Characterization and identification of specific EGFR mutations in circulating tumor cells (CTCs) isolated from non-small cell lung cancer patients using an antibody independent method, ApoStream™: An update report

E.B. Roarty[1], H. T. Tran[1], V. O. Melnikova[2], A. S. Tsao[1], F. V. Fossella[1], F. M. Johnson[1], V. Papadimitrakopoulou[1], M. Garza[2], C. Neal[2], D. Hasegawa[2], A. Kruempel[1], G. Wu[1], K. Richardson[3], M. E. Lewis[3], B. L. Legendre Jr[3], K. L. Anderes[2], D. W. Davis[2], J. Heymach[1]

[1]UT MD Anderson Cancer Center, Houston, TX; Department of Thoracic, Head & Neck Medical Oncology; [2]ApoCell, Inc., Houston, TX; [3]Transgenomic, Inc., Omaha, NE

Abstract

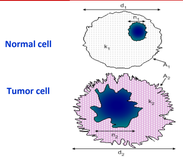
Background: A variety of methods for capture of rare CTCs of epithelial origin are available; most employ antibodies to epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK). Using a classic phenotypic definition, a CTC is a nucleated, CK(+), CD45(-) cell. However, some CTCs may elude capture 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 32 NSCLC patients and 3 healthy volunteers were processed. ApoStream™ isolated 0 to 65 CK(+)/CD45(-) CTCs(n=32) and CellSearch® isolated 0 to 13 EpCAM(+)/CK(+)/CD45(-) CTCs(n=7). 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. EGFR mutations [exon 19 deletions and exon 21 L858R] were determined and found to be concordant when compared to tumor tissue analysis by Sanger sequencing.

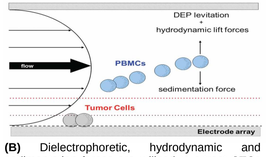
Conclusions: The ApoStream™ platform enriched EpCAM(+) and EpCAM(-) CTCs from the blood of NSCLC patients demonstrating utility in recovering cancer cells with multiple phenotypes. From recovered CTCs, detection of EGFR mutations was possible indicating the clinical relevance and potential utility of CTCs as an alternative to tissue biopsy. Complete mutation analysis will be presented.

ApoStream™ Technology

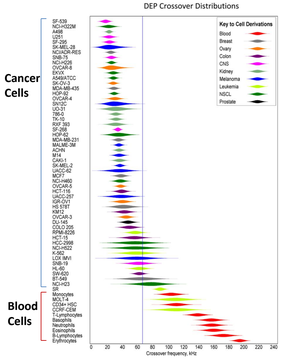


(A) Dielectric properties (polarizability) of cells are dependent upon cell diameter, membrane morphology and conductivity.

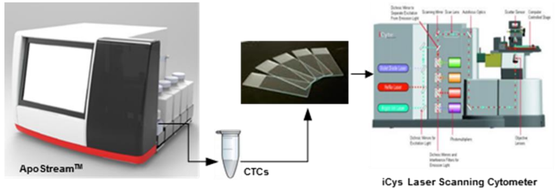
Inherent differences in morphology of CTCs and normal cells result in different dielectric polarization charges when exposed to an AC electric current.



(B) Dielectrophoretic, hydrodynamic and sedimentation forces are utilized to attract CTCs and repel normal cells from the chamber floor. CTCs are collected through a port located in the chamber floor while normal cells flow into a waste port.



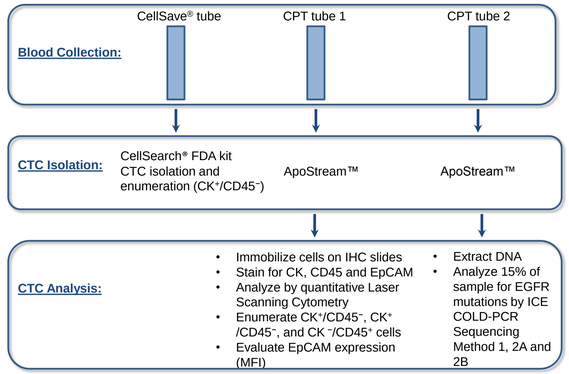
(C) Cross-over frequencies from different tumor cell types including breast, colon, ovarian, lung and melanoma cell lines and from peripheral blood mononuclear cells (PBMCs) were determined.[2] The differences in cross-over frequencies between cancer and normal cells enable ApoStream™ to separate CTCs from normal cells.



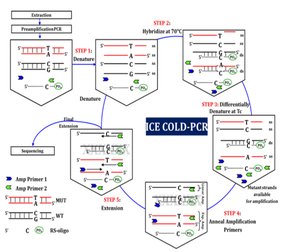
Methods

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

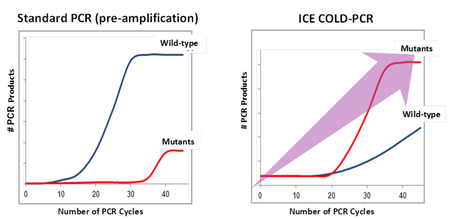
Study Design



ICE COLD-PCR Sequencing



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.



Results

Sample ID	ApoStream™ CTC Count			UT MDACC EGFR Mutation Tissue Pathology Report	Transgenomic® EGFR Mutation Results, All Methods	Transgenomic® EGFR Mutation Results			
						EGFR Exon 19			EGFR Exon 21
	CK+/CD45− Cells (% EpCAM+)	CK−/CD45− Cells (% EpCAM+)	CK+/CD45+ Cells (% EpCAM+)			Method 1	Method 2A	Method 2A	Method 1
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.P753fs, 100%**	delATCTCCGAAAGCCAACAAGGAAATC; p.P753fs, 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	delGAGAAGCAACATCTCCGAinsACATCTCCCGp.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)	EGFT 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 - L858R 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_T753delinsP; 85%	NVD	FAIL
MDACC-051	0 (NA)	4 (0)	45 (73)	KDR	N/T	N/T	N/T	N/T	N/T

*Extrapolated from visual confirmation of 50 cells
** Data not available due to staining/processing issue
*** N/T: not tested, Analysis are ongoing for these specific molecular alterations

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%).
Method 1: Standard ICE COLD-PCR was performed using 15% of CTC DNA specimen.
Method 2A,B: Modified ICE COLD-PCR was performed using 15% of CTC DNA specimen. (A) Modified pre-amplification PCR combined with a hemi-nested PCR followed by sequencing. (B) Modified pre-amplification PCR combined with the standard ICE COLD-PCR.
* NVD, No Variant Detected, ** % mutant allele, *** NA, no data collected

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 out of 7
Median number (range) of CK+/CD45− cells detected by CellSearch®	1 (0 to 13)
Number of samples with CK+/CD45− cells detected by ApoStream™	4 out of 7
Median number (range) of CK+/CD45− cells detected by ApoStream™	6 (0 to 29)
ApoStream™ CTC/Putative CTC Yields per 7.5 mL of Blood	
NSCLC	
Total number of NSCLC samples analyzed successfully	36
Number of NSCLC samples with CK+/CD45− cells detected	26
Median number (range) of CK+/CD45− cells detected in NSCLC samples	3 (0 to 65)
Number of NSCLC samples with CK−/CD45− cells detected	35
Median number (range) of CK−/CD45− cells detected in NSCLC samples	268 (2 to 3536)
Number of samples with CK+/CD45+ cells detected in NSCLC samples	35
Median number (range) of CK+/CD45+ cells detected in NSCLC samples	37 (1 to 10702)
Healthy Donor Blood	
Total number of healthy donor blood samples analyzed successfully	3
Number of healthy donor samples with CK+/CD45− cells detected	1
Median number (range) of CK+/CD45− cells detected in healthy blood	0 (0 to 4)
Number of healthy donor samples with CK−/CD45− cells detected	3
Median number (range) of CK−/CD45− cells detected in healthy blood	107 (28 to 457)
Number of healthy donor samples with CK+/CD45+ cells detected	3
Median number (range) of CK+/CD45+ cells detected in healthy blood	15 (7 to 26)
EGFR Exon 19 ICE-COLD PCR	
Total number of samples analyzed	33
Total number of EGFR Exon 19 deletion tissue positive cases analyzed for CTC mutations	17
Number of cases with EGFR Exon 19 deletion in tissue successfully analyzed by CTC Method 1	17
Number of specimens with EGFR Exon 19 CTC mutations detected by Method 1	0 out of 17 (0%)
Number of cases with EGFR Exon 19 deletion in tissue successfully analyzed by CTC Method 2A	17
Number of specimens with EGFR Exon 19 CTC mutations detected by Method 2A	5 out of 17 (29%)
Number of cases with EGFR Exon 19 deletion in tissue successfully analyzed by CTC Method 2B	8
Number of specimens with EGFR Exon 19 CTC mutations detected by Method 2B	4 out 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 3 out of 6 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. Additional samples are being recruited to establish CTC enumeration cut-offs.
- Percent cells expressing EpCAM varied from 0 to 100% in CK+/CD45− cells, from 0 to 7% in CK−/CD45− cells, and from 10 to 93% 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, two 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, both mutations were found plus an additional for which the tissue analysis was EGFR Exon 19 with 15bp deletion.
- Analysis for EML4-ALK and RET from isolated CTCs are on-going.
- In summary, successful isolation of NSCLC CTCs and detection of EGFR mutations by an integrated ApoStream™ - ICE COLD-PCR approach enables exploration of the clinical utility of CTCs as an alternative to tissue biopsy.

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.