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Accessibility
Gender-Specific Molecular and Clinical Features underlie Malignant Pleural Mesothelioma

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Abstract

Malignant pleural mesothelioma (MPM) is an aggressive cancer that occurs more frequently in men, but is associated with longer survival in women. Insight into the survival advantage of female patients may advance the molecular understanding of MPM and identify therapeutic interventions that will improve the prognosis for all MPM patients. In this study, we performed whole-genome sequencing of tumor specimens from 10 MPM patients and matched control samples to identify potential driver mutations underlying MPM. We identified molecular differences associated with gender and histology. Specifically, single-nucleotide variants of BAP1 were observed in 21% of cases, with lower mutation rates observed in sarcomatoid MPM (p<0.001). Chromosome 22q loss was more frequently associated with the epithelioid than that
non-epitheliod histology (p=0.037), whereas CDKN2A deletions occurred more frequently in non-
epitheliod subtypes among men (p=0.021) and were correlated with shorter overall survival for
the entire cohort (p=0.002) and for men (p=0.012). Furthermore, women were more likely to
harbor TP53 mutations (p=0.004). Novel mutations were found in genes associated with the
integrin-linked kinase pathway, including MYH9 and RHOA. Moreover, expression levels of
BAP1, MYH9, and RHOA were significantly higher in non-epitheliod tumors, and were
associated with significant reduction in survival of the entire cohort and across gender subgroups.
Collectively, our findings indicate that diverse mechanisms highly related to gender and histology
appear to drive MPM.

Keywords
Mesothelioma; Gender; Sequencing; ILK pathway; TP53

Introduction
Malignant Pleural Mesothelioma (MPM) is a rare, aggressive cancer, associated with prior
exposure to asbestos (1). Approximately 3,200 new cases are diagnosed in United States
annually, and the incidence worldwide is estimated to rise during the next two decades (2).
Prognosis is poor for most patients with a reported median survival between 4 and 12
months because there are few effective systemic treatments for this malignancy (3). Three
major histological types are recognized in MPM: epitheliod, sarcomatoid, and biphasic (5).
The incidence of MPM is higher in men than women, likely because of the more common
occupational asbestos exposure in men, whereas women are usually subject to secondary
exposure through spouses’ clothing, low-level environmental exposure, and other sources
(6,7). A recent study suggests that female patients with MPM live significantly longer
compared to men even after adjustment for potential confounders such as age, stage, and
treatment (6).

High-throughput sequencing techniques have revealed that each cancer genome appears to
have a unique genetic profile acquired through cumulative genetic alterations, including
driver mutations, that may confer advantageous survival phenotypes on the cancerous cells
and represent precise therapeutic targets (8). To date, the genetic landscape of MPM has
been primarily described in terms of chromosomal rearrangement events investigated mostly
in epitheliod and biphasic human specimens and cell lines (9). The cyclin-dependent kinase
inhibitor 2A (CDKN2A), the neurofibromatosis type 2 (NF2), and the BRCA1-associated
protein-1 (BAP1) genes have been shown to be the most frequently mutated tumor
suppressor genes in MPM (11–14). Recently, two independent investigations have identified
BAP1 as frequently mutated in the chromosomal region 3p21.1 in sporadic and familial
MPM indicating the importance of genetic factors in MPM susceptibility (12,14,15).
Although BAP1 mutations have been associated with age (12) and with the epitheliod
subtype (16), no other significant correlation of these genes to demographic, clinical or
pathological variables has so far been identified.
In the current study, a comprehensive genome-wide approach was applied to discover novel genetic alterations in MPM. Whole-genome sequencing data of tumor specimens from 10 MPM patients and their matched normal tissue were analyzed to identify novel somatic point mutations. A bioinformatic approach was employed to prioritize genes to further analyze by focused sequencing in close to three hundred additional MPM tumor samples.

**Methods**

**Specimens and cell lines**

All tumor and normal lung specimens were collected at surgery with patient consent, fresh frozen, stored, and annotated by the institutional tumor bank with Institutional Review Board approval at Brigham and Women’s Hospital (BWH; Boston, MA). High quality, tumor enriched (19) samples were prepared as previously described (18). Tumor cell enrichment (>83% for whole-genome sequencing experiments; >40% for targeted re-sequencing experiments) was confirmed by reviewing H&E-stained frozen sections. Eleven mesothelioma cell lines [H2052, MSTO-211H, H2452, H28, and the epithelial virus transformed MET5A from the American Type Culture Collection (ATCC, Manassas, VA), JMN and JMN1B from Cell Culture Core (BWH), and MESO257, MESO428, MESO589, MS924 from the laboratory of JAF] were cultured in RPMI (Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum (ATCC, Manassas, VA) and maintained at 37°C in 5% CO₂. Cells were collected at 70% confluency and frozen for DNA extraction or placed in RNA extraction buffer (Triazol reagent; Invitrogen) for RNA extraction. DNA was isolated using the DNeasy Tissue kit (Qiagen, Valencia, CA), and RNA using the Trizol (ThermoFisher) method in combination with RNeasy kit (Qiagen). RNase or DNase I (Qiagen) treatments were conducted according to the manufacturer’s instructions. Matched normal DNA was prepared from peripheral blood or lung tissue. Nucleic acids were quantified using an ND-1000 spectrophotometer (ThermoFisher). The integrity of the DNA and RNA were determined using Qubit 2.0 Fluorometer (ThermoFisher) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), respectively.

**Whole-Genome Sequencing**

Whole-genome sequencing (WGS) of 10 tumor and 10 matched normal genomic DNA (obtained from blood) samples (3μg) was accomplished using a Complete Genomics platform (20). Mean coverage of tumor and normal genomes were 187× and 188× respectively (Supplementary Table 1). All the Pair-End reads were filtered based on average read quality score ≥ 20. Candidate somatic mutations were detected using the SynaAlign program (Synamatix, Kuala Lumpur, Malaysia). Briefly, sequencing reads were first aligned to the human reference genome (hg19) using short read mapper SXmapper (Synamatix) with default parameter settings. The numbers of reads containing single nucleotide variants (SNVs) and indels in both tumor and germline samples were counted and the quality score of each base and the read direction information were collected. This information and the mapping statistics for each read across the genome (reads in repeat regions etc.) were used to call SNVs and indels. The null hypothesis of equal allele frequencies in tumor and germline samples was tested using the two-tailed Fisher’s exact test on quality-filtered SNVs.
and indels. Data were also collected for each sample on larger scale structural variations, loss of heterozygosity, and copy number variations.

**Validation of SNV by Sanger Sequencing**

Selected candidate SNVs were further characterized using PCR and conventional Sanger sequencing to confirm tumor-specific single nucleotide mutations. Gene-specific primer-pairs were used to PCR amplify genomic DNA from tumor and normal specimens (Supplementary Table 2) as previously described (18). For TP53 analysis, primers were chosen in the flanking intronic region of each exon. Twenty-two samples were analyzed using both re-sequencing and Sanger methods. Discordant results were found in six cases, probably due to tissue heterogeneity. In discordant cases, the mutation was called as present for each sample.

**Karyotype and Fluorescence In Situ Hybridization (FISH) analysis**

Metaphase chromosome spreads from diagnostic MPM samples were prepared and G-banded according to standard procedures as part of the routine BWH pathological evaluation of all MPM cases. Additional FISH analyses were performed in those samples either with no metaphases or normal karyotype in the context of pathologic mesothelioma positive report. FISH analysis was performed using the Vysis LSICDKN2A/CEP9 Dual Color Probe Set (Abbott Molecular, Des Plaines, IL) for the CDKN2A locus at 9p21, CEP9 at the chromosome 9 centromeric region, and Vysis TUPLE 1/LSI ARSA Dual color Probe set (Abbott) for TUPLE 1 at 22q11.2, and ARSA at 22q13. Observed aberration in ≥2% of observed nuclei (minimum 50 per case) was reported as consistent with a clonal, neoplastic, cellular proliferation.

**Additional Dual-color FISH Analysis**

For a subset of cases, dual-color FISH analysis was performed on frozen sections (5 µm) to assess two distinct regions of chromosome 22. The sections were fixed at room temperature for two hours in freshly prepared 3:1 methanol-acetic acid, then air-dried.

Bacterial artificial chromosome (BAC) DNA probes (CHORI, BAC PAC Resource, Oakland, CA) containing most or all of the coding sequence for NF2 (RP11-155B12, 159 kb) and MYH9 (RP11-105I18, 161 kb) were selected and labeled by nick translation (Abbott), NF2 with Orange dUTP and MYH9 with Green dUTP. Slides were pretreated with pepsin (Digest-All 3, Invitrogen) 5–15 minutes at 37°C, rinsed in Phosphate-buffered saline (PBS) 5 minutes at RT, post-fixed 1 minute in 10% formalin, rinsed in PBS, dehydrated in ethanol series, and air-dried. FISH procedure followed standard protocols. Scoring was performed by two technologists, each scoring Orange (O, NF2) and Green (G, MYH9) signals present on 50 nuclei, for 100 nuclei total per specimen in an area determined by a pathologist in parallel H&E stained sections to have a minimum tumor involvement of 40%. Negative controls were a normal lymphoblast line with metaphases to confirm localization, and sections from four tumors with no histologically apparent tumor in the scored areas. Each deletion signal pattern (1O1G, 1O2G, 2O1G) was determined to be present if its frequency exceeded the mean plus 3 standard deviations of the same pattern in the normal sections.
**Target capture, sequencing, and data analysis**

Library preparation, custom exome capture, and next generation sequencing were performed at the Center for Cancer Computational Biology, Boston, MA, USA. Library construction and custom exome capture were performed using the SureSelect XT2 for Illumina protocol (Agilent) according to the manufacturer’s instructions. Libraries were combined into 3 library pools, diluted to a 2nM working stock and sequenced at a final concentration of 12pM on a paired end flowcell with 50 cycles in each direction. Sequencing was done on a HiSeq 2000 (Illumina, San Diego, CA, USA) according to the manufacturer’s protocols. Sequencing reads were demultiplexed using the Illumina CASAVA package and aligned to the hg19 assembly of the human genome using BWA aln with default parameters and a mismatch penalty of 1 (21). SNV and indel discovery was performed using HaplotypeCaller within the GATK package with default parameters (22). Variants with a minimum phred-scaled confidence threshold under 10 were excluded, and those under 30 were flagged as low quality. Resulting variant calls were annotated for potential genetic impact using the SnpEff package (23).

**Microarray analysis**

Expression levels of specific genes were explored using microarray data from a parallel project. Epithelioid samples (N=129) were selected from the International Mesothelioma Program Tumor Bank (Supplementary Figure 1). This sample set consisted of male and female samples matched by age and nodal status (Coleman MH, and Bueno, R personal communication). In addition, 19 sarcomatoid and 3 biphasic MPM samples were included in the analysis. To determine the levels of transcripts in each sample, 0.25 µg of total RNA was amplified using the Ambion WT Expression Kit (ThermoFisher). The cRNA was hybridized to Affymetrix® Human Gene 1.1 ST Array (ThermoFisher), subsequently labeled with GeneChip WT Terminal Labeling Kit (ThermoFisher), and scanned with a GeneAtlas™ Workstation (ThermoFisher). Hybridization, washing, staining and scanner procedures were performed per manufacturer recommendations. For quality control across platforms, two MAQC samples were included in the analysis (24). A blind control was also added to check the variability of the expression values across the chips. The probe intensity distribution was examined for quality control, and outliers were removed. Expression levels for BAP1, NF2, TP53, MYH9, MYH6, MYH10, PIK3C2A, RHOA, and TNFRSF1A for this cohort are included in Supplementary Table 3.

**Statistical analysis of mutation status and gene expression versus clinicopathologic variables**

Asbestos exposure was analyzed as a binary covariate with background exposure defined as asbestos body counts in lung tissue ≤50 fibers per gram of wet lung tissue. Fisher’s exact test was used to compare histology, gender and asbestos exposure between mutation and wild-type groups, whereas Wilcoxon rank-sum test was used to analyze age as a continuous covariate. Overall survival was defined from the date of definitive surgery until the date of death or was censored at the date of last follow-up for patients who had not died at their latest contact. Overall survival was estimated by the Kaplan-Meier method, with group differences assessed by the log-rank test. The normalized expression level of each candidate
gene was initially grouped into quartiles for exploratory survival analysis. Patient subgroups with comparable survival were combined for further analysis in order to estimate the meaningful differences. Proportional hazards regression was used to estimate the hazard ratio for reporting a survival difference, including adjusting for the gender effect. All p-values are based on a two-sided hypothesis, with p<0.01 used to identify potentially significant results in order to be conservative on false positives. Data analysis was performed using SAS 9.4 (SAS Institute, Cary, NC).

Results

Experimental subjects

Ten MPM tumor and matched normal DNA samples were analyzed by WGS (Table 1). Matched MPM and normal DNA from 283 additional patients was subjected to focused sequencing (Table 2).

Tumor Somatic DNA variants

Point mutations, small (<30 bp) structural variations, and copy number alterations were discovered in 10 pairs of WGS MPM tumor and germline DNA samples using computational approaches. Single nucleotide variants (N=146; SNVs) mapping in the amino acid coding regions of annotated exons and generating non-synonymous amino acid changes were further re-sequenced in the tumor and normal DNA by Sanger methodology. Eighty-five were confirmed to be tumor-specific (Supplementary Table 2). Each patient’s sample pair displayed a unique small number of point mutations ranging from three to 20 per tumor. Seventy-eight SNVs were missense, and seven were nonsense mutations. TP53 was found mutated in 2 out of 10 tumors (20%). In both cases, the tumors came from female patients with asbestos body counts in the background range as measured in resected lung tissue (data not shown). Three of the mutated genes (RNF43, TP53 and MYH9) were listed in the ‘Catalogue Of Somatic Mutation In Cancer’ (COSMIC, Release v69) database (25).

Functional Analysis of Point Mutations

To identify clusters of genes associated with known pathways, functional enrichments of genes affected by point mutations were performed utilizing Ingenuity Pathway Analysis (Qiagen) using Biological Process Gene Ontology terms and canonical pathways. Among 216 canonical pathways identified, 51 were significantly enriched (p<0.05) for the mutated genes (Supplementary Table 4). Mutations affecting genes involved in the integrin-linked kinase (ILK) pathway were the most significantly (p = 4.9e-5) enriched. Specifically, 5 of the 10 sequenced MPM samples showed point mutations in at least one of 6 identified genes associated with this pathway (MYH9, MYH6, MYH10, PIK3C2A, RHOA, and TNFRSF1A) (Figure 1A).

Validation of the candidate driver genes in an independent cohort

To further assess the prevalence of mutations observed herein and in previous studies (11–14), a panel of 147 MPM tumors (including 81 epithelioid, 41 biphasic, 24 sarcomatoid, and 1 desmoplastic) and 11 previously established MPM cell lines were further analyzed using targeted re-sequencing of BAP1, NF2, TP53, MYH9, MYH6, MYH10, PIK3C2A, RHOA, and
TNFRSF1A. This cohort included tumor samples from women (n = 30) to evaluate the frequency of mutations with respect to gender. In addition, clinical karyotype analysis and FISH data for CDKN2A and 22q were available for most of the patients. Targeted re-sequencing revealed 116 non-synonymous SNVs and/or INDELs (Supplementary Table 5A, B, C). Seven of these were confirmed to be single nucleotide polymorphisms occurring at high frequency (>1–2%) in the normal population. The remaining 109 occurring in 86 samples were further examined in normal and tumor DNA pairs by Sanger sequencing. One or more somatic mutations were confirmed in 81 of 147 samples (55%).

*BAP1* was mutated in 31 of 147 (21%) samples (Supplementary Table 5A). Twenty-six tumor specific variants and 5 germline variants (present in tumor and normal matching DNA) were observed. In six samples, multiple *BAP1* mutations were identified. The mutations occurred mostly in epithelioid (23%) and biphasic (24%) samples, whereas only two out of 24 sarcomatoid (8%) samples had tumor specific *BAP1* mutations. No significant correlation was found between mutation in *BAP1* and gender, exposure to asbestos, or survival. *BAP1* mutations were associated with marginally older age compared to wild-type tumors (median 67 versus 64 years; p = 0.050). *BAP1* mutations were also observed in cell lines H2452 and H28. Table 3 describes the cases carrying *BAP1* germline mutations.

*NF2* point mutations occurred in 21 (14%) of 147 analyzed samples (Supplementary Table 5A). In addition, karyotype and/or FISH analyses available for 133 of these 147 samples showed that 75 samples (56%) had loss of chromosome 22q. Eleven of 133 (8%) had both *NF2* point mutation and 22q deletion. Compared to the cases with two copies of 22q, the deletion of 22q was more frequently associated with the epithelioid than non-epithelioid histology (p=0.037). The difference was greater in women but did not reach strict statistical significance due to low power (p=0.141). Mutations in *NF2* were not correlated with any of the other clinical or pathologic variables examined. *NF2* mutation was found in H2052.

Karyotyping and/or FISH analyses of *CDKN2A* were available for 133 of 147 samples. Eighty-two (62%) of 133 samples had deletion of the *CDKN2A* region on 9p (determined by karyotyping) and/or confirmed *CDKN2A* deletion (as determined by FISH with a *CDKN2A* probe). Forty-one of 74 (55%) epithelioid, 29 of 41 (71%) biphasic, 11 of 17 (65%) sarcomatoid, and one of one desmoplastic samples had *CDKN2A* deletion. Compared to the cases with two copies of *CDKN2A*, cases with *CDKN2A* deletion appeared to be more frequently associated with the non-epithelioid histology (p=0.117). When this analysis was repeated for gender, the association between histology and *CDKN2A* loss was statistically significant only for the men (p=0.021). Interestingly, deletions in CDKN2A were correlated with shorter overall survival in the whole cohort (p=0.002), but the difference was statistically significant only in the men (p=0.012).

*TP53* was mutated in 22 cases (15%) in this sample set (Supplementary Table 5B). Three mutations (D49N, C176F, A86T) were present in both the tumor and the normal DNA (Table 3). Notably, one sample showed only one germline *TP53* mutation (D49N) and no mutation in any of the other genes investigated herein. None of the cell lines examined exhibited *TP53* mutations.
No difference was observed in overall survival between the group of 62 patients with point mutation in at least one of the established MPM-related genes (BAP1, NF2, TP53) and the group wild type for these genes (p = 0.646).

Forty SNVs in 31 samples (21%) occurred in ILK pathway-associated genes (TNFRSF1A, PIK3C2A, MYH10, MYH6, MYH9, and RHOA) (Supplementary Table 5C), of which 35 (88%) were germline. Fifteen patients (43 %) carrying a germline mutation in one or more of these genes had familial history of cancer. Only one SNV, in TNFRSF1A, is known to occur frequently (>1–2%) in the general population (26). This SNV occurred in 5 patients and was predicted to have a “probably damaging” effect (PolyPhen: http://genetics.bwh.harvard.edu/pph2/) on the corresponding protein. Seven germline mutations were identified in PIK3C2A. Five were predicted to be benign and two “possibly damaging”, one of which resides in the Phosphoinositide 3-kinase C2 domain. Twenty-six SNVs resided in three myosin heavy chain genes. Two samples displayed germline mutation of MYH10. Although functional analyses predict a benign effect, these mutations occur near mutations identified in other cancers (27). Nine germline mutations were detected in MYH6, two of which had a predicted “probably damaging” effect and one caused a reading frame shift. Twelve samples exhibited germline mutations in MYH9; seven had a predicted “possibly or probably damaging” effect. Although many mutations of MYH9 have been identified in several other cancers, only R1576Q identified in our series has been previously described (27).

In 4 patients, tumor specific mutations of ILK pathway-associated genes were identified: 3 in MYH9 (1.4%) (Q738*, K737N, A1197fs) and 2 in RHOA (1.4%) (Y66N, A161V). One patient had in MYH9 one germline and two tumor-specific mutations. No significant correlation was found between the mutations in the ILK pathway-associated genes and clinical or pathological variables. No cell line exhibited mutations in these genes. Figure 1B depicts the mutations found in the myosin heavy chain genes and RHOA. Aberrations identified in BAP1, NF2, TP53, MYH9, MYH6, MYH10, PIK3C2A, RHOA, TNFRSF1A, 9p/CDKN2A, and 22q are illustrated in Supplementary Figure 2.

**Assessment of NF2 and MYH9 copy number status**

Given the proximity of NF2 and MYH9 on the chromosome, 22q copy number status was explored. Although allelic loss in this region is common in MPM, the probes used for standard clinical FISH analysis do not distinguish copy number independently for these two genes. Dual-color FISH analysis using MYH9- and NF2-specific probes was undertaken for the 13 cases carrying MYH9 mutation. Of the 13 primary tumors in which the two markers could be assessed, 2 (15%) exhibited no aberrations involving NF2 or MYH9; 1 (8%) was abnormal by losing NF2 only; 7 (54%) had loss of both markers; and 3 (23%) samples exhibited extra copies of both markers likely due to polyploidy (Supplementary Table 5D) (28). Two of the 7 cases displaying loss of both markers had tumor specific mutation in both MYH9 and NF2, whereas 5 had tumor-specific mutation of MYH9 only. Furthermore, the case exhibiting NF2 loss only presented two tumor specific mutations in addition to one germline mutation in MYH9. Examples of FISH analysis are shown in Figure 1C.
Mutual exclusivity

In MPM, tumor-specific mutations in RHOA, MYH9 and TP53 were mutually exclusive events that occurred more frequently in women (30%) than in men (12%) (p=0.023). We examined the relationship between RHOA and MYH9 in other cancers using the cBioPortal for Cancer Genomics (accessed 7/13/2015) (27). Notably, in 26 of 31 (84%) tumor types that had mutations in both genes, RHOA and MYH9 mutations had a strong tendency toward mutual exclusivity (Supplementary Table 6).

Analysis of TP53 in additional MPM cases

Given that mutations of TP53 were found more commonly in women, and that women with MPM generally have lower levels of asbestos exposure than men, we expanded our validation cohort to power exploration of these associations. Mutational status of TP53 was analyzed by Sanger sequencing in 136 additional MPM samples. Asbestos counts were available for 148 of 283 cases in the combined cohort, including tumors from 88 women and 67 patients with asbestos body counts in lung tissue consistent with background exposure (≤ 50 fibers/g lung tissue) (29). Thirty-eight of 283 patients (13%) had TP53 point mutations, including 18 men (9%) and 20 women (23%), (p=0.004; Supplementary Table 5B). Expanding the cohort added one case (total 4/283) with germline TP53 mutation (R248W; Table 3). Although TP53 mutations were found more frequently in tumors with background versus higher asbestos exposure, the analysis did not have sufficient power to exclude exposure level as a risk factor in either the whole cohort (p = 0.068) or among women (p = 0.498). No significant correlation of mutations in TP53 was found to either survival, or histological subtype.

Expression analysis of the candidate genes

A comparative exploratory analysis of MPM transcriptomic profiles was conducted to determine whether histological subtype, survival or gender are correlated with specific expression patterns in BAP1, NF2, TP53, MYH9, MYH6, MYH10, PIK3C2A, RHOA, CDKN2A and TNFRSF1A (Supplementary Figure 1 and Supplementary Table 3). This cohort comprised 85 men and 66 women. Ten samples were also included in the sequencing analysis; these were pure epithelioid tumors showing no mutation in BAP1, MYH9 or RHOA.

Non-epithelioid (versus epithelioid) tumors expressed significantly higher levels of BAP1 (p<0.001), MYH9 (p<0.001), RHOA (p<0.001), and MYH10 (p=0.001). RHOA was more highly expressed in men than women (p=0.001). Expression levels of other genes examined were not correlated with gender or histology.

Patients with lower BAP1 expression had improved overall survival (p<0.001) (Figure 2A). The highest quartiles of BAP1 expression were associated with twice the risk of death [hazard ratio (HR)=2.31] compared to the lowest quartile, after adjusting for the independent effect of gender. The survival difference was similar across gender subgroups (male: HR =2.20; p=0.005; and female: HR=2.38; p=0.004) (Figure 2B). Similarly, the highest quartile of MYH9 expression was associated with increased gender-adjusted risk of death (HR=2.23) compared to the lower three quartiles (p<0.001) (Figure 2C). MYH9 expression was
associated with twice the risk of death in both males (HR=2.26; p=0.001) and females (HR=2.17; p=0.022) (Figure 2D). In the entire cohort, lower RHOA expression was associated with improved survival (p<0.001) (Figure 2E). The highest quartile of RHOA expression was associated with twice the gender-adjusted risk of death (HR=1.95) compared to the lower three quartiles. Analysis by gender subgroups showed that the highest quartile of RHOA expression was associated with increased risk of death (HR=2.22; p<0.001) compared to the lower three quartiles in males (Figure 2F). No significant association was demonstrated between RHOA expression and the risk of death (HR=1.42; p=0.385) in females, possibly due to the relatively few females with tumor highly expressing RHOA (Figure 2F). Survival analyses for BAP1, MYH9, and RHOA restricted to the epithelioid tumors showed similar conclusions. Expression levels of the other genes examined were not associated with survival either in the entire cohort or within gender subgroups.

**Discussion**

Realizing the potential of precision medicine requires identification of subgroups of patients deemed likely to respond to specific biologically-based therapies. Such stratification only becomes possible as the genetic features that contribute to disease heterogeneity for a given tumor are elucidated (30).

Refinements of molecular sub-classification based on comprehensive analysis of the genetic landscape are required to personalize treatment of MPM. Until recently, the genetics of MPM was primarily described in terms of deletions of specific chromosomal regions, particularly within 1p, 3p, 6q, 9p, 13q, 15q, and 22q [reviewed in (31)]. CDKN2A and NF2 are the tumor suppressor genes most commonly found mutated (9,11,32), and recently, evidence for a role for BAP1 was demonstrated (12,14). During the preparation of this work, a whole-exome sequencing study using DNA from 22 MPMs and matched blood samples found 517 somatic mutations across 490 mutated genes, confirmed frequent mutation in BAP1, NF2, and CDKN2A, and identified alterations of CUL1 in 2/22 cases (33,34). However, the small number of patients analyzed neither allowed for clinical correlation, nor suggested a basis for patient stratification.

The current study validated candidate driver genes (TP53, BAP1, NF2 and CDKN2A) in large cohort of MPM cases. Although CUL1 was not sequenced among the validation cases, no CUL1 mutations were observed among the 10 whole-genome sequenced cases. Novel molecular alterations in MYH9, MYH6, MYH10, PIK3C2A, RHOA, and TNFRSF1A were documented, and bioinformatic analyses found these genes to be linked.

TP53 mutations occurred in 13% of MPM cases analyzed here. Mutations in this gene were more than twice as frequent in women than in men. In one prior study, TP53 genotype was predictive of survival in women following adjuvant therapy for colon cancer (35). In another study of 152 TP53-germline mutation carriers, TP53 PIN3 polymorphism was found to have a sex-specific effect, conferring cancer risk in men (P = 0.0041) but not women (36). To our knowledge, this is the first report of TP53 mutation in tumor and germline DNA in sporadic MPM or in patients with familial history of cancer. Interestingly, only germline mutations in women had predicted deleterious functional impact on p53, whereas the mutations identified

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in men had predicted neutral impact on the protein. No evidence was found in the clinical profile of patients carrying these TP53 mutations to suggest Li-Fraumeni syndrome (37). Although women with MPM have TP53 mutations at a higher rate compared to men, no significant correlation of TP53 status was found to survival in this cohort.

A recent study reports BAP1 mutation in ~70% of MPM and suggests that large chromosomal deletions commonly inferred by immunohistochemistry are missed by Sanger sequencing (38). This may explain why BAPI was found mutated in 21% of the cases by targeted sequencing in the current study. Five cases showed germline BAPI mutations of which 4 had familial history of cancer. To date, BAPI germline mutations have been considered very rare in patients with sporadic MPM (39), and a recent study has shown that 23 MPM patients with BAPI germline mutations had an overall 7-fold increased long-term survival, independent of sex and age (40). In the current study, non-epithelioid MPM was associated with higher expression and less frequent mutation of BAPI. Furthermore, elevated BAPI expression was associated with twice the risk of death after adjusting for the independent effect of sex.

Several investigators have suggested that p16 protein expression may be predictive of favorable prognosis, and that down-regulation of p16 is significantly correlated with poor patient survival in various cancers (41,42). In the current study, deletion of the p16 gene, CDKN2A, was correlated with shorter survival. CDKN2A deletions were more frequent in the non-epithelioid subtype among men. By contrast, 22q loss was more frequently associated with epithelioid subtype, but was not associated with survival.

In this study, 26 SNVs were identified in three myosin heavy chain genes (MYH6, MYH9, MYH10). Myosins are required for actin-based intracellular mobility and have been linked to cell proliferation, adhesion and migration (43). The heavy chain, type A, myosin MYH9 was the most commonly mutated in the current MPM cohort. This gene has been previously associated with cancer cell migration, invasion, and metastasis (44). In addition, it has been shown that the protein product, myosin IIA, is required for both stability and nuclear retention of p53 (44). Fifteen mutations of this gene were identified in 13 (9%) MPM patients of which 6 had familial history of cancer. Three of the 15 mutations were tumor specific and 12 were germline. One patient carried two tumor specific and one germline mutation of MYH9.

Loss of MYH9 alleles was also common. MYH9 maps to 22q13.1 telomeric to the region where NF2 is located (22q12.2). Loss of chromosome 22 is the single most consistent numerical cytogenetic change in MPM (11). It has been suggested that cancer genes other than NF2 may be involved in some malignancies associated with chromosome 22 allelic losses in which NF2 mutations are not detected or found at very low frequencies (11). The probes used for clinical FISH analysis to detect 22q deletion hybridize to 22q11.2 (TUP1L) and 22q13 (ARSA). Because both NF2 and MYH9 are within this region, deletions encompassing both probes indicate loss of both genes. However, rearrangements associated with deletion of only one of the probes do not necessarily indicate deletion of either of these genes, and, conversely, cases lacking deletion of these probes could still have NF2 and/or MYH9 deletions. In the current study, dual-color FISH analysis using probe sets specific for...
each gene indicated that 45% of the samples with \textit{MYH9} mutation and wild-type \textit{NF2} had loss of one allele for both genes. This finding suggests that mutated \textit{MYH9} may contribute to tumorigenesis in some 22q-deletion cases.

\textit{RHOA} encodes a Rho family GTPase, known to regulate the actin cytoskeleton in the formation of stress fibers and focal adhesions, as well as having a significant involvement in cancer signaling cascades (45–49). Two tumor specific mutations have been identified in the validation cohort of this study. \textit{RHOA} resides on 3p21.3 close to \textit{BAP1} (3p21.1), another region frequently deleted in MPM.

The observations that \textit{RHOA} signaling leading to \textit{MYH9} activation in the processes of hemostasis and thrombosis (50), and the observed mutual exclusivity of \textit{MYH9} and \textit{RHOA} support the functional association of these two genes. The significance of \textit{RHOA} and \textit{MYH9} in MPM is supported by their association to clinical factors including gender, histology and patient outcome after surgical therapy. The common relation of these and other genes found mutated in this study to the ILK pathway is intriguing. However, the role of the ILK pathway per se in MPM remains unclear.

In conclusion, the analysis of 10 genome pairs has highlighted novel molecular features in the molecular biology of MPM. The molecular events leading to MPM tumorigenesis appear complex, involving alterations in different genes in subgroups of patients. Chromosomal regions of common allelic loss may contain more than one gene involved in the transformation of a mesothelial cell. Some of the genetic abnormalities are associated with clinicopathological variables such as histological subtype, gender, and survival. Experience in other cancers suggests that studying much larger numbers of MPM genomes may be required for comprehensive molecular understanding of MPM.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


Figure 1.
A) Ingenuity Pathway Analysis was used to connect a subset of 6 genes (MYH9, MYH6, MYH10, PIK3C2A, RHOA, and TNFRSF1A), with tumor specific mutations in 5 MPM samples, mapping in the integrin-linked kinase pathway. Four genes (UBC, MDM2, CLTC, and SRC) were added to show the indirect relationship. The connectivity map was generated from available published data (Ingenuity Systems). Each symbol for gene denotes the function of the interacting protein. Symbols representing specific categories of cellular molecules as well as interactive relationships are depicted in the legend. B) Schematic representation of the SNVs identified in MYH9, MYH10, MYH6, and RHOA. Nucleotide-binding domains and protein domains are included. C) Dual color FISH analysis of MYH9 and NF2 in MPM samples. Negative control (normal lymphoblast line with metaphases to confirm localization) is in the top left panel. The orange probe is specific for NF2, the green for MYH9.
Figure 2.
Kaplan-Meier analysis of tumor-specific survival in patients with MPM according to \textit{BAP1}, \textit{MYH9}, and \textit{RHOA} expression
Overall survival of 151 MPM patients grouped into quartiles (lowest Q1, Q2, Q3, Q4 highest) of \textit{BAP1} (A, B), \textit{MYH9} (C, D), and \textit{RHOA} (E, F) transcriptomic profiles for all tumors (A, C, E) and by gender (B, D, F).
Table 1

Clinical and molecular characteristics of the 10 MPM samples included in the whole genome sequencing analysis

<table>
<thead>
<tr>
<th>Tumor Sample</th>
<th>Histology</th>
<th>Gender</th>
<th>Asbestos Body Counts</th>
<th>9p (FISH)</th>
<th>22q (FISH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG3</td>
<td>BIPHASIC</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CG5</td>
<td>BIPHASIC</td>
<td>M</td>
<td>87</td>
<td>Abnormal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>CG4</td>
<td>EPITHELIAL</td>
<td>M</td>
<td>25</td>
<td>Abnormal</td>
<td>N/A</td>
</tr>
<tr>
<td>CG10</td>
<td>BIPHASIC</td>
<td>M</td>
<td>N/A</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>CG9</td>
<td>EPITHELIAL</td>
<td>M</td>
<td>934</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>CG12</td>
<td>BIPHASIC</td>
<td>M</td>
<td>1382</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>CG14</td>
<td>EPITHELIAL</td>
<td>F</td>
<td>6</td>
<td>Abnormal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>CG16</td>
<td>EPITHELIAL</td>
<td>F</td>
<td>3</td>
<td>Abnormal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>CG18</td>
<td>EPITHELIAL</td>
<td>F</td>
<td>0</td>
<td>Normal</td>
<td>N/A</td>
</tr>
<tr>
<td>CG20</td>
<td>EPITHELIAL</td>
<td>M</td>
<td>25</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
</tbody>
</table>

N/A = not available
Table 2
Clinical and histopathologic characteristics of patients in mutational and expression analyses

<table>
<thead>
<tr>
<th></th>
<th>Mutational</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluable for analysis</td>
<td>283</td>
<td>151</td>
</tr>
<tr>
<td>Alive at last follow-up</td>
<td>34 (12%)</td>
<td>6 (4%)</td>
</tr>
<tr>
<td>Follow-up from surgery, months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>45 (2 – 106)</td>
<td>59 (4 – 99)</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>61 (17 – 84)</td>
<td>59 (17 – 75)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>195 (69%)</td>
<td>85 (56%)</td>
</tr>
<tr>
<td>Female</td>
<td>88 (31%)</td>
<td>66 (44%)</td>
</tr>
<tr>
<td>Histologic subtype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelioid</td>
<td>205 (72%)</td>
<td>129 (85%)</td>
</tr>
<tr>
<td>Biphasic</td>
<td>52 (18%)</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>Sarcomatoid</td>
<td>25 (9%)</td>
<td>19 (13%)</td>
</tr>
<tr>
<td>Desmoplastic</td>
<td>1 (&lt;1%)</td>
<td>0</td>
</tr>
<tr>
<td>Asbestos body counts per gram wet lung tissue*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 fibers</td>
<td>67 (45%)</td>
<td>31 (37%)</td>
</tr>
<tr>
<td>&gt; 50 fibers</td>
<td>81 (55%)</td>
<td>53 (63%)</td>
</tr>
</tbody>
</table>

* Asbestos data missing for 135 patients in mutational analysis and for 67 patients in expression analysis
Table 3
Protein alteration and clinical information of the cases carrying *BAP1* and *TP53* germline mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein alteration</th>
<th>Functional Classification</th>
<th>Gender</th>
<th>Other cancer (self)</th>
<th>Other cancer in the family</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP1</td>
<td>W196*</td>
<td></td>
<td>Male</td>
<td>no</td>
<td>No</td>
</tr>
<tr>
<td>BAP1</td>
<td>W202C</td>
<td>Probably damaging (score 1)*</td>
<td>Male</td>
<td>no</td>
<td>Nasal (father), Breast (sister)</td>
</tr>
<tr>
<td>BAP1</td>
<td>V171*</td>
<td></td>
<td>Male</td>
<td>no</td>
<td>Renal and gastric (mother), lung (grandfather)</td>
</tr>
<tr>
<td>BAP1</td>
<td>G41S</td>
<td>Benign (score 0.078)*</td>
<td>Male</td>
<td>no</td>
<td>Lang (father)</td>
</tr>
<tr>
<td>BAP1</td>
<td>Splice site acceptor</td>
<td></td>
<td>Female</td>
<td>no</td>
<td>Prostate (father), Meningioma (mother), Bladder (sister)</td>
</tr>
<tr>
<td>TP53</td>
<td>R248W</td>
<td>Deleterious ++</td>
<td>Female</td>
<td>no</td>
<td>Prostate (father), Rhabdomyosarcoma (grandmother)</td>
</tr>
<tr>
<td>TP53</td>
<td>D49N</td>
<td>Neutral ++</td>
<td>Male</td>
<td>Skin Cancer</td>
<td>No</td>
</tr>
<tr>
<td>TP53</td>
<td>C176F</td>
<td>Deleterious ++</td>
<td>Female</td>
<td>Myelodysplasia, Acute myeloid leukemia</td>
<td>No</td>
</tr>
<tr>
<td>TP53</td>
<td>A86T</td>
<td>Neutral ++</td>
<td>Male</td>
<td>no</td>
<td>Unknown (two sisters and grandparents)</td>
</tr>
</tbody>
</table>

*Polyphen (http://genetics.bwh.harvard.edu/pph2/)

++ http://p53.iarc.fr/