Advances in Brief

Multiple Regions of Chromosome 4 Demonstrating Allelic Losses in Breast Carcinomas[1]

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Abstract

Allelotyping studies suggest that allelic losses at one or both arms of chromosome 4 are frequent in several tumor types, but information about breast cancer is scant. A recent comparative genomic hybridization analysis revealed frequent losses of chromosome 4 in breast carcinomas. In an effort to more precisely locate the putative tumor suppressor gene(s) on chromosome 4 involved in the pathogenesis of breast carcinomas, we performed loss of heterozygosity studies using 19 polymorphic microsatellite markers. After precise microdissection of archival surgical cases, we analyzed DNA obtained from 44 breast carcinomas for loss of heterozygosity. In addition, DNA from tumor cell lines derived from 14 of these 44 breast carcinomas were also analyzed. We observed deletions of chromosome 4 at multiple sites in both tumor cell lines and breast carcinomas. The deletions in cell lines and their corresponding tumors were extensive in nature, whereas they were more localized in noncultured breast carcinomas. The localized deletions in the noncultured breast carcinomas clearly defined four nonoverlapping regions of frequent deletions: 4q33–34 (76%); 4q25–26 (63%); 4p15.1–15.3 (57%); and 4p16.3 (50%). Our results suggest that there may be multiple putative tumor suppressor genes, located on both arms of chromosome 4, whose inactivation is important in the pathogenesis of breast cancer.

Introduction

Breast cancer is the most frequent neoplasm among women (1). It is now well recognized that tumorigenesis is a multistep process resulting from the accumulation of sequential genetic alterations (2). In addition to oncogene activation, inactivation of tumor suppressor genes has been shown to play an important role in tumorigenesis (2). Allelic deletion manifested as LOH[3] at polymorphic loci is recognized as a hallmark of tumor suppressor genes, whose other allele is inactivated by point mutations or by some other mechanism (3–6). The delineation of such genetic alterations that occur in breast cancer may be important for both early detection and prognosis.

In breast cancers, LOH has been described on one or both arms of multiple chromosomes (3, 7–13). However, with the exception of 8p, 11q, 16q, and 17p, the highest reported frequencies of LOH were usually less than 50%. Detection of allelic loss in human breast cancer is hindered by the fact that cancer tissues frequently contain a high percentage of nonneoplastic cells. As demonstrated by Fujii *et al.* (14), microdissection permits the detection of allelic loss at higher frequencies than recognized previously.

Previous allelotyping studies have documented allelic loss on one or both arms of chromosome 4 in several neoplasms including bladder, cervical, colorectal, hepatocellular, and esophageal cancers and in squamous cell carcinomas of head and neck and of the skin (6, 15–20). Our recent studies demonstrated frequent losses at three nonoverlapping sites located on both arms of chromosome 4 in MMs and SCLCs (21). Phenotypic tumor suppression has been observed by the introduction of chromosome 4 into human glioma cells (22). Thus, chromosome 4 likely contains one or more tumor suppressor genes that are frequently inactivated in several types of cancers, although data from breast cancers are sparse. CGH studies on breast carcinomas (23–26) describe a complex pattern of gains and losses at many chromosomal locations. However, a recent CGH report found frequent losses at chromosome 4q in breast carcinomas analyzed in this study, with a distal region of 4q (~4q28-ter), as the smallest overlapping region deleted (27). CGH is not as sensitive as allelotyping, and the technique does not detect smaller detections.

The present study was undertaken to determine the presence and locations of regions of frequent deletions involving chromosome 4 in breast carcinomas. Using a precise microdissection methodology, DNA from archival paraffin-embedded tissue sections of breast carcinomas, and DNA from cell lines derived from some of these breast carcinomas, we studied the incidences of LOH on the p and q arms of chromosome 4.

Materials and Methods

Tumors and Cell Lines. Paraffin-embedded archival material from surgical resections of 44 breast carcinomas acquired from Parkland Memorial Hospital (Dallas, TX) were used for this study. Fourteen paired breast cancer and lymphoblastoid cell lines corresponding to 14 of the tumors were also studied. Additional details about these cell lines have been published elsewhere (28).

Microdissection and DNA Extraction. Areas of malignant cells were identified by pathological review of all of the cases of breast carcinomas. These areas were precisely microdissected under microscopic visualization, avoiding contamination by nonmalignant cells (29). Stromal cells from the microdissected slides provided a source of constitutional DNA. Approximately 500-1000 tumor and stromal cells were microdissected from each case. The dissected cells were digested using the proteinase K method described previously (29), and 5 μl of the DNA samples were used directly for each multiplex PCR reaction.

LOH Analyses. Microsatellite analysis was used to determine the frequency and pattern of allelic loss on chromosome 4 using 19 polymorphic markers, 11 markers on 4q and 8 markers on 4p. Primer pairs that were used to identify specific dinucleotide and tetranucleotide repeat polymorphisms in genomic DNA were obtained from Life Technologies, Inc. (Gaithersburg, MD). The primer sequences used for LOH studies were obtained from the Genome Database. The relative order of these markers was ascertained from the Genethon map of chromosome 4 (30).

Two rounds of PCR (multiplex PCR followed by uniplex PCR) were performed to amplify each marker used in this study (4). A 10°C "touch down" strategy was used spanning the primers annealing temperature, followed by 25

[3] The abbreviations used are: LOH, loss of heterozygosity; CGH, comparative genomic hybridization; MM, malignant mesothelioma; SCLC, small cell lung carcinoma.

cycles at the optimal annealing temperature. The final products were separated on a 6% denaturing polyacrylamide gel and subjected to autoradiography. LOH was scored by visual detection of the absence of the upper or lower allele of informative cases.

Statistical Analysis. Fisher's exact two-tailed test was used for statistical evaluation of the differences between the percentage of loss of two different regions or their combinations. $P < 0.05$ was regarded as statistically significant.

Results

Microsatellite analysis was used to determine the frequency of allelic loss from chromosome 4 in breast carcinomas and breast carcinoma cell lines. The patterns of LOH and the frequencies of allelic loss at different critical regions are shown in Fig. 1.

The overall frequencies of allelic loss at any chromosome 4 site in the breast carcinomas and breast carcinoma cell lines were high, with 40 of 44 (91%) breast carcinomas and 11 of 14 (79%) cell lines showing LOH at one or more informative markers. In 9 of 14 (64%) breast carcinomas and their corresponding cell lines, the chromosome 4 deletions were extensive, with a loss of all or most of both arms (Figs. 1 and 2). There was an excellent correlation between the extent of the losses in tumors and the extent of the losses in the corresponding cell lines (Fig. 2), although in three cases (HCC1500, HCC1143, and HCC70), the deletions in the cell lines were more extensive. In noncultured breast carcinomas, the deletions were more localized, except in three cases. The extent of the partial deletions was used to identify four discrete minimal regions of nonoverlapping deletions (two on 4q and two on 4p; Figs. 1 and 2). Representative autoradiographs for cases with partial loss of chromosome 4 demonstrating loss and retention of neighboring alleles in noncultured breast carcinomas are illustrated in Fig. 3. There was no marked bias in the allele loss pattern, with loss of the upper allele in 56% of the cases and loss of the lower allele in 44% of the cases, as illustrated in Fig. 3.

At 4q, the region between markers D4S408 and D4S171 (4q33–34) was a minimal region of deletion previously designated R1 (21), with allelic loss in 27 of 34 (79%) breast carcinomas (Fig. 1). The other minimal region of deletion on 4q was observed between the markers D4S1586 and D4S175 (4q25–26; designated R2; Ref. (21), with allelic loss in 26 of 39 (67%) tumors (Fig. 1).

We found two minimal regions of deletion at 4p: (*a*) one previously identified by us as R3 (21); and (*b*) one other region. The region between markers D4S1546 and D4S404 (4p15.1–15.3; designated R3) demonstrated allelic loss in 26 of 41 tumors (63%; Fig. 1). The newly identified region of deletion on 4p (4p16.3; designated R4) lay between markers D4S43 and D4S127 and demonstrated allelic loss in 22 of 38 (58%) tumors (Fig. 1).

Table 1 shows data on patterns of allelic loss involving the four
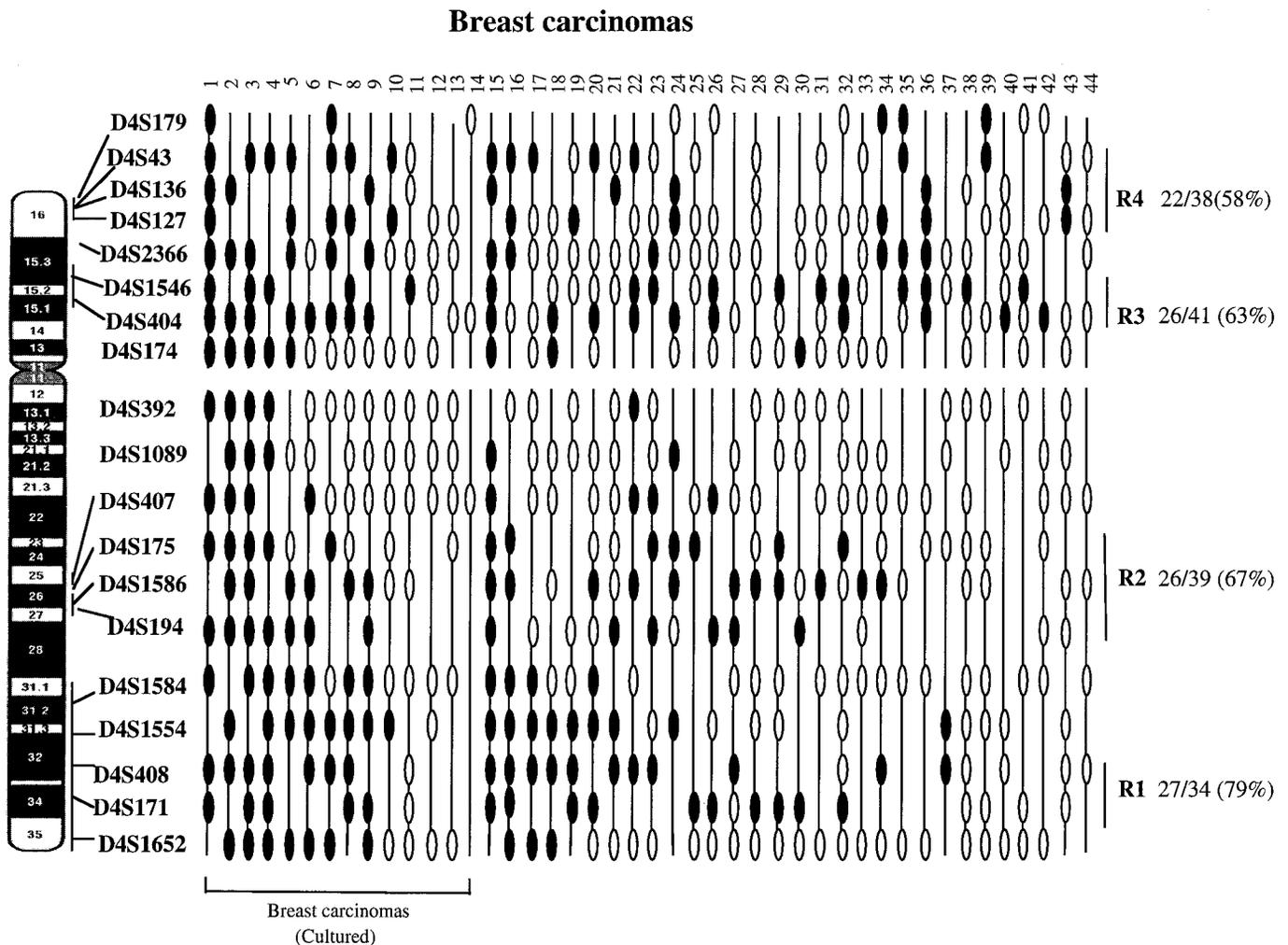
Breast carcinomas

Fig. 1. Patterns of LOH in breast carcinomas, exhibiting deletions of 4q and 4p. The cases have been arranged from left to right in decreasing order of chromosome 4 deletions (cases *1–14* and cases *15–44*). Cases 1–14 represent breast carcinomas from which the cell lines were derived. Markers are placed in the predicted order from 4pter-qter. Regions (*R1–R4*) showing frequent allelic losses are identified by *bars* at the *right*. *Closed ellipses*, allelic loss; *open ellipses*, retention of heterozygosity; *line*, noninformative.
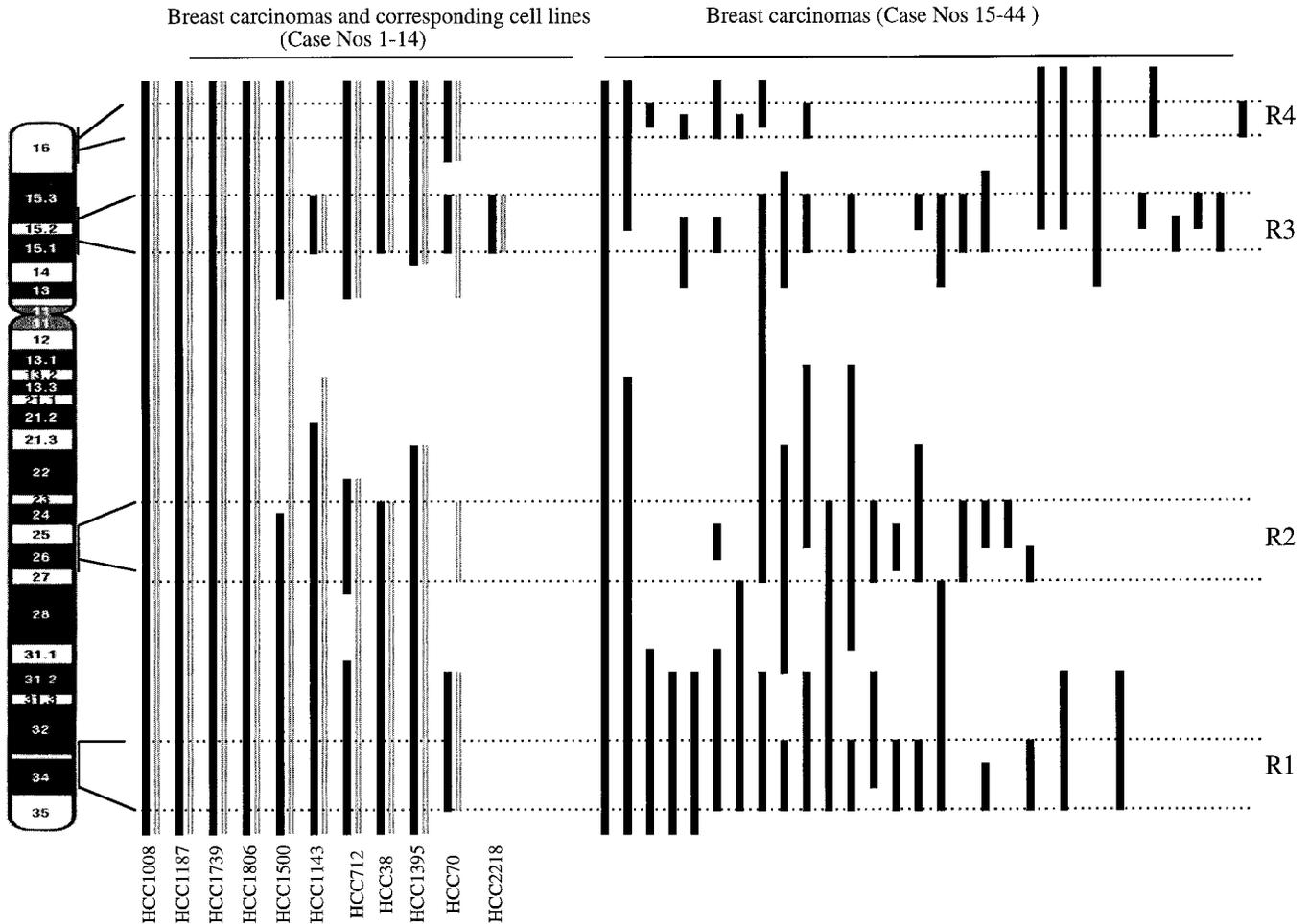
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Fig. 2. Diagrammatic representation of chromosome 4 deletions in breast cell lines, their corresponding cultured breast carcinomas, and sporadic breast carcinomas. The cases are arranged in decreasing order of the extent of chromosome 4 deletions (as described in the Fig. 1 legend). The comparison of the deletions in cultured breast carcinomas and the corresponding cell lines is shown in the *left portion* of the figure. The *hollowgram bars* on the *right* represent breast carcinoma cell lines, whereas the *solid bars* on the *left* represent the corresponding breast carcinomas from which the cell lines were derived. The cell line numbers are shown at the *bottom*. The deletions in noncultured breast carcinomas (*solid bars*) are shown in the *right* portion of the figure. The *dotted lines* represent minimal regions of deletions. Differences in lengths and positions of bars within a region of minimal deletions are based on the number of markers in that region and the orientation (telomeric or centromeric) of deletions. Three cell lines (HCC1685, HCC1569, and HCC2157) and their corresponding breast carcinomas (cases 12–14 in Fig. 1) and one noncultured breast carcinoma (case 44 in Fig. 1) did not show LOH at any of the informative markers; therefore, they are not shown in Fig. 3.

chromosome 4 regions. Region R1 was lost by itself in 2% of the cases, whereas together with regions R2, R3, and R4, it was lost in 73%, 63%, and 57% of the cases. Region R2 was lost by itself in 2% of the cases, whereas together with regions R3 and R4, it was lost in 59% and 47% of the cases. Region R3 was lost by itself in 8% of the cases, whereas together with region R4, it was lost in 49% of the cases. Region R4 was lost by itself in 4% of the cases. The data demonstrate that the most frequently observed pattern was loss of two or more regions. However, each of the four regions may be lost independent of the other three regions.

Discussion

Several previously published reports have documented that allelic losses at chromosome 4 are present in several tumor types (6, 15–21), although breast cancers have not been studied in detail. At least three discrete regions of frequent loss that we have termed R1 (4q 33–34), R2 (4q 25–26), and R3 (4p 15.1–15.3; Ref. 21) have been identified in mesotheliomas and SCLCs. We performed allelotyping to identify the critical regions of allelic loss on chromosome 4 in breast carcinomas. Frequent losses were noted at the three previously identified sites, as well as at a newly identified region at 4p16.3 (termed R4). In

noncultured cancers, the losses were relatively small and discrete, whereas in successfully cultured tumors, they were larger and involved all or most of the chromosome. Of interest, the deletions in breast cancer cell lines and their corresponding tumors were similar in size. We have previously noted that the phenotypic and genotypic properties of successfully cultured breast cancers and their corresponding cell lines demonstrate a high degree of concordance. However, the cultured tumors (when compared to noncultured tumors) had several features indicating that they were derived from a subset of aggressive tumors having a poor prognosis (28). The findings of the present study demonstrating that cultured tumors have larger deletions with frequent loss of all four critical regions support our previously reported results. Cell lines provide reagents for testing candidate oncogenes in these critical regions.

One major difficulty encountered when using DNA isolated from a small number of microdissected cancer cells from tumor tissue for PCR analysis of microsatellite repeat length alleles is artifactual allelic loss resulting from too little PCR target template. Artifactual allelic losses preferentially target the larger-sized microsatellite repeat sequence. Our data are not likely to have been affected by this problem for two reasons: (*a*) allelic loss data from tumor tissue DNA
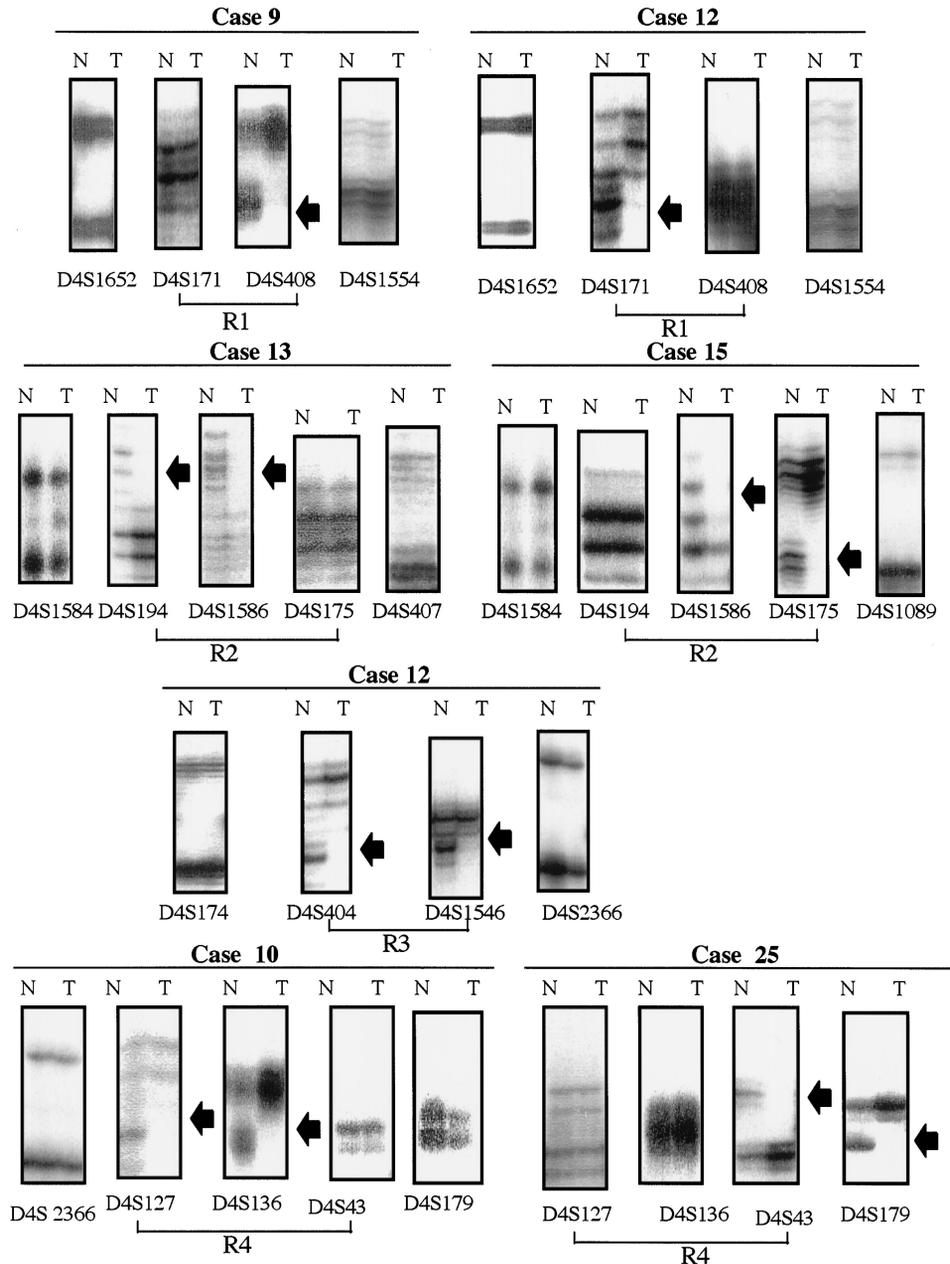
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Fig. 3. Representative autoradiographs for cases with discrete allelic loss at chromosome 4 demonstrating loss and retention of neighboring alleles in noncultured breast carcinomas. Het, retention of heterozygosity; Ni, noninformative. Case 9 (D4S1652-Het, D4S171-Ni, D4S408-LOH, D4S1554-Het) and case 12 (D4S1652-Het, D4S171-LOH, D4S408-Ni, D4S1554-Het) define the region R1 between D4S408 and D4S171. Case 13 (D4S1584-Het, D4S194-LOH, D4S1586-LOH, D4S175-Ni, D4S407-Het) and case 15 (D4S1584-Het, D4S194-Ni, D4S1586-LOH, D4S175-LOH, D4S1089-Het) define the region R2 between D4S175 and D4S194. Case 12 (D4S174-Het, D4S404-LOH, D4S1586-LOH, D4S2366-Het) defines region R3 between D4S174 and D4S2366. Case 10 (D4S2366-Het, D4S127-LOH, D4S136-LOH, D4S43-Ni, D4S179-Het) and case 25 (D4S127-Het, D4S136-Ni, D4S43-LOH, D4S179-LOH) define the region R4 between D4S43 and D4S127. N, normal; T, tumor. DNAs were assayed as described in "Materials and Methods" using the microsatellite markers indicated. Arrowheads, LOH.

and cell line DNA (which is not limited) correspond with each other; and (b) there was no preferential loss of the upper allele (in 56% of the cases, the upper allele was lost, whereas in 44% of the cases, the lower allele was lost; Fig. 3).

In breast carcinomas, the most frequently deleted region (79%) was R1 (4q33–34). This region, which is about 14-cM long, is also frequently deleted in MM; SCLC; bladder, cervical, and esophageal cancers; and squamous cell carcinomas of the head and neck and the skin (6, 15–20, 21). Of interest, the recent CGH study (27) detected a distal 4q region (~4q28-ter) as the smallest overlapping region lost in the breast carcinomas that were analyzed in that study. Although this region is longer than R1, it is within the 4q region that includes R1. CGH is not as sensitive as allelotyping, and the technique does not detect smaller deletions. Nevertheless, the CGH findings reported recently (27) are in agreement with our observations. The other critical region on 4q (R2) includes markers D4S194, D4S1586, and D4S175 located at 4q25–26. Previous studies have shown a common region of deletion at or near this site in squamous cell carcinomas of

both the head and neck and the skin, MM, and SCLC (19, 20, 21). Presently, there are no known candidate suppressor genes in regions R1 and R2.

The R3 region on 4p is delineated by markers D4S1546 and xD4S404 (4p15.1–15.3). This region, which is about 3 cM in length, was previously found to be frequently deleted in bladder cancer, MM,

Table 1 *Patterns of allelic loss on chromosome 4*

The chromosomal regions are: R1, regions between D4S171 and D4S408 (4q33–34); R2, region between D4S175, D4S1586, and D4S194 (4q25–26); R3, region between D4S1546 and D4S404 (4p15.1–15.3); R4, region between D4S43 and D4S127 (4p16.3). The data in the table are combined data from tumors and cell lines ($n = 49$). All values for loss of only one region were significantly different from the values for loss of the same region in combination with other regions ($P < 0.05$).

	R1	R2	R3	R4
R1	1 (2%)	36 (73%)	31 (63%)	28 (57%)
R2		1 (2%)	29 (59%)	23 (47%)
R3			4 (8%)	24 (49%)
R4				2 (4%)

and SCLC (6, 21). Presently, there are no known candidate suppressor genes in this region. The other critical region on 4p lies between D4S43 and D4S127 and was reported to be frequently deleted in bladder carcinoma (31). This region is only 750-kb long and is located near the Huntington's disease gene. There are at least three genes (ring finger gene, SH3BP2 gene, and adducin gene) known to be present within this 750-kb region that are candidate suppressor genes (32–34).

In summary, deletions of chromosome 4 are frequent in breast carcinomas. Our data identify four distinct regions of loss on chromosome 4 in breast carcinomas. These four regions closely overlap the regions previously reported to be frequently lost in several different cancers, suggesting that there may be four putative suppressor genes on chromosome 4, the inactivation of which may be important in the pathogenesis of breast cancer. We are currently performing fine deletion mapping of chromosome 4 in breast carcinomas. These efforts should eventually aid in the identification of the putative tumor suppressor genes in these regions.

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