high-risk group on the basis of estrogen receptor (ER) status and only the high-risk patients received adjuvant therapy.

The node-negative group was studied with a view to find an independent prognostic factor that could identify patients likely to have an early relapse. In the multivariate analysis, DNA content has come out to be the only sig-

nificant important prognostic indicator in the node-negative group ($P < 0.05$). Of the 28 node-negative tumors nine were diploid and 19 were nondiploid. Eight of 19 nondiploid tumors (42%) recurred whereas there were no recurrences in the diploid group. This shows the advantage of using ploidy analysis in identifying a group within the node-negative group that is more likely to recur. This prognostic indicator could be used to select a group that may benefit from adjuvant therapy.

It is concluded that nuclear DNA content is an objective marker of tumor aggressiveness and can significantly enhance our prognostic capabilities. This can also help in defining subsets of patients with a high probability of short-term relapse especially in the node-negative group, thus making them candidates for adjuvant therapy.

REFERENCES

1. Fisher ER, Gregorio RM, Fisher B *et al.* The Pathology of invasive breast cancer: A syllabus derived from findings of the National Surgical Adjuvant Breast Project (protocol no. 4). *Cancer* 1975; 36:1–85.
2. Fisher ER, Sass R, Fisher B. Pathological finding from the National Surgical Adjuvant Project for Breast Cancer (protocol no. 4): X. Discriminants for tenth year treatment failure. *Cancer* 1984; 53:712–723.
3. Hedley DW, Rugg CA, Ng ABP, Taylor IW. Influence of cellular DNA content on disease free survival of Stage II breast cancer patients. *Cancer Res* 1984; 44:5395–5398.
4. Thorud E, Fossa SD, Vaage S *et al.* Primary breast cancer: Flow cytometric DNA pattern in relation to clinical and histopathological characteristics. *Cancer* 1986; 57:808–811.
5. Baildam AD, Zaloudik J, Howell A *et al.* DNA analysis by flow cytometry: Response to endocrine treatment and prognosis in advanced carcinoma of the breast. *Br J Cancer* 1987; 55:553–559.
6. Kallioniemi OP, Blanco G, Alavaikkom M *et al.* Tumour DNA ploidy as an independent prognostic factor in breast cancer. *Br J Cancer* 1987; 56:637–642.
7. Dowel CS, Owainati AA, Robins A *et al.* Prognostic significance of DNA content of human breast cancer. *Br J Surg* 1987; 74:133–136.
8. Kallioniemi OP, Blanco G, Alavaikko M *et al.* Improving the prognostic value of DNA flow cytometry in breast cancer by combining DNA index and S-phase fraction: A proposed classification of DNA histograms in breast cancer. *Cancer* 1988; 62:2183–2190.
9. Hedley DW, Friedlander ML, Taylor IW, Rugg C, Musgrove EA. Method for analysis of cellular DNA content of paraffin embedded pathological material using flow cytometry. *J Histochem* 1983; 31:1333–1335.
10. Toikkanen S, Joensuu H, Klemi P. The prognostic significance of nuclear DNA content in invasive breast cancer: A study with long term follow-up. *Br J Cancer* 1989; 60:693–700.
11. Bonadonna G, Brussamolina M, Valagussa P *et al.* Combination chemotherapy as an adjunct treatment in operable breast cancer. *N Engl J Med* 1976; 294:405–410.
12. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. *Br J Cancer* 1957; 11:359–377.
13. Hedley DW, Rugg CA, Gelber RD. Association of DNA ploidy as an independent prognostic factor in breast cancer. *Br J Cancer* 1987; 47:4729–4735.
14. Fisher B, Redmond C, Dimitrov NV *et al.* A randomized clinical trial evaluating sequential methotrexate and fluorouracil in treatment of patients with node negative breast cancer who have estrogen receptor negative tumors. *N Engl J Med* 1989; 320:473–478.
15. Fisher B, Redmond C, Dimitrov NV *et al.* A randomized clinical trial evaluating tamoxifen in the treatment of patient with node negative breast cancer who have estrogen receptor negative tumors. *N Engl J Med* 1989; 320:479–484.
16. Coulson PB, Thornwaite JT, Wooly TW, Sugarbakar EV, Seckinger D. Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer patient survival. *Cancer Res* 1987; 44:4187–4196.
17. Uyterlinde AM, Schipper NW, Baak JPA, Petrese H, Matze E. Limited prognostic value of cellular DNA content to classical and morphometrical parameters in invasive ductal breast cancer. *Am J Clin Pathol* 1988; 89:301–307.
18. Owainati AA, Robins RA, Hinton C *et al.* Tumour aneuploidy, prognostic parameters and survival in primary breast cancer. *Br J Cancer* 1987; 55:449–454.
19. Moran RE, Black MM, Alpert L, Stuart, MJ. Correlation of cell cycle kinetics, hormone receptors, histopathology, and nodal status in human breast cancer. *Cancer* 1984; 54:1586–1590.
20. Kute TE, Muss HB, Hopkins M, Marshall R, Case D, Kammire L. Relationship of flow cytometry results to clinical and steroid receptor status in human breast cancer. *Breast Cancer Res Treat* 1985; 6:113–121.
21. McDivitt RW, Stone KR, Craig RB, Palmer JO, Meyer JS, Bauer WCA. Proposed classification of breast cancer based on kinetic information. *Cancer* 1986; 57:269–276.
22. Dressler LG, Seamer LC, Owens MA, Clark GM, Mcguire WL. DNA flow cytometry and prognostic factors in 1331 frozen breast cancer specimens. *Cancer* 1988; 61:420–427.