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Mutational Analysis of Mismatch Repair Genes, *hMLH1* and *hMSH2*, in Sporadic Endometrial Carcinomas with Microsatellite Instability

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Microsatellite instability, monitored by replication error (RER), has been observed in both sporadic and hereditary types of endometrial carcinoma. In the hereditary tumors, this instability is considered to be caused by a germline defect in the DNA mismatch-repair system. We previously reported that nearly one-quarter of sporadic endometrial carcinomas examined revealed an RER-positive phenotype at multiple microsatellite loci. To investigate the role of genetic alterations of DNA mismatch-repair genes in sporadic endometrial carcinomas, we screened 18 RER(+) endometrial carcinomas for mutations of *hMLH1* and *hMSH2*. Although we found no germline mutations, we detected two somatic mutations of *hMLH1* in a single endometrial cancer; these two mutations had occurred on different alleles, suggesting that two separate mutational events had affected both copies of *hMLH1* in this particular tumor. These data implied that mutations of *hMLH1* or *hMSH2* play limited roles in the development of sporadic endometrial carcinomas, and that the tumors with genetic instability might have alterations of other mismatch-repair genes, such as *hPMS1* and *hPMS2*, or of unknown genes related to the mismatch-repair system.

Key words: Two hits — Mismatch repair gene — *hMLH1* — *hMSH2* — Replication error

Recent studies have demonstrated that alterations in allele sizes at microsatellite loci, caused by replication error (RER), frequently occur in tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC) as well as in sporadic colorectal cancers.[1–3] The hereditary trait of microsatellite instability is considered to be generated by germline mutation in one of the genes involved in the DNA mismatch-repair system.[4–8] Disruption of one of DNA mismatch-repair genes significantly increases RER in prokaryotes and yeast.[9] RER has also been detected in sporadic tumors arising in various human tissues, but at a lower frequency than is found among tumors developed in HNPCC patients.[10–13] The proportion of tumors showing RER among all sporadic endometrial carcinomas is estimated to be approximately 20%.[14,15] The mechanism by which sporadic tumors exhibit the RER(+) phenotype is not well understood. We previously reported observation of RER at two or more loci in 18 of 77 endometrial carcinomas examined among five microsatellite loci, and showed that the frequency of RER tended to be higher in tumors of early onset (<50 of age) than in those of late onset (>60 of age).[16] These results implied that patients who develop endometrial carcinomas with RER(+) may be carrying germline mutations in a DNA mismatch-repair gene. To investigate whether hereditary defects in the DNA mismatch-repair system may have contributed to development of these seemingly sporadic tumors, we analyzed the 18 endometrial carcinomas with RER(+) from the former study for mutations in the two genes most commonly predisposing to HNPCC, *hMLH1* and *hMSH2*.

MATERIALS AND METHODS

Samples Tumors and noncancerous tissues had been obtained at surgery from 18 patients with endometrial carcinomas whose tumor DNAs were subsequently shown to carry the RER(+) phenotype.[16] All tissues were dissected in the operating room, frozen immediately, and stored at −80°C until the DNA was isolated. Genomic DNA was purified as described previously.[17]

Analysis of single-strand conformation polymorphism (SSCP) To screen the *hMLH1* and *hMSH2* genes for variant sequences, SSCP analysis was performed as described by Orita et al.[18,19] Polymerase chain reaction (PCR) primers for amplification of each exon of *hMLH1* were described previously,[20] and primer sets for *hMSH2* are listed in Table I. In this study, each 25-μl reaction mixture contained 1×PCR buffer [6.7 m*M* Tris (pH 8.8), 16.6 m*M* $(NH_4)_2SO_4$, 6.7 m*M* EDTA, 10 m*M* β-mercaptoethanol, 6.7 m*M* $MgCl_2$], 25 pmol of each primer, 50 ng of genomic DNA, 0.2 pmol of each dNTP, 2 μCi of α^{32}P-dCTP (3000 Ci/mmol, 10 mCi/ml), and 0.25 units of Taq DNA polymerase (Boehringer

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Table I. Primers Used for Amplification of All Exons of *hMSH2*

	Sense (5′→3′)	Antisense (5′→3′)	Length (bp)
Exon 1	TCGCGCATTTTCTTCAACCA	TCCCTCCCCAGCACG	284
Exon 2	TTTTTTGAGCAAAGAATCTGC	ACCTTATATGCCAAATACCAATC	162
Exon 3	TTAGGCTTCTCCTGGCAATC	CCTTTCCTAGGCCTGGAATC	332
Exon 4	CTTATTCCTTTTCTCATAGTAGT	TTGTAATTCACATTTATAATCCATG	221
Exon 5	GCTATAGGAAATCTTCGATTTTTA	TACCTGAAAAAGGTTAAGGGCTC	193
Exon 6	TGAGCTTGCCATTCTTTCTATT	TGGGTAACTGCAGTTACATAAA	225
Exon 7	TTCAGATTGAATTTAGTGGAAGC	ACCTTCATGTTTTTCCAGAGC	207
Exon 8	TTTGTTTTACTACTTTCTTTTAGG	AAGTATATTGCATACCTGATCC	148
Exon 9	TAATTTCTGTCTTTACCCATTATTT	CAACCTCCAATGACCCATTC	204
Exon 10	TGGTAGTAGGTATTTATGGAATAC	ATCATGTTTAAGAGCATTTAGGG	264
Exon 11	TACACATTGCTTCTAGTACAC	AGCCAGGTGACATTCAGAAC	202
Exon 12	ATTATTCAGTATTCCTGTGTAC	ACCCCCACAAAAGCCCAAA	326
Exon 13	ATTTATTAGTAGCAGAAAGAAGTT	AAGGGACTAGGAGATGCAC	287
Exon 14	GTTACCACATTTTATGTGATGG	TTCTGAATTTAGAGTACTCC	329
Exon 15	TCTCATGCTGTCCCCTCAC	AAGTTAAACTATGAAAACAAACTG	247
Exon 16	ACTAATGGGACATTCACATGTG	TCAATATTACCTTCATTCCATTAC	232

Mannheim, Mannheim). Reaction mixtures were heated to 94°C for 2 min and then cycled 35 times; each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and strand elongation at 72°C for 30 s. After PCR, 10 μl of reaction mixture was transferred into 40 μl of loading buffer (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were heated at 80°C for 5 min and then quickly cooled on ice. A 2-μl aliquot of each mixture was loaded onto a 6% polyacrylamide gel (ratio of acrylamide/bis-acrylamide, 19:1) containing 50 m*M* Tris-borate (pH 8.3), 4 m*M* EDTA, and 5% glycerol. Electrophoresis was carried out at 16°C, then the gels were dried and subjected to autoradiography for 12–16 h at room temperature.

DNA sequencing PCR products showing aberrant migration patterns on the SSCP gels were cloned into a plasmid vector (pBluescript SK, Stratagene). At least 50 subclones were pooled and DNAs were extracted for a sequencing template. DNA sequences were determined by the dideoxynucleotide-termination method.[21]

PCR of the long DNA fragment To clarify whether both somatic mutations occurred on the same allele, PCR reactions with the Expand Long Template PCR System (Boehringer Mannheim) were performed between exon 1 and exon 2 (3.2 kb) as described elsewhere,[22, 23] with some modifications. The reaction was performed in 50-μl volumes of a mixture containing 1×buffer [50 m*M* Tris (pH 9.2), 16 m*M* $(NH_4)_2SO_4$, 1.75 m*M* $MgCl_2$], 30 pmol of each primer, 350 m*M* of each dNTP, 50 ng of genomic DNA, and 3.5 units each of Taq and Pwo DNA polymerases. Reaction mixtures were heated to 92°C for 2 min and then cycled 30 times in a Perkin Elmer Cetus machine, as follows: cycles 1–10 consisted of 10 s at 92°C for denaturation, 30 s at 65°C for annealing, and 2 min at 68°C for strand elongation; cycles 11–30 consisted of 10 s at 92°C, 30 s at 65°C, and 2 min 20 s to 8 min 40 s at 68°C (the elongation time in each successive cycle was prolonged by 20 s), and 7 min at 68°C for final elongation.

RESULTS

We performed SSCP analysis of the entire coding regions of two mismatch repair genes, *hMLH1* and *hMSH2*, in DNA samples from 18 endometrial carcinomas known to exhibit RER(+) at multiple loci. All DNAs which showed aberrant mobilities on the gels were subcloned and sequenced. Fig. 1A shows SSCP analysis of PCR products amplified from exon 1 of the *hMLH1* gene. A band showing altered mobility was observed, and DNA sequence analysis revealed that this DNA contained a 9-bp insertion at codon 37 (Fig. 1B). In the same DNA sample (patient E-24), a second mutation was detected in exon 2 of *hMLH1* (Fig. 2), i.e., a frameshift caused by a 4-bp deletion (GTGA) at codon 49. As neither of these mutations was observed in DNAs isolated from the normal tissue of this patient, we assume that both the 9-bp insertion and the 4-bp deletion had occurred as somatic events. In this case, the patient's age at diagnosis of endometrial carcinoma was 51 years, and she reported no history of cancer among first- or second-degree relatives. To clarify whether these mutations had occurred on the same or different alleles of *hMLH1*, we amplified the 3.2-kb genomic DNA fragment including both exons 1 and 2, and sequenced each of ten subclones of the PCR products; seven clones contained either the 9-bp insertion (4 clones) or the 4-bp deletion (3 clones), but none had both mutations, suggesting that these two

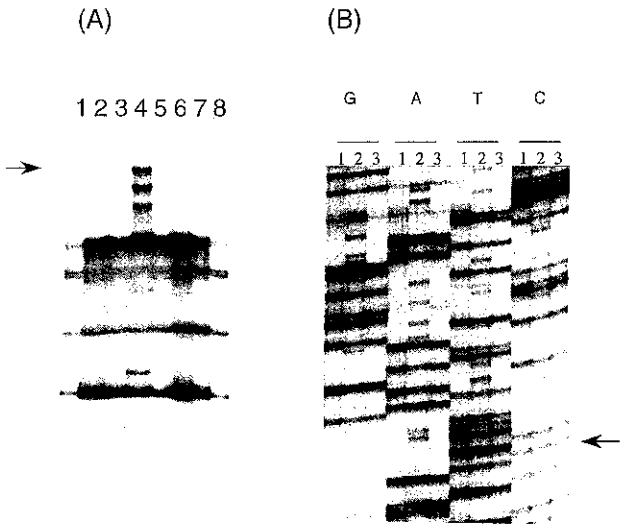

Fig. 1. Mutational analysis in exon 1 of the *hMLH1* gene. (A) SSCP analysis of amplified DNAs from eight of the 18 sporadic RER(+) endometrial carcinomas. A band in lane 4 showing altered mobility is marked with an arrow. (B) Reverse-primer sequence analysis of DNA corresponding to the aberrant band in (A). A 9-bp insertion (CTC AAT CAT) begins at codon 37 in DNA from the tumor (lane 2, at the point marked with an arrow). Lanes 1 and 3, containing constitutional DNAs from this patient (E-24) and from an individual without cancer, respectively, represent the normal *hMLH1* sequence.

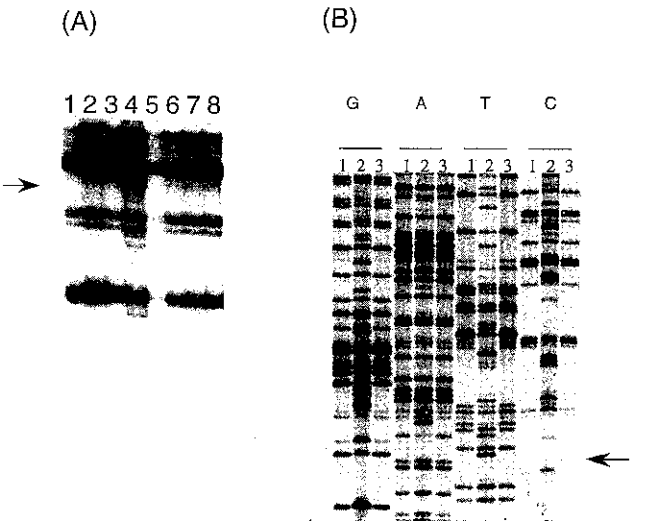

Fig. 2. Mutational analysis in exon 2 of *hMLH1* gene. (A) SSCP analysis of eight tumor DNAs. The amplified DNA of tumor E-24 (lane 4) shows aberrant migration patterns (marked with an arrow). (B) Sequence analysis of DNAs from patient E-24 and an individual without cancer. A 4-bp (GTGA) deletion begins at codon 49 in DNA from tumor tissue (lane 2, at the arrow). Lanes 1 and 3, containing DNA from normal tissue of patient E-24 and the control, respectively, represent the normal *hMLH1* sequence.

mutations had occurred on different alleles (data not shown). Hence, we concluded that both alleles of the *MLH1* gene were inactivated in the tumor tissue. In the course of sequencing other SSCP variants, we found one polymorphism causing an amino-acid substitution (Val to Asp at codon 384 of *hMLH1*) that was observed in 3% of the 70 control individuals tested. One somatic mutation (GGC to GGT at codon 596 in *hMLH1*) that did not affect the amino-acid sequence was also detected.

DISCUSSION

The RER phenotype is associated with deficient strand-specific correction of slipped-strand and base-base mismatches, and is considered to be the result of a genetic mismatch-repair defect.[9] We previously reported that nearly one-quarter of 77 sporadic endometrial carcinomas examined revealed the RER(+) phenotype[16] and suggested that the 18 patients whose tumors were RER(+) might carry germline mutations of genes involved in the mismatch repair system, such as *hMLH1* and *hMSH2*. However, in those 18 patients we found no germline mutations in either of these genes, which are considered to be major predisposing genes for HNPCC, and we detected only two somatic mutations of *hMLH1*, both in a single tumor. Similarly, Liu *et al.*[24] and we (unpublished data) found that sporadic colorectal cancers with RER(+) had no association with germline mutations of the mismatch repair genes; those tumors had acquired somatic changes that presumably did not directly affect cell growth, but resulted only in genetic instability.[24] We have described here two somatic mutations of *hMLH1* in a single endometrial carcinoma, one a 9-bp insertion in exon 1 and the other a 4-bp deletion in exon 2, each on a separate allele. As the former mutation would result in deletion of three amino acids but no frame shift, it is uncertain whether this change would significantly affect the biological activity of the *hMLH1* protein. However, as the 4-bp deletion would result in a frame shift and yield a truncated product, it is certain that this mutation inactivated *hMLH1*. To our knowledge, this is the first report on mutational analysis of mismatch repair genes in sporadic RER(+) endometrial carcinomas.

Recent *in vitro* studies demonstrated that heterozygosity for mutation in a mismatch-repair gene, unlike homozygosity, did not severely affect mismatch repair activity.[9] Hemminki *et al.*[25] reported two mutations of

hMLH1 within some HNPCC tumors, on the basis of evidence that in some cases DNAs from patients with an identified *hMLH1* germline mutation showed loss of heterozygosity of markers within the *hMLH1* gene on chromosome 3p. Although we found only one case of somatic mutation in *hMLH1*, the fact that both alleles were altered is likely to have played a crucial role in development of this tumor. However, we cannot exclude the possibility that its genetic instability may reflect a germline alteration of some other mismatch-repair gene. In either case, our results do imply that the genetic basis of the mechanism causing RER(+) in sporadic endometrial tumors is likely to be different from that in HNPCC.

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