Frequent Somatic Imbalance of Marker Alleles for Chromosome 1 in Human Primary Breast Carcinoma

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ABSTRACT

Loss of heterozygosity at particular chromosomal loci in the tumor cell, as evidenced by restriction fragment length polymorphism analysis, has been taken as a hallmark of the presence of tumor suppressor genes. Recent studies of breast carcinoma have suggested that such genes might be located on the short as well as on the long arm of chromosome 1. We report here that comparison of constitutional and tumor genotypes of 84 breast tumors at 7 polymorphic chromosome 1 loci indicates a frequent imbalance of alleles on both 1p (12 of 61 informative patients) and 1q (37 of 71 informative patients). In about one-half of these cases, however, this imbalance was consistent with a gain in copy number of one allele in tumor DNA relative to normal DNA, rather than loss of the other. In 10 tumors we performed chromosome 1 enumeration in the interphase nucleus using in situ hybridization with a probe detecting the heterochromatin region at 1q12. These experiments confirmed the supernumary presence of region 1q12 in those tumors showing an allelic copy number gain of 1q. We suggest that there are several genes on chromosome 1 serving as targets for these changes, some of them associated with breast cancer development through their deletion and others through an increase in copy number.

INTRODUCTION

The involvement of chromosome 1 in breast carcinoma has been a continuous source of speculation ever since banding techniques allowed unambiguous chromosome identification (1). Although the total number of fully karyotyped primary breast tumor specimens is at present still limited due to various technical difficulties (2), the involvement of chromosome 1 in marker formation generally concerns simple chromosome addition (i.e., trisomy) or structural rearrangements with breaks occurring in the short arm as well as the long arm (3–8). An apparent clustering of translocation breakpoints in the region immediately adjacent to the heterochromatin has been suggested (4, 9). Recurrent interstitial deletions of particular chromosome 1 regions, e.g., 1p32, 1p22, 1p13, and 1q23, have been noted (4, 5, 8), although in many cases it remained unclarified whether or not this lost material was residing in unidentified marker chromosomes. By contrast, a frequent overrepresentation of 1q, and to a lesser extent of 1p, through isochromosome formation or trisomy, has been reported in several studies (1, 4–7).

Molecular genetic analyses, using polymorphic DNA markers to pairwise screen normal and tumor DNA from a series of breast cancer patients, provide a means to address this paradox. Three recent studies have hinted at the frequent (>25%) occurrence of heterozygous deletions at loci on 1p (10) and 1q (11, 12). Nonrandom somatic loss of heterozygosity at chromosomal regions is generally taken as an indication for the presence of a putative tumor suppressor gene in that region (13, 14). However, a gain in signal intensity of alleles, expected in at least a few cases on the basis of the cytogenetic data, was noted in just one study (12) and in a very low frequency (4%).

We report here an RFLP3 analysis of 84 primary breast carcinomas with seven chromosome 1 DNA markers. In addition, a chromosome 1 enumeration was conducted in 10 cases using in situ hybridization to breast tumor interphase nuclei (15). The combined results of these two approaches suggest that loss of heterozygosity does occur on 1p as well as on 1q in breast carcinoma but that a gain of marker alleles can be observed in comparable frequencies.

MATERIALS AND METHODS

Patients. Breast tumor patients were referred to two local hospitals between 1983 and 1988 for either mastectomy or tumor excision. Formalin-fixed, paraffin-embedded sections of all tumors were stained with hematoxylin and eosin and the tumors were classified histologically according to the criteria of the World Health Organization. Among the 84 carcinomas thus obtained were 74 infiltrating ductal, 5 infiltrating lobular, 2 intraductal, 1 colloid, 1 medullary, and 1 undifferentiated carcinoma.

Tissue Handling. Immediately after surgical removal, cells were isolated by scraping the freshly cut surface of the tissue. One-half of these were prepared for DNA flow cytometry (16), the other one-half were fixed in methanol/acetic acid (3:1) for in situ hybridizations. Part of the tissue was paraffin embedded, the remainder was snap-frozen in cold isopentane and stored at —70°C. For isolation of genomic DNA, frozen material was sliced into 40-μm sections and processed as described before (17). Thin 5-μm sections were taken at various positions and stained with hematoxylin and eosin to assess the percentage of nonmalignant cells.

DNA Probes. All polymorphic DNA markers used in this study, their associated RFLPs and chromosomal assignments, have been documented in the Human Gene Mapping Proceedings 10 (18). All probes yielded restriction patterns identical to those reported therein.

RFLP Analysis. DNA extraction from tumor specimens was performed as described previously (17). Isolation of DNA from 20-ml blood samples taken from breast cancer patients was done according to the salting out procedure (19). Methods for restriction endonuclease digestions, gel electrophoresis, transfer to Biotrace nylon filters (Gelman Sciences Inc.), and probe labeling have been described previously (17). Hybridization was allowed overnight at 65°C in 70% sodium dodecyl sulfate–0.5 M sodium phosphate, pH 7.3–1 mm EDTA (20). After exposure of the filters, the bound probe was removed by incubating the filters for 20 min at 90°C in 0.1% standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate. Autoradiographs were scanned with an LKB 2202 Ultrascan laser densitometer coupled to an LKB 2220 recording integrator.

The abbreviations used are: RFLP, restriction fragment length polymorphism; ISH, in situ hybridization; VNTR, variable number of tandem repeats.
Classification of Allelic Imbalance. The percentage of nonmalignant cells in tumor tissues was visually estimated by scoring representative histological sections at low magnification. In aneuploid tumors, this fraction was also calculated from DNA flow histograms by integrating peak areas (15). The estimated fractions of tumor cells in the 84 tumors examined were 70–90% in 45 cases, 50–70% in 27 cases, and 30–50% in 12 cases. Any imbalance in the ratio of allele intensities in the tumor sample relative to that in lymphocyte DNA was scored positive if it was roughly consonant with the percentage of tumor cells. For example, an imbalance of a factor 2.50 is expected in a tumor with allele loss in all tumor cells and containing 40% nonmalignant cells (1:0.4 in tumor versus 1:1 in lymphocyte DNA), while a factor of 1.43 (1:0.7) is expected in a sample containing 70% normal DNA. A decrease of this factor may be caused by the heterogeneity with which the imbalance occurred among tumor cells. An arbitrary factor of 1.3 was taken as a cutoff below which any imbalance was considered inconclusive.

Interpretation of allelic imbalance in terms of allelic gain or loss is dependent on the amounts of normal and tumor DNAs loaded on the gel. Potential unequal loading was controlled in two ways: (a) by visual inspection of ethidium bromide-stained gels of separated DNA samples and (b) by comparing the results obtained with a single DNA filter by its repeated probing, stripping, and reprobing (17) with a variety of DNA markers. In our laboratory we are currently screening tumor samples with DNA markers for all nonacentric chromosomes, and a DNA filter may be probed as many as 10 times during this procedure. Therefore, most patients will be homozygous for at least one RFLP, providing a reference for the amounts of DNA blotted onto the filter.

In Situ Hybridization. Plasmid DNA of probe pUC1.77 (21) was nick-translated with biotin-11-dUTP (22). Isolation and preparation of nuclei from breast tumor tissues and methods for ISH have been described previously (15). The probe pUC1.77 detects the heterochromatin region at 1q12 (23), and hybrids were visualized with an avinidin-fluorescein complex (24). Approximately 250 nuclei were scored independently by two or three observers who had no advance knowledge of results of histological examination and DNA flow analysis of the tumor. Nuclei with very bad morphology, “doublet” or “blurred” signals (15, 23, 25), or clumped nuclei were discarded. No discrimination was made on the basis of cytology during spot counting.

RESULTS

Loss or Gain of Chromosome 1 Alleles Occurs Approximately Equally Frequently. We performed an RFLP analysis of 84 paired normal (lymphocyte) and breast tumor DNA samples with 4 polymorphic DNA markers for chromosome 1p and 3 for 1q. In 41 cases we found an increase in the ratio of allele intensities in tumor relative to normal DNA with either 1p or 1q markers or both. This increase, operationally defined as allelic imbalance, was quantified by densitometry analysis. Reprobing of the filter with polymorphic DNA markers for other chromosomes, some which will be fortuitously homozygous in that patient, provided a control for the amounts of blotted normal and tumor DNA and enabled interpretation of the imbalance in terms of signal gain or loss. The data presented in Fig. 1 and Table 1 exemplify this procedure in more detail.

Using probe pMUC10, which detects the MUC1 locus at 1q21, we found the signal of one allele to be significantly reduced relative to normal DNA in several tumors, while the signal of the other allele was retained (e.g., cases 8, 194, 211; Fig. 1A). Homozygous controls indicated that approximately equal amounts of normal and tumor DNA were loaded for case 211 but that relatively less tumor DNA was present for cases 8 and 194 (control in Fig. 1A). Therefore, we concluded that the loss of the signal of the lower allele in tumor 211 was due to allelic loss, while in tumors 8 and 194 this signal loss was caused by unequal loading. Consequently, the allelic imbalance in these tumors was interpreted as an actual gain in copy number of the opposite allele (Table 1).

A gain in signal intensity of one allele in conjunction with an unchanged signal of the other was also observed (e.g., at MUC1 in tumors 35, 67, 156, 161, 169, 183, 201; Fig. 1A). Here, homozygous controls indicated approximately equal loading of normal and tumor DNA pairs, supporting the interpretation of the imbalance in these tumors as allelic gain (Table 1). Comparable results were obtained with markers for the short arm of chromosome 1, e.g., loss of an allele in tumor 123 and gain in tumor 176 at locus D1S57 (Fig. 1B; Table 1). Note that quantified imbalances were always greater than the cutoff value of 1.3, and, with few exceptions, in the order of magnitude expected on the basis of the percentage of nonmalignant cells present in the tumor sample. This supports the validity of our quantification method which is apparently not influenced by the unequal intensities of alleles in constitutional DNA sometimes generated by probes detecting a VNTR type of polymorphism (Fig. 1, N lanes). Finally, some tumors exhibited an increase in intensity of one allele with a concomitant decrease in intensity of the other (e.g., at MUC1 in tumors 64 and 125, Fig. 1A).

Table 2 presents an overview of the results thus obtained with all seven chromosome 1 markers. Of the cases heterozygous for at least one marker, 20% showed allelic imbalance on the short arm, and 52% showed allelic imbalance on the long arm of chromosome 1. Among the tumors examined, allelic gain on either the short or the long arm of chromosome 1 occurred about equally frequently as loss (Table 2; 5 versus 7 cases on 1p, 21 versus 16 cases on 1q).

Mapping of Chromosome Regions Affected by Allelic Imbalance. Most tumors with an allelic imbalance at a particular locus showed comparable shifts in signal ratio of the two alleles at informative flanking loci. Compare, for example, allelic imbalances at MUC1 in tumors 125 and 183 (Fig. 1A) with those at D1S61 (Fig. 1C). However, in two tumors (67, 169) the allelic imbalance was noted at the MUC1 locus (1q21) (Fig. 1A) but not distally at D1S61 (1q24-q31) (Fig. 1C). No statistically significant association was found between the detected alterations on 1p and 1q using x2 analysis (not shown).

Rearrangement of the MUC1 Locus. In one tumor we obtained evidence to suggest that the MUC1 locus had undergone a rearrangement (Fig. 1D). Patient 130 was constitutionally homozygous at the MUC1 locus, as indicated by the single band in lymphocyte DNA. In tumor DNA from this patient two bands were observed, one at a new position and one of the same size as that of the constitutional alleles. Hybridization of the same filter with a number of highly polymorphic DNA markers from other chromosomes verified the consanguinity of the DNA samples in that restriction patterns were always identical (not shown).

In Situ Hybridization Indicates a Frequent Supernumary Presence of 1q12. We have used the probe pUC1.77 in an ISH to highlight the subregion 1q12 in interphase nuclei harvested directly from tumor tissues. The target sites of pUC1.77 become visible as distinct spots in the nucleus. The peak in the distribution of numbers of spots/nucleus has been shown earlier to reflect the modal number of chromosomes present in that population of nuclei (15, 23, 25). Of the 84 tumors investigated with RFLPs, fresh nuclei were available from 10 cases (Table 3). Two observers, although at odds with respect to the sizes of the populations, both counted major populations with either 3 or 4 signals in nuclei from tumor 289 (Fig. 2). Such discordance
SOMATIC IMBALANCE OF MARKER ALLELES FOR CHROMOSOME 1

Fig. 1. Southern analysis of constitutional normal (N) and tumor (T) DNA pairs. (A) probe pMUC10 (MUC1), EcoR I digest; (B) pYNZ2 (D1S57), BamHI digest (left), HindIII digest (right); (C) pMLJA-1 (D1S61), TaqI digest; (D) pMUC10 (MUC1), EcoR I digest. Bottom abscissa, patient identification numbers; ordinates, size of marker fragments in kilobases (kb). Control probes were: p17H8 (D17Z1) for tumors 8, 35, 64, 67; pK083 (D4S10) for tumor 125; p68RS2.0 (RBI) for tumors 169, 183, 194, 201; pHM2.6 (MYB) for tumor 211.

Table 1. Quantification and classification of allelic imbalances shown in Fig. 1 versus percentage of nonmalignant cells in tumor samples

<table>
<thead>
<tr>
<th>Tumor</th>
<th>% nonmalignant cells</th>
<th>Probe</th>
<th>Expected* Observed*</th>
<th>Classification of allele changes</th>
</tr>
</thead>
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<tr>
<td>8</td>
<td>30</td>
<td>pMUC10</td>
<td>1.7</td>
<td>Gain upper allele</td>
</tr>
<tr>
<td>35</td>
<td>20</td>
<td>pMUC10</td>
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<td>Gain (upper)</td>
</tr>
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<td>64</td>
<td>30</td>
<td>pMUC10</td>
<td>5.6</td>
<td>Loss/gain (upper/lower)</td>
</tr>
<tr>
<td>67</td>
<td>50</td>
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<td>1.5</td>
<td>Gain (upper)</td>
</tr>
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<td>10</td>
<td>pYNZ2</td>
<td>10.0</td>
<td>Loss (upper)</td>
</tr>
<tr>
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<td>30–50</td>
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<td>6.9</td>
<td>Loss/gain (upper/lower)</td>
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<tr>
<td>156</td>
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<td>1.7</td>
<td>Gain (lower)</td>
</tr>
<tr>
<td>161</td>
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<td>1.6</td>
<td>Gain (upper)</td>
</tr>
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<td>169</td>
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<td>Gain (lower)</td>
</tr>
<tr>
<td>176</td>
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<td>pYNZ2</td>
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<tr>
<td>130</td>
<td></td>
<td>pMLJA-1</td>
<td>1.2</td>
<td>No change</td>
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* Extent to which the ratio of allele intensities in the tumor will differ from that in normal DNA, assuming that a loss or a duplication of an allele occurred in all tumor cells (see also “Materials and Methods”).

DISCUSSION

In this study we have combined two approaches to address the involvement of chromosome 1 in breast cancer pathogenesis: (a) a molecular genetic approach, using polymorphic DNA markers for both the short and long arm, to allow the identification of loss or gain of chromosome homologues and (b) in situ hybridization of nondividing interphase nuclei harvested directly from tumor specimens, using a probe detecting the heterochromatin region at 1q12, to assess the modal number of target sites in that population of cells (15, 23, 25). Our RFLP analysis shows that a remarkably high proportion (52%) of the informative tumors have alterations of the long arm of chromosome 1, while the short arm was similarly involved in 20% of the cases. In both instances, about one-half of the alterations concern a gain of chromosomal material rather than loss. The fact that pUC1.77 and pMUC10 are located in the two adjacent regions...
The gain of heterozygosity at the MUC1 locus detected by us explain the findings in tumors 267 and 286 where we found between the two probes pUC1.77 and pMUC1.0. This may bladder cancer (25, 26) were found to contain populations of nuclei with multiple target bands Iq12 and Iq21, respectively, warrants a direct comparison of the results obtained with both probes. In two tumors with a gain of Iq alleles, we were able to demonstrate the supernormal presence of Iq12 by ISH with pUC1.77 in significant populations of nuclei. In addition, four tumors that showed no detectable changes in the ratio of allele intensities were found to contain populations of nuclei with multiple target sites for pUC1.77. This suggests that the frequency of chromosome 1 alterations detected by an RFLP screening represents a minimal estimate. Notably, the presence of extra chromosome 1 material is not necessarily reflected in DNA flow cytometry by aneuploidy, a finding that has also been documented for bladder cancer (25, 26).

Several explanations can be suggested for the inability of RFLP analysis to detect in some cases the extra copies of Iq12 as evidenced by ISH: (a) a simple duplication of both chromosome 1 homologues would not alter the allelic ratio, (b) extreme intratumor cell heterogeneity with regard to spot/nucleus content (e.g., tumors 197 and 266) may obscure or neutralize any imbalance in this ratio, and (c) chromosome translocations with a break between Iq12 and Iq21 may disrupt the physical link between the two probes pUC1.77 and pMUC1.0. This may explain the findings in tumors 267 and 286 where we found allele loss on Iq in conjunction with multiple target sites for pUC1.77. The region Iq12-q21 has been proposed to be a translocation breakpoint cluster region in breast tumors (4, 9).

The gain of heterozygosity at the MUC1 locus detected by us in tumor 130 may also have been generated by a translocation with the breakpoint occurring in one of the constitutional EcoRI fragments, although replacement of one constitutional allele by an allele in which a point mutation has affected one of the EcoRI sites cannot be excluded by our data. Gendler et al. (12) also found evidence for rearrangements at the MUC1 locus in 2 of 88 tumors investigated.

In four tumors we observed a loss of signal intensity of one MUC1 allele and a concomitant gain of the other. One possible explanation for this is a somatic recombination event proximal of the MUC1 locus (27). The residual signal at the position of the lost allele may be due to variable proportions of contaminating normal DNA in the tumor sample, originating from stroma cells or infiltrating lymphocytes, or intratumor heterogeneity for this event.

The finding that chromosome 1 marker alleles can be over-represented as well as lost in breast tumors is in agreement with cytogenetic studies which have indicated the occurrence of both deletions and duplications of chromosome 1 regions. Notably, multiple different rearrangements affecting distinct regions may coexist in the same tumor (5, 8). Because rearrangements of chromosome 1 in breast tumors are often complex, cytogenetic studies have not yet been able to decisively delineate a common region for either of these alterations. We observed two tumors in which alterations of Iq affected only the proximal half of the long arm, more specifically the region proximal of D1S61 and including the MUC1 locus. Interestingly, the same segment of Iq was cytogenetically found to be duplicated in one breast tumor (4).

The results presented here are in striking contrast with three recent reports on loss of heterozygosity on chromosome 1 in breast cancer. The specific involvement of the region Iq32-p34 in allelic losses was reported in 15 cases (41%) (10). All these events were attributed to simple deletions and virtually no alterations were detected with a marker for Iq. Subsequently,
in a series of 48 tumors, it was shown that about 25% carried a heterozygous deletion involving at least the region 1q23-q32, while a deletion on 1p was found only once (11). Finally, Gendler et al. (12) found alterations of the MUC1 locus in 24 of 70 informative cases (34%), of which 21 concerned allelic loss and 1 showed a gain of allele intensity. Especially the latter two reports are in remarkable contrast with cytogenetic data presented by Gerbault-Seureau et al. (7), who demonstrated 3–5 copies of 1q in 7 of 12 cases examined. Others (1, 4–6) have also noted the recurrent presence of extra copies of 1q in substantial numbers of tumors examined. Differences in classification and interpretation of the observed imbalances in allele intensities may have contributed to the differences between the findings reported here and in previous RFLP studies of breast tumors. Our quantification results were in most cases in the range expected on the basis of the percentage of nonmalignant cells present in the tumor. In some cases with allele loss, the imbalance factor was smaller, suggesting tumor heterogeneity for the detected event (e.g., case 211, Table 1), while in some tumors with allelic gain this factor was greater, suggesting that more than one copy was acquired.

The question arises whether the detected alterations of chromosome 1 in breast cancer reflect the involvement of a locus of crucial importance for its development. We show here that particular chromosome 1 regions may be found overrepresented in one tumor but deleted in another. Studies of somatic cell hybrids have suggested the presence of one or more growth-regulating genes on chromosome 1 (28, 29), while more recently the region 1q23-qter was shown to harbor a gene associated with the induction of a limited life span when introduced into an immortal Syrian hamster cell line (30). On the other hand, it has been suggested (12) that one or more of the eight oncogenes located on chromosome 1 may be involved in breast cancer tumorigenesis, either by mutation or by amplification. One possibility is that in each tumor a distinct set of genes on chromosome 1 is associated with breast cancer development. In that case, allelic loss would signify the presence of a growth-regulating gene, while allelic gain would involve a growth-promoting gene. Consequently, different selective pressures could be operating on different regions of chromosome 1, perhaps forcing it into complex rearrangements.

Thus, it seems that the role of chromosome 1 in allelic loss is not as comprehensible as that of other chromosomes identified in this way in previous studies. Allele losses have been noted to occur on 3p, 11p, 13q, and 17p (17, 31–34). On 3p, 13q, and 17p, the deletions define a common region (17, 34, 35), which has led to the identification of the RB1 gene on 13q and the p53 gene on 17p as candidate target genes for the deletions (36–38). Furthermore, it is becoming increasingly evident that multiple chromosomes can be involved in allelic loss simultaneously in a single tumor (17). This study and those by others (10–12) indicate that chromosome 1 should be added to this list, although its complex rearrangements will severely hamper attempts to isolate the target genes through deletion mapping and “reverse genetics” procedures.

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