

Analysis of microsatellite instability in stool DNA of patients with colorectal cancer using denaturing high performance liquid chromatography

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Abstract

AIM: To evaluate the usefulness of denaturing high performance liquid chromatography (DHPLC) for analyzing microsatellite instability (MSI) status in stool DNA of patients with colorectal cancer.

METHODS: A total of 80 cancer tissues from patients with primary sporadic colorectal tumor (proximal cancer: 27, distal cancer: 53) and matched stool (which were employed for comparison with the tissues) were analyzed for MSI status in BAT 26. DNA samples extracted from stool were evaluated by nested polymerase chain reaction (PCR) and DHPLC for MSI analysis.

RESULTS: Six cases (7.5%) of MSI were identified in BAT 26 from 80 cancer tissues. All the stool DNA samples from patients whose cancer tissue showed MSI also displayed MSI in BAT 26.

CONCLUSION: As MSI is one of the established fecal DNA markers to screen colorectal cancer, we propose to use DHPLC for the MSI analysis in fecal DNA.

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Key words: Colorectal cancer; Denaturing high performance liquid chromatography; Fecal DNA; Microsatellite instability; Stool

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INTRODUCTION

Colorectal cancer, one of major causes of tumor-induced death in the Western population, becomes prevalent in Korea. The mortality rate from colorectal cancer has increased rapidly from 3.9 per 100 000 individuals in 1983 to 11.4 in 2003 in Korea^[1]. Due to its orderly natural history, location within readily accessible organs, and high lifetime incidence, colorectal cancer is suitable for mass screening. It has been estimated that more than 50% of mortality due to colorectal cancer has been prevented through screening tests^[2]. Fecal occult blood tests are non-invasive and useful, particularly as an adjunct to sigmoidoscopy^[3]. However, the relatively high false positive rates and other problems have necessitated a search for more specific non-invasive tests. In this regard, assays for mutations in fecal DNA are particularly promising^[4]. Following the initial identification of mutant *K-ras* in stools^[5], many investigators have conducted molecular genetic analyses of stools. In view of the heterogeneity of mutations in colorectal cancer, multiple genetic targets have been screened. Dong *et al*^[6] found that analysis of a combination of *p53*, BAT 26 and *K-ras* mutations in stools identified 71% cases of colorectal cancer. Ahlquist and colleagues^[7] improved the detection sensitivity to 91% by using a panel consisting of *p53*, BAT 26, APC, and *K-ras*.

MSI is caused by a failure of the mismatch repair system to correct errors that occur during DNA replication. This phenomenon is characterized by the presence of novel alleles in DNA of cancer tissues that are absent in matched repetitive sequences in normal tissues designated 'microsatellites'^[8,9]. The MSI status has notable biological significance. For instance, colorectal cancer patients with MSI show better prognosis^[9-11]. Cancer detection via microsatellite analyses has been reported for several malignancies, including renal cell carcinoma^[12], uterine cervical carcinoma^[13] and head and neck squamous

cell carcinoma^[14]. Koshiji *et al*^[15] reported the results of MSI analysis of fecal DNA for colorectal cancer detection, while Traverso *et al*^[16] demonstrated the practical application of the fecal BAT 26 assay with sigmoidoscopy.

In view of the importance of MSI analysis, these techniques required substantial improvement^[17]. One particular concern was that the database would be limited if optimal high-throughput assays for running and interpreting microsatellite assays were not developed^[18]. Thus, simple and automatic methods are required that make MSI analysis more systematic and convenient, and the many clinical approaches to cancer therapy feasible.

DHPLC has emerged as one of the most versatile technologies for the evaluation of genetic variations. The most patent advantage of this technique is the feasibility of automatic high-throughput analysis using computer-controlled systems. We have developed and reported a novel protocol for MSI analysis in cancer tissues that utilizes DHPLC^[19]. In this study, we extended the protocol for MSI analysis using DHPLC to stool samples of patients with colorectal cancer.

MATERIALS AND METHODS

Patients

Eighty cancer tissues, taken from 27 patients with cancer of the proximal colon (i.e., between the cecum and splenic flexure) and from 53 patients with distal cancer were analyzed. Matched stool samples from the 80 patients were also analyzed. None of the patients investigated had familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer. All cancer tissues and stool were collected from the Center for Colorectal Cancer, National Cancer Center, Korea. The tumor stage was determined using the American Joint Committee on Cancer TNM system^[20]. All patients gave informed consent prior to entry into the study.

DNA preparation

Genomic DNA was extracted from surgical specimens, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's specifications. Stool samples were obtained before commencing laxative treatment to prepare for surgery or colonoscopy. Solid stools were weighed into 10 g lots, and stored at -70°C. After defrosting, we employed a QIAamp Stool Mini Kit (QIAGEN, Hilden, Germany) for DNA preparation. DNA was extracted according to the manufacturer's instructions. Briefly, 180-220 mg of stool was prepared in a 2 mL microcentrifuge tube, and 1.6 mL Buffer ASL was added for homogenization of samples. Following centrifugation at full speed for 1 min, supernatants were transferred a new 2 mL tubes, and one InhibitEX tablet was added. The tablet was thoroughly dissolved in the stool solution, followed by a second round of centrifugation for 3 min. The supernatant was transferred to a new 1.5 mL tube with 25 µL of proteinase K. AL buffer (600 µL) was added to the prepared solution, and incubated at 70°C for 10 min. After the addition of 600 µL of ethanol, solutions (600 µL) were applied to the QIAamp spin column, and centrifuged three times. The column was washed with 500

µL of AW1 and AW2 and stool DNA samples eluted using 200 µL of AE buffer.

Primer sequences for amplifying microsatellite marker, BAT 26 were obtained from the GDB Human Genome Database (www.gdb.org). Stool DNA was initially amplified with the outer primers, forward, 5'-TTTAGGTTGCAGT TTCATCA-3', and reverse, 5'-ACCATTC AACATTTT AAGCC-3'. The outer PCR product (1 µL) was employed for a second inner round of amplification. Inner PCR primers for BAT 26 were follows as: forward primer, 5'-ACTGTCTGCGGTAATCAAGT-3'; and reverse primer, 5'-CCATTCAACATTTTAAAGCC-3'. Amplification for DHPLC analysis was performed with 1 µL of the first PCR product, 1X PCR buffer supplemented with 1.5 mmol/L MgCl₂, 10 pmol/µL of primers, 50 µmol/L dNTPs (each), and 0.25 U of *Taq* polymerase (QIAGEN, Hilden, Germany) in a total volume of 25 µL. For heteroduplex formation, crude PCR products were denatured at 95°C for 5 min, followed by gradual cooling from 95°C to 25°C over a period of 1 h.

MSI analysis using DHPLC

DHPLC was performed with a fully automated system (WAVE, Transgenomic Inc., Omaha, NE, USA) as previously reported^[19]. Amplified products were automatically injected into a DNA Seq® cartridge (Transgenomic Inc., Omaha, NE, USA), and eluted at a flow rate of 0.9 mL/min through a linear gradient of acetonitrile with 0.1 mol/L triethylammonium acetate (TEAA). Buffers A (0.1 mol/L triethylammonium acetate (TEAA) solution) and B (0.1 mol/L TEAA containing 25% acetonitrile) were automatically adjusted to obtain the best running conditions. The portion of Buffer A was 53% and Buffer B was 47%. The sample injection volume per analysis was 5 µL of PCR product. Regardless of the markers and samples, the running temperature was set to 50°C for double-stranded DNA analysis in WAVEMAKER™ software (Transgenomic Inc., Omaha, NE, USA). The analysis of individual samples took an average of 9 min from sample injection to the finished result. UV detection was performed at 260 nm. DHPLC buffer conditions for the marker were automatically calculated. MSI analysis by DHPLC took 9 min per amplified DNA sample. Since no matched normal tissue DNA was required for quasimonomorphic markers of BAT 26, the MSI status was determined within 9 min. Results were analyzed using WAVEMAKER software (Transgenomic, Omaha, NE, USA.) in real time. No additional adducts (i.e., radioisotope or fluorescent-labeled primers) were employed other than the conventional PCR reaction, and no extra software was required.

Statistical analysis

Statistical analysis was performed using the Pearson's Chi-square test, Fisher's exact test, or Student's *t* test, depending on the nature of the data. Two-tailed *P* < 0.05 was considered statistically significant.

RESULTS

We evaluated the status of the microsatellite from surgical

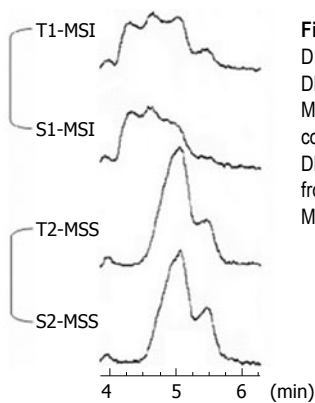


Figure 1 MSI analysis in BAT 26 using DHPLC. Chromatograms of four different DNA samples are shown. T2-S2 displayed MSS, while T1-S1 exhibited MSI in both colorectal cancer tissue and stool DNA. T, DNA from colorectal cancer tissue; S, DNA from stool; MSI, microsatellite instability; MSS, microsatellite stability.

specimens of all 80 cancers by DHPLC. DNA of adequate quality was recovered from all the lesions. We identified six cases (7.5%) of MSI in BAT 26 from 80 cancer tissues, 4 of 27 proximal colon cancers and 2 of 53 distal colorectal cancers.

To determine the degree of concordance between BAT 26 alterations in cancer tissue and those in matched fecal DNA, we evaluated the status of the microsatellite from matched stool DNA samples of all 80 patients by nested PCR and DHPLC analysis. Seven cases of MSI in BAT 26 were identified from 80 stool DNA samples. Among 7 stool DNA samples with MSI, 6 stool samples corresponded to tissue MSI and the size of the BAT 26 alteration in the tumor and fecal DNA was identical in each patient (Figure 1). Cancer tissue from one proximal colon cancer did not display MSI, but displayed MSI repeatedly in the matched stool DNA.

DISCUSSION

MSI induced as a result of failure of the mismatch repair system has significant clinical importance in the diagnosis and prognosis of colorectal cancer. Radioactive gel-based electrophoresis and fluorescent-labeled sequencing analysis have been used for MSI analysis. However, these methods have critical limitations for use in high-throughput analysis in a clinical setting. Thus, there is a need for improved MSI analysis techniques. We have developed a novel protocol for MSI analysis in cancer tissues that utilizes DHPLC^[19]. In the proposed method, standardized experimental conditions facilitate robust, high-throughput MSI analysis, and consistent results are obtained with high specificity, regardless of sample type or individual researchers' skills. DHPLC can separate PCR products that differ by as little as a few base pairs^[21]. This allows the size-dependent separation of double-stranded DNA fragments. In cases where there is a deletion in a microsatellite leading to a shorter allele, the resulting chromatogram is shifted to the left compared with that of the original allele. All the MSI samples in this investigation displayed left-shifted bands, indicating deletion alterations. The main advantage of DHPLC is that automatic high-throughput analysis is feasible using its computer-controlled systems^[21].

The BAT 26 marker is employed as an indicator of microsatellite instability, since its mononucleotide tract is altered in nearly all mismatch-deficient tumors^[22]. We

initially attempted to amplify stool DNA using original BAT 26 markers, but failed to detect mutated DNA in DHPLC. Consequently, we devised a nested PCR analysis method by adding an outer amplification step. A comparison of the outer and inner PCR experiments disclosed that outer PCR followed by BAT 26 amplification led to the best results. Regarding the correlation between MSI status of stools and clinical data, MSI (+) status has been associated with proximal location^[23]. Consistent with this, MSI (+) stools were significantly associated with proximal location in our data ($P = 0.040$).

Our data confirm the reliability of stool DNA for MSI analysis of colorectal cancers. Significantly, all 6 cases with BAT 26 mutations in their tissues displayed a positive stool DNA test. In one case with only MSI in stool DNA, it was possible that tumor heterogeneity influenced the result of tissue MSI. We used a part of tissue instead of whole tumor. Baisse *et al.*^[24] suggested that a molecular heterogeneity in tumors could modify MSI detection.

Main limitation of stool MSI was a low sensitivity. Colorectal cancers with MSI are less frequent even in the proximal colon compared with those without MSI. Using only MSI analysis is not suitable as a screening method. An analysis of a combination of several DNA alterations including MSI status using BAT 26 in stools improved the sensitivity over 90%^[7].

In this work, we have extended the DHPLC method for MSI analysis to stool DNA and verified the reliability of stool DNA in the MSI analysis for one of the multiple markers of the genetic screening of colorectal cancers. As MSI is one of the established fecal DNA markers to screen colorectal cancer, we propose to use DHPLC for the MSI analysis in stool DNA.

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