Genetic Heterogeneity and Clonal Evolution Underlying Development of Asynchronous Metastasis in Human Breast Cancer

Tuula Kuukasjärvi, Ritva Karhu, Minna Tanner, Marketta Kähkönen, Alejandro Schäffer, Nina Nupponen, Sari Pennanen, Anne Kallioniemi, Olli-Pekka Kallioniemi, and Jorma Isola

Department of Pathology [T. K.] and Laboratory of Cancer Genetics [T. K., R. K., M. T., M. K., N. N., S. P., A. K., O.-P. K., J. J.], Tampere University Hospital and Institute of Medical Technology, University of Tampere, P. O. Box 2000, FIN-33521 Tampere, Finland, and National Center for Human Genome Research, NIH, Bethesda, Maryland 20892-4407 [A. S., A. K., O.-P. K.]

ABSTRACT

To understand the genetic basis and clonal evolution underlying metastatic progression of human breast cancer in vivo, we analyzed the genetic composition of 29 primary breast carcinomas and their paired asynchronous metastases by comparative genomic hybridization and fluorescence in situ hybridization. The mean number of genetic changes by comparative genomic hybridization was 8.7 ± 5.3 in primary tumors and 9.0 ± 5.7 in their metastases. Although most of the genetic changes occurred equally often in the two groups, gains of the Xq12–q22 region were enriched in the metastases. According to a statistical analysis of shared genetic changes and breakpoints in paired specimens, 20 of the metastases (69%) showed a high degree of clonal relationship with the corresponding primary tumor, whereas the genetic composition of 9 metastases (31%) differed almost completely from that of the paired primary tumors. In both groups, however, chromosome X inactivation patterns suggested that the metastatic lesions originated from the same clone as the primary tumor. Fluorescence in situ hybridization analysis with probes specific to metastatic clones usually failed to find such cells in the primary tumor sample. In conclusion, detailed characterization of the in vivo progression pathways of metastatic breast cancer indicates that a linear progression model is unlikely to account for the progression of primary tumors to metastases. An early stem line clone apparently evolves independently in the primary tumor and its metastasis, eventually leading to multiple, genetically almost completely different, clones in the various tumor locations in a given patient. The resulting heterogeneity of metastatic breast cancer may underlie its poor responsiveness to therapy and explain why biomarkers of prognosis or therapy responsiveness measured exclusively from primary tumors give a restricted view of the biological properties of metastatic breast cancer.

INTRODUCTION

A stepwise accumulation of genetic changes underlies the gradual transition of normal epithelium to invasive carcinoma through a number of intermediate lesions (1, 2). These genetic aberrations lead to the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis, and genetic stability (1–4). However, relatively little is known of the genetic changes underlying metastatic dissemination in spite of the fact that metastasis is the most important factor in determining survival of the cancer patient. Because cancer therapies often aim at eliminating metastatic cells or preventing their dissemination, understanding the molecular mechanisms of the metastatic progression could help to improve therapy.

The biology of metastasis has been extensively studied in rodent model systems (5–9). For example, the nm23 gene (9) was originally discovered based on its reduced expression in highly metastatic murine melanoma cell lines. Loss of this gene has been associated with poor prognosis in human breast cancer (9). Metastatic progression in human cancer has also been studied by evaluating the expression in primary tumors of certain candidate genes as predictors of the likelihood of subsequent metastasis. Besides nm23, other members of the metalloproteinase gene-family and their inhibitors have been extensively studied as predictors of the likelihood of metastasis (10, 11). Metalloproteinases are involved in the proteolytic degradation of the extracellular matrix and include genes such as collagenase IV, urokinase X, cathepsin D, and stromelysin. Expression levels of many of these genes have been shown to correlate with the length of metastasis-free survival in breast cancer (10–14). However, it is unknown to what extent the expression of these genes in the primary tumors reflects the metastatic process or other associated events, such as tumor invasiveness. Finally, studies of genetic changes in metastatic tumor deposits have also been reported. LOH (15) studies have suggested that primary tumors and synchronous lymph node metastases have a common clonal origin. Cytogenetic analyses have in turn revealed evidence for increased prevalence of some karyotypic alterations, such as those involving chromosomes 1, 7, and 11, as the disease progresses (16), as well as demonstrating extreme genetic heterogeneity in breast cancer (17–19). Multiple clonally unrelated clones have also been found in primary breast carcinomas by cytogenetics (17–19).

Although the model systems, candidate gene approaches, and studies by LOH and cytogenetics have all substantially advanced the knowledge of the mechanisms of the metastatic progression, critical questions remain unanswered. Very little is known of the actual genetic changes present in the metastatic tumor cells, especially in asynchronous metastases. Assessing genetic changes directly in the metastatic lesions rather than in the more commonly targeted primary tumors could shed light into the molecular mechanisms of the metastatic progression. Studies to compare changes in the metastases with those found in the corresponding primary tumors in the same patient would be informative in revealing subtle genetic differences between primary and metastatic lesions and thereby pinpointing genetic events that could have predisposed to metastatic dissemination. The analysis of paired specimens would also make it possible to assess the degree of clonal evolution and genetic heterogeneity that characterizes the metastatic process. The extent to which the primary and metastatic cell clones are different from one another is an important question for both tumor biology and clinical oncology.

Here, we have taken the advantage of the vast archives of formalin-fixed, paraffin-embedded tumor specimens to collect samples for detailed genetic studies from primary human breast carcinomas and their paired asynchronous metastases, detected up to 19 years after operation of the primary tumor. In particular, we aimed at determining the degree of CR between primary and metastatic cells, as well as at

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2 To whom requests for reprints should be addressed, at Laboratory of Cancer Genetics, Tampere University Hospital, University of Tampere, P. O. Box 2000, FIN-33521 Tampere, Finland. Fax: 358-3-247-4168; E-mail: bhltu@uta.fi.

3 The abbreviations used are: LOH, loss of heterozygosity; CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization; DP, PCR, degenerate oligonucleotide-primer PCR; PGK, phosphoglycerate kinase; CR, clonal relationship.
the identification of genetic changes enriched in the metastases. CGH, with a recently optimized protocol for archival tissue (20, 21), was used for analysis of the genetic changes. CGH is ideal for this kind of a study because it allows comprehensive surveying of the entire tumor genome for DNA sequence copy number changes and is not dependent on the availability of fresh tissue metaphase cells specific probes or prior knowledge on the involvement of candidate genes (20–24). Because CGH is not able to detect cell subpopulations or assess monoclonal origin of the tumors, we also performed interphase FISH analysis of the tumor cells with specific probes for the loci implicated by CGH analysis, as well as chromosome X inactivation studies to assess monoclonality of the specimens.

PATIENTS AND METHODS

Patients. Breast cancer patients were selected for this study from a population based data base containing 4000 cases using the following three criteria: (a) both primary breast cancer and asynchronous metastatic lesions had been biopsied from the patient; (b) formalin-fixed, paraffin-embedded histologically representative specimens (containing >50% tumor cells) were available from both sites; and (c) the patients had not received any chemotherapy or endocrine therapy prior to the diagnosis of the metastasis. The median age of the patients at the time of diagnosis was 49 years (range, 24–79). Surgical treatment consisted of mastectomy or segmental resection with axillary node evacuation. Postoperative radiation therapy was given to 14 (48%) patients. Median time from diagnosis of the primary tumor to appearance of the metastasis (disease-free interval) was 19 months (range, 3–228). The distribution of anatomical sites of the metastases was as follows: locoregional (15 cases), supraclavicular area (3), lungs (3), pelvis or abdominal cavity (5), bone marrow (2), and distant soft tissue (femoral muscle; 1). In two cases, specimens from both the primary tumor and multiple asynchronous metastases were also available.

Histopathological type and grade of the carcinomas were reclassified according to the WHO criteria (25). Twenty-one of the 29 tumors (72%) were invasive ductal carcinomas, 5 (17%) were invasive lobular carcinomas, 2 were medullary carcinomas, and 1 was a tubular carcinoma. The percentage distribution of histological grades was as follows: I, 14%; II, 55%; and III, 31%. H&E-stained sections were used to evaluate the histological representativeness of the samples, and if necessary, specimens were trimmed to reduce the normal tissue contamination. DNA was extracted from formalin-fixed, archival tissues according to previously published protocols (20).

CGH. CGH was carried out as described elsewhere (20, 21), with minor modifications. Briefly, normal female DNA (normal reference DNA) was labeled by nick translation with Texas red-dUTP (DuPont, Boston, MA) and the tumor DNAs with FITC-dUTP (DuPont). In five cases in which the quality of extracted DNA was not sufficient for direct labeling with nick-translation, the tumor DNA was amplified and labeled by DOP-PCR using UNI primers (26). DOP-PCR was performed according to a recently described protocol (27) optimized for formalin-fixed tissue and extensively validated with normal DNA specimens and specimens from known abnormalities. Labeled test DNA (600 ng of nick-translated DNA or 5 μl of the labeled DOP-PCR product) and normal reference DNA (600 ng), as well as 10 μg of unlabeled Cot-1 DNA (Life Technologies, Inc., Gaithersburg, MD), were hybridized to denatured normal lymphocyte metaphase spreads. For each batch of hybridizations, two control experiments were performed. These consisted of hybridizations of normal male DNA against normal female DNA and of DNA from previously characterized breast cancer cell lines (MCF-7 or BT474) against normal female DNA.

Hybridizations were analyzed using a digital image analysis system as described previously (21, 28). Relative DNA sequence copy number changes were determined by quantitating intensities of green (tumor DNA) and red fluorescence (normal DNA) from per to qter along the length of all metaphase chromosomes. Four to eight carefully selected high-quality metaphase spreads were analyzed from each slide. After background subtraction and normalization of the intensities of the two colors, the results were expressed as a set of green to red fluorescence ratio profiles for each target chromosome from per to qter. Chromosomal regions for which the mean green to red ratio (and plus 1 SD of this ratio) was less than 0.85 were considered lost, whereas gains were defined as regions for which the mean ratio (and minus 1 SD of this ratio) was greater than 1.15. These cutoff values were determined based on negative control experiments. In CGH experiments with normal male versus female DNA, the mean green to red ratio, as well as its 1 SD, remained between 0.85 and 1.15 for all autosomes. Heterochromatic regions and p arms of acrocentric chromosomes were always excluded from the analysis. The male versus female control hybridization was also used to ascertain that the difference between one and two X chromosomes was always detectable by determining the green to red ratio of the X chromosome.

Clonality Analysis Using the X-Chromosome Inactivation Method. Clonality analysis was based on RFLPs of the X-chromosome-linked PGK gene and on the differential methylation of the PGK gene due to random inactivation of the two X-chromosomes by methylation (29). This analysis can be applied as a test of monoclonoality (29, 30) because one of the X-chromosomes in normal female tissues is randomly inactivated. Furthermore, if the same X chromosome is inactivated between pairs of specimens (e.g., primary tumor and its metastasis) from the same patient, this suggests that the two specimens had originated from the same common ancestor clone. Here, 26 paired DNA specimens, representing both primary tumors and metastases from 13 patients, were used to determine whether the same X chromosome was inactivated between the primary and metastatic specimens. The cases studied included both those with a clear CR by CGH and those with no evidence of a significant number of shared genetic changes. The same DNA specimen applied in the CGH analyses was also used in clonality tests. Normal female DNA, heterozygous for the RFLP-marker tested, was used as a positive control.

FISH. Two-color FISH of tumor interphase nuclei with directly (Texas Red or FITC-dUTP) or indirectly (digoxigenin-11-dUTP or biotin-14-dATP) labeled probes was used to validate CGH results essentially as described previously (31, 32). Thirty-six cases (consisting of 18 primary tumors and 18 metastases) were evaluated with locus-specific probes for 8q24 and 20q13, as well as with peri-centromeric alaphoid probes for chromosomes 12 and X. In addition, five other chromosomal regions (1cen, 1q21.2, 1q43, 1q33, and 17q21–q23) were analyzed in selected tumor specimens. The probes included α-satellite repeat probes for chromosomes 1 (puc177), 12 (pA1218H), and X (BamX7), as well as locus-specific P1, bacterial artificial chromosome, or cosmids probes for 1q (RMCR01P01 and RMCR01P03), 8q24 (C-MYCX, RMCR08P01 and BAC probes 293F4 and 392G7), 13q33 (RMC31P016), 17q21–q23 (D17S578 and CK17.84), and 20q11–13 (RMC20P01, RM20C002, and PTPN1). The FISH probes came from the Resource of Molecular Cytogenetics (Berkeley, CA), and the two 8q-specific BAC probes were provided by J. Korenberg (Cedars Sinai Medical Center, Los Angeles, CA).

Statistical Analyses. Statistical differences in the prevalence of the 13 most common gains and losses between the primary and metastatic tumors were analyzed using Fisher’s exact test. P values were not adjusted for multiple comparisons.

The degree of CR between primary tumors and metastases by CGH was based on a probabilistic model developed by a mathematician (A. S.). Intuitively, the primary tumors and metastases should share a CR if they share a set of gains and losses not likely to be shared at random. The following probabilistic model was developed to quantify this intuition: first, let a1, a2, a3, and so forth be the specific abnormalities. The probability that a1 occurs is p(a1), the number of occurrences of a1 in tumor samples. Furthermore, P (a1) = 1 − X, where X is the proportion of the common chromosome. Thus, if the tumors have no events in common, the product defining X has no terms; it is standard in probability theory that such an empty product is defined as 1. The standard definition makes sense here because the probability of a CR is estimated as 1 − X. Thus, when the tumors have no events in common, the probability of a CR is estimated as 0. One could use more precise models, but in our data, this model gives a clear dichotomy between these two tumors pairs with a value of 1 − X near 1 and those far from 1. This model uses only data from shared abnormalities and assumes that the paired specimens must be both losses or both gains and that the breakpoint along the chromosome arm is the same.
RESULTS

Overview of Genetic Changes in Primary Breast Carcinomas and Their Metastases by CGH. Genetic changes were detectable by CGH in all but one of 29 primary breast carcinomas and in all of their metastases. The mean number (± SD) of changes per specimen was 8.7 ± 5.3 in the primary tumors (range, 0–20) and 9.0 ± 5.7 in the metastatic lesions (range, 1–24). In 13 cases, the metastasis had more genetic aberrations than the corresponding primary tumor, whereas in 12 cases, fewer genetic alterations were found in the metastasis.

The frequencies of involvement of the various chromosomal regions in gains (Table 1) and losses (Table 2) were remarkably similar in the primary tumors and in the metastases. For example, no significant differences were found in the frequencies of the most common gains (such as those involving 1q, 8q, and 17q) or losses (such as those involving 8p, 16q, and 17p). In contrast, 5 of the 29 metastases (17%) showed a gain of chromosome X (minimal region, Xq12–q22). This was not detected in any of the primary tumors (P = 0.05) by CGH nor by FISH with a specific probe (Fig. 1). There was also a tendency for some genetic changes to be more common in primary tumors than in the metastases. For example, gain of 6q22 was seen in 21% of primary tumors and 3% of metastases.

Degree of CR between Primary Tumors and Their Metastases:

Pairwise Analyses. Despite the fact that individual primary tumors and their metastatic lesions had very similar genetic changes when evaluated as groups, pairwise analysis revealed that the two specimen pairs were never identical. The degree of CR between the primary and metastatic cell clones varied substantially from one patient to another (Fig. 2). For example, several tumors and their metastases shared 3–7 genetic changes with identical breakpoints and had only one different aberration. This constitutes strong evidence for a close CR. At the other end of the spectrum, there was a metastasis with 23 genetic changes, none of which could be recovered from the corresponding primary tumor.

The number of shared genetic changes is a rough estimate of the degree of CR. Commonly occurring genetic changes in breast cancer are often likely to be shared between two specimens, whereas more infrequent changes and unique breakpoints provide strong evidence for a CR. A mathematical model was developed to more accurately quantify the degree of CR by estimating the probability that shared genetic changes in the paired specimens are not likely to be shared by chance alone. According to this analysis, 20 of the 29 cases (69%) had a high probability (greater than 0.95) for having a CR. In 17 of these cases, the probability was >0.999 (Fig. 2). In nine patients (31%), the genetic changes were almost completely different between the primary and metastatic lesions, so that no significant probability of CR emerged. In these cases, the few shared genetic changes between the two specimens were likely to be attributable to chance alone.

In an effort to evaluate whether the degree of CR between the metastasis and the primary tumor is reflected in the clinical behavior and pattern of metastatic dissemination of the cancer, we evaluated whether the clinicopathological features of the disease differed significantly in the two patient groups defined based on the degree of CR. No significant differences were found in any of the parameters studied (age at diagnosis, hormone receptor status, histological grade, the site of metastasis, or the time from diagnosis to metastasis) between patients with and without a CR.

Clonality Analyses by the X-Chromosome Inactivation Test. Clonality analyses were performed for 26 tumors representing paired specimens from 13 patients. Eight of the patients were informative (heterozygous) for the PGK alleles. Primary and metastatic specimens from all eight of these patients exhibited inactivation of the same X chromosome, suggesting that in each patient, the primary and metastatic specimens had the same clonal origin (Fig. 3). These eight pairs consisted of three cases with a clear CR by CGH and five with no CR. The findings indicate that despite of the substantially different genetic changes by CGH, primary tumors and their metastases are still likely to originate from the same precursor clone. Normal female DNA included as a control of nonneoplastic tissue showed polyclonality (Fig. 3).

Construction of Individual Clonal Evolution and Tumor Progression Pathways. It is possible to construct the genetic composition of a common progenitor cell clone for breast cancer based on the assumption that genetic changes shared between a primary tumor and its metastasis occurred early on during tumor progression, prior to the divergence of the primary and metastatic tumor clones. Subsequently, one can plot hypothetical genetic tumor progression pathways that lead to the development of the primary tumor or the metastasis (Fig. 4). This pathway construction was most informative in two cases, where both the primary tumor and two different metastatic lesions from different anatomical sites were available from the same patient for CGH analysis. In case 1 (Fig. 4A), the primary tumor (diagnosed in 1989), as well as the locoregional (diagnosed in 1991) and sternum (diagnosed in 1995) metastases, all showed gains of 9q and 17q21–q23, suggesting that these gains had already been present in the progenitor cell clone common to all of these specimens. However, subsequent genetic divergence and tumor progression had led to cell clones that were substantially different from one another in all the three tumor sites. Similarly, in case 4 (Fig. 4B), gains of 8q, 17q21–qter, and 19q13–qter were all detected in the primary tumor (diagnosed in 1988), as well as in metastases to both the lungs (diagnosed in 1990) and lymph nodes (diagnosed in 1990), suggesting that these aberrations had been present in the stemline clone common to all these lesions. Subsequently, however, the primary tumor showed loss of 11q14–qter and gain of 1q, whereas the lymph node metastasis only contained the former and the lung metastasis only the latter aberration.

Other examples of complex progression pathways are shown in Fig. 4.

Table 1 Most common gains (>15%) in primary breast cancers and their metastases

<table>
<thead>
<tr>
<th>Chromosomal region</th>
<th>Primary tumor</th>
<th>Metastatic tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1q</td>
<td>66% (19/29)</td>
<td>52% (15/29)</td>
</tr>
<tr>
<td>3cen–q22</td>
<td>21% (6/29)</td>
<td>7% (2/29)</td>
</tr>
<tr>
<td>3q26–qter</td>
<td>21% (6/29)</td>
<td>14% (4/29)</td>
</tr>
<tr>
<td>5p</td>
<td>10% (3/29)</td>
<td>17% (5/29)</td>
</tr>
<tr>
<td>5q22–q31</td>
<td>10% (3/29)</td>
<td>17% (5/29)</td>
</tr>
<tr>
<td>6q22</td>
<td>21% (6/29)</td>
<td>3% (1/29)</td>
</tr>
<tr>
<td>7p21–pter</td>
<td>21% (6/29)</td>
<td>14% (4/29)</td>
</tr>
<tr>
<td>8q</td>
<td>59% (17/29)</td>
<td>69% (20/29)</td>
</tr>
<tr>
<td>14q12–q21</td>
<td>17% (5/29)</td>
<td>17% (5/29)</td>
</tr>
<tr>
<td>16p13</td>
<td>14% (4/29)</td>
<td>17% (5/29)</td>
</tr>
<tr>
<td>17q22–q24</td>
<td>31% (9/29)</td>
<td>41% (12/29)</td>
</tr>
<tr>
<td>17q12</td>
<td>10% (3/29)</td>
<td>17% (5/29)</td>
</tr>
<tr>
<td>Xq2*</td>
<td>0%</td>
<td>17% (5/29)</td>
</tr>
</tbody>
</table>

a P = 0.05, Fisher's exact test.

Table 2 Most common losses (>15%) in primary breast cancers and their metastases

<table>
<thead>
<tr>
<th>Chromosomal region</th>
<th>Primary tumor</th>
<th>Metastatic tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p</td>
<td>17% (5/29)</td>
<td>28% (8/29)</td>
</tr>
<tr>
<td>6cen–q22</td>
<td>14% (4/29)</td>
<td>17% (5/29)</td>
</tr>
<tr>
<td>7cen–q22</td>
<td>17% (5/29)</td>
<td>3% (1/29)</td>
</tr>
<tr>
<td>8p</td>
<td>52% (15/29)</td>
<td>45% (13/29)</td>
</tr>
<tr>
<td>9p</td>
<td>21% (5/29)</td>
<td>24% (7/29)</td>
</tr>
<tr>
<td>11q14–qter</td>
<td>45% (13/29)</td>
<td>31% (9/29)</td>
</tr>
<tr>
<td>13cen–q14</td>
<td>21% (6/29)</td>
<td>21% (6/29)</td>
</tr>
<tr>
<td>14q23–qter</td>
<td>17% (5/29)</td>
<td>14% (4/29)</td>
</tr>
<tr>
<td>16q</td>
<td>24% (7/29)</td>
<td>31% (9/29)</td>
</tr>
<tr>
<td>17q</td>
<td>52% (15/29)</td>
<td>38% (11/29)</td>
</tr>
<tr>
<td>19p</td>
<td>17% (5/29)</td>
<td>24% (7/29)</td>
</tr>
<tr>
<td>22q</td>
<td>31% (9/29)</td>
<td>28% (8/29)</td>
</tr>
<tr>
<td>Xp</td>
<td>21% (6/29)</td>
<td>24% (7/29)</td>
</tr>
<tr>
<td>Xq</td>
<td>14% (4/29)</td>
<td>17% (5/29)</td>
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C–D, in cases in which a CR was found. Fig. 4, E–F, illustrates two examples of metastases that had only one shared change with the corresponding primary tumor but did show inactivation of the same X chromosome.

Evaluation of Cell Subpopulations by Interphase FISH Analysis. FISH analysis with specific probes was essential to complement the CGH results for two reasons. First, FISH provided validation for the findings obtained by the CGH analysis. Second, FISH made it possible to evaluate the degree of intratumor heterogeneity of tumor specimens. Altogether, 65 FISH experiments on 18 primary tumors and 18 metastases were performed to evaluate the concordance between CGH and FISH results. As indicated in Table 3, the concordance rates ranged from 95 to 100% for the 8q24 and 20q13 loci and for the pericentric region of chromosome X. The high prevalence of chromosome X gains in the metastases could be validated by FISH (Fig. 1).

We then used FISH to determine whether genetic changes that were only seen in the metastases were present in a subpopulation of cells of the primary tumor. For this analysis, we picked metastatic specimens with a prominent amplification in the metastasis by CGH with no evidence of amplification in the primary tumor. Not only were >200 cells scored from each slide, but the entire slide was scanned visually for individual highly amplified cells. In only a few cases were we able to find the metastatic clone present in the corresponding primary tumors as minute subpopulations. For example, the metastasis of case 22 showed gain of 1q, 8q24, and 17q23 and loss of 11q14–qter, none of which were found in the corresponding primary tumor. Using FISH, individual cells with amplifications of 8q24 and 17q23 were found as minute subpopulations (<5% of 300 cells scored) in the primary tumor, whereas the changes affecting 1q and 11q were not. Similarly, in case 18, FISH analysis of the primary tumor indicated a small subpopulation of 20q13 amplified cells, whereas all the cells in the metastasis were 20q13 positive. However, in most cases, such subpopulations with genetic characteristics of the metastatic cells were not all detectable in the primary tumors. For example, in case 23, gains of 8q and 13q seen by CGH and FISH in the metastatic tumor were not detectable in any cells of the primary tumor. Similarly, the gain of chromosome X was very prominent by CGH and FISH in the metastasis of case 11, but no X-amplified cells could be recovered in the primary tumor (Fig. 1). Because the gain of chromosome X appeared to be specifically enriched in the metastases, we intensely searched for these cells in the primary tumors. In none of the specimen
pairs, where metastases were amplified for chromosome X, could we detect these X-amplified cells in the primary tumor by FISH.

DISCUSSION

The ability of CGH to provide detailed comparisons of genetic changes in paired specimens of primary breast carcinomas and their metastases (taken up to a decade apart) has enabled the study of the metastatic progression of human breast cancer in vivo. The present study emphasizes this novel molecular cytogenetic view of tumor progression and metastasis, as achieved by the combination of CGH and FISH techniques. Similar longitudinal follow-up information has previously been difficult, if not impossible, to obtain from any other source than animal models. Furthermore, although the concept of clonal evolution of cancer is well known (33–35), it has not been possible to achieve detailed genetic characterization of the significance of this process for human cancer progression and metastasis. Here, CGH analyses indicated that primary tumors and their asynchronous metastases are often clearly derived from the same precursor clone. However, based on the often extensive differences in their genetic composition, clonal diversification and heterogeneity emerged as prominent characteristics of the breast cancer progression and metastasis process. This diversity is likely to derive from genetic instability of breast cancer cells and to be enforced by the different host selection pressures that the cancer cells must face in the different growth environments. Some genetic changes, such as the enrichment of gains of the Xq12–q22 region in metastases, may give clues to the basic molecular mechanisms providing a selective advantage for the metastatic process. Overall, the results have implications to understanding the biological basis of metastasis in human cancer in vivo, as well as the management of patients with metastatic breast cancer.

The monoclonal origin of cancer of the breast and other solid tumors has been established by X chromosome inactivation studies and genetic evidence (29, 30, 33–35). However, recent classical cytogenetic studies on breast cancer have suggested that up to 70% of breast carcinomas are cytogenetically polyclonal, i.e., characterized by multiple cytogenetically unrelated clones (17–19). Because we found by CGH some metastases to be clearly clonally related with the corresponding primary tumor and others showing no apparent relation compatible with the multiclonehypothesis, the same cases were also evaluated by X chromosome inactivation for monoclonality and CR. Our results supported the common clonal origin between all pairs of primary and metastatic lesions studied. This indicates that the substantial differences in the genetic composition between primary and metastatic tumors are likely to reflect genetic instability, clonal divergence, and selection of different cell subpopulations derived from a common progenitor cell clone.

The findings from those patients with multiple specimens available for analysis from both the primary and metastatic sites were particularly informative in suggesting that when a hypothetical common "progenitor" tumor precursor clone begins to expand and disseminate, it evolves to form a family of more and more genetically distant clones that then populate the different tumor regions and metastatic sites. The CGH results from paired specimens of the same patient allowed us to define a hypothetical clonal precursor clone. The definition of such a progenitor clone was based on the assumption that genetic changes present in both primary and metastasis are substan-
Fig. 4. A hypothetical common stemline and subsequent clonal evolution and tumor progression pathways for primary tumors and their metastases in 6 different breast cancer patients (A-F) who all had metastases that were clearly clonally related to the primary tumor. A hypothetical common stemline clone was first constructed based on the shared genetic changes between all specimens evaluated from a patient. The subsequent progression pathways and genetic changes characterizing these progression events are indicated with open arrows. DNA sequence gains are shown with a + and losses with a −. Subchromosomal breakpoints are not included in the figure. Localization and type of the tumor/metastasis and time of diagnosis are indicated. A, primary tumor (diagnosed in 1989), locoregional metastasis (diagnosed in 1991), and sternum metastasis (diagnosed in 1995) all showed a gain of 17q, suggesting that this aberration was already present in the stemline cell clone common to all specimens from case 1. Subsequently, the cell clones diverged and evolved independently in the different anatomical sites, leading to marked genetic heterogeneity. B, although a common stemline clone could be defined for all the specimens from case 4 based on three identical genetic changes, gain of 1q and losses of 8p and 11q were found in the primary tumor; the lung and lymph node metastases contained only one or two of these changes. C–F, clonal diversification pathways constructed for tumors from four other patients. The exact progression patterns varied from one case to another, but in all cases the resulting pattern of genetic changes was markedly different in the primary tumor than in the metastasis.
Comparison of the present results with those obtained previously must take into account the technical differences between all of the different technologies available for cancer genetic studies. CGH is similar to LOH analyses in that only the predominant genetic composition of a heterogeneous tumor cell population can be evaluated. CGH is better than LOH studies in that the entire genome is screened at once, that amplifications are reliably distinguished from losses, and that CRs can be ascertained. However, CGH cannot detect very small losses and amplifications and it does not discriminate between alleles. Thus, loss of one allele followed by duplication of the other would remain undetected by CGH. Although technically very demanding, conventional cytogenetic analysis is similar to CGH in surveying the entire genome at once. Karyotyping can also reveal tumor cell subpopulations, but only within the limits of the numbers of metaphase spreads available from solid tumors. Interphase FISH analysis extracts information about the copy number of specific loci in all cells of the tumor, but it still has only a limited ability to detect very small subpopulations, such as possible precursors of metastatic cells in the primary tumor (39). Whereas the present study has combined information from both CGH and FISH, as well as from X chromosome inactivation studies, additional studies of similar pairs of specimens from primary tumors and metastases by other techniques are warranted. Finally, the present study was based on a single specimen collected from the primary and metastatic tumors. Because all tumor sites may be heterogeneous, even more extensive sampling could be informative to conclusively trace the clonal evolution of cancer throughout the cancer tissue and its metastatic lesions.

In addition to providing in vivo evidence of the complex genetic basis and cell population dynamics underlying breast cancer progression and metastatic dissemination, the results may have some clinical implications. First, on the genetic level, metastatic breast cancers were proven to be extremely heterogeneous, not only between patients, but specifically between the individual tumor sites in a single patient. This heterogeneity may increase the adaptability of the tumor to different environmental challenges and may underlie the propensity of metastatic breast cancer to develop therapy resistance. Second, measurement of prognostic markers and markers of therapy responsiveness is usually performed from the primary tumors. This may often result in a very limited view of the spectrum of biological properties that the individual cells in the different metastatic sites may possess. Because metastatic cells often have a completely different genetic composition, their phenotype, including aggressiveness and therapy responsiveness, may also vary substantially from that seen in the primary tumors. As we recently showed in breast cancer, the predictive accuracy of estrogen receptor assays is substantially improved if specimens from the metastases are also available (40). After all, the metastatic cells represent the prime targets of cancer therapy. Finally, follow-up studies to pinpoint chromosomal regions and genes that are systematically differentially represented in the metastatic lesions are warranted as they may reveal potential targets that could be used therapeutically. The appearance of Xq12-q22 gains in several metastatic lesions identifies one candidate region for further study.

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