KRAS Genotypic Changes of Circulating Tumor Cells during Treatment of Patients with Metastatic Colorectal Cancer

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Abstract

Introduction: Circulating tumor cells (CTCs) could represent a non-invasive source of cancer cells used for longitudinal monitoring of the tumoral mutation status throughout the course of the disease. The aims of the present study were to investigate the detection of KRAS mutations in CTCs from patients with metastatic colorectal cancer (mCRC) and to compare their mutation status during treatment or disease progression with that of the corresponding primary tumors.

Materials and Methods: Identification of the seven most common KRAS mutations on codons 12 and 13 was performed by Peptide Nucleic Acid (PNA)-based qPCR method. The sensitivity of the assay was determined after isolation of KRAS mutant cancer cells spiked into healthy donors’ blood, using the CellSearch Epithelial Cell kit. Consistent detection of KRAS mutations was achieved in samples containing at least 10 tumor cells/7.5 ml of blood.

Results: The clinical utility of the assay was assessed in 48 blood samples drawn from 31 patients with mCRC. All patients had PIK3CA and BRAF wild type primary tumors and 14 KRAS mutant tumors. CTCs were detected in 65% of specimens obtained from 74% of patients. KRAS mutation analysis in CTC-enriched specimens showed that 45% and 18.7% of patients with mutant and wild type primary tumors, respectively, had detectable mutations in their CTCs. Assessing KRAS mutations in serial blood samples revealed that individual patient’s CTCs exhibited different mutational status of KRAS during treatment.

Conclusions: The current findings support the rationale for using the CTCs as a dynamic source of tumor cells which, by re-evaluating their KRAS mutation status, could predict, perhaps more accurately, the response of mCRC patients to targeted therapy.

Introduction

The association between KRAS mutations and response to EGFR inhibitors has been established in multiple studies; consequently, KRAS genotyping is recommended in all patients with metastatic colorectal cancer (mCRC) before any therapy that utilizes the EGFR-targeted monoclonal antibodies, cetuximab or panitumumab [1]. Nevertheless, not all patients with KRAS wild type tumors respond to EGFR-targeted therapies and the majority of the initially responsive patients experienced disease progression within 5 to 6 months [2].

Considering that most of the studies have been conducted using tissue obtained from the primary tumor whereas EGFR monoclonal antibodies have been used to treat the metastatic disease, it is possible that the lack of efficacy and/or the emergence of subsequent resistance may be due to genetic diversification of metastatic cells compared to their primary tumor counterparts or to dynamic variations in tumor genotype or phenotype that emerge during treatment.

Several studies have shown discordant mutation status between primary tumors and corresponding metastasis in a proportion (5%–30%) of CRC patients [3,4,5,6]. Furthermore, recent studies suggest that acquired resistance is partly achieved by the selection...
of pre-existing minor sub-clones harboring mutations conferring resistance to anti-EGFR therapy [7,8]. Because invasive biopsies of metastatic sites are not always feasible and cannot be easily performed repeatedly, circulating tumor cells (CTCs) in the peripheral blood of cancer patients, which are thought to mediate the hematogenous spread of disease to distant sites, may represent an alternative source of metastasizing tumor cells.

It is well documented that CTCs, as defined by the FDA-approved CellSearch System, could serve as a marker of micrometastatic tumor load associated with patients’ prognosis and can accurately predict effectiveness of therapy in metastatic breast, colorectal, prostate and lung cancer [9,10,11,12]. Previous studies in metastatic colorectal cancer suggested that the absolute number and the numerical variations of CTCs during disease progression or therapy can provide valuable information for the clinical outcome and the efficacy of administered treatments [13,14,15,16,17,18]. However, CTCs cannot always be identified in metastatic patients, emphasizing the need to develop more sensitive and cancer type-specific CTC detection assays [19]. In this context, the identification of oncogenic mutations in CTCs could contribute to the improvement of existing detection methods. Moreover, genotyping of CTCs could possibly improve the monitoring of response to targeted therapies by identifying genomic profiles predictive of disease recurrence prior to clinical disease progression [20,21,22,23,24].

The aim of this study was to investigate the feasibility of detecting KRAS mutations in CTC-enriched fractions in patients with mCRC. Additional objectives were to evaluate whether KRAS mutation status of CTCs correlates with that of corresponding primary tumors and examine the genetic heterogeneity of CTCs in respect to KRAS mutation status during treatment.

Materials and Methods

Patients

Thirty-one patients with metastatic colorectal cancer were enrolled in the current study. In all patients, diagnosis was confirmed by histologic examination of the primary tumor before the initiation of any systemic therapy. All but one patient were treated with 5-FU-based first-line combination chemotherapy, with or without a biological agent (bevacizumab or panitumumab). Nineteen (55%) patients received an irinotecan-based combination and 11 (37%) an oxaliplatin-based regimen in the first-line setting (one patient did not receive any treatment). Additionally, 25 (83%) patients received bevacizumab and two (7%) panitumumab. At the time of analysis, 19 patients presented disease progression to first-line treatment and 12 of them were treated with a second-line chemotherapy regimen; five out of these 12 patients were treated with panitumumab in combination with chemotherapy.

Peripheral blood was analyzed for the presence of CTCs before initiation of first-line treatment in 12 patients, at the time of progression on first-line treatment in 9 and at any time during treatment in 18 patients.

All patients as well as 16 healthy blood donors, who had no known illness and no history of malignant disease, were tested for the presence of CTCs using the CellSearch Epithelial Cell Kit (Veridex LLC). Peripheral blood was obtained from mCRC patients (7.5 ml) and healthy donors (23 ml, for use in spiking experiments) by vein puncture in the specific CellSave tubes; in order to avoid contamination of the samples with epithelial cells the first 5 ml of the blood was discarded. All tests were performed within 72 hours from the blood draw according to the manufacturer’s instructions. Cells which were CD45−/CD45+/CD20− and had a DAPI-positive intracellular nucleus were characterized as CTCs by experienced biologists (E.P. and S.A.).

Since this study was a pilot feasibility study, there was no statistically sample size estimation and specimen collection was based on the availability of CellSearch cartridges.

Ethics statement

All patients gave their written informed consent to participate in the study which has been approved by the Ethics and Scientific Committees of the University General Hospital of Heraklion, Crete, Greece; the manuscript was prepared according to the REMARK criteria [25].

Cell lines

Cancer cell lines harboring KRAS mutations [LS174T, Human colon adenocarcinoma, c.35G>A (p.G12D); HCT116, Human colon adenocarcinoma, c.38G>A (p.G13D); HUP-T3, Human pancreatic adenocarcinoma, c.34G>C (p.G12R); KYSE410, Human oesophageal squamous cell carcinoma, c.34G>T (p.G12C); A549, Human alveolar adenocarcinoma, c.34G>A (p.G12S); SW403, Human colon adenocarcinoma, c.35G>T (p.G12V) and RPMI8226, Human myeloma, c.35G>C (p.G12A)] or wild type for KRAS (HT-29, Human colon adenocarcinoma) originated from the American Type Culture Collection (ATCC, USA) and were kindly provided from Prof. A. Jung (Institute of Pathology, Ludwig-Maximilian-University, Munich, Germany). All cell lines were cultured in flasks according to supplier’s recommendations, before subsequent harvesting using 0.25% trypsin and 5 mmol/L EDTA (GIBCO-BRL). Authentication was done by determining the KRAS mutational status of each cell line by Sanger sequencing. All cell lines except SW403 were heterozygous for KRAS mutations and revealed the expected genotype.

DNA isolation from CTC-enriched fractions and tissues

Following CTC counting, the captured cells were transferred from the chamber to a 2 ml tube and subjected to a Proteinase K digestion at 65 °C for 2–5 h; DNA isolation was performed using the Epicentre MasterPure Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturers’ instructions and the extracted DNA was quantified using a NanoDrop ND1000 (NanoDrop Technologies, USA) and stored at −20 °C until used. The average yield of DNA was ~1.5 μg; however, DNA quantification by UV spectrophotometry is not precise due to the detection of single stranded DNA, free nucleotides or RNA in the sample.

Formalin-fixed paraffin-embedded (FFPE) primary tumor-tissue was evaluated histologically by a pathologist (MT) and microdissection was performed to increase the percentage of tumor cells. Three 5 μm tissue sections were deparaffinised by xylene and ethanol washes and subjected to a Proteinase K digestion overnight at 56 °C DNA was then purified using a QIAamp DNA Micro Kit (Qiagen, Germany).

Mutation analysis in primary tumors

In consenting patients, primary tumors were evaluated for mutations in KRAS, PIK3CA and BRAF by both Sanger sequencing and methodologies with high sensitivity as previously described [26].

KRAS mutation assay

A mutation assay that combined the peptide nucleic acid (PNA)-mediated PCR clamp with TaqMan-MGB allelic discrimination
assays has been previously developed to detect the seven more common \textit{KRAS} mutations \cite{6}. The thermal conditions for PNA mediated PCR clamping for each of the seven \textit{KRAS} mutant templates were further optimized by using a PNA labeled with a fluorescent dye as both sensor probe and PCR clamp according to Luo JD et al \cite{27}. The greatest selectivity, i.e. high \textit{Ct} value in the wild-type probe and low \textit{Ct} value in the mutant probe was achieved at 62°C with 200 nM PNA for the c.35G>A (p.G12D), c.38G>A (p.G13D) and c.35G>T (p.G12V) and 150 nM PNA for the c.34G>C (p.G12R), c.34G>T (p.G12C), c.34G>A (p.G12A) and c.35G>C (p.G12A) \textit{KRAS} mutant templates respectively. The reactions were performed on Applied Biosystems 7900HT Real-Time PCR System in 384-well plates in a total volume of 5 \textmu l containing 2 \textmu l (approximately 1/8 of total extracted DNA from CTC-enriched specimens and 20 ng extracted DNA from FFPEs) DNA, 2.5 \textmu l 2X TaqMan genotyping master mix and 150 or 200 nm PNA; in parallel, a non-PNA-clamp reaction was performed. The PCR conditions were 95°C for 10 min, followed by two-step cycling: 50 cycles of 92°C for 15 s, and 62°C for 90 s. All samples were run at least in duplicates. \textit{Ct} values were obtained from the instrument's real-time PCR data collection software using 0.2 manual threshold. A positive control for each \textit{KRAS} mutation (model samples that comprised mixtures of cell lines with mutated/wild type ratios 1/100 and 1/500) and a negative control (\textit{KRAS} wild type cell line) in total input of 50 ng were included in each run.

The $\Delta$\textit{Ct} = \textit{Ct}mut probe (+PNA) - \textit{Ct}wt probe (-PNA) value was computed for each sample; \textit{Ct}mut probe (+PNA) and \textit{Ct}wt probe (-PNA) denoted the \textit{Ct} (cycle threshold \textit{Ct}, is termed as the cycle number at which a signal is detected above background fluorescence) values for the mutant and wt probes of the reactions with and without PNA, respectively. The \textit{Ct}wt probe (-PNA) reflects the amount of \textit{KRAS} mutant DNA within the sample while the \textit{Ct}mut probe (+PNA) values for the mutant and wt probes of the reactions with and without PNA, respectively. The $\Delta$\textit{Ct} = \textit{Ct}wt probe (-PNA) - \textit{Ct}mut probe (+PNA) reflects the amount of amplifiable template derived from the varying numbers of contaminating leukocytes in CTC fraction \cite{28}.

\textbf{Assay validity} \\
Validity of the assay result for each sample was determined by the $\Delta$\textit{Ct} value that should be $24 \leq \Delta$\textit{Ct} < 32; if the \textit{Ct}wt probe (-PNA) in a sample is greater than 32, the assay result is not reliable because of a low amount of DNA or failed target amplification. Efficient PCR PNA clamping was confirmed by a \textit{Ct}mut probe (+PNA) value > 45. The \textit{KRAS} mutation status was determined by comparison of the $\Delta$\textit{Ct} values to previously defined $\Delta$\textit{Ct} cutoff values for each of the seven more common \textit{KRAS} mutations. The cutoff values for each mutation assay have been determined from analysis of 16 healthy donors’ blood samples following CellSearch analysis. As cutoff value was defined the $\Delta$\textit{Ct} corresponding to the maximum specificity; that is, no mutation detected in 100% (16/16) of healthy donors (Table 1, Figure 1).

\textbf{Analytical specificity and sensitivity} \\
The analytical specificity of the seven assays was individually evaluated using genomic DNA (gDNA) isolated from \textit{KRAS} wild-type cell line (HT29) or positive for specific \textit{KRAS} mutations; the lower limit of detection for all assays was 0.015 ng. To assess analytical sensitivity, each of the seven \textit{KRAS} mutant cell line gDNA was serially diluted over a range of three different concentrations (100 ng, 50 ng and 20 ng) of wild-type gDNA provided by the HT-29 cell line to give mutation/wild-type ratios of 100%, 50%, 10%, 2%, 1%, 0.5%, 0.1% and 0%. All assays have a sensitivity of 0.1%, keeping the total input constant at

| Table 1. Analytical sensitivity of \textit{KRAS} mutation detection assay. |
|-------------------|-------------------|-------------------|-------------------|
| \% of mutant/wild type DNA | 100 | 50 | 10 |
| 1 | 24.8 | 19.4 | 14.3 |
| 0.5 | 25.4 | 19.9 | 14.8 |
| 0.1 | 26.4 | 20.2 | 14.8 |
| 0.01 | 27.4 | 20.9 | 14.8 |
| 0.001 | 28.4 | 21.9 | 14.8 |
| Cut off | 12.5 | 12.5 | 12.5 |

For every assay, 0.01% to 100% of mutant cell line gDNA was mixed into a background wild type gDNA keeping the total input constant at 20 ng. The numbers indicate the $\Delta$\textit{Ct} value, the cutoff value mutuated threshold for each.
20 ng, except the assays for the detection of p.G13D and p.G12V that have a sensitivity of 0.5% (Table 1).

Cross reactivity
Cross-reactivity tests were performed for all mutation assays that potentially identify another assay’s template due to imperfect base-pairing and read-through. The results indicate that the G12D assay shows significant cross-reactivity with the G12V and G12A mutant templates, the G13D assay with the G12S mutant template, the G12R assay with the G12C and G12S mutant templates and the G12C assay with the G12S mutant template (Table 2).

Intra- and inter-assay precision
Intra-assay precision has been evaluated by analyzing model samples run in triplicate in the same experiment using serial dilution (100%, 50%, 10%, 2%, 1%, 0.5% and 0.1%) of KRAS mutant DNAs, as mentioned above, in 20 ng of background KRAS wild type DNA. The ΔCt coefficients of variation for the KRAS mutated DNA ranged between 0.3% and 2.5%. Inter-assay reproducibility has been similarly measured by calculating the ΔCt coefficients of variation of triplicate samples run in different days using the same serial dilutions of mutated DNA. The ΔCt coefficients of variation for the KRAS mutated DNA ranged between 0.4% and 2.5%. Furthermore, in model samples with a mutated/wild type ratio 0.5%, the overall mutation assay failure rate was 0%.

Results
Mutation assay optimization
The sensitivity of our experimental approach to determine accurately the KRAS mutation status in a limited number of
CTCs present among normal blood cells in circulating blood was validated by spiking KRAS mutant cancer cells into the blood of healthy donors. Approximately 100 and 10 tumor cells were spiked into 7.5 ml of blood in CellSave tubes and analyzed by CellSearch within 2 days, in at least 3 independent experiments. The average percent recovery for the 100 and 10 spiked cells [LS174T, c.35G>A (p.G12D), n = 5 experiments; HCT116, c.38G>A (p.G13D), n = 4 experiments; SW403, c.35G>T (p.G12V), n = 4 experiments and KYSE410, c.34G>T (p.G12C), n = 3 experiments] was 95% (range, 70%–100%) and 92.5% (range, 25%–130%), respectively. The broad range of recovery can be attributed to the error associated with spike-in low numbers of cells. Following CTC enumeration by the CellSearch Epithelial Cell kit, DNA was extracted from the captured cells and specimens were analyzed for all seven KRAS mutations. By this assay, KRAS mutations could be consistently identified from 10 spiked tumor cells.

Patients’ characteristics and evaluation of CTCs

Patient’s demographics, clinical and pathological characteristics are listed in Table 3. KRAS mutations were identified in the primary tumors of 45% of patients; eight (57%) patients’ primary tumor harbored the c.35G>A (p.G12D) mutation, three (21%) the c.35G>T (p.G12V), one (7%) the c.38G>A (p.G13D), one (7%) the c.34G>T (p.G12C) and one (7%) the c.35G>C (p.G12A). All tumors were PIK3CA and BRAF wild type. The median time between surgical resection of the primary tumor and analysis of CTCs was 6 months (range 1 to 134).

A total of 46 blood samples were obtained from 31 patients for CTC enumeration using CellSearch (Table 4). Twelve (39%) patients were chemotherapy naive at the time of first blood draw. Twelve (71%) and 11 (75%) patients with KRAS wild type and mutant primary tumors, respectively, had detectable CTCs; a median of 9 (range 2 to 660) and 7 (range 1 to 865) CTCs were detected in patients with KRAS wild type and mutant primary tumors, respectively. In total, 1 or more CTCs could be detected in 31 (65%) blood samples obtained from 23 (74%) patients (Table 4). KRAS mutation analysis on codons 12 and 13 was performed in all specimens with detectable CTCs (Figure 1).

KRAS mutations in patients’ CTC-enriched samples

KRAS mutations could be identified in the CTCs of five (45%) patients whose primary tumors had mutated KRAS. In particular, before the initiation of 1st line chemotherapy only two of the six chemotherapy naive patients (#1 and #5) harbored CTCs with detectable mutations while at the time of disease progression, mutations could be identified in one (#8) of the two patients; mutations were also detectable during the follow-up of 1st line treatment in two patients (#6 and #10) (Table 4).

KRAS mutations could also be identified in the CTCs of two (17%) patients (#15 and #16) whose primary tumors had no detectable mutations; in both patients, CTCs were collected during the monitoring of 1st line chemotherapy (Table 4).

KRAS mutational status of CTCs in serial blood samples

Four serial blood samples of patient #1 had detectable CTCs; KRAS mutations were documented even after the administration of the first chemotherapy cycle despite the important decrease of the number of CTCs; treatment continuation (after the 3rd cycle) resulted in further decrease of the CTC number and undetectable KRAS mutations in CTC-enriched specimens while assessment of treatment response revealed a partial response; after the 8th chemotherapy cycle, the number of CTCs was increased and

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Table 2. Cross reactivity pattern of KRAS mutation detection assay.

| KRAS mutation detection assay | G12D | G12A | G12C | G12S | G12V | G12R |
|------------------------------|------|------|------|------|------|------|
| LS174T (c.35G>A; p.G12D)    | 1.6  | —    | —    | —    | 0.5  | —    |
| HCT116 (c.38G>A; p.G13D)    | —    | 1.7  | —    | —    | 0.9  | —    |
| HUPT3 (c.34G>C; p.G12R)     | —    | —    | 1.4  | —    | 5.8  | —    |
| KYSE410 (c.34G>T; p.G12C)   | —    | —    | 0.5  | —    | 6.4  | —    |
| A549 (c.34G>A; p.G12S)      | —    | —    | —    | —    | 4.5  | —    |
| SW403 (c.35G>T; p.G12V)     | —    | —    | —    | —    | 0.5  | —    |
| RPMI8226 (c.35G>C; p.G12A)  | —    | —    | —    | —    | —    | 0.6  |

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Table 3. KRAS mutations identified in the primary tumors of patients.

| Patient ID | KRAS mutation |
|------------|---------------|
| #1         | c.35G>A (p.G12D) |
| #2         | c.38G>A (p.G13D) |
| #3         | c.35G>T (p.G12V) |
| #4         | c.34G>T (p.G12C) |
| #5         | c.35G>C (p.G12A) |

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Table 4. Summary of CTC enumeration in patients.

| Patient ID | CTCs detected | Detected Mutations |
|------------|---------------|--------------------|
| #1         | Yes           | KRAS c.35G>A (p.G12D) |
| #2         | Yes           | KRAS c.38G>A (p.G13D) |
| #3         | Yes           | KRAS c.35G>T (p.G12V) |
| #4         | No            | No                 |
| #5         | Yes           | KRAS c.35G>C (p.G12A) |

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Table 5. KRAS Mutations in CTC-Enriched Samples.

| Patient ID | CTCs detected | Detected Mutations |
|------------|---------------|--------------------|
| #1         | Yes           | KRAS c.35G>A (p.G12D) |
| #2         | Yes           | KRAS c.38G>A (p.G13D) |
| #3         | Yes           | KRAS c.35G>T (p.G12V) |
| #4         | No            | No                 |
| #5         | Yes           | KRAS c.35G>C (p.G12A) |

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KRAS mutations could be detected again while the patient still remained in partial response (Table 4).

In patient #15 who had a KRAS wild type primary tumor, no KRAS mutations could be detected in the CTC-enriched specimen when the patient was in partial response and under treatment with maintenance panitumumab; however, after 4 months of treatment, at the time of disease progression, as documented by the radiological worsening of hepatic lesions and clinical appearance of ascites, the number of CTCs was increased and KRAS mutations could be identified in CTCs-enriched cell fraction. KRAS mutations were further detectable after the 2nd cycle of salvage chemotherapy despite the decrease of the CTC number (Table 4).

**Discussion**

Mutations in KRAS result in the constitutive activation of the RAS/MAPK pathway and predict lack of response to anti-EGFR monoclonal antibodies. To date, therapy decisions rely mainly on KRAS mutation status of the primary tumor; however, genetic changes occurring during disease progression and acquired resistance to treatment may alter tumor’s biology. Therefore, an important question concerns the possibility to use CTCs as a non-invasive alternative of a new biopsy which could be more representative of the current biological status of tumor cells and might be used as a biomarker of either sensitivity or acquired resistance to EGFR targeted therapy.
**Table 4. KRAS mutation status in primary tumor and corresponding CTC-enriched samples in mCRC patients.**

| Patient ID | KRAS status | CTC enriched fraction | KRAS status | Time point |
|------------|-------------|-----------------------|-------------|------------|
| 1          | c.35G>A; p.G12D | 102 c.35G>A; p.G12D | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 12         | c.35G>A; p.G12D | 2 Post cycle 1 of 1<sup>st</sup> line |
| 9          | NVD | Post cycle 3 of 1<sup>st</sup> line |
| 42         | c.35G>A; p.G12D | Post cycle 8 of 1<sup>st</sup> line |
| 2          | c.35G>A; p.G12D | 243 NVD | Prior 1<sup>st</sup> line Folfox |
| 3          | c.35G>A; p.G12D | 1 NVD | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 4          | NVD | Post cycle 3 of 1<sup>st</sup> line |
| 5          | c.34G>T; p.G12C | 2 NVD | Prior 1<sup>st</sup> line Folfox |
| 6          | c.35G>G; p.G12D | 3 c.35G>G; p.G12D | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 7          | c.35G>G; p.G12A | 11 c.35G>G; p.G12A | Post cycle 3 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 8          | c.35G>G; p.G12V | 7 c.35G>G; p.G12V | Progression on 1<sup>st</sup> line Folfox/Bevacizumab |
| 9          | c.35G>A; p.G12D | 0 - | Post cycle 8 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 10         | c.38G>G; p.G13D | 9 c.38G>G; p.G13D | Post cycle 6 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 11         | c.35G>G; p.G12D | 0 - | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 12         | c.35G>G; p.G12D | 5 NVD | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 13         | c.35G>G; p.G12D | 4 NVD | Post cycle 1 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 14         | c.35G>G; p.G12D | 0 - | Post cycle 6 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 15         | wt | 3 NVD | Maintenance therapy with Panitumumab |
| 17         | wt | 0 - | Progression on 1<sup>st</sup> line therapy |
| 18         | wt | 10 NVD | Without treatment |
| 19         | wt | 660 NVD | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 20         | wt | 3 NVD | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 21         | wt | 0 - | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 22         | wt | 2 NVD | Post cycle 3 of 1<sup>st</sup> line therapy |
| 23         | wt | 0 - | Prior 1<sup>st</sup> line Folfox/Bevacizumab |
| 24         | wt | 17 NVD | Post cycle 6 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 25         | wt | 0 - | Follow up |
| 26         | wt | 173 NVD | Post cycle 2 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 27         | wt | 11 NVD | Post cycle 6 of 1<sup>st</sup> line Folfox/Bevacizumab |
| 28         | wt | 8 NVD | Post cycle 6 of 1<sup>st</sup> line Xeloda/Bevacizumab |
| 29         | wt | 4 NVD | Progression on 1<sup>st</sup> line Folfox/Bevacizumab |
| 30         | wt | 3 NVD | Progression on 1<sup>st</sup> line Folfox/Bevacizumab |
| 31         | wt | 0 - | Progression on 1<sup>st</sup> line Folfox/Bevacizumab |

*CTC count using CellSearch system depicted as number of cells per 7.5 mL whole blood. Abbreviations: wt; wild type, NVD; no variant detected.

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The results of the current study clearly indicate the feasibility to determine the KRAS mutation status in CTC-enriched blood samples in the context of routine clinical practice, following CTCs enumeration by the CellSearch system. The presented data strongly support the hypothesis that CTCs may represent a real-time source of liquid biopsy which may allow the dynamic genotyping of tumor cells during treatment. The data also indicate that the established assay provides a detection sensitivity of approximately 10 mutated cells/7.5 ml of blood, without the need of whole genome amplification, and offers the possibility for a non-invasive, rapid and low-cost serial monitoring of the KRAS mutation status in codons 12 and 13 during the course of treatment.

KRAS mutations were identified even in clinical specimens containing as low as 3 CTCs/7.5 ml of blood; we cannot exclude that this may be due to the presence of KRAS mutations in CTC fragments which are not considered as real CTCs during the documentation and enumeration of CTCs by the CellSearch system and/or cell free DNA (cfDNA) adsorbed to the surface of contaminating leukocytes [29]. It is to note that prior studies have shown that both CTCs and CTC fragments correlate with patient outcomes in prostate cancer [30].

Despite the small sample size and the heterogeneous patient population analyzed, the present study provides evidence that the KRAS mutation status of CTCs may substantially differ from that of the corresponding primary tumor. CTCs with no detectable KRAS mutations were obtained from six out the 11 patients with mutant primary tumors; this could be explained by the intratumoral heterogeneity and/or the enrichment of minor preexisting clones with increased metastatic potential during disease progression. Nevertheless, in three of the six discordant cases, the resection and examination of the primary tumor for KRAS mutations was performed only one month before the analysis of CTCs (data not shown), indicating possibly a model of parallel evolution of the primary tumor and metastasis. Previous studies have shown that the mutation status of CTCs does not always reflect that of the corresponding metastasis [31]; therefore, the comparison of the KRAS mutation status of primary tumor, corresponding metastases and serial CTC-enriched blood specimens might shed light to the origin of metastases.

Although the conditions and the rate of genotype conversion are not well understood, the current study suggests that CTCs of different mutation status may arise during treatment in the same patient (patients #1 and #15). Indeed, it could be hypothesized that treatment, with or without targeted agents, by eliminating some chemotherapy-sensitive clones may allow the emergence of other low frequency clones that differ from the predominant tumoral cells in respect to the mutation status. This hypothesis is strongly suggested by the presence of KRAS mutations in CTCs of two out the 12 patients with KRAS wild type primary tumors who were treated with regimens incorporating panitumumab or bevacizumab (patients #15 and #16). Nevertheless, the failure of detecting mutations in the CTC-enriched samples could also be attributed to the low frequency of CTCs harboring mutations as well as to methodological limitations; the FDA approved CellSearch Epithelial Cell Kit, which is reported to be inferior to CellSearch Epithelial Cell Profile kit in terms of CTCs’ molecular characterization [32]. However, it has been shown that CTCs captured with the CellSearch Epithelial Cell Kit can be successfully analyzed by next-generation sequencing methodologies [33]. Furthermore, a subpopulation of CTCs could not be detected by CellSearch due to insufficient expression of EpCAM and/or Cytokeratins; though, a recent study in SCLC demonstrated that CTCs, expressing EpCAM, captured with CellSearch system can form tumors in immunocompromised mice [34].

Previous studies have used different methodologies in order to isolate and molecularly characterize the CTCs. In metastatic breast and prostate cancer, genomic profiling of CTCs isolated by immunomagnetic enrichment and fluorescence activated cell sorting revealed new genomic changes occurring during disease progression [35,36]. Using a microfluidic CTC capture device, the TMPRSS2-ERG fusion, the TKIs sensitizing EGFR activating mutations and the EGFR T790M TKI-resistance mutation were detected in CTCs from patients with prostate and lung cancer metastatic disease respectively, whereas mutations of the AR, KRAS and BRAF genes have been identified in CTC-enriched samples isolated from prostate and mCRC patients, respectively, using the CellSearch Profile Kit [24,37,38,39]. Genotyping of single CTCs isolated by the CellSearch or the IsoFlux system in patients with mCRC confirmed an intra- and inter-patient heterogeneity based on the PIK3CA and KRAS mutation status [22,31]; moreover, different genetic alterations on single CTCs have already been reported in patients with breast and esophageal cancer [23,40].

Previous reports suggested a link between the presence of CTCs and the cell free DNA (cfDNA) in the serum or plasma of cancer patients [41]. KRAS mutations detected in the serum of patients with mCRC have been proposed to monitor response to treatment before the clinical appearance of disease progression whereas in NSCLC patients it has been reported a higher sensitivity for EGFR mutation detection in cfDNA than in CTCs [7,8,42]. Nevertheless, the origin of cfDNA is not well understood since it is derived from apoptotic tumor cells, apoptotic leukocytes produced by the cytotoxic therapy as well as by exosomes or CTCs released from tumor cells in the bloodstream. Although the isolation and analysis of cfDNA is simpler and more reproducible than isolating and genotyping CTCs, the molecular characterization of CTCs in addition to being useful for monitoring treatment failure or disease relapse may also provide information to guide optimal treatment selection and/or development of novel therapiess.

In conclusion, the presented data describe a simple methodology based on the daily use of the CellSearch platform for the identification of KRAS mutational status of CTCs from patients with metastatic colorectal cancer and provide a rationale for considering re-assessment of KRAS mutational status in CTCs in order to better predict response to anti-EGFR therapy.

Author Contributions
Conceived and designed the experiments: AV. Performed the experiments: AK HP SA AV. Analyzed the data: AK AV. Contributed reagents/materials/analysis tools: NG. Contributed to the writing of the manuscript: AK AV. Contributed to the collection and assembly of clinical data: EP JS. Acquired and managed patients and participated clinically in sample provision and data interpretation: JS MT VG DM. Were involved in critical review and edited the manuscript: VG DM.

References
1. Jonker DJ, O’Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, et al. (2007) Cetuximab For The Treatment Of Colorectal Cancer. N Engl J Med 357: 2040-2048.
2. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, et al. (2010) Effects Of Kras, Raf, Nras, And Pik3ca Mutations On The Efficacy Of Cetuximab Plus Chemotherapy In Chemotherapy-Refractory Metastatic
Colorectal Cancer: A Retrospective Consortium Analysis. Lancet Oncol 11: 753–762.

3. Balslev SE, Schaefer KL, Engers R, Hartleb D, Stoecklein NH, et al. (2010) Prevalence And Heterogeneity Of Kras, BRAF, And PIK3CA Mutations In Primary Colorectal Adenocarcinomas And Their Corresponding Metastases. Clin Cancer Res 16: 790–799.

4. Vermaas JS, Nijman JI, Koudij M, Gerrits FL, Scherer SJ, et al. (2012) Primary Colorectal Cancers And Their Subsequent Hapetic Metastases Are Genetically Different: Implications For Selection Of Patients For Targeted Treatment. Clin Cancer Res 18: 688–699.

5. Watanabe T, Kobunai T, Yamamoto Y, Matsuda K, Ishihara S, et al. (2011) Heterogeneity Of Kras Status May Explain The Subset Of Discordant Kras Status Between Primary And Metastatic Colorectal Cancer. Dis Colon Rectum 54: 1170–1178.

6. Voutsina A, Tzardis M, Kallikakis Z, Zafeiriou Z, Papadimitriaki E, et al. (2012) Combined Analysis Of Kras And PIK3CA Mutations, Met And Etn Expression In Primary Tumours And Corresponding Metastases In Colorectal Cancer. Mod Pathol 26: 302–313.

7. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, et al. (2012) The Molecular Evolution Of Acquired Resistance To Targeted Egr8 Blockade In Colorectal Cancer. Nature 466: 532–536.

8. Danila DC, Slinger R, Priest L, Lancashire L, Hou JM, et al. (2011) Evaluation Of Circulating Tumor Cells As Biomarkers In Prostate Cancer: Clin Cancer Res 17: 3903–3912.

9. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, et al. (2006) Circulating Tumor Cells At Each Follow-Up Time Point During Therapy Of Metastatic Breast Cancer Patients Predict Progression-Free And Overall Survival. J Clin Oncol 24: 4219–4224.

10. Serrano Fernandez MJ, Alvarez Merino JC, Martinez Zabiaure I, Fernandez Garcia A, Sanchez Ravilla P, et al. (2009) Clinical Relevance Associated To The Analysis Of Circulating Tumor Cells In Patients With Solid Tumours. Clin Transl Oncol 11: 659–666.

11. Krebs MG, Soane R, Priest L, Lancashire L, Hou JM, et al. (2011) Evaluation And Prognostic Significance Of Circulating Tumor Cells In Patients With Non-Small-Cell Lung Cancer. J Clin Oncol 29: 1536–1563.

12. Saure J, Maestro ML, Gomez-Espana A, Rivero F, Valladares M, et al. (2012) Circulating Tumor Cell Count Is A Prognostic Factor In Metastatic Colorectal Cancer Patients Receiving First-Line Chemotherapy Plus Bevacizumab: A Spanish Cooperative Group For The Treatment Of Digestive Tumors Study. Oncology 79: 497–505.

13. Allen JE, El-Deyrie WS (2010) Circulating Tumor Cells And Colorectal Cancer: Curr Colorectal Cancer Rep 6: 212–220.

14. Cohren SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, et al. (2008) Relationship Of Circulating Tumor Cells To Tumor Response, Progression-Free Survival, And Overall Survival In Patients With Metastatic Colorectal Cancer. J Clin Oncol 26: 3213–3221.

15. Garrigos N, Gallego J, Guillen-Ponce C, Guaraz P, Garcia-Bautista M, et al. (2010) Circulating Tumor Cell Analysis As An Early Marker For Relapse In Stage II And Iii Colorectal Cancer Patients: A Pilot Study. Clin Transl Oncol 12: 142–147.

16. Tol J, Koopman M, Miller MC, Tibbe A, Cats A, et al. (2009) Circulating Tumor Cells Early Predict Progression-Free And Overall Survival In Advanced Stage Ii And Iii Colorectal Cancer Patients: A Pilot Study. Clin Transl Oncol 11: 659–668.

17. Heitzer E, Auer M, Gasch C, Pichler M, Uta P, et al. (2013) Complex Tumor Genomes Inferred From Single Circulating Tumor Cells By Array-Cgh And Next-Generation Sequencing. Cancer Res 73: 2965–2973.

18. Moster D, Jong Y, Sieuwerts AM, Wang H, Bol-D de Vries J, et al. (2013) Kras And BRAF Mutation Status In Circulating Colorectal Tumor Cells And Their Correlation With Primary And Metastatic Tumor Tissue. Int J Cancer 133: 130–141.

19. Mesnage LM, Ahnman DG, Saurerwein W, Taube SE, Gien M, et al. (2005) Reporting Recommendations For Tumor Marker Prognostic Studies (Remark). Nat Clin Pract Urol 2: 416–422.

20. Voutsina A, Tzardis M, Kallikakis Z, Zafeiriou Z, Papadimitriaki E, et al. (2012) Combined Analysis Of Kras And PIK3CA Mutations, Met And Etn Expression In Primary Tumours And Corresponding Metastases In Colorectal Cancer. Mod Pathol.

21. Lai JD, Chan EC, Shih CL, Chen TL, Liang Y, et al. (2006) Detection Of Rare Mutant K-Ras Dna In A Single-Stage Reaction Using PiggyboAcid As Both Pcr Clamp And Sensor Probe. Nucleic Acids Res 35: E12.

22. Sieuwerts AM, Kraan J, Bol-D de Vries J, Van Der Spoel P, Moster D, et al. (2009) Molecular Characterization Of Circulating Tumor Cells In Large Quantities Of Contaminating Leukocytes By A Multiplex Real-Time Pcr. Breast Cancer Res Treat 118: 453–460.

23. Skovsotova TE, Rykova EY, Tamkovich SN, Bryzgunova OE, Starikov AV, et al. (2006) Cell-Free And Cell-Bound Circulating Dna In Breast Tumours: Dna Quantification And Analysis Of Tumour-Related Gene Methylation. Br J Cancer 94: 1492–1496.

24. Coumans FA, Doggen CJ, Attard G, De Bon J, Van Der Spoel P, Moster D, et al. (2011) Kras Mutations And Acquired Resistance To Anti-Egrf Therapy In Colorectal Cancer. Nature 486: 532–536.

25. Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-De Vries J, et al. (2013) Genetic Disparity Between Primary Tumours, CTC-Enriched Samples And Corresponding Metastases In Colorectal Cancer: A Retrospective Consortium Analysis. Lancet Oncol 11: 753–762.

26. Schneewind K, Blasi G, Mier-Sieben F, Neves RP, Jann I, et al. (2013) Combined Analysis Of Circulating Tumor Cells In Metastatic Breast Cancer Patients. Mol Oncol.

27. Di Fiore F, Charbonnier F, Lebreure B, Laurent M, Le Pessot F, et al. (2008) Clinical Interest Of Kras Mutation Detection In Blood For Anti-Egrf Therapies In Metastatic Colorectal Cancer. Br J Cancer 99: 551–552.

28. Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeth M, et al. (2012) Heterogeneity Of Epidermal Growth Factor Receptor Status And Mutations Of Kras/Pik3ca In Circulating Tumor Cells Of Patients With Colorectal Cancer. Clin Chem 59: 252–260.

29. Heitzer E, Auer M, Gasch C, Pichler M, Uta P, et al. (2013) Complex Tumor Genomes Inferred From Single Circulating Tumor Cells By Array-Cgh And Next-Generation Sequencing. Cancer Res 73: 2965–2973.

30. Moster D, Jong Y, Sieuwerts AM, Wang H, Bol-D de Vries J, et al. (2013) Kras And BRAF Mutation Status In Circulating Colorectal Tumor Cells And Their Correlation With Primary And Metastatic Tumor Tissue. Int J Cancer 133: 130–141.