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Elevated Breast Cancer Risk in Irradiated BALB/c Mice Associates with Unique Functional Polymorphism of the *Prkdc* (DNA-dependent Protein Kinase Catalytic Subunit) Gene[1]

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Abstract

Female BALB/c mice are unusually radiosensitive and more susceptible than C57BL/6 and other tested inbred mice to ionizing radiation (IR)-induced mammary tumors. This breast cancer susceptibility is correlated with elevated susceptibility for mammary cell transformation and genomic instability following irradiation. In this study, we report the identification of two BALB/c strain-specific polymorphisms in the coding region of *Prkdc*, the gene encoding the DNA-dependent protein kinase catalytic subunit, which is known to be involved in DNA double-stranded break repair and post-IR signal transduction. First, we identified an A → G transition at base 11530 resulting in a Met → Val conversion at codon 3844 (M3844V) in the phosphatidylinositol 3-kinase domain upstream of the *scid* mutation (Y4046X). Second, we identified a C → T transition at base 6418 resulting in an Arg → Cys conversion at codon 2140 (R2140C) downstream of the putative leucine zipper domain. This unique $Prkdc^{BALB}$ variant gene is shown to be associated with decreased DNA-dependent protein kinase catalytic subunit activity and with increased susceptibility to IR-induced genomic instability in primary mammary epithelial cells. The data provide the first evidence that naturally arising allelic variation in a mouse DNA damage response gene may associate with IR response and breast cancer risk.

Introduction

IR[3] is a well-characterized carcinogen capable of inducing breast cancer in humans and animals (1). The interaction between IR and germ-line genes has been implicated in human carcinogenesis although the overall importance of such interactions remains poorly understood (2). Women with inherited germ-line mutations in several known breast cancer susceptibility genes, *Tp53*, *BRCA1*, *BRCA2*, and possibly *ATM*, are predisposed to breast cancer (3). Since the protein products of the above susceptibility genes are believed to be involved in IR-induced damage signaling and repair (3), gene carriers may also be at an increased risk of IR-induced malignancy following the diagnostic and therapeutic use of radiation (4). Such highly expressing familial genetic disorders are rare whereas other minor deficiencies and polymorphisms in DNA damage response genes are expected to be more common in the human population (4). These may, however, escape detection because of their relatively low penetrance and the difficulties in identifying suitable study populations (5). Thus, although a number of investigations are suggestive of a relatively common association between heritable human cellular response to IR and breast cancer risk (6, 7), little progress has been made toward the specific identification of candidate polymorphic genes. In this respect mouse models can be of potential value in establishing proof-of-principle evidence on the involvement of specific gene polymorphisms in IR response and tumor risk.

The BALB/c mouse is unusually sensitive to the tissue-damaging effects of IR (8, 9) but, more importantly, postirradiation breast cancer risk in BALB/c is markedly higher than that recorded in C57BL/6 and the other common inbred strains that have been examined (10). For example, BALB/c shows an ~10-fold greater post 1 Gy γ-ray breast cancer risk than C57BL/6 and hybrids of the two strains ($CB6F_1$) because of an inherent hypersensitivity of BALB/c mammary epithelial cells to IR-induced transformation manifested as ductal dysplasia in a cell transplantation assay (11). This differential susceptibility to induced breast cancer has also been shown to correlate with two additional postirradiation cellular phenotypes. First, cytogenetic studies of *in vivo* or *in vitro* irradiated mammary epithelial cells showed BALB/c to have an ~3-fold elevation over C57BL/6 and $CB6F_1$ of chromatid-type aberrations arising during postirradiation cell culture (12). The development of such persistent genomic instability is believed to be an important early step in the postirradiation genesis of murine breast cancer and may therefore act as a cellular marker for susceptibility to neoplastic transformation (13–15). Second, cells from BALB/c have been shown to be partially deficient in the postirradiation repair of DNA DSB in comparison to C57BL/6, $CB6F_1$, three other inbred strains, and *scid* mice controls (9). Moreover, this repair deficiency of BALB/c correlated with significantly reduced abundance and activity of the DNA-PKcs (9). This protein has cellular IR response functions which include nonhomologous end joining of DNA breaks, apoptosis, and signal transduction (16); it is also involved in V(D)J recombination and in the control of chromatin structure and telomeric integrity (17).

On the basis of the above correlations and DNA-PKcs functions, we have proposed (9) that the IR phenotype of BALB/c mice may associate with a partial defect in the structure or expression of the *Prkdc* gene encoding DNA-PKcs. Here, we report a unique $Prkdc^{BALB}$ allele having two coding sequence polymorphisms distinct from the *scid* mutation. On the basis of genetic data, we associate this variant gene with reduced DNA-PKcs protein and elevated postirradiation genomic instability in preneoplastic mammary epithelium.

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[3] The abbreviations used are: IR, ionizing radiation; DSB, double-stranded break; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; RI, recombinant inbred; YAC, yeast artificial chromosome.

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Materials and Methods

Animals and Cells. Virgin female BALB/cByJ, C57BL/6ByJ, $CB6F_1$, and RI mice were obtained fromThe Jackson Laboratory (Bar Harbor, ME). Backcross mice were generated by crossing female $CB6F_1$ with male BALB/cByJ mice. All animals were maintained in the University of Texas Medical Branch Animal Resource Center.

Primary epithelial cells were obtained as previously described (12). Briefly, mammary glands 4 and 5 were removed from anesthetized mice, minced with scalpels, and suspended in Medium 199 containing Type III collagenase (200 units/ml) and dispase (1 mg/ml). The suspension was incubated at 37°C for 3 h with gentle agitation, followed by extensive washes with Medium 199. The cells were plated in 2% fetal bovine serum for 90 min to allow unwanted fibroblasts to attach. The supernatant, containing epithelial cells, was then collected, counted, and plated in collagen-coated culture dishes. Cells were grown under 10% CO_2 in JRH Ham's F-12 medium supplemented with insulin, hydrocortisone, transferrin, epidermal growth factor, Fungizone, gentamicin, and 5% fetal bovine serum.

Primary kidney cells were established by digesting minced kidney with Type III collagenase followed by extensive washing in PBS. The cells were plated and maintained in α-MEM medium containing 10% fetal bovine serum, antibiotics, and Fungizone (9).

Irradiation. Irradiation was carried out using a ^{137}Cs irradiator with a dose rate of 6.70 Gy min^{-1}.

DNA and PCR/RFLP Analyses. DNA for analyses were prepared from mice bred at the University of Texas Medical Branch or, for other inbred strains, from stocks commercially available from The Jackson Laboratory or from other investigators. PCR amplifications were carried out using a Qiagen kit (Qiagen, Chatsworth, CA) in a Perkin-Elmer 9600 thermal cycler (Norwalk, CT). The primer sequences for M3844V were: (F) tgtcacaagaggagaaagtggc and (R) tgtacattagcacataggctcc; PCR cycling conditions were: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 45 cycles, followed by a final extension at 70°C for 10 min. PCR product was digested with *Hph*I at 37°C for 3 h according to the manufacturer's instructions. The primer sequences for R2140C were: (F) gccatgatccttagcaagtg and (R) gcctaaggtaaggtgctgta; PCR cycling conditions were 94°C for 30 s, 49°C for 30 s, and 72°C for 30 s for 40 cycles, followed by a final extension at 70°C for 10 min. The PCR product was digested with *Bsm*BI at 55°C for 3 h according to the manufacturer's instructions. Microsatellite analyses were performed using the PCR primers and conditions specified by Research Genetics (Hunstville, AL). In all cases, PCR products were analyzed on 2% agarose gels. The WI/Mit 820 mouse YAC library (18) was purchased from Research Genetics and used according to their instructions.

Western Blotting and Kinase Assay. Total cell lysates were extracted as previously described (17). Twenty to 100 μg of protein/sample were separated on 8% or 10% SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with different antibodies followed by enhanced chemiluminescence detection. The sources of antibodies used are NeoMarkers (DNA-PKcs Ab-4, Ku80 Ab-3, Ku70 Ab-4) and Oncogene (actin).

DNA-PK kinase activity was measured using a "pull-down" assay with modification as described previously (9).

Cytogenetic Instability Analysis. Mammary cell dissociation, culture, and cytogenetic analysis were conducted as previously described (12).

Results

Uniquely Low DNA-PKcs Level in BALB/c Mice. We previously observed that primary kidney cells from BALB/c mice expressed a lower level of DNA-PKcs than those from C57BL/6 (9). To investigate whether there is common variation in DNA-PKcs abundance among different inbred mice, we determined protein levels of all three components of the DNA-PK complex by Western blot analysis of primary kidney cells from a number of commonly used laboratory mouse strains (Fig. 1*a*). No apparent difference in the protein levels of Ku80 and Ku70 among these strains was found. In contrast, BALB/cByJ mice expressed significantly lower yet detectable levels of DNA-PKcs protein than the other tested strains, whereas the BALB/c *scid* mutant showed undetectable DNA-PKcs protein as expected (Fig. 1*a*). To determine whether the lower DNA-PKcs expression in BALB/cByJ mice is tissue dependent, we further examined DNA-PKcs protein levels in brain, heart, kidney, liver, mammary glands, and spleen from BALB/cByJ and C57BL/6ByJ mice as well as their $CB6F_1$ progeny. Although variations were observed among different organs, with the mammary gland having the lowest level, DNA-PKcs protein level in each organ from BALB/cByJ mice was always lower than in its counterpart from C57BL/6ByJ mice (Fig. 1*b*). The detection of the lower DNA-PKcs protein level in BALB/cByJ mice is unlikely due to the affinity of the protein to monoclonal anti-DNA-PKcs antibody, since the use of a rabbit polyclonal DNA-PKcs antibody yielded similar results (data not shown).

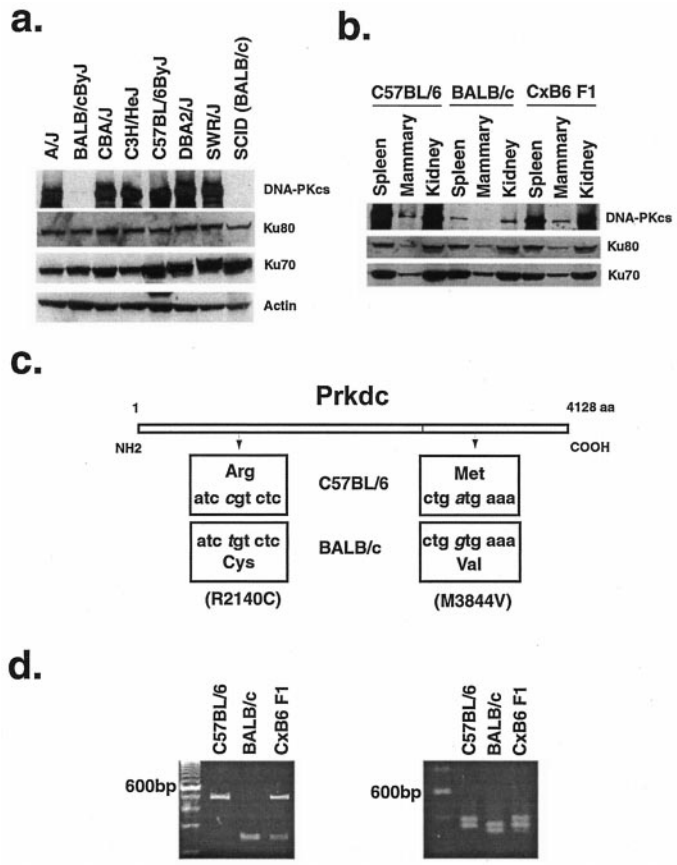

Fig. 1. DNA-PKcs expression and *Prkdc* genotype. *a*, expression of DNA-PKcs, Ku80, and Ku70 in mouse kidney epithelium by Western blot analysis. *b*, expression in selected tissues. *c*, schematic of R2140C and M3844V *Prkdc* polymorphisms. *d*, PCR/RFLP genomic analysis of R2140C (*Bsm*BI) and M3844V (*Hph*I) polymorphisms.

Northern blot analysis revealed no detectable difference in the mRNA level of *Prkdc* between BALB/cByJ and C57BL/6ByJ mice (data not shown). This issue was further investigated by reverse transcription-PCR analysis of *Prkdc* gene expression with β-actin and glucose-6-phosphate dehydrogenase as controls. No difference was detected between BALB/cByJ and C57BL/6ByJ in the mRNA levels of all three genes (data not shown). The low abundance of DNA-PKcs in BALB/c may therefore be associated with structural variation in *Prkdc*, which reduces DNA-PKcs protein stability (9).

Identification of *Prkdc* Coding Sequence Polymorphisms in BALB/c Mice. *Prkdc* maps to mouse chromosome 16 in a 1–2-cM segment flanked by microsatellites *D16 Mit73* and *D16 Mit34*.[4] Within this segment BALB/c differs in allele size from other recorded

[4] Mouse Genome Informatics at http://www.informatic.jax.org.

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strains for the *D16 Mit34* and *D16 Mit56* loci.[5] Genetic linkage was sought by PCR screening of the WI/MIT 820 mouse YAC library (18). This showed YAC 357-F-7 containing *D16 Mit34*, *D16 Mit56* loci along with the *Prkdc* sequence. Linkage of these loci was confirmed by PCR analysis of DNA from the isolated 357-F-7 YAC clone and from an independent *Prkdc*-positive murine YAC clone kindly provided by P. A. Jeggo (University of Sussex, UK; data not shown). Microsatellite allele sizes were subsequently determined for 14 inbred strains (A/J, AKR, BALB/cByJ, BALB/cANJ, CBA/J, C3H/HeJ, C57BL/6ByJ, DBA2/J, FVB/NHsd, NZB, RFM, SJL SWR/J, and 129S6/SvEvTac.); they were concordant for all except the two BALB/c strains for which *Prkdc* was associated with unique alleles for both *D16 Mit34* (110 bp *versus* 138 bp) and *D16 Mit56* (89 bp *versus* 76 bp).[5] These data, along with those on BALB/c-specific deficiencies in DNA-PKcs and DNA DSB repair, support the proposition that BALB/c harbors a small genomic segment encoding a variant *Prkdc* gene inherited from a founding ancestral mouse. Data on mouse genealogy (19) allow for such unique genetic inputs to BALB/c and this issue is the subject of a further investigation.

The entire open reading frame of the *Prkdc* was sequenced in cDNA fragments prepared from BALB/cByJ and C57BL/6ByJ mRNA and comparison was made with published and unpublished data as given below. Two nonconservative DNA bp differences between C57BL/6ByJ and BALB/cByJ were detected (Fig. 1, *c* and *d*). First, we identified an A → G transition at base 11530 creating a novel *Hph*1 restriction site resulting in a Met → Val conversion at codon 3844 in the phosphatidylinositol 3- kinase domain upstream of the *scid* mutation (Y4046X) (20). Second, we identified a C → T transition at base 6418 abolishing a *Bsm*B1 restriction site and resulting in an Arg → Cys conversion at codon 2140 downstream of the putative leucine zipper domain. In full support of a founder effect, PCR/RFLP analysis showed that of the 14 inbred strains tested (A/J, AKR, BALB/cByJ, BALB/cANJ, CBA/J, C3H/HeJ, C57BL/6ByJ, DBA2/J, FVB/NHsd, NZB, RFM, SJL, SWR/J, and 129S6/SvEvTac.), the two BALB/c strains were the only ones not carrying $Prkdc^{C57BL}$ (Fig. 1*d*). Therefore, the presence of these two DNA bp changes in coding sequence characterizes a unique variant *Prkdc* locus ($Prkdc^{BALB}$) in BALB/c mice.

Published nucleotide sequences[6] for the human, mouse, and hamster genes in combination with unpublished sequences kindly provided by K. Meek (Michigan State University, MI) (for horse) and P. A. Jeggo (for hamster) were used to assess the degree of amino acid conservation around the M3844V and R2140C polymorphism that characterize $Prkdc^{BALB}$ (Fig. 2). The relatively poor amino acid conservation around and including M3844V, also noted by others (20), is taken as preliminary evidence that this codon change will have little functional consequence. By the same reasoning, the high degree of conservation around and including R2140C suggests that this unique codon change characteristic of $Prkdc^{BALB}$ may well determine functionally variant and/or unstable DNA-PKcs protein.

***Prkdc*^{BALB} Dictates Reduced DNA-PKcs Protein Expression.** To determine whether the variant $Prkdc^{BALB}$ gene is indeed responsible for the reduced DNA-PKcs protein level, we biochemically phenotyped F_2 backcross mice and CXB RI mouse strains (21). Of the 50 F_2 backcross mice, 27 were $Prkdc^{BALB/BALB}$ homozygotes while the remaining 23 were $Prkdc^{BALB/C57BL}$ heterozygotes. Significantly, all homozygous backcross mice had BALB/c-like low levels of DNA-PKcs protein as well as kinase activity, whereas the heterozygous

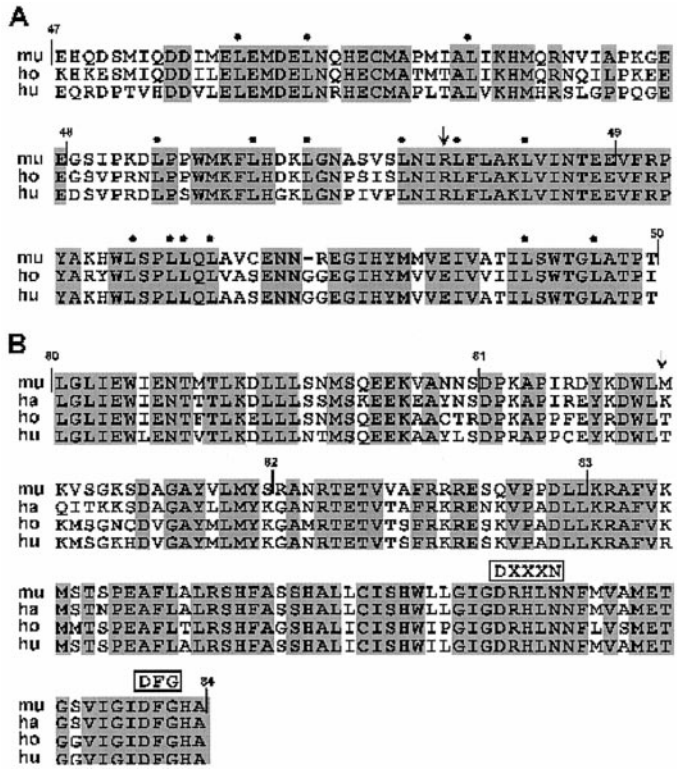

Fig. 2. Interspecies amino acid conservation in DNA-PKcs domains that encompass $Prkdc^{BALB}$ polymorphisms. *mu*, murine; *ha*, hamster (data not available for A); *ho*, horse; *hu*, human. Shaded blocks denote full conservation between species; murine exon boundaries are indicated by vertical lines giving exon numbers. *A*, conservation relating to R2140C (murine site *arrowed*) with conserved leucine residues noted to highlight the putative leucine zipper domain of exon 48. *B*, conservation relating to M3844V (murine site *arrowed*) with DXXXN/DFG denoting the phosphatidylinositol 3-kinase motif of exon 83.

backcross mice displayed higher DNA-PKcs protein levels as seen in $CXB6F_1$ mice (Fig. 1*b*). Further support for the correlation between the $Prkdc^{BALB/BALB}$ genotype and low DNA-PKcs protein level came from studies on 13 commercially available strains of CXB RI mice. Each RI line is a different inbred strain, homozygous at each locus, with different combinations of genetic input from C57BL/6 and BALB/c founder strains (21, 22). Of 13 CXB RI mice, 9 were $Prkdc^{BALB/BALB}$ and, as predicted, all showed lower levels of DNA-PKcs than the 4 $Prkdc^{C57BL/C57BL}$ strains (data not shown).

***Prkdc*^{BALB} Enhances IR-induced Genomic Instability in Mammary Cells.** Previous studies had demonstrated that mammary epithelial cells from breast cancer–prone BALB/c mice express elevated sensitive to IR-induced neoplastic transformation (11). These same cells are also more susceptible to IR-induced genomic instability (12), and we sought evidence on whether this breast cancer-associated phenotype is also determined by the $Prkdc^{BALB/BALB}$ genotype. Female F_2 backcross mice were subjected to a 1 Gy γ-ray irradiation and their mammary glands were removed 1 month later. Mammary epithelial cell cultures were initiated and karyotyped after 10 *in vitro* population doublings. In these proliferating cells, chromatid aberrations in mitotic cells represent genomic events occurring in the cell cycle prior to mitosis. Therefore, an increase in such aberrations is a measure of the development of ongoing genomic instability in the clonal progeny of irradiated mammary epithelial cells preceding overt neoplastic transformation. Of 20 irradiated backcross mice, 11 were $Prkdc^{BALB/BALB}$ and all showed BALB/c-like levels of chromatid damage elevated ~3-fold over that seen in the remaining 9 heterozygotes (Fig. 3). By reference to parallel studies on protein expression (data not shown), it is evident that DNA-PKcs and chromatid damage

[5] Whitehead Institute Mouse Physical database at http://www.genome.wi.mit.edu/edu/cgi-bin/mouse/index.

[6] GenBank accession nos. NM006904 (human) and AB007544 (mouse) at http://www.ncbi.nlm.nih.gov/.

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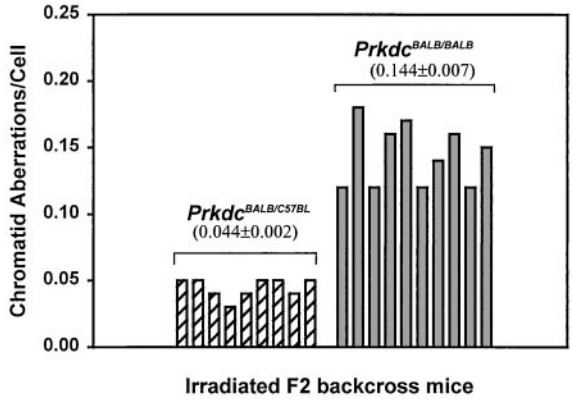

Fig. 3. Radiation-induced genomic instability and *Prkdc* genotype. Mammary cells were isolated from individual $CB6F_1 \times$ BALB/cByJ backcross mice 4 weeks after irradiation, cultured for 10 population doublings, and scored for cytogenetic instability. Each *bar* on the histogram represents an individual mouse. Numbers in parentheses are means ± SD. In four unirradiated controls, the values were between 0.03 and 0.04 regardless of genotype.

levels in F_2 backcross mice are inversely correlated. In nonirradiated controls, the *Prkdc* genotype did not measurably affect the low endogenous levels of chromatid breakage. Thus, postirradiation expression of genomic instability is genetically associated with both the $Prkdc^{BALB/BALB}$ genotype and DNA-PKcs deficiency ($P < 0.001$).

Discussion

The unusual *in vivo* radiosensitivity of the BALB/c mouse and its particular predisposition to induced breast cancer point clearly toward a substantial genetic component to *in vivo* IR response. Quantitative traits such as radiation response in a given set of mouse strains will be determined by the relative balance between susceptibility and resistance alleles. However, we have argued elsewhere (9) that these *in vivo* data along with those on (*a*) DNA DSB repair, (*b*) the induction of neoplastic transformation, and (*c*) induced chromatid instability might be explained if BALB/c were to carry a functionally important recessive allele conferring *in vivo* radiosensitivity, particularly with respect to breast cancer induction. These recent data also highlighted a possible association between the IR phenotype of BALB/c and reduced abundance/activity of the DNA repair-related protein DNA-PKcs (9). The molecular genetic study reported here adds considerable weight to these proposals.

First, the finding of the two BALB/c-specific *Prkdc* coding sequence polymorphisms (Fig. 1), the close linkage of the variant gene to unusual *D16 Mit34* and *D16 Mit56* flanking microsatellite alleles, and published genealogical data (19) allow for the unique inheritance, by BALB/c, of an important variant gene for IR response. In this way the IR response of BALB/c might be expected to be readily distinguishable from that of other inbred mice. Second, the study of F_2 backcross and CXB RI mice provided strong evidence that the variant *Prkdc* gene of BALB/c is phenotypically characterized by reduced DNA-PKcs protein abundance and activity which would be consistent with the partial DNA DSB repair deficiency of this mouse strain (9). These genetic studies do not, however, comment upon the functional specificity of the *Prkdc* M3844V and R2140C polymorphisms which, as expected, were not separated in the recombinants that were studied. Consideration of amino acid sequence conservation in and around the two sites (Fig. 2) tends to place functional emphasis on R2140C, which is included in a well-conserved region upstream of the putative leucine zipper domain; this initial judgment needs to be experimentally confirmed. Third, with respect to the possible implications of functional *Prkdc* variation for the IR response of mammary epithelium, the study of F_2 backcross mice provided genetic evidence that excess IR-induced persistent chromatid instability is primarily determined by *Prkdc* polymorphism; there was little phenotypic variance between $Prkdc^{BALB/BALB}$ genotypes (Fig. 3). The mechanistic basis of such genomic instability in murine mammary epithelial cells has yet to be resolved and, here, we use this end point as a simple cellular measure of susceptibility to neoplastic development in the breast (13–15). On this basis, these cytogenetic data support an association between variant *Prkdc* and the breast cancer susceptibility of BALB/c mice. Relevant to this are the data in Fig. 1*b* which show that, as in humans (23), DNA-PKcs abundance is intrinsically low in mammary gland tissue. This feature may provide an explanation as to why the partial DNA-PKcs deficiency of BALB/c tends to target induced neoplasia to the breast.

To our knowledge, these data provide the first evidence that naturally arising functional polymorphisms of a specific DNA damage response gene in the mouse can be associated with elevated IR sensitivity and breast cancer susceptibility. Studies on postirradiation life span breast cancer risk in *Prkdc* F_2 backcross recombinants and on the origin and functional role of the two polymorphisms are under way and should clarify remaining uncertainties. However, taking the existing data as preliminary, proof-of-principle evidence, it is reasonable to project that similar functional polymorphisms and partial deficiencies in IR damage response genes will be present within human populations. Although potentially lacking overt phenotypes, these may well confer a degree of susceptibility to radiation tumorigenesis. A recent study suggesting a relationship between chromosomal radiosensitivity and breast cancer predisposition supports this view (7). The mouse data presented here further suggest that the human gene encoding DNA-PKcs would be an important germ-line candidate for breast cancer risk.

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