Chapter 8
Germline and Somatic Mutations in Human Mesothelioma and Lessons from Asbestos-Exposed Genetically Engineered Mouse Models

Mitchell Cheung, Craig W. Menges, and Joseph R. Testa

Abstract Like cancer generally, malignant mesothelioma is a genetic disease at the cellular level. Specific genes most frequently linked to mesothelioma include the tumor suppressor genes *BAP1*, *CDKN2A*, and *NF2*. Somatic (acquired) mutations of these and other tumor suppressor genes often occur in combination in a given mesothelioma, suggesting that a cascade of genomic alterations is involved in the pathogenesis of this deadly disease. Overall, only a small fraction of individuals exposed to asbestos fibers develop the disorder, suggesting that inherited genetic factors may play a role in predisposing to mesothelioma. A person who is genetically predisposed to mesothelioma carries a DNA variant in one or possibly more genes, but the disease may not be triggered unless there is exposure to asbestos—perhaps even minimally—or some other relevant carcinogenic environmental factor. For example, clustering of mesothelioma cases has been documented in some, but not all, families with a germline inactivating mutation of *BAP1*. People without a genetic predisposition also develop the disease when exposed to asbestos, but studies in humans and genetically engineered mouse models indicate that the risk is likely to be much lower. In this review, we highlight the current understanding of the role of both hereditary and somatic mutations in human malignant mesothelioma, as well as what has been learned from experimental studies of asbestos-exposed rodent models of mesothelioma.

Keywords Mesothelioma • Genetics • Tumor suppressor genes • Somatic and germline mutations • BAP1 syndrome • Mouse models of mesothelioma

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Mitchell Cheung and Craig W. Menges have contributed equally to this review.

M. Cheung • C.W. Menges • J.R. Testa (✉)
Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA
e-mail: joseph.testa@fccc.edu

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8.1 Somatic Mutations in Malignant Mesothelioma

Several prominent sites of somatic (non-germline) chromosomal loss have been identified in human mesothelioma, including recurring deletions of chromosomes 3, 9, and 22, specifically at genomic locations ("bands") 3p21, 9p21, and 22q12, respectively. These acquired chromosome abnormalities often occur in combination in a given tumor, suggesting a multi-step pathogenetic process (Murthy and Testa 1999). Work performed in the 1990s implicated tumor suppressor loci at two of these chromosomal sites: *CDKN2A* (Cheng et al. 1994; Xio et al. 1995; Altomare et al. 2005), located at 9p21, and *NF2* (Bianchi et al. 1995; Sekido et al. 1995), residing at 22q12. The identity of the critical 3p21 gene in mesothelioma had remained a mystery until 2011, when somatic point mutations of the BRCA1 associated protein-1 gene, *BAP1*, located at 3p21.3, were identified in 20–25% of sporadic mesotheliomas (Bott et al. 2011; Testa et al. 2011). In 2012, Yoshikawa and colleagues uncovered biallelic alterations of *BAP1*, including homozygous deletions of part or all of this tumor suppressor gene as well as sequence-level mutations, in 61% of sporadic mesotheliomas (Yoshikawa et al. 2012). Subsequent studies with newer next generation and multiplex ligation-dependent probe amplification platforms confirmed a similarly high incidence of *BAP1* mutations in this disease (Lo Iacono et al. 2015; Nasu et al. 2015).

In vivo work has demonstrated that the BAP1 protein is a bona fide tumor suppressor (Kadariya et al. 2016a). Functionally, BAP1 and its *Drosophila* homolog Calypso are nuclear-localized deubiquitinating enzymes and members of the polycomb-group of highly conserved transcriptional repressors, which are required for long-term silencing of genes that regulate stem cell pluripotency, cell fate determination, and other developmental processes (Gaytan de Ayala Alonso et al. 2007). BAP1 has also been shown to play an important role in double-strand break repair by homologous recombination, thereby suggesting a potential mechanism by which *BAP1* mutations might contribute to genomic instability and tumor formation (Ismail et al. 2014). Further functional implications of *BAP1* mutations in mesothelioma are described in the following section.

The *CDKN2A* locus encodes two important tumor suppressors, p16INK4A and p14ARF, which are known to regulate the critical Rb and p53 cell cycle regulatory pathways, respectively. Deletions of *CDKN2A* have been reported in 75–90% of mesothelioma samples and tumor-derived cell lines (Cheng et al. 1994; Xio et al. 1995; Altomare et al. 2005). Re-expression of p16INK4A in p16INK4A-null mesothelioma cells has been shown to cause cell cycle arrest and tumor suppression or regression (Frizelle et al. 1998), while re-expression of p14ARF in mesothelioma cells induced G1-phase cell cycle arrest and apoptosis (Yang et al. 2000). As noted above, p14ARF is an integral component of the p53 pathway, and mutations of the p53 gene, *TP53*, have also been reported in a subset of mesotheliomas (Cote et al. 1991; Altomare et al. 2005). For example, we identified *TP53* mutations in 3 of 20 (15%) malignant mesotheliomas we tested. Notably, two of these three tumor samples with *TP53* mutations did not show homozygous loss of *p14(ARF)*, indicating

that alterations of the p53 pathway in mesothelioma can occur in connection with defects in either gene, thereby providing further support for a critical role of this pathway in mesothelioma pathogenesis.

Mutations of *NF2* have been reported in 20–55% of mesothelioma specimens and tumor-derived cell lines (Bianchi et al. 1995; Sekido et al. 1995; Cheng et al. 1999; Bott et al. 2011). Interestingly, mesotheliomas have been reported in several individuals with neurofibromatosis type 2 (Baser et al. 2002; Baser et al. 2005), an autosomal dominant disorder caused by germline mutation of *NF2*. In genetically engineered mouse models, there is ample evidence demonstrating that germline mutations of *Nf2*, like mutations of *Cdkn2a* or *Bap1*, contribute significantly to mesothelioma development (see below). The *NF2* product, merlin, is known to repress cyclin D1 expression, and merlin loss in mesothelioma cells leads to cell cycle progression via up regulation of cyclin D1 (Xiao et al. 2005). In our studies, adenovirus-mediated re-expression of merlin in *NF2*-deficient mesothelioma cells resulted in decreased expression of cyclin D1 mRNA (Xiao et al. 2005). Merlin has also been proposed to regulate cyclin D1 post-transcriptionally through the activation of mTORC1 (James et al. 2009; Lopez-Lago et al. 2009). Merlin also inhibits Rac/Pak and FAK signaling, which are implicated in cell migration and spreading, respectively, and inactivation of *NF2* in mesothelioma cells was found to promote invasiveness and spreading (Xiao et al. 2002; Poulikakos et al. 2006). More recently, deregulation of the Hippo signaling pathway, one of the downstream cascades regulated by merlin, has been implicated in mesothelioma. Notably, in addition to *NF2*, alterations of genes encoding several other components in the Hippo pathway have been reported in mesothelioma. These include somatic mutations of the tumor suppressor genes *LATS1* and *LATS2*, and upregulation of the transcriptional coactivator YAP, the main downstream effector of the pathway and a putative oncogene (Bott et al. 2011; Murakami et al. 2011). Given their frequent involvement in human mesothelioma, alterations of *CDKN2A*, *NF2,* and *BAP1* appear to be primary drivers in this malignancy.

Recently, investigators have begun to use massively parallel next-generation sequencing for comprehensive genomic analysis of malignant mesotheliomas, which has confirmed and extended earlier single gene studies on mesothelioma. In one report, Guo and colleagues performed whole-exome sequencing on 22 pleural malignant mesotheliomas and matched blood, which revealed frequent alterations in *BAP1*, *NF2*, *CDKN2A*, and *CUL1* (Guo et al. 2015). In a much larger study, Bueno and colleagues analyzed transcriptomes, whole exomes and targeted exomes from a total of 216 pleural mesotheliomas (Bueno et al. 2016). Using exome analysis, they found *BAP1*, *NF2*, *TP53*, *SETD2*, *DDX3X*, *ULK2*, *RYR2*, *CFAP45*, *SETDB1,* and *DDX51* to be significantly mutated. Besides the more commonly mutated genes, i.e., *BAP1*, *NF2,* and *TP53*, mutation of *SETD2* (8%) was the most frequently mutated of the remaining seven genes newly implicated in this disease. The remaining six newer genes were mutated in 4% of less of cases. Whole-genome sequencing revealed additional structural variations that resulted in loss of gene function or copy number loss. For example, of the 20 samples analyzed using whole-genome sequencing, chromosomal rearrangements within *BAP1*, *NF2,* or

CDKN2A were identified in 9 (45%) cases, and recurrent gene fusions and splice alterations appeared to be common mechanisms for the inactivation of *NF2*, *BAP1,* and *SETD2* (Bueno et al. 2016).

While most mesotheliomas investigated genetically have involved the pleura, a recent report by Borczuk and colleagues included a series of peritoneal mesotheliomas (Borczuk et al. 2016). The group compared DNA array-based findings from 48 epithelioid peritoneal mesotheliomas and 41 epithelioid pleural mesotheliomas to identify similarities and differences in DNA copy number alterations. Recurrent losses in 3p (including *BAP1*), 9p (*CDKN2A*), and 22q (*NF2*) were seen in mesotheliomas from both tumor sites, although losses of *CDKN2A* and *NF2* occurred more frequently in pleural disease. Interestingly, whereas DNA copy number losses were more frequent in pleural mesotheliomas, copy number gains were more common in the peritoneal tumors, with regions of gain encompassing genes encoding receptor tyrosine kinase pathway members. Also noteworthy, among the peritoneal tumors, deletions in chromosome arms 6q, 14q, 17p, and 22q, and gain of 17q were observed in asbestos-associated cases but not in cases in which the disease was linked to radiation exposure. The investigators concluded that the pattern of genomic imbalances suggests both overlapping and distinct molecular genetic pathways in mesothelioma of the pleura and peritoneum, and that differences in causation—i.e., asbestos versus radiation—may explain some of the site-dependent genomic differences observed (Borczuk et al. 2016).

8.2 Germline Mutations in Malignant Mesothelioma

Since only a small percentage of asbestos-exposed individuals develop mesothelioma, and because familial clustering of the disease occurs in some families, it was proposed that genetic factors are likely to play a role in the etiology of mesothelioma (Roushdy-Hammady et al. 2001). The following section provides compelling evidence in support of this idea.

8.2.1 Early Evidence for the Possible Involvement of Germline Mutations in Mesothelioma

An early report suggesting that genetic susceptibility may play a role in mesothelioma development was published in the late 1970s (Li et al. 1978). The paper described the presence of mesothelioma in the wife and daughter of a man who was a pipe insulator at a shipyard. The man developed asbestosis and died from metastatic lung adenocarcinoma, possibly linked to heavy smoking. Family members reported that this individual frequently came home from work with his clothes covered with white dust, possibly asbestos, and the authors suggested "… the dosage of

asbestos to induce mesothelioma in susceptible persons may be low and hardly noticed." In another report, a statistically significant number of families of female mesothelioma patients with non-occupational asbestos exposure were found to have parents with gastric or intestinal carcinomas (Vianna and Polan 1978). Since the authors did not find any evidence of asbestos exposure in this group of parents, they proposed that these individuals were from cancer-prone families. On a much larger scale, genetic susceptibility to mesothelioma has been proposed to explain the high incidence of mesothelioma in certain villages in Cappadocia, Turkey, where a mesothelioma epidemic was first reported by Baris and colleagues in 1978 (Baris et al. 1978). The authors later proposed the possibility that an asbestos-like mineral fiber, erionite, may be the cause of this epidemic (Baris et al. 1981). A role for genetic susceptibility to mesothelioma was later proposed when investigators found a high incidence of the disease in some homes but not in other adjacent homes made with the same type of erionite-containing building blocks (Roushdy-Hammady et al. 2001; Carbone et al. 2007). However, to date a candidate gene(s) involved in this epidemic has not been discovered.

8.2.2 Clues Incriminating the BAP1 Gene in Mesothelioma

Carbone, Testa and colleagues initiated studies to identify a mesothelioma predisposition gene(s) using linkage analysis and/or a candidate gene approach in families from Cappadocia, Turkey, as well as in two unrelated families residing in two different states, Wisconsin and Louisiana (W and L families, respectively), in the USA (Testa et al. 2011). Chromosome microarray analysis on tumor samples from the W and L families, and later linkage analysis on germline DNA, led to the implication of a candidate gene at chromosome band 3p21 (Testa et al. 2011). Notably, Harbour, Bowcock, and colleagues had previously uncovered inactivating somatic mutations of *BAP1* in 26 of 31 (84%) metastasizing uveal melanomas, and one of their patients had a germline mutation in *BAP1*, suggesting the existence of a tumor susceptibility allele in this individual (Harbour et al. 2010). This finding piqued our interest for three reasons: (1) our chromosome microarray analysis on two mesothelioma samples from the W and L families had uncovered genomic alterations that either encompassed *BAP1* (homozygous deletion) or involved a chromosomal break within the *BAP1* locus; (2) our group's L family had two individuals with uveal melanomas (including one with metastasis to the liver); and (3) the *BAP1* gene is located at 3p21, a chromosomal site previously implicated in our extensive earlier cytogenetic and loss of heterozygosity (LOH) studies of sporadic mesotheliomas (Flejter et al. 1989; Lu et al. 1994). Spurred by these clues, we took a candidate approach which led to the discovery of germline inactivating mutations of *BAP1* in both the W and the L families (Testa et al. 2011). Interestingly, none of the members in these two families reported any occupational exposure to asbestos, and only trace amounts of asbestos were found in their homes. In family W, a germline splice site mutation in intron 6 was discovered in five family members with mesothelioma and

three members with kidney, breast, or ovarian carcinomas. This mutation was shown to result in an aberrantly spliced mRNA leading to nonsense-mediated mRNA decay or a truncated protein due to a frameshift and premature stop codon. In the L family, a different germline *BAP1* alteration, a nonsense mutation, was found in seven family members with mesothelioma, one individual with a uveal melanoma, and one other with both a mesothelioma and a uveal melanoma. Immunohistochemistry performed on mesothelioma specimens from these families revealed loss of BAP1 nuclear expression and only weak cytoplasmic staining (Testa et al. 2011). Interestingly, germline *BAP1* mutations were also discovered in 2 of 26 sporadic mesotheliomas tested, and both of these mutation carriers were previously diagnosed with uveal melanoma.

Intriguingly, in the same issue of *Nature Genetics* as our report (Testa et al. 2011), Wiesner, Speicher, and colleagues reported two families in which germline inactivating mutations of *BAP1* were connected with predisposition to melanocytic tumors (Wiesner et al. 2011). Both of their families were characterized by high incidence of benign melanocytic tumors with some overlapping features common to cutaneous melanoma. A frameshift germline *BAP1* mutation was found to co-segregate with affected individuals in family 1, including one person who also developed UM. The second family had multiple relatives with benign melanocytic lesions as well as the co-occurrence of cutaneous melanoma in three family members, and a uveal melanoma in a fourth individual. Affected members of this family had a germline acceptor splice site mutation in *BAP1*, which leads to a predicted frameshift and truncation of the protein. Also notable, at the time of publication, there were no mesotheliomas reported in these two families. After the simultaneous publications by Testa et al. and Wiesner et al. in the same journal issue, communication between the two groups was initiated. This led to the reexamination and discovery of one member (previously known to have benign melanocytic lesions and cutaneous melanoma) of family 2 who was later found to have peritoneal mesothelioma with no known asbestos exposure (Wiesner et al. 2012). One month after the initial reports of BAP1 families, a report was released online by Abdel-Rahman and colleagues, who provided further evidence for a novel hereditary cancer syndrome caused by germline *BAP1* mutation (Abdel-Rahman et al. 2011). They reported a family with predisposition to uveal melanoma, lung carcinoma, meningioma, and possibly other cancers in connection with a germline *BAP1* nonsense mutation. Although not emphasized in this report, it is very noteworthy that one mutation carrier in this family had a mesothelioma, and that individual's son and a nephew both had mesothelioma and second tumors, although *BAP1* sequencing results were not available in these two cases. As in other studies (Testa et al. 2011; Wiesner et al. 2011), DNA analysis and immunohistochemical studies revealed loss of the wild type *BAP1* allele in tumor specimens from the germline mutation carriers.

8.3 BAP1 Cancer Predisposition Syndrome

Since the time of these initial reports, many other studies have uncovered germline *BAP1* mutations in familial mesothelioma and other cancers (Wiesner et al. 2012; Njauw et al. 2012; Wadt et al. 2012; Cheung et al. 2013; Hoiom et al. 2013; Popova et al. 2013; Wadt et al. 2014; Carbone et al. 2015; Cheung et al. 2015a; de la Fouchardiere et al. 2015; Ohar et al. 2016). Collectively, these findings suggest a single BAP1-related tumor predisposition syndrome in which affected families are predisposed to mesothelioma, uveal and cutaneous melanoma, benign melanocytic tumors, renal cell carcinoma, meningioma, basal cell carcinoma, cholangiocarcinomas, and potentially other less common cancers. In Table 8.1, we summarize the frequency of each tumor type reported to date in connection with germline *BAP1* mutations. The numbers in parentheses represent additional individuals with the indicated type of malignancy in the same families as the probands, but whose germline DNA was not directly sequenced. To date, the two most common types of cancers observed among *BAP1* mutation carriers are mesothelioma and uveal melanoma (Table 8.1). The fact that biallelic inactivation of *BAP1* has been documented in multiple tumors from these high-risk families implies that *BAP1* acts as a classical tumor suppressor gene. Consistent with these exciting genetic discoveries, BAP1 has also been found to exhibit tumor suppressor activity in cell-based transfection assays, and such tumor suppression requires both nuclear localization and BAP1 deubiquitinase activity (reviewed in Fang et al. 2010).

Over the past 5 years, a number of remarkable clinical features have been reported with regard to mesotheliomas seen in *BAP1* mutation carriers. While approximately 20% of all sporadic mesotheliomas are found in the peritoneum (Faig et al. 2015), a higher proportion of peritoneal mesothelioma has been observed in *BAP1* mutation carriers (Baumann et al. 2015; Cheung et al. 2015a; Ohar et al. 2016). Second, our large-scale study of 150 mesothelioma patients revealed a 10-year younger age of mesothelioma diagnosis among germline *BAP1* mutation

Table 8.1 Summary of tumors reported in connection with germline BAP1 mutations

Tumor type	Number of cases[a]
Malignant mesothelioma	55 (+42) = 97 total
Uveal melanoma	57 (+36) = 93 total
Cutaneous melanoma	32 (+28) = 60 total
Renal cell carcinoma	18 (+26) = 44 total
Melanocytic tumor	34 (+3) = 37 total
Breast carcinoma	12 (+16) = 28 total
Basal cell carcinoma	16 (+9) = 25 total
Lung carcinoma	7 (+15) = 22 total

[a]Numbers in parentheses represent individuals with the indicated tumor type in the same families as the probands, but whose germline DNA was not directly sequenced

carriers when compared to mesothelioma patients without a germline mutation (Ohar et al. 2016). This early age of cancer onset phenotype is typical of other cancer predisposition syndromes such as Li-Fraumeni syndrome (Li et al. 1988; Malkin et al. 1990), hereditary breast and ovarian cancer syndrome (Lynch et al. 2013), familial adenomatous polyposis (Tezcan et al. 2016), and Lynch syndrome (Tezcan et al. 2016). Third, *BAP1* mutation carriers who develop mesothelioma have a 3.5- to 7-fold improved survival rate after tumor diagnosis compared to non-carriers (Baumann et al. 2015; Ohar et al. 2016). The reason(s) for the improved survival among *BAP1* mutation carriers may be connected with the younger age and/or higher proportion of the more treatable peritoneal tumor in this group (Cheung et al. 2015a; Ohar et al. 2016). Sporadic mesotheliomas also appear to have a better prognosis when there is loss of BAP1 immunohistochemical staining (Farzin et al. 2015). This is in stark contrast to the situation in sporadic uveal melanomas (Harbour et al. 2010) and clear cell renal cell carcinomas (Minardi et al. 2016; Pena-Llopis et al. 2012), where BAP1 loss is associated with a poor prognosis. Finally, we and others have observed a high number of mutation carriers having more than one type of primary cancer (Cheung et al. 2015a; Ohar et al. 2016), a phenomenon that mirrors what is seen in individuals with Li-Fraumeni syndrome (Hisada et al. 1998). An example is depicted in Fig. 8.1, showing a family in which five *BAP1* mutation carriers with mesothelioma had at least one other primary cancer. This is particularly pronounced in individual III-09 who has three different types of cancers: mesothelioma, basal cell carcinoma, and meningioma.

Despite the multiple studies implicating *BAP1* as an important player in familial mesothelioma, there are likely other genes that when mutated in the germline can lead to a similar cancer syndrome. In our study involving Sanger sequencing of *BAP1* in germline DNA from 150 mesothelioma patients with a family history of cancer, we uncovered 9 (6%) individuals who were *BAP1* mutation carriers (Ohar et al. 2016). Whether families of the remaining 141 patients harbor germline mutation of some other cancer predisposition gene is not known. Similarly, we reported a large family with eight mesotheliomas in which no *BAP1* germline mutation was identified (Cheung et al. 2015b). One recent paper described a family in which both mesothelioma and cutaneous melanoma cases were found to have a deleterious germline missense mutation in the *CDKN2A* gene (c.301G > T; p.Gly101Trp) (Betti et al. 2016). The proband with this mutation, a female who developed cutaneous melanoma at the age of 42, has a mother who also carried the mutation and developed cutaneous melanoma at age 62 and mesothelioma at 65 (Betti et al. 2016). The exposure level for the mother was determined to be of the "low exposure" category, which was defined as non-occupational exposure. This missense mutation has been previously reported in other familial cutaneous melanoma cases (reviewed in Goldstein 2004). Considering that the *CDKN2A* locus at 9p21 is the most frequently site of somatic deletions in sporadic mesotheliomas (Cheng et al. 1994; Murthy and Testa 1999), it is plausible that germline mutations in the *CDKN2A* gene could also lead to increased risk for developing mesothelioma. Germline mutations in the *TP53* tumor suppressor gene have also been reported in mesothelioma. A germline missense mutation (p.Arg213Gln) was reported in a large family characterized by a

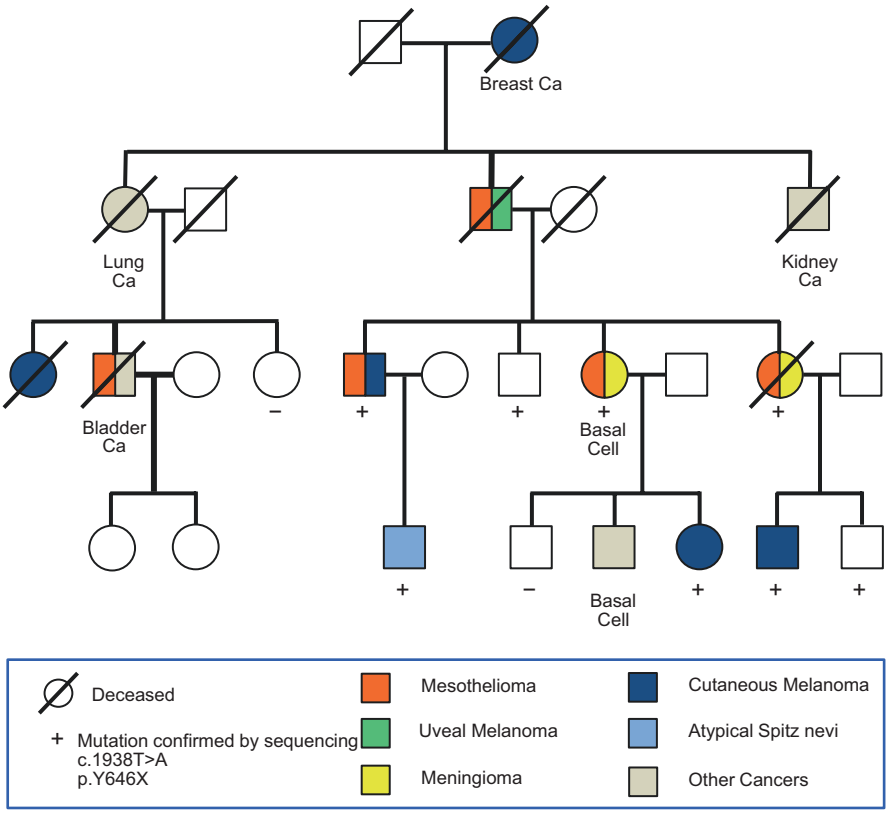


Fig. 8.1 Pedigree of BAP1 syndrome family with high incidence of multiple primary cancers. Affected family members have germline nonsense mutation in *BAP1* exon 15 (c.1938T>A). The family exhibits many neoplasms, including six family members with two or more primary tumors. Predominant cancers include mesothelioma and cutaneous melanoma, each observed in five family members. Modified from Cheung et al. (2015a); Cancer Lett 369:261–265. Copyright (2015), with permission from Elsevier

number of cancers, including breast and colon cancers (Ruijs et al. 2006). One mutation carrier in this family developed mesothelioma at the age of 55, with no asbestos exposure history described by the authors. Furthermore, a peritoneal mesothelioma was reported in an unrelated 60-year-old patient with Li-Fraumeni syndrome, in which a germline *TP53* missense mutation (p.R158H) was found (Ceelen et al. 2011). No known exposure to asbestos was reported for this individual. Finally, germline mutations in *NF2* have been reported in two studies. In the earlier report, an individual with NF2 disorder developed bilateral vestibular schwannomas and numerous spinal tumors in his 20s, and he later developed peritoneal mesothelioma at the age of 40 (Baser et al. 2002). Single-strand conformational polymorphism analysis did not reveal a germline mutation in the *NF2* gene; however, comparative

genomic hybridization analysis and immunohistochemistry staining of the mesothelioma tissue revealed loss of the gene and protein product, respectively. This patient may have been exposed to asbestos occupationally, as he had worked as an auto mechanic. A second patient, with a missense mutation in *NF2* (p.Phe62Ser), developed bilateral vestibular schwannomas at the age of 66 and spinal tumors at age 74 (Baser et al. 2005). A pleural mesothelioma was discovered in this same individual 1 year later. He was reported to have an occupational asbestos exposure while working as a gas fitter for 25 years (Baser et al. 2005).

8.4 Animal Models of Malignant Mesothelioma

Animal models are invaluable tools for basic research on embryonic development and disease states such as cancer. Rodents, especially rat (*Rattus norvegicus*) and mouse (*Mus musculus*), are often employed to evaluate the role of asbestos in the induction of malignant mesothelioma, as well as for the unbiased genetic assessment of the role of mutant tumor suppressor genes in mesothelioma pathogenesis. In this section of our review, we focus on murine models but also provide a historic perspective of knowledge gained from other rodent models.

8.4.1 Chronic Asbestos Exposure Studies and Mesothelioma Pathogenesis in Rats

A strong epidemiological link between risk of mesothelioma and exposure to asbestos dust was established in the 1960s (reviewed in Gilson 1966). During the same decade, researchers began to formally test this hypothesis experimentally in laboratory rats by evaluating the carcinogenicity of both amphibole and serpentine mineral fibers (Wagner and Berry 1969). In 1962, Wagner and colleagues initiated a comprehensive study, in which 600 standard and 600 specific pathogen-free rats were inoculated intrapleurally with either amosite, chrysotile, crocidolite, extracted crocidolite (where oils were removed from the fibers with cyclohexane), silica or saline (~100 animals/arm/rat strain) and then followed until death (Wagner and Berry 1969). All carcinogenic mineral fibers tested were found to cause mesothelioma in a significant percentage of the animals irrespective of rat strain, thereby providing compelling experimental evidence for a causal relationship between asbestos and mesothelioma formation. This initial study was performed using a single inoculation of mineral fibers into the pleural space. However, in the occupational setting, humans typically inhale asbestos dust over a protracted time frame, different than what had been formally tested in rats. To address this discrepancy experimentally, in a subsequent investigation rats were chronically exposed to amphiboles and serpentine fibers using inhalation chambers. Animals were exposed

to asbestos for 5, 8, and 10 weeks, as well as over longer time periods of 3, 6, 12, and 24 months, and then monitored for asbestos deposition in the lung and for disease onset (Wagner et al. 1974). In this study, a marked difference in lung deposition was observed between amphibole and serpentine fibers, with little to no deposition found in the lungs of chrysotile-exposed animals (Wagner et al. 1974). This observation led some to speculate that the serpentine shape of the chrysotile fibers makes it easier to expel in sputum from the lung (Bernstein et al. 2013). In the inhalation studies, a smaller percentage of mesotheliomas was observed compared to the previous study where asbestos was inoculated into the pleura, with Rhodesian chrysotile causing no mesotheliomas at all (Wagner et al. 1974). Asbestosis was a predominant disease observed in all exposed rats, although the authors also concluded that there was a positive correlation between asbestos exposure and mesothelioma development. Erionite, a zeolite mineral fiber implicated in the mesothelioma epidemic seen in several villages in Cappadocia, Turkey (Baris et al. 1978; Baris et al. 1981), was demonstrated to cause a very high rate of mesothelioma in rats when intrapleurally inoculated and was more carcinogenic than any amphibole or serpentine fiber tested in chronic inhalation experiments (Wagner et al. 1985).

These early studies in rats provided compelling evidence for the causal role of mineral fiber exposure in mesothelioma pathogenesis, a risk association previously identified in humans occupationally exposed to asbestos (Gilson 1966). Thus, rodents were established as an important tool for studies on the carcinogenicity of various types of mineral fibers.

8.4.2 Chronic Asbestos Exposure Studies and Mesothelioma Pathogenesis in Laboratory Mice

Initial rodent studies modeling asbestos-induced mesothelioma pathogenesis favored the rat over the mouse, due to the rat's larger pleural space for asbestos inoculation and lung capacity for mineral fiber inhalation studies. In some of the early investigations that did use mice, intrapleural inoculation of amphiboles and serpentine mineral fibers only caused granulomas and fibrosis (Davis 1970). Long-term asbestos inhalation studies in mice mainly caused fibrosis with occasional papillary carcinomas (Reeves et al. 1974). It was also demonstrated that very small amounts of asbestos deposition occur in the lung of mice via inhalation, presumably due to the mouse's smaller, more contorted nasal passages, such that asbestos fibers were less likely to enter the lung.

The first comprehensive mouse study testing the carcinogenicity of intraperitoneally inoculated asbestos and zeolite fibers was reported in the mid-1980s; in this investigation, more than 700 mice were injected with various concentrations and types of mineral fibers (Suzuki and Kohyama 1984). Malignant mesotheliomas were observed in 23.6% of the mice. This was a significantly higher incidence of

mesothelioma than observed in mice either injected intrapleurally with mineral fibers or exposed to asbestos via inhalation (Davis 1970; Reeves et al. 1974; Suzuki and Kohyama 1984). Moreover, different laboratories have reported differing mesothelioma incidence and disease-free survival rates in wild type mice inoculated intraperitoneally with asbestos, due at least in part to differing types, amounts and fiber dimensions, variability of injection schedules, as well as differences in the genetic background of the strains of animals used (Marsella et al. 1997; Vaslet et al. 2002; Fleury-Feith et al. 2003; Altomare et al. 2005; Robinson et al. 2006; Altomare et al. 2009; Altomare et al. 2011; Chow et al. 2012; Xu et al. 2014; Menges et al. 2014; Kadariya et al. 2016a; Kadariya et al. 2016b; Pietrofesa et al. 2016). For example, laboratories that perform multiple injections of asbestos over time may observe a higher incidence of mesothelioma and shorter tumor-free survival than laboratories that inject mice with a single bolus of mineral fibers. Injection of asbestos into the peritoneal space induces an acute inflammation. This inflammatory response appears to contribute to mesothelioma pathogenesis via the repeated release of chemokines such as IL-1β, IL-6, and TNFα (Kadariya et al. 2016b; Pietrofesa et al. 2016). The acute inflammatory reaction to intraperitoneal injection of asbestos fibers declines after 21 days (Macdonald and Kane 1997). They found that a 3-week recovery period decreased the extent of fibrosis induced following repeated injections that can cause premature death in exposed mice before mesotheliomas have time to develop. Pilot studies using doses of 100, 200, or 500 μg revealed a dose-dependent increase in the acute inflammatory response (Macdonald and Kane 1997), and for most of our publications we selected repeated injections at a dose of 400 μg, which induced mesotheliomas in about 50% of wild-type mice (Altomare et al. 2005).

8.4.3 Chronic Asbestos Exposure Studies and Mesothelioma Pathogenesis in Genetically Engineered Mouse (GEM) Models

GEM models are mice that have had their genome altered through the use of genetic engineering, including gene knock-out and knock-in techniques, and have been used in science to understand embryonic development and to model disease states such as cancer. Genes frequently activated or inactivated in human cancers can be mutated or deleted in mice to unravel genetically the role of oncogenes and tumor suppressor genes, respectively, in development or tumorigenesis (Gopinathan and Tuveson 2008). Cytogenetic and molecular genetic analysis of human mesotheliomas by many different laboratories have revealed recurrent losses at chromosomal sites harboring known or suspected tumor suppressor loci (Tiainen et al. 1988; Flejter et al. 1989; Cheng et al. 1993; Taguchi et al. 1993; Lu et al. 1994; Lee et al. 1996; Bell et al. 1997; Bjorkqvist et al. 1997), and as noted above, mutations of several different tumor suppressor genes, especially *CDKN2A*, *BAP1,* and *NF2*, are

considered to be driving events in this disease (Bianchi et al. 1995; Sekido et al. 1995; Cheng et al. 1999; Illei et al. 2003; Bott et al. 2011; Testa et al. 2011).

To determine if loss/inactivation of these tumor suppressor genes accelerates asbestos-induced mesothelioma development, various groups have conducted asbestos carcinogenicity studies with GEM models carrying germline mutations of these genes, along with wild type littermates that serve as controls. The first such studies involved a mouse model deficient for the *Tp53* gene, which encodes the tumor suppressor p53 (Marsella et al. 1997; Vaslet et al. 2002). Mice (129/Sv strain) with heterozygous (+/−) or homozygous (−/−) mutation of *Tp53*, along with wild type (+/+) littermates, were injected intraperitoneally with crocidolite every week. After 22 weekly injections, 76% of asbestos-exposed $Tp53^{+/-}$ mice developed mesothelioma (median latency: 44 weeks) compared to 32% of asbestos-exposed genetically normal ($Tp53^{+/+}$) mice (median latency: 67 weeks). Only 1 of 8 (12.5%) $Tp53^{-/-}$ mice developed a mesothelioma, with the remainder of this cohort dying of thymic lymphomas or hemangiosarcomas, which are known to arise spontaneously in such mice (Donehower et al. 1995).

The tumor suppressor gene *NF2* is mutated in up to 55% of mesotheliomas (Cheng et al. 1999). Two groups evaluated whether mice heterozygously deficient for *Nf2* ($Nf2^{+/-}$) have increased susceptibility to asbestos-induced carcinogenesis (Fleury-Feith et al. 2003; Altomare et al. 2005). Both studies found that asbestos-injected $Nf2^{+/-}$ mice developed a higher incidence of peritoneal mesotheliomas and a shorter latency compared to mineral fiber-exposed wild-type cohorts. Interestingly, the second, wild-type copy of *Nf2* was lost in a high percentage of mesotheliomas from asbestos-exposed *Nf2*-deficient mice, a finding that mirrors what is observed in many human mesotheliomas (Altomare et al. 2005). Mesothelioma cell lines from asbestos-exposed $Nf2^{+/-}$ mice exhibited somatic genetic and cell signaling alterations recapitulating those of the human disease counterpart. Together, these data demonstrated that *Nf2* is a bona fide tumor suppressor gene and that, upon exposure to asbestos, *Nf2* inactivation can act as a primary driver in mesothelioma pathogenesis.

The next GEM model that was used to investigate mesothelioma formation was a transgenic model (C57/Bl 6 J background) expressing SV40 large T antigen (TAg) in the mesothelial lining (Robinson et al. 2006). Transgenic mice expressing a single copy or multiple copies of SV40 TAg, as well as non-transgenic littermates, were exposed to asbestos and followed for development of mesothelioma. TAg transgenic mice developed mesothelioma with a shorter latency than did wild type mice, with a direct relationship found between transgene copy number and survival after exposure to asbestos. This study demonstrated that SV40 TAg, presumably through its disruption of Rb and p53 pathways, can contribute to asbestos-induced mesothelioma pathogenesis, although this model has limited relevance to human disease, given that the proposed link between SV40 infection and human mesothelioma now appears unlikely (Lopez-Rios et al. 2004).

As mentioned earlier, the *CDKN2A* locus encodes two tumor suppressors, p16INK4A and p14ARF (p19Arf in mice), and deletion of this locus occurs in most mesotheliomas (Cheng et al. 1994; Xio et al. 1995; Altomare et al. 2005). Because

that the genes encoding p16INK4A and p14/p19ARF share exon 2, the exon most often lost in mesotheliomas, deletions of *CDKN2A* usually inactivate both tumor suppressors. It was not clear until recently that both tumor suppressors contribute significantly to mesothelioma tumorigenesis. To address the potential role of p14/p19ARF loss in mesothelioma development, we used mice harboring a mutation in exon 1β of the *Cdkn2a* locus, which specifically inactivates p19Arf but not p16Ink4a (Altomare et al. 2009). Asbestos-exposed *p19Arf*$^{+/-}$ mice showed a higher incidence, and shorter latency, of mesothelioma formation compared to asbestos-exposed wild-type littermates. Mesotheliomas from *p19Arf*$^{+/-}$ mice showed recurrent genomic losses of chromosome 4, band C6, encompassing the Fas associated factor 1 gene, *Faf1*, which is known to play a role in apoptosis and cell death (Altomare et al. 2009; Menges et al. 2009). Additionally, its homolog, *FAF1*, is down-regulated in many human mesothelioma specimens and tumor-derived cell lines that were tested (Altomare et al. 2009). We also found that Faf1 regulates TNF-α-mediated NF-κB signaling, a signaling node that has been implicated in asbestos-induced carcinogenesis (Yang et al. 2006).

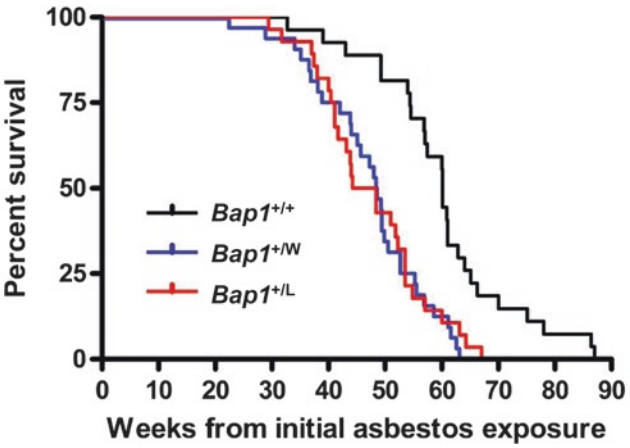
We next tested the relative contributions of both p16Ink4a and p19Arf to mesothelioma pathogenesis using mice with heterozygous deletions of one or the other genes (loss of exons 1α or 1β of *Cdkn2a*, respectively), or with a deletion knocking out both genes (loss of *Cdkn2a* exon 2) (Altomare et al. 2011). Asbestos-treated *p16Ink4a*$^{+/-}$ and *p19Arf*$^{+/-}$ mice each showed increased incidence and shorter latency of mesothelioma formation relative to wild type littermates. Mice deficient for both p16Ink4a and p19Arf showed accelerated asbestos-induced mesothelioma formation relative to mice deficient for *p16Ink4a* or *p19Arf* alone, and the resulting tumors exhibited biallelic loss of both tumor suppressor genes. Thus, these findings provided in vivo evidence indicating that both *Cdkn2a* gene products suppress asbestos carcinogenicity. Moreover, simultaneous inactivation of both genes appeared to cooperate to accelerate asbestos-induced tumor development and progression.

As stated above, human mesotheliomas frequently harbor alterations of both *NF2* and *CDKN2A*, suggesting cooperativity between losses of these two tumor suppressor genes. Tumorigenic cooperativity between these genes in mesothelioma pathogenesis was recently demonstrated in asbestos-exposed mice with germline mutation of one allele of each of these genes. These *Nf2*$^{+/-}$;*Cdkn2a*$^{+/-}$ mice showed markedly accelerated onset and progression of asbestos-induced mesothelioma compared to asbestos-exposed *Nf2*$^{+/-}$ or wild-type mice (Menges et al. 2014). Interestingly, ascites from the doubly mutant mice sometimes harbored large tumor spheroids, and tail vein injections of tumor-derived cell lines established from *Nf2*$^{+/-}$;*Cdkn2a*$^{+/-}$ mice, but not from *Nf2*$^{+/-}$ or wild type mice, produced numerous tumors in the lung, suggesting an increased metastatic potential. Additionally, these mesothelioma cell lines had increased markers of cancer stem cells (CSC) and formed CSC spheroids in vitro more efficiently than tumor cells from wild type or *Nf2*$^{+/-}$ mice. The mesothelioma cells from *Nf2*$^{+/-}$;*Cdkn2a*$^{+/-}$ mice had elevated levels of c-Met expression and activation, which was partly dependent on p53-mediated regulation of the microRNA miR-34a. This signaling axis appeared to be required for tumor migration, invasiveness and maintenance of the CSC population in the

tumor cells from *Nf2*$^{+/-}$;*Cdkn2a*$^{+/-}$ mice. Collectively, these studies implicate in vivo cooperativity between *Nf2* and *Cdkn2a* losses in the development of aggressive mesotheliomas that exhibit enhanced metastatic potential and an increased CSC population in connection with p53/miR-34a-dependent activation of c-Met (Menges et al. 2014). Thus, we proposed that cooperativity between losses of *Nf2* and *Cdkn2a* plays a fundamental role in driving the highly aggressive tumorigenic phenotype considered to be a hallmark of malignant mesothelioma.

At the time of the initial studies of germline *BAP1* mutations in high-risk cancer families (Testa et al. 2011; Wiesner et al. 2011), it was unclear why mesothelioma was the main malignancy seen in some of these families (Testa et al. 2011), whereas melanocytic tumors predominated in other families (Wiesner et al. 2011). To address these questions experimentally, we generated GEM mice harboring a *Bap1* deletion (designated *Bap1*$^{+/-}$ from here on) to assess whether *Bap1* heterozygosity in the germline predisposes to asbestos-induced mesothelioma. *Bap1*$^{+/-}$ mice exhibited a significantly higher incidence of asbestos-induced mesotheliomas than wild type littermates (73% vs. 32%, respectively), and tumors arose at an accelerated rate in *Bap1*$^{+/-}$ mice as compared to wild type animals (median survival, 43 weeks vs. 55 weeks after initial exposure, respectively) (Xu et al. 2014). Mesothelioma cells from the *Bap1*$^{+/-}$ mice demonstrated biallelic inactivation of *Bap1*, consistent with the gene's proposed role as a recessive cancer susceptibility gene. Unlike the situation in wild type mice, mesothelioma cells from *Bap1*$^{+/-}$ mice did not require homozygous loss of *Cdkn2a*. Interestingly, normal mesothelial cells and mesothelioma cells from *Bap1*$^{+/-}$ mice showed down-regulation of Rb through a p16Ink4a-independent mechanism, suggesting that predisposition of *Bap1*$^{+/-}$ mice to mesothelioma may be facilitated, in part, by cooperation between Bap1 and Rb (Xu et al. 2014). In a subsequent study, a significantly higher incidence of mesothelioma was reported in *Bap1*$^{+/-}$ mice upon exposure to minimal doses of asbestos that rarely caused the disease in wild type mice, potentially connected with a deregulated inflammatory response (Napolitano et al. 2015). In another study (Fig. 8.2), we reported that knock-in mice harboring point mutations identical to those found in our first two BAP1 syndrome families (Testa et al. 2011) also demonstrated accelerated asbestos-induced mesothelioma development as compared to identically exposed wild-type littermates (Kadariya et al. 2016a). Taken together, these findings suggest that *BAP1* carriers have markedly enhanced susceptibility to the carcinogenic effects of asbestos, even minimal doses (Napolitano et al. 2015), in comparison to the general population.

Finally, we should point out that BAP1's putative role in cancer has been somewhat perplexing, because *BAP1* knockdown has been reported to inhibit cell proliferation and/or tumorigenicity. For example, Bott and colleagues reported that mesothelioma cell lines containing wild-type BAP1 showed decreased proliferation upon *BAP1* knockdown, and that the reintroduction of wild-type *BAP1* in *BAP1*-null mesothelioma cells resulted in an *increase* in cell proliferation, enigmatic findings for a putative tumor suppressor gene (Bott et al. 2011). Similarly, knockdown of *BAP1* in breast cancer was shown to *inhibit* cell proliferation, tumorigenicity, and



	Bap1 $^{+/+}$	*Bap1* $^{+/W}$	*Bap1* $^{+/L}$	Significance
Median % disease-free (weeks)	60	48	46	$p < 0.01$ for Bap1$^{+/+}$ mice vs. either *Bap1*$^{+/W}$ or *Bap1*$^{+/L}$ mice
Mesothelioma	35%	74%	71%	$p < 0.01$ for Bap1$^{+/+}$ mice vs. either *Bap1*$^{+/W}$ or *Bap1*$^{+/L}$ mice

Fig. 8.2 GEM mice with clinically relevant germline mutations of *Bap1* show increased susceptibility to the carcinogenic effects of asbestos. *Top panel,* Kaplan-Meier survival curves showing markedly decreased survival (all deaths) in asbestos-exposed *Bap1*-mutant mice than in asbestos-exposed wild type (*Bap1*$^{+/+}$) littermates. *Bap1*$^{+/W}$ mice have germline knock-in mutation identical to that of a BAP1 syndrome family from Wisconsin (W family), whereas *Bap1*$^{+/L}$ mice have the same germline mutation seen in a BAP1 syndrome family from Louisiana (L). *Lower panel,* Table summarizing statistically significant differences in disease-free time duration and mesothelioma incidence between *Bap1*$^{+/+}$ mice and *Bap1*-mutant cohorts. Modified from Kadariya et al. (2016a); Cancer Res 2016; 76:2836–2844. Copyright (2016), with permission from the American Association for Cancer Research (AACR)

metastasis (Qin et al. 2015). To examine experimentally whether germline *Bap1* mutations act as potent cancer susceptibility alleles, we monitored spontaneous tumor formation in three different heterozygous *Bap1*-mutant mouse models, including two with knock-in mutations identical to those reported in human BAP1 cancer syndrome families (Kadariya et al. 2016a). Spontaneous malignant tumors were observed in 54 of 93 (58%) *Bap1*-mutant mice versus only 4 of 43 (9%) wild-type littermates. The three *Bap1*-mutant models had a high incidence and similar spectrum of cancers, predominantly ovarian sex cord stromal tumors, lung and mammary carcinomas, as well as spindle cell tumors. Intriguingly, malignant mesotheliomas were seen in two *Bap1*-mutant mice but not in any wild-type mice, although this difference was not statistically significant. Together, these findings

provided unbiased genetic evidence that *Bap1* is indeed a bona fide tumor suppressor gene, which when mutated in the germline predisposes to a wide spectrum of tumors, including occasional mesotheliomas, although high penetrance of mesothelioma was shown to require exposure to carcinogenic fibers (Kadariya et al. 2016a).

Declaration of Interests J.R.T. has served as a genetics consultant, and in one instance, as an expert witness for the plaintiff in a case involving the role of germline mutations of *BAP1* in mesothelioma. C.W.M. and M.C. declare no potential conflict of interest.

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