

# Monitoring colorectal cancer following surgery using plasma circulating tumor DNA

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**Abstract.** Postoperative monitoring for patients with colorectal cancer (CRC) requires sensitive biomarkers that are associated with medical response and adjuvant therapy following surgery. Conventional tumor biomarkers [including carcinoembryonic antigen (CEA), CA19-9 and CA125] are widely used, but none of the markers provide high sensitivity or specificity. Previous studies indicated that circulating tumor DNA (ctDNA) is useful for postoperative monitoring of patients with cancer. However, the majority of previous studies involved patients with lung cancer, and therefore further studies are required which investigate patients with CRC. The present study enrolled 11 patients with CRC. All patients underwent surgery, and a number of patients were treated with postoperative chemotherapy. Tumor tissues and serial blood samples were collected from each patient, and somatic mutations of each sample were obtained using next-generation sequencing. The mutation landscape and dynamic changes in mutations for each patient were analyzed, and these results were compared with the changes of CEA levels. A number of driver genes were selected, including tumor protein P53 (TP53), APC and KRAS, to monitor the postoperative outcome of the 11 patients with CRC. Driver mutations were detected in preoperative plasma in 7 patients, with markedly decreased mutation rates detected in postoperative plasma compared with preoperative plasma. Driver mutations were not detected in 4 patients in

the preoperative or postoperative plasma. In 1 patient with metastatic rectal cancer, the rate of TP53 mutation increased from 8.95 (preoperative) to 71.4% (postoperative), and a new phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit  $\alpha$  mutation emerged. This patient succumbed to mortality six months following surgery, however there were no marked changes in CEA levels during periodic detection of CEA levels. In summary, ctDNA has a high sensitivity and specificity in prediction of the prognosis of patients with CRC.

## Introduction

Colorectal cancer (CRC) is one of the most common types of gastrointestinal cancer in China (1). CRC increased from being the fifth most common cause of mortality in 2000 to the third most common cause in 2015 (2). As colonoscopy is not a popular screening method in China, a majority of patients are diagnosed at advanced stages of cancer (3). In addition, effective treatment protocols for patients following surgery or chemotherapy have not developed successfully (4,5). The patients that are diagnosed at an early-stage CRC have a better prognosis, and the five-year survival rate is ~90% following surgery (6). Radical resection has been the primary treatment for early-stage CRC (including stage I-II) (7). However, adjuvant chemotherapy has additionally become an important treatment (8-10). Regardless of whether patients receive adjuvant therapy, dynamic monitoring following surgery is associated with the improved survival rates of patients with early-stage CRC (11-13). The sensitivity of existing tumor biomarkers [including carcinoembryonic antigen (CEA), cancer antigen 125 (CA125) and carbohydrate antigen 19-9] and other tests [including computed tomography (CT)] used for monitoring CRC is low (14-16). Therefore, identifying a highly sensitive clinical biomarker for the detection of CRC is of utmost importance.

Circulating tumor DNA (ctDNA) is fragmented DNA that is released from exfoliated tumor cells into the blood (17). ctDNA carries genetic information from the primary tumor and displays the genetic mutations of the tumor, which overcomes the problem of tumor heterogeneity (18). CtDNA is additionally used to monitor responses to treatment and to make decisions on further therapy during follow-up visits (19-23). Previous studies have revealed that mutations

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in plasma ctDNA and tumor biopsies were similar for patients with early breast cancer and CRC, and the majority of patients (89%) with driver mutations detected in ctDNA would recur 2-4 weeks postoperatively (24,25).

As a noninvasive detection method, ctDNA as a marker reveals distinct advantages in patients with advanced cancer over other detection methods. However, using ctDNA analysis to monitor therapy is a less sensitive method for patients in the early stages of cancer, which is likely as early-stage tumors release only a few DNA fragments into the blood (26). However, other sequencing methods additionally have problems that limit early detection (17,27). In the present study, next generation sequencing (NGS) technology was used to reveal significant associations between changes in ctDNA and different stages of CRC progression in order to examine the value of ctDNA in the clinical diagnosis and treatment of patients with CRC.

### Materials and methods

**Ethics statement.** The present study was approved by the Shenzhen People's Hospital Ethics Committee, and the protocols were performed in accordance with the approved guidelines (28). Written informed consent was obtained from each patient for the use of blood and tumor tissues with the approval of the Shenzhen People's Hospital Ethics Committee (Shenzhen, China). All samples and clinical data used in the present study were double-blind.

**Patients and collection of clinical samples.** The present study recruited 11 patients with primary CRC who underwent radical resection in the Department of Gastrointestinal Surgery at the Shenzhen People's Hospital from September 2016 to April 2017. Exclusion criteria: i) Pregnant or lactating women; ii) patients who had received prior treatment with a MEK inhibitor, and iii) a history of clinically significant interstitial lung disease or pneumonitis. Tumor tissues were collected during surgery, quickly frozen in liquid nitrogen and stored at -80°C. Blood samples (20 ml) for ctDNA and CEA analysis were collected from 4-10 weeks postoperatively, with serial twice-monthly blood sample collection, which lasted for up to six months (Fig. 1). Adjuvant chemotherapy was used at the discretion of the treating clinician, who was blinded to the ctDNA results. As per protocol, follow-up included a twice-monthly clinical review, detection of CEA levels and CT imaging for six months. Subsequently, follow-up visits were performed according to the standard of care at participating institutions, which included three-month and six-month CT visits for two years.

**DNA extraction and sequencing.** Tumor DNA was isolated from surgically resected CRC specimens. Blood samples were collected in tubes containing EDTA and centrifuged at 1,600 x g for 10 min at 4°C within 2 h of collection. Peripheral blood lymphocyte (PBL) debris was stored at -20°C until further use. The supernatants were further centrifuged at 10,000 x g for 10 min at 4°C, and plasma was harvested and stored at -80°C until further use. PBL DNA was extracted using the RelaxGene Blood DNA system (Tiangen Biotech Co., Ltd., Beijing, China), and ctDNA was extracted from

Table I. Colorectal cancer gene panel (85 genes).

Gene name	GenBank accession number
ABCB1	NM_000927
AKAP9	NM_005751
AKT1	NM_005163
APC	NM_000038
ARID1A	NM_006015
ATIC	NM_004044
ATM	NM_000051
AXIN2	NM_004655
BARD1	NM_000465
BMPR1A	NM_004329
BRAF	NM_004333
BRCA1	NM_007294
BRCA2	NM_000059
BRIP1	NM_032043
BUB1	NM_004336
C8orf34	NM_052958
CBR3	NM_001236
CDA	NM_001785
CDH1	NM_004360
CHEK2	NM_007194
CREBBP	NM_004380
CTNNB1	NM_001904
CYP19A1	NM_000103
CYP2D6	NM_000106
DPYD	NM_000110
EGFR	NM_005228
EIF3E	NM_001568
EPCAM	NM_002354
ERBB2	NM_004448
ERBB4	NM_005235
ERCC1	NM_001983
FBXW7	NM_018315
GREM1	NM_013372
GSTP1	NM_000852
HRAS	NM_005343
KDR	NM_002253
KMT2C	NM_170606
KMT2D	NM_003482
KRAS	NM_004985
MET	NM_000245
MLH1	NM_000249
MLH3	NM_014381
MRE11A	NM_005590
MSH2	NM_000251
MSH3	NM_002439
MSH5	NM_002441
MSH6	NM_000179
MTHFR	NM_005957
MTRR	NM_002454
MUTYH	NM_012222
NBN	NM_002485
NF1	NM_000267
NRAS	NM_002524
PALB2	NM_024675
PIK3CA	NM_006218
PMS1	NM_000534
PMS2	NM_000535
POLD1	NM_002691
POLE	NM_006231
PTCH1	NM_000264
PTEN	NM_000314
PTPRK	NM_002844

Table I. Continued.

Gene name	GenBank accession number
RAD50	NM_005732
RAD51C	NM_002876
RAD51D	NM_002878
RB1	NM_000321
RRM1	NM_001033
RSPO2	NM_178565
RSPO3	NM_032784
SLIT1	NM_003061
SMAD2	NM_005901
SMAD4	NM_005359
SOD2	NM_000636
STK11	NM_000455
STMN1	NM_005563
TOP2A	NM_001067
TP53	NM_000546
TRRAP	NM_003496
TUBB3	NM_006086
TYMP	NM_001953
TYMS	NM_001071
UGT1A1	NM_000463
UMPS	NM_000373
XPC	NM_004628
XRCC1	NM_006297

≥2 ml of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. DNA was quantified using the Qubit 2.0 fluorometer and the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

DNA was collected from PBLs and was sheared using the enzyme dsDNA Fragmentase (New England BioLabs, Inc., Ipswich, MA, USA). Size selection of the DNA fragments (150-250 bp) was then performed using Ampure XP beads (Beckman Coulter, Inc., Brea, CA, USA), which has the additional benefits of higher recovery and greater speed. DNA fragments and ctDNA were used for library construction using the KAPA Library Preparation kit (Kapa Biosystems, Inc., Wilmington, MA, USA) according to the manufacturer's protocol. Agencourt AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA) were used for all the cleanup steps. End repair and 3'-end A-tailing were performed following DNA fragmentation. Notably, T-tailed adapters were used and a 3'dA overhang was added enzymatically onto the fragmented DNA sample. Ligation was performed for 15 min at 20°C. Single-step size selection was performed by adding 50 µl (x1) of PEG/NaCl SPRI Solution buffer (Beckman Coulter, Inc.) to enrich ligated DNA fragments. The ligated fragments were then amplified using 1x KAPA HiFi Hot Start Ready mix (Kapa Biosystems, Inc.) and Pre-LM-PCR Oligos (Kapa Biosystems, Inc.) in 50 µl reactions, and 7-8 PCR cycles were performed depending on the amount of DNA input. The thermocycling conditions were as follows: Initial Denaturation, 98°C for 30 sec; 7-8 cycles at 98°C for 10 sec; 60°C for 10 sec; 68°C for 30 sec, and the final extension at 68°C for 60 sec. The purity and concentration of the DNA fragments were assessed using the Qubit 2.0 fluorometer and the Qubit

dsDNA HS Assay kit. Fragment length was determined on a 4200 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) using the DNA 1000 kit (Agilent Technologies, Inc., Santa Clara, CA, USA). DNA sequencing was then performed on the Illumina NextSeq 500 system according to the manufacturer's recommendations at a depth of 7,000x.

**Variant calling and analysis.** Sequenced genes and their genebank accession numbers are provided in Table I (Colorectal cancer gene panel, 85 genes). Sequencing data were de-multiplexed and aligned to the hg19 genome (GRch37) using Burrows-Wheeler Aligner (29) version 0.7.15-r1140 using default settings. Pileup files for properly paired reads with mapping quality ≥60 were generated using Samtools (<http://www.htslib.org/>) (30). Somatic variants call were created using VarScan2 (<http://varscan.sourceforge.net/>) (31) [the minimum read depth at a position=100; the variant allele frequency (VAF) is the proportion of reads at a site which contain the variant allele. VAF threshold ≥0.05; somatic-P-value ≤0.01; strand-filter ≥1; otherwise, default parameters]. Allele frequencies were calculated for all Q30 bases. Using a custom Python script, previously identified tumor DNA mutations were intersected with a Samtools mpileup file generated for each plasma DNA sample; the number and frequency were then calculated for each mutation. For detection of mutations from plasma DNA without tumor DNA present, a mutation was identified if ≥4 mutant reads were identified in plasma and ≥1 mutant read was identified on each strand. No mutant reads were observed in matched genomic DNA from white blood cells.

**Statistical analysis.** A likelihood ratio test was used for comparing the difference of the number of nonsynonymous mutations between cancer stages. P<0.05 was considered to indicate a statistically significant difference. Analyses were performed using the R (version 3.1.2, <https://www.R-project.org>) (32).

## Results

**Patient characteristics.** A total of 11 patients with CRC were enrolled, including 9 patients with colon cancer and 2 patients with rectal cancer. The cohort included 4 patients with stage I, 3 patients with stage II, 3 patients with stage III and 1 patient with stage IV using a pathological staging system (33). CT imaging revealed that 1 patient with CRC had liver metastasis at surgery. A total of 9 patients received postoperative chemotherapy following surgery. In total, 11 primary tumor tissues and 42 plasma samples were collected. Complete information is presented in Tables II and III.

**Somatic mutations in tumor tissue samples.** VarScan2 (somatic P-value=0.01, minimum variant frequency=5%, strand filter=true, and otherwise default parameters) was used to detect tumor somatic single nucleotide variants (SNVs) and short insertion/deletion events captured by a panel of 85 genes (0.03% of the human genome; Table I). Within this small target, the number of nonsynonymous mutations detected in tumors ranged from 1 to 15, with a median of 5, including SNVs (59-86%) and small deletions

Table II. Clinical characteristics of 11 patients with CRC.

Patient no.	Sex	Age at surgery (years)	Histology	TNM	Tumor stage	Size of tumor tissue (cm)	Maximum diameter (cm)	Adjuvant chemotherapy at surgery	Family history of cancer
H1001	Male	69	Colon	T4bN0M0	IIC	4.0x3.0x2.0	4.0	N	N
H1002	Female	50	Colon	T2N1aM0	IIIA	2.1x1.5x0.6	2.1	Oxaliplatin+5-FU (20 weeks)	N
H1003	Male	73	Rectum	T3N2aM1b	IVB	5.5x4.0x0.8	5.5	Capecitabine (10 weeks)	Y
H1004	Female	49	Rectum	T2N0M0	I	2.0x2.0x0.5	2.0	Oxaliplatin+5-FU (2 weeks) Capecitabine (12 weeks)	N
H1005	Female	60	Colon	T3N1cM0	IIIB	2.2x1.8x1.0	2.2	Oxaliplatin (16 weeks)	NA
H1006	Female	69	Colon	T1N0M0	I	1.5x1.5x1.0	1.5	N	Y
H1007	Male	53	Colon	T2N0M0	I	4.3x2.5x1.2	4.3	Oxaliplatin+5-FU (4 weeks) Capecitabine (18 weeks)	N
H1008	Female	61	Colon	T1N0M0	I	7.5x4.0x3.5	7.5	Capecitabine (18 weeks)	N
H1009	Male	67	Colon	T3N0M0	IIA	11.0x4.0x1.2	11	Capecitabine (18 weeks)	N
H010	Female	35	Colon	T3N1aM0	IIIB	3.0x2.5x0.7	3	Oxaliplatin+5-FU (20 weeks)	N
H1011	Female	48	Colon	T3N0M0	IIA	2.5x4.5x1.5	4.5	Capecitabine (24 weeks)	N

N, no; Y, yes; TNM, tumor-node metastasis; 5-FU, fluorouracil; NA, unknown.

(10-14%; Fig. 2; Table IV). The number of nonsynonymous mutations was not associated with the CRC stage ( $P=0.451$ ). Additionally, it was identified that A>C/T>G transversion occurred most frequently (24 and 41%, respectively), followed by C>T/G>A transition (Fig. 3).

Different driver gene mutations revealed inter-individual tumor genetic heterogeneity. For example, KRAS mutations in codons 12 and 13 were observed in 3 patients and 1 patient, respectively. Tumor protein p53 (TP53) mutations in exons 5, 7 and 8 were observed in 3, 2 and 2 patients, respectively. A heatmap was constructed in order to demonstrate the somatic mutations occurring in the tumor tissues (Fig. 4). Different mutation loci and their corresponding mutation rates indicated intratumor genetic heterogeneity, implying tumor evolution (Fig. 5).

*Somatic mutation detection in preoperative plasma.* Next, ctDNA levels in preoperative plasma were examined. At a mean sequencing depth of  $\times\sim 7,000$  (pre-duplication), a custom Python script was used to identify whether somatic tumor mutations were present in preoperative plasma. For a total of 7 patients, at least one mutation in tumor tissue was detected in preoperative plasma, and VAF in plasma ranged from 0.11 to 9.42% (Fig. 6). For the other 4 patients (H1002, H1005, H1006 and H1010), who were diagnosed with stage II or stage III cancer, no mutations that matched the tumor mutations were detected in the plasma.

*Dynamic monitoring of postoperative patients with CRC using ctDNA.* In order to determine prognosis for patients with CRC and decide whether or not to administer adjuvant chemotherapy, ctDNA levels were monitored periodically for six months. Driver gene mutations were detected at low mutation rates in preoperative plasma ctDNA from 7 patients (7/11; 63.6%), whilst no driver mutations were detected in the remaining 4 patients (4/11; 36.4%; patient nos. ID H1002, H1005, H1006 and H1010; see Table IV).

Following surgery, the number of mutations and the corresponding mutation rate decreased compared with preoperative

Table III. Patient and tumor characteristics.

Characteristics	Patients (n=11)
Age at surgery	
Years, median (range)	57.6 (35-73)
Sex, n (%)	
Male	4 (36.4)
Female	7 (63.6)
Tumor stage, n (%)	
I	4 (36.3)
II	3 (27.3)
III	3 (27.3)
IV	1 (9.1)
Localization, n (%)	
Colon	9 (81.8)
Rectum	2 (18.2)
Adjuvant chemotherapy, n (%)	
Yes	9 (81.8)
No	2 (18.2)
Family history, n (%)	
Yes	2 (18.2)
No	8 (72.7)
NA	1 (9.1)

NA, unknown.

plasma in postoperative plasma in the majority of patients. Driver mutations were not detected in 6 patients (6/11; 54.5%). To illustrate this, the changes in the percentage of ctDNA and CEA levels in patient H1001 were plotted (Fig. 7A and B). In patient H1001, the CEA levels initially declined and then increased slowly, but the levels remained in the normal range ( $<5 \mu\text{g/l}$ ; Fig. 7B).

The VAF values for patient H1003 are presented in Fig. 8A. For patient H1003, the mutation rate of NM\_0,011,26113.2 (TP53):c.857A>G (p.Glu286Gly) in ctDNA increased from 8.95 (preoperation) to 11.32% (post-operation) (Fig. 8A). Liver metastases were detected in patient H1003 at the



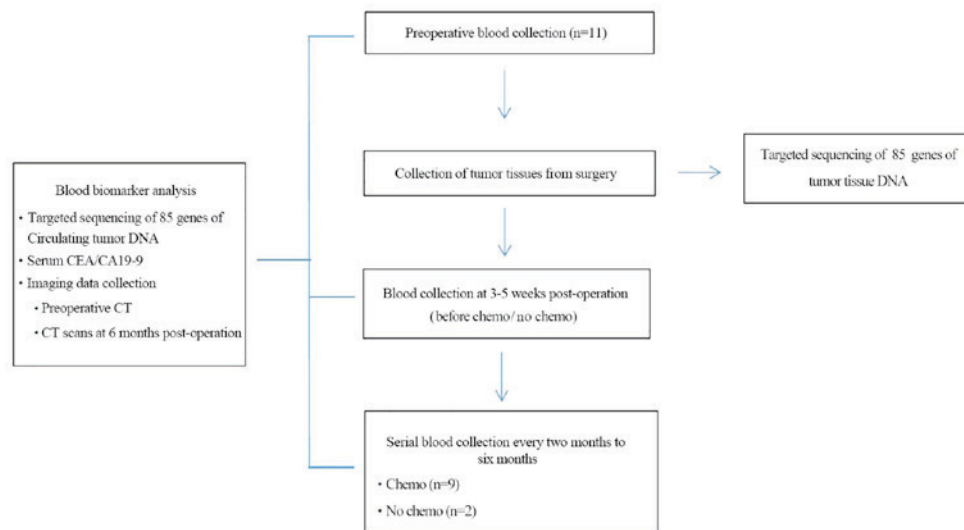


Figure 1. Patient enrolment and sample collection. Flow chart of patient enrollment and sample collection. CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CT, computed tomography; chemo, chemotherapy.

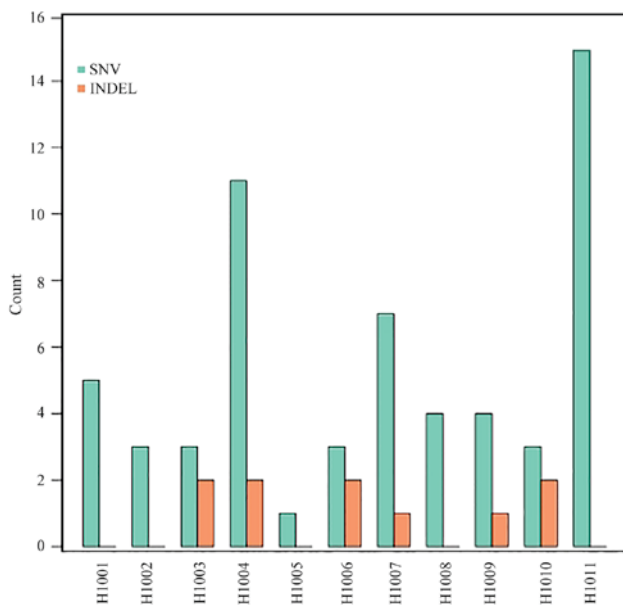


Figure 2. Mutation statistics of tumor samples from 11 patients with colorectal cancer. The y-axis indicates the number of mutations and the x-axis indicates the patient ID. The type of mutations (SNV and INDEL) is indicated in green and orange, respectively. SNV, single nucleotide variants; INDEL, small insertions and deletions.

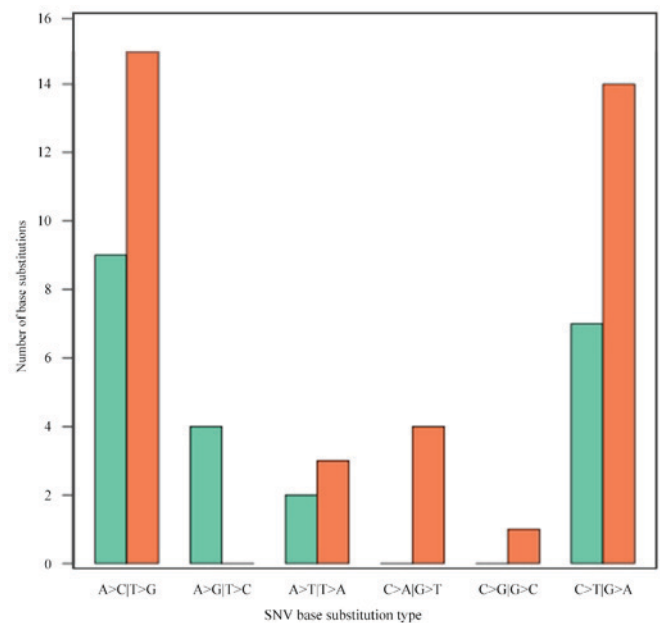


Figure 3. SNV base substitution statistics. Counts are indicated in columns. The base substitution types are indicated in rows, and different subtypes of base substitutions are represented in aquamarine and coral. SNV, single nucleotide variant.

time of surgery (Fig. 8B). Following surgery, patient H1003 received one round of chemotherapy and two rounds of microwave ablation for liver metastases, but treatments were subsequently terminated. The periodic monitoring of plasma ctDNA in patient H1003 revealed an increase in the mutation rate of TP53, and the development of a new mutation, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit  $\alpha$  (PIK3CA). NM\_006218.3 (PIK3CA):c.1624G>A (p.Glu542Lys) (Fig. 8A). In the final ctDNA sample from this patient, the mutation rate of TP53 and PIK3CA reached 71.4 and 19.96%, respectively (Fig. 8A). Patient H1003 succumbed to mortality six months following surgery. Furthermore, CEA levels were lower compared to the threshold (cut-off, 5  $\mu\text{g/l}$ )

in the three tests prior to mortality (2.63, 2.02 and 3.45  $\mu\text{g/l}$ ), which was not revealed to be associated with the patient's condition. However, the CEA level increased at the time of last sampling (11.12  $\mu\text{g/l}$ ; Fig. 8B). This finding suggests that ctDNA is more sensitive compared with CEA as a biomarker for predication of disease progression.

## Discussion

In the present study, somatic mutations in tumor tissues and preoperative plasma were highly associated as represented by the status of KRAS gene. In previous studies, ctDNA detection rates for patients in the early stages of cancer were



Table IV. Continued.

Case	Gene	Chromosome	Transcript	Exon	Nucleotide variation	Amino acid variation	Mutation frequency of tumor tissue (%)	Cosmid	Mutation frequency of preoperative ALT-plasma (%)		Mutation frequency of 1 month postoperative ALT-plasma (%)		Mutation frequency of 3 month postoperative ALT-plasma (%)		Mutation frequency of 5 mo postoperative ALT-plasma (%)	
									Depth	ALT-Depth	Depth	ALT-Depth	Depth	ALT-Depth	Depth	ALT-Depth
H1007	ATM	chr11	NM_000051	exon17	c.T2570G	p.L857R	14.45		0	0	0	0	0	0	0	0
H1007	SMAD2	chr18	NM_005901	exon9	c.1009_1024del	p.R337fs	29.56		0	0	0	0	0	0	0	0
H1008	KRAS	chr12	NM_004985	exon3	c.A183T	p.Q61H	79.42	COSM555	3.26	52	1,593	0	0	0	0	0
H1008	SMAD4	chr18	NM_005359	exon12	c.T1619G	p.L540R	68.95	COSM14165	0	0	0	0	0	0	0	0
H1008	UGT1A1	chr2	NM_000463	exon1	c.G323A	p.R108H	36.8	COSM1018132	0.46	7	1,508	0.2	4	1,992	0	0
H1008	ATIC	chr2	NM_004044	exon6	c.G515A	p.R172H	18.27	COSM1183927	0	0	0	0	0	0	0	0
H1009	KRAS	chr12	NM_004985	exon2	c.G35A	p.G12D	33.98	COSM521	0.54	3	553	0	0	0	0	0
H1009	RB1	chr13	NM_000321	exon1	c.A13C	p.T5P	7.94		0	0	0	0	0	0	0	0
H1009	ARID1A	chr1	NM_006015	exon1	c.A317C	p.N106T	6.77		0	0	0	0	0	0	0	0
H1009	MSH6	chr2	NM_000179	exon1	c.A256C	p.T86P	5.56		0	0	0	0	0	0	0	0
H1009	ARID1A	chr1	NM_006015	exon20	c.5125_5131del	p.L1709fs	21.35		0	0	0	0	0	0	0	0
H1010	KMT2B	chr19	NM_014727	exon1	c.T266G	p.V89G	7.46		0	0	0	0	0	0	0	0
H1010	KMT2B	chr19	NM_014727	exon28	c.T6746G	p.V2249G	6.78		0	0	0	0	0	0	0	0
H1010	KMT2D	chr12	NM_003482	exon11	c.T2987G	p.V996G	5.41		0	0	0	0	0	0	0	0
H1010	TP53	chr17	NM_000546	exon7	c.7115_720del	p.239_240del	11.72	COSM45055	0	0	0	0	0	0	3	1,624
H1010	APC	chr5	NM_000038	exon16	c.2025delA	p.T675fs	7.35		0	0	0	0	0	0	0	0
H1011	TP53	chr17	NM_000546	exon8	c.C844T	p.R282W	43.17	COSM10704	0.39	6	1,533	0	0	0	0	0
H1011	PIK3CA	chr3	NM_006218	exon5	c.T1035A	p.N345K	40.51	COSM754	0	0	0	0	0	0	0	0
H1011	RB1	chr13	NM_000321	exon1	c.A13C	p.T5P	8.73		9.42	13	138	0	0	0	0	0
H1011	TP53	chr17	NM_000546	exon5	c.G404A	p.C135Y	8.28	COSM10801	0.19	3	1,548	0	0	0	0	0
H1011	KMT2B	chr19	NM_014727	exon1	c.T248G	p.L83R	7.96		0	0	0	0	0	0	0	0
H1011	KMT2B	chr19	NM_014727	exon28	c.T6746G	p.V2249G	7.06		0	0	0	0	0	0	0	0
H1011	ERBB2	chr17	NM_004448	exon1	c.T25G	p.W9G	6.37		0	0	0	0	0	0	0	0
H1011	KMT2B	chr19	NM_014727	exon28	c.T6737G	p.V2246G	5.67		0	0	0	0	0	0	0	0
H1011	CREBBP	chr16	NM_004380	exon30	c.A4931C	p.N1644T	5.61		0	0	0	0	0	0	0	0
H1011	APC	chr5	NM_000038	exon16	c.G4057T	p.E1353X	5.57	COSM19048	0	0	0	0	0	0	0	0
H1011	PTCH1	chr9	NM_000264	exon1	c.A26C	p.E9A	5.41		0	0	0	0	0	0	0	0
H1011	PTPRK	chr6	NM_002844	exon15	c.C2432T	p.T811I	5.39		0	0	0	0	0	0	0	0
H1011	TYMP	chr22	NM_001953	exon8	c.T941G	p.L314R	5.31		0	0	0	0	0	0	0	0
H1011	RB1	chr13	NM_000321	exon1	c.A24C	p.K8N	5.29		0	0	0	0	0	0	0	0
H1011	TYMP	chr22	NM_001953	exon9	c.T1175G	p.V392G	5.22		0	0	0	0	0	0	0	0

ALT, alteration.

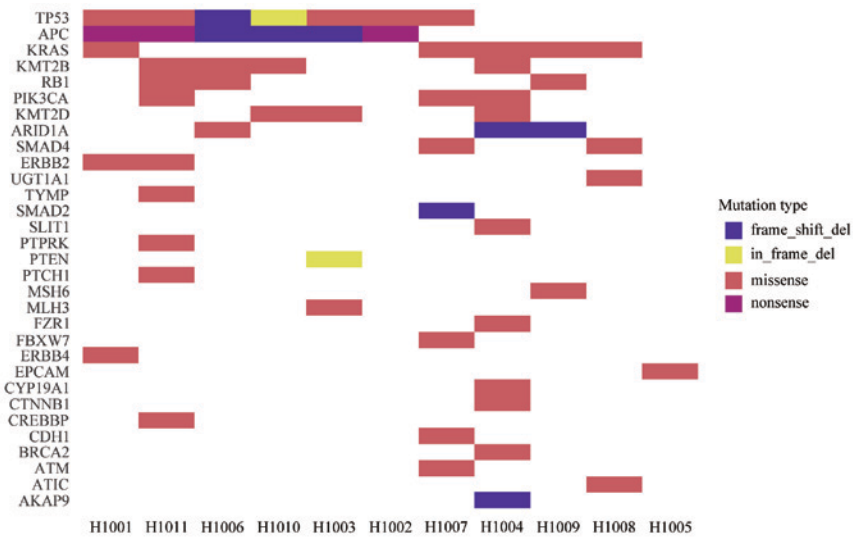


Figure 4. Mutation heatmap. Mutation spectrum of tumor tissues from 11 patients with colorectal cancer. The mutation frequencies of genes with high mutation rates among these 11 patients are indicated. The names of the mutated genes are indicated in the y-axis, and the patient no. is shown in the x-axis. The type of mutations, including frame-shift deletion, in-frame deletion, missense, nonsense and frame-shift insertions are represented by the colors blue, yellow, red, orange and pink, respectively.

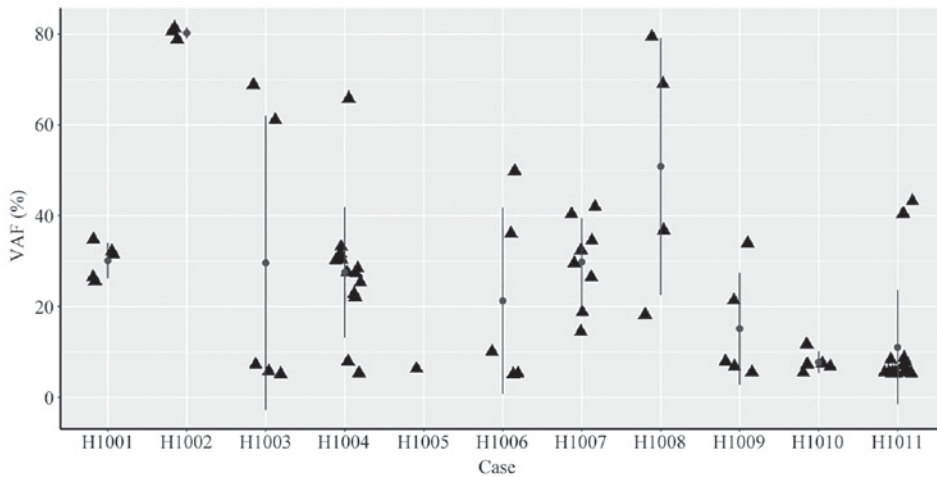


Figure 5. Summary of VAF in 11 tumor cases. Scatter plots of the VAF in tumor tissues from 11 patients with colorectal cancer. The VAF values are indicated in the y-axis, and the patient ID is shown in the x-axis. The vertical line with dots represent the mean  $\pm$  standard deviation of VAF. VAF, variant allele fraction.

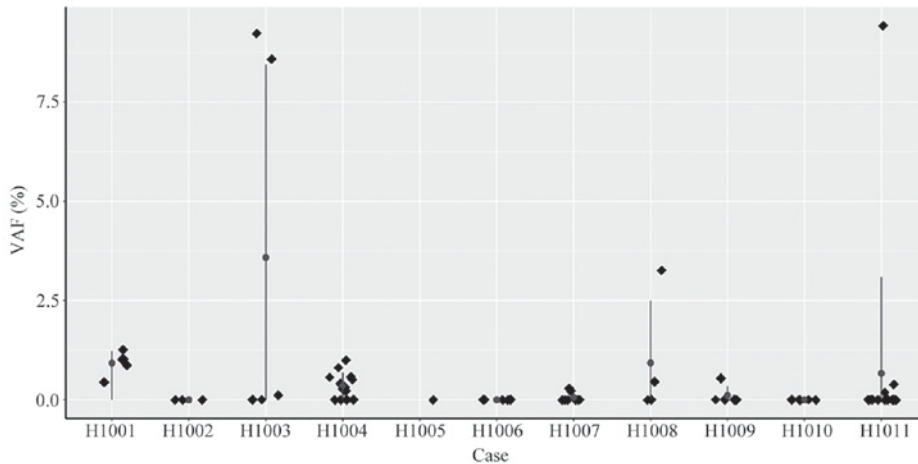


Figure 6. VAF in pre-operative plasma from 11 patients with CRC. Scatter plots of VAF in ctDNA of pre-operative plasma from 11 patients with CRC. The patient ID is presented on the x-axis, and the VAF is indicated on the y-axis. The vertical line with dots represent the mean  $\pm$  standard deviation of the VAF. CRC, colorectal cancer; VAF, variant allele fraction.



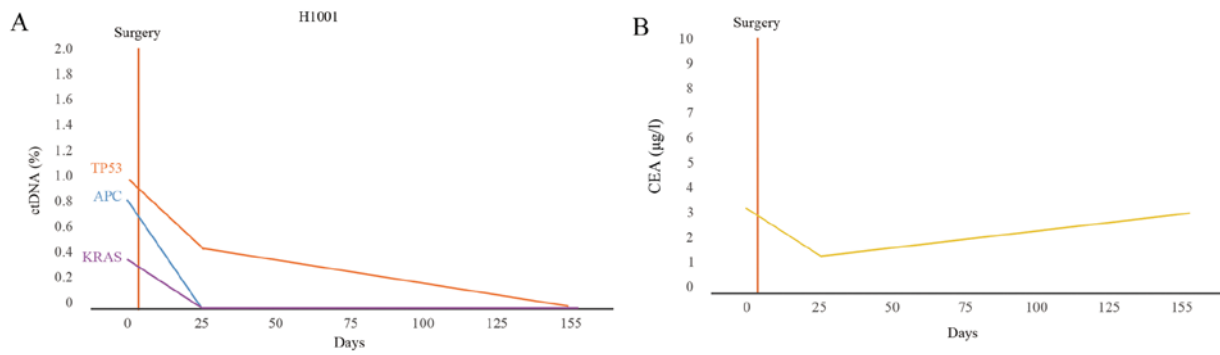


Figure 7. Dynamic ctDNA monitoring of patient H1001. (A) ctDNA and (B) CEA levels were assessed preoperatively and postoperatively for patient no. H1001. Patient no. H1001 exhibited no recurrence. The number of days following surgery is indicated on the x-axis. (B) The concentration of CEA is indicated on the y-axis. ctDNA, circulating tumor DNA; CEA, carcinoembryonic antigen.

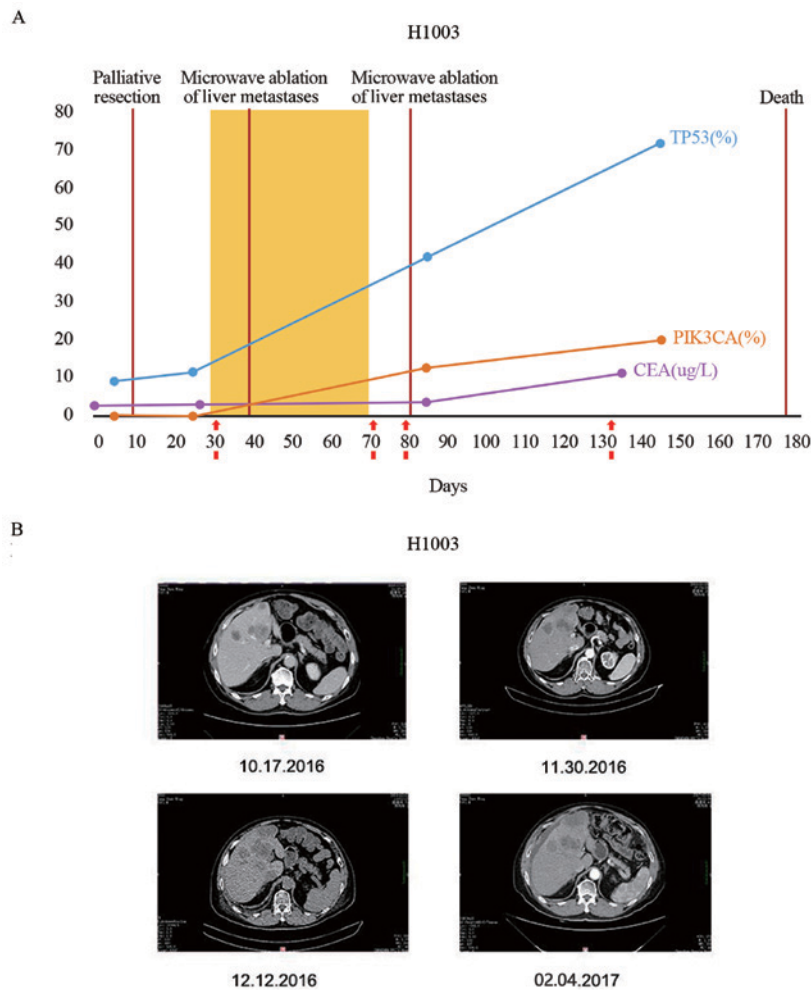


Figure 8. Dynamic ctDNA monitoring of patient H1003. (A) ctDNA and CEA levels were assessed pre-operatively and postoperatively for patient H1003. H1003 presented with recurrence during colonoscopy and in contrast-enhanced CT. For patient H1003, the TP53 and PIK3CA mutation rate changes in ctDNA are presented at the indicated times, and compared with CEA. The number of days following surgery is indicated on the x-axis is indicated on the x-axis. The VAF values are indicated on the y-axis. (B) CT images of liver metastases in patient H1003, in whom the level of ctDNA was four times compared with other patients in the study without metastases. Images were captured on October 17th, 2016 (30 days after surgery), November 30th, 2016 (43 days after surgery), December 12th, (55 days after surgery) and February 4th, 2017 (98 days after surgery). CT, computed tomography; ctDNA, circulating tumor DNA; CEA, carcinoembryonic antigen; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; TP53, tumor protein P53; VAF, variant allele fraction.

low (16,27,34). Detection was improved in the present study by increasing the depth of ctDNA sequencing (~7,000X), which allowed for the monitoring of ctDNA dynamically to

follow disease progression. ctDNA analysis using NGS was demonstrated to be sensitive for monitoring early therapeutic responses of CRC and metastatic breast cancer (35). Driver

mutations in genes such as TP53, KRAS and adenomatous polyposis coli tumor suppressor as observed in the 11 patients in the present study, were previously reported in patients with CRC (36). These mutations, thus, appear to be useful biomarkers for postoperative monitoring of CRC. As the sample size is relatively small, statistical bias is difficult to avoid.

For patients with early-stage CRC, administration of adjuvant chemotherapy following surgical resection remains controversial. Highly sensitive and specific biomarkers to predict the response to surgery are urgently required. Biomarkers for the early detection of recurrences would also be clinically valuable. In addition to previous research (16), the present study indicates that plasma ctDNA analysis has the potential for the detection of the early stages of cancer. Tie *et al* (37) demonstrated that ctDNA detection subsequent to stage II (33) colon cancer resection provided evidence of residual disease and identified patients at a substantially high risk of recurrence. Similar results were observed in the present study. However, the previous study by Tie *et al* (37) made predictions by targeting 15 genes and one single mutation in the ctDNA was selected for further analysis. By contrast, the present study examined preoperative and postoperative ctDNA for a whole spectrum of mutations. By comparison, the present study not only predicted the risk of recurrence, but was indicative of the tumor evolution for patients with cancer recurrence, which increases the current understanding of mechanism underlying recurrence in cancer.

As tumor-specific DNA is only a small fraction of cell-free DNA, ctDNA mutations are difficult to detect in patients with early stage cancer (37). For the patients in the present study with no preoperative driver gene mutations, we hypothesized that tumor cells may have invaded the serous layer of the intestinal wall. During the six months of monitoring, no changes were observed in the blood of patients with early-stage cancer. These findings indicate a good surgical outcome consistent with a greater survival of patients with early-stage CRC following surgery (37,38).

With the rapid progression of patients with late-stage cancer, driver mutations may quickly and substantially change in the blood. A high frequency of TP53 somatic mutation was detected in preoperative blood and tumor tissue of patient H1003. One month after surgery, the rate of TP53 mutations increased, which was associated with the presence of liver metastases observed on CT imaging subsequent to surgery. Conversely, decreased VAF of TP53 mutation were observed in all the other patients that did not have metastases following surgery. These results suggest that ctDNA ought to be analyzed in plasma at least every six months following surgery in order to monitor for disease progression in patients with early-stage cancer. Plasma CEA levels of patients with early or even advanced CRC were often below the normal threshold, implying that ctDNA has a higher sensitivity and specificity for detecting CRC compared with conventional CEA analysis.

Sequencing error rates are reported to be >0.1% in data analysis (39). In the present study, the correct mutations were selected by increasing the sequencing depth and a VAF threshold of <5% was used to filter the mutations detected. According to the tissue mutation results, ctDNA mutations were filtered using a VAF threshold of <0.1%, which may limit

the sensitivity of ctDNA and omit a number of low-frequency mutations. Although the number of patients in the present study is small, it demonstrates the potential of using ctDNA to monitor the medical response and disease progression following surgery. ctDNA analysis will be applied to a greater number of clinical applications in the future.

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