Breast Cancer Research and Treatment **28**: 9–20, 1993.
© 1993 *Kluwer Academic Publishers. Printed in the Netherlands.*

Report

DNA ploidy, S-phase, and steroid receptors in more than 127,000 breast cancer patients

Charlotte R. Wenger,[1] Susan Beardslee,[1] Marilyn A. Owens,[2] George Pounds,[2] Teri Oldaker,[2] Patricia Vendely,[2] M.R. Pandian,[2] Douglas Harrington,[2] Gary M. Clark[1] and William L. McGuire[1,3]
[1] *University of Texas Health Science Center, Department of Medicine/Medical Oncology, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7884, USA; and* [2] *Nichols Institute Reference Laboratories, San Juan Capistrano, California 92675, USA*

Key words: breast cancer, flow cytometry, hormone receptors, prognosis

Summary

Several potential prognostic factors are available today for patients with breast cancer, and many more are being identified and studied. To evaluate the clinical utility of these factors, it will be necessary to measure them on a large number of patients, and then follow these patients so that multivariate survival analyses can be performed.

The Oncology Research Network was established in 1986 by the University of Texas Health Science Center at San Antonio and Nichols Institute Reference Laboratories in order to evaluate the clinical utility of new prognostic factors for patients with primary breast cancer. The first generation of prognostic factors included steroid receptors, along with DNA ploidy and S-phase fraction determined by flow cytometry. Currently, laboratory results have been obtained from more than 127,000 patients, and follow-up information is available on a subset of more than 25,000 of these patients.

S-phase fraction was related to the ploidy status of the tumor. An increased incidence of aneuploidy and higher S-phase fractions were found in estrogen and progesterone receptor negative tumors, tumors from patients with positive axillary lymph nodes, tumors greater than 2 cm in diameter, and patients younger than 35 years of age. Preliminary survival analyses suggest that S-phase fraction and DNA ploidy, in combination with other prognostic factors, are powerful predictors of early disease relapse.

The Oncology Research Network provides an important resource for examining the clinical significance of new laboratory assays and for expediting improvements in existing laboratory techniques.

Introduction

Prognostic factors are important in the treatment decision process for patients with breast cancer [1]. As we learn more about the available prognostic factors, we will be able to incorporate this knowledge into more accurate estimates of disease recurrence for individual patients.

DNA flow cytometry is a relatively new technology that has been used to evaluate the nuclear DNA content of many types of human tumors, including breast cancer [2, 3]. It can be performed on fresh

[3] We regret to report that Dr. William L. McGuire died on March 25, 1992, after this work was largely completed

Address for offprints: G.M. Clark, University of Texas Health Science Center, Department of Medicine/Medical Oncology, 7703 Floyd Curl Drive, San Antonio, TX 78284-7884, USA

tissue specimens, frozen biopsy samples, needle aspirates taken directly from the tumor, or paraffin-embedded tumor tissues. DNA flow cytometry provides a measure of DNA content (DNA ploidy) and a measure of proliferative activity (S-phase fraction). Numerous researchers have demonstrated the feasibility of routinely performing DNA flow cytometry on specimens from patients with primary breast cancer to obtain additional prognostic information that could be used to determine the most appropriate treatment strategies. Several retrospective, correlative studies have now been published that show relationships between flow cytometric parameters and other prognostic factors [4–25], and, more recently, associations with disease-free and overall survival [10, 14, 17, 23–26].

We have previously shown in a pilot study of 1331 breast cancer specimens that DNA flow cytometry results are associated with other established prognostic factors for breast cancer, including steroid receptor status, tumor size, and axillary lymph node status [11]. In order to evaluate the clinical utility of DNA flow cytometry based on a large patient population, the University of Texas Health Science Center at San Antonio (UTHSCSA) and Nichols Institute Reference Laboratories established the Oncology Research Network (ORN) in 1986.

In 1986, clients who were sending breast cancer specimens to Nichols Institute Reference Laboratories for flow cytometry were identified, contacted, and invited to participate in the pilot phase of the ORN. Nearly 80 hospitals and health care institutions responded to this initial request by submitting clinical data (the patient's age, tumor size, nodal status, and tumor histology) for over 1200 cases. As the number of institutions and the monthly volume of assays increased, the scope of the project was expanded to incorporate this growth. Participation has now increased to more than 300 health care institutions and physicians who have contributed clinical history and follow-up data to the Oncology Research Network.

This large data base provides a unique resource for examining relationships between ploidy, S-phase fraction, and other prognostic factors. As continued follow-up information is collected, we will be able to assess the prognostic significance of these new factors.

Methods

Receptor assays

Tumor specimens were frozen immediately after excision and shipped on dry ice to the laboratory. Assays were performed using a dual-label, ligand-binding procedure as described elsewhere [11]. Tumor specimens were considered estrogen receptor positive (ER+) or progesterone receptor positive (PgR+) if they contained at least 3 or 5 fmol of specific binding sites per mg of protein, respectively.

Flow cytometry

The DNA content of the tumor cells and the S-phase fraction (SPF) were determined as described elsewhere [27]. In brief, approximately 100 mg of frozen pulverized tumor was manually homogenized, filtered, and centrifuged at 750 × g for 45 minutes. Chicken red cells in phosphate-buffered saline were added as an internal standard. Cells were lysed and stained for DNA by incubation in a modified Krishan hypotonic sodium citrate staining buffer containing propidium iodide as the DNA fluorochrome. DNA-stained nuclei were prepared and run on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Fifty thousand tumor events were acquired on a single-parameter 256-channel integrated fluorescence histogram. Doublets were minimized using integrated versus peak fluorescence dual parameter bit mapping.

Cell-cycle distributions of the presynthetic growth phase (G_0/G_1), synthetic phase (SPF), and postsynthetic and mitotic growth phases (G_2M) were evaluated along with the DNA content. If the G_0/G_1 peak was within ± 10 channels of the expected diploid position, stained human peripheral-blood lymphocytes (hPBL) were added to the tumor sample to confirm ploidy. The DNA index, a value that expresses the amount of DNA content relative to normal, is calculated as the ratio of the peak chan-

nel number of the tumor G_0/G_1 peak to the peak channel number of the normal content G_0/G_1 peak. By definition, the DNA index for a diploid population is 1.0. DNA content was defined as aneuploid if two discrete G_0/G_1 peaks could be confirmed after the addition of hPBL. In addition, the aneuploid G_0/G_1 peak had to contain at least 10% (20% in the tetraploid region) of the 50,000 sample events collected and have a corresponding identifiable G_2M peak. Aneuploid tumors were further classified based on their DNA index (DI): hypodiploid (DI < 1.0); near-diploid ($1.0 < DI < 1.2$); hyperdiploid ($1.2 \leq DI < 1.85$); tetraploid ($1.85 \leq DI < 2.05$); hypertetraploid ($DI \geq 2.05$). When ploidy status could not be determined because of poor sample quality or insufficient resolution to distinguish two peaks (coefficient of variation greater than 6%), the histograms were considered uninterpretable for ploidy status.

Prior to 1988, the PARA 1 and PARA 2 software programs (Coulter Electronics, Hialeah, FL) were used in concert with a modeling system developed for heterogeneous tumors with overlapping cell populations [27]. Beginning in 1988, the MODFIT program (Verity Software House, Inc., Topsham, ME), using the same parameter settings from the PARA 1 and PARA 2 analyses, was used for cell-cycle analysis. G_0/G_1 and G_2M components were modeled as Gaussian components in all MODFIT models. The G_2M was allowed to float provided the resulting G_2M to G_0/G_1 ratio was in the range 1.85 to 2.05. Otherwise, it was fixed at 1.95 in accordance with the linearity of the flow cytometer. SPF components were modeled as single trapezoids. Debris was modeled with an exponential equation with slope -0.05 for diploid tumors and -0.04 for aneuploid tumors. For tetraploid and near-diploid histograms, a combined SPF was used. Neither the autoanalysis feature nor aggregate correction was used since these options are recent additions to the software. User-defined regions were set manually to assign peak ranges.

Patients

Breast tumor samples were sent from hospitals to Nichols Institute for routine steroid receptor and flow cytometry analyses. Eligible patients for this study were women with primary breast cancer whose biopsy or mastectomy specimen was analyzed for both hormone receptors and flow cytometry. Additionally, the women were without distant metastases at the time that the assay was performed.

Hospitals and health care institutions were initially contacted by telephone. After the appropriate contact person was identified and interest in the project was expressed, complete information about the ORN was provided to the health care facility. The proposal was presented to the institution's cancer research committee for consideration and approval. After approval of the project, data forms for recording the abstracted clinical data were sent to the institution. All requests for data were directed to the participating health care institution's designated data coordinator or to a collaborating physician.

The data were abstracted from either institutional medical records or tumor registry records. The initial data request forms provide space for recording patient data (age, birthdate, menopausal status), tumor characteristics (tumor size, histology, lymph node status), therapy information, and follow-up data for eligible cases. The data base is reviewed on a continuing basis to identify cases requiring updated follow-up. Data request forms are computer generated and sent to the collaborator. The forms include the last known follow-up status and the initial tumor characteristics. Printing the historical data on the follow-up forms is a quality control mechanism to facilitate verification of previously submitted data. It also allows for the completion of any missing data items. The collaborator amends the survival and the disease recurrence information with more recent follow-up dates, verifies the clinical data, and returns the updated forms. The process of updating and enlarging the data base is a continuous one. Virtually all participants are contacted at least once a year for updated or additional data.

Steroid receptor and flow cytometry assay results are transferred at regular intervals from the computer at Nichols Institute, via electronic media, to

the computer at UTHSCSA for long-term storage. Laboratory results from more than 127,000 breast cancer patients are currently stored in the data base. Follow-up data have been collected for a subset of more than 25,000 of these patients. The length of follow-up ranges from 0 to 94 months, with a median of 26 months. This cohort of cases will continue to grow and mature as participants are contacted and provide additional clinical and follow-up data for tumor specimens sent from their institutions.

Table 1. Ploidy status distribution

Ploidy status	N	(%)
Euploid		
Diploid	62,381	(49%)
Near-diploid	5,169	(4%)
Aneuploid		
Tetraploid	11,033	(9%)
Hyperdiploid	37,025	(29%)
Hypertetraploid	5,108	(4%)
Hypodiploid	2,600	(2%)
Multiploid	3,904	(3%)
Total	127,220	(100%)

Results

DNA ploidy status was determined for 127,220 specimens from patients with breast cancer (Table 1). The total number of specimens received for flow cytometric evaluation has not been accurately recorded throughout the time period of this study, but currently approximately 3% of samples received are uninterpretable for DNA ploidy status. About 5% of specimens require repeated analyses to obtain evaluable results. A total of 53% of the DNA histograms were euploid (diploid or near-diploid with DNA index between 1.0 and 1.2), while the remaining 47% exhibited various types of aneuploidy. The most common type of aneuploidy was the simple hyperdiploid DNA pattern which contained a diploid population of cells and a single population of aneuploid cells with DNA index between 1.2 and 1.85. However, 18% of the tumors had other types of aneuploid histograms (tetraploid, 9%; hypertetraploid, 4%; hypodiploid, 2%; multiploid, 3%).

Table 2 shows the relationships between ploidy status and several other factors that are known to have prognostic significance for patients with primary breast cancer. Since flow cytometry and ste-

Table 2. ER, PgR, nodal status, tumor size, and age by ploidy status

Ploidy status	% ER+	% PgR+	% Node−	% ≤ 2 cm	% > 50 yr
Euploid					
Diploid	86% (n = 60260)	65% (n = 60230)	66% (n = 10696)	60% (n = 10994)	76% (n = 12116)
Near-diploid	89% (n = 5021)	71% (n = 5018)	63% (n = 980)	57% (n = 994)	76% (n = 1087)
Aneuploid					
Tetraploid	86% (n = 10683)	63% (n = 10682)	57% (n = 2001)	48% (n = 2133)	76% (n = 2258)
Hyperdiploid	73% (n = 35829)	51% (n = 35827)	57% (n = 6496)	46% (n = 6717)	71% (n = 7221)
Hypertetraploid	71% (n = 4932)	47% (n = 4930)	57% (n = 947)	45% (n = 996)	76% (n = 1054)
Hypodiploid	66% (n = 2506)	43% (n = 2505)	59% (n = 466)	51% (n = 482)	57% (n = 522)
Multiploid	79% (n = 3788)	62% (n = 3783)	54% (n = 768)	42% (n = 809)	74% (n = 857)
Total	81% (n = 123019)	60% (n = 122975)	62% (n = 22354)	53% (n = 23125)	74% (n = 25115)

Table 3. Two-year actuarial relapse rates by ploidy status

Ploidy status	N	Two-year relapse rate	95% confidence interval
Euploid			
Diploid	11346	6.3	(5.8– 6.8)
Near-diploid	1025	5.1	(3.6– 6.6)
Aneuploid			
Tetraploid	2124	8.8	(7.4–10.2)
Hyperdiploid	6776	11.4	(10.5–12.3)
Hypertetraploid	984	12.5	(10.1–14.9)
Hypodiploid	501	12.7	(9.4–16.0)
Multiploid	807	11.8	(9.3–14.3)

Median follow-up = 26 months.

Table 4. S-phase fraction by ploidy status

Ploidy status	Evaluable for SPF	Median SPF
Euploid		
Diploid	58,705 (94%)	3.3
Near-diploid	2,547 (49%)	5.5
Aneuploid		
Tetraploid	10,269 (93%)	9.1
Hyperdiploid	34,060 (92%)	11.0
Hypertetraploid	4,349 (85%)	12.4
Hypodiploid	0 (0%)	–
Multiploid	0 (0%)	–
Total	109,930 (86%)	

roid receptor assays are generally performed at the same time, approximately 123,000 samples were available for these analyses. Diploid, near-diploid, and tetraploid tumors were most often ER+ (≥ 85%) and PgR+ (≥ 63%). It is of interest that multiploid tumors, thought by many to have poor prognosis, also have a relatively high frequency of positive receptors. Tumors with other types of aneuploidy had significantly lower rates of positive steroid receptors.

Axillary lymph node status, tumor size, and the age of the patient were obtained through the Oncology Research Network for a subset of approximately 25,000 of these patients. Patients with euploid tumors more frequently had negative axillary lymph nodes (≥ 63%) compared to patients with aneuploid tumors (≤ 59%). No significant differences were observed among the various aneuploid classifications, although the lowest incidence of node-negativity was for patients with multiploid tumors. A similar pattern was observed for tumor size. Sixty percent of the euploid tumors were less than 2 cm in diameter, compared to less than half of the aneuploid tumors. Again, multiploid tumors had the lowest percentage (42%) of small tumors. Despite the high rate of positive steroid receptors, tetraploid tumors were very similar to other aneuploid tumors with respect to positive lymph nodes and tumor size. The percentage of patients older than 50 years of age was quite similar for the two ploidy groups (76% of patients with euploid tumors, compared to 74% with aneuploid tumors). However, patients with hypodiploid tumors were significantly younger than patients with other ploidy classifications with only 57% older than 50, indicating that hypodiploidy may be associated with more aggressive tumors often found in younger, pre-menopausal women.

Since the Oncology Research Network was only established in 1986 and patients have been continuously enrolled, the median follow-up of these patients is only 26 months. Therefore, definitive correlations with clinical outcomes must await additional follow-up. Nevertheless, we have performed preliminary survival analyses to compare short-term relapse rates among the various ploidy classifications. Table 3 presents two-year actuarial relapse rates and 95% confidence intervals by ploidy status. Patients with euploid tumors have significantly lower relapse rates than patients with aneuploid tumors (6.2% and 11.1%, respectively). Patients with tetraploid tumors have a relapse rate intermediate between the two major ploidy groupings. No other significant differences were observed within the subclassifications of euploidy or aneuploidy.

Evaluable S-phase fractions were obtained for 86% of all specimens (Table 4). The evaluability rates for SPF depended on the ploidy status of the tumor. SPF was obtained for more than 90% of the most common types of tumors (diploid, hyperdiploid, tetraploid), but in only 49% of near-diploid tumors due to the closeness of the overlapping populations. The evaluability rate for near-diploid tumors has increased with the introduction of more

sophisticated modeling procedures. SPF was not estimated for multiploid tumors or hypodiploid tumors.

The SPF values also differed significantly by ploidy status. The median SPF for euploid tumors was 3.4 compared to 10.7 for all aneuploid tumors. The distributions of SPF are displayed by ploidy status in Fig. 1. Not only is the median SPF of the euploid tumors (3.4) significantly lower than the median of the aneuploid tumors (10.7) but the shape of the distributions is also quite different. This suggests that evaluations of correlations between SPF and other prognostic factors should take into account the interactive, and potentially confounding relationship with ploidy status. Table 5 shows the relationships between SPF and other prognostic factors separately for euploid and aneuploid tumors.

A strong inverse relationship exists between SPF and steroid receptor positivity within both ploidy classifications. Tumors that contain both ER and PgR have relatively low SPF, and the SPF increases significantly as the tumors lose these receptors from ER+/PgR+ to ER+/PgR− to ER−/PgR+ to ER−/PgR−.

Patients with negative axillary lymph nodes have significantly lower SPF than patients with positive nodes. The median SPF increased as the number of positive nodes increased for both ploidy groups, although the magnitude of the increases was quite modest. This was confirmed by the relatively weak Spearman rank correlation coefficients between SPF and the number of positive nodes (+ 0.13 and + 0.07, respectively, for euploid and aneuploid tumors).

The relationship between SPF and tumor size is partially dependent on ploidy status. Euploid tumors greater than 2 cm in diameter have a significantly higher SPF than smaller tumors, but there does not appear to be a gradient of increasing SPF with increasing tumor size. In contrast, there is a direct relationship between SPF and tumor size among aneuploid tumors. SPF is inversely related to the age of the patient. Although the number of patients younger than 35 is relatively small, their

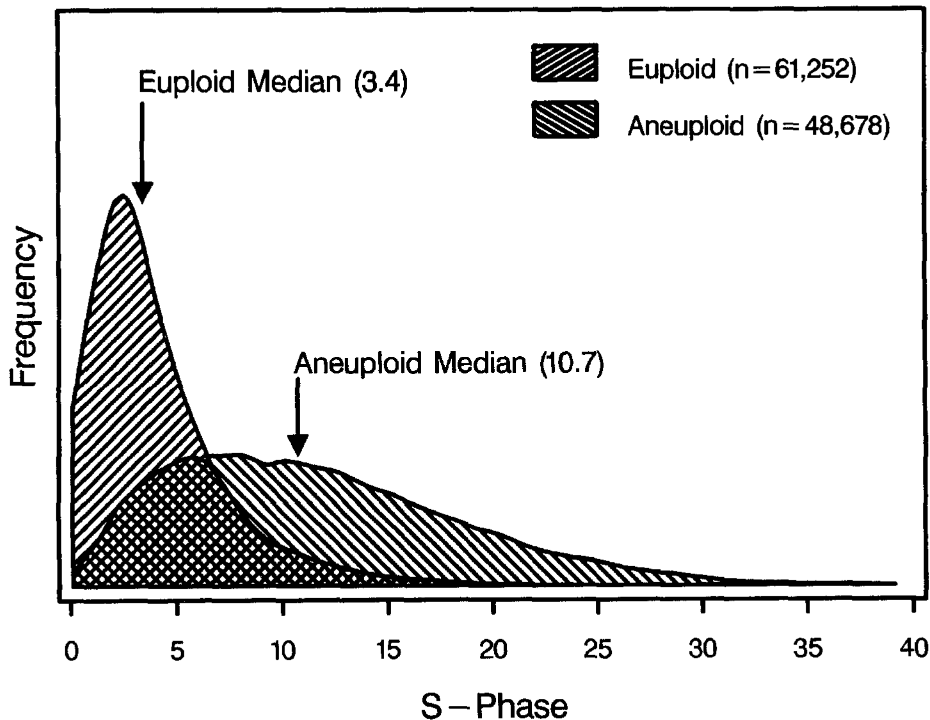

Fig. 1. Distribution of SPF by ploidy status. The median SPF for euploid tumors (3.4) was significantly lower than the median SPF for aneuploid tumors (10.7), $p < 0.0001$.

Table 5. Median S-phase fraction by ploidy and other prognostic factors

	Euploid			Aneuploid		
	N	Median SPF		N	Median SPF	
Steroid receptors						
ER+/PgR+	37,173	3.1		23,289	8.5	
ER+/PgR−	14,107	3.7	p = 0.0001	12,492	11.4	p = 0.0001
ER−/PgR+	1,712	3.9	p = 0.0218	1,756	13.0	p = 0.0001
ER−/PgR−	6,175	5.1	p = 0.0001	9,560	15.3	p = 0.0001
Positive nodes						
0	6,611	3.2		4,686	10.0	
1–3	2,043	3.8	p = 0.0001	1,898	10.7	p = 0.0005
4–10	927	4.0	p = 0.0040	1,076	10.8	p = 0.42
> 10	475	4.4	p = 0.0167	583	11.6	p = 0.0206
Tumor size						
≤ 1 cm	1,734	3.1		827	8.1	
1–2 cm	4,448	3.2	p = 0.0012	3,159	9.7	p = 0.0001
2–5 cm	3,640	3.8	p = 0.0001	3,990	11.2	p = 0.0001
> 5 cm	531	3.9	p = 0.56	614	12.2	p = 0.0428
Age						
< 35	280	4.9		305	14.4	
35–65	5,864	3.6	p = 0.0001	5,260	11.4	p = 0.0001
> 65	5,248	3.2	p = 0.0001	3,631	8.8	p = 0.0001

Due to multiple comparisons within subgroups, p-values should only be considered 'statistically significant ($p < 0.05$)' if the reported pairwise p-values are less than 0.008.

SPFs are quite high, consistent with the poor clinical prognosis observed for many of these patients.

Complete information (flow cytometry and steroid receptor results, lymph node status, tumor size, age) were available for 15,877 patients with primary breast cancer. Each of these factors was significantly related to disease-free survival in univariate analyses ($p < 0.0001$). The lymph node status, tumor size, age, ER, PgR, and ploidy were dichotomized, while the logarithm of SPF was analyzed as a continuous factor. A preliminary multivariate analysis produced the Cox model displayed in Table 6. As expected, the strongest predictive factor was lymph node status, followed by tumor size, SPF, ER status, PgR status, and age. Once the results of these factors were known, the additional knowledge of ploidy status provided no additional significant information for predicting clinical outcome. It must be cautioned that the optimal representations of these factors and their relative weights in multivariate models will require additional follow-up of these patients.

Although SPF was significantly associated with disease-free survival when it was expressed as a continuous factor, it is often useful to dichotomize factors into low and high ranges. Due to the relationship described above between SPF and ploidy, we examined different cutpoints for euploid and aneuploid tumors. A wide range of possible cutpoints within each ploidy group yielded statistically significant differences between patients with low and high SPF. All cutpoints between 2.6 and 9.8 for euploid tumors had log-rank p-values $< 10^{-9}$, and all cutpoints between 11.0 and 12.4 for aneuploid tumors had p-values $< 10^{-5}$. Figure 2 displays the disease-free survival curves for node-negative and node-positive patients using our previously established cutpoint of 6.7 for euploid tumors and 11.0 for aneuploid tumors. The 3-year actuarial relapse rates were 6% and 12%, respectively, for node-neg-

Table 6. Multivariate disease-free survival (n = 15,877)

Factors in the model	Relative risk	(95% C.I.)	p-value
Nodal status (+ vs –)	2.4	(2.2–2.7)	< 0.0001
Tumor size (≤ 2 cm vs > 2 cm)	1.7	(1.5–1.9)	< 0.0001
Log (SPF)*	1.4	(1.3–1.5)	< 0.0001
ER (– vs +)	1.4	(1.2–1.6)	< 0.0001
PgR (– vs +)	1.2	(1.1–1.4)	0.0004
Age (≤ 50 vs > 50)	1.2	(1.1–1.4)	0.0006

* Relative risk evaluated at median SPF for aneuploid tumors (10.7) vs median SPF for euploid tumors (3.4).

ative patients with low and high SPF, and 17% and 29%, respectively, for node-positive patients.

It must be stressed that these survival analyses are very preliminary due to the short follow-up and a variety of adjuvant therapies that were often based on the prognostic factors included in this study. With continued follow-up, we will be able to make definitive statements about the prognostic significance of ploidy and SPF, and determine the most useful representation of their results for clinical treatment planning.

Discussion

The Oncology Research Network has proven to be an effective way to gather and assess a large volume of data on primary breast cancer cases. Through this network we have rapidly amassed a data base representing breast cancer patients from across the nation. Laboratory results on specimens from more than 127,000 patients and clinical information on a subset of more than 25,000 patients are currently in the data base. Statistical analyses on these large numbers have demonstrated meaningful relationships between flow cytometric parameters, steroid receptors, and other prognostic factors. As the follow-up time lengthens, important relationships with disease-free and overall survival can also be examined.

We have analyzed over 123,000 breast tumor specimens for hormone receptors, ploidy, and SPF. Approximately half (49%) of the specimens were diploid, which agrees closely with our published pilot study [11]. Some investigators have reported less than 40% of tumors to be diploid [14, 18, 19, 23], while others have found 41% to 48% to be diploid [13, 20, 21, 28]. One study [24] reported a higher per-

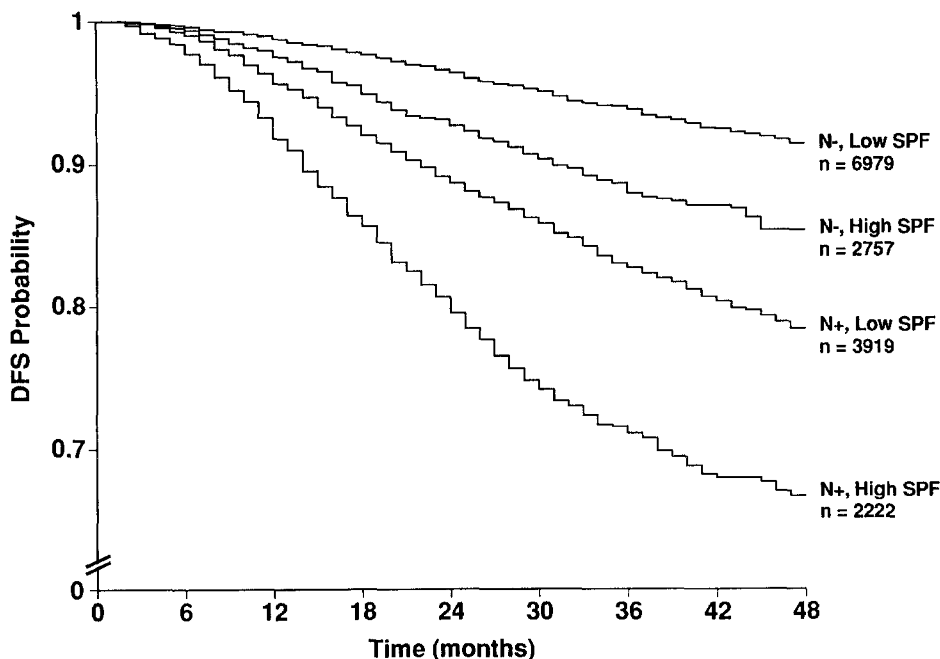

Fig. 2. Disease-free survival by lymph node status and SPF. Node-negative patients with high SPF had worse disease-free survival than node-negative patients with low SPF ($p < 0.0001$). A similar relationship was observed for node-positive patients ($p < 0.0001$).

centage of diploid tumors (59%). Some of the variability among these studies is due to the heterogenous patients that were included in the analyses, but the relatively small sample size in many of these studies probably explains most of the discrepancies.

Our overall median SPF (5.5), diploid median SPF (3.4) and aneuploid median SPF (10.7) were also in close agreement with our earlier results (5.8, 2.6, 10.3, respectively), and correlate well with other published studies [9, 14, 17]. However, other studies have reported higher median SPF [13, 20, 21, 28]. The SPF can be dramatically affected by the computer modeling algorithms that are used to estimate cell-cycle components and by the type of flow instrumentation. Failure to adjust for nuclear debris that is almost always found in frozen breast specimens can artificially inflate the observed SPF.

We found that steroid receptor negative tumors were more often aneuploid, and had a higher median SPF than receptor positive tumors. The strong inverse relationship between steroid receptor status and ploidy has also been reported by other investigators [6, 9–12, 17, 19, 24], but not all [4, 7, 13, 15, 20, 21, 25]. Most studies [6, 9, 11, 13, 15, 17, 19, 22, 25] have shown the inverse relationship between steroid receptor status and SPF, but a few of the earlier studies failed to observe this finding [4, 7, 10, 21]. Of particular interest is the ER–/PgR+ subgroup of tumors. This fairly rare syndrome is often thought to be a laboratory artifact reflecting a bad ER assay, and these patients are frequently considered to be receptor positive for treatment decisions. However, the SPF in ER–/PgR+ tumors is significantly higher than either ER+/PgR+ or ER+/PgR– tumors, suggesting that this unusual steroid receptor syndrome may be the result of genetic alterations that are not yet fully understood [28].

There have been conflicting reports regarding the relationship between ploidy and axillary lymph node status. Several investigators have reported that no correlations exist [6, 8, 19, 21, 30, 31]. However, Ewers *et al.* [5], Kallioniemi *et al.* [9, 17], and Joensuu *et al.* [23], have all found a trend for an increased frequency of aneuploidy in node-positive patients. Lykkesfeldt *et al.* [12] found that all of his 'high-risk' patients with diploid tumors were node-positive, and Hedley [10] showed that patients with four or more positive nodes were more likely to have an aneuploid tumor. Correlations between SPF and nodal status have not been reported although several studies have addressed this issue [9, 10, 17, 19, 21, 22]. We found that node-negative patients more often had diploid tumors with low SPF compared to node-positive patients, but that the relationship between SPF and the actual number of positive nodes was weak.

We previously reported that aneuploidy was not related to age although there was a higher SPF in tumors from younger women [11]. In the present study, we found that younger women more often have aneuploid tumors compared to older women, and the tumors of younger women have a higher median SPF than tumors in older women. Most studies [13, 19, 20, 24] have found no association between ploidy and age, but some have observed this relationship [9, 23, 25]. Similarly, correlations between SPF and age have been reported by some investigators [13, 25], but not all [9, 17, 22]. Christov *et al.* [16] did not find an association between age and SPF for diploid tumors, but they did show that women who were less than 50 years of age with an aneuploid tumor had higher SPF.

There is no consensus regarding relationships between tumor size and ploidy or SPF. A few investigators have reported associations between ploidy and tumor size [19, 23, 24], but most studies have concluded that no relationship exists [9, 10, 12, 13, 17, 21, 25, 31]. A correlation between SPF and tumor size was found in two studies [19, 25], but not in most [9, 10, 13, 17, 21, 22]. With our large series of patients, we were able to demonstrate that large tumors are more likely to be aneuploid and have a higher median SPF than small tumors.

Most investigators [9, 10, 14, 20, 23–25] agree that patients with diploid or low SPF tumors have a longer disease-free survival and overall survival than patients with aneuploid or high SPF tumors, but a few [12, 13, 20, 21] failed to confirm these results. Due to our short overall median follow-up of 26 months it is impossible to make definitive statements about disease-free or overall survival. But preliminary analyses of early disease recurrence, without adjustments for adjuvant therapy, indicate that SPF and ploidy are significant predictors of dis-

ease-free survival, and that SPF must be evaluated in the context of the ploidy status of the tumor. Additional follow-up will be necessary to learn the ultimate prognostic significance of these flow cytometric factors.

In summary, we have established the Oncology Research Network as an effective way to gather a large volume of data on primary breast cancer. With these data we have demonstrated relationships between flow cytometric factors and receptor status, lymph node status, tumor size, and age of the patient. It is now possible to study similar relationships between newer factors, such as HER-2/neu and cathepsin D, and other traditional factors. The ORN actively continues to contact participants and collects necessary follow-up data. Longer follow-up times will allow for the definitive evaluation of disease-free and overall survival in relationship to DNA ploidy and SPF and other factors. Collaborators understand the importance of these follow-up data and have incorporated this responsibility into their decision and commitment to participate. The ORN provides an important resource for examining the clinical significance of laboratory assay results, and for expediting research of improvements in existing laboratory techniques.

Acknowledgements

This work was supported in part by NIH Grant CA30195 and Cancer Center Support Grant P30 CA54174. We are indebted to David G. Mascorro for his technical expertise in preparation of the graphs and figures, and to all the hospitals and clinicians who participated in our study.

Appendix

Each of the following collaborating institutions has provided follow-up data for more than 50 of the patients included in these analyses:

Alachua General Hospital, Gainesville, FL; Alexian Brothers Medical Center, Elk Grove Village, IL; AMI Tarzana Regional Medical Center, Tarzana, CA; Anaheim Memorial Hospital, Anaheim, CA; Anderson Memorial Hospital, Anderson, SC; Bannock Regional Medical Center, Pocatello, ID; Baptist Medical Center, Oklahoma City, OK; Baptist Medical Center, Birmingham, AL; Baptist Medical Center, Kansas City, MO; Baptist Medical Center, Columbia, SC; Bay Harbor Hospital, Harbor City, CA; Baylor University Medical Center, Dallas, TX; Bergan Mercy Hospital, Omaha, NE; Bethany Medical Center, Kansas City, KS; Bloomington Hospital, Bloomington, IN; Brick Hospital, Brick, NJ; Cabrini Medical Center, New York, NY; Centinela Hospital, Inglewood, CA; Chippenham Hospital, Richmond, VA; Christ Hospital, Cincinnati, OH; Columbia Presbyterian Medical Center, New York, NY; Community Hospital-East, Indianapolis, IN; Community Hospital of Roanoke Valley, Roanoke, VA; Condell Memorial Hospital, Libertyville, IL; Danbury Hospital, Danbury, CT; Daniel Freeman Memorial Hospital, Inglewood, CA; Deaconess Hospital, St. Louis, MO; Deaconess Hospital, Oklahoma City, OK; DePaul Hospital, Norfolk, VA; Dover General Hospital and Medical Center, Dover, NJ; Downey Community Hospital, Downey, CA; Doylestown Hospital, Doylestown, PA; Easton Hospital, Easton, PA; Englewood Hospital, Englewood, NJ; Erlanger Medical Center, Chattanooga, TN; Fair Oaks Hospital, Fairfax, VA; Florida Hospital, Orlando, FL; Floyd Memorial Hospital, New Albany, IN; Forsyth Memorial Hospital, Winston-Salem, NC; Fountain Valley Regional Hospital, Fountain Valley, CA; Frederick Memorial Hospital, Frederick, MD; Fresno Community Hospital, Fresno, CA; General Hospital Center at Passaic, Passaic, NJ; Georgia Baptist Hospital, Atlanta, GA; Goldston Regional Tumor Registry, Amarillo, TX; Good Samaritan Hospital, San Jose, CA; H. Lee Moffitt Cancer Center, Tampa, FL; Hackensack Medical Center, Hackensack, NJ; Harris Methodist Hospital, Fort Worth, TX; HCA Presbyterian Hospital, Oklahoma City, OK; HealthSouth Medical Center, Richmond, VA; Henrico Doctors' Hospital, Richmond, VA; Hillcrest Medical Center, Tulsa, OK; Hinsdale Hospital, Hinsdale, IL; Hoag Memorial Hospital, Newport Beach, CA; Holy Cross Hospital, Chicago, IL; Holy Cross Hospital, Mission Hills, CA; Holy Name Hospital, Teaneck, NJ; Holy Redeemer Hospital, Meadowbrook, PA; Humana Hospital, Overland Park, KS; Humana Hospital Sunrise, Las Vegas, NV; Humana West Hospital, Anaheim, CA; Immanuel Medical Center, Omaha, NE; Irving Health Care System, Irving, TX; Jersey Shore Medical Center, Neptune, NJ; Johnston-Willis Hospital, Richmond, VA; Jupiter Hospital, Jupiter, FL; Kennestone Hospital, Marietta, GA; Kuakini Medical Center, Honolulu, HI; Lee Memorial Hospital, Ft. Myers, FL; Lewis-Gale Hospital, Salem, VA; Little Company of Mary Hospital, Torrance, CA; Long Beach Memorial Medical Center, Long Beach, CA; Los Robles Regional Medical Center, Thousand Oaks, CA; Lovelace Medical Center, Albuquerque, NM; Maricopa Medical Center, Phoenix, AZ; Martin Luther Hospital, Anaheim, CA; Medical Center Hospital, Tyler, TX; Medical College of Virginia Hospital, Richmond, VA; Memorial Southwest Hospital, Houston, TX; Mercy General Hospital, Sacramento, CA; Methodist Hospital, St. Louis Park, MN; Methodist Hospital, Lubbock, TX; Methodist Medical Center of Illinois, Peoria, IL; Metropolitan Health Medical Center, Cleveland, OH; Mid-Maine Medical Center, Waterville, ME; Mills Memorial Hospital, San Mateo, CA; Mission Hospital Regional Med-

ical Center, Mission Viejo, CA; Morristown Memorial Hospital, Morristown, NJ; Moses H. Cone Memorial Hospital, Greensboro, NC; Mother Francis Hospital, Tyler, TX; Mount Carmel Medical Center, Columbus, OH; Mount Zion Hospital, San Francisco, CA; Norman Regional Hospital, Norman, OK; North Florida Regional Hospital, Gainesville, FL; North Penn Hospital, Lansdale, PA; Northeast Georgia Medical Center, Gainesville, GA; Northside Hospital, Atlanta, GA; O'Connor Hospital, San Jose, CA; Overlook Hospital, Summit, NJ; Palos Community Hospital, Palos Heights, IL; Parkview Community Hospital, Riverside, CA; Peninsula Hospital, Burlingame, CA; Phoenix Memorial Hospital, Phoenix, AZ; Presbyterian Hospital, Charlotte, NC; Presbyterian Intercommunity Hospital, Whittier, CA; Providence Medical Center, Seattle, WA; Providence Memorial Hospital, El Paso, TX; Providence St. Margaret Health Center, Kansas City, KS; Ravenswood Hospital Medical Center, Chicago, IL; Resurrection Hospital, Chicago, IL; Riverside Regional Medical Center, Newport News, VA; Roper Hospital, Charleston, SC; Rose Medical Center, Denver, CO; Saddleback Memorial Medical Center, Laguna Hills, CA; San Jose Medical Center, San Jose, CA; Santa Rosa Memorial Hospital, Santa Rosa, CA; Schumpert Medical Center, Shreveport, LA; Sentara Leigh Memorial Hospital, Norfolk, VA; Sentara Norfolk General Hospital, Norfolk, VA; Sequoia Hospital, Redwood City, CA; Shawnee Mission Medical Center, Shawnee Mission, KS; Sibley Memorial Hospital, Washington, DC; Silver Cross Hospital, Joliet, IL; Southern Baptist Hospital, New Orleans, LA; Sparks Regional Medical Center, Fort Smith, AR; Spartanburg Regional Medical Center, Spartanburg, SC; Spring Branch Memorial Hospital, Houston, TX; St. Agnes Medical Center, Fresno, CA; St. Anthony Hospital, Oklahoma City, OK; St. Bernardine Medical Center, San Bernardino, CA; St. Elizabeth Hospital, Beaumont, TX; St. Francis Hospital, Blue Island, IL; St. Francis Hospital, Tulsa, OK; St. Francis Medical Center, Pittsburgh, PA; St. John Medical Center, Tulsa, OK; St. Joseph Hospital, Reading, PA; St. Joseph Medical Center, Stockton, CA; St. Joseph's Hospital, Fort Worth, TX; St. Joseph's Hospital, Atlanta, GA; St. Joseph's Hospital, Orange, CA; St. Joseph's Hospital, Lorain, OH; St. Jude Medical Center, Fullerton, CA; St. Mary Desert Valley Hospital, Apple Valley, CA; St. Mary Medical Center, Long Beach, CA; St. Mary of Nazareth Hospital Center, Chicago, IL; St. Mary's Hospital, Richmond, VA; St. Vincent's Medical Center, Jacksonville, FL; Stevens Memorial Hospital, Edmonds, WA; Southwest Comprehensive Cancer Center, Palos Heights, IL; The Medical Center of Ocean County, Point Pleasant, NJ; Thomas Jefferson University Hospital, Philadelphia, PA; University Hospitals of Cleveland, Cleveland, OH; Valley Presbyterian Hospital, Van Nuys, CA; Virginia Beach General Hospital, Virginia Beach, VA; Waukesha Memorial Hospital, Waukesha, WI; West Florida Regional Medical Center, Pensacola, FL; West Hudson Hospital, Kearny, NJ; Western Medical Center, Santa Ana, CA; Western Pennsylvania Hospital, Pittsburgh, PA; Whittier Hospital, Whittier, CA.

References

1. McGuire WL, Tandon AK, Allred DC, Chamness GC, Clark GM: How to use prognostic factors in axillary node-negative breast cancer patients. J Natl Cancer Inst 82: 1006–1015, 1990
2. McGuire WL, Dressler LG: Emerging impact of flow cytometry in predicting recurrence and survival in breast cancer patients. J Natl Cancer Inst 75: 405–410, 1985
3. Merkel DE, McGuire WL: Ploidy, proliferative activity and prognosis. DNA flow cytometry of solid tumors. Cancer 65: 1194–1205, 1990
4. Bichel P, Poulsen S, Anderson J: Estrogen receptor content and ploidy in human mammary carcinoma. Cancer 50: 1771–1774, 1982
5. Ewers S-B, Langstrom E, Baldetorp B, Killander D: Flow-cytometric DNA analysis in primary breast carcinomas and clinicopathological correlations. Cytometry 5: 408–419, 1984
6. Moran R, Black M, Alpert L: Correlation of cell-cycle kinetics, hormone receptors, histopathology, and nodal status in human breast cancer. Cancer 54: 1586–1590, 1984
7. McDivitt RW, Stone KR, Cragi RB, Palmer JG, Meyer JS, Bauer WC: A proposed classification of breast cancer based on kinetic information: derived from a comparison of risk factors in 168 primary operable breast cancers. Cancer 57: 269–276, 1986
8. Thorud E, Fossa SD, Vaage S, Kaalhus O, Knudsen OS, Bormer O, Shoaib MC: Primary breast cancer: Flow cytometric DNA pattern in relation to clinical and histopathological characteristics. Cancer 57: 808–811, 1986
9. Kallioniemi O-P, Blanco G, Alavaikko M, Hietanen T, Mattila J, Lauslahti K, Koivula T: Tumour DNA ploidy as an independent prognostic factor in breast cancer. Br J Cancer 56: 637–642, 1987
10. Hedley DW, Rugg CA, Gelber RD: Association of DNA index and S-phase fraction with prognosis of node positive early breast cancer. Cancer Res 47: 4729–4735, 1987
11. Dressler LG, Seamer LC, Owens MA, Clark GM, McGuire WL: DNA flow cytometry and prognostic factors in 1331 frozen breast cancer specimens. Cancer 61: 420–427, 1988
12. Lykkesfeldt AE, Balslev I, Christensen IJ, Larsen JK, Molgaard H, Rasmussen BB, Thorpe S, Rose C: DNA ploidy and S-phase fraction in primary breast carcinomas in relation to prognostic factors and survival for premenopausal patients at high risk for recurrent disease. Acta Oncol 27: 749–756, 1988
13. Muss HB, Kute TE, Case LD, Smith LR, Booher C, Long R, Kammire L, Gregory B, Brockschmidt JK: The relation of flow cytometry to clinical and biological characteristics in women with node negative primary breast cancer. Cancer 64: 1894–1900, 1989
14. Clark GM, Dressler LG, Owens MA, Pounds G, Oldaker T, McGuire WL: Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. N Engl J Med 320: 627–633, 1989
15. Helin HJ, Helle MJ, Kallioniemi OP, Isola JJ: Immunohis-

tochemical determination of estrogen and progesterone receptors in human breast carcinoma. Correlation with histopathology and DNA flow cytometry. Cancer 63: 1761–1767, 1989

16. Christov K, Miley A, Todorov V: DNA aneuploidy and cell proliferation in breast tumors. Cancer 65: 673–679, 1989
17. Kallioniemi O-P, Blanco G, Alavaikko M, Hietanen T, Mattila J, Lauslahti K, Lehtinen M, Koivula T: 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 62: 2183–2190, 1988
18. Winchester DJ, Duda RB, August CZ, Goldschmidt RA, Wruck DM, Rademaker AW, Winchester DP, Merkel DE: The importance of DNA flow cytometry in node-negative breast cancer. Arch Surg 125: 886–889, 1990
19. Visscher DW, Zarbo RJ, Jacobsen G, Kambouris A, Talpos G, Sakr W: Multiparametric deoxyribonucleic acid and cell cycle analysis of breast carcinomas by flow cytometry. Clinicopathologic correlations. Lab Invest 62: 370–378, 1990
20. Keyhani-Rofagha S, O'Toole RV, Farrar WB, Sickle-Santanello B, DeCenzo J, Young D: Is DNA ploidy an independent prognostic indicator in infiltrative node-negative breast adenocarcinoma? Cancer 65: 1577–1582, 1990
21. Kute TE, Muss HB, Cooper MR, Case LD, Buss D, Stanley V, Gregory B, Galleshaw J, Bocher K: The use of flow cytometry for the prognosis of stage II adjuvant treated breast cancer patients. Cancer 66: 1810–1816, 1990
22. Vielh P, Chevillard S, Mosseri V, Donatini B, Magdelenat H: Ki67 index and S-phase fraction in human breast carcinomas. Comparison and correlations with prognostic factors. Am J Clin Path 94: 681–686, 1990
23. Joensuu H, Toikkanen S, Klemi PJ: DNA index and S-phase fraction and their combination as prognostic factors in operable ductal breast carcinoma. Cancer 66: 331–340, 1990
24. Lewis WE: Prognostic significance of flow cytometric DNA analysis in node-negative breast cancer patients. Cancer 65: 2315–2320, 1990
25. Sigurdsson H, Baldetorp B, Borg A, Dalberg M, Ferno M, Killander D, Olsson H: Indicators of prognosis in node-negative breast cancer. N Engl J Med 322: 1045–1053, 1990
26. Cornelisse CJ, van de Velde CJH, Caspers RJC, Moolenaar AJ, Hermans J: DNA ploidy and survival in breast cancer patients. Cytometry 8: 225–234, 1987
27. Dressler LG, Seamer L, Owens MA, Clark GM, McGuire WL: Evaluation of a modeling system for S-phase estimation in breast cancer by flow cytometry. Cancer Res 47: 5294–5302, 1987
28. Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Malley BW, McGuire WL: Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 51: 105–109, 1991
29. Olsson H, Ranstam J, Baldetorp B, Ewers S-B, Ferno M, Killander D, Sigurdsson H: Proliferation and DNA ploidy in malignant breast tumors in relation to early oral contraceptive use and early abortions. Cancer 67: 1285–1290, 1991
30. Horsfall DJ, Tilley MD, Orell SR, Marshall VE, McKant EL: Relationship between ploidy and steroid hormone receptors in primary invasive breast cancer. Br J Cancer 53: 23–28, 1986
31. Uyterlinde AM, Schipper NW, Baak JP: Comparison of extent of disease and morphometric and DNA flow cytometric prognostic factors in invasive ductal breast cancer. J Clin Pathol 40: 1432–1436, 1987