Supplementary Information

Multiomic Analysis of Dried Single-Drop Plasma Sample by Integrated Mass Spectrometry Approach

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Experimental details

Sample collection and storage. Human plasma was provided by Shenzhen People's Hospital (Shenzhen, China). All of the experiments in this study were performed in compliance with the Declaration of Helsinki and the relevant laws of the People's Republic of China, and approved by Committee of Medical Ethics of Shenzhen People's Hospital (Shenzhen, China). Informed consent was obtained from the human subjects prior to obtaining the plasma. Plasma was obtained as previously reported.[1] Briefly, plasma was obtained from venous blood by centrifuging at 4 °C and stored at −80 °C. One healthy volunteer and five colon cancer patients were enrolled in this study.

Design of multiomic analysis of dried single-drop plasma sample. The multiomic analysis was integrated by seamless sample preparation of metabolomics and proteomics and data acquirement by uniform nano-LC-MS/MS as shown in **Figure 1**. The detail procedures were described as below.

Sample preparation. A 200 μL pipet tip was packed with three plugs of C18 disk (3 M Empore, USA) and, then packed with 500 μg C18 beads and 2mg of SCX/SAX mixed beads (20 μm, Applied Biosystems, U.S.A.).

The cartridge tip was activated and equilibrated by washing with 80 μL methanol (MeOH) and 40 μL of 10 mM ammonium bicarbonate (ABC) (pH 7.8), respectively. One microliter of plasma was diluted to 60 μL by 20 mM ABC and equivalent to 1 μg protein per microliter. 50 μL of diluted plasma was loaded onto the conditioned cartridge twice. The cartridge was completely dry after centrifugation and stored at room temperature for following experiments. For metabolomic analysis, the cartridge was eluted by 20 μL isopropanol (IPA) twice. Eluted IPA solution was collected and dried in vacuum. The sample was redissolved in 80% (v/v) MeOH in water for metabolomics analysis. After that, the cartridge was sequentially washed by 40 μL acetonitrile (ACN). Proteins captured in mixed beads were processed for proteomics under standard SISPROT protocol.[2]

For optimization LC-MS system, metabolites was processed from large amount of plasma as conventional pre-treatment procedures[3]. Briefly, 100 μL plasma was thawed in ice water, then was added with 400 μL of ACN. The samples were centrifuged at 13000 g for 10 min at 4 °C after vortex. The supernatant was dried in vacuum and dissolved in 300 μL of 80% MeOH/water (v/v) for MS analysis.

LC–MS/MS analysis. The metabolome and proteome were both analyzed by using quadrupole-orbitrap tandem mass spectrometer (Q-Exactive, Thermo) coupled with an Easy-nLC 1000 system (Thermo) as previously reported with slight modification on LC gradient for metabolite analysis.[4]

Metabolites were eluted off the column by buffers 0.1% (v/v) formic acid (FA) in water (buffer A) and in ACN (buffer B) at a flow rate of 250 nL/min in a 60-min gradient: 5% B keeping for 1 min, 5 to 43% B for 4 min, 43 to 100% B for 43 min, and 100% B keeping for 12 min. In data dependent acquisition (DDA) mode, the mass spectrometer was operated with the following parameters. The full scan was acquired from m/z 80-1000 for metabolomic analysis with a resolution of 70,000 in top 10 mode; MS/MS scans were performed with an 1.6 Da isolation window, higher energy collisional dissociation fragmentation with stepped collision energy of 20, 35 and 70, and dynamic exclusion was correspondingly set to 6s.

Proteins were eluted off the column by buffers as same as metabolite at same flow rate in a 70-min gradient: 3 to 7 % B for 2 min, 7 to 22% B for 50 min, 22 to 35% B for 10 min, 35 to 90% B for 2 min, and 90% B keeping for 6 min. In DDA mode, the mass spectrometer was as the following. The full scan was acquired from m/z 166.7 to 2500 for proteomic analysis with a resolution of 70,000 in top 10 mode; MS/MS scans were performed with an 1.6 Da isolation window, higher energy collisional dissociation fragmentation with 27 collision energy, and dynamic exclusion was correspondingly set to 40 s.

As the expansion of our developed approach, multiple reaction monitoring (MRM) assay was utilized to boosting up the MS sensitivity. The LC-MS system was triple quadrupole mass spectrometer (TSQ Vantage, Thermo) coupled with micro-flow LC. The LC separation used a micro-flow column (2.1mm × 200 mm) packed with 5 μm C18 resins at a flow rate of 250 μL/min. The column temperature was set to 55 °C.

The separation buffer, 0.1% (v/v) FA in water (A) and 0.1% (v/v) FA in ACN (B), eluted peptides with following gradient: 3 to 7% B for 2 min, 7 to 22% B for 12.5 min, 22 to 35% B for 2.5 min, 35 to 90% B for 2 min, 90% B keeping for 4 min, 90 to 3% B for 1 min, 3% B keeping for 2 min. The mass spectrometer was set at MRM scanning mode. The normalized collision energy was set to 28, the trigger was 1000, and the spray voltage of the positive ions was set to 4000. Nine known cancer biomarkers (APOA2, AFP, CEA, CNDP2, EGFR, FCGBP, HMGB1, LGALS4, MUC1) were selected as previously reported.[5-7]

To develop our approach, nano LC and micro-flow LC coupled with same quadrupole-orbitrap tandem mass spectrometer (Q Exactive) were compared to analyze same metabolites. The micro-flow LC-MS was performed as below. Metabolites processed by conventional method was separated by micro-flow LC system (UltiMate 3000, Thermo). An Acquity BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters) was used and maintained at 50 °C in column oven. The separation buffers were the same as that used in nano LC system. Metabolites were separated with a 30 min gradient: 5% B keeping for 0.5 min, 5 to 100% B for 23.5 min, 100% B keeping for 4 min, and 2 min B for equilibration. Then, the eluate from micro-flow LC was analyzed by quadrupole-orbitrap tandem mass spectrometer (Q Exactive). Then, the same metabolites were analyzed by using nano-LC coupled with same mass spectrometer.

Data analysis.

Proteomic analysis For untargeted proteomic analysis (DDA mode), raw data were searched against the database of homo sapiens containing 68485 entries (June 01, 2016, Uniprot) by using Sequest HT algorithm within Proteome Discoverer (PD) software (Version 1.4, Thermo) as previously reported.[13]

For targeted proteomic analysis in MRM mode, the MS results was analyzed manually by extracting LC peaks of transitions, calculating peak areas, and labeling the peak retention time, area, and signal-to-noise ratio (S/N). For SRMatlas database searching, the number of incomplete digestion sites was set to 0, and the peptides have no special modification. The b or y ions with high abundance were chosen. **Table S1 and Figure S2** shows the transitions for colon cancer biomarker proteins.

The functional annotation of identified plasma proteins were determined by Gene Ontology Biological Process (GOBP) from DAVID (version 6.8). The threshold of p value was set to 0.05 for GO categories.[8]

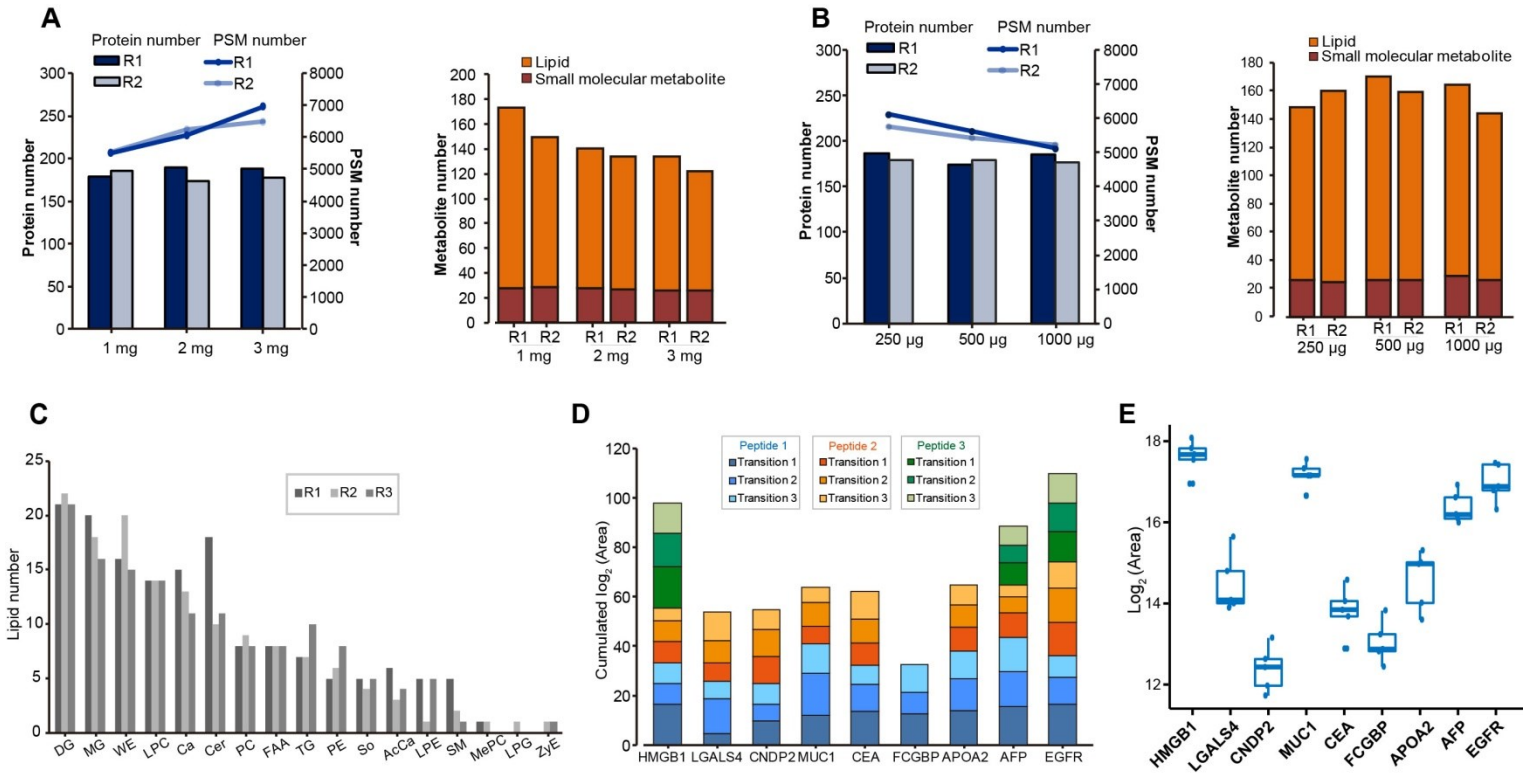
Metabolite analysis Lipids and small molecular matabolites were processed separatedly. LipidSearch software v4.1.16 (Thermo, CA) was used for identification and quantification of lipids, in which lipid identification was based on MS/MS spectrum as reported previously.[9] The parameter of software was set as following: 8 ppm and 15 ppm for mass tolerance of precursor and fragment ion, respectively. Grades A to D were used for ID quality filter. To identify small molecular metabolites, raw data files were converted to mzML files by using MSconvertGUI tool included in Proteowizard software. Metabolite structures were determined by using report databases for plasma metabolome profiling,[3] and searched with a 5 ppm mass tolerance for precursor ion.[10]

Supporting tables

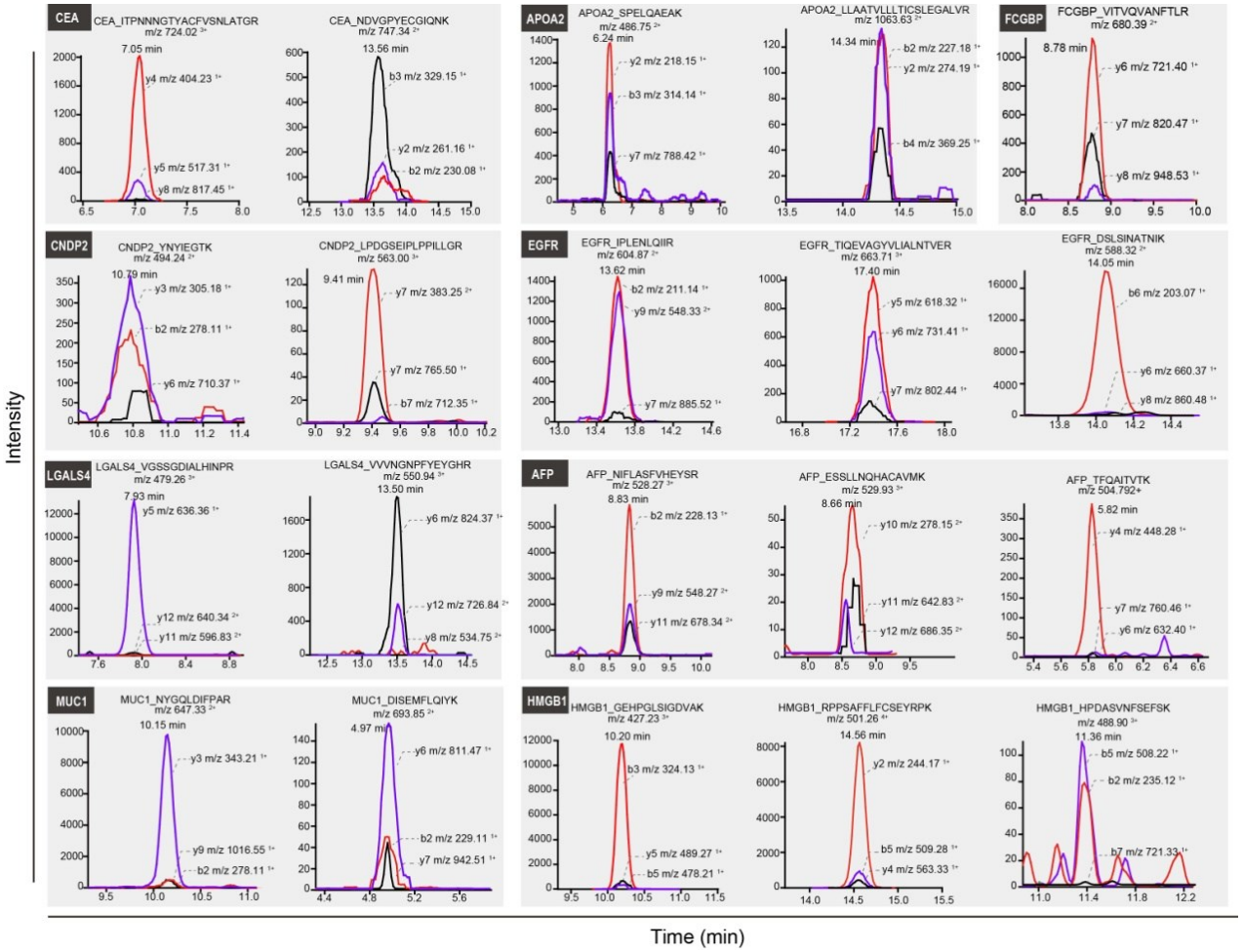
Table S1. Untargeted proteomics analysis of 1 μL plasma samples from 5 colon cancer patients.

Table S2. Metabolomic analysis of 1 μL plasma samples from 5 colon cancer patients.

Table S3. Targeted analysis of biomarker proteins from 1 μL plasma samples of 5 colon cancer.



Supporting Figure 1. (A) Optimization of SCX/SAX mixed beads amount, and (B) C18 beads amount. (C) Repeatability of lipid classes of newly developed method from 1 µL plasma sample. R1, R2, R3 represent replicate 1, 2, 3, respectively. (D) Cumulated area of transition for each peptide of nine biomarkers in colon cancer plasma by MRM mode. (E) Relative abundance and correlation of 9 biomarker proteins identified by micro flow LC-MS in MRM mode.



Supporting Figure 2. All chromatography of 60 transitions from 30 peptides of 9 marker proteins by MRM assay.

Notes and references

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