

Mutational Analysis of Promoters of Mismatch Repair Genes *hMSH2* and *hMLH1* in Hereditary Nonpolyposis Colorectal Cancer and Early Onset Colorectal Cancer Patients: Identification of Three Novel Germ-line Mutations in Promoter of the *hMSH2* Gene¹

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Abstract

The human DNA mismatch repair genes *hMSH2* and *hMLH1* are responsible for the development of hereditary nonpolyposis colorectal cancer (HNPCC). Although genetic alteration of the coding region of *hMSH2* and *hMLH1* has been well investigated in HNPCC patients, the regulatory regions of these genes have been poorly investigated, though recent studies have defined and characterized the core promoter regions of *hMSH2* and *hMLH1*. Therefore, to investigate the presence of germ-line mutations, we screened the core promoter regions of *hMSH2* and *hMLH1* from 157 nonmalignant control individuals, 40 cases of HNPCC, 56 suspected HNPCC cases, and 45 sporadic early onset colorectal cancer patients. Three novel germ-line mutations of the *hMSH2* promoter were identified in two suspected HNPCC cases and one sporadic early onset colorectal cancer patient but not in the 157 nonmalignant controls, namely, an A insertion at position –80, a G-to-A transition at position –190, and a G-to-C transversion at position –225. Tumors from patients containing the promoter mutations displayed microsatellite instability. The A insertion at –80 is within a sequence homologous to the consensus sequence for E1AF and very close to the major transcription start point. Luciferase assay demonstrated that the –80A insertion and the –190A allele decreased the transcriptional efficiency by 82 and 77%, respectively, and the –225C allele increased the transcriptional efficiency by 466%. The –80A insertion allele was detected only in affected members within the family and showed novel transcription factor binding ability. Furthermore, the loss of single nucleotide polymorphism allelic expression was identified in blood of the patient containing the –80A insertion. Our results indicate that mutations in the promoter region of *hMSH2* have a limited role in development of suspected HNPCC and sporadic early onset colorectal cancer.

Introduction

HNPCC⁴ is the most common hereditary condition and predisposing factor for the development of cancer of multiple organs. Susceptibility to HNPCC is caused by mutations in one of five genes with the MMR function (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, and *hMSH6*). Among these MMR genes, germ-line mutations in the coding regions

of *hMSH2* and *hMLH1* are known to be responsible for up to 45–64% of HNPCCs (1, 2).

Although genetic alteration in coding regions of *hMSH2* and *hMLH1* has been well investigated in HNPCC, the genetic alteration in the regulatory regions of these genes has been poorly investigated. It has been documented that point mutations in the regulatory region of the *Rb* gene cause hereditary retinoblastoma (3). Recent studies have defined and characterized the core promoter regions of *hMSH2* (from –300 to –17) and *hMLH1* (from –220 to +39 Refs. 4 and 5). These observations prompted us to clarify whether or not a germ-line mutation in the promoter regions of *hMSH2* and *hMLH1* is implicated in the tumorigenesis of HNPCC and sporadic early onset colorectal cancer. Therefore, we screened the core promoter regions of *hMSH2* and *hMLH1* from 40 HNPCC patients, which satisfied the ICG-HNPCC criteria, and 56 patients suspected of having HNPCC but who did not fulfill the ICG-HNPCC criteria (6, 7). Forty-five early onset colorectal cancer patients (who developed colorectal cancer before the age of 40 years) without any family history of colorectal cancer were also examined.

Materials and Methods

Subjects and DNA Isolation from Blood Samples. Forty cases of HNPCC, 56 cases of suspected HNPCC, and 45 sporadic early onset colorectal cancer patients registered in the Korean Hereditary Colorectal Cancer Registry were used in this study. All patients enrolled in this study were Korean and originated from unrelated families. Peripheral blood (20 ml) from each patient was used to prepare genomic DNA from white blood cells, as described elsewhere (8).

Blood samples (157) were taken as controls from nonmalignant individuals (55 healthy normal, 100 hemorrhoid patients, and 2 anal fissure patients). None of the individuals in the control group had a family history which suggested HNPCC or the development of colon cancer at an earlier age.

Mutational Analysis of the Core Promoter Regions of *hMSH2* and *hMLH1*. The PCR-SSCP method was used to screen for mutations of the core promoter regions of *hMSH2* and *hMLH1*, which have been described previously (4, 5). For PCR-SSCP of the *hMSH2* promoter, a 282-bp fragment from –299 to –17, and the *hMLH1* promoter, a 259-bp fragment from –220 to +39, were amplified by PCR using the primers described in Table 1. When abnormal patterns were detected by PCR-SSCP analysis, the PCR products were purified using a QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA) and then sequenced directly with a Taq dideoxy terminator cycle sequencing kit on an ABI 377 automatic DNA sequencer (Perkin-Elmer, Foster City, CA).

Analysis of MSI. Genomic DNA was extracted from the tissues using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. MSI status was determined by screening the polyadenine sequence, BAT-26, which proved 99.4% efficient at detecting MSI-associated cancer (9). The repeat sequence was amplified, and products were separated on 15% polyacrylamide gels. After gel electrophoresis, PCR products were visualized using a Silverstar staining kit (Bioneer, Seoul, Korea)

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⁴ The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; SNP, single nucleotide polymorphism; MMR, mismatch repair; ICG, international collaborative group; PCR-SSCP, PCR-single-strand conformational polymorphism; EMSA, electrophoretic mobility shift assay; RFLP, restriction fragment length polymorphism; MSI, microsatellite instability.

Table 1 Sequences of oligonucleotides used for PCR and EMSA^a

Oligonucleotides	Sequences
For PCR	
hMSH2PS	5'- ⁻²⁹⁰ GCTGAGTAAACACAGAAA ⁻²⁸² -3'
hMSH2PA	5'- ⁻¹⁷ CTCCTGGTTGAAGAAAATGC ⁻³⁶ -3'
hMLH1PS	5'- ⁻²²⁰ AGTAGCCGCTTCAGGGA ⁻²⁰⁴ -3'
hMLH1PA	5'- ⁺³⁹ CTCGTCCAGCCGCCGAATAA ⁺²⁰ -3'
For EMSA	
-80wt	5'- ⁻⁹⁰ GCTCGGGGGACGTGGGAGGGG ⁻⁷⁰ -3'
-80mt	5'- ⁻⁹⁰ GCTCGGGGGAAACGTGGGAGGGG ⁻⁷⁰ -3'
-190wt	5'- ⁻¹⁹¹ GCTGGGCCGCTCTGCTTAT ⁻¹⁷² -3'
-190mt	5'- ⁻¹⁹¹ GCTGGGCCGATCTGCTTAT ⁻¹⁷² -3'
-225wt	5'- ⁻²³³ GCAGGCATCGCAGTAGCTA ⁻²¹⁴ -3'
-225mt	5'- ⁻²³³ GCAGGCATCCGAGTAGCTA ⁻²¹⁴ -3'

^a The mutation sites are in bold type.

according to the manufacturer's instructions. The primers sequences and the detailed condition for amplification have been described elsewhere (9).

Construction of Luciferase-Reporter Plasmid Containing Wild-type or Mutant Promoters of hMSH2. The core wild-type promoter region of hMSH2 (from -299 to -17) was amplified by PCR using normal DNA. Three mutant hMSH2 promoters were also amplified from the DNA of patients SNU-H1011, SNU-H1018, and SNU-YC23. The PCR products were inserted upstream of the luciferase gene in the pGL3-Basic vector (Promega, Madison, WI), and the construct was confirmed by sequencing.

Transient Transfection and Luciferase Assay. Promoter activity was measured using the Dural Luciferase Reporter Assay System (Promega). Hela cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. $\sim 5 \times 10^4$ cells/well in a 24-well plate were inoculated and cultured for 24 h before transfection. The pRL-SV40 and the pGL3-Basic vectors with or without the three mutant hMSH2 promoters were cotransfected using Lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD). The pRL-SV40 vector that provides constitutive expression of Renilla luciferase served as internal control to normalize luciferase activity. Cells were collected 48 h after transfection, and cell lysates were prepared according to Promega's instruction manual. Luciferase activity was measured using a luminometer (Promega) and normalized using the activity of the Renilla luciferase.

EMSA. EMSA was performed using a Gel Shift Assay System (Promega). Complementary oligonucleotide pairs corresponding to the hMSH2 promoter sequence (from -90 to -70; from -191 to -172; and from -233 to -214) were synthesized (Table 1), and each of the oligonucleotide pairs was annealed and labeled with [γ -³²P]ATP. Binding reactions were carried out with Hela nuclear extract (HeLaScribe Nuclear Extract; Promega) according to the manufacturer's instruction manual. After completing the binding reaction, the DNA-protein complexes were resolved by electrophoresis in a 4% nondenaturing acrylamide gel. After electrophoresis, the gels were dried and subjected to autoradiography.

PCR-RFLP. To determine genotype of an SNP of C/A at 471 (G157G) in exon 3 of hMSH2, we used the fact that the SNP of C/A at 471 in the hMSH2 gene resulted in loss of the existing HaeIII restriction site. Exon 3 of hMSH2 was amplified using an intronic sense primer (5'-TTAGGCTTCTCCTG-GCAATC-3') and an intronic antisense primer (5'-CCTTTCCTAGGCCTG-GAATC-3'), and the expected amplification product was 350 bp. hMSH2 cDNA encompassing a part of exons 2-3 was also amplified by reverse transcription-PCR. Randomly primed cDNAs were reverse-transcribed from 5 μ g of total RNA from white blood cells using Superscriptase (Life Technologies, Inc., Grand Island, NY) in a 40- μ l mixture. The cDNA mixture (2 μ l) was used to determine the level of the hMSH2 transcript. The hMSH2 cDNA

was amplified using a sense primer from exon 2 (5'-AGGAGAATGATTGG-TATTTGGC-3') and an antisense primer from exon 3 (5'-TCTCCAG-CAGTCTCTCCTC-3'). The expected amplification product for hMSH2 mRNA was 288 bp. The PCR product was then digested with HaeIII enzyme and analyzed on 2% agarose gel electrophoresis.

Results and Discussion

Identification of Germ-line Mutations in the Core Promoter Regions of hMSH2 and hMLH1 and the Status of MSI in Tumors from the Patients. We screened a 282-bp core promoter region of hMSH2 (from -299 to -17) and a 259-bp core promoter region of hMLH1 (from -220 to +39) by PCR-SSCP and sequencing analysis. Three novel germ-line mutations were identified in the hMSH2 promoter but not in the hMLH1 promoter. Abnormal band patterns were revealed in the hMSH2 promoter of two suspected HNPCC patients (SNU-H1011 and SNU-H1018) and a sporadic early onset colorectal cancer patient (SNU-YC23), in which germ-line mutations in the coding and splicing regions of hMSH2 and hMLH1 have not been detected by direct sequencing (Fig. 1). Subsequent sequencing analysis of the hMSH2 promoter revealed that SNU-H1011 had a G-to-C transversion at position -225 and that SNU-H1018 contained an A insertion at position -80 (Fig. 2). In a sporadic early onset colorectal cancer patient (SNU-YC23), a G-to-A transition at position -190 was detected (Fig. 2). These mutations were not found in 157 unrelated nonmalignant control individuals.

Patient SNU-H1011 was diagnosed as having gastric cancer at age

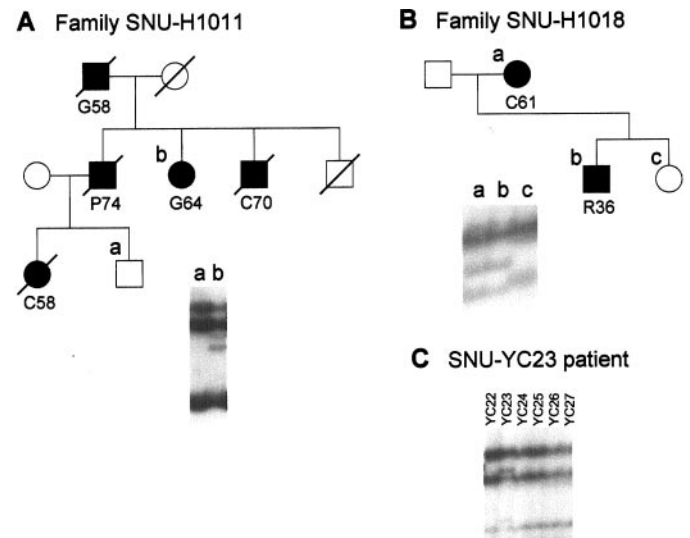
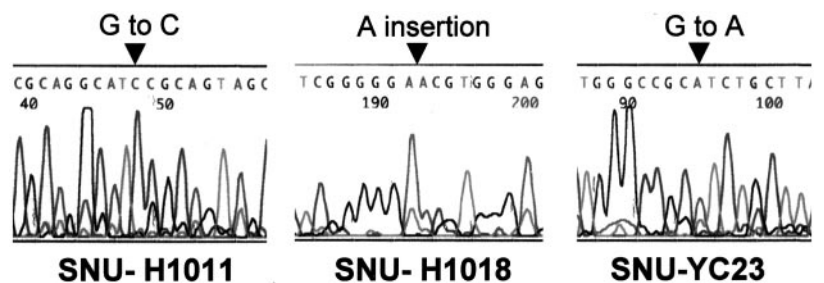


Fig. 1. Pedigree and PCR-SSCP analysis of families SNU-H1011 and SNU-H1018 and patient SNU-YC23. A, family SNU-H1011. \square , males; \circ , females; filled symbols, cancer occurred; open symbols, no cancer occurred; G, gastric cancer; P, pancreatic cancer; C, colon cancer; and numbers symbols, age at cancer. B, family SNU-H1018. R, rectal cancer. C, patient SNU-YC23.

Fig. 2. Three novel germ-line mutations identified in the hMSH2 promoter. SNU-H1011 contained a G-to-C transversion at position -225. SNU-H1018 harbored an A insertion at position -80. SNU-YC23 had a G-to-A transition at position -190.



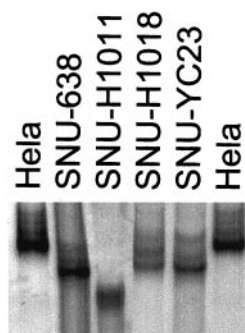


Fig. 3. Analysis of MSI in tumors from the patients containing promoter alterations. Alteration in the BAT-26 repeat sequence was found in SNU-H1011, SNU-H1018, and SNU-YC23. Helix and SNU-638 (human gastric carcinoma cell line) were used as a negative and a positive control for MSI, respectively.

51. Although SNU-H1011's family did not meet the ICG-HNPCC criteria, her father and brother were diagnosed with gastric and colon cancer, respectively (Fig. 1A). Her niece, whose father had died of pancreatic cancer, also had colon cancer. Because the blood of the affected family members was not available, we could not examine the mutation. Patient SNU-H1018 had colon cancer at 61 years of age, and her son was diagnosed with rectal cancer at age 36 (Fig. 1B). We investigated the possibility of A insertion mutation at -80 in the *hMSH2* promoter from the genomic DNAs of her affected son and daughter, and an identical mutation to that found in SNU-H1018 was detected in the DNA of her affected son only (Fig. 1B). Since germ-line mutations in the *hMSH2* and *hMLH1* genes were not detected, the promoter mutation of *hMSH2* was found only in the affected family members, and this mutation was not found in 157 unrelated nonmalignant control individuals, we speculated that the promoter mutation of the *hMSH2* gene is pathogenic in the tumorigenesis of these families.

Furthermore, to verify whether the tumors containing the promoter mutations are defective in MMR genes, we determined the MSI status of the tumors from patients by using BAT-26 sequence. As shown in Fig. 3, all tumors showed abnormal band patterns in the tested repeat sequence, indicating MSI.

In addition, we also found the previously reported single nucleotide polymorphisms of a C-to-T at -118 in *hMSH2* and a G-to-A at -93

in *hMLH1* (4, 5). As shown in Table 2, the allelic difference was not statistically associated with the tested disease groups.

Effects of the Mutations on the Promoter Activity of *hMSH2*.

As shown in Fig. 4A, among the patient specific mutations, the mutation in the family of SNU-H1018 is within a sequence homologous to the consensus sequence (NGGAYGT) for E1AF and very close to the major transcription start point (4). However, there are no known transcription factor binding sites around the mutations found in SNU-H1011 and SNU-YC23 (4).

We next investigated the effects of the germ-line mutations on the promoter activity of *hMSH2* by luciferase assay. We cloned wild-type and three mutant *hMSH2* promoters upstream of the luciferase gene in the pGL3-Basic plasmid. The promoter activities of mutants were compared with that of wild-type promoter by transient transfection assay in the HeLa cell line. As shown in Fig. 4B, the promoter activities of the -80A insertion and the -190A allele were significantly decreased by 82 and 77%, respectively. However, the -225C allele increased the transcriptional efficiency by 466%.

Since the down-regulation of genes is known to be one of the inactivating mechanisms, the effect of A insertion at -80 and G-to-A transition at position -190 on the pathogenesis can be explained in this aspect. In particular, transcriptional reduction caused by the A insertion at -80 may be explained by the presence of the transcription start point at -85nt (4). Although the involvement of overexpression of *hMSH2* in the inactivation of the MMR pathway has not been reported, a recent study demonstrated that MMR deficiency could arise through the overexpression of hMSH3 protein (10).

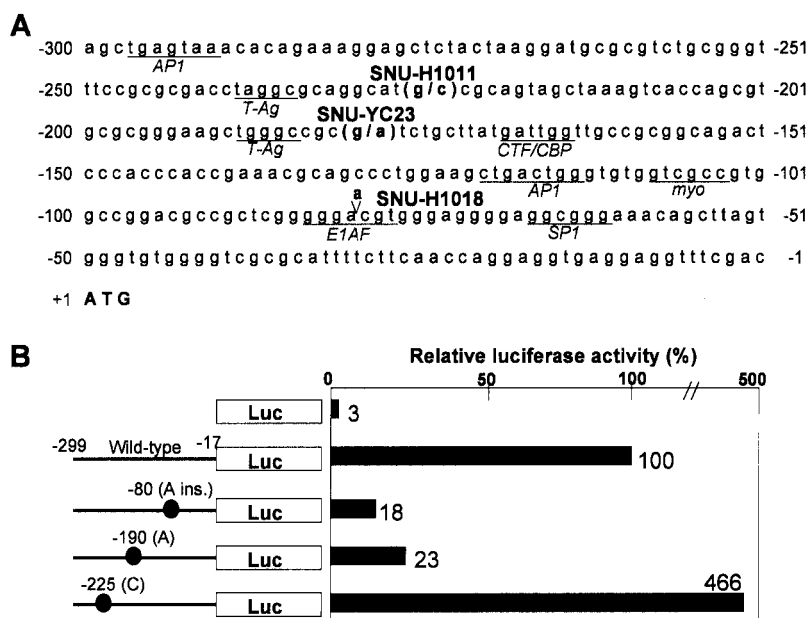
Table 2 Single nucleotide polymorphisms of the *hMSH2* and *hMLH1* promoters in nonmalignant controls, cases of HNPCC, suspected cases of HNPCC, and sporadic early onset colorectal cancer patients

	Frequency (%)					
	<i>hMSH2</i> (-118)			<i>hMLH1</i> (-93)		
	C/C	C/T	T/T	G/G	G/A	A/A
Control (n = 157)	3.3	30.3	66.4	26.7	47.3	26
ICG-HNPCC (n = 40)	0	29.1	70.8	16	40	44
S-HNPCC (n = 56)	0	40.6	59.3	31	38	31
Early CRC ^b (n = 45)	0	29.6	70.3	23	53	23

^a Suspected HNPCC patient.

^b Sporadic early-onset colorectal cancer patient.

Fig. 4. A, Locations of *cis*-acting elements (4) and the three germ-line mutations identified in the *hMSH2* promoter. The A insertion at -80 found in SNU-H1018 located in the E1AF binding site and very close to the major transcription start point (-85nt). The G-to-A transition at -190 found in SNU-YC23 and the G-to-C transversion at -225 found in SNU-H1011 located in unknown transcriptional factor binding sites. B, luciferase assay for wild-type and mutant type *hMSH2* promoter. The *hMSH2* promoter corresponding to the region from -299 to -17 was cloned from normal subjects and 3 patients upstream of the luciferase promoter gene in the pGL3-Basic vector. Each constructed report vector was transiently cotransfected with pRL-SV40 into HeLa cells. The luciferase activity of each construct was normalized versus the activity of *Renilla* luciferase.



Effects of the Mutations on the Binding Activity of Nuclear Proteins. To determine whether the identified mutations affect the binding activity of nuclear factors, three synthetic mutant double-stranded DNA oligomers and their corresponding wild-type double-stranded DNA oligomers were subjected to EMSA. A nuclear extract from HeLa cells was incubated with matched sets of DNA oligomers according to the normal and mutant sequences of the promoter region of *hMSH2*. Two mutant oligomers (G/A at -190 and G/C at -225) did not affect the DNA binding activity of nuclear factor compared with their corresponding wild-type oligomers (data not shown). However, the -80A insertion oligomer showed a specific DNA-protein complex (complex I), which was not detected in the wild-type oligomer (Fig. 5A). To verify the specificity of complex I, competition assay was performed with unlabelled wild-type and mutant oligomers (Fig. 5B). When unlabelled wild-type oligomer was used as a competitor with labeled mutant oligomer to form complex I (Lanes 1-4). However, when unlabeled mutant oligomer was used as a competitor with labeled mutant oligomer, it effectively competed with the labeled mutant oligomer to form complex I (Lanes 5-8). This result indicates that complex I is the result of a specific DNA-protein interaction between the -80A insertion oligomer and transcription factor(s).

In Vivo Effects of the Promoter Alterations in the Expression of *hMSH2* mRNA. We identified a SNP of C/A at 471 (G157G) in exon 3 of *hMSH2* from the SNU-H1018 patient. Allele frequency of the SNP was 0.02 in normal population, and the SNP did not show significant difference from the frequency determined in the controls and patients. The G157G SNP in the *hMSH2* gene eliminated the *Hae*III restriction site. We then used the *Hae*III site to detect genotypes by RFLP analysis. A single undigested band at 350 bp represents a SNP allele, and two bands at 200 and 150 bp represent a wild-type allele. We amplified exon 3 of *hMSH2* from genomic DNA of SHU-H1018 with intronic sequences, and performed *Hae*III RFLP. As shown in Fig. 6A, the RFLP assay revealed bands at 350, 200, and 150 bp in *Hae*III-digested SNU-H1018 sample, indicating the presence of wild-type and the SNP allele (Fig. 6A).

To confirm the *in vivo* effect of the promoter mutation found in

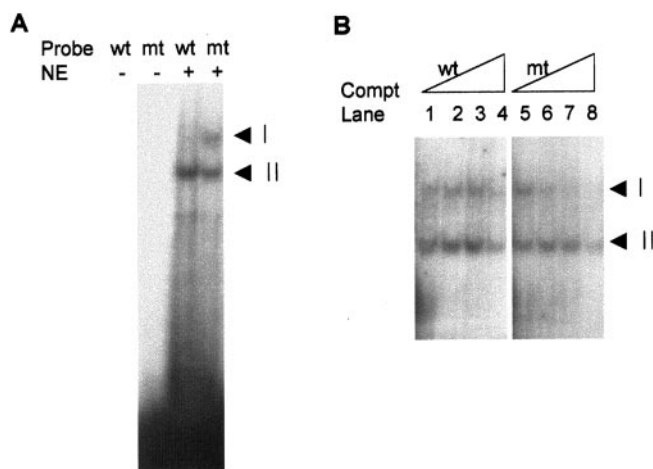


Fig. 5. EMSA with HeLa nuclear extract and -80wt or -80mt oligonucleotide probes. A, binding activity of the wild-type (*wt*) and mutant (*mt*) oligonucleotide. The assay was performed in the presence (+) or absence (-) of HeLa nuclear extract (NE). Arrowheads, DNA-protein complexes, I and II. The free probe is shown at the bottom of each lane. In B, the competition assay was performed with unlabelled -80wt or -80mt oligonucleotides. Excess unlabelled wild-type oligonucleotides (0-, 10-, 50-, and 100-fold) were added to the binding reaction as competitor in Lanes 1-4. Excess unlabelled mutant oligonucleotides (0-, 10-, 50-, and 100-fold) were added to the binding reaction as competitor in Lanes 5-8. *Compt*, competitor.

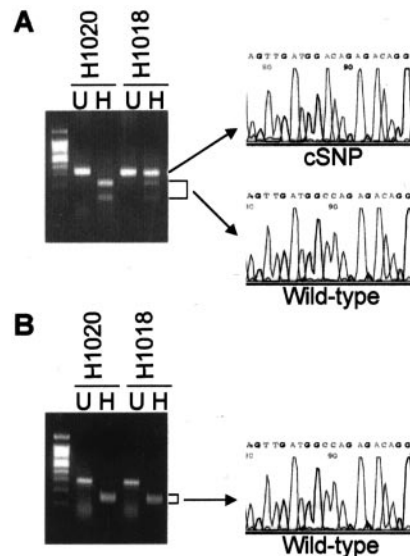


Fig. 6. PCR-RFLP analysis of the *hMSH2* SNP in the SNU-H1018 patient. In A, Exon 3 of *hMSH2* was amplified with genomic DNA isolated from white blood cells of the patients. The PCR product was undigested (U) or digested with *Hae*III (H). The samples were then analyzed on 2% agarose gel electrophoresis. A single undigested band at 350 bp represents a SNP allele, and two bands at 200 and 150 bp represent a wild-type allele. Bands at 350, 200, and 150 bp in *Hae*III-digested SNU-H1018 sample were revealed, indicating the presence of wild-type and the SNP allele. In B, *hMSH2* cDNA encompassing a part of exons 2-3 was amplified by reverse transcription PCR. The PCR product was undigested (U) or digested with *Hae*III (H). The samples were then analyzed on 2% agarose gel electrophoresis. A single undigested band at 288 bp represents a SNP allele, and two bands at 134 and 154 bp represent a wild-type allele. Bands at 134 and 154 bp in *Hae*III-digested SNU-H1018 sample were observed, indicating the loss of *hMSH2* expression from the SNP allele.

SNU-H1018 on *hMSH2* expression, we then used the fact that the SNU-H1018 patient had two different alleles. Therefore, to examine whether two alleles are expressed, we synthesized cDNA from white blood cells of SNU-H1018, amplified *hMSH2* mRNA, and performed *Hae*III RFLP. A single undigested band at 288 bp represents a SNP allele, and two bands at 134 and 154 bp represent a wild-type allele. The RFLP assay revealed bands at 134 and 154 bp in *Hae*III-digested SNU-H1018 sample, indicating the loss of *hMSH2* expression from the SNP allele (Fig. 6B). We were unable to determine the *in vivo* effects of the promoter mutations found in SNU-H1011 and SNU-YC23.

In summary, we report on three novel germ-line mutations of the *hMSH2* promoter in suspected cases of HNPCC and sporadic early onset colorectal cancer patients. MSI was observed in tumor samples obtained from the 3 patients containing the promoter mutations. These mutations were not found in nonmalignant control individuals and affected the transcriptional activity of the *hMSH2* promoter. The pathogenic effects of the A insertion at position -80 were evident in four ways: (a) the mutation was not found in the 157 members of the nonmalignant control group and found in affected family members; (b) the mutation significantly decreased the promoter activity of *hMSH2*; (c) the mutation affected the transcription start site and the transcriptional factor binding site, resulting in a novel DNA-protein complex; and (d) the loss of *hMSH2* expression from the SNP allele was observed in blood samples of SNU-H1018. Our results indicate that mutations in the promoter region of *hMSH2* are responsible for tumorigenesis in minor groups of suspected HNPCC and sporadic early onset colorectal cancer patients.

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