

Molecular Pathogenesis of Genetic and Inherited Diseases

Genetic Mapping in Mice Identifies *DMBT1* as a Candidate Modifier of Mammary Tumors and Breast Cancer Risk

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Low-penetrance breast cancer susceptibility alleles seem to play a significant role in breast cancer risk but are difficult to identify in human cohorts. A genetic screen of 176 N2 backcross progeny of two *Trp53*^{+/-} strains, BALB/c and C57BL/6, which differ in their susceptibility to mammary tumors, identified a modifier of mammary tumor susceptibility in an ~25-Mb interval on mouse chromosome 7 (designated *SuprMam1*). Relative to heterozygotes, homozygosity for BALB/c alleles of *SuprMam1* significantly decreased mammary tumor latency from 70.7 to 61.1 weeks and increased risk twofold ($P = 0.002$). *Dmbt1* (deleted in malignant brain tumors 1) was identified as a candidate modifier gene within the *SuprMam1* interval because it was differentially expressed in mammary tissues from BALB/c-*Trp53*^{+/-} and C57BL/6-*Trp53*^{+/-} mice. *Dmbt1* mRNA and protein was reduced in mammary glands of the susceptible BALB/c mice. Immunohistochemical staining demonstrated that *DMBT1* protein expression was also significantly reduced in normal breast tissue from women with breast cancer (staining score, 1.8; $n = 46$) compared with cancer-free controls (staining score, 3.9; $n = 53$; $P < 0.0001$). These experiments

demonstrate the use of *Trp53*^{+/-} mice as a sensitized background to screen for low-penetrance modifiers of cancer. The results identify a novel mammary tumor susceptibility locus in mice and support a role for *DMBT1* in suppression of mammary tumors in both mice and women. (Am J Pathol 2007, 170:2030–2041; DOI: 10.2353/ajpath.2007.060512)

Approximately 27% of breast cancer risk has been attributed to genetic factors.¹ 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 less than one-third of hereditary breast cancer and fewer than 5% of breast cancer cases in the general population.^{2–5} 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; however, the search remains primarily unfulfilled. This has stimulated interest in the role of low-penetrance modifier alleles in heritable breast cancers. Low-penetrance alleles may be very frequent in the population, but 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 con-

Supported by the National Institutes of Health (grant R01CA105452 to D.J.J.); the Department of Defense Breast Cancer Program (grant DAMD17-00-1-0632 to D.J.J. and DAMD17-01-1-0315 to A.C.B. and L.Z.H.); the Susan G. Komen Breast Cancer Foundation (grant PDF0601103 to L.Z.H.); the National Health and Medical Research Council Australia (grant 179842 to A.C.B.); the Deutsche Kerbshilfe (1835-Mol to J.M.); the Wilhelm Sander-Stiftung (grant 99.018.2 to J.M.); and the Heimholtz Association (J.M.).

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Accepted for publication February 23, 2007.

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tribute to a significant fraction of what is presently recognized as sporadic breast cancer.^{6,7} Modeling of the genetic basis of breast cancer risk indicated that more than 50% of breast cancers may originate from the 12% of the population that are highly susceptible to cancer,⁸ suggesting that most of the genetic predisposition to breast cancer might be connected to these low-penetrance risk alleles that are common among the general population.

The significance of low-penetrance alleles is also evident in breast cancer families with known mutations. Although mutations in *BRCA1* and *BRCA2* seem to cause strikingly similar clinical disease in twins,⁹ the penetrance of breast cancer varies between 28% and 85% among different populations carrying *BRCA1* susceptibility alleles.^{6,10,11} Therefore, genetic background can alter latency and clinical course of breast cancer. 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 females. Penetrance of breast cancer also varies considerably among women with identical *TP53* mutations^{12,13} that may be caused by low-penetrance alleles that modify cancer phenotypes. 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 in one study.¹⁴

Although 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 genetic deletion of a tumor suppressor gene or with transgenic oncogene expression can be used 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*) that affects tumor multiplicity and size of intestinal tumors.^{15,16} Expression of the polyoma middle T-antigen oncogene has also been used to identify tyrosine kinase signaling pathways that alter mammary tumor latency.¹⁷⁻²¹

In addition to predisposing women to breast cancer when mutated in the germline, somatic mutation or loss of the p53 tumor suppressor gene is a common feature of breast cancer. Therefore, we used *Trp53*^{+/-} mice as a model system to identify genes that modify breast cancer susceptibility and may be relevant to both hereditary and sporadic breast cancer. The frequency of mammary tumors in *Trp53*^{+/-} mice is highly strain-dependent, with a high frequency (55%) occurring only on a BALB/c background,²² whereas occurrence of mammary tumors was <1% in C57BL/6- and 129/Sv-*Trp53*^{+/-} mice.^{23,24} [C57BL/6 × BALB/c]-F1-*Trp53*^{+/-} mice exhibited an incidence of mammary tumors intermediate to the parental strains, indicating both dominant and recessive components contributing to mammary tumor susceptibility in BALB/c mice.²⁵ We have used ([C57BL/6 × BALB/c] × BALB/c)-N2-*Trp53*^{+/-} backcross mice to genetically map a locus on the distal part of chromosome 7 that contributed to increased mammary tumor susceptibility and have examined *Dmbt1* as a candidate modifier gene within this locus.

Materials and Methods

Mice and Breeding Strategy

BALB/c-*Trp53*^{+/-} mice were generated previously²⁶ by backcrossing C57BL/6 × 129/Sv *Trp53*^{+/-} mice onto the BALB/cMed strain for 11 generations. The C57BL/6 × BALB/c intercross populations have been described previously.²⁵ 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 × BALB/cMed]-F1-*Trp53*^{+/+} females × N11 BALB/cMed-*Trp53*^{-/-} males. Nineteen female (C57BL/6 × BALB/c)-F1-*Trp53*^{+/-} mice and 224 female [(C57BL/6 × BALB/c) × BALB/c]-N2-*Trp53*^{+/-} mice were monitored weekly for tumor development or morbidity and palpated for mammary tumors. Mice were sacrificed before tumors reached 1 cm in size or when signs of morbidity were observed. Tumor tissues were fixed overnight in neutral-buffered formalin, processed, and stained with hematoxylin and eosin (H&E) for histological assessment. All tumors were examined by a pathologist to confirm diagnosis. Mammary glands without tumors were whole mounted 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 from mammary tumor-bearing ($n = 85$) and nonmammary tumor-bearing mice ($n = 91$). For this analysis, only mice with definite mammary epithelial lesions—adenocarcinoma ($n = 67$), adenosquamous carcinoma ($n = 7$), mixed adenocarcinoma and adenosquamous carcinoma ($n = 8$), or mammary intraepithelial neoplasia ($n = 3$)—were included in the mammary tumor pool. This excluded nine mice with carcinosarcomas of the mammary gland and four mice with adenocarcinomas of ambiguous mammary or salivary gland origin. The nonmammary tumor pool was restricted in two ways to include only the mice at lowest risk of developing mammary tumors. The nonmammary tumor pool was restricted by age, with mice succumbing to other tumor types younger than 30 weeks ($n = 12$) being excluded because this was too early for a mammary phenotype to be observed. An additional five mice were excluded that showed unusual ductal growth patterns in whole-mounted 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 mice, all of which had developed mammary tumors in other glands. Because these features appeared associated with tumor susceptibility, the mice were excluded from the nonmammary tumor pool. The pooled DNA samples were analyzed quantitatively for SNPs at 150 markers throughout the genome.²⁷ The significance of differences in allele

Table 1. Locations of Markers Used for Localizing *SuprMam1* Locus

Chr	Position (Mb)	SNP ID	-log P value	Human Chr
7	14.1	S46665_1	0.198	19
7	28.7	M-09441_1	0.280	19
7	60.3	M-10051_3	0.266	15
7	72.9	Mfge8_3_JC5236_2	0.874	15
7	74.3	Blm_1_JC5359_1	0.867	15
7	85.2	M-00295_1	1.156	11
7	107.9	M-09671_1	2.921	11
7	109.2	M-08779_3	2.509	11
7	113.2	M-10217_1	2.456	16
7	119.6	M64879_201_1	3.523	16
7	120.5	X67140_BS2_1	2.796	16
7	137.1	U71085_JC2185_2	1.471	11

Data based on Ensembl (build 34).

frequency between the two groups was calculated for each individual marker using the z-test and plotted as a LOD score (-logP). A significance threshold of -logP >3.3 was used as described previously.²⁸ In regions of significant association (-logP >3.3), markers were genotyped in individual mice for confirmation, and finer mapping with additional markers (Table 1) 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 entire group of animals was used for survival analysis. 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 reaction was performed using 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 ~36,000 full-length mouse genes and expressed sequence tag 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 twofold expression differences, with intensity values >150 in one strain, and a P value <0.05 were considered to be differentially expressed.

Reverse Transcriptase-Polymerase Chain Reaction (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, Valencia, CA) following the manufacturer's manual. One μ g of each tissue total RNA was reverse-transcribed using AMV reverse transcriptase (Seikagaku America, East Falmouth, MA) in a 20- μ l reaction mix. A 5- μ l aliquot of the cDNA products was then amplified using two sets of forward and reverse primers using CUB5 (5'-AGCA-CAAGTCTCCATCACCCAAACA-3') and ZP3 (5'-GATTG-GTGGTGTATTGTCAAAGTC-3') as well as TM5 (5'-ATCTTTGGCGGAGTCTTCTCTGG-3') and mUTR3 (5'-GTTGGCTATACATGGGGAAAAGGG-3'). The annealing temperature for the primer pairs was 60°C, and product sizes were 761 and 418 bp, respectively. Mouse β -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 405-bp product. PCR was performed with 300 pmol/ml of each primer in a 20- μ l reaction volume containing 1 \times PCR buffer (Sigma, St. Louis, MO), 2 mmol/L MgCl₂ (Sigma), 250 μ mol/L dNTPs (Sigma), and 0.5 U of *Taq* polymerase (Sigma) and amplified 30 cycles (94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute). 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 adenovirus E6 (76N + hTERT, 76N + p53mt, and 76N + E6, respectively; a generous gift from Dr. Vimla Band, Northwestern University, Chicago, IL)^{29,30} using QIAzol reagent (Qiagen). Human breast cancer RNA samples were from a panel of infiltrating ductal carcinomas (grades I to III) collected previously from the frozen tissue bank at Baystate Medical Center, Springfield, MA.³¹ For *DMBT1* Q-PCR, the primer pair 5'-ATTGTGCTGCACCTGGTCAT-3' and 5'-AGCGGGAAGAGGGGTCATA-3' was used to amplify a 263-bp product. Total RNA (90 ng) was reverse-transcribed and amplified using the Applied Biosystems (Foster City, CA) rTth DNA polymerase in a single tube. Reactions were run on GeneAmp 5700 sequence detection system (Applied Biosystems) with an RT cycle of 50°C for 2 minutes, 95°C for 1 minute, 60°C for 30 minutes, followed by 45 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 10 minutes. The products were measured using SYBR green. Human β -actin was amplified as a loading control. For RT-PCR, total RNAs were used as a template to amplify a 780-bp fragment of *DMBT1* mRNA with the forward primer ZP5 (5'-TTCCTGTATCCTGTGACCAG-3') and reverse primer hUTR3 (5'-GCAGTTTCACCAAAATTCCTTTATG-3'). Human GAPDH was also amplified using primers 5'-TTCACCACCATG-GAGAAGGC-3' (forward) and 5'-TGCATGGACTGTGGT-CATGA-3' (reverse) as the loading control. The reaction conditions were 30 PCR cycles at 94°C for 45 seconds, 51°C for 45 seconds, 72°C for 1 minute.

Northern Blot Analysis

Northern blot analysis was performed as described previously.²⁶ Total RNA (10 μ g) from each tissue were subjected to electrophoresis on a 1.2% agarose-formaldehyde gel and then immobilized onto nylon membranes (CUNO Laboratory Products, Meridan, CT). The membranes were hybridized to cDNA probes labeled with [³²P]dCTP. The probe covers mouse *Dmbt1* nucleotides 4479 to 5250 in the coding region, which was obtained through RT-PCR using CUB5 + ZP3 primer set. The hybridization signals were visualized by Cyclone phosphor imager (Packard Bioscience, Downers Grove, IL). The results were quantified using Optiquant image analysis software (Packard Bioscience). 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

Polyclonal antibodies raised against the sodium dodecyl sulfate-denatured human salivary agglutinin/gp340 isoform (anti-SAG1529, provided by D.M.) were used for immunohistochemical staining of mouse tissues at a dilution of 1:100 with DAKO (Carpinteria, CA) anti-rabbit polymer for detection. The immunohistochemical expression of DMBT1 was evaluated in benign breast glandular epithelium from 53 patients without a history of carcinoma [46 reduction mammoplasties and seven 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 (six ductal carcinoma *in situ*, six infiltrating lobular carcinomas, and 34 infiltrating ductal carcinomas). All cases were formalin-fixed, paraffin-embedded specimens retrieved from the surgical pathology archives of Department of Pathology, Baystate Medical Center, Springfield, MA. The specimens were stripped of identifiers in accordance with procedures approved by the Institutional Review Board at Baystate Medical Center and the University of Massachusetts. The primary diagnoses were confirmed by review of the original H&E-stained slides. The DMBT1 protein expression was evaluated by immunohistochemistry with anti-DMBT1h12 monoclonal antibody³² using standard protocols on a DAKO autostainer. 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 quantified using percentage of staining as well as staining intensity. Staining percentage was quantified as follows: 0, = no staining; 1, <5%; 2, 6 to 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 (see Figure 6C). The scores for staining percentage and intensity were combined for an overall score. The staining score was as-

essed by two pathologists independently (J.C. and R.S.J.R.) with consensus recorded. Cases with combined scores of 0 to 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 (StataCorp, College Station, TX).

Results

Identification of *SuprMam1* on Chromosome 7

To identify loci contributing to mammary tumor susceptibility, 224 ([C57BL/6 \times BALB/c] \times BALB/c)-N2-*Trp53*^{+/-} female mice were palpated weekly for mammary tumor development for 18 months. After histological confirmation of the mammary gland phenotypes, two groups of mice were defined—mice with mammary tumors ($n = 85$) and mice without mammary tumors ($n = 91$). Individual DNA samples were prepared and then pooled within each group. 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 occurrence of mammary tumors in *Trp53*^{+/-} mice (Figure 1A). Analysis of the DNAs from individual mice using additional SNP markers confirmed a locus on chromosome 7. The association was greatest ($-\log P = 3.5$) with marker M64879_201_1 (Figure 1B) with significant linkage extending between markers 107.9 ($-\log P = 2.92$; -09671_1) and 120.5 Mb ($-\log P = 2.79$; X67140_BS2_1). This chromosome 7 locus was designated *SuprMam1* for suppressor of mammary tumors. 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 affecting susceptibility.

The genotype of N2 mice at M64879_201_1 significantly altered the prevalence of mammary tumors during the observation period. Of the mice homozygous for BALB/c alleles, 63 of 116 (54%) developed mammary tumors compared with 26 of 92 (28%) among those heterozygous for C57BL/6 and BALB/c alleles ($P < 0.0001$, χ^2 test). Mice that did not succumb to mammary tumors developed other tumor types typical of *Trp53*^{+/-} mice,^{24,25} primarily lymphoma, adrenal gland tumors, and osteosarcomas. However, in contrast to mammary tumors, the occurrence of these tumor types was not altered by the M64879_201_1 genotype (Table 2). This suggests that homozygosity for BALB/c alleles may increase the incidence of mammary tumors.

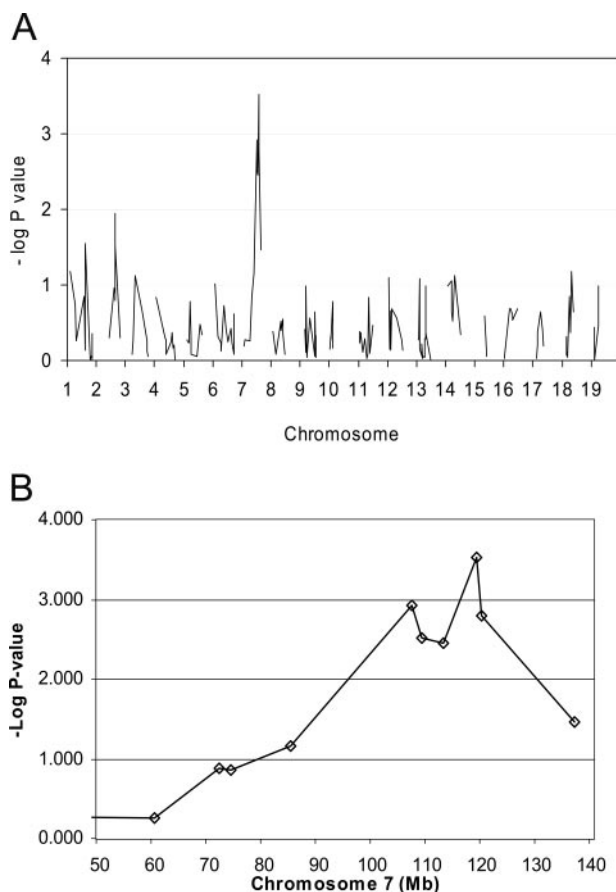


Figure 1. Linkage analysis results for mammary tumor susceptibility alleles. **A:** A complete genome scan was conducted comparing mammary tumor-bearing versus nonmammary tumor-bearing N2-*Trp53*^{+/-} mice. Using 150 SNPs distributed across the mouse genome, significant linkage was detected on chromosome 7. **B:** Additional SNPs were used to define an interval of strong linkage on chromosome 7. The *SuprMam1* locus was considered to span the region from 100 to 125 Mb. Locations of markers are based on the Ensembl mouse map, build 34.

The magnitude of the effect of *SuprMam1* on latency and risk was analyzed using Kaplan-Meier survival estimates in which frequencies are adjusted for the number of mice surviving at each time. Homozygosity for the BALB/c alleles at M64879_201_1 significantly decreased mammary tumor-free survival ($P = 0.002$). The median

Table 2. Occurrence of Tumors for *SuprMam1* Genotypes

	Number TT genotype	Number CT genotype
Total number of mice	116	92
Tumor types		
Mammary	63	26
Lymphoma	25	19
Adrenal gland	14	15
Osteosarcoma	11	17
Mammary carcinosarcoma	3	6
Other tumors/illnesses	18	23
Total tumors*	142	98
Tumor-free	2	2

Genotypes for the *SuprMam1* locus were determined using marker M64879_201_1.

*Multiple tumors develop in mice, frequently causing the tumor number to exceed the number of mice.

age of mammary tumor occurrence decreased from 70.7 weeks in heterozygotes to 61.1 week in homozygotes, with homozygotes having a twofold increase in risk of developing a mammary tumors compared with heterozygotes (hazard ratio, 1.93; 95% confidence interval, 1.26 to 2.95; $P = 0.002$) (Figure 2). These results suggest that homozygosity for BALB/c alleles for the *SuprMam1* locus decreases the latency of mammary tumors that may explain the higher frequency of mammary tumors in homozygotes. It is possible that, in the absence of other tumor types, heterozygotes may go on to develop a similar frequency of mammary tumors to homozygotes, but with considerably longer latency. Thus, in the context of germline deficiency in *Trp53* and the occurrence of tumors in other tissues, it is not possible to distinguish the effect of the *SuprMam1* locus on latency versus incidence of mammary tumors. The effect of this locus was specific for mammary tumors because tumor-free survival for either all tumor types or for any other major tumor type (lymphoma, osteosarcoma, adrenal gland tumor) did not differ (hazard ratio, 1.14; 95% confidence interval, 0.87 to 1.51; $P = 0.3$ for all tumor types). Overall, these results identify a genetic susceptibility locus on the distal portion of mouse chromosome 7 that alters latency, and possibly the incidence, of mammary tumors in *Trp53*^{+/-} mice.

Dmbt1 as a Candidate Genetic Modifier

The interval between 100 to 125 Mb of mouse chromosome 7 (*SuprMam1*) encodes more than 200 expressed or predicted genes. To identify candidate genetic modifiers within this interval, gene expression in mammary glands obtained from C57BL/6- and BALB/c-*Trp53*^{+/-} mice was profiled using oligonucleotide microarrays. Within the interval between 100 and 125 Mb, two expressed sequence tags and three genes were found to be significantly different in expression between the two strains (intensity >150, $P < 0.05$, change more than twofold). Of these, *Hbb-b2* and *Spon1* were higher in BALB/c, whereas *Trim12* (tripartite motif protein 12), *Dmbt1* (deleted in malignant brain tumors 1) and the *Thumpd1* (a predicted protein) were decreased in BALB/c. Of these genes, only *Dmbt1* has been described as a putative tumor suppressor gene.^{32,33} *Dmbt1* mRNA showed 3.8-fold difference in expression with lower levels in the susceptible BALB/c strain, consistent with the expectations for a tumor suppressor gene. Therefore, *Dmbt1* was selected as a candidate gene for further analysis.

Northern blotting was performed to characterize expression of *Dmbt1* in mammary tissues from both strains. A single transcript of ~6 kb was observed, which was an expected result. *Dmbt1* expression was more than fivefold higher in mammary glands from C57BL/6-*Trp53*^{+/-} mice than in BALB/c-*Trp53*^{+/-} ($P = 0.002$; Figure 3, A and B). This difference in expression was mammary gland-specific because levels of *Dmbt1* mRNA were equivalent level in small intestines of C57BL/6 and BALB/c mice (Figure 3, A and B). Alternative usage of the

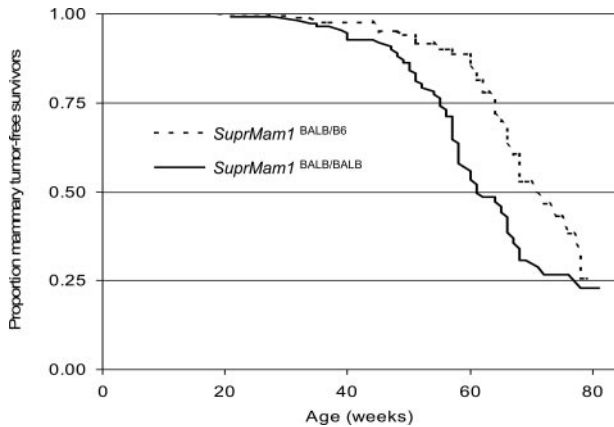


Figure 2. Kaplan-Meier mammary tumor-free survival plots of N2-*Trp53*^{+/-} mice segregated according to genotype at *SuprMam1*. Median age of occurrence of mammary tumors among mice that were heterozygous for the susceptibility allele (*SuprMam1*^{BALB/B6}) was 70.7 weeks compared with 61.1 weeks for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

3' exons of *Dmbt1* in mouse results in transcripts that contain the transmembrane spanning domain (*Dmbt1* β) or lack this region (*Dmbt1* α). Because this small size difference would not be detected by Northern blot, specific primers were designed to analyze the 3' ends of the transcripts using RT-PCR. A primer pair spanning the CUB and ZP domains amplified the same amount of *Dmbt1* mRNA in small intestine of both strains and confirmed that BALB/c mammary tissue had reduced but detectable *Dmbt1* mRNA expression (Figure 4). RT-PCR analysis performed using primers specific for the transmembrane and 3' UTR regions produced similar results (Figure 4). These data demonstrate that *Dmbt1* β is the major transcript expressed in both strains and that only the levels of expression differ between the strains.

Levels of DMBT1 protein were also examined in both strains. DMBT1 protein was detected in the majority of ducts in mammary glands from C57BL/6-*p53*^{+/-} mice but not in the mammary stroma. Punctate staining was observed within the epithelial cells (Figure 5A). In contrast, DMBT1 was detected in a minority of the ducts in

mammary glands from BALB/c-*Trp53*^{+/-} mice. When detectable (Figure 5B), levels were substantially lower in the mammary epithelium of BALB/c-*Trp53*^{+/-} compared with C57BL/6-*p53*^{+/-}. Despite striking differences in the mammary gland, expression of DMBT1 was similarly expressed in the crypts of the small intestines of both strains (Figure 5, C and D). Expression of DMBT1 protein in mammary tumors also reflected strain differences. Because C57BL/6-*p53*^{+/-} mice do not develop mammary tumors, expression of DMBT1 was assessed in spontaneous mammary tumors in F1 females ([C57BL/6 \times BALB/c]-F1-*Trp53*^{+/-}). Focal expression of DMBT1 was observed in three of the mammary tumors in F1 females, whereas three F1 tumors lacked detectable expression (Figure 5E, i and ii). Analysis of spontaneous mammary tumors in BALB/c-*Trp53*^{+/-} females generally lacked detectable DMBT1 ($n = 5$). Weak focal staining was present in one of six tumors from BALB/c-*Trp53*^{+/-} females (Figure 5E, iii and iv). Therefore, it seems that either tumors arise more frequently from ducts that lack expression or expression is often lost during tumorigenesis. In either case, loss of expression is not essential because half of the tumors arising in F1 mice retained detectable levels of DMBT1. These observations are consistent with expectations for a low-penetrance modifier of susceptibility.

Low DMBT1 Expression Is Associated with the Occurrence of Human Breast Cancer

We next wanted to determine whether reduced *DMBT1* expression was a characteristic of human breast cancer. Normal and tumor tissues were examined by Q-PCR. Of the normal tissues tested, significant expression of DMBT1 mRNA was only observed in lung. The remaining tissues had low to undetectable levels. Because normal breast tissue is composed of heterogeneous cell types with epithelial cells being a minority (<30% of tissue), direct comparisons between breast tumors, which are enriched for epithelium, can be misleading. Therefore, immortalized normal breast epithelial cells derived from reduction mammoplasty were used in these compari-

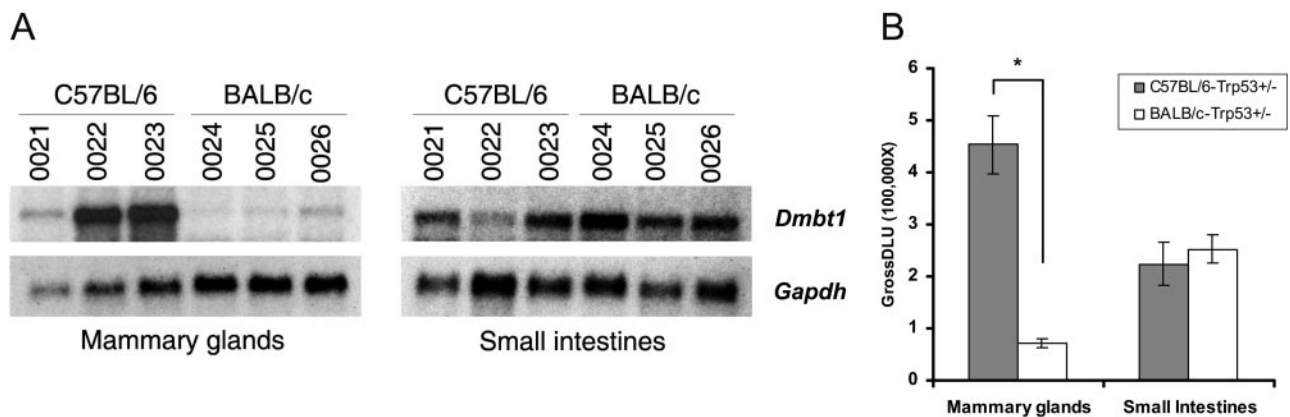


Figure 3. Expression of *Dmbt1* in small intestines and mammary glands from BALB/c-*Trp53*^{+/-} and C57BL/6-C57BL/6-*p53*^{+/-} mouse strains. **A:** Northern blots were hybridized with a ³²P-labeled probe from the 3' end of the mouse *Dmbt1* cDNA. The blots were stripped and rehybridized with a ³²P-labeled *Gapdh* cDNA. **B:** The levels of *Dmbt1* mRNA expression in small intestine and mammary gland were quantified using phosphor imaging and normalized relative to *Gapdh* to control for loading variations. Levels of *Dmbt1* were significantly reduced in mammary tissue from BALB/c compared with C57BL/6 (* $P = 0.002$).

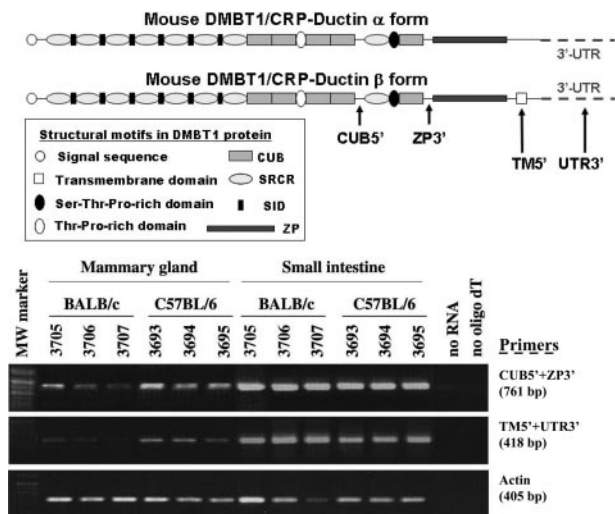


Figure 4. RT-PCR was used to analyze the structure of the 3' end of *Dmbt1* transcripts in mammary gland and small intestine. Top: The structure of *DMBT1* protein and the location of the sequences of primers used for PCR. Bottom: Both strains of mice express sequences consistent with *Dmbt1* β . Only the levels of expression seem to differ between the strains.

sons. Levels of *DMBT1* mRNA were quite high in normal, immortalized breast epithelial cell lines (76N + p53mt, 76N + hTERT, and 76N + E6; Figure 6A). In contrast, expression of *DMBT1* was below the limit of detection in all of the primary human breast cancers tested (Figure 6A). Equivalent levels of *DMBT1* mRNA were also detected in MCF10A cells (Figure 6B), which represent spontaneously immortalized, nontumorigenic breast epithelial cells. In contrast, breast cancer-derived cell lines (MCF-7, T47-D) were devoid of *DMBT1* expression when analyzed by RT-PCR. Expression was retained in HeLa cells (ovarian carcinoma), which were used as a positive control. Loss of *DMBT1* expression is not invariant as 21PT cells derived from a primary breast cancer retained expression. However, expression of *DMBT1* was lost in the 21MT cell line derived from a metastatic tumor in the same patient (Figure 6B, 21PT versus 21MT). Thus, consistent with tumor suppressor function, decreased expression of *DMBT1* mRNA occurs frequently in breast cancers.

Because 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 immunohistochemistry 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 6C. Intracytoplasmic granular staining in the epithelium was the most common pattern but varied among women. *DMBT1* staining was often very focal, with some ducts staining strongly while adjacent ducts were negative (Figure 6C). Positive staining was observed in benign epithelium adjacent to tumors as well

as in benign epithelium that was far away from cancer. No consistent staining pattern in terms of the location was noted in specimens from either cancer or cancer-free patients.

The *DMBT1* staining within the benign glandular epithelium was semiquantified using percentage of staining as well as staining intensity to generate a staining score. Overall, women without breast cancer were more likely to show occurrence of significant positive staining with scores >4 and showed a greater degree of staining ($P = 0.008$ and $P < 0.0001$, respectively; Table 3). There was no significant association of age with staining score in either the cancer or cancer-free group ($P = 0.19$ and 0.75 , respectively). Furthermore, adjusted analyses performed to control for age (restricted to the women aged 40 to 55 years) resulted in very similar findings to the overall results ($P = 0.002$, Table 3). Thus, the *DMBT1* staining score in normal breast epithelium was lower among patients with breast cancer compared with the cancer-free controls.

Discussion

Although ~30% of breast cancer risk has been attributed to heritable factors,¹ the known breast cancer genes account for fewer than 5% of the breast cancer cases^{2,4,5} 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,^{8,34} but linkage studies in human pedigrees have limited power to detect these alleles.

Here we describe the use of the *Trp53*^{+/-} mouse model of breast cancer to identify *DMBT1* as a modifier of susceptibility to mammary tumors in mice and humans. *DMBT1* was originally characterized as a gene deleted in malignant brain tumors³³ and down-regulated in breast cancer^{35,36} as well as other cancers with epithelial origins, including cancers in the brain, skin, lung, and digestive tract.^{33,37-39} These studies indicate a potential role for *DMBT1* as a tumor suppressor protein; however, *DMBT1* has not previously been considered as a breast cancer susceptibility gene. In the present study, *Dmbt1* was identified as a candidate susceptibility modifier gene because it is located within *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 twofold increase in the risk of developing mammary tumors compared with heterozygotes (Figure 2). Analysis of gene expression within the *SuprMam1* locus revealed that expression of full-length *Dmbt1* mRNA was diminished specifically in the mammary tissues of BALB/c mice (Figure 3). BALB/c mice seem to express a full-length transcript and protein, but at levels that are greatly reduced (Figures 4 and 5). Therefore, a decrease in basal levels of *Dmbt1* seems sufficient to render mammary tissue susceptible to tumor development. Because the reduction in *Dmbt1* expression is specific to the mammary gland in BALB/c mice, enhancers that bind tissue-specific transcription factors

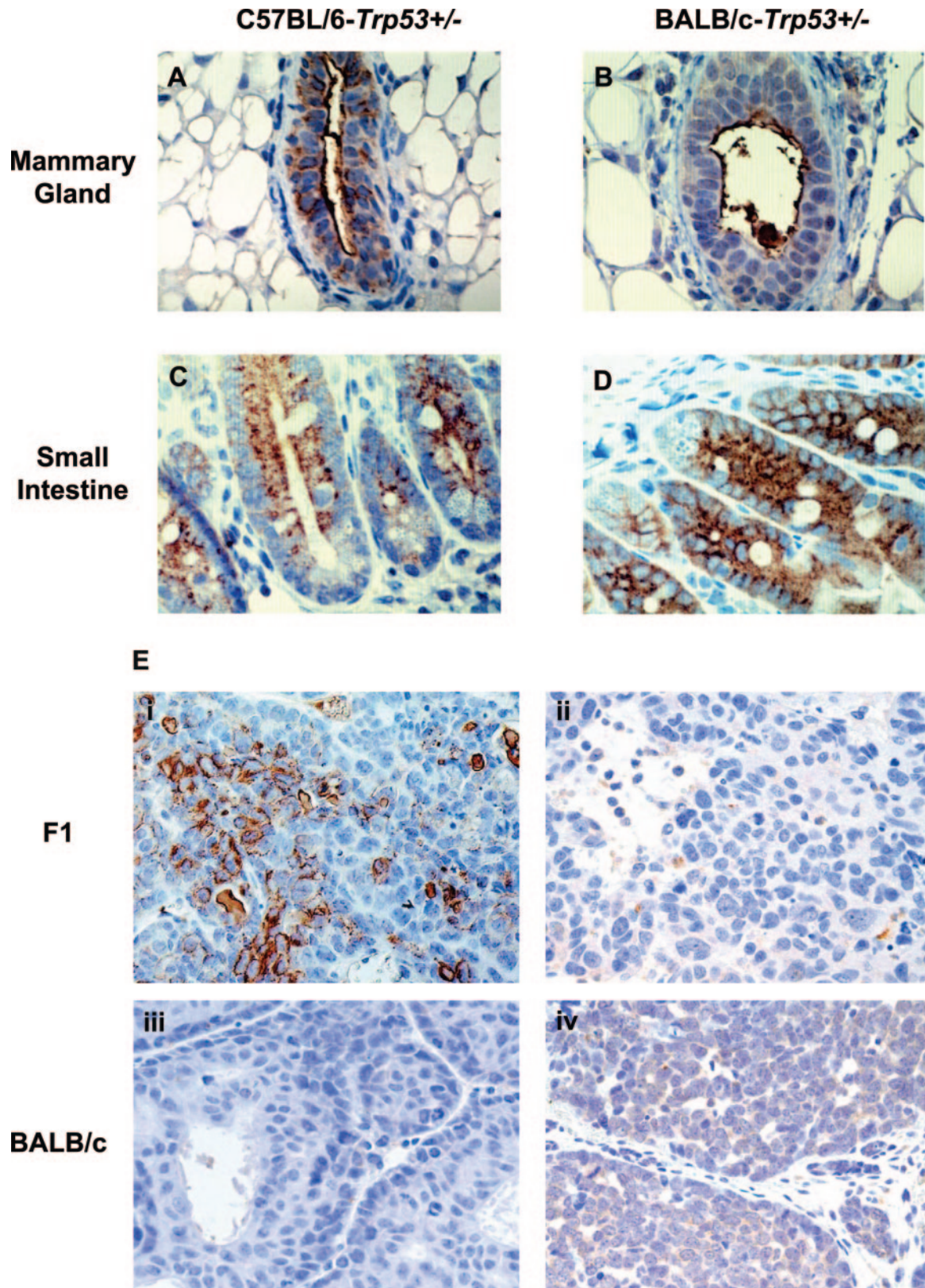


Figure 5. Expression and localization of DMBT1 protein in mammary glands and small intestines. Tissues were collected from nulliparous BALB/c-*Trp53*^{+/-} and C57BL/6-*p53*^{+/-} mice and analyzed by immunohistochemistry. **A:** Punctate staining was present within the mammary epithelium of C57BL/6-*p53*^{+/-} mice. Secretions were also evident within the lumen. **B:** The BALB/c-*Trp53*^{+/-} mice were primarily devoid of staining in the mammary epithelium, but detectable levels were observed in some ducts as shown. In contrast, levels of expression were similar in the small intestines from both the C57BL/6-*p53*^{+/-} (C) and BALB/c-*Trp53*^{+/-} (D) strains. **E:** Expression of DMBT1 protein was examined in spontaneous mammary tumors in [C57BL/6 × BALB/c]-F1-*Trp53*^{+/-} (F1) and BALB/c-*Trp53*^{+/-} mice. Among the mammary tumors in F1 mice, there were equal numbers with significant expression as well as tumors with no detectable protein (i and ii, respectively). The mammary tumors from the BALB/c-*Trp53*^{+/-} mice were primarily devoid of staining (iii) with only one that had very weak staining (iv).

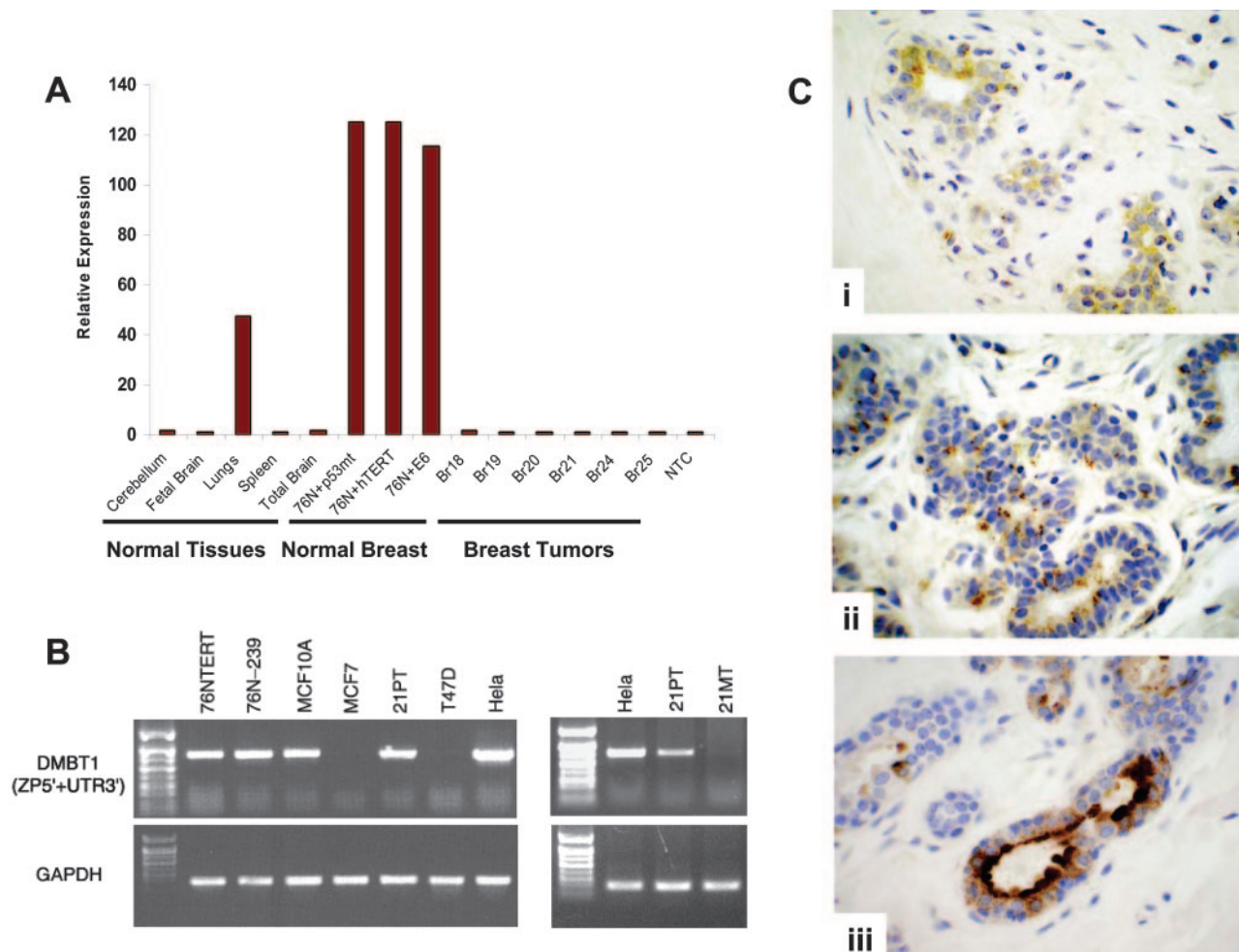


Figure 6. Expression of *DMBT1* mRNA in human tissues. **A:** Levels of *DMBT1* expression were compared in normal human tissues using Q-PCR. Only lung expressed significant levels. The immortalized normal breast epithelial cells (76N series) also expressed high levels of *DMBT1* mRNA. In contrast, expression of *DMBT1* mRNA was undetectable in the primary breast cancers (BR18, BR19, BR20, BR21, BR24, BR25). NTC, no template control. All samples were analyzed for β -actin to ensure integrity of RNA. **B:** RT-PCR was used to analyze expression of *DMBT1* mRNA in normal, immortalized breast epithelial cell lines (76N + Tert, 76N + 239, MCF10A), breast cancer cell lines (MCF-7, T47-D), and isogenic cells representing the primary breast tumor (21PT) and metastatic tumor (21MT) from a single patient. HeLa cells were used as a positive control. **C:** Immunohistochemistry for *DMBT1* in benign human mammary epithelium. Immunohistochemical staining revealed variable patterns and intensities of staining among individuals. **i:** Diffuse cytoplasmic staining that was low intensity of reaction was considered score = 1. **ii:** Intense expression within the supranuclear region and polarized toward the luminal surface was considered score = 2. **iii:** Intense staining polarized toward the luminal surface was considered score = 3.

are likely to be disrupted in the BALB/c allele. The 3 kb upstream of exon 1 were sequenced, but no polymorphisms were detected between BALB/c and C57BL/6, suggesting that polymorphic regulatory elements reside at more distant sites upstream or within introns of the mouse *Dmbt1* gene.

When normal human breast epithelium was studied, variable expression in the extent and intensity of *DMBT1* immunostaining was 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 3. Analysis 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 \pm SD)	3.9 \pm 1.2	1.8 \pm 1.8	<0.0001
Age (mean \pm SD)	38.6 \pm 12.6	59.5 \pm 15.0	<0.0001
n	53	46	
Age 40 to 55			
Occurrence of significant staining (score 4)	72%	23%	0.001
Staining score (mean \pm SD)	4.1 \pm 1.0	1.9 \pm 1.8	0.7
Age (mean \pm SD)	46.9 \pm 3.9	46.3 \pm 4.0	
n	18	13	

(Table 3). As reported previously,^{35,36} there was limited staining in tumor cells whereas the adjacent normal breast epithelium retained DMBT1 expression. We did not observe localized induction of DMBT1 in the breast epithelium immediately adjacent to the tumors as reported by Mollenhauer and coworkers.³⁶ This difference may reflect the selection of tissues. In our study, tumors were selected to have clear margins as the unperturbed adjacent epithelium was our focus. In contrast, Mollenhauer and coworkers³⁶ included hyperplastic epithelium flanking the cancers in an effort to address the hypothesis that DMBT1 expression is induced by pathophysiological processes such as inflammation and cancer. Although women show varying basal levels of DMBT1 expression in our study, it remains possible that women with low DMBT1 expression are still able to induce expression of DMBT1 as part of an inflammatory response.

At least eight common alleles of *DMBT1* have been described in humans, based on Southern blotting and sequencing of exons.^{32,39–41} These polymorphisms may act as low-penetrance modifiers of cancer susceptibility. Some of the polymorphisms involve deletion of a portion of the highly homologous scavenger receptor cysteine rich domains. Although these proteins would still be detectable with the antibodies used, the deletion could render the protein unstable resulting in lower levels of protein in breast tissues. Alternatively, differences in promoter elements may exist in linkage with the variants in coding regions and alter basal expression levels. This may contribute to the variation in expression levels observed between patients because protein function may be compromised by lower levels of mRNA as well as alleles that are hypomorphic with respect to function.

The exact role of DMBT1 as a tumor suppressor is not well 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.^{42,43} In the mouse, hensin seems to support terminal differentiation of columnar epithelia during early embryogenesis.⁴⁴ DMBT1 is also induced early in response to certain pathological stresses and may play an unidentified protective role. These stresses include tissue injury from paracetamol toxicity or hepatitis B infection in the liver⁴⁵ and carcinogen exposure in lung.³⁷ The presence of hypomorphic alleles of *DMBT1* resulting in disrupted differentiation or stress responses are likely to contribute to tumorigenesis.

Although DMBT1 is an excellent candidate and the evidence presented here supports its potential role in breast cancer susceptibility, we cannot exclude the possible involvement of other genes lying in this linkage region. Cancer development in other tissues has also been linked to regions of mouse chromosome 7^{46–50}; however, these do not overlap physically or functionally with *SuprMam1*. The interval of *SuprMam1* corresponds with syntenic portions of three human chromosomes: 11p15 (81 to 110 Mb), 16p11-p12 (111 to 122 Mb), and 10q25-q26 (122 to 135 Mb). These human regions contain the tumor suppressors *PTEN* (10q24) and *TSG101* (11p15). However, these do not map to *SuprMam1*, but

rather lie on mouse chromosome 19 and the proximal region of mouse chromosome 7 (34 Mb), respectively. Therefore, *SuprMam1* represents a novel locus containing gene(s) conferring susceptibility to mammary tumors in mice.

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 susceptibility modifier gene. Association in both mice and humans of lower DMBT1 expression in normal mammary epithelium with increased likelihood of breast cancer 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. Identifying the function of DMBT1 that is relevant to cancer suppression will be important in understanding which alleles confer increased risk. With its role in terminal differentiation of epithelial cells, DMBT1 presents us with a new pathway, that of cell fate and differentiation, which can influence breast cancer risk and may be a target for preventive strategies.

Acknowledgments

We thank Jeff Wood of the Statistical Consulting Unit of the Graduate School, Australian National University for assistance; Carol Bigelow of the Statistical Consulting Center (Biostatistics and Epidemiology, University of Massachusetts) for assistance with the analysis of DMBT1 expression in human tissues; and Dr. Vimla Band for generously providing immortalized breast epithelial cells (76N series) and cancer cell lines (21PT, 21MT).

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