Detection of Chromosomal Aneuploidies and Gene Copy Number Changes in Fine Needle Aspirates Is a Specific, Sensitive, and Objective Genetic Test for the Diagnosis of Breast Cancer

Kerstin Heselmeyer-Haddad, Nadia Chaudhri, Patricia Stoltzfus, Joseph C. Cheng, Kim Wilber, Larry Morrison, Gert Auer, and Thomas Ried

ABSTRACT

Fine needle aspiration cytology is central to the evaluation of clinically or mammographically detected suspicious lesions of the breast. On the basis of results from studies of >500 breast cancers by comparative genomic hybridization we have developed protocols and designed probe sets that allow one to visualize recurrent chromosomal aneuploidies, amplification of oncogenes, and deletion of tumor suppressor genes directly in cytological preparations using multicolor fluorescence in situ hybridization. The fluorescence in situ hybridization probes are specific for chromosome arm 1q, the c-MYC and HER2 oncogenes, the tumor suppressor gene p53 and, as controls for chromosome ploidy of each cell, the centromeres of chromosomes 8, 10, and 17. Application of these diagnostic mixtures to 20 invasive breast cancers, 7 mastopathias, and 2 fibroadenomas demonstrates that a highly sensitive, specific, and objective diagnosis of breast cancer is now possible on cytological preparations obtained by minimally invasive fine needle aspiration.

INTRODUCTION

FNA cytology is an integral part of the diagnostic evaluation of suspicious breast lesions (1). In many cases this minimally invasive procedure allows one to collect representative material for cytological evaluation. The accuracy by which lesions can be targeted has been improved using systems guided by ultrasound or other imaging techniques. However, the identification of aberrant cells in cytological preparations is more difficult than histomorphological diagnosis, because information from the surrounding tissue and its context is lost. Accordingly, precise diagnosis and prognostication remains a challenge even for experienced cytopathologists, in particular regarding small preinvasive lesions and highly differentiated tumors. This problem could be overcome by complementing the evaluation of cellular morphology with objective markers for invasive cancer. Such markers should be highly specific for breast cancer cells and have a high level of sensitivity. As such they should not be present in normal tissue or benign breast disease but in most or all cancers.

CGH is a screening test for genomic imbalances and chromosomal aneuploidies (2). The application of CGH to virtually all human cancers has revealed that each tumor type can be described by a specific distribution of chromosomal gains and losses (3). CGH analyses of >500 breast cancers have revealed that tumor-specific chromosomal imbalances could be mapped to 1q, 8q, 11q, 17, and 20q (gains) and 17p (loss; Ref. 4). We could also show that fibroadenomas maintain a stable genome and that diploid breast carcinomas specifically reveal copy number increases on chromosome 1q (5). Prompted by this knowledge of tumor-specific chromosomal aneuploidies that are not found in normal tissue, we eagerly developed a translational assay to complement the cytomorphological diagnosis of breast cancer using tumor-specific and sensitive genetic markers. Numerical chromosome aberrations can be readily visualized in intact interphase nuclei using in situ hybridization with DNA probes labeled either with fluorochromes or chromogenic dyes (6, 7). Termed interphase cytogenetics, this technique has typically used probes containing centromere-specific repeat sequences or individual oncogenes (8–10). We now demonstrate that interphase cytogenetics with three custom-designed triple color probe sets can be used to simultaneously visualize oncogene amplification, tumor suppressor gene deletion, and cancerspecific aneuploidy in fine needle aspirates from breast lesions. The use of these probe panels, of which the design was based on breast cancer-specific patterns of genetic aberrations, unambiguously and objectively distinguishes aspirates obtained from carcinomas from those of benign breast disease.

MATERIALS AND METHODS

Cytological Preparations. FNA biopsy was performed using a 23-gauge needle attached to a 10-ml syringe and inserted into a syringe holder. The aspirates were smeared on microscope slides for cytopathological evaluation. Samples for FISH analysis were produced by washing the needle and syringe with 1 ml 1% BSA in 1× PBS. The solution was collected in an Eppendorf tube. Each sample (100 μl) was loaded into a Cytospin (Shandon, Pittsburgh, PA) using the Shandon Cytofunnel Disposable Sample Chambers. The slides were dehydrated in an ethanol series (70%, 90%, and 100%) for 5 min each and stored at 4°C.

FISH Probes. Three probe panels were designed based on our own and previously published CGH results (4). The mixtures consisted of DNA probes specific for the following chromosomal regions and genes: centromere of chromosomes 10 (CEP10), 8 (CEP8), the c-MYC oncogene (Panel 1), CEP17, the oncogene HER2 (Panel 2), CEP10, CEP1, and the tumor suppressor gene p53 (Panel 3, see also Fig. 1). All of the probes were provided by Vysis, Inc. (Downers Grove, IL). In all of the mixtures, the gene-specific probe was labeled with SO; CEP10 with SG; and CEP1, CEP8, and CEP17 with SA. Probe labeling was performed chemically as described previously (11). Slides were pretreated with RNase digestion followed by pepsin digestion and fixation in an ethanol series. Slides were denatured in 70% formamide, 2× SSC for 3 min at 80°C. After overnight hybridization at 37°C, the coverslips were removed gently, slides washed four times in 50% formamide/2× SSC at 45°C (3 min and 3 × 7 min), followed by washes in 2× SSC (45°C, 5 min) and 0.1% NP40 in 2× SSC (45°C, 5 min). The slides were counterstained with DAPI and embedded in an antifade solution. Images were acquired using a Leica DMRXA microscope (Leica, Wetzlar, Germany) equipped with custom optical filters for DAPI, SA, SG, and SO (Chroma Technologies, Brattleboro, VT) with a 40× Plan Apo (NA 1.25) objective. The microscope was connected to an ORCA ER (Ideen1394 UF) digital camera (Hamamatsu, Bridgewater, NJ).

Signal Enumeration. For benign breast diseases such as mastopathia and fibroadenoma, signals were evaluated by screening the entire slide visually for the oncogene and tumor suppressor gene probes (using the optical filters...
GENETIC DIAGNOSIS OF BREAST CANCER IN FNAs

RESULTS

In human breast cancers, as in other carcinomas, the pattern of chromosomal aneuploidies and resulting genomic imbalances is strongly selected for and strictly conserved (4). Extensive CGH studies of human breast carcinomas have shown that virtually all breast carcinomas have gains of chromosome arms 1q, 8q, 11q, 17q, and 20, and losses on 17p, either alone or in combination (4). The target genes acquired and maintained by these specific aneuploidies are known in some instances and include the c-MYC (8q24), cyclinD1 (11q13), and HER2 (17q21) oncogenes, and the tumor suppressor gene p53 (17p13). Guided by the pattern and distribution of these specific imbalances, we designed three triple color probe sets for the visualization of genetic aberrations directly in interphase nuclei of cells obtained by FNA. The chromosomal locations, the fluorochromes and the composition of the probe mixtures are presented in Fig. 1. A summary of representative CGH results. Whenever possible, we have used the most likely candidate genes on the chromosomes subject to copy number variations. Therefore, the genespecific probe for chromosome 8 is c-MYC, HER2 on 17q, and p53 on 17p. All of the probe panels contained a centromere 10 probe, because this chromosome is only rarely subject to copy number changes in breast cancers. Such a probe is important to establish a baseline for the ploidy of the cells, and hence, for the interpretation of relative copy number changes. Panels 1 and 2 also include a probe corresponding to the centromeric heterochromatin of the chromosome on which the oncogene resides. This again serves as an internal control for signal enumeration. An example of the hybridization of probe panel 1 to metaphase chromosomes and interphase nuclei from the breast cancer cell line SKBR3 confirms the copy number changes enumerated previously by spectral karyotyping and CGH analysis of this cell line (12). Amplification of the c-MYC oncogene embedded in giant marker chromosomes is readily visible, and signal copy enumeration can be performed for CEP8 and CEP10 in interphase cells (Fig. 2A). Before analyzing copy number in unknown samples, it was imperative that we establish the hybridization efficiency of the probe panels and the rate of aneuploidy in normal diploid human lymphocytes. Between 200 and 300 nuclei from two healthy donors were enumerated for each of the probe panels. Metaphase spreads from these donors had been used extensively as controls for both spectral karyotyping and CGH, and invariably revealed a normal karyotype (46,XY and 46,XX, respectively). Assuming that all of the interphase cells also have a diploid chromosome count, the hybridization efficiency of the probe panels is in the range of 92%–96% on methanol/acetic acid fixed interphase nuclei from peripheral blood lymphocytes. Aberrant signal numbers for the oncogene probes and the probe for p53 were detected in <3% of the cells (data not shown).

The usefulness of the probe panels as a diagnostic tool is dependent on their ability to unambiguously distinguish normal breast epithelium, benign breast disease, and invasive carcinomas. This relies on both the sensitivity, as defined by the percentage of invasive carcinomas that show aberrant copy numbers, and the specificity, defined by the percentage of normal breast epithelium and benign breast disease specimens with a normal signal count. Therefore, we have decided to analyze two fibroadenomas and seven mastopathias. For all of the cases, between 200 and 2623 interphase nuclei were enumerated visually by two investigators. Images were acquired from those cells that revealed copy number aberrations with the oncogene probes and the probe for p53. The percentage of cells with copy numbers other than two ranged from 1 to 2% among the different cases. Applying a threshold of 2%, the specificity of the test is 1 (95% CI, 77.91–100). In one case of a fibroadenoma, we could detect a tetraploid clone that did not contain any genomic imbalances relative to the ploidy (4n) of the cells. Such cases were not categorized as aberrant. An example of a hybridization is shown in Fig. 2B. The results show that fibroadenomas and mastopathias retain a stable genome, consistent with previous CGH results and cytogenetic analyses (5, 13). Signals diverging from a diploid or tetraploid count were present in percentages similar to those observed in normal human lymphocytes.

Twenty carcinomas were analyzed. The histological diagnosis, tumor grade and size, nuclear DNA content, and cyclin A levels as a marker for proliferative activity are summarized in Table 1. Six of the cancers revealed a diploid DNA content (DNA index = 1) as established by image cytometry on the same samples used for in situ hybridization. Fourteen carcinomas were diagnosed as aneuploid with DNA indexes varying from 1.1 to 1.9. Probe panel 1 was successfully hybridized to 19 of 20 cases, panel 2 to all cases, and panel 3 to 19 of 20 cases. Examples of the interphase FISH images typically obtained for quantification are provided in Fig. 2, C–F. Therefore, the hybridization efficiency of the combined probe panels was 96%, and at least two probe panels could be evaluated in all of the cases. Two of the diploid tumors (case #10 and #20) revealed normal copy numbers for all of the probe panels. Four diploid tumors revealed aberrations in 8–90% of the cells analyzed, including amplifications of c-MYC and HER2 in case #36. Interestingly, two of the diploid tumors contained a gain of chromosome 1 as the sole anomaly (Fig. 2D), consistent with previous CGH results (5).

All of the aneuploid carcinomas clearly revealed copy number changes with one or more of the three probe panels used, mostly in the
The majority of the cells (Table 1). The enumeration of approximately 200–400 nuclei/case revealed a tremendous degree of intercellular chromosomal heterogeneity; however, it also revealed a surprising degree of stability when considering the consequences of chromosomal instability, i.e., the acquisition and retention of specific genomic imbalances, to the tumor cell population as a whole. For instance, tumor #9 revealed 26 different hybridization patterns with probe panel 3, but only five major clones could be discerned, all of which showed a relative loss of p53, yet in different permutations. This means that p53 was lost in >93% of the cells (Fig. 3). In general, probe copy numbers corresponded well with measurements of the nuclear DNA content. In case #9 the seemingly normal 2c peak in the DNA histogram (inset in Fig. 3) reflects the population of diploid cells with the loss of one copy of p53 (2-2-1). Inclusion of the HER2 probe allowed us to establish the amplification status of this growth factor receptor in all instances. c-MYC and HER2 gain or amplification occurred concurrently in 10 tumors, and c-MYC gain alone in 5 tumors. HER2 amplification in the absence of c-MYC gain was not observed. p53 was lost in 12 cases, mostly (10 of 12) accompanied by gain of at least one of the oncogenes. The gain of CEP1 was the sole anomaly in two diploid tumors, whereas one diploid tumor (case #33) showed loss of one copy of p53 as the only copy number change.

Our results demonstrate that the use of specially designed probe panels alone is sufficient to diagnose breast cancer from fine needle aspirates in 100% of tumors identified previously as aneuploid by DNA image cytometry and in 66% of the seemingly diploid (2n) tumors, independent of all of the other parameters evaluated. The specificity of this test for the diagnosis of aneuploid carcinomas is 100% (95% CI, 80.74–100), and the specificity for the diagnosis of all cancers combined is 90% (95% CI, 62.11–96.79).

DISCUSSION

Disease-free survival and prognosis of patients with breast cancer improves with early detection (14). Screening programs including mammography, as successfully established for instance in Sweden,
have considerably reduced breast cancer-associated mortality. The difference in mortality between Sweden and Germany, a country in which mammography is not part of breast cancer screening programs, is significant (15). Using FNA biopsy, a minimally invasive method, suspicious lesions can be evaluated morphologically. In Stockholm and suburbs, the average size of cancerous lesions at the time of diagnosis is clearly smaller compared with 10 years ago. This trend is likely to continue with improved imaging techniques and the identification of high-risk individuals based on family history or genetic markers for cancer diagnosis will become increasingly important and standard of care.

The analysis of some 500 breast cancers by CGH has revealed a specific distribution of chromosomal gains and losses (aneuploidies), and genomic imbalances. We have chosen to apply this knowledge to clinical practice by designing probe panels that allow the visualization of these breast cancer-specific genetic aberrations directly in cytologic samples. The proliferative activity was determined by measuring percentage of cells positive for cyclin A. The numbers indicate the enumeration of three probes in one panel (e.g., 2-3-2). Amp refers to copy numbers more than five. Whenever possible, the major clone was indicated in addition to the percentage of cells carrying a gain of MYC of HER and loss of p53, e.g., for case 31 a MYC gain occured in 71% of all cells whereas the major clone was characterized by a 2-4-4 aberration pattern.

Table 1 Clinical features and Interphase FISH results with three breast cancer-specific probe sets on fine needle aspirates from 20 carcinomas

The proliferative activity was determined by measuring percentage of cells positive for cyclin A. The numbers indicate the enumeration of three probes in one panel (e.g., 2-3-2). Amp refers to copy numbers more than five. Whenever possible, the major clone was indicated in addition to the percentage of cells carrying a gain of MYC of HER and loss of p53, e.g., for case 31 a MYC gain occured in 71% of all cells whereas the major clone was characterized by a 2-4-4 aberration pattern.

<table>
<thead>
<tr>
<th>Case</th>
<th>Histology</th>
<th>Grade</th>
<th>Size</th>
<th>DNA ploidy</th>
<th>Cyclin A</th>
<th>CEP10-CEP8-MYC</th>
<th>CEP10-CEP17-HER</th>
<th>CEP10-CEP1-p53</th>
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<td>8</td>
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<td>2</td>
<td>24mm</td>
<td>diploid</td>
<td>0%</td>
<td>normal (5% 4-4-4)</td>
<td>normal (6% 4-4-4)</td>
<td>28% CEP1 gain (2-3-2)</td>
</tr>
<tr>
<td>10</td>
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<td>2</td>
<td>12mm</td>
<td>diploid</td>
<td>2%</td>
<td>normal</td>
<td>normal (6% 4-4-4)</td>
<td>28% CEP1 gain (2-3-2)</td>
</tr>
<tr>
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<td>1</td>
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<td>diploid</td>
<td>5%</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>28</td>
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<td>33mm</td>
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<td>13%</td>
<td>normal</td>
<td>normal</td>
<td>9% CEP1 gain (2-3-2)</td>
</tr>
<tr>
<td>33</td>
<td>tubular</td>
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<td>0%</td>
<td>normal</td>
<td>normal</td>
<td>8% p53 loss</td>
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<td>36</td>
<td>ductal</td>
<td>3</td>
<td>20mm</td>
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<td>25%</td>
<td>95% MYC gain, 81% 2-3-3</td>
<td>95% HER amp, 86% 2-2-amp</td>
<td>13% CEP1 gain (2-3-2)</td>
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<td>82% MYC gain</td>
<td>50% loss of 17 and HER</td>
<td>11% CEP1 gain (2-3-2)</td>
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<tr>
<td>9</td>
<td>comedo (ductal)</td>
<td>3</td>
<td>&gt;30mm</td>
<td>tetraploid</td>
<td>2%</td>
<td>18% MYC gain, 4-4-4, 4-4-3, 4-4-3, 3-3-3</td>
<td>22% HER gain, 4-4-4, 4-3-4</td>
<td>28% CEP1 gain, 93% p53 loss</td>
</tr>
<tr>
<td>13</td>
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<td>18mm</td>
<td>aneuploid</td>
<td>11%</td>
<td>n.d.*</td>
<td>50% 3-2-2</td>
<td>61% CEP1 gain, 54% p53 loss, 17% 4-3-2</td>
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<tr>
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<td>tetraploid</td>
<td>1%</td>
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<td>61% 4-3-3</td>
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<tr>
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<td>comedo (ductal)</td>
<td>3</td>
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<td>aneuploid</td>
<td>17%</td>
<td>62% 2-2-2, 20% &gt;6MYC</td>
<td>67% 2-2-2, 15% HER amp</td>
<td>27% 2-2-2, 46% 2-3-2, 17% &lt;2p53</td>
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<tr>
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<td>42% MYC gain</td>
<td>4% HER amp, 3.5% HER gain, 14.6% HER loss</td>
<td>41% CEP1 gain, 47% p53 loss, 16% 2-3-1</td>
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<td>diploid</td>
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<td>35% MYC gain, 46% MYC amp</td>
<td>78% HER gain</td>
<td>36% CEP1 gain, 83% p53 gain</td>
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<td>17mm</td>
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<td>36% MYC gain, 30% 3-3-3</td>
<td>33% HER gain, 23% 3-3-3</td>
<td>27% CEP1 gain, 68% p53 loss, 28% 3-3-1</td>
</tr>
<tr>
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<td>aneuploid</td>
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<td>71% MYC gain, 22% 2-4-4</td>
<td>77% HER gain, 57% 2-2-3</td>
<td>79% p53 loss, 55% 2-2-1</td>
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<td>87% MYC gain</td>
<td>68% HER loss, 8% HER gain normal</td>
<td>26% CEP1 gain, 64% p53 loss</td>
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<td>96% MYC gain</td>
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<td>21% MYC gain, 33% 3-3-3, 15% 4-3-3</td>
<td>96% HER gain</td>
<td>59% CEP1 gain, 99% p53 loss</td>
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</table>

* n.d., not determined.

Fig. 3. Intratumor heterogeneity in an aneuploid breast cancer (case 9). The enumeration of probe panel 3 revealed 26 different hybridization patterns; however, the common loss of the p53 tumor suppressor gene (copy numbers are below the numbers for the ploidy probe, CEP10) is common in the majority of the cells. The five major clones, of which the enumeration patterns are indicated in the graph, represent >75% of the cells. Insets: a, percentage of cells with reduced copy number of p53 relative to the copy number of CEP10; b, histogram of the DNA ploidy measurement by image cytometry on fine needle aspirates of the same preparations used for interphase FISH. The FISH signal numbers correlate well with the DNA content measurements.
cical preparations from fine needle aspirates. Probes were chosen that correspond to those portions of the genome frequently subject to chromosomal imbalances. The high sensitivity with which we can detect breast cancer confirms the usefulness of these probe panels. However, one could envision that expansion of the panels to other relevant oncogenes, such as cyclin D1, and to genes or candidate genes on 17q23 and 20q, other areas frequently amplified in breast cancer (16, 17), will additionally increase the sensitivity of the assay. This might be particularly useful for the diagnosis of diploid tumors. However, the possibility exists that a subgroup of diploid breast cancers does indeed not carry any genomic imbalances but is defective in its mismatch repair machinery, a pathway toward cancer described in some colorectal carcinomas (18, 19). We have, however, no information on the mismatch repair status on these tumors. Both tumors that were negative for any of the probe panels were relatively small. Therefore, one could also argue that the failure to detect aberrant signal numbers lies in the lack of representative cancer cells in the fine needle aspirates.

In this study three different probe panels were used so that control probes for both the ploidy of the cells as well as the copy number of those chromosomes containing the gene-specific probes could be included. These panels could otherwise be combined into a single panel containing probes for the c-MYC and HER2 oncogenes, and chromosome 1q. The use of different fluorochromes or fluorochrome combinations can increase the number of simultaneously discernible targets (20, 21). We have chosen not to venture in this direction in this proof of principle study, because the visual review of questionable hybridization signals was helpful in some instances. This would not have been possible if fluorochromes emitting in the near-infrared (such as Cy5 or Cy5.5) were used. On the basis of the experience gained from the present study we would also discourage the use of combinatorial labeling strategies to increase the number of simultaneously discernible targets. Overlapping signals are more difficult to deconvolute, especially in relatively flat cytospin preparations. Future technological developments, such as hardware and software for automated signal enumeration, are being developed and will greatly facilitate the use of probe sets as diagnostic tools in cytological laboratories.

In summary, we present here probe panels for FISH analysis of cells after FNA of breast lesions. The use of these probe panels allows one to objectively diagnose breast cancer with high sensitivity and specificity independent of any other markers. Benign breast diseases, such as mastopathia and fibroadenoma, did not reveal numerical chromosomal aberrations. As such, they were readily discernable from carcinomas. Therefore, we suggest that the use of these probes panels will increase the diagnostic precision with which breast cancer and its premalignant precursor lesions can be diagnosed on cytological preparations. The direct visualization of relevant genetic markers, such as the amplification status of the HER2 oncogene, is not only useful for the improvement of disease prognostication but will contribute to individualize therapeutic strategies.

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