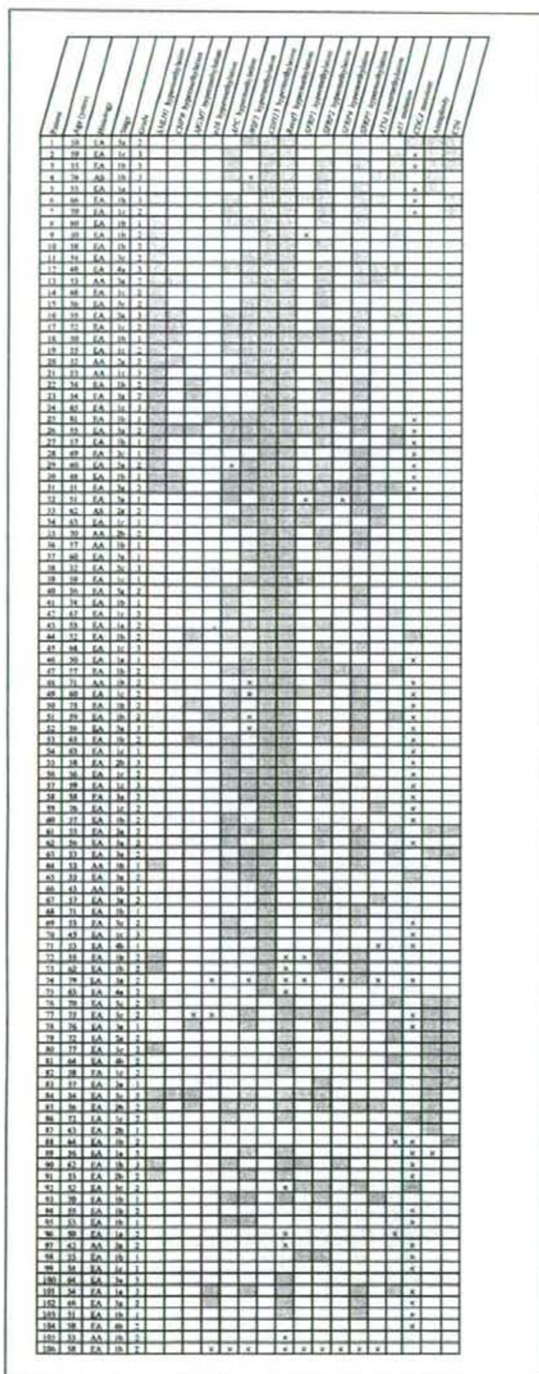

Fig. 1. Diagram of clinicopathologic, epigenetic, and genetic alterations in 106 endometrial cancers. Shading indicates the presence of a molecular alteration, × indicates unavailable data, and an open cell indicates absence of the alteration.

Table 1. Frequency of hypermethylation of genes in normal endometrium and endometrial cancer

Gene	Hypermethylation	
	Endometrium	Cancer
	Frequency (%)	Frequency (%)
hMLH1	1/27 (3.7)	34/106 (32.1)
CHFR	2/25 (8.0)	9/106 (8.5)
MGMT	1/27 (3.7)	9/105 (8.6)
p16	2/27 (7.4)	4/103 (3.9)
APC	2/26 (7.7)	43/104 (41.3)
WIF1	3/25 (12.0)	43/99 (43.4)
CDH13	4/27 (14.8)	75/106 (70.8)
Rassf1	6/25 (24.0)	80/97 (82.5)
SFRP1	2/27 (7.4)	16/101 (15.8)
SFRP2	4/25 (16.0)	54/105 (51.4)
SFRP4	0/27 (0.0)	5/103 (4.9)
SFRP5	4/22 (18.2)	50/105 (47.6)
ATM	7/20 (35.0)	11/103 (10.7)

endometrium and endometrial cancer are shown in Table 1. Hypermethylation of *CDH13* and *Rassf1* was observed frequently in endometrial cancer (70.8% and 82.5%, respectively) as described previously (17). The quantitative analysis of *CDH13* methylation showed that endometrial cancer had higher levels of *CDH13* methylation than normal endometrium (Fig. 2A). The mean methylation level was 40.3% for endometrial cancer and 7.1% for the normal endometrium

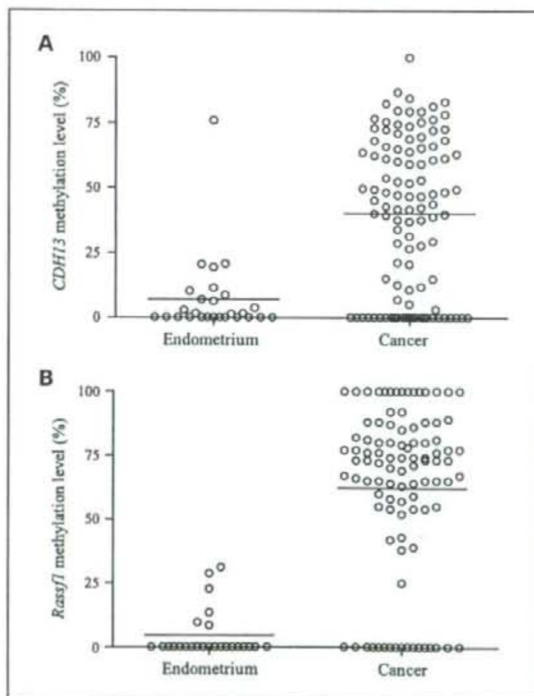

Fig. 2. Distribution of *CDH13* and *Rassf1* methylation (%) in normal endometrium and endometrial cancer. The horizontal lines represent the mean methylation level in each group.

($P < 0.0001$). Mean methylation level of *Rassf1* was also greater in endometrial cancer than in normal endometrium (62.3% versus 4.6%; $P < 0.0001$; Fig. 2B). These data suggest that detection of *CDH13* and *Rassf1* methylation using cytologic materials may be useful as a screening test for endometrial cancer as reported previously (17).

p53 and *CDC4* mutation were observed in 21 of 104 (20.2%) and 5 of 60 (8.3%) endometrial cancers, respectively. Aneuploidy and CIN were found in 19 of 106 (17.9%) and 16 of 106 (15.1%) endometrial cancers, respectively.

Comparison of gene methylation,* p53 *mutations, and* CDC4 *mutations with aneuploidy and CIN. Aneuploidy and CIN were highly concordant with an expected relation in 91.4% of the carcinomas ($P < 0.0001$) as reported previously (29). Thirteen of 19 (68.4%) cases with aneuploidy had CIN, whereas 84 of 87 (96.6%) cases without aneuploidy did not have CIN [odds ratio (OR), 60.7; 95% confidence interval (95% CI), 13.5-273.1; $P < 0.0001$].

Association between various factors, including clinicopathologic variables, gene hypermethylation, and gene mutation, and the presence of aneuploidy is shown in Table 2. In addition, these factors were also compared with CIN for validation (Table 2). Hypermethylation of *CDH13* was shown to have an inverse association with aneuploidy and CIN. In other words, lack of *CDH13* hypermethylation associated positively with aneuploidy and CIN. Only 7 of 75 (9.3%) tumors with *CDH13* hypermethylation had aneuploidy, whereas 12 of 31 (38.7%) tumors without *CDH13* hypermethylation had aneuploidy ($P = 0.0007$). Similarly, 7 of 75 (9.3%) tumors with *CDH13* hypermethylation had CIN, whereas 9 of 31 (29.0%) tumors without *CDH13* hypermethylation showed CIN ($P = 0.0160$). *p53* mutation related positively with aneuploidy and CIN. Eleven of 21 (52.4%) tumors with *p53* mutations had aneuploidy, whereas 8 of 83 (9.6%) cases without *p53* mutations had aneuploidy ($P < 0.0001$). In addition, 9 of 21 (42.9%) tumors with *p53* mutations had CIN, whereas only 6 of 83 (7.2%) cases without *p53* mutations had CIN ($P = 0.0003$). Mean age was significantly higher in cases with aneuploidy and CIN than in those without. Mean age was 62.0 years for cases with

Table 2. Aneuploidy and CIN in relation to clinicopathologic features, hypermethylation of genes, and mutation of genes

Variable		Aneuploidy			CIN		
		Frequency (%)	OR (95% CI)	*P*	Frequency (%)	OR (95% CI)	*P*
Grade	1	4/27 (14.8)	1.0 (Reference)		2/27 (7.4)	1.0 (Reference)	
	2	11/59 (18.6)	1.3 (0.4-4.6)	0.7672	11/59 (18.6)	2.9 (0.6-13.9)	0.2138
	3	4/20 (20.0)	1.4 (0.3-6.6)	0.7073	3/20 (15.0)	2.2 (0.3-14.6)	0.6377
Stage	I	6/60 (10.0)	1.0 (Reference)		6/60 (10.0)	1.0 (Reference)	
	II	3/8 (37.5)	5.4 (1.0-28.5)	0.0652	1/8 (12.5)	1.3 (0.1-12.3)	1.0000
	III	9/33 (27.3)	3.4 (1.1-10.6)	0.0406	8/33 (24.2)	2.9 (0.9-9.2)	0.0773
	IV	1/5 (20.0)	2.3 (0.2-23.6)	0.4453	1/5 (20.0)	2.3 (0.2-23.6)	0.4453
hMLH1	H	4/34 (11.8)	0.5 (0.2-1.7)	0.2931	3/34 (8.8)	0.4 (0.1-1.7)	0.2590
	U	15/72 (20.8)			13/72 (21.1)		
CHFR	H	1/9 (11.1)	0.5 (0.1-4.7)	1.0000	0/9 (0.0)	0.3 (0.0-4.7)	0.3493
	U	18/97 (18.6)			16/97 (16.5)		
MGMT	H	3/9 (33.3)	2.7 (0.6-12.0)	0.1818	1/9 (11.1)	0.7 (0.1-6.3)	1.0000
	U	15/96 (15.6)			14/96 (14.6)		
p16	H	0/4 (0.0)	0.5 (0.0-9.5)	1.0000	0/4 (0.0)	0.6 (0.0-11.8)	1.0000
	U	18/99 (18.2)			15/99 (15.2)		
APC	H	6/43 (14.0)	0.6 (0.2-1.7)	0.4423	5/43 (11.6)	0.6 (0.2-1.9)	0.4000
	U	13/61 (21.3)			11/61 (18.0)		
WIF1	H	8/43 (18.6)	1.1 (0.4-2.9)	1.0000	5/43 (11.6)	0.5 (0.2-1.7)	0.4098
	U	10/56 (17.9)			11/56 (19.6)		
CDH13	H	7/75 (9.3)	0.2 (0.1-0.5)	0.0007	7/75 (9.3)	0.3 (0.1-0.8)	0.0160
	U	12/31 (38.7)			9/31 (29.0)		
Rassf1	H	16/80 (20.0)	1.2 (0.3-4.6)	1.0000	13/80 (16.3)	0.9 (0.2-3.6)	1.0000
	U	3/17 (17.6)			3/17 (17.6)		
SFRP1	H	2/16 (12.5)	0.6 (0.1-2.8)	0.7297	2/16 (12.5)	0.7 (0.1-3.5)	1.0000
	U	17/85 (20.0)			14/85 (16.5)		
SFRP2	H	9/54 (16.7)	0.8 (0.3-2.2)	0.8016	7/54 (13.0)	0.7 (0.2-2.0)	0.5917
	U	10/51 (19.6)			9/51 (17.6)		
SFRP4	H	0/5 (0.0)	0.4 (0.0-7.0)	0.5812	0/5 (0.0)	0.5 (0.0-8.6)	1.0000
	U	19/98 (19.4)			16/98 (16.3)		
SFRP5	H	6/50 (12.0)	0.4 (0.2-1.3)	0.1367	6/50 (12.0)	0.6 (0.2-1.8)	0.4260
	U	13/55 (23.6)			10/55 (18.2)		
ATM	H	2/11 (18.2)	1.0 (0.2-5.0)	1.0000	1/11 (9.1)	0.5 (0.1-4.3)	1.0000
	U	17/92 (18.5)			15/92 (16.3)		
p53	M	11/21 (52.4)	10.3 (3.4-31.8)	<0.0001	9/21 (42.9)	9.6 (2.9-32.0)	0.0003
	W	8/83 (9.6)			6/83 (7.2)		
CDC4	M	2/5 (40.0)	2.4 (0.4-16.0)	0.5817	1/5 (20.0)	1.5 (0.1-14.9)	0.5699
	W	12/55 (21.8)			8/55 (14.5)		

Abbreviations: H, hypermethylated; U, unmethylated or lack of hypermethylation; M, mutated; W, wild-type.

aneuploidy and 57.1 years for cases without aneuploidy ($P = 0.0448$) and 63.8 years for cases with CIN and 56.9 years for cases without CIN ($P = 0.0074$). According to multivariate analysis, lack of *CDH13* hypermethylation and *p53* mutation related positively to aneuploidy (Table 3). Similarly, age, lack of *CDH13* hypermethylation, and *p53* mutation were associated significantly with CIN (Table 3).

We also studied correlations between methylated genes in endometrial cancer (Supplementary Table S2). Five markers, including *hMLH1*, and four genes (*CHFR*, *WIF1*, *Rassf1*, and *SFRP4*), which were methylated concurrently with *hMLH1* methylation, were used to determine methylation phenotype. Endometrial cancer was classified as having methylator phenotype if 50% or more of the evaluated markers were methylated. Relation of the methylator phenotype with aneuploidy, CIN, and *p53* mutation was shown in Supplementary Table S3. Interestingly, the methylator phenotype correlated negatively with CIN in endometrial cancer, as shown for colon cancer (35). None of 16 (0.0%) cancers with CIN has methylator phenotype, whereas 26 of 90 (28.9%) cancers without CIN have methylator phenotype ($P = 0.0105$).

Risk factors for nonsurvival. Results of univariate analysis of risk factors for nonsurvival are shown in Table 4. Regardless of differences in follow-up time, variables including stages III and IV, CIN, and aneuploidy related significantly to death from the disease ($P = 0.0451$, 0.0296, 0.0280, and 0.0120, respectively). In addition, *CDH13* hypermethylation associated negatively with unfavorable outcome ($P = 0.0143$). In other words, lack of *CDH13* hypermethylation was associated with negative prognosis. Age of the patients did not correlate with death from endometrial cancer. Mean age of the patients who died was 61.6 years; mean age of patients who survived was 57.2 years ($P = 0.1851$).

Multivariate analysis showed that the stage in combination with either DNA aneuploid type or lack of *CDH13* hypermethylation was significantly predictive of death from the disease (Table 5A and B). However, CIN and *Rassf1* methylation were not independent prognostic markers (data not shown).

Based on the hierarchical clustering result, we estimated the number of clusters at 2 (Supplementary Fig. S2). The cluster 2 related significantly to death from the disease. Frequency of nonsurviving patients was 4 of 75 (5.3%) for the cluster 1, whereas it was 5 of 17 (29.4%) for the cluster 2 (OR, 7.4; 95% CI, 1.7-31.5; $P = 0.0097$).

Table 3. Results of multivariate analysis of risk factors for aneuploidy and CIN in patients with endometrial cancer

Factor	OR (95% CI)	P
A. Risk factors for aneuploidy		
Age	1.1* (1.0-1.1)	0.0546
Lack of *CDH13* hypermethylation	11.8 (2.8-49.9)	0.0007
p53 mutation	18.7 (4.1-84.2)	0.0001
B. Risk factors for CIN		
Age	1.1* (1.0-1.2)	0.0164
Lack of *CDH13* hypermethylation	4.0 (1.0-15.6)	0.0434
p53 mutation	12.4 (3.1-50.5)	0.0004

NOTE: Variables were considered for the multivariable models if their univariable *P* value was <0.05.
*OR for an age interval for 1 y.

Discussion

In the current study, we found that aneuploidy was a useful prognostic marker in patients with endometrial cancer, which is consistent with the results of previous studies (5-10). Interestingly, DNA aneuploidy was associated with increased mortality even for stage I endometrial cancer (5, 10). Furthermore, Susini et al. (9) have reported results of a 10-year prospective study in which the presence of aneuploidy in endometrial cancer identified high-risk cases among patients considered to be at 'low risk' based on stage and grade of differentiation. Thus, DNA aneuploidy can be a useful prognostic marker in patients with endometrial cancer. Although our finding is not new, this is the first study that evaluated the usefulness of laser scanning cytometric DNA analysis for predicting patient outcomes. The utility of flow cytometric measurements of DNA ploidy in various carcinomas remains controversial (36). Retrospective studies of large numbers of patients and the exchange of samples among laboratories are hampered by the relative scarcity of archival frozen material and difficulties in transporting frozen specimens (37). However, LSC can resolve these problems. Touch preparations for LSC require only small amounts of fresh material (11) and can be stored and transported at 4°C or even at room temperature. Furthermore, another advantage of LSC is that cells selected visually can be quantified. Conversely, cells selected by LSC measurement can be relocated and examined visually. This is important for analyzing cellular properties of malignant tumors because contamination with normal cells is inevitable. Thus, LSC seems more feasible for clinical laboratory use than flow cytometry. Further studies with larger sample sizes and multiple laboratory groups are needed to confirm the feasibility of laser scanning cytometric DNA analysis as a clinical laboratory test.

We found that lack of *CDH13* hypermethylation is positively associated with aneuploidy, CIN, and an unfavorable outcome. Evaluation of methylation level of *CDH13* may assist in therapeutic decision for patients with endometrial cancer. To our knowledge, ours is the first report on the relation of lack of *CDH13* hypermethylation to aneuploidy, CIN, and death from endometrial cancer. CDH13 (also known as H-cadherin or T-cadherin) is an atypical glycosylphosphatidylinositol-anchored member of the cadherin superfamily (38). Down-regulation of *CDH13* gene expression related to promoter hypermethylation has been reported frequently for breast, lung, and colon carcinomas (39, 40). In contrast, in primary hepatocellular carcinomas, *CDH13* is globally overexpressed in approximately half of all tumor specimens (38). Although CDH13 can mediate weak homophilic cell-cell aggregation (41), accumulating evidence suggests that CDH13 might not function as a true intercellular adhesion molecule (42, 43). Because the function of CDH13 is not yet known, further studies are needed.

Aneuploidy, a state of abnormal chromosome number and content, is characteristic of many human cancers, and it plays an important role in tumor formation and development (44). Genetic change leading to aneuploidy is termed CIN, which is defined by continuous and conspicuous changes in

Table 4. Univariate analysis of risk factors for nonsurvival

Factor		Frequency of nonsurviving patients (%)	OR (95% CI)	P
Grade	1	1/26 (3.8)	1.0 (Reference)	
	2	4/53 (7.5)	2.0 (0.2-19.3)	1.0000
	3	4/20 (20.0)	6.3 (0.6-61.1)	0.1512
Stage	I	2/57 (3.5)	1.0 (Reference)	
	II	0/7 (0.0)	1.5 (0.1-33.9)	1.0000
	III	5/30 (16.7)	5.5 (1.0-30.3)	0.0451
	IV	2/5 (40.0)	18.3 (1.9-179.1)	0.0296
hMLH1	H	4/34 (11.8)	1.6 (0.4-6.4)	0.4888
	U	5/65 (7.7)		
CHFR	H	1/9 (11.1)	1.3 (0.1-11.6)	0.5920
	U	8/90 (8.9)		
MGMT	H	1/9 (11.1)	1.5 (0.2-13.5)	0.5512
	U	7/89 (7.9)		
p16	H	0/4 (0.0)	1.1 (0.1-22.6)	1.0000
	U	8/93 (8.6)		
APC	H	3/43 (7.0)	0.6 (0.1-2.6)	0.7269
	U	6/54 (11.1)		
WIF1	H	5/42 (11.9)	1.6 (0.4-6.5)	0.5068
	U	4/52 (9.5)		
CDH13	H	3/71 (4.2)	0.2 (0.0-0.7)	0.0143
	U	6/28 (21.4)		
Rassf1	H	9/77 (11.7)	4.3 (0.2-77.9)	0.3460
	U	0/15 (0.0)		
SFRP1	H	3/16 (18.8)	3.4 (0.7-16.1)	0.1294
	U	5/79 (6.3)		
SFRP2	H	5/52 (9.6)	1.1 (0.3-4.4)	1.0000
	U	4/46 (8.7)		
SFRP4	H	0/5 (0.0)	0.9 (0.0-17.8)	1.0000
	U	8/92 (8.7)		
SFRP5	H	3/47 (6.4)	0.5 (0.1-2.2)	0.4902
	U	6/51 (11.8)		
ATM	H	1/11 (9.1)	1.0 (0.1-8.6)	1.0000
	U	8/86 (9.3)		
p53	M	3/21 (14.3)	2.0 (0.4-8.7)	0.3983
	W	6/77 (7.8)		
CDC4	M	0/5 (0.0)	0.7 (0.0-13.5)	1.0000
	W	6/53 (11.3)		
CIN	+	4/15 (26.7)	5.8 (1.3-24.7)	0.0280
	-	5/84 (6.0)		
Aneuploidy	+	5/19 (26.3)	6.8 (1.6-28.5)	0.0120
	-	4/80 (5.0)		

Abbreviations: +, present; -, absent.

chromosome structure and number (45). It is well known that CIN is associated with aneuploidy (29, 30, 46). It is also widely accepted that tumor cells become aneuploid as a result of aberrant mitotic divisions that are caused by errors in centrosome duplication, chromosome cohesion, spindle attachment, or cytokinesis (12). Indeed, mitotic checkpoint defects lead to aneuploidy in cultured cells (13) and mouse models (14). However, mutations of such mitotic checkpoint genes have been detected in only a fraction of human cancers (15). This prompted us to speculate that multiple factors, such as DNA methylation and chromatin modification, are key players in tumor cell aneuploidization. Several studies have shown a possible association between DNA methylation status and CIN in cancer cells. For example, hypomethylation of pericentromeric satellite sequences predisposes to chromosomal breakage and recombination, leading CIN in tumor cells (47, 48). Interestingly, *CDH13* hypomethylation is associated with satellite DNA hypomethylation in ovarian and breast cancers (49, 50). Thus, lack of *CDH13* hypermethylation may be a predictive marker for aneuploidy via satellite DNA hypomethylation.

We found that mutation of *p53* is related to aneuploidy and CIN as found in previous studies (9). Normal p53 function imposes a barrier to genomically unstable cells by stimulating G_1-S or G_2-M checkpoint responses, mitotic catastrophe, and apoptotic cell death (51). In contrast, cells with inactivated p53 may overcome these barriers and maintain proliferative activity despite CIN (52). The most frequent form of genomic instability in human cancer, aneuploidy, often coincides with loss of p53. However, there is mounting evidence that a defect in p53 does not have a direct role in CIN but instead promotes CIN indirectly (52–54). In addition, the present finding that *p53* mutation correlates with aneuploidy and CIN but not patient outcomes suggests that *p53* mutation alone may be only an early event in the CIN pathway and have little effect on the malignant behavior of tumor cells.

In the current study, *CDC4* mutations were not associated with aneuploidy or CIN. Rajagopalan et al. (55) reported that

Table 5. Results of multivariate analysis of risk factors for nonsurvival in patients with endometrial cancer

Variable		OR (95% CI)	P
A. Risk factors for nonsurvival			
Aneuploidy		7.2 (1.4-36.4)	0.0167
Stage	II	0.0 (NA)	0.9787
	III	3.6 (0.6-21.9)	0.1601
	IV	19.5 (1.7-221.5)	0.0167
B. Risk factors for nonsurvival			
Lack of *CDH13* hypermethylation		7.0 (1.5-33.8)	0.0153
Stage	II	0.0 (NA)	0.9792
	III	5.3 (0.9-31.2)	0.0645
	IV	17.9 (1.5-212.8)	0.0225

NOTE: Logistic regression was used to test multivariate associations of stage and aneuploidy (A) or lack of *CDH13* hypermethylation (B) with cancer death. Variables were considered for the multivariable models if their univariable *P* value was <0.05.
Abbreviation: NA, not applicable.

CDC4 (F-box and WD40 domain protein 7, *FBW7, FBXW7*) is a CIN gene for human cancer. However, the association between *CDC4* mutation and CIN is controversial. Hubalek et al. (56) screened for *CDC4* mutations in endometrial cancers. When they excluded isoform-specific changes, they found mutations in 6 of 12 aneuploid and/or polyploid cancers and in none of 3 diploid lesions. Although these data were suggestive of a link between *CDC4* mutations and CIN, the association was not statistically significant ($P = 0.19$, Fisher's exact test). Furthermore, Kemp et al. (57) screened 244 colorectal tumors and 40 cell lines for *CDC4* mutations and CIN. They found that 18 of 284 (6%) tumors, including near-diploid (CIN$^-$) lesions, harbored *CDC4* mutations and that there was no association between *CDC4* mutations and CIN. These results suggest that *CDC4* mutations are not associated with CIN.

In conclusion, we found a significant relation between DNA aneuploidy and lack of *CDH13* hypermethylation in endometrial cancer. Furthermore, we observed a significant association of DNA aneuploidy and lack of *CDH13* hypermethylation with negative prognosis. The validity of an individualized approach to management of endometrial cancer patients, including determination of tumor ploidy and *CDH13* methylation status, should be properly evaluated in prospective randomized trials.

Disclosure of Potential Conflicts of Interest

The authors declare that there is no conflict of interest.

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Phase I Study of Irinotecan Combined with Mitomycin-C and 5-Fluorouracil for Gynecological Malignancies: The JGOG Study

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Abstract. *Background: A phase I study to evaluate combined therapy with irinotecan (CPT-11), mitomycin-C (MMC), and 5-fluorouracil (5-FU) was performed in patients with gynecological malignancy, especially non-squamous cell carcinoma of the uterine cervix. Materials and Methods: Eligibility for the study included patients with previously untreated, chemotherapy-naïve cervical and ovarian carcinoma. CPT-11 and MMC were administered on days 1 and 15 by intravenous infusion, while 5-FU was given on days 3 to 7. This regimen was repeated after 5 weeks. Four escalating dose levels were carried out (CPT-11/MMC: 120/5, 120/6, 150/6, and 150/7 mg/m^2; 5-FU 600 mg/m^2 fixed). Results: Fourteen patients were enrolled in the study. Although all the patients had no previous chemotherapy, three patients had undergone a simple hysterectomy and nine had a radical hysterectomy performed before this chemotherapy. The maximum tolerated dose was not reached by using CPT-11 150 mg/m^2, MMC 7 mg/m^2, and 5-FU 600 mg/m^2 because none of the patients experienced any hematological or non-hematological toxicities of grade 4 during the first cycle. Conclusion: The recommended doses of this new regimen are CPT-11 150 mg/m^2, MMC 7 mg/m^2, and 5-Fu 600 mg/m^2 which can be well tolerated for gynecological malignancies.*

The major treatments for gynecological malignancies consist of surgery, radiotherapy, and chemotherapy. In patients with epithelial ovarian carcinomas, the histological subtype is one of the most important prognostic factors. It is widely acknowledged that the prognosis of clear cell carcinoma and mucinous adenocarcinoma of the ovary is poorer than that of serous and endometrioid adenocarcinoma (1, 2). The platinum-based regimens such as paclitaxel (PTX) plus carboplatin (CBDCA) therapy (TC), which has been introduced broadly as a standard regimen for ovarian cancer (3, 4), may not be the optimal chemotherapy in patients with mucinous and clear cell carcinoma (1, 2). Thus new combined chemotherapies including irinotecan hydrochloride (CPT-11) have been examined (5) and a randomized phase III trial of TC compared with CPT-11 plus cisplatin (CDDP) for clear cell carcinoma is an ongoing study (international collaborative study: GCIG/JGOG3017).

Although the number of patients with cervical cancer has decreased recently, the incidence of adenocarcinoma of the cervix has increased. A recent report of SEER data demonstrated that the rate of non-squamous cell carcinoma has increased by 29.1% over the last 20 years (6). Most patients with locally advanced squamous cell carcinoma of the uterine cervix are commonly treated with radiotherapy or chemoradiotherapy (7-9), but other types of cervical carcinomas are less sensitive to radiotherapy (10, 11) and have a poorer prognosis (12-15). However, there have been few studies of the efficacy of adjuvant chemotherapy for cervical adenocarcinoma (16, 17). CDDP has commonly been used in patients with gynecological malignancies and the response rate of CDDP alone was 20% and for combined chemotherapy with CDDP plus PTX was 60% in non-squamous carcinoma of the cervix (18, 19). However, patients with advanced and recurrent cervical carcinoma often have complications such as ureteral stenosis and renal dysfunction and since it is known that CDDP can cause renal disorders, a new combined regimen to achieve clinical benefits needs to be developed.

CPT-11 is a semisynthetic derivative of camptothecin, a plant alkaloid obtained from *Camptotheca acuminata* (20).

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Key Words: New combined chemotherapy, phase I study, gynecological malignancy, CPT-11, MMC, 5-FU.

The antitumor effects of CPT-11 are related to the inhibition of DNA topoisomerase I (21). CPT-11 has shown strong activity against various experimental tumors with little renal toxicity because CPT-11 is excreted into the gastrointestinal tract (22). In patients with gynecological malignancies, monotherapy and combined chemotherapy using CPT-11 have been performed (23-28). 5-Fluorouracil (5-FU) is a key drug for colorectal adenocarcinoma, which is histologically like cervical adenocarcinoma and mucinous ovarian carcinoma. Combined therapy with CPT-11 plus 5-FU/ leucovorin (LV) is one of the most standard regimens for primary and metastatic colon cancer (29-31). 5-FU had an effect on cervical adenocarcinoma, with a response rate of 14% (32). In our preliminary study (JGOG 1057, unpublished data), the response rate was 2 out of 17 (11.8%) patients with CPT-11 (150 mg/m^2, days 1 and 15) plus 5-FU (600 mg/m^2, days 3 to 7). Grade 4 leukopenia, thrombocytopenia, and diarrhea using this combined chemotherapy was present in 21.2%, 5.3%, and 5.3% patients, respectively. This combination chemotherapy was well tolerated and there appears to be the possibility of adding another antitumor agent. In *in vitro* experiments using cervical adenocarcinoma cell lines, mitomycin-C (MMC) is one of the most effective antitumor agents (33). It has been reported that MMC is a modulator of CPT-11 activity because it increases topoisomerase I expression (34).

Non-squamous cell carcinoma of the uterine cervix and mucinous or clear cell adenocarcinoma of the ovary are diseases with poor prognosis, and are not effectively treated with the present chemotherapy. Therefore, the cervical cancer committee members of the Japanese Gynecologic Oncology Group (JGOG) performed a phase I trial of CPT-11, MMC and 5-FU for gynecological malignancies, especially non- squamous cell carcinoma of the cervix, in order to determine the maximum tolerated dose (MTD) and the dose-limiting toxicity (DLT).

Patients and Methods

Patient selection. Patients enrolled in this study were required to fulfil the following eligibility criteria: histologically proven non-squamous cell carcinoma of the cervix or clear cell or mucinous adenocarcinoma of the ovary; no prior chemotherapy or radiotherapy; age ≤75 years; performance status (WHO) ≤2 and life expectancy ≥3 months. The patients were also required to meet all of the following laboratory criteria: an adequate bone marrow reserve (leukocyte count of 4.0-12.0×10^3/μl, platelet count ≥100×10^3/μl, and hemoglobin ≥9.5 g/dl), and adequate renal and hepatic function (24-hour creatinine clearance ≥60 ml/min, serum creatinine ≤1.5× the upper limit of normal, serum total bilirubin ≤1.5 mg/dl, and AST/ALT ≤2× the upper limit of normal). All the participants gave written informed consent for the study. Patients were excluded for any of the following reasons: metachronous or synchronous other carcinomas, concurrent infection, pre-existing diarrhea, ileus, or bowel obstruction, interstitial pneumonia or pulmonary fibrosis, massive ascites, pleural effusion, uncontrolled diabetes, or a history of severe drug hypersensitivity. This trial was approved by the Review Board of the Japanese Gynecologic Oncology Group and by the Institutional Review Board of each participating hospital.

Table I. *Dose escalation scheme.*

Dose level	Irinotecan mg/m^2	Mitomycin-C mg/m^2	5-Fluorouracil mg/m^2	No. of patients	No. of cycles
1	120	5	600	4	7
2	120	6	600	4	4
3	150	6	600	3	5
4	150	7	600	3	3

Regimen. On day 1, MMC dissolved in 20 ml physiological saline was administered as a bolus infusion, after which CPT-11 (in 500 ml of normal saline or 5% glucose solution) was also administered intravenously over 90 min. On days 3 to 7, 5-FU (600 mg/m^2) was given intravenously over 12 hours. On day 15, MMC dissolved in 20 ml physiological saline was administered as a bolus infusion, after which CPT-11 (in 500 ml of normal saline or 5% glucose solution) was also administered intravenously over 90 min. Granulocyte colony-stimulating factor (G-CSF) was administered if grade 3 neutropenia occurred with a fever ≥38.5°C or if grade 4 neutropenia developed with or without fever. This treatment schedule was repeated every 5 weeks.

The doses and the treatment schedule were modified to avoid severe side-effects. CPT-11 and MMC were not given on day 15 if the leukocyte count was <3.0×10^3/μl or the platelet count was <100×10^3/μl. The treatment was also withheld if the patient developed diarrhea ≥grade 2 according to the Eastern Cooperative Oncology Group scale (35). Before the next course was started, the patient condition was evaluated and the starting criteria included a leukocyte count of ≥4.0×10^3/μl and a platelet count ≥100×10^3/μl. In addition, there had to be no diarrhea and the renal function of eligibility criteria had to be within acceptable limits. Dose modification was not carried out for low blood cell counts or diarrhea during the same course.

Dose escalation plan and toxicity evaluation. The starting dose of CPT-11 was 120 mg/m^2 and MMC was 5 mg/m^2 and the dose of 5-Fu was fixed at 600 mg/m^2. Four escalating dose levels of CPT-11/MMC (120/5, 120/6, 150/6, and 150/7 mg/m^2) were studied (Table I). Intra-patient dose modification was not permitted. Toxicity was evaluated by the Japan Clinical Oncology Group (JCOG) Criteria (36), except for diarrhea which was assessed by the Eastern Cooperative Oncology Group scale (35). The DLT was defined as any grade 3 or higher nonhematological toxicity (except alopecia, nausea or vomiting, appetite loss and general fatigue) and hematological toxicity of leukopenia grade 4 (>5 days), leukopenia grade 3 with a fever ≥38.5°C, thrombocytopenia grade 4 or thrombocytopenia grade 3 with severe bleeding. Three patients were initially enrolled at each dose level, however, the number of patients at levels 1 and 2 was 4 because the last 2 patients were registered on the same day. If none of the patients experienced DLT during the first treatment cycle, the next cohort of three patients was tested at the next higher dose level. If any DLT was observed in one of the

patients, an additional three patients were enrolled at the same dose level. If two patients in the first cohort or three or more of all the patients at each dose level experienced any DLT, the MTD had been reached and the dose level below the MTD was considered to be the recommended dose for further study. The determination of MTD was based on the toxicity observed in the first cycle of each patient. Toxicity in the patients was evaluated if the patients received at least one full course of the protocol therapy, except for an omission of therapy on day 15.

Results

Patient demographics. Between 2001 and 2006, 14 patients with carcinomas of the cervix or ovary who had no prior chemotherapy entered this trial in four cooperative institutions in Japan. The characteristics of the patients are listed in Table II. Among the 14 eligible patients, 8 were diagnosed with adenocarcinoma of the cervix, 3 with adenosquamous carcinoma of the cervix, 2 with clear cell carcinoma of the ovary, and 1 with mucinous adenocarcinoma of the ovary. The median age was 57 (39-70) years and all the patients had a performance status of 0 to 1. In the patients with cervical carcinoma, the following clinical stages based on the International Federation of Gynecology and Obstetrics (FIGO) were found; five patients had stage Ib, two patients had stage IIa, and four patients had stage IIb. The patient with mucinous ovarian carcinoma was diagnosed as stage Ic and the two patients with clear cell carcinoma had stage IIIb and IIIc disease. All three patients with ovarian carcinoma received simple hysterectomy and bilateral adnexectomy before this protocol chemotherapy. Nine out of the 11 patients with cervical carcinoma had radical hysterectomy performed before this chemotherapy. Only two patients received this chemotherapy in a neoadjuvant setting. Thus, 12 out of the 14 patients did not have any measurable disease.

Treatment under study. Out of a total of 19 cycles of this protocol therapy, 4 dose levels were administered in 14 patients (Table I). Ten patients (71%) received one cycle of protocol therapy and the other patients received 2 or 3 cycles (Table II). All the patients were assessable for toxicity. Ten out of the 14 patients received at least one complete cycle of protocol therapy, whereas four patients (dose level 1, one patient; dose level 2, one patient; dose level 4, two patients) were not administered CPT-11 and MMC on day 15. Only the two patients with cervical adenocarcinoma who received this chemotherapy in a neoadjuvant setting had a tumor >4 cm in diameter in the uterine cervix at the time of treatment. After cycle 1, one patient showed no change (NC) and the other was a partial response (PR) and these patients underwent radical hysterectomy after this protocol therapy. There were no treatment-related deaths. The median overall survival (OS) period was 47.5 months (range: 6-65). The reasons for protocol termination were as follows: eight patients were due to finish adjuvant therapy; two patients were due to undergo surgical procedures; three patients requested to discontinue the study therapy and one patient with clear cell carcinoma of the ovary had increased ascites during the treatment period. Among the 14 patients, only two patients who had been diagnosed with adenocarcinoma of the cervix with stage Ib and IIb disease died of the disease at 21 months and 30 months after this protocol therapy, respectively.

Table II. *Characteristics of the eligible patients.*

	No. of patients	(%)
Overall	14	100
Age (years)		
Median (range)	57 (39-70)	
WHO performance status score		
0	5	36
1	9	64
Histology		
Adenocarcinoma of the uterine cervix	8	57
Adenosquamous carcinoma of the uterine cervix	3	21
Clear cell adenocarcinoma of the ovary	2	14
Mucinous adenocarcinoma of the ovary	1	7
Prior therapy		
None	2	14
Simple hysterectomy	3	21
Radical hysterectomy	9	64
No. of cycles		
1	10	71
2	3	21
3	1	7

Toxicity observed in cycle 1. Tables III and IV list the major toxicities encountered during the first cycle. At dose level 1, three patients developed grade 3 leukopenia and two of them received G-CSF. Two patients experienced grade 2 diarrhea and one of them received loperamide and hangeshashinto. Only one patient was not administered CPT-11 and MMC on day 15, because grade 2 leukopenia was still present. At dose level 2, one patient developed grade 3 leukopenia and administration of CPT-11 and MMC was not carried out on day 15. At dose level 3, one patient developed grade 3 neutropenia and grade 2 diarrhea and the patient received G-CSF and hangeshashinto. Another patient showed grade 3 leukopenia and grade 4 neutropenia and was administered G-CSF. At dose level 4, tow out of the three patients developed grade 3 leukopenia and the administration of CPT-11 and MMC was cancelled on day 15. As mentioned above, leukopenia was the major toxicity, but no DLTs were observed at any of the dose levels. Therefore, the MTD had not been reached and the maximum dose level (CPT-11/MMC, 150/7 mg/m^2) was considered to be the recommended dose for further study.

Table III. *Hematological toxicity: Cycle 1.*

Dose level (%)	No. of patients	Leukopenia					Anemia					Thrombocytopenia				
		G1	G2	G3	G4	G 3/4 (%)	G1	G2	G3	G4	G 3/4 (%)	G1	G2	G3	G4	G 3/4
1	4	0	1	3	0	75	2	2	0	0	0	1	0	0	0	0
2	4	0	3	1	0	25	2	1	0	0	0	0	0	0	0	0
3	3	0	1	1	0	33	0	2	0	0	0	0	1	0	0	0
4	3	0	1	2	0	67	1	0	0	0	0	0	1	0	0	0

Table IV. *Nonhematological toxicity: Cycle 1.*

Dose level	No.of patients	Diarrhea				Nausea/vomiting				Hepatotoxicity				Alopecia			
		G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4
1	4	0	2	0	0	2	1	0	0	0	0	0	0	3	1	0	0
2	4	0	1	0	0	1	0	0	0	2	0	0	0	3	1	0	0
3	3	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
4	3	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0

Table V. *Hematological toxicity: Overall.*

Dose level (%)	No. of cycles	Leukopenia					Anemia					Thrombocytopenia				
		G1	G2	G3	G4	G 3/4 (%)	G1	G2	G3	G4	G 3/4 (%)	G1	G2	G3	G4	G 3/4
1	7	1	3	3	0	47	3	3	0	0	0	3	0	0	0	0
2	4	0	3	1	0	25	2	1	0	0	0	0	0	0	0	0
3	5	0	1	3	0	60	0	3	1	0	20	0	1	1	0	20
4	3	0	1	2	0	67	1	0	0	0	0	0	1	0	0	0

Table VI. *Nonhematological toxicity: Overall.*

Dose level	No. of cycles	Diarrhea				Nausea/vomiting				Hepatotoxicity				Alopecia			
		G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4
1	7	3	2	0	0	4	1	0	0	0	0	0	0	4	3	0	0
2	4	0	1	0	0	1	0	0	0	2	0	0	0	3	1	0	0
3	5	1	1	0	0	0	2	0	0	0	0	0	0	1	0	0	0
4	3	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0

Toxicity observed in overall treatment cycles. In our series, grade 3 leukopenia was noted in 9 cycles (47%), but no grade 4 leukopenia occurred (Table V). Grades 3 and 4 neutropenia were observed in 7 out of 11 (63%) estimated cycles and 4 cycles (36%), respectively. G-CSF was administered in 6 out of 19 (32%) cycles. Only one patient in the second cycle treated with dose level 3 demonstrated grade 3 anemia and thrombocytopenia and none of the patients had any other hematological grade 4 toxicities. Grade 1 diarrhea was observed in 5 out of the 19 cycles and grade 2 diarrhea was observed in 4 out of the 19 cycles (Table VI). Three patients were treated with loperamide and/or hangeshashinto. No grade 3 or 4 non-hematological toxicities occurred in any of the cycles. Fifteen out of the 19 cycles (79%) completed the full scheduled dosage, while CPT-11 and MMC were omitted in 4 cycles (21%) on day 15 because of the delay in the recovery of toxicities.

Discussion

Cervical cancer is the second most common cancer among women worldwide Histological analysis revealed that the incidence of adenocarcinoma has risen in recent years (37, 38). It is commonly accepted that the prognosis for advanced or recurrent cervical adenocarcinoma is poorer than that of squamous cell carcinoma (10, 11, 13). Eifel *et al.* demonstrated that the overall 5-year survival rates for FIGO stage Ib patients with squamous cell carcinoma and adenocarcinoma were 81% and 72%, respectively, and that adenocarcinoma of the cervix had an estimated risk of death 1.9 times that of patients with squamous cell type (11). The efficacy of chemotherapy for advanced or recurrent adenocarcinoma of the cervix has been examined because radiotherapy is less effective. Papadimitriou *et al.* reported that clinical responses of the combination of PTX and CDDP occurred in 6 out of 10 (60%) patients with metastatic and recurrent non-squamous carcinoma (19). However, advanced and recurrent cervical carcinoma in often complicated by ureteral stenosis or obstruction and it seems to be difficult to use CDDP. In our preliminary study (unpublished data), the response rate was 2 out of 17 (11.8%) patients for adenocarcinoma of the cervix treated with CPT-11 and 5-FU. These previous reports of chemotherapy for cervical adenocarcinoma have only been studied in a small population and the efficacy of chemotherapy is controversial. Therefore, a new effective regimen without CDDP would be desirable in patients with non-squamous cell carcinoma of the cervix.

The incidence of clear cell carcinoma is higher in Japan (15-20%) than that in Europe and the United States (5-10%). Sugiyama *et al.* reported that CDDP-based chemotherapy was effective in only 3 out of 27 (11%) cases of clear cell carcinoma, but was effective in 79 out of 109 (73%) cases of serous adenocarcinoma (1). Enomoto *et al.* showed that the response rate with TC was significantly lower in clear cell (18%, 2/11) and mucinous (13%, 1/8) carcinomas than in serous (81%, 61/75) and endometrioid (89%, 16/18) carcinomas (2). Thus, there is a need to evaluate potentially more effective regimens for mucinous and clear cell carcinoma of the ovary.

In the current phase I study against non-squamous cell carcinoma of the cervix and clear cell and mucinous carcinoma of the ovary, DLTs were not observed in any of the three patients studied at dose level 4 (CPT-11, 150 mg/m^2; MMC, 7 mg/m^2; 5-FU, 600 mg/m^2). Therefore, the MTD had not been reached and that dose level was recommended for further study. The incidence of grade 3 leukopenia in the first cycle was 7 out of 14 (50%) cycles. Other grade 3/4 adverse events were not observed. Diarrhea was the most important non-hematological toxic effect of CPT-11. Grade 2 diarrhea occurred in 3 out of 14 (21%) cycles, but no grade 3/4 diarrhea was observed during cycle 1. Only one patient treated with dose level 2 showed grade 1 renal toxicity in the first cycle. Therefore, this new regimen can be well tolerated for gynecological malignancies. In the present series, one patient with cervical adenocarcinoma was diagnosed as FIGO stage Ib with a tumor 8.5 cm in diameter showed a cervical lesion reduced by 18% after 2 cycles of protocol treatment, and was still alive without disease for 61 months after radical surgery. Another patient who had stage IIb disease showed a partial response (50% reduction) after this therapy, and has survived over 25 months without disease after radical surgery and adjuvant radiotherapy. These data suggested that this protocol regimen may be effective for cervical adenocarcinoma.

The combined chemotherapy with CPT-11 (15 mg/m^2 on days 1 and 15), MMC (7 mg/m^2 on days 1 and 15) and 5-FU (600 mg/m^2 from days 3 to day 7) is well tolerated for non-squamous cell carcinoma of the cervix and mucinous and clear cell carcinoma of the ovary. It is possible that this protocol treatment may be an option for gynecological malignancy with renal disorders and platinum resistance. A phase II study using this protocol regimen will be required to evaluate the response rate and survival benefit for advanced or recurrent gynecological malignancies, especially for non-squamous cell carcinoma of the uterine cervix.

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Antiproliferative effects of the major tea polyphenol, (−)-epigallocatechin gallate and retinoic acid in cervical adenocarcinoma

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Abstract

Objective. To investigate the combined effect of the major tea polyphenol, (−)-epigallocatechin gallate (EGCG) and retinoic acid (RA) on cervical adenocarcinoma.

Methods. Cell growth rate was examined after treatment for 4, 7 and 10 days with 0–100 μM EGCG and/or 1 μM RA in two cervical adenocarcinoma cell lines, HeLa and TMCC-1. The effect of EGCG treatment was examined for the induction of apoptosis by DNA ladder assay and caspase-3-related protease activity in cell lysate. Telomerase activity was detected by stretch PCR telomere extension assay. hTERT expression levels were quantified by a real-time PCR system.

Results. Combining EGCG and RA increased the antiproliferative effect in adenocarcinoma cell lines, whereas EGCG or RA treatment alone caused a less sensitive response in these cells. Neither EGCG nor RA treatment alone affected apoptosis and telomerase activity. The combination treatment of EGCG and RA induced apoptosis and inhibited telomerase activity in adenocarcinoma cell lines. These results were consistent with those of an antiproliferative effect of EGCG and/or RA in cervical adenocarcinoma cells.

Conclusion. Our data suggest that EGCG and RA combined to prevent the carcinogenesis of cervical adenocarcinoma, induce apoptosis and inhibit telomerase activity. The treatments of combining EGCG and RA may be effective in preventing or treating cervical adenocarcinoma.
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Keywords: EGCG; Retinoic Acid; Cervical adenocarcinoma

Introduction

Cervical cancer is the second most common cancer in the world. Adenocarcinoma, including adenosquamous carcinoma, represents only 15–20% of all primary carcinoma of the cervix, but this percentage is increasing greatly [1,2]. It has been suggested that adenocarcinoma of the uterine cervix tends to metastasize earlier to lymph nodes and is less sensitive to radiation therapy and to chemotherapy than is squamous cell carcinoma [3,4]. Radical surgery seems to be the treatment of choice for this tumor; however, treatments for advanced or recurring cases have been most often unsuccessful.

Green tea is one of the most common beverages consumed worldwide, and its possible beneficial health effects have received much attention. A number of epidemiological and rodent carcinogenesis studies have provided evidence that green tea has chemopreventive effects for a wide range of malignancies [5–7]. Green tea contains a variety of polyphenols known as catechins. (−)-Epigalocatechin gallate (EGCG) is a major component of polyphenols in green tea [8]. The antitumor effect of EGCG has been demonstrated [5,6,8–11]. Previously, we reported that the treatment with EGCG prevented the carcinogenesis of cervical cancer, induced apoptosis and inhibited telomerase activity [12,13]. It is also reported that EGCG inhibits the growth of human squamous cervical cancer cell lines through apoptosis [12]. EGCG was effective in HPV-18 immortalized endocervical cell lines (premalignant cell) but less effective in cervical adenocarcinoma cell lines. These results suggest that the sensitivity to

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Table 1
Origin and human papillomavirus status of cervical cells and cell lines

Cell line	Origin	HPV DNA type	Tumorigenicity
HEN-18	Endocervix	HPV-18	(−)
HEN-18-S[a]	Endocervix	HPV-18	(−)
HeLa	Cx. adenocarcinoma	HPV-18	(+)
TMCC-1	Cx. adenocarcinoma	HPV-18	(+)

HEN = human endocervical cells; − = negative; + = positive.
[a] Non-tumorigenic, adapted to growth in serum.

EGCG decreases with the progression of the carcinogenic process [12]. The inhibitory effect in cell lines derived from cervical adenocarcinoma cells was less than that from cervical squamous carcinoma cells [12], although several studies have shown that EGCG possesses anticarcinogenic effects in adenocarcinoma, including stomach, colon and breast cancer [5–10]. Recently, Tachibana et al. [14] reported that the combination of EGCG and vitamin A increased expression of the receptor that mediates the anticancer activity of EGCG and enhances the anticancer activity of EGCG.

In the present study, we investigated whether the combined use of EGCG and retinoic acid (RA), the direct acting vitamin A metabolite, possesses growth inhibitory properties in human cervical adenocarcinoma cell lines. Furthermore, we investigated whether the mechanism involves the induction of apoptosis and inhibition of telomerase activity.

Materials and methods

Cells, cell culture and cell growth assay

Primary human end cervical cells (HEN) were prepared as described previously [15,16]. Cervical cell lines are summarized in Table 1. Keratinocyte serum-free medium (K-SFM, GIBCO, Grand Island, NY) was used for growth of HEN; HPV-18-immortalizes HEN. Other cell lines were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (GIBCO). The uterine cervix adenocarcinoma cell line, TMCC-1, was kindly provided by M. Sakamoto (Tokyo Medical College, Tokyo, Japan). For growth assay, EGCG (Wako Pure Chemical Industries, Osaka, Japan) and RA (Sigma, St. Louis, NY) were diluted in 100% ethanol and stored at −20 °C before use. Cells seeded at 2×10^5 cells per 5 cm plate were incubated with or without 0–100 μM EGCG and/or 1 μM all-trans RA, which was changed every other day. Cell growth rate was determined by counting the number of cells with a Coulter counter and expressed with the following formula:

$$\text{Growth rate (\%)} = \frac{\text{cell number (experiment)}}{\text{cell number (control)}}$$

DNA ladder apoptosis assay

Cells were cultured in medium with 0 μM (control) EGCG and 50–100 μM EGCG and/or 1 μM all-trans RA for 4 days. High molecular weight DNA was extracted, resolved by 1.5% agarose gel electrophoresis and stained using an apoptosis ladder detection kit (Wako Pure Chemicals Industries, Osaka, Japan).

Caspase-3 activity

Caspase-3-related protease activity in cell lysate was determined using a caspase-3 assay kit, fluorometric (Sigma, St. Louis, MO). Briefly, cell lysate was mixed with assay buffer and the caspase-3 substrate, Ac-DEVD-AMC, followed by incubation at room temperature for 1.5 h. Absorbance was then read with a plate fluorometer. The excitation and emission wavelengths were 360 nm and 460 nm, respectively. Caspase-3 activity was expressed as the fluorescence value relative to that for untreated cells (control).

Telomerase assay

Telomerase activity was quantified with the overlap extension PCR assay method using TeloChaser (Toyobo Co., Osaka, Japan). Briefly, 2×10^4 cells grown in medium with 0 μM (control) EGCG and 50–100 μM EGCG and/or 1 μM all-trans RA for 4 days were obtained and suspended in Lysis solution. Cell extracts were assayed in extension mixture. After 30 min incubation at 30 °C for telomerase extension, the telomerase products were purified by Clean-Up solution, followed by isopropyl alcohol precipitation. Recovered pellets were mixed with 30 μl of PCR mixture, heated at 95 °C for 150 s and then subjected to 30 PCR cycles of 95 °C for 30 s, 68 °C for 30 s and 72 °C for 45 s. PCR products were resolved by electrophoresis in a 7% polyacrylamide gel and visualized with SYBR Green I nucleic acid stain (Molecular Probes, Inc., Eugene, USA).

Quantitation of hTERT mRNA

hTERT expression levels were quantified by a real-time PCR system, Light Cycler Telo TAGGG hTERT Quantification kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacture's instructions. Briefly, cells grown in medium with 0 μM (control) EGCG and 50–100 μM EGCG and/or 1 μM all-trans RA for 4 days were obtained. Two hundred nanograms total RNA of each sample was analyzed. hTERT encoding mRNA was reversely transcribed and amplified in a one-step RT-PCR protocol using the LightCycler instrument. Relative expression levels were calculated by dividing the amount of the housekeeping gene PBGD. The resulting ratio is an hTERT value normalized to the expression of PBDG. Duplicates for each sample were performed. PCR products were resolved by electrophoresis in a 7% polyacrylamide gel and visualized with SYBR Green I nucleic acid stain (Molecular Probes, Inc., Eugene, USA).

Table 2
Effect of epigallocatechin gallate (EGCG) and/or retinoic acid on cell growth rate of endocervical and cervical adenocarcinoma cell lines

Cell line	Treatment	Growth rate (% of untreated control)		
		Day 4	Day 7	Day 10
HEN-18	EGCG 100 μM	15.4±4.1*	9.5±0.8*	9.0±0.8*
	EGCG 50 μM	35.3±1.8*	10.9±0.3*	8.2±1.4*
	EGCG 10 μM	51.4±4.1*	9.2±4.8*	7.8±1.3*
HEN-18S	EGCG 100 μM	42.1±0.8*	14.6±0.8*	8.6±0.9*
	EGCG 50 μM	94.2±1.8***	68.2±6.9***	66.6±3.1**
	EGCG 10 μM	120±12.6****	89.3±1.5***	91.1±4.3****
TMCC-1	EGCG 100 μM	72.8±4.5***	92.5±1.1**	108±2.1***
	EGCG 50 μM	89.8±4.5****	106.2±2.3**	99.5±3.1****
	RA	79±5.1**	57.7±2.3*	62.2±4.2*
	EGCG 50 μM+ RA	53.0±00**	22.4±00*	25.4±00*
HeLa	EGCG 100 μM	78.3±5.6***	93.2±1.6***	63.1±1.2*
	IEGCG 50 μM	89.5±3.8***	103±1.5****	101.4±3.8****
	RA	66.4±5.8**	80.1±3.8**	78±4.3**
	EGCG 50 μM+ RA	62.1±7.2**	17.5±5.4*	20.2±6.3*

HEN-18 = human papillomavirus type 18-immoratalized human endocervical cell; HEN-18S = non-tumorigenic serum adapted HEC-18; RA = retinoic acid; EGCG = epigallocatechin gallate. The results represent the mean ± standard deviation of percent. P is the statistical significance of difference in cell growth rate between each day treated and untreated control cells. *$P<0.001$, **$P<0.01$, ***$P<0.05$, ****not significant.

Results

To investigate the effect of EGCG and/or RA on premalignant and malignant cervical adeno cell lines (Table 1), we treated cells for 4, 7 and 10 days with EGCG and/or RA. Cell growth inhibition assays demonstrated that the combination of EGCG and RA treatment inhibited 75–80% growth in cervical adenocarcinoma cell lines, HeLa and TMCC-1, whereas EGCG or RA treatment alone caused a less sensitive response in these cells (Table 2). TMCC-1 was less sensitive than HeLa in EGCG treatment, but the inhibitory effect of RA in TMCC-1 was more than that in HeLa.

The effect of EGCG or RA treatment on apoptosis was examined by DNA ladder apoptosis assay. Cells treated with 0 and 100 μM EGCG or 1 μM RA for 4 days showed that neither EGCG nor RA treatment resulted in the formation of DNA fragments in HeLa and TMCC-1 (Fig. 1, lanes 2, 3, 6 and 7). In comparison with these treatments, the combination of EGCG and RA treatment induced the formation of DNA fragments (Fig. 1, lanes 4 and 8). These results are consistent with those of an antiproliferative effect of EGCG and/or RA in these cell lines.

As caspase-3 plays an important role in apoptotic events, the influence of EGCG and RA on the caspase-3 activity was examined by using a specific flurogenic substrate for caspase-3. The TMCC-1 and HeLa treated with the combination of EGCG and RA had significantly higher activity of caspase-3 than that of no treatment (Fig. 2). These results showed that the activation of caspase-3 was associated with the induction of apoptosis in the combination treatment of EGCG and RA.

Telomerase has been proposed to represent a novel and potentially selective target for cancer therapy. It is also reported that the inhibition of telomerase is one of the major mechanisms

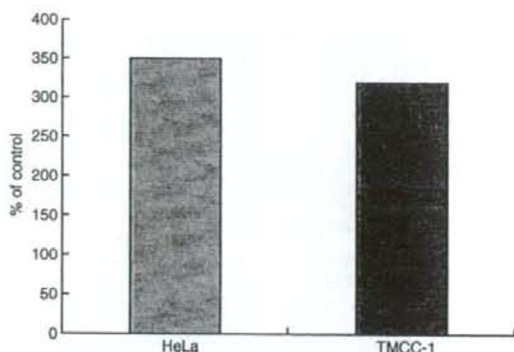

Fig. 2. Effect of epigallocatechin gallate (EGCG) and/or retinoic acid (RA) on caspase-3 activity in HeLa and TMCC-1.

of the anticancer effects of EGCG. We studied whether EGCG and RA inhibit telomerase activity by the stretch PCR assay methods. Telomerase was dramatically decreased in HeLa with the combination of EGCG and RA treatment (Fig. 3, lanes 4). Neither EGCG nor RA treatment inhibited the telomerase activity.

A strong correlation between telomerase activity and the expression level of hTERT mRNA has been described in cancer cells. We investigate hTERT expression level by a real-time PCR system. EGCG and RA treatment showed that hTERT expression was decreased in HeLa and TMCC-1 (Fig. 4, lanes 2 and 4). These

Fig. 1. Effect of epigallocatechin gallate (EGCG) and/or retinoic acid (RA) on apoptosis in TMCC-1 and HeLa. Apoptosis was assayed by DNA ladder formation analysis. TMCC-1 (lanes 1, 2, 3 and 4) and HeLa (lanes 5, 6, 7 and 8) were cultured in medium with EGCG and/or RA. M: 123 bp ladder marker; lanes 1 and 5: control medium; lanes 2 and 6: 1 μM of RA; lanes 3 and 7: 100 μM of EGCG; lanes 4 and 8: 1 μM of RA and 100 μM of EGCG. DNA by ladder formation obtained from cells after EGCG and RA treatment confirmed apoptosis.

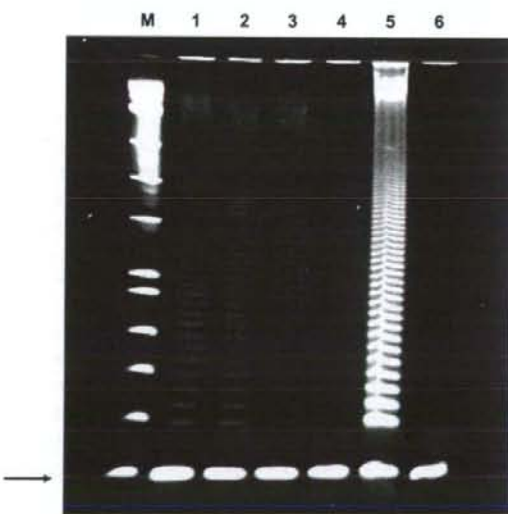

Fig. 3. Effect of epigallocatechin gallate (EGCG) and/or retinoic acid (RA) on telomerase activity in HeLa. Semiquantitative telomerase activity was assayed by stretch PCR assay method. HeLa was cultured in medium with 1 μM of RA and/or 100 μM of EGCG M:φX174/HinfI marker; lane 1: control medium; lane 2: 1 μM of RA; lane 3: 100 μM of EGCG; lane 4: 1 μM of RA and 100 μM of EGCG; lane 5: positive control (HeLa cell); lane 6: negative control. The arrow indicates the internal standard DNA (65 bp).

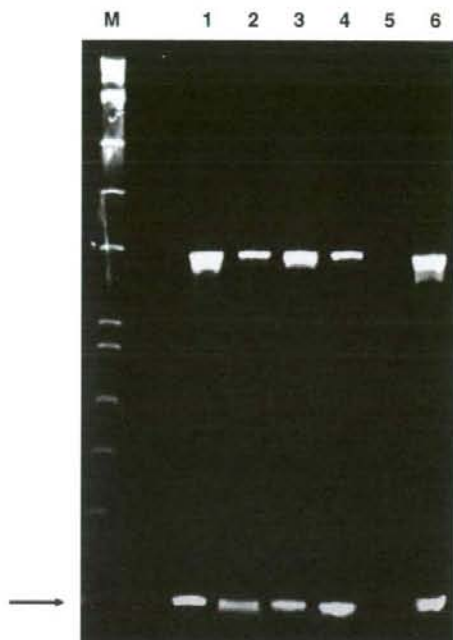

Fig. 4. Effect of epigallocatechin gallate (EGCG) and/or retinoic acid (RA) on the expression of hTERT mRNA in HeLa (lanes 1 and 2) and TMCC-1 (lanes 3 and 4). hTERT expression levels were quantified by a real-time PCR system. Lanes 1 and 3: no treatment; lanes 2 and 4: 1 μM of RA and 100 μM of EGCG; lane 5: negative control; lane 6: positive control (HeLa). The arrow indicates the PBDG.

results are consistent with those of telomerase activity by stretch PCR assay.

Discussion

It has been suggested that cervical adenocarcinoma has relatively more aggressive biological activity, poor prognosis, earlier metastasis and less sensitivity to radiation and chemotherapy, compared to squamous cell carcinoma [3,4]. It is necessary to develop a new anticancer agent for treatment of cervical adenocarcinoma. Polyphenols derived from green tea, particularly EGCG, have been reported to control the proliferation of various cancers and possess the anticarcinogenic and chemopreventive effects in various cancers both in vitro and in vivo [5–7,9–11,17–19]. Previously, we reported that EGCG prevented the carcinogenesis of cervical cancer [12]. It is also reported that the inhibitory effect was less in cervical adenocarcinoma cell lines than squamous cell carcinoma cell lines [12]. EGCG may be effective for treatment of cervical cancer, but, alone, it is inadequate for cervical adenocarcinoma.

Recently, Tachibana et al. [14] reported that all-trans-retinoic acid (RA) enhanced the binding of EGCG to the cell surface of cancer cells. It is also revealed that the cell surface 67-kDa laminin receptor (67 LR) is the target for EGCG and acts as the receptor for antitumor action of EGCG. The growth inhibitory activity of EGCG correlated with the binding strength of EGCG to the cell surface. Our previous study showed that RA EGCG inhibited cell growth for cell lines derived from the endocervix and cervical adenocarcinoma generally less effectively than those derived from their squamous cell counterparts [12]. In the present study, we investigated the efficacy of EGCG and/or RA in cervical adenocarcinoma cells. Our study showed that the combination treatment of EGCG and RA increased the antiproliferative effect in cervical adenocarcinoma cell lines. It is reported that the expression of 67 LR was enhanced by RA treatment [14]. The enhancement of 67 LR by RA treatment may result in the combination effect of EGCG and RA.

The mechanism of cancer inhibition of EGCG is not clear, but several hypotheses have been proposed [20]. It has been reported that EGCG induced apoptosis, and G1 or G2-M arrest of the cell cycle [13,17,21–25]. We previously reported that EGCG treatment resulted in DNA ladder formation in the cell lines from squamous cell, but not the adenocarcinoma counterparts [12]. EGCG treatment induced DNA ladder formation in HPV-18 immortalized endocervical cells, but not in serum adapted HPV-18 immortalized endocervical cells, which confers greater cervical cell growth potential and higher grade cervical lesion [12]. These results supported the hypothesis that the sensitivity to EGCG and induction of apoptosis by EGCG in the carcinogenesis of cervical adenocarcinoma decreases according to the progression to the carcinogenic process. The present study demonstrated that the combination treatment of EGCG and RA induced apoptosis in adenocarcinoma cells. EGCG or RA treatment alone, which was less effective in growth inhibition, did not induce apoptosis. We previously found that RA did not induce apoptosis [26]. These results suggest that the induction of apoptosis by the combination treatment of EGCG and RA is one of the important mechanisms for the EGCG-mediated anticancer effects in cervical adenocarcinoma. The induction of 67 LR by RA may be associated with the induction of apoptosis thorough the enhancement of the binding strength of EGCG to the cell surface. Although the mechanism of EGCG-induced apoptosis is not clear, the activation of caspases has been shown in cancer cells treated with EGCG [27–29]. In the present study, caspase-3 activity was induced by combination treatment of EGCG and RA. These results were consistent with those of an antiproliferative effect of EGCG and/or RA in adenocarcinoma cells.

The activation of telomerase has been proposed to be a critical event in the immortalization of human cells and is characteristic of most human cancer cell lines and tumors, including cervical cancer carcinogenesis [30–32]. It is reported that telomerase inhibition could be one of the major mechanisms in the anticancer effects of EGCG [20,33–36]. Previously, we reported that EGCG treatment inhibited telomerase activity in immortalized cervical cell lines, as well as non-transformed, serum-adapted HPV-18 immortalized endocervical cell lines and transformed HPV-18 immortalized ectocervical cell lines [12]. In this study, we demonstrated that EGCG treatment did not inhibit telomerase activity in cervical adenocarcinoma cells, but the combination treatment of EGCG and RA did. The effect was associated with a decrease of the hTERT expression.

With regard to in vivo studies, EGCG has been reported to prevent the formation of various solid tumors. The effective EGCG

levels were lower than those of in vitro models. The concentration of EGCG shown to have an effect in these previous in vitro studies (10–200 μM) is much higher than those observed in the blood or tissue after drinking tea [37], although the concentration of RA, 1 μM, was a peak plasma level of oral retinoid therapy. There is the disparity between the concentrations needed to achieve the various effects observed in vitro and the plasma levels at which significant anticancer and chemopreventive effects were observed in animal and epidemiological studies. Several mechanisms of cancer inhibition by EGCG in vivo have been proposed [38–40]. It is reported that EGCG inhibited angiogenesis and matrix metalloproteinase in vivo [38,40]. Recently, Tachibana et al. [14] reported that the 67-kDa laminin receptor (67 LR) was associated with EGCG responsiveness to cancer cells at physiologically relevant concentrations. It is also reported that the combination of EGCG and vitamin A increased the expression of the 67 LR that mediates the anticancer activity of EGCG and enhances the anticancer activity of EGCG. Vitamin A in plasma may result in lower levels of effective EGCG in animal and epidemiological studies. Considering our results and these reports, the combination treatment of EGCG and RA could therefore be a promising strategy in treatment for cancer, including cervical adenocarcinoma.

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Uterine Artery Embolization Followed by Dilation and Curettage for Cervical Pregnancy

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BACKGROUND: Cervical pregnancy can be a life-threatening condition due to the risk of severe hemorrhage. Progression of ultrasonographic diagnostic technology has allowed the early detection of cervical pregnancy. However, a standard treatment protocol for fertility preservation has not yet been established.

CASE: Two women with cervical pregnancy presented with cardiac activity at 6 and 7 weeks of gestation. They were treated with transfemoral uterine artery embolization followed by dilation and curettage with minimal bleeding. One patient gave birth to a healthy neonate 20 months after the procedure.

CONCLUSION: Early cervical pregnancies were treated with dilation and curettage after uterine artery embolization. This treatment can be considered as conservative management for patients who desire to preserve their fertility.

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Cervical pregnancy can be a life-threatening condition due to the risk of severe hemorrhage. Cervical pregnancy is the implantation of a developing conceptus in the endocervical mucosa. Diagnosis and treatment of cervical pregnancy has changed dramatically in recent decades. Before 1980, the diagnosis was commonly made when dilation and curettage (D&C) for presumed incomplete abortion resulted in unexpected catastrophic hemorrhage. Emergency hysterectomy usually ensued.[1] Cervical pregnancy is now commonly diagnosed on a routine transvaginal ultrasound tomography examination in first-trimester pregnant patients without bleeding. However, a protocol for conservative management in early cervical pregnancy patients has not been established, as yet.

Below, we present two cases of cervical pregnancy in which uterine artery embolization was performed as a prophylactic procedure before D&C. According to our experience and review of published reports written about the management of cervical pregnancy, new suggestions are made regarding the potential role of uterine artery embolization before D&C in the treatment of cervical pregnancy.

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CASE 1

A 27-year-old, gravida 2, para 0, with an ultrasonographic diagnosis of cervical pregnancy at the sixth week of gestation was referred to our hospital in 2005. She presented with painless fresh vaginal bleeding. Transvaginal ultrasonography showed a yolk sac with a positive fetal heart beat in a 13.2-mm-sized gestational sac located within the cervical canal (Fig. 1.). The urine human chorionic gonadotropin (hCG) was 3,951 milli-International Units/mL. Her vital signs were stable, and findings from a general physical examination were unremarkable. In the uterine cavity, there were no endometrial signs of an intrauterine pregnancy. As the patient expressed a strong desire to preserve her fertility and presented a moderate amount of flesh uterine bleeding, conservative management with uterine artery embolization followed by D&C was planned with informed consent. Under fluoroscopic control, each uterine artery was embolized with minced gel foam. After the procedure, uterine bleeding was immediately decreased. To avoid severe postembolization ischemic pain, continuous epidural analgesic reagent injection was started before uterine artery embolization. Twenty-four hours after the uterine artery embolization, D&C of the cervical pregnancy was performed under general anesthesia. The estimated blood loss was 20 mL. After the evacuation, no uterine bleeding occurred. Three days after D&C, a ultrasound examination showed a normal structure of the uterine cervix and body. Pathological examination of the intracervical curettage specimen confirmed the products of conception. The urinary hCG titer was decreased to normal range within 4 weeks. Seventy-six days after the procedure, the patient resumed regular menstrual cycles.

A natural intrauterine pregnancy was confirmed 12 months after the procedure. The course of pregnancy was unremarkable except the patient received prophylactic cervical cerclage for suspected cervical incompetency with a wedge-shaped cervix detected by transvaginal ultrasound tomography at 19 weeks of gestation on routine screening. At 36 weeks of gestation, her membranes ruptured spontaneously, the cerclage was removed, and she gave birth to a healthy neonate after a spontaneous labor. The newborn (male, weight 3,016 g, Apgar scores 8 and 9) and the mother's course were uneventful after delivery.

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