Original Study

Plasma Dynamics of *RAS/RAF* Mutations in Patients With Metastatic Colorectal Cancer Receiving Chemotherapy and Anti-*EGFR* Treatment

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Abstract

This study aimed to investigate the clinical aspects of *RAS/RAF* mutations during chemotherapy and anti-*EGFR* treatment in tumor wild-type patients. Blood samples were collected from 46 patients at every treatment cycle until progressive disease or censoring. Emergence of mutations was not correlated with treatment effect.

Background: *RAS* and *RAF* mutations in colorectal cancer (CRC) hold value in precision medicine. Liquid biopsy is an alternative to tumor tissue biopsy, and circulating tumor DNA (ctDNA) has been intensively investigated, but the clinical relevance of *RAS* and *RAF* mutations in plasma is yet to be determined. This study aimed to investigate the clinical aspects of *RAS/RAF* mutations during combination treatment. **Patients and Methods:** Patients with *RAS/RAF* tumor wild-type metastatic CRC treated with combination chemotherapy and an *EGFR* inhibitor were included. Blood samples were collected at baseline and every treatment cycle and analyzed for 31 *RAS*, *RAF*, and *EGFR* mutations until progressive disease or censoring using droplet digital PCR. **Results:** Forty-six patients were prospectively enrolled onto the study. At baseline, 7% had detectable *RAS/RAF* mutations in ctDNA. During the treatment course, the fraction of patients with mutated ctDNA increased to 22%. The emergence of mutations did not correlate with response or risk of progression while receiving treatment ($P = 1.0$). **Conclusion:** Emergence of plasma *RAS/RAF* mutations was not correlated with the effect of combination chemotherapy and *EGFR* inhibition in patients with *RAS/RAF* wild-type metastatic CRC.

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Introduction

Determination of *RAS* and *RAF* mutations has been a major step in personalized treatment of colorectal cancer (CRC); these mutations are now well-established predictive biomarkers in clinical practice.[1] Treatment with monoclonal *EGFR* antibodies is standard in metastatic CRC (mCRC) without *RAS/RAF* mutations (wild type) either as monotherapy or combined with chemotherapy. Despite the initial response to treatment, patients' disease will inevitably progress while receiving treatment.[2]

The molecular profile of CRC is routinely assessed in tumor tissue, which has several limitations. A high-ranking one is heterogeneity driven by different subclones and divergence between primary tumor and metastases.[3,4] Furthermore, the composition can evolve over time and during a treatment course.[4,5] A major barrier to testing the hypothesis of resistance caused by evolving mutations is the limited access to posttreatment tumor tissue.

Recent years have facilitated establishment of a tumor's mutational status by analyzing blood—that is, liquid biopsy. The benefits of such a minimally invasive procedure, such as accessibility at any time and sufficient material, are appealing, also from the patient's point of view. A clinically important application of liquid biopsies is analysis of circulating tumor DNA (ctDNA). Previous studies have indicated a

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high degree of concordance between mutations detected in tumor tissue and blood in patients with CRC.[6,7]

The development of drug resistance is a key limitation of the treatment of patients with cancer. Acquired resistance to treatment with *EGFR* inhibitors is presumably linked to the emergence of *RAS*, *RAF*, and *EGFR* mutations, which are detectable in the blood.[8-11] Prior studies have reported that approximately 40% of patients who initially had tumor *KRAS* wild type developed mutations detectable in the blood during anti-*EGFR* therapy.[5,10,12] However, questions have recently been raised as to the clinical importance of changes in the plasma mutational profile during treatment with anti-*EGFR* therapy.[5,7,13]

The aim of the present study was to monitor plasma *RAS/RAF* and *EGFR* mutational status in patients with mCRC during treatment with chemotherapy and an *EGFR* inhibitor, and relate the changes to treatment response.

Patients and Methods

Study Population

Patients with tumor *RAS/RAF* wild-type mCRC receiving chemotherapy and anti-*EGFR* treatment were offered inclusion in a prospective biomarker study at the Danish Colorectal Cancer Center South, Vejle Hospital, Denmark. The inclusion criteria were adenocarcinoma in the colon or rectum, recurrent or primary metastatic disease, measurable disease according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1, tumor *RAS/RAF* wild type at baseline according to standard procedures as indicated in the Supplemental Material in the online version, life expectancy more than 3 months, planned treatment with cetuximab or panitumumab, age > 18 years, and Eastern Cooperative Oncology Group performance status 0 to 2. Treatment was discontinued in case of progressive disease (PD), unacceptable toxicity, death, cancer surgery, or patient request, or as decided by the treating physician.

Plasma samples were collected at baseline and sequentially before every treatment cycle throughout the treatment course until PD or censoring. Treatment effect was evaluated by computed tomographic (CT) scans at every fourth cycle of treatment according to RECIST 1.1, by personnel unaware of the patient's mutational status. One patient underwent only the baseline CT scan, thus leaving 45 (98%) of 46 patients for response evaluation.

Written informed consent was obtained from all patients. The study was approved by the Regional Committees on Health Research Ethics for Southern Denmark (S-20150103), and the investigation is reported in accordance with the REMARK criteria.[14]

Blood Sample Analyses

Blood samples at baseline and at progression or censoring were screened for 24 *RAS/RAF* mutations as previously reported,[15] with subsequent quantitative analysis of positive samples. Furthermore, 7 *EGFR* mutations were analyzed in single assays as described by Arena et al.[16] The specific mutations are listed Supplemental Table 1 in the online version. If a mutation was detected in the baseline blood sample of a patient, the tumor sample was reanalyzed for that specific mutation using droplet digital PCR (ddPCR), as described in the Supplemental Material in the online version.

The last blood sample obtained from each patient was also screened, and if a mutation was found, it was followed by a retrospective screening of the blood samples preceding progression or censoring to identify the time point when the mutation became detectable. The screening method has previously been published[15] and is briefly described in the Supplemental Material in the online version.

All analyses were carried out by staff unaware of the clinical status of the patients.

Statistical Analysis

Descriptive statistics included median and range for continuous variables, and number and percentage for categorical variables.

The clinical database was last updated in February 2018. The median duration of treatment was estimated by inverse Kaplan-Meier analysis. The relative risk was calculated on the basis of the presence of PD or death during treatment in the 2 groups of patients with and without plasma mutations. Statistical significance was calculated by the Fisher exact test. Response was defined as partial or complete according to RECIST 1.1, and a response rate was calculated.

All reported P values were 2 sided, and $P < .05$ was considered statistically significant. Statistical analyses were carried out by NCSS 10 statistical software (2015) (NCSS, Kaysville, UT; http://ncss.com/software/ncss).

Results

Patient Characteristics

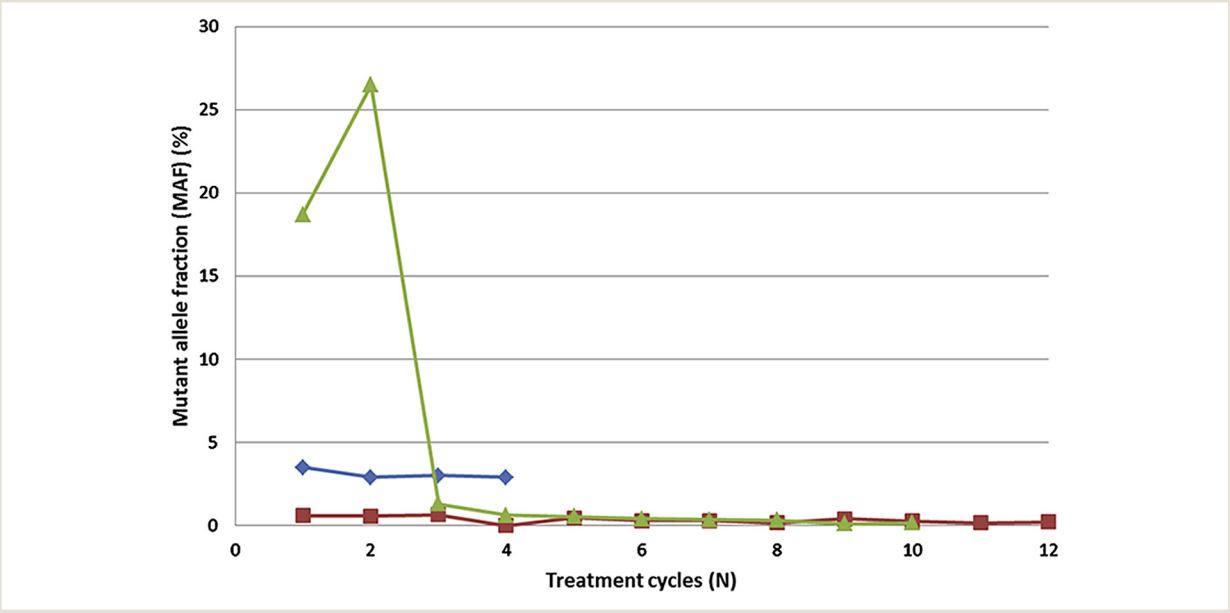
The study enrolled 46 patients between November 2015 and November 2017. Baseline patient characteristics are listed in Table 1. Chemotherapy and anti-*EGFR* treatment were

Table 1 Baseline Characteristics of 46 Patients

Characteristic	Value
Age (y), median (range)	63 (45-79)
Gender	
Female	19 (41)
Male	27 (59)
Performance Status	
0	30 (65)
1-2	16 (35)
Location of Primary Tumor	
Right/transverse colon	8 (17)
Left colon	22 (48)
Rectum	15 (33)
Unknown	1 (2)
Primary disseminated disease	29 (63)
No. of Metastatic Sites	
1-2	40 (87)
>2	6 (13)
Prior treatment regimens	
0	23 (50)
1	15 (33)
≥ 2	8 (17)

Data are presented as n (%) unless otherwise indicated.

Figure 1 MAF During Treatment in 3 Patients With Baseline Plasma Mutations



Abbreviation: MAF = mutated allele fraction.

administered as first-line treatment in half of the patients and as second- or third-line treatment in the other half. All but one patient were anti-*EGFR* treatment naive, and 7 patients underwent cancer surgery after chemotherapy. The disease of 2 patients was still responding to treatment at the time of data analysis, and only analysis of their baseline samples were included.

The median time from tumor tissue analysis of *RAS/RAF* mutations to start of treatment was 3.6 months (range, 0-20 months).

All 46 patients had at least a baseline blood sample drawn and contributed with a total of 481 serially collected plasma samples. In 2 patients, only the baseline sample was available because of withdrawal of consent and non-adherence to blood sampling for unknown reasons.

Baseline Plasma Analysis

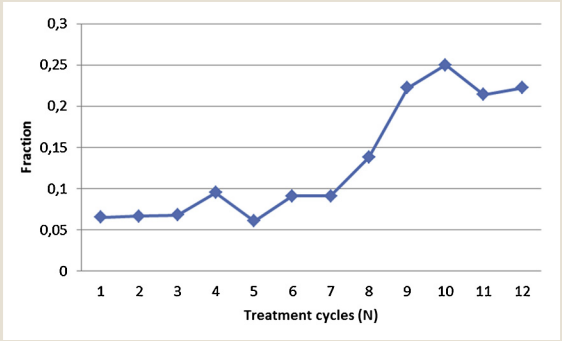
The baseline samples from 3 of 46 (7%) tumor *RAS/RAF* wild-type patients showed the presence of plasma mutations. Two were tumor wild type when reanalyzed by ddPCR. In the third sample, a *KRAS* G12D mutation was found, indicating a sampling error in the standard method. The plasma mutations found in these 3 cases were *NRAS* G12D, *KRAS* G12C, and *KRAS* G12D. The patients were kept in the study for further analyses. Assessing their characteristics did not reveal common features (data not shown). In all 3 cases, the plasma mutation allele fraction (MAF) at baseline was higher than at the end of treatment (Figure 1). None of the patients experienced disease progression while receiving treatment.

Emergence of Mutations During Treatment

The fraction of patients with mutated ctDNA along the treatment course remained rather stable during the first 7 cycles (Figure 2). From this point, a steep increase in the fraction of patients with mutated ctDNA was observed. Twenty-five percent (4/16) of the patients harbored mutations at treatment cycle 10, and in patients receiving 12 cycles of treatment, the fraction with detectable mutated ctDNA was 22% (2/9). Two patients (4%) had multiple detectable mutations during treatment. In both, the second *RAS* mutation fluctuated with low MAF and disappeared before the end of treatment.

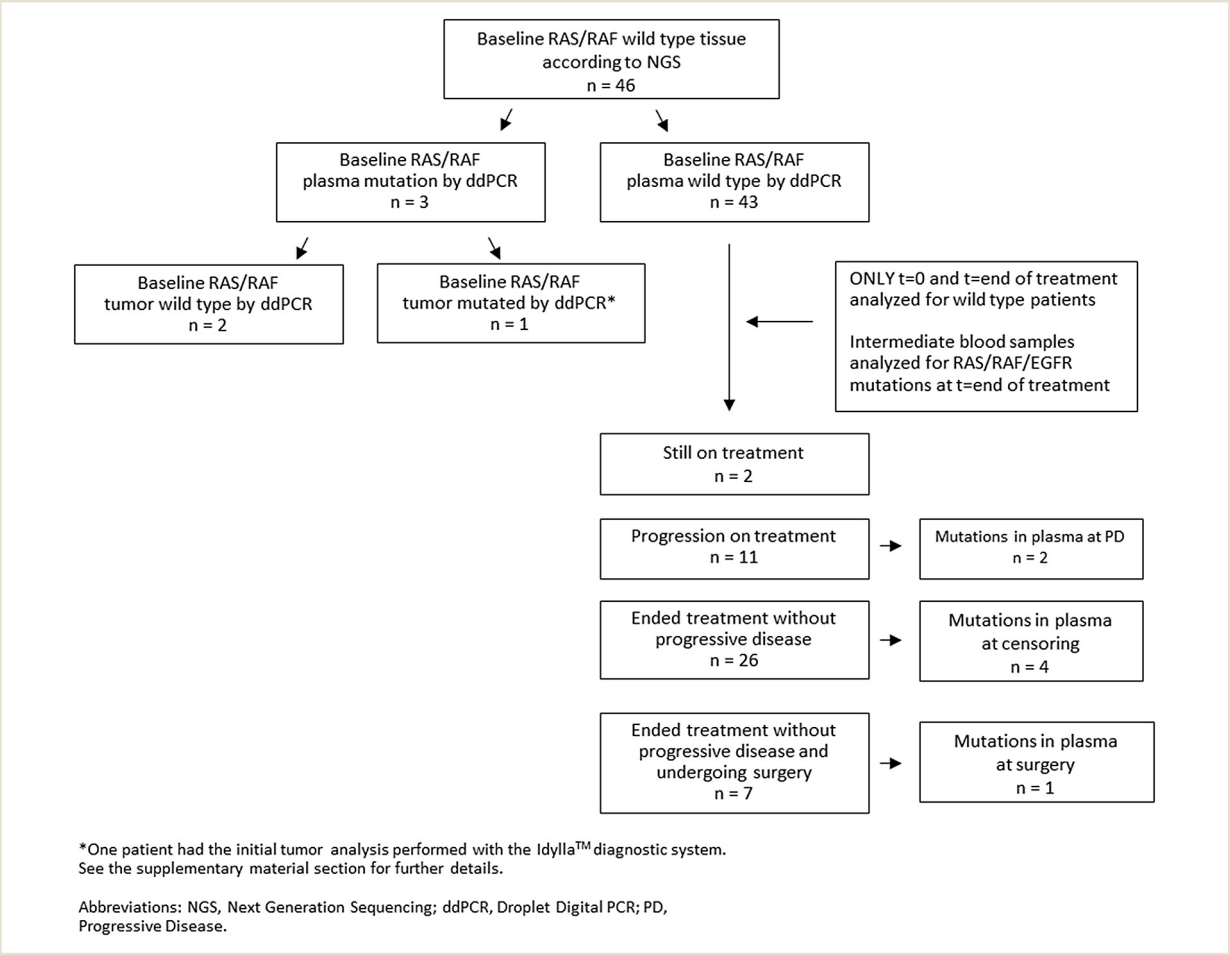
EGFR mutations were found only in one patient, who harbored multiple detectable mutations (ie, *KRAS* Q61HC, *NRAS* Q61L, *EGFR* G465R, *EGFR* A464L). The mutations fluctuated in MAF during treatment but reached a higher level at the time of progression than at occurrence. The first mutation to occur was *KRAS* Q61HC, with a MAF of 0.083% at treatment cycle 6.

Figure 2 Fraction of Patients With Mutated ctDNA During Treatment Course



Abbreviation: ctDNA = circulating tumor DNA.

Figure 3 Study Flowchart



*One patient had the initial tumor analysis performed with the Idylla™ diagnostic system.
See the supplementary material section for further details.

Abbreviations: NGS, Next Generation Sequencing; ddPCR, Droplet Digital PCR; PD, Progressive Disease.

Correlation With Clinical Outcome

Eleven patients experienced disease progression while receiving treatment. Twenty-six patients ended treatment without disease progression. Seven patients underwent cancer surgery and were therefore censored (Figure 3).

The median duration of treatment was 4.2 months (95% confidence interval, 3.5-4.8). The relative risk of PD or death during treatment was based on 11 events in the whole cohort of 46 patients.

The emergence of plasma mutations did not seem to influence the treatment effect. The relative risk of PD or death in the group of patients with plasma mutations compared to the group without plasma mutation was 1.24 and was not statistically significant ($P = 1.0$).

The first and second evaluations resulted in a response rate of 24% (11/45) and 45% (15/33), respectively. At the third and final evaluation, the response rate was 47% (8/17). Five of the patients had mutated ctDNA; of these, 3 patients ended treatment without PD. The patient previously treated with anti-*EGFR* therapy experienced disease progression after 4 cycles of treatment, but no emergence of detectable *RAS/RAF/EGFR* mutations was observed.

Discussion

This prospective study of patients with mCRC receiving chemotherapy and anti-*EGFR* treatment in a daily clinical setting indicates that the fraction of patients with plasma *RAS/RAF* mutations increased during the treatment course. Furthermore, mutational status does not seem to correlate with treatment effect.

Establishment of mutational status is important in precision medicine in order to offer targeted treatment. It is well known that a tumor *RAS/RAF* mutation in colorectal cancer correlates with resistance to anti-*EGFR* monotherapy.[17] The predictive value of tumor tissue mutations, however, has been questioned regarding the administration of *EGFR* inhibitors and combination chemotherapy. A large study published in 2017 with a follow-up of 5 years reported no additional effect on overall response rate, progression-free survival, or overall survival when adding cetuximab to combination chemotherapy in patients with *RAS/RAF* wild-type tumors.[18]

The clinical application of mutation analyses in the blood as a replacement for tumor tissue analysis is extensively investigated for several reasons. The time from receipt of tumor tissue sample to communicating the results varies among centers, but one study has reported a favorable turnaround time when comparing analysis of

blood and tumor tissue biopsy samples.[7] Furthermore, previous publications have reported a high concordance between tumor tissue and blood in the detection of mutations, and have suggested liquid biopsies as a surrogate in selecting patients for targeted therapy.[5,6,19]

In the present study, we supplemented the standard procedure of tumor tissue analysis with ddPCR. We found a concordance of 95.6% (43/45; 2 patients were tumor mutation negative and plasma mutation positive) between tumor and plasma as analyzed by this modality. The level of agreement will inevitably differ among studies depending on the sensitivity of the selected methods, but similar results have been reported.[5,20]

According to our results, plasma mutational status does not appear to have predictive value regarding the effect of combination chemotherapy and *EGFR* inhibition. Even patients with baseline mutations received numerous cycles of treatment with clinical benefit, as determined by response-evaluating CT scans. Similar results have recently been presented. Baseline *RAS/RAF* mutated patients benefitted from treatment and some even experienced loss of detectable mutations at PD after chemotherapy and anti-*EGFR* treatment.[21] Vidal et al[5] recently described 4 patients whose disease was characterized as tissue *RAS* wild-type/plasma *RAS* positive. Three patients experienced partial response (75%), and one had PD, as revealed by combination chemotherapy and anti-*EGFR* treatment. In the present nonrandomized study, it is not possible to differentiate between the effect of chemotherapy and *EGFR* inhibitors.

During chemotherapy and anti-*EGFR* treatment, some patients developed detectable mutations. This could indicate that patients with plasma mutations receive treatment longer than plasma wild-type patients, or that mutations will eventually emerge. Several studies support the observation that plasma mutations appear during treatment.[5,10,22]

We found that the emergence of mutations did not correlate with treatment outcome. In agreement with our findings, Siravegna et al[11] described that *KRAS* mutations emerged in the blood of CRC patients with disease responding to anti-*EGFR* treatment. Siena et al[13] found similar evidence concerning the absence of correlation between clinical outcome and plasma mutations. However, they found the *RAS* mutation level to increase in most cases during treatment, as opposed to our findings. The authors suggested that the combination of *EGFR* inhibitors and irinotecan influences patient sensitivity and treatment selection pressure. In the present study, all patients received combination chemotherapy and anti-*EGFR* treatment, which may blur the specific effect of the targeted therapy and may also explain the lack of increase in MAF.

Other studies have reported the emergence of *RAS/RAF* mutations to correlate with PD in the majority of mCRC patients as a sign of secondary resistance, although the duration of treatment was longer than that of the patients in our study. It is postulated that resistance inevitably will occur, as undetectable subclones are present at baseline and will repopulate the tumor over time.[9,12] More than a third of the patients enrolled in the study by Thierry et al[22] had received anti-*EGFR* treatment previously, and 67% harbored mutated ctDNA at baseline. Pietrantonio et al[23] investigated 11 patients with postprogression tumor biopsy and ctDNA samples, and found that 5 patients had developed *RAS/RAF* mutations at PD. It is, however, not stated how many of them were anti-*EGFR* treatment naive, since 50% of the whole cohort of 22 patients had previously received an *EGFR* inhibitor. Additionally, there was a time span of up to 4 months from verified PD to blood sample collection. Van Emburgh et al[24] found that the majority of patients harbored *RAS* mutations at the time of progression. However, 62% of the patients who developed *RAS* mutations had stable disease for more than 16 weeks as best response, compared to 44% in the whole cohort. These conflicting results are underlined in the recently published joint review on ctDNA in cancer by the American Society of Clinical Oncology and the College of American Pathologists.[25]

The most frequently acquired mutation in our study was *KRAS* Q61H A > C, which is in line with the literature reporting higher frequency of this specific mutation in patients treated with *EGFR* inhibitors compared to anti-*EGFR* treatment−naive patients.[22] *EGFR* mutations only occurred in one patient during therapy and did not correlate with progression of disease while receiving treatment. The low frequency of *EGFR* mutations in this cohort is in accordance with several other studies.[10,23]

We did not identify a statistically significant difference in the relative risk of PD or death determined by plasma mutational status. Prior reports on patients with mCRC receiving combination chemotherapy and *EGFR* inhibitors report divergent results. Diaz et al[12] reported no difference in survival between patients who developed *KRAS* mutations during therapy and those who did not. Normanno et al[6] found a significant difference in median progression-free survival between patients with plasma *RAS*-mutant and wild-type disease, but they saw a lower concordance rate between mutations in tumor and blood (78.3%) and described their study as an "unplanned evaluation of a small cohort."

The current study has several limitations. The sample size is small, and the results are therefore hypothesis generating and need validation. However, the results correlate with recent publications identifying the lack of clinical benefit by *EGFR* inhibitors and the presence of *RAS/RAF* mutations in the blood. Previous studies report divergent results on the topic, and it is important to note that several of them examine the value of anti-*EGFR* monotherapy, analyze blood samples during and after discontinuation of treatment, or only include patients who experienced disease progression while receiving treatment.

This study was conducted prospectively with ddPCR analysis of consecutive plasma samples in baseline tumor *RAS/RAF* wild-type mCRC patients. We collected plasma samples during treatment in a clinical cohort and screened the samples for relevant *RAS*, *RAF*, and *EGFR* mutations, thus displaying a clinically relevant application of ctDNA. Whether our findings are due to the treatment pressure of combination therapy needs to be addressed in larger prospective and preferably randomized trials.

Conclusion

The fraction of patients with mutated ctDNA increased during the course of treatment with chemotherapy and an *EGFR* inhibitor. In the present hypothesis-generating study, plasma *RAS/RAF* mutational status was not associated with clinical outcome in patients with mCRC.

Clinical Practice Points

- It is widely accepted that the presence of tumor *RAS/RAF* mutations in patients with mCRC correlates with resistance to anti-*EGFR* monotherapy. The predictive value of plasma *RAS/RAF* mutations has been discussed, but a few recent studies have presented a very small number of patients with plasma-mutated disease who apparently experienced effect of anti-*EGFR* treatment.
- In this study with 46 baseline tumor wild-type patients, one fourth of the patients developed plasma *RAS/RAF* mutations during chemotherapy combined with an *EGFR* inhibitor. However, the development of mutations did not influence the treatment effect.
- The effect of *RAS/RAF* mutations detected in a liquid biopsy during combination treatment should be further discussed because the predictive value in plasma seems to differ from that in tumor.

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Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

Supplemental material and figures accompanying this article can be found in the online version at https://doi.org/10.1016/j.clcc.2018.10.004.

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Supplemental Material

Tissue Analyses According to Standard Procedures

Tumor tissue samples of all patients but one were analyzed by next-generation sequencing on the Illumina platform with the Illumina TruSight Tumor 15 (TST15) panel (Illumina, San Diego, CA). One patient had initial tumor analysis performed outside Vejle Hospital with the Idylla (Biocartis, Mechelen, Belgium) diagnostic system, which is based on real-time PCR.

Blood Analyses

Blood samples were collected in three 9 mL EDTA tubes, and plasma was isolated by centrifugation at 2000 × *g* for 10 minutes within 4 hours of sampling and stored at −80°C until use. The plasma was centrifuged again at 10,000 × *g* for 10 minutes before purification, and cysteine-rich polycomb-like protein 1 (CPP1) DNA fragments were added as exogenous internal controls.[1] DNA was purified from 4 × 1.0 mL plasma on the QIAsymphony SP instrument using the QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA from plasma was pooled and analyzed by real-time quantitative PCR for the number of CPP1 and gB2M alleles, as previously described.[1]

The remaining DNA was concentrated to 20 µL and preamplified for 10 cycles with Q5 Mastermix (New England Biolabs, Ipswich, MA, USA) and a preamplification primer mix (primers from DNA Technology, Aarhus, Denmark) in 50 µL reaction volumes according to the manufacturer's instructions. Primers are listed in Supplemental Table 2 in the online version. Samples were diluted 50 times before PCR amplification.

Droplet Digital PCR

After PCR amplification in 20 µL reactions, samples were analyzed in a droplet reader QX100 droplet reader (Bio-Rad, Hercules, CA), and data were analyzed with Quantasoft (BioRad, Hercules, CA) 1.7 droplet digital PCR software. Samples positive in the multiplex analyses were further analyzed in singleplex analysis to identify the exact mutation. Negative controls (water and genomic DNA) were preamplified and analyzed simultaneously with preamplification of the samples. Positive controls were gBlocks or fragments generated by site-directed mutagenesis diluted in genomic DNA.

Quantasoft software calculated the number of wild-type and mutated copies of DNA per microliter of reaction based on the number of FAM- and HEX-positive droplets and the Poisson distribution. The mutant allele fraction was calculated as copies of mutated DNA/(copies of mutated DNA + copies of wild-type DNA). Samples were categorized as positive if the lower limit of the 95% confidence interval for the mutant allele fraction of a multiplex reaction was above the upper limit of the 95% confidence interval of the corresponding multiplex for negative controls. In case of overlapping confidence intervals, the sample was deemed negative.

Reference

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Supplemental Table 1	Mutations Analyzed	
BRAF	**Exon 15**	**V600E**
KRAS	Exon 2	G12A
KRAS	Exon 2	G12C
KRAS	Exon 2	G12D
KRAS	Exon 2	G12R
KRAS	Exon 2	G12S
KRAS	Exon 2	G12V
KRAS	Exon 2	G13D
KRAS	Exon 3	Q61H A > C
KRAS	Exon 3	Q61H A > T
KRAS	Exon 3	Q61L
KRAS	Exon 3	Q61R
KRAS	Exon 4	A146P
KRAS	Exon 4	A146T
KRAS	Exon 4	A146V
NRAS	Exon 2	G12C
NRAS	Exon 2	G12D
NRAS	Exon 2	G12V
NRAS	Exon 2	G13D
NRAS	Exon 2	G13R
NRAS	Exon 3	Q61H
NRAS	Exon 3	Q61K
NRAS	Exon 3	Q61L
NRAS	Exon 3	Q61R
EGFR		R451C
EGFR		S464L
EGFR		G465E
EGFR		G465R
EGFR		K467T
EGFR		I491M
EGFR		S492R_1476C > A

Supplemental Table 2 Primers Used for Targeted Preamplification

Codon	Forward Primer	Reverse Primer
BRAF.p.V600	GATCCAGACAACTGTTCAAA	CATGAAGACCTCACAGTAAA
KRAS.p.G12_13	AATTAGCTGTATCGTCAAGG	TATAAGGCCTGCTGAAAATG
KRAS.p.Q61	CAGTCCTCATGTACTGGT	ACCTGTCTCTTGGATATTCT
KRAS.p.A146	CAGATCTGTATTTATTTCAGTGT	CAGGACTTAGCAAGAAGTTA
NRAS.p.G12_13	ACAAAGTGGTTCTGGATTAG	AATGACTGAGTACAAACTGG
NRAS.p.Q61	CATGTATTGGTCTCTCATGG	AAACCTGTTTGTTGGACATA
EGFR.p.R451	TCGTCAGCCTGAACATAAC	ATTATCACATCTCCATCACTTAT
EGFR.p.S464_G465_K467	TCAAGGAGATAAGTGATGGA	ACAGTTTTTTCCAGTTTATTGT
EGFR.p.I491_S492	CTGTTTGGGACCTCCG	AGAAAGCGGTGACTTACT