Identification of Subjects at Risk for Colorectal Carcinoma Through a Test Based on K-*ras* Determination in the Stool

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Background & Aims: **The gold standard for screening for colorectal carcinoma is colonoscopy. The aim of this study was to compare endoscopic results with those obtained using the noninvasive screening test of K-*ras* determination in the stool in a large population of patients undergoing colonoscopy.** ***Methods:*** **Two hundred thirty consecutive patients were studied by K-*ras* amplification on stool-derived DNA using polymerase chain reaction and oligomer-specific hybridization.** ***Results:*** **Wild-type K-*ras* was amplified in 103 of 230 patients (44.8%), the rate of amplification being directly proportional to the presence of an organic disease of the intestine characterized by hyperproliferating mucosa. In 30 of these 103 patients (29.1%), a K-*ras* mutation was found. Four of 5 with early colorectal carcinoma, all who had K-*ras* mutations in the tumor, were identified. In first-degree relatives of patients with colorectal carcinoma, all subjects either carrying adenomas >1 cm in diameter or multiple smaller adenomas were identified. In patients with inflammatory bowel disease, the test identified the only patient with neoplastic transformation.** ***Conclusions:*** **The sensitivity and specificity of K-*ras* determination on stool-derived DNA in patients with colorectal carcinoma, in first-degree relatives of patients with colorectal carcinoma, and in patients with inflammatory bowel disease support the opportunity of a large-scale trial to validate its use as a screening test.**

Sporadic colorectal cancer (CRC) occurs in Western countries at a frequency second only to lung cancer.[1] If diagnosed at an early stage, CRC can be cured either by endoscopic excision or by surgery. The use of occult blood tests as a screening test for early identification of neoplastic lesions has shown controversial results[2–8]; although all trials showed an increased discovery of early colorectal cancers, only one showed a reduction in mortality.[3] Therefore, there is still uncertainty about the sensitivity and specificity of this test, with some authors suggesting that a significant proportion of cancers detected may be chance discoveries.[7,9] Colonoscopy remains the neoplastic lesions; however, it cannot be used for mass screening because of its cost and invasiveness. Studies using colonoscopy for screening are in progress in selected populations such as patients older than 60 years of age.[10]

Apart from genetically determined colonic tumors, several changes in proto-oncogenes and tumor-suppressor genes have been shown in sporadic colorectal carcinogenesis[11] and, recently, also in colitis-associated tumors.[12] Although the model proposed for tumorigenesis suggests that it is the accumulation of multiple alterations rather than the specific order of them that is important for the development of tumor, K-*ras* mutations occur earlier than *p53* mutations and are observed with a similar prevalence in carcinomas and in intermediate-stage adenomas.[11] Furthermore, most of the reported mutations cluster in a rather short fragment of DNA (i.e., the first exon of K-*ras* gene), making the exploration of possible mutations in genomic material derived from intestinal mucosa easier, whereas mutations of *APC* or *p53* gene are dispersed in a rather longer fragment of DNA.[11]

Sidransky et al.[13] were first to report the possibility of using DNA extracted from the stool for identifying mutations of DNA derived from intestinal mucosa; they found mutations in 8 patients with colorectal tumor. We tested this method as a noninvasive screening test for colorectal tumors or colorectal adenomas in a large population of nonselected patients undergoing diagnostic colonoscopy.

Materials and Methods

Patients

Stool specimens were collected from 230 nonselected patients undergoing diagnostic colonoscopy from September 1993 to June 1994. The indications for colonoscopy were rectal bleeding in 6 patients (1 first-degree relative of a patient with

Abbreviations used in this paper: CRC, colorectal cancer; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

Table 1. Demographic Characteristics of Patients in the Different Categories

	CRC	History of CRC	Adenomas (including hyperplastic polyps)	History of adenomas	IBD	Lymphocytic colitis	No organic disease
No. of patients	5	31	55	67	20	6	46
Age (*yr*, mean ± SD)	62 ± 16	67 ± 8	59 ± 11	62 ± 10	41 ± 13	67 ± 6	50 ± 14
M/F	3/2	21/10	37/18	50/17	11/9	2/4	14/32
Personal history of tumors other than CRC	0/5	0/31	1/42[a]	1/67[b]	0/20	0/6	1/46[c]
Family history of CRC	1/5	4/31	20/42	10/67	0/20	0/6	19/46
Family history of tumors other than CRC	0/5	0/31	2/42[a,d]	4/67[a,b,d]	0/20	0/6	2/46[b,e]

[a]Prostate.
[b]Lung.
[c]Uterus.
[d]Stomach
[e]Pancreas.

CRC); surveillance endoscopy after surgery for CRC in 31 patients (4 first-degree relatives of a patient with CRC); surveillance endoscopy for previously excised adenomas in 67 patients (10 first-degree relatives of patients with CRC); surveillance endoscopy for inflammatory bowel disease (IBD) follow-up in 19 patients; symptoms of irritable bowel syndrome, diarrhea, or constipation in 68 patients; and screening for family history of CRC in 39 patients.

The goal of the study was explained to the patients, and they were asked to bring a sample of stool 1 week after colonoscopy before excision of the tumor or polyp. Samples were collected and coded by personnel unaware of the results of endoscopy. Results of the molecular analysis (performed under code) and endoscopy were matched only at the end of the investigation. Stool specimens were frozen at −80°C until use.

All patients agreed to participate in the study; in a subgroup of patients with CRC, bioptic specimens were obtained for comparison with the results of the stool test. All of these patients gave written informed consent for use of the bioptic specimens not for diagnostic purposes but for use in the present study. The study was approved by the Institutional Human Research Committee of the University of Modena.

Each patient completed a questionnaire with the following information: age, sex, results of previous colonoscopies (when present), histology of adenomas (when present), previous surgery in the gastrointestinal tract for tumors, family history of intestinal tumors, and family history of tumors elsewhere than the gastrointestinal tract. These data are summarized in Table 1.

Detection of K-*ras* Mutations

DNA was extracted from stool specimens according to the method described by Sidransky et al.[13] with minor modifications. Different amounts of stool specimens (100 mg, 500 mg, 1 g, and 5 g) were extracted in a pilot experiment (data not shown) to evaluate the optimal quantity of stool to use. The best results, in terms of number of positive amplifica-

Therefore, this amount was routinely used. Samples of stool were diluted in 300 μL lysis buffer (500 mmol/L Tris-HCl, 16 mmol/L ethylenediaminetetraacetic acid, and 10 mmol/L NaCl (pH 9.0), and particulate material was removed by centrifugation at 12,000*g* for 2 minutes. Proteins were digested with sodium dodecyl sulfate (SDS)–proteinase K (20 mg/mL) overnight at 37°C or for 1 hour at 65°C, extracted twice with phenol-chloroform, and ethanol precipitated. The extracted DNA (0.5–9 μg) was incubated in a total volume of 100 μL of a solution containing 10 mmol/L Tris-HCl (pH 7.2); 50 mmol/L KCl; 2 mmol/L $MgCl_2$; 500 μmol/L each of deoxyadenosine triphosphate, deoxyguanine triphosphate, deoxycytidine triphosphate, and deoxyribosylthymine triphosphate; 100 ng of primers located in the first *ras* exon (sense primer: 5′ AGG AAT TCA TGA CTG AAT ATA AAC TTG 3′; antisense primer: 5′ ATC GAA TTC CTC TAT TGT TGG ATC ATA TTC 3′, giving rise to an amplified fragment of 202 base pairs), and 1 U of *Taq* DNA polymerase (Boehringer Mannheim, Milan, Italy). Forty-five cycles (30 minutes at 95°C, 3 minutes at 58°C, and 2 minutes at 70°C) were performed. Amplification products were electrophoresed through 1.8% agarose gel and ethidium bromide stained. Electrophoresed polymerase chain reaction (PCR) products were transferred to nylon membranes (Hybond N; Amersham, Milan, Italy) and prehybridized in a mixture containing 50% formamide, 5× standard saline citrate (SSC) (0.75 mol/L NaCl and 0.075 mol/L sodium citrate), 5× Denhardt's solution, 0.1% Ficoll (type 400; Pharmacia, Milan, Italy), 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin (fraction V; Sigma, Milan, Italy), and 50 mmol/L $NaHPO_4$ (pH 7.0) at 42°C for at least 2 hours. Hybridization was performed under stringent conditions in a mixture containing 50% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt's, and 20 mmol/L $NaPO_4$ (pH 7.0) in the presence of 100 μg/mL of denatured salmon sperm DNA at 42°C overnight. As probes, specific oligonucleotides located between the amplification primers and specific for each mutation, terminally labeled with [γ-^{32}P]

were used. Stringent washing conditions were used; after rinsing the nylon membranes in 3× SSC plus 0.1% SDS at room temperature for 5 minutes, they were washed in 3× SSC plus 0.1% SDS for 30 minutes at approximately 10°C below the estimated melting temperature for each probe. Autoradiography was performed for 6, 12, and 36 hours at −80°C with an intensifying screen; samples were considered positive when a clear band was present after 36 hours of exposure. No cross-hybridization was observed between the mutated probes.

As internal control for DNA origin and quality, in an initial group of 30 patients, β-globin was amplified using the following primers: sense, 5′ GGT TGG CCA ATC TAC TCC AGG 3′; antisense, 5′ TGG TCT CCT TAA ACC TGT CTT G 3′, which give rise to an amplified fragment of 270 base pairs.

To reduce the risk of contamination and false-positive results, a number of precautions were taken as already described.[14] As positive control, DNA extracted from surgical specimens of normal and neoplastic colon mucosa, known to be positive for K-*ras* mutation, were used in each run of experiment.

In 5 patients with CRC at colonoscopy, bioptic specimens of the tumor were obtained and frozen at −80°C until processed for DNA extraction. In 10 patients who had undergone surgery for CRC, tumor tissue embedded in paraffin was available for amplification; DNA was extracted as described previously[14] and amplified as described in Materials and Methods.

Statistical Analysis

Data were analyzed using the χ^2 test with Yates' correction when appropriate or using two-tailed Fisher's Exact Test. A P value of <0.05 was considered significant.

Results

DNA obtained from 100 mg of stool ranged from 0.5 to 9 μg. Differences were present among the various groups of patients studied concerning both the purity and the total quantity recovered (Table 2).

K-*ras* fragments were amplified from DNA extracted from stool in 103 of 230 patients (44.7%). A K-*ras* mutation was identified in 30 of these 103 patients (29.1%); the mutation identified was Asp^{12} in 21 of 30 patients (70.0%; Figure 1), Val^{12} in 5 (16.6%), and Asp^{13} in 4 (13.3%). Table 3 shows the percentages of amplified K-*ras* fragments and identified mutations in the patients divided according to endoscopic diagnosis.

Table 2. Characteristics of DNA Extracted From Stool

	A_{260}/A_{280}	Total amount of DNA obtained from 100 mg stool (μg)
CRC	2.0	7.3 ± 1.1
Patients undergoing surgery for CRC		
Without recurrence of polyps	1.9	3.4 ± 2.2
With recurrence of polyps	1.8	5.9 ± 2.1
Adenomas	1.9	6.4 ± 2.8
Active IBD	2.0	7.5 ± 1.6
Quiescent IBD	1.9	2.9 ± 0.8

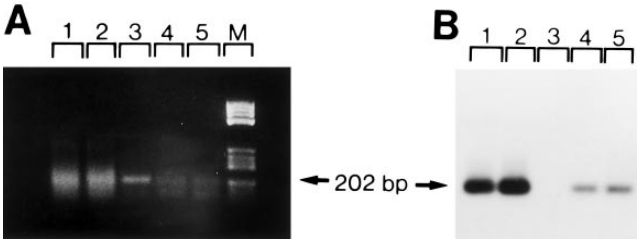

Figure 1. (*A*) Ethidium bromide staining and (*B*) Southern blot analysis of K-*ras* fragments on DNA from the stool. DNA was amplified by PCR as described in Materials and Methods and hybridized with Asp^{12}-specific probe. *Lanes 1* and *2*, patients with colorectal carcinoma; *lane 3*, patient with ulcerative colitis; *lanes 4* and *5*, patients with adenomas, belonging to the subgroup of CRC relatives. M, molecular weight marker; pBR 322 DNA, *Hae* III digested.

In a subgroup of 30 patients (4 with CRC, 8 with adenomas [5 with a diameter of >1 cm, 3 with <1 cm], 4 with IBD, and 14 without endoscopic abnormalities), DNA was also amplified with β-globin primers. β-Globin was successfully amplified in all patients with CRC, in the 5 patients with adenomas >1 cm in diameter, and in the 4 patients with IBD. No amplification was obtained in patients with adenomas <1 cm in diameter, whereas a positive amplification was found in 4 of 14 patients (28.5%) without endoscopic abnormalities.

In 4 of 5 patients in whom a carcinoma (4 in T1 stage and 1 in T2 stage according to Hutter et al.[15]) was found, K-*ras* fragments were amplifiable; Asp^{12} mutation was found in 3 of these patients, and Asp^{13} mutation was found in 1 patient.

In patients who had undergone surgery for CRC, the rate of amplification of K-*ras* fragments was significantly higher in those who had a recurrence of adenomas during follow-up than in patients without adenomas found during follow-up ($P < 0.01$) (Table 3). A higher, although not significantly different, number of K-*ras* mutations was found in patients with recurrent adenomas compared with patients without recurrences (3 of 7 vs. 3 of 24).

Among 42 subjects with adenomas at colonoscopy, K-*ras* was amplifiable in 30 (71.4%). In 19 of these patients, the polyp was single and the diameter was ≥1; in the other 11 patients, several small adenomas (4–7 mm in diameter) were present. A mutation was present in 12 patients (28.5%): 11 with adenomas ≥1 cm and 1 with multiple small adenomas (4–7 mm). In the 12 subjects with adenomas in whom K-*ras* fragments were not amplified, polyps were single and <8 mm in diameter.

Table 3. Results of K-*ras* Amplification in All Patients Studied

	n	K-*ras* fragments (%)	Mutated K-*ras* (%)
Patients with colon cancer	5	5 (100)	4 (80.0)
Patients undergoing surgery for colon cancer	31	19 (61.2)	6 (19.3)
With adenomas during follow-up	7	7 (100)[a]	3 (42.8)
Without adenomas during follow-up	24	12 (50.0)[a]	3 (12.5)
Patients with adenomas	42	30 (71.4)	12 (28.5)
Patients with history of adenomas	67	11 (16.4)	2 (2.9)
Patients with hyperplastic polyps	13	5 (38.4)	1 (7.7)
IBD	20	13 (65.0)	3 (15.0)
Lymphocytic colitis	6	6 (100)	0
No organic diseases	46	15 (32.6)	2 (4.3)
Total	230	104 (45.2)	30 (13.0)

[a] $P < 0.01$.

no adenomas were found; K-*ras* fragments were amplifiable in only 11 and a mutation was present in only 2 (2.9%). In 13 other patients with an adenoma excised in the past, only hyperplastic polyps were found; K-*ras* was amplified in 5 (38.4%), but a mutation (Asp^{13}) was detected in only 1.

First-Degree Relatives of Patients With Colon Cancer

Fifty-four subjects were first-degree relatives of patients with colon cancer. The distribution of results in this subgroup of patients is shown in Table 4. The difference in the rate of amplification of K-*ras* fragments is particularly evident, although not significant, in patients with previous surgery for colon cancer and in patients without organic disease at colonoscopy. The difference becomes significant both in the rate of amplification of K-*ras* ($P < 0.05$) and in the frequency of K-*ras* mutations ($P < 0.01$) when the whole group of relatives of patients with colon cancer is compared with that of nonrelatives (Table 4).

In patients with adenomas, the rate of amplification of K-*ras* fragments was not significantly different between the two subgroups (relatives and nonrelatives), whereas the difference in the rate of demonstration of K-*ras* mutations was statistically significant ($P < 0.01$) (Table 4). The diameter of the adenomas found at colonoscopy in CRC kindred was ≥1 cm in 12 of 20 patients; 10 of these 12 patients had K-*ras* mutations (83.3%). In nonrelatives, only 7 of 22 subjects had polyps >1 cm and only 1 subject had mutations (14.0%) ($P < 0.05$, relatives vs. nonrelatives).

IBD

In patients with IBD, independently from having ulcerative colitis or Crohn's disease, there was a marked difference between patients with active and widespread disease and patients with inactive disease; K-*ras* fragments were amplified in a very high percentage of patients with active disease and in none with inactive disease (Table 5). Mutations were found in only 3 patients, all with pancolitis. One patient had Crohn's disease diagnosed 14 years earlier (Asp^{13}). The other 2 patients (both with evidence of Asp^{12} mutation) had ulcerative colitis;

Table 4. Results of K-*ras* Amplification in DNA From Stool From Different Categories of Patients Divided According to Presence of History of Colon Cancer in a First-Degree Relative

	CRC kindred			Nonrelatives		
	n	K-*ras* fragments (%)	Mutated K-*ras* (%)	n	K-*ras* fragments (%)	Mutated K-*ras* (%)
Patients with colon cancer	1	1 (100)	1 (100)	4	3 (75.0)	3 (75.0)
Patients undergoing surgery for colon cancer	4	4 (100)	2 (50.0)	27	15 (55.5)	4 (14.8)
Patients with adenomas	20	13 (65.0)	10 (50.0)[a]	22	17 (77.2)	2 (9.0)[a]
Patients with history of adenomas	10	2 (20.0)	1 (10.0)	57	9 (15.8)	1 (1.7)
No organic diseases	19	9 (47.3)	1 (5.2)	27	6 (22.2)	1 (3.7)
Total	54	29 (53.7)[b]	15 (27.7)[c]	137	50 (36.9)[b]	11 (8.0)[c]

Table 5. Percentage of K-*ras* Fragments and Mutated K-*ras* in Patients With IBD and Lymphocytic Colitis

	n	K-*ras* fragments (%)	Mutated K-*ras* (%)
Ulcerative colitis	15		
Active disease	10	9 (90.0)	2 (20.0)
Inactive disease	5	0	0
Crohn's disease	5	4 (80.0)	1 (20.0)
Lymphocytic colitis	6	6 (100)	0

NOTE. Among the patients with ulcerative colitis, all but 1 with active disease had pancolitis.

1 patient, a 46-year-old man with ulcerative colitis diagnosed 13 years earlier, underwent colectomy for deep impairment of clinical conditions. He did not have endoscopic evidence of neoplasm, but microscopic examination of the excised colon showed three small, plaque-like carcinomas about 2 cm in diameter in the right colon. The other patient, a 55-year-old man, was diagnosed at first colonoscopy performed for rectal bleeding. Histological analysis showed polymorphs infiltrating the epithelium, mucin depletion, and a slight increase in the number of nuclei, which were also hyperchromatic. These changes were considered indefinite for dysplasia, and colectomy was delayed.

Six patients (2 men and 4 women; mean age, 67 ± 6 years) had a diagnosis of lymphocytic colitis[16,17]; these patients had a normal endoscopic appearance apart from slight hyperemic changes. Histologically, mild inflammatory infiltrates of the surface epithelium and of the lamina propria were present. K-*ras* fragments were amplifiable in all of these patients, but none had evidence of K-*ras* mutation (Table 5).

On the whole, K-*ras* fragments were amplified in 54 of 56 patients (96.4%) with conditions associated with hyperproliferating mucosa and increased cellular shedding into the intestinal lumen (5 CRCs, 19 adenomas >1 cm, 11 multiple adenomas, 15 active IBD, and 6 lymphocytic colitis). Instead, this gene has been amplified in only 15 of 63 patients (23.8%) either with no organic disease (46 subjects) or with substantially benign conditions (12 small adenomas <8 mm in diameter or 5 inactive IBD). The difference is highly significant ($P < 0.0001$). K-*ras* mutations were also differently distributed in the two subgroups (19 of 56 vs. 2 of 62; $P < 0.001$).

Presence of K-*ras* Mutation in DNA From Stools and in Cancer Tissue

Colonic tissue derived from the tumoral lesion was available for investigation of K-*ras* mutation in all who had undergone surgery for CRC, 6 of whom had a K-*ras* mutation. In patients with actual CRC at colonoscopy, results of amplification on DNA from stool and from colonic tissue were the same, whereas in patients with previous surgery for CRC, PCR on DNA from stool failed to identify a K-*ras* mutation in 1 of 6 (16.6%) (Table 6). The only patient characterized by Asp^{13} mutation had a recurrence during the period of the study, whereas all others remained tumor-free.

Discussion

K-*ras* mutations have been found to occur during colorectal carcinogenesis in a stage that roughly corresponds to transition from early to intermediate adenoma; they could be involved in the conversion of small adenomas to more dysplastic ones with progression to carcinoma.[11,18] They are mostly localized on codon 12 and, at a lesser degree, on codon 13. This clustering in a short fragment of DNA, about 150 base pairs in size, is very convenient for reliable and easy PCR amplification, even on DNA derived from stool (which could be more degraded than that derived directly from the intestinal mucosa).[13]

Table 6. Comparison Between K-*ras* Findings in the DNA Extracted From Stool and From Tumor Tissue of Patients With CRC and Patients With Previous Surgery for CRC

	K-*ras*	
	Tumor tissue	Stool
Patients with CRC		
1	Wild type	Wild type
2	Asp^{12}	Asp^{12}
3	Asp^{12}	Asp^{12}
4	Asp^{12}	Asp^{12}
5	Asp^{13}	Asp^{13}
Patients with previous surgery for CRC		
1	Wild type	Wild type
2	Wild type	Wild type
3	Wild type	Wild type
4	Wild type	Wild type
5	Asp^{12}	Asp^{12}
6	Asp^{12}	Asp^{12}
7[a]	Asp^{12}	Asp^{12}
8[a]	Asp^{12}	Asp^{12}
9[a]	Asp^{13}	Asp^{13}
10	Val^{12}	Negative

NOTE. Patients with CRC were studied by PCR on DNA derived from fresh-frozen tumor tissue and patients with previous surgery for CRC by DNA extracted from paraffin-embedded tumor tissue.

[a]Patients 7–9 had a recurrence of adenomas during follow-up. Patient 9 had also local tumor recurrence diagnosed 1 1/2 years after surgery and 1 year after collection of stool for K-*ras* testing. No tumor tissue

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