Characterization of Mutator Pathway in Younger-age-onset Colorectal Adenocarcinomas

INTRODUCTION

Colorectal cancer is known to involve multiple genetic and epigenetic alterations (1). \( hMSH2 \) and \( hMLH1 \) are the two main mismatch repair genes frequently mutated in hereditary nonpolyposis colorectal cancer (HNPCC). Genomic instability at short tandem repeats in the DNA, referred to as microsatellite instability (MSI), occurs from the mutational inactivation of the DNA mismatch repair genes, i.e. \( hMSH2 \) and \( hMLH1 \) in HNPCC, as well as from epigenetic inactivation of \( hMLH1 \) in sporadic colorectal tumors. The mutator pathway including microsatellite instability, \( hMLH1 \) promoter methylation, and \( hMSH2 \) and \( hMLH1 \) mutation patterns were identified in 21 sporadic colorectal adenocarcinoma patients younger than 30 yr excluding HNPCC. More than half of tumors showed MSI, with five MSI-H and six MSI-L (low-frequency microsatellite instability). Three of six MSI-H tumors showed the \( hMLH1 \) promoter methylation and did not express the \( hMLH1 \) protein. On the other hand, all MSI-L and all MSS (microsatellite stable) tumors expressed both \( hMSH2 \) and \( hMLH1 \) proteins. Two novel mutations, i.e. a missense mutation in \( hMLH1 \) and a splice-site alteration in \( hMSH2 \), were identified in two patients respectively. Although mutator pathway was implicated in younger-age-onset colorectal carcinogenesis, many tumors appeared to evolve from different genetic events other than \( hMSH2 \) and \( hMLH1 \) mutations frequently identified in HNPCC.

\textbf{Key Words :} Colorectal Neoplasms; DNA Repair; Microsatellite Repeats; Methylation

MATERIALS AND METHODS

Patients

Twenty-one sporadic colorectal cancer patients younger than 30 yr were prospectively included from the Colorectal Cancer Registry (Asan Medical Center, Seoul, Korea). Familial adenomatous polyposis and HNPCC were excluded. The mean age of the patients was 25 (19-30) yr and the male to female ratio was 15:6. There were six cases of right-sided, two cases of left-sided colon cancers, and 13 cases of rectal cancers. There were four patients of stage I, two of stage II, ten of stage III, and five of stage IV regarding AJCC tumor stage.

DNA from peripheral lymphocytes, tumors, and normal colonic tissues was extracted using a standard method. Normal colonic tissue was acquired at least 10 cm from the tumor. This study was performed under the approval of the Institutional Review Board.
Detection of hMSH2 and hMLH1

For PCR-SSCP analysis, previously defined oligonucleotide primers were used (12). The reaction mixture consisted of approximately 200 ng of template DNA in a volume of 50 µL, 1 x standard PCR buffer (Promega, Madison, WI), 1.5 mM Mg<sup>2+</sup>, 0.5 mM dNTPs, and 0.4 mM each specific oligonucleotide primer. One-unit Taq polymerase was added per reaction. Amplification was performed using the following protocol: 94 °C for 4 min at the start, then 35 cycles of 94 °C for 1 min, respective annealing temperature for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min.

PCR products were heated to 90 °C for 5 min and quenched on ice. Electrophoresis was performed in 10% non-denaturing polyacrylamide gel with an acrylamide:bisacrylamide ratio of 30:0.8 containing 10% glycerol at 20 mA for 18 hr. DNA bands of distinct mobility were detected by silver staining. DNA alterations identified on a PCR-SSCP were then determined by direct sequencing. The PCR products were sequenced on an ABI 377 DNA sequencer (Applied Biosystems, Foster city, CA, U.S.A.).

Microsatellite Instability (MSI)

The MSI was identified on a PCR using primer sets of five microsatellite loci according to the NCI recommendations for colorectal cancer, i.e. BAT25, BAT26, D17S250, D5S346, and D2S123 (13). Standard PCR was carried out in a 25 µL reaction mixture, containing 10 pmol of respective primers, 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L each of dNTP, and 0.5 units of Taq DNA polymerase (Promega, Madison, WI). Consecutive PCR consisted of an initial denaturation step at 94 °C for 4 min, followed by 34 cycles of 94 °C for 30 sec, 50-60 °C for 30 sec, and then 72 °C for 30 sec, and a final extension step at 72 °C for 7 min. Denaturation of PCR products, gel electrophoresis, and silver staining were performed as previously described (14). Tumor DNA showing alleles that were not present in the corresponding normal DNA were classified as MSI positive. Tumors were defined as MSI-H (high-frequency MSI) when MSI was identified in at least two loci, as MSI-L (low-frequency MSI) in one locus, and as MSS (microsatellite stable) in no locus.

Methylation-specific PCR (MSP)

The DNA methylation pattern in the CpG islands of the hMLH1 promoter region was determined by sodium bisulfite modification and subsequent MSP as described previously (6). Primer sequences of hMLH1 for unmethylated DNA were 5'-TTT TGA TGT AGA TGT TTT ATT AGG GTT GT-3' (sense) and 5'-ACC ACC TCA TCA TAA CTA CCC ACA-3' (antisense), and for methylated DNA were 5'-ACG TAG ACG TTT TAT TAG GGT CGC-3' (sense) and 5'-CCT CAT CGT AAC TAC CCG CG-3' (antisense). The PCR mixture contained 10 x PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM MgCl<sub>2</sub>, and 10 mM 2-mercaptoethanol), dNTPs (each 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) in a final volume of 50 µL. The reaction was performed using hot start at 95 °C for 5 min followed by addition of 1.25 units Taq polymerase. Amplification was carried out using a PCR Express Thermal Cycler (Hybaid, Middlesex, U.K.) for 35 cycles (30 sec at 95 °C, 30 sec at 53 °C, and then 30 sec at 72 °C), followed by final 4 min extension at 72 °C. The colon cancer cell line SW48, known as completely methylated at the hMLH1 promoter region, was used as a positive control. Ten µL of each PCR reaction product were directly loaded onto non-denaturing 6% polyacrylamide gels. Gels were stained with ethidium bromide and visualized under UV transilluminator.

Any indeterminate results were repeatedly verified for MSI and methylation analyses.

Immunohistochemistry

Slides with 5-µm sections were deparaffinized in xylene, rehydrated in graded alcohols, and washed in ddH<sub>2</sub>O. Endogenous peroxidase activity was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub>. Antigen retrieval was accomplished in 10 mM citrate buffer (pH 6.0) by boiling in a microwave for 15 min. After treatment with 10% normal goat serum for 10 min to block nonspecific protein binding, mouse monoclonal antibody against hMLH1 (PharMingen, San Diego, CA) and hMSH2 (PharMingen, San Diego, CA) were applied. Antibody-antibody reactions were visualized using the avidin-biotinylated horseradish peroxidase complex (DAKO LSAB kit, Los Angeles, CA) and diaminobezidine as chromogen. Slides were counterstained with hematoxylin. Normal tissues adjacent to respective tumor was used as an internal positive control. A distinct nuclear staining more than 10% of all nuclei was interpreted as positive staining for hMSH2 and hMLH1.

RESULTS

Patterns of hMSH2 and hMLH1 mutations

All coding exons and exon-intron borders of hMSH2 and hMLH1 genes were examined using genomic DNA samples. Two different novel mutations were found in two patients (Fig. 1). One missense mutation in the hMLH1 exon 10 (845 C→G, A282G) was identified. The other mutation was splice-site alteration in the hMSH2 intron 10 (1661 +6, T→C).

MSI and MSP

There were five cases (23.8%) of MSI-H, six cases (28.6%) of MSI-L, and 10 cases (47.6%) of MSS in 21 colorectal ade-
nocarcinomas (Fig. 2). When MSI-H tumors were compared with MSI-L and MSS tumors together, they did not differ in sex, tumor location, histologic differentiation, or cancer stage (Table 1). Three of MSI-H tumors showed \( hMLH1 \) promoter methylation, whereas all MSI-L tumor and MSS tumors did not. MSI-H tumors were significantly associated with \( hMLH1 \) promoter methylation \( (p<0.001\), Fisher’s exact test). \( hMSH2 \) promoter methylation was not identified in any of the tumors.

**Fig. 1.** \( hMLH1 \) and \( hMSH2 \) mutations in the younger age-onset sporadic colorectal cancer. A missense mutation in exon 10 \( (A282G, 845\ C–G) \) of \( hMLH1 \) in case 5 (left) and a splice-site alteration in intron 10 \( (1661+6, \ T–C) \) of \( hMSH2 \) in case 11 (right).

**Table 1.** Clinicopathologic characteristics regarding various mutator phenotypes in younger-age-onset sporadic colorectal adenocarcinomas

| Case No. | Tumor location* | Differentiation† | hMLH1/hMSH2 mutation | MSI‡ | Promoter methylation§ | IHC⊥ |
|----------|-----------------|-----------------|----------------------|------|------------------------|------|
|          |                 |                 |                      |      | hMLH1 | hMSH2 | hMLH1 | hMSH2 |
| 1        | RC              | MUC             | -                    | H (2) | M     | U     | -     | +     |
| 2        | R               | MD              | -                    | S     | U     | U     | +     | +     |
| 3        | RC              | MUC             | -                    | L     | U     | U     | +     | +     |
| 4        | RC              | MUC             | -                    | S     | U     | U     | +     | +     |
| 5        | R               | MD              | +                    | S     | U     | U     | +     | +     |
| 6        | RC              | PD              | -                    | S     | U     | U     | +     | +     |
| 7        | LC              | MD              | -                    | S     | U     | U     | +     | +     |
| 8        | R               | MD              | -                    | L     | U     | U     | +     | +     |
| 9        | R               | MD              | -                    | H (4) | U     | U     | +     | +     |
| 10       | R               | MD              | -                    | S     | U     | U     | +     | +     |
| 11       | R               | MD              | +                    | S     | U     | U     | +     | +     |
| 12       | R               | MD              | -                    | S     | U     | U     | +     | +     |
| 13       | R               | MUC             | -                    | L     | U     | U     | +     | +     |
| 14       | RC              | MD              | -                    | H (3) | M     | U     | -     | +     |
| 15       | R               | MD              | -                    | L     | U     | U     | +     | +     |
| 16       | RC              | WD              | -                    | H (4) | U     | U     | -     | +     |
| 17       | R               | MD              | -                    | S     | U     | U     | +     | +     |
| 18       | R               | WD              | -                    | L     | U     | U     | +     | +     |
| 19       | R               | MD              | -                    | H (4) | M     | U     | -     | +     |
| 20       | R               | MUC             | -                    | L     | U     | U     | +     | +     |
| 21       | LC              | SRC             | -                    | S     | U     | U     | +     | +     |

*RC, right colon (cecum - transverse colon); LC, left colon (descending-sigmoid colon); R, rectum. †WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; MUC, mucinous; SRC, signet ring cell. ‡H, MSI-H; L, MSI-L; number of unstable markers in the parenthesis. §U, unmethylated; M, methylated. ⊥IHC, Immunohistochemical staining; +, normal nuclear expression; −, absent nuclear expression.
Immunohistochemistry

Three tumors of MSI-H with hMLH1 promoter methylation did not express hMLH1 protein and one MSI-H tumor without promoter methylation expressed both hMSH2 and hMLH1 proteins. The other MSI-H tumor did not express hMSH2 with profuse expression of hMLH1. All MSI-L and MSS tumors showed profuse expression of both hMSH2 and hMLH1 proteins. In patients younger than 20 y, all tumors showed both hMSH2 and hMLH1 protein expression.

DISCUSSION

MSI-H was identified in less than one quarter of younger-age-onset sporadic colorectal carcinoma in our study. MSI-H cancers constitute 10-15% of sporadic colorectal cancers and virtually all cancers of the HNPPC (15). HNPPC and sporadic MSI-H cancers came to be regarded as familial and sporadic counterparts of the same mutator pathway, respectively, in their tumorigenesis (15). Both the frequency and extent of de novo methylation has been shown to increase strikingly in the colon cancer with MSI-H. A significantly lower frequency of hMLH1 promoter methylation has been identified in HNPPC than in MSI-H sporadic colorectal cancers, showing none in 30 HNPPC cancers with MSI-H and 80% in 40 sporadic colorectal cancers with MSI-H (16). In sporadic colorectal cancers with MSI-H, hMLH1 promoter methylation is extremely frequent and often accompanied by down-regulation of hMLH1 gene expression (17).

In our study, more than half of MSI-H cancers included hMLH1 promoter methylation without hMLH1 protein expression. Many investigations reported that a large portion of the MSI-H in sporadic colorectal cancer occurred from hMLH1 promoter methylation (1, 6). The immunohistochemistry of hMSH2 and hMLH1 has been known to predict MSI-H with great sensitivity (92.3%) and specificity (up to 100%) (18, 19). All tumors without either hMSH2 or hMLH1 expression were MSI-H in our study. On the other hand, all MSI-L and MSS tumors showed both hMSH2 and hMLH1 protein expression. One MSI-H cancer without hMLH1 promoter methylation showed hMSH2 and hMLH1 protein expression in our study. Several investigations proposed that some tumors with MSI-H showing normal expressions of hMSH2 and hMLH1 possibly occur from the wild type allele or hMSH6 mutation (17, 20). The promoter methylation has been known to correlate with the CpG island methylation phenotype (CIMP) in subsets of cancer specific genes, i.e. P16INK4a and THBS1 in sporadic colorectal carcinomas (2).

Approximately 70% of HNPPC patients with MSI tumors have been found to have germline mutations in one of the mismatch repair genes (18). Although some of the MSI positive sporadic colorectal cancers demonstrated mutations in one of the mismatch repair genes (21), the majority of these cancers had no identifiable mutation (22, 23). Two germline mutations were identified in two patients of our study (9.5%). The other study including colorectal cancer patients younger than 30 yr showed hMSH2 or hMLH1 mutation in two of 14 patients with no family history of any cancers (24). However, missed mutations, i.e. large deletions with genomic rearrangement, cannot be excluded with PCR-SSCP analysis. The efficiency of PCR-SSCP in detection of single-base substitution is known to be more than 95% in fragments of less than 300-350 bp (25). One missense mutation in hMLH1 and the other splice-site alteration in hMSH2 did not alter hMSH2 and hMLH1 protein expression in our study. As these alterations may frequently show functional significance and immunologic reactivity to poorly functional proteins (18, 26), their pathogenic significance cannot be excluded. Furthermore, these alterations have not been reported in healthy individuals (IGC-HNPPC database 2003, http://www.nfahr.nl) and our missense mutation included non-conservative amino acid change from non-polar alanine to polar glycine. MSI cancers have been reported to have different characteristics in terms of proximal colonic location, mucinous and undifferentiated histology (13). In our study, these clinicopathologic features did not differ by MSI status in a multiple regression analysis, probably due to the limited sample size.

The mutator pathway other than hMSH2 and hMLH1 mutations appeared to be implicated in many younger-age-onset colorectal cancers. It can be included in the category of a variant form of sporadic colorectal cancer rather than that of HNPPC regarding genetic and epigenetic phenotypes.

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