Pathology (April 2016) **48(3)**, pp. 261–266

MOLECULAR PATHOLOGY

Comparison of whole-exome sequencing of matched fresh and formalin fixed paraffin embedded melanoma tumours: implications for clinical decision making

Ricardo De Paoli-Iseppi[1], Peter A. Johansson[2], Alexander M. Menzies[1,3,4], Kerith-Rae Dias[5], Gulietta M. Pupo[6], Hojabr Kakavand[1,3], James S. Wilmott[1,3], Graham J. Mann[1,3,6], Nicholas K. Hayward[2], Marcel E. Dinger[5], Georgina V. Long[1,3,4] and Richard A. Scolyer[1,7,8]

[1]*Melanoma Institute Australia, North Sydney, NSW,* [2]*Oncogenomics Laboratory, QIMR Berghofer Medical Research Institute, Royal Brisbane and Women's Hospital, Brisbane, Qld,* [3]*Discipline of Medicine, Sydney Medical School, The University of Sydney,* [4]*Department of Medical Oncology, Royal North Shore Hospital, St Leonards,* [5]*Garvan Institute of Medical Research, Darlinghurst,* [6]*Centre for Cancer Research, The University of Sydney at Westmead Millennium Institute, Westmead,* [7]*Discipline of Pathology, Sydney Medical School, The University of Sydney, and* [8]*Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Camperdown, NSW, Australia*

Summary

The identification of recurrent driver mutations by whole-exome sequencing (WES) of fresh-frozen human cancers and the subsequent development of novel targeted therapies have recently transformed the treatment of many cancers including melanoma. In routine clinical practice, fresh-frozen tissue is rarely available and mutation testing usually needs to be carried out on archival formalin fixed, paraffin embedded (FFPE) tissue, from which DNA is typically fragmented, cross-linked and of lower quality.

In this study we aimed to determine whether WES data generated from genomic DNA (gDNA) extracted from FFPE tissues can be produced reliably and of clinically-actionable standard.

In this study of ten melanoma patients, we compared WES data produced from analysis of gDNA isolated from FFPE tumour tissue with that isolated from fresh-frozen tumour tissue from the same specimen. FFPE samples were sequenced using both Illumina's Nextera and NimbleGen SeqCap exome capture kits. To examine mutations between the two tissue sources and platforms, somatic mutations in the FFPE exomes were called using the matched fresh tissue sequence as a reference.

Of the 10 FFPE DNA samples, seven Nextera and four SeqCap samples passed library preparation. On average, there were 5341 and 2246 variants lost in FFPE compared to matched fresh tissue utilising Nextera and SeqCap kits, respectively. In order to explore the feasibility of future clinical implementation of WES, FFPE variants in 27 genes of important clinical relevance in melanoma were assessed. The average concordance rate was 43.2% over a total of 1299 calls for the chosen genes in the FFPE DNA. For the current clinically most important melanoma mutations, 0/3 BRAF and 6/8 (75%) NRAS FFPE calls were concordant with the fresh tissue result, which was confirmed using a Sequenom OncoCarta Panel.

The poor performance of FFPE WES indicates that specialised library construction to account for low quality DNA and further refinements will be necessary before this approach could be used for routine clinical decision making over currently preferred techniques.

Key words: BRAF; NRAS; exome sequencing; formalin fixed paraffin embedded; management; melanoma; mutation testing; pathology; treatment; melanoma; molecular.

Received 24 March, revised 22 December 2015, accepted 3 January 2016
Available online 9 March 2016

INTRODUCTION

As whole-genome sequencing (WGS) and WES[1] of human genomic DNA (gDNA) becomes more feasible due to reduced costs[2] and delivery speed for translational research,[3] large reserves of formalin fixed, paraffin embedded (FFPE) tissue blocks are becoming a focus of many laboratories as a valuable source of material. Whilst fresh tissue or blood is preferred for the majority of molecular tests, these samples are rarely routinely collated outside of specialist centres and have complex and expensive storage and handling requirements.[4] Consequently, in routine clinical practice, formalin fixation of tissue remains the standard protocol within the majority of pathology laboratories. Formalin fixation is known to cause extensive DNA damage due to the creation of DNA-protein crosslinks resulting in possible sequence aberrations[5,6] and incorrect interpretation of data. Recently the successful use of FFPE derived DNA in next generation sequencing (NGS) applications was reported.[7,8] A subsequent study reported results of a limited panel of genes tested by NGS of fresh-frozen and FFPE material and concluded that there are detectable but non-compromising effects of FFPE on NGS data.[9]

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: http://dx.doi.org/10.1016/j.pathol.2016.01.001

WES involves the capture of all protein coding regions by hybridising DNA to oligonucleotide probes that cover human exonic regions. The isolated regions are then sequenced using NGS technology.[10] This approach has expanded knowledge of the genetic landscape of many tumours and in some instances has provided new therapeutic targets and novel efficacious treatment options.[11] Recently, Van Allen and colleagues reported that they were able to identify clinically relevant alterations in approximately 90% of patient samples analysed in a translational WES study, supporting the potential use of FFPE tissue DNA for rapid, high precision clinical decision making.[12] Melanoma xenograft FFPE DNA used in targeted massively-parallel sequencing has also been shown to successfully identify a number of clinically important mutations in genes described in the COSMIC cancer mutation database. The study by Wong *et al.* reports no marked difference in the ability for this method to detect the BRAF V600E variant from DNA derived from FFPE or cell lines (un-fixed control).[13] These results are yet to be verified and a study on melanoma, a disease where targeting driver mutations has recently transformed clinical care, comparing WES data from fresh and FFPE material, has not been previously reported to the best of our knowledge.

In this study, we carried out a comparison of WES data generated from ten DNA samples derived from paired fresh-frozen and FFPE melanoma specimens to determine the potential effects of routine clinical tissue handling on standard WES data and its utility for clinical decision making.

METHODS

Specimen collection

All tissue samples analysed in this study were obtained from the Melanoma Institute Australia's (MIA) Biospecimen Bank, accrued prospectively with written informed patient consent and institutional review board approval by the Sydney South West Area Health Service institutional ethics review committee. Clinical and follow-up details were collected on all patients. Following routine clinical practice, fresh tissue samples were sent to the Royal Prince Alfred Hospital (RPAH) Pathology Department (e.g., a lymph node metastasis). Following inspection, a small piece was cut for fresh tissue collection, generally adjacent to the tumour sample sent for routine FFPE storage. FFPE blocks collected by the Pathology Department were stored in racks at room temperature and away from direct sunlight. FFPE samples were placed in formalin and later embedded. Fresh snap-frozen samples of ten surgically resected lymph node melanoma metastases were selected, and matched with the routinely collected FFPE tumour tissue blocks from the same specimen.

DNA extraction and quality control

Fresh-frozen tumour samples were sectioned on a cryostat (CM1520; Leica Biosystems, Germany) and stained with Mayer's haematoxylin and eosin (H&E) and scored by a pathologist (RS) to evaluate the following parameters: degree of pigmentation, percentage necrosis, percentage tumour content, predominant cell size and shape, and immune infiltrate density and distribution, as previously described.[14] The minimum tissue criteria required for inclusion in the study was a dissectible tumour area containing greater than 80% tumour content and less than 30% necrosis. Fresh-frozen tumour DNA was extracted at Westmead Millennium Institute (WMI) using Qiagen QIAmp DNA Mini Kits (C#:51304; Qiagen, Germany) according to the manufacturer's instructions. FFPE tumour DNA was extracted at RPAH utilising a NucleoSpin FFPE DNA Kit (REF#:740980.50; Machery-Nagel, Germany) according to the manufacturer's instructions. All samples were quantified using the PicoGreen dsDNA Quantification Reagent (Invitrogen, USA) or Qubit 2.0 (Life Technologies, USA) and fragmentation evaluated with gel electrophoresis.

Whole-exome sequencing

WES of fresh tissue was performed at Macrogen (South Korea) and FFPE specimens at the Garvan Institute of Medical Research (Australia). Library construction was carried out using a TruSeq Exome Enrichment Kit (Illumina, USA) for fresh-frozen DNA, whilst the Nextera Rapid Capture Expanded Exome Kit (Illumina) and NimbleGen SeqCap EZ Exome +UTR Kit (Roche, USA) were used for FFPE DNA according to the manufacturers' instructions. Briefly, 1 µg of DNA was fragmented by nebulisation, the fragmented DNA was then repaired and adapters ligated to the fragments. The size-selected product was PCR amplified, and the final product assessed using an Agilent Bioanalyser. The libraries were then enriched using the appropriate enrichment kit protocol. Briefly, the DNA libraries were hybridised with probes to exonic regions, then washed using streptavidin beads to capture the probes containing targeted regions of interest. Non-specifically bound beads were washed away and the enrichment libraries were eluted from the beads. Libraries underwent a second hybridisation, wash and elution step to further enrich for targeted regions, and were then amplified using sample preparation PCR primer cocktail followed by library validation, clustering and sequencing on a HiSeq 2000.

Sequence data analysis

Data were aligned against the human reference genome using the Burrows-Wheeler Aligner,[15] duplicate reads were marked with Picard, reads were re-aligned against known indels and base-qualities were re-calibrated using the Genome Analysis Toolkit (Broad Institute, USA).[16] Single nucleotide variants (SNVs) in the fresh-frozen tissue and FFPE samples were called jointly with samtools/bcftools.[17] To identify discordant variants, we used the phred-scaled constrained likelihood ratio (CLR), which takes into account coverage, number of variant reads, and base call qualities as described by Li.[18] We defined discordant variants as variants with CLR $\geq$60, which implies the likelihood of getting the data given the called combination of genotypes is a million times greater than the likelihood getting the data given that the genotypes are identical in the two samples. Regions were annotated using ANNOVAR.[19]

For patients sequenced by both Nextera and SeqCap we wanted to compare the two capture kits, and to avoid any bias introduced by differences in sequencing coverage, we randomly removed reads from the higher coverage sample such that the exonic coverage was the same in the two samples. These samples were SNP called and compared with fresh-frozen samples; this procedure was repeated ten times to minimise randomisation effects.

Statistical analysis

GraphPad Prism 6.04 (GraphPad, USA), Adobe Illustrator CS6 and Adobe Photoshop CS6 (Adobe, USA) and OpenRefine utilising GREL and JSON script were used to analyse the sequence data following variant calling and present the data. Differences between groups were determined by paired t-tests with significance set at $p < 0.05$. Multiple comparisons were adjusted by the Holm-Šídák method.

RESULTS

Analysis of sequencing results and quality of fresh-frozen and FFPE samples

To determine whether DNA from fresh-frozen and FFPE tumour samples yielded similar sequence data, we compared the number of reads generated, mapping results and insert size by three techniques (Table 1). WES resulted in a mean of 63, 90 and 140 million reads for fresh-frozen and FFPE tissues captured with the Nextera and SeqCap kits, respectively. FFPE samples produced a significantly (Nextera $p \leq 0.01$; SeqCap $p = 0.036$) lower fraction of mapped reads compared with the fresh-frozen samples (frozen 99%, FFPE Nextera 55% and FFPE SeqCap 69%). Fresh-frozen and FFPE samples showed a similar fraction of mapped on-target and properly paired reads indicating correct genomic configuration. The percentage of uniquely mapped reads (confidence in alignment to correct region) was significantly lower in Nextera