Analysis of candidate target genes for mononucleotide repeat mutation in microsatellite instability-high (MSI-H) endometrial cancer

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Abstract. Microsatellite instability (MSI) is an indicator of DNA instability and is caused by abnormalities in DNA mismatch repair (MMR) genes such as *hMLH1*, *hMSH2* and *hMSH6*. MSI occurs frequently in endometrial cancer (in approximately 30% of cases), and accumulation of gene mutations due to MSI may therefore have a major role in the mechanism of malignant transformation. However, a responsible target gene has not been identified in endometrial cancer. In this study, we analyzed mutations in 11 cancer-related genes with mononucleotide repeats susceptible to MSI in a coding region [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6 in exon 7), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] in 22 patients with MSI-H sporadic endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-βRII* (A10) were found most frequently, at rates of 36.3% (8/22) each. Mutations of *BAX* (G8) and *TCF-4* (A9), which are common in MSI-positive colorectal cancer, occurred at rates of 22.7 and 0%, respectively, which suggests that the MSI target gene may differ between endometrial and colorectal cancers. Mutations in *hMSH6* (C8) were correlated with reduced protein expression (p=0.042) and patients with these mutations had significantly more mutations in mononucleotide repeats in other cancer-related genes compared to patients without *hMSH6* (C8) mutations (p=0.042). This suggests the possibility of a novel cascade in carcinogenesis of endometrial cancer in which MSI mutates *hMSH6* (C8), increases gene instability, and leads to accumulation of mutations in other cancer-related genes. To our knowledge, this is the first report to show that *hMSH6* (C8) has an important role as an MSI target gene in sporadic endometrial cancer.

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Introduction

Microsatellite instability (MSI) is an indicator of genetic instability at the DNA level (1,2). MSI can be evaluated by PCR-based detection of errors in replication of DNA sequences called microsatellites, which consist of repeating units of 1-2 base pairs. MSI has been found in many carcinomas and is particularly common in patients with hereditary non-polyposis colorectal cancer (HNPCC), a familial colon and endometrial cancer that is frequently MSI-positive (3). The mutated genes associated with HNPCC, *hMLH1* (4,5), *hMSH2* (6,7), *hMSH3* (8), *hMSH6* (9,10), *hPMS1* and *hPMS2* (11), are mismatch repair (MMR) genes that repair errors during DNA replication. In HNPCC patients, germline mutations in these genes cause abnormalities in the MMR system, which results in frequent errors in target genes. In addition, approximately 15% of patients with non-hereditary sporadic colon cancer are MSI-positive (3). This may be due to inactivation of the *hMLH1* gene promoter by aberrant hypermethylation, which causes abnormalities in the MMR system similar to that in HNPCC and results in unstable MSI-positive genes (12,13). About 30% of patients with sporadic endometrial cancer are also MSI-positive (14,15) and this may also be due to inactivation of hypermethylated *hMLH1* (16).

In somatic cells, replication errors are likely to occur in DNA regions including repeat sequences. MSI-based mutations accumulate in target genes with repeat sequences, resulting in malignant transformation of cells. In particular, mutation of tumor suppressor genes with a mononucleotide or dinucleotide repeats (repeating unit of one or two base pairs, respectively) may be strongly associated with malignant transformation of cells. Cancer-related genes including mononucleotide repeats (i.e., candidate MSI-target genes) include *TGF-βR II* (17) and *PTEN* (18), which are related to cell growth inhibition; apoptosis-related *BAX* (19) and *Caspase-5* (20); *TCF-4* (21), *EPHB2* (22) and *AXIN2* (23), which are components of the Wnt-signaling pathway; and *HDAC2* (24), which codes for a histone deacetylase. *hMSH3* (25) and *hMSH6* (26), which are MMR genes, and *MBD4* (27), which codes for the methyl-CpG binding protein, also have a mononucleotide repeat sequence and are also candidate MSI-target genes. In MSI-positive sporadic colorectal cancer, mutations of *TGF-βRII* (A10) and *BAX* (G8) have been found in 90% (28) and 45%

Table I. Primer sequences used in gene mutation analysis.

Gene	Repeat	Sense	Antisense
hMSH3	A8	AGATGTGAATCCCCTAATCAAGC	ACTCCCACAATGCCAATAAAAAT
hMSH6	C8	GGGTGATGGTCCTATGTGTC	CGTAATGCAAGGATGGCGT
TGF-βRII	A10	CTTTATTCTGGAAGATGCTGC	GAAGAAAGTCTCACCAGG
MBD4	A10	TGACCAGTGAAGAAAACAGCC	GTTTATGATGCCAGAAGTTTTTTG
BAX	G8	ATCCAGGATCGAGCAGGGCG	ACTCGCTCAGCTTCTTGGTG
PTEN	A6	CCTGTGAAATAATACTGGTATG	CTCCCAATGAAAGTAAAGTACA
HDAC2	A9	ACCTCCGATTCCGAGCTTT	CCGCTCACCGTCGTAGTAGT
EPHB2	A9	CACGAGACGTCACCAAGAAA	CGCAAGAACAGTCATTGCTTT
Caspase-5	A10	CAGAGTTATGTCTTAGGTGAAGG	ACCATGAAGAACATCTTTGCCCAG
TCF-4	A9	GCCTCTATTCACAGATAACTC	GTTCACCTTGTATGTAGCGAA
Axin2	G7	CCTACCCCTTGGAGTCTGC	CAGGGTCCTGGGTGAACA

(29) of cases, respectively, which suggests that MSI plays an important role in malignant transformation in this cancer. However, the mutation frequency of target genes varies between carcinoma types and a responsible MSI-target gene has not been identified in endometrial cancer. Mutation of the tumor suppressor gene *PTEN* has been found in MSI-positive endometrial cancer (18). However, genes including mononucleotide repeats have not been investigated in endometrial cancer.

In this study, we analyzed mutations of 11 cancer-related genes with mononucleotide repeat sequences [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6 in exon 7), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] in MSI-positive sporadic endometrial cancer, in order to identify MSI-target genes that contribute to the pathogenic mechanism of endometrial cancer.

Materials and methods

Clinical specimens. The subjects were 69 patients with endometrial cancer (G1, 32; G2, 17 and G3, 20) who gave informed consent to collection of tissue specimens. Of these patients, 59 had endometrioid adenocarcinoma and 10 had adenosquamous carcinoma. The grade of histological differentiation (G1-G3) and the cancer stage at surgery were determined based on the Guidelines for Endometrial Cancer published by the Japan Society of Obstetrics and Gynecology.

Microsatellite instability (MSI) analysis. Genomic DNA was extracted from normal and tumor tissue samples collected from the 69 patients with endometrial cancer using a Get Pure DNA kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The genomic DNA was PCR amplified at the microsatellite repeat loci D2S123, D5S346, D17S250, BAT26 and BAT25. PCR reactions were performed in a total volume of 25 μl containing 10X buffer, 0.125 mM deoxynucleoside triphosphate, 0.2 μM of each primer and 0.25 Units of TaqDNA polymerase. The PCR conditions were as follows: 94˚C for 10 min; 30 cycles at 94˚C for 45 sec, 58˚C for 45 sec, and 72˚C for 40 sec; followed by a final extension step at 72˚C for 10 min. After PCR, 1 μl of the product was mixed with 12 μl of loading buffer containing formamide and Rox size standards. This mixture was denatured at 95˚C for 2 min and cooled on ice before loading onto an ABI 310 Prism sequencer (Applied Biosystems, Foster City, CA). The results were analyzed using Genescan software (Applied Biosystems). Tumors were classified as MSI-H when ≥30% of the markers showed MSI in accordance with the recent recommendation of the National Cancer Institute Workshop. Tumors in which <30% of the markers showed MSI were included in the MSI-L category. Alteration of even one microsatellite region led to definition of the patient as MSI-positive.

Determination of frameshift mutations of mononucleotide repeats in 11 cancer-related genes. DNA was extracted from tumor tissue from patients with MSI-H endometrial cancer using a Get Pure DNA kit (Dojindo Molecular Technologies). Somatic frameshift mutations in 11 cancer-related genes [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] were determined using two gene-specific oligonucleotide primer pairs designed for PCR amplification of mononucleotide repeat regions. The oligonucleotide primers for sequencing of the 11 genes are shown in Table I. Each mononucleotide region was amplified by PCR using 0.5 μg of template DNA, sense and antisense primers, and an AmpliTaq Gold PCR kit (Applied Biosystems). A 50-μl reaction mixture was prepared according to the manufacturer's instructions and PCR was started at 94˚C for 3 min; followed by 35 cycles of 94˚C for 30 sec, 64˚C or 60˚C for 30 sec, and 72˚C for 1 min; with a final extension step for 5 min. The PCR products were purified using an UltraClean PCR Clean-up kit (Mobio Laboratories, Solana Beach, CA) and subjected to direct sequencing using purified products and the same sets of primers in a capillary automatic sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) software located at the National Center for Biotechnology Information web site (http:/ www.ncbi.nlm.nih.gov).

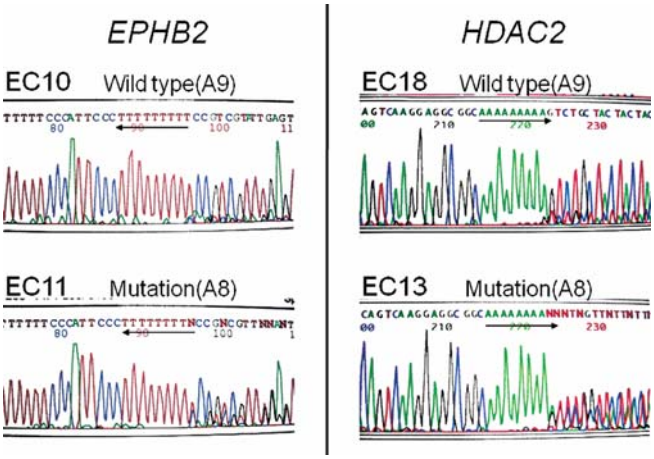

Figure 1. Analysis of mutations in MSI-H endometrial cancer. Frameshift mutations were observed in *EPHB2* (A9) in case EC11 and *HDAC2* (A9) in EC13.

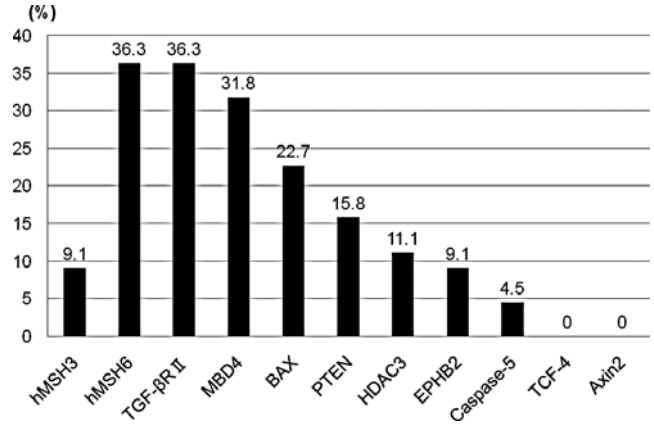

Figure 2. Frequency of mutations in mononucleotide repeats in cancer-related genes in tissue samples from patients with MSI-H endometrial cancer. Mutations in *hMSH6* and *TGF-βRII* were found most frequently (36.3%).

Immunohistochemistry. Immunohistochemical staining was performed on 2-μm sections of formalin-fixed, paraffin-embedded tissues using standard procedures. Slides were cleaned in xylene and dehydrated in graded alcohols. Antigen retrieval was performed with 10-min microwave treatment in 10 mM citrate buffer (pH 7.0). Endogenous peroxidase was blocked by dipping sections in 0.3% H_2O_2 in methanol for 10 min. Slides were incubated with mouse monoclonal antibody to hMSH6 (clone44; BD Transduction Laboratories, San Jose, CA) (1:500) for 90 min at room temperature. Immunostaining was performed by the avidin-biotin-peroxidase complex technique with an Elite ABC kit (Vector Laboratories, Burlingame, CA), using 3,3-diaminobenzidine as a chromogen and H_2O_2. Slides were counterstained with hematoxylin, dehydrated in graded alcohol, dried and cover-slipped. The normal staining pattern for hMSH6 is nuclear, and nuclei in stromal cells were used as internal positive controls. For the purpose of the study, staining of tumor nuclei for hMSH6 was evaluated as positive (+) or negative (-).

Statistical analysis. The association of frameshift mutations in the mononucleotide repeat region of *hMSH6* (C8) in MSI-H endometrial cancer specimens with mutations in the other 10 genes was analyzed using a Mann-Whitney test. The statistical association between mutations in *hMSH6* (C8) and hMSH6 protein expression was analyzed using a Fisher's exact test.

Results

MSI was determined by PCR in 69 patients with endometrial cancer and 22 cases (31.8%) were diagnosed as MSI-H. Mutations in mononucleotide repeats in 11 cancer-related genes [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] were examined in the 22 cases of MSI-H endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-βRII* (A10) were found most frequently, each in 36.3% (8/22) of the cases. For the other genes, the percentages of cases with mutations were 9.1% (2/22) for *hMSH3* (A8), 31.8% (6/18) for *MBD4* (A10), 22.7% (5/22) for *BAX* (G8), 15.8% (3/19) for *PTEN* (A6), 11.1% (2/18) for *HDAC2* (A9), 9.1% (2/22) for *EPHB2* (A9), and 4.5% (1/22) for *Caspase-5* (A10). No mutation was found in the mononucleotide repeat regions of *TCF-4* (A9) or *Axin2* (G7) (Figs. 1 and 2, Table II).

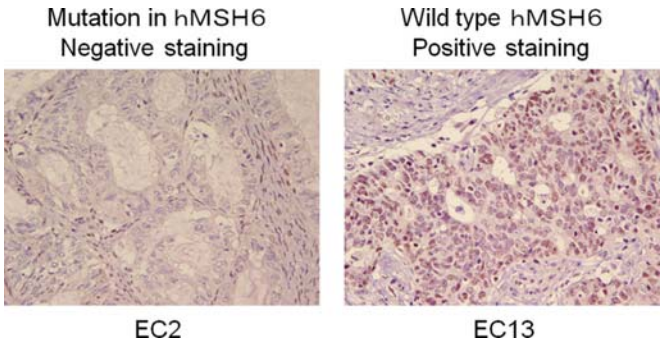

Figure 3. Immunohistochemical analysis of hMSH6 protein in endometrial cancer. Reduced expression of hMSH6 in tumor regions was found in case EC2, which had mutations in *hMSH6*. In contrast, hMSH6 showed clear staining in tumor cell nuclei in EC13, in which there were no mutations in *hMSH6*. In both specimens, normal nuclei surrounding the tumor are normally stained.

Mutations were found most frequently in mononucleotide repeats in *hMSH6* (C8) among the 11 genes that were analyzed. Further analysis in patients with mutations in *hMSH6* (C8) showed a statistically significant tendency for accumulation of mutations in mononucleotide repeats in one or more genes other than *hMSH6* (p=0.012, Tables II and III). Furthermore, tumors with mutations in *hMSH6* showed significant negative immunostaining for hMSH6 protein (p=0.042, Fig. 3, Table IV), indicating that mutations in hMSH6 correlated with reduced *hMSH6* protein expression.

Discussion

Microsatellite instability (MSI) is an indicator of genetic instability at the DNA level. MSI can be evaluated by detecting errors in replication of DNA regions referred to as

Table II. Analysis of mutations in mononucleotide repeats in 11 cancer-related genes in tissue samples from patients with MSI-H endometrial cancer.

Case	hMSH3 A8	hMSH6 C8	TGF-ßRII A10	MBD4 A10	BAX G8	PTEN A6	HDAC2 A9	EPHB2 A9	Caspase-5 A10	TCF-4 A9	Axin2 G7
EC1	-	+	-	+	-	ND	ND	-	-	-	-
EC2	-	+	+	+	-	ND	-	-	-	-	-
EC3	-	+	-	-	-	+	-	-	-	-	-
EC4	-	+	+	ND	-	-	-	+	-	-	-
EC5	-	+	+	ND	+	-	-	-	-	-	-
EC6	-	+	+	+	-	+	-	-	-	-	-
EC7	+	+	+	-	-	+	-	+	-	-	-
EC8	-	+	+	-	+	-	-	-	-	-	-
EC9	-	-	-	-	+	ND	+	-	-	-	-
EC10	-	-	+	-	-	-	-	-	-	-	-
EC11	-	-	+	-	-	-	ND	-	-	-	-
EC12	-	-	-	+		-	ND	-	-	-	-
EC13	-	-	-	-	-	-	+	-	-	-	-
EC14	-	-	-	-	-	-	-	-	-	-	-
EC15	-	-	-	-	-	-	-	-	+	-	-
EC16	-	-	-	-	-	-	-	-	-	-	-
EC17	+	-	-	-	-	-	-	-	-	-	-
EC18	-	-	-	ND	-	-	-	-	-	-	-
EC19	-	-	-	-	-	-	-	-	-	-	-
EC20	-	-	-	ND		-	ND	-	-	-	-
EC21	-	-	-	+	+	-	-	-	-	-	-
EC22	-	-	-	+	+	-	-	-	-	-	-

+, mutated; -, wild-type and ND, not done.

Table III. Association of mutations in *hMSH6* in MSI-H endometrial cancer with mutations in 10 other cancer-related genes (p=0.012, Mann-Whitney test).

	No. of mutations in 10 genes (other than hMSH6)				
	0	1	2	3	4
Mutation in hMSH6	0	2	4	0	2
No mutation in hMSH6	4	7	2	1	0

Statistical analysis was performed by Mann-Whitney test (p=0.012).

microsatellites, which consist of a sequence of repeating units of 1 or 2 base pairs. HNPCC is a familial tumor that is very frequently MSI positive and is probably caused by germline mutations in DNA mismatch repair (MMR) genes that cause abnormalities in the MMR system. This results in frequent replication errors of various target genes followed by malignant transformation. In MSI-positive colorectal cancer, mutations of *TGF-ßRII* and *BAX* tumor suppressor genes are frequently found and these genes are considered to be MSI target genes. *TGF-ßRII* and *BAX* include mononucleotide repeats susceptible to MSI and are likely to be mutated in MSI-positive tumors; therefore, these mutations are suspected to be involved in malignant transformation of cells.

Approximately 30% of MSI-positive endometrial cancer is defined as MSI-H, but a responsible MSI-target gene has not been identified in endometrial cancer. In this study, we analyzed MSI in 69 patients with endometrial cancer and 22 (31.8%) were diagnosed as MSI-H. This result is similar to those in previous studies. Mutations in mononucleotide repeats in 11 cancer-related genes were analyzed in the 22 cases of MSI-H endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-ßRII* (A10) were found most frequently (36.3%), whereas no mutation was found in *TCF-4* (A9) or *Axin2* (G7), which are components of the Wnt-signaling pathway. Mutations in *PTEN* (A6), which has a high frequency of mutations in MSI-positive endometrial cancer, were found in 15.8% of the 22 cases.

TGF-ß inhibits growth of epithelial cells and TGF-ßRII transmits growth inhibitory signals; therefore, a loss of the function of these proteins may lead to malignant transformation of cells. In a previous study, mutations of *TGF-ßRII* (A10) were found in 90% of MSI-positive colorectal cancer, whereas no mutation was found in MSI-negative colorectal cancer, which suggests that *TGF-ßRII* plays an important role in malignant transformation as an MSI-target gene (28). Similarly, mutations of *BAX* (G8), which is involved in apoptosis, have been found in 45% of cases of MSI-positive colorectal cancer and *BAX* is thought to be related to malignant

Table IV. Association of mutations in *hMSH6* in MSI-H endometrial cancer with reduced hMSH6 protein expression.

	Positive	Negative	Total
Mutation in hMSH6	2	6	8
Wild-type in hMSH6	11	1	12
Total	13	7	20

Statistical analysis was performed by Fisher's exact test (p=0.042).

transformation in this cancer as an MSI target gene (29). The mutation rates of *TGF-βRII* (A10) and *BAX* (G8) in MSI-H endometrial cancer have been shown to be 12 and 33%, respectively (29), whereas we found rates of 36.3 and 22.7%, respectively, with these rates being the highest and third highest among the 11 genes analyzed. However, both rates are much lower than those found in MSI-positive colorectal cancer. Mutations in *TCF-4* (A9), a component of the Wnt-signaling pathway, were not found in our specimens, but occur at a frequency of 39% in MSI-positive colorectal cancer (29). This suggests that the frequency of mutations in mononucleotide repeats differs substantially between colorectal and endometrial cancers, and that MSI target genes and the mechanism of malignant transformation may also differ between these cancers.

Mutations in *PTEN* are found in about 60% of cases of MSI-positive endometrial cancer and about 30% of cases of MSI-negative endometrial cancer (30,31). The significantly higher rate in MSI-positive endometrial cancer suggests an association with MSI. Mutations in mononucleotide A repeats in exons 7 and 8 of *PTEN* are found in 27% of cases of MSI-positive endometrial cancer, which suggests that *PTEN* is an MSI-target gene (31), but PTEN mutation patterns vary and another study found mutations in the mononucleotide A repeats in only 3% of cases of endometrial cancers with microsatellite instability (32). The results of our study showed a 15.8% mutation rate for *PTEN* (A6), which was lower than those for *hMSH6* and *TGF-βRII*.

The function of hMSH6 is to detect deletion or insertion of a base pair in a mononucleotide repeat sequence and to initiate repair by forming a complex with hMSH2, hPMS2 and hMLH1. Reduced expression of hMSH6 due to mutation of *hMSH6* damages MMR function and induces MSI, which may result in malignant transformation of cells. Hendriks *et al* investigated families with germline mutations in *hMSH6* and showed that carriers of these mutations had a significantly higher risk of endometrial cancer than carriers of an *hMSH1* or *hMSH2* mutation (33). Furthermore, 69% of cases of endometrial cancer among *hMSH6* mutation carriers were MSI-H and immunohistochemistry showed that 97.5% were negative for hMSH6, indicating reduced expression of *hMSH6* (33). These results suggest that reduced expression of *hMSH6* caused by germline mutation induces MSI and is associated with development of hereditary endometrial cancer. Somatic mutations in *hMSH6* in MSI-positive sporadic endometrial cancer patients have been shown in several studies, but it is unclear if these mutations have an important role (34,35). Goodfellow *et al* found somatic mutations in *hMSH6* in 16 of 60 patients (26.6%) with MSI-H endometrial cancer and frameshift mutations in C8 in 12 of 16 patients with a somatic mutation, but no somatic mutation in *hMSH6* in MSI-negative patients. In the current study, frameshift mutations in *hMSH6* (C8) were found in 36.3% of patients, higher than the rate in Goodfellow *et al*, and immunohistochemical analysis showed that mutation of *hMSH6* correlated with reduced protein expression. These results suggest that mutation in *hMSH6* plays an important role in development of MSI-H sporadic endometrial cancer, similarly to hereditary endometrial cancer.

The current results also showed that patients with mutations in *hMSH6* (C8) had a tendency for accumulation of mutations in mononucleotide repeats in other genes. This tendency was found only in patients with mutations in *hMSH6* (C8). In MSI-positive colorectal cancer, Ikeda *et al* found that mutations in *E2F4* (CAG13), which codes for a transcriptional activator, were often associated with mutations in *hMSH3* (A8), an MMR gene that repairs dinucleotide and trinucleotide repeats, and proposed an interesting hypothesis in which mutations in trinucleotide repeats in *E2F4* are induced by mutations in *hMSH3*, with a subsequent reduction in expression (36). A cascade of malignant transformation with a similar mechanism to this hypothesis may also occur in MSI-H endometrial cancer, with mutations in *hMSH6*, a repair gene for mononucleotide repeats, inducing mutations in tumor suppressor genes that include mononucleotide repeats, such as *TGF-βRII* (A10), *BAX* (G8) and *β* (A6).

Collectively, the results of this study suggest the possibility of a novel cascade in endometrial cancer, in which MSI caused by reduced expression of *hMLH1* due to aberrant hypermethylation (epigenetic change) leads to mutation of *hMSH6* (C8), an MSI target gene, and reduced expression of hMSH6 subsequently increases gene instability and leads to accumulation of mutations in other cancer-related genes (genetic change), resulting in malignant transformation. This is the first study to show that *hMSH6* (C8) has an important role in the mechanism of malignant transformation in MSI-H sporadic endometrial cancer as a target gene, and further studies on the proposed cascade may provide new drugs and preventive approaches for endometrial cancer.

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