Germline Mutation of Bap1 Accelerates Development of Asbestos-Induced Malignant Mesothelioma

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Abstract

Malignant mesotheliomas are highly aggressive tumors usually caused by exposure to asbestos. Germline-inactivating mutations of BAPI predispose to mesothelioma and certain other cancers. However, why mesothelioma is the predominate malignancy in some BAPI families and not others, and whether exposure to asbestos is required for development of mesothelioma in BAPI mutation carriers are not known. To address these questions experimentally, we generated a Bap1+/− knockout mouse model to assess its susceptibility to mesothelioma upon chronic exposure to asbestos. Bap1+/− mice exhibited a significantly higher incidence of asbestos-induced mesothelioma than wild-type (WT) littermates (73% vs. 32%, respectively). Furthermore, mesotheliomas arose at an accelerated rate in Bap1+/− mice than in WT animals (median survival, 43 weeks vs. 55 weeks after initial exposure, respectively) and showed increased invasiveness and proliferation. No spontaneous mesotheliomas were seen in unexposed Bap1+/− mice followed for up to 87 weeks of age. Mesothelioma cells from Bap1+/− mice showed biallelic inactivation of Bap1, consistent with its proposed role as a recessive cancer susceptibility gene. Unlike in WT mice, mesotheliomas from Bap1+/− mice did not require homozygous loss of Cdkn2a. However, normal mesothelial cells and mesothelioma cells from Bap1+/− mice showed downregulation of Rb through a p16(Ink4a)-independent mechanism, suggesting that predisposition of Bap1+/− mice to mesothelioma may be facilitated, in part, by cooperation between Bap1 and Rb. Drawing parallels to human disease, these unbiased genomic findings indicate that BAPI mutation carriers are predisposed to the tumorigenic effects of asbestos and suggest that high penetrance of mesothelioma requires such environmental exposure.

Cancer Res; 74(16); 4388–97. ©2014 AACR.

Introduction

Malignant mesotheliomas are a highly aggressive, treatment-resistant cancer (1) causally related to asbestos exposure (2–4). Even three decades after peak commercial use of asbestos in the United States, about 3,200 malignant mesothelioma cases are diagnosed here annually (5), and deaths because of malignant mesothelioma are expected to increase by 5% to 10% per year in Europe over the next 25 years (4, 6). Moreover, a marked increase in malignant mesothelioma is predicted in developing countries, where use of asbestos is increasing exponentially (7).

Some individuals develop malignant mesothelioma following exposure to small amounts of asbestos, whereas others exposed to heavy amounts do not (6), suggesting that genetic factors influence risk of this disease. In 1998, Jensen and colleagues identified a novel protein, BRCAl-associated protein 1 (BAP1), a nuclear-localized deubiquitylase, and intragenic homozygous rearrangements and deletions of BAP1 were found in several lung carcinoma cell lines (8). More than a decade later, frequent somatic BAPI mutations were reported in metastatic uveal melanoma, with one mutation observed in germline DNA (9). Shortly thereafter, recurring somatic mutations of BAPI were reported in malignant mesothelioma (10–12). Indeed, the high incidence (~25%–60%) of somatic BAPI mutations reported in malignant mesothelioma implicates defects of this putative tumor suppressor gene along with frequent alterations of CDKN2A (13, 14), which encodes the tumor suppressors p16(INK4A) and p14(ARF), as well as NF2 (15, 16) as the main drivers of malignant mesothelioma tumorogenesis identified to date.

Importantly, in 2011, germline BAPI mutations were found in two families with multiple malignant mesotheliomas (one also having two uveal melanomas) as well as in two sporadic cases affected by both malignant mesothelioma and uveal melanoma (11), the first demonstration that a hereditary defect can affect risk of malignant mesothelioma. Concurrently, Wiesner and...
colleagues described germline BAPI mutations in two families with atypical melanocytic tumors, uveal melanoma and cutaneous melanoma (17), and similar findings were reported by others (18). Collectively, the observations in these high-risk cancer families have led investigators to propose that germline BAPI mutations cause a novel cancer susceptibility syndrome characterized by a high incidence of malignant mesothelioma, uveal melanoma, cutaneous melanoma, and probably additional cancers (19, 20). The latter possibility is now certain, based on the subsequent identification of multiple BAPI families with one or more renal cell carcinomas (21, 22). Other studies suggest that the clinical phenotype of the BAPI syndrome may be expanded to other cancers such as lung adenocarcinoma, meningioma, breast carcinoma, and paraganglioma (23–25).

We have hypothesized that when families with germline BAPI mutations are exposed to asbestos, malignant mesothelioma may represent the predominant cancer observed (11). However, it is noteworthy that members of neither of the malignant mesothelioma families we reported had any obvious occupational exposure to asbestos, and only traces of asbestos were found in their homes. Similarly, Wiens et al. (2012) reported a germline BAPI mutation in a European family with four malignant mesotheliomas, none with any known exposure to asbestos (26). Thus, it is possible that exposure to carcinogenic mineral fibers may not be required for development of malignant mesothelioma in BAPI mutation carriers. To test this hypothesis experimentally, mouse models would be invaluable. Although conditional, whole-body (except brain) homozygous deletion of Bap1 in adult mice recapitulates features of human myelodysplastic syndrome (MDS; ref. 27), studies of germline heterozygous mutation of Bap1 have not been described to date. We report here the generation of a Bap1–/– knockout mouse model used to assess predisposition to asbestos-induced malignant mesothelioma. Although no spontaneous malignant mesotheliomas or MDS were seen in unexposed Bap1–/– mice followed for up to 20 months of age, such haploinsufficient mutant mice exhibited a markedly higher incidence and accelerated onset of asbestos-induced malignant mesothelioma when compared with wild-type (WT) littermates. Mechanistically, we also demonstrate that predisposition of Bap1+/– mice to malignant mesothelioma may be facilitated, in part, by cooperation between Bap1 and p16(ink4a)-independent epigenetic inactivation of Rb.

Materials and Methods

Generation of Bap1 knockout mice

Bap1 knockout mice in FVB genetic background were developed by zinc finger nucleases (ZFN) technology (28, 29), with the assistance of the Fox Chase Cancer Center (FCCC) Transgenic Mouse Facility, directed by Dietmar Kappes. Custom ZFNs targeting Bap1 were designed and validated in mammalian cells by Sigma-Aldrich. Binding and cutting site of the ZFNs were TGCTCCCCCGTACGCTGAAGGGTGGCGCAGC, with spacer between binding sites underlined. ZFN expression plasmids were linearized at the XbaI site located at the 3' end of the Fok1 ORF. 5' capped and 3' poly(A)-tailed message RNAs were prepared using MessageMAX T7 ARCA-Capped Message Transcription (Cellscript) and Poly(A) Tailing Kits (Epigenetix) and purified using an Ambion MEGAClear Kit. The ZFN mRNAs were combined, microinjected into FVB blastocysts, which were implanted into pseudopregnant female mice. A schematic diagram showing the cutting site of the ZFNs and a portion of the Bap1 knockout allele are depicted in Fig. 1A. Tail sections resulting pups were genotyped by PCR amplification and sequencing to verify correct targeting (Fig. 1B). A Bap1 knockout allele with deletion of exons 6 and 7 was identified, which results in a frameshift and predicted premature truncation of the Bap1 protein. The net effect is similar, but not identical, to that observed in a human family having an intron 6 splice site mutation in BAPI that results in loss of exon 7 (11).

All mouse studies were performed according to NIH’s Guide for the Care and Use of Laboratory Animals. The FCCC Committee on the Ethics of Animal Experiments approved the protocol for studies using asbestos.

Genotyping

Tail DNA samples were obtained through digestion and purification with a Gentra DNA Extraction Kit (Qiagen). The genotyping primers were as follows: forward, 5’-AGGGTTTGCTGCTAAAATGAGA-3’; reverse, 5’-CCCTGAGACCCAGAAAATCA-3’. PCR cycling conditions were: 95°C (5 minutes), followed by 35 cycles at 95°C (30 seconds), 58°C (30 seconds), 72°C (45 seconds), and 72°C (10 minutes). PCR products were resolved on gels and purified for DNA sequence analysis.

Asbestos injections

To assess susceptibility of Bap1+/– mice to the tumorigenic effects of asbestos, animals were injected intraperitoneally (i.p.) with UICC crocidolite (SPI Supplies) per our usual method (30–32). Representative examples of scanning electron microscopy (SEM) images of crocidolite fibers, and graphs illustrating fiber length distribution, are depicted in Supplementary Fig. S1. Briefly, male Bap1+/– and WT littermate mice (25 per arm) at 8 to 10 weeks of age were injected i.p. with 800 μg/0.5 mL of crocidolite fibers in PBS every 21 days for a total of 4 injections (total, 3.2 mg/mouse). Mice were examined daily and sacrificed upon evidence of difficulty in breathing, severe weight loss, or when tumor burden was otherwise obvious. Upon detection of illness, mice were sacrificed by CO2 asphyxiation, and internal organs were harvested and fixed in formalin for pathologic analysis. When available, ascitic fluid was collected to generate tumor cell cultures.
mesothelioma, immunohistochemistry (IHC) was performed for various markers of malignant mesothelioma, including calretinin, cytokeratin 18/19, vimentin, and WT1 using antibodies from Sigma and a mesothelin antibody from Santa Cruz. Following incubation of slides with the designated antibodies, detection was with biotinylated secondary antibodies. Sections were stained with DAB and counterstained with hematoxylin. To assess cell proliferation and apoptosis, IHC staining of tumors was performed with antibodies for Ki-67 (Dako) and cleaved caspase-3 (Cell Signaling), respectively. Tumors were also stained with a BAP1 antibody from Bethyl to evaluate the presence and localization of Bap1.

In malignant mesotheliomas with limited tissue available for IHC, reverse transcriptase-PCR (RT-PCR) analysis of tumor tissue and/or primary cell cultures was occasionally used. Mice were scored as having malignant mesothelioma if tumor cells exhibited a combination of three or more malignant mesothelioma markers, including mesothelin, E-cadherin, N-cadherin, and cytokeratin 18/19 as assessed by RT-PCR (30). Control Gapdh was used to assess template integrity.

Hematologic assessment of Bap1+/− mice

In several Bap1+/− mice not exposed to asbestos, hematologic assessment was performed on bone marrow and peripheral blood to rule out the possibility of MDS, a phenotype observed in Bap1 conditional knockout mice (27), or other hematologic disease. After sacrificing mice, bone marrow aspirates were obtained from femurs. Blood was collected for complete blood counts and cytologic evaluation.

Primary tumor and normal mesothelial cell cultures

Primary malignant mesothelioma cells were isolated from ascitic fluid and/or peritoneal lavage from Bap1+/− and WT mice as described (30). Cells were cultured and maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS, 2 mmol/L L-glutamine, and penicillin (50 units/mL)/streptomycin (50 µg/mL). Cell cultures used for molecular analyses were from passages ≤10. PCR analysis was carried out to confirm expression of mesothelial markers.

Primary mesothelial cells were obtained from 6- to 8-week-old Bap1+/− and WT mice that were not exposed to asbestos, according to the method of Bot and colleagues (33). Mesothelial cell cultures used for molecular analyses were from passages ≤3.

Immunoblotting

Immunoblots were prepared with 50 µg of protein/sample, as described (31). Primary antibodies against Bap1 (Bethyl; 1:1,000 dilution), Rb (BD Biosciences; 1:1,000 dilution), and β-actin (Santa Cruz; 1:1,000 dilution) were used. Appropriate secondary antibodies (anti-rabbit-, anti-mouse- and anti-goat-HRP; Santa Cruz) were used at a 1:2,000 dilution.

Figure 1. Targeting of Bap1 locus and induction of asbestos-induced malignant mesotheliomas in Bap1+/− and WT mice. A, schematic diagram showing intronic cutting site (arrow) of ZFNs targeting Bap1, which resulted in loss of exons (boxes) 6 and 7 in Bap1 knockout allele. Targeted region of Bap1 locus corresponds to a portion of the UCH domain of BAP1 protein shown at the top. ASXL1/2, additional sex combs like proteins 1 and 2 interaction domain; BRCA1, BRCA1 interaction domain; HBM, HCF1-binding motif; NLS, nuclear localization signal; UCH, ubiquitin C-terminal hydrolase; ULD, UCH57-like domain. B, genotyping of WT (+/+) and heterozygous (+/−) Bap1 mice. Size of WT allele is 634 bp, and size of mutant allele (arrow) is 158 bp. C, Kaplan–Meier survival curves showing increased susceptibility to malignant mesothelioma and shorter survival in asbestos-treated Bap1+/− mice than in asbestos-treated WT littermates. Survival differences were highly significant (P < 0.0001).
Array-based comparative genomic hybridization analysis

Array-CGH (aCGH) with Agilent 244K genomic DNA arrays was performed on asbestos-induced malignant mesotheliomas from Bap1+/– and WT mice as previously described (31). Briefly, genomic DNA was isolated, restriction enzyme digested, fluorescently labeled, purified, and hybridized to Agilent arrays. After scanning of chips on an Agilent scanner, data were extracted using Feature Extraction Software, and output was imported into CGH Analytics for DNA Copy Number Analysis (Agilent).

Cell viability assay

Malignant mesothelioma cells were seeded onto 96-well plates at a density of 2,000 cells/well. Cells were immediately treated with 20 μmol/L HLM006474, 5 μmol/L LY2835219, or DMSO vehicle for 72 hours. MTS reagent was added, and absorbance was determined at 490 nm as a read out of cell viability.

Immunoprecipitation and Ub-AMC assay

Whole cell lysates were extracted with NP40 lysis buffer. Bap1 antibody was precoated on protein A/G PLUS-Agarose (Santa Cruz) in binding buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.25% NP40, 2% BSA) at 4°C overnight. Five milligrams of each whole cell lysate was used for immunoprecipitation (IP) with precoated anti-Bap1 agarose beads in 2 mL reaction volume at 4°C for 2 hours. IP complex resins were washed with buffer three times, with final wash done in 1× ubiquitin 7-amido-4-methylcoumarin (Ub-AMC) assay reaction buffer. The Ub-AMC activity reaction was performed with IP complex resin in 1× Ub-AMC assay reaction buffer (20 mmol/L HEPES, pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA, 5 mmol/L DTT, and 0.05% [w/v] Tween-20) and 10 nmol/L Ub-AMC (total volume, 50 μL) and incubated at 25°C for 15 minutes. The resin was spun down, and 25 μL of supernatant was used for the Ub-AMC assay. Fluorescence was measured at excitation and emission wavelengths of 355 and 460 nm, respectively, with hydrolysis rate corrected for background signal (no enzyme).

5-Aza-2′-deoxycytidine treatment and methylated DNA immunoprecipitation assay

To assess epigenetic modification of the Rb1 gene in malignant mesothelioma cells, initially total Rb expression was evaluated by treatment of cells with 10 and 20 μmol/L of 5-Aza-2′-deoxycytidine (5-Aza-CdR) for 72 hours. Methylated DNA immunoprecipitation (MeDIP) was carried out according to the manufacturer’s protocol (Active Motif). For each sample, a 1 μL aliquot was used for PCR analysis of the Rb1 promoter, and nested PCR was carried out. Primers used in the first PCR were as follows: Forward: 5′-CTGTTTCTCTCCAGAGGGCCAC-3′, reverse: 5′-TACAAAA-TATGGAACGTCCC-3′ at 95°C (5 minutes) followed by 45 cycles of 95°C (30 seconds), 60°C (30 seconds), and 72°C (10 minutes). Primers used in the second PCR were as follows: Forward: 5′-ACGACGCAGCGAGAC-3′, reverse: 5′-TCAGTGGAACGTCCC at 95°C (5 minutes) followed by 45 cycles of 95°C (30 seconds), 60°C (30 seconds), and 72°C (10 minutes).

Results

Phenotype of Bap1 knockout mice

To assess spontaneous tumor formation, 27 Bap1+/– and 30 WT mice were followed long term. As reported previously (27), homozygous loss of Bap1 was found to be lethal in early embryogenesis (post conception day 7.5–8.5), whereas heterozygous Bap1+/– mice (genotyping shown in Fig. 1B) are viable with no obvious anatomical abnormalities. To date, only 1 spontaneous tumor, a squamous cell carcinoma (SCC) of mammary origin, has been detected in our Bap1+/– mice, although most of the remaining untreated animals are still only 11 to 20 months of age. Peripheral blood smears and complete blood counts of four 3-month-old Bap1+/– mice and three 18-month-old Bap1+/– mice (including the mouse with the SCC) were comparable to that observed in WT littermates. Bone marrow smears from the three 18-month-old Bap1+/– mice showed no evidence of MDS or other hematologic disease, and no macroscopic and microscopic abnormalities of the uvea, skin, mesothelial tissues, or other organs were observed except for the single SCC. To date, one tumor, a thymic lymphoma, has been observed in WT littermates.

Bap1+/– mice are predisposed to development of asbestos-induced malignant mesotheliomas

Bap1+/– mice and WT littermates were chronically injected i.p. with crocidolite asbestos fibers starting at 8 to 10 weeks of age. Animals were monitored continuously over 16 months. Abdominal swelling was observed in Bap1+/– mice as early as 20 weeks after the first injection of asbestos, whereas WT animals did not begin showing this effect until 27 weeks after the initial injection. Eventually, 90% of the asbestos-exposed Bap1+/– mice showed abdominal distention, and ~60% of these were found to have ascites when sacrificed. A few others had abdominal swelling because of intestinal distention. Almost all of these mice showed liver abnormalities, pancreatic fibrosis, intestinal adhesions, and thickenings of the peritoneum, mesentery, and dia- phragm. The Bap1+/– mice were found to succumb to disease earlier than their WT littermates, with a median survival of 43 weeks from the time of the first asbestos injection in Bap1+/– mice versus 55 weeks in WT mice (Fig. 1C; Wilcoxon 2-sample test, P < 0.0001). Deaths because of peritoneal malignant mesothelioma occurred in 73% of Bap1+/– mice that could be pathologically evaluated compared with only 32% of WT animals (Fisher exact test, P < 0.01). Other deaths in WT and Bap1+/– mice occurred mainly because of plaque-related organ failure or intestinal obstructions related to fibrosis. It is also noteworthy that although all asbestos-exposed Bap1+/– mice succumbed from malignant mesothelioma and other asbestos-related disease by 57 weeks, 5 (20%) of WT mice were still alive and asymptomatic at this time, 69 weeks after initial exposure to asbestos.
Asbestos-induced malignant mesotheliomas in Bap1+/− mice show increased aggressiveness

Examples of the histopathology of asbestos-induced malignant mesotheliomas from WT and Bap1 knockout mice are shown in Supplementary Fig. S2. Notably, asbestos-induced malignant mesotheliomas seen in Bap1+/− mice were consistently larger and more aggressive than those found in WT littermates, often with invasion to the pancreas, liver, and/or intestinal smooth muscle (Supplementary Fig. S3); occasional metastasis to the lungs was also observed in the Bap1+/− mice. Moreover, tumors in Bap1+/− mice were more proliferative based on Ki-67 staining (Supplementary Fig. S2). Immunohistochemical staining of tumors from both WT and Bap1+/− mice revealed strong staining for markers of malignant mesothelioma, such as mesothelin (Supplementary Fig. S4). Importantly, Bap1 staining was observed in malignant mesotheliomas from WT mice but was absent in malignant mesotheliomas from Bap1+/− mice (Supplementary Fig. S4).

Malignant mesothelioma cells from Bap1+/− mice show biallelic inactivation of Bap1 and Cdkn2a-independent downregulation of Rb

We were able to generate primary malignant mesothelioma cell cultures from ascites or peritoneal lavage of several Bap1+/− and WT mice, and multiple analyses were performed on early passage cells, as summarized below. Verification of the mesothelial origin of the cells was performed by semiquantitative RT-PCR using primers against three malignant mesothelioma markers: mesothelin, N-cadherin, and cytokeratin 18 (Fig. 2A). We also performed semiquantitative RT-PCR using primers against Bap1. All three malignant mesothelioma cultures derived from asbestos-exposed WT mice showed abundant expression of WT Bap1 mRNA, whereas malignant mesothelioma cells from Bap1+/− mice lacked expression of WT Bap1 but did retain expression of a truncated form of Bap1 mRNA (Fig. 2A). Sequence analysis of the RT-PCR products confirmed that the truncated Bap1 mRNA did not contain exons 6 and 7, consistent with its derivation from the germline mutant Bap1 allele. Western blot analysis demonstrated abundant expression of Bap1 protein in malignant mesotheliomas from WT mice but loss of Bap1 expression in malignant mesotheliomas from Bap1+/− mice. To ascertain why the tumor cells from the Bap1+/− mice did not show Bap1 protein expression, we designed PCR primers encompassing the Bap1 knockout site to assess whether there was loss of the WT Bap1 allele. PCR analyses on genomic DNA isolated from cells lacking Bap1 protein expression demonstrated that the WT allele of Bap1 was absent (Fig. 2B), consistent with loss of heterozygosity.

In addition, assessment by Ub-AMC assay revealed Bap1 deubiquitinating enzyme activity in malignant mesothelioma cells from WT mice but not in malignant mesothelioma cells from Bap1+/− mice (Fig. 2C). Intriguingly, aCGH analysis revealed that all three malignant mesothelioma cell cultures derived from WT mice had homozygous deletions of the Cdkn2a/Cdkn2b loci, whereas malignant mesothelioma cells from two Bap1+/− mice did not (Fig. 3A). Semiquantitative RT-PCR analyses confirmed that expression of Cdkn2a (p16Ink4a and p19Arf) and Cdkn2b (p15Ink4b) was absent in tumor cells from WT mice but present in cells from Bap1+/− mice (Fig. 2A). This result prompted us to examine the expression and phosphorylation status of the Rb tumor suppressor, because inactivation of Rb by hyperphosphorylation is the major downstream effect resulting from p16Ink4a loss. Interestingly, expression of Rb was strikingly decreased in malignant mesothelioma cells from Bap1+/− mice, which showed no expression of Bap1 protein, when compared with Bap1-expressing malignant mesothelioma cells from WT tumors that lacked expression of p16Ink4a (Fig. 3B).

To determine the reason why Rb phosphorylation was drastically reduced, we first assessed the expression of total Rb protein. Surprisingly, expression of total Rb protein was markedly decreased in tumor cells from Bap1+/− mice. Quantitative RT-PCR revealed that the mRNA expression levels of Rb1 in malignant mesothelioma cells from the Bap1+/− mice were less than half that of tumor cells from WT mice (Fig. 3B). Intriguingly, expression of Rb1 mRNA and Rb protein was also found to be downregulated in early passage (≤3) normal mesothelial cells isolated from Bap1+/− mice compared with those from WT mice (Fig. 3C), suggesting that reduced expression of Rb may contribute to the enhanced susceptibility to the carcinogenic effects of asbestos observed in Bap1+/− mice.

Aberrant expression of Rb in malignant mesothelioma cells from Bap1+/− mice occurs by epigenetic regulation

Because aCGH analysis did not uncover any DNA copy number changes at the Rb1 locus in any of the malignant mesothelioma cell cultures tested, we hypothesized that the decreased Rb1 expression was because of aberrant epigenetic modification of the Rb1 gene in malignant mesothelioma cells from Bap1+/− mice. In addition, BAP1 mutation and decreased BAP1 expression have been shown to be associated with increased methylation of PRC2 target genes (34), and GC-rich DNA can itself suffice to recruit PRC2 even in the absence of more complex DNA sequence motifs (35). An analysis of the Rb1 promoter sequence found in GenBank revealed a promoter CpG island containing 101 CpG and GpC dinucleotides within 300 bp upstream or downstream of the transcription initiation site of the Rb1 gene. Thus, reduced expression of Rb protein in tumor cells from Bap1+/− mice could be because of inactivation of PRC2, resulting from loss of the Bap1 protein. Indeed, we found that treatment of Bap1-null malignant mesothelioma cells with 5-Aza-Cdr significantly increased the expression of Rb (Fig. 4A). Moreover, analysis by MeDIP assay revealed that methylation modifications of the Rb1 promoter were markedly elevated in Bap1-null malignant mesothelioma cells compared with malignant mesothelioma cells from WT mice (Fig. 4B). Collectively, these findings suggest that the lower expression of Rb1 in Bap1-null malignant mesothelioma cells is because of hypermethylation of the Rb1 promoter.

To test whether Rb inactivation leads to increased proliferative capacity that might contribute to the malignant phenotype of malignant mesothelioma cells from Bap1+/− mice, the cells were treated with either an E2f inhibitor or a Cdk4/6 dual inhibitor (Fig. 4C). Interestingly, we found malignant mesothelioma cell cultures from both WT and Bap1+/− mice
were sensitive to the E2f inhibitor, but only the cells derived from WT mice were highly sensitive to the Cdk4/6 inhibitor. p16(Ink4a) is known to inhibit the activity of a complex composed by cyclin D, Cdk4, and Cdk6, leading to inactivation of Rb by phosphorylation, thereby abolishing binding between Rb and E2f, leading to activation of E2f target genes. Thus, an inhibitor of Cdk4/6 would be expected to block the inhibition of Rb, resulting in enhanced binding of Rb to E2f and to Rb and E2f, leading to activation of E2f target genes. Indeed, we found that WT tumor cells lacking expression of p16(Ink4a), but retaining expression of Bap1, were much more sensitive to the Cdk4/6 inhibitor than Bap1-null cells that retained expression of p16(Ink4a) (Fig. 4C). These results suggest that Bap1-deficient malignant mesothelioma cells are still dependent on E2f activation, but through a mechanism independent of p16 (Ink4a) loss.

**Discussion**

Although we have seen evidence of only a single spontaneous tumor in Bap1−/− mice to date, our mice are still <20 months of age at this time. Thus, we cannot rule out the possibility that spontaneous malignant mesotheliomas and various other tumors will arise as our mice age further. Germane to this, mice with heterozygous germine inactivation of Nf2, another tumor suppressor gene commonly mutated in malignant mesothelioma, developed a variety of spontaneous malignant tumors late in life (10–30 months of age); most animals died of osteosarcomas, with a median survival of 19 months in females and 23 months in males (36). Continued study of our cohort of untreated Bap1−/− mice will permit us to determine if older mice are predisposed to a particular spectrum of solid tumors or hematopoietic diseases, possibly including malignant mesothelioma or MDS, the latter reported in homozygous conditional Bap1 knockout mice (27).

Upon exposure to asbestos fibers, we found a high prevalence of malignant mesothelioma in Bap1−/− mice when compared with WT littermates. Importantly, malignant mesothelioma development was significantly accelerated and more aggressive in the Bap1−/− mice, providing unbiased genetic evidence supporting a fundamental role of Bap1 loss in malignant mesothelioma pathogenesis. The accelerated rate of death because of asbestos-related disease in Bap1−/− mice compared with WT mice (median survival 43 weeks vs. 55 weeks in WT mice, respectively) is similar to the accelerated onset and shorter median survivals that we observed previously in mice with haploinsufficiency for other tumor suppressor genes—Cdkn2a/p16(Ink4a), Cdkn2a/p19Arf, and Nf2—that are thought to act as key drivers in malignant mesothelioma pathogenesis (30, 31, 37). Moreover, like the malignant mesotheliomas in our mice, the spontaneous mesotheliomas in Bap1−/− mice share similar pathological characteristics (38), and both are severely compromised in their capacity to grow in immunocompromised hosts (39). These findings strongly suggest that Bap1 is a critical component of the malignant mesothelioma cell

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**Figure 2.** Molecular analysis of asbestos-induced malignant mesotheliomas in Bap1−/− and WT mice. A, RT-PCR analysis of expression of Bap1, various mesothelial markers, as well as p16(Ink4a), p15(Ink4b), and p19(Ink4b) in normal mesothelial cells and malignant mesothelioma cells derived from asbestos-induced tumors from Bap1−/− and WT mice. Normal (+/+), normal mesothelial cells isolated from untreated WT mice. Normal (+/-), normal mesothelial cells isolated from untreated Bap1−/− mice. Tumor (+/-), malignant mesothelioma cells derived from asbestos-exposed WT mice, retaining both alleles of Bap1 and expressing WT Bap1. Tumor (-/-), malignant mesothelioma cells derived from asbestos-exposed Bap1−/− mice, showing loss of expression of WT Bap1. B, PCR analysis of genomic DNA from Bap1 (+/+) and (-/-) malignant mesothelioma cells derived from WT and Bap1−/− mice, respectively. Note loss of WT allele in tumor cells from Bap1−/− mice. C, Ub-AMC assay demonstrating activity of Bap1 in malignant mesothelioma cells from WT mice but not in malignant mesothelioma cells from Bap1−/− mice. The Bap1 status shown refers to that of the tumor cells, that is, +/- in malignant mesothelioma cells from WT and -/- in malignant mesothelioma cells from Bap1 heterozygous mice.
mesotheliomas from these other mutant mouse models, malignant mesotheliomas from Bap1−/− mice showed biallelic inactivation of the predisposing gene, indicating that Bap1 acts as a bona fide tumor suppressor gene in malignant mesothelioma pathogenesis. Thus, the experimental data presented here are consistent with the notion that Bap1 inactivation plays an important role in MM development.

With regard to tumor histology, it is noteworthy that all of the malignant mesotheliomas in our Bap1−/− and WT mice seemed to be biphasic, whereas sarcomatoid malignant mesotheliomas were predominant in our earlier studies of Cdkn2a/p16(Ink4a)+/+ and Cdkn2a/p19(Arf)+/+ mice (37). In our report of human BAP1 families (11), all of the available malignant mesothelioma specimens seen in BAP1 mutation carriers were of the epithelial type, although the significance of this finding could not be established because of the relatively small number of cases evaluated. In sporadic malignant mesothelioma, several groups have reported that somatic mutations of BAP1 occur primarily in epithelioid tumors (11, 12, 38), whereas Zauderer and colleagues could find no obvious difference in tumor histology among malignant mesotheliomas with and without somatic BAPI mutations (39).

Collectively, the experimental findings summarized here indicate that germline Bap1 mutation predisposes to the tumorigenic effects of asbestos. By using an unbiased genetic model system and chronic exposure to asbestos, we have been able to demonstrate that germline haploinsufficiency for Bap1 causes increased susceptibility to asbestos-induced malignant mesothelioma formation, which may be facilitated, at least in part, by a p16(Ink4a)-independent mechanism involving epigenetic dysregulation of Rb. Although loss of p16(INK4A) expression, via homozygous deletion of CDKN2A, has long been known to be a frequent finding in human malignant mesothelioma (13, 14), a recent integrated genomic analysis of 53 sporadic pleural malignant mesotheliomas revealed that 6 of 12 tumors with BAPI mutations did not exhibit allelic losses of CDKN2A; moreover, 17 of 20 tumors with homozygous losses of CDKN2A did not show point mutations in RAP1 (10). It is also
Bap1−/− Mice Predisposed to Asbestos-Induced Mesothelioma

Figure 4. Decreased expression of Rb results from aberrant epigenetic modification of the Rb1 gene in malignant mesothelioma cells from Bap1−/− mice, and Rb inactivation contributes to the malignant phenotype of these malignant mesothelioma cells. A, total Rb expression in Bap1−/− malignant mesothelioma cells treated with 0 (control), 10 μmol/L, and 20 μmol/L of 5-Aza-CdR for 72 hours. B, methylation analysis of Rb1 promoter in Bap1++/− and Bap1−/− malignant mesothelioma cells from WT and Bap1−/− mice, respectively, by MeDIP assay followed by semiquantitative PCR. Note that genomic DNA samples subjected to MeDIP show increased methylation of Rb1 promoter in Bap1−/− malignant mesothelioma cells compared with that of Bap1++/− cells. Input controls depict equivalent amounts of Rb1 promoter DNA in all samples. C, treatment of malignant mesothelioma cells with E2f inhibitor HLM006474 (20 μmol/L) and analyzed by MTS assay after incubation at 37°C for 72 hours. Note that malignant mesothelioma cells from both WT and Bap1−/− mice were sensitive to the E2f inhibitor, whereas tumor cells from WT animals, which lacked expression of p16(Ink4a), were much more sensitive to a Cdk4/6 inhibitor than Bap1−/− cells that retained expression of p16(Ink4a).

Noteworthy that our earlier aCGH analysis of malignant mesotheliomas from two unrelated BAP1 mutation carriers revealed no homozygous losses of CDKN2A, although one of the tumors did exhibit monosomy 9, resulting in heterozygous loss of CDKN2A (11). Thus, our findings in an experimental model system, together with these clinical findings, indicate that BAP1 inactivation in malignant mesothelioma may not require homozygous loss of CDKN2A/p16(Ink4A). Importantly, however, our data suggest that dysregulation of the Rb pathway may be required for Bap1-driven malignant mesothelioma pathogenesis, at least in an experimental model. The fact that expression of Rb is diminished in both normal mesothelial cells and malignant mesothelioma cells from Bap1−/− mice suggests that Rb loss may contribute mechanistically to accelerated malignant mesothelioma onset and increased proliferation of tumor cells observed in Bap1−/− mice. Relevant to this possibility, germline heterozygous mutations of other cancer susceptibility genes, such as the tumor suppressor gene VHL connected with hereditary renal cancer, have been shown to alter the mRNA expression profiles of primary cultures of phenotypically normal epithelial cells in a gene-specific manner, and in some instances the expression data confirmed what is known about key tumorigenic pathways affected by biallelic mutations of such tumor suppressor genes (40). In Bap1−/− mice, heterozygous ("one hit") mutation is associated with alteration in the expression of a central node (Rb) in a cellular corridor—the p16(Ink4a)-Rb pathway—that is strongly implicated in malignant mesothelioma pathogenesis generally. Whether this novel finding translates to its human counterpart will be the subject of future investigation.

Our findings in our Bap1−/− mouse model suggest that early onset and high penetrance of malignant mesothelioma may require exposure to carcinogenic mineral fibers. Germane to this possibility, the initial two reports of families with germline BAP1 mutations were paradoxical, with our group reporting two families with multiple (5 and 7) malignant mesotheliomas (11), and a second group describing two families with atypical melanocytic neoplasms but no malignant mesotheliomas (17), although a follow up study uncovered a single malignant mesothelioma in one of the latter families (26). Rather than
representing two dissimilar cancer-related syndromes, it became apparent that germline BAP1 mutations are associated with a spectrum of neoplasms, suggesting that BAP1 has a critical tumor suppressor function in a variety of tissues (41, 42). The first reports of BAP1 families (17, 26) involved a selection bias, in the sense that the investigators were drawn to their families because of the high incidence of a particular cancer type, but subsequent work has revealed that malignant mesothelioma and atypical melanocytic neoplasms are not always the predominant tumor types observed in BAP1 families (19, 23). Thus, as originally proposed, malignant mesothelioma may only predominate when there is exposure to asbestos (11). Based on our earlier work on two BAP1 families with multiple malignant mesotheliomas (11), it seems that the level of asbestos exposure need not be high in BAP1 mutation carriers, as occupational histories on those families did not suggest any obvious exposure, and only modest or trace levels of asbestos were found in their homes. In the *in vivo* tumorigenicity studies reported here, the level of asbestos exposure used was sufficient to induce deaths in all mice with germline haploinsufficiency for *Bap1* but not in all WT animals. Drawing parallels to humans, our findings provide experimental support for the idea that *BAP1* mutation carriers may be highly susceptible to malignant mesothelioma even at modest levels of asbestos exposure that would be considerably less tumorigenic in the general population and, thus, would require close clinical monitoring with the goal of early detection and intervention.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


