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Combined Analysis of Microsatellite Instability and K-*ras* Mutation Increases Detection Incidence of Normal Samples from Colorectal Cancer Patients

Toshinari Minamoto,[1] Hiroyasu Esumi, Atsushi Ochiai, Gennady Belitsky, Masayoshi Mai, Takashi Sugimura, and Ze'ev Ronai[2]

The Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York 10029-6574 [T. M., Z. R.]; National Cancer Center Research Institute, Tokyo 104, Japan [H. E., A. O., T. S.]; Russian Academy of Medical Sciences, Moscow 115478, Russian Federations [G. B.]; and Cancer Research Institute, Kanazawa University, Kanazawa 921, Japan [M. M.]

ABSTRACT

Microsatellite instability (MI) and K-*ras* oncogene mutation have been widely used as biomarkers of genetic changes in colorectal cancer (CRC). Each of these biomarkers was independently found in normal-appearing colonic mucosa at stages preceding the development of CRC, albeit at a relatively low incidence. To assess the potential value of combined MI and K-*ras* mutation analysis in the detection of normal-appearing colonic mucosa samples taken from patients with CRC, we have chosen to analyze multiple (3–7) normal colonic mucosa samples and the respective colorectal tumor tissues from 20 patients with CRC. As a control, we have used 54 normal mucosa samples obtained from 9 autopsies of patients without CRC. In at least 1 of 5 loci analyzed, MI was found in 8 of 20 patients via analysis of multiple normal-appearing colonic mucosa samples from each patient. Combined analysis of MI and mutant *ras* alleles in normal-appearing colonic mucosa samples enabled the identification of 11 of 20 patients with CRC. None of the 54 normal colonic mucosa samples obtained from 9 patients without CRC were found to carry mutant *ras* or MI. The ability to detect 55% of patients with CRC via the analysis of normal mucosa samples provides an important advance in our approach toward early detection of individuals who may be at risk to develop this tumor type.

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[2] To whom requests for reprints should be addressed, at The Ruttenberg Cancer Center, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1130, New York, NY 10029-6574. Phone: (212) 824-8193; Fax: (212) 849-2446; E-mail: zeev_ronai@smtplink.mssm.edu.

INTRODUCTION

Because specific genetic changes are known to underlie the multistep colon carcinogenesis process, biomarkers that can be associated with such instability have the potential to identify individuals at high risk for developing CRC.[3] Among the genetic alterations associated with the multistep process of colon cancer development are mutations in the K-*ras* oncogene (1) and in *p53, APC, DCC,* and *MCC* tumor suppressor genes (2–4). Involvement of genomic instability is also implicated via instability in microsatellite repeats and LOH (5, 6). Each of these genetic changes was previously evaluated for its potential to serve as a biomarker of CRC risk. Mutations in the K-*ras* oncogene were found in 50% of tumors (1, 7) and in 25% of normal-appearing mucosa from patients with colorectal tumors (8); such mutations were also identified in up to 40% of colonic effluents obtained from patients at high risk for developing CRC (9), qualifying this oncogene as a biomarker that can be used for early detection of this tumor type. Mutations in *p53* tumor suppressor genes were found in about 50% of colorectal tumors (10), but not at the earlier stages, mainly due to technical limitations of early identification methods of mutations at unknown sites. Similarly, mutations in other tumor suppressor genes occurred at lower incidence and, in most cases, were restricted to the tumor site (11). MI was reported in 15–20% of patients with CRC (sporadic non-hereditary nonpolyposis colorectal cancer forms), in most cases, at the primary tumor site (5, 6). Due to the involvement of multiple genetic changes during colon cancer development, the presence of multiple biomarkers in a given sample was also examined. To this end, analysis of p53 and *ras* mutation or p53 and MI did not reveal a major difference in the incidences found for each of the two biomarkers alone (12, 13). In contrast, analysis of MI together with LOH identified a significantly higher incidence (up to 95%) of patients with bladder cancer (14). To further explore the combined analysis of several biomarkers, we have addressed two independent questions: (*a*) we have determined the concurrent incidence of MI, LOH, and mutant K-*ras* alleles in colorectal tumors; and (*b*) we have analyzed multiple samples of normal-appearing colonic mucosa taken from patients with CRC for changes in the degree of MI and mutant K-*ras* alleles, a combination that may facilitate early detection of patients at risk for developing this type of tumor.

PATIENTS AND METHODS

Tissue samples, including tumors and normal-appearing mucosa, were obtained from the fresh surgical specimens of 20

[3] The abbreviations used are: CRC, colorectal cancer; LOH, loss of heterozygosity; MI, microsatellite instability; EPCR, enriched PCR.

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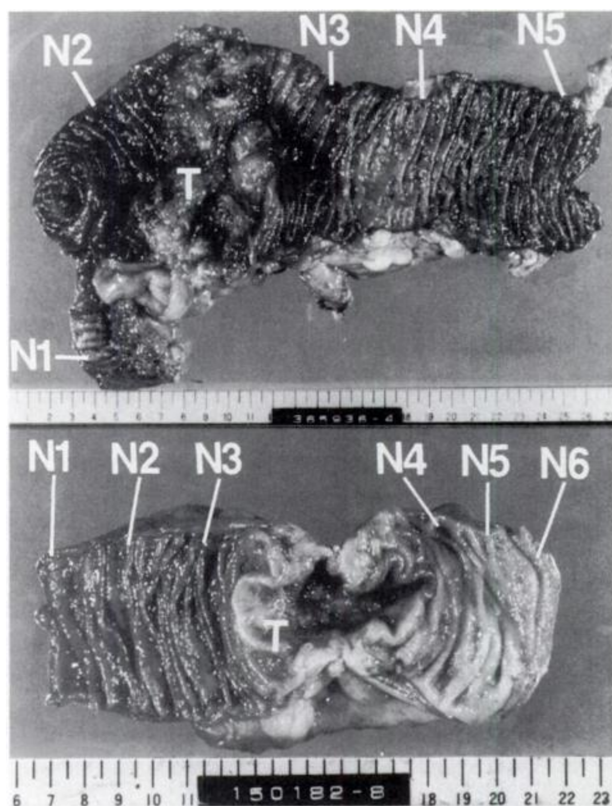

Fig. 1 Representative figures of surgical specimens indicate the location of the colorectal tumor sites (*T*) and normal-appearing mucosa sites (*N*) used for analysis. *Sample numbers* in the *upper panel* (patient 18) correspond to those shown in Fig. 2*A*. *Lower panel*, samples taken from patient 16.

patients with sporadic CRC who underwent surgery at the National Cancer Center Hospital (Tokyo, Japan). These patients consisted of 14 men and 6 women, ages 51–89 years, who did not have a family history of CRC. Multiple samples (three to seven) of normal-appearing mucosa harvested at a distance from each other (Fig. 1) were taken from the surgical specimen; they were then divided for DNA preparation for PCR analysis and for histopathological examination. All histopathological examinations of normal mucosal samples confirmed their normal morphology and that they do not consist of small morphological changes such as aberrant crypt foci.

Multiple samples of normal colonic mucosa from patients without colon cancer were collected from nine autopsy patients (six samples from each) who had been diagnosed with either myocardial infarction, brain infarction, or arteriosclerosis. All samples were collected postmortem (within 24 h of death) at Russian hospitals within the metropolitan Moscow area. Informed consent was obtained from each subject or his/her relative for all cases studied here. This investigation was approved by the Institutional Review Board for Human Studies.

In all cases, DNA was prepared by phenol chloroform extraction followed by ethanol precipitation under standard conditions. Special attention was paid to the avoidance of contamination when samples were taken from surgical specimens of patients with tumors. Specifically, before extracting DNA, these tissues were extensively washed with cold PBS to remove desquamated tumor cells. Each sample was homogenized in individual mortars that were washed beforehand with a chromic acid mixture. DNA preparation and PCR reactions were performed in different settings.

Analysis for K-*ras* mutation in codon 12 was performed with the aid of the EPCR procedure (15). This is a two-step amplification in which the 5′ primer incorporates a restriction enzyme site into the normal alleles but not into the mutant alleles. Twenty cycles of amplification were performed in this first step using 70 ηg of 5′ and 3′ wild-type primers, 1 μg of genomic DNA, 0.2 mM deoxynucleotide triphosphates, and 2.5 units of Taq DNA polymerase (Perkin-Elmer) in a total volume of 75 μl in the presence of Ampliwax. After the first round of amplification, a 1.0-μl aliquot of the amplified material was subjected to restriction enzyme (*Mva*I) digestion (in a total volume of 10 μl), followed by reamplification of mutant-enriched material. The second round of amplification was performed with 1.0 μl of digested material as template DNA and 100 ng of 5′ and 3′ primers. Final analysis was performed via RFLP through ethidium bromide-stained gels. This method was previously shown to enable the detection of 1 mutant allele in 1000 normal alleles (8, 15, 16), leading to the detection of mutant K-*ras* genes in colonic effluent (9) and sputum (17) samples taken from patients at risk for developing colorectal or lung tumors, respectively. Validation of mutation identified via EPCR was performed through sequencing that confirmed the presence of the mutation and identified the nature of the mutation. Each positive result was independently reproduced at least twice.

Analysis for MI was performed with primers designed for amplification of the five loci indicated. Primer sequences were adapted from previous publications (18, 19). In all cases, the upstream primer was labeled using a standard kinase reaction (with the aid of polynucleotide kinase and 3000 mCi/mmol [γ-^{32}P]ATP). Labeled primers were purified and used (50 ng/reaction) together with 3′ primers for PCR amplification, which also consisted of 100–200 ng of template test DNA, 0.125 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 0.25 unit of Taq enzyme. Amplification consisted of 35 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) followed by a 10-min extension at 72°C. Amplified material (10 μl) was mixed with formamide dye (10 μl), denatured, and separated on a sequencing gel (7% acrylamide and 32% formamide in the presence of 5.6 M urea) that was run at 90 W for 1 h. Gels were then exposed to film for 24 h at −80°C.

In each case, positive identification of MI was reproduced at least twice via limited dilutions of sample DNA before PCR amplification.

RESULTS

K-*ras* Mutation in Normal-appearing Mucosa and Tumor Samples of Patients with CRC. DNA prepared from the samples of normal mucosa as well as DNA from the tumor tissues of the same patients was analyzed via EPCR, a highly sensitive method that enables low-incidence (1 in 1000) detection of a K-*ras* oncogene mutation. As shown in Table 1 in 11 of 20 patients (55%) were found to harbor mutant K-*ras* alleles in codon 12. Five of 20 cases were found to contain mutant *ras* alleles in the normal-appearing mucosa tissues as well. Such

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Table 1 K-*ras* mutation, MI, or LOH found in normal-appearing tissues and tumors of patients with CRC

Patient No.	Age/sex	Site of tumor[a]	Histology[b]	Dukes' stage	Mutant K-*ras*[c] N	Mutant K-*ras*[c] T	*D2S123*[d] N	*D2S123*[d] T	*D3S1067* N	*D3S1067* T	*D5S107* N	*D5S107* T	*D17S261* N	*D17S261* T	*D18S34* N	*D18S34* T
1	59/F	R	MD	B	+	+	−	MI	−	−	−	−	−	LOH	MI	MI
2	58/M	R	WD	C	−	+	−	−	−	−	−	−	−	−	−	−
3	69/M	R	WD	C	−	+	−	−	MI	MI	−	LOH+MI	−	−	MI	−
4	57/F	R	MD	C	−	+	−	−	−	−	−	−	−	LOH	−	−
5	65/F	A	WD	C	+	+	−	MI	−	MI	−	−	−	−	−	−
6	57/M	R	MD	C	−	+ (EPCR)	−	−	−	−	−	−	−	−	−	MI
7	75/M	S	WD	B	+[e]	−	−	−	−	−	−	LOH	−	LOH	MI	−
8	53/M	R	MD	C	−	−	−	−	−	−	−	−	−	−	−	−
9	55/F	R	WD	C	−	+ (EPCR)	MI	MI	−	MI	−	−	MI	LOH	−	MI
10	66/F	S	WD	B	−	+	−	−	−	−	−	−	−	−	−	−
11	70/F	R	MD	B	−	+	−	−	−	−	−	−	−	−	−	LOH
12	51/F	R	MD	B	−	+	−	−	−	−	−	−	−	−	−	−
13	73/M	S	WD	B	−	−	−	−	MI	−	−	LOH	−	LOH	−	−
14	60/M	Tr	MD	C	−	−	−	−	−	MI	−	−	−	−	−	−
15	77/M	R	WD	B	−	−	−	−	−	−	−	LOH	−	LOH	−	LOH
16	89/M	S	WD	B	+	−	−	MI	−	−	−	−	−	−	−	−
17	76/M	R	MD	C	−	−	−	−	−	−	−	−	−	−	−	−
18	83/M	A	Muc	B	+	+	−	MI	−	−	−	−	−	MI	−	MI
19	58/M	R	MD	C	−	−	MI	−	−	−	−	−	−	−	−	−
20	58/M	S	MD	C	−	−	MI	−	−	−	−	−	−	−	−	−

[a] A, ascending colon; Tr, transverse colon; S, sigmoid colon; R, rectum.
[b] MD, moderately differentiated adenocarcinoma; WD, well-differentiated adenocarcinoma; Muc, mucinous adenocarcinoma.
[c] N, normal mucosa; T, tumor; +, mutation; −, no mutation or no instability.
[d] Of 90 normal-appearing tissue samples, 3 for *D2S123* and 2 each for *D3S1067*, *D5S107*, *D17S261*, and *D18S34* were noninformative.
[e] Multiple normal-appearing samples from patient 7 were found to have K-*ras* mutations.

mutations were found only in a small fraction of the multiple samples analyzed (*i.e.*, one of four samples) and did not always correlate with mutation in the tumor site, indicative of an independent or late event in the course of CRC development. The presence of mutant *ras* alleles in normal-appearing mucosa was confined to patients with CRC, because none of the normal mucosa samples of patients without CRC were found to harbor this mutation (20). As a control, we analyzed 54 colonic mucosa samples that were obtained from 9 patients without CRC. None of the multiple samples analyzed were found to harbor mutant K-*ras* codon 12 alleles using the sensitive EPCR technology (data not shown).

Genomic Instability in Colorectal Tumor Samples. We elected to perform analysis of genomic instability in five loci that were previously found to exhibit changes in colorectal tumors (5, 6, 19). The loci, present on chromosomes 2, 3, 5, 17, and 18, were analyzed individually in duplicate reactions. The results were grouped as those clearly exhibiting instability in microsatellite repeats and those found to exhibit LOH (Fig. 2). Shown in Fig. 2*A* is an example of a sporadic replication error-positive tumor of patient 18 that is also likely to represent patients 9, 5, and 1, all of whom share MI in multiple loci (Table 1).

Overall, the incidence of MI in the tumor samples was found to be 50 or 25% when when one or at least two of the five loci analyzed were found to exhibit MI, respectively. LOH was found in 30% of the tumor samples (6 of 20 samples) for 1 of 5 loci analyzed and in 3 of 20 samples (15%) for multiple (at least 2) loci (Table 1). When these 2 parameters were combined, genomic instability in at least 1 locus was found in the tumor samples of 13 of 20 patients (65%) and in 10 of 20 patients (50%) when 2 of 5 loci were considered.

MI in Multiple Samples of Normal Mucosa of Patients with CRC. The availability of multiple samples of normal mucosa from each patient and the fact that these specimens represent various regions distant from the tumor site (Fig. 1) enabled us to determine the incidence and possible heterogeneity of the instability of microsatellite repeats. Of the five loci tested, three (*D2S123*, *D3S1067*, and *D18S34*) contained such instability in the samples obtained from normal mucosa. Eight of 20 patients (40%) were found to harbor MI in at least 1 of the multiple (3–7) normal mucosa samples analyzed. MI found in the normal mucosa of patients with CRC was also confirmed by two independent experimental approaches: (*a*) limited dilution (up to 1:10) of the original DNA sample was performed before the PCR reaction, and only samples that exhibited a positive MI signal were considered (Table 1); and (*b*) to ensure the identity of the MI band, a Southern blot analysis was carried out according to the methods of Litt *et al.* (21), using the purified amplicon of the respective loci as a ^{32}P-labeled probe for hybridization with PCR products after their separation on a sequencing gel and transfer to a nylon membrane (data not shown).

In light of identifying MI in normal-appearing mucosa of patients with CRC, we have analyzed the incidence of MI in normal mucosa samples of patients who were free of CRC. To this end, analysis of 54 normal mucosa samples that were obtained from 9 autopsy patients did not identify MI in any of the 3 loci that were found to harbor such instability in the patients with CRC. None of the MI observed in normal tissues (Fig. 2*C*) was seen in the normal-appearing mucosa of patients free of CRC (data not shown).

Combined Analysis of MI and *ras* Mutation. The data were further analyzed by determining the combined incidence of

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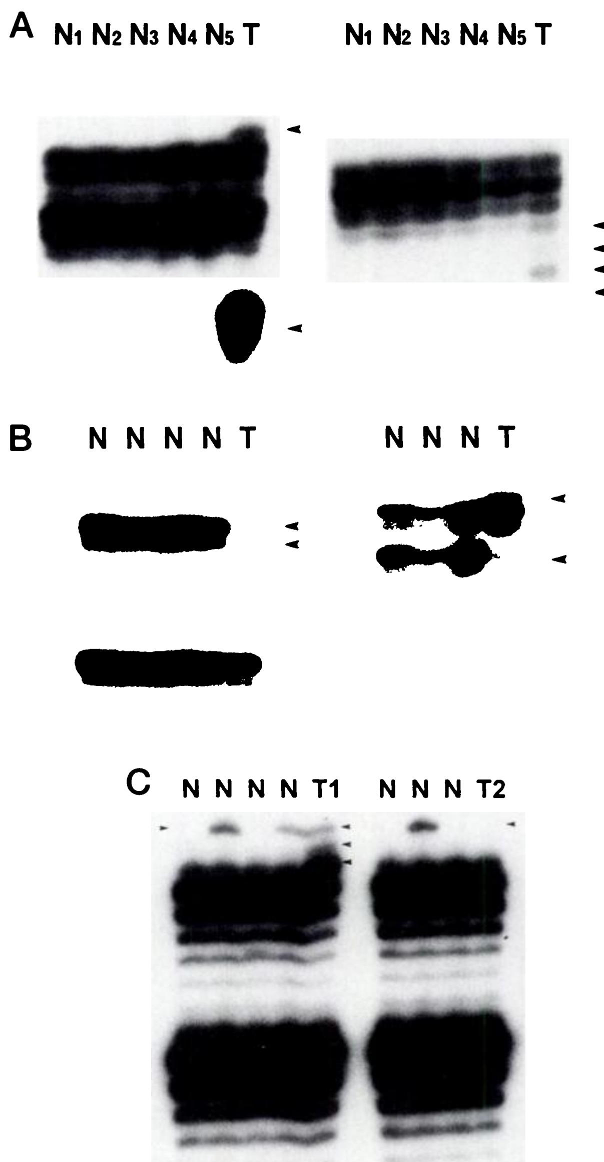

Fig. 2 Representative analysis of MI and LOH. *A*, analysis of multiple normal mucosa samples (*N*) and the corresponding tumor site (*T*) taken from patient 18 (*upper panel*, Fig. 1). MI: *left panel*, locus *D2S123*; *right panel*, locus *D18S34*. One of the normal-appearing samples, N3, harbored a K-*ras* mutation. *B*, representative LOH (*left panel*, patient 7, locus *D5S107*; *right panel*, patient 3, locus *D5S107*). *C*, heterogeneity among normal-appearing tissues in a set of samples taken from two CRCs excised from different locations (and their respective normal-appearing mucosa) in the same patient (patient 3, locus *D3S1067*). *Arrows*, position of the respective changes.

Table 2 Incidence of K-*ras* mutation and genomic instability determined by independent or combined analysis

	Tumor tissues	Normal-appearing mucosa
K-*ras* mutation	11/20 (55%)	5/20 (25%)
GI[a] ≥ 1 locus	13/20 (65%)	8/20 (40%)
GI ≥ 2 loci	10/20 (50%)	1/20 (5%)
K-*ras* mutation + GI ≥ 1 locus	16/20 (80%)	11/20 (55%)
K-*ras* mutation + GI ≥ 2 loci	14/20 (70%)	6/20 (30%)

[a] GI, genomic instability including MI and LOH in the case of tumor tissues and MI in the case of normal-appearing mucosa.

these biomarkers in tumor tissues and in normal mucosa. As shown in Table 2, when using the criterion of genomic instability found in one of five loci tested, 80% of the tumor tissues analyzed were found to harbor at least one of these three biomarkers. When detection of genomic instability in at least 2 of 5 loci was used as a criterion, combined with the presence of *ras* mutation, 14 of 20 tumor samples (70%) were identified. Whereas genomic instability in multiple loci was found only in 50% of the tumors analyzed, adding *ras* mutation as a third biomarker raised the rate of detection to 70%. Furthermore, there seems to be a relationship between the presence of mutant *ras* and genomic instability, because all 11 cases that were found to harbor mutant *ras* allele also exhibited genomic instability in at least 1 of the 5 loci examined. Our ability to detect changes in normal mucosa from patients with CRC increased from 40 to 55% when detection of MI in one of the five loci and *ras* mutation were combined. When two of five loci were set as the minimum for combined analysis with mutant *ras* alleles, 30% of the normal-appearing samples were found to harbor these changes (Table 2).

DISCUSSION

In this study, we have found that up to 80% of colorectal tumors could be identified via combined analysis of LOH, MI, and *ras* oncogene mutation. The contribution of multiple biomarkers to the overall detection rate is clearly noted when multiple loci are considered for genomic instability. For example, our present analysis allowed us to identify 70% of the colorectal tumors that exhibited either mutant *ras*, LOH, or instability in microsatellite repeats in at least two of the five loci analyzed, whereas only 50–55% of the tumors could be identified when each of these biomarkers was used independently. When considering only one of the five loci selected for the present analysis as a positive indicator for genomic instability, 80% of the tumor samples could be identified. The observation that all tumors found to harbor mutant *ras* alleles also contain genomic instability points to the possible existence of shared/common mutator phenotype(s). Although this is the highest rate demonstrated to date, it is likely that this rate can be further increased by examining additional loci that may exhibit a high incidence of instability of microsatellite repeats in this tumor type.

The analysis of multiple samples of normal-appearing colonic mucosa revealed that the combined set of biomarkers used here can detect up to 55% of the patients with CRC. When considering the 0% background as determined *via* the analysis of normal mucosa from patients without CRC, detecting 55% of patients by analysis of normal-appearing tissues is remarkably high.

The examination of multiple normal-appearing colonic mucosa samples from the same patient allowed us to increase the possible detection of such an occurrence. Two of the five loci analyzed did not exhibit any instability in any of the normal-appearing mucosa samples, suggesting that early occurrences of instability appear in a selective locus. It is important to note the lower incidence of MI in normal tissues when compared with that

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in tumor samples, as judged by the need to analyze multiple normal samples to identify a sample with MI. The latter may be characteristic of the normal-appearing samples analyzed (*i.e.*, small pockets of MI in normal tissues). The observation that MI observed in tumors was not always observed in the corresponding normal mucosa and *vice versa* indicates that MI occurred independently of the existing tumor, similar to the occurrence of K-*ras* mutation (8, 9, 11, 16, 17, 20). Further analysis to quantify the incidence of MI in normal tissues and to identify additional loci that exhibit MI in normal tissues is likely to yield a higher rate of detection of normal-appearing mucosa on a quantitative basis.

Our results also point to the need for a proper sampling method, because more than one sample of normal mucosal tissue needs to be analyzed to identify both MI and mutant *ras* alleles. Among the alternate sampling methods are colonic effluents (enemas) that were previously shown to provide an adequate representation of the colon. Analysis of colonic effluent samples enabled the detection of mutant *ras* alleles in patients at high risk for developing CRC (9).

MI in nonneoplastic mucosa was previously reported in 50% of patients with inflammatory conditions such as ulcerative colitis (22), indicating that conditions associated with inflammation (such as high duplication rate) may also prevail in selected normal-appearing colonic mucosa regions of patients with CRC. MI was reported in 30–40% of gastric cancer, adenomas, and intestinal metaplasia mucosa of the stomach (23) as well as in 49% of normal-appearing bronchial mucosa and cytological specimens of patients with lung cancer (24), further indicating the appearance of this marker at early stages of the neoplastic transformation process.

Because the biomarkers studied here were found (albeit at low incidence, as studied independently) in the biopsies or effluent samples of patients without CRC, the ability to reach a detection rate of 55% of patients with CRC via the analysis of normal mucosa samples, with no background, as determined via analysis of the control samples, provides an important advance in our approach toward the early detection of individuals with CRC and possibly of individuals that may be at risk of developing this tumor type.

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