Chapter 12
Recent Advances in the Genomic and Proteomic Researches on Mesothelioma: What Are Novel Insights into Mesothelioma Biology?

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Abstract Malignant mesothelioma is an aggressive tumor that has been associated with exposure to asbestos fibers. The discovery that germline heterozygous mutations of the gene encoding the deubiquitylase BRCA-associated protein 1 (BAP1) leads to inheritable higher susceptibility to mesothelioma underscores the relevance of gene x environment (GxE) interactions. Carriers of *BAP1* germline mutations are affected by the BAP1 cancer syndrome, a high penetrance Mendelian disorder, characterized by earlier development of mesothelioma and specific types of other cancers. Numerous next-generation sequencing (NGS) analyses have been recently conducted searching for both germline and somatic alterations in patients affected by mesothelioma and associated cancers, and their relatives. *BAP1* resulted in the more frequently germline mutated gene; however, other genes involved in DNA repair and homologous recombination were also identified. The pattern of chromothripsis, or chromosome staggering, which has been somatically identified in mesothelioma by several groups, may explain the frequent occurrence of noncontiguous biallelic genome alterations. Moreover, transcriptome studies in mesothelioma showed also the occurrence of fusion transcripts involving tumor suppressor genes. The complete knowledge of the genetic background associated with the GxE interactions involved in the pathogenesis of mesothelioma will be further improved by future genetic and genomic studies, allowing to develop better strategies for the prevention and treatment of this malignancy.

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T. Nakano, T. Kijima (eds.), *Malignant Pleural Mesothelioma*, Respiratory Disease Series: Diagnostic Tools and Disease Managements,
https://doi.org/10.1007/978-981-15-9158-7_12

Keywords Mesothelioma · *BAP1* · NGS · GxE interaction · Chromothripsis

1 Introduction

Malignant mesothelioma is an aggressive tumor whose pathogenesis is associated closely with occupational exposure to asbestos. The populations of workers handling asbestos, such as miners, manufacturing, or shipyard workers displayed a higher incidence of mesothelioma than the general population [1, 2].

The latency period between the exposure to mineral fibers to the development of asbestos-associated pleural mesothelioma is on average of 30–60 years [3]. Therefore, the incidence of mesothelioma is still increasing despite the legal bans on the use of asbestos in the Western countries at the end of the last century [4]. The majority of emerging countries are still using asbestos in their manufacturing activities, thus mesothelioma incidence in these counties is expected to keep increasing in the future [5].

Asbestos refers to a family of six mineral fibers that were used commercially until the 1970s and 1980s, which are classified into two subgroups: the amphiboles, a group of rod-like fibers including amosite, or brown asbestos, crocidolite or blue asbestos, anthophyllite, actinolite, and tremolite; and the serpentine group, consisting of chrysotile or white asbestos [6]. Exposure to the naturally occurring asbestos-like mineral fibers, such as erionite, antigorite, and others, as well as irradiation, account for further environmental risk factors for mesothelioma.

It has been observed that human mesothelial cells are particularly susceptible to cytotoxicity induced by asbestos, of which major mechanism of cell death appears to be in the form of necrosis rather than apoptosis. Then, a large amount of high mobility group box 1 (HMGB1) protein that belongs to the damage-associated molecular protein (DAMP) family, gets released by mesothelial cells, recruiting macrophages to sustain chronic inflammation [7]. Owing to the prolonged chronic inflammation microenvironment, surviving mesothelial cells accumulate genetic alterations after prolonged asbestos exposure. The accumulation of such genetic alterations might cause those mesothelial cells to develop mesothelioma after long latency [8]. However, the observation that among the workers with a long history of exposure to asbestos, only ~5% developed mesothelioma led to speculate that genetic component may also confer addition to occupational and environmental risks [5].

2 Germline Mutations of the *BAP1* Gene

About 20 years ago, Michele Carbone discovered apparent autosomal dominant transmission of mesothelioma susceptibility in some Turkish families, who have resided and have been exposed to erionite in the soil for a long time [9, 10].

Furthermore, Carbone and coworkers discovered germline mutations in the gene encoding the BRCA1-associated Protein 1 (BAP1), located in chromosome 3p21.3, in families with a high incidence of both pleural and peritoneal mesothelioma as well as uveal melanomas (UVMs), cutaneous melanoma, and clear cell renal carcinoma [11]. Subsequently, families of similar phenotypes with *BAP1* germline mutations have been reported in various ethnicities with an elevated risk of developing several other malignancies, such as cholangiocarcinoma, basal cell carcinoma, meningioma (reviewed in [12]). These findings established the concept of the "BAP1 cancer syndrome," as an autosomal familial cancer syndrome. An extended family with over nine generations inheriting mesothelioma, UVM, and other cancers since the 1700s established the inheritance mode of BAP1 cancer syndrome [11].

BAP1 encodes a nuclear ubiquitin carboxy-terminal hydrolase (UCH) functioning as a deubiquitinating enzyme. BAP1 is unique among UCH family members because of its long C-terminal tail, which contains two nuclear localization signals [13]. Both nuclear localization and deubiquitinating activity of BAP1 protein are postulated to be necessary for the maintenance of tumor suppressor activity [14]. BAP1 is implicated in the regulation of cell cycle, cellular differentiation, gluconeogenesis, chromatin remodeling, gene transcription, and DNA repair [12].

At the clinical level, the discovery of the BAP1 cancer syndrome emphasizes the necessity for genotyping the DNA of patients with mesothelioma for mutations, to determine the presence of germline mutations in the *BAP1* gene and other yet unidentified additional genes to acquire more complete information on the inherited predisposition to cancers like mesothelioma.

3 NGS Analysis in the Search for Germline Mutations in Other Genes

Several Next-Generation Sequencing (NGS) studies have been performed following the identification of *BAP1* in mesothelioma and other cancers to investigate germline variants individuals at risk of mesothelioma or in patients with this aggressive cancer [12]. Patients (n = 89) who developed pleural mesothelioma because of ascertained cumulative exposure to asbestos were screened for the presence of germline pathogenic truncating nonsense or frameshift variants (PTVs), by targeting 94 genes known for predisposition to cancer. *BAP1* germline PTVs were identified in four patients with mesothelioma, while germline PTVs were found also in *CDKN2A* or DNA repair genes. The asbestos exposure was significantly higher in patients with familial mesothelioma and PTVs in tumor suppressor genes than the patients with no germline variants in the 94 cancer-predisposing genes [15, 16].

A different approach, aimed at studying the inheritance of germline mutations of *BAP1* or other genes, was used to select a cohort of 79 individuals to be investigated. This population consisted of 52 unrelated probands with familial mesothelioma and their 27 first- and second-degree relatives, and was selected for

possible genetic predisposition, based on the following four criteria: (1) mesothelioma in first- or second-degree relatives; (2) diagnosis of cancers typical of *BAP1*$^{+/-}$carriers (uveal melanoma, cutaneous melanoma, clear-cell renal cell carcinoma) in the probands or at least one first- or second-degree relative; (3) family history of multiple cancers; and (4) early cancer onset less than 50 years old. *BAP1* Sanger sequencing and tNGS of more than other additional 50 cancer susceptibility genes were performed in this population. The results of this study showed that most of the patients were carriers of *BAP1*$^{+/-}$ with familial mesothelioma (43/79). Germline PTVs involving the following cancer susceptibility genes other than *BAP1* were also identified in this group: *ARID1A*, *ARID2*, *BAP1*, *CREBBP*, *KDR*, *MLH1*, *NCOR1*, *RAD50*, *RBM6*, *SETD2*, *SMARCA2*, *SMARCA4*, *SMARCE1*, *SMO*, *TP53*. Survival of 77 patients were compared with data from the mesothelioma in general, using dataset of the Surveillance, Epidemiology, and End Results (SEER) cohort (https://seer.cancer.gov), revealing a significant improvement of survival and earlier age at diagnosis (5 years and 54 years of age, respectively) in the selected population compared with the SEER cohort (8 months and aged 72 years, respectively). In the selected patients with familial mesothelioma and wild-type *BAP1*, survival was even more favorable (9 years) and diagnosis occurred earlier (45 years). These data point at the selected criteria as helpful in identifying patients and family members who are more susceptible to develop additional cancers [17].

Another study performed targeted NGS (tNGS) in 198 germline DNAs from patients with different types of mesothelioma, analyzing 85 cancer susceptibility genes. Germline mutations of *BAP1* other genes involved in homologous recombination (HR) and DNA repair were found in 12% of cases. Age, cancer diagnosis, and asbestos exposure were examined by multivariate analysis, revealing that young age and a second diagnosis of cancer were significantly associated with the occurrence of germline mutations in cancer susceptibility genes, for which minimal or no asbestos exposure turned out to be the most significant predictor [18].

The joint effort of two large centers of the National Cancer Institute (NCI) and the University of Chicago (UC) allowed studying the relationship of germline mutations in tumor suppressor or DNA repair genes with responsiveness to platinum-based chemotherapy in 385 patients with different types of mesothelioma. A multi-gene panel BROCA v10, containing 73 target genes associated with DNA repair and/or with inherited predisposition to develop solid cancers was used for genotyping. The analysis of the NCI/UC cohort identified at least a mutation in one of the targeted genes in 12% of patients. *BAP1* was the most altered gene (16 mutations), while the other 12 mutations involved the following genes: *CHEK2*, *PALB2*, *BRCA2*, *MLH1*, *POT1*, *TP53*, and *MRE11A*. In patients with pleural mesothelioma (not with peritoneal type) mutated *BAP1*, or a mutation in the other targeted genes, was significantly associated with improved overall survival (OS), compared with wild-type patients [19].

Interestingly, within a large exon tNGS study of 168 genes associated with hereditary cancer in a cohort of more than 600 patients with different cancers, the results obtained in 12 mesotheliomas revealed the highest frequency of pathogenic variants (7/12, 58%) in genes regulating HR DNA repair, with the genes of the

Table 12.1 NGS studies of germline mutations in patients with mesothelioma

Study	Target genes (*n*)	Adopted criteria	Mutated genes (no. of patients)	Total patients
(a)	Cancer-predisposing genes (94)	• Truncating variants • Asbestos exposure	*BAP1* (4), *ATM*, *BRCA1*[a], *BRCA2*, *CDKN2A*, *FANCC*, *FANCF*, *FANCI*[a], *PALB2*, *PMS1*, *SLX4*, *XPC* (1 each)	89
(b)	Cancer linked genes (56)	• Allele frequency • CADD[b] score > 20 • Family history of cancers • Early diagnosis	*BAP1* (43/79[c]), *MLH1* (3), *SMARCA2* (2), *ARID1A*, *ARID2*, *CREBBP*, *KDR*, *NCOR1*, *RAD50*, *RBM6*, *SETD2*, *SMARCA4*, *SMARCE1*, *SMO*, *TP53* (1 each)	45
(c)	Cancer-predisposing genes (85)	• Allele frequency • ACMG/AMP[d] guidelines	*BAP1* (6), *BRCA2* (3), *CHEK2* (3), *CDKN2A* (2), *ATM* (2), *BRCA1*, *MRE11A*, *TP53*, *MSH6*, *TMEM127*, *SDHA*, *VHL*, *WT1* (1 each)	198
(d)	DNA repair and/or cancer-predisposing genes (73)	• Protein damaging variants	*BAP1* (16), *CHECK2* (5), *PALB2* (2), *BRCA2*, *MLH1*, *POT1*, *TP53*, *MRE11A* (1 each 1)	239
(e)	Hereditary cancer genes (168)	• Allele frequency • ACMG/AMP[d] guidelines	*BAP1*, *BRCA2*, *FANCA*, *FANCC*, *FANCD2*, *FANCM*, *XPC* (each 1)	12

(a) Betti et al., Cancer Lett 405:38–45, 2017
(b) Pastorino et al., J Clin Oncol 36:3485–3494, 2018
(c) Panou et al., J Clin Oncol 36:2863–2871, 2018
(d) Hassan et al., Proc Natl Acad Sci U S A 116(18):9008–9013, 2019
(e) Bertelesen et al., NPJ Genom Med 4:13, 2019
[a]Occurring in the same patient
[b]Combined Annotation Dependent Depletion
[c]16 $BAP1^{+/-}$ patients +27 relatives
[d]American College of Medical Genetics/Association for Molecular Pathology

pathway of Fanconi anemia (*BRCA2* or *FANCD1*, *FANCA*, *FANCC*, *FANCD2*, and *FANCM*) particularly represented [20].

The results of all these studies (summarized in Table 12.1) clearly indicate that at least 10%–12% of mesothelioma cases were associated with germline mutations in *BAP1* or in other HR genes and displayed better prognosis and chemosensitivity than patients with wild-type genetic background.

4 Somatic Mutations of *BAP1*

Frequent somatic mutations in BAP1 have been observed in highly metastatic uveal melanomas, 26 of 31 (84%) metastasizing tumors [21]. The majority (63.6%) of sporadic mesotheliomas contain somatic *BAP1* mutations/inactivation [22]. These

findings confirmed our previous data on *BAP1* inactivation in epithelioid type mesothelioma accompanied by loss of heterozygosity (LOH) [23], and are supported by two NGS studies of the mesothelioma genome that revealed that *BAP1* was somatically mutated in 41% [24] and 58% [25] of mesotheliomas, respectively. Therefore, the *BAP1* gene undergoes biallelic inactivation in tumors, thus, meeting the criteria of classical two-hit inactivation theory for tumor suppressor genes.

5 Chromothripsis in Mesothelioma Genome

Frequent observation of loss of heterozygosity on 3p21 in malignant mesothelioma led us and others to focus on *BAP1* as a target gene of somatic inactivation. In 2011 a study found that *BAP1* was inactivated by somatic mutations in mesothelioma [26], while in metastatic clear cell renal carcinoma the minimal common deletion region at 3p21.1 contained *BAP1* and *PBRM1* at 3p21 [27]. The genomic pattern of peritoneal mesothelioma is similar to that of pleural mesothelioma [28]. We performed a comprehensive tumor genome analysis targeting the 3p21 region by performing high-density array comparative genomic hybridization (CGH; average probe interval: 254 bp) detecting multiple minute simultaneous biallelic deletions in this region, especially in *BAP1* (8/33, 24%), *SETD2* (7/33, 21%), *PBRM1* (3/33, 9%), and *SMARCC1* (2/33, 6%) [29]. Overall, 46 genes in this region were found to contain biallelic deletions in at least one biopsy specimen out of 33 mesothelioma specimens examined. Breakpoints of these genomic deletions were different in different cases. Many of these deletions were not contiguous but alternated with segments showing oscillating copy number changes along the 3p21 region. This may be because of chromothripsis (derived from the Greek word "chromos" for chromosome and "thripsis" for shattering into pieces) [30], a phenomenon characterized by numerous genomic rearrangements caused by a single catastrophic event in multiple cancer samples. The catastrophic genetic event known as chromothripsis consists of the fragmentation of a segregated single chromosome that is then rearranged leading to incorrect reassembling or loss of certain DNA sequences. Therefore, a single chromothripsis event may cause a high number of alterations in the genome after a short number of cell replications, leading to oncogenic activations or to loss of tumor suppressor functions, eventually favoring tumorigenesis [30].

Interestingly, noncontiguous biallelic genome alterations with the characteristic pattern of chromothripsis have been observed in mesothelioma [29], later confirmed by other groups [31], also with the potential consequence of neoantigen expression and tumor immunogenicity [31].

NGS alone hardly detects larger-sized DNA deletions (>30 bp). Conventional array CGH alone cannot detect smaller-sized deletions (<3000 bp). In other words, these analyses overlook genomic alteration in the size range of 30–3000 bp. Our comprehensive genome analysis combining high-density array CGH (average probe interval: 254 bp in the 3p21 region) and targeted NGS disclosed to or at higher frequencies than frequencies of sequence-level mutations [29]. Genomic alterations in

mesothelioma usually include genomic rearrangements that induce complex and multiple deletions. Digital MLPA, which analyzes the copy number of approximately 600 exons simultaneously by using NGS-based MLPA, shall become a reliable method for high-throughput detection of multiple segmental deletions in small amounts of DNA in mesothelioma specimens to complement NGS analysis.

6 LOH, CDKN2A, NF2

The chromosomal changes of malignant mesothelioma are complex and heterogeneous, and more losses than gains of genetic material are observed. Losses of chromosomes 1p, 3p, 4q, 6q, 9p, 13, 14q, and 22 were detected in the majority of the abnormal cases [32–34]. Homozygous deletion of 9p21.3 is most frequently detected for the genetic alteration of mesothelioma and occurs in more than 90% of established cell lines. Deletion region involves *CDKN2A*, *CDKN2B* (cyclin-dependent kinase inhibitor 2B), and often adjacent *MTAP* (methylthioadenosine phosphorylase) and *MIR31* genes. The *CDKN2A* gene generates at least three alternatively spliced variants encoding distinct proteins: p16INK4A, p16gamma, and p14ARF. These products encoded by this gene play an essential role in cell cycle and senescence regulation through two major tumor-suppressing pathways of retinoblastoma protein (RB) and p53 in the cell. Fluorescence in situ hybridization (FISH) of *CDKN2A* would be useful for the diagnosis of mesothelioma because this analysis could differentiate pleural mesothelioma cells from reactive mesothelial cells [35, 36]. Accumulating information shows that the homozygous deletion of *CDKN2A* is a predictor of poor survival [37].

The *NF2* (Neurofibromin 2) gene responsible for neurofibromatosis type 2 familial cancer syndrome was shown to be the target gene of 22q12 loss. This gene is inactivated by homozygous deletion or heterozygous deletion/point mutation in a total of 40–50% of mesotheliomas [38, 39]. NF2 protein acts upstream of SAV1, LATS1/2, and yes-associated protein (YAP) in the Hippo tumor suppressor pathway. In addition to *NF2* inactivation, deletions/mutations in *SAV1* and *LATS2* genes are found in mesothelioma [40]. Hippo tumor suppressor pathway plays a vital role in controlling proper organ sizes, cell contact inhibition, stem cell function, and regeneration. Studies with this pathway would hide the possibility of causing a new therapeutic strategy.

7 Fusion Transcripts, Altered Splicing, MicroRNA

Transcriptome analysis by next-generating sequencing (n = 211) showed fusion transcripts involving tumor suppressor genes in mesothelioma: 13 fusions in *NF2*, 7 in *BAP1*, 8 in *SETD2*, 7 in *PBRM1*, 2 in *PTEN,* and 6 in others [41]. The reports on fusion transcripts in mesothelioma have been accumulating [42, 43], but the gene

pairs of fusion and the braking-region of these transcripts were different among patients with mesothelioma. Then the detection of fusion transcripts has not yet to be exploited as a diagnostic tool. Many of these fusions and aberrant splicing variants are derived from the genes in chromosomes 3p21, 9p21.3, 13q12, and 22q12, frequently deleted regions in mesothelioma. These gene regions might be fragmented by chromothripsis and lead to extensive rearrangements causing fusion genes or aberrant splicing variants. In addition, the mutation of the *SF3B1* gene, encoding subunit 1 of the splicing factor 3b protein complex, was found at ~2% of frequency (4/216) [41] and the mutations in this splicing factor gene were associated with specific alterations in mRNA splicing.

Because mutations in the genes encoding proteins associated with histone modification and chromatin remodeling, including BAP1, SETD2, and PBRM1, occur predominantly in mesothelioma, diverse gene expression changes induced by aberrant epigenetic regulation are estimated. Most of the deregulated genes in mesothelioma belong to the following pathways: angiogenesis, cell adhesion, p53 signaling, integrin signaling, MAPK signaling, apoptosis, and cell cycle regulation [44]. A special set of genes could differentiate mesothelioma from others. The set of 26 genes could distinguish pleural mesothelioma from others, normal pleura, sarcomas, renal cell carcinoma, and thymoma, with high sensitivity and specificity [45]. It was also reported that two gene sets, one including 22 genes and the other 40 genes, narrowed down from 117 genes selected from previous reports could be discriminate malignant from benign pleural proliferations [44].

MicroRNAs (miRNAs) are short noncoding RNAs of approximately 18–22 nucleotides in length, which function as posttranscriptional regulators of gene expression. It is known that miRNA expression is dysregulated in human cancer through various mechanisms, including amplification or deletion of miRNA genes, abnormal transcriptional control of miRNAs, dysregulated epigenetic changes, and defects on biogenesis components. MiR-31 expression was shown to be reduced in mesotheliomas in most cases via deletion combined with the *CDKN2A* gene at 9p21.3. MiR-34b and miR-34c, sharing a common primary transcript, were silenced by methylation in the majority (85%) of mesothelioma tumors. The miR-15/16 family has also been shown to be significantly downregulated in mesothelioma compared with those from normal pleura. MiR-193a-3p and the miR-200 family showed a statistically significant down-expression in mesothelioma tumors compared to normal pleura. The miRNAs including let-7 and miR-21 have been reported several times from different groups. These findings are reviewed in [46]. MiRNA mimics are small, double-stranded RNA molecules, designed to mimic endogenous mature miRNA molecules when transfected into cells. In order to deliver miRNAs, the minicells, known as EDVTMnanocells (EDVs) derived from asymmetric bacterial cell division were used. The therapy, dubbed TargomiRs, comprises patented miRNA mimics based on the miR-15/107 consensus sequences, packaged in EDVs that are targeted with an anti-EGFR-specific antibody. The trial was designed to test TargomiRs in patients with pleural MM or advanced NSCLC (ClinicalTrials.gov Identifier: NCT02369198). The drug showed early signs of activity [47].

Comprehensive molecular profiling, including exome sequencing, copy-number arrays, mRNA sequencing, noncoding RNA profiling, DNA methylation, and

Table 12.2 Association between prognosis and the four distinct integrated subtypes of pleural MM by the multiplatform molecular profiling

iCluster	Enriched histology	Molecular profiling characteristics	Prognosis
1	Epithelioid	Low somatic copy-number alteration, low *CDKN2A* homozygous deletions, high DNA methylation, high *BAP1* alterations	Best
2	Epithelioid	Low *BAP1* alteration, low DNA methylation	
3	Biphasic	High *CDKN2A* homozygous deletion, low *CLDN1* expression	
4	Biphasic & Sarcomatoid	High *MSLN* promoter methylation, high *LATS2* mutations, high *CDKN2A* homozygous deletions, gene expression showing epithelial-mesenchymal transition (high mRNA expression of *VIM*, *PECAM1*, and *TGFB1*, and low miR-200 family expression)	Worst

reverse-phase protein arrays, identified four distinct integrated subtypes of mesothelioma [48]. The results of the study (summarized in Table 12.2) indicate that survival was significantly different across the 4 clusters ($P < 0.0001$) [48]. Cases in the poor-prognosis subset showed higher *AURKA* mRNA expression and upregulation of the PI3K and mTOR signaling pathways. This study showed a strong expression of the immune-checkpoint gene *VISTA* in epithelioid pleural mesothelioma. These new findings integrated into the biology of mesothelioma could lead to new therapeutic strategies.

8 Conclusions

Since the discovery of *BAP1* as a predisposition gene to mesothelioma and a number of other different cancers, grouped in the BAP1 cancer syndrome, numerous germline analyses were performed in patients with mesothelioma and in subjects individuals who have experienced environmental or occupational exposure to

carcinogenic fibers and are therefore at high risk of developing mesothelioma. The knowledge of the molecular mechanisms underlying the pathogenesis of malignant mesothelioma will benefit from the future results of further studies required to complete the information on the prevalence of germline and somatic variants present in cancer susceptibility genes.

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