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Multi-Analyte Profiling Reveals Relationships Among Circulating Biomarkers in Colorectal Cancer

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INTRODUCTION

- Blood-based tests hold great promise as cancer diagnostics, but most current tests are restricted to the analysis of a single class of molecules (e.g., circulating tumor DNA, circulating mRNA, circulating proteins)[1-4]
- The ability to analyze multiple analytes simultaneously from the same biological sample may increase the sensitivity and specificity of such tests by exploiting independent information among signals[5]

OBJECTIVE

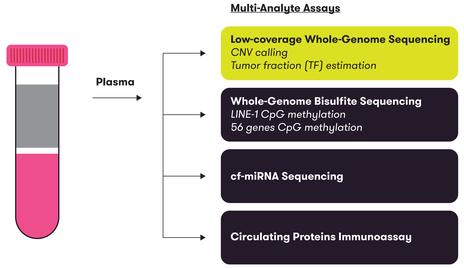
- Develop and implement an experimental and analytical system for the integrated analysis of multiple analytes from a single blood sample

METHODS

Multi-Analyte Approach (Figure 1)

- De-identified blood samples were obtained from healthy individuals and individuals with non-advanced adenomas (non-AAs), advanced adenomas (AAs), and stage I-IV colorectal cancer (CRC)
- After plasma separation, multiple analytes were assayed as follows:
  - Cell-free DNA (cfDNA) content was assessed by low-coverage whole-genome sequencing (lcWGS) and whole-genome bisulfite sequencing (WGBS)
  - Cell-free microRNA (cf-miRNA) was assessed by small-RNA sequencing
  - Levels of circulating proteins were measured by quantitative immunoassay

FIGURE 1. Overview of Multi-Analyte Approach for 'Liquid Biopsy'



Multi-Analyte Assays

Low-coverage Whole-Genome Sequencing
CNV calling
Tumor fraction (TF) estimation

Whole-Genome Bisulfite Sequencing
LINE-1 CpG methylation
56 genes CpG methylation

cf-miRNA Sequencing

Circulating Proteins Immunoassay

Whole blood was collected in K3-EDTA tubes, and double-spun to isolate plasma. Plasma was split into aliquots for cfDNA lcWGS, WGBS, cf-miRNA sequencing, and quantitative immunoassays. CNV, copy number variation; LINE-1, long interspersed nuclear element 1.

Analysis

- Sequenced cfDNA, WGBS, and cf-miRNA reads were analyzed as follows:
  - cfDNA (lcWGS): Aligned to Genome Reference Consortium Human Build 38 (GRCh38) using Burrows-Wheeler Aligner (BWA) software. Fragments that aligned within annotated genomic regions were counted and normalized for depth of sequencing to produce a 30,000-dimensional vector per sample
    - Samples with high TF were identified via manual inspection of large-scale CNV
  - WGBS: Aligned to GRCh38 using BWA-meth software. Percentage of methylation was calculated per sample across LINE-1 CpGs and CpG sites in targeted genes (56 genes)
  - cf-miRNA: Aligned to miRbase 21 using Bowtie 2. Fragments that aligned to annotated miRNA genomic regions were counted and normalized for depth of sequencing to produce a 1700-dimensional vector per sample
- Absolute protein abundances were determined using standard curves
- Principal component analysis (PCA) was performed per analyte

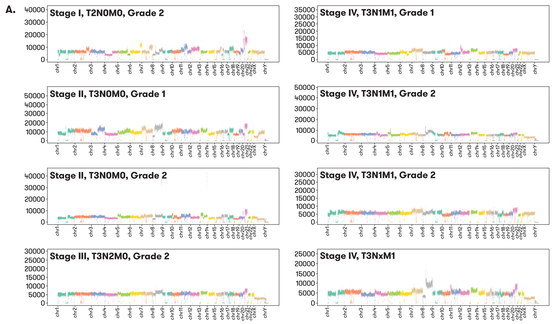
RESULTS

cfDNA: lcWGS

- lcWGS of plasma cfDNA was able to identify CRC samples with high TF on the basis of large-scale CNV across the genome (**Figure 2**)
- High TFs, while more frequent in late-stage CRC samples, were observed in some stage I and II samples (**Figure 2**). High TFs were not observed in samples from healthy individuals or those with non-AAs or AAs

FIGURE 2. Distribution of High TF Samples, as Inferred by CNV, Across Clinical Stage

A.



B.

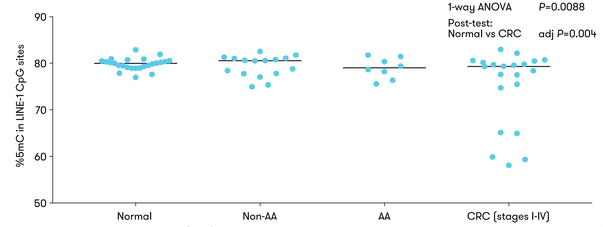
	Healthy	Non-AA	AA	Stage I CRC	Stage II CRC	Stage III CRC	Stage IV CRC
N with high TF	0	0	0	1	2	1	4
Total N	26	13	10	3	7	4	5

(A) CNV plots for individuals with high TF based on cfDNA-seq data. (B) Distribution of high TF cfDNA samples across clinical stage. High TF samples do not necessarily correspond to samples clinically classified as late stage. Chr, chromosome.

Methylation: WGBS

- Genome-wide hypomethylation at LINE-1 CpG loci was only observed in individuals with CRC (**Figure 3**)
- Hypomethylation was not observed in samples from healthy individuals or those with non-AAs or AAs

FIGURE 3. CpG Methylation Analysis at LINE-1 Sites

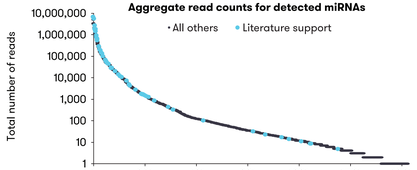


Significance was assessed by 1-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. Only significant adjusted *P* values are shown. CpG hypomethylation of LINE-1 was only observed in CRC cases. 5mC, 5-methylcytosine.

miRNA

- miRNAs suggested as potential CRC biomarkers in the literature tended to be present in higher abundance relative to other miRNAs (**Figure 4**)

FIGURE 4. cf-miRNA Sequencing Analysis



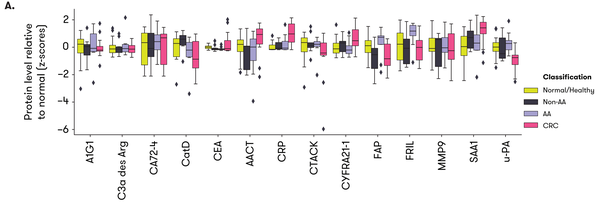
Shown are the number of reads mapping to each miRNA after pooling reads from all samples. miRNAs indicated in blue have been suggested as potential CRC biomarkers in the literature. Adapter-trimmed reads were mapped to mature human microRNA sequences (miRBase 21) using bowtie2. More than 1800 miRNAs were detected in plasma samples with at least 1 read, while 375 miRNAs were present at higher abundance (detected with an average of ≥10 reads per sample).

Proteins

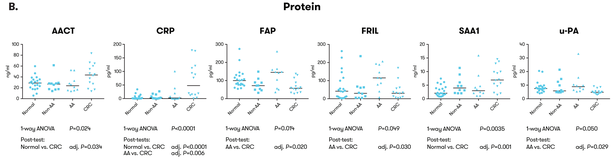
- In CRC samples, circulating levels of alpha-1-antichymotrypsin (AACT), C-reactive protein (CRP), and serum amyloid A1 (SAA1) proteins were elevated, while urokinase-type plasminogen activator (u-PA) levels were lower compared with healthy controls (**Figure 5**)
- In AA samples, circulating levels of fibroblast activation protein (FAP) and Flt3 receptor-interacting lectin precursor (FRIL) proteins were elevated, while CRP levels were lower compared with CRC samples (**Figure 5**)

FIGURE 5. Circulating Protein Biomarker Distribution

A.



B.

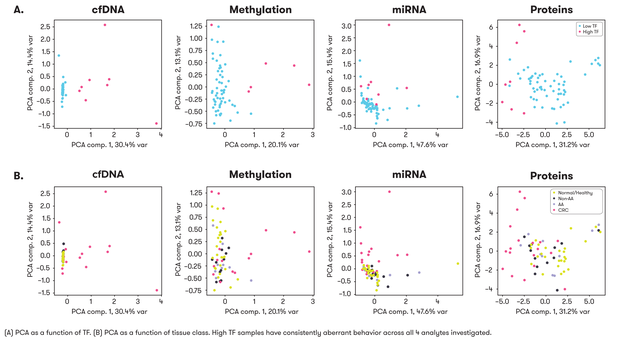


(A) Levels of all circulating proteins assayed. (B) Proteins which show significantly different levels across clinicopathologic stages according to 1-way ANOVA followed by Sidak's multiple comparison test. Only significant adjusted *P* values are shown. Proteins measured using SIMOA (Quanterix): ATP-binding cassette transporter A1/G1 (A1G1), aciation stimulating protein (C3a des Arg), cancer antigen 72-4 (CA72-4), carcinoembryonic antigen (CEA), cytokeratin fragment 21-1 (CYFRA21-1), FRIL u-PA. Proteins measured by ELISA (Abcam): AACT, cathepsin D (CATD), CRP, cutaneous T-cell-attracting chemokine (CTACK), FAP, matrix metalloproteinase-9 (MMP9), SAA1.

Multi-analyte

- Strikingly, aberrant profiles across analytes were indicative of high TF (as estimated from cfDNA CNV), rather than cancer stage (**Figure 6**)
- All individual analyte profiles were discordant between CRC samples and normal controls

FIGURE 6. PCA of cfDNA, CpG Methylation, cf-miRNA and Protein



(A) PCA as a function of TF. (B) PCA as a function of tissue class. High TF samples have consistently aberrant behavior across all 4 analytes investigated.

CONCLUSIONS

- These data suggest that TF is correlated with cancer stage, but has a large potential range, even in early stage samples
- We found that aberrant profiles among cfDNA CpG methylation, cf-miRNA, and circulating protein levels were more strongly associated with high TF than with late stage. This may explain the variability reported in cancer detection rates for other screening tests[6-8]
- These findings suggest that some positive "early stage" detection results may in fact be "high TF" detection results[7,8]
- The presence of low TF CRC outlier data in non-cfDNA analytes, including miRNA and protein, suggests that assaying multiple analytes from a single sample may enable the development of classifiers that are reliable at low TF

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