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Circulating Cell–Free DNA from Colorectal Cancer Patients May Reveal High KRAS or BRAF Mutation Load1,2

Florent Mouliere*,†,‡,§, Safia El Messaoudi*,†,‡,§, Celine Gongora*,†,‡,§, Anne-Sophie Guedj†, Bruno Robert*,†,‡,§, Maguy Del Rio*,†,‡,§, Franck Molina‡, Pierre-Jean Lamy#, Evelyne Lopez-Crapez#, Muriel Mathonnet**, Marc Ychou††, Denis Pezet‡‡, and Alain R. Thierry*,†,‡,§,¶

Institut de Recherche en Cancérologie de Montpellier, Montpellier, France; †Institut National de la Santé et de la Recherche Médicale, U896, Montpellier, France; ‡Université Montpellier 1, Montpellier, France; §Institut régional du Cancer Montpellier, Montpellier, France; ††Centre Régional de Lutte contre le Cancer Val d’Aurelle-Paul Lamarque, Montpellier, France; ‡‡Centre Hospitalier Universitaire Estaing, Unité d’Oncologie Digestive, Clermont Ferrand, France

Abstract

We used a novel method based on allele-specific quantitative polymerase chain reaction (Intplex) for the analysis of circulating cell–free DNA (ccfDNA) to compare total ccfDNA and KRAS- or BRAF-mutated ccfDNA concentrations in blood samples from mice xenografted with the human SW620 colorectal cancer (CRC) cell line and from patients with CRC. Intplex enables single-copy detection of variant alleles down to a sensitivity of ≥0.005 mutant to wild-type ratio. The proportion of mutant allele corresponding to the percentage of tumor-derived ccfDNA was elevated in xenografted mice with KRAS homozygous mutation and varied highly from 0.13% to 68.7% in samples from mutation-positive CRC patients (n = 38). Mutant ccfDNA alleles were quantified in the plasma of every patient at stages II/III and IV with a mean of 8.4% (median, 8.4%) and 21.8% (median, 12.4%), respectively. Twelve of 38 (31.6%) and 5 of 38 (13.2%) samples showed a mutation load higher than 25% and 50%, respectively. This suggests that an important part of ccfDNA may originate from tumor cells. In addition, we observed that tumor-derived (mutant) ccfDNA was more fragmented than ccfDNA from normal tissues. This observation suggests that the form of tumor-derived and normal ccfDNA could differ. Our approach revealed that allelic dilution is much less pronounced than previously stated, considerably facilitating the noninvasive molecular analysis of tumors.

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Address all correspondence to: Alain R. Thierry, PhD, Institut National de la Santé et de la Recherche Médicale, U896, Montpellier, F-34298, France. E-mail: alain.thierry@inserm.fr

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2This article refers to supplementary materials, which are designated by Tables W1 to W3 and Figures W1 and W2 and are available online at www.transonc.com.

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Materials and Methods

ccfDNA cell or tissue origins.

in a CRC mouse xenograft model and in 38 CRC patient plasma sam-
portion of mutant (tumor cell
of quantifying mutant ccfDNA. In this study, we determined the pro-
method that demonstrated the unprecedented sensitivity and specificity
individuals [18]. From these observations, we set up a Q-PCR
criminate plasma from CRC patients and from plasma from healthy
dependent on the target size [17]: The ccfDNA size pattern can dis-
overlap in the ccfDNA level between healthy individuals and cancer
attractive tool for detecting the presence of mutations. ccfDNA dy-
is easy to set up and relatively noninvasive, ccfDNA represents a very
enlarging the targeted therapy--resistant patient population stratum.

Significant amounts of circulating cell–free DNA (ccfDNA) are pres-
ent in the plasma of cancer patients [5,6]. As blood analysis of ccfDNA is easy to set up and relatively noninvasive, ccfDNA represents a very attractive tool for detecting the presence of mutations. ccfDNA dynamics can be easily modeled using xenografted mice [7,8]. Plasma ccfDNA in cancer patients may originate from three sources: 1) healthy normal cells, 2) tumor stromal cells, and 3) tumor cells [9]. A partial overlap in the ccfDNA level between healthy individuals and cancer patients has been observed in the literature [10,11]. Quantification of ccfDNA exclusively derived from tumor cells represents an obvious interest with regard to monitoring or following up tumor progression in the course of cancer patient management. Only a few reports have described the systematic quantification of tumor-derived ccfDNA [12–16] based on assays for quantifying ccfDNA harboring the point mutation that characterizes the tumor. Thus, mutant ccfDNA has been found previously as a tiny fraction of the total ccfDNA [13–15].

We have previously demonstrated that quantifying ccfDNA by quantitative polymerase chain reaction (Q-PCR) analysis is largely dependent on the target size [17]: The ccfDNA size pattern can discriminate plasma from CRC patients and from plasma from healthy individuals [18]. From these observations, we set up a Q-PCR–based method that demonstrated the unprecedented sensitivity and specificity of quantifying mutant ccfDNA. In this study, we determined the proportion of mutant (tumor cell–derived) alleles from ccfDNA analysis in a CRC mouse xenograft model and in 38 CRC patient plasma samples. The results are related to the as-yet-unknown contribution of the ccfDNA cell or tissue origins.

Introduction

KRAS is an essential activator within the signaling cascade induced by
the activation of the endothelial growth factor receptor (EGFR) gene. KRAS
plays a central role in tumor development by regulating the expression of
the proteins that are involved in cell proliferation and survival, metastatic
spread, and angiogenesis. KRAS point mutations lead to a constitutively
active guanosine triphosphate (GTP)-bound protein that conforms the sig-
nal to BRAF and the subsequent downstream activation of the mitogen-
activated protein kinase (MAPK) pathway. The presence of some KRAS
point mutations leads to its constitutional activation and renders the
treatment of colorectal cancer (CRC) patients with potent inhibitors of
EGFR, such as cetuximab and panitumumab, ineffective [1]. Therefore,
all patients eligible for anti-EGFR treatments must first be tested for
KRAS mutations (present in 35–45% of CRC cases) before starting such
therapies. BRAF point mutations in CRC cells (8–14% of CRC cases)
might also cause resistance to targeted therapies. BRAF and KRAS muta-
tions are considered as mutually exclusive in CRC [2]. Although the
detection of BRAF mutations is also associated with poorer prognosis
[3,4], the BRAF mutational status has not been incorporated into the
treatment guidelines in force because of conflicting results among studies.
Currently, analysis of the KRAS mutational status of a patient is carried
out from tumor sections by various methods including sequencing. It
represents one of the first molecular assessments of personalized medi-
cine in oncology. BRAF V600E mutation is examined in the case of a negative KRAS mutation status in several national guidelines, thus
enlarging the targeted therapy–resistant patient population stratum.

Significant amounts of circulating cell–free DNA (ccfDNA) are pres-
ent in the plasma of cancer patients [5,6]. As blood analysis of ccfDNA is easy to set up and relatively noninvasive, ccfDNA represents a very attractive tool for detecting the presence of mutations. ccfDNA dynamics can be easily modeled using xenografted mice [7,8]. Plasma ccfDNA in cancer patients may originate from three sources: 1) healthy normal cells, 2) tumor stromal cells, and 3) tumor cells [9]. A partial overlap in the ccfDNA level between healthy individuals and cancer patients has been observed in the literature [10,11]. Quantification of ccfDNA exclusively derived from tumor cells represents an obvious interest with regard to monitoring or following up tumor progression in the course of cancer patient management. Only a few reports have described the systematic quantification of tumor-derived ccfDNA [12–16] based on assays for quantifying ccfDNA harboring the point mutation that characterizes the tumor. Thus, mutant ccfDNA has been found previously as a tiny fraction of the total ccfDNA [13–15].

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Materials and Methods

Cell Lines

The human colorectal adenocarcinoma HCT-116, SW620, LS174T,
SW1116, and HT29, the human lung adenocarcinoma A549, and
the human pancreatic MiaPaca2 cell lines were obtained from ATCC
(Manasas, VA). They were grown in RPMI 1640 supplemented with
10% fetal calf serum and 2 mmol/l l-glutamine at 37°C in a humidified
atmosphere with 5% CO₂.

Mouse Xenograft Model

Female athymic nude mice (6–8 weeks of age, n = 8) were xenografted
with human SW620 CRC cells as previously described [18]. Three nude mice were not grafted and were used as controls. The mice were sacrificed using CO₂. Blood collection and tumor weighing were carried out at different times post graft. All experiments were performed by an accredited person (Dr B. ROBERT, No. 34-156) and they complied with the current national and institutional regulations and ethical guidelines.

Human Blood Samples

Blood samples were collected from patients (n = 38) with CRC
(metastatic or not), irrespective of their CRC stage or relapse (stage IV,
n = 33; stage III, n = 1; stage II, n = 4). CRC patients did not receive
chemotherapy or radiotherapy for at least 1 month before the blood
collection. The clinical features [Tumor Nodule Metastasis (TNM)
staging] and the treatment for each CRC patient in this study before
blood collection are summarized in Tables 1 and W1, respectively.
Positive mutation status was retrospectively or prospectively carried out
in tissue (primary tumor or metastasis) in the context of the standard
management care of CRC patients. All plasma samples included in this
study were analyzed correctly (100% success rate). Plasma analysis was
performed in a blinded manner and only once for each of the 38 patients
considered for treatment or surgery. Written, informed consent was ob-
tained from all participants before the onset of the study. The protocols
for the use of blood samples from healthy volunteers used in this study
were approved by the "Etablissement Français du Sang" Ethics Com-
mittee (EFS-PM agreement: 21/PVNT/MTP/CNR14/2010-0029).

Plasma Isolation and ccfDNA Extraction

Blood samples were collected in EDTA tubes: 2 ml of blood was col-
lected from human patients and 0.8 to 1 ml from mice. The blood was
centrifuged at 1200g at 4°C in a Heraeus Multifuge LR centrifuge for
10 minutes. The supernatants were isolated in sterile 1.5-ml Eppendorf
tubes and centrifuged at 1200g for 10 minutes. The supernatants were either immediately handled for DNA extraction
or stored at –80°C. ccfDNA was extracted from 1 ml of plasma using
the QiAmp DNA Mini Blood Kit (Qiagen, Hilden, Germany) according
to the "Blood and body fluid protocol." DNA samples were kept at
–20°C until use.

ccfDNA Quantification by Q-PCR

The methodology and the data description were carried out accord-
ing to the MIQE guidelines [19]. Q-PCR amplifications were carried
out at least in duplicate in a 25-μl reaction volume on a Chromo4
instrument using the MJ Opticon Monitor 3 software (Bio-Rad, Her-
cules, CA). Each PCR mixture was composed of 12.5 μl of PCR mix
(Bio-Rad Supermix SYBR Green), 2.5 μl of each amplification primer
(0.3 pmol/μl), 2.5 μl of PCR-analyzed water, and 5 μl of DNA extract.
Thermal cycling consisted of three repeated steps: a 3-minute hot-start
polymerase activation-denaturation step at 95°C followed by 40 re-
cycled cycles at 95°C for 10 seconds and then at 60°C for 30 seconds.
Melting curves were obtained by increasing the temperature from 55 to
90°C with a plate reading every 0.2°C.
Intplex Mutation Analysis

We established an allele-specific blocker [20] Q-PCR–based method specific for ccfDNA analysis (named Intplex) to enable the detection of point mutation and to determine mutant allele concentration. This method combined the use of 1) allele-specific Q-PCR with blocking 3’-phosphate–modified oligonucleotide [20], 2) low Tm primers with mutation in 3’, 3) an integrated primer design, 4) routine internal positive and negative controls, and 5) optimal analytical procedures.

Table 1. Determination by Q-PCR of the ccfDNA Concentration (refA) and of the Proportion of Mutant Allele (mA%) in Plasma Samples from CRC Patients with KRAS- or BRAF-Mutated Tumors.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TNM Status</th>
<th>Mutation</th>
<th>refA (ng/ml Plasma)</th>
<th>mA%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRC1</td>
<td>T3N0M0</td>
<td>G13D</td>
<td>2.6</td>
<td>12.69</td>
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<tr>
<td>CRC2</td>
<td>T3N2M1</td>
<td>G12V</td>
<td>32.5</td>
<td>0.89</td>
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<td>T0N0M1</td>
<td>G12D</td>
<td>24.6</td>
<td>7.67</td>
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<tr>
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<td>G12A</td>
<td>167</td>
<td>32.76</td>
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<tr>
<td>CRC5</td>
<td>T4N2M1</td>
<td>BRAF V600E</td>
<td>177.9</td>
<td>46.22</td>
</tr>
<tr>
<td>CRC6</td>
<td>T3N2M1</td>
<td>G12D</td>
<td>55.9</td>
<td>64</td>
</tr>
<tr>
<td>CRC7</td>
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<td>BRAF V600E</td>
<td>359.3</td>
<td>44.77</td>
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<td>CRC8</td>
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<td>G12D</td>
<td>25.3</td>
<td>5</td>
</tr>
<tr>
<td>CRC9</td>
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<td>G13D</td>
<td>28.2</td>
<td>4.05</td>
</tr>
<tr>
<td>CRC10</td>
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<td>BRAF V600E</td>
<td>35.4</td>
<td>10</td>
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<tr>
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<td>G12S</td>
<td>75</td>
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<tr>
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<td>45.2</td>
<td>10.72</td>
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<tr>
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<td>G12C</td>
<td>143.3</td>
<td>2.56</td>
</tr>
<tr>
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<td>BRAF V600E</td>
<td>31</td>
<td>26.6</td>
</tr>
<tr>
<td>CRC15</td>
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<td>G12C</td>
<td>23.7</td>
<td>0.332</td>
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<tr>
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<td>G13D</td>
<td>41.7</td>
<td>13.12</td>
</tr>
<tr>
<td>CRC17</td>
<td>T4N2M1</td>
<td>G12S</td>
<td>160.9</td>
<td>43.5</td>
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<tr>
<td>CRC18</td>
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<tr>
<td>CRC19</td>
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<td>175.8</td>
<td>64.16</td>
</tr>
<tr>
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<td>G12D</td>
<td>89.8</td>
<td>18</td>
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<tr>
<td>CRC21</td>
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<td>G12V</td>
<td>200.5</td>
<td>4.57</td>
</tr>
<tr>
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<td>G13D</td>
<td>25.1</td>
<td>12.18</td>
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<tr>
<td>CRC23</td>
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<td>G13D</td>
<td>21</td>
<td>0.711</td>
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<tr>
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<td>G12V</td>
<td>54.4</td>
<td>17.5</td>
</tr>
<tr>
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<td>G12D</td>
<td>1386.9</td>
<td>36.6</td>
</tr>
<tr>
<td>CRC26</td>
<td>T3N2M1</td>
<td>G13D</td>
<td>42.2</td>
<td>4.15</td>
</tr>
<tr>
<td>CRC27</td>
<td>T4N1M0</td>
<td>G12S</td>
<td>29.4</td>
<td>8.36</td>
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<tr>
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<td>G12D</td>
<td>13.1</td>
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<tr>
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<td>G12V</td>
<td>13.6</td>
<td>6.59</td>
</tr>
<tr>
<td>CRC30</td>
<td>T4N1M0</td>
<td>G13D</td>
<td>10.3</td>
<td>56.36</td>
</tr>
<tr>
<td>CRC31</td>
<td>T4N1M0</td>
<td>G12A</td>
<td>25.1</td>
<td>8.5</td>
</tr>
<tr>
<td>CRC32</td>
<td>T4N1M0</td>
<td>G12V</td>
<td>6.2</td>
<td>68.77</td>
</tr>
<tr>
<td>CRC33</td>
<td>T4N1M0</td>
<td>G12C</td>
<td>23.9</td>
<td>9.26</td>
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<tr>
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<td>T4N1M0</td>
<td>G12D</td>
<td>47.1</td>
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<td>29.18</td>
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<td>G13D</td>
<td>87.2</td>
<td>56.91</td>
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<td>G12D</td>
<td>6.7</td>
<td>5.05</td>
</tr>
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<td>CRC38</td>
<td>T3N2M1</td>
<td>G12V</td>
<td>220.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Mutational status was confirmed by tumor section analysis as described in Materials and Methods section. TNM represents the Tumor Nodule Metastasis classification. refA corresponds to the total allele concentration (in ng/ml plasma) of a reference allele (Figure 1). mA% was calculated as the percentage of mutant allele from refA.

Serial dilutions of genomic DNA from human placenta cells (Sigma, Munich, Germany) were used as standard for quantification and their concentration and quality was assessed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Each sample was analyzed in duplicate or in triplicate and each assay was repeated at least once. The ccfDNA concentrations obtained with each primer set were normalized to the precise concentration of a genomic DNA sample amplified using the same primer set. The coefficient of variation of the concentration value due to ccfDNA extraction and Q-PCR analysis was calculated as 24% from two experiments (n = 12). Quantification of mutated concentrations.

The blocking oligonucleotides were modified by adding a phosphate group in the 3’ extremity to increase the specificity of Q-PCR by blocking the nonspecific extension of wild-type (WT) sequences. Mutational status was determined by detection of the specific amplification of the mutant sequence through melting analysis: only samples with amplification showing Tm within a 0.4°C range compared to the internal positive control from mutated cell line DNA was designated as positive for the mutation. The concentration was calculated from Cq detected by Q-PCR and a control standard curve on DNA of known concentration and copy number (Sigma-Aldrich). Each concentration of sample used for standard curve was controlled by nanodrop evaluation.

Intplex primer system design is presented in Figure 1. The concentration obtained when targeting the mutated sequence corresponds to the concentration of the alleles bearing the mutation (mA%). The concentration obtained when targeting the WT sequence located at 300 bp from the position of the point mutation corresponds to total ccfDNA (WT + mutated ccfDNA) and is called refA. The proportion of mutant allele (mA%) was determined by quantifying the relative ratios between mA and refA (Figure 1).

We adapted Intplex for the detection of the KRAS hot-spot mutations at the 2nd exon (G12V, G12A, G12D, G12S, G12C, and G13D), which combined corresponds to 96% of KRAS-mutated tumors in CRC (Cosmic Sanger analysis), and also for the detection of the human BRAF 15th exon sequence containing the V600E point mutation, which corresponds to 97.8% of BRAF-mutated tumors in CRC (Cosmic Sanger analysis). The proportion of mutant allele (mA%) was determined for the fragments >60 to 64 bp for KRAS and >97 bp for BRAF (Figure 1).

Figure 1. Primer designs used in the study: primers targeting a KRAS region (A) or a BRAF region (B) for quantifying ccfDNA WT and mutated concentrations.

A

Human KRAS
(exon 2)

6126

6188

6379

6445

5’

3’

produced amplicons

amplior

mutant specific primer

mutation location

primer

phosphate-modified oligonucleotide blocker

B

Human BRAF
(exon 15)

171019

171923

172010

172108

5’

3’

produced amplicons

amplior

mutant specific primer

mutation location

primer

phosphate-modified oligonucleotide blocker
Positive control DNA was extracted from cell lines bearing the KRAS and BRAF mutations. The respective correspondence between cell lines and the corresponding mutation was further detailed: HCT-116 for the G13D KRAS mutation, SW620 for the G12V KRAS mutation, A549 for the G12S KRAS mutation, LS174T for the G12D KRAS mutation, MiaPaca-2 for the G12C mutation, SW1116 for the G12A KRAS mutation, and HT29 for the V600E BRAF mutation.

Every mutational status, summarized in Table 1, was further validated by comparing it with that obtained from the genomic DNA analysis of tumor sections by either sequencing (>50% of tumor cells) or by high resolution melt (HRM) and pyrosequencing (20% to 50% of tumor cells) [21].

Sensitivity and Specificity Analysis of the Quantification of Mutant ccfDNA

Evaluation of the sensitivity level of our method was first conducted on genomic DNA. DNA from cells harboring a specific mutation was serially diluted six times into high concentrated WT genomic DNA from human placenta (Sigma-Aldrich) up to dilution of 0.2 mutated copies in 20,000 copies (1/100,000 ratio). All the experimental points were obtained in triplicate.

Primer Design

The sequences and characteristics of the selected primers are presented in Table W2. The primers were designed using the Primer 3 software and all sequences were checked for self-molecular or intermolecular annealing with nucleic acid folding software (mfold and oligoAnalyzer 1.2). We performed local alignment analyses with the BLAST program to confirm the specificity of the designed primers. Oligonucleotides were synthesized and purified on high performance liquid chromatography (HPLC) by Eurofins (Ebersberg, Germany) and quality control of the oligonucleotides was performed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF).

ccfDNA Size Allele Analysis

We used a set of primers that amplifies targets of increasing size (82, 138, 200, 300, 355, and 390 bp) within the hot-spot region of the KRAS gene (codons 12 and 13 of exon 2) and in which the forward primer of each primer pair specifically targets a point mutation of this region or the WT sequence (Table W2 and Figure W1). Therefore, the concentration of the allele bearing either the point mutation (mA) or not (wtA) was directly compared to the ccfDNA fragment size. The plasma of the CRC patients with KRAS G13D (CRC1) and KRAS G12D (CRC3 and CRC6) mutations was examined. The efficiency of the PCR primer system was normalized with known amount of genomic DNA (in ng) from cell lines harboring KRAS G12D (LS174T) or G13D (HCT116) mutations.

Integrity Index Analyses

Intplex method allowed calculation of the ccfDNA integrity index. The degree of ccfDNA integrity was assessed by calculating an index we termed the DNA integrity index (DII). The DII was determined by calculating the ratio of the concentration determined by using the primer set amplifying the large (circa 300 bp) target to the concentration determined by using the primer set amplifying the short (<100 bp) target (Figure 1).

Results

Sensitive and Specific Quantification of Mutant ccfDNA by a Q-PCR–Based Method

We set up a Q-PCR method combining several refinements for allele-specific discrimination and a primer system design specific to ccfDNA. The integrated primer design of our test involves amplification at the extremities of a 300-bp region of two shorter regions of the same size (in the range of 60–100 bp, ±10%), one corresponding to a mutated sequence and another to a WT sequence (Figure 1, A and B). We demonstrated previously the crucial importance of the primer set design by demonstrating that ccfDNA quantification by Q-PCR is much higher and more accurate when amplifying regions shorter than 100 bp [18]. Varying PCR target size of both short amplicons of more than 10% generates detectable differences between the determined respective ccfDNA amounts. Serial dilutions of mutant DNA to WT ratio revealed detection of the mutant at a level of 1/25,000 for each KRAS and BRAF mutation, as illustrated in Figure W2. Intplex enables single-copy detection of variant alleles down to a sensitivity of ≥0.005% mutant to WT ratio.

As a result, this system design allows the accurate determination of the percentage of mutant allele (mA%). In this study, the lowest mA% found in mCRC plasma was 0.13% (Table 1).

Up to 63% of Total ccfDNA in Mice Xenografted with Cells Harboring a Homozygous KRAS G12V Mutation Originates from Tumors

We used athymic nude mice xenografted with human SW620 CRC cells to clearly distinguish between non-tumor-derived (mouse origin) and mutated or nonmutated tumor-derived (human origin) ccfDNA (Figure 2 and Table W3). These cells were homozygous for the KRAS...
G12V mutation. We first confirmed several observations that we have described previously [18]: 1) the nontumor-derived ccfDNA amount in mouse plasma (Figure 2A) did not vary with tumor size, 2) the control mouse ccfDNA amount (mean, 8.6 ± 3.8 ng/ml; median, 6.6 ng/ml) was similar to the nontumor-derived ccfDNA amount (mean, 7.4 ± 2.6 ng/ml; median, 7.5 ng/ml; Figure 2A), 3) the tumor-derived ccfDNA amount appeared to increase with tumor size (Figure 2, B and D), and 4) no ccfDNA was detected when targeting
the BRAF V600E point mutation (Figure 2C), highlighting the high specificity of our analysis to quantify and detect the presence of ccfDNA alleles with a specific point mutation.

Moreover, our data showed that the amount of the ccfDNA derived from the tumor, as determined when targeting the human KRAS second intron, was found to be somewhat similar to the amount of human KRAS second exon G12V-mutated ccfDNA (Figure 2, B and D). This observation was consistent both with the clonogenic property of the SW620 cells used for the xenografts and with the same allele being carried by each member of the pair of homologous chromosomes in these cells (homozygous mutation). In addition, this demonstrates consequently that our method for determining the concentration of KRAS second exon G12V-mutated ccfDNA was accurate. Tumor-derived ccfDNA represented a prevalent proportion (35–44%) of the total ccfDNA concentration (derived from both mouse and human cells, as quantified in this study) in mice bearing tumors between 100 and 500 mg in weight; this amount ranged from 44% to 63% in mice with 500 to 1200 mg of tumors (Figure 2, E–G). Hence, we concluded that more than half of the total concentration of ccfDNA in xenografted mice may be derived from the tumor, which can be quantified by detecting KRAS-mutant ccfDNA.

0.13% to 69% of KRAS-Mutant Allele Was Observed in ccfDNA from the Plasma of KRAS-Mutated CRC Patients

On the basis of these results, we examined the quantification of selected KRAS- and BRAF-mutated ccfDNA in the plasma of CRC patients. Table 1 and Figure 3 present the mutation detection and the percentage of mutated ccfDNA that we found in 38 plasma samples. This number of KRAS/BRAF-mutated samples could correspond to a nonselected cohort of 85 to 95 (negative and positive) CRC patient blood samples. The mutational status of all samples was further validated by comparing it with that obtained from the genomic DNA analysis of the tumor section either by sequencing or by HRM and pyrosequencing. Hence, mutant ccfDNA alleles could be quantified in the plasma of every patient at stages II, III, and IV (Table 1). The proportion of mutated ccfDNA alleles varied from 0.13% to 68.8% (mean, 20.05%; median, 10.36%) and, more precisely, at stages II and III (mean, 8.39%; median, 8.36%; n = 5) and at stage IV (mean, 21.81%; median, 12.04%; n = 33). Note that we found six plasma samples with 5% to 46.22% ccfDNA bearing the BRAF V600E point mutation.

The KRAS-Mutated and WT ccfDNA Fragment Size Profiles Markedly Differ

We observed previously that the total ccfDNA from the CRC patients’ plasma is much more fragmented than that from healthy individuals [18]. Since our preliminary results revealed a high proportion of tumor-derived ccfDNA, we examined its size pattern in comparison with nontumor-derived ccfDNA from CRC patients (Figure 4). The size profile of the ccfDNA fragments bearing a mutation detected in CRC6, CRC3, and CRC1 plasma was similar. Similarity in the size profile was also observed in these three CRC plasmas when detecting the WT sequence. In all cases, the concentration of ccfDNA was correlated inversely with the amplicon size. It appeared very low or non-detectable when targeting 355 and 390 bp. Note that the sum of wtA and mA was 92%, 54%, and 146% of refA (Figure 4), suggesting a somewhat equivalency between wtA + mA and refA, inasmuch as the coefficient of variation of the plasma ccfDNA concentration as determined by our method was calculated as 24%.

Subsequently, we determined the percentage of mutant ccfDNA (mA%) relative to refA by using the primer set described in Figure 1B. mA% was 26.6%, 17.4%, and 20.6% in CRC6, CRC3, and CRC1, respectively (Figure 5), which confirmed the results determined with the primer set that amplified two 60-bp sequences in the same region of the KRAS exon 2 (Figure 1 and Table 1). Schmidt and Diehl [22] found that high fragmentation was not due to the storage conditions or the procedure used to prepare the samples.
Furthermore, among the 82- to 138-bp ccfDNA fragments, very few WT fragments (Table 2) were observed in contrast to the very high proportion of mutant fragments (70.1%, 74.0%, and 89.3% in CRC6, CRC3, and CRC1, respectively). This was confirmed by using the plasma sample of another CRC patient harboring the KRAS G12V mutation (CRC2) in which 52.1% of the 82- to 138-bp ccfDNA fragments carried the mutation (Figure 5). By quantifying the proportion of KRAS-mutated ccfDNA, we demonstrated that tumor-derived ccfDNA could be discriminated from ccfDNA originating from nonmalignant cells, assuming that the mutant allele is in great excess among all tumor malignant cells [23].

Discussion

The Mutation Loads in the Xenografted Mouse Animal Model and in CRC Plasma Differ Markedly

ccfDNA analysis showed the very high proportion of mutant allele (up to 63%) present in xenografted mice and demonstrated that the mutant tumor-derived ccfDNA amount corresponds to the total tumor-derived ccfDNA amount. This suggests tumor cell clonogenicity and a very low proportion of tumor microenvironment surrounding cells. These observations highlight some of the crucial differences between this experimental mouse model and the clinical setting when compared with our data on CRC plasma samples, which showed, in some cases, a very low proportion of the mutant allele.

The Mutation Load in CRC Patients’ Plasma Is Highly Variable

Only two papers from the same group based on a cohort study and another two based on one case report have reported previously the examination of the proportion of mutant ccfDNA alleles among total circulating DNA alleles. Diehl et al. [13–15] developed a highly sensitive approach (BEAMing) to detect and quantify ccfDNA harboring APC mutations in CRC patients’ plasma samples [13]. Assuming that they calculated the mutant APC fragment percentage as the mA% value, they detected lower amounts of mutant allele (median of 0.04% for samples from patients with Dukes A, n = 8; 1.28% for patients with Dukes B, n = 8; and 8.05% for patients with Dukes D, n = 6, cancers) in comparison with our data. In another study, this group evaluated the median percentage of mutant allele as 0.18% in plasma from 16 Dukes D CRC patients [14,15]. Furthermore, only 0.3% of ccfDNA with the KRAS G12V mutation was detected in the plasma sample from a patient with metastatic CRC using this technique [16]. The high mA% variation observed by this group was confirmed in our study, despite the higher median values we obtained. In particular, our study revealed, for the first time, several cases (5 of 38) where more than half of total ccfDNA amount was mutated (Figure 5). This observation indicated that, in some cases, ccfDNA may originate mainly from tumor cells. Twelve of 38 (31.6%) samples showed a mutation load higher than 25% (Figure 4). As a consequence, the tumor-derived ccfDNA did not correspond to “a tiny fraction” as previously stated [13–16] and it may not have derived mainly from stroma cells.

The higher level of mutation load as observed here may be due to the use of targeting low size sequence (<100 bp) in exon 2 and exon 15 of KRAS and BRAF, respectively. Targeting low size sequence for ccfDNA was based on previous work [18] in which a ccfDNA size distribution study indicated a much higher (four- to six-fold) efficacy in detecting WT intron fragments (total ccfDNA). The approach of comparing amplification data of a specific sequence with another sequence of a similar target size (+10 bp) within a 300-bp region, as carried out here (Figure 1), enabled the direct comparison between the concentration of the mutant and the WT KRAS sequence (Figure 5) and, subsequently, accurate determination of mutation load (Figure 3). In addition, the combination of allele-specific priming, blocking of WT unspecific amplification, and melting analysis provides unsurpassed sensitivity and specificity for a Q-PCR–based method. Inplex can be adapted to all point mutations of interest.

The higher percentage of mutant ccfDNA detected with our Q-PCR method may be due to the use of the KRAS and the BRAF mutations as biomarkers and also to our Q-PCR method. Its use revealed that allelic dilution appears less pronounced than previously stated [13–15], easing the detection of resistance-associated point mutations and the quantification of mutant ccfDNA alleles. Alternatively to the hypothesis of Diehl and Vogelstein [13], the observation of mA% higher than 25% in more than one-third of the plasma samples provides evidence that ccfDNA does not originate only from microenvironment cells but rather that a significant proportion of ccfDNA could be tumor derived. It was shown that phagocytosis by macrophages or other scavenger cells of apoptotic or necrotic neoplastic cells.
could lead to DNA release in the circulation of patients with cancer [24]. This seems to occur especially in invasive tumors, which generally contain large regions of necrosis due principally to hypoxia. Interestingly, the CRC tumor was among the tumors with a higher cell loss factor (94–98%) [25], which might explain the high ccfDNA level observed in CRC patients.

mA% was highly variable (0.13–68.77%), as determined from ccfDNA by Intplex among the positive KRAS or BRAF mutational status CRC plasma samples (n = 38). This raises crucial questioning about the cell origin of ccfDNA release, tumor cell composition, and mutant allele specific imbalance. In CRC patients, we can discriminate three cells from which the release of ccfDNA originates: 1) malignant tumor cells, 2) tumor microenvironment cells (stromal, epithelial, or inflammatory cells), and 3) nontumor-derived cells, which produce the basal ccfDNA level in healthy individuals (about 5 ng/ml, [18]).

The highest mA% (68.77%) is more than 500-fold greater than the lowest mA% (0.13%) observed. As it seems unlikely that the proportion of the microenvironment cells reaches up to more than 90%, the low mA% observed in a significant proportion of the plasma samples (for instance, 18/38 being lower than 10%) was not only due to a low malignant cell proportion but also to poor tumor cell clonogenicity. West et al. [26] demonstrated that the median proportion of cells from primary CRC tumors was 57.1% (interquartile range from 47.4% to 66.4%) and 9% at the lowest in pT1 stage cancer.

The proportion of mutated tumor cells within a tumor may be expressed by the mutation load, which can be determined by quantifying the number of mutated cells in a tumor section [23,26] or by quantifying the mutant alleles from circulating DNA, as performed in this study. It depends on the mutation zygosity status, being directly equivalent to the mutant allele percentage in case of homozygosity or being twice as much as the mutant allele percentage in case of heterozygosity. We determined through the Cosmic Sanger database and several reports that 25% and none of the colorectal adenocarcinoma tumor samples that tested positive showed a KRAS and BRAF homozygous mutation, respectively. Since the zygosity status of the patient tumors was unknown in our study, we were unable to quantify precisely their mutation load. Nevertheless, we can assume that the five CRC plasma samples showing a mutant allele percentage superior to 50% correspond to a tumor-bearing KRAS homozygous mutation. Given the heterozygosity of the BRAF 600E mutation, the allele mutation load is equivalent to half of the genome-equivalent mutation load, and as a result, the proportion of malignant cells at least ranged from 10% to 92.4%.

Our data might suggest a very high variation in the proportion of malignant cells in colorectal tumors and/or low tumor cell clonogenicity. However, the direct relationship between these data and the proportion of malignant cells is currently uncertain as the mechanism of ccfDNA release determines the yield (necrosis, apoptosis, or active release). Therefore, interindividual comparison of the proportion of mutant allele determined from circulating DNA could appear somewhat more difficult to interpret; however, this parameter could be an efficient biomarker for monitoring or following up CRC patients.
and could be combined with size fraction analysis, as demonstrated in this study, to provide a deeper examination toward this goal.

**ccfDNA Size Fraction Analysis Characterizes Tumor-Derived ccfDNA**

ccfDNA is thought to be released through various mechanisms such as necrosis, phagocytosis, apoptosis, or active release [27]. ccfDNA seems to be present in the circulation, especially in the form of complexes (through electrostatic bonds) with cell constituents (mononucleosomes or oligonucleosomes or nucleolipoproteins, [28,29]), or inserted in apoptotic vesicles that make it relatively stable. As apoptosis leads to high intermuncleosomal DNA fragmentation, fragments as small as 180 to 200 bp (the length of mononucleosome DNA) may be found following this release mechanism [27,28]. Apoptosis results to the release of shorter fragments compared to necrosis or phagocytosis (larger than 10,000 bp, [13]); therefore, the estimation of ccfDNA fragmentation could provide evidence on the ccfDNA release mechanism.

To specify cancer patients’ ccfDNA and study its origin, previous studies have evaluated their fragmentation level. However, as stated by Jung et al. [30], experimental bias has led to large discrepancies when drawing general conclusions. Given the ability to quantifying mutant ccfDNA in this work, we studied various fragmentation indexes. Hence, in addition to the integrity index (corresponding to the proportion of DNA longer than the length of the mononucleosome DNA among the total ccfDNA) as previously examined [6,31], the proportion of various ccfDNA size fractions of the plasma from four CRC patients with a KRAS point mutation were determined (Figure 5).

In CRC6, CRC3, CRC1, and CRC2 samples, preliminary results indicate that mutant ccfDNA was composed mostly of fragments <138 bp, while nonmutant ccfDNA was mostly constituted of fragments in the 138- to 300-bp range and very few fragments <138 bp. Conversely, Schmidt and Diehl [22] recently observed that mutant ccfDNA has almost no influence on DNA integrity, indicating that mutant ccfDNA presents a minor fraction of the total ccfDNA. This highlights the numerous discrepancies found in the literature on ccfDNA in cancer patients [30]; in this regard, we proved herein that careful methodology design with better knowledge of the ccfDNA structure would provide a more accurate observation.

Altogether, our data suggest that ccfDNA deriving from normal tissue or CRC patient tumors vary in their form, and consequently, their mechanism of release might be different. It is difficult to hypothesize on the high fragmentation of CRC tumor cell–derived ccfDNA, which could result in the release of either intermuncleosomal breaks, as a result of apoptosis, or large-sized ccfDNA, subsequent to necrosis or phagocytosis [28]. Nevertheless, these data indicate that the specific detection of KRAS-mutated ccfDNA enables its discrimination from the WT ccfDNA through the analysis of the ccfDNA fragment size profile. In addition, size fraction analysis could give information about the nature of the ccfDNA release mechanism. Size fraction analysis should be further studied by using a large cohort to validate our results and to determine whether it might be generalized for the analysis of other gene mutations.

**Clinical Implications and Perspectives**

In addition to the diagnosis and monitoring of CRC patients, close indication of tumor evolution through the quantification of tumor (or mutant) ccfDNA by blood testing could allow the control and adaptation of chemotherapy or radiotherapy. mA% determination by ccfDNA analysis should be further examined in a large cohort to study whether an mA% threshold could be defined under which the use of targeted therapy could be beneficial. Patient monitoring by examining KRAS/BRAF-mutant ccfDNA would be reduced to about half of CRC cases; nevertheless, analyzing ccfDNA-bearing mutations on other genes is likely in CRC as well as in other cancers. Determination of the mutation load might be of great interest: A recent study demonstrated that a low proportion of malignant cells compared to epithelial and stromal cells in CRC tumors is related to poor cancer survival [26]. Prospective trials should further investigate the clinical impact of this parameter and aim to evaluate whether it might also serve as a prognostic biomarker. Targeting highly fragmented ccfDNA revealed high level of mutant ccfDNA in a significant proportion of CRC plasmas. This provides clues for monitoring cancer progression with the aim of developing personalized treatment and to better circumscribe the potential biologic roles of ccfDNA-bearing specific oncogene point mutations.

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<td>CT palliative 1 (oxaliplatin-egafur uracil-bevacizumab), CT 2 palliative (LFT-irinotecan-bevacizumab)</td>
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</tr>
<tr>
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<td>CRC18</td>
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RT, radiotherapy; CT, chemotherapy; the number indicates the sequence of treatment.
Table W2. Characteristics of the Selected Primers and of the Amplicons Obtained.

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<Figure W1. Design of the primers used for the ccfDNA size distribution study.>
Table W3. Numerical Values of ccfDNA Quantification Obtained in the Xenografted Mice Experiments.

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<th>Mouse No.</th>
<th>mWT</th>
<th>hWT</th>
<th>hKRASm</th>
<th>hBRAFm</th>
<th>Tumor Weight (mg)</th>
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</table>

Data are expressed as ng/ml plasma.
ND, not determined.

Figure W2. Analysis of the sensitivity of the method for detecting BRAF V600E point mutation. mA% represents the estimated mutation load and Cq represents the quantification cycle when the amplified amplicon is detected during the Q-PCR experiment. Each point corresponds to a specific amplification of the targeted sequence as determined by melting analysis. DNA from HT29 cells harboring a specific mutation was serially diluted six times into high concentrated WT genomic DNA from human placenta up to dilution of 0.2 mutated copies in 20,000 copies (1/100,000 ratio). Assay is carried in triplicate determination.