Genetic profiling of tumours using both circulating free DNA and circulating tumour cells isolated from the same preserved whole blood sample

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\textbf{ABSTRACT}

Molecular information obtained from cancer patients’ blood is an emerging and powerful research tool with immense potential as a companion diagnostic for patient stratification and monitoring. Blood, which can be sampled routinely, provides a means of inferring the current genetic status of patients’ tumours via analysis of circulating tumour cells (CTCs) or circulating tumour DNA (ctDNA). However, accurate assessment of both CTCs and ctDNA requires all blood cells to be maintained intact until samples are processed. This dictates for ctDNA analysis EDTA blood samples must be processed within 4 h of blood draw, severely limiting the use of ctDNA in multi-site trials. Here we describe a blood collection protocol that is amenable for analysis of both CTCs and ctDNA up to four days after blood collection. We demonstrate that yields of circulating free DNA (cfDNA) obtained from whole blood CellSave samples are equivalent to those obtained from conventional EDTA plasma processed within 4 h of blood draw. Targeted and genome-wide NGS revealed comparable DNA quality and resultant sequence information from cfDNA within CellSave and EDTA samples. We also demonstrate that CTCs and ctDNA can be isolated from the same patient blood sample, and give the same patterns of CNA enabling direct analysis of the genetic status of patients’ tumours.

In summary, our results demonstrate the utility of a simple approach that enabling robust molecular analysis of CTCs and cfDNA for genotype-directed therapies in multi-site clinical trials and represent a significant methodological improvement for clinical benefit.

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1. Introduction

Technological advances in blood borne cancer biomarkers now make it possible to routinely analyse RNA and DNA from single cells (Rothwell et al., 2014; Ramskold et al., 2012; Guzvic et al., 2014) including isolated circulating tumour cells (CTCs) and the minute amounts of tumour derived DNA present in patient blood samples (reviewed in Krebs et al., 2014; Diaz and Bardelli, 2014). Circulating cell-free DNA (cfDNA) analysis is emerging as a relatively simple yet powerful biomarker for monitoring disease status and reporting mechanisms of treatment resistance in cancer patients, with the important advantage of being minimally invasive and suitable for longitudinal sampling (Murtaza et al., 2013). CTCs have also been shown to be clinically informative with CTC enumeration recognised as a prognostic biomarker by the FDA in metastatic breast, prostate and colorectal cancers (Cristofanilli et al., 2004; de Bono et al., 2008; Cohen et al., 2008). More recently, CTCs have been expanded in vitro and in vivo providing valuable insights into tumour biology (Hodgkinson et al., 2014) and have the potential to provide a minimally invasive opportunity to study tumour genetic profiles, drug resistance mechanisms and evaluate tumour heterogeneity.

However, for accurate and sensitive analysis of both CTCs and cfDNA, it is important to ensure that blood collection, transport and processing do not result in cell damage or lysis resulting in loss of CTCs or dilution of cfDNA by lysed white blood cell (WBC) contents. Dilution of cfDNA due to WBC lysis may hinder the ability to detect clinically important tumour associated mutations, or lead to misleading estimates of the mutant fraction of cfDNA, thereby impairing studies of residual disease and emergent mechanisms of treatment resistance (Luke et al., 2014; De Mattos-Arruda et al., 2013). In standard cfDNA protocols, WBC lysis is minimized by preparation of plasma within a short time from the blood draw (typically 1–4 h), which may be challenging in non-specialized sites and busy clinics. This requirement for immediate processing of the blood sample severely limits the scope of the use of cfDNA in a larger clinical setting, including genotype-directed multi-centre clinical trials where samples need to be shipped to central laboratories. Recently, the use of dedicated blood collection tubes containing a preservative which allows transport of whole blood at ambient temperature for several days prior to cfDNA isolation has been shown to extend the window within which samples can be used for cfDNA extraction (Norton et al., 2013). For CTC analysis, the gradual loss of cell integrity with prolonged storage of a standard EDTA blood sample is overcome by using a CellSearch® CellSave Preservation tube. This preserves cells in whole blood for up to 4 days at room temperature and allows international transport of blood samples and a standardized workflow without the need for sample processing at collection sites. Using the CellSearch CellSave system, CTCs can be fluorescently labelled and enumerated (Hou et al., 2012), and isolated and genetically characterized by whole genome sequencing (WGS) (Hodgkinson et al., 2014). Analysis of CTCs and cfDNA from the same whole blood sample would extend the molecular information extracted from a single sample and enable a direct comparison of CTCs, that provide information at the single cell level and cfDNA, which represents a global molecular picture of the disease (Kidess and Jeffrey, 2013). Here, we describe the isolation of CTCs and cfDNA from CellSave blood samples, followed by genome-wide and focused next generation sequencing (NGS) to establish reliable and effective analysis of both CTCs and cfDNA from whole blood transported up to 4 days at ambient temperature. This potentially enables non-specialized clinical sites to ship blood samples to central laboratories for expert processing and analysis, reducing the time required for blood processing in busy clinics, minimizing variability in the resultant molecular data obtained and opening molecular analysis of CTCs and cfDNA to large multi-centre clinical trials.

2. Material and methods

2.1. Healthy normal volunteer (HNV) and patient blood sample collection

Paired blood samples were collected in a CellSave and an EDTA vacutainer and transferred to the Clinical and Experimental laboratory for processing. All samples were collected either from HNVs (persons recruited from within the CR-UK Manchester Institute that were not currently suffering or being treated for cancer) or cancer patients following receipt of informed consent in compliance with the Declaration of Helsinki under ethics 07/H1014/96 after approval from the Internal Review and the Ethics Boards of the Christie Hospital NHS Trust. Throughout the study a total of 20 HNV, 11 small cell lung cancer (SCLC) and 34 melanoma patients were recruited and cfDNA isolated.

2.2. cfDNA preparation and quantification

For both EDTA and CellSave blood samples plasma was separated from whole blood by means of two sequential centrifugations (2,000 g, 10 min) and stored at −80 °C in 1 ml aliquots. cfDNA was isolated from 1 ml of double spun plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) as per manufacturer’s instructions. Following isolation the cfDNA yield was quantified using the TaqMan® RNase P Detection Kit (Life Technologies) as per manufacturer’s instructions.

2.3. Enrichment and isolation of CTCs

CTCs and WBCs (pre-stained with antibody to CD45, pan-CK and DAPI) were aspirated from the CellSearch cartridge used for the CTC enumeration, and single cells were isolated using the DEPArray system (Silicon Biosystems) as per manufacturer’s instructions. WGA of single CTCs and WBCs was performed using the Ampli1 WGA kit (Silicon Biosystems) according to manufacturer’s instructions.

2.4. NGS library preparation and sequencing

Whole genome sequencing (WGS) of CTC derived explant tumours (CDX), CTCs and WBCs was carried out as previously
described (Hodgkinson et al., 2014). Focused NGS of samples with a minimum of 8 ng cfDNA was performed using the Qiagen GeneRead Lung Cancer v1 Panel (Qiagen) as described by the manufacturer, except input was reduced to as low as 8 ng DNA (ensuring ≥2 ng input into each of the 4 Qiagen GeneRead multiplex PCR reactions). This panel covered 20 genes commonly mutated in lung cancer (MTOR, NRAS, PFGS2, PTEN, HRAAS, KRAS, RB1, AKT1, TP53, ERBB2, STK11, ALK, CTNNB1, PIK3CA, PDGFRα, KIT, EGFR, MET, BRAF, CDKN2A). WGS of cfDNA was carried out using the NEBNext Ultra™ DNA Library Prep Kit for Illumina® kit (NEB) using 5 ng DNA input. NGS for both focused GeneRead libraries and WGS cfDNA libraries was carried out using an Illumina® MiSeq desktop sequencer.

2.5. Targeted NGS analysis

Analysis of the GeneRead NGS data was performed on the Qia- gen Cloud-Based DNAseq Sequence Variant Analysis software according to the manufacturer’s instructions. For WGS, analysis paired-end sequence reads were aligned to the human reference genome GRCh37/hg19 using the Burrows–Wheeler alignment tool (BWA, version 0.7.4) with default parameters and the BWA-MEM algorithm. The alignments were sorted and indexed by chromosome coordinates using SAMtools (version 0.1.19), followed by PCR duplicates removal using Picard tools MarkDuplicates function (version 1.96) (http://picard.sourceforge.net). Single nucleotide variants (SNVs) were identified using VarScan2 (version 2.3.7) with the following settings: min-coverage = 8, min-reads2 = 2, min-avg.qual = 15, min-var-freq = 0.01, p-value = 0.01.

2.6. Copy number aberration analysis from WGS data

Paired-end sequence reads were aligned to the human reference genome GRCh37/hg19 using SMALT aligner (version 0.7.1, http://www.sanger.ac.uk/resources/software/smalt/). SMALT index was built by setting k = 20 and b = 13. The alignments were sorted and indexed by chromosome coordinates using SAMtools (version 0.1.18). Copy number variations were predicted by using Control-FREEC (version 6.4) with the following settings: coefficientOfVariation = 0.1, ploidy = 2, mateOrientation = FR. Control-FREEC produces different window sizes according to the sequencing depth in each sample. In order to cluster the samples by their copy number profiles, we decomposed the overlapping windows into disjoint (i.e. non-overlapping) windows. The newly formed bins inherited the copy number status that was assigned to its parental window before decomposition. After this operation, we obtained a matrix with equal number of bins across samples. Then the samples were hierarchically clustered by their copy number profiles based on the Euclidian distance and the Ward linkage method in R.

2.7. Evaluation of NGS error rates

Two metrics were used to infer mutation rate in the CellSave and EDTA samples: the first was calculated as the number of SNV detected divided by total number of bases in the pileup file; the second metric was calculated by dividing the number of SNV detected by number of bases with at least 8× coverage in the pileup file.

To account for variation in sequencing depth between samples, we performed 100 down-sampling of the aligned data, keeping 1 million read pairs in each iteration. We re-calculated the mutation rates by averaging the output from all iterations. A two-tailed t-test was performed to assess if the mutation rate is significantly different between CellSave and EDTA samples.

3. Results

3.1. Isolation of cfDNA from EDTA and CellSave HNV blood samples

Our objective was to evaluate the ‘real life’ utility of CellSave preserved whole blood collection for analysis of cfDNA and CTCs as applied to blood samples obtained in multiple sites and shipped to a centralised laboratory for analysis. This had the wider goal of developing a standardised protocol to facilitate the generation of consistent, molecular analysis of both cfDNA and CTCs in clinical samples. To determine the effect of WBC lysis on cfDNA yields following long term storage (>24 h) of whole blood in EDTA, we isolated plasma from blood within 1 h of collection in a standard EDTA vacutainers tube and then at 24, 48, 72 and 96 h post-draw. Following isolation, the cfDNA yield was determined using the RNaseP real-time PCR assay (Figure 1A). Increasing amounts of cfDNA were detected over time, with almost a 3-fold increase seen by 24-h post-draw, increasing to over 60 fold by 96 h, which could reduce the ability to detect the ctDNA fraction within clinical samples.

To evaluate the suitability of using CellSave to reduce WBC lysis and facilitate cfDNA analysis, we undertook a 20 healthy normal volunteers (HNV) study where each HNV donated two EDTA and two CellSave blood samples. For each HNV donor cfDNA was isolated from one EDTA and one CellSave tube within 4 h post blood draw (isolation range 2.0–3.3 h, mean = 2.8 h). The remaining EDTA and CellSave tubes were sent through the British postal system back to the host institute using a Royal Mail Safe Box™, then maintained at ambient temperature storage for up to 96 h post-draw (isolation range 93.3–95.3 h, mean = 94.5 h) (Figure 1B). The yield of cfDNA from all samples was determined using an RNaseP real-time PCR assay, and showed no significance difference between the 4 h EDTA, 4 h CellSave and 96 h CellSave samples (Figure 1C). As expected, a significant increase in cfDNA was seen in the 96 h EDTA sample compared to both the CellSave samples and the 4 h EDTA sample, reflecting extensive WBC lysis.

3.2. Evaluation of EDTA and CellSave cfDNA NGS error rates

Although the CellSave preservative significantly reduced the level of WBC lysis, thereby maintaining the ctDNA fraction within samples, it is possible that the components of the CellSave tube could act as a DNA damaging agent and effectively increase background sequencing errors. To test this,
standard EDTA and 96 h CellSave cfDNA samples from the 20 HNV were subjected to WGS. To estimate the overall mutation burden low pass WGS Illumina MiSeq sequencing data were generated from three technical replicates of each sample set, with pooled cfDNA of each sample set being used to obtain the 5 ng cfDNA input. Over $1.0 \times 10^8$ bases were sequenced for each library with approximately $9.5 \times 10^3$ single nucleotide variants (SNVs) identified per sample when analysed against the Hg19 genome. No significant difference was found between the overall quality of the NGS data in terms of overall coverage, mapability, duplicates and total reads, and mutation rates of the CellSave (60.4 SNV per million bases) compared to the EDTA samples (58.9 SNV per million bases) indicating CellSave cfDNA is compatible with extended NGS strategies (Figure 2A and 2C). Analysis of the types of SNV detected within the cfDNA in each collection tubes was also performed, with similar frequencies of transitions and transversions seen in both sample type suggesting no effect of CellSave preservative on cfDNA integrity (Figure 2B).

3.3. Isolation of cfDNA from EDTA and CellSave patient blood samples

CellSave vacutainers are routinely used for CTC enumeration using the CellSearch® platform and molecular analysis of CTCs retrieved from CellSearch® cartridges can be achieved using both focused and genome wide NGS (Hodgkinson et al., 2014; Gasch et al., 2013; Heitzer et al., 2013). Since the CellSearch® system requires 7.5 ml blood input and the CellSave vacutainer can hold up to 10 ml there is often surplus

![Figure 1](image1.png)

Figure 1 – A. Graph showing increase in cfDNA levels in plasma from EDTA blood left at room temperature for up to 96 h post-draw. B. Schematic of EDTA and CellSave cfDNA stability study. C. cfDNA yields from 20 HNV blood samples collected in EDTA or CellSave and processed either 4 h or 96 h post-draw. No significant difference in overall yields between the 4 h EDTA, 4 h CellSave and 96 h CellSave samples with a highly significant increase in cfDNA yield following 96 h in EDTA.
blood, which can be used for additional analyses. To test the suitability of CellSave for cfDNA analysis of clinical samples, we compared yields of cfDNA obtained from surplus CellSave blood to yields of cfDNA obtained from sample obtained from a parallel EDTA blood sample processed to plasma within 4 h from two clinical cohorts. Analysis of 11 SCLC and 34 melanoma patient samples showed comparable yields of patient cfDNA from 4 h EDTA plasma (hereafter referred to as standard EDTA) to cfDNA isolated from CellSave blood kept at room temperature for up to 96 h (Figure 3A). This mirrored the results from the HNV experiment and showed CellSave blood to be stable source of both cfDNA and CTCs for clinical sample analysis.

3.4. Targeted NGS of EDTA and CellSave cfDNA

With the drive to utilise cfDNA to identify disease-associated mutations we next tested the suitability of CellSave cfDNA for targeted NGS analysis of clinical samples. To this end, 5 of the 11 SCLC patients with above 8 ng of cfDNA available for both standard EDTA and CellSave cfDNAs were analysed using the Qiagen GeneRead Lung Cancer Panel. This panel consists of 4 pools of PCR-based amplicons that covers 20 lung cancer associated genes. Analysis of the NGS data was carried out and compared to a corresponding germline sample for each EDTA and CellSave sample. In keeping with the high frequency of TP53 mutations in SCLC (Peifer et al., 2012; Rudin et al., 2012; George et al., 2015), somatic TP53 mutations were identified in 4 of the 5 SCLC patients analysed with essentially identical results observed for both EDTA and CellSave matched samples (Figure 3B). For 1 patient (SCLC-03) who did not harbour a detectable TP53 mutation, an ALK mutation was detected, again with similar levels seen for both EDTA and CellSave matched samples. For patient SCLC-05, in addition to a TP53 mutation, a second lower frequency mutation in ERBB2 was also consistently identified in both EDTA and CellSave samples suggesting possible tumour heterogeneity within this patient.

3.5. Whole genome copy number alteration (CNA) of matched cfDNA and CTCs

As well as the identification of tumour associated mutations, low depth whole genome sequencing (WGS) of cfDNA can be used to characterise CNA patterns arising from the circulating tumour DNA (ctDNA) present in the total cfDNA (Leary et al., 2012; Mohan et al., 2014). Since we and others have shown CNA analysis can be readily applied to CTCs isolated following CellSearch enrichment (Hodgkinson et al., 2014; Gasch et al., 2013; Heitzer et al., 2013), use of CellSave for cfDNA isolation would enable combined CTC and cfDNA analysis from the same collection tube (Figure 4A), thereby maximizing the potential clinical information that can be elucidated.
To establish combined CTC and cfDNA analysis, 7.5 ml of a CellSave whole blood sample was used for CTC isolation via CellSearch and DEParray as previously described (Hodgkinson et al., 2014) and the remaining CellSave blood (typically 1–2.5 ml) was used to prepare cfDNA. WBCs were used as a germline control for CTC CNA analysis and WGS of whole blood DNA served as a germline control for the cfDNA samples. For one patient, we were also able to generate CDX tumours in an immune-compromised mouse following CTC enrichment of a parallel EDTA blood sample. We have previously shown that these enriched CTCs can give rise to Circulating tumour cell Derived Xenografts (CDX tumours) that provide tumour material (Hodgkinson et al., 2014), to compare CNA patterns from both CTCs and cfDNA obtained from the corresponding CellSave blood sample. Figure 4 shows the comparison of CNA profiles generated from isolated CTCs, EDTA cfDNA, CellSave cfDNA, two CDX tumours, germline gDNA and isolated WBC DNA from 2 SCLC patients. The results show a clear tumour related CNA patterns in matched CTC, CDX and cfDNA with similar patterns seen for both CellSave and EDTA cfDNA. The pattern of gain and loss in the two CDX tumours in patient 1 (Figure 4B) are consistent with previously published studies on CNA in SCLC (Peifer et al., 2012; Rudin et al., 2012) with regions containing RASSF1 and FHIT being lost and regions containing SOX2 and BCL2 showing amplification. The CDX tumours also show amplification of regions of chromosomes 2 and 14, with this pattern also observed in both CTCs and all cfDNA samples. In patient 2 (Figure 4C) there was no CDX tumour available, but regions of loss and gain in the CTCs correspond well with published data, including loss of chromosome 17 (TP53) and amplification of chromosome 3 (SOX2). A similar pattern of loss and gain is also seen in the CNA of the cfDNA samples, with good correlation between the EDTA and CellSave samples showing CellSave cfDNA to be suitable for NGS CNA and compatible with combined CTC collection and analysis.
Figure 4 – A. Schematic showing procedure for processing of a single blood sample to give NGS analysis of cfDNA and CTCs. B & C. Unsupervised, hierarchical clustering of CNA profiles in two SCLC patients. CNA profiles were generated from isolated CTCs, EDTA cfDNA, CellSave cfDNA, two CDX tumours (A only), germline gDNA and isolated WBC. Matching patterns of gain (regions of red) and loss (regions of blue) were seen across all tumour material and were absent from germline controls. Arrows indicate location of common copy number aberrations found in SCLC with red indicating gain and blue loss. Dark filled arrows indicate loci altered in the patient sample.
4. Discussion

The utilisation of blood borne biomarkers such as ctDNA and CTCs for the molecular profiling of tumours and longitudinal sampling has immense clinical potential that is starting to be realised (Murtaza et al., 2013; Garcia-Murillas et al., 2015). However, to fully realise this potential it is important that the biomarkers are applicable to multi-centre studies and accurately represent the molecular status of the tumour at the time of collection. In an attempt to ensure this we have evaluated the use of preserved CellSave whole blood as a source of both CTCs and cfDNA.

Initial experiments looking at cfDNA yields from CellSave bloods were consistent with both an effective reduction in WBC lysis and efficient cfDNA isolation from whole blood CellSave samples kept at ambient temperature for up to 96 h. In addition, WGS NGS analysis of overall mutational burden following storage in CellSave showed no significant difference to the 4 h EDTA sample. Both of these results suggest that CellSave whole blood is a viable source of cfDNA.

Targeted NGS of matched EDTA and CellSave patient cfDNA samples in 5 SCLC patients identified the same mutations with similar tumour allele frequencies supporting the suitability of CellSave whole blood collection for cfDNA molecular analysis of clinical samples. Tumour specific mutations were identified in both EDTA and CellSave isolated ctDNA, with TP53 mutations, which are commonly associated with SCLC, seen in 4 of the 5 patients. An ALK mutation was identified in both the EDTA and CellSave ctDNA in the remaining patient. Although ALK mutations at this locus have not been previously reported, low frequency ALK translocations have been observed in SCLC (Toyokawa et al., 2013) raising the possibility that the detected mutation is involved in the pathology of the disease.

A major benefit of CellSave blood is that it enables the analysis of both CTCs and cfDNA from the same tube. CTC analysis provides molecular analysis of the tumour at the single cell level and has the potential to give insight into tumour heterogeneity and EMT and mechanisms of metastatic spread of the disease. ctDNA analysis provides a global picture of the genetic status of the disease with ctDNA being released from all disease sites enabling possible longitudinal detection of tumour evolution and resistance mechanisms. This analysis is interesting as it enables direct comparison of the two as potential liquid biopsies and allows an evaluation of the importance of determining whether genetic alterations picked up by ctDNA assessment are co-expressed in single CTCs.

In summary, we have demonstrated the suitability of whole blood CellSave samples for both CTC and cfDNA molecular analysis. The ability to generate informative molecular profiles of both CTCs and cfDNA from a simple whole blood sample shipped at ambient temperature for up to 4 days represents a significant methodological improvement for clinical benefit. The ability to process samples at a single recipient site avoids site-to-site variability, a major confounding issue in cfDNA analysis (Gormally et al., 2004, 2007). Furthermore, the use of a simple blood collection protocol does not require specialised equipment, such as centrifuges or even refrigeration, extending the number of clinical sites that can participate in patient evaluation via liquid biopsies to anywhere where a blood draw is taken. For example, following initial cancer therapy, patients in remission can be monitored via a blood draw at a local medical practice rather than necessitating often lengthy/expensive trips to a specialised oncology centre.

In September 2015, the first ctDNA companion diagnostic assessing EGFR mutation for patient stratification was approved by the EDA (Douillard et al., 2014). We posit that as minimally invasive, liquid biopsies become increasingly employed for cancer patient management, the ability to routinely and simply draw blood and ship samples to accredited biomarker assessment laboratories will facilitate the dawn of this new development in the delivery of personalised cancer medicines.

Conflicts of interest

No conflicts of interest.

Authors’ contributions

GB devised the study and co-wrote the manuscript. DGR co-wrote the manuscript, planned and ran cfDNA NGS experiments. YL and HS performed the bioinformatic analysis. NS devised and ran the NHV study. DM isolated the patient ctDNA. AH, JA and MA generated and ran some of the cfDNA NGS experiments. LC collected clinical samples and analysed data. FHB and LK oversaw ethical permission and patient consent for blood samples. CM and CD designed and supervised the project. All authors read and approved the final manuscript.

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