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Molecular characterization of pleomorphic mesothelioma: a multi-institutional study

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The molecular alterations of pleomorphic mesotheliomas are largely unknown. In the present study, we performed whole-exome sequencing (WES) on 24 pleomorphic mesotheliomas in order to better characterize the molecular profile of this rare histologic variant. BAP1 protein expression and *CDKN2A* deletion by FISH were also evaluated. Significantly mutated genes included *BAP1* (35%), *NF2* (13%), *LATS2* (8%), *TP53* (5%), and *LATS1* (3%). *BAP1* alterations most frequently co-occurred with deletions of chromosomes 4, 9, and 13. Other important genetic alterations in pleomorphic mesotheliomas included truncating mutations in *NF2* (3 of 24; 12.5%), *LATS2* (2 of 24; 8%), *TP53* (1 of 24; 4%), and *PBRM1* (1 of 24; 4%). Focal losses of chromosome 9p21 were most common copy number alterations (11 of 24 cases; 46%), and were assessed by WES and targeted FISH. The second most common were deletions of chromosome 4 (8 of 24; 33% pleomorphic mesotheliomas). Three cases of pleomorphic mesothelioma did not show any mutations, copy number alterations, or LOH. This first WES analysis of pleomorphic mesotheliomas did not identify novel or unique mutations. In contrast to transitional mesothelioma that was reclassified as sarcomatoid variant based on transcriptome data, pleomorphic mesotheliomas are molecularly heterogeneous and therefore their reclassification into single subtype is more difficult.

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INTRODUCTION

Mesotheliomas are classified into three major histologic subtypes, including epithelioid, sarcomatoid, and biphasic[1]. This histologic classification is of prognostic and therapeutic significance[2]. Epithelioid mesotheliomas are associated with better prognosis when compared to sarcomatoid and biphasic subtypes. The clinical significance of histological subtyping has been recognized for many years, and recently published guidelines for the diagnosis of mesotheliomas recommended documenting the histologic subtype in pathology reports[3,4].

Morphological heterogeneity of epithelioid mesothelioma has been addressed in the World Health Organization (WHO) classification. Similar to what was demonstrated in lung adenocarcinoma, architectural patterns of epithelioid mesothelioma may have a prognostic value[5–7]. Two architectural patterns, transitional and pleomorphic, that were classified under epithelioid subtype showed a poor prognosis similar to sarcomatoid and biphasic subtypes, while solid epithelioid subtype which is considered to be associated with clinically more aggressive behavior showed significantly better survival[5,8]. One of the main questions is if those prognostically adverse patterns are genomically different from other epithelioid patterns. Recently published RNA-seq-based unsupervised clustering analysis revealed that transitional mesothelioma grouped together and were closely related to sarcomatoid than to epithelioid mesothelioma[8]. These results together with clinically poor outcomes provided a rationale for reclassification of transitional mesothelioma as a sarcomatoid variant. In contrast, molecular characteristics of pleomorphic mesotheliomas are largely unknown.

In the present study we performed whole-exome sequencing (WES) of pleomorphic mesothelioma in order to characterize the molecular profile of this histologic variant.

MATERIALS AND METHODS

Study samples

A total of 40 cases of surgically resected mesothelioma including 24 pleomorphic, 9 sarcomatoid, and 7 epithelioid were selected for the study from the case files of the MESOPATH center and MESOBANK database and three co-authors (A.C., R.A., and S.D.). The sarcomatoid and epithelioid comparison cases were carefully inspected to be sure that they contained no pleomorphic areas and were otherwise typical mesotheliomas. The mesotheliomas were classified according to the 2021 WHO morphologic and immunophenotypic criteria[1]. Morphologic criteria used for classification of pleomorphic mesothelioma included at least 10% of tumor cells with prominent anaplastic nuclei and bizarre nuclei often containing multinucleated tumor giant cells (Fig. 1). Pleomorphic morphology was identified in 18 epithelioid, 3 biphasic, and 2 sarcomatoid mesothelioma subtypes. Formalin-fixed paraffin-embedded (FFPE) mesothelioma samples with sufficient tumor quantity and if available matched normal tissue were selected for WES.

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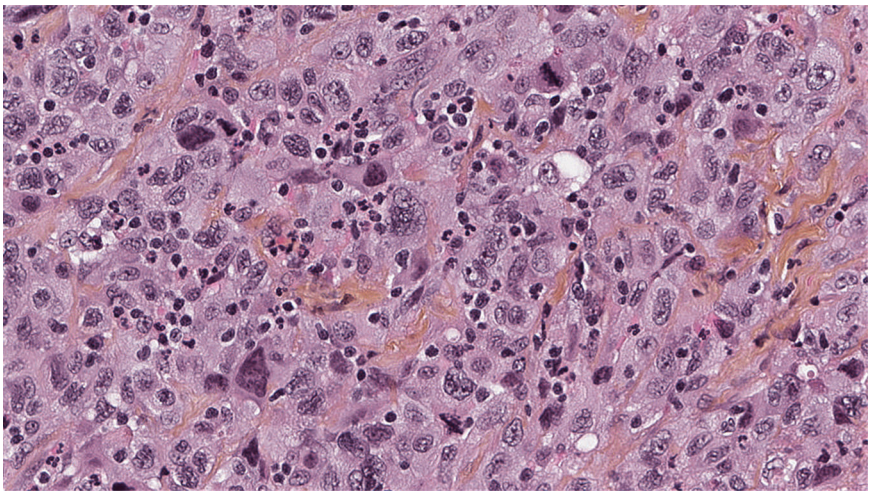


Fig. 1 Pleomorphic mesothelioma morphology. An example of pleomorphic mesothelioma with prominent anaplastic and bizarre nuclei (H&E, x40).

WES and bioinformatics analysis

Specimen processing and DNA isolation. For all samples, the hematoxylin and eosin-stained histologic sections were reviewed for microdissection to target areas with the highest proportion of viable tumor cells. After deparaffinization of the unstained histologic sections, DNA was extracted using the Zymo Quick DNA FFPE extraction kit (Zymo, Irvine, CA, catalog #: D3067). Briefly, deparaffinized tissue from each sample was incubated in digestion buffer with proteinase K and incubated. DNA extraction was performed according to the manufacturer's instructions.

Library preparation and sequencing. Genomic DNA from both tumor–normal pairs underwent exome library prep using the Swift Accel-NGS 2S Hyb DNA library kit (Swift, Ann Arbor, MI, catalog#: 23024) assay for pre-hybridization library construction. Exome capture was performed using the Agilent Human all exon V6 baits (Agilent, Santa Clara, CA, catalog#: 5190-8863) and post hybridization library prep was performed using Agilent's SureSelect XT hybridization and blocking reagents (Agilent, Santa Clara, CA, catalog#: 930672) and the Swift XT Compatibility Module (Swift, Ann Arbor, MI, Catalog# 26424) following Agilent's SureSelect XT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library protocol (Agilent, Santa Clara, CA, Version C3, September 2019). Both PCR steps were conducted using the Herculase II Fusion DNA Polymerase kit (Agilent, Santa Clara, CA, catalog #: 600679) and following the Illumina (Illumina, San Diego, CA) NextSeq library dilution and denature guide for sequencing (catalog #: 15048776, March 2020).

After obtaining an average DNA fragment size of approximately 150 bp, using the Covaris S-1 (Covaris, Woburn, MA), end repair was performed using the Swift Accel-NGS 2S Hyb DNA Library Kit reagents. After magnetic bead cleanup adaptor-ligated library was amplified using the R-XT reagent (Swift, Ann Arbor, MI, XT Compatibility Module, catalog# 26424) and further cleaned using the AMPure XP beads. The pre-hybridization PCR product was evaluated for quality control using the High Sensitivity Qubit Kit (Thermo Fisher Scientific, Pittsburgh, PA, catalog#: 32854) and the Agilent High sensitivity DNA Bioanalyzer kit (Agilent, Santa Clara, CA, catalog#: 5067-4626). The PCR product was then subjected to 24-h hybridization for target enrichment followed by library amplification and adaptor ligation. The pooled library was run on the Illumina NextSeq 500 150 cycle high output kit using paired-end sequencing run (75 bp × 2, with single 8 base pair index).

Bioinformatics analysis. Bioinformatics analysis was performed using a custom protocol developed for tumor and paired normal and tumor only analysis. First, base call files (BCL) were converted to FASTQ files using bcltofastq (Illumina, San Diego, CA). Per lane FASTQ files were merged into FASTQ files for each read pair (R1 and R2), as per the manufacturer's recommendation. Each set of read pair FASTQs were aligned to the human reference genome (GRCh37.p13; GCF_000001405.25) using BWA MEM[1] and encoded into a BAM (binary sequence alignment) format using Samtools. RG (read group) tags for each sample were added at the time of sequence alignment. The raw BAM files were sorted, indexed, and PCR duplicates marked using Sambamba. Pre-variant calling processing included concurrent local realignment around regions of known indels (COSMIC v89 and Clinvar) for both tumor and normal aligned reads using GATK. Subsequently, variant calling was performed on the realigned BAMs using Varscan2 for SNV and short Indel detection and VarDict for larger Indel detection. Briefly for Varscan2, realigned BAM files for both tumor and normal or tumor only were used to generate a paired tumor–normal sequence mpileup or a tumor only mpileup, respectively. Subsequently, the mpileups were used for calling variants using Varscan2 in somatic and single-sample mode. Potential false positives were marked in the VCF files using Varscan2's fpfilter. Variants marked as somatic and high confidence by the variant caller were prioritized. For large indel detection, VarDict was used in somatic (paired tumor–normal) and single-sample (tumor only) mode. Variants were represented using VCF format v4.2 (https://samtools.github.io/hts-specs/VCFv4.2.pdf, last accessed 4/2/2020). Variant calls from both callers were integrated, normalized, and annotated using custom python modules and hgvs python package. Variants were prioritized based on reported minor allele frequency in population databases (1000genomes, ExAC, Exome server variant [http://evs.gs.washington.edu/EVS/, last accessed 4/2/2020]), variant location (coding vs non-coding), in silico prediction algorithms (SIFT, PolyPhen2) for missense variants, reported incidence in public somatic and germline variant databases (COSMIC v90, ClinVar). After variant annotation using custom algorithm and publicly available databases, the genetic alterations were prioritized using the AMP/CAP/ASCO guidelines[9]. Integrative Genomics Viewer (IGV, Broad Institute) was used for manual review of detected variants. Copy number and Loss of heterozygosity (LOH) analysis was performed using FACETS and CNVkit algorithms. For FASTQ and BAM files, QC metrics were generated by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and QualiMap, respectively. For variant calling, sequence reads with minimum base quality score (*Q*-score) of 30 and minimum mapping quality score of 20 were used. Only variants with variant quality score of 20 (corresponding p value <0.01) and at least 80 or more variant-supporting reads were included for variant prioritization and further analysis.

BAP1 immunohistochemistry and *CDKN2A* FISH

BAP1 immunohistochemistry and *CDKN2A* FISH were performed as previously described[10]. Immunohistochemical labeling was performed on 4-µm unstained paraffin sections subjected to antigen retrieval using heated citrate solution (pH 9.0) at 100 °C for 4 min. BAP1 (C-4 mouse monoclonal, Santa Cruz, CA; at 1:50 dilution) nuclear staining was considered positive (nuclear expression retained) and negative (complete loss of staining with a positive internal control).

Fluorescence in situ hybridization (FISH) for *CDKN2A* (Abbott Molecular, Des Plains, IL, USA) was considered positive for deletion if homozygous deletion was identified in at least 20% of tumor cells.

RESULTS

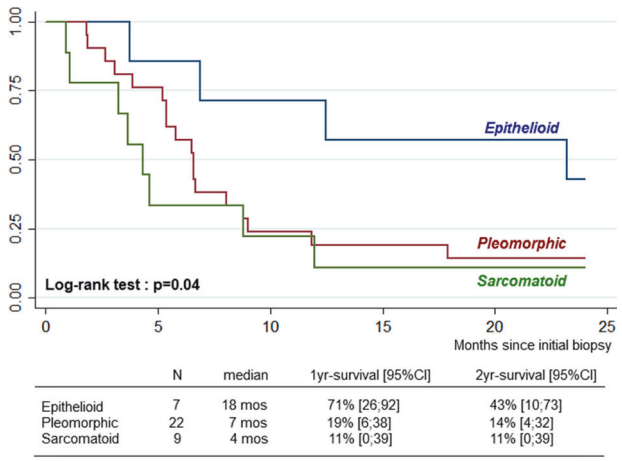
Cohort characteristics

The 40 patients had a mean age of 73 years (range, 50–90 years); 30 were male and 22 had an asbestos exposure history. Exposure

history was unknown in 12 patients. Patients with pleomorphic mesothelioma were mostly male. The mean age of patients with pleomorphic mesothelioma was 64 (range, 50–73) and nine had asbestos exposure history. History of asbestos exposure was uncertain in nine patients. Pleomorphic mesotheliomas showed survival similar to sarcomatoid subtype (Fig. 2). Survival data were not available for two patients with pleomorphic mesothelioma.

WES results

A total of 40 samples were analyzed using WES, of which 11 samples had paired normal tissue. The WES of all samples provided a mean coverage of 210X across the exome target regions. Significantly mutated genes included *BAP1* (35%), *NF2* (13%), *LATS2* (8%), *TP53* (5%), and *LATS1* (3%) (Fig. 3).



	N	median	1yr-survival [95%CI]	2yr-survival [95%CI]
Epithelioid	7	18 mos	71% [26;92]	43% [10;73]
Pleomorphic	22	7 mos	19% [6;38]	14% [4;32]
Sarcomatoid	9	4 mos	11% [0;39]	11% [0;39]

Fig. 2 Overall suvival and mesothelioma histology. Pleomorphic mesotheliomas showed survival similar to sarcomatoid subtype.

Somatic alterations in *BAP1* gene were most commonly observed in pleomorphic mesotheliomas (9 of 24; 37.5%) that were otherwise epithelioid and biphasic subtypes. No *BAP1* alterations were identified in pleomorphic mesothelioma that were sarcomatoid subtype. *BAP1* alterations across all tumors included frameshift (8 of 14; 57%), splice site (2 of 14; 14%), missense (2 of 14; 14%), inframe (1 of 14; 7%), and a structural variant (1 of 14; 7%). Three cases of pleomorphic mesothelioma harbored *BAP1* mutation as the only clinically significant genetic alterations. *BAP1* alterations in pleomorphic mesothelioma most frequently showed co-occurrence with deletions of chromosomes 4, 9, and 13. One case of pleomorphic mesothelioma showed *BAP1* mutation and deletion of chromosome 3 along with deletions of chromosomes 9, 13, and 14. Another case harbored mutation in *BAP1, RYR1*, and *SETD2* genes and deletion of chromosome 4. Interestingly, one of the epithelioid mesothelioma demonstrated a structural alteration that involved the rearrangement of exons 3 and 5 of the *BAP1* gene, which upon comparison to the protein expression demonstrated complete loss, supporting this rearrangement to be a loss of function mutation.

Other important genetic alterations in pleomorphic mesotheliomas included truncating mutations in *NF2* (3 of 24; 12.5%), *LATS2* (2 of 24; 8%), *TP53* (1 of 24; 4%), and *PBRM1* (1 of 24; 4%). Three cases of pleomorphic mesothelioma did not show any mutations, copy number alterations, or LOH.

As expected in mesotheliomas, copy number alterations included chromosomal losses, but no amplifications. Copy number losses were observed in chromosomes 4, 9, 6,13, and 14 (Figs. 3 and 4). Focal losses of chromosome 9p21 were most common (21 of 40 cases (52.5%) across all histologies including 6 (67%) sarcomatoid, 11 (46%) pleomorphic, and 4 (57%) epithelioid mesotheliomas. Copy number losses of 9p chromosome include combined WES and targeted FISH results. The second most common were deletions of chromosome 4 (8 of 24; 33% pleomorphic mesotheliomas). Similarly, 3 of 8 (37.5%) epithelioid mesotheliomas showed the loss of chromosome 4, while no changes were observed in sarcomatoid mesotheliomas. Chromosome 6 deletions were observed in epithelioid and sarcomatoid mesotheliomas, but not in pleomorphic ($p = 0.02$).

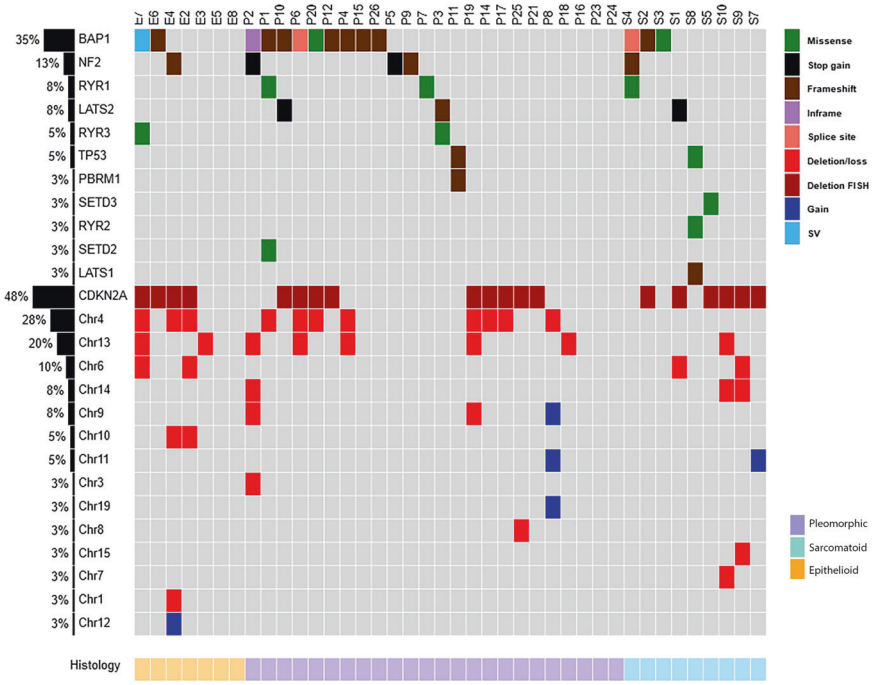


Fig. 3 COMUT plot showing gene mutations and copy number alterations of the study mesothelioma cohort. Each column represents an individual case. Samples are displayed based on histology. Note: All cases with *BAP1* alterations showed loss of BAP1 expression by immunohistochemistry.

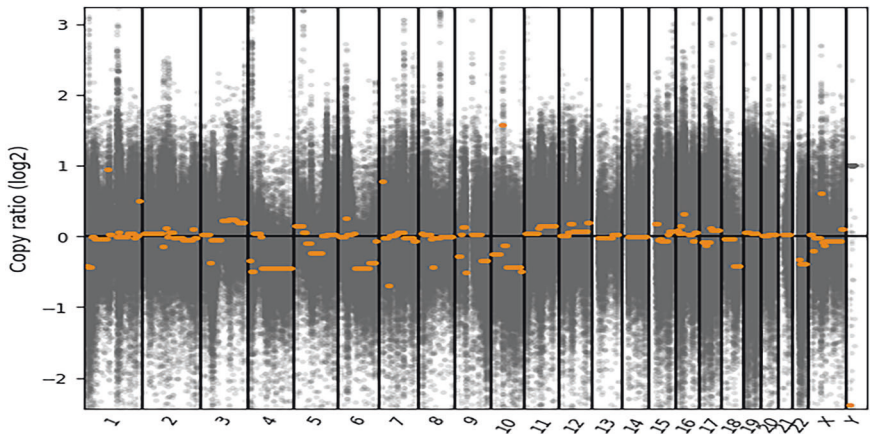


Fig. 4 Copy number alterations in mesothelioma. An example of epithelioid mesothelioma with multiple copy number alterations including loss of chromosome 4 that was also commonly observed in pleomorphic mesothelioma.

DISCUSSION

Molecular studies in mesotheliomas are limited, and mostly focused on improved prognostic classification of three major histologic subtypes[9,11–14]. Clinical and morphological heterogeneity of mesothelioma, particularly epithelioid, is well known. Recently, large-scale genomic studies have identified additional molecular subtypes (i.e. four prognostically different transcriptome subtypes) that do not entirely fit into the current histological classification[9,11,14,15].

Our study focused on the pleomorphic pattern, a pattern known to be associated with a poor prognosis. Survival of the subset of cases included in this study has been published previously[16]. Published studies did not provide detailed morphological assessment beyond three major subtypes and therefore a link between mutation profile and histological patterns of epithelioid mesothelioma is uncertain[11,12,17]. Therefore, we decided to use a comprehensive WES approach on a morphologically highly selected cohort of pleomorphic mesotheliomas.

The most common genomic alterations in this cohort of pleomorphic mesotheliomas were identified in the *BAP1* gene and included mutations and copy number loss. This finding is similar to previously published studies, in which *BAP1* alterations have been reported in over 50% of mesotheliomas more often in epithelioid subtype[11,12,18]. It is not surprising that *BAP1* alterations are the most common. We previously demonstrated in clinicopathologically defined cases of mesothelioma in situ by WES that *BAP1* alterations are the initiating and early event in a subset of epithelioid mesotheliomas[19].

Interestingly, in three cases of pleomorphic mesothelioma *BAP1* frameshift mutation was the only alteration identified suggesting that perhaps other mechanisms such as DNA methylation may be involved in the progression of those cases. Similar to prior reports, the most common mutations were frameshift mutations resulting in a complete loss of BAP1 protein expression by immunohistochemistry[12,18]. De Rienzo et al.[18] recently compared different BAP1 staining patterns (nuclear, cytoplasmic) and *BAP1* mutation type and found no association. BAP1 staining patterns can be heterogeneous in mesotheliomas, but that was not observed in our study cohort. In terms of the prognostic significance of *BAP1* alterations, published studies including data based on exome sequencing of the entire *BAP1* gene did not reveal different prognosis between wild type and *BAP1* mutated cases[18]. Therefore, *BAP1* alteration alone cannot provide an explanation for more aggressive behavior of pleomorphic mesotheliomas. All mutations were somatic, which is not unusual in the age group represented in this study[20]. To our knowledge none of the study patients have evidence of other tumors associated with *BAP1* tumor predisposition syndrome[21].

Overall, the number of mutations was low. The mutation spectrum was similar across the study cases and lacked distinctive or novel findings. *LATS2* mutations were found in two cases, but no co-mutations of *LATS2* and *NF2* genes known to be associated with poor prognosis were found[17]. Similarly, a single case showed *TP53* mutation. However, a genetic subtype with *TP53* and *SETDB1* mutations and extensive LOH phenotype that was reported by TCGA to be associated with aggressive behavior of mesothelioma was not identified[12]. This particular molecular subtype shows female predominance and younger age at diagnosis. Pleomorphic mesotheliomas in our cohort predominantly occurred in older men.

In addition to mutations, we assessed copy number alterations. Consistent with prior reports, 9p21 (*CDKN2A*) deletions were the most common alteration[11,12,22]. *CDKN2A* is a well-established molecular poor prognostic factor in mesotheliomas and its frequency depends on histologic subtype[12,22,23]. In addition to WES, FISH for 9p21 deletion was also performed in order to enrich the detection of small CNV events. Although we demonstrated 9p21 deletions by WES, FISH assay allowed identification of focal alterations that could be challenging for detection by WES. Copy number loss of 9p co-occurred with gene mutations and copy number losses on other chromosomes.

The second most common copy number alteration that frequently co-occurred with 9p loss was loss of the long arm of chromosome 4. Loss of chromosome 4 in mesothelioma was previously reported[24–26]. Bjorkqvist et al.[26] reported CGH study of 27 mesotheliomas of which three, including one epithelioid and two biphasic, showed loss of 4q. In the same study, 9p loss was reported similar to other reports in about 60% of mesotheliomas across different histologic subtypes[26]. In our study, one-third of pleomorphic mesotheliomas showed 4q loss. Although loss of 4q has been reported in epithelioid mesothelioma, its impact on survival is unknown. In this study, loss of 4q did not show prognostic significance. Earlier studies in several cancer types including bladder, esophagus, and colorectal among others provided evidence for tumor suppressor genes on chromosome 4. Thus, chromosome 4 likely harbors one or more tumor suppressor genes that are frequently inactivated in several cancer types including mesothelioma[27].

Our study is the first WES analysis of pleomorphic mesotheliomas. Although our study did not identify novel mutations that would be unique for pleomorphic mesotheliomas, copy number alterations, particularly on chromosome 4q, are intriguing and require further investigation. In contrast to transitional mesothelioma that was reclassified as sarcomatoid variant based on transcriptome data, pleomorphic mesotheliomas are molecularly heterogeneous and therefore their reclassification into single subtype is more difficult.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

S.R., F.G.-S., A.C., R.A., and S.D. performed study concept and design, interpretation of data, writing and review of the paper. N.L.S. provided statistical analysis. M.A.L. provided technical support.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL/CONSENT TO PARTICIPATE

The study protocol was reviewed and approved by the institutional review board of the University of Pittsburgh (PRO18010420).

ADDITIONAL INFORMATION

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