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Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer

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Abstract: Development of a Q-PCR-based assay for the high-performance analysis of circulating cell-free DNA (ccfDNA) requires good knowledge of its structure and size. In this work, we present the first visual determination of ccfDNA by Atomic Force Microscopy (AFM) on plasma samples from colorectal cancer (CRC) patients and healthy donors. In addition to the examination of fragment size distribution profile as performed by Q-PCR, this analysis confirms that ccfDNA is highly fragmented and that more than 80 % of ccfDNA fragments in CRC plasma are below 145 bp. We adapted an Allele-Specific Blocker (ASB) Q-PCR to small ccfDNA fragments to determine simultaneously the total ccfDNA concentration, the presence of point mutation, the proportion of mutated allele, and a ccfDNA integrity index. The data validated analytically these four parameters in 124 CRC clinical samples and 71 healthy individuals. The multi-marker method, termed Intplex, enables sensitive and specific non-invasive analysis of tumor ccfDNA, which has great potential in terms of cost, quality control, and easy implementation in every clinical center laboratory.

1. Introduction

Circulating cell-free DNA (ccfDNA) is an emerging biomarker in cancer diagnosis and theragnosis (Schwarzenbach, Hoon, and Pantel 2011). The use of ccfDNA presents conceptual advantages compared to classic genetic analysis via tumor-tissue sampling. CcfDNA analysis is non-invasive and enables day-to-day patient follow-up (Diehl, Schmidt, Choti, et al. 2008, Dawson et al. 2013) and monitoring of treatment response (Gorges et al. 2012). CcfDNA also exhibits the genetic and the epigenetic alterations from its tumor of origin (Stroun et al. 2001). Analysis of these alterations could provide valuable information to tailor the clinician's choice of treatment given the restrictions of the new targeted therapies: *KRAS* mutation status has been found to be predictive of response to cetuximab, an anti-EGFR (epidermal growth factor receptor) monoclonal antibody (mAb) in colorectal cancer (CRC) (Lièvre et al. 2006) and patients with metastatic colorectal cancer (mCRC) bearing *KRAS* mutations do not respond to cetuximab and panitumumab anti-EGFR mAbs, either as single agent or in combination with chemotherapy (Van Cutsem et al. 2011, Amado et al. 2008).

In CRC, the *KRAS* oncogene is often mutated (~35 % of cases, Cosmic Sanger Analysis (Bamford et al. 2004)). Although the V600E *BRAF* mutation is observed less frequently in CRC patients (5-12 %, Cosmic Sanger (Bamford et al. 2004)), it is a strong indicator of poor prognosis (Di Nicolantonio, Martini, et al. 2008). The presence of *KRAS* or *BRAF* point mutations is associated with clinical resistance to anti-EGFR receptor mAb treatment (Lièvre et al. 2008, Di Nicolantonio, Arena, et al. 2008). Consequently, *KRAS* status assessment is mandatory in patients with mCRC before administering targeted therapy. CcfDNA also appears to be an interesting non-invasive biomarker for determining *KRAS* mutation (Spindler et al. 2012). In addition, ccfDNA analysis offers the possibility for monitoring the real-time evolution of CRC during and after treatment.

Numerous investigations have shown an increased mean ccfDNA concentration, but ccfDNA concentration alone seems insufficient for high-diagnostic performance or to establish it as a diagnosis biomarker (van der Vaart and Pretorius 2010). The structure and size of tumor ccfDNA is poorly characterized systematically in the literature. Our previous work has surprisingly shown that ccfDNA is highly fragmented and mainly of size smaller than 100 bp (Mouliere et al. 2011), which is smaller than the observed size between 145 and 180 bp reported in the literature (Schwarzenbach, Hoon, and Pantel 2011, Jung, Fleischhacker, and Rabien 2010, Fleischhacker et al. 2011). This specific tumor ccfDNA fragmentation pattern was observed using a Q-PCR-based method on human CRC plasma samples and confirmed on human CRC-cell line xenografted mice plasma samples (Thierry et al. 2010). When targeting small-sized sequences, the observed proportions of *KRAS* and *BRAF* mutant ccfDNA were between 0.13 and 69 % (Mouliere et al. 2013) in contrast to previous reports establishing that mutant ccfDNA represents only a tiny fraction of total ccfDNA (Diehl et al. 2005, Diehl, Schmidt, Choti, et al. 2008). As detection of mutation from a blood test (Diehl, Schmidt, Choti, et al. 2008, Guha et al. 2013, Forsheew et al. 2012) requires high-sensitivity methods, we present here the first use of Atomic Force Microscopy (AFM) to observe ccfDNA fragments from healthy donors and CRC patients and confirm the specific size pattern of tumor-derived ccfDNA.

Using multi-marker approach to study ccfDNA could be a good way to increase the discriminative performance of a diagnosis test. Thus, based on the specific size fragmentation of tumor ccfDNA, we designed and developed an Allele-Specific Blocker (ASB) Q-PCR method where an original primer system enabled specific ccfDNA multiparametric examination. The goal of this work was firstly to confirm and build on our previous observations that ccfDNA is highly fragmented and small in size in the blood of CRC patients

(Mouliere et al. 2011) and secondly, to validate the use of four different ccfDNA parameters: total ccfDNA concentration, point mutation detection, mutant fragment concentration, and fragmentation. We developed a robust Q-PCR-based method, denoted Intplex, dedicated to tumor ccfDNA analysis and the characterization of these four parameters for clinical validation as biomarkers for CRC diagnosis.

2. Materials and Methods

2.1. Blood samples

Blood samples were collected from CRC patients (n=124), irrespective of their CRC stage or relapse. CRC patients did not receive chemotherapy, radiotherapy, or anti-EGFR antibody treatment for at least one month before blood collection. Plasma samples were taken prior surgical resection of the tumor. Written, informed consent was obtained from all participants prior to the onset of the study. The protocols for the use of blood samples from healthy volunteers (n=71) used in this study were approved by the “Etablissement Français du Sang” (EFS) Ethics Committee (EFS-PM agreement: 21/PVNT/MTP/CNR14/2010-0029).

2.2. Plasma isolation and ccfDNA extraction

A sample of 4 ml of blood was collected from patients in EDTA tubes. The blood was centrifuged at 1200 g at 4°C in a Heraeus Multifuge LR centrifuge for 10 min. The supernatants were isolated in sterile 1.5 ml Eppendorf tubes and centrifuged at 16000 g at 4°C for 10 min (Mouliere et al. 2011). Subsequently, the plasma was 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, CA) according to the “Blood and body fluid protocol” and our detailed protocol (Mouliere et al. 2013). DNA samples were kept at -20°C until use.

2.3. AFM preparation

Freshly cleaved muscovite mica was incubated in a mixture of a 1-(3-aminopropyl) silatrane (APS) solution for 30 min to prepare APS-mica. 2 µl of ccfDNA sample in 10 mM Tris, 0.5 mM EDTA buffer was deposited onto the APS-mica and air-dried for 2 min (Pang et al. 2011). The sample was then washed with deionized water and subsequently dried with nitrogen gas. It was then mounted on a NanoScope IIIa AFM for imaging (Veeco/Digital Instruments, Santa Barbara, CA). Images were acquired in tapping mode in air. Silicon tapping mode probes (TESP; Veeco, Santa Barbara, CA) were used. The probes have a nominal spring constants of ~60 N/m and resonant frequency of ~245 Hz. The scan size was 2x2 µm². Ten images were acquired for each ccfDNA sample. Acquired AFM images were analyzed using commercially available software, Femtoscan Online (Advanced technologies Center, Moscow, Russia), for the measurement of ccfDNA fragment lengths. Measured ccfDNA fragment lengths ranged from 15 nm to 63 nm. A total of ~1300 fragments were measured. Measured DNA fragment sizes were binned into six groups of 0-10 nm, 10-20nm, 20-30 nm, 30-40 nm, 40-50nm, and 50-60 nm, and the fragment size distribution was obtained by dividing the number of fragments in each bin by the total number of fragments. DNA fragments size conversions were made on the assumption that 1 bp was equivalent to a length of 0.34 nm. Dalong et al. (45) previously used AFM to characterize the short fragments of DNA produced after ionization of cells.

2.4. Primer design

Primers were designed using the Primer 3 software and all sequences were checked for self- or inter-molecular 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 HPLC

by Eurofins (Ebersberg, Germany) and quality control of the oligonucleotides was performed by MALDI TOF. The sequences and characteristics of the selected primers are presented in the Supplementary Table 1.

2.5. *CcfDNA quantification by Q-PCR*

Our Q-PCR experiments followed the MIQE guideline (Bustin et al. 2009). Q-PCR amplifications were carried out at least in duplicate in a 25 μ l reaction volume on a CFX96 instrument using the CFX manager software (Bio-Rad). Each PCR reaction mixture was composed of 12.5 μ l PCR mix (Bio-Rad Super mix SYBR Green), 2.5 μ l of each amplification primer (0.3 pmol/ μ l), 2.5 μ l PCR-analyzed water, and 5 μ l DNA extract. Thermal cycling consisted of three repeated steps: a 3-min Hot-start Polymerase activation-denaturation step at 95°C, followed by 40 repeated cycles at 95°C for 10 sec, and then at 60°C for 30 sec. Melting curves were obtained by increasing the temperature from 55°C to 90°C with a plate reading every 0.2°C. The concentration was calculated from Cq detected by Q-PCR and also a control standard curve on DNA of known concentration and copy number (Sigma-Aldrich). Serial dilutions of genomic DNA from human placenta cells (Sigma) were used as a standard for quantification and their concentration and quality was assessed using a Qubit spectrofluorimeter (Invitrogen). The amount profile of ctDNA fragments of different size was assessed using an integrated PCR system that targeted intronic sequences within the same region. For the human wild-type specific primer set, the designed amplification sizes were 60 bp, 73 bp, 101 bp, 145 bp, 185 bp, 249 bp, 300 bp, 357 bp, and 409 bp (the reverse primer was the same for each amplification system). These primers targeted intron 2 of the human *KRAS* (Supplementary Table 1).

2.6. *Intplex general design*

Intplex is an ASB Q-PCR based on specific and original primer construction. We focused on the size of the targeted sequence for each primer pair. One primer pair targeted a specific sequence of less than 100 bp. In a 300-bp area, we compared the results of this primer pair with another primer pair targeting a sequence of almost the same size (\pm 10 bp). In one of the two primer pairs, we targeted a point mutation (on the *KRAS* or *BRAF* gene). This primer construction also enabled calculation of a fragmentation index by comparing the concentration obtained with one of the primer pairs targeting a short size sequence (< 100 bp) with a primer pair targeting a longer sequence (approximately 300 bp).

2.7. *Mutation analysis*

Intplex Q-PCR analysis enables the detection of seven *KRAS* 2nd exon-point mutations (G12V, G12A, G12D, G12S, G12C, G12R, and G13D) and a *BRAF* 15th exon-point mutation (V600E). Those mutations correspond to the seven most frequent somatic *KRAS* mutations (corresponding to 90% of all *KRAS* mutations determined by the Sanger Cosmic data base) and the *BRAF* V600E mutation (corresponding to 98% of all *BRAF* mutations). The Intplex primer system design is presented in Fig. 2. The concentration obtained when targeting the mutated sequence corresponded to the concentration of the alleles bearing the mutation (mA). The concentration obtained when targeting the wild-type (WT) sequence located at 300 bp from the position of the point mutation corresponded to the total cfDNA (WT + mutated cfDNA), denoted refA. The proportion of mutant allele (mA%) was determined by quantifying the relative ratios between mA and refA (Fig. 2).

Each Q-PCR experiment was carried out after validating the internal technical controls. Non-template controls for each primer set targeting *KRAS* or *BRAF* mutations were included in every experimental run in this study. Positive-control DNA was extracted from cell lines bearing the targeted *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, MiaPaca2 for the G12C mutation, SW1116 for the G12A *KRAS* mutation, and HT29 for the V600E *BRAF* mutation. Synthetic DNA bearing the *KRAS* sequence of interest (Horizon Discovery Ltd.) was used as a positive control for *KRAS* G12R. Every mutational status, summarized in Table 1, was further validated by comparing it to that obtained from the genomic DNA analysis of tumor sections either by sequencing (>50%, tumor cells) or by HRM (High Resolution Melt analysis) and pyrosequencing (20 to 50% of tumor cells) (Alain R. THIERRY Accepted in Nature Médecine, 2013).

2.8. Sensitivity and specificity analysis of mutant ccfDNA quantification

Evaluation of the sensitivity level of our method was firstly conducted on genomic DNA. From each targeted mutation, a corresponding positive control was added and its sensitivity was evaluated. DNA from the cells harboring targeted mutation was serially diluted six times into high-concentrated WT genomic DNA from human placenta (Sigma Aldrich) up to a dilution of 0.2 mutated copies in 20,000 WT copies. All the experimental points were obtained in triplicate. The non-specificity of the different primer systems for each mutation was evaluated on plasma ccfDNA from WT-confirmed human mCRC patients and it was expressed as the ratio between mA and refA. Non-template controls were conducted for each experiments and each primer pair.

2.9. Integrity Index analysis

The degree of ccfDNA integrity was assessed by calculating the DNA Integrity Index (DII) using the Intplex method. The DII was determined by calculating the ratio of the concentration determined by using the primer set amplifying a large target (circa 300 bp) to the concentration determined by using the primer set amplifying a short target (<100 bp) (Fig. 2). The DII was evaluated simultaneously in the *KRAS* and *BRAF* gene of each cancerous plasma sample in our Intplex assays.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.02 software. The Student's t-test was used for comparison of means. A probability of less than 0.05 was considered to be statistically significant; *p # 0.05, **p # 0.01, ***p # 0.001. We investigated the diagnostic performance of each potential biomarker by means of the area under the ROC curve (AUC). This curve measures the accuracy of biomarkers when their expression is detected on a continuous scale, displaying the relationship between sensitivity (true-positive rate, y-axes) and 1-specificity (false-positive rate, x-axes) across all possible threshold values of the considered biomarker. The AUC is a useful way to summarize the overall diagnostic accuracy of the biomarker, the value of which is expected to be 0.5 in absence of predictive capability, whereas it tends to be 1.00 in the case of high predictive capacity. Values greater than 0.8 were considered as having high predictive capacity. The diagnostic performance of cfdNA analysis was compared to tumor-tissue analysis (the current gold standard) for its sensitivity and its specificity in distinguishing between mutated and non-mutated individuals. Sensitivity (Se) reflected the diagnostic performance for the detection *KRAS* and *BRAF* mutations, and specificity (Sp) reflected the performance for the detection of samples determined as WT for the tested mutations.

3. Results

3.1. *CcfDNA is highly fragmented in the blood of CRC patients*

Fig. 1A shows that the amount of ccfDNA was greater when targeting small-size amplicons and that amplifying these amplicons provided higher quantifications (Mouliere et al. 2011). We observed that the proportion of ccfDNA fragments below 100 bp was greater in CRC patients than in healthy individuals (53.2 % vs 24.5 %, respectively) (Fig1, B). Inversely, the proportion of ccfDNA fragments larger than 300 bp was greater in healthy individuals than in CRC patients (17.4 % vs 3.8 %). We found that concentrations were greater in CRC plasma than in the ccfDNA of healthy individuals, as described previously in a shorter cohort (plos).

AFM was used to confirm our previous observations. We performed AFM analysis on the previously tested samples (Fig 1,A and B) and obtained the first visual determination of ccfDNA (Fig1, C). We chose six metastatic CRC samples with an elevated level of ccfDNA (~1 ng/μl after extraction) estimated by Q-PCR. In the six cases, we observed, without any difficulty, DNA fragments of various short sizes. We estimated the size distribution of ccfDNA fragments and our data revealed that ccfDNA was highly fragmented in the plasma of both CRC patients and healthy individuals (Fig1, D). A slight difference was observed in the median-size distribution of 135 bp for CRC patients and 180 bp for healthy individuals (Fig1, D). More than 80 % of ccfDNA fragments were below 145 bp in the plasma of cancerous patients. No ccfDNA fragments were found at sizes greater than 300 bp in the plasma of CRC patients in contrast to healthy individuals where 10 % of the fragments were greater than 300 bp. Healthy individuals also presented a highly fragmented profile with more than 65 % of ccfDNA fragments below 145 bp.

3.2. *Mains characteristic of the Intplex system:*

3.2.1. *General design of Intplex primer system*

Intplex design is based on the observation that ccfDNA fragment quantification is greatly dependent on the length of the targeted sequence (Mouliere et al. 2011). Intplex targets low-size amplicons and is, therefore, dedicated to ccfDNA study. More precisely, it simultaneously analyzes by Q-PCR three targeted amplicons. Two of these amplicons are of low-size (< 100 bp); one is specific to a mutated sequence, and another is specific to a non-mutated distant point of mutation (Fig 2). The third amplicon, with a size of 300 bp, is used for determining the DII. Intplex is an ASB Q-PCR-based method specific for ccfDNA analysis to enable the detection of point mutation and to determine mutant allele concentration. This method combines the use of (1) allele-specific Q-PCR with blocking 3'-phosphate modified oligonucleotide; (2) low T_m primers with mutation in 3'; (3) an integrated primer design; (4) routine internal positive and negative controls; (5) optimal pre-analytical and analytical procedures. In this study, the blocking oligonucleotides were modified by adding a phosphate group in the 3' end to increase the specificity of the Q-PCR reaction by blocking the non-specific extension of WT sequences. Mutational status was determined by detection of the specific amplification of the mutant sequence. The specificity of the targeted amplified mutation was confirmed by comparing the experimental T_m with a positive control bearing the sequence of interest.

3.2.2. *Technical validation of ccfDNA concentration as a biomarker*

The ability to quantify with confidence ccfDNA from blood is the central parameter of Intplex analysis. During an Intplex run, the total ccfDNA concentration, as well as the mutated ccfDNA concentration, is evaluated. We tested the repeatability of our analysis by repeating 10 times in the same run the ccfDNA quantification from CRC plasma with the same primer set (*KRAS* B1B2 - 67 bp). We also tested the reproducibility of our experiments (Fig3, A) by evaluating ccfDNA in 10 repeated runs using another primer set (*BRAF* A1A2 - 105 bp). We

also validated the reproducibility of our quantification method on different genes (*KRAS* and *BRAF*) and on different sequences (exon and/or intron) of the same gene (Fig3, B). These 10 experiments were carried out by two different manipulators. The results of these experiments showed no significant difference in the quantification of *KRAS* and *BRAF* genes, irrespective of the targeted sequence (Fig3, C). In 10 experiments, we repeated the extraction and quantification of ccfDNA fragments using the same primers and Q-PCR experimental conditions and observed a coefficient of variation of 21.3 % of the DNA concentration in our wells. In previous work, we estimated a median coefficient of variation at 24 % (with 19 % caused by the Q-PCR experiment and 5 % by DNA extraction) (Mouliere et al. 2011). We also tested our quantification on different genes, or targeted sequence of the same gene, and found equivalent concentrations (for instance, for one CRC patient the median concentration observed on five different genetic loci was 279.6 ± 23.5 ng/mL plasma). We also investigated the variability of our measurements on different samples of increasing concentration (Fig3, D). CcfDNA concentration of the *KRAS* and *BRAF* gene was strongly correlated as indicated by Spearman analysis ($r=0.966$; $p<0.001$).

3.2.3. Technical validation of ccfDNA mutation as a biomarker

A data analysis workflow for determining mutation positivity was designed according to the mutations tested (Alain R. THIERRY Accepted in Nature Médecine, 2013). The quantification cycle (Cq) and the melting temperature (Tm) were the two main elements establishing correct mutation determination. One of the features of the Intplex system is not only to enable the accurate determination of the mutation presence and type, but also the calculation of the percentage of mutant allele (mA%). The mA value was determined up to a dilution of two mutated copies in 20,000 copies of WT DNA (0.01%) as observed using genomic DNA bearing the *KRAS* G12C mutation (Fig. 4). The others mutations tested with Intplex exhibited equivalent or greater sensitivity: 0.005 % for *KRAS* G13D, G12V, G12S, G12A, and G12R primer sets, 0.01 % for *KRAS* G12D primer set, and 0.004 % for *BRAF* V600E primer set (Alain R. THIERRY Accepted in Nature Médecine, 2013). This sensitivity was confirmed with all the mutations of interest in our study. The lowest mA % found in mCRC plasma samples was 0.037 % (Table 1). The specificity of the different primer systems was evaluated on plasma ccfDNA from WT-confirmed human mCRC patients using the corresponding refA amplicon and healthy donor ccfDNA as references. The mean percentage of non-specificity was calculated as 0.01 % for *KRAS* G13D primer set, 0.008 % for *KRAS* G12V primer set, 0.009 % for *KRAS* G12D primer set, 0.005 % for *KRAS* G12A primer set, 0.001 % for *KRAS* G12C primer set, 0.012 % for *KRAS* G12S primer set, less than 0.01 % for *KRAS* G12R and for *BRAF* V600E primer sets (Alain R. THIERRY Accepted in Nature Médecine, 2013).

3.2.4. Technical validation of a ccfDNA fragmentation index

CcfDNA fragmentation was evaluated in each Q-PCR run by calculating the DII corresponding to the ratio of ccfDNA concentration determined on a large targeted sequence (300 bp) and the concentration of ccfDNA determined on a short targeted sequence (60 bp). We validated the repeatability and reproducibility of the DII analysis on ccfDNA samples (Fig.5). We compared the DII determined from two different genes (*KRAS* and *BRAF*) on 98 clinical mCRC samples and observed a linear repartition of the DII data of these genes (Fig. 5A). The Spearman correlation between *KRAS* and *BRAF* DII was estimated at 0.707. The DII was also estimated on two exon sequences of *BRAF* and on 2 exon sequences and one intron sequence of *KRAS*. The observed ccfDNA fragmentation varied with the targeted sequence (Fig. 5B). This is consistent with data in the literature: various DII were found depending on the targeted gene and the cancer (Schwartz, Meshorer, and Ast 2009). However, the DII ratio

between healthy individuals and cancerous patients seemed to be constant irrespective of the targeted sequence (Fig. 5C). This indicates that ccfDNA fragmentation has potential interest as a biomarker, although the cohort size used in this study is too small draw any definitive conclusions.

3.3. Clinical application on CRC patient plasma

After validating all the technical parameters of our Intplex test, we investigated its diagnostic performance by clinical application. A total of 124 plasma samples of CRC patients from stage I to IV (Table 1) were tested and 71 healthy individuals plasma were used as controls (data not shown). CcfDNA concentration, ccfDNA fragmentation, mutation presence, and the proportion of mutated ccfDNA were examined for each sample. All clinical samples were tested in duplicate. In the 124 samples tested, ccfDNA concentration of the short fragment (refA) was quantifiable in all cases (median concentration 26.0 ng/mL plasma). CcfDNA concentration was detectable in the samples of all healthy individuals tested (median concentration 4.7 ng/mL plasma, n=71). CcfDNA fragmentation (represented by the DII) was also calculated in a large majority of CRC samples (three DII were undetermined in our CRC samples). However, the ccfDNA DII was only obtained partially in healthy individuals. *KRAS* or *BRAF* mutation was detected in 47/124 CRC samples, which represented 37.9 % of the samples (similar to the Cosmic mutation frequency reported for this type of cancer). No mutation was detected in healthy individuals. The proportion of mutated ccfDNA was between 0.037 % and 64 % of the total ccfDNA for this size of amplicon. No statistically significant difference in the mutation proportion was observed when the mutation was located on either the *KRAS* or *BRAF* gene (p=0.803; Supplementary Figure 1). This observation seems in concordance with previous works (ForsheW et al. 2012, Mouliere et al. 2013).

Each tested parameter may independently be diagnostically important. The concentration observed in mCRC patients is significantly greater than in healthy individuals (Mann-Whitney, p=0.0022), (Fig. 6, A). ROC curve univariate analysis of the AUC for each parameter predicted a satisfactory level of diagnostic predictive capacity (Fig. 6, B), in particular, ccfDNA total concentration, which had a high predictive capacity (AUC=0.91) for discriminating mCRC patients (n=98) and healthy individuals (n=71). CcfDNA fragmentation (estimated with the DII) study could have been more conclusive since sample number may appear insufficient to discriminate with statistical significance healthy individuals from CRC patients, and between the different CRC stages. Low concentrations observed in healthy individuals also hampered the estimation of DNA fragmentation and the subsequent comparison with the DII of cancer patients. Increasing the number of samples in the cohort, and working on larger volume of plasma (in order to recover more ccfDNA), could help to resolve this issue.

4. Discussion

Numerous studies on ccfDNA have suggested its capacity as biomarker of early stage detection, prognosis, therapy monitoring, tumor follow-up, and theragnosis choice (Fleischhacker et al. 2011) following on from the pioneering work of Leon et al. (Leon et al. 1977). Alternatively to previously required advanced and/or sophisticated technologies (Diehl et al. 2005, Diehl, Schmidt, Durkee, et al. 2008, Diehl, Schmidt, Choti, et al. 2008, Heitzer et al. 2013, Murtaza et al. 2013, Chan et al. 2013), we propose here a multiparametric method using an adapted Q-PCR technique, based on a specific primer design, which can be implemented in all clinical laboratories. This method, denoted Intplex, is based on our previous work on the structure and origin of ccfDNA (Mouliere et al. 2011).

Firstly, we investigated the size profile distribution of the amounts of ccfDNA in the plasma of CRC patients and healthy individuals using multiple integrated Q-PCR systems (Mouliere et al. 2011). Five to six fold more ccfDNA amounts were quantified when targeting a 60 bp sequence compared to a 150 bp sequence (Mouliere et al. 2011). This led us to hypothesize that ccfDNA is mostly composed of short fragments. CcfDNA size and structure are highly dependent on the mechanism of release of ccfDNA in blood and on its subsequent modifications (degradation by blood nucleases, phagocytosis, or complex formation with other macromolecules). Tumor ccfDNA fragment size could be considered as a "signature" of the ccfDNA release mechanism. For instance, apoptosis leads to the release of ccfDNA of a size between 145 and 180bp (or multiples of these sizes) corresponding to the size of the DNA wrapped around the nucleosome (Schwarzenbach, Hoon, and Pantel 2011). Necrosis, on the other hand, may release more irregular and larger-sized ccfDNA (up to 10 kbp). However, apoptosis alone cannot explain the ccfDNA size distribution we observed here in the plasma of CRC patients. We hypothesize that ccfDNA is released in the bloodstream by apoptosis and is subsequently fragmented by the action of nucleases, mononucleosome breakdown, or by a phagocytosis (Rykova et al. 2012). In any cases, a better knowledge of ccfDNA size and structure is required to optimize for a particular cancer type any ccfDNA analysis requiring a PCR amplification or pre-amplification.

In this study, AFM enabled us to achieve precise ccfDNA fragment sizing and to visualize for the first time the short ccfDNA fragments in CRC patient plasma and healthy individuals. The size pattern observed by AFM indicated a high fragmentation of ccfDNA with more than 80 % of the fragments below 145 bp and the median size of fragments was greater (~130 bp) than that observed by Q-PCR (~100 bp). This slight discrepancy could be due to the difference in sensitivity between AFM and Q-PCR, or on the minimal amount of ccfDNA required in both cases. We observed with AFM a slight difference in the ccfDNA size pattern distribution between healthy individuals and CRC patients with longer ccfDNA fragments observed in healthy individuals. The proportion of long ccfDNA fragments was greater when observed by Q-PCR, but the presence of long fragments is also more frequently observed in healthy individuals than in CRC patients. Another possibility is that AFM could not distinguish ccfDNA from mitochondrial DNA (mtDNA), which cannot be detected by using Q-PCR and primers targeting nuclear genomic sequences. Subsequent experiments would help us to further investigate this point. Note that the AFM images shown here examine DNA fragment length and theoretically represent naked ccfDNA, which should be inserted in supra-molecular structures potentially implying that cellular proteins or histones are eliminated during the extraction process. This visual examination should be extended to other cancer types before generalizing about tumor ccfDNA fragmentation in patient plasma.

CcfDNA in the blood of CRC patients has a higher mean concentration than in healthy individuals (Stroun et al. 1987). Nevertheless, examining total ccfDNA concentration alone was found to be insufficient to discriminate healthy and cancerous patients as a large overlap between the two groups has been observed (van der Vaart and Pretorius 2010). Detecting amplicon size below 100 bp is more suited to quantifying tumor ccfDNA with higher specificity. In this work, a ROC analysis enables discrimination of healthy individuals from late stage CRC patients with high-diagnostic performance (AUC: 0.91). Thus, determination of the ccfDNA concentration reveals that this test might have a prognosis capacity, especially when considering the potential of implementing multiparametric analysis by associating the DII, the detection of a driving mutation, and the mutation load parameters. Higher performance of the diagnostic power by a multiparametric approach would certainly be necessary in case of stage I or II CRC patients in which the total plasma ccfDNA

concentration is lower. A wider analysis could help answer other biological questions, such as the putative physiological role of ccfDNA in oncogenesis or metastasis (García-Olmo et al. 2010). Specifically targeting tumor ccfDNA under the precise standard operating procedures used here (El Messaoudi et al. 2013) may help to better estimate the value of ccfDNA concentration as biomarker for early diagnosis, or determine a risk of recurrence after tumor resection. In both cases, the corresponding tumor mass may be low with few tumor cells releasing ccfDNA, leading to a low concentration of tumor ccfDNA in blood.

Our recent blinded, prospective, multicentric study (n=106) compared the *KRAS* and *BRAF* mutation status data obtained from the analysis of mCRC tumor tissue using standard care methods and paired plasma DNA using Intplex. The results showed 92% and 100% sensitivity for the seven exon 2 *KRAS* and *BRAF* V600E mutations (Alain R. THIERRY Accepted in Nature Medicine, 2013). In comparison, *KRAS* mutation detection sensitivity in paired FFPE tumors and plasma in a non-blinded study is comprised between 50-78% (Diehl, Schmidt, Durkee, et al. 2008, Perkins et al. 2012). Intplex is a practical, fast, and robust alternative method for measuring with low proportions down to 0.015 – 0.005 % of mutated ccfDNA in clinical samples, which is unprecedented among Q-PCR methods.

Intplex, as for other PCR derived techniques for detecting point mutation on ccfDNA, such as COLD-PCR or digital PCR, can be adapted easily to all mutations of interest, and it is applicable to testing several well-identified mutations. This advantage is less obvious when targeting genes exhibiting more dispersed and less frequent mutations (for example *TP53*) because each mutation should be targeted with a specific primer pair when using Q-PCR. Intplex primer design could be implemented by using digital PCR system. However dPCR system might be more suited for the detection of copy number variations by providing absolute quantification of the DNA copy number (Whale et al. 2012, Chan et al. 2013). Next-generation sequencing (NGS) appears to be better optimized for monitoring the clonal evolution of the tumor and for the evaluation of unknown mutations leading to therapeutic resistance (Forshew et al. 2012, Dawson et al. 2013), and efforts are underway to enhance mutation detection sensitivity (Narayan et al. 2012). However, the NGS approach is still time consuming and requires computational analysis. Furthermore, in light of the data presented here, only a few reports, have provided point mutation test sensitivity values determined from clinical samples while various reports on other methods have described only *in vitro* evaluations (Taly et al. 2012). However, current mutation testing from ccfDNA sequencing (sensitivity ~2%) would have failed to detect approximately 20 % of the patients harboring *KRAS/BRAF* mutation in our blinded, prospective, multicentric assay (Alain R. THIERRY Accepted in Nature Medicine, 2013); Intplex analysis exhibits a median lower than 0.01% (0.005-0.012%) sensitivity for detecting point mutations (Alain R. THIERRY Accepted in Nature Medicine, 2013). The turnaround time is much faster using Intplex with clinical decision making in ~2 days compared to ~28 days when using standard mutation-status testing on tumor tissue (Pascal Artru 2012), and 13 and 2 days when testing is done with FFPE carried out in the same clinical center (Alain R. THIERRY Accepted in Nature Medicine, 2013). Hence, using Q-PCR-derived techniques saves time compared to genomic approach requiring computational data analysis. Moreover, conventional Q-PCR (and derivatives) is currently approximately 1/20th of the cost of an equivalent digital PCR analysis (Whale et al. 2012), but the cost of analysis per sample will decrease for digital PCR in the coming years. Mutation testing by ccfDNA analysis could replace advantageously tumor-resection analysis due to a rapid data turnaround in a time-frame suitable for clinical decision making, cost effectiveness, and non-invasiveness when compared to a tissue biopsy. Note, Intplex may be adapted to all point mutations or short deletions. Detection of the rare *KRAS*

and *NRAS* mutations in CRC patients is under investigation, as determining *RAS* mutation status is now recommended for selecting targeted therapies (Douillard et al. 2013).

The high variation in mutation load that we previously observed may be explained by the high variation in the number of malignant cells compared to the micro-environment cells (West et al. 2010) or in the proportion of cells harboring the mutation (Mouliere and Thierry 2012, Mouliere et al. 2013). The roles of the micro-environment and tumor clonal heterogeneity are two main, crucial aspects, especially in regard to tumor resistance to therapy (Holohan et al., 2013). We previously demonstrated (Mouliere et al. 2013) that the proportion of mutant ccfDNA in stage IV CRC patients is greater than initially thought (Diehl et al. 2005, Diehl, Schmidt, Choti, et al. 2008). In case of known tumor-positive mutational status, precise determination of the mutation load could allow dynamic follow-up of the presence or the regression of the number of the mutant malignant cells following tumor resection or during drug treatment, respectively.

We established a correlation between ccfDNA fragmentation or apoptosis as it generates high DNA fragmentation, and cancer diagnosis and tumor progression in CRC patients. Q-PCR analysis of plasma samples from xenografted mice and cancer patients showed that tumor-derived ccfDNA exhibits significant higher ccfDNA fragmentation (Mouliere et al. 2011). In regards to fragment size distribution, major discrepancies exist in the literature due to various experimental bias, such as patient selection (different type and stage of cancer), heteroclonality and diversity of the clonal cell population of the tumor, serum vs. plasma, extraction chemistry, pre-analytical conditions, use of various DNA fragmentation indexes (especially the shortest size examined), DII not optimized for ccfDNA analysis (non-nested target sequences, involvement of different genes, etc.), and method sensitivity (especially respective to the different fragment sizes). The major part of the previous studies showed an apparent higher DII in cancer patients compared to healthy individuals (Jung, Fleischhacker, and Rabien 2010), and the minor part showed apparent equivalence (Schmidt et al. 2008) with a few showing an apparent lower DII (Mouliere et al. 2011, Thierry et al. 2010, Ellinger et al. 2009). Note, the lower the short target sequence is compared to the long one, the lower the DII value observed for cancer patients is, with respect to healthy subjects. Thus, we found in a short cohort that the fragmentation level of ccfDNA in metastatic colorectal patients (n=12) was statistically different ($p < 0.01$, mean nearly five-fold higher) compared with healthy individuals (n=16) (Mouliere et al. 2011). However, this parameter seems more sensitive to low variation in tumor biology and experimental conditions. A large and well-defined patient cohort, and high study sample standardization are required to properly evaluate, independently or in combination, its potential.

5. Conclusion

We arbitrarily designed a simple, cost effective method that can be implemented in every clinical laboratory. Intplex is optimized for ccfDNA analysis in the blood of CRC patients and data revealed that it provides at least equivalent sensitivity and specificity as more sophisticated methods. The test singularly analyzes simultaneously four potentials biomarkers: total cfDNA concentration, point mutation detection, mutation load, and fragmentation level (Mouliere et al. 2011, Mouliere and Thierry 2012). Alone, each of these biomarkers may be informative, but multiparametric calculation could increase the diagnostic performance of the method for discriminating cancer patients and healthy individuals. A multi-marker based-strategy was previously explored (Perkins et al. 2012) and a recent report on melanoma proposed that a multiparametric approach by analyzing separately several ccfDNA factors could lead to increased diagnostic performance. (Salvianti et al. 2012). This

test makes the repetitive examination possible during patient follow-up toward personalized medicine in cancer. This robustness makes that Intplex would be adaptable to the sequential liquid biopsy examination for the follow-up of cancer patients in case of recurrence after surgery, or to evaluate tumor response, such as revealing emerging point mutations leading to CRC anti-EGFR therapy (Misale et al. 2012), or during treatment monitoring. Furthermore, it is reasonable to assume that this multimarker assay might be beneficial to CRC patient management care in terms of prognosis. Clinical validation of Intplex toward these objectives is undergoing.

Supplementary Materials

Table S1. List and characteristics of the primers used in this study.

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Author contributions: F.M. and A.R.T. designed the study, F.M., S.E.M. and A.R.T. developed Intplex methodology, F.M and S.E.M. performed Intplex experiments and analyzed the data, D.P. and A.D. developed and performed AFM experiments, F.M., S.E.M., D.P., A.D. and A.R.T. interpreted the data, F.M. and A.R.T. wrote the manuscript, S.E.M., D.P. and A.D. critically review the manuscript.

Competing interests: The *Centre National de la Recherche Scientifique* (CNRS) has a patent application in which A.R.T. is an author, on circulating DNA analytical method.

Figures:

Sample number	Total ccfDNA concentration (ng/ml plasma)	DII	Mutational status	mA %
CRC 1	24	0.49	WT	
CRC 2	21.019	0.457	G13D	0.59
CRC 3	11.67	0.04	WT	
CRC 4	4.59	0.035	WT	
CRC 5	14.946	0.269	G12V	1.72
CRC 6	5.994	0.39	WT	
CRC 7	35.42	0.1	WT	
CRC 8	93.7	0.258	WT	
CRC 9	42.24	0.21	G13D	4.14
CRC 10	42.56	0.15	WT	
CRC 11	6.14	0.012	WT	
CRC 12	4.98	0.017	WT	
CRC 13	100.9	0.14	G12S	43.5
CRC 14	200.47	0.04	G12V	4.57
CRC 15	2.55	0.28	G12A	20.75
CRC 16	483.466	0.043	G12D	0.11
CRC 17	44.59	0.223	WT	
CRC 18	4.507	0.27	WT	
CRC 19	25.14	0.47	G13D	5.69
CRC 20	29.4	0.36	G12S	8.37
CRC 21	7.75	ND	WT	
CRC 22	91.97	0.1	WT	
CRC 23	1386.9	0.04	G12D	36.6
CRC 24	1.3	0.4	WT	
CRC 25	28.55	0.025	WT	
CRC 26	11.92	0.1	WT	
CRC 27	12.7	0.006	WT	
CRC 28	7.23	0.11	WT	
CRC 29	506.49	0.93	WT	
CRC 30	89.79	0.07	G13D	18.02
CRC 31	195.93	0.019	WT	
CRC 32	175.8	0.013	G13D	64.11
CRC 33	8	0.27	WT	
CRC 34	10.98	0.201	WT	
CRC 35	47.43	0.09	G12D	12.05
CRC 36	10.56	0.25	WT	
CRC 37	2.58	0.064	G13D	12.79
CRC 38	3.4	0.48	WT	
CRC 39	28.6	0.28	WT	
CRC 40	25.8	0.17	G12V	1.12
CRC 41	18.7	0.48	G12D	0.39
CRC 42	10.5	0.34	WT	
CRC 43	30.02	0.44	WT	
CRC 44	6.8	0.036	WT	
CRC 45	17.97	0.085	WT	
CRC 46	24.645	0.014	G12D	7.67
CRC 47	522.9	0.024	G13D	0.037
CRC 48	12.729	0.337	WT	
CRC 49	166.965	0.019	G12A	32.76
CRC 50	20.76	0.036	WT	
CRC 51	59.679	0.26	WT	
CRC 52	11.429	0.39	WT	
CRC 53	19.87	0.78	WT	
CRC 54	13.04	0.13	WT	
CRC 55	231	0.18	V600E	46.23
CRC 56	55.88	0.11	G12D	64.23
CRC 57	85.692	0.074	WT	
CRC 58	359.28	0.11	V600E	44.76
CRC 59	15.16	0.22	WT	
CRC 60	14.19	0.04	WT	
CRC 61	25.7	0.32	WT	
CRC 62	25.3	0.17	WT	
CRC 63	44	0.07	WT	
CRC 64	70.79	0.021	WT	
CRC 65	36.4	0.26	WT	
CRC 66	15.06	0.51	WT	
CRC 67	8.625	0.41	WT	
CRC 68	34.75	0.49	WT	
CRC 69	28.2	0.011	G13D	4.04
CRC 70	9.4	0.16	WT	
CRC 71	15.47	0.45	V600E	10.28
CRC 72	273.72	0.2	WT	
CRC 73	26.022	0.88	WT	
CRC 74	47.3	0.12	G12S	12.04
CRC 75	47.748	0.02	WT	
CRC 76	12.9	0.25	WT	
CRC 77	64	0.12	WT	
CRC 78	33.508	0.19	WT	
CRC 79	56.133	0.42	WT	
CRC 80	38.96	0.2	WT	
CRC 81	15.7	0.39	WT	
CRC 82	952.2	0.11	WT	
CRC 83	251.16	0.08	WT	
CRC 84	45.28	0.48	V600E	10.72
CRC 85	267.63	0.011	WT	
CRC 86	10.94	0.097	WT	
CRC 87	7.49	0.25	WT	
CRC 88	8.93	0.054	WT	
CRC 89	9.7	0.08	WT	
CRC 90	6.5	0.38	WT	
CRC 91	143.34	0.81	G12C	2.56
CRC 92	16.2	0.28	WT	
CRC 93	51.124	0.117	WT	
CRC 94	26.31	0.099	V600E	26.6
CRC 95	23.73	0.2	G12C	0.51
CRC 96	43.78	0.1	G13D	13.12
CRC 97	6.1	0.112	G12A	4.5
CRC 98	106	1.45	G12S	1.3
CRC 99	27.4	0.024	G12D	16.5
CRC 100	7.6	0.12	G13D	0.57
CRC 101	143	0.032	G12V	16.1
CRC 102	7.5	0.003	G12V	0.53
CRC 103	513	0.072	G12S	61
CRC 104	5.22	0.166	WT	
CRC 105	5.28	0.359	WT	
CRC 106	12.8	0.367	G12V	1.25
CRC 107	21	0.06	WT	
CRC 108	15.5	0.015	WT	
CRC 109	4.8	0.21	G12A	2.2
CRC 110	7.3	0.21	WT	
CRC 111	18.8	0.018	G13D	15.4
CRC 112	20.9	0.015	G13D	21
CRC 113	23.7	0.008	WT	
CRC 114	1.39	0.489	WT	
CRC 115	2.99	0.036	WT	
CRC 116	10.7	ND	WT	
CRC 117	110.13	0.036	G12D	3
CRC 118	6.8	0.05	G12D	8.3
CRC 119	109	0.028	G12D	14.6
CRC 120	25.6	0.04	G13D	1.4
CRC 121	50	0.009	G12D	4.6
CRC 122	202	0.027	WT	
CRC 123	6.2	0.49	G13D	4.1
CRC 124	9.4	ND	WT	

Table 1. Determination by Intplex Q-PCR of the ccfDNA concentration (refA), the DNA Integrity Index (DII) and the nature and the proportion of mutant allele (mA%) in plasma samples from CRC patients (n=124). *KRAS* or *BRAF*-mutated tumors were found in 47 of these patients (37.9% of the samples). mA% was calculated as the % of mutant allele from the total concentration of ccfDNA. WT, wild type for the tested mutations.

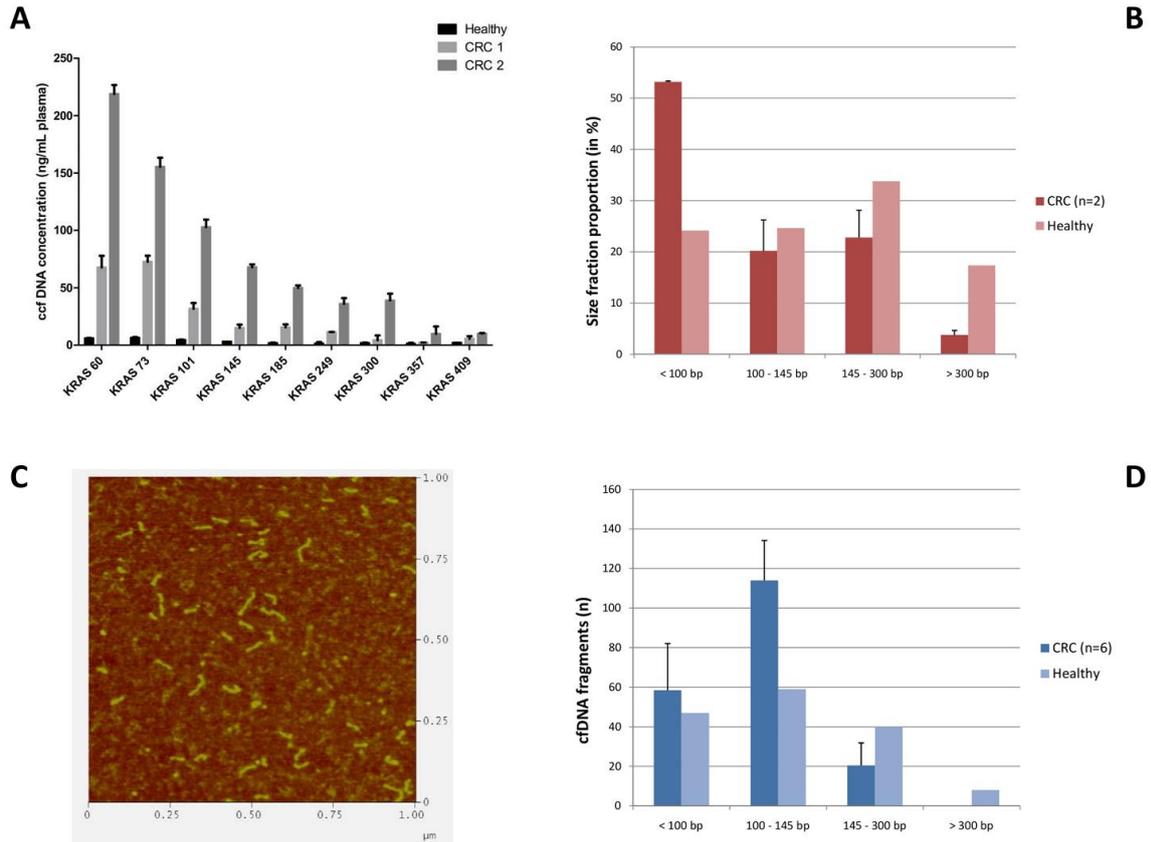


Figure 1. CcfDNA is highly fragmented. **A.** Size profile distribution of ccfDNA amounts in CRC (colorectal cancer) of two patients (named CRC1 and CRC2) and healthy individual plasma. The size profile distribution was evaluated with a Q-PCR experiment and corresponding primer of targeting amplicons of increasing size. **B.** Proportions of ccfDNA per size range in % of the total observed ccfDNA with Q-PCR. **C.** AFM visualization of ccfDNA fragments extracted and purified from stage IV CRC patient plasma. A representative visual determination of ccfDNA is shown here. **D.** Size profile distribution of ccfDNA fragments (n) measured with AFM experiments.

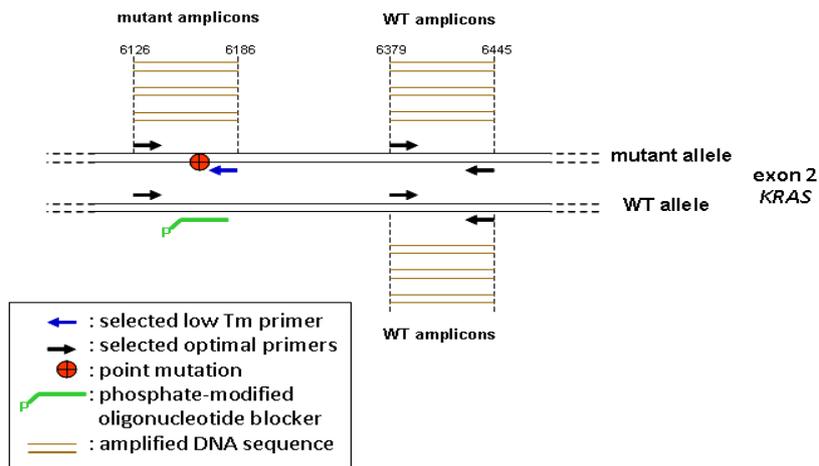


Figure 2. Schematic representation of Intplex primer system, localization of each primer set on a hypothetical DNA template and the oligoblocking primer. This schema is given as an illustration and an equivalent Intplex construction was carried out when we analyzed the V600E mutation of the *BRAF* gene.

A

	Target	Mean (Cq)	Median (Cq)	95 % CI of mean	SD
Repetability (n=10 in the same experiment)	KRAS B1B2	27.01	26.82	26.73 - 27.28	0.39
	BRAF A1A2	26.66	26.69	26.49 - 26.82	0.23
Reproducibility (n=10 in different experiments)	KRAS B1B2	25.81	25.8	25.57 - 26.04	0.3
	BRAF A1A2	25.45	25.34	25.22 - 25.69	0.31

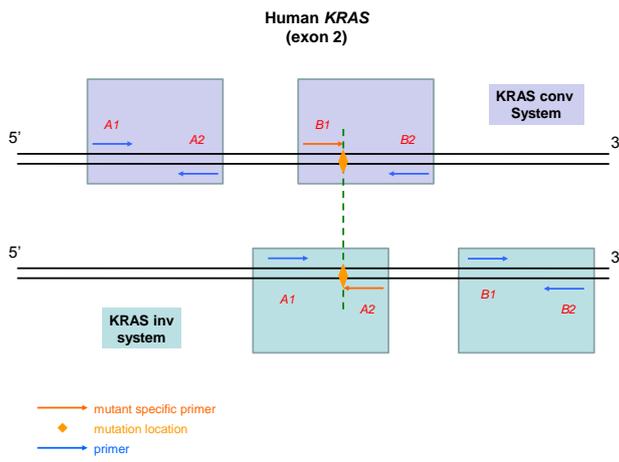
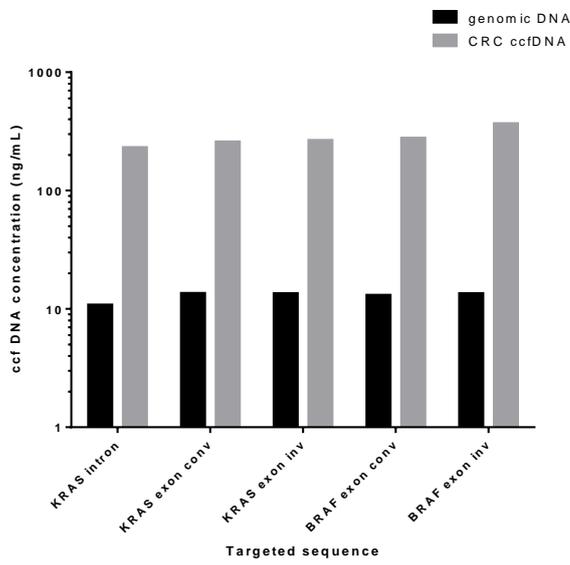
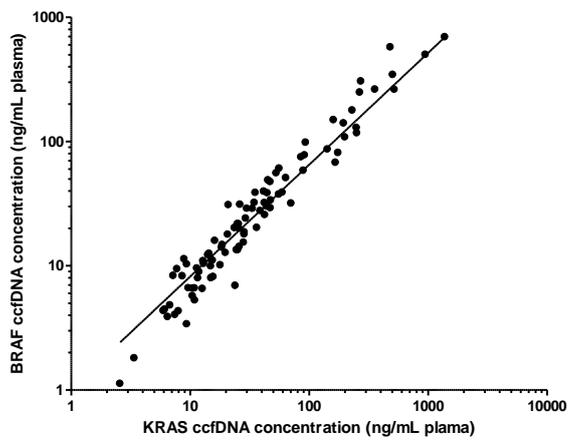
B**C****D**

Figure 3. CcfDNA concentration measurement with Intplex. **A.** Representation of the repeatability and reproducibility of the ccfDNA quantification results with the Intplex primer system on ccfDNA extracted with a QIAgen Blood mini kit. **B.** Schematic representation of the targeted *KRAS* and *BRAF* sequences in Fig.3C, and respective localization of the primers. **C.** Quantification with Intplex of genomic DNA (black) and quantification of ccfDNA from a mCRC patient (grey). DNA concentration was determined with different primer sets: one specific to an intronic sequence of *KRAS*, two specific to two sequences on an exonic area of *KRAS*, and two specific to two *BRAF* sequences. **D.** Evaluation of the ccfDNA concentration with the Intplex Q-PCR system on two different genes (*KRAS* and *BRAF*) and at different concentrations (n=98, corresponding to CRC plasma samples).

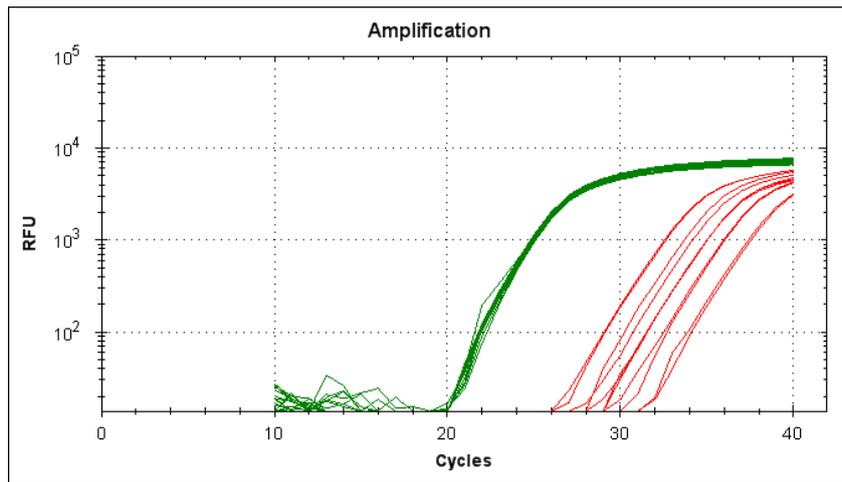


Figure 4. Technical validation of the ccfDNA mutation measurement with Intplex. Q-PCR raw amplification curve of genomic DNA from the mutant cell line containing the tested mutation (red) was serially diluted five times into high-concentrated WT gDNA (green) from human placenta (Sigma). All the experimental points were obtained in duplicate. In this figure, only the *KRAS* G12A dilution is shown as a representative example of our sensitivity data.

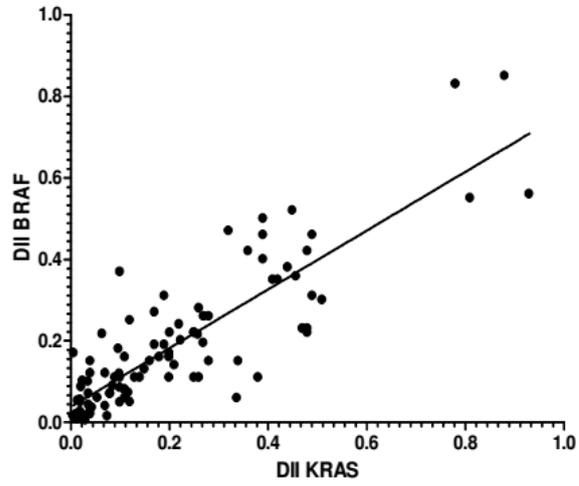
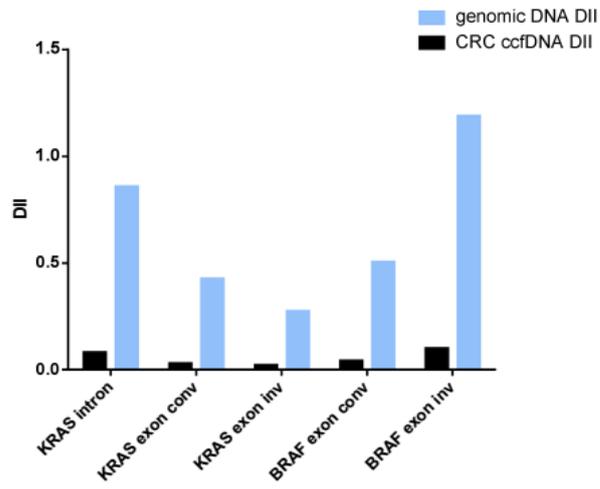
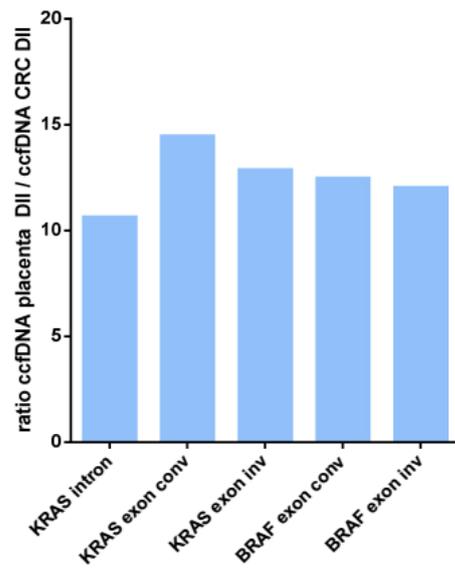
A**B****C**

Figure 5. Technical validation of the ccfDNA fragmentation measurement with Intplex. The fragmentation is calculated with a representative index, the DII (DNA Integrity Index). The DII was estimated from the ratio of the DNA concentration obtained by targeting a 300-bp sequence and a 60-bp sequence in a same locus. **A.** Evaluation of the DII with Intplex Q-PCR system on two different genes (*KRAS* and *BRAF*). Correlation between the *KRAS* and *BRAF* DII was evaluated with a Spearman analysis ($r=0.817$, $p<0.001$). **B.** Estimation of the DII on the targeted gene (*KRAS*, *BRAF*) and the sequence within this gene (exon, intron). The denominations "conv" and "inv" refer to a particular primer system schematized in Fig3, B. The ccfDNA used for the DII evaluation was isolated from a mCRC patient and from a placenta DNA extract (to serve as the genomic DNA control). **C.** Ratio of ccfDNA DII from genomic DNA control and from CRC plasma extract.

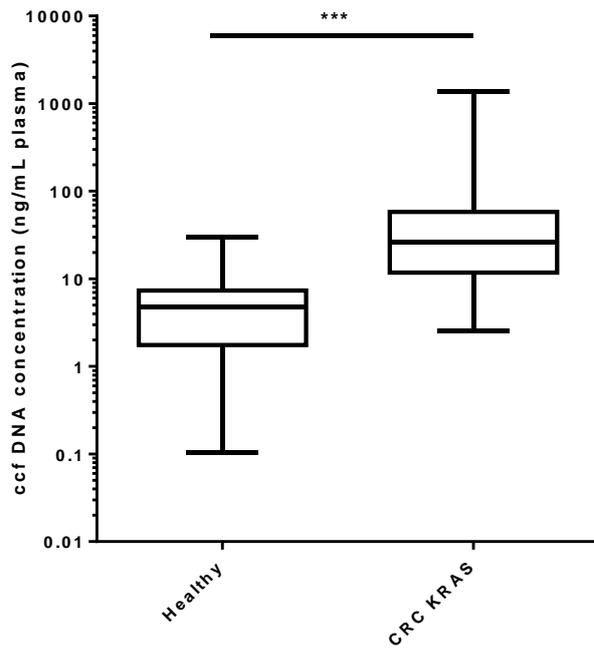
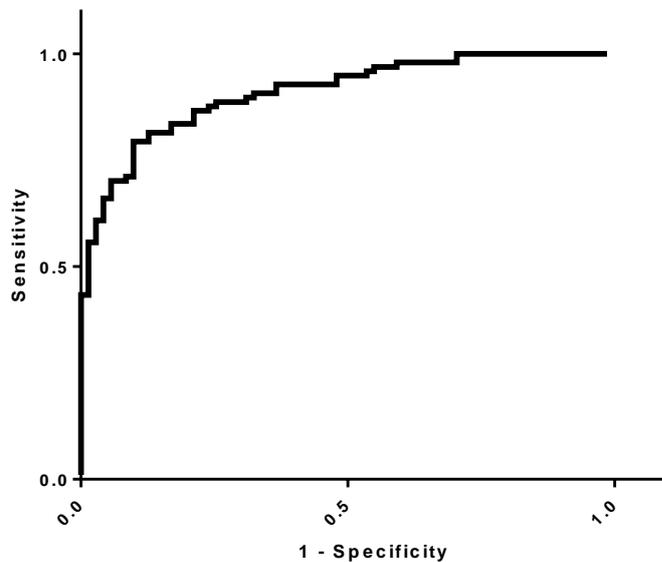
A**B**

Figure 6. A: Comparison of the quantification of plasma ccfDNA from healthy individuals (n=71) and mCRC patients (n=98). The concentration observed in mCRC patients is significantly greater than those of healthy individuals (p=0.0022). The data are expressed in ng/mL plasma. B: Diagnosis predictive capacity of total ccfDNA concentration to distinguish plasma from mCRC patients and healthy subjects. ROC Curve representation deriving from the univariate logistic analysis corresponding to the total ccfDNA (AUC = 0.91). The observed concentration of total ccfDNA from late stage mCRC patients (n=98) was compared to the total ccfDNA concentration of healthy individuals (n=71). This curve measures the accuracy of biomarkers displaying the relationship between sensitivity (true-positive rate, y-axes) and 1-specificity (false-positive rate, x-axes).

Supplementary Materials:

Supplementary Table 1: List and characteristics of the primers used in this study.

Gene	Primer name	Direction	Sequence 5'-3'	Tm (°C)	Amplicon size (bp)
KRAS	Kras 60 Hf	sense	GCCTGTGACCTACAGTGAAAA	57.9	60
	Kras 73 Hf	sense	CCCTACACGTAGAGCCTGTGA	61.8	73
	Kras 101 Hf	sense	GAGATGGTGGGAAGAACAGGTG	59.8	101
	Kras 145 Hf	sense	TGGGCTGTGACATTGCTG	60.42	145
	Kras 185 Hf	sense	ATCTGCCTCCGAGTTCCTG	58.8	185
	Kras 249 Hf	sense	TGGAAGAGCATAGGAAAGTGC	57.9	249
	Kras 300 Hf	sense	GGTCCTTTTCCGTGTGTAGG	59.45	300
	Kras 357 Hf	sense	GGCATCTCTAGGACGAAGGT	59.4	357
	Kras 409 Hf	sense	GCCTCCCTTCCAGCACT	58.2	409
	Kras 60-409 re	antisense	TGACCAAGCAAAAACAGACCA	60.28	-
BRAF	Braf A1 conv k	sense	TTATTGACTCTAAGAGGAAAGATGAA	53.2	105
	Braf A2 conv k	antisense	GAGCAAGCATTATGAAGAGTTTAGG	56.9	-
	Braf V600E conv k	sense	GATTTTGGTCTAGCTACAGA	59.3	97
	Braf B2 conv k	antisense	TAGCCTCAATTCTTACCATCCACA	59.7	-
	Braf blocker	sense	GCTACAGTGAATCTCGATGG - PHO		
KRAS	Kras A1 inv k	sense	GCCTGCTGAAAATGACTGA	54.5	61
	Kras G12D Inv k	antisense	CTCTTGCCCTACGCCAT	51.7	-
	Kras G12A Inv k	antisense	CTCTTGCCCTACGCCAG	54.3	-
	Kras G12S Inv k	antisense	TCTTGCCCTACGCCACT	51.7	60
	Kras G12C Inv k	antisense	TCTTGCCCTACGCCACA	51.7	-
	Kras G13D Inv k	antisense	GCACTCTTGCCCTACGT	51.7	64
	Kras G12V Inv k	antisense	CTCTTGCCCTACGCCAA	51.7	61
	Kras G12R Inv k	antisense			
	Kras B1 inv k	sense	CCTTGGGTTTCAAGTTATATG	54.0	67
	Kras B2 inv k	antisense	CCCTGACATACTCCCAAGGA	59.4	-
Kras blocker	antisense	GCCTACGCCACCAGCTC - PHO			

