FACULTY OF MEDICINE IN PILSEN
CHARLES UNIVERSITY

BIOMEDICAL CENTER

ANALYSIS OF CIRCULATING CELL-FREE RNAs IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA

Klieber R[1,2], Holubová M[1,3], Macečková D[1,4], Dostálová K[1,4], Lysák D[1,3], Ostašov P[1,4]

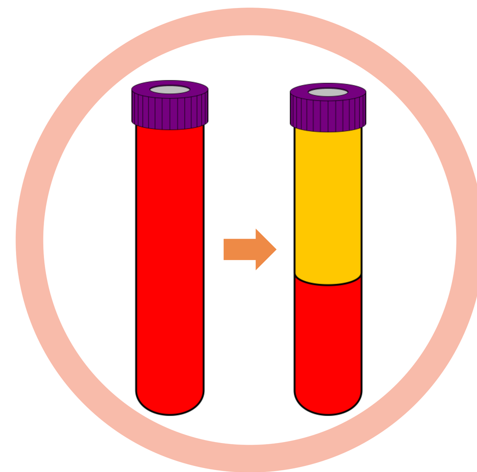
1 Biomedical center, Faculty of Medicine in Pilsen, Charles University in Prague, Pilsen, Czech Republic
2 Department of Biology, Faculty of Medicine in Pilsen, Charles University in Prague, Pilsen, Czech Republic
3 Department of Hematology and Oncology, University Hospital in Pilsen, Pilsen, Czech Republic
4 Department of Histology and Embryology, Faculty of Medicine in Pilsen, Charles University in Prague, Pilsen, Czech Republic

INTRODUCTION

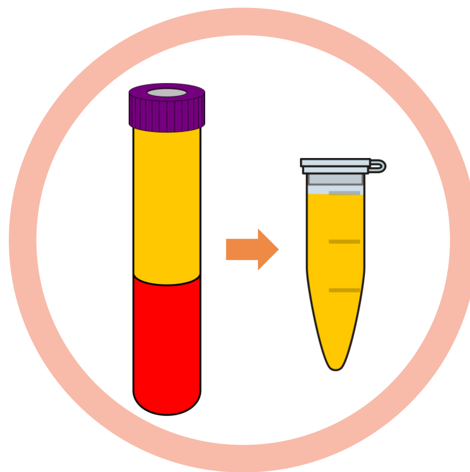
Diffuse large B-cell lymphoma (DLBCL) is a malignant lymphoproliferative disease characterized by clonal B cell proliferation affecting bone marrow, peripheral blood, nodes and spleen. Cell-free long RNAs are potential and prominent molecular prognostic and minimal residual disease markers that can be studied by undemanding and non-invasive blood collection as a liquid biopsy. In our study, we focused on analyzing 3' end polyadenylated RNA molecules in DLBCL patients' plasma. Messenger RNA transcripts and some long non-coding RNAs then were analyzed by high-throughput RNA sequencing.

METHODS

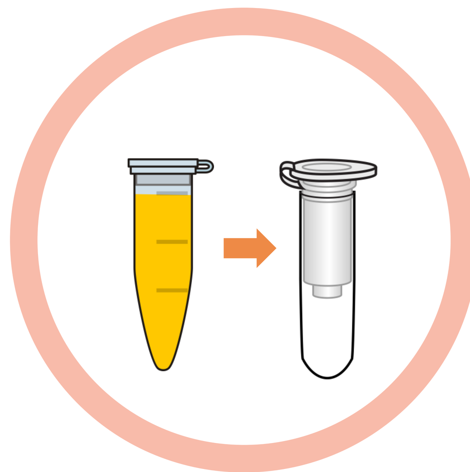
Blood samples from ten patients, diagnosed with DLBCL, were obtained before and after chemotherapy. Blood was collected in K3EDTA tubes and within two hours centrifuged twice to obtain platelet poor plasma and to remove cell debris. Total RNA from 1 mL of plasma was isolated using miRNeasy Serum/Plasma kit (Qiagen), fluorescently quantified using Ribogreen (Thermo Fisher Sc.), treated with DNase I (ArcticZymes Tech.), and 3' mRNA sequencing libraries were prepared (Lexogen). To exclude PCR amplification bias, Unique Molecular Identifiers (UMIs) also provided by Lexogen were used in the process of library preparation. Libraries were pooled and sequenced on Illumina NovaSeq 6000 (2×75 cycles, paired-end).



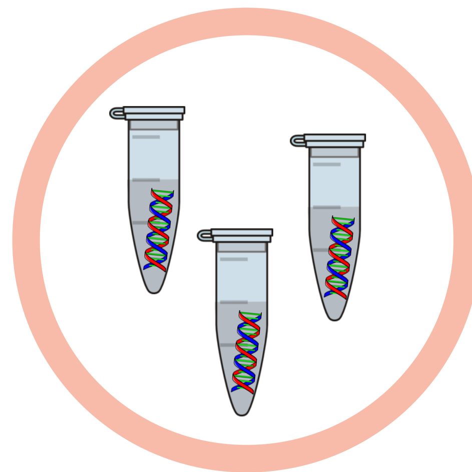
1. Whole blood centrifugation
   - 10 minutes 3000 × g



2. Plasma recentrifugation
   - 15 minutes 3000 × g



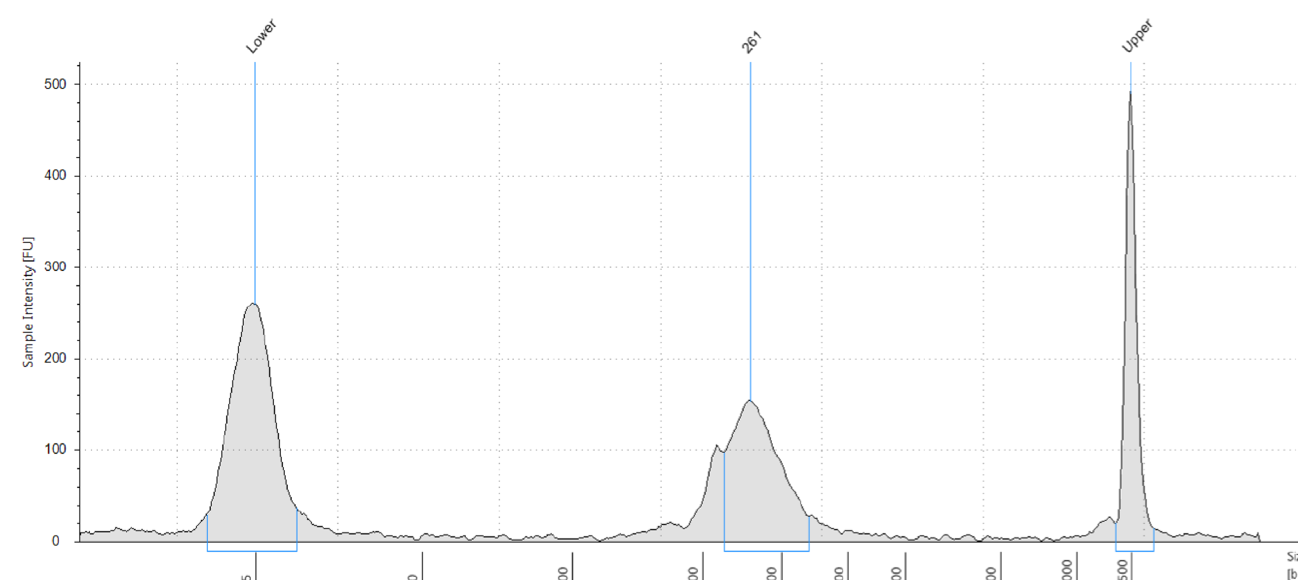
3. Cell-free RNA isolation
   - Qiagen miRNeasy serum/plasma kit



4. NGS Library preparation
   - Lexogen Quantseq 3'mRNA library prep kit for Illumina + UMIs

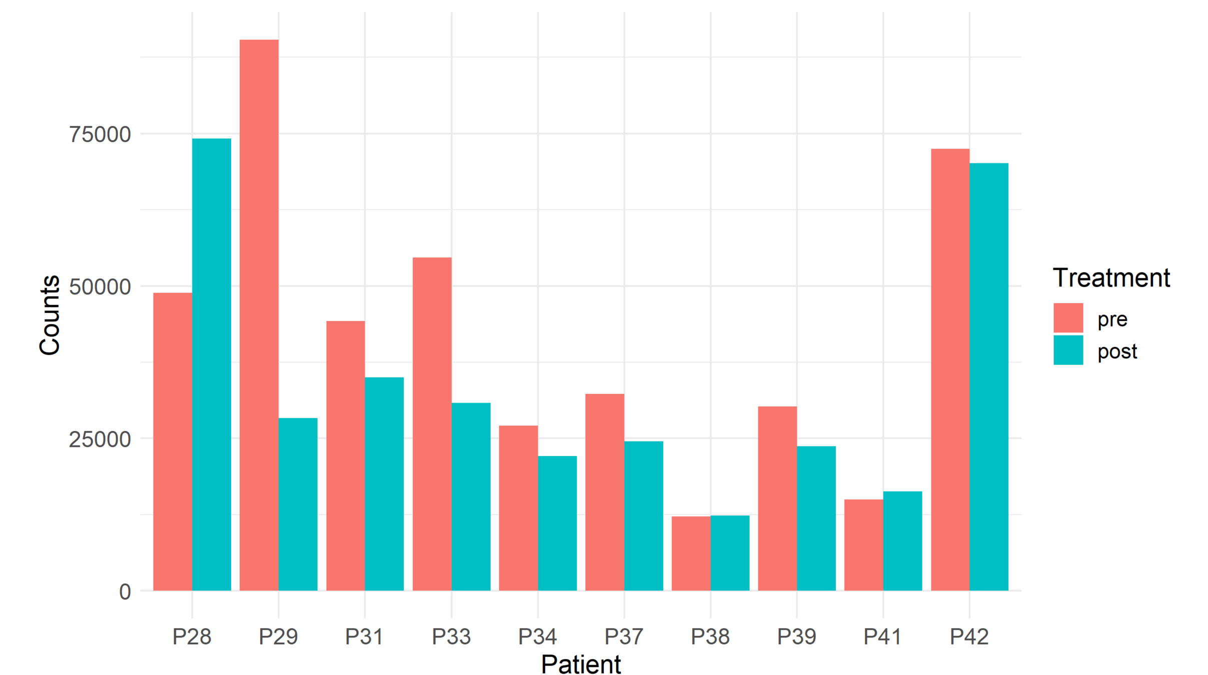
5. High-throughput sequencing
   - Illumina NovaSeq 6000

RESULTS



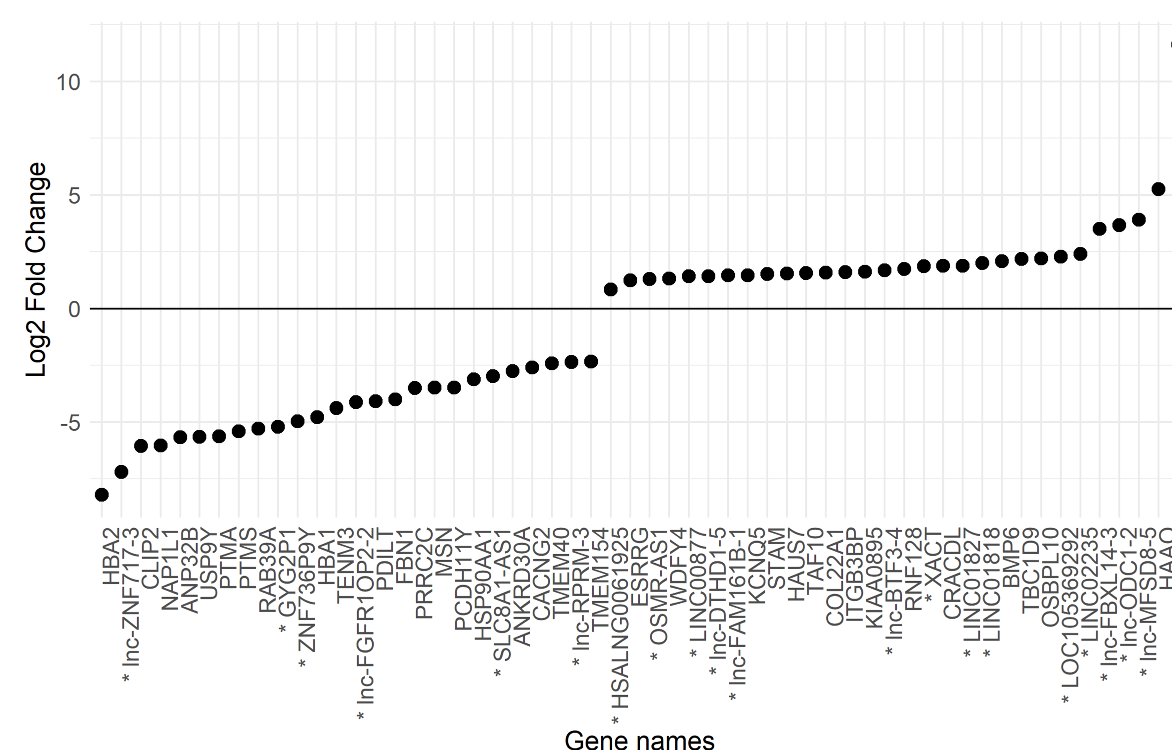
1. POOL OF CELL-FREE RNA NGS LIBRARIES

Twenty 3'mRNA libraries were prepared (Lexogen QuantSeq) and quality was measured by automated capillary electrophoresis analyzer (Agilent Tapestation 2200). Mean library fragment was 261 base pairs.



2. NUMBER OF RAW READ COUNTS PER PATIENT

Sequencing data were processed using Lexogen QuantSeq pipeline. The PCR amplification bias was removed using unique molecular identifiers (UMI_tools). Low quality reads and adapters were trimmed (Trimmomatic) and reads were aligned to the GRCh38 reference genome (STAR). Represented read counts were determined by HTseq-count.



3. DIFFERENTIALLY EXPRESSED GENES IN THE CELL-FREE RNA FROM DLBCL PATIENTS

Differential gene expression analysis based on the negative binomial distribution was determined by DESeq2 between plasma cell-free RNA samples before and after chemotherapy treatment in patients with DLBCL. Only statistically significant genes with p-value < 0.05 are shown. Long non-coding RNAs are marked *.

	Gene	Biological pathway / Gene function
Upregulated	TRAF3IP3	Cell growth by modulating the c-Jun N-terminal kinase signal transduction pathway
	BMP6	Activation of cAMP-Dependent PKA and Akt Signaling
	RNF128	Deubiquitination and Calcineurin-regulated NFAT-dependent transcription in lymphocytes
	ITGB3BP	Chromosome Maintenance and p75 NTR receptor-mediated signalling
	COL22A1	Collagen chain trimerization and Degradation of the extracellular matrix
	STAM	Endocytosis and EGF/EGFR Signaling Pathway
Downregulated	PTMA	Validated targets of C-MYC transcriptional activation
	RAB39A	Metabolism of proteins and Vesicle-mediated transport
	FBN1	Degradation of the extracellular matrix and ECM-receptor interaction
	MSN	Developmental Biology and Glial Cell Differentiation
	HSP90AA1	MAPK Pathway and IL2 signaling events mediated by PI3K
	CACNG2	Activation of cAMP-Dependent PKA and Apoptotic Pathways in Synovial Fibroblasts

4. EXAMPLES OF BIOLOGICAL PATHWAYS OF DIFFERENTIALLY EXPRESSED GENES IN THE CELL-FREE RNA FROM DLBCL PATIENTS

Selected genes and their function in pathways involving cell signaling and extracellular matrix rearrangements.

CONCLUSIONS

Analysis of cell-free RNA identified changes in gene expression of 36 protein-coding transcripts and 20 lncRNAs. Genes downregulated after chemotherapy were found to be involved in the MAPK signaling pathway, IL2 signaling pathway and vesicle-mediated transport, while upregulated ones were involved in Akt signaling pathway, degradation of extracellular matrix, endocytosis and EGF/EGFR signaling pathways. High-throughput RNA sequencing is the predominant method for analyzing suitable molecular markers. The most informative molecules may then serve as a prognostic markers and as a sensitive tool for minimal residual disease evaluation.

ACKNOWLEDGMENT

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