Multiple Detection of Genetic Alterations in Tumors and Stool

Claudia Rengucci, Pasquale Maiolo, Luca Saragoni, Wainer Zoli, Dino Amadori, and Daniele Calistri
Istituto Oncologico Romagnolo [C. R., D. C.], Divisione di Gastroenterologia, Ospedale Morgagni [P. M.], and Divisioni di Anatomia Patologica [L. S.] e Oncologia [D.A.], Ospedale Pierantoni, Forlì, Italy

ABSTRACT
Detection of genetic alterations in exfoliated intestinal cells in stool could represent an alternative, noninvasive tool for the screening of colorectal tumors. To verify this, we analyzed p53 and K-ras mutations and microsatellite instability on 46 cases of colorectal cancer and compared the presence of molecular alterations in tumor tissue and stool samples from individual patients. p53 exons 5–8 and K-ras exons 1–2 were analyzed by denaturing gradient gel electrophoresis. For the microsatellite instability, a set of 5 microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26) was evaluated. In the 18 healthy individuals, no genetic alterations in either tissue or stool were detected. p53 mutations were detected in 17 (37%), K-ras alterations in 15 (33%), and microsatellite instabilities in 5 (11%) of the 46 tumors analyzed. In a side study, we analyzed the correlation in genetic alteration profiles between tumors and macroscopically normal or healthy tissue from the same patient. The presence of at least one molecular alteration in tumor was observed in 31 (67%) of the cases. p53, K-ras mutations, and microsatellite instabilities were detected in stool samples in 18, 40, and 60% of patients with tumors harboring the same alterations. Due to the largely complementary presence of p53 and K-ras mutations in tumors, the use of highly sensitive procedures for stool analysis could offer a means competitive with colonoscopy and the fecal occult blood test.

INTRODUCTION
Colon cancer is an important cause of death in Western countries (1). Early diagnosis is perhaps even more important than for other tumor histotypes because early stage cancers can be cured by surgery, whereas patients with advanced and resectable disease have a poor prognosis on account of their high intrinsic resistance to the majority of antitumor drugs (2).

Colonoscopy is the gold standard for the detection of colorectal carcinoma and preneoplastic lesions, but its invasive-ness and relatively high cost do not permit a population-based screening. On the other hand, the noninvasive and low cost FOBT² allows for an early diagnosis and, as a consequence, an improvement in patient clinical outcome (3–5). However, the sensitivity and specificity of the test are wide-ranging in different studies (6), and the accuracy still remains to be defined.

A number of genetic alterations have been associated with colorectal cancer, and a multistep model of cancer development has been proposed (7) in which the most frequent alterations involve tumor suppressor gene p53 and proto-oncogene K-ras.

Mutations of the p53 gene are the most diffuse alterations in human tumors and have been detected in a very high fraction of sporadic colorectal cancers. p53 gene mutation seems to represent a late event in colorectal carcinogenesis and is often associated with the progression from adenoma to carcinoma (7).

Mutations of K-ras proto-oncogene occur in the transition from early to intermediate adenoma and may be involved in the conversion of a small adenoma to a larger and more dysplastic one (7). They are mostly localized on codon 12 and to a lesser degree on codons 13 and 61.

Recently, DNA replication errors in microsatellite sequence have been observed in the majority of tumors from patients with hereditary nonpolyposis colorectal syndrome and in a subgroup of patients with sporadic colorectal carcinomas (8–12).

In the last few years, research has aimed at verifying whether mutations observed in tumors can be detected in exfoliated intestinal cells in stool. In particular, p53 (13) and K-ras (14–19) mutations have been singly determined in colorectal cancers and compared with those detected in the stool from the same patient, providing important information on the specificity of this analysis. Results from these studies have indicated the high potential of the detection of molecular alterations in stool but have also shown that the determination of a single marker is not sufficiently sensitive to justify a population screening.

In the present study, we determined the presence of p53 and K-ras gene mutations and microsatellite instability in the same series of patients and performed a pair comparison between tumor tissue and stool in individual patients, as a potential approach to a noninvasive screening for colorectal tumor diagnosis.

MATERIALS AND METHODS
Case Series. Biopptic tumor samples from 46 patients and normal colon tissue from 18 healthy individuals were taken during colonoscopy. Diagnosis of cancer was histologically confirmed. Pathological stage was defined according to Dukes' classification: 4 tumors were classified as stage A; 19 as stage B; 20 as stage C; and 3 as stage D. Stool specimens were collected from all 64 cases after colonoscopy to identify cancer or normal individuals and to avoid too large a number of normal samples, as would have happened for a consecutive series of cases. In particular, this was

_____________
1 To whom requests for reprints should be addressed, at Laboratorio Biologico, Divisione de Oncologia Medica, Ospedale Pierantoni, Via Forlanini 34, 47100 Forlì, Italy. Phone: +39 0543 731623; Fax: +39 0543 731736; E-mail: ropinz@tin.it.

2 The abbreviation used is: FOBT, fecal occult blood test.

Received 8/7/00; revised 12/26/00; accepted 12/27/00. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
done 72 h after endoscopic analysis to permit a repopulation of intestinal cells. All samples were stored at −70°C until use or for a maximum of 2 months.

Mutation Analysis. Approximately 100 mg of frozen stool were diluted with 200 μl of guanidine thiocyanate 6 M buffer containing 1% SDS and 500 μg of proteinase K and incubated overnight at 37°C. The DNA from tissue and fecal samples was extracted by QIAamp tissue kit (Qiagen, Hilden, Germany).

p53 exons 5–8 and K-ras exons 1–2 were analyzed by denaturing gradient gel electrophoresis. The sequence of the primers used for p53 gene amplification has been described in previous reports (20, 21). Briefly, the amplification was performed on 50 ng of genomic DNA from tissue or on 1–2 μl of DNA from stool in 25 μl, 0.4 μM concentrations of each primer, 200 μM concentrations of deoxynucleotide triphosphates, 1× reaction buffer with 3.5 mM MgCl₂, and 1 unit of Taq polymerase (KlenTaq1 Ab Peptides) for exon 5, 0.5 unit for exon 6, and 0.2 unit for exons 7 and 8 of p53. The reaction mixture was subjected to 38 PCR cycles: 60 s at 94°C and then 60 s at 58°C for exons 5 and 7; 56°C for exon 6; and 54°C for exon 8 of p53.

For the detection of K-ras, 0.4 unit of Taq polymerase (KlenTaq1 Ab Peptides) for exon 1 and 0.5 unit for exon 2 were used. The reaction mixture was subjected to 38 PCR cycles: 60 s at 94°C and then 60 s at 54°C for exon 1; and 56°C for exon 2 of K-ras; followed by an incubation at 72°C for 60 s. The PCR products were analyzed in the running conditions previously described (22).

All mutations were confirmed by sequencing with 373A DNA sequencer (Applied Biosystems) according to the supplier’s instructions.

Microsatellite Analysis. Biological samples were tested for a set of five microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26) (23). PCR amplifications were performed on 100 ng of genomic DNA from tissue or 1–2 μl of DNA from stool in 25 μl, 0.2 μM concentrations of each primer, 200 μM concentrations of deoxynucleotide triphosphates, 1 μM fluorescent dUTP, 1× reaction buffer with 3.5 mM MgCl₂, and 0.5 unit of Taq polymerase (KlenTaq1 Ab Peptides). The reaction mixture was subjected to 38 PCR cycles: 60 s at 94°C and then 120 s at 58°C followed by an incubation at 72°C for 70 s. PCR products were analyzed by 6% polyacrylamide-7 M urea gel in a 373A DNA sequencer (Applied Biosystems) and GENESCAN 672 software. Microsatellite instability was defined by the presence of unequivocal novel bands in tumor DNA in at least 1 locus.

RESULTS

The determination performed on the 18 healthy individuals failed to detect any p53 or K-ras mutations or microsatellite instabilities in tissue and stool samples. Conversely, in the 46 cancer patients, p53 mutations were detected in 17 (37%) of the tumor tissue samples evaluated (Table 1; Fig. 1). In particular, single mutations in exon 5 were observed in five tumors, in exon 6 in two tumors, in exon 7 in six tumors and in exon 8 in four tumors. In stool samples, mutations were detected in only three patients, two in exon 6 and one in exon 8, and all were identical with those observed in their tumor counterparts. p53 mutations in both tumor and stool samples from individual patients were detected in 18% of cases.

K-ras mutations were observed in 15 of the 46 tumors (33%) (Table 1; Fig. 2); specifically, 13 alterations were observed in exon 1 and only 2 in exon 2. In stool samples, K-ras alterations were observed in 6 patients, 2 in exon 2 and 4 in exon 1, with a 40% agreement between tumor and stool. With regard to microsatellite profile, alterations were observed in five tumors (11%) (Table 1; Fig. 3). Microsatellite instability of D5S346 was detected in three tumors and in corresponding stool. BAT25, D2S123, and D17S250 instability was found in one tumor and D17S250 instability in one tumor, but not in the corresponding stool. The overall agreement between tumor and stool was 60%.

The low frequency of cases in stages A and D does not permit a statistical evaluation of results. However, no p53 mu-

| Table 1 Presence of molecular alterations in colorectal cancer patients |
|----------------------|--------|--------|--------|--------|
|                     | Tumor  | Stool  |
| Alterations         | No.    | %     | No.    | %     |
| p53                 | 17     | 37    | 3      | 7     |
| K-ras               | 15     | 33    | 6      | 13    |
| Microsatellite instability | 5 | 11 | 7 |

![Fig. 1](https://via.placeholder.com/150)

Fig. 1 Examples of p53 exon 6 and 8 mutation analysis. DNA derived from stool (S) and tumor (T) from different patients are analyzed in parallel. Parentheses and arrows, bands that migrated to different positions from the normal ones. Exon 6: the same alterations are visible in tumor and corresponding stool. Exon 8: the alterations visible in two of three tumor cases are not detectable in corresponding stool.

![Fig. 2](https://via.placeholder.com/150)

Fig. 2 Example of K-ras exon 1 mutation analysis. DNA derived from stool (S) and tumor (T) from different patients are analyzed in parallel. Parentheses and arrow, presence of new bands derived from the mutated sequence. From the left: three cases with mutation pattern visible in tumor and corresponding stool; one case with only the tumor showing new bands; and one case with no alterations.
tations were observed in four stage A tumors, in 35–40% of stage B and C tumors, and in two of the three stage D tumors (Table 2). Conversely, there was no evidence of a trend between K-ras mutations or microsatellite instability and tumor stage, in either tumor or stool.

Moreover, p53 mutations were associated with K-ras mutation in only three cases (7%) and with microsatellite instability in only two cases (4%). The copresence of K-ras mutation and microsatellite instability was observed in only one case (2%). These findings show a largely unrelated and complementary pattern of the different types of genetic alterations (Fig. 4).

Overall, the presence of at least one marker alteration was observed in 31 of the 46 cases, i.e., in 67% of tumors. The frequency increased from 25% in Dukes’ stage A to 74, 70, and 67% in stages B, C, and D, respectively.

**DISCUSSION**

A noninvasive method as an alternative to colonoscopic examination for early colon cancer detection is undoubtedly warranted. At present, the only widely used and important noninvasive method is FOBT. Results from randomized screening trials using FOBT have demonstrated a reduction in the incidence of Dukes’ stage D cancer and in parallel, a substantial, statistically significant decrease in colorectal cancer mortality (3–5). However, the accuracy in terms of its sensitivity and specificity has yet to be precisely defined (6).

Another potential and promising approach is the detection of gene mutations in exfoliated tumor cells in stool. The simultaneous determination of multiple genetic alterations on the same series of tumors, which, to our knowledge, has no precedent, showed that p53 and K-ras mutations are largely complementary events, covering about two-thirds of colorectal tumors and that microsatellite instability, albeit a very rare event, is important because it is present in tumors lacking in p53 and K-ras mutations. The multiple alteration profile observed in the tumor, if reproducible in stool, could be very important for a noninvasive, diagnostic approach. However, in our study, the frequency of p53 and K-ras mutations in stool is even lower than that reported in other studies. This may be due to different reasons such as the use of molecular procedures that are less sensitive than radioactive approaches (14–15); selected and not consecutive series of cases (17), as in our study; or small case series with a prevalence of advanced tumor stages (11, 18).

In conclusion, the approach we propose in the present technical conditions is not, in itself, routinely applicable, but in consideration of the complementarity of different genetic alterations.

### Table 2  Presence of molecular alterations as a function of tumor stage

<table>
<thead>
<tr>
<th>Dukes’ stage</th>
<th>Total cases</th>
<th>p53 Tumor</th>
<th>p53 Stool</th>
<th>K-ras Tumor</th>
<th>K-ras Stool</th>
<th>Microsatellite instability Tumor</th>
<th>Microsatellite instability Stool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>5</td>
<td>26</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>19</td>
<td>7</td>
<td>1</td>
<td>5</td>
<td>35</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>35</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>33</td>
<td></td>
<td>1</td>
<td>67</td>
</tr>
</tbody>
</table>

**Fig. 3** Microsatellite instability at different loci. PCR products from tumor (T), normal mucosa (N), and stool (S) DNA were loaded in parallel. Arrows, additional bands visible in tumors and corresponding stool at the D5S346 locus (patients A, B, and D), and only in tumor samples at the D17S250 locus (patients E and F).
and the lack of false positives, there is reason to believe that the use of more sensitive procedures that are capable of increasing sensitivity in stool could provide an important screening strategy for the early detection of colorectal cancer.

Moreover, although the analysis of microsatellite instability would not appear to be sufficient for detecting sporadic colorectal cancer, it could be important for tumors derived from inherited nonpolyposis colorectal cancer because they exhibit this instability in ~90% of cases (8, 24, 25).

REFERENCES

Fig. 4 Molecular alteration in tumor tissue.
Multiple Detection of Genetic Alterations in Tumors and Stool

Claudia Rengucci, Pasquale Maiolo, Luca Saragoni, et al.

Clin Cancer Res 2001;7:590-593.

Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/3/590

Cited articles
This article cites 24 articles, 6 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/3/590.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/7/3/590.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.