Q-PCR and sequence analysis of DNA template from a microfluidic CTC isolation platform.

CYNVENIO biosystems

FOUNDATION MEDICINE

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Abstract

Sequence analysis and quantitative allele specific PCR (QPCR) methods permit genetic profiling of cancer for targeted therapeutic selection; such personalized treatments have been associated with improved outcomes in cancer. Circulating tumor cells (CTC) offer a minimally-invasive opportunity for serial patient sampling, and potentially a means of tracking the molecular evolution that underlies the behavior and response phenotype of the disease including potential therapeutic response markers. Acquiring these types of patient profiles requires a platform and workflow providing reliable detection and recovery of small numbers of mutation-bearing CTC from a blood sample. Availability of such an enabling platform is a necessary prerequisite to the clinical correlation studies needed to demonstrate the utility of mutation-bearing CTC to patient care .

We have successfully purified CTCs and converted them into DNA template of sufficient purity and quality to support multiple non-overlapping advanced molecular characterizations. Beginning with whole human blood spiked with defined numbers of cultured cancer cells as surrogates for CTCs, cells were successfully fluid-phase labeled using an anti-EpCAM antibody ferrofluid. Using a proprietary microfluidic sheath flow technology, EpCAM positive, cytokeratin staining cells were selected from 2 to 4 ml of labeled blood. This method produced sufficient DNA template for multiple analyses per patient sample. QPCR analysis of these templates demonstrated reproducible detection of fewer than 1% target cells in a background of non-target cells, allowing detection of the KRAS G12S mutation from as few as 5 recaptured cancer cells.

The same DNA templates were then used for hybrid capture and next-generation sequencing of a panel of more than 200 cancer-related genes. This sequencing platform was able to detect multiple somatic mutations in genomic DNA templates produced from samples containing as few as 10 cancer cells per milliliter of blood. Together these data provide initial proof-of-concept for a system capable of detecting and characterizing mutations across any specified set of genes within purified CTC populations.

Material and Methods

Model System – Cell Line Spike-ins

- Cultured A549 lung cancer cells were added at a range of abundances to aliquots of fresh human whole blood.
- A549 cells were recovered using Cynvenio CTC flow cell technology (Figure 1).
- DNA was isolated from cells and then amplified using Φ-29 WGA (GE Healthcare).

Patient Specimens

- Patients consented to participate in and donate blood for an IRB-approved protocol designed to demonstrate isolation of CTCs from the blood of patients diagnosed with primary, node-positive, or metastatic breast carcinoma and subsequent molecular analysis of the CTC genome.
- Patient CTCs were recovered using Cynvenio CTC flow cell technology (Figure 1) and enumerated using a combination of CD45, DAPI and cytokeratin staining.
- DNA was isolated from CTCs and amplified using Φ-29 WGA (GE Healthcare).

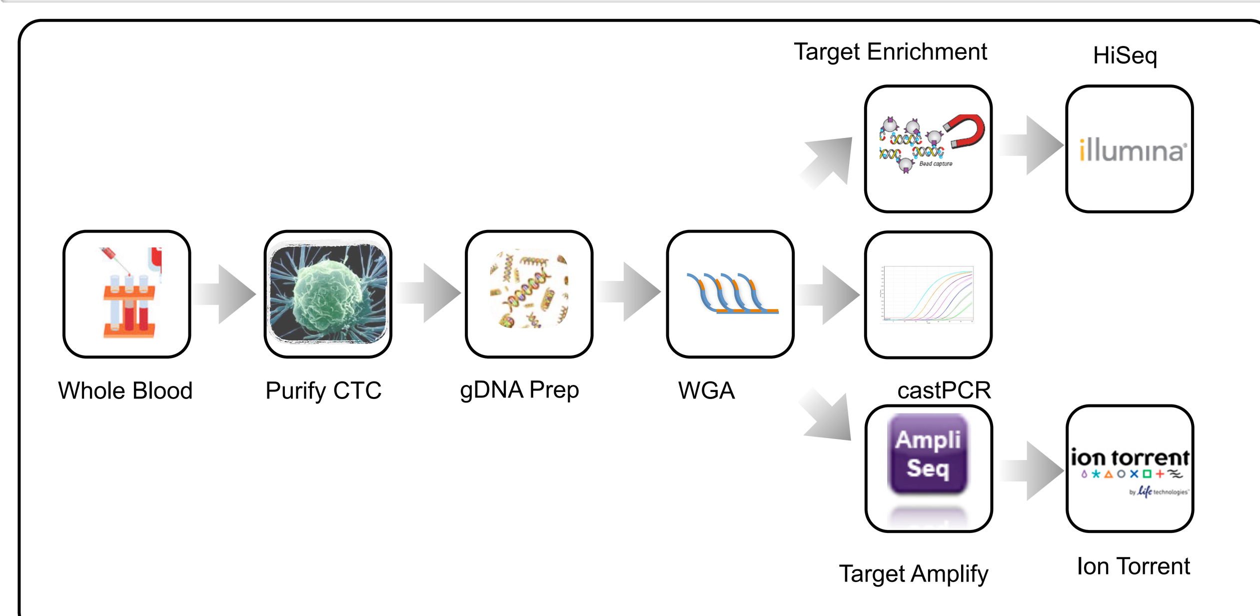

Figure 2. Sample prep and analysis pipeline. Tumor cells (either from patient samples or engineering spiked tumor cell lines) are isolated and amplified template prepared. Template is then analyzed by castPCR or massively parallel sequencing. The sequencing analysis pipeline determines point mutations (Bayesian algorithm), short InDels (local assembly) and annotates by comparison to dbSNP and COSMIC for identification of germline variants and known tumor-associated mutations respectively.

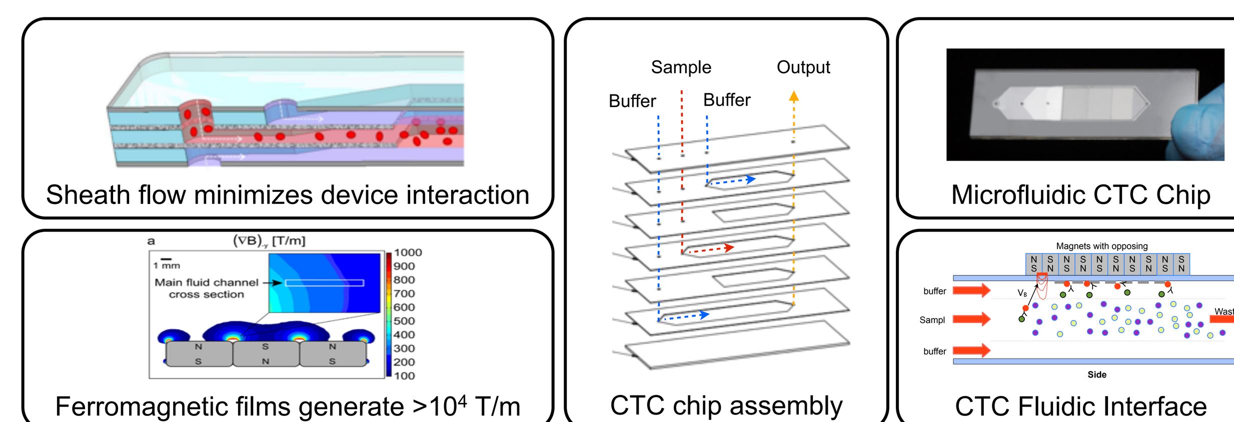

Figure 1. Tumor cells are isolated from whole blood using the Cynvenio CTC Flow Cell

DNA Sequencing: *Two different sequencing methods were employed:*

Method I

- Sequencing libraries were constructed directly from WGA products by ultrasonic shearing and barcode adaptor ligation.
- Library molecules corresponding to all exons of 182 cancer-associated genes were selected using a custom Agilent SureSelect^{TM} bait reagent.
- Sequencing used 2x50bp paired-end reads on the Illumina HiSeq2000^{TM} platform.
- The Foundation Medicine analytical pipeline was used to identify and annotate point mutations, (Figure 2).

Method II

- Amplified DNA was enriched using the Ampliseq Cancer Panel from LIFE Technologies.
- Emulsion based enrichment using the One Touch technology (LIFE) was followed by sequencing using ION TORRENT PGM.

Competitive Allele Specific TaqMan PCR (castPCR)

- castPCR was performed in triplicate 5µl reactions in an ABI ViiA7 Platform with a 384w plate.
- Mutation detection assay and reference probes were run for each sample. Each contains an allele specific primer, a locus specific Taqman probe, a locus specific reverse primer and an allele-specific MGB blocker.
- Data are presented as the difference in threshold cycle between the mutant and reference probes (∂Ct).

Results

Microfluidic capture of cultured epithelial tumor cells introduced into whole human blood was dependent on expression of EpCAM, the ligand of our capture antibody. We chose to develop our proof-of-concept experiments around low-expressing A549 cells (average 25% recovery; Figure 3A) in hopes of modeling a majority of real-world CTC populations. ISMAC separations reproducibly yielded a background capture of 200-600 white blood cells (WBC; Figure 3B). Recovered A549 cells from spike-ins prepared for sequencing ranged from 2% to 14% of total cells captured; the remainder are presumed to be WBC.

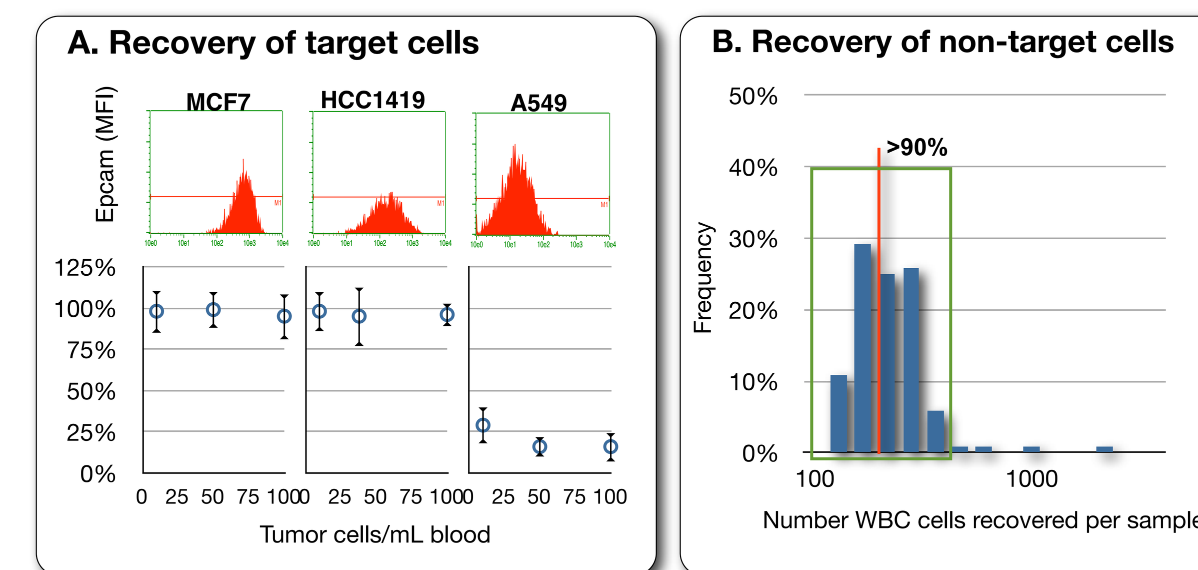

Figure 3. (A) Tumor cells (MCF7, HCC1419 and A549) expressing varying Epcam levels were added in 10, 50 or 100c/ml inputs to normal donor blood. 2mL aliquots were processed using the Cynvenio protocol. Recovery and purity were assessed using DAPI, cytokeratin and CD45 staining to distinguish target (CD45-, CK+, DAPI+) from non-target (CD45+, CK-, DAPI+) populations. (B) Mean non-target capture from 132 experiments.

Allele specific CAST PCR analysis of CTC Flow cell purified tumor cells

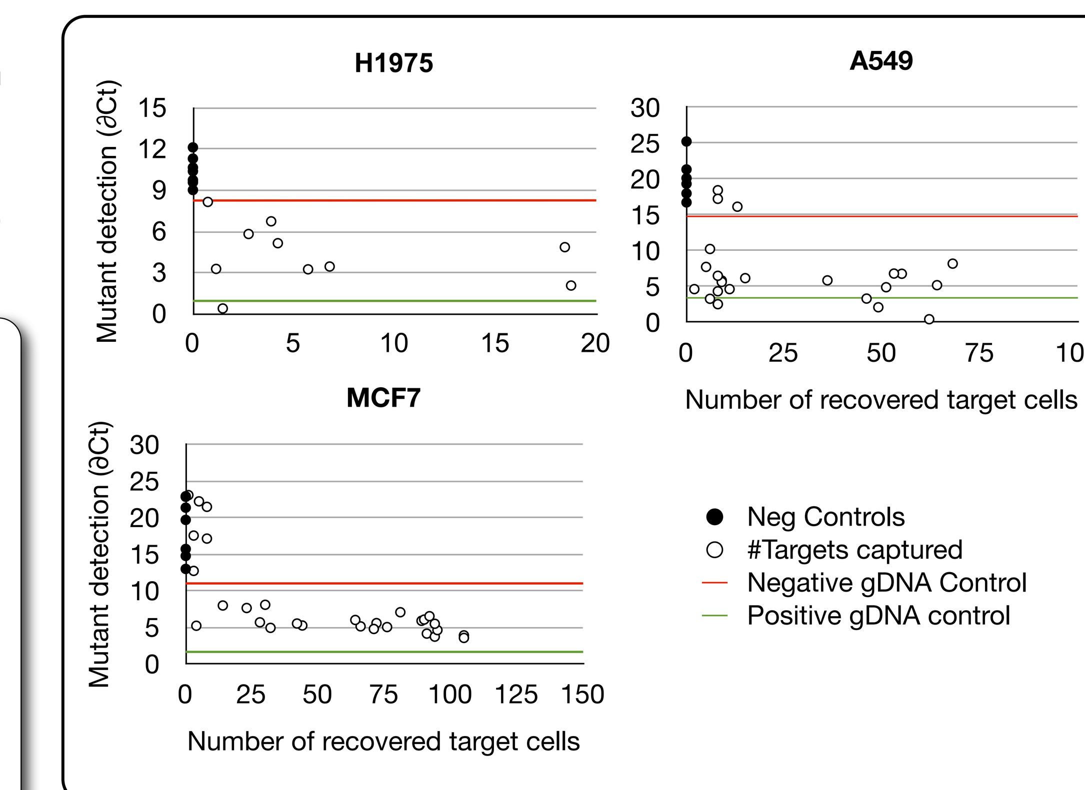

Figure 4. Tumor cells (MCF7, H1975 and A549) expressing varying Epcam levels were added in different inputs to normal donor blood. 2mL aliquots were processed using the Cynvenio protocol. Recovery and purity were assessed using DAPI, cytokeratin and CD45 staining to distinguish target (CD45-, CK+, DAPI+) from non-target (CD45+, CK-, DAPI+) populations. Cells were eluted from ISMAC device, DNA isolated and amplified using Φ-29 WGA (GE Healthcare). 50-60 ng of DNA used in QPCR assays. Assays consisted of allele specific CAST assays, KRAS G12S, PIK3CA E545K, EGFR T790M, (LIFE technologies). Ct values between controls and experimental samples were used to calculate delta-CT.

Sequence analysis of DNA amplified from recovered spike-in cells identified all four of the known cancer-associated mutations present in A549 cells in specimens where 7-14% of recovered cells were A549. Analysis of specimen CYN361, where only 2% of recaptured cells were A549, revealed only two of four known mutations (Table 1).

Sample	N A549 Capture	Total Cells Captured	A549 Purity	FLT1 E726del		KRAS G12S		SMARCA4 Q729fs*4		STK11 Q37*	
				Frq MT	Tot Read	Frq MT	Tot Read	Frq MT	Tot Read	Frq MT	Tot Read
A549-gDNA	N/A	N/A	100.0%	70.0%	201	100.0%	661	100.0%	112	100.0%	134
CYN-353	50	357	14.0%	9.8%	112	8.3%	48	5.3%	322	9.0%	105
CYN-355	61	555	11.0%	5.2%	213	17.0%	106	5.0%	579	19.0%	270
CYN-356	40	444	9.0%	12.0%	116	20.0%	60	6.7%	565	12.0%	222
CYN-492	8	96	7.7%	0.5%	817	10.0%	1138	0.1%	2663	0.1%	92
CYN-358	15	214	7.0%	8.0%	293	14.0%	110	8.0%	484	1.4%	71
CYN-489	13	173	7.0%	1.3%	524	5.3%	847	0.1%	1430	2.8%	177
CYN-488	9	143	5.9%	2.1%	526	6.0%	302	0.1%	1304	15.8%	152
CYN-487	8	149	5.1%	0.3%	689	2.3%	793	0.1%	1844	0.1%	355
CYN-493	11	239	4.4%	1.3%	922	5.3%	931	0.1%	1705	3.6%	56
CYN-490	8	204	3.8%	1.0%	624	3.6%	604	0.1%	1252	0.1%	134
CYN-496	5	197	2.5%	0.7%	769	1.3%	839	0.1%	2004	0.1%	250
CYN-361	9	450	2.0%	1.5%	132	0.0%	58	0.1%	713	0.0%	280
CYN-479	7	423	1.6%	0.3%	572	0.7%	696	0.2%	1935	1.0%	305

Table 1. Number of mutant / wildtype sequence reads for each of four known cancer-associated mutations present in A549 lung adenocarcinoma cells. Model specimens are ranked by the fraction of total recovered cells determined to be A549.

CTC sequencing from primary breast carcinoma patients

Fifteen total blood specimens provided by 11 breast carcinoma patients were subjected to CTC isolation using the Cynvenio ISMAC platform (Table 2). An average of six putative CTCs were identified per blood specimen (range 0-22), versus an average total cell harvest including WBC of 245 (range 82-890). Seven commonly-reported cancer associated mutations were observed among the 11 patients, including mutations in KRAS, FLT4, JAK3, MUTYH and four different mutations in TP53.

Patient	TNM Stage	CTC Sample	N CTC Counted	Total Cells Captured	Estimated Purity	Median Target Coverage	Mutation 1	Percent Mutant Reads	Total Reads	Mutation 2	Percent Mutant Reads	Total Reads	Mutation 3	Percent Mutant Reads	Total Reads	Mutation 4	Percent Mutant Reads	Total Reads
01-02	T2-N3-M0	BC02	16	362	4.2%	510	TP53 Y220C	18%	1313	FLT4 del34 splice	39%	33	TP53 A74fs*47	21%	478	JAK3 R395C	20%	283
		BC04	14	344	3.9%	242	TP53 Y220C	3%	466	FLT4 del34 splice	30%	30						
01-14	T2-N1b-M1	BC50	4	184	2.1%	202												
		BC51	1	121	0.8%	325												
01-13	T3-N1-Mx	BC47	4	245	1.6%	139	MUTYH S501F	16%	249									
		BC48	0	125	0.0%	594	MUTYH S501F	42%	337									
01-12	T3-N3-Mx	BC44	5	231	2.1%	151												
		BC45	2	141	1.4%	299												
01-10	T4-Nx-Mx	BC36	5	283	1.7%	23												
01-11	T1-N1-Mx	BC40	2	82	2.4%	182												
		BC41	2	113	1.7%	30	TP53 Q167*	11%	65									
01-08	T4-N3-Mx	BC08	6	210	2.3%	92	KRAS G12S	13%	46									
						796	KRAS G12S	8%	51									
01-04	T4-N3-Mx	BC11	2	890	1.0%	62												
01-06	T2-N3-M0	BC17	22	267	8.6%	137												
01-08	T2-N0-M0	BC28	5	245	2.0%	261												
01-16	T1c-N1bIII-M0	BC55	5	118	4.2%		TP53 C176W	4	1825									
01-17	T1-N2-M0	BC57	2	196	1.0%													

Table 2. Mutations identified in CTCs captured from 11 breast carcinoma patients using HiSeq and IonTorrent platforms.

Conclusions

- CTC flow cell separation technology can reproducibly recover low-EpCAM expressing cells. Cells expressing (such as A549) 1 x 10^3 - 5 x 10^3 EpCAM receptors can be efficiently captured and recovered from whole human blood.
- CTC Flow cell technology can reproducibly enrich CTCs from whole fixed blood. The routine effective enrichment using the CTC Flow cell is >5 x 10^6 with a purity sufficient for molecular analysis (QPCR & DNA sequencing).
- Whole-genome amplification using Φ-29 polymerase is an effective means of making very small amounts of DNA, such as those recovered using rare-cell separation techniques like the CTC flow cell, available for next-generation sequencing library construction.
- This separation technology yielded putative CTCs from 18/20 blood samples provided by breast carcinoma patients.
- castPCR analysis of model CTC samples prepared by spiking either H1975, MCF-7, or A549 cells into whole blood showed allele specific signals after ISMAC purification when target purity was >5%.
- Sequence analysis of model CTC samples prepared by spiking A549 cells into whole blood supported recovery of 4/4 known cancer-associated mutations in every case where the cancer cells were ≥7% of the total captured cell population.
- Analysis of isolated CTCs using the Foundation Medicine NGS platform revealed familiar cancer-associated mutations in 4/11 patients, including mutations in TP53, KRAS, FLT4, JAK3 and MUTYH.
- Estimates of mutant allele frequency based on sequence read depth indicate that CTC enumeration using CD45/CK/DAPI staining might underestimate the total number of tumor-derived cells captured.
- These results suggest that circulating tumor cells may represent a viable substrate for a minimally-invasive tumor sequencing

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