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Malignant cells from pleural fluids in malignant mesothelioma patients reveal novel mutations

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ABSTRACT

Objectives: Malignant mesothelioma (MM) is an asbestos related tumour affecting cells of serosal cavities. More than 70% of MM patients develop pleural effusions which contain tumour cells, representing a readily accessible source of malignant cells for genetic analysis. Although common somatic mutations and losses have been identified in solid MM tumours, the characterization of tumour cells within pleural effusions could provide novel insights but is little studied.

Materials and methods: DNA and RNA were extracted from cells from short term cultures of 27 human MM pleural effusion samples. Whole exome and transcriptome sequencing was performed using the Ion Torrent platform. Somatic mutations were identified using VarScan2 and SomaticSniper. Copy number alterations were identified using ExomeCNV in R. Significant copy number alterations were identified across all samples using GISTIC2.0. The association between tumour intrinsic properties and survival was analyzed using the Cox proportional hazards regression model.

Results: We identified *BAP1*, *CDKN2A* and *NF2* alterations in the cells from MM pleural effusions at a higher frequency than what is typically seen in MM tumours from surgical series. The median mutation rate was 1.09 mutations/Mb. *TRAF7* and *LATS2* alterations were also identified at a high frequency (66% and 59% respectively). Novel regions of interest were identified, including alterations in *FGFR3*, and the regions 19p13.3, 8p23.1 and 1p36.32.

Conclusion: Short term cultures of tumour cells from MM pleural effusions offer an accessible alternative to surgical tumour biopsies in the study of MM genomics and reveal novel mutations of interest. Pleural effusion tumour cells provide an opportunity for the monitoring of tumour dynamics, treatment response and the clonal evolution of MM tumours.

1. Introduction

Malignant mesothelioma (MM) is an aggressive cancer with poor survival caused primarily by exposure to asbestos, affecting the cells of the pleural and peritoneal surfaces [1–3]. Treatment options for pleural MM include surgery, chemotherapy, radiotherapy and immunotherapy; however, results are modest. Current molecular studies aim to reveal new treatment strategies, including the identification of druggable targets as well as clinically useful biomarkers within the tumour mutanome.

To date, sequencing and gene expression studies have been performed primarily on solid MM tumour samples. Several common

Abbreviations: −x, −fold coverage; cpm, counts per million; MM, malignant mesothelioma

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genetic changes have been found, including loss of *CDKN2A* (> 70% of cases) [4], loss, and/or mutation of the *BAP1* gene (40–60% of cases) [5] and loss or mutation of *NF2* (40% of cases) [6,7]. More recent studies have identified low frequency mutations in *LATS2, CUL1*, *TP53*, and *SETD2* [8,9].

Surgical tumour samples, while informative, may not provide a complete picture of MM genetics due to a number of important factors. Sequencing of surgical samples generally requires a high level of tumour cellularity, for The Cancer Genome Atlas project a 60% minimum tumour cell content threshold was required (https://cancergenome.nih.gov/cancersselected/biospeccriteria). Such a criterion results in the exclusion of samples of other potentially highly informative phenotypes, such as those that contain heavy infiltrates of lymphocytes, fibroblasts or macrophages. Sampling is also limited to those cases suitable for surgical resection, which represents a minority of all cases [10]. Additionally, single site sampling may limit analyses due to extensive tumour heterogeneity, resulting in reduced sensitivity in identifying sub clonal or low abundance mutations [11].

Most pleural MM cases present with a build up of tumour-associated fluid, that is an effusion that usually contains malignant cells [1]. These pleural effusions may potentially overcome the limitations posed by surgical samples. Therefore, as an alternative to sequencing solid tumour, we explored the use of low passage tumour cell cultures generated from MM pleural effusion samples. We performed whole exome and transcriptome sequencing of the tumour cells derived from pleural effusions of 27 MM patients, characterizing the mutational landscape of these pleural effusion tumour cells. We compared the mutations landscape to what has been reported for surgical MM tumour samples in order to determine if they are representative of the underlying solid tumor. We found novel mutations not previously identified in solid MM tumours. Characterizing the mutations in tumour cells found in pleural effusion samples will allow us to understand the genetics and biology of these cells which may lead to improvement in MM patient outcomes.

2. Materials and methods

2.1. Samples

In this study we analysed 27 primary MM short-term tumour cell cultures (median time in culture 7 passages, range 3–10). Cell cultures were established from pre-treatment pleural effusion specimens collected between 2002 and 2014 based on previously described methods [12]. All cell cultures were confirmed to be *Mycoplasma spp.* free by polymerase chain reaction. No matched surgical or biopsy samples from the corresponding primary tumours were available. Blood was collected from all individuals in either Vacutainer K2EDTA plasma or sodium heparin tubes (BD Bioscience, New Jersey, USA). This study was approved by the Human Research Ethics Committee of at Sir Charles Gairdner Hospital and the University of Western Australia (Perth, Western Australia). Written informed consent from all participants was obtained.

2.2. Nucleic acid extraction

Genomic DNA and RNA were isolated from MM cell cultures growing in logarithmic phase from these early passages. Germline genomic DNA was isolated from whole blood. DNA and RNA quality were determined using the Bioanalyser 2100 (Agilent Technologies, Santa Clara, California, USA); sequencing was performed on unfragmented DNA, and RNA samples with RIN values > 7.

2.3. Whole exome sequencing and targeted sequencing of BAP1, CDKN2A and NF2

Whole exome sequencing (WES) libraries were prepared using an Ion Ampliseq Exome RDY Kit (Life Technologies, Welshpool, Western Australia) according to the manufacturer's instructions. Additionally, libraries for a 3854 base pair (bp) region of *BAP1*; 2228 bp region of *CDKN2A;* and 7245 bp region of *NF2* including all exons and 3′ and 5′ untranslated regions were prepared. Sequencing on the Ion Torrent (Thermofisher Scientific, Malaga, Western Australia) and alignment to the human (hg19) reference genome were performed as previously described [13]. Local realignment around known indels and base quality score recalibration was performed using the Genome Analysis Toolkit (GATK) [14] to produce analysis ready binary alignment (BAM) files.

2.4. Transcriptome sequencing

Ribosomal RNA was depleted from 2.5ug of total RNA using a RiboZero Gold kit (EpiCentre, Madison, Wisconsin, USA) according to the manufacturer's instructions. Remaining RNA was quantified by fluorometry on a Qubit (Thermofisher Scientific) using a high sensitivity RNA kit (Invitrogen, Malaga, Western Australia). A total of 40 ng of rRNA-depleted RNA was used to prepare total RNA libraries using an Ion Total RNA sequencing kit V2 (Ambion, Malaga, Western Australia) according to the manufacturer's instructions. The libraries were diluted to 100–120pM and attached to Ion Sphere Particles (ISPs) using an Ion Chef (Thermofisher Scientific). Two samples were pooled on a single P1 chip. Samples were sequenced for 520 cycles on an Ion Proton sequencer (Thermofisher Scientific). Torrent Suite 5.0 was used to collect data and perform QC, including removal of polyclonal reads, removal of low quality reads and 3′ end trimming. The process generated 30–45 million reads per sample.

Adapters were trimmed using CutAdapt [15]. A two-step alignment process was then performed for transcriptome data for each sample. Firstly, reads were mapped to the hg19 RefSeq transcriptome using Tophat2 [16]. Secondly, unmapped reads from the first step were remapped to the hg19 genome using bowtie 2 [17]. The BAM files from each step were merged using Picard tools. Quality control and alignment metrics were determined using Qualimap [18]. Expression was measured in normal expected count values generated by Trimmed Mean of *M*-values (TMM) normalisation method in edgeR [19], converted to counts per million (cpm) and log transformed where appropriate.

2.5. Somatic mutation detection

Somatic single nucleotide variants (SNVs) were identified using two publically available variant detection tools: VarScan2 [20] and SomaticSniper [21]. A mapping quality of at least 1 and a minimum of 8 reads in the normal sample and 10 reads in the tumour sample were required for SNV detection at each position for both tools. Variants reported in dbSNP (version 137) and that were located in clusters near indels were filtered out. Candidate SNVs from each tool were pooled using vcftools (v0.1.10). Indels were identified using VarScan2 with the same parameters as above. Indels required a minimum variant allele frequency of 35% to be considered for further analysis. Orthologous validation of candidate SNVs and indels was performed by counting alleles at each variant position in the corresponding patient transcriptome. Expressed loci containing only wild-type alleles were removed from the analysis. All candidate indels were screened against all other samples. If the indel was called in more than one sample it was considered a sequencing or alignment artifact and discarded from downstream analysis. Mutation rates were determined as the number of exonic SNVs and indels divided by the size of the exome library (57.7 million bases). SNVs and indels were annotated using snpEff [22] and converted from variant call format (VCF) to mutation annotation format (MAF) using vcf2maf (https://github.com/mskcc/vcf2maf). Significantly mutated genes were identified using MutSigCV [23] where a q value of < 0.1 was determined to be significant.

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