Induction of Renal Tumorigenesis with Elevated Levels of Somatic Loss of Heterozygosity in Tsc1+/− Mice on a Blm-Deficient Background

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Abstract

A Bloom’s deficient mouse model (Blm+/m3/m3) has been shown to induce colorectal tumorigenesis when crossed with Apc+/−/Min mice. Here, we investigated whether the Blm+/m3/m3 genotype could induce tumorigenesis in extracolonic tissues in tuberous sclerosis 1–deficient (Tsc1+/−) mice that are predisposed to renal cystadenomas and carcinomas. Genotyping of offspring from Tsc1+/−/Blm+/m3/m3 intercrosses showed that a ~24% excess of Tsc1+/− over Tsc1+/− mice died before weaning (P = 0.016), although Blm deficiency had no cumulative effect on Tsc1+/− survival. Tsc1+/−/Blm+/m3/m3 mice had significantly more macroscopic and microscopic renal lesions at 3 to 6 months compared with Tsc1+/−/Blm+/m3/m3 mice (P < 0.0003 and 0.0203, respectively), and their tumors showed significantly increased levels of somatic loss of heterozygosity (LOH) of the wild-type Tsc1 (Tsc1wt) allele compared with those from Tsc1+/−/Blm+/m3/m3 mice (P < 0.0001). Tsc1+/−/Blm+/m3/m3 mice did not show significantly more renal lesions compared with Tsc1+/−/Blm+/m3/m3 animals; however, their lesions still showed significantly increased levels of somatic LOH of the Tsc1wt allele (P = 0.03). Ninety-five percent (19 of 20) of lesions from Tsc1+/−/Blm+/m3/m3 mice retained the wild-type Blm (Blmwt) allele, indicating that the increased somatic LOH at Tsc1 was mediated by Blm haploinsufficiency. Renal lesions from a Blm-deficient background generated significantly more mitotic recombination on chromosome 10 compared with those from a normal Blm background (P < 0.05). Immunohistochemistry using anti-phospho-S6 ribosomal protein (Ser240/244) suggested that these lesions develop through the normal pathway of Tsc-associated tumorigenesis. This work shows the use of the Blm+/m3/m3 mice for inducing renal tumorigenesis, and the high levels (~87%) of LOH in the resultant tumors will help facilitate mapping of loci involved in tumor progression. (Cancer Res 2005; 65(22): 10179-82)

Introduction

Bloom syndrome is a unique hereditary cancer predisposition disorder in which patients are predisposed to the development of many cancer types (1). The Bloom syndrome gene, BLM, encodes an ATP-dependent, 3′ to 5′ helicase with homology to RecQ DNA helicase (2). BLM resolves Holliday junctions, suppresses recombination in vitro, and is required for the fidelity of DNA double-strand break repair (3–5). Loss of BLM activity causes genomic instability characterized by an elevated frequency of the sister chromatid exchange (6–9). Recently, several investigators have exploited the high rate of mitotic recombination in Blm-deficient embryonic stem cells to allow powerful recessive genetic screens to be carried out in vitro (10, 11). Furthermore, Luo et al. (12) have generated a mouse model of the human syndrome (Blm+/m3/m3) that is prone to a wide variety of cancers and can be used to induce colorectal tumorigenesis when crossed onto an Apc+/−/Min background. Here, we investigated whether Blm+/m3/m3 mice could induce tumorigenesis in extracolonic tissue by crossing with tuberous sclerosis 1–deficient (Tsc1+/−) mice that are predisposed to renal cystadenomas and carcinomas (13). Renal tumors develop in Tsc1+/− mice according to Knudson’s “two-hit” hypothesis with somatic inactivation of the Tsc1wt allele required for tumor development (13). Normally, a diverse range of mechanisms (including nonsense, frameshift and deletion mutations, and epigenetic silencing) are implicated in the somatic inactivation of the wild-type allele of tumor suppressor genes, and this diversity of somatic alterations makes genetic mapping of loci involved in tumor progression problematic. We hypothesized that by crossing Tsc1+/− mice onto a Blm-deficient background, we would increase the frequency of somatic loss of heterozygosity (LOH), thereby providing a convenient means of directly mapping genes involved in Tsc-associated tumorigenesis.

Materials and Methods

Animal care, genotyping, necropsy, and pathology. All procedures with animals were carried out in accordance with Home Office guidelines. Tsc1+/− mice on a BALB/c background (backcross, N ≥ 3; ref. 13) were crossed with Blm+/m3/m3 mice on a C57BL6 background (12) and Tsc1+/−/Blm+/m3/m3 offspring were intercrossed to generate mice for subsequent analyses. DNA was extracted from mouse tail tips using the Qiagen (Chatsworth, CA) QIAamp DNA mini kits and genotyping was done as previously described (12, 13). Necropsy analysis included macroscopic examination of the brain, heart, lungs, kidneys, liver, spleen, and uterus in 20 Tsc1+/−/Blm+/m3/m3 and 20 Tsc1+/−/Blm+/m3/m3 mice at 3 to 6 and 9 to 12 months. Half of each organ was fixed and processed into paraffin wax, sectioned at 4 μm, and stained with H&E for microscopic inspection (tissues from 10 mice per genotype were analyzed microscopically at 3-6 and 9-12 months). Estimations of the average number of kidney lesions per mouse were determined as previously described (13). The other half of the organs were snap-frozen in liquid nitrogen–cooled isopentane for laser capture microdissection (LCM).

Immunohistochemistry. Immunohistochemistry using anti-phospho-S6 ribosomal protein (Ser240/244; Cell Signaling Technologies, Beverly, MA) was done as previously described (13).

Somatic mutation analysis. Snap-frozen tissue was sectioned at 10 μm onto PEN (PALM) membrane covered slides and stained with toluidine blue. Tumor and normal tissue was microdissected (PALM LCM) and DNA extracted using the QIAamp DNA micro kits (Qiagen). LOH at the Tsc1 locus was assayed as previously described (13), using wild type/mutant peak ratios of <0.67 or ≥1.3 as indicative of LOH (14). To search for somatic intragenic Tsc1 mutations, the entire Tsc1 open reading frame (ORF) was

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amplified as 23 fragments (primer sequences are available upon request) and sequenced directly. LOH at the *Bln* locus was assayed by amplifying and directly sequencing the region encompassing the variant 4109-152 G>A, located on intron 18 of the *Bln* gene,1 using the primers rs8248591/2F 5′-ACAGTTCTGAGAGGGCTCA-3′ and rs8248591/2R 5′-CTAGCTTTCA-CAGGCCACT-3′. LOH of the *Blm* allele was determined by visual inspection of the 4109-152 G>A variant on the sequencing chromatograms by two independent investigators.

**Simple sequence length polymorphism markers flanking Tsc1.** Six highly polymorphic simple sequence length polymorphism (SSLP) markers flanking *Tsc1* (D2Mit194, D2Mit651, D2Mit652, D2Mit63, D2Mit433, and D2Mit431) were analyzed in 18 and 14 mice with LOH of the *Tsc1* allele from *Tsc1*/— *Bln*<sup>m3/m3</sup> and *Tsc1*/— *Bln*<sup>wt</sup> mice, respectively. Amplification of DNA from tumors was done as previously described (13), using a 25-cycle PCR reaction. Two microliters of PCR products were mixed with an ABI 5000 internal size standard and formamide loading buffer and run on an ABI3100 genetic analyzer. Results were analyzed using Genescan v.3.7 software, and LOH was determined as previously described (14).

**Statistical analysis.** Comparisons of numbers of mice were done using the χ² test. Lesion counts per mouse were compared using the Mann-Whitney confidence interval tests, and the frequency of LOH was compared using Fisher’s exact test.

**Results and Discussion**

**Blm deficiency has no effect on early *Tsc1*/— mortality.** *Tsc1*/— *Bln*<sup>m3/m3</sup> mice were intercrossed. Genotyping showed that of the 360 offspring that survived until weaning, 204 were *Tsc1*/— and 134 were wild type (22 failed to be genotyped), indicating that there was no cumulative effect on early mortality for both *Tsc1* and *Blm* deficiency.

**Induction of renal tumors in *Tsc1*/— mice on a *Blm*-deficient background.** We examined the kidneys of mice at 3 to 6 months by microscopic inspection and microscopic analysis of five sections ~200 μm apart. We observed a significant increase in renal tumors in *Tsc1*/— mice on a *Blm*-deficient background: 70% (14 of 20) of *Tsc1*/— *Bln*<sup>m3/m3</sup> mice developed macroscopically visible renal lesions (average of 4.2 lesions per mouse) compared with only 15% (3 of 20) of *Tsc1*/— *Bln*<sup>wt</sup> mice (average of 0.2 lesions per mouse; P = 0.0016; Table 1; Fig. 1). At a microscopic level, *Tsc1*/— *Bln*<sup>m3/m3</sup> mice had an average of 23.2 lesions per mouse compared with 5.4 for *Tsc1*/— *Bln*<sup>wt</sup> mice (P = 0.00203). Renal lesions from *Tsc1*/— *Bln*<sup>m3/m3</sup> mice stained positively with anti-phospho-S6 ribosomal protein (Ser<sup>240/244</sup>), indicating that these lesions develop through the normal pathway of *Tsc1*-associated tumorigenesis (13).

**Somatic mutation analysis of *Tsc1* and mechanism of loss of heterozygosity.** DNA was extracted from LCM kidney cystadenomas and carcinomas and analyzed for LOH at the *Tsc1* locus using an IRES (mutant specific) and exon 8 (wild type specific) quantitative PCR assay, as previously described (13). We observed significantly increased levels of somatic LOH of the *Tsc1*<sup>wt</sup> allele in renal lesions from *Tsc1*/— *Bln*<sup>m3/m3</sup> mice (53 of 61, 87%) compared with renal lesions from *Tsc1*/— *Bln*<sup>wt</sup> mice (61 of 118, 52%; P < 0.0001; Fig. 2). To determine the mechanism of increased somatic LOH in these lesions, we analyzed the extent of the LOH events using six SSLP markers on mouse chromosome 2, on which *Tsc1* resides. Two of 18 tumors showing loss of the *Tsc1*<sup>wt</sup> allele from *Tsc1*/— *Bln*<sup>m3/m3</sup> mice remained heterozygous at the marker D2Mit431 located ~5 cm upstream of *Tsc1*, whereas no tumors with loss of the *Tsc1*<sup>wt</sup> allele from *Tsc1*/— *Bln*<sup>wt</sup> mice retained heterozygosity at any of the markers tested. This suggested that the LOH on a *Bln*-deficient background was mediated by mitotic recombination in agreement with Luo et al. (12). We sought intragenic somatic *Tsc1* mutations in DNA from the eight tumors from *Tsc1*/— *Bln*<sup>m3/m3</sup> mice that retained the *Tsc1*<sup>wt</sup> allele, by direct sequence analysis of the entire *Tsc1* ORF. We generated high-quality sequencing for ≥91% of the *Tsc1* ORF for each tumor and identified a single intragenic somatic mutation, characterized as a 2-bp deletion in exon 22 (Tsc1 c.2872_2873 delTG). Therefore, in total, we identified ~89% (54 of 61) of second-hit mutations in *Tsc1*/— *Bln*<sup>m3/m3</sup> mice and clearly show that LOH mediated by mitotic recombination is the major mechanism of somatic mutagenesis in these animals. Further studies are warranted to determine the nature of the somatic mutations in the 11% of tumors from *Tsc1*/— *Bln*<sup>wt</sup> mice without LOH or coding region variations.

**Do *Tsc1*/— *Bln*<sup>m3/m3</sup> mice show increased tumor burden?** Goss et al. (15) showed that mice heterozygous for a targeted null mutation of *Bln* developed lymphoma earlier than wild-type littermates when challenged with murine leukemia virus and developed twice the number of intestinal tumors when crossed with *Apc*/—/*Bln* mice. Furthermore, Gruber et al. (16) showed that human carriers of a *BLM* mutation have an increased risk for colorectal cancer. In contrast, we did not observe significantly more renal lesions in *Tsc1*/— *Bln*<sup>m3/m3</sup> mice at 3 to 6 months when compared with *Tsc1*/— *Bln*<sup>wt</sup> mice that had previously been backcrossed (N > 3) onto two different backgrounds (BALB/c and C57BL/6; ref. 13). However, we did observe significantly increased levels of somatic LOH of the *Tsc1*<sup>wt</sup> allele in renal lesions from *Tsc1*/— *Bln*<sup>m3/m3</sup> mice (23 of 32, 71%; P = 0.03). To determine whether tumors from *Tsc1*/— *Bln*<sup>m3/m3</sup> mice had lost the *Bln*<sup>wt</sup> allele, we analyzed the polymorphism 4109-152 G>A, which lies within intron 18 of the *Bln* gene. Nineteen of 20 (95%) renal lesions from *Tsc1*/— *Bln*<sup>m3/m3</sup> mice that had lost the *Bln*<sup>wt</sup> allele, and were informative for this marker retained the *Bln*<sup>wt</sup> allele, indicating that the increased somatic LOH at the *Tsc1* locus in these mice was mediated by *Bln* haploinsufficiency.

<table>
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<th>Type of analysis</th>
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<th>Mean no. of tumors per mouse</th>
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<td></td>
<td>m3/*m3</td>
<td>116</td>
<td>23.2</td>
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</tr>
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</table>

NOTE: Numbers are based on the analysis of 20 *Tsc1*/— mice for macroscopic inspection and 10 *Tsc1*/— mice for microscopic inspection.
McDaniel et al. (17) recently showed that the Blm<sup>m3/m3</sup> allele is in fact hypomorphic and expresses a low level of the Blm protein. This may explain our failure to find significantly increased tumor burden in Tsc1<sup>+/−</sup> Blm<sup>m3/m3</sup> mice. However, our results indicate that the previously reported tumor predisposition associated with Blm haploinsufficiency (15, 16) may be mediated by increased somatic LOH.

**Does the nature of the second hit influence the spectrum and distribution of Tsc-associated lesions?** We examined Tsc1<sup>+/−</sup> Blm<sup>m3/m3</sup> and Tsc1<sup>+/−</sup> Blm<sup>m3/m3</sup> mice at 9 to 12 months and compared these with Tsc1<sup>+/−</sup> Blm<sup>+/m3</sup> mice to see whether the increased frequency of LOH at Tsc1 in the Blm-deficient animals influenced the spectrum and distribution of their tumors. Although Blm<sup>m3/m3</sup> mice are prone to a wide variety of cancers, the incidence of lesions is very low (3%) before 1 year (12). Renal tumors were again more frequent in Tsc1<sup>+/−</sup> Blm<sup>m3/m3</sup> mice compared with Tsc1<sup>−/−</sup> Blm<sup>m3/m3</sup> mice (P = 0.0027). Consistent with the increased numbers of tumors noted at 3 to 6 months, Tsc1<sup>+/−</sup> Blm<sup>m3/m3</sup> mice had a higher proportion of more advanced renal tumors (with a solid to cystic tumor ratio of 1:5) compared with Tsc1<sup>+/−</sup> Blm<sup>+/m3</sup> mice (ratio of 1:1.5), with a single renal lesion metastasizing to the lungs.

The spectrum and distribution of extrarenal tumors in Tsc1<sup>+/−</sup> mice on Blm-proficient (Blm<sup>+/+</sup>), haploinsufficient (Blm<sup>m3/m3</sup>), and deficient (Blm<sup>m3/m3</sup>) backgrounds was not significantly different to Tsc1<sup>+/−</sup> mice on a Blm<sup>+/+</sup> background. Only an isolated liver hemangioma was observed in a single Tsc1<sup>+/−</sup> Blm<sup>m3/m3</sup> mouse, and no other lesions were seen after macroscopic and microscopic examination of the brain, heart, lungs, spleen, and uterus. Renal lesions have also been induced in other Tsc1<sup>−/−</sup> Blm<sup>m3/m3</sup> mice (18) by using chemical mutagens, which cause a high frequency of somatic point mutations on the wild-type Tsc1 allele in the tumor (bottom) but not in adjacent normal tissue (top). WT, wild-type allele; Mut, mutant allele. Unshaded trace, an ABI GS500 internal size standard. B, % lesions with LOH versus those without LOH from Tsc1<sup>+/−</sup> Blm<sup>+/+</sup>, Tsc1<sup>−/−</sup> Blm<sup>m3/m3</sup>, and Tsc1<sup>−/−</sup> Blm<sup>m3/m3</sup> mice. P<sub>i</sub> reflect differences in the frequency of LOH in tumors from Tsc1<sup>+/−</sup> Blm<sup>m3/m3</sup> and Tsc1<sup>−/−</sup> Blm<sup>m3/m3</sup> mice compared with Tsc1<sup>−/−</sup> Blm<sup>+/+</sup> mice.

The spectrum and distribution of extrarenal tumors in Tsc1<sup>−/−</sup> mice on Blm<sup>m3/m3</sup> or Blm<sup>+/m3</sup> backgrounds was not significantly different to Tsc1<sup>−/−</sup> mice on a Blm<sup>+/+</sup> background. Only an isolated liver hemangioma was observed in a single Tsc1<sup>−/−</sup> Blm<sup>m3/m3</sup> mouse, and no other lesions were seen after macroscopic and microscopic examination of the brain, heart, lungs, spleen, and uterus. Renal lesions have also been induced in other Tsc1<sup>−/−</sup> Blm<sup>m3/m3</sup> mice (18) by using chemical mutagens, which cause a high frequency of somatic point mutations on the wild-type allele, and consistent with our observations, these animals do not seem to develop any unusual extrarenal tumors. These data indicate that the nature of the second hit does not affect the spectrum and distribution of extrarenal lesions in Tsc1<sup>−/−</sup> mice.

In conclusion, our study confirms the use of the Blm<sup>m3/m3</sup> mouse for inducing tumorigenesis in cancer-prone mouse models. The high levels of LOH in the resultant tumors (and consequently low level of intragenic mutations) will help facilitate the mapping of loci that may play a role in tumor progression.

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References


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