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Targeted Next-Generation Sequencing of Cancer Genes in Advanced Stage Malignant Pleural Mesothelioma: A Retrospective Study

Lo Iacono M[1], Monica V, Righi L, Grosso F, Libener R, Vatrano S, Bironzo P, Novello S, Musmeci L, Volante M, Papotti M, Scagliotti GV

Introduction

Malignant pleural mesothelioma (MPM) is a rare malignant disease, and the understanding of molecular pathogenesis has lagged behind other malignancies.

Methods

A series of 123 formalin-fixed, paraffin-embedded tissue samples with clinical annotations were retrospectively tested with a commercial library kit (Ion AmpliSeq Cancer Hotspot Panel v.2, Life Technologies, Grand Island, NY) to investigate 50 genes plus other two, BRCA1-associated protein-1 (BAP-1) and neurofibromatosis-2 (NF2), frequently altered in MPM. DNA was obtained from tissues after manual microdissection and enriched for at least 50% cancer cells. Variations affecting protein stability or previously correlated to cancer, more frequently identified (≥25 patients with at least 10% of allelic frequency), were subsequently evaluated by Sanger sequencing. Immunohistochemistry staining for BAP1 and NF2 proteins was also performed.

Results

The commonest genetic variations were clustered in two main pathways: the p53/DNA repair (*TP53*, *SMACB1*, and *BAP1*) and phosphatidylinositol 3-kinase–AKT pathways (*PDGFRA*, *KIT*, *KDR*, *HRAS*, *PIK3CA*, *STK11*, and *NF2*). PIK3CA:c.1173A>G mutation, STK11:rs2075606 (T>C), or TP53:rs1042522 (Pro/Pro) was significantly associated with time to progressive disease (TTPD; all *p* values < 0.01). Furthermore, the accumulation of genetic alterations correlated with shorter TTPD and reduced overall survival (TTPD *p* value = 0.02, overall survival *p* value = 0.04). *BAP1* genetic variations identified were mainly located in exons 13 and 17, and BAP1 nonsynonymous variations were significantly correlated with BAP1 protein nuclear localization.

Conclusion

Next-generation sequencing was applied to a relatively large retrospective series of MPM using formalin-fixed, paraffin-embedded archival material. Our results indicate a complex mutational landscape with a higher number of genetic variations in the p53/DNA repair and phosphatidylinositol 3-kinase pathways, some of them with prognostic value.

Key Words

- Malignant pleural mesothelioma;
- Genetic variation;
- Next-generation sequencing;
- Genetic characterization;
- *BAP1* gene;
- *NF2* gene;
- *PI3K* gene

Malignant pleural mesothelioma (MPM) is a highly lethal cancer with limited therapeutic options.[1] Most of newly diagnosed patients present with advanced disease and first-line chemotherapy extends survival of 3 months, whereas there is no approved agent for second-line chemotherapy.[2] Prognostication in MPM has been approached by studying several clinical variables, radiological parameters at presentation and molecular/pathological findings, mainly in retrospective studies with a limited number of patients, and most of the findings remain not validated.[3] Prognostic scoring systems have been proposed, but they are not routinely implemented in daily clinical practice.[4] Moreover, even in the context of a specific MPM histological subtype, there are differences in the clinical course including long surviving patients, whose tumors apparently do not differ morphologically from conventional, highly aggressive MPM.[5]

According to the COSMIC database, the most frequently mutated genes in MPM include *CDKN2A*, *neurofibromatosis-2* (*NF2*), and *BRCA1-associated protein-1* (*BAP-1*). [6, 7 and 8]*BAP1* germline gene mutations have been identified and associated with a cancer syndrome that includes MPM, ocular or cutaneous melanoma, and other cancers. [9, 10 and 11]

The expanding application of next-generation sequencing (NGS) offers the opportunity to accurately map the type and extent of genetic variations in MPM and to provide correlation with morphological and prognostic parameters of potential therapeutic relevance.

In this retrospective study, 123 formalin-fixed, paraffin-embedded (FFPE) MPM tissue samples with clinical annotates, collected from two institutions, were analyzed by NGS with the aim of generating knowledge about tumor-specific genetic profile and to investigate any potential correlation of the most frequently detected genetic variations with clinical pathological variables and survival outcomes.

PATIENTS AND METHODS

Patients and Tissue Samples

Between November 2003 and December 2012, 123 consecutive cases of MPM diagnosed at two institutions (Orbassano, n = 93; Alessandria, n = 30) with enough leftover tissue available and detailed clinical annotates were retrospectively collected. The available tissues were FFPE biopsy samples obtained through pleuroscopy or videothoracoscopy from patients (pts) with stages III (limited to T3, any stage) and IV[12] MPM, treated with platinum-based plus pemetrexed chemotherapy. None of the patients received surgery or radiation therapy at any time. During chemotherapy, patients were evaluated for tumor response by chest-computed tomography scans every two cycles and during the follow up according to local policy using modified response evaluation criteria in solid tumors.[13]

All samples were reviewed and classified according to the World Health Organization classification.[5] Informed consent was obtained from each patient, and the Institutional Review Board of the participating institutions approved the study. All samples were de-identified, and cases anonymized by a pathology staff member were not involved in the study.

Genomic DNA Extraction

DNA was obtained from tissues after manual microdissection with enrichment for neoplastic cells (at least 50%). Genomic DNA (gDNA) was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. gDNA was quantified using fluorometer Qubit platform (Invitrogen, Carlsbad, CA), and the DNA quality was tested amplifying a 200-base pair region of the β-actin gene.

Next-Generation Sequencing

NGS analyses were performed on the Ion Torrent Personal Genome Machine (PGM, Life Technologies, Grand Island, NE). Tumor samples were tested with a commercial library kit (Ion AmpliSeq Cancer Hotspot Panel v.2) to investigate 50 cancer-associated genes (see Supplemental Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A753). In addition, because the panel did not include *BAP1* and *NF2*, two genes frequently mutated in MPM, a custom NGS library was designed including 58 amplicons, covering all the exonic sequences. Each amplicon library was generated starting from 10 ng of gDNA, as indicated by the manufacturer, and barcoded with Ion Xpress Barcode Adaptors Kit (Life Technologies). DNA library quantification was performed using the polymerase chain reaction (PCR) quantification kit and the 7900HT real time PCR system (Life Technologies), diluted in nuclease-free water to obtain a final concentration of 100 pM. Emulsion PCR was performed on Ion PGM Template OneTouch 2 system (Life Technologies). The quality of the emulsion PCRs was measured using the Qubit IonSphere Quality control kit (Life Technologies). Ionsphere particles with DNA were isolated and sequenced on Ion 316 chip using the Ion PGM Sequencing 200 Kit (Life Technologies). Only sample sequences with at least a quality score of AQ20 (1 misaligned base per 100 bases) were considered for further analyses. The coverage target for each sample was set at a minimum average deep of 100 reads for each amplicon.

Variant Caller and Annotation

Variant Caller plugin included in Torrent Suite Software (v.3.6; Life Technologies) was used to identify variations in target regions, and genetic annotation was performed with Annovar software (http://www.openbioinformatics.org/annovar/). Each of the identified genetic variation was coded according to “plus strand” of Human Genome assembly hg19. More frequent genetic variations (≥25 patients with ≥10% allele frequency [AF]) affecting protein stability or previously correlated to cancer by COSMIC database (v.64) were validated by means of Sanger sequencing.

BAP1 and NF2 immunohistochemistry

FFPE tissue blocks were cut into serial 4-μm thick sections and collected onto charged slides for staining. Immunohistochemistry (IHC) reaction was performed as previously described.[11] Primary antibodies used were as follows: anti-BAP1 (mouse monoclonal, clone C-4, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-NF2 (rabbit polyclonal, code A-19, Santa Cruz Biotechnology). Stromal endothelial cells were the internal reference control for BAP1; for NF2 an external control of colon cancer was used as indicated by manufacturer's instructions. The BAP1 staining was considered positive in the presence of a nuclear immunoreactivity, whereas NF2 displayed a cytoplasmic staining. The semiquantitative H-score evaluation method was used, as previously described.[14]

Statistical Analysis

Statistical correlation between gene variations with AF ≥10% and clinical pathological features were investigated by Fisher exact test. Time to progressive disease (TTPD) was defined as the time from the diagnosis until the first evidence of disease progression.

Overall survival (OS) was calculated from the date of diagnosis to death or last follow up. The log rank test was used to assess differences between groups. The Cox proportional hazards regression model was performed to analyze independent predictors of MPM and OS. Only the variables that were found to be significant in the univariate analyses ($p < 0.05$) were entered into the multivariable analysis to determine the most significant factor for predicting disease outcome. Statistical analysis was elaborated using R statistical software (http://www.r-project.org/).

RESULTS

Patients

MPM tissue samples included 96 epithelioid, 22 biphasic and 5 sarcomatous subtypes; 86 were males and 37 females. Median age at diagnosis was 66.5 years (range 36–82). Sixty-one patients were current smokers, 56 were never or former smokers; for six patients the smoking status information was not available. After a mean follow up of 21.1 months, 99 of 123 were died. At the univariate analyses, only age and histological subtype were significantly associated with disease outcomes.

Gene Variations in the 52 Cancer Associated Genes

DNA was successfully amplified from all samples. The large number of variations detected in our study requested the selection of an arbitrary cutoff value, mainly based on the amount of generated NGS data and the sample size of our population. Based on these assumptions, the genes mutated in at least 20% of our samples (corresponding to 25 patients) was chosen. Twenty genes harbored variations (including intronic, synonymous, nonsynonymous, and regulative) in more than 25 patients with ≥10% AF. Some of these genes had a large number of mutations, and the nonsynonymous were strongly underrepresented (Fig. 1). A small number of samples (Fig. 1) showed a high variation rate in the top 20 genes. The other 32 genes included K/N-*RAS*, *PTEN*, *ERBB2*, *AKT1*, *B-RAF*, and *FGFR1*/2 genes reported to be altered in other types of tumors (see Supplemental Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A753).

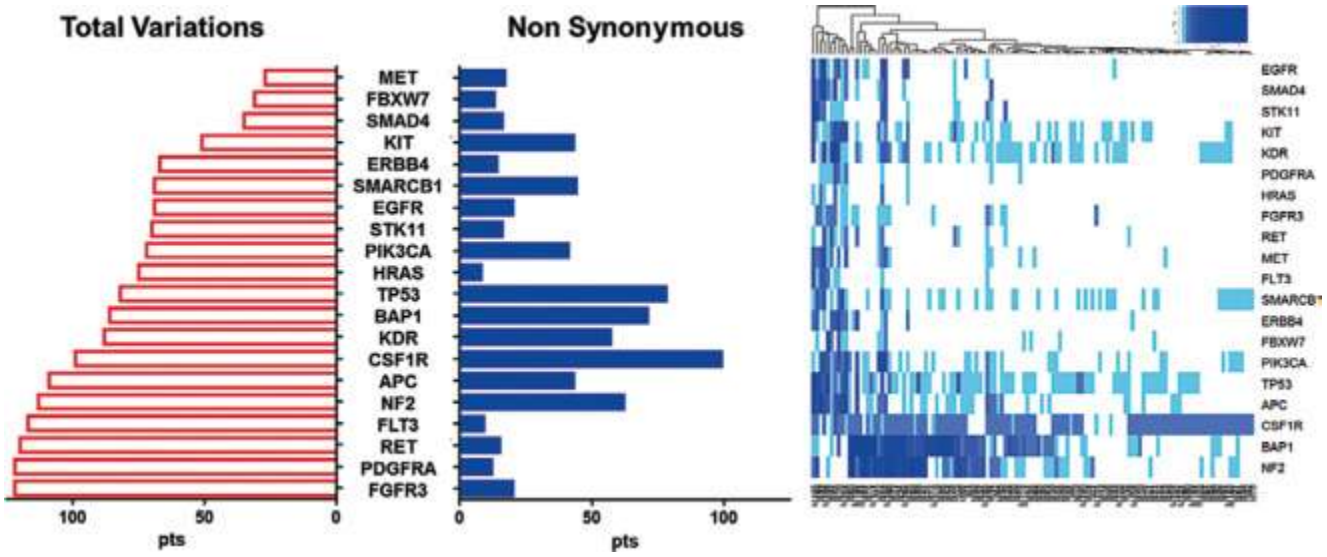

FIGURE 1.

Summary of genetic variations identified by next-generation sequencing. In the left panel, the variations identified in the top 20 genes (≥25 patients mutated with at least 10% of allelic frequency) are summarized: in red the malignant pleural mesothelioma (MPM) samples (pts) with at least a variation (intronic, synonymous, nonsynonymous, and regulative), whereas in blue the MPM samples with at least one nonsynonymous/regulative variation in the top 20 genes. The right panel shows the heatmap of MPM samples with only the nonsynonymous/regulative variations at ≥10% AF. The blue blocks identify variations, although the color intensity is proportional to number of mutations observed in each MPM sample.

Figure options

BAP1 genetic variations were mainly located in exon 13 accounting for 54 variations detected in 47 patients (38%) and in exon 17 (44 variations in 31 patients, 25%). On the contrary, variations identified in *NF2* gene were distributed homogeneously throughout the sequenced regions (Fig. 2; see Supplemental Tables, Supplemental Digital Content 1, http://links.lww.com/JTO/A753 and Supplemental Digital Content 2, http://links.lww.com/JTO/A754).

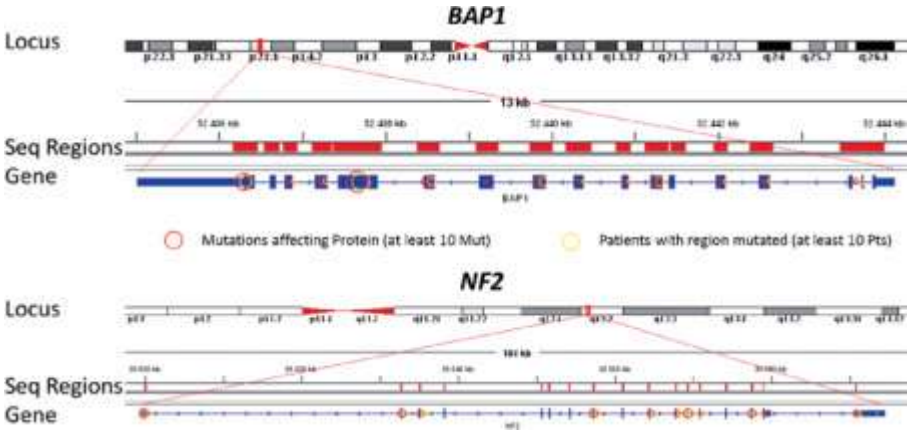

FIGURE 2.

BAP1/NF2 panel: BAP1 variations were enriched mainly in exons 13 and 17. Loci of *BAP1* and *NF2* are shown. The blue blocks represent the exons, whereas the red blocks indicated the regions amplified and sequenced with BAP1/NF2 custom panel. The red and yellow circles indicate the regions with at least 10 variations (red) or mutated samples (yellow). The size of circles is proportional to variations/samples counted for each region. The genetic variations identified in *NF2* were distributed throughout the sequenced exons. Conversely, the *BAP1* genetic variations were enriched mainly in exons 13 and 17.

Figure options

BAP1 and NF2 IHC

BAP1 IHC expression was assessed in 116 of the 123 considered cases. Sixty tumors (52%) had nuclear expression in mesothelial neoplastic cells with diffuse and intense staining; 13 of 116 (11%) did not show nuclear positivity but had a weak granular cytoplasmic positivity, whereas 43 of 116 (37%) were completely negative in tumor cells with a positivity in stromal cells, used as an internal control. Nuclear BAP1 protein expression was correlated to nonsynonymous variations detected by BAP1/NF2 custom panel. The lack of BAP1 IHC nuclear expression significantly correlated with the detection of nonsynonymous variations with allelic frequency of 10% or 25% (Fisher test *p* value < 0.01 for both allelic frequencies; Fig. 3). No correlations were found between BAP1 protein loss and each of the available clinical pathological characteristics.

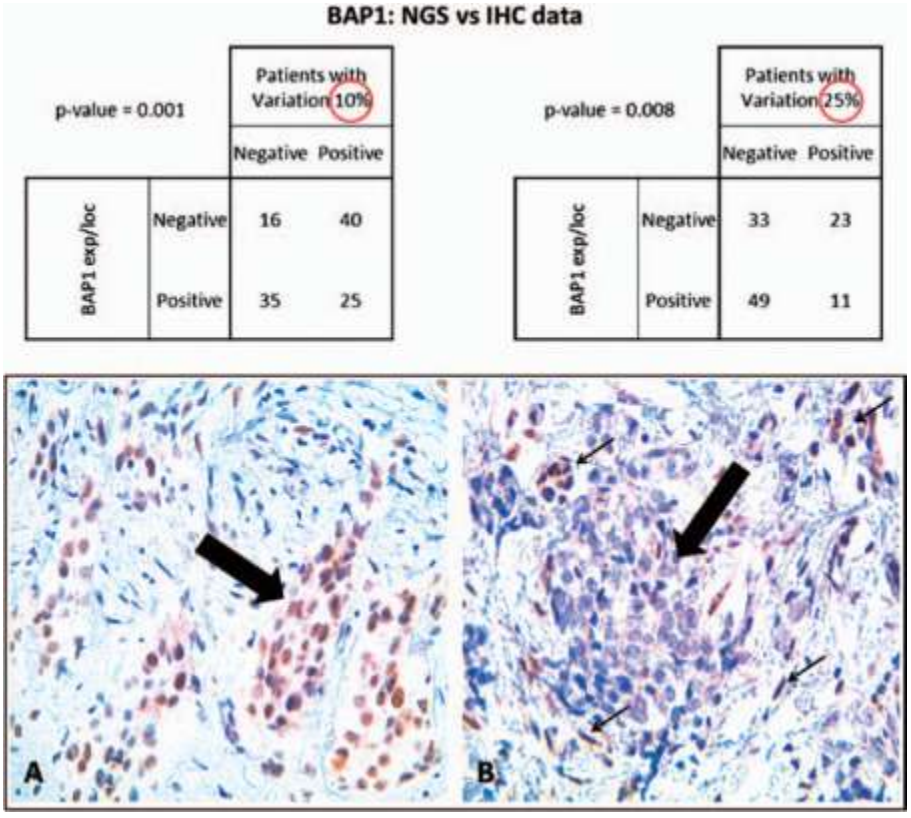

FIGURE 3.

BAP1 nuclear protein localization correlates with next-generation sequencing results. Top panel: contingency tables show the correlation between nuclear BAP1 protein expression and nonsynonymous variations detected by BAP1/NF2 custom panel. The lack of BAP1 protein expression was significantly correlated with the detection of nonsynonymous variations identified with allelic frequency of 10% or 25% (Fisher test *p* value < 0.01 for both 10% and 25% of allelic frequency). Bottom panel: BAP1 immunohistochemistry. *A*, malignant pleural mesothelioma with nuclear expression of BAP1 (arrow). *B*, malignant pleural mesothelioma with loss of BAP1 nuclear expression in neoplastic cells (thick arrow) and maintained BAP1 expression in endothelial and stromal normal cells (thin arrows; immunoperoxidase staining, ×400).

Figure options

NF2 reactivity was positive in the cytoplasm in 107 of 116 cases (92%) ranging from weak to intense diffuse staining and with an heterogeneous pattern of positivity in different tumor areas (median H-score value = 90, range 10–300); 9 of 116 cases (8%) were completely negative. There was no correlation between NF2 protein expression and *NF2* gene variations detected by NGS or clinical pathological characteristics. In the 62 tumor samples harboring *NF2* gene nonsynonymous variations (see Supplemental Tables, Supplemental Digital Content 1, http://links.lww.com/JTO/A753 and Supplemental Digital Content 2, http://links.lww.com/JTO/A754), IHC loss of NF2 protein (H-score = 0) was reported in nine cases only.

Selection of Genes and Validation of NGS Data using Sanger Sequencing

All genetic variations documented in at least 25 patients and with ≥10% AF were further evaluated according to two main criteria: (1) the presence of genetic variation in COSMIC database and associated with cancer or/and (2) the detection of a statistical association with clinical pathological characteristics. Nine variations detected at NGS in ≥25 patients and with ≥10% AF were further analyzed by Sanger sequencing (see Supplemental Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A753). In eight out of these genes (*STK11*, *HRAS*, *SMARCB1*, *PDGFRA*, *TP53*, *PIK3CA*, *KIT*, and *KDR*), the genetic variations were confirmed (Table 1), whereas that initially identified in the APC gene was an artifact because of software bias.

Table 1.

Genetic Variations Identified by Next-Generation Sequencing and Validate by Sanger Sequencing in Malignant Pleural Mesothelioma Patient Cohort

Amplicon	Nucleotide more Freq. Variated	Patients with Var. (≥10% AF)	Cosmic ID	Allelic Freq. (Mean, Median)
Synonymous or Intronic Genetic Variations				
STK11.2	chr19:1220321	—	—	0.54, 0.52
HRAS.1	chr11:534242	72 (58%)	COSM249860	0.54, 0.48
SMARCB1.4	chr22:24176287	38 (31%)	COSM1090	0.51, 0.46
PDGFRA.4	chr4:55152040	28 (23%)	COSM22413	0.52, 0.47
Nonsynonymous genetic variations				
TP53.2	chr17:7579472	70 (57%)	—	0.84, 0.90
PIK3CA.4	chr3:178927410	31 (25%)	COSM328028	0.48, 0.53
KIT.3	chr4:55593464	32 (26%)	COSM28026	0.50, 0.49
KDR.3	chr4:55972974	48 (39%)	COSM149673	0.52, 0.51

Table options

The most frequent nonsynonymous genetic variations identified in our tumor samples were mainly two single nucleotide polymorphisms (SNPs) within the CSFR1 gene, reported with the same allelic frequency as indicated in 1000 genomes database, not previously correlated with cancer and not enriched in MPM. No correlation with clinical pathological characteristics was identified.

Those genetic alterations not affecting protein expression, initially identified by NGS and then validated, had a high incidence. Three of these variations, all in heterozygosis, already associated to cancer and annotated in COSMIC database, have the following IDs: COSM249860 (HRAS p.H27H, 58% patients, mean AF 54%), COSM1090 (SMARCB1 intronic, 31% patients, mean AF 51%), and COSM22413 (PDGFRA p.V824V, 23% patients, mean AF 52%). The polymorphism rs2075606 (T>C) identified in the *STK11* intron (43% MPM samples, mean AF 54%) and not yet correlated to cancer was significantly associated with early disease progression (see Supplemental Fig., Supplemental Digital Content 3, http://links.lww.com/JTO/A755], log-rank *p* value less than 0.01).

Similarly, a significant association between some of the validated nonsynonymous genetic variations (Fig. 4) and disease progression was identified. In the *TP53* gene, the polymorphism rs1042522 was C:C (corresponding to amino acids arginine/arginine [Arg/Arg]) in 33% of considered samples, C:G (arginine/proline [Arg/Pro]) in 61%, and G:G in 6% (proline/proline [Pro/Pro]). A correlation between *TP53* rs1042522 genotype, TTPD, and OS was documented; the upper right graphs in Figure 4 show the Kaplan–Meier curves according to NGS data, whereas the lower graphs those using Sanger sequencing data. In both groups, estimated survival curves show the same trend. With NGS data, a strong correlation between the G:G genotype and early TTPD (median 8.1 months), compared with C:C and C:G genotypes (median 21.5 and 15.6 months, respectively), was observed (log-rank *p* value < 0.01), whereas the association with OS was of borderline significance (*p* value = 0.042). In the 60 cases validated at Sanger sequencing, the association with TTPD was maintained (*p* value = 0.042), whereas there was only a trend for OS (*p* value = 0.087).

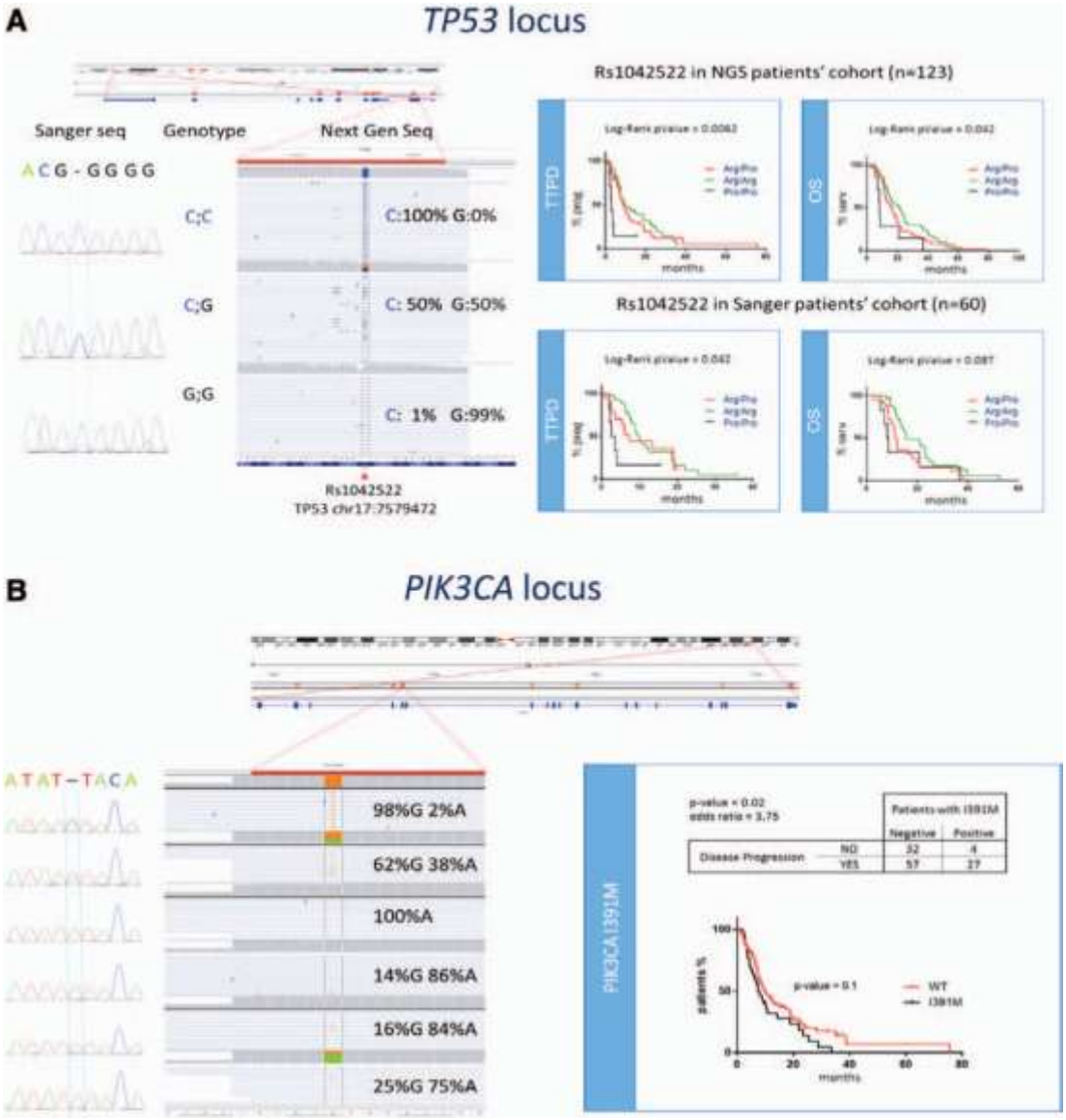

FIGURE 4.

TP53 and *PIK3CA* genetic variations identified by next-generation sequencing (NGS) and correlation with time to progressive disease or overall survival (OS) of malignant pleural mesothelioma patients. *A*, TP53 rs1042522 genotype and time to progressive disease/OS. In left part of panel, the genotypes of three patients identified by NGS are shown and confirmed by Sanger sequencing. In details, the left panel shows Sanger electropherograms for rs1042522 homo- (C:C), hetero- (C:G), and homo-zygote (G:G) samples. In the middle panel, the same samples were represented by NGS sequence BAM files uploaded in IGV software. In the right panel, KaplanMeier curves show the association between rs1042522 genotype and the early disease progression or reduced survival (log-rank *p* value indicated in figure). *B*, PIK3CA mutation I391M and patient progression/OS. Left part of panel shows the high correlation between Sanger and

NGS data when the allelic frequency for mutation was ≥15% at NGS. In the right part of the figure. KaplanMeier curves and contingency table show associations between PIK3CA p.I391M mutation and early disease progression (log-rank and Fisher test *p* values indicated in the figure).

Figure options

In 31% of the MPM samples, the PIK3CA mutation I391M, corresponding to COSM328028 (c.1173A>G), was detected and associated with disease progression after two cycles (Fisher test *p* value = 0.03), although the median TTPD was not significantly different (log-rank *p* value = 0.1). With the two sequencing methods, concordant results were reported when the allelic frequency at NGS was ≥15% (Fig. 4B).

Multivariable Cox regression analysis indicated STK11 (rs2075606) and TP53 (rs1042522; G:G genotype) as independent predictors of TTPD (STK11: hazard ratio [HR] = 1.78; 95% confidence interval [CI] = [1.12–2.81]; *p* value = 0.01 and TP53: HR = 3.9; 95% CI = [1.54–9.79]; *p* value <0.01). STK11 and TP53 were also independently associated to OS (STK11: HR = 1.55; 95% CI = [1.04–2.33]; *p* value = 0.03 and TP53: HR = 2.65; 95% CI = [0.15–6.10]; *p* value = 0.02).

Patients with multiple validated variations (*STK11*, *TP53*, *PIK3CA*, *KIT*, and *KDR*) had a shorter TTPD and reduced OS (Fig. 5). Interestingly, an inverse correlation between TTPD or OS and the number of allelic variations (0, 1, 2, and ≥3 genetic variations) in these genes was observed (TTPD log-rank *p* value = 0.02; OS log-rank *p* value=0.04; Fig. 5).

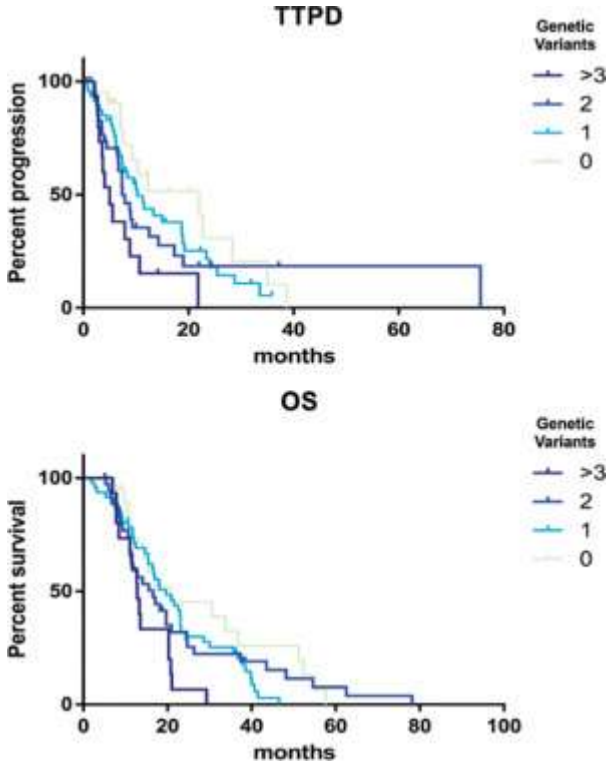

FIGURE 5.

Correlation between the number of identified genetic variations and time to progressive disease (TTPD) or overall survival (OS). KaplanMeier curves show associations between one or more genetic validated variations identified (STK11: rs2075606, TP53: rs1042522, PIK3CA: COSM328028, KIT: COSM28026, and KDR: COSM149673) and TTPD or OS. In particular, an inverse correlation between the number of alterations identified in these genes (0, 1, 2, and ≥3 genetic variations) and TTPD or OS was observed (log-rank *p* value = 0.02 and 0.04, respectively). Median TTPD group with more than three genetic variations, 5 months; two genetic variations, 7.4 months; one genetic variation, 10.3 months; zero genetic variation, 22 months. Median OS group with more than three genetic variations, 12.8 months; two genetic variations, 16.6 months; one genetic variation, 19.7 months; zero genetic variation, 20.8 months.

Figure options

DISCUSSION

Over the past few years, NGS boosted biological and biomedical knowledge facilitating multigene mutational profiling using extremely small amount of DNA, available also by FFPE samples. The expanding application of NGS techniques has the potential for accurately mapping the type and extent of gene mutations in MPM.

In this retrospective study in MPM, the feasibility of assessing mutational changes by NGS using FFPE tissue samples was investigated using a commercially available panel of key cancer-associated genes. Our results showed that NGS was feasible, despite the disadvantage of DNA fragmentation and reduced DNA amounts obtained from FFPE samples.

Our data do not indicate the presence of a specific mutation in a single driver gene, favoring the hypothesis of the accumulation of several nondriver mutations that could explain the extremely long latency phase of this asbestos-related disease.

Most frequently identified and validated genetic variations were clustered in two main and partially overlapped pathways: the p53/DNA repair and the receptor tyrosine kinase–phosphatidylinositol 3-kinase (PI3K)–AKT pathways (see Supplemental Fig., Supplemental Digital Content 4, http://links.lww.com/JTO/A756).

In the p53/DNA pathway, genetic variations were mainly identified in *TP53*, *SMARCB1*, *BAP1*, and *CDKN2A*. [15] Interestingly, *TP53* and *CDKN2A* had the highest nonsynonymous/synonymous genetic variations ratio (see Supplemental Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A753), suggesting that mutations in this pathway could be relevant for MPM carcinogenesis and/or evolution. Furthermore, *TP53* SNP rs1042522 was observed, and the Pro/Pro genotype was associated to shorter TTPD and reduced OS (Fig 4A, p value <0.01 and <0.04, respectively). According to previously reported data, the Arg72 genotype induces more efficiently apoptosis, whereas the Pro72 genotype preferentially induces G1 arrest, leading to the activation of p53-dependent DNA repair.[16,17] Early studies indicate that patients with the Pro/Pro genotype are more prone to have primary resistance to cisplatin-based chemotherapy, compared with those with Arg/Arg or Arg/Pro variants.[18,19] In advanced gastric cancer patients treated with paclitaxel and cisplatin, the *TP53* codon 72 SNP was predictive of chemotherapy response and correlated with TTPD. [20]

As expected, several genetic variations were documented in the *BAP1* gene. Two hotspot regions with genetic variations were identified: one in exon 13 (54 variations, 38% patients) and the other in exon 17 (44 variations, 25% patients; Fig. 2). Ongoing research in our laboratory is focused to better understand the functional consequences of the genetic variations in the exons 13 and 17 of BAP1 gene. The detected nonsynonymous variations were significantly correlated to the absence of BAP1 nuclear protein expression as assessed by IHC (Fig. 3). These data suggest that nonsynonymous variations identified affect BAP1 protein production and/or its stability and, according to published studies, suggest that the lack of BAP1 protein is involved in MPM carcinogenesis.[8] Furthermore, a recent study indicated that the absence of BAP1 causes accumulation of mutations and chromosomal aberrations, leading to genomic instability.[21]

In the receptor tyrosine kinase–PI3K–AKT pathway, already known cancer-associated mutations, such as *PDGFRA* (COSM22413), [22]*KIT* (COSM28026), [23] and *KDR* (COSM149673), were identified in 38, 26, and 38 samples, respectively. A HRAS silent genetic variation previously reported in bladder cancer (COSM249860) [24] was identified in 72 of 123 (58.5%) patients with MPM. More relevantly, a mutation in the catalytic subunit of PI3K was detected and associated to disease progression. Indeed, 27 of 31 patients carrying the *PIK3CA* mutation I391M (COSM328028) had progressive disease as the best response to chemotherapy, suggesting a potential pathological role of this mutation in the natural history of MPM. These data match with previously published studies in which PI3K–AKT signaling was found activated in MPM, and a marker of therapy response. The detected genetic variation PIK3CA requires additional studies to evaluate if the change in the amino acid sequence may affect or deregulate the PIK3CA protein function and, as suggested by our data, associated with a more aggressive type of MPM. Cacciotti et al. [25] suggested that PI3K–AKT pathway is involved in asbestos-related mesothelioma carcinogenesis, increasing the survival of mesothelial cells after amosite exposure. Kim et al.[26] demonstrated that the inhibition of AKT activation through double PI3K–mTOR inhibitors enhanced apoptosis in human mesothelioma spheroids. In our study, *PIK3CA* nonsynonymous genetic variations with ≥10% AF were identified in 41 patients (33%), and these findings potentially support further studies specifically investigating the activity of PI3K–mTOR inhibitors in this subgroup of MPM. Phase I/II studies are currently evaluating the role of double mTOR–PI3K inhibitors in several types of solid tumors, [27] including MPM. GDC-0980, an oral PI3K–mTOR inhibitor, showed antitumor activity in MPM as evidenced by tumor regression and prolonged disease control.[28]

Mutations in two genes that affect mTOR regulation, *STK11* and *NF2*, were also detected. Of note, the SNP variation rs2075606 in *STK11* gene was correlated with shorter TTPD (see Supplemental Fig., Supplemental Digital Content 3, http://links.lww.com/JTO/A755). *NF2* that encodes for a protein called merlin, [29,30] has been previously associated to MPM, whose inactivating mutations have been reported in up to 40% of the cases.[29]*NF2* nonsynonymous genetic variations were detected in 62 (50%) patients, and 15 had at least one mutation annotated in the COSMIC database (see Supplemental Table, Supplemental Digital Content 1, http://links.lww.com/JTO/A753). No significant correlation was observed between *NF2* genetic variations and, respectively, merlin IHC expression, others genes validated through Sanger sequencing and patients' clinical pathological variables considered. These data might indicate that *NF2* genetic variations identified and scattered across the gene could deregulate NF2 without affecting protein expression/stability.

The interpretation of the results of this retrospective study has some limitations. First, there is a selection bias due to tissue availability because of asbestos workers' compensation cases usually filed. The associated long-standing litigation frequently comes with the request to provide MPM tissue blocks for external pathological confirmation or additional analyses leading to tissue exhaustion. Second, although all known *BAP1* and *NF2* gene mutations were investigated through a custom-made panel, all the other genes were only assessed for the mutations included by manufacturer in Ion AmpliSeq Cancer Hotspot Panel v.2. Third, another limitation of the our study is represented by the assessment of gene mutations only, whereas other types of genetic changes, such as amplification, [31] wide genetic losses,[8] or gene translocations,[32] are not detected by the chosen technology. This makes complicated to compare the results of our study with those reported by others using a different sequencing technique. A previously reported study identified several nonsense and indels mutations often associated to protein changes,[8] whereas, in the

present study, we identified several point mutations that require additional functional characterization to assess putative effects on BAP1 protein. Lastly, any observed association between a specific gene variation and the clinical outcome should be considered of borderline significance, especially in light of multiple potential interactions among mutations.

In conclusion, this is the first relatively large retrospective study of NGS for genetic characterization of MPM using FFPE archival material. Our results indicate a complex mutational status shooting mainly p53/DNA repair and PI3K–AKT pathways. In the present series, some of the genetic variations and their accumulation were associated with early progression of the tumor. Future studies should prospectively validate the prognostic role of these genetic variations and, if confirmed, should be included among the stratification factors in future MPM studies.

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Address for correspondence: Giorgio V. Scagliotti, MD, Department of Oncology, S. Luigi Hospital, University of Torino, Regione Gonzole 10, 10043 Orbassano, Torino, Italy