Targeted Next-Generation Sequencing of Cancer Genes in Advanced Stage Malignant Pleural Mesothelioma

A Retrospective Study

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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.

(*J Thorac Oncol.* 2015;10: 492–499)

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*).[6–8] *BAP1* germline gene mutations have been identified and associated with a cancer syndrome that includes MPM, ocular or cutaneous melanoma, and other cancers.[9–11]

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

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Disclosure: Dr. Scagliotti has received honoraria from Roche, Astra Zeneca, Eli Lilly, Pfizer and Clovis Oncology, He participated in speakers' bureau for Eli Lilly and had travel accommodations by Bayer. Dr. Papotti has received honoraria from Clovis Oncology, Boehringer Ingelheim, Pfizer and Novartis. Dr. Novello has received honoraria from Roche, Astra Zeneca, Boehringer ingelheim, Eli Lilly, MSD. Dr. Volante has received honoraria and research funding by Novartis Pharma. All other authors declare no conflict of interest.

This study was partially supported by grants from the Istituto Superiore di Sanità, Rome and HEALTH-F2-2010-258677-CURELUNG from the European Community's Seventh Framework Program (FP7/2007–2013).

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DOI: 10.1097/JTO.0000000000000436

ISSN: 1556-0864/15/1003-0492

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 left-over 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/).

