ORIGINAL PAPERS

Noninvasive Detection through REMS-PCR Technique of K-*ras* Mutations in Stool DNA of Patients with Colorectal Cancer

Francisc Mixich[1], Mihai Ioana[1], Florea Voinea[2], Adrian Săftoiu[3], Tudorel Ciurea[3]

1) Department of Molecular and Cellular Biology, University of Medicine and Pharmacy of Craiova. 2) Department of Gastroenterology, 1st Internal Medical Clinic, Ovidius University, Constanta. 3) Department of Gastroenterology, University of Medicine and Pharmacy of Craiova

Abstract

Background and aims. Tumor exfoliated cells that shed into stool are attractive targets for molecular screening and early detection of colon malignancies. Many studies have suggested that the detection of activated *ras* may have diagnostic or prognostic importance. The aim of this study was to establish the suitability for use in diagnostic laboratories of the noninvasive screening test of K-*ras* mutation determination in the stool and its routine prognostic value in colorectal cancer. **Methods.** Paired stool and tissue specimens obtained after polypectomy and colorectal biopsy from 28 patients diagnosed solely by histopathological findings with primary colorectal carcinoma, were prospectively studied for K-*ras* codon 12 mutations by restriction endonuclease-mediated selective (REMS)-PCR. **Results.** DNA was obtained in 28 of tissue samples (100%) and 26 of stool samples (92.8%). K-*ras* codon 12 mutation was seen in 14 (50.0%) paired stool and tissue samples. Mutation detection was possible in 1000-fold excess of wild-type sequence. These results may be important in the design of genetic screening programs, determination of prognosis, early detection and treatment for patients with colon malignancy. **Conclusions.** The sensitivity and specificity of K-*ras* determination on stool-derived DNA in patients with colorectal carcinoma, support the opportunity of a large-scale trial to validate its use as a screening test. REMS- PCR is not labor intensive, but a sensitive, rapid, and robust assay for the detection of point mutations, and was introduced by us in a routine diagnostic laboratory.

Key words

Colorectal cancer – REMS PCR – K-*ras* mutation – stool DNA – noninvasive screening test

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Address for correspondence: Dr. Mihai Ioana
Univ. Med. Pharmacy Craiova
Petru Rareş Str., No 2
Craiova, Romania
E-mail: imihai@umfcv.ro

Introduction

Activating point mutations at codon 12, or 13 of the *ras* proto-oncogenes occur frequently in human tumors (1). In colon and pancreatic cancer, more than 90% of the *ras* mutations occur in the K-*ras* gene, and most of these are found in codon 12 (2, 3). The analysis of tumor exfoliated cells that shed into stool is a noninvasive method of screening for colorectal cancer. Although there are many techniques available for the detection of K-*ras* mutations (6) most of them show low sensitivity if applied to DNA extracted from feces. More than 95% of DNA from stool is of nonhuman origin and only 0.5–5% of the human DNA will show a mutation in the *ras* gene of tumor cells in the sample (4). Indeed, K-*ras* mutations were detected in DNA isolated from stool obtained from symptomatic and asymptomatic patients with colorectal cancer, suggesting a novel approach for a noninvasive screening procedure (5). Simple extraction procedures, as well as sensitive and specific amplification and detection techniques, are required to make the molecular screening of stool samples available for routine diagnosis (7).

Only limited conclusions can be drawn from the literature data. Most studies were made on small numbers of archived frozen stools. The majority of patients in all studies had cancers in Duke's B and C stages, and only a few studies examined patients with premalignant lesions. Also, many control subjects were selected from a population of symptomatic individuals who were endoscopically normal or had minimal lesions (8). Nevertheless, preliminary data suggest that fecal DNA markers have the ability to detect premalignant and malignant lesions in all locations and at all stages with great sensitivity and with a very high specificity. It has been proposed that fecal DNA could be used as an adjunct to endoscopy, lengthening the interval between colono-scopies, or added to a program of one-time colonoscopy, or as surveillance after therapy for colonic neoplasia (9).

The aim of this study was to establish the suitability for use in diagnostic laboratories of the noninvasive screening test of K-*ras* mutation determination in the stool and its

routine prognostic value in colorectal cancer. Paired stool and tissue specimens obtained after polypectomy and colorectal biopsy from 28 patients diagnosed solely by histopathological findings for primary colorectal carcinoma, were prospectively studied for K-*ras* codon 12 mutations by restriction endonuclease-mediated selective (REMS)-PCR. Moreover, to verify the existence of amplifiable human DNA in our probes, we compared the correspondence of some human STR loci between the DNA extracted from feces and tissue samples respectively, in six selected patients.

Material and method

Patient samples

After informed consent was obtained paired stool and tissue specimens obtained after plypectomy and colorectal biopsy from 28 individuals from Craiova and Constanța University Hospitals were enrolled in this prospective study between 2005 and 2006. Fresh samples of colorectal biopsy and of feces were immediately frozen at –20°C. A total of 28 paired specimens were assayed from 19 males and 9 females with ages ranging from 18 to 69. For all tissue samples, the histopathological diagnosis, stage and tumor size were determined independently by specialists from Departments of Pathology, University of Medicine and Pharmacy - Craiova and Faculty of Medicine - Constanța.

DNA preparation from fresh tissues and stool

For preparation of DNA from fresh tissues the Wizard Genomic DNA Purification Kit from Promega (USA) was used. The principle for DNA extraction with this kit is the digestion of 25 mg of tissue with Proteinase K and subsequent extraction of DNA according to producer instructions. DNA yield from 25 mg of starting tissue was 20-40 mg.

DNA from stool samples was extracted from 200 mg of stool material by a column-based method (QIAamp® DNA Stool Mini Test Kit; Qiagen). Purified DNA was suspended in 200 µL of 10 mmol/L Tris-HCl (pH 7.4)–1 mmol/L EDTA buffer.

Validation of human origin of DNA extracted from stool by STR loci typing

STR loci correspondence analysis between the DNA extracted from feces and tissue samples, was performed with three GenePrint™ STR multiplex systems provided by Promega (USA). Allele variants of a total of nine STR loci: CSF1PO, TPOX, TH01, F13A01, FESFPS, vWA, D16S539, D7S820, and D13S317, were determined. DNA extracted from feces and tissue samples respectively, in 6 selected patients was amplified according to Promega protocol. Amplified STR alleles were detected by 6% denaturing polyacrylamide gel electrophoresis, with the CBS (USA) research sequencing gel electrophoresis system, according to the protocol recommended by Promega. Detection of PCR products after separation by polyacrylamide gel electrophoresis was performed by silver staining method.

REMS-PCR mutational analysis

Restriction endonuclease-mediated selective (REMS)-PCR (10-17) allows detection of point mutations, deletions, and insertions. Reactions require concurrent activity of a restriction endonuclease (RE) and a DNA polymerase, both of which must be sufficiently thermostable to retain activity during thermocycling. The inclusion of the RE in REMS-PCR inhibits amplification of sequences containing the RE recognition site, thus producing selective amplification of sequences that lack the RE site.

Mutations at the first and second bases of codon 12 of the K-*ras* gene were detected according to Ward et al (16). Each PCR reaction contained three sets of primers (Table I).

Table I Primer sequence for REMS-PCR

Primers	Forward primer	Reverse primer
Diagnostic primers	5'-TAT AAA CTT GTG GTA GTT GGA CCT-3'	5'-CGT CCA CAA AAT GAT TCT GA-3'
PCR control primers	5'-GTA CAC ATG AAG CCA TCG TAT A-3'	5'-CCA CTT GTA CTA GTA TGC CTT AAG-3'
RE control primers	5'-CTA GAA CAG TAG ACA CAA ACC A-3'	5'-GAT TTT GCA GAA AAC AGA TC-3'

A REMS-PCR protocol using *Bst*NI was used to detect mutations in the first two bases within codon 12 of the K-*ras* gene. These reactions contained three sets of primers: (*a*) diagnostic primers, which amplify an 82-bp region of exon 1 of the K-*ras* gene (forward primer induce a *Bst*NI site spanning codon 12 in wild-type, but not mutant amplicons); (*b*) RE control primers, which amplify a 130-bp region in exon 3 of the K-*ras* gene (forward primer induce a *Bst*NI site in all RE control amplicons); and (*c*) PCR control primers, which amplify a 215-bp region of exon 4b of the K-*ras* gene (this amplicon contains no *Bst*NI sites).

In the reactions containing *Bst*NI, the presence of the 82-bp fragment was diagnostic for the existence of K-*ras* codon 12 mutations. This fragment was not visible in reactions containing wild type K562 DNA. The RE control fragment was not visible in any reactions containing *Bst*NI, indicating that the RE activity was sufficient throughout the PCR to inhibit amplification of all fragments containing *Bst*NI sites. The 215-bp PCR control fragment was visible in all reactions, including the reaction containing K562 DNA, confirming that the reaction conditions were adequate for amplification.

The PCR reaction was performed using 0.1 to 0.6 mg of DNA extracted from tissue or 20 to 30 mg of DNA from stool samples. PCR reaction mixtures also contained the following: 40 pmoles of each diagnostic primer, 2 pmoles of each RE control primer, 20 pmoles of each PCR control primer, 1 mmol/L dithiothreitol, 50 mmol/L deoxynucleotide triphosphate, 4 mmol/L Mg^{2+}, 40 U of *Bst*NI (New England Biolabs), and 5 U of *Taq* DNA polymerase (Promega, USA). The final reaction

volume was brought to 25 ml in PCR buffer (50 mmol/L Tris-HCl and 100 mmol/L NaCl, pH 8.3.

The reactions were cycled in a MiniCycler™ (MJ Research, USA) as follows: 94°C for 2 minutes (one cycle) and 58°C for 60 seconds followed by 92°C for 20 seconds (30 cycles); the reactions were then held at 4°C before analysis. Samples were analyzed by electrophoresis on a 5% agarose gel (Promega, USA). In all assays, DNA from human K562 cell line, known to be homozygous wild type for K-*ras*, was amplified.

Samples were considered mutant when the diagnostic and PCR control bands were present and the RE control band was absent. They were considered wild type when the PCR control band and the diagnostic band were absent.

Results

Only a minor part of the DNA isolated from stool is of human origin. To control the effectiveness of the DNA-extraction procedure, 9 human control STR loci were coamplified in a duplex PCR protocol, using the DNA extracted from feces and from tissue biopsy sampled from six selected patients. A missing amplification of the internal control STR or no matching between STR alleles in paired samples was taken as an indication that not enough human DNA was extracted or originally present in the stool sample or that PCR-inhibiting substances were present. Correspondence of nine STR alleles in all six patients was absolute (Fig.1, Table II).

Using the QIAamp DNA Stool Mini Kit gave high DNA yields from 200 mg stool samples, according to the manufacturer DNA yields are typically 4–48 µg (18). As we proved bellow, PCR of the purified DNA showed that it was of high quality, and no inhibition of PCR was observed.

The ability of the REMS-PCR assay to detect mutations of K-*ras* codon 12 was determined in paired stool and tissue specimens obtained after polypectomy and colorectal biopsy from 28 patients diagnosed for primary colorectal carcinoma (Table III). DNA was obtained in 28 of tissue samples (100%) and 26 of stool samples (92.8%). K-*ras* codon 12 mutation was seen in 14 (50,0%) paired stool and tissue samples.

Table II Allelic constitutions for STR typed loci using DNA from stool and tissue samples in six patients

Loci	Patients/samples Case 1		Case 2		Case 3		Case 4		Case 5		Case 6	
CSF1PO	1b	1f	2b	2f	3b	3f	4b	4f	5b	5f	6b	6f
TPOX	9,10	9,10	10,10	10,10	10,12	10,12	9,11	9,11	11,12	11,12	11,12	11,12
TH01	11,11	11,11	8,9	8,9	9,9	9,9	8,8	8,8	8,8	8,8	8,8	8,8
D16S539	6,10	6,10	7,7	7,7	9,9	9,9	7,7	7,7	9,9	9,9	7,7	7,7
D7S820	11,11	11,11	11,12	11,12	13,14	13,14	9,11	9,11	12,12	12,12	9,12	9,12
D13S317	11,11	11,11	10,11	10,11	10,10	10,10	11,12	11,12	9,9	9,9	8,9	8,9
F13A01	11,13	11,13	11,12	11,12	9,13	9,13	11,11	11,11	8,9	8,9	8,9	8,9
FESFPS	7,7	7,7	5,6	5,6	6,7	6,7	6,7	6,7	7,7	7,7	7,7	7,7
vWA	10,11	10,11	11,12	11,12	11,11	11,11	12,13	12,13	9,13	9,13	11,12	11,12
	14,18	14,18	15,18	15,18	14,18	14,18	15,17	15,17	15,16	15,16	16,17	16,17

1b, 2b, ... 6b – biopsy tissue samples; 1f, 2f, ... 6f – stool samples

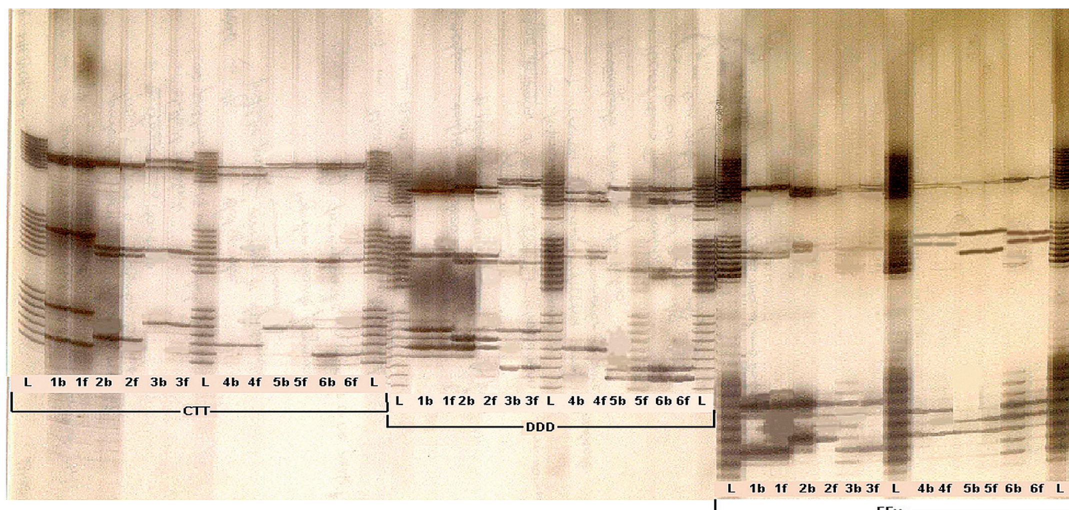

Fig.1 Polyacrylamide gel electrophoresis of PCR products for STR amplified loci and detected using silver staining. CTT: loci CSF1PO, TPOX and TH01 (from top to bottom); DDD: loci D16S539, D7S820 and D13S317 (from top to bottom); FFv: loci F13A01, FESFPS and vWA (from top to bottom). Lanes labeled (L) contain allelic ladders for the respective loci. Individual genomic DNA samples in lanes labeled with 1b, 2b, ... 6b represent PCR products obtained from tissue (b) and stool (f) samples from corresponding patients named in Table II.

Table III Analysis of K-ras status of DNA extracted from biopsy tissue samples and stool in patients studied (A plus sign indicates the presence of mutation, while minus denotes the abscence of mutation**)**

Sample		Patients																											
		01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Tissue	DNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mutation	+	+	−	−	+	−	+	−	−	+	+	+	+	−	−	+	+	−	+	−	+	+	+	+	−	−	−	−
Stool	DNA	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mutation	+	+	−	−	+	−	+	?	−	+	+	+	?	−	−	+	+	−	+	−	+	+	+	+	−	−	−	−

When performed on DNA from wild type K562 cell line, the assay resulted only in diagnostic amplicon of 214 bp (Fig.2), thus indicating the absence of a K-*ras* codon 12 mutation. When *Bst*NI was omitted from this reaction, three amplicons were seen. Absence of the RE control band of 130 bp in reactions containing *Bst*NI indicated that the enzyme was sufficiently active during these reactions (Fig.2).

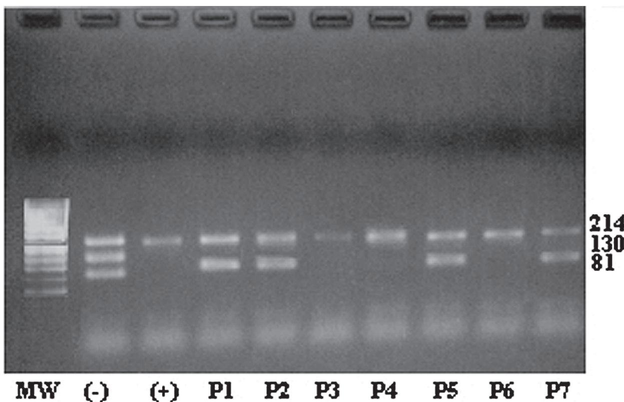

Fig.2 Detection of mutations on K-ras codon 12 in DNA from Tissue samples. (+) Wild type K562 cell line; (-) Negative control without *Bst*NI in the reaction. P1-P7 Tissue specimens DNA extracted from patients 1-7. PCR amplicons are shown on a 5% agarose gel stained with ethidium bromide.

Of the 28 patients in whom both stool and tissue samples obtained by colorectal biopsy were analyzed, 13 were wild type in both assays, 14 were mutant, and in 1 case, mutation was detected in the tissue sample, but we were unable to obtain DNA from the stool.

Discussion

At the moment, a number of methods for the detection of single base mutations in DNA sequences have been described. Those methods include the amplification refractory mutation system (20), single-strand conformation polymorphism (SSCP) and allele-specific amplification (21), mismatch amplification mutation assay (22), oligonucleotide ligation assay (23), ligation chain reaction (24), enriched PCR (25), PCR primer-introduced restriction analysis with enrichment of mutant alleles (26), PCR-based assays (27), point mutation detection using exonuclease amplification-coupled capture technique (28), restriction fragment length polymorphism (RFLP) (19, 29) and denaturing gradient gel electrophoresis (DGGE) (30). These techniques have several disadvantages of automating DNA diagnosis, which in some cases include the requirement for various hybridization conditions, the use of two PCR amplifications and, finally, the need for electrophoresis. No ideal method for diagnosis, in which minimal steps are required to give maximal sensitivity, is in universal use for cases of known mutations in a single codon.

The strategy of REMS-PCR represents a significant advance over existing enriched PCR approaches, producing a robust assay suitable for use in the diagnostic laboratory. To be applicable in routine laboratories, it is important that the assay be reliable as well as rapid and sensitive. The main advance of the REMS procedure comes from the greatly reduced handling of the amplified product. Unlike conventional enriched PCR, in which the two rounds of PCR are separated by an enzyme digestion step, all reactions in the REMS-PCR occur concurrently in one tube. This reduced handling has important implications for the applicability of the assay as a diagnostic procedure.

When assayed with methods of appropriate sensitivity, the frequency of K-*ras* codon 12 mutations in colorectal cancer has been reported to vary between 20 and 50% (31, 32). Our results with REMS-PCR are clearly in agreement with these reports and suggest that the assay reflects the true incidence in the analyzed population, and show an effective method for detecting K-*ras* mutations in human stool.

An average of approximately 20 μg of DNA were isolated from a 200 mg stool sample. This corresponds to less than 50 ng of DNA of interest, which is equivalent to approximately 40 gene copies per microliter of eluted DNA (18). Therefore the quality of the isolated DNA is especially critical. Using this method, mutations were successfully detected and were in concordance with tissue determinations at the same individuals, indicating the high quality of the DNA from the stool.

Although there is a consensus that K-*ras* mutations serve as a marker for colorectal cancer or cancer prestages (18,33) the detection of a single marker is not sensitive enough to detect all cases of these malignancies. Several other markers and techniques were previously tested to improve the sensitivity for the diagnosis of colorectal cancer (promoter methylation, microsatellite instability, DNA

integrity assay, etc.) (34). However, because of the molecular heterogenity of colorectal carcinoma, no single marker has yielded perfect sensitivity. One combination of markers used in the multi-target DNA assay panel (MTAP) includes mutations in the *p53*, K-*ras*, and *APC* genes, a microsatellite instability marker (BAT-26), and the DNA integrity assay (DIA), a marker of loss of apoptosis (35). The MTAP tests were suggested to increase the sensitivity for the detection of advanced colorectal neoplasia to more than 60% [36].

However, further studies including an increased number of patients from multiple centers, using additional markers or techniques, have to be designed to further increase the sensitivity of fecal DNA testing.

Conclusions

The sensitivity and specificity of K-*ras* codon 12 mutation determination on stool-derived DNA in patients with colorectal carcinoma support the opportunity of a large-scale trial to validate its use as a screening test.

REMS-PCR is not labor intensive, but a sensitive, rapid and robust assay for the detection of point mutations, and was introduced by us a routine diagnostic laboratory.

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