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Advances in Brief

Germ-Line Mutation of the *hMSH6/GTBP* Gene in an Atypical Hereditary Nonpolyposis Colorectal Cancer Kindred[1]

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Abstract

A germ-line mutation of *hMSH6* (also called *GTBP*) was found in a hereditary nonpolyposis colorectal cancer (HNPCC)-like patient in whom germ-line mutations of *hMSH2*, *hMSH3*, or *hMLH1* had not been detected. The patient had rectal cancer and two colon adenomas at 62 years of age and a weak family history of gastrointestinal tumors, indicating atypical HNPCC. Somatic mutations of *hMSH6* were observed in three colorectal tumors from the patient, indicating two-hit inactivation. Microsatellite instabilities at mononucleotide repeats were detected in all three tumors. These data suggest that *hMSH6* is responsible for tumorigenesis in atypical HNPCC.

Introduction

HNPCC[3] is a common cancer susceptibility syndrome characterized by the early onset of colorectal cancers (1). Cancer DNA of such patients mostly demonstrates alterations in microsatellite sequences that reflect a malfunction in DNA repair termed MI or replication error (1). Until now, four causative genes for HNPCC (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*) have been identified as homologues of bacterial DNA MMR genes (2). Two other MMR genes, *hMSH6* (also called *GTBP*) and *hMSH3*, have been cloned as bacterial *mutS* homologues (3–5) as well. Although somatic mutations in the *hMSH6* and *hMSH3* genes were detected in HNPCC cancers, sporadic cancers, and cancer cell lines with MI (6–9), germ-line mutations of them have not yet been reported in HNPCC. In yeast and man, there are two pathways of MSH2-dependent MMR: (*a*) an MSH2-MSH6 complex that recognizes both single-base mispairs and insertion/deletion loops; and (*b*) an MSH2-MSH3 complex that recognizes larger insertion/deletion loops (3, 4, 10–12). These results suggested that MSH6 and MSH3 might be redundant in MMR and supported the possibility that germ-line mutations of *hMSH6* and *hMSH3* might account for the small subset, if any, of HNPCC patients. Recently, the genomic organization of *hMSH6*, which consists of 10 exons, was elucidated (10). To clarify the tumorigenic role of *hMSH6*, we screened for germ-line mutations in all exons of *hMSH6* in six classical HNPCC patients satisfying the Amsterdam criteria (1) and in one HNPCC-like patient. Although the HNPCC-like patient did not fulfill the Amsterdam HNPCC criteria, he had three colorectal tumors and a weak family history of gastrointestinal tumors, indicating atypical HNPCC. Because germ-line mutation of *hMSH6* was found in the atypical HNPCC patient in this study, we further examined for somatic mutations of *hMSH6* as well as of *hMSH3* mononucleotide repeat (7–9) and analyzed MI using 11 microsatellite markers in the atypical HNPCC tumors.

Materials and Methods

Subjects. Lymphocytes and/or normal colonic mucosa were obtained from affected members in seven kindreds. All seven patients had not yet been detected for germ-line mutations in *hMSH2*, *hMSH3*, or *hMLH1* by PCR-SSCP and sequencing analyses. A total of three tumors, *i.e.*, one rectal cancer and two colonic adenomas, from patient H82 were collected. Genomic DNA was extracted from frozen or paraffin-embedded tissues as described previously (13).

Analysis of *hMSH6* for Mutations. According to the exon-intron boundary sequences (GenBank numbers U73732–U73737), 25 sets of primers were designed to amplify the entire coding region, including each splicing site, of the *hMSH6* gene. The sequences of these primers used for the amplification and sequencing of *hMSH6* are available from the authors on request. PCR was performed in 25-μl reaction mixtures comprising 20–100 ng of template DNA, 5 pmol of each oligonucleotide primer pair, 2.5 units of Taq DNA polymerase (Biotech International, Ltd., Bentley, Australia), 2.5 μl of 10× buffer, and 4 μl of 1.25 mM deoxynucleotide triphosphate (Pharmacia, Uppsala, Sweden). Each PCR comprised 35 cycles of 94°C (1 min), 55°C–60°C (2 min), and 72°C (1 min), with a final 10-min extension at 72°C. PCR-SSCP analysis was performed as described previously (13). Briefly, the PCR products were denatured and then electrophoresed on 12.5–15% nondenaturing polyacrylamide gels containing 10% glycerol in Tris-glycine buffer [25 mM Tris-HCl and 200 mM glycine (pH 8.3)]. After electrophoresis, the gels were stained with silver (Dai-ichi Co., Ltd., Tokyo, Japan).

Analysis of the *hMSH6* (C)8 and *hMSH3* (A)8 Repeats. Because tracts of (C)8 and (A)8 are present in *hMSH6* exon 5 and *hMSH3* exon 7, respectively, we analyzed frameshift mutations in these regions by PCR-SSCP analysis as described above except for the cycle number (30 cycles). The PCR primers were 5′-GGGTGATGGTCCTATGTGTC-3′ and 5′-CGTAATGCAAGGATGGCGT-3′ for the (C)8 repeat in *hMSH6* (7) and 5′-TGACTGATACTTCTACCAGC-3′ and 5′-AACATTTGTTCCTCACCTGC-3′ for the (A)8 repeat in *hMSH3*.

Sequencing. When abnormal patterns were observed by SSCP analysis, the PCR products were purified with a QIA-quick spin PCR purification kit (Qiagen, Inc., Chatsworth, CA) and then sequenced directly with a cycle sequencing kit (Takara Shuzo Co., Ltd., Kyoto, Japan). For simple repeated sequences in *hMSH6* exon 5 and *hMSH3* exon 7, the PCR products were cloned into the pT7Blue(R) T-vector (Novagen, Madison, WI). Several clones were sequenced with a Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH).

Analysis of MI. We examined DNA for MI using loci containing the $(A)_n$ repeat (BAT25 and BAT26) and loci containing the $(CA)_n$ repeat (D2S119, D2S123, D3S1029, D10S197, and D13S175; Refs. 9 and 13). Two trinucleotide repeats (transcription factor IID and DM-1) and two tetranucleotide

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[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MI, microsatellite instability; MMR, mismatch repair; SSCP, single-strand conformational polymorphism, (C)8, eight cytosines; (A)8, 8 adenines; TGF-β RII, transforming growth factor β type II receptor; (A)10, 10 adenines.

repeats (D16S310 and D17S846; Ref. 9) were also analyzed. MI was examined as described previously (9, 13).

Results

Analysis of *hMSH6* Germ-Line Mutations in HNPCC and Atypical HNPCC Patients. We screened for germ-line mutations in all exons of *hMSH6* in normal blood cells from six classical HNPCC patients and from the atypical HNPCC patient (Fig. 1*a*) by PCR-SSCP and sequencing analyses. Only the atypical HNPCC patient (H82) was found to exhibit an abnormal SSCP pattern for exon 5, *i.e.*, the six classical HNPCC patients did not exhibit one. Because exon 5 contains the (C)8 repeat in codons 1085–1087, and because frequent somatic mutations in the region were reported earlier (7), we also screened for mutations at the (C)8 repeat with a specific primer set (7) by PCR-SSCP. An abnormal SSCP pattern was also detected in patient H82, but not in the other six patients, in this region (Fig. 1*b*). To confirm whether the altered pattern is specific or not, we examined for mutations in DNA from his normal colonic mucosa and cDNA prepared from lymphoblastoid cells. All of the PCR products showed the same SSCP pattern (data not shown). Patient H82 had rectal cancer and two adenomas at 62 years of age. Although patient H82 did not fulfill the Amsterdam HNPCC criteria, his father and mother suffered from gastric and rectal cancers at 71 and 72 years of age, respectively (Fig. 1*a*). We screened for the mutation at this region in normal cells from five family members (his father, two brothers, one sister, and one daughter). Only the father's DNA showed a SSCP pattern similar to that of H82 (Fig. 1*b*). His mother died of rectal cancer at 72 years of age. Because her rectal cancer was not resected surgically, we could not examine the mutation at the (C)8 repeat of her DNA.

The PCR product showing the abnormal SSCP pattern was cloned into a plasmid vector, pT7Blue(R), and then sequenced. Patient H82 was heterozygous due to one C insertion in codons 1085–1087 (Fig. 1*c*). This insertion is predicted to encode a truncated protein in codon 1092 through a frameshift mutation. We further confirmed the mutation in patient H82 and his father by direct sequencing (data not shown).

Somatic Mutations of *hMSH6* in Atypical HNPCC Tumors. To determine whether or not two-hit inactivation is necessary to abolish hMSH6 function, we screened for somatic mutations in the entire coding region of *hMSH6* in a rectal cancer (T798) and two colon adenomas (T796 and T797) from patient H82. Two of the tumors (T796 and T798) clearly exhibited the same mutant SSCP band at the (C)8 repeat in both alleles (Fig. 1*b*). Direct sequencing revealed that the somatic mutation in exon 5 comprised one C deletion at the (C)8 repeat. This deletion results in a new stop codon located 5 bp downstream. These two tumors thus contained frameshift mutations in both *hMSH6* alleles, a germ-line one due to an insertion and a somatic one (Table 1). The other colon adenoma (T797) showed an abnormal SSCP pattern for exon 7, and the variant was only observed in tumor DNA, indicating a somatic mutation (Fig. 1*d*). Direct sequencing analysis of the PCR product of this adenoma demonstrated an A→C transversion at codon 1214, resulting in the substitution of alanine for glutamic acid (Table 1). Because the mutation created a new *Nsi*I restriction enzyme site, we confirmed the mutation further by *Nsi*I digestion (data not shown). The mutation was located in a highly conserved codon among the homologues of bacterial *mutS*, suggesting that it is functionally important.

Analysis of MI. Because MMR gene mutations induce MI, we analyzed MI in the three *hMSH6* defective colorectal tumors of patient H82. Eleven microsatellite DNA markers, *i.e.*, two $(A)_n$, five $(CA)_n$, and two trinucleotide and two tetranucleotide repeats, were used to detect MI. Alterations of the electrophoretic patterns of microsatellite repeats in tumor DNA were analyzed in comparison with those in normal DNA. MI in $(A)_n$ repeats was detected in all three tumors. Cancer T798 revealed alterations in most of two or more nucleotide repeats, whereas the two adenomas showed only a subtle change in one $(CA)_n$ repeat, *i.e.*, no alterations in the trinucleotide and tetranucleotide repeats (Fig. 2; Table 1).

Somatic Mutation of *hMSH3* in an Atypical HNPCC Cancer. When we screened for *hMSH3* mutations in the three tumors from patient H82 by PCR-SSCP, only the rectal cancer (T798) showed alterations at the (A)8 repeat (data not shown). Sequencing revealed that the alteration was heterozygous for a two-A deletion (Fig. 3; Table 1). The two-A deletion is predicted to encode a truncated hMSH3 at codon 402 through a frameshift mutation.

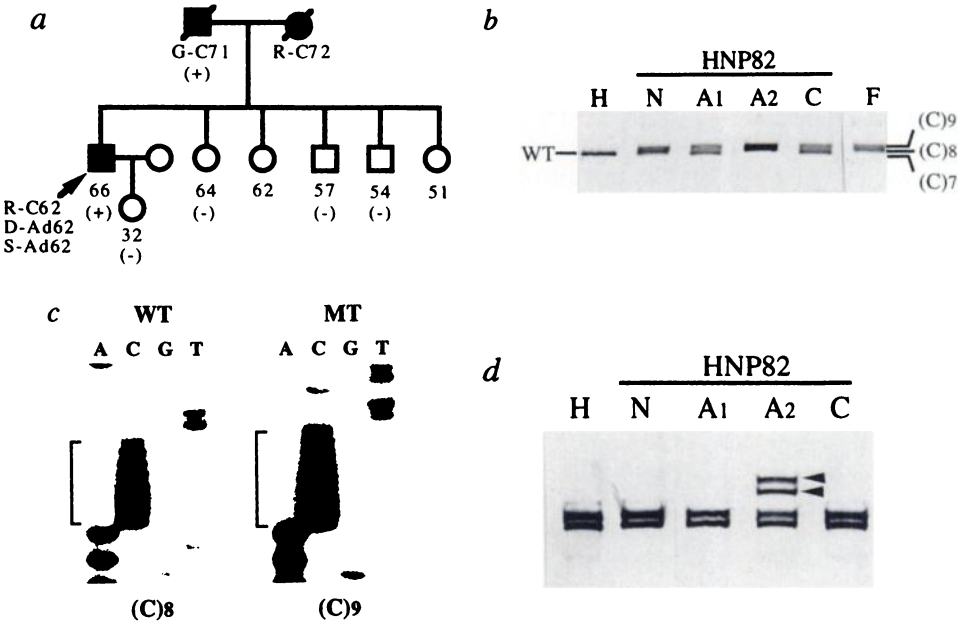

Fig. 1. Detection of *hMSH6* mutations in the atypical HNPCC patient. *a*, pedigree of the atypical HNPCC family with an *hMSH6* germ-line mutation. Patient H82 is indicated by an *arrow*. *Squares*, males; *circles*, females; *filled symbols*, cancer occurred in patients with an *hMSH6* germ-line mutation; *open symbols*, no cancer occurred; *G-C*, gastric cancer; *R-C*, rectal cancer; *D-Ad*, descending colon adenoma; *S-Ad*, sigmoid colon adenoma; *numbers under symbols*, age at cancer or adenoma diagnosis; +, *hMSH6* germ-line mutation positive; −, *hMSH6* germ-line mutation negative. Although the mother had a rectal cancer, as shown by a *shaded symbol*, we could not obtain her specimen. *b*, PCR-SSCP analysis of the (C)8 repeat in *hMSH6* exon 5. Mobility shifts were detected in normal cells from patient H82 (*N*) and his father (*F*), as shown by *(C)9*. Other mobility shifts indicating a somatic mutation were detected in colonic adenoma T796 (*A1*) and rectal cancer T798 (*C*). *H*, healthy individual; *A2*, colonic adenoma T797. *c*, mutant *hMSH6* sequences in normal cells from patient H82. The PCR product containing the (C)8 repeat region was subcloned and then sequenced. *WT*, wild type; *MT*, mutant; *d*, PCR-SSCP analysis of *hMSH6* exon 7 in patient H82; *H*, healthy individual; *N*, normal cells; *A1*, adenoma T796; *A2*, adenoma T797; *C*, rectal cancer T798. Mutant bands are indicated by *arrowheads*.

Table 1 *Summary of the somatic mutations of hMSH6 and MI in the tumors of patient H82*[a]

			hMSH6 somatic mutations				MI[d]				
Tumor no.	Ad/Ca[b]	Site[c]	Exon	Codon	Base change	Amino acid change	Mono	Di	Tri	Tetra	*hMSH3*[e] (A)8
796	Ad	S	5	1085–1087	(C)8 to (C)7	Frameshift	2/2	0/5	0/2	0/2	–
797	Ad	D	7	1214	GAA to GCA	Glu to Ala	2/2	1/5	0/2	0/2	–
798	Ca	R	5	1085–1087	(C)8 to (C)7	Frameshift	2/2	5/5	2/2	1/2	(A)6:8

[a] The germ-line mutation in patient H82 comprised a one-C insertion at the (C)8 repeat in *hMSH6* exon 5.
[b] Ad/Ca, adenoma/cancer.
[c] Sites of tumors: S, sigmoid colon; D, descending colon; R, rectum.
[d] Total number of microsatellite markers with MI/total number of markers examined. Mono, mononucleotide repeats (BAT25 and BAT26); di, dinucleotide repeats (D2S119, D2S123, D3S1029, D10S197, and D13S175); tri, trinucleotide repeats (transcription factor IID and DM-1); tetra, tetranucleotide repeats (D16S310 and D17S846).
[e] Somatic mutation at the (A)8 repeat in *hMSH3* exon 7. (A)6:8, heterozygous for a two-A deletion; –, wild type.

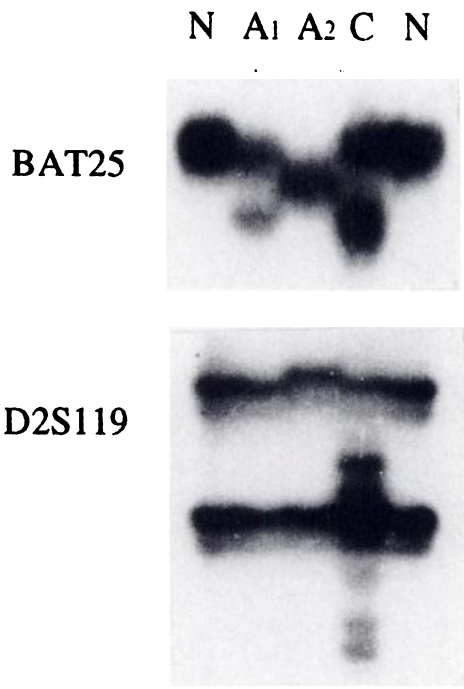

Fig. 2. Representative results of MI in tumors from patient H82. The microsatellite markers BAT25 and D2S119 were used to detect MI. *N*, normal colonic mucosa; *A1*, adenoma T796; *A2*, adenoma T797; *C*, rectal cancer T798.

Discussion

In this study, we found a germ-line mutation of *hMSH6* in an atypical HNPCC patient but not in six classical HNPCC patients satisfying the Amsterdam criteria. Patient H82 and his father, who had rectal and gastric cancer, respectively, were found to have the same frameshift mutation, indicating that the *hMSH6* germ-line mutation segregated with the kindred. Two patients with an *hMSH6* germ-line mutation had rectal or gastric cancer, respectively, at more than 60 years of age. According to the Amsterdam HNPCC criteria, at least one patient who has colorectal cancer should be diagnosed at under 50 years of age (1). Germ-line mutations of *hMSH2* or *hMLH1* are found in most HNPCC patients satisfying the Amsterdam criteria, whereas germ-line mutations of *hPMS1* or *hPMS2* are rare (2). As for patients with sporadic colorectal cancers with MI, it was reported that germ-line mutations of the *hMSH2* or *hMLH1* gene were found in the majority of young patients (less than 35 years of age; Ref. 14). Our data suggest that the tumorigenic tendency of atypical HNPCC patients with an *hMSH6* germ-line mutation might be weaker than those of classical HNPCC and young patients with mutations in *hMSH2* or *hMLH1*.

Loss or somatic mutations of residual wild-type alleles in MMR genes have been found in HNPCC tumors, indicating two-hit inactivation (15, 16). The human colorectal cancer cell line DLD-1 was reported earlier to have a two-hit mutation in *hMSH6* (6). In this study, somatic mutations of *hMSH6* were detected not only in one cancer but also in two adenomas from patient H82. Two and one of the three tumors showed somatic frameshift and missense mutations, respectively, indicating that *hMSH6* shares the two-hit inactivation with *hMSH2* and *hMLH1* and that mutations of it occur at an early stage of tumorigenesis.

Mutations of major MMR genes such as *hMSH2* and *hMLH1* induce hypermutability in microsatellite repeat sequences (2). Frequent MI in dinucleotide repeats was detected in most HNPCC tumors (2), whereas *hMSH6*-defective tumors showed alterations primarily in mononucleotide repeats (6). These data suggested that the function of the hMSH6 protein might be different from those of other MMR proteins. In our study, two adenomas showed alterations in $(A)_n$ repeats, with very few alterations in two or more nucleotide repeats, which is consistent with previous data (6). Interestingly, the rectal cancer from patient H82 showed alterations in most of two and more nucleotide repeat markers.

In biochemical and genetic studies on yeast, *MSH6-MSH3* double mutant cells showed higher mutability in the $(CA)_n$ repeat than *MSH6* mutant cells (11). Such double mutations were also found in some colon tumors and an endometrial cancer cell line with MI, most of which showed frameshift mutations at the (A)8 repeat in *hMSH3* exon

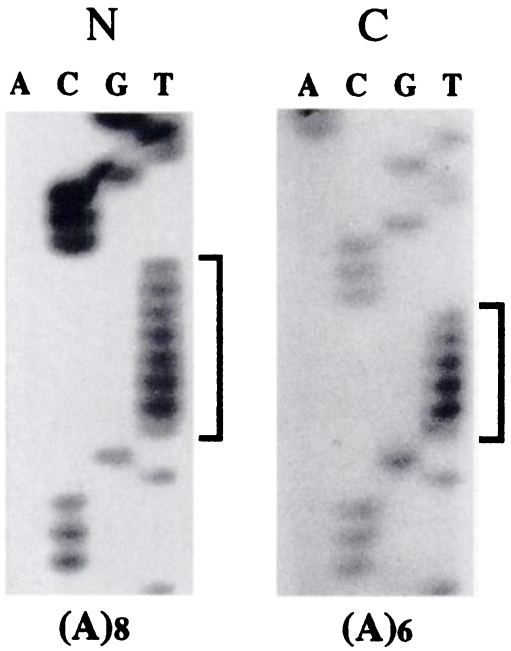

Fig. 3. Sequencing analysis of the *hMSH3* PCR products containing the (A)8 repeat region. The PCR products from normal (*N*) and rectal cancer (*C*) DNA from patient H82 were subcloned and then sequenced. In the case of cancer, only the mutant allele is shown. The sequence should be read in the antisense way.

7 (7, 8). We also found frequent frameshift mutations at the *hMSH3* (A)8 repeat in HNPCC tumors (9). Therefore, mutations at the (A)8 repeat may be strongly associated with MI. Mutations at the (A)8 repeat were found only in a rectal cancer with MI at two or more nucleotide repeats, *i.e.*, not in two adenomas with $(A)_n$ repeat instability, in this study. Our data suggest that mutations in two MMR genes enhance MI and further support the idea that "a mutator mutates another mutator" (17).

Can *hMSH6* mutations cause sufficient MI to result in a predisposition to tumor formation in atypical HNPCC? Mutations in the (A)10 of the *TGF-β RII* gene were reported to be highly related to HNPCC (18). This region was also mutated in sporadic colorectal cancers and cancer cell lines, including DLD-1, with MI (2, 13, 19). Moreover, it was reported that cancer cells reduced malignancy when the wild-type *TGF-β RII* gene was transfected (20). Therefore, the *TGF-β RII* gene (A)10 repeat is a major target of MI, and the *TGF-β RII* gene behaves like a tumor suppressor gene during colorectal cancer development. We previously reported frameshift mutations at the (A)10 repeat as well as additional missense mutations at the kinase domain of the *TGF-β RII* gene in the rectal cancer (T798) and one adenoma (T797) of patient H82 (18). These results, together with the data obtained in this study, indicate that the *TGF-β RII* gene is also a critical target of MI induced by a defective *hMSH6* and suggest that the tumorigenic mechanism in atypical HNPCC tumors may be similar to that in most HNPCC tumors.

In conclusion, a defective *hMSH6* may have been responsible for tumorigenesis in the atypical HNPCC patient. Although the atypical HNPCC kindred did not fulfill the Amsterdam criteria, two patients with an *hMSH6* germ-line mutation in the kindred had tumors related to HNPCC. Several reports have demonstrated that a part of sporadic colorectal cancers exhibited widespread MI (2, 13), some of which were shown to have mutations of *hMSH2* or *hMLH1* (2, 13). When mutations of these MMR genes were not identified in such cancers, it is likely that they may have been associated with *hMSH6* germ-line or somatic mutations.

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