Critical Insights into Total Transcriptome Profiling using Next Generation Sequencing Technology

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What is RNAome

I. RNAome is understood as genomewide profiling of functional RNA population expressed in a cell / tissue.

II. Functional RNA population include mRNA, miRNA, siRNA, LnCRNA, enhancer RNAs, anti-sense RNA etc

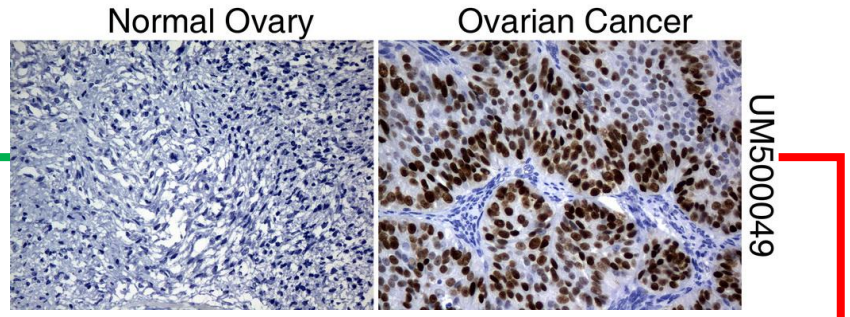
Applications of RNAome in cancer research

I. Diagnostics

II. Transcriptional and Post Transcriptional gene regulation of cancer establishment

III. Gene Regulatory Networks Underlying cancer progression

Deep Sequencing Applications for profiling RNAome



Total RNA (Without Column Clean Up)

Deep Sequencing of Small RNA Population

Deep Sequencing of Long RNA Population

Expression Profiling and Analysis

Integrome Analysis for Transcriptional and Post Transcriptional Gene Regulation

Gene Regulatory Network Modelling

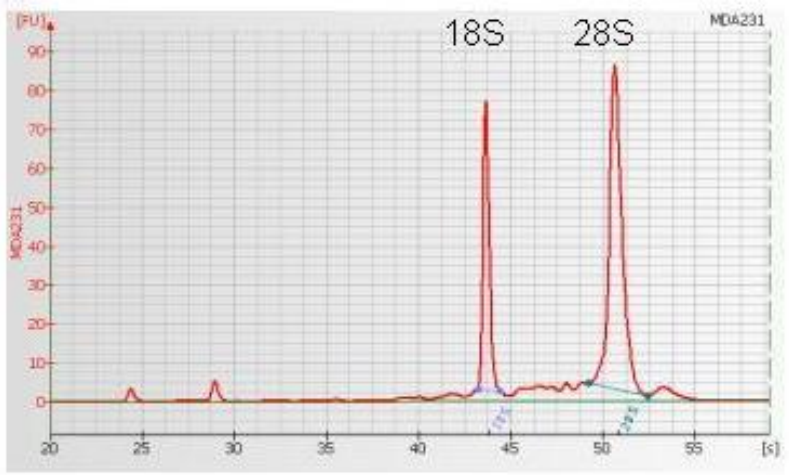
Critical metrics representative of a largely TRUE RNAOme - using sequence reads from Illumina Platform

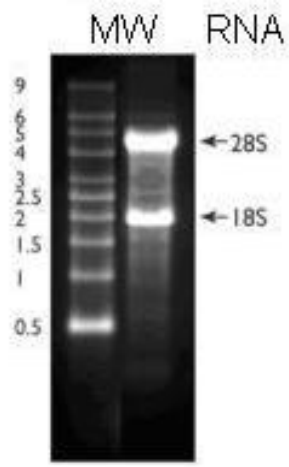
1. Homogeneity / Heterogeneity in libraries for sequencing
2. SD (Standard Deviation) in the total number of HQ reads in libraries to be merged for building transcriptome
3. Total RNA Quality and Purity
4. Total number of full length transcripts detected
5. Percentage of reads mapping to reference genome
6. Recovery of conserved, widely expressed genes
7. Percentage of Splice variants / modified transcripts
8. Total number of novel transcripts expressed
9. Coverage vs Depth criteria
10. Percentage of novel transcripts / fusion transcripts

Preparing for a Total RNAome Profiling using Illumina Platform

Considerations for RNA Quality

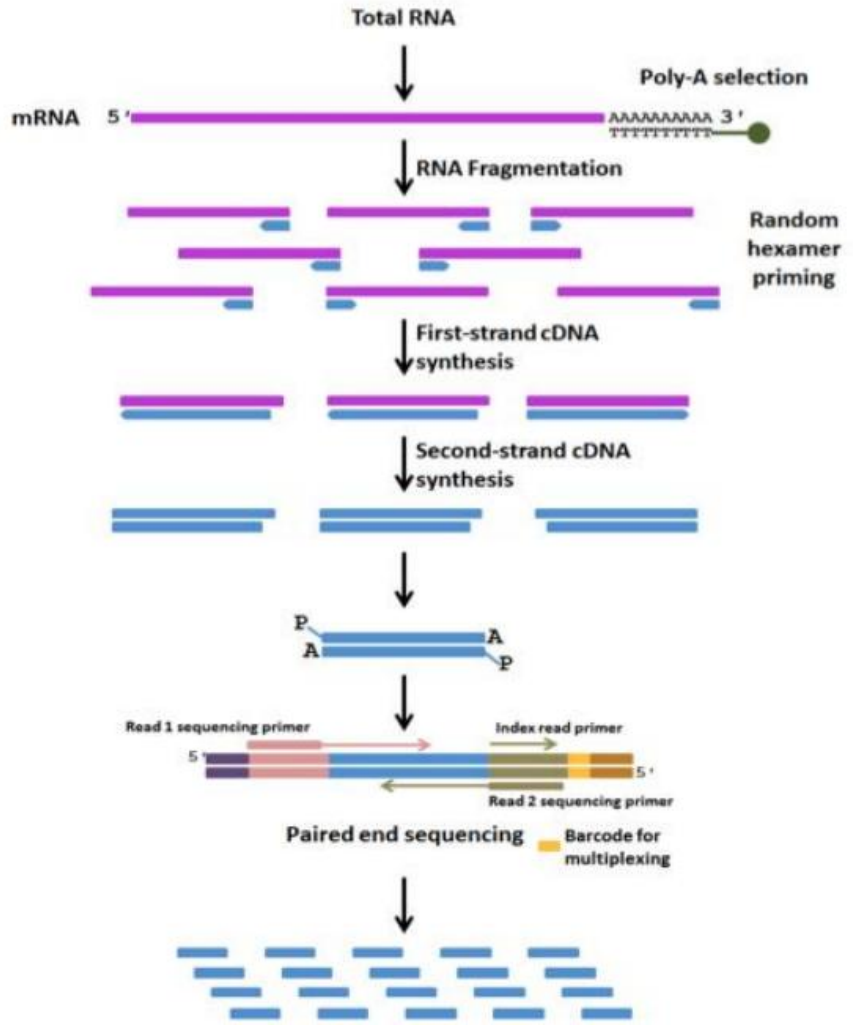
1. Total RNA (without column purification)
2. 2-3 microgram (if there is no dearth for cells)
3. 20-50 nanogram (if very few cells are only available – Alternative linear amplification protocol)
4. 50 ng/ul for option 2 and 5ng/ul for option 3
5. RNA in RNAse free water / Ethanol Precipitation for 4deg shipping
6. Agilent 2100 Bioanalyzer QC of Total RNA using NanoChip (Option 2) or PicoChip (Option 3)

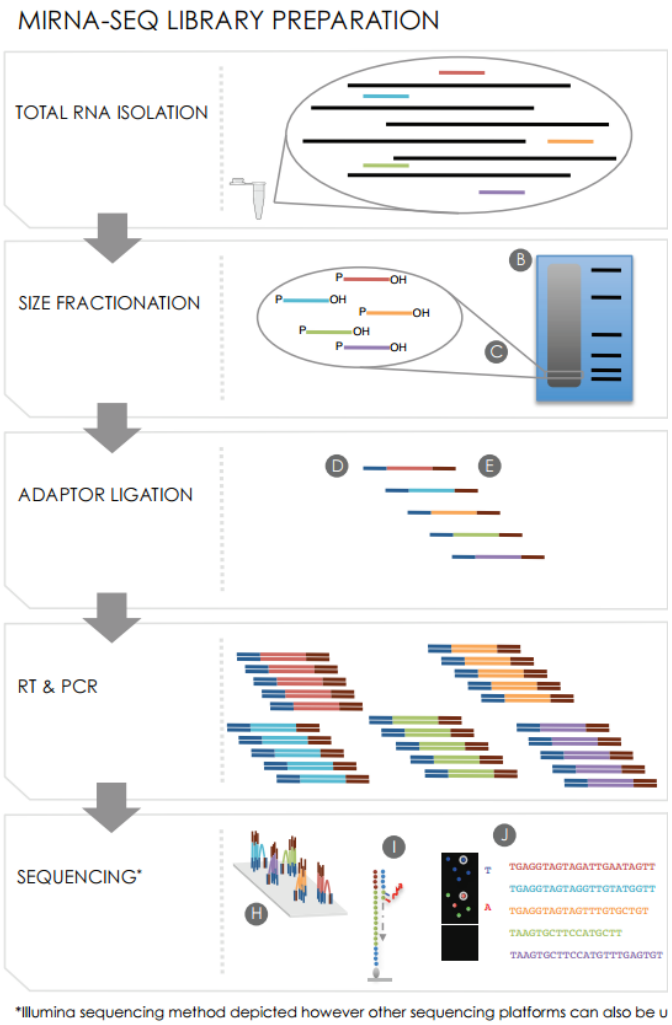




Sequencing Library preparation for a complete RNAOme Profiling using Illumina Platform

1. Libraries prepared using Illumina Recommended TrueSeq Protocol
2. 100bp + reads mandate for long RNA sequencing
3. 36bp - 50bp reads for small RNA sequencing
4. Paired end sequencing module is preferred for long RNA sequencing
5. Upto 24 samples can be multiplexed in HiSEQ platform
6. Minimum of 20-30 million reads required for optimal coverage and depth of transcriptome (Large / Small)
7. Coverage is indicative of number of transcripts profiled
8. Depth is indicative of sensitivity in copy number detection





Typical results from long RNA profiling

1. Full Length mRNAs
2. Known Splice Variants
3. Novel Modifications in mRNAs
4. Known Fusion Transcripts
5. Novel Fusion Transcripts
6. Long Non-coding RNAs
7. Precursor miRNAs
8. Novel Transcripts (Arising from known non-protein coding regions)

Typical results from small RNA profiling

1. Known miRNAs
2. Novel miRNAs
3. Novel Modifications in mRNAs
4. Known siRNAs
5. piRNAs
6. SnoRNAs

Case Study – RNAOme Profiling of Retinoblastoma

Bioinformatics and Biology Insights

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ORIGINAL RESEARCH

Molecular Insights on Post-chemotherapy Retinoblastoma by Microarray Gene Expression Analysis

Venkatesan Nalini[1,2], Ramya Segu[3], Perinkulam Ravi Deepa[4], Vikas Khetan[5], Madavan Vasudevan[6] and Subramanian Krishnakumar[1]

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Abstract

Purpose: Management of Retinoblastoma (RB), a pediatric ocular cancer is limited by drug-resistance and drug-dosage related side effects during chemotherapy. Molecular de-regulation in post-chemotherapy RB tumors was investigated.

Materials and Methods: cDNA microarray analysis of two post-chemotherapy and one pre-chemotherapy RB tumor tissues was performed, followed by Principle Component Analysis, Gene ontology, Pathway Enrichment analysis and Biological Analysis Network (BAN) modeling. The drug modulation role of two significantly up-regulated genes ($P \leq 0.05$) — *Ect2* (Epithelial-cell-transforming-sequence-2), and *PRAME* (preferentially-expressed-Antigen-in-Melanoma) was assessed by qRT-PCR, immunohistochemistry and cell viability assays.

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ORIGINAL RESEARCH

Identification and Insilico Analysis of Retinoblastoma Serum microRNA Profile and Gene Targets Towards Prediction of Novel Serum Biomarkers

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Abstract: Retinoblastoma (RB) is a malignant tumor of the retina seen in children, and potential non invasive biomarkers are in need for rapid diagnosis and for prognosticating the therapy. This study was undertaken to identify the differentially expressed miRNAs in the serum of children with RB in comparison with the normal age matched serum, to analyze its concurrence with the existing RB tumor miRNA profile, to identify its novel gene targets specific to RB, and to study the expression of a few of the identified oncogenic miRNAs in the advanced stage primary RB patient's serum sample. MiRNA profiling was performed on 14 pooled serum from children with advanced RB and 14 normal age matched serum samples, wherein 21 miRNAs were found to be upregulated (fold change $\geq +2.0$, $P \leq 0.05$) and 24 to be downregulated (fold change ≤ -2.0, $P \leq 0.05$). Furthermore, intersection of 59 significantly deregulated

Case Study – RNAOme Profiling of Retinoblastoma

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PLOS ONE

RNAi Mediated Tiam1 Gene Knockdown Inhibits Invasion of Retinoblastoma

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Abstract

T lymphoma invasion and metastasis protein (Tiam1) is up-regulated in variety of cancers and its expression level is related to metastatic potential of the type of cancer. Earlier, Tiam1 was shown to be overexpressed in retinoblastoma (RB) and we hypothesized that it was involved in invasiveness of RB. This was tested by silencing Tiam1 in RB cell lines (Y79 and Weri-Rb1) using siRNA pool, targeting different regions of Tiam1 mRNA. The cDNA microarray of Tiam1 silenced cells showed gene regulations altered by Tiam1 were predominantly on the actin cytoskeleton interacting proteins, apoptotic initiators and tumorogenic potential targets. The silenced phenotype resulted in decreased growth and increased apoptosis with non-invasive characteristics. Transfection of full length and N-terminal truncated construct (C1199) clearly revealed membrane localization of Tiam1 and not in the case of C580 construct. F-actin staining showed the interaction of Tiam1 with actin in the membrane edges that leads to ruffling, and also imparts varying invasive potential to the cell. The results obtained from our study show for the first time that Tiam1 modulates the cell invasion, mediated by actin cytoskeleton remodeling in RB.

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Bioinformatics and Biology Insights

Integrated Analysis of Dysregulated miRNA-gene Expression in *HMGA2*-silenced Retinoblastoma Cells

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ABSTRACT: Retinoblastoma (RB) is a primary childhood eye cancer. *HMGA2* shows promise as a molecule for targeted therapy. The involvement of miRNAs in genome-level molecular dys-regulation in *HMGA2*-silenced RB cells is poorly understood. Through miRNA expression microarray profiling, and an integrated array analysis of the *HMGA2*-silenced RB cells, the dysregulated miRNAs and the miRNA-target relationships were modelled. Loop network analysis revealed a regulatory association between the transcription factor (*SOX5*) and the deregulated miRNAs (*miR-29a, miR-9*, miR-9-3*). Silencing of *HMGA2* deregulated the vital oncomirs (*miR-7, miR-331, miR-26a, miR-221, miR-17~92* and *miR-106b~25*) in RB cells. From this list, the role of the *miR-106b~25* cluster was examined further for its expression in primary RB tumor tissues (n = 20). The regulatory targets of *miR-106b~25* cluster namely *p21* (cyclin-dependent kinase inhibitor) and *BIM* (pro-apoptotic gene) were elevated, and apoptotic cell death was observed, in RB tumor cells treated with the specific antagomirs of the *miR-106b~25* cluster. Thus, suppression of *miR-106b~25* cluster controls RB tumor growth. Taken together, *HMGA2* mediated anti-tumor effect present in RB is, in part, mediated through the *miR-106b~25* cluster.

KEYWORDS: Retinoblastoma, High mobility group proteins *(HMG)A2*, *miR-106b-25* cluster, Integrated mRNA-miRNA analysis, Antagomirs

Case Study – RNAOme Profiling of Retinoblastoma

Computational and *in vitro* Investigation of miRNA-Gene Regulations in Retinoblastoma Pathogenesis: miRNA Mimics Strategy

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ABSTRACT

PURPOSE: Retinoblastoma (RB), a primary pediatric intraocular tumor, arises from primitive retinal layers. Several novel molecular strategies are being developed for the clinical management of RB. miRNAs are known to regulate cancer-relevant biological processes. Here, the role of selected miRNAs, namely, *miR-532-5p* and *miR-486-3p*, has been analyzed for potential therapeutic targeting in RB.

METHODS: A comprehensive bioinformatic analysis was performed to predict the posttranscriptional regulators (miRNAs) of the select panel of genes [Group 1: oncogenes (*HMGA2, MYCN, SYK, FASN*); Group 2: cancer stem cell markers (*TACSTD, ABCG2, CD133, CD44, CD24*) and Group 3: cell cycle regulatory proteins (*p53, MDM2*)] using Microcosm, DIANALAB, miRBase v 18, and REFSEQ database, and RNA hybrid. The expressions of five miRNAs, namely, *miR-146b-5p, miR-532-5p, miR-142-5p, miR-328*, and *miR- 486-3p*, were analyzed by qRT–PCR on primary RB tumor samples ($n = 30$; including 17 invasive RB tumors and 13 noninvasive RB tumors). Detailed complementary alignment between 5' seed sequence of differentially expressed miRNAs and the sequence of target genes was determined. Based on minimum energy level and piCTAR scores, the gene targets were selected. Functional roles of these miRNA clusters were studied by using mimics in cultured RB (Y79, Weri Rb-1) cells *in vitro*. The gene targets (*SYK* and *FASN*) of the studied miRNAs were confirmed by qRT-PCR and western blot analysis. Cell proliferation and apoptotic studies were performed.

RESULTS: Nearly 1948 miRNAs were identified in the in silico analysis, From this list, only 9 upregulated miRNAs (*miR-146b-5p, miR-305, miR-663b, miR-299, miR-532-5p, miR-892b, miR-501, miR-142-5p*, and *miR-513b)* and 10 downregulated miRNAs (*miR-1254, miR-328, miR-133a, miR-1287, miR-1299, miR-375, miR-486-3p, miR-720, miR-98*, and *miR-122*)* were found to be common with the RB serum miRNA profile. Downregulation of five miRNAs (*miR-146b-5p, miR-532-5p, miR-142-5p, miR-328*, and *miR-486-3p*) was confirmed experimentally. Predicted common oncogene targets (*SYK* and *FASN*) of *miR-486-3p* and *miR-532-5p* were evaluated for their mRNA and protein expression in these miRNA mimic-treated RB cells. Experimental overexpression of these miRNAs mediated apoptotic cell death without significantly altering the cell cycle in RB cells.

CONCLUSION: Key miRNAs in RB pathogenesis were identified by an in silico approach. Downregulation of *miR-486-3p* and *miR-532-5p* in primary retinoblastoma tissues implicates their role in tumorigenesis. Prognostic and therapeutic potential of these miRNA was established by the miRNA mimic strategy.

KEYWORDS: bio-informatics analysis, miRNA-mRNA, mimics, retinoblastoma

ARTICLE

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Global Gene Deregulations in *FASN* Silenced Retinoblastoma Cancer Cells: Molecular and Clinico-Pathological Correlations

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ABSTRACT

Activation of fatty acid synthase (FASN) enzyme in the de novo lipogenic pathway has been reported in various cancers including retinoblastoma (RB), a pediatric ocular cancer. The present study investigates lipogenesis-dependent survival of RB cancer cells and the associated molecular pathways in *FASN* silenced RB cells. The siRNA-mediated FASN gene knockdown in RB cancer cells (Y79, WERI RB1) repressed FASN mRNA and protein expressions, and decreased cancer cell viability. Global gene expression microarray analysis was performed

Case Study – RNAOme Profiling of Sarcoma

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Data in Brief

Global gene expression profiling data analysis reveals key gene families and biological processes inhibited by Mithramycin in sarcoma cell lines

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ABSTRACT

The role of Mithramycin as an anticancer drug has been well studied. Sarcoma is a type of cancer arising from cells of mesenchymal origin. Though incidence of sarcoma is not of significant percentage, it becomes vital to understand the role of Mithramycin in controlling tumor progression of sarcoma. In this article, we have analyzed the global gene expression profile changes induced by Mithramycin in two different sarcoma lines from whole genome gene expression profiling microarray data. We have found that the primary mode of action of Mithramycin is by global repression of key cellular processes and gene families like phosphoproteins, kinases, alternative splicing, regulation of transcription, DNA binding, regulation of histone acetylation, negative regulation of gene expression, chromosome organization or chromatin assembly and cytoskeleton.

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Anticancer drug mithramycin interacts with core histones: An additional mode of action of the DNA groove binder

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ABSTRACT

Mithramycin (MTR) is a clinically approved DNA-binding antitumor antibiotic currently in Phase 2 clinical trials at National Institutes of Health for treatment of osteosarcoma. In view of the resurgence in the studies of this generic antibiotic as a human medicine, we have examined the binding properties of MTR with the integral component of chromatin – histone proteins – as a part of our broad objective to classify DNA-binding molecules in terms of their ability to bind chromosomal DNA alone (single binding mode) or both histones and chromosomal DNA (dual binding mode). The present report shows that besides DNA, MTR also binds to core histones present in chromatin and thus possesses the property of dual binding in the chromatin context. In contrast to the MTR–DNA interaction, association of MTR with histones does not require obligatory presence of bivalent metal ion like Mg^{2+}. As a consequence of its ability to interact with core histones, MTR inhibits histone H3 acetylation at lysine 18, an important signature of active chromatin, *in vitro* and *ex vivo*. Reanalysis of microarray data of Ewing sarcoma cell lines shows that upon MTR treatment there is a significant down regulation of genes, possibly implicating a repression of H3K18Ac-enriched genes apart from DNA-binding transcription factors. Association of MTR with core histones and its ability to alter post-translational modification of histone H3 clearly indicates an additional mode of action of this anticancer drug that could be implicated in novel therapeutic strategies.

Case Study – RNAOme Profiling of Oral Squamous Cell Carcinoma

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Discussion..

1. Illumina is the widely used sequencing platform for whole transcriptome studies.
2. With hundreds of whole transcriptome published with majority using Illumina sequencing platform, the integrity and resolution of the transcriptome remain un-addressed.
3. The choice of the platform, aligner and sample size and study design largely determines the sensitivity and specificity of the resolved transcriptome.
4. The most important step in RNA-seq analysis is alignment of generated short reads into full-length transcripts.
5. Depending on the choice of the aligner it is very critical to identify and profile both known and novel transcripts for integrated analysis of the transcriptome.

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