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Peripheral Circulating Tumor DNA Detection Predicts Poor Outcomes After Liver Resection for Metastatic Colorectal Cancer

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Abstract

Background—Liver resection can be curative for well-selected metastatic colorectal cancer (CRC) patients. Circulating tumor DNA (ctDNA) has shown promise as a biomarker for tumor dynamics and recurrence following CRC resection. This prospective pilot study investigated the use of ctDNA to predict disease outcome in resected CRC patients.

Methods—Between November 2014 and November 2015, 60 patients with CRC were identified and prospectively enrolled. During liver resection, blood was drawn from peripheral (PERIPH), portal (PV), and hepatic (HV) veins, and 3–4 weeks postoperatively from a peripheral vein (POSTOP). Kappa statistics were used to compare mutated (mt) genes in tissue and ctDNA. Disease-specific and disease-free survival (DSS and DFS) were assessed from surgery with Kaplan-Meier and Cox methods.

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Results—For the 59 eligible patients, the most commonly mutated genes were TP53 (mtTP53, 47.5%) and APC (mtAPC, 50.8%). Substantial to almost-perfect agreement was seen between ctDNA from PERIPH and PV (mtTP53:89.8%, $\kappa=0.73$, 95%CI:0.53-0.93; mtAPC:94.9%, $\kappa=0.83$, 95%CI:0.64-1.00) as well as HV (mtTP53:91.5%, $\kappa=0.78$, 95%CI:0.60-0.96; mtAPC:91.5%, $\kappa=0.73$, 95%CI:0.51-0.95). Tumor mutations and PERIPH ctDNA had fair-to-moderate agreement (mtTP53:72.9%, $\kappa=0.44$, 95%CI:0.23-0.66; mtAPC:61.0%, $\kappa=0.23$, 95%CI:0.04-0.42). Detection of PERIPH mtTP53 was associated with worse 2-year DSS (mt+ 79% vs. mt– 90%, $P=0.024$).

Conclusions—Peripheral blood reflects the perihepatic ctDNA signature. Disagreement between tissue and ctDNA mutations may reflect the true natural history of tumor genes or an assay limitation. Peripheral ctDNA detection prior to liver resection is associated with worse DSS.

INTRODUCTION

The rates of diagnosis and death from colorectal cancer (CRC) are rising worldwide.¹ Death from CRC primarily occurs from metastatic disease, and the liver is the most frequently invaded site.^{2,3} The greatest improvement in survival over the last decade for patients with colorectal liver metastases (CRLM) is attributed to multimodality therapy including chemotherapy, ablation, and resection. For well-selected candidates, median five-year survival following CRLM resection exceeds 70%.⁴ Although some will be cured following liver resection, 70% will recur within 2 years.⁵ Current biomarkers, like cross-sectional imaging and serum carcinoembryonic antigen lack the sensitivity to determine which patients have aggressive disease increasing their risk to recur.^{6,7} Emerging genomic studies of resected tumor specimens suggest that detection of certain mutations in genes like BRAF and KRAS can be useful for prognostication.^{8–11}

Circulating tumor DNA (ctDNA)-DNA fragments found in the circulation following tumor cell breakdown^{12,13}—is potentially a non-invasive prognostic indicator of risk for CRC recurrence. Early studies reported that ctDNA levels predict tumor dynamics in CRC patients,^{14,15} and strong concordance exists between mutations identified in resected tumor specimens and ctDNA in metastatic CRC patients.^{16–18} Additionally, ctDNA has been demonstrated to predict progression-free and overall survival.^{15,19}

Prior work has investigated ctDNA samples drawn from peripheral veins but not centrally. Notably, circulating tumor cells (CTC) are found in perihepatic blood.^{20,21} Little is known about the clinical value of ctDNA obtained from the portal (PV) or hepatic veins (HV) nor has ctDNA been studied in well-selected, resectable CRLM patients. The primary objective of this prospective pilot study was to identify mutations in perihepatic blood via ctDNA analysis and compare to peripheral levels and known tumor mutations. Our secondary objective was to assess the relationship between ctDNA with outcomes.

MATERIALS AND METHODS

Patient Selection and Sample Acquisition

Between November 2014 and November 2015, CRLM patients were enrolled if they met the following inclusion criteria: no preoperative extrahepatic metastases, no cytotoxic therapy within 2 weeks or anti-angiogenic therapy 6 weeks before planned hepatic resection.

Exclusion criteria included known bleeding or clotting diatheses, chronic inflammatory diseases, evidence of portal venous hypertension or thrombosis, immunomodulating agent use within 8 weeks of enrollment, or daily use of anti-inflammatory medications. This study was approved by the Memorial Sloan-Kettering Institutional Review Board ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01749332) ID:NCT01749332). Venous blood samples were collected in 10-mL Cell-Free DNA BCT tubes (STRECK) intraoperatively prior to liver manipulation from a peripheral vein (PERIPH). Prior to hepatic resection, samples from the PV and HV were also obtained directly. Within 3-4 weeks, another peripheral blood sample was obtained (POSTOP).

Next-Generation Sequencing of Tumor Mutations

Tumor and companion normal tissue from resected primary colon or liver specimens underwent next-generation sequencing using the Memorial Sloan-Kettering Integrated Mutation Profiling of Actionable Cancer Targets assay (MSK-IMPACT) or the mass array-based iPLEX assay (Sequenom, San Diego, California). For the former, mutational burden was adjusted by megabase (mB) per IMPACT panel size (IMPACT-341=1.2mB, IMPACT-410=1.38mB).^{22,23} Array-based iPLEX is a single-base primer extension assay in which multiplexed PCR and extension primers are constructed for a custom gene panel.²⁴ Following PCR and extension reactions, extension products are analyzed with a matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer. Genomic data was stored on a secure, institutionally-licensed server for large-scale cancer genomics data (cBioPortal for Cancer Genomics).^{25,26}

Circulating Tumor DNA Extraction and Sequencing

Whole blood yielded DNA using previously described techniques.^{27,28} To limit operator-dependent variation, all samples were batched and sent for DNA extraction simultaneously. Library construction and target enrichment were performed using the KAPA Hyper Prep Kit (Kapa Biosystems, Wilmington, MA) followed by hybridization capture with custom baits (Integrated DNA Technologies, Coralville, IA); the custom panel included APC, BRAF, EGFR, ERBB2, KRAS, NRAS, PIK3CA, SMAD4, and TP53. Post-capture libraries were pooled in equimolar concentrations and sequenced on an Illumina HiSeq 2500 system (Illumina, San Diego, CA).

Positions with mutations known from tissue data but not discovered de-novo through variant calling were genotyped with custom pileup scripts. The following cutoffs defined mutation detection based on plasma variant allele frequency (VAF): 1) 0.01 if the mutation was identified in tumor specimen; 2) 0.02 if the mutation was absent in tumor specimen but detected in ctDNA at a hotspot locus; 3) 0.05 if the mutation was absent in tumor specimen and not at a hotspot locus.^{29,30} Patients with VAF below these thresholds were considered negative whereas patients missing VAF data for a mutation were excluded from further analysis. Samples from 5 patients undergoing resection of benign pancreatic lesions were included as controls to calibrate the sensitivity of ctDNA mutation calling.

The union of all mutations was generated, and germline variants were eliminated using DNA from matched blood. Each alteration identified de-novo was reviewed manually to remove false positives.

Statistical Analysis

Formal statistical tests were conducted on ctDNA genes where 5 patients had mutations. Agreement between the perioperative blood draws and between tumor mutations was assessed with Cohen's kappa (k) and percent agreement for pairwise comparisons of perioperative measurements was defined based on precedent literature.³¹ The relationship between clinicopathologic variables with ctDNA detection and VAF levels were assessed with Fisher's Exact and Wilcoxon Rank-Sum tests.

Disease-specific survival (DSS) was assessed for all patients from time of hepatic resection until death from disease. Patients alive at last follow-up were censored. Disease-free survival (DFS) was assessed from the time of hepatic resection until recurrence or death from disease. Patients alive and with no evidence of disease (NED) were censored. Only those who had NED after first or second-stage resection were included in DFS analyses. Kaplan-Meier methods estimated DSS and DFS and the log-rank test assessed the relationship between categorical ctDNA and outcomes. Univariable Cox regression assessed the relationship between the cfDNA concentration with outcomes.

Two-sided p-values less than 0.05 were considered statistically significant. Due to the exploratory and preliminary nature of these analyses, no corrections were made for multiple testing. All analyses were performed with SAS 9.4 (The SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

Of 60 patients enrolled, one had inaccessible perihepatic vessels at surgery and was thus excluded. Table 1 summarizes the preoperative features of the evaluable 59 patients. The majority enrolled were male (41, 69%) and the median age was 52.2 years (range(r): 34.4-92.5). Most had T3-T4 (53, 90%) and primary node-positive disease (36, 61%). The primary tumor was present at the time of hepatic resection in 24 patients (41%), and all but one underwent colectomy simultaneously.

ctDNA Mutation Profile

Thirty-eight patients (67%) had at least one mutation detected in ctDNA. Figure 1 summarizes the mutations identified across the 4 sources of ctDNA and resected tumor tissue. All mutations detected in ctDNA were present on tissue analysis. TP53 and APC were the two most commonly mutated genes in all ctDNA sources detected in 19 (32%) and 16 (27%), respectively. KRAS mutation was detected in ctDNA in one patient out of 15 bearing a tumor with that mutation. Given their greater relative frequency compared to others, further analysis was restricted to mutations in TP53 (mtTP53), APC (mtAPC), and any ctDNA.

Intraoperative ctDNA Agreement

When mtTP53 was detected in PERIPH blood, PV agreed in 89.8% of samples ($\kappa=0.73$, 95%CI:0.53-0.93) and HV in 91.5% ($\kappa=0.78$, 95%CI:0.60-0.96). Almost-perfect agreement in mtTP53 was also found between the two perihepatic sources (98.3%, $\kappa=0.95$, 95%CI:0.87-1.00). Similarly, PERIPH mtAPC detection agreed with PV blood in 94.9% ($\kappa=0.83$, 95%CI:0.64-1.00) and HV in 91.5% ($\kappa=0.73$, 95%CI:0.51-0.95). PV and HV mtAPC detection agreed in 93.2% ($\kappa=0.78$, 95%CI:0.57-0.99).

ctDNA and Tissue Agreement

In comparing blood to matched tissue, slight-to-moderate agreement was found between tissue mtTP53 and PERIPH (72.9%, $\kappa=0.44$, 95%CI:0.23-0.66), PV (72.9%, $\kappa=0.44$, 95%CI:0.24-0.65), HV (74.6%, $\kappa=0.48$, 95%CI:0.28-0.68), and POSTOP blood (61.0%, $\kappa=0.19$, 95%CI:0.02-0.36). Similarly, slight-to-fair agreement was found between tissue and ctDNA mtAPC from PERIPH (61.0%, $\kappa=0.23$, 95%CI:0.04-0.42), PV (62.7%, $\kappa=0.26$, 95%CI:0.08-0.44), HV (66.1%, $\kappa=0.33$, 95%CI:0.14-0.52), and POSTOP blood (57.6%, $\kappa=0.16$, 95%CI:0.00-0.32).

Measuring Tumor Burden with ctDNA

Detection of ctDNA was associated with larger CRLM tumor size (Table 3). Patients with mtAPC had larger tumors by comparison in PERIPH ($P=0.002$), PV ($P=0.004$), and HV blood ($P=0.002$). Similar results were obtained for mtTP53.

Thirty-eight and 39 patients had mtTP53 and mtAPC VAF measured, respectively. Patients with primary tumor present had significantly higher POSTOP mtTP53 VAF (median:0.0018, $r:0-0.4077$) compared to patients without their primary tumor (median:0.0000, $r:0-0.1253$, $P=0.040$), and significantly higher POSTOP mtAPC VAF (median:0.0008, $r:0-0.3239$) compared to patients without their primary tumor (median:0, $r:0-0.1192$, $P=0.024$). Detection of perioperative mtTP53 or mtAPC was not significantly associated without primary tumor presence at the time of hepatic resection ($p=0.11-0.21$).

Association of ctDNA with Outcomes

The median follow-up of survivors was 30 months ($r:3-36$). No patients died of unknown or other causes. Twelve (20.3%) died of metastatic disease with a 2-year DSS of 87% (95%CI:75-94%). Median DSS was not reached. By the end of follow-up, 34 of 49 initially NED patients recurred or died with a 2-year DFS of 30% (95%CI:18-43%) and a median DFS of 13.1 months (95%CI:8.6-17.8 months).

Detection of PERIPH mtTP53 was associated with worse DSS (2y:79%, 95%CI:48-93%) compared to patients lacking detection (2y:90%, 95%CI:77-96%, $P=0.02$) (Figure 2). However, the association between PERIPH mtAPC and 2-year DSS (mt(+):80% vs mt(-):89%, $P=0.11$) or between any ctDNA on PERIPH and 2-year DSS (mt(+):83% vs mt(-):90%, $P=0.09$) was not significant. HV ctDNA ($P=0.29-0.50$) and PV ctDNA ($P=0.28-0.46$) were not significantly associated with DSS.

As shown in Figure 3a, patients with any ctDNA detected in PV blood had better DFS (2y: 60%, 95%CI:25-83%) compared to patients without any ctDNA (2y:22%, 95%CI: 11-36%, $P=0.04$); however, no significant association was found between mtTP53 ($P=0.23$) or mtAPC ($P=0.15$) in PV blood and DFS. In the PERIPH blood, no significant associations were found for mtTP53 ($P=0.66$), mtAPC ($P=0.06$), or any ctDNA ($P=0.58$) with DFS. Additionally, no significant associations were found between HV measurements and DFS ($P=0.22-0.43$).

Associations of cfDNA with Outcomes

Higher concentrations of cfDNA were associated with worse DSS for PERIPH (HR:1.05, 95%CI: 1.00-1.11, $P=0.048$), PV (HR:1.05, 95%CI:1.01-1.10, $P=0.011$), and HV (HR:1.04, 95%CI:1.00-1.08, $P=0.034$). Similarly, higher concentrations of cfDNA were associated with higher hazard of DFS for PV (HR:1.07, 95%CI: 1.02-1.13, $P=0.005$) and HV blood (HR:1.08, 95%CI:1.03-1.13, $P=0.003$), but not PERIPH blood (HR:1.05, 95%CI:0.99-1.11, $P=0.09$).

DISCUSSION

Although most prior work has focused on ctDNA in peripheral blood, this study is the first to consider ctDNA detected in perihepatic blood. This interest stems from the observation that more CTCs are detected in perihepatic than peripheral blood as well as the association between DFS and overall survival with the number of perihepatic (but not peripheral) CTCs.^{20, 21} Our results demonstrate strong agreement between perihepatic and peripheral sources of ctDNA for select genes.

There is great interest in using “liquid biopsies” to determine mutations found in primary or metastatic tumors. In this study, slight-to-fair agreement was found between ctDNA from all blood locations and tumor mutations. In the literature, there are mixed reports regarding this relationship. Several studies of metastatic CRC patients with mtKRAS found agreement between ctDNA and resected tumors to be 85-97%.^{17,18,32-34} In this study, only one of 15 patients with mtKRAS in tumor tissue had it detected on ctDNA. Although tumor tissue analysis has many limitations, KRAS mutation status can alter treatment decisions. The low sensitivity seen in this study may indicate that ctDNA testing alone for KRAS, as a liquid biopsy, may be insufficient. Other reports comparing mutations in ctDNA and primary tumors of stage I-III disease note agreement of 28-68%.^{35,36} Kidess et al (2015) studied ctDNA drawn from 38 patients in which half had metastases.¹⁶ Therein, high concordance between plasma and tumor mutations was reported in metastatic patients (93%), whereas agreement with tumor mutations in nonmetastatic patients was lower (54%) suggesting dependency on disease stage. In support of this, Lin et al (2014) noted better sensitivity for mutation detection in ctDNA with progressively higher stage CRC.³⁷ Moreover, Vakiani et al (2012) found that mutations identified in primary and metastatic CRC tumors had over 90% agreement, however, mtTP53 was significantly more common in metastases (53% vs 30%) suggesting it may be associated with disseminated disease.³⁸ The noninvasive and preoperative aspects of ctDNA make it a favorable tool to pathology to identify patients with

actionable mutations.³⁹ However, detection of mutations may be contingent on the stage of disease.

Earlier work demonstrated that fluctuations in ctDNA levels reflect tumor dynamics during surgery and systemic therapy.¹⁴ In the present study, mtTP53 and mtAPC ctDNA detection and VAF were associated with tumor burden. Larger tumors were also associated with detection and higher VAF of mtTP53 in PERIPH and perihepatic blood. Of note, only mtAPC detection was associated with larger tumors.

This study also identified potential for cfDNA to predict survival as higher levels were associated with worse DSS when detected in PERIPH and perihepatic blood. Additionally, higher cfDNA was associated with worse DFS when detected in perihepatic blood. These findings concur with other reports.^{15,18,19,40} PERIPH mtTP53 detection was also found to be associated with worse DSS. In contrast, detection of any ctDNA in PV blood was associated with better DFS. In support of this, Jorissen et al (2015) similarly found that detection of mtAPC in microsatellite stable proximal CRC tumors was associated with a lower risk of recurrence.⁴¹ Of note, however, mtAPC detection in PERIPH blood was not found to be significantly associated with better DFS in this study.

Several limitations apply to this study. First, this was a pilot study so we are limited in our ability to draw definitive conclusions from our exploratory hypotheses. Our inclusion and exclusion criteria created a heterogeneous genomic sample; different mutation rates may be seen if ctDNA collection was standard of care for all patients. Second, most studies of ctDNA investigate the significance of mtKRAS given its implications on the choice of chemotherapy,^{8,9} however, this was not possible in our cohort due to its infrequency. Given its predilection for appearing in metastases rather than primary tumors,³⁸ mtTP53 may be better to study as it may reflect the biology of metastatic disease more reliably than other mutations. Comparison to mtAPC, a mutation seen equally across stages^{37,42,43} and locations,⁴⁴ is then beneficial in this regard. Third, sequencing a portion of a tumor may not represent the complete molecular makeup of the cancer. For example, Vietsch et al (2017) reported that 71% of mutations detected in ctDNA were absent on tumor analysis suggesting that detection from plasma may be more representative than a single tumor sample.³⁶ Studies of cfDNA and VAF, however, can be marred by the influence of aneuploid or triploid mutations erroneously increasing or decreasing their levels. There is also the potential that our ctDNA sequencing assay was not sensitive enough to detect the true landscape of plasma mutations. Finally, our study was underpowered to examine the association between change in ctDNA and outcomes; future studies should include a longer follow-up period and a larger sample to allow clinical factors associated with prognosis to be controlled such as receipt of preoperative chemotherapy, components of the clinical risk score, and margin status. Additionally, comparison of percutaneously and intraoperatively-acquired samples may reveal effects that result from liver manipulation to access the perihepatic blood stream.

CONCLUSIONS

Mutations found in ctDNA were not highly representative of mutations in the corresponding tumor tissue. This discrepancy may reflect differences in the prognostic utility of mtTP53

and mtAPC, a limitation of our ctDNA sequencing assay, or the true natural history of the tumor. Despite this, we found evidence that preoperative peripheral ctDNA detection was associated with worse DSS. ctDNA may be a worthy prognostic marker, but additional studies are needed to determine the utility of ctDNA in clinical management.

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SYNOPSIS

Contemporary biomarkers unreliably predict recurrence after resection of colorectal liver metastases. Circulating tumor DNA is a potentially effective and novel biomarker. Peripheral and perihepatic plasma tumor DNA are compared as markers for tumor burden and disease outcomes.

Figure 2:

Disease-specific survival based on detection of (a) any ctDNA from intraoperative peripheral vein blood, (b) mutated TP53 in ctDNA from intraoperative peripheral vein blood, (c) mutated APC in ctDNA from intraoperative peripheral vein blood.

Figure 3:

Disease-free survival based on intraoperative detection in portal vein blood of any ctDNA mutations (a), mutated TP53 (b), or mutated APC (c); intraoperative detection in hepatic vein blood of any ctDNA mutations (d), mutated TP53 (e), or mutated APC (f); intraoperative detection in peripheral vein blood of any ctDNA mutations (g), mutated TP53 (h), or mutated APC (i).

Table 1:**Clinicopathologic Characteristics**

N=59		
Gender	Female	18 (31%)
	Male	41 (69%)
Age at Surgery, years	Median (range)	52.2 (34.4-92.5)
BMI, kg/m ²	Median (range)	26.5 (15.8-38.5)
Comorbidities		
	HTN	21 (36%)
	DM	5 (8%)
	CAD	4 (7%)
Pathologic Primary T-Stage	T1	2 (3%)
	T2	2 (3%)
	T3	39 (66%)
	T4a	13 (22%)
	T4b	1 (2%)
	TX	2 (3%)
Pathologic Primary N-Stage	N0	22 (37%)
	N1	22 (37%)
	N2	14 (24%)
	NX	1 (2%)
Preoperative CEA, ng/mL	Median (range)	8.5 (0.5-357.7)
Number of CRLM	Median (range)	3.0 (1.0-11.0)
Size of Largest CRLM, cm *	Median (range)	3 (1-9)
Clinical Risk Score	High Risk (3-5)	27 (46%)
	Low Risk (0-2)	32 (54%)
Chemotherapy		
	Preoperative Systemic	38 (64%)
	Adjuvant Systemic	50 (85%)
	Preoperative HAIP	10 (17%)
	Adjuvant HAIP	33 (56%)
Became NED		449 (83%)

Numbers represent frequency with percent of total sample unless otherwise specified; BMI=Body Mass Index, HTN=hypertension, DM=diabetes mellitus, CAD=coronary artery disease, T and N stage correspond to the 8th edition of the American Joint Committee on Cancer classification, CEA=carcinoembryonic antigen, CRLM=Colorectal liver metastasis, HAIP=Hepatic artery infusion pump, NED=No evidence of disease.

* Size determined from surgical pathology reports available for 50 patients following liver resection

Table 2:

Associations between Mutation Status and Tumor Size

	Mutation Status	Median Tumor Size (Range)	P-value
PERIPH mtTP53	Mt(+)	4.0 (1.8-6.4)	0.014
	Mt(-)	2.5 (0.7-9.2)	
PV mtTP53	Mt(+)	4.6 (2.0-6.4)	0.002
	Mt(-)	2.5 (0.7-9.2)	
HV mtTP53	Mt(+)	4.5 (2.0-6.4)	0.001
	Mt(-)	2.4 (0.7-9.2)	
POSTOP mtTP53	Mt(+)	3.1 (2.1-3.7)	0.55
	Mt(-)	2.8 (0.7-9.2)	
PERIPH mtAPC	Mt(+)	4.3 (3.0-6.4)	0.002
	Mt(-)	2.4 (0.7-9.2)	
PV mtAPC	Mt(+)	4.8 (3.0-6.4)	0.004
	Mt(-)	2.5 (0.7-9.2)	
HV mtAPC	Mt(+)	4.3 (3.1-6.4)	0.002
	Mt(-)	2.4 (0.7-9.2)	
POSTOP mtAPC	Mt(+)	3.1 (2.1-3.7)	0.55
	Mt(-)	2.8 (0.7-9.2)	

PERIPH=pre-resection intraoperative peripheral vein blood, PV=pre-resection peripheral portal vein blood, HV=pre-resection peripheral hepatic vein blood, POSTOP=post-resection peripheral vein blood