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Mesothelioma Families without Inheritance of a *BAP1* Predisposing Mutation

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Running title:

BAP1-unmutated Familial Mesothelioma

Abstract

Familial malignant mesothelioma clusters are ideal candidates to explore *BAP1* genomic status as a predisposing risk factor. We report data on *BAP1* analysis in four families with multiple mesothelioma cases to investigate possible *BAP1* alterations associated with an inherited cancer syndrome. We also recorded family history of cancer and assessed asbestos exposure. By genomic direct sequencing, we found no evidence of a *BAP1* germline mutation in tumor DNA samples (one mesothelioma per family: n=3 epithelioid; n=1 biphasic). On the other hand, we identified a novel *BAP1* somatic alteration (c.329_335delinsTC) in exon 5 (n=1 biphasic), and we hypothesized the occurrence of somatic inactivating events not identifiable by sequencing in the other cases (n=3 epithelioid), as demonstrated by the loss of nuclear BAP1 immunostaining. History of other cancers was in sites not typical of the *BAP1* cancer syndrome. Asbestos exposure was occupational (n=2 clusters), household (n=1), and unknown (n=1). These family units without inheritance of a *BAP1* predisposing mutation expand the number of unmutated germline *BAP1* families with multiple mesothelioma cases. This suggests that besides the exposure to asbestos other currently unknown genetic or epigenetic factors may be responsible for the high incidence of mesothelioma in *BAP1*-unmutated families.

Key words: malignant mesothelioma; asbestos; familial cancer; *BAP1* germline mutation; *BAP1* somatic mutation

Introduction

Malignant mesothelioma (MM) occurs as sporadic cancer (one affected individual in the family) generally in association with asbestos exposure. Its occurrence as clusters among blood-relatives (two or more individuals are affected) suggests increased genetic susceptibility.

BAP1 (BRCA1-associated protein 1) is a tumor suppressor gene located on chromosome 3p21.1, encoding for a deubiquitinating enzyme that requires nuclear localization [1]. *BAP1* regulates cell cycle control, target genes transcription, and DNA damage repair [2]. The germline mutation in the *BAP1* gene is associated with a hereditary tumor predisposition syndrome (BAP1-TPDS, OMIM#614327) that occurs in family members with several cancer types: MM, uveal/cutaneous melanoma, renal cell carcinoma, basal cell carcinoma and other cancers [3-16]. As of March 2016, forty-six families with multiple cases of MM have been analyzed for *BAP1* germline alterations. Some families were proven to carry *BAP1* germline alterations in association with family history of either typical BAP1-TPDS cancers [9, 10, 17] or not typical BAP1-TPDS cancers [9, 10, 18, 19]. In addition, families with multiple cases of MM without inheritance of a predisposing germline *BAP1* mutation have been reported [20]. Germline mutations described to date are nucleotide-level mutations.

Germline *BAP1* alterations are a rare event in sporadic MM [10, 18, 21-23]. The majority are benign polymorphisms (SNPs) or variants of unknown significance (VUS) [4, 5, 23]. Those of pathogenic significance have been described in association with BAP1-TPDS malignancies [4, 21, 24-28]. On the other hand, somatic mutations of *BAP1* are a frequent event in MM [26, 28, 29], with a variable rate of occurrence depending on the molecular assay used. Somatic mutations described to date are both nucleotide-level and chromosomal mutations [30].

MM familial clusters among blood-relatives are ideal candidates to identify *BAP1* germline carriers associated with the putative BAP1 cancer syndrome. Familial mesothelioma is defined by the diagnosis of two or more blood-relatives affected by MM. Over the past 30 years, we have detected 13 MM familial clusters in a population-based study [19]. One of these, an asbestos-exposed family with multiple cases of pleural MM and without inheritance of a predisposing *BAP1* mutation has been recently reported [30]. We have decided to investigate further if additional MM clusters are associated or not with inheritance of a *BAP1* germline mutation.

Materials and Methods

Familial cases and Samples

Families were identified through a regional section of the Italian network of the Mesothelioma Registry (www.deplazio.net) and a pathology-based archive. We selected 4 clusters (family C, D, G, and O) whose pedigrees were previously reported [30] on the basis of availability of samples from at least two first-degree relatives with MM. Peripheral blood samples were unavailable, and only limited formalin-fixed paraffin-embedded (FFPE) tissues specimens were available for mutational and immunohistochemical (IHC) studies. As indicator of a genetic predisposition to cancer, we assessed personal and family history of other primary malignancies that was positive in 3 families (stomach, pancreas, lung, prostate, colon). Asbestos exposures were assessed by interviewing patients and/or the next of kin through a standard questionnaire and coded according to a classification relating to modalities of exposure as 'occupational (n=2 families) household (n=1) and unknown (n=1)' (<https://www.inail.it>).

Sequencing analysis

BAP1 mutational analysis was performed on one case for each family considering the index cases [31]. Since DNA was not available from peripheral blood, genomic DNA was obtained from FFPE tissues (3 epithelioid and 1 biphasic MM), using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Neoplastic tissue was selected to be analyzed separately from the normal tissue portions. For all samples, we made sure that the neoplastic cell population was above 80%. Furthermore, the biphasic MM was manually microdissected in the two neoplastic populations (epithelioid and sarcomatoid) that were analyzed separately.

The rationale of the molecular approach was the following: 1) to analyze the tumor-only population in the index case since tumor-only testing can reveal germline mutation [26, 28]; 2) in the case of a *BAP1* mutation detection, to expand the mutational analysis, matching normal and tumor DNA for a correct assessment of a somatic versus a germline mutation; 3) in the case of a germline *BAP1* mutation, to expand the mutational analysis, sequencing normal DNA of the blood-relative.

The entire *BAP1* coding sequence was amplified with primers designed on the flanking intronic/exonic regions using Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>) Because of the

fragmentation of DNA fixed in formalin, 32 amplicons were used to amplify small DNA fragments.

Primers and PCR conditions are available on request. Bidirectional Sanger sequencing was performed by an external commercial service using standard protocols (Eurofins MWG Operon, Ebesberg, Germany). Electropherogram results were analyzed by using FinchTV software (Geospiza, Seattle, WA) and BLASTN tool for alignment analyses to the human reference genome

(<https://blast.ncbi.nlm.nih.gov/>). Putative *BAP1* variants were validated via bidirectional re-sequencing of an independent PCR amplification. Variants were annotated according to the longest isoform RefSeqs from the Genome Reference Consortium Human Build 37.3 (NM_004656.3) and described according to the Human Genome Variation Society guidelines (HGVS, <http://www.hgvs.org/mutnomen/>). Putative genetic alterations were classified as polymorphism (neutral or germinal) or somatic pathogenic (or of unknown pathogenicity) by considering the following: (1) the reported frequency in the following polymorphic databases: dbSNP (build 131; <http://www.ncbi.nlm.nih.gov/projects/SNP/>), 1000 Genomes (<http://www.1000genomes.org/>), NHLBI GO ESP (<http://evs.gs.washington.edu/EVS/>); (2) the presence in somatic mutational COSMIC databases (<http://cancer.sanger.ac.uk/cosmic>); (3) evolutionary conservation of the involved residue at both nucleotide and amino acid level, calculated by multiple alignments of 46 vertebrate species and (4) *in-silico* prediction of functional effect by SIFT (<http://sift.jcvi.org/>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>), SNPs3D (<http://www.snps3d.org/>), Panther (<http://www.pantherdb.org/>), MutPred (<http://mutpred.mutdb.org/>), NetGene (<http://www.cbs.dtu.dk/services/NetGene2/>).

Immunohistochemical analysis

IHC detection of BAP1 was performed using a primary anti-human BAP1 antibody (C-4, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an automated immunostainer (BenchMark, Ventana Medical System, Tucson, AZ) on FFPE tumor sections in 8 out of 9 MM cases (6 epithelioid, 1 sarcomatoid, and 1 biphasic MM). One case (1 sarcomatoid, G-II-9) was not available. Tumor tissues were assessed as positive or negative on the basis of the presence or absence of BAP1 nuclear staining. It was also assessed the staining of the cytoplasm (and intensity, as strong or faint). The fraction of

BAP1 positive stained cells was evaluated for nuclear and cytoplasmic sub-cellular localization (<25%, 25-75% or >75%). Normal stromal endothelial or lymphoid cells served as positive internal control.

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Results

BAP1 sequencing and IHC findings are summarized in Table 1 and illustrated in Figures 1 and 2. Sequencing analysis showed no *BAP1* gene mutation neither in heterozygosis nor in homozygosis in three of the four index cases analyzed (C-II-2, G-II-6 and O-III-1, all epithelioid MM). Conversely, in the fourth index case (D-II-4, biphasic MM), a complex non-frameshift insertion/deletion (InDel) in exon 5 (c.329_335delinsTC) was detected in heterozygosis, as shown in Figure 2C. This leads to the loss of two aminoacids (p.Pro110_Ser111del) resulting in a shorter BAP1 protein of 727 aminoacids, and involves the UCH (Ubiquitin carboxyl-terminal hydrolase, UniProtKB - Q92560) catalytic domain. This alteration was identified both in the epithelioid and in the sarcomatoid component with two different allelic frequencies. The electropherogram relating to sarcomatoid cells showed clear-cut peak heights lower than those of epithelioid cells (Figure 2C). The mutational analysis of normal tissue from this case lacked the *BAP1* alteration, indicating a somatic origin (Figure 2C).

By IHC, a complete loss of nuclear BAP1 staining was seen in all epithelioid MM (C-II-1, C-II-2, D-II-3, G-II-6 and O-III-1) and in the epithelioid cells of biphasic MM (D-II-4). BAP1 nuclear immunoreactivity was retained only in the sarcomatoid MM (G-II-8) and in sarcomatoid cells of the biphasic MM (D-II-4), with a variable percentage of stained cells. BAP1 cytoplasmic staining was observed in all epithelioid tumors, with a variable number of stained cells, uneven intensity and a coarse granular pattern. Similarly, BAP1 cytoplasmic staining was observed in the fraction of sarcomatoid cells that lost their nuclear reactivity.

Discussion

In the present study, we demonstrated that no germline *BAP1* alterations could be identified in the tissues of four families affected by multiple MM, thus speculating that in these individuals there is no evidence of an inheritance linked to *BAP1* mutations. The absence of a germline mutation in coding regions and intron-exon boundaries of *BAP1* gene was proven in the normal tissue DNA belonging to one index case (D-II-4) and by excluding single point mutations and small InDels in tumor DNA belonging to the other three index cases (C-II-2, O-III-1, G-II-6). We cannot exclude large deletions of *BAP1* that are undetectable by Sanger sequencing [3, 9, 11, 19]. However, large deletions of *BAP1* have never been detected in germline configuration in families either with multiple MM [3, 9, 11, 32-37] or without MM [26, 28, 29]. The loss of nuclear BAP1 immunostaining suggests that these tumors may harbor somatic alterations of the *BAP1* gene, a frequent event in sporadic epithelial MM [21, 24, 26, 27]. Although most *BAP1* somatic mutations are nucleotide-level mutations, additional technical approaches would have been necessary to detect gross deletions at exon level. Unfortunately, a limit of the present study includes the unavailability of sufficient amount of tumor DNA to perform a comprehensive analysis of *BAP1* gene alterations.

Furthermore, we identified -at the somatic level only- a complex InDel p.Pro110_Ser111del (exon 5) in case D-II-4 that has never been previously reported. This subject had a history of occupational asbestos exposure as his brother (Table 1, case D-II-3). Somatic mutations in exon 5 occur in about 5% of all tumors (COSMIC; <http://cancer.sanger.ac.uk/cosmic>) and in 6% considering sporadic MM, only [38]. This novel *BAP1* alteration leads to a probably impaired deubiquitinase activity of BAP1 protein, suggesting its pathogenic role. This hypothesis is supported by *in-silico* function predictions and by the absence of such *BAP1* variant in control populations (MAF<0.01). Interestingly, the same mutation is present in the DNA of both epithelial and sarcomatoid MM components, with a heterogeneous allelic frequency. Loss of nuclear BAP1 and presence of cytoplasmic staining in epithelial cells is in line with our identified mutation in the UCH domain, which promotes BAP1 cytoplasmic retention [39]. Instead, the retaining of BAP1 nuclear staining in a prevailing fraction of neoplastic spindle cells (Figure 2C and Table 2) is in line with a lower allelic frequency of mutant alleles in sarcomatoid MM DNA compared to that of the epithelial DNA, that might

be due to an heterogeneous population of mutated and wild type sarcomatoid cells. Alternatively, loss of heterozygosity (LOH) could be taken into account. Indeed, Nasu and co-workers found different and sometimes-coexisting mechanisms of gene alteration in their MM series, depending on the assay used. In two samples, they detected large deletions in one allele by MLPA and nucleotide-level mutations in the other allele by Sanger sequencing, in association with loss of BAP1 nuclear protein by IHC, thus supporting LOH [26]. Cigognetti and co-workers provided evidence of homozygous deletions in association with heterozygous deletions of the *BAP1* locus by FISH analysis in a few cases among their IHC BAP1-negative MM series [40]. Intra-tumor differences in *BAP1* LOH were recently reported in sarcomatoid and carcinomatous elements in clear renal cell carcinomas [41].

We were not able to find any somatic mutations in the other tumors. However, BAP1 IHC results give us some further information. Loss of BAP1 nuclear staining in association with a cytoplasmic staining could indicate a mutant *BAP1* allele [42, 43]. The accumulation of BAP1 mutant protein in the cell could inactivate the wild-type protein with a consequently altered protein localization and/or loss of function. This is likely due to a dominant-negative effect of the mutant protein, a distinctive characteristic of somatic events of tumor suppressor genes [44], in the same way as also discussed for *BAP1* [26]. Failing to detect this mutation by Sanger sequencing is not a surprise, based on the results of Nasu et al. [26]. There are several possible explanations. First of all, a large *BAP1* deletion (or insertion) which can involve the portion of gene codifying the nuclear localization signal (exons 16-17) or the UCH domain. On the other hand, this large deletion (or insertion) could be associated with LOH of the other allele thus leading to a cytoplasmic accumulation of the BAP1 deleted protein. BAP1 could be modified by post-translational modifications inhibiting its autodeubiquitination activity that lead to a cytoplasmic sequestration of BAP1 mediated by regulatory proteins, without any export signal in *BAP1* gene. As a matter of fact, several ubiquitination sites of *BAP1* were identified in vitro study by Mashtalir and coworkers [38].

Regarding family members with MM whose DNA was not sequenced (Table 1), by inference we can likely exclude *BAP1* germline mutation being first-degree relatives affected by the same cancer of index cases whose tumor DNA was sequenced and found not mutated. IHC findings in three epithelioid MM cases (C-II-1, D-II-3, O-II-3) suggest that the loss of nuclear staining occurred via somatic mutation

(see Figure 1). BAP1 nuclear staining retained in one sarcomatoid MM (G-II-8) is in agreement with other studies reporting data of positive BAP1 staining on sarcomatoid MM [25] and on the absence of *BAP1* mutation [9, 10, 18, 19]. Considering all families with multiple cases of MM analyzed to date for *BAP1* germline mutations (see Table 2), those described in the present study without a predisposing germline *BAP1* mutation are similar to those reported by other investigators [9, 10, 19]. Most familial clusters have asbestos exposure, and a family history of malignancies other than those typical of TPDS [9]. This suggests that other genetic or epigenetic factors may be responsible for the high incidence of MM in these families, besides asbestos exposure. By contrast, most families with multiple cases of MM and with *BAP1* germline mutations have typical BAP1-TPDS malignancies (uveal melanoma, skin melanoma, renal cell carcinoma, basal cell carcinoma, etc.) and are heterogeneously linked to asbestos exposure. In our families, no TPDS syndrome malignancies were recorded. In the literature, there are significant differences between unmutated and mutated *BAP1* gene families for history of TPDS malignancies ($p=0.00$, Fisher-Yates test), history of other cancer ($p=0.014$), and history of asbestos exposure ($p=0.008$). When asbestos exposure is unknown or when this information is missing [4, 6, 9, 19], the differences are of borderline statistical significance ($p=0.046$). Heterogeneity of *BAP1* status in familial MM (mutated *versus* unmutated *BAP1*) can be attributed to a bias in patient recruitment. Investigators have examined *BAP1* in families: i) with high incidence of MM [3, 7, 9, 11, 14]; ii) referred to genetic or cancer centers for uveal melanoma [9, 12, 15] or other melanocytic tumors [13, 17]; iii) affected by basal cell carcinoma [16]; iv) having a family history suggestive of the BAP1 cancer syndrome [18]. Other families were examined as part of asbestos surveillance program [10]. Our families were actively searched based on the Italian Mesothelioma Registry, and detected because the proband (index case) and at least one blood-relative were diagnosed with MM according to the Registry available information. Therefore, the recruitment of cases is on a population basis, as those reported by other Italian investigators [10, 18, 19].

The present study contributes to understanding the role of *BAP1* in familial MM, expanding the number of unmutated-*BAP1* families, and to suggesting that other genes may determine cancer susceptibility in some familial clusters with multiple MM. Very recently, a large study analyzing

germline *CDKN2A* and *BAP1* mutations in families with both melanoma and mesothelioma, melanoma (without mesothelioma), mesothelioma (without melanoma) has been published [45]. All *BAP1* sequenced families with mesothelioma (but without melanoma) showed a wild-type configuration of the gene and asbestos exposure, in agreement with our present findings. Moreover, the findings in the unmutated-*BAP1* families described in this study and in others reported to date provide evidence of the importance of exposure to asbestos (Table 2 and [45]). As far as *BAP1* gene is concerned in somatic configuration, these familial MM cases should be considered equivalent to “sporadic” MM, because they harbor non-inherited genomic *BAP1* alterations, a common event in MM, especially of the epithelial and biphasic types.

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Legend to Figures

Figure 1 BAP1 immunostaining (brown chromogen). A, B, and C) Epithelioid mesothelioma cells show the loss of nuclear expression associated with a granular staining of the cytoplasm (cases: C-II-1; C-II-2; G-II-6). D) Sarcomatoid mesothelioma cells show staining heterogeneity: cells retaining nuclear staining are intermingled with others showing the loss of nuclear expression, and strong and diffuse cytoplasmic reactivity (Case: G-II-8).

Figure 2 BAP1 immunostaining and BAP1 sequencing in case D-II-4 (biphasic mesothelioma). A) Absence of nuclear staining in epithelioid cells. B) Retained nuclear staining in >75% of sarcomatoid cells. C) Forward electropherograms depicting a non-frameshift InDel of *BAP1* in heterozygosis (exon 5: c.329_335delinsTC) in both epithelioid and sarcomatoid DNA with different allelic frequency. Normal patient's tissue and wild-type sequences of exon 5 are shown for comparison. WT, wild-type sequence; N, normal tissue DNA; T-Epit, epithelial DNA; T-Sarc, sarcomatoid DNA.

Table 1 Clinical information, histopathological and genetic findings in 4 families with multiple cases of malignant mesothelioma

Family ^a	Case	Gender/Age at diagnosis	Histology		BAP1 tumor sequencing status	BAP1 Immunostaining			
						Nucleus	% stained cells	Cytoplasm	% stained cells
C ⁰	II-1	M-69	Epithelioid		No testing done	Negative	-	Positive-strong	>75
	II-2	M-66	Epithelioid		No mutation	Negative	-	Positive-strong	>75
D ⁰	II-3	M-66	Epithelioid		No testing done	Negative	-	Positive-faint	>75
	II-4	M-70	Biphasic	epithelioid	c.329_335delinsTC	Negative	-	Positive-faint	25-75
				sarcomatoid	c.329_335delinsTC ^b	Negative	25 ^c	Positive-faint	25
G ^H	II-6	F-63	Epithelioid		No mutation	Negative	-	Positive-strong	>75
	II-8	M-55	Sarcomatoid		No testing done	Negative	25-75 ^d	Positive-strong	25-75
	II-9	M-56	Sarcomatoid		No testing done ^e				
O ^{Uk}	II-3	M-78	Epithelioid ^f		No testing done	Negative	-	Positive-faint	>75
	III-1	F-45	Epithelioid		No mutation	Negative	-	Positive-faint	>75

^aAscoli, et al 2014 [29]; Asbestos exposure circumstances: O, occupational; H, household; Uk, unknown (interview available)

^bLower allelic frequency in respect to the epithelioid component of the same tumor (Figure 2C)

^cThe fraction of cells with retained BAP1 nuclear staining showed negative cytoplasmic reactivity (Figure 2B)

^dThe fraction of cells with retained BAP1 nuclear staining showed negative cytoplasmic reactivity (Figure 1D)

^eNo tissue specimen was available

^fPeritoneal mesothelioma

F, female; M, male

TABLE 2. Families with Multiple cases of Malignant Mesothelioma, History of Cancer and Asbestos Exposure Analyzed for BAP1 Germline Mutations

	Families (n=46)	BAP1 unmutated (n=20)	Reference(s)	BAP1 mutated (n=26)	Reference(s)
History of cancer					
Common TPDS ^a cancer	21	1	[8]	20	[2-6, 8, 10-16, 22]
Other cancer ^b	12	9	[8-9, 18] present study	3	[8-9, 22]
Negative	5	2	[8-9] present study	3	[7-9]
Missing data	8	8	[17]	0	-
Asbestos Exposure					
Yes	23	16	[9, 17-18] present study	7	[3-4, 9, 13-14, 22]
No	3	0	-	3	[5, 7, 15]
Missing data	20	4	[8] present study	16	[2, 6, 8, 10-12, 16]

^a TPDS, tumor predisposition syndrome (OMIM, #614327)

^b Leukemia, prostate, lung, thyroid, breast, larynx, colon, stomach, pancreas (BAP1 unmutated families); Breast, salivary gland, stomach, throat (BAP1 mutated families). Some authors report breast cancer as potential BAP1 syndrome-related cancer [22]