研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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REVIEW ARTICLE

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Association of HNPCC and endometrial cancers

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Abstract Hereditary nonpolyposis colorectal cancer (HNPCC) is among the representative familial cancers that are autosomally dominant inherited disorders. Because endometrial cancers develop at high rates in women with HNPCC, it is suggested that some endometrial cancers are familial cancers that are induced by mutations of the DNA mismatch repair (MMR) genes, as in HNPCC. To understand the clinical pathology of familial endometrial cancers that are associated with HNPCC, we surveyed the family histories of 385 patients with endometrial cancer and found that 0.5% of endometrial cancers met the new diagnostic criteria of HNPCC. From molecular and biological analyses, we found microsatellite instability in 30.8% of endometrial cancers and germline mutations of MMR genes in 8.3%. These results suggest a close relationship of MMR gene mutations to the development of endometrial cancers. For a better understanding of the clinical pathology of HNPCC-associated familial endometrial cancers, it is critical for gynecologists to perform a large multicenter study, including detailed family histories.

Key words HNPCC · Endometrial cancer · Revised Amsterdam Criteria · *hMLH1* · *hMSH2*

Introduction

Among gynecological malignant tumors, the incidence of endometrial cancers has increased in Japan in recent years (due to the westernization of our lifestyle and changes in the environment), rising to about 40% of all uterine cancers in Japan. As the number of cases diagnosed and the incidence have increased in Japan and are projected to continue to increase worldwide, the prevention of endometrial cancers is a most important issue for gynecologists. However, the mechanisms of their development and progression remain unclear. It has been assumed that risk factors, such as obesity and high estrogen states, play important roles in the development of endometrial cancers. On the other hand, it has been suggested that genetic factors are closely involved in their development, because multiple cancers occur in endometrial cancer patients, and there are many instances in which family members of endometrial cancer patients also develop cancer. Recently, it was found that endometrial cancer occurred at a high rate in women with hereditary nonpolyposis colorectal cancer (HNPCC; a representative familial tumor inherited as an autosomal dominant trait), showing the relation of familial endometrial cancers with HNPCC.

Hereditary nonpolyposis colorectal cancer (HNPCC) comprises a group of inherited diseases (with high risks of cancers in the colorectum) which are transmitted by autosomal dominant inheritance. In 1913, Wartin et al. reported for the first time the clustered occurrences of colorectal cancers within certain families. After a detailed analysis of the families, the disease concept, "cancer family syndrome", was proposed by Lynch and Krush in 1971.[1,2] In 1991, the International Collaborative Group (ICG) proposed HNPCC clinical diagnostic criteria, called the "Amsterdam Minimum 0Criteria":[3] (1) colorectal cancer (pathologically verified) is diagnosed in at least three relatives. (2) One is a first degree relative of the other two. (3) At least two successive generations are affected. (4) One colorectal cancer is diagnosed before the age of 50 years.

In 1993, it was discovered that HNPCC was inherited through the DNA mismatch repair genes, the so-called DNA mismatch repair (MMR) gene group. The Amsterdam Minimum Criteria were amended by the ICG-HNPCC in 1999 to include the diagnosis of endometrial cancers.[4] Thus, the association of endometrial cancers and

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Table 1. Hereditary nonpolyposis colon cancer (HNPCC) clinical criteria

Amsterdam Minimum Criteria (1991)
1. At least three relatives with colorectal cancer (pathologically verified)
2. One is a first-degree relative of the other two
3. At least two successive generations should be affected
4. One colorectal cancer is diagnosed before the age of 50 years
5. FAP should be excluded
New Amsterdam Criteria (1999)
1. At least three relatives with an HNPCC-associated cancer (cancer of colorectum, endometrium, small bowel, urethra, or renal pelvis)
2–5: Same as for Amsterdam Minimum Criteria

Four types of cancer, including uterine cancer, were added to the HNPCC-associated cancers in the revision made in 1999 (from Banno et al. [2003],[5] with permission)

HNPCC has been recognized internationally, suggesting that, like HNPCC, some endometrial cancers develop from mutations involving one of the MMR genes.

New clinical criteria of HNPCC

After the Amsterdam Minimum Criteria were proposed as the HNPCC clinical diagnostic criteria in 1991, several other clinical standards, such as the Japanese Criteria and the Bethesda Criteria, were established, inviting confusion in the clinical diagnosis of HNPCC from time to time. In 1999, the Amsterdam Minimum Criteria were modified, and the New Amsterdam Criteria were approved by the ICG-HNPCC[5] (Table 1). Of particular note in the New Amsterdam Criteria is that the old diagnostic criteria apply only to colorectal cancers, and the new ones extend to endometrial, intestinal, urethral, and renal cancers. With this revision, cases that do not meet the Amsterdam Minimum Criteria may meet the New Amsterdam Criteria, resulting in an increase in the number of cases that are diagnosed as HNPCC. In addition, it will become possible to identify cases of HNPCC by surveying the family histories of patients with endometrial cancer in detail. On the other hand, there remains a possibility that only a proportion of hereditary endometrial cancers will be identified by the New Amsterdam Criteria, because ovarian, breast, and gastric cancers, which are suggested to be associated with HNPCC, are not included.

Responsible genes in HNPCC

Mutations of DNA in cells are induced by external factors such as radiation and mutagens, as well as by errors during DNA replication. These DNA changes can result in serious consequences, including the carcinogenesis of cells. The DNA repair system provides a mechanism for removing these changes in DNA. One element is the MMR mechanism that detects and repairs errors during DNA, replication, which is carried out by the DNA MMR enzymes. This mechanism was originally studied using *Escherichia coli*, and the DNA MMR genes (*Mut S, Mut L*) were identified. MMR genes were found to be conserved among species, and six kinds of MMR genes (*hMSH2, hMLH1, hMSH3, hMSH6, hPMS1, and hPMS2*) have been identified. These MMR genes were found to be the genes responsible for the development of HNPCC. It is suggested that these MMR genes function in a multisubunit complex in human. During DNA replication, hMSH2 recognizes a mismatch and repairs it in a complex with other MMR proteins. Abnormalities in one to two bases are recognized by the complex of hMSH2 and hMSH6, and two to four base defects and insertions are recognized by the complex of hMSH2 and hMSH3, followed by repair in concert with hMLH1 and hPMS2 further recruited into the complex. These repair mechanisms remain to be defined further.[5]

DNA mismatch repair (MMR) genes and microsatellite instability (MSI)

When there are abnormalities in the MMR genes, mismatch bases generated during DNA replication cannot be corrected, generating DNA chains with different lengths. This phenomenon tends to occur particularly in regions with repeated sequences of several bases in the human genome and is called microsatellite instability (MSI). In the presence of MSI, the incidence of genetic abnormalities in the genes involved in carcinogenesis increases. MSI has been identified in about 10% of all colorectal cancers. It is suggested that about 25% of MSI-positive colorectal cancers are varieties of HNPCC and that MSI analysis could be an effective screening method for HNPCC. Among the six MMR genes, germline mutations of the *hMLH1* gene on chromosome 3 and the *hMSH2* gene on chromosome 2 are suggested to contribute to the majority (about 60%) of HNPCC cases. It remains to be determined if similar mechanisms or genetic abnormalities are involved in hereditary forms of uterine cancer. *TGF-typeIIR*, which is involved in the regulation of cell proliferation, and *BAX*, which is involved in the induction of apoptosis, are reported as candidates for target genes in the MMR mechanism.[6,7] Other candidates for target genes include the *E2F* gene[8,9] and *TCF-4* gene.[10] Abnormalities in these genes occur very infrequently in endometrial cancers, suggesting that target genes that cause an abnormality in the MMR mechanism differ in each organ, and that other specific target genes may be responsible for endometrial cancers.

Clinical characteristics of HNPCC

The exact occurrence rate of HNPCC is unclear due to differences in reports, but it is suggested to be about 5% among all colorectal cancers.[11–17] The clinico-pathological characteristics of HNPCC are reported to include: (1) autosomal dominant inheritance; (2) approximately 85% of

gene penetrance by age 80; (3) young onset; (4) a higher occurrence on the right side of the colorectum; (5) a high frequency of cases of mucinous or poorly differentiated carcinoma; (6) diploidy on cytometric analysis; (7) significant lymphocyte infiltration; (8) MSI positive; (9) high risk of endometrial, urethral, and intestinal cancers; and (10) a favorable prognosis.[18] It is not understood why the prognosis is favorable even though there are many cases of mucinous and poorly differentiated carcinomas, but lymphocyte infiltration into the tumors and a low rate of lymphatic metastasis are suggested to contribute to the favorable prognosis. It has been reported recently that abnormalities of the MMR genes are associated with a decrease in sensitivity to chemotherapy such as cisplatin.[19]

HNPCC-associated gynecological cancers

In regard to patients with HNPCC, the incidence of other cancers in first-degree relatives is highest for endometrial cancers, at 9%–19%, followed by 6%–14% for gastric cancers, and 5%–7% for ovarian cancers.[20] A relation between HNPCC and ovarian cancers is suggested, although it is not as strong as that for endometrial cancers. Recently, a relation has also been suggested for breast cancers. Muir-Torre syndrome, a hereditary disease associated with sebaceous tumors (adenoma, epithelioma, and carcinoma) and malignant tumors of internal organs, is assumed to be transmitted by autosomal dominant inheritance. Among malignant tumors of internal organs, the occurrence of colorectal cancers and urethral/genital organ cancers is high, followed by breast cancers and hematological malignancies. The probability of developing breast cancer is about 12%. Mutations of the *hMSH2* gene, which is an MMR gene and a gene responsible for HNPCC, were reported in 2 two patients with Muir-Torre syndrome.[21,22] It is suggested that this disease is one of the hereditary breast cancers and a subtype of HNPCC.

Molecular epidemiological analysis of HNPCC-associated endometrial cancers

To study the status and clinicopathological characteristics of familial endometrial cancers, the family history and disease history in 385 patients who received treatments for endometrial cancer at our clinic between 1994 and 2002 were studied. After obtaining informed consent from the patients, MSI analysis was performed in 39 of these patients. From the study of family history, 2 of the 385 patients met the New Amsterdam Criteria of HNPCC, and, therefore, about 0.5% of all endometrial cancers were HNPCC-associated tumors[23] (Figs. 1 and 2). The familial cancer clustering in the relatives (890 persons; 439 male and 451 female) of the 39 patients with endometrial cancers was examined. The occurrence of endometrial, colorectal, and ovarian cancers, in which the incidence of HNPCC is high, appeared to be high, suggesting possible involvement of common genetic factors between endometrial cancers and HNPCC.

In MSI analysis using one to five microsatellite markers (Table 2), MSI was identified with at least one marker in 12 of the 39 patients with endometrial cancer (30.8%; Fig. 3).[23] Thus, the occurrence of MSI was significantly

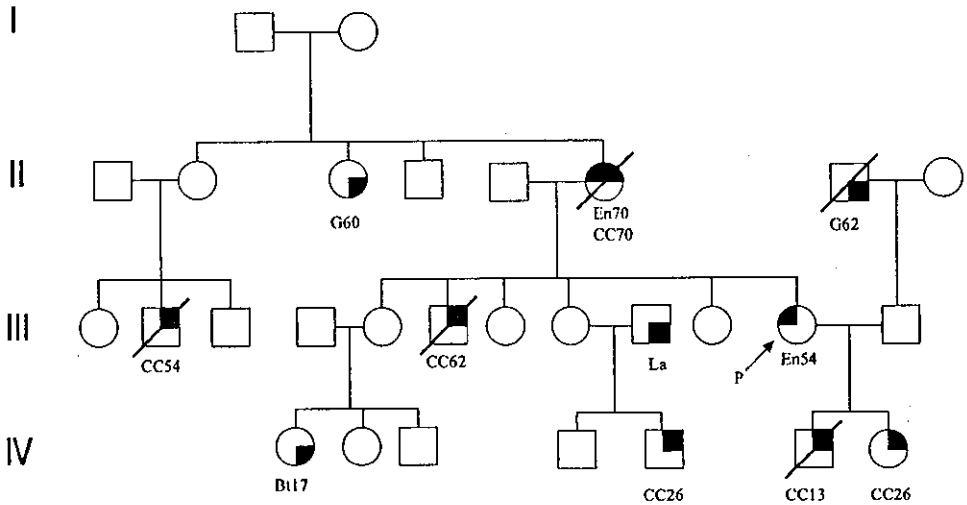

Fig. 1. Pedigree of case A, which meets the New Amsterdam Criteria. The *arrow* shows the proband (*p*) with uterine cancer. ○, female; □, male. ◴ and *En* are uterine cancers. ◔ and *CC* are large-bowel cancers. ◕, other cancers. (*G*, *Bt*, and *La*, respectively, show gastric cancer, brain tumor, and laryngeal cancer.) The *numbers* show the age at the time of diagnosis. The *diagonal lines* show deceased family members. The *Roman numerals on the left* show the generation numbers. Five cases of hereditary nonpolyposis colon cancer (HNPCC)-associated tumors, including the original carrier, were found within the first-degree relatives. The mother of the original carrier had multiple cancers (uterine and large-bowel cancers) (from Banno [2004],[23] with permission)

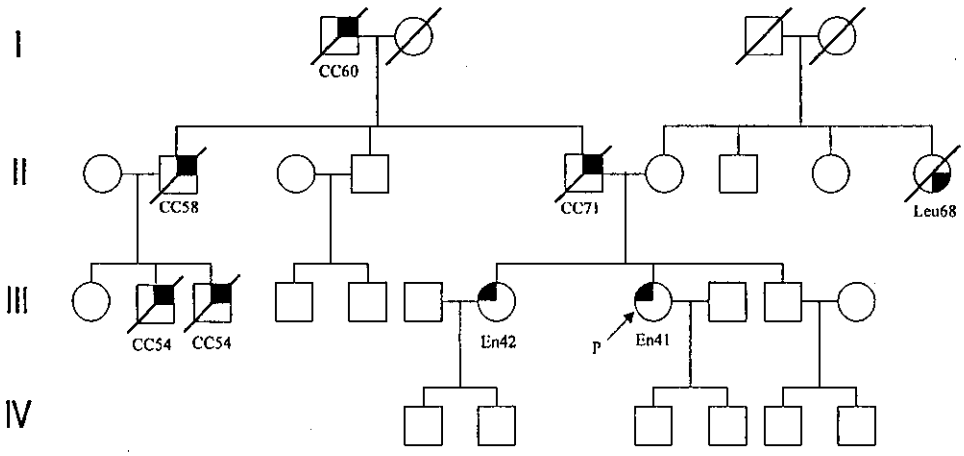

Fig. 2. Pedigree of case B, which meets the New Amsterdam Criteria. The *arrow* is the proband (*p*) with uterine cancer. ○, female; □, male. ◴ and *En* are uterine cancers. ◔ and *CC* are large-bowel cancers. ◶ is other cancers (*Leu*, leukemia). The *numbers* show the age at the time of diagnosis. The *diagonal lines* show deceased family members. The *Roman numerals on the left* show the generation numbers. The original carrier developed uterine cancer at the relatively young age of 41 years. Three cases, including the original carrier of HNPCC-associated tumors, were found within the first-degree relatives. (from Banno et al. [2004],[23] with permission)

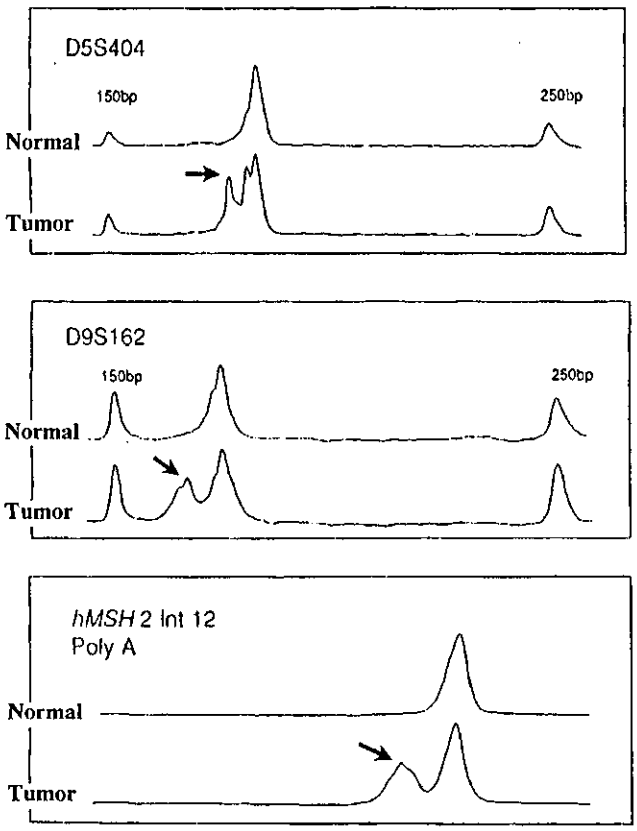

Fig. 3. Microsatellite instability (MSI) analysis in case 1. The *arrow* shows an abnormal wave pattern observed in the tumor-derived DNA. Five areas at the most were analyzed with microsatellite markers. MSI was judged positive when an abnormal wave pattern due to replication error was observed in one area or more *Int, intron* (from Banno et al. [2003],[24] with permission)

higher than that in cancers of other organs, proving that abnormal MMR genes play important roles in the development of endometrial cancers. Statistical analysis of the relationship between MSI-positive endometrial cancers and clinopathological characteristics failed to show a correlation with early onset and multiple cancers, although this was generally observed in familial cancers. On the other hand, histopathological association with tumor differentiation was shown, as in HNPCC, by the significant number of poorly differentiated G2 and G3 adenocarcinomas in MSI-positive endometrial cancers (Table 3). There was no difference in the prognosis of MSI-positive and MSI-negative patients.

In 12 patients with MSI-positive endometrial cancer, germline mutations involving the *hMLH1* and *hMSH2* genes, both of which are DNA MMR genes, were analyzed, after approval by the institutional review board and obtaining informed patient consent (Fig. 4). The results showed germline mutations of the *hMLH1* and *hMSH2* genes in 3 of the 12 patients.[24] Mutation of one base was identified in the *hMSH2* gene in two cases, in which ATG was replaced by ATA at codon 688 (Met to Ile) and CTT was replaced by TTT at condon 390 (Leu to Phe). A nonsense mutation (Arg to stop codon) was identified in the other case, with CGA replaced by TGA at codon 100 of the *hMLH1* gene (Table 4). The mutations of one base in the *hMSH2* gene in two cases were not functional mutations but genetic polymorphisms in Japanese.

In the patient with a nonsense mutation in the *hMLH1* gene, a mucinous adenocarcinoma was present in the ileocecum, and hMLH1 protein expression was decreased in the endometrial and ileocecal cancers. A similar germline mutation was identified in a sister of the patient (Fig. 5). All these results clearly indicate that this is a functional germline mutation. It appears to be the case that germline mutations of the *hMLH1* gene disrupt the MMR mechanism, resulting in carcinogenesis of the endometrium

Table 2. Primer sequences of microsatellite markers used in the present study

Locus	Forward primer sequence	Reverse primer sequence	Size (bp)
D2S123	5′ AAACAGGATGCCTGCCTTTA 3′	5′ GGACTTTCCACCTATGGGAC 3′	197–227
D3S1284	5′ GGAATTACAGGCCACTGCTC 3′	5′ GGAATTACAGGCCACTGCTC 3′	155–177
D5S404	5′ GATCACCACATTCCACCTAAT 3′	5′ GATCACCACATTCCACCTAAT 3′	180–198
D9S162	5′ GCAATGACTTAAGGTTC 3′	5′ GCAATGACCAGTTAAGGTTC 3′	172–196
hMSH2 intron12	5′ GATGTTCCACATCATTACTG 3′	5′ GTGGTTCCACATCATTACTG 3′	182

The primer sequences and polymerase chain reaction (PCR) products are shown for the five different micro-satellite markers used in this study. The *hMSH2* intron 12 is a mononucleotide marker, and the other four markers are dinucleotide markers (from Banno et al. [2003],[24] with permission)

Table 3. Microsatellite instability (MSI) analysis in 39 patients with endometrial cancers

Case no.	Age at operation (years)	Histological type	Grade	Stage	Double cancer	D2	D3	D5	D9	IN
1	58	E	G2	IIIa	–	+	+	+	+	+
2	56	E	G1	Ia	–	+	+	–	+	+
3	55	AS	G2	IIIc	–	+	+	+	–	+
4	55	AS	G3	Ic	OC	+	+	+	+	–
5	60	E	G1	Ib	–	+	–	+	–	+
6	62	E	G2	IIIc	–	+	+	–	+	–
7	55	E	G3	IIIc	–	+	–	+	+	–
8	54	E	G1	Ia	CC	+	+	+	–	–
9	50	E	G1	IIb	OC	–	+	–	+	–
10	56	E	G2	Ic	–	–	–	–	–	+
11	66	E	G1	Ia	–	–	–	–	–	+
12	44	E	G2	IIIc	CC	+	ND	ND	ND	ND
13	59	E	G1	IIa	BC, OC	–	–	–	–	–
14	48	E	G2	Ib	–	–	–	–	–	–
15	58	E	G2	IIIc	–	–	–	–	–	–
16	65	E	G3	IIc	–	–	–	–	–	–
17	72	E	G2	IVb	–	–	–	–	–	–
18	55	E	G1	Ib	–	–	–	–	–	–
19	65	E	G1	Ic	–	–	–	–	–	–
20	56	E	G1	Ib	–	–	–	–	–	–
21	69	E	G1	Ia	–	–	–	–	–	–
22	68	E	G1	Ic	–	–	–	–	–	–
23	34	E	G1	Ib	–	–	–	–	–	–
24	50	E	G1	Ib	–	–	–	–	–	–
25	61	E	G1	Ic	–	–	–	–	–	–
26	57	E	G1	Ic	–	–	–	–	–	–
27	55	E	G1	Ia	BC	–	–	–	–	–
28	64	E	G3	IIIa	–	–	–	–	–	–
29	56	E	G1	Ic	–	–	–	–	–	–
30	44	E	G1	Ib	–	–	–	–	–	–
31	45	E	G1	Ib	OC	–	–	–	–	–
32	53	E	G1	Ib	–	–	–	–	–	–
33	69	E	G1	Ia	CC	–	–	–	–	–
34	81	E	G1	IIIa	–	–	–	–	–	–
35	58	E	G1	Ib	–	–	–	–	–	–
36	60	E	G3	Ic	–	–	–	–	–	–
37	64	E	G1	IIIc	–	–	–	–	–	–
38	66	S	G2	IIIa	–	–	–	–	–	–
39	62	S	G2	IIIa	–	–	–	–	–	–

This table summarizes the results of MSI analysis and clinical pathological information (age at operation, clinical stage, differentiation grade, and presence or absence of multiple cancers) for 39 patients with uterine cancers. MSI-positive tumors were identified in 12 patients (30.8%) (from Banno et al. [2003],[24] with permission)
E, endometrioid adenocarcinoma; AS, adenosquamous carcinoma; S, serous adenocarcinoma; OC, ovarian cancer; BC, breast cancer; CC, colorectal cancer; D2, D2S123; D3, D3S1284; D5, D5S404; D9, D9S162; IN, *hMSH2* intron 12; ND, not done

and ileocecum. This case did not meet the New Amsterdam Criteria, but this case was part of a familial cancer cluster. The fact that this case did not meet the New Amsterdam Criteria suggests the possibility that the clinical criteria may identify only some HNPCC-associated endometrial cancers.

To examine the correlation between MSI and inactivation of one of the DNA MMR genes, immunohistochemical analyses were performed, using anti-hMLH1 antibody in 12 cases of MSI-positive endometrial cancer. A decrease in staining with anti-hMLH1 antibody, indicating a decrease of hMLH1 protein expression, was observed in 8 of 12 cases

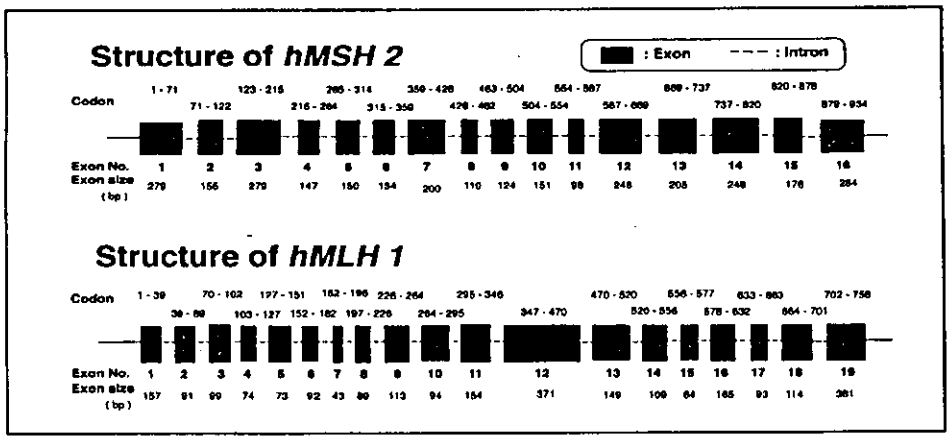

Fig. 4. DNA structures and primer sequences for *hMLH1* and *hMSH2* genes. The DNA structure and the location of the primers for DNA sequencing are shown in the *upper* and *lower pants*, respectively. The *hMSH2* and *hMLH1* genes are composed of 16 and 19 exons, respectively. *PCR*, polymerase chain reaction (from Banno et al. (2003),[24] with permission)

Primer sequences for *hMSH2*

	Sense primer sequence		Antisense primer sequence		Size of PCR products (bp)
Exon1	1a	5' TCGCGCATTTTCTTCAACC 3'	1b	5' GTCCCTCCCCAGCACCG 3'	285
Exon2	2a	5' TTGAACATGTAATATCTCAAATCTGT 3'	2b	5' AAAGGAAGATAATTACCTTATATGC 3'	220
Exon3	3a	5' TCAAGAGTTTGTTAAATTTTTAAAA 3'	3b	5' CTAGGCCTGGAATCTCCTCT 3'	363
Exon4	4a	5' TTCCTTTTCTCATAGTAGTTTAAAC 3'	4b	5' TTGTAATTCACATTTATAATCCATG 3'	216
Exon5	5a	5' CCAGATGGTATAGAAATCTTCG 3'	5b	5' CCATTCAACATTTTTAACCCTT 3'	240
Exon6	6a	5' GCTTGCCATTCTTTCTATTTTATT 3'	6b	5' GCAGGTTACATAAAACTAACGAAAG 3'	214
Exon7	7a	5' CATTAATTCAAGTTAATTTATTTCA 3'	7b	5' CATTAATTCAAGTTAATTTATTTCA 3'	246
Exon8	8a	5' TGAGATCTTTTTATTTGTTTGTTTT 3'	8b	5' TTTGCTTTTTAAAAATAACTACTGC 3'	200
Exon9	9a	5' GGATTTTGTCACTTTGTTCTGTT 3'	9b	5' TCCAACCTCCAATGACCCAT 3'	178
Exon10	10a	5' TGGAATACTTTTTCTTTTCTTCTT 3'	10b	5' GCATTTAGGGAATTAATAAAGGG 3'	235
Exon11	11a	5' ATAAAACTGTTATTTCGATTTGCA 3'	11b	5' CCAGGTGACATTCAGAACATT 3'	164
Exon12	12a	5' TTATTATTCAGTATTCCTGTGTACA 3'	12b	5' CCCACAAAGCCCAAAAACC 3'	325
Exon13	13a	5' ATAATTTGTTTTGTAGGCCCC 3'	13b	5' TTTCTATCTTCAAGGGACTAGGAG 3'	255
Exon14	14a	5' CCACATTTTATGTGATGGGAA 3'	14b	5' CCAATAGTACATACCTTTCTTCACC 3'	307
Exon15	15a	5' GTCCCCTCACGCTTCCC 3'	15b	5' AAACTATGAAAACAAACTGACAAAC 3'	232
Exon16	16a	5' AATGGGACATTCACATGTGTT 3'	16b	5' CCATGGGCACTGACAGTTAA 3'	306

Primer sequences for *hMLH1*

	Sense primer sequence		Antisense primer sequence		Size of PCR products (bp)
Exon1	1a	5' ACATCTAGACGTTTCCTTGG 3'	1b	5' AAGTCGTAGCCCTTAAGTGA 3'	195
Exon2	2a	5' TTTTCTGTTTGATTGCCAG 3'	2b	5' GACTCTTCCATGAAGCGC 3'	162
Exon3	3a	5' TGGGAATTCAAAGAGATTTG 3'	3b	5' CAACAGGAGGATATTTTACAC 3'	211
Exon4	4a	5' GAAGCAGCAGTTCAGCTAAG 3'	4b	5' ATGAGTAAAAGAAGTCAGAC 3'	203
Exon5	5a	5' GGGATTAGTATCTATCTCTCTACTG 3'	5b	5' CAACAATTTACTCTCCATGTAC 3'	158
Exon6	6a	5' GTCAGTGCTTAGAACTGTGCTG 3'	6b	5' TCTCAGAGACCCACTCCCAG 3'	262
Exon7	7a	5' CTAGTGTGTGTTTTTGGCAAC 3'	7b	5' CCTTATCTCCACCAGCAAAC 3'	179
Exon8	8a	5' AATCCTTGTGTCTTCTGCTG 3'	8b	5' TAGGTTATCGACATACCGAC 3'	137
Exon9	9a	5' TTTTGTAATGTTTGAGTTTTGAGTA 3'	9b	5' GTTTCCTGTGAGTGGATTTC 3'	214
Exon10	10a	5' TCCTGAGGTGATTTCATGAC 3'	10b	5' CTGTTCCTTGTGAGTCTTGG 3'	232
Exon11	11a	5' TCCCACTATCTAAGGTAATTG 3'	11b	5' AGAAGTAGCTGGATGAGAAG 3'	231
Exon12	12a	5' CTTATTCTGAGTCTCTCC 3'	12c	5' GGTTTGCTCAGAGGCTGCAG 3'	First PCR 474
	12b	5' CCAGATGGTTCGTACAGATTCC 3'	12d	5' GAGGTAGGCTGTACTTTTCC 3'	Second PCR 240,300
Exon13	13a	5' CACAGAGAAGTTGCTTGCTCC 3'	13b	5' TTGAGCCCTATCATCCCATG 3'	289
Exon14	14a	5' GGGTTGGTAGGATTCTATTAC 3'	14b	5' GGACCATTGTTGTAGTAGCTC 3'	214
Exon15	15a	5' CAACTGGTTGTATCTCAAGC 3'	15b	5' GAAACGATCAGTTGAAATTTC 3'	175
Exon16	16a	5' GCTTGCTCCTTCATGTTCTTG 3'	16b	5' GATTACAGCCATGAGCCACC 3'	278
Exon17	17a	5' GACAGCATTATTTCTTGTTCCC 3'	17b	5' CGAAATGCTTTTTAGTATCTGCTTG 3'	168
Exon18	18a	5' AATTCGGGGTACCTATTTTGAGG 3'	18b	5' ATTGTATAGGCCTGTCCTAG 3'	202
Exon19	19a	5' ACCAGTGTAGGTTGGGATGC 3'	19b	5' AAGAACACATCCCACAGTGC 3'	259

• First PCR : 12a + 12d , Second PCR : 12a + 12c or 12b + 12d

(66.7%) of MSI-positive endometrial cancer. Thus, MSI was strongly associated with negative staining with anti-hMLH1 antibody in endometrial cancers (Fig. 6).[5] These results showed that another mechanism of inactivation of the *hMLH1* gene, possibly including methylation, but not germline mutation, was involved in the MSI in many cases of endometrial cancer, and that immunohistochemical methods could take the place of MSI analysis.

Summary and future directions

It is important to understand the biological characteristics of endometrial cancers, for which the mechanism of carcinogenesis is not fully known. Some endometrial cancers are familial tumors, and abnormalities in the DNA MMR genes are significantly involved in their carcinogenesis. We believe it is very important to identify and analyze these

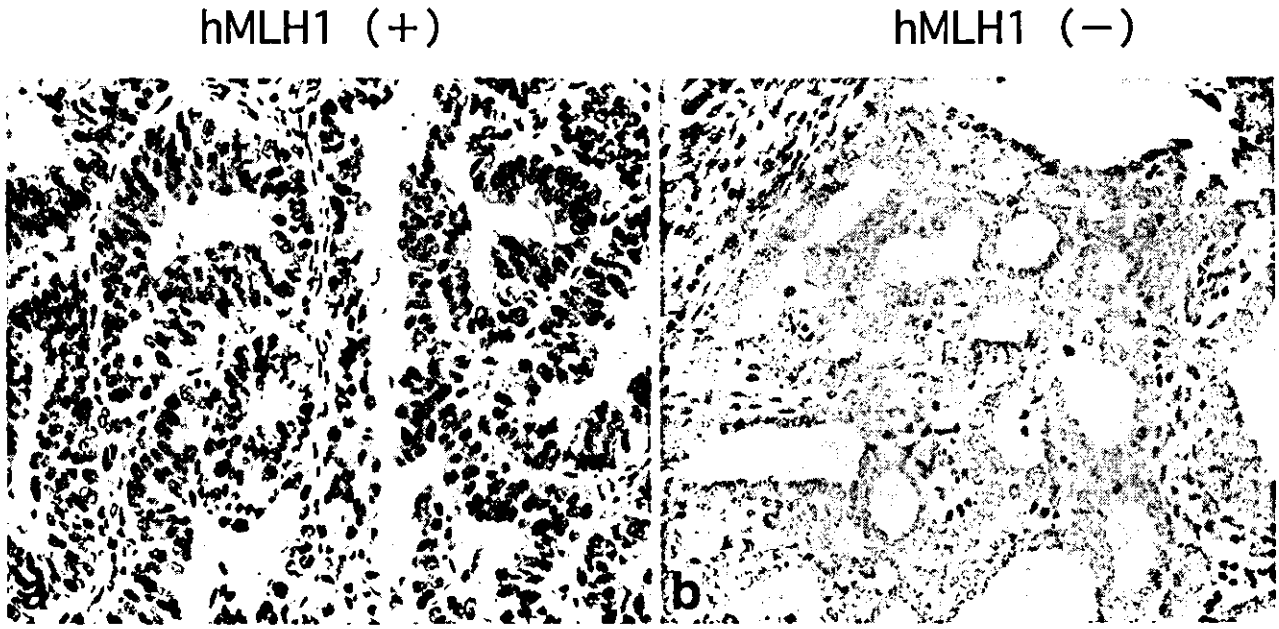

Fig. 5a,b. Reduced expression of hMLH1 protein in uterine cancers (endometrioid adenocarcinona). Immunohistochemical staining with anti-hMLH1 antibody is shown for an MSI-negative case (**a**; case 13; stage IIa, G1) and an MSI-positive case (**b**; case 5; stage Ib, G1). Staining with anti-hMLH1 antibody is absent in the nuclei of endometrial tumors in case 5 (**b**). In both cases, staining is positive in the normal area surrounding tumors. **a** and **b** ×100 (from Banno et al. [2003],[5] with permission)

Table 4. Germline mutation analysis of *hMLH1* and *hMSH2* genes

Case no.	Exon affected	Genomic DNA alteration	Predicted effects (codon)
1	Exon 13 (*hMSH2*)	ATG to ATA	Met (688) Ile
11	Exon 7 (*hMSH2*)	CTT to TTT	Leu (390) Phe
12	Exon 3 (*hMLH1*)	CGA to TGA	Arg (100) Stop

Germline mutation of an MMR gene was detected in 3 out of 12 patients with MSI-positive uterine cancers. Mutations of one base were identified at codon 688 of the *hMSH2* gene in case 1 and at codon 390 in case 11. Both cases were not functional mutations but genetic polymorphisms in Japanese. In case 12, a nonsense mutation was identified at codon 100 of the *hMLH1* gene (from Banno et al. [2003],[24] with permission)

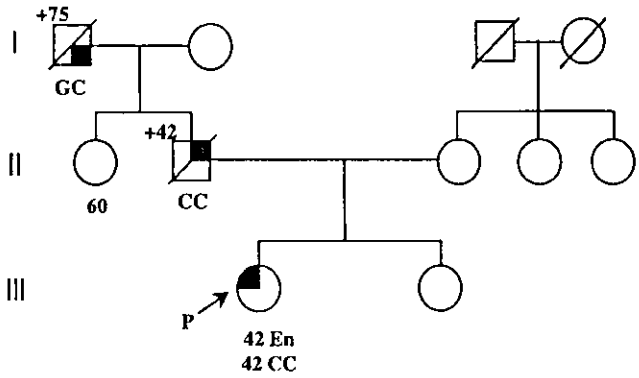

Fig. 6. Pedigree of case 12 with germline mutation in *hMLH1*. This is the family tree of case 12, with a germline gene mutation at codon 100 of the *hMLH1* gene. The *arrow* shows the original carrier of uterine cancer (*p*). ○, female; □, male. ◐ and *En* are uterine cancers, ◔ and *CC* are colorectal cancers. ◒ and *GC* are gastric cancer. The *diagonal lines* show deceased family members. *The numbers* are the ages at onset or death. *The Roman numerals on the left* show the generation numbers (from Banno et al. [2003],[5] with permission)

endometrial cancer cases. However, familial endometrial cancers constitute only about 0.5% of the total, and it is essential to examine family histories in detail. In addition, gynecologists must be accurately informed, and it is important to perform large-scale, multicenter studies nationwide and internationally. Furthermore, there is an urgent need for systems of genetic analysis and genetic counseling of patients. If the significance of surveillance methods and MSI could be understood from pathological studies of HNPCC-associated tumors, new diagnostic and therapeutic methods applicable to all endometrial cancers could be established in the future.

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Int J Gynecol Cancer 2005, 15, 70–80

Diagnostic clinical application of two-color fluorescence *in situ* hybridization that detects chromosome 1 and 17 alterations to direct touch smear and liquid-based thin-layer cytologic preparations of endometrial cancers

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Abstract. Susumu N, Aoki D, Noda T, Nagashima Y, Hirao T, Tamada Y, Banno K, Suzuki A, Suzuki N, Tsuda H, Inazawa J, Nozawa S. Diagnostic clinical application of two-color fluorescence *in situ* hybridization that detects chromosome 1 and 17 alterations to direct touch smear and liquid-based thin-layer cytologic preparations of endometrial cancers. *Int J Gynecol Cancer* 2005;15:70–80.

We performed two-color fluorescence *in situ* hybridization (FISH) on direct touch smears and liquid-based thin-layer (ThinPrep) cytological preparations of endometrial tumors to detect alterations of chromosome 1 and 17 that present with high incidence in endometrial cancers. The DNA probes used for two-color FISH analysis were a combination of the probes designed for 17cen (cCI 17-321) and 17p13.3 (*D17S34*), and a combination of the probes designed for 1q12 (*D1Z1*) and 1p36 (cCI1-5335). Numerical or structural alterations of chromosome 1 and/or 17 were detected in 95% (19 of 20 cases) of the direct touch smears obtained from endometrial cancer, while these alterations were also detected in 93% (12 of 13 cases) of samples obtained from grade 1 endometrioid adenocarcinoma cases, including three cases that could not be diagnosed as positive by conventional Papanicolaou cytopathologic staining. Using ThinPrep cytopathologic preparations, numerical or structural abnormalities were found in 26 (90%) and five (100%) cases, respectively, of samples obtained transcervically from 29 endometrial cancer and five atypical endometrial hyperplasia cases. Therefore, two-color FISH may be a useful diagnostic method for endometrial adenocarcinoma and premalignant lesions that demonstrate only slight cellular atypia in conventional cytopathologic preparations.

KEYWORDS: cytological diagnosis, endometrial cancer, liquid-based cytology, two-color FISH.

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In Japan, endometrial cytology testing has been widely performed for women who present with irregular genital bleeding to test for the possibility of endometrial cancers. Compared to endometrial tissue

biopsies, cytological screening is less painful during patient sampling, and diagnostic results are obtained in a shorter time[1,2]. It has been shown that endometrial cytology examinations are useful for the early diagnosis of endometrial neoplasias[3] and that its sensitivity for endometrial cancers is 70–95%, if both cytologically suspicious cases and positive cases are combined and compared with histologically true-positive cases[1,4–6]. A major drawback in cytology examinations, however, is the difficulty in the diagnosis of grade 1 endometrioid adenocarcinoma cases that present with only slight nuclear atypia and cellular overlapping[1]. Thus, equivocal cases, defined as cases in which the differentiation between benign and malignant is difficult, are relatively common. In addition, interinstitutional differences in the sensitivity of cytological diagnosis, due to the subjective nature of cytological diagnosis, are often cited as problem points in the implementation of cytological screening examinations.

We aimed to develop a new objective method to diagnose endometrial cytology specimens based on alterations in specific chromosomes in cell specimens. Several cytogenetic studies have shown that chromosome 1 is often highly altered[7–9] and that the 1p36 region is often deleted, as detected by two-color FISH[10] in endometrial cancers. In addition, 17p has also been reported to be deleted in 35% of endometrial cancers[11]. Interestingly, p73, a tumor-suppressor gene (p53 homologue)[12], is located on 1p36, while the tumor-suppressor gene p53[13,14] and the HIC-1 (hypermethylation in cancer) gene [15] are located on the 17p13 region. We demonstrate in the current study that two-color fluorescence *in situ* hybridization (FISH) using DNA probes of a centromeric region and of a specific locus in the same chromosome can detect both numerical and structural chromosomal alterations.

In the present study, our initial objective was to detect numerical and structural alterations in chromosome 1 and chromosome 17 in endometrial cancers by two-color FISH. This method was applied to direct imprinted specimens to demonstrate the frequency of these alterations in tumors. Next, this two-color FISH method was applied to endometrial cytology specimens obtained by liquid-based thin-layer ThinPrep method (the ThinPrep slide preparation system, Cytyc Corporation, Boxborough, MA)[16], a method widely used for cervical cancer screening in the United States[17,18]. Fixation in liquid fixative was incorporated in this investigation due to its usefulness in reducing overlap of glandular cells[19].

Materials and methods

Tissue samples

Endometrial tumor tissues were obtained from 45 women who underwent hysterectomies (30 patients) or dilation and curettage (15 patients) at Keio University Hospital, Tokyo, Japan. With respect to histology, the tissue specimens consisted of 30 cases of well-differentiated grade 1 endometrioid adenocarcinoma, six cases of moderately differentiated grade 2 endometrioid adenocarcinoma, and four cases of poorly differentiated grade 3 endometrioid adenocarcinoma in addition to five cases of atypical endometrial hyperplasia complex, a precancerous lesion. All samples were pathologically diagnosed using surgical specimens. As controls, 10 cases of benign endometrial tissue were obtained from women who underwent hysterectomies due to a diagnosis of myoma uteri in addition to 10 cases of benign endometrial tissue obtained by curettage from outpatients. Pathological diagnoses were based on WHO criteria[20]. All tissues were sampled following informed consent from the patients.

As endometrial cancer tissue samples, FISH cytology slides were prepared by direct touch smear of tissue samples from 20 hysterectomy patients followed by fixation with Carnoy's solution and drying. Cytology slides were also prepared by transcervical sampling from nine of these patients just before hysterectomy with a Uterobrush[2] (Medscand, Malmö, Sweden and Teikoku Hormone, Tokyo, Japan), followed by a liquid-based cell suspension thin-layer slide technique. In brief, endometrial cells were collected using a Uterobrush and dispersed in preservation fluid (PreserveCyt, Cytyc Corporation)[18], following removal of debris and red blood. Cells were again dispersed before cell collection onto a filter by negative pressure. The cells on the filter were then transferred to a glass slide by positive pressure using a ThinPrep processor (Cytyc Corporation). In addition, FISH cytology slides were made from transcervical samplings from 10 patients with endometrial cancer undergoing hysterectomy and from 10 patients with endometrial cancers who underwent only dilation and curettages before conservative progestin therapy. In total, 20 direct touch smears and 29 liquid-based smear samples were used as specimens. The majority (>90%) of the constituent cells in each sample were confirmed to be tumor cells by microscopic observations. Similarly, with atypical endometrial hyperplasia complex samples, specimens were obtained from patients undergoing dilation and curettage before conservative

progestin therapy. With benign endometrial tissue samples, cytology slides were prepared by direct touch smears before hysterectomy and by transcervical preparation with liquid-based cytological methods.

DNA probes

The DNA probes used were as follows: plasmid clones pUC1.77 (*D1Z1*; 1q12)[21], p144D6 (*D17S34*; 17p13.3)[22], and cosmid clones cCI-1-5335 (1p36)[23], cCI-17-321 (17 centromere)[24]. Two satellite DNA probes (pUC1.77 and cCI 17-321) were labeled with digoxigenin-11-dUTP, and two locus-specific DNA probes (cCI 1-5335 and p144D6) were labeled with biotin-16-dUTP using a nick translation kit (Boehringer Mannheim, Mannheim, Germany). These labeled DNA probes were precipitated in ethanol with 20 μg each of salmon testis DNA (Sigma Chemical Co., St. Louis, MO) and *Escherichia coli* tRNA (Sigma), dried, and dissolved in 20 μl of formamide.

Procedure for two-color FISH

The combinations of probe DNAs used for two-color FISH are presented in Table 1. The cell samples on glass slides were digested with 0.2–0.5 μg/ml of proteinase K (Merck, Darmstadt, Germany) for 30 min at 37 °C, fixed with 4% paraformaldehyde for 15 min, denatured in 70% formamide/×2 standard saline citrate (SSC) (0.3 mol/l NaCl, 0.03 mol/l sodium citrate) at 75 °C for 2 min, cooled, and dehydrated through an ethanol series. A total of 1 μg of the two DNA probes (Table 1) was mixed at a predetermined ratio with 2.5 μg of Cot-1 DNA (Gibco BRL, Grand Island, NY), denatured at 70 °C for 10 min, cooled on ice, mixed with an equal volume of ×2 SSC/20% dextran sulfate, incubated at 37 °C for 15 min, and hybridized with the samples on glass slides at 37 °C for 12–16 h. After washing, the slides were reacted with avidin-fluorescein isothiocyanate (FITC) and antidigoxigenin rhodamine (Boehringer Mannheim); following a second washing, the slides were counterstained with 4,6-diamidino-2-phenylindole and mounted in antifade solutions[25].

Interpretation of signals in interphase FISH images

Figure 1 shows a simplified schematic interpretation of two-color FISH signals in interphase images in relation to metaphase images. In this schematic representation, a red signal indicates the centromere and a green signal indicates the telomeric locus-specific signal on the short arm. As described previously[26], we categorized the signal patterns of each tumor cell as follows: disomy (D) presents with two centromeric signals and two telomeric signals; disomy with loss of the short arm (D + L) presents with two centromeric signals and one telomeric signal; and polysomy (P) presents with equal numbers of three or more centromeric and telomeric signals. In addition, polysomy with loss of the short arm (P + L) presents with three or more centromeric signals and a lesser number of telomeric signals (eg, 3 to 1, 3 to 2, or 4 to 2). Monosomy (M) presents with one centromeric signal and one telomeric signal, and gain of the short arm (G) presents with more telomeric signals than centromeric signals. We interpreted disomy with loss, monosomy, and polysomy with loss to be deletions of the short arm. The 1p36 or 17p13.3 region was interpreted to be deleted when the sum of the ratios of the tumor cells with a signal pattern of D + L, M, or P + L exceeded the mean plus 3SD value of the sum of the ratios of control endometrial cells with a signal pattern of D + L, M, or P + L. If two or more of the deletions, polisomies, and/or gains of the short arm were interpreted to coexist as significant clones, the category of the highest percentage was assigned to represent the pattern of chromosome 1 or chromosome 17 of the tumor. Overlapping nuclei were excluded from the analysis. Scattered signals or two small neighboring signals were interpreted as a single signal, and any yellow signals were reconfirmed with a single-band pass filter to determine whether it consisted of an overlapping green signal and red signal. The fluorescent signals of 100–200 nuclei were counted through a triple-band pass (D-F-T) filter, a dual-band pass (F-T) filter, an fluorescein isothiocyanate (FITC) filter, and a rhodamine filter (Chroma, Brattleboro, VT) using a fluorescence microscope (model BX50, Nikon, Tokyo, Japan).

Results

Ten benign endometrial samples were used as two-color FISH controls. Table 1 summarizes the percentages of abnormal categories in these benign endometrial glandular cells. Based on the above criteria, the cut-off values were estimated to be 26.5% for the loss of 1p36 and 36.7% for the loss of 17p13.3.

Figure 2 shows representative images of two-color FISH in metaphase spreads of normal lymphocytes after short-term culture. Figure 2A shows two pairs of signals for 1p13.6 (FITC signals) and 1 centromere (rhodamine signals), and Figure 2B shows a single pair of signals for 17p13.3 (an FITC signal) and 17

Table 1. Combinations of DNA probes used for two-color fluorescence *in situ* hybridization (FISH) analyses and their cut-off values calculated from the mean and standard deviation of 10 control specimens

	Chromosome 1 (cCI-1-5335, pUC1.77)							Chromosome 17 (p144D6, cCI-17-321)						
		Percentage of signals							Percentage of signals					
Categories of signal pattern	Number of counted cells	D+L	M	P	P+L	G	Deletion (D+L, M, P+L)	Number of counted cells	D+L	M	P	P+L	G	Deletion (D+L, M, P+L)
1	191	4.2	2.1	2.1	9.4	3.7	15.7	180	11.7	4.4	4.4	15.0	2.8	31.1
2	190	4.2	4.2	6.3	11.6	1.1	20.0	194	10.8	1.5	9.3	16.5	2.1	28.9
3	108	5.6	1.9	6.5	5.6	6.5	13.0	110	6.4	6.4	8.2	9.1	1.8	21.8
4	105	4.8	2.9	6.7	6.7	1.9	14.3	104	8.7	4.8	3.8	4.8	4.8	18.3
5	198	3.0	2.0	7.1	9.1	7.1	14.1	115	7.8	2.6	3.5	7.0	8.7	17.4
6	102	3.9	3.9	9.8	4.9	9.8	12.7	196	8.2	4.1	4.1	6.1	5.1	18.4
7	150	5.3	2.7	3.3	6.7	2.7	14.7	111	11.7	2.7	4.5	13.5	1.8	27.9
8	177	7.3	3.4	6.8	11.9	3.4	22.6	150	12.7	2.7	8.0	2.7	5.7	18.0
9	203	3.9	3.0	6.9	7.9	2.0	14.8	163	9.8	2.5	4.3	11.0	2.0	23.3
10	160	6.3	3.1	6.3	8.1	3.1	17.5	177	7.3	2.3	10.2	5.1	7.0	14.9
Mean		4.9	3.0	6.2	8.1	4.2	16.1		9.8	3.7	6.0	9.1	4.3	21.6
SD		1.18	0.75	2.18	2.49	2.90	3.47		2.70	1.38	2.63	5.62	2.58	5.03
Mean+3SD (cut-off value)		8.4	5.2	12.7	15.6	12.9	26.5		17.9	7.8	13.9	26.0	12.0	36.7

The two-color FISH signals correspond to the patterns of signals shown in Fig. 1. D+L (disomy with loss of the short arm) represents two centromeric signals and one telomeric signal; M (monosomy) represents one centromeric signal and one telomeric signal; P (polysomy) represents equal numbers of three of more centromeric and telomeric signals; P+L (polysomy with loss of the short arm) represents three or more centromeric signals and a lesser number of telomeric signals (eg, 3 to 1, 3 to 2, and 4 to 2); G (gain) represents three or more telomeric signals and a lesser number of centromeric signals (eg, 1 to 3, 2 to 3, and 2 to 4).

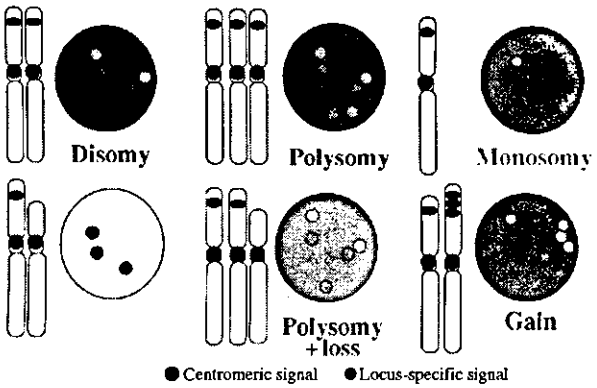

Fig. 1. Interpretation of two-color fluorescence *in situ* hybridization signals in interphase images of endometrial materials in relation to metaphase images.

centromere (a rhodamine signal). The signals were specific, and no cross-reaction was observed.

Figure 3A shows the interphase images of two-color FISH using direct touch smear preparations of normal endometrial tissues hybridized with the chromosome 1 probes. In 10 cases of normal endometrial tissue, no abnormal categories or deletions were detected based on the criteria described in Figure 1 by counting nuclei hybridized with chromosome 1 probes or chromosome 17 probes. In endometrial adenocarcinomas samples, cells with a P+L pattern in specimens hybridized with chromosome 1 probes (Fig. 3B) and cells with a P+L pattern in specimens hybridized with chromosome 17 probes (Fig. 3C) were detected. In specimens hybridized with chromosome 17 probes, cells with a D+L pattern were detected (Fig. 3D).

Analysis of the 20 adenocarcinoma cases demonstrated interpretable FISH data for chromosome 1 in 19 cases with alterations of chromosome 1 in 16 cases (84.2%). Deletions of 1p36 were detected in 11 cases, gains of 1p36 were detected in four cases, and polysomy of chromosome 1 was detected in one case. FISH

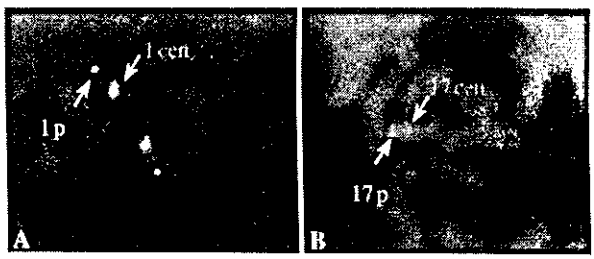

Fig. 2. Metaphase image of two-color fluorescence *in situ* hybridization (FISH). (A) FISH image of a normal lymphocyte hybridized with chromosome 1 probes (original magnification ×4000). (B) FISH image of a normal lymphocyte hybridized with chromosome 17 probes (original magnification ×5000).

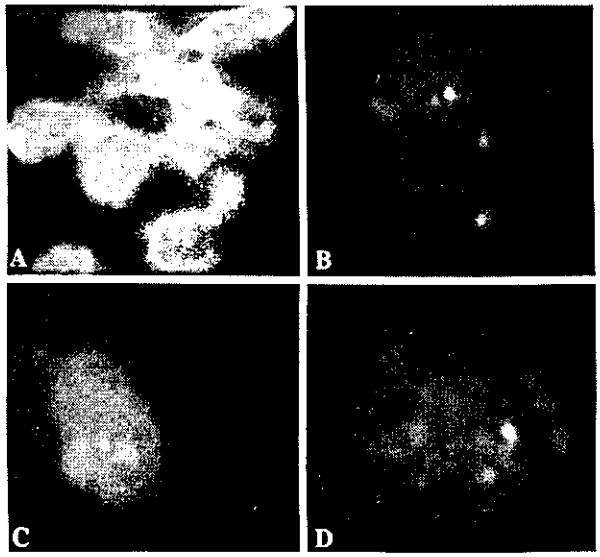

Fig. 3. Interphase images of two-color fluorescence *in situ* hybridization (FISH). (A) FISH image of a normal endometrial cell hybridized with chromosome 1 probes (original magnification ×1000). (B) FISH image of a well-differentiated endometrioid adenocarcinoma cell hybridized with chromosome 1 probes. Three fluorescein isothiocyanate (FITC) signals for the locus on 1p and four rhodamine signals for the centromere of chromosome 1 can be seen (original magnification ×3000). (C) FISH image of a well-differentiated endometrioid adenocarcinoma cell hybridized with chromosome 17 probes. One FITC signal for the locus on 17p and three rhodamine signals for the centromere of chromosome 17 can be seen (original magnification ×3000). (D) FISH image of a well-differentiated endometrioid adenocarcinoma cell hybridized with chromosome 17 probes. One FITC signal for the locus on 17p and two rhodamine signals for the centromere of chromosome 17 can be seen (original magnification ×3000).

data on chromosome 17 were interpretable for 16 cases, with alterations of chromosome 17 in 10 cases (62.5%). Deletions of 17p13.3 were detected in nine cases, and monosomy of chromosome 17 was detected in one case.

Table 2 summarizes the data for chromosome 1 and chromosome 17 alterations in 20 direct touch smear samples of endometrial adenocarcinomas. The two-color FISH signal patterns correspond to the categories of signals shown in the schematic representation in Figure 1. The main abnormal patterns were polysomy with loss of 1p and disomy with loss of 17p. Thus, in 19 of 20 endometrial adenocarcinoma cases (95.0%), either chromosome 1 or 17 showed alterations.

These chromosomal alterations were detected by this two-color FISH method in 12 of 13 grade 1 endometrioid adenocarcinoma cases (92.3%), in all three grade 2 endometrioid adenocarcinoma cases (100%), and in all four grade 3 endometrioid adenocarcinoma

Table 2. The results of two-color fluorescence *in situ* hybridization (FISH) using direct touch smear preparations of endometrial cancer tissues

			Chromosome 1				Chromosome 17			
				Percentage of signals				Percentage of signals		
Case	Grade	Cytology	Number of counted cells	Abnormal pattern	Disomy pattern	Deletion	Number of counted cells	Abnormal pattern	Disomy pattern	Deletion
1	G1	Suspicious	101		D (75)	− (25)	107		D (72)	− (20)
2	G1	Positive	132		D (81)	− (10)	122	M (13)	D (66)	− (19)
3	G2	Positive	120	G (34)	D (53)	− (8)			Not done	
4	G3	Positive	100	G (41)	D (46)	− (8)			Not done	
5	G1	Positive	106	P (42)	D (46)	− (10)			Not done	
6	G2	Positive	104	G (21), P (19)	D (60)	− (1)	121	D+L (50)	D (46)	+ (54)
7	G1	Positive	102		D (78)	− (10)	112	D+L (42)	D (43)	+ (50)
8	G1	Positive	102	G (18), P (15)	D (62)	− (6)	111	D+L (30)	D (58)	+ (42)
9	G2	Positive	Not done				124	P+L (28)	D (20)	+ (76)
10	G1	Positive	135	D+L (20)	D (52)	+ (33)	102	D+L (19)	D (66)	− (28)
11	G3	Positive	110	D+L (35)	D (52)	+ (42)	110	D+L (21)	D (65)	− (27)
12	G1	Negative	102	P+L (23)	D (74)	+ (27)	130		D (58)	− (25)
13	G1	Positive	148	P+L (89)	D (9)	+ (89)	106	D+L (21)	D (63)	− (26)
14	G3	Positive	147	P+L (26)	D (61)	+ (29)	146		D (72)	− (24)
15	G3	Positive	100	P+L (27)	D (52)	+ (27)	105	D+L (26)	D (66)	− (33)
16	G1	Suspicious	109	P+L (27)	D (63)	+ (27)	120		D (68)	− (31)
17	G1	Positive	120	P+L (39)	D (59)	+ (38)	Not done			
18	G1	Positive	148	P+L (81)	D (5)	+ (88)	120	D+L (29)	D (47)	+ (40)
19	G1	Suspicious	102	P+L (73)	D (26)	+ (74)	102	D+L (37)	D (53)	+ (48)
20	G1	Positive	103	P+L (50)	D (40)	+ (53)	118	D+L (31)	D (49)	+ (47)

The two-color FISH signals correspond to the patterns of signals shown in Fig. 1. D, disomy; P, polysomy; M, monosomy; D+L, disomy with loss of the short arm; P+L, polysomy with loss of the short arm; G, gain; G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3.

cases (100%). These results suggest that two-color FISH is a very useful method to diagnose endometrial cancers, showing high sensitivity (95.0%) in direct touch smears.

Table 3 summarizes the results of two-color FISH on ThinPrep specimens obtained by transcervical sampling of endometrial cancers. In all of the 29 adenocarcinomas, interpretable FISH data on chromosome 1 and 17 were obtained. Alterations of chromosome 1 were detected in 22 cases (75.9%), and deletions of 1p36 were detected in 13 cases (44.8%). The main abnormal patterns for 29 cases of adenocarcinoma were polysomy with loss of 1p36 (detected in 12 of 29 cases, 41.4%), gain (G) of 1p36 (detected in seven of 29 cases), and polysomy(P) of chromosome 1 (detected in five of 29 cases). Alterations of chromosome 17 were detected in 24 cases (82.8%), and deletions of 17p13.3 were detected in 17 cases (58.6%). The main abnormal patterns for 29 cases of adenocarcinoma were disomy with loss of 17p13.3 (detected in 22 of 29 cases, 75.9%) and polysomy with loss of 17p13.3 (P+L) (detected in three of 29 cases). Thus, in 26 of the 29 adenocarcinoma cases (89.7%), either chromosome 1 or 17 demonstrated alterations. By grade of differentiation, two-color FISH detected chromosome alterations in 22 of 25 grade 1 adenocarcinoma cases and in all four cases of grade 2 adenocarcinomas. Table 4 summarizes the frequencies of deletion or abnormal signal patterns detected by two-color FISH in chromosome 1 or 17.

Table 5 summarizes the frequency of alterations of chromosome 1 or 17 in ThinPrep specimens from cases that presented with either negative or suspicious cytologic diagnosis by Papanicolaou staining. All specimens were grade 1 endometrioid adenocarcinomas. All the four negative cases demonstrated a D+L pattern of chromosome 17 as summarized in Table 3. Among 10 suspicious cases, seven demonstrated abnormal patterns; these included P+L of chromosome 1 in five cases, D+L of chromosome 17 in seven cases, G of chromosome 1 in one case, and polysomy of chromosome 1 in two cases. Moreover, these chromosomal alterations were detected by two-color FISH in 11 of 14 cases for which cytological diagnosis was rendered as false-negative or equivocal. In atypical endometrial hyperplasia complex cases, alterations of 1p or 17p were detected in all five cases. The main abnormal patterns were P+L of

Table 3. The results of two-color fluorescence *in situ* hybridization (FISH) using transcervical sampling smear preparations of endometrial cancer tissues

			Chromosome 1				Chromosome 17			
				Percentage of signals				Percentage of signals		
Case	Histology and grade	Cytology	Number of counted cells	Abnormal pattern	Disomy pattern	Deletion	Number of counted cells	Abnormal pattern	Disomy pattern	Deletion
1	AEH	Suspicious	120		D (68)	− (16)	115	D + L (18)	D (62)	− (30)
2	AEH	Suspicious	105	P + L (21)	D (53)	− (21)	108	D + L (20)	D (49)	− (30)
3	AEH	Suspicious	110	P + L (20)	D (55)	− (26)	114	D + L (24)	D (53)	+ (40)
4	AEH	Suspicious	121	P + L (26), P (17)	D (51)	+ (32)	103	D + L (26)	D (62)	− (33)
5	AEH	Suspicious	131	P + L (30)	D (53)	+ (42)	120	D + L (22)	D (43)	+ (39)
6	G1	Suspicious	102		D (78)	− (17)	104		D (85)	− (15)
7	G1	Suspicious	103		D (78)	− (7)	116		D (68)	− (30)
8	G1	Suspicious	111		D (85)	− (6)	131		D (79)	− (10)
9	G2	Positive	147	G (45)	D (54)	− (1)	150		D (88)	− (8)
10	G1	Suspicious	110	D + L (9)	D (71)	− (23)	105	D + L (18)	D (71)	− (22)
11	G1	Positive	120	P (21)	D (60)	− (19)	105	D + L (19)	D (71)	− (19)
12	G1	Negative	115		D (85)	− (6)	121	D + L (35)	D (52)	+ (45)
13	G1	Negative	145		D (70)	− (17)	152	D + L (34)	D (42)	+ (41)
14	G1	Negative	104		D (68)	− (22)	108	D + L (30)	D (50)	+ (42)
15	G1	Positive	104		D (59)	− (23)	115	P + L (30)	D (55)	+ (40)
16	G1	Positive	160	G (38)	D (38)	− (24)	117	P + L (35)	D (52)	+ (42)
17	G1	Suspicious	156	G (32)	D (59)	− (6)	141	D + L (28)	D (52)	+ (40)
18	G1	Positive	158	G (22)	D (63)	− (11)	144	D + L (21)	D (55)	+ (40)
19	G2	Positive	122	G (35)	D (51)	− (6)	126	D + L (40)	D (51)	+ (49)
20	G1	Positive	104	G (32)	D (63)	− (4)	107	D + L (28)	D (54)	+ (46)
21	G2	Positive	100	G (30)	D (60)	− (6)	105	D + L (51)	D (29)	+ (71)
22	G2	Positive	145	P + L (27)	D (62)	+ (27)	147		D (82)	− (7)
23	G1	Positive	104	D + L (28)	D (58)	+ (32)	103	D + L (22)	D (68)	− (31)
24	G1	Positive	132	P + L (28)	D (58)	+ (35)	128	D + L (26)	D (65)	− (28)
25	G1	Suspicious	166	P + L (23)	D (60)	+ (31)	145	D + L (23)	D (56)	− (29)
26	G1	Positive	133	P + L (32)	D (55)	+ (40)	148	D + L (21)	D (69)	− (25)
27	G1	Suspicious	122	P + L (25), P (13)	D (56)	+ (31)	119	D + L (26)	D (60)	− (32)
28	G1	Positive	108	P + L (36)	D (56)	+ (42)	103	D + L (23)	D (57)	+ (38)
29	G1	Positive	102	P + L (30)	D (64)	+ (35)	147	D + L (28)	D (54)	+ (39)
30	G1	Suspicious	125	P + L (23)	D (50)	+ (30)	105	D + L (35)	D (55)	+ (41)
31	G1	Negative	133	P + L (23), P (20)	D (45)	+ (32)	109	D + L (30)	D (57)	+ (40)
32	G1	Positive	110	P + L (28), P (13)	D (50)	+ (35)	131	D + L (34)	D (51)	+ (42)
33	G1	Suspicious	122	P + L (23), P (17)	D (53)	+ (27)	128	D + L (30)	D (58)	+ (39)
34	G1	Suspicious	101	P + L (28)	D (53)	+ (40)	109	P + L (27), D + L (18)	D (45)	+ (52)

The two-color FISH signals correspond to the patterns of signals shown in Fig. 1. D, disomy; P, polysomy; M, monosomy; D + L, disomy with loss of the short arm; P + L, polysomy with loss of the short arm; G, gain; AEH, atypical endometrial hyperplasia complex; G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2.

Table 4. Frequency of deletion or abnormal signal patterns detected by two-color fluorescence *in situ* hybridization with ThinPrep method obtained by transcervical sampling of endometrial carcinomas

	1p	17p	1p or 17p
Deletion	13/29 (44.8%)	17/29 (58.6%)	23/29 (79.3%)
Expression of abnormal categories	22/29 (75.9%)	24/29 (82.8%)	26/29 (89.7%)

Table 5. Relation between deletion or abnormal signal patterns in 1p or 17p detected by two-color fluorescence *in situ* hybridization and cytological diagnosis of endometrial cancers

	Endometrial cytology		
	Negative	Suspicious	Positive
Deletion	4/4 (100%)	6/10 (60%)	13/15 (87%)
Expression of abnormal categories	4/4 (100%)	7/10 (70%)	15/15 (100%)

1p36 (detected in four of five cases) and D+L of 17p13.3 (detected in five of five cases).

Discussion

The pathological differentiation of atypical endometrial hyperplasia complex from well-differentiated (grade 1) endometrioid adenocarcinoma is often confusing and lacks consensus in diagnostic criteria even among pathologists who specialize in the field of gynecological malignancies. Some pathologists emphasize nuclear atypia, such as enlarged nuclei, increased nuclear chromatin, enlarged nucleoli, irregular thickening of the nuclear membrane, irregular intranuclear distribution of chromatin, and frequent mitotic figures, as well as other nuclear features, whereas other pathologists place greater emphasis on architectural abnormalities such as the degree of crowded glands, irregular contour of glands, high degree of pseudostratification of glandular cells, as well as other architectural features. The criteria advocated by Blaustein[27] places the greatest diagnostic emphasis on stromal invasion such as cribriform pattern growth in the differential diagnosis between grade 1 endometrioid adenocarcinomas and atypical endometrial hyperplasia complex. However, uniform diagnostic criteria to cytologically discriminate the two diseases have not been firmly established based on conventional Papanicolaou staining.

Recently, novel methods for distinguishing endometrial carcinoma from nonmalignant endometrial lesions have been reported. Langer *et al.*[28] reported that transvaginal ultrasonography had 90% sensitivity for detecting endometrial cancer, but that the specificity was only 48% and that some difficulties remained concerning the discrimination of endometrial cancer and endometrial hyperplasia using various cut-off criteria. Simil3ar attempts to define diagnostic methods to detect endometrial cancer have failed to achieve high sensitivity (>80%) and high specificity (>80%). Therefore, we aimed to develop a new, less-invasive diagnostic method for detecting endometrial cancer with both high sensitivity and high specificity. We objectively evaluated the significance of chromosomal alterations in endometrial adenocarcinoma by two-color fluorescence *in situ* hybridization (FISH) using liquid-based thin-layer cytological preparations.

There have been few reports on cytogenetic analysis methods for endometrial cancers. Numerical and structural rearrangements have been reported in chromosomes 1, 3, 6, 7, 10, 12, 17, and the X chromosome. Fujita *et al.*[7] demonstrated alterations of chromosome 1 in five of six cases, while Milatovich *et al.*[9] demonstrated chromosome 1 alterations in 10 of 14 stage I tumors with deletion of 1p36 or translocated regions involving 1p36 in four of 10 cases. Recently, using comparative genomic hybridization (CGH), Kiechle *et al.*[29] reported a high incidence of loss of 1p36-pter region in endometrial hyperplasia and suggested that loss of putative tumor-suppressor genes located within this region may be involved in the initiation and progression of endometrial hyperplasia complex. Based on restriction fragment length polymorphisms (RFLP) analysis, Jones *et al.*[11] reported frequent loss of heterozygosity on 17p, 3p, 19q, and 10q. Similarly, using CGH, Pere *et al.*[30] demonstrated that gains of 8q, especially 8q23-qter, are associated with aggressive phenotypes such as lymph node metastasis and adnexal tissue involvement in

endometrial carcinomas. In contrast, Hirasawa *et al.*[31] demonstrated no obvious difference in the frequency of 8q gain among different tumor grades. Based on a comprehensive overview of all available evidence, we determined that specific analyses of chromosomes 1 and 17 by two-color FISH would be an effective tool to detect chromosomal alterations in endometrial carcinomas.

The specific detection of large fluorescent signals by fluorescent microscopy requires selection of probe DNA fragments with a highly repeated sequence in a localized region of the sample DNA. Variable number tandem repeat DNA (VNTR DNA) is commonly used as probe DNA for detection in fluorescent techniques. Thus, we used VNTR DNA probes for chromosome 1 and chromosome 17, more specifically p144D6 (*D17S34*; 17p13.3) and cosmid cCI-1-5335(1p36) clones.

With regard to the clonality of normal endometrial glandular cells, the altered chromosomal patterns are random and they are derived from inevitable technical problems. DNA probes, avidin-FITC, and anti-digoxigenin rhodamine cannot always equally penetrate through the nuclear membrane of all examined cells treated with proteinase K solution. In addition, due to the different composition of the nucleic acids of centromere DNA probe and the locus-specific DNA probe, the hybridization states are subtly different. Furthermore, the fluorescent signals are often overlapped, and the number of FITC signals or rhodamine signals is often underestimated. Owing to these inevitable technical problems, normal endometrial glandular cells do not always reveal a disomy pattern. To avoid diagnosing these random chromosomal alterations as significant clonal chromosomal alteration, the alterations were analyzed using cut-off values of mean + 3SD values, values that have often been adopted in many FISH analyses.

Two-color FISH using the ThinPrep method with Uterobrush sampling detected numerical or structural alterations in either 1p or 17p in 89.7% of endometrial carcinoma cases, in 100% of atypical endometrial hyperplasia cases, and in 0% of 10 normal endometrium cases. In this study, the cut-off level was set at mean + 3SD, a standard that has been frequently adopted for the detection of genomic alterations by two-color FISH in various solid tumors[26,32–36]. Using this cut-off level, our method demonstrated a sensitivity of 89.7% and a specificity of 100%, which as a diagnostic method is one of the highest values reported for endometrial cytology screening[1,5,6]. However, the number of examined cases is still small, and further study is required for further validation of this method. In general, the low sensitivity in the detection of endometrial adenocarcinoma is believed to be due to difficulties associated with objectively distinguishing the slight nuclear atypia in well-differentiated grade 1 endometrioid adenocarcinoma from benign nuclear atypia due to inflammatory change. This is clinically important as the latter nuclear atypia often occurs with the repetitive samplings necessary to determine the cause of abnormal uterine bleeding. Based on the sensitivity and specificity of this method, our results suggest that this cut-off setting was appropriate for detecting these chromosomal alterations in endometrial malignancies.

Numerical or structural chromosomal alterations were frequently found in endometrial adenocarcinoma ThinPrep cytologic preparations. In 11 of 14 grade 1 adenocarcinoma cases with an initial negative or equivocal cytological diagnosis, alterations of chromosome 1 and/or 17 were objectively detected by two-color FISH. These 11 cases included four negative cases and seven suspicious but not positive cases by cytological diagnosis with Papanicolaou staining. These results suggest that two-color FISH may be useful as a supplemental diagnostic method in the detection of endometrial precancerous lesions or low-grade endometrial cancers.

With regard to the clonality of tumor cells with abnormal chromosomal alterations, the majority of examined cases demonstrated clonal expression of abnormal chromosomal alterations in addition to normal disomy patterns in chromosome 1 or 17, but a number of cases demonstrated simultaneous expressions of two different categories of abnormal chromosomal alterations. For example, cases 6 and 8 in Table 2 demonstrated both polysomy and gain of chromosome 1, while cases 4, 27, 31, 32, and 33 in Table 3 demonstrated both polysomy and polysomy with loss of short arm in chromosome 1, and case 34 demonstrated both polysomy with loss of short arm and disomy with loss of short arm in chromosome 17. Based on the present data, it is not possible to determine whether the coexpression of abnormal chromosomal alterations is derived from two different clonal expansions or random chromosomal alterations. Further studies of clonality analysis[37] will be necessary to address this problem.

Further studies are also necessary to elucidate the biological significance of the numerical and structural alterations in chromosome 1 and chromosome 17. The p73 tumor-suppressor gene, a p53 homologue[12], is located on 1p36. p73 has been reported to be deleted with high incidence in various brain tumors[38,39]. Tumor-suppressor genes such as p53 and HIC