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Betti M[1], Aspesi A[1], Biasi A[1], Casalone E[2,3], Ferrante D[4], Ogliara P[5],Gironi LC[6], Giorgione R[6], Farinelli P[6], Grosso F[7], Libener R[8], Rosato S[9], Turchetti D[10], Maffè A[11], Casadio C[12], Ascoli V[13], Dianzani C[14], Colombo E[6], Piccolini E[15], Pavesi M[16], Miccoli S[10], Mirabelli D[17,18], Bracco C[5], Righi L[19], Boldorini R[20], Papotti M[21], Matullo G[2,3], Magnani C[4,18],Pasini B[2,5,^], Dianzani I[1,18,^,*]

[1]Department of Health Sciences, University of Piemonte Orientale, Novara, Italy

[2]Department of Medical Sciences, University of Turin, Turin, Italy

[3]Human Genetics Foundation, HuGeF, Turin, Italy

[4]CPO-Piemonte and Unit of Medical Statistics and Epidemiology, Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy

[5]Medical Genetics Unit, AOU Città della Salute e della Scienza, Turin, Italy

[6]Dermatology Clinic, Department of Clinical and Experimental Medicine, University of Piemonte Orientale, Novara, Italy

[7]Division of Medical Oncology, SS.Antonio e Biagio General Hospital, Alessandria, Italy

[8]Pathology Unit, SS.Antonio e Biagio General Hospital, Alessandria, Italy

[9]Department of Obstetric, Gynecologic and Pediatric, Section of Clinical Genetics, Arcispedale S. Maria Nuova-IRCCS, Reggio Emilia, Italy

[10]Medical Genetics, Policlinico Sant'Orsola-Malpighi, Bologna, Italy

[11]Molecular Genetics and Biology Unit, Santa Croce e Carle Hospital, Cuneo, Italy

[12]Thoracic Surgery Unit, Azienda Ospedaliero-Universitaria Maggiore della Carità, Novara, Italy

[13]Department of Radiological, Oncological and Pathological Sciences, Sapienza University, Rome, Italy

[14]Department of Dermatology, Campus Biomedico, University of Rome, Rome, Italy

[15]Pneumology Unit, Santo Spirito Hospital, Casale Monferrato, Italy

[16]Pathological Anatomy Unit, Santo Spirito Hospital, Casale Monferrato, Italy

[17]Unit of Cancer Epidemiology, CPO-Piemonte and University of Turin, Turin, Italy

[18]Interdepartmental Center for Studies on Asbestos and other Toxic Particulates "G. Scansetti", University of Turin, Turin, Italy

[19]Department of Oncology, University of Turin at San Luigi Hospital, Orbassano, Turin, Italy

[20]Department of Health Sciences, Section of Pathological Anatomy, University of Piemonte Orientale, Novara, Italy

[21]Department of Oncology, University of Turin, AOU Città della Salute e della Scienza di Torino, Turin, Italy

^ Barbara Pasini and Irma Dianzani contributed equally to this work.

*Corresponding author:
Irma Dianzani
Department of Health Sciences, University of Piemonte Orientale
Via Solaroli 17, 28100 Novara, Italy
E-mail: irma.dianzani@med.uniupo.it

Abstract

BAP1 germline mutations predispose to a cancer predisposition syndrome that includes mesothelioma, cutaneous melanoma, uveal melanoma and other cancers. This co-occurrence suggests that these tumors share a common carcinogenic pathway. To evaluate this hypothesis, we studied 40 Italian families with mesothelioma and/or melanoma. The probands were sequenced for *BAP1*and for the most common melanoma predisposition genes (i.e. *CDKN2A*, *CDK4*, *TERT*, *MITF* and *POT1*) to investigate if these genes may also confer susceptibility to mesothelioma.

In two out of six families with both mesothelioma and melanoma we identified either a germline nonsense mutation (c.1153C>T, p.Arg385*) in *BAP1* or a recurrent pathogenic germline mutation (c.301G>T, p.Gly101Trp) in *CDKN2A*.

Our study suggests that *CDKN2A*, in addition to *BAP1*, could be involved in the melanoma and mesothelioma susceptibility, leading to the rare familial cancer syndromes. It also suggests that these tumors share key steps that drive carcinogenesis and that other genes may be involved in inherited predisposition to malignant mesothelioma and melanoma.

1. Introduction

Monogenic cancer predisposition syndromes provide key insights into the complex stepwise mechanisms of carcinogenesis. Identified in 2011, the inherited cancer predisposition syndrome caused by germline mutations in the tumor suppressor gene *BAP1* (BRCA1-Associated Protein 1) has shed light for the first time on monogenic malignant mesothelioma (MM) predisposition [1-6]. However, the *BAP1* syndrome spectrum (MIM#614327) includes also other tumors, e.g. cutaneous melanoma (CM), uveal melanoma (UM) [7-18], epithelioid atypical Spitz tumors [19], lung adenocarcinoma, meningiomas [20], neuroendocrine tumors, breast cancer, cholangiocarcinoma, prostate cancer, paraganglioma, renal cell carcinomas [12,21-23] and basal cell carcinoma (BCC) [24-25]. Recently, our group showed an additional tumor type associated with *BAP1* germlinemutation, the rare mucoepidermoid carcinoma [26].

So far 67 families with the *BAP1* cancer predisposition syndrome and 56*BAP1* germline mutations have been described [5-6,15-18].

More information is needed to ascertain the phenotype of this syndrome, including the evolution of malignant tumors and their response to therapy. Information gained on other cancer-prone syndromes has shown that peculiar response to treatment should influence treatment choice in carriers of germline mutations as compared with sporadic tumors (for example, breast and ovarian cancer due to *BRCA1* and *BRCA2*) [27].

Thus, to better characterize the *BAP1* cancer predisposition syndrome, we identified and studied 40 Italian families with a suspected predisposition for mesothelioma and/or melanoma. *BAP1* gene was sequenced in a representative subject of each family and the same index cases were also studied for the most common melanoma predisposition genes (i.e. *CDKN2A*, *CDK4*, *TERT*, *MITF*, *POT1*) [28-29] to investigate if these genes may also confer susceptibility to mesothelioma.

2. Materials and Methods

2.1 Probands and Families

We focused only on melanoma and mesothelioma among the *BAP1*-related tumors because of our clinical and epidemiological expertise focused on these tumors.

The probands with melanoma were collected from the melanoma databases of the Dermatology Clinic, AOU Maggiore della Carità (Novara), the Medical Genetics Unit, AOU Città della Salute e della Scienza (University of Turin), the Medical Genetics Unit, Policlinico S.Orsola-Malpighi

(University of Bologna), the Clinical Genetics Unit of the Department of Obstetric, Gynecologic and Pediatric, Arcispedale S. Maria Nuova (Reggio Emilia) and of the Unit of Dermatology &Plastic Surgery, Campus Biomedico University (Rome).

The probands with mesothelioma were collected from the databases of Mesothelioma Biobank (Alessandria), Molecular Genetics and Biology Unit, Santa Croce and Carle Hospital (Cuneo), Thoracic Surgery Unit, AOU Maggiore della Carità (Novara) and Pathology Unit, Sapienza University (Rome).

Selection criteria for melanoma patients were the following: diagnosis of melanoma with family history for tumors associated with *BAP1* mutations (i.e. uveal melanoma, mesothelioma, renal cell carcinoma) or juvenile melanoma with family history for melanoma or multiple melanomas with family history for melanoma. Mesothelioma selection criteria were: familial mesothelioma or mesothelioma with family history for *BAP1* associated tumors or melanoma and mesothelioma in the same proband or mesothelioma and other tumors occurring in the same subject.

Overall, we identified 40 Italian families classified into three groups (Table I): 6 families with both mesothelioma and melanoma, 23 families with melanoma (without mesothelioma) and with familiarity for *BAP1* associated tumors and 11 families with mesothelioma (without melanoma) and with familiarity for *BAP1* associated tumors. Details on proband's tumors with age at diagnosis and family history are reported in Table I. Pedigrees are shown in Figure 1 (A1,B1,C1) and Supplementary Figure 1 B1. Histological details, asbestos exposure, Fitzpatrick skin phototype and other clinical information are described in Supplementary Table I.

All the probands were Caucasian of Italian ancestry and signed an informed consent to participate in the research project.

2.2 Histological diagnosis

The diagnosis of melanoma was based on histological and immunochemistry findings. Histological criteria were related to the evaluation of morphological and cytological features; antibodies against Human Melanoma Black-45 (HMB-45), S100 protein and p16 were used in equivocal cases. Melanomas were classified in subtypes, and staged on the basis of the American Joint Committee on Cancer (AJCC) TNM classification (7^{th} edition) [30].

The diagnosis of mesothelioma was based on standard histological and immunohistochemical criteria, including positivity to calretinin, vimentin, cytokeratins 5 and 6, and WT1 and negativity to carcinoembryonic antigen, thyroid transcription factor 1 and Ber-EP4. The

MMs were classified on the basis of the WHO classification of pleural tumors [31].

2.3 Information on asbestos exposure of mesothelioma patients

Since asbestos exposure is the main risk factor for MM [32], it was carefully evaluated in all MM probands. Information on asbestos exposure at work, at home and in the general environment for index cases included in the Malignant Mesothelioma Registry of the Piedmont Region (RMM) was collected by RMM using a standardized questionnaire [33], that was administered by trained interviewers. For familial cases identified in the proband's pedigree, information was gathered from their attending clinician's reports, clinical records and data included in the RMM. Information was collected during the personal interview of the proband if available. Asbestos exposure was classified as: high exposure, low exposure, no exposure, unknown exposure. Details are reported in Betti et al. 2015 [26].

2.4 Information on risk factors of melanoma probands

The role of UV exposure is well known in the development of melanoma. People with pale skin are at higher risk for this tumor. Dysplastic and/or melanocytic nevi are considered premalignant lesions in melanoma carcinogenesis [34]. Thus, the professional or chronic UV exposure, the occurrence of dysplastic and/or melanocytic nevi and Fitzpatrick skin phototype were evaluated in melanoma probands.

2.5 DNA extraction and Sequencing analyses

Blood samples were collected in vacutainers with ethylenediaminetetraacetic acid (EDTA) and stored at -20°C until use. Genomic DNA was extracted from peripheral blood using QIAamp® DNA Blood Maxi Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's protocol.

The 17 exons, intron-exon boundaries and promoter region (~1000bp upstream of the ATG) of*BAP1* (NM_004656.2), the exons and intron-exon boundaries of *CDKN2A* (NM_000077.4), the exon 2 of *CDK4* (NM_000075.3), the promoter of *TERT* (NM_198253.2), the missense variant p.Glu318Lys of *MITF* (NM_000248.3) and the exon 10 of *POT1* (NM_015450) were amplified. The primers were designed using the reference sequences provided by NCBI or Ensembl databases (Supplementary Table II).

PCR reactions were performed in a 25 µL volume using GoTaq® Flexi Polymerase (PROMEGA, Madison, WI, USA) for *BAP1* fragments amplification and Taq Gold 360+GC enhancer for fragments amplification of melanoma predisposition genes. Sanger sequencing was done using ABI PRISM 3130xl Genetic Analyzer or by IGA Technology Services (Udine, Italy). Sequences were read by aligning with the reference sequence, using the GENESTREAM II Align tool.

Each variant was confirmed on DNA obtained from a second independent blood vial.

Segregation analysis of three variants was done on DNA extracted from blood samples or tumor FFPE of key relatives.

2.6 Loss-of-heterozygosity analysis

To assess *loss-of-heterozygosity (LOH)*, we performed Sanger sequencing and microsatellite analysis on DNA obtained from tumor portions of FFPE (Formalin-fixed paraffin-embedded) biopsy specimens. For each sample three serial 10µm thick sections were cut using microtome. The slides were heated at 60°C for 30 min. The paraffin-embedded tissues were processed with xylene and absolute ethanol. Deparaffinized tissues were scraped from the slides and placed in 1.5 ml-Eppendorf tubes. DNA was purified using QIAamp® DNA FFPE Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol.

Sanger sequencing of the mutated region was then performed.LOH was indicated by loss of the wild type allele in the mutation site.

Microsatellite analysis was performed using three markers (D3S3026, D3S3561 and D3S1578) flanking the *BAP1* gene [20]. The analysis was carried out on genomic and tumor DNAs. Amplified PCR products were separated with ABI 3130xl Genetic Analyzer and fragments were analyzed using GeneMapper. LOH was indicated by apparent homozygosity at the microsatellite sites.

2.7 Immunohistochemistry analysis

To ascertain the role of germline *BAP1* or *CDKN2A* mutations, protein expression was evaluated by immunohistochemistry (IHC) using a standard automated immunostainer (BenchMark, Ventana Medical System, Tucson AZ).

Specific primary antibody against the anti-human BAP1 (mouse monoclonal, clone C-4, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-$p16^{INK4a}$ (mouse monoclonal, CLONE E6H4, Ventana Medical System, Tucson, AZ) were used, respectively.

BAP1 was considered positive when a weak-to-strong nuclear positivity was shown. The specificity of BAP1 stain was validated in serial negative control sections by omitting the primary antibody for each immunohistochemical run. Non-neoplastic cells, such as vascular endothelium or inflammatory cells, were considered as internal positive controls.

To detect the p16 protein, every tumor was given a score according to the intensity of the nuclear or cytoplasmic staining (no staining; weak staining; moderate staining; strong staining). A stained slide with a high-degree intraepithelial lesion of the uterine cervix was used as a positive control.

2.8 In silico prediction analyses

Pathogenicity assessment of missense variants is an important problem in cancer predisposition syndrome [35].

To address the problem concerning *BAP1* missense variants, we have considered the following parameters: results of LOH assay, results of IHC, frequency in 1000 Genomes Project and ExAC databases, disease-causing potential using seven different prediction tools (Mutation taster, SIFT, Provean, Polyphen-2, Mutation assessor, Condel, Phyre2), segregation analysis.

Since a change in a coding sequence may activate a cryptic splice site, we have also evaluated splice site prediction using seven different tools (Human Splicing Finder, MaxEnt, BDGP, NetGene2, GeneScan, FGENESH 2.6, FSPLICE 1.0).

As controls for the prediction programs, we used the five most frequent missense variants in ExAC, (these variants are expected to be silent changes, since they are commonly found in normal individuals).

We considered LOH, IHC or splice site prediction as the most important parameters. If LOH and IHC data were not available, we considered as pathogenic a missense variant that has a disease causing potential for at least 5/7 tools.

Segregation should be evaluated taking into consideration that frequent cancers may also occur as phenocopies, i.e. patient with cancer but without the mutation identified in other affected family members. Phenocopies are events not so rare in inherited cancer predisposition syndromes.

2.9 Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was used to evaluate the presence of copy number variations. MLPA assays were performed on 100-200ng of genomic DNAs using the SALSA MLPA P417 BAP1 probemix and the SALSA MLPA probemix P419-A1 CDKN2A/2B-CDK4 Melanoma according to instructions

provided by the manufacturer (MRC-Holland, Amsterdam, The Netherlands). Amplified PCR products were separated with ABI 3130xl Genetic Analyzer and fragments were analyzed using Coffalyser.NET software (MRC-Holland).

3. Results

3.1 Risk factors

Information on asbestos exposure was available for 10 out of 12 mesothelioma probands (Supplementary Table I). All of them showed asbestos exposure, in agreement with literature [32]. Possible occupational exposure was reported for four probands, para-occupational or household exposure forfive patients and both possible occupational and household exposure for one proband.

One out of 28 melanoma probands showed professional or chronic UV exposure. Information regarding Fitzpatrick skin phototype was available for 16 out of 28 probands: one had phototype I, 10 had phototype II and 5 had phototype III. Information about dysplastic and/or melanocytic nevi was available for 13 patients. Whereas one proband showed dysplastic nevi, 13 showed melanocytic nevi (7 probands <10, 2 probands 10-50, 4 probands >50). In total, 17 out of 28 probands showed at least well-documented risk factor (Supplementary Table I). Even if information is lacking for 11 probands, our data confirms the high occurrence of well-documented risk factors in melanoma patients [34].

3.2 Mutation analysis

Among the 40 probands with a family history of cancer we identified four *BAP1* germline variants and a recurrent pathogenic germline mutation in *CDKN2A* in three and one index cases, respectively (Table I).

Two *BAP1* and one *CDKN2A* germline variants were found in families with both mesothelioma and melanoma. Two other *BAP1* germline variants were identified in a patient with multiple cutaneous amelanotic melanomas (Table I).

The proband of family ID5 carried a heterozygous nonsense mutation in *BAP1* gene (c.1153C>T, p.Arg385*, exon 12) (II-4 PB, Figure 1 A2). The mutation seemed heterozygous also in the tumor samples (II-4 CM, II-4 MM, Figure 1 A2) and microsatellite analysis did not reveal evidence of LOH (data not shown). However, IHC did not reveal any BAP1 nuclear expression in both melanoma and mesothelioma cells (II-4 CM, II-4 MM, Figure 1A3). It is thus possible that the second inactivating “hit” was either a somatic point mutation or a methylation event. This mutation

has been previously reported in three families with Spitz tumors and ocular and cutaneous melanoma [8,14], but this is the first report in association with MM. The proband developed two independent cutaneous melanomas (53, 57yrs), a meningioma (37yrs) and an epithelioid pleural mesothelioma (53yrs) (Figure 1 A1, Table I). The mutation was transmitted to the proband's healthy daughter (III-2 PB, Figure 1 A2) while the proband's brother died because of a basal cell carcinoma, but no sample was available for segregation analysis.

The proband of melanoma family ID16 developed two cutaneous amelanotic melanomas and showed a *BAP1* missense variant (c.1700A>C, p.Asp567Ala, exon 13) (IV-1 PB, Figure 1 B2) and a 5bp duplication in the promoter region (c.-594dupCCCGT) (IV-1 PB, Supplementary Figure 1 A1). Sequencing analysis of both parents confirmed that these variants were inherited *in cis* from the mother (III-5 PB, Figure 1 B2 and Supplementary Figure 1 A1), who presented with a non-Hodgkin cutaneous lymphoma. A healthy maternal aunt and a proband's healthy sister were also carriers (III-6 PB, IV-2 PB respectively, Figure 1 B2 and Supplementary Figure 1 A1). On the other hand, the proband's healthy father and the paternal uncle affected by cutaneous melanoma did not carry the variant allele. Thus, the cutaneous pigmented melanoma presented by the proband's paternal uncle was not due to the aforementioned *BAP1* germline variants.

The missense variant appeared in heterozygous state also in the two melanomas from the proband (IV-1 CM_1, IV-1 CM_2, Figure 1 B2).

Microsatellites analysis, performed on genomic and tumor DNAs, showed a decreased amount of the shorter paternal allele for the only informative marker (D3S3026) (IV-1 CM_1, IV-1 CM_2, Figure 1 B3).Considering a stromal cell contamination of tumor DNA from melanoma samples (determined as 64% in average, 15-90%) [36], this result suggests the loss of the wild type allele (LOH).

The presence of BAP1 protein was ascertained by IHC on FFPE pertaining to the two different melanomas from the proband (IV-1 CM_1, IV-1 CM_2, Figure 1 B4). BAP1 was normally expressed in the nucleus, showing that both the promoter duplication and the missense variant do not drastically affect protein levels. Since IHC is not quantitative, this result does not conflict with a tumor genotype that includes an expressed allele with a missense variant and a somatic deletion that abolishes the expression of the wild type protein.

To assess the pathogenicity of this missense variant, we have comparedthe p.Asp567Ala with all the eleven *BAP1* missense variants reported so far in patients with familiarity for *BAP1*

associated tumors (Supplementary Table III) using the parameters reported in the Materials and Methods section.

Using these parameters, 7/12 variants could be considered as disease causing. Two of them are predicted to cause aberrant splicing. One of these was experimentally confirmed [21], the second was not tested by the authors [6]. The other missense variants should be considered as *variant of unknown significance* (VUS), including p.Asp567Ala.

We have also performed a prediction of the three dimensional structure of the variant protein according to Protein Homology/analogy Recognition Engine v.2 (Phyre2). The mutant hydrophobic Ala567 is predicted to be buried within the globular portion of the protein and the relevant domains of the BAP1 protein seem to assume a different position (Supplementary Figure 2).

In conclusion, a functional assay is strongly needed to assess the pathogenicity of missense variants including p.Asp567Ala.

The duplication in the promoter was predicted to remove a MAZ (MYC-Associated Zinc Finger Protein) binding site by PROMO (TRANSFAC), but has no effect on transcription factor binding by AliBaba 2.1. The duplication is not reported in public databases (NCBI dbSNPs, 1000 Genomes Project phase 3, Exome Variant Server, Exome Aggregation Consortium at march 2015). Thus, considering that the protein is expressed, our data overall suggest that the duplication is a likely neutral variant.

The proband of family ID4 showed a heterozygote 19bp duplication (c.-247dupCCTTCGCCCCCGTCCCTCC) in the *BAP1* promoter region (II-1 PB, Supplementary Figure 1 B2) not reported in the above mentioned databases. This variant is predicted to generate an ETF binding site by AliBaba 2.1, but has no effect on transcription factor binding site by PROMO (TRANSFAC). IHC performed on the mesothelioma tissue showed nuclear expression of BAP1 protein (data not shown), supporting the hypothesis that this variant does not significantly alter gene transcription and could be considered as VUS. This family included both melanoma and MM in its tumor spectrum, but additional specimens were not available for further studies.

Probands from the other families were analyzed using the same strategy, but no other germline variant was found in *BAP1* gene.

Conversely, a recurrent pathogenic germline mutation (c.301G>T, p.Gly101Trp, exon 2) in *CDKN2A* was found in the family ID3 showing both melanoma and mesothelioma. The *CDKN2A* germline mutation was carried by the proband affected by cutaneous melanoma and by her mother affected by both cutaneous melanoma and mesothelioma (III-3 PB, II-3 FFPE non-tumor tissue

respectively, Figure 1 C2). This mutation was found in heterozygosity with the wild type sequence also in mesothelioma from the proband's mother (II-3 MM, Figure 1 C2).

Mesothelioma cells showed nuclear staining at IHC with anti-p16 antibody (II-3 MM, Figure 1 C3) although the intensity of nuclear expression was slightly lower in less differentiated tumor cells.

MLPA analysis was performed on germline DNA from all the probands, but no copy number variations has been identified.

The probands from the other families under study were sequenced for the most common melanoma predisposition genes (*CDKN2A, CDK4* exon 2*, TERT* promoter*, MITF* exon 9*, POT1* exon 10*),* but no additional germline variant was found. Details of the results for each proband are reported in Table 1.

4. Discussion

Malignant mesothelioma is a rare and aggressive tumor that mainly arise from the pleura and the peritoneum. The main risk factor is asbestos exposure [32]. Over 80% of MM patients have a history of asbestos exposure, but only 10-17% of individuals heavily exposed to asbestos develop MM [37]. Moreover, several families with multiples cases of MM have been described [38-41]. These observations suggest the involvement of inherited factors in the pathogenesis of MM. A single high penetrance MM predisposition gene, *BAP1*, has been so far reported in familial cases of MM [1]. Previous observations from our group and other researchers [26,42-43] limit the role of the *BAP1* predisposition to the familial cases, as the mutation detection rate among sporadic cases showing the mutations is less than 2% [26].

Cutaneous melanoma (CM) and uveal melanoma (UM) are also aggressive tumors that originate from melanocytes of the skin and the eye, respectively. CM is responsible for 75% of deaths from skin cancer [34] whereas UM accounts for less than 4% of all melanoma cases [44]. Although UV radiations are awell known environmental risk factor, 10% of melanomas are familial regardless sun exposure or skin phototype. A subset of families (50%) carries mutations in one of the known high penetrance melanoma predisposition genes: *CDKN2A, CDK4, POT1, TERT, MITF* and *BAP1* [45].

Thus, germline mutations in *BAP1* are thought to be responsible for both MM and familial melanoma. Within the 67 families identified so far as affected by this syndrome, 18 showed both melanoma and mesothelioma [5-6,15-18]. Interestingly, six families from our series included patients with mesothelioma and melanoma.

To better characterize the *BAP1* cancer syndrome we sequenced *BAP1* in 40 Italian families in which multiple cases of either MM or melanoma, or cases of both malignancies occurred. Moreover, we reasoned that given the shared predisposition for these tumors due to *BAP1*, other genes predisposing to melanoma could also predispose to MM. Thus, we also analysed *CDKN2A*, *CDK4*, *TERT*, *MITF* and *POT1* in the MM families.

We identified a germline *BAP1* truncating mutation (c.1153C>T, p.Arg385*) in a patient who developed both MM and melanoma (Family ID5). This nonsense mutation has been previously identified in three families with familial melanoma [8,14], but never in MM. This patient is still alive five years after the diagnosis of epithelioid MM and was not heavily exposed to asbestos. Epithelioid histology, long survival and low asbestos exposure have all been described in patients with MM carrying germline *BAP1* mutations [1,46-48]. Further studies are needed to ascertain whether these aspects are typical of the *BAP1* cancer syndrome. However, it is possible that studies on familial predispositions are biased towards an over representation of long-survivors while short survival might hamper sample collection and proper investigation.

A further patient (Family ID4), whose father died of MM, developed cutaneous melanoma and breast cancer and carried a germline 19bp duplication in *BAP1* promoter region (c.-247dup CCTTCGCCCCCGTCCCTCC). This VUS is not included in public databases, but could not be traced in segregation analysis because key relatives were not available.

Our study also stresses the need of a reliable functional assay to evaluate the pathogenicity of missense variants. In fact, the use of multiple *in silico* prediction tools did not allow to assess the pathogenicity of the p.Asp567Ala variant found in a patient with multiple melanomas (family ID16).

Finally, looking for mutations in the familial melanoma genes we found a germline *CDKN2A* mutation in family ID3 (c.301G>T, p.Gly101Trp). This mutation has been frequently identified in familial melanoma (44%) [49]. The proband was diagnosed with cutaneous melanoma, whereas her mother had both cutaneous melanoma and MM. Mesothelioma tissue of the proband's mother showed p16 nuclear expression. A retained p16 expression for this mutation is reported in literature for melanoma [50]. Ghiorzo et al., reported that 28 out of 30 Gly101Trp-positive melanoma samples retained p16 expression and in 25 of these, p16 expression was predominantly nuclear.

Asbestos exposure at work of the proband's mother was assessed as 'low exposure', a category including all occupational exposures occurring outside industries were asbestos was directly used as a raw material.

Patients with *BAP1* mutations seem to develop mesothelioma even after a low asbestos exposure [1]. Our finding suggests that the *CDKN2A* p.Gly101Trp behaves as *BAP1* mutations: the mutation carrier is at high risk for tumors and the type of carcinogen exposure is important for the cancer type that is developed. In conclusion, our study suggests for the first time that also *CDKN2A* may predispose to MM.

Although the coincidence of two rare tumors in a single patient promptly suggests a common origin, we cannot exclude the independent occurrence of a malignant mesothelioma in a patient otherwise predisposed to melanoma. Indeed, we failed to identify additional *CDKN2A* germline mutations in our series but the same is true for *BAP1* whose mutations are very rare and do not explain most familial clustering of tumors investigated in this work, as well as cases or families with both melanoma and renal cell cancer.

Both *BAP1* and *CDKN2A* genes are found somatically deleted or mutated in MM tissues [51]. This study further supports that loss of either BAP1 or CDKN2A is an important step in both melanoma and MM carcinogenesis and individuals that carry a germline mutation in either of these two genes might have an increased risk for both tumors.

Three other families (IDs 1,2,6) showed co-occurrence of MM and melanoma. The fact that none of these families showed germline mutations in either *BAP1* or *CDKN2A*, *CDK4*, *TERT*, *MITF* and *POT1* suggests that other genes may be involved in melanoma and MM shared predisposition.
Other cancer predisposition syndromes have been reportedly associated with both tumors. Patients with Li Fraumeni syndrome due to *TP53*mutations frequently develop melanoma [52-53], more rarely MM [54]. Moreover, the occurrence of both melanoma and mesothelioma has been reported in a patient with neurofibromatosis type 2, due to heterozygous germline mutations in *NF2* [55-57]. Both *TP53* and *NF2* are often somatically deleted in MM. Thus, at least four genes that cause inherited cancer predisposition syndromes, including MM and melanoma in their spectrum, are often subjected to inactivating somatic mutations or deletions both in melanoma and MM [7,58-59].
An occurrence of MM as a second tumor after melanoma was reported two-fold more frequently than expected ($p<0.05$, in males) in a recent survey performed on Italian cancer registries [60]. A genetic susceptibility may explain the statistically significant co-occurrence of the two tumors in the same individual.

A common denominator between mesothelioma and melanoma is also the well-defined role of environmental carcinogens. In mice, germline *Bap1* mutations increase the susceptibility to asbestos-induced mesothelioma formation [61] and *BAP1* mutation carriers may be prone to UV carcinogenesis and melanoma development [5].

Our study suggests that *CDKN2A*, in addition to *BAP1*, could be involved in melanoma and mesothelioma susceptibility and these tumors share key steps that drive carcinogenesis and may cause familial aggregations of cases. Our hypothesis should be confirmed on a larger patients serie. Our study also suggests that other unknown genes may be involved in familial MM or familial melanoma, including uveal melanoma. Exome-sequencing analysis in families that show both MM and melanoma could help to identify new genes involved in the shared susceptibility between these tumors.

Acknowledegments

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Figure 1 - Families with germline mutations

The probands for each family are denoted by a black arrow. A plus symbol indicates individuals who carry germline mutations, a minus symbol indicates wild type individuals. A grey arrow denotes the mutation in the electropherograms. PB (peripheral blood), CM (cutaneous melanoma), MM (mesothelioma), FFPE (formalin-fixed paraffin-embedded).

A. Family ID5. A1. Pedigree: the proband (II-4) had multiple cancers, her brother (II-1) had basal cell carcinoma of the skin and her mother (I-2) had gastric cancer. A2. Electropherograms: *BAP1* germline mutation (c.1153C>T p.Arg385*) was carried in PB and in both tumors of the proband (II-4 PB, II-4 CM, II-4 MM). The proband's healthy daughter (III-2 PB) also carries the mutation. Other family members were not available. A3. IHC: II-4 CM: cutaneous melanoma cells showed the expression of specific Melanoma Antigen (Melan A); the same cells showed the loss of BAP1 nuclear expression(thin arrow), while BAP1 nuclear staining was retained in normal cells of the surrounding skin (thick arrow) and infiltrating inflammatory cellsII-4 MM: epithelioid mesothelioma cells showed the expression of specific mesothelial antigen (Calretinin); the same cells showed the loss of BAP1 nuclear expression (thin arrows), while BAP1 nuclear staining was retained in the normal endothelial cells and infiltrating inflammatory cells (thick arrows).

B. Family ID16. B1. Pedigree: the proband (IV-1) had two cutaneous amelanotic melanomas, her mother (III-5) had non-Hodgkin cutaneous lymphoma and her paternal uncle (III-2) had cutaneous melanoma. B2. Electropherograms: a *BAP1* missense mutation (c.1700A>C p.Asp567Ala) was found in the proband (IV-1 PB), in a sister (IV-2 PB), in her mother (III-5 PB) and in her maternal aunt (III-6 PB). The mutation was heterozygous also in both cutaneous amelanotic melanomas of the proband (IV-1 CM_1, IV-1 CM_2). B3. Microsatellite marker (D3S3026): a decreased amount of paternal allele was shown in a specimen from the second melanoma developed by the proband (IV-1 T, grey arrow) as compared with genomic DNA. B4. IHC: BAP1 was normallyexpressed in the nucleus of both cutaneous amelanotic melanomas of the proband (IV-1 CM_1 and IV-1 CM_2).

C. Family ID3. C1. Pedigree: the proband (III-3) had cutaneous melanoma, the proband's mother (II-3) had cutaneous melanoma and mesothelioma. C2. Electropherograms: *CDKN2A* germline mutation (c.301G>T p.Gly101Trp) was detected in the proband (III-3 PB) and in the proband's mother (II-3 FFPE non-tumor tissue, II-3 MM). Melanoma specimen of II-3 wasnotavailable. C3. IHC: epithelioid mesothelioma from the proband's mother (II-3 MM) showed intranuclear positivity to anti p16 antibody in neoplastic cells (left panel). The intensity of nuclear expression was slightly lower in less differentiated tumor cells (right panel).

682 **Figure 1**
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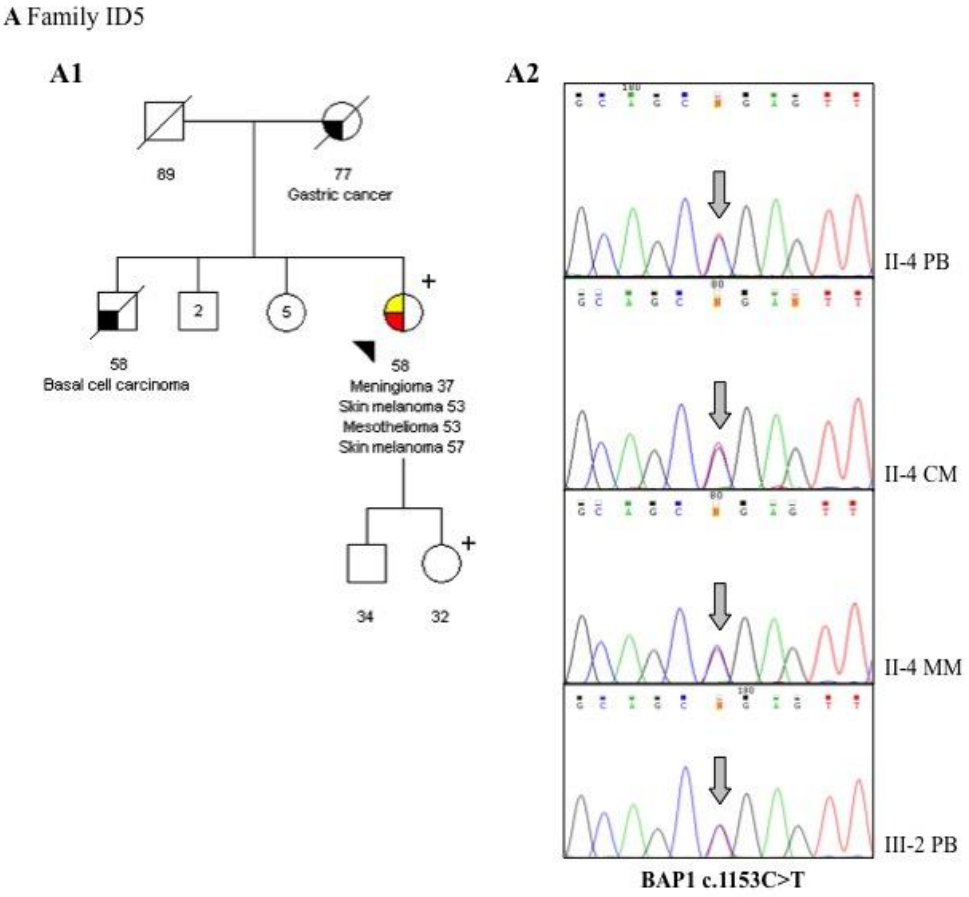
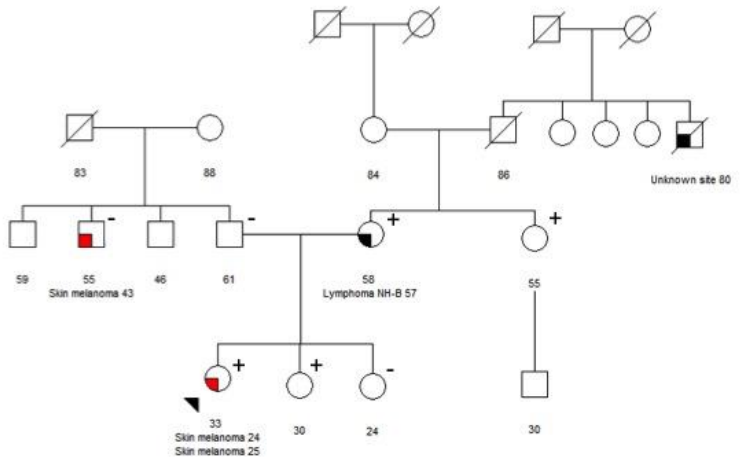


684

B Family ID16

B1



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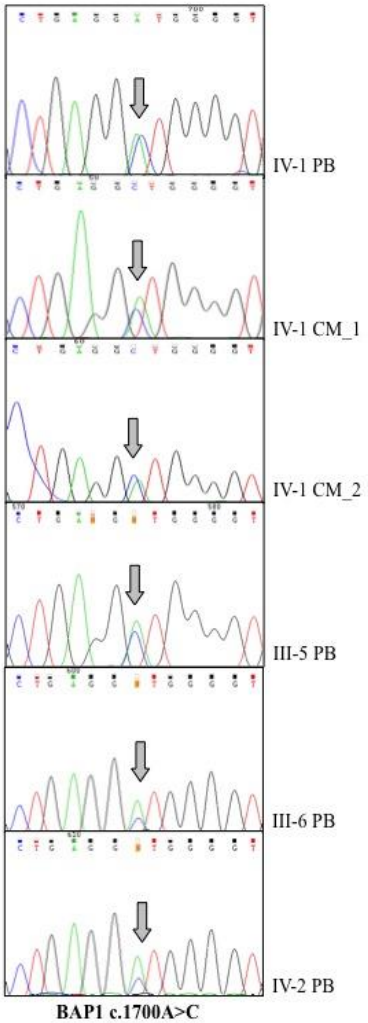
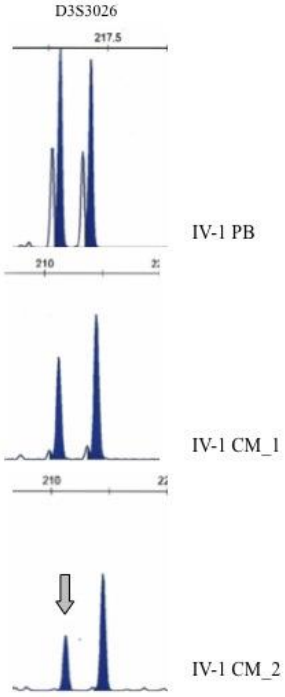


BAP1 c.1700A>C

B3



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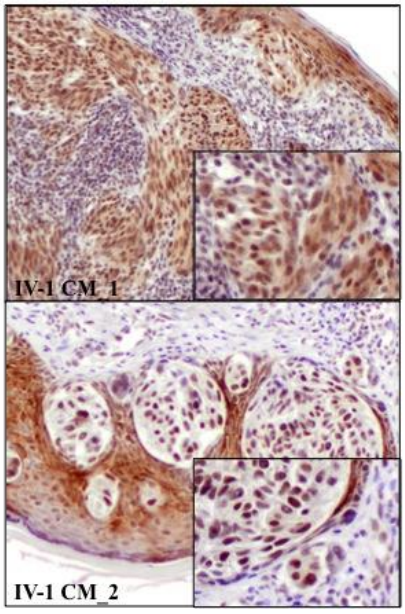

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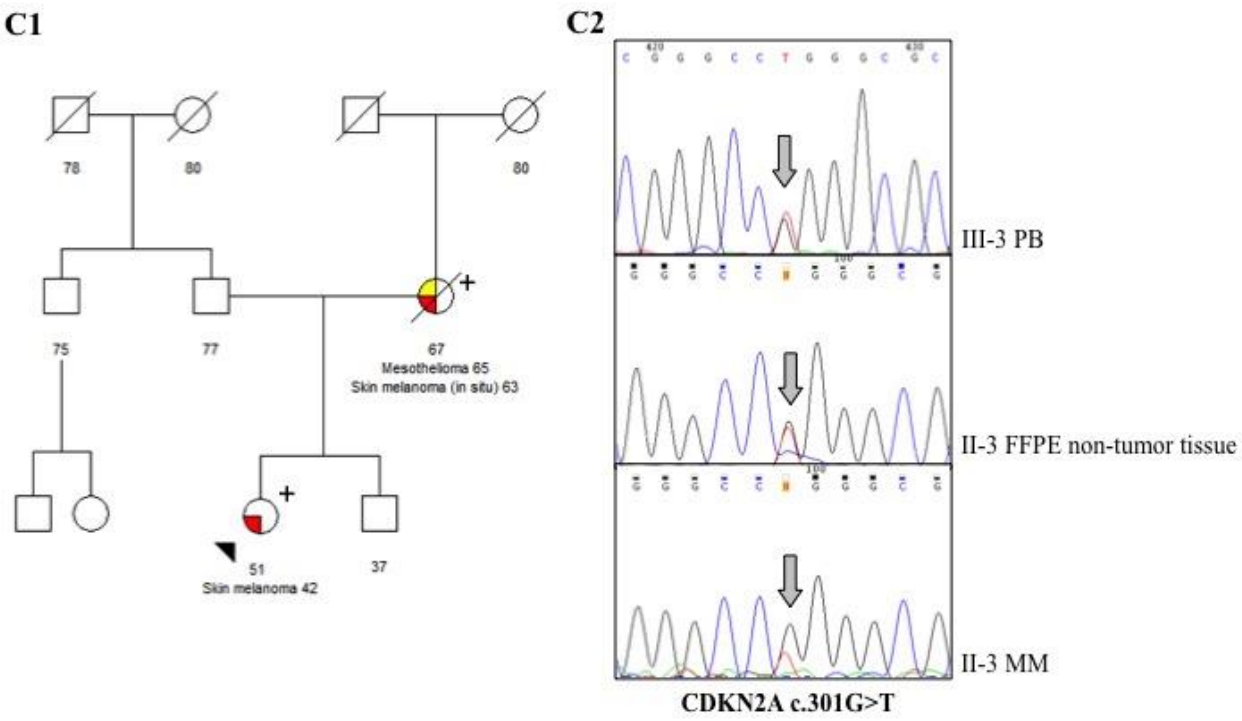
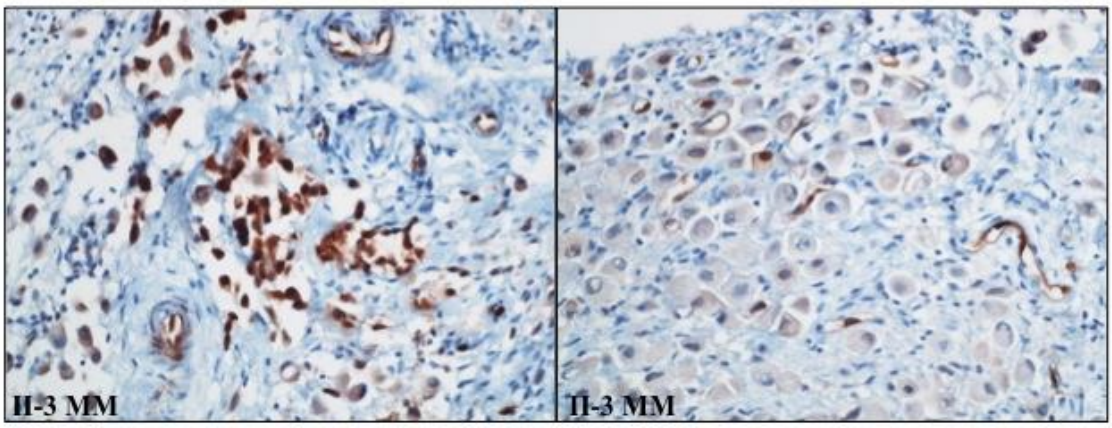
C Family ID3



C3



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690 **Table I –** Concise clinical details and testing results of the 40 probands
691

Family ID (sex)	Proband's tumors (age at diagnosis)	Family history	BAP1	CDKN2A	CDK4 (ex2)	TERT (prom)	MITF (p.Glu318Lys)	POT1 (ex10)	Other genes
Probands/families with both mesothelioma and melanoma									
1 (F)	Cutaneous melanoma (33)	Biphasic mesothelioma (mother) Breast cancer (maternal grandmother)	wt	wt	wt	wt	wt	wt	
2 (F)	Nasopharynx carcinoma (24) Cutaneous melanoma (43) Rectal carcinoma (56)	Mesothelioma (father) other tumors in relatives of the paternal family	wt	wt	wt	wt	wt	wt	TP53 wt
3 (F)	Cutaneous melanoma (42)	Mesothelioma and cutaneous melanoma (mother)	wt	**c.301G>T p.Gly101Trp**	wt	nd	wt	nd	-
4 (F)	Cutaneous melanoma (30) Breastcancer (58)	Mesothelioma (father), breast cancer (mother), papillary renal cell carcinoma type 2 (brother)	**c.-247dupCCTTCGCCCCCGTCCCTCC**	wt	wt	wt	wt	nd	-
5 (F)	Meningioma (37) Cutaneous melanoma (53) Epithelioid pleura lmesothelioma (53) Cutaneous melanoma (57)	Gastric cancer (mother), basal cell carcinoma of the skin (brother)	**c.1153C>T p.Arg385***	wt	wt	wt	wt	wt	-
6 (M)	Epithelioid mesothelioma (65)	Melanoma (brother)	wt	wt	wt	wt	wt	nd	-
Probands/families with melanoma (without mesothelioma) and features of inherited cancer predisposition									
7 (F)	Bilateral uveal melanoma (25, 30) Breast cancer (*in situ*, 40)	Acustic neurinoma (sister), bladder (mother), head/neck tumors in both maternal and paternal families	wt	wt	wt	wt	wt	wt	BRCA1 BRCA2 wt
8 (M)	Iris-Uveal melanoma (31)	Negative	wt	wt	wt	wt	wt	wt	-
9 (F)	Uveal melanoma (67) Cutaneous melanoma (72)	Cutaneous melanoma (daughter, niece)	wt	wt	wt	wt	wt	nd	-
10 (F)	Choroidal melanoma (63) Bilateral breast cancer (60, 65)	Breast cancer (sister, maternal cousin, paternal aunt), renal cancer (maternal aunt)	wt	wt	wt	wt	wt	wt	TP53 BRCA1 BRCA2 wt
11 (F)	Cutaneous melanoma (46)	Ocular melanoma (uveal, father),	wt	wt	wt	wt	wt	wt	-

		colon cancer							
12 (F)	Renal cell cancer (41) Two synchronous cutaneous melanomas (43)	Negative	wt	wt	wt	wt	wt	wt	-
13 (M)	Lentigo maligna melanoma (65)	Cutaneous melanoma, renal cell cancer	wt	wt	wt	wt	wt	wt	-
14 (M)	Lentigo melanoma (54) Two synchronous cutaneous primary melanomas (58)	Cutaneous melanoma (mother), renal cell cancer + uterus cancer + breast cancer (sister), pancreatic carcinoma, liver cancer, prostate cancer	wt	wt	wt	wt	wt	wt	-
15 (F)	Two cutaneous primary melanomas (34, 40)	Cutaneous melanoma (paternal uncles), renal cell cancer (father), lung cancer (two paternal uncles)	wt	wt	wt	wt	wt	wt	-
16 (F)	Two cutaneous amelanotic melanomas (24, 25)	Cutaneous melanoma (paternal uncle), non-Hodgkin cutaneous lymphoma (low grade, CD20+, mother)	**c.1700A>C (p.Asp567Ala) c.-594dupCCCGT**	wt	wt	wt	wt	wt	-
17 (F)	Two cutaneous primary melanomas (31, 33)	Cutaneous melanoma (father)	wt	wt	wt	wt	wt	wt	-
18 (F)	Two cutaneous melanomas (56, 57)	Cutaneous melanoma (father)	wt	wt	wt	wt	wt	wt	-
19 (F)	Two synchronous cutaneous primary melanomas (67) Facial basal cell carcinoma (69)	Cutaneous melanoma (brother), breast cancer, CNS, prostate cancer	wt	wt	wt	wt	wt	wt	-
20 (M)	Cutaneous melanoma (20)	Cutaneous melanoma (mother), breast and colon cancer	wt	wt	wt	wt	wt	wt	-
21 (M)	Cutaneous melanoma (24)	Cutaneous melanoma (mother), prostate cancer, vulvar cancer	wt	wt	wt	wt	wt	wt	-
22 (F)	Cutaneous melanoma (*in situ*, 27)	Cutaneous melanoma (father), prostate cancer	wt	wt	wt	wt	wt	wt	-
23 (M)	Cutaneous melanoma (32)	Cutaneous melanoma (sister)	wt	wt	wt	wt	wt	wt	-
24 (F)	Cutaneous melanoma (35)	Cutaneous melanoma + thyroid cancer (father)	wt	wt	wt	wt	wt	wt	-
25 (F)	Cutaneous melanoma (39)	Cutaneous melanoma + colon cancer (brother)	wt	wt	wt	wt	wt	wt	-

26 (M)	Cutaneous melanoma (82)	Cutaneous melanoma (brother), pancreatic cancer (sister)	wt	wt	wt	wt	nd	nd	-
27 (M)	Testicular teratoma (27) Multinodular goiter Ten cutaneous primary melanomas (38 to 52)	Breast cancer (mother)	wt	p.Thr77Ala§	wt	wt	wt	wt	-
28 (M)	Lipoma (27) Adrenal cortex adenoma (53) Three cutaneous primary melanomas (57, 58)	Prostate cancer (father), liver cancer (paternal uncle and paternal grandfather)	wt	wt	wt	wt	wt	wt	-
29 (M)	Cutaneous melanoma (61)	Breast cancer (sister and two paternal aunts), lung cancer (paternal uncle)	wt	wt	wt	wt	wt	nd	-
Probands/families with mesothelioma (without melanoma) and features of inherited cancer predisposition									
30 (F)	Epithelioid pleural mesothelioma (46)	Mesothelioma (mother)	wt	wt	wt	wt	wt	nd	-
31 (M)	Biphasic peritoneal mesothelioma(49)	Mesothelioma (three sisters and brother)	nd^,*	wt	wt	nd*	nd*	nd*	-
32 (M)	Biphasic pleural mesothelioma (53)	Mesothelioma (paternal uncle and two cousins), leukemia (twin brother of the father)	wt	wt	wt	wt	wt	nd	-
33 (M)	Epithelioid pleural mesothelioma (55)	Mesothelioma (paternal grandmother), prostate cancer (father)	wt	wt	wt	wt	wt	nd	-
34 (M)	Epithelioid pleural mesothelioma (68)	Mesothelioma (sister and nephew)	wt	wt	wt	wt	wt	nd	-
35 (M)	Epithelioid pleural mesothelioma (70)	Mesothelioma (both parents), papillary thyroid carcinoma (daughter)	wt	wt	wt	wt	wt	nd	-
36 (M)	Sarcomatoid mesothelioma (66)	Mesothelioma (father and cousin)	wt	wt	wt	wt	wt	nd	-
37 (M)	Mesothelioma Neuroblastoma	Negative	nd^,*	nd*	nd*	nd*	nd*	nd*	-
38 (F)	Epithelioid mesothelioma (28) Hodgkin linfoma (18)	Negative	wt	wt	wt	wt	wt	nd	-
39 (M)	Epithelioid mesothelioma Basalioma	Negative	nd^,*	nd*	nd*	nd*	nd*	nd*	-
40 (F)	Epithelioid mesothelioma (69)	Lung cancer (uncertain diagnosis,	wt	nd	nd	nd	nd	nd	-

		mother)							

wt (wild type), nd (not done)

^IHC: normal nuclear expression; *Germline DNA not available or very scanty

§ This patient was reported by Pastorino et al. (2008) [1]. The authors considered this variant as possibly damaging, using a single prediction tool (Polyphen). We have integrated this *in silico* analysis using six prediction tools, i.e. Mut taster, SIFT, Provean, Mut assessor, Condel, and Phyre2. To assess a possible aberrant splicing we used Human Splicing Finder, MaxEnt, BDGP, NetGene2, GeneScan, FGENESH 2.6 and FSPLICE 1.0. Four out of seven disease-causing prediction tools reported the p.Thr77Ala variant as neutral. No data are reported for LOH, IHC and segregation analysis. Moreover, none of the 7 splice-site prediction tools has shown the creation of alternative splice sites for this variant. Thus, we concluded that the p.Thr77Ala variant should be considered as a VUS and the patient was considered eligible for mutation analysis in other genes.

[1] Pastorino L, Bonelli L, Ghiorzo P, Queirolo P, Battistuzzi L, Balleari E, Nasti S, Gargiulo S, Gliori S, Savoia P, Abate Osella S, Bernengo MG, Bianchi Scarrà G. 2008. CDKN2A mutations and MC1R variants in Italian patients with single or multiple primary melanoma. Pigment CellMelanomaRes 21(6):700-9. doi: 10.1111/j.1755-148X.2008.00512.x.

Supplementary Figure 1- *BAP1* germline duplications

The proband is denoted by a black arrow. A plus symbol indicates individuals with germline variant. A grey arrow denotes the mutation in the electropherogram. PB (peripheral blood).

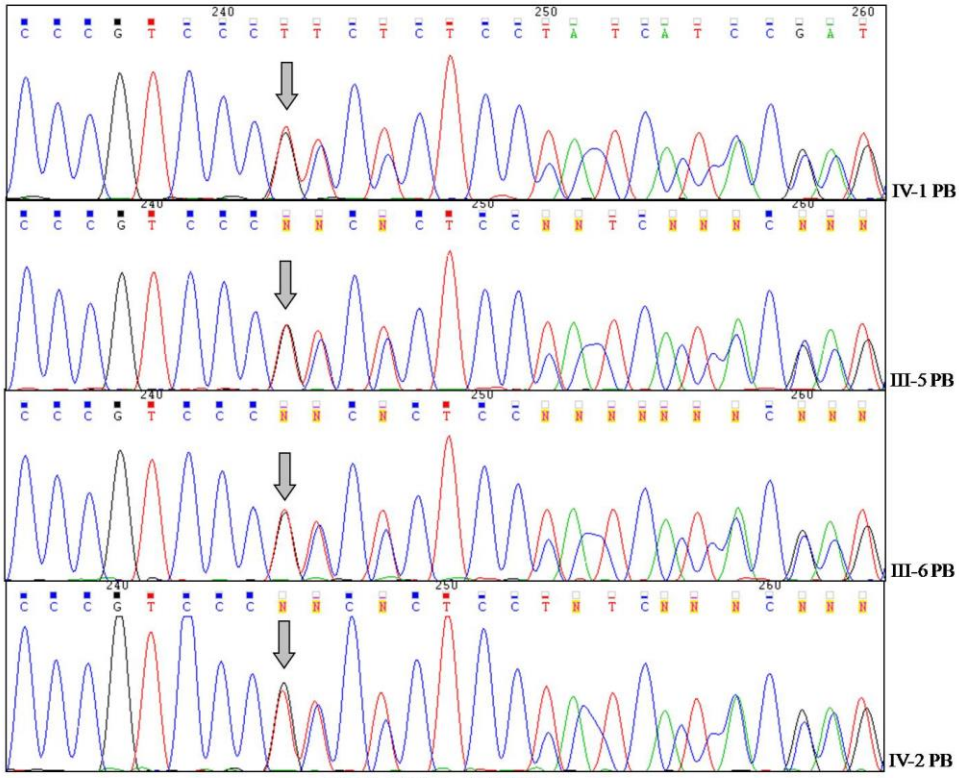
A. Family ID16. Pedigree is reported in Figure 1B. A1. Electropherogram: a new germline 5bp duplication (c.-594dupCCCGT) in the *BAP1* promoter region was found in the proband (IV-1 PB), in a sister (IV-2 PB), in her mother (III-5 PB) and in her maternal aunt (III-6 PB). This duplication is *in cis* with the p.Asp567Ala missense variant.

B. Family ID4. B1. Pedigree: the proband (II-1) had cutaneous melanoma and breast cancer, the brother (II-2) had papillary renal cell carcinoma type 2, the father (I-1) died for mesothelioma and the mother (I-2) had breast cancer. B2. Electropherogram: a new germline 19bp duplication (c.-247dupCCTTCGCCCCCGTCCCTCC) in the *BAP1* promoter region was found in the proband (II-1 PB). No specimens from relatives were available for segregation analysis.

Supplementary Figure 1

A Family ID16

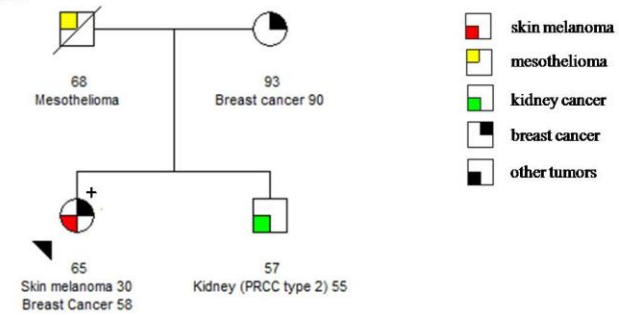
A1



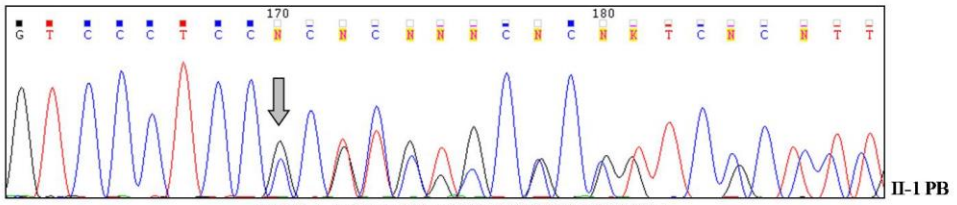
BAP1 c.-594dupCCCGT

B Family ID4

B1



B2



BAP1 c.-247dupCCTTCGCCCCCGTCCCTCC

Supplementary Figure 2 - Predicted structure of BAP1 protein

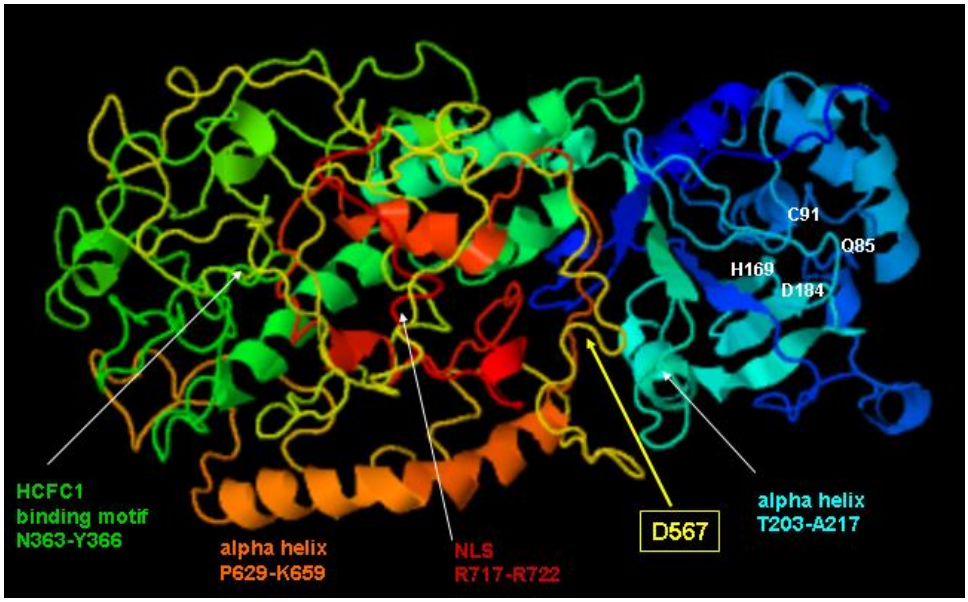
Predicted structure of BAP1 protein according to Protein Homology/analogY Recognition Engine v 2.0 (Phyre2). The yellow arrows indicate the position of D567 (a) or the mutant A567 (b).

The wild type negative charged D567 seems to localize at the interface with the ubiquitin carboxy-terminal hydrolase domain (residues 1-240) depicted in dark to light blue with the catalytic sites Q85, C91, H169 and D184 shown in white. Known domains are indicated with different colours according to their position: HCFC1 binding motif in green (residues 363-366), the long alpha helix of the BRCA1 binding domain in orange (residues 629-659/669) and the Nuclear Localization Signal (NLS, residues 717-722) in red.

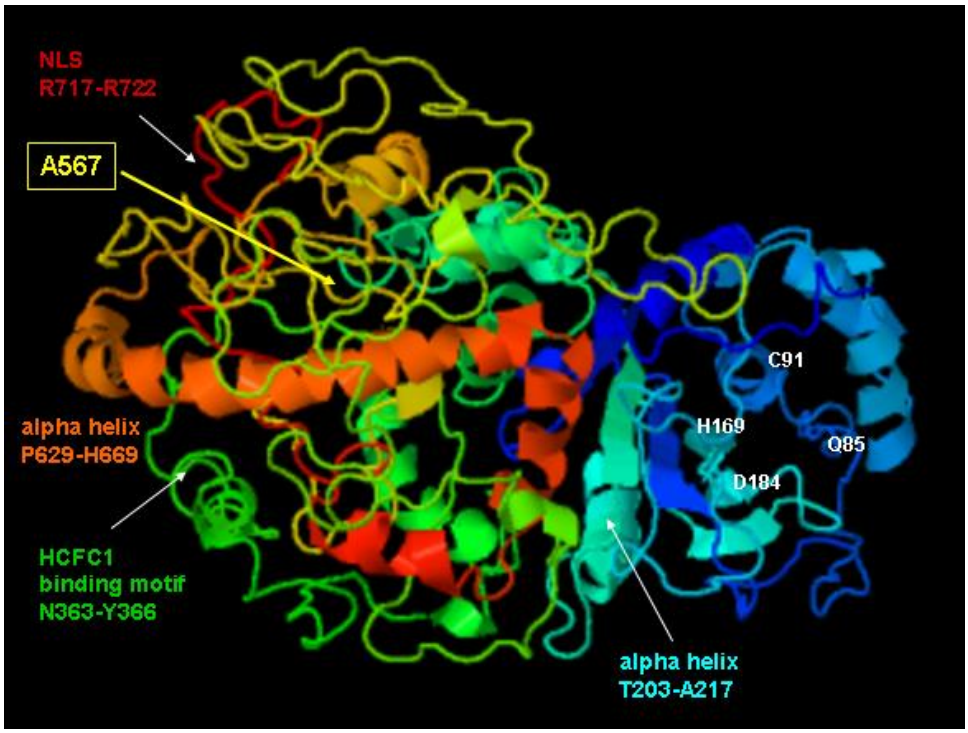
The mutant hydrophobic A567 is predicted buried within the globular portion of the protein and the relevant domains of the BAP1 protein seem to assume a different position.

Supplementary Figure 2

a)



b)



Supplementary Table I – Probands clinical data

Family ID (sex)	Age at diagnosis	Proband's tumors	Other clinical information	Asbestos exposure	Follow-up (years)	Family history
Cases/families with the association of cutaneous melanoma and mesothelioma						
1 (F)	33	Cutaneous melanoma of the left leg (superficial spreading melanoma, 0.46 mm Breslow thickness, Stage IA)	No dysplastic nevi, 10-50 melanocytic nevi, Fitzpatrick skin phototype II	Not registered (mother)	No relapse (5)	Mesothelioma (mother), breast cancer (maternal grandmother)
2 (F)	43	Cutaneous melanoma of the abdomen (superficial spreading melanoma, 0.20 mm Breslow thickness, Stage pT1)	High grade naso pharynx carcinoma with lymph node metastasis (24) Rectal carcinoma (56)	Not registered (father)	Exitus for metastatic rectal carcinoma (14)	Mesothelioma (father) and other tumors in the paternal family
3 (F)	41	Cutaneous melanoma (superficial spreading melanoma, 1.00 mm Breslow thickness Stage IB)	Previous excision of several dysplastic nevi Fitzpatrick skin phototype II	Possible occupational exposure as cotton weaver for 5 years (mother)	No relapse (10)	Mesothelioma and cutaneous melanoma (mother)
4 (F)	30	Cutaneous melanoma of the left leg (superficial spreading melanoma, Breslow thickness not available)	Breast cancer (58)	Not registered (father)	Subcutaneous metastasis (35)	Mesothelioma (father), breast cancer (mother), papillary renal cell carcinoma type 2 (brother)
5 (F)	53	Cutaneous melanoma of the ear (53) (superficial spreading melanoma, 1.1 cm Breslow thickness Stage pT2a), ephitelioid pleural mesothelioma (53), cutaneous melanoma of the scalp (57)	Meningioma (37)	No exposure to asbestos could be identified, but residential history was incomplete	No relapse (5)	Gastric cancer (mother), basal cell carcinoma of the skin (brother)
6 (M)	65	Epithelioid mesothelioma	-	No interview available	(3)	Melanoma (brother), colon cancer (father)
Cases/families with melanoma (without mesothelioma) and with familiarity for *BAP1* associated tumors (i.e. early onset melanoma, either ocular or cutaneous, multiple primary tumors, positive family history, development of other tumors except mesothelioma)						

Family ID (sex)	Age at diagnosis	Proband's tumors	Other clinical information	Asbestos exposure	Follow-up (years)	Family history
7 (F)	25 and 30	Bilateral ocular melanoma (uvea)	*In situ* ductal breast tumor (40)	-	No relapse (23)	Acustic neurinoma (sister), larynx tumor (father and two paternal relatives), bladder tumor (mother)
8 (M)	31 and 33	Ocular melanoma (iris-uvea)	No dysplastic nevi <10 melanocytic nevi Fitzpatrick skin phototype III	-	No relapse (5)	Negative
9 (F)	67 and 72	Ocular melanoma (uvea) and cutaneous melanoma (1st Clark level)	Thyroidectomy for goiter (58) Schwannoma of the auditory nerve - facial dx (71)	-	No relapse (1)	Cutaneous melanoma (daughter and niece), lung cancer (father)
10 (F)	63	Ocular melanoma (choroid)	Bilateral breast cancer (60, 65)	-	No relapse (7)	Breast cancer (sister, maternal cousin, paternal aunt), renal cancer (maternal aunt)
11 (F)	46	Cutaneous melanoma of the abdomen (superficial spreading melanoma, 0.20 mm Breslow thickness, Stage IA)	No dysplastic nevi, <10 melanocytic nevi	-	No relapse (4)	Ocular melanoma (uveal, father), colon cancer
12 (F)	43	Two synchronous cutaneous melanomas of left and right leg (superficial spreading melanoma, 0.20 and 0.28mm Breslow thickness, Stage IA)	No dysplastic nevi, <10 melanocytic nevi, Fitzpatrick skin phototype III Renal cell cancer, clear cells with focal areas of papillary architecture (41)	-	No relapse (4)	Negative
13 (M)	65	Lentigo maligna melanoma of the right forearm (0.23mm Breslow thickness, Stage IA)	No dysplastic nevi Fitzpatrick skin phototype II	-	No relapse (13)	Cutaneous melanoma (daughter, nephew), renal cell carcinoma (brother)
14 (M)	54 and 58	Three cutaneous primary melanomas: lentigo melanoma of the left cheekbone (Stage 0); two synchronous cutaneous melanomas of the left shoulder and lumbar region (superficial spreading melanoma 0.65 mm Breslow thickness, Stage IA and 2.34 mm Breslow thickness, Stage IIIB)	No dysplastic nevi, >50 melanocytic nevi, Fitzpatrick skin phototype I	-	Exitus for metastatic melanoma (5)	Cutaneous melanoma (mother), renal cell carcinoma + uterus cancer + breast cancer (sister), pancreatic carcinoma, liver, prostate

Family ID (sex)	Age at diagnosis	Proband's tumors	Other clinical information	Asbestos exposure	Follow-up (years)	Family history
15 (F)	34 and 40	Two cutaneous primary melanomas of the supraclavear left and left breast regions (superficial spreading melanomas, 0.64 and 0.23 mm Breslow thickness, Stage IA)	No dysplastic nevi, > 50 nevi melanocytic nevi, Fitzpatrick skin phototype III	-	No relapse (10)	Cutaneous melanoma (paternal uncles), renal cell cancer (father), lung cancer (two paternal uncles)
16 (F)	24 and 25	Two cutaneous amelanotic melanomas of the left shoulder and of the right arm (nodular melanoma, 1.80 mm Breslow thickness, Stage IB and superficial spreading melanoma, 0.30 mm Breslow thickness, Stage IA)	No dysplastic nevi, >50 clinical atypical melanocytic nevi Fitzpatrick skin phototype II	-	No relapse (9)	Cutaneous melanoma (paternal uncle), non-Hodgkin cutaneous lymphoma (low grade, CD20+, mother)
17 (F)	31 and 33	Two cutaneous primary melanomas (superficial spreading melanoma, Breslow thickness not available)	-	-	Not available	Cutaneous melanoma (father)
18 (F)	56 and 57	Two cutaneous melanomas (superficial spreading melanoma, Breslow thickness not available)	-	-	Not available	Cutaneous melanoma (father)
19 (F)	67	Two synchronous cutaneous melanomas of the right hip and of the right arm (superficial spreading melanoma, 0.20 mm Breslow thickness, Stage IA and melanoma *in situ*, Stage 0)	No dysplastic nevi, <10 melanocytic nevi Fitzpatrick skin phototype II Facial basal cell carcinoma (69)	-	No relapse (4)	Cutaneous melanoma (sister), breast cancer, CNS, prostate cancer
20 (M)	20	Cutaneous melanoma of left leg (superficial spreading melanoma, 0.52 mm Breslow thickness, Stage IA)	No dysplastic nevi, <10 melanocytic nevi Fitzpatrick skin phototype II	-	No relapse (3)	Cutaneous melanoma (mother), breast and colon cancer
21 (M)	24	Cutaneous melanoma of the abdomen (superficial spreading melanoma, 0.26 mm Breslow thickness, Stage IA)	No dysplastic nevi, <10 melanocytic nevi, Fitzpatrick skin phototype II	-	No relapse (6)	Cutaneous melanoma (mother), prostate cancer, vulvar cancer
22 (F)	27	Cutaneous *in situ* melanoma on the back (Stage 0)	Dysplastic nevi, >50 melanocytic nevi, Fitzpatrick skin phototype II	-	No relapse (5)	Cutaneous melanoma (father), prostate cancer
23 (M)	32	Cutaneous melanoma of the sternum (nodular melanoma, 2.10 mm Breslow thickness, Stage IIB)	No dysplastic nevi, <10 melanocytic nevi, Fitzpatrick skin phototype II	-	No relapse (3)	Cutaneous melanoma (sister)

Family ID (sex)	Age at diagnosis	Proband's tumors	Other clinical information	Asbestos exposure	Follow-up (years)	Family history
24 (F)	35	Cutaneous melanoma of the right scapula (superficial spreading melanoma, 0.90 mm Breslow thickness, Stage IB)	No dysplastic nevi, 10-50 melanocytic nevi, Fitzpatrick skin phototype II	-	No relapse (3)	Cutaneous melanoma + thyroid cancer (father)
25 (F)	39	Cutaneous melanoma (superficial spreading melanoma, Breslow thickness not available)	-	-	Not available	Cutaneous melanoma + colon cancer (brother)
26 M	82	Cutaneous melanoma of the back, (0.83 mm Breslow thickness, 3rd Clark level)	Fitzpatrick skin phototype III, chronic sun exposure	-	No relapse (1)	Cutaneous melanoma (brother), pancreatic cancer (sister)
27 (M)	38 to 52	Ten cutaneous primary melanomas	Testicular teratoma (27) multinodular goiter	-	Not available	Breast cancer (mother)
28 (M)	57 and 58	Three cutaneous primary melanomas	Lipoma (27) Adrenal cortex adenoma (53)	-	Not available	Prostate cancer (father), liver cancer (paternal uncle and paternal grandfather)
29 (M)	61	Cutaneous melanoma of the back (4.6mm Breslow thickness, 5th Clark level)	Fitzpatrick skin phototype III, sun exposure	-	No relapse (2)	Breast cancer (sister and two paternal aunts), lung cancer (paternal uncle)
Cases/families with mesothelioma (without melanoma) and with familiarity for *BAP1* associated tumors (other mesothelioma cases among relatives, development of other tumors except melanoma)						
30 (F)	46	Epithelioid pleural mesothelioma	-	Household (low)	No relapse (10)	Biphasic mesothelioma (mother), lung cancer (father), bone cancer (maternal aunt), bowel cancer (maternal grandmother)
31 (M)	49	Biphasic peritoneal mesothelioma	-	Household	Exitus (7 months)	Mesothelioma (three sisters and brother)
32 (M)	53	Biphasic pleural mesothelioma	-	Household (low)	Exitus (5)	Mesothelioma (paternal uncle and two cousins), leukemia (twin brother of the father)
33 (M)	55	Epithelioid pleural mesothelioma	-	Possible occupational/ household (low)	Not available	Mesothelioma (paternal grandmother), prostate cancer (father)
34 M	68	Epithelioid pleural mesothelioma	-	No interview available	No relapse (1)	Mesothelioma (sister and nephew)

Family ID (sex)	Age at diagnosis	Proband's tumors	Other clinical information	Asbestos exposure	Follow-up (years)	Family history
35 (M)	70	Epithelioid pleural mesothelioma	-	Household (low)	No relapse (3)	Mesothelioma (both parents), papillary thyroid carcinoma (daughter)
36 (M)	66	Sarcomatoid mesothelioma	-	Possible occupational exposure as baker 1962-1974 and residential exposure 1947-1977 (in Stradella)	No relapse (2)	Mesothelioma (father and cousin), lung cancer (paternal uncle)
37 (M)	Not available	Mesothelioma, neuroblastoma	-	Possible occupational exposure as pipe-fitter 2003-2004, radiotherapy 1985 (for neuroblastoma)	Not available	Negative
38 (F)	28 and 18	Epithelioid mesothelioma, Hodgkin linfoma	-	Para-occupational exposure 1985-1993 (father boiler and pipe-fitter), radiotherapy 2003 (for NHL)	Exitus (5 months)	Lung cancer (maternal cousin), leukemia (maternal cousin's daughter)

Family ID (sex)	Age at diagnosis	Proband's tumors	Other clinical information	Asbestos exposure	Follow-up (years)	Family history
39 (M)	Not available	Epithelioid mesothelioma, basalioma	-	Possible occupational exposure as house-builder 1944 and 1951-1983	Not available	Negative
40 (F)	69	Epithelioid mesothelioma with metastasis on the scalp	-	Possible occupational exposure as cotton warper 1950-1983 and household exposure 1982 (removal of asbestos-cement roofing)	No relapse (12)	Lung cancer (uncertain diagnosis, mother)

Supplementary Table II – Primer sequences for the amplification of *BAP1*, *CDKN2A*, *CDK4*, *MITF*, *TERT*, *POT1* genes.

GENE	PRIMERS	SEQUENCES	AMPLICON LENGTH (bp)
	BAP1_prom1F BAP1_prom2R	GCTTTAGTCGTTGACACAGG GGGAGAAAAGGCTCTTACCG	1040
BAP1	BAP1_ex1-3F BAP1_ex1-3R	GAAGACGAGCCCAGAGG CGTAGGGTTCCTGGCACTGTC	619
	BAP1_ex4bisF BAP1_ex4bisR	TTCATAAGGAGACTGGGTGGA GACACAGAGAGTGGACTCAG	597
	BAP1_ex5F BAP1_ex5R	TGGGTATTTGGTAGGTGCTTG CTTTCCCCGCAACTGCATC	486
	BAP1_ex6-7F BAP1_ex6-7R	TCTGTGTTCCTTCCGATTCC GGTCGGGCAATATGGTGTAG	562
	BAP1_ex8F BAP1_ex8R	CGACCAGCTCCTGATTCC CCTGATCTTGCCAGATTCACC	313
	BAP1_ex9F BAP1_ex9R	CAGGTCTGCTGGTTCACTTCC CTATTCTCCCTCCCCACTCC	531
	BAP1_ex10F BAP1_ex10R	GGAAAGGTGGGACTTGGAG AGGGGCCTGTGGTAACAGC	531
	BAP1_ex11F BAP1_ex11R	GGCTGGGCTGTTCTTCTCTG GGAACCACATGGGAAAATTGC	374
	BAP1_ex12F BAP1_ex12R	CAGTGTAAGTGGGTGGCAGC CAAACTCCGCAGGTGCTCAAC	425
	BAP1_ex13F BAP1_ex13R	GGCTTAGCATGGCTAGTTCAAG GCAGGACACTTTGTGGTCAC	715
	BAP1_ex14F BAP1_ex14R	CTTGGACTGGCTCACTGGC GAAAGTCTTCTGGCACATGGC	408
	BAP1_ex15-16F BAP1_ex15-16R	GAAGACTTTCTGGGTTGGGTGG CCTGCGAAGAGGTAGAGACC	591
	BAP1_ex17F BAP1_ex17R	CCTGAGGCTTGAGCAGACCTTG GCTGTGCCCCAACTCCAGATG	466
CDKN2A	p16_ex 1α F p16_ex 1α R	GAAGAAAGAGGAGGGGCTG GCGCTACCTGATTCCAATTC	340
	p14_ex 1β F p14_ex 1β R	CGCTCAGGGAAGGCGGGTG ACCAAACAAAACAAGTGCCG	407
	p16_ex 2F p16_ex 2R	GGGGCTTGTGTGGGGGTCTG GTGCTGGAAAATGAATGCTCTG	483
	p16_ex 3F p16_ex 3R	CGGTAGGGACGGCAAGAGAG CCTGTAGGACCTTCGGTGACTGA	179
CDK4	CDK4_ex 2F CDK4_ex 2R	GCTGCAGGTCATACCATCCT ATCATCACACCCCACCTATAGG	372
MITF	MITF_ex 9F MITF_ex 9R	TGTGCTCTGCCTATTTCAGTG AAGAAAACCCCTTCAGGTAAGTT	340
TERT	Prom F Prom R	GTCCTGCCCCTTCACCTT AGCACCTCGCGGTAGTGG	488
POT1	POT1_ex10F POT1_ex10R	AGCTGATATTCAACCACACTCGT GCACAAAAGGCTAGGGAACTATC	435

Supplementary Table III – *In silico* analyses of *BAP1* exonic mutations

***BAP1* missense variants (exon) Genomic position on chr3**				**[Ref] Case ID Tumors in patients with *BAP1* variants (Age of diagnosis) and in untested family members**								
1000G ExAC	**Mut taster**	**SIFT**	**Provean**	**PolyPhen HumVar** (HumDiv)	**Mutation assessor**	**Condel**	**Phyre2**	**LOH**	**IHC**	**Segregation analysis**	**Splice site prediction**	**Conclusion**
c.41T>A (**p.Leu14His)** (exon 2) 3:52443756A>T				[23] NCI-1326 RCC (40, 44, 46, 54, 57) in carriers / RCC in other family members								
-	0,9999 diseasecausing	0,000 damaging	-6,04 deleterious	1,00 probablydamaging (1,00)	2,48 medium impact	0,55568 deleterious	likely to affect function (highest score)	clear LOH in proband's tumors	Loss of BAP1 proteinexpression	Variant cosegregates with the RCC predisposition (LOD score 1.2)	No alterations	Disease causing
c.277A>G (**p.Thr93Ala**) (exon 5) 3:52442072T>C				[22] A Adenocarcinoma of Unknown Primitive Tumor, Breast Ca x3, RCC (37, 39, 40, 47, 47), Cervical Ca, CaSU in carriers / Esophagus Ca x2, Head and Neck Carcinoma, Lung Ca, Breast Ca in other family members								
-	0,9999 diseasecausing	0,001 damaging	-4,56 deleterious	0,989 probablydamaging (0,999)	3,875 higt impact	0,73011 deleterious	likely to affect function (highest score)	RCC sample from the index case shows LOH for the 3pter-14.1 chromosomal region (genomic position 0-64Mb) as the unique acquired large genomic deletion in this tumor	Loss of BAP1 proteinexpression	Variant segregates with RCC (Segregation analysis based on identity by descend -IBD-)	New cryptic acceptor site (Human Splicing Finder, MaxEnt)	Disease causing

***BAP1* missense variants** **(exon) Genomic position on chr3**				**[Ref] Case ID** **Tumors in patients with *BAP1* variants (Age of diagnosis) and in untested family members**								
1000G ExAC	**Mut taster**	**SIFT**	**Provean**	**PolyPhen HumVar** (HumDiv)	**Mutation assessor**	**Condel**	**Phyre2**	**LOH**	**IHC**	**Segregation analysis**	**Splice site prediction**	**Conclusion**
c.299T>C (**p.Leu100Pro**) (exon 5) 3:52442050A>G				[11] UM (45, 56), Cholangiocarcinoma (71), Urothelial Carcinoma (48) in carries / RCC, Brain Ca, Leukemia, Uterine Ca in other family members								
-	0,9999 diseasecausing	0,000 damaging	-6,62 deleterious	1,00 probablydamaging (1,00)	3,77 higt impact	0,69994 deleterious	likely to affect function (highest score)	LOH of 8 markers, consistent with monosomy 3	nd	Variant segregates with UM (mother-son)	No alterations	Disease causing
c.539T>C (**p.Leu180Pro**) (exon 7) 3:52441231A>G				[16] FUM124 UM (60), CM (72), MM (71), BCC (56, 65, 68) in carriers / Breast Ca x2, BCC x6, SCC, Non-melanoma Skin Ca, CM x2, Prostate Ca, Uterine Ca, Liposarcoma, Melanoma (meningeal), Cervical Ca, CaSU x3, MM, GI TractCancer in otherfamilymembers								
-	0,9999 diseasecausing	0,000 damaging	-6,90 deleterious	1,00 probablydamaging (1,00)	3,725 higt impact	0,70362 deleterious	likely to affect function (highest score)	nd	nd	Variant does not unconditionally track with the disease	No alterations	Disease causing
c.1327A>T (**p.Asn443Tyr**) (exon 13)3:52437834T>A				[14] 16 UM in carrier / CaSU in otherfamilymembers								
-	0,9997 diseasecausing	0,005 damaging	-0,89 neutral	0,855 possiblydamaging (0,994)	0,55 neutral	0,46382 neutral	unlikely to affect function (highest score)	nd	nd	no data	No alterations	VUS

***BAP1* missense variants** **(exon) Genomic position on chr3**				**[Ref] Case ID** **Tumors in patients with *BAP1* variants (Age of diagnosis) and in untested family members**								
1000G ExAC	**Mut taster**	**SIFT**	**Provean**	**PolyPhen HumVar** (HumDiv)	**Mutation assessor**	**Condel**	**Phyre2**	**LOH**	**IHC**	**Segregation analysis**	**Splice site prediction**	**Conclusion**
c.1445C>T, **p.Ser482Leu** (exon 13)3:52437716G>A				[14] 19 UM in carrier / CM, Lung Ca x3 in otherfamilymembers								
- 1 het 60.463 All	0,9999 diseasecausing	0,104 tolerated	-0,71 neutral	0,057 benign (0,760)	0,895 low impact	0,472555 neutral	unlikely to affect function (highest score)	nd	nd	no data	New acceptor site (MaxEnt, NetGene2)	VUS
c.1708C>G **p.Leu570Val** (exon 13)3:52437453G>C				[21] UM (18, 46, 62), CM (27, 27, 33), MM (47), Peritoneal MM (84), Lung Ca (46), Paraganglioma (42), Breast Ca (75), Malignant Fibrous Histiocytoma (45) in carriers / Prolactinoma in other family member								
-	0,6937 diseasecausing	0,054 tolerated	-0,52 neutral	0,016 benign (0,075)	0,695 neutral	0,469014 neutral	unlikely to affect function (highest score)	LOH in UM and PGL	nd	Variant segregates in this multi-cancer family	Activation of a cryptic donor site 22bp before the end of the exon (Human Splicing Finder, MaxEnt, BDGP, NetGene2, GeneScan,FGENESH 2.6, FSPLICE 1.0)	Disease causing
c.518A>G **(p.Tyr173Cys)** (exon 7)3:52441252T>C				[17] UM (72), MM, adenocarcinoma in carriers / MM x2, RCC, ALL in otherfamilymembres								

***BAP1* missense variants** **(exon) Genomic position on chr3**				**[Ref] Case ID** **Tumors in patients with *BAP1* variants (Age of diagnosis) and in untested family members**								
1000G ExAC	**Mut taster**	**SIFT**	**Provean**	**PolyPhen HumVar** (HumDiv)	**Mutation assessor**	**Condel**	**Phyre2**	**LOH**	**IHC**	**Segregation analysis**	**Splice site prediction**	**Conclusion**
-	0,9999disease causing	0,000damaging	-7,75 deleterious	0,999probablydamaging(1,000)	3,27medium	0,662034deleterious	likely to affect function (highest score)	nd	Loss of nuclear BAP1 labeling in adenocarcinoma but nuclear labeling for BAP1 was retained in the MM (No UM specimen available)	no data	Activation of an exonic cryptic donor site (Human Splicing Finder, MaxEnt)	Disease causing
c.233A>G(**p.Asn78Ser**) (exon 4) 52442512T>C				[6] ABS2570 Kidney Ca, MM in carriers / Gastic Ca x4 in otherfamilymembres								
-	0,9999disease causing	0,06tolerated	-3,80 deleterious	0,608possiblydamaging(0,767)	0,7 neutral	0,442072neutral	unlikely to affect function (high score)	nd	nd	no data	Activation of an exonic cryptic donor site (Human Splicing Finder, MaxEnt, BDGP, NetGene2, GeneScan, FSPLICE); Activation of an exonic acceptor site, with one or more cryptic branch point(s) (Human Splicing Finder	Disease causing
c.1748C>T (**p.Ser583Leu**) (exon 14)3:52437296G>A				[6] ABS3313 MM (67), BCC in carrier / CM in otherfamilymember								

***BAP1* missense variants** **(exon) Genomic position on chr3**				**[Ref] Case ID** **Tumors in patients with *BAP1* variants (Age of diagnosis) and in untested family members**								
1000G ExAC	**Mut taster**	**SIFT**	**Provean**	**PolyPhen HumVar** (HumDiv)	**Mutation assessor**	**Condel**	**Phyre2**	**LOH**	**IHC**	**Segregation analysis**	**Splice site prediction**	**Conclusion**
- 1 het 6503 All	0.9871diseasecausing	0,38tolerated	-0,84 neutral	0,001benign (0,001)	0,345 neutral	0,451540neutral	unlikely to affect function (high score)	nd	nd	no data	New acceptor site (MaxEnt)	VUS
c.1147C>T (**p.Arg383Cys**) (exon 12)3:52438572G>A				[6] ABS3023 MM in carrier / Breast Ca in otherfamilymember								
1 het 6502 All5 het (4.471e-05)	0,9999diseasecausing	0,03 damaging	-2,51 deleterious	0,990probablydamaging(1,000)	1,845 low impact	0,489999neutral	unlikely to affect function (high score)	nd	nd	no data	No alterations	VUS
c.1700A>C (**p.Asp567Ala)** (exon 13) 52437461T>G				[this work] ID16 CM (28, 28), Cutaneous lymphoma NH-B (57) in carriers /								
-	0,9999 diseasecausing	0,003 damaging	-1,71 neutral	0,696 possiblydamaging (0.976)	0,975 low impact	0,48069 neutral	unlikely to affect function (high score)	Melanoma sample show a decreased amount of the paternal allele, suggesting the loss of the wild type allele	Two different melanomas of the index case show the normal BAP1 expression in the nucleus	Variant does not unconditionally track with the disease	No alterations	VUS
More frequent missense variants reported in ExAc (heterozygote, homozygote and total individuals of the population at higher frequency)												

***BAP1* missense variants (exon) Genomic position on chr3**				**[Ref] Case ID Tumors in patients with *BAP1* variants (Age of diagnosis) and in untested family members**								
1000G ExAC	**Mut taster**	**SIFT**	**Provean**	**PolyPhen HumVar** (HumDiv)	**Mutation assessor**	**Condel**	**Phyre2**	**LOH**	**IHC**	**Segregation analysis**	**Splice site prediction**	**Conclusion**
c.121G>A (**p.Gly41Ser**) (exon 3) 3:52443571C>T				nn								
78 het 2.506 Asn	0,9999 diseasecausing	0,369 tolerated	-3,12 deleterious	0.078 benign (0,078)	0,049 neutral	0,458715 neutral	unlikely to affect function (high score)	-	-	-	New cryptic donor site (MaxEnt)	VUS / Likely benign
c.869A>G (**p.Asn290Ser**) (exon 10) 3:52439843T>C				nn								
54 het 5.772 Lat	0,9998 poly-morphism	1,000 tolerated	-0,57 neutral	0.000 benign (0,000)	neg score neutral	0,332615 neutral	unlikely to affect function (highest score)	-	-	-	No alterations	Likely benign
c.1201T>G (**p.Tyr401Asp**) (exon 12)3:52438518A>C				nn								
20 het 60.059 All	0,9999 diseasecausing	0,537 tolerated	-2,12 neutral	0.186 benign (0,437)	0,095 neutral	0,491095 neutral	unlikely to affect function (highest score)	-	-	-	No alterations	VUS / Likely benign
c.1786A>G (**p.Ser596Gly**) (exon 14) 3:52437258T>C				nn								

***BAP1* missense variants (exon) Genomic position on chr3**				**[Ref] Case ID Tumors in patients with *BAP1* variants (Age of diagnosis) and in untested family members**								
1000G ExAC	**Mut taster**	**SIFT**	**Provean**	**PolyPhen HumVar** (HumDiv)	**Mutation assessor**	**Condel**	**Phyre2**	**LOH**	**IHC**	**Segregation analysis**	**Splice site prediction**	**Conclusion**
740 het 30 hom 5.201 Afr	0,9999 poly-morphism	0,393 tolerated	-0,01 neutral	0.000 benign (0,000)	neg score neutral	0,390318 neutral	unlikely to affect function (highest score)	-	-	-	No alterations	Benign
c.1838C>T (**p.Thr613Met**) (exon 14) 52437206G>A				nn								
128 het 1 hom 5.202 Afr	0,9999 poly-morphism	0,063 tolerated	-0,34 neutral	0.001 benign (0,001)	0,000 neutral	0,426829 neutral	unlikely to affect function (highest score)	-	-	-	No alterations	Benign

This table was modified from Rai et al. 2016 [16]

RCC renal cell carcinoma, CaSU, Cancer site unknown, UM uveal melanoma, MM malignant mesothelioma, CM cutaneous melanoma, BCC basal cell carcinoma, ALL Acute lymphocytic leukemia, LOH loss of heterozygosity, IHC immunohistochemistry, Nd not done, NN none, VUS variant of unknown significance.