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Evaluation of three advanced methodologies, COLD-PCR, microarray and ddPCR, for identifying the mutational status by liquid biopsies in metastatic colorectal cancer patients

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A B S T R A C T

A major effort has been focused on the detection of oncogenes' mutations in diverse types of clinical specimens including formalin-fixed and paraffin embedded tissues, presently the gold–standard samples, up to plasma, that constitute a noninvasive alternative source of tumor DNA. The reliable detection of mutations in circulating tumor DNA requires a high analytical sensitivity. Here, we applied three different highly sensitive methodologies (COLD-PCR, a microarray–based approach and the droplet digital PCR, ddPCR) to identify mutations in the plasma of 30 metastatic colorectal cancer patients previously genotyped on tissue biopsy.

The methods showed a modest concordance rate with respect to the results obtained on tissue biopsies: 63.3% by ddPCR, 63% by microarray and 55.6% by COLD-PCR. This could be ascribed either to the different timing between tissue and liquid biopsy collection, which could reflect a different stage of disease progression or to the diverse sensitivity of the methodologies applied. Indeed, if we compare the results obtained on plasma samples, the concordance rates were higher especially by comparing ddPCR versus COLD-PCR (92.6%). Thus, we consider both methodologies as useful procedures easily transferable in a clinical setting. Notably, the ddPCR allows a quantitative assessment of the fractional abundance of the mutation.

1. Introduction

Nowadays, there is a great attention in mutation detection for the genotype-driven personalized treatment of tumor patients. One of the main clinical challenge remains the ability to detect mutations with very high sensitivity in all types of clinical specimens starting from the formalin-fixed and paraffin embedded (FFPE) tissues, presently the gold–standard procedure, up to plasma samples. Circulating tumor DNA (ctDNA) in plasma represents an accessible noninvasive source of DNA alternative to tissue biopsy, being repeatable along the patient's history enabling testing for specific genetic biomarkers thus providing a whole and real-time assessment of the cancer mutation status. However, the analysis of ctDNA in plasma still requires a detailed clinical evaluation. The major technical issues in analyzing ctDNA are the low abundance of the mutated allele, which is highly variable in different patients, and the wide dynamic range. Most of the ordinary techniques (quantitative PCR, sequencing and pyrosequencing) used for DNA mutation detection lack the analytical sensitivity to accurately measure ctDNA in plasma. This is extremely important because false-negative results, especially when ctDNA results are used for therapeutic decision making or in detecting occult disease, could turn out to be detrimental.

On the contrary, the most recently developed highly sensitive techniques based on deep sequencing approach are too expensive, time-consuming and require bioinformatics expertise [1,2]. Based on these considerations we compared 3 different highly sensitive methodologies which are also cost effective and time-saving: CO-amplification at Lower Denaturation temperature-PCR (COLD-PCR) either in *fast* or *full* format coupled with Sanger sequencing, an amplicon down microarray–based approach [3] and finally, droplet digital PCR (ddPCR).

In particular, *full* COLD-PCR enriches all possible mutations along the sequence, although the enrichment is reported generally lower than with *fast* COLD-PCR [4–7]. Concerning the microarray, we previously described an innovative microarray format based on a silicon oxide layered slide coated with a functional copolymer (dimethylacrylamide

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(DMA), N-acryloyloxysucinimide (NAS) and meta-acryloy propyl trimethoxy silane (MAPS), copoly(DMANAS-MAPS). This format proved to be optimal for the detection of low abundant sequences in complex matrices displaying a detection limit of about 0.1% [3].

Finally, ddPCR is one of the newly developed methods based on the original digital PCR technique described by Vogelstein and Kinzler in 1999 [8], that allows for enumeration of rare mutant variants in complex mixtures of DNA (wild-type and mutant) based on emulsion droplet technology. Mutation-specific amplification of the template subsequently occurs in each individual droplet, and counting the positive droplets gives precise and absolute target quantification.

In this study, we applied these methodologies to principally investigate the *KRAS* gene due to its clinical relevance. The absence of a *KRAS* gene mutations in the primary tumor is a prerequisite for treatment of metastatic colorectal cancer (CRC) patients with *EGFR*-targeted therapies. Gain-of-function missense mutations in the *KRAS* gene are often somatically acquired prevalently at the hot spots represented by codons 12–13, and with minor frequency, in codons 61 and 146 [9].

Our aim was to compare COLD-PCR, microarray and ddPCR methodologies performing a retrospective pilot study on plasma ctDNA in a cohort of patients with metastatic CRC and a previously performed molecular genotyping on tissue biopsy.

The outcome of the results will be discussed to show the advantages and the drawbacks of the three technologies.

2. Material and methods

2.1. Sample collection and processing

We collected 10 mL of peripheral blood in EDTA vacutainer tubes from 30 metastatic CRC patients followed at the Department of Oncology at San Raffaele Hospital at different times after primary or metastatic tissue biopsy. We considered synchronous 11 samples in which the distance between tissue biopsy and blood collection was less or equal to 6 months and asynchronous 14 samples for which the time between the two biopsies was longer than 6 months. For the remaining 5 samples the precise date of the tissue biopsy was unknown (Table 1). The study was approved by the Institutional Review Board of the San Raffaele Hospital in Milan. Patients were enrolled selectively by clinicians to be included in the study samples which resulted to be mutated or wild-type for codons 12–13 in exon 2, codon 61 in exon 3 and codon 146 in exon 4 of the *KRAS* gene by molecular analysis on tissue biopsy (Table 1). The biopsy on tumor tissue was performed by the Departmentof of Pathology at the San Raffaele Hospital (with the exception of 3 patients) by MassARRAY (Sequenom, San Diego, CA, USA), that is reported to have a sensitivity of 5% in the detection of KRAS mutations.

Plasma samples were separated after double centrifugation within two hours from collection, one at 1600*g* for 10 min and the second at 16000 g for 10 min. Plasma samples were frozen at −20 °C. Circulating free DNA was extracted from 500 μL of plasma using QIAamp DSP Virus kit (Qiagen, Gmbh, Hilden, Germany) and eluted with 55 μl of Elution Buffer. For each of the methodologies applied we have performed a dedicated DNA extraction session, thus, for every sample, we have extracted 3 aliquots of plasma.

2.2. COLD-PCR and Sanger sequencing

For the analysis of the mutational hot-spots in KRAS codon 12–13, for which more than 90% of sequence variations are known to be Tm-reducing substitutions [10], we developed the *fast* COLD-PCR protocol. However, due to its theoretical enhancement detection of any type of nucleotide variations the *full* COLD-PCR protocol was developed for all the remaining mutations (ie. codon 61 and 146 of the KRAS gene).

We analyzed 27 samples referred to be mutated or wild-type for KRAS codons 12–13 in tissue biopsy by *fast* COLD-PCR, while the last 3 samples reported as mutated in codons 61 or 146 were analyzed by *full* COLD-PCR protocol. For all the three amplicons generated, the correct critical temperature (Tc) was determined experimentally as previously described [11].

Cycling conditions for *fast* and *full* COLD-PCR, primer sequences, amplification length, annealing temperature (Ta) and Tc gradient for the *KRAS* exon 2,3 and 4 amplification are detailed in Supplemental Data and in Table 1 Supplemental Data.

2.3. Microarray

2.3.1. PCR conditions

Amino-modified amplicons were obtained with the primer set as previously reported [3]. PCR reagents and cycling conditions were reported in Supplemental Data.

2.3.2. Microarray preparation and analysis

Untreated silicon slides 1000A Thermal Oxide (14 × 14 mm^2) were supplied by Silicon Valley Microelectronics Inc. (Santa Clara, CA, USA). Slides were functionalized by physical adsorption of copoly(DMA-NAS-MAPS) (Lucidant Polymers Inc. Sunnyvale CA) which provides a polymeric active surface on which amino-modified PCR amplicons were covalently immobilized.

Four hundred pL of amino-modified amplicons were printed in 6 replicates using a piezoelectric spotter, SciFLEXARRAYER S5 (Scienion, Johannisthal, Germany), on coated silicon slides. Spotting was carried out at +20 °C and 50% humidity.

Amino-modified PCR amplicons were then hybridized with fluorescently labeled oligonucleotide probes specific for mutant and wild-type sequences. Samples in microarrays were characterized according to a "target/amplicon down" detection protocol. Presence or absence of codon 12–13 *KRAS* mutations (G12A, G12C, G12D, G12R, G12S, G12 V and G13D) were detected by fluorescence. The sensitivity observed for each mutation in study has already been published [3] and reposted in Table 2.

Silicon chip coating, microarray preparation, reporter and stabilizer sequences, hybridization conditions, thermal stringency, fluorescence detection, image-scanning and data-analysis have already been previously reported [3].

2.4. Droplet digital PCR (ddPCR)

We employed the QX100™ Droplet Digital™ PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Eight μL of eluted DNA plasma sample was mixed with primers and fluorophore labeled commercial probes (FAM for the mutated allele and HEX for the wild-type allele) specific for each of the mutations analyzed (assays ID were reported in Supplemental Table 2). Commercial PrimePCR™ ddPCR™ Mutation Assays were available for all the tested mutations and respective wild-type counterparts, except for the c.180_c.181 TC > AA (p.Q61K) mutation, a complex - compound substitution, for which a custom assay was specifically designed. A technical validation for sensitivity, reported to be 0.1%, and specificity was performed by the manufacturer using appropriate mutant cell line. For the samples reported as wild-type for *KRAS* in tissue biopsy and COLD-PCR analysis, we performed the ddPCR™ *KRAS* Screening multiplex kit (ID 1863506) able to screen the samples for all the seven *KRAS* mutations in study in a single well. The sensitivity of the ddPCR™ *KRAS* Screening multiplex kit in detecting mutated allele was reported to be 0.2% by the manufacturer. ddPCR reagents and cycling conditions were reported in Supplemental Data.

3. Results

Considering the medical report of the tissue biopsy as a reference, we performed the mutational analysis in matched ctDNA plasma

Table 1
Patients enrolled and corresponding mutational analysis on tissue biopsy and liquid biopsy by COLD-PCR, microarray and ddPCR approaches.

Sample ID	Tissue biopsy	Liquid biposy			Therapy status
		COLD-PCR	Microarray	ddPCR (mutated allele%)	
1[a]	G12D	G12D	G12D (3/3)	G12D = 18.7% (16.5%–20.8%)	nd
2[b]	wt	G12 V + G12C (G12F)	wt (3/3)	G12 V = 1.6% (0.9%–2.4%) G12C = 0% wt	Rechallenge in 4th line with oxaliplatin and 5FU (FOLFOX) (5FU = 5-Fluorouracil)
3[b]	G12S	G12S	G12S (3/3)	G12S = 3.4% (1.7%–5%)	Rechallenge in 4th line with 5FU per os + Irinotecan (XELIRI) (5FU per os = capecitabine)
4[a]	wt	wt	wt (3/3)	wt	nd
5[b]	G12A	wt [d]	G12A (3/3)	G12A = 3.30% (0.8%–5.8%)	2nd line therapy with FOLFIRI
6 [a]	G13D	G13D	G13D (3/3)	G13D = 17.20% (15.4%–19%)	nd
7	wt	G13D	G13D (3/3)	wt	Rechallenge in 4th line with 5FU per os + oxaliplatin (XELOX)
8[a]	G12D	G12D	G12D (3/3)	G12D = 3.52% (1.2%–5.1%)	nd
9[b]	G12C	G12C	G12C (1/3)	G12C = 0.8% (0%–1.9%)	Maintenance therapy with capecitabine + Bevacizumab
10[a]	G12 V	G12 V	wt (3/3)	G12 V = 0.16% (0%–0.32%)	nd
11[b]	wt	wt	wt (3/3)	wt	3rd line therapy with Panitumumab
12[b]	wt	G12D	wt (3/3)	G12D = 0.9% (0.3%–1.6%)	2nd line therapy with Panitumumab
13[b]	G12D	G12D	G12D (3/3)	G12D = 1.20% (0.9%–1.5%)	2nd line therapy with FOLFIRI + Aflibercept
14[b]	G12 V	G12 V	G13D (3/3)	G12 V = 0.18% (0%–0.46%)	2nd line therapy with FOLFOX
15[b]	G12D	wt	wt (3/3)	wt	1st line therapy with XELIRI + Bevacizumab
16[b]	wt	wt	wt (3/3)	wt	Maintenance therapy with capecitabine + Bevacizumab
17[b]	G13D	wt [d]	G12R (3/3)	G12R = 1.9% (0.5%–3.3%)	2nd line therapy with XELIRI + Bevacizumab
18	G13D	n.a.	wt (3/3)	G13D = 0.5% (0%–1.6%)	1st line therapy with XELOX
19	wt	G13D	wt (3/3)	G13D = 0.15% (0%–0.51%)	1st line therapy with Folfox + Bevacizumab
20[b]	G13D	G13D	G13D (3/3)	G13D = 0.9% (0.1%–1.7%)	2nd line therapy with FOLFIRI
21	G12D	G12D	G12D (3/3)	G12D = 5.8% (3.5%–7.54%)	1st line therapy with XELOX + Bevacizumab
22	G12 V	wt	wt (3/3)	wt	1st line therapy with XELOX + Bevacizumab
23	G13D	wt	wt (3/3)	wt	1st line therapy with Capecitabine + Bevacizumab
24	Q61K	un	[c]wt (3/3)	Q61K = 2.4% (0.5%–5.35%)	1st line therapy with FOLFOX + Bevacizumab followed by Radiation therapy (RT) to treat minimal persistence of disease to the chest wall (blood sample collected during RT)
25	G13D	wt	wt (3/3)	wt	1st line therapy with XELIRI + Bevacizumab
26[b]	G13D	wt	wt (3/3)	wt	Maintenance therapy with Bevacizumab
27[b]	A146 T	wt	[c]wt (3/3)	wt	Concomitant radiotherapy and chemotherapy with Capecitabine
28	A146T	wt	[c]wt (3/3)	wt	1st line therapy with FOLFOX + Bevacizumab
29	wt	wt	wt (3/3)	wt	1st line therapy with FOLFOX + Bevacizumab
30	wt	wt	wt (3/3)	wt	Maintenance therapy with Capecitabine + Bevacizumab

n.d = date not available, CT = chemotherapy; RT = radiotherapy.
[a] Samples from which the date of the tissue biopsy were unknown.
[b] Asynchronous samples, wt = wild-type samples for KRAS gene.
[c] Samples identified as wild-type by microarray analysis since no specific reporter was designed for these specific mutations. [d] Samples classified as wild-type by *fast* COLD-PCR, indeed the G12A (c.35G > C) and G12R (c.34G > C) mutations could not be detectable by *fast* COLD-PCR protocol. n.a. = no amplified sample; un = uninterpretable result.

Table 2
Limit of detection of the COLD-PCR, Microarray and ddPCR methodologies.

KRAS mutations	*Fast* COLD-PCR (%)	Microarray (%)	ddPCR (%)
G12A (c.35G > C)	na	0.4	0.1
G12C (c.34G > T)	0.4	0.05	0.07
G12D (c.35G > A)	0.2–0.4	0.1	0.1
G12R (c.34G > C)	na	0.025	0.06
G12S (c.34G > A)	0.2–0.1	0.4	0.15
G12 V (c.35G > T)	0.1	0.8	0.1
G13D (c.38G > A)	0.1	0.01	0.1
KRAS mutations	*Full* COLD-PCR		
p.Q61H	1.25–2.5	–	–
p. A146T	1.25	–	–

na = protocol not applicable for identifying this mutation.

samples by COLD-PCR, microarray and ddPCR.

3.1. Cold-PCR

The sensitivity of the assay was evaluated on serial dilutions of mutated DNA opportunely mixed with wild-type DNA. In particular, DNA from a heterozygous reference standard (50% mutated allele) by Diatech Pharmacogenetics was mixed with wild-type DNA up to 0.1%. The detection limits of each mutation analyzed are reported in Table 2. In Fig. 1 the electropherograms of the dilution curves of the G12 V mutation is shown.

The results obtained by analyzing 27 out of 30 plasma samples by *fast* COLD-PCR protocol to cover the hot spot mutations in codon 12–13 of the KRAS gene are shown in Table 1.

Some of the results obtained are reported in Fig. 2. For sample n.2, we found a double mutated peak (Fig. 2B). For sample n.18 that was classified as a “hemolytic sample”, no amplification was obtained using the *fast* COLD-PCR protocol.

Samples n.24 and n.27 – n.28 were analyzed by *full* COLD-PCR protocol for the p.Q61K and p.A146T mutations, respectively. For sample n. 24 we obtained an uninterpretable result due to the background noise of the sequencing, while for samples n.27 and 28 no mutated peaks were visible. For both the mutations, the limits of detection were reported in Table 2.

3.2. Microarray

The results obtained by analyzing all of the 30 samples by microarray are reported in Table 1. The samples in which at least one replica out of three showed the mutant allele were considered as “mutated”. As an example, microarray results for the G12A and G12D *KRAS* mutations

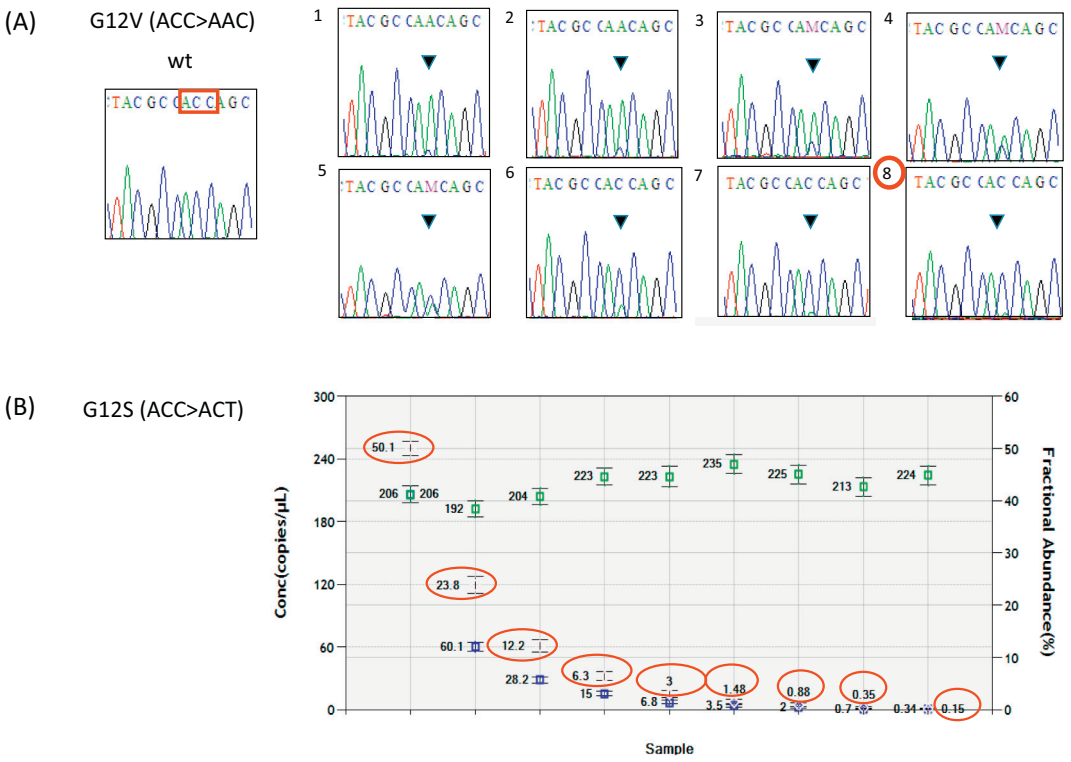

Fig. 1. Sensitivity of the COLD–PCR and ddPCR assays. (A) *Fast* COLD-PCR sequence profiles of G12 V mutated DNA serially diluted with wild-type DNA (1 = 12.5%, 2 = 6.25%, 3 = 3.12%, 4 = 1.56%, 5 = 0.78%, 6 = 0.39%, 7 = 0.2%, 8 = 0.1% of mutated DNA). The antisense sequence is shown. (B) Sensitivity of the ddPCR G12S assay in discriminating different proportions of mutated alleles on serial dilutions starting from 50% up to 0.1% of mutated allele. The respective percentages of fractional abundance obtained for each point are circled in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

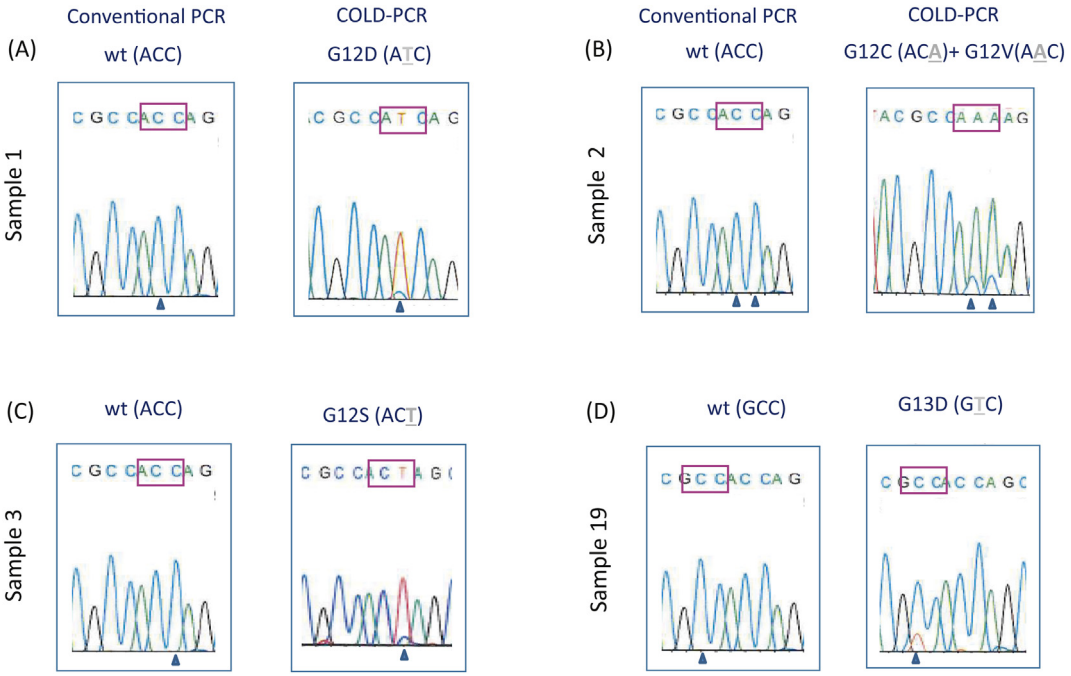

Fig. 2. Non-invasive identification of *KRAS* mutations in plasma of four mCRC cancer patients: sequence profiles of *fast* COLD-PCR products compared with conventional PCR. By conventional PCR, no mutated sequence is visible. For all the four mutations, the antisense sequence is shown.

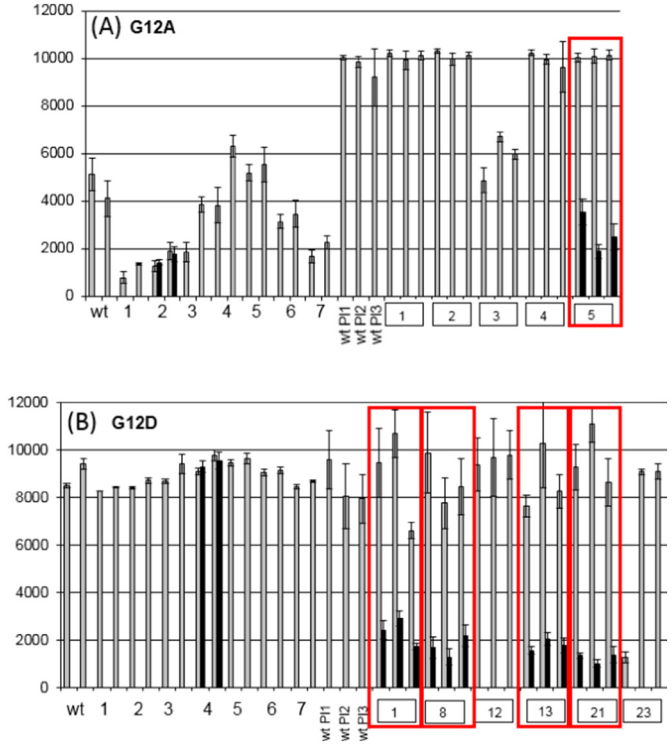

Fig. 3. Microarray analysis of 2 different KRAS mutations (G12A, panel A and G12D, panel B) in the plasma of colon cancer patients analyzed in triplicate compared with a wild-type sample. The grey and black histograms represent the wild-type and the mutant form of the mutation, respectively. Left: control genomic DNA wild-type (wt) and heterozygous samples (1 = G12C, 2 = G12A, 3 = G12S, 4 = G12D, 5 = G12R, 6 = G12 V and 7 = G13D), each control is analyzed in duplicate. Right: wild-type plasma samples (wt pl) and tumor patients plasma samples. Normalized fluorescence signals for mutant (mut) and wild-type (wt) alleles are shown.

are shown in Fig. 3.

3.3. ddPCR

3.3.1. ddPCR analysis of the KRAS gene

We tested the sensitivity of the codon 12–13 *KRAS* mutations assays by serial dilutions of mutated DNA opportunely mixed with wild-type DNA as reported for COLD-PCR. The results obtained were summarized in Table 2 and showed in Fig. 1 (panel B).

Twenty-one out of the 30 samples were analyzed using the specific probes for the mutations referring to the tissue biopsies. The results obtained are reported in Table 1. Fig. 4 displays some examples of the results obtained. For the remaining 9 samples reported as wild-type in tissue specimens we proceeded as follows. For 5 out of the 9 wild–type samples reported as wild-type also by COLD-PCR and microarray approaches (n.4, 11, 16, 29, 30) we performed a ddPCR analysis using the ddPCR™ *KRAS* Screening multiplex kit. All samples were found to be wild-type also by ddPCR analysis.

For the remaining 4 samples (n.2, 7, 12, 19) we performed a ddPCR analysis directly for the mutations previously detected by COLD-PCR and/or microarray.

3.4. Concordance rate between tissue and plasma molecular analysis results and intra-liquid biopsy protocol

A comparison of the results obtained is shown in Table 3.

The overall concordance between COLD-PCR, microarray and ddPCR is 75% (18 out of 24 samples), while the best concordance rate (92.6%) was observed by comparing ddPCR and COLD-PCR.

We excluded from the analysis samples n.5, n.17, n.18, n.24, n.27 and 28 from which at least one method was not performed. In the comparison with tissue biopsy we considered 27 out of 30 samples analyzed by COLD-PCR. We excluded samples n.5 and n.17 because the nucleotide involved in the mutation (G > C) could not be enriched by fast COLD-PCR protocol, and sample n.18 because no amplification was obtained by this hemolytic sample. Moreover, we considered 27 out of the 30 samples analyzed by microarray. We excluded samples n.24, n.27 and n.28 since no specific reporter was designed for the p.Q61K and p.A146T mutations.

4. Discussion

The aim of our work was to compare the performance of three non-invasive methodologies in the accurate identification of low abundance mutated alleles undetectable by conventional approaches in liquid biopsies.

For this purpose we compared different techniques based on either enrichment protocols (COLD-PCR), or a hybridization protocol (microarray) or digital amplification (ddPCR) all reported to detect around 0.1% of the mutated allele in a background of wild-type DNA which could be easily applied in a clinical setting. Using metastatic CRC patients as a model system, the study started by identifying the tumor-specific somatic mutations previously identified in tissue samples (as usually occurs in cancer management) in plasma specimens. Current National Comprehensive Cancer Network (NCCN) guidelines specifically highlight *RAS* mutational status and *BRAF* mutational status (in patients with *RAS* wt) as the key biomarkers to be assayed at the time of diagnosis of stage IV disease [12].

In the cohort of our study 4 patients (n.2, n.7, n.12 and n.19) who had been classified as wild-type for *KRAS* mutations in tissue biopsies were found to be mutated in plasma from 1 month up to 36 months later by at least two different non-invasive approaches. Among the samples collected from these patients, 2 samples could be considered synchronous with the tumor biopsy (the plasma samples were taken 1 or 6 months later) thus, the different mutational status detected in the *KRAS* gene might be ascribed to the different sensitivity of the methodologies used (5% by tissue biopsy instead of less than 1% by liquid biopsy techniques) For the two remaining patients sampled 31 or 36 months later than tissue biopsy, respectively, the emergence of *RAS* pathway mutations might be due to the acquired resistance to *EGFR*-specific antibodies suggesting that the *KRAS* gene mutation was not present in the primary tumor but had presumably arisen in a small population of cells within a metastatic lesion and expanded under the influence of the *EGFR* blockade [13–16]. We can also consider that ctDNA studies may identify somatic mutations derived from intrinsic factors (tumor clonal heterogeneity or progression) almost undetectable in cancer biopsies, as we supposed for patient number 17 from whom we found a *KRAS* mutation (G12R) in liquid biopsy different from the one found in tissue biopsy (G13D). On the contrary, we detected a wild-type genotype in liquid biopsy for 6 patients (n.22, n.23, n.25, n.26, n.27 and n.28), referred to be mutated in tissue biopsy. In these patients ctDNA concentration correlates with disease staging and progression. Indeed, at the moment of blood drawing the clinical history in 5 out of the 6 patients was reported as “free from disease” or “disease in regression”.

In a previous study, a concordance rate between solid and liquid biopsy mutation detection for testing *KRAS* codon 12 mutations by ddPCR–based technologies was reported at about 82% in patients with metastatic CRC or even higher [17–19]. In our study, the 3 methods for liquid biopsy mutational analysis showed a lower concordance rate with respect to those obtained on tissue biopsies: 63.3% by ddPCR, 63% by microarray and 55.6% by COLD-PCR. This could be ascribed to the different timing between tissue and liquid biopsy collection for most patients of our cohort that could reflect a different mutational profile at different stages of disease. Moreover, we used different methodologies

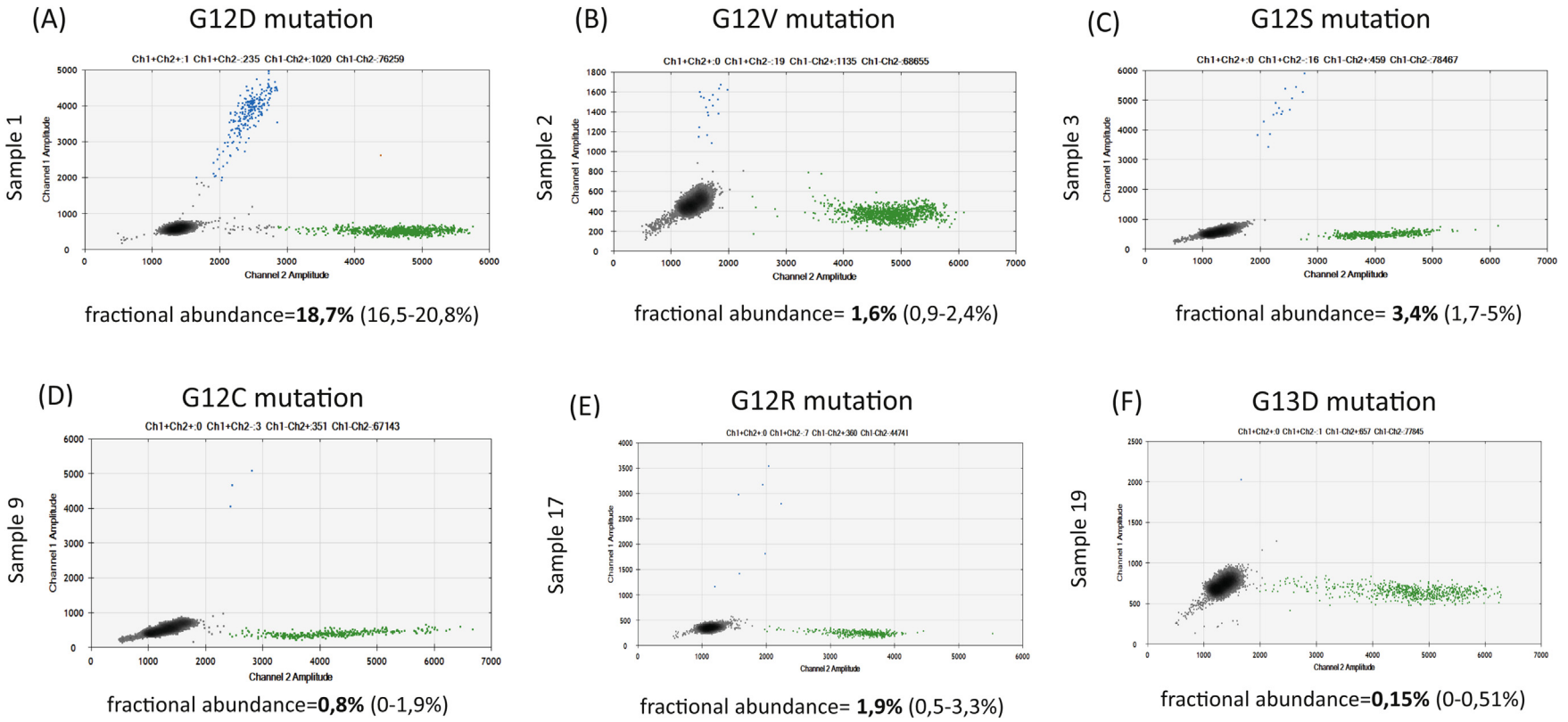

Fig. 4. Examples of ddPCR plot for six tumor patients and respective fractional abundance of the mutated KRAS allele: G12D mutation (panel A), G12 V (panel B), G12S (panel C), G12C (panel D), G12R (panel E) and G13D (panel F). Mutants are clustered in the upper left corner with high FAM fluorescent intensities (blue), wild-type is clustered in lower right corner with high HEX fluorescent intensities (green) while mutant plus wild-type sequences are clustered in the upper right corner (orange). The empty droplets are represented in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Concordance rate between tissue and plasma molecular analysis results and intra-liquid biopsy protocol.

	COLD-PCR	Microarray	ddPCR
Tissue biopsy	15/27 (55.6%) s = 3/10 (30%) as = 7/12 (58.3%) n.d = 5/5 (100%)	17/27 (63%) s = 4/9 (44.4%) as = 9/13 (69.2%) n.d = 4/5 (80%)	19/30 (63.3%) s = 6/11 (54.5%) as = 8/14 (57.1%) n.d = 5/5 (100%)
COLD-PCR	–	19/24 (79.2%)	25/27 (92.6%)
Microarray	19/24 (79.2%)	–	20/27 (74%)
ddPCR	25/27 (92.6%)	20/27 (74%)	–

s = synchronous samples; as = asynchronous samples; n.d = not determined, samples for which the date of the tissue biopsy was unknown.

to perform tissue biopsy and liquid biopsy with diverse sensitivity. Notably, if we compare in our study the results obtained on the same plasma sample by the three liquid biopsy methodologies the concordance of the results obtained was higher especially if we matched COLD-PCR versus ddPCR (92.6%).

Our results confirm that the three methodologies performed on liquid biopsies have a complementary role on the basis of their limits and powers, especially COLD-PCR and ddPCR which seems to be the most useful approach to be transferred to a diagnostic setting [20–22].

In particular, concerning COLD-PCR, advantages include its relative simplicity, the preferential amplification of mutant-containing DNA without the need for cumbersome protocols or additional reagents, and the possibility to subsequently sequence the amplified product to identify the mutation by Sanger sequencing or even before NGS-based amplicon resequencing [23]. Moreover, the strength of this method is its ability to detect all types of mutations (single-nucleotide substitutions, insertions, and deletions) in the amplified locus and no a priori knowledge of the mutations present is required, thus rendering COLD-PCR a useful discovery tool. Moreover, for COLD-PCR an initial pre-amplification was performed using regular PCR cycling, followed by a characteristic COLD-PCR step. It would seem reasonable to perform a multiplexed pre-amplification step to include all three amplicons, followed by separate COLD-PCR in the second reaction, as this would consume less cfDNA.

Interestingly, in our cohort of patients, COLD-PCR was the only method able to identify a double nucleotide variation. This molecular alteration could be considered as a double mutation in *trans* composed by G12V plus G12C mutations. The development of different mutations in the same patients is not surprising given that each of these patients had multiple metastatic lesions [17]. However, we cannot exclude the possibility of a single double mutation in *cis,* reported in COSMIC as G12F mutation since sequencing does not allow for assigning a specific genotype to each allele. However, setting up COLD-PCR reactions may be a little cumbersome since it requires the optimization of the protocol based on the identification of the correct Tc for each amplicon and a dedicated thermal cycler with a very strict thermal control. Moreover, the enrichment step does not allow for an exact quantification of the mutational level.

In contrast, in ddPCR the sensitivity is only limited by the number of molecules that can be analyzed and the false-positive rate of the mutation detection assay. In our opinion, based on this assumption, ddPCR is the methodology that better suits the comprehensive characteristics of liquid biopsy since this methodology could provide real time information not only in the assessment of the molecular tumor genotype (qualitative analysis) but also on the existing tumor burden (quantitative analysis). Moreover, it was demonstrated that ddPCR can offer reproducible quantitative data in different laboratories [24].

Additionally, plasma separation and DNA extraction are unavoidable steps preceding any analytical methodology; thus, in an attempt to keep as short as possible the “time to result” variable, considered as the time taken to get the result once the technology for mutation testing is applied, ddPCR seems to be the faster protocol to be coupled with the previous steps. At present, microarrays may be considered as more time consuming compared with both the other two methods since several “runs” are necessary to cover the multiple genetic variations of *KRAS*. Moreover, this methodology showed a lower sensitivity especially for the detection of the G12V mutation (0.8%) and analyzing some plasma samples in our cohort in this study, we did not identify the mutation even in samples in which the fractional abundance assessed by ddPCR was higher than the limit of detection previously reported [3]. This could probably be due to the presence of PCR inhibitors derived from the DNA extraction step, and at a lower concentration of the plasma DNA, in comparison to the optimized conditions obtained analyzing cell lines or plasmids carrying the mutations [3]. Moreover, it is important to underline that microarray reveals a high concordance with tissue biopsy probably due to its low sensitivity. Indeed, it doesn't identify in 3 plasma samples referred as wild-type in tissue biopsy (n.2, 12, 19) the presence of the mutation while the ddPCR and COLD-PCR did this. We demonstrated that by applying a dedicated analytical methodology ctDNA it could be a reliable non-invasive biomarker for several mutations in mCRC patients allowing to monitor the disease load and to provide clinically relevant information compared to archive biopsies. Finally, in our opinion, between low cost and time-saving methodologies, the ddPCR is the most useful procedure allowing also a quantitative assessment of the fractional abundance of the mutation.

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Appendix A. Supplementary data

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