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GRANT NUMBER DAMD17-94-J-4078

TITLE: Genomic Instability at Premalignant and Early Stages of Breast Cancer Development

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REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Aug 96 - 31 Jul 97

4. TITLE AND SUBTITLE
Genomic Instability at Premalignant and Early Stages of Breast Cancer Development

5. FUNDING NUMBERS
DAMD17-94-J-4078

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

We have developed techniques that allow the analysis of multiple chromosomal loci from single paraffin sections from breast cancer lesions. This approach is being used to allelotype small preinvasive breast cancer lesions. We identified the chromosome arms most frequently affected by allelic losses and imbalances at preinvasive stages of breast carcinogenesis and those allelic losses involved in more advanced stages of progression. We have now performed a high resolution allelotype of chromosome 16q. This allowed us to refine the location of specific subchromosome regions containing putative tumor suppressor genes of relevance on early breast carcinogenesis.

We also performed a chromosome 16 high resolution allelotype of a panel of human breast cancer lines in order to identify areas of hemizygosity and homozygous loss. As a natural and important extension of these studies we have built a contig of yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones spanning the chromosome 16q region in which frequent allelic losses were detected. In studies in progress we have isolated numerous cDNA clones from a human breast epithelial library that map to the region of interest.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	13
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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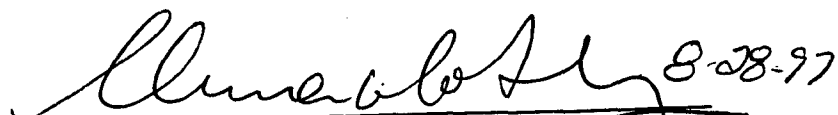

8-28-97

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Genomic instability at premalignant and early stages of breast cancer development.

INRODUCTION

Numerous studies have focused on the identification and analysis of specific gene mutations and chromosome abnormalities in sporadic cancer, but to date no clear model of the critical events or delineation of primary abnormalities have emerged. The best obvious source of material for the identification of the various stages of breast cancer progression is available from paraffin-embedded tissues used in routine diagnostic procedures. A first phase of this project consisted in the optimization of a comprehensive technical approach for allowing a multiparametric analysis of human breast cancer lesions from paraffin-embedded tissue sections. Thus, numerous chromosomal loci can be analyzed from single tissue sections by means of microsatellite length polymorphism analysis. DNA samples from normal and breast cancerous tissue can be obtained from the same section by means of microdissection. This allows to correlate the allelotype of specific lesions with other markers of prognostic and diagnostic significance. These results were reported in a previous publication (Chen et al., Breast Cancer Res. & Treat. 39:177-185, 1996).

Loss of heterozygosity (LOH) at specific chromosomal loci has been considered as part of the indirect evidence for postulating the existence of possible tumor suppressor genes within those specific chromosome regions. In order to better understand the timing for presentation of allelic losses in breast carcinogenesis, in a second phase of our project, we compared the allelotypic profile of *in situ* ductal carcinomas with that of invasive ductal tumors. One of the goals of this study was to determine which of the chromosome areas most commonly affected by allelic losses or imbalances in breast cancer are involved at preinvasive stages of breast carcinogenesis.

Ductal carcinoma *in situ* (DCIS) of the breast is known as a preinvasive stage of breast cancer and is probably the precursor of infiltrating breast cancer. Genetic alterations shown at this stage might indicate association with early events in malignancy or invasiveness. We observed that involvement of chromosome arms 1p, 3p, 3q, 6p, 16p, 18p, 18q, 22q, and possibly 6q and 11p appear to be late events in breast cancer progression since allelic losses or imbalances affecting these areas were observed with very low frequency at the *in situ* stage. On the other hand allelic imbalances and losses affecting chromosome arms 7p, 16q, 17p and 17q appear to be early abnormalities since they were observed in a significant number of DCIS lesions. These results were reported in Aldaz et al. (Cancer Res. 55:3976-3981, 1995).

Loss of heterozygosity on chromosome 16q has been previously reported in breast and prostate cancer with various frequency. In additional studies we further explored the extent of involvement of chromosome 16q in DCIS. To precisely define the minimum region of LOH, we generated a high-resolution allelotype of 35 ductal carcinoma *in situ* cases and completed a deletion map of chromosome 16q by means of paraffin-embedded tissue microdissection and PCR microsatellite analysis of 22 markers. We observed a strikingly high frequency of LOH affecting 16q, with 31 of 35 tumors (89%) affected. We identified three distinctive areas with high LOH. Two areas were described previously and correspond to 16q21 and 16q24.2-qter. The third and most commonly affected area spanned the region

from marker *D16S515* to marker *D16S504*. the most affected locus was at *D16S518*, in which LOH was observed in 20 of 26 informative cases (77%), and we estimate that it lies in subregion q23.3-q24.1. The region of highest LOH spanned approximately 2 Mb, as determined by a yeast artificial chromosome contig covering this region (reported in Chen et al. Cancer Res. 56:5605-5609, 1996). Such a high frequency of LOH at a preinvasive stage of breast cancer suggests that a candidate tumor suppressor gene or genes at this location may play an important role in breast carcinogenesis.

To extend these studies we performed a chromosome 16 high resolution allelotype of a panel of human breast cancer lines in order to identify areas of hemizygosity and homozygous loss. As a natural and important extension of these studies we have built a contig of yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones spanning the chromosome 16q region in which frequent allelic losses were detected. In studies in progress we have isolated numerous cDNA clones from a human breast epithelial library that map to the region of interest.

We are currently extending our analysis of the 16q region to earlier premalignant lesions such as atypical hyperplasias.

MATERIALS AND METHODS

Tumor Samples

Atypical hyperplasia samples were obtained from paraffin embedded blocks from the archives of the Department of Pathology of The University of Texas M. D. Anderson Cancer Center. We did not include any atypical hyperplasia with infiltrating components.

Paraffin tissue microdissection

The basic technical approach has been described previously (Chen et al., Breast Cancer Res. & Treat. 39:177-185, 1996). Minor modifications were introduced to improve efficiency of microdissection. Briefly, one to three five to eight micron thick paraffin sections were stained and used for microdissection. Using companion H&E stained slides as reference first tumor cells were microdissected using a fine point surgical blade (No. 11) under an inverted microscope. The edges of tumor area and stroma were cleared of debris using the same blade and blown with a stream of compressed air. A new blade was then used to dissect normal tissue the same way.

DNA preparation

Samples were rehydrated and DNA was extracted by incubating in 200 µl Instagene chelex matrix solution (BioRad) containing 60 µg of proteinase K in a shaking incubator at 37°C overnight. After proteinase K digestion, samples were boiled for 10 min., vortexed, and centrifuged at >7,000 G for 5 minutes. 5 µl aliquots of the supernatant were used for PCR amplification.

PCR microsatellite analysis

Primers for highly polymorphic human microsatellite repeats were purchased from Research Genetics (Huntsville, AL), as listed in (Chen et al. Cancer Res. 56:5605-5609, 1996). Prior to PCR reactions, the forward primer was end labeled using T4 polynucleotide kinase (Promega) and [γ32P]ATP (NEN 6,000 Ci/mmol). PCR reactions were performed in

a 20 µl reaction volume containing 150 µM each dNTP, 1 unit Taq polymerase and 1X Taq buffer (Promega), 1.5 mM MgCl2, 1 pmole labeled primer and 2.5 pmole unlabelled forward and reverse primers. A hot start procedure was used in which template and primers were denatured at 96°C for 5 min, after which the remaining reaction constituents are added for 35-40 cycles at 94°C for 40 sec.; 55°C for 30 sec and 72°C for 30 sec. and a final elongation step of 72°C for 5 min. Products were electrophoresed on a 7% polyacrylamide sequencing gel at 90 watts constant power for 2-3 hrs. Gels were dried at 65-70°C for 1-2 hrs and exposed to X-ray film from 4 hrs to overnight. If necessary for certain primer sets the amplification conditions were further optimized by adjusting the MgCl2 concentration in the reaction buffer.

The sample was considered to have partial loss of heterozygosity, or allelic imbalance, if the signal intensity of one allele was diminished by approximately one-half or more of its normal intensity (i.e., in normal tissue) in relation to the remaining allele. Complete loss of heterozygosity was defined as a decrease of 90% or more in the signal intensity of one allele reactive to the other.

Yac and Bac clones spanning the region of interest were identified and obtained from Research Genetics (Huntsville, AB).

Novel STS were generated by sequencing the extremes of BAC clones. cDNA clones were obtained by using a modification of the Method described by (Futeral et al., Human Mol. Genet. 3:1359-1364, 1994).

RESULTS AND DISCUSSION

Most of the studies described in this section are currently under progress. We are performing and allelotypic study of the chromosome 16q subregion in Atypical hyperplasias, which constitutes and earlier and very likely precursor of Ductal Carcinoma in situ. We utilized same of the same microsatellite markers employed in our previous study on DCIS (Chen et al. Cancer Res. 56:5605-5609, 1996). As can be observed in Table 1, we observed some allelic imbalances and losses affecting the same chromosome 16q regions in these earlier preinvasive lesions. These results indicate and confirm that allelic losses affecting the distal portions of the chromosome 16q arm are early events in breast cancer development.

To extend these studies we performed a high resolution allelotype of the chromosome 16q area in a panel of 23 human breast cancer lines. We utilized highly polymorphic markers with high heterozygosity scores (~ 0.70 or more). Since to perform these studies we lack the normal matching control tissue corresponding to each breast cancer line, it is very difficult to distinguish between loss of heterozygosity or homozygosity. Nevertheless the presence of large areas with lack of heterozygosity in various marker, given the high polymorphism of the loci investigated, very likely represent hemizygosity as a consequence of allelic loss. As can be observed in Figure 1, numerous breast cancer lines showed evidence of hemizigosity affecting all or almost all of the chromosome 16q arm. These results are in strong agreement with our previous findings in preinvasive and invasive breast cancer (Aldaz et al and Chen et al.). The heterozygosity scores calculated at the various loci is shown in the rightmost column. Again, in agreement with our previous studies the lowest scores point to the same areas previously identified as hot spots for allelic loss. In order to isolate the

putative breast cancer suppressor gene residing in the area of interest we built a contig of YAC and BAC clones spanning the target region (Figure 2) . Interestingly, we have identified three breast cancer lines which showed homozygous losses affecting markers in this region. This indicates that the target gene is very likely contained within this region.

We have isolated numerous clones from a human breast cDNA library and are currently characterizing several of these expressed sequences mapping to two of the BAC clones (249 and 286) spanning the area of interest. cDNA clones were isolated following a modification of the methods described by Futreal et al. (Futreal et al., Human Mol. Genet. 3:1359-1364, 1994). Once the clones were obtained after sequencing we confirmed mapping by PCR and by hybridization to the BACs DNA. As can be observed (Figure 3) some of the clones mapped to BAC 249 and others to BAC286 as detected by hybridization of 32P labeled BAC DNA to duplicate membranes containing spotted samples of the various cDNAs isolated.

We are currently characterizing and obtaining the whole length cDNA clones mapping to this region.

Table 1

Sample	D16s390	D16s518	D16s516	D16s504	D16s402	D16s413
AH1	NI	NI	✳	○	✳	✳
AH2	○	○	○	○	✳	○
AH3	NI	✳		●	S	○
AH4	✳	✳	✳	✳	●	NI
AH5	○	○	✳	○	○	○
AH6	○	○	✳	NI	○	○
AH7	NI	S		●		✳
AH8	○	○	○	○	○	○
AH9	NI	○	NI	NI	○	○
AH12					●	
AH13					○	
AH14					✳	
AH15					○	
AH16					○	
AH17					NI	

○ heterozygous

● LOH

✳ allelic imbalance

NI non informative,

S shift novel allele

blank, not done yet

Current on going studies on allelic losses affecting the chromosome 16q region in breast epithelial atypical hyperplasias (AH)

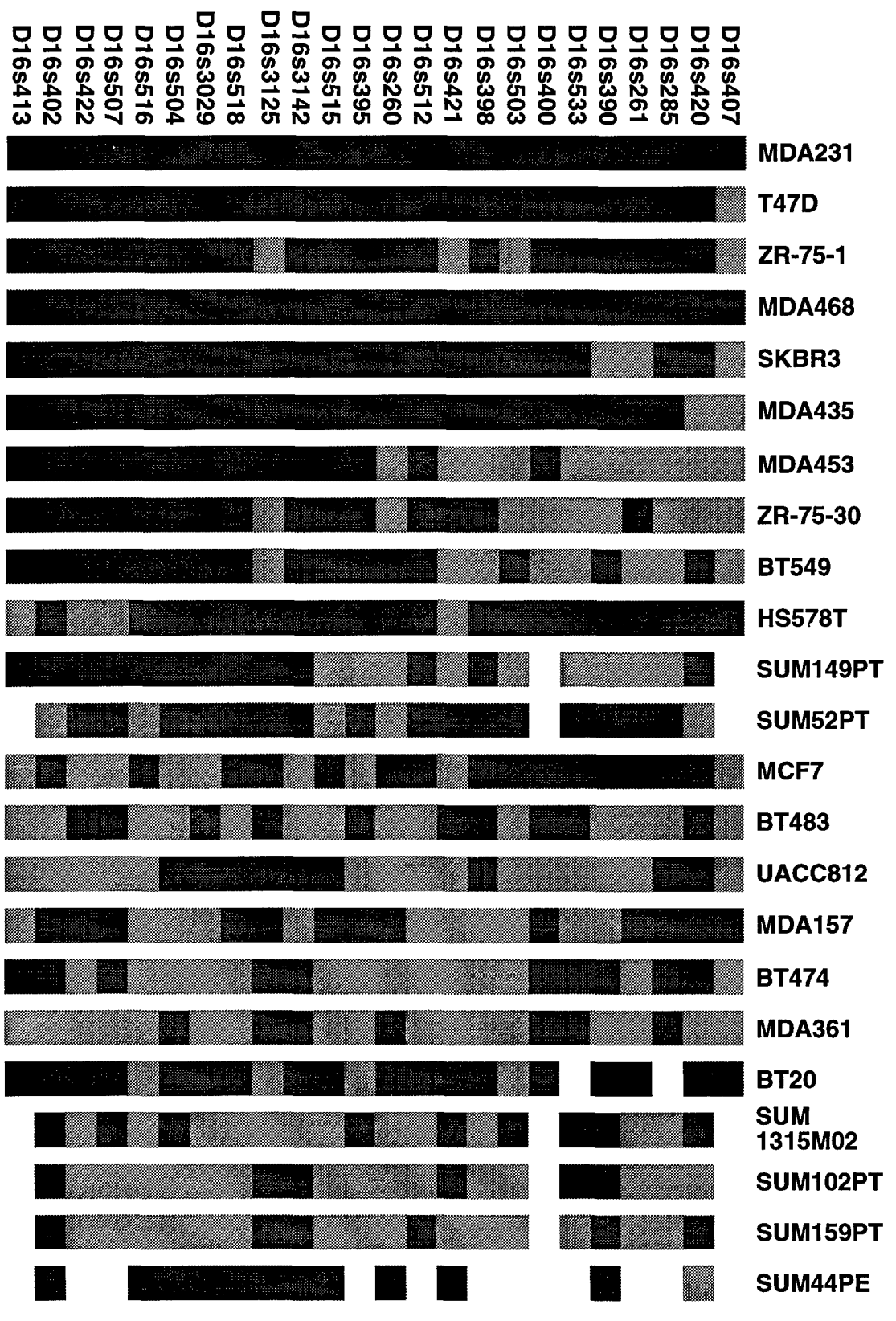

Figure 1

High resolution allelotype of chromosome 16 microsatellite markers in breast cancer cell lines. Markers arranged in mapping and linkage order as previously described in Chen et al. (Cancer Res. 56:5605-5609, 1996)

Dark gray blocks indicate that a single allele was observed at the corresponding locus (i.e. hemi or homozygosity), light gray areas indicates heterozygosity preserved. Blank indicates not done.

Numbers at right represent the heterozygosity scores calculated at each specific locus from analyzing this breast cancer panel.

In agreement with our previous findings, note that several breast cancer lines showed hemizygosity affecting all or most of the chromosome 16q arm.

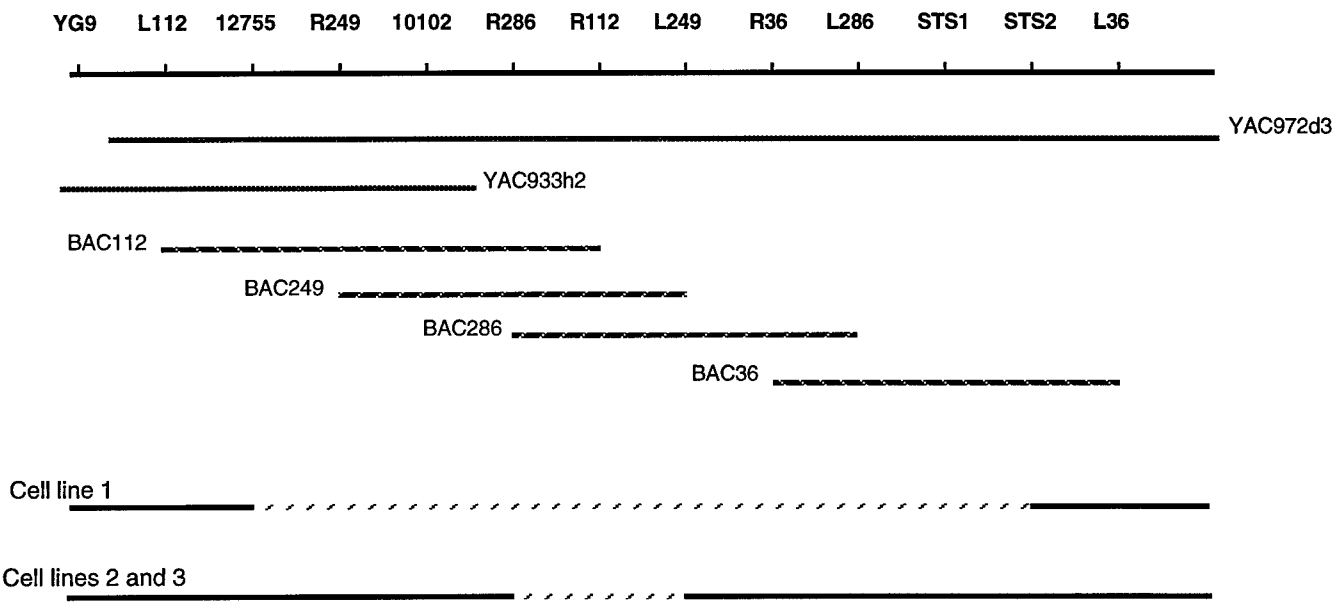

Figure 2
YAC and BAC contig spanning a region with observed homozygous loss in three breast cancer lines. The various STSs and ESTs identified and used to built this contig are shown. The represented distance between STSs is not to scale. At the bottom of the figure the homozygous losses in the breast cancer lines are represented by a discountinued line.

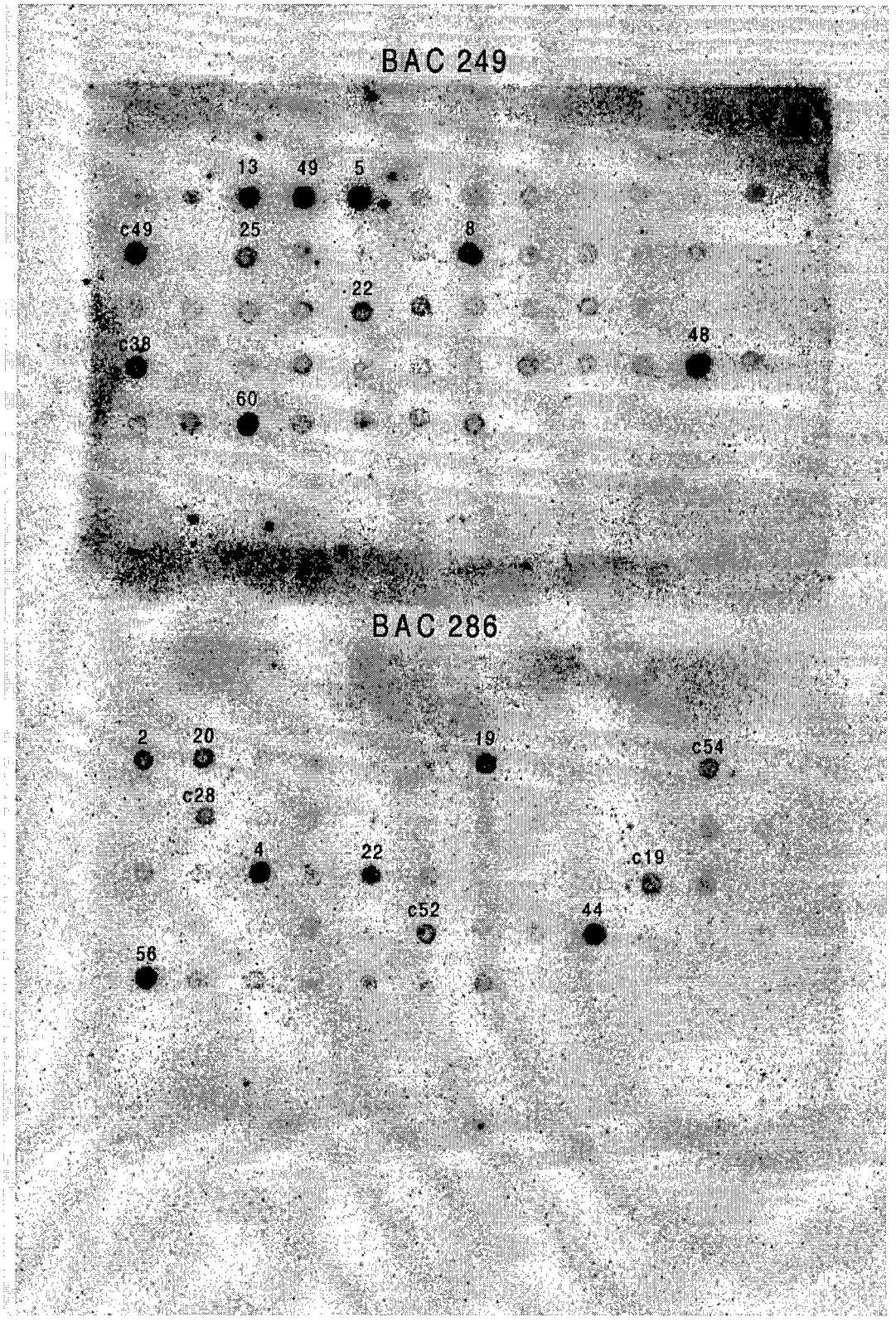

Figure 3
Dot blot hybridization of various cDNAs isolated from the region of interes (Figure 2) using as probes the labeled BAC DNAs as indicated.

CONCLUSIONS

We have identified the occurrence of allelic losses affecting specific regions of the chromosome 16q at a very likely early preinvasive stage of breast tumor development, since we observed these abnormalities in various Atypical hyperplasia samples.
This extends and confirms our recent findings in Ductal Carcinoma in situ (Chen et al. Cancer Res. 56:5605-5609, 1996).

In agreement with these observations in preinvasive breast lesions and previous studies (Aldaz et al. Cancer Res. 55:3976-3981, 1995) in invasive cancer we determined that the same regions of chromosome 16q showed evidence of hemizygosity in the majority of a panel breast cancer lines analyzed. Furthermore we have identified three breast cancer lines that showed evidence of homozygous loss affecting markers spanning one of the regions of interest. This indicates that it is likely that a putative breast cancer suppressor gene resides in this chromosome area which is commonly deleted during tumor development. By positional cloning strategies we are currently attempting to identify and clone this gene.

LIST OF PUBLICATIONS

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