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

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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.

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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]

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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

Table 1 Comparison of sequencing results and quality statistics (fresh versus FFPE)

Reads	Frozen		FFPE-Nextera			FFPE-SeqCap		
	Mean	Range	Mean	Range	*p* value[a]	Mean	Range	*p* value[a]
Total reads, in millions	63.4	56.7–67.9	90.4	20.8–163.9	0.026	140.0	82.8–203.8	<0.01
Mapped reads, %	99.0	98.4–99.3	55.1	12.4–69.6	<0.01	68.9	49.0–77.6	0.036
Mapped on-target reads, %	69.8	59.1–79.2	80.5	79.2–86.3	0.51	92.4	92.0–93.1	0.137
Properly paired reads, %	99.7	99.6–99.8	81.2	54.3–85.7	0.158	84.5	75.2–87.6	0.382
Unique reads (≥Q20[b]), %	92.9	90.0–95.6	42.3	33.0–47.0	<0.01	73.2	71.5–74.4	0.213
Mean insert size, bp	237	212–271	132	115–147	<0.01	131	124–139	<0.01
Samples, *n*	7	–	7	–		4	–	

FFPE, formalin fixed paraffin embedded.
[a] *p* value is adjusted for multiple comparisons (Dunnett).
[b] Phred scaled quality of at least 20.

($p \leq 0.01$) FFPE samples compared to fresh-frozen samples (Supplementary Fig. 1). Gel electrophoresis of FFPE samples showed significant degradation of fixed DNA. As fixation is known to damage DNA via crosslinking, we compared library insert sizes; FFPE samples generated significantly shorter (frozen 237, FFPE Nextera 132, FFPE SeqCap 131) library inserts when compared with the matched fresh-frozen sample (Supplementary Fig. 2). Tissue samples were collected following surgery in the years 1999–2003 and were an average of 13.1 years old at the time of exome sequencing.

FFPE versus fresh tissue only variants

Variants in each FFPE sample were called using the matched fresh-frozen WES as 'normal', allowing us to determine whether a variant was concordant, or observed in the fresh or FFPE sequence only. Patients 1–4 were successfully sequenced with both FFPE kits and the paired raw variants lost in FFPE tissues are presented in Fig. 1. Analysis revealed high numbers of FFPE -only variants for patients 1–7 with an average of 5250 reads (1283–11238) in the Nextera kit. SeqCap capture had an average of 1316 reads (795–2203). Fresh sequence only calls were comparatively low with an average of 163 (0–719) and 176 reads (95–225) for Nextera and SeqCap, respectively. While there was a trend towards the Nextera kit reporting greater discordance with the matched fresh-frozen sequence compared to SeqCap, a paired t-test did not show a significant difference ($p = 0.064$) between the kits. Raw concordant reads are shown in Table 2. The range of total concordant calls varied widely from 2237 to 23,897. Combined results from both kits show a clear trend ($p = 0.005$) of increased FFPE-only calls compared to fresh.

FFPE WES for detection of clinically relevant mutations in melanoma

To assess the value of FFPE derived WES data for use in clinical decision making, a list of 27 genes recently identified as being of clinical importance in melanoma[20] was examined for differences between matched fresh-frozen and FFPE tissue. Figure 2A presents the raw number of concordant calls for each whole exome sequenced patient. Figure 2B shows the results for all 27 clinically important genes in melanoma and presents concordance of the identified mutational changes in each sequence. The results indicate that in general there is a trend towards higher variation between sequences

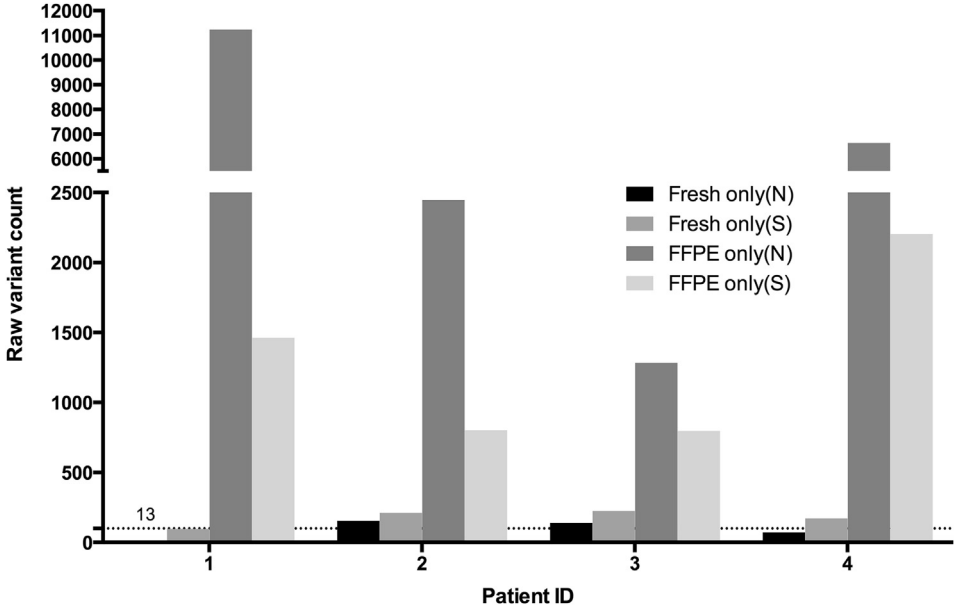

Fig. 1 The count of variants seen only in fresh and FFPE tissue sequences per successfully sequenced patient in both Nextera (N) and SeqCap (S) exome kits. The significant difference between FFPE and fresh only calls can be clearly seen here with FFPE sequencing in both kits displaying relatively high levels of introduced calls.

Table 2 Whole -exome sequencing concordance (FFPE versus fresh tissue)

Patient ID	Raw calls		
	Concordant	Fresh only calls	FFPE only calls
1N	12,716	13	11,239
1S	14,603	95	1463
2N	23,677	154	2446
2S	22,034	211	801
3N	23,897	139	1284
3S	21,400	226	796
4N	15,949	72	6646
4S	17,667	172	2204
5	20,668	719	2415
6	2237	0	3346
7	11,123	46	9382

FFPE, formalin fixed paraffin embedded.

as the number of raw variations increase. From a total of 1299 high quality fresh-tissue calls amongst patients 1–7 in the 27 melanoma genes, we found an average FFPE concordance rate of 43.2% with a wide variation in concordant calls across patients (1–85.7%). The average discordance rate was 1% (range 0–4.9%) and low quality, indicating a low number of reads, or no-coverage (NA) calls accounted for remaining differences between sequences (mean 55.8%, range 14.3–98.4%). On average the SeqCap platform had a higher concordance rate (56.4%) and fewer low quality scores (43.1%) compared to Nextera WES, with 35.6% and 63.1%, respectively. For the clinically important melanoma mutations in BRAF and NRAS, none of three BRAF (V600E) calls made in FFPE sequences were concordant (low number of reads led to low confidence in calls), while NRAS (Q61K and Q61R) demonstrated a concordant result in six of eight cases correctly reporting the known mutation in at least one kit for four of five patients. Mutation status of all patients was confirmed with the use of an OncoCarta Panel.

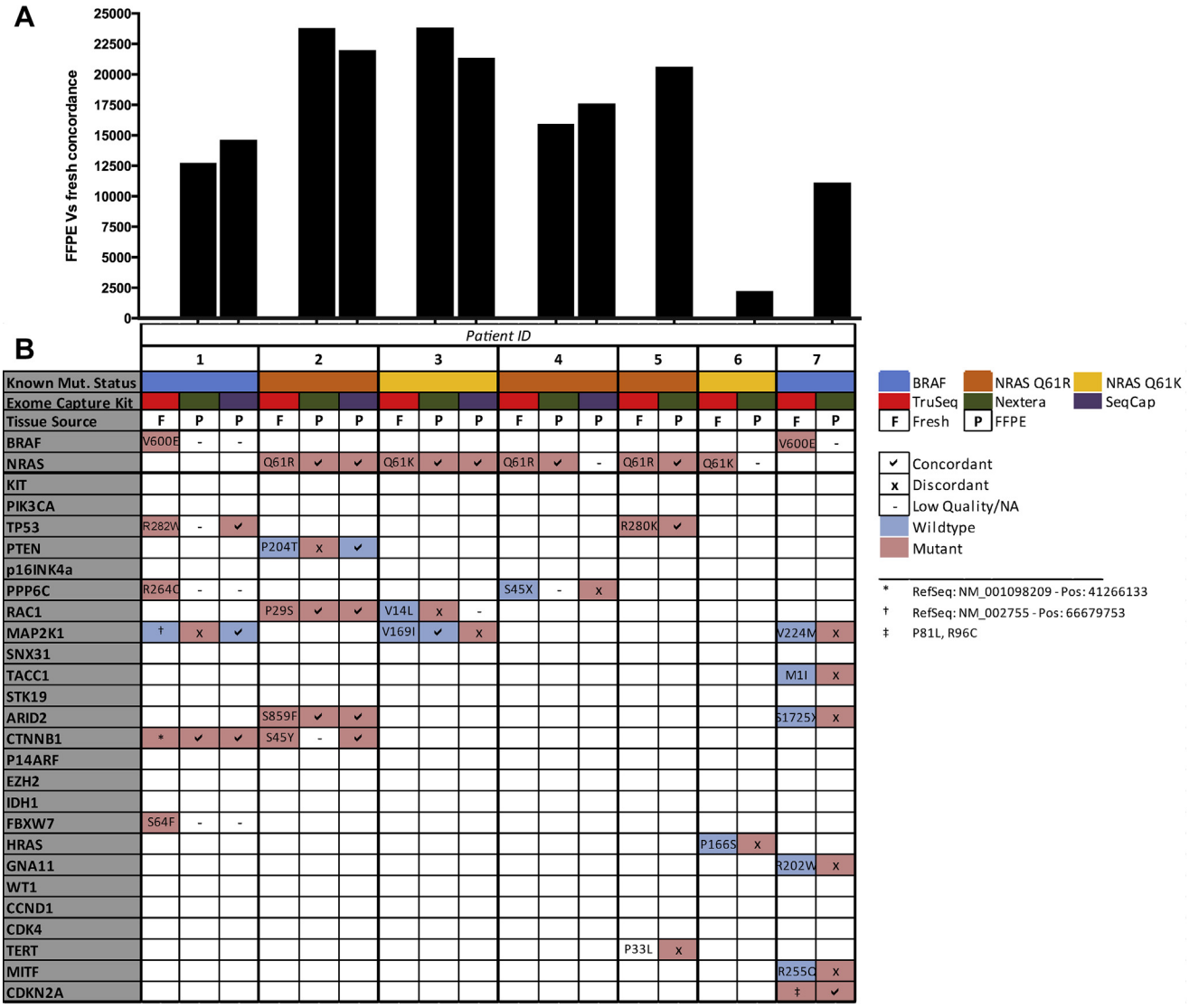

Fig. 2 Discordance between fresh-frozen and FFPE melanoma samples for clinically relevant genes. (A) Raw number reads that were concordant between the FFPE sequence and its matched fresh tissue counterpart. (B) High quality sequence comparison of mutations called between each FFPE capture kit and its matched fresh-frozen tissue counterpart. A tick (✓) indicates concordance of the FFPE call with the matched fresh-frozen (F) sequence while a cross (×) indicates discordance [e.g., a high quality wild-type call, (a sample matched with the reference), and a high quality mutation call]. Reads that were of a low quality and therefore not powered to support clinical actions or outside the kit capture range are shown with a dash (–).

DISCUSSION

Targeted sequencing of carefully selected genes using FFPE samples has become possible in recent years and is regularly used to inform clinical decisions in the management of patients with metastatic melanoma.[21] As the era of personalised medicine evolves, high quality WES offers a number of advantages over targeted sequencing including its ability to prospectively inform clinical decision making as the landscape of gene aberrations that can be therapeutically targeted grows. Another advantage of WES is that as new drugs become available, panel style sequencing may need to be continually updated to accommodate new cancer genes. This may involve lengthy wait times, testing may need to be repeated for the additional genes, and precious DNA may not be available for serial testing, all of which may result in adverse consequences for patient management. Ideally, fresh-frozen tissue is used to for WGS and WES, however such material is usually unavailable in routine clinical practice. Whilst FFPE tissue is a comparatively abundant source of gDNA for clinical WES, attaining the amount of DNA used in this study (1 μg) can be difficult. Additionally, the fixation process, storage temperatures and times have an effect on the quality of the DNA that can be obtained. These factors can contribute to poor quality WES results from the FFPE derived DNA. However, these samples represent the type of conditions present in many pathology laboratories and accurately reflect the samples that are routinely used for clinical molecular testing. As sample storage, DNA extraction, library preparation and sequencing technologies improve, the DNA requirements and accuracy will improve.

A previous study on a single matched prostate cancer tumour reported an overall concordance rate of 84.9% ($n = 1$) between FFPE and fresh-frozen SNV profiles.[22] Recently, another study reported at least one clinically relevant alteration in each of 15 of 16 patients, leading to clinical trial enrolment of one patient. The approach used an altered library construction step, replacing Illumina's paired-end adapters with palindromic forked adapters containing unique barcode sequencing for later pooling.[12] Our study is the first to our knowledge to compare fresh-frozen and FFPE WES data in melanoma patients, and demonstrates the difficulty in attaining actionable FFPE WES results utilising standard library construction methods. Whilst seven of ten patients passed library preparation and were ultimately sequenced in one of the two FFPE kits, the final sequence concordance of 54.5% ($n = 11$) between fresh-frozen and FFPE calls in genes commonly mutated in melanoma was disappointingly poor. However, complete concordance between the paired samples is unlikely due to tumour heterogeneity.[23] As discussed by Gerlinger *et al.*, advances in technology have shown that intra-tumoural genetic heterogeneity can have serious implications for diagnostic biomarker approaches.[23] Recent advances in laboratory technology can help to reduce error when selecting tumour tissue for DNA or RNA extraction. One such piece of equipment, the CryoXtract, allows cores (1.5 and 3 mm) of frozen tissue to be taken from the larger whole with laser guidance to ensure that marked areas on QC slides are successfully sampled. Nevertheless, based on our results, a higher degree of confidence is required before clinical management of patients can be altered based upon WES data produced from FFPE tumour tissue.

In an attempt to address the inadequacies of FFPE for generating WES data, the PAXgene tissue system (PreAnalytix, Switzerland) technology has been investigated as a formalin free alternative method for tissue fixation that can be employed in routine pathology practice. A study comparing PAXgene and formalin fixed tissue in melanoma has previously reported conserved morphology, lower intensity and overall staining by immunohistochemistry and less fragmentation of DNA.[24] However, the authors concluded that the evidence for observed advantages did not outweigh the adverse consequences associated with a large change in routine pathology practice. PAXgene tissue was also shown to have improved reproducibility compared to FFPE tissue for genome-wide methylation analysis utilising Illumina's 450K BeadChip.[25]

In conclusion, FFPE sourced gDNA currently shows little promise for use in clinical WES. For standard library preparation in seven melanoma samples, we observed a mutation concordance rate that was too inaccurate to confidently inform clinical decisions. Appropriately validated targeted NGS is currently the preferred method in routine clinical practice for investigating tumour mutation status as it has high sensitivity and specificity for targetable alterations. Methodological and technical refinements will be necessary to facilitate the routine clinical use of rapid and dependable FFPE WES as a tool in clinical management in the personalised medicine era. Refinements that begin to produce accurate WES results will be invaluable to the field, given the lack of fresh-frozen tissue available and the considerable expense required to store it.

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APPENDIX A: SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pathol.2016.01.001.

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