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Original Article

High concordance of mutation patterns in 10 common mutated genes between tumor tissue and cell-free DNA in metastatic colorectal cancer

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Abstract: The concordance of mutation patterns between cell-free DNA (cfDNA) and tumor DNA varies in colorectal cancers (CRCs). Next-generation sequencing (NGS) by targeted sequencing can detect novel genes. We aimed to use NGS to test the concordance between cfDNA and tumor DNA in metastatic CRCs. A total of 95 paired tumor and peripheral blood samples from metastatic CRC patients were included. The tumor DNA and cfDNA were analyzed with a 10-gene NGS panel (Illumina HiSeq2500 system). The median number of mutations in tumor samples was 3 (range 0-7). The most commonly mutated gene was *TP53* (63.2%), followed by *APC* (49.5%), *KRAS* (35.8%) and *FAT4* (15.8%). The concordance of mutation patterns in these 10 genes was as high as 91% between cfDNA and tumor samples in these metastatic CRC patients. A sensitivity of 88.2% and specificity of 100% was found when using *KRAS* mutation status of cfDNA to predict *KRAS* mutation in tumor tissue. For tumor DNA with *TP53*, *KRAS, or APC* mutations, right-sided CRCs were more likely to develop peritoneal metastases, while for tumor DNA with *TP53* mutations, left-sided tumors were more likely to have lung metastases. For cfDNA with *TP53* or *KRAS* mutations, right-sided CRCs were more likely to have peritoneal metastases. Due to the high concordance of mutation patterns between cfDNA and tumor samples, monitoring the mutation pattern of cfDNA may be applicable in the treatment of metastatic CRC.

Keywords: Tumor DNA, cfDNA, NGS, CRC, mutational pattern, concordance

Introduction

In Taiwan, colorectal cancer (CRC) is the most common cancer type and the third leading cause of cancer death. More than 15,000 new CRCs cases are diagnosed every year [1]. Approximately 20% of CRCs were diagnosed with distant metastases and were associated with a poor prognosis [2, 3]. The management of metastatic CRCs has improved the overall survival of these metastatic CRCs in recent decades with the introduction of multidisciplinary team approach, application of various target therapies in addition to systemic chemotherapy, and aggressive surgical resection of metastatic lesion [4]. Genetic test is important for target therapy because *KRAS*-mutated CRC is resistant to anti-EGFR antibody therapy and is a predictive biomarker of treatment response [5]. In addition, *BRAF* mutation is a strongly related to poor prognosis in metastatic CRC [5]. Accurate genetic analysis from tumor samples either from surgical specimen or endoscopic biopsy is essential but somehow not practical for patients with metastatic CRC when disseminated metastatic status prohibits surgical treatment or when sample of endoscopic biopsy is inadequate. During the treatment of molecular targeted therapy, clonal evolution of the tumor occurs resulting in temporal heterogeneity of tumor genetic profile [6]. A simple and real-time method to monitor the genetic

information is necessary for current personalized medicine in order to provide better choices of molecular therapy.

Circulating cell-free DNA (cfDNA) is useful in cancer monitoring and management, including that of CRC [7]. cfDNA derives from both normal and malignant cells and could be easily retrieved from the bloodstream, which is also known as liquid biopsy and is thought a non-invasive method for cancer monitoring because cfDNA derived from malignant cells may contain cancer-specific mutations [8, 9]. Our previous studies [10, 11] investigating the role of cfDNA in cancer of the gastreointestinal tract showed that cfDNA levels were more sensitive than CEA levels in predicting recurrence. In addition, cfDNA levels were higher in stage IV disease and were associated with treatment responses in CRC patients, including chemotherapy and surgery.

To date, few studies have used next-generation sequencing (NGS) to investigate the concordance of genetic mutation patterns between tumor tissue DNA and cfDNA [12, 13]. Although high concordance of genetic mutation patterns was reported between tumor tissue DNA and cfDNA, some mutant variants were found in tumor tissue DNA only, and some were found in cfDNA only. The discrepancy in genetic mutations between primary tumor DNA and cfDNA was considered to be due to tumor heterogeneity. However, the patient numbers in these studies were limited and the relationship between genetic alterations and sites of metastases in stage IV CRC is still obscure.

In the current study, we evaluated the concordance of mutation patterns between tumor tissue DNA and cfDNA samples in stage IV CRC patients using NGS. In addition, we analyzed the relationship between the mutational patterns and the sites of metastases.

Materials and methods

Tumor and preoperative serum samples were collected from 95 metastatic CRC (stage IV) patients from Taipei Veterans General Hospital Biobank. Only stage IV CRC patients with available both tumor and preoperative serum samples in the biobank were enrolled in this study. The exclusion criteria included patients who had stage I-III CRC, who received emergent surgery, or who did not have available tumor or preoperative serum samples in the biobank. All samples were anonymized and obtained with written informed consent. The Institutional Review Board of Taipei Veterans General Hospital has approved the present study.

Tumor DNA was extracted from tissue specimens by using QIAamp DNA Tissue Kit (Qiagen, Valencia, CA, USA) [14], and 1,000 ul of plasma was used to extract cell free DNA by using the QIAamp MinElute cfDNA Kits (Qiagen). The preparation of plasma from 2 mL blood was carried out according to the previous study [9]. After DNA extraction, DNA quantity was measured by using the Qubit Fluorometer and Qubit dsDNA High-Sensitivity assay (Thermo Scientific).

Illumina HiSeq2500 DNA sequencer was used to profile the mutation information of ten well-known CRC-related genes, namely, *TP53*, *APC*, *KRAS*, *FAT4*, *ARID1A*, *FBXW7*, *SMAD4*, *PIK3CA*, *NRAS* and *BRAF*, of both tumor DNA and cfDNA of each case.

Amount of 250 ng DNA of each tumor tissue was used to construct NGS library using IDT Lotus Library Preparation Kit (IDT, USA). Each DNA were fragmented and then used to prepare DNA library by performing end-repairing, a-overhang adding, adaptor ligation and size selection (250~350 bp) [15]. The 15 ul of each cfDNA was used to construct library by using xGen Prism DNA Library Prep Kit (IDT). Target DNA of exonic regions were enriched using the probe-based methods. The probes were synthesized by Integrated DNA Technologies (USA) according to our previously-designed probe sequences, and the capture procedure was performed following the IDT guideline. After the probe-based enrichment, library of each pool was amplified with 14 cycles. The amplified libraries were quantified using LC480 qPCR system (Roche), and were pooled to a new 1.5 ml tube as 10 nM pooled DNA library. The final pool was used for sequencing (Illumina Hiseq2500 sequencer, 2×150 bp). The raw output of each tumor tissue were > 0.5 Gb, and the average depth of target regions were > 250×. The raw output of each cfDNA were > 5 Gb, and the average depth of target regions were > 2000×. We used the Illumina Basespace Dragen somatic mutation pipeline (https://www.illumina.com/products/by-type/informat-

Table 1. Clinicopathological features of 95 colorectal cancer patients

Clinicopathological features	Metastatic CRC (n=95) (%)
Age	67.2 ± 13.5
Gender (M/F)	52/43
Tumor location	
Right side	25 (26.3)
Left side	70 (73.7)
Poor differentiation	18 (18.9)
Lymphovascular invasion	58 (61.1)
Mucinous histology	9 (9.5)
Metastatic site	
Liver	65 (68.4)
Lung	27 (28.4)
Peritoneum	32 (33.7)
Bone	2 (2.1)
Ovary	4 (4.2)
Distant lymph node	7 (7.4)
Metastatic status	
Single organ	67 (70.5)
Multiple organs	28 (29.5)

ics-products/basespace-sequence-hub/apps/edico-genome-inc-dragen-somatic-pipeline.html) to perform variant calling and annotated all variants by using Illumina variant interpreter (https://variantinterpreter.informatics.illumina.com/home).

Statistical analysis

IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY, USA) was used to perform statistical analyses. The χ^2 test with Yates' correction or Fisher's exact test was used to compare the categorical data. The frequency of the specific gene mutation was calculated using the number of the patients carrying the specific gene mutation divided by total number of the patients and was presented as percentage. Statistical significance was defined as a *P* value less than 0.05.

Results

Clinicopathological characteristics

All 95 metastatic CRC patients were stage IV disease and their clinicopathological features are shown in **Table 1**. The sex ratio (male to female) was 1.21. The median age was 67 years old. The most common metastatic location was the liver, followed by the peritoneum and lung. Among the 95 metastatic CRC patients, 28 (29.5%) patients had metastases in multiple organs.

Investigation of the mutated genes in CRC tumor samples

As shown in **Figure 1**, the median number of mutated genes s in the 95 CRC tumor samples was 3 (range 0-7). Among them, most patients had 2 or 3 mutated genes. As shown in **Figure 2**, the most commonly mutated gene was *TP53* (63.2%), followed by *APC* (49.5%), *KRAS* (35.8%), *FAT4* (15.8%), *ARID1A* (14.7%), *FBXW7* (11.6%), *SMAD4* (9.5%), *PIK3CA* (8.4%), *BRAF* (7.4%), and *NRAS* (4.2%). As demonstrated in **Figure 3**, the most frequent mutation was *KRAS* c.35G > T (12.6%), followed by *KRAS* c.35G > A (9.5%), *TP53* c.524G > A (8.4%), *KRAS* c.38G > A (6.3%), and *TP53* c.637C > T (5.3%).

As shown in **Table 2**, there was no significant difference in the frequency of any of the genetic mutations between right and left-sided CRC tumors.

As shown in Table S1, patients with mucinous adenocarcinoma were more likely to have genetic mutations in *TP53* (88.9% vs. 50.0%, *P*=0.026), *APC* (88.9% vs. 44.2 %, *P*=0.011), and *BRAF* (33.3% vs. 4.7%, *P*=0.002) genes than patients with non-mucinous adenocarcinoma.

Concordance of genetic mutations between cfDNA and tumor samples

Totally, 260 mutation spots among these 10 genes were found in tumor samples. Among these 95 patients, 23 mutation spots in tumor samples in 12 patients (12.6%) could not be detected in cfDNA. The concordance of mutation spots in these 10 genes was as high as 91% (237/260) between cfDNA and tumor samples in these metastatic CRC patients. **Figure 4** demonstrates the high concordance of mutation patterns in these 10 genes between cfDNA (**Figure 4A**) and tumor samples (**Figure 4B**).

As shown in **Table 3**, regarding the three major mutated genes (*TP53*, *KRAS* and *APC*) between tumor DNA and cfDNA, using cfDNA to detect mutation status in tumor sample would

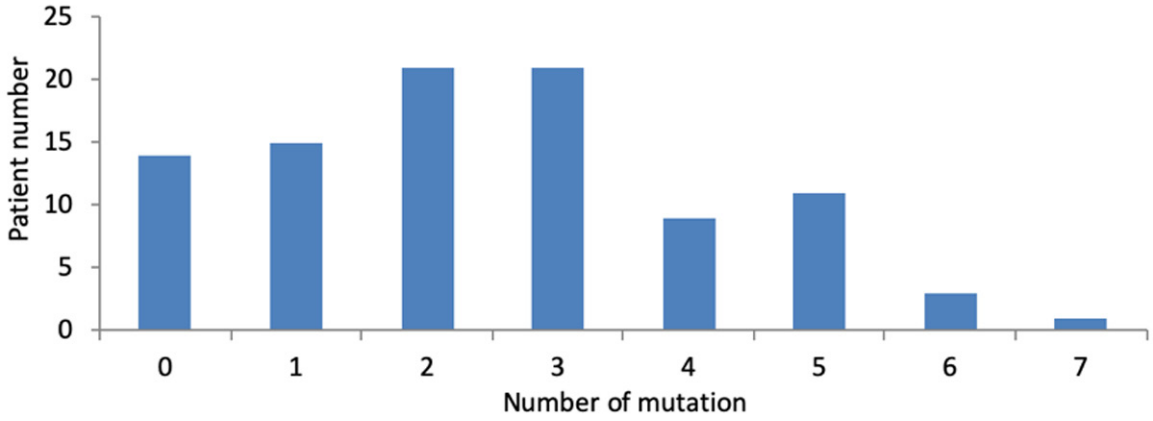

Figure 1. The distribution of patient number according to the number of genetic mutations.

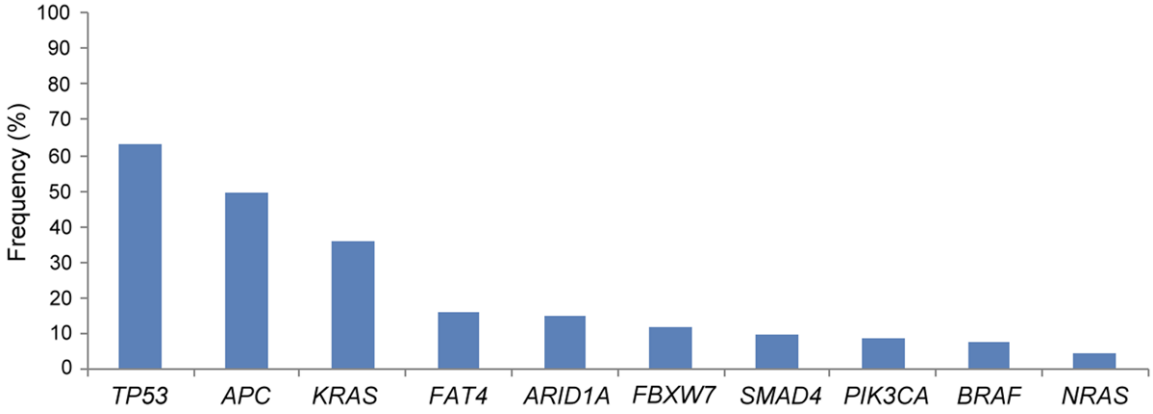

Figure 2. The frequency of genetic mutations in each of the ten genes.

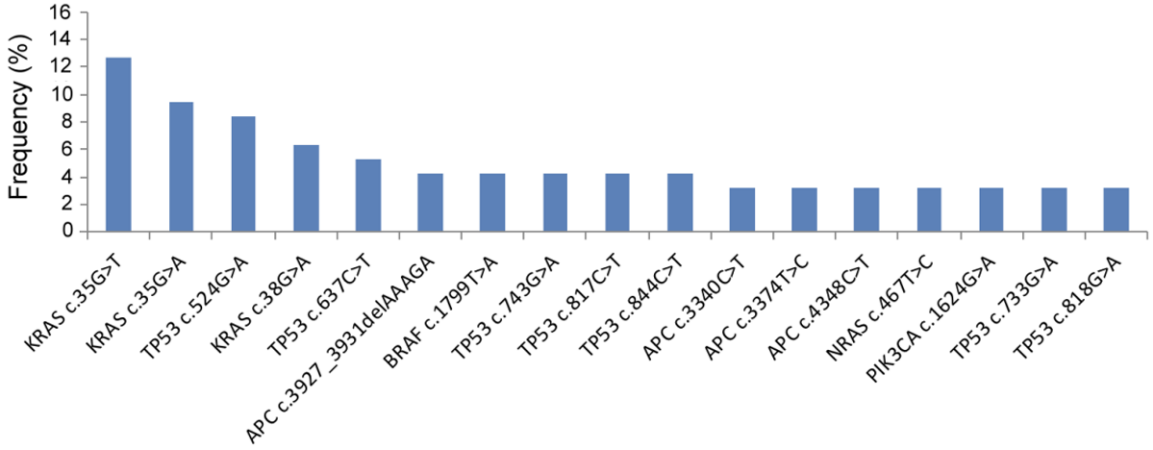

Figure 3. The frequency of the hot spot genetic mutations.

have a sensitivity of 85.0, 88.2% and 95.8%, and the specificity was as high as 100% for these three genes. The positive predictive value (PPV) was also as high as 100% for these three genes, and the negative predictive value (NPC) was 70%, 92.2% and 94.3%, respectively.

Table 2. The correlation of genetic mutations and tumor location

Genetic mutation	Right-sided colon n=25 n (%)	Left-sided colon n=70 n (%)	*P* value
TP53	14 (56.0)	37 (53.9)	0.787
APC	12 (48.0)	34 (48.6)	0.961
KRAS	6 (24.0)	24 (34.3)	0.342
FAT4	5 (20.0)	16 (22.9)	0.768
ARID1A	6 (24.0)	14 (20.0)	0.674
FBXW7	3 (12.0)	7 (10.0)	0.780
SMAD4	2 (8.0)	3 (4.3)	0.475
PIK3CA	2 (8.0)	5 (7.1)	0.888
BRAF	3 (12.0)	4 (5.7)	0.302
NRAS	1 (4.0)	6 (8.6)	0.453

The correlation between the mutation patterns of tumor samples and the metastatic sites and number of metastatic organs

As shown in **Figure 5**, for patients with peritoneal metastasis, the frequency of genetic mutations was the highest in *TP53*, followed by *APC*, *KRAS*, *SMAD4*, etc. For patients with ovary metastasis, the frequency of genetic mutations was the highest in *TP53*, followed by *APC*, *FBXW7*, *KRAS*, *ARID1A*, etc. For patients with distant lymph node metastasis, the frequency of genetic mutations was the highest in *TP53*, followed by *APC*, *KRAS*, *FBXW7*, etc. *KRAS* mutations were common in hematogenous metastases, including the liver, lung, and bone. *TP53* was the most common mutated gene in patients with metastasis in the liver, lung, ovary, peritoneum, and distant lymph node.

As shown in **Figure 6**, patients with metastases in multiple organs had more genetic mutations in the *TP53*, *APC*, *KRAS*, *FBXW7*, and *SMAD4* genes than patients with metastasis in a single organ.

The correlation between genetic mutations and primary tumor locations and metastatic organs

Regarding tumor DNA genetic mutations (**Table 4**), for patients with *TP53* mutations, left-sided CRCs were more likely to have lung metastases than right-sided CRCs (36.4% vs. 6.7%, *P*=0.028), while right-sided CRCs were more likely to have peritoneal metastases than left-sided CRCs (53.3% vs. 25.0%, *P*=0.043). For patients with *KRAS* mutations, right-sided CRCs were more likely to have peritoneal metastases than left-sided CRCs. For patients with *APC* mutations, right-sided CRCs were more likely to have peritoneal metastases than left-sided CRCs.

Regarding cfDNA genetic mutations (**Table 5**), for patients with *TP53* mutations, right-sided CRCs were more likely to have peritoneal metastases than left-sided CRCs. For patients with *KRAS* mutations, right-sided CRCs were more likely to develop peritoneal metastases than left-sided CRCs.

Discussion

In the present study, there are two valuable findings. First, the concordance of mutation patterns in these 10 genes was as high as 91% between cfDNA and tumor samples from stage IV CRC patients. Second, the sensitivity, specificity, PPV and NPV were high between tumor DNA and cfDNA in stage IV CRC. Third, the mutations of tumor DNA and cfDNA were associated with metastatic sites and location of primary tumor in CRC.

Regarding the concordance of mutation patterns between cfDNA and tumor samples, Takeda et al [12] reported that only 50.9% of the mutations detected in tumor samples were identified in cfDNA, while Shi et al [13] reported that 92% of the mutations detected in tumor samples were identified in cfDNA, which was similar to the 91% shown in our results. The major discrepancy between the results is possibly because only stage IV CRC patients were included in the present study and the study of Shi et al [13], but stage II-IV CRC patients were included in the study of Takeda et al [12]. The sensitivity of *TP53*, *APC* and *KRAS* mutations between tumor tissue DNA and cfDNA was 66.7%, 62.5% and 70.0%, respectively in Takeda's study, while the specificity for these three mutations was 59.1.5%, 73.1% and 79.2%, respectively. They also detected 20 mutations in cfDNA that were not identified in tumor tissue DNA. But in our study, all mutations which were detected in cfDNA could also be identified in tumor tissue DNA. There are several reasons which may explain the discrepancy. First, in the present study, with the use of whole exome sequencing with NGS method, the quality of genetic sequencing was relatively

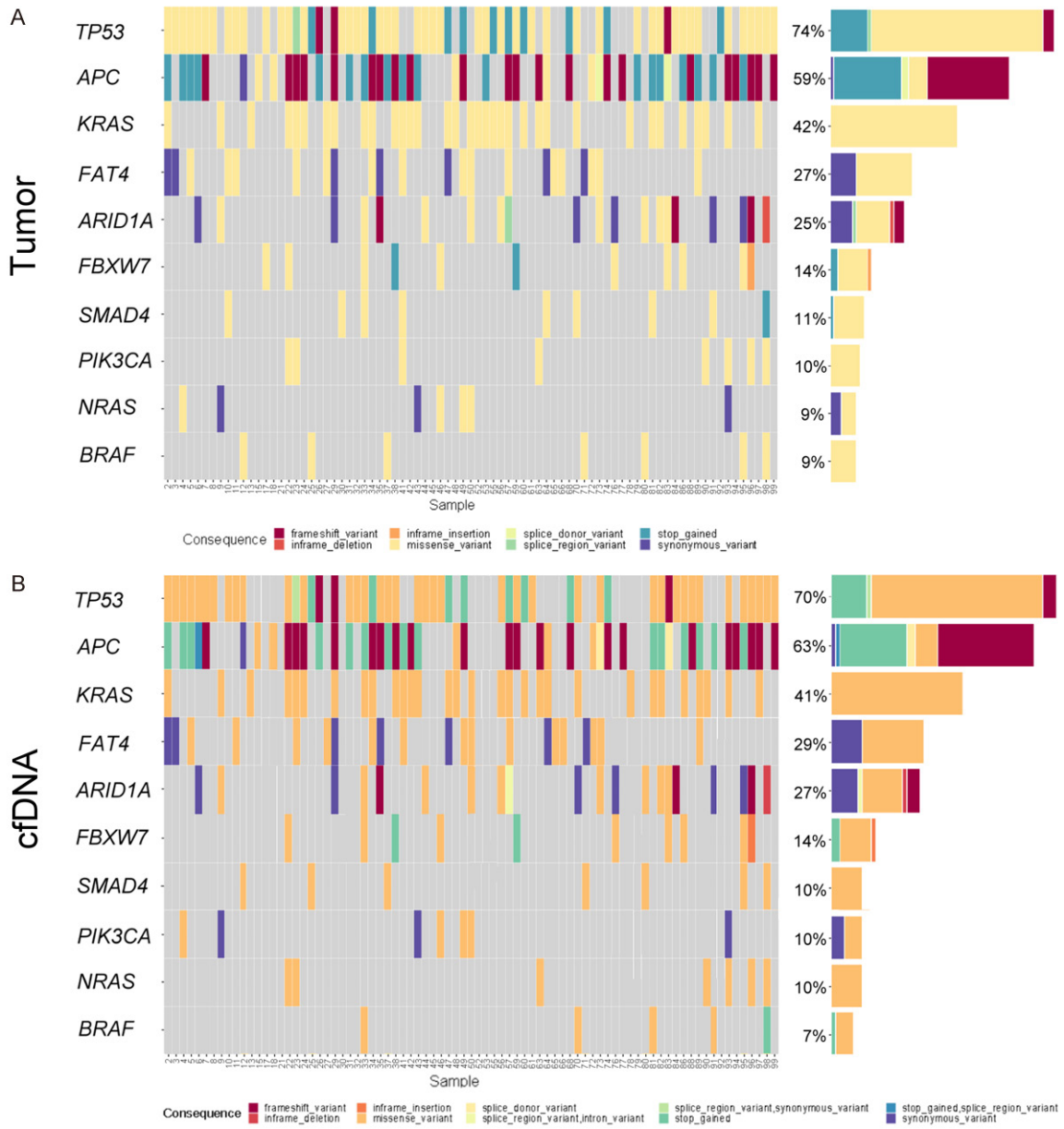

Figure 4. The mutational patterns were similar between the (A) tumor tissue and (B) cfDNA.

well with high coverage rate and high uniformity of the studied genes. In order to call reliable variants of cfDNA, we took the advantages of Illumina Basespace Dragen pipeline to identify variants with filter="PASS" based on the high sequencing depth of each base (average depth > 2000× and > 98% uniformity). Sequencing quality, depth and calling algorithm were key factors contributing the variant calling, and determine the percent of false-positive and false-negative variants. In this study, base quality (> Q30), mapping (MAPQ > 50) and variant qualities (GQ > 20) were all considered and filtered for the following comparisons. Second, most importantly, all of our patients are stage IV CRC, which may increase the detection rate of genetic mutations in cfDNA. As mentioned in our previous reports [10, 11], cfDNA levels were significantly higher in stage IV CRC patients and in patients with tumor recurrence. Consequently, the genetic analysis of cfDNA is more applicable in clinical use for stage IV tumors or for patients with tumor recurrence.

A worthy clinical application of cfDNA is to monitor cancer genomes in plasma for early detec-

Table 3. PPV, NPV, sensitivity and specificity of three major genetic mutations (*TP53*, *KRAS* and *APC*) between tumor DNA and cfDNA of CRC patients

	TP53		*KRAS*		*APC*	
	Tumor DNA mutation		Tumor DNA mutation		Tumor DNA mutation	
	Yes	No	Yes	No	Yes	No
cfDNA mutation						
Yes	51	0	30	0	46	0
No	9	21	4	47	2	33
Sensitivity	85.0%		88.2%		95.8%	
Specificity	100%		100%		100%	
PPV	100%		100%		100%	
NPV	70.0%		92.2%		94.3%	

cfDNA: cell-free DNA; NPV: negative predictive value; PPV: positive predictive value.

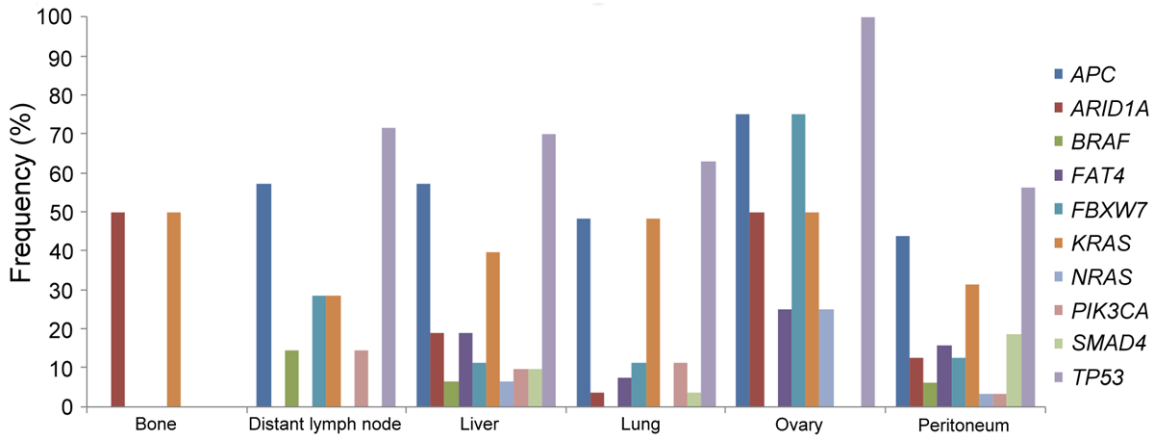

Figure 5. The frequency of genetic mutations in different metastatic organs.

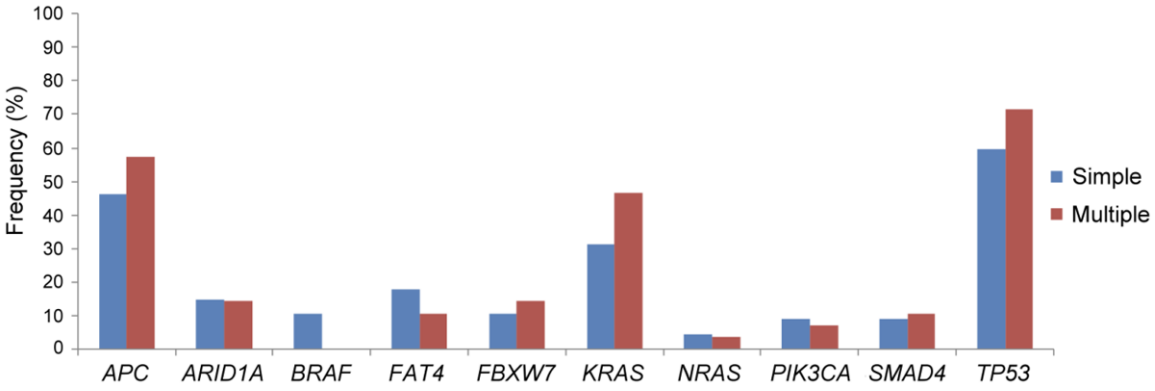

Figure 6. The frequency of genetic mutations in patients with single or multiple organ metastases.

tion of acquired resistance to target agents [16]. For patients with *KRAS* wild-type metastatic CRC, anti-EGFR target therapy in addition to systemic chemotherapy could provide better overall survival, progression-free survival and response rate compared with chemotherapy alone [17]. A previous study found that secondary resistant to anti-EGFR therapy in CRC is associated with emergence of *KRAS* mutation in tumors [18]. Monitoring *KRAS* status of

Table 4. The correlation of tumor location, tumor DNA mutations, and metastatic organ of CRC patients

Metastatic sites	*TP53* mutation (n=59)			*KRAS* mutation (n=35)			*APC* mutation (n=45)		
	Right-sided colon n=15	Left-sided colon n=44	*P* value	Right-sided colon n=7	Left-sided colon n=28	*P* value	Right-sided colon n=13	Left-sided colon n=32	*P* value
Hematogenous	11 (73.3)	36 (81.8)	0.479	6 (85.7)	24 (85.7)	1.000	9 (69.2)	24 (75.0)	0.692
Liver	11 (73.3)	30 (68.2)	0.708	5 (71.4)	20 (71.4)	1.000	9 (69.2)	21 (65.6)	0.816
Lung	1 (6.7)	16 (36.4)	0.028	2 (28.6)	10 (35.7)	0.722	1 (7.7)	10 (31.3)	0.096
Bone	0	2 (4.5)	0.401	0	1 (3.6)	0.612	0	2 (6.3)	0.356
Peritoneum	8 (53.3)	11 (25.0)	0.043	4 (57.1)	3 (10.7)	0.006	8 (61.5)	9 (28.1)	0.036
Distant LN	2 (13.3)	4 (9.1)	0.639	1 (14.3)	2 (7.1)	0.546	2 (15.4)	4 (12.5)	0.796
Ovary	1 (6.7)	2 (4.5)	0.747	0	2 (7.1)	0.466	1 (7.7)	2 (6.3)	0.860

Table 5. The correlation of tumor location, cfDNA mutations, and metastatic organs of CRC patients

Metastatic sites	*TP53* mutation (n=49)			*KRAS* mutation (n=30)			*APC* mutation (n=43)		
	Right-sided CRC n=13	Left-sided CRC n=36	*P* value	Right-sided CRC n=6	Left-sided CRC n=24	*P* value	Right-sided CRC n=12	Left-sided CRC n=31	*P* value
Hematogenous	10 (76.9)	30 (83.3)	0.609	5 (83.3)	20 (83.3)	1.000	8 (66.7)	23 (74.2)	0.622
Liver	10 (76.9)	26 (72.2)	0.742	5 (83.3)	16 (66.7)	0.426	8 (66.7)	20 (64.5)	0.894
Lung	1 (7.7)	13 (36.1)	0.052	1 (16.7)	7 (29.2)	0.536	1 (8.3)	9 (29.0)	0.150
Bone	0	2 (5.6)	0.386	0	1 (4.2)	0.611	0	2 (6.5)	0.368
Peritoneum	7 (53.8)	8 (22.2)	0.034	3 (50.0)	3 (12.5)	0.040	7 (58.3)	9 (29.0)	0.075
Distant LN	2 (15.4)	4 (11.1)	0.687	1 (16.7)	2 (8.3)	0.543	2 (16.7)	4 (12.9)	0.749
Ovary	1 (7.7)	2 (5.6)	0.783	0	2 (8.3)	0.464	1 (8.3)	2 (6.5)	0.828

tumor tissue through repeated endoscopic biopsy at colon or image guided biopsy at metastatic sites is not clinical practical. Studies showed that early detection of *KRAS* mutation in the blood during the treatment of anti-EGFR therapy is possible up to 10 months before conventional methods [18, 19]. In our study, we also found that a sensitivity of 88.2% and specificity of 100% was found when using cfDNA to predict *KRAS* mutation status in tumor tissue. Using this non-invasion method of liquid biopsy, continuous monitor of cancer genome by cfDNA is practical and could provide real-time response to the occurrence of secondary resistance to target agent.

Based on the number of mutations, CRC can be defined as hypermutated or non-hypermutated tumors [20]; however, the mutational patterns are distinct between them. Approximately 15% of the genetic alterations in hypermutated tumors were associated with microsatellite instability (MSI) and *POLE* mutations. Approximately 85% of the genetic mutations in non-hypermutated tumors include were associated with activation of *KRAS* and *PIK3CA* and mutation of *APC* and *TP53*. In the present study, *KRAS*, *APC*, and *TP53* mutations were associated with non-hypermutated tumors and were among the top commonly mutated genes in both tumor DNA and cfDNA. Similar to our findings, Strickler et al [21] demonstrated that the top three most common genetic mutations in cfDNA were *TP53*, *APC*, and *KRAS*, which were also observed in the tissue samples according to The Cancer Genome Atlas (TCGA) database. In addition, these three genes were more common in patients with metastases in multiple organs than in patients with metastasis in a single organ, which was an interesting finding of the present study. Further in vitro and in vivo studies and studies in which more patients are enrolled are required to validate our results.

Our results showed that for tumor DNA with *APC, KRAS, or TP53* mutations, right-sided CRCs were more likely to have peritoneal metastases than left-sided CRCs, which has not yet been reported. Meanwhile, left-sided CRCs with *TP53* mutations were more likely to have lung metastasis. For cfDNA with *TP53* mutations, right-sided CRCs were more likely to have peritoneal metastases. Our previous study showed that RAS pathway mutation was associated with lung and peritoneal metastasis in colon cancer, but not in rectal cancer [22].

The association of mutation patterns of tumor DNA and cfDNA with metastatic sties and location of primary tumor in CRC has seldom been mentioned before. Further evaluation with increased case number is necessary.

Mucinous adenocarcinoma was reported to be associated with more MSI-H and mutations in *BRAF*, *KRAS*, and *PIK3CA* than non-mucinous adenocarcinoma in CRC [23]. In the present study, our results (Table S1) demonstrated that mucinous adenocarcinoma had more genetic mutations in *TP53*, *APC*, and *BRAF* than non-mucinous adenocarcinoma but only 9 cases of mucinous adenocarcinoma were included in our study. The discrepancy between the present study and other studies [23] might be due to race, sample size, and the cause of CRC. More patients enrolled from different races and countries are required to validate our results.

There are limitations in the current study. First, this is a retrospective study in a single institute, and selection bias exists. Second, although some genetic mutations were significantly associated with tumor location and metastatic sites, the patient number was limited and bias could occur. Studies in which more patients and different races enrolled from different countries are required to validate the results in the present study.

Conclusions

Our results showed that the concordance of genetic mutation patterns is high between cfDNA and tumor tissue DNA in CRC patients. Genetic mutations are associated with tumor locations and metastatic sites in both tumor DNA and cfDNA. The mutation pattern of cfDNA may serve as a surrogate for monitory secondary resistance of tumor during target therapy in metastatic CRC in the future.

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Disclosure of conflict of interest

None.

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Table S1. The correlation of genetic mutations according to the histological types

Genetic mutation	Non-mucinous adenocarcinoma n=86 n (%)	Mucinous adenocarcinoma n=9 n (%)	*P* value
TP53	43 (50.0)	8 (88.9)	0.026
APC	38 (44.2)	8 (88.9)	0.011
KRAS	28 (32.6)	2 (22.2)	0.526
FAT4	19 (22.1)	2 (22.2)	0.993
ARID1A	17 (19.8)	3 (33.3)	0.342
FBXW7	10 (11.6)	0	0.279
SMAD4	4 (4.7)	1 (11.1)	0.409
PIK3CA	6 (7.0)	1 (11.1)	0.651
BRAF	4 (4.7)	3 (33.3)	0.002
NRAS	7 (8.1)	0	0.374