A Study of Hypermethylated Circulating Tumor DNA as a Universal Colorectal Cancer Biomarker

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BACKGROUND: Circulating tumor DNA (ctDNA) has emerged as a good candidate for tracking tumor dynamics in different cancer types, potentially avoiding repeated tumor biopsies. Many different genes can be mutated within a tumor, complicating procedures for tumor monitoring, even with highly sensitive next-generation sequencing (NGS) strategies. Droplet-based digital PCR (dPCR) is a highly sensitive and quantitative procedure, allowing detection of very low amounts of circulating tumor genetic material, but can be limited in the total number of target loci monitored.

METHODS: We analyzed hypermethylation of 3 genes, by use of droplet-based dPCR in different stages of colorectal cancer (CRC), to identify universal markers for tumor follow-up.

RESULTS: Hypermethylation of WIF1 (WNT inhibitory factor 1) and NPY (neuropeptide Y) genes was significantly higher in tumor tissue compared to normal tissue, independently of tumor stage. All tumor tissues appeared positive for one of the 2 markers. Methylated ctDNA (MetctDNA) was detected in 80% of metastatic CRC and 43% of localized CRC. For samples with detectable markers in ctDNA, MetctDNA and mutant ctDNA (MutctDNA) fractions were correlated. During follow-up of different stage CRC patients, MetctDNA changes allowed monitoring of tumor evolution.

CONCLUSIONS: These results indicate that MetctDNA could be used as a universal surrogate marker for tumor follow-up in CRC patients, and monitoring MetctDNA by droplet-based dPCR could avoid the need for monitoring mutations.

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The ability to perform noninvasive dynamic monitoring of tumors has long been a challenge for clinicians. A rapidly growing body of work has raised the prospect of performing serial analyses of circulating tumor DNA (ctDNA) in body fluids, and this approach is now being explored as a potential marker of cancer evolution. Technologic advances have facilitated the development of methods for highly sensitive and quantitative detection of ctDNA, including microfluidic droplet-based digital PCR (dPCR) and optimized next-generation sequencing (NGS) strategies. Building on previous bulk dPCR strategies, droplet-based dPCR uses millions of water-in-oil droplets for parallel amplification of millions of individual DNA fragments, leading to highly sensitive mutation detection. This method is truly quantitative and highly sensitive.
precise, allowing finely tuned monitoring of ctDNA levels (3, 15), paving the way for early cancer recurrence detection, identification of resistant subclones as well as early and cost-effective evaluation of treatment efficacy (16–20).

Two strategies generally have been followed for ctDNA analysis by dPCR. The first strategy has relied on the detection of well-known mutations associated with sensitivity or resistance to treatment (and generally part of companion diagnostics tests), whereas, the second strategy has been based on the identification of one specific mutation identified from the patient’s tumor DNA to monitor the presence of ctDNA (17). The latter strategy has several drawbacks: first, when using targeted sequencing, all tumors do not present well-represented mutant allele(s) that can be easily tracked to follow disease evolution and, second, even when a mutated allele is detected, there may not be readily available assays for its detection. Moreover, although tumor-specific mutations represent highly specific cancer markers, the sheer number of potentially mutated genes as well as the many different base positions that can be mutated for the same gene have compromised the utility (21) and the cost-effectiveness of this approach. Highly sensitive NGS strategies that address some of these challenges have been described, but still require cumbersome and hands-on procedures to be successfully implemented in the laboratory. Consequently, new biomarkers are being investigated for screening a large fraction, if not all, cancer patients. Among these markers, a large focus has been placed on the analysis of circulating DNA integrity (22–24), dysregulation of microRNA expression (25), and DNA hypermethylation (26).

DNA hypermethylation in the regulatory regions of specific genes has been described as a potential early cancer marker with strategies for diagnostic or cancer screening procedures being developed (27). Most methods developed for DNA methylation analysis have used conventional quantitative PCR (qPCR) strategies with a few reports describing the use of droplet-based digital approaches for this purpose (21, 28, 29).

Here, we have explored whether universal markers for colon cancer follow-up could be developed by combining the simplicity of picoliter droplet-based dPCR and pertinent hypermethylated sequence monitoring. In addition, we considered whether monitoring ctDNA evolution through the analysis of such markers could provide a surrogate for the follow-up of tumor-specific mutations.

Materials and Methods

STUDY DESIGN

The main goal of this study was to explore the presence of methylated DNA in colorectal cancer (CRC) patients’ tumors and plasma, with 3 markers: WIFI (WNT inhibitory factor 1)15, NPY (neuropeptide Y), and PENK (proenkephalin), using droplet-based dPCR technology. The workflow of the study is described in Fig. 1. Four prospective cohorts of patients presenting a CRC were included, with a total of 70 metastatic CRC patients and 78 localized CRC patients, with 240 samples. First, methylation was evaluated in tumor samples of 56 patients, for either comparison with healthy tissue, or for correlation with tumor stage. Secondly, plasma samples from 127 patients were analyzed for the presence of methylated ctDNA (MetctDNA). Comparison was done between the detection of methylation in tumor tissue and plasma for 41 patients. Data were available for 53 patients for analysis and comparison of fractions of mutant ctDNA (MutctDNA) and MetctDNA. Nine patients were selected for MutctDNA and MetctDNA comparison for the follow-up. The samples presenting a low fraction of targeted allele(s) or ambiguous results [i.e., presenting droplet cluster with very low amounts of droplets close to the limit of blank (LOB) or nonclustered population of droplets] were submitted to the appropriate duplex assay(s) to confirm the presence of the targeted allele(s).

SAMPLE COLLECTION AND DNA PREPARATION

Patients and healthy study participants. Two hundred and forty CRC samples were selected from 4 cohorts of patients (n = 148 patients) and pooled for this study. Among these 148 patients, 63 had a metastatic CRC among which 20 of them proceed from the CETRAS study and 43 from the PLACOL study both approved by the Ile-de-France ethics committee number 2 (CPP Ile-de-France 2. 2007–03–01–RCB 2007-A00124–49, AFSSAPS A70310–31 for the CETRAS study and ID CRB: 2013-A00680–45 for the PLACOL study). The CETRAS study included mCRC patients treated with anti-EGFR (epidermal growth factor receptor) therapy. For the 20 CETRAS patients, primitive tumor DNA was analyzed as well as plasma DNA obtained at progression of metastatic disease and before the start of the anti-EGFR therapy. The PLACOL study included mCRC patients treated with chemotherapy. For the 43 PLACOL, primitive tumor samples were available as well as plasma samples drawn at inclusion and before each chemotherapy treatment (3 patients, 22 samples excluding the first sample at inclusion). For these patients, 33 were selected at the start of their first line of chemother-

15 Human genes: WIFI, WNT inhibitory factor 1; NPY, neuropeptide Y; PENK, proenkephalin; ALB, albumin; KRAS, Kirsten rat sarcoma viral oncogene homolog; BRAF, B-Raf proto-oncogene, serine/threonine kinase; TP53, tumor protein p53; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, NRAS, neuroblastoma RAS viral (v-ras) oncogene homolog, APC, adenomatous polyposis coli.
apy, 6 at the start of their second line of chemotherapy, 2 at the start of their third line of chemotherapy, 1 at the start of their fourth line of chemotherapy and 1 at the start of their fifth line of chemotherapy. Seventy-four patients were selected from the ALGECOLS protocol also approved by the Ile-de-France ethics committee number 2 (NCT01198743), and had a localized CRC. The ALGECOLS study included patients with localized CRC patients. Plasma samples were drawn at the time of surgery and/or 5 days after surgery, then every 3–5 months during follow-up, for up to 3 years. For these patients, primitive tumor DNA was analyzed for 25 patients as well as corresponding healthy tissue DNA for 11 of these patients and plasma DNA was analyzed before surgery (n = 49), 5–21 days after surgery (n = 22 from which 18 were drawn at day 5, 1 at day 6, 1 at day 7, 1 at day 8 and 1 at day 21) and during follow-up (6 patients, 28 samples excluding samples before and after surgery). Finally 11 patients with stage II to IV cancer were included (INSERM, CPP-IDF IX-11–019) (see also (30)). Primitive tumor DNA and corresponding normal tumor DNA were analyzed for these 11 patients. All patients signed an informed consent form. The mean age at inclusion was 65, and the male/female ratio was 1:7.

Fifty-two patients with either localized or metastatic cancer presented a mutation within their tumor tissue for which we had developed a picoliter-droplet dPCR assay. These mutations include the 7 most frequent mutations

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**Fig. 1. Workflow of the study.**

A total of 276 samples from 142 CRC patients were analyzed for this study. Among these patients, 78 frozen tumor samples (56 patients) were analyzed for the presence and fraction of the target methylated sequences (A). First, 44 matched tumor and normal adjacent tissues (22 patients) were analyzed to understand the specificity of the markers for tumor tissues. Thirty-four additional frozen tumor tissues were analyzed and results of these 56 cancer tissues compiled to analyze the prevalence of methylated DNA in tumor tissues in function of tumor stage. For 41 of these patients*, plasma samples were analyzed for the presence of MetctDNA (B). Plasma samples of additional 85 patients were analyzed for the presence of MetctDNA (B). For 53 of these patients, we compared the detected fraction of MetctDNA and MutctDNA. For 22 patients, we both analyzed plasma samples collected before and after surgery (5–21 days after surgery). Finally, MetctDNA and MutctDNA were compared for the follow-up of 9 patients (**68 samples for MutctDNA and 68 matched samples for MetctDNA). In addition, 46 plasma samples from noncancer patients were also tested (described in online Supplemental Table 4).
of the KRAS (Kirsten rat sarcoma viral oncogene homolog) oncogene, KRAS Q61H, KRAS A146T, BRAF V600E, TP53 R175H, TP53 R248Q, TP53 R273C, TP53 R273H, PIK3CA E545K, or NRAS Q61L. Circulating plasma DNA of these patients was tested for the presence of the mutations.

In addition to these patients’ samples, plasma samples from 30 healthy individuals were ordered from Biological Specialty Corporation. The mean age was 38 (13) years. The male/female ratio was 2:3. Sixteen controls from the case controls of the Cecile study (noncancer patients, female) were also included (31).

**Tumor sample preparation, storage, DNA extraction, and quantification.** Tumors were flash frozen in liquid nitrogen immediately after resection until further analysis. Each tumor was reviewed by a pathologist (J.-F. Emile) and the tumor cell content was assessed by hematoxylin-eosin-safran staining. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. DNA concentration was measured with a Qubit 2.0 fluorometer (Thermo Fisher) by use of the dsDNA BR Assay Kit (Invitrogen). Extracted DNA samples were stored frozen at −20 °C before testing.

**Plasma sample preparation, storage, DNA extraction, and quantification.** Blood samples were collected in EDTA tubes. Plasma samples were immediately separated from the cellular fraction by centrifugation at 3000g (4 °C) and stored frozen at −80 °C before DNA extraction. Plasma samples of healthy volunteers (see above) were received in dry ice, aliquoted and immediately frozen at −80 °C before DNA extraction. Before extraction, plasma samples were centrifuged for 10 min at 3000g. Plasma DNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) from 2 mL of sample according to the manufacturer’s instructions. The quantity of DNA was measured by Qubit 2.0 fluorometer (Invitrogen, Life Technologies) using the dsDNA HS Assay Kit (Invitrogen). Extracted DNA samples were stored frozen at −20 °C before testing.

**Genomic DNA preparation.** Universal hypermethylated DNA was purchased from Zymo Research and normal genomic DNA from Promega. These DNAs were used as controls and both fragmented to an average size of 600–800 pb using the S2 Focused-Ultrasonicator (Covaris), according to manufacturer’s recommended protocol. DNA concentration was determined using the Qubit 2.0 fluorometer using the dsDNA BR Assay Kit.

Hypermethylated universal control DNA was serially diluted and mixed with human normal genomic DNA to test the lower limit of hypermethylated DNA detection with the developed picoliter droplet-based assays. Human genomic DNA was also used as negative control to calculate the LOB and limit of detection (LOD) of the assay.

**Bisulfite conversion.** DNA extracted was modified by bisulfite, using the EZ DNA Methylation-Gold Kit (Zymo Research). In brief, bisulfite reaction was carried out in a thermocycler at 98 °C for 10 min and 53 °C for 4 h. The cleanup of bisulfite-converted DNA followed the recommendations of the manufacturer, and converted DNA was eluted in M-Elution Buffer and stored at −20 °C.

**NGS, PICOLITER DROPLET-dPCR, DATA ANALYSIS, AND STATISTICAL ANALYSIS**

For a description see the Supplemental materials provided in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue8.

**Results**

**MULTIPLEX dPCR TO DETECT SPECIFIC DNA HYPERMETHYLATED SEQUENCES**

Aberrant methylation of NPY, PENK, and WIF1 promoter regions has been described in CRC tissues in comparison with healthy tissues and proposed as a promising marker for blood- or stool-based diagnosis (30). We developed a 2-panel assay targeting these markers with albumin sequence (ALB) as a reference (Fig. 2 and online Supplemental Table 1). The LOB and LOD determination are described in the Materials and Methods sections. The dynamic range and sensitivity of the assays were determined as described previously (32–34). The measured concentration of hypermethylated DNA matched the anticipated concentration from 10% to 0.1% (see online Supplemental Fig. 1). A higher false positive rate was observed for PENK marker (see online Supplemental Fig. 2). In the following text, only WIF1 and NPY markers’ results will be presented.

**PICOLITER DROPLET-BASED PCR ANALYSIS OF TUMOR TISSUE SAMPLES WITH DIFFERENT STAGE DISEASES**

Hypermethylation of the targeted sequences was assessed in a set of 22 paired tumor and adjacent normal frozen tissue samples (Fig. 2, B and C). When analyzing the frequency of hypermethylated alleles (Fig. 3, A and B, and online Supplemental Table 2), tumor tissues appeared significantly different from normal tissues (P values <0.0001, paired nonparametric Wilcoxon test). To confirm the prevalence of these markers in CRC, additional tumors were analyzed. In total, 56 stage I to IV CRC samples were analyzed (see online Supplemental Tables 2 and 3). All samples appeared positive for WIF1 and/or NPY markers (94.3% were positive for both markers) with a good correlation between the observed
Fig. 2. Multiplex analysis of methylated tumor DNA (A).
Schematic representation of assay workflow. An aqueous phase containing TaqMan® assay reagents and genomic DNA is emulsified. Probes specific for the different sequences are present. A 2-panel assay has been developed. The first panel targets methylated WIF1 and unmethylated ALB sequences (3a) when the second panel targets methylated PENK and NPY sequences (3b). After thermal cycling, the endpoint fluorescence of each target containing droplet is measured, allowing the identification of the target sequences. (B and C), 2-dimensional histogram of the 2-panel assay for the analysis of DNA extracted from tumor tissue (B) or an unmethylated healthy tissue (C). DNA extracted from tumor tissue or adjacent healthy tissue was encapsulated in droplets and submitted to the procedure described in Panel A. 6-FAM, 6-carboxyfluorescein; AU, arbitrary units; VIC, a proprietary dye of Applied Biosystems/Life Technologies.
methylated marker fractions in the tested samples (see online Supplemental Fig. 3, D–F). It is noteworthy that 2 samples presented a percentage of \textit{WIF1} methylated DNA higher than 100% meaning that a higher number of \textit{WIF1} sequences were detected as compared to \textit{ALB} sequences. Such results could be due to tumor genetic rearrangement leading to amplification of the targeted sequence. No significant differences were observed either between the tumor stage and the presence or absence of hypermethylated sequences, or between the stage and the fraction of the 3 markers (Kruskal–Wallis nonparametric test) (see online Supplemental Fig. 3, A–C).

PICOLITER DROPLET-BASED PCR ANALYSIS OF MATCHED TUMOR/PLASMA SAMPLES OF PATIENTS WITH DIFFERENT STAGE DISEASES

Among these patients, we analyzed 41 plasma samples from 20 patients with metastatic CRC (mCRC, plasma obtained at treatment progression and before the start of anti-EGFR therapy) and 21 patients with localized CRC (plasma obtained at diagnosis and before surgery) (see online Supplemental Table 3). The ratio of the targeted markers in tumor tissue and plasma was correlated (\textit{NPY}/\textit{WIF}; \( R^2 = 0.69, P = 0.001 \)). Within the samples described above, 23 samples were selected such as the patient’s tumor was bearing a mutation for which a dPCR assay had been developed and validated (see Materials and Methods) and, of these, 18 presented MutctDNA when analyzed by picoliter droplet-based dPCR (34) (average of 12.7% or 1.48% for mCRC or localized cancers patients respectively) (see online Supplemental Table 3). The fractions of MutctDNA and MetctDNA were correlated (\( R^2 = 0.87 \) and 0.94 for \textit{WIF1} and \textit{NPY} markers, respectively, \( P \text{ values} <0.0001 \), Spearman nonparametric test). To ensure specificity of the assay, 46 control plasma samples from noncancer patients were analyzed (see Materials and Methods). Three samples presented a low number of \textit{NPY} droplets (higher than LOB but lower than LOD; see online Supplemental Table 4 and Supplemental Fig. 4).

To further confirm these results, 85 additional plasma samples were analyzed (43 collected at diagnosis of localized CRC and 42 collected from mCRC patients under follow-up) (see online Supplemental Table 5). The samples were chosen such that 28 presented a mutation within their tumor for which a dPCR assay had been developed and validated (see Materials and Methods section and online Supplemental Table 5). Among these patients, 23 presented detectable MutctDNA (0.02%–5.8% for localized cancer patients and 0.77%–51.9% for mCRC patients). Nineteen patients with detectable MutctDNA were positive for \textit{WIF1} or \textit{NPY} markers with the fraction of MetctDNA ranging from 0.01% to approximately 100% (4 patients had insufficient amplifiable genomes to be detected). The analysis of a higher quantity of ctDNA probably would have allowed detection in these patients.

A high correlation between MutctDNA and MetctDNA was observed (Fig. 4, A and B, \( R^2 = 0.89 \) and 0.85 for \textit{WIF1} and \textit{NPY} markers respectively, \( P \text{ values} <0.0001 \), nonparametric Spearman test). Samples without detectable MutctDNA (\( n = 11 \)) were also negative for the presence of MetctDNA. As expected from the correlation with MutctDNA, the fraction of MetctDNA was significantly higher in mCRC compared to localized CRC (Fig. 3C, Mann–Whitney test, \( P <
In addition, we observed clearance or decrease of MetctDNA after tumor resection in 73% of the cases (plasma DNA of 22 patients analyzed, see online Supplemental Table 6). These results were confirmed by MutctDNA analysis confirming specificity of the developed assay.

Overall, among the samples that were not tested for MutctDNA by droplet-based dPCR, [i.e., patient samples that had no mutations for which a dPCR assay had been developed and validated (see Materials and Methods section)] within the tumor or not analyzed (samples from 38 localized CRC and 35 mCRC patients), MetctDNA was detected in 80% of mCRC patients (68.6% of the samples with number of observed droplets higher than LOD) and 44.7% of localized CRC patients (28.9% of the samples with number of observed droplets higher than LOD).

Online Supplemental Table 7 summarizes the results described here for the MetctDNA analysis.

**Fig. 4.** Correlation between MutctDNA and MetctDNA. (A and B), correlation between the fraction of MutctDNA (%) and WIF1 MetctDNA (%) (A) or NPY MetctDNA (%) (B) for patients with CRC. (C), Fraction of WIF1 MetctDNA (%), NPY MetctDNA (%), and MutctDNA (%) for stage II to IV CRC patients.
plasma DNA samples of 9 patients with KRAS, BRAF (B-Raf proto-oncogene, serine/threonine kinase) or TP53 (tumor protein p53) mutated tumors and for whom the fraction of MutctDNA was measured during treatment follow-up. Fig. 5 and Fig. 6 show that the variations the MetctDNA and MutctDNA fractions were correlated, further validating the chosen approach. The 6 first patients (Fig. 5, A–F) presented a localized CRC and were evaluated for tumor recurrence. For these patients, tumor tissue was available at diagnosis, plasma samples were available at diagnosis after surgery, and then every 3–5 months for up to 3 years follow-up. Within these patients, patient 1A (Fig. 5A) presented MetctDNA (and MutctDNA) at diagnosis but no ctDNA was detected after surgery and during the 3-year follow-up and this patient did not present a recurrence of

Fig. 5. Monitoring of MutctDNA (%), purple) and WIF1 MetctDNA (%), light blue) for 6 patients (A–F) with localized CRC.

Fig. 6. Monitoring of MutctDNA (%), purple) and WIF1 MetctDNA (%), light blue) for 3 patients with advanced (A–C) CRC.
SD, stable disease; PD, progressive disease.
his cancer during this period. Interestingly, even 7 years after the end of the follow-up this patient did not present a recurrence of the disease. For the 5 other patients (Fig. 5, B–F), an increase of ctDNA was observed during follow-up before the clinical detection of the cancer recurrence. Four of them (Fig. 5, B, C, E and F) had detectable of both MetctDNA and MutctDNA at diagnosis, followed by a decrease of ctDNA and then an increase before recurrence diagnosis. One of them (Fig. 5D) had no detectable MetctDNA (and MutctDNA) at diagnosis, but ctDNA was detectable before recurrence assessment. The 3 patients described in Fig. 6, A–C presented a metastatic disease and were followed for tumor progression under chemotherapy treatment. For these 3 patients, an increase of both MetctDNA and MutctDNA was observed before detection of disease progression (RECIST).

Discussion

Detection of ctDNA is one of the most promising approaches for improving tumor monitoring in different types of cancers. MutctDNA, so far, has been the most studied and these studies have highlighted the great potential of this marker (35). However, there are clear limitations to approaches based on the monitoring of MutctDNA, including the necessity to previously identify specific mutation in the archived tumor tissue. In addition, even when a tumor-specific mutation is identified, it may not be usable for routine monitoring (no available or set-up assays for example). Universal biomarkers are thus needed for ctDNA detection and monitoring.

Based on the work presented here, MetctDNA determination using WIF and NPY markers appears to be a promising surrogate biomarker of tumor burden in CRC patients. First of all, we showed that methylated DNA in cancer tissue was detectable throughout the development of the disease, even in early stage tumors, suggesting that these hypermethylated loci appear as an early and persistent event in tumorigenesis. These results are appealing, since, in contrast, analysis of primitive tumors of CRC patients as described in cBioPortal, for 12 most frequently reported mutations [i.e., KRAS p.G12D, p.G12V, p.G13D, p.A146T, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA) p.E545K, BRAF p.V600E, TP53 p.R175H, p.R248W, p.R213*, neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) p.G61K, and adenomatous polyposis coli (APC) p.R1450*] revealed that only 46.2% of the patients were positive for one of these mutations in tumor tissue (36, 37).

Secondly, we found that MetctDNA could be detected in 80% of metastatic CRC patients and 44.7% of localized CRC patients (stage II and III). Moreover, MetctDNA and MutctDNA fractions appeared strongly correlated. To our knowledge, there is no biological relation between MetctDNA and the presence of any tested mutation; therefore, the observed correlation suggests that the detection of MetctDNA is owing to the presence of ctDNA. Our newly developed test brings the information regarding the presence of circulating DNA independently of the a priori knowledge the tumor mutation and can be used for the quantification of ctDNA during patient follow-up. This test is not directed at the detection of druggable mutations. These fractions of positive patients are comparable to results obtained when screening MutctDNA for tumor-specific mutations (when such a mutation was detected and an assay developed) with the advantage of detecting a significantly higher fraction of patients with just 2 assays. Indeed, we previously described that, MutctDNA (34) can be detected in 84% of mCRC patients with KRAS or BRAF mutated tumors and more recently that, the fraction of stage II–III CRC patients with detectable MetctDNA was 21.6% (17/779) (38). As for the detection of MutctDNA, we found that the fraction of MetctDNA was correlated to tumor stage (Fig. 4C) (39).

These results are highly complementary to those in the large body of literature demonstrating the pertinence of ctDNA monitoring. As compared to conventional procedures based on MutctDNA monitoring, which require a large number of assays, our method based on MetctDNA allows the same efficiency for detecting ctDNA to be reached by screening for just 2 markers in all patients. In this work, the analysis of 105 tumors by targeted sequencing highlighted that the use of 45 different assays would have allowed 95 patients to be followed (10 tumors remaining with undetected mutations). The gain in terms of validation time and cost for the implementation of such markers in routine laboratories is important and advocates for our method. With WIFI and NPY markers being found to be hypermethylated in a large fraction of tumor tissues, independently of the stage of the cancer, our results suggest that ctDNA monitoring can be performed without analysis of the tumor DNA (which may be inaccessible or of poor quality). In addition, these results suggest that monitoring of MetctDNA was as efficient as the monitoring of MutctDNA for the detection of CRC recurrence in plasma of patients with localized disease, and for monitoring treatment efficiency for patients with advanced CRC (18, 40).

In conclusion, our data indicate that the monitoring of ctDNA could be performed using just 2 methylation markers instead of tumor-specific mutations. MetctDNA appears to be a universal approach to follow tumor burden of CRC patients, as compared with MutctDNA, which requires previous tumor mutation identification. These results demonstrate that the combination of picoliter droplet-based dPCR and pertinent methylation markers allows the development of a highly
useful tool for ctDNA monitoring of CRC patients and this method could be extended to other tumor types.

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