Loss of *Blm* enhances basal cell carcinoma and rhabdomyosarcoma tumorigenesis in *Ptch1*^{+/-} mice

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Basal cell carcinomas (BCCs) have relative genomic stability and relatively benign clinical behavior but whether these two are related causally is unknown. To investigate the effects of introducing genomic instability into murine BCCs, we have compared ionizing radiation-induced tumorigenesis in *Ptch1*^{+/-} mice versus that in *Ptch1*^{+/-} mice carrying mutant *Blm* alleles. We found that BCCs in *Ptch1*^{+/-} *Blm*^{tm3Brd/tm3Brd} mice had a trend toward greater genomic instability as measured by array comprehensive genomic hybridization and that these mice developed significantly more microscopic BCCs than did *Ptch1*^{+/-} *Blm*^{+/tm3Brd} or *Ptch1*^{+/-} *Blm*^{+/+} mice. The mutant *Blm* alleles also markedly enhanced the formation of rhabdomyosarcomas (RMSs), another cancer to which *Ptch1*^{+/-} mice and *PTCH1*^{+/-} (basal cell nevus syndrome) patients are susceptible. Highly recurrent but different copy number changes were associated with the two tumor types and included losses of chromosomes 4 and 10 in all BCCs and gain of chromosome 10 in 80% of RMSs. Loss of chromosome 11 and 13, including the *Trp53* and *Ptch1* loci, respectively, occurred frequently in BCCs, suggesting tissue-specific selection for genes or pathways that collaborate with *Ptch* deficiency in tumorigenesis. Despite the quantitative differences, there was no dramatic qualitative difference in the BCC or RMS tumors associated with the mutant *Blm* genotype.

Introduction

Basal cell carcinomas (BCCs) comprise ~80% of all non-melanoma skin cancers and as such are the most common of human cancers. Despite their locally invasive nature, BCCs metastasize extraordinarily rarely (1,2), and the mechanism underlying this extremely low incidence is unclear. Suggested barriers to metastasis have included BCCs' relative genomic stability, which contrasts with the nearly uniform genomic instability of more malignant, more metastatic visceral cancers (3). Such genomic instability may generate more mutations and hence more opportunities to produce cells able to overcome the barriers to more extensive local invasion and more widespread dissemination (4). However, there has been no direct assessment of the influence of the level of genomic instability on the behavior of BCCs and so the connection remains hypothetical.

One autosomal recessive heritable condition with chromosomal instability is the Bloom syndrome (5), which is caused by mutations in the

RecQ helicase *BLM*. Helicases unwind complementary strands of DNA for processes such as transcription of RNA, DNA repair and recombination and replication in which single-stranded DNA is required. Mutations in other *RecQ* helicase family members underlie Werner syndrome (*WRN*), Rothmund–Thomson syndrome, (*RAPADILINO*) and Baller–Gerold syndrome (*RECQ5*) (6–13). Mutations in each of these three helicases lead to spontaneous chromosome instability, elevated mutations and elevated predisposition to cancers, findings which are common to Bloom, Werner and Rothmund–Thomson syndromes (14–16).

In this study, we assessed whether the loss of genetic stability induced by *Blm* dysfunction would affect tumorigenesis arising in the *Ptch1*^{+/-} mouse model of hedgehog-driven carcinogenesis (17). Several mutant murine *Blm* alleles have been engineered (18–20), and the degree of genomic instability and the severity of cancer predisposition in mice carrying these alleles vary inversely according to the degree of loss of *Blm* protein function (21). Hence, we mated *Ptch1*^{+/-} mice to mice carrying a hypomorphic *Blm* allele (*Blm*^{tm3Brd}) (20) and assessed ionizing radiation-induced carcinogenesis in the compound mutant mice. We found that reduced *Blm* function indeed significantly increases tumorigenesis of BCCs and RMSs, two tumor types that are characteristic of *Ptch1*^{+/-} mice and *PTCH1*^{+/-} human (basal cell nevus syndrome) patients. Despite the increased tumorigenesis, tumors arising in *Blm* wild-type and deficient mice could not be distinguished histologically.

Materials and methods

Mice

Ptch1^{+/-} heterozygous mice (17) were bred with *Blm*^{tm3Brd/tm3Brd} mice to produce *Blm*^{+/+} wild-type, heterozygous and homozygous mutant mice (20). We enrolled 90 mice in these studies—29 *Ptch1*^{+/-} *Blm*^{+/+} mice, 30 *Ptch1*^{+/-} *Blm*^{+/tm3Brd} mice and 31 *Ptch1*^{+/-} *Blm*^{tm3Brd/tm3Brd} mice. Mice of all three tested genotypes were littermates and were of mixed FVB/N/J, C57BL/6 and DBA/2 backgrounds. Mice were maintained in plastic cages with metal lids at 21–23°C, 50% humidity and on a 12 h white fluorescent light, from 34 W bulbs, and 12 h dark cycle. They had access to laboratory chow (5008; Purina, St Louis, MO) and tap water *ad libitum*. At the age of 2 months, all of the mice were exposed to 5 Gy of γ -radiation, using a Best Industries (Springfield, VA) ¹³⁷Cs radiation device (half-value layer, 0.60 cm Pb; dose, 0.94 Gy/min).

At the age of 9 months, 1 cm² of dorsal skin was excised, divided into 5 equal slivers and fixed in 10% buffered formalin for β -galactosidase staining. All biopsies were performed under anesthetic, using a 1:1 solution of 20 mg/ml xylazine and 100 mg/ml ketamine. During the study, all mice were observed for macroscopic tumor formation. Mice exceeding the animal welfare guidelines were euthanized. Tumors and a 1 cm² piece of back skin lacking visible tumor were excised and fixed for histological assessments. For β -galactosidase staining, samples were fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D galactopyranoside in iron buffer solution (22).

Microscopic BCCs quantification

A single investigator, blinded to the study groups, counted the number of BCC-like tumors and measured the two-dimensional cross-sectional size of these tumors in five slivers of the dorsal skin taken from each mouse. Total BCC area was determined by summing the cross-sectional areas of all BCC-like tumors observed in the five sections of the 1 cm² skin samples.

Comparative genomic hybridization

Array comprehensive genomic hybridization (CGH) and imaging was performed as described previously using arrays of 2870 mouse bacterial artificial chromosome and P1 clones (23) and a custom imaging system (24). Data analysis was carried out as described previously (25). Genomic DNA was isolated from paraffin-embedded blocks (six BCC samples and five RMS samples from separate mice) by standard techniques. In addition, genomic DNA was extracted from paraffin-embedded liver tissue from each mouse to be used as the normal reference. The tumor and reference DNAs were labeled by random priming to incorporate Cy3- and Cy5-deoxyuridine triphosphate, respectively. The array CGH data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database, accession number GSE19880.

Abbreviations: BCC, basal cell carcinoma; CGH, comparative genomic hybridization; RMS, rhabdomyosarcoma.

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Fig. 1. Influence of *Blm* genotype on survival and tumor latency. (a) Overall survival for *Ptch1*^{+/-} mice that were also *Blm*^{+/+}, *Blm*^{+/-tm3Brd} and *Blm*^{tm3Brd/tm3Brd}. Overall survival for *Blm*^{+/+}, *Blm*^{+/-tm3Brd} and *Blm*^{tm3Brd/tm3Brd} were 598 ± 39, 450 ± 32 and 409 ± 34 days, respectively. Significant differences were observed between *Blm*^{+/+} versus *Blm*^{+/-tm3Brd} (*P* value: 0.04) and *Blm*^{+/+} and *Blm*^{tm3Brd/tm3Brd} (*P* value: 0.002) but not between *Blm*^{+/-tm3Brd} and *Blm*^{tm3Brd/tm3Brd} (*P* value: 0.2). (b) Visible BCC free duration for *Blm*^{+/+}, *Blm*^{+/-tm3Brd} and *Blm*^{tm3Brd/tm3Brd} mice. Mean visible BCC free duration was 607 ± 42 days for *Blm*^{+/+}, 533 ± 26 days for

Statistics

SPSS version 11.5 for Windows software (SPSS, Chicago, IL) was used to analyze the data. Significance was assigned to *P* values equal to or <0.05. One-way analysis of variance and post hoc tests were used for bivariate analysis of quantitative data. Kaplan-Meier plots were used to determine the survival probabilities by occurrence of the macroscopic BCCs or RMSs, being BCC free or being RMS free. Log-rank test was used to compare the survival times among study groups. Quantitative data are reported as mean ± 1 SD. Survivals are reported as mean ± 1 SE. χ^2 test (or Fisher's exact test, where necessary) was used to compare the differences of categorical variables among genetic groups.

Results

Blm deficiency enhances tumorigenesis in a tissue-specific manner

Ptch1^{+/-} mice of wild-type, heterozygous mutant, or homozygous mutant *Blm* genotypes were treated with ionizing radiation at age 8 weeks, biopsied at age 9 months to assess microscopic BCC-like tumor formation and observed subsequently for visible BCC and RMS development. Irradiated *Ptch1*^{+/-} mice with homozygous or heterozygous *Blm*^{tm3Brd} alleles had significantly shorter survival than did those with wild-type *Blm* alleles (Figure 1a).

BCC

Visible BCCs were present in similar numbers of mice in each of the three genotypes, and there were no significant differences in the visible BCC free survival (Figure 1b). The morphology of these tumors varied from BCC-like with almost no follicular differentiation, to trichoblastoma-like with advanced follicular differentiation, and the histologic findings did not differ in *Blm* wild-type versus *Blm* mutant mice (Figure 2a). However, reduction of *Blm* gene function was associated with increased microscopic BCC-like tumor size, tumor number per unit area and total BCC involved area with a greater increase in *Blm*^{tm3Brd/tm3Brd} compared with *Blm*^{+/-tm3Brd} animals. Both the larger size and increased numbers of microscopic BCC-like tumors in *Blm*^{tm3Brd/tm3Brd} mice compared with *Blm*^{+/+} animals (*P* = 0.01) were statistically significant (Figure 3). There was a trend toward enhanced microscopic BCC-like tumor formation in the *Blm* heterozygotes.

Metastasis of BCC-like tumors was not detected in any mice of the three studied genotypes.

RMS

Formation of RMS tumors was significantly enhanced in both *Blm*^{+/-tm3Brd} and *Blm*^{tm3Brd/tm3Brd} genetic backgrounds suggesting that *Blm* is haploinsufficient for RMS development. Ten-fold more RMS tumors developed in *Ptch1*^{+/-} mice carrying one or two mutant *Blm* alleles compared with *Blm*^{+/+} mice (32% of *Blm*^{tm3Brd/tm3Brd}, 30% of *Blm*^{+/-tm3Brd} versus 3% of *Blm*^{+/+} mice; *P* = 0.004 and 0.01 for homozygotes and heterozygotes versus wild-type, respectively). In addition, the age of onset of RMS was reduced in these genetic backgrounds compared with *Ptch1*^{+/-} *Blm*^{+/+} animals (Figure 1c). Nevertheless, as for BCCs, we found no significant histologic differences in RMS tumors arising in mice of the three genotypes including features such as infiltrative growth, necrosis, mitotic activity, heterologous differentiation, atypical mitoses and vascular invasion (Figure 2b).

Tissue-specific copy number aberrations in BCC and RMS tumor genomes

Genomic copy number was analyzed by array CGH on six BCC and five RMS tumors from *Ptch1*^{+/-} mice carrying wild-type, heterozygous or knockout *Blm* alleles. Hierarchical clustering revealed two

Blm^{+/-tm3Brd} and 536 ± 34 days for *Blm*^{tm3Brd/tm3Brd}. No statistically significant differences in the age of onset of visible BCCs were observed between *Blm*^{+/+} and *Blm*^{+/-tm3Brd} mice (*P* value: 0.7) or *Blm*^{+/+} or *Blm*^{tm3Brd/tm3Brd} mice (*P* value: 0.6). (c) Rhabdomyosarcoma free duration for *Blm*^{+/+}, *Blm*^{+/-tm3Brd} and *Blm*^{tm3Brd/tm3Brd} mice. The average RMS free duration for *Blm*^{+/+}, *Blm*^{+/-tm3Brd} and *Blm*^{tm3Brd/tm3Brd} were 739 ± 22, 481 ± 31 and 440 ± 35 days, respectively. Tumors appeared significantly earlier in *Ptch1*^{+/-} *Blm*^{+/-tm3Brd} (*P* value: 0.01) and in *Blm*^{tm3Brd/tm3Brd} (*P* value: 0.001) than in *Blm*^{+/+} mice.

Fig. 2. Histology of microscopic BCC-like and RMS tumors. (a) Morphology of microscopic BCC-like tumors varies from almost no follicular differentiation to trichoblastoma-like with advanced follicular differentiation. (b). RMS tumor is composed of sheets of eosinophilic spindle cells with abundant cytoplasm growing in intersecting fascicles confirming rhabdomyoblastic differentiation. Higher magnification demonstrates dense eosinophilic cytoplasm, and cells varying from strap-like cytomorphology to larger, bizarre epithelioid cells often with multinucleation characteristic of atypical rhabdomyoblasts. Mitotic figures are present. (Hematoxylin and eosin stain).

distinct clusters separating the BCC and RMS samples (Figure 4). All of the BCC samples had losses of the entire chromosome 13 (the site of the *Ptch1* gene) as well as of distal chromosome 4. In addition, three BCCs, two from *Blm*^{+/-tm3Brd} mice and one from a *Blm*^{tm3Brd/tm3Brd} mouse each had copy number loss at the *Trp53* locus on chromosome 11. Losses involving portions of chromosome 10 were also present in three BCCs and included *Gli1*, one of the transcription factors mediating hedgehog signaling, in one case. In contrast, a gain of chromosome 10, including *Gli1*, was present in all five RMS tumors from *Ptch1*^{+/-} mice with *Blm*^{+/-tm3Brd} or *Blm*^{tm3Brd/tm3Brd} genotypes but not in the one RMS tumor arising in a *Ptch1*^{+/-} *Blm*^{+/+} animal. The latter RMS harbored focal deletions at distal chromosomes 6 and 12. One of two RMS tumors arising in *Ptch1*^{+/-} *Blm*^{tm3Brd/tm3Brd} mice had partial loss of chromosome 13 including the *Ptch1* locus. Overall, the differences in CGH-detectable changes according to *Blm* genotype did not reach statistical significance (i.e. not <0.05). However, the fraction of the genome altered was higher in the BCCs from *Ptch1*^{+/-} *Blm*^{tm3Brd/tm3Brd} mice (0.22 and 0.24) than was the fraction in tumors from the *Ptch1*^{+/-} *Blm*^{+/-tm3Brd} or *Blm*^{+/+}

mice (0.11, 0.13, 0.13 and 0.16), although the difference could not be statistically tested due to inadequate sample size. This finding suggests a possible contribution of *Blm* haploinsufficiency, perhaps via enhancement of *Trp53* loss (present in three of these four tumors), to copy number genomic instability in BCCs. A similar analysis was not done for RMS because only one tumor with *Blm* wild-type genotype was analyzed.

Discussion

We found that *Blm* deficiency reduced the survival of *Ptch1*^{+/-} mice, largely via significantly enhanced RMS tumor formation, with reduced tumor latency and increasing tumor number. *Blm* deficiency also significantly enhanced the development of microscopic BCC-like tumors, albeit not of visible BCCs. Similar to our findings of mutant *Blm* alleles enhancing tumorigenesis in *Ptch1*^{+/-} mice, mutant *Blm* alleles have been reported to enhance by 2- to 3-fold intestinal tumor formation in *Apc*^{+/-min} mice but, as in our study, did not alter the histology of the tumors (19,20). Similarly, *Blm*^{tm3Brd/tm3Brd} mice

Fig. 3. Characteristics of microscopic BCC-like tumors in *Ptch1*^{+/-} mice of differing *Blm* genotypes. (a) Tumor size. Mean size of BCC-like tumors was higher in *Blm*-deficient mice compared with wild-type mice. The difference was significant between *Blm*^{tm3Brd/tm3Brd} and *Blm*^{+/+} mice (*P value: 0.01). (b) BCC-like tumor number. *Blm*-deficient mice developed significantly more BCC-like tumors compared with *Blm*^{+/+} mice (*P value: 0.006 and 0.0003, respectively, for *Blm*^{+/-} and *Blm*^{tm3Brd/tm3Brd}). (c) BCC-like tumor area. A larger BCC-like tumor involved area was observed in *Blm*-deficient mice. The difference was considerable between *Blm*^{+/+} and *Blm*^{tm3Brd/tm3Brd} mice (*P value: 0.0004) as well as *Blm*^{+/-} and *Blm*^{tm3Brd/tm3Brd} mice (*P value: 0.01). Data are shown as mean ± SD.

develop far more hematologic malignancies than do *Blm* wild-type mice, an increase similar to that induced by ionizing radiation, and the incidence is enhanced in a multiplicative fashion by the two (26). Thus, genetically engineered mice with mutated *Blm* alleles develop cancers of the same types as occur in *Blm*^{+/-} mice. Similarly, human Bloom syndrome patients have an abnormally high incidence of cancers, and of 100 such cancers in 168 persons, the range of tumor types was not significantly different from that in sporadic cases (27). One

Fig. 4. Hierarchical clustering of BCC and RMS samples. Columns represent individual tumor samples. Rows represent individual genome probes (bacterial artificial chromosome and P1 clones) that are ordered from chromosome 1 to 19 and then chromosome X from proximal to distal. Genome copy number losses are indicated in red and copy number gains are indicated in green. Yellow dots represent high-level genome amplifications. The positions of some hedgehog pathway genes are indicated on the right of the heatmap. The *Blm* genotypes of the samples are indicated by +/+ = wild-type, +/- = *Blm*^{+/-}*tm3Brd* and -/- = *Blm*^{tm3Brd/tm3Brd}.

Bloom syndrome patient with many BCCs has been reported (28), but no data on *BLM* sequences in BCCs have been published. Patients heterozygous for a mutant *BLM* allele have been reported to have an increased incidence of cancers of various types (29,30). Hence, *Blm* appears to act as a 'caretaker' with respect to many types of cancers as opposed to a 'gatekeeper' whose loss enhances the incidence of a more limited range of cancers.

Age and hair cycle phase at irradiation time point can affect murine *Ptch1*^{+/-} BCC carcinogenesis, with increased BCCs if ionizing radiation is given specifically during anagen (31). In our study, we did not control specifically for follicular phase.

Bloom syndrome cells have an increased frequency of somatic mutations (32,33), micronucleus formation (34) and homologous recombination (35). Chromosome instability in this syndrome is characterized by a striking tendency for spontaneous exchanges between DNA strands. These exchanges occur either within chromosomes, termed sister-chromatid exchanges or between chromosomes at homologous sites (36). Sister-chromatid exchanges, the genetic hallmark of Bloom syndrome, cause chromosomal rearrangements such as duplications, deletions and translocations if they occur either unequally using identical sequences or between non-identical repeat sequences (37). The somatic recombination leads to homozygosity, hemizygosity or partial loss of the expression, e.g. of tumor suppressor genes in Bloom Syndrome cells (33). Mitotic recombination in Bloom Syndrome cells that are heterozygous for tumor suppressor genes may result in daughter cells homozygous or hemizygous for a mutant allele (38,39), and this increased tendency for loss of wild-type alleles may explain at least part of the cancer predisposition in Bloom syndrome patients (40).

Blm appears to function primarily in DNA repair, especially in reducing error-prone homologous recombination, e.g. following double-strand DNA breaks. Blm protein localizes to foci at double-strand DNA breaks with p-ATM, p-Chk1 and 53BP1 and is capable of binding directly to 53BP1 and Chk1(41). Cells lacking Blm protein appear to have increased activation of their endogenous DNA damage response with increased numbers of the latter such foci (42), and this finding suggests that Blm protein normally participates in repair of DNA damage and that its absence delays such resolution.

Like the *Blm* gene, hypomorphic alleles of the gene encoding another enzyme particularly active in double-strand DNA breakage repair, the Nijmegen breakage syndrome gene (*Nbs1*^{ΔB}), also induced relatively little qualitative change in intestinal tumorigenesis in *Apc*^{min/+min} mice. Although they did not test for chromosomal instability, the authors of that study hypothesized that reduction in *Nbs1* activity may not lead to genetic instability in epithelial cells of the intestine. (43). Indeed, knockdown of Blm protein expression in human colorectal carcinoma cell lines did not cause chromosomal aberrations (35,44). These observations are consistent with our findings of a lack of extensive DNA copy number aberrations in either BCC or RMS tumors in our *Ptch1*^{+/-} mice with mutant *Blm* alleles (Figure 3); however, CGH does not detect all types of genome instability.

The BCC and RMS tumors differed markedly in their spectra of copy number alterations, including loss of chromosomes 4 and 13 in all BCCs and gain of chromosome 10 in 80% of RMS tumors. In addition, losses of chromosomes 10 and 11, including the *Gli1* and *Trp53* loci, respectively, were observed in 50% of BCCs (Figure 3). Since *Gli1* is one of the transcriptional effectors of hedgehog signaling, selection for its loss in BCCs would not be expected. Indeed, in only one of the three cases was the entire chromosome lost, in the other two *Gli1* was excluded from the large minimal region of deletion defined by the extent of the copy number loss. The marked difference we found in loss of chromosome 13, the site of the murine *Ptch1* gene, between BCC and RMS tumors is consistent with past findings of frequent, albeit not uniform, retention of the wild-type gene in RMS tumors. Some evidence suggests that in such RMS tumors with both wild-type and mutant alleles, most of the expressed *Ptch1* messenger RNA is encoded by the mutant allele, perhaps reflecting epigenetic silencing of the wild-type allele (45,46).

A role for Blm protein in maintaining genomic integrity is certain, but the mechanism by which *Blm* loss enhances BCC and RMS tumorigenesis in *Ptch1*^{+/-} mice and the connection between genomic stability and tumor behavior remain open to conjecture (47,48).

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