Integration of RNA and DNA Blood Borne Cancer Biomarkers

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Background

- Conventional tumour biopsies are expensive, invasive and severely limited both spatially and longitudinally
- Increasingly liquid biopsies, including **circulating tumour DNA (ctDNA)** and **circulating tumour cells (CTCs)** provide an alternative to determine the genetic profile of cancer patients
- Here we describe approaches which allow the analysis of ctDNA, CTC DNA and CTC RNA from the same blood sample

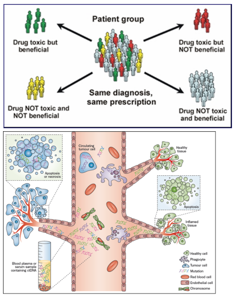


Figure 1. A. Stratification of patients in a personalised therapy approach B. Generation of ctDNA and CTCs in patients' circulation (Taken from Crowley et al. 2013)

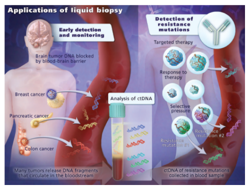


Figure 2. Applications of cfDNA in a clinical setting (Taken from Bettegowda et al. 2014)

Approaches

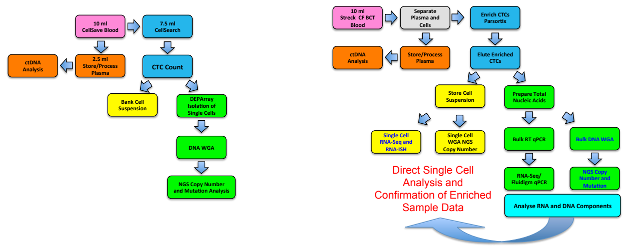


Figure 3. Workflows showing fractionation and analysis of the cellular and plasma components of clinical whole blood samples. Blue text indicates studies in progress.

EDTA vs CellSave ctDNA

- White cell lysis (WCL) increases ctDNA levels in EDTA blood samples up to 4 days post-draw
- Collection in CellSave tubes prevents WCL and maintains the cfDNA levels
- Clinical samples showed no difference in ctDNA yield between conventional EDTA and CellSave blood

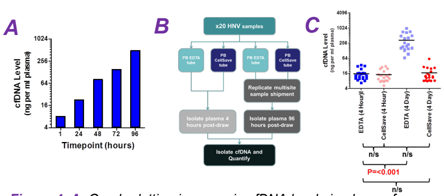


Figure 4. A. Graph plotting increase in cfDNA levels in plasma from EDTA blood left at room temperature for up to 96 hours. B. Schematic of HNV EDTA and CellSave cfDNA stability study. C. cfDNA yields from 20 HNV blood samples collected in EDTA or CellSave and processed either 4 hours or 96 hours post-draw. No significant difference in overall yields between the 4 hour EDTA, 4 hour CellSave and 96 hour CellSave samples with a highly significant increase in cfDNA yield following 96 hours in EDTA

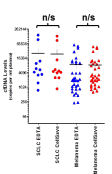


Figure 5. Yields of cfDNA from duplicate clinical samples collected in EDTA and CellSave bloods from a cohort of 11 SCLC and 34 melanoma patients. No significant difference was found between each collection type.

EDTA vs CellSave NGS

- No measurable differences were seen when comparing NGS data from cfDNA obtained from either CellSave or EDTA blood

A

Sample	Bases in pileup	SNP predicted value (p-value<0.01)	# SNV per Million bases
EDTA 1	157057344	9345	59.501
EDTA 2	158379699	9210	58.152
EDTA 3	157007320	9249	58.908
CellSave 1	161532233	9725	60.205
CellSave 2	159561656	9816	61.519
CellSave 3	161275644	9649	59.829

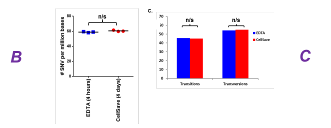


Figure 6. A. Triplicate NGS libraries were generated from HNV cfDNA isolated from 4-hour EDTA and 96-hour CellSave cfDNA. Low resolution sequencing was then used to determine the mutation rate for each sample. B. No significant difference was found between mutation rates in 4-hour EDTA and 96-hour CellSave libraries. C. No significant difference was found in the repertoire of transitions and transversions between the two sample sets.

			TP53 7578212 G>A Stop gain		TP53 7577022 G>A Stop gain		TP53 7577574 A>G Y236C		TP53 7578281 C>A Stop P190T		ERBB2 37884019 G>T G1164C		ALK 29416481 A>G K1491R	
Patient Sample	Sample type	cfDNA input (ng/rxn)	Mutation Detected	Mutation frequency	Mutation Detected	Mutation frequency	Mutation Detected	Mutation frequency	Mutation Detected	Mutation frequency	Mutation Detected	Mutation frequency	Mutation Detected	Mutation frequency
SCLC-01	WT gDNA	20.0	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA
	EDTA cfDNA	10.4	Mut	0.47	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA
	CellSave cfDNA	14.7	Mut	0.48	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA
SCLC-02	WT gDNA	2.0	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA
	EDTA cfDNA	2.0	WT	NA	WT	NA	Mut	0.26	WT	NA	WT	NA	WT	NA
	CellSave cfDNA	2.0	WT	NA	WT	NA	Mut	0.31	WT	NA	WT	NA	WT	NA
SCLC-03	WT gDNA	2.0	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA
	EDTA cfDNA	2.0	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	Mut	0.34
	CellSave cfDNA	2.0	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	Mut	0.42
SCLC-04	WT gDNA	20.0	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA
	EDTA cfDNA	22.5	WT	NA	Mut	0.63	WT	NA	WT	NA	WT	NA	WT	NA
	CellSave cfDNA	19.5	WT	NA	Mut	0.65	WT	NA	WT	NA	WT	NA	WT	NA
SCLC-05	WT gDNA	2.0	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA	WT	NA
	EDTA cfDNA	2.0	WT	NA	WT	NA	WT	NA	Mut	0.79	Mut	0.08	WT	NA
	CellSave cfDNA	2.0	WT	NA	WT	NA	WT	NA	Mut	0.71	Mut	0.09	WT	NA

Figure 7. Mutations identified in five SCLC patient samples using a targeted NGS approach. Germline gDNA, EDTA cfDNA and CellSave cfDNA was analysed for each patient. Mutations were called with read counts >200 and frequency >10%. Mutated samples are indicated by red fill with WT alleles indicated in green.

Combined cfDNA and CTCs

- Comparable CNA profiles were found in both CTCs and cfDNA from the same patient, which also matched tumour (CDX) CNA profiles

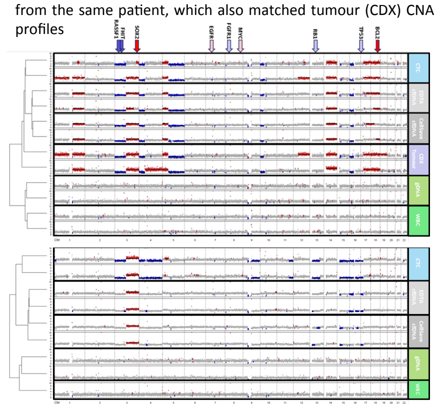


Figure 8. Copy number analysis (CNA) of two SCLC patients. CNA profiles were generated from isolated single CTCs, EDTA cfDNA, CellSave cfDNA, and two CDX tumours (lower panel). Matching patterns of gain (red) and loss (blue) were seen across all tumour material. Loci commonly amplified (red arrow) or deleted (blue arrows) are shown above each plot with common SCLC associated aberrations detected in both samples.

Data from this section now published: Rothwell et al (2015). "Genetic profiling of tumours using both circulating free DNA and circulating tumour cells isolated from the same preserved whole blood sample." Mol Oncol. Epub ahead of print

DNA and RNA Analysis of Cells Retrieved from Parsortix

- High quality WGA products obtained from single tumour cells spiked into HNV blood and enriched via Parsortix
- Analysable RNA obtained post Parsortix enrichment of clinical samples

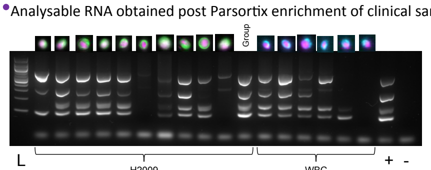
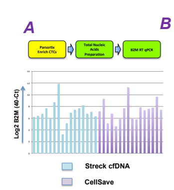


Figure 9. The figure shows the results of single cell whole genome amplification (WGA) of H2009 cells spiked into HNV blood and subsequently enriched using Parsortix, fluorescently labelled (KRT 18/19, CD45 and DAPI) and isolated using DEPArray. Following WGA the quality of amplification was assessed using a PCR based Genomic Integrity Assay (GI Index Silicon Biosystems) where the presence of 4 bands indicates high quality. Pictures of isolated cells are shown above the gel lane used to run the matched GI Index.



B

	Parsortix Prostate 1	Parsortix Prostate 2	Parsortix Prostate 3	Parsortix Prostate 4	Parsortix Prostate 5	White Blood Cells	Parsortix NSCLC 2.9
SPINT2	10.9	6.2	9.1	ND	ND	ND	15.0
ANXA1	ND	8.8	7.6	14.0	ND	9.7	14.6
ANXA9	ND	ND	10.4	ND	ND	ND	26.4
B2M	17.5	17.5	18.8	20.9	12.3	18.1	ND
CALM2	12.8	13.8	14.1	14.4	8.5	12.3	23.6
CD2	ND	10.9	ND	13.9	ND	11.4	13.9
CDH1	ND	ND	ND	5.9	ND	ND	ND
CLDN10	ND	ND	ND	ND	ND	ND	8.8
CXCL10	ND	ND	ND	ND	ND	ND	7.9
EGR1	ND	ND	ND	ND	ND	ND	26.0
EPCAM	ND	6.0	ND	ND	ND	ND	24.1
FXYD3	4.3	ND	ND	ND	ND	ND	19.2
FYB	12.1	12.9	14.4	15.3	ND	13.1	1.1
GRB14	10.0	9.3	9.4	8.8	ND	8.7	ND
IDO1	ND	ND	ND	11.7	ND	ND	5.0
IFI44L	ND	ND	ND	6.1	ND	ND	ND
IGFBP7	ND	8.4	ND	7.6	ND	ND	ND
ITGAL	ND	2.6	ND	10.3	ND	ND	ND
KRT19	ND	ND	ND	ND	ND	ND	23.0
KRT5	ND	ND	ND	2.1	ND	ND	14.5
MX1	ND	ND	ND	8.5	ND	ND	ND
NAT1	ND	ND	ND	8.5	ND	ND	ND
NLRC5	ND	ND	ND	4.9	ND	ND	ND
PRAME	ND	ND	ND	ND	ND	ND	15.2
S100P	9.3	ND	12.4	18.7	ND	12.7	15.3
SLPI	ND	9.9	ND	12.4	ND	9.6	25.3
VIM	7.8	10.6	13.4	18.1	9.3	12.6	5.2

Figure 10. A. RT qPCR of the reference gene B2M applied to RNA extracted from Parsortix bulk CTC enrichment of samples from patients with colorectal cancer. Whole blood samples were obtained using either Streck cfDNA (blue bars) or CellSave (purple bars) blood collection tubes and maintained at RT for up to 4 days prior to Parsortix enrichment and analysis. B Fluidigm RT-qPCR applied to RNA extracted from Parsortix bulk CTC enrichment of samples from patients with locally advanced prostate cancer (Prostate 1-5), a known CTC positive NSCLC sample (NSCLC 2.9) and RNA prepared from a blood mononuclear fraction (White Blood Cells).

Summary

- CellSave collection tubes suitable for ctDNA analysis as well as CTC analysis
- Streck cfDNA collection tubes suitable for CTC analysis as well as cfDNA analysis (also see Chudziak et al 2016 for additional data on Parsortix CTC enrichment using Streck cfDNA tubes)
- Equivalent NGS data obtained with ctDNA obtained either from CellSave or EDTA plasma
- Measurable RNA obtained following Parsortix CTC enrichment from clinical samples supplied in either Cellsave or Streck cfDNA tubes
- Ongoing RNA-Seq and droplet digital PCR studies aimed at establishing the quality, consistency and utility of RNA obtained from Cellsave and Streck cfDNA blood samples

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