

Enabling Circulating Cell-free mRNA Profiling to Empower Cancer Early Detection

Chen-Hsiung Y*

Circulogene Theranostics, 3125 Independence Drive, Suite 301, Birmingham, AL, 35209, USA

Abstract

DNA methylation occurs early in tumorigenesis and is highly pervasive across cancer types, making it suitable for early detection and intervention of cancer. Gene expression at the transcription level is reflected by epigenetic regulation of chromosome especially DNA methylation, therefore providing a parallel approach to achieve the same goal. Non-invasive and real-time gene expression profiling was performed on plasma circulating cell-free mRNA (cell free m-RNA) enriched from cancer patients using proprietary high sensitivity RT-qPCR assays. A plasma cell free m-RNA expression database covering 750 genes in 9 major cancer pathways was established with multiple layers of cancer type-specific characteristics: (i) the distribution of cell free m-RNA species across 9 major cancer pathways; (ii) the differential expression of target genes (high, medium or low expression); (iii) the global cell free m-RNA expression landscape in circulation; and (iv) the unique cell free m-RNA signatures to differentiate lung, breast, and pancreatic cancer. These novel blood-based metrics and biomarkers can be deployed for early detection and stratification of cancer.

Keywords: Gene expression • Pancreatic cancer • Biomarkers • Protein-coding mRNAs

Introduction

The emergence of relatively non-invasive liquid biopsy as a complementary approach to surgical biopsies has fueled intensive research effort and investment. Circulating cell-free nucleic acids in this respect have revolutionized cancer diagnosis in recent decade, allowing non-invasive, real-time and longitudinal interrogation for genomic alterations using a single sample of blood [1]. Consequently, the use of cell-free nucleic acids as biomarkers could facilitate the early detection of diseases such as cancer and enable simple, specific monitoring of disease progression.

In addition to DNAs, protein-coding mRNAs from the tumor tissues are released into the blood, enriched over time and can reflect changes in tumor-specific gene expression. Plasma cfDNA methylation and mutation events are less dynamic and likely provide limited information on tissue homeostasis and disruption. In contrast, circulating cell-free mRNA (cell free m-RNA) profiling could provide richer molecular content compared to other non-invasive biomarkers and constitutes a unique non-invasive interrogation of tissue function in scenarios such as early detection of disease, early drug engagement and response in patients. Combined with advances in molecular diagnostics, systematic profiling of cell free m-RNA can improve our understanding of cancer pathology and identify novel biomarkers for early detection, without the need for invasive biopsy. The potential clinical utility of cell free m-RNA has been demonstrated in patients with various malignant cancers [2-4]. Understanding the mechanisms underlying the presence of mRNA transcripts in circulation is essential to interpret their clinical value.

Methodology

Circulating cell free m-RNA abundance can be influenced by physiological state, level of nucleases in the blood, the half-life of individual cell free m-RNA

***Address for Correspondence:** Dr. Chen-Hsiung Yeh, Circulogene Theranostics, 3125 Independence Drive, Suite 301, Birmingham, AL, 35209, USA, E-mail: cyeh@circulogene.com

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and the clearance rate by immune system, liver and kidney [2]. Certain cell free m-RNA species are in complexed forms that protects them from degradation by RNases. This ensures their unique stability in the circulation, in contrast to complex-free RNA, which is rapidly degraded [5]. Therefore, key challenges in the cell free m-RNA testing include its extremely low abundance, susceptible to degradation, relatively unstable and poor extraction efficiency. To circumvent these limitations, a signal amplification step following cell free m-RNA extractions should be performed as we discovered in our studies. Circulating cell free m-RNA carries information from human tissues; the pattern of cell free m-RNA expression reflects dysregulation of cancer immunity, tumor cell growth, proliferation and stromal interaction, which makes cell free m-RNA expression signature a promising biomarker for early diagnostic, prognostic and therapeutic purposes [6,7].

Circulating cell free m-RNA in plasma is usually made up of degraded small fragments with size smaller than 200 nucleotides, very low concentration (average lower than 10 ng/mL), and with different terminal modification, these properties make it difficult to investigate [8-10]. The current molecular techniques employed for the detection and characterization of cell free m-RNA include microarrays, RT-qPCR and next-generation sequencing (NGS; RNA-Seq) [11-14]. Microarrays had been widely used to define circulating microRNA expression. However, due to their limited sensitivity microarrays can only screen the most abundant RNA in biofluids. On the contrary, both RT-qPCR and NGS can detect low abundant cell free m-RNA and remain currently the methods of choice. NGS has been used for cell free m-RNA studies, but some intrinsic problems were not solved, including labor-intensive, time-consuming, requirement of large volume of blood, no standard sequencing method for all RNA fractions, high cost for large scale RNA library preparation, as well as low mapping rate and thus low sensitivity [15-17]. RT-qPCR is a more convenient, sensitive and cost-effective approach, with pre-loaded custom plates, further enhancing its capability as an automate and high throughput platform. Developing a simple, highly reliable, cost-efficient and non-invasive diagnostic cell free m-RNA test system to screen and identify early stages without the use of a tissue biopsy would significantly reduce both the mortality and the economic burden associated with cancer.

Tissue biopsy and associated approaches which are highly dependent on skills of an operator and the availability of costly equipment could hardly fit into a model of point-of-care diagnostics. The absence of clear alternatives prompts the development and validation of functionalized gene signatures where each individual gene would, ideally, reflect certain pathophysiological process contributing to specific cancer progression in a given individual and predict its outcome. Although multiple research reports have demonstrated amazing promises of circulating cell free m-RNA for diagnostic application, this

field is still in its infancy. It is imperative and of paramount interest to harness highly sensitive cell free m-RNA detection technologies and establishing unique expression signatures as early-stage fingerprints of oncogenesis in biological fluids. The fundamental advantage of circulating cell free m-RNA over protein biomarkers is that, unlike proteins, nucleic acids can be detected by a PCR which has the detection threshold of a single molecule. A cell free m-RNA-based expression signature could, if necessary, be augmented by other blood-based biomarkers including cancer-specific cfDNA. The cell free m-RNA expression profiling can be considered as a compendium of transcripts collected from all organs. Some of these circulating transcripts correspond to "true" tissue-specific or cancer type-specific genes, strongly supporting interrogation of these biomolecules to dynamically monitor early pathological changes of tissues and organs. In contrast to poorly functional annotation of non-coding RNA, the coding cell free m-RNA expression profiling provides direct access to both genetic information and functional information pertaining to the tissue of origin and its physiology. Previous studies have reported transcripts in circulation encoding functional information of the liver, brain, immune system, or fetal development [7,11,12]. Therefore, cell free m-RNA expression pattern has the capability of integrating functional and genetic information of tissues, highlighting this analyte's unique potential as a non-invasive biomarker.

Our comprehensive cell free m-RNA profiling data here provides circulating transcript snapshots of gene expression signatures in patients with lung, pancreatic or breast cancer. The cell free m-RNA expression signature will allow non-invasive delineation of cancer type, early detection, and progression monitoring. Our data further provide promising proof of concept of using cell free m-RNA profiling to monitor early onset cancer activity, which could lead to improved therapeutic management of cancer patients, and eventually alleviate the need for invasive biopsies.

Results and Discussion

While microarrays and RNA-Seq were previously benchmarked for

differential expression and prediction model development, the complexity of quantifying low-abundance cell free m-RNA is compounded by the presence of high and variable levels of globin mRNA and ribosomal RNA (rRNA). Although rRNA depletion and globin reduction have been shown to mitigate some of these issues, they require a large amount of total cfRNA pool and may induce biases in the quantification of gene expression [18]. To address these limitations, we applied targeted expression profiling methods based on multiplex RT-qPCR amplification followed by quantitative analysis of cell free m-RNA abundance by delta Ct, the difference of Ct values between reference gene (18S) and target gene.

Circulating cell free m-RNA was extracted from pooled plasma cohorts of patients with lung, pancreatic or breast cancer. Total 750 cancer-associated genes were profiled and categorized into 9 major cancer signaling pathways: immune response (IR), transcription factors (TF), DNA repair (DR), oncogenesis (ONC), tumor metastasis (TM), TP53 signaling (TS), MAP kinases (MK), cell surface markers (CSM) and DNA methylation (DM). The distribution of detected cell free m-RNA species in these 9 categories from the lung cancer cohort was demonstrated in Figure 1. We identified eighteen genes belonged to cell surface markers (21%), eleven genes involved in DNA repair (13%), fourteen genes are MAP kinases (17%), eighteen genes involved in TP53 signaling (21%), four genes are transcription factors (5%), five genes involved in immune response (6%), seven genes correlated with oncogenesis (8%), six genes associated with tumor metastasis (7%) and two gene related to DNA methylation (2%).

For quantification of cell free m-RNA expression levels, genes with delta Ct values between 0-15 was classified as "high expression" (blue); delta Ct values between 15-20 was interpreted as "medium expression" (green); and delta Ct values of 20-30 was called "low expression" (red). The genes with delta Ct values >30 were not color coded. Figure 2 illustrated a global cell free m-RNA expression and functional landscape we established in non-small cell lung cancer (NSCLC). Among the 9 key cancer pathways, the circulating cell-free transcriptome composition of TP53 signaling, MAP kinases and cell surface markers were particularly dominant in NSCLC cohort.

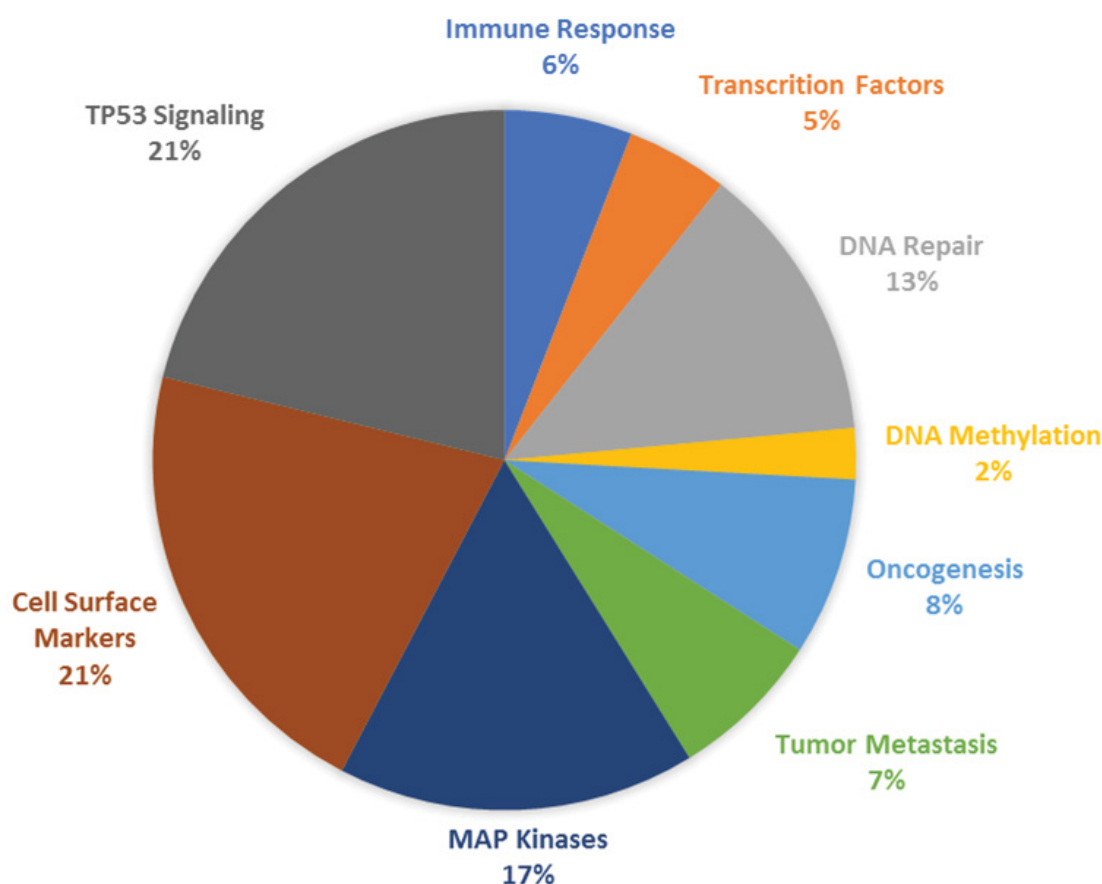


Figure 1. Distribution of detected cell free m-RNA species in NSCLC.

IR	TF	DR	DRc	TM	TS	TK	CSM
IL10	RRLP0	SPRY1	SL1	UPC	WAL1	ARJF	ALC8M
IL19	HMG2	STM	UKT1	RRM1	UKT1	WTF2	CGR1
IL2	TBP	UTR	UKT2	CGSP8	UKT2	ARJF	CD160
IL3	PGM1	SRD1	UPC	COL7	UKT2	CCND1	CD169
IL4	USC	BRD1	SOX	CD44	STM	CCNE1	CD19
IL5	PP1A	BRD2	CDR1	CD42	UTR	CDCA3	CD1A
IL6	SR	CCND	COL3	CDH1	SM	CDKN1B	CD1C
IL7	SRNT	CHK1	COLL1	CDKN2A	SM	CDKN1C	CD1D
IL8	WTF1	CHK2	COLL11	CEACAM1	COL2	CHUK	CD2
IL9	WTF2	COLR1A	ID	CTRP1	CDCA1	COL1A1	CD209
IL10	WTF3	RRCC1	ARJF	CTNNA1	CDSP1	CRS3	CD22
IL15	WTF4	RRCC2	CDSP8	CTSK	CDSP10	CRS3SP	CD24
IL16	CDSP6	RRCC3	CDSP9	CMCL12	CDSP2	CDSP1	CD244
IL18	CDSP8	RRCC4	CCND1	CDCA4	CDSP9	DUSP10	CD247
IL19	CDSP9	RRCC5	CCND2	DJPK1	CDSP4	DUSP16	CD28
IL17A	CRS3	RRCC6	CCND3	DCC	CDSP5	DUSP2	CD27
IL18	CRS3SP	RRCC8	CDN2	EPH2	CDSP6	DUSP4	CD28
COL3	CTNNA1	FANCA	CDCA2	RRM2	CDSP7	DUSP5	CD30
COL19	DR1	FANCC	CDH1	ETR	CDSP8	DUSP9	CD33
COL3	EIF1	FANCD3	CDK2	FAT1	CDSP9	DUSP7	CD4
COL2	EIF9	FANCD5	CDK4	FGF2	CCND1	DUSP8	CD43
CCR2	GGR1	FANCF	CDKN1A	FGR4	CDN5	DUSP9	CD40LG
CCR4	SLK1	FANCG	CDKN1B	FN	CDK2	FOG	CD5
CCR5	GGR1	FBN1	CDKN2A	FXYD2	CDK8	ARRL	CD6
CCR7	WTF	GADD45A	CDKN2B	GHRH1	CDKN1A	HR23	CD63
CDCA9	WTF2	GADD45B	COL1A1	HGF	CHK1	HGF1	CD69
CDML10	FO2	GADD45G	CRK	AP2	CHK2	KR29	CD7
CDML11	FO3D2	GTF2H1	CTNNA1	HR23	CDKN1D	MA2D	CD70
CDP1	FOXD1	GTF2H3	CYCS	WALTP2	DDR1	MSP2K1	CD72
CDP2	GA101	HUG1	DVL1	HGF1	EIF1	MSP2K2	CD74
CDP3	GA102	HGF1	EIF1	IL18	EIF2	MSP2K2	CD74B
GA103	GA103	LIG1	EIF2	IL18	EIF3	MSP2K2	CD74B
NFKB2	GTF2B	LIG2	ELK1	CDK2	EIF4	MSP2K4	CD80
HMGK9	GTF2F1	LIG4	ERBB2	KG2F	EIF5	MSP2K5	CD80
CD95	HAND1	MSPK10	FDD	GGP1	EIF6	MSP2K6	CD86
CD4	HAND2	MSPK11	FOG	KR29	EP00	MSP2K7	CD86
CD86	HDJC1	MSPK12	FJGL	LDNA1	FOG	MSP2K1	CD88
CD19	HIF1A	MURK14	FGF2	LYPD3	FOG	MSP2K10	CD89
IL3RA	HNF4A	MSPK8	FN1	MCM1	GADD45A	MSP2K11	CD97
CD28	HDJ2	MSPK9	FOG	NET	GADD45B	MSP2K2	COL1A1
CD98	HGF1	MDD4	FYN	MG27E	GADD45G	MSP2K3	COL1A2
CD40	ID1	MDD2	FZD1	MMP1	GGX2	MSP2K4	CR2
PTPRC	IRF1	MGMT	GRB2	MMP10	HDJ2	MSP2K5	CSF1R
CD44	JUN	MRE11A	GGK3	MMP14	HDJ10	MSP2K9	CTLA4
CD80	JUNB	MRE11	HGF	MMP2	HDJ11	MSP2K7	DP4
CD86	JUND	MRE11	HR23	MMP9	HDJ2	MSP2K8	DNF
CTLA4	MX	MRE11	HGF1	MMP7	HDJ3	MSP2K9	FOG
CD40LG	MRE11	MRE11	HGF1R	MMP9	HDJ4	MSP2K1	CD80
HLA-DRA	MRE11	NTL1	ITGA2B	MT1	HDJ5	MSP2K2	CD80
HLA-DRA1	MRE11	OGG1	ITGA1V	MT2	HDJ6	MURK1	FCGR1A
TGFB1	MYB	CDSP1	ITGA1	MT2	HDJ7	MSPK10	HLA-D
TNFRSF16	MYC	PCNA	ITGA5	MYC	HDJ8	MSPK11	HLA-DRA
FOO2	MYF9	PNKP	JUN	NCAM1	HDJ9	MSPK12	CD112
NO2	MYO1	POLA1	KDR	NF2	HGF1	MSPK13	L13R9
COL2	NFAT2	POLA2	KIT	NME1	HRK2	MSPK14	L1R2
COL12A1	NFATC1	POLA1	KR29	NR4A9	KAT2B	MSPK15	L3R3
MAX	NFATC2	POLA2	LEF1	PCCAMH	MSPK10	MSPK9	ITGA1
FOAM1	NFATC3	POLA1	MSPK1	PNN	MSPK11	MSPK4	ITGA2
GLP	NFATC4	POLK	MSPK5	PSC3	MSPK12	MURK1	ITGA3
GLP2	NFKB1	POLO	MSPK1	PTEN	MSPK13	MSPK7	KLRB1
HMOX1	NFYB	POLR1B	MSPK14	PTGS2	MSPK14	MSPK8	KLRC1
PTGS2	NR3C1	POLR1C	MSPK3	RG1	MSPK6	MSPK&P1	KLRD1
LRP2	PO3	POLR2B	MSPK6	RGL1	MSPK9	MSPK&P2	KRT19
CYP11B2	POU3F1	POLR2B	MX	RGL2	MDD12	MSPK9	KRT5
CYP11A2	PP1A	POLR2C	MDD2	RET	POG1	MSPK&P3	KRT6
IFNG	PP1A2	PRKDC	MYC	RAOC	PRKDC	MSPK&P2	USP11
PRF1	RP1	PRLN3	NFKB1	G100M	P2M2	MSPK&P3	MYH10
GZMB	REL	PRLN10	NFKB2	GERPNB5	P2M3	MSP20	MURK1
GZML	RELA	PRLN5	NFKB1A	GERPNE1	P2M4	MSP20	MYOCD
FOS	RELB	PRLN6	NR2A	SET	P2M5	MURK1	NCAM
FOSL2	SMAD1	PRLN7	PINCH3	GADD2	P2M6	MURK2	NO2
TGFB1	SMAD4	PRLN8	PINCH1	GADD6	P2M7	MYC	NT5E
SMAD9	SMAD5	RAD1	PTEN	GADD3	P2M8	NFK	PCAM1
SMAD7	SMAD6	RAD17	PTK2	SETD2	P2M9	NR2A	RETN
SH	SP1	COL1A2	PTK2B	SH	P2M10	RAD1	SH2B3
FN1	SP2	RAD50	RAD1	SPCAM	P2M11	SH2	GLP
CS	STAT1	RAD51	RAD1	TGF20	P2M12	P2M3	STAT6L1
TNF	STAT2	RAD52	RAD1	TGF21	PTEN	P2M4	SPCAM
LTA	STAT3	RAD53	RELA	TGF22	SH	PTK2B	TG
ACE	STAT4	RAD54	RAD1	TGFB1	SH	RAD1	TNFRSF4
VGGF3	STAT5A	RP29	GHC1	TIMP1	GR1	SH2	TNFRSF9
CD94	STAT5B	SMUG1	SMAD4	TIMP2	GR2	RELA	TRAF3
STAT1	STAT6	TGFB1	OGG1	TIMP4	GR3	PP2M2	VCAM1
STAT2	TBP	TREX1	GPP1	TNFRSF4	GR4	PP2M3	VWF
CDN1	HNF1A	TREX2	GR1	TNFSF10	GR5	PP2M4	
LIF	TGFB1	XBP2	TCF9	TP29	GR6	PP2M5	
LY96	TRAP2A	XPA	TGFB1	TP29	TRAF1	PP2M6	
MF	TGFB1	XPC	TGFB1	TGFB1	TNFRSF10	PP2M7	
NFATC4	TP29	RRCC1	TGFB1	TP29	PP2M8		
NFATC4	YY1	XRCC4	TP53	VGGF3	USP	GHC1	
PP1		XRCC5	MRE11	VGGF3	USP	TP53	
MYK		XRCC6	WNT1	WSP1	USP	ZAK	

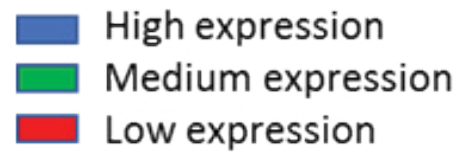


Figure 2. Fingerprint of cell free m-RNA functional clusters detected in NSCLC.

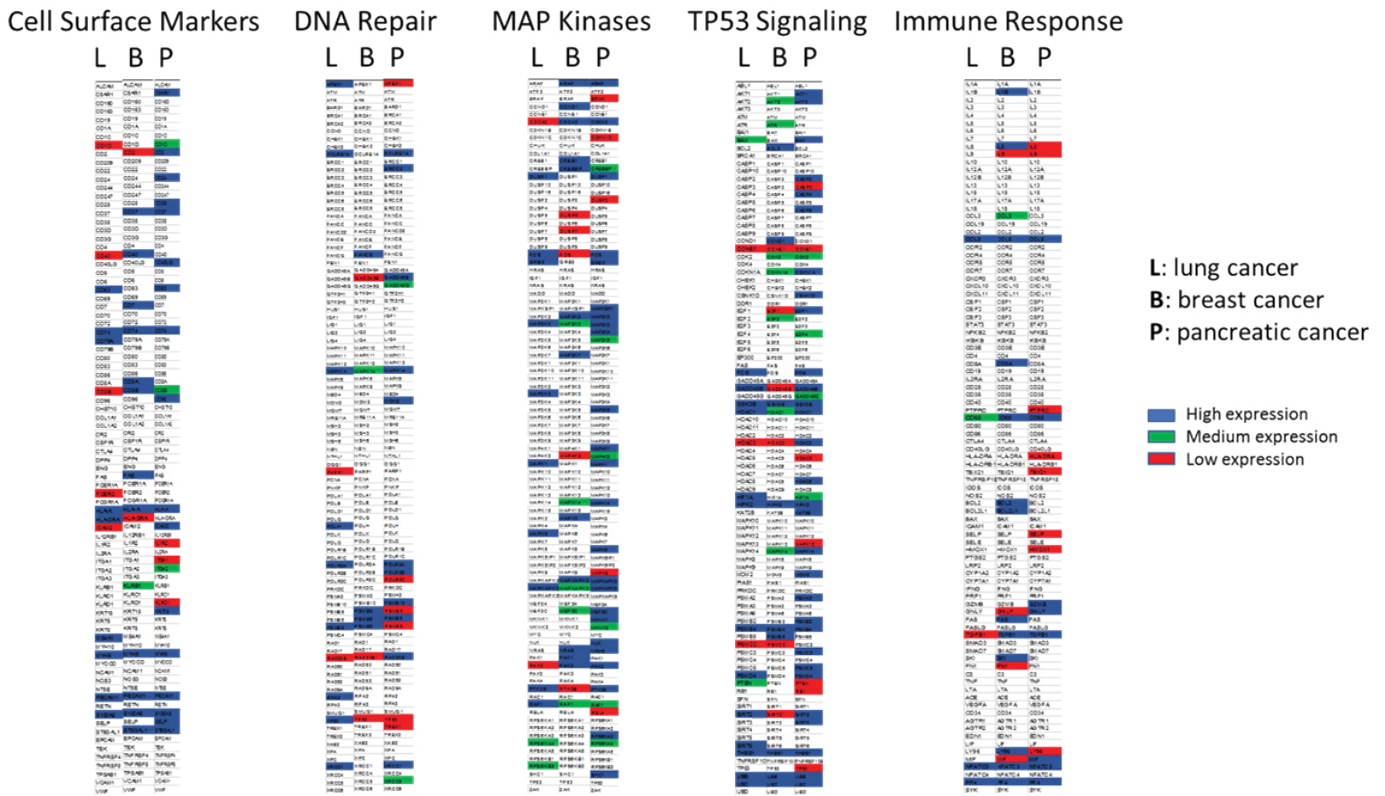


Figure 3. Cancer type-specific cell free m-RNA expression profiles can be used to identify novel biomarkers for early detection and stratification of cancer.



Figure 4. Blood-based cell free m-RNA fingerprint – a non-invasive, real-time and longitudinal liquid biopsy for early-stage cancer detection.

From the representative cell free m-RNA expression heatmaps shown in **Figure 3**, differentially expressed cancer type-specific genes can be easily identified in the same cluster, for example, ERCC2, MDM2, POLR2B, PSMB10 in DNA repair cluster are highly expressed and are pancreatic cancer-specific genes; while FANCG is breast cancer-specific gene; and POLH, RPA2 are strongly expressed as lung cancer-specific genes. Among cell surface markers, C5AR1, CD24, CD28, CD40LG, CD96, KRT18, SELP are highly expressed as pancreatic cancer-specific genes; whereas CD7, CD8A, FAS are breast cancer-specific genes; and CD79A and MS4A1 are strongly expressed as lung cancer-specific genes. Together, we have obtained highly distinct cell free m-RNA expression profiles and functional clusters specific for NSCLC, breast cancer, and pancreatic cancer. Pancreatic cancer revealed the largest heterogeneity of gene expression as a wide spectrum of cell free m-RNA molecules are produced by its transcriptional machinery. In contrast, NSCLC

has relatively low cell free m-RNA heterogeneity and fewer specific genes that contribute to the total cell free m-RNA composition.

Multiple pathways are responsible for transducing mechanical and growth stimuli into changes in gene expression during cancer development. In this project we have established an unprecedented functional cell free m-RNA database which will lead to (i) classification of cell free m-RNA species by their functions, (ii) identification of differentially expressed cell free m-RNA in a particular cancer type, (iii) illustration of a comprehensive landscape of cell free m-RNA in circulation, and (iv) establishment of specific cell free m-RNA expression signatures for different cancer types. The cell free m-RNA expression profiles identified in this study represent gene fingerprints in circulation for specific cancer types, thus offering the exciting possibility of detecting early stage cancer (**Figure 4**).

Conclusion

We have comprehensively explored cell free m-RNA expression profiles and signatures of different cancer types, thereby establishing a plasma-based functional transcriptomic databank, including differential gene expression, classification, functional clustering and cancer type-specific signatures. We believe that our work has research, clinical, and diagnostic value, and provides greater dimensionality to the current landscape of cell free m-RNA research and makes a relevant jump into understanding and devising strategies to tackle early cancer detection.

Future Perspectives

Prior studies have laid a solid foundation for tissue-based gene expression signatures in cancer diagnosis and prognosis. However, gene expression signatures for early cancer detection were hampered by tissue unavailability and limited bioinformatics tools, and in many ways spurred renewed interest in developing non-invasive blood-based high-dimensional analytical algorithms. With our cell free m-RNA gene expression database, it is now possible to derive highly accurate and sensitive cancer type-specific signatures that are amenable to large-scale clinical validation. Our profiling model comprised of 750 cancer-associated genes has the potential to be translated into widely applicable assays. While the clinical validity of cell free m-RNA signatures will ultimately be proven by cross-validation in prospective studies, the accurate extraction of information from genomic and/or epigenomic studies is of vital importance for guiding such studies.

The establishment of cancer type-specific cell free m-RNA expression signatures is necessarily an ongoing and dynamic process, in which, with the inclusion of more early-stage patient samples with consistent clinical information, an early detection signature will be continuously refined. Due to biological and technical limitations, cell free m-RNA-based expression signatures may not be able to achieve 100% accuracy, yet the application of advanced feature selection algorithms and the combination of genetic and clinical data will enable their robust performance with dramatically reduced complexity.

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