

# World Journal of *Gastroenterology*

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## Basic Study

# Detection of *KRAS* G12D in colorectal cancer stool by droplet digital PCR

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## Abstract

### AIM

To assess *KRAS* G12D mutation detection by droplet digital PCR (ddPCR) in stool-derived DNA from colorectal cancer (CRC) patients.

### METHODS

In this study, tumor tissue and stool samples were collected from 70 patients with stage I-IV CRC diagnosed by preoperative biopsy. *KRAS* mutational status was determined by pyrosequencing analysis of DNA obtained from formalin-fixed paraffin-embedded (FFPE) tumor tissues. The *KRAS* G12D mutation was then analyzed by ddPCR in FFPE tumors and stool-derived DNA from patients with this point mutation. Wild-type (WT) tumors, as determined by pyrosequencing, were included as controls; analysis of FFPE tissue and stool-derived DNA by ddPCR was performed for these patients as well.

### RESULTS

Among the total 70 patients included, *KRAS* mutations were detected by pyrosequencing in 32 (45.71%), whereas 38 (54.29%) had WT tumors. The frequency of *KRAS* mutations was higher in left-sided tumors (11 located in the right colon, 15 in the left, and 6 in the rectum). The predominant point mutation was *KRAS* G12D (14.29%,  $n = 10$ ), which was more frequent in early-stage tumors (I-IIA,  $n = 7$ ). In agreement with pyrosequencing results, the *KRAS* G12D mutation was detected by ddPCR in FFPE tumor-derived DNA, and only a residual number of mutated copies was found in WT controls. The *KRAS* G12D mutation was also detected in stool-derived DNA in 80% of all fecal samples from CRC patients with this point mutation.

### CONCLUSION

ddPCR is a reliable and sensitive method to analyze *KRAS* G12D mutation in stool-derived DNA from CRC patients, especially at early stages. This non-invasive approach is potentially applicable to other relevant biomarkers for CRC management.

**Key words:** Droplet digital PCR; *KRAS*; Stool; Formalin-fixed paraffin-embedded; Pyrosequencing; Colorectal cancer

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**Core tip:** The potential of droplet digital PCR (ddPCR) to detect *KRAS* G12D mutation in stool DNA from colorectal cancer (CRC) patients was examined as a proof-of-concept for the applicability of this technology to study DNA biomarkers in stool-derived DNA. It was

shown that *KRAS* G12D detection in stool-derived DNA from CRC patients by ddPCR is feasible and provides comparable results to the analysis of formalin-fixed paraffin-embedded tissue by pyrosequencing. These results suggest that analysis of *KRAS* mutations and other molecular biomarkers in stool by ddPCR could represent a complementary non-invasive approach to standard screening tests for CRC.

Olmedillas-López S, Lévano-Linares DC, Aúz Alexandre CL, Vega-Clemente L, León Sánchez E, Villagrasa A, Ruiz-Tovar J, García-Arranz M, García-Olmo D. Detection of *KRAS* G12D in colorectal cancer stool by droplet digital PCR. *World J Gastroenterol* 2017; 23(39): 7087-7097 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i39/7087.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i39.7087>

## INTRODUCTION

Colorectal cancer (CRC) is the second and third most common cancer in women and men, respectively, with more than one million cases diagnosed each year worldwide<sup>[1]</sup>. Current therapeutic options have increased overall survival (OS), but have also made clinical decisions more complex, especially in patients with an initial diagnosis of metastatic colorectal cancer (mCRC)<sup>[2]</sup>. Biological agents targeting the epidermal growth factor receptor (EGFR), such as cetuximab and panitumumab, used either as monotherapy or in combination with standard chemotherapy, are indicated in mCRC patients with *RAS* (*KRAS* and *NRAS*) or *KRAS* wild-type (WT) tumors, respectively<sup>[3,4]</sup>. These strategies significantly improve progression-free survival (PFS) and OS in *KRAS* WT mCRC patients depending on the therapeutic regimen applied (chemotherapy and line of treatment)<sup>[2,5]</sup>.

*KRAS* oncogene mutations, mostly found in codons 12 and 13<sup>[6]</sup>, have been described in approximately 30%-40% of CRC tumors<sup>[7-9]</sup>. These mutations are associated with absence of response to therapy with biological agents<sup>[10,11]</sup> and have been correlated with worse prognosis<sup>[12,13]</sup>. In fact, in Europe and the United States, monoclonal antibody-based therapy has been restricted to patients with WT tumors<sup>[14]</sup>, as when administered in association with standard chemotherapy, this treatment may result in an increased cost and toxicity<sup>[10]</sup>. Drug resistance can occur months after start of combined therapy, likely due to intratumoral heterogeneity and proliferation of small sub-groups of clonal cells carrying resistance mutations that are difficult to identify by most of the currently applied methods<sup>[14,15]</sup>. Therefore, highly sensitive and specific methodologies are needed to detect and quantify molecular markers, including *KRAS* mutations, which play a pivotal role in early detection and clinical management of CRC patients.

Droplet digital PCR (ddPCR) is increasingly seen as one of the most powerful techniques to accurately detect a wide variety of genetic alterations in many cancer types. These molecular biomarkers have been analyzed by ddPCR in different body fluids such as blood, urine, cerebrospinal fluid, pleural effusions, ascites and sputum (reviewed in<sup>[16]</sup>).

Stool-derived DNA is a potential non-invasive alternative source of DNA for tumor genotyping in CRC due to the high rate of exfoliation of tumor cells into the bowel lumen<sup>[17]</sup>. Digital PCR was first described in 1999 by Vogelstein and Kinzler in a study aimed at identifying *KRAS* in DNA obtained from fecal samples of CRC patients<sup>[18]</sup>. Based on the isolation of single molecules by limiting dilution of DNA samples and individual amplification by PCR, mutations were detected using fluorescent probes. However, this methodology was found to be quite laborious and difficult to translate into clinical practice<sup>[19]</sup>. The introduction of new instrumentation involving nanofluidic devices and improved emulsion chemistries has allowed for more widespread use of digital PCR, giving way to the current commercially available platforms<sup>[19]</sup>; of these, emulsion-based ddPCR has undergone huge growth in cancer research. In fact, a recent study has investigated the application of ddPCR to quantify mRNA biomarkers in stool from patients with CRC as a potential non-invasive screening test<sup>[17]</sup>. Thus, analysis of DNA obtained from fecal samples in patients with CRC may complement currently used procedures for diagnosis and disease follow-up.

In our experience, ddPCR has shown high sensitivity for detection of mutated *KRAS* alleles in circulating cell-free DNA (cfDNA) in plasma from CRC patients<sup>[20]</sup>. However, early-stage patients sometimes have undetectable levels of circulating tumor DNA (ctDNA)<sup>[20,21]</sup>. The aim of this study was to evaluate the feasibility of *KRAS* G12D mutation detection in stool-derived DNA from CRC patients by ddPCR, including early-stage patients.

## MATERIALS AND METHODS

### Patients

Seventy CRC patients were consecutively included in this study from 2014 to 2015 in the Department of General Surgery at Fundación Jiménez Díaz University Hospital (Madrid, Spain). Inclusion criteria were endoscopic histological diagnosis of CRC and eligibility for primary tumor resection with curative intent. Patients with primary tumors located in the rectum who had received prior neoadjuvant treatment were excluded. All subjects signed an informed consent in accordance with a protocol approved by the Ethics Committee for Clinical Research of this institution (PIC 63/2016\_FJD).

### Fecal sample collection

All fecal samples were collected during hospitalization before surgery without any bowel preparation (with the exception of patient 17, who was subjected to cathartic preparation due to an oversight). Stool samples were collected in sterile containers and stored at -20 °C until analysis.

### DNA extraction

A total amount of 200-500 mg of fecal sample was used for DNA extraction. DNA was isolated using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA from the LS-174T cell line (kindly provided by the Translational Oncology Division, OncoHealth Institute, IIS-FJD, which had previously purchased this cell line from the American Type Culture Collection, ATCC in Manassas, VA, United States) and DNA from peripheral blood mononuclear cells of a healthy donor were extracted with the QIAamp DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA from formalin-fixed paraffin-embedded (FFPE) tumors was extracted using the Cobas DNA Sample Preparation Kit (Roche Molecular Systems, Inc., Branchburg, NJ, United States) following the manufacturer's instructions. The quantity and purity of the DNA obtained was estimated by NanoDrop (ND-2000 UV-Vis Spectrophotometer; Nanodrop Technologies Inc., Waltham, MA, United States).

### Mutation detection in FFPE tumor samples by pyrosequencing

Quantitative analysis of *KRAS* mutations was performed by pyrosequencing in accordance with routine practice at the Department of Pathology at Fundación Jiménez Díaz University Hospital, using the CE-IVD marked Therascreen *KRAS* Pyro kit (Qiagen), according to the manufacturer's protocols. Each PCR product was analyzed by pyrosequencing using the Therascreen *KRAS* Pyro reagents (Qiagen), Streptavidin Sepharose High Performance (GE Healthcare Bio-Science AB, Uppsala, Sweden), and a PyroMark Q24 instrument (Qiagen).

### Mutation detection in FFPE tumor tissues and stool samples by ddPCR

ddPCR assays were performed using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, United States) with the Prime-PCR™ ddPCR™ Mutation Detection Assay Kit (Bio-Rad); amplicon size was 57 bp. DNA from LS-174T, a human colon adenocarcinoma *KRAS* G12D heterozygous cell line, was used as a positive control. *KRAS* WT control DNA was obtained from peripheral blood mononuclear cells of a healthy donor. Background was measured by adding water to the reaction mixture instead of

DNA. The PCR reaction mixture (20  $\mu$ L) contained 10  $\mu$ L of ddPCR Supermix (no dUTP) for probes, 1  $\mu$ L of each primer/probe mix (target and reference, labeled with HEX and FAM fluorophores, respectively), and 2–8  $\mu$ L of stool-extracted DNA. Different amounts of stool-derived DNA were assayed per sample, as the proportion of human DNA with respect to bacterial DNA was unknown and could vary among patients. A total amount of 100 ng of cell-derived control DNA was added per well. In case of DNA from FFPE tumors, 50 ng per well was used. Thermal cycling consisted of 10 min at 95  $^{\circ}$ C, 40 cycles of 94  $^{\circ}$ C for 30 s, and 55  $^{\circ}$ C for 60 s. Results were analyzed using QuantaSoft v.1.7 software (Bio-Rad) and reported as number of copies per 20  $\mu$ L reaction as well as copies per ng of DNA. Three to four replicates of each stool sample were analyzed. FFPE tumors and controls were assayed in duplicate.

### Statistical analysis

A non-parametric Mann-Whitney test for significance was performed using R software.

## RESULTS

### Patient characteristics

A total of 70 stool and tissue samples from CRC patients were collected and included in the study. All patients were classified according to the distance between the primary tumor and anal margin (cm) reported in the preoperative colonoscopy. Forty-one (58.57%) were male, with a median age of 73 years. Only 5 (7.14%) had a diagnosis of mCRC at baseline.

The patients included in this study were representative of all tumor locations, with 28 (40%) located in the right colon, 34 (48.57%) in the left colon, and 8 (11.43%) in the rectum. Interestingly, 45 (64.29%) patients were diagnosed at an early stage (I–IIA). Clinical and pathological features are summarized in Table 1.

### *KRAS* mutations in FFPE tumor samples analyzed by pyrosequencing

*KRAS* mutations were found in the tumors of 32 patients (45.71%), including 11 in the right colon, 15 in the left, and 6 in the rectum. Most mutations were located at codon 12 ( $n = 17$ , 53.12%) or codon 13 ( $n = 6$ , 18.75%). The most prevalent mutation was G12D ( $n = 10$ , 14.29%). Two were located in the right colon, 6 in the left colon, and 2 in the rectum. This mutation was found more frequently in early-stage tumors (I–IIA,  $n = 7$ ). The incidence of the different types of mutations is shown in Table 2.

### *KRAS* G12D detection in FFPE tumor samples by ddPCR

FFPE tumors from patients found to have a *KRAS* G12D mutation by pyrosequencing were analyzed

using ddPCR. Five CRC patients with WT *KRAS* exon 2 tumors were selected as controls. One control carried a *KRAS* Q61L mutation (exon 3) that did not interfere with our assays. Results obtained from ddPCR analysis of FFPE tumor DNA from these 15 CRC patients were in agreement with pyrosequencing results. A residual number of *KRAS* G12D copies was found in WT tumors. Due to this level of unspecific background signal, mean copies/ng DNA from control patients plus 2 standard deviation (SD) was considered as a threshold for positivity (0.41 copies/ng DNA). All samples from patients with G12D-positive tumors were above this threshold and showed a significantly higher number of mutant copies/ng DNA than patients with WT *KRAS* tumors (median, 106 and 0.19 copies/ng DNA, respectively;  $P = 0.001$ ). However, the difference in number of WT *KRAS* copies/ng DNA between both groups was not statistically significant (210.00 copies/ng vs 208.40 copies/ng DNA, median;  $P = 0.699$ ).

### *KRAS* G12D detection in stool samples by ddPCR

Subsequently, we analyzed the presence of the *KRAS* G12D mutation by ddPCR analysis of fecal samples from the 10 patients with mutated tumors by pyrosequencing. Stool DNA from 5 patients with tumors carrying the WT *KRAS* exon 2 were also included as controls. A limited number of *KRAS* G12D-positive events were detected in stool DNA from control WT *KRAS* exon 2 samples. Consequently, mean control value plus 2 SD was established as the positivity threshold. Thus, in our study, stool samples were required to contain more than 1.9 copies/20  $\mu$ L of reaction to be considered positive for the mutation. According to this threshold, which was equivalent to  $> 3$  positive events per sample, the *KRAS* G12D mutation was detected in 8 of 10 patients. Of these 8 positive samples, 6 were from early-stage tumors. Samples from Patients 46 and 64 were considered negative because they had values less than or equal to the positivity threshold. *KRAS* G12D mutation levels in stool samples are shown in Tables 3 and 4. The median number of copies of *KRAS* G12D/20  $\mu$ L of reaction as well as copies/ng of stool DNA in control patients differed significantly from those with mutated tumors ( $P = 0.017$ ). The *KRAS* WT sequence was also detected in stool samples of all CRC patients, though there were no significant differences in the median number of *KRAS* WT copies/ng of stool DNA between both groups ( $P = 0.129$ ).

In summary, the results of pyrosequencing were in 100% agreement with ddPCR analysis in FFPE tissues, whereas ddPCR detected the *KRAS* mutation in 8 out of 10 stool samples.

## DISCUSSION

Despite the advances made in CRC research, the



Table 1 Patient characteristics

		Colorectal cancer patients, <i>n</i> = 70		
		Right colon, <i>n</i> = 28	Left colon, <i>n</i> = 34	Rectum, <i>n</i> = 8
Sex	Female	13	13	3
	Male	15	21	5
Age (mean)		76.89	72.26	70.63
pT	T1	3	4	2
	T2	6	9	2
	T3	16	18	3
	T4	3	3	1
pN	N0	20	22	8
	N+	8	12	-
Stage	I	9	10	4
	II a	9	10	3
	II b	1	1	1
	III a	1	3	-
	III b	4	5	-
	III c	2	2	-
	IV	2	3	-
KRAS status	Mutant	11	15	6
	Wild-type	17	19	2

disease remains a major cause of death worldwide. Recently, the analysis of *KRAS* oncogene mutations has taken on a major prognostic role in CRC clinical management<sup>[22]</sup> owing to the fact that the presence of these mutations, which have been described in approximately 30%-40% of cases<sup>[7,9,23]</sup>, could determine the absence of response to anti-EGFR therapies and worse outcome in cases of metastatic disease<sup>[10-13]</sup>. The most frequent *KRAS* mutations are located at codons 12 and 13; of these, G12D and G13D are particularly relevant, representing around 13%-14% and 6%-7% of all cases, respectively<sup>[7,23]</sup>. Moreover, worse prognosis has been documented among patients with tumors with the G12D mutation<sup>[8,23]</sup>. In agreement with previous observations, the incidence of *KRAS* G12D mutation in our study population was 14.29%.

Molecular biomarkers in blood and stool may be used as a complementary screening strategy and prognostic tool for the prediction of clinical outcome in patients with or at high risk for CRC<sup>[24]</sup>. Not only in CRC, but also in many other human malignancies, the analysis of molecular biomarkers in plasma and other body fluids is attracting increasing interest as a highly valuable, non-invasive predictive tool for monitoring disease progression and response to treatment<sup>[16]</sup>. In CRC, *KRAS* mutations have been analyzed in blood, both in DNA obtained from circulating tumor cells<sup>[25]</sup> and in cfDNA<sup>[26]</sup>. *KRAS*-mutation detection by digital PCR has been described using several commercially available platforms<sup>[14,27-32]</sup>, most of which are focused on detecting mutations in plasma. One of these strategies is ddPCR, which has shown a remarkably

high sensitivity when detecting these minority *KRAS* alleles present at low levels in plasma DNA.

In a previous study, using ddPCR, we detected the *KRAS* G12V mutation in plasma cfDNA from 9 of 10 patients whose tumors were also mutated<sup>[20]</sup>. In this study, we found that metastatic patients had a significantly higher number of mutated copies in circulating cfDNA than M0 patients. The only negative sample was obtained from a T1N0M0 patient. These results are in line with other studies: Bettegowda *et al.*<sup>[21]</sup> also reported that ctDNA in plasma increases with disease stage, and only 47% of early-stage patients with a wide variety of cancers had detectable levels of ctDNA. Similarly, Galanopoulos *et al.*<sup>[26]</sup> recently described that the *KRAS* codon 12 mutation rate in cfDNA is significantly higher in CRC patients compared to healthy subjects, though this methodology seems to have limited potential for predicting the existence of premalignant lesions (neoplastic colonic polyps). Taken together, these findings suggest that at early disease stages, levels of mutated copies in circulating cfDNA may be, in some cases, too low for detection. Thus, alternative non-invasive methods are still needed.

Interestingly, in a very recent study, ddPCR was also used to quantify an mRNA biomarker, *ITGA6*, in stool from patients with CRC<sup>[17]</sup>. Tumor-derived nucleic acids present in stool samples come from the exfoliation of tumor cells of the intestinal mucosa and are a non-invasive, alternative source of genetic material for *KRAS* oncogene mutation screening in CRC patients<sup>[33]</sup>. Exfoliation of colonocytes into the large bowel lumen is a continuous, naturally-occurring phenomenon that seems to be exacerbated in tumors<sup>[34,35]</sup>. Thus, colonocyte shedding from malignant lesions is more frequent than from healthy mucosa<sup>[36,37]</sup>. Hypothetically, DNA from colorectal tumors should be shed into the bowel fecal content before reaching the bloodstream. This would make testing stool DNA for CRC screening more time-sensitive than plasma or other biological fluids<sup>[38]</sup>.

The proof-of-concept study for stool DNA analysis for CRC detection screened 15 point mutations in several genes, including *KRAS*<sup>[39]</sup>. Subsequently, several case-control and prospective studies<sup>[40-42]</sup> led to the 2014 approval by the United States Food and Drug Administration of a fecal DNA analysis system called Cologuard<sup>™</sup> (Exact Sciences Corporation, Madison, WI, United States) for CRC detection. This system includes an immunochemical assay for human hemoglobin and molecular biomarkers associated with CRC, such as methylation markers (*BMP3* and *NFRG4* gene promoter regions), *KRAS* mutations, and  $\beta$ -actin. The test is based on amplification and detection by Quantitative Allele-specific Real-time Target and Signal

**Table 2** *KRAS* mutational status by pyrosequencing

		<i>KRAS</i> wild-type											
		Codon 12				Codon 13				Others			
		G12D	G12V	G12R	G12S	G13D	G13R	A146V	A146T	A59T	Q61R	Q61H	Q61L
Right colon	17	2	0	1	0	3	1	1	1	0	1	1	0
Left colon	19	6	5	0	0	1	0	1	1	1	0	0	0
Rectum	2	2	0	0	1	1	0	0	0	0	0	1	1
Total	38 (54.29%)	10	5	1	1	5	1	2	2	1	1	2	1
		32 (45.71%)											

Amplification (QuARTS™) technology. However, this system still has limited application in clinical practice due to its elevated cost. Additionally, the technical difficulties of this test include the need for a large volume of stool sample and the high rate of false-positive results, creating a need for more confirmative colonoscopies and additional costs<sup>[38]</sup>. Further studies evaluating the cost-effectiveness of this test for large-scale population screening are needed<sup>[43]</sup>.

Fecal DNA analysis for mutation detection has also been reported using several digital PCR systems<sup>[18,27,44-48]</sup>, the first of which were the studies carried out by Vogelstein and Kinzler<sup>[18,44]</sup>, which led to the development of BEAMing (named for 'beads, emulsions, amplification, and magnetics')<sup>[27]</sup>. Other examples are target-enriched multiplex PCR (Tem-PCR)<sup>[47]</sup>, MDHB (multiplex digital PCR coupled with hydrogel bead-array)<sup>[46]</sup>, and MLPA-DABA (multiplex ligation-dependent probe amplification-digital amplification coupled with hydrogel bead-array)<sup>[48]</sup>. It is worth mentioning that, to date, none of these systems has been further developed and subjected to clinical validation for stool DNA screening.

In our study, DNA from fecal samples of CRC patients was successfully obtained in all cases. Presence of the *KRAS* G12D mutation was determined by pyrosequencing of FFPE tissue as a reference standard. Results of *KRAS* G12D mutation detection in FFPE tumors using ddPCR were in total agreement with pyrosequencing analysis. This was expected, given the fact that ddPCR has been proven to achieve higher sensitivity than pyrosequencing<sup>[49]</sup>. Once the *KRAS* G12D mutation had been screened in tumor tissues, DNA from stool samples obtained from the same patients prior to surgery was also analyzed. Thus, we were able to detect the *KRAS* G12D mutation in 8 out of 10 stool samples from patients known to carry this mutation in their tumors using both methods. It is noteworthy that 6 of these 8 samples were from early-stage patients (I-IIA), highlighting the potential of this approach to identify *KRAS* mutations at the initial stages of the disease.

Absorbance at a wavelength of 230 nm has been reported as an indicator of the level of potential PCR inhibitors in fecal samples<sup>[50]</sup>, and it should be

noted that the two negative samples showed peak of absorbance at this same wavelength. Thus, the sensitivity of detection in our assay could have been greatly reduced by the presence of PCR inhibitors in these samples.

The sample from Patient 17 is noteworthy for its remarkably high concentration of both mutated and WT copies. It is worth mentioning that this patient was subjected to cathartic preparation prior to sample collection due to an oversight. The rest of the samples were collected without any bowel preparation. We hypothesize that purging could have increased the exfoliation of tumor cells into the bowel lumen. This unexpected observation raises the question of whether bowel preparation could be advisable prior to sample collection to increase the sensitivity of detection in stool screening of *KRAS* mutations by ddPCR.

To our knowledge, this is the first study to evaluate the feasibility of detection of the *KRAS* G12D mutation in stool DNA from CRC patients using this particular ddPCR platform. Our results are in line with the above-mentioned recently published study by Herring *et al.*<sup>[17]</sup>, reporting the detection and accurate quantification of an mRNA biomarker in stool from CRC patients using the same ddPCR system. In light of these results, the analysis of CRC biomarkers in stool using ddPCR merits further study in larger cohorts of patients to evaluate the clinical utility of this approach.

We analyzed only the most prevalent *KRAS* mutation (G12D) in our population, as it was the only one with a sufficient number of samples available for analysis ( $n = 10$ ). Another reason for choosing G12D as a target was that it has the highest incidence in CRC patients worldwide and is associated with poor clinical outcome<sup>[8,23]</sup>. For lower-incidence mutations, such as G12V, there were too few samples in our study population to provide conclusive results. The analysis of *KRAS* G12D performed in this study represents a proof-of-concept of the feasibility of this strategy as a first step prior to the screening of other relevant hotspot mutations.

This preliminary study demonstrates the capability of ddPCR to detect *KRAS* mutations in stool-derived DNA, acting as a complementary approach to tissue biopsy for tumor genotyping. These results pave the

Table 3 *KRAS* G12D mutation levels in DNA from stool samples of wild-type control patients

Patient	Tumor location	Tumor stage	G12D						Wild-type					
			Single			Merged			Single			Merged		
			Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction	Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction	Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction	Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction
53	Left	I	1	0.1	2	3	0.07	1.40	0.011	9	180	469	10.50	210.00
			2	0.17	3.4					10.8	216			
			0	0	0					9.9	198			
56	Left	II A	0	0	0					12.1	242			
			1	0.08	1.6	1	0.02	0.40	0.003	17	28	76	1.56	31.20
			0	0	0					2.1	42			
63	Right	II A	0	0	0					1.3	26			
			0	0	0					1.4	28			
			1	0.08	1.6	1	0.02	0.42	0.003	20.4	408	957	20.30	406.00
71	Right	III B	0	0	0					22.1	442			
			0	0	0					19	380			
			0	0	0					19.9	398			
96	Left	IV	1	0.08	1.6	3	0.06	1.20	0.009	6.7	134	377	7.20	144.00
			2	0.15	3					7.9	158			
			0	0	0					6.9	138			
96	Left	IV	0	0	0					7.3	146			
			1	0.09	1.8	3	0.06	1.20	0.009	3	60	133	2.84	56.80
			1	0.08	1.6					2.6	52			
96	Left	IV	1	0.09	1.8					3	60			
			0	0	0					2.9	58			
			0	0	0									

way for the ddPCR analysis of other molecular biomarkers of CRC in stool, including other *KRAS*, *NRAS* and *BRAF* mutations. A multiplex assay simultaneously covering all *KRAS* mutations relevant for anti-EGFR therapy decision-making would maximize the benefits and optimize the cost-effectiveness of this approach. Further studies involving larger cohorts of patients and samples collected at different time points throughout the progression of the disease should be performed in order to confirm the prognostic value and economic viability of this tool before implementation in clinical practice.

This study is the first to describe the detection of *KRAS* G12D mutation in stool-derived DNA from CRC patients using a commercially available ddPCR platform, including individuals at early stages of the disease. We hypothesized that ddPCR could be a reliable and sensitive method to analyze *KRAS* mutations in stool-derived DNA providing reproducible and accurate results. Our findings suggest this approach, which is fast, simple and affordable, could be adaptable to the detection of other clinically relevant molecular biomarkers for CRC management. These advantages with respect to other previously described stool-based strategies, together with instrumentation and protocols easily adoptable by any lab, make our approach more feasible for implementation into routine clinical practice. In light of our results, it could be proposed that biomarker analysis by ddPCR of stool samples may complement current CRC screening methods; stool-derived nucleic acid testing by ddPCR offers an alternative tool to tissue genotyping and blood-based biomarker quantification, being less invasive than the former and, probably, more time-sensitive than

Table 4 *KRAS* G12D mutation levels in DNA from stool samples of patients with *KRAS* G12D mutated tumors

Patient	Tumor location	Tumor stage	G12D						Wild-type					
			Single			Merged			single			Merged		
			Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction	Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction	Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction	Positive events	Copies/ $\mu$ L	Copies/20 $\mu$ L reaction
12	Rectum	I	4	0.32	6.4	14	0.37	7.40	0.051	155	12.6	252	13.10	262.00
			5	0.39	7.8					158	12.5	250		
			5	0.39	7.8					180	14.1	282		
17	Left	I	1147	95	1900	4849	97.20	1944.00	41.362	4684	446	8920	456.00	9120.00
			1243	98	1960					4996	458	9160		
			1269	99	1980					5094	460	9200		
			1190	97	1940					4890	461	9220		
29	Left	IIIB	5	0.41	8.2	11	0.30	6.00	0.024	94	7.7	154	7.10	142.00
			3	0.24	4.8					90	7.4	148		
			3	0.25	5					73	6.2	124		
30	Right	I	6	0.57	11.4	18	0.54	10.80	0.017	238	22.8	456	23.10	462.00
			7	0.64	12.8					268	24.8	496		
			5	0.43	8.6					251	21.8	436		
43	Right	IIIC	6	0.59	11.8	19	0.59	11.80	0.035	418	42	840	44.10	882.00
			8	0.7	14					492	45.5	910		
			5	0.46	9.2					483	44.8	896		
46 <sup>1</sup>	Left	IIC	1	0.09	1.8	3	0.09	1.80	0.014	81	7	140	7.80	156.00
			1	0.09	1.8					97	8.3	166		
			1	0.09	1.8					87	8.2	164		
51	Rectum	I	13	1	20	47	0.90	18.00	0.295	1292	103	2060	102.60	2052.00
			12	0.9	18					1291	102	2040		
			9	0.68	13.6					1297	102	2040		
			13	1	20					1230	103	2060		
64 <sup>1</sup>	Left	I	0	0	0	0	0.00	0.00	0.000	141	12	240	12.20	244.00
			0	0	0					163	12.1	242		
			0	0	0					154	11.4	228		
			0	0	0					195	13.3	266		
70	Left	I	11	1.1	22	45	1.37	27.40	0.036	2515	282	5640	294.00	5880.00
			24	1.9	38					3313	305	6100		
			10	1	20					2688	291	5820		
75	Left	IIA	0	0	0	11	0.33	6.60	0.032	304	27.2	544	29.00	580.00
			2	0.18	3.6					326	29.8	596		
			9	0.8	16					334	30.1	602		

<sup>1</sup>Patients 46 and 64 were considered negative because they had values less than or equal to the positivity threshold.



the later, especially at early stages, as tumor DNA will reasonably reach the fecal content more quickly than the bloodstream, at least during the initial phases of cancer development.

## ARTICLE HIGHLIGHTS

### Research background

Clinical management of colorectal cancer (CRC) requires analysis of molecular biomarkers, such as *KRAS* or *NRAS* mutations, which are associated with the emergence of resistance to therapy with biological agents. Tumor genotyping is usually performed using DNA from tissue biopsies, and, in recent years, from blood as well. However, at early disease stages, levels of mutated copies in circulating cell-free DNA may be, in some cases, too low for detection. Thus, extremely sensitive and non-invasive alternative methods are still needed to improve detection and achieve accurate quantification of these biomarkers.

### Research motivation

Stool is an alternative and non-invasive source of genetic material for tumor genotyping in CRC. To date, several strategies based on analysis of molecular markers in fecal samples have been proposed, though their application in clinical practice remains limited due to their elevated cost and reduced sensitivity at early stages of disease.

### Research objectives

The aim of this study was to assess the potential of droplet digital PCR (ddPCR) to detect the *KRAS* G12D mutation in stool-derived DNA from CRC patients as a proof-of-concept for the applicability of this technology as a non-invasive method of studying clinically relevant DNA biomarkers in stool.

### Research methods

*KRAS* mutations were determined by pyrosequencing in DNA obtained from formalin-fixed paraffin-embedded (FFPE) tumor tissues. Then, *KRAS* G12D mutation was analyzed by ddPCR in FFPE tumors and stool-derived DNA in samples obtained from patients carrying this point mutation.

### Research results

The *KRAS* G12D mutation was detected by ddPCR in FFPE tumor-derived DNA and in stool-derived DNA in 80% of all fecal samples from CRC patients with this mutation.

### Research conclusions

This is the first study to describe the detection of the *KRAS* G12D mutation in stool-derived DNA from CRC patients using a commercially available ddPCR platform, including in individuals with early stages of the disease. ddPCR served as a reliable tool for detecting this clinically relevant mutation in stool-derived DNA from CRC patients. Several stool-based strategies involving digital PCR have been investigated to analyze relevant mutations for CRC management. However, none of these approaches has been further developed and subjected to clinical validation for stool DNA screening to date.

The advantages of ddPCR technology, together with instrumentation and protocols easily adoptable by any lab, support a potential translation of this approach to clinical scenarios. Our results show that *KRAS* G12D detection in stool-derived DNA from CRC patients by ddPCR is feasible and suggests this technology might be useful for the analysis of other molecular markers in stool. The authors hypothesized that ddPCR could be a reliable and sensitive method of analyzing *KRAS* mutations in stool-derived DNA, providing reproducible and accurate results.

This study proposed a new strategy based on detecting *KRAS* mutations in stool-derived DNA using a commercially available ddPCR platform. ddPCR is an emulsion-based amplification technology with fluorescently labelled probes. *KRAS* G12D mutation detection in stool-derived DNA by ddPCR is a fast, simple, and affordable approach which could be adapted to detect other clinically relevant molecular biomarkers for CRC management. This technique is more feasible for implementation into routine clinical practice than other

previously described stool-based strategies.

ddPCR provided sensitive, accurate, and reproducible results for detection of the *KRAS* G12D mutation in stool-derived DNA from CRC patients, especially at early stages of the disease. In light of our results, it could be proposed that biomarker analysis by ddPCR in stool samples may complement current CRC screening methods; stool-derived nucleic acid testing by ddPCR offers an alternative to tissue genotyping and blood-based biomarker quantification, is a less invasive tool than the former and is likely more time-sensitive than the latter, especially at early stages, as tumor DNA will reasonably reach the fecal content more quickly than the bloodstream, at least during the initial phases of cancer development.

### Research perspectives

*KRAS* mutations are analyzable by ddPCR in stool-derived DNA from CRC patients, including early-stage patients. This observation merits further studies aimed at evaluating and improving the efficiency of this approach prior to its clinical application. These results pave the way for ddPCR analysis of other molecular biomarkers of CRC in stool. Further studies involving larger cohorts of patients and samples collected at different time points throughout the progression of the disease should be performed in order to confirm the prognostic value and economic viability of this tool before implementation in clinical practice. A multiplex assay simultaneously covering all *KRAS* mutations relevant for anti-EGFR-therapy decision-making would maximize the benefits and optimize the cost-effectiveness of this approach. This strategy should be further investigated as a complementary screening test for early detection of CRC.

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