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Abstract

Breast caner is the most common cancer type in women carrying *p53* germline mutations which lead to multiple cancer types. Frequent observation of mammary tumors in BALB/c-*Trp53*$^{+/-}$ (*p53*) female mice but rare in C57BL/6-*Trp53*$^{+/-}$ females suggested that inherited breast cancer susceptibility also exists in mice. *p53* heterozygosity in these mice provides a sensitized background for screening alleles that contribute to cancer susceptibility. By multiple genetic crosses, we conducted a mouse genome scan and isolated the *SuprMam1* region on mouse chromosome 7 that could be linked to mammary tumor susceptibility in BALB/c-*Trp53*$^{+/-}$ mice. Microarray gene expression profiling identified *Deleted in Malignant Brain Tumors 1* (*DMBT1*) as a leading candidate. Loss or down-regulation of *DMBT1* has been found in many cancer types as well as in breast cancer. Furthermore, the basal level of *DMBT1* mRNA in the mammary glands was dramatically reduced in the susceptible BALB/c mice compared to C57BL/6. In normal human breast tissues, an overall reduction DMBT1 protein was statistically linked to women with breast cancer history compared to those who had no breast cancer before. The data suggests that DMBT1 could be a low-penetrance allele that contribute to breast cancer susceptibility.

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Introduction

Tumor suppressor *p53* is a key inhibitor for tumor development in human and animals. Breast cancer is the most frequent tumor observed in women carrying *p53* germline mutations. In mice, mammary tumors are common among BALB/c-*Trp53*$^{+/-}$ females but rare among C57BL/6-*Trp53*$^{+/-}$ females (1,2). The inherited difference in cancer susceptibility between the two strains provides a model system to study genetic modifiers (both high- and low-penetrance alleles) that contribute to breast cancer susceptibility. In the previous report, we have mapped a 10Mb region on mouse chromosome 7 that contains a recessive-acting gene that could be responsible for the mammary tumor susceptibility in BALB/c mice. Gene microarray analysis further revealed that *Deleted in Malignant Brain Tumors 1 (DMBT1)* is a leading candidate. Loss or reduction in DMBT1 expression has been broadly reported in various of epithelial cancers, including breast cancer (3-6). In this report, we provided evidence that low DMBT1 expression is associated with tumor susceptibility in mice and human.

Body
See appendices 2 (attached).

Key Research Accomplishments

Aim 1: Genetic alterations leading to mammary tumorigenesis in BALB/c-*Trp53*+/- mice.
Status: Completed, Data published in Blackburn *et al.*, 2004.

Aim 2: Mapping genetic modifiers of mammary tumor susceptibility in BALB/c-*Trp53*+/- mice.
Status: Partially completed, Manuscript in preparation (Blackburn *et al.*, 2005).
- Genetic mapping identified SuprMam1, a ~15cM interval on mouse chromosome 7 is linked to mammary tumor susceptibility in BALB/c-*Trp53*+/- mice. Microarray gene profiling further identified *DMBT1* as a leading candidate.
- Northern and RT-PCR analysis confirmed that lowered *DMBT1* gene expression is specifically observed in the mammary gland of the susceptible BALB/c mice compared to the resistant C57BL/6.
- Immunocytochemistry detected that in normal breast tissues, low DMBT1 protein level is statistically significantly associated with women with breast cancer history compared to women who are cancer-free.

Reportable Outcomes

Publications:
Blackburn, A.C., McLary, S.C., Naeem, R., Luszcz, J., Stockton, D.W., Donehower, L.A., Soferr, T., Naber, S.P., Otis, C.N., and **Jerry, D.J.** (2004). Loss of heterozygosity occurs via homologous recombination in Trp53+/- mice and associates with mammary tumor susceptibility of the BALB/c strain. *Cancer Res.* 64: 5140-5147, 2004.

Manuscripts in preparation:

Blackburn, A.C., **Hill, L.Z.**, Roberts, A.L., Wang, J., Aud, D., Jung, J., Nicolcheva, T., Allard, J., Otis, C.N., Cao, J., Ricketts, R.S.J., Naber, S.P., Mollenhauer, J., Dickinson, E., Bigelow, C., Peltz, G., and **Jerry, D.J.** (2005). Genetic mapping in mice identifies *DMBT1* as tumor suppressor protein associated with cancer susceptibility. *Proc Natl. Acad. Sci. USA* (Submitted).

Poster Presentations:
Blackburn, A.C., **Hill, L.Z.**, Roberts, A.L., Dickinson, E., Wang, J., Aud, D., Jung, J., Nicolcheva, T., Allard, J., Peltz, G., Otis, C.N., Cao, J., Ricketts, R.S.J., Marconi S., Naber, S.P., and **Jerry, D.J.** (2005). Genetic mapping of low-penetrance modifier loci and identification of candidate genes that influence susceptibility to breast cancer. Department of Defence Breast Caner Research Program Meeing: The 4th Era of Hope in Philadelphia, Pennsylvania, June 8-11, 2005.

Development of cell lines and tissue repositories:
Mouse mammary epithelial cell line COMMA-D expressing mouse DMBT1 cDNA has been established. The cell line will be used for transplantation experiments to evaluate the relationship between DMBT1 expression and mammary tumor incidence.

Grants:
NIH, 12/1/05-12/1/08, The role of DMBT1 in breast cancer susceptibility (submitted).

Conclusions

- In the 10Mb *SuprMam1* interval, *DMBT1* is a strong candidate for a potential low-penetrance genetic modifier for mammary tumor susceptibility in BALB/c-*Trp53+/-* mice.
- Low basal level of *DMBT1* mRNA is specifically associated with the mammary glands from tumor susceptible strain BALB/c-*Trp53+/-* compared to the resistant strain C57BL/6.
- In normal breast tissues, reduced DMBT1 protein level is associated with women who had breast cancer compared to women with no breast cancer history. The difference is statistically significant.

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Cancer Detect. Prev. 26, 266-274.

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Appendices

1-Reprint of published article is attached.
2-2005 Manuscript is attached.

Loss of Heterozygosity Occurs via Mitotic Recombination in $Trp53^{+/-}$ Mice and Associates with Mammary Tumor Susceptibility of the BALB/c Strain

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ABSTRACT

Loss of heterozygosity (LOH) occurs commonly in cancers causing disruption of tumor suppressor genes and promoting tumor progression. BALB/c-$Trp53^{+/-}$ mice are a model of Li-Fraumeni syndrome, exhibiting a high frequency of mammary tumors and other tumor types seen in patients. However, the frequency of mammary tumors and LOH differs among strains of $Trp53^{+/-}$ mice, with mammary tumors occurring only on a BALB/c genetic background and showing a high frequency of LOH, whereas $Trp53^{+/-}$ mice on a 129/Sv or (C57BL/6 × 129/Sv) mixed background have a very low frequency of mammary tumors and show LOH for $Trp53$ in only ~50% of tumors. We have performed studies on tumors from $Trp53^{+/-}$ mice of several genetic backgrounds to examine the mechanism of LOH in BALB/c-$Trp53^{+/-}$ mammary tumors. By Southern blotting, 96% (24 of 25) of BALB/c-$Trp53^{+/-}$ mammary tumors displayed LOH for $Trp53$. Karyotype analysis indicated that cells lacking one copy of chromosome 11 were present in all five mammary tumors analyzed but were not always the dominant population. Comparative genomic hybridization analysis of these five tumors indicated either loss or retention of the entire chromosome 11. Thus chromosome loss or deletions within chromosome 11 do not account for the LOH observed by Southern blotting. Simple sequence length polymorphism analysis of (C57BL/6 × BALB/c) F1-$Trp53^{+/-}$ mammary tumors showed that LOH occurred over multiple loci and that a combination of maternal and paternal alleles were retained, indicating that mitotic recombination is the most likely mechanism of LOH. Nonmammary tumors of BALB/c mice also showed a high frequency of LOH (22 of 26, 85%) indicating it was not a mammary tumor specific phenomenon but rather a feature of the BALB/c strain. In (C57BL/6 × BALB/c) F1-$Trp53^{+/-}$ mice LOH was observed in 93% (13 of 14) of tumors, indicating that the high frequency of LOH was a dominant genetic trait. Thus the high frequency of LOH for $Trp53$ in BALB/c-$Trp53^{+/-}$ mammary tumors occurs via mitotic recombination and is a dominant genetic trait that associates with the occurrence of mammary tumors in (C57BL/6 × BALB/c) F1-$Trp53^{+/-}$ mice. These results further implicate double-strand DNA break repair machinery as important contributors to mammary tumorigenesis.

INTRODUCTION

Loss of heterozygosity (LOH) plays an important role in carcinogenesis in both sporadic and familial cancers. LOH is a means by which mutations, germline or sporadic, in tumor suppressor genes can become homozygous leading to tumor predisposition in the affected cells (1, 2). Mutations in the p53 tumor suppressor gene are commonly observed in human cancers and occur together with LOH at the *TP53* locus (3, 4). Although the p53 tumor suppressor gene (*TP53* in humans or *Trp53* in mice) is critical for inhibiting tumor development in many tissues, the breast epithelium appears particularly dependent on proper p53 function. This is evident from the high frequency of mutations in *TP53* in sporadic human breast cancers (5) and the high frequency of breast cancer in Li-Fraumeni syndrome patients (LFS), of whom approximately half carry mutations in one allele of *TP53* (6). Even in the context of mutations in the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, high rates of p53 mutation are found (7, 8) and inactivation of p53 is frequent in the development of mammary tumors in *Brca1* or *Brca2* conditional mutant mice (9, 10). Thus p53 mutation and LOH appear to play particularly prominent roles in the development of breast cancer.

Mice heterozygous for the *Trp53* null allele develop a spectrum of tumors similar to LFS patients (11, 12). However, only when the *Trp53* null allele was backcrossed onto the BALB/c genetic background was the high frequency of mammary tumors seen in LFS patients recapitulated (13). Therefore BALB/c-$Trp53^{+/-}$ mice serve as a unique model for breast cancer in LFS. The BALB/c susceptibility to $Trp53^{+/-}$ mammary tumors has both dominant and recessive genetic components, as determined by breeding with the C57BL/6 strain (14). Female BALB/c-$Trp53^{+/-}$ mice developed mammary tumors at a frequency of 55% and a latency of 8–14 months, with the majority being adenocarcinomas that exhibit karyotypic instability and are often aneuploid (13). Mammary tumors arising in BALB/c-$Trp53^{+/-}$ mice exhibited a high frequency of loss of the wild-type *Trp53* allele (13), whereas other tumor types in other strains displayed lower frequencies of LOH (15). Examination of the *TP53* locus in LFS tumors also revealed frequent loss of the wild-type allele (16). The mechanism by which the wild-type *Trp53* allele is lost in BALB/c-$Trp53^{+/-}$ mammary tumors and the extent of chromosomal loss around *Trp53* are unknown.

Various mechanisms can result in LOH that may or may not lead to changes in gene copy number, thus affecting our ability to detect LOH. Loss of an entire chromosome by missegregation will lead to LOH along the entire chromosome length, and unless accompanied by reduplication, this loss will result in only one copy of that chromosome being present. Recombination between homologous chromosomes rather than sister chromatids during mitosis (mitotic recombination, MR) will result in LOH occurring over the distance between the recombination break points, with multiple rounds of this over numerous cell divisions producing a mosaic effect of retained heterozygosity and LOH at different loci within the one chromosome,

although maintaining two copies of each gene. Deletion events, perhaps occurring as a result of nonhomologous end joining (NHEJ) of double-strand DNA breaks, will result in LOH and a reduction in gene dosage for loci within the deleted region.

In this study, *Trp53*^{+/−} mice of BALB/c and mixed genetic background were used to compare frequencies of LOH at *Trp53* in mammary and nonmammary tumors and examine the mechanisms leading to LOH. Karyotype analysis was used to detect large chromosomal alterations, whereas comparative genomic hybridization (CGH) microarray analysis was used to detect changes in copy number in regions as small as a few megabases. Simple sequence length polymorphism (SSLP) analysis together with Southern blotting was used to determine the identity of the alleles present in tumors. Together these analyses provided insights into the mutagenesis processes leading to LOH in the mouse mammary gland.

MATERIALS AND METHODS

Mice. BALB/c-*Trp53*^{+/−} mice were generated previously (17) by backcrossing (C57BL/6 × 129/Sv) *Trp53*^{−/−} mice onto the BALB/cMed strain for 11 generations. F1 intercross mice were *Trp53*^{+/−} offspring of inbred C57BL/6J-*Trp53*^{+/+} female and BALB/cMed-*Trp53*^{−/−} male mice. N2 backcross mice were the offspring of (C57BL/6J × BALB/cMed) F1-*Trp53*^{+/+} females × BALB/cMed-*Trp53*^{−/−} males. Ninety-seven virgin female BALB/c-*Trp53*^{+/−}, 19 virgin female (C57BL/6 × BALB/c) F1-*Trp53*^{+/−} study mice, and 224 virgin female [(C57BL/6 × BALB/c) × BALB/c] N2-*Trp53*^{+/−} study mice were monitored weekly for tumor development or morbidity and were palpated for mammary tumors. The survival from and the occurrence of mammary and other tumors in these mouse populations has been described previously (14).

Tumors from male and female *Trp53*^{+/−} mice of mixed [C57BL/6 × 129/Sv] background analyzed for LOH are those that have been described previously (15, 18). Kaplan-Meier plots of survival ($n = 96$ females, $n = 113$ males) were analyzed by the log-rank test (Mantel-Cox) for significant differences.

Isolation of Genomic DNA. Genomic DNA for Southern blotting, PCR, and comparative genomic hybridization were extracted from tumors and normal tail tissues of *Trp53*^{+/−} mice. The tissues were snap frozen in liquid nitrogen at the time of necropsy, later minced and digested overnight with 100 μg/ml proteinase K in 100 mM Tris, 5 mM EDTA, 0.2% SDS, and 200 mM NaCl. Genomic DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1).

Trp53 Genotyping and LOH. The *Trp53*^{−/−} males used for generating the *Trp53*^{+/−} mice were genotyped by multiplex PCR as described previously (11, 17). LOH in tumors at the *Trp53* locus was determined by Southern blotting as described previously (13). Briefly, genomic DNA was digested with *Stu*I and *Eco*RI, blotted and hybridized with a probe spanning exon 7 to exon 9 of the *Trp53* gene. The intensity of the wild-type and null bands was quantitated using a phosphorimager (Cyclone; Packard Bioscience, Boston MA) and the OptiQuant software package. The ratio of wild-type:null band hybridization values was calculated. Loss of the wild-type allele was defined as wild-type: null <0.5. Statistical significance for the frequency of LOH was determined by Fisher's exact test.

Genotyping at SSLP Loci. The normal and tumor-derived DNA samples from F1- and N2-study mice were genotyped using fluorescently labeled PCR primers that amplify five SSLP markers on chromosome 11 (Applied Biosystems, Foster City, CA and Research Genetics, Huntsville, AL). Reaction volumes of 7.5 μl were used containing 1.5 μl of sample DNA at 20 ng/μl, 1.5 μl of locus specific primer mix at 4 μM concentration each, 4.5 μl of TrueAllele PCR premix (Applied Biosystems). Amplifications were performed on a tetrad thermocycler (MJ Research, Waltham, MA) with an initial melt at 95°C for 12 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, then a final hold of 72°C for 7 min. All amplifications included positive control DNA from the parental inbred strains as well as a negative control where sterile water was substituted for the template DNA. Diluted PCR product (1.5 μl) was combined with a mixture containing 1 μl of deionized formamide, 0.5 μl of loading buffer (50 mg/ml blue dextran, 25 mM EDTA), 0.5 μl commercial size standard Genescan500 or Genescan400HD (Applied

Biosystems), heated at 95°C for 5 min, and cooled on ice. Then 1.5 μl of the mixture was loaded in a 5% denaturing polyacrylamide gel and electrophoresed for 2.5 h on an ABI PRISM 377 DNA Analyzer to determine the sizes of the PCR products. GeneScan version 3.1 was used to analyze the raw data, to identify and to determine the size of each DNA fragment on the gel. Genotyper version 2.5 was used for analysis of the experimental fragments at each locus to assign the genotypes (Applied Biosystems). The areas of the allele peaks were determined and the ratio calculated. LOH was defined as called when the ratio of peak areas was ≥2-fold the value obtained for corresponding heterozygous normal tail DNA.

Comparative Genomic Hybridization. CGH was performed using 3 megabase resolution genomic DNA microarray slides, the Mouse SpectralChip Microarray, from Spectral Genomics (Houston, TX). Mouse tumor DNA and reference tail genomic DNA were digested with *Eco*RI for 16 h at 37°C and repurified by Clean and Concentrator (Zymo Research, Orange, CA). The tail and tumor DNAs were labeled with Cy3 and Cy5 by Invitrogen's BioPrime random labeling kit, making the majority of the probe between 100–500 bp in size. The Cy3-labeled reference DNA and Cy5-labeled test DNA samples were combined with 50 μg of blocking DNA for repeat sequences. This mix was precipitated with ethanol, rinsed in 70% ethanol and air-dried. The same procedure was repeated with the Cy5-labeled tail and Cy3-labeled tumor DNAs. The pellets were dissolved in 10 μl of distilled water and mixed with 30 μg of hybridization solution (50% formamide, 10% dextran sulfate in 2 × SSC). The labeled DNAs were denatured at 72°C for 10 min followed by incubation at 37°C for 30 min to block repetitive sequences. Oppositely labeled DNA mixes (Cy3-labeled test and Cy5-labeled reference DNA, Cy3-labeled reference and Cy5-labeled test DNA) were added onto duplicate microarray slides. Hybridization as per the Spectral Genomics protocol was overnight at 37°C. Slides were washed at room temperature 2 × SSC for 3–5 min, then washed at 50°C for 20 min with shaking in 50% formamide/2 × SSC. The wash step was repeated with prewarmed (50°C) 0.1%NP40/2 × SSC for 20 min and with 0.2 × SSC for 10 min at 5°C. The microarrays were briefly rinsed with distilled water at room temperature for 3–5 s and immediately centrifuged for 3 min at 500 × g for drying. Hybridized microarray slides were scanned with GenePix 4000B scanner (Axon Ins. Inc., Union City, CA), and the data obtained were analyzed using the Spectralware 1.0 software (Spectral Genomics). SpectralWare was used to normalize the Cy5:Cy3 intensity ratios for each slide such that the summed Cy5 signal equals the summed Cy3 signal. The normalized Cy5:Cy3 intensity ratios were computed for each of the two slides and plotted together for each chromosome. Gains in DNA copy number at a particular locus are observed as the simultaneous deviation of the ratio plots from a modal value of 1, with the blue ratio plot showing a positive deviation (upward) whereas the red ratio plot shows a negative deviation at the same locus (downward). Conversely, DNA copy number losses show the opposite pattern.

Karyotypic Analysis of Mammary Tumors. Primary mammary epithelial cell cultures were prepared from mammary tumors arising in BALB/c-*Trp53*^{+/−} mice. Tumor tissue was minced finely with razor blades and digested in mammary epithelial cell media [consisting of DMEM/F12 (Sigma) plus 25 mM HEPES, 1.2g/L NaHCO, 10 μg/ml insulin, 5 ng/ml EGF] supplemented with 2% adult bovine serum and 0.4% collagenase type III (Life Technologies, Inc.) at 37°C for 3–4 h with gentle agitation. The digested cell suspension was then washed several times in PBS, and the finer clumps of cells were plated in flasks with mammary epithelial cell plus 2% adult bovine serum and grown 1–2 nights until a monolayer was formed. Cells were then grown in mammary epithelial cell media plus 5% fetal bovine serum overnight followed by treatment with Colcemid (Life Technologies, Inc.) overnight at 30 ng/ml or 3–5 h at 100 ng/ml. After Colcemid treatment, cells were harvested with trypsin, lysed with 0.068 M KCl hypotonic solution and the nuclei fixed with methanol:acetic acid (3:1). Chromosome spreads were prepared and stained with Giemsa for G-banding. At least 20 cells were scored for each tumor and at least 90 cells for wild-type and *Trp53*^{+/−} control cultures derived from 5 individual 1-year-old mice of each genotype.

Cultures were also grown in chamber wells and checked for epithelial cell content by performing immunohistochemistry for cytokeratin. This confirmed that over 95% of cells in these cultures were epithelial cells.

RESULTS

Tumor Spectrum. The tumor free survival and the spectrum of tumors occurring in the three $Trp53^{+/-}$ mouse populations used in this study have been analyzed in detail previously (14). In brief, mammary tumors were the most common tumor type observed in BALB/c-, [(C57BL/6 × BALB/c) × BALB/c] N2-, and (C57BL/6 × BALB/c) F1-$Trp53^{+/-}$ mice, although the frequency decreased and latency increased with decreasing BALB/c genetic component, indicating that both dominant and recessive alleles were contributing to the BALB/c mammary tumor susceptibility. The age of overall tumor free survival increased with decreasing BALB/c background. The remainder of the tumor spectrum observed in BALB/c-, F1-, and N2-$Trp53^{+/-}$ study mice included the tumor types most commonly reported in $Trp53^{+/-}$ mice on other genetic backgrounds, including lymphoma and osteosarcoma. Adrenal gland tumors were also observed as a major tumor type in the N2-$Trp53^{+/-}$ mice, a tumor type restricted to BALB/c background (14).

LOH for Trp53 in Tumors. In the initial report of BALB/c-$Trp53^{+/-}$ mammary tumors, 7 of 7 mammary tumors examined showed LOH for $Trp53$ wild-type allele (13). To confirm this result, 25 additional mammary tumors from virgin BALB/c-$Trp53^{+/-}$ mice were analyzed by Southern blotting for LOH at the $Trp53$ locus (Fig. 1). It was found that 22 of 25 showed >50% loss of the wild-type allele and 2 tumors (V05 and V15) showed 35% and 45% loss of wild-type signal, respectively. Histologically, V05 and V15 contained more stromal tissue, which may account for the presence of more wild-type allele in the sample, with one being a papillary ductal hyperplasia and the other being a solid adenocarcinoma but with a significant fibrous stromal component. Interestingly, the remaining tumor (V15) showed complete loss of the null allele, indicating that genetic changes had occurred but not with the usual outcome. Thus loss of the wild-type allele was detected in 24 of 25 (96%) mammary tumors by Southern blotting.

This high frequency of LOH contrasts with previous reports of 50–70% of spontaneous tumors from $Trp53^{+/-}$ mice showing LOH for $Trp53$ (11, 15, 18, 19). To determine whether this high frequency of LOH was particular to mammary tumors, other tumor types arising in BALB/c-$Trp53^{+/-}$ mice were examined for LOH. Lymphomas, sarcomas, and adrenal gland tumors collected from female virgin and breeder BALB/c-$Trp53^{+/-}$ mice showed a high frequency of LOH (22 of 26 tumors) that was similar to the mammary tumors ($P = 0.35$; Table 1). Thus the high frequency of LOH is not restricted to mammary tumors.

There are two differences between the mice used in this study and the previous studies reporting lower frequencies of LOH in tumors.

Table 1 LOH^a for Trp53 *locus in tumors from* Trp53$^{+/-}$ *mice*

Tumor category	LOH		P value
	n	%	
BALB/c, female			
Total BALB/c female	46/51	90	
Mammary	24/25	96	
Nonmammary	22/26	85	0.350^b
Lymphoma	13/14		
Other	9/12		
129/Sv or (C57BL/6 × 129/Sv)			
Nonmammary	24/56	43	
Female	19/32	59	$0.046,^c 0.002^d$
Male	5/24	21	0.006^e
(C57BL/6 × BALB/c)-F1, female			
Total [C57BL/6 × BALB/c], female	13/14	93	0.035^e
Mammary	5/6	83	
Nonmammary	8/8	100	
[(C57BL/6 × BALB/c) × BALB/c]-N2, female			
Mammary			
Young (mean 36.7 wk)	10/12	83	
Old (mean 69.4 wk)	14/14	100	0.203^f
Total mammary	53/57	93	

^{a} LOH, loss of heterozygosity.
^{b} P values are for comparison with BALB/c mammary.
^{c} P values are for comparison with BALB/c non-mammary.
^{d} P values are for comparison with BALB/c female total.
^{e} P values are for comparison with 129/Sv or (C57BL/6 × 129/Sv) non-mammary female.
^{f} P values are for comparison with Young.

The previous studies used mice of 129/Sv or mixed (C57BL/6 × 129/Sv) background and examined both males and females, whereas this study used all BALB/c female mice; therefore, both strain and gender could be contributing to the difference in LOH frequency. To determine whether gender affects the frequency of LOH, tumors analyzed previously for LOH (15) were segregated according to gender, and the frequency of LOH was calculated. Tumors from females showed a significantly higher frequency of LOH compared with males, with 59% of tumors from females showing LOH compared with only 21% of male tumors ($P = 0.006$). The frequency of LOH in the female (C57BL/6 × 129/Sv) tumors was still significantly lower than in female BALB/c tumors ($P = 0.046$; Table 1). Thus, both a strain effect and a gender effect contribute to the high frequency of LOH in female BALB/c-$Trp53^{+/-}$ tumors.

Loss of the wild-type allele of $Trp53$ has been suggested to accelerate tumor formation (15). To determine whether the rate of tumorigenesis correlated with the frequency of LOH, the survival of male and female $Trp53^{+/-}$ mice of mixed [C57BL/6 × 129/Sv] background was analyzed (Fig. 2). Female mice were found to have a significantly shorter survival time than their male counterparts, with median survival times of 70 and 80 weeks respectively ($P < 0.0001$).

+/+ +/- -/- V01 V03 V04 V06 V07 V09 V10 V14 V15 V17 V18

null ➤

wt ➤
pseudo ➤
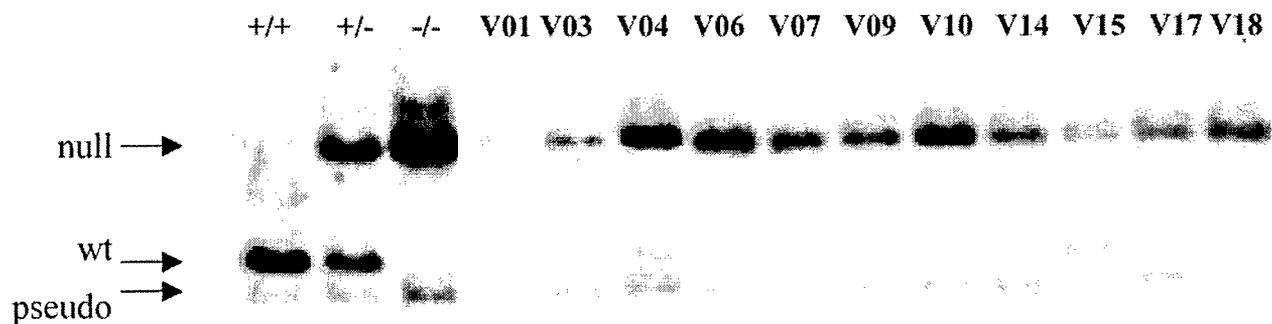
Fig. 1. Southern blotting of BALB/c-$Trp53^{+/-}$ mammary tumor DNA for the $Trp53$ locus. *Lanes 1–3*, control tail DNA. *Lanes 4–14*, tumor DNA. The majority of tumors show almost complete loss of signal from the wild-type allele. The ratio wild-type:null band for V15 was >0.5, indicating retention of the wild-type allele. A p53 pseudogene also weakly hybridizes with the probe (*pseudo*). *Wt*, wild-type.
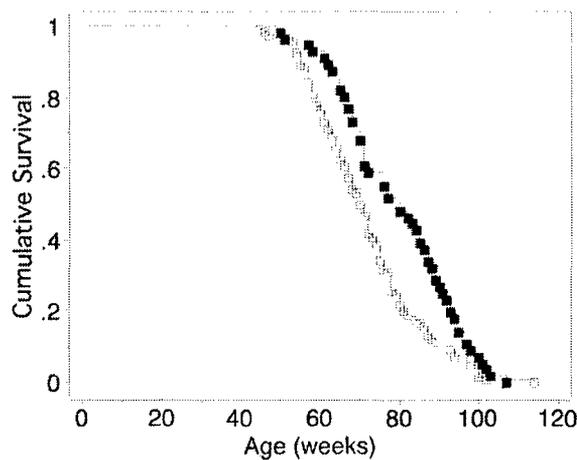
Fig. 2. Kaplan-Meier survival curves for male (■; n = 113) and female (□; n = 96) Trp53$^{+/-}$ mice of mixed (C57BL/6 × 129/Sv) background.

This difference in survival was not accounted for by gender-specific tumors because there were essentially no mammary, ovarian, or uterine cancers in the Trp53$^{+/-}$ females included in this analysis. Wild-type females did not show statistically significant differences in survival compared with wild-type males, albeit the numbers of wild-type mice analyzed were low (data not shown).

To determine whether the genetic factors leading to LOH in BALB/c-Trp53$^{+/-}$ mice were dominant or recessive, tumors arising in the (C57BL/6 × BALB/c) F1-Trp53$^{+/-}$ mice were analyzed for LOH. With the exception of the benign sclerosing adenosis of the mammary gland, all tumors examined showed loss of the wild-type allele (Table 1) giving a frequency of 93%. This was not different from the frequency for all BALB/c-Trp53$^{+/-}$ tumors (90%) and was significantly different from the (C57BL/6 × 129/Sv) female frequency (59%, P = 0.035) indicating that it was a dominant genetic trait.

To determine whether the frequency of retention of the wild-type allele increased with age, mammary tumors from [(C57BL/6 × BALB/c) × BALB/c] N2-Trp53$^{+/-}$ mice (acquired previously in an experiment genetically mapping recessive factors contributing to mammary tumor susceptibility; Ref. 14), were examined. Because of the size of this study population and the mixed genetic background of mice, considerable numbers of mammary tumors from older mice were able to be collected. LOH was analyzed in the earliest 12 (21.4–46.9, mean latency 36.7 weeks) and the latest 14 (66.9–77.6, mean latency 69.4 weeks) occurring mammary tumors. Interestingly, two of the early-onset tumors (21.4 and 40 weeks) retained the wild-type allele, whereas all other mammary tumors, old and young, had lost the wild-type allele (Table 1).

Karyotype Analysis and Comparative Genomic Hybridization of Mammary Tumors. The occurrence of aneuploidy was studied in five mammary tumors arising in BALB/c-Trp53$^{+/-}$ mice by karyotyping using short-term culture methods and by CGH microarray analysis.

Karyotype analysis revealed significant genetic instability in each of the tumors with the proportion of diploid cells ranging from 25–70% in the tumors (Table 2) compared with >90% diploid or tetraploid in normal mammary epithelial cells from one-year-old Trp53$^{+/-}$ or wild-type females. The remainder of cells in the tumors were either hypodiploid or near-tetraploid. Loss of one copy of chromosome 11 was observed in the hypodiploid population of each tumor (Fig. 3). In contrast, loss of one entire copy of chromosome 11 was detected in only three of five tumors when analyzed by CGH (tumors MTuV04, MTuV14, MTuV17; Table 2). These results are,

however, consistent with the karyotype of the dominant cell population within the metaphase samples. Thus, MTuV02, containing 50% diploid cells shows no loss on chromosome 11 by CGH, whereas MTuV14 containing 65% aneuploid cells shows loss of the entire chromosome 11 by CGH (Table 2; Fig. 3). This CGH trace is typical of those obtained from the other tumors. The small difference in the normal and tumor signal in the CGH results, contributed to by the diploid population of cells present in the tumor, is further diluted by the presence of variable numbers of chromosome 11 in the near-tetraploid cell population. Of note, CGH analysis of these tumors rarely found losses or gains at particular clones of the array, but rather, it found loss across the entire chromosome 11 or no loss at all. Thus, deletion of portions of chromosome 11 is not a common feature of these tumors.

Marker chromosomes bearing translocations (Fig. 3) and/or expanded regions of heterogeneously staining segments were also observed in the hypodiploid cells and in the polyploid cells. Therefore, the hypodiploid population appears to be the progenitor of the polyploid population of cells. In other tumors, the polyploid cells also show evidence of further genomic instability with double minutes present that are characteristic of amplified segments of the genome.

In contrast to both the karyotype and CGH results, Southern blotting indicated the unambiguous loss of one allele of Trp53 in 4 of 5 of these tumors, including MTuV14 (Table 2, Fig. 1). This was especially the case in V02, where the population of hypodiploid cells was only 25% of the total, loss along chromosome 11 was not detectable by CGH (Fig. 3), and yet >90% loss of one Trp53 allele was detected by Southern blot hybridization (Table 2). This is indicative of LOH occurring by a mechanism other than chromosomal loss and before the development of aneuploidy.

LOH at SSLP Markers. The F1 and N2 mice are heterozygous for C57BL/6 and BALB/c polymorphic markers throughout the genome, allowing more extensive analysis of LOH in tumors arising in these mice. Because the number of F1 mammary tumors available was small, informative N2 mammary tumors were analyzed in addition to all of the F1 tumors. SSLP markers spanning 1.1–37 cM of chromosome 11 were used to analyze 21 tumors from F1- and N2-Trp53$^{+/-}$ mice. The genotyping of the normal tail DNA from F1 mice was used to define the haplotype of the paternal chromosome bearing the Trp53 null allele (Fig. 4A). The paternal chromosome 11 carried BALB/c alleles for all markers except D11MIT4 (located at 37 cM). This was expected because the Trp53 null allele (located at 39 cM) was generated in embryonic stem cells from 129/Sv mice (11). The markers were all informative in the F1 mice, and therefore were used to deduce the haplotypes of tumors arising in these mice. Tumors from F1 mice

Table 2 *Karyotype of mammary tumors from BALB/c-*Trp53$^{+/-}$ *mice*

	Karyotype (% cells)					
Sample	Diploid	Hypo-diploid	Hyper-diploid	Tetraploid[c]	CGH[a] on chr 11	Southern % LOH Trp53
Tumors						
MTuV02	50[b]	25		25	No loss or gains	>90[d]
MTuV04	31	38		31	Loss of entire chr 11, gain on one clone	91
MTuV14	35	40		25	Loss of entire chr 11	92
MTuV15	70	10		20	No loss or gains	45
MTuV17	25	50		25	Loss of entire chr 11	92
Normal						
Wild-type	73	5	4	17		
Trp53$^{+/-}$	73	2	4	19		

[a] CGH, comparative genomic hybridization; chr, chromosome; LOH, loss of heterozygosity.
[b] Numbers in bold indicate the dominant cell population.
[c] In tumors, these cells were near-tetraploid, presumably derived from the hypodiploid cells.
[d] Loss of the null allele.
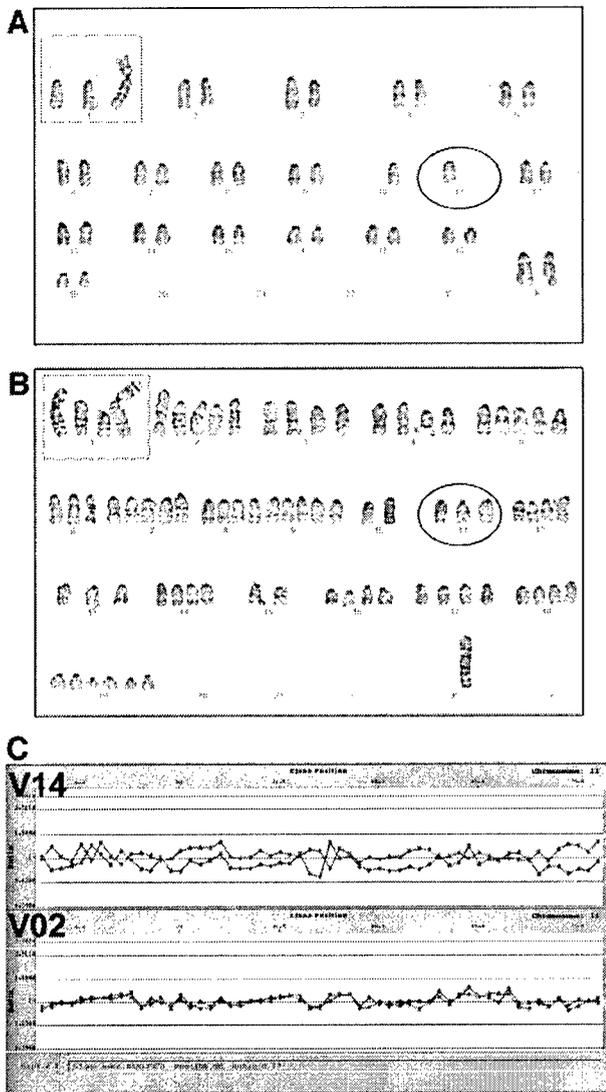
Fig. 3. Analysis of chromosome 11 by karyotyping and comparative genomic hybridization (CGH). *A*, two karyotypes from mammary tumor V02. The hypodiploid cell has only one copy of chromosome 11 (*circle*); however, this was not the dominant population (Table 2). A translocation of chromosome 10 to chromosome 1 (t1:10) has occurred (*box*) providing a "marker chromosome" in the karyotype. *B*, the polyploid cell is near tetraploid. The presence of the t1:10 marker chromosome suggests that the polyploid cells are derived from the hypodiploid population. *C*, CGH ratio plots from mammary tumors showing loss (V14) or no loss (V02) of chromosome 11.

included five mammary adenocarcinomas, one benign sclerosing adenosis of the mammary gland and seven tumors from other tissues. An initial screen of normal tail DNA from 13 N2 mice bearing mammary tumors was performed and the eight mice bearing the most informative polymorphisms were selected for further analysis. LOH and haplotypes of N2 tumor alleles were determined by comparison with normal tail DNA from the same mouse.

Examination of five SSLP markers allowed the tumors to be classified into four groups, as shown in Fig. 4, *B–E*: (*B*) retention of heterozygosity (no LOH) at all markers; (*C*) LOH at all markers with the paternal alleles being retained; (*D*) LOH at all markers with a mixture of maternal and paternal alleles being retained; and (*E*) LOH at some markers and a mixture of maternal and paternal alleles being retained. These classifications are based on the markers analyzed that are assumed to be indicative of the rest of the chromosome. Two tumors were found to have retained heterozygosity at all markers (Fig.

4*B*), consistent with the Southern blotting results for the *Trp53* locus. Where LOH was present by Southern blotting, SSLP analysis demonstrated that LOH was not restricted to the *Trp53* locus but was present at many of the SSLP loci examined in the tumors (Fig. 4, *C–E*). In the majority of tumors that showed LOH at *Trp53*, one or more adjacent loci also exhibited LOH. Thus, LOH around the *Trp53* locus spanned at least 2–22 cM, indicating that small deletions involving just the *Trp53* locus are an unlikely mechanism of loss of the wild-type allele of *Trp53*. This is consistent with the absence of partial chromosome losses in the CGH analysis.

Loss of the wild-type *Trp53* allele by missegregation and chromosome loss would be expected to be accompanied by loss of all of the maternal C57BL/6 alleles, with retention of the paternal BALB/c and 129/Sv alleles of chromosome 11. The allele pattern present in tumors 264Adr, 267Adr, and 268Lym (Fig. 4*C*) is consistent with this process. In mammary tumors 192, 252, 257, and 266, LOH was observed at all loci examined, suggesting that one copy of chromosome 11 was lost from these tumors (Fig. 4*D*). However, a combination of maternal and paternal alleles was retained, indicating that mitotic recombination had occurred during tumorigenesis before loss of one copy of chromosome 11. In the majority (12 of 21) of tumors, a fourth pattern was detected in which LOH was observed at some loci with retention of heterozygosity at other loci (Fig. 4*E*). Where LOH was present, a mixture of maternal and paternal alleles were retained, intermingled with retention of heterozygosity at other loci. These results are most consistent with the occurrence of multiple recombination events in cells retaining two copies of chromosome 11.

Of note, the three tumors that had undergone LOH without recombination (264Adr, 267Adr and 268Lym) were not of mammary origin (Fig. 4*C*). Thus, the number of syntenic regions of homozygosity or heterozygosity detected per tumor, indicative of recombination break points, was compared between mammary tumors ($n = 13$, excluding the benign sclerosing adenosis 259Msa) and other tumor types ($n = 7$). Mammary tumors had 2.92 ± 0.86 regions per tumor compared with 1.86 ± 0.90 regions per tumor in all other tumors ($P = 0.02$), suggesting that recombination is a more common event in mammary tumors than in other tumor types.

DISCUSSION

The initial report on mammary tumors in BALB/c-*Trp53*^{+/−} mice found a high frequency (seven of seven) of LOH for the wild-type *Trp53* allele (13). The current report confirms and expands that finding with the analysis of 57 more spontaneous mammary tumors from both BALB/c and mixed (C57BL/6 × BALB/c) genetic backgrounds using Southern blotting, karyotyping, CGH, and SSLP analysis to examine the mechanism of LOH in these mammary tumors. Southern blotting demonstrated that a high degree of LOH for *Trp53* was found in 93% of mammary tumors (Fig. 1; Table 1). Karyotype analysis indicated that cells lacking one copy of chromosome 11 were present in all five mammary tumors analyzed but were not always the dominant population (Table 2), suggesting that loss of chromosome 11 was not an early event in tumorigenesis and could not account for the high degree of LOH observed by Southern blotting. CGH analysis indicated either loss or retention of the entire chromosome 11, thus eliminating deletions within chromosome 11 as a mechanism of LOH (Fig. 3). SSLP analysis showed that LOH occurred over multiple loci, and that a combination of maternal and paternal alleles were retained, indicating that MR is the most likely mechanism of LOH (Fig. 4). Thus we propose a model for the genetic progression of spontaneous *Trp53*^{+/−} mammary adenocarcinomas whereby loss of the wild-type allele of *Trp53* occurs as a result of MR, which may be followed by missegregation and aneuploidy promoted by the absence of p53.

A

Pat Mat

B
144 259
MTu Msa

C
264 267 268
Adr Adr Lym

D
192 257 252 266
MTu MTu MTu MTu

D11MIT71 (1.1)
D11MIT227 (2)

D11MIT152 (13)
D11MIT231 (17)

D11MIT4 (37)
Trp53 (39)

E
9a 23 28 129 175 211 254 254 255 266 267 268
MTu MTu MTu MTu MTu MTu MTu Sar Lym Lip MTu Lu

D11MIT71 (1.1)
D11MIT227 (2)

D11MIT152 (13)
D11MIT231 (17)

D11MIT4 (37)
Trp53 (39)

Legend:
BALB/c
C57BL/6
129/Sv
Heterozygous
LOH, Paternal allele retained
LOH, Maternal allele retained
Uninformative

Fig. 4. Patterns of loss of heterozygosity (LOH) on chromosome 11 in spontaneous mammary tumors. *A*, haplotypes of the paternal (*Pat*) *Trp53* null bearing chromosome and maternal (*Mat*) C57BL/6 chromosome of F1 mice are depicted. On the basis of these haplotypes, the parental origin of alleles present in tumors of N2 (9–211) and F1 (252–268) mice was deduced. *B*, tumors showing no LOH and no chromosome loss. *C*, tumors showing LOH at all loci and retention of the paternal haplotype indicating loss of the maternal chromosome. *D*, tumors showing LOH at all loci with retention of some maternal alleles, indicating mitotic recombination occurred followed by chromosome loss. *E*, tumors showing LOH at some loci and retention of a mixture of maternal and paternal alleles. *Adr*, adrenal gland tumor; *Lip*, liposarcoma; *Lu*, lung tumor; *Lym*, lymphoma; *Msa*, benign mammary sclerosing adenosis; *MTu*, mammary tumor; *Sar*, sarcoma.

Mitotic recombination is thought to be responsible for the majority of LOH that occurs spontaneously in normal somatic cells. This is indicated by studies on LOH for the HLA locus in cultured human lymphocytes (20), on the pink-eyed unstable locus in mice (21) and on the *Aprt* locus in human lymphocytes and mouse fibroblasts (22, 23). The gender bias observed in LOH frequency in *Trp53*^{+/−} tumors (59% in females *versus* 21% in males, Table 1) is consistent with LOH occurring by MR as it has been shown in human lymphocytes that MR rates are 2.5-fold higher in females compared with males (20). Numerous studies have reported that wild-type p53 suppresses homologous recombination, measured as intrachromosomal recombination in integrated plasmid substrates with short tracts of homology (24, 25). In this context, repression of recombination may occur independently of other p53 functions

such as transcriptional transactivation and cell cycle control (26–28) and *Trp53*^{+/−} mouse fibroblasts had 3-fold the frequency of homologous recombination compared with wild-type cells, indicating a moderate haploinsufficiency for suppression of homologous recombination (28). However, repression of recombination is much more modest, if detectable, in the context of endogenous loci and interchromosomal recombination events (21, 29). Even without elevated rates of recombination, the background rate of recombination in *Trp53*^{+/−} tissues may be amplified because of haploinsufficiency for p53 transcriptional activation, cell cycle arrest and apoptosis (30–32), allowing the accumulation of more recombination events over time compared with wild-type cells. Mitotic recombination frequencies are inhibited by a high degree of chromosomal divergence as exists between some mouse strains (33);

however BALB/c × C57BL/6 hybrid cells were not affected in this manner.

Once the wild-type allele of *Trp53* has been lost, general genomic instability and aneuploidy will occur as is characteristic of p53-deficient tumors (34). Chromosomal missegregation without reduplication could leave the cells with only one copy of chromosome 11 and lead to deficiency in other tumor suppressor genes such as *Brca1* which lies at 60 cM on mouse chromosome 11. However, the inconsistent demonstration of loss of chromosome 11 by CGH in the mammary tumors argues against the existence of a strong selective pressure for cells possessing only one copy of chromosome 11. This is further supported by Southern blotting analysis, which indicated no significant loss of alleles at the *Brca1* locus in BALB/c-*Trp53*^{+/−} mammary tumors (13). These results support the proposed model in which LOH for *Trp53* by MR occurs as an earlier and more significant event in mammary tumorigenesis than loss of chromosome 11 in this mouse model.

A comparison of the LOH results from this study with what is known in LFS patients is favorable for this mouse model being relevant to the human setting. In a detailed study of LOH for *TP53* in LFS tumors, Varley et al. (16) found LOH occurring in 44% of tumors. Examination of allelic imbalance along chromosome 17 by microsatellite analysis produced similar findings to the mouse tumors of this study. Although some tumors showed LOH or allelic imbalance at all loci, consistent with loss of an entire chromosome, the majority of tumors showed LOH for only part of the chromosome. Varley et al. note that the relative frequency of this pattern of LOH is higher in their LFS tumors than that reported for other tumor types, such as retinoblastoma. This may be because of haploinsufficiency for suppression of MR by p53 in the heterozygous LFS patients. Interestingly, when LOH frequency in LFS breast cancers alone was considered, 11 of 14 or 79% (seven of eight in the Varley et al. study and four of six in other published studies; Ref. 16) of tumors show LOH. Thus, LOH for *TP53* occurs at a much higher frequency in breast cancer than in other tumor types. The effect of gender on LOH in LFS cannot be assessed from these reports because the published genders of these LFS patients is incomplete. However, female carriers of p53 mutations have been shown to have a consistently higher cancer risk compared with male carriers (35), even after the exclusion of cases of breast, ovarian, and prostate cancer. This aspect of LFS is recapitulated in the *Trp53*^{+/−} mouse model (Fig. 2) and correlates with the higher frequency of LOH in female mice (Table 1). Hwang et al. (35) do not speculate on the mechanism for the sex difference in cancer risk, but it is tempting to suggest that a higher frequency of LOH of *TP53* occurring in female patients contributes to earlier onset cancers than in males, although the influence of estrogen as a tumor promotor generally cannot be excluded.

Analysis of nonmammary tumors arising in BALB/c-*Trp53*^{+/−} mice demonstrated that LOH occurred at a similar high frequency to mammary tumors (Table 1). Thus, rather than being restricted to mammary tumors, the high frequency of LOH is characteristic of the BALB/c strain. One potential contributor to this trait is the hypomorphic BALB/c allele of *Prkdc* (36), the gene encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PK). DNA-PK has been reported to suppress induced and spontaneous homologous recombination (37). DNA-PK is also an essential component of the NHEJ pathway for DNA double-strand break repair (38). A deficiency in NHEJ may increase the proportion of double-strand break repaired by alternate pathways such as recombination. BALB/c mice have been found to have two missense mutations in *Prkdc*, resulting in decreased protein levels, DNA-PK activity and double-strand break joining activity compared with most other mouse strains (36, 39, 40). This deficiency in DNA-PK function may promote

MR, contributing to the higher frequency of LOH via MR for *Trp53* observed in the BALB/c strain.

The finding of a comparable frequency of LOH in the F1-*Trp53*^{+/−} tumors indicates that the elevated frequency of LOH in the BALB/c strain is a dominant genetic trait. We have reported previously that the occurrence of BALB/c-*Trp53*^{+/−} mammary tumors also has a dominant genetic component, with 32% of female (C57BL/6 × BALB/c) F1-*Trp53*^{+/−} developing mammary tumors compared with <1% in published reports for C57BL/6 mice (14). Thus, the high frequency of tumor LOH for *Trp53* associates with the occurrence of mammary tumors. Although LOH for *Trp53* occurred equally among tumor types, the number of recombination events that occurred in the tumors was not equal. SSLP analysis of paternal and maternal alleles retained within the tumors indicated that recombination events may be more common in mammary tumors than in other tumor types. This would be consistent with the higher frequency of LOH for p53 in LFS breast cancer compared with other tumor types. Tumor-specific LOH may be the result of several different mechanisms, discussed in Monteiro (41), including tissue-specific differences in recombination rates. Studies on MR in mouse fibroblasts and T-lymphocytes support the notion that MR is regulated in a tissue-specific manner (33, 42). If MR determines the rate of LOH in spontaneous tumors of this model, and mammary tumors undergo higher rates of MR than other tissues, then a further increase because of strain differences in the MR rate may greatly amplify the LOH rate in the mammary gland over other tissues, thereby specifically accelerating mammary tumor development. Thus, we hypothesize that elevated MR rates may contribute to the high susceptibility of BALB/c-*Trp53*^{+/−} mice to mammary tumors compared with other strains.

If reduced DNA-PK activity is contributing to a higher frequency of LOH, this may have a particularly strong impact on the mammary gland because DNA-PK activity is already low in normal mouse (39) and human (43) mammary glands compared with other tissues. *Prkdc* has been suggested as the gene responsible for BALB/c susceptibility to radiation-induced mammary tumors (39). We have demonstrated clearly that the *Prkdc* allele is not a major recessive locus contributing to spontaneous mammary tumor susceptibility in BALB/c-*Trp53*^{+/−} mice (14), but its dominant actions have not been characterized. In humans, a study has recently been published examining breast cancer risk and polymorphisms in five NHEJ genes, *Ku70*, *Ku80*, *DNA-PKcs*, *Ligase IV*, and *XRCC4* (44). In a multigenic analysis, increased risk of developing breast cancer was found in women harboring a greater number of putative high-risk alleles of NHEJ genes, which was stronger and more significant in women thought to have a greater exposure to estrogen (*i.e.*, no full-term pregnancies; Ref. 44). This is consistent with our hypothesis for BALB/c mice, which stated that elevated MR and decreased DNA-PK activity contribute to mammary tumor susceptibility. The shortcoming of this population study is the lack of functional information for the single nucleotide polymorphism alleles examined. It would be valuable to test this hypothesis with polymorphisms of known functional consequence, either in mouse models or in population studies, to confirm the mechanism responsible for the risk association identified in Fu et al. (44) and in our study.

Other genes and pathways are potentially involved in the dominant mammary tumor phenotype observed in these mice. However, the finding that recombination may be responsible for loss of the wild-type allele of *Trp53* in spontaneous mammary tumors in *Trp53*^{+/−} mice implicates the recombination machinery in the tumorigenic process of the BALB/c mouse mammary gland. Both *BRCA1* and *BRCA2* have been shown to be involved in repair of double-strand break particularly via recombination (38, 45). Additional studies are required to determine the underlying reason for the high frequency of

LOH and recombination observed in these tumors and the relationship to the breast cancer susceptibility of the BALB/c strain.

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Genetic mapping in mice identifies *DMBT1* as a modifier of breast cancer risk

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ABSTRACT

Heritable breast cancer is a complex disease with multiple genes contributing to overall risk. A genetic screen using intercross progeny from strains of *Trp53+/-* mice that differ in incidence of mammary tumors identified a recessive modifier of mammary tumor susceptibility on mouse chromosome 7 (designated *SuprMam1*). Relative to heterozygotes, homozygosity for BALB/c alleles of *SuprMam1* significantly decreased latency of mammary tumors. *Dmbt1* (deleted in malignant brain tumor 1) was identified as a candidate because it was the only gene within the *SuprMam1* interval that showed reduced expression in mammary glands of the susceptible BALB/c mice and was a putative tumor suppressor. DMBT1 protein expression was also significantly reduced in breast epithelium among women with breast cancer compared with cancer-free controls.

SIGNIFICANCE

Low-penetrance breast cancer susceptibility alleles appear to play a significant role in modifying breast cancer risk. However, analysis of human cohorts has very limited statistical power to identify modifier genes with low-penetrance. These experiments demonstrate the use of $Trp53^{+/-}$ mice as a sensitized background to screen for novel low-penetrance modifiers of cancer. The results identify a new class of breast cancer susceptibility alleles that are inherited as recessive traits and support a role for *DMBT1* in suppression of mammary tumors in both mice and women. As low levels of *DMBT1* expression are associated with increased risk of breast cancer, it provides a novel tool with which to assess risk of recurrence among breast cancer patients as well as providing a target for therapeutics.

INTRODUCTION

Approximately 27% of breast cancer risk has been attributed to genetic factors (Lichtenstein et al., 2000). Highly penetrant, dominant alleles of genes such as *BRCA1*, *BRCA2* and *TP53* confer a high risk of developing breast cancer; however, mutant alleles such as these occur at a low frequency accounting for <1/3 of hereditary breast cancer and only 3% of breast cancer cases in the general population (Anglian Breast Cancer Study Group, 2000; Ford et al., 1998; Newman et al., 1998; Whittemore et al., 1997). Therefore, the susceptibility alleles underlying the majority of heritable breast cancer remain to be identified. Intensive efforts during the past decade have sought additional highly penetrant genes (hypothetical "*BRCA3*" gene), however the search remains largely unfulfilled.

The inability to detect additional high-penetrance breast cancer susceptibility alleles has stimulated interest in the role of low-penetrance modifier alleles in heritable breast cancers. Though low-penetrance alleles may be very frequent in the population, breast cancer would occur in only a small fraction of individuals carrying these alleles. In this situation it would be difficult to recognize familial clustering. Therefore, low-penetrance alleles are likely to contribute to a significant fraction of what is presently recognized as sporadic breast cancer (Nathanson et al., 2001; Ponder, 2001). Modeling the genetic basis of breast cancer indicated that more than 50% of breast cancers may originate from the 12% population that are highly susceptible to cancer (Pharoah et al., 2002) This suggests that most of the genetic predisposition to breast cancer may be connected to low-penetrance risk alleles which are common among the general population.

The significance of low-penetrance modifers is also evident in breast cancer families with known mutations. While mutations in *BRCA1* and *BRCA2* appear to cause strikingly similar clinical disease in twins (Delgado et al., 2002) the penetrance of breast cancer varies between 28% and 85% among different populations carrying *BRCA1* susceptibility alleles (Fodor et al., 1998; Nathanson et al., 2001; Warner et al., 1999). Patients carrying germline mutations in *TP53* suffer from Li-Fraumeni syndrome and are at high risk of developing multiple tumor types with early-onset breast cancer being the most common cancer in female Li-Fraumeni patients. Penetrance of breast cancer also varies considerably among women with identical mutations in *TP53* (Varley et al., 1997b; Varley et al., 1997a) which may be caused by low-penetrance alleles that modify cancer phenotypes (Ponder, 2001). Indeed, a single nucleotide polymorphism (SNP) in the promoter of *MDM2*, an inhibitor of p53 function, decreased the age-at-onset by 10 years of hereditary breast cancer in Li-Fraumeni Syndrome patients (Bond et al., 2004)

Though evidence suggests a strong role for low-penetrance modifiers of breast cancer risk, genetic

mapping in human populations has limited power. In contrast, inbred mouse strains bearing knockout alleles of tumor suppressor genes or oncogenic transgenes can be used to examine variations in tumor phenotypes among strains and to identify genetic loci that modify cancer risk. Mice bearing a mutation in the *Apc* gene (*Min*) have been used to discover a "modifier of Min" (*Mom1* or *Pla2g2a*) which affects tumor multiplicity and size of intestinal tumors (Cormier et al., 1997; Dietrich et al., 1993). Expression of the polyoma middle T-antigen oncogene have been used to identify pathways that collaborate with tyrosine kinase signaling and alter the latency of mammary tumors (Le Voyer et al., 2000; Le Voyer et al., 2001; Lifsted et al., 1998; Rose-Hellekant et al., 2002).

As mutation and loss of the p53 tumor suppressor gene is a common feature of breast cancer, we have used *Trp53+/-* mice as a model in which to screen for genes that modify breast cancer risk. The frequency of mammary tumors is highly strain dependent in *Trp53+/-* mice (Backlund et al., 2001; Kuperwasser et al., 2000b). The incidence of spontaneous mammary tumors is ~55% among BALB/c-*Trp53+/-* females (Blackburn et al., 2003; Kuperwasser et al., 2000a). In contrast, spontaneous mammary tumors were rare (<1% incidence) among *Trp53+/-* mice on C57BL/6- and 129/Sv backgrounds mice (Donehower et al., 1992; Harvey et al., 1993). The incidence of mammary tumors among (C57BL/6 x BALB/c)F1-*Trp53+/-* females was intermediate compared to the the parental strains, indicating both dominant and recessive components contributing to mammary tumor susceptibility in BALB/c mice (Blackburn et al., 2003). In this study, progeny from a backcross of (C57BL/6 x BALB/c)F1 to BALB/c-*Trp53-/-* mice (N2-*Trp53+/-*) were analyzed to genetically map a recessive locus on the distal portion of chromosome 7 that contributed to the increased incidence of mammary tumors in BALB/c-*Trp53+/-* mice. Gene expression profiling identified *DMBT1* as a candidate genetic modifier. Decreased expression in mammary tissue is associated with increased susceptibility to breast cancer in humans and mice.

RESULTS

Genome scanning for mammary tumor modifiers

A total of 224 female N2-backcross mice were monitored for tumors as described in the Experimental Procedures. After histological confirmation of the mammary tumors, mice were divided into two phenotypic groups — mice with mammary tumors (n=85) and mice without mammary tumors (N=102). A genome scan was performed on the two pools of DNA to identify mammary tumor modifier loci. The results indicated a single genomic region on chromosome 7 that had a significant association with the occurrence of mammary tumors in *Trp53+/-* mice (Figure 1A). Subsequent analysis performed using additional SNP markers indicated an interval between 101.4

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Mb (-logP=2.92, marker M-09671_1) and 113.9 Mb (-log P=2.79, marker X67140_BS2) with a peak LOD score of 3.5 at 113.0 Mb (marker M64879_201_1). This chromosome 7 locus was designated *SuprMam1* for **suppressor** of **mam**mary tum**ors**. Of note, there were potentially two peaks within this linkage region. This raises the possibility that there may be more than one gene within the interval that influences mammary tumor susceptibility. This region had a very significant effect on incidence of mammary tumors in progeny. More than half of the N2 mice (60/101) mice homozygous for the BALB/c *SuprMam1* allele developed mammary tumors, compared to only 25/74 in the heterozygous mice (p<0.0001, χ^2 test).

The specificity and magnitude of the genetic effect of *SuprMam1* was analyzed using Kaplan-Meier survival estimates. The genotypes at marker M64879_201_1 were used for this analysis. Homozygosity for the BALB/c alleles significantly decreased mammary tumor-free survival (p=0.002). The median age of mammary tumor occurrence decreased from 70.7 weeks in heterozygotes to 61.1 weeks in homozygotes (Figure 2) with homozygotes having a 2-fold increase in risk of developing a mammary tumor compared to heterozygotes (hazard ratio of 1.93, 95% confidence interval 1.26-2.95, p=0.002). This result is consistent with expectations for low-penetrance alleles. This effect was specific for mammary tumor development, as analysis of tumor-free survival for all tumor types or for any of the other major tumor types (lymphoma, osteosarcoma, adrenal gland tumor) did not show any significant decrease in survival in N2 backcross mice (hazard ratio 1.14, 95% confidence interval 0.87-1.51, p=0.3 for all tumor types). These results identify a genetic susceptibility locus with a pattern of inheritance on the distal portion of mouse chromosome 7 that specifically modifies risk of developing mammary tumors in mice.

Dmbt1 as a candidate genetic modifier of breast cancer risk

Significant linkage spans the interval between 90-125 Mb of mouse chromosome 7 (*SuprMam1*) which encodes over 200 expressed or predicted genes. However, none of the genes within this region were known mammary tumor susceptibility factors, nor were there any obvious candidate genes based upon known functions. To identify candidate genetic modifiers within this interval, gene expression in mammary glands obtained from C57BL/6- and BALB/c-*Trp53*+/- mice were profiled using oligonucleotide microarrays. Within the *SuprMam1* locus 10 genes were found to be significantly different in expression between the two strains (fold change >1.6; p<0.05), including 7 ESTs and 3 genes: *Tead1* (TEA domain family member 1), *Dmbt1* (Deleted in malignant brain tumors 1) and *Nucb2* (nucleobindin 2). Of these known genes, only *Dmbt1* had more than 2-fold difference in expression

with lower levels in the susceptible BALB/c strain, consistent with the expectations for a recessive-acting modifier. As *Dmbt1* has been described as a putative tumor suppressor gene (Mollenhauer et al., 2000), it was selected as a candidate gene for further analysis.

The difference in expression between the strains was confirmed by Northern blot hybridization. A single transcript in of ~6 kb was detected in both strains which was consistent with published reports and reference sequences in UniGene. Expression in mammary glands from C57BL/6-*Trp53+/-* mice was over 5-fold higher than the levels in BALB/c-*Trp53+/-* (p=0.009, Figure 3A and 3B). This effect was tissue-specific because levels of *Dmbt1* mRNA were equivalent in small intestines of C57BL/6 and BALB/c mice (Figure 3A and B). Alternative usage of the 3' exons of *Dmbt1* in mouse can result in transcripts that contain the transmembrane spanning domain (*Dmbt1β*) or lack this region (*Dmbt1α*). As small differences in the transcript size would be undetectable by Northern blot, specific primers were designed to define the structure of the 3' ends of the transcripts using RT-PCR. A primer pair spanning the CUB and ZP domains amplified a region common to both α and β forms. The results show detectable levels of *Dmbt1* mRNA in both strains and confirms the reduced expression in BALB/c mammary tissue (Figure 3C). Amplification of *Dmbt1* mRNA using primers specific for the transmembrane region and 3' UTR also yielded a single band at the expected size (418 bp). No differences in amplification products were detected in small intestine. These data demonstrate that *Dmbt1β* is the major transcript expressed in both strains and that levels of expression are the only difference between the strains.

Low DMBT1 expression is associated with human breast cancer

We next determined if expression of *DMBT1* was altered in human breast cancers. Expression of *DMBT1* mRNA was analyzed in normal human tissues as well as breast cancers using quantitative RT-PCR (Q-PCR). As normal breast tissue is composed of heterogeneous cell types with epithelial cells being a minority (<30% of tissue), direct comparisons with breast tumors which are enriched for epithelium can be misleading. Therefore, immortalized breast epithelial cells derived from reduction mammoplasty were used. *DMBT1* mRNA was low to undetectable in several normal tissues. Significant levels of *DMBT1* mRNA were detected only in the lung and immortalized breast epithelial cell lines (76N cell lines provided by Vimla Band, Figure 4). In contrast, expression of *DMBT1* was below the limit of detection in 6 primary human breast cancers tested (Figure 4). The decreased expression of *DMBT1* mRNA in breast cancers is consistent with the proposed tumor suppressor function in breast tissue.

As decreased expression of *Dmbt1* was associated with increased mammary tumor susceptibility in mice, we hypothesized that lower levels of expression of DMBT1 in normal human breast epithelium may be associated with increased risk, and therefore, may be more common among women with breast cancer. To address this, variations in the staining patterns of normal, benign breast tissue for DMBT1 protein were examined by immunohistology in a sample of 99 women, 46 with breast cancer and 53 with no history of breast cancer or other breast disease. Examples of weak, moderate and strong staining (staining intensity score 1, 2 and 3 respectively) are shown in Figure 5. Intracytoplasmic granular staining in the epithelium was the most common pattern, but varied among women. DMBT1 staining was often focal and variable, with some ducts staining strongly while adjacent ducts were negative (Figure 5C). Positive staining was observed in benign epithelium adjacent to tumors as well as in benign epithelium that was distant from the cancer. No consistent staining pattern in terms of the location of benign epithelium in relationship to cancer was noted in specimens from patients with history of cancer.

The DMBT1 staining within the benign glandular epithelium was semi-quantified using percent of staining as well as staining intensity to generate a staining score. Overall, women without breast cancer were more likely to show significant positive staining with scores ≥ 4 (Table 1; 72% of controls versus 26% of breast cancer patients, p=0.008, Fisher's Exact test) and showed a greater degree of staining (Table 1; staining summary score median=3.9 versus median=1.8, p<0.0001, Wilcoxon Rank Sum Test). There was no significant association of age with staining score in either the cancer or no cancer group (p=0.19 and 0.75 respectively). Furthermore, adjusted analyses performed to control for age (restricted to the women aged 40-55 years) resulted in very similar findings (p=0.002, Table 1). Thus, DMBT1 staining score in normal breast epithelium was lower among patients with breast cancer compared to the cancer-free controls.

DISCUSSION

Though nearly 30% of breast cancer risk can be attributable to heritable factors (Lichtenstein et al., 2000), the known breast cancer genes account for fewer than 5% of the breast cancer cases (Anglian Breast Cancer Study Group, 2000; Newman et al., 1998; Whittemore et al., 1997) leaving a large fraction of heritable breast cancer for which the genes remain to be identified. A polygenic model with low-penetrance risk alleles provides the best fit to explain the residual familial risk of breast cancer (Antoniou et al., 2002; Pharoah et al., 2002). Therefore, identification of low-penetrance risk alleles are likely to significantly improve the precision of risk assessment, but linkage studies in human pedigrees have limited power to detect these alleles.

Here we describe the use of the *Trp53+/-* mouse models of breast cancer to identify *DMBT1* as a low-penetrance modifier of susceptibility to mammary tumors in mice and humans. *DMBT1* was originally characterized as a gene deleted in malignant brain tumors (Mollenhauer et al., 1997) and is down-regulated in breast cancer (Braidotti et al., 2004; Mollenhauer et al., 2004) as well as other cancers of epithelial origins, including brain, skin, lung and digestive tract (Mollenhauer et al., 1997; Mollenhauer et al., 2002; Mori et al., 1999; Wu et al., 1999). These studies indicate a potential role for *DMBT1* as a tumor suppressor, however *DMBT1* has not previously been considered as a breast cancer susceptibility gene. In the present study, *DMBT1* was identified as a candidate because it is located within the *SuprMam1*, a mammary tumor susceptibility locus on mouse chromosome 7. Homozygosity for the BALB/c allele at *SuprMam1* resulted in a 10 week reduction in latency and a 2-fold increase in the risk of developing mammary tumors compared to heterozygotes (Figure 2). Expression profiling of genes within the *SuprMam1* locus revealed diminished expression of *Dmbt1* mRNA specifically in mammary tissues of the susceptible BALB/c mice. Though basal expression of *Dmbt1* mRNA was very low in mammary tissues from BALB/c mice in our experiments (Figure 3) as well as those by Mollenhauer and co-workers, it appears that the *Dmbt1* regulatory elements in BALB/c mammary epithelium remain responsive to physiologic stresses such as carcinogen treatment (Mollenhauer et al., 2004). Therefore, decreased basal levels of *Dmbt1* in mouse mammary epithelium appear sufficient to render the tissue susceptible to tumor development. As the reduction in *Dmbt1* mRNA expression is specific to the mammary gland (Figure 3), enhancers which bind tissue-specific transcription factors are likely to be disrupted in the BALB/c allele. We have sequenced 3 kb upstream of exon 1 in *Dmbt1* and did not find any polymorphisms between BALB/c and C57BL/6, suggesting that these polymorphic regulatory elements are present large distances upstream or within introns of the gene that lead to diminished basal expression.

Variable expression of DMBT1 protein in normal human breast epithelium was also observed. A significant association was found between low expression of DMBT1 in normal human breast tissue and the occurrence of breast cancer among patients that were unselected for family history (Table 1). In this study we sought to evaluate the basal levels of DMBT1 expression in cancer patients. In contrast, Mollenhauer and co-workers included hyperplastic epithelium flanking cancers in an effort to address the hypothesis that DMBT1 expression is induced by pathophysiological processes such as inflammation and cancer (Mollenhauer et al., 2004). The high frequency of positive staining (17/20) in breast cancer patients analyzed by Mollenhauer et al. suggests that, similar to the BALB/c mouse, women at risk of developing breast cancer are still able to induce DMBT1 expression. Therefore,

reduced basal levels of DMBT1 protein in normal breast epithelium among women with breast cancer supports a significant role of DMBT1 in modifying risk of breast cancer.

At least 8 common alleles of *DMBT1* have been described in humans, based on Southern blotting and sequencing of exons (Mollenhauer et al., 2000; Wu et al., 1999). These polymorphisms may act as low-penetrance modifiers of cancer susceptibility. Some of these are thought to involve deletion of scavenger receptor cysteine rich (SRCR) domains. The highly repetitive structure of the SRCR domains raises the possibility that multiple alleles have arisen due to unequal crossing during meiosis. As none of the alleles are predicted to eliminate protein expression, they would be expected to be hypomorphic with respect to their functions. Alternatively, differences in promoter elements that alter basal expression may exist in linkage with the variants in coding regions. This may contribute to the variation in expression levels observed among individuals.

The mechanism by which DMBT1 acts as a tumor suppressor is not understood, but may be mediated through its role in epithelial cell differentiation. Secreted hensin, the DMBT1 homolog in rat, polymerizes in the extracellular matrix through galectin-3 and directs terminal differentiation of rat kidney cells (Hikita et al., 2000; Takito et al., 1999) In the mouse, hensin null embryos die at embryonic day 5.5 due to the requirement for hensin in terminal differentiation of columnar epithelia during early embryogenesis (Takito and Al Awqati, 2004). DMBT1 is induced rapidly in response to certain pathological stresses which may mediate an unidentified protective role within epithelial cells. These stresses include tissue injury from paracetamol toxicity and hepatitis B infection in the liver (Bisgaard et al., 2002), carcinogen exposure in the lung (Mollenhauer et al., 2002) or mammary gland (Mollenhauer et al., 2004). Therefore, the presence of hypomorphic alleles resulting in disrupted differentiation or stress responses are likely to contribute to tumorigenesis.

We have used the *Trp53+/-* mouse model of breast cancer and transcriptional profiling to identify a novel mammary tumor susceptibility locus and candidate low-penetrance modifier gene. Association of lower DMBT1 expression in normal mammary epithelium with increased likelihood of breast cancer in both mice and humans indicates the discovery of a relevant modifier of breast cancer risk. Given that genetic variants of *DMBT1* are common, this gene has the potential to contribute significantly to breast cancer risk in the general population. However, the diversity of alleles of *DMBT1* raises the possibility that multiple variants may act as modifiers. Therefore, identifying the function of DMBT1 that is relevant to cancer suppression may be necessary to ascertain which alleles confer susceptibility. With its role in terminal differentiation of epithelial cells, DMBT1 presents us with a new pathway, that of cell fate and differentiation, that can influence breast cancer risk and may be a target for

preventive strategies.

EXPERIMENTAL PROCEDURES

Mice and Breeding Strategy: BALB/c-*Trp53+/-* mice were generated previously (Jerry et al., 1998) by backcrossing C57BL/6 x 129/Sv *Trp53+/-* mice onto the BALB/cMed strain for 11 generations. The C57BL/6 x BALB/c intercross populations have been described previously (Blackburn et al., 2003). The F1 intercross mice were *Trp53+/-* offspring of inbred C57BL/6J-*Trp53+/+* female and N11 BALB/cMed-*Trp53-/-* male mice. N2 backcross mice were the offspring of [C57BL/6J x BALB/cMed]-*Trp53+/+* F1 females x N11 BALB/cMed-*Trp53-/-* males. Nineteen female (C57BL/6 x BALB/c)-*Trp53+/-* F1 mice and 224 female [(C57BL/6 x BALB/c) x BALB/c]-*Trp53+/-* N2 mice were monitored weekly for tumor development or morbidity and palpated for mammary tumors. Mice were sacrificed before tumors reached 1cm in size or when signs of morbidity were observed. Tumor tissues were fixed overnight in neutral-buffered formalin, processed, and stained with H&E for histological assessment. All tumors were examined by a qualified pathologist for diagnosis. Mammary glands without tumors were wholemounted for examination of ductal tree structure. All procedures involving animals were in accordance with institutional and national guidelines for the use of animals and were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts-Amherst.

Genome scanning for mammary tumor susceptibility alleles: The initial genome scan was performed on two pools of DNA: mammary tumor (n=85) and non-mammary tumor (n=102) bearing mice. For this analysis, only mice with definite mammary epithelial lesions (adenocarcinoma, adenosquamous carcinoma or mammary intraepithelial neoplasia (MIN)) were included in the mammary tumor pool. This excluded 9 mice with carcinosarcomas of the mammary gland and 4 mice with adenocarcinomas of ambiguous mammary or salivary gland origin. The non-mammary tumor pool was restricted by age, with mice succumbing to other tumor types younger than 30 weeks (n=12) being excluded, as this was too early for a mammary phenotype to be observed. A further 5 mice were excluded which showed unusual ductal growth patterns in wholemounted mammary glands. This growth pattern, a secondary tree of very fine ducts originating from the nipple between ducts of the original outgrowth, was observed in 16 other mice, all of which had developed mammary tumors in other glands.

The pooled DNA samples were analyzed quantitatively for single nucleotide polymorphisms (SNP) at 150 markers throughout the genome (Grupe et al., 2001). The significance of each allele frequency difference between the two groups was calculated using the z-test and plotted as a LOD score (-logP). In regions of significant

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association (-logP > 3.3), markers were genotyped in individual mice for confirmation, and finer mapping with additional markers was performed. Using the genotype at the marker with the highest LOD score, Kaplan-Meier estimates of the tumor-free survival curves were calculated and plotted for homozygotes and heterozygotes. The median time to tumor was used for comparison of latencies and the significance of differences in latency (tumor-free survival times) and were analyzed by the log-rank test.

Expression Microarray Analysis: Mammary glands from C57BL/6- and BALB/c-*Trp53+/-* mice were collected from 12 week old virgins and snap frozen in liquid nitrogen. Total RNA was reverse transcribed using a T7- promoter coupled oligo(d)T primer (GeneChip T7-Oligo(d)T Promoter Primer Kit, Affymetrix, Santa Clara, CA). After the second-strand cDNA synthesis, an in vitro transcription (IVT) reaction was performed using an Enzo BioArray High Yield RNA transcript labeling kit (Affymetrix). The labeled samples were hybridized to the Murine Genome U74v2 set that contains probe sets for approximately 36,000 full-length mouse genes and EST clusters from the UniGene database (Affymetrix). GeneChips were scanned using the GS2500 scanner and images were analyzed by Affymetrix software (Microarray Analysis Suite version 5.0). Four mice of each strain were analyzed with pairwise comparisons. Genes showing at least 2-fold expression differences and with p value < 0.05 were considered to be differentially expressed.

RT-PCR for mouse Dmbt1: Mammary glands and small intestines from C57BL/6- and BALB/c-*Trp53+/-* mice were collected from 8 week-old virgin females as described above. Total RNA was extracted from these tissues using QIAzol reagent (QIAgen, CA) following the manufacturer's manual. 1 μg of each tissue total RNA was reverse transcribed using AMV (Seikagaku America, MA) in a 20 μl reaction mix using protocols developed by Roche (CA). 5 μl of the cDNA products were then amplified using 2 sets of forward and reverse primers with: CUB5 (5'-AGCACAAGTCTCCATCACCCAAACA-3') and ZP3 (5'-GATTGGTGGTGTTATTGTCAAAGTC-3'); TM5 (5'-ATCTTTGGCGGAGTCTTCCTGG-3') and UTR3 (5'-GTTGGCTATACATGGGGAAAAGGG-3'). The annealing temperature for the primer pairs was 60°C and product sizes were 761 and 418 bp, respectively. Mouse actin was also amplified as a control using primers Actin5F (5'-TGCTGTCCCTGTATGCCTCT-3') and Actin3R (5'-TGCCACAGGATTCCATACCC-3'), which anneal to the cDNA template at 60°C and produce a 405bp product. Polymerase chain reaction (PCR) was performed with 300 pmol/ml of each primer in a 20 μl reaction volume containing 1 x PCR buffer (Sigma), 2 mM MgCl$_2$ (Sigma), 250 μM dNTPs (Sigma) and 0.5 units of Taq polymerase (Sigma). 30 cycles of [94°C 1 min, 60°C 1 min, 72°C 1 min] were performed. The products were run on agarose

gels and viewed by ethidium bromide staining.

RT-PCR for human DMBT1: RNA from normal human tissues was purchased from Clontech (Palo Alto, CA). RNA was isolated from the 76N breast epithelial cell line series, normal cells immortalized with telomerase, mutant p53 or E6 (76N+hTERT, 76N+p53mt and 76N+E6 respectively) (Cao et al., 1997; Gao et al., 1996) using QIAzol reagent (QIAgen, CA). Human breast cancer RNA samples were from a panel of infiltrating ductal carcinomas (grades I-III) collected previously from the frozen tissue bank at Baystate Medical Center (Springfield, MA) (Pinkas et al., 1999) RT-PCR for *DMBT1* was performed using the primer pair 5'-ATGACCAGGTGCAGCCAAT-3' and 5'-AGCGGGAAGAGGGGTCATA-3', giving a 263 bp product. Total RNA (90 ng) was reverse transcribed (RT) and amplified using the Applied Biosystems (Foster City, CA) rTth DNA polymerase in a single tube reaction containing 200 nM of each primer. Reactions were run on GeneAmp 5700 sequence detection system (Applied Biosystems) with a RT cycle of [50°C 2 min, 95°C 1 min, 60°C 30 min], followed by 45 cycles of [95°C 20 sec, 60°C 20 sec] and 72°C 10 min. The products were measured using SYBR green.

Northern blot analysis: Northern blot analysis was preformed as described previously (Jerry et al., 1998). 10 µg of total RNA from each tissue were subject to electrophoresis on a 1.2% agarose-formaldehyde gel, then immobilized onto nylon membranes (CUNO Laboratory Products, CT). The membranes were hybridized to cDNA probes labeled with ^{32}P-dCTP (Sigma, MO). The probe covers mouse *Dmbt1* nucleotides 4479-5250 in the coding region, which is obtained through RT-PCR using CUB5+ZP3 primer set. The hybridization signals were visualized by Cyclone phosphor imager after exposing the membrane to phosphor screen (Packard Instrument Co., CT) for 24 hours. The results were quantified using Optiquant image analysis software (Packard, CT). Band intensities for *Dmbt1* were standardized for loading by comparison to bands hybridized with a probe for *Gapdh*, and the significance of differences was determined by t-tests.

Immunohistochemistry for DMBT1: The immunohistochemical expression of DMBT1 was evaluated in benign breast glandular epithelium from 53 patients without a history of carcinoma (46 reduction mammoplasties and 7 excisional breast biopsies for ectopic (axillary) mass or calcifications found to be benign) and in benign breast glandular epithelium from 46 patients with a history of breast carcinoma (6 ductal carcinoma in-situ, 6 infiltrating lobular carcinomas, and 34 infiltrating ductal carcinomas). All cases were formalin fixed paraffin embedded specimens retrieved from the surgical pathology archives of Baystate Medical Center Department of Pathology, Springfield, MA. The specimens were stripped of identifiers in accordance with procedures approved by the

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Institutional Review Board at Baystate Medical Center and the University of Massachusetts. The primary diagnoses were confirmed by review of the original hematoxylin and eosin stained slides. The DMBT1 protein expression was evaluated by immunohistochemistry with anti-DMBT1h12 monoclonal antibody provided by Jan Mollenhauer, (Mollenhauer et al., 2000) utilizing standard protocols on a DAKO automated platform. The primary antibody was used at a dilution of 1:250 for 60 minutes at room temperature. The antibody complex was detected using a polymer-based amplification system (Envision+, DAKO, Carpinteria, CA).

Intracytoplasmic granular staining in the epithelium was the most common pattern of staining and was regarded as positive. Immunoreactivity in inflammatory cells and stroma was infrequent and was not considered in this study. The immunohistochemical expression of DMBT1 within the glandular epithelium was semi-quantified using percent of staining as well as staining intensity. Staining percentage was quantified as follows: 0 = no staining; 1 = <5%; 2 = 6-50%; 3 = >50% of cells with positive staining. A separate score was given for staining intensity; 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining). The scores for staining percentage and intensity were combined for an overall score. The staining score was assessed by two pathologists independently (Q.J.C. and R.S.J.R.) with consensus recorded. Cases with combined scores of 0-2 were considered negative. Cases with a score ≥ 4 (with 6 being the maximum) were considered significantly positive.

Comparisons of the occurrence of significant positive staining (score ≥ 4) and the average staining score in women with breast cancer versus cancer-free controls were made using Fisher's exact tests and Wilcoxon rank sum tests respectively. Adjusted analyses to control for age were performed in addition to analyses on the whole set of results, and the association of age with staining was examined by Fisher's exact test on subgroups of the samples. The variance of each group was compared using variance ratio tests. All analyses were performed using the STATA statistical software package.

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FIGURE LEGENDS

Figure 1: Linkage analysis results for recessive mammary tumor susceptibility alleles. **(A)** A complete genome scan was conducted comparing mammary tumor versus non-mammary tumor bearing N2-*Trp53+/-* mice. Using 150 SNPs distributed across the mouse genome, significant linkage was detected on chromosome 7. **(B)** Additional SNPs were used to define an interval of strong linkage on chromosome 7 (between 101 Mb and 114 Mb=*SuprMam1* locus).

Figure 2: Kaplan-Meier mammary tumor-free survival plots of N2-*Trp53+/-* mice segregated according to genotype at *SuprMam1*. Median age of occurrence of mammary tumors among mice that were heterozygous for the susceptibility allele (*SuprMam1*$^{BALB/B6}$) had a median survival of 70.7 weeks compared to 61.1 weeks for mice that were homozygous for the susceptibility alleles (*SuprMam1*$^{BALB/BALB}$).

Figure 3: Expression of *Dmbt1* in small intestines and mammary glands from BALB/c-*Trp53+/-* and C57BL/6-*Trp53+/-* mice. **(A)** Northern blots were hybridized with a ^{32}P-labeled probe from the 3' end of the mouse *Dmbt1* cDNA spanning the CUB and ZP domains. The blots were stripped and rehybridized with a ^{32}P-labeled *Gapdh* cDNA. **(B)** The levels of *Dmbt1* mRNA expression in small intestine and mammary gland were quantified using phosphor imaging. Levels of *Dmbt1* were significantly reduced in mammary tissue from BALB/c compared to C57BL/6 ($P<0.05$). **(C)** RT-PCR was used to analyze the structure of the 3' end of *Dmbt1* transcripts in mammary gland and small intestine. The upper panel describes the structure of DMBT1 protein and the location of the sequences of primers used for PCR. The lower panel shows that both strains of mice express sequences consistent with *Dmbt1*-β. Only the levels of expression appear to differ between the strains.

Figure 4: Expression of *DMBT1* mRNA in human tissues. Levels of *DMBT1* expression were compared in normal human tissues using Q-PCR. Only lung expressed significant levels. The immortalized normal breast epithelial cells (76N series) also expressed high levels of *DMBT1* mRNA. In contrast, expression of *DMBT1* mRNA was undetectable in the primary breast cancers. (NTC=no template control)

Figure 5: Immunohistochemistry for *DMBT1* in benign human mammary epithelium. Immunohistochemical staining revealed variable patterns and intensities of staining among individuals. **(A)** Diffuse cytoplasmic staining that was low intensity of reaction was considered "score =1". **(B)** Intense, focal expression within the supranuclear region and polarized toward the luminal surface was considered "score=2". **(C)** Intense staining polarized toward the luminal surface was considered "score=3".

Table 1: Aanalysis of DMBT1 staining in normal breast epithelium.

	No Cancer	Cancer	p value
Overall			
Occurrence of significant staining (score ≥4)	72%	26%	0.008
Staining score (Mean ± SD)	3.9±1.2	1.8±1.8	<0.0001
Age (Mean ± SD)	38.6±12.6	59.5±15.0	<0.0001
n	53	46	
Age 40-55			
Occurrence of significant staining (score ≥4)	72%	23%	
Staining score (Mean ± SD)	4.1±1.0	1.9±1.8	0.001
Age (Mean ± SD)	46.9±3.9	46.3±4.0	0.7
n	18	13	

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Figure 1A

Figure 1B

Figure 1B. Plot of -log P value versus Chromosome 7 (Mb).

Figure 2

Proportion mammary tumor-free survivors

Age (weeks)

SuprMam1 BALB/B6

SuprMam1 BALB/BALB
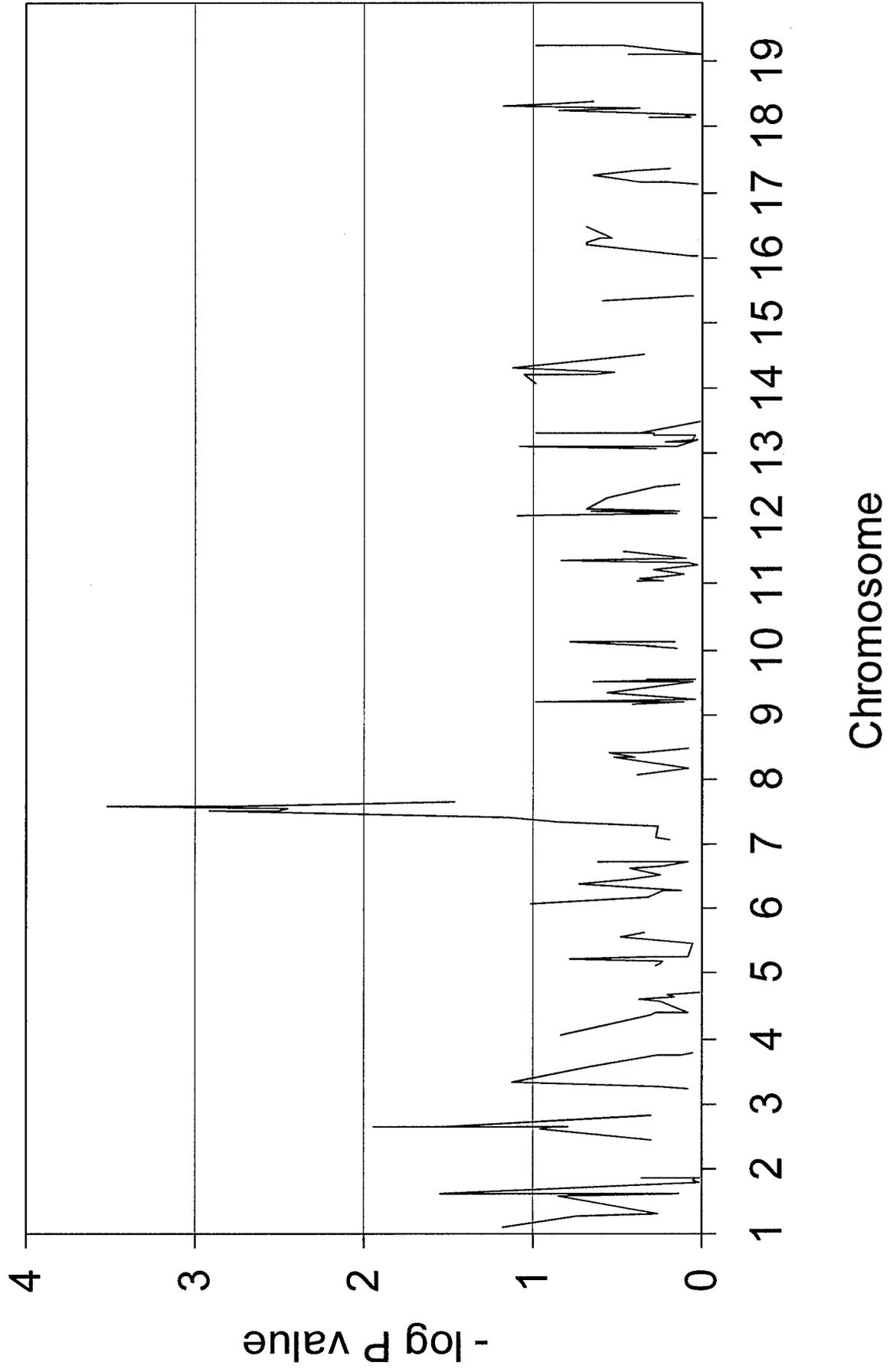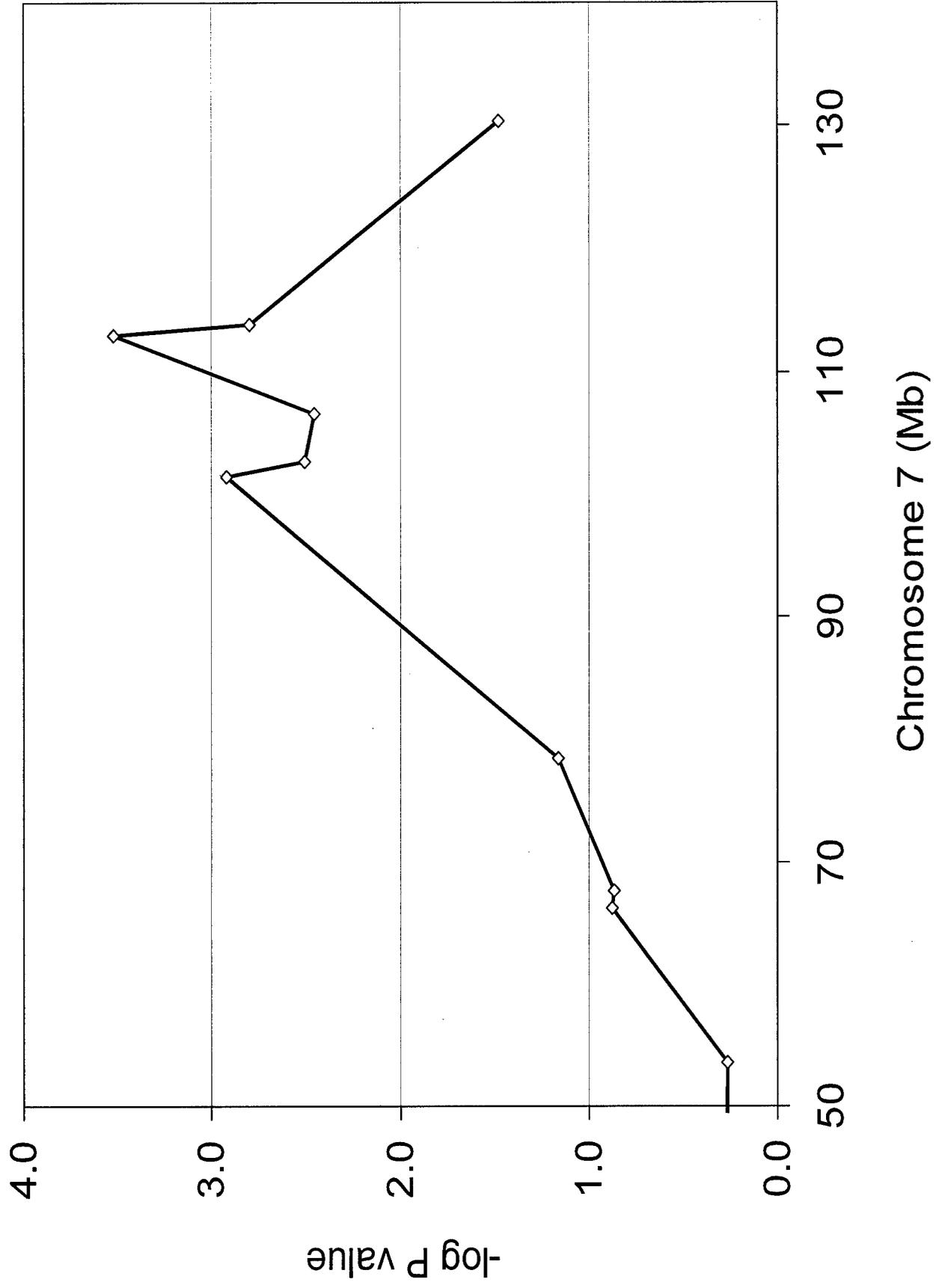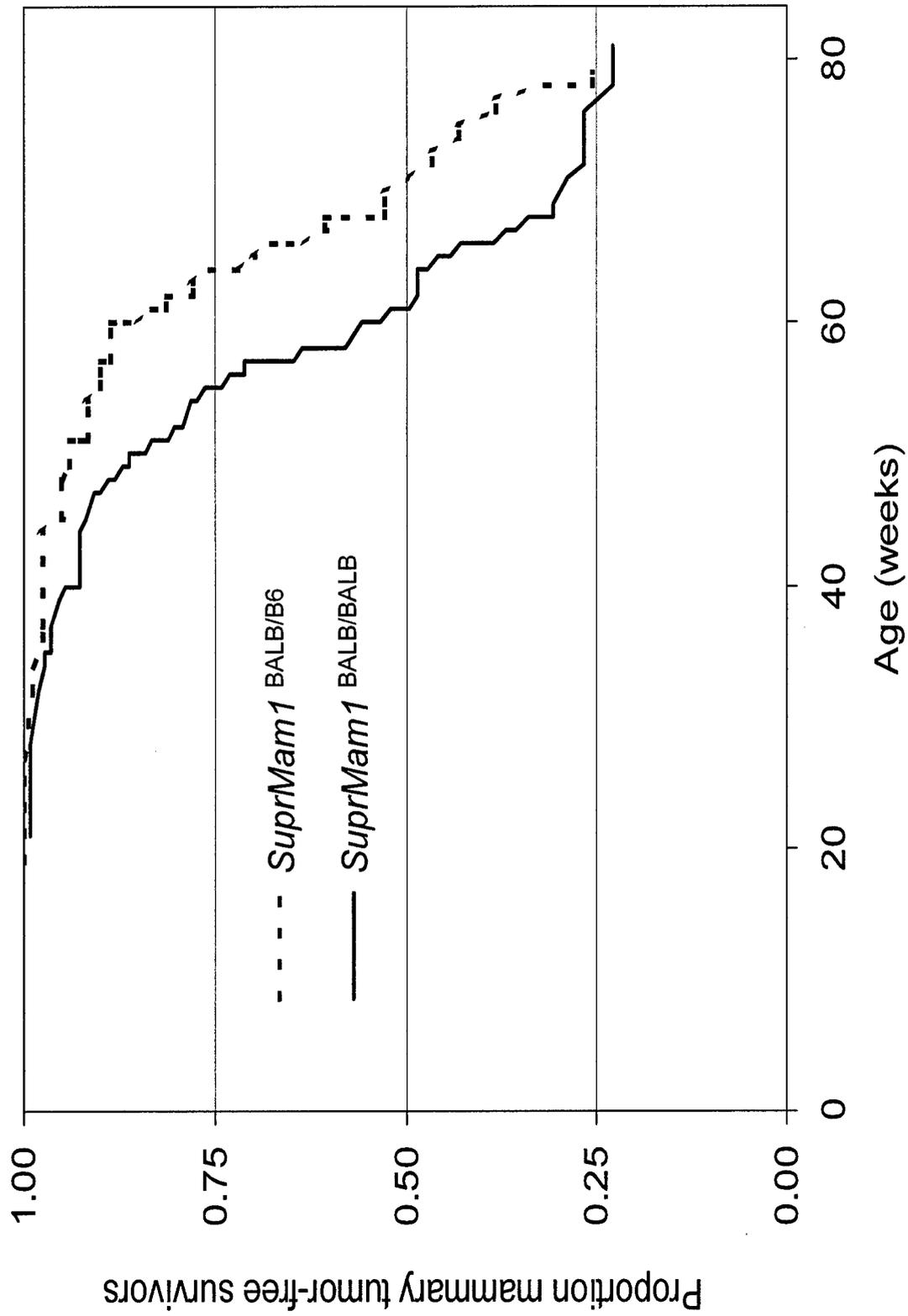
Figure 3A

Figure 3B

Figure 3C

Mouse DMBT1/CRP-Ductin α form

Mouse DMBT1/CRP-Ductin β form

Structural motifs in DMBT1 protein

Symbol	Domain	Symbol	Domain
○ Signal sequence		▮ CUB	
▪ Transmembrane domain		⬭ SRCR	
○ Ser-Thr-Pro-rich domain		▮ SID	
⬭ Thr-Pro-rich domain		▭ ZP	

3'-UTR

CUB5'   ZP3'   TM5'   UTR3'

Primers

CUB5'+ZP3' (761 bp)

TM5'+UTR3' (418 bp)

Actin (405 bp)

Mammary gland — BALB/c (3705, 3706, 3707), C57BL/6 (3693, 3694, 3695)

Small intestine — BALB/c (3705, 3706, 3707), C57BL/6 (3693, 3694, 3695)

no RNA, no oligo dT

MW marker

Figure 4

Relative Expression (y-axis, values: 0, 20, 40, 60, 80, 100, 120, 140)

Categories (x-axis): Cerebellum, Fetal Brain, Lungs, Spleen, Total Brain, 76N+p53mt, 76N+hTERT, 76N+E6, Br18, Br19, Br20, Br21, Br24, Br25, NTC

Normal Tissues | **Normal Breast** | **Breast Tumors**

Figure 5