Brca1-Associated Protein 1 (BAP1) Immunohistochemical Expression as a Diagnostic Tool in Malignant Pleural Mesothelioma Classification: a Large Retrospective Study

BRCA1-ASSOCIATED PROTEIN 1 (BAP1) IMMUNOHISTOCHEMICAL EXPRESSION AS A DIAGNOSTIC TOOL IN MALIGNANT PLEURAL MESOTHELIOMA CLASSIFICATION: A LARGE RETROSPECTIVE STUDY

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ABSTRACT

Background: Malignant pleural mesothelioma (MPM) is a highly aggressive disease with limited therapeutic options. Histology remains among the most reliable prognostic factors, since epithelioid is associated with the best and sarcomatoid subtype with the worst prognosis. Biphasic subtype has an intermediate prognosis, but its definitive histological diagnosis may be challenging due to the difficult assessment of the neoplastic nature of the stromal component. Recent data identified BRCA1-Associated Protein 1 (*BAP1*) as one of the most frequently mutated genes in MPM. Immunohistochemistry for BAP1 has been proposed to be predictive for the detection of *BAP1* mutation in neoplastic cells. The aim of the present study was to define the diagnostic usefulness of BAP1 immunohistochemical determination in MPM, with clinical-pathological correlation.

Methods: A series of 143 MPMs was investigated for BAP1 protein expression in correlation with clinical and pathological data, including a newly proposed nuclear grade. A pilot series of twenty selected cases were also investigated for *BAP1* mutational status.

Results: Nuclear negative staining for BAP1 occurred in 62% of MPMs (including 27% of cytoplasmic pattern) and was significantly associated with the presence of *BAP1* mutation, epithelioid subtype and a better prognosis. In a subgroup of cases, the pattern of expression of BAP1 in stromal cells supported their distinction into reactive vs neoplastic, thus helping the correct classification of biphasic histology.

Conclusion: We showed that BAP1 protein determination is a diagnostic tool to correctly distinguish biphasic MPM from epithelial subtypes with an atypical/activated reactive stroma and is an independent prognostic parameter in MPM.

KEY WORDS: malignant mesothelioma, pleura, BAP1 mutation, prognosis, histology

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare, highly aggressive, relatively chemo- and radio-resistant type of cancer with limited therapeutic options[1]. In patients with advanced stage disease treated with cisplatin and pemetrexed median survival time is approximately 12 months, long-term survivors are occasionally seen[2,3] and, disappointingly, there is no approved agent for second-line chemotherapy[4]. In MPM, proposed prognostic factors include clinical variables, radiological parameters at presentation, molecular/pathological findings, but the vast majority of them are not fully validated[5] and the proposed scoring systems (Cancer and Leukemia Group B and European Organization for Research and Treatment of Cancer[6,7]) are not widely used. Histology remains among the most reliable prognostic factors, since epithelioid subtype is associated with the best prognosis and the sarcomatoid subtype with the worst[8]. While the biphasic/mixed subtype has usually an intermediate prognosis, sometimes its definitive histological diagnosis may be cumbersome, due to the sometimes problematic grade assessment of nuclear atypia in the stromal component. Furthermore, high grade MPM with pleomorphic features has controversial histologic classification: although according to guidelines is classified as epithelioid MPM[8,9], clinical and pathological findings suggest an association with sarcomatoid subtype[10,11].

Recently, in the epithelioid subtype only, a nuclear grading system based on nuclear atypia and mitotic count has been proposed and it was shown to be associated to prognosis[12].

Next-Generation Sequencing (NGS) data indicate cyclin-dependent kinase Inhibitor 2A (*CDKN2A*), neurofibromatosis 2 (*NF2*) and BRCA1-Associated Protein 1 (*BAP1*) as the most frequently mutated genes in MPM[13-15]. BAP1 is a nuclear de-ubiquitinating enzyme[16], recently suggested to be a tumor suppressor gene, with a role in cell proliferation and growth inhibition[17]. *BAP1* gene is located on

chromosome 3p21, a region that harbors germ-line mutations associated to an inherited multi-cancer syndrome with a dominant autosomal transmission[18]. So far, BAP1 is the first and only gene that is proposed in influencing environmental carcinogenesis: when germ-line BAP1 exists, it leads to a higher susceptibility to asbestos favoring the clinical onset of MPM[17, 19-21]. In addition BAP1 is the most frequently mutated gene in sporadic MPM[13-15,22]; the mutational status is associated to a less aggressive tumor phenotype and improved prognosis in familial mesothelioma[19] and probably also in sporadic mesothelioma[23-25].

The loss of *BAP1* gene, independently of the underlying mechanism (e.g. gene deletion or insertion, point mutation, gain or loss), translates into nuclear negativity for BAP1 expression at immunohistochemistry (IHC), with a high concordance between the two techniques[13,22,26]. Loss of nuclear BAP1 protein expression is useful in differentiating both malignant mesothelioma *versus* pleural malignant mimickers (e.g. lung and ovary cancers) and reactive *versus* malignant mesothelial proliferation, with a high specificity, despite the variable sensitivity[25,27].

The aim of the present study was: a) to clarify the diagnostic usefulness of BAP1 IHC in characterizing MPM biphasic subtype with molecular confirmation and b) to correlate in sporadic MPM BAP1 protein expression with clinical-pathological and outcome data to validate its prognostic role.

Because of the challenging differential diagnosis between biphasic and epithelioid MPM with atypical reactive stroma[8] and in consideration that the cellular distribution of BAP1 IHC expression patterns among different MPM histotypes is not clearly established, we investigated the role of BAP1 IHC in 143 cases of MPM (including 101 surgical resected cases) aiming to further characterize the current histotypes of MPM. Furthermore, we performed molecular analysis of *BAP1* gene status in a pilot study series of 20 MPM with different IHC staining pattern and then

separately in epithelial and stromal component of three cases of morphologically biphasic MPM to correlate both BAP1 protein and gene status. Finally, we correlate BAP1 IHC with clinical-pathological and survival data.

We detected that a) BAP1 protein nuclear expression was lost in approximately two thirds of epithelial and biphasic cases (and in 20% of sarcomatoid MPM) and BAP1 mutated tumors showed either a complete loss of the protein expression or a cytoplasmic staining pattern in epithelioid MPM; b) atypical stromal cells associated to BAP1 negative epithelioid MPMs retained BAP1 expression and molecular analysis of this stromal cell component confirmed the expected wild type status; c) higher disease stage, high nuclear grade and BAP1 expression are independent predictor of poor prognosis, irrespective of the histotype.

MATERIALS AND METHODS

Tissue collection: 101 consecutive resected samples of MPM diagnosed between 2000 and 2012 and with enough left-over tissue were retrieved from the pathology files of the Pathology Units of the University of Torino at San Luigi Hospital (Orbassano, Turin) and Città della Salute e della Scienza (Torino); furthermore to enrich the study population for sarcomatoid and biphasic MPM cases we also collected 42 consecutive thoracoscopic biopsies from Pathology Unit files of San Luigi Hospital. For all cases, the main clinical-pathological data were obtained and analyzed. Relevant clinical pathological findings included: mean age: 60 years, male/female ratio: 108/35. For surgical cases IMIG tumor stage I-II/III were: 16/55, stage IV: 30. Median overall survival for all patients was 15 months. The study received ethical approval from the local Review Board of our Institutions.

Morphological revision and grading: All haematoxylin-eosin stained slides available were reviewed by two pathologists (MP and LR) and classified according to the 2015 WHO classification criteria[8]. Additional collected morphological features included the nuclear grading of the epithelioid component both in epithelioid and biphasic MPM according to the grading system proposed by Kadota et al.[12] Briefly, this is a three-tier nuclear grade score based on the sum of nuclear atypia score – i.e., 1) low, 2) mild and 3) high - and mitotic count score – i.e 1) 0-1 mitoses/10HPF, 2) 2-5 mitoses/10HPF and 3) >5 mitoses/10HPF. Furthermore, morphological atypia of the tumour-associated stroma was also reported assessing stromal cellularity (increase of stromal spindle cells), nuclear pleomorphism, size and hypercromasia and assessed as low, moderate and high, as follows: low stromal atypia characterized by slightly increase in spindle cellularity, abundant fibrous tissue, small wrinkled nuclei with packed chromatin and smooth nuclear contours; moderate stromal atypia indicated a mild cellularity with some overlapped nuclei, little variation in nuclear size, irregular and sharp nuclear contours and inconspicuous nucleoli; high stromal atypia indicated marked hypercellularity with densely overlapped nuclei, marked variation in size, coarse chromatin and irregular nuclear membranes with evident nucleoli[28]

Immunohistochemistry: IHC was performed in all cases. Three µm thick serial paraffin sections from representative paraffin blocks were processed using an automated immunostainer (Ventana BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ, USA) with a primary antibody against BAP1 (clone-C4, rabbit monoclonal, Santa-Cruz Biotechnology, Santa Cruz, CA, USA). Non-neoplastic cells, such as vascular endothelium or inflammatory cells, acted as internal positive controls. BAP1 was considered positive when a weak-to strong nuclear positivity was shown.

Mutational analysis –A series of 20 MPM cases (16 epithelioid and 4 biphasic subtypes), selected based on the yield of BAP1 IHC staining (10 cytoplasmic, 9 nuclear negative and 1 nuclear positive)

was investigated by Sanger direct sequencing for mutational *BAP1* gene status. Briefly, genomic DNA was extracted from formalin-fixed paraffin-embedded tissues, as previously reported [29]. The entire BAP1 coding sequence was amplified with primers designed on the flanking intronic/exonic regions using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). Primers and PCR conditions are available on request. Bidirectional Sanger sequencing was performed by an external commercial service using standard protocols (Eurofins MWG Operon, Ebesberg, Germany) to screen genetic alterations in coding and in exonic/intronic junctions of gene. Putative mutant variants were validated via bidirectional re-sequencing of independent PCR amplifications. Variants were annotated according to the longest isoform RefSeqs from the Genome Reference Consortium Human Build 37.3 (NM_004656.3) and reported according to the Human Genome Variation Society guidelines. Variants characterization and bio-informatic analyses were performed according to reference databases (i.e dbSNP - build 131; http://www.ncbi.nlm.nih.gov/projects/ SNP/; 1000 Genomes - http://www.1000genomes.org/; NHLBI GO ESP - http://evs.gs. washington.edu/EVS/; somatic mutational COSMIC databases), while *in silico* prediction of functional effect was performed by SIFT (http://sift. jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SNAP (http:// rostlab.org/services/snap/) databases. Furthermore, three cases of biphasic MPM having a differential BAP1 expression in the epithelioid and atypical stromal components were separately micro-dissected and analyzed after sample enrichment of the epithelial and stromal components.

Fluorescence in situ hybridization (FISH) analysis – To further study those MPM cases that showed discrepancy in BAP1 status between IHC and direct sequencing, FISH analysis was performed on 4µm of formalin-fixed, paraffin embedded tissue sections. Briefly, slides were treated using the Invitrogen Spot-light tissue pretreatment kit (Invitrogen Corporation, Camarillo, USA), then

digested with pepsin (Invitrogen, Carlsbad, CA, USA) and dehydrated before hybridisation with FISH probes. FISH using a dual colour probe for *BAP1* gene (3p21.1) (Texas Red-labeled) / CEN3q (FITC-labeled) (Abnova, Walnut, CA, USA) was carried out according to the manufacturers' protocol. The slides were incubated with *BAP1/CEN3q* probe, co-denatured in HYBrite System at 75°C for 5 min and hybridized overnight at 37°C. Slides were then washed, dehydrated and counter-stained with 4'6' -diamidino-2-phenylindole (DAPI) (Abnova). Three to five tumor areas on each slide were selected and automated acquisition was performed with the motorized Metafer Scanning System (Carl Zeiss MetaSystems GmbH, Jena, Germany) and AxioImager epifluorescence microscope (one focus plane for DAPI and 13 focus planes for green and red spots). Analysis of the *BAP1/CEP3* probes was performed by counting red (*BAP1*) and green (*CEN3q*) spots on images taken by Metafer, and transferred into the ISIS software. The *BAP1/CEN3q* probe labels the chromosome 3 centromere green (G) and the *BAP1* gene red (R). In normal interphase cells, two green and two red signals (2G–2R) can be clearly detectable. Considering recents reports[27] and based on the evaluation of a range between 100 and 140 nuclei, only samples harboring *BAP1* gene deletion signal in ≥30% of cells were designated as positive. In addition *BAP1* homozygous or heterozygous deletion was defined as follows: homozygous deletion when at least one green without red signals (1/2 G-0R or >2G-0R) and heterozygous deletion when two green with a single red signal (2G–1R, or green more numerous than red signals, G>R) were found.

Statistical analysis – The Fisher test was used to analyze the dependence between categorical variables and nonparametric tests; Wilcoxon and Mann-Whitney were used to test for differences between subgroups in quantitative variables. Univariate analyses for survival were performed for all clinical and pathological variables; Kaplan–Meier estimating survival distributions were performed and survival curves were compared using the log–rank test. The Cox proportional

hazards regression model was used for multivariate analyses. Statistical analyses were performed using the free software R (http://www.r-project.org/) and the significance level was set at 0.05.

RESULTS

Morphological subtyping and nuclear grading – The main clinical and pathological features of the whole series of 143 cases are represented in Figure 1**.** Upon revision, cases were classified as follows: 107 epithelioid (including 12 pleomorphic), 13 biphasic and 23 sarcomatoid MPM. Excluding the 12 pleomorphic MPM among the remaining 95 epithelioid MPM, 39 had a relevant associated stromal component[8] with low-to-moderate atypia in the spindle cells (Figure 2). Kadota nuclear grade of the epithelial component was assessed in all the non-sarcomatous MPM (including the epithelial component of the biphasic MPM). A significant difference in the distribution of the nuclear grade was detected, with the 95 epithelioid MPM mostly segregated in the GI group, while pleomorphic and biphasic MPMs were mainly grouped in the GII or GIII categories ($p<0.0001$) (Table 1). In addition, the distribution of the stromal atypia significantly differed among histotypes, having all the epithelioid MPMs a low-to-moderate stromal atypia, while for the majority of those cases diagnosed as pleomorphic or biphasic MPMs the grading was high ($p<0.0001$) (Table 1).

BAP1 expression **-** Details about BAP1 expression according to histology are reported in Table 1. Overall, the lack of nuclear reactivity for BAP1 in MPM cells was reported in 89 out of 143 (62%) cases, including 24 cases (27%) with a granular cytoplasmic positive staining (Figure 3). BAP1 negativity in MPM cells showed a significant distribution among histotypes ($p<0.0001$) ranging from 22% in sarcomatoid MPM to 75% in pleomorphic MPM. Regarding stromal cells, all epithelioid MPM with morphologically atypical stroma had BAP1 positive staining in the spindle

cells. Among pleomorphic MPMs, only 1 out of 12 (8%) samples was BAP1 negative both in atypical stromal spindle cells and in the neoplastic epithelioid component. All together, in these two groups, 35 out of 51 (69%) samples showed a discrepancy between BAP1 expression in the epithelial and stromal component: 27 epithelioid and 8 pleomorphic MPMs were BAP1 negative in epithelioid neoplastic cells (including 11 with cytoplasmic pattern), but positive in stromal cells. In sarcomatoid MPM, five cases were completely negative (22%) and six out of 23 (26%) had a heterogeneous reactivity in malignant spindle cells (Figure 4). Other considered clinical-pathological variables were not significantly correlated with BAP1 expression (data not shown).

Differential BAP1 expression in biphasic MPM components – In biphasic MPM a differential BAP1 expression in the epithelioid and atypical stromal areas was identified. While the expression was concordant in both cellular components in eight (3 positive and 5 negative) in the remaining five cases BAP1 was negative in the epithelioid component and positive in the atypical spindle cell component, suggesting a reactive rather than neoplastic nature of such atypical stromal cells. This IHC profile suggests a potential re-classification of these five cases among the epithelioid MPMs with an atypical stromal component (Figure 5).

Validation of BAP1 IHC expression by mutational analysis – All ten cases (100%) with BAP1 nuclear negativity and cytoplasmic positivity harbored genotypic alterations (including missense mutations) in exons 2 to 12; only 6 out of 9 cases (66%) with globally nuclear and cytoplasmic BAP1 negativity also showed BAP1 mutations, but in three remaining cases no mutations were detectable (Supplementary Table 1). The only case with BAP1 IHC nuclear positivity had a wild type genotype.

Furthermore, in the three biphasic MPM with a discordant BAP1 protein expression in which the two compartments were separately micro-dissected and genotyped BAP1 mutations were detected in the epithelioid areas only, but not in the atypical spindle cell components (Figure 5).

Fluorescence in situ hybridization (FISH) analysis – In those three cases that were wild type at Sanger sequencing but showed a complete negativity of IHC protein expression, FISH analysis for *BAP1* gene was performed. Heterozygous deletions (2G–1R or G>R) were found in all three cases in 34%, 59% and 66% of the analyzed nuclei, respectively.

Survival analyses- At the time of the present report, all patients had died because of their disease. Follow-up, available for all patients, ranged from 1 to 114 months (median overall survival: 15 months). As expected, advanced age and stage were significantly associated with a poor prognosis (Log rank test, p=0.0083 and p=0.002), while there was no correlation between sex and survival (p=0.87).

Differences in survival of the three WHO MPM histological subtypes were confirmed (Kaplan Mayer, log rank test p<0.0001) (Figure 6A). Interestingly, a comparable survival was found either between pleomorphic and biphasic MPM (Figure 6B) or with epithelioid MPMs with or without atypical reactive stroma (Table 2A).

The nuclear grading score (also evaluated in the epithelioid component of biphasic MPMs) was a significant predictor of poor survival at the univariate analysis (log rank test, p<0.0001, Figure 6C and Table 2A).

The stromal component grading showed a significant difference in poor survival, only comparing those cases with high stromal atypia (N=16, namely pleomorphic and biphasic MPMs) with low-to-moderate atypia (log rank test, p=0.0004, Figure 6D and Table 2A) thus confirming that only high grade morphological atypia of the stromal cells could be predictive or poor outcome. Furthermore,

in our series, when combining the nuclear grade of the epithelial component with the grading score of the tumor stroma, only one case with a low Kadota score associated with a high stromal atypia was found and that showed a rather long survival; on the other hand none cases with a high epithelioid grade and a low grade stromal component associated were found (Figure 6E).

Finally, overall MPM cases with nuclear positivity for BAP1 expression (n=54) had a worse prognosis than those with BAP1 nuclear negativity (n=36), epithelial cell cytoplasmic BAP1 granularity (n=24) or with a discordant BAP1 expression between epithelial (negative) and stromal (positive) components (log rank test, p=0.0006, Figure 6F). This finding was confirmed also considering BAP1 positive nuclear expression as opposed to any other type of BAP1 IHC pattern ($p<0.0001$, Figure 6G)

At multivariate analysis, only stage and Kadota grading score resulted significant independent prognostic factors of poor prognosis (Table 2B), although BAP1 IHC showed a borderline significance (p=0.055).

DISCUSSION

In this retrospective study of 143 cases of MPM, we demonstrated that in mutated MPM, BAP1 immunohistochemical determination is a reliable tool to distinguish the true biphasic from epithelioid MPM with prominent atypical but reactive stroma; furthermore we confirmed that not only the lack of nuclear expression of BAP1 but also the cytoplasmic staining is correlated with *BAP1* mutation, as previously reported[22] and we described the prevalence of BAP1 protein distribution in the different MPM subtypes. Nuclear BAP1 loss was observed in 62% of the current MPM series, a finding well compared with the literature that reports BAP1 protein loss, corresponding to BAP1 double hit mutation/inactivation, in approximately 50 to 67% of MPM[22,30-32]. Indeed, of such BAP1 altered tumors, only 75% of these were completely negative by

immunohistochemistry, while the remaining 25% had a variable granular cytoplasmic reactivity. The cytoplasmic pattern has been already reported by other Authors[22,25,27,33], but only Nasu et al[22] demonstrated that this type of reactivity was associated with BAP1 genetic abnormalities.

Based on the IHC results, we randomly selected 20 cases, independently from histology, to further investigate the BAP1 gene status in cases with a pure cytoplasmic BAP1 IHC positive pattern as compared to cases with nuclear negativity. Although the genetic investigation was performed on a limited number of cases and not representative of the entire series, our data showed that 100% of cytoplasmic positive MPM cases were mutated thus confirming that only BAP1 positivity in the nucleus is associated to BAP1 wild type status, as opposed to any other pattern of immunoreactivity (complete negativity or cytoplasmic staining). Furthermore, in our series, 67% of nuclear negative BAP1 cases had point mutations, or insertions or deletions, while in the remaining three cases lacking nuclear or cytoplasmic BAP1, no genetic anomalies detectable by Sanger direct sequencing were documented[22]. In these three cases an altered BAP1 gene pattern was shown by means of FISH analysis. Although deletion was found only in one of the two alleles, it could be hypothesized that co-occurring inactivating somatic alterations of the other *BAP1* allele may exist as previously reported[22]. Alternatively, somatic epigenetic silencing of *BAP1* gene that could lead to protein loss may have occurred, even if not demonstrated to date. This complexity confirms that IHC is the most reliable and easily available tool to detect BAP1 genetic abnormalities, independently from the underlying genetic mechanism.

The issue of correctly classifying MPM has relevant clinical implications because histological subtyping has constantly been reported to be one of the most significant prognostic factors[34]. After stratifying the present series of MPM according to classical subtypes, BAP1 loss has been most frequently detected in the epitheliod and biphasic rather than in the sarcomatoid subtype, in

agreement with previous reports[25,27]. In our series, a heterogeneous nuclear BAP1 reactivity within stromal spindle cells, with intermingling of negative and positive elements, was mainly observed in sarcomatoid MPM, with a relatively low number of cases with complete loss of BAP1 reactivity (see below).

If sarcomatoid subtype is an immediate diagnosis in the vast majority of cases, for epithelial and biphasic subtypes, the differential diagnosis was challenging especially in the case of epithelioid MPMs with prominent atypical spindle cell stroma[8]. In a subset of BAP1 negative biphasic MPMs, McGregor and coworkers documented that associated spindle cells could be either negative or positive, suggesting a retained BAP1 expression at least in a fraction of cells[25]. In our series, among BAP1 negative epithelioid MPMs, those cases with atypical spindle cell reactive stroma that could mimic a biphasic MPM were identified. All such MPM cases consistently retained BAP1 nuclear reactivity in the spindle cell component, thus confirming the epithelioid subtype. Conversely, we observed that in 5 out of 13 (38%) cases morphologically classified as biphasic (with morphologically malignant spindle cells) and having a BAP1 negative epithelioid component, the apparently neoplastic spindle cells were consistently BAP1 positive in their nuclei, thus questioning the initial diagnosis. For three of these cases, BAP1 mutational analysis was separately performed in microdissected areas of epithelioid and spindle cell components, and BAP1 mutations were only detected in the epithelioid component, but not in the presumed malignant spindle component, in agreement with the IHC results. This is a new piece of information[25] because the underlying genetic profile of such stromal cells was never assessed.

These findings may be interpreted in two different ways. These cases could be epithelioid MPM that mimicked biphasic MPM, due to a borderline morphology. The retained BAP1 immuno-reactivity in the atypical spindle cells may assist the pathologist in the correct classification of an

epithelioid histotype, at least in the two thirds of cases expected to bear BAP1 mutations. This view is supported by the occurrence of low Kadota grade in the epithelial component and the presence of bland or moderate atypias in the reactive stroma in all such cases having discrepant BAP1 expression in the two tissue components. An alternative interpretation is possible when the epithelial cell component shows a high nuclear grade and the spindle cell component is more frequently morphologically malignant (high stromal grade). In this case, it cannot be unequivocally demonstrated that stromal cells are not neoplastic, and these tumors could be true biphasic MPM having a BAP1 negative epithelioid compartment associated to a BAP1 positive malignant spindle cell component, or could belong to the rare pleomorphic variant of MPM (not different from "biphasic" MPM in terms of survival). Indeed, in this context, the term "biphasic" should be more appropriately replaced by "combined" MPM, since the two neoplastic populations probably represent the collision of two tumor clones, rather than the result of a monoclonal epithelioid–mesenchymal transition process, as currently accepted in biphasic MPM[35]. In fact, in this latter hypothesis it would be unlikely that the progression of a BAP1 mutated epithelioid mesothelioma to a de-differentiated sarcomatoid neoplastic population is associated with *BAP1* gene wild type status. On the other hand the first hypothesis of a collision tumor is supported also by previous evidence of a polyclonal origin of MPM[36]. In agreement with Comertpay et al. the heterogeneity of BAP1 IHC in our sarcomatoid subtype cases could be explained by the polyclonal transformation of multiple mesothelial cells.

Specifically designed for epithelioid MPM, the Kadota nuclear grading system[12] is based on nuclear atypia and mitotic count, and is useful in stratifying patients into three groups with distinct clinical outcome. Recently, BAP1 loss has been associated with an improved survival[23-25]. In our series, although histology, nuclear grade and BAP1 were all relevant prognostic factors at univariate

analysis for survival, surprisingly, only nuclear grade (and stage), but not histology, retained prognostic value for survival at multivariate Cox proportional hazard regression analyses. BAP1 had a borderline significance as an independent prognostic factor for survival. Therefore, it seems that in non sarcomatous MPM (i.e. epithelioid and biphasic MPM), a risk of death was firstly based on nuclear grade of the epithelial component and secondly on BAP1 expression. It can therefore be envisaged that the prognostic evaluation of MPM needs to be implemented, adding to the conventional classification of the three histotypes, also data on grading, staging and the genetic profile, being BAP1 gene the most relevant at this time.

In conclusions, we showed that BAP1 IHC is a reliable tool to predict *BAP1* mutation both in case of nuclear lack and cytoplasmic localization. Furthermore, in BAP1 mutated MPM, BAP1 IHC determination contributed in the differential diagnosis between epithelioid and biphasic subtypes and restricted the diagnosis of biphasic subtype to rare cases that had BAP1 nuclear protein loss in both tumor cell populations (3,5% in our series), as opposed to conventional epithelioid MPM with an atypical reactive (non neoplastic/non mutated) stroma. Finally, a prognostic impact was confirmed for BAP1 expression in MPM together with Kadota nuclear grading and stage.

Further studies are needed to definitely establish whether the biphasic subtype is a real entity or if a two-tier classification into non-sarcomatous and sarcomatous MPM, followed by grading and molecular profile determinations, is rather more appropriate in MPM management.

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FIGURE LEGENDS

Figure 1. Schematic representation of main clinical and pathological features in 143 MPM cases. Abbreviations: BIO: biopsies; EPI: epithelioid; BIPH: biphasic; SARCO: sarcomatous; NA: not applicable; IHC: immunohistochemistry; POS: positive; NEG: negative; Nuclear grading score according to Kadota et al [12].

Figure 2: A: epithelioid MPM with scant associated stroma; **B:** epithelioid MPM with relevant associated stromal component with low-to-moderate atypia in the spindle cells; **C:** epithelioid MPM with relevant associated stromal component with severe atypia in the spindle cells.

Figure 3: A: epithelioid MPM showing nuclear BAP1 immunonegativity in neoplastic and nuclear BAP1 immunopositivity in associated non neoplastic cells (internal control); **B:** epithelioid MPM showing BAP1 cytoplasmic immunopositivity and nuclear negativity in neoplastic cells.

Figure 4: A: epithelioid MPM showing nuclear BAP1 immunonegativity in neoplastic cells and BAP1 positivity in atypical stromal cells (insert: high power); **B:** pleomorphic MPM showing nuclear BAP1 immunonegativity in neoplastic cells and nuclear BAP1 immunopositivity in stromal associated cells; **C:** sarcomatoid MPM showing heterogeneous BAP1 immunoreactivity in malignant spindle cells (thick arrow: BAP1 positive atypical spindle cell; thin arrow: BAP1 negative atypical spindle cell).

Figure 5: Upper panels: MPM case with epithelioid neoplastic and atypical stromal component. **A:** epithelioid component (blue square) was micro-dissected and analyzed for BAP1 protein and gene status; **B:** BAP1 immunonegativity of the epithelioid component with positive internal control; **insert:** electropherogram of the *BAP1* mutational analysis showing the presence of a point mutation. **C:** atypical stromal component (red square) was micro-dissected and analyzed for BAP1 protein and gene status; **D:** BAP1 immunopositivity of the stromal component; **insert:**

electropherogram of the *BAP1* mutational analysis showing a wild type status.

Lower panel: In the table the mutational analysis results of biphasic MPM cases with differential BAP1 immunohistochemical expression analyzed separately in the epiothelioid and stromal components.

Abbreviations: E: epithelioid S: sarcomatous; IHC: immunohistochemistry; POS: positive; NEG: negative; mut: mutation; WT: wild type; NA: not annotated.

Figure 6: A: survival curves of MPM main histological subtypes; **B:** survival curves of pleomorphic MPM cases compared to biphasic and epithelioid MPM. **C:** survival curves of nuclear grade groups according to Kadota et al[12] in MPM with epithelioid component (biphasic type included); **D:** survival curves of stromal grade groups; **E:** paired comparison between epithelial and stromal grading score groups; **F:** survival curves of different BAP1 immunohistochemical pattern groups; **G:** survival curves of BAP1 immunohistochemical positive and negative (including cytoplasmic positive) groups .

Abbreviations: EPI: epithelioid; BIPH: biphasic; SARCO: sarcomatous; PLEO: pleomorphic MPM; mod: moderate; POS: positive; NEG: negative; CYTO: cytoplasmic.

Table 1. Histological and BAP1 immunohistochemical features of 143 MPM.

	Nuclear Grade				BAP1 IHC in tumor cells			BAP1 IHC in stromal cells		
Histology by morphology, only (#143)	**Tumor cells# (%)**	***p***	**Stromal cells (%)**	***p***	**Positive (%)**	**Negative (NN or NN/CP)(%)**	***p***	**Positive (%)**	**Negative (NN or NN/CP)(%)**	***p***
Epithelioid MPM (#95)	GI: 59 (62) GII: 35 (37) GIII: 1 (1)	***	low: 18 (46) mod: 21 (54) high: 0	***	30 (32)	NN: 65 (68) NN/CP: 44/21	***	39 (41)	0	***
Pleomorphic MPM (#12)	GI: 0 GII: 10 (83) GIII: 2 (17)		low: 0 mod: 3 (25) high: 9 (75)		3 (25)	NN: 9 (75) NN/CP: 7/2		11 (92)	NN: 1 (8)	
Biphasic MPM (#13)	GI: 4 (31) GII: 9 (69) GIII: 0		low: 0 mod: 6 (46) high: 7 (54)		3 (23)	NN: 10 (77) NN/CP: 9/1		8 (62)	NN: 5 (38)	
Sarcomatoid MPM (#23)	*na*		*na*		18 (78)	NN: 5 (22) NN/CP: 5/0		*na*	*na*	
Total	**GI: 63 (53) GII: 54 (45) GIII: 3 (2)**		**low: 18 (28) mod: 30 (47) high: 16 (25)**		**54 (38)**	**89 (62) NN/CP: 65/24**		**58 (91)**	**6 (9)**	

Abbreviations: BAP1: BRCA1-Associated Protein 1; IHC: immunohistochemistry; MPM: Malignant Pleural Mesothelioma; G: grade; mod: moderate; na: not applicable; NN: nuclear negativity; CP: cytoplasmic positivity*;* ***:<0.0001; #According to Kadota et al[12]

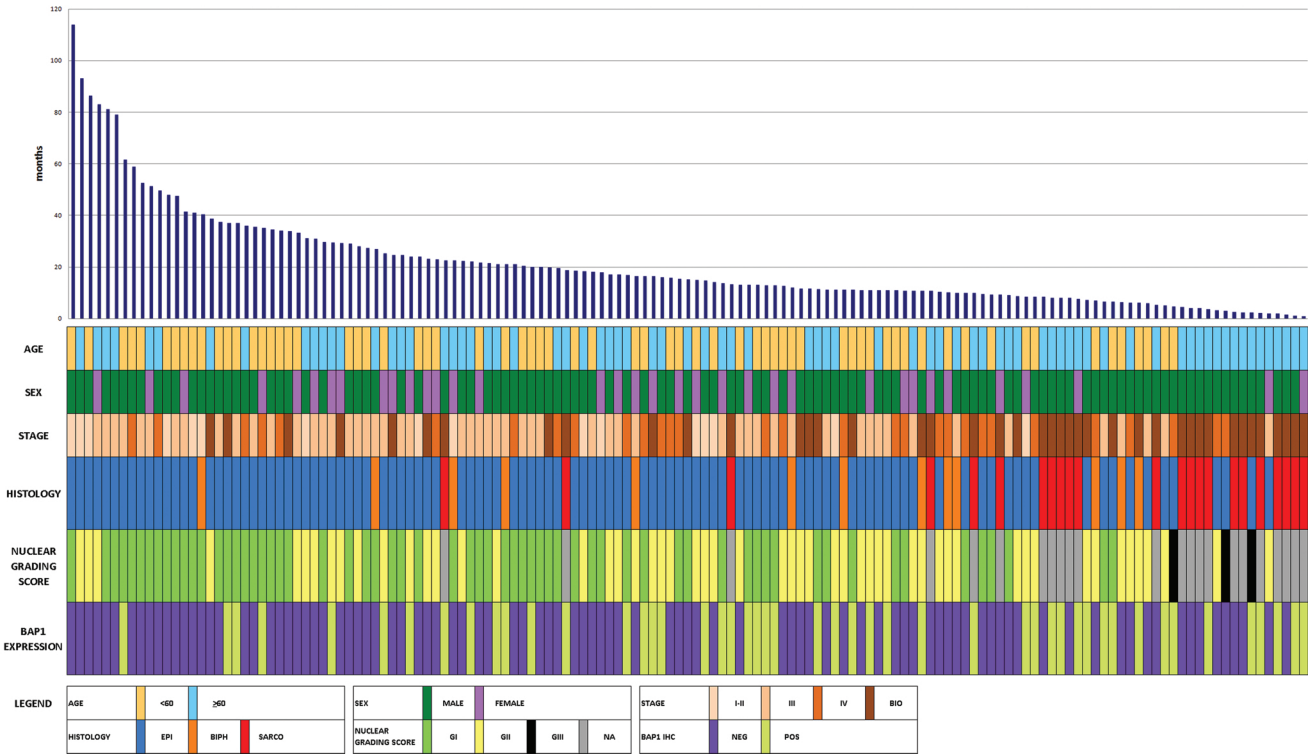
Table 2 A) Univariate analyses of clinico-pathological variables in 143 MPM

	Hazard Ratio	**95%CI**	**P**
Age	1.56	1.12 – 2.18	0.0083
Stage			
III vs I-II	1.84	0.99 – 3.41	0.053
IV vs I-II	2.84	1.45 – 5.54	0.002
Bio vs I-II	4.64	2.42 – 8.91	<0.0001
Histology			
EPIstr vs Epi	1.35	0.88 – 2.08	0.17
PLEO vs Epi	3.13	1.62 – 6.02	0.0006
Biph vs Epi	2.25	1.20 – 4.19	0.011
Sarco vs Epi	7.56	4.38 – 13.04	<0.0001
Nuclear Grade			
II-III vs I	1.97	1.36 – 2.85	0.00033
Stromal Grade			
mod vs low	1.11	0.62 – 1.99	0.74
high vs low	4.22	2.35 – 7.58	<0.0001
BAP1 IHC	0.49	0.34 – 0.69	<0.0001

Table 2B) Multivariate analysis of clinico-pathological variables in 143 MPM

	Hazard Ratio	**95%CI**	**P**
Age	1.10	0.73 – 1.66	0.64
Stage			**0.0022**
III vs I-II	2.09	1.09 – 4.02	0.027
IV vs I-II	3.63	1.77 – 7.44	0.0004
Bio vs I-II	3.19	1.34 – 7.57	0.0085
Histology			0.69
EPIstr vs Epi	1.27	0.71 – 2.26	0.43
PLEO vs Epi	2.13	0.73 – 6.24	0.17
Biph vs Epi	2.79	0.89 – 8.78	0.08
Sarco vs Epi	4.61	1.25 – 17.03	0.02
Nuclear Grade*			
II-III vs I	2.03	1.31 – 3.16	**0.0016**
Stromal Grade			0.28
mod vs low	0.63	0.31 – 1.29	0.21
high vs low	1.14	0.35 – 3.73	0.83
BAP1 IHC	0.67	0.45 – 1.01	***0.055***

Abbreviations: Bio: biopsies; EPIstr: epithelioid with atypical stroma; PLEO: pleomorphic; EPI: epitelioid; Biph: biphasic; Sarco: sarcomatoid; mod: moderate; BAP1: BRCA1-Associated Protein 1; IHC: immunohistochemistry; MPM: Malignant Pleural Mesothelioma. *According to Kadota et al[12]


months
120
100
80
60
40
20
0
AGE
SEX
STAGE
HISTOLOGY
NUCLEAR GRADING SCORE
BAP1 EXPRESSION
LEGEND
AGE
<60
≥60
HISTOLOGY
EPI
BIPH
SARCO
SEX
MALE
FEMALE
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GI
GII
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I-II
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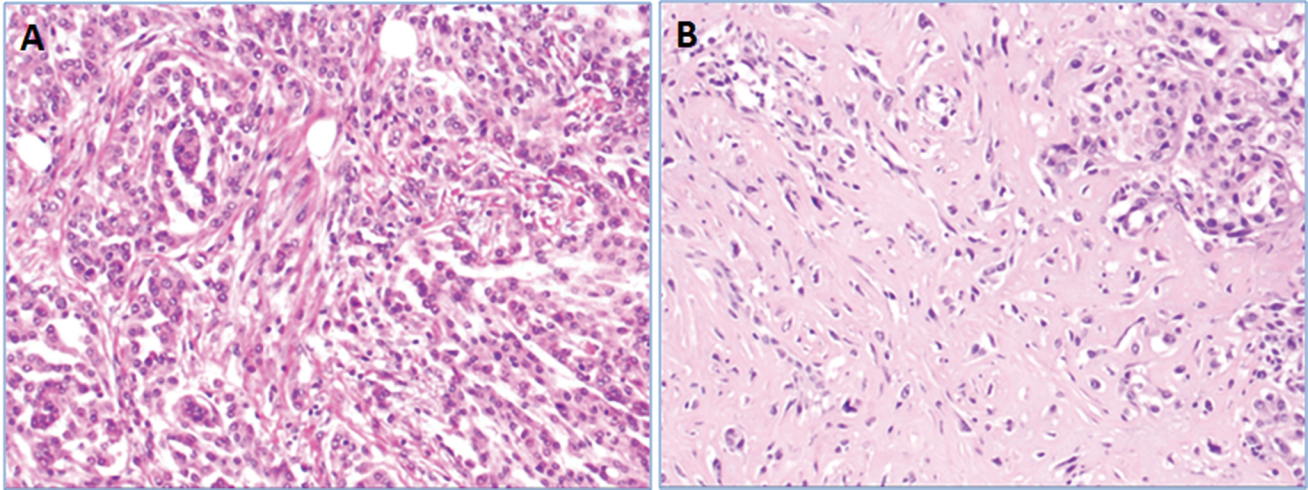
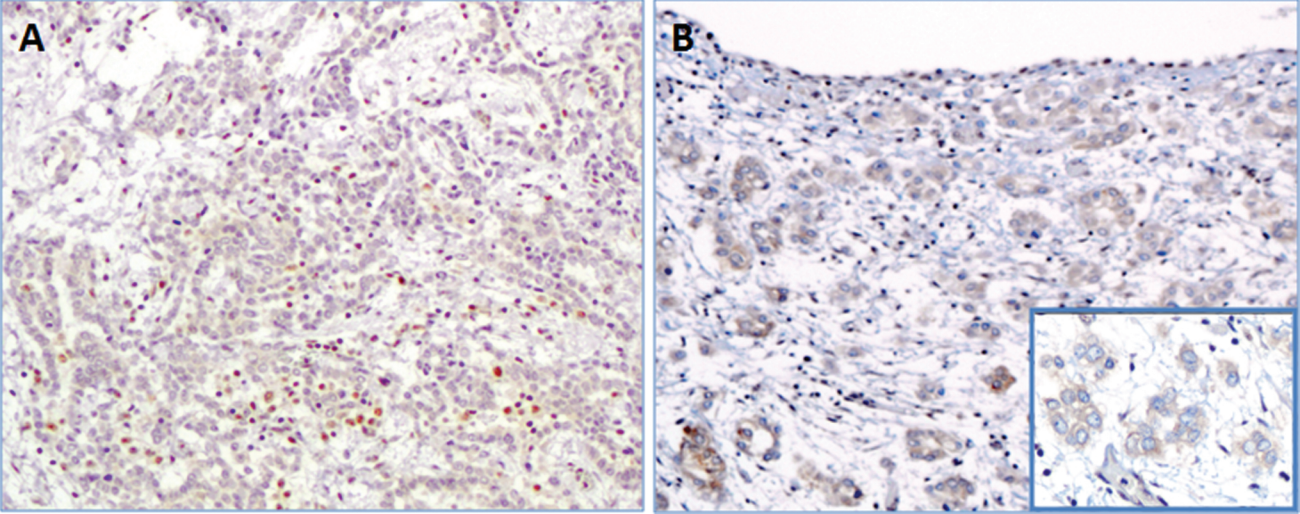
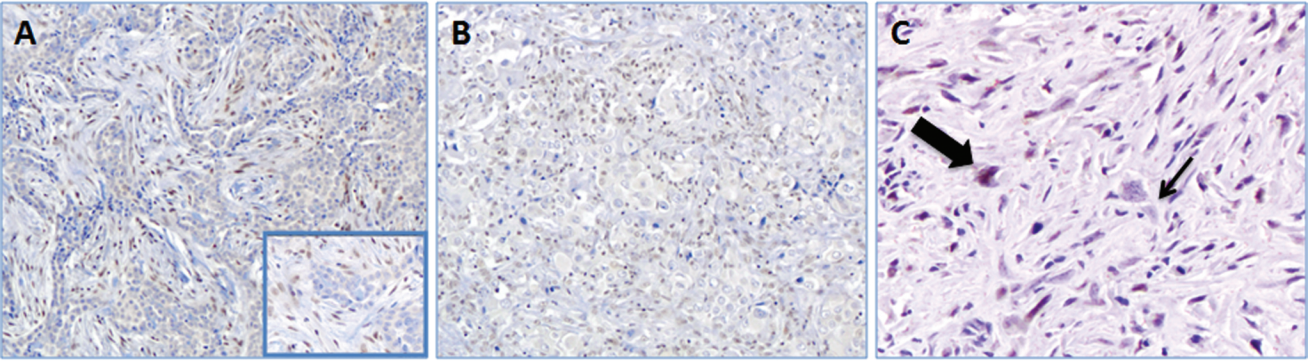
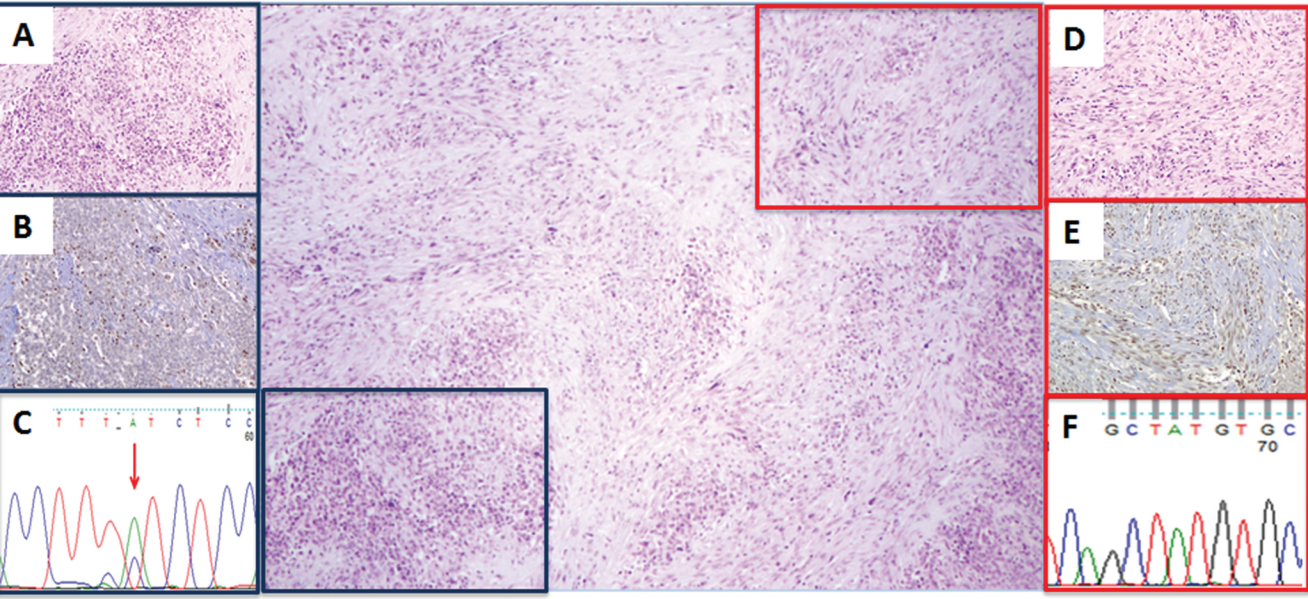
BAP1 IHC
NEG
POS

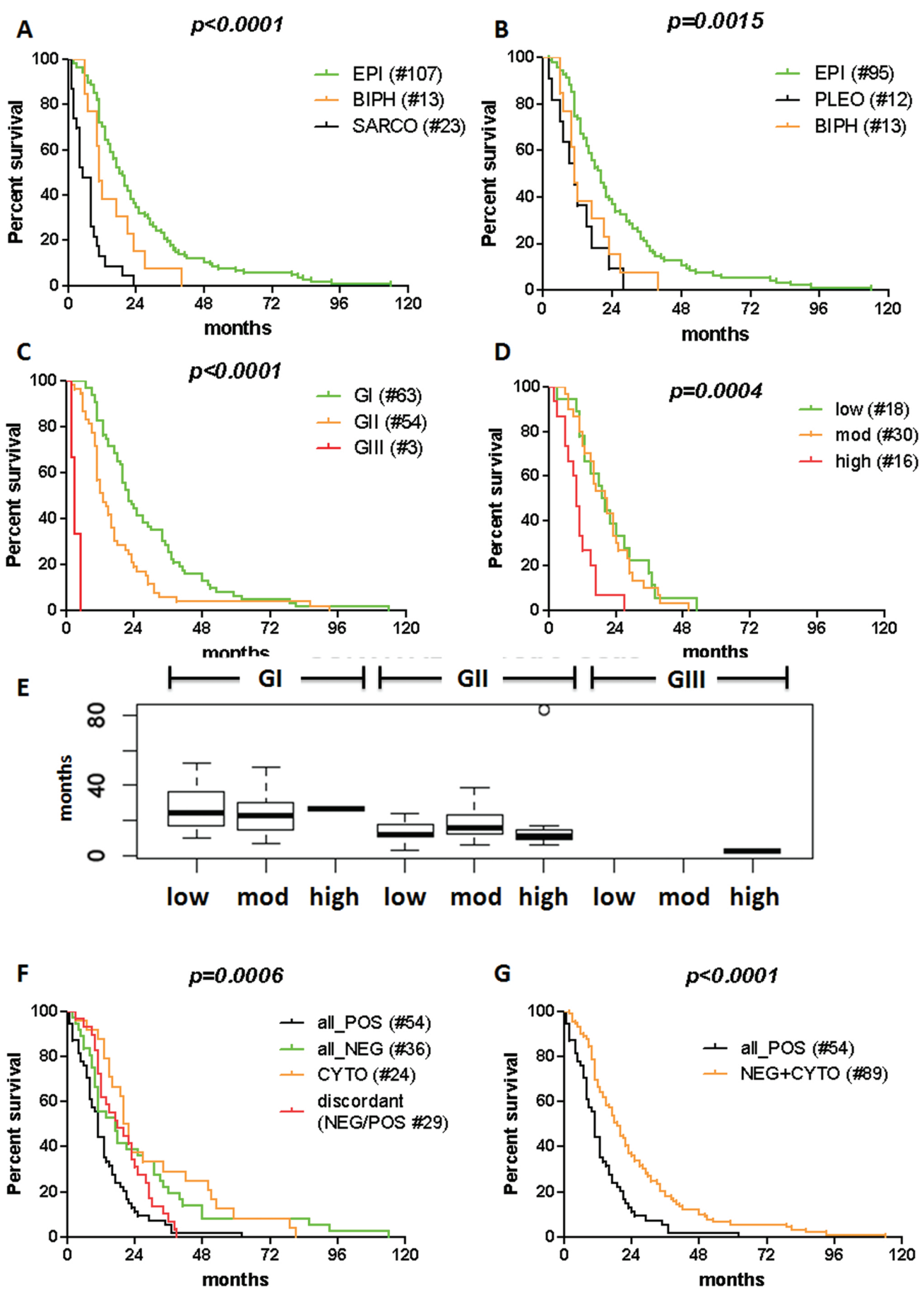

A
B


A
B


A
B
C



ID	BAP IHC	EXON	NUCLEOTYDE	TYPE	PROTEIN	CODING EFFECT	ANNOTATION	IN SILICO ANALYSIS
#21E	NEG	12	c.1184C>G	point mut	p.S395*	non-sense	NA	Deleterious
#21S	POS	/	/	/	WT	/	/	/
#22E	NEG	14+17	c.1810G>A c.2095C>T	point mut point mut	p.V604M p.R699W	missense missense	COSM4119058 NA	Probably damaging
#22S	POS	/	/	/	WT	/	/	/
#23E	NEG	10	c.919G>A	point mut	p.G307S	missense	NA	Probably damaging
#23S	POS	/	/	/	WT	/	/	/


A
p<0.0001
EPI (#107)
BIPH (#13)
SARCO (#23)
Percent survival
months
B
p=0.0015
EPI (#95)
PLEO (#12)
BIPH (#13)
C
p<0.0001
GI (#63)
GII (#54)
GIII (#3)
D
p=0.0004
low (#18)
mod (#30)
high (#16)
E
GI
GII
GIII
low
mod
high
F
p=0.0006
all_POS (#54)
all_NEG (#36)
CYTO (#24)
discordant (NEG/POS #29)
G
p<0.0001
all_POS (#54)
NEG+CYTO (#89)