The Use of an Antibody Independent Method, ApoStream®, to Isolate Circulating Tumor Cells (CTCs) Isolated from Non-Small Cell Lung Cancer Patients and Identification of EGFR Mutations

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Abstract # C16

Background: A variety of methods for capture of rare CTCs of epithelial origin are available; most employ antibodies to epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK). Using a classic phenotypic definition, a CTC is a nucleated, CK^+, $CD45^-$ cell. However, some CTCs may elude capture as they originate from primary tumor cells that have undergone epithelial-mesenchymal transition (EMT). We report here the use of ApoStream®, a novel dielectrophoresis field-flow-assisted, antibody-free method to isolate CTCs from blood.

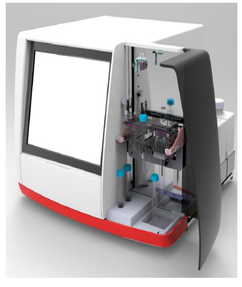
Methods: Blood was collected from consented NSCLC patients and processed using ApoStream®. For CTC enumeration comparison, the CellSearch® FDA-approved kit was used. Isolated cells were evaluated with a multiplexed immunofluorescent assay and laser scanning cytometry was applied to identify multiple combinations of positive and/or negative staining for CK/CD45/DAPI and EpCAM. To determine specific EGFR mutations from captured CTCs, samples were analyzed using Improved and Complete Enrichment with CO-amplification at Lower Denaturation temperature (ICE COLD-PCR).

Results: Blood samples from 40 NSCLC patients and 12 healthy volunteers were processed. In the normal, healthy volunteers, ApoStream® isolated 0-1 $CK^+/CD45^-$ cells and 0-33 $CK^+/CD45^+$ cells. From the 38 of 40 NSCLC patients, ApoStream® identified 0-65 $CK^+/CD45^-$ CTCs, 2 samples failed in processing. Additionally, ApoStream® recovered 37-3536 $CK^-/CD45^-$ and 4-10702 $CK^+/CD45^+$ cells. EpCAM expression was detected in 7-100% of $CK^+/CD45^-$ and 0-5% of $CK^-/CD45^-$ cells, and 18-100% of $CK^+/CD45^+$ cells. In comparison, CellSearch® isolated 0-13 $EpCAM^+/CK^+/CD45^-$ CTCs in 7 patient samples tested. From our whole-blood spiked cancer cell (H1600, H1975) experiments, CTC recovery ranged from 13% to 60% with detection of EGFR mutations in as low as 10 recovered cells by ICE COLD PCR. Overall, from the isolated CTCs ApoStream®, ICE COLD PCR correctly identified mutation status in 15 cases (EGFR exon 19 deletions (5), exon 21 – L858R (3) and wild type in 7 cases).

Conclusions: The ApoStream® platform enriched $EpCAM^+$ and $EpCAM^-$ CTCs from the blood of NSCLC patients utility recovering cancer cells with multiple phenotypes. From a subset of samples, higher number of $CK^+/CD45^-$ cells were recovered by ApoStream® than CellSearch®. Furthermore, recovered CTCs, detection of EGFR mutations in recovered CTCs was possible indicating the clinical relevance and utility of CTCs as an alternative to tissue biopsy.

Methods

ApoStream® Technology



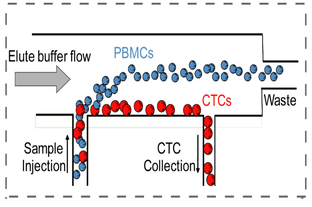
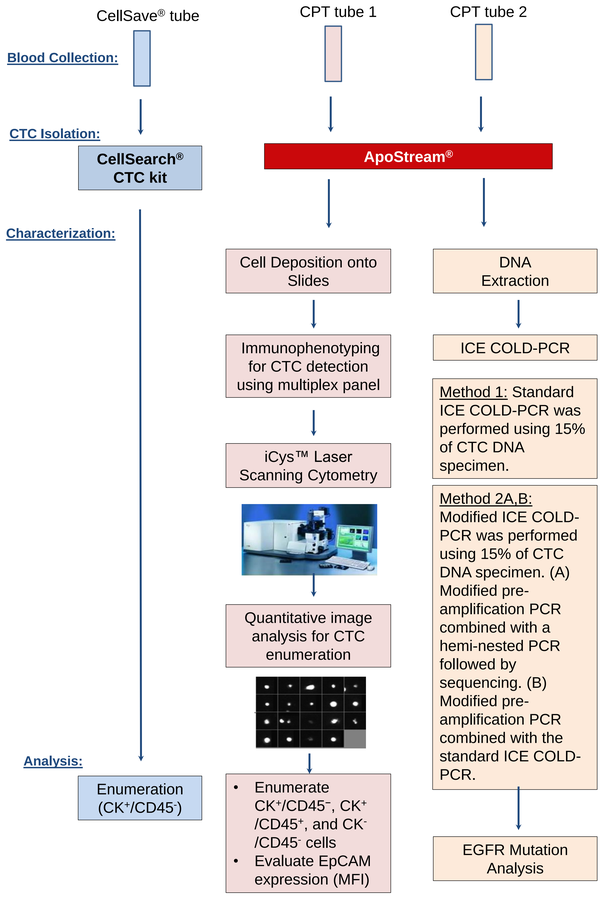


Figure 1. (Left) ApoStream® system; (Right) Schematic illustrating CTC separation from PBMCs in ApoStream® flow chamber [1,2]

Study Design and Work Flow

Figure 3: CellSearch® and ApoStream® CTC Enrichment and Downstream Analysis Workflows

All patients and healthy volunteers provided signed, written informed consent for this laboratory-based research study approved by UTMDACC IRB (LAB11-0490).



Results

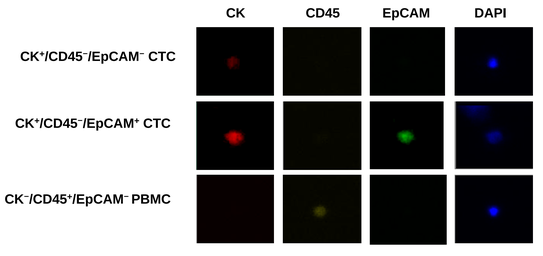
Table 1: ApoStream® CTC Counts and EGFR Mutation Status in Tissue Pathology Report and CTCs

Sample ID	ApoStream® CTC Count			UT MDACC EGFR Mutation Tissue Pathology Report	Transgenomic® EGFR Mutation Results in CTCs, All Methods	EGFR Exon 19			EGFR Exon 21
	$CK^+/CD45^-$ Cells (% $EpCAM^+$)	$CK^-/CD45^-$ Cells (% $EpCAM^+$)	$CK^+/CD45^+$ Cells (% $EpCAM^+$)			Method 1	Method 2A	Method 2B	Method 2B
MDACC-007B	20 (35)	1968 (0.2)	118 (33)	Exon 20, 9 bp insertion	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-012	6 (17)	1037 (0.5)	11 (64)	Exon 18, codon 719 (GGC to TGC, G719C)	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-012B	65 (95)	92 (0)	115 (83)	Exon 18, codon 719 (GGC to TGC, G719C)	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-013	10 (0)	479 (1)	4 (25)	Exon 19, 15 bp deletion	NVD* - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-014	2 (0)	609 (0)	26 (85)	Exon 19, deletion	Exon 19 deletion	NVD	delATCTCCGAAAGCCAACAAGGAAATC; p.P753Is, 100%**	delATCTCCGAAAGCCAACAAGGAAATC; p.P753Is, 100%	NVD
MDACC-015	0 (NA)	1109 (0.1)	1 (100)	Exon 19, 18 bp insertion	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-016	4 (50)	100 (1)	64 (28)	Exon 20	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-017	3 (33)	321	6 (83)	no known mutations	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-018	8 (0)	289 (0)	11 (73)	Exon 19, 15 bp deletion	Exon 19 deletion	NVD	delATCTCCGAAAGCCAACAAGGAAAT; p.S752-I759del, 100%	delATCTCCGAAAGCCAACAAGGAAAT; p.S752-I759del, 50%	NVD
MDACC-019	9 (100)	962 (7)	132 (61)	Exon 19, deletion	Exon 19 deletion	NVD	delATCTCCGAAAGCCAACAAGGAAAT; p.S752-I759del, 40%	delATCTCCGAAAGCCAACAAGGAAAT; p.S752-I759del, 100%	NVD
MDACC-020	18 (6)	330 (0)	56 (23)	Exon 19, 18 bp deletion	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-021	18 (89)	145 (5)	201 (89)	EGFR Exon 18, R705K; KRAS, G12D	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-022	0 (NA)	51 (0)	590 (93)	Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-023	1 (NA)	1139 (0.1)	10702 (90)	Exon 19, L747P	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-024	1 (100)	24 (0)	6 (67)	Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-028	3 (NA***)	54 (NA)	619 (NA)	Exon 19, insertion/deletion	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-029	1 (NA)	37 (NA)	347 (NA)	Exon 20, 6 bp insertion	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-030	7 (NA)	231 (NA)	55 (NA)	Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-032	1 (NA)	263 (NA)	33 (NA)	Exon 20, 9 bp insertion	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-033	0 (NA)	335 (NA)	5 (NA)	Exon 19, deletion	Exon 19 deletion/insertion	NVD	NVD	delGAGAAGCAACATCTCCGAinsACATCTCCG p.R748-K754delinsNISE, 40%	NVD
MDACC-034	0 (NA)	372 (NA)	41 (NA)	Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-035	0 (NA)	442 (1.6)	27 (44)	EGFR WT; KRAS, codon 12	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-036	12 (8)	2125 (0.1)	33 (36)	no known mutations	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-037B	N/A			Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	FAIL	NVD
MDACC-038	5 (60)	397 (5)	117 (78)	Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-039	10 (20)	114* (0)	15 (60)	EGFR WT	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-040	2 (0)	57* (0)	14 (14)	Exon 19, 15 bp deletion	Exon 21	NVD	NVD	NVD	c.A>G; p.K860E, 25%
MDACC-041	1 (100)	22 (0)	3 (33)	Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-042	11 (27)	273* (0)	93* (10)	Exon 19, 15 bp deletion	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-043	56 (14)	1677* (0)	1431* (41)	EGFR WT/EML4-ALK	N/T	N/T	N/T	N/T	N/T
MDACC-044	0 (NA)	2 (0)	1 (0)	Exon 20 - T790M, Exon 21 - L858R	Exon 21	NVD	NVD	NVD	c.A>G; p.K860K, 50%
MDACC-045	0 (NA)	227* (0)	9 (11)	EGFR WT	NVD - Exon 19/21	NVD	NVD	NVD	NVD
MDACC-046	NA**	NA**	NA**	EGFR WT/RET	N/T	N/T	N/T	N/T	N/T
MDACC-047	0 (NA)	82 (4)	76 (78)	Exon 21 - L658R and L861Q	Exon 21	NVD	NVD	NVD	c.C>T; p.A859V, 25%
MDACC-048	1 (0)	0 (NA)	0 (NA)	EGFR WT/EML4-ALK	N/T	N/T	N/T	N/T	N/T
MDACC-049	22 (5)	406 (0)	70 (24)	EGFR WT/EML4-ALK	N/T	N/T	N/T	N/T	N/T
MDACC-050	0 (NA)	2 (0)	32 (50)	Exon 19, 15 bp deletion	Exon 19, 15 bp deletion	NVD	c.2239_2251delTTAAGAGAAGCAAinsC; p.L747_T751delinsP, 80%	NVD	FAIL
MDACC-051	0 (NA)	4 (0)	45 (73)	EGFR WT/KDR	N/T	N/T	N/T	N/T	N/T

Table 1: UTMDACC EGFR Mutation Tissue Pathology Analysis: PCR-based EGFR exon 18 to 21 DNA sequencing analysis was performed on DNA extracted from paraffin-embedded tumor tissue blocks. The lower limit of sensitivity of detection is approximately one mutated cell per five total cells in sample (20%).
* NVD, No Variant Detected, ** % mutant allele, *** NA, no data collected

Results

Figure 4: Representative Immunofluorescent Images of Cells Isolated by ApoStream®



Results

Table 2: Summary of CTC Enumeration and Mutation Analysis Results

Side-by-Side CellSearch® and ApoStream® analysis	
Total number of NSCLC Samples Analyzed Successfully by Both Methods	7
Number of Samples with $CK^+/CD45^-$ Cells Detected by CellSearch®	4 of 7 (57%)
Median Number (Range) of $CK^+/CD45^-$ Cells Detected by CellSearch®	1 (0 – 13)
Number of Samples with $CK^+/CD45^-$ Cells Detected by ApoStream®	4 of 7 (57%)
Median Number (Range) of $CK^+/CD45^-$ Cells Detected by ApoStream®	6 (0 – 29)
ApoStream® CTC / Putative CTC Yields per 7.5mL of Blood	
NSCLC	
Total Number of NSCLC Samples Analyzed Successfully	38
Number of NSCLC Samples with $CK^+/CD45^-$ Cells Detected	25
Median Number (Range) of $CK^+/CD45^-$ Cells Detected in NSCLC Samples	3 (0 – 65)
Number of NSCLC Samples with $CK^-/CD45^-$ Cells Detected	30
Median Number (Range) of $CK^-/CD45^-$ Cells Detected in NSCLC Samples	354 (24 – 3536)
Number of Samples with $CK^+/CD45^+$ Cells Detected in NSCLC Samples	30
Median Number (Range) of $CK^+/CD45^+$ Cells Detected in NSCLC Samples	46 (1 – 10702)
Healthy Donor Blood	
Total Number of Healthy Donor Blood Samples Analyzed Successfully	12
Number of Healthy Donor Samples with $CK^+/CD45^-$ Cells Detected	2
Median Number (Range) of $CK^+/CD45^-$ Cells Detected in Healthy Blood	0 (0 – 1)
Number of Healthy Donor Samples with $CK^-/CD45^-$ Cells Detected	12
Median Number (Range) of $CK^-/CD45^-$ Cells Detected in Healthy Blood	75 (2 – 753)
Number of Healthy Donor Samples with $CK^+/CD45^+$ Cells Detected	4
Median Number (Range) of $CK^+/CD45^+$ Cells Detected in Healthy Blood	0 (0 – 33)
EGFR Exon 19 ICE COLD-PCR	
Total Number of Samples Analyzed	25
Total Number of EGFR Exon 19 Deletion Tissue Positive Cases Analyzed for CTC Mutations	15
Number of Cases with EGFR Exon 19 Deletion in Tissue Successfully Analyzed by CTC Method 1	15
Number of Specimens with EGFR Exon 19 CTC Mutations Detected by Method 1	0 of 15 (0%)
Number of Cases with EGFR Exon 19 Deletion in Tissue Successfully Analyzed by CTC Method 1	15
Number of Specimens with EGFR Exon 19 CTC Mutations Detected by Method 1	3 of 15 (27%)
Number of Cases with EGFR Exon 19 Deletion in Tissue Successfully Analyzed by CTC Method 1	8
Number of Specimens with EGFR Exon 19 CTC Mutations Detected by Method 1	4 of 8 (50%)

Summary & Clinical Significance

- A novel, antibody-independent platform ApoStream® successfully isolated CTCs from the blood of patients with advanced NSCLC. In a side-by-side comparison, ApoStream® isolated more $CK^+/CD45^-$ NSCLC CTCs compared to the CellSearch® platform in 4 out of 7 NSCLC patient samples with detectable $CK^+/CD45^-$ cells; neither system detected CTCs in 1 patient sample.
- Phenotypic immunofluorescent analysis of cells isolated by ApoStream® revealed the presence of $CK^+/CD45^-$ CTCs as well as $CK^-/CD45^-$ and $CK^+/CD45^+$ cells. Median of 3 $CK^+/CD45^-$ CTCs was detected in NSCLC samples as compared to 0 in healthy donor blood.
- Percent cells expressing EpCAM varied from 0 to 100% in $CK^+/CD45^-$ cells, from 0 to 7% in $CK^-/CD45^-$ cells, and from 0 to 100% in $CK^+/CD45^+$ cells, thus confirming that ApoStream® isolates $EpCAM^-$ cells that would be undetected by EpCAM-based technologies.
- The use of ICE COLD-PCR coupled with standard Sanger sequencing allowed detection of EGFR Exon 19 mutations in CTCs isolated by ApoStream®. Method modifications led to increases in the sensitivity of detecting EGFR Exon 19 mutations in CTCs from tissue-positive patients from 0% with standard ICE COLD-PCR (Method 1) to 27% and 50% with Methods 2A and 2B respectively. Note that only a portion of the extracted DNA was used per pre-amplification PCR, therefore, the entire template population of the sample was not tested with each assay; this could have led to some discrepant results.
- For EGFR Exon 21, no mutations were observed in the tumor tissue from this set of patients. Using standard ICE COLD-PCR followed by Sanger sequencing on the template DNA extracted from the CTCs isolated by ApoStream®, no mutations were found, thus the results from the ICE COLD-PCR analysis were 100% concordant with the tumor samples with no false positives observed. Development of a modified ICE COLD-PCR approach (Method 2) for EGFR Exon 21 CTC mutation analysis is ongoing.
- Overall, from the isolated CTCs using ApoStream®, ICE COLD-PCR identified mutation status in 15 cases (EGFR exon 19 deletions (5), exon 21 – L858R (3) and wild type in 7 cases) in concordance to tumor tissue analysis by Sanger sequencing.
- The mutation detection of other samples (n=23) did not match tumor tissue mutation status by Sanger sequencing at the time of collection.

ICE COLD-PCR Sequencing

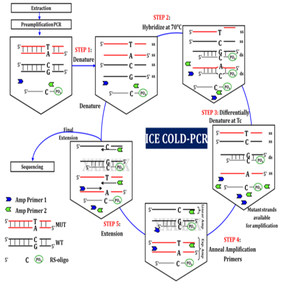
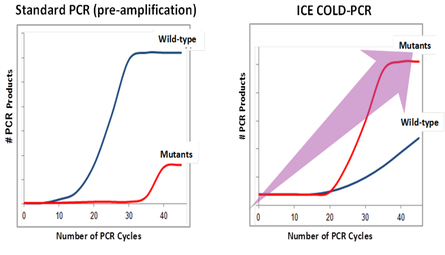


Figure 2. Overview of ICE COLD-PCR Process (L) and Theory (R)

Step 1: All DNA is denatured to single strands.
Step 2: The RS-oligo binds to one strand of the wild-type and mutant sequences: mutant:RS-oligo forms a heteroduplex.
Step 3: The reaction is incubated at the Tc: the mutant:RS-oligo denatures but the wild-type:RS-oligo remains bound.
Step 4: Anneal the PCR primers. The forward and reverse PCR primers will bind to both strands of the mutant DNA, but only one strand of the wild-type.
Step 5: Extension of the PCR primers along the mutant and wild-type DNA sequences. The mutant sequence will undergo exponential amplification while the amplification of the wild-type sequence will be linear.
Step 6: Perform standard Sanger Sequencing reactions.
Step 7: Analyse using a DNA sequencer.



References:
[1]Vishal Gupta, et al. ApoStream™, a new dielectrophoretic device for antibody independent isolation and recovery of viable cancer cells from blood. Biomicrofluidics 6, 024133 (2012).
[2]Sangjo Shim, et al. Dielectrophoresis has broad applicability to marker-free isolation of tumor cells from blood by microfluidic systems. Biomicrofluidics, 7, 011808, 2013.