Circulating tumor cells
Exploring intratumor heterogeneity of colorectal cancer

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The hypothesis of the "liquid biopsy" using circulating tumor cells (CTCs) emerged as a minimally invasive alternative to traditional tissue biopsy to determine cancer therapy. Discordance for biomarkers expression between primary tumor tissue and circulating tumor cells (CTCs) has been widely reported, thus rendering the biological characterization of CTCs an attractive tool for biomarkers assessment and treatment selection. Studies performed in metastatic colorectal cancer (mCRC) patients using CellSearch, the only FDA-cleared test for CTCs assessment, demonstrated a much lower yield of CTCs in this tumor type compared with breast and prostate cancer, both at baseline and during the course of treatment. Thus, although attractive, the possibility to use CTCs as therapy-related biomarker for colorectal cancer patients is still limited by a number of technical issues mainly due to the low sensitivity of the CellSearch method. In the present study we found a significant discordance between CellSearch and AdnaTest in the detection of CTCs from mCRC patients. We then investigated KRAS pathway activating mutations in CTCs and determined the degree of heterogeneity for KRAS oncogenic mutations between CTCs and tumor tissues. Whether KRAS gene amplification may represent an alternative pathway responsible for KRAS activation was further explored. KRAS gene amplification emerged as a functionally equivalent and mutually exclusive mechanism of KRAS pathway activation in CTCs, possibly related to transcriptional activation. The serial assessment of CTCs may represent an early biomarker of treatment response, able to overcome the intrinsic limit of current molecular biomarkers represented by intratumor heterogeneity.

Introduction

Molecular targeted therapies hold the promise of a personalized cancer treatment based on the knowledge of single patient's tumor profile. As a consequence of the improved understanding of molecular biology of cancer, significant steps have been made toward the individualization of treatment for patients with advanced colorectal cancer in the past few years. Despite the introduction of an increasing number of molecular agents into clinical practice, the improvement in survival for metastatic colorectal cancer (mCRC) patients is modest, with a median survival still not exceeding 24 mo. Research has demonstrated that the identification of predictive biomarkers is crucial in the selection of patients who might most likely benefit from targeted agents. Inhibitors of the vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) are both key approaches in the treatment of mCRC patients, improving the efficacy of chemotherapy alone.[1] While none of the proposed biomarkers is currently validated for predicting the response to anti-angiogenic treatment, KRAS mutational status

has been established as a major biomarker of resistance to anti-EGFR monoclonal antibodies and KRAS testing is required before starting the treatment with these compounds.[2] Point mutations in the KRAS gene are causally associated with both primary and acquired resistance to anti-EGFR drugs in metastatic colorectal cancer.[3,4] Nevertheless, a large proportion of patients with KRAS wild-type (wt-KRAS) tumors are still not responsive to the treatment. Additional oncogenic activations of downstream genes of EGFR signaling pathway, such as the RAS-RAF-MAPK and PI3K-PTEN-AKT pathways, are emerging as promising biomarkers, although they have not been yet validated for routine clinical use.[5,6] The complexity of the molecular alterations driving resistance to EGFR-targeted treatments is further enhanced by recent evidence on spatial heterogeneity in KRAS mutations between primary tumor samples, which in turn raises the question on whether or not appropriate sampling protocols are currently in place to enable detection of low frequency KRAS mutations. Although not conclusive, data have been reported on a temporal heterogeneity of activating KRAS mutations, due to positive or negative selective pressure on KRAS mutant tumor
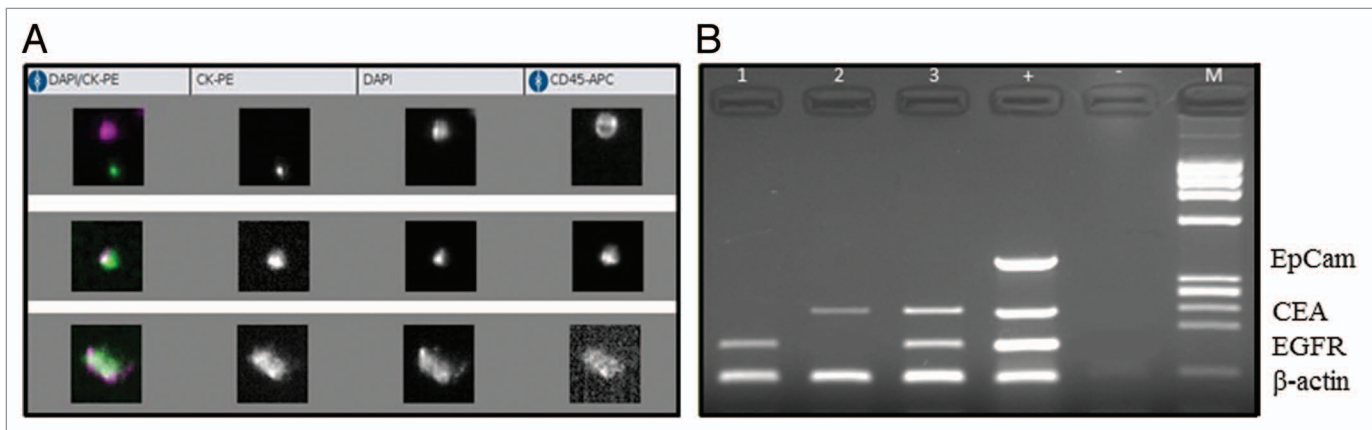
Figure 1. CellSearch analysis (**A**) and multiplex PCR performed after isolation of CTCs with AdnaTest (**B**) in three exemplificative patients with metastatic colorectal cancer. The figure shows that the absence of CTC at the CellSearch analysis is consistent with lack of EpCAM expression, as visualized at the AdnaTest analysis. Conversely, the approach with the multiplex PCR allows the identification of CTC underestimated by CellSearch, through the positive expression of EGFR and CEA.

cells over the course of the disease.[7,8] Heterogeneity in the expression of predictive biomarkers creates challenges in the diagnostic accuracy and, consequently, in the therapeutic decision making process for cancer patients. The availability of minimally invasive approaches to monitor the dynamic evolution of cancer represents a significant step toward the individualization of therapeutic approaches, to improve treatment outcomes. In this context, and taking into consideration the frequent genetic discordance between primary tumors and corresponding metastases, the hypothesis of the "liquid biopsy" using CTCs emerged as a minimally invasive alternative to traditional tissue biopsy in determining cancer therapy.[9] To date, the CellSearch system is the only FDA-cleared test for CTCs assessment with prognostic purpose in metastatic breast, prostate, and colorectal cancer.[10]

In metastatic colorectal cancer, the CellSearch system received US FDA clearance for routine clinical use with a threshold CTC count ≥3/7.5 mL of blood, to stratify patients into favorable and unfavorable prognostic groups.[11-14] Nevertheless, the studies performed in colorectal cancer patients using CellSearch demonstrated a much lower yield of CTCs in this tumor type compared with breast and prostate cancer, both at baseline and in course of treatment.[15] The PCR-based assay developed by AdnaGen makes use of RT-PCR to identify putative transcripts of genes in EpCAM-positive cells isolated by a magnetic separation method. The immunomagnetic cell capture technology, combined with multiplex RT-PCR in AdnaTest may potentially allow the detection of a broad range of molecular abnormalities in CTCs supporting the design of future studies testing CTCs directed therapies.

Aims of the present study were: (1) to compare CellSearch and AdnaTest in their ability to detect CTCs in mCRC patients; (2) to investigate whether the KRAS pathway activating mutations can be identified in CTCs and to determine the degree of heterogeneity for KRAS oncogenic mutations between CTCs and tumor tissue; and (3) to explore the correlation between KRAS gene point mutations and KRAS gene amplification as alternative alterations responsible for KRAS pathway activation.

Results

CTCs detection rate: comparison between CellSearch and AdnaTest

Forty-one (41) consecutive patients were initially recruited, with 2 later excluded for inadequate samples. In each patient CTCs were isolated with both CellSearch system and AdnaTest.

CTCs were found in 8/39 patients (20.5%) using CellSearch system. The rate of patients found positive for CTCs presence with AdnaTest was 81% with a positive expression rate of 81% for EGFR, 48% for CEA and 19% for EpCAM respectively (**Fig. 1**). Statistical analysis found no correlation between the two isolation methods (Chi square test P = 0.306; Mann–Whitney and Spearman tests P = 0.659 and 0.678, respectively) (**Table 1**). No significant correlation was found between CTCs presence and clinico-pathological characteristics of patients.

Correlation between KRAS mutational status in tissue samples and CTCs

The further molecular analysis of CTCs referred to KRAS mutational status, KRAS overexpression, and EGFR expression was then performed in CTCs isolated through AdnaTest.

KRAS gene mutational status in CRC tissue was available for 24 out of 32 CTCs positive samples; in this group of patients KRAS sequence analysis was performed in CTCs as well. KRAS codon 12 mutation was detected in 7 of the 24 tumor tissue samples analyzed (29%), whereas in 5/24 cases (21%) CTCs harboring the same mutation were identified. The correlation between KRAS mutation in tumor tissue and CTCs was found not statistically significant (P = 0.580 and 0.672 at Chi square and Fisher exact test, respectively) (**Table 2**).

Correlation between KRAS overexpression and KRAS mutation in CTCs

In the same series of patients, KRAS overexpression in CTCs was detected in 8/24 cases (33%). None of these eight samples displayed a concomitant KRAS activating mutation in CTCs or in tumor tissue, suggesting that the pathway activation by mutation or gene overexpression might be functionally equivalent and

Table 1. Detection rate of CTC in metastatic colorectal cancer: comparison between CellSearch and Adnatest

	CTC AdnaTest		Total
	Negative	Positive	
CTC Veridex 0 count	5	26	31
% within CTC Veridex	16.1%	83.9%	100.0%
% within CTC AdnaTest	71.4%	81.2%	79.5%
% of total	12.8%	66.7%	79.5%
CTC Veridex 1 count	1	2	3
% within CTC Veridex	33.3%	66.7%	100.0%
% within CTC AdnaTest	14.3%	6.2%	7.7%
% of total	2.6%	5.1%	7.7%
CTC Veridex 2 count	1	0	1
% within CTC Veridex	100.0%	0.0%	100.0%
% within CTC AdnaTest	14.3%	0.0%	2.6%
% of total	2.6%	0.0%	2.6%
CTC Veridex 3 count	0	1	1
% within CTC Veridex	0.0%	100.0%	100.0%
% within CTC AdnaTest	0.0%	3.1%	2.6%
% of total	0.0%	2.6%	2.6%
CTC Veridex 5 count	0	2	2
% within CTC Veridex	0.0%	100.0%	100.0%
% within CTC AdnaTest	0.0%	6.2%	5.1%
% of total	0.0%	5.1%	5.1%
CTC Veridex 11 count	0	1	1
% within CTC Veridex	0.0%	100.0%	100.0%
% within CTC AdnaTest	0.0%	3.1%	2.6%
% of total	0.0%	2.6%	2.6%
Total count	7	32	39
% within CTC Veridex	17.9%	82.1%	100.0%
% within CTC AdnaTest	100.0%	100.0%	100.0%
% of total	17.9%	82.1%	100.0%

thus mutually exclusive. Of note, when tissue KRAS codon 12 mutations and KRAS gene point mutations or overexpression in CTCs were all combined, KRAS activating genetic alterations were globally found in 20/24 patients with a prevalence of 83%, confirming the key role of KRAS pathway in the biology of CRC. No statistically significant correlation was found between KRAS mutation and KRAS overexpression in CTCs (P = 0.075 and 0.104 at Chi square and Fisher exact test, respectively) (**Table 3**).

Correlation between KRAS mutation/overexpression and EGFR expression in CTCs

No correlation was found between KRAS mutation and EGFR expression in CTCs (P = 0.459 and 0.646 at Chi square and Fisher exact test, respectively), as well as between KRAS overexpression and EGFR (P = 0.217 and 0.252 at Chi square and Fisher exact test, respectively) (**Table 4**).

Correlation between KRAS mutation in tissue samples and KRAS overexpression in CTCs

The correlation between KRAS mutation in tissues and KRAS overexpression in CTCs was found not statistically significant (P = 0.368 and 0.508 at Chi square and Fisher exact test, respectively) (**Table 5**).

Discussion

The EGFR signaling pathway has been broadly characterized over the last few years and currently represents a therapeutically relevant target in metastatic colorectal cancer. Anti-EGFR monoclonal antibodies have demonstrated clinical benefit in metastatic colorectal cancer patients. Molecular alterations leading to a constitutive activation of the EGFR signal transduction have been associated with resistance to anti-EGFR targeted therapies. Among these, KRAS point mutations, which occur in approximately 40% of mCRC patients, are a well-established negative predictive biomarker for resistance to cetuximab and panitumumab.[16,17] Particularly, KRAS codon 12 and 13 mutations are routinely tested to select patients candidate for anti-EGFR targeted therapy. Despite robust clinical data that have demonstrated the negative predictive value of KRAS mutations, a heterogeneous response rate has been observed in patients with KRAS wild-type CRC. Activating mutations in EGFR downstream effectors have been demonstrated as additional determinants of response to anti-EGFR monoclonal antibodies.[6,18] Furthermore, KRAS gene amplification, despite being an infrequent event, accounting for only 2% of primary CRC, has been recently proposed as an alternative regulator of KRAS activation and associated with reduced progression free survival in mCRC patients treated with cetuximab.[19,20]

The success of individualized cancer treatments is furthermore limited by the significant variability of biomarkers expression within primary and metastatic tumors. Increasing evidence for regional and temporal heterogeneity of cancer is indeed emerging and clinical implications of the branched evolutionary tumor model are becoming evident.[21,22] To overcome the intrinsic limits of a personalized approach to cancer treatment based on the assessment of biomarkers in the primary tumor at the time of diagnosis, minimally invasive and repeatable methods have been developed to monitor the dynamic evolution of the disease over the course of tumor progression. Circulating tumor cells have a recognized prognostic and predictive value in metastatic colorectal cancer patients and are gathering increasing interest as an easily accessible source of biological information. A real-time biomarkers detection through the molecular analysis of CTCs might allow a dynamic selection of patients and guide the individual therapeutic decision making process. Although attractive, the possibility to use CTCs as therapy-related biomarkers for cancer patients is still limited by a number of technical issues. Ideal markers for the identification of CTCs have not yet been identified and the need to distinguish between different cancer types is gaining growing attention. Stringent criteria by which a CTCs is defined in the CellSearch system manufacturer's instructions

may preclude the identification of subpopulations of cells in which a downregulation of epithelial markers occurred.[23]

In the present study, using the AdnaTest Colon Cancer, which combines immunomagnetic capture of CTCs with a multiplex expression analysis of the tumor associated mRNA markers EpCAM, CEA and EGFR, CTCs were found in 81% of mCRC patients evaluated. Compared with the lower detection rate of CTCs found using CellSearch in our cohort of patients (21%), the higher proportion of CTC-positive patients seems not surprising and might be attributed to the panel of markers included in the AdnaTest Colon Cancer. The higher expression rate of EGFR in CTCs positive samples, as well as the low EpCAM expression rate, seems to indicate that EGFR is the most accurate marker for the detection of CTCs in metastatic colorectal cancer patients, as demonstrated by the significantly lower detection rate of CTCs in the same cohort of patients with the CellSearch system. Hepatic filtration via the portal circulation has been suggested to explain the relatively low number of CTCs found in patients with metastatic colorectal cancer, compared with patients with other cancer types. As a possible explanation for the downregulation of epithelial markers expression in CTCs from mCRC patients, an activation of the epithelial to mesenchymal transition (EMT) program could be advocated.[24] In parallel, the KRAS pathway activation due to gene point mutations, or alternatively to gene overexpression, might be implicated in the induction and maintenance of the EMT program in circulating tumor cells, as suggested by a number of preclinical models.[25,26] International guidelines recommend to test KRAS mutations on primary colorectal tumors and/or corresponding metastases, as literature has shown that the KRAS mutations are similar in both specimen types.[27] Few studies have explored in small series of mCRC patients the correlation between KRAS mutational status in primary or metastatic tumor and in CTCs, revealing further mutational discordance, possibly complicating the selection of patients candidate for anti-EGFR treatments.[28-33] Our results confirm a divergent KRAS status in a large proportion of cases, highlighting that KRAS wild-type CTCs

Table 2. Correlation between KRAS mutational status in tumor tissues and CTC

	KRAS CTC wild-type mutated		Total
KRAS tissue wild-type count	13	4	17
% within KRAS tissue	76.5%	23.5%	100.0%
% within KRAS tissue	54.2%	66.7%	56.7%
% of total	43.3%	13.3%	56.7%
Mutated count	11	2	13
% within KRAS tissue	84.6%	15.4%	100%
% within KRAS tissue	45.8%	33.3%	43.3%
% of total	36.7%	6.7%	43.3%
Total count	24	6	30
% within KRAS tissue	80.0%	20.0%	100.0%
% within KRAS tissue	100.0%	100.0%	100.0%
% of total	80.0%	20.0%	100.0%

are frequently detected in peripheral blood of patients harboring mutated primary tumors. As potential mechanism to explain the relative prevalence of wild type KRAS CTCs,[34] the generation of hypoxia in large advanced tumors or induced by treatments has been evoked. Particularly, hypoxia-induced oxidative stress may exert a negative selection against KRAS mutant cells through higher levels of ERK activation in KRAS mutant cells compared with the wild type counterpart, that cause cell growth arrest.[35]

Alternatively, the continuous mutagenesis in primary tumors may be proposed. Although codon 12 and 13 KRAS mutations in tumor tissue specimen are routinely tested, we decided to restrict the analysis to codon 12 mutations in CTCs, due to recent data suggesting the unclear association between codon 13 KRAS mutation and response to anti-EGFR monoclonal antibodies.[36] A number of possible explanations exists for the reported discrepancy between KRAS mutational status in primary tumor and

Table 3. Correlation between KRAS overexpression and KRAS mutation in CTC

			KRAS overexpression CTC		Total
			Negative	Positive	
KRAS overexpression CTC	Negative	Count	12	6	18
		% within KRAS overexpression CTC	66.7%	33.3%	100.0%
		% within K-RAS CTC	48.0%	85.7%	56.2%
		% of total	37.5%	18.8%	56.2%
	Positive	Count	13	1	14
		% within KRAS overexpression CTC	92.9%	7.1%	100.0%
		% within KRAS CTC	52.0%	14.3%	43.8%
		% of total	40.6%	3.1%	43.8%
Total		Count	25	7	32
		% within KRAS overexpression CTC	78.1%	21.9%	100.0%
		% within KRAS CTC	100.0%	100.0%	100.0%
		% of total	78.1%	21.9%	100.0%

Table 4. Correlation between KRAS mutation/KRAS overexpression and EGFR expression in CTC

			KRAS CTC		Total
			Wild type	Mutated	
EGFR CTC	Negative	Count	7	1	8
		% within EGFR CTC	87.5%	12.5%	100.0%
		% within KRAS CTC	28.0%	14.3%	25.0%
		% of total	21.9%	3.1%	25.0%
	Positive	Count	18	6	24
		% within EGFR CTC	75.0%	25.0%	100.0%
		% within KRAS CTC	72.0%	85.7%	75.0%
		% of total	56.2%	18.8%	75.0%
Total		Count	25	7	32
		% within EGFR CTC	78.1%	21.9%	100.0%
		% within KRAS CTC	100.0%	100.0%	100.0%
		% of total	78.1%	21.9%	100.0%
			K-RAS CTC		Total
			Negative	Positive	
EGFR CTC	Negative	Count	3	5	8
		% within EGFR CTC	37.5%	62.5%	100.0%
		% within KRAS overexpression CTC	16.7%	35.7%	25.0%
		% of total	9.4%	15.6%	25.0%
	Positive	Count	15	9	24
		% within EGFR CTC	62.5%	37.5%	100.0%
		% within KRAS overexpression CTC	83.3%	64.3%	75.0%
		% of total	46.9%	28.1%	75.0%
Total		Count	18	14	32
		% within EGFR CTC	56.2%	43.8%	100.0%
		% within KRAS overexpression CTC	100.0%	100.0%	100.0%
		% of total	56.2%	43.8%	100.0%

CTCs. First of all, a sampling bias must be taken into consideration causally related to intratumor heterogeneity, small mutated cell fractions and consequent underestimation of mutation frequency in the analysis of tumor specimen. Tumor heterogeneity also implicates that mixed KRAS wild-type and mutated sub-clones of cells might coexist in the same tumor and compete each other for metastatic dissemination. Finally, dynamic changes in the sub-clonal architecture of tumor due to selective pressure under treatment might increase genetic instability and lead to the emergence of biological determinants for resistance to therapies. KRAS mRNA overexpression in CTCs was in parallel investigated in the present work and emerged as a functionally equivalent and mutually exclusive mechanism of KRAS pathway activation, possibly related to transcriptional activation. The lack of data on KRAS overexpression in tissue samples may represent a major drawback of the present study. The clinical relevance of KRAS gene amplification and mRNA overexpression in predicting response to anti-EGFR targeted therapies is still largely under investigated and deserves further attention.

Although preliminary, our results show that the serial assessment of CTCs may represent an early biomarker of treatment response, providing the opportunity to assess clonal dynamics over the course of a single patient disease and overcoming the intrinsic limit of current molecular biomarkers represented by the intratumor spatial and temporal heterogeneity.

Patients and Methods

A total of 41 stage IV colorectal cancer patients were enrolled in this study. All patients were enrolled at Sapienza University—Policlinico Umberto I of Rome. Clinical characteristics of patients are summarized in **Table 6**. All patients provided a written informed consent and the study was approved by the institutional review board.

KRAS gene mutational status in tissue samples

DNA was extracted from FFPE tissue sections using a DNA minikit (Qiagen), following the manufacturer's instructions. A 321-bp PCR fragment of the exon 2 KRAS gene, including codons

Table 5. Correlation between KRAS mutation in tissue samples and KRAS overexpression in CTC

| | | | KRAS overexpression CTC | | Total |
			Negative	Positive	
K-RAS tissue	Wild type	Count	10	10	20
		% within KRAS tissue	50.0%	50.0%	100.0%
		% within KRAS overexpression CTC	47.6%	62.5%	54.1%
		% of total	27.0%	27.0%	25.0%
	Mutated	Count	11	6	17
		% within KRAS tissue	64.7%	35.3%	100.0%
		% within KRAS overexpression CTC	52.4%	37.5%	45.9%
		% of total	29.7%	16.2%	45.9%
Total		Count	21	6	37
		% within KRAS tissue	56.8%	43.2%	100.0%
		% within KRAS overexpression CTC	100.0%	100.0%	100.0%
		% of total	56.8%	43.2%	100.0%

12 and 13, was amplified by the following primer pair: KRAS F: 5′-TTTGAAATAA TTTTTCATAT AAAGGTGAG-3′ and KRAS R: 5′-TCATGAAAAT GGTCAGAGAA ACC-3′. PCR conditions were as follows: initial denaturation for 5 min at 95 °C, cyclic denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, elongation at 72 °C for 1 min for 35 cycles, and final extension at 72 °C for 7 min. Following PCR the fragments were purified by Exostar 1-Step (VWR International) according to the manufacturer's instructions. Sequencing reactions were performed for both DNA strands by the Big Dye Terminator v1.1 (Applied Biosystems) on a total of 10 ng of purified PCR products. Dye purification was performed by Centrisep Spin columns (Princeton Separation) according to the manufacturer's instructions. Sequence analysis was performed on an Applied Biosystems 3130XL Genetic Analyzer.

Isolation of circulating tumor cells

In order to isolate CTCs from peripheral blood, CellSearch and AdnaTest ColonCancer Select/Detect were used. For AdnaTest, blood samples (5 mL) were taken using AdnaCollect blood collection tubes (AdnaGen) and immediately placed on ice, according to the manufacturer's instructions. For CellSearch analysis, 7.5 mL of blood were drawn in CellSave Vacutainer tubes (Becton Dickinson), kept at room temperature and processed within 96 h, as described.

CellSearch

The CellSearch Circulating Tumor Cell system uses a combination of immunomagnetic labeling and automated digital microscopy to identify and enumerate the number of circulating tumor cells in a peripheral blood specimen. It consists of a CellTracks AutoPrep system which isolates CTCs and a CellTracks Analyzer, which differentiates the tumor cells from nonspecific cells. The CellSearch Epithelial Cell Kit which is used for CTCs capture contains ferrofluids labeled with the epithelial cell adhesion molecule (EpCAM), the staining reagents 2-diamidino-2-phenylindole, dihydrochloride (DAPI), CD45-Allophycocyan (CD45-APC), and cytokeratin 8, 18, and 19

Table 6. Patient's characteristics

Patient's characteristics	N (%)
Age	
Range	45–83
Median	63.92
Gender	
Male	25/40 (62.5)
Female	15/40 (37.5)
Therapy	
Bevacizumab	25/40 (65)
Cetuximab	9/40 (25)
No therapy	6/40 (10)
Line of therapy	
First	20/34 (59)
Second	10/34 (29)
Third	4/34 (12)

Phycoerythrin (CK-PE). The whole blood specimen is centrifuged and placed on the CellTracks AutoPrep system. The plasma is aspirated and the remaining cellular component is mixed with buffer and ferrofluid reagent conjugated with monoclonal epithelial cell adhesion molecule (EpCAM) antibodies. The ferrofluid/antibody complex attaches specifically to epithelial cells. Magnets then attract the ferrofluid-bound cells to the side of the tube, the remaining fluid and any unlabeled cells are aspirated and the magnets are removed. The remaining cells are then resuspended in buffer. The resulting epithelial-enriched fluid is then placed in a cell presentation device (MagNest) that attracts the magnetically labeled epithelial cells to the surface of the cartridge. The cartridge is placed on the CellTracks Analyzer, a fluorescence-based microscopy system that scans the surface of the cartridge to acquire cell images to visualize DAPI-labeled nuclei, PE-labeled CK, and APC-labeled CD45. A gallery of images is reviewed by
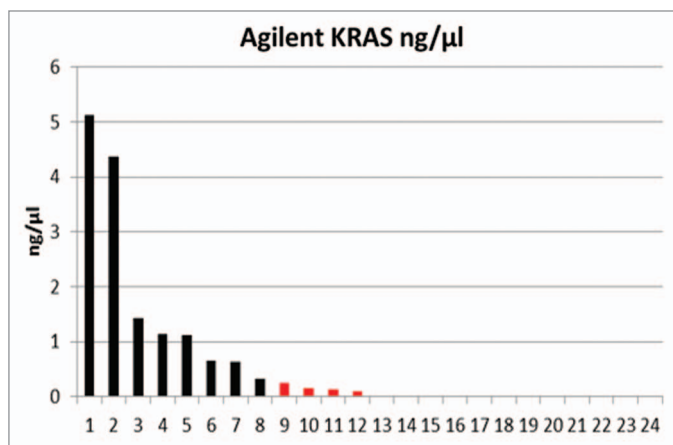
Figure 2. KRAS expression profile in a cohort of 24 CRC patients initially detected to be CTC positive based on the AdnaTest ColonCancer.

a technologist who identifies tumor cells based on the circulating tumor cell phenotype (positive DAPI and CK staining with an absence of CD45 staining)

AdnaTest ColonCancer Select

Colon Select Beads (100 µL) were added to 5 mL of blood and incubated for 30 min at room temperature (5 rpm). After incubation, cells were repeatedly washed with PBS and lysed by adding a Lysis/Binding buffer (AdnaGen). The supernatant was recovered. The AdnaTest ColonCancer provides a specificity of >90% as well as a recovery of 2 CRC cells which has been proven by analyzing healthy donor blood samples as well as CRC cell spiking experiments respectively.[37]

AdnaTest Colon Cancer Detect

mRNA was subsequently recovered by a magnetic separation using Oligo(dT)25 Dynabeads. The total mRNA/bead mixture (29.5 µL) was reverse transcribed using 0.5 µL of RNase inhibitor (40 U µL^{-1}; Promega), 4 µL of RT buffer, 4 µL of dNTPs, and 2 µL of Sensiscript Reverse Transcriptase (Qiagen). Reverse transcription was performed in a one-step reaction (60 min at 37 °C, 5 min at 93 °C). The mixture was then chilled down on ice and stored at –20 °C. The analysis of tumor-associated mRNA isolated from CTC tumor cells was performed in a multiplex polymerase chain reaction (PCR) for three tumor-associated transcripts (EGFR, CEA, and GA733-2) and one housekeeping gene (β-actin). GA 73.3 refers to the epithelial cell adhesion molecule (EpCAM). The primers generate fragments in positive samples for the GA733-2, 383 bp; CEA, 226 bp; EGFR, 161 bp; β-actin, 114 bp (internal PCR control) sizes.

For the analysis of tumor-associated mRNAs, a multiplex PCR was performed. The primer mixture consisted of four specific primer pairs for the amplification of CEA, EGFR, and GA733-2.

PCR analyses were performed in a final volume of 50 µL PCR mixture, containing 8 µL of cDNA, 4 µL primer mixture (PrimerMix Colon Detect; AdnaGen), 25 µL of Hot Star Taq Master Mix (Qiagen) and 13 µL of distilled water. PCR analyses were performed as follows: pre-denaturation at 95 °C for 15 min, followed by 38 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 45 s

and a final extension step at 72 °C for 10 min. Visualization of the PCR fragments was performed with a 2100 Bioanalyzer (Agilent Technologies Inc.) by using DNA 1000 LabChips and the Expert Software Package (version B.02.03.SI307). The test was considered CTCs-positive if a PCR fragment of at least one tumor-associated transcript (EGFR, GA 773-2, or CEA) and a fragment of the control gene β-actin (internal PCR control) were clearly detected. For negative controls, mRNA and cDNA were replaced by water in the reverse transcription and PCR experiments. The PCR products were then analyzed by electrophoresis on a 4% agarose gel.

KRAS gene mutational status and overexpression in circulating tumor cells

For the KRAS pre-amplification HotStarTaq Master Mix Kit (Qiagen) and self-designed Primers (MWG) for the amplification of the region between exon 2 and exon 4 was used. RT-PCR and KRAS pre-amplification was created with an Arctic Thermocycler (Thermo scientific). Pre-amplification was performed as follows: 95 °C for 15 min, followed by 39 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 45 s and a final extension step at 72 °C for 10 min. Resulting PCR fragment concentrations were quantified using the Agilent Bioanalyzer 2100 (Agilent). The KRAS expression as observed in the 24 CTCs positive patient samples ranged from 0 to 5.12 ng/µL. Overexpression was defined as all values above 5% of the maximum value identified in this setting 0.255 ng/µL rounded to 0.3 ng/µL. The values excluded by choosing this cut-off ranged from 0 to 0.25 ng/µL (**Fig. 2**). Following the pre-amplification the detection of G12X mutations was executed with Ampli-set-K-Ras, Version 007 of 06-04-2011 (Diachem s.r.l.).

The protocol of the G12X amplification was modified. We replaced 28 µL water with template, for a total template volume of 30 µL, instead of original 2 µL template volume + 28 µL water, because of a low cDNA concentration. Amplification was performed as follows: 95 °C for 10 min, followed by 50 cycles of denaturation at 94 °C for 40 s, annealing at 52 °C for 40 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 10 min.

The protocol of the enzymatic digestion with MVA I enzyme was modified. We replaced 3 µL water with template, for a total template volume of 13 µL, instead of original 10 µL template volume + 3 µL water, because of the low concentrations. The MVA I digestion was proceeded overnight and the products of digestion were visualized in 4% agarose gel.

Statistical analysis

Categorical variables were compared with the Fisher exact test. Continuous variables were compared with the Mann–Whitney U test, which does not assume an underlying normal distribution of the dependent variable and is more robust in small and potentially skewed samples than parametric tests. Statistical significance was set at the 2-tailed 0.05 level, and *P* values unadjusted for multiplicity are reported throughout. Computations were performed with SPSS 20 (IBM).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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