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Mammary tumor modifiers in BALB/cJ mice heterozygous for *p53*

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Abstract BALB/c mice are predisposed to developing spontaneous mammary tumors, which are further increased in a *p53* heterozygous state. C57BL/6J mice are resistant to induced mammary tumors and develop less than 1% mammary tumors in both wild-type and $p53^{+/-}$ states. To map modifiers of mammary tumorigenesis, we have established F_1 and F_2 crosses and backcrosses to BALB/cJ (N2-BALB/cJ) and C57BL/6J (N2-C57BL/6J) strains. All cohorts developed mammary carcinomas in $p53^{+/-}$ females, suggesting that multiple loci dominantly and recessively contributed to mammary tumorigenesis. We mapped two modifiers of mammary tumorigenesis in the BALB/cJ strain. *Mtsm1* (mammary tumor susceptibility modifier), a dominant-acting modifier, is located on chromosome 7. *Mtsm1* is suggestive for linkage to mammary tumorigenesis ($p = 0.001$). We have analyzed the *Mtsm1* region to locate candidate genes by comparing it to a rat modifier region, *Mcs3*, which shares syntenic conservation with *Mtsm1*. Expression data and SNPs were also taken into account. Five potential candidate genes within *Mtsm1* are *Aldh1a3*, *Chd2*, *Nipa2*, *Pcsk6*, and *Tubgcp5*. The second modifier mapped is *Mtsm2*, a recessive-acting modifier. *Mtsm2* is located on chromosome X and is significantly linked to mammary tumorigenesis ($p = 1.03 \times 10^{-7}$).

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Introduction

Breast cancer is the most frequent type of cancer in women in the United States (Wingo et al. 1998). Several genes have been identified that, when inherited, increase a woman's likelihood of developing breast cancer. These include, but are not limited to, *BRCA1*, *BRCA2*, *PTEN*, and *TP53* and account for only approximately 5%–10% of breast cancers (Balmain et al. 2003).

Most patients with Li-Fraumeni syndrome (LFS) inherit *TP53* mutations (Malkin et al. 1990; Srivastava et al. 1990). LFS patients develop many types of tumors, including soft tissue sarcomas, osteosarcomas, breast carcinomas, and brain tumors, with breast carcinomas being the most frequent tumor type in women (Evans and Lozano 1997; Kleihues et al. 1997). Different patients within a family with the same *TP53* mutation develop different types of cancers. While a majority of LFS patients develop cancer at a young age, the latency of tumorigenesis also varies widely within families. The latency and occurrence of LFS-related cancers are affected by environmental causes such as smoking (Hwang et al. 2003). Generation or birth cohort effects can also affect latency of breast cancer in LFS patients (Brown et al. 2005; Trkova et al. 2002). Thus, additional genes, or modifiers, may predispose patients to a particular type of tumor or modify the latency of that tumor. These modifiers may also be present in the general population and could contribute to ''spontaneous'' breast cancer (Balmain et al. 2003).

Recently, a modifier of cancer risk in Li-Fraumeni syndrome was discovered. *Mdm2* encodes a negative regulator of p53 and contains a single nucleotide polymorphism (SNP) that alters a T to G at nucleotide 309 in the promoter region of *Mdm2* (Bond et al. 2004). SNP309 causes an increase in *Mdm2* RNA and protein levels, which in turn decreases p53 activity. SNP309 has been associated with an earlier median age of breast cancer onset in women with Li-Fraumeni syndrome, with breast cancer occurring ten years earlier in patients with G/T or G/G genotypes. A similar decrease in latency was seen in patients with no evidence of an inherited predisposition to cancer (Bond et al. 2004, 2005).

To model the Li-Fraumeni syndrome, mice with *p53* deletions have been made on three different inbred strains. Each strain shares many similarities in tumor development; all three strains develop sarcomas, lymphomas, and some carcinomas. However, each strain also develops a unique tumor type. The 129Sv strain develops teratomas (Harvey et al. 1993), while the BALB/cMed strain develops mammary carcinomas (Kuperwasser et al. 2000), both of which are rarely seen in the C57BL/6J strain (Donehower et al. 1992, 1995; Harvey et al. 1993; Jacks et al. 1994; Kuperwasser et al. 2000). Harvey et al. (1993) suggested that the 129Sv strain was already predisposed to tumors, specifically teratomas, and that loss of *p53* only served to accelerate the occurrence of these tumors. They also suggested that genetic background plays a role in both the type of tumor that develops and tumor latency. Indeed, wild-type 129Sv mice develop teratomas, and wild-type BALB/c mice develop mammary tumors, albeit at low levels with long latency (Altman and Katz 1979; Heston and Vlahakis 1971; Kuperwasser et al. 2000). Loss of *p53* in both strains decreases the latency and increases the occurrence of these tumor types in their respective strains (Harvey et al. 1993; Kuperwasser et al. 2000). Differences in tumor development between mouse strains can be exploited to map modifying, low-penetrance genes involved in specific tumor types and possibly be extrapolated to patients with Li-Fraumeni syndrome.

BALB/c mice have been used to study the propensity of mammary tumorigenesis. Twenty percent of BALB/c mice develop spontaneous mammary tumors at an average latency of 16.7 months (Heston and Vlahakis 1971). BALB/cMed mice that are heterozygous for *p53* have increased incidence of mammary tumors at 42% in a cohort of males and females (Kuperwasser et al. 2000). In a cohort with only virgin female BALB/cMed $p53^{+/-}$ mice, 65% develop mammary tumors with a latency of 8–14 months (Blackburn et al. 2003; Kuperwasser et al. 2000). C57BL/6 mice develop less than 1% mammary tumors in both the wild-type and $p53^{+/-}$ states (Heston and Vlahakis 1971; Jacks et al. 1994). Blackburn et al. (2003) have shown that F_1 mice with 50% C57BL/6 and 50% BALB/c background developed mammary tumors at a lower incidence (32%) than BALB/c mice, and that N_2 mice with 75% BALB/c background developed an intermediate level of mammary tumors (45%). These data suggest that both dominant and recessive modifiers are involved in the BALB/c susceptibility to mammary tumors. However, no mapping data are available from these studies.

We have chosen to map modifiers that increase the occurrence and decrease latency of mammary tumors in mice. We crossed the C57BL/6J (resistant) and BALB/cJ (susceptible) strains and followed inheritance of the mammary tumor phenotype with inheritance of alleles from the BALB/c strain to map regions of the genome that are likely to contain modifiers that increase susceptibility to mammary tumorigenesis. We have located two potential modifiers of mammary tumorigenesis in the BALB/cJ strain. One locus, named *Mtsm1* (mammary tumor susceptibility modifier 1) is on chromosome 7, and a second locus, named *Mtsm2*, is located on chromosome X.

Method

Mice and tumors

C57BL/6J $p53^{+/-}$ (formally named B6.129S2-*Trp53*^{tm1Tyj}/J) and BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in a conventional specific pathogen-free colony with a 12-h light cycle and were fed *ad libitum*. Genotyping for *p53* was performed as previously described (Jacks et al. 1994). The mice were monitored for tumors daily and were sacrificed when a tumor was visible or the mouse was moribund. A small section of each tumor was fixed in 10% buffered formalin, and any remaining sections of tumors were frozen and stored at –80°C. The fixed tissues were paraffin embedded, sectioned, and stained with hematoxylin and eosin in the histology core facility at M.D. Anderson Cancer Center. All mouse protocols were approved by the IACUC.

Marker analysis

ABI Prism Mouse Mapping Primers (Applied Biosystems, Foster City, CA) were selected using the Center for Inherited Disease's software tool for STRP mapping panels (http://www.cidr.org). The centimorgan location of these markers was taken from The Jackson Laboratory's Mouse Genome Informatics website (http://www.informatics.jax.org), while megabase locations were taken from Ensembl version 38 (http://www.ensembl.org). Each marker was verified for polymorphic PCR amplification using genomic DNA from BALB/cJ and C57BL/6J wild-type mice.

Genomic DNA from each mouse was extracted from tail biopsies at weaning and/or normal liver tissues after sacrifice. PCR was performed separately for each mouse and primer. The PCR products were then multiplexed and run on an ABI 3100 16-capillary genetic analyzer to detect the polymorphic size differences. Data were analyzed using Applied Biosystem's Genotyper 3.5 NT program.

Additional markers for fine mapping were selected from the Whitehead Institute/MIT Center for Genome Research Genetic Maps (http://www.broad.mit.edu/cgi-bin/mouse/index) and purchased from Research Genetics (MapPairs primers). PCR for most of these markers was performed as follows: 95°C for 1 min; 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec; followed by 72°C for 5 min. Markers *D7Mit321* and *D7Mit353* required an annealing temperature of 59°C. The products were run on a 4% NuSieve 3:1 agarose gel (Cambrex, East Rutherford, NJ).

Statistical analysis

Prior to statistical analysis the markers were aligned according to megabase positions along the chromosomes, based on Ensembl version 38. Any markers that showed double recombination with flanking markers were flagged and regenotyped to confirm results. We then used Kaplan-Meier survival analysis to assess the relationship between time to onset for mammary tumors and the genotypes at each marker. To assess significance with the Kaplan-Meier analyses, we used a log rank test. To evaluate the joint effects of both markers on time to onset for cancer, we used Cox proportional hazards models and tested for significance using a Wald test. To evaluate evidence for interactions, we applied Cox proportional hazards models that included interaction terms among SSLPs on chromosomes X and 7 that were found in univariable analyses to significantly predict time to onset for mammary tumors. To evaluate the dominance or recessivity of alleles at the chromosome X and 7 loci, we used Cox regression analysis to compare the hazard ratios for the homozygous animals to those of the heterozygous animals. Under the recessive model, the hazard for mice homozygous for C57BL/6J alleles equals the hazard for heterozygous animals, while the BALB/cJ homozygous mice would have a higher hazard ratio. Under a dominant model, the homozygous BALB/cJ and heterozygous mice have similar hazards that are higher than those of the C57BL/6J homozygous mice. All analyses were conducted using STATA version 8 (Stata Corporation, College Station, TX). Of the 75 animals genotyped for the entire genome, 55 were F_2 mice, 13 were N_2 C57BL/6J, and 7 were N_2 BALB/cJ. Some analyses were restricted to include only F_2 intercross animals to insure similar results to our mixed cohort study. Eighty-six mice, which included ten additional F_2 and one N_2 BALB/cJ mice with mammary tumors, were genotyped for *D7Mit91*, *D7Mit201*, *D7Mit350*, and *DXMit68*.

Candidate gene selection

The rat modifier *Mcs3* (Shepel et al. 1998) was defined using Ensembl v38 (http://www.ensemble.org) and the Rat Genome Database release 7.7 (http://www.rgd.mcw.edu). The rat modifier is surrounded by *D1Mit11* and *D1Mit2* at 102,532,145 and 134,980,527 bp. SoftBerry's (http://www.softberry.com/berry.phtml) Rat-Mouse Synteny for chromosome 1 of rat was used to determine regions of syntenic conservation between rat and mouse in the *Mcs3* region. Gene names and abbreviations, if not listed directly on SoftBerry, were determined by searching Ensembl (mouse and/or rat versions), Mouse Genome Informatics (http://www.informatics.jax.org) (Eppig et al. 2005), or

NCBI (http://www.ncbi.nlm.nih.gov) using other information provided by SoftBerry, and confirmed by position on the rat and/or mouse chromosomes in Ensembl.

Single nucleotide polymorphisms (SNPs) between BALB/cJ and C57BL/6J strains in the *Mtsm1* region (27.4–71.8 Mb) were identified using the ''Strains and Polymorphisms'' link on The Jackson Laboratory's Mouse Genome Informatics website (http://www.informatics.jax.org), which is based on NCBI dbSNP build 125.

Expression data

The fourth inguinal mammary glands, with the intramammary lymph nodes removed, were collected from 12-week-old virgin C57BL/6J $p53^{+/-}$ and BALB/cMed $p53^{+/-}$ mice 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 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 1.5-fold expression differences, with intensity values greater than 150 in one strain and $p < 0.05$ were considered to be differentially expressed.

Real-time RT-PCR

The fourth inguinal mammary glands with the intramammary lymph nodes removed were isolated from 3–12-week-old C57BL/6J and BALB/cJ wild-type animals and snap-frozen in liquid nitrogen. Total RNA was extracted using the Qiagen RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed using a T7 promoter-coupled oligo(d)T primer (First Strand cDNA Synthesis Kit, Amersham Biosciences, Piscataway, NJ). The primer sequences for *Gapdh* were previously described (Iwakuma et al. 2004). The following primers sets were used: *Aldh1a3*, TGTGTGGACAGATCATCCCG and TCCAGGTGAACATCAGCAGG, *Chd2*, CCTGTGGACCCTGAGGAAAA and TTTGACTC GGCAGGTTAGGC, *Nipa2*, GTGGGCTGGACTGCTGTCA and GCAGCAAAATTGGCCACTTC, *Pcsk6*, AGCAATGCCGATGAGACCTT and GCAGAGCCGATTGG ACTTCA, and *Tubgcp5*, TGTTGGCCATCAACTTTGCA and CGTGAAAGTCGCTCTGCTCC. Each sample was analyzed in triplicate and expression of each target gene was normalized to expression of *Gapdh* in that reaction. Fold change was determined by dividing the mean of the three C57BL/6J animals (in triplicate) by the BALB/cJ mean for each animal.

Results

Previous studies using BALB/c $p53^{+/-}$ mice have indicated an increased susceptibility of BALB/c mice to mammary tumors compared with C57BL/6J mice (Blackburn et al. 2003; Kuperwasser et al. 2000). We therefore set up four cohorts of mice to map mammary tumor modifiers in the BALB/cJ strain. B6.129S2-*Trp53tm1Tyj/J* mice containing a *p53* null allele in the C57BL/6J background (called C57BL/6J I $p53^{+/-}$ in this study) were crossed with BALB/cJ mice to create an F_1 cohort of 58 female $p53^{+/-}$ mice. F_1 mice were then crossed to create an F_2 cohort of 155 female $p53^{+/-}$ mice. Two backcross cohorts were also established by crossing F_1 mice to BALB/cJ mice to create an N_2-BALB/cJ cohort with 50 female $p53^{+/-}$ mice and to C57BL/6J mice to create an N_2-C57BL/6J cohort with 37 female $p53^{+/-}$ mice.

All cohorts developed the expected types of tumors, including lymphomas, sarcomas, and some carcinomas (Table 1). In addition, glandular and cystic hyperplasias of the uterus and ovaries were found in our cohorts that have not been previously reported in any *p53* mouse model. All cohorts developed mammary tumors, which were primarily adenocarcinomas with some adenosquamous carcinomas. In addition, we observed increased mammary tumor development and decreased latency in mice with a greater percentage of BALB/cJ alleles (Table 2). In the F_1 cohort (50% BALB/cJ and 50% C57BL/6J alleles), 28% of the females developed mammary tumors with a median latency of 489 days. The presence of mammary tumors in the F_1 cohort indicates the presence of at least one dominant mammary tumor susceptibility modifier in the BALB/cJ background. In the N_2-BALB/cJ cohort (75% BALB/cJ and 25% C57BL/6J alleles), 40% of the females developed mammary tumors with a median latency of 391 days. The increased number of mammary tumors and decreased latency in the N_2-BALB/cJ cohort, compared to the F_1 cohort, indicates the presence of recessive modifiers for mammary tumorigenesis in the BALB/cJ strain.

In the initial mapping study, 75 virgin female mice, including 28 with mammary tumors and 47 without mammary tumors, were genotyped with 166 microsatellite markers across the genome. Fifty-five of these mice were from the F_2 cohort (19 with mammary tumors), with the remaining 20 from backcross cohorts [13 N_2 C57BL/6J (4 with mammary tumors) and 7 N_2 BALB/cJ (5 with mammary tumors)]. The markers are ABI Prism Mouse

Table 1 Tumors in mixed background $p53^{+/-}$ females

Tumor types	F_1	F_2	N_2-BALB/cJ	N_2-C57BL/6J
Carcinomas	36% (23)[a]	26% (43)	43% (24)	13% (5)
Mammary	25% (16)	18% (30)	36% (20)	11% (4)
Lung	8% (5)	5.5% (9)	5% (3)	0
Other	3% (2)	2.5% (4)	2% (1)	3% (1)
Sarcomas	42% (26)	46% (75)	32% (18)	60% (22)
Osteosarcomas	22% (14)	26% (43)	16% (9)	38% (14)
Angiosarcomas	10% (6)	8% (13)	3.5% (2)	6% (2)
Histiocytic	0	3% (4)	3.5% (2)	0
Other	10% (6)	9% (15)	9% (15)	16% (6)
Lymphomas	22% (14)	28% (45)	25% (14)	27% (10)
Total tumors	63	163	56	37
Total mice	58	155	50	37
Mice with hyperplasia of uterus and/or ovary	22% (13)	17% (27)	12% (6)	27% (10)

[a] Number of tumors are shown in parentheses

Table 2 Mammary tumor incidence and latency in female $p53^{+/-}$ mice

	F_1	F_2	N_2-BALB/cJ	N_2-C57BL/6J
Median latency of mammary tumors(days)	489	445	391	385[a]
% of mice with mammary tumors	28% (16/58)	19% (30/155)	40% (20/50)	11% (4/37)

[a] Incidence and latency are skewed due to low number of mice in cohort

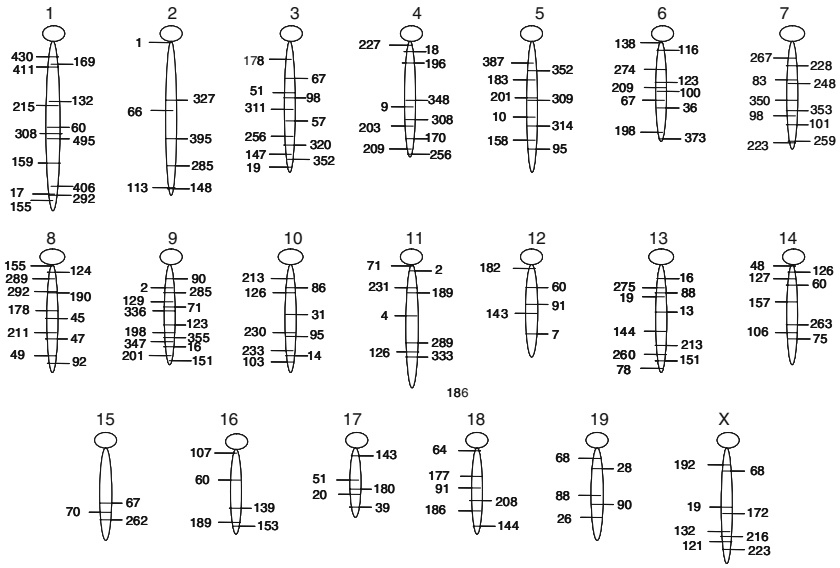

Fig. 1 Positions of markers on mouse genome used in genome-wide modifier screen. Numbers reflect the last number in the marker name. For example, the first marker on chromosome 1 is *D1Mit430*. Each marker was placed on the chromosome at its relative centimorgan location

Mapping Primers and are an average of 8.4 cM apart, with the median distance being 6 cM. The largest gap is on chromosome 15 from 0 to 40.9 cM at the centromeric end (Fig. 1). The statistical analyses were based on the presence or absence of mammary tumors and time to onset.

Initial mapping of the cohort described above showed that chromosomes 6, 7, 17, 18, and X each contained one or more markers with $p < 0.05$, suggesting the presence of at least five possible modifiers (Table 3). While a p value of 0.05 cannot be reported as definitive evidence for linkage, it has been set by Lander and Kruglyak (1995) as the upper limit of p that should be reported from a complete genome scan. These criteria adjust for inherent multiple testing when analyzing data from the genome-wide scans. On

Table 3 Log rank tests from survival analyses of markers from genome-wide scan

Marker[a]	cM	Mb	*p* value
D6Mit274			0.0288
D7Mit83	26.5	39.0	0.0177
D7Mit248	27.8	nm[b]	0.0123
D7Mit350	41.0	70.7	0.0140
D17Mit245			0.0062
D18Mit144			0.0474
DXMit192	16.0	45.4	0.0217
DXMit68	17.2	45.8	0.00004

[a] A total of 75 mice were genotyped at 166 markers, 55 F_2 of which 19 had mammary tumors, 13 N_2-C57BL/6J of which 4 had mammary tumors, and 7 N_2-BALB/cJ of which 5 had mammary tumors

[b] nm = not mapped to Ensembl

chromosome 7, initial mapping indicated that several markers had $p < 0.05$ (Table 3). We then focused on chromosome 7 and increased the density of the markers by adding eight markers between *D7Mit83* and *D7Mit321*, a region containing three consecutive markers with $p < 0.05$. The original 75 mice were genotyped for these new markers, after which *D7Mit201* had the lowest *p* value (0.0025) (Table 4). An additional ten F_2 mice and one N_2-BALB/cJ mouse with mammary tumors, which were also $p53^{+/-}$ virgin females, were genotyped for *D7Mit201* and the two flanking markers, *D7Mit91* and *D7Mit350* (Table 4). These 86 mice included all those with mammary tumors (39) and a comparable number of mice that developed other tumor types (47). The remaining mice in the cohort lacked mammary tumors and were not analyzed. A previous simulation showed no change in relative risks for disease when excluding up to 50% of observations that were censored at random (Vitezica et al. 2005). Given limitations on genotyping resources and the likely lack of additional statistical significance, we did not test additional mice lacking mammary tumors.

Expanded mapping revealed that marker *D7Mit201* was suggestive for linkage to earlier onset of mammary tumorigenesis ($p = 0.001$) (Table 4). Marker *D7Mit201* was shown by statistical analysis to be a dominant modifier, which decreases time to onset of mammary tumors (Fig. 2). At the *D7Mit201* locus there was no significant difference between heterozygous mice and mice homozygous for BALB/cJ ($p = 0.14$, hazards ratio = 0.57 ± 0.224),

Table 4 Linkage of markers to mammary tumor susceptibility

Marker[a]	cM	Mb	*p* values		
			75 mice[b]	86 mice[c]	65 F_2 mice
Chromosome 7					
D7Mit228[a,c]	18.0	27.4	0.1049		
D7Mit82	24.0	39.0	0.0105		
D7Mit83	26.5	39.0	0.0177		
D7Mit120	26.2	41.5	0.0164		
D7Mit86	25.1	42.9	0.0164		
D7Mit211	26.2	45.1	0.0164		
D7Mit91	27.3	46.1	0.0164	0.0085	0.0497
D7Mit201	28.4	61.4	0.0025	0.0011	0.0069
D7Mit350	41.0	70.7	0.0140	0.0261	0.1331
D7Mit62	31.7	71.8	0.0317		
D7Mit301	33.9	78.8	0.0248		
D7Mit321	36.1	85.1	0.0168		
D7Mit353[a]	37.2	86.8	0.0735		
Chromosome X					
DXMit192	16.0	45.4	0.0217		
DXMit68	17.2	45.8	4.23×10^{-5}	1.03×10^{-7}	5×10^{-6}
DXMit19[a]	43.2	nm	0.1278		
DXMit172[a]	48.7	113.4	0.4665		

nm = not mapped to Ensembl

[a] Markers are flanking markers with $p > 0.05$

[b] See Table 3 for description of these 75 mice

[c] This included the original 75 mice and an additional 10 F_2 and 1 N_2-BALB/cJ mice with mammary tumors

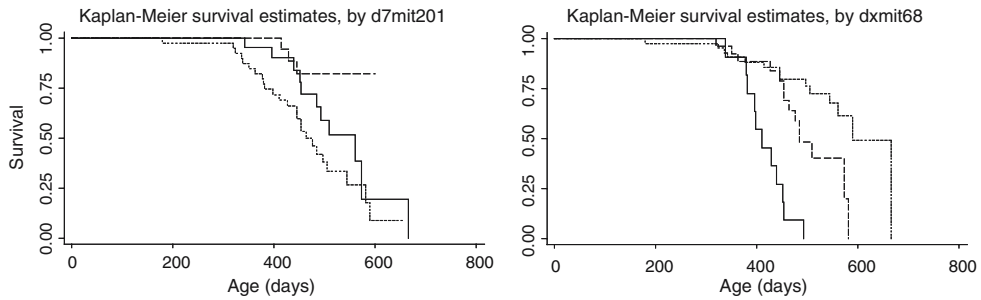

Fig. 2 Kaplan-Meier analyses to assess the relationship between time to onset and genotypes. Eighty-six mice were genotyped with markers *D7Mit201* (**A**) and *DXMit68* (**B**). Dotted lines represent heterozygous mice with one allele of each BALB/cJ and C57BL/6J. Mice homozygous for BALB/cJ alleles are depicted as solid lines, and mice homozygous for C57BL/6J alleles are represented as dashed lines

but heterozygous mice had a higher risk than C57BL/6J mice ($p = 0.007$, hazard ratio = 4.28 ± 2.32). The surrounding markers *D7Mit228* ($p = 0.1049$) and *D7Mit353* ($p = 0.0735$) were not significantly linked, or suggestive for linkage, to mammary tumorigenesis. These markers map to 27.4 and 86.8 Mb on chromosome 7 on Ensembl (http://www.ensembl.org). We have named this region *Mtsm1* for mammary tumor susceptibility modifier 1.

The additional ten F_2 mice and one N_2-BALB/cJ mouse with mammary tumors were also genotyped for the suggestive marker on chromosome X, *DXMit68*. After the addition of these mice, marker *DXMit68* was statistically significant for linkage to earlier onset of mammary tumorigenesis ($p = 1.03 \times 10^{-7}$) (Table 4) and was shown to behave primarily as a recessive modifier that decreases time to onset of mammary tumors by statistical analysis (Fig. 2). At the *DXMit68* locus there was a minimally significant difference between heterozygous mice and mice homozygous for C57BL/6J ($p = 0.025$, hazards ratio = 0.41 ± 0.16), but BALB/cJ mice had a much higher risk than heterozygous mice ($p < 0.001$, hazards ratio = 8.14 ± 3.90). No markers closer to the centromere than *DXMit68* had $p > 0.05$. Moving toward the telomeric end of chromosome X from *DXMit68*, the next closest marker, *DXMit19* ($p = 0.1278$), was not significantly linked or suggestive for linkage to mammary tumorigenesis, so the modifier locus is between 0 and 43.2 cM. *DXMit19* has not been mapped on Ensembl, so the megabase location is unknown. The next closest marker in our mapping panel that has been mapped to Ensembl, which was also not linked to mammary tumorigenesis, was *DXMit172*, located at 113.4 Mb and 48.7 cM. We have named the modifier locus around *DXMit68 Mtsm2*.

Restriction of the analyses to only F_2 intercross animals yielded similar results to those obtained on the entire sample set (Table 4). On chromosome 7 the *p* values for markers *D7Mit91*, *D7Mit201*, and *D7Mit350* were 0.05, 0.007, and 0.13, respectively. On chromosome X for marker *DXMit68*, $p = 0.000005$.

There was no evidence of interaction between the loci on chromosomes 7 and X in influencing time to onset for mammary tumors. The log likelihood model, including only marginal effects from genotypes of *D7Mit201* and *DXMit68*, was 123.52 compared to a log likelihood of 122.18 for a model that included the genotypic effects and all interactions. Comparing the models yields a χ^2 test of 2.68 with 4 degrees of freedom, $p = 0.61$.

Candidate genes

The modifier region on mouse chromosome 7, *Mtsm1*, overlaps a previously mapped rat modifier of mammary carcinoma susceptibility, *Mcs3* (Shepel et al. 1998). *Mcs3* affects the susceptibility of rats to DMBA-induced mammary carcinomas. We hypothesized that these two modifier regions could contain the same gene or genes responsible for mammary tumorigenesis, whether it develops spontaneously, with loss of *p53*, or is induced by DMBA. Therefore, we searched for syntenic conservation between rat and mouse genes that were contained in both the *Mcs3* and *Mtsm1* modifier regions (Fig. 3). Forty-one genes met this criterion.

To further reduce the list of candidate genes for *Mtsm1*, we compared the list of conserved genes with expression data. Gene expression data of BALB/cMed $p53^{+/-}$ and C57BL/6J $p53^{+/-}$ normal mammary glands were compared using the Affymetrix Murine U74v2 chip set (Supplementary Table 1). These expression data revealed differential expression of 5 of the 41 conserved genes (Fig. 3). *Aldh1a3* was expressed 4.8-fold and *Pcsk6* was expressed 1.5-fold higher in C57BL/6J $p53^{+/-}$ mammary glands. *Chd2*, *Nipa2*, and *Tubgcp5* were expressed 1.7-, 1.5-, and 1.8-fold lower in C57BL/6J $p53^{+/-}$ mammary glands than in the BALB/c $p53^{+/-}$ mammary glands. We then searched for known SNPs in these genes between BALB/cJ and C57BL/6J, using the strains and polymorphisms database on The Jackson Laboratory's informatics website (http://www.informatics.jax.org). Only *Pcsk6* contained known

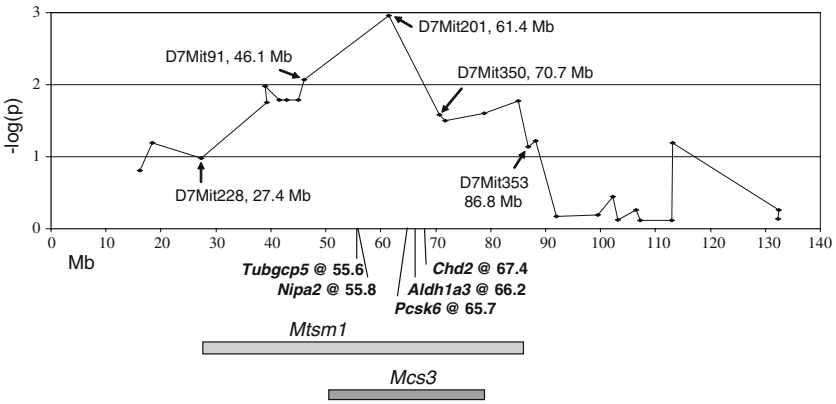

Fig. 3 Linkage analysis of markers on chromosome 7. Five selected candidate genes, *Aldh1a3*, *Pcsk6*, *Tubgcp5*, *Nipa2*, and *Chd2*, with their location in megabases (in parentheses), are located near *D7Mit201*, the peak marker in *Mtsm1*. These genes are also located within the rat modifier *Mcs3*, show syntenic conservation between rat and mouse, and are differentially expressed between BALB/cMed $p53^{+/-}$ and C57BL/6J $p53^{+/-}$ mammary glands

SNPs; one SNP is located in the 3′ UTR (untranslated region) (rs4226647) and the second SNP is intronic (rs16805862). To verify the Affymetrix array data, the five genes identified above were analyzed by real-time RT-PCR using mammary glands from BALB/cJ and C57BL/6J wild-type mice. All five genes were differentially expressed and the data replicated the data obtained from Affymetrix arrays (Fig. 4). Based on the evidence presented above, we propose that *Aldh1a3*, *Pcsk6*, *Chd2*, *Nipa2*, and *Tubgcp5* are good candidate genes for *Mtsm1* and should be further examined. Only two genes in this region, cDNA sequence AY078069 and *Mfge8*, contained coding SNPs. These should also be further examined.

Discussion

In this study we have characterized crosses between mammary tumor-susceptible (BALB/cJ) and -resistant (C57BL/6J) $p53^{+/-}$ mice and mapped two potential modifiers. Similar to previously published data (Blackburn et al. 2003), a decrease in mammary tumor incidence in mice with less BALB/c alleles and an increase in latency in cohorts with more C57BL/6J alleles were observed. The frequency of mammary tumors was similar in these two studies, with 28% of our F_1 mice developing mammary tumors compared with 32% of Blackburn's cohort. In addition, 40% of our N_2-BALB/c backcross mice developed mammary tumors compared with 45% (Blackburn et al. 2003). The latency of the mammary tumors was decreased in our cohorts compared to Blackburn's cohorts. Our F_1 cohort developed mammary tumors at a median of 69.8 weeks compared with 74.7 weeks for Blackburn's, and our N_2-BALB/c cohort developed mammary tumors at a median of 55.7 weeks compared with 66.4 weeks. While tumor incidence was similar in the two studies, we did not find any pituitary or adrenal gland tumors, while Blackburn's cohorts developed 14% and 11% adrenal gland tumors and 2% and 11% pituitary tumors in the N_2-BALB/c backcross and F_1 backcross, respectively. We also found glandular and cystic hyperplasias in the uterus and ovaries in our cohorts that have not been previously reported in any *p53* mouse model.

A possible explanation for these differences is that subtle modifications in the BALB/c substrains exist. Blackburn's cohorts were created with a substrain of BALB/c mice, BALB/cMed. These mice have been bred at Baylor College of Medicine for many years specifically for use in mammary tumor studies. We believe the BALB/cJ and BALB/cMed mice may harbor some similar modifiers of mammary tumorigenesis. However, latencies in the two strains are not the same, so each substrain may harbor some unique modifiers of mammary tumor latency.

From our crosses two potential loci for mammary tumor modifiers in the BALB/cJ strain have been detected. One locus on chromosome 7 near marker *D7Mit201*, designated *Mtsm1*, is a dominant-acting modifier. This modifier overlaps *Mcs3*, a rat modifier of mammary carcinomas. *Aldh1a3*, *Pcsk6*, *Chd2*, *Nipa2*, and *Tubgcp5* are likely

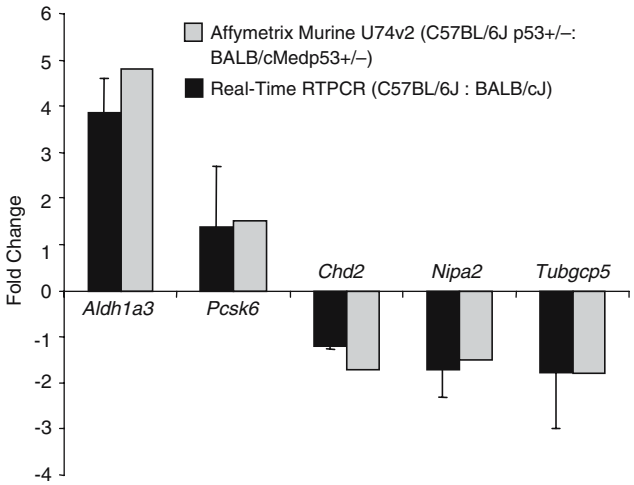

Fig. 4 Differential expression of candidate genes *Aldh1a3*, *Pcsk6*, *Chd2*, *Nipa2*, and *Tubgcp5*. Bar graphs depict real-time RT-PCR data from C57BL/6J mice compared with BALB/cJ mice (black bars). For comparison, gray bars depict data for the same genes from the Affymetrix Murine Genome U74v2 chip for C57BL/6J $p53^{+/-}$ compared with the BALB/cMed $p53^{+/-}$ strain

candidate genes in *Mtsm1* because they are located within both *Mtsm1* and *Mcs3* and are differentially expressed in mammary glands of BALB/cMed $p53^{+/-}$ and C57BL/6J $p53^{+/-}$ mice. *Pcsk6* also contains two known SNPs between these strains.

Two genes were upregulated in C57BL/6J mice. *Aldh1a3*, aldehyde dehydrogenase family 1 subfamily a3 (*ALDH6* in humans), is necessary for oxidation of retinol to retinoic acid. Many breast cancer cell lines lack the ability to oxidize retinol to retinoic acid, and MCF-7 cells in particular lack expression of *ALDH6* (Mira et al. 2000; Rexer et al. 2001). *Pcsk6* (*PACE4* in humans), proprotein convertase subtilisin/kexin type 6, cleaves precursor proteins such as hormones, adhesion molecules, growth factors, receptors, and matrix metalloproteinases into their active forms. In human breast cancer tissues, correlation has been found between *PACE4* expression and estrogen receptor content (Cheng et al. 1997).

Three genes showed decreased expression in C57BL/6J mice. *Tubgcp5*, tubulin gamma complex associated protein 5, is a subunit of the tubulin gamma complex (Murphy et al. 2001), which nucleates microtubulin polymerization during mitosis. *Nipa2*, nonimprinted in Prader-Willi/Angelman syndrome 2, is a relatively unknown gene (Chai et al. 2003). *CHD2*, chromodomain helicase DNA binding protein 2, contains a chromatin organization modifier domain near the N terminus and a SNF2-related helicase/ATPase domain near the center of the protein (Woodage et al. 1997). Any of these genes could be involved in mammary tumorigenesis and will be further studied in this model.

A second locus on chromosome X near marker *DXMit68*, designated *Mtsm2*, is a recessively-acting modifier of mammary tumorigenesis. *Mtsm2* is highly significant for mammary tumorigenesis. *Timp1*, *Pnck*, and *Ar* are genes in this modifier region that are involved in mammary growth and/or differentiation that could be potential candidate genes. The androgen receptor (*Ar*) was also expressed 1.49-fold higher in the C57BL/6J $p53^{+/-}$ mammary glands than in BALB/cMed. The androgen receptor has already been implicated in an increased breast cancer incidence and decreased latency in BRCA1-related patients (Rebbeck et al. 1999).

Mtsm1 and *Mtsm2* are linked to mammary tumorigenesis in the BALB/cJ strain, but these are probably not the only mammary tumor modifiers present. *Mtsm1* and *Mtsm2* did not account for all of the mammary tumors seen in our model, as 3 of the 39 total mice with mammary tumors genotyped did not have any BALB/c alleles present at the dominant modifier locus on chromosome 7 or homozygosity for BALB/c at the recessive modifier locus on chromosome X. Another 29 mice that did not develop breast cancer did contain BALB/c alleles at these modifier loci. These mice may have succumbed to other tumor types prior to the onset of breast cancer due to *p53* heterozygosity. The complexity and cooperativity of low penetrance modifiers could also contribute to our observations.

While the regions that contain modifier loci *Mtsm1* and *Mtsm2* remain large and the data suggest that these are not the only modifiers involved in mammary tumorigenesis, we hope that the mapping of these modifier loci and identification of candidate genes will allow future studies to pinpoint genes in these regions that are involved in mammary tumorigenesis. Pinpointing these genes should aid in finding corresponding modifiers of human breast cancer. The identification of novel genes and pathways will further our understanding of the complex pathways involved in susceptibility to breast cancer both in the general population and in inherited cases such as Li-Fraumeni syndrome.

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