

# Prognostic Value of Circulating Mutant DNA in Unresectable Metastatic Colorectal Cancer

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**Objective:** No validated biologic prognostic marker is presently available in metastatic colorectal cancer (MCRC). We prospectively evaluated the prognostic value of circulating mutant DNA in 31 patients presenting an unresectable MCRC treated by chemotherapy, and we used, as tumor markers, *KRAS* mutations and methylation of the *RASSF2A* promoter.

**Methods:** Detection in the serum of *KRAS* mutation and *RASSF2A* methylation were performed using sensitive methods, respectively, real-time polymerase chain reaction (PCR) performed in the presence of a peptide nucleic acid specific of the wild-type sequence and methyl-specific PCR after bisulfite treatment.

**Results:** Among 29 MCRC patients for whom DNA from the primary tumor was available, 23 (79%) presented at least one of the markers in their primary tumor, and 12 of them presented the same alteration in serum. For the 2 remaining patients, *RASSF2A* methylation was detected in serum indicating that this alteration was present in the primary tumor. These 14 patients with a detectable tumor marker in their serum were designed sDNA+ patients. After 6 months of follow-up, 11/14 (79%) sDNA+ and 1/11 (9%) sDNA- patients presented a progressive disease ( $P = 0.001$ ). The median progression free survival was 5 months in sDNA+ patients versus 14 months in sDNA- patients ( $P = 0.004$ ). After 1 year of follow-up, 2 of 14 (14%) sDNA+ and 8 of 11 (73%) sDNA- patients presented no signs of disease progression ( $P = 0.005$ ).

**Conclusions:** This study suggests that the presence of circulating mutant DNA in unresectable MCRC patients, which can be detected using simple methods such as methylation-specific PCR or real-time PCR, is highly predictive of clinical outcome.

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Colorectal cancer (CRC) is one of the most common causes of cancer death in Western countries.<sup>1</sup> Twenty percent of patients with CRC have synchronous metastases at the time of diagnosis and, for the other patients with an initial non metastatic disease, approximately 50% of them will develop metachronous metastases during the follow-up.<sup>2</sup> It has been established that 80% to 90% of metastatic colorectal

cancer (MCRC) patients have an unresectable metastatic disease and therefore chemotherapy represents the main treatment option.<sup>2</sup> Although the development of new combinations of conventional chemotherapies and introduction of targeted therapies has recently led to an improvement of the overall survival of patients with MCRC, with an increase of the median overall survival from 12 to approximately 20 months, the 2 years survival rates in MCRC remains below 40%.<sup>2,3</sup>

The prognostic evaluation of MCRC patients is based on clinical and radiologic exams and, as recently highlighted, the large variability in the survival of MCRC patients underlines the need to identify new prognostic markers.<sup>2</sup> Development of reliable molecular markers with a prognostic value should facilitate, in particular, among the patients who cannot undergo curative surgery, identification of those who might really benefit from a chemotherapy or an optimization of chemotherapy protocols.

Several studies have clearly demonstrated the presence, in the blood of cancer patients, of free circulating DNA resulting from the lysis of circulating cancer cells or tumor necrosis and apoptosis.<sup>4–11</sup> Higher levels of circulating DNA have been reported in CRC patients than in healthy subjects, but no clear correlation has been established between the level of circulating DNA and either the size and site of the tumor, or the clinical course of the disease.<sup>5–9</sup> In contrast, detection of circulating mutant DNA, which is a technical challenge considering the dilution in the blood of mutant DNA by wild-type DNA, appears a more promising approach, since mutant DNA is specific of the tumor. Presence in the blood of mutant DNA, either detected by DNA sequencing or allele specific polymerase chain reaction (PCR) amplification, has already been shown to have a prognostic value in CRC patients,<sup>6,7</sup> and sensitive detection of circulating mutant DNA can now be achieved by new methods with higher sensitivity such as SnaPshot,<sup>12</sup> PCR-ligase chain reaction assays,<sup>12</sup> allele-specific real-time PCR,<sup>10,13</sup> pyrosequencing,<sup>14</sup> or the BEAming method.<sup>11</sup> In CRC, several genetic and epigenetic alterations present within the primary tumor and their metastases can be used as reliable molecular tumor markers.<sup>15</sup> Among genetic alterations, *KRAS* point mutations are of particular interest because of their frequency estimated to around 40% in CRC, and their location restricted, in most of tumors, to codons 12 and 13 within exon 2, which facilitates their routine detection.<sup>15</sup> Aberrant methylation of promoters is commonly observed in colorectal cancers, and methylation of the *RASSF2A* gene has recently been reported to be one the most frequent epigenetic alterations observed in colorectal adenoma and carcinoma.<sup>16,17</sup>

The aim of our study was to evaluate, in treated unresectable MCRC patients, the prognostic value of circulating mutant DNA and we chose, as tumor markers, *KRAS* point mutations and *RASSF2A* methylation.

## PATIENTS AND METHODS

### Patients

Included patients presented either a newly diagnosed unresectable MCRC or a MCRC which had previously been treated by chemotherapy. Metastases were synchronous or metachronous of the

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CRC. Patients met the following eligibility criteria: histologically proven adenocarcinoma, at least 1 bidimensionally measurable tumor target lesion; absence of prior or concomitant other malignancy; absence of severe concomitant medical condition. Patients with familial history of colorectal cancer suggestive of familial adenomatous polyposis or Lynch syndrome were excluded. Patients were evaluated with the Köhne predictive score developed for MCRC patients and determined according to the following items: World Health Organization performance status; white blood cell count, alkaline phosphatase, and number of metastatic sites.<sup>18</sup> Patients were then divided into the 3 Köhne predictive score risk groups.<sup>18</sup> Chemotherapy response was evaluated every 3 months blindly with regards to molecular results, according to the response evaluation criteria in solid tumors, and was classified as complete response, partial response (PR), stable disease (SD), or progressive disease (PD).<sup>19</sup> The study was approved by local ethical committees, and all patients provided signed informed consent.

### DNA Extraction

Primary tumor DNA was extracted either from paraffin embedded tumor tissue samples, using Takara's extraction kits (Madison, WI) or Ambion's kits (Huntingdon, Cambridgeshire, United Kingdom), according to the manufacturer's instructions or from frozen tissue samples using QiAamp DNA Mini KIT (Qiagen, Westburg Leudsen, The Netherlands), following the standard protocol. Peripheral blood (5 mL) was collected, in untreated patients, before the first cycle of chemotherapy and in previously treated patients, at least 1 month after chemotherapy, into a serum separator tube containing a clot activation additive and a barrier gel. Serum was immediately separated from the cellular fraction by centrifugation at 4000 rpm for 10 minutes and then stored at  $-80^{\circ}\text{C}$ . DNA was extracted from 200  $\mu\text{L}$  of serum, using the Nucleospin Plasma XS Kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacturer's instructions, eluted into a final elution of 20  $\mu\text{L}$  and stored at  $-20^{\circ}\text{C}$ .

### Detection of KRAS Mutations

*KRAS* exon 2 was PCR-amplified from tumor DNA using the sense primer 5'ATTAAGGTTACTGGTGGAG3' and the antisense primer 5'CTATTGTTGGATCATATTCG3'. PCR was performed on a GeneAmp 9700 Thermocycler (Applied Biosystems, Foster City, CA), using Hot Star TaqDNA polymerase (Qiagen), with an initial denaturation for 15 minutes at  $95^{\circ}\text{C}$ , followed by 10 cycles of  $95^{\circ}\text{C}$  for 15 seconds,  $60^{\circ}\text{C}$  for 15 seconds with a decrease of  $1^{\circ}\text{C}$  per cycle,  $72^{\circ}\text{C}$  for 15 seconds, followed by 30 cycles of  $95^{\circ}\text{C}$  for 15 seconds,  $50^{\circ}\text{C}$  for 15 seconds,  $72^{\circ}\text{C}$  for 15 seconds, and a final extension for 10 minutes at  $72^{\circ}\text{C}$ . After purification using the Montage PCR plate extraction kit (Millipore, Molsheim, France), PCR products were sequenced using the Big Dye Terminator v3.0 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI3100 DNA sequencer (Applied Biosystems). All sequencing analyses were performed at least twice on 2 independent PCRs. Sensitive detection of *KRAS* mutation in serum was ensured, using, as previously described,<sup>10</sup> real time PCR performed in the presence of a Peptide Nucleic Acid (PNA) sequence specific for the wild-type *KRAS* codons 12 and 13, which inhibits amplification from the wild-type template.<sup>20</sup> Briefly, PCR was performed twice, with and without PNA, in a 25  $\mu\text{L}$  reaction mixture containing 12.5  $\mu\text{L}$  of the power SybrGreen kit (Applied Biosystems), 0.4  $\mu\text{M}$  of the sense 5' CCTGCTGAAAATGACTGAATAT 3' and antisense 5' CTGTATCAAAGAATGGTCCTGC 3' primers, 0.4  $\mu\text{M}$  of PNA and 5  $\mu\text{L}$  of eluted DNA extracted from serum. PCR was performed on a 9700 Taqman (Applied Biosystems), with a denaturation at  $95^{\circ}\text{C}$  for 10 minutes, followed by 50 cycles ( $95^{\circ}\text{C}$  for 15 seconds,  $70^{\circ}\text{C}$  for 15 seconds,  $60^{\circ}\text{C}$  for 1 minute). In this method, the same number of

wild-type and mutant *KRAS* copies in a sample results into a difference of Cycle threshold (Ct) of 1, when the PNA is added to the reaction since only the mutant allele is amplified (see supplementary material). To validate this method, we therefore performed limit dilutions of tumor genomic DNA harboring a heterozygous *KRAS* mutation and a  $\Delta\text{Ct}$  of 1 was systematically observed down to 50 ng/mL of genomic DNA. When the same experiment was performed with a control genomic DNA containing only wild-type *KRAS* alleles, addition of PNA resulted in a  $\delta\text{Ct}$  of 8, indicating that PCR-induced mutations were amplified. Since mutant DNA copies, when present in serum, are diluted to a large excess of wild-type copies, a  $\Delta\text{Ct}$  below 6 was considered as indicative of the presence of mutant alleles. In each case, the presence and nature of the *KRAS* mutation were systematically verified by DNA sequencing performed on PCR products obtained in the presence of PNA.

### Detection of RASSF2A Methylation

*RASSF2A* methylation was detected using a classic methylation-specific assay exploring the *RASSF2A* promoter after bisulfite treatment. Briefly, DNA extracted from tumors or serum was sodium bisulfite-modified using the EZ DNA Methylation Kit TM (ZYMO RESEARCH, Orange, CA) following the manufacturer's instructions. For methylation-specific PCR (MSP) and unmethylation-specific PCR, modified DNAs were amplified using primers that were described previously.<sup>17</sup> PCR was performed on a GeneAmp 9700 Thermocycler (Applied Biosystems), using Hot Star TaqDNA polymerase (Qiagen), with an initial denaturation for 15 minutes at  $95^{\circ}\text{C}$ , followed by 45 cycles of  $95^{\circ}\text{C}$  for 15 seconds,  $60^{\circ}\text{C}$  for 15 seconds (for MSP) or  $59^{\circ}\text{C}$  for 15 seconds (for unmethylation-specific PCR),  $72^{\circ}\text{C}$  for 15 seconds, and a final extension for 10 minutes at  $72^{\circ}\text{C}$ .

### Statistical Analysis

The aim of the study was to investigate the association between the presence of tumor DNA in serum and progression free survival (PFS). PFS was calculated as the period from blood sampling to the first observation of disease progression or to death. The secondary objectives were survival at 1 year and response status at 3 and 6 months after blood sampling. Patients with complete response or PR or SD were considered as patients with controlled disease (CD). The duration of follow-up was calculated from the date of the first chemotherapy infusion after blood sampling to the cut-off date of the study. Duration of survival was the interval from the date of blood sampling to the date of death or last follow-up. Clinical evaluation and the molecular analysis of the patients were blindly performed by independent investigators. Qualitative variables were compared with the Fisher exact test and quantitative variables were compared with the Mann-Whitney *U* test. PFS were estimated using Kaplan-Meier method and compared with the log-rank test. A *P* value less than 0.05 (2-tailed) was considered significant. Statistical analyses were performed with the STATVIEW software (Statview for Windows, SAS Institute, version 5, NC).

## RESULTS

### Patient Characteristics

Thirty-one patients treated for MCRC, including 19 men and 12 women with a median age of 71 years (range: 49–84), were prospectively enrolled. Patient's characteristics are summarized in Table 1. Among the 31 MCRC patients (1 patient having presented 2 primary CRC), DNA from the primary tumor was available for 29. We detected a *KRAS* point mutation and a *RASSF2A* promoter methylation in the primary tumors of 14 (48%) and 22 (76%) patients, respectively, and 23 of 29 (79%) patients presented at least 1 alteration in their primary tumor (Table 2). We observed no

**TABLE 1.** Characteristics of 31 MCRC Patients

Site of primary CRC*	
Right colon	8/36 (23%)
Left colon	21/36 (58%)
Rectum	7/36 (19%)
Synchronous metastatic disease	19/31 (61%)
WHO performance status	
0	12/31 (39%)
1	9 (29%)
>1	10 (32%)
Median number of metastasis	2 (range: 1–4)
Metastatic sites	
Liver	28/31 (90%)
Lung	8/31 (26%)
Peritoneum	9/31 (29%)
Previously treated patients for MCRC	17/31 (55%)

\*One patient, previously described,<sup>10</sup> presented 2 primary colorectal tumors.

significant difference, in term of age, gender, tumor stage, localization of the primary tumor or survival, between patients with and without *KRAS* mutation or *RASSF2A* promoter methylation in the primary tumor.

### Detection of Tumor Marker in Serum

Among the 23 patients with a detectable tumor marker in their primary tumor, 12 presented the same alteration in their serum (52%). Among the 12 patients harboring a *KRAS* mutation in their primary tumor, 7 (58%) presented the same mutation in their serum, and among the 23 patients with *RASSF2A* methylation in the primary tumor, 11 (48%), presented this tumor marker in their serum (Table 2). For the 2 patients whose primary tumor DNA was not available, *KRAS* and *RASSF2A* analyses performed in serum revealed the presence of *RASSF2A* methylation, suggesting that this alteration was present in their primary tumor. These 14 patients with a detectable tumor marker in their serum were designed “sDNA+ patients.” For the 6 patients without detectable marker in the primary tumor, molecular analyses confirmed the absence of *KRAS* mutation and *RASSF2A* methylation in their serum. The 11 patients with a detectable marker in their primary tumor but without marker in the serum were designed “sDNA- patients.” As shown in Table 3, no difference was observed between the sDNA+ and sDNA- groups, for age, gender, metastatic site, number of organ involved, World Health Organization performance status, Köhne predictive score,

**TABLE 2.** Molecular Analysis and Treatment Evaluation of 25 MCRC Patients Presenting in Their Primary Tumor and/or Serum a *KRAS* Point Mutation and/or a *RASSF2A* Promoter Methylation

Patient N°	<i>KRAS</i> Status		<i>RASSF2A</i> Status		Presence/Absence of DNA in Serum	Chemotherapy Regimen Evaluated	Response Evaluation	
	Primary Tumor	Serum	Primary Tumor	Serum			3 mo	6 mo
1	c.38G > A	c.38G > A	M*	M	+	Folfiri	S <sup>†</sup>	PD <sup>‡</sup>
2	c.35G > C	c.35G > C	M	M	+	Irinotecan-cetuximab	PD	PD
3	Wt <sup>§</sup>	Wt	M	M	+	Folfiri	PR	PD
4	Wt	Wt	M	M	+	Folfiri	PD	D <sup>¶</sup>
5	c.35G > T	c.35G > T	U <sup>  </sup>	U	+	Folfiri	PR**	PR
6	c.34G > T	c.34G > T	M	M	+	Folfiri bevacizumab	S	PD
7	ND <sup>††</sup>	Wt	ND	M	+	Folfiri	S	PD
8	c.35G > C	c.35G > C	M	M	+	Folfiri bevacizumab	PR	S
9	Wt	Wt	M	M	+	Folfiri	PD	D
10 <sup>**</sup>	c.38G > A Wt	Wt	M M	M	+	Irinotecan-cetuximab	PR	PD
11	c.35G > T	c.35G > T	M	M	+	Folfiri	PD	D
12	ND	Wt	ND	M	+	Folfiri-	PR	PR
13	c.34G > T	c.34G > T	M	M	+	Folfox	D	D
14	Wt	Wt	M	M	+	Folfiri bevacizumab	PD	D
15	c.38G > A	Wt	M	U	–	Xelox	S	S
16	c.35G > T	Wt	M	U	–	Folfox	S	S
17	Wt	Wt	M	U	–	Folfiri	S	PR
18	Wt	Wt	M	U	–	Folfiri bevacizumab	S	S
19	c.34G > T	Wt	M	U	–	Xelox	PD	PD
20	c.34G > T	Wt	M	U	–	Folfiri	PR	S
21	Wt	Wt	M	U	–	Folfiri bevacizumab	PR	S
22	c.35G > T	Wt	M	U	–	Folfiri	S	S
23	Wt	Wt	M	U	–	Folfiri	S	S
24	c.35G > T	Wt	M	U	–	Folfiri	PR	S
25	Wt	Wt	M	U	–	Folfiri	S	S

\*M indicates methylated; <sup>†</sup>S, stable disease; <sup>‡</sup>PD, progressive disease; <sup>§</sup>Wt, wild-type; <sup>¶</sup>D, deceased; <sup>||</sup>U, unmethylated; \*\*PR, partial response; <sup>††</sup>ND, not done; <sup>\*\*</sup>patient 10, previously described,<sup>10</sup> had developed 2 primary CRC and the analysis of both primary tumors is presented. For this patient, the results of serum analyses suggested that metastases derived from the CRC without *KRAS* mutation.

**TABLE 3.** Comparison of MCRC Patients With (sDNA+) or Without (sDNA-) Tumor Marker in Serum

Characteristics	sDNA + Patients (n = 14)	sDNA- Patients (n = 11)	P
Age at diagnostic*	68.43 ± 9.8	67.5 ± 10	NS†
Synchronous metastases	9/14 (64%)	7/11 (64%)	NS
Metachronous metastases‡	16.12 ± 8.09	12.75 ± 7.4	NS
Patients with more than one metastatic site	8/14 (54%)	6/11 (54%)	NS
Liver metastases	14/14	9/11 (82%)	NS
WHO performance status			
0	7/14 (50%)	3/11 (27%)	NS
1 and >1	7/14 (50%)	8/11 (73%)	
Kohne score			
Low	4/14 (29%)	3/11 (27%)	NS
Intermediate	4/14 (29%)	1/11 (9%)	NS
High	6/14 (43%)	7/11 (64%)	NS
Time between diagnosis§ of the metastatic disease and blood sampling	12.04 (2–35)	12.46 (1–40)	NS
Untreated patients	8/14 (57%)	7/11 (64%)	NS
Previously treated patients with chemotherapy <90 d	2/14 (14%)	3/11 (27%)	NS
Chemotherapy regimen evaluated after blood sampling			
Folfiri	8/14 (57%)	6/11 (54%)	NS
Folfox	1/14 (7%)	1/11 (9%)	
Folfiri-bevacizumab	3/14 (21%)	2/11 (18%)	
Xelox	0	2/11 (18%)	
Irinotecan-cetuximab	2/14 (15%)	0	

\*Expressed in years as mean ± SD; †NS, non significant; ‡expressed in months as mean ± SD; §expressed in days (mean, range).

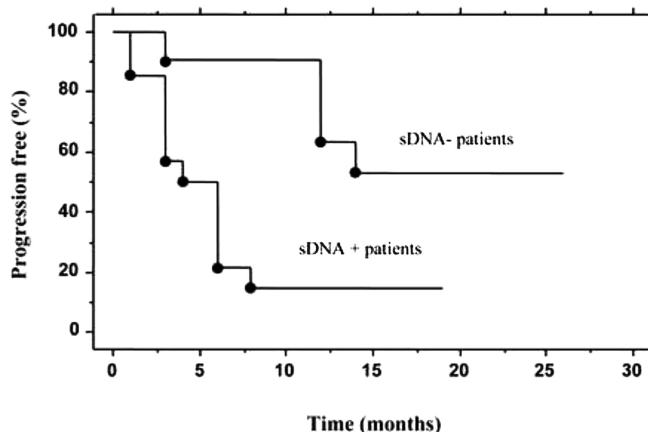
**TABLE 4.** Response Status to Chemotherapy at 3 and 6 mo According to Presence (sDNA+) or Absence (sDNA-) of Tumor Marker in Serum

	3 Months		P	6 Months		P
	Controlled Disease	Progressive Disease		Controlled Disease	Progressive Disease	
sDNA + (n = 14)	8 (57%)	6 (43%)	NS	3 (21%)	11 (79%)	0.001
sDNA- (n = 11)	10 (91%)	1 (9%)		10 (91%)	1 (9%)	

prior chemotherapy regimens, median time since last chemotherapy and chemotherapy regimen.

**Correlation With Clinical Outcome**

Among the 25 patients with a marker in their primary tumor (n = 23) or for whom primary tumor DNA was not available (n = 2), after 3 months of chemotherapy, a PR was observed in 8 patients, a SD in 10 patients, and a PD in 7 patients. No significant correlation was observed between the presence of tumor DNA in serum and the 3 months chemotherapy response (Table 4). After 6 months of



**FIGURE 1.** PFS curves according to presence (sDNA+) or absence (sDNA-) of tumor marker in the serum.

chemotherapy, a PR was observed in 3 patients, SD in 10 patients, and PD in 12 patients. As shown in Table 4, among the 14 sDNA+ patients, 11 presented a PD (79%), whereas among the 11 sDNA- patients, 1 showed a PD (9%, P = 0.001). As shown in Figure 1, the median PFS was 5 months in sDNA+ patients (range: 1–19), versus 14 months in sDNA- patients (range: 3–26; P = 0.004). After 1 year of follow-up, 2 of 14 (14%) sDNA+ and 8 of 11 (73%) sDNA- patients presented no signs of disease progression (P = 0.005). At 1 year after detection in the blood of the tumor markers, 13 of 25 (52%) MCRC patients were still alive. After a median follow-up of 11.5 months, 11 of 14 sDNA+ (79%) and 1 of 11 (9%) sDNA- patients were deceased (P = 0.001).

**DISCUSSION**

This study indicates that the presence of tumor mutant DNA in the serum of unresectable MCRC has a prognostic value, in term of PFS, disease progression, and mortality. If the prognostic value of circulating DNA had already been evaluated in localized CRC<sup>6,7,21</sup> or in MCRC patients who underwent surgery,<sup>11,22</sup> to our knowledge, it has never been investigated specifically in unresectable MCRC. In the study published by Lecomte et al,<sup>6</sup> the 2-year overall survival rate in nonmetastatic CRC was 48% in the group of patients with circulating tumor DNA in serum and 100% in the group of patients without detectable circulating tumor DNA. Preoperative detection of mutant KRAS and TP53 in the serum of CRC patients undergoing resective surgery has also been shown to be predictive of disease recurrence.<sup>21</sup> In MCRC patients, the probability to have a curative surgical resection has been shown to be lower in patients with detectable KRAS mutation in their serum.<sup>22</sup> Recently, Diehl et al<sup>11</sup> used, in 18 MCRC patients who underwent surgical resection of their metastases, a highly sensitive quantitative assay for circulating mutant DNA based on the combination of real-time PCR to quantify the number of circulating DNA fragments and on the BEAming method to determine the fraction of mutant DNA. They observed that detection of circulating mutant DNA after surgery was highly predictive of disease recurrence. In our study performed on 31 unresectable MCRC patients, analysis of only 2 easily detectable alterations associated with cancer, ie, KRAS mutation and RASSF2A methylation, allowed us to determine a tumor marker for 80% of the CRCs. The respective frequencies of these alterations, 47% and 77%, are in agreement with previous studies indicating that our series, although of limited size, is representative of CRC. Indeed KRAS mutations and RASSF2A methylation had previously been reported to be present, respectively, in 20% to 50%,<sup>6,7,12–15</sup> and

73%<sup>16,17</sup> of CRC. *KRAS* mutation and *RASSF2A* methylation are probably relevant tumor markers since the presence of these alterations is maintained during cancer development. Indeed, the key role of activating *KRAS* mutations in colorectal carcinogenesis has been clearly established,<sup>23</sup> and several studies have shown that in MCRC all patients with a *KRAS* mutation in their primary tumor have the same mutation in their metastases.<sup>24,25</sup> In cellular models, expression of the *RASSF2A* protein has been shown to induce apoptosis and prevent malignant transformation and inactivation of *RASSF2A* induces oncogenic transformation mediated by the *KRAS* oncogene, suggesting that *RASSF2A* can be considered as a tumor suppressor gene.<sup>26</sup> Furthermore, the high frequency of this alteration not only in colorectal adenocarcinomas but also in adenomas strongly suggest that *RASSF2A* gene promoter methylation might play an important role in the early development of colorectal carcinogenesis.<sup>16</sup> In 12 of the 23 MCRC patients with *KRAS* and/or *RASSF2* methylation within their primary tumor, we could detect the presence of circulating mutant DNA in the serum, and direct analysis of the serum of 2 patients in whom analysis of the primary tumor was not available revealed the presence of the marker. We used in this study the highly specific MSP method which has already been used to detect mutant DNA in the blood of cancer patients.<sup>27</sup> To detect *KRAS* mutations in serum, we used real-time PCR performed in the presence of PNA, which is able to prevent PCR amplification of wild-type templates and therefore to unmask mutant templates present, even in very small amounts. We could estimate the sensitivity of the assay to 50 ng/mL corresponding to less than 10 copies of mutant genomic DNA per  $\mu$ L. Interestingly, in the 12 patients presenting in their primary tumor both a *KRAS* mutation and *RASSF2A* methylation, the results of both analyses in the serum were concordant indicating that the sensitivity of both methods are within the similar range. Since the presence of mutant DNA in the blood of cancer patients is probably explained not only by the lysis of circulating cancer cells, but mostly by DNA leakage resulting from tumor necrosis or apoptosis,<sup>4,8,11</sup> it is tempting to speculate that in a patient with metastatic cancer the amount of circulating tumor DNA depends on the total number of malignant cells present in the patient and reflects, as already suggested, the aggressiveness of the disease and the systemic tumor burden.<sup>11</sup> This would explain why the presence of circulating mutant DNA is associated with a worse prognostic, and we think that this parameter is probably a more relevant prognostic parameter than the number of circulating cancerous cells. The prognostic value of circulating DNA in term of disease progression clinical outcome that we report here in MCRC has already been reported in metastatic melanoma. Indeed, in 50 stage IV melanoma patients, Mori et al reported that detection of *RASSF1A* in serum was the only factor that significantly correlated with overall survival.<sup>28</sup>

In conclusion, this study suggests that the presence of circulating mutant DNA in MCRC may be a relevant biologic parameter evaluating the severity of the metastatic disease, and therefore may be of clinical interest to determine the most appropriate therapeutic protocol in MCRC patients. As shown by this study, sensitive detection of *KRAS* mutation in serum can be achieved using real-time PCR performed in the presence of PNA, and detection of methylated promoters can easily be achieved using MSP, a simple method widely used in molecular diagnostic laboratories. In MCRC patients, detection in the blood of *KRAS* mutations could have therefore 2 clinical interests, first, as we previously showed, identification of patients who are resistant to anti-epidermal growth factor receptor monoclonal antibodies<sup>10</sup> and, second, prognostic evaluation. While *RASSF2A* methylation could be detected in approximately half of the CRC analyzed in this series, analysis of additional promoters, commonly methylated in CRC, should cover all the cases. Therefore, we think that our results should lead to prospective

studies on larger MCRC patient series to confirm the clinical relevance of the detection of circulating tumor DNA in MCRC.

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