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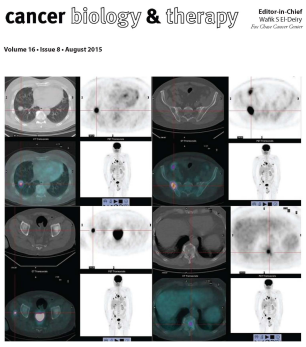

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Cancer Biology & Therapy
Publication details, including instructions for authors and subscription information:
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Detection of KRAS mutations in circulating tumor cells from patients with metastatic colorectal cancer

Marcilei EC Buim[ab], Marcello F Fanelli[c], Virgilio S Souza[c], Juliana Romero[a], Emne A Abdallah[a], Celso AL Mello[c], Vanessa Alves[a], Luciana MM Ocea[a], Natália B Mingues[a], Paula NVP Barbosa[d], Chiang J Tyng[d], Rubens Chojniak[d] & Ludmilla TD Chinen[a]

[a] International Reserch Center; AC Camargo Cancer Center; São Paulo, Brazil

[b] Department of Health; Universidade Nove de Julho; São Paulo, Brazil

[c] Department of Clinical Oncology; AC Camargo Cancer Center; São Paulo, Brazil

[d] Department of Image; AC Camargo Cancer Center; São Paulo, Brazil

Accepted author version posted online: 07 Aug 2015.Published online: 07 Aug 2015.

To cite this article: Marcilei EC Buim, Marcello F Fanelli, Virgilio S Souza, Juliana Romero, Emne A Abdallah, Celso AL Mello, Vanessa Alves, Luciana MM Ocea, Natália B Mingues, Paula NVP Barbosa, Chiang J Tyng, Rubens Chojniak & Ludmilla TD Chinen (2015): Detection of KRAS mutations in circulating tumor cells from patients with metastatic colorectal cancer, Cancer Biology & Therapy, DOI: 10.1080/15384047.2015.1070991

To link to this article: http://dx.doi.org/10.1080/15384047.2015.1070991

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Cancer Biology & Therapy 16:9, 1–7; September 2015; © 2015 Taylor & Francis Group, LLC

Detection of KRAS mutations in circulating tumor cells from patients with metastatic colorectal cancer

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[1]International Reserch Center; AC Camargo Cancer Center; São Paulo, Brazil; [2]Department of Health; Universidade Nove de Julho; São Paulo, Brazil; [3]Department of Clinical Oncology; AC Camargo Cancer Center; São Paulo, Brazil; [4]Department of Image; AC Camargo Cancer Center; São Paulo, Brazil

Keywords: circulating tumor cell, colorectal cancer, KRAs mutation

Abbreviations: ASB-PCR, allele specific PCR with a blocking reagent; CEA, carcinoembryonic antigen; cftDNA, circulating free tumor DNA; CRC, colorectal cancer; CTCs, circulating tumor cells; EDTA, Ethylenediamine tetraacetic acid; EGFR, Endothelial Growth Factor Receptor; ESMO, European Society for Medical Oncology; ISET-Isolation by Size of Epithelial Tumors; JSCCR, Japanese Society for Cancer of the Colon and Rectum; mCRC, metastatic colorectal cancer; NCCN National Comprehensive Cancer Network; PFS-progression free survival; WT, wild type; MT, mutation; OS, overall survival.

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Background: Quantification of Circulating Tumor Cells (CTCs) as a prognostic marker in metastatic colorectal cancer (mCRC) has already been validated and approved for routine use. However, more than quantification, qualification or characterization of CTCs is gaining importance, since the genetic characterization of CTCs may reflect, in a real time fashion, genetic profile of the disease. **Objective:** To characterize *KRAS* mutations (codon 12 and 13) in CTCs from patients with mCRC and to compare with matched primary tumor. Additionally, correlate these mutations with clinical and pathological features of patients. **Methods:** Blood samples were collected from 26 patients with mCRC from the AC Camargo Cancer Center (São Paulo-Brazil). CTCs were isolated by ISET technology (Isolation by Size of Epithelial Tumors; Rarecells Diagnostics, France) and mutations analyzes were performed by pyrosequencing (QIAGEN). **Results:** *KRAS* mutation was detected in 7 of the 21 cases (33%) of samples from CTCs. In matched primary tumors, 9 of the 24 cases (37.5%) were found *KRAS* mutated. We observed that 5 of the 9 samples with *KRAS* mutation in their primary tumor had also *KRAS* mutation in CTCs, meaning a concordance of 71% of matched cases ($P = 0.017$). *KRAS* mutation neither on primary tumor nor in CTCs was associated with clinical-pathological parameters analyzed. **Conclusion:** Faced with a polyclonal disease like colorectal cancer, which is often treated with alternating and successive lines of chemotherapy, real time genetic characterization of CTCs, in a fast and feasible fashion, can provide important information to clinical management of metastatic patients. Although our cohort was limited, it was possible to show a high grade of concordance between primary tumor and CTCs, which suggests that CTCs can be used as surrogate of primary tumors in clinical practice, when the knowledge of mutation profile is necessary and the primary tumor is not available.

Introduction

The analysis of Circulating Tumor Cells (CTCs) in the era of personalized medicine is a promising tool for early detecting response to therapy. Nowadays, the identification of specific biomarkers that brings information to the target therapy selection is generally made with specimens collected at the moment of initial diagnosis. Frequently, the material is not available or is found insufficient for molecular analysis in advanced stages of the disease.[1] In addition, initial primary tumor specimens are not always representative of the metastasis that can occur many years after the resection of the primary tumor.[2] After primary tumor biopsy or surgical removal, many other mutations can be acquired and other specific abnormalities found in the primary tumor can be lost. In this way, the CTC analysis can provide a non-invasive way of evaluating a tumor genotype and better correlate with the dynamic process of tumor progression and resistance to chemotherapeutic drugs.[3]

The development of target therapies has created a need for rapid and robust molecular characterization of cancer. *KRAS* gene codes for an essential protein involved in the activation of the pathway within the signaling cascade induced by the activation of the *Endothelial Growth Factor Receptor* (*EGFR*) gene. *KRAS* gene plays a central role in tumor development by

*Correspondence to: Ludmilla TD Chinen; Email: ludmillatdchinen@hotmail.com
Submitted: 03/03/2015; Revised: 05/26/2015; Accepted: 07/03/2015
http://dx.doi.org/10.1080/15384047.2015.1070991

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regulating the expression of proteins that are involved in cell proliferation and survival, metastasis and angiogenesis.[4-6]

In colorectal carcinoma, *KRAS* mutations are present in 30-40% of patients.[7] Activating mutations in *KRAS* are responsible to anti-EGFR therapy resistance (Cetuximab or Panitumumab) in metastatic colorectal cancer (mCRC). More recently, around 18% of patients with wild type *KRAS* - exon 2, were find to carry mutation in exon 3, 4 of *KRAS* gene and exon 2, 3, 4 of *NRAS* gene. Thus, only wild-type *RAS* (*KRAS* and *NRAS*) patients can receive anti-EGFR therapy for mCRC, according to Treatment Guidelines for CRC published by the National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO) and Japanese Society for Cancer of the Colon and Rectum (JSCCR).[8] Metastatic process is dynamic, so, longitudinal monitoring of CTCs can provide a noninvasive and real time evaluation of potential biomarkers related to resistance to specific target therapies.

In this sense, our study had the objective to characterize *KRAS* mutations on CTCs isolated by ISET from patients with mCRC and to compare *KRAS* results with primary tumors. We also tried to correlate *KRAS* mutations with clinical and pathological features of patients with mCRC.

Results

CTCs were identified in 23/26 patients, with a median CTC count of 2 CTCs/mL (range 0-14 CTCs/mL). **Figure 1** shows CTCs isolated by ISET and residual leukocytes from a patient with mCRC. Three patients had no detectable CTC.

We evaluated the *KRAS* (codons 12 and 13, exon 2) in CTCs samples obtained from 23/26 patients with mCRC. Then, we matched their results with the results of *KRAS* mutations from primary tumors obtained from medical records.

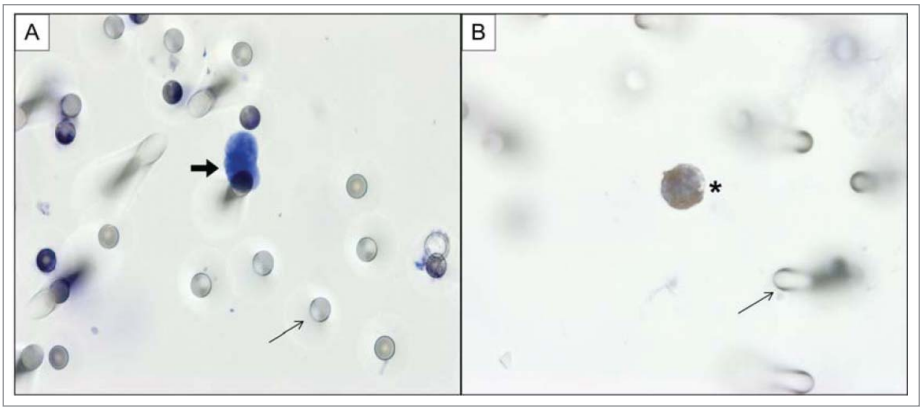

Figure 1. (A) CTC (thick arrow) isolated by ISET from patient with mCRC stained with hematoxylin-eosin. (B) Residual leukocyte (asterisk), without any CTC visualized on the ISET membrane. Spiked cells were stained with antibody against CD45 to identify leukocytes, visualized with DAB (3,3′- Diaminobenzidine) and counterstained with haematoxylin. Images were taken using a light microscope (Axioskop 40; Carl Zeiss Meditec, Jena, Germany) coupled to a digital camera (Sony Cyber-Shot Dsc-s75; Sony, Tokyo, Japan) at 60× magnification. Fine arrows represent pores of ISET membrane.

In 5 samples of the 23 patients there was no PCR amplification. We found KRAS mutation in 7 samples of CTCs (7/21) (33%). All mutations of KRAS in CTC were found in codon 12.

In matched primary tumors, we observed *KRAS* mutation in 9 cases (9/24) (37.5%). In six primary tumors samples, *KRAS* mutation was observed in codon 12 and in 3 samples the mutation was observed in codon 13. There was concordance between *KRAS* mutations in CTCs and primary tumor in 5 samples (71%; $P = 0.017$) (patients 2, 7, 13, 14 and 20) (**Table 2**). Additionally, *KRAS* mutations in CTCs were found in 2 patients that were *KRAS* wild type (wt) in matched primary tumor (patients 4 and 22). **Table 1** shows mutational status of *KRAS* in CTCs samples and primary tumors.

We observed *KRAS* wt in 3 cases without counting CTC, reflecting the presence of leukocytes in samples, as observed by immunocytochemistry with anti-CD45 (**Fig 1**).

The *KRAS* status and its association with clinical-pathological parameters are shown in **Table 2**. *KRAS* mutation was not associated with the clinical-pathological parameters analyzed (histological grade, lymph node status, diagnosis, tumor progression, carcinoembryonic antigen (CEA). The progression-free survival (PFS) was not statistically different between patients with *KRAS* mutation and *KRAS* wild type neither on primary tumor samples nor on CTC's samples ($P = 0.143$ and $P = 0.185$, respectively). We did not find association between PFS and CTC's counts ($P = 0.194$), histological grade ($P = 0.381$) and CEA level ($P = 1.000$).

We observed that 9 of 23 patients analyzed here showed disease progression after inclusion in our study (all of them were included after a diagnosis of metastasis or protocol change and the blood was collected before first or line second line of chemotherapy). In 4/9 patients we could not assess the KRAS status in CTC because there was no PCR amplification. In 3/9 patients we observed KRAS mutation in CTCs and 2 of them died in 3.18 and 9.18 months after inclusion in study. In **Table 3** we show status of KRAS mutation (codon 12/13) gene in primary tumors and in CTCs in these 9 patients that showed disease progression after inclusion in our study, as also types of treatment.

Discussion

Approximately half of patients with mCRC carry mutations in *KRAS* and *NRAS* genes, and theses mutations result in resistance to anti-EGFR therapy. Although, 50% of patients with wild type *RAS* present better response to Cetuximab and Panitumumab therapy, not all patients benefit equally from these specific and expensive drugs. Other biomarkers have already been linked to drug resistance, for example *PI3KCA, PTEN, Amphiregulin, Epiregulin*.[9] Several studies have shown that CTC

Table 1. Status mutation of KRAS (codon 12/13) and BRAF (V600E) genes, tumor site, CTC count, in CTC and primary tumor of patients with mCRC

Patients	Site	CTC /mL	Tumor progression	KRAS status in CTCs	KRAS status in primary tumor
1	colon	3.0	No	NA	WT
2	rectum	1.0	Yes	MT codon 12	MT codon 12
3	rectum	0	No	WT	WT
4	rectum	5.0	No	MT codon 12	WT
5	colon	1.0	Yes	NA	WT
6	colon	1.0	Yes	NA	WT
7	rectum	6.0	Yes	MT codon 12	MT codon 12
8	colon	1.0	Yes	NA	MT codon 13
9	colon	5.0	Yes	WT	WT
10	rectum	0	No	WT	WT
11	colon	1.0	Yes	NA	MT codon 12
12	colon	14.0	No	WT	WT
13	colon	4.0	No	MT codon 12	MT codon 12
14	colon	2.0	No	MT codon 12	MT codon 13
15	rectum	2.0	No	WT	WT
16	rectum	0	No	WT	WT
17	rectum	5.0	No	WT	MT codon 13
18	colon	5.0	No	WT	WT
19	colon	2.0	No	WT	WT
20	colon	2.0	Yes	MT codon 12	MT codon 12
21	colon	12.0	No	WT	WT
22	rectum	1.0	No	MT codon 12	WT
23	colon	2.0	No	WT	MT codon 12
24	rectum	4.0	No	WT	WT
25	colon	5.0	Yes	WT	WT
26	colon	2.0	No	WT	WT

NA- not analyzed; WT- wild type; MT- mutation

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detection is an important prognostic factor in patients with mCRC.[10-14] High levels of CTCs are correlated with worse prognosis in breast cancer and their decrease after treatment are related to better survival.[15] More recently, liquid biopsy technology, such as CTCs, have shown to be able to provide real time information about the tumor biology. Genotyping CTCs may be

Table 2. Correlation between clinical-pathological parameters and KRAS mutations in CTC and primary tumors in patients with mCRC

	KRAS CTC			KRAS primary tumor		
Parameters	**No Mutation (%)**	**Mutation (%)**	**P value**	**No Mutation (%)**	**Mutation (%)**	**P value**
CTC Count						
< 2 cells	03 (21)	02 (29)	1.000	06 (40)	03 (33)	0.547
≤ 2 cells	11 (79)	05 (71)		09 (60)	06 (67)	
Histological grade						
Well-differentiated	00 (00)	01 (17)	0.353	00 (00)	01 (14)	0.368
Poorly/moderated differentiated	11 (100)	05 (83)		12 (100)	06 (86)	
Diagnosis						
Colon carcinoma	08 (57)	03 (43)	0.659	09 (60)	06 (86)	1.000
Rectum carcinoma	06 (43)	04 (57)		06 (40)	03 (33)	
Progression of tumor						
No (1)	12 (86)	04 (57)	0.280	11 (73)	04 (44)	0.157
Yes (2)	02 (14)	03 (43)		04 (27)	05 (56)	
Lymph nodes status						
No	01 (14)	02 (33)	0.559	02 (25)	01 (12.5)	1.000
Yes	06 (86)	04 (67)		06 (75)	07 (87,5)	
CEA						
< 13	07 (58)	04 (57)	1.000	08 (61.5)	03 (33)	0.387
≥ 13	05 (42)	03 (43)		05 (38.5)	06 (67)	
KRAS mutation in primary tumor						
No	10 (83)	02 (29)	0.017			
Yes	02 (17)	05 (71)				

CTC: circulating tumor cell; CEA: carcinoembryonic antigen.

Table 3. Status of KRAS mutation (codon 12/13) gene in primary tumors and in CTCs, forms of treatment and time to progression after CTC analysis of 9 mCRC patients that showed disease progression after inclusion in our study

Patients	CTC /mL	KRAS status in CTCs	KRAS status in primary tumor sample	Treatment after the CTC collection	Progression after CTC collection	Treatment after progression	Time of progression (month)	Cuurent Status
2	1.0	MT codon 12	MT codon 12	Folfiri (C2) Folfiri + Bevacizumab (C12)	Yes	Folfox (C5)	13.21	Stable disease
5	1.0	NA	WT	Folfiri + Bevacizumab (C7) Folfox + bevacizumab (C2)	Yes	Irinotecan+ Cetuximab	7.00	Death
6	1.0	NA	WT	Folfox (C6)	Yes	Irinotecan+ Cetuximab (C10)	23.1	Death
7	6.0	MT codon 12	MT codon 12	Folfox (C6)	Yes	Irinotecan+ Cetuximab (C4)	9.18	Death
8	1.0	NA	MT codon 13	Folfiri + Bevacizumab (C1)	Yes		4.26	Death
9	5.0	WT	WT	Folfiri + Cetuximab (C12)	Yes		6.22	Death
11	1.0	NA	MT codon 12	Folfiri + Cetuximab (C4)	Yes	Folfox	4.22	Death
20	2.0	MT codon 12	MT codon 12	Folfiri (C1)	Yes		3.18	Death
25	5.0	WT	WT	Folfiri + Cetuximab (C8)	Yes	Capecitabine+ Bevacizumab (C16)	5.8	Stable disease

NA- not analysed; WT- wild type; MT- mutation, C- number of cycles.

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an early manner to detect mutations that may have therapeutic implication.[10,16-18]

Here, we performed analysis of *KRAS* mutations in CTCs samples and *KRAS* status in matched primary tumor of patients with mCRC. In matched cases, we found a concordance of *KRAS* status between CTC and primary tumors of 71%. Similar results were also found by other groups.[7,19-21] Fabbri et al.,[19] using a dielectrophoresis-based device, had analyzed *KRAS* mutation (exon 2), and observed 50% of concordance between *KRAS* mutation in CTCs and matched primary tumor of mCRC. Mostert et al.[7] found 5 of the 42 (12%) mCRC patients with *KRAS* mutation (codon 12/13) in CTCs by nested ASB-PCR. This group also observed that 4 of the 5 patients analyzed had the same *KRAS* mutation in CTC and their metastasis tissue. Raimondi et al.[21] investigated *KRAS* mutation in CTCs and tumor tissue of CRC patients and observed 29% of correlation of *KRAS* mutation between CTC and tumor tissue matched. Here, we also observed that 33% of CTCs samples showed *KRAS* mutation. This result is similar to previous findings published by Gasch et al.,[20] that verified the presence of mutation in *KRAS* in 1 of the 5 CTCs of mCRC patient studied and the same mutation was observed in primary tumor.

Concerning this level of agreement found by our group and others[7,19,20] between CTCs and primary tumors, it has been hypothesized that the development of resistance to EGFR therapy is caused by rare cells with *KRAS* mutations that pre exist at low levels in mCRC with apparent *KRAS* wt.[22] Diaz et al.[22] tested this hypothesis by analyzing 24 patients with mCRC, all of them with *KRAS* wt in primary tumor and resistant to anti-EGFR therapy. They showed by circulating free tumor DNA (cftDNA) that 9 of the 24 (38%) patients showed *KRAS* mutation in their serum. These authors suggested that *KRAS* mutations were present in subclones before the initiation of treatment. In this way, this mutation could be a mediator of acquired resistance to EGFR therapy. It is expected that cells containing *KRAS* mutation would be released into the circulation being possible to detect by non-invasive manner, as cftDNA[22] or CTC technique.[18] Although our study had fewer numbers of patients, we could observe CTCs containing *KRAS* mutations in 2 patients whose primary tumors were *KRAS* wt. As described by Diaz et al.,[22] our result suggests that these patients could develop resistance to anti-EGFR treatment by *KRAS* mutation present in subclones in primary tumor.

In favor of dynamic process that evolves tumor progression, in 2 patients from our study, we found differences in *KRAS* mutation in CTCs samples (mutation in codon 12) and matched primary tumors (mutation in codon 13). This result suggests that more than one subclone could be present in tumor. Other reports also found discrepancy of concordance between types of *KRAS* mutations in CTCs and primary tumor or metastasis tissue. Again, it can be explained by the cellular heterogeneity found in epithelial malignancies, which can be reflected by the heterogeneity found in CTCs.[7,19,22,23] Another explanation for our findings is the parallel progression model, which suggests that it may be possible to have various metastatic subclones which are disseminated early during disease development and remain dormant for years.[24]

Additionally, we tried to correlate our findings with clinical-pathological characteristics of patients. It is presumed that patients with *KRAS* mutations are more likely to develop progression,[12] but we could not associate this mutation with progression or another clinical-pathological feature and progression-free survival (PFS) in our CTC samples neither on primary tumors. The lack of association may be due to the small number of cases analyzed and the relatively poor frequency of *KRAS* mutation in both, CTCs ($n = 7$) and primary tumors ($n = 9$). Another group, recently, showed that CTCs count and *KRAS* status in tumor were independent prognostic factors for patients with mCRC treated with monoclonal antibody (bevacizumab plus chemotherapy). In this study, patients with less CTC's count (<3 CTCs/7.5mL) and *KRAS* wt in tumor had higher PFS and overall survival (OS) than patients with more CTCs (>3 CTCs/7.5mL) and KRAS mutation in tumor ($P = 0.001$ and $P = 0.004$, respectively).[17]

The presence of heterogeneous cell population in the blood may be an obstacle to the detection of gene mutation in CTCs. We can note that the leukocyte contamination of CTCs sample and their retention on membrane surface could camouflage the results. We observed than in 3 cases without CTC score, *KRAS* wt has been observed, reflecting the presence of other cells, in addition to the CTC, in the ISET membrane. Maybe, a viable alternative is to make laser microdissection of ISET membranes. In the case of *KRAS*, which is detected in 30–40% of patients with CRC,[7] we believe that the laser microdissection is not necessary and contamination with leukocytes is not sufficient to disrupt the reading. The pyrosequencing is a sensitive technique, being able to identify the mutation in a few cells. Several studies have shown its sensitivity.[25,26] However, we should give attention to leucocyte contamination when a qualitative method of sequencing is been employed.

Our results show that molecular characterization of CTCs, such as *KRAS* status, are relevant, as they show that these cells can be used as "liquid biopsy," since samples from primary tumors are not always available to make molecular analysis. Moreover, CTCs can be a useful tool to determine real time molecular profile of mCRC patients in order to characterize the evolution of a determined biomarker responsible for resistance. Here, we used pyrosequencing, after isolating CTCs by ISET technology. ISET has been used for research purpose only, but it underwent technical.[27,28] and clinical validation.[29] In addition, this technique has been show to be extremely sensible and specific as cells with malignant phenotype were found only in patients with cancer and never in healthy volunteers when tested on ISET (reviewed by Chinen et al, 2014).[30] Thus, we suggest that the method described here using CTCs isolated by ISET can be used for the most common mutations, as it is faster, cheaper and practical. However, despite its feasibility, molecular analysis after CTCs isolation by ISET, must be tested in a larger cohort of patients in order to validate our findings.

In conclusion, we demonstrated that CTC can be characterized based on molecular profile and genetic analysis. Moreover, for mCRC, there are discrepancies among KRAS mutation both, in CTCs and primary tumor. The hypothesis that this disease presents dynamic changes from the point of view of molecular abnormalities is reinforced by our data.

Materials and Methods

Patients

This study included 26 patients with mCRC obtained from the A C Camargo Cancer Center, São Paulo-Brazil, between February and November (2013). It was approved by local Ethics Committee (study n° 1708/12). Patients with stage IV CRC were recruited at Clinical Oncology Department. The majority of patients (18/26) (69.2%) had received previous therapy (first line chemotherapy) and were receiving monoclonal therapy with anti-EGFR antibody (17/26) (63.4%) before the blood collection for CTC analysis. The most common site of metastasis was liver (8/26) (30.8%), followed by liver and lung (7/26) (29.6%)

Evaluation of tumor response to therapy followed the radiologic criteria of RECIST (version 1.1).[31] Clinical-pathological characteristics of patients are summarized in **Table 4**.

CTCs were isolated by ISET technology (Isolation by Size of Epithelial Tumors; Rarecells Diagnostics, Paris, France). Blood samples (8mL) from patients were collected in EDTA tubes, stored at room temperature for up to 4 hours under homogenization and processed by ISET according to manufacturer's instructions. ISET methodology isolates intact CTCs from blood through direct filtration without using antibodies, as it exploits the larger size of tumor cells as compared with leukocytes. Recently, we reported the advantage of ISET, as compared with a "marker-related" approach, in showing progression of a metastatic lung cancer before the progression became evident by imaging.[32] ISET uses a polycarbonate membrane with 8-μm-diameter cylindrical pores. We used procedures of this platform and CTCs definition criteria according to previous detailed reports.[33] ISET membrane spots were cut and submitted to immunocytochemistry with anti-CD45 antibody (1:100; clone 2B11 + PD7/26, Dako™), a surface leukocyte marker, in order to differentiate them from the CTCs. For CTCs counting, 8 spots from the ISET filter were used (corresponding to 8 mL of blood), after staining with hematoxylin and eosin. Counting was made per mL of blood. Cells were considered as CTCs if they were negative for CD45, have presented with hypercromatic nucleus, irregular shape, high cytoplasm nucleus ratio (>0.5) and nuclear size

Table 4. Clinical and pathological characteristics of patients with mCCR

Clinico-pathological parameters	Total (%)
Sex	
Male	18 (69.2)
Female	8 (30.8)
Lymph nodes	
N0	3 (11.5)
N1-3	14 (53.8)
No lymph node dissection	9 (34.6)
Localization	
Colon	16 (61.5)
Rectum	10 (38.5)
Histological grade	
Well-differentiated	1 (3.8)
Poorly/moderately differentiated	20 (76.9)
Not available	5 (19.2)
Progression	
No	17 (65.4)
Yes	9 (34.6)
Metastases status	
No	2 (7.7)
Yes	19 (73.1)
Not available	5 (19.2)
CEA	
< 13	12 (46.2)
≥ 13	12 (46.2)
Not available	2 (7.7)
Size	
T1/T2	3 (11.5)
T3	16 (61.5)
T4	3 (11.5)
Not available	4 (15.5)

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≥12 μm. Images of results obtained with this technique were taken using a light microscope (Research System Microscope BX61– Olympus, Tokyo, Japan) coupled to a digital camera (SC100–Olympus, Tokyo, Japan) at 40× and 60× magnification.

Although ISET has passed through technical,[27,28] and clinical validation,[32,34] we made our own tests of its sensitivity and specificity, as described previously by Chinen et al., (2014).[30] Briefly, we spiked different numbers of cells HCT 116 (colorectal carcinoma cell line) in 1 ml of blood from healthy subject. We made the dilutions in a counting chamber before spiking the cells. Then, we filtered the blood by using the ISET. We made the experiment with 3 concentrations, tested in triplicate, in one ml of blood: 50, 100 and 150 cells. Each spot was stained with antibody against CD45 to visualize leucocytes, counterstained with haematoxilin and read in a light microscope. We found a mean number of HCT 116 cells closely consistent with the number of cells we added to the blood before ISET.

Paraffin-embedded primary tumor samples from the same patients included for CTCs´ analysis were evaluated for *KRAS* mutation by Anatomic Pathology Department, as the analysis of *KRAS* status is standard practice in AC Camargo Cancer Center.

Analysis of Kras mutation by pyrosequencing

We performed DNA extraction of CTC in polycarbonate membrane obtained by ISET technique (after quantification of CTCs) by QIAamp DNA Micro Kit (Qiagen, Valencia, CA USA). The spot's membrane (4 spots per patient) were cut in small pieces and transferred into the lyses buffer and proteinase K supplied by Kit and the extractions were conducted according to protocol provided by the manufacturer. For analysis of primary tumor, total DNA was isolated from formalin-fixed paraffin embedded tissue with QIAamp DNA Micro Kit (Qiagen, Valencia, CA USA). All pyrosequencing reactions included DNA sample without mutation of *KRAS* gene, supplied by kit. We could not perform analyses of *KRAS* gene in metastasis samples from our patients because the majority of them were not available.

PCR amplification and pyrosequencing targeted for *KRAS* codon 12 and 13 were done by PyroMark Q24 *KRAS* (24), version 2.0, version 2.0 (QIAGEN, Valencia, CA USA), respectively, using the instrument PyroMark Q96 (QIAGEN, Valencia, CA USA).

The results from *KRAS* status in primary tumor samples were retrieved from medical records. *KRAS* analysis is currently made in mCRC patients as approved test to help in choice of anti-EGFR therapies.[35] The evaluation of *KRAS* status in these samples was performed by the same methods described above for CTCs, by the Anatomic Pathology Department of the AC Camargo Cancer Center.

Statistical analysis

Association between mutational status of *KRAS* and clinical and pathological parameters were assessed by the X^2-test. The progression free survival (PFS) graphics were constructed according to the Kaplan-Meier statistical method and the difference between curves was analyzed by log-rank method.

All p-values were on 2-tailed statistical analysis and p value ≤0.05 was considered as statistically significant. Statistical analysis were performed using the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL USA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil for financial support.

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