

Quantitative fluorescence determination of long-fragment DNA in stool as a marker for the early detection of colorectal cancer

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Abstract. *Background:* A variety of molecular markers have been evaluated for the development of a non-invasive approach to the diagnosis of colorectal cancer. We aimed to validate the diagnostic accuracy, using the same threshold as in the previous pilot study, of fluorescent long DNA test as a relatively simple and inexpensive tool for colorectal cancer detection.

Methods: A case-control study was conducted on 100 healthy subjects and 100 patients at first diagnosis of colorectal cancer. Human long-fragment DNA in stool was quantified by fluorescence primers and a standard curve and expressed in DNA nanograms.

Results: We validated the 25-ng value, which emerged as the most accurate cut-off in the pilot study, obtaining 79% (95% CI, 71–87%) sensitivity and 89% (95% CI, 83–95%) specificity. Specificity was very high for all cut-off values (15–40 ng) analyzed, ranging from 78 to 96%. Sensitivity was only slightly lower, reaching 84% at the lowest cut-off and maintaining a good level at the higher values. Diagnostic potential was independent of gender, age and tumor site.

Conclusion: Fecal DNA analysis is a non-invasive and fairly simple test showing high diagnostic potential. These characteristics, together with the small amount of stool required, make it potentially suitable to be used alongside or as an alternative to current non-invasive screening approaches. Our next step will be to validate these results in a large-scale cohort study of a screening population, which is needed prior to implementation into clinical practice.

Keywords: Fluorescence long DNA, stool, colorectal cancer, diagnosis

1. Introduction

Colorectal cancer (CRC) is the third most common malignancy in the world [16]. Screening for CRC has great potential to reduce morbidity and mortality from the disease, but currently only a small portion of eligible individuals undergo testing for early diagnosis [11]. Moreover, there is ongoing debate about the best and most effective method to use in CRC screening programs. With the exception of the Fecal Occult Blood

Test (FOBT), all currently available tools are both invasive and expensive [11]. Molecular stool testing could provide a valid alternative to conventional methods in terms of compliance and practicability. New tests based on molecular markers and aimed at detecting neoplastic cells or cell products in stool have therefore been developed and are currently being evaluated. A variety of genetic and epigenetic alterations, which commonly occur during evolution from normal colon mucosa to adenoma and carcinoma, such as K-ras, p53, APC gene mutations, microsatellite instability or epigenetic events, have been investigated [1,4,6,12–15, 19–22,24]. The results from these studies have shown that individual molecular alterations are present in the stool of only a fraction of patients and are therefore

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characterized by low sensitivity in detecting colorectal cancer. Multiple mutation analyses have improved test sensitivity, but with a corresponding increase in costs and test execution time [6,14,20]. For these reasons, none of the above methods can be used in early diagnosis programs [3,23].

DNA integrity analysis of genomic DNA extracted from stool represents a moderately inexpensive and relatively rapid test [5,26,28]. In particular, the fluorescence long DNA (FL-DNA) method, which was developed in our laboratory and is based on the quantification of stool DNA using fluorescent primers, showed interesting results in a pilot case-control study [5].

In the present confirmatory study, we aimed to validate the results from the pilot study, using the same threshold as before, in a larger and independent case series.

2. Materials and methods

2.1. Case series

One hundred patients with primary CRC at first diagnosis and 100 healthy individuals were entered onto the study. Patients were enrolled from the Departments of Gastroenterology of Morgagni-Pierantoni Hospital (Forlì, Italy) and Bufalini Hospital (Cesena, Italy), the Department of Oncology of Infermi Hospital (Rimini, Italy), and the Department of Surgery and Oncology, University of Palermo (Palermo, Italy) from January 2005 to December 2006. Stool was collected 5–7 days after colonoscopy in order to avoid traumatic artifacts due to bleeding or inflammation, which could produce falsely elevated long DNA levels, and to allow restoration of normal bowel function. Healthy donors were recruited over the same period from laboratory staff members of the same hospitals and their families, and frequency matched by sex and age (≤ 55 , 56–70, >70 years). All healthy donors had a negative FOBT. Stools from patients and healthy donors were frozen immediately and stored at -80°C for a maximum of 2 months.

Diagnosis of cancer was histologically confirmed and pathological stage was defined according to Dukes' classification: 17 tumors were classified as stage A, 45 as stage B, 29 as stage C and 7 as stage D. Information was not available for 2 patients. Moreover, 34 cancers were located in the ascending colon, 45 in the descending colon, and 5 in the transverse colon, while 16 were rectal cancers.

The study protocol was reviewed and approved by the local Ethics Committee of each center taking part.

2.2. DNA purification

Approximately 200 mg of stool were thawed at room temperature and homogenized with 10 ml of TE-9 buffer (0.5 mol/l Tris-HCl pH 9, 20 mmol/l EDTA and 10 mmol/l NaCl). After centrifugation at 5000g for 15 min to remove all particulate matter, the supernatant was transferred to a tube containing 350 μl of ammonium acetate 7.5 mol/l (M-Medical, Florence, Italy) and 19 ml of ethanol 100% (Carlo Erba, Milan, Italy). DNA was recovered by centrifugation at 5000g for 15 min at room temperature, suspended in 1.6 ml of ASL buffer and purified by QIAamp DNA Stool Kit (Qiagen, Hilden, Germany).

2.3. Fluorescence long DNA (FL-DNA) analysis

The fluorescence intensity of each sample-specific PCR product was determined with fluorescent-labelled primers, as previously described [5]. Briefly, p53 exons 5–8 and fragments 1–4 of APC exon 15 were amplified in a final volume of 25 μl containing 2 μl of stool DNA, 0.4 μM of each primer, 200 μM of deoxynucleotide (Takara Bio Inc, Shiga, Japan), 1 \times reaction buffer with 3.5 mM MgCl_2 (Qiagen), and 1 U of Taq polymerase (Qiagen). The reaction mixture was subjected to 32 cycles: 60 s at 94°C and then 60 s at 60°C for p53 exons, and 58°C for APC fragments, followed by incubation at 72°C for 60 s. Primers used were end-labeled with fluorochromes provided by Applied Biosystems (Foster City, CA, USA).

The p53 exons were amplified simultaneously in a single reaction mixture. *Primer sequences were:* exon 5: 5S FAM-CTC TTC CTG CAG TAC TCC CCT GC and 5AS GCC CCA GCT GCT CAC CAT CGC TA; exon 6: 6S GAT TGC TCT TAG GTC TGG CCC CTG and 6AS HEX-GGC CAC TGA CAA CCA CCC TTA ACC; exon 7: 7S FAM-GTG TTG TCT CCT AGG TTG GCT CTG and 7AS CAA GTG GCT CCT GAC CTG GAG TC; exon 8: 8S ACC TGA TTT CCT TAC TGC CTC TGG C and 8AS HEX-GTC CTG CTT GCT TAC CTC GCT TAG T. The four APC fragments were amplified in two different mixes (mix 1, fragments 3 and 4; mix 2, fragments 1 and 2). Sequences were as follows: fragment 3: 3S GAT GTA ATC AGA CGA CAC AG and 3AS HEX-GGC AAT CGA ACG ACT CTC AA; fragment 4: 4S FAM-CAG TGA TCT TCC AGA TAG CC and 4AS AAA TGG CTC ATC GAG GCT CA; fragment 1: 1S AAC TAC CAT CCA GCA ACA GA and 1AS HEX-TAA TTT GGC ATA AGG CAT AG; fragment 2: 2S FAM-CAG TTG AAC

TCT GGA AGG CA and 2AS TGA CAC AAA GAC TGG CTT AC. DNA from each sample was quantified on a standard curve of genomic DNA (1, 2, 5, 10 and 20 ng) normalized to 100, and expressed as nanograms.

Electrophoresis was carried out using a 3100 Avant Genetic Analyzer (Applied Biosystems) equipped with GeneScan Analysis 3.7. The final FL-DNA value was obtained by analyzing the fluorescence intensity of each sample-specific PCR product. The quantification of each sample was calculated by reference to a standard curve (1, 2, 5, 10 and 20 ng) of genomic DNA and expressed as nanograms. We evaluated three different standard curves, one for p53 exons 5–8, one for APC fragments 1–2 and one for APC fragments 3–4.

All samples were run in duplicate and only inter-sample variations of less than 15% were accepted. In all other cases the determination was performed on a third sample, and was required in <10% of the entire series. All samples were amplified with a mix containing 25 attograms of a plasmid with a control sequence to test for the presence of Taq inhibitors.

2.4. Statistical analysis

The objective of this validation study was to confirm sensitivity and specificity of the DNA test using the best cut-off value identified in the pilot study [5]. The population size was based on the results of the pilot study, which reported 76% sensitivity and 93% specificity using the best cut-off of 25 ng. It was estimated that, with 100 patients and 100 controls, the two-sided 95% confidence intervals, using the normal approximation, would extend 0.08 and 0.05 from the expected sensitivity and specificity, respectively.

The analysis of the FL-DNA concentration, considered as a continuous variable, was carried out by receiver operating characteristic (ROC) curve analysis. In the ROC curve, the true positive rates (sensitivity) were plotted against the false positive rates (1-specificity) for all classification points. Sensitivity, specificity and relative 95% confidence intervals (95% CI) were calculated using different cut-off values. The median values of amplified DNA levels in controls and patients were compared using the non-parametric median test.

A breakdown analysis for clinical and pathological subgroups was performed with explorative intent. Subgroup sensitivity was analyzed by the chi-square test.

3. Results

Values of amplified DNA ranged from 0 to 246 ng in healthy donors and from 0 to 731 ng in patients, with median values of 0 and 64 ng, respectively ($p < 0.0001$) (Table 1). FL-DNA median values were independent of gender and age. In patients, fecal DNA levels were also independent of tumor size and site and slightly lower, albeit not significantly, in the few patients with Dukes' stage D tumors compared to those with earlier stage tumors (Table 1).

ROC curve analysis showed an area under the curve (AUC) of 0.870 (95% CI: 0.82–0.92) (Fig. 1). The diagnostic potential of different FL-DNA cut-off values from 15 to 40 ng was evaluated (Table 2). Specificity was very high for all the cut-offs analyzed, varying from 78 to 96%. Sensitivity was only slightly lower, reaching 84% at the lowest cut-off (15 ng) and maintaining good levels up to the highest cut-off (40 ng). The most accurate cut-off was 25 ng, with 79% sensitivity and 89% specificity and an overall accuracy of 84% (95% CI: 0.79–0.89) (Table 2).

Breakdown analysis showed that the sensitivity of the fecal DNA test was similar for females and males and was not influenced by age when a cut-off of 69 years, generally adopted as the upper limit in Italian screening programs, or any other age value was used (Table 3). Moreover, sensitivity was not significantly influenced by tumor size or site, and, already high for tumors in the descending (76%) and ascending (79%) colon and rectal tract (81%), reached 100% for the small number of transverse tumors. Finally, there was a trend towards lower sensitivity for more advanced stage tumors (chi square for trend, $p = 0.057$), with the lowest true positive rate for patients with stage D tumors.

4. Discussion

In recent years, new tests based on the molecular analysis of fecal DNA have been proposed as promising tools for the early diagnosis of colorectal cancer. These tests have the advantage of being non-invasive, of not requiring bowel preparation, and of being capable of detecting tumors along the entire length of the colon and rectum.

Among the molecular targets tested up to now, genomic DNA integrity extracted from stool has proven to be potentially useful for discriminating between colorectal cancer and normal epithelial cells [5,26,28].

Table 1
FL-DNA fecal levels as a function of clinical and pathological characteristics

	Healthy donors			Patients			<i>p</i>
	No.	Median (ng)	Range	No.	Median (ng)	Range	
All	100	0	0–246	100	64	0–731	<0.0001
Gender							
F	57	0	0–246	43	64	0–731	
M	43	0	0–207	57	64	0–612	n.s.
Age (years)							
≤69	81	0	0–246	59	68	0–612	
>69	19	3	0–38	41	59	0–731	n.s.
Tumor site							
Descending				45	58	0–731	
Ascending				34	63	0–612	
Transverse				5	78	53–157	
Rectum				16	72	0–266	n.s.
Dukes' stage							
A				17	77	0–375	
B				45	61	0–612	
C				29	66	0–731	
D				7	28	0–133	n.s.
Tumor size (cm)							
0.1–3.9				41	53	0–731	
≥4.0				47	80	0–612	n.s.

Note: n.s. – not significant.

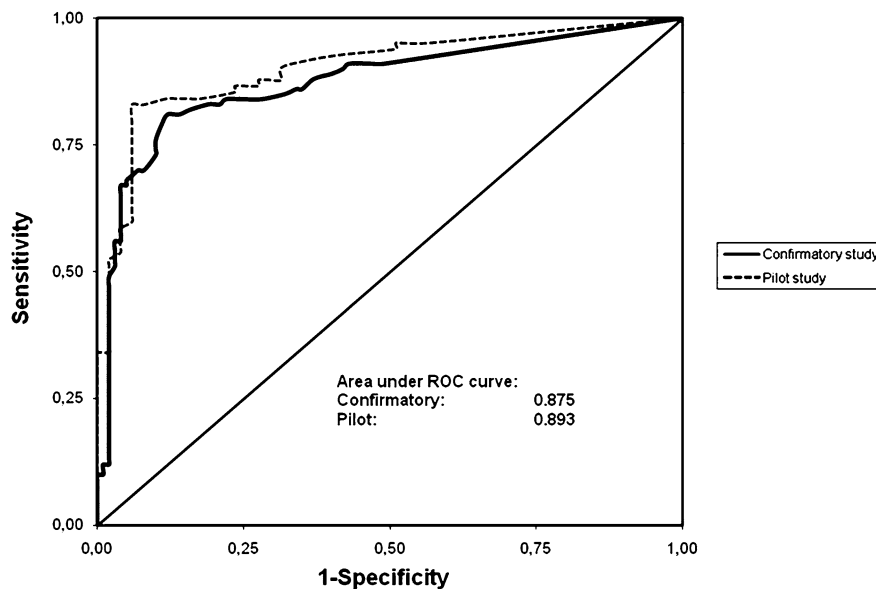


Fig. 1. Receiver operating characteristic (ROC) curve of FL-DNA analysis of the pilot and confirmatory studies.

The results reported by different studies on the diagnostic accuracy of fecal DNA are not consistent, probably due to the different methodologies used [6,20,25]

and to biases occurring during the pre-analytical phase. Bleeding and inflammation consequent to colonoscopy can cause falsely elevated long DNA levels and bowel

Table 2
Sensitivity and specificity of FL-DNA analysis

DNA levels	Healthy donors		Patients		% Sensitivity (95% CI)	% Specificity (95% CI)	% Accuracy (95% CI)
	Positive	Negative	Positive	Negative			
Cut-offs (ng)							
15	22	78	84	16	84 (77–91)	78 (70–86)	81 (76–86)
20	16	84	82	18	82 (76–90)	84 (77–91)	83 (78–88)
25	11	89	79	21	79 (71–87)	89 (83–95)	84 (79–89)
30	8	92	70	30	70 (61–79)	92 (87–97)	81 (76–86)
35	5	95	68	32	68 (59–77)	95 (91–99)	82 (77–87)
40	4	96	65	35	65 (56–74)	96 (92–100)	81 (76–86)

Table 3
Sensitivity and specificity* as a function clinical and pathological characteristics

	Healthy donors	Positive	Negative	% Specificity (95% CI)	Patients	Positive	Negative	% Sensitivity (95% CI)	<i>p</i>
Gender									
F	57	8	49	86 (77–95)	43	36	7	84 (73–95)	
M	43	3	40	93 (85–100)	57	43	14	75 (64–86)	n.s.
Age (years)									
≤69	81	8	73	90 (83–97)	59	47	12	80 (70–90)	
>69	19	3	16	84 (68–100)	41	32	9	78 (65–91)	n.s.
Tumor site									
Descending					45	34	11	76 (64–88)	
Ascending					34	27	7	79 (65–93)	
Transverse					5	5	0	100	
Rectum					16	13	3	81 (62–100)	n.s.
Dukes' stage									
A					17	15	2	88 (73–100)	
B					45	37	8	82 (71–93)	
C					29	21	8	72 (56–88)	
D					7	4	3	57 (20–95)	n.s.
Tumor size (cm)									
0.1–3.9					41	30	11	73 (59–87)	
≥4.0					47	38	9	81 (70–92)	n.s.

*Cut-off value: 25 DNA ng.

n.s. – not significant.

purgation may alter normal colon flora and interfere with DNA degradation. In our study, the pre-analytical phase was thus standardized to overcome these potential biases. In particular, as previously described, fecal samples from patients in our study were generally obtained several days after colonoscopy to allow for the restoration of normal bowel function. Moreover, stool samples were immediately frozen and stored at -80°C for a maximum of 2 months.

In Loktionov's study [18], it was observed that the quantity of DNA extracted from stool was higher in colorectal cancer patients than in healthy individuals,

suggesting that this approach could be useful for the early diagnosis of colorectal cancer. Recently, other studies have highlighted the possibility of distinguishing between colorectal cancer patients and healthy individuals using stool DNA integrity analysis [26,28]. We obtained interesting results of diagnostic relevance using quantitative long DNA fragment analysis developed in our laboratory [5].

The use of specific markers such as APC and p53 to determine DNA integrity rather than random genomic sequences was a logical follow-on to our previous papers. Our first study on molecular markers for the diag-

nosis of colorectal cancer was based on the analysis of gene alterations [4,21] and on a DNA integrity analysis comparing a non-quantitative approach, developed by Ahlquist et al. [1], with our fluorescent methodology [5], based on the analysis of DNA integrity of these specific oncogenes as target. In view of the interesting results obtained from these studies, and to maintain a correct confirmatory study design, we did not change the methodological approach in the present work.

In the present confirmatory case-control study, we validated the sensitivity and specificity of this test in a large and independent case series, and also confirmed the highest overall diagnostic potential for the 25-ng cut-off, observed in the pilot study [5]. The diagnostic potential of the molecular test appears to be independent of gender, age and tumor size, in agreement with the results from other studies [14,15,20]. Moreover, it was highly effective in detecting transverse colon and rectal tumors and more successful at detecting early rather than advanced cancers. This latter finding, which requires confirmation in a large series of stage D tumors, could be ascribable to a lower cell exfoliation of primary colorectal cancer in very advanced stages, as supported by the lower levels of DNA detected in stool from these patients.

Interestingly, the sensitivity of long DNA evaluation in detecting colorectal tumors is in line with or slightly better than that obtained with the most accurate molecular approaches currently available, which are mainly based on expensive multi-parametric analyses [3,6,14,15,28]. On the basis of these results and of cost-benefit considerations, a comparison study is currently being carried out on the same series of patients to evaluate the diagnostic potential of circulating DNA, another simple, rapid and non-invasive approach, which in our experience has shown to have a high diagnostic accuracy [8].

Our next objective will be to verify the diagnostic potential of the quantitative stool DNA test in symptomatic patients and high-risk individuals and to define its potential usefulness within large-scale FOBT screening programs. Moreover, in view of the long natural history of colorectal cancer evolution [6], a further interesting application of the test could be for the detection of high risk premalignant lesions. It is known that a large fraction of high grade adenomas actually progress into carcinomas. In fact, the sensitivity in detecting these lesions ranges from 20 to 45%, using non-invasive diagnostic tests based on fecal occult blood [2,9,17], and only few data are available on molecular tests [3,6]. The development of a qualitative analysis

of long DNA fragments from stool to identify high-risk adenomas would therefore constitute an important step forward in the area of early diagnosis [3,7,27].

In conclusion, too few results are available at present to propose this molecular test as an alternative to FOBT in screening programs. For this reason, our next step will be to validate these findings in a large-scale cohort study of a screening population, which is necessary prior to implementation into clinical practice. Ongoing studies are currently exploring the potential of this molecular approach for unmasking false FOBT positives to spare patients from invasive colonoscopy.

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