Flow Cytometric DNA Analyses of Benign Breast Lesions Detected by Screening Mammography

Paul C. Stomper,1 James R. DeBloom II, Rose Marie Budnick, and Carleton C. Stewart
Division of Diagnostic Imaging [P. C. S.] and Department of Flow Cytometry [R. M. B., C. C. S.], Roswell Park Cancer Institute, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, and Department of Natural Sciences, Roswell Park Division of the State University of New York at Buffalo [J. R. D.], Buffalo, New York 14263

ABSTRACT
There is little information regarding flow cytometric DNA analyses of benign breast lesions. This prospective study consists of mammographic and pathological correlation of DNA flow cytometric analyses of specimen mammography-guided fine-needle aspirates (FNAs) of 189 consecutive benign breast lesions and 114 FNAs of adjacent normal tissue as a control. Clinical follow-up was also performed. Aneuploidy was detected in 14 of 189 (7%) benign lesion specimen mammography-guided FNAs and in only 1 of 114 (0.9%) FNAs of adjacent normal tissue (P = 0.01). Aneuploidy was detected in two (33%) benign intramammary lymph nodes compared with four (12%) benign lesions with atypia, one benign lesion (3%) with hyperplasia, four benign lesions (10%) with adenosis, and three (4%) other benign lesions (P = 0.01). There were no significant associations between DNA content and S-phase percentage and patient age, mammographic appearance, or extent. During a median follow-up of 40 months (range, 6-84 months), 2 of 13 (15%) patients with aneuploid benign lesions developed ipsilateral breast carcinoma compared with 5 of 175 (3%) patients with diploid benign lesions (odds ratio, 6.18; 95% confidence interval, 1.08-35.56). Our data suggest that aneuploidy, which is detected in a variety of benign breast lesions, may be associated with a higher risk of development of breast carcinoma. The combined techniques of specimen mammography-guided fine-needle aspiration and flow cytometry provide a practical translational research method for the study of benign breast disease.

INTRODUCTION

The increased utilization of screening mammography has led to increased numbers of either excisional or percutaneous core needle biopsies of clinically occult lesions that subsequently prove to be benign. Whereas efforts should continue to be made to decrease the number of false positive mammograms and benign biopsies, the study of this biopsy material may lead not only to further understanding of benign and premalignant breast disease but also to a possible means of risk assessment for the subsequent short- or long-term development of breast carcinoma for women who undergo benign breast biopsies.

There is little information in the literature regarding ploidy and S-phase percentage analysis in benign breast lesions (1-6). There is extensive literature regarding flow cytometric DNA analysis of invasive breast carcinoma, and there are several reports of DNA analysis of ductal carcinoma in situ (5, 7-14). We have previously reported the use of specimen mammography-guided fine-needle aspiration of clinically occult lesions within excised specimens for DNA analysis by flow cytometry (15, 16).

This report analyzes a prospective pilot study that was performed: (a) to determine the feasibility of flow cytometric DNA analysis of specimen mammography-guided FNAs of clinically occult lesions detected during screening mammography that proved to be benign after wire localization and excision; (b) to explore associations with mammographic and pathological features of the benign lesions; (c) to explore the frequency of aneuploidy in benign lesions; and (d) to assess any subsequent short-term risk of development of breast carcinoma.

MATERIALS AND METHODS

The study group consists of 188 consecutive patients who underwent biopsies of 189 clinically occult lesions detected by screening mammography that showed benign pathology at pathological examination. The median age of the patients in the study group was 62 years (range, 31-85 years). Eighty-four (44%) of the patients were less than 50 years old; 105 (56%) were 50 years or older. Written informed consent was obtained.

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 mammography center. Each of the calcified lesions was imaged in vivo with accessory magnification projections. Mammographically guided needle localization procedures were performed using the method described by Kopans et al. (17). Radiography of the specimen was performed using the compression and magnification technique (x1.85) in each case with the clinical DMR mammography unit.

The surgical staff was notified immediately of the confirmation of the excision of lesions. After the removal of compression, the mammographer performed a 20-gauge needle as-

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1 To whom requests for reprints should be addressed, at Division of Diagnostic Imaging, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-5796; Fax: (716) 845-8759; E-mail: stomper@sc3102.med.buffalo.edu.

The abbreviations used are: FNA, fine-needle aspirate; LCIS, lobular carcinoma in situ.
piration within the suspect mammographic lesion in the operative specimen (16). Needle guidance techniques based on the radiography of the specimen included: (a) the use of circumferential coordinates or surface landmarks on the specimen for larger lesions; and (b) the 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. The needle aspirate was then sent for flow cytometric DNA analysis. Excisional biopsy specimens were sent to the cancer institute department of pathology.

For 114 consecutive benign lesions, a second needle was used to aspirate adjacent normal breast tissue at least 2 cm from the index lesion within the excised specimen under mammographic guidance. These samples also underwent flow cytometric DNA analysis.

The needle aspirates sent for flow cytometric DNA analysis were dispersed by means of repeated aspiration through a 25-gauge needle and then fixed in 70% ethanol and stored at −20°C. For analysis, the cells were centrifuged, and the pellet was stained with propidium iodide (50 μg/ml) in Kristen buffer (0.1% sodium citrate, 0.02 mg/ml RNase A, and 0.37% NP40 (pH 7.4)). Samples of lysed whole blood from a healthy donor were used to aspirate adjacent normal breast tissue at least 2 cm from the index lesion. The cytometer. A minimum of 1000 cells from the needle aspirates were analyzed on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) with a double t discriminator module and an excitation beam at 488 nm. Data were collected for the fluorescence 2 channel, measuring propidium iodide emission through a 585/42 band-pass filter. 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 healthy donor blood peaked at channel 200. A threshold of 28 was set on this channel to eliminate cellular debris but include RBCs. All samples were collected ungated.

Data were first analyzed with the Lysis II program (Becton Dickinson) to exclude doublets and debris. The resulting histogram files were further analyzed with Multicycle (Phoenix Flow 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 of either the standard or the aneuploid cells: the peak G1 channel of diploid cells. The BAD index and coefficient of variation for the sample are a measure of the quality of the sample staining. All specimens exhibited a BAD of less than 25% and a coefficient of variation of less than 6%. The DNA index for the sample is a measure of the degree of aneuploidy in the abnormal population. Without knowledge of mammographic and pathological findings, coauthor C. C. S. performed all flow cytometry and interpreted the results.

Cell populations with a DNA index of 1.0 ± 0.1 (2 SDs) 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 33% percentiles determined for the study group.

Histological material derived from excisional biopsy was stained with H&E stain. All pathological interpretations were performed prospectively by the cancer institute department of pathology staff without knowledge of the flow cytometric findings. Retrospective pathological review of benign lesions in patients who subsequently developed ipsilateral breast carcinoma was also performed. There was no evidence of malignancy in the benign excisional biopsies during each pathological review. Histological assessment of the entire biopsy specimen was made with the 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.

Benign lesions were grouped into five pathological categories: (a) atypical hyperplasia/LCIS; (b) hyperplasia; (c) adenosis; (d) intramammary lymph nodes; and (e) other benign lesions. These pathological diagnosis categories were then combined as proliferative (categories a–c) and nonproliferative (categories d and e). Prospective mammographic interpretations were categorized by appearance: (a) soft tissue mass content or with calcifications; or (b) microcalcifications only. Mammographic lesion extent was categorized as 1–10, 11–20, and >20 mm.

Clinical follow-up to determine the subsequent development of breast carcinoma after the initial benign biopsies was performed by mammographic and clinical chart review. Statistical analyses were performed with χ2 and odds ratio analysis (18). P < 0.05 were considered significant. The odds ratio was defined as the relative risk of carcinoma in patients with aneuploid benign lesions relative to that in patients with diploid benign lesions and was considered significant if the lower limit of the 95% confidence interval was greater than 1.
Table 3  Associations between clinical mammographic features and flow cytometric DNA analyses of benign lesions

<table>
<thead>
<tr>
<th>DNA analyses</th>
<th>n</th>
<th>Aneuploid</th>
<th>Diploid</th>
<th>Low S-phase %</th>
<th>High S-phase %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age (yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤49</td>
<td>84 (100)</td>
<td>8 (10)</td>
<td>76 (90)</td>
<td>30 (36)</td>
<td>23 (27)</td>
</tr>
<tr>
<td>≥50</td>
<td>105 (100)</td>
<td>6 (6)</td>
<td>99 (94)</td>
<td>34 (32)</td>
<td>38 (36)</td>
</tr>
<tr>
<td>Mammographic appearance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcification only</td>
<td>91 (100)</td>
<td>8 (9)</td>
<td>83 (91)</td>
<td>32 (35)</td>
<td>31 (34)</td>
</tr>
<tr>
<td>Soft tissue mass</td>
<td>98 (100)</td>
<td>6 (6)</td>
<td>92 (94)</td>
<td>32 (33)</td>
<td>31 (32)</td>
</tr>
<tr>
<td>Mammographic soft tissue only lesion extent (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–10</td>
<td>16 (100)</td>
<td>1 (6)</td>
<td>15 (94)</td>
<td>3 (19)</td>
<td>5 (31)</td>
</tr>
<tr>
<td>1–20</td>
<td>47 (100)</td>
<td>2 (4)</td>
<td>45 (96)</td>
<td>16 (34)</td>
<td>14 (30)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>19 (100)</td>
<td>0 (0)</td>
<td>19 (100)</td>
<td>8 (42)</td>
<td>7 (37)</td>
</tr>
<tr>
<td>Mammographic calcification lesion extent (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–10</td>
<td>56 (100)</td>
<td>4 (7)</td>
<td>52 (93)</td>
<td>18 (32)</td>
<td>19 (34)</td>
</tr>
<tr>
<td>1–20</td>
<td>15 (100)</td>
<td>2 (13)</td>
<td>13 (87)</td>
<td>6 (40)</td>
<td>5 (33)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>20 (100)</td>
<td>2 (10)</td>
<td>18 (90)</td>
<td>8 (40)</td>
<td>7 (35)</td>
</tr>
</tbody>
</table>

* No significant associations were present.
* This includes 15 soft tissue masses with calcifications.

Table 4  Associations between histological diagnoses of benign mammographic lesions and flow cytometric DNA analyses

<table>
<thead>
<tr>
<th>DNA analyses</th>
<th>n</th>
<th>Aneuploid</th>
<th>Diploid</th>
<th>Low S-phase %</th>
<th>High S-phase %</th>
</tr>
</thead>
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<tr>
<td>Histological diagnoses</td>
<td></td>
<td></td>
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<tr>
<td>Atypia/LCIS</td>
<td>34 (100)</td>
<td>4 (12)</td>
<td>30 (88)</td>
<td>12 (35)</td>
<td>13 (38)</td>
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<td>Hyperplasia</td>
<td>31 (100)</td>
<td>1 (3)</td>
<td>30 (97)</td>
<td>11 (35)</td>
<td>7 (23)</td>
</tr>
<tr>
<td>Adenosis</td>
<td>41 (100)</td>
<td>4 (10)</td>
<td>37 (90)</td>
<td>12 (29)</td>
<td>19 (46)</td>
</tr>
<tr>
<td>Intrammary lymph nodes</td>
<td>6 (100)</td>
<td>2 (33)*</td>
<td>4 (67)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other benign lesions</td>
<td>77 (100)</td>
<td>3 (4)</td>
<td>74 (96)</td>
<td>29 (38)</td>
<td>23 (30)</td>
</tr>
<tr>
<td>Proliferative*</td>
<td>106 (100)</td>
<td>9 (8)</td>
<td>97 (92)</td>
<td>35 (33)</td>
<td>40 (38)</td>
</tr>
<tr>
<td>Nonproliferative*</td>
<td>83 (100)</td>
<td>5 (6)</td>
<td>78 (94)</td>
<td>29 (35)</td>
<td>21 (25)</td>
</tr>
</tbody>
</table>

* P = 0.014 comparing the aneuploid rate of intrammary lymph nodes with all other diagnoses.
* Atypia/LCIS, hyperplasia, and adenosis.
* Intrammary lymph nodes and other benign lesions.

RESULTS

Flow cytometric DNA analysis of specimen mammography-guided FNAs was possible on 189 consecutive clinically occult benign lesions that underwent excision prompted by screening mammography. Fourteen of 189 (7%) benign lesions contained aneuploidy. The median S-phase percentage of the study group was 2.0; the lower 33rd percentile S-phase percentage was ≤1.6, and the higher 33rd percentile S-phase percentage was ≥4.1 (Table 1).

DNA analysis of specimen mammography-guided FNAs in adjacent normal mammographic tissue with benign histological findings showed aneuploidy in only 1 of 114 (0.9%) cases within the study. As shown in Table 2, the aneuploid rate in the normal tissue control group was significantly lower than that of the benign lesions (P = 0.01).

Associations between clinical and mammographic features and DNA analyses of the benign lesions are shown in Table 3. DNA content and S-phase percentage showed no significant associations with patient age, mammographic appearance, and mammographic soft tissue or calcification extent.

Associations between histological diagnoses and DNA analyses of the benign lesions are shown in Table 4. Benign histological diagnoses included nonproliferative benign lesions (77 lesions, 41%), benign intrammary lymph nodes (6 lesions, 3%), the proliferative changes of adenosis (41 lesions, 22%), and lesions containing hyperplasia (31 lesions, 16%) or atypia or LCIS (34 lesions, 18%). Aneuploidy was detected in all categories of benign lesions. The aneuploid rate for benign intrammary lymph nodes (2 of 6 lesions, 33%) was greater than the aneuploid rate of all other benign diagnoses combined (12 of 183 lesions, 6.5%; P = 0.01). There was no significant association between aneuploid rates and the combined proliferative histological diagnoses as compared with the nonproliferative diagnoses. There was no significant association between high and low S-phase percentage and benign histological diagnoses.

The risk of development of subsequent breast carcinoma in patients with aneuploid and diploid benign lesions is shown in Table 5. During a median follow-up of 40 months (range, 6–84 months), 2 of 13 (15%) patients with aneuploid benign lesions...
developed ipsilateral breast carcinoma. One patient developed a new mammographic lesion showing ductal carcinoma in situ, and another patient developed a mammographic lesion showing infiltrating lobular carcinoma in different quadrants of the same breast as the index benign biopsies within 8 and 34 months, respectively. In comparison, 5 of 175 (3%) patients with diploid benign lesions developed ipsilateral breast carcinomas. The calculated odds ratio was 6.18 (95% confidence interval, 1.08–35.56). No patients developed contralateral breast carcinoma during the period of the study. One patient with an initial aneuploid benign lesion did not develop carcinoma but underwent a subsequent biopsy of benign calcification that contained atypia and aneuploidy.

DISCUSSION

During this study, DNA flow cytometry was successfully performed on 189 consecutive specimen mammography-guided FNAs of clinically occult lesions detected by screening mammography that proved to be benign at histological examination. Aneuploid DNA content was detected in 7% of the benign lesions, most commonly in benign intramammary lymph nodes, but also in benign atypical and hyperplastic lesions, adenosis, and other nonproliferative benign lesions. The observation that, in comparison, aneuploidy was detected in only 1% of FNAs of adjacent normal tissue (P < 0.01) shows that the detection of aneuploidy was not a laboratory artifact.

There are little data in the literature regarding DNA flow cytometry of benign breast lesions (1–6). To our knowledge, this is the largest series of DNA flow cytometry of benign breast lesions. The relative lack of aneuploidy in the normal adjacent breast tissue controls suggests some biological significance of aneuploidy in benign breast lesions. Whether aneuploidy predates or is an obligate precursor of light microscopic malignancy is unresolved. Our S-phase percentage data show a wide range of proliferative activity for benign breast lesions that is independent of the specific histological diagnoses.

To our knowledge, this is the first study showing the risk of subsequent development of ipsilateral breast carcinoma in patients who underwent benign breast biopsies with aneuploidy as compared to patients who underwent benign breast biopsies with diploid DNA content. There is one anecdotal report of a patient who developed ipsilateral breast cancer 2.5 years after a benign biopsy showing atypia and aneuploidy (1), but the relationship of these occurrences and the importance of this isolated report were uncertain. The patients in our study with aneuploid benign lesions who have not yet developed malignancy may represent patients whose potential malignancy was completely removed by the excisional biopsy or whose follow-up is not yet long enough. Due to the small numbers and short follow-up in our pilot study, a study of a larger number and longer follow-up of patients with benign breast biopsies would be necessary to validate the risk prediction of aneuploid DNA content for the subsequent development of ipsilateral breast carcinoma. Determination of whether the risk associated with aneuploidy in benign biopsies is ipsilateral or bilateral would also be of potential clinical significance.

In light of the increasing interest in breast cancer risk assessment, partially attributed to recent breakthroughs in genetic testing for BRCA1 and BRCA2 mutations (19), there is an increasing need for intermediate risk markers for the development of breast carcinomas, i.e., markers that would predict the development of breast carcinoma within 5–10 years rather than over a lifetime. A study of in vivo FNAs of normal breast tissue in high- and low-risk women was performed by Fabian et al. (20), who performed eight clinically guided fine-needle aspirations/breast. They evaluated the aspirates for overexpression of estrogen receptor, epidermal growth factor, mutant p53, and HER-2/neu by immunocytochemistry and for aneuploidy by image analysis. With their technique and the criteria for aneuploidy, aneuploidy was detected in 32% of high-risk women as compared with 0% of low-risk women.

With the increasing numbers of benign breast biopsies performed as a result of screening mammography, the in vitro specimen mammography-guided fine-needle aspiration technique is a practical clinical research method that provides mammographic lesion-specific fresh cell samples immediately after wire localization and excision. Fresh cell samples are preferred by both our flow cytometry and molecular genetic laboratories and others (21–23). Core biopsy washings retrieved during stereotactic core breast biopsies of clinically occult lesions detected by mammography have also been reported as a source of fresh cellular material for flow cytometry (24). FNAs and scrapings of palpable breast carcinoma have also been used for flow cytometry (4–6, 25), but these techniques are limited to larger tumors only and exclude smaller clinically occult lesions detected by screening mammography. We are now performing combined staining with fluorescein-labeled anticytokertatin antibodies that allows the DNA analysis of epithelial cells to be separated from that of other elements. The development of multiple marker immunophenotyping panels by flow cytometry in breast disease has potential use in risk assessment in cells obtained from normal breast tissue or benign lesions using.
fine-needle aspiration, because multiple tests can be performed simultaneously on the same low number of cells.

In conclusion, DNA flow cytometry can be performed successfully on specimen mammography-guided FNAs of mammary-detected lesions that prove to be benign at biopsy. Our pilot study data suggest that aneuploidy, which is seen in a variety of benign breast lesions, is associated with a higher short-term risk of development of ipsilateral breast carcinoma than benign lesions with diploidy. The combined techniques of specimen mammography-guided FNA and flow cytometry provide a practical translational research method for the further study and molecular and genetic risk assessment of benign breast disease.

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Flow cytometric DNA analyses of benign breast lesions detected by screening mammography.

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