Mammary Tumor Formation in p53- and BRCA1-deficient Mice

Victoria L. Cressman, Dana C. Backlund, Elizabeth M. Hicks, Lori C. Gowen, Virginia Godfrey, and Beverly H. Koller

Curriculum in Genetics and Molecular Biology [V. L. C., L. C. G.], and Departments of Pathology [V. G.] and Medicine [D. C. B., E. M. H., B. H. K.], University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Abstract
The inheritance of a mutant copy of the BRCA1 gene greatly increases a woman’s lifetime risk for ovarian and breast cancer. While a homologous gene has been identified in mouse, mice carrying mutations in this gene do not display a detectable increase in tumor formation. To determine whether mutations in p53 might increase the incidence of tumors associated with the loss of BRCA1 function in mice, we have generated mice carrying mutations at both of these loci. We report here that the presence of a mutant Brca1 allele does not alter survival of either p53/- or p53+/+ mice. Although the tumor spectrum was not dramatically altered, an increased incidence of mammary tumors was observed in the Brca1+/+p53/- group whereas only one such tumor was seen among the p53/+- control group. In addition, although the presence of a mutant Brca1 allele did not alter the survival rate or the incidence of most tumor types in the p53/+- mice, 5 of the 23 tumors isolated from the Brca1+/+p53/- mice treated with ionizing radiation were of mammary epithelial origin, and 3 of these had lost expression of the wild-type Brca1 gene. In contrast, no such tumors were observed in the irradiated p53/+- controls. Although the number of mammary tumors observed in these animals is small, these results are suggestive of a role for BRCA1 in mammary tumor formation after exposure to specific DNA damaging agents.

Introduction
Approximately 5% of breast cancer cases are believed to result from the inheritance of high penetrance risk factors (1). Mutations in the BRCA1 gene have been identified as the risk factor responsible for disease in >90% of individuals from families with a high incidence of both breast and ovarian cancer as well as in approximately 45% of hereditary breast cancer cases (2–4).

Although the BRCA1 protein and its murine homologue display only 58% overall homology, in specific regions of the protein (such as the putative zinc finger domain and the C-terminus) conservation of the protein sequence is >80% (5). This high degree of conservation suggests that not only do these regions represent important functional domains but at least some functions of this protein are conserved between mouse and man (6). It was, therefore, anticipated that the introduction of mutations similar to those seen in the human population into the murine homologue would result in a predisposition to tumor formation in these animals. Mice carrying a mutant copy of Brca1 were generated from ES cells in which a portion of exon 11 of the Brca1 gene was replaced with a marker gene by homologous recombination (7). Brca1 +/- mice develop normally, are fertile, and, surprisingly, fail to show the increased susceptibility to tumors seen in humans carrying similar mutations. The failure of mutations in Brca1 to lead to increased tumor susceptibility has been documented by several groups, each of which generated mouse lines carrying different Brca1 mutations (7–11). Thus the mouse differs markedly from the human, in which the presence of a mutant BRCA1 allele results in an estimated 80–90% overall lifetime risk for developing breast and/or ovarian cancer (3).

Differences between humans and mice in the phenotype resulting from mutations in tumor suppressor genes are not unique to BRCA1. For example, while mutations in the WT1 gene predispose to tumors in humans, no tumors are seen in the mouse (12, 13). Deletions and mutations in the p53 gene are observed in 40–60% of all sporadic breast cancers, and Li-Fraumeni patients, who carry a mutant copy of p53, have an increased susceptibility to many types of tumors, especially mammary carcinomas (14–17). Mammary adenocarcinomas are rare in p53/+- and p53/- mice and have been reported by only one group with a frequency of <2% of the tumors examined, whereas sarcomas are seen at a high frequency both in mice deficient in p53 and in Li-Fraumeni patients (18).

These observed differences between species seem less surprising when one considers that, despite the broad tissue distribution of a number of these tumor suppressor genes, inheritance of the mutated allele leads to very specific types of tumors. For example, although high levels of RB can be demonstrated in virtually all cell types, inheritance of a mutant RB allele in humans leads primarily to tumors of retinal neuronal and osteoblastic origin (19). Similarly, BRCA1 is expressed in a wide variety of actively dividing human cells, but mutations in BRCA1 are associated predominantly with an increased risk for breast, ovarian, and prostate cancer (20). The mechanisms that
underlie the differential impact of the loss of function of a ubiquitously expressed gene on a cell’s susceptibility to malignant transformation are poorly understood. However, a number of explanations for these findings have been proposed, including the theory that cell-to-cell and species-to-species variations reflect differences in lifetime exposure to environmental agents (such as ionizing radiation and carcinogens) or simply differences in the growth profiles of specific populations of cells (21, 22). Differences between species in the role of specific genes in mammary tumorigenesis may be related to the observation that the contribution of hormones to human and murine mammary tumor formation may be somewhat different. In mice, although prolonged exposure to high levels of prolactin and progesterone results in an increased incidence of mammary tumors, exposure of mice to high levels of estrogen itself does not alter the frequency with which these tumors are observed (23). It is also possible that parallel pathways and/or functional redundancy may be factors that contribute to both differences in tumor susceptibility of different cell types within a species as well as differences between species (24–26).

To generate better models of human disease and to begin to define the relationship between various oncogenes and tumor suppressor genes in vivo, investigators have intercrossed the mouse lines carrying mutant alleles of these genes. Often this results in either a change in the tumor types observed or in the latency of tumor formation (27, 28). We have, therefore, initiated a similar approach to examine tumor formation in mice carrying a mutant Brca1 allele. Specifically, we have examined the impact of deficiency of p53 on mammary tumor formation in these animals.

Recent studies support multiple functions for p53 in protecting animals from tumor formation (24, 29). These studies demonstrate that p53 deficiency leads to increased chromosomal instability, the premature entry of cells with DNA damage into S phase, and, in certain circumstances, the loss of the apoptotic response. A number of lines of evidence suggest that p53 interacts with BRCA1. Embryos homozygous for mutations in the Brca1 gene are severely growth retarded and die between embryonic days 7 and 13 (7, 9–11). Examination of these embryos suggested that there is a change in the expression of the p53-regulated genes p21 and mdm2 in cells deficient in BRCA1 (9). In addition, BRCA1-deficient embryos that are also deficient in either p53 or p21 survive several days longer (11, 30). A relationship between BRCA1 and the p53 pathway is further supported by studies demonstrating a striking coincidence between the cell cycle distribution of BRCA1 and p53 (31, 32). Recent reports demonstrate that BRCA1 and p53 directly interact and that BRCA1 requires the presence of p53 for the enhancement of transcriptional activation (33, 34). There is evidence to suggest that BRCA1 may play a critical role in DNA repair, especially after exposure to γ irradiation, UV light, or mitomycin C (35). It is, therefore, possible that the loss of BRCA1 impairs the ability of cells to efficiently repair DNA and that this in turn results in increased p53 activity, perhaps through alterations in MDM2 expression. If this interaction between the p53 pathway and BRCA1 is important in mammmary epithelium, then the deficiency in p53 may increase the frequency of malignant transformation of cells that have impaired DNA repair pathways as a result of somatic loss of the wild-type Brca1 allele.

To determine whether a deficiency in p53 and BRCA1 has a synergistic effect in tumor formation, particularly in mammary tumorigenesis, we have generated Brca1<sup>+/−</sup> mice that carry mutations in either one or both of their p53 alleles. The p53 mutation introduced into these mice is a null allele, and tumor formation in p53<sup>+/−</sup> and p53<sup>−/−</sup> has been described in detail previously (36, 37). The Brca1 mutation that these mice carry results in a complete loss of the expression of the native full length Brca1 gene (7). However, a splice variant mRNA, 4 kb smaller than the wild-type transcript, can be seen in the ES cells heterozygous and homozygous for this mutation. We have designated this mutation Brca1<sup>Δ223−763</sup> to indicate that the DNA encoding amino acids 223 to 763 are deleted in this mutation. Because the splice acceptor site for exon 11 is also deleted, the splice variant mRNA found in cells carrying this mutation does not include any coding sequence from exon 11. Amino acids 223 through 1364 would be absent from any protein transcribed from this mRNA. This region of the protein has been shown to include two nuclear localization signals, a RAD51-binding domain, a p53-binding domain, and a phosphorylation site (34, 38–41). Domains outside exon 11 in BRCA1 include the RING finger domain located in the NH₂ terminus and the BRCT domains in the COOH terminus. The RING finger domain binds BARD1 and BAP1, while the BRCT domains contain three RNA helicase A binding domains, the minimal transcriptional activation domain, and another p53-binding domain (33, 42–45). Similar to all of the other BRCA1-deficient lines generated to date, embryos homozygous for this mutation die early in gestation (7). However, although embryonic lethality in the majority of other homozygous embryos occurs between days 6 and 8 of gestation, those carrying the Brca1<sup>Δ223−763</sup> mutation survive until embryonic days 8 to 10 (7, 9–11). This increased survival is likely to reflect the more heterogeneous genetic background on which this mutation was studied. A similar impact of genetic background has been observed on analysis of mice carrying Brca2 mutations (11, 46–48). However, we cannot at this point exclude the possibility that extended survival reflects some function imparted by protein encoded by the splice variant mRNA present in these embryos. Recently we have shown that BRCA1 has a role in transcription-coupled repair (49). No activity was seen in cells homozygous for the mutant allele despite high levels of expression of the splice variant. However, despite the severe developmental phenotype and the complete loss of transcription-coupled repair observed in mice and cells homozygous for the Δ223–763 mutation, it will not be possible to ascertain whether this mutation represents a null allele until all of the functions of BRCA1 have been defined. For simplicity, we will refer to mice carrying this mutation as Brca1<sup>+/−</sup> throughout the manuscript.

In this report, we compare the tumor type and rate of tumor formation in Brca1<sup>−/−</sup> and p53-deficient mice to animals carrying p53 mutations alone. We also examined the effect of ionizing radiation on tumor formation in these populations.
These animals were again intercrossed and the genotype of intercrossed to obtain animals heterozygous for both alleles. failed to reveal obvious differences between the two groups prevalence of this tumor in the (37). The presence of a mutant type observed in the (Table 1). As previously reported, the predominant tumor Brca1 (11, 30). However, consistent with results reported with other embryonic development (7). Deficiency in p53 has been re- for the mutant Brca1 16 offspring were observed. Genotyping of the pups identified ever, this was true of both Brca1 mice were present in lower numbers than expected; how- ever, this was true of both Brca1 and Brca1 female mice deficient in p53 (50, 51). Mouse embryos homozygous for the mutant Brca1 allele fail to progress past day 10 of embryonic development (7). Deficiency in p53 has been reported to extend the survival of the embryos by several days (11, 30). However, consistent with results reported with other Brca1 mutant lines, p53-deficiency did not rescue the embryonic lethality of the BRCA1-deficient animals. Of nine litters generated from Brca1/p53 matings, no Brca1 offspring were observed. Genotyping of the pups identified 16 Brca1 and 40 Brca1 animals.

Thirty-eight Brca1/p53 and 40 Brca1/p53 animals were monitored biweekly for the development of tumors and killed when behavior and appearance indicated that the death of the animals was imminent. As can be seen in Fig.1, latency to tumor formation was similar in both groups of animals. By 19 weeks of age, ~50% of both Brca1 and Brca1 mice on a p53 background had been killed because of tumor formation. Tumors were classified both by observation of anatomical location and by histological ex- amination of tumor biopsies by a trained pathologist (V. G.). This analysis indicated that the distribution of tumors arising in the mice carrying a Brca1 mutation did not differ signifi- cantly from those observed in the Brca1/p53 animals (Table 1). As previously reported, the predominant tumor type observed in the p53 mice is the thymic lymphoma (36, 37). The presence of a mutant Brca1 allele did not change the prevalence of this tumor in the p53 animals. Histological examination of lung, kidney, and liver from these mice also failed to reveal obvious differences between the two groups in the extent of metastases to other organ systems.

Mammary tumors were observed in both Brca1 and Brca1 mice with mutations in both p53 alleles. Although only one mammary tumor was seen in the Brca1 mice (which is comparable to the frequency of such tumors seen in p53 mice by other investigators), 4 of the 23 Brca1 mice developed mammary tumors (18, 37). Two of these tumors were classified as papillary adenocarcinomas, and a third was classified as a tubular mammary carcinoma. Al- though histopathological examination established that a fourth tumor seen in this group of animals was of mammary gland origin, the characteristics of the tumor did not allow classification into established tumor subtypes. Although suggestive of an increased incidence of mammary tumor formation in mice heterozygous for a Brca1 mutation on a p53 background, the overall low number of mammary tumors in Brca1 and Brca1 mice did not permit mean- ingful statistical evaluation (Fisher’s exact test, P = 0.17).

Analysis of Brca1 Gene Expression in Brca1/p53 Tumors. Loss of the wild-type Brca1 allele is almost always observed on examination of DNA prepared from tu- mors arising in patients that have inherited a single mutant copy of the gene (52–54). The loss of heterozygosity of Brca1 in the cells of these patients is most likely an impor- tant event in mammary tumor formation. Although the similar tumor latency and spectrum in the Brca1 and the Brca1 mice did not suggest a general role for this gene in tumor formation in the mouse, we wished to verify this by examination of RNA and DNA from these tumors for the expression of the wild-type Brca1 allele. The mutation intro- duced into the Brca1 gene by homologous recombination results in a smaller RNA transcript than that produced from the wild-type allele. When the wild-type Brca1 transcript was not seen, the presence of this mutant transcript served as an internal control and allowed us to distinguish between loss of transcription of Brca1 and the loss of function of the wild- type gene. In tumors where analysis of RNA was not possible because of the postmortem changes in the tissue or in cases in which neither the wild-type nor mutant Brca1 transcripts were seen by Northern blot analysis, DNA was prepared and ana- lyzed by Southern blot for the presence of the wild-type allele. Analysis of 22 tumors from Brca1/p53 mice identified only 2 tumors in which loss of the wild-type allele could be detected (Table 1 and Fig. 2A, Lane 2, and Fig. 2C). As can be seen in Fig. 2A, Northern analysis of mRNA prepared from the first of these two tumors failed to reveal a band corresponding to the wild-type Brca1 transcript. In contrast, a transcript of approximately 4 kb from the mutated allele is present at high levels in the tumor RNA. Loss of heterozygosity in this tumor was confirmed by DNA analysis (Fig. 2B). In the second tumor, Northern analysis again failed to reveal expression of the native Brca1 transcript, whereas the tran- scription from the targeted allele was easily detected. In addition, a novel transcript, smaller in size than the wild-type Brca1 transcript, was also present in the RNA isolated from this tumor. Presumably this transcript originates from the wild-type allele that mutated during tumorigenesis (Fig. 2C). Both of the tumors that no longer expressed wild-type Brca1 mRNA were thymic lymphomas (Table 1). None of the mam-
Mammary Tumor Formation in p53- and BRCA1-deficient Mice

A cell line was established from the thymic lymphoma expressing Brca1 mRNA derived only from the targeted allele. RNA and DNA derived from this cell line confirmed the loss of the wild-type Brca1 allele in this tumor (Fig. 2A, Lane 1). p53-deficient thymic lymphomas have been previously reported to be CD4+8+ “double positive” or a mixed population of CD8+/CD4+8+ T cells. Flow cytometry revealed a mixed population phenotype (CD4+/8+ and CD8+) in this Brca1+/p53−/− thymic lymphoma cell line (data not shown).

Tumorigenesis in Brca1+/+ and Brca1+/− Mice on a p53-Heterozygous Background. We considered the possibility that the high incidence and early appearance of thymic lymphomas leading to early death of the majority of the Brca1+/+/p53−/− and Brca1+/−/p53−/− mice might obscure our ability to observe cooperativity between these two tumor suppressor genes in mammary tumorigenesis. Although p53−/− mice are also predisposed to tumors, the tumor latency and life span of p53−/− mice are longer (36, 37). We therefore examined tumor formation in Brca1+/+ and Brca1+/− mice on a p53−/− background. Twenty-three mice heterozygous for both the p53 and Brca1 mutation were generated, and survival and tumor formation were compared with 23 mice carrying only a single copy of the mutant p53 gene. Germ line inactivation of one copy of Brca1 does not alter the survival or the types of tumors that arise in the p53−/− animals (Table 2 and Fig. 3).

RNA was isolated from six tumors that arose in the Brca1+/−/p53−/− mice. In all of the cases, Northern analysis revealed the presence of both the wild-type Brca1 transcript and the transcript originating from the targeted allele (Table 2).

Table 1 Tumor spectrum in Brca1+/+ versus Brca1+/− mice on a p53−/− background

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Brca1+/+</th>
<th>Brca1+/−</th>
<th>No. of Brca1+/− tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Thymic lymphoma</td>
<td>28</td>
<td>54.9</td>
<td>19</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>9</td>
<td>17.6</td>
<td>10</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>1</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
<td>3</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>Cerebellar developmental defect</td>
<td>2</td>
<td>3.9</td>
<td>2</td>
</tr>
<tr>
<td>Adenocarcinoma of the Hardarian gland</td>
<td>1</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>1</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>2</td>
<td>3.9</td>
<td>2</td>
</tr>
<tr>
<td>Teratoma</td>
<td>1</td>
<td>2.0</td>
<td>4</td>
</tr>
<tr>
<td>Adenocarcinoma of rete ovari</td>
<td>1</td>
<td>2.0</td>
<td>1</td>
</tr>
<tr>
<td>Carcinoma of unknown origin</td>
<td>1</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>No visible or metastatic tumors at death</td>
<td>2</td>
<td>3.9</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>

*LOH, loss of heterozygosity.

Mammary tumors examined had lost expression of the wild-type Brca1 gene.

Consistent with published work (21), our observations revealed the presence of both the wild-type Brca1 allele. RNA and DNA derived from this line confirmed the loss of the wild-type Brca1 allele in this tumor (Fig. 2A, Lane 1). p53-deficient thymic lymphomas have been previously reported to be CD4+8+ “double positive” or a mixed population of CD8+/CD4+8+ T cells. Flow cytometry revealed a mixed population phenotype (CD4+/8+ and CD8+) in this Brca1+/p53−/− thymic lymphoma cell line (data not shown).

Consistent with published work (21), our observations revealed the presence of both the wild-type Brca1 allele. RNA and DNA derived from this line confirmed the loss of the wild-type Brca1 allele in this tumor (Fig. 2A, Lane 1). p53-deficient thymic lymphomas have been previously reported to be CD4+8+ “double positive” or a mixed population of CD8+/CD4+8+ T cells. Flow cytometry revealed a mixed population phenotype (CD4+/8+ and CD8+) in this Brca1+/p53−/− thymic lymphoma cell line (data not shown).

BRCA1 Expression in Mammary Tumors Arising in Irradiated Brca1+/+/p53−/− Animals. Five tumors arising in the irradiated Brca1+/−/p53−/− population were examined for loss of heterozygosity at both the p53 and Brca1 locus. By Southern blot analysis, the genomic fragment corresponding to the wild-type p53 allele was absent in all of the five tumors (data not shown).

Loss of heterozygosity at the Brca1 locus was examined as described above using RNA isolated from tumors. North-
ern analysis of RNA prepared from three ovarian tumors, a single histiocytoma, a pilomatrixoma, and a hemangiosarcoma showed that expression of the Brca1 wild-type allele was unaltered in these tumors (Table 3). Two of the mammary carcinomas also showed expression of both the mutant and the wild-type Brca1 genes in the tumor tissue (data not shown). Analysis of a third mammary tumor, classified as a papillary adenocarcinoma, indicated that the wild-type Brca1 allele was no longer expressed, whereas transcripts originating from the targeted allele were easily visible (Fig. 2). Loss of heterozygosity was demonstrated by analysis of DNA obtained from the tumor, as only the band corresponding to the mutant allele was observed on Southern analysis (Fig. 2).

Fig. 2. Loss of Brca1 heterozygosity in p53-deficient lymphomas. RNA was collected from tumor samples and cell lines derived from tumors and was examined for loss of Brca1 expression by Northern blot analysis. The lower band (mutant) is the RNA derived from the targeted allele. Its presence acts as an internal control, verifying that the transcriptional machinery required to produce Brca1 mRNA is present in the cell. A, RNA from one thymic lymphoma reveals the deletion of the wild-type copy of Brca1 (Lane 2). RNA from the cell line derived from this tumor revealed that it was also BRCA1-deficient (Lane 1). B, DNA derived from this tumor confirms the loss of the Brca1 allele. C, Northern analysis reveals that another thymic lymphoma line deleted a portion of Brca1. D, RNA made from one of three mammary tumors from irradiated Brca1-/-p53-/- mice that had lost the wild-type Brca1 allele. E, Southern analysis confirmed the loss of heterozygosity of Brca1 in this tumor.

The loss of wild-type BRCA1 expression in three of the five mammary tumors in the irradiated Brca1-/-p53-/- mice and the lack of mammary tumors in the Brca1-/-p53-/- mice suggests a correlation between Brca1 status and the formation of mammary tumors.

Discussion

We have generated mice that are carrying mutations in both Brca1 and p53 to determine whether mutations in both of these genes have a cooperative effect on tumorigenesis. These cooperative effects could result in a change in the latency of tumor formation already observed in the p53-deficient animals, or in the appearance of new tumor types arising in animals carrying mutations at both loci. We report that there is no alteration in the age at which p53-/- or p53-/- animals heterozygous for the mutant Brca1 allele succumb to tumors. Consistent with this observation, only 2 of 23 tumors examined from the Brca1-/-p53-/- population had lost expression of the wild-type Brca1 gene. These two Brca1-/- thymic lymphomas did not differ from the other tumors in any
of the parameters examined, including latency of tumor formation, histopathology, or the stage of T cell development from which they arose. These results are perhaps not surprising when one considers the results obtained in other model systems. For example, mice carrying a Wnt-1 oncogene driven by the MMTV-LTR and homozygous for a null p53 allele developed tumors earlier than mice carrying the transgene on a wild-type background (28). However, the failure to see an alteration in tumor latency in the p53<sup>-/-</sup> animals in this same model system raised the possibility that the decrease in tumor latency in the Wnt-1 transgenic, p53<sup>-/-</sup> mice was caused by non-cell-autonomous factors. These authors proposed that the inhibitory influence of normal cells on the growth of tumor cells may be compromised in the p53<sup>-/-</sup> animals. The latency of tumor formation in mice carrying mutations in both the p53 gene and the Rb gene was not substantially different from animals carrying mutations in only one of the genes (27). The age at which pituitary tumors formed in the Rb<sup>-/-</sup>p53<sup>-/-</sup> animals was not greatly altered in comparison to mice with the Rb mutation alone, and loss of the wild-type p53 allele in these tumors was rare.

In most model systems, the strongest evidence for a cooperative effect between mutations in two different loci in tumorigenesis comes from the appearance of novel tumors in mice carrying both mutations but not in mice carrying only one of the two mutations. For example, mice heterozygous for both Rb and p53 mutations present with novel tumors of endocrine origin, such as islet cell carcinomas and medullary thyroid carcinomas, which are not seen in either the Rb- or the p53-deficient animals (27). Examination of DNA from these tumors from Rb<sup>-/-</sup>p53<sup>-/-</sup> mice revealed that in most cases loss of the wild-type alleles had occurred. Novel tumors seen in Rb<sup>-/-</sup>p53<sup>-/-</sup> animals also resulted from the loss of the wild-type Rb allele. In contrast to these findings, we report that the tumor spectrum of Brca1<sup>+/+</sup>p53<sup>-/-</sup> mice is similar to that seen in p53<sup>-/-</sup> animals. A survey of RNA from tumors arising in these animals also failed to show any loss of the wild-type Brca1 allele. Thus, analysis of

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Table 2  Tumor spectrum in Brca1<sup>+/+</sup> versus Brca1<sup>−/−</sup> mice on a p53<sup>−/−</sup> background

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Brca1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Brca1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>7</td>
<td>36.8</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>Reticulum cell sarcoma</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>Bronchoalveolar carcinoma</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>Keratoacanthoma</td>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>Basal cell carcinoma</td>
<td>2</td>
<td>8.7</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>4</td>
<td>17.4</td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
<td>2</td>
<td>8.7</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>1</td>
<td>4.3</td>
</tr>
<tr>
<td>Benign spindle cell tumor</td>
<td>1</td>
<td>4.3</td>
</tr>
<tr>
<td>No visible or metastatic tumors at death</td>
<td>3</td>
<td>15.8</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

*LOH, loss of heterozygosity.

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Fig. 3.  Germline loss of Brca1 does not affect the survival rate of p53<sup>-/-</sup> mice. Brca1<sup>+/+</sup> and Brca1<sup>−/−</sup> mice on a p53<sup>−/−</sup> background were carefully monitored for ill health. Moribund mice were killed and necropsy was performed.

Fig. 4. γ irradiation of mice alters the overall tumor spectrum and latency of p53<sup>-/-</sup> mice but affects Brca1<sup>+/+</sup> and Brca1<sup>−/−</sup> mice similarly. Female Brca1<sup>+/+</sup> and Brca1<sup>−/−</sup> mice on a p53<sup>−/−</sup> background were irradiated with 5 Gy between 4 and 6 weeks of age. Moribund mice were killed and necropsied. Although the overall survival was decreased in both groups of p53<sup>-/-</sup> mice, the viability of mice was similar in the Brca1<sup>+/+</sup> and Brca1<sup>−/−</sup> mice.
this population of mice did not provide evidence for a cooperative effect between p53 and BRCA1 in the formation of these tumors.

The failure to observe cooperative effects between p53 and BRCA1 in the initial populations of animals examined is perhaps even more surprising in light of the recent publication demonstrating mutations in p53 in all of the eight tumors examined from \(Brca1^{+/−}\) patients (55). The phenotype of the mouse embryos suggests that BRCA1 is essential for normal cell growth. It might, therefore, be expected that permissive mutations such as the loss of p53 would be important events in tumorigenesis before the loss of BRCA1 expression. However, in contrast to the results obtained with the \(Brca1^{+/−}\)/p53\(^{+/−}\) mice, results obtained on the analysis of tumors arising in the \(Brca1^{+/−}\)/p53\(^{−/−}\) mice were suggestive of a role for cooperative effects between the two loci. Four mammary tumors were observed in this population of mice, whereas only one mammary tumor was observed in the p53\(^{−/−}\) population. Mammary tumors, although frequent in Li-Fraumeni patients, are uncommon in mice carrying similar p53 mutations. Jacks et al. (37) did not observe mammary tumors on analysis of 56 p53\(^{−/−}\) mice, while Harvey et al. (18) reported one in 56 p53\(^{−/−}\) mice analyzed. In contrast, we observed more mammary tumors in mice heterozygous for \(Brca1\) compared with control \(Brca1^{+/−}\) mice on a p53\(^{−/−}\) background. Differences between the number of mammary tumors in the \(Brca1^{+/−}/p53^{−/−}\) and the p53\(^{−/−}\) populations did not achieve statistical significance because of the small overall proportion of mammary tumors found in the \(Brca1^{+/−}/p53^{−/−}\) animals. A study with larger groups of animals of each genotype would be necessary to establish whether a statistically significant difference exists in the development of mammary tumors in \(Brca1^{+/−}\) animals.

Examination of the mammary tumors for the wild-type allele of \(Brca1\) did not support a role for this gene in tumorigenesis, inasmuch as RNA transcripts corresponding to both the mutant and wild-type \(Brca1\) allele were present in all of the tumors. However, this may reflect the fact that point mutations occur more frequently than mutations that result in loss of the transcription of the \(Brca1\) gene.

BRCA1 has been found to colocalize with RAD51 in discrete nuclear foci during S phase (40). DNA damaging agents, such as ionizing radiation, result in the dispersal of these nuclear foci (35). If BRCA1 is involved in the response of cells to DNA damaging agents, it might be expected that a role for this gene in tumorigenesis may be dependent on exposure of the organism to these agents. The short life span of mice and the controlled environment in which they are raised may not result in a sufficient lifetime exposure to DNA damaging agents to allow BRCA1-deficiency to have a significant impact on tumor formation. We, therefore, examined the effect of BRCA1-deficiency on tumor latency in p53\(^{+/−}\)/p53\(^{−/−}\) mice after exposure to \(γ\)-irradiation. We found that in general the tumor spectrum was not altered on comparison of the irradiated \(Brca1^{+/−}/p53^{−/−}\) and \(Brca1^{+/−}/p53^{−/−}\) mice. However, when we specifically examined the formation of mammary tumors in the two groups, interesting differences in the populations emerged. If BRCA1 is involved in cellular pathways such as DNA repair, it is possible that a 50% decrease in BRCA1 expression in cells heterozygous of the mutant allele results directly in an increased tumor formation rate in the \(Brca1^{+/−}/p53^{−/−}\) mice.

Consistent with the previous reports, no mammary tumors were seen in the irradiated p53\(^{+/−}\) mice (21). However, five mammary tumors were isolated from \(Brca1^{+/−}/−\) mice (21). In contrast, we observed more mammary tumors in mice heterozygous for \(Brca1\) compared with control \(Brca1^{+/−}\) mice on a p53\(^{−/−}\) background. Differences between the number of mammary tumors in the \(Brca1^{+/−}/p53^{−/−}\) and the p53\(^{−/−}\) populations did not achieve statistical significance because of the small overall proportion of mammary tumors found in the \(Brca1^{+/−}/p53^{−/−}\) animals. A study with larger groups of animals of each genotype would be necessary to establish whether a statistically significant difference exists in the development of mammary tumors in \(Brca1^{+/−}\) animals.

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loss of expression of p53 in tumors from BRCA1 +/- patients (55).

If studies with a larger group of animals confirm these results, it would suggest that cooperativity between BRCA1-deficiency and p53-deficiency can occur in mammary tumorigenesis but only with the exposure of the mammary gland to DNA damaging agents. This could reflect a direct role for BRCA1 in the response to DNA damage in mammary epithelial cells and would be consistent with the finding that BRCA1 is associated with RAD51 in the nucleus (35, 40). It is also possible that BRCA1 is itself a target for radiation-induced mutations. Exposure to radiation would then simply result in an increased frequency of loss of the wild-type Brca1 allele. Other investigators have shown that the loss of the wild-type allele and duplication of the mutant locus are more likely to occur in tumors from irradiated p53 +/- mice than in tumors arising spontaneously in this population of animals (56). The genetic mechanisms leading to loss of heterozygosity are poorly understood, and it remains unclear whether the frequency of this event will differ between different loci and at the same loci in different species. Some investigators have suggested that the high numbers of Alu repeats present in the human BRCA1 gene may contribute to loss of heterozygosity at this locus during tumorigenesis (57). Differences in the structure and the number of repetitive elements in the mouse genome may make this a rare event unless animals are exposed to ionizing radiation. Alternatively, exposure to radiation may simply result in additional mutations that synergistically act with mutations in Brca1 and p53 in tumorigenesis. Finally, radiation damage may lead to initiating events critical for tumor development.

While the low frequency of mammary tumors precludes the establishment of the statistical significance of this finding, this is the first report of loss of the wild-type Brca1 gene during mammary tumorigenesis in mice. With the caveat that a large scale loss of heterozygosity that is not specifically targeted at Brca1 may underlie the observed loss of BRCA1 expression, these studies suggest that a combination of genetic and environmental factors may allow the development of a mouse model to study the role of BRCA1 in tumorigenesis in the mouse.

Materials and Methods

Mice. p53 heterozygotes were obtained from Jackson Labs and were bred to Brca1 heterozygotes generated in our colony (7, 37). Genomic DNA was recovered from tail biopsy, and genotypes were determined by PCR amplification, as described previously (7, 37). A subset of Brca1 +/- and Brca1 /- female mice on a p53 +/- background were treated with 5 Gy of ionizing radiation at 4–6 weeks of age. Mice killed because of health were necropsied, and tumor samples were frozen at –80°C for DNA extraction or fixed in 10% phosphate-buffered neutral formalin (pH 7.0) for histological examination. In most cases, cell lines were successfully derived from tumor samples. Briefly, nonneoplastic portions of each tumor were rinsed in PBS and then dispersed in DMEM-H medium containing 15% fetal bovine serum, penicillin, streptomycin, and gentamicin. The homogenized tumor samples were then plated directly on plastic 100-mm plates or on plates containing irradiated fibroblasts. Growth was monitored daily and nonadherent cells were separated from adherent cells when these two populations arose from the same tumor.

The Fisher’s exact test was used for all of the statistical analyses.

Tumor Analysis/ Histopathology. Tumor samples fixed in 10% phosphate-buffered neutral formalin were dehydrated and embedded in paraffin. Sections (3 μm) were processed for H&E staining. Classification was done in a blinded fashion by a veterinary pathologist (V. G.). Cytokeratin 18 levels were examined by Northern blot analysis. Total cellular RNA was isolated from frozen tumor samples with RNAzol B (TelTest, Inc., Friendswood, TX), according to the manufacturer’s instructions. Twenty μg of RNA were electrophoresed in a 1.2% agarose formaldehyde gel, blotted to a immobilon-NC transfer membrane (Millipore, Bedford, MA) and UV-cross-linked. The membranes were hybridized with a 32P-labeled cytokeratin 18 cDNA for 1 h at 68°C using Quikhyb reagent (Stratagene, La Jolla, CA) and blots were washed twice with 2× SSC-0.1% SDS and then with once with 0.2× SSC-0.1% SDS at 42°C for 15 min each. The cytokeratin 18 cDNA was isolated through reverse-transcription followed by PCR of total RNA isolated from the mammary carcinomas (Invitrogen, Carlsbad, CA), using the primers 5′-ATGCCACCAATCTCAGGAC-3′ and 5′-AACCGGCCAGTTG-GCAAATCTC-3′. The PCR fragment was subcloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. A 772-bp-labeled probe was made and hybridized to a Northern blot containing total RNA from mammary tumors.

Loss of Heterozygosity. Total cellular RNA was isolated from frozen tumor samples as described above and transferred to nitrocellulose membranes. The membranes were probed with a 32P-labeled Brca1 cDNA containing exons 7 through 10 of Brca1 (7) and hybridized as described above. Genomic DNA was prepared from additional tumor samples using Nucleon (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. DNA was digested with EcoRV and analyzed by Southern blot with a probe containing a portion of intron 9 and exon 10 of Brca1. Hybridization was then done using a 32P-labeled probe specific for exons 7 through 9 of p53 genomic sequence. This probe was prepared from murine genomic DNA using the primers 5′-GGGGGGAGAG-3′ and 5′ -CTTTT-GCCGGGAGAGG-3′.

Flow Cytometry. Cells derived from a BRCA1-deficient, p53-deficient thymic lymphoma cell line and a p53-deficient thymic lymphoma control cell line were stained with phycoerythrin-conjugated anti-CD4 and fluoroscein isothiocyanate-conjugated anti-CD8 antibodies (PharMigen, San Diego, CA) and analyzed using a FACSscan cell sorter (San Jose, CA) and Cytometry, Inc. (Fort Collins, CO) data acquisition software.

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