Dried Feces Spots as an Alternative DNA Source: Detection of K-ras Mutations in Colorectal Cancer Screening

Akihiro Yamaguchi[1,4,5], Naoko Hashimoto[1], Wataru Tsutae[1], Kuniyoshi Seino[2], Hiroyuki Osanai[3], Naoya Yamamoto[3], Takashi Tokino[4], Noriyuki Sato[5], and Kokichi Kikuchi[1,2,5]

[1]*Sapporo Immunodiagnostic Laboratory Sinkawa 2-2-12-20, Kita-ku, Sapporo 001-0922, Japan*
[2]*Cytodiagnostic Center, Hokkaido Cancer Society N 26 E 14, Higashi-ku, Sapporo 065-0026, Japan*
[3]*Sapporo Memorial Surgery Hospital S 23 W 15, Chuo-ku, Sapporo 064-0923, Japan*
[4]*Department of Molecular Biology, Cancer Research Institute,*
[5]*Department of Pathology, Sapporo Medical University S 1 W 17, Chuo-ku, Sapporo 060-8556, Japan*

ABSTRACT

Detection of genetic alterations in exfoliated intestinal cells into feces is expected to be a novel approach to colorectal cancer screening. We aimed here to detect cancer-related gene mutations in routine fecal specimen; dried feces spots (DFS) on a filter paper especially designated for an automated fecal occult blood (FOB) test. DNA was simply extracted from a 3 mm disc of DFS (including around 10 mg feces) with a boiling procedure followed by a cationic surfactant extraction. K-ras common mutations, as the typical cancer-related gene alterations, were detected with a nested allele specific PCR with microfluorometry (ASP/MFL). Adequate DNA was recovered from 363/451 (80.5%) DFS specimens tested. The K-ras mutations were detected in 2/24 (8.3%) from patients with colorectal cancer and 13/315 (4.1%) from screening participants with positive FOB. No mutations were detected in 24 DFS specimens with negative FOB. The results of K-ras mutation frequency were not encouraging to achieve practically a specific and reliable method for colorectal cancer screening. However this report, identifying for the first time a cancer related gene alteration in feces using identical samples with routine FOB test, will be a step to realize a molecular genetic testing for the cancer screening.

Key words : Stool, Noninvasive sample, Fecal occult blood, Colorectal cancer, K-ras mutation, Genetic screening

INTRODUCTION

Colorectal cancer is a typical form of curable cancer if diagnosed at an early stage. Population screening, based on fecal occult blood (FOB) test, has been successfully introduced and improved the clinical outcome of the

Address correspondence and reprint requests to Kokichi Kikuchi, M.D., Ph.D., Sapporo Immunodiagnostic Laboratory, Shinkawa 2-2-12-20, Kita-ku, Sapporo, 001-0922 Japan, Tel: +81-11-762-3610, Fax: +81-11-764-2042
E-mail: kkikuchi@remus.dti.ne.jp

patient[1-3]. The FOB test is noninvasive and economical, however there remains an obscurity in sensitivity and specificity being caused by frequent false negative and positive cases.

In the last decade, extensive research has aimed to identify cancer related gene alterations (such as K-ras, p53 and BAT26 mutations) in cells shed from the tumors into stools[4-7]. Such a molecular genetic approach is still not a practical alternative to the FOB test. Nevertheless, it has great potential to improve the efficacy of colorectal cancer screening.

Most of the previous studies on genetic testing of stool specimens were limited to the use of samples obtained from patients already diagnosed as having colorectal cancer. In contrast, we aimed here to detect K-ras mutations (as one of the most typical gene alterations) in routine fecal specimen. This was done by obtaining dried feces spots (DFS) on a filter paper especially designated for an automated FOB test[8].

K-ras has been preferentially targeted for genetic alteration because most mutations, found in 30 - 40% of colorectal cancer, are clustered at codons 12 and 13. Several methods, applicable to stool samples, were reported based on PCR amplification with a variety of detection techniques[6,9-11]. We adopted and optimized here a nested allele specific PCR (ASP) with microfluorometry (MFL) for K-ras mutations in DFS. ASP/MFL is a simple and practical technique developed by combined use of ASP amplification and subsequent fluorogenic intercalation to the amplicon[12]. The method, designated to work on a 96-well microplate scale, is especially suitable for screening a multitude of samples and produces objective results.

MATERIALS AND METHODS

DFS specimens

The DFS specimens with positive FOB test were kept at 4℃ for 3 to 6 months until colonoscopic evaluation was completed. DFS specimens with negative FOB were also used as control. A total of 422 routine DFS, obtained from 211 participants in two successive days, was analyzed. In addition, DFS from 29 patients with colorectal cancer, not screened but found clinically, were obtained prior to surgery. All participants in the study had given their informed consent, and experiments were carried out after approval by the local ethics committee.

DNA extraction from DFS specimens

Scraps of stool surface taken with a swab were obtained by participants, and applied onto the filter paper. Each specimen, including 3 discs of 5 mm diameter with around ten mg stool / disc, was kept dry and away from the light in a closed aluminum bag (according to the manufacturer's instruction). One of the fecal discs was submitted for the automated FOB test (SanAuto Hemo Kit & FE system, Sanko Pure Parmaceurical, Tokyo, Japan). Another disc, for DNA extraction, was placed in a 0.5 mL microtube using a tweezer. Two hundred μL of cetyltrimethylammonium bromide (CTAB) - lysis buffer (0.5% CTAB, 0.5 M Tris, 16 mmol/L EDTA, 1.4 M NaCl, pH 9.0) was added, and vortexed vigorously. By this step, the dried feces were spread out in the sol of the filter paper matrix (with highly water absorbent material). The microtubes were boiled at 95℃ for 15 min using a GeneAmp 9600 PCR System (Perkin Elmer, Norwalk, CT, U.S.A.). The tubes were centrifuged for 5 min at 15,000 rpm with the resultant supernatant (around 120 μL) being transferred to a new tube. The excess CTAB was removed by vigorous vortexing for 1 min with 200 μL chloroform followed by centrifugation for 5 min at 15,000 rpm. The aqueous layer was transferred to a new tube. Another 200 μL chloroform was added, and the CTAB removal step was repeated. DNA was precipitated by the addition of 100 μL 2-propanol to the final aqueous layer, and the mixture left standing for 30 min at -20℃. DNA was collected by centrifugation at 15,000 rpm for 10 min at 4℃ and the supernatant was discarded. The DNA was then washed with 400 μL 70% ethanol and finally dissolved in 50 μL of TE buffer solution (10 mmol/L Tris, 1

mmol/L EDTA, pH 8).

ASP/MFL for K-ras mutations

Template DNA of the DFS extracts was first amplified using a PCR primer pair; KrasF-1st (forward: 5'-agg cct gct gaa aat gac tga ata) and reported KrasR (reverse: 5'-tga gct gtc gct taa ttg ctc)[13]. Eighteen μl of PCR reaction mixture was added to each microtube containing 2 μl of the DNA. The final mixture consisted of 10 mmol/L of Tris-HCl (pH 9.0), 50 mmol/L of KCl, 1.5 mmol/L of MgCl2, 0.2 mmol/L of each dNTP, 0.5 units of Taq DNA Polymerase (Amersham Pharmacia, Piscataway, NJ, U.S.A.) modified with 0.7 pmol of TaqStartTM Antibody (Clontech, Palo Alto, CA, U.S.A.), and 5 pmol of both KrasF-1st and KrasR primers. The mixtures were denatured initially for 5 minutes at 95℃, then alternatively cycled at 95℃, 60℃ and 72℃ for 30 second periods over 38 cycles. This was followed by a final extension at 72℃ for 7 minutes using a GeneAmp 9600 PCR System (Perkin Elmer, Norwalk, CT, U.S.A.). After spin down the plate, a 10 μl-portion of the amplicon was transferred with a multichannel micropipette to a well of a 96-well white plate for fluorometry (Nunc, Rochester, NY, U.S.A.). The residual 10 μl of the amplicon was diluted 10 times by the addition of 90 μl TE buffer solution (the cap was replaced to avoid leaking).

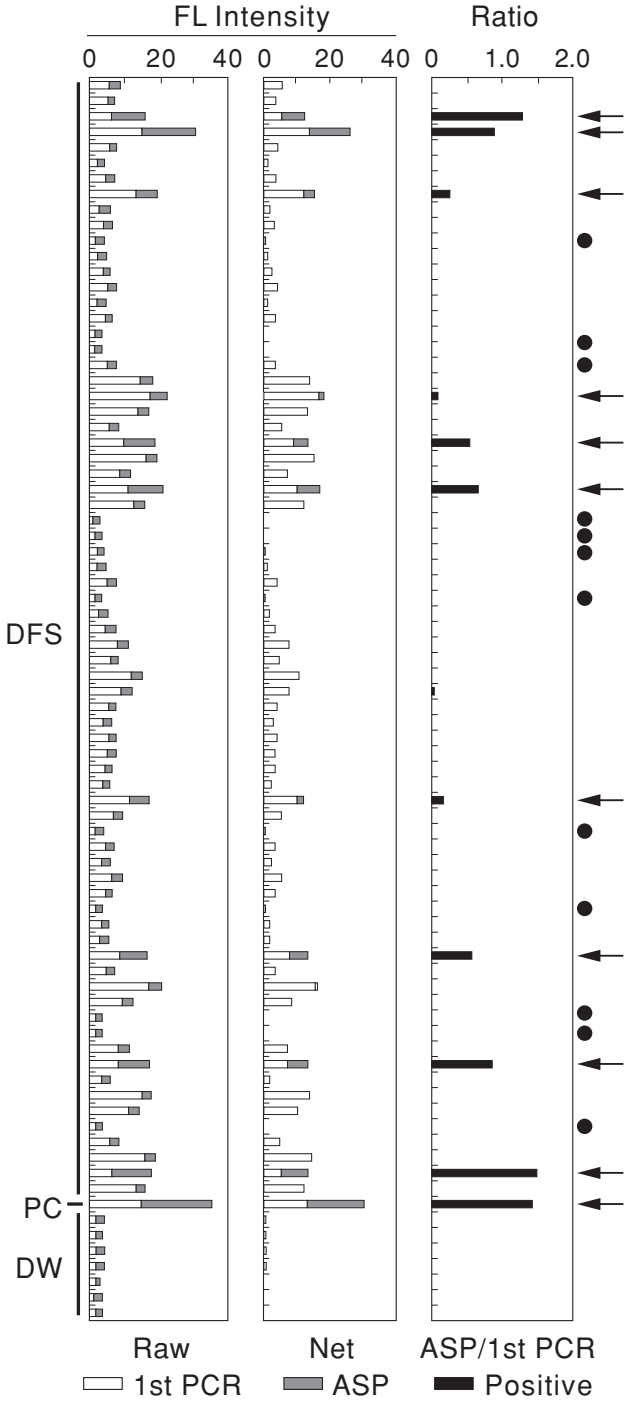
A two μl-portion of the diluted amplicon was submitted for nested ASP. The ASP primers were particularly designed for highly specific detection of 3 major K-ras mutations; G12D (ggt>gat), G12V (ggt>gtt) and G13D (ggc>gac). The primer sequence were as follows: Kras12A-3g (5'-ctt gtg gta gtt gga gGt ga) for G12D, Kras 12T-3g (5'-ctt gtg gta gtt gga gGt gt) for G12V and Kras13A-1a (5'-gtg gta gtt gga gct ggt Aa) for G13D. A mismatch sequence (capitalized) was introduced to achieve optimum specificity at 1 or 3 bp upstream of each mutant position from the 3' terminal. The nested ASP condition was the same as that described in the first PCR section except for the cycle numbers being changed to 15, the reaction volume being reduced to 10 μl, and the primer pair being replaced by an ASP primer set (every 2.5 pmol of Kras12A-3g, Kras12T-3g and Kras13A-1a with the common KrasR). For the typing of the individual mutation, one of the corresponding forward ASP primers was used with the common KrasR primer instead of the ASP primer set.

A working solution of fluorogenic intercalator, SYBR Green I (Molecular Probes, Eugene, U.S.A.) was prepared by diluting the stock solution at a ratio of 5,000 to 1 with TAE buffer solution (0.04 M Tris, 1 mmol/L EDTA, pH 7.8 adjusted with acetic acid). To each well of the white plate, including the first PCR product, 100 μl SYBR Green I was added and mixed by pumping using a multichannel micropipette, and the mixture was then left standing for 10 minutes at room temperature. Meanwhile, SYBR Green I was added and mixed to the nested PCR products in the PCR tubes, 100 μl as well. The 100 μl-portion of the mixture was transferred to the white plate. The specific fluorescence was measured by a fluorometric microplate reader Fluoroskan II (Labsystems, Helsinki, Finland) at 485/538nm of excitation/emission wavelength.

RESULTS AND DISCUSSION

The typical output of the ASP/MFL is shown in Fig. 1. The fluorescence intensities derived from the first PCR and nested ASP corresponded to K-ras alleles with [wild plus mutant] and [mutant], respectively. The DFS samples were defined as being positive when the intensity ratio of the following criteria were satisfied; [mutant]/ [reagent blank] > 2 and [mutant] / [wild plus mutant] > 0.1. The DFS sample was considered as insufficient DNA, without further evaluation, when the fluorescence ratio [wild plus mutant] / [reagent blank] < 2. The positive result was confirmed by the typing protocol for the individual mutation from the first PCR step. The sample, shown to be successively positive by this repetitive test, was finally recognized as harboring the mutations.

Based on these criteria, adequate DNA was



recovered from 363 out of 451 DFS specimens (80.5%), and the K-ras mutations were detected in 2 out of 24 patients (8.3%) and 13 out of 315 DFS specimens (4.1%) from screening participants with positive FOB test (Table 1). The routine samples were analyzed in duplicate using the discs obtained in two successive days. Therefore, the mutation frequency per person was calculated as being nearly twice the rate since no case was shown to be successively positive in both discs. Similarly, no mutations were detected in 24 DFS samples or 12 participants with negative FOB test.

Given the difficulties of using DFS as a DNA source, such as stools with complex matrix, small sampling amounts and samples not being freshly obtained (stored at 4℃ for a long period), 80% recovery of DNA is an adequate rate. All of the previous studies required stool samples freshly obtained and frozen stored[4,6-7,9-11].

On the other hand, DNA degradation by apoptosis is supposed to be less efficient in cells shed by tumors compared with fully differentiated cells[5]. In addition, Machiels et al.[14] recently reported that DNA could be recovered from stools kept dry in a closed tube for two days at room temperature. The dehydration is likely to inactivate enzymes related to degradation of DNA. The SanAuto Hemo kit is used to stabilize hemoglobin in stool. It is easy to use and transport. Thus, the use of DFS will be more preferable for routine genetic testing than the system of hydrated stool samples in a buffer solution adopted by the majority of FOB kits.

The procedure for DNA extraction was simplified by boiling the fecal disc in the pres-

Fig. 1 Detection of K-ras mutations in DFS samples by nested ASP/MFL. The DNA was extracted from DFS samples by CTAB/boil method. K-ras exon 1, encompassing codons 12 and 13, was amplified by the first PCR. The nested ASP was targeted for 3 major mutations; G12D, G12V and G13V using a mixed ASP primer set. Fluorogenic intercalator, SYBR Green I was added to the first PCR and the nested ASP products, then the specific fluorescence was measured by a conventional fluorometric microplate reader at 485/538nm. DFS = Dried feces spots, PC = Cloned positive control for the 3 mutations, DW = Deionized water as reagent, FL = Fluorescence intensity in arbitrary, Raw = Fluorescence versus air blank, Net = Fluorescence subtracted the background level of S/N 2, Open bar = Signal for the first PCR, Hashed bar = Signal for the nested ASP, Closed bar & Arrow = Positive cases of K-ras mutation under the criteria both with [S/N>2 of the Raw signals] and with [the Ratio of the Net signals; ASP/1st PCR >0.1], Closed circle = Samples with insufficient DNA.

Table 1 K-ras Mutations detected in DFS specimens from patients with colorectal cancer and screening participants with FOB tests

DFS	Tested	DNA adequate	K-ras mutation detected				
			No.	%	G12D	G12V	G13D
Colorectal cancer	29	24	2	8.3	1	0	1
Screening participant[1]							
FOB positive[2]							
Cancer	72	54	3	5.6	1	0	2
Polyp	174	149	3	2.0	1	1	1
Negative	144	112	7	6.2	3[3]	2[3]	3
FOB negative	32	24	0	-	-	-	-

1 Tested duplicate samples per person obtained in two successive days.
No cases of mutations identified in both samples.
2 Subgrouped by clinical diagnosis with colonoscopy.
3 Including one case with heterogeneous mutations.

ence of a cationic surfactant, CTAB. The excess CTAB as well as stool debris and filter paper matrix was efficiently removed at once by subsequent chloroform extraction. The removal of fecal anionic polysaccharides by ion-pairing with CTAB was an essential step for preventing PCR inhibition, as previously pointed out[15].

The condition of the nested ASP/MFL was examined for the detection of K-ras common mutations. The best counterpoise in sensitivity and specificity was achieved by a combination of 38 cycle numbers for the first PCR and 15 for the nested ASP. The introduction of a mismatch sequence, in each ASP primer, was effective to improve specificity while maintaining moderate sensitivity. The adoption of ASP, in conjunction with MFL detection, has great potential, especially for robust and economical application in population-based studies[12].

The three major K-ras mutations were targeted here since our preliminary study, as well as other studies, detected them in 30 to 40 % of tissue specimens from colorectal cancer. The 8.3% frequency of the mutations, detected in DFS from patients with colorectal cancer, is lower than that generally accepted (Table 1); and this is primarily due to the smaller amounts of stool samples (around 10 mg). Another possible reason may be the rather strict criteria adopted for positive results in our study; although paired tumors were not available and therefore could not be tested. Most of the other reports on genetic testing of stool samples did not clearly explain their criteria for possible results.

The main focus of this paper is to assess the possibilities of a genetic test for routine screening of colorectal cancer. The FOB positive DFS samples were recovered and tested for the K-ras mutations under uniform conditions. The samples were classified into 3 subgroups according to clinical diagnosis by colonoscopy. K-ras mutations were detected from all the subgroups of Cancer, Polyp and Negative (without any specific observations) in 5.6, 2.0 and 6.2 % of samples, respectively (Table 1). The frequencies for the Cancer and Polyp groups may be appropriate in comparison to the 8.3% in the typical patients and a similar frequency is noted even in the Negative group including a case with heterogeneous mutations. In terms of K-ras mutation frequencies, however, the age of the DFS samples must be borne in mind. All DFS samples from screening participants, had been

obtained 3 to 6 months before colonoscopy and the tumor and/or genetic status might have changed during this time lag. Moreover, K-ras status with heterogeneous mutations is also frequently reported in adenomas (30-40%)[16]. These data may support recent findings showing that K-ras mutations are not centrally involved in the malignancy of tumors, but just related to the morphological phenotype of the adenoma[5,10,16].

In FOB tests, successive stool sampling is recommended because bleeding from the tumor is known to be intermittent. Our results suggest similar limitations to FOB tests in genetic testing also. Regarding K-ras detection in this study, there were no cases which were positive in successive DFS samples, even though the corresponding FOB tests showed positive in most cases. This suggests inadequate sensitivity of the detection of K-ras mutations in colorectal cancer screening under present circumstances.

Somatic mutations in tumors, like K-ras as we experienced it here, are often hard to detect and still not widely acknowledged in the clinical setting. Conversely, germline mutations in familial cancer, such as in APC or p53, will be easier to detect and more open to definitive clinical action. However, a different methodological approach will be needed for the detection of their dispersed mutations. We are now trying to detect APC mutations in DFS with positive FOB results as a secondary test. When an APC mutation is identified, colonoscopy should be strongly recommended.

The final goal of achieving a specific and reliable method of molecular genetic testing for cancer screening is perhaps some way in the future yet. Nevertheless this report, identifying for the first time a cancer related gene alteration in stool using identical samples with routine FOB test, is a step in the right direction. Furthermore, the potential advantage of DFS specimens as a novel DNA source for genetic testing is clarified.

REFERENCES

1. Kronborg O, Fenger C, Olsen J, Jorgensen OD, Sondergaard O. Randomized study of screening for colorectal cancer with faecal-occult-blood test. Lancet 1996, 348: 1467–71.
2. Hardcastle JD, Chamberlain JO, Robinson MH, Moss SM, Amar SS, Balfour TW, James PD, Mangham CM. Randomized controlled trial of faecal-occult-blood screening for colorectal cancer: results for first 107,349 subjects. Lancet 1996, 348: 1472–7.
3. Mandel JS, Church TR, Ederer F, Bond JH. Colorectal cancer mortality: effectiveness of biennial screening for fecal occult blood. J Natl Cancer Inst 1999, 91: 434–7.
4. Sidransky D, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P, Vogelstein B. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. Science 1992, 256: 102–5.
5. Atkin W, Martin JP. Stool DNA-based colorectal cancer detection: finding the needle in the haystack. J Natl Cancer Inst 2001, 93: 798–9.
6. Dong SM, Traverso G, Johnson C, Geng L, Favis R, Boynton K, Hibi K, Goodman SN, D'Allessio M, Paty P, Hamilton SR, Sidransky D, Barany F, Levin B, Shuber A, Kinzler KW, Vogelstein B, Jen J. Detecting colorectal cancer in stool with the use of multiple genetic targets. J Natl Cancer Inst 2001, 93: 858–65.
7. Rengucci C, Maiolo P, Saragoni L, Zoli W, Amadori D, Calistri D. Multiple detection of genetic alterations in tumors and stool. Clin Cancer Res 2001, 7: 590–3.
8. Arisue T, Hojo K, Ito T, Kudo S, Nagasawa S, Hiwatashi N, Ikeda S, Mochizuki F, Itasaka S, Sakai J, Hiratsuka H, Ogoshi K, Tsukioka M, Imai S, Baba S, Kasugai T, Murakami R, Matsuoka A, Sumii K, Takeuchi Y, Sagara Y, Yao T, Kawamoto K, Fujiyoshi T, Kusano K. The evaluation of automated immunological fecal occult blood test for Colorectal cancer mass screening. Shoukaki Shudan Kenshin (J Gastroenterol

Mass Survey) 1996, 34: 514–25 (in Japanese).
9. Nollau P, Moser C, Weinland G, Wagener C. Detection of K-ras mutations in stools of patients with colorectal cancer by mutant-enriched PCR. Int J Cancer 1996, 66: 332–6.
10. Ahlquist DA, Skoletsky JE, Boynton KA, Harrington JJ, Mahoney DW, Pierceall WE, Thibodeau SN, Shuber AP. Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. Gastroenterology 2000, 119: 1219–27.
11. Doolittle BR, Emanuel J, Tuttle C, Costa J. Detection of the mutated K-ras biomarker in colorectal carcinoma. Exp Mol Pathol 2001, 70: 289–301.
12. Yamaguchi A, Nepote JA, Kadivar M, Tagami Y, Fukushi M, Kikuchi Y, Sato N, Kikuchi K. Allele specific PCR with microfluorometry: application to the detection of del F508 mutation in cystic fibrosis. Clin Chim Acta 2002, 316: 147–54.
13. Takeda S, Ichii S, Nakamura Y. Detection of K-ras mutation in sputum by mutant-allele-specific amplification (MASA). Hum Mutat 1993, 2: 112–7.
14. Machiels BM, Ruers T, Lindhout M, Hardy K, Hlavaty T, Bang DD, Somers VA, Baeten C, von Meyenfeldt M, Thunnissen FB. New protocol for DNA extraction of stool. Biotechniques 2000, 28: 286–90.
15. Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, Cabrita J, Megraud F. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J Clin Microbiol 1997, 35: 995–8.
16. Ohnishi T, Tomita N, Monden T, Ohue M, Yana I, Takami K, Yamamoto H, Yagyu T, Kikkawa N, Shimano T, Monden M. A detailed analysis of the role of K-ras gene mutation in the progression of colorectal adenoma. Br J Cancer 1997, 75: 341–7.

(Accepted for publication, Nov. 21, 2001)