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Deletion of Two Separate Regions on Chromosome 3p in Breast Cancers[1]

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ABSTRACT

We have characterized the copy number of various loci on chromosome 3p in a series of breast cancers. To determine the precise region(s) involved, restriction fragment length polymorphism (RFLP) analysis for loss of heterozygosity (LOH) was performed using a panel of RFLP probes at 3p13–14, 3p21–22, and 3p24–26. The incidence of LOH at the three loci was 41, 32, and 45%, respectively. To validate the LOH data and to gain insights into the mechanisms resulting in LOH, chromosome 3 pericentromeric and 3p region-specific DNA probes were used to determine the DNA copy number by fluorescence *in situ* hybridization (FISH). Among 22 cases examined, 15 showed loss by both LOH and FISH, indicating that the dominant mechanism of LOH at 3p in breast cancer is a physical deletion. Two of the 22 cases showed loss by RFLP analysis but not by FISH, suggesting either mitotic recombination or loss and endoreduplication. In three cases, RFLP analysis indicated allelic imbalance, which was incorrectly interpreted as LOH, since a gain of one allele was suggested by FISH. By constructing a deletion map, we found that 2 separate regions, 3p13–14 and 3p24–26, were independently deleted in some breast cancers. Additionally, four cases had break points within the 3p24–26 region and one case had a homozygous deletion at 3p13, further supporting the hypothesis that there are tumor suppressor genes at both 3p13–14 and 3p24–26. Although high frequency of LOH was observed at the 3p21–22 region, there was no direct evidence supporting the existence of a breast cancer tumor suppressor gene there as opposed to codeletion with either the proximal or distal region.

INTRODUCTION

Substantial evidence suggests that functional inactivation of tumor suppressor genes may play an important role in the pathogenesis of human cancers (1, 2). Inactivation of such genes often involves mutation of one allele and physical deletion or replacement of a chromosomal segment containing the other allele. Such allelic loss is detected by RFLP[3] analysis as LOH. Chromosomal regions which show frequent LOH in tumor cells are thought to harbor putative tumor suppressor genes (1, 2).

There are a number of confounding variables which may technically limit LOH analysis by RFLP. Because tumor specimens may contain contaminating normal stromal cells, one allele is rarely completely absent. Often, LOH is inferred from diminished density of one allele in the tumor DNA compared to the same person's normal DNA. Under these conditions, it is possible to falsely interpret gain of one allele (3) as LOH with diminished density of the other allele. Furthermore, an LOH may be overlooked by RFLP analysis if the tumor sample contains too many contaminating normal cells or if the tumor is heterogeneous so that the loss is present in only a portion of the tumor cells.

FISH of interphase nuclei can also be used to detect gene loss or gain (4, 5). Since FISH examines each nucleus individually, neither intratumor heterogeneity nor contaminating normal cells will lead to ambiguous results. When the mechanism of allelic loss is a physical deletion, it can be easily detected by FISH as loss of the hybridization signals. However, FISH cannot identify allelic losses that arise from other mechanisms, such as mitotic recombination. Therefore, combining RFLP with FISH will be more powerful than either method alone for identifying the chromosomal regions with frequent losses indicative of tumor suppressor genes.

LOH at chromosome 3p occurs in many human malignancies, including breast cancers (6–8), small cell lung carcinomas (9), renal cell carcinomas (10), and cervical carcinomas (11). It is uncertain, however, which region within 3p is involved. For breast cancer, Ali *et al.* (6), reported a 30% LOH of markers at 3p21–25. In contrast, Sato *et al.* (8), identified a more proximal region of chromosome 3p as the most likely location for a chromosome 3 tumor suppressor gene in breast cancers, finding LOH at 3p12–14 in 47% of cases but only 14% at chromosome 3p25.

To more precisely determine the location of a putative tumor suppressor gene(s) on chromosome 3p in breast cancer, we used a panel of probes detecting RFLP at 3p13–14, 3p21–22, and 3p24–26. The same breast cancer specimens were further characterized by FISH to confirm the allelic losses. In this paper, we demonstrate (*a*) how combining FISH and RFLP can clarify interpretations of LOH; (*b*) that the dominant mechanism of allelic loss at 3p in breast cancer is by physical deletion; and (*c*) that, in some cases, 2 separate regions, 3p13–14 and 3p24–26, can be independently deleted.

MATERIALS AND METHODS

Tumor Specimens. Breast tumor patients were referred to several hospitals in the San Francisco Bay Area between 1985 and 1993 for either mastectomy or lumpectomy. Among the 108 tumors, 98 were infiltrating ductal, 6 were infiltrating lobular, and 4 were carcinoma *in situ*. Tumor staging was according to the criteria of the American Joint Committee on Cancer (12): 29% were classified as stage I tumors, 56% were classified as stage II, 11% were classified as stage III, and 4% were classified as stage IV tumors. Fifty % of the tumors analyzed were positive for lymph node involvement. Fifty-five % of the tumors analyzed were positive for both estrogen and progesterone receptors. Among the rest, 10% each were positive for only one of the hormone receptors, and 25% were negative for both receptors.

DNA Probes. DNA probes used for RFLP analysis on Southern blot were as follows: D3S642 (3p13), D3S734 (3p21–22), D3S617 (3p24–26), and D3S726 (3p25–26). The information concerning these probes is described in Ref. 13. All of the probes were labeled by random priming (14) with [^{32}P]dCTP. Primers used for PCR-RFLP analysis were as follows: D3S6 (3p14), D3S30 (3p13–14), D3S2 and H3H2 (3p21), and THRB (3p24). The sequences and other information concerning these primers were in the "Genome Database," William H. Welch Medical Library, The John Hopkins University. The cosmid probes for FISH analysis were cCI3–373 (3p13), cCI3–800 (3p21.33–32), and cCI3–839 (3p24–25). The chromosome 3-specific α-satellite repetitive probe was phage probe pα3–5. The cosmid probes

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[3] The abbreviations used are: RFLP, restriction fragment length polymorphism; LOH, loss of heterozygosity; FISH, fluorescence *in situ* hybridization; PCR, polymerase chain reaction.

Table 1 *Summary of allelic loss by RFLP and FISH analyses*

RFLP analysis[a]		FISH analysis							
		No. of chromosome 3 centromeric signals/no. of 3p cosmid signals[b]							
		No. of cases without deletion[c]			No. of cases with deletion[d]				
Chromosomal region	No. of cases with LOH/ no. of informative cases (%)	No. of cases with deletion/ no. of cases analyzed (%)	2/2	3/3	4/4	2/0	2/1 or 1/1	4/2 or 3/2	4/3	6/2
3p13–14	15/37 (41)	22/67 (33)	29	8	8	1	5	14	1	1
3p 21–22	12/37 (32)	26/58 (45)	24	2	6	0	8	16	1	1
3p 24–26	32/71 (45)	24/58 (41)	23	7	6	0	6	17	0	1

[a] Combined data from Southern blot and PCR-RFLP analyses.
[b] Representing the predominant population of tumor nuclei.
[c] Whenever the number of cosmid signals was equal to the number of centromeric signals, the sample was considered to have no deletion. Exceptions to this rule were the cases with chromosome 3 monosomy (one centromere and one cosmid signal) which were included among cases with deletion.
[d] Whenever the number of centromeric signals was greater than the number of cosmid signals, or monosomic, the sample was considered to have a deletion.

for FISH analysis were kindly provided by Dr. Y. Nakamura (Cancer Institute, Tokyo). The cosmid probes were with biotin-11-dUTP, and the pericentromeric probe was labeled with digoxigenin-11-dUTP by nick translation.

RFLP Analysis. High molecular weight DNA was from pulverized skin biopsies, and frozen tumor tissue pieces were isolated by proteinase K and phenol-chloroform treatment as described previously (15). RFLP analysis was performed by Southern blot and PCR-RFLP. The procedures for Southern blot analysis and quantification of the hybridization signals were previously described (16). PCR-RFLP analysis was performed as described before (17). In brief, 200 ng of DNA was amplified by PCR in a 50-μl reaction. After PCR reaction, 5 μl of the PCR product were digested with 10 units of the proper restriction enzyme in a 10-μl reaction. The samples were then analyzed on 2% agarose gels. LOH was determined by measuring the intensity of the polymorphic bands from normal and tumor DNA of the same patient. In most cases, the difference in intensities of the 2 alleles was obvious and could be visually determined by the ethidium bromide-stained gel. Otherwise, fragments were labeled with [^{32}P]dCTP, and the autoradiograms were scanned by densitometer as in the cases of autoradiograms obtained by Southern blot analysis.

FISH. Slides for FISH analysis were prepared by touching freshly sliced tumor tissue to clean slides to release loosely adherent tumor cells or by dropping a cell suspension prepared from mechanically disaggregated fresh tissues (4). Normal peripheral blood lymphocytes were used as a control for hybridization specificities. Details of the fixation, *in situ* hybridization, immunological staining of the slides, and scoring of signals were described previously (4, 18).

RESULTS

Frequency of Aberration on Chromosome 3p in Breast Cancers. To determine the frequency of allelic loss at 3 regions on the short arm of chromosome 3 (3p13–14, 3p21–22, and 3p24–26), we used RFLP to detect LOH and FISH to reveal chromosomal deletions. To increase the number of informative cases for RFLP analysis, we combined results using RFLP probes on Southern blots with results using primers to amplify by PCR short chromosomal regions which are polymorphic when cut by a specific restriction enzyme. The frequencies of LOH detected by RFLP and deletions detected by FISH are summarized in Table 1. For all markers tested, incidences of loss obtained by RFLP and allelic deletion were comparable, ranging from 32–45%.

Further Analysis of FISH Results. Each tumor specimen was hybridized simultaneously with a digoxigenin-labeled pericentromeric probe and a biotin-labeled cosmid probe. For each specimen, the number of centromeric and cosmid hybridization signals were analyzed by FISH for approximately 100 individual tumor cells. The distribution of cases according to centromeric and cosmid copy number of the predominant tumor cell population is summarized in Table 1. Tumors with an equal number of centromeric and cosmid signals, except monosomy, were interpreted as having no deletion. The majority of such tumors had 2 centromeric and 2 cosmid signals, and the remainder had either 3 or 4 centromeric and cosmid signals. Monosomic tumors and tumors with more centromeric than cosmid signals were scored as having a deletion at the cosmid region tested. The majority of these cases had either 4 or 2 centromeres, although tumors were also seen with 1, 3, or 6 centromeric copies. Approximately one-third of the tumors considered to have a deletion retained only one copy of the chromosome; the remainder retained 2 cosmid signals with 3 or more centromeric signals. There was one tumor with a homozygous deletion at the 3p13 region.

Comparison of LOH and FISH Results. Of the cases analyzed by FISH, 42 were informative by RFLP analysis. The results of these 42 cases are summarized in Table 2. Thirty-five of the 42 cases (83%) gave consistent results with both assays (20 tumors showed no deletion or LOH, and 15 tumors had deletions by both assays). Of the 15 cases with deletions, 4 had only one cosmid signal, while 11 cases had 2 cosmid signals with 3 or more centromeric signals.

Seven of the 42 cases gave inconsistent results between the 2 methods. Four of the 7 cases showed decreased density of one allele by RFLP analysis but had equal numbers of centromeric and cosmid copies by FISH. (Two of the 4 cases had 3 centromeric signals and 3 cosmid signals; the other 2 cases had 2 centromeric and 2 cosmid signals.) As illustrated in Fig. 1, tumor sample B318 and another case which had 3 centromeric and 3 cosmid signal showed allelic imbalance by either Southern blot (*A, lanes 1* and *2*) or RCR-RFLP (*B, lanes 1* and *2*) analysis. These cases represent allelic gain. Tumor B317 and another tumor that had 2 centromeric and 2 cosmid signals also showed LOH by Southern blot (*A, lanes 3* and *4*) or PCR-RFLP analysis (*B, lanes 3* and *4*). These 2 cases represent allelic loss followed by chromosomal duplication. Three additional cases showed no LOH by RFLP but had 2 cosmid signals and 4 centromeric signals.

Presence of a Break Point at 3p24–26 Region. LOH was detected by both Southern blot and PCR-RFLP analyses. Within each 3p region tested, the exact locations of the probes and primers used in these 2 techniques differed. In 30 cases, the results obtained with Southern and PCR-RFLP analyses were consistent, and there were no discrepancies at either 3p13–14 or 3p21–22. However, in 4 cases, the

Table 2 *Comparison of FISH results with LOH*[a]

	LOH	Deletion (by FISH)	No. of cases	Interpretation
Consistent cases	No	No	20	No loss
	Yes	Yes	15	Physical deletion
Inconsistent cases	Yes	No	2	Chromosomal reduplication
	Yes	No	2	Allelic gain
	No	Yes	3	Other[b]

[a] LOH was detected by either Southern blot or PCR-RFLP analysis.
[b] See "Discussion."

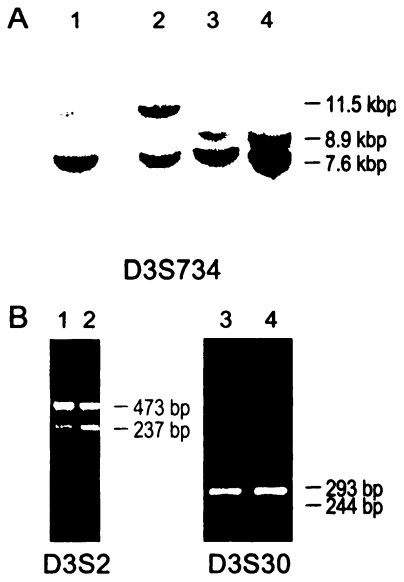

Fig. 1. Examples showed that tumors B318 (*lanes 1* and *2*) and B317 (*lanes 3* and *4*) had an LOH by Southern blot analysis (*A*) and PCR-RFLP analysis. *Lanes 1* and *3* in *A* and *lanes 1* and *4* in *B* represent tumor DNA. *Lanes 2* and *4* in *A* and *lanes 2* and *3* in *B* represent skin DNA from the same patient. Tumor B318 had 3 pericentromeric signals and 3 cosmid signals at these regions. Tumor 317 had 2 pericentromeric signals and 2 cosmid signals at these regions. The allele length is indicated on the right in base pairs (bp). The loci tested are listed at the bottom.

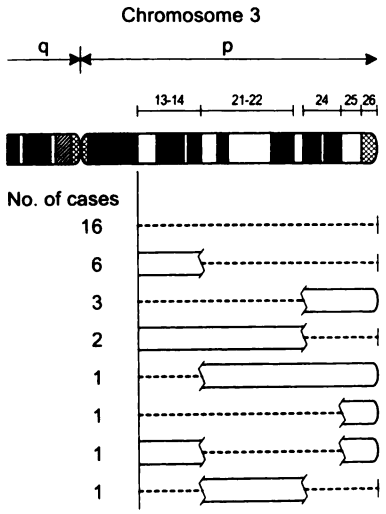

Fig. 2. Deletion map of chromosome 3 in breast cancer. The chromosome map was taken from the "Eleventh International Workshop on Human Gene Mapping" (13). *Dashed lines*, chromosomal regions that were deleted. *Vertical zig-zag lines*, beginning or end of deletions.

results varied depending on the probes used at chromosome 3p24–26. In 3 of the 4 cases, LOH was detected with a probe located at 3p24 but not at 3p24–26. In 2 of these cases, LOH at 3p24 was confirmed by FISH using a cosmid probe located at 3p24. The fourth case had an LOH at 3p24–26, but none was detected using probes located at 3p24 or 3p21. These data suggest that breaks might occur between loci THRB (3p24) and D3S617 (3p24–26), resulting in deletion of chromosomal segments either proximal or distal to the break points.

Deletion Map of Chromosome 3p. When we combined the results obtained by RFLP and FISH analyses, 31 tumors were informative at all three 3p regions tested and had deletion and/or LOH in at least one region. Fig. 2 illustrates the deletion map of these 30 cases. Sixteen of the 30 cases (52%) had physical deletion involving all 3 chromosomal regions. Six cases deleted the 3p21–22 and 3p24–26 regions but retained the 3p13–14 region. Three cases retained the telemeric region (3p24–26) and deleted the 3p13–14 and 3p21–22 regions. Two other cases had the reverse condition, in which the telemeric region (3p24–26) was deleted but the rest of the 3p arm was retained. One case deleted only the 3p13–14 region but not the rest of the arm. In addition, 2 cases had break points located distal to the THRB (3p24) locus. While one of these 2 cases deleted almost the whole arm but retained the small telemeric region, the other case also retained the 3p13–14 region. One tumor showed deletion of the 3p13–14 and 3p24–26 regions but retained the middle 3p21–22 region. None of the tumors deleted only the middle region (3p21–22) while retaining the 3p13–14 and 3p24–26 regions.

DISCUSSION

Functional inactivation of a tumor suppressor gene often involves deletion of the normal allele to unmask the mutated allele. Therefore, chromosomal regions with frequent deletions are thought to harbor putative tumor suppressors. RFLP and FISH analyses are commonly used to detect chromosomal deletions in tumor cells. However, the results obtained by either method could give false-positive or false-negative information. In this study, we have used both RFLP and FISH analyses to determine the regions on chromosome 3p that are deleted in breast cancer. We demonstrated that, by comparing the results obtained with both methods, one can interpret the results with minimal ambiguity. In most cases, the results obtained by FISH and RFLP analyses were consistent (Table 2). All 20 cases that showed no LOH had equal numbers of centromeric and cosmid signals. In 15 of 22 cases that showed LOH by RFLP analysis, the predominant population of tumor nuclei had fewer cosmid signals than chromosome 3 centromeric signals. Thus, we conclude that the LOH detected in these 15 cases represent physical deletions of chromosome 3p regions. These cases are candidates for having a classic tumor suppressor gene since they retain only one allele.

Inconsistencies between the 2 methods were seen in 7 cases. Two cases that had 2 centromeric signals and 2 cosmid signals showed LOH by both Southern blot and PCR-RFLP. Since LOH was present, the 2 cosmid signals must represent alleles with the same parental origin. These tumors may arise by mitotic recombination or by deletion, followed by reduplication of the remaining allele. These results also are consistent with the hypothesis that the tumors have a putative tumor suppressor gene at the involved region, assuming that 2 copies of the mutated allele are the ones retained.

In 2 cases, the tumor cells were shown to have 3 centromeric and cosmid signals at all 3 regions tested, indicating trisomy. These cases were incorrectly scored as having an LOH with some contaminating normal cells since there was a considerable difference in density between the 2 alleles by RFLP analysis. The biological significance of trisomy of chromosome 3p is unknown; however, gaining an additional allele does not necessarily indicate selection of cells with an inactivated putative tumor suppressor gene. This type of misclassification occurred in only 2 of 22 cases scored as having an LOH, indicating that allelic loss at chromosome 3p is, in fact, a common occurrence in breast cancers. Since the allelic gain in these 2 cases represented the whole 3p arm, the misinterpretation by RFLP analysis did not affect the break point analysis (Fig. 2) which indicated the there might be 2 different target suppressor genes on 3p since there were 2 common regions of overlapping deletion.

Three cases had four 3p centromeric signals and 2 cosmid signals by FISH but no LOH by RFLP analysis. This type of inconsistency may occur if there were intratumor heterogeneity among different portions of the same tumor in either the amount of normal cell contamination or the presence of tumor cell subpopulations with the

deletion. A more complicated but possible explanation for the inconsistency is that 2 independent deletions may have occurred in a tetraploid tumor. If the deletions were in different alleles, the remaining 2 cosmid signals would represent alleles derived from each parent. Under these conditions, RFLP analysis would not reveal an LOH. Since we could not distinguish between these possibilities, these 3 cases were excluded from deletion mapping.

By constructing a deletion map (Fig. 2), we found that 2 separated regions, 3p13–14 and 3p24–26, were independently deleted in some breast cancers. The finding that 4 cases had break points within the 3p24–26 region and that one case had a homozygous deletion at 3p13 adds additional support for the presence of tumor suppressor genes at these 2 regions. Since allelic loss at 3p12 was not analyzed in this study, we do not know whether the deletion observed at 3p13–14 extends to the 3p12 region. However, the retaining of 3p centromeres as shown by FISH analysis suggests that the deletion does not extend to the q arm of chromosome 3. While we confirmed the finding by Sato *et al.* that there is a tumor suppressor gene at 3p13–14, we also found a high frequency of LOH at 3p24–26. Since only a single probe at 3p25 was used by Sato *et al.*, they may have inadvertently missed an additional LOH at 3p24–26. Although 3p21 is involved in several types of human cancers and a high frequency of LOH was observed at 3p21–22, we did not find any case that showed independent deletion of this region. Therefore, there was no direct evidence supporting the existence of a tumor suppressor gene in breast cancer at 3p21–22 as opposed to codeletion with either the proximal or distal region.

By comparing the results obtained with both RFLP and FISH analyses, one can understand the mechanism underlying the LOH. Our results suggest that the chromosomal mechanisms underlying LOH seem to be chromosome specific in breast cancers. Of the 34 cases which show allelic loss by FISH at any 3p region, approximately two-thirds of the cases had 2 copies of the cosmid signals and 3 or more copies of the centromeric signals. Only 2 cases were found to be monosomic for chromosome 3. This result is different from our findings at the *p53* locus (chromosome 17p13) or at the *Rb* locus (chromosome 13q14) in breast cancer. The majority of breast cancers with LOH at 17p13 showed only one 17p13 cosmid signal per nucleus, representing physical deletions without duplication or additional deletion after duplication (4). In contrast, the predominant mechanism underlying the LOH at the *Rb* locus is not a physical deletion but rather mitotic recombination or endoreduplication of the mutated allele after deletion of the normal allele (19).

In summary, we have demonstrated the benefit of combining FISH and RFLP analyses to characterize allelic imbalances. When both methods are applied together, one can more precisely define which cases with allelic imbalance truly represent allelic deletion, an indicator for the presence of a putative tumor suppressor gene. Additionally, FISH and RFLP together lead to a clearer understanding of the mechanisms underlying an observed LOH.

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