Dynamic Monitoring of Circulating Tumor DNA in Patients With Metastatic Colorectal Cancer

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ABSTRACT

PURPOSE Plasma circulating tumor DNA (ctDNA) is a valuable resource for tumor characterization and for monitoring of residual disease during treatment; however, it is not yet introduced in metastatic colorectal cancer (mCRC) routine clinical practice. In this retrospective exploratory study, we evaluated the role of ctDNA in patients with mCRC treated with chemotherapy plus bevacizumab.

MATERIALS AND METHODS Fifty-three patients were characterized for *RAS* and *BRAF* status on tumor tissue before the start of treatment. Plasma was collected at baseline, at first clinical evaluation, and at disease progression. ctDNA analysis was performed using Oncomine Colon cfDNA Assay on the Ion S5 XL instrument.

RESULTS At baseline, from a plasma sample, *RAS*, *BRAF*, or *PIK3CA* mutations were detected in 44 patients. A high correspondence was observed between ctDNA and tumor tissue mutations (*KRAS* 100%, *NRAS* 97.9%, *BRAF* 97.9%, *PIK3CA* 90%). Low baseline variant allele frequency (VAF) was found to be associated with longer median progression-free survival (PFS) compared with those with high VAF (15.9 *v* 12.2 months, $P = .02$). A higher PFS {12.29 months (95% CI, 9.03 to 17.9) *v* 8.15 months (95% CI, 2.76 to not available [NA]), $P = .04$} and overall survival (34.1 months [95% CI, 21.68 to NA] *v* 11.1 months [95% CI, 3.71 to NA], $P = .003$) were observed in patients with large decline in VAF at first evaluation.

CONCLUSION ctDNA analysis is useful for molecular characterization and tumor response monitoring in patients with mCRC. Quantitative variations of released ctDNA are associated with clinical outcomes.

ACCOMPANYING CONTENT

Appendix

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INTRODUCTION

Colorectal cancer (CRC) is the third most frequent cancer and the second leading cause of cancer mortality worldwide.[1] Metastatic CRC (mCRC) accounts for almost half of the newly diagnosed CRC cases and is associated with poor prognosis.[1]

Fluoropyrimidine-based chemotherapy regimens in combination with bevacizumab or monoclonal antibodies directed against the epidermal growth factor receptor (anti-EGFR mAB), such as cetuximab and panitumumab, are approved worldwide as first-line treatment. The determination of *RAS* (*KRAS/NRAS*) and *BRAF* mutational status is essential when metastatic disease is diagnosed, to optimize the treatment choice and patient management.[2] Moreover, immune checkpoint inhibitors are shown to be effective among patients with mCRC harboring high microsatellite instability and/or mismatch repair deficiency.[3] *RAS* mutations predict a lack of response to the anti-EGFR mAb. This is particularly true for left-sided tumors, whereas right-sided CRCs appear to derive less benefit from EGFR downregulation regardless of the presence of *RAS* mutations.[4,5] Also, for *BRAF*-mutated mCRC, the standard first-line treatment is chemotherapy plus bevacizumab,[6] whereas in the second line, an anti-BRAF kinase inhibitor (encorafenib) and cetuximab are recommended.[7] Tumor tissue is routinely used to detect such mutations, but high interpatient variability and high spatial heterogeneity, which characterize mCRC, could affect results.[8]

Liquid biopsy, with the circulating tumor DNA (ctDNA), represents a noninvasive approach to investigate these alterations and monitor their evolution during treatment. Among various malignancies, mCRC harbors the highest amount of ctDNA.[9] ctDNA detection requires highly sensitive and specific approaches. Targeted next-generation sequencing (NGS) represents a reliable technology for

CONTEXT

Key Objective

An unmet need exists to provide guidance toward accelerating the integration of circulating tumor DNA (ctDNA) into the routine care of patients with metastatic colorectal cancer (mCRC). The purpose of our exploratory study was to compare the detection of mutations in ctDNA and tumor tissue and to evaluate the role of liquid biopsy in predicting prognosis and monitoring patients with mCRC receiving first-line bevacizumab-based chemotherapy.

Knowledge Generated

A high correspondence was shown between ctDNA and tumor tissue mutations. ctDNA levels at baseline and at the first reassessment are associated with clinical outcomes, and the monitoring of ctDNA dynamics during treatment can provide important insights into the response to therapy and, potentially, anticipate radiologic progression.

Relevance

Mutational analysis performed on ctDNA is useful for the molecular characterization of colorectal cancer. The study provides additional data on the ctDNA use, which is still not routine clinical practice in the management of patients with mCRC.

characterizing tumors. The use of NGS for ctDNA analyses may reveal novel therapeutic targets for the application of personalized therapy and represents a promising tool for the management of patients with mCRC.[10]

Given the many proposed uses of ctDNA in patients with CRC,[11] an unmet need exists to provide guidance toward accelerating the integration of ctDNA into the routine care of patients with mCRC.

The purpose of our study was to investigate the detection of druggable gene mutations in ctDNA and tumor tissue, and to evaluate whether liquid biopsy can be used to predict prognosis and to monitor patients with mCRC treated with first-line bevacizumab based-chemotherapy.

MATERIALS AND METHODS

Patient's Selection and Treatment

One hundred eighty-two patients were enrolled in the IRSTB038 study between 2014 and 2018 by IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori," and for 53 of these patients, liquid biopsies collected at baseline and at predefined time points were available (Fig 1).

Main inclusion criteria for IRSTB038 trial were as follows: patients with histologically confirmed diagnosis of mCRC; older than 18 years; a performance status Eastern Cooperative Oncology Group score of 0-2 and a life expectancy of >3 months; at least one measurable lesion according to modified RECIST (mRECIST) 1.0; no previous treatment for metastatic disease (previous adjuvant fluorouracil with or without oxaliplatin treatment was allowed); patients candidate to one of the following chemotherapeutic regimens in association with bevacizumab: fluorouracil, leucovorin, and irinotecan (FOLFIRI), fluorouracil, leucovorin, and oxaliplatin (FOLFOX), and capecitabine and oxaliplatin (CAPOX); and adequate bone marrow, liver, and renal function.

All patients underwent evaluation of *RAS* and *BRAF* mutational status as routine molecular diagnostic characterization. Tumor evaluations were performed using computed tomography (CT) scan every 8-12 weeks, and tumor response was evaluated by mRECIST.

The Local Ethical Committees of Area Vasta Romagna (CEIIAV) approved this study under reference number IRSTB038, and all patients signed written informed consent for study participation and liquid biopsy and tumor tissue collection for translational research.

Plasma Collection and ctDNA Isolation

We collected longitudinal blood samples for each patient at three different time points: at baseline (before the first treatment cycle, T0), at the first clinical evaluation (8-12 weeks after the first dose of treatment, T1), and at disease progression (TPD). When available, additional intermediate time points were collected (Ti). The EDTA vacutainer tubes were centrifuged at room temperature, first for 10 minutes at 2800 $\times$ *g*, followed by centrifugation of plasma for 10 minutes at 3100 $\times$ *g*. Plasma samples were stored at −80°C until use. Plasma samples were available for ctDNA analysis at baseline for 53 patients, at the first clinical evaluation for 34 patients (64%), and at disease progression for 14 patients (26%).

Isolation of ctDNA started from 1 mL of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany), and ctDNA quantity was assessed using the Qubit dsDNA HS Assay Kit (Thermo Fisher, Waltham, MA). The quality of extracted ctDNA was checked on the Agilent 2100

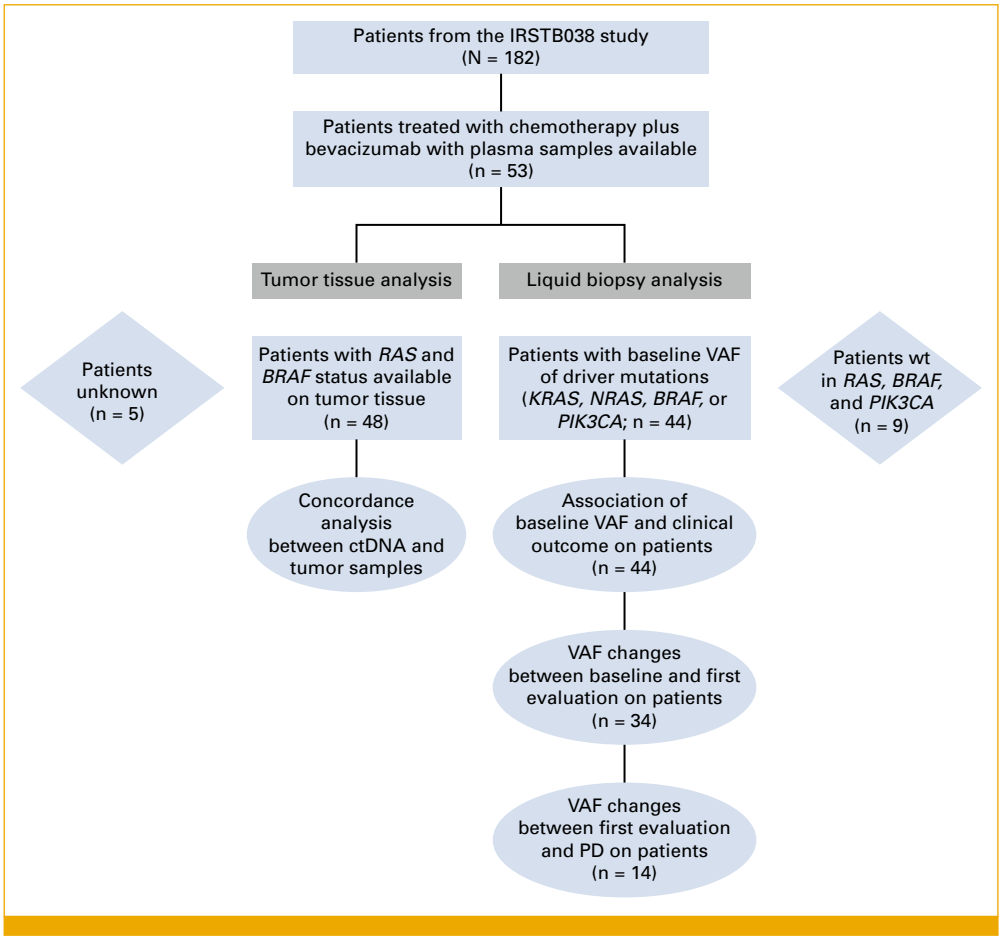

FIG 1. Patient flow diagram. ctDNA, circulating tumor DNA; PD, disease progression; VAF, variant allele frequency; wt, wild-type.

BioAnalyzer using the Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA) to exclude the presence of genomic contamination.

Analysis of ctDNA by Targeted NGS

For library preparation, 20-50 ng of ctDNA was used using the Oncomine Colon cfDNA Assay kit (Thermo Fisher), which enables the analysis of single-nucleotide variants and short indels that are frequently mutated in colon/gastrointestinal cancers. Fourteen genes were covered (*AKT1*, *BRAF*, *CTNNB1*, *EGFR*, *ERBB2*, *FBXW7*, *GNAS*, *KRAS*, *MAP2K1*, *NRAS*, *PIK3CA*, *MAD4*, *TP53*, *APC*). With tag sequencing technology, which incorporates unique molecular identifiers (UMIs) during library preparation, a limit of detection (LOD) down to 0.1% could be achieved. After quality checks, libraries were sequenced on Ion S5 XL (Thermo Fisher), reaching an average sequencing depth of 41,000× per sample with a mean molecular coverage of 3,500× (UMI deduplicated).

We defined variant allele frequency (VAF) as the number of mutant molecules over the total number of wild-type molecules, and for each patient, we considered the highest VAF of the putative driver mutations (*KRAS*, *NRAS*, *BRAF*, or *PIK3CA*) as the measure of released ctDNA of the plasma sample.

NGS Data Analysis

Torrent Suite software (Thermo Fisher) was used for sequencing analysis. Then, aligned bam files were processed using Ion Reporter cloud (Thermo Fisher) with standard Oncomine Colon Liquid Biopsy-w1.6 workflow for mutational calling (Ion Reporter version 5.14). It uses hg19 as reference genome and calls single-nucleotide variants or indels with a minimum frequency of 0.001 and a minimum alternative coverage of three. Then, for each mutation identified, it reports their specific LOD dependent by the level of molecular amplicon coverage of the locus obtained in the sample. Mutations with VAF greater than their specific LOD were retained.

Statistics

Data were summarized as median, minimum, and maximum values for continuous variables and as absolute frequencies

and percentages for categorical variables. The association between baseline VAF and clinical categorical variables was assessed by means of the chi-squared test or Fisher's exact test, when appropriate, and between baseline VAF and clinical continuous variables by means of the Wilcoxon rank-sum test. Concordance rate between the tumor sample and liquid biopsy was evaluated using only the baseline time point. When the same amino acid change was found in the same gene in both tumor and plasma of the same patient, the two samples were considered as concordant.

The primary end point was progression-free survival (PFS), defined as the time since diagnosis until disease progression or death by any cause. The secondary end point was overall survival (OS) defined as the time since diagnosis until death by any cause. The Kaplan-Meier method and the log-rank test were used to compare PFS and OS between groups of patients. The median PFS (mPFS) and OS values and corresponding 95% CIs are reported. A univariate recursive partitioning analysis was implemented to find cutoffs for VAF and changes in VAF that define class risk for PFS. The value that determines the first split from the root was selected as the cutoff. Landmark analysis was performed to evaluate the association between relative changes in VAF and PFS or OS.

All statistical analyses were performed using R studio software, version 4.0.4.

RESULTS

Patient Characteristics and Concordance Analysis Between ctDNA and Tumor Samples

Fifty-three patients receiving chemotherapy plus bevacizumab were available for our analysis. Thirty-four (64%) were males and 19 (36%) were females with the median age at diagnosis of 70 years (range, 38-85). The main demographic and clinical characteristics of the study population are summarized in Table 1. The median follow-up was 52.3 (95% CI, 50.3 to not available [NA]) months. The mPFS and median OS (mOS) were 12.9 months (95% CI, 11.5 to 15.4) and 25.3 months (95% CI, 17.7 to 38.6), respectively. All the deaths occurred were cancer-related deaths. Five patients (9%) were lost to follow-up.

We performed univariate analyses regarding PFS and OS data in relation to baseline patient characteristics (Appendix Table A1). In particular, we found that patients with peritoneal metastasis showed worse OS than patients without peritoneal metastasis (11.7 *v* 29.1 months; hazard ratio [HR], 2.13; 95% CI, 1.07 to 4.27; $P = .03$).

Mutations identified on baseline liquid biopsy showed a good concordance with molecular analysis performed on tumor tissue. In particular, considering the 48 patients with available *KRAS*, *NRAS*, and *BRAF* status on tumor tissue, we observed a concordance in 100% (48 of 48), 97.9% (47 of 48), and 97.9% (47 of 48), between liquid biopsy and tumor tissue, for each gene, respectively. Tissue *PIK3CA* mutation status was available for 30 cases, and the concordance with liquid biopsy was 90% (27/30). In addition to *RAS*, *BRAF*, and

TABLE 1. Patient Characteristics

Variable	Overall (n = 53)
Age, years	
Median (range)	70 (38-85)
Sex, No. (%)	
Female	19 (35.8)
Male	34 (64.2)
Grading, No. (%)	
1	2 (3.8)
2	21 (39.6)
3	16 (30.2)
4	4 (7.5)
Unknown	10 (18.9)
Tumor localization, No. (%)	
Rectum	9 (17)
Right colon	19 (35.8)
Left colon	24 (45.3)
Others	1 (1.9)
Metastasis, No. (%)	
Liver	34 (64.2)
Lung	24 (45.3)
Peritoneum	15 (28.3)
Bone	4 (7.5)
Lymph node	24 (45.3)
Chemotherapy regimen, No. (%)	
FOLFOX	39 (73.6)
FOLFIRI	5 (9.4)
CAPOX	7 (13.2)
Capecitabine	2 (3.8)
Previous surgery, No. (%)	
Yes	37 (69.8)
Previous adjuvant chemotherapy, No. (%)	
Yes	14 (26.4)
KRAS, No. (%)	
Wt	19 (35.8)
Mutated	29 (54.7)
Unknown	5 (9.4)
NRAS, No. (%)	
Wt	44 (83.0)
Mutated	4 (7.5)
Unknown	5 (9.4)
BRAF, No. (%)	
Wt	40 (75.5)
Mutated	8 (15.1)
Unknown	5 (9.4)

Abbreviations: CAPOX, capecitabine and oxaliplatin; FOLFIRI, fluorouracil, leucovorin, and irinotecan; FOLFOX, fluorouracil, leucovorin, and oxaliplatin; Wt, wild-type.

PIK3CA mutations, other gene alterations were reported by the NGS panel on ctDNA. Specifically, one or more inactivating mutations per patient were detected in *TP53* and *APC* genes in 38.9% and 31.5% of cases, respectively. Moreover, few sporadic mutations were found to be affecting *FBXWT* (3.7%) and *SMAD4* (1.9%; Appendix Table A2).

Association of Baseline VAF ctDNA and Clinical Outcome

We analyzed the correlation between the quantitative variations of the different alterations at baseline. For each plasma sample, we used the VAF of putative driver mutations (*KRAS*, *NRAS*, *BRAF*, or *PIK3CA*). In total, 44 patients of 53 carried at least one detectable mutation on these genes in baseline plasma samples (Fig 1).

Baseline VAF level was tested for association with patients' clinical features (site of metastases, sex, chemotherapy regimen, previous adjuvant treatment); however, no significant associations were found (Appendix Table A3).

Recursive partitioning analysis identified a VAF of 0.29% as the optimal cutoff within the chemotherapy plus bevacizumab-treated population, and this stratification (ie, VAF < 0.29%—low VAF *v* VAF ≥ 0.29%—high VAF) was used for subsequent analysis regarding baseline VAF.

Baseline VAF was found to be significantly associated with longer PFS. In particular, the mPFS was 15.9 months (95% CI, 10.84 to NA) and 12.2 months (95% CI, 8.87 to 16.1) for patients with low (7 patients) and high (37 patients) baseline VAF, respectively (log-rank $P = .02$; HR low *v* high, 0.31; 95% CI, 0.10 to 0.89; Fig 2A).

No significant associations were found between baseline VAF and OS (Fig 2B). The mOS was 24.6 months (95% CI, 16.9 to 37.9) for patients with high baseline VAF and not reached for patients with low baseline VAF (log-rank $P = .09$; HR low *v* high, 0.37; 95% CI, 0.11 to 1.23).

VAF Changes and Their Association With Clinical Outcome

At first evaluation, VAFs of driver mutations were available for 36 of 44 patients and were compared with baseline values (Fig 1). The entity of the variation was calculated as the delta VAF between baseline and first evaluation normalized against the baseline VAF. Recursive partitioning analysis identified a relative change in VAF of 98% as the optimal cutoff within the chemotherapy plus bevacizumab-treated population, and this stratification (ie, deltaVAF ≤ 98%—low decreasing VAF *v* delta VAF > 98%—high decreasing VAF) was used for subsequent analysis regarding the delta VAF. No association emerged between baseline patient characteristics and VAF dynamics (Appendix Table A4). At first evaluation, the entity of VAF reduction with respect to baseline was found to be associated with both PFS and OS. The mPFS was 12.29 months (95% CI, 9.03 to 17.9) and 8.15 months (95% CI, 2.76 to NA) for patients with high (27 patients) and low (7 patients) decreasing VAF, respectively (log-rank $P = .04$; HR low *v* high, 2.45; 95% CI, 10.1 to 5.93; Fig 3A). The mOS was 34.1 months (95% CI, 21.68 to NA) and 11.1 months (95% CI, 3.71 to NA) for patients with high and low decreasing VAF, respectively (log-rank $P = .001$; HR low *v* high, 3.9; 95% CI, 1.46 to 10.43; Fig 3B).

Since 50% of patients (7 of 14) had a cleared VAF at first evaluation (Fig 1), we evaluated the absolute change in VAF between first evaluation and progressive disease (PD) rather than the relative change that otherwise would have been undefined in

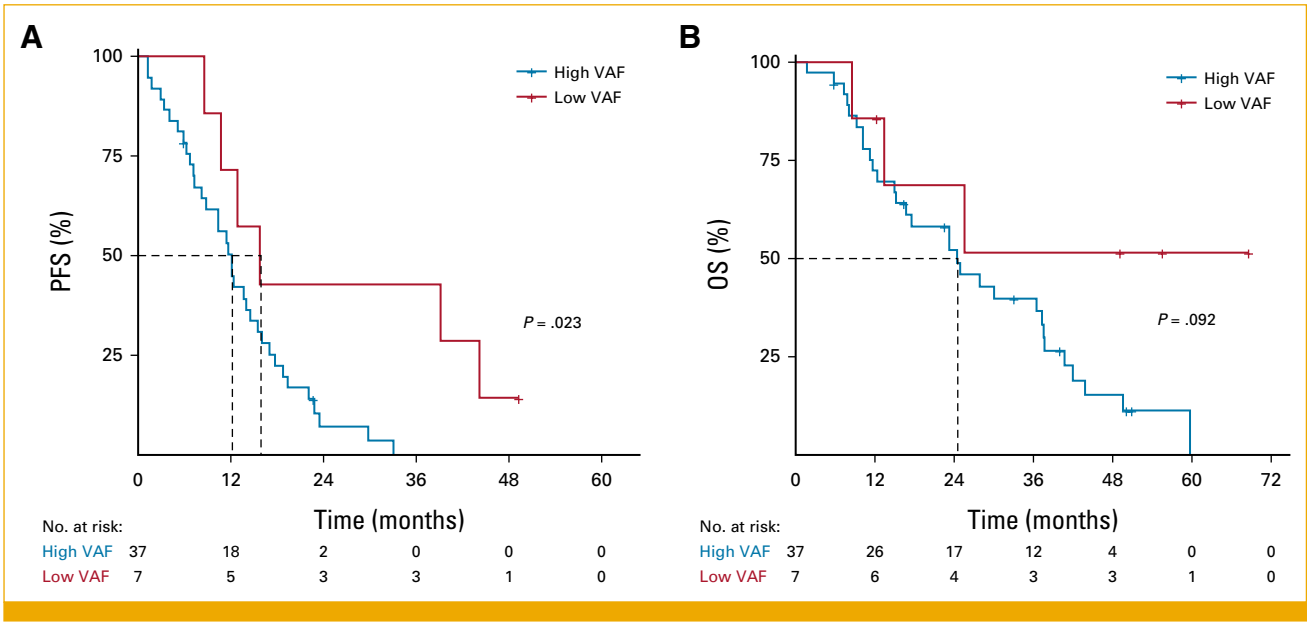

FIG 2. Baseline VAF in relation to (A) PFS and (B) OS. OS, overall survival; PFS, progression-free survival; VAF, variant allele frequency.

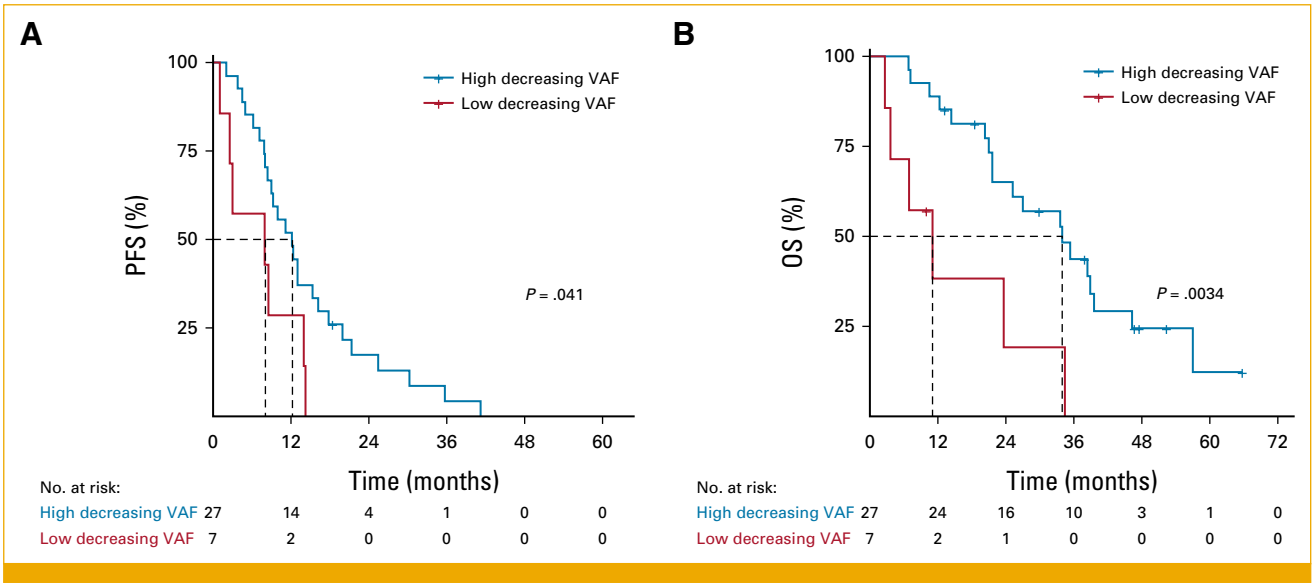

FIG 3. VAF changes between baseline and first evaluation in relation to (A) PFS and (B) OS. OS, overall survival; PFS, progression-free survival; VAF, variant allele frequency.

50% of the patients. No significant association was found between VAF change from first evaluation to PD and OS (data not shown).

Monitoring ctDNA Release During Treatment

For eight patients, in addition to T0, T1, and TPD, ctDNA released in other intermediate time points (Ti) was analyzed. Interestingly, in seven of eight cases, an increase in ctDNA levels was seen in the intermediate time points before the radiologic PD onset. Among cases carrying more than one mutation, the increase in ctDNA was detected in all mutations (driver and *TP53*/*APC*) before the PD onset in four of six patients (Appendix Table A2).

We selected four representative cases that displayed contrast-enhanced computed tomography (CECT) scan images at different time points with parallel detection of ctDNA mutations (Fig 4). The tumor size was determined by the sum of longest diameters (SLDs) of all target lesions by using the mRECIST. In most patients, although levels of ctDNA were already high at baseline, we observed a dramatic decrease in ctDNA at the first CECT re-evaluation, along with a decrease in the SLD tumor size. Then, when the tumor progressed, there was an increase in ctDNA, along with a partial increase in the SLD tumor size. For these patients, we can observe an increase in ctDNA levels before the radiologic PD onset (Fig 4).

DISCUSSION

In this study, we investigated the role of liquid biopsy in tumor characterization and in monitoring patients with mCRC treated with first-line bevacizumab-based chemotherapy.

Our findings showed that low baseline VAF of druggable gene mutations was associated with longer PFS; moreover, the entity of the decrease in VAF at first evaluation with respect to baseline was found to be associated with both PFS and OS.

Consistent with our results, many authors observed high concordance rate between ctDNA and tumor tissue and most of these studies are focused on the analysis of *RAS* genes as their alterations are useful for predicting the response to anti-EGFR treatments.[12-14]

In our study, the mPFS of all patients was 12.9 months, whereas the mOS was 25.3 months. These results are in line with those of the main clinical studies that involved the use of bevacizumab in combination with first-line chemotherapy.[15-17]

Our results indicated that baseline VAF correlates significantly with PFS. A number of studies have described the potential impact of ctDNA on the prognostic outcome of patients with mCRC in terms of both PFS and OS. Similar to our results, in Vidal's study, patients with high baseline ctDNA levels are associated with a worse prognosis in terms of PFS and OS.[12] On the other hand, others demonstrated that patients with higher levels of ctDNA and higher mutational burden for mutations found in *KRAS* and *BRAF* genes had a significantly shorter OS (18 *v* 28.5 months).[18] In Spindler's study, the data showed worse OS with increasing baseline ctDNA levels (mOS, 10.2 *v* 5.2 months).[19]

Moreover, we evaluated the correlation between the quantitative variations of the different alterations during treatment and the response to therapy. The deltaVAF also significantly correlated with PFS and OS.

In agreement with our study, Lim's study demonstrated that the change in median VAF between baseline and first evaluation was shown to be correlated with PFS. Patients who have a decrease in VAF at the first evaluation showed a PFS

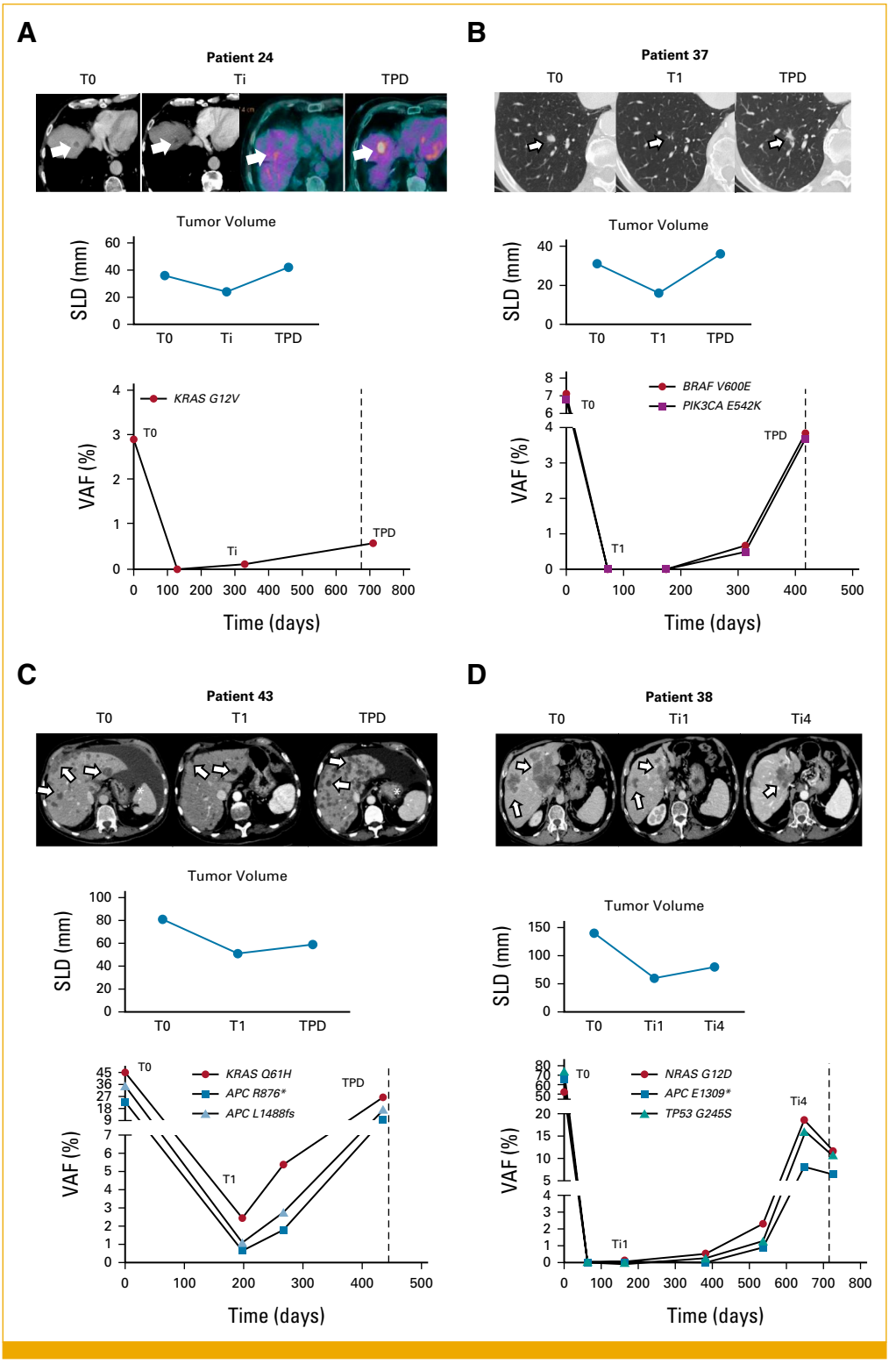

FIG 4. Representative CECT scan images of four selected patients at different time points with parallel detection of circulating tumor DNA mutations. Lesions are pointed out (arrows) in the upper panel, whereas the volume of all lesions is represented as the SLD. The dashed lines in the VAF plot represent the radiologic PD. The asterisk in (C) indicates the presence of ascites. For patient 24, CECT shows hypodense liver metastases at the baseline scan (T0); during following CECT scans (Ti), (A) liver metastases showed a significant size reduction confirmed also by PET-CT scan. At TPD, the PET-CT scan showed liver metastases with increased uptake of radiotracer because of disease progression. For patient 37, baseline (T0) CECT scans showed lung metastases, which demonstrated a significant reduction in size in the subsequent (T1) CECT scans. (B) After 1 year of treatment (TPD), an increase in the lesions (continued on following page)

FIG 4. (Continued). can be observed. (C) For patient 43, baseline CECT scan (T0) showed multiple hypodense liver metastases and severe ascites (asterisk). At the next CECT scan (T1), a resolution of the ascites and a substantial reduction of liver metastases can be observed. (C) At TPD, after 1 year and half from the beginning of chemotherapy, CECT scan demonstrated a PD with the abrupt increase in liver metastases and the recurrence of ascites. (D) For patient 38, up to seven plasma samples were collected and analyzed during a follow-up of almost 2 years. Baseline CECT scan (T0) showed several confluent hypodense liver metastases. At the subsequent CECT scan, liver metastases showed a significant reduction in size and number (Ti1), which were stable in the next three CECT scans. (D) After 1 year and half from the beginning of chemotherapy, CECT scan (Ti4) demonstrated a disease progression with the abrupt increase in liver metastases. CECT, contrast-enhanced computed tomography; CT, computed tomography; PD, progressive disease; PET, positron emission tomography; SLDs, sum of longest diameters; T0, baseline; T1, time point at the first clinical evaluation; Ti1, intermediate time point 1; Ti4, intermediate time point 4; TPD, time point at disease progression; VAF, variant allele frequency.

significantly better than patients with higher VAF (mPFS, 11.9 months *v* 5.5 months).[20]

By evaluating the dynamics of the VAF, we noticed that VAF decreases strongly at the first evaluation, reflecting the response to treatment, whereas VAF increases at PD together with tumor activity.

This is in line with Vidal's study, where *RAS* was longitudinally monitored in 21 patients with baseline *RAS* mutations.[12] In another study involving 53 patients, the authors conclude that early changes in ctDNA levels during first-line chemotherapy treatment can predict the subsequent radiologic response.[21] In agreement with the previous study, we observed, in some patients, how the increase in the ctDNA can be detected before the onset of radiologic PD.

The proof-of-concept study by Parikh et al[22] also revealed that serial ctDNA monitoring may be an early and accurate predictor of treatment response and therapeutic benefit to systemic therapy in gastrointestinal malignancies, which is consistent with our findings. Several clinical trials on the basis of ctDNA testing are ongoing in the adjuvant setting to understand how ctDNA may be used to inform clinical decision (CIRCULATE ClinicalTrials.gov identifier: NCT05174169; COBRA ClinicalTrials.gov identifier: NCT04068103, Dynamic III ACTRN12617015663325). In metastatic context, ctDNA testing can be used to guide clinical decision making, detect intratumoral and temporal heterogeneities, and identify acquired resistance mechanisms. An ongoing phase II trial studies ctDNA testing to guide treatment with regorafenib or TAS-102 in patients with CRC (ClinicalTrials.gov identifier: NCT03844620).

The sensitivity of the various ctDNA detection methods is different, and the perfect methodology and modality for quantifying the ctDNA is still unknown. Moreover, the choice of the mutation to be monitored, as an indicator of ctDNA release, can be different in various studies.[12,19,20]

Using NGS, we analyzed several genes, however, to have more reproducible results, and we opted to use only the mutations on oncogenes as an estimate of the amount of ctDNA and not to consider the mutations on tumor suppressors (eg, *TP53*, *APC*) whose presence and ploidy have not been evaluated on the tumor tissue. From our research, it is clear that liquid biopsy is increasingly improving and implementing our knowledge on CRC research. The results from our study show how the mutational analysis performed on liquid biopsy is useful for the characterization of CRC, confirming an almost absolute concordance rate with the analysis performed on tumor tissue.

Moreover, our data show that ctDNA levels at baseline and at the first reassessment are associated with clinical outcomes and the monitoring of ctDNA dynamics during treatment can provide important insights into the response to therapy and, potentially, anticipate radiologic progression.

Our study has, however, a main limitation, and it is a retrospective study with a small sample size. In addition, serial blood sample collection was available only in a limited number of patients. The consequence is a flawed statistical analysis not always able to detect associations. The cutoffs are just explorative, calculated with the purpose of analyzing whether VAF values can divide patients into groups with divergent outcomes. All results should therefore be considered as hypothesis-generating and not conclusion making.

This study does not change the clinical practice, but provides additional data on the use of liquid biopsy, which is still not routine clinical practice in the management of patients with CRC.

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EQUAL CONTRIBUTION

P.U. and A.P. contributed equally to this work.

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DATA SHARING STATEMENT

Data supporting the findings are reported in the Supplementary material. Data not included in the Supporting Information are available from the corresponding authors (giorgia.marisi@irst.emr.it) upon request with explanation for use.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

TABLE A1. OS in Relation to Baseline Patient Characteristics

Characteristic	Median OS (95% CI)	Log-Rank Test	HR (95% CI)	*P*
Liver metastasis				
Yes	25.9 (14.3 to 42.1)		1.10 (0.55 to 2.20)	.78
No	25.3 (22.6 to NA)	0.80	1.00	
Lung metastasis				
Yes	16.2 (13.9 to 41.4)		1.58 (0.83 to 3.02)	.16
No	31.5 (22.6 to 52.1)	0.20	1.00	
Peritoneum metastasis				
Yes	11.7 (8.54 to NA)		2.13 (1.07 to 4.27)	**.03**
No	29.1 (22.64 to 47.7)	0.03	1.00	
Bone metastasis				
Yes	19.3 (9.36 to NA)		2.82 (0.95 to 8.41)	.06
No	31.5 (22.64 to 44.4)	0.05	1.00	
Lymph node metastasis				
Yes	25.9 (17.8 to 52.1)		1.05 (0.54 to 2.06)	.87
No	31.5 (16.2 to NA)	0.90	1.00	
Sex				
Female	23.4 (11.9 to NA)		1.00	
Male	29.1 (22.6 to 44.4)	0.50	0.81 (0.42 to 1.57)	.54
Adjuvant chemotherapy				
Yes	29.1 (22.6 to NA)		0.83 (0.41 to 1.67)	.59
No	24.6 (15.2 to 42.1)	0.60	1.00	
Chemotherapy regimen				
FOLFOX/CAPOX	23.4 (15.2 to 38.6)		1.00	
FOLFIRI/CAPIRI	39.0 (25.3 to NA)	0.10	0.36 (0.09 to 1.52)	.17

NOTE. *P* value in bold indicate statistical significance.
Abbreviations: CAPIRI, capecitabine and irinotecan; CAPOX, capecitabine and oxaliplatin; FOLFIRI, fluorouracil, leucovorin, and irinotecan; FOLFOX, fluorouracil, leucovorin, and oxaliplatin; HR, hazard ratio; NA, not available; OS, overall survival.

TABLE A2. Genetic Variant and VAF

Patient Code	Time Point	*KRAS* (VAF%)	*NRAS* (VAF%)	*BRAF* (VAF%)	*PIK3CA* (VAF%)	*APC* (VAF%)	*TP53* (VAF%)	Other Gene Mutations (VAF%)
24[a]	T0	G12V (2.89)	wt	wt	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
	Ti	G12V (0.12)				wt	wt	
	TPD	G12V (0.59)	wt	wt	wt	wt	wt	
25	T0	wt	wt	wt	wt	wt	wt	
	TPD	wt	wt	wt	wt	wt	wt	
26	T0	G13D (12.69)	wt	wt	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
27	T0	G12D (6.48)	wt	wt	wt	p.R1114[a] (2.57); p.E1309DfsTer4 (2.34)	P190L (4.42)	
	T1	G12D (0.1)	wt	wt	wt	p.R1114[a] (0.00); p.E1309DfsTer4 (0.32)	P190L (0.37)	
	Ti	G12D (1.67)	wt	wt	wt	p.R1114[a] (0.97); p.E1309DfsTer4 (1.33)	P190L (2.1)	
	TPD	G12D (4.72)	wt	wt	wt	p.R1114[a] (1.40); p.E1309DfsTer4 (2.14)	P190L (5.24)	
28	T0	G12C (31.14)	wt	wt	wt	wt	wt	
	T1	G12C (0.28)	wt	wt	wt	wt	wt	
	TPD	G12C (11.8)	wt	wt	wt	wt	C275Y (0.14)	
29	T0	wt	wt	wt	wt	wt	Y220N (3.31)	
	TPD	wt	wt	wt	wt	wt	Y220N (0.71)	
30	T0	G12C (10.23)	wt	wt	wt	R1450[a] (5.9)	wt	
	T1	wt	wt	wt	wt	wt	wt	
	TPD	G12C (1.03)	wt	wt	wt	R1450[a] (0.33)	wt	
31	T0	G12D (0.13)	wt	wt	wt	R1450[a] (0.11)	wt	
32	T0	Q61R (0.11)	wt	wt	wt	wt	R273C (0.13)	
35	T0	G12V (36.6)	wt	wt	wt	p.Y935[a] (38.05); p.R1450[a] (31.13)	wt	
	TPD	G12V (18.6)	wt	wt	wt	p.Y935[a] (15.65); p.R1450[a] (15.99)	wt	
36	T0	wt	wt	wt	E542K (51.9)	wt	p.E180RfsTer; 6 (0.10) p.R158H (0.051) p.R158H (0.13)	
	T1	wt	wt	wt	E542K (31.9)	wt		
37[a]	T0	wt	wt	V600E (7.16)	E542K (6.8)	wt	wt	FBXW7 R465H (0.1)
	T1	wt	wt	wt	wt	wt	wt	
	Ti1	wt	wt	wt	wt	wt	wt	
	Ti2	wt	wt	V600E (0.7)	E542K (0.49)	wt	wt	
	TPD	wt	wt	V600E (3.88)	E542K (3.66)	wt	wt	
38[a]	T0	wt	G12D (51.32)	wt	wt	E1309[a] (65.46)	G245S (74.95)	
	T1	wt	wt	wt	wt	wt	wt	
	Ti1	wt	G12D (0.09)	wt	wt	wt	wt	
	Ti2	wt	G12D (0.50)	wt	wt	wt	G245S (0.28)	
	Ti3	wt	G12D (2.32)	wt	wt	E1309[a] (0.89)	G245S (1.21)	
	Ti4	wt	G12D (18.5)	wt	wt	E1309[a] (8.3)	G245S (16)	
	TPD	wt	G12D (11.68)	wt	wt	E1309[a] (6.51)	G245S (10.93)	

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TABLE A2. Genetic Variant and VAF (continued)

Patient Code	Time Point	*KRAS* (VAF%)	*NRAS* (VAF%)	*BRAF* (VAF%)	*PIK3CA* (VAF%)	*APC* (VAF%)	*TP53* (VAF%)	Other Gene Mutations (VAF%)
40	T0	wt	wt	wt	wt	wt	wt	
41	T0	wt	wt	wt	wt	wt	C176F (26.8)	
42	T0	G12D (0.54)	wt	wt	wt	wt	G245D (0.23)	
	T1	wt	wt	wt	wt	wt	wt	
43[a]	T0	Q61H (45.52)	wt	wt	wt	p.R876[a] (22.86); p.L1488FfsTer26 (35.4)	wt	
	T1	Q61H (2.45)	wt	wt	wt	p.R876[a] (0.69); p.L1488FfsTer26 (1.13)	wt	
	Ti	Q61H (5.42)	wt	wt	wt	p.R876[a] (1,85); p.L1488FfsTer26 (2.76)	wt	
	TPD	Q61H (26.6)	wt	wt	wt	p.R876[a] (9.5); p.L1488FfsTer26 (17.2)	wt	
44	T0	G12D (4.44)	wt	wt	wt	R876[a] (4.42)	wt	
	T1	wt	wt	wt	wt	wt	wt	
45	T0	G12D (28.9)	wt	wt	Q546R (33.2)	wt	wt	
	T1	G12D (0.11)	wt	wt	Q546R (0.96)	wt	wt	
	TPD	G12D (18.17)	wt	wt	Q546R (22.87)	wt	wt	
46	T0	wt	wt	wt	wt	wt	p.Y220C (50.46); p.E180RfsTer; 6 (0.1)	
47	T0	G13D (0.16)	wt	wt	wt	A1492PfsTer15 (0.14)	wt	MAP2K1 E203K (0.09)
	T1	G13D (0.39)	wt	wt	wt	A1492PfsTer15 (0.4)	wt	
	TPD	G13D (2.11)	wt	wt	wt	A1492PfsTer15 (1.62); p.R1114[a]; (0.58)	C277Y (0.13)	
48	T0	Q61H (2.18)	wt	wt	E542K (0.16)	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
49	T0	A146T (0.12)	wt	wt	wt	S1465WfsTer3 (0.097)	wt	
	T1	wt	wt	wt	wt	wt	F134L (0.78)	
	TPD	A146T (10.2)	wt	wt	E545K (2.5)	S1465WfsTer3 (4.9)	F134L (2.1)	
50	T0	wt	wt	V600E (7.4)	wt	wt	E286K (6.83); R282W (3.08)	FBXW7 R465C (2.27)
	T1	wt	wt	wt	wt	wt	wt	
	TPD	wt	wt	V600E (13.9)	wt	wt	p.E286K (19.67); p.G245D (0.076)	
51	T0	G12S (16.9)	wt	wt	wt	wt	R248Q (7.35)	
	T1	wt	wt	wt	wt	wt	wt	
	TPD	G12S (0.61)	wt	wt	wt	wt	R248Q (0.53)	
52	T0	G12D (33.84)	wt	wt	wt	wt	R248W (46.0)	
	T1	wt	wt	wt	wt	wt	wt	
	Ti	G12D (0.75)	wt	wt	wt	wt	wt	
	TPD	G12D (6.02)	wt	wt	wt	wt	R248W (3.96)	
53	T0	wt	wt	wt	E542K (0.09)	wt	E180RfsTer6 (0.11)	
	T1	wt	wt	wt	wt	wt	P177_C182d el (0.29)	
54	T0	G13D (19.8)	wt	wt	wt	wt	G245S (0.5)	
	T1	G13D (0.18)	wt	wt	wt	wt	L194P (0.22)	
55	T0	G12R (2.8)	wt	wt	wt	wt	R282W (3.58)	
	T1	G12R (0.27)	wt	wt	wt	wt	R282W (0.29)	

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TABLE A2. Genetic Variant and VAF (continued)

Patient Code	Time Point	*KRAS* (VAF%)	*NRAS* (VAF%)	*BRAF* (VAF%)	*PIK3CA* (VAF%)	*APC* (VAF%)	*TP53* (VAF%)	Other Gene Mutations (VAF%)
56	T0	G12V (45.02)	wt	wt	wt	wt	R156AfsTer1 4 (0.16)	
57	T0	wt	wt	V600E (18.6)	wt	wt	wt	
	T1	wt	wt	V600E (0.15)	wt	wt	wt	
58	T0	G12V (10.39)	wt	wt	G1049R (0.87)	wt	H179R (0.26)	
	T1	wt	wt	wt	wt	wt	wt	
	TPD	wt	wt	wt	wt	wt	wt	
59	T0	wt	G12D (4.99)	wt	wt	E1309fs (1.63)	wt	
	T1	wt	G12D (0.03)	wt	wt	wt	wt	
	Ti1	wt	wt	wt	wt	wt	wt	
	Ti2	wt	G12D (0.07)	wt	wt	wt	wt	
	TPD	wt	G12D (10.28)	wt	wt	E1309fs (2.44)	wt	
61	T0	G12D (0.71)	wt	wt	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
	TPD	G12D (0.08)	wt	wt	wt	wt	V272L (0.09)	
63	T0	wt	wt	wt	wt	wt	wt	
64	T0	wt	wt	V600E (11.7)	wt	wt	wt	
	T1	wt	wt	V600E (0.2)	wt	wt	wt	
	TPD	wt	wt	V600E (26.7)	wt	wt	wt	
65	T0	G13D (3.96)	wt	wt	wt	Q1294Ter (1.00)	wt	
66	T0	wt	wt	V600E (26.1)	wt	wt	R282W (43.3)	
67	T0	G12V (12.11)	wt	wt	wt	E1379Ter (8.87)	p.I195NfsTer 14(11.68); p.R273H (0.43); p.P151A (0.50)	
	TPD	G12V (1.81)	wt	wt	wt	E1379Ter (1.52)	p.I195NfsTer 14 (1.26); p.R273H (1.66); p.P151A (1.02)	
68	T0	wt	wt	wt	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
	Ti	wt	wt	wt	wt	wt	R175H (0.05)	
	TPD	wt	wt	wt	wt	wt	R175H (74.26)	
69	T0	wt	wt	wt	wt	wt	wt	
	TPD	wt	wt	wt	wt	wt	wt	
70	T0	G13D (0.48)	wt	wt	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
	TPD	G13D (0.47)	wt	wt	wt	wt	wt	
72	T0	Q61L (0.32)	wt	wt	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
	Ti	wt	wt	wt	wt	wt	wt	
	TPD	Q61L (0.15)	wt	wt	wt	wt	wt	

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TABLE A2. Genetic Variant and VAF (continued)

Patient Code	Time Point	*KRAS* (VAF%)	*NRAS* (VAF%)	*BRAF* (VAF%)	*PIK3CA* (VAF%)	*APC* (VAF%)	*TP53* (VAF%)	Other Gene Mutations (VAF%)
74	T0	G12A (11.52)	wt	wt	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
	Ti	wt	wt	wt	wt	wt	wt	
76	T0	wt	G12D (43.64)	Wt	wt	wt	R175H (77.12)	
	T1	wt	G12D (14)	wt	wt	wt	R175H (16.7)	
	TPD	wt	G12D (59.66)	wt	wt	wt	R175H (62.71)	
77	T0	wt	wt	V600E (2.37)	wt	wt	wt	
	T1	wt	wt	wt	wt	wt	wt	
78	T0	wt	wt	V600E (0.17)	wt	wt	wt	ERBB2 G776V (0.13)
	T1	wt	wt	wt	wt	wt	wt	
79	T0	G13D (0.8)	wt	wt	wt	R1450[a] (0.59)	wt	
	T1	wt	wt	wt	wt	wt	wt	
80	T0	wt	wt	wt	wt	Q1378[a] (54.3)	R175H (51.2)	
	T1	wt	wt	wt	wt	wt	R175H (0.13)	
	Ti	wt	wt	wt	wt	wt	wt	
	TPD	wt	wt	wt	wt	Q1378[a] (0.22)	R175H (0.17)	
81	T0	G12D (22.91)	wt	wt	wt	L1488fs (26.29)	wt	
	T1	wt	wt	wt	wt	wt	wt	
82	T0	wt	wt	V600E (5.58)	E545K (5.15)	R876[a] (5.47)	R181C (0.22)	SMAD4 R361H (5.8)
83	T0	G12A (11.35)	wt	wt	wt	R876[a] (11.7); Q1367[a] (7.00)	wt	
	T1	wt	wt	wt	wt	wt	wt	
	TPD	wt	wt	wt	wt	wt	wt	
84	T0	G12D (0.2)	wt	wt	E545K (0.25)	wt	wt	
	T1	wt	wt	wt	E545K (0.07)	wt	wt	

Abbreviations: T0, baseline; T1, time point at the first clinical evaluation; Ti, intermediate time point; TPD, time point at disease progression; VAF, variant allele frequency; wt, wild-type.
[a]Four representative cases displayed in Figure 4.

TABLE A3. Association Between Baseline VAF and Patient Characteristics

Characteristic	Median VAF (range)	*P*
Overall	7.28 (0.09-51.9)	—
Liver metastasis		
Yes	10.31 (0.09-51.9)	
No	5.74 (0.11-33.2)	.18
Lung metastasis		
Yes	4.99 (0.13-43.64)	
No	10.39 (0.09-51.9)	.51
Peritoneum metastasis		
Yes	5.01 (0.11-45.02)	
No	8.82 (0.09-51.9)	.59
Bone metastasis		
Yes	5.22 (0.13-7.16)	
No	10.23 (0.09-51.9)	.33
Lymph node metastasis		
Yes	5.58 (0.09-51.9)	
No	8.82 (0.11-51.32)	.41
Sex		
Female	8.69 (0.09-43.64)	
Male	6.94 (0.12-51.9)	.64
Adjuvant chemotherapy		
Yes	1.59 (0.12-22.91)	
No	10.87 (0.09-51.9)	.06
Chemotherapy regimen		
FOLFOX/CAPOX	7.4 (0.09-51.9)	
FOLFIRI/CAPIRI	22.91 (0.11-33.2)	.62
RAS		
Mut	8.36 (0.11-51.32)	
Wt	7.4 (0.09-51.9)	.59
BRAF		
Mut	7.16 (0.17-18.6)	
Wt	10.23 (0.09-51.9)	.74

Abbreviations: CAPIRI, capecitabine and irinotecan; CAPOX, capecitabine and oxaliplatin; FOLFIRI, fluorouracil, leucovorin, and irinotecan; FOLFOX, fluorouracil, leucovorin, and oxaliplatin; mut, mutation; VAF, variant allele frequency; wt, wild-type.

TABLE A4. Association Between Change in VAF Between Baseline and First Evaluation and Patient Characteristics

Characteristic	High Decreasing VAF (n = 29)	Low Decreasing VAF (n = 7)	*P*
Liver metastasis, No.			
Yes	22	4	
No	7	3	.37
Lung metastasis, No.			
Yes	13	3	
No	16	4	1.00
Peritoneum metastasis, No.			
Yes	6	2	
No	23	5	.64
Bone metastasis, No.			
Yes	2	0	
No	27	6	1.00
Missing		1	
Lymph node metastasis, No.			
Yes	13	4	
No	16	2	.40
Sex, No			
Female	10	4	
Male	19	3	.39
Adjuvant chemotherapy, No.			
Yes	9	1	
No	20	6	.65
Chemotherapy regimen, No.			
FOLFOX/CAPOX	25	6	
FOLFIRI/CAPIRI	2	1	
Others	2	0	.69
RAS, No.			
Mut	21	4	
Wt	8	1	1.00
Missing		2	
BRAF, No.			
Mut	6	0	
Wt	23	6	.56
Missing		1	

Abbreviations: CAPIRI, capecitabine and irinotecan; CAPOX, capecitabine and oxaliplatin; FOLFIRI, fluorouracil, leucovorin, and irinotecan; FOLFOX, infusional fluorouracil, leucovorin, and oxaliplatin; mut, mutation; VAF, variant allele frequency; wt, wild-type.