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MRP1 expression in CTCs confers resistance to irinotecan-based chemotherapy in metastatic colorectal cancer

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Circulating tumor cells are important markers of tumor progression and can reflect tumor behavior in metastatic colorectal cancer (mCRC). Identification of proteins that confer resistance to treatment is an important step to predict response and better selection of treatment for patients. Multidrug resistance-associated protein 1 (MRP1) and Multidrug resistance-associated protein 4 (MRP4) play a role in irinotecan-resistance, and Excision Repair Cross-Complementation group 1 (ERCC1) expression can confer resistance to platinum compounds. Here, we included 34 patients with mCRC and most of them received FOLFIRI or FOLFOX chemotherapy (91.1%). CTCs were isolated by ISET® Technology and identified in 30 patients (88.2%), with a median of 2.0 CTCs/mL (0–31.0). We analyzed the immunocytochemical expression of MRP1, MRP4 and ERCC1 only in patients who had previously detectable CTCs, accordingly to treatment received ($n = 19$, 15 and 13 patients, respectively). Among patients treated with irinotecan-based chemotherapy, 4 out of 19 cases with MRP1 positive CTCs showed a worse progression free survival (PFS) in comparison to those with MRP1 negative CTCs (2.1 months *vs*. 9.1 months; $p = 0.003$). None of the other proteins studied in CTCs had significant association with PFS. We analyzed also histological sections of primary tumors and metastases by immunohistochemistry, and found no association with clinicopathological characteristics or with PFS. Our results show MRP1 as a potential biomarker of resistance to treatment with irinotecan when found in CTCs from mCRC patients. This is a small proof-of-principle study and these early findings need to be validated in a larger cohort of patients.

Colorectal cancer (CRC) is the third most common tumor in men and the second in women worldwide.[1] Despite the great advance in overall survival for those patients with metastatic disease in recent years, the majority of patients cannot be cured due to progression of disease and death. Unfortunately, even after initial response to chemotherapy, most of tumors develop mechanisms of drug resistance and it leads to treatment failure. Circulating Tumor Cells (CTCs) are reported to cause distant metastasis and are a prominent field of investigation. Previous studies have demonstrated that high CTCs levels are correlated with poor progression free survival and overall survival (OS) in many neoplasms.[2–4]

In a previous study[5] of our group, we investigated the role of Thymidylate Synthase (TYMS) expression, a protein correlated to tumor resistance to 5-FU-based therapy, in CTCs from patients treated with FOLFIRI/FOLFOX/5-Fluorouracil (5-FU). All of them received 5-FU-based chemotherapy, so we focused on TYMS expression. However, we observed that patients without TYMS expression in their CTCs also had disease progression after a period of treatment. Therefore, we decided to search for different proteins that could be involved in drug resistance by analyzing the CTCs in these patients.

Multidrug Resistance (MDR) is a relevant phenomenon that can occur in cancer cells, as per overproducing drug-transporting proteins, increasing efflux of a broad class of hydrophobic cytotoxic drugs.[6] Multidrug resistance proteins (MRP) belong to the ATP binding cassette (ABC) transporter family[7] located in the cell membrane and act modulating absorption, distribution and excretion of many chemical compounds. MRPs expel cytotoxic molecules, like chemotherapeutic agents and protect the target

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What's new?
Wouldn't it be nice to know right away when a patient's cancer becomes drug-resistant? New results suggest that a molecular marker, MRP1, on circulating tumor cells could provide just such a tip. These authors studied patients with metastatic colorectal cancer who were receiving irinotecan therapy. They tested the patients' circulating tumor cells for various marker proteins, and when the cells carried MRP1, irinotecan resistance was more likely.

cell from death. The multidrug resistance-associated protein 1 (MRP1; *ABCC1* gene) has been described as a contributor to the transport of folate-based antimetabolites, anthracyclines, plant alkaloids, antiandrogens and camptothecins, such as irinotecan and topotecan.[8–10] MRP4 belongs to the same family of drug transporters; however, it confers resistance to irinotecan, topotecan and methotrexate.[9] Excision repair cross-complementation Group 1 (ERCC1) is a protein involved in DNA damage and repair process, that is described to confer resistance to platinum-based chemotherapy.[11,12] Thereby, these three proteins cooperate to tumor resistance to the two agents most commonly combined with 5-Fluorouracil in the treatment of metastatic colorectal cancer (mCRC) patients: oxaliplatin and irinotecan. We hypothesized that by analyzing these proteins in CTCs, primary tumors and metastasis, we could identify good responders to chemotherapy in mCRC patients.

Therefore, our objective was to verify if the expression of multidrug resistance proteins MRP1, MRP4 and ERCC1 in CTCs, primary tumors and metastasis by immunocytochemistry/immunohistochemistry (ICC/IHC) could predict response to FOLFIRI or FOLFOX chemotherapy in mCRC patients, combined or not with monoclonal antibodies.

Material and Methods

This was a single center, pilot, prospective study, conducted with mCRC patients underwent treatment with systemic chemotherapy. The analysis was made by blood collection (8 mL) from mCRC patients, in order to isolate CTCs and study the MRP1, MRP4 and ERCC1 proteins staining in these cells and in paraffin embedded tissues (primary tumor and metastasis) from the same patient population.

Patients and samples

This study had been approved by institutional ethics committee and all the patients signed informed consent term before any procedure. Blood from mCRC patients treated at Medical Oncology Department of A.C. Camargo Cancer Center, São Paulo, Brazil were prospectively collected from July 2012 to December 2013. Blood was collected (8 mL) in EDTA tubes before the beginning of irinotecan/oxaliplatin/5-FU-based chemotherapy (at diagnosis of metastases, disease progression or protocol change). Inclusion criteria were: stage IV CRC patients who were initiating a new line of chemotherapy, with ECOG 0–2, without organ dysfunction, measurable disease by RECIST (Response Evaluation Criteria in Solid Tumors) criteria (version 1.1).[13] Patients who had been submitted to any surgical procedure within 3 weeks before CTC detection were excluded.

CTC Isolation

We isolated CTC using the ISET® technology as previously reported.[5] In summary, 8 mL blood was diluted with the ISET® buffer, then transferred to the ISET® block coupled to a polycarbonate membrane with 10 spots, containing calibrated, 8 micrometres diameter, cylindrical pores. The samples were filtered by negative pressure through ISET® device. The majority of leucocytes were thus eliminated by filtration. Membranes were maintained at −4°C until time of immunostaining analysis. CTCs fixed on spots from ISET membranes were stained by ICC and counterstained with hematoxylin. CTCs were counted in 4 spots of the membrane and quantified in 1 mL of blood in accordance to Krebs *et al.*[14] As negative control, we used healthy donor filtered by ISET®, and as positive control, healthy donor spiked with HCT-116 (colorectal carcinoma) cell line.

CTC immunostaining

To characterize CTCs as resistant or sensitive to treatment, we performed an ICC assay using a protocol previously described.[5] The following antibodies were chosen accordingly to the treatment: anti-MRP1 (ABCC-1, Polyclonal, Sigma 1:100, code: HPA002380), and anti-MRP4 (ABCC-4, Polyclonal, Sigma 1:100, code: HPA002476) were used in order to verify if CTCs from patients who underwent irinotecan/5-FU-based chemotherapy expressed these proteins on cell surface or cytoplasm. We used anti-ERCC1 (Polyclonal, Sigma-Aldrich 1:100, code: SAB4500795) in CTCs from patients who underwent oxaliplatin/5-FU-based chemotherapy. For all ICC reactions and all antibodies (MRP1, MRP4 and ERCC1), we used A-549 cell line, which, accordingly to The Human Protein Atlas (http://www.proteinatlas.org/)[15] express these proteins. A-549 cells were "spiked" in healthy donor and filtered on ISET® as a positive control (Figs. 2*b*, 2*f* and 2*j*). As negative control of ICC, we used the same cell-line, omitting the primary antibody, to ensure the exclusion of cross-reactivity. To confirm that CTCs analyzed were not leucocytes, we used anti-CD45 antibody (Polyclonal, Sigma 1:100, code: HPA000440). The detailed information about antibodies is shown in Supporting Information Table 1.

The evaluation of the immunostaining results was made manually on Research System Microscope BX61—Olympus coupled to SC100 high-resolution digital color camera—Olympus.

Table 1. CRC patients' clinicopathological characteristics

Variable	N°.	%
Total number of patients	34	100
Age at entry study, years		
Median (range)	54.5 (30–81)	
Gender		
Male	20	58.8
Female	14	41.2
Location of primary tumor		
Colon	26	76.5
Rectum	8	23.5
Histological grade (data available in 29/34 patients)		
Well-differentiated	3	10.3
Moderately differentiated	26	89.7
Treatment		
FOLFIRI	19	55.9
FOLFOX	10	29.4
Irinotecan	2	5.9
Other[1]	3	8.8
Cetuximab		
Yes	8	23.5
No	26	76.5
Bevacizumab		
Yes	14	41.2
No	20	58.8
Line of chemotherapy		
First	12	35.3
Second or more	22	64.7
KRAS status (data available in 31/34 patients)		
Wild-type	19	61.3
Mutant	12	38.7
Median CTC/mL number (range)		
Baseline	2.3 (0–31.25)	
Median CEA serum level (ng/mL) (range)		
Baseline (30/34)	15.2 (1.1–9531)	

Abbreviations: CTC: circulating tumor cells; CEA: carcinoembryonic antigen.
[1]Other agents: XELIRI, XELOX and Regorafenib.

Immunohistochemistry

The primary tumors and metastases paraffin blocks were obtained from A.C. Camargo Cancer Center Tissue Bank Archive. Slides obtained from these blocks were submitted to IHC staining. All reactions were accompanied by a positive control, in known positive tissue for each antibody (Supporting Information Table 1), and a negative control (removal of the primary antibody and withdrawal of the secondary complex).

The histological section was deparaffinized in xylene, three baths of 5 min each and rehydrated in alcohol 100%, 4 baths of 20 sec each, and then running water for 5 min. Antigen retrieval was done using a citrate buffer (pH 6.0) and heated in a pressure cooker for 15 min.

The slides were placed three times (5 min each) in 3% hydrogen peroxide (10V) to block endogenous peroxides, and then washed in running water for 5 min. The sections were subjected to blocking nonspecific protein casein (Protein Block Serum-Free, DakoCytomation, Carpinteria) for 20 min at room temperature in a humid chamber.

The primary antibody was diluted in diluent containing 0.05 mol/L Tris-HCl buffer and 0.1% Tween 20 (Antibody Diluent with Background Reducing Components, DakoCytomation, Carpinteria) and the slides were incubated at 4°C, overnight (12–14 hr) in a humid chamber. After three washes with a PBS 1X buffer for 5 min each, the slides were incubated with a secondary antibody, containing a pool of anti-mouse, anti-rabbit or anti-goat antibodies using the Kit Advance TM HRP (DakoCytomation, Carpinteria) for 1 hr in the darkroom, and washed with PBS three times for 5 min each. Staining was performed by using 3,3' diaminobenzidine tetrachloride (DakoCytomation, Carpinteria). The specimens were counterstained with haematoxylin, dehydrated with alcohol and xylene and then mounted on slide.

The evaluation of the IHC study results was made for each antibody manually on Research System Microscope BX61—Olympus coupled to C100 high-resolution digital color camera—Olympus.

Statistical analysis

A descriptive analysis was performed for each clinical-pathological variable and group of treatments. To evaluate differences between groups (those that expressed MRP1 and those that did not express), the $\chi 2$ test was used for categorical variables and Fisher's exact test was considered for small numbers. Survival curves were analyzed by Kaplan–Meier method and the difference between curves was calculated by the Log Rank test. The Cox proportional hazards regression model was used to run the multivariate analysis. All statistical analyses were performed using the SPSS program for Windows, version 15. The p values were considered significant when ≤ 0.05.

Results

Patients

Thirty-four patients who underwent FOLFIRI- or FOLFOX-based chemotherapy were included, 19 patients received FOLFIRI, 10 patients received FOLFOX regimen; two patients received only Irinotecan with or without monoclonal antibodies. Three patients received other agents (capecitabine plus irinotecan, capecitabine plus oxaliplatin and Regorafenib). The clinicopathological characteristics are available in Table 1. Twenty patients (58.8%) were male and primary tumor was predominantly found in the colon ($n = 26$; 76.5%). All tumors were classified as adenocarcinomas and 89.7% (29/34) of the

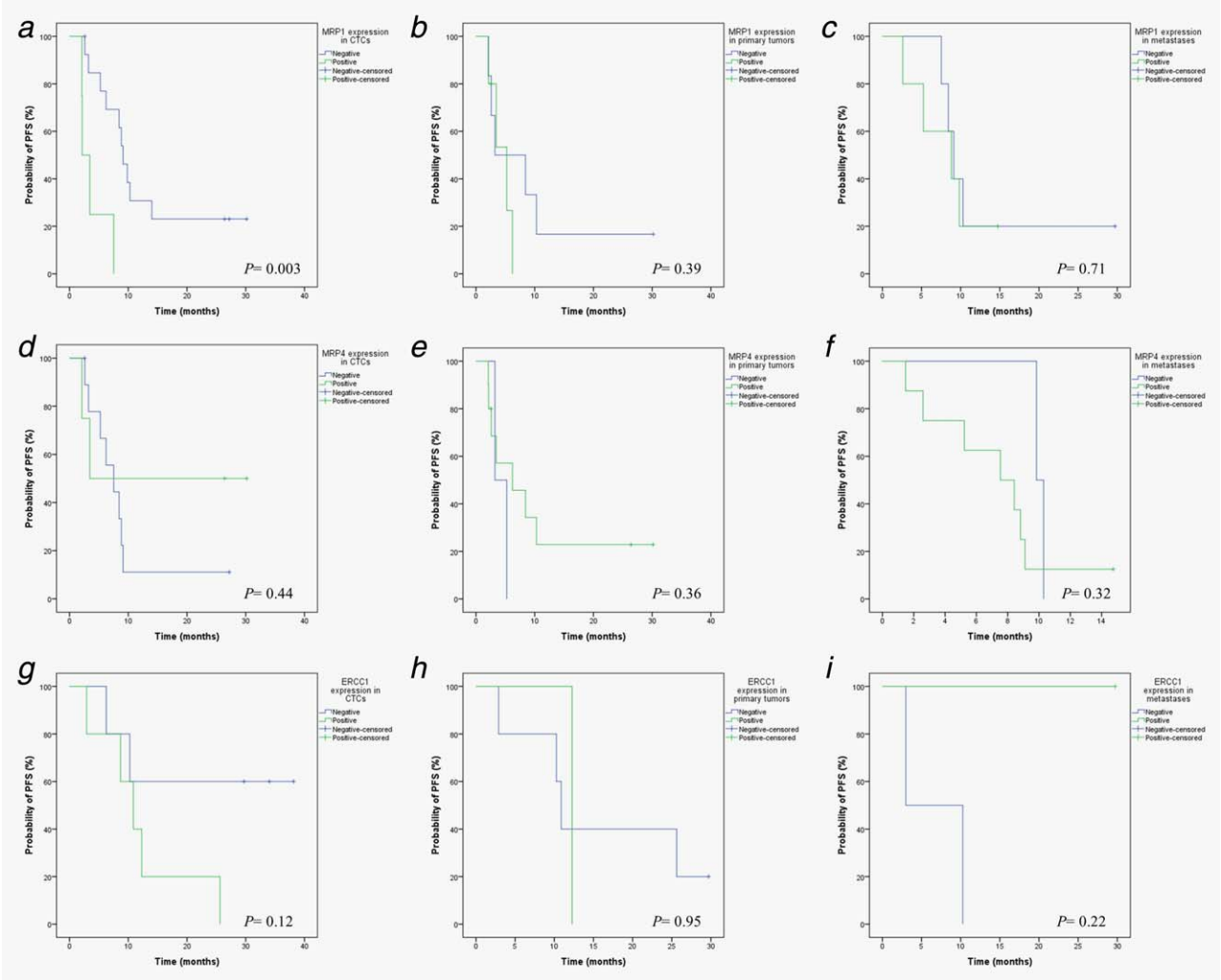

Figure 1. Progression free survival (PFS) of mCRC patients in relation to protein expression in CTCs. These cells were collected before the beginning of chemotherapeutic treatment and tested by immunocytochemistry for MRP1, MRP4 and ERCC1 antibodies. (***a***) CTCs negative for MRP1: median PFS of 9.1 months *versus* CTCs positive to MRP1: 2.1 months; $p = 0.003$. (***b***) Primary tumors negative for MRP1: median PFS of 3.2 months *versus* primary tumors positive for MRP1: 5.2 months; $p = 0.39$. (***c***) Metastases negative for MRP1: median PFS of 9.1 months *versus* metastases positive for MRP1: 8.8 months; $p = 0.71$. (***d***) CTCs negative for MRP4: median PFS of 7.5 months *versus* CTCs positive to MRP4: 3.4 months; $p = 0.44$. (***e***) Primary tumors negative for MRP4: median PFS of 3.2 months *versus* primary tumors positive for MRP1: 6.2 months; $p = 0.36$. (***f***) Metastases negative for MRP4: median PFS of 9.8 months *versus* metastases positive for MRP4: 7.5 months; $p = 0.32$. (***g***) CTCs negative for ERCC1: median PFS not achieved *versus* CTCs positive to ERCC1: 10.8 months; $p = 0.12$. (***h***) Primary tumors negative for ERCC1: median PFS of 10.8 months *versus* primary tumors positive for ERCC1: 12.2 months; $p = 0.95$. (***i***) Metastases tested for ERCC1: No median PFS was computed because all cases were censored. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

patients with data available were classified as moderately differentiated. Twenty-two patients (64.7%) were treated with monoclonal antibodies wherein 8 (23.5%) used cetuximab and 14 (41.2%) used bevacizumab. Twenty-two out of 34 patients (64.7%) were submitted to two or more lines of therapy before the blood drawn used for the CTC assay. The status of KRAS in the tumor was available in 31 medical records and 38.7% ($n = 12$) were found as mutant KRAS (MT KRAS). Median follow-up time was 9.1 months (95% CI: 7.2 – 11 months).

CTCs count and CTCs protein expression

CTCs counts were performed by morphological analysis and leucocyte-population exclusion by ICC with anti-CD45 antibody. We analyzed the membranes from all patients ($n = 34$) and no CTCs were found in 4 (11.7%) of them. The median number of CTCs per mL was 2.0 (0 – 31.0). None of the healthy donors presented CTCs.

ICC with proteins conferring treatment resistance was performed in patients who have previously detectable CTCs, and each antibody was tested accordingly with the treatment received by the patients, in an individual immunocytochemical assay. MRP1 was tested in 19 samples by ICC and found positive in 4 (Fig. 2*a*). Patients who had positive MRP1 CTC had shorter progression free survival (PFS) when compared with those with negative MRP1 CTC, 2.1 *versus* 9.1 months ($p = 0.003$) as demonstrated in Figure 1*a*.

Table 2. Patients' outcome according to CTC prevision by MRP1 staining ($n = 19$)

Patient ID	Tumor site	Treatment regimen	MRP1 assay	CTC prevision	Follow-up	CTC count/mL	Time to DP (days)
1	Rectum	FOLFIRI + Cetuximab	−	Sensitivity	SD	0.6	-
2	Rectum	FOLFIRI	−	Sensitivity	DP	11.2	159
3	Colon	Irinotecan + Cetuximab	−	Sensitivity	[1]	10	-
4	Rectum	FOLFIRI + Bevacizumab	−	Sensitivity	DP	5.9	256
5	Colon	FOLFIRI + Bevacizumab	−	Sensitivity	DP	1.6	426
6	Colon	FOLFIRI	−	Sensitivity	SD	13.7	-
7	Colon	FOLFIRI + Bevacizumab	−	Sensitivity	DP	1.2	98
8	Colon	FOLFIRI + Cetuximab	−	Sensitivity	DP	4.6	189
9	Colon	FOLFIRI + Cetuximab	−	Sensitivity	SD	4.3	-
10	Rectum	FOLFIRI + Bevacizumab	−	Sensitivity	DP	0	268
11	Colon	Irinotecan + Cetuximab	−	Sensitivity	DP	0.8	299
12	Colon	FOLFIRI	+	Resistance	DP	1.6	64
13	Colon	FOLFIRI + Bevacizumab	+	Resistance	DP	1.3	65
14	Colon	FOLFIRI + Cetuximab	−	Sensitivity	DP	0	313
15	Rectum	FOLFIRI + Bevacizumab	−	Sensitivity	SD	0	-
16	Colon	FOLFIRI + Bevacizumab	−	Sensitivity	DP	2.5	277
17	Colon	FOLFIRI + Cetuximab	+	Resistance	DP	11.8	105
18	Rectum	FOLFIRI + Bevacizumab	+	Resistance	DP	0.8	229
19	Colon	FOLFIRI + Bevacizumab	−	Sensitivity	DP	1.2	79

Abbreviations: MRP1: Multidrug Resistance Protein 1; CTC: Circulating Tumor Cell; FOLFIRI: Irinotecan, 5-Flourouracil, Leucovorin; SD: Stable Disease; DP: Disease Progression.
[1]Loss of follow up.

We analyzed the effect of CTCs positive for MRP1 on disease progression and time to progress for each patient included in the present study. As it can be seen in Table 2, we found 44% of concordance with these data (MRP1 staining and disease progression; we did not analyze the Case 3, because of lost of follow-up). We also observed that the median number of days for disease progression for patients with CTCs negative for MRP1 was 262 days, compared to 85 days in the group of patients with CTCs positive for MRP1. Of the four patients with CTCs positive for MRP1, three (75%) had colon as primary tumor site and were MT KRAS (75%). Two patients with both CTCs positive for MRP1 and tumor MT KRAS progressed to death on an average time of 96 days.

Regarding the cases with CTC negative for MRP1, 10 out of 15 (66.6%) had colon as primary tumor site. Four (26.6%) of them were tumor MT KRAS and only two (13.3%) progressed to death (average time of 267 days). There was no statistical significant difference between the groups of patients, in relation to MRP1 staining and mutational status of KRAS ($p = 0.11$, $\chi 2$ test).

Concerning some bias of analyzing the same population of a work previously reported,[5] we performed a Fisher's exact test in order to verify if the patients resistant to irinotecan (who had MRP1 expression in CTCs) belonged to a subpopulation of the patients resistant to 5-FU (who had TYMS expression in CTCs). We found that the resistant population was different between these two groups. None of patients with positive MRP1 CTC had also CTC positive for TYMS.

We attempted to detect MRP4 expression in CTCs for patients treated with irinotecan, and ERCC1 for patients treated with oxaliplatin and found 5 out of 15 (33.3%) and 5 out of 10 (50%) tumors with positive expression, respectively (Figs. 2*E* and 2*I*). None of these proteins had significant association with PFS or any clinical-pathological characteristics, as shown in Table 3.

Immunohistochemistry

To verify MRP1 expression in tumors specimens, we analyzed 12 available tumor samples and 10 available metastasis samples by IHC. We found cytoplasmic MRP1 staining in 5/12 (41.6%) of the primary tumors (Fig. 2*c*), and in 5/10 (50%) of the metastases samples (Fig. 2*d*), with no association to clinic-pathological characteristics or PFS (Table 3). Regarding MRP4 expression in tumors specimens, we evaluated 13 primary tumors samples 11 metastases samples. We found cytoplasmic staining in 11/13 (84.6%) of primary tumors (Fig. 2*g*) and in 9/11 (81.8%) of metastases tissues (Fig. 2*h*); in both cases, there was no difference in PFS between those negative and positive staining, as can be seen in Table 3.

In the case of ERCC1, we have analyzed 6 primary tumors and 3 metastatic tissues. We have found 1/6 (16.6%) of

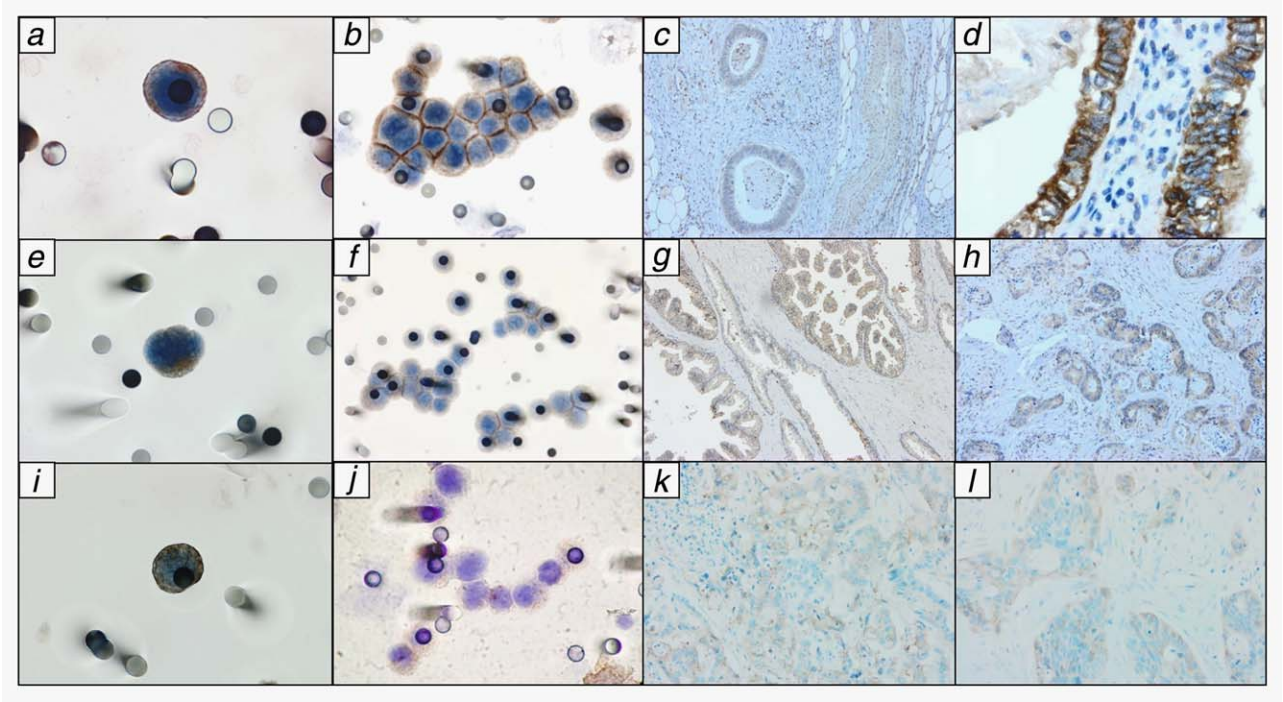

Figure 2. Immunostaining of CTCs, positive controls, primary tumors and metastases specimens. (***a***) CTC from mCRC patient positive for MRP1 (x60). (***b***) Positive control, A-549 cell line "spiked" in healthy blood and stained for MRP1 (x60). (***c, d***) Primary tumor (x10) and metastasis (x60) positive for MRP1. (***e***) CTC from a mCRC patient positive for MRP4 (x60). (***f***) Positive control, A-549 cell line "spiked" in healthy blood and stained for MRP4 (x20). (***g, h***) Primary tumor (x10) and metastasis (x10) positive for MRP4. (***i***) CTCs from mCRC patient positive for ERCC1 (x60). (***j***) Positive control, A-549 cell line "spiked" in healthy blood and stained for ERCC1 (x40). (***k, l***) Primary tumor (x20) and metastasis (x20) weakly positive for ERCC1. All photomicrographies were taken using a light microscope (Research System Microscope BX61—Olympus, Tokyo, Japan) coupled to a digital camera (SC100—Olympus, Tokyo, Japan).

Table 3. Median PFS (months) of patients with negative and positive expression of the markers in CTCs, primary tumors and metastases

	MRP1−	MRP1+	*p*	MRP4-	MRP4+	*p*	ERCC1-	ERCC1+	*p*
CTCs	9.1	2.1	0.003	7.5	3.4	0.44	-	10.8	0.12
Primary tumors	3.2	5.2	0.39	3.2	6.2	0.36	10.8	12.2	0.95
Metastases	9.1	8.8	0.71	9.8	7.5	0.32	[1]	[1]	0.22

- No median PFS was achieved.
[1]No statistics were computed because all cases were censored.

primary tumors positive (Fig. 2*k*), and 1/3 (33.3%) of metastases tissues positive (Fig. 2*l*).

The slides were analyzed by a single pathologist (VPA) who found a wide difference in the staining pattern between the different tumors, but not much in the same tumor. Some tumors had focal staining and others showed diffuse staining, however, the intensity of staining within each tumor was homogeneous.

Comparison between the expression of MRP1, MRP4 and ERCC1 in CTCs, primary tumors and metastases

We could compare the expression of MRP1 in the three sites (CTC, primary tumor and metastases) in 4 patients and found 50% (2/4) of concordance between them. Looking only at MRP1 expression in CTCs and primary tumors ($n = 11$), we observed 63.6% (7/11) of concordance. When we looked into CTCs and metastases ($n = 8$), the concordance decreased to 37.5% (3/8).

For MRP4, only three patients had the three sites evaluated for immunostaining and none of them had concordance. The same was observed in comparison between CTCs and metastases ($n = 6$), none of the patients expressed concordant MRP4 in these sites. On the other hand, looking into the expression of MRP4 in CTCs and primary tumors ($n = 10$), we found 60% (6/10) of concordance.

We made the comparison between the three sites for ERCC1 expression in three patients and found 66.6% (2/3) of concordance. Interestingly, these numbers remained the same when we compared CTCs with metastases ($n = 3$; 66.6%). Focusing in the comparison between CTCs and primary tumors, the number of patients analyzed increased ($n = 8$) and we found 50% (4/8) of concordance.

Table 4. Multivariate regression analysis of factors associated with disease progression, showing number of metastatic sites as an independent prognostic factor

Variable	Category	HR	CI (95%)	*p* values
KRAS status	Wild-type	1	–	
	Mutant	2.9	0.7 – 11.3	0.11
MRP1 expression in CTCs	Negative	1	–	
	Positive	2.8	0.6 – 13.0	0.16
Number of metastatic sites	One	1	–	
	Two or more	5.9	1.0 – 34.0	0.04

Multivariate analysis

We used the Cox proportional hazards regression model to run the multivariate analysis. We included those variables with the p values >0.25 in the PFS analysis. There were included: the CEA levels at CTC collection time (cut-off: 5 ng/mL; $p = 0.21$), lines of chemotherapy before the CTC collection ($p = 0.07$), number of metastatic sites (1 *vs.* 2 or more; $p = 0.01$); MRP1 expression in CTCs ($p = 0.003$), and KRAS status in the primary tumor ($p < 0.001$). We verified that number of metastatic sites remained as independent prognostic factor to disease progression (HR: 5.9; $p = 0.04$; Table 4).

Discussion

So far, there are described 49 members of the ATP-binding cassette transporter family, which are grouped into 7 subfamilies.[16,17] These membrane proteins have been reported as strong contributors to treatment failure in different tumor types, such as breast cancer,[18] primary central nervous system lymphoma,[19] laryngeal squamous cell carcinoma[20] and also colorectal carcinoma.[17,21] ABC transporters have also been reported as important biomarkers in neuroblastomas[22] and glioblastomas,[23] because of their capability to actively extrude a wide sort of drugs and/or their respective substrates out of the cell. Specifically, in healthy tissues, MRP1 is extensively involved in the protection of cells from toxic xenobiotics and endogenous metabolites.[24]

MRP1 protein analysis can be useful in clinical practice to select patients who will not benefit from irinotecan-based chemotherapy. Irinotecan is a topoisomerase I inhibitor, a semisynthetic drug derived from camptothecin.[25] It is the conventional option combined with 5-FU and folic acid (the FOLFIRI regimen), for first- and second-line treatment of mCRC.[26]

Our results with MRP1 expression in CTCs brought out some interesting information. We found that patients with negative MPR1 CTC had a significant better PFS (9.1 *vs.* 2.1 months; $p = 0.003$). We can suggest that in this compartment of the tumor (liquid biopsy), MRP1 could be acting by removing the drug from the cells that are able to migrate, and may be responsible for tumor seeding in distant organs. Moreover, these cells with expression of MRP1 can be resistant to irinotecan and it can explain a shorter time to progression. Although only a small percentage of patients with CTCs were positive for MRP1 (21%), this factor was a strong predictor of worse PFS. Our results corroborate previous studies in the literature. Gazzaniga *et al.*[9] investigated this marker in CTCs using a combination of immunomagnetic separation and reverse transcriptase polymerase chain reaction (RT-PCR) and performed a molecular prediction in conformity to individually chemosensitivity profile. They found that the absent MRP1 expression was associated with improved chemotherapy response.

The same group[27] evaluated MRP1 and MRP2 messenger RNA expression in CTCs by RT-PCR from metastatic breast cancer patients treated with conventional anthracyclines or nonpegylated liposomal doxorubicin, and observed that patients who had CTC with higher levels of these markers showed a significantly shorter PFS. The expression of genes related to drug sensitivity has been considered the next step towards the real personalized medicine. The higher levels of expression of MRP1 and MRP2 in colorectal tumors in comparison with normal tissues were already reported.[17]

Here, we also analyzed MRP1 expression by IHC in primary tumors specimens and the findings did not correlate with PFS such as observed in CTCs. Similarly, Moureau-Zabotto *et al.*,[28] investigated by semiquantitative RT–PCR the expression of MRP1, MDR1 (Multidrug Resistance Protein 1) and GSTP1 (Glutathione S-Transferase Pi 1) in frozen samples of breast cancer tissue and did not find impact in neither PFS nor OS, excepting for GSTP1. Burger *et al.*[29] evaluated mRNA levels of BCRP (breast cancer resistance protein), LRP (Lung Resistance-Related Protein), MRP1, MRP2 (Multidrug Resistance-associated Protein 2) and MDR1 in breast tumors. They found a correlation between the expression of BCRP and MRP1 and clinical outcome in the patients treated with anthracycline-based chemotherapy. Regarding colorectal cancer, Micsik *et al.*,[30] measured functional activity of MDR1 and MRP1 by modified calcein-assay in primary colorectal tumors and compared to normal mucosa. They found no difference in MRP1 activity between these sites. Curiously, the activity of MDR1 was increased in normal mucosa when they compared with tumors. Altogether, these results show that maybe the primary tumor is not the best compartment to be analyzed when drug resistance has to be tested. Furthermore, these studies show the role of MRP1 in primary tumors. Our work and the studies of

Gazzaniga[9] and Gradilone[27] are the only ones that searched for MRP1 expression in CTCs. However, we used different methods to isolate these cells. While they used beads coated with EpCAM to identify CTCs, we used a size-based assay, which is capable of selecting cells in an independent marker manner. In addition, ISET® relies on cytology, which is part of common good clinical practice. Our findings are the first one to compare MRP1 expression in CTCs, primary tumor and metastasis and strongly suggest that CTCs are the best tumor compartment to inform about drug resistance in mCRC.

Reinforcing the concept of different resistance mechanisms, we previously carried out a subpopulation of this study for the presence of TYMS staining in CTCs, to associate with tumor resistance to 5-FU agents.[5] Interestingly, we found that none of the patients irinotecan-resistant belonged to the population of 5-FU resistant, indeed highlighting the different mechanisms of resistance.

Curiously, 75% of patients with CTCs positive for MRP1 had also MT KRAS (the reason why they could not be treated with cetuximab) and patients CTC MRP1 negative were prevalently wild-type KRAS (WT KRAS) (69.3%). It reinforces the strong predictive value of this drug transporter protein. This finding could suggest an interaction between these pathways, and more studies are necessary to address if any connection really exists. Furthermore, colorectal cancer is a heterogeneous disease, with several prognostic factors,[31] and, maybe, MRP1 expression analysis in CTCs comes to add another prognosis marker to this scenario. We know that we worked with a small number of patients and that a more robust study is necessary to corroborate our findings.

We compared the expression of MRP4 and ERCC1 proteins in CTCs, primary tumors and metastases specimens. We observed a two-fold increased PFS in patients who had CTCs negative for MRP4 compared with those positive, although without statistical significance (7.5 *vs.* 3.4 months, respectively; $p = 0.44$). The inverse result was seen in primary tumors for MPR4 (3.2 *vs.* 6.2 months; $p = 0.36$). For metastases, we found an increase of 2.3 months of PFS in the patients negative for MRP4 in relation to those positive, also without statistical significance (9.8 *vs.* 7.5 months, respectively; $p = 0.32$). MRP4 is able to transport drugs, and plays multiple physiological functions, as protecting the brain from cytotoxic effects from topotecan.[32] Gazzaniga *et al.*[9] verified by RT-PCR the MRP expression profile in CTCs, including MRP1 as cited above, and also MRP4 in CTCs from patients treated with irinotecan. They showed no MRP4 mRNA expression in three patients who responded and weak MRP4 mRNA expression in three patients who did not respond to treatment with irinotecan, indicating that maybe this protein can be a good indicator of response to this chemotherapy.

Regarding ERCC1 expression, because of small cases analyzed, we did not reach sample enough to perform PFS analysis. ERCC1 has been demonstrated in the literature as a potential biomarker to select responders to treatment with platinum-based chemotherapy, mainly in Non-Small Cell Lung Cancer (NSCLC), when evaluated in tumors specimens.[33,34] Focusing in CTCs, ERCC1 was already evaluated in NSCLC,[11] in breast cancer,[34] and in ovarian cancer,[12] looking this expression in patients treated with platinum. The role of ERCC1 in CTCs is still controversial. Somlo *et al.*[35] found poor correlation between ERCC expression in CTCs and primary tumors ($N = 11$), and also between primary tumor and metastasis ($N = 8$). On the other hand, Das *et al.*[11] verified that the expression of ERCC1 in CTCs was correlated with worst PFS in NSCLC ($p < 0.02$, HR: 4.2). Kuhlmann *et al.*[12] analyzed the expression of ERCC1 in CTCs and in primary tumors of patients with ovarian cancer. Patients with CTCs positive for ERCC1 had poor PFS and OS, when compared with CTCs negative ($p = 0.026$ and $p = 0.009$, respectively). The expression of ERCC1 was correlated with platinum resistance ($p = 0.01$).

Altogether, our results have some limitations concerning the number of samples analyzed as well the availability of tumor tissues from primary and metastatic sites. Even considering these limitations, we could show a consistent data, mostly, in relation to CTC's isolation and analysis. CTCs have a very heterogeneous and plastic phenotype. However, the methodology we used here to isolate and to characterize these cells (ISET®, Rarecells, France) is considered feasible, as we isolate them in a marker independent manner and by this, do not lose cells due to downregulation of epithelial markers during epithelial-to-mesenchymal transition process that CTCs can suffer.[36] According to Krebs *et al* (2012)[14] there are more advantages than disadvantages using ISET® in comparison to CellSearch®. One of the major concern is about the cells that can be lost, as ISET® does not isolate cells smaller than 8 μm. At the same time, some recently published works have demonstrated a high sensitivity (around 95%) of ISET® in recapture culture cells spiked in healthy blood donor.[37–40] Moreover, cytopathology can distinguish circulating nontumor epithelial cells from circulating tumor cells, making it an additional advantage. In our previous report,[5] we made a review including the pros and cons of ISET® technology.

In the genomic research era, the personalized care challenge persists. It is necessary to understand tumor biology and chemotherapeutic resistance to overcome tumor heterogeneity. Here, knowing all limitations of our hypothesis generating study, we advocate that CTCs is a feasible and noninvasive manner to better select treatment for patients with mCRC. We believe that it is urgent to conduct studies with large cohorts of patients to define the real role of CTCs and the expression of MRP1 in these cells in the clinical practice.

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