Mutational Profiling of Driver Tumor Suppressor and Oncogenic Genes in Brazilian Malignant Pleural Mesotheliomas

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Keywords

Next-generation sequencing · Oncogenes · Tumor suppressor genes · Mutation · Malignant mesothelioma · Asbestos

Abstract

Background: Malignant pleural mesothelioma (MPM) is a highly lethal disease comprising a heterogeneous group of tumors with challenging to predict biological behavior. The diagnosis is complex, and the histologic classification includes 2 major subtypes of MPM: epithelioid (~60% of cases) and sarcomatous (~20%). Its identification depends upon pathological investigation supported by clinical and radiological evidence and more recently ancillary molecular testing. Treatment options are currently limited, with no known targeted therapies available. **Objectives:** To elucidate the mutation profile of driver tumor suppressor and oncogenic

genes in a cohort of Brazilian patients. **Methods:** We sequenced 16 driver genes in a series of 43 Brazilian malignant mesothelioma (MM) patients from 3 distinct Brazilian centers. Genomic DNA was extracted from formalin-fixed paraffin-embedded tumor tissue blocks, and the *TERT* promoter region was amplified by PCR followed by direct capillary sequencing. The Illumina TruSight Tumor 15 was used to evaluate 250 amplicons from 15 genes associated with solid tumors (*AKT1*, *GNA11*, *NRAS*, *BRAF*, *GNAQ*, *PDGFRA*, *EGFR*, *KIT*, *PIK3CA*, *ERBB2*, *KRAS*, *RET*, *FOXL2*, *MET*, and *TP53*). Library preparation with the TruSight Tumor 15 was performed before sequencing at the MiSeq platform. Data analysis was performed using Sophia DDM software. **Results:** Out of 43 MPM patients, 38 (88.4%) were epithelioid subtype and 5 (11.6%) were sarcomatoid histotype. Asbestos exposure was present in 15 (39.5%) patients with epithelioid MPM and 3 (60%) patients with sarcomatoid MPM. We found a *TERT* promoter mutation in 11.6% of MM, and the c.-146C>T mutation

was the most common event. The next-generation sequencing was successful in 33 cases. A total of 18 samples showed at least 1 pathogenic, with a median of 1.8 variants, ranging from 1 to 6. The most mutated genes were *TP53* and *ERBB2* with 7 variants each, followed by *NRAS*, *BRAF*, *PI3KCA*, *EGFR* and *PDGFRA* with 2 variants each. *KIT*, *AKT1*, and *FOXL2* genes exhibited 1 variant each. Interestingly, 2 variants observed in the *PDGFRA* gene are classic imatinib-sensitive therapy. **Conclusions:** We concluded that Brazilian MPM harbor mutation in classic tumor suppressor and oncogenic genes, which might help in the guidance of personalized treatment of MPM.

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Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor arising from mesothelial cells forming a lubricated and nonadhesive surface that cover and protect the lungs, abdomen, and heart [1–3]. MPM is a rare and a universally lethal cancer [4] in which the most common form arises in the pleura of the lung (80% of cases) [1, 2], and much more rarely in the peritoneum and tunica vaginalis [2]. Following the World Health Organization (WHO), the number of new MPM cases worldwide in 2018 was 30,443, and the number of deaths was 25,576 [5]. In Brazil, there is an impressive lack of studies and an underreporting of MPM cases, making it difficult to make public health decisions [6]. Pedra et al. [7] studied MPM mortality in Brazil, from 1980 to 2003, and found the death frequency increased from an average of 68.4 per year in the 1980s to 110 per year in the following decade, and 157 per year in 2000–2003.

MPM is a direct causal relationship between exposure to an environmental carcinogen, such as asbestos, and the transformation of mesothelial cells and the development of the tumor [2]. Asbestos is a generic name referring to a family of 6 mineral fibers, with high tensile strength and resistance to thermal and chemical degradation, very popular in the industry [1, 8]. Although the use of asbestos has already been prohibited in 54 countries worldwide, its extraction and use are still ongoing in many developing countries as Russia, China, Kazakhstan, and Brazil [2, 9]. One of the peculiarities of MPM is the long-term latency period between asbestos exposure and tumor development, ranging from about 25 to 40 years [10, 11].

Histologically, MPM are classified into 2 major types: epithelioid mesotheliomas, which constitute about 60%

of mesotheliomas and have the most extended survival (12–27 months) and sarcomatoid mesotheliomas, which constitute around 20% of mesotheliomas and are characterized by their spindle cell morphology and have the worst survival (7–18 months) [2, 12, 13].

For the last 15 years, first-line chemotherapy combines pemetrexed and cisplatin or pemetrexed and carboplatin [14]. The benefits are usually modest at best, and prognosis remains poor: the median survival is <1 year from the time of diagnosis [10, 11]. Recently, the major advance in MPM treatment is the identification of bevacizumab in addition to pemetrexed and cisplatin chemotherapy, probably by modifying the tumor microvasculature [2, 15].

Genetic and epigenetic alterations are observed in mesothelial cells. The most frequently altered tumor suppressor genes are BRCA-associated protein 1 (BAP-1), neurofibromatosis type 2 (NF-2), cyclin-dependent kinase inhibitor 2A (CDKN2A), large tumor suppressor kinase 2 (LATS2), and SET domain containing 2 (SETD2) [1, 16]. These alterations drive cell proliferation, resistance to apoptosis, and local immunosuppression, providing the rationale for some new targeted therapies nowadays [17, 18]. Approximately 65% of mesotheliomas harbor inactivation of the tumor suppressor *BAP1* and, although rare, germline mutation in *BAP1* confers a higher risk of mesothelioma development [19, 20]. While several prognostic factors have been proposed, only a few have been independently validated.

Recently, genome-wide somatic mutations of MPM were profiled using next-generation sequencing (NGS) methods, identifying genomic subtypes harboring mutations in *TP53*, *TERT*, and other driver genes [16, 21]. Nevertheless, useful predictive biomarkers for therapy are yet to be found, increasing the need to elucidate and deepen the complexities of MPM biology and heterogeneity. In the present study, we evaluated the mutation profile of driver genes that are frequently mutated in solid tumors, in order to correlate the mutation status with malignant mesothelioma (MM) patients' clinical-pathological features and to identify potential clinically actionable genetic alterations in Brazilian MM.

Materials and Methods

Patients

Information on 43 patients diagnosed with MPM between 2008 and 2018 at Barretos Cancer Hospital (Barretos, SP, Brazil), Cancer Institute of São Paulo (ICESP; São Paulo, SP, Brazil) and from the files of a large reference pathology laboratory located in

Table 1. Clinical characteristics of patients with malignant mesothelioma

Characteristic	Epithelioid (<i>n</i> = 38)	Sarcomatoid (<i>n</i> = 5)	<i>p</i> value
Median age, years	60	56	0.33
Sex			
Male	26 (68.4)	1 (20)	0.45
Female	12 (31.6)	4 (80)	
Asbestos exposure			
No	23 (60.5)	2 (40)	0.51
Yes	15 (39.5)	3 (60)	
Topography			
Pleural	38 (100)	5 (100)	
<i>TERT</i> status			
Mutation	5 (15.2)	0	0.94
Wild type	28 (84.8)	5 (100)	

Data are presented as *n* (%) unless otherwise indicated.

São Paulo (SP, Brazil) was collected through the Thoracic Surgery mesothelioma database and the Department of Pathology data file.

Pathologic diagnosis was based on standard histologic, histochemical, and immunohistochemical criteria [22]. As a positive marker of immunohistochemistry for MPM, we used calretinin, WT-1, cytokeratin 5/6, and D2-40. As negative markers for MPM, we used MOC31, BerEP4, and thyroid transcription factor-1. In cases where positive mesothelial markers were not yet available, negative markers were used for making the diagnosis of MPM. A review of pathological reports and confirmation by 2 experienced pathologists yielded 38 epithelioid and 5 sarcomatoid MPM. Variables recorded in the database included age, gender, and histologic types (Table 1).

Histologic Evaluation

Two pathologists reviewed all available hematoxylin and eosin-stained slides of MPM, which included a median of 9 slides per case. Histologic classification for epithelioid and sarcomatoid MPM was done according to the 2015 WHO classification [3] (Fig. 1).

DNA Isolation

Representative formalin-fixed paraffin-embedded tumor samples from 43 MPM patients were retrieved from the Pathology Department of Barretos Cancer Hospital. DNA from formalin-fixed paraffin-embedded tissues was retrieved from 10- μ m cuts, after careful macrodissection of the tumor area using a sterile needle and ensuring the presence of >50% of neoplastic cells. DNA was isolated using the QIAamp DNA Micro Kit (Qiagen, Germany) according to the manufacturer's instructions and as previously described by our group [23, 24]. The quality and concentration of DNA were measured in a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) or Qubit Fluorometric Quantitation (Thermo Fisher Scientific) followed by storage at -20 °C until molecular analysis.

Fig. 1. Hematoxylin and eosin-stained slides of MPM, with different histologic classification: epithelioid (a), sarcomatoid (b), biphasic with the epithelioid component (c), and biphasic showing a sarcomatoid component (d).

TERT Sanger Sequencing

A fragment of the *TERT* promoter region was amplified in all 43 samples by PCR using the primers 5'-AGTGGATTCGC-GGGCACAGA-3' and 5'-CAGCGCTGCCTGAAACTC-3', resulting in a PCR product of 235 bp, which contained the sites of the c.-124C>T and c.-146C>T mutations as previously described [25–27]. PCR was performed with an initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 90 s, elongation at 72 °C for 30 s, and final elongation at 72 °C for 7 min. The quality of PCR products was confirmed by gel electrophoresis. The sequencing of the PCR product was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI PRISM 3500 $\times$ L Genetic Analyzer (Applied Biosystems).

The sequencing reaction was performed in the forward direction. An independent PCR amplification/sequencing, in the forward direction, was performed in positive samples or samples that were inconclusive.

Targeted NGS

NGS-based mutation analysis was performed using the Illumina TruSight Tumor 15 on the MiSeq instrument according to the manufacturer's instructions (Illumina, USA). It provides a mutation profile of 15 therapy driver genes *AKT1*, *GNA11*, *NRAS*, *BRAF*, *GNAQ*, *PDGFRA*, *EGFR*, *KIT*, *PIK3CA*, *ERBB2*, *KRAS*, *RET*, *FOXL2*, *MET*, and *TP53*. The read alignment and variant calling were performed with BaseSpace BWA Enrichment version 2.1 (Illumina) and Sophia DDM[®] software version 4.2 (Sophia Genetics SA, Switzerland). Several steps were used to filter variants

Fig. 2. Electropherograms showing sequence of *TERT* promoter region with 2 hot-spot mutations c.-124C>T and c.-146C>T. **a** Heterozygous c.-124C>T *TERT* promoter mutation (arrow). **b** Heterozygous c.-146C>T *TERT* promoter mutation (arrow).

Table 2. *TERT* status and mutations according to histotype

<i>TERT</i> p status	Histotype	Mutated genes
c.-146 C>T	Epithelioid	No
c.-146 C>T	Epithelioid	Undetermined
c.-146 C>T	Epithelioid	<i>ERBB2</i> exon 29 (Pro1105Ser) <i>ERBB2</i> exon 21 (Gly674Arg) <i>PI3KCA</i> exon 21 (*Arg1023Gln) <i>TP53</i> exon 6 p. (Gly187Val)
c.-124 C>T	Epithelioid	<i>TP53</i> exon 6 p. (Leu194Arg)
c.-124 C>T	Epithelioid	<i>TP53</i> exon 8 p. (Glu294*)

identified in the screening. First, intronic variants and synonymous single nucleotide variants (SNVs) were excluded. Subsequently, polymorphisms were excluded using the frequency of 1% in both databases – 1000 Genomes Project and GO Exome Sequencing Project (ESP5400). Finally, this set was further filtered by excluding all variants showing a poor quality (read depth <500×), low variant allele frequency (variant frequency analysis <10%), and without clinical significance available in Sophia DDM® software version 4.2 (Sophia Genetics SA). In addition, 10 bp were considered in the initial and final portion of introns for variant analyses at the splice site of each exon.

Results

Cohort Description

The clinical characteristics of the patients in our MPM cohort are summarized in Table 1 by histologic type. Out of 43 MPM patients, 38 (88.4%) were epithelioid subtype

and 5 (11.6%) were sarcomatoid histotype. A similar distribution of age and sex was found between the histologic types. Asbestos exposure was present in 15 (39.5%) patients with epithelioid MPM and 3 (60%) patients with sarcomatoid MPM. Pleural topography was found in 38 (100%) patients with epithelioid histology and 5 (100%) patients with sarcomatoid histology.

Mutation Profile

We found a *TERT* promoter mutation in 11.6% (5/43) of the MPM (Table 2; Fig. 2). The c.-146C>T mutation was present in 3 cases and the c.-124C>T mutation in 2 cases. The 2 mutations occur in a mutually exclusive manner. All *TERT* mutated cases were histologically classified as epithelioid (Table 2).

In NGS-based mutation analysis, only 33 samples were successfully sequenced due to DNA quality issues. A total of 18 samples showed at least 1 variant (54.5%), and the number of variants per sample ranged from 1 to 6, with a median of 1.8 variants (Table 3). The top altered genes were as follows: *TP53* with 7 different variants in 7 cases (7/33 cases representing 21.2%), *ERBB2* with 7 different variants in 6 cases (6/33; 18.2%), *BRAF* and *PDGFRA* with 2 variants in 2 cases each (2/33; 6%), *NRAS* and *EGFR* with 2 variants in 1 case each (1/33; 3%), and *KIT*, *AKT1*, *PIK3CA*, and *FOXL2* with 1 variant each (1/33; 3%; Table 3).

Fourteen samples showed 1 variant (77.7%), 2 samples showed 2 variants (11.1%), one sample showed 3 variants (5.6%), and 1 sample showed 6 variants (5.6%; Table 3). In total, 27 different variants were identified: 23 were missense, 2 nonsense, and 2 frameshift (Table 3). In addition, 24 were SNVs and 3 INDELs. In summary, 10 of the 27

Table 3. NGS-based mutation analysis in 18 samples that showed at least one variant

Sample	TERTp status	Mutated genes	COSMIC ID	Clinical significance ^a	Mutation consequence
1	WT	TP53 exon 5 (c.488A>G)	COSM10808	Most likely pathogenic	missense (SNV)
4	WT	AKT1 exon 3 (c.67C>T)	–	Potentially pathogenic	missense (SNV)
5	WT	ERBB2 exon 29 (c.3316delC)	–	Most likely pathogenic	frameshift (INDEL)
		ERBB2 exon 20 (c.1967C>T)	–	Potentially pathogenic	missense (SNV)
		TP53 exon 8 (c.809T>G)	COSM43621	Potentially pathogenic	missense (SNV)
7	WT	BRAF exon 15 (c.1816G>A)	COSM27640	Potentially pathogenic	missense (SNV)
		EGFR exon 20 (c.2409_2410delGGinsAA)	–	Potentially pathogenic	missense (INDEL)
		EGFR exon 20 (c.2335G>T)	COSM22942	Potentially pathogenic	missense (SNV)
		ERBB2 exon 21 (c.2020G>A)	COSM3378168	Potentially pathogenic	missense (SNV)
		PDGFRA exon 18 (c.2449C>T)	–	Potentially pathogenic	missense (SNV)
		PIK3CA exon 21 (c.3052G>T)	–	Potentially pathogenic	missense (SNV)
11	WT	ERBB2 exon 20 (c.1870_1873delA)	CpA_inins6G2T4C.Ile625delinsValVal	Potentially pathogenic	missense (SNV)
12	WT	PDGFRA exon 18 (c.2449C>T)	–	Potentially pathogenic	missense (SNV)
20	WT	TP53 exon11 (c.1136G>A)	COSM44189	Potentially pathogenic	missense (SNV)
21	WT	BRAF exon 15 (c.1807C>T)	COSM33729	Most likely pathogenic	nonsense (SNV)
22	c.-124 C>T	TP53 exon 6 (c.581T>G)	COSM44571	Potentially pathogenic	missense (SNV)
23	WT	ERBB2 exon 21 (c.2046G>T)	–	Potentially pathogenic	missense (SNV)
25	WT	KIT exon 10 (c.1552C>T)	–	Potentially pathogenic	missense (SNV)
27	WT	ERBB2 exon 20 (c.1958G>A)	–	Potentially pathogenic	missense (SNV)
34	WT	TP53 exon 5 (c.488A>G)	COSM10808	Most likely pathogenic	missense (SNV)
35	c.-124 C>T	TP53 exon 8 (c.880G>T)	COSM10856	Most likely pathogenic	nonsense (SNV)
36	WT	ERBB2 exon 24 (c.2450C>T)	–	Potentially pathogenic	missense (SNV)
		PDGFRA exon 14 (c.1980G>T)	–	Potentially pathogenic	missense (SNV)
39	WT	NRAS exon 3 (c.239G>A)	–	Potentially pathogenic	missense (SNV)
		NRAS exon 3 c.251T>C)	–	Potentially pathogenic	missense (SNV)
40	WT	FOXO2 exon 1 (c.436C>A)	–	Potentially pathogenic	missense (SNV)
42	WT	TP53 exon 5 (c.492delG)	–	Most likely pathogenic	frameshift (INDEL)

SNV, Single nucleotide variant; INDEL, insertion deletion polymorphism. ^a Clinical significance in accordance with Sophia DDM reports.

variants are known COSMICs (catalog of somatic mutations in cancer), and 21 were reported as potentially pathogenic and 6 as most likely pathogenic in accordance with Sophia DDM reports (Table 3).

To assess the therapeutic implications of molecular events in our set of variants, we used Database of Evidence for Precision Oncology (DEPO; <http://depo-dinglab.ddns.net>) that focuses on specific mutations (STAR Methods) and casts therapeutic projections based on FDA-approved therapies, clinical trials, and published clinical evidence [28]. Of note, 2 variants in the *PDGFRA* gene (p.[Arg817Cys] in exon 18 and p.[Leu660Phe] in exon 14) found in MM were already described as a target of imatinib and an FDA-approved targeted therapy for GIST patients.

Discussion/Conclusion

MPM is a lethal cancer of the lung caused by human exposure to asbestos fibers [29]. Asbestos fibers may be inhaled by workers who deal directly with the fibers, by family members who are unintentionally exposed through workers' clothing, and by inhabitants of areas close to work sites where asbestos is processed or used [7]. In the year 2000, only 6 countries were responsible for almost all global asbestos production. Brazil is among these countries and produces approximately 250,000 tons/year, which ranks Brazil as the third greatest worldwide asbestos consumer [7].

MPM is highly refractory to conventional therapies, and the median survival of patients is 9–12 months after diagnosis, even with a combination of aggressive surgical intervention and multimodality strategies [1, 29]. Recently, MPM studies have identified genetic subtypes with a distinct profile of alterations in driver genes, increasing the need for understanding MPM biology for the successful development of personalized therapeutic modalities.

We analyzed the mutational profile in 16 driver genes by NGS or Sanger sequencing in 43 Brazilian MPM. Using Sanger sequencing, we analyzed *TERT* promoter mutations and found 11.6% mutated tumors. The c.-146C>T mutation was present in 7% and c.-124C>T in 4.6% of MPM. The 2 mutations occur in a mutually exclusive manner, and all *TERT*-mutated samples were histologically classified as epithelioid mesothelioma. In 2014, Tallet et al. [21] analyzed 132 MM and found 15.2% of *TERT* mutation (20/132), all in the c.-124 C>T region, and with a higher frequency in sarcomatoid histologic subtype. They screened *TERT* promoter mutations by Sanger sequencing and in-

cluded 61 samples derived from MM culture cells and 71 from tumor samples. Considering only the tumor samples, they found a mutation frequency similar to ours – 11.3% of the tumors had *TERT* promoter mutation.

In NGS-based mutation analysis, we found a total of 18 samples that showed at least 1 variant (representing 54.5% in a total of 33 cases studied). The range of variants was from 1 to 6 per sample, with a median of 1.8 variants. In total, 27 different variants were identified, and *TP53* and *ERBB2* were the most altered genes with 7 different variants each.

Mutations in *TP53* have been reported in MPM. Previous studies reported a range of *TP53* mutations from 5.7 to 19% in MPM cases [16, 29–34]. Here, we found 21.2% of cases with *TP53* mutation. In 2016, Bueno et al. [29] published a large series with 202 MPM cases and found *TP53* mutations in 8% of cases. Recently, a TCGA cohort comprising 74 tumors revealed 13.5% of *TP53* mutations [16]. Hmeljak et al. [16] showed that *TP53* is one of the MPM driver genes, together with *BAP1*, *SETD2*, and *NF2*, and is associated with aggressive behavior [16, 28]. They showed 13 *TP53* mutations, mainly SNV type, missense type, and in exons 5–8 (all these alterations were found in 10/13 mutated cases), as found by us, but in different nucleotides [16]. Interestingly, they suggested that *TP53* mutations occur early and presumably permit the catastrophic loss of chromosomes [16]. Here, we confirmed the impact of *TP53* mutations in many MPM cases (about 21%), but we did not evaluate *BAP1*, *SETD2*, and *NF2* genes.

Additionally, by targeted NGS, we found 18.2% of cases with at least 1 variant in the *ERBB2* gene. Despite *ERBB2* mutations occurring in multiple cancers [28], this is the first report in MPM. *ERBB2* is a member of the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases, of which overexpression results in the oncogenic transformation of cells [35]. The *ERBB2* gene is amplified in 15–20% of breast cancers and is associated with aggressive disease behavior [35]. It is known that some mutations in the *ERBB2* transmembrane domain, mainly in the 656–660 region in protein sequence, promote significant cell survival by increasing the stability of the receptor and keeping it in its activated state [28]. Interestingly, here, we found 2 *ERBB2* mutations specifically localized in 656 and 660 residues.

Other variants found in MPM cases were *PDGFRA*, *BRAF*, *NRAS*, and *EGFR* with 2 variants each, and *KIT*, *AKT1*, *PIK3CA*, and *FOXL2* with one variant each. Searching for mutations that are targeted by drugs that are now available, we used the DEPO platform and found 2 variants in *PDGFRA* (p.[Arg817Cys] in exon 18 and

p.[Leu660Phe] in exon 14), targeted by imatinib that could improve outcomes for patients with mesothelioma. In accordance, Bailey et al. [28] also found that very few MM mutations are druggable (using DEPO, they found 1%). In addition, they found in about 50% of cases that the nucleotide changes occur in C>T. Here, we found that almost 30% of nucleotide changes occurred in C>T.

One limitation of our study is that we did not have the opportunity to correlate the asbestos exposure history from MPM cases with the clinical pathological and mutational profile.

In conclusion, we showed that MPM are highly complex and heterogeneous neoplasms. By Sanger sequencing and targeted NGS, we described a somatic mutation profile composed of the top altered genes *TP53*, *ERBB2*, and *TERT*. Interestingly, clinically actionable alterations were found in 2 cases, suggesting that these patients could benefit from this therapeutic modality.

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Statement of Ethics

This study was approved by the local Ethics Committees (CEP-1033/2015), and due to the retrospective nature of the study design signed patient consent was waived.

Disclosure Statement

The authors declare no competing financial interests.

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Author Contributions

N.C.C. and R.M.R. wrote the manuscript. R.M.R. designed the study. E.C.S., G.D., F.L.V., M.B., R.C., R.V.M.L., H.C.S.S., and V.L.C. organized and/or reviewed the clinical samples. N.C.C., F.E.P., and G.N.B. performed the experiments. N.C.C., G.N.B., F.E.P., and R.M.R. contributed to data analysis and interpretation. All authors reviewed the manuscript.

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