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

Case no.	Age (yrs)	Grade	FIGO stage	UICC[a] classification			Invasion		Peritoneal cytology	Endometrial hyperplasia	Genetic alternation		*MLH1* methylation status	Gain	
				pT	pN	pM	Myometrium[b]	Vascular space			CI	MI		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*, *TGF*β*RII*, *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′-ACGTAGACGTTTTATT-AGGGTCGC-3′ (sense) and 5′-CCTCATCGTAACTAC-CCGCG-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.

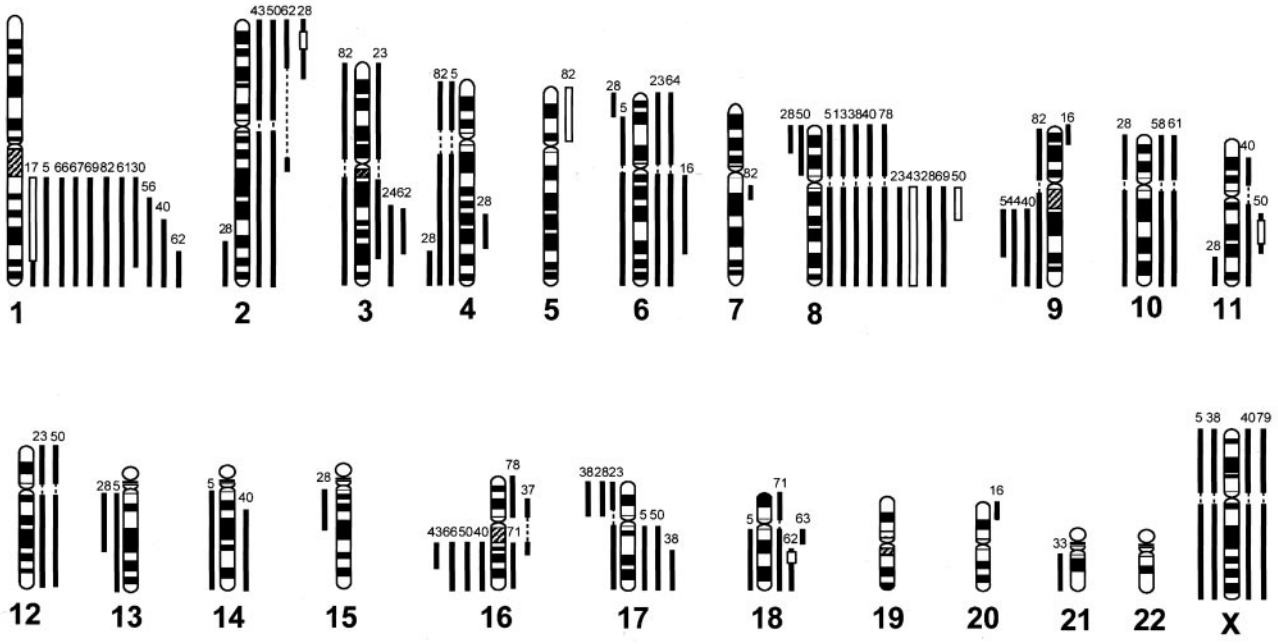

Fig. 1 Summary of genomic imbalances detected by CGH in 43 primary EACs. The 23 autosomes are represented by ideograms showing G-banding patterns. *Vertical lines* on the left of each ideogram show losses of genomic material in the tumors indicated by their numerical identifiers; those on the right correspond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

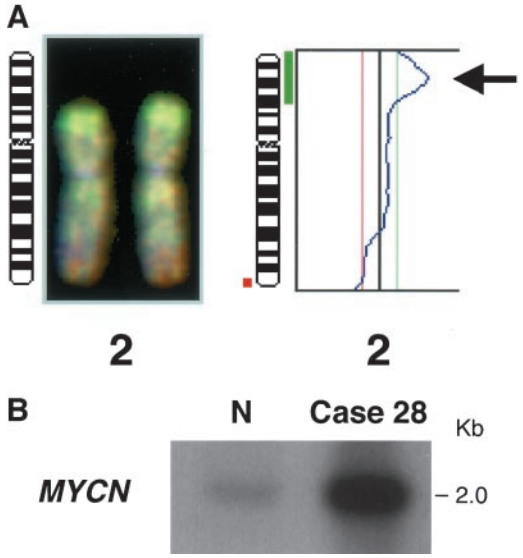

Fig. 2 *A*, a CGH image of chromosome 2 and the corresponding green-to-red ratio profile, illustrating HLGs (*arrow*) at 2p24 in case no. 28. *B*, amplification of *MYCN* in tumor no. 28 detected by Southern blotting. N, normal (control) DNA.

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] *P*s 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] *P*s 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] *P*s 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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