Evaluation of Full Spectrum cell-free RNA in Peripheral Blood as Potential Biomarker for Acute myeloid leukemia Profiling

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ABSTRACT

Introduction: Acute myeloid leukemia is one of the most aggressive hematological malignancies with high relapse/ refractory rate. To date, studies are lacking on extracellular RNA profiling in AML as cell free RNA is relatively unstable but could contain potential biomarkers. In this study, we investigated the potential of total cell-free RNA from small volumes of blood plasma as a potential biomarker for AML disease profiling.

Methods: Peripheral blood was obtained after informed consent from *de novo* AML patients. Plasma was separated and cfRNA isolated using four different platforms. cfRNA quality was checked by Tape station. Low input total RNA library was generated using cfRNA (QIAseq FastSelect RNA Library kit). In parallel, RNA isolated from paired PBMCs was subjected to library preparation using the same protocol. Sequencing was performed on Novaseq 6000 and data analysis was done using R software.

Results: The yield of cfRNA from peripheral blood ranged between 6.42 to 380ng/ml plasma. With an average RIN value of 2.2, the cfRNA showed low molecular weight RNA profile. Next generation sequencing analysis of paired cfRNA and cell-associated peripheral blood RNA revealed >2000 protein coding transcripts with >10 TPM value and a high concordance (0.84-0.94) between the paired samples was observed (Figure below). Amongst the transcripts with highest TPM values were ferroptosis related (FTL, FTH1) and extracellular S100 proteins (S100A8 and S100A9), that regulate immune homeostasis, inflammation and serve as danger signals. In addition, long-non coding RNA (MALAT1, RMRP_1, GAS5) and small nucleolar RNA known to play a crucial role in the development of cancer were detected.

Conclusion: Our findings provided the basis for the utilization of cfRNA to enhance our understanding of the AML biology as well as identify potential biomarkers relevant to the diagnosis and prognosis of AML patients.

INTRODUCTION

Acute Myeloid Leukaemia (AML) is a genetically heterogeneous blood cancer that relies on the invasive, risk-prone and painful procedure of bone marrow biopsy for diagnosis and disease monitoring. Genomic DNA derived from bone marrow biopsy is the gold standard for genetic alteration detection. Liquid biopsy has revolutionised the field of clinical oncology as it offers ease of tumor sampling, reflect the tissue of origin, real-time monitoring, screening for therapy resistance and therapeutic-decision making.

Hematopoietic cells are a major contributor to plasma cell-free nucleic acids (Figure 1), which contain both leukemia-specific aberrations in the circulating tumor-derived DNA (ctDNA) fraction and cell free RNA (cfRNA) that reflects the tumor microenvironment. Moreover, a tumor's genotype can influence its microenvironment, specifically immune phenotype that could potentially be reflected by the cfRNA.

cfRNA is released by tissues into circulation through apoptosis, microvesicle shedding, and exosome signaling. Alterations in cfRNA transcripts expression is a dynamic process that can serve as an indicator of patient status, in terms of tumor origin, cancer stage, and immune response that are essential parameters during cancer treatment (e.g., immune and neoadjuvant therapies). The measurements of cfRNA reflect tissue-specific changes in gene expression, intercellular signaling, cell death and thus provide extensive clinical value.

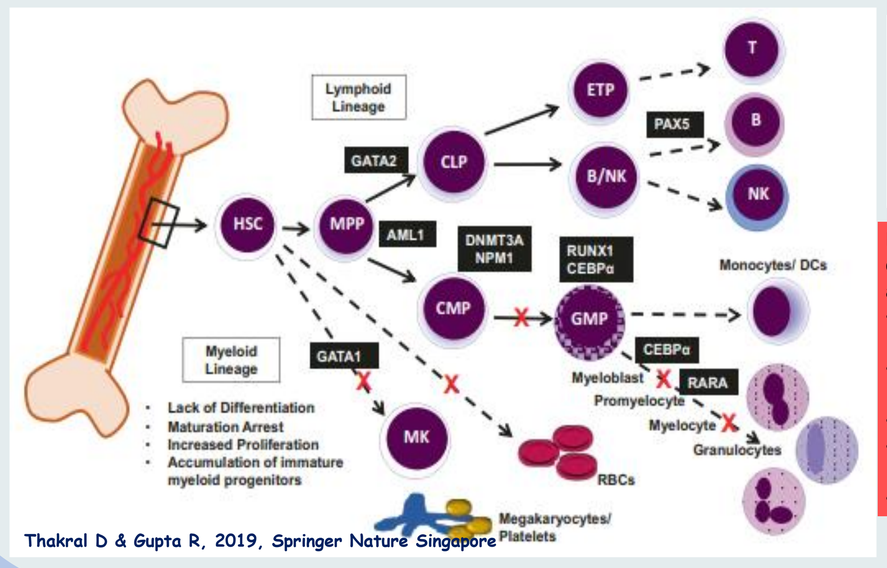

Figure 1: Full spectrum of circulating cell free nucleic acids-
- Protein-coding fragmented (mRNA)
- Small non-coding (miRNA, snRNA, snoRNA, piRNA)
- Long-noncoding RNA
- Non-coding RNA (rRNA, tRNA)
- cfDNA

AIM & OBJECTIVES

- **To determine the concordance between the profile of plasma-derived cell free RNA and PBMC RNA from AML patient samples.**
- **To evaluate the diagnostic potential of total cell free RNA as a minimally invasive approach for AML disease profiling.**

MATERIALS & METHODS

Sample Collection and processing: In this prospective study, newly diagnosed *de novo* AML patients (excluding acute promyelocytic leukemia) ≤ 70 years of age, undergoing frontline chemotherapy were recruited. Ethical clearance was obtained from the Institute Ethics Committee, AIIMS, New Delhi (IEC-277/07.05.2021, RP-13/2021). Approx. 10ml peripheral blood was collected from all the AML patients at the time of diagnosis and healthy controls were included. Plasma was separated and cells were resuspended in Trizol. Total RNA was extracted from MNCs and in parallel peripheral blood plasma-derived cell free RNA (cfRNA) was isolated using commercially available kits (four different protocols were tested).

cfRNA Characterization: The cfRNA fragment analysis was performed using TapeStation. The fragment size and RIN values were recorded.

RNAseq: Total RNA isolated from PBMC (100ng) and plasma-derived cfRNA (5-10ng) were converted into cDNA and NGS libraries were generated using low input QIAseq FastSelect kit (with rRNA HMR and rRNA globin kits for cytoplasmic and mitochondrial ribosomal RNA and globin mRNA removal) as shown in the workflow (Figure 2). After library QC, sequencing was performed on Illumina sequencing at 50million reads per sample. Read sequences were aligned to the human reference genome GRCh38. Gene expression levels (TPM values) were inferred from de-duplicated BAM files using RSEM (v1.3.0). Data analysis was performed using R software.

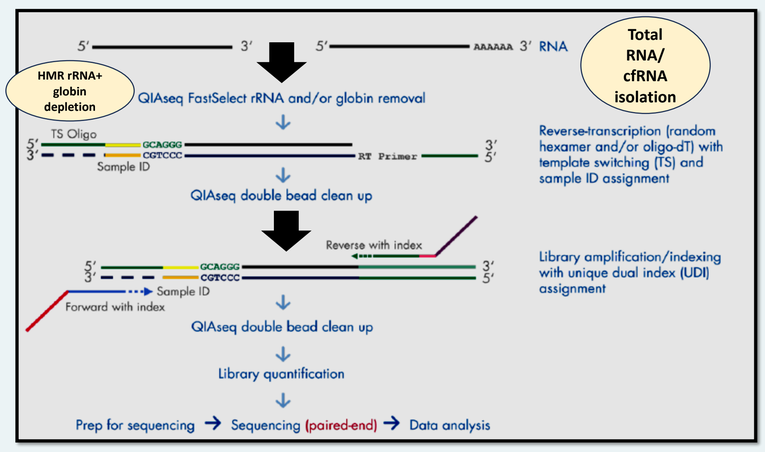

Figure 2: Workflow of QIAseq FastSelect RNA library preparation.

RESULTS

Characterization of Plasma derived cfRNA profile in AML: cfRNA was successfully isolated from peripheral blood plasma obtained from AML samples and healthy controls. Small RNA fragments in the range of 25-150nt were observed as shown in Figure 3.

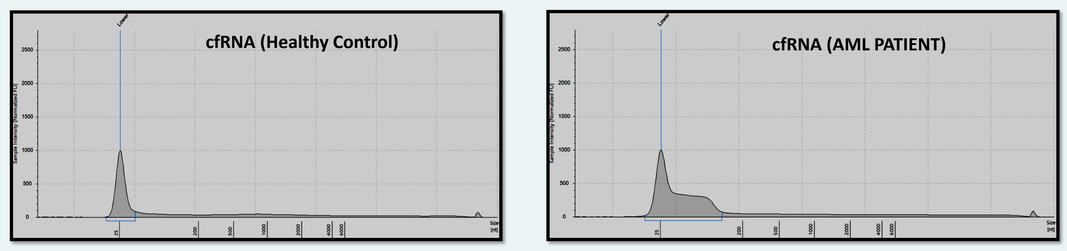

Figure 3: cell free RNA profiles extracted from peripheral blood plasma. RNA was extracted from samples by Method 1 and run on Tape station. Representative of samples collected from a healthy volunteer (left panel) and AML patient (right panel) are shown.

cfRNA isolation using Method 1 showed maximum recovery of the four methods tested (Figure 4; left panel). Subsequent samples were isolated using Method 1. The yield of cfRNA from plasma of AML samples ranged between 6.42 to 380ng/ml plasma with a median of 65.8ng/ml (Figure 4; right panel) as opposed to 9.8ng/ml in control samples .

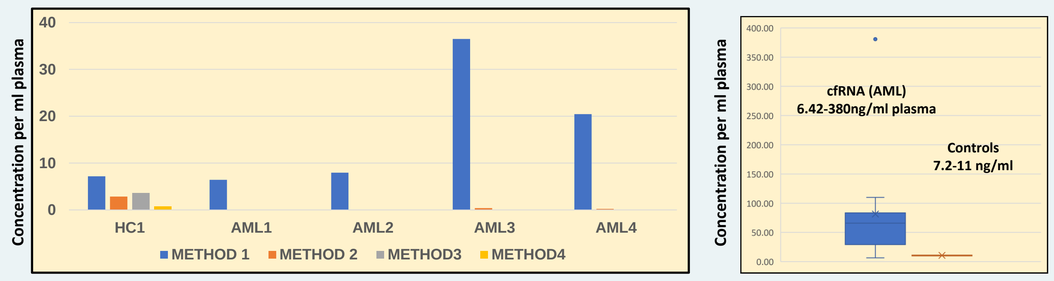

Figure 4: Comparison showing yield of cell free RNA concentration per ml plasma extracted using four different methods (left panel). RNA was extracted from AML samples (n=12) and healthy controls (n=3) using Method 1. Range of cfRNA is shown in box whisker plot (right panel).

Quality check of RNAseq libraries generated from PBMC RNA and cfRNA from AML samples: The average peak size obtained from total cfRNA libraries was 335bp (Figure 5; left panel) as compared to 454bp for PBMC RNA (Figure 5; left panel).

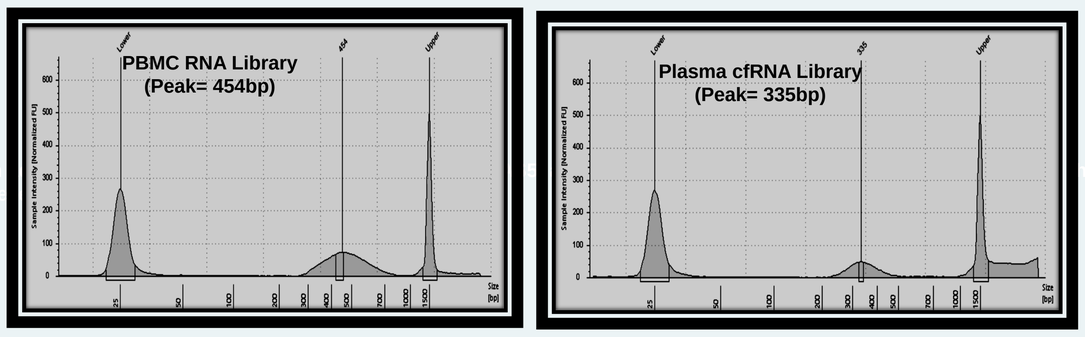

Figure 5: PBMC RNA library (left panel) and cfRNA library (right panel) quality check showing average peak sizes of a representative sample.

RNAseq Analysis showed high concordance between cfRNA and PBMC RNA transcripts: Paired cfRNA and PBMC RNA revealed >2000 protein coding transcripts with >10 TPM value and a high concordance (0.84-0.94) between the paired samples was observed (Figure 6; top left panel). Amongst the transcripts with highest TPM values in cfRNA compartment were ferroptosis related (FTL, FTH1), thymosin and extracellular S100 proteins (S100A8 and S100A9, S100A10, S100A11, S100A4) that regulate immune homeostasis, inflammation and serve as danger signals (Figure 6; bottom panel). Transcripts of few CD markers CD52, CD68 and CD74 associated with tumor immune microenvironment were identified. In addition, lncRNA implicated in oncogenesis and chemoresistance were identified (Figure 6; bottom right panel).

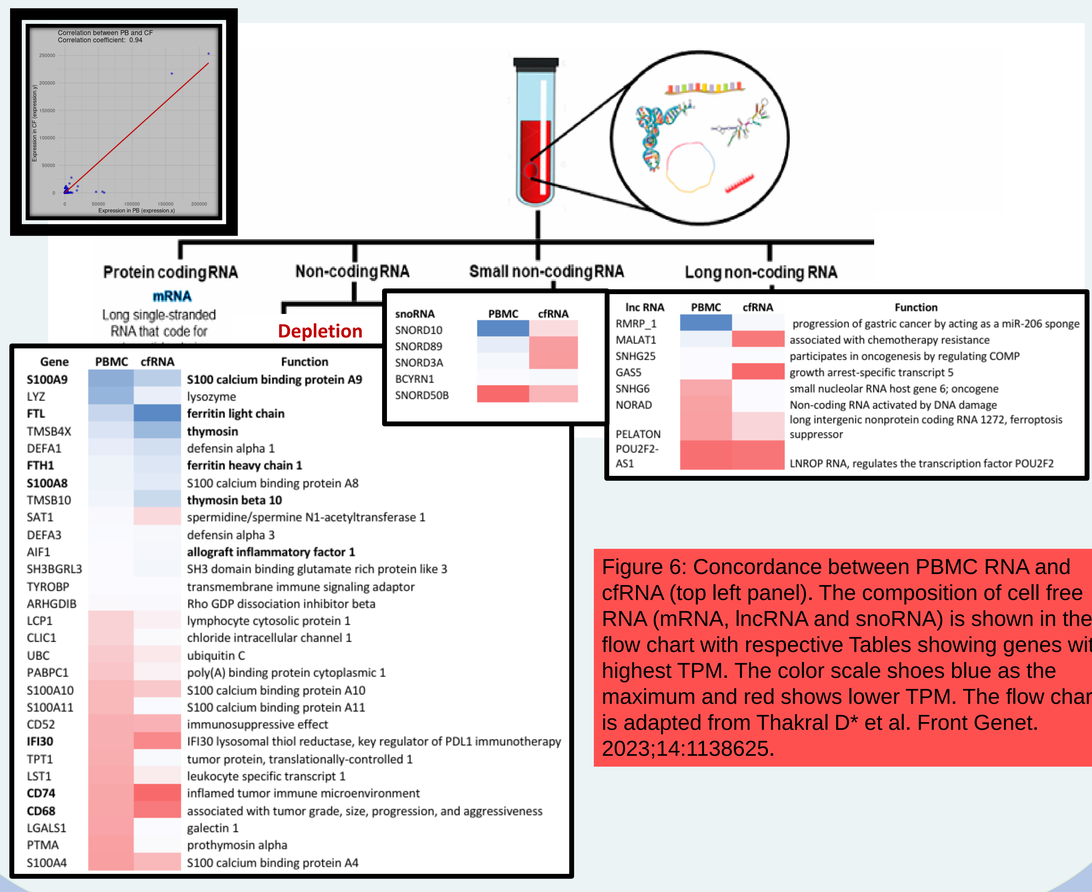

Figure 6: Concordance between PBMC RNA and cfRNA (top left panel). The composition of cell free RNA (mRNA, lncRNA and snoRNA) is shown in the flow chart with respective Tables showing genes with highest TPM. The color scale shoes blue as the maximum and red shows lower TPM. The flow chart is adapted from Thakral D* et al. Front Genet. 2023;14:1138625.

CONCLUSION & FUTURE DIRECTIONS

- cfRNA concentration was significantly higher in AML samples relative to healthy controls.
- The difference in the average library size of cfRNA than PBMC indicated fragmented circulating RNA.
- High concordance between the RNA profiles of cfRNA and PBMC RNA holds great potential in future translation for AML disease profiling.
- The gene signatures inferred from plasma cfRNA sequencing data warrant further investigation of the targets for the mechanisms that lead to disparate treatment responses.
- Potential gene targets identified by RNAseq analysis are under evaluation using cfRNA and correlation with clinical outcome.
- Combining cfRNA with ctDNA might improve the prognostic assessment of AML disease in real-time and warrants further evaluation.

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