ELSEVIER

Clinica Chimica Acta 318 (2002) 107–112

www.elsevier.com/locate/clinchim

A simple method of detecting K-*ras* point mutations in stool samples for colorectal cancer screening using one-step polymerase chain reaction/restriction fragment length polymorphism analysis

Takashi Nishikawa [a,*], Kentaro Maemura [a], Ichiro Hirata [a], Ryouichi Matsuse [b], Hiroshi Morikawa [a], Ken Toshina [a], Mitsuyuki Murano [a], Keiichi Hashimoto [a], Yoshihito Nakagawa [a], Osamu Saitoh [a], Kazuo Uchida [b], Kenichi Katsu [a]

[a] *Second Department of Internal Medicine, Osaka Medical College, Takatsuki, Japan*
[b] *Kyoto Medical Science Laboratory, Kyoto, Japan*

Received 17 July 2001; received in revised form 20 November 2001; accepted 10 December 2001

Abstract

Background: We examined a technique for detecting point mutations of K-*ras* codon 12 in stool samples using one-step polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analysis, in order to determine whether it could be used to screen for colorectal cancer. *Methods*: DNA was extracted from 200-mg stool specimens of 5 healthy controls and 31 colorectal cancer patients. A 107-base-pair fragment of exon 1 of K-*ras* was amplified by PCR using mismatched primers. PCR products were digested with B*st* NI and analyzed by gel electrophoresis followed by silver staining. Specificity of one-step PCR/RFLP was examined by using synthetic oligonucleotides. The detection limit of K-*ras* codon 12 mutations was determined by using SW480 and HT29 cells. *Results*: The K-*ras* gene was successfully amplified from all healthy controls and colorectal cancer patients studied. Mutations of K-*ras* codon 12 were not detected in any of the healthy controls, but were identified in 13 (41.9%) of the 31 patients with colorectal cancer. Mutations were detectable in all six synthetic mutant DNAs, while none were detected among the wild type. The detection limit of this method was $\geq 0.1\%$. *Conclusions*: PCR/RFLP analysis could be used in mass screening for colorectal cancer, because it is highly specific, has a low detection limit, and is simpler than conventional methods for detecting genetic abnormalities.

Keywords: K-*ras*; Colorectal cancer; Stool; PCR/RFLP

1. Introduction

Recent advances in research into the molecular biology of colorectal cancer have identified a close relationship between oncogenes, abnormalities of tumor suppressor genes (such as *APC*, K-*ras*, *p53*, and *DCC*), and the development and progression of colorectal cancer. K-*ras* is an oncogene that exists on chromosome 12p12.1 and is activated by point mutations at codons 12, 13, and 61 [1]. The products of K-*ras* are GTP/GDP-binding proteins that have a molecular weight of approximately 21 kDa and are found on the inner side of the cell membrane where they act as transmitters for cell proliferation and differentiation signals

* Corresponding author. 7676 Phoenix Drive, #1509 Houston, TX 77030, USA. Tel.: +1-713-796-8680.
E-mail address: tnishika@mdanderson.org (T. Nishikawa).

0009-8981/02/$ - see front matter

[1]. Activation of K-*ras* by point mutations appears to play a role in carcinogenesis by increasing the active GTP-bound form, which increases the activity of transcription factors through binding to target proteins (Raf, PI3kinase, and RalGDS) [2–5]. K-*ras* mutations are common in various cancers, with a prevalence of 40–50% in colorectal cancer [6–9], 75–93% in pancreatic cancer [9], 22–33% in pulmonary adenocarcinoma [9]. In colorectal cancer, most (70–80%) of these mutations occur at codon 12 [6–9]. In colorectal cancer, K-*ras* point mutations occur early in the adenoma–carcinoma sequence and are believed to contribute to the growth and increased atypia of adenomas [8,10].

At present, fecal occult blood testing is commonly used in mass screening for colorectal cancer. However, this method is not specific for cancer because it detects the secondary phenomenon of bleeding and is therefore also positive for bleeding caused by inflammation or hemorrhoids. For a diagnostic tool to be specific for cancer, it must be capable of the direct detection of genetic alterations in cancer cells within stool samples. Since Sidransky et al. [11] first reported that K-*ras* point mutations were detectable in stool samples, several groups have analyzed specimens using modified techniques such as mutant allele-specific amplification [12,13] and mutant-enriched PCR [14]. However, these methods cannot be readily applied to mass screening because of contamination and low reproducibility due to the complexity of the procedures. In an attempt to overcome these problems, we used one-step polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) to detect K-*ras* point mutations in stool samples, and we investigated the specificity and the mutation detection limit of this method.

2. Materials and methods

2.1. Cell culture

A human colon cancer cell line (HT29) was maintained at 37 °C in McCoy's 5A medium (Dai-Nippon Pharmaceutical, Japan) supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Another human colonic cancer cell line (SW480) was maintained at 37 °C in L-15 medium (Dai-Nippon Pharmaceutical) supplemented with 10% fetal bovine serum and penicillin/streptomycin.

2.2. Patients and specimens

Stool and tissue specimens were collected from 31 patients with colorectal cancer. As a normal control, stool specimens were obtained from five patients who had no evidence of malignant tumors on colonoscopy, gastroduodenoscopy, and upper abdominal ultrasonography. All of these patients gave informed consent for our study. The stool specimens were immediately frozen at −80 °C until use.

Tissue samples were obtained either from formalin-fixed, paraffin-embedded tissue blocks or from biopsy materials. Paraffin-embedded tissue samples were serially cut into 10-μm sections. All specimens were reviewed by a pathologist in our department.

2.3. DNA extraction from stool specimens

Approximately 200 mg of stool was resuspended in 5 ml of TEN buffer (500 mmol/l Tris buffer, pH 9.0, containing 29 mmol/l EDTA and 10 mmol/l NaCl) and incubated for 1 h at room temperature. The supernatant obtained by centrifugation at 3000 rpm for 10 min was digested with 1 mg/ml proteinase K at 56 °C overnight in the presence of 1% sodium dodecylsulfate (SDS). Then, extraction was performed with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), and DNA was precipitated with isopropyl alcohol. The DNA pellet was obtained by centrifugation at 15,000 rpm for 5 min and dried under a vacuum. For cetyltrimethylammonium bromide (CTAB) treatment, the DNA pellet was dissolved in 500 μl of TE buffer (pH 8.0) containing 0.35 mol/l NaCl and 1% (w/v) CTAB, and then the reaction mixture was incubated at 65 °C for 15 min. After extraction with chloroform/isoamyl alcohol (24:1), DNA was precipitated with isopropyl alcohol and dissolved in 30 μl of distilled sterilized H_2O.

2.4. DNA extraction from tissue sections

Paraffin-embedded tissue sections were deparaffinized with xylene and dehydrated in a graded ethanol series. Subsequently, cancerous regions were separated from noncancerous regions using a razor blade.

The samples thus obtained were treated with SDS/proteinase K and DNA was extracted and purified as described above, except for CTAB treatment.

2.5. PCR/RFLP analysis

To detect K-*ras* gene alterations, PCR/RFLP analysis was performed according to the protocol described elsewhere with several modifications. The oligonucleotide primers used for PCR amplification were

5′ -GACTGAATATAAACTTGTGGTAGTTGGACCT-3′ , (sense) and
5′ -CTATTGTTGGATCATATTCGTCC-3′ (antisense).

The sense primer was designed to introduce a base substitution that created a B*st* NI recognition site only for the wild-type codon 12. A 107-base-pair (bp) fragment of exon 1 of K-*ras* was amplified. The PCR mixture consisted of 5 μl of DNA and 45 μl of 10 mmol/l Tris–HCl buffer (pH 8.3) containing 3 mmol/l $MgC1_2$, 50 mmol/l KCl, 0.01% (w/v) gelatine, 20 mmol/l of each dNTP, 1.25 U of Taq DNA polymerase (AmpliTaq Gold™; Perkin Elmer, USA), and 0.5 mmol/l of each primer. PCR was carried out in a Thermal Cycler (TaKaRa TP3000, Japan), with preheating at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 30 s. An aliquot (10 μl) of PCR products was digested with 30 units of B*st* NI at 60 °C for 3 h, and analyzed by polyacrylamide gel electrophoresis followed by silver staining. If there was no mutation of K-*ras* codon 12, the 107-bp fragment was cleaved into 77- and 30-bp fragments.

2.6. Specificity of one-step PCR/RFLP analysis

We examined whether mutations could be detected by the PCR/RFLP method using six synthetic oligonucleotides containing different mutations of K-*ras* codon 12. The sequences of the mutated codon were AGT, TGT, CGT, GAT, GTT, or GCT. In addition, oligonucleotides containing wild-type codon 12 (GGT) were synthesized.

2.7. Detection limit of one-step PCR/RFLP analysis

To establish the detection limit of our method, SW480 cells (which have two mutant alleles at codon 12 of the K-*ras* oncogene) were mixed with HT29 cells (which have the wild-type K-*ras* oncogene). SW480 cells were added at dilutions from 1:1 to 1:1,000,000. Subsequently, DNA was extracted and subjected to PCR/RFLP analysis.

3. Results

3.1. Detection of K-ras gene mutations in stool specimens from colon cancer patients

In all cases, an intense band for the 107-base-pair fragment was detected with ethidium bromide staining after the one-step PCR (data not shown). In normal controls, no extra band indicating K-*ras* mutation was obtained by B*st* NI digestion (Fig. 1a). On the other hand, an extra band at 107 bp was clearly detected in some colon cancer patients (Fig. 1b).

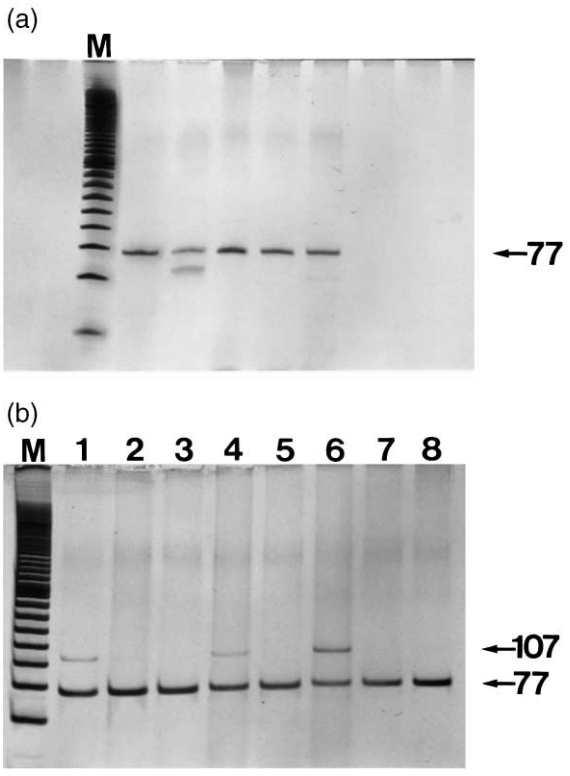

Fig. 1. Detection of K-*ras* codon 12 point mutation. One-step PCR/RFLP products were electrophoresed in a 12% polyacrylamide gel followed by silver staining. (a) In all normal controls, only a 77-bp fragment was detected. (b) In some colorectal cancer patients, a 107-bp fragment as well as a 77-bp fragment were detected (lanes 1, 4, 6). M: molecular weight marker.

3.2. K-ras codon 12 mutations in stool or tissue specimens from colon cancer patients

The results of PCR/RFLP analysis of K-*ras* codon 12 mutations in stool and tissue specimens are summarized in Table 1. Among the 31 cancer patients examined, we found somatic K-*ras* mutations in stool from 13 patients (41.9%) and 13 patients had mutations in their cancer tissues. Eleven of these patients had mutations in both their stool specimen and the tumor. In 16 patients, no mutations were observed in both the stool specimen and the tumor. However, two patients only had mutations in the stool specimen and another two patients only had mutations in the tumor.

Table 1
Summary of K-*ras* codon 12 mutations in stool or tissue samples of colon cancer patients

Case	Sex	Age	Tumor			K-*ras* mutation	
			Site	Size (mm)	Stage	Stool	Tissue
1	M	42	A	52 × 42	Dukes C	+	+
2	M	66	D	15 × 9	Dukes A	−	−
3	F	64	S	20 × 19	Dukes A	−	−
4	M	70	S	33 × 29	Dukes B	+	+
5	M	42	R	29 × 27	Dukes C	−	−
6	F	41	S	70 × 55	Dukes C	+	−
7	M	57	S	11 × 9	Dukes A	−	−
8	M	64	S	30 × 27	Dukes C	−	−
9	M	66	S	14 × 8	Dukes A	+	+
10	M	59	S	10 × 10	Dukes A	−	−
11	M	65	D	20 × 20	Dukes B	−	−
12	F	73	A	92 × 81	Dukes C	+	+
13	F	58	S	60 × 55	Dukes B	−	−
14	M	64	S	51 × 42	Dukes C	+	+
15	M	69	A	52 × 34	Dukes C	+	+
16	F	69	S	40 × 31	Dukes A	−	−
17	F	72	S	32 × 23	Dukes A	+	+
18	F	70	S	20 × 19	Dukes A	+	+
19	F	59	R	60 × 60	Dukes C	−	+
20	M	67	R	60 × 58	Dukes C	+	+
21	F	51	S	20 × 18	Dukes B	+	+
22	M	70	R	60 × 45	Dukes B	−	−
23	F	80	R	55 × 51	Dukes C	−	−
24	F	52	R	57 × 55	Dukes C	−	−
25	M	75	A	54 × 50	Dukes C	−	−
26	M	71	D	27 × 25	Dukes C	+	−
27	M	40	R	25 × 25	Dukes A	−	−
28	M	57	S	42 × 40	Dukes C	−	−
29	F	48	D	35 × 30	Dukes C	−	−
30	M	61	S	20 × 20	Dukes A	−	+
31	F	74	T	32 × 30	Dukes C	+	+

A — ascending colon; D — descending colon; S — sigmoid colon; R — rectum; T — transverse colon.

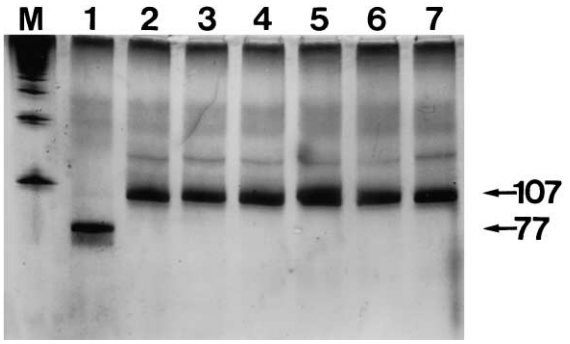

Fig. 2. Specificity of one-step PCR/RFLP analysis. Digested synthetic oligonucleotide containing wild type K-*ras* codon 12 (lane 1: GGT) shows a 77-bp fragment. Synthetic oligonucleotides containing mutant type K-*ras* codon 12 (lane 2: CGT, lane 3: AGT, lane 4: GAT, lane 5: GTT, lane 6: TGT, lane 7: GCT) show a 107-bp fragment even after B*st* NI digestion. M: molecular weight marker.

In the ascending colon, 3 out of 4 (75%) tumors, as well as 1 out of 1 (100%) in transverse colon, 1 out of 4 (25%) in the descending colon, 8 out of 15 (53.3%) in the sigmoid colon, and 2 out of 7 (28.6%) in the rectum were found to have K-*ras* mutations. Mutations were found in 1 out of 4 (25%) tumors that measured 1–2 cm in diameter, as well as in 4 out of 8 (50%) tumors measuring 2–3 cm, and 10 out of 19 (52.6%) tumors measuring >3 cm. The following were found to have mutations: 4 out of 10 (40%)

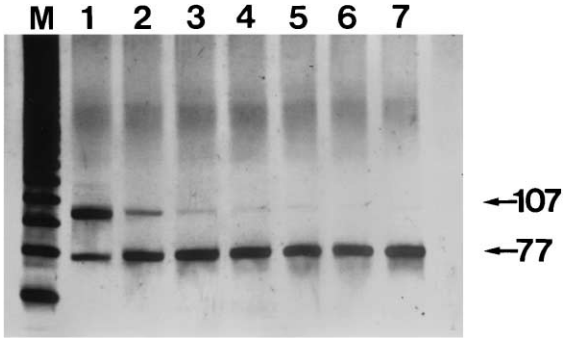

Fig. 3. Detection limit of one-step PCR/RFLP analysis. SW480 cells were mixed with HT29 cells in the following ratios. Lane 1—1:1. Lane 2—1:10. Lane 3—1:100. Lane 4—1:1000. Lane 5—1:10,000. Lane 6—1:100,000. Lane 7—1:1,000,000. Detection limit of this method was 1:1000 mutant type cells in wild type cells. Mutations were detected when at least 0.1% of the cells were SW480 cells.

Dukes A tumors, 2 out of 5 (40%) Dukes B tumors, and 9 out of 16 (56.3%) Dukes C tumors.

3.3. Specificity of one-step PCR/RFLP analysis

Six synthetic oligonucleotides containing K-*ras* codon 12 mutations were not digested by B*st* NI, although synthetic oligonucleotides having the wild-type sequence were digested and yielded smaller fragments (Fig. 2).

3.4. Detection limit of one-step PCR/RFLP analysis

Mutations were detected when at least 0.l% of the cells were SW480 cells (Fig. 3).

4. Discussion

The detection rate of K-*ras* mutations in stool specimens from our patients with colorectal cancer was 41.9% (13:31). Among patients who were positive for mutations in tumor tissue, the detection rate in stool specimens was high (84.6%, 11:13). This positive rate is well compatible to results reported previously [11,14,15]. Mutations were identified only in the stool specimens of two patients (Nos. 6 and 26) and only in the tissue specimens of other two patients (Nos. 19 and 30). This was probably because K-*ras* mutations are not uniform (heterogenous) within the same tumor. In addition, K-*ras* mutations are reported to be detected with high rate in aberrant crypt foci [16,17] and have also been detected in some hyperplastic polyps [18,19] and normal-appearing tissue [18,20], which may account for the false positives in some stool specimens. Contamination by large amounts of enteric bacteria, food residue, and hemoglobin may also have interfered with DNA extraction and may have led to dilution of cells shed by the tumor, thereby causing false negative results.

Although there was no significant relationship between the mutation rate and the tumor site, the mutation rate tended to be higher for large or advanced cancers, suggesting that activation of K-*ras* promote tumor cell proliferation.

In the present study, we were able to detect point mutations of K-*ras* codon 12 in stool samples using a one-step PCR/RFLP. Many investigators have used this method to detect K-*ras* mutations [21–23], but we could find only one report on stool samples [15]. This is probably because extracting and purifying very small quantities of human DNA from stool is difficult, hindering subsequent PCR amplification and detection of mutations. In a preliminary study aimed at overcoming this problem, we were able to detect point mutations of codon 12 in stool specimens simply and with high sensitivity. In the present study, DNA was successfully extracted from all patients using standard SDS/proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction, isopropyl alcohol precipitation, and treatment with CTAB. It has been traditional to use CTAB for extracting and purifying DNA from plants and bacteria [24], and it appears to facilitate DNA extraction by removing polysaccharides derived from enteric bacteria and plant residue in the stool. The efficiency of analyzing K-*ras* mutations was also increased by using a hot-start PCR employing the DNA polymerase AmpliTaq Gold™ (Perkin Elmer) to improve specific amplification. We also used silver staining to detect mutations, because this is more sensitive than ethidium bromide staining [25,26]. Through the combined use of these techniques, we were able to employ the one-step PCR in all of our patients.

A study with synthetic DNA confirmed the appearance of bands specific for the six mutant sequences, and investigation using cell lines showed a detection limit of 0.1%. This is higher than is generally reported for mutant allele-specific amplification (0.01–0.001%) [27] or mutant-enriched PCR (<0.01%) [28]. However, the specific amplification technique aims to specifically amplify mutant alleles involving one base located at the 3′ end of the forward primer, and it is therefore possible that amplification of the wild-type allele will proceed if there is a slight change in conditions such as the annealing temperature [29]. Another problem with the PCR itself is that incorrect reading of nucleotides cannot be avoided during the amplification process, giving rise to mutations. Mutant-enriched PCR has certain disadvantages when used to detect genetic mutations, because there is a high risk that mutations arising during the PCR process will be detected and every effort must be made to avoid cloning of the PCR product.

The one-step PCR/RFLP method therefore has a higher reproducibility and is simpler than other techniques. As a result, it should be more useful for screening.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.