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Significance of Liquid Biopsy for Monitoring and Therapy Decision of Colorectal Cancer

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Abstract

PURPOSE: Despite therapeutic improvements, all patients with nonresectable metastatic colorectal cancer (mCRC) acquire resistance to treatment probably due to the growth of mutated clones. In contrast to tissue-based studies, liquid biopsies have enabled the opportunity to reveal emerging resistance to treatment by detecting mutated clones and noninvasively monitoring clonal dynamics during therapy. *METHODS:* The courses of three patients with mCRC who were initially RAS wild-type were monitored longitudinally using liquid biopsy with long-term follow-up of up to 20 sequential samples. Detection of fragmented RAS mutated circulating cell-free DNA (cf)DNA in plasma was performed by BEAMing. In addition, plasma digital droplet PCR was used to detect and quantify BRAF and PIK3CA mutated cfDNA. Changes of mutational load were correlated with imaging data. *RESULTS:* A combination of liquid biopsy and radiological imaging enabled visualization of the occurrence of clonal redistribution after discontinuation of anti-EGFR mAb therapy, as well as emerging RAS mutations during therapy with anti-EGFR mAb indicating resistance. Furthermore, we found that growth of RAS mutated clones is independent of direct selective pressure by anti-EGFR therapy, which is a significant and new finding of this study. *CONCLUSIONS:* Our findings demonstrated the whole spectrum of clonal selection and redistribution of mutated cell clones leading to acquired resistance. Given our observation that the growth of RAS mutated clones can evolve even in the absence of anti-EGFR mAb therapy, there is a clear imperative to monitor RAS mutations in serial blood draws in all RAS wild-type patients in general and independent of the therapy.

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Introduction

Colorectal cancer (CRC) represents a major cause of cancer death in the Western world. Indeed, CRC is the second most prevalent cancer in Europe, responsible for approximately 12% of cancer deaths [1]. Despite ongoing efforts of early preventive screening of the disease, CRC is diagnosed at advanced stage in 20% to 30% of the patients; relapse occurs in 40% to 50% of those diagnosed in early stages [2]. In the last few years, diverse combinations of cytotoxic chemotherapies such as irinotecan, oxaliplatin, and fluoropyrimidine together with monoclonal antibodies including cetuximab, panitumumab, bevacizumab, aflibercept, and ramucirumab have significantly improved median overall survival (OS) in metastatic CRC (mCRC). Currently, median OS exceeds 30 months in patients having all-RAS wild-type mCRC [3]. Despite improvements in treatment, all nonresectable mCRC patients sooner or later acquire resistance and show disease progression during cytotoxic and targeted therapies.

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The growths of tumor cells having driver mutations in certain oncogenes are thought to be the main cause of secondary resistance. For instance, mutations in KRAS, NRAS, BRAF, and PIK3CA oncogenes lead to secondary treatment resistance against anti-EGFR antibodies [4].

In order to optimize currently available and future targeted therapies, careful treatment selection is needed, taking into consideration the increasing importance of clinical and biological disease factors such as (secondary) resistance genetic mechanisms that occur during the disease course.

To gain a better mechanistic understanding of acquired resistance in CRC, we closely monitored the disease courses of three mCRC patients with serial blood sampling/liquid biopsy with long-term follow-up. These patients initially presented with RAS wild-type status as assessed by tumor biomarker testing of primary tumor tissue.

Material and Methods

Patients, Sample Preparation, and DNA Isolation

The University of Bochum Ethical Committee approved collection and analysis of samples for this study (registration number**:** 16-5961; ethics committees of Ruhr-University of Bochum). Patients' informed written consent was obtained prior to sample collection and analysis. Peripheral blood samples were collected in STRECK BCT tubes or in K2-EDTA Vacutainer tubes (Becton Dickenson). Subsequently, plasma samples were prepared within 3 days or within 4 hours, respectively, by centrifugation as recommended for the different types of collection tubes. After removal of cell debris, plasma samples were stored in aliquots at −80°C until further analyses. CtDNA was isolated using 2 to 3 ml of plasma by means of QIAamp circulating nucleic acid kit purification kit following the manufacturer's protocol (Qiagen, Hilden, Germany). The isolated ctDNAs were stored at −20°C until use in BEAMing or ddPCR assays (maximal 4 weeks).

Mutation Detection in Tissue Samples

Formalin-fixed and paraffin-embedded tissue sections derived from primary tumors and from metastasized tumor tissue were analyzed by sequencing according to the procedures established for routine clinical use (Institute for Pathology of the Ruhr-University Bochum, Germany).

RAS-Mutation Detection in Plasma Samples

Two different methods to monitor dynamic changes of the mutational status in plasma samples were used. First, all relevant RAS variants were generally monitored using the BEAMing technology. The procedure of RAS-mutation detection by BEAMing has recently been described by our group [5]; the procedures involved in the testing by BEAMing were performed according to the OncoBEAM RAS CRC CE-IVD kit instructions for use (Sysmex Inostics GmbH, Hamburg, Germany) [5]. In brief, samples were analyzed by an emulsion digital PCR procedure for the presence of 34 different mutations in exons 2, 3, and 4 of KRAS and NRAS, respectively. Codon-specific labeled mutant or wild-type bead numbers were detected by flow cytometry, and the fraction of mutant beads in plasma samples was determined after analysis of 3×10^6 beads per measurement. The cutoff to detect a RAS mutation was set at 0.02% as recently published [5].

Standard plasma volumes for cfDNA isolation by QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) were 3 ml for BEAMing and 3 or 1 ml for ddPCR. The eluation volumes were adjusted to plasma volumes of 140 µl or 70µl, respectively. The fractional abundance measured by ddPCR experiments did not depend on the sample volume used for cfDNA isolation (data not shown).

cfDNA Quantification in Plasma Samples

In addition, we analyzed the E545K mutant variant of PIK3CA, the V600E mutant variant of BRAF, and the KRAS 2 and 3 mutation variants using ddPCR assays.

All ddPCR assays were carried out in duplicate according to manufacturer's instructions (Bio-Rad Laboratories Inc.). For each reaction, a standard volume of 20 µl was prepared using 6 µl DNA eluate and 14 µl mastermix (containing 10 µl 2× supermix, 1 µl mutation assay 1, 1 µl wild-type assay 2, 2 µl DNA-free water). Standard plasma volumes for cfDNA isolation by QIAamp circulating nucleic acid kit were 1 or 3 ml for ddPCR. The eluation volumes were adjusted to plasma volumes of 70 µl or 140 µl, respectively. The results of ddPCR experiments did not depend on the sample volume used for cfDNA isolation (data not shown). PIK3CA E545K and KR2-12/13 multiplex assays were proved by titration of standard samples purchased from Horizon discovery (H701, Wien; Supplementary Figure S4). The measured values of mutant fraction abundance were stable if the input DNA amount is higher than 0.1 ng cfDNA in KRAS-12/13 multiplex assay and higher than 1 ng using PIK3CA E545K assay (Supplementary Figure S4).

Digital droplet (dd)PCR assays offer the opportunity to quantify cfDNA amounts directly isolated from plasma [6]. Therefore, ddPCR assays (Biorad) were used to monitor the absolute amount of cfDNA during therapy in order to support the BEAMing assay results in the same plasma sample. The sum of wild-type and mutated copies measured by ddPCR assays was calculated considering the assay volume of cfDNA and plasma sample (equation in Supplementary Figure S4). PIK3CA E545K and KR2-12/13 multiplex assays were proved by titration of standard samples purchased from Horizon discovery (H701, Wien, Austria; Supplementary Figure S4). The measured values of mutant fraction abundance were stable if the input DNA amount is higher than 0.1 ng cfDNA in KRAS-12/13 multiplex assay and higher than 1 ng using PIK3CA E545K assay (Supplementary Figure S4). The examples of PIK3CA E545K and KRAS Q61 assay results of patient samples given in Supplementary Figure S5 show the specificity of mutation detection as seen in the 2D amplitude blots.

Measurement of Total Tumor Load

Patient total tumor masses were determined by measuring the tumor volumes using the radiograph taken as the efficiency control of patient's treatment strategies.

All CT measurements were done manually on commercial PACS workstations (Jive-X-PACS, Bochum, Germany) by an experienced radiology specialist. The reconstructed slice thickness was 4 mm or less for volumetry of liver and lung metastases. Due to the irregular shape of metastases, the contours were marked manually, and the surface area of the different layers was integrated to determine the total volume.

The cfDNA amount was then correlated with the size of metastases visualized in the images. Although there was some temporal discontinuity between the timing of blood draws with respect to that of images taken to measure tumor size, an exact relationship between cfDNA quantity results and tumor burden was not possible;

however, our analyses demonstrate use of cfDNA analyses within the restrictions and practicalities normally experienced in a clinical setting.

Results

First, primary tissue samples derived from three mCRC patients were classified as wild-type for all RAS variants. Accordingly, treatment with anti-EGFR mAb therapy was initiated. Corresponding to the tumor tissue results, monitoring of RAS, BRAF, and PIK3CA mutational status by liquid biopsy analyses was also started at the same time. RAS mutations were detected by BEAMing assays [5,7], whereas PIK3CA E545K and BRAF V600E mutations were analyzed by ddPCR assays at diagnosis and at several time points during treatment.

In this study, blood-based mutational monitoring reflected that dynamical changes of mutational states (loads) of tumor cells could be detected in plasma samples of each patient. Due to association of tumor load with the concertation of cfDNA, we anticipate that the cfDNA is in total equal to cf tumor DNA. As shown in Figures 1-3, changes in mutational loads appeared over time as well as during different treatment phases. The individual profiles for the three mCRC patients being monitored were as follows:

Case 1 This 37-year-old woman had been diagnosed with rectal adenocarcinoma and synchronous hepatic and pulmonary metastases. The initial molecular genetic examination of tumor tissue revealed RAS and BRAF wild-type status in this patient. Microsatellite instability was also ruled out. Following deep anterior rectum resection, a combined therapy with cetuximab and FOLFIRI was initiated. Due to repeated confirmation of a partial tumor response, this treatment was continued for altogether 33 cycles.

During this period, no RAS mutation was detectable by liquid biopsy (Figure 1*A*). Due to significant tumor response, a partial hepatectomy was performed (Figure 1*A*, *arrow*), and chemotherapy was paused. Three months later, chemotherapy was reinitiated recognizing cancer progression. After four additional cycles, KRAS codon 61 mutations were detectable at low frequency by liquid biopsy for the first time (Figure 1*A*). During the following two treatment cycles, the KRAS codon 61 mutational load increased more than 10-fold. As a response, we brought forward the follow-up imaging. As expected, it revealed progression of hepatic metastases (Figure 3*B*; Supplementary Figure S3). Subsequently, anti-EGFR therapy treatments were stopped, and anti-VEGFR therapy with aflibercept in combination with chemotherapy was started. Directly after therapy, there was a noted change in the whole amount of cfDNA, predominantly containing wild-type DNA, which increased four-fold (Figures 1*A* and 3*C*). Simultaneously, the number of KRAS mutated fragments declined rapidly within 6 weeks (2 cycles).

Follow-up imaging after 6 and 12 cycles of aflibercept with FOLFIRI in combination revealed stable disease, demonstrated by a reduction in size of two liver metastases (nearly 25% shrinkage). After 16 cycles (5 months) of anti-VEGFR therapy, the number of KRAS codon 61 mutated fragments increased again, and progressive disease was then visualized in follow-up imaging (Figure 1*A*). Measurement of PIK3CA mutations revealed that PIK3CA mutated fragments increased about 12-fold during this period (Figure 1*B*). Interestingly, tissue biopsy of the liver metastasis with the strongest growth was performed and showed no RAS mutations but showed a PIK3CA E545K mutation (Figure 1*B* and Supplementary Figure S3).

In summary, this case illustrates that RAS-mutated clones declined after the discontinuation of anti-EGFR treatment. We directly demonstrated the relative decrease of mutational fraction in the absence of selective pressure of an anti-EGFR treatment (Figure 1*C*). The absolute amount of mutated cfDNA fragments did not change significantly and remained stable, but the number of wild-type fragments increased after discontinuation of the anti-EGFR treatment.

Case 2 A 50-year-old woman had been diagnosed with deep-seated rectal carcinoma (4 cm ab ano) including synchronous liver metastases. The initial molecular genetic examination of tumor tissue revealed a RAS and BRAF wild-type status. Microsatellite instability had been ruled out. Neoadjuvant combined radiochemotherapy (5-fluoruracil) was initiated, resulting in dissociative response with regression of the primary tumor size but progressive disease of the hepatic metastases. A planned operation was not performed, and palliative chemoimmunotherapy with panitumumab and FOLFIRI was initiated. After an initial partial response, this treatment was continued for 12 cycles (7 months) until disease progression was then noted. Second-line chemoimmunotherapy comprised of cetuximab combined with FOLFOX was subsequently discontinued due to progressive tumor disease after four cycles (at 3 months). At this time point, liquid biopsy analyses of relevant RAS mutations confirmed the RAS wild-type status as originally defined by primary tumor tissue analysis (Figure 2*A*). Next, intensified chemotherapy with FOLFOXIRI and anti-VEGFR treatment with bevacizumab followed. However, disease progression was observed after only six cycles of this treatment (4 months). At this time point, KRAS codon 12 mutations became detectable for the first time in plasma samples. Interestingly, three additional mutations in KRAS codon 61, NRAS codon 12, and NRAS codon 61 were also detected following two cycles (2 months) of subsequent chemoimmunotherapy treatment with cetuximab and FOLFIRI (Figure 2*A*). No additional mutation of PIK3CA or BRAF could be detected. We reacted immediately and changed the therapy to aflibercept and FOLFIRI. Nonetheless, fatal disease progression was observed. During this final treatment phase, the mutation load of acquired KRAS codon 12, KRAS codon 61, NRAS codon 12, and NRAS codon 61 mutations quickly decreased (Figure 2*A*). On the contrary, the concentration of whole tumor cfDNA increased simultaneously with clinical disease progression (Figure 2*B*; Supplementary Figure S1). Twenty months after initial diagnosis, the patient died due to mCRC.

Case 3 In this 66-year-old man, rectum adenocarcinoma and hepatic metastases were diagnosed. Considering the initial RAS wild-type status of the primary tumor, initial treatment consisted of cetuximab and FOLFIRI. Despite treatment response (PR), cetuximab was withdrawn due to extensive skin toxicity after the sixth treatment cycle. Three cycles of FOLFIRI were administered subsequently. However, early progressive disease was addressed by switching therapy to bevacizumab and FOLFOX. Partial remission was observed after six cycles of this schedule. Liquid biopsy (BEAMing) at this time point revealed no RAS mutations (Figure 2). Progressive disease occurred following 12 additional cycles of bevacizumab and FOLFOX. Hence, therapy was again modified to FOLFIRI. Following four cycles of FOLFIRI and a radioablation of liver metastases, chemotherapy was stopped for 4 months. Now, staging demonstrated progressive disease, and liquid biopsy revealed KRAS codon 12 and NRAS codon 61 mutations (Figure 3; Supplementary Figure S2). No other mutation in RAS, PIK3CA, or BRAF oncogenes was detected. Strikingly, KRAS codon 12 and NRAS codon 61

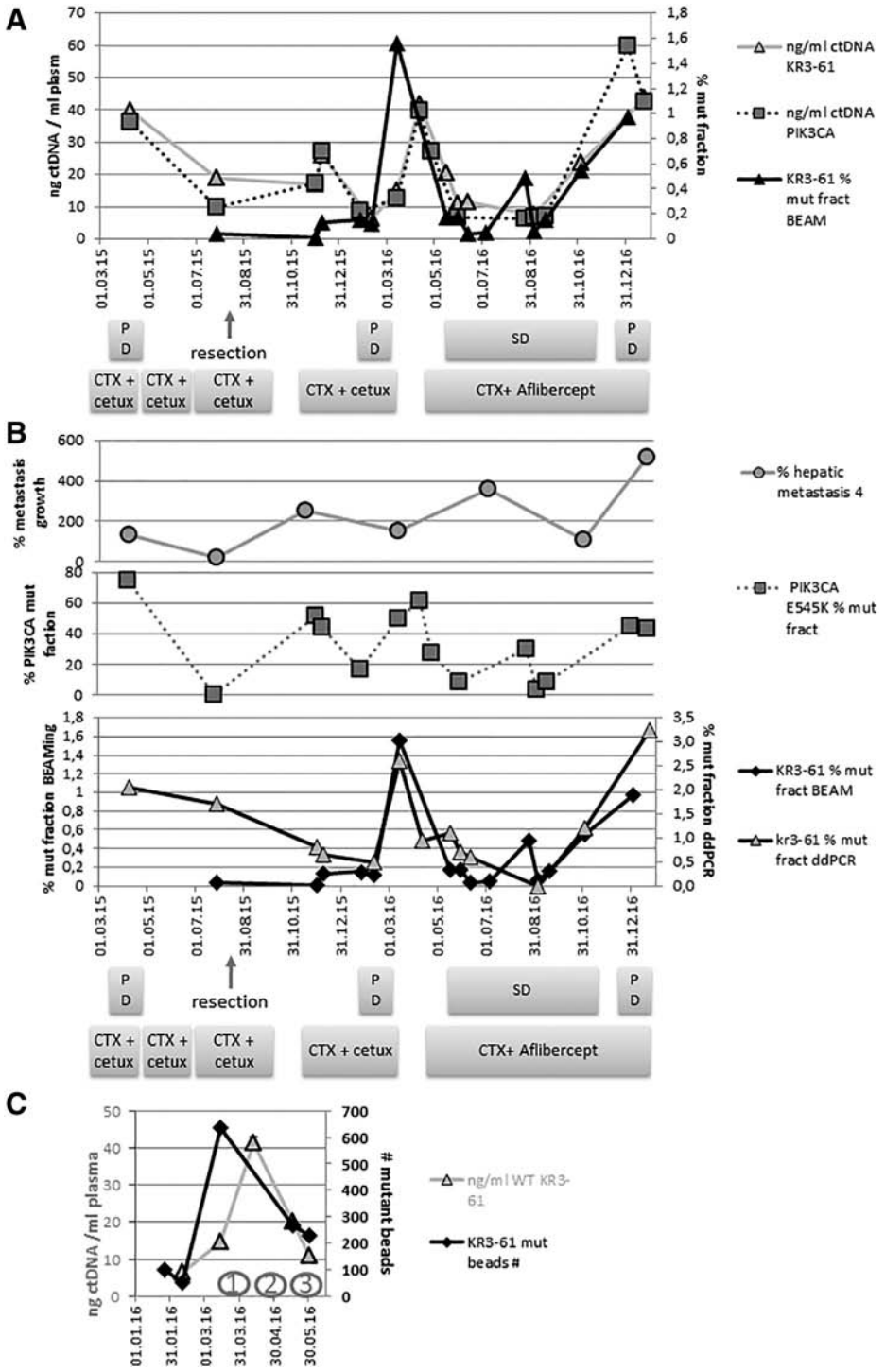

Figure 1. (A) Dynamics of KRAS and PI3KCA mutant clones in plasma samples of the patient in case 1. BEAMing results of KRAS Codon 61 mutation and PI3KCA mutations and amount of whole cf tumor DNA measured by ddPCR. The solid black line indicates the frequency of KRAS exon 3 mutation (percentage of alleles) and the doted line the frequency of PI3KCA mutation detected in cf DNA at the indicated time points. Progressive disease occurred after the long-term treatment (37 cycles) with anti-EGFR antibodies. Recently, the KRAS exon 3 mutation load increased more than 10-fold. After interruption of anti-EGFR therapy, the KRAS mutation load distinctly declined and rose again after 5 months of anti-VEGFR therapy simultaneously with progressive disease. (B) Dynamics of KRAS and PI3KCA mutant clones in plasma samples of the patient in case 1. Both lower curves show the load of KRAS codon 61 mutation (percentage of alleles) obtained by BEAMing and by ddPCR in a direct comparison. Both upper curves demonstrate the size progression of metastasis 4 and the course of PI3KCA mutation measured by ddPCR, which exhibit similar course. The tissue biopsy of the liver metastasis was performed and confirmed a PI3KCA mutation. (C) Clonal redistribution after discontinuation of therapy with anti-EGFR mAb. The black curve shows KRAS exon 3 mutations obtained by BEAMing and the green curve the amount of RAS-wild-type DNA measured by ddPCR. After the more than 10-fold increase of KRAS exon 3 mutation load, the anti-EGFR therapy was stopped, and an anti-VEGFR therapy in combination with CTX was initiated (see time point 1). As expected, the load of RAS-mutated DNA decreased in the blood rapidly (see time point 3). But remarkably, the amount of RAS-wild-type DNA increased at the same time four-fold (see time point 2). Due to simultaneous reduction in the size of measurable tumor masses, the increase of RAS wild-type DNA is the specific effect of therapy change.

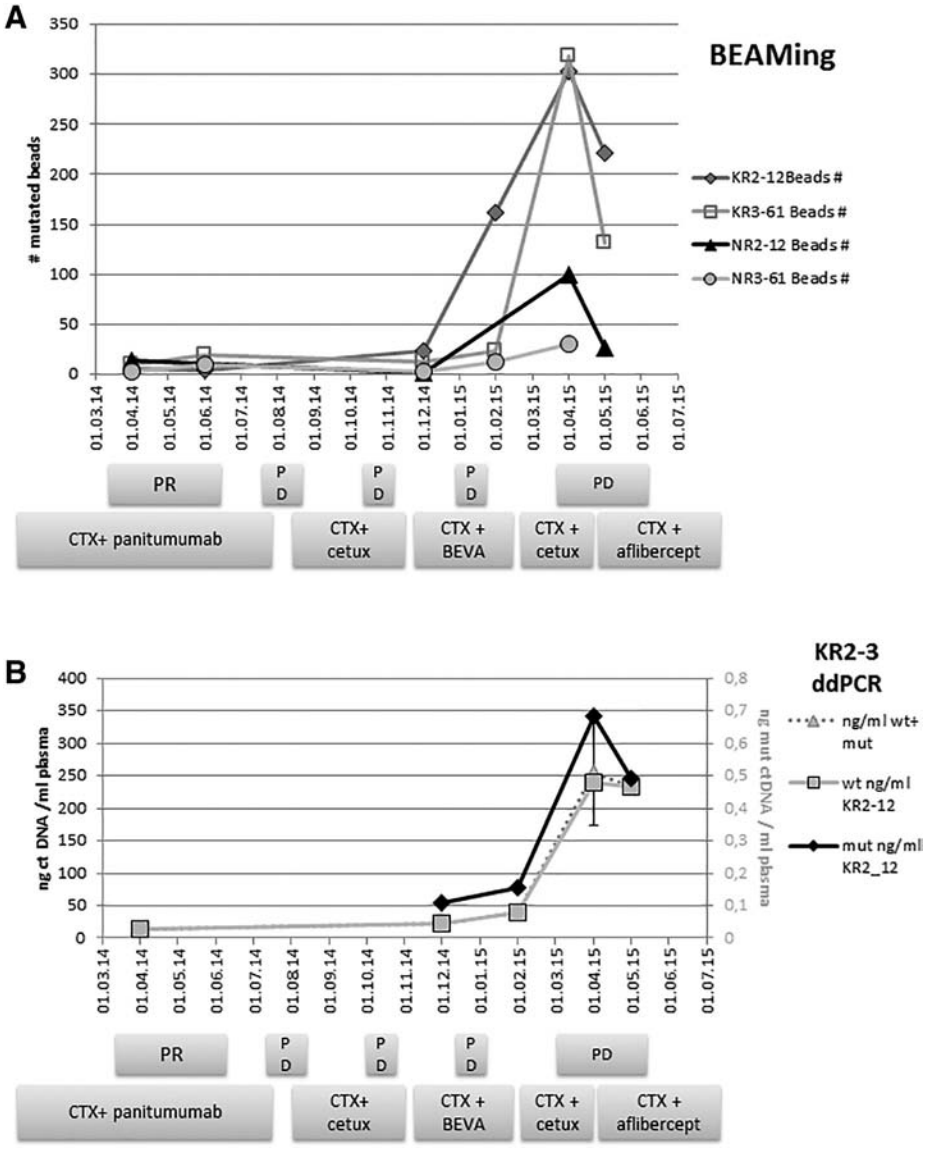

Figure 2. Dynamics of KRAS mutant cfDNA in plasma samples of the patient in case 1. (A) BEAMing results. (B) Results of ddPCR. After the initial tumor tissue testing revealed a RAS wild-type, chemotherapy (CTX) with panitumumab was continued for 7 months. After progression, second-line CTX with cetuximab was applied for 3 months. At that time, no RAS mutations were found in plasma. Intensified chemotherapy with FOLFOXIRI with bevacizumab followed and resulted in progress after 4 months. Only at this moment did KRAS codon 12 mutants become detectable for the first time in plasma. Furthermore, three additional RAS mutations (KRAS codon 61, NRAS codon 12, and NRAS codon 61) were measured after 2 months of application of subsequent CTX with cetuximab. After progression, aflibercept and CTX followed and resulted in massive progress 3 months afterward. The mutation load of KRAS codon 12 and KRAS codon 61 as well as NRAS codon 12 and NRAS codon 61 mutations decreased most rapidly due to the absence of continued selection pressure by anti-EGFR mAb treatment. Otherwise, the entire amount of cfDNA increased simultaneously with the progression of resistant metastases. Thus, RAS mutations emerged during treatment with cytostatic agents several months after anti-EGFR therapy was discontinued.

mutations became detectable despite the lack of mutant selective anti-EGFR agents for more than 1 year.

Discussion

Genomic instability is a key factor both in the emergence of cancer and in the evolution of cancer resistance properties toward anticancer agents. Genomic instability causes initially cellular heterogeneity that leads to intra- and intertumoral heterogeneity. Recent data indicate that RAS mutation status of primary tumors and metastases of colorectal cancer can differ up to 20% due to the intrinsic molecular heterogeneity of cancer [8–11]. Clonal diversity offers a selective advantage for cancer cell survival due to the higher probability of preexisting individual resistant clones and thus acquired resistance. Chemotherapies and especially targeted therapies mostly result in partial clinical responses and because the therapeutic treatment is a selective pressure that can subsequently induce clonal redistribution. Due to tumor heterogeneity, clonal redistribution is difficult to grasp in tissue sample that is fixed in time. Moreover, it is neither practical nor feasible to perform multiple tissue biopsies so as to visualize such clonal redistribution in time. Such restrictions are alleviated by employing liquid biopsies of serially collected blood samples from patients. Blood-based detection of tumor-specific alterations allows

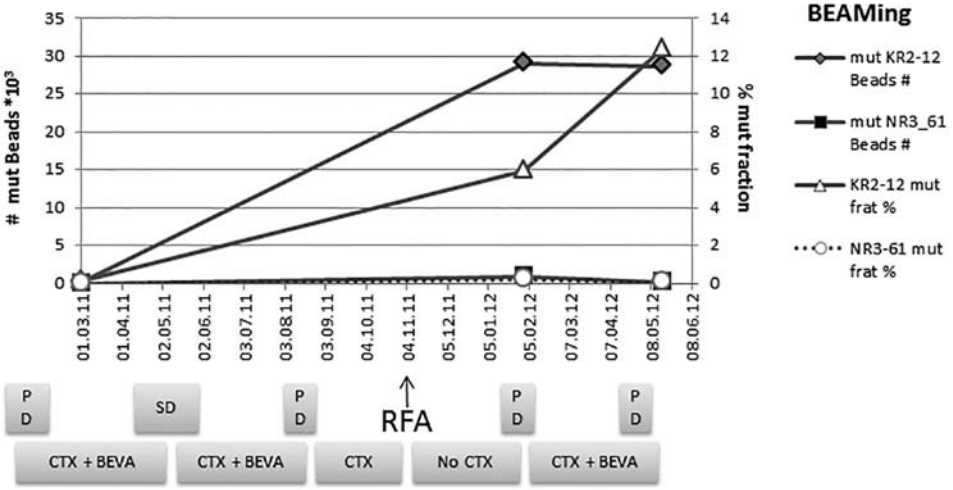

Figure 3. Dynamics of KRAS mutant clones of the patient in case 2 measured by BEAMing in plasma samples. After determination of RAS wild-type in tissue, six cycles of cetuximab and CTX resulted in a progress. The switch to anti-VEGFR mAb and CTX followed. The liquid biopsy after six cycles of subsequent treatment with bevacizumab and CTX showed no RAS mutations and confirmed the mutational statue based on tissue analysis 1, which occurred 5 years ago. Due to PD after further 12 cycles of bevacizumab and CTX, the therapy was changed to another CTX. After further four cycles CTX and RFA of the liver metastases, a chemotherapy-free phase followed for 4 months. The staging thereafter demonstrated PD. In the liquid biopsy from that time, KRAS codon 12 and NRAS codon 61 mutations were detected by beaming for the first time. The period between both liquid biopsies amounts to 1 year, and no anti-EGFR therapy was applied in this time.

characterization and monitoring of tumor heterogeneity as well as treatment-related dynamic changes of molecular profiles independently of tumor location.

The procedure of liquid biopsy (i.e., blood-based mutation analysis of cell-free circulating tumor DNA) is well established [5,12,13]. Within our study, we took the next step and compared the two most sensitive methods. We demonstrated that the OncoBEAM RAS measurement is more sensitive than the ddPCR. The value of RAS mutation frequencies differed between the two methods, but the changes followed the same time course. However, the ddPCR measurements delivered quantitative findings without any bias of PCR preamplification step.

A classic example for the characterization of clonal redistribution is given in setting of anti-EGFR mAb therapy of colorectal cancer: Newly detected KRAS mutations occur during the anti-EGFR mAb treatment and are predictive for reduced benefit from anti-EGFR mAb therapy [7,14–16]. This is reasoned by predominant suppression of RAS wild-type clones during anti-EGFR mAb therapy and, in this manner, indirect selection of RAS mutated clones. However, after the discontinuation of EGFR inhibition, RAS mutational load rapidly decreases within a few weeks, probably due to the lack of selective pressure by the anti-EGFR mAb therapy [7,15–17]. Supported by data of Santini et al., retreatment (aka rechallenge) with anti-EGFR mAb therapy is currently under active investigation in clinical trials [18].

The previous reports dealing with the detection of emerged RAS mutations in periodically collected plasma samples differ considerably from our study. Morelli et al. and Siravegna et al. focused on the evidence of emerging RAS mutations and decrease of mutational load after discontinuation of anti-EGFR mAb therapy [14,15]. Toledo et al. addressed in particular the question of prognostic value of the emerged RAS mutations and a limit of RAS mutant frequency [16].

The data presented in the current study show that the theoretical concepts of anti-EGFR rechallenge have a number of practical considerations based on the therapy history and trajectory of both mutational and tumor loads of each patient. The three presented clinical cases represent highly individualized profiles within a diverse spectrum of clonal selection and redistribution possibilities that result in acquired resistance. RAS mutated subclones are clearly selected during the course anti-EGFR therapy. The application of ddPCR enabled us to quantify the wild-type ctDNA and to be the first to make the clonal redistribution after discontinuation of therapy with anti-EGFR mAb directly visible (Figure 1*C*). Therein, we demonstrated not only the expected decrease of RAS-mutated DNA in blood but also the increase of the amount of RAS wild-type DNA at the same time. Due to simultaneous reduction in the size of measurable tumor masses, the increase of RAS wild-type DNA is the specific effect of therapy change. Altogether, using the measurement of RAS wild-type DNA, we finally succeed to monitor the initially invisible cancer cells quasi "the dark side of the moon."

Remarkable findings were observed in cases 2 and 3: RAS mutated subclones became detectable several months after anti-EGFR therapy was discontinued (Figures 1-3). To our knowledge, our observation of emerged RAS mutations occurring without direct selective pressure by anti-EGFR therapy is unique and poses a number of challenges in the clinical setting (Figures 2 and 3). Certainly, further studies are needed to determine whether these RAS mutated subclones arise through a targeted selection or they appear as a rare event brought about by generally mutagenic cytostatic treatment. Due to the high specificity of BEAMing and ddPCR, the observed new mutations are true and not artifactual.

The example of patient 1 reveals the many-faceted nature of the cancer and discloses the weak points of sole tissue findings. Even though multiple molecular tissue studies were carried out within 4 years of therapy, some crucial facets or mutations of the cancer still remained hidden. The primary tumor and six metastases were either removed or biopsied. Nonetheless, the RAS mutations that play a decisive part in the course of disease were not found. These data on the effective use of liquid biopsy monitoring during therapy demonstrate the need to reevaluate and to determine more precise timing as well as anatomical sampling of tissue biopsies taken during the course of disease of colorectal cancer. Indeed, blood-based liquid biopsy longitudinal monitoring enables the

detection of all actual therapy-relevant driver mutations at different times so as to have better temporal sampling of tumor evolution as well as relating selective pressures to the emergence of certain clones during progressive disease.

If we link the changes of mutation load with the radiological follow-up data, we receive indirect evidence about the mutational profiles of the single metastases. In case 1, the development of liver metastases in contrast to lung metastases correlated with the levels of PIK3CA mutations. The biopsy of liver metastasis, which closely correlated with the PIK3CA mutation load, revealed the PIK3CA mutation and confirmed our hypothesis (Figure 1*C*). These findings may be useful for selective surgical or ablative treatments of individual metastases, for example, by pointing out the metastases with unfavorable molecular profile (e.g., with BRAF or PIK3CA mutation).

The fact that *de novo* RAS mutated subclones arise during the anti-EGFR mAb therapy may be an explanatory model for the results of FIRE-3-study, which showed the outstanding significance of the sequence of drug application [19]. In patients with RAS wild-type tumors, first-line application of anti-EGFR mAb therapy demonstrated a favorable condition for promoting effective subsequent therapies compared to an anti-VEGFR containing first-line therapy [3]. These data comply with previous reports proposing that anti-EGFR mAb therapy was considerably less effective when applied subsequently after anti-VEGFR containing first-line therapy [20,21]. According to our proposal, patients that develop RAS mutations due to anti-VEGFR therapy reveal reduced response to subsequent anti-EGFR–based therapies.

Another consequence of emergence of *de novo* RAS mutations due to therapies without anti-EGFR mAb is that we need to monitor RAS mutations in all RAS wild-type patients in general and independent of the therapy.

Certainly, the presented data raise more questions than answers. Nevertheless, these new observations create awareness of the obstacles clinicians need to overcome so as to offer highly individualized treatment plans based on current tumor mutational data that are correlated with imaging results. Definitely, the presented data need the validation on larger collective.

In summary, it can be emphasized that liquid biopsy is a perfectly suited method to detect emerging mutations in mCRC. In contrast to tissue biopsy, it reflects tumor heterogeneity and clonal evolution. The joint reflection of liquid biopsy and radiological imaging allows the allocation of mutation profile of individual metastases. Emerging RAS mutations during therapy with anti-EGFR mAb reflect resistance to anti-EGFR mAb. Moreover, the development of RAS mutated clones may proceed regardless of direct selective pressure by anti-EGFR therapy. These insights have to be taken into account in decision making about the optimal therapy for patients with mCRC.

Authorship

Contribution: Data generation: C. E. M., S. K. S., M. M., A.B. Analyses of the data: S. K. S., A. B. Contribution to the discussion: M. P., R. S., W. S. Wrote the manuscript: S. K. S., R. S., A. B.

Conflict-of-interest disclosure: A. B. has received honoraria for lectures or travel grants from Sysmex, Takeda, Servier, and Merck Serono. W. S. consulted for AiCuris, Amgen, Apceth, AstraZeneca, Indivumed, Merck Serono, Roche, and 4SC and received honoraria for lecture from Abbott, Falk Foundation, GSB GmbH, Lilly Deutschland GmbH, Merck Serono, Pfizer, Roche, and Sanofi Aventis. The remaining authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2017.12.010.

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