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Original Article

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Maura Menghi is an employee of Diatech Pharmacogenetics. None of the other authors have any conflicts of interest to declare.

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Abstract

Background: DNA integrity analysis could represent an alternative approach to the early detection of colorectal cancer (CRC). Previously, fluorescence long DNA (FL-DNA) in stools was extracted using a manual approach and analysed by capillary electrophoresis assay (CE FL-DNA). We aimed to improve diagnostic accuracy using a simpler and more standardised method (Real Time PCR FL-DNA [RT FL-DNA]) for the detection of early malignant lesions in a population undergoing CRC screening.

Methods: From 241 stool samples, DNA was extracted using manual and semi-automatic extraction systems and analysed using FL-DNA tests by CE and RT assays. The RT FL-DNA approach showed slightly higher sensitivity and specificity compared to the CE FL-DNA method. Furthermore, we compared the RT FL-DNA approach with the iFOBT report.

Results: Non-parametric ranking statistics were used to analyse the relationship between the median values of RT FL-DNA and the clinico-histopathological characteristics. The median values of both variables were significantly higher in cancer patients than in patients with non-cancerous lesions. According to the Fagan nomogram results, iFOBT and FL-DNA methods provided more accurate diagnostic information and were able to identify subgroups at varying risks of cancer.

Conclusions: The combination of the semi-automatic extraction system and RT FL-DNA analysis improved the quality of DNA extracted from stool samples.

Impact: RT FL-DNA shows great potential for colorectal cancer diagnosis as it is a reliable and relatively easy analysis to perform on routinely processed stool samples in combination with iFOBT.

Introduction

Colorectal cancer (CRC) is the third most common form of cancer and the second leading cause of deaths among cancers worldwide (1). Sporadic colon cancer, which represents 70% of newly diagnosed cases, develops via the progressive accumulation of multiple mutations that affect tumour suppressor genes, as well as oncogenes or mismatch repair genes (MMR) (2).

Several studies have shown that colorectal cancer screening programmes are able to reduce cancer mortality (3-5). Strategies used in screening programmes, which differ according to geographical areas, can be classified into three broad categories: stool tests (faecal occult blood test [FOBT]), endoscopic examinations (flexible sigmoidoscopy and colonoscopy) and imaging tests (double contrast barium enema or computed tomographic colonography) (3, 6).

Nevertheless, none of these methods is truly optimal due to different technical limits. FOBT is a cheap, non-invasive test but it has several limitations, such as: low sensitivity, especially in detecting pre-cancerous lesions, and low ability to distinguish benign and malignant pre-cancerous lesions compared to endoscopic examinations (7). Moreover, the low specificity of occult blood test leads to a high number of unnecessary colonoscopies (8). All patients with a positive iFOBT are invited to undergo a colonoscopy examination, but it is estimated that only 50% of individuals at average risk of the development of CRC comply with current screening guidelines and agree to the medical examination (9, 10). Conversely, endoscopic and imaging examinations are more accurate but are more expensive and invasive, thus reducing compliance in screening programmes (6). The main difficulties involved in undergoing colonoscopies include psychological barriers, such as fear of the procedure and embarrassment, as well

as procedural problems, such as the requirement for a cathartic preparation, sedation, and the inherent risks of complications and discomfort associated with colonoscopies (11). All these points represent potential reasons for individuals to avoid undergoing this crucial preventive health test (12), which has been shown to be able to reduce mortality related to the CRC disease (13).

Many new molecular non-invasive screening tests have been developed and investigated for the detection of CRC. Faecal DNA tests have been designed to detect molecular abnormalities present in pre-cancerous or cancerous lesions: chromosomal instability due to abnormalities in mutational hotspots like *APC, KRAS*, and *TP53*; microsatellite instability (MSI); and alteration of DNA methylation status (14-15). The factors that limit the widespread diffusion of these methodologies are related to time-consuming approaches and poor cost-effectiveness compared to other screening tests. In fact, despite good sensitivity and specificity compared to iFOBT, the actual costs for analyses with molecular tests are too high to suggest their use in screening programmes (6).

In order to identify a new approach, which is relatively cheap and not time-consuming, able to increase accuracy in detecting colorectal lesions, in recent years, we studied stool DNA integrity as a molecular marker that could help to improve the identi-fication of colorectal cancers (CRC) and to determine a patient's risk of harbouring a pre-neoplastic or neoplastic lesion (16-19).

For this purpose, we carried out a quantitative evaluation based upon fluorescence amplification of different genomic DNA targets and quantification by capillary electrophoresis and reference standard curve, fluorescence long DNA (FL-DNA) (16-19). After completing pilot and confirmation case-control studies (17, 19) and

further to an initial evaluation of the combination of this test with iFOBT (19), the aim of this study is to devise a standardised method, based upon Real-Time PCR analysis combined with a semi-automatic extraction of stool DNA, which is simpler and easier to perform than previously described approaches, so as to improve the accuracy of FL-DNA in detecting pre-malignant and malignant lesions (16-19).

Materials and Methods

Patient sample

All study subjects were recruited from the Gastroenterology and Digestive Endoscopy Units of the Morgagni-Pierantoni" Hospital (Forlì, Italy) and the "Castel San Pietro Terme" Hospital (Bologna, Italy) by two methods: a regional screening programme or direct access to the Medical Unit. Informed consent was obtained from all individuals agreeing to take part in the study. A total of 241 individuals were enrolled in the study with a medical report of colonoscopy within 45 days of the result of the iFOBT test. Of these, 23 were diagnosed with adenocarcinomas, 34 with high-risk adenomas and 35 with low-risk adenomas. One hundred and forty- nine individuals did not show any malignant or premalignant lesion. All individuals were submitted to endoscopic examination in order to confirm the diagnosis. The lesion type was histologically confirmed and, in cancer patients, the pathologic stage was defined in accordance with Dukes' classification. Pre-neoplastic lesions were classified as low or high-risk according to the National Comprehensive Cancer Network guidelines (20).

Specifically, all patients were considered at high-risk when they had high-risk dysplasia, >3 adenomatous villous or tubulovillous polyps, at least one of which with a diameter of ≥1 cm, or an *in situ* carcinoma, whereas those who presented <3 tubular polyps with a diameter <1 cm were considered at low-risk (19). The study protocol was reviewed and approved by the local ethics committee.

Sampling

Stool samples were collected using the OC-Sensor device (Alfa Wassermann, Bologna, Italy). Subjects were provided with instructions for collecting the faecal matter at home and were informed that the samples had to be brought to the analysis laboratory

within 24 hours. In accordance with regional guidelines for colorectal cancer screening, test positivity was defined as a haemoglobin value ≥100 ng/ml. Haemoglobin values were determined using an immunochemical technique. The same specimen was used for iFOBT and molecular analyses. Immediately after occult blood tests, samples were processed for DNA extraction or stored at -20°C for a maximum of two months on the basis of results from preliminary experiments on DNA stability (19).

DNA extraction:

Manual approach

A QIAamp DNA Stool Kit (Qiagen, Hilden, Germany) was used for stool DNA purification as previously described (19).

Semi-automatic extraction

Five hundred microliters of helix tissue buffer (Diatech Pharmacogenetics, Jesi, Italy) were added to the frozen pellet and after solution homogenisation, the samples were centrifuged at 13000 rpm for 1 minute. A volume of 450 µl of supernatant was transferred to a new collection tube containing 8 µl of Helix Proteinase K (Diatech Pharmacogenetics) and mixed thoroughly for 15-20 seconds. The solution was then were then incubated at 65°C for30 minutes, agitating constantly (V=500 rpm). The samples were then left to cool at room temperature and mixed for 15-20 seconds. After brief centrifugation, 400 µl from each sample was transferred into a HES Lysis Plate. From this step onwards the "*HELIX DNA strip vc400-ve60 v200807_stool*" protocol was applied using the Helix Extraction System (Diatech Pharmacogenetics).

FL-DNA analysis:

Capillary electrophoresis (CE FL-DNA)

FL-DNA was determined by PCR with fluorescent-labelled primers and capillary electrophoresis as previously described (19). All samples were run in duplicate and only inter-sample variations of <15% were accepted. In all other cases (15% of the series), the determination was performed again and only <10% variations were accepted for the entire series. No samples showed variations >10% at this third evaluation.

Real-Time PCR (RT FL-DNA)

FL-DNA was analysed by Real-Time PCR. The following reagents were added at the stool sample 5 µl used: Eurogentec MESA GREEN 1X 12.5 µl and Oligo-MixA 2 µl or Oligo-MixB 2 µl. Water5.5 µl was added to reach the final volume of 25 µl. Oligo-MixA is composed of fragments 2 and 3 of APC exon 15 and exon 8 of *p53*. Oligo-MixB is composed of fragment 4 of APC exon 8 and exons 5 and 7 of *p53*. Two mixture reactions were amplified simultaneously in the same programme composed of 41 cycles: one cycle at 95°C for 5 minutes and 40 cycles at 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 30 seconds. Fluorescence was acquired during PCR at 77°C for Oligo-Mix A and at 80°C for Oligo-Mix B to select only signals coming from specific amplification products. The reaction specificity was further checked by a post-PCR Melting Curve. Reaction was carried out using a Rotor Gene 6000 (Qiagen) equipped with Rotor Gene 6000 Series Software 1.7 (Build 87). The final FL-DNA value was obtained by analysing the fluorescence intensity of each sample-specific PCR product against a reference standard curve (5, 0.5 and 0.1 ng/reaction) of genomic DNA, expressed as ng/reaction. All samples were run in duplicate and only inter-sample variations of <15% were accepted.

Statistical analysis

The objective of this study was to compare manual and semi-automatic systems to

identify the best and least labour-intensive extraction system. FL-DNA concentrations were considered as a continuous variable and the median values between these methodologies were compared using the non-parametric ranking median test. The analysis of the FL-DNA concentration in the two methodologies was carried out by receiver operating characteristic (ROC) curve analysis. In the ROC curves, true positive rates (sensitivity) were plotted against false positive rates (1-specificity) for all classification points. Sensitivity, specificity and their relative 95% Confidence Intervals (95% CI) were calculated using different cut-off values and the FL-DNA accuracy was measured using the Area Under ROC curve (AUC). Median values of RT FL-DNA and iFOBT between different types of lesions were compared using the Kruskal-Wallis test. Non-parametric ranking statistics (median test) were used to analyse the relationship between the median values of RT FL-DNA and the clinico-histopathological characteristics. In order to estimate post-test probability, i.e., the probability of disease in a subject after the diagnostic test results are known, we first estimated the pre-test probability and determined the likelihood ratio. The pre-test probability is the chance of having the disease prior to testing and this is usually related to the disease prevalence. The likelihood ratio is the ratio of the probability of the specific test result in people who do have the disease to the probability in people who do not. The results were divided into three classes according to different cut-off values (0-9, 10-30, and ≥30 ng/reaction) to determine the FL-DNA likelihood ratio, which was calculated by dividing the percentage of patients with colorectal cancer by the percentage without the disease in each class. Finally, post-test probability was calculated by multiplying the likelihood ratio of the diagnostic test by the pre-test probability. All *P* values were two-sided and values ≤ 0.05 were considered statistically

significant. Statistical analyses were carried out using SAS Statistical Software (version 9.3, SAS Institute, Cary, NC, USA).

Results

In the previous works (16-19), analyses were performed using a manual approach to extract DNA from stool and the CE FL-DNA analysis method was used to evaluate DNA integrity. In an effort to improve this methodological approach, we divided our study into two phases. In the set-up phase, we detected the best stool DNA extraction method between the manual and semi-automatic systems, in combination with the best analytic tools between CE and RT FL-DNA. Secondly, we compared the efficiency of the semi-automatic extraction system and the RT FL-DNA analysis method, the tool found to be best, with the current screening test used, iFOBT. We performed both steps on the overall series of 241 individuals.

DNA extraction optimisation

In order to set up the best DNA extraction method, the new semi-automatic approach and the standard manual protocol were tested in parallel. Starting with an amount of 10 mg of faeces per sample, the DNA obtained using these two extraction approaches was amplified by two different multi-locus PCR and analysed by gel electrophoresis showing that the semi-automatic system allows for higher yields of amplification products (data not shown). In order to verify whether any Taq inhibitors were present in the DNA solution, the DNA samples were analysed by inhibition plasmid control. To this purpose, 25 ag of a plasmid containing a 150-bp non-human insert flanked by hybridisation regions for APC fragment 3 primers were added to each sample and amplified according to the CE FL-DNA protocol. In the absence of Taq inhibition, the 150-bp fragment was detectable by capillary electrophoresis. Approximately 23% of DNA samples extracted by manual approaches presented DNA inhibition. For these samples it was necessary to make a further precipitation with ammonium-

acetate/isopropanol to remove all inhibitors. Using the semi-automatic extraction method, the percentage of inhibition was reduced to only 3%.

FL-DNA analysis evaluation: comparison between the two methods

DNA integrity was evaluated for all 241 stool samples using two methods: FL-DNA analysis was performed by CE and by RT FL-DNA. ROC curve analysis for CE FL-DNA showed an area under the curve (AUC) of 0.81 (95% CI: 0.71-0.92); similarly, ROC curve analysis for RT-DNA showed an AUC of 0.82 (95% CI: 0.70-0.94) (Figure 1).

The best cut-offs seem to range from 10 to 30 ng for both approaches. The capillary electrophoresis approach seems to confirm the previous best cut-off of 25 ng in detecting tumours (18), with 57% (37-74; 95% CI) sensitivity and 84% (79-89; 95% CI) specificity and 82% (76-86; 95% CI) accuracy (Table 1). Conversely, using the RT FL-DNA method, the best cut-off seems to be slightly lower. In particular, the cut-off of 15 ng showed 70% (49-84; 95% CI) sensitivity in detecting tumours, 87% (82-91; 95% CI) specificity and 85% (80-89; 95% CI) accuracy. With a higher cut-off of 20 ng, the sensitivity decreased to 61% (41-78; 95% CI) but, conversely, an increase of specificity 91% (87-85; 95% CI) and accuracy 88% (84-92; 95% CI) was observed (Table 1). In addition, considering the accuracy of the two approaches in detecting not only tumour patients but also high-risk adenomas, the RT FL-DNA approach confirms a slightly higher sensitivity and specificity (Table 1).

Comparison between iFOBT and RT FL-DNA values in relation to clinical pathologic characteristics

Our series in this work, in accordance with the conclusions of the 2010 study

performed by Calistri et al. (19) consists of individuals with positive and negative and iFOBT values. Between positive iFOBT the median value is 432 ng/ml values, ranging from 100 to 3811 ng/ml. Individuals with no lesions and low-risk adenomas patients showed the lowest median iFOBT value of 4 ng/ml, both ranged from 0-1000 ng/ml. In patients with high-risk adenomas a higher median value was recorded of 13 ng/ml; ranging from 0-1000 ng/ml. Considered overall, the median iFOBT value for these three subgroups was much lower than that observed for cancer patients 1000 ng/ml, ranging from 0 to 3811 ng/ml ($P < 0.0001$) (Table 2).

Similar results were observed for the RT FL-DNA values. In particular, median values were comparable for individuals with no lesions (2 ng/reaction, ranging from 0-2140 ng/reaction) or with low and high-risk adenomas (1 ng/reaction ranging from 0-31 ng/reaction and from 0-75 ng/reaction, respectively), and were > 4-fold higher (49 ng/reaction ranging from 0-1304 ng/reaction) in cancer patients ($P < 0.0001$) (Table 2).

A breakdown analysis for clinical and pathological subgroups was performed with explorative intent. No differences were noted between healthy donors without any benign diseases or lesions and healthy donors with diverticula, haemorrhoids, inflammatory bowel disease or benign polyps (data not shown). Moreover, there are no significant differences in the FL-DNA value in patients with tumour or adenomas as a function of characteristics such as size, stage, dimension, localisation and number of lesions.-The relationship between iFOBT and RT FL-DNA values within the different clinical and pathologic subgroups was investigated separately in adenomas and cancer patients, but no significant differences were detected (Tables 3 and 4).

iFOBT and FL-DNA combination analysis

Finally, we evaluated whether the combination of iFOBT and FL-DNA could improve our ability to predict the presence of a tumour and/or high risk adenomas. According to the diagnostic relevance of faecal haemoglobin and FL-DNA as independent variables, we tested whether or not, and to what extent, the FL-DNA assay could improve iFOBT diagnostic accuracy (Table 5 and Supplementary Fig. S1A and S1B). In contrast to our previous work, (19) in this study, the analysis was extended negative iFOBT values. All iFOBT values were divided into three main subgroups: 0-99 ng/ml; 100-432 ng/ml and > 432 ng/ml, while FL-DNA results were divided into three classes according to different cut-off values (0-9, 10-30, and ≥ 30 ng/reaction), suggested in the previous paper (19). In the negative-iFOBT subgroup, the pre-test probability of there being a tumour was around 13%, but FL-DNA did not add any useful information. Furthermore, in the intermediate positive iFOBT subgroup, with its 12% overall probability of having cancer, the breakdown analysis as a function of the higher RT FL-DNA subgroup brings the probability of having a tumour to 76%. Specifically, in the last iFOBT subgroup, with its 38% overall probability of having cancer, breakdown analysis as a function of the last RT FL-DNA subgroup highlighted the probability of having colorectal cancer as 93% (Table 5). Interestingly, the combination between CRC and high risk adenomas increased the post-test probability values of having a disease in association with the higher RT FL-DNA values at 76%, 85% and 94% for all the three main iFOBT subgroups, respectively (Table 5).
In view of the fact that the best RT FL-DNA cut-off was slightly lower than that of CE FL-DNA, we performed the iFOBT and FL-DNA combination analysis considering different cut-off ranges (0-14; 15-24; ≥25 ng/reaction). Substantially different results were not observed (Supplementary Table S1).

Discussion

iFOBT is the most widely used method in screening programmes, although it presents some limits in terms of accuracy. The most important hallmark of iFOBT is bleeding, which may be intermittent and a largely unspecific event and may lead to diagnostic errors (21). Conversely, a high number of cells are continuously released into the intestinal lumen every day and biomolecular analysis of genomic DNA extracted from stool specimens could be an alternative approach to improve the early diagnosis of colorectal pre-neoplastic and neoplastic patients (22).

In previous studies, we demonstrated that DNA integrity analysis of stools extracted by a manual approach could represent an alternative tool to the early detection of colorectal lesions (17, 18). In this work, we developed a more user-friendly approach to analysing DNA integrity based upon semi-automatic DNA extraction and Real Time PCR. Our results show that DNA integrity status evaluated using the RT FL-DNA assay and extracted using a semi-automatic approach could be considered as a sensitive and specific marker for early CRC detection. Moreover, we observed that RT FL-DNA was more accurate than the previous CE FL-DNA method in detecting high risk-adenomas.

This new RT FL-DNA method was compared not only with positive iFOBT values, but also with negative values of the diagnostic iFOBT so as to evaluate if a multiple approach could increase predictive accuracy in detecting tumours and high-risk adenomas, thus overcoming the limitations of the occult blood test detection. In the positive-iFOBT values subgroups, faecal RT FL-DNA provided more accurate diagnostic information and identified subgroups with different probability of having a tumour. Interesting results were also obtained by evaluating high-risk adenoma and tumour subgroups together. iFOBT values in combination with subsequent higher

values of RT FL-DNA in the Fagan nomogram improved the risk of disease in terms of post-test probability.

Our results would seem to indicate that this molecular method could be a useful addition to the conventional iFOBT in CRC screening programmes. However, the transfer of new diagnostic approaches to clinical practice is often hindered by problems relating to time-consuming methodologies and costs of individual tests. Song et al. (23) estimated the costs of faecal molecular tests as being between $350 and $795, whereas the cost of colonoscopies ranges from $1,200 to $1,800, depending upon the localisation of the lesions.

Studies assessing the best cost-benefit ratio through the creation of a computer simulation of screening for CRC and polyps indicate that no useful results are yet available for molecular DNA tests considering the current price. Using simulation models, it has been calculated, for example, that a molecular test submitted every two years and with a sensitivity of 65% for CRC and 40% for advanced adenoma, with a specificity of 95%, could be an alternative to colonoscopy only if it costs less than $200 (24). In all likelihood, the cost of the RT FL-DNA test is significantly less than the hypothetical costs suggested by Song et al. Moreover, this cost could probably be further reduced in the case of its large-scale use, as was the case for the hepatitis B virus in 1990 (23).

In conclusion, the limitations of this approach include the unknown frequency at which the tests should be carried out and the number of stool samples that need to be analysed at specific time points for each individual. It should be noted that the adenoma risk classification was based only upon pathologic parameters, which needed to be improved. Its evaluation through clinical multicenter trials in order to verify its real

effectiveness with standard approaches such as iFOBT, colonoscopy and sigmoidoscopy, before it can be implemented into clinical practice, may be an important starting point. It could also be used to enhance the personalised surveillance intervals in individuals undergoing the current standard CRC screening methods. Innovative and personalised diagnoses and therapies against cancer are the main aims of all future clinical trials.

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approaches. Gastroenterology 2005;128:192-206.

Table 1. A comparison between CE FL-DNA and RT FL-DNA to evaluate the best valid method to quantify FL-DNA from stool samples in detecting tumour and tumour and high risk adenomas patients.

	CE FL-DNA					RT-FL-DNA				
Cut-offs (ng)	**CRC** Positive	**Others[1]**	**% Sensitivity (95% CI)**	**% Specificity (95% CI)**	**% Accuracy (95% CI)**	**CRC** Positive	**Others[1]**	**% Sensitivity (95% CI)**	**% Specificity (95% CI)**	**% Accuracy (95% CI)**
≥10	21	123	91(73-97)	44 (37-50)	48 (42-54)	17	44	74 (53-87)	80 (74-85)	79 (74-84)
≥15	18	71	78 (58-90)	67 (61-73)	68 (62-74)	16	29	70 (49-84)	87 (82-91)	85 (80-89)
≥20	16	46	70 (49-84)	79 (73-84)	78 (72-83)	14	19	61 (41-78)	91 (87-85)	88 (84-92)
≥25	13	34	57 (37-74)	84 (79-89)	82 (76-86)	13	12	57 (37-74)	94 (91-97)	91 (87-94)
≥30	12	21	52 (33-71)	90 (86-94)	87 (82-90)	13	5	57 (37-74)	98 (95-99)	94 (90-96)
	CRC + HRA Positive	**Others[2]**				**CRC + HRA** Positive	**Others[2]**			
≥10	40	104	71 (58-82)	44 (37-51)	50 (44-55)	23	38	41 (29-54)	79 (73-85)	71 (65-76)
≥15	27	62	48 (36-61)	66 (59-73)	62 (56-68)	22	23	39 (28-52)	88 (82-92)	76 (71-81)
≥20	23	39	41 (29-54)	79 (72-84)	70 (64-76)	17	16	30 (20-43)	91 (86-95)	77 (72-82)
≥25	18	29	32 (21-45)	84 (78-89)	72 (66-77)	15	10	27 (17-40)	95 (90-97)	79 (74-84)
≥30	15	18	27 (17-40)	90 (85-94)	76 (70-81)	15	3	27 (17-40)	98 (95-99)	82 (76-86)

Abbreviation: CE-FL DNA: capillary electrophoresis fluorescence long DNA; RT FL-DNA: real time PCR fluorescence long DNA; 95% CI: 95% confidence intervals; CRC: colorectal cancer patients; HRA: high risk adenomas patients; Other[1]: high and low risk adenomas and healthy subjects; Other[2]: low risk adenomas and healthy subjects. Sensitivity: true positive rates; Specificity: true negative rates; Accuracy: number of true positive plus number of true negative, divided by the total series.

Table 2. RT FL-DNA and iFOBT values in 241 individuals with malignant, pre-malignant or no lesions

	N	**RT FL-DNA**	**iFOBT**
	241	**Median value (ng/reaction) (range)**	**Median value (ng/ml) (range)**
No lesions	149	2 (0-2140)	4 (0-1000)
Low-risk adenomas	35	1 (0-31)	4 (0-1000)
High-risk adenomas	34	1 (0-75)	13 (0-1000)
Colorectal cancer	23	49 (0-1304)	1000 (0-3811)
		$P < 0.0001$	$P < 0.0001$

Abbreviation: RT FL-DNA, real time PCR fluorescence long DNA; iFOBT, immunochemical faecal occult blood test.

Table 3. RT FL-DNA and iFOBT values in adenoma patients according to clinical-pathological characteristics

	Cases	RT FL-DNA (ng/reaction)		iFOBT (ng/ml)	
	(*N*=69)	Median (range)	*P*	Median (range)	*P*
Gender					
Male	43	0.68 (0-30.6)		9 (0-1000)	
Female	26	1.37 (0-74.5)	0.871	8 (0-1000)	0.663
Patient classification					
Low-Risk	35	1.45 (0-30.6)		4 (0-1000)	
High-Risk	34	0.65 (0-74.5)	0.379	13 (0-1000)	0.138
Lesion dimension					
0-0.9 cm	40	1.47 (0-30.6)		4.5 (0-1000)	
≥ 1 cm	27	0.62 (0-74.5)	0.566	16 (0-753)	0.144
Number of lesions					
Single	48	0.88 (0-74.5)		7 (0-1000)	
Multiple	20	1.37 (0-30.6)	0.446	10.5 (0-753)	0.361
Lesion localisation					

Descending+transverse	11	0.50 (0-26.8)		2 (0-75)	
Ascending	22	0.29 (0-30.6)		15 (0-1000)	
Mixed	5	1.05 (0-19.9)	0.637	1 (0-485)	0.169

Abbreviation: RT FL-DNA, real time PCR fluorescence long DNA; iFOBT, immunochemical faecal occult blood test.

Table 4. RT FL-DNA and iFOBT values in CRC patients according to clinical-pathological characteristics

	Cases	RT FL-DNA (ng/reaction)		iFOBT (ng/ml)	
	(*N*=23)	Median (range)	*P*	Median (range)	*P*
Gender					
Male	17	40.23 (0.0-1303.95)		1000 (1-3707)	
Female	6	60.39 (0.0-387.14)	0.528	1000 (0-3811)	0.807
Duke's stage					
A	7	40.23 (1.88-323.04)		1000 (219-2786)	
B	11	64.46 (1.56-1303.95)		1000 (1-3811)	
C+D	2	33.49 (8.60-58.38)	0.345	1000 (1000-1000)	0.960
TNM classification					
T1	7	40.23 (1.88-323.04)		1000 (219-2786)	
T2	5	113.94 (51.5-387.14)		534 (1-1000)	
T3	8	38.17 (1.56-1303.95)	0.170	1464 (396-3811)	0.052
Lesion dimension					
0-0.9 cm	5	64.46 (40.23-202.57)		2018 (217-3707)	
≥1 cm	13	58.38 (1.88-1303.95)	0.628	1000 (241-3811)	0.922

Abbreviation: RT FL-DNA, real time PCR fluorescence long DNA; iFOBT, immunochemical faecal occult blood test; N.A., not applicable; TNM, tumour-node-metastasis.

Table 5. Colorectal cancer and colorectal cancer plus high risk adenoma prevalence as a function of FL-DNA evaluation and negative iFOBT and positive iFOBT separated by the median value of all positive iFOBT detected.

Colorectal cancer						
RT FL-DNA	Yes *N* (%)	No *N* (%)	Likelihood ratio (95% CI)	iFOBT <100 Post-test probability (pre-test=0.0128)	iFOBT 100-432 Post-test probability (pre-test=0.116)	iFOBT >432 Post-test probability (pre-test=0.381)
0-9	6 (26.1)	174 (79.8)	0.327 (0.156-0.683)	0.004	0.041	0.168
10-30	4 (17.4)	39 (17.9)	0.972 (0.398-2.374)	0.012	0.113	0.374
≥30	13 (56.5)	5 (2.3)	24.643 (17.210-35.288)	0.242	0.764	0.938
Total	23 (100)	218 (100)				

Colorectal cancer and high-risk adenoma						
RT FL-DNA	Yes *N* (%)	No *N* (%)	Likelihood ratio (95% CI)	iFOBT <100 Post-test probability (pre-test=0.160)	iFOBT 100-432 Post-test probability (pre-test=0.256)	iFOBT >432 Post-test probability (pre-test=0.500)
0-9	34 (59.7)	146 (79.4)	0.752 (0.527-1.072)	0.125	0.206	0.429
10-30	8 (14.0)	35 (19.0)	0.738 (0.387-1.408)	0.123	0.203	0.425
≥30	15 (26.3)	3 (1.6)	16.140 (10.449-24.931)	0.755	0.847	0.942
Total	57 (100)	184 (100)				

Abbreviation: RT FL-DNA, real time PCR fluorescence long DNA; iFOBT, immunochemical faecal occult blood test; 95% CI, 95% confidence intervals.

Figure legends

Figure 1. ROC curve. ROC curve of FL-DNA and RT-DNA analyses for the complete series of stool samples.

Figure 1