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Puzzling Results from *BAP1* Germline Mutations Analysis in a Group of Asbestos-Exposed Patients in a High-risk Area of Northeast Italy

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Abstract. *Background: Germline mutations of the oncosuppressor gene breast cancer 1-associated protein 1 (BAP1) were recently related to an autosomal-dominant tumor predisposition syndrome (BAP1-TPDS), characterized by uveal melanoma, malignant mesothelioma (MM), cutaneous melanoma, and other malignancies. The demonstration that BAP1 mutations are strongly associated with MM has provided a real breakthrough in the study of genetic predisposition in MM, that may explain why only a fraction of asbestos-exposed individuals go on to develop MM. Materials and Methods: To evaluate the possible role of BAP1 mutations in the epidemiology of sporadic MM, and their relationship with asbestos exposure, we determined the prevalence of germline BAP1 mutations by the Sanger method in a group of 29 asbestos-exposed patients, 21 of which were diagnosed with MM. They were residents of Trieste, a ship-building town in Northeast Italy with a very high incidence of mesothelioma. Results: We identified non-obviously pathogenetic germline sequence variants of BAP1 in 3/29 patients and in 2/21 MM cases (10%). Conclusion: Non obviously pathogenic germline sequence variants of BAP1 were found. Nevertheless, limitations of predictive web tools allowed us to comment on some interesting peculiarities of our findings.*

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Malignant mesothelioma (MM) is a highly aggressive malignancy that arises from mesothelial cells of serosal surfaces, primarily the pleura and peritoneum, and less often the pericardium or *tunica vaginalis*, with a median survival of 1 year from diagnosis (1). The single most important risk factor for MM is exposure to asbestos (2-4). Nevertheless, the observation that only a fraction (10-17%) of asbestos-exposed individuals actually go on to develop MM (5) and the identification of clusters of MM cases within certain families suggest that genetics influence carcinogenesis from mineral fibers (6-9). The demonstration that mutations in the tumor-suppressor gene *breast cancer 1-associated protein 1* (*BAP1*) are strongly associated with MM has provided a real breakthrough in the study of genetic predisposition to MM (8). In families with BAP1 mutation, there is a dramatically increased incidence of malignant tumors overall, often developed at an earlier age than observed in the general population. Recently, germline mutations of *BAP1* have been related to a hereditary tumor predisposition syndrome characterized by uveal melanoma, MM, cutaneous melanoma, and possibly several other malignancies (8-12).

The molecular functions of BAP1, as well as the penetrance and the phenotypic spectrum of germline mutations of the gene, however, have still to be clarified, as does the role of gene–lifestyle/environmental interactions in the development of these tumors. To give our contribution in shedding light on the possible role of *BAP1* mutations in the epidemiology of sporadic MM, and their relationship with asbestos exposure, we determined the prevalence of germline *BAP1* mutations in an unselected sample of 29 asbestos-exposed patients, 21 of which were diagnosed with MM. The patients were residents of Trieste, a ship-building town of about 200,000 inhabitants in Northeast Italy that may be considered as hyperendemic for

mesothelioma. The standardized incidence rate (European population) due to mesothelioma among male residents in the provinces of Trieste and Gorizia was 13.2 (13, 14) compared to national rates ranging from 3.2 and 2.0 per 100,000 persons per year (15).

Materials and Methods

Tissue samples. Tissues samples were obtained from 29 autopsy cases randomly selected from the archives of the Institute of Forensic Medicine of the University of Trieste. All the cases were subjected to legal medical advice to establish the cause of death and a possible correlation to professional exposure to asbestos and to the presence of asbestos-related diseases. Clinical features were extracted including sex, age at death, asbestos exposure, cause of death and concurrent diseases including malignancies, and in patients with MM, age at diagnosis, histology, stage, and treatment including surgery, chemotherapy, and radiation, as well as the duration of survival.

MM cases were histologically diagnosed either during their clinical course or at post-mortem examination, according to the standard histological and immunohistochemical criteria, and classified according to the WHO classification of pleural tumors (16). Diagnosis of asbestosis was made according to the criteria reported by the Asbestosis Committee of the College of American Pathologists and Pulmonary Pathology Society (17).

The quantification of asbestos bodies in the lung parenchyma was performed in accordance with the method of Smith and Naylor with slight modifications (18).

Nucleic acid extraction and polymerase chain reaction (PCR) amplification. Genomic DNA of 29 patients was extracted from frozen tissues using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. PCR amplification of 17 *BAP1* exons and of all their exon-intron boundaries (NCBI accession number: NM_004656.3) was performed as previously described by Bortot *et al*. (19). Briefly, primer design was performed using Primer3web v4.0.0 on-line software (20, 21) (Table I). All amplifications were carried out using KAPA2G Fast ReadyMix (Kapa Biosystems, Cape Town, South Africa) in a 96-well PCR plate and sharing a common annealing temperature in a two-cycle step touchdown protocol: primary, an initial denaturation step at 96˚C for 3 minutes was followed by a touchdown step planning to decrease the temperature by 0.5˚C/cycle, through 10 cycles: 95˚C for 15 seconds, 62˚C for 15 sec, and 72˚C for 1 second; secondary, an endpoint-PCR step through 30 cycles was performed: 95˚C for 10 sec, 59˚C for 10 sec, and 72˚C for 1 second.

Total RNA was isolated from frozen tissues using EuroGOLD Total RNA Kit (EuroClone, Milan, Italy). Reverse transcription (RT) reaction was performed using the GoScript Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacture's protocol. Briefly, RT was performed in a reaction volume of 5 μl containing 5 μg of total RNA and 15 pM of each sequence-specific primer (Table I). Primers were designed using the on-line NCBI Primer-BLAST tool (22) and their specificity was checked on the RefSeq mRNA database. Finally, cDNA PCR amplification was performed according to the above described touchdown protocol using the same sequence-specific primers of each RT reaction.

Sanger sequencing and mutation data handling. PCR products were purified using Illustra ExoStar 1-Step kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol. Sequencing of the PCR fragments was carried out using BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Foster City, CA, USA) following the manufacturer's instructions. Automated electrophoresis was performed on an ABI 3500Dx Genetic Analyzer and the sequencing results were analyzed using SeqScape v2.7 software (Life Technologies).

All identified variants were annotated using a custom bioinformatics pipeline basic on Annovar software (23) and referring to several databases as reported in Table II. To detect alterations in exon-intron boundary regions and splicing motifs due to nucleotide changes, the bioinformatics tools for splice site prediction HSF (24), NNSplice (25), NetGene (26) and SPANR (27) were used. All selected variants were reconfirmed either on genomic DNA or, in case of splicing site mutations, on cDNA by Sanger sequencing.

An *in silico* mutation analysis was performed for the non-synonymous selected mutation to predict if the amino acid substitution involves a structural modification of BAP1 protein. A web-based resource for template-based modeling, Phyre2 v2.0 (28), was used. Wild-type amino acid sequence (NCBI accession number: NP_004647.1) was submitted through the intensive method in order to obtain a pdb structure. The outcome wild-type protein structure present a structural homology at 100% of confidence with the ubiquitin carboxyl-terminal hydrolase isozyme l5 (PDB ID: 3IHR) from amino acids 5 to 238 of the query sequence. The modeling of the remaining amino acid sequence was predicted. As a second attempt, the mutated amino acid sequence was aligned using the same tool on the wild-type protein obtained previously. Analyses of all docked poses were performed using the molecular visualization software UCSF-Chimera v1.10.2 (29).

Multiplex ligation-dependent probe amplification (MLPA). The *BAP1* gene was analyzed by MLPA (30) using the SALSA MLPA P471-BAP1 - LOT1011 (B1) probe mix (MRC Holland, Amsterdam, the Netherlands). MLPA reactions were performed , using 75 ng of genomic DNA according to the manufacturer's instructions. The products were separated by capillary electrophoresis on an ABI 3130XL Genetic Analyzer and controlled using the Gene Mapper v4.0 software (Life Technologies). Copy number variations were predicted for each patient using the Coffalyzer.Net v140721 software (MRC Holland).

Results

Of 29 patients, there were 26 men (90%) and three women (10%). At the time of death, the patients' ages ranged from 58 to 91 years (mean±SD: 76±9 years). They included 21 cases of MM (20 pleural and one peritoneal), two cases of squamous cell carcinoma and one of adenocarcinoma of the lung, and one case of squamous cell carcinoma of the oral cavity metastasized to the lungs. Four patients had non-neoplastic diseases including two cases of asbestosis, one of pulmonary fibrosis, who had also an incidental renal cell carcinoma, and one had pleural plaques only.

Table I. *List of all primers used for Sanger sequencing of DNA and cDNA target regions.*

Exon number	Forward 5' to 3'	Reverse 5' to 3'
BAP1, NG_031859.1		
1	gttcgccttcgagcgcatg	cacgagcagggtgaagaggc
2 and 3	gaataagggctggctggagctg	gccctgttctctgggaccttc
4	cacagcaaggacacctgagtgatg	cttcctccatttccacttcccaagc
5	gttgtccagatatgactgacctgctc	catgtggtagcattcccagtgg
6 and 7	cgtctgtgttccttccgattcctg	gctggtcgggcaatatggtgtag
8	ctacaccatattgcccgaccagc	cccatgatctaagcctgatcttgcc
9	tgccaggatatctgcctcaacct	gctgaagcccagatctacaagagagt
10	gaatgggtagagccaaggcc	agactttccctgtttaggcctccc
11	gcttgctgactcccattgcac	accacatgggaaaattgcctgttg
12	gactcagtctggaaaaccatgttggc	aggtgctcaacattatctgctgca
13	gtcgggatgtatttaagccattctgggt	tgcaggacactttgtggtcacttg
14	gtgatctgggtcctgtcatcagc	aggcaaggatgagcagcgagtc
15 and 16	ctcgctgctcatccttgcct	caaggtctgctcaagcctcagga
17	tcctgaggcttgagcagaccttg	agggcacgatggaaggaatgtg
Exons and cDNA start-end position	Forward 5' to 3'	Reverse 5' to 3'
BAP1 NM_004656.3, CCDS2853.1		
Exons 9-11, 715 - 941	atcaagtatgaggccaggctg	tctgcaccatctgtgtggttg
Exons 10-12, 921- 1127	cgctggtgctggaagcaaac	tcttcttcctcctgcatggg
Exons 13-14, 1628 - 1845	agcctgctgcgtgttgactg	catccccgtcttctctctgctgtc

Of the 21 patients with MM, 18 were men (86%) and three were women (14%). At the time of death, the patients' ages ranged from 58 to 88 years (mean±SD: 76±9). MM was the cause of death in 18 cases, while in three cases, death was attributable to other causes. In some of the 21 patients with MM, other malignancies were documented such as prostatic carcinoma, oral squamous cell carcinoma, bladder carcinoma and squamous cell carcinoma of the contralateral lung. Of the 20 cases of pleural MM, 15 were epithelioid, and four biphasic. In one case, the histotype of MM was not available. The case of peritoneal MM was of the epithelioid type. A summary of clinical features is reported in Table III.

Table II. *List of all tools and databases used in the present study for the annotation of all identified variants.*

RefSeq database version 69
ExAC 65000 exome version 0.3
NIH-NHLBI 6500 exome database version 2
1000 Genomes Project 2014 Oct version
NCBI dbSNP build 142
NCBI ClinVar version 20150330
HGMD professional version 2014.4
COSMIC version 70
CADD, SIFT, Polyphen2, LRT, MutationTaster, MutationAssessor, FATHMM, VEST3, MetaSVM and MetaLR from dbNSFP version 2.6
PhyloP, phastCons, GERP++ and SiPhy from dbNSFP version 2.6
Mutalyzer version 2.0.10

Among these 29 patients with a history of asbestos exposure, Sanger sequencing of *BAP1* gene identified one non-synonymous variant and two intronic variants. MLPA analysis did not reveal significant copy number variations at the exon level in any of patient samples.

In detail, patient 4 heterozygously carried a known intronic variation 8 bases downstream of exon 13 (c.1729+8T>C) (Figure 1). This variant has already been described as a single-nucleotide pleomorphism (rs150945583) with a minor allelic frequency of between 0.0030 and 0.0055 (31, 32). Web tools for prediction of alternative splicing reported this as a possible alteration of an intronic splicing site. Sanger sequencing of cDNA did not detect alternative splicing forms. This patient was affected by pulmonary fibrosis, and died from respiratory failure. The quantification of asbestos bodies in the lung parenchyma revealed the presence of 104 asbestos bodies per gram of dry lung tissue. At autopsy, a clear cell renal cell carcinoma of 40 mm in the greatest diameter was evidenced as an incidental finding.

Patient 9 heterozygously carried a missense variant (c.T1028C; p.L343P) at exon 11 (Figure 2A). Web tools for prediction of mutation pathogenicity reported this variant as being possibly benign. Sanger sequencing of cDNA revealed no alternative splicing due to the nucleotide change. *In silico*

Table III. *Summary of clinical features of the 29 patients of the study sample. Patients harboring of breast cancer 1 (BRCA1)-associated protein 1 (BAP1) gene genetic variants are shown in bold.*

Patient no.	Gender	Age at diagnosis (years)	Age at death (years)	MM survival (months)	Cancer	MM histotype	Other diseases	Treatment of MM	Asbestos exposure	Occupation	Asbestos bodies (n/g)
1	M	77.9	77.9	0.5	Pleural mesothelioma; prostatic carcinoma	Epithelioid	/	Palliative	Occupational	Dockworker	13
2	M	87.3	87.6	2.6	Pleural mesothelioma	Epithelioid	/	Palliative	Occupational	Ship mechanic	34300
3	M		72.2		Laryngeal carcinoma; prostatic carcinoma; lung carcinoma; oral carcinoma with lung metastases	/	/	/	Occupational	Sailor	8400
4	**M**		**70.5**		**Renal cell carcinoma**	**/**	**Pulmonary fibrosis**	**/**	**Occupational**	**Chemical factory worker**	**104**
5	F	84.9	85.0	0.5	Pleural mesothelioma	Biphasic	/	Palliative	NA	NA	94
6	M	71.5	72.9	16.8	Pleural mesothelioma (circumscribed non evolutive); oral carcinoma	Epithelioid	/	None	Occupational	Dockworker	5100
7	M	82.1	82.6	5.23	Pleural mesothelioma	Epithelioid	/	Palliative	Occupational	Engine factory worker	20000
8	M	57.9	68.7	126.6	Pleural mesothelioma (healed); bladder carcinoma; contralateral lung carcinoma	NA	/	Pneumectomy	NA	Italian finance police	55
9	**M**	**68.3**	**71.8**	**41.1**	**Pleural mesothelioma**	**Epithelioid**	**Diffuse pleural fibrosis; desmoid-type fibromatosis**	**Pleurectomy and decortication, multiple pulmonary resections; chemo- and radiotherapy**	**Occupational**	**Ship engine room worker, shipyard worker**	**229**
10	F	68.4	69.9	17.6	Pleural mesothelioma	Epithelioid	/	Pleuropneu-mectomy; chemo- and radiotherapy	Household	Housewife	121
11	M	81.5	81.5	0.6	Pleural mesothelioma	Biphasic	/	Palliative	NA	Steel mill worker	231
12	M	82.1	83.6	17.5	Pleural mesothelioma	Epithelioid	/	Chemo- and radiotherapy	NA	Electrician	513
13	M		78.6		/	/	Asbestosis	/	Occupational	Dockworker	5025
14	M		77.1		/	/	Asbestosis	/	Occupational	Ship cook	6580
15	**M**	**62.6**	**64.1**	**18.1**	**Pleural mesothelioma**	**Epithelioid**	**/**	**Chemotherapy?**	**Occupational**	**Welder**	**31330**
16	M	66.5	69.4	34.2	Pleural mesothelioma	Epithelioid	/	Pleurectomy	Occupational	Dockworker	19840
17	M		69.9		/	/	/	/	Occupational	Engine factory worker	6690
18	M	57.3	60.1	32.9	Peritoneal mesothelioma	Epithelioid	/	Intestinal resection; chemotherapy	NA	NA	25
19	M	82.7	82.9	2.1	Pleural mesothelioma	Epithelioid	/	Chemotherapy	Occupational	Shipyard worker	119
20	M	64.3	65.5	14.5	Lung carcinoma	/	/	/	Occupational	Glass factory worker	136
21	M	64.4	70.5	72.1	Pleural mesothelioma	Epithelioid	/	Pleurectomy and decortication; chemotherapy	Occupational	Elevator technician	485
22	M	74.3	74.4	1.4	Pleural mesothelioma	NA	/	Palliative	NA	NA	75

Table III. *Continued*

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Patient no.	Gender	Age at diagnosis (years)	Age at death (years)	MM survival (months)	Cancer	MM histotype	Other diseases	Treatment of MM	Asbestos exposure	Occupation	Asbestos bodies (n/g)
23	M	55.5	58.0	29.4	Pleural mesothelioma	Epithelioid	/	Pleurectomy and decortication; chemotherapy	Occupational	Occupational health and safety technician	18
24	M	86.8	87.0	3.0	Pleural mesothelioma	Epithelioid	/	Palliative	Occupational	Navy officer	1660
25	M	86.8	87.3	5.7	Pleural mesothelioma	Biphasic	/	Palliative	Occupational	Sailor	1320
26	M	65.1	65.8	8.2	Lung adenocarcinoma	/	/	/	Occupational	Dockworker	11150
27	M	85.7	88.4	31.1	Pleural mesothelioma	Biphasic	/	None	NA	NA	1270
28	F	78.9	79.9	12.3	Pleural mesothelioma (regressed)	Epithelioid	/	Chemo- and radiotherapy	NA	Teacher	0
29	M		91.1		Lung carcinoma; colorectal carcinoma	/	Asbestosis	/	NA	Fisherman	1670

M, Male; F, female; MM, malignant mesothelioma; NA, not available.

mutation analysis was performed for a predicted protein structure of BAP1 protein without any significant possible effect of the amino acid change (Figure 2B). This was a case of pleural epithelioid MM, in a patient with a history of heavy occupational exposure to asbestos, in association with diffuse pleural fibrosis associated with areas of desmoid-type fibromatosis. The patient underwent a multimodality therapy, and died 41 months after diagnosis from advanced-stage MM with pulmonary, pericardial, mediastinal, diaphragmatic, peritoneal and osseous metastases. The quantification of asbestos bodies in the lung parenchyma revealed the presence of 229 asbestos bodies per gram of dry lung tissue.

Finally, for patient 15, a heterozygous intronic variation 8 bases upstream of the exon 10 (c.784-8G>A) (Figure 3) was identified. Alternative splicing prediction web tools gave contradictory results. Sanger sequencing of cDNA did not detect alternative splicing forms. This was a case of pleural epithelioid MM in a patient with a history of occupational exposure to asbestos. He underwent an unspecified chemotherapy treatment and died 18 months after diagnosis from advanced-stage MM with pulmonary, pericardial, mediastinal, diaphragmatic, peritoneal and osseous metastases. The quantification of asbestos bodies in the lung parenchyma revealed the presence of 31,330 asbestos bodies per gram of dry lung tissue.

All three patients with variants were male and had no other malignancies in their clinical history.

Discussion

BAP1 is a tumor-suppressor gene that encodes a 90-kDA nuclear de-ubiquitinating enzyme. BAP1 protein acts through its de-ubiquitinase activity, regulating target genes in transcription, cell-cycle control, DNA damage repair and cellular differentiation [reviewed in (33)]. *BAP1* is located on chromosome 3p21, in a region that is often lost or deleted in various cancer types, including MM (8, 34-41).

Somatic *BAP1* mutations and loss of BAP1 expression have been reported in presumably sporadic MM with frequencies ranging between 20 and 60% (8, 37, 40-48). These differences appeared related to either methodological or ethnical differences across various studies (12, 41). Previously, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and neurofibromin 2 (*NF2*) have been considered the most commonly mutated tumor-suppressor genes in MM [reviewed in (49)]. Now, however, *BAP1* has been identified as the gene with the highest rate of non-synonymous mutations in pleural MM (37, 41). These findings are supported by two next-generation sequencing studies of the MM genome, which revealed that various inactivating mutations occur randomly and are rarely shared among MM biopsies, with the exception of *BAP1*, and to a lesser extent *NF2, CDKN2A*, and possibly cullin 1 (*CUL1*) (46, 47, 50). The observation that *BAP1* mutation is such a frequent somatic event has also been confirmed in peritoneal MM (49, 51). Unlike MM occurring in germline *BAP1* mutations carriers, data concerning the clinical phenotype of MM with somatic *BAP1* mutation are controversial (37, 40, 41, 44, 45, 48, 51, 52).

Germline mutation of *BAP1* has been related to a recently identified autosomal dominant tumor predisposition syndrome (*BAP1*-TPDS) (8-10, 12, 53) characterized by an increased risk for a specific skin lesion - atypical Spitz tumor - and various malignancies, mainly uveal melanoma, MM, cutaneous melanoma and renal cell carcinoma (54). Because of the limited number of families reported to date, the penetrance, natural history, and frequencies of *BAP1*-

associated tumors are yet to be determined (54). The demonstration of a strong association with *BAP1* mutations, however, has most likely paved the way for comprehension of genetic predisposition to MM, which could account for the observation that only a small fraction of asbestos-exposed individuals develop MM (5). A recent comprehensive review of published research into *BAP1*-TPDS reported that 22% of patients with germline mutations were diagnosed with MM (55). MM occurring in individuals carrying germline *BAP1* mutations hasdistinct clinicopathological features, namely predilection for peritoneal involvement, earlier mean age of onset, less aggressive clinical course, and improved long-term survival (12, 54-57). Mutations in *BAP1* result in either complete absence of protein expression or cytoplasmic sequestration of BAP1, which can be detected by immunohistochemistry (41, 43-45, 48, 51).

In regard to the additional possible role of germline *BAP1* mutations in epidemiology of sporadic (non-familial) MM, Testa *et al*. suggested that these mutations are also frequent in sporadic MM because they found them in 2/26 (7.7%) of patients with MM (8). Subsequent studies, however, have observed no *BAP1* mutations in truly unselected MM (58-61) so that, collectively, the prevalence of germline *BAP1* mutations in patients with sporadic MM can be considered lower than 1-2% (59). Thus, taken together, the screenings performed to date suggest a minor role of germline *BAP1* mutations in the pathogenesis of sporadic MM.

In our sample, we identified germline sequence variants of *BAP1* in 3/29 asbestos-exposed patients and in 2/21 sporadic MM cases. Web tools for prediction of alternative splicing and mutation pathogenicity, including *in silico* analysis, did not demonstrate any obvious functional significance of these variations. Thus, our results seem to be in agreement with most of the recent studies that support the notion that sporadic germline *BAP1* mutations are not relevant to genetic susceptibility to MM (58-61).

Nevertheless, it is recognized that tools available for predicting the pathogenicity of splicing alteration and amino acid change caused by point mutations have some limitations. Splicing regulatory elements act in concert, and their interactions and dependencies play an important role in splice site functionality, but the meaningful combination of cis-regulatory elements and splice site scores into a functional measure still remains to be achieved (62). Analogously, despite the availability of high-quality web-based tools, predicting the effect of missense mutations remains a challenging biophysics and bioinformatics problem. The main reason for this is that mutations can simultaneously affect several highly interrelated or correlated structural and physico-chemical characteristic of proteins, making it difficult to decouple them from one another (63, 64). Therefore, there may be aberrant splicing or missense

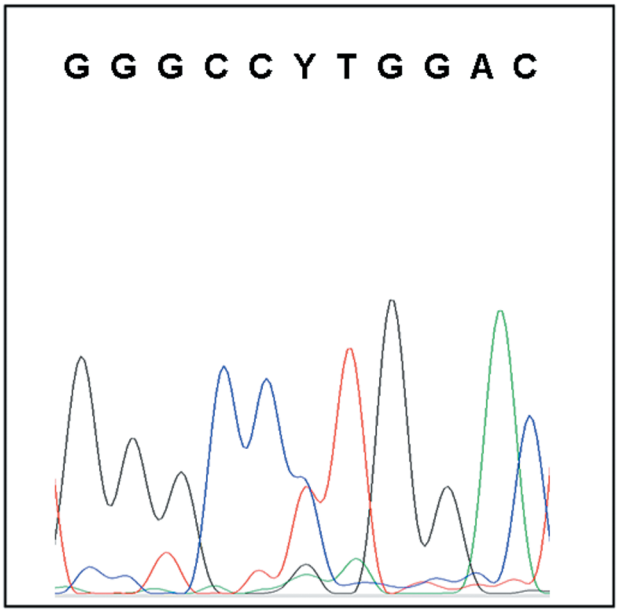

Figure 1. *Partial electropherograms of the c.1729+8T>C mutation of breast cancer 1-associated protein 1 (BAP1) gene in patient 4.*

mutations that have an undetected impact on protein expression, conformation or function, so that the relevance of these changes for predisposition to tumor development remains to be explored (59, 62).

In the light of the above considerations, our findings present some peculiarities that are worth emphasizing and provide the opportunity to make some observations.

Patient 4 carried c.1729+8T>C mutation and was affected by pulmonary fibrosis. He had no malignancies in his clinical history but, at autopsy, a clear cell renal cell carcinoma was evidenced as an incidental finding. It is clearly established that germline *BAP1* mutations predispose to renal cell carcinoma (65-67), and that renal cell carcinoma belongs to the phenotypic spectrum of *BAP1*-TPDS [reviewed in (54, 55)]. *BAP1* is most commonly mutated in sporadic clear cell renal cell carcinoma, with an incidence rate of 6-17%. The inactivation of BAP1 has been associated with high tumor grade, rhabdoid/sarcomatoid transformation and poor prognosis (33).

Patient 9, who carried a non-synonymous mutation (c.T1028C; p.L343P) at exon 11, presents some intriguing peculiarities in his clinical history. MM developed in the form of multiple nodulations relatively circumscribed and distinct from one another, in a background of a widespread and marked pleural fibrosis, with features of desmoid-type fibromatosis. Diffuse pleural fibrosis is a benign pleural disease characterized by non-circumscribed fibrous thickening

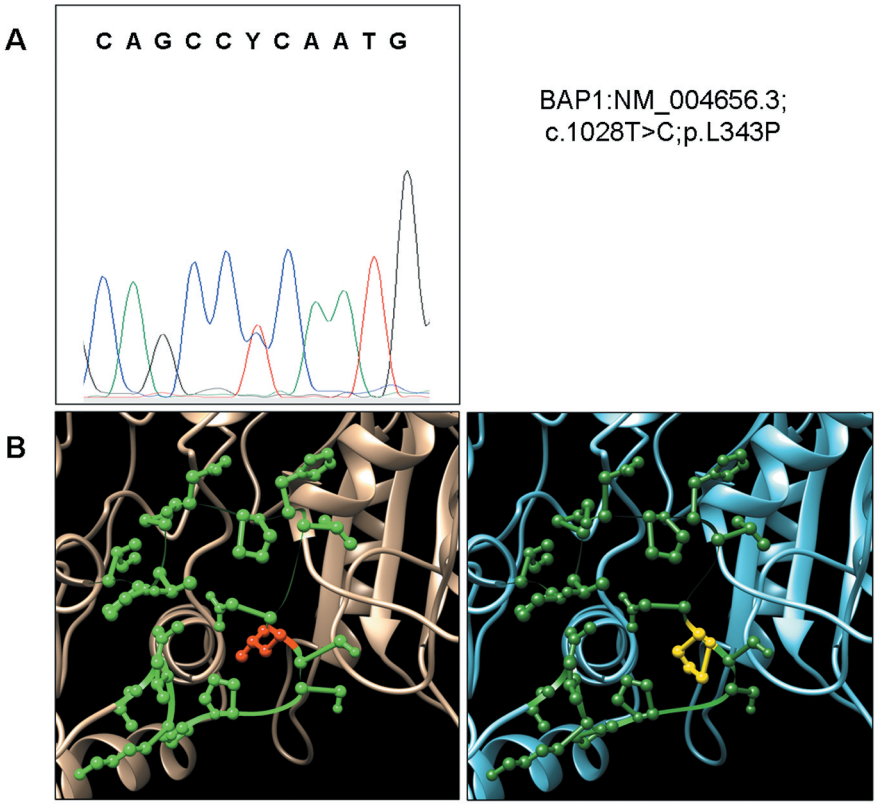

Figure 2. *Partial electropherograms of the non-synonymous mutation of breast cancer 1-associated protein 1 (BAP1) gene in patient 9 (A). Wild-type and mutated amino acids are also evidenced on the structure reconstruction of the BAP1 protein. (B). Left, Wild-type leucine in red; right, mutated proline in yellow.*

of variable cellularity involving mainly the visceral pleura, often described in association with moderate or heavy exposure to asbestos. The mechanism of development remains unclear, although it is thought to be a consequence of benign asbestos-induced pleuritis with effusion (3). On the other hand, primary desmoid-type fibromatosis arising primarily in the pleura is rare, and has never been reported in association with asbestos exposure to our knowledge. It is a locally aggressive but non-metastasizing myofibroblastic neoplasm that typically arises in deep soft tissues and often exhibits mutations in the gene encoding β-catenin (*CTNNB1*) (68, 69). After diagnosis, the patient was subjected to pleurectomy and decortication, multiple pulmonary resections, chemotherapy and radiotherapy and survived for 41 months. Trimodality treatment may have contributed to prolonging survival because the stage of the disease allowed adequate radical surgical resection. In patients diagnosed at earlier stages and treated with extrapleural pneumonectomy followed by radiation and chemotherapy, survival rates of 38% after 2 years and 15% after 5 years have been reported (3). Nevertheless, considering the recent observation that *BAP1*

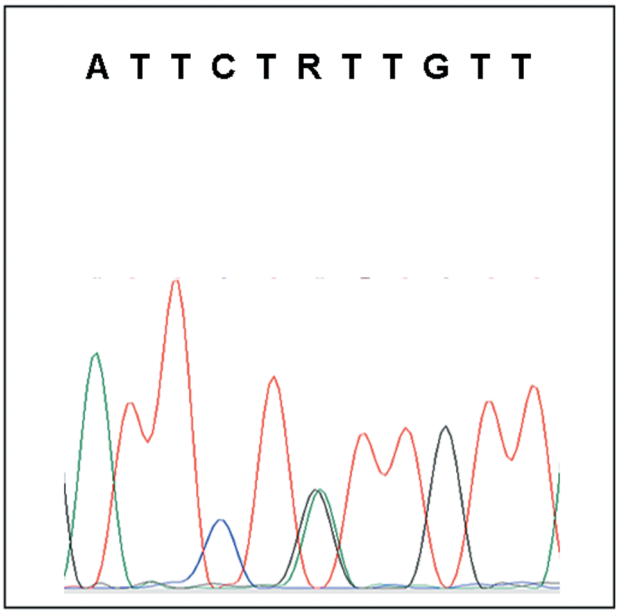

Figure 3. *Partial electropherograms of the c.784-8G>A mutation of breast cancer 1-associated protein 1 (BAP1) gene in patient 15.*

germline mutation is associated with an improved long-term survival in MM (12, 54-57), it might also be speculated that there a relationship between such a peculiar clinical presentation and prolonged survival and the presence of missense variant in exon 11 of *BAP1*.

Patient 15 carried c.784-8G>A mutation and was affected by pleural epithelioid MM. Although he did not undergo surgery, survival from diagnosis was 18 months, in any case longer than the mean survival rates of patients with MM, which remains poor and typically around 12 months (1).

The role of asbestos exposure in germline *BAP1* mutation carriers is unclear (55). In the report by Testa *et al*., all family members affected by MM in both of the described families with germline mutations of *BAP1* lived in an asbestos-containing home, and patients with sporadic MM carrying *BAP1* deletions had a history of exposure to asbestos (8). Studies in mice support a well-defined role of environmental carcinogens. When *BAP1* was deleted, mice developed myeloid transformation, but not MM, uveal or cutaneous melanoma (70), suggesting that germline *BAP1* mutations might increase the susceptibility to mineral fiber-induced and UV light-induced carcinogenesis (12). In mice with germline mutation of *BAP1*, exposure to asbestos accelerates development of MM, also with involvement of dysregulation of the retinoblastoma pathway, thus confirming that high penetrance of MM requires such environmental exposure (71, 72). BAP1 acts as an important DNA damage signaling and repair enzyme (33), and may help prevent environmental carcinogenesis caused by asbestos or UV light. This would explain the very high incidence of MM, melanoma and basal cell carcinoma (rather than other not environmental related cancer types) among *BAP1* germline mutant carriers (73).

In order to investigate the role of germline *BAP1* mutation in asbestos-induced MM, Ohar *et al*. determined the prevalence of germline *BAP1* mutation in a population of asbestos-exposed MM cases. They found *BAP1* alterations in 6% of patients, also confirming that *BAP1* mutation carriers develop MM at an earlier age that is more often peritoneal than pleural, and exhibit improved long-term survival compared to MM patients without *BAP1* mutations (61). The finding of an increase prevalence of inherited *BAP1* mutations in asbestos-exposed patients with MM *versus* asbestos-exposed controls appears to be consistent with the hypothesis that germline mutation in *BAP1* may contribute to susceptibility to MM in asbestos-exposed individuals through a mechanism that involves a gene–environment interaction (8, 12). Napolitano *et al*. have suggested a novel, complex model of asbestos-induced MM pathogenesis that implicates tumor-suppressor effects of BAP1 mediated *via* the microenvironment, in which the asbestos-induced chronic inflammatory response can have preferentially anti-tumoral or pro-tumoral roles, depending on the cellular and soluble mediators involved (74). Thus, BAP1 mutation carriers may be highly susceptible to MM even at modest background levels of asbestos exposure that would be considerably less tumorigenic for the general population (56, 57, 71, 74). This could explain observations of minimal or no evidence of exposure to asbestos among patients carrying *BAP1* germline mutations who developed MM (8, 11, 58). An additional role of gene–asbestos interaction on MM susceptibility also emerges from a study examining interactions between asbestos exposure and a set of candidate single nucleotide polymorphisms deriving from a genome-wide association study on pleural MM (75, 76). Alternatively, these MMs might be totally unrelated to exposure to asbestos (8, 56, 57). Indeed, no statistically significant effect of asbestos exposure on BAP1 protein expression has been found (44).

In our series, both mesothelioma cases (patients 9 and 15) harboring *BAP1* variants had a story of occupational exposure to asbestos. For patient 9, despite the long lasting and high level of asbestos exposure (he was been employed as a fireman and lubricator in the engine room of a ship and then, for a long time, in the technical office of shipyards in Trieste and Monfalcone), there were only 229 asbestos bodies per gram of dry lung tissue. According to guidelines, over 1000 asbestos bodies per gram of dry tissue are required in order to identify persons with a high probability of exposure to asbestos dust (3), and a relationship between asbestos burden and survival in pleural MM has also been demonstrated (77). Nevertheless, the mechanism responsible for this dose–response association with survival is unclear and possibly attributable once again to genetic susceptibility (77), which might at the same time be able to influence the process of formation of asbestos bodies as an epiphenomenon. Patient 15 had been a welder for 43 years and the asbestos content of his lungs (31,330 asbestos bodies per gram of dry lung tissue) was consistent with a high level of exposure.

In conclusion, we analyzed the prevalence of germline *BAP1* mutations in a group of asbestos-exposed patients who had lived and worked in Trieste, a ship-building town in Northeast Italy with a very high incidence of mesothelioma. As far as we are aware, this is the first published study dealing with the topic in this high-risk area. In 3/29 patients and in 2/21 MM cases, we found non obviously pathogenic germline sequence variants of *BAP1*. Nevertheless, limitations of web tools for prediction impose reservations in the interpretation of some puzzling features of our findings.

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