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Loss or Somatic Mutations of *hMSH2* Occur in Hereditary Nonpolyposis Colorectal Cancers with *hMSH2* Germline Mutations

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Hereditary nonpolyposis colorectal cancer (HNPCC) is a major cancer susceptibility syndrome known to be caused by the inheritance of mutations in DNA mismatch repair genes, such as *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2*. To investigate the role of genetic alterations of *hMSH2* in HNPCC tumorigenesis, we analyzed 36 Japanese HNPCC kindreds as to *hMSH2* germline mutations. Moreover, we also examined somatic mutations of *hMSH2* or loss of heterozygosity at or near the *hMSH2* locus in the tumors from the *hMSH2*-related kindreds. Germline mutations were detected in five HNPCC kindreds (5/36, 14%). Among them, three were nonsense mutations, one was a frameshift mutation and the other was a mutation in an intron where the mutation affected splicing. Loss of heterozygosity in four and somatic mutations in one were detected among the eight tumors with *hMSH2* germline mutations. All these alterations were only detected in genomic instability(+) tumors, i.e., not in genomic instability(−) ones, indicating that mutations of *hMSH2* were responsible for at least some of the tumors with genomic instability. These data establish a basis for the presymptomatic diagnosis of HNPCC patients, and constitute further evidence that both DNA mismatch repair genes and tumor suppressor genes may share the same requirement, i.e., two hits are necessary to inactivate the gene function.

Key words: Hereditary nonpolyposis colorectal cancer — *hMSH2* — Germline mutation — Somatic mutation — Loss of heterozygosity

Hereditary nonpolyposis colorectal cancer (HNPCC) is one of the most common cancer predisposition syndromes[1] and is often divided into two major subgroups: hereditary site-specific colon cancer (Lynch syndrome I) and the cancer family syndrome (Lynch syndrome II).[2] Cancer DNA of such patients mostly shows alterations in microsatellite sequences that reflect a malfunction in the replication or repair of DNA, termed genomic instability or replication errors (RER).[3–8] These phenomena led to the cloning of a group of human mismatch repair (MMR) genes, *hMSH2*,[9,10] *hMLH1*,[11,12] *hPMS1*[13] and *hPMS2*,[13] of which germline mutations are responsible for the hereditary susceptibility to colorectal and related cancers. Among them, *hMSH2* seems to be the main causative gene, accounting for at least 40%[14] of HNPCC kindreds.

HNPCC is inherited in an autosomal dominant manner with normal cells from affected individuals containing one functional and one defective allele of one of the MMR genes.[15] Since *in vitro* studies[16] showed that the presence of a single functional copy of *hMSH2* is sufficient for normal development, the transformation from normal tissue to a tumor may require the loss or a mutation of the wild-type allele. Surprisingly, early analysis of DNA from HNPCC tumors did not reveal loss of heterozygosity (LOH) at or near the regions of the MMR genes.[4,17] However, using many more microsatellite markers including an intragenic one, LOH was recently found at or near the *hMLH1* locus in *hMLH1*-related HNPCC families.[18] Meanwhile, a slight excess of LOH was also seen in the region of the *hMSH2* locus in a few patients, though the germline mutations were not determined.[18] These results suggested that *hMSH2*-related loss might also occur as in the case of *hMLH1*.

To examine further the role of *hMSH2* genetic alterations in HNPCC tumorigenesis, we screened a panel of HNPCC from 36 Japanese kindreds for the presence of germline and somatic mutations of the *hMSH2* gene by means of polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) and cycle sequencing. LOH at or near this locus was also examined by means of a number of techniques, including PCR-microallelotyping analysis, PCR-restriction fragment length polymorphism (RFLP), PCR-SSCP, and sequencing.

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MATERIALS AND METHODS

Subjects We collected a total of 36 Japanese HNPCC kindreds. Among them, 12 families had Lynch syndrome I, and 24 Lynch II. Genomic DNA was isolated from peripheral blood karyocytes, Epstein-Barr virus-transformed lymphoblastoid cells and tumor tissues using standard methods.[19]

Reverse transcription (RT)-PCR and PCR mRNA was prepared from lymphoblastoid cell lines using a QuickPrep Micro mRNA purification kit (Pharmacia Biochemicals Inc., Sweden), and cDNA was synthesized with Superscript II (RNase H(−) reverse transcriptase; Life Technologies Inc., Gaithersburg, MD). A longer RT-PCR flanking the region of codons 253 to 784 of the *hMSH2* mRNA sequence (GenBank, #U04045) was applied to screen a large deletion in this area. RT-PCR flanking three conserved coding regions, codons 253 to 352, codons 608 to 705 and codons 684 to 784, of *hMSH2* cDNA was also used for screening. In the case of genomic DNA, we designed six pairs of primers to screen variants in exons 5, 7 and 12–15 of *hMSH2*. The PCR primers for the genomic DNA or cDNA are shown in Table I. PCR comprised 35 cycles of 94°C (1 min), 53–60°C (2 min), and 72°C (1 min) in standard solutions containing 1 μl (20 ng) of genomic or cDNA template, 5 μl of 10× PCR buffer (Wako Chemicals, Osaka), 1 μl of 1.25 m*M* dNTP, 10 pmol of each primer, 0.3 μl of Taq DNA polymerase (5 U/μl) and doubly distilled H_2O, in a total volume of 50 μl.

PCR-SSCP analysis Non-radioisotopic SSCP was performed according to the method described previously.[20] Briefly, PCR products (4 μl) and 11 μl of a denaturing stop solution (95% formamide, 10 m*M* EDTA, 0.25% bromophenol blue and xylene cyanol FF) were denatured for 5 min at 80°C and then cooled rapidly on ice. Electrophoresis was carried out on 12.5% to 15% polyacrylamide gels supplemented with 10% glycerol in a Tris-glycine buffer (25 m*M* Tris/HCl-200 m*M* glycine, pH 8.3) using a commercially available minislab gel (90 ×70×1.0 mm) apparatus (ATTO Co., Ltd., Tokyo). The running conditions were 300 V for 2.5 h at 15°C, and the gels were stained with silver (Silver Stain Kit, Daiichi Co., Ltd., Tokyo).

PCR-microallelotyping analysis for RER or LOH We determined RER at five microsatellite markers containing the (CA)n repeat localized to chromosome arms 2p (*D2S123*,[21] CA21[18]), 3p (*D3S1611*[18]), 10p (*D10S19*[21]), and 13p (*D13S175*[21]). Another microsatellite marker containing the (A)n repeat in *hMSH2* intron 5 was also used.[14] PCR amplification of these microsatellite sequences was performed as described previously.[8, 18, 21] The PCR products were run on 8% to 12.5% polyacrylamide gels and then stained with silver. RER(+) was determined with more than one positive marker. LOH was examined using two markers close to the *hMSH2* locus, i.e., *D2S123*[21] and CA21.[18] LOH was scored when a major reduction or the total loss of one allele could be observed in tumor DNA compared to the corresponding normal tissue sample. In addition to homozygosity in normal tissues, all loci showing the RER phenomenon were scored as uninformative.

PCR-RFLP analysis for mutations or LOH Since some mutations change the restriction enzyme site of the corresponding region, these changes offer the possibility of using RFLP for the confirmation of mutations as well as for the detection of LOH, especially when microallelotyping analysis does not offer information. PCR-RFLP studies were performed using the following restriction enzymes: *Nde* I for patient H110, *Mse* I for patient H129, *Alw*N I for patient H62, and *Eco*N I for patient H10 (Table III). All the enzymes were bought from New England Biolabs (Beverly, MA). The digestion was carried out according to the protocol of the manufacturer. The digested products were run on 10 to 15% polyacrylamide gels and then stained with ethidium bromide.

Sequencing PCR products were purified using a QIA quick-spin PCR purification kit (Qiagen Inc., Chatsworth, CA), and directly sequenced with a cycle

Table I. Genomic and cDNA Primers of *hMSH2* for PCR Amplification and SSCP Analysis

Primer pair	DNA	Size (bp)	Codon	Sense	Antisense
C1	cDNA	296	253–352	5′-GAATAGTGCTGTATTGCCAG-3′	5′-CCATGAGAGGCTGCTTAATC-3′
Exon 5	Genomic	245	265–314	5′-AGTGGCTATAGGAAATCTTC-3′	5′-ACCATTCAACATTTTTAACCC-3′
Exon 7	Genomic	220	359–426	5′-AGATTGAATTTAGTGGAAGC-3′	5′-CAAAATCACTTGTTACCTTC-3′
Exon 12	Genomic	253	587–669	5′-AGGCTATGTAGAACCAATGC-3′	5′-TACCAGTAATGATGTGGAAC-3′
C2	cDNA	296	608–705	5′-TTGCTCACGTGTCAAATGGA-3′	5′-CCACAATGGACACTTCTGC-3′
C3	cDNA	304	684–784	5′-GTGATAGTACTCATGGCCC-3′	5′-AAAATGGGTTGCAAACATGC-3′
Exon 13	Genomic	232	669–737	5′-TT(T/C)TGTAGGCCCCAATATGG-3′	5′-CTAGGAGATGCACTTACCTG-3′
Exon 14	Genomic	290	737–820	5′-TCAGGTAATTATGTGCTTCAG-3′	5′-GTACTCCAATAGTACATACC-3′
Exon 15	Genomic	235	820–878	5′-TGTCTCTTCTCATGCTGTCC-3′	5′-ACTGACAAACCTCTCTTTCC-3′

sequencing kit (Takara, Kyoto) using end-labeled primers and the conditions specified by the manufacturer. The sequencing primers used were: 5′-ATGATTCCAACTTTGGACAG-3′ and 5′-CTGAAGTCAAAAGTAGTCAG-3′ for exon 5; 5′-CAGATCTTAACCGACTTGC-3′ and 5′-CAATCTTGTAAGTTTGCTGC-3′ for exon 7; 5′-TTGCTCACGTGTCAAATGGA-3′ and 5′-ACACAAGCATGCCTGGATG-3′ for exon 12; 5′-GTGATAGTACTCATGGCCC-3′ and 5′-CCACAATGGACACTTCTGC-3′ for exon 13; 5′-ATGGATTTGGGTTAGCATGG-3′ and 5′-AAAATGGGTTGCAAACATGC-3′ for exon 14; for exon 15, the sequencing primers were the same as the primers for PCR amplification.

RESULTS

Mutations of the *hMSH2* gene A total of 15 SSCP variants from 36 normal and 26 tumor DNA samples was initially detected, representative examples being shown in Fig. 1. In patient H62 (kindred 22), a mobility shift was observed in tumor T769 but not in normal cells, indicating a somatic mutation. In patient H129 (kindred 32), a mobility shift was seen in both normal and tumor DNA, which suggested a germline mutation (Fig. 1). Cycle sequencing was performed using the PCR product of each variant as a template. In all cases, sequence alterations could be detected.

Germline mutations were found in five HNPCC families, i.e., kindreds 22, 30, 32, 39 and 41. All of them had Lynch syndrome II except kindred 32 (Lynch I). As for the nature of the mutations, three kindreds (K22, K32 and K41) had nonsense mutations, and one (K30) a

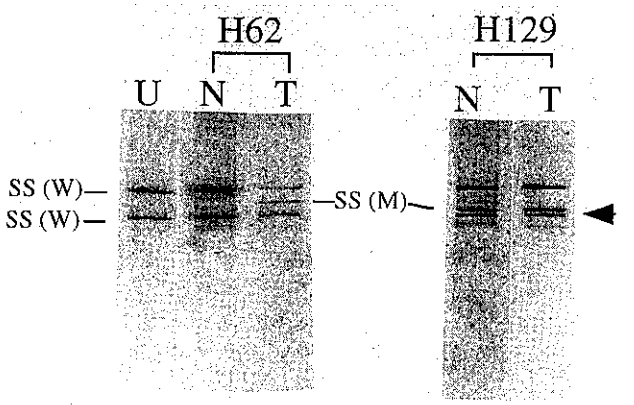

Fig. 1. SSCP analysis of exon 14 in patients H62 and H129. The products shown were obtained from normal cells (N) or tumor tissue (T). U: Unaffected individual. SS (W) and SS (M) indicate a wild-type allele and a mutant-type allele of single-stranded DNA, respectively. Loss of the wild-type allele was also demonstrated in the tumor (T819) of H129, as the arrowhead shows.

Table II. *hMSH2* Alterations in HNPCC Patients

ID[a]	Exon/Intron affected[b]	Nucleotide position[b]	Genomic DNA alteration		cDNA alteration[e]	Designation of mutations[f]	Predicted effects	Role of mutations
			Normal DNA[c]	Cancer DNA[d]				
K22	Exon 7	1255	CAG to TAG	CAG to TAG	ND[g]	Gln419ter	Nonsense	Germline
K41	Exon 12	1857	TAT to TAG	TAT to TAG	ND	Tyr619ter	Nonsense	Germline
K32	Exon 14	2432	TTA to TGA	TTA to TGA	ND	Leu811ter	Nonsense	Germline
K30	Exon 15	2507	TTC to TC	TTC to TC	ND	2507del1	Frame-shift, stop at codon 840	Germline
K39	Intron 5	splicing site	gta to gtt	ND	codons 265–314 deleted	Exon5del	In-frame deletion of 50 amino acids	Germline
T823	Exon 5	934	CTT	CTT to TT	ND	934del1	Frameshift, stop at codon 330	Somatic
T769	Exon 14	2270	TAC	TAC to TAT	ND	Tyr757Tyr	Silent	Somatic
T769	Intron 5	splicing site	gta	gta to gtt	ND	Exon5del	In-frame deletion of 50 amino acids	Somatic
K39	Exon 12	1873	ND	ND	TTG to GTG	Leu625Val	Missense	Polymorphism
K44	Exon 12	1873	ND	ND	TTG to GTG	Leu625Val	Missense	Polymorphism

a) K, kindred number; T, tumor number. T823 is a tumor from patient H10 in kindred 8. T769 is a tumor from patient H62 in kindred 22.
b) Exon, intron and nucleotide positions were reported by Liu *et al.*[14] and Leach *et al.*[10]
c) DNA from lymphoblastoid cells or karyocytes of HNPCC patients.
d) DNA from cancer tissues.
e) cDNA were synthesized from lymphoblastoid cell lines of HNPCC patients.
f) Designation of mutations is according to the nomenclature recommended by Beaudet & Tsui,[25] i.e., an amino acid followed by a codon number designates a missense or nonsense mutation, and a number without a prefix designates the nucleotide positions of deletions (del), then the number of deleted nucleotides follows that number.
g) ND, not done.

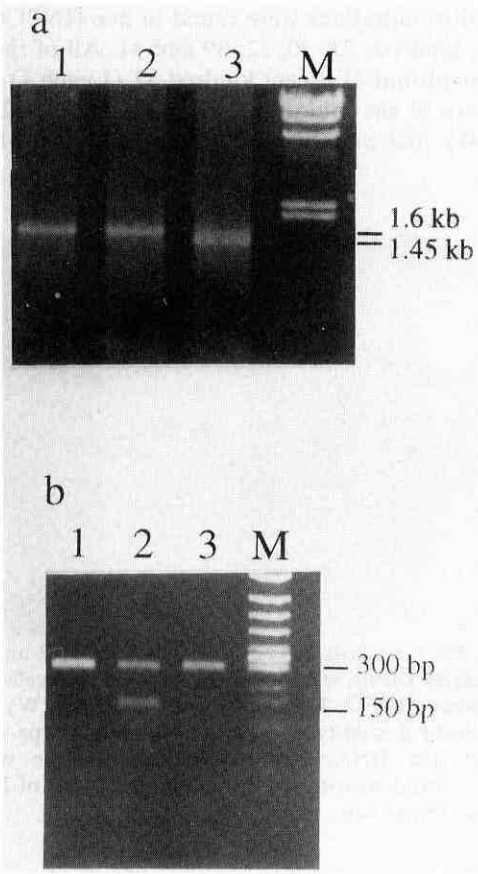

Fig. 2. (a) A longer RT-PCR containing codons 253 to 784 for screening a larger deletion in HNPCC kindreds. An abnormal band was detected in kindred 39 (lane 3). Lanes 1 and 2, kindreds 8 and 13, respectively. M: marker λ/*Hin*d III. (b) RT-PCR amplification of codons 253 to 352. Kindred 39 (lane 2) showed an abnormal 150 bp band. Lanes 1 and 3, kindreds 22 and 25, respectively. M: marker ϕx174/*Hin*c II.

frame-shift mutation. All four were new kinds of germline mutations. One kindred (K39) had a mutation in a splicing site, which was reported before.[14, 22–24] All these germline mutations are summarized in Table II.

Kindred 22 had a nonsense mutation at nucleotide position 1255 of the reported cDNA sequence, and this transition of C to T in codon 419 changed glutamine to a stop codon. In kindred 41, a transversion of T to G occurring in codon 619 changed tyrosine to a stop codon. A substitution of T to G was also detected in kindred 32, that changed leucine to a stop codon in codon 811. Kindred 30 had a 1 bp deletion at nucleotide position 2507, resulting in a stop codon 12 bp downstream from the site of deletion. Kindred 39 had an A to T transversion at the splicing donor site of intron 5, by which an aberrant transcript may be synthesized during the splicing process. In fact, a longer RT-PCR including codons 253 to 784 for screening showed an abnormal transcript in this kindred (Fig. 2a). A product of RT-PCR including codons 253 to 352 indicated that the mutation resulted in the deletion of exon 5 in the transcript (Fig. 2b). Sequencing of this cDNA product further confirmed the complete loss of exon 5 (data not shown).

Three somatic mutations were detected in two tumors of HNPCC kindreds (Table II). The first two mutations occurred in one tumor (T769) of patient H62 (kindred 22) who already carried a Gln419ter germline mutation in one allele. Of these two somatic mutations, one occurred in the splicing site of intron 5, which is the same as the germline mutation in kindred 39, and thus an abnormal transcript with an exon 5 deletion is also expected. The other is a silent mutation in codon 757 that cannot play a functional role in the inactivation. Another mutation occurred in tumor T823 of patient H10, i.e., a 1 bp deletion at nucleotide position 934 that created a

Table III. Germline Mutations, Somatic Mutations and LOH in the RER(+) Tumors from HNPCC Patients

Tumor No.	832	833	834	835	819	769	809	810	823	841	698	608	743	763	765	784	798
Patient No.	H110	H110	H110	H110	H129	H62	H130	H130	H10	H116	H1	H13	H13	H28	H28	H85	H82
Kindred No.	K41	K41	K41	K41	K32	K22	K30	K30	K8	K45	K1	K3	K3	K11	K11	K25	K27
Germline mutations	+[a]	+[a]	+[a]	+[a]	+[b]	+[c]	+[d]	+[d]	UI	UI	UI	UI	UI	UI	UI	UI	UI
Somatic mutations	UI	UI	UI	UI	UI	+[e,f]	UI	UI	+[g]	UI	UI	UI	UI	UI	UI	UI	UI
LOH studies																	
SSCP	—	o	o	o	o	—	—	—	—	NA	NA	NA	NA	NA	NA	NA	NA
RFLP	—	o	o	o	o	—	NA	NA	—	NA	NA	NA	NA	NA	NA	NA	NA
Sequencing	—	o	o	o	o	—	—	—	—	NA	NA	NA	NA	NA	NA	NA	NA
CA 21	NI	NI	NI	NI	NI	—	NI	NI	—	o	—	NI	NI	NI	NI	NI	NI
D2S123	NI	o	o	o	o	NI	NI	NI	NI	—	NI	NI	NI	—	NI	NI	NI

Germline mutations and enzymes for the RFLP study were as follows: *a*) Tyr619ter, *Nde* I. *b*) Leu811ter, *Mse* I. *c*) Gln419 ter, *Alw*N I. *d*) 2507del1, no enzyme site.
Somatic mutations and enzymes for the RFLP study were as follows: *e*) Intron 5, gta to gtt, *Mse* I. *f*) Silent mutation, Tyr757 Tyr, no enzyme site. *g*) 934del1, *Eco*N I.
+, mutation positive; o, LOH positive; —, LOH negative; UI, unidentified; NA, not applicable; NI, not informative, due to a homozygous state and/or RER(+) phenotype.

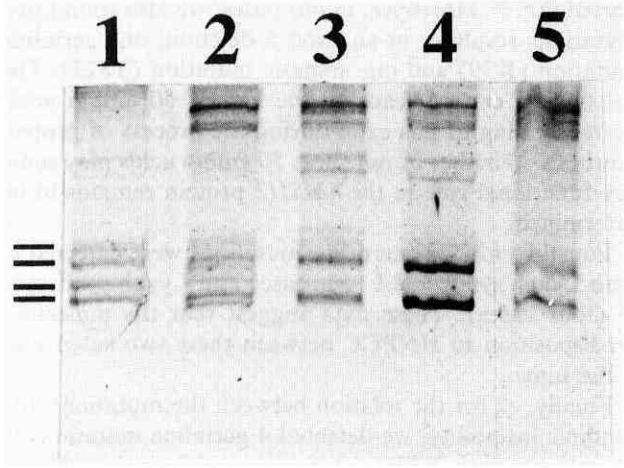

Fig. 3. LOH analysis using microsatellite marker *D2S123* in multiple cancers from patient H110. Normal DNA of patient H110 (lane 1) revealed a heterozygous state at this locus. Tumor T832 (lane 2) showed a RER(+) phenotype, while tumors T833 (lane 3), T834 (lane 4) and T835 (lane 5) showed LOH.

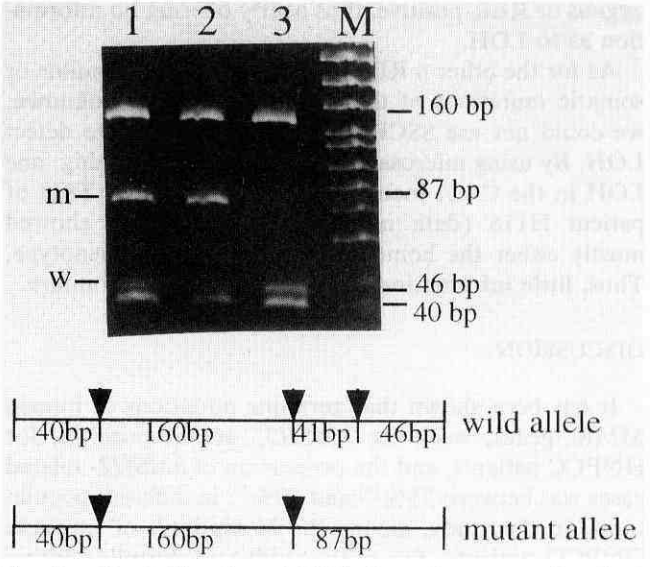

Fig. 4. *Mse* I digestion of DNA from the tumor of patient H129. Both normal (lane 1) and tumor DNA (T819, lane 2) showed a mutant band. Normal cells showed heterozygosity while tumor T819 showed the loss of the wild-type allele. Lane 3, a healthy individual. M: marker ϕx174/*Hin*f I. m, mutant allele; w, wild allele.

stop signal at codon 330. However, the germline mutation in this patient has not yet been identified.

Sequence variants of the *hMSH2* gene Sequencing of two SSCP variants in cDNA products flanking codons 608 to 705 derived from kindreds 39 and 44 showed a T-to-G transversion in codon 625 that changed leucine to valine (Table II). Since the germline mutation that occurred in the splicing site of intron 5 had already been determined in kindred 39, and the Leu625Val change was on the same allele as the intron mutation (using the cDNA sequence in exon 5 as a primer for sequencing), this change might be a polymorphism.

LOH in tumors of HNPCC patients Twenty-six tumors were examined as to LOH at or near the *hMSH2* locus. LOH was detected in 29% (5/17) of RER(+) tumors, but not in any of the RER(−) tumors (0/9). Among the RER(+) tumors, we found LOH in 4 of the 8 (50%) carrying *hMSH2* germline mutations. As for the other nine tumors without identified germline mutations of the *hMSH2* gene, we detected LOH in one (Table III).

In patient H110, there were four independent tumors along the patient's colon, i.e., T832 at the transverse proximal colon, T833 at the transverse distal colon, T834 at the sigmoid colon and T835 at the rectum. As shown in Fig. 3, normal DNA showed a heterozygous state in the *D2S123* locus, T832 showed the RER(+) phenotype, while T833 , T834 and T835 showed LOH. RFLP or SSCP analysis also showed LOH in these tumors. No information was obtained for the CA21 locus since all the tumors were in the RER(+) state at this site. Sequencing of these areas further revealed that, unlike tumor T832, in which the mutation in codon 619 was found to be in a heterozygous state (T/G), the mutation was in a homozygous state containing G in tumors T833, T834 and T835 (data not shown).

In the case of patient H129, since microallelotyping analysis did not offer information as to LOH, we used RFLP for this analysis. A Leu811ter germline mutation in this patient resulted in the loss of one *Mse* I restriction enzyme site, and thus the 46 bp band represented the wild-type allele. As shown in Fig. 4, this wild-type band of tumor T 819 (lane 2) is very faint compared with that in the case of the normal DNA (lane 1) or a healthy individual (lane 3), indicating the loss of the wild-type allele in this tumor. The abnormal SSCP pattern (Fig. 1) also showed that loss of the wild-type allele had occurred. The sequencing data demonstrated that a heterozygous state (T/G) existed in normal DNA, but a homozygous one (one G) in tumor DNA of this patient.

RFLP studies on patients H62 (*Alw*N I) and H10 (*Eco*N I) showed that both wild-type and mutant-type alleles existed in the tumor DNA. In patient H130, RFLP could not be applied for analysis, since the mutation did not alter the restriction site. The SSCP analysis and sequencing data for all tumors of these three patients showed a heterozygous state. Moreover, the locus status of markers CA21 and *D2S123* was mostly either homo-

zygous or RER-positive, thus nearly offering no information as to LOH.

As for the other 8 RER(+) tumors, since germline or somatic mutations of these tumors remained unknown, we could not use SSCP, RFLP or sequencing to detect LOH. By using microsatellite markers for searching, one LOH in the CA21 locus was detected in tumor T841 of patient H116 (data not shown). The others showed mostly either the homozygous or RER(+) phenotype. Thus, little information was obtained on these tumors.

DISCUSSION

It has been shown that germline mutations of human MMR genes, such as *hMSH2*, are responsible for HNPCC patients, and the proportion of *hMSH2*- related cases was between 21%[26] and 40%[14] in different populations. In our study, among the 36 kindreds of Japanese HNPCC patients, five cases (14%) of germline mutations were detected in the 6 exons of the *hMSH2* gene. These exons represent the most conserved part among various species, but occupy about 45% of the whole coding region.[9,10] Since only about a half of the coding region was examined in our study, we could not determine the exact proportion of *hMSH2*-related cases in the Japanese population. However, several interesting points stand out as to germline mutations of the *hMSH2* gene.

Firstly, all five germline mutations in our study were either nonsense mutations (three cases), a frame-shift mutation (one case) or a mutation in the splicing site (one case). These data, combined with the previous reports of *hMSH2* germline mutations,[9,10,14,24,26-28] showed that nearly all germline mutations result in truncation of the predicted protein product. In this respect, the *hMSH2* gene in HNPCC is to some extent similar to the *APC* gene in familial adenomatous polyposis and the *BRCA1* gene in familial breast cancer, in which about 92%[29] and 70%,[30] respectively, of the germline mutations result in loss or premature termination of protein synthesis.

Secondly, in our study, the strategy for detecting *hMSH2* mutations was based on SSCP screening and a longer RT-PCR for detecting a larger deletion of the transcripts. On the other hand, Wijnen *et al.*[26] used denaturing gradient gel electrophoresis (DGGE) for screening. Both SSCP and DGGE are sensitive methods for detecting any mutations, including missense ones. However, the results of both groups showed that all the mutations resulted in truncation of the protein. In this case, an *in vitro*-synthesized protein technique [31] seems to be a faster way to detect any mutation resulting in truncation of the protein.

Thirdly, the deletion of exon 5 is a hot spot mutation. Several cases of such mutations have already been reported.[14,22-24] Moreover, in our cases, we also found two mutations resulting in an exon 5 deletion, one germline mutation (K39) and one somatic mutation (T823). The deletion of exon 5 leads to the loss of 50 amino acids corresponding to this exon during the process of protein synthesis. Whether or not these 50 amino acids play some key functional role in the *hMSH2* protein remains to be determined.

Fourthly, *hMSH2* germline mutations were detected in both Lynch syndrome I (one case) and Lynch syndrome II (four cases). These data suggest that the molecular predisposition to HNPCC between these two subgroups is the same.

Finally, as for the relation between the mutations and genomic instability, we detected 4 germline mutations in 11 patients with RER(+) tumors, while we could not detect such mutations in 9 patients without RER(+) tumors. These results suggest that *hMSH2* mutations are responsible for at least some RER(+) tumors, while there might be a different carcinogenetic process in RER(−) HNPCC cases.

There is compelling evidence that a loss of mismatch repair proficiency is the primary step in the development of HNPCC tumors.[3-8,16,32] However, *in vitro*[16] analysis showed that a germline mutation may not be sufficient for inactivation of the *hMSH2* gene, and hence questions arise regarding the nature and number of the subsequent somatic events. According to the "two hits" scenario,[33] the hereditary form of cancer syndromes might exhibit the same mode. This mechanism has been well illustrated in most tumor suppressor genes in cancer-predisposing syndromes.[34] However, whether or not it is also valid for the MMR genes in HNPCC is unclear.

After the localization of HNPCC susceptibility genes on chromosomes 2p[35] and 3p,[17] several studies involving microsatellite markers for the detection of LOH gave negative results.[4,17] In contrast, the RER(+) phenotype existed in most cases of HNPCC.[3-8] Recently, LOH in the *hMLH1* locus was detected.[18] However, to our knowledge, no finding regarding LOH in the *hMSH2* gene has been reported so far. In our study, at least 50% of the tumors with *hMSH2* germline mutations showed LOH at or near the *hMSH2* locus. These data indicated that LOH can act as the "second hit," inactivating the wild-type allele of the *hMSH2* gene.

The result is not incompatible with the report that showed no LOH at this locus previously, because previous studies involved microsatellite markers that often showed the RER(+) phenotype or a homozygous state.[4,17] In both cases, LOH information could not be gained. In our studies, we also applied SSCP, RFLP and sequencing analysis for detection. On the other hand, using microsatellite markers, only 4/17 (24%) of CA21 and 6/17 (35%) of *D2S123* were informative. For exam-

ple, the CA21 locus showed the RER(+) phenotype in T819, and thus it could not offer LOH information, even though LOH in this tumor was detected with other methods. Additionally, no intragenic markers in *hMSH2* have been reported. Most LOH occurring in *hMLH1* was detected when an intragenic marker on chromosome 3p, *D3S1611*,[18] was used. Therefore, an intragenic marker in the *hMSH2* locus, such as *D3S1611* in *hMLH1*-related cases, may be useful for LOH detection.

Patient H110 is of special interest, because there were four independent colorectal tumors at different sites along the patient's colon simultaneously. LOH at or near the *hMSH2* locus was detected in three of these tumors (T833, T834 and T835), but not in the fourth tumor (T832). These results suggest that the same or very similar independent changes leading to LOH at the same region of the *hMSH2* gene occurred in these three tumors, while the fourth tumor had a different type of inactivating mutation in *hMSH2*.

In our study, 4 of the 8 (50%) RER(+) tumors with *hMSH2* germline mutations showed LOH at the *hMSH2* locus. This proportion is slightly higher than that of LOH at the *hMLH1* locus (44%)[18] in tumors associated with *hMLH1*. These data show that LOH at the *hMSH2* locus is a common mechanism for gene inactivation. LOH was also detected in 1 of the 9 RER(+) tumors without identified germline mutations. In this case, it is more likely that the *hMSH2* gene was the causative gene rather than other MMR genes. In the other 8 tumors, one of the MMR genes other than *hMSH2* may be responsible.

Somatic mutations were detected in two RER(+) tumors (T769 and T823), of which one (T769) exhibited a germline mutation of *hMSH2*. These data, combined with the LOH data mentioned above, indicate that somatic events, either through the loss or mutation of the *hMSH2* gene, can be the second hit for impairment of the mismatch repair system, and lead to the hypothesis that the MMR genes may share the same inactivation mechanism as tumor suppressor genes.

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