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BALB/c-p53+/-Mice: Detection of Early Genetic
Alterations & the Mapping of BALB/c Susceptibility Genes

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13. ABSTRACT (Maximum 200 Words) The TP53 tumor suppressor gene is defective in the majority of sporadic breast cancers, and breast cancer is the most frequent tumor type in women with Li-Fraumeni syndrome and bear germline mutations in TP53. We have used BALB/c-Trp53+/- mice as a model of Li-Fraumeni syndrome to follow the pathogenic changes in mammary glands of BALB/c-Trp53+/- mice and map genes that can alter sensitivity to mammary tumors. Normal mammary tissues from BALB/c-Trp53+/- mice did not reveal gross karyotypic instability or gross hyperproliferative changes. Hyperplastic tissues and intraepithelial neoplasias retained the wild type allele of Trp53 and expressed estrogen receptors. However, transition to invasive lesions was accompanied by a loss of the wild type allele of Trp53 and loss of estrogen receptor. Though BALB/c-Trp53+/- mice develop spontaneous mammary tumors, C57BL/6-Trp53+/- mice are resistant. A genome scan has identified a low-penetrance modifier locus on mouse chromosome 7. Fine mapping of the region of interest is being undertaken.				
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Introduction

The TP53 tumor suppressor gene is defective in the majority of sporadic breast cancers, and breast cancer is the most frequent tumor type in women with Li-Fraumeni syndrome who inherit germline mutations in TP53. This suggests that p53 is fundamental to the growth regulation and prevention of tumor formation in mammary epithelial cells. Our laboratory has backcrossed the p53-null allele in mice onto the BALB/c genetic background. We have recently described the occurrence of mammary tumors in 55% of female BALB/c-p53^{+/-} mice with a latency of 8-14 months(1). This is in contrast to C57BL/6- and 129/Sv-p53^{+/-} mice, which rarely develop mammary tumors (2), suggesting that the BALB/c-p53^{+/-} mice serve as a unique model for Li-Fraumeni syndrome (LFS). The experiments proposed in this fellowship are designed to characterize the BALB/c-p53^{+/-} mouse model of breast cancer with respect to the progression of the glands towards tumor formation, and with respect to genetic contributions towards tumor susceptibility which are particular to this strain of mouse.

Key Research Accomplishments

- The sequence of molecular events involved in pathogenesis of mammary tumors in BALB/c-*Trp53*^{+/-} mice have been defined.
 - Hyperplastic lesions develop in both wild type and *Trp53*^{+/-} mice.
 - Loss of the wild type allele of *Trp53* is associated with progression and coincides with loss of ER expression.
 - Approximately 30% of spontaneous mammary tumors showed overexpression of HER2 protein by western blot.
- Genome scanning has identified linkage of mammary tumor susceptibility to mouse chromosome 7.
- Dr. Sallie Smith resigned from the project. A no-cost extension has been filed to allow time to search for a successor who can complete the fine-mapping of the recessive-acting mammary tumor susceptibility locus.

Reportable Outcomes

Publications:

Blackburn, A.C., Brown, J.S., Naber, S.P., Otis, C.N. Wood, J.T., and Jerry, D.J. 2003. BALB/c alleles for *Prkdc* and *Cdkn2a* interact to modify tumor susceptibility in *Trp53*^{+/-} mice. *Cancer Res.* 63:2364-2368, 2003.

A. C. Blackburn and D. J. Jerry. Knock-out and transgenic mice of *Trp53*: what have we learned about p53 in breast cancer? *Breast Cancer Res.* (2002) 4:101-111.

Manuscripts in preparation:

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. 2003. Loss of heterozygosity occurs via homologous recombination in Trp53^{+/-} mice and associates with mammary tumor susceptibility of the BALB/c strain. *Cancer Res.* (Submitted)

Poster Presentations:

None

Development of cell lines and tissue repositories:

ABV14 mouse mammary adenocarcinoma cell line has been developed and is being used by collaborators to investigate cell signalling pathways involved in tumor cell death.

A panel of DNA samples has been collected from the mapping mice population of mice and are available for investigation of further candidate genes which may be involved in susceptibility to mammary adenocarcinoma or other tumor types which occur in p53^{+/-} mice.

Grants:

PHS/NIH, 10/1/04-9/31/09, *Genetic modifiers of mammary tumor susceptibility.* (submitted)

Susan G. Komen Breast Cancer Foundation, 5/1/2004-5/30/2007. *Modifiers of mammary tumor susceptibility that interact with p53 status.* (under review)

Conclusions

- These results demonstrate the utility of BALB/c-Trp53^{+/-} as a model of mammary tumorigenesis.
- Though breast cancer susceptibility genes identified to date are inherited as autosomal dominant genes, our results identify a recessive-acting susceptibility locus in BALB/c mice.
- Impact --- Mapping modifiers of mammary tumor susceptibility in mice will provide novel markers to with which to assess risk and identifies pathways that can be targeted to compensate for p53-deficiency.

References

1. Kuperwasser, C.; Hurlbut, G.; Kittrell, F.; Medina, D.; Naber, S.; Jerry., D. (2000) Development of mammary tumors in BALB/c p53 heterozygous mice: A model for Li-Fraumeni Syndrome. *Am. J. Pathol.* 157:2151-2159.
2. Donehower LA, Harvey M, , Vogel H, McArthur MJ, Montgomery CA Jr, Park SH, Thompson T, Ford RJ, Bradley A. (1995) Effects of genetic background on tumorigenesis in p53-deficient mice. *Molecular Carcinogenesis* 14:16-22.

Appendices

- 1-Reprint of published article is attached.
- 2-Resignation letter from Dr. Sallie Smith.

BALB/c Alleles for *Prkdc* and *Cdkn2a* Interact to Modify Tumor Susceptibility in *Trp53*^{+/-} Mice¹

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Abstract

In mice heterozygous for p53 (*Trp53*^{+/-}), the incidence of mammary tumors varies among strains, with C57BL/6 being resistant and BALB/c being susceptible. Mammary tumor phenotypes were examined in female *Trp53*^{+/-} F1 mice (C57BL/6 × BALB/c; *n* = 19) and N2 backcross mice [(C57BL/6 × BALB/c) × BALB/c] (*n* = 224). Susceptibility to mammary tumors segregated as a dominant phenotype in F1 females, but a higher frequency and shorter latency in N2 mice indicated a contribution from recessive-acting modifiers. Segregation of the hypomorphic BALB/c alleles for DNA-dependent protein kinase catalytic subunit (*Prkdc*) and p16^{INK4A} (*Cdkn2a*) was analyzed in the N2 mice. The time to first tumor (considering all tumor types) was significantly different among the four genotype combinations (*P* = 0.01). *Cdkn2a* had a strong effect (*P* = 0.008) but was restricted to *Prkdc*^{B/B} mice (*P* = 0.001), indicating a strong interaction between the loci. Differences in mammary tumor occurrence among genotypes for *Prkdc* and *Cdkn2a* in N2 mice were not statistically significant. This study indicates that BALB/c *Prkdc* and *Cdkn2a* alleles do modify tumor incidence in *Trp53*^{+/-} mice and highlights the complexity of gene interaction effects in determining cancer phenotypes but discounts these alleles as major recessive loci contributing to spontaneous mammary tumor susceptibility.

Introduction

Breast cancer is a complex disease with many environmental and genetic factors contributing to an individual's risk of developing the disease. In the presence of rare, highly penetrant alleles, such as *BRCA1* and *BRCA2*, genetics can play a major role in determining an individual's risk. More common alleles may modify the risk associated with these high penetrance alleles, as is suggested by the relatively low penetrance of *BRCA1* mutations present in Ashkenazi Jewish population (1, 2). In the absence of high penetrance alleles, a study of twins has suggested that as much as 27% of breast cancer risk may still be attributed to genetic factors (3). Low penetrance alleles, which are common in the population, are likely to contribute to risk in this setting (1) and overlap with the modifiers of high penetrance alleles (4). Thus, the study of modifier genes in the setting of a high

penetrance allele may point to alleles contributing to cancer risk in the general population.

Germ-line mutations in the tumor suppressor gene *TP53*, which have been found in approximately half of Li-Fraumeni Syndrome families, also confer a high risk of breast cancer (5). Variation in the age of onset of disease between siblings carrying the same mutation also suggests the existence of modifier genes altering breast cancer risk. Li-Fraumeni Syndrome is characterized by the occurrence of various cancers at an early age, including breast cancer, soft tissue sarcomas, osteosarcomas, and adrenocortical carcinoma (6). Similarly, *Trp53*^{+/-} mice succumb to a range of tumor types; however, only on a BALB/c genetic background do they have a significant incidence of mammary adenocarcinomas, indicating that BALB/c mice possess alleles of modifier genes which increase their susceptibility to mammary tumors (7). The BALB/c strain also displays increased susceptibility to radiation- and carcinogen-induced mammary tumors compared with C57BL/6 mice (8, 9). The susceptibility to radiation-induced tumors has been demonstrated to be a recessive trait of the mammary epithelium, as C57BL/6 × BALB/c F1 epithelium showed the same low rate of mammary tumor formation as C57BL/6 epithelium (8). Thus, we undertook to identify recessive alleles present in BALB/c which would confer increased risk of spontaneous mammary tumors in the *Trp53*^{+/-} breast cancer model.

Two candidate genes have been described for which mutant alleles in the BALB/c strain may confer increased risk to mammary tumors. The *Prkdc* locus, encoding the catalytic subunit of the DNA double-strand break repair protein DNA-PK,⁵ contains two point mutations in BALB/c which result in increased radiation-induced genomic instability in mammary epithelial cells and thus may be involved in increased susceptibility to radiation-induced mammary tumors (10). The BALB/c allele of the *Cdkn2a* locus, encoding p16^{INK4A} and p19^{ARF}, also contains two point mutations which result in decreased ability of p16^{INK4A} to inhibit Rb phosphorylation and induce growth arrest but do not appear to alter p19^{ARF} function (11, 12). Therefore, these candidate genes were examined for their contribution to the formation of mammary and other tumor types in a [C57BL/6 × BALB/c] × BALB/c backcross population of female *Trp53*^{+/-} mice.

Materials and Methods

Mice and Breeding Strategy. BALB/c-*Trp53*^{+/-} mice were generated previously (13) 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]-*Trp53*^{+/+} F1 females × BALB/cMed-*Trp53*^{-/-} males. Nineteen virgin female (C57BL/6 × BALB/c)-*Trp53*^{+/-} F1-study mice and 224 virgin female

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⁵ The abbreviations used are: DNA-PK, DNA-dependent protein kinase; Rb, retinoblastoma.

[(C57BL/6 × BALB/c) × BALB/c]-*Trp53*^{+/-} N2-study mice were monitored weekly for tumor development or morbidity and palpated for mammary tumors. Tissues for DNA extraction were collected from 218 N2 mice. Four N2 mice survived to 18 months of age tumor free. 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 H&E for histological assessment. Mammary glands without tumors were whole mounted for examination of ductal tree structure. Ninety-seven virgin female BALB/c-*Trp53*^{+/-} were monitored similarly, but healthy mice were removed at set time points for ≤1 year for a study of precancerous changes, such that 56 mice were sacrificed for time points and 41 mice for tumors. No BALB/c-*Trp53*^{+/-} mice survived tumor free; the last tumor was found at 54 weeks of age.

Analysis of the Genotypes at the *Prkdc* and *Cdkn2a* Loci. Normal tail tissues from each mouse were snap frozen in liquid nitrogen at the time of necropsy. The tissue was digested overnight with 100 μg/ml proteinase K in 100 mM Tris, 5 mM EDTA, 0.2% sodium dodecyl sulfate, and 200 mM NaCl, and genomic DNA was isolated by phenol:chloroform:isoamyl alcohol extraction. PCR followed by restriction digestion was performed using published primers and cycling temperatures to distinguish between the C57BL/6 and BALB/c alleles at the *Prkdc* (10) and *Cdkn2a* (11) loci. PCR was performed with 10–50 ng of genomic DNA with 300 pmol/ml each primer in a 20-μl reaction volume containing 1 × PCR buffer (Sigma), 2 mM MgCl (Sigma), 250 μM deoxynucleotide triphosphates (Sigma), and 0.5 unit of Taq polymerase (Sigma). The allelic variants in exon 1 of *Cdkn2a* were examined after digestion with *Nla*III (New England Biolabs, Beverly, MA) at 37°C, and fragments were separated by PAGE using 10% acrylamide in 1 × Tris-borate EDTA and stained with ethidium bromide. The allelic variants at position 2140 of *Prkdc* were examined after digestion with *Bsm*BI (New England Biolabs) at 55°C, and fragments were separated on 1.5% agarose gels with 1 × Tris-borate EDTA and stained with ethidium bromide.

Statistical Analysis. Mice were categorized according to tumor types observed and alleles present at the *Prkdc* and *Cdkn2a* loci. Kaplan-Meier estimates of the tumor-free survival curves were calculated and plotted (This analysis takes into account the removal of healthy BALB/c mice at various times). The median time to tumor with 95% confidence limits was used for comparison of latencies where possible, avoiding biases caused by rare events at early and late ages that can influence mean results. The significance of differences in latency (tumor-free survival times) was analyzed by the Log-rank test. To test the effect of a single locus, the test was stratified by homozygosity or heterozygosity at the other locus. Latency of mammary tumors was defined as the age when a palpable lump was first detected in the mammary gland. Latency for lymphomas was defined as the age at which enlarged nodes or spleen were first detected or the age when first signs of illness were observed, which progressed to morbidity and sacrifice of the mouse and consequent diagnosis of lymphoma. The majority of osteosarcomas occurred on peripheral limbs; thus, the latency for osteosarcomas was simply defined as the age at which the tumor was first observed. When analyzing mammary tumors, mice bearing carcinosarcomas of the mammary gland were excluded to focus on tumors of definite epithelial origin.

Results

Survival and Tumor Spectrum. The tumor-free survival of female *Trp53*^{+/-} mice was altered considerably by genetic background of the three populations studied. BALB/c mice succumbed to tumors at the earliest age, whereas N2 and F1 study mice survived significantly longer ($P < 0.001$ for BALB/c versus either N2 or F1). The median time to tumor was 47.4, 57.6, and 61 weeks (95% confidence limits 45.6–52.9, 56.3–59.7, and 53–74) for BALB/c-, N2-, and F1-*Trp53*^{+/-} mice, respectively (Fig. 1A). The F1 mice survived slightly longer than N2 mice; however, this was not significantly different ($P = 0.1$) because of the small number of F1 mice studied. All F1-*Trp53*^{+/-} mice succumbed to tumors or morbidity by 18 months of age, whereas 4 N2-*Trp53*^{+/-} mice remained healthy at 18 months.

The most frequent tumor type observed in all three populations was mammary tumors. This has been reported previously for BALB/c-

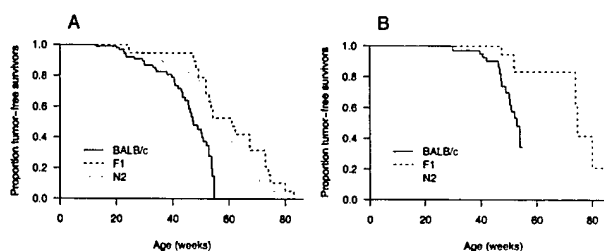


Fig. 1. Kaplan-Meier tumor-free survival curves for *Trp53*^{+/-} mice. Probability of tumor-free survival (all tumor types; A) and mammary tumor-free survival (B) for BALB/c (C57BL/6 × BALB/c)-F1 and [(C57BL/6 × BALB/c) × BALB/c]-N2 *Trp53*^{+/-} mouse populations.

Trp53^{+/-} mice but is a characteristic that was not observed in other genetic backgrounds (7, 14). The BALB/c data from the present study cannot be directly compared with the published data of Kuperwasser *et al.* because of the inclusion of males and breeder females in the previous study. However, when just the virgin female mice of the previous study were analyzed, 65% of mice developed mammary tumors at a median age of 54 weeks ($n = 17$), consistent with the current study. BALB/c mice were significantly more susceptible to mammary tumors than N2 and F1 mice ($P < 0.001$ and 0.005, respectively; Fig. 1B), with median times to first mammary tumor of 52.9, 66.4, and 74.7 weeks (95% confidence limits 50.4–NA, 64.6–68.3, and 74–NA, with NA indicating not available because of a lack of sufficient tumors occurring after the median age) for BALB/c-, N2-, and F1-*Trp53*^{+/-} mice, respectively. Mammary tumors occurred in 98 of 218 (45%) N2 mice, displaying both a lower incidence and increased latency compared with the BALB/c study mice, indicating the presence of recessive BALB/c alleles contributing to mammary tumor susceptibility. N2 and F1 mice were not statistically significant because of the small number of F1 mice ($P = 0.1$). Mammary adenocarcinoma was found in 6 of 19 (32%) F1 study mice (Fig. 1B). The occurrence of mammary tumors in the F1-*Trp53*^{+/-} mice compared with the lack of reports of occurrences in C57BL/6 mice indicates the presence of dominant BALB/c alleles contributing to BALB/c-*Trp53*^{+/-} mammary tumor susceptibility. Thus, both dominant and recessive BALB/c alleles contribute to spontaneous mammary tumor susceptibility in *Trp53*^{+/-} mice.

The mammary tumors arising in the three populations had similar histological characteristics, although the extent of squamous differentiation appeared to vary with genetic background. In the present study, 16 of 17 (94%) mammary tumors arising in BALB/c-*Trp53*^{+/-} mice were adenocarcinomas, with one case of mammary intraepithelial neoplasia and no adenosquamous carcinomas. In the F1 mice, of the 6 mammary tumors, 4 (66%) were acinar or alveolar adenocarcinomas, and 2 (33%) were adenosquamous in character. An additional 2 mice displayed significant mammary epithelial lesions in the form of sclerosing adenosis, a benign lesion, and adenosquamous hyperplasias, a likely precursor to adenosquamous carcinoma. The N2 mammary tumors consisted mainly of adenocarcinomas (76 of 98, 78%), with 9 (9%) cases of carcinosarcomas, 10 (10%) adenosquamous carcinomas, and 3 (3%) mammary intraepithelial neoplasias. Thus, in addition to increased latency and decreased frequency of mammary tumors, the introduction of C57BL/6 alleles into *Trp53*^{+/-} mice increased the extent of adenosquamous differentiation occurring within mammary tumors from none to 10 and 33% in the BALB/c, N2, and F1 mice, respectively.

The remainder of the tumor spectrum observed in F1- and N2-*Trp53*^{+/-} study mice included the tumor types most commonly reported in *Trp53*^{+/-} mice on other genetic backgrounds (14). Lymphoma was the second most frequent tumor type in both F1- and

Table 1 Analysis of tumor-free survival in *N2-Trp53*^{+/-} mice according to *Prkdc* and *Cdkn2a* genotypes

Tumor type	Genotype groups/latency ^{a,b} in weeks (n)	Effect of <i>Prkdc</i> ^c		Effect of <i>Cdkn2a</i> ^c		Interaction effects ^c			
		1 <i>P</i> ^{+/-} / <i>C</i> ^{+/-}	2 <i>P</i> ^{+/-} / <i>C</i> ^{B/B}	3 <i>P</i> ^{B/B} / <i>C</i> ^{+/-}	4 <i>P</i> ^{B/B} / <i>C</i> ^{B/B}	(1 + 2) vs. (3 + 4) <i>P</i> ^{+/-} vs. <i>P</i> ^{B/B}	(1 + 3) vs. (2 + 4) <i>C</i> ^{+/-} vs. <i>C</i> ^{B/B}	1 vs. 2 <i>P</i> ^{+/-}	3 vs. 4 <i>P</i> ^{B/B}
All ^a	214	57.7 (54)	57.6 (50)	64.0 (57)	55.5 (53)	0.94	0.008	0.60	0.001
Osteosarcoma ^b	28	77.6 (2)	72.1 (9)	76.3 (9)	72.6 (8)	0.24	0.01	0.01	0.25
Lymphoma ^b	44	73.8 (7)	71.8 (9)	73.9 (13)	68.8 (15)	0.13	0.09	0.49	0.10
Mammary ^{a,d}	89	67.6 (24)	66.0 (19)	68.1 (25)	65.6 (21)	0.93	0.35	0.79	0.11
Adrenal gland ^b	30	72.7 (11)	76.0 (4)	76.3 (9)	74.7 (6)				

^a Median latency.

^b Mean latency is listed where the probability of tumor-free survival did not fall below 0.5 in all groups.

^c *P*s determined from analysis of tumor-free survival curves (Fig. 2) by the Log-rank test. Boldface in Genotype group columns indicates the groups compared with the boldface *P*s.

^d Excluding carcinosarcomas.

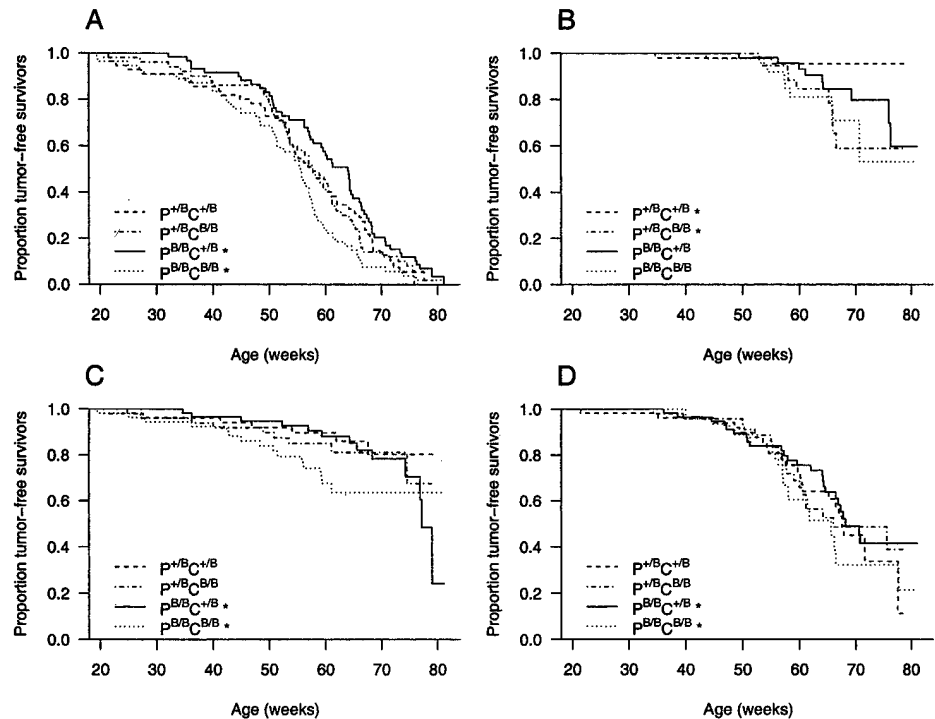
^e *P*, *Prkdc*.

^f *C*, *Cdkn2a*.

N2-populations, occurring in 4 of 19 (21%) F1 mice and 44 of 218 (20%) N2 mice. Osteosarcomas were observed in 28 of 218 (13%) N2 mice and in only 1 (5%) F1 mouse. However, in addition to these commonly observed tumors, two tumor types not reported previously in *Trp53*^{+/-} mice occurred in the F1 and N2 mice. Adrenal gland tumors were observed in two F1 mice (11%) at 73 and 78 weeks of age and were the third most abundant tumor type in the N2 mice (31 mice, 14%), also occurring with a latency of >70 weeks (Table 1). Four mice with adrenal tumors displayed bilateral adrenal gland tumors. Histologically, the tumors were poorly differentiated carcinomas. The adrenal gland tumors varied in size at time of detection depending on whether they were found by palpation or on necropsy. Because of this inconsistent determination of latency, adrenal gland tumors were not subject to statistical analysis. Pituitary adenomas were observed in 2 F1 mice (11%) and 4 (2%) N2 mice occurring at 51–75 weeks of age (mean 62 weeks). Secretion of prolactin by these tumors was inferred from the abundant milky secretions in the mammary glands. No other tumor type affected >5% of the N2-*Trp53*^{+/-} mice.

Modifier Effects of *Cdkn2a* and *Prkdc* in N2 Mice. Polymorphisms at the *Prkdc* and *Cdkn2a* loci, which alter gene function, have been identified in the BALB/c strain. To determine whether the BALB/c alleles contributed to tumor occurrence, the genotypes of the N2 mice were determined at these two loci by PCR-RFLP analysis. The tumor-free survival curves for the four different genotype combinations differed significantly (Fig. 2A; *P* = 0.01). Analysis of the occurrence of all tumors within the N2 population according to single loci suggested that *Prkdc* had no significant effect (*P* = 0.94; Table 1) but that the differences were attributable entirely to the *Cdkn2a* locus (*P* = 0.008; Table 1). However, examination of the two loci in combination indicates that *Prkdc* genotype was not without effect. Analysis of the interaction of *Prkdc* and *Cdkn2a* revealed an 8-week delay in tumor appearance in *Prkdc*^{B/B}, *Cdkn2a*^{+/-} mice compared with *Prkdc*^{B/B}, *Cdkn2a*^{B/B} mice (*P* = 0.001; Table 1). In contrast, *Cdkn2a* genotype makes no significant difference to survival in *Prkdc*^{+/-} mice (*P* = 0.60; Fig. 2A). Thus, when considering all tumor types, a strong modifying effect of the *Cdkn2a* locus is detected among mice homozygous for the BALB/c *Prkdc* allele.

Fig. 2. Kaplan-Meier tumor-free survival curves for *Trp53*^{+/-} mice. Probability of tumor-free survival (all tumor types; A), osteosarcoma-free survival (B), lymphoma-free survival (C), and mammary tumor-free survival (D) of N2-*Trp53*^{+/-} mice segregated according to genotypes at both the *Prkdc* (*P*) and *Cdkn2a* (*C*) loci. *, comparisons highlighted in Table 1.



Interactions between these two loci were also observed when examining individual tumor types (Table 1). Osteosarcoma-free survival was modified by *Cdkn2a* ($P = 0.01$) but not *Prkdc* ($P = 0.24$). Considering interactions between *Cdkn2a* and *Prkdc*, *Cdkn2a* heterozygosity significantly increased osteosarcoma-free survival in *Prkdc*^{+/^B} mice ($P = 0.01$), with *Prkdc*^{+/^B}, *Cdkn2a*^{+/^B} mice having a particularly low incidence of osteosarcomas. The other genotypes all displayed similar latencies and probabilities for developing osteosarcoma (Fig. 2B). Analysis of mice bearing lymphoma was suggestive of *Cdkn2a* being a modifier of lymphoma-free survival ($P = 0.09$). Considering interactions, this effect was most prominent in *Prkdc*^{B/^B} mice ($P = 0.1$), with *Cdkn2a*^{+/^B} genotype being associated with an increased average latency of 5 weeks and a higher probability of developing lymphoma at an older age (Fig. 2C).

Analysis of mice bearing mammary tumors, considering either *Prkdc* or *Cdkn2a* alone, did not produce statistically significant results. However, when considering interactions between the loci, a suggestion of *Cdkn2a* being a modifier was observed in *Prkdc*^{B/^B} mice, with the *Prkdc*^{B/^B}, *Cdkn2a*^{B/^B} genotype reducing the median latency of mammary tumor development by 2.5 weeks compared with *Prkdc*^{B/^B}, *Cdkn2a*^{+/^B} mice ($P = 0.11$; Table 1). Interestingly, this effect was limited to mice developing mammary tumors at >55 weeks of age, where the latency difference was generally 5–7 weeks (Fig. 2D).

Discussion

We undertook to identify recessive alleles present in BALB/c which increase risk of breast cancer in *Trp53*^{+/-} mice. The multiple tumor phenotype in *Trp53*^{+/-} mice allowed analysis of the effects of modifiers on other tumor types also. The BALB/c alleles of *Cdkn2a* and *Prkdc* were shown to interact and alter tumor latency in the experiments reported, but their effects differed among tissues, such that strong effects were seen on osteosarcomas, whereas no significant effects were seen on mammary tumors. *Cdkn2a* and *Prkdc* are both logical candidates for genes that would modify a tumor phenotype resulting from p53 deficiency (10, 11, 12). *Prkdc* encodes the catalytic subunit of DNA-PK. This kinase, which senses double-strand DNA breaks, is one of the kinases which stabilizes and activates p53 protein by phosphorylation (15). Thus, in cells already haploinsufficient for p53 function (16), decreased DNA-PK activity may further attenuate the p53 tumor suppression response. The p53 response can include induction of cell cycle arrest at both G₁ and G₂ checkpoints. p16^{INK4A}, one of the products of the *Cdkn2a* gene, also inhibits cell cycle progression through the G₁-S restriction point by preventing the phosphorylation of Rb by Cdk4, thereby preventing the release from Rb of the transcription factor E2F, which is necessary for transcription of genes involved in progression to S phase of the cell cycle (17). Thus, a less functional *Cdkn2a* allele will further diminish the fidelity of the G₁-S-phase checkpoint in *Trp53*^{+/-} cells.

The effect on all tumor types of these two hypomorphic alleles in combination was that mice homozygous for BALB/c alleles at both loci displayed the shortest time to first tumor, as might be expected, because the combination of decreased DNA repair and reduced cell cycle control would be complementary in allowing the survival of mutation-bearing cells, which can progress on to become cancer. However, the strength of the modifying effects and particular genotype combination of greatest difference varied between tumor types, with *Prkdc*^{+/^B}, *Cdkn2a*^{+/^B} mice standing out for low incidence of osteosarcomas (Fig. 2B) and *Prkdc*^{B/^B}, *Cdkn2a*^{+/^B} mice standing out for high incidence of lymphomas, whereas *Prkdc*^{B/^B}, *Cdkn2a*^{B/^B} mice had a noticeably shorter latency for lymphomas (Fig. 2C). The intricacies of these interactions depend on the level of expression of

DNA-PK and p16^{INK4A} in a particular tissue and on the integration of the signals and activities of many different mechanisms involved in determining the outcome of p53 activation. A p53 response can result in cell cycle arrest, stimulation of DNA repair, and apoptosis, and it is the balance between cell survival and maintenance of genomic integrity that is critical in protection against cancer. The p53 response of a cell can also vary with the proliferative status of a tissue, as is the case in the mammary gland (18); thus, the complex web of p53 regulation and cell fate decisions means that the effect of modifier alleles could be highly cell type, cell cycle, and carcinogen specific. The integration of these factors is complex and subtle in the controlled experimental setting of the *Trp53*^{+/-} model, indicating that the discovery of low penetrance alleles contributing to cancer phenotypes within the highly variable human population will be a very difficult task. However, the study of cancer susceptibility in animal models provides the opportunity to identify novel loci for investigation in the human disease setting.

The occurrence of mammary tumors in the F1 population of *Trp53*^{+/-} mice and increased latency of mammary tumors in the N2 population compared with the BALB/c mice indicate that both dominant and recessive alleles contribute to BALB/c susceptibility to mammary tumor formation. Both *Cdkn2a* and *Prkdc* have been suggested as mammary tumor susceptibility loci; however, neither locus made a major contribution as recessive alleles to the occurrence of mammary tumors in this model. The BALB/c allele of *Prkdc* was discovered as a gene contributing to the radiation sensitivity of mammary epithelial cells in BALB/c mice (10) and suggested to be involved in mammary tumorigenesis of this strain. Although *Prkdc* is not a recessive contributor to spontaneous *Trp53*^{+/-} mammary tumors, the possibility that it could be a dominant contributor cannot be ruled out. DNA-PK is a particularly attractive candidate for breast cancer susceptibility because of its already low expression level in the mammary epithelium (19), its involvement in DNA double-strand break repair, and the mounting evidence of the involvement of these repair pathways in breast cancer (20, 21).

Several human studies of melanoma families with mutations in *CDKN2A* have suggested that these families may also be at greater risk of developing breast cancer (22, 23). The *Cdkn2a*^{B/^B} genotype, when present with *Prkdc*^{B/^B}, decreased mammary tumor latency in this study by 5–7 weeks in the older mammary tumor-bearing mice. Although not statistically significant, this is of interest because the absence of a modifying effect on the younger onset mammary tumors suggests differences in the mechanism of tumorigenesis between early and late onset mammary tumors in the N2 population. This may point to an unknown allele which accelerates mammary tumor development and renders any contribution from *Cdkn2a*^{B/^B} genotype insignificant. It will be of interest to examine any locus identified in future studies to be involved in mammary tumor susceptibility for interactions with *Cdkn2a*.

Two tumor types novel for *Trp53*^{+/-} mice, pituitary adenomas and adrenal gland tumors, were observed in the F1- and N2-*Trp53*^{+/-} mice of this study. Pituitary adenomas have been described in *Trp53*^{+/-} mice only when also deficient in Rb, where *Rb*^{+/-} mice characteristically develop pituitary adenomas (24). Perhaps the hypomorphic BALB/c *Cdkn2a* allele resulting in reduced Rb activity, combined with the decreased susceptibility to mammary tumors allowing longer survival times, contribute to the occurrence of this tumor type in the F1- and N2-*Trp53*^{+/-} mice. The occurrence of adrenal gland tumors in the F1- and N2-*Trp53*^{+/-} mice is of particular interest because adrenocortical carcinomas are strongly associated with Li-Fraumeni Syndrome while rare in the general population (6). Although these tumors were not observed in the BALB/c-*Trp53*^{+/-} mice of this study, they were observed at 59–73 weeks and often

bilateral in retired breeders kept for monitoring,⁶ suggesting that more time is required to develop these long latency tumors. The occurrence of adrenal tumors further supports the idea that BALB/c alleles contribute toward making BALB/c-*Trp53*^{+/-} mice a unique model for Li-Fraumeni Syndrome.

The current study demonstrates clearly that although *Cdkn2a* may be a weak modifier of *Trp53*^{+/-} mammary tumor susceptibility, homozygosity at either *Cdkn2a* or *Prkdc* cannot account for the susceptibility of BALB/c mice to spontaneous mammary tumors in this model. Genome scans on the N2-*Trp53*^{+/-} mice are currently underway in the search for other alleles responsible for BALB/c mammary tumor susceptibility.

Acknowledgments

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References

- Nathanson, K. L., and Weber, B. L. "Other" breast cancer susceptibility genes: searching for more holy grail. *Hum. Mol. Genet.*, *10*: 715-720, 2001.
- Fodor, F. H., Weston, A., Bleiweiss, I. J., McCurdy, L. D., Walsh, M. M., Tartter, P. I., Brower, S. T., and Eng, C. M. Frequency and carrier risk associated with common BRCA1 and BRCA2 mutations in Ashkenazi Jewish breast cancer patients. *Am. J. Hum. Genet.*, *63*: 45-51, 1998.
- Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A., and Hemminki, K. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.*, *343*: 78-85, 2000.
- Ponder, B. A. Cancer genetics. *Nature (Lond.)*, *411*: 336-341, 2001.
- Kleihues, P., Schauble, B., zur Hausen, A., Esteve, J., and Ohgaki, H. Tumors associated with p53 germline mutations: a synopsis of 91 families. *Am. J. Pathol.*, *150*: 1-13, 1997.
- Birch, J. M., Alston, R. D., McNally, R. J., Evans, D. G., Kelsey, A. M., Harris, M., Eden, O. B., and Varley, J. M. Relative frequency and morphology of cancers in carriers of germline TP53 mutations. *Oncogene*, *20*: 4621-4628, 2001.
- Kuperwasser, C., Hurlbut, G. D., Kittrell, F. S., Medina, D., Naber, S. P., and Jerry, D. J. Development of mammary tumors in BALB/c p53 heterozygous mice: a model for Li-Fraumeni Syndrome. *Am. J. Pathol.*, *157*: 2151-2159, 2000.
- Ullrich, R. L., Bowles, N. D., Satterfield, L. C., and Davis, C. M. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. *Radiat. Res.*, *146*: 353-355, 1996.
- Medina, D. Mammary tumorigenesis in chemical carcinogen-treated mice. I. Incidence in BALB/c and C57BL mice. *J. Natl. Cancer Inst. (Bethesda)*, *53*: 213-221, 1974.
- Yu, Y., Okayasu, R., Weil, M. M., Silver, A., McCarthy, M., Zabriskie, R., Long, S., Cox, R., and Ullrich, R. L. Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the *Prkdc* (DNA-dependent protein kinase catalytic subunit) gene. *Cancer Res.*, *61*: 1820-1824, 2001.
- Zhang, S., Ramsay, E., and Mock, B. *Cdkn2a*, the cyclin-dependent kinase inhibitor encoding p16^{INK4α} and p19^{ARF}, is a candidate for the plasmacytoma susceptibility locus. *Pctrl*. *Proc. Natl. Acad. Sci. USA*, *95*: 2429-2434, 1998.
- Zhang, S. L., DuBois, W., Ramsay, E. S., Bliskovski, V., Morse, H. C., Tadesse-Heath, L., Vass, W. C., DePinho, R. A., and Mock, B. A. Efficiency alleles of the *Pctrl* modifier locus for plasmacytoma susceptibility. *Mol. Cell. Biol.*, *21*: 310-318, 2001.
- Jerry, D. J., Kuperwasser, C., Downing, S. R., Pinkas, J., He, C., Dickinson, E., Marconi, S., and Naber, S. P. Delayed involution in the mammary epithelium in BALB/c-p53^{null} mice. *Oncogene*, *17*: 2305-2312, 1998.
- Donehower, L. A., Harvey, M., Vogel, H., McArthur, M. J., Montgomery, C. A., Jr., Park, S. H., Thompson, T., Ford, R. J., and Bradley, A. Effects of genetic background on tumorigenesis in p53-deficient mice. *Mol. Carcinog.*, *14*: 16-22, 1995.
- Lakin, N. D., and Jackson, S. P. Regulation of p53 in response to DNA damage. *Oncogene*, *18*: 7644-7655, 1999.
- Venkatachalam, S., Tyner, S. D., Pickering, C. R., Boley, S., Recio, L., French, J. E., and Donehower, L. A. Is p53 haploinsufficient for tumor suppression? Implications for the p53^{+/-} mouse model in carcinogenicity testing. *Toxicol. Pathol.*, *29* (Suppl.): 147-154, 2001.
- Sherr, C. J. The *INK4a/ARF* network in tumour suppression. *Nat. Rev. Mol. Cell. Biol.*, *2*: 731-737, 2001.
- Minter, L. M., Dickinson, E. S., Naber, S. P., and Jerry, D. J. Epithelial cell cycling predicts p53 responsiveness to γ -irradiation during post-natal mammary gland development. *Development*, *129*: 2997-3008, 2002.
- Moll, U., Lau, R., Sypes, M. A., Gupta, M. M., and Anderson, C. W. DNA-PK, the DNA-activated protein kinase, is differentially expressed in normal and malignant human tissues. *Oncogene*, *18*: 3114-3126, 1999.
- Khanna, K. K., and Jackson, S. P. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat. Genet.*, *27*: 247-254, 2001.
- Venkitaraman, A. R. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell*, *108*: 171-182, 2002.
- Borg, A., Sandberg, T., Nilsson, K., Johannsson, O., Klinker, M., Masback, A., Westerdahl, J., Olsson, H., and Ingvar, C. High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families. *J. Natl. Cancer Inst. (Bethesda)*, *92*: 1260-1266, 2000.
- Plna, K., and Hemminki, K. High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families. *J. Natl. Cancer Inst. (Bethesda)*, *93*: 323-325, 2001.
- Harvey, M., Vogel, H., Lee, E. Y., Bradley, A., and Donehower, L. Mice deficient in both p53 and Rb develop tumors primarily of endocrine origin. *Cancer Res.*, *55*: 1146-1151, 1995.

⁶ A. C. Blackburn, personal observation.

Subject: termination of fellowship
From: ssmith@vasci.umass.edu
Date: Thu, 04 Sep 2003 11:24:18 -0400
To: jjerry@vasci.umass.edu

Dear Joe,

As you know, I was offered and have accepted a research faculty position at the Baystate Medical Center/UMASS-Amherst Biomedical Research Institute. My acceptance of this position at the institute makes me ineligible for the award. Though I look forward to our continued collaborations on genetic modifiers of mammary tumor susceptibility and related projects involving hormonal regulation of p53, I must terminate the DOD postdoc fellowship. I do hope that you will be able to transfer it to another who will be able to benefit from working on this project.

I do appreciate your support and guidance as I have moved to my independent lab.

Sincerely,

Sallie

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