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

Short Communication

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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 distinct approach to achieve the same goal. Noninvasive and real-time gene expression profiling was performed on plasma circulating cell-free mRNA (cfmRNA) enriched from cancer patients using proprietary high sensitivity RT-qPCR assays. A plasma cfmRNA expression database covering 750 genes in 9 major cancer pathways was established with multiple layers of cancer type-specific characteristics: (i) the distribution of cfmRNA species across 9 major cancer pathways; (ii) the differential expression of target genes (high, medium or low expression); (iii) the global cfmRNA expression landscape in circulation; and (iv) the unique cfmRNA 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.

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 (cfmRNA) 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 cfmRNA 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

cfmRNA 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.

Circulating cfmRNA abundance can be influenced by physiological state, level of nucleases in the blood, the half-life of individual cfmRNA and the clearance rate by immune system, liver and kidney (2). Certain cfmRNA 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 cfmRNA testing include its extremely low abundance, susceptible to degradation, relatively unstable, and poor extraction efficiency. To circumvent these limitations, a signal amplification step following cfmRNA extractions should be performed as we discovered in our studies. Circulating cfmRNA carries information from human tissues, the pattern of cfmRNA expression reflects dysregulation of cancer immunity, tumor cell growth, proliferation and stromal interaction, which makes cfmRNA expression signature a promising biomarker for early diagnostic, prognostic and therapeutic purposes (6, 7).

Circulating cfmRNA 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 (8-10), these properties make it difficult to investigate. The current molecular techniques employed for the detection and characterization of cfmRNA 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 cfmRNA and remain currently the methods of choice. NGS has been used for cfmRNA 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 cfmRNA 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 cfmRNA for diagnostic application, this field is still in its infancy. It is imperative and of paramount interest to harness highly sensitive cfmRNA detection technologies and establishing unique expression signatures as early-stage fingerprints of oncogenesis in biological fluids. The fundamental advantage of circulating cfmRNA 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 cfmRNA-based expression signature could, if necessary, be augmented by other blood-based biomarkers including cancer-specific cfDNA. The cfmRNA 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 cfmRNA 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, cfmRNA 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 cfmRNA profiling data here provides circulating transcript snapshots of gene expression signatures in patients with lung, pancreatic or breast cancer. The cfmRNA 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 cfmRNA 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 cfmRNA 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 cfmRNA abundance by delta Ct, the difference of Ct values between reference gene (18S) and target gene.

Circulating cfmRNA 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 cfmRNA 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 cfmRNA 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 cfmRNA expression and functional landscape we established in non-small cell lung cancer (NSCLC). Among the 9 key caner pathways, the circulating cell-free transcriptome composition of TP53 signaling, MAP kinases and cell surface markers were particularly dominant in NSCLC cohort.

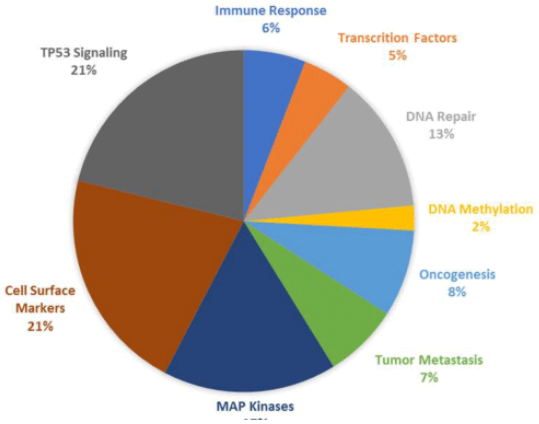

Figure 1: Distribution of detected cfmRNA species in NSCLC.

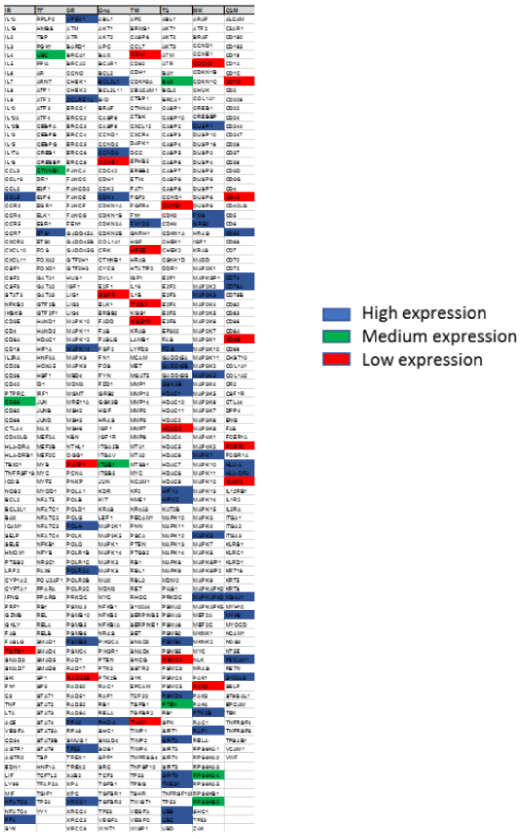

Figure 1: Fingerprint of cfmRNA functional clusters detected in NSCLC.

From the representative cfmRNA 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 cfmRNA 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 cfmRNA molecules are produced by its transcriptional machinery. In contrast, NSCLC has a relatively low cfmRNA heterogeneity and fewer specific genes that contribute to the total cfmRNA composition.

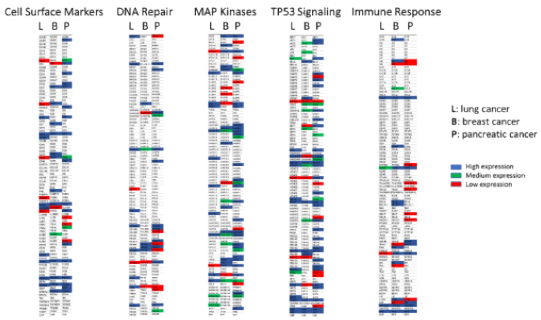

Figure 1: Cancer type-specific cfmRNA expression profiles can be used to identify novel biomarkers for early detection and stratification of cancer.

Figure 1: Blood-based cfmRNA fingerprint – a noninvasive, real-time and longitudinal liquid biopsy for early-stage cancer detection.

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 cfmRNA database which will lead to (i) classification of cfmRNA species by their functions, (ii) identification of differentially expressed cfmRNA in a particular cancer type, (iii) illustration of a comprehensive landscape of cfmRNA in circulation, and (iv) establishment of specific cfmRNA expression signatures for different cancer types. The cfmRNA 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 cfmRNA 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 cfmRNA research and makes a relevant jump into understanding and devising strategies to tackle early cancer detection.

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