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FOREWORD

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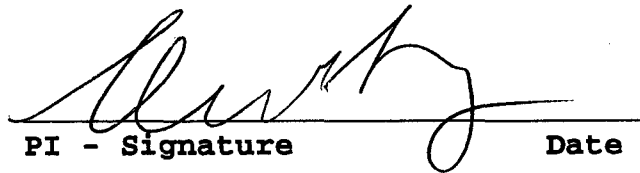
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Genomic Instability at Premalignant and Early Stages of Breast Cancer Development

INTRODUCTION

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. 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.

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 already been previously reported in breast and prostate cancer with high frequency, indicating the existence of a putative tumor suppressor gene(s) located in this chromosome arm. We observed that the most commonly affected area spanned the region from marker *D16S515* to marker *D16S504*. Within this region the most affected locus was at *D16S518*, in which LOH was observed in 20 of 26 informative cases (77%). We have estimated that the area of interest 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 agreement with our previous findings, most breast cancer lines showed evidence of hemizygosity affecting all or almost all the chromosome 16q arm. One breast cancer line showed a homozygous loss affecting markers from this area, indicating that a likely target gene for inactivation may reside within this region.

To extend mapping of this deleted area we built a series of new STS markers according to DNA sequences obtained from ends of BAC clones. Using a high density STS map we determined that the deletion is approximately 300 kb in size (Figure 2).

In studies in progress we have isolated numerous cDNA clones from a human breast epithelial library that match to this region.

In summary, we have constructed a comprehensive physical map of the region of interest, and we have now the critical resources for the positional cloning of the putative breast cancer suppressor gene.

RESULTS OF ONGOING STUDIES

The ultimate goal of the ongoing studies is the identification and isolation of genes from chromosome 16 that can play a role in breast carcinogenesis.

Previously we have reported the frequent occurrence of loss of heterozygosity on chromosome 16q loci at preinvasive stages of breast cancer development. This suggests that the inactivation of a putative tumor suppressor gene or genes at this location may be important at early stages of breast carcinogenesis. We have now defined a relatively small region of homozygous loss located in area between markers *D16S515* and *D16S504*, that may contain this gene.

We propose to use standard positional cloning strategies to identify and clone this putative breast cancer suppressor gene.

We have already characterized a panel of 23 breast cancer cell lines using STS markers as reported in Chen et al. (*Cancer Res.* 56:5605-5609, 1996). We utilized highly polymorphic markers with high heterozygosity scores (~0.70 or more). Given the high polymorphism of the loci investigated, the presence of large areas with lack of heterozygosity in various markers, very likely represent hemizygosity as a consequence of allelic loss. As can be observed in Figure 1, numerous breast cancer lines showed evidence of hemizygosity affecting all or almost all of the chromosome 16q arm. These results were reported at the last AACR meeting (Bednarek and Aldaz). These results are in strong agreement with our previous findings in preinvasive and invasive breast cancer (Aldaz et al and Chen

et al.). 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. Interestingly, we have identified one breast cancer line which showed homozygous losses affecting markers in this region (Figure 2). This indicates that the target gene is very likely contained within this region. Using new STS markers generated from BAC insert ends DNA sequences, we predict that the deleted region is approximately 300 kb in length.

In order to isolate cDNAs encoded in this region we are utilizing a solution hybrid capture method. 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. Once the clones were obtained after sequencing we confirmed mapping by PCR and by hybridization to the BACs DNA. Several clones were found to be homologous to previously reported ESTs (underlined in Table 1). Interestingly, two of the most abundant cDNA clones showed no homology to any known sequences. We are currently working to isolate a full length cDNA. In total, we identified five previously unreported cDNA clones which are encoded in this region.

As a second approach to identify genes encoded in this region, we are performing shotgun sequencing of BAC inserts spanning the deleted area. We have presently sequenced approximately 150 kb. Figure 3 schematically represents the genomic sequence spanning BAC 286, of approximately 100 kb in size, assembled from contigs obtained by shotgun sequencing. The figure also depicts the relative position of various isolated cDNAs and ESTs that map to this region. Preliminary analysis (using BLAST algorithm) of this genomic sequence identified a total of 29 previously reported ESTs (Table 1). Additionally, we predicted 22 putative exons, according to GRAIL-1.3 cutoff > 0.5. We are currently obtaining and characterizing whole length cDNA clones mapping to this region. Although these results are preliminary, the above data allowed us to predict that in this region may be encoded genes important in the process of breast carcinogenesis i.e., tumor suppressors.

MATERIAL AND METHODS

Genomic Sequencing Approach

The primary goal is to determine the genomic nucleotide sequence of the homozygously deleted region located in the area between markers D16S515 and D16S504 (Figure 2). To do this we will perform shotgun sequencing of the isolated BAC clones. This will be done using the library of randomly overlapping clones prepared from BAC inserts. We have already define a BAC contig spanning this area and we have mapped the homozyous deletion between BAC markers L112 and L36 (Figure 2).

STS analysis is performed as previously described in Chen et al. (*Cancer Res.* 56:5605-5609, 1996). Prior to PCR reactions, the forward primer is end-labeled using T4 polynucleotide kinase (Promega) and [$\gamma^{32}\text{P}$]ATP. PCR reactions are performed in a 20 μl reaction volume containing 150 μM each dNTP, 1 unit Taq polymerase and 1X Taq buffer (Promega), 1.5 mM MgCl_2 , 1 pmole labeled primer and 2.5 pmole unlabeled forward and reverse primers. A hot start procedure is used in which template and primers are denatured at 96°C for 5 min, after which the remaining reaction constituents were 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.

For deriving BAC insert ends-specific sequences, we performed manual sequencing. BAC ends are presently been sequenced using SequiTherm EXCEL II DNA Sequencing Kit (Epicentre Technologies). Reactions are performed with ^{32}P end-labeled primers T7 and SP6 and 5 μg of BAC DNA. Cycling conditions were 95°C for 5 min. and then 35 cycles of 95°C for 30 s, 33 °C for 30 s and 70 °C for 1 min. Primers suitable for PCR were designed using Amplify version 2.53 β r (Software for PCR © Bill Engels, University of Wisconsin).

The shotgun sequencing strategy was designed after identification of BAC clones spanning the deleted region. The library of randomly overlapping clones was constructed from BAC 286 DNA using partial DNAase I digestion and cloned into pZErO 1 vector (Invitrogen). Clones are now been screened by PCR and inserts longer than 300 bp are sequenced using primer pZF16 (5'-CCTCTAGATGCATGCTCGAGCGGC-3') and BigDye Cycle Sequencing Kit (Perkin Elmer/Applied Biosystems). Products are now been analyzed on an ABI 377 automated fluorescent sequencer (Perkin Elmer/Applied Biosystems). DNA sequences are align using SequencherTM (Gene Codes Corporation).

The same strategy will be used for sequencing of all BAC clones which cover deleted region. DNA sequences are currently analyzed to search similarities with DNA and protein sequences available in computer databases. We are using BLAST, PSI-BLAST and PowerBLAST programs (available at <ftp://ncbi.nlm.nih.gov/Home.html>), GRAIL-1.3 and XGRAIL (available at <http://compbio.ornl.gov/tools/index.shtml>). Exons predicted by GRAIL analysis will be used to design primers for PCR directed cDNA library screening.

cDNA's Isolation and Characterization

In order to isolate cDNA clones transcribed from the region of interest, we performed a modification of the solution hybrid capture method described by Futreal et al. (*Human Mol. Genet.* 3:1359-1364, 1994). DNA from BACs 249 and 286 was used as a selector. As a cDNA source we use Human Mammary Gland 5'-STRETCH cDNA Library from CLONTECH Laboratories, Inc. (Palo Alto, CA). Clones are currently sequenced and analyzed. The homology of the isolated clones to BAC inserts have been confirmed using PCR and dot hybridization (Figure 3).

To perform a full length cDNA isolation we will use PCR based screening of the cDNA libraries. Primers for this screening will be designed from nucleotide sequence obtained from partial cDNA clones. As an alternative method we will use primers designed from putative exons predicted with GRAIL analysis. Isolated full length cDNAs will be sequenced and analyzed for open reading frames (ORF). As a source for full length cDNA clones we will use Marathon-Ready cDNA libraries (CLONTECH Laboratories, Inc.) and Rapid-Screen cDNA Library Panels (OriGene Technologies, Inc.), both already adopted and working in our laboratory.

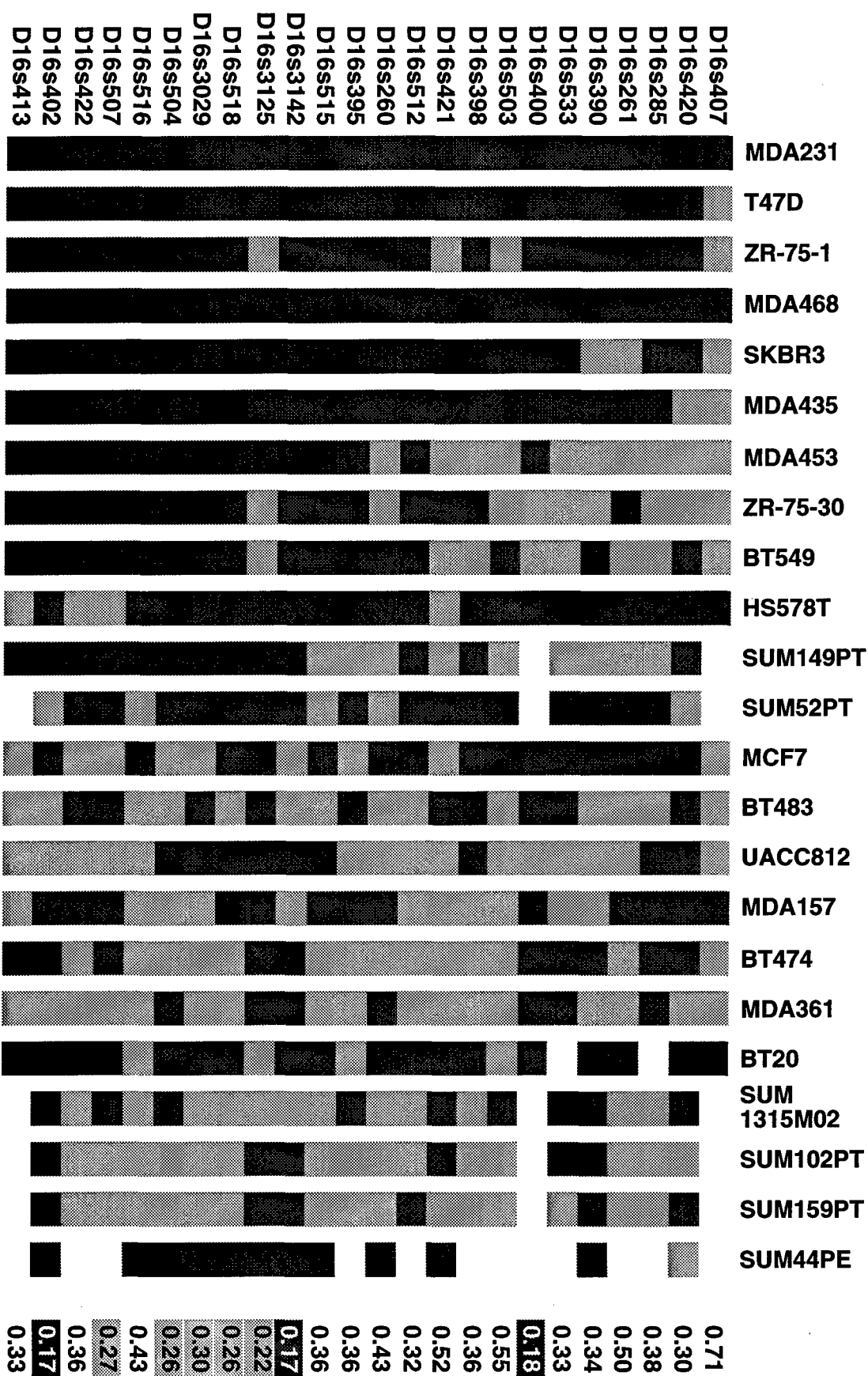
In order to perform a preliminary characterization of the putative tumor suppressor gene cDNA and predicted aminoacid sequences will be screened for homology and analyzed using computer databases. We will seek specifically for aminoacids domains characteristic for homology with transmembrane proteins (membrane receptors), kinases domains and DNA binding domains. We are currently using internet access's to several databases;

<http://genemark.biology.gatech.edu/GeneMark/>, <http://www.ncbi.nlm.nih.gov/BLAST/>,
<http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html>,
<http://protein.toulouse.inra.fr/prodom/prodom.html>, <http://www.blocks.fhcrc.org/>,
<http://bimas.dcrn.nih.gov/molbio/index.html>, <http://genome.cs.unc.edu/online.html>,
<http://gdbwww.gdb.org/>, <http://www.ncbi.nlm.nih.gov/ncicgap/>,
<http://compbio.ornl.gov/tools/index.shtml>, <http://www.ncbi.nlm.nih.gov/UniGene/index.html>,
<http://www-shgc.stanford.edu/Mapping/>.

Such comprehensive analysis could give some information about a predicted protein structure and function.

The final analysis will be to characterize an expression pattern and possible mutations of the putative tumor suppressor gene(s). To do this we will utilize Northern blot hybridization using mRNA isolated from breast cancer cell lines, tumors and normal tissues. Additionally we will develop a RT-PCR system for fast expression screening.

In order to characterize possible mutations and polymorphisms in interesting genes we will do a direct sequencing analysis. A single-strand conformation polymorphism (PCR SSCP) and BESS T-Scan Kit (Epicentre Technologies) will be adopted for fast tumor samples analysis. In order to further characterize these transcripts, *in vitro* translation and Western blot analysis will be performed.

**Figure 1**

High resolution allelotyping 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 hemizyosity affecting all or most of the chromosome 16q arm.

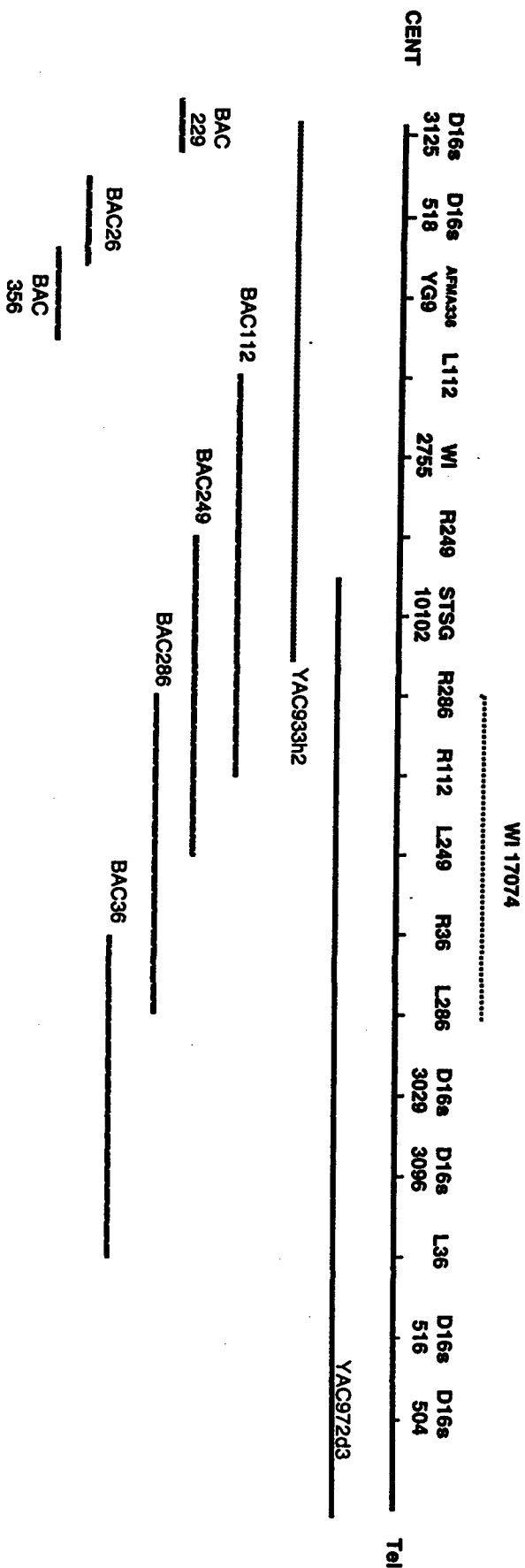
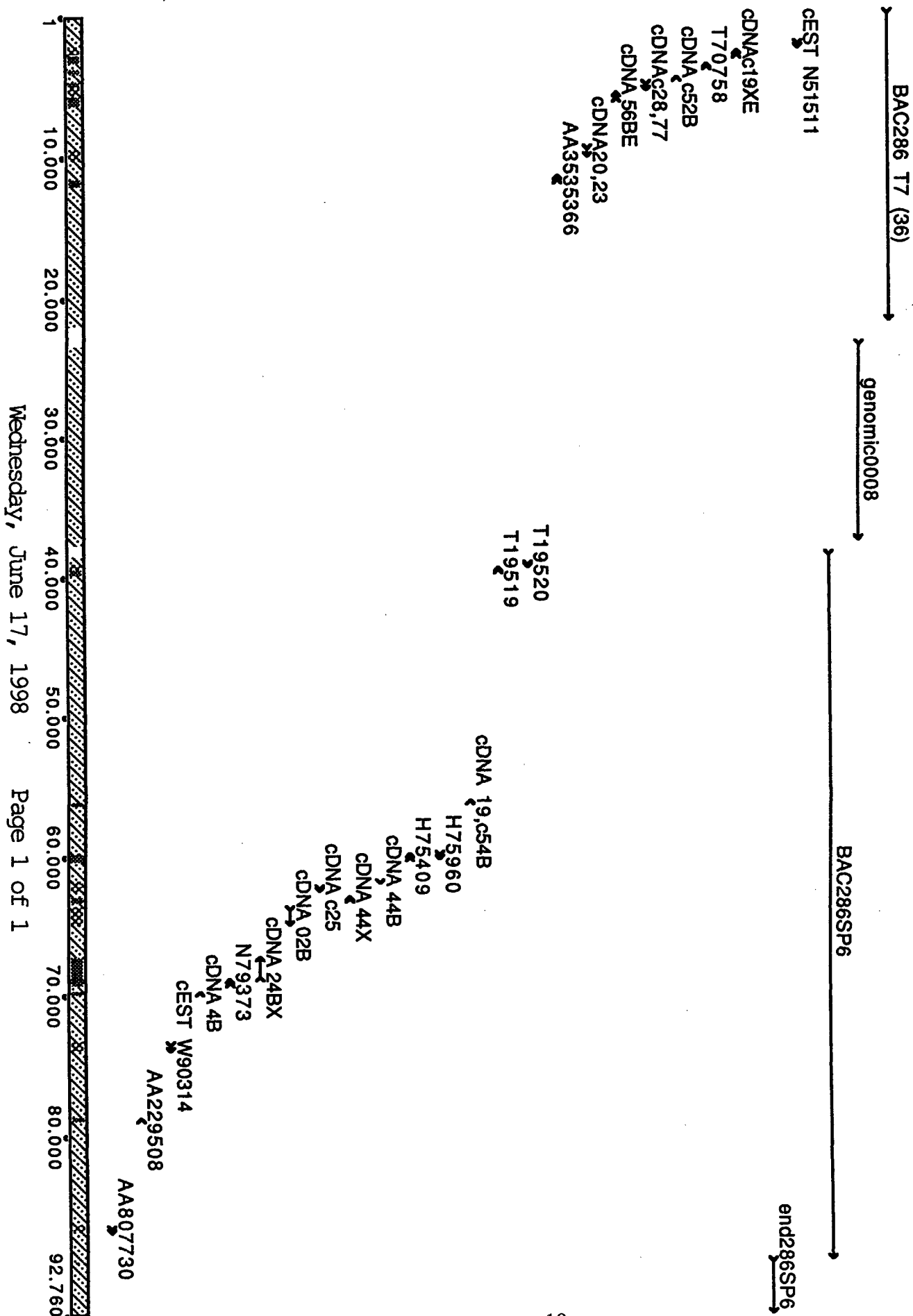


Figure 2.

YAC and BAC contig spanning a region with observed homozygous loss in one breast cancer line. The various STSs identified and used to build this contig are shown. The represented distance between STSs is not to scale. The homozygous deletion have been defined in the region between STS markers L112 and L36.

BAC286
Sequencher™ "cDNA versus Genomic"



Genomic DNA clone	ESTs Accession Number
BAC286 	W90314 W90671 T62694 AA778207 AA604596 T62845 <u>R05832</u> <u>W86864</u> <u>AA693565</u> <u>W86667</u> N51511 T69772 AA166628 AA229508 AA353536 AA560960 AA570335 AA807730 H75409 H75960 N53108 <u>N62411</u> <u>N79373</u> T19519 T19520 T70758 W79095
BAC 249 	<u>T50247</u> <u>H54363</u>

Table1.

List of ESTs homologous to BACs DNA.

ESTs were identified by BLAST algorithm using the genomic sequence and/or by analysis of cDNAs isolated by a solution hybrid capture from human mammary gland cDNA library.

Underlined ESTs were found to be expressed in human breast.

CONCLUSIONS

High resolution allelotype of chromosome 16 in Breast Ductal Carcinoma *in situ* shows a very high frequency of allelic losses affecting markers on the long arm of this autosome. The most common region of overlapping allelic losses spans the region 16q23.3 - q24.1, observed affected in 60 - 70% of *in situ* breast lesions. Losses of heterozygosity affecting this region are also observed in even earlier preinvasive lesions such as Atypical Ductal Hyperplasias.

Positional cloning strategies are currently being employed to clone the putative tumor suppressor gene. We have defined approximately 300 kb of a homozygous deletion within the region q23.3 - q24.1 of chromosome 16 in a breast cancer cell line. A total of 29 previously reported ESTs were mapped by us to this region. Five new cDNA clones were isolated and mapped. The analysis of 150 kb of the genomic sequence from this deletion showed 22 putative exons.

The identification of this gene as well as the use of molecular markers from this chromosome region will provide tools of potential diagnostic-prognostic significance.

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