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ORIGINAL ARTICLE

Circulating cell-free DNA mutation patterns in early and late stage colon and pancreatic cancer

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Cancer is a heterogeneous disease harboring diverse subclonal populations that can be discriminated by their DNA mutations. Environmental pressure selects subclones that ultimately drive disease progression and tumor relapse. Circulating cell-free DNA (ccfDNA) can be used to approximate the mutational makeup of cancer lesions and can serve as a marker for monitoring disease progression at the molecular level without the need for invasively acquired samples from primary or metastatic lesions. This potential for molecular analysis makes ccfDNA attractive for the study of clonal evolution and for uncovering emerging therapeutic resistance or sensitivity. We assessed ccfDNA from colon and pancreatic adenocarcinoma patients using next generation sequencing of 56 cancer-associated genes at the time of primary resectable disease and metastatic progression and compared this to the mutational patterns of the primary tumor. 28%–47% of nonsynonymous mutations in the primary tumors were also detected in the ccfDNA while 71%–78% mutations found in ccfDNA were not detected in the primary tumors. ccfDNA collected at the time of progression harbored 3–5 new mutations not detected in ccfDNA at the earlier collection time points. We conclude that incorporation of ccfDNA analysis provides crucial insights into the changing molecular makeup of progressive colon and pancreatic cancer.

Keywords Liquid biopsies, circulating tumor DNA, circulating cell-free DNA (ccfDNA), clonal evolution, pancreatic cancer, colon cancer

Introduction

Genomic mutations are one of the hallmarks of cancer (1). The molecular characterization of a given cancer relies on the analysis of tissue specimen from a primary or metastatic lesion typically obtained at a single time point. However, due to intratumoral heterogeneity, the selection of cell subpopulations during cancer evolution and metastasis, the analysis of a single tissue specimen will provide only a limited characterization of the molecular makeup of the disease (2,3). Monitoring the molecular characteristics of cancer by serial analyses of circulating cell-free DNA (ccfDNA) enables capture of emerging heterogeneity of the disease and may support treatment decisions (4,5). ccfDNA analysis has evolved since its inception with improvements in the technologies and detection limits (6,7) and represents a set of research tools that appear poised to enter routine clinical care (8,9). The recent FDA approval of a ccfDNA assay for the EGFR T790M mutation in lung cancer supports this notion (10).

Whether ccfDNA should complement tissue analyses in all cancer types remains to be studied, especially in early stage diseases (9,11). However, ccfDNA may be superior to tumor tissue DNA in the assessment of cancer heterogeneity and evolution during disease progression (12,13). Here we study the mutational landscape of ccfDNA at diagnosis and disease recurrence and compare it to that of DNA from the primary tumor tissues in 10 patients with colon and pancreatic cancer.

Materials and methods

Patient samples

Patients with newly diagnosed colon adenocarcinoma (colon AC) or pancreatic ductal adenocarcinoma (PDAC) were recruited for blood and tissue collection under the IRB protocol 2007-345

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"Establishment of the High Quality Tumor Biobank and Clinical Database" and the Non-Therapeutic Subject Registry (NTSR) Shared Resource protocol Pr000000007 at the Lombardi Comprehensive Cancer Center at Georgetown University after obtaining informed consent. Ten patients were retrospectively selected with the following inclusion criteria: initial diagnosis of treatment-naïve resectable primary adenocarcinoma (n = 5 colon; n = 5 pancreatic), surgical resection of the primary tumor, and development of progressive disease after surgery. None of the patients had a previous malignant disease.

Peripheral venous blood samples were collected in EDTA plasma tubes before the surgical removal of the primary tumors as well as at time of metastatic disease progression (1–70 months after surgery). The blood samples were centrifuged at ≤1300 RCF for 10 min within 2 h of blood collection, after which plasma was separated and stored at –80 °C until further analysis.

Surgical specimens of the primary tumors were frozen in O.C.T. and cryosectioned into 20 μm scrolls and examined by a pathologist for the presence of cancer cells.

DNA isolation

The plasma samples were thawed on ice and circulating cell-free DNA (ccfDNA) was isolated from 2 × 100 μL plasma per patient, using the DNA extractor SP Kit (Wako cat. # 296-60501), following the manufacturer's protocol. In brief, 200 μL Enzyme Reaction Solution and 5 μL Protein Digestion Solution was added to 100 μL plasma and mixed by vortexing. The samples were incubated at 56 °C for 10 min. Thereafter 300 μL of Sodium Iodide Solution and 600 μL Alcohol Solution were added and mixed by vortexing. After 10 min incubation at room temperature, the samples were centrifuged at 16,000 × g for 10 min at room temperature. The supernatant was discarded and ccfDNA pellets were washed with 1 mL Washing Solution A by vortexing. After 5 min centrifugation at 16,000 × g the supernatant was discarded. The ccfDNA pellets were washed with 1 mL Washing Solution B and centrifuged once more for 5 min. The supernatant was discarded again and DNA pellets were allowed to dry. The ccfDNA was diluted in 15 μL of ultra pure water and quantitated with the NanoDrop 2000c (Thermo Scientific) and the Promega Quantifluor ONE dsDNA Fluorescence Assay (Promega).

Two frozen tumor tissue scrolls of 20 μm thickness per patient with an average weight of 75 μg and surface of 1.15 cm^2 were used for genomic DNA isolation. DNA was isolated using the PrepEase Genomic DNA Isolation Kit (USB), following the manufacturer's protocol. In brief, the tissue was homogenized in 240 μL Homogenization Buffer in MagNA Lyser Green Beads (Roche) in the MagNA Lyser (Roche). A mixture of 200 μL Chloroform/Isoamyl Alcohol (24:1), as well as 800 μL Protein Precipitation Buffer were added to the lysates. Samples were mixed by vortexing and centrifuged at 13,000 × g for 4 min at room temperature. 880 μL of the upper aqueous phase of the sample was transferred to a new microcentrifuge tube containing 620 μL isopropanol. The samples were mixed by inverting the tubes and centrifuged at 13,000 × g for 4 min. The supernatant was discarded and DNA pellets were washed with 1 mL of 70% ethanol by vortexing. The samples were centrifuged for 2 min and DNA pellets were allowed to dry. The tumor DNA was diluted in 15 μL of ultra pure water and quantitated with the NanoDrop 2000c (Thermo Scientific) and the Promega Quantifluor ONE dsDNA Fluorescence Assay (Promega).

56G oncology panel sequencing library preparation

DNA mutation analysis was conducted using a Targeted Next Generation Sequencing Library Preparation Kit that is compatible with circulating cell-free DNA and the Illumina MiSeq Platform: the 56G Oncology Panel v2 from Swift Biosciences (Cat. # AL-56248). This panel contains 263 amplicons sized 92–184 bp that covers hotspots, exonic SNPs and contiguous regions of 56 human genes. The list of genes and number of amplicons is provided in Table 1. The kit contains a DNA standard with a set of 11 defined allelic frequencies for major oncology targets to be used as a sequencing control and DNA from HCT116, RKO and SW48 colon cancer cell lines. The 56G Oncology library was prepared according to the manufacturer's protocol. In brief, 10 ng DNA per sample was used for the Multiplex PCR Step using the Reaction Mix, and the following Thermocycler Program: 30 s at 98 °C, 4 cycles of 10 s at 98 °C, 5 min at 63 °C, 1 min at 65 °C, followed by 21 cycles of 10 s at 98 °C, 1 min at 64 °C, followed by 1 min at 65 °C and hold at 4 °C. The resulting amplicons were purified using SPRIselect beads (Beckman Coulter, Cat. #B23318) and

Table 1 Genes included in the analysis. The amplicon panel is from Swift Biosciences "Accel-Amplicon 56G Oncology Panel v2". The number of amplicons (# ampl) for each gene is shown

Gene name	# Ampl	Gene name	# Ampl
ABL1	5	*IDH2*	2
AKT1	2	*JAK2*	2
ALK	2	*JAK3*	3
APC	9	*KDR*	9
ATM	19	*KIT*	14
BRAF	2	*KRAS*	3
CDH1	3	*MAP2K1*	5
CDKN2A	2	*MET*	6
CSF1R	2	*MLH1*	1
CTNNB1	1	*MPL*	1
DDR2	1	*MSH6*	4
DNMT3A	1	*NOTCH1*	3
EGFR (HER1)	9	*NPM1*	1
ERBB2 (HER2)	4	*NRAS*	3
ERBB4 (HER4)	8	*PDGFRA*	4
EZH2	1	*PIK3CA*	11
FBXW7	6	*PTEN*	14
FGFR1	2	*PTPN11*	2
FGFR2	4	*RB1*	12
FGFR3	6	*RET*	6
FLT3	4	*STK11*	5
FOXL2	1	*SMAD4*	10
GNA11	2	*SMARCB1*	4
GNAQ	2	*SMO*	5
GNAS	2	*SRC*	1
HNF1A	4	*TP53 (P53)*	21
HRAS	2	*TSC1*	1
IDH1	1	*VHL*	3