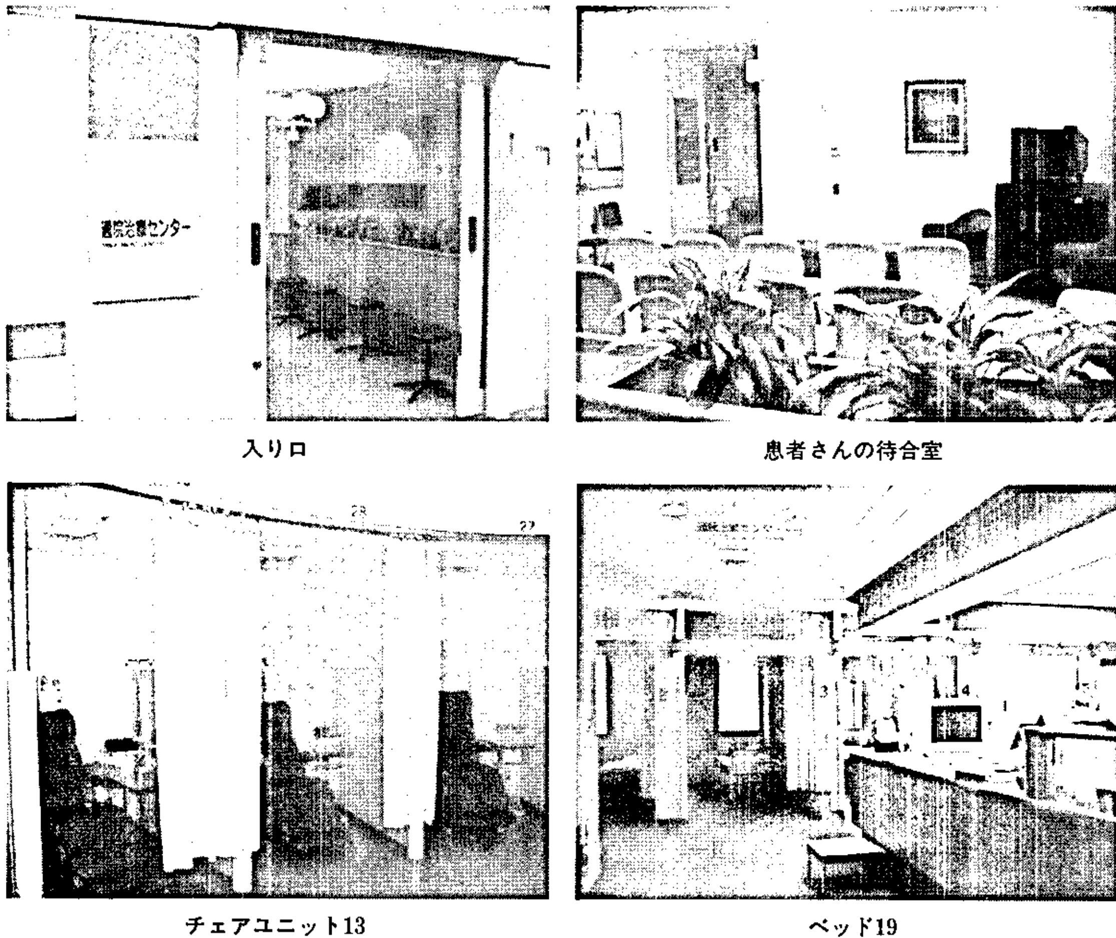

入り口　　患者さんの待合室

チェアユニット13　　ベッド19

図1　国立がんセンター中央病院の通院治療センター

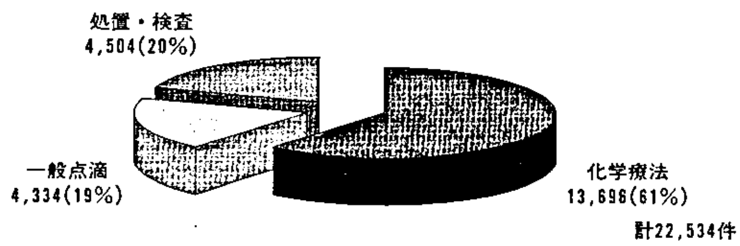

図2　通院治療センター業務の内訳
(2000年度)

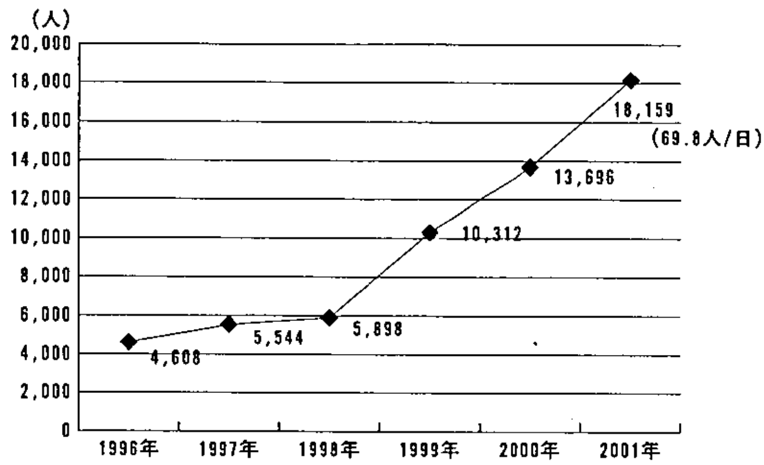

図3　通院治療センターにおける化学療法総数（のべ数）

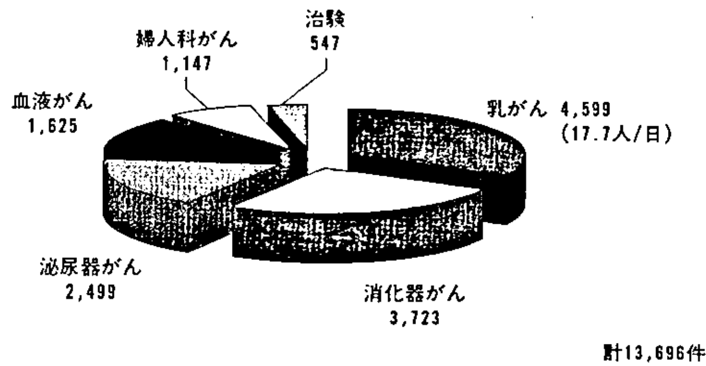

図4　疾患別化学療法の患者の割合
(2000年度)

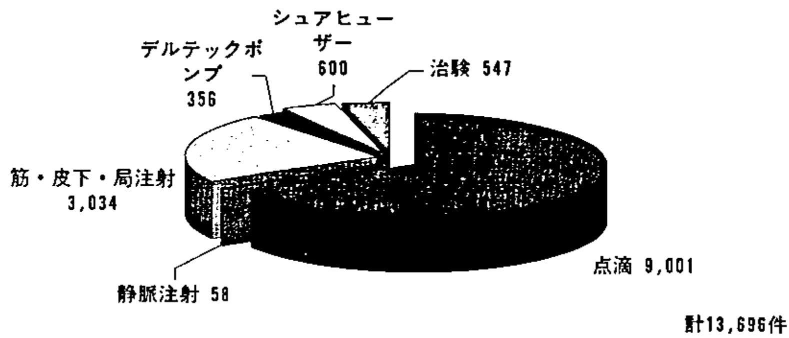

図5　化学療法の投与方法
(2000年度)

近年ではほとんどが外来投与可能となっているためであろうと考えられる．化学療法の投与方法では点滴が一番多かった（図5）．これにはゾラデックスなどの皮下注射も含まれる．このようにルーチン化された外来化学療法を行うためには専門ブースの設置は必須であると考えられる．

2. オーダーのコンピュータ化

安全に化学療法を実施するためには，化学療法をオーダーする際のミスを防ぐことも大切である．投与オーダーミスを防ぐためには，医師・看護師によるダブルチェックを強化する，あらかじめプリントしたものを使用する，スタンプ形式にする，などの方法もあるが，本院ではオーダーをコンピュータ化することによって投与オーダーミスを防ぐようにしている．使用する化学療法レジメンをすべてコンピュータへ登録し，投与量の上限，投与インターバルの制限を行うことにより，投与オーダーミスを防いでいる．登録されたレジメン以外の化学療法はオーダーできないようになっている．

3. 専門職種（腫瘍内科医，専門看護婦，専門薬剤師）によるマネージメント

外来化学療法を行うためには専門職種の役割も大きい．腫瘍内科医はすべての化学療法のレジメンの作成，登録，投与オーダーを中心的に行っており，また実際に投与する際には，腫瘍内科医（スタッフ29名，レジデント25名）が当番制で点滴当番を午前3時間，午後3時間の交替制で行っており，急性期の副作用（吐き気，アレルギー，点滴漏出など）に対応をしている．また，通院治療センターに配属された看護師7名は化学療法に対して専門的知識をもっており，点滴，処置の介助をするだけでなく，患者教育・オリエンテーション，精神面，社会面への看護，急性期副作用への対応などを行っている．外来化学療法のための専門薬剤師は2名配属されており，医師の投与オーダーの後，レジメンの内容，投与量などをもう一度チェックされ，クリーンベンチにて化学療法剤を厳密に調整が行われる．そして，調整が済んだ薬剤点滴ボトルにシールにしたラベルが貼り付けられる．外来化学療法を安全に円滑に行うためには，こうした専門職種による人的役割も欠かせないと考えられる．

4. リスクマネージメントの実際

外来化学療法におけるリスクマネージメントは3．で示したような専門職種が実際にリスクマネージメントを行うことになる．専門職をおいてもミスは起こり得ることであるので，現場でのリスクマネージメントも大切である．現場でのリスクを回避するための工夫として，通院治療センターマニュアルを医師用，看護師用と作成し，新人教育に使用している．また，実際のインシデント/アクシンデントが起こった際にはどんなに小さなものが起こっても必ず報告させるようにし,起こったインシデント/アクシンデントに関しては，月毎に対策をリスクマネージメント委員会へ報告するようにしている．

5. EBM（Evidence-based Medicine）に基づいた治療，適切な支持療法などの確立

EBMに基づいた治療を実践する際には，腫瘍内科医の役割は大きい．年々進歩する化学療法の分野にupdateしていく必要があるし，最新の治療を現場に取り入れ，少しでも患者さんの利益に通じるようにEBMを実践していくことは重要なことである．支持療法の進歩は，入院治療が原則であると考えられてきた化学療法を，外来治療を可能とさせるようになった．入院治療を強いられるもっとも大きな要因は吐き気と骨髄抑制であるが，吐き気に対しては，セロトニン拮抗剤，ステロイドの使用により，急性の悪心・嘔吐はかなり抑えることができるようになった[1]．また骨髄抑制に対しては，G-CSFの登場以来，化学療法を安全に投与できるようになったことは言うまでもない．しかし，G-CSF製剤は毎日皮下注射しなければならないので入院が必要となる場合が多い．また，G-CSF製剤の過剰投与が世界的にも問題となり，ASCO（米国腫瘍学会）でevidenceに基づいたガイドラインが作成された[2]．ASCOガイドライ

ンでは，初回治療から予防投与は原則として行わず，CSFを使用しないと40%以上の確立でneutropenic feverをきたすと予想される場合，または，先行化学療法を行った際に，neutropenic feverが認められた場合にG-CSF製剤の投与を考慮することがすすめられる，としている．我が国のG-CSF製剤の保険適応は，「好中球数1,000／mm^3未満で発熱(原則として38℃以上)あるいは好中球数500／mm^3未満が観察され，引き続き同一のがん化学療法を施行する症例に対しては，次回以降のがん化学療法施行時には好中球数1,000／mm^3未満が観察された時点から投与する」となっているため，ASCOのガイドラインよりも甘い規準となっている．乳がんに使用する化学療法レジメンで40%以上のneutropenic feverをきたすと予想されるレジメンはほとんど皆無である．そうなると，乳がんの患者さんにはほとんどの場合G-CSF投与を行う必要がなくなってくる．また，発熱がみられた場合も，適格にrisk assessmentを行いlow risk（感染源がない，PS良好，食事・水分摂取が可能など）の場合は，外来での経口抗生物質で十分管理可能であるというevidenceも報告されている[3,4]．このように最新のevidenceを応用することは外来治療をより安全に効率よく可能にさせると考えられる．

21世紀のがん化学療法は，ハーセプチンに代表される分子標的治療が中心となることによって，より副作用が少なく外来での化学療法が広く行われるようになると考えられる．患者のQOL重視，医療経済的観点からも外来治療が望まれることは言うまでもない．乳癌治療においても今後ますます外来治療が中心となってくると考えられる．既に欧米では外来化学療法は常識となっており，我が国でも整備されていくことが望まれる．そのために，ソフト面，ハード面両面からの整備をしていくことが重要課題であると考えられる．

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Unfavorable Prognostic Factors Associated with High Frequency of Microsatellite Instability and Comparative Genomic Hybridization Analysis in Endometrial Cancer

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ABSTRACT

Purpose: **Although many articles have been published regarding chromosomal instability (CI) and microsatellite instability (MI) in endometrial adenocarcinoma, the relationship between prognostic factors and the biological mechanisms accounting for genetic instability in these tumors has not yet been precisely defined. To do that, it will be necessary to clarify the molecular mechanisms involved in endometrial carcinogenesis.**

Experimental Design: **Tissue samples from 43 human primary endometrioid endometrial adenocarcinomas (EACs) were analyzed for CI and MI status using comparative genomic hybridization and 11 microsatellite loci, respectively. Methylation status of the promoter of *MLH1* was also determined. We analyzed all three of these parameters in relation to each other and to clinicopathological factors.**

Results: **Sixty-five percent of the EACs we examined had detectable CI. Frequent copy number gains were seen at 1q25–41 (23%), 8q11.1-q21.1 (23%), 8q21.3-qter (21%); 28% of these tumors exhibited high-frequency MI (MSI-H); Methylation of the *MLH1* promoter was observed in 92% of EACs with MSI-H. Southern blotting showed amplification of *MYCN* in one tumor, which has been documented for the first time in a primary human EAC.**

Conclusions: **MSI-H was correlated with histological grade, International Federation of Gynecologists and Obstetricians (FIGO) stage, myometrial invasion, and lymphonode metastasis. Our comparative genomic hybridization results demonstrated that the number of chromosomes involved in genomic alterations in EACs was distinctively fewer than those in other types of tumor. The carcinogenetic process leading to EAC appears to be highly complex; for example, MI and CI may act synergistically, whereas CI and/or MI are likely to be linked with tumor heterogeneity.**

INTRODUCTION

EC[5] is the most common gynecologic neoplasm in Western countries and has been increasing over the past several decades in Japan (1). Proposed prognostic factors for survival include age of the patient, histological type and grade of differentiation of the tumor, the degree of nuclear atypia, myometrial invasion, invasion of vascular space, tumor size, peritoneal cytology, hormone receptor status, DNA ploidy, and type of therapy (surgery *versus* radiation), as well as the surgical staging systems of the FIGO (2). Potential relationships between these factors and the biological mechanisms that account for genetic instability in endometrial tumors have never been precisely defined. EAC, the most common subtype in EC, accounts for over three-fourths of all cases of uterine corpus carcinoma, but the pattern of progression varies from one patient to another. Therefore, it is important to better understand the molecular mechanisms involved in EC, especially in EAC.

Two apparently independent mechanisms of genomic instability, CI and MI, have been identified in several kinds of carcinoma, especially in CRC (3). Certain CRC cell lines exhibiting a CI phenotype are defective in a kinetocore-checkpoint function, which may facilitate chromosome nondisjunction; some of those cell lines also harbor mutations in *hBUB1*, a gene encoding one component of the mitotic checkpoint (3). On the other hand, MI is characteristic of the vast majority of HNPCCs (3, 4). In general, an inverse correlation tends to exist between CI and MI phenotypes of CRC cells *in vitro*, and there are significant differences in clinicopathological features between CRCs with CI as opposed to colorectal tumors with MI phenotypes.

EC is the most common extracolonic neoplasm among

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[5] The abbreviations used are: EC, endometrial adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; EAC, endometrioid endometrial adenocarcinoma; CRC, colorectal cancer; EMH, endometrial hyperplasia; CGH, comparative genomic hybridization; CI, chromosomal instability; HLG, high-level gain; MI, microsatellite instability; MSI-H, high-frequency MSI; MSS, MSI-negative; HNPCC, hereditary nonpolyposis colorectal cancer.

patients with HNPCC, and some sporadic ECs also display the MI phenotype (5). These data have suggested that the genetic background for EC might be similar to that of CRC. Toward a comprehensive understanding of associations between genetic alterations and clinicopathological features in EC, here we examined genetic and epigenetic alterations associated with CI and MI in 43 primary EACs. CI status was examined using CGH, whereas the MI status of each tumor was assessed using 11 microsatellite repeat loci. Furthermore, we examined methylation status of the promoter of *MLH1*, a gene that is frequently involved in MI of sporadic EC (6). Taken together, we analyzed relationships among CI, MI, and methylation of the *MLH1* promoter, as well as clinicopathological factors for EAC, including the presence of EMHs, lesions that are generally regarded as precursors of EAC (7).

MATERIALS AND METHODS

Tumor Specimens and Extraction of DNA. Tissue specimens were obtained from 43 EAC patients and frozen at the time of surgery at the Keio University Hospital after informed consent was obtained for the study. Clinical data for all cases are summarized in Table 1. The mean age of the patients was 59 years (range, 39–83 years). None had received cytotoxic

Table 1 Summary of clinicopathological data and genetic aberrations in 43 primary endometrial adenocarcinomas

				UICC[a] classification			Invasion				Genetic alternation			Gain	
Case no.	Age (yrs)	Grade	FIGO stage	pT	pN	pM	Myometrium[b]	Vascular space	Peritoneal cytology	Endometrial hyperplasia	CI	MI	*MLH1* methylation status	1q	8q
16	54	1	Ia	1a	0	0	0	–	–	+	3	MSS	–		
17	63	1	IIIa	3a	0	0	3	+	–	–	1	MSS	N.T.	+	
23	71	1	Ib	1b	0	0	1	–	–	+	5	MSS	–		+
30	60	1	Ib	1b	0	0	3	–	–	–	1	MSS	–	+	
40	72	1	Ib	1b	0	0	2	–	–	+	7	MSS	–	+	+
43	74	1	Ic	1c	0	0	2	+	–	–	3	MSS	–		+
49	55	1	Ib	1b	0	0	2	+	–	+	0	MSS	–		
51	58	1	IIa	2a	0	0	1	+	–	+	0	MSS	+		
55	53	1	Ib	1b	0	0	1	–	–	–	0	MSS	–		
56	47	1	Ia	1a	0	0	0	–	–	+	1	MSS	–	+	
57	65	1	Ib	1b	0	0	1	+	–	+	0	MSI-H	+		
60	49	1	Ia	1a	0	0	0	–	–	–	0	MSI-H	+		
64	63	1	Ib	1b	0	0	1	–	–	+	1	MSS	–		
67	55	1	Ia	1a	0	0	0	–	–	–	1	MSS	–	+	
69	61	1	Ic	1c	0	0	2	–	–	+	2	MSS	+	+	+
71	49	1	IIIa	3a	0	0	1	–	+	+	2	MSS	+		
73	47	1	Ia	1a	0	0	0	–	–	+	0	MSS	–		
74	55	1	Ib	1b	0	0	1	–	–	+	0	MSS	+		
78	80	1	Ic	1c	0	0	3	–	–	–	2	MSS	N.T.		+
79	53	1	Ic	1c	0	0	2	–	–	–	1	MSI-H	+		
80	57	1	IVb	3a	1	1	3	+	+	+	0	MSI-H	+		
82	39	1	Ib	1b	0	0	2	–	–	+	6	MSS	N.T.	+	
83	47	1	Ib	1b	0	0	1	–	–	+	0	MSS	–		
5	63	2	IIb	2b	0	0	2	+	–	–	10	MSS	–	+	+
13	60	2	Ib	1b	0	0	1	–	–	+	1	MSI-H	+		+
37	50	2	Ib	1b	0	0	1	–	–	+	1	MSS	N.T.		
38	64	2	IIIa	3a	0	0	3	–	–	+	4	MSS	+		+
48	60	2	Ib	1b	0	0	2	+	–	–	0	MSS	N.T.		
63	56	2	IVb	4	0	1	1	+	+	–	1	MSS	+		
66	83	2	Ib	1b	0	0	1	–	–	–	2	MSS	–	+	
68	46	2	IIa	2a	0	0	1	–	–	+	0	MSS	+		
75	58	2	IIIa	3a	0	0	3	+	–	+	0	MSI-H	+		
24	77	3	IIa	2a	0	0	2	+	–	+	1	MSS	+		
28	60	3	Ib	1b	0	0	1	+	–	–	12	MSS	+		+
31	50	3	Ib	1b	0	0	1	+	–	–	0	MSI-H	+		
33	67	3	IIIc	2a	1	0	3	+	–	–	1	MSI-H	+		
44	59	3	IIIa	3a	0	0	3	+	–	–	1	MSS	–		
46	58	3	IIIa	3a	0	0	3	+	–	+	0	MSI-H	+		
50	58	3	Ib	1b	0	0	1	–	–	–	7	MSS	–		+
58	57	3	IIIa	3a	0	0	3	–	+	–	1	MSI-H	–		
61	62	3	IIIc	1c	1	0	3	+	–	+	2	MSI-H	+	+	
62	66	3	IIIc	3a	1	0	3	+	–	–	5	MSS	+	+	
77	51	3	IIIc	1c	1	0	3	+	+	–	0	MSI-H	+		

[a] UICC, International Union against Cancer; CI, total number of chromosomal aberrations detected by CGH; MI, Microsatellite instability; N.T., not tested.
[b] 0, endometrium only; 1, inner third; 2, middle third; 3, outer third.

or radiation therapy before tumor resection or had reported a family history of other types of cancers, including HNPCC. All tumors were stained with H&E. All tumors were histologically classified before any additional analysis by two experienced gynecological pathologists in Keio University Hospital independently using the standard WHO criteria (8), and only cases with histological subtype agreement of two pathologists were used for the present study. All tumors examined were EAC, and all specimens contained >60% tumor cells. We evaluated each case for the presence of EMH lesions because according to Sherman *et al.* (7) most hyperplasias without atypia probably represent early, highly reversible lesions in the pathogenesis of EAC, whereas atypical EMH is considered the immediate precursor of EAC. Stage and grade were determined using the surgical staging systems of FIGO (9). The 43 EAC samples consisted of 23 grade 1 tumors, 9 grade 2 tumors, and 11 grade 3 tumors; 26 were stage I, 4 were stage II, 11 were stage III, and 2 were stage IV. Among them, 23 cases (53.5%) included EMH lesions. Genomic DNAs were extracted from frozen specimens according to the standard procedure.

CGH Analysis. CGH experiments were performed using DNAs labeled directly with fluorochrome as described previously (10, 11). Hybridized chromosomes were analyzed with a digital imaging system (Quip CGH software; Vysis, Chicago, IL). Target regions were determined according to the green-to-red profiles of fluorescence intensity and by visual inspection of the images. Chromosomal regions where the mean ratio fell below 0.8 were considered to reflect losses of DNA (underrepresented), whereas regions where the mean ratio exceeded 1.2 were considered gained (overrepresented) in the tumor genome. Overrepresentations were considered to be HLGs indicative of gene amplification when the fluorescence ratio exceeded 1.5 (10, 11). Heterochromatic regions near the centromeres and Y chromosome were excluded from the analysis. In our study, CI-positive cases were defined as tumors exhibiting chromosomal aberration in one or more loci on CGH analysis.

MI. Genomic DNAs extracted from the samples were PCR amplified at microsatellite repeat loci *D2S123*, *D5S346*, *D17S250*, *BAT26*, *BAT25*, *MSH3*, *MSH6*, *TGFβRII*, *BAX*, *MBD4A10*, and *MBD4A6*. Among them, 3 microsatellite markers were dinucleotide (CA) repeats, and 8 markers were mononucleotide repeats. PCR reactions were performed in a total volume of 25 μl containing 10× 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 of 94°C for 45 s; 58°C for 45 s; 72°C for 40 s; followed by a final extension 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). Results were analyzed by using Genescan software (Applied Biosystems). Tumors were classified as MSI-H, when ≥30% of these markers showed MSI, in accordance with the recent recommendation of the National Cancer Institute Workshop (12). Low-frequency MSI (<30% of 11 markers) is included in the category of MSS.

Methylation in the *MLH1* Promoter Region. The methylation pattern in the CpG island upstream of *MLH1* was determined by methylation-specific PCR experiments, according to methods described elsewhere (13). Genomic DNA from each tumor was treated with sodium bisulfite; this procedure converts all unmethylated cytosine residues to uracil, which is then converted to thymidine in a subsequent PCR. Primers for either methylated or unmethylated versions of *MLH1* were designed for the CpG island in the 5′-untranslated region of this gene; primer sequences for the unmethylated reaction were 5′-TTTTGATGTAGATGTTTTATTAGGGTTGT-3′ (sense) and 5′-ACCACCTCATCATAACTACCCACA-3′ (antisense) and, for the methylated reaction, 5′-ACGTAGACGTTTTATTAGGGTCGC-3′ (sense) and 5′-CCTCATCGTAACTACCCGCG-3′ (antisense) (13).

The SW480 CRC cell line served as a positive control for the methylated primer sets for *MLH1* because these cells lack *hMLH1* expression and the relevant CpG island is hypermethylated (14). DNA from normal peripheral blood cells was used as negative control. Amplified products were separated on 3.0% agarose gels and visualized by ethidium bromide staining and UV illumination.

Southern Blotting. In tumor no. 28, CGH revealed HLG indicative of gene amplification at 2p23-24, the region harboring *MYCN* as the most likely target gene. We performed a Southern analysis to ascertain whether *MYCN* itself was amplified, as described previously (10). The prehybridized membrane was hybridized overnight with pNB-1, which contains parts of intron 1 and exon 2 of *MYCN* (15).

Statistical Analysis. χ^2 or Fisher's exact test was used to determine the relationships between each clinicopathological risk factor of EAC and CI or MI status, CI and MI status, and MI status and methylation status of the *MLH1* promoter. Differences were considered significant when $P < 0.05$.

RESULTS

CI. Twenty-eight of the samples examined (65%) had detectable chromosomal imbalances (Fig. 1, Table 1). Of those 28 tumors, 14 were among the 23 diagnosed as grade 1 (61%); 6 were among the 9 tumors of grade 2 (67%); and 8 were among the 11 of grade 3 (73%). On average, 1.3 (range, 0–5) gains and 0.4 (range, 0–6) losses were observed/case; overall, we detected 1.3 (range, 0–5) gains and 0.1 (range, 0–1) losses in tumors of grade 1, 1.1 (range, 0–3) gains and 0.6 (range, 0–2) losses in tumors of grade 2, and 1.6 (range, 0–5) gains and 0.9 (range, 0–6) losses in tumors of grade 3. The most frequent copy number gains were at 1q25-41 (23%), 8q11.1-q21.1 (23%), and 8q21.3-qter (21%); HLGs were detected at 1q21-q35, 2p23-p24 (Fig. 2*A*), 8q11.2-q13, 8q, 11q13-q21, and 18q21. The most frequent losses were at 16q11.2-q22 (9.3%). In accord with previous studies (16–18), our CGH results demonstrated that the number of chromosomes involved in genomic alterations in EAC was distinctively fewer than those in other types of tumor (19).

MI. MSI-H was detected in 27.9% (12 of 43) of EACs, and MSS was in 72.1% (31 of 43). None of the cases showed MSI-L. MI status, together with CI status and clinicopathological features in each case, are summarized in Table 1.

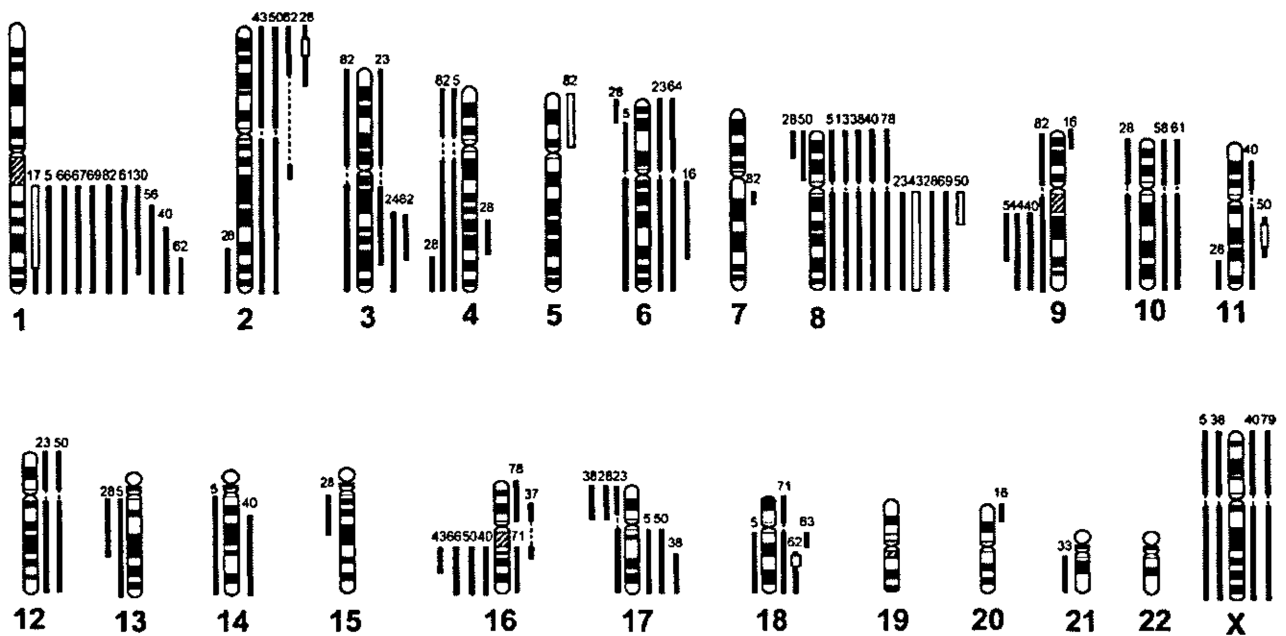

Fig. 1 Summary of genomic imbalances detected by CGH in 43 primary EACs. The 23 autosomes are represented by ideograms showing G-banding patterns. *Vertical lines* on the left of each ideogram show losses of genomic material in the tumors indicated by their numerical identifiers; those on the right correspond to gains in copy number. HLGs are represented as *open rectangles*.

Relationship between Clinicopathological Factors and CI or MI. The results of correlative analysis are shown in Table 2. The frequency of the CI-positive phenotype was significantly higher in women > 60 years of age ($P = 0.0038$). MSI-H was correlated with histological grade, FIGO stage, myometrial invasion, and lymphonode metastasis. Using the grading criteria of FIGO, 6 of 11 (55%) grade 3 tumors were MSI-H in contrast to 6 of 32 tumors (19%) of grades 1 or 2. In terms of surgical stages, 7 of 13 stage III or IV tumors (54%) were MSI-H in contrast to 5 of 30 tumors (17%) of stages I or II. Seven of the 13 cases (54%) where the outer third of the myometrium had been invaded were MSI-H, in contrast to only 5 of the 30 cases (17%) where invasion affected only the inner two-thirds. Four of 5 cases showing lymphonode metastasis (80%) were MSI-H, in contrast to 8 of 38 negative cases (21%).

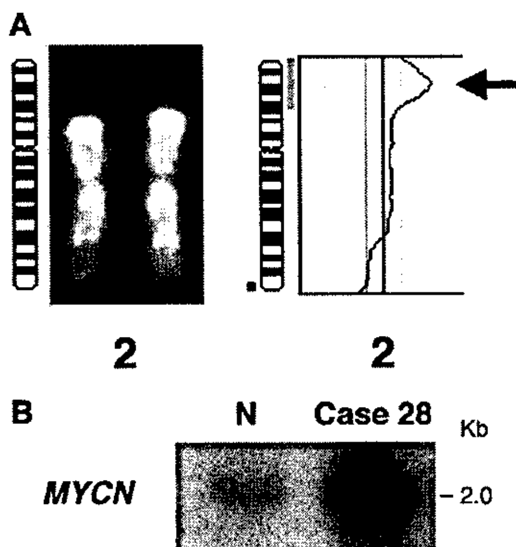

Fig. 2 *A*, a CGH image of chromosome 2 and the corresponding green-to-red ratio profile, illustrating HLGs (*arrow*) at 2p24 in case no. 28. *B*, amplification of *MYCN* in tumor no. 28 detected by Southern blotting. N, normal (control) DNA.

Relationship between CI and MI in EACs. We found a significant correlation was observed between chromosomal aberration and MI when the 12 cases with MSI-H were divided into two groups, *i.e.*, chromosomal aberrations fewer than one or more than two ($P = 0.0403$, Table 3). MSI-H was never observed in tumors having CI at >3 loci.

No statistically significant relationship was observed overall between CI and MI if cases were divided into two groups, *i.e.*, chromosomal aberrations existed or not existed.

Methylation of the *MLH1* Promoter. We were able to evaluate 38 of the 43 EACs for methylation of the *MLH1* promoter by methylation-specific PCRs. Of these 38 tumors, 21 (55%) showed hypermethylation of the *MLH1* promoter region (Table 1). Furthermore, 11 of those 21 (53%) also showed MSI-H. Methylation of the *MLH1* promoter was observed in 92% of EACs with MSI-H. The correlation of the *MLH1* promoter methylation with the presence of MI in the corresponding tumor reached significance ($P = 0.0039$, Table 4).

Amplification of *MYCN*. Tumor no. 28 showed the most remarkable changes in chromosomal copy numbers, including a HLG at 2p23-24 in CGH analysis (Fig. 2*A*). *MYCN* is known as the most likely target gene within this amplified region, and its

Table 2 Relation between clinicopathological features and CI or MI status in 43 primary endometrial adenocarcinomas

		CI[a] (1)			CI (2)			MI		
	n	CI = 0 (n = 15)	CI ≧ 1 (n = 28)	P^b	CI ≦ 2 (n = 33)	CI ≧ 3 (n = 10)	P^b	MSS + MSI-L (n = 31)	MSI-H (n = 12)	P^b
Age										
60 yrs >	24	13	11	**0.0038**	21	3	0.0793	16	8	0.4995
60 yrs ≦	19	2	17		12	7		15	4	
Grade										
G1 + G2	32	12	20	0.1790	25	10	>0.9999	26	6	**0.0468**
G3	11	3	8		8	3		5	6	
FIGO stage										
I + II	30	11	19	>0.9999	22	8	0.6963	25	5	**0.0241**
III + IV	13	4	9		11	2		6	7	
Vascular space invasion										
Negative	24	6	18	0.1262	18	6	>0.9999	20	4	0.0915
Positive	19	9	10		15	4		11	8	
Depth of myometrial invasion										
Endometrium − middle third	30	11	19	>0.9999	22	8	0.6963	25	5	**0.0241**
Outer third	13	4	9		11	2		6	7	
Peritoneal cytology										
Negative	38	13	25	>0.9999	28	9	>0.9999	29	9	0.1230
Positive	5	2	3		5	1		2	3	
Endometrial hyperplasia										
Negative	20	5	15	0.3363	15	5	>0.9999	14	6	>0.9999
Positive	23	10	13		18	5		17	6	
Lymphonode metastasis										
Negative	38	13	25	>0.9999	29	9	>0.9999	30	8	**0.0168**
Positive	5	2	3		4	1		1	4	
Extrauterine invasion										
Negative	31	11	20	>0.9999	23	8	0.6983	25	6	0.0634
Positive	12	4	8		10	2		6	6	
Cervical involvement										
Negative	32	11	21	>0.9999	25	7	0.6983	23	9	>0.9999
Positive	11	4	7		8	3		8	3	

[a] CI, total number of chromosomal aberrations detected by CGH.
[b] Ps were calculated by χ^2 or Fisher's exact test and statistically significant when <0.05. Statistically significant values are in boldface type.

Table 3 Relation between CI and MI in 43 primary endometrial adenocarcinomas

	MI		
	MSS + MSI-L	MSI-H	P^a
CI[b]			
0	8	7	0.0739
≧1	23	5	
CI			
≦2	21	12	**0.0403**
≧3	10	0	

[a] Ps were calculated by χ^2 or Fisher's exact test and statistically significant when <0.05. Statistically significant values are in boldface type.
[b] CI, total number of chromosomal aberrations detected by CGH.

amplification has been reported mainly from tumors of neuronal origin. In addition, amplification of *MYCN* gene was recently reported in rat uterine endometrial carcinoma model (20). Therefore, we next examined the amplification status of this gene by Southern blot to determine whether *MYCN* was an actual target of amplification and may involve in the tumorigenesis of EAC. Consequently as shown in Fig. 2*B*, *MYCN* was clearly amplified in tumor no. 28.

DISCUSSION

Bockman (21) first described two main clinicopathological types, type I and type II, of EC. Type I ECs are low-grade and estrogen-related EAC, which usually develop in pre- and perimenopausal women and coexist with or are preceded by EMH. On the other hand, type II ECs are aggressive and estrogen-unrelated nonendometrioid carcinomas, which largely occur in older women and occasionally arise in endometrial polyps or from precancerous lesions that develop in atrophic endometrium. Because molecular alternations involved in the development of type I EC/EAC have been shown to differ from those of type II EC/nonendometrioid carcinomas, a dualistic model of

Table 4 Relation between MI and *MLH1* methylation status

	MI		
	MSS + MSI-L	MSI-H	P^a
MLH1 methylation[b]			
−	16	1	0.0039
+	10	11	

[a] Ps were calculated by Fisher's exact test and statistically significant when <0.05.
[b] *MLH1* methylation status were determined by methylation-specific PCR.

endometrial carcinogenesis was proposed (22, 23). Normal endometrial cells would transform into EAC through four molecular alternations, *i.e.*, MI and mutations of the *PTEN*, *KRAS*, and *CTNNB1* genes in type I EC, whereas type II EC, especially *de novo* type, would develop through p53 alteration and loss of heterozygosity on several chromosomes (23, 24). However, there are many cases having overlapping clinicopathological and molecular features of these two types of EC. Here, we have focused on analyzing genetic alterations and comparing them with clinicopathological features in EAC for better understanding the molecular pathogenesis of this type of cancer.

In accord with previous studies (16–18, 25), we found frequent gains on chromosome arms 1q and 8q in our EACs. When Kiechle *et al.* (26) performed CGH on EMHs and invasive EC, using a microdissection technique, they found that overrepresentations of 1q and 8q were rare or absent in EMH lesions and suggested that gains of 1q and 8q might define the transition from complex atypical hyperplasia to invasive adenocarcinoma of the endometrium. In our own CGH study, 8 of 23 (35%) EACs with coexisting EMH lesions showed gains of 1q or 8q, and either or both of those alterations occurred in 10 of 20 (50%) EACs without EMH. Table 5 indicates a correlation between 1q or 8q status and coexistence of EMH. Our findings suggest that copy number gains of 1q and/or 8q are frequently involved in EAC regardless of whether EMH lesions are present and that gains in these regions are of paramount importance if normal endometrium or EMH lesions are to become EC. Furthermore, Kiechle's group defined regions of overlap for losses at chromosomes 1p36-pter, 20q13.1-q13.2, and 16p13.1 in EMH and suggested that loss of putative tumor-suppressor genes located within those regions might be involved in the initiation and progression of complex hyperplasia (26). If so, CGH analysis of EACs coexistent with EMH would be likely to show frequent losses in the same chromosome regions. However, in our experiments, we never detected losses at 1p36-pter, 20q13.1-q13.2, and 16p13.1 in EACs, with or without EMH. Clearly additional studies are needed to resolve any significant relationship between losses of those chromosomal regions and the pathogenesis of EAC.

Table 5 Correlation between 1q, 8q status and coexistence of endometrial hyperplasia

	Endometrial hyperplasia		
	Negative	Positive	P^{a}
1q gains			
−	14	18	0.7279
+	6	5	
8q gains			
−	15	18	>0.9999
+	5	5	
1q or 8q gains			
−	10	15	0.3652
+	10	8	
1q and 8q gains			
−	19	21	0.9999
+	1	2	

[a] *P*s were calculated by Fisher's exact test and statistically significant when <0.05.

Pere *et al.* (17) and Suehiro *et al.* (18) reported that gains of 8q, especially 8q23-qter, were associated with aggressive phenotypes of EC such as lymph node metastasis, adnexal tissue involvement, positive peritoneal cytology, and cervical involvement. In our study, however, the frequency of 8q gain was not obviously different among tumor grades: 10 of 23 (43.5%) in grade 1; 4 of 9 (44.4%) in grade 2; and 4 of 11 (36.4%) in grade 3, regardless of whether the tumors exhibited aggressive phenotypes and/or coexisting EMH. Thus, an important goal must be to identify the actual target genes at 1q or 8q in EAC. To our knowledge, no gene except *c-MYC* has been identified or proposed as a potential target for 1q or 8q amplification in EAC (16–18). In other types of tumors, however, several possible target genes for 1q or 8q amplification were reported. In esophageal squamous cell carcinoma, we identified *ATF3* and *CEMPF* as target genes within 1q32 amplicon (27). As possible target genes for 8q amplification except *c-MYC*, several genes such as *E2F5*, *TPD52*, *EIF3S3*, and *FAK* have been identified in various tumors, including breast cancer (28–30). Additional examination will be necessary to identify target genes for 1q and 8q amplification involved in the pathogenesis of EAC.

Amplification of specific genes is frequently detected in advanced stages of various types of cancer. *MYCN* amplification has been reported mainly in tumors of neural origin, for example in ~30% of advanced neuroblastomas (31), this gene is amplified also in a significant number of other cancers, including gastric cancers and breast cancers (32). In neuroblastomas, the degree of *MYCN* amplification is significantly correlated with poor prognosis (31). In our study, one EAC (case no. 28) showed HLG indicative of gene amplification at 2p23-24, and Southern blotting clearly showed amplification of *MYCN*. Interestingly, amplification and consequent overexpression of *Mycn* was reported recently in ECs of BDII rats, (20), a laboratory model that is genetically predisposed to estrogen-dependent EC. Although amplification of *c-MYC*, which encodes similar protein of MYCN, was observed in EC even infrequent, our case no. 8 is the first human EC shown to bear *MYCN* amplification thus far as we know. Therefore, it is important to survey this gene in ECs, including EAC, and to verify the possible involvement of *MYCN* in the progression of this disease in a subset of human cases.

MI, a hallmark of the DNA replication error phenotype, reflects inactivation of mismatch repair genes. To evaluate MI status in our panel of EACs, we chose 11 microsatellite loci for greater precision. Among our EAC samples, we found a significant association between the MI phenotype and histological grade ($P = 0.0468$). The frequency of MSI-H cases was remarkably higher in grade 3 EACs than in tumors of grades 1 or 2. An association of MI status with high grade had already been reported in both ECs (33, 34) and CRCs (35). In CRCs, however, the MI phenotype has been linked also with favorable prognosis and the absence of metastases at diagnosis (35, 36) because of postulated to result from peritumoral inflammatory infiltrates representing a host response (35). On the other hand, the prognosis of grade 3 EACs was poorer than that of grade 1 or 2 EACs, although many grade 3 EACs were also accompanied by the peritumoral inflammatory reactions. The reason of this difference between CRCs and EACs remains unclear. However, in our study, MSI-H and MSS patients showed equivalent

prognoses; there was no significant difference in overall survival (data not shown). Because insufficient evidence is available in the current literatures to reach a conclusion regarding the impact of MI as a prognostic factor in EAC (33, 34, 37, 38), additional examination using larger set of cases will be necessary.

In the present study, hypermethylation of the *MLH1* promoter was observed in 92% of MSI-H EACs and showed a significantly positive correlation with MSI status ($P = 0.0039$), suggesting that methylation of the *MLH1* promoter region and subsequent inactivation of genes, especially tumor suppressor genes, may play a crucial role in the development of MSI-H EACs.

The finding that MI was never observed in tumors having CI at >3 loci (Table 3) was very interesting. Taking all available evidence together, we conclude that the molecular determinants of EAC may be highly complex regardless of prognostic features and that MI and CI may play important roles in the multistep carcinogenesis of endometrial tissue, independently or synergistically. Furthermore, CI and/or MI are likely to be linked with tumor heterogeneity, and this heterogeneity will necessarily hamper development of therapeutic strategies. In addition, because instabilities reflect defects in cellular processes that maintain the integrity of the genome, they can be expected to generate sensitivity to ionizing radiation or particular chemical agents. Therefore, we expect that defining the molecular and physiological bases of both types of instability will eventually yield entirely new approaches to treating any genetic types of EAC.

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Identification of germline *MSH2* gene mutations in endometrial cancer not fulfilling the new clinical criteria for hereditary nonpolyposis colorectal cancer

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Abstract

Endometrial cancer is the second most common malignancy in patients with hereditary nonpolyposis colorectal cancer (HNPCC). This cancer is caused by germline mutations in one of the DNA mismatch repair (MMR) genes. The present study was undertaken to analyze the relation between microsatellite instability (MSI) and germline mutations of MMR genes. We analyzed MSI in 38 cases of endometrial cancer. MSI was present in one or more (out of 5 examined) regions in 11 (29%) cases. Furthermore, alterations in *MLH1* and *MSH2*, two culprit genes representative of HNPCC, were examined in the 11 MSI-positive patients using polymerase chain reaction–single-strand conformation polymorphism and sequencing. Germline mutations, namely, 1) a missense mutation at codon 688 (ATG→ATA, Met→Ile) and 2) a missense mutation at codon 390 (CTT→TTT, Leu→Phe) of the *MSH2* gene, were found in 2 of the 11 patients (18%). Although these two cases do not fulfill the new Amsterdam criteria, they had strong family histories of colorectal and endometrial carcinoma. Our results show that genetic testing is important in cases of endometrial cancer with a history suggestive of HNPCC even if the new Amsterdam criteria are not fulfilled. © 2003 Elsevier Inc. All rights reserved.

1. Introduction

The concept of hereditary nonpolyposis colorectal cancer (HNPCC), one of the most significant hereditary cancer syndromes, was proposed by H.T. Lynch in 1971 [1], and understanding of the disease advanced rapidly during the last 10 years of the 20th century [2]. In 1991, the International Collaborative Group on HNPCC (ICG-HNPCC) established the diagnostic criteria defining HNPCC, known as the Amsterdam criteria [3], which require that 1) histologically verified colorectal cancer is diagnosed in three or more family members, at least two of whom must be first-degree relatives; 2) the disease affects family members from at least two successive generations; and 3) one of the colorectal cancers must occur prior to age 50.

Hereditary nonpolyposis colorectal cancer is an autosomally dominantly inherited disorder with a significantly increased incidence of colon, uterine, ovarian, stomach, and other cancers among affected families [4]. Earlier research performed worldwide on HNPCC and its frequency in patients with colorectal carcinoma indicated that it accounts for 5%–10% of all colon cancers [5–11].

The diagnosis of HNPCC is based on the classical Amsterdam criteria; however, a recognized disadvantage of these criteria is that HNPCC-associated extracolonic tumors such as cancer of the endometrium, stomach, small bowel, renal pelvis, and ureter are not included. This may lead to a delay in the diagnosis of these tumors in HNPCC families. To resolve this problem with the criteria, new HNPCC criteria that include extracolonic cancers were recently proposed by the International Collaborative Group on HNPCC (ICG-HNPCC) in 1999 [12]. The new clinical HNPCC criteria, known as the Amsterdam II criteria, are as follows. There should be at least three relatives with an HNPCC-associated cancer (colorectal cancer, cancer of the endometrium, small

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bowel, ureter, or renal pelvis), and all the following criteria should be present: 1) one should be a first-degree relative of the other two; 2) at least two successive generations should be affected; 3) at least one should be diagnosed before age 50; 4) familial adenomatous polyposis should be excluded in colorectal cases, if any; and 5) the tumor diagnosis should be verified by pathologic examination.

Our study was undertaken to analyze the relation between microsatellite instability (MSI) and germline mutations of DNA mismatch repair (MMR) genes. We conducted a detailed examination of the family and past histories of 102 Japanese women treated for endometrial cancer from 1994 to 1997 at Keio University Hospital. Paired peripheral blood and tumor tissue samples were collected from 38 of the 102 patients after obtaining their consent to perform genetic testing including MSI analysis, which is reported to be positive in 92% of HNPCC cases 13]. Additionally, in MSI-positive (MSI+) cases analysis was performed for germline mutations in *MLH1* and *MSH2*, the two main genes responsible for HNPCC.

2. Materials and methods

2.1. Analysis of family and past histories

Detailed family and past histories were collected after obtaining informed consent from 102 endometrial cancer patients treated at the Department of Obstetrics and Gynecology, Keio University Hospital, from 1994 to 1997. The patients were asked about their closest relatives within the third degree of consanguinity; the recorded data included sex, age at diagnosis, and tumor site, together with current age or age at death of such relatives. Unreliable data were excluded from the study. A search for HNPCC-affected families fulfilling the new Amsterdam criteria was performed using valid family records.

2.2. Genomic DNA extraction from endometrial carcinoma and normal tissue

To obtain uterine cancer tissue samples, part of the tumor was surgically excised from 38 endometrial cancer patients at the Department of Obstetrics and Gynecology of Keio University Hospital and was aseptically transferred into liquid nitrogen. Normal tissue samples and peripheral blood were collected from each patient, after obtaining informed consent. White blood cells were separated using LeukoPrep centrifugation tubes and the samples were kept at −20°C. Similarly, genetic tests including MSI analysis were performed after obtaining the patient's consent.

DNA extraction was performed with SS phenol–chloroform after digesting the samples with protein kinase K. Following phenol extraction, DNA was precipitated by adding 3 mol/L sodium acetate and ethanol. After drying, the pellet was resuspended in Tris and EDTA buffer and kept at 4°C.

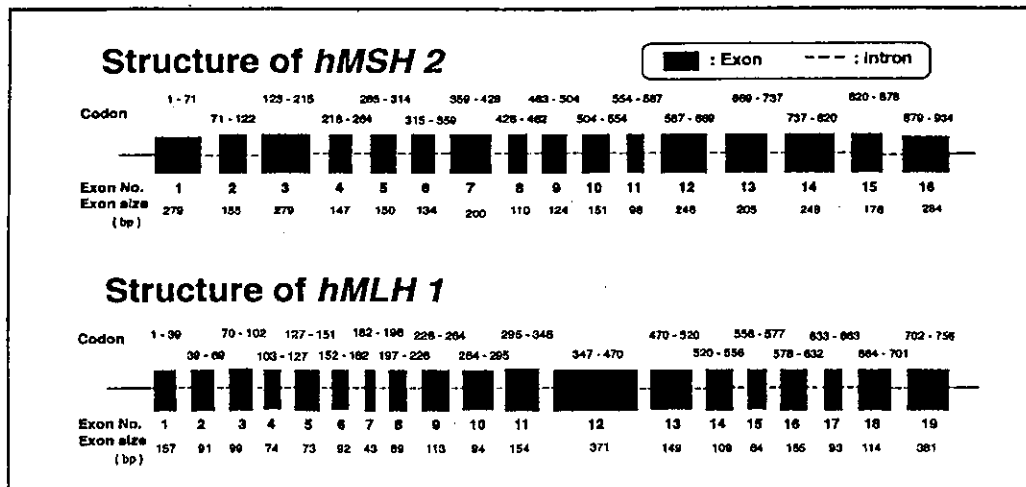
2.3. MSI analysis

DNA of five loci (D2S123, D3S1284, D5S404, D9S162, and *MSH2* intron 12) was amplified by polymerase chain reaction (PCR) of DNA extracted from both normal and tumor tissue samples of endometrial cancer patients (Table 1). The D2S123, D3S1284, D5S404, and D9S162 regions are microsatellite loci containing CA repeats. The PCR primers for these regions were purchased from Research Genetics (Huntsville, AL); the antisense primers contained a fluorescent marker, Cy5 amidite (indodicarbocyanine), at their 5′ ends. Specific primers for *MSH2* intron 12, a polyA-sequence-containing microsatellite locus originally used in our laboratory, were synthesized on a DNA synthesizer; both sense and antisense primers contained the fluorescent marker, Cy5 amidite, at their 5′ ends. AmpliTaq polymerase and AmpliTaq buffer (Perkin Elmer, Boston, MA) were used in the PCR reaction, which was run according to the following protocol: 1 μL of sample DNA (template, 0.1 μg/μL) was added to 24 μL of premixture (distilled water, 16.125 μL; 1.25 mmol/L dNTP, 4 μL; 10× BPCR buffer, 2.5 μL; optical density (OD) 2.2 forward primer, 0.625 μL; OD 2.2 reverse primer, 0.625 μL; and Taq polymerase, 0.125 μL); the total reaction volume was 25 μL. Forward and reverse PCR primers specific for the D2S123, D3S1284, D5S404, D9S162, and *MSH2* intron 12 regions were used. DNA denaturation at 95°C for 5 minutes was followed by 40 cycles of 30 seconds at 95°C, 40 seconds at 55°C, and 40 seconds at 72°C. The reaction mixture was then warmed to 72°C for 7 minutes, then cooled and stored at 4°C.

To detect MSI in the D2S123, D3S1284, D5S404, and D9S162 loci, the amplified DNAs were separated on 8% acrylamide gel containing 7 mol/L urea following denaturation with formamide; electrophoresis was run on an ALFred DNA sequencer (Amersham Pharmacia Biotech, Tokyo, Japan). After denaturation in formamide, the amplified

Table 1
Primer sequences for amplification of each microsatellite marker

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
MSH2 intron 12	5′ GATGTTCCACATCATTACTG 3′	5′ GTGGTTCCACATCATTACTG 3′	182



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' TTTTCTGTTTGATTTGCCAG 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

Fig. 1. DNA structures and primer setting for *MSH2* and *MLH1*

MSH2 intron 12 DNAs were electrophoretically separated in 12% acrylamide gel matrix on the ALFred DNA sequencer. The lengths of amplified DNA fragments for each microsatellite locus in normal and tumor-tissue-derived DNA samples were compared. Electrophoretic results were analyzed with Fragment Manager software (Pharmacia).

Hence, to screen for replication error, each patient had five microsatellite loci assayed for replication errors, and alteration of even one microsatellite region led to definition of the patient as MSI+.

2.4. Mutation analysis of *MLH1* and *MSH2*

In MSI+ cases, the presence or absence of germline mutations in *MLH1* and *MSH2*, the main genes responsible for HNPCC, was studied using the PCR–single-strand conformational polymorphism (SSCP) method. Primers were designed for each exon of the *MLH1* or *MSH2* genes, and PCR was run on DNA derived from normal tissue (Fig. 1).

First, 1 μL of sample DNA (template, 0.1 μg/μL) was added to 24 μL of premixture (distilled water, 16.125 μL; 1.25 mmol/L dNTP, 4 μL; 10× BPCR buffer, 2.5 μL; OD 2.2 forward primer, 0.625 μL; OD 2.2 reverse primer, 0.625 μL and Taq polymerase, 0.125 μL); the total reaction volume was 25 μL. Five minutes of denaturation of DNA at 95°C was followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. The reaction mixture was then warmed to 72°C for 7 minutes, then cooled and stored at 4°C.

Amplicons of each of the *MLH1* exons were separated by electrophoresis in 7.5% acrylamide gel (AA:BIS = 30:1, 10× Tris–glycine), run for 2 hours at 200 V and 15°C. Amplicons of each of the *MSH2* exons were separated by electrophoresis in 10% acrylamide gel. Following electrophoresis, the gels were stained with silver staining and examined for the presence of altered bands. The temperature and time of electrophoresis varied among the *MSH2* exons; the conditions are summarized in Table 2.

Table 2
Electrophoretic conditions for PCR-SSCP analysis of *MSH2*

Exon	Time (h)	Water temperature (°C)
1	4	18
2	4	18
3	6	18
4	4	18
5	3	18
6	4	18
7	4	18
8	3	18
9	4	18
10	6	10
11	3	18
12	4	18
13	6	10
14	4	16
15	4	16
16	4	16

2.5. DNA sequencing

The DNA sequences that produced altered bands were obtained by sequencing on an auto sequencer. They were compared with known normal DNA sequences.

3. Results

As shown in Fig. 2, regions of microsatellite DNA extracted from normal (N) and tumor (T) tissues were amplified by PCR and separated by electrophoresis. An abnormal pattern on electrophoresis was considered to indicate MSI (Fig. 2). MSI was detected in 11 (29%) of the 38 cases examined, in which alteration of at least one of five microsatellite loci was identified, and such patients were considered to be MSI+ (Table 3).

According to the family history of MSI+ cases, none of the 11 families complied with the new Amsterdam criteria.

All exons of *MLH1* and *MSH2* were amplified by PCR on normal tissue DNAs obtained from the 11 MSI+ cases. The PCR reaction was followed by SSCP separation and silver staining. No patient had a *MLH1* gene germline mutation; however, germline mutations of *MSH2* were detected in 2 (18%) of the 11 MSI+ cases (Fig. 3).

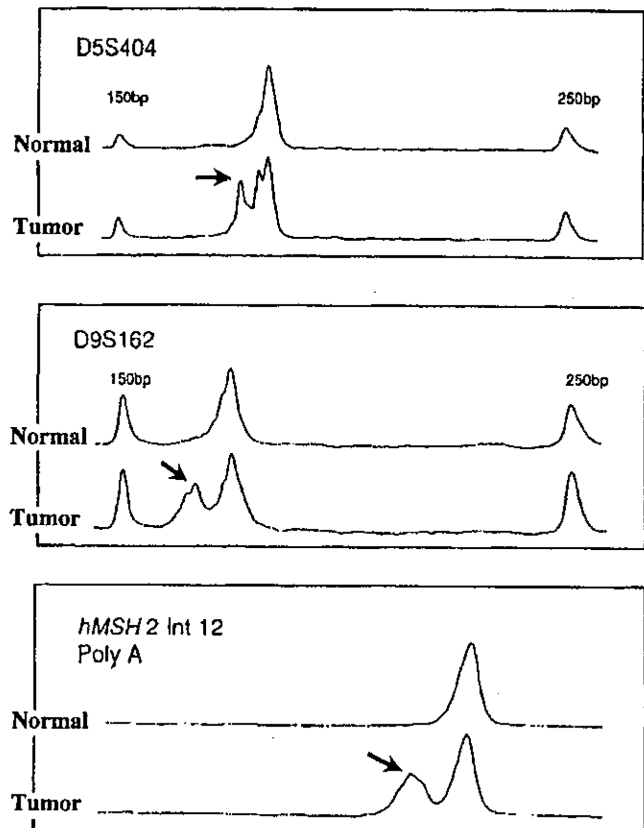


Fig. 2. Analysis of MSI in case 1. Abnormal bands in tumor DNA are indicated by arrowheads.

Table 3
Microsatellite alterations in all examined cases

Case no.	Age at operation (y)	Histologic 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	IIc	OC	–	+	–	+	–
10	56	E	G2	Ic	–	–	–	–	–	+
11	66	E	G1	Ia	–	–	ND	ND	–	+
12	59	E	G1	IIa	BC,OC	–	–	–	–	–
13	48	E	G2	Ib	–	–	–	–	–	–
14	58	E	G2	IIIc	–	–	–	–	–	–
15	65	E	G3	IIIc	–	–	–	–	–	–
16	72	E	G2	IVb	–	–	–	–	–	–
17	55	E	G1	Ib	–	–	–	–	–	–
18	65	E	G1	Ic	–	–	–	–	–	–
19	56	E	G1	Ib	–	–	–	–	–	–
20	69	E	G1	Ia	–	–	–	–	–	–
21	68	E	G1	Ic	–	–	–	–	–	–
22	34	E	G1	Ib	–	–	–	–	–	–
23	50	E	G1	Ib	–	–	–	–	–	–
24	61	E	G1	Ic	–	–	–	–	–	–
25	57	E	G1	Ic	–	–	–	–	–	–
26	55	E	G1	Ia	BC	–	–	–	–	–
27	64	E	G3	IIIa	–	–	–	–	–	–
28	56	E	G1	Ic	–	–	–	–	–	–
29	44	E	G1	Ib	–	–	–	–	–	–
30	45	E	G1	Ib	OC	–	–	–	–	–
31	53	E	G1	Ib	–	–	–	–	–	–
32	69	E	G1	Ia	CC	–	–	–	–	–
33	81	E	G1	IIIa	–	–	–	–	–	–
34	58	E	G1	Ib	–	–	–	–	–	–
35	60	E	G3	Ic	–	–	–	–	–	–
36	64	E	G1	IIIc	–	–	–	–	–	–
37	66	S	G2	IIIa	–	–	–	–	–	–
38	62	S	G2	IIIa	–	–	–	–	–	–

Abbreviations: AS, adenosquamous carcinoma; BC, breast cancer; CC, colorectal cancer; D2, D2S123; D3, D3S1284; D5, D5S404; D9, D9S162; E, endometrioid adenocarcinoma; IN, MSH2 intron 12; ND, not done; OC, ovarian cancer; S, serous adenocarcinoma.

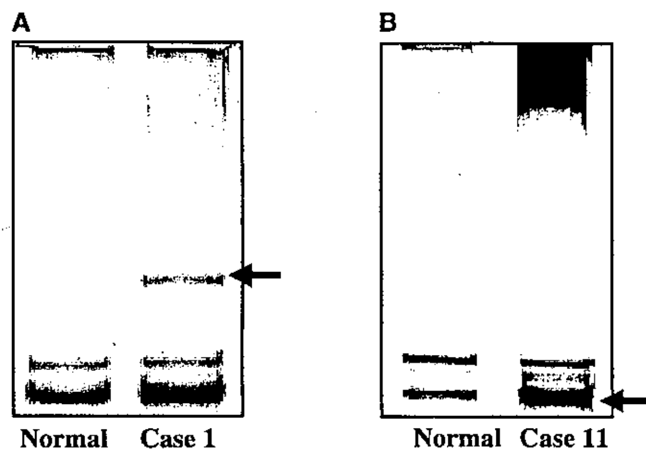


Fig. 3. Results of PCR-SSCP analysis of *MSH2* exon 13 in case 1 (*A*) and exon 7 in case 11 (*B*). Mutant bands (arrowheads) were seen in only two cases.

Exons of the *MSH2* gene showing abnormal SSCP patterns (2 cases) were sequenced on an automatic sequencer. Two missense mutations were identified: at codon 688 (ATG→ATA, Met→Ile) and codon 390 (CTT→TTT, Leu→Phe) (Table 4). These germline mutations of *MSH2* have already been reported in HNPCC patients 14,15] and have been registered in the Human Gene Mutation Database (HGMD) (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.htm).

Table 4
MSH2 germline mutations in endometrial cancer patients

Case no.	Exon affected	Genomic DNA alteration	Predicted effects (codon)
1	Exon 13	ATG→ATA	Met (688) Ile
11	Exon 7	CTT→TTT	Leu (390) Phe

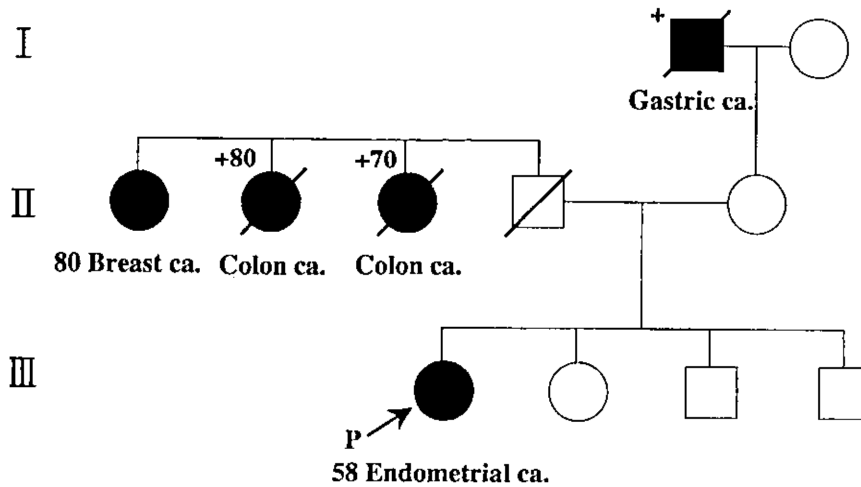


Fig. 4. Pedigree of case 1 with germline mutation in *MSH2*. ● and ■, affected subjects; ○ and □, asymptomatic individuals; cross-line, deceased; arrow, proband.

Although these two cases do not fulfill the Amsterdam II criteria, the patients had strong family histories of colorectal and endometrial carcinoma (Figs. 4 and 5). Both sisters of one patient had uterine carcinoma and were found to carry the identical *MSH2* mutation, which is considered to be of germline origin (data not shown).

4. Discussion

To date, analysis of HNPCC has focused mainly on patients with colon cancer; however, considering the high incidence of uterine carcinoma among patients with HNPCC 12], and the higher rate of MSI+ patients in HNPCC 16] and higher survival rate of such patients, uterine carcinoma would appear to be a disease deserving of more studies than colorectal cancer, especially when examined from the perspective of better treatment policies towards secondary cancers and care for relatives affected by familial cancer syndromes.

There were 11 (29%) MSI+ patients among the 38 uterine cancer patients in our study; this finding was almost identical to those previously reported for other cancers, such as colon (19%), hepatoma (14%), and breast cancer (4%) 17]. The high MSI+ rate among uterine cancer patients, compared with those for other cancers, suggests a high correlation with mutations of MMR genes; however, the significance of positive MSI in endometrial cancers is not completely understood.

Although a report exists stating that MSI+ tumors and the occurrence of double cancers are correlated 16], we did not detect any significant relationship between the two; MSI+ cases did not show a stronger tendency to develop double cancers. In fact, there would appear to be a need to focus more attention on this aspect of clinical pathology in the future, since the follow-up period after uterine cancer treatment is still relatively short. Recently, the relationship of MSI and susceptibility to chemotherapeutic drugs such as cisplatin has received considerable attention. Adducts of platinum drugs, such as cisplatin, form bridges with cellular

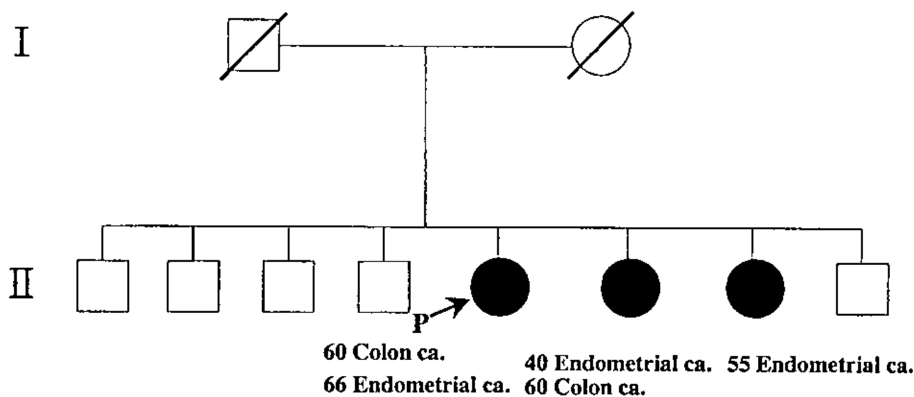


Fig. 5. Pedigree of case 11 with germline mutation in *MSH2*. ● and ■, affected subjects; ○ and □, asymptomatic individuals; cross-line, deceased; arrow, proband.

DNA and induce significant distortion of DNA. In the presence of an abnormal MMR system, the DNA adducts cannot be recognized, rescuing the cell from G2/M blockage and death [18].

In this study, none of the 11 MSI+ patients met the new Amsterdam II diagnostic criteria for HNPCC. This result suggests that the frequency of developing HNPCC in patients with endometrial cancers is very low compared with that in patients with colorectal cancers.

Analysis of MMR genes, and of *MLH1* and *MSH2* in particular, performed in the 11 MSI+ patients revealed two germline mutations (18%) of the *MSH2* gene. Family trees of the two patients carrying mutations at codon 688 (ATG→ATA, Met→Ile; case 1) or 390 (CTT→TTT, Leu→Phe; case 11) are shown in Fig. 3. Germline mutations in these two *MSH2* genes have been reported in other HNPCC patients, suggesting them to be functional missense mutations [14,15]. Neither of the two families met the new Amsterdam criteria for HNPCC, but cancer morbidity in both families was considerably high. Two sisters of one of the probands (case 11) consented to cooperate in the genetic analyses, which revealed that all three sisters carried the identical missense mutation at codon 390 (CTT→TTT, Leu→Phe), which is considered to be of germline origin (Fig. 5). With their prior consent, all three sisters were informed of their missense mutation and of the increased risk of developing colorectal cancer. One year later, one of the proband's sisters was diagnosed with early-stage colon carcinoma and was treated appropriately. In this case, the possibility of gene polymorphism could not be completely excluded; however, such a characteristic family history suggested strong involvement of genetic factors as well.

Germline mutations of the *MSH2* gene were observed in 18% of MSI+ endometrial cancer patients, suggesting an important role of the *MSH2* gene in the development of endometrial tumors. On the other hand, there was no germline mutation of the *MLH1* gene in this study. Nonetheless, it cannot be concluded from this study that the *MLH1* gene is not involved in the development of endometrial tumors. Since it was reported that hypermethylation of the *MLH1* gene occurred in a large number of endometrial tumors [19], *MLH1* gene mutations other than germline mutations could be involved. In addition, other MMR genes besides the *MSH2* and *MLH1* genes could be involved. Since germline mutations of the *MSH6* gene were reported in endometrial cancer patients [20], further study is required to understand the role of MMR genes in the development of endometrial tumors.

This study identified germline mutations of the *MSH2* gene associated with HNPCC in endometrial cancer patients who did not meet the Amsterdam II criteria for HNPCC. This result suggests, similarly to colorectal cancers, the importance of genetic testing in patients suspected of having hereditary endometrial cancers, even if they do not meet the new Amsterdam criteria [21].

Research on HNPCC is continuously developing, but many areas remain obscure. Nevertheless, given that one individual among hundreds carries the responsible genes [22], and that the disease is autosomally dominantly inherited and develops at a young age [5], the advantage of elucidating the relationship between HNPCC and colon or uterine cancer for the many affected patients appears to be unquestionable.

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