Flow Cytometric DNA Analysis of Invasive Carcinomas Detected by Screening Mammography: Use of Specimen Mammography-guided Fine-Needle Aspirates

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ABSTRACT
Clinical studies of flow cytometric DNA analysis of breast carcinoma are often limited by the lack of fresh tissue samples from smaller, nonpalpable carcinomas. In addition, most studies measuring DNA in the current literature focus on larger palpable masses that may have less relevance to the smaller, nonpalpable lesions. A prospective study of flow cytometric DNA analysis of in vitro specimen mammography-guided fine-needle aspirates (FNAs) of 103 consecutive nonpalpable invasive carcinomas detected by screening mammography was performed to determine efficacy and explore associations with mammographic and pathological features. For 62 (60%) lesions for which DNA analysis on both FNA and standard tissue incision samples was performed, there was excellent (89%) agreement for ploidy determinations (κ = 0.77) and poor agreement for S-phase percentage determinations (κ = 0.23). Specimen mammography-guided FNA analysis detected aneuploidy in 36% of lesions overall, including 34% of 41 lesions for which standard tissue procurement was not possible. Mammographic microcalcifications had a higher aneuploid rate (14 of 28 lesions, 50%) as compared with soft tissue masses (22 of 75 lesions, 29%), P < 0.01. Lobulated masses with indistinct margins had a higher aneuploid rate (5 of 6 lesions, 83%) as compared with more irregular, spiculated masses (7 of 27 lesions, 26%), P < 0.01. The aneuploidy rate was independent of specific histological diagnosis, lesion size, nuclear grade, or nodal or estrogen receptor status. Flow cytometric DNA analysis of mammographic lesion-specific, fresh, cellular FNA samples obtained under specimen mammographic guidance can assess early invasive carcinomas when gross fresh tissue procurement is not possible. This technique could be incorporated into larger clinical follow-up studies to determine the prognostic significance of flow cytometric DNA analysis for these very early breast carcinomas.

INTRODUCTION
The frequency of early invasive breast carcinomas has increased with the widespread utilization of screening mammography. Recent therapeutic trends toward adjuvant systemic therapy in node-negative breast cancer patients have increased the need for better prognostic assessment for these early lesions. DNA ploidy and S-phase percentage are among the prognostic markers that are being evaluated for invasive breast carcinomas. Prior clinical studies suggest that S-phase percentage has greater prognostic significance than does aneuploidy (1–19). However, clinical studies of flow cytometric DNA analysis are often limited by the lack of fresh tissue samples from smaller invasive carcinomas detected by screening mammography, resulting in a lack of understanding of the prognostic significance of this test in an increasingly relevant group of patients.

We have been performing flow cytometric DNA analysis on specimen mammography-guided FNAs2 of lesions within excised breast specimens after wire localization and surgical excision of clinically occult lesions detected by mammography (20, 21). To our knowledge, there is little information in the literature regarding flow cytometric DNA analysis of clinically occult invasive breast carcinomas using this technique of procurement of mammographic lesion-specific fresh cell samples. There are also little published data regarding mammographic and pathological associations with flow cytometric DNA analysis features of screening-detected nonpalpable invasive breast carcinomas.

This prospective pilot study was undertaken to determine the DNA ploidy and S-phase percentage of specimen mammography-guided FNAs of screening mammography-detected invasive carcinomas by flow cytometry and to explore associations between mammographic and pathological features with ploidy and S-phase percentages.

MATERIALS AND METHODS
The study group consists of 103 consecutive patients who underwent biopsies of clinically occult lesions detected by screening mammography that showed invasive breast carcinoma...
at pathological exam. The median age of the patients in the study group was 62 years (range, 32–88 years). Twenty-three (22%) of the patients were less than 50 years old; 80 (78%) of the patients were 50 years of age or older. All patients had negative clinical breast examinations performed by breast surgeons.

Mammograms were obtained with a Senographe 600 T or DMR unit (General Electric Medical Systems, Milwaukee, WI) using Min-R cassettes and Min-R film (Eastman Kodak, Rochester, NY) at a cancer institute mammmography center. Each of the calcified lesions was imaged in vivo with accessory magnification projections. Mammographically guided needle localization procedures were performed with the method described by Kopans et al. (22). Radiography of the specimen was performed with the compression and magnification technique (×1.85) in each case with the clinical DMR mammography unit.

The surgical staff was notified immediately of the confirmation of the excision of lesions. Subsequently, after the removal of compression, the mammographer performed a 20-gauge needle aspiration within the suspect mammographic lesion in the operative specimen. Needle guidance techniques based on the radiography of the specimen included: (a) use of circumferential coordinates or surface landmarks on the specimen for larger lesions; and (b) insertion of a localizing needle and repeat radiography of the uncompressed specimen for smaller lesions. Fine-needle aspiration was performed throughout the mammographically depicted lesion to provide adequate lesion sampling (20). The needle aspirate was then sent for flow cytometric DNA analysis.

Excisional biopsy specimens were sent to the cancer institute department of pathology. If a palpable mass was present within the operative specimen, the pathologist obtained a gross sample of tissue from the mass by incisional biopsy and submitted the tissue sample for flow cytometric analysis.

The needle aspirate sent for flow cytometric DNA analysis was dispersed by means of repeated aspiration through a 25-gauge needle and then fixed in 70% ethanol and stored at −20°C. For analysis, cells were centrifuged, and the pellet was stained with propidium iodide (50 μg/ml) in Kristen buffer [0.1% sodium citrate, 0.02 ms/ml RNase A, and 0.37% NP40 (pH = 7.4)]. Samples of lysed whole blood from a healthy donor and chick RBCs processed in identical fashion were also stained. Cells (10,000 total) were analyzed on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) with a doublet discriminator module and an excitation beam at 488 nm/liter. Data were collected for the fluorescence 2 channel measuring propidium iodide emission through a 585/42 band pass filter. Lysis II software was used only to acquire the data using its pulse processing capability to reduce aggregates, no data analysis was performed using this program. Chick RBCs were added to the samples as an internal standard. The photomultiplier tube voltage for the fluorescence 2 channel was set so that the cells in the healthy donor blood peaked at channel 200. A threshold of 28 was set on this channel to eliminate cellular debris. All samples were collected ungated.

The histogram files were analyzed with Multicycle (Phoenix Flo Systems, San Diego, CA). This program calculated the percentage of cells in the G0-G1, S, and G2 phases for each cycling cell population present as well as the DNA index of the standard (chick RBCs) and any aneuploid peak present compared with that of the diploid peak. The DNA index is the ratio of the peak G1 channel for either the standard or the sample cells/to that of diploid cells. The DNA index for the standard is a measure of the quality of the sample staining. The DNA index for the sample is a measure of the degree of aneuploidy in the abnormal population. Coauthor C. C. S. performed all flow cytometry and interpreted results without knowledge of mammographic and pathological findings.

Cell populations with a DNA index of 1.0 ± 0.1 (SD) were considered diploid. All others were considered to have aneuploidy present. All needle aspirates, including those that had aneuploid populations, had some diploid cells present. When aneuploid cells were present, the aneuploid S-phase percentage was used for S-phase percentage analysis rather than the diploid cell population S-phase value. Cases were then designated as having either a low, intermediate, or high S-phase percentage based on their placement within the lowest, middle, and highest thirds determined for the study group.

Histological material derived from excisional biopsy was stained with H&E. All pathological interpretations were performed prospectively by the cancer institute department of pathology staff without knowledge of flow cytometric findings. Histological assessment of the entire biopsy specimen was made with knowledge of the presence and location of the mammographic abnormality within the specimen so that specific anatomical correlation could be made in each case. For calcified lesions, the specimen was sliced and reradiographed to aid localization during pathological examination.

For pathological features, histological diagnosis, invasive tumor size, nuclear grade, and axillary node status were recorded and categorized. The pathological diagnoses were categorized as invasive ductal carcinoma, invasive ductal carcinoma with greater or less than 90% DCIS, and invasive lobular carcinoma. Pathological invasive tumor sizes were categorized as 1–10, 11–20, or >20 mm. Nuclear grade I or II invasive carcinomas were combined for the purpose of this analysis. Estrogen and progesterone receptor assays for breast carcinoma were performed with immunohistochemical techniques. For the immunohistochemical assay, 20% or more nuclear staining was considered positive.

Lesions were prospectively categorized by mammographic appearance and size before biopsy. Lesions were categorized as calcifications only or soft tissue masses (with or without calcifications). Mammographic lesions were also subcategorized for appearance by shape and margin. Mammographic calcifications were categorized as predominantly linear or predominantly granular. Soft tissue lesions were categorized as spiculated, irregular/poorly defined, indistinct lobulated, or indistinct round/oval masses or as areas of architectural distortion. The features are based on prior mammographic pathological correlation studies (23, 24). Mammographic measurement of the greatest dimension of each lesion was categorized as 1–10, 11–20, or >20 mm. For spiculated masses, the greatest dimension of the central mass not including the extent of spiculations was used.

Statistical analyses were performed with χ2 analysis, κ tests, and Wilcoxon signed-rank tests (25). Ps < 0.05 were considered significant.
Table 1  Flow cytometric ploidy agreement in 62 invasive cases where both FNA and tissue procurement were performed

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<th>Diploid</th>
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<tr>
<td>Tissue</td>
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* Agreement rate = 89%; $\kappa = 0.77$ (95% confidence interval, 0.61–0.93); $P < 0.0001$.

### RESULTS

For 62 of the 103 (60%) excisional biopsy specimens in the study, a gross tissue sample was procured from the lesion by palpation-guided incision in addition to the specimen mammography-guided fine-needle aspiration. These were performed independently of each other by the mammographer and pathologist. The agreement rate for ploidy between the two procurement techniques was 89% (Table 1). Using the $\kappa$ test, a $\kappa$ value of 0.77 (95% confidence interval, 0.61–0.93) and a $P$ of $<0.0001$ were derived for the ploidy agreement rate between flow cytometric analysis of gross tissue samples when available and the FNAs. It is significant that in 41 of our 103 (40%) invasive carcinomas, no gross tissue could be procured. Only specimen mammography-guided fine-needle aspiration could be performed, yielding an aneuploid rate of 34%. The evaluation of S-phase percentage was also performed for all 62 specimens for which both gross tissue and FNA were available. The agreement between flow cytometric analysis of gross tissue samples and FNAs for S-phase percentage was not significant ($\kappa = 0.23$; Table 2). Overall, 51% of the specimens were in different percentile groups.

The remainder of the analyses in this study are based on the FNA flow cytometric data only. For the 103 screening mammography-detected invasive carcinomas in this study, aneuploidy was present in 36% (37 of 103) of the FNAs. The median S-phase percentage was 4.3% (range, 0–46.7%). The lower 33rd percentile was 2.7%; the higher 33rd percentile was 6.5%.

**Associations with Mammographic and Pathological Features.** Mammographic and pathological features with significant associations with ploidy in S-phase percentage are shown in Table 3. For mammographic features, invasive carcinomas that manifested themselves as microcalcifications had a significantly higher rate of aneuploidy (14 of 28, 50%) as compared with soft tissue abnormalities (23 of 75, 31%), $P < 0.01$. Among mammographic soft tissue subtypes, lobulated masses with indistinct margins had a greater association with aneuploidy (5 of 6, 83%) as compared with either stellate masses ([7 of 27, 26%] $P < 0.01$) or round or oval masses with indistinct margins [2 of 15, 13%; $P < 0.01$]. Lobulated masses with indistinct margins or lesions manifested by architectural distortion were also less likely to have low S-phase percentages [1 of 16 (17%) and 2 of 12 (17%), respectively] as compared with round or oval masses with indistinct margins (10 of 15, 67%), $P < 0.01$.

For pathological features, nuclear grade was assessed for 96 of the 103 (93%) invasive carcinomas. Of the seven (7%) cases not graded, six were pure invasive lobular carcinomas, and one was a predominately invasive lobular carcinoma for which nuclear grade was not assessed. Nuclear grade III carcinomas were more likely associated with aneuploidy (20 of 38, 53%) as compared with nuclear grade I or II carcinomas (14 of 48, 29%), $P < 0.01$. Nuclear grade III carcinomas were more likely associated with high S-phase percentage (18 of 38, 47%) as compared with nuclear grade I or II carcinomas (11 or 48, 23%), $P < 0.05$. Nuclear grade III carcinomas were also less likely to be associated with low S-phase percentage (7 of 38, 18%) as compared with nuclear grade I or II carcinomas (19 of 48, 40%), $P < 0.05$. Progesterone receptor-negative carcinomas were more likely to be associated with high S-phase percentage (15 of 30, 50%) as compared with progesterone receptor-positive carcinomas (12 of 55, 22%), $P < 0.05$.

Mammographic and pathological features without significant associations with DNA ploidy or S-phase percentage are shown in Table 4. There were no significant associations between DNA ploidy or S-phase percentage and patient age, the pathological diagnoses of invasive ductal carcinoma, mixed invasive ductal carcinoma and DCIS, or invasive lobular carcinoma or estrogen receptor status. There were also no significant associations between DNA ploidy and S-phase percentage and either mammographic or pathological lesion sizes.

### DISCUSSION

Flow cytometric analysis of specimen mammography-guided FNAs of nonpalpable invasive carcinomas detected by screening mammography in this study showed an aneuploidy rate of 36%. This is in the lower range of aneuploidy rates reported for invasive carcinomas of the breast (1, 17). Most prior studies, some of which required gross tissue samples for analysis, included or predominately consisted of larger clinically palpable carcinomas or tumors that were palpable within the excised pathological specimen. Also, DNA analysis methodology and interpretive criteria vary between studies. Whereas aneuploidy has not been shown to have major prognostic value for invasive carcinomas to date, it may have prognostic value for the earlier invasive carcinomas detected by screening mammography. Further clinical evaluation of this DNA analysis technique is necessary to determine the prognostic significance of the findings from this pilot study.

For these screening-detected invasive carcinomas, lesions with the mammographic feature of microcalcifications only showed significantly greater aneuploidy as compared with soft tissue lesions. Among soft tissue abnormalities, indistinct lobulated masses with indistinct margins showed significantly
greater aneuploidy and less low S-phase percentages as compared with other soft tissue lesions, including spiculated masses. The pathological feature of high nuclear grade was also significantly associated with higher S-phase percentages. No significant associations were seen between DNA analysis and patient age, tumor size, specific histological diagnosis (i.e., lobular versus ductal carcinoma, associated DCIS), and axillary node or estrogen receptor status. Due to the small numbers in this pilot study, these findings are not conclusive.

For these clinically occult carcinomas, the specimen mammography-guided fine-needle aspiration technique enabled DNA analysis in 40% of lesions for which standard incisional biopsy tissue procurement could not be performed due to the lack of palpable tumor within the excised specimen. For the 60% of lesions for which both fine-needle aspiration and incisional biopsy tissue were analyzed, the agreement in ploidy results was excellent (89%). The level of agreement is especially significant due to the fact that each method of procurement was done blinded and independent of each other. Martelli et al. (26) showed an 82% agreement rate (82 of 100) for the detection of aneuploidy in a study comparing flow cytometric analysis of in vivo FNAs of larger clinically palpable masses and subsequent gross palpable tissue incision of the surgical biopsy specimens. In our study, four of the six cases in which aneuploidy was detected by gross tissue procurement and not detected in the FNAs were lesions manifested by larger mammographic abnormalities (greater than 2–3 cm) within which a smaller palpable invasive tumor existed. Because the specimen mammography-guided FNAs were obtained with imaging guidance only without palpation, it is possible that DCIS adjacent to the palpable invasive carcinomas was aspirated. Conversely, palpation alone does not insure adequate sampling of the invasive carcinomas as seen in the case of aneuploidy detected in the FNA but not in the tissue sample. A combined imaging and palpation-guided FNA technique would seem to offer the optimal comparison to gross fresh tissue procurement.

Because the optimal stratification of lesions into different groups by S-phase percentage has not been determined, we used three groups instead of two groups to decrease the chance of misclassifying tumors with borderline values. The lack of significant correlation between the S-phase percentages of FNAs and gross tissue samples is probably related to the variable mixtures of tumor cells, benign epithelium, and stromal cells within different samples. Whether specimen mammography-guided fine-needle aspiration provides a more accurate assessment of the S-phase percentage of early invasive carcinomas than gross tissue incision, when possible, is yet to be determined. We know of no published data comparing S-phase percentages determined on separate gross incision tissue samples from the same lesions. We are now performing combined staining with fluorescein-labeled anticytokeratin antibodies that allows the DNA content of epithelial cells to be separated from that of other elements. We are also studying multiple marker immunophenotyping with flow cytometry. Although there are no published data showing the benefit of the combined staining technique, we feel that this will further improve the prognostic significance of the S-phase percentage in breast carcinoma. Investigators who have reviewed the clinical utility of flow cytometric DNA analysis in breast carcinoma note that because S-phase percentage is a continuous rather than a dichotomous variable, each laboratory must validate the prognostic significance of its own S-phase values (1). We concur that the prognostic significance of S-phase values obtained by this technique of cell procurement and flow cytometric DNA analysis, like other methods of procurement and analysis for S-phase determinations, must be validated by larger clinical follow-up studies. The lack of association between DNA ploidy or S-phase percentage and size among the screening-detected invasive carcinomas in this study suggests that these DNA features of early breast carcinomas are established at a premammographic phase and do not change within the screening mammography spectrum.
DNA analysis of \textit{in vivo} stereotactic FNA and \textit{in vitro} FNA of frozen tissue samples of nonpalpable breast carcinomas has been performed using microspectrophotometry (27, 28). DNA flow cytometry has been performed on paraffin-embedded tissue and fresh tissue samples, needle aspirates, or scrapings from larger tumors that were palpable at clinical or pathological exam (5, 18, 20, 29–35). We and other investigators prefer fresh tissue, rather than paraffin-embedded tissue, because the fixation process may render the analysis less accurate (36–38).

Aside from the advantages of providing mammographic lesion-specific fresh cell samples for clinically occult lesions, the specimen mammography-guided fine-needle aspiration technique offers several advantages: (a) it is \textit{in vitro} and does not directly involve the patient; (b) no significant histological artifacts have been reported. This is partially attributable to the lack of bleeding with the \textit{in vitro} technique; (c) it takes less than 5 min of the mammographer’s time during the routine and mandatory performance of specimen radiography after localization and excision of clinically occult lesions; and (d) for cost-effectiveness in both clinical practice and research, needle aspiration samples could be stored until a final histological diagnosis of the lesion is made and analyzed only if a selected diagnosis of malignancy or specifically invasive carcinoma is made. We have reported a prior study of the specimen mammography-guided FNA technique in which a second needle aspirate of each lesion was sent for cytological examination (21). If cytological evaluation is also desired, alternatively, a second needle aspirate could be obtained, and a portion of the pooled cells could be submitted for cytological evaluation.

The observation that early invasive carcinomas manifested by mammographic lobulated masses with indistinct margins have a significantly greater association with aneuploidy and higher S-phase percentage than spiculated masses should increase caution with regard to these lesions by mammographers. Whereas lesser circumscription of tumors has been considered a sign of tumor aggressiveness, this may not always be the case. Spiculations, associated with greater predictive value for the presence of malignancy, pathologically represent predominately fibrosis. The lack of fibrinous spiculations in indistinct lobulated masses may reflect the aggressive growth of the tumor itself, precluding the opportunity for development of large fibrinous spiculations. Whereas larger numbers of lesions must be studied to validate this finding, careful margin analysis of the more circumscribed mammographic masses and prompt biopsy of solid masses with indistinct margins are further supported by this data.

On the basis of this pilot study data, we conclude that analysis of specimen mammography-guided FNAs can be used in prognostic marker evaluations such as flow cytometry DNA analysis for early invasive carcinomas detected by screening mammography in which gross tissue procurement and fresh tissue analysis is often not otherwise possible. The correlations

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Feature} & \textbf{\(n\) (%)} & \textbf{Aneuploidy (%)} & \textbf{\(P\)} & \textbf{Low S-phase % no. (%)} & \textbf{\(P\)} & \textbf{High S-phase % no. (%)} & \textbf{\(P\)} \\
\hline
\textbf{Mammographic soft tissue extent (mm\textsuperscript{a})} & & & & & & & \\
1–10 & 38 (57) & 13 (34) & & 18 (47) & & 10 (36) & \\
11–20 & 20 (30) & 5 (25) & & 3 (15) & & 6 (30) & NS \\
\geq 21 & 9 (13) & 4 (44) & & 4 (44) & & 2 (22) & \\
\textbf{Mammographic calcification extent (mm)} & & & & & & & \\
1–10 & 10 (36) & 5 (50) & & 3 (30) & & 5 (50) & \\
11–20 & 7 (25) & 4 (57) & & 1 (14) & & 5 (71) & NS \\
\geq 21 & 11 (39) & 5 (45) & & 2 (18) & & 5 (45) & \\
\textbf{Pathological invasive tumor size (mm\textsuperscript{b})} & & & & & & & \\
1–10 & 59 (50) & 23 (39) & & 23 (39) & & 20 (34) & \\
11–20 & 30 (30) & 11 (37) & NS & 9 (30) & NS & 12 (40) & NS \\
\geq 21 & 11 (11) & 3 (27) & NS & 1 (9) & & 1 (9) & \\
\textbf{Patient age (yr)} & & & & & & & \\
<50 & 23 (22) & 11 (48) & & 4 (17) & & 11 (48) & \\
\geq 50 & 80 (78) & 26 (33) & NS & 30 (38) & NS & 23 (29) & NS \\
\textbf{Histological diagnosis} & & & & & & & \\
Invasive ductal & 46 (45) & 16 (35) & NS & 16 (35) & NS & 13 (28) & NS \\
Invasive ductal, <90\% DCIS & 39 (38) & 16 (41) & & 11 (28) & & 14 (36) & \\
Invasive ductal, >90\% DCIS & 11 (11) & 4 (36) & & 3 (27) & & 6 (55) & \\
Invasive lobular & 7 (7) & 1 (14) & & 4 (57) & & 1 (14) & \\
\textbf{Axillary nodes\textsuperscript{c}} & & & & & & & \\
Positive & 21 (33) & 9 (42) & NS & 5 (24) & NS & 9 (43) & NS \\
Negative & 43 (67) & 12 (28) & & 16 (37) & & 10 (23) & \\
\textbf{Estrogen receptor\textsuperscript{d}} & & & & & & & \\
Positive & 63 (74) & 19 (30) & NS & 21 (33) & NS & 19 (30) & NS \\
Negative & 22 (26) & 12 (55) & & 6 (27) & & 11 (50) & \\
\hline
\end{tabular}
\caption{Mammographic and pathological features without significant associations with ploidy or S-phase percentage for invasive carcinomas detected by screening mammography}
\end{table}

\textsuperscript{a} For stellate masses, the central mass diameter was used.

\textsuperscript{b} NS, not significant.

\textsuperscript{c} Pathological tumor size was difficult to assess for three lesions.

\textsuperscript{d} Values shown are for 64 patients who underwent axillary node dissection.

\textsuperscript{e} Receptor assays were not performed on 18 lesions.
between DNA analysis and mammographic features increase our understanding of the mammographic features of early invasive carcinomas. We feel that this technique of mammographic lesion-specific fresh cell procurement is easy to perform, and that the flow cytometric DNA analysis data obtained can stratify early invasive breast carcinomas and could be incorporated into larger clinical trials to ultimately determine the prognostic significance of the findings.

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Flow cytometric DNA analysis of invasive carcinomas detected by screening mammography: use of specimen mammography-guided fine-needle aspirates.


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