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Research Article

Improved Stool DNA Integrity Method for Early Colorectal Cancer Diagnosis

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Abstract

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

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

Results: Nonparametric ranking statistics were used to analyze the relationship between the median values of RT FL-DNA and the clinicohistopathologic characteristics. The median values of both variables were significantly higher in patients with cancer than in patients with noncancerous lesions. According to the Fagan nomogram results, the 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 semiautomatic 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. *Cancer Epidemiol Biomarkers Prev; 23(11); 2553–60. ©2014 AACR.*

Introduction

Colorectal cancer 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 tumor-suppressor genes, as well as oncogenes or mismatch repair genes (MMR; ref. 2).

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

Nevertheless, none of these methods is truly optimal due to different technical limits. FOBT is a cheap, noninvasive test but it has several limitations, such as low sensitivity, especially in detecting precancerous lesions, and low ability to distinguish benign and malignant precancerous lesions compared with 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 immunochemical FOBT (iFOBT) are invited to undergo a colonoscopy

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examination, but it is estimated that only 50% of individuals at average risk of the development of colorectal cancer 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 programs (6). The main difficulties involved in undergoing colonoscopies include psychologic 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 colorectal cancer (13).

Many new molecular noninvasive screening tests have been developed and investigated for the detection of colorectal cancer. Fecal DNA tests have been designed to detect molecular abnormalities present in precancerous 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 methods are related to time-consuming approaches and poor cost effectiveness compared with other screening tests. In fact, despite good sensitivity and specificity compared with iFOBT, the actual costs for analyses with molecular tests are too high to suggest their use in screening programs (6).

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 identification of colorectal cancers and to determine a patient's risk of harboring a preneoplastic 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; refs. 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 standardized method, based upon real-time PCR analysis combined with a semiautomatic 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 premalignant 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 program 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 to confirm the diagnosis. The lesion type was histologically confirmed and, in patients with cancer, the pathologic stage was defined in accordance with Dukes' classification. Preneoplastic 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 $\geq$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). Subjects were provided with instructions for collecting the fecal 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 hemoglobin value $\geq$100 ng/mL. Hemoglobin 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 2 months on the basis of results from preliminary experiments on DNA stability (19).

DNA extraction

Manual approach. A QIAamp DNA Stool Kit (Qiagen) was used for stool DNA purification as previously described (19).

Semiautomatic extraction. Five hundred microliters of helix tissue buffer (Diatech Pharmacogenetics) was added to the frozen pellet and after solution homogenization, the samples were centrifuged at 13,000 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 to 20 seconds. The solution was then incubated at 65°C for 30 minutes, agitating constantly ($V = 500$ rpm). The samples were then left to cool at room temperature and mixed for 15 to 20 seconds. After brief centrifugation, 400 μL from each sample was transferred into a HES Lysis Plate. From this step onward, the "*HELIX DNA*

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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-labeled primers and capillary electrophoresis as previously described (19). All samples were run in duplicate and only intersample 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. FL-DNA was analyzed by real-time PCR (RT FL-DNA). The following reagents were added to the stool sample (5 μL) used: Eurogentec MESA GREEN 1× 12.5 μL and Oligo-MixA 2 μL or Oligo-MixB 2 μL. Water (5.5 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 program 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 analyzing 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 intersample variations of <15% were accepted.

Statistical analysis

The objective of this study was to compare manual and semiautomatic systems to identify the best and least labor-intensive extraction system. FL-DNA concentrations were considered as a continuous variable and the median values between these methods were compared using the nonparametric ranking median test. The analysis of the FL-DNA concentration in the two methods 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 cutoff 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. Nonparametric ranking statistics (median test) were used to analyze the relationship between the median values of RT FL-DNA and the clinicohistopathologic characteristics. To estimate posttest probability, i.e., the probability of disease in a subject after the diagnostic test results are known, we first estimated the pretest probability and determined the likelihood ratio. The pretest probability is the chance of having the disease before 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 cutoff 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, posttest probability was calculated by multiplying the likelihood ratio of the diagnostic test by the pretest 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).

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 methodologic 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 semiautomatic systems, in combination with the best analytic tools between CE and RT FL-DNA. Second, we compared the efficiency of the semiautomatic 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 optimization

To set up the best DNA extraction method, the new semiautomatic approach and the standard manual protocol were tested in parallel. Starting with an amount of 10 mg of feces per sample, the DNA obtained using these two extraction approaches was amplified by two different multi-locus PCR and analyzed by gel electrophoresis showing that the semiautomatic system allows for higher yields of amplification products (data not shown). To verify whether any Taq inhibitors were present in the DNA solution, the DNA samples were analyzed by inhibition plasmid control. For this purpose, 25 ag of a plasmid containing a 150-bp non-human insert flanked by hybridization 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 CE. 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 semiautomatic

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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 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; Fig. 1).

The best cutoffs seem to range from 10 to 30 ng for both approaches. The CE approach seems to confirm the previous best cutoff of 25 ng in detecting tumors (18), with 57% (95% CI, 37–74) sensitivity and 84% (95% CI, 79–89) specificity, and 82% (95% CI, 76–86) accuracy (Table 1). Conversely, using the RT FL-DNA method, the best cutoff seems to be slightly lower. In particular, the cutoff of 15 ng showed 70% (95% CI, 49–84) sensitivity in detecting tumors, 87% (95% CI, 82–91) specificity, and 85% (95% CI, 80–89) accuracy. With a higher cutoff of 20 ng, the sensitivity decreased to 61% (95% CI, 41–78) but, conversely, an increase of specificity 91% (95% CI, 87–85) and accuracy 88% (95% CI, 84–92) was observed (Table 1). In addition, considering the accuracy of the two approaches in detecting not only tumor 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 clinicopathologic characteristics

Our series in this work, in accordance with the conclusions of the 2010 study performed by Calistri and colleagues (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 3,811 ng/mL. Individuals with no lesions and low-risk adenomas patients showed the lowest median iFOBT value of 4 ng/mL, both ranged from 0–1,000 ng/mL. In patients with high-risk adenomas, a higher median value was recorded of 13 ng/mL, ranging from 0 to 1,000 ng/mL. Considered overall, the median iFOBT value for these three subgroups was much lower than that observed for patients with cancer (1,000 ng/mL), ranging from 0 to 3,811 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 0–1,304 ng/reaction) in patients with cancer ($P < 0.0001$; Table 2).

A breakdown analysis for clinical and pathologic subgroups was performed with explorative intent. No differences were noted between healthy donors without any benign diseases or lesions and healthy donors with diverticula, hemorrhoids, inflammatory bowel disease, or benign polyps (data not shown). Moreover, there are no significant differences in the FL-DNA value in patients with tumor or adenomas as a function of characteristics such as size, stage, dimension, localization, 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).

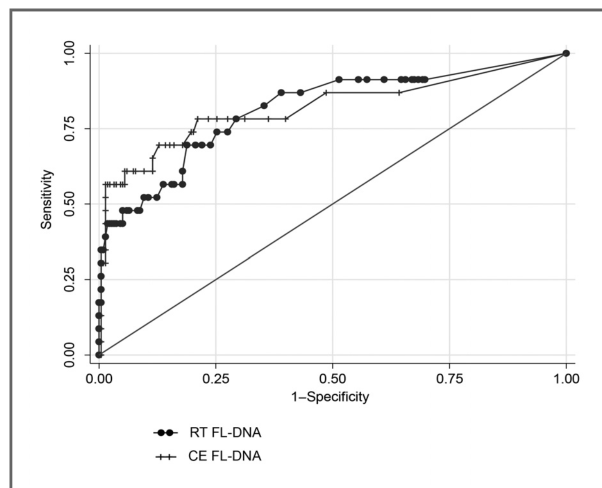

Figure 1. ROC curve. ROC curve of FL-DNA and RT-DNA analyses for the complete series of stool samples.

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 tumor and/or high-risk adenomas. According to the diagnostic relevance of fecal hemoglobin 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 with our previous work (19), in this study the analysis was extended to negative iFOBT values. All iFOBT values were divided into three main subgroups: 0–99 ng/mL, 100–432 ng/mL, and >432 ng/mL, whereas FL-DNA results were divided into three classes according to different cutoff values (0–9, 10–30, and ≥30 ng/reaction), suggested in the previous article (19). In the negative-iFOBT subgroup, the pretest probability of there being a tumor 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 tumor 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 colorectal

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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 tumor and tumor and high-risk adenoma patients

	CE FL-DNA					RT FL-DNA				
Cutoffs (ng)	CRC	Others[a]	% Sensitivity (95% CI)	% Specificity (95% CI)	% Accuracy (95% CI)	CRC	Others[a]	% Sensitivity (95% CI)	% Specificity (95% CI)	% Accuracy (95% CI)
	Positive					Positive				
≥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	**Others[b]**				**CRC + HRA**	**Others[b]**			
	Positive					Positive				
≥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)

NOTE: Sensitivity, true-positive rates; specificity, true-negative rates; accuracy, number of true positive plus number of true negative, divided by the total series.

Abbreviations: CRC, colorectal cancer patients; HRA, high-risk adenoma patients.

[a]Other, high- and low-risk adenomas and healthy subjects.

[b]Other, low-risk adenomas and healthy subjects.

cancer and high-risk adenomas increased the posttest 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 cutoff was slightly lower than that of CE FL-DNA, we performed the iFOBT and FL-DNA combination analysis considering different cutoff ranges (0–14, 15–24, and ≥25 ng/reaction). Substantially different results were not observed (Supplementary Table S1).

Discussion

iFOBT is the most widely used method in screening programs, 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 preneoplastic 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 analyzing DNA integrity based upon semiautomatic DNA extraction and RT PCR. Our results show that DNA integrity status evaluated using the RT FL-DNA assay and extracted using a semiautomatic approach could be considered as a sensitive and specific marker for early colorectal cancer detection. Moreover, we observed that RT FL-DNA was more accurate than the previous CE FL-DNA method in detecting high-risk adenomas.

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

	N	RT FL-DNA	iFOBT
	241	**Median value (ng/reaction; range)**	**Median value (ng/mL; range)**
No lesions	149	2 (0–2,140)	4 (0–1,000)
Low-risk adenomas	35	1 (0–31)	4 (0–1,000)
High-risk adenomas	34	1 (0–75)	13 (0–1,000)
Colorectal cancer	23	49 (0–1,304)	1000 (0–3,811)
		P < 0.0001	*P* < 0.0001

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

	Cases ($N = 69$)	RT FL-DNA (ng/reaction) Median (range)	P	iFOBT (ng/mL) Median (range)	P
Gender					
Male	43	0.68 (0–30.6)		9 (0–1,000)	
Female	26	1.37 (0–74.5)	0.871	8 (0–1,000)	0.663
Patient classification					
Low-risk	35	1.45 (0–30.6)		4 (0–1,000)	
High-risk	34	0.65 (0–74.5)	0.379	13 (0–1,000)	0.138
Lesion dimension					
0–0.9 cm	40	1.47 (0–30.6)		4.5 (0–1,000)	
≥ 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 (0–1,000)	
Multiple	20	1.37 (0–30.6)	0.446	10.5 (0–753)	0.361
Lesion localization					
Descending + transverse	11	0.50 (0–26.8)		2 (0–75)	
Ascending	22	0.29 (0–30.6)		15 (0–1,000)	
Mixed	5	1.05 (0–19.9)	0.637	1 (0–485)	0.169

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 tumors and high-risk adenomas, thus overcoming the limitations of the occult blood test detection. In the positive-iFOBT-value subgroups, fecal RT FL-DNA provided more accurate diagnostic information and identified subgroups with different probability of having a tumor. Interesting results were also obtained by evaluating high-risk adenoma and tumor 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 posttest probability.

Our results would seem to indicate that this molecular method could be a useful addition to the conventional iFOBT in colorectal cancer screening programs. However, the transfer of new diagnostic approaches to clinical practice is often hindered by problems relating to time-consuming methods and costs of individual tests. Song

Table 4. RT FL-DNA and iFOBT values in patients with colorectal cancer according to clinicopathologic characteristics

	Cases ($N = 23$)	RT FL-DNA (ng/reaction) Median (range)	P	iFOBT (ng/mL) Median (range)	P
Gender					
Male	17	40.23 (0.0–1,303.95)		1,000 (1–3,707)	
Female	6	60.39 (0.0–387.14)	0.528	1,000 (0–3,811)	0.807
Duke stage					
A	7	40.23 (1.88–323.04)		1,000 (219–2,786)	
B	11	64.46 (1.56–1,303.95)		1,000 (1–3,811)	
C+D	2	33.49 (8.60–58.38)	0.345	1,000 (1,000–1,000)	0.960
TNM classification					
T1	7	40.23 (1.88–323.04)		1000 (219–2,786)	
T2	5	113.94 (51.5–387.14)		534 (1–1,000)	
T3	8	38.17 (1.56–1,303.95)	0.170	1,464 (396–3,811)	0.052
Lesion dimension					
0–0.9 cm	5	64.46 (40.23–202.57)		2,018 (217–3,707)	
≥1 cm	13	58.38 (1.88–1,303.95)	0.628	1,000 (241–3,811)	0.922

Abbreviation: TNM, tumor-node metastasis.

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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 Posttest probability (pretest = 0.0128)	iFOBT 100–432 Posttest probability (pretest = 0.116)	iFOBT >432 Posttest probability (pretest = 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 Posttest probability (pretest = 0.160)	iFOBT 100–432 Posttest probability (pretest = 0.256)	iFOBT >432 Posttest probability (pretest = 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)				

and colleagues (23) estimated the costs of fecal molecular tests as being between $350 and $795, whereas the cost of colonoscopies ranges from $1,200 to $1,800, depending upon the localization of the lesions.

Studies assessing the best cost–benefit ratio through the creation of a computer simulation of screening for colorectal cancer 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 2 years and with a sensitivity of 65% for colorectal cancer 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 and colleagues. 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 analyzed 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 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 personalized surveillance intervals in individuals undergoing the current standard colorectal cancer screening methods. Innovative and personalized diagnoses and therapies against cancer are the main aims of all future clinical trials.

Disclosure of Potential Conflicts of Interest

M. Menghi is an employee of Diatech Pharmacogenetics. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: C. Rengucci, F. Falcini, D. Calistri
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Rengucci, P. Fusaroli, G. Caletti, F. Falcini
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Rengucci, G. De Maio, E. Scarpi, D. Calistri
Writing, review, and/or revision of the manuscript: C. Rengucci, G. De Maio, M. Menghi, E. Scarpi, D. Amadori, D. Calistri
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References

1. Garcia-Bilbao A, Armananzas R, Ispizua Z, Calvo B, Alonso-Varona A, Inza I, et al. Identification of a biomarker panel for colorectal cancer diagnosis. BMC Cancer 2012;12:43.
2. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67.
3. Levin B, Lieberman DA, McFarland B, Andrews KS, Brooks D, Bond J, et al. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. Gastroenterology 2008;134:1570–95.
4. Mandel JS, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, et al. The effect of fecal occult-blood screening on the incidence of colorectal cancer. N Engl J Med 2000;343:1603–607.
5. Atkin WS, Edwards R, Kralj-Hans I, Wooldrage K, Hart AR, Northover JM, et al. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial. Lancet 2010;375:1624–33.
6. Quintero E, Castells A, Bujanda L, Cubiella J, Salas D, Lanas Á, et al. Colonoscopy versus fecal immunochemical testing in colorectal-cancer screening. N Engl J Med 2012;366:697–706.
7. Hol L, van Leerdam ME, van Ballegooijen M, van Vuuren AJ, van Dekken H, Reijerink JC, et al. Screening for colorectal cancer: randomised trial comparing guaiac-based and immunochemical fecal occult blood testing and flexible sigmoidoscopy. Gut 2010;59:62–8.
8. Van Rossum LG, van Rijn AF, Laheij RJ, van Oijen MG, Fockens P, van Krieken HH, et al. Random comparison of guaiac and immunochemical fecal occult blood tests for colorectal cancer in a screeningpopulation. Gastroenterology 2008;135:82–90.
9. Yeazel MW, Church TR, Jones RM, Kochevar LK, Watt GD, Cordes JE, et al. Colorectal cancer screening adherence in a general population. Cancer Epidemiol Biomarkers Prev 2004;13:654–57.
10. Subramanian S, Klosterman M, Amonkar MM, Hunt TL. Adherence with colorectal cancer screening guidelines: a review. Prev Med 2004;38:536–50.
11. Weitzman ER, Zapka J, Estabrook B, Goins KV. Risk and reluctance: understanding impediments to colorectal cancer screening. Prev Med 2001;32:502–13.
12. Moawad FJ, Maydonovitch CL, Cullen PA, Barlow DS, Jenson DW, Cash BD. CT colonography may improve colorectal cancer screening compliance. AJR Am J Roentgenol 2010;195:1118–23.
13. Baxter NN, Goldwasser MA, Paszat LF, Saskin R, Urbach DR, Rabeneck L. Association of colonoscopy and death from colorectal cancer. Ann Intern Med 2009;150:1–8.
14. Bosch LJ, Carvalho B, Fijneman RJ, Jimenez CR, Pinedo HM, van Engeland M, et al. Molecular tests for colorectal cancer screening. Clin Colorectal Cancer 2011;10:8–23.
15. Ahlquist DA. Molecular detection of colorectal neoplasia. Gastroenterology 2010;138:2127–39.
16. Calistri D, Rengucci C, Bocchini R, Saragoni L, Zoli W, Amadori D. Fecal multiple molecular tests to detect colorectal cancer in stool. Clin Gastroenterol Hepatol 2003,1:377–83.
17. Calistri D, Rengucci C, Lattuneddu A, Francioni G, Polifemo AM, Nanni O, et al. Detection of colorectal cancer by a quantitative fluorescence determination of DNA amplification in stool. Neoplasia 2004;6:536–40.
18. Calistri D, Rengucci C, Molinari C, Ricci E, Cavargini E, Scarpi E, et al. Quantitative fluorescence determination of long-fragment DNA in stool as a marker for the early detection of colorectal cancer. Cell Oncol 2009;31:11–17.
19. Calistri D, Rengucci C, Casadei Gardini A, Frassineti GL, Scarpi E, Zoli W, et al. Fecal DNA for noninvasive diagnosis of colorectal cancer in immunochemical fecal occult blood test-positive individuals. Cancer Epidemiol Biomarkers Prev 2010;19:2647–54.
20. Available from: http://www.nccn.org/professionals/physician_gls/PDF/colorectal_screening.pdf [accessed June 2014].
21. Sonnenberg A, Delcò F, Inadomi JM. Cost effectiveness of colonoscopy in screening for colorectal cancer. Ann Intern Med 2000;133:573–84.
22. Burch JA, Soares-Weiser K, St John DJB, Duffy S, Smith S, Kleijnen J, et al. Diagnostic accuracy of fecal occult blood tests used in screening for colorectal cancer: a systematic review. J Med Screen 2007;14:132–37.
23. Song K, Fendrick AM, Ladabaum U. Fecal DNA testing compared with conventional colorectal cancer screening methods: a decision analysis. Gastroenterology 2004;126:1270–79.
24. Osborn NK, Ahlquist DA. Stool screening for colorectal cancer: molecular approaches. Gastroenterology 2005;128:192–206.

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