New Method for Colorectal Cancer Diagnosis Based on SSCP Analysis of DNA from Exfoliated Colonocytes in Naturally Evacuated Feces

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Abstract. *Background: Establishment of a sensitive, reliable and non-invasive method for the diagnosis of early-stage colorectal cancer is necessary, because colorectal cancer can be cured surgically if diagnosed early. The aim of the present study was to evaluate the feasibility of using PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) analysis of the DNA extracted from the colonocytes isolated from naturally evacuated feces, in order to detect colorectal cancer. Materials and Methods: Colonocytes exfoliated into feces, retrieved from 33 patients with colorectal cancer and 63 healthy volunteers, were analyzed for the presence of mutations in their DNA. The DNA extracted from the colonocytes was examined for mutations of the APC, K-ras and p53 genes using direct sequence analysis, and also subjected to PCR-SSCP analysis. Results: Genetic alterations were detected in the colonocytes isolated from the feces of 12 out of 33 patients with colorectal cancer (36.4%) by direct sequence analysis. PCR-SSCP analysis using the same DNA samples revealed abnormal signals in 18 of the 33 patients (54.5%). However, 3 and 7 among the 63 healthy volunteers were also found to have abnormal genetic alterations by direct sequence and PCR-SSCP analysis, respectively. Conclusion: The present study indicated the feasibility of using PCR-SSCP analysis for the detection of mutations in the DNA extracted from colonocytes isolated from naturally evacuated fecal samples.*

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Colorectal cancer is the second leading cause of death from cancer not only in Japan (1) but also worldwide. Survival of patients with colorectal cancer is highly dependent on the stage at the time of diagnosis, with the 5-year survival of patients with early-stage colorectal cancer being more than 90% (2). Therefore, it is important to undertake mass screening for the detection of early-stage colorectal cancer. A mass screening method for cancer detection should be simple, economic and non-invasive, such as fecal occult blood testing (FOBT) which is widely used for colorectal cancer screening (3-5). FOBT every 1-2 years has yielded a 15-33% reduction in the cumulative colorectal cancer mortality rate (6-8). However, it is not sufficiently sensitive for the detection of early colorectal cancer (9-11). To date, several screening methods for colorectal cancer based on the detection of mutated DNA in feces have been reported (11-24). These methods are, however, time-consuming and not sufficiently sensitive.

A new effective methodology that allows simple isolation of exfoliated colonocytes with well-maintained morphology from the feces, not only from the surface, but also the central portion of the fecal specimens has been developed. Following the isolation of colonocytes from naturally evacuated feces, we applied a molecular biological tool to detect DNA alterations in these cells for cancer diagnosis. Subsequently, we reported direct-sequence analysis of the genomic DNA extracted from colonocytes with a sensitivity and specificity for the detection of colorectal cancer of 71% (82/116) and 88% (73/83), respectively (25).

However, since direct-sequence analysis involves a complex procedure, we considered that PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) analysis may be a simpler, less expensive and, therefore, more suitable technique for mass screening than direct-sequence analysis (26-27). The purpose of the present study was to determine the feasibility of PCR-SSCP analysis use for the detection of mutations in DNA extracted from colonocytes isolated from fecal samples, as compared with that of direct-sequence analysis, for colorectal cancer screening.

Materials and Methods

Magnetic beads. DynaBeads® (Dynal, Norway) magnetic beads, 4.5 μm in diameter, and coated with mouse IgG1 monoclonal antibody (Ber-EP4) specific for the glycopolypeptide membrane antigen, Ep-CAM, expressed in human epithelial tissues, were used for isolating colonocytes from the feces (28-30).

Patients. The study population consisted of 33 patients with colorectal cancer and 63 healthy volunteers. The characteristics of the patients and healthy volunteers are summarized in Table I. The patients with colorectal cancer had undergone curative surgery at the National Cancer Center Hospital, Tsukiji, Japan, between June 2005 and December 2005. The healthy volunteers had no abnormalities, such as adenoma or carcinoma (including hyperplastic polyp) of the colon, as determined by a total colonoscopy performed at the National Cancer Center Research Center for Cancer Prevention and Screening between June 2005 and November 2005. All patients and healthy volunteers were thoroughly informed about the procedure and intent of the study, which was approved by the Institutional Review Board of the National Cancer Center, Japan.

Stool samples and isolation of exfoliated cells. Naturally evacuated feces, evacuated without the intake of any laxatives, were used as the fecal samples. Such fecal samples were obtained from 33 patients with colorectal cancer, prior to their undergoing surgical resection, and 63 healthy volunteers who had undergone total colonoscopy more than 7 days prior to the day of the specimen collection. Approximately 3 g of naturally evacuated feces were used to isolate exfoliated cells following a previously reported method (25). The stool samples were collected into a Stomacher Lab Blender bag (Seward, Thetford, UK) and homogenized with a buffer (30 mL) containing Hanks' solution, 10% fetal bovine serum (FBS), and 25 mM Hepes buffer (pH 7.35) at 200 rpm for 1 minute in the Stomacher. The homogenates were then filtered through a nylon filter with a pore size of 512 μm. Subsequently, 240 μL of DynaBeads® were added to the homogenized sample solution and the mixture was incubated for 30 minutes under gentle rolling in a mixer at room temperature. The samples on the magnet were then incubated on a shaking platform for 15 minutes at room temperature. The isolated colonocytes were centrifuged and the sediments were stored at –80˚C until DNA extraction.

Tissue samples. Fresh tissue samples were obtained from the surgically resected specimens of the 33 patients with colorectal cancer. The samples were stored in liquid nitrogen until analysis.

DNA extraction. Genomic DNA for mutation analysis was extracted from the colonocytes isolated from the feces using the QIAamp DNA Stool Mini kit (QIAGEN, Valencia, USA), in accordance with manufacturer's instructions. Genomic DNA was also extracted from each tumor tissue specimen using the DNeasy kit (QIAGEN), in accordance with the manufacturer's instructions.

Table I. *Characteristics of patients and healthy volunteers.*

	Patients	Healthy volunteers
Number of individuals	33	63
Age (years)		
Mean (range),	61.0 (39-78)	58.0 (41-75)
Gender		
Male	24	34
Female	9	29
Tumour location		
Cecum	2	
Ascending colon	4	
Transeverse colon	3	
Descending colon	0	
Sigmoid colon	9	
Rectum	15	
Size		
Mean (range), mm	4.5(2-8.6)	
Histology		
W/D	26	
M/D	7	
Depth		
T1	1	
T2	4	
T3	27	
T4	1	
Duke's stage		
A	5	
B	17	
C	11	

W/D: Well-differentiated adenocarcinoma; M/D: Moderately differentiated adenocarcinoma.

Nested PCR amplification. The DNA-based analysis was targeted to 10 regions from 3 genes (p53, K-*ras* and APC). Table II shows the primer sets used in this study. The second primers for the nested PCR were fluorescence (X-rhodamine isothiocyanate)-labeled. Genomic DNA from the colonocytes and standard human genomic DNA (Roche, Switzerland) as control, were amplified by nested PCR using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The First PCR amplification was carried out in a 20 μl volume of the reaction mixture containing 0.5 U Novataq™ Hot Start DNA polymerase and 2xAmpdirect® Plus buffer (Shimazu, Japan), 0.375 μM of each of the primers, and 3 μl of the template DNA. The second nested PCR amplification was carried out in a 20 μl volume of the reaction mixture containing 0.5 U Amplitaq Gold® Hot Start DNA polymerase and 10xbuffer (Applied Biosystems, USA), 0.2 mM each dNTP mixture, 2 mM $MgCl_2$, 0.375 μM of each primers, and 2 μl of the first PCR products diluted 100-fold. The cycling conditions were 94˚C for 3 minutes followed by 35 cycles at 95˚C for 20 seconds, each annealing temperature for 40 seconds, and extension at 72˚C for 1 minute. Genomic DNA obtained from the tumor tissues was amplified by 1-step PCR under the same conditions as the second PCR for the DNA extracted from the exfoliated colonocytes.

Table II. *PCR primer sets of genes analyzed in this study.*

Region	Amplification	Forward Primer sequences 5'-3'	Reverse	Product size (bp)	Annealing temperature (˚C)
p53ex5	1st	TGCCGTGTTCCAGTTGCTTTAT	CACTCGGATAAGATGCTGAG	370	55
	2nd	ACTTGTGCCCTGACTTTCAAC	AACCAGCCCTGTCGTCTCT	266	58
p53ex6	1st	CCACCATGAGCGCTGTGCTCAG	TTGCACATCTCATGGGGTTATAGG	350	58
	2nd	CAGGGTCCCCAGGCCTCTGATTC	GGGCCACTGACAACCACCCTTAACC	212	63
p53ex7	1st	TGGGCGACAGAGCGAGATTC	GGGAGCAGTAAGGAGATTCC	374	68
	2nd	AAGGCCTCCCCTGCTTGCCACAG	CACAGCAGGCCAGTGTGCAGGGTG	222	68
p53ex8	1st	TTGGGAGTAGATGGAGCCTGGTT	CTGGGGAGAGGAGCTGGTGTTG	410	63
	2nd	ATTTCCTTACTGCCTCTTGCTTCTC	GCATAACTGCACCCTTGGTCTC	231	58
K-*ras*	1st	CTGGTGGAGTATTTGATAGTG	CCCAAGGAAAGTAAAGTTCCC	388	50
	2nd	TTTTCATTATTTTTATTATAAGG	GTCCTGCACCAGTAATAT	183	50
APC ex15-1	1st	CTCCAATATGTTTTTCAAGATGTAGTT	TGGTTCTAGGGTGCTGTGACA	539	55
	2nd	CTCCAATATGTTTTTCAAGATGTAGTT	TGGTTCTAGGGTGCTGTGACA	198	58
APC ex15-2	1st	TGAAGATCCTGTGAGCGAAGTT	AGTGGGGTCTCCTGAACATAGTG	500	55
	2nd	TGAAGATCCTGTGAGCGAAGTT	AGTGGGGTCTCCTGAACATAGTG	198	63
APC ex15-3	1st	AGCGAAATCTCCCTCCAAAA	TTGGTGGCATGGTTTGTCC	459	58
	2nd	AGCGAAATCTCCCTCCAAAA	TTGGTGGCATGGTTTGTCC	230	63
APC ex15-4	1st	ATTGCCAGCTCCGTTCAGA	TGCTTAGGTCCACTCTCTCTCTTT	373	58
	2nd	ATTGCCAGCTCCGTTCAGA	TGCTTAGGTCCACTCTCTCTCTTT	206	63
APC ex15-5	1st	GCATTATAAGCCCCAGTGATCT	GAACCTGGACCCTCTGAACTG	406	58
	2nd	GCATTATAAGCCCCAGTGATCT	GAACCTGGACCCTCTGAACTG	198	55

Direct sequence analysis. The PCR products were sequenced by direct sequencing with the Big Dye Terminator v 3.1/1.1 cycle kit (Applied Biosystems, Forester City, USA), in accordance with the manufacturer's instructions. All sequences obtained were aligned with previously published sequences [National Center for Biotechnology Information (NCBI) Genebank accession numbers M74088 (APC), M54968 (K-ras), X54156 (p53)] for each of the target genes and analyzed by Phred/Phrp/DNASIS Pro (Hitachi Software Engineering, Tokyo, Japan).

SSCP analysis. A volume of 2 μl of the fluorescence-labeled DNA fragments were diluted 7-fold with a loading buffer mainly consisting of formamide (99.75% formamide, 0.5 mM EDTA, 0.005% Fuchsin). The DNA samples were denatured at 90˚C for 1.5 minutes and cooled immediately on ice. Three microliters of each sample were applied onto 14%T-1%C polyacrylamide gel and electrophoresis was performed at 1200 V for 2-4 hours. The electrophoresis conditions for each fragment are given in Table III. The appearance of the extra bands that seemed to be aberrantly migrating in comparison with bands obtained from control DNA was considered to represent abnormal signals.

Statistical analyses. All statistical analyses employed the χ^2 test to compare proportions. All the reported *p*-values are two-sided and values of $p<0.05$ were considered statistically significant.

Table III. *Electrophoretic conditions for each fragment.*

Primer	Temperature (˚C)	Time (h)
p53_ex5	30	2
p53_ex6	15	3
p53_ex7	20	2
p53_ex8	15	4
K-*ras*	20	2
APC-1	10	4
APC-2	10	4
APC-3	10	4
APC-4	10	4
APC-5	10	2

Results

Analysis of the tumor tissue samples.

i) Direct sequence analysis. Genetic alterations were detected in the samples from 22 out of the 33 patients (66.7%) with colorectal cancer. Among the 33 patients, 4 out of 5 Duke's stage A colorectal cancer patients and 5 out of 9 proximal colorectal cancer patients showed genetic alterations (Table IV).

ii) SSCP analysis. A representative electrophoretogram of the PCR products for K-*ras* is shown in Figure 1. Abnormal signals were detected in the samples from 24 out of the 33 patients (72.7%) with colorectal cancer, including 4 out of 5 Duke's stage A colorectal cancer patients and 6 out of 9 (66.7%) proximal colorectal cancer patients (Table V).

Analysis of the colonocytes isolated from the fecal samples.

i) Direct sequence analysis. Genetic alterations were detected in the colonocyte DNA of 12 out of the 33 patients with

Table IV. *Detection of some genetic alteration by direct sequencing analysis, according to tumor location and tumor stage.*

	Patients with colorectal cancer					Healthy volunteers		
	Tissues			Colonocytes		Colonocytes		
	Total (n)	Positive (n)	Sensitivity, %	Positive (n)	Sensitivity, %	Total (n)	Positive (n)	Specificity, %
Overall	33	21	63.6	12	36.4	63	3	95.2
Duke's A	5	4	80	3	60			
Duke's B	17	10	58.8	5	29.4			
Duke's C	11	8	72.7	4	36.4			
Proximal colon	9	5	55.6	2	22.2			
Distal colon	24	16	66.7	10	41.7			

Table V. *Detection of some abnormal signs by SSCP analysis, according to tumor location and tumor stage.*

	Patients with colorectal cancer					Healthy volunteers		
	Tissues			Colonocytes		Colonocytes		
	Total (n)	Positive (n)	Sensitivity, %	Positive (n)	Sensitivity, %	Total (n)	Positive (n)	Specificity, %
Overall	33	24	72.7	18	54.5	63	7	88.9
Duke's A	5	4	80	3	60			
Duke's B	17	12	70.6	9	52.9			
Duke's C	11	7	63.6	6	54.5			
Proximal colon	9	6	66.7	3	33.3			
Distal colon	24	18	75	15	83.3			

colorectal cancer, including 3 out of 5 Duke's stage A colorectal cancer patients and 2 out of 9 proximal colorectal cancer patients. On the other hand, abnormal gene alterations were also detected in the DNA of 3 out of the 63 healthy volunteers (Table IV).

ii) SSCP analysis. Abnormal signals were detected in the colonocyte DNA of 18 out of the 33 patients with colorectal cancer, including 3 out of 5 Duke's stage A colorectal cancer patients and 3 out of 9 with proximal colorectal cancer. On the other hand, abnormal signals were also detected in the DNA of 7 out of the 63 healthy volunteers (Table V).

Statistical analysis. The χ^2 test revealed no statistically significant differences in sensitivity between direct sequence and SSCP analysis for colorectal cancer tissue samples ($p=0.428$), colonocytes isolated from colorectal cancer patients ($p=0.138$), or healthy volunteers ($p=0.187$).

Discussion

The idea of isolating cancer cells from the feces was originally prompted by a study that described abnormal expression of the CD44 gene in many tumors, including colon and bladder carcinomas (31-33). In the course of a series of studies, we hypothesized that normal mucous cells would die and be exfoliated during normal turnover and that the cancer cells were likely to survive for prolonged periods of time, even in the feces. Consequently, we developed a new method for isolating colonocytes from naturally evacuated feces (25).

However, it appeared that cytological examination of these colonocytes was not a suitable method for cancer diagnosis because of the low sensitivity (32%) (25). The colonocytes isolated from the feces were then subjected to DNA analysis. Direct sequence analysis of the APC, K-*ras* and p53 genes in colonocytes isolated from the feces showed a sensitivity of 71% (82/116) and specificity of 88% (73/83) (25) for the diagnosis of colorectal cancer. Direct sequence analysis is, however, a complex and time-consuming procedure. We, therefore, devised a PCR-SSCP analysis system instead for the detection of mutations, as we considered it to be a simpler and less expensive method as compared to direct sequence analysis for the diagnosis of colorectal cancer.

In the present study, direct sequence analysis and PCR-SSCP analysis of the genomic DNA extracted from the fecal colonocytes showed a sensitivity of 36.4% (12/33) and 54.5% (18/33), respectively. Both the figures were lower than the

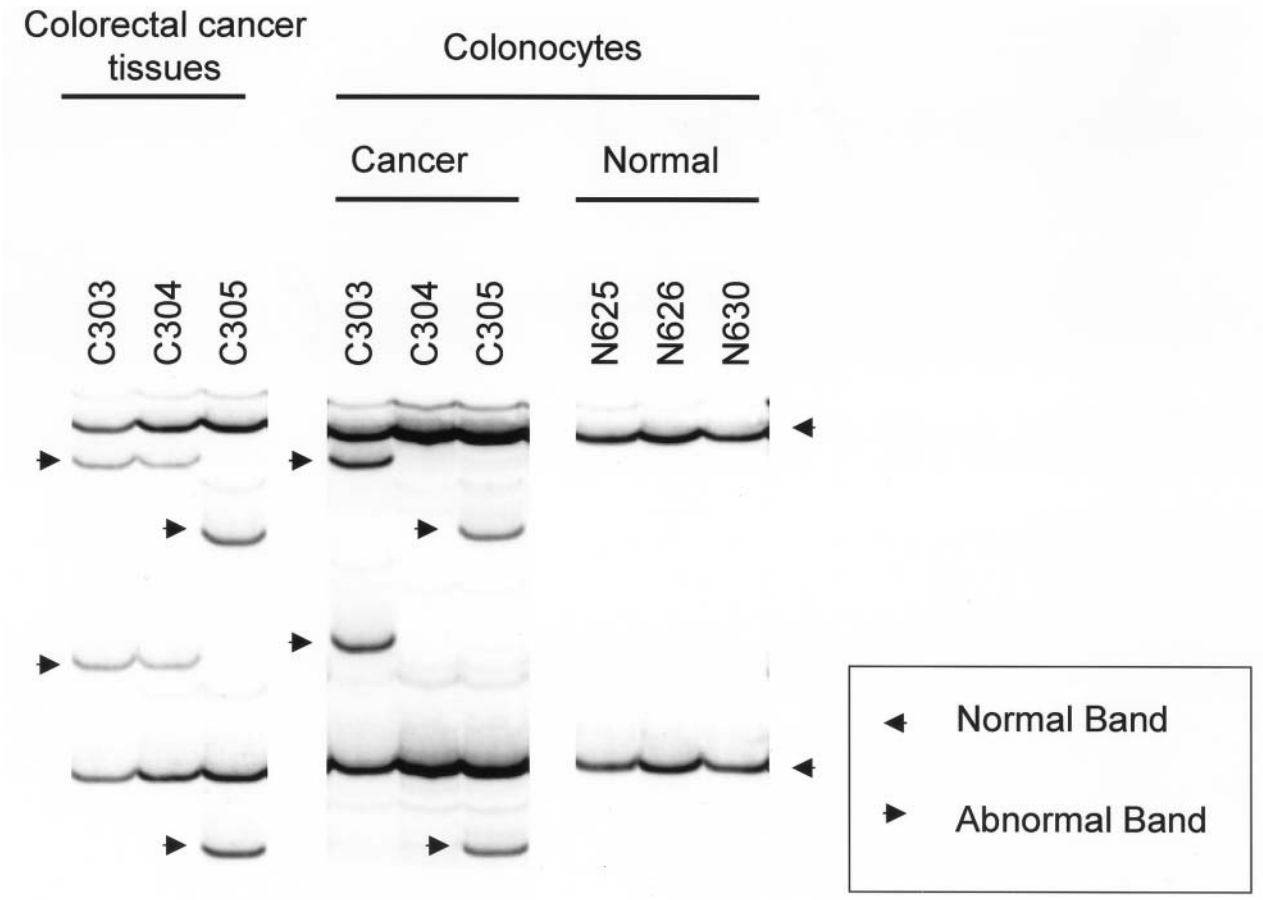

Figure 1. *A representative electrophoretogram of the PCR products for K-ras. C303 and C305 had an abnormal band in both cancer tissue specimens and colonocytes. C304 had an abnormal band only in the cancer tissue specimen. N625, N626 and N630 had no abnormal bands.*

figure we reported previously (71%) (25). This could possibly be explained by the detection of genetic alterations at an incidence of only 66.7% (22/33) in the original cancer tissues in the present study, whereas the corresponding incidence was 80% (93/116) in the previous study. Obviously, genetic alterations would not be expected in the colonocytes isolated from the feces if the original cancer tissue did not contain genetic alterations. Another problem is the relatively lower specificity of this PCR-SSCP analysis (89%; 56/63) as compared with that of direct sequence analysis (95%; 60/63); we have no clear explanation for this result. However, it is easy to speculate that direct sequence analysis may be more accurate than PCR-SSCP analysis because of the higher frequency of technical errors in the latter. We believe, however, that PCR-SSCP analysis may be more suitable for the mass screening of colorectal cancer as compared to direct sequence analysis because it can be easily automated and mechanized, and may be more economical.

Although the PCR-SSCP analysis was not sufficiently sensitive nor specific for colorectal cancer diagnosis in this study, we may be able to improve the sensitivity and specificity by enhancing the colonocyte retrieval rate from feces using the novel and more potent immunobeads we developed recently.

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References

1 The Editorial Board of Cancer Statistics in Japan; Cancer Statistices IN Japan-2005. http://www.fpcr.or.jp.
2 Bosman FT: Prognostic value of pathological characteristics of colorectal cancer. Eur J Cancer *31*: 1216-1221, 1995.
3 Towler B, Irwig L, Glasziou P, Kewenter J, Weller D and Silagy C: A systematic review of the effects of screening for colorectal cancer using the faecal occult blood test, hemoccult. BMJ *317*: 559-565, 1998.

4 Winawer S, Fletcher R, Rex D, Bond J, Burt R, Ferrucci J, Ganiats T, Levin T, Woolf S, Johnson D, Kirk L, Litin S and Simmang C; Gastrointestinal Consortium Panel: colorectal cancer screening and surveillance: Clinical guidelines and rationale-Update based on new evidence. Gastroenterology *124*: 544-560, 2003.

5 Mandel JS, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, Snover DC and Schuman LM: The effect of fecal occult-blood screening on the incidence of colorectal cancer. N Engl J Med *343*: 1603-1607, 2000.

6 Mandel JS, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM and Ederer F: Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. N Engl J Med *328*: 1365-1371, 1993.

7 Hardcastle JD, Chamberlain JO, Robinson MH, Moss SM, Amar SS, Balfour TW and James PD and Mangham CM: Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. Lancet *348*: 1472-1477, 1996.

8 Kronborg O, Fenger C, Olsen J, Jorgensen OD and Sondergaard O: Randomised study of screening for colorectal cancer with faecal-occult-blood test. Lancet *348*: 1467-1471, 1996.

9 Lieberman DA and Weiss DG: Veterans Affairs Cooperative Study Group 380: One-time screening for colorectal cancer with combined fecal occult-blood testing and examination of the distal colon. N Engl J Med *345*: 555-560, 2001.

10 Sung JJY, Chan FKL, Leung WK, Wu JCY, Lau JYW, Chang J, TO KF, Lee YT, Luk YW, Kung NNS, Kwok SPY, Li MKW and Chung SCS: Screening for colorectal cancer in Chinese: comparison of fecal occult blood test, flexible sigmoidoscopy, and colonoscopy. Gastroenterology *124*: 608-614, 2003.

11 Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA and Ross ME; Colorectal Cancer Study Group: Fecal DNA *versus* fecal occult blood for colorectal-cancer screening in an average-risk population. N Engl J Med 351: 2704-2714, 2004.

12 Sidransky D, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P and Vogelstein B: Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. Science *256*: 102-105, 1992.

13 Hasegawa Y, Takeda S, Ichii S, Koizumi K, Maruyama M, Fujii A, Ohta H, Nakajima T, Okuda M, Baba S and Nakamura Y: Detection of K-ras mutations in DNAs isolated from feces of patients with colorectal tumors by mutant-allele-specific amplification (MASA). Oncogene *10*: 1441-1445, 1995.

14 Smith-Ravin J, England J, Talbot IC and Bodmer W: Detection of c-Ki-ras mutations in faecal samples from sporadic colorectal cancer patients. Gut *36*: 81-86, 1995.

15 Eguchi S, Kohara N, Komuta K and Kanematsu T: Mutations of the p53 gene in the stool of patients with resectable colorectal cancer. Cancer *77*: 1707-1710, 1996.

16 Nollau P, Moser C, Weinland G and Wagener C: Detection of K-ras mutations in stools of patients with colorectal cancer by mutant-enriched PCR. Int J Cancer *66*: 332-336, 1996.

17 Ratto C, Flamini G, Sofo L, Nucera P, Ippoliti M, Curigliano G, Ferretti G, Sgambato A, Merico M, Doglietto GB, Cittadini A and Crucitti F: Detection of oncogene mutation from neoplastic colonic cells exfoliated in feces. Dis Colon Rectum *39*: 1238-1244, 1996.

18 Deuter R and Muller O: Detection of APC mutations in stool DNA of patients with colorectal cancer by HD-PCR. Hum Mutat *11*: 84-89,1998.

19 Ahlquist DA, Skoletsky JE, Boynton KA, Harrington JJ, Mahoney DW, Pierceall WE, Thibodeau SN and Shuber AP: Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. Gastroenterology *119*: 1219-1227, 2000.

20 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 and Jen J: Detecting colorectal cancer in stool with the use of multiple genetic targets. J Natl Cancer Inst *93*: 858-865, 2001.

21 Rengucci C, Maiolo P, Saragoni L, Zoli W, Amadori D and Calistri D: Multiple detection of genetic alterations in tumors and stool. Clin Cancer Res *7*: 590-593, 2001.

22 Traverso G, Shuber A, Olsson L, Levin B, Johnson C, Hamilton SR, Boynton K, Kinzler KW and Vogelstein B: Detection of proximal colorectal cancers through analysis of faecal DNA. Lancet *359*: 403-404, 2002.

23 Traverso G, Shuber A, Levin B, Johnson C, Olsson L, Schoetz DJ Jr, Hamilton SR, Boynton K, Kinzler KW and Vogelstein B: Detection of APC mutations in fecal DNA from patients with colorectal tumors. N Engl J Med *346*: 311-320, 2002.

24 Boynton KA, Summerhayes IC, Ahlquist DA and Shuber AP: DNA integrity as a potential marker for stool-based detection of colorectal cancer. Clin Chem *49*: 1058-1065, 2003.

25 Matsushita H, Matsumura Y, Moriya Y, Alasu T, Fujita S, Yamamoto S, Onouchi S, Saito N, Sugito M, Ito M, Kozo T, Minowa T, Nomura S, Tsunoda H and Kakizoe T: A New Method for Isolating Colonocytes From Naturally Evacuated Feces and Its Clinical Application to Colorectal Cancer Diagnosis. Gastroenterology *129*: 1918-1927, 2005.

26 Orita M, Suzuki Y, Sekiya T and Hayashi K: Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction Genomics *5*: 874-879, 1989.

27 Hayashi K: PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods Appl 1: 34-38, 1991.

28 Winter MJ, Nagtegaal ID, van Krieken JH and Litvinov SV: The epithelial cell adhesion molecule (Ep-CAM) as a morphoregulatory molecule is a tool in surgical pathology. Am J Pathol *163*: 2139-2148, 2003.

29 Balzar M, Prins FA, Bakker HAM, Fleuren GJ, Warnaar SO and Litvinov SV: The structural analysis of adhesions mediated by Ep-CAM. Exp Cell Res *246*: 108-121, 1999.

30 Salem RR, Wolf BC, Sears HF, Lavin PT, Ravikumar TS, DeCoste D, D'Emilia JC, Herlyn M, Schlom J and Gottlieb LS: Expression of colorectal carcinoma-associated antigens in colonic polyps. J Surg Res *55*: 249-255, 1993.

31 Yamao T, Matsumura Y, Shimada Y, Moriya Y, Sugihara K, Akasu T, Fujita S and Kakizoe T: Abnormal expression of CD44 variants in the exfoliated cells in the feces of patients with colorectal cancer. Gastroenterology *114*: 1196-1205, 1998.

32 Matsumura Y and Tarin D: Significance of CD44 gene products for cancer diagnosis and disease evaluation. Lancet *340*: 1053-1058, 1992.

33 Matsumura Y, Hanbury D, Smith J and Tarin D: Non-invasive detection of malignancy by identification of unusual CD44 gene activity in exfoliated cancer cells. BMJ *308*: 619-624, 1994.

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