Role of Circulating Tumor DNA in Evaluating Clinical Tumor Burden and Predicting Survival in Chinese Metastatic Colorectal Cancer Patients

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Abstract

Background: The aim of this study was to further explore the utility of ctDNA in the evaluation of clinical tumor burden and survival in Chinese metastatic CRC (mCRC) patients and to preliminarily summarize some metastatic characteristics associated with mutations in ctDNA.

Patients and Methods: A four-gene mutation testing panel covering a total 197 mutation hotspots of KRAS, NRAS, BRAF and PIK3CA was used to evaluate the gene mutation status via next-generation sequencing of plasma from patients with mCRC. Clinical markers including CEA, CA199, CA125, NSE and LDH in serum and the sum of all tumor diameters on CT or PET/CT were collected to indicate clinical tumor burden. The correlations between cfDNA levels and clinical tumor burden were analyzed using Pearson correlation and linear regression models. The median PFS and 1-year OS rates were calculated by Kaplan–Meier (K–M) survival analysis.

Results: The study enrolled 126 mCRC patients for ctDNA testing. Wild-type patients accounted for 54.8% (69/126) of the total patients, and mutant-type patients accounted for 45.2% (57/126) of the total patients. Mutations in KRAS, NRAS, BRAF and PIK3CA were detected in 37.3% (47/126), 1.6% (2/126), 3.2% (4/126) and 13.5% (17/126) of patients, respectively. The overall concordance rate of gene status between ctDNA and matched tissues was 78.6% (99/126). The concentration of cfDNA was significantly correlated with the levels of clinical markers, especially CEA (P<0.0001, Pearson r = 0.81), LDH (P<0.0001, Pearson r = 0.84) and the sum of tumor diameters (P<0.0001, Pearson r = 0.80). The median PFS (11.7 versus 6.6 months, P<0.0001) and 1-year OS rates (94% versus 56%, P<0.0001) were significantly different between patients with a low cfDNA concentration (≤17.91 ng/ml) and those with a high cfDNA concentration (>17.91 ng/ml). The most common metastatic site was liver (77.8%), followed by lymph nodes (62.7%), lung (40.5%), peritoneum (14.3%) and bone (10.3%), in all patients. There was no significant difference in metastasis regardless of gene status.

Conclusion: These results suggested that the level of cfDNA could be a quantitative biomarker of tumor burden and could predict survival in Chinese patients with mCRC. Patients with gene mutations had a similar metastatic pattern to those with wild-type genes.

1. Background:

Colorectal cancer (CRC) is the fourth leading cause of cancer-related death worldwide, and the incidence has increased in the past decade with an aging and growing population[1]. With the development of liquid biopsies, circulating tumor DNA (ctDNA) has been increasingly investigated as a promising biomarker for cancer management[2]. ctDNA testing may improve diagnostic efficiency, better evaluate prognosis and preemptively predict recurrence and treatment response in CRC[3]. Furthermore, monitoring genomic alternations in RAS, BRAF and other cancer-related genes by ctDNA analysis can guide targeted therapeutic strategies and especially optimize anti-epidermal growth factor receptor (EGFR) therapy for metastatic colorectal cancer (mCRC)[4, 5].
The change in tumor burden is an essential feature in the clinical evaluation of cancer therapeutics. However, there is no clear definition and uniform assessment method for overall tumor burden in patients with CRC. The image-based Response Evaluation Criteria in Solid Tumors (RECIST) guidelines are widely used to measure partial disease load and evaluate sequential treatment responses based on the assessment of anatomical tumor burden\cite{4,5}. In addition, circulating biomarkers and clinical symptoms are also significant for the effective surveillance of tumor burden in clinical practice. These serum markers include carcinoembryonic antigen (CEA), carbohydrate antigen 199 (CA199), carbohydrate antigen 125 (CA125), neuron-specific enolase (NSE), lactate dehydrogenase (LDH), etc. The potential of exploiting quantified levels and mutation load of ctDNA to monitor tumor burden in CRC patients has also been reported in a few studies\cite{7,8}.

The aims of this study were to further explore the utility of ctDNA in the evaluation of tumor burden and survival in Chinese patients with mCRC. We also summarized and preliminarily analyzed some metastatic characteristics according to mutation status in ctDNA.

2. Methods

2.1 Patients

Patients with mCRC treated in the Oncology Department, ZhongShan Hospital, Fudan University, Shanghai, China, from April to November 2018 were enrolled in this study. The inclusion criteria were adenocarcinoma in the colon or rectum, recurrent or primary metastatic disease, planned treatment with first-line chemotherapy, life expectancy more than 3 months, age >18 years, and Eastern Cooperative Oncology Group performance status 0-1. Plasma samples were collected at baseline. This study was approved by the ethics committee of Zhongshan Hospital. Written informed consent was obtained from all patients.

2.2 Sample collection, DNA extraction and sequencing of ctDNA

A total of 20 ml of venous blood was collected from each patient and then centrifuged at 1,900’g for 10 min at 4°C to separate the plasma from peripheral blood cells within 2 h of receipt. Plasma was then further centrifuged at 16,000’g for 10 min at 4°C to pellet any remaining cells. Circulating free DNA (cfDNA) was extracted from 8.0 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, 55114) according to the manufacturer’s instructions and quantified using the Qubit Fluorometer 3.0 (Life Technologies, Grand Island, NY) and 2100 bioanalyzer (Technologies, Palo Alto, CA). The quality control criteria for cfDNA were as follows: the concentration of the cfDNA was less than 0.9 ng/μl as measured by the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32854); the total amount of cfDNA was greater than 20 ng; and the fragment distribution feature detected by the 2100 bioanalyzer had a typical peak at 160-180 bp and a small peak at 320 - 360 bp. The extracted cfDNA was immediately subjected to the next step or cryopreservation. The cfDNA was not stored at -20±5°C for more than one week; for long-
term storage, the DNA was placed at -80°C for a period of not more than 6 months. Attempts were made to keep the number of repeated freeze-thaw cycles of cfDNA samples ≤ 3.

Plasma cfDNA (20 ng) was subjected to Firefly™ amplicon-based NGS technology using Accu-Kit™ CRC-01 (AccuraGen, Shanghai, China) [8]. The panel covered a total of 197 hotspots in exons 2, 3 and 4 of KRAS, exons 2, 3 and 4 of NRAS, exons 9 and 20 of PIK3CA, and exon 15 of BRAF (Supplemental Table 1). NGS libraries were generated using the KAPA Sequencing Library Construction Kit (Kapa Biosystems, Boston, MA, USA). The quality control criteria for the cfDNA libraries were as follows: the concentration of the library was less than 0.5 ng/μl as measured by the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32854); and the fragment distribution feature detected by the 2100 bioanalyzer had a typical peak at 600-1,300 bp, and the average length was 900-1,000 bp. Libraries were then sequenced using 2×250 pair-end reads on an Illumina MiSeq Dx sequencer (Illumina, San Diego, CA, USA). Unique sequencing reads were determined by using an AccuraGen proprietary algorithm including quality control, read collapse, read alignment, and variant calling. The average coverage depth for all probes in plasma was nearly 30,000X, and the sensitivity of variant detection in this study was 0.2%. For the details of this laboratory-developed test, refer to the reported articles [10]. Generally, sequence reads were aligned to the hg19/GRCh37 human reference sequence, and background noise introduced by random NGS error was removed by AccuraGen proprietary algorithms. The firefly algorithm used a 2-proportion Z-test [11] to reduce the false positive mutations from background noise, and mutations passing the Z-test were reported. The cfDNA yield was calculated from the extraction process as cfDNA yield = cfDNA concentration×cfDNA volume, and the cfDNA concentration was determined by the Qubit Fluorometer 3.0. The ctDNA yield was calculated as cfDNA yield×the maximum allele frequency of detected variants.

2.3 Identification of mutation status in tissue samples

The isolation and purification of genomic DNA from formalin-fixed, paraffin-embedded (FFPE) tissue sections was performed using an AmoyDx® FFPE DNA Kit (Xiamen, China). FFPE specimen tissue sections were first deparaffinized with the xylene/ethanol method and then incubated in buffer DTL and proteinase K solution to release DNA from the sections. A short incubation in DES buffer at a high temperature partially reversed formalin crosslinking of the released nucleic acids, improving DNA yield and quality as well as DNA performance in downstream assays. The lysate was mixed with DTB buffer and ethanol to provide appropriate binding conditions for DNA. Then, the mixture was applied to a DNA spin column, where the DNA bound to the membrane and impurities were removed with wash buffer. The DNA was eluted in DTE buffer. A human KRAS/NRAS/BRAF/PIK3CA gene mutation fluorescence polymerase chain reaction (PCR) diagnostic kit (Amoy Diagnostics, Xiamen, China) based on amplification refractory mutation system (ARMS) technology approved by the China Food and Drug Administration was used to analyze genomic DNA from tissue samples. The list of mutations detectable with this panel is shown in Supplemental Table 2. The assay was carried out according to the manufacturer’s protocol for the ABI7500 real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). We defined a cut-off of 1% mutation content as a sample quality check according to the minimum requirement of ARMS technology (approximately 1% analytical sensitivity).
2.4 Assessment of serum markers

Carcinoembryonic antigen (CEA) was measured using an enzyme-linked immunosorbent assay (CanAg CEA EIA). Carbohydrate antigen 199 (CA199), carbohydrate antigen 125 (CA125), and neuron-specific enolase (NSE) were measured by electrochemical fluoroimmunoassay (Roche Elecsys series). Lactate dehydrogenase (LDH) was detected by the stronger the correlation degree was. The optimal cutoff level of cfDNA was determined by receiver operating curve (ROC) analysis. Median progression-free survival (PFS) and 1-year overall survival (OS) were calculated by Kaplan-Meier (K-M) survival analysis, and the significance was evaluated by the log-rank test. Tumor metastatic sites with different gene statuses were compared using the chi square test. Statistical tests provided two-sided \( P \) values, and a significance level of \( P < 0.05 \) was used. Statistical analyses were carried out using SPSS 24.

3. Results

3.1 Patient characteristics

A total of 128 patients were enrolled in this study. Because the ctDNA or the library did not meet the quality control standards in 2 patients, blood samples from 126 patients were collected for ctDNA analysis of four genes (\( \text{KRAS}, \text{NRAS}, \text{BRAF} \) and \( \text{PIK3CA} \)) before treatment. All patients had gene results for matched tissues and complete clinical information. The flow chart is shown in Fig. 1. The clinical characteristics are summarized in Table 1. The median age was 58 years (26–84 years). There were 90 males and 36 females. The most common locations of primary lesions were sigmoid colon and rectum (38.9% and 35.7%, respectively). Ninety-one (72.2%) patients had received surgery before.

3.2 Gene mutation status in ctDNA and tissues

The results showed that 69 patients (54.8%) had wild type genes and 57 patients (45.2%) had gene mutations in plasma samples (Table 2). \( \text{KRAS} \) mutations were found in a total of 47 patients (37.3%), \( \text{NRAS} \) mutations were found in 2 patients (1.6%), \( \text{BRAF} \) mutations were found in 4 patients (3.2%), and \( \text{PIK3CA} \) mutations were found in 17 patients (13.5%). Thirteen patients (10.3%) harbored double mutations. The most common mutation sites were G12 (52.6%) and G13 (12.3%) in exon 2 of the \( \text{KRAS} \) gene. Some rare mutations, including the V14I mutation in exon 2 of \( \text{KRAS} \) and the K117N and the K117R mutations in exon 4 of \( \text{KRAS} \), were detected in this study (Table 3 and Fig. 3A). In matched tissue samples, 74 patients had no mutations (58.7%) and 52 patients had mutations in the analyzed genes (41.3%), including \( \text{KRAS} \) mutations in 42 patients (33.3%), \( \text{NRAS} \) mutations in 3 patients (2.4%), \( \text{BRAF} \) mutations in 3 patients (2.4%) and \( \text{PIK3CA} \) mutations in 5 patients (4.0%). The mutation status in tissues and ctDNA was consistent in 99 patients (78.6%). Of the 27 patients with inconsistency in mutation status, 11 patients had mutations in tissues but not plasma, and 16 patients had mutations in plasma but not in tissues.
3.3 Correlation between cfDNA and tumor burden

The median concentration of cfDNA was 10.4 ng/ml (2.2–33.5 ng/ml), and the median content of cfDNA was 105 ng (8.8–438.4 ng). The results showed that the concentration of cfDNA was significantly correlated with the levels of CEA, CA199, CA125, NSE, and LDH and the sum of tumor diameters, especially CEA ($P < 0.0001$, Pearson $r = 0.81$), LDH ($P < 0.0001$, Pearson $r = 0.84$) and the sum of tumor diameters ($P < 0.0001$, Pearson $r = 0.80$). The cfDNA content was strongly correlated with the levels of CEA ($P < 0.0001$, Pearson $r = 0.84$), LDH ($P < 0.0001$, Pearson $r = 0.86$) and the sum of tumor diameters ($P < 0.0001$, Pearson $r = 0.76$) (Table 4 and Fig. 2A). The ctDNA mutation abundance was moderately correlated with the levels of CA199 ($P < 0.0001$, Pearson $r = 0.66$), LDH ($P < 0.0001$, Pearson $r = 0.61$) and the sum of tumor diameters ($P < 0.0001$, Pearson $r = 0.64$) (Table 4). The optimal cutoff level of cfDNA concentration was determined as 17.91 ng/ml based on the 9-month PFS through ROC curve analysis (Fig. 2B). The patients with a low cfDNA concentration ($\leq 17.91$ ng/ml) had a longer PFS (11.7 months vs 6.6 months, $P < 0.0001$) and a higher 1-year overall survival rate (94% versus 56%, $P < 0.0001$) than those with high cfDNA concentrations ($\geq 17.91$ ng/ml) (Fig. 2C and D). Two typical mCRC patients in this study were included, and the changes in cfDNA levels were correlated with the effect of therapy (Fig. 2E).

3.4 Gene status in ctDNA and tumor metastasis

The most common metastatic site was liver (77.8%), followed by lymph nodes (62.7%), lung (40.5%), peritoneum (14.3%) and bone (10.3%), in all patients. Other rare metastatic sites included adrenal gland, spleen and some soft tissues. Whether the gene status was wild type or mutant, the sites of metastasis were similar (Supplemental Table 3). In the patients with gene mutations, the top three metastatic sites were also liver, lymph nodes and lung, regardless of the mutation site. Bone metastasis was found in patients with G12 mutations in exon 2 of KRAS, A146 and K117 mutations in exon 4 of KRAS, Q61 mutations in exon 3 of NRAS, E542 mutations in exon 9 of PIK3CA and V600 mutations in exon 15 of BRAF. Peritoneal metastasis was found in patients with KRAS or PIK3CA mutations (Fig. 3B).

4. Discussion:

In this study, the mutation status of four genes was detected by liquid biopsy technology in 126 patients with mCRC. The correlation between ctDNA and tumor burden was analyzed. In recent years, as an accurate, noninvasive, dynamic tool, ctDNA analysis has been gradually used to guide management and monitor treatment response in some cancers, such as lung cancer, breast cancer, and CRC[3]. It has been reported that the concordance rate of RAS/RAF mutations between plasma and tissue is approximately 70–90% in CRC[12–15]. In this study, the concordance rate was 78.6%. In a subgroup of 92 patients from the CAPRI-GOIM clinical trial, both NGS analysis of tumor tissue and plasma testing with BEAMing identified a concordance rate of 78.3%[16], similar to our results. In this study, the subgroup of 11 patients with mutations in tissue and no mutations in plasma ctDNA often had a lower tumor burden (N = 9). In
the other two patients with high tumor burden, the metastatic sites were predominantly lymph nodes, bone and peritoneum. In the subgroup of 16 patients with mutations in plasma ctDNA and no mutations in tissues, the discrepancy may have mainly resulted from different sampling times and the difficulty in detecting low-frequency mutations in tissues. Eleven of these patients had their primary lesion removed before blood sample collection. Therefore, liquid biopsies in these patients indicated a metastatic mutation status; interestingly, the mutation status of \textit{KRAS} has been reported to be different in primary and metastatic lesions, with an inconsistency rate of 5% or higher\cite{17}.

This study further analyzed the effect of ctDNA on tumor burden. At present, there is no uniform quantitative gold standard for evaluating tumor burden. Serological biomarkers and tumor diameters are mainly used as references, and RECIST guidelines are widely applied to evaluate the therapeutic effect in clinical trials. In this study, we found that cfDNA concentration and cfDNA content were positively correlated with the levels of CEA, CA199, NSE, LDH, and the sum of tumor diameters (including all tumors assessable). Therefore, cfDNA might be used as a quantitative tool for assessing tumor burden. In this study, we also found that the higher the cfDNA concentration, the shorter the PFS was, with an optimal threshold of 17.91 ng/ml. Reinert et al also found that ctDNA could be used to monitor the response to adjuvant therapy and incipient recurrence in patients following colorectal cancer surgery\cite{7}. Another potential application of ctDNA testing is to dynamically evaluate the therapeutic effect. As seen in Fig. 2E, we found that changes in cfDNA were correlated with the early effect of first-line bevacizumab plus XELOX (oxaliplatin and capecitabine) therapy in two patients in this study. An early decrease in cfDNA concentration was related to a good therapeutic effect. Thomsen et al reported that a low level of ctDNA after chemotherapy, prior to radiological imaging evaluation, was associated with a low risk of progression\cite{18}.

In this study, liver, lung and lymph nodes were the most common metastatic sites in mCRC regardless of the gene mutation status. We also observed some interesting metastatic patterns in clinical practice. For example, one patient with a high-frequency Q61K mutation of 28\% in exon 3 of \textit{NRAS} had mandible metastasis. One patient had metastasis in soft tissues, such as chest wall, anterior sacrum, and psoas major muscle, and he carried both the V14I and G12A mutation in exon 2 of \textit{KRAS}. One patient with a K117R mutation in exon 4 of \textit{KRAS} had only systemic lymph node metastases but no other organ metastasis. His cfDNA concentration was more than 50 ng/ml, but his PFS was more than 20 months. These patients with rare mutation sites may have a unique mechanism of metastasis and further influence survival. This phenomenon might be an interesting problem worthy of further research.

5. Conclusion

In conclusion, our results suggested that cfDNA could be a quantitative biomarker to evaluate tumor burden and predict the survival of mCRC patients. Liver, lung and lymph nodes were the most common metastatic sites in mCRC regardless of the gene mutation status. Patients with rare mutation sites may have a unique mechanism of metastasis.
Abbreviations

Circulating tumor DNA (ctDNA); circulating free DNA (cfDNA); metastatic colorectal cancer (mCRC); next-generation sequencing (NGS); amplification-refractory mutation system (ARMS); carcinoembryonic antigen (CEA); carbohydrate antigen 199 (CA199); carbohydrate antigen 125 (CA125); neuron-specific enolase (NSE); lactate dehydrogenase (LDH); positron emission tomography/computed tomography (PET/CT); progression-free survival (PFS); overall survival (OS); epidermal growth factor receptor (EGFR); response Evaluation Criteria in Solid Tumors (RECIST); formalin-fixed paraffin-embedded (FFPE); receiver operating curve (ROC).

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Zhongshan Hospital. Written informed consent was obtained from all patients.

Consent for publication

Not Applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflicts of interest.

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Authors’ contributions

XX, WG and TL conceived and designed the study. XX, YY enrolled the patients and MS performed the experiments. SW provided the results of serum markers. ML, LL, FH and CZ collected the clinical information. XX, YY and MS analyzed and wrote the paper. WG and TL reviewed and edited the manuscript. All authors read and approved the manuscript.

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References

1. Fitzmaurice C, Allen C, Barber RM, Barregard L, Bhutta ZA, Brenner Het al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and Disability-Adjusted life-years for 32 cancer groups, 1990 to 2015: A systematic analysis for the global burden of disease study. Jama Oncol. 2017; 3(4): 524-48.doi: 10.1001/jamaoncol.2016.5688.

2. Wan J, Massie C, Garcia-Corbach J, Mouliere F, Brenton JD, Caldas Cet al. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nat Rev Cancer. 2017; 17(4): 223-38.doi: 10.1038/nrc.2017.7.
3. Thierry AR, Mouliere F, El MS, Mollevi C, Lopez-Crapez E, Rolet F et al. Clinical validation of the detection of \textit{KRAS} and \textit{BRAF} mutations from circulating tumor DNA. Nat Med. 2014; 20(4): 430-5. doi: 10.1038/nm.3511.

4. Vidal J, Muinelo L, Dalmases A, Jones F, Edelstein D, Iglesias M et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Ann Oncol. 2017; 28(6): 1325-32. doi: 10.1093/annonc/mdx125.

5. Van Emburgh BO, Arena S, Siravegna G, Lazzari L, Crisafulli G, Corti G et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. Nat Commun. 2016; 7: 13665. doi: 10.1038/ncomms13665.

6. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer. 2009; 45(2): 228-47. doi: 10.1016/j.ejca.2008.10.026.

7. Reinert T, Scholer LV, Thomsen R, Tobiasen H, Vang S, Nordentoft I et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut. 2016; 65(4): 625-34. doi: 10.1136/gutjnl-2014-308859.

8. Yi X, Ma J, Guan Y, Chen R, Yang L, Xia X. The feasibility of using mutation detection in ctDNA to assess tumor dynamics. Int J Cancer. 2017; 140(12): 2642-7. doi: 10.1002/ijc.30620.

9. Wang B, Wu S, Huang F, Shen M, Jiang H, Yu Y, et al. \textit{Analytical and clinical validation of a novel amplicon-based NGS assay for the evaluation of circulating tumor DNA in metastatic colorectal cancer patients.} Clin. Chem. Lab. Med. 2019; 57(10) DOI 10.1515/cclm-2019-0142

10. Kastrisiou, M., et al., Clinical Application of Next-Generation Sequencing as A Liquid Biopsy Technique in Advanced Colorectal Cancer: A Trick or A Treat? Cancers, 2019. 11(10): p. 1573.

11. Newman, A.M., et al., Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotechnol, 2016. 34(5): p. 547-555.

12. Schmiegel W, Scott RJ, Dooley S, Lewis W, Meldrum CJ, Pockney P et al. Blood-based detection of RAS mutations to guide anti-EGFR therapy in colorectal cancer patients: Concordance of results from circulating tumor DNA and tissue-based RAS testing. Mol Oncol. 2017; 11(2): 208-19. doi: 10.1002/1878-0261.12023.

13. Grasselli J, Elez E, Caratu G, Matito J, Santos C, Macarulla T et al. Concordance of blood- and tumor-based detection of RAS mutations to guide anti-EGFR therapy in metastatic colorectal cancer. Ann Oncol. 2017; 28(6): 1294-301. doi: 10.1093/annonc/mdx112.

14. Bachet JB, Bouche O, Taieb J, Dubreuil O, Garcia ML, Meurisse A et al. RAS mutation analysis in circulating tumor DNA from patients with metastatic colorectal cancer: The AGEO RASANC prospective multicenter study. Ann Oncol. 2018; 29(5): 1211-9. doi: 10.1093/annonc/mdy061.

15. Grasselli J, Elez E, Caratu G, Matito J, Santos C, Macarulla T et al. Concordance of blood- and tumor-based detection of RAS mutations to guide anti-EGFR therapy in metastatic colorectal cancer. Ann Oncol. 2017; 28(6): 1294-301. doi: 10.1093/annonc/mdx112.
16. Normanno N, Esposito AR, Lambiase M, Forgione L, Cardone C, Iannaccone A et al. RAS testing of liquid biopsy correlates with the outcome of metastatic colorectal cancer patients treated with first-line FOLFIRI plus cetuximab in the CAPRI-GOIM trial. Ann Oncol. 2018; 29(1): 112-8. doi: 10.1093/annonc/mdx417.

17. Baldus SE, Schaefer KL, Engers R, Hartleb D, Stoecklein NH, Gabbert HE. Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. Clin Cancer Res. 2010; 16(3): 790-9. doi: 10.1158/1078-0432.CCR-09-2446.

18. Thomsen CB, Hansen TF, Andersen RF, Lindebjerg J, Jensen LH, Jakobsen A. Monitoring the effect of first line treatment in RAS/RAF mutated metastatic colorectal cancer by serial analysis of tumor specific DNA in plasma. J Exp Clin Cancer Res. 2018; 37(1): 55. doi: 10.1186/s13046-018-0723-5.

**Tables**

Due to technical limitations, tables are only available as a download in the supplemental files section.

**Figures**
Figure 1

Overview of patient enrollment
Figure 2

Correlation between cfDNA and tumor load (A) The concentration and content of cfDNA were significantly correlated with the levels of CEA, LDH and the sum of tumor diameters. (B) Through ROC curve analysis, the optimal cutoff level of cfDNA concentration was determined to be 17.91 ng/ml. (C)
The median progression-free survival (11.7 versus 6.6 months, P<0.0001) was significantly different between patients with a low cfDNA concentration (≤17.91 ng/ml) and those with a high cfDNA concentration (>17.91 ng/ml). (D) Patients with a low cfDNA concentration had a higher 1-year overall survival rates (94% versus 56%, P<0.0001) than those with a high cfDNA concentration. (E) An early decrease in cfDNA was related to a good therapeutic effect in two typical mCRC patient.

**Figure 3.**

Mutation types in ctDNA and tumor metastasis (A) Mutation sites detected in ctDNA. (B) Liver, lung and lymph nodes were the most common metastatic sites in mCRC regardless of the gene mutation site.

**Supplementary Files**
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