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Prognostic Value of DNA Ploidy and S-Phase Fraction in Relation to Estrogen Receptor Content and Clinicopathological Variables in Primary Breast Cancer

OLLE STÅL,* STEN WINGREN,* JOHN CARSTENSEN,*† LARS ERIK RUTQVIST,† LAMBERT SKOOG,‡ CLAES KLINTENBERG* and BO NORDENSKJÖLD*

*Department of Oncology, University Hospital, Linköping, Oncologic Center, †Radiumhemmet, Department of Tumor Pathology, ‡Division of Cytology, Karolinska Hospital, Stockholm, Sweden

Abstract—*Tumors from 472 women with primary breast cancer were analyzed by flow cytometry. Divided into four categories, DNA ploidy showed significant association with disease recurrence and mortality. When allowance was made for its correlation with nodal status and estrogen receptor (ER) content, DNA ploidy did not add prognostic information. S-phase fraction was estimated in 290 DNA histograms. In contrast, it was significantly related to recurrence and mortality when controlling for nodal status, tumor size and ER content. When the follow-up was divided into two periods DNA ploidy and S-phase fraction showed association with disease recurrence in the first period only (<2.5 years), while the association with mortality was valid for both periods. Light scatter was measured in 234 samples. A low light scatter variability for the stemline nuclei was related to a high recurrence rate during the early follow-up period. In conclusion, DNA flow cytometry adds prognostic information concerning breast cancer patients.*

INTRODUCTION

NODAL STATUS and tumor size are important prognostic variables in primary breast cancer. Several reports have also dealt with cellular parameters such as hormone receptors, DNA content and the fraction of S-phase cells (SPF). An increased recurrence rate was found for breast cancers with a low estrogen receptor (ER) content [1–7], in tumors with aneuploid DNA content [8–13] and in tumors with a high fraction of S-phase cells, measured either as thymidine labelling index (TLI) [14–17] or by flow cytometry [4, 11, 13]. High SPF values are often found in DNA aneuploid and ER negative tumors, indicating that these factors are correlated and differing results concerning their relative importance have been reported [4, 8, 10, 11, 15, 17, 18]. However, differences in regard to length of follow-up, number of patients, stage distribution and adjuvant treatment may help to explain some of the inconsistency in these results.

The present study concerns 472 women with primary breast cancer. The prognostic significance of DNA content and proliferative activity analyzed by flow cytometry was assessed in relation to ER content and traditional histopathological parameters. In addition, nuclear size as measured by forward light scatter (FLS) was analyzed for its potential prognostic value and as an attempt to improve S-phase estimates by excluding normal host cells from diploid histograms.

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Address for correspondence: Olle Stål, Department of Oncology, University Hospital, S-58185 Linköping, Sweden.
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MATERIALS AND METHODS

Patients

The group studied comprised 472 patients with primary, invasive breast cancer whose operations were a modified radical mastectomy (94%) or a partial mastectomy with axillary dissection. Reasons for exclusion from the study were: bilateral breast cancer, previous history of cancer, preoperative therapy, non-radical surgery and evidence of distant dissemination at diagnosis. The patients were diagnosed in Stockholm County from February 1978 to December 1981. Data were collected on age, menopausal status, clinical stage, primary surgery, histopathological tumor size and nodal involvement, and adjuvant therapy.

A total of 107 patients (23%) received adjuvant systemic treatment; either CMF (10 patients), tamoxifen (79 patients) or a combination of both (18 patients). All patients treated with a partial

mastectomy were given postoperative radiotherapy to the breast (50 Gy/5 weeks), 145 patients received postmastectomy radiation to the chest wall and regional nodes (46 Gy/4 1/2 weeks).

Follow-up

Follow-up visits took place once every three months during the first 2 years, every 6 months for 2–5 years and yearly thereafter. Routinely these visits included only a physical examination. Chest X-rays, bone scans, blood tests, etc. were performed if clinical signs or symptoms indicated possible relapse. Disease recurrence was confirmed when possible by biopsy. Bone and visceral metastases, however, were sometimes established on unequivocal radiological evidence. Recurrence was dated from the first evidence of relapse based on physical, histological or imaging data. Following recurrence, treatment was decided individually for each patient by the responsible clinician. Patients who did not come to a scheduled follow-up visit were sought using official death registers, a computerized register of in-patient care which covers all hospital admissions in Stockholm County, or were contacted by letter. The ending date for the current follow-up was 1 October 1987. No patient was lost to follow-up. A total of 186 patients developed a loco-regional and/or distant recurrence during the follow-up. Of these, 144 died before the closing date. In addition, 53 patients died without a reported recurrence and were treated as censored observations in the analysis.

Estrogen receptor analysis

All specimens were collected from fresh surgical resections and stored below −70°C until analyzed for DNA pattern and estrogen receptors.

Cytosol receptor for estradiol was measured as described by Wrange *et al.* [19] by using incubation with 5 nM [^{3}H]estradiol and isoelectric focusing of the receptor in polyacrylamide gel. Three hundred and fifty-three of the tumors (75%) were judged as ER-positive with a cut-off value of 0.10 fmol/μg DNA.

DNA flow cytometry

Small pieces from the tumors were minced in 0.6 ml citrate buffer and trout and chicken red blood cells were added as internal marker cells. A suspension of isolated nuclei was prepared as described by Vindelöv *et al.* [20] with Nonidet P 40 as detergent and trypsin treatment, with stabilization by sperminetetrahydrochloride. After addition of RNAase the suspension was filtered through a 41 μm nylon mesh and stained with propidium iodide. Imprints from the tissues were examined to ascertain the presence of tumor cells in the samples.

Cell suspensions were analyzed with a Leitz MPV FLOW flow cytometer (Leitz GmbH, Wetzlar, F.R.G.) interfaced to a Monroe OC8888 microcomputer (Litton Business, U.S.A.). The software used for data acquisition and dual-parameter analysis was developed in our laboratory. Illumination from a high-pressure mercury lamp was used with light filtered through an AL interference filter with peak transmission at 546 nm and with a 20 nm bandwidth. Emission was recorded through a dicroic mirror TK 580 and a 590 nm long-pass filter. Forward light scatter (FLS) was collected by a microscope objective in a darkfield configuration established by a circular field stop in the objective used for illumination as described by Steen [21].

DNA histograms, usually with 20,000 cells, were recorded. DNA index was calculated assuming that chicken and trout erythrocytes show 35% and 80% respectively of the fluorescence of human diploid cells stained with propidium iodide [22]. The coefficient of variation (CV) of DNA peaks was estimated from the width of the peak at half-maximum peak height. Mean CV of the tumor DNA peaks was 4.1% (range 2.1–8%). The percentage of S-phase cells was estimated using a rectangular model. The number of S-phase cells was calculated by multiplying the number of channels beween the $G_{0/1}$ and G_2-M peaks by the mean number of cells in channels in the S-phase region interactively selected by the operator. In this way small extra peaks in the S-phase region could be excluded when the SPF was calculated by the program.

In 234 tumors the forward light scatter was analyzed as a measure of nuclear size. Calculations were restricted to nuclei belonging to the stemline peaks, i.e. $G_{0/1}$ cells. To obtain a relative measure of nuclear size, the mean FLS value of the tumor cells was related to the mean FLS of the trout nuclei. Also, the variability in nuclear size was calculated as the coefficient of variation of the light scatter (FLS-CV) for the same tumor cells. Variability in size ratio due to the preparation procedure and the light scatter measurement was assessed by calculating the ratio between chicken and trout nuclear size. This ratio ranged roughly between 0.6 and 0.8 with a mean of 0.71 and a CV of 8.8%.

Statistical methods

The recurrence-free interval and survival was estimated using life-table methods [23]. The prognostic value of DNA ploidy, S-phase fraction, FLS, ER content, tumor size and nodal status was analyzed using the proportional hazards method of Cox [24].

RESULTS

DNA ploidy

The distribution of DNA index (DI) was bimodal with one peak in the diploid range and another in the tri- to tetraploid range. Six DNA histogram types were identified (Table 1). Tumors with DI in

the diploid range (0.9–1.1) were classified as either hypodiploid or hyperdiploid if two peaks could be distinguished in the interval.

In Fig. 1 the recurrence-free interval curves are shown for the different DNA ploidy types. The relationship between DNA ploidy and disease recurrence was significant (Table 2). The group comprising hypodiploid, hypertetraploid and multiple aneuploid tumors had the highest recurrence rate. Tetraploid tumors showed the lowest rate, even lower than that of diploid tumors. In Fig. 2 the breast cancer survival curves are shown for the same ploidy groups. As shown in Table 2, the association of DNA ploidy with survival was also significant. The prognostic impact was further analyzed by dividing the follow-up into two periods (Table 3). A significant relationship between DNA ploidy and recurrence was only observed during the first period. On the other hand, a significant association with survival was observed during both periods.

Table 4 shows the relationship between DNA ploidy and other variables. Significantly more of the node positive tumors were classified as aneuploid compared with node negative tumors. Furthermore, tumors with a low ER content were more often aneuploid than those with a high ER content.

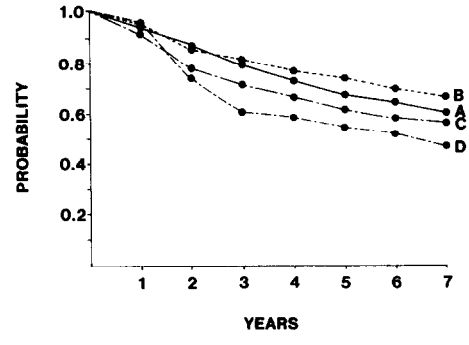

Fig. 1. Recurrence-free survival related to DNA ploidy. A = diploid, B = tetraploid, C = hyperdiploid and D = other aneuploid.

Table 1. The distribution of different DNA histogram types from a total of 472 breast cancer patients

DNA histogram type	DI (range)	No. of patients	Percentage
1. Diploid	0.9–1.1	174	37
2. Tetraploid	1.9–2.1	59	13
3. Hyperdiploid	1.1–1.9	188	40
4. Multiple hyperdiploid	>1.0	21	4
5. Hypodiploid	<1.0	8	2
6. Hypertetraploid	>2.0	22	5

Table 2. Relation of disease recurrence and mortality to different FCM characteristics in women operated for primary breast cancer. Univariate analysis according to the Cox model

FCM characteristic category	No. of patients	No. of recurrences	Rate ratio*	Test for trend†	No. of cancer deaths	Rate ratio*	Test for trend†
DNA ploidy							
Diploid	174	63	1.00		44	1.00	
Tetraploid	59	18	0.82	$\chi^2 = 4.17$;	12	0.77	$\chi^2 = 8.03$;
Hyperdiploid	188	79	1.24	$P = 0.041$	67	1.51	$P = 0.0044$
Other aneuploid	51	26	1.56		21	1.85	
SPF							
<5%	73	25	1.00		16	1.00	
5–10%	126	48	1.28	$\chi^2 = 8.15$;	38	1.71	$\chi^2 = 16.7$;
>10%	91	45	1.98	$P = 0.0043$	40	3.11	$P < 0.0001$
FLS ratio							
<1.25	81	29	1.00		20	1.00	
1.25–1.50	76	37	1.42	$\chi^2 = 2.49$;	26	1.46	$\chi^2 = 4.38$;
>1.50	77	38	1.48	$P = 0.11$	31	1.81	$P = 0.0036$
FLS-CV							
<15%	87	41	1.00		30	1.00	
15–20%	87	36	0.76	$\chi^2 = 1.56$;	32	0.94	$\chi^2 = 3.65$;
>20%	55	24	0.74	$P = 0.21$	12	0.50	$P = 0.056$

*Rate ratio in a given category to that in the reference category (the first category for each characteristic).
†The categories are coded 1, 2, 3 etc.

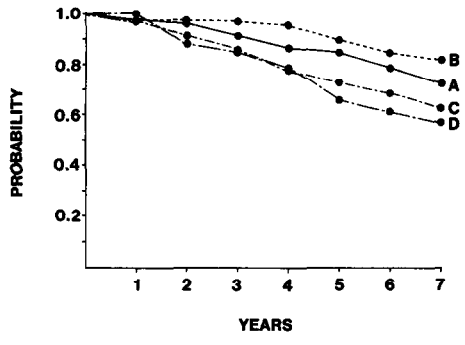

Fig. 2. Survival related to DNA ploidy. A = diploid, B = tetraploid, C = hyperdiploid and D = other aneuploid.

Tumor size, age and menopausal status seemed not to be associated to DNA ploidy.

When related to disease recurrence, DNA ploidy did not show a significant prognostic value in addition to that of tumor size, node status and ER content. The same was true when the association with breast cancer mortality was analyzed multivariately.

S-phase fraction

In 290 DNA histograms the estimated S-phase fraction was judged to be reliable. In most cases of hypodiploid and multiple aneuploid tumors the SPF was not estimated since the histogram showed overlapping populations. Furthermore, the calculated SPF was judged to be unreliable if the number of tumor cells was small compared to that of other cells and registrations derived from debris. The range of the estimated S-phase fractions was 0.7% to 33% with a mean of 8.5% and a median at 7.7%.

SPF was significantly associated with disease recurrence and mortality (Figs. 3, 4, Table 2). It was a strong predictor of early relapse while the rate of late events was equal for groups with low and high SPF values (Table 3). Concerning mortality, the predictive value was significantly higher during the first period although a significant trend in rate ratio was found in the late period. Larger tumors, tumors with a low ER content and non-diploid tumors had a significantly higher fraction of cells in S-phase (Table 4). There was also a trend towards a higher SPF among patients with four or more positive nodes. On the other hand, neither age nor menopausal status showed an association to SPF.

When controlling for DNA ploidy, S-phase fraction showed significant associations with disease recurrence and mortality while the prognostic significance of DNA ploidy was lost. SPF had a prognostic impact regarding survival and early relapse when adjusted for tumor size, nodal status and ER content (Table 5). However, the relationship between disease recurrence and S-phase was not significant over the entire follow-up period while controlling for the other variables.

Table 3. Relation of disease recurrence to different FCM characteristics, by period of follow-up. Univariate analysis according to the Cox model

FCM characteristic category	<2.5 years No. of recurrences	Rate ratio*	Test for trend†	>2.5 years No. of recurrences	Rate ratio*	Test for trend†	Test of difference between periods
DNA ploidy							
Diploid	28	1.00		35	1.00		
Tetraploid	10	1.03	$\chi^2 = 8.30$;	8	0.66	$\chi^2 = 0.07$;	$\chi^2 = 4.70$;
Hyperdiploid	47	1.64	$P = 0.0030$	32	0.92	$P = 0.80$	$P = 0.030$
Other aneuploid	18	2.28		8	0.94		
SPF							
<5%	9	1.00		16	1.00		
5–10%	25	1.72	$\chi^2 = 16.0$;	23	1.05	$\chi^2 = 0.11$;	$\chi^2 = 8.00$;
>10%	33	3.67	$P = 0.0003$	12	0.93	$P = 0.95$	$P = 0.0047$
FLS ratio							
<1.25	19	1.00		10	1.00		
1.25–1.50	19	1.05	$\chi^2 = 1.00$;	18	2.16	$\chi^2 = 1.59$;	$\chi^2 = 1.03$;
>1.50	24	1.36	$P = 0.32$	14	1.70	$P = 0.21$	$P = 0.33$
FLS-CV							
<15%	31	1.00		10	1.00		
15–20%	20	0.56	$\chi^2 = 8.31$;	16	1.38	$\chi^2 = 2.11$;	$\chi^2 = 7.93$;
>20%	9	0.38	$P = 0.0039$	15	1.80	$P = 0.15$	$P = 0.0049$

*Rate ratio in a given category to that in the reference category (the first category for each characteristic).
†The categories are coded 1, 2, 3 etc.

Table 4. Relation of DNA ploidy, S-phase fraction and nuclear size variability (FLS-CV) to tumor size, nodal status, ER content, age and menopausal status in women operated for primary breast cancer

	DNA ploidy		SPF(%)		FLS-CV(%)	
	No. of patients	Percentage aneuploid*	No. of patients	Mean	No. of patients	Mean
Tumor size						
<21 mm	241	50	153	7.9	111	17.3
21–30 mm	151	52	88	8.6‡	73	16.4‡
>30 mm	80	52	49	10.4	45	15.3
No. of positive nodes						
0	248	45	149	8.1	128	16.7
1–3	141	59†	88	8.6	59	17.5
>3	83	54	53	9.5	42	15.1
ER content (fmole/μg*DNA*)						
<0.10	119	67	60	11.4	66	17.0
0.10–0.99	183	42‡	118	7.9§	89	16.3
>0.99	170	48	112	7.6	74	16.7
Age						
<50 years	87	53	52	8.7	39	16.2
50–64 years	178	48	107	8.6	84	16.6
>64 years	207	52	131	8.3	104	16.8
Menopausal status						
Premenopausal	96	51	60	8.5	42	16.0
Postmenopausal	376	50	230	8.5	187	16.8
DNA ploidy						
Diploid			138	6.2	88	18.4
Tetraploid			35	8.9	27	15.0
Hyperdiploid			100	10.9§	88	16.0§
Other aneuploid			17	12.3	26	14.6
SPF						
<5%					27	16.3
5–10%					69	16.2
>10%					45	16.3

*The diploid and tetraploid tumors were treated as one group and the rest of the tumors were regarded as DNA aneuploid.
†$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$. The P-values refer to tests for trend.

Light scatter

For FLS ratio a wide range was obtained (0.9–3.0) with a mean of 1.4 and it was to some extent related to DNA index ($r = 0.66$). FLS-CV ranged between 8% and 35% with a mean of 17%. Larger tumors and non-diploid tumors had a significant lower FLS-CV (Table 4). However, FLS-CV was not related to any other variable.

In a few diploid samples (5%) with a high FLS ratio two populations could be identified in a two-parameter plot. One of these is illustrated in Fig. 5. The percentage of S-phase cells, first estimated to be 7.9%, was 11.2% when corrected for the presence of cells judged as normal host cells.

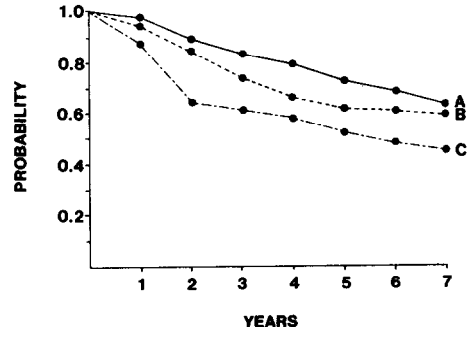

Fig. 3. Recurrence-free survival related to S-phase fraction. A = <5%, B = 5–10% and C = >10%.

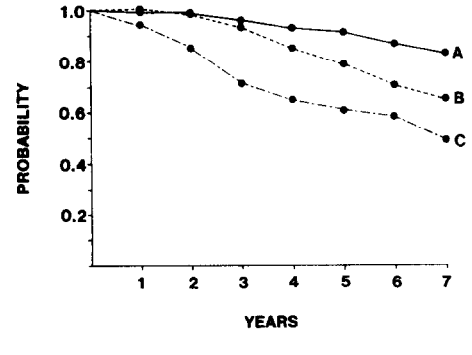

Fig. 4. Survival related to S-phase fraction. A = <5%, B = 5–10% and C = >10%.

Table 5. Relation of disease recurrence (<2.5 years) and mortality to S-phase fraction and other characteristics in women operated for primary breast cancer. Multivariate analysis according to the Cox model

Characteristic category	Recurrence; <2.5 years: No. of patients	No. of recurrences	Adjusted rate ratio*	Test for trend†	Mortality: No. of cancer deaths	Adjusted rate ratio*	Test for trend†
SPF							
<5%	73	9	1.00		16	1.00	
5–10%	126	25	1.59	$\chi^2 = 4.14$;	38	1.64	$\chi^2 = 4.81$;
>10%	91	33	2.84	$P = 0.044$	40	1.98	$P = 0.029$
Tumor size							
<21 mm	153	22	1.00		30	1.00	
21–33 mm	88	20	1.27	$\chi^2 = 9.29$;	36	1.81	$\chi^2 = 9.87$;
>30 mm	49	25	2.68	$P = 0.0023$	28	2.40	$P = 0.0017$
No. of positive nodes							
0	149	21	1.00		23	1.00	
1–3	88	21	1.53	$\chi^2 = 14.1$;	33	2.60	$\chi^2 = 37.3$;
>3	53	25	3.21	$P = 0.0002$	38	5.27	$P < 0.0001$
ER content (fmole/μg*DNA*)							
<0.10	60	39	1.00		32	1.00	
0.10–0.99	118	25	0.47	$\chi^2 = 17.5$;	37	0.45	$\chi^2 = 13.7$;
>0.99	112	13	0.25	$P < 0.0001$	25	0.33	$P = 0.0002$

*Adjusted rate ratio in a given category to that in the reference category (the first category for each characteristic). The rate ratio is adjusted for all other variables listed.
†The categories are coded 1, 2, 3.

There was a tendency to a higher recurrence rate among tumors with high FLS ratios and a significant prognostic value regarding survival (Table 2). No additional significant value could be seen if adjusting for node status, tumor size and ER content. There was both a tendency to a higher recurrence rate and a higher death rate among patients with a low FLS-CV. Regarding early relapse the trend was significant (Table 3), even when controlling for all other variables.

DISCUSSION

Measurement of cellular DNA content by flow cytometry is a simple and rapid technique which provides information about tumor ploidy and proliferative activity. DNA aneuploidy was shown to be significantly related to shorter recurrence-free survival in several studies [8, 10–13]. In the present study this relation was not significant independent of nodal status, tumor size and ER content which is in accordance with the report of Hedley *et al.* [11] and our previous study [4]. Cornelisse *et al.* [8] found a significant predictive value for DNA aneuploidy in node-positive postmenopausal women when controlling for other variables. The same was true in a microspectrophotometric study of Fallenius [10] including tumors with various nodal and menopausal status. In both studies the distant relapse-free survival was considered. While controlling for nodal status, size, grade and progesterone receptor content, Kallioniemi *et al.* [25] found a significant association with survival.

The way in which DNA ploidy groups are defined based on DNA index is one factor which may affect the significance obtained in the analysis. In the present study tetraploid tumors showed a better prognosis than other hyperdiploid tumors which is in agreement with other studies [4, 10, 25, 26]. Dowle *et al.* [18] did not find a significant difference in survival between patients with diploid and hyperdiploid tumors after 7 years. However, if tetraploid and hypertetraploid tumors were excluded from the hyperdiploid group a significant difference was obtained. Fallenius [10] found a highly significant difference between DNA diploid and aneuploid tumors when classifying a great proportion of the tumors as tetraploid with a DI range between 1.7 and 2.3. Coulson *et al.* [27] defined a high risk group consisting of hypodiploid, hypertetraploid and multiple aneuploid tumors and found a poor prognosis for this group. Our data support this finding. Thus, it may be advantageous to divide the non-diploid tumors into several groups.

Like several authors [5, 8, 11, 13, 25, 28–31], we found an inverse correlation between ER status and DNA ploidy while others did not observe a significant relation [4, 12, 18, 32] in spite of large series. However, in the latter reports tetraploid tumors were not distinguished from DNA aneuploid tumors. A significant association between DNA ploidy and nodal status was found in this study in

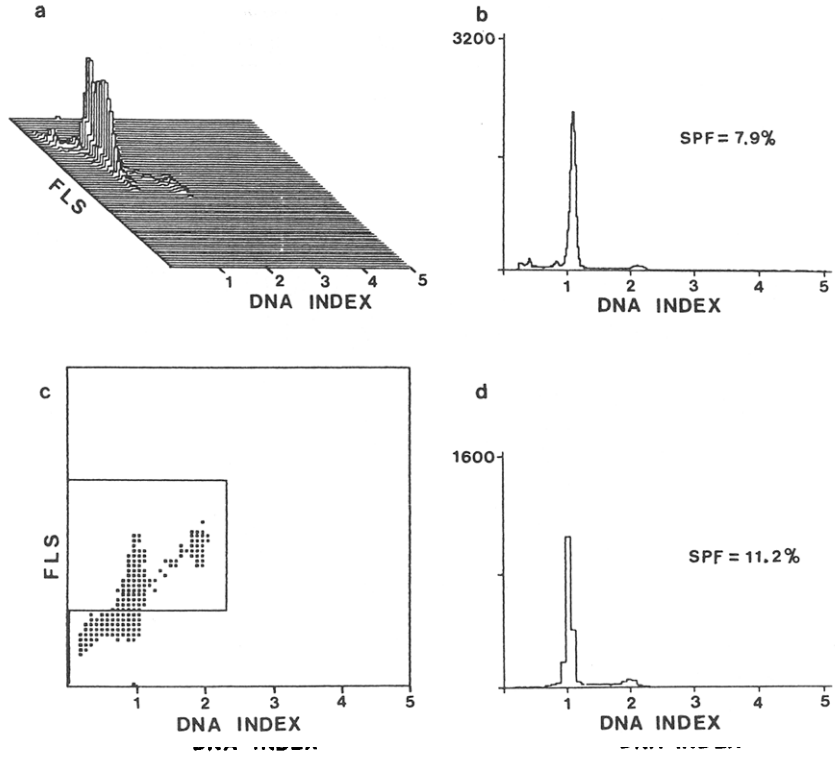

Fig. 5. A DNA diploid breast tumor analyzed by flow cytometry. In (a) forward light scatter is plotted against DNA content, (b) illustrates the corresponding DNA histogram, (c) shows gated cells judged as tumor cells and (d) shows the DNA histogram of the gated cells.

accordance with some other reports [11–13, 25, 28]. A correlation between DNA ploidy and tumor size reported by some investigators [9, 10, 18, 28] was not found in this study. Few authors have observed a relation between ploidy and age [25, 33] or between ploidy and menopausal status [11, 18].

Mainly consistent results have been reported by different investigators concerning the correlations between fraction of S-phase cells and other prognostic variables. In agreement with the present study all studies cited herein showed that SPF was related to DNA ploidy if both factors were evaluated. The same is true for SPF and estrogen receptor content with two exceptions [11, 14]. In addition, there is a trend that high SPF values are found in node-positive and large tumors. The latter relationship was significant in a study by Meyer *et al.* [34] as well as in the present study. This may reflect that tumors with a high S-phase percentage probably grow to a larger size before surgery.

The mean SPF values obtained for both DNA diploid and aneuploid tumors are in agreement with data reported by McDivitt *et al.* [32], who obtained good correlation between flow SPF and thymidine labelling index. The mean values also correspond to those obtained in a comparative study of FCM and static cytofluorometry [35]. The fact that normal cells were excluded from the diploid peak in a few samples in the present series did not produce a noticeable change of the mean SPF value. Thus, the attempt to improve SPF estimates from DNA diploid tumors in general was unsuccessful.

The S-phase fraction measured by thymidine autoradiography [14–17] or flow cytometry [4, 11, 13] has been shown to influence the disease-free survival in breast cancer. This has been observed in node-negative [14, 16] as well as in node-positive series [11]. The prognostic impact independent of nodal status is also confirmed by multivariate analyses [4, 15, 17]. However, since SPF is related to histologic grade [11, 15] and ER status, its additional prognostic value may be questioned. In the study of Hedley *et al.* [11] SPF failed to show prognostic significance when adjusting for both nodal status and grade while it demonstrated an additional value in two other studies [15, 17]. In our previous study [4] the fraction of S-phase cells showed predictive value in addition to that of nodal status and ER content. In the present study, the same was true regarding survival at 7 years and recurrence at 2.5 years, but it was obvious that the prognostic impact of SPF regarding disease recurrence decreased with time. In fact, a similar trend was observed for ER status which is in agreement with other studies [36–39].

Some prognostic information was found for the factors derived from the light scatter analyses. The risk predicted by the FLS ratio was related to the correlation between this factor and DNA index, i.e. DNA aneuploid tumors more often had a large

mean nuclear size. The observation that a low FLS-CV was related to early recurrence, independently of the other variables, is to our knowledge novel. Light scatter, analyzed in the same way, combined with SPF has been used in discriminating between different grades of non-Hodgkin's lymphoma [40].

In the majority of DNA diploid samples it could not be concluded from the FLS analysis whether the sample contained mostly tumor cells or a mixture of host and tumor cells. The higher FLS-CV found in DNA diploid tumors may be an indication of heterogeneous populations. It should be stated that the preparation procedure is optimized with regard to reliable quantitation of DNA content rather than to size measurements. Light scatter analysis of fixed nuclei not treated with enzymes may yield a different result.

In conclusion, properties such as fraction of S-phase cells and estrogen receptor status help to predict the risk of recurrence and death from disease in breast cancer. In many countries an increased use of mammography screening contributes to the finding of an increasing proportion of low stage tumors which emphasizes the need for new prognostic variables. The predictive power of SPF seems to be stronger than that of DNA ploidy. However, a classification based on both factors as proposed by McDivitt *et al.* [32] might be beneficial. The prediction of recurrence primarily concerns early relapse. Extensive studies will be necessary to evaluate if measurement of cellular parameters may help to identify subgroups of patients who should be candidates for different adjuvant therapies.

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