



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

Introduction: Malignant pleural mesothelioma (MPM) is a highly aggressive disease with limited therapeutic options. Histological subtype remains among the most reliable prognostic factors, because the epithelioid subtype associated with the best prognosis and the sarcomatoid subtype with the worst. The biphasic subtype has an intermediate prognosis, but its definitive histological diagnosis may be challenging owing to the difficulty of assessing the neoplastic nature of the stromal component. Recent data identified BRCA1-associated protein 1 gene (*BAP1*) as one of the most frequently mutated genes in MPM. Immunohistochemical testing for BRCA1-associated protein 1 (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 immunohistochemical determination of BAP1 in MPM, with clinicopathological correlation.

Methods: A series of 143 MPMs were investigated for BAP1 protein expression in correlation with clinical and pathological data, including with a newly proposed nuclear grade. A pilot series of 20 selected cases were also investigated for *BAP1* mutational status.

Results: Negative nuclear staining for BAP1 occurred in 62% of MPMs (including 27% with a 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 as reactive versus neoplastic, thus helping achieve the correct classification of biphasic histological subtype.

Conclusions: 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 an independent prognostic parameter in MPM.

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Keywords: Malignant mesothelioma; Pleura; BAP1 mutation; Prognosis; Histology

Introduction

Malignant pleural mesothelioma (MPM) is a rare, highly aggressive, relatively chemotherapy- and radiotherapy-resistant type of cancer with limited therapeutic options.¹ In patients with advanced-stage disease treated with cisplatin and pemetrexed, median survival time is

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approximately 12 months, long-term survivors are seen only occasionally,^{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, and molecular/pathological findings, but the vast majority of them are not fully validated⁵ 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. Histological subtype remains among the most reliable prognostic factors because the epithelioid subtype is associated with the best prognosis and the sarcomatoid subtype with the worst.⁸ Although the biphasic/mixed subtype usually has an intermediate prognosis, sometimes its definitive histological diagnosis may be cumbersome owing to the sometimes problematic grade assessment of nuclear atypia in the stromal component. Furthermore, high-grade MPM with pleomorphic features has a controversial histological classification; although according to the guidelines, it is classified as epithelioid MPM,^{8,9} clinical and pathological findings suggest an association with the 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 shown to be associated with prognosis.¹²

Next-generation sequencing data indicate cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*), neurofibromatosis 2 gene (*NF2*), and BRCA1-associated protein 1 gene (*BAP1*) as the most frequently mutated genes in MPM.^{13–15} *BAP1* is a nuclear deubiquitinating enzyme¹⁶ that was 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 germline mutations associated with an inherited multicancer syndrome with a dominant autosomal transmission.¹⁸ So far, *BAP1* is the first and only gene that has been proposed as influencing environmental carcinogenesis: when a germline *BAP1* mutation 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 with a less aggressive tumor phenotype and improved prognosis in familial mesothelioma¹⁹ 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 immunohistochemical (IHC) staining, 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 ovarian cancers) and reactive versus malignant mesothelial proliferation with a high specificity despite the variable sensitivity.^{25,27}

The aim of the present study was to (1) clarify the diagnostic usefulness of *BAP1* IHC in characterizing MPM biphasic subtype with molecular confirmation and (2) correlate sporadic MPM *BAP1* protein expression with clinicopathological and outcome data to validate its prognostic role.

Because of the challenging differential diagnosis between biphasic and epithelioid MPM with atypical reactive stroma⁸ and in consideration of the fact that the cellular distribution of *BAP1* IHC expression patterns among different MPM histotypes is not clearly established, we investigated the role of *BAP1* IHC determination in 143 cases of MPM (including 101 surgically 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 MPMs with different IHC staining patterns and then separately in the epithelial and stromal component of three cases of morphologically biphasic MPM to correlate both *BAP1* protein and *BAP1* gene status. Finally, we correlated *BAP1* IHC determination with clinicopathological and survival data.

We discovered that (1) *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; (2) atypical stromal cells associated with *BAP1*-negative epithelioid MPMs retained *BAP1* expression, and molecular analysis of this stromal cell component confirmed the expected wild-type status; (3) higher disease stage, high nuclear grade, and *BAP1* expression are independent predictors of poor prognosis irrespective of the histotype.

Materials and Methods

Tissue Collection

A total of 101 consecutive resected samples of MPM diagnosed between 2000 and 2012 and with enough leftover tissue were retrieved from the pathology files of the pathology units of the University of Torino at San Luigi Hospital (Orbassano, Turin, Italy) and City of Health and Science Hospital (Turin); furthermore, to enrich the study population for sarcomatoid and biphasic MPM cases we also collected 42 consecutive thoracoscopic biopsy samples from pathology unit files of San Luigi Hospital. For all cases, the main clinicopathological data were obtained and analyzed. Relevant clinical pathological findings included a mean age of 60 years and male-to-female ratio of 108:35. Of the surgical

cases, 16 were International Mesothelioma Interest Group (IMIG) tumor stage I or II, 55 were IMIG stage III, and 30 were IMIG stage IV. 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 hematoxylin and eosin-stained slides available were reviewed by two pathologists (M.P. and L.R.) and classified according to the 2015 WHO classification criteria.⁸ Additional collected morphological features included the nuclear grading of the epithelioid component in both 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 the nuclear atypia score (1 = low, 2 = mild, and 3 = high) and mitotic count score (1 = 0–1 mitoses per 10 high-power fields [HPFs], 2 = 2–5 mitoses per 10 HPFs, and 3 = >5 mitoses per 10 HPFs). Furthermore, morphological atypia of the tumor-associated stroma was also reported for assessment of stromal cellularity (an increase in stromal spindle cells), nuclear pleomorphism, size, and hyperchromasia, which was assessed as low, moderate, and high as follows: low stromal atypia was characterized by a slight 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; and high stromal atypia indicated marked hypercellularity with densely overlapped nuclei, marked variation in size, coarse chromatin, and irregular nuclear membranes with evident nucleoli.²⁸

IHC Analysis

IHC analysis was performed in all cases as follows: 3- μ m-thick serial paraffin sections from representative paraffin blocks were processed using an automated immunostainer (Ventana BenchMark AutoStainer [Ventana Medical Systems, Tucson, AZ] with a primary antibody against BAP1 (clone C4, rabbit monoclonal [Santa Cruz Biotechnology, Santa Cruz, CA]). Nonneoplastic 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 four biphasic subtypes) that were selected on the basis of the yield of BAP1 IHC staining (10 cytoplasmic, nine nuclear negative, and one nuclear positive) were 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.³⁰ Primers and polymerase chain reaction 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 through bidirectional resequencing of independent polymerase chain reaction 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 bioinformatic analyses were performed according to reference databases (i.e., dbSNP-build 131,³¹ 1000 Genomes,³² National Heart, Lung and Blood Institute Grand Opportunity Exome Sequencing Project,³³ and somatic mutational COSMIC databases), whereas in silico prediction of functional effect was performed by the SIFT,³⁴ PolyPhen-2,³⁵ and SNAP³⁶ databases.

Furthermore, three cases of biphasic MPM having a differential BAP1 expression in the epithelioid and atypical stromal components were separately microdissected and analyzed after sample enrichment of the epithelial and stromal components.

FISH Analysis

To further study those MPM cases that showed discrepancy in BAP1 status between IHC analysis and direct sequencing, fluorescence in situ hybridization (FISH) analysis was performed on 4- μ m-thick formalin-fixed, paraffin-embedded tissue sections. Briefly, slides were treated using the Invitrogen Spot-Light Tissue Pretreatment Kit (Invitrogen, Camarillo, CA) and then digested with pepsin (Invitrogen, Carlsbad, CA) and dehydrated before hybridization with FISH probes. FISH using a dual colour probe for *BAP1* gene (3p21.1) (Texas Red-labeled)/CEN3q (fluorescein isothiocyanate-labeled) (Abnova, Walnut, CA) was carried out according to the manufacturers' protocol. The slides were incubated with *BAP1/CEN3q* probe, codenatured in the HYBrite System (Abbott Diagnostics, Lake Forest, Illinois) at 75°C for 5 minutes and hybridized overnight at 37°C. Slides were then washed, dehydrated, and counterstained with 4'6'-diamidino-2-phenylindole (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 4'6' -diamidino-2-phenylindole 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 Integrated Set of Information Systems 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 recent reports²⁷ and on the basis of evaluation of a range from 100 to 140 nuclei, only samples harboring *BAP1* gene deletion signal in 30% or more 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 tests 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 estimations of 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 (R Foundation for Statistical Computing, Vienna, Austria) 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 MPMs. Excluding the 12 pleomorphic MPMs among the remaining 95 epithelioid MPMs, 39 had a relevant associated stromal component⁸ with low to moderate atypia in the spindle cells (Fig. 2). Kadota nuclear grade of the epithelial component was assessed in all the nonsarcomatous MPMs (including the epithelial component of the biphasic MPMs). A significant difference in the distribution of the nuclear grade was detected, with the 95 epithelioid MPMs mostly segregated in the GI group, whereas pleomorphic and biphasic

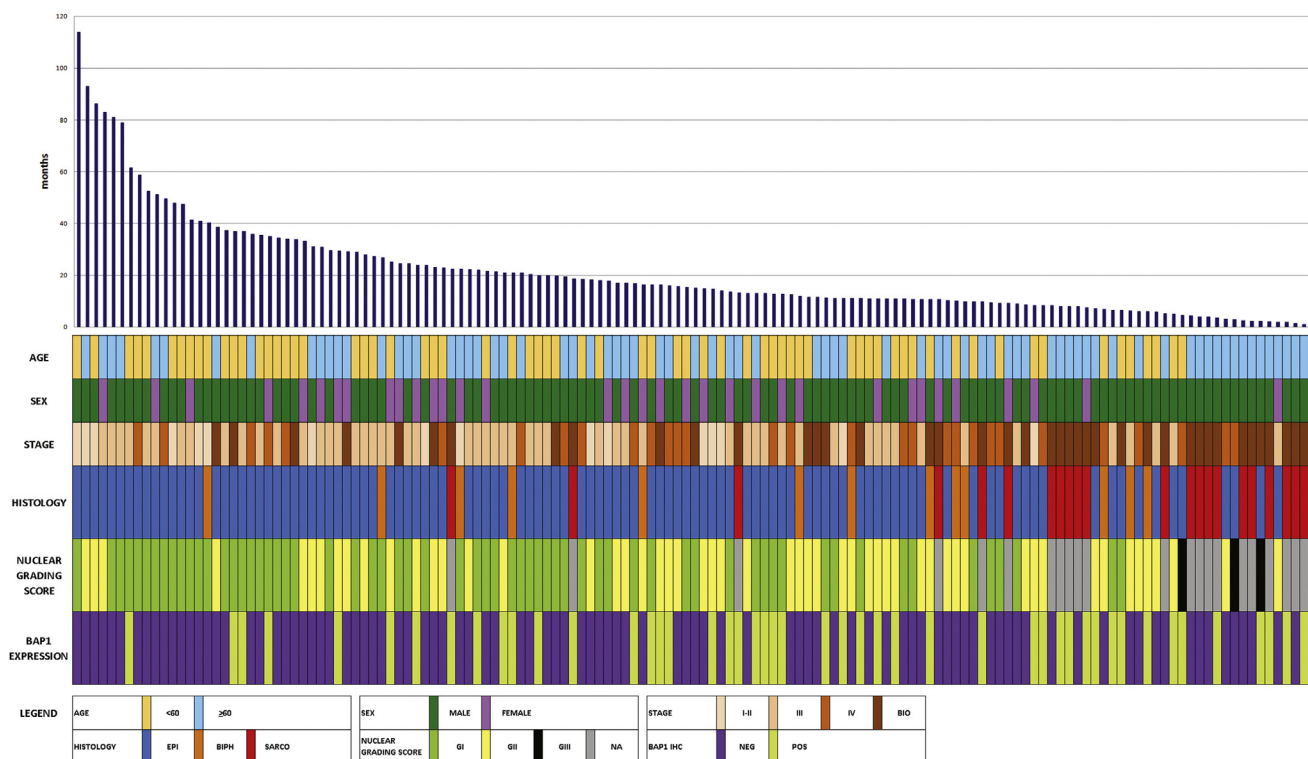


Figure 1. Schematic representation of main clinical and pathological features in 143 malignant pleural mesothelioma cases. BIO, biopsies; EPI, epithelioid; BIPH, biphasic; SARCO, sarcomatous; NA, not applicable; IHC, immunohistochemistry; POS, positive; NEG, negative. Nuclear grading score according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

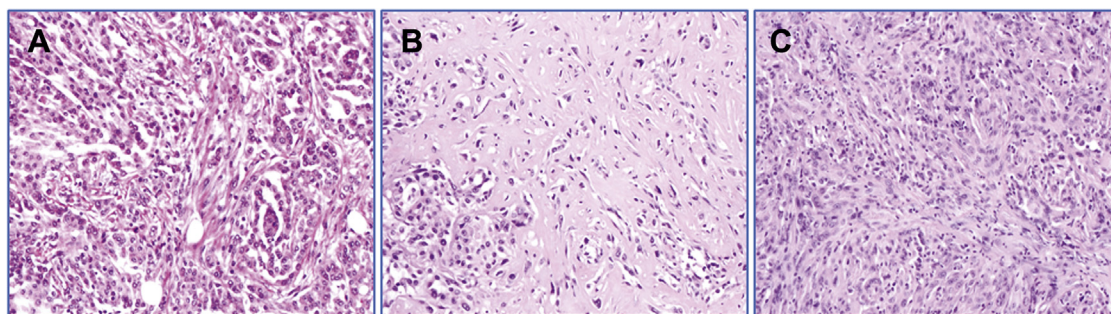


Figure 2. (A) Epithelioid malignant pleural mesothelioma (MPM) with scant associated stroma. (B) Epithelioid MPM with relevant associated stromal component with low to moderate atypia in the spindle cells. (C) Epithelioid MPM with relevant associated stromal component with severe atypia in the spindle cells.

MPMs were mainly grouped in the GII or GIII categories ($p < 0.0001$) (see [Table 1](#)). In addition, the distribution of the stromal atypia differed significantly among histotypes, with all the epithelioid MPMs having a low to moderate stromal atypia, whereas for most of those cases diagnosed as pleomorphic or biphasic MPMs the grading was high ($p < 0.0001$) (see [Table 1](#)).

BAP1 Expression

Details about BAP1 expression according to histotype are reported in [Table 1](#). Overall, the lack of nuclear reactivity for BAP1 in MPM cells was reported in 89 of 143 cases (62%), including 24 cases (27%) with a granular cytoplasmic-positive staining ([Fig. 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 one of 12 samples (8%) was BAP1 negative both in atypical stromal spindle cells and in the neoplastic epithelioid component. All together, in these two groups, 35 of 51 samples (69%) showed a discrepancy between BAP1 expression in the epithelial and stromal components: 27 epithelioid and eight pleomorphic MPMs were BAP1 negative in epithelioid neoplastic cells (including 11 with a cytoplasmic pattern) but positive in stromal cells. In sarcomatoid MPM, five cases were completely negative (22%) and six of 23 (26%) had a heterogeneous reactivity in malignant spindle cells ([Fig. 4](#)). Other considered clinicopathological 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.

Although the expression was concordant in both cellular components in eight cases (three positive and five 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 reclassification of these five cases among the epithelioid MPMs with an atypical stromal component ([Fig. 5](#)).

Validation of BAP1 IHC Expression by Mutational Analysis

All 10 cases (100%) with BAP1 nuclear negativity and cytoplasmic positivity harbored genotypic alterations (including missense mutations) in exons 2 to 12; only six of nine 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 MPMs with a discordant BAP1 protein expression in which the two compartments were separately microdissected and genotyped, *BAP1* mutations were detected in the epithelioid areas only and not in the atypical spindle cell components (see [Fig. 5](#)).

FISH Analysis

In the 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 of their disease. Follow-up, which was available for all patients, ranged from 1 to 114 months (median overall

Table 1. Histological and BAP1 Immunohistochemical Features of 143 MPMs

Histological Subtype by Morphological Examination Only (N = 143) ^a	Nuclear Grade		Stromal Cells,		p Value		BAP1 IHC Staining in Tumor Cells		BAP1 IHC Analysis in Stromal Cells	
	Tumor Cells, n (%) ^a	p Value	n (%)	p Value	Positive, n (%)	Negative (NN or NN/CP), n (%)	Positive, n (%)	Negative (NN or NN/CP), n (%)	Positive, n (%)	p Value
Epithelioid MPM (n = 95) ^a	GI: 59 (62) Gill: 35 (37) Gill: 1 (1)	p < 0.0001	Low: 18 (46) Mod: 21 (54) High: 0	p < 0.0001	30 (32)	NN: 65 (68) NN/CP: 44/21	39 (41)	0	39 (41)	p < 0.0001
Pleomorphic MPM (n = 12) ^a	GI: 0 Gill: 10 (83) Gill: 2 (17)		Low: 0 Mod: 3 (25) High: 9 (75)		3 (25)	NN: 9 (75) NN/CP: 7/2	11 (92)	NN: 1 (8)	11 (92)	
Biphasic MPM (n = 13) ^a	GI: 4 (31) Gill: 9 (69) Gill: 0		Low: 0 Mod: 6 (46) High: 7 (54)		3 (23)	NN: 10 (77) NN/CP: 9/1	8 (62)	NN: 5 (38)	8 (62)	
Sarcomatoid MPM (n = 23) ^a	NA		NA		18 (78)	NN: 5 (22) NN/CP: 5/0	NA	NA	NA	
Total	GI: 63 (53) Gill: 54 (45) Gill: 3 (2)		Low: 18 (28) Mod: 30 (47) High: 16 (25)		54 (38)	89 (62) NN/CP: 65/24	58 (91)	6 (9)	58 (91)	

^aParentetical numbers from Kadota et al.¹² BAP1, BRCA1-associated protein 1; IHC, immunohistochemical; MPM, malignant pleural mesothelioma; G, grade; Mod, moderate; NA, not applicable; NN, nuclear negativity; CP, cytoplasmic positivity.

survival 15 months). As expected, advanced age and stage were significantly associated with a poor prognosis (log-rank test, $p = 0.0083$ and $p = 0.002$), whereas there was no correlation between sex and survival ($p = 0.87$).

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

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

The stromal component grading showed a significant difference in poor survival when those cases with high stromal atypia ($n = 16$ [namely, pleomorphic and biphasic MPMs]) and those with low to moderate atypia were compared (log rank test, $p = 0.0004$ [Fig. 6D and Table 2]), thus confirming that only high-grade morphological atypia of the stromal cells could be predictive of poor outcome. Furthermore, in our series, when the nuclear grade of the epithelial component was combined 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 it showed a rather long survival; on the other hand, no cases with a high epithelioid grade and a low-grade stromal component associated were found (Fig. 6E).

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

At multivariate analysis, only stage and Kadota grading score resulted in significant independent prognostic factors of poor prognosis (Table 3), although BAP1 IHC expression showed a borderline significance ($p = 0.055$).

Discussion

In this retrospective study of 143 cases of MPM, we demonstrated that in mutated MPM, BAP1 IHC 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

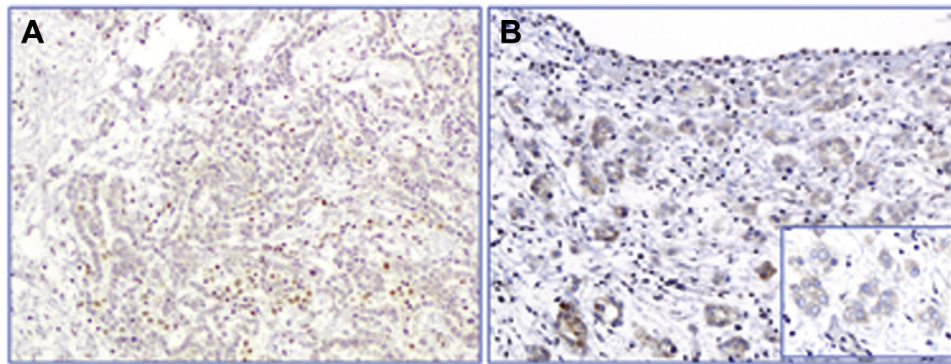


Figure 3. (A) Epithelioid malignant pleural mesothelioma showing nuclear BRCA1-associated protein 1 (BAP1) immunonegativity in neoplastic and nuclear BAP1 immunopositivity in associated nonneoplastic cells (internal control). (B) Epithelioid malignant pleural mesothelioma showing BAP1 cytoplasmic immunopositivity and nuclear negativity in neoplastic cells.

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 that compared well with the literature reporting BAP1 protein loss, corresponding to *BAP1* double-hit mutation/inactivation, in approximately 50% to 67% of MPMs.^{22,37-39} Indeed, only 75% of such *BAP1*-altered tumors were completely negative by IHC determination, whereas the remaining 25% had a variable granular cytoplasmic reactivity. The cytoplasmic pattern has been already reported by others,^{22,25,27,40} but only Nasu et al.²² demonstrated that this type of reactivity was associated with *BAP1* genetic abnormalities.

On the basis of the IHC results, we randomly selected 20 cases, independently from histotype, to further investigate the *BAP1* gene status in cases with a pure cytoplasmic BAP1 IHC determination-positive pattern as compared with 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 with

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, whereas 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 in only 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 determination is the most reliable and easily available tool to detect BAP1 genetic abnormalities independently from the underlying genetic mechanism.

The issue of correctly classifying MPMs has relevant clinical implications because histological subtyping has constantly been reported to be one of the most significant prognostic factors.⁴¹ After stratification of the

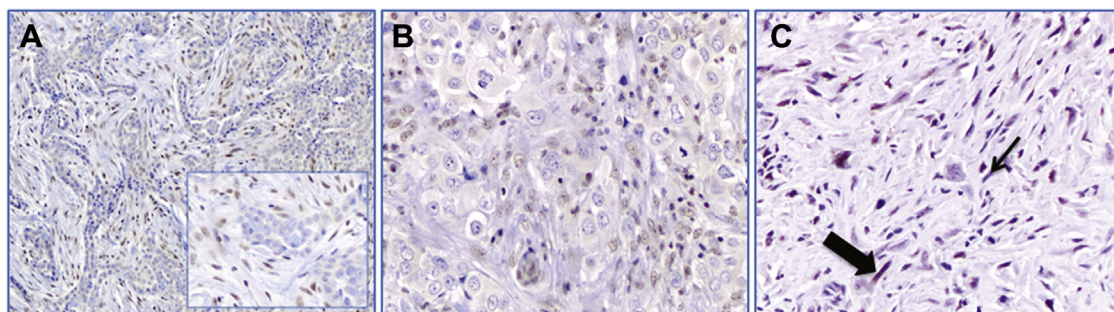


Figure 4. (A) Epithelioid malignant pleural mesothelioma (MPM) showing nuclear BRCA1-associated protein 1 (BAP1) immunonegativity in neoplastic cells and BAP1 positivity in atypical stromal cells (*insert*: high power). (B) Pleomorphic MPM showing nuclear BAP1 immunonegativity in neoplastic cells and nuclear BAP1 immunopositivity in stromal associated cells. (C) Sarcomatoid MPM showing heterogeneous BAP1 immunoreactivity in malignant spindle cells (*thick arrow* indicates BAP1-positive atypical spindle cell; *thin arrow* indicates BAP1-negative atypical spindle cell).

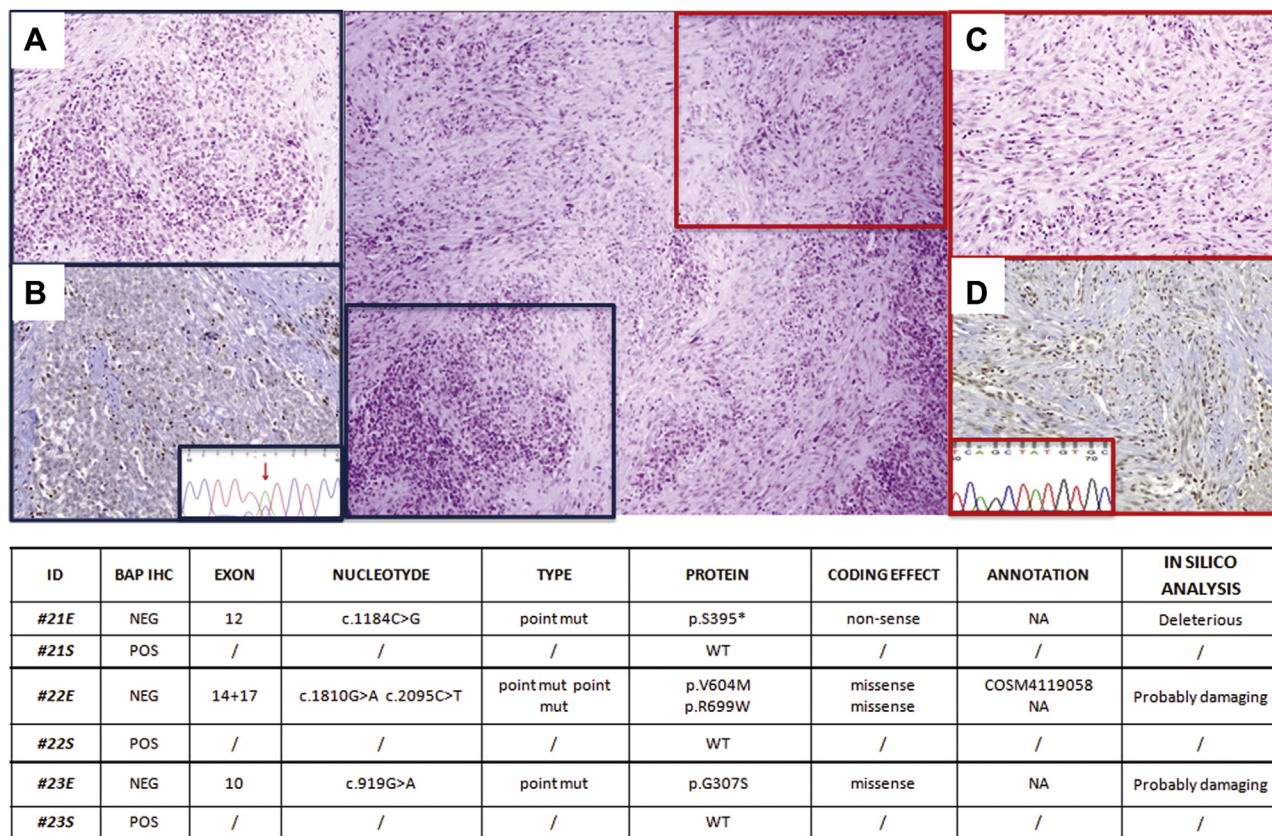


Figure 5. (Upper panels) Malignant pleural mesothelioma case with epithelioid neoplastic and atypical stromal component. (A) Epithelioid component (blue square) was microdissected and analyzed for BRCA1-associated protein 1 (BAP1 protein) and BRCA1-associated protein 1 gene (*BAP1*) status. (B) BAP1 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 microdissected and analyzed for BAP1 protein and *BAP1* 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 malignant pleural mesothelioma cases with differential BAP1 immunohistochemical expression analyzed separately in the epithelioid and stromal components. E, epithelioid; S, sarcomatous; IHC, immunohistochemical; POS, positive; NEG, negative; mut, mutation; WT, wild type; NA, not annotated.

present series of MPMs according to classical subtypes, BAP1 loss has been most frequently detected in the epithelioid and biphasic rather than in the sarcomatoid subtype, which is 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 later).

If sarcomatoid subtype is an immediate diagnosis in the vast majority of cases, for epithelial and biphasic subtypes the differential diagnosis was especially challenging in the case of epithelioid MPMs with prominent atypical spindle cell stroma.⁸ In a subset of BAP1-negative biphasic MPMs, McGregor et al. 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 five of 13 cases (38%) 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 calling the initial diagnosis into question. 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, which is in agreement with the IHC

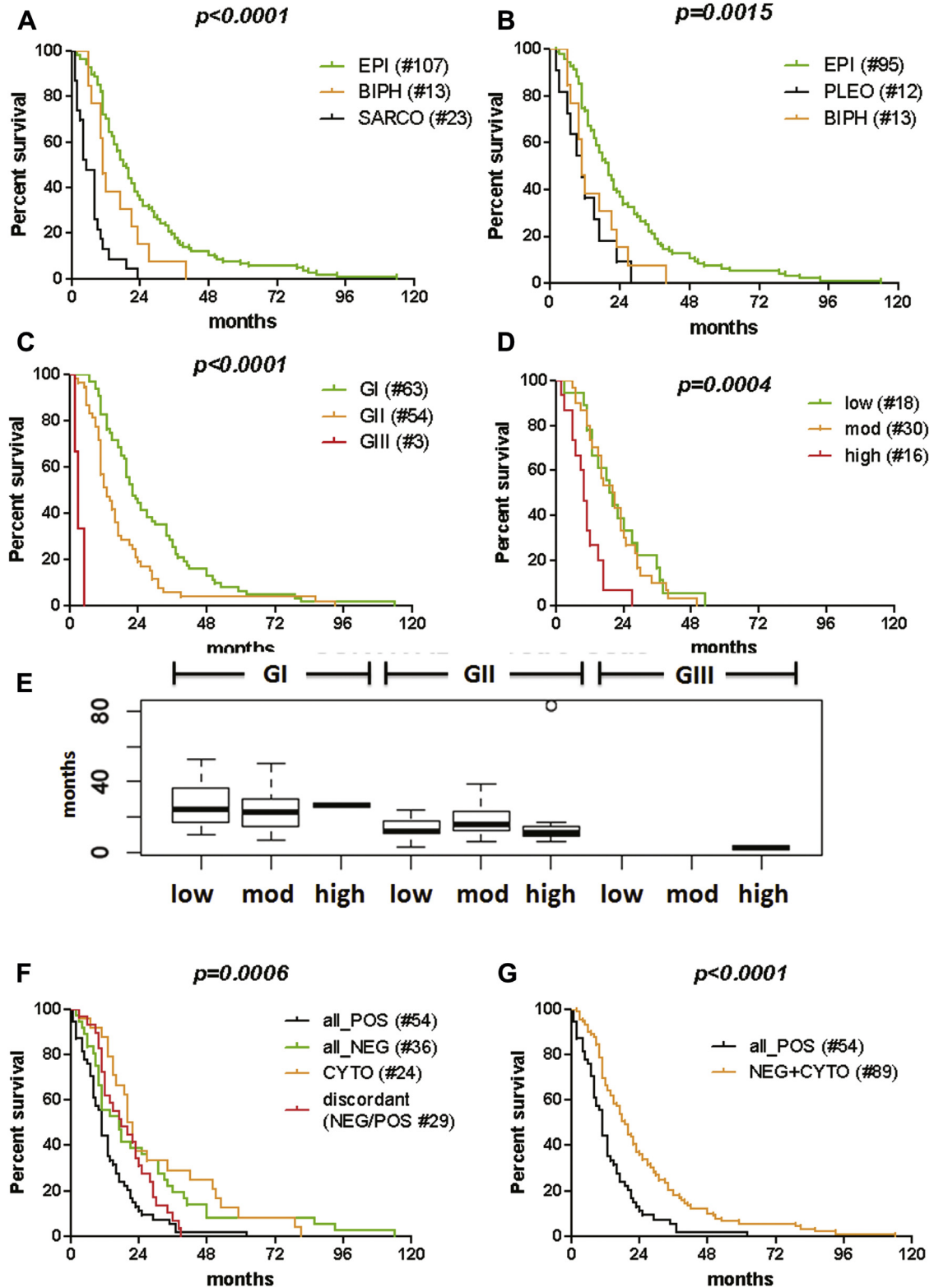


Figure 6. (A) Survival curves of malignant pleural mesothelioma (MPM) main histological subtypes. (B) Survival curves of pleomorphic MPM cases compared with biphasic and epithelioid MPM. (C) Survival curves of nuclear grade groups according to Kadota et al¹² in MPM with epithelioid component (biphasic type included). (D) Survival curves of stromal grade groups. (E) Paired comparison between epithelial and stromal grading score groups. (F) Survival curves of different BRCA1-associated protein 1 immunohistochemical pattern groups. (G) Survival curves of BRCA1-associated protein 1 immunohistochemical expression-positive and immunohistochemical expression-negative (including cytoplasmic positive) groups. EPI, epithelioid; BIPH, biphasic; SARCO, sarcomatous; PLEO, pleomorphic MPM; mod, moderate; POS, positive; NEG, negative; CYTO, cytoplasmic.

Table 2. Univariate Analyses of Clinicopathological Variables in 143 MPMs

Characteristic	Hazard Ratio	95% CI	p Value
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
Histological subtype			
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 analysis	0.49	0.34-0.69	<0.0001

MPM, malignant pleural mesothelioma; CI, confidence interval; Bio, biopsies; EPIstr, epithelioid with atypical stroma; Epi, epithelioid; PLEO, pleomorphic; Biph, biphasic; Sarco, sarcomatoid; BAP1, BRCA1-associated protein 1; IHC, immunohistochemical.

determination results. This is a new piece of information²⁵ 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 MPMs that mimicked biphasic MPMs owing to a borderline morphology. The retained BAP1 immunoreactivity 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 MPMs having a BAP1-negative epithelioid compartment associated with a BAP1-positive malignant spindle cell component, or they 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 because the two neoplastic populations probably represent the collision of two tumor clones rather than the result of a monoclonal epithelioid-mesenchymal transition process, as is currently accepted in biphasic MPM.⁴² In fact, in this latter hypothesis it would be

Table 3. Multivariate Analysis of Clinicopathological Variables in 143 MPMs

Characteristic	Hazard Ratio	95% CI	p Value
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
Histological subtype			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 ^a			
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 analysis	0.67	0.45-1.01	0.055

Note: Significant p values (<0.05) are depicted in bold. Borderline significant p values are depicted in italics.

^aAccording to Kadota et al.¹²

MPM, malignant pleural mesothelioma; CI, confidence interval; Bio, biopsies; EPIstr, epithelioid with atypical stroma; EPI, epithelioid; PLEO, pleomorphic; Biph, biphasic; Sarco, sarcomatoid; Mod, moderate; BAP1, BRCA1-Associated Protein 1; IHC, immunohistochemical.

unlikely that the progression of a BAP1-mutated epithelioid mesothelioma to a dedifferentiated 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.⁴³ 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.⁴³

Specifically designed for epithelioid MPM, the Kadota nuclear grading system¹² is based on nuclear atypia and mitotic count, and it 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 histological subtype, nuclear grade, and BAP1 were all relevant prognostic factors at univariate analysis for survival, surprisingly, only nuclear grade (and stage), and not histological subtype, 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 nonsarcomatous MPM (i.e., epithelioid and biphasic MPM), a risk for death was based first on nuclear grade of the epithelial component and second on BAP1 expression. It can therefore be envisaged that the prognostic evaluation of MPM needs to be implemented: to the conventional classification of the three histotypes,

also needed are data on grading, staging and the genetic profile, being BAP1 gene the most relevant at this time.

In conclusion, we showed that BAP1 IHC determination is a reliable tool to predict BAP1 mutation in the case of both nuclear lack and cytoplasmic localization. Furthermore, in BAP1-mutated MPM, BAP1 IHC determination contributed to 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 (nonneoplastic/nonmutated) 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 whether a two-tier classification into nonsarcomatous and sarcomatous MPM, followed by grading and molecular profile determinations, is rather more appropriate in MPM management.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at <http://dx.doi.org/10.1016/j.jtho.2016.06.020>.

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