

Detection of K-ras gene mutation in fecal samples from elderly large intestinal cancer patients and its diagnostic significance

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Abstract

AIM: To study the diagnostic significance of K-ras gene mutations in fecal samples from elderly patients with large intestinal cancer.

METHODS: DNA was extracted in the fecal and tissue samples from 23 large intestinal cancer patients, 20 colonic adenomatoid polypus patients and 20 healthy subjects. The K-ras gene mutations at the first and second bases of codon 12 were detected by the allele specific mismatch method.

RESULTS: The K-ras gene mutation was 56.52%(13/23) in the large intestinal cancer patients, which was notably higher than that in the normal subjects whose K-ras gene mutation was 5%(1/20) ($\chi^2=12.93$, $P<0.001$). There was no significant difference in comparison with that of colonic adenomatoid polypus patients whose K-ras gene mutation was 30%(6/12) ($\chi^2=3.05$, $P>0.05$). The K-ras gene mutation at the second base of codon 12 was 92.13%(12/13) in the large intestinal cancer patients. There was no significant difference between the detection rate of K-ras gene mutation in the fecal and tissue samples ($\chi^2=9.35$, $P<0.01$).

CONCLUSION: Our results indicate that detection of the K-ras gene mutations in fecal samples provides a non-invasive diagnostic method for the elderly large intestinal cancer patients. Its significance in the early diagnosis of large intestinal cancer awaits further studies.

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INTRODUCTION

Large intestinal cancer is one of the common malignant tumors in China. Its incidence has been increasing in the elderly, and its death rate is approximately 60% in large intestinal cancer patients over 60 years old. In China, large intestinal cancer is often resulted from the malignancy of colonic adenomas, because the incidence of large intestinal polypus is high in the elderly. It was reported that the detectable rate of large intestinal polypus and adenomatoid polypus was as high as 62.1% and 67.9%, respectively^[1]. Therefore, early detection of cancerous

adenomas is of great significance in decreasing the incidence and death rate of large intestinal cancer. At present, colonoscopy is the most ideal diagnostic method for large intestinal cancer^[2,3], the reported detectable rate of early large intestinal cancer was 36.5% in the elderly^[1]. Since colonoscopy is an invasive method and the examined subjects would have some suffering, it has therefore become a topic of general interest to find a non-invasive diagnostic method for large intestinal cancer patients^[4-10]. The K-ras gene mutations in fecal samples from the elderly were detected by the allele specific mismatch method, and its diagnostic significance in the large intestinal cancer patients was discussed.

MATERIALS AND METHODS

Reagents

Taq DNA polymerase, dNTPS, DNA fragments, agarose, DNA extraction kits were the products of Promega (Madison, USA). Proteinase K was the product of Merck.

Specimens

The patients enrolled in this study were 23 cases of large intestinal cancer (19 males, 4 females, averaging 68.8 years), 20 cases of colonic adenomatoid polypus and 20 healthy subjects. Their diagnoses were confirmed by endoscopy and biopsy. Of the 23 cases of large intestinal cancer, 5 had well differentiated adenocarcinomas, 10 had moderately differentiated adenocarcinomas, 6 had poorly differentiated adenocarcinomas, and 2 had mucinous adenocarcinomas. The fecal samples were collected from the above patients before undergoing surgery and stored at -30 °C.

DNA extraction

DNA was extracted from the fecal samples using the DNA extraction kits. The fecal samples were processed according to the following procedures: 100-200 g of the fecal samples was diluted in 500 μ L of phosphate buffered saline (PBS), pH7.5, and homogenized for 2 min at 1 000 r/min. Then, 500 μ L supernatant after the addition of 50 μ L hydrolytic buffer was homogenized for 5 min, and centrifuged at 6 000 r/min for 2 min. The precipitate was placed into 500 μ L cleaning solution and centrifuged at 6 000 r/min for 2 min. After washed twice and addition of 50 μ L lysate and covered by paraffin oil, the precipitate was boiled for 5 min, centrifuged at 13 000 g for 10 min and stored at -30 °C before it was used. The DNA in the 16 cancer tissue samples was extracted by proteinase K (10 g/L, thermostatic water bath at 37 °C for 24 h), and purified by phenol-chloro-isopentanol extraction, and dissolved in TE buffer after ethanol precipitation for use.

PCR reaction

The oligonucleotide primer was synthesized by the Oligo 1000DNA synthesizer (Beckman). The DNA amplification PCR reaction was carried out in a total volume of 50 μ L buffer containing 5 μ L of diluted DNA templates, 10 mmol/L Tris-HCl (pH8.8), 1.5 mmol/L MgCl₂, 1g/L Triton X-100, 200 mmol/L dNTPs, 50 pmol/L of each primer and 1U of Taq DNA

polymerase. The PCR conditions were as follows: denaturing at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 3 min. The amplification was performed for 30 cycles. L14841 and H15149 are the primers specific for human cytochrome B gene, COI and ND2 are the primers specific for human mitochondrial cytochrome oxidase COI subunit. These primers were also used in the detection of K-ras gene mutations.

The point mutation at the first and second bases of codon 12 of the K-ras gene was detected by the allele specific mismatch method^[11]. The amplification PCR reaction was performed for 45 cycles in a total volume of 50 µL of buffer, but the conditions were slightly modified as the follows: denaturing at 95 °C for 1 min, annealing at 58 °C for 2 min, extension at 72 °C for 1 min. The A set and B set primers were used to detect the presence of K-ras gene mutations at the first and second bases of codon 12. Besides one general primer found in the 2 groups, both Ag and Bc primers were specific for the wild-type K-ras gene. The other primers were used to amplify various mutations of the K-ras gene. The control bands of DNA fragments from the corresponding tumors were validated using an ultraviolet detector after the PCR products were analyzed on a 30g/L agarose gel and visualized by ethidium bromide staining.

The nucleotide sequences of human cytochrome B gene primers and human mitochondrial cytochrome oxidase COI subunits used in the amplification reactions were as follows: L14841: 5' AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA3', H15 149: 5' AAAGTGCAGCCCCCTCAGAATGATATTTGTCCTCA3', COI: 5' ACGATGTCTAGTGATGAGTTGCTA3', ND2: 5' ACGCCTAATCTACTCCACCTCAATC 3'.

A 300-bp PCR amplification product was detected using L14841 and H15149, and a 1 400 bp PCR amplification product was detected using COI and ND2. The K-ras gene mutation detected at the first base of codon 12 using the A set primers included K-ras A: 5' CAGAGAAACCTTTATCTG 3', K-ras Aa: 5' TGGTAGTTGGAGCTA 3'. K-ras Ac, K-ras Ag and K-ras At differed from K-ras Aa in the last nucleotide, which was replaced by C, G and T respectively^[11], and produced a 146 bp DNA amplification fragment.

The K-ras gene mutations detected using the B set primers at the second base of codon 12 included K-ras B: 5' GTACTGGTGGAGTATTT3' and K-ras Ba: 5' ACTCTTGCCTACGCCAA3'. K-ras Bc, K-ras Bg and K-ras Bt differed from K-ras Ba in the last 3' nucleotide, which was replaced by C, G and T respectively^[11], and generated a 161 bp DNA amplification fragment.

RESULTS

Sequence of specific human DNA in fecal extraction

The DNA of human cytochrome B gene (a 300 bp amplification fragment) and human mitochondrial cytochrome oxidase COI (a 1 400 bp amplification fragment) in the extraction of 10 fecal samples was amplified using the pair of L14841 and H15149 primers and the pair of COI and ND2 primers. A 300 bp and a 1 400 bp electrophoretic bands were observed in 9 fecal samples after the PCR products were analyzed on a 15g/L agarose gel and visualized by ethidium bromide staining.

Consistency of K-ras gene mutations in fecal and tissue samples

The K-ras gene mutations in the fecal and tissue samples of 16 large intestinal cancer patients were detected. Of the 16 large intestinal cancer patients, 9 had identical K-ras gene mutation detected both in fecal and tissue samples, none had K-ras gene mutation detected both in fecal and tissue samples, and only 2 had K-ras gene mutation in tissue samples. The consistency test showed that the K-ras gene mutations in fecal and tissue samples were well correlated ($\chi^2=9.35$, $P<0.01$).

K-ras gene mutation in fecal samples

The K-ras gene mutation was detected in the fecal samples from 23 large intestinal cancer patients. The K-ras gene mutation rate was 56.25% (13/23), which was significantly higher than that (5%, 1/20) in the healthy subjects ($\chi^2=12.93$, $P<0.001$). There was no significant difference in comparison with that (30%, 6/20) of colonic adenomatoid polypi ($\chi^2=3.05$, $P>0.05$). The K-ras gene mutation rate was 40% (2/5), 60% (6/10), 66.67% (4/6), and 50% (1/2) in the well, moderately and poorly differentiated adenomas and mucinous adenomas, respectively. The K-ras gene mutation was detected in 2 patients with cancerous colonic adenomatoid polypus, its mutation rate was 30% (6/20) in the fecal samples from colonic adenomatoid polypus patients, which was significantly higher than that in the healthy subjects ($\chi^2=4.33$, $P<0.05$). The mutation rate of K-ras gene was 23.08% (3/13), 40% (2/5), and 50% (1/2) in the polypi with a diameter less than 1 cm, a diameter of 1-2 cm, a diameter larger than 2 cm, respectively. Among the 13 large intestinal cancer patients, the K-ras gene mutation was detected at the second base of codon 12 in 12 patients (92.31%), GGT was mutated into GAT and GTT in 9 and 3 patients, respectively. The K-ras gene mutation site was observed at the first base of codon 12 in 1 patient, whose GGT was mutated into GTT.

DISCUSSION

A healthy adult excretes approximately 10^{10} epithelial cells every day. A large number of tumor cells will renew and exfoliate into the intestinal cavity of colonic cancer patients every day. A certain amount of DNA can maintain its stability due to the resistance of intestinal tumor cells to various degradation enzymes or due to the impairment of apoptotic mechanism of tumor cells^[12]. Based on the above findings, Sidransky *et al*^[13] detected the K-ras gene mutation in the fecal samples from early large intestinal cancer patients in 1992, and found the K-ras gene mutation in the fecal and tissue samples from tumor patients. Since then, several scholars have carried out some similar studies^[11,14-16]. However, their research findings have not been popularized and applied due to low PCR amplification rate of DNA in fecal samples. We used the conventional phenol-chloroform extraction method to extract DNA in 10 fecal samples. The PCR amplification product was observed only in 3 faecal samples using the primers specific for mitochondrial DNA of human eukaryotic cells. Then, the PCR amplification reaction was performed in the DNA extraction kits, the amplification rate was as high as 90% (9/10) when the DNA extraction kits used in the detection of DNA in fecal samples were washed twice in hydrolysate to remove the hybrid proteins. In the experiment, we found that the number of templates had a certain effect on the PCR amplification reaction, and could dilute the extract stock to some extent. The results indicated that the amplification rate increased with increase of dilution strength, suggesting that the amount of DNA was quite suitable to its amplification. The effect of some PCR inhibitors present in the PCR amplification reaction was significantly reduced due to the increase of dilution strength. Berndt *et al*^[17] held that these PCR inhibitors were the bile salts and bilirubin present in the fecal samples. Villa *et al*^[14] recovered the purified DNA using purification columns after the absorbent was added to the extract from the fecal samples, and found that the amplification rate was significantly increased, but the cost was rather high. In our experiment, the amplification rate was increased when the DNA extract was approximately diluted, which simplified the procedures of DNA preparation and reduced the cost. However, as the amount of templates in the DNA extract stock from the fecal samples was uncertain, its dilution factor exerted an effect on the DNA

amplification stability. Therefore, the fecal samples with a poor amplification result should be amplified again after the dilution factor of the extract stock was adjusted. This would no doubt increase the cost and time in detecting some fecal samples. In the present study, we detected the K-ras gene mutation in tissue and fecal samples from 16 large intestinal cancer patients, and achieved a rather good consistency ($P < 0.01$), indicating that detection of the K-ras gene mutation could reflect the presence of its mutation in the tissues. Since it is much easier to obtain tissue samples than fecal samples in clinic, this method will make it clinically possible to screen colorectal cancer.

Researches showed that the K-ras gene mutation in oncogenes was most frequently found in large intestinal cancer, accounting for 40-50%^[18-20]. Smith-Ravin *et al.*^[11] detected the K-ras gene mutation in the fecal samples from large intestinal cancer patients using the allele specific mismatch method, and found that the mutation rate was 50%. Xiao *et al.*^[21] detected the K-ras gene mutation in the fecal samples from large intestinal cancer patients using PCR-RFLP, and found the mutation rate was 36.4%. In our study, the detection rate of K-ras gene mutation was 56.25% and 5% in the fecal samples from large intestinal cancer patients and healthy subjects, respectively. There was a very significant difference between the two groups ($P < 0.001$), but there was no significant difference in comparison with that of colonic adenomatoid polypus patients ($P > 0.05$), suggesting that adenomatoid polypus, a precancerous lesion of large intestinal cancer, is closely related with the development of colonic cancer. According to the literature reports, 90% of large intestinal cancers were resulted from large intestinal adenomatoid polypi. Therefore, early detection and resection of large intestinal adenomatoid polypi could greatly reduce the incidence of large intestinal cancer^[2,22]. However, the diagnostic significance of large intestinal cancer at its early stages should be further studied in a larger number of large intestinal cancer patients. Many researchers^[23-29] held that the mutation rate of K-ras gene in colonic adenomas would increase with the growth of adenomas. In our study, among the two cases of adenomas with a diameter larger than 2 cm, the K-ras gene mutation was detected in one case, and was also detected in the 2 cases after their colonic adenomatoid polypi were found to be cancerous. This was consistent with the K-ras gene mutation found at the early stages of large intestinal cancer reported both in Chinese and foreign literature. Further researches showed that the K-ras gene mutation was usually resulted from the mutation of GGT to GAT of its codon 12, and 84% K-ras gene mutations were observed at the second base of codon 12. In our study, the mutation site of K-ras gene was observed at the second base of codon 12, accounting for 92.31% (12/13), 69.23% of which was resulted from the mutation of GGT to GAT, and 23.08% was resulted from the mutation of GGT to GTT. This was consistent with that reported in the literature^[11,30,31].

The non-invasive method for the detection of K-ras gene mutation in fecal samples reported in this paper is simple to operate and the samples are easy to collect. Its preliminary application in clinic has shown that it is of significance in the diagnosis of elderly large intestinal cancer patients, and can be used in combination with colonoscopy to screen the high risk population for colorectal cancer. It should be further improved because its PCR reaction time is long, which leads to the prolonged detection and is disadvantageous to the detection of large samples.

REFERENCES

- 1 **Wan J**, Zhang ZQ, Zhu C, Wang MW, Zhao DH, Fu YH, Zhang JP, Wang YH, Wu BY. Colonoscopic screening and follow-up for colorectal cancer in the elderly. *World J Gastroenterol* 2002; **8**: 267-269
- 2 **Pignone M**, Rich M, Teutsch SM, Berg AO, Lohr KN. Screening for colorectal cancer in adults at average risk: a summary of the evidence for the U.S. Preventive Services Task Force. *Ann Intern Med* 2002; **137**: 132-141
- 3 **Xiang DN**, Xiang P, Zheng SB, Cao XY, Wang GS. Diagnostic value of colonofibroscopy in 1507 elderly patients of lower gastrointestinal bleeding. *Laonian Yixue Yu Baojian* 2002; **8**: 35-36
- 4 **Atkin W**. Options for screening for colorectal cancer. *Scand J Gastroenterol Suppl* 2003; **237**: 13-16
- 5 **Meng W**, Li SR, Li L. Effect of the combined sequent program for screening colorectal carcinoma. *Linchuang Xiaohuabin Zazhi* 2000; **11**: 157-158
- 6 **Lou CY**, Li SY, Zhu XG. The detection of the rearrangements of bcl-2 gene in the cancer tissues and stool of the patients with colorectal carcinoma by semi-nest PCR. *Zhonghua Shiyian Waikexi* 1999; **16**: 197-198
- 7 **Fan RY**, Li SR, Wu ZT. The detection of K-ras mutations in stools and tissue samples from patients with colorectal cancer. *Zhonghua Xiaohua Zazhi* 2001; **21**: 445-446
- 8 **Fan RY**, Li SR, Wu X, Wu ZT, Chen ZM, Deng YJ, Cao JB, Zhang HG. The expression of P53 in shedding cells from patients with colorectal cancer. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 814-815
- 9 **Mandel JS**, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, Snover DC, Schuman LM. The effect of fecal occult-blood screening on the incidence of colorectal cancer. *N Engl J Med* 2000; **343**: 1603-1607
- 10 **Ito S**, Hibi K, Nakayama H, Kodera Y, Ito K, Akiyama S, Nakao A. Detection of tumor DNA in serum of colorectal cancer patients. *Jpn J Cancer Res* 2002; **93**: 1266-1269
- 11 **Smith-Ravin J**, England J, Talbot IC, Bodmer W. Detection of c-Ki-ras mutations in faecal samples from sporadic colorectal cancer patients. *Gut* 1995; **36**: 81-86
- 12 **Ratto C**, Flamini G, Sofo L, Nucera P, Ippoliti M, Curigliano G, Ferretti G, Sgambato A, Merico M, Doglietto GB, Cittadini A, Crucitti F. Detection of oncogene mutation from neoplastic colonic cells exfoliated in feces. *Dis Colon Rectum* 1996; **39**: 1238-1244
- 13 **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**: 102-105
- 14 **Villa E**, Dugani A, Rebecchi AM, Vignoli A, Grottola A, Buttafoco P, Losi L, Perini M, Trande P, Merighi A, Lerose R, Manenti F. Identification of subjects at risk for colorectal carcinoma through a test based on K-ras determination in the stool. *Gastroenterology* 1996; **110**: 1346-1353
- 15 **Tagore KS**, Lawson MJ, Yucaitis JA, Gage R, Orr T, Shuber AP, Ross ME. Sensitivity and specificity of a stool DNA multitarget assay panel for the detection of advanced colorectal neoplasia. *Clin Colorectal Cancer* 2003; **3**: 47-53
- 16 **Ito Y**, Kobayashi S, Taniguchi T, Kainuma O, Hara T, Ochiai T. Frequent detection of K-ras mutation in stool samples of colorectal carcinoma patients after improved DNA extraction: comparison with tissue samples. *Int J Oncol* 2002; **20**: 1263-1268
- 17 **Berndt C**, Haubold K, Wenger F, Brux B, Muller J, Bendzko P, Hillebrand T, Kottgen E, Zanol J. K-ras mutations in stools and tissue samples from patients with malignant and nonmalignant pancreatic diseases. *Clin Chem* 1998; **44**: 2103-2107
- 18 **Okulczyk B**, Piotrowski Z, Kowalcuk O, Niklinski J, Chyczewski L. Evaluation of K-RAS gene in colorectal cancer. *Folia Histochem Cytobiol* 2003; **41**: 97-100
- 19 **van Engeland M**, Roemen GM, Brink M, Pachen MM, Weijenberg MP, de-Bruine AP, Arends JW, van den Brandt PA, de Goeij AF, Herman JG. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* 2002; **21**: 3792-3795
- 20 **Kislitsin D**, Lerner A, Rennert G, Lev Z. K-ras mutations in sporadic colorectal tumors in Israel: unusual high frequency of codon 13 mutations and evidence for nonhomogeneous representation of mutation subtypes. *Dig Dis Sci* 2002; **47**: 1073-1079
- 21 **Xiao SX**, Xu AM, Wang DB, Wu L, Liu HP, Zeng B. The detection of codon 12 mutations of K-ras gene in feces by nested PCR-RFLP. *Zhongguo Shengwu Zhipin Zazhi* 1998; **11**: 103-105

- 22 **Hermesen M**, Postma C, Baak J, Weiss M, Rapallo A, Sciutto A, Roemen G, Arends JW, Williams R, Giaretti W, De Goeij A, Meijer G. Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. *Gastroenterology* 2002; **123**: 1109-1119
- 23 **Scott N**, Bell SM, Sagar P, Blair GE, Dixon MF, Quirke P. p53 expression and K-ras mutation in colorectal adenomas. *Gut* 1993; **34**: 62162-62164
- 24 **Blum HE**. Colorectal cancer: future population screening for early colorectal cancer. *Eur J Cancer* 1995; **31A**: 1369-1672
- 25 **Hosaka S**, Aoki Y, Akamatsu T, Nakamura N, Hosaka N, Kiyosawa K. Detection of genetic alterations in the p53 suppressor gene and the K-ras oncogene among different grades of dysplasia in patients with colorectal adenomas. *Cancer* 2002; **94**: 219-227
- 26 **Rashid A**, Houlihan PS, Booker S, Petersen GM, Giardiello FM, Hamilton SR. Phenotypic and molecular characteristics of hyperplastic polyposis. *Gastroenterology* 2000; **119**: 323-332
- 27 **Lamlum H**, Papadopoulou A, Ilyas M, Rowan A, Gillet C, Hanby A, Talbot I, Bodmer W, Tomlinson I. APC mutations are sufficient for the growth of early colorectal adenomas. *Proc Natl Acad Sci U S A* 2000; **97**: 2225-2228
- 28 **Rashid A**, Zahurak M, Goodman SN, Hamilton SR. Genetic epidemiology of mutated K-ras proto-oncogene, altered suppressor genes, and microsatellite instability in colorectal adenomas. *Gut* 1999; **44**: 826-833
- 29 **Glarakis IS**, Savva S, Spandidos DA. Activation of the ras genes in malignant and premalignant colorectal tumors. *Oncol Rep* 1998; **5**: 1451-1454
- 30 **Nishikawa T**, Maemura K, Hirata I, Matsuse R, Morikawa H, Toshina K, Murano M, Hashimoto K, Nakagawa Y, Saitoh O, Uchida K, Katsu K. 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. *Clin Chim Acta* 2002; **318**: 107-112
- 31 **Prix L**, Uciechowski P, Bockmann B, Giesing M, Schuetz AJ. Diagnostic biochip array for fast and sensitive detection of K-ras mutations in stool. *Clin Chem* 2002; **48**: 428-435

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