Genome-based Mutational Analysis by Next Generation Sequencing in Patients with Malignant Pleural and Peritoneal Mesothelioma

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Abstract. *Background/Aim: Malignant mesothelioma is a rare malignancy with limited therapeutic options. Exome-based next-generation sequencing (NGS) techniques may direct the future of molecular targeting and improve systemic therapies for patients with mesothelioma. Materials and Methods: Eleven patients with NGS testing were selected, with a total of 236 somatic cancer-related mutations analyzed. Descriptive and Kaplan-Meier statistics were applied. Results: The median age was 65 years (range=27-73 years); 4 (36%) patients were females. Seven (64%) and four patients (36%) had pleural and peritoneal mesothelioma, respectively. Detectable mutations were found in 86% of the pleural and 50% of the peritoneal mesothelioma patients (overall, 73% of patients). The families of BAP1 (36%), CDKNA2A/B (27%) and NF2 (27%) represented the most frequently mutated genes. The median overall survival for all patients was 20.8 months, with 1- and 2-year survival rates of 91% and 40%, respectively. Conclusion: Genomic alterations as potential therapeutic targets were found by NGS. These findings will help in the development of new screening tools and targeting therapies,*

and in turn impact the standard-of-care and potentially lengthen disease control and survival periods in the future. Malignant mesothelioma is a rare and aggressive malignancy primarily arising from the pleural or peritoneal cavity linings (1). Asbestos exposure is a significant risk factor in the development of mesothelioma, and a history of asbestos exposure can be found in more than 80% of mesothelioma patients (2). There are also other known risk factors, such as genetic predisposition, Simian Virus 40 infection, radiation therapy, and erionite exposure (3-6). The mesothelioma incidence has greatly increased starting from the 1970's, when the past asbestos exposure effects became evident; still, no signs of decline in the United States has been observed, despite the fact that asbestos use has been banned for a number of decades now (7).

By average, the exposure to diagnosis time is approximately or longer than 40 years, which explains why incidence is still rising in many countries although asbestos ban has already taken place (8). Because of the long latency period, morbidity is expected to peak within the next two decades in industrialized countries such as the United States and the European Union countries (9).

Based on the WHO (World Health Organization) mortality database between 1994-2008, in total, 92,253 mesothelioma deaths were reported in 83 countries and the age-adjusted and crude mortality rates were 6.2 and 4.9 per million population, respectively, representing an age-adjusted mortality rate increase by 5.4% per year (10). Since mesothelioma patients are often diagnosed at locally advanced and metastatic stages and they do not respond well to conventional therapies, their prognosis is very limited (11); the median overall survival ranges from 4 to 13 months for untreated patients, and 6 to 18 months for the treated ones (12, 13).

Overall, the therapeutic strategy for mesothelioma is lagging behind its recent advances in molecular biology (14). Trimodality therapy including radical surgery with extrapleural

pneumonectomy, neoadjuvant chemotherapy and adjuvant hemithoracic radiotherapy has been associated with encouraging long-term disease controls mainly in phase II settings (14); new therapeutic approaches are urgently needed (15). To date most clinical trials have focused on cytotoxic agents rather than targeted therapies (16); just as identifying somatic mutations in other malignancies has led to development of effective disease-specific therapeutics, the potential identification of similar targetable mutations in mesothelioma is attractive (17). According to the COSMIC database, the most frequently mutated genes in mesothelioma include cyclin-dependent kinase inhibitor 2A (*CDKN2A*), neurofibromatosis type 2 (*NF2*), and BRCA1-associated protein-1 (*BAP-1*) (18-20).

Next-generation DNA sequencing (NGS) has the potential of replacing traditional technologies in diagnosing and evaluating genetic and oncologic disorders and its basic principles, impact and various applications have previously been reviewed (21-24). The expanding application of NGS offers the opportunity to accurately map-out the type and extent of genetic variations in mesothelioma and provide correlation with morphological and prognostic parameters of therapeutic relevance and importance (25). Since exome-based NGS techniques may impact on the management of molecular targeting and systemic therapies in this group of patients, we aimed to retrospectively review our institutional experience with NGS in clinical practice. The molecular profiles exhibited in malignant mesothelioma patients are reported.

Materials and Methods

Patient selection and treatment. The study was approved by the Institutional Review Board at the Mayo Clinic. Eleven consecutive patients with prior completion of NGS for mesothelioma were selected for this study; the patients must have follow-up information after NGS query, and with available clinical notes for outcome analyses. These patients were diagnosed between June 2011 and October 2013. A detailed retrospective medical record review was completed in August 2014 for the 11 patients.

Mutational analyses and detection. Previously, these patients all had their entire tissue samples tested by NGS. To do so, archival FFPE slides were first obtained, and cut into a number of small sections. DNA mutations were then tested using a standard platform provided by Illumina HiSeq2000 (Foundation Medicine, Cambridge, MA, USA). The technique can reliably test 236 oncologic genes which included a large number of exons (over 3,000) and 47 introns which were often rearranged or changed in human cancer lines. It is well known that NGS technology is capable of genotyping individual base pairs. The Foundation Medicine performed a proprietary data analysis that included mapping to a reference human genome and also comparing the entire genomic DNA landscape in a controlled fashion (26).

Statistical methods. Descriptive statistics were used to summarize patient and tumor characteristics. We reported median, range, number and/or frequency, with or without percentages. $p \leq 0.05$ was considered statistically significant. Survival since diagnosis was calculated using the standard log-rank Kaplan–Meier method. SPSS version 20.0 for Windows users was used for statistical analysis.

Results

Clinical and tumor characteristics. Selected patient and tumor characteristics are shown in Table I. The median age was 65 years, with a range of 27 to 73 years. Seven (64%) patients were male, and four (36%) were female. Ten patients were Caucasian, and one being African American. Seven patients had a family history of cancer in a parent or sibling and four had more than one first-degree relative with a malignancy. Two patients were previously diagnosed with lymphoma, one with Hodgkin's and another one with Non-Hodgkin's lymphoma. One of them had radiation treatment to the neck region 13 years prior to the diagnosis of mesothelioma. Three patients had limited asbestos exposure in the past. Nine patients were current or past smokers. Of the 11 patients, seven (64%) and four (36%) had pleural and peritoneal mesothelioma, respectively. Nine patients had epithelioid subtype, and 2 had the biphasic subtype.

Shortness of breath (57%) was the most common presenting symptom for patients with pleural mesothelioma, and abdominal discomfort or pain (50%) for the peritoneal mesothelioma patients. Five (46%) patients presented with metastatic disease at diagnosis. For the pleural mesothelioma patients, one had resection, four had insertion of pleural catheter for symptomatic needs (PleurX®, Denver Biomedical, Inc., part of Cardinal Health, Inc.; Golden, CO) and one had talc pleurodesis. Three patients with peritoneal mesothelioma had surgery. Three pleural mesothelioma patients participated in a phase 1 study with the use of oncolytic measles virus. One of the pleural mesothelioma patients received a total of 6 weeks of consolidative radiation therapy. A patient with peritoneal mesothelioma received stereotactic body radiation therapy for lung metastases. One patient was observed after surgery and radiotherapy, and nine patients received platinum-based chemotherapy along with pemetrexed.

Genomic alterations. At least one genomic alteration was identified in 8 of 11 patients (73%), with a mean average of 1.9 mutations per patient (range=0-5 mutations). Detectable mutations were found in 86% of the pleural and 50% of the peritoneal mesothelioma patients (Figure 1).

The families of *BAP1* (36%, p53/DNA repair pathway), *CDKNA2A/B* (27%, cell cycling pathway) and *NF2* (27%, phosphatidylinositol 3-kinase-AKT pathway) represented the most frequently mutated genes (Figure 2). *CDKNA2A/B* mutations were detected only in pleural mesothelioma patients. One or more variants of genetic significance, be it unknown or additionally, were also detected in 10 (91%) patients' tumors.

Table I. *Patient and disease characteristics, along with their associated NGS-derived mutations.*

| Case	Histology	Origin	Age at diagnosis (years)/ gender	Stage at diagnosis	All detected genomic alterations	Variants of unknown or additional significance	Patient status
1	Malignant Mesothelioma, Epithelioid subtype	Pleural	69/M	IB	*CDKN2A/B (loss)*	*RET (V262A); SF3B1 (R625G); TSC1 (H732Y)*	Alive
2	Malignant Mesothelioma, Epithelioid subtype	Pleural	65/M	II	*BAP1(N142fs*12); SF3B1(Y623C)*	*ASXL1 (E727K); FLT4 (G866S); GNAS (A237D); MAP3K1 (S939C)*	Alive
3	Malignant Mesothelioma, Biphasic subtype	Pleural	70/M	III	*CDKN2A/B (loss)*	None	Deceased
4	Malignant Mesothelioma, Epithelioid subtype	Pleural	63/F	III	No reportable genomic alterations were detected	*BCL6 (R459C); EP300 (A1412_H1216del); FLT3 (S963L); GNAS (G406G); TSC2 (L1190M); WT1 (G321A)*	Deceased
5	Malignant Mesothelioma, Biphasic subtype	Pleural	73/M	IV	*NF2 (E427); PTCH1 (deletion, exons 6-14); BAP1 (S460); MYD88 (L220P); SETD2 (E1756fs*33)*	*BLM (T1015I); FAT3 (R3408W); GRIN2A (T1064A); NOTCH3 (R1014H); PARP2 (R370C)*	Deceased
6	Malignant Mesothelioma, Epithelioid subtype	Pleural	27/M	IV	*MAP2K1 (E203K); NF2 (V24fs*25); TP53 (S314F)*	*BRIP1 (R865Q); CDK12 (S569del); CDKN2A (R58R); FANCD2 (I450V); FGFR3 (K517R); MLL2 (P3448S); WT1 (A100G); ZNF217 (A631S)*	Deceased
7	Malignant Mesothelioma, Epithelioid subtype	Pleural	73/F	II	*STK11 (T24fs*138); BAP1 (splice site 659+1G>T); CDKN2A/B (loss)*	*CHEK2 (L375F); ESR1 (S118P); FGF3 (A113T); FGFR1 (M456I); RPTOR (A765T)*	Alive
8	Malignant Mesothelioma, Epithelioid subtype	Peritoneal	64/F	IV	No reportable genomic alterations were detected	*AR (G457_G469del); JAK1 (F482Y); KEAP1 (Q619del); MET (V378I)*	Alive
9	Malignant Mesothelioma, Epithelioid subtype	Peritoneal	61/M	IV	No reportable genomic alterations were detected	*CDK12 (S323L); EPHB1 (R485K); ESR1 (Y73C); FGF6 (A2V);* KDR (E78G)	Alive
10	Malignant Mesothelioma, Epithelioid subtype	Peritoneal	29/M	IV	*NF2 (splice site 1341-6_1347del13)*	*IRS2 (P1033del); JAK1 (P144A); MRE11A (L675_ N678>K); MTOR (A1828_ A1831del); NSD1 (R632Q); SETD2 (D1321N, E1478K); TSC1 (R284H)*	Alive
11	Malignant Mesothelioma, Epithelioid subtype	Peritoneal	71/F	IV	*BAP1(loss ; KDM6A (L338fs*26); ASXL1 (L764fs*8); BRIP1 (K998fs*5); SETD2 (D653fs*5)*	*DAXX (G606V); MLL (S1325N)*	Alive
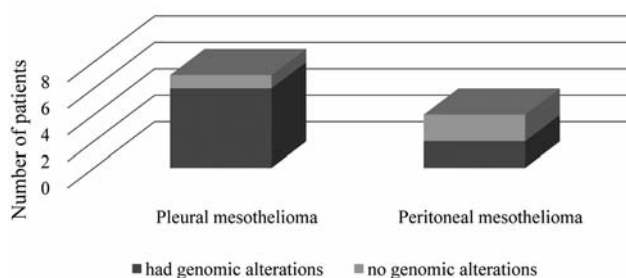
Figure 1. *Detectable number of mutations in patients with pleural and peritoneal mesothelioma.*

Although there are no FDA-approved therapies specifically for the reported genomic alterations in these patients with mesothelioma, mutations identified in four (36%) patients did have existing FDA-approved clinical trials currently open for another type of malignancy or histology. One patient enrolled in a relevant experimental therapy (using vorinostat as an off-label use); his tumor had *NF2, BAP1, PTC, MYD 88* and *SETD2* mutations; he survived for an additional of 14 months after the initiation of therapy.

Survival. Median follow-up time was 19.8 months (range=3.7-29.6 months). At the time of analysis, four patients (36%) had died. Three died due to disease progression, and the last death was related to hematological toxicity from myelosuppression. The median overall survival for this group of 11 patients was 20.8 months (95%CI=15.7-25.9 months), with 1 and 2-year survival rates of 91% and 40%, respectively. The Kaplan-Meier survival curve for the cohort of 11 patients is shown in Figure 3. There was no statistical difference in overall survival in patients with or without a genomic alteration (16.4 *vs.* 20.8 months, *p*>0.05, Figure 4).

Discussion

In this study, we retrospectively reported a detailed analysis of a comprehensively genomic-based, tumor-related genetic profile of mutations for a small group of 11 consecutive patients with malignant mesothelioma, a rare but very aggressive tumor of the body pleura. Through identifying the commonly-based genetic alterations in malignant mesothelioma from the basis, the goal remains to develop new and rational targeted therapies in the future. The scientific community should also focus on identifying key genetic alterations that lead to development of mesothelioma, and factors (with or without asbestos) that may predispose to it (27).

BAP1 is an important tumor suppressor gene that has been implicated in malignant mesothelioma; it has been shown that somatic inactivating mutations in *BAP1* could be found in

23% of mesotheliomas (28). *BAP1* exhibits tumor suppressor activity by binding to the RING finger domain of *BRCA1* and catalyzes the removal of ubiquitin chains from ubiquitinated proteins (29). BAP1 gene alterations including bi-allelic variations have been found in mesothelioma tumors before (30). In a relatively large retrospective study of NGS for genetic characterization of malignant pleural mesothelioma by Lo Iacono *et al.* two major clusters of gene mutational pathways were found: the p53/DNA repair (*BAP1, TP53* and *SMACB1*) pathway, and the phosphatidylinositol 3 kinase AKT (*NF2, STK11, KIT, KDR, HRAS* and *PIK3CA*); their results for the *BAP1* and *NF2*-led pathway discovery certainly agreed with our results (31).

Peritoneal mesothelioma is a rare form of mesothelioma that constitutes about 10% of the new diagnoses, however, less is known on its genetic variation and pathways (32). Alakus *et al.* analyzed the somatic mutational landscape of 12 peritoneal mesothelioma patients and found that *BAP1* was the most frequently inactivated gene; however, contrary to our results they found a lack of alterations in *NF2* and *CDKNA2* (32). In the study by Sheffield *et al.* from two patients with peritoneal mesothelioma, their derived whole genome sequences, RNA expression profiles, and targeted deep sequencing data also identified mutations in known mesothelioma-related genes such as *NF2, CDKN2A, LATS2* (33). We did note that the *BAP1* was the most frequently mutated gene that was found in our patients (36%), in one of the peritoneal mesothelioma patients the gene was mutated.

CDKNA2A gene is one of the most frequently inactivated tumor suppressor gene in mesothelioma and deletion of *CDKN2A* is a negative prognostic factor (34). *CDKNA2A* gene is located on chromosome 9p21.3 and since the targeted region is often large, other genes located in the same gene cluster such as *CDKNA2B* can also get co-deleted; this is thought to be responsible for the development of more malignant mesothelioma phenotypically (35). The high prevalence of deletion in *CDKN2A* gene makes it an interesting target for gene therapy, and *CDKN2A* deletion in mesothelioma has been proposed before as a diagnostic marker for distinguishing reactive versus neoplastic mesothelial cells in pleural effusion (36). The three patients of ours with *CDKNA2A/B* loss all had pleural mesothelioma. On the other hand, Alakus *et al.* did not observe *CDKNA2A* alterations in any of the 12 peritoneal mesothelioma patients (32). The *CDKN2A/B* mutation may play an important role in the pathogenic difference between peritoneal and pleural mesotheliomas. To link the two of our commonly found mutation in this study, it was also previously studied that *BAP1* inactivation in malignant mesothelioma might not require homozygous loss of *CDKN2A* (37).

NF2 gene plays an important role in the development of familial and spontaneous tumors of neuroectodermal origin,
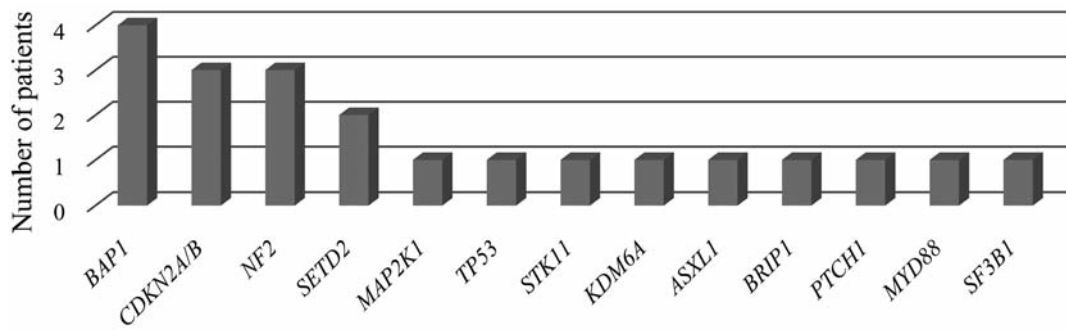
Figure 2. *Number of patients with the affected genetic mutations found in mesothelioma.*
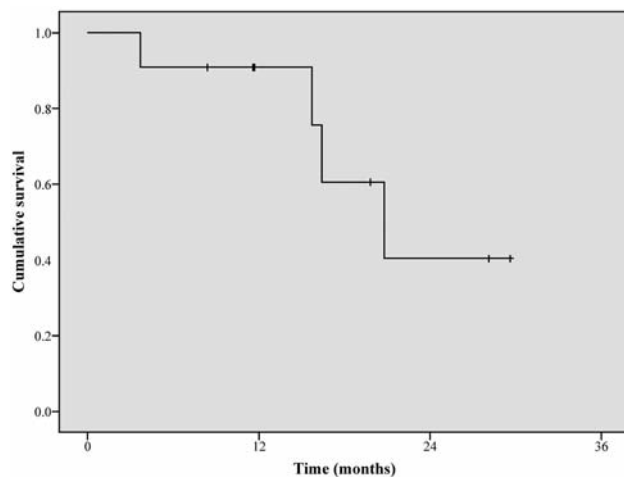

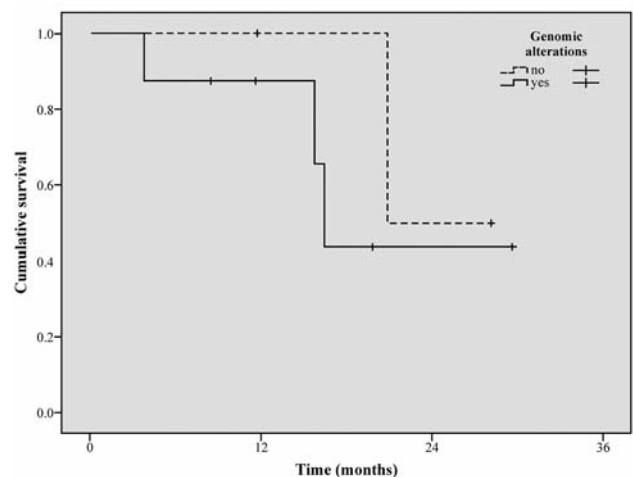Figure 3. *Overall survival in 11 patients with mesothelioma.*

Figure 4. *Overall survival in 11 patients with mesothelioma, with or without a detectable genomic alteration (p>0.05).*

which encodes a domain for the Merlin protein and in turn suppresses tumorigenesis on chromosome 22q (38). A study has found that 38% of malignant pleural mesothelioma samples displayed *NF2* mutation and 29% with deletions, while no *NF2* mutation was found in non-small cell lung cancer patients (39). Inactivated mutations in the *NF2* gene have been reported in 35-40% of malignant pleural mesothelioma patients which was originally implicated in the pathogenesis of familial neurofibromatosis (40).

Other genomic alterations noted in our study included *SF3B1, PTCH1, MYD88, SETD2, MAP2K1, TP53, STK11, KDM6A, ASXL1*, and *BRIP1*. These less studied genetic alterations may suggest potentially new therapeutic targets or diagnostic markers in addition to the most frequently known genetic mutations in *NF2, CDKN2A*, and *BAP1* that were found in our cohort of patients with mesothelioma. Our data agreed with the COSMIC database (http://cancer.sanger.ac.uk, 41).

The median overall survival of locally advanced or metastatic disease without treatment is 4-13 months but, during recent years, some improvement (albeit still very limited) in survival has been achieved due to improvement with better palliative care, systemic chemotherapy, surgery and improved diagnostics methods (42). For the treatment of mesothelioma, the development of systemic therapies is currently plateaued with pemetrexed that was first approved in 2002 (43, 44). Patients with mesothelioma appear to have optimal outcomes with multimodal therapy, adjuvant radiation therapy after extrapleural pneumonectomy has been shown to decrease locoregional recurrence to 13% (45, 46). In the last decade, emerging molecular targeted therapies changed the landscape of non-small cell lung cancer treatment and shifted to the focus to personalized medicine (47). In contrast to lung cancer, oncogenic driver mutations are much less known in the malignant mesothelioma. It is, therefore, necessary to identify

the signaling pathways that drive malignant mesothelioma and develop new therapeutics which specifically involve targeting the molecules (48). The development of targeted therapy hinges on the exploration of pathways that are related to either the loss of tumor suppressor genes or other known proto-oncogene targets (8). A number of malignant pleural mesothelioma molecular therapies have been tried as monotherapies or in combination with other modalities, mainly chemotherapeutic agents but with limited success (9). Although there were no FDA-approved therapies specifically for the reported genomic alterations in our patients, mutations identified in 4 (36%) patients did have existing FDA-approved clinical trials currently open for another type of malignancy or histology; one patient who had *NF2, BAP1, PTC, MYD 88* and *SETD2* mutations enrolled in a relevant experimental therapy (vorinostat, off-label), and survived for additional 14 months. Vorinostat is a histone deacetylase inhibitor, and the possible involvement of *BAP1* in modulating histone modifications and counteracting the expression profile of *BAP1*-deficient uveal melanomas has been reported previously (49). In a randomized trial by Krug *et al*. comparing vorinostat, a histone deacetylase inhibitor, with placebo, given as a second-line or third-line therapy did not improve overall survival and reported that it cannot be generally recommended as a therapy for patients with advanced malignant pleural mesothelioma (50); however, patient selection factors based on known genetic mutations should be considered for individualized therapy.

Asbestos exposure is a significant risk factor in the development of mesothelioma, and a history of asbestos exposure can be found in more than 80% of mesothelioma patients (2). In the study by Dogan *et al*. pedigree and mineralogical studies indicated that the malignant mesothelioma epidemic was caused by erionite exposure in genetically predisposed individuals and that was the first time that genetics was shown to influence mineral fiber carcinogenesis (3).

One or more variants of unknown or additional genetic significance were also detected in 91% of our patients. NGS is becoming more commonly used clinically particularly for malignant neoplasms, however, most mutations identified by NGS are still categorized as variants of unknown clinical significance (51). Hundreds of loss-of-function variants and thousands of variants of unknown significance in each person's genome are recognized. Prioritizing these variants remains a significant challenge (52). On the contrary, we also noticed that 3 of our 11 patients carried no detectable mutations by next-generation sequencing at all; coincidentally, at the time of analysis, 2 of these patients were alive with stable disease. It is possible that additional genetic aberrations can cause further genomic instability, which in turn is a poor prognostic marker independent of the specific mutations the tumor may harbor in the oncologic process.

A number of limitations existed in our study; first, our sample size was small in this retrospective study. As we aimed to identify more novel mutations that may guide future research directions, clearly, a larger patient sample size will be helpful. Combining patients who are enrolled in clinical trials should be considered in the future. Clinical centers from around the world should collaborate for the generation of meaningful clinical data, including molecularly, for this rare cancer, and establishing an international collaboration for tissue banking and registry will be valuable. At the time of this study, NGS was an expensive technology and not every patient can afford the testing cost; now, it has become more affordable clinically but is still not commonly covered by medical insurances (53). Further study focusing on defining molecular profiles in mesothelioma is needed to allow identification of subgroups of patients who may derive the most benefit from aggressive multimodality treatment, as not all detectable mutations can be targeted.

Conclusion

In our study, we found tumor-related mutations in 73% of our patients afflicted by mesothelioma. There were detectable mutations in 86% of the pleural and 50% of the peritoneal mesothelioma patients, respectively; the families of *BAP1, CDKNA2A/B* and *NF2* were the most frequently mutated genes. Using NGS technology, genetic mutations with therapeutic potential for future drug development were found in a majority of our patients with pleural and peritoneal mesotheliomas. The detection of mutations for which specific therapies are readily available may provide valuable clinical trial options for selected patients in the future. Additionally, NGS-mediated identification of novel mutations can be applied to the development of new screening tools and systemically or molecularly targeted therapies, which may have therapeutic and prognostic impact in the research and oncologic management of malignant mesothelioma in the future for which the current therapeutic options are unfortunately very limited.

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