Combining Cell-Free RNA (cfRNA) with Cell-Free Total Nucleic Acid (cfTNA) as a new Paradigm for Liquid Biopsy

Maher Albitar1, Hong Zhang1, Ahmad Charifa1, Andrew IP2, Ivan De Dios1, Wanlong Ma1, James McCloskey2, Michele Donato2, David Siegel2, Stanley Waintraub2, Martin Gutierrez2, Andrew Pecora2, Andre Goy2

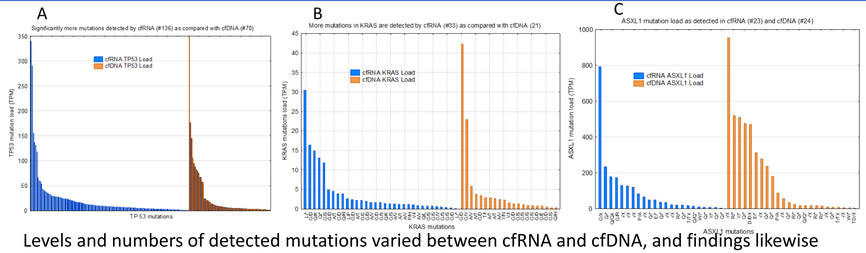
1 Genomic Testing Cooperative, LCA, Irvine, CA, USA, 2 John Theurer Cancer Center at Hackensack University Medical Center, Hackensack, NJ, USA

Background: RNA is typically considered less stable than DNA and is more prone to degradation, especially in the peripheral blood circulation. Therefore, it is assumed that it is difficult to analyze cell-free RNA (cfRNA). However, the principle underlying NGS technology is to use fragmented DNA or RNA for sequencing in order to align these fragments against the reference genome, and RNA analysis from formalin-fixed paraffin-embedded (FFPE) tissue using NGS is routinely used in clinical testing and has been shown to be highly informative and reliable. NGS technology relies on the fragmentation of DNA or RNA prior to sequencing. Aligning the sequence of the fragmented DNA or RNA with a proper bioinformatics system allows for the analysis and quantification of the sequenced nucleic acids. Therefore, sequencing fragmented cfRNA should be attainable by using methodology similar to that used for RNA sequencing in FFPE tissue . Developing methods for analyzing cfRNA is an unmet need that may change how liquid biopsies are currently performed and used in clinical settings. We evaluated whether an analysis of both cfRNA and cfDNA significantly enhances the sensitivity of liquid biopsy. We hypothesized that our results would demonstrate that this method can provide information that is highly useful for accurately detecting clinically relevant mutations, tumor-specific biomarkers, chromosomal structural abnormalities, and copy number variations (CNV), as well as for evaluating fusion data.

Methods: Each patient's cfDNA and cfRNA were processed in parallel using the KAPA RNA HyperPrep Kit. The steps for processing cfRNA were as follows: 1) first-strand synthesis, 2) second-strand synthesis and A-tailing, 3) ligating adapters, 4) library amplification, 5) cleaning, 6) quantification, 7) preparing the 8-plex DNA sample library pool, 8) adding the designed KAPA target enrichment probes (1,459 genes, Supplementary Table S1) for overnight hybridization, 9) implementing KAPA beads in order to capture a multiplexed DNA library, 10) amplifying the enriched multiplex DNA library, 11) cleaning the amplified enriched multiplexed DNA library, 12) checking the multiplexed library by running TapeStation, 13) normalizing and pooling, and 14) denaturing and loading libraries on a NovaSeq 6000 sequencer. Similar steps were followed for cfDNA, with the exception of the initial reverse transcription step.

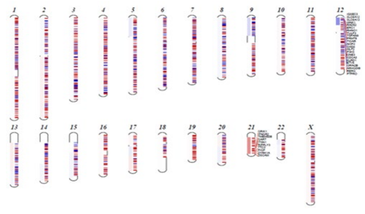
Conclusions

- With few exceptions, more mutations and higher variant allele frequency are detected using cfRNA than cfDNA.
- Combined cfRNA and cfDNA is optimal approach for liquid biopsy to evaluate mutations, fusion genes and chromosomal structural abnormalities

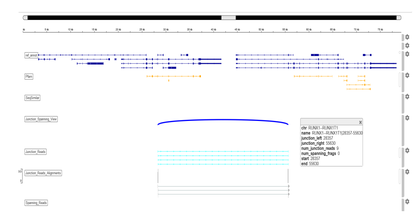


Levels and numbers of detected mutations varied between cfRNA and cfDNA, and findings likewise varied according to the types of mutated genes and the types of mutations. A. A statistically significantly higher number of mutations in *TP53* were detected using cfRNA as compared with cfDNA. B. Only 21 mutations in the *KRAS* gene were detected in cfDNA, while 33 mutations were detected in cfRNA. C. Slightly more mutations in the *ASXL1* gene were detected in cfDNA as compared with cfRNA (24 vs. 23). The levels of these mutations are shown on the Y-axis.

Detection of Chromosomal abnormalities: Loss and gain by cfDNA and fusions fusion by cfRNA



Illustrative example of chromosomal structural loss and gain detected using cfDNA. Diagram resulting from a CNVkit analysis of cfDNA, showing a 12p deletion and a trisomy chromosome 21 in one of the tested samples.



A RUNX1-RUNX1T1 fusion transcript was detected in cfRNA from a patient with acute myeloid leukemia.

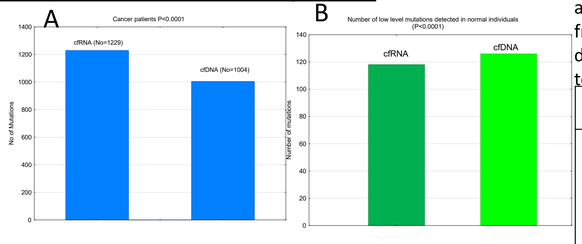
Disease	Fusion	MANTA score	Chromosome 1 Position	Chromosome 2 Position	Mutation (VAF%)
Lymphoma	DDX5--BCL6	0.931638	17:64506076:-	3:187734907:-	CARD11(0.51), SUFU (1.56)
Lung Cancer	SLC34A2--ROS1	0.905027	4:25664330:+	6:117326230:-	None*
AML	RUNX1--RUNX1T1	0.952367	21:34859474:-	8:92017363:-	U2AF1 (38.6%), DNMT3A (27.78%), TP53(7.41
ALL	RUNX1--ETV6	0.932005	21:35048842:-	12:11884445:+	CBL (7.69), TET2(4.17)
ALL	EP300--ZNF384	0.996816	22:41131633:+	12:6679525:-	ERBB3(6.9), SDHC (15.38)
AML	DEK--NUP214	0.981224	6:18236452:-	9:131159383:+	WT1 (50), PPM1D (6.9)
AML	RUNX1--ZFPM2	1	21:34834410:-	8:105801047:+	SRSF2 (42.26), NRAS(5.01), ASXL1(36.44)
AML	RARA--PML	0.295816	17:40331396:+	15:74022828:+	NCAM1(32.88)

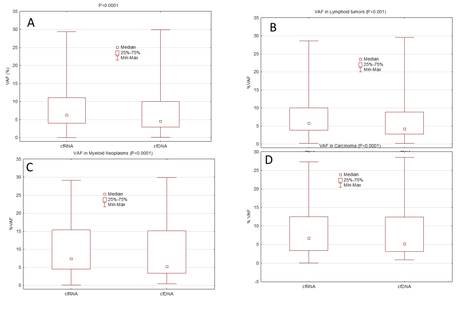
*TET2 mutation as CHIP was detected

Results: Higher number of mutations detected using cfRNA

A higher number of mutations were detected in cfRNA as compared with cfDNA in patients with various cancer types.

A. Bar graphs showing a statistically significantly higher number of mutations detected in cfRNA than in cfDNA.
B. B. Bar graphs showing a statistically significantly higher number of CHIP mutations detected in cfDNA than in cfRNA.





Higher variant allele frequency (VAF) detected in cfRNA as compared with cfDNA. A. Box plot showing overall comparison between cfRNA and cfDNA. B. Box plot showing the difference in VAF between cfRNA and cfDNA in lymphoid neoplasms. C. Box plot showing the difference in VAF between cfRNA and cfDNA in myeloid neoplasms. D. Box plot showing the difference in VAF between cfRNA and cfDNA in solid tumors.

Future Directions for Research:

We are working on integrating RNA levels (transcriptome) in the diagnostic evaluation of liquid biopsy (see our poster on using cfRNA as biomarkers). Transcriptome along with mutation profiling when used in artificial intelligent models can provide remarkable insight on the neoplastic disease and the host. Our plans are to combine liquid biopsy transcriptome with mutation profiling to develop models for diagnosis, predicting prognosis, selecting therapy and monitoring relapse and MRD.