Targeted Sequencing of Circulating Tumor DNA to Monitor Genetic Variants and Therapeutic Response in Metastatic Colorectal Cancer

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Abstract

Substantial improvements have been made in the management of metastatic colorectal cancer (mCRC) in the last two decades, but disease monitoring remains underdeveloped. Circulating tumor DNA (ctDNA) is a promising prognostic and predictive biomarker; however, ctDNA as a marker for mCRC patients is not well established, and there is still no consensus about how to utilize it most cost-effectively. In this study, we aim to investigate plasma ctDNA levels as a biomarker for therapeutic response of mCRC patients. We performed next-generation sequencing (NGS) by using a 12-gene panel to identify genetic variants in 136 tumor tissue and ctDNA samples from 32 mCRC patients. Genetic variants were detected in approximately 70% of samples, and there was a high concordance (85%) between tumor tissue and plasma ctDNA. We observed ctDNA changes in 18 follow-up patients, including the emergence of new variants. Changes in ctDNA levels significantly correlated with tumor shrinkage (P = 0.041), and patients with a ctDNA decrease >80% after treatment had a longer progression-free survival compared with patients with a ctDNA decrease of <80% (HR, 0.22; P = 0.015). The objective response rate among patients with a ctDNA decrease of >80% was better than those with a ctDNA decrease <80% (OR, 0.026; P = 0.007). In conclusion, this study demonstrates that monitoring of genetic ctDNA variants can serve as a valuable biomarker for therapeutic efficacy in mCRC patients, and that using a moderate-sized 12-gene NGS panel may be suitable for such clinical monitoring.

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Introduction

About 1.4 million new colorectal cancer cases and 700,000 deaths are reported annually (1). Although improved screening and treatments have resulted in decreased case numbers in some geographic areas, such as the United States (1, 2), an increased mortality is observed in parts of Asia (3). Patients with distant metastasis continue to have low 5-year survival rates (4). To ensure an appropriate follow-up, it is important to monitor treatment outcome in an effective manner.

Carcinoembryonic antigen (CEA) is a widely used marker that may be used for follow-up; however, sensitivity and specificity remain issues limiting its clinical usefulness (5–7). CT imaging may yield false positive results, involves the use of radiation, and is not performed frequently (8). Recently, circulating tumor DNA (ctDNA) has been shown to be a promising marker that is particularly easily detectable in patients with metastatic cancers (9). Changes in ctDNA levels have been reported to determine therapeutic response earlier than CEA levels and CT imaging (10), and information may be gained regarding drug resistance (11).

ctDNA can be detected by PCR-based methods that are highly sensitive, such as droplet digital PCR (ddPCR). However, the number of detected mutations is limited for this method, and information may not always be able to detect mutations of interest (10, 12–14). Using broader next-generation sequencing (NGS) sequencing panels may overcome these limitations. Panels using 54 to 70 genes cover about 80% of cases (15, 16), but such an approach may be rather expensive for routine clinical monitoring. Unfortunately, the number of studies utilizing NGS gene panels of different size in colorectal cancer patients is limited (13, 17–20). Furthermore, the prognostic value of ctDNA in metastatic colorectal cancer (mCRC) is not yet widely established, although several studies indicate its usefulness (10, 21). Therefore, there is a need to further characterize ctDNA as a prognostic and predictive mCRC marker, and to determine how to utilize it most cost-effectively.

In our study, we characterized the performance of a moderate-sized 12-gene NGS panel by sequencing a total of 136 tumor tissue and plasma ctDNA samples from 32 mCRC patients. The panel was designed to cover a high patient proportion for different
types of cancers and is particularly suitable for colorectal cancer patients. We aimed to investigate the value of the gene panel to monitor plasma ctDNA levels and predict therapeutic efficacy in patients with mCRC.

Materials and Methods

Study patients and samples

A total of 32 patients with mCRC were enrolled at the Chang Gung Memorial Hospital, Taiwan. Each subject or the closest relative gave written informed consent for the study, which had been approved by the institutional review board (IRB) and ethics committee of our hospital (IRB 201600209B0). Genetic variants from a total of 136 samples were analyzed, including 32 formalin-fixed paraffin-embedded (FFPE) tumor tissues and 104 paired plasma ctDNA samples. One tumor tissue sample was analyzed per patient, and 29 of the 32 samples were obtained from primary tumors from the colon and 3 samples were obtained from metastatic sites. Plasma ctDNA samples included samples obtained before and after anticancer treatment. We used bevacizumab/FOLFIRI or cetuximab/FOLFIRI as initial treatment regimens. The analysis of an association of ctDNA levels with tumor change from baseline, response, and progression-free survival (PFS) was performed retrospectively for all evaluable follow-up patients, who were patients with available posttreatment samples who had detectable ctDNA variants in their pretreatment samples. Therapeutic response of mCRC was evaluated by CT scans using RECIST criteria 1.1.

DNA extraction and NGS

The tumor purity of FFPE samples was determined by H&E staining, and samples with a tumor purity of less than 30% were enriched by macroresoration. DNA from FFPE samples was isolated using the QiAamp DNA FFPE Tissue Kit (Qiagen). Blood samples were collected by Cell-Free DNA BCT tubes (Streck). Circulating cell-free DNA (cfDNA) was isolated from 2.5–4 mL plasma with the QiAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s protocol. The lower limit of plasma volume for inclusion in the study was 2 mL; however, no subject had an obtained plasma volume lower than 2.5 mL. The extracted DNA from both tissue and plasma samples was quantified with the Quanti-IT dsDNA HS Assay (Invitrogen) and qualified by investigating the integrity of genomic DNA using the Fragment Analyzer (Advanced Analytical Technologies, Inc.). The DNA was subsequently amplified by multiplex PCR with 31 primer pairs, covering frequently mutated regions in the genes AKT1, BRAF, CDKN2A, CTNNB1, EGRF, HRAS, KRAS, NRAS, IDH1, IDH2, PIK3CA, and TP53. PCR products were ligated to barcode adapters and underwent further amplification, followed by emulsion PCR on the OneTouch System (Applied Biosystems). For ultra-deep NGS, the Ion PGM sequencer and the Ion 318 chip (Life Technologies) were used. The coverage for plasma ctDNA and tumor samples were approximately 30,000× and 60,000×, and mean uniformities were 95% and 93%, respectively.

Variant analysis

The analyzed variants included single-nucleotide variants and small insertions and deletions. The human genome sequence hg19 was used as the reference genome, and alignment and base calling were performed with the Torrent Suite Server version 4.4. Annotated plasma variants had to have at least 20 reads and the allelic fraction had to be above a background threshold of 7 Z-

Scores from the mean of healthy donors. Annotated tumor variants had to have a variant frequency of at least 5% or to be known hotspot variants identified as true variants after raw data analysis. For annotation, COSMIC (v74), dbSNP 138, and 1000 Genomes (phase I) were used.

Correlation of ctDNA levels with clinical parameters

ctDNA changes were defined as the difference between the frequency of the ctDNA variant with the highest variant frequency at treatment initiation and the frequency of the ctDNA variant with the highest variant frequency at the next available follow-up time point. To be evaluable for quantitative ctDNA decrease analysis, patients with undetectable ctDNA variants at follow-up had to have a ratio of pretreatment variant frequency to variant detection threshold that allowed a detection of a ctDNA frequency decrease of at least 80%. For Pearson correlation between ctDNA and tumor changes from baseline, only patients with response evaluation after an available posttreatment sample were considered. For analyzing the correlation of ctDNA with CEA levels, the time difference between ctDNA and CEA samples had to be no longer than 15 days, and CEA levels before therapy had to be at least 5 ng/mL. PFS was calculated as the time difference between the cetuximab/FOLFIRI or bevacizumab/FOLFIRI treatment start date and the earlier of the first date of progression by CT imaging or the last CT imaging follow-up date.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (v. 6.0; GraphPad Inc.). CtDNA correlation analysis with CEA and tumor change from baseline was performed by Pearson correlation. Categorical outcomes were compared by Fisher exact test, and survival analysis was performed by the log-rank test. Differences were considered statistically significant at P values of less than 0.05.

Results

Patients and samples

A total of 32 patients with mCRC were included in the study. Among them, all 18 patients with detectable ctDNA mutations in their pretreatment samples and available posttreatment samples were further included in the "follow-up patients" subgroup. The demographic and clinical characteristics of the enrolled mCRC patients are listed in Table 1. The median age in our cohort was 58.5 years. The majority of patients were male, had undergone a resection of their primary tumor before obtaining the first plasma sample, and exhibited a synchronous metastasis pattern (each 66%, N = 21). One tumor sample was analyzed for each of the 32 patients. Most tumor samples used for sequencing were primary tumors isolated from the colon (91%, N = 29), while 9% (N = 3) were isolated from metastases. The time between the isolation of the tumor and plasma ctDNA sample for concordance analysis was 41 weeks on average. A similar distribution of clinical parameters was present in the follow-up subgroup. Among the 18 follow-up patients, each 50% (N = 9) were treated with cetuximab/FOLFIRI or bevacizumab/FOLFIRI.

NGS detects most ctDNA variants well above the detection threshold

A total of 136 samples (including tumor tissues and plasma ctDNA) from 32 patients with mCRC were sequenced and
analyzed. We used a 12-gene panel that was designed for ctDNA testing in different types of cancers and is particularly suitable for colorectal cancer patients. Hotspots included covered by the used 12-gene panel are listed in Supplementary Table S1. One tumor and at least one plasma sample were obtained for all 32 patients. Plasma ctDNA samples were first obtained before therapy with bevacizumab/FOLFIRI or cetuximab/FOLFIRI for all patients. In addition to those 32 pretreatment plasma samples, 72 posttreatment samples of 27 patients were analyzed, including 50 samples from 18 follow-up patients with detectable ctDNA in their pretreatment samples. Deep sequencing was performed for all plasma ctDNA samples, the average sequencing depth in this study being 31,807×. The ctDNA variant detection limit in our panel depends on the background signal in healthy volunteers and additionally requires at least 20 reads per sample (see Materials and Methods). The background threshold for healthy volunteers of variants detected in pretreatment samples varied between 0.00% and 0.89%. All variants detected in tumor and pretreatment plasma samples and their allelic frequencies are depicted in Fig. 1. Details for all variants detected in the study are listed in Supplementary Table S2. Of the 35 variants detected in pretreatment plasma samples, 83% (N = 29) were detected more than 1% above the detection limit, and three of the remaining six variants were detected at frequencies of approximately 0.5% above the detection limit. Only three variants were detected at frequencies very close to the detection limit (patient 4, BRAF V600E; patient 20, TP53 R273C; and patient 22, KRAS G12V), and all the patients had their primary tumors resected. Five patients harbored tumor-specific variants that were undetectable in plasma (patients 19–23), and 4 of these patients had their primary tumors resected. Three of these patients had tumor and plasma samples taken at least 48 weeks apart (Supplementary Table S2). For patients 19 and 20, who each harbored a tumor variant detected in plasma, the tumor variant undetected in plasma had a lower frequency in tumor tissue than the detected variant.

High concordance between tumor and plasma ctDNA samples

The majority of the 32 study patients (72%, N = 23) harbored at least one detectable variant, and most patients harbored TP53 and KRAS variants (50%, N = 16 and 41%, N = 13, respectively). Other genetic variants detected were PIK3CA, BRAF, AKTI, and CTNNB1 variants in 16% (N = 5), 13% (N = 4), 3% (N = 1), and 3% (N = 1) of patients, respectively (Fig. 1A). All 23 patients with detectable variants harbored tissue variants (Fig. 1B). Complete concordance of tumor tissue and plasma results was observed in 17 (53%) enrolled patients with detectable variants and in 9 (28%) patients without detectable variants. For the overall study cohort, there was complete concordance in 81% (53% + 28%; N = 26) of patients. Patients with nonconcordant results either had detectable tumor variants but no plasma variants, or harbored shared as well as unique tumor and plasma variants (Fig. 1A and B). The proportions of mCRC patients harboring no, one, two, or three variants in tumor and plasma ctDNA samples are shown in Fig. 1C and D. The proportion of patients harboring two or three variants was 47% (41% + 6%) for tumor samples, compared with 34% (25% + 9%) for plasma ctDNA samples (Fig. 1C and D). In total, 40 and 35 variants were detected in tumor tissues and plasma ctDNA samples, respectively (Fig. 1A and E). The sensitivity to detect tumor tissue variants in plasma ctDNA was 85% (34/40).

The aforementioned calculations are restricted to pretreatment tumor tissue and plasma ctDNA samples. However, four new variants emerged in samples obtained after treatment initiation. Those variants were PIK3CA 1427T (patient 11), KRAS G61R (patient 13), TP53 R273H (patient 28), and CDKN2A E61 (patient 30), the last two being detected in patients with previously undetectable variants in both tumor and pretreatment plasma ctDNA samples.

Plasma ctDNA levels predict therapeutic outcome for mCRC patients

We next analyzed possible relationships of changes in ctDNA levels with CEA levels and tumor shrinkage. Tumor shrinkage in follow-up patients was evaluated by CT imaging and RECIST criteria 1.1 analysis, with results displayed in Figs. 2A and 3A. The results showed that changes of ctDNA levels did significantly correlate with changes in tumor shrinkage (Fig. 2A, P = 0.041, r² = 0.551). However, there was no significant correlation between the decrease of ctDNA levels and CEA levels (Fig. 2B, P = 0.232, r = 0.416). For the prediction of treatment response in our cohort, a cutoff value of >80% for ctDNA decrease allowed the best stratification, with a sensitivity of 100% and a specificity of 71% (Fig. 2C; P = 0.048, AUC = 81.63%).

Tumor shrinkage according to ctDNA decrease status was further illustrated in a waterfall plot (Fig. 3A). The objective response rate among patients with ctDNA decrease >80% was 78% (7/9) versus 0% (0/6) for patients with a ctDNA decrease <80% [Fig. 3A; odds ratio (OR), 0.026; 95% confidence interval (CI), 0.001–0.637; P = 0.007, by Fisher exact test]. In contrast to a ctDNA decrease >80%, undetectable ctDNA in first posttreatment samples was not associated with therapeutic response (Fisher exact test, P = 0.637). We further evaluated the survival in our study cohort. Our result revealed that patients with a ctDNA decrease >80% had a significantly longer PFS, the median PFS...
 Detected variants in tumor and pretreatment plasma samples of 32 mCRC patients using a 12-gene sequencing panel. Variants in tumor and pretreatment plasma samples of all 32 study patients were evaluated. Genes with variants and the variant distribution among study patients are displayed (A), and concordance results between tumor and plasma samples are summarized in a pie chart (B). The proportions of study patients harboring no, one, two, or three variants in tumor tissue (C) and plasma ctDNA (D) samples are depicted in pie charts. The allelic frequencies for all variants are displayed for tumor and plasma samples (E).
being 23.8 weeks compared with 41.6 weeks in patients with a ctDNA decrease < 80% (Fig. 3B; HR, 0.22; 95% CI, 0.03–0.59; P = 0.015, by log-rank test).

Plasma ctDNA levels allow monitoring in mCRC patients

To further evaluate the clinical utility of the 12-gene panel for monitoring of mCRC, we performed a more detailed ctDNA follow-up analysis for patients with available posttreatment samples who also had detectable ctDNA variants in their pretreatment samples. Details about the time course of ctDNA plasma samples, ctDNA variant detection status, therapies and their outcomes are listed in Supplementary Table S3. Patients were treated with either bevacizumab/FOLFIRI or cetuximab/FOLFIRI, and therapeutic responses were evaluated by CT images. An example of CT images demonstrating the partial response observed for one of the patients (patient 5) is displayed in Fig. 4A. The displayed follow-up times start from the time of ctDNA pretreatment samples (week 0) and range from 13 to 44 weeks, the median follow-up time being 31 weeks (Figs. 4 and 5).

Four of 9 patients had partial responses (PR) at the first response assessment of bevacizumab/FOLFIRI (Fig. 4B), whereas 5 of 9 patients were found to respond to therapy for cetuximab/FOLFIRI, and therapeutic responses were evaluated by CT images. An example of CT images demonstrating the partial response observed for one of the patients (patient 5) is displayed in Fig. 4A. The displayed follow-up times start from the time of ctDNA pretreatment samples (week 0) and range from 13 to 44 weeks, the median follow-up time being 31 weeks (Figs. 4 and 5).

Four of 9 patients had partial responses (PR) at the first response assessment of bevacizumab/FOLFIRI (Fig. 4B), whereas 5 of 9 patients were found to respond to therapy for cetuximab/FOLFIRI.
Figure 4.
CtDNA courses in mCRC patients with PR, SD or progressive disease (PD) upon bevacizumab/FOLFIRI treatment. CtDNA courses for a total of 9 follow-up patients with detectable ctDNA levels before treatment with bevacizumab/FOLFIRI were analyzed. The partial response of one of the patients (patient 5) is shown (A). Patients were categorized according to their response status at the first posttreatment response assessment, responses being PR (B), SD (C), or progressive disease (D). Treatment responses were evaluated by CT images. The time axis is displayed in relation to the first plasma ctDNA sample analyzed (week 0). CtDNA variant frequencies and CEA levels are displayed on the right and left vertical axes, respectively. Variants of TP53 (blue line), KRAS (red or orange line), and other genes (brown line) are depicted.
Figure 5. CtDNA courses in mCRC patients with PR, SD or progressive disease (PD) upon cetuximab/FOLFIRI treatment. CtDNA courses for a total of 9 follow-up patients with detectable ctDNA levels before treatment with cetuximab/FOLFIRI were analyzed. Patients were categorized according to their response status at the first posttreatment response assessment, responses being PR (A), SD (B) or progressive disease (C). Treatment responses were evaluated by CT images. The time axis is displayed in relation to the first plasma ctDNA sample analyzed (week 0). CtDNA variant frequencies and CEA levels are displayed on the right and left vertical axis, respectively. Variants of TP53 (blue line), KRAS (red or orange line), and other genes (brown line) are depicted.
FOLFIRI treatment (Fig. 5A). For these patients, responses were preceded by sharp drops in ctDNA frequencies and/or a drop of variants below the detection limit. There were 4 and 3 patients with stable disease (SD) at initial response assessment in bevacizumab/FOLFIRI and cetuximab/FOLFIRI groups, respectively (Figs. 4C and 5B). In patients with SD, ctDNA levels either decreased by varying degrees or stabilized before first response evaluation. Finally, a total of 2 patients had progression of disease after bevacizumab/FOLFIRI and cetuximab/FOLFIRI treatment (Figs. 4D and 5C). In addition to those patients, there still were 6 patients with initial responses or SD who progressed at later time points (patients 1, 6, 7, 11, 12, and 22). A rise in ctDNA levels before progression could not be detected in all patients; however, the same was true for CEA levels.

Taken together, response status correlated with ctDNA changes in most patients, although it failed to identify some progressors.

Discussion

This study investigated the feasibility of using a 12-gene NGS panel to detect plasma ctDNA mutations and monitor disease burden in patients with mCRC. To characterize this panel, we compared pretreatment plasma ctDNA samples with tumor tissue samples of 32 mCRC patients. Genetic variants were detectable in 72% (N = 23) of patients, and at least one ctDNA variant could be detected in 66% (N = 21) of patients. Only one detected pretreatment plasma ctDNA variant was undetectable in tumor tissue. There was a high concordance between tumor and plasma ctDNA samples, and the sensitivity of ctDNA variant detection was 85% (34/40). Taken together, these results indicate the suitability of the panel for disease monitoring in a substantial proportion of mCRC patients. Although the lack of plasma-specific variants may be in part due to plasma samples being taken before further treatment-induced clonal evolution could occur (11), the results are remarkable considering the substantial genetic heterogeneity of tumors from different locations observed in many, but not all colorectal cancer studies (22–25).

Larger sequencing cohorts show the detection of tissue variants in genes covered by our panel in almost 30% of patients (26) and demonstrate a strong correlation between tissue and ctDNA prevalence for the most genes (15). Therefore, the variant detection rate of 72% of our study is slightly lower than expected. However, no remarkable differences in gene mutation frequencies were observed between our and other cohorts (15, 16, 26). APC, a gene not included in our assay, was among the two most frequently mutated genes in those studies. However, in most patients APC mutations co-occur with mutations in genes included in our panel (26), indicating potential benefit from our assay. Furthermore, truncating APC variants are distributed over a wide range of amino acids, making variants more difficult to detect by smaller gene panels.

The size of gene panels is an important consideration for routine clinical use, because ctDNA gene panels need to be cost-effective while covering a high proportion of mCRC patients. Previous colorectal cancer studies focusing on the 3-gene analysis of RAS/BRAF were only able to detect mutant ctDNA in 19% to 40% of patients (13, 14). CtDNA panels covering 54 to 70 genes detected ctDNA in about 80% of cases (15, 16); however, smaller gene panels might provide a more cost-effective solution. We chose a moderate gene panel size of 12 and were able to detect plasma ctDNA variants in 66% of our enrolled patients, obtaining a similar proportion as a 22-gene panel (19). We therefore suggest that the panel size of 12 genes may be clinically feasible.

In our study 28% (N = 9) of patients had no detectable variants in tumor or pretreatment plasma ctDNA samples. For such patients, a more comprehensive panel for ctDNA analysis or the individualized follow-up with mutations identified from tumor tissues could be considered.

Furthermore, genetic variants may be below the detection limit. In our cohort, reasons for the inability to detect tumor variants in pretreatment plasma samples might be tumor resection (19), a prolonged time difference between obtaining tumor and plasma samples (27), and variant subclonality, resulting in variants of lower frequency in tumor to be undetectable in plasma. However, the majority of ctDNA variants in this study was detected at frequencies well above the detection threshold.

Studies have demonstrated that plasma ctDNA monitoring is superior to CEA in detecting response and relapse timely and reliably (10, 12, 28, 29). Those studies used ddPCR (28), BEAMing (29), or NGS based on previously identified tumor mutations (10, 12). Unfortunately, monitoring studies using NGS, especially for mutations not restricted to those previously identified in tumor tissue, are scarce (18, 20). Our results demonstrate that using NGS-based ctDNA detection for monitoring mCRC patients’ treatment outcome is feasible. Similar to previous studies using ddPCR or sequencing of predefined mutations for ctDNA analysis, tumor shrinkage was correlated with ctDNA level decrease (10, 30). We noted that a ctDNA decrease of >80% predicted through CEA lesions were not associated with prolonged PFS. This suggests that plasma ctDNA is a useful biomarker for therapeutic efficacy, and using a 12-gene NGS panel may be suitable for clinical monitoring of mCRC patients.

The magnitude for ctDNA decrease necessary to predict response in our cohort is comparable to other previous studies (10, 31). Absolute ctDNA levels were reported to be inferior to predict response compared with relative changes (10), and the decrease of ctDNA levels below the detection limit was not associated with response in our cohort.

Rising ctDNA levels did not preclude progression of disease in all our patients. The reason is likely the growth of subclonal variants not detectable with our panel. Although CEA levels were more informative than ctDNA levels in those patients, these results indicate that plasma ctDNA monitoring should be used together with other methods of clinical follow-up.

Mutations of genes such as TP53, KRAS, NRAS, BRAF, and PIK3CA have implications for the outcome of targeted therapy and chemotherapy (18, 32–37), and the analysis of KRAS, NRAS, and BRAF mutations is recommended in the NCCN guidelines to guide cetuximab treatment. In our cohort, all 21 patients with detectable ctDNA variants in pretreatment samples harbored at least one variant in the therapeutically relevant genes TP53, KRAS, BRAF, and PIK3CA. When evaluating anti-EGFR eligibility, we excluded patients with tumor KRAS exon 2, 3, and 4 mutations, as detected by an independent clinical test. Therefore, a patient with a KRAS mutation in plasma only (A146V) and a patient with an exon 1 mutation (K5N) were still treated with anti-EGFR, and the additional variant Q61R emerged upon treatment of the second patient. Although all those mutations might lead to cetuximab resistance (18, 35, 38, 39), they remained at low frequency and the patients responded to therapy. Another patient harbored the...
References


Disclosure of Potential Conflicts of Interest

N. Lapke has ownership interest (including stock, patents, etc.) in ACT Genomics. No potential conflicts of interest were disclosed by the other authors.

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