HHS Public Access

Author manuscript

Cancer Genet. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:

Cancer Genet. 2015 October ; 208(10): 502–507. doi:10.1016/j.cancergen.2015.07.004.

An asbestos-exposed family with multiple cases of pleural malignant mesothelioma without inheritance of a predisposing *BAP1* mutation

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Abstract

We report a family with domestic exposure to asbestos and multiple cancers, including eight pleural malignant mesotheliomas and several other lung/pleural tumors. DNA sequence analysis revealed no evidence for an inherited mutation of *BAP1*. Sequence analysis of other potentially relevant genes, including *TP53*, *CDKN2A* and *BARD1*, also revealed no mutations. DNA microarray analysis of two mesotheliomas revealed multiple genomic imbalances including consistent losses of overlappping segments in 2q, 6q, 9p, 14q, 15q and 22q, but no losses of chromosome 3 harboring the *BAP1* locus. However, immunohistochemistry demonstrated loss of nuclear BAP1 staining in 3 of 6 mesotheliomas tested, suggesting that somatic alterations of BAP1 occurred in a subset of tumors from this family. Since mesothelioma could be confirmed in only a single generation, domestic exposure to asbestos may be the predominant cause of mesothelioma in this family. Given the existence of unspecified malignant pleural tumors and lung cancers in a prior generation, the possibility that some other tumor susceptibility or modifier gene(s) may contribute to the high incidence of mesothelioma in this family is discussed. Because the incidence of mesothelioma in this family is higher than expected even in heavily exposed asbestos workers, we conclude that both asbestos and genetic factors have played a role in the high rate of mesothelioma and potentially other pleural/lung cancers seen in this family.

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Conflict of Interest Disclosure Statement:
All other authors declare no potential conflict of interest.

Keywords

malignant mesothelioma; asbestos; familial cancer; cancer predisposition; BAP1

Introduction

Malignant mesothelioma (MM) is an uncommon cancer associated with asbestos exposure. Familial clustering in close relatives has been described in multiple reports and cannot be explained by chance alone (1). In addition to shared asbestos exposures, MM clustering in some families may suggest the contribution of inherited genes (low-penetrance alleles) in the development of this malignancy. A genetic factor predisposing to MM was recently discovered, namely germline mutation of the *BAP1* (BRCA1-associated protein 1) gene in two families with a high incidence of MM and only modest exposure to asbestos (2,3). The association of germline *BAP1* mutations with familial MM has been confirmed in a series of recent reports (4-7). Germline *BAP1* mutations have also been repeatedly associated with other tumor types, including uveal melanomas, cutaneous melanomas, benign melanocytic tumors, kidney cancers, and basal cell carcinomas (3-14). Somatic mutations and/or deletions have been described in MMs of both individuals with germline *BAP1* mutations (3) and in sporadic cases lacking a germline mutation (3,15). While Sanger sequencing revealed point mutations in 20-25% of sporadic MMs in these earlier studies (3,15), Yoshikawa et al. found biallelic gene alterations, including homozygous deletions of part or all of the *BAP1* gene as well as sequence-level mutations, in 61% of sporadic MMs (16). Subsequent studies with newer next generation and multiplex ligation-dependent probe amplification (MLPA) platforms confirmed a similarly high incidence of *BAP1* mutations in this disease (17,18). Based on the reported connection between familial MM and inheritance of a *BAP1* mutation, we decided to analyze the *BAP1* status in a family with many cases of pleural MM and asbestos exposure.

Materials and methods

Family History and Patient Samples

Over the past 18 years, since the identification of the index patient (III-2) in 1996 and the descripton of four MM cases (19), we have continued to follow this Italian family with multiple cases of MM and other malignancies (Fig. 1). Metaphase-based comparative genomic hybridization (CGH) analysis on tumor samples uncovered DNA losses involving 1p, 6q, 9p, 13q, and 14q (20), each of which is a chromosomal arm that is commonly lost in sporadic MM (21). An update of the history of this extended family was published in 2014 (2). Between 1987 and 2012, there were six women (mean age 62 yrs) and two men (mean age 67 yrs) who developed pleural MM in generation III. In addition to the eight confirmed MMs, two female family members in a prior generation (II-3 and II-8) had pleural cancers (highly suspicious of MM but unconfirmed), without radiological evidence of a primary tumor in the lung or elsewhere. The kindred had exposure to asbestos in the domestic setting; we demonstrated the presence of asbestos in lung tissue from three MM cases, using transmission electron microscopy (III-5: crocidolite, elevated fiber burden, and asbestos bodies) and optical microscopy (III-2 and III-17: asbestos bodies). Furthermore, there was

radiological and/or histological evidence of bilateral pleural plaques in six subjects (III-2, III-5, III-6, III-7, III-15 and III-17). For the remaining two MM patients (III-1 and III-4: diagnosed in 1987 and 1988, respectively), X-rays and CT scans were not available for review. Notably, family member II-4 was a brick furnace worker who had significant, lengthy exposure to asbestos occupationally and likely brought asbestos fibers home on his clothes. All seven of his children developed one or more cancer(s) in adulthood, including six with MM. Other family members who developed MM or a pleural cancer of unknown histology, including II-8, III-15 and III-17, were almost daily visitors to the home of II-4.

All MM patients in this family are deceased, and only very limited samples were available for mutation and/or immunohistochemical studies. Most specimens were formalin-fixed, paraffin-embedded (FFPE) tissues (from six MM cases). In addition, for one MM case (III-5), a pleural fluid sample was available that lacked any detectable genomic imbalances, based on a chromosome microarray analysis; moreover, ~90% of the cells were cytologically normal as determined by cytopathologic assessment of a corresponding cytospin preparation.

Sequence Analysis

Our main goal was to identify a predisposing (germline) mutation in this family, but unfortunately peripheral blood samples were unavailable; therefore, sequence analysis was performed on three FFPE specimens and one pleural fluid sample from a total of four MM cases. PCR products encompassing all exons and intronic splice regions of *BAP1* were amplified for sequencing. Primers consisted of M13-F and M13-R sequences incorporated at the 5’ end to facilitate sequencing. PCR products were gel purified and sequenced using M13-For (GTAAAACGACGGCCAGT) and M13-Rev (CAGGAAACAGCTATGAC) primers. Primers for *BAP1* and *CDKN2A* are shown in Table 1. Due to the inherent DNA fragmentation that can be found when using FFPE samples, primers were chosen to amplify small DNA fragments for Sanger sequencing. Satisfactory sequence data were obtained with all samples tested.

Immunohistochemistry

Immunohistochemical detection of BAP1 in FFPE tumor tissues was performed using a BAP1 antibody (C4, from Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (15).

Chromosome Microarray Analysis (CMA)

CMA was performed using Affymetrix Oncoscan arrays. Total genomic DNA from each test sample was digested with *Nsp*I restriction enzyme and ligated to adapters that recognize cohesive 4-basepair (bp) overhangs. A generic primer that recognizes the adapter sequence was then used to amplify the adapter-ligated DNA fragments. Amplification products were purified using magnetic beads, fragmented, biotin-labeled, and hybridized to arrays according to the manufacturer's recommendations. The hybridized array was then washed and scanned with a GeneChip Scanner 3000 7G. Intensities of probe hybridization were analyzed by using Affymetrix's GeneChip Command Console, and copy number and

genotyping analyses were performed using Affymetrix Chromosome Analysis Suite software with default settings.

Multiplex Ligation-Dependent Probe Amplification (MLPA) Analysis

To assess the possibility of a heterozygous or homozygous deletion of all or part of the *BAP1* gene, MLPA studies were perfomed on the pleural effusion from subject III-5 by myGenomics (Alpharetta, GA) using a commercially available *BAP1* MLPA kit (P417, MRC-Holland, Amsterdam, Netherlands).

Results

To ascertain the potential involvement of a *BAP1* mutation, Sanger sequencing was performed on samples from family members III-1, III-2, III-5, and III-7. No point mutations were identified. MLPA analysis of the pleural effusion from case III-5, consisting of mostly normal cells, revealed no deletions in *BAP1*. We also sequenced the *CDKN2A* gene in one specimen (III-5) to assess the possibility of a germline point mutation. No mutation was identified, although we did detect a common polymorphism (designated rs11515) in the 3'UTR that does not appear to affect the gene in any obvious way. Additionally, no mutations were identified in *TP53* in four tumor tumor tissues from this family.

CMA studies of two MMs revealed multiple genomic imbalances in each tumor. No DNA copy number losses or loss of heterozygosity of chromosome 3, site of the *BAP1* locus (3p21), were observed in either tumor. However, overlapping losses of segments in 2q, 6q, 9p, 14q, 15q and 22q were identified. Sequence analysis of the BRCA1-associated ring domain 1 gene, *BARD1*, located at 2q35 – within the region of overlapping deletions in 2q– revealed no mutation in this gene in pleural fluid cells from case III-05.

In three tumors (cases III-2, III-5, and III-6), BAP1 immunohistochemistry showed nuclear staining (Fig. 2A; III-2 not shown). The other three tumors tested (III-1, III-7 and III-17) showed loss of BAP1 nuclear staining (Fig. 2B; III-7 not shown). Cytoplasmic staining for BAP1 was observed in four tumors, two in association with nuclear positivity (III-5, III-6; Fig. 2A) and two in tumors lacking nuclear staining (III-1, III-7; Fig. 2B; III-7 not shown). Histopathological and genetic findings of six family members with MM for whom detailed imformation was available are shown in Table 2.

Discussion

Recent studies have revealed germline mutations of *BAP1* in familial MM (3-7). In the family presented here, no evidence for an inherited *BAP1* mutation was identified, although loss of nuclear BAP1 staining was observed in 3 (III-1, III-7 and III-17) of six MMs tested, suggesting that somatic genetic inactivation of *BAP1* occurred in a subset of tumors. Thus, while sequencing of *BAP1* in MMs from individuals III-1 and III-7 did not reveal any mutation, loss of nuclear BAP1 staining in these two samples, and in tumor from III-17 – which was not sequenced, may have occurred via somatic deletions of the gene, which would not be identified by Sanger sequencing. Alternatively, somatic epigenetic silencing of *BAP1* may have occurred in these cases. A recent immunohistochemistry study

demonstrated that sporadic MM patients with loss of expression of BAP1 have a prolonged survival (22). Similarly, our patients with a relatively long survival (III-1, III-7, and III-17; Table 2) showed negative nuclear staining for BAP1.

The high incidence of MM in this family occurred within one generation and did not appear to be transmitted vertically. However, the presence of other cancers in generation II leaves open the possibility of vertical transmission of a cancer susceptibility locus or loci. The family is known to have exposure to asbestos in recent generations, which presumably accounts, at least in part, for the MM clustering among blood-related relatives. By interviewing individual III-17, it emerged that she and her cousin (III-15), both of whom developed MM, had during their childhood spent a great deal of time at the proband's home (III-2), whereas individuals III-14 and III-16 did not. As noted earllier, the proband's father (II-4) had significant, lengthy exposure to asbestos occupationally. The occurrence of two pleural cancer (highly suspicious of MM) in subjects blood-unrelated between them (II-3, proband's mother and II-8, proband's aunt) highlights the possibility of shared domestic exposure, because they frequented the same home. Nevertheless, the aggregation of MM in generation III and the finding of lung cancer (II-5), liver cancer (II-6) and pleural cancer (II-8) in generation II in subjects blood-related to the proband is very striking and suggests the involvement of one or more unknown genetic factors. Since germline *BAP1* mutations have been shown to be involved in a tumor syndrome consisting of not just MM but also other tumor types such as cutaneous melanoma, and given that germline mutations of *CDKN2A* mutations have been identified in some families with a high incidence of cutaneous melanoma (23), sequencing of *CDKN2A* was performed in one case (III-5) to determine whether a germline *CDKN2A* mutation might be responsible for the high incidence of MM in this family. The identified polymorphism in *CDKN2A* is designated by the Single Nucleotide Polymorphism Database (dbSNP) as rs11515. According to information provided in the 1000 Genomes Project, this minor allele occurs with a frequency of 11.5% in the general population. Given the high frequency of this allele, it is unlikely that this variant has a role in MM susceptibility observed in the family presented here. Also, a literature search was performed, using the terms ‘rs11515’ and ‘cancer’ (http://www.ncbi.nlm.nih.gov/pubmed?cmd=search&term=rs11515++cancer), and nearly all the published work indicates that this polymorphism does not increase a person's risk for any type of cancer. Since somatic mutations of *TP53* occur in ~15% of MMs (24), and germline mutation of *TP53* predisposes to a variety of cancers in patients with Li-Fraumeni Syndrome, we also searched for mutations in this gene in MMs from four members of the family reported here. No point mutations in *TP53* were observed.

In summary, an inherited *BAP1* mutation does not appear to be involved in the high incidence of MM in the family reported here. Since MM does not appear to be transmitted vertically from one generation to the next, it appears likely that domestic asbestos exposure is the predominant cause of MM in this extended family. However, it is still possible that another susceptibility locus may contribute to the high incidence of MM and other pleural and lung cancers seen in this family. Experimental evidence with knockout mouse models demonstrate that heterozygous germline mutations of tumor suppressor genes such as *Cdkn2a* and *Bap1* are more prone to the development of asbestos-induced MM than

genetically normal (wild-type) littermates, but given a sufficient amount of exposure to these carcinogenic fibers, even wild-type mice will develop a significant number of MMs (25, 26). Given that our proband's immediate family experienced certain asbestos exposure in a domestic setting, exposure alone might have been sufficient to cause a high incidence of MM. Notably, however, the proportion of asbestos-exposed individuals who develop MM, even among those who have been heavily exposed occupationally, generally is relatively small. While familial risk for MM has not yet been fully determined to date, an increased risk has been reported among blood relatives in cohorts wherein estimates of asbestos exposure levels for families of workers are known (27). Thus, we speculate that both asbestos and genetic factors have played a role in the high rate of MM and pleural/lung cancers seen in the family presented here.

Acknowledgments

JRT and Fox Chase colleagues are supported by National Cancer Institute grants CA175691 and P30 CA06927, NIEHS grant P42 ES023720 (UPenn Superfund Research and Training Program Center), and a gift from the Local No. 14 Mesothelioma Fund of the International Association of Heat and Frost Insulators & Allied Workers. MC was also supported by a grant from the Mesothelioma Applied Research Foundation. JRT, MC, and JP have a pending patent application on *BAP1* mutation testing. JRT has provided consultation regarding genetic aspects of mesothelioma.

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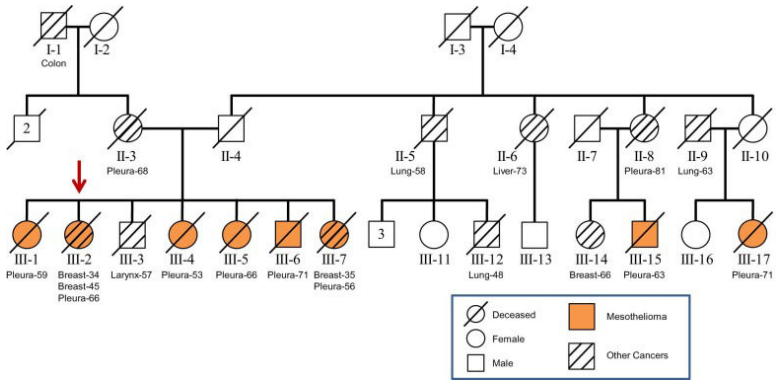

Figure 1.

Pedigree showing multiple family members with pleural malignant mesothelioma (MM) in first-degree (6 cases) and third-degree (2 cases) relatives of the proband (arrow) as well as additional cancers in relatives of both paternal and maternal lines. Other malignancies involved the pleura (II-3 and II-8), larynx (III-3), lung (II-5, II-9, and III-12), breast (III-14), liver (II-6), and colon (I-1). Breast cancer also occurred in two MM patients (III-2, III-7) at early ages.

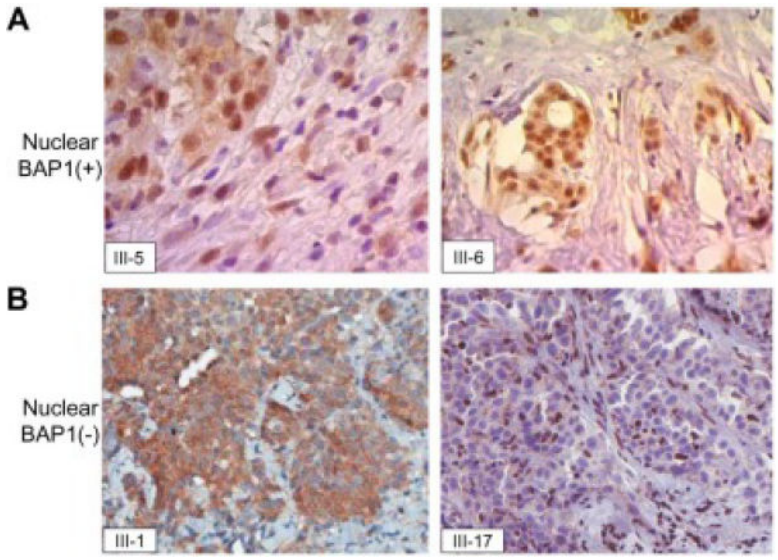

Figure 2.

BAP1 immunostaining of MM cells. A) BAP1 nuclear positive staining in cases III-5 and III-6. B) Lack of BAP1 nuclear staining in MM cells from cases III-1 and III-17. Note cytoplasmic immunostaining in MM cells associated either with BAP1 nuclear positivity (III-5 and III-6 in A) or negativity (III-1 in B); note BAP1 nuclear staining in normal stromal cells and lymphocytes (III-17 in B).

Table 1

Bap1 Exons	Forward primer	Reverse Primer
1	GTAAAACGACGGCCAGTGAGCCCAGAGGCGGAGCAG	CAGGAAACAGCTATGACGTCAGGCAGGCGCGTC
2	GTAAAACGACGGCCAGTGACGCGCCTGCCTGAC	CAGGAAACAGCTATGACCTTGACACCTGCGATGAGGAA
3	GTAAAACGACGGCCAGTCTCACTCATCAGGGGCTGTC	CAGGAAACAGCTATGACCAGCACTCTGGGTGTAAGGG
4	GTAAAACGACGGCCAGTAGTGATGACGCAGTGCAAAG	CAGGAAACAGCTATGACCTCCATTTCCACTTCCCAAG
5	GTAAAACGACGGCCAGTGAGGGGTGCTGTGTATGGG	CAGGAAACAGCTATGACCTGTGAGCCAGGATGAAGGC
6	GTAAAACGACGGCCAGTTGTGTTCCTTCCGATTCCTGG	CAGGAAACAGCTATGACAAACAGAGTCAGGGCCCAAAA
7	GTAAAACGACGGCCAGTGGTGGGAGTAGGGGGAGTATC	CAGGAAACAGCTATGACGGTAGGCAGAGACACCCAAC
8	GTAAAACGACGGCCAGTCAGGGTTTCCTTCTCGCTGA	CAGGAAACAGCTATGACCCCAAAGTAGGTACAGCTCCAG
9	GTAAAACGACGGCCAGTCCTGCCAGGATATCTGCCTC	CAGGAAACAGCTATGACTCAGAGACAAATGCTGTGGG
10	GTAAAACGACGGCCAGTAGGTCCTCAGCCCTTAGCTATT	CAGGAAACAGCTATGACTCAGACATTAGCGGGTGGCTC
11	GTAAAACGACGGCCAGTGGAGGTCCTGCCTGTGTTC	CAGGAAACAGCTATGACTCAAGTAGAGAATCCTGCAAGGG
12	GTAAAACGACGGCCAGTCCGAGCAGCACTTGTTTGTA	CAGGAAACAGCTATGACGGGATCCGAAGCACCTAGAAC
13a	GTAAAACGACGGCCAGTCGTTCCCTTGCTTCACATCTTCT	CAGGAAACAGCTATGACCCGCTGCTAGTCTTGATGGA
13b	GTAAAACGACGGCCAGTTGGCTGAGAAGCTCAAAGAGTC	CAGGAAACAGCTATGACCGCGTCGGGTTGGCTG
13c	GTAAAACGACGGCCAGTAGTACAGACACGGCCTCTGA	CAGGAAACAGCTATGACGGTTGTAGCGTATGCAGTCAAC
13d	GTAAAACGACGGCCAGTCCCACATCTCCAAGGTGCTT	CAGGAAACAGCTATGACCCTCCTGGGTGCACCAA
14	GTAAAACGACGGCCAGTAAAGTGTCCTGCACTCTGATGATT	CAGGAAACAGCTATGACGCCTTACCCTCTGCCAGGATTA
15	GTAAAACGACGGCCAGTGCATGGACTCGCTGCTCATC	CAGGAAACAGCTATGACTGGGTCCTTCTCTGGTCATCAA
16	GTAAAACGACGGCCAGTTCTGGCAAGATTGGCTCCAG	CAGGAAACAGCTATGACCTCAGCAGGGCATTCCAGTTA
17	GTAAAACGACGGCCAGTCATGAGAGCCTCAGCTCCT	CAGGAAACAGCTATGACGCAAGAGTGGGCTGCAGAG

CDKN2A Exons	Forward primer	Reverse Primer
1b	GTAAAACGACGGCCAGTGCGCGCTCAGGGAAGG	CAGGAAACAGCTATGACACAAAACAAGTGCCGAATGC
1a	GTAAAACGACGGCCAGTGAGCGCGGCTGGGAG	CAGGAAACAGCTATGACCAGAGTCGCCCGCCATC
2	GTAAAACGACGGCCAGTTTAGACACCTGGGGCTTGTG	CAGGAAACAGCTATGACTGGAAGCTCTCAGGGTACAA
3	GTAAAACGACGGCCAGTTTTCAATGCCGGTAGGGACG	CAGGAAACAGCTATGACAAACGATGCTGTCTTCCATGC

Table 2

Histopathological and genetic findings in six family members with MM.

Case	Histology	Age at dx.	Survival (months)	BAP1 nuclear staining	*BAP1* tumor sequencing status	*CDKN2A* sequencing status	Chromosome Microarray Losses§	Chromosome Microarray Gains§
III-1	Epithelial	59	34	negative	No mutation	Not done	X, 2q, 6q13-qter, 8pter-q12, 9p, 9q, 14, 15q11-q21.1, 22	6pter-q13, 8q12-q24, 15q21.1-qter
III-2	Sarcomatoid (desmoplastic)	66	11	positive	No mutation	Not done	Not done	Not done
III-5	Mixed	66	9	positive	No mutation	Polymorphism 500C>G in 3′UTR	1p, 1p, 2q, 4, 6q, 9p, 10q, 11p, 14, 15q11-q21.1, 18p, 20p, 22	15q21.1-qter
III-6	Epithelial	71	7	positive	Not done	Not done	Not done	Not done
III-7	Epithelial	56	50	negative	No mutation	Not done	Not done	Not done
III-17	Epithelial	71	34	negative	Not done	Not done	Not done	Not done