In consequence, whatever process of tumor shrinkage works, the key to achieving local control of tumors associated with residual tumor masses is to increase the target dose. The use of chemoradiotherapy in place of RT treatment alone likely enhances the RR during EBRT.[22] The effect of chemotherapy on RR was, however, not analyzed in this study because of the small number of patients and the heterogeneity of their backgrounds, such as age. In addition to routine use of chemoradiotherapy, we currently further intensify treatment by performing more than three ICRTs with shortening of the ICRT interval or the addition of interstitial RT for those patients in whom post-EBRT MR imaging reveals a residual mass of substantial size.

Post-ICRT volume correlated significantly with the post-EBRT volume, whereas the pre-RT volume did not predict the post-EBRT volume. This result shows that RR during EBRT represents the RR during both EBRT and ICRT. Therefore, the appropriate timing of MR imaging from the standpoint of clinical practice is immediately before the start of ICRT (post-EBRT), as was done in the present study. This finding supports the results of Mayr et al.[23] Post-EBRT MR imaging is also useful for specifying the target volume in ICRT, whereas post-ICRT imaging is beneficial only in patients with a substantial residual mass observed on post-EBRT MR images.

Recent advances in target volume specification using MR imaging, as well as in dose distribution optimization using a computerized high-dose-rate ICRT unit, have allowed the introduction of image-guided ICRT for cervical cancer.[24–26] Regardless of tumor radioresponse, this and other innovations in ICRT technique can facilitate personalized treatment by increasing the minimum target dose in accordance with the individual target volume and contribute to improvements in local tumor control without an increase in the incidence of complications.

Conclusion

The difference in the impact of physical treatment was not directly shown as a difference in RR of cervical cancer during EBRT and ICRT. This inconsistency probably is due not only to error-causing factors such as approximate tumor-volume estimation but also to biological factors such as tumor clearance mechanisms. Whatever mechanisms work in regard to tumor shrinkage, post-EBRT MR imaging is useful not only for assessing the tumor volume before ICRT (used to estimate the minimum target doses via ICRT) but also for predicting the residual tumor mass after ICRT because a strong correlation was seen between the post-ICRT and post-EBRT tumor volumes.

References

1. Perez CA, Grigsby PW, Chao KS, Mutch DG, Lockett MA. Tumor size, irradiation dose, and long-term outcome of carcinoma of uterine cervix. Int J Radiat Oncol Biol Phys 1998;41:307–17.
2. Eifel PJ, Morris M, Wharton TJ, Oswald MJ. The influence of tumor size and morphology on the outcome of patients with FIGO stage IB squamous cell carcinoma of the uterine cervix. Int J Radiat Oncol Biol Phys 1994;29:9–16.
3. Grossman I, Kurohara SS, Webster JH, Gorge FW 3rd. The prognostic significance of tumor response during radiotherapy in cervical carcinoma. Radiology 1973;107:411–5.
4. Hardt N, van Nagell JR, Hanson M, Donaldson E, Yoneda J, Maruyama Y. Radiation-induced tumor regression as a prognostic factor in patients with cervical cancer. Cancer 1982;49:35–9.
5. Ohara K, Tsunoda H, Tanaka YO, Onishi K, Nemoto K, Hashimoto T, et al. Explanation for the failure of neoadjuvant chemotherapy to improve outcomes in radiotherapy for locally advanced uterine cervical cancer from the standpoint of tumor regression rate. Radiat Med 2007;25:53–9.
6. Hong JH, Chen MS, Lin FJ, Tang SG. Prognostic assessment of tumor regression after external irradiation for cervical cancer. Int J Radiat Oncol Biol Phys 1992;22:913–7.
7. Saibishkumar EP, Patel FD, Sharma SC, Karunanidhi G, Ghoshal S, Kumar V, et al. Prognostic value of response to external radiation in stage IIIB cancer cervix in prediction clinical outcomes: a retrospective analysis of 556 patients from India. Radiother Oncol 2006;79:142–6.
8. Hricak H, Quivey J, Campos Z, Gildengorin V, Hindmarsh T, Bis KG, et al. Carcinoma of the cervix: predictive value of clinical and magnetic resonance (MR) imaging assessment of prognostic factors. Int J Radiat Oncol Biol Phys 1993;27:791–801.
9. Mayr NA, Yuh WT, Zheng J, Ehrhardt JC, Sorosky JI, Magnotta VA, et al. Tumor size evaluated by pelvic examination compared with 3-D MR quantitative analysis in prediction of outcome for cervical cancer. Int J Radiat Oncol Biol Phys 1997;39:395–404.
10. Ohara K, Oki A, Tanaka YO, Onishi K, Fukumitsu N, Hashimoto T, et al. Early determination of uterine cervical squamous cell carcinoma radioresponse identifies high- and low-response tumors. Int J Radiat Oncol Biol Phys 2006;64:1179–82.
11. Ohara K, Tanaka YO, Tsunoda H, Sugahara S, Hashimoto T, Kagei K, et al. Nonoperative assessment of nodal status for locally advanced cervical squamous cell carcinoma treated by radiotherapy with regard to patterns of treatment failure. Int J Radiat Oncol Biol Phys 2003;55:354–61.
12. Nag S, Erickson B, Thomadsen B, Orton C, Demanes JD, Petereit D. The American Brachytherapy Society recommendations for high-dose-rate brachytherapy for carcinoma of the cervix. Int J Radiat Oncol Biol Phys 2000;48:201–11.
13. Gong QY, Tan LT, Romaniuk CS, Jones B, Brunt JN, Roberts N. Determination of tumor regression rates during radiotherapy for cervical carcinoma by serial MRI: comparison of two measurement techniques and examination of intraobserver and interobserver variability. Br J Radiol 1999;72:62–72.

14. Mayr NA, Yuh WT, Taoka T, Wang JZ, Wu DH, Montebello JF, et al. Serial therapy-induced changes in tumor shape in cervical cancer and their impact on assessing tumor volume and treatment response. AJR Am J Roentgenol 2006;187: 65–72.
15. Ohara K, Hayakawa Y, Fuji H, Tatsuzaki H, Itai Y. Impact of biological clearance on tumor radioresponsiveness. Int J Radiat Oncol Biol Phys 1996;34:389–93.
16. West CM, Davidson SE, Roberts SA, Hunter RD. The independence of intrinsic radiosensitivity as a prognostic factor for patient response to radiotherapy of carcinoma of the cervix. Br J Cancer 1997;79:1187–90.
17. Lim K, Chan P, Dinniwell R, Fyles A, Haider M, Cho YB, et al. Cervical cancer regression measured using weekly magnetic resonance imaging during fractionated radiotherapy: radiobiologic modeling and correlation with tumor hypoxia. Int J Radiat Oncol Biol Phys 2007;70:126–33.
18. Lin LL, Yang Z, Mutic S, Miller TR, Grigsby PW. FDG-PET imaging for the assessment of physiologic volume response during radiotherapy in cervix cancer. Int J Radiat Oncol Biol Phys 2006;70:177–181.
19. Paley PJ, Goff BA, Minudri R, Greer BE, Tamimi HK, Koh WJ. The prognostic significance of radiation dose and residual tumor in the treatment of barrel-shaped endophytic cervical carcinoma. Gynecol Oncol 2000;76:373–9.
20. Azria E, Morice P, Haie-Meder C, Thoury A, Pautier P, Lhomme C, et al. Results of hysterectomy in patients with bulky residual disease at the end of chemoradiotherapy for stage IB2/II cervical carcinoma. Ann Surg Oncol 2005;12: 332–7.
21. Eifel PJ, Thoms WW Jr, Smith TL, Morris M, Oswald MJ. The relationship between brachytherapy dose and outcome in patients with bulky endocervical tumors treated with radiation alone. Int J Radiat Oncol Biol Phys 1994;28:113–8.
22. Ohara K, Tanaka YO, Tsunoda H, Oki A, Satoh T, Onishi K, et al. Preliminary estimation of treatment effect on uterine cervical squamous cell carcinoma in terms of tumor regression rate: comparison between chemoradiotherapy and radiotherapy alone. Radiat Med 2005;23:25–9.
23. Mayr NA, Taoka T, Yuh WT, Denning LM, Zhen WK, Paulino AC, et al. Method and timing of tumor volume measurement for outcome prediction in cervical cancer using magnetic resonance imaging. Int J Radiat Oncol Biol Phys 2002; 52:14–22.
24. Nag S, Cardenes H, Chang S, Das IJ, Erickson B, Ibbott GS, et al. Proposed guidelines for image-based intracavitary brachytherapy for cervical cancer: report from image-guided brachytherapy working group. Int J Radiat Oncol Biol Phys 2004;60:1160–72.
25. Haie-Meder C, Pötter R, Van Limbergen E, Briot E, De Brabandere M, Dimopoulos J, et al. Recommendations from Gynaecological (GYN) GEC-ESTRO Working Group (I): concepts and terms in 3D image based 3D treatment planning in cervix cancer brachytherapy with emphasis on MRI assessment of GTV and CTV. Radiother Oncol 2005;74: 235–45.
26. Pötter R, Haie-Meder C, van Limbergen E, Barillot I, De Brabandere M, Dimopoulos J, et al. Recommendations from gynaecological (GYN) GEC-ESTRO working group (II): concepts and terms in 3D image-based 3D treatment planning in cervix cancer brachytherapy—3D dose volume parameters and aspect of 3D image-based anatomy, radiation physics, radiobiology. Radiother Oncol 2006;78:67–77.

Aneuploidy Predicts Outcome in Patients with Endometrial Carcinoma and Is Related to Lack of *CDH13* Hypermethylation

Yutaka Suehiro,[1] Toshiyuki Okada,[1] Takae Okada,[2] Keiko Anno,[3] Naoko Okayama,[1] Koji Ueno,[1] Mikako Hiura,[1] Mikiko Nakamura,[1] Tomoko Kondo,[2] Atsunori Oga,[2] Shigeto Kawauchi,[2] Kei Hirabayashi,[4] Fumitaka Numa,[4] Takehisa Ito,[4] Toshiaki Saito,[5] Kohsuke Sasaki,[2] and Yuji Hinoda[1]

Abstract **Purpose:** Many investigators have reported that aneuploidy detected by flow cytometry is a useful prognostic marker in patients with endometrial cancer. Laser scanning cytometry (LSC) is a technology similar to flow cytometry but is more feasible for clinical laboratory use. We evaluated the usefulness of DNA ploidy detected by LSC as a prognostic marker in patients with endometrial cancer and investigated genetic and epigenetic factors related to aneuploidy.

Experimental Design: Endometrial cancer specimens from 106 patients were evaluated. The methylation status of *CDH13, Rassf1, SFRP1, SFRP2, SFRP4, SFRP5, p16, hMLH1, MGMT, APC, ATM,* and *WIF1* and mutations in the *p53* and *CDC4* genes were investigated. LSC was carried out to determine DNA ploidy. Fluorescence *in situ* hybridization was done with chromosome-specific centromeric probes to assess chromosomal instability.

Results: Univariate and multivariate analyses revealed that *p53* mutation and lack of *CDH13* hypermethylation associated positively with aneuploidy. Univariate analysis showed that aneuploidy, chromosomal instability, and lack of *CDH13* hypermethylation as well as surgical stage were significantly predictive of death from endometrial cancer. Furthermore, multivariate analysis revealed that stage in combination with either DNA aneuploidy or lack of *CDH13* hypermethylation was an independent prognostic factor.

Conclusion: These results suggest that analysis of DNA ploidy and methylation status of *CDH13* may help predict clinical outcome in patients with endometrial cancer. Prospective randomized trials are needed to confirm the validity of an individualized approach, including determination of tumor ploidy and methylation status of *CDH13*, to management of endometrial cancer patients.

Endometrial cancer is one of the most common genital cancers in women worldwide. The highest incidences are observed in western Europe and North America (1), whereas the incidence is lower but rapidly rising in Japan (1, 2). The number of endometrial cancer patients in Japan was estimated as 1.5 to 1.7 per 100,000 women in the 1970s; however, it has been steadily increasing and is now estimated as 6 to 7 cases per 100,000 women in the 2000s (2). Changes in diet and lifestyle related to obesity, a risk factor for endometrial cancer (3), may account for the drastic increase in endometrial cancer in Japan.

Surgical stage is used routinely to guide patient treatment. Histologic grade is also used because the prognosis for low-grade endometrial cancers is better than for high-grade endometrial cancers (4). Improved tumor classification is needed, however, because patients with tumors that are identical in grade and stage often have significantly different clinical outcomes or responses to therapy. In previous studies, flow cytometric analysis of DNA ploidy has been shown to provide stronger, independent prognostic information (5–10). Laser scanning cytometry (LSC) is a technology similar to flow cytometry, but LSC generates data from analysis of successive microscopic fields and is advantageous for certain clinical and research applications (11). In general, the advantages of LSC include reduced specimen size requirements, simplified methodologies, the ability to examine individual cells, microscopically allowing for direct comparison between cytologic morphology and objective fluorescence measurements, and the ability to scan the same cells within an individual specimen repeatedly (11). Although LSC seems more suitable for clinical application, the relation between DNA ploidy detected by LSC and the clinicopathologic features in patients with endometrial cancer has not been studied.

Tumor cells can become aneuploid as a result of aberrant mitotic divisions that are caused by errors in centrosome duplication, chromosome cohesion, spindle attachment, or

Authors' Affiliations: Departments of [1]Laboratory Medicine, [2]Pathology, and [3]Obstetrics and Gynecology, Yamaguchi University Graduate School of Medicine; [4]Department of Obstetrics and Gynecology, Tokuyama Central Hospital, Yamaguchi, Japan; and [5]Department of Gynecology, National Kyushu Cancer Center, Fukuoka, Japan

Received 10/11/07; revised 1/28/08; accepted 2/26/08.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Yutaka Suehiro, Department of Laboratory Medicine, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan. Phone: 81-836-22-2337; Fax: 81-836-22-2338; E-mail: ysuehiro@yamaguchi-u.ac.jp.

doi:10.1158/1078-0432.CCR-07-4609

cytokinesis (12). 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.

In the present study, we evaluated the efficacy of DNA ploidy determined by LSC as a prognostic marker in patients with endometrial cancer and investigated clinicopathologic, genetic, and epigenetic factors related to aneuploidy and patient outcomes. We found that aneuploidy and lack of *CDH13* hypermethylation as well as surgical stage were predictive of death from endometrial cancer and that age, *p53* mutation, and lack of *CDH13* hypermethylation were related to aneuploidy.

Materials and Methods

Specimens. We evaluated endometrial cancer specimens from 106 patients who underwent surgical resection of the uterus. Mean age of the patients was 58.0 y (range, 32-81 y). Mean follow-up time was 34.8 mo (range, 3.2-89.4 mo). Stage, grade, and histologic type were determined with the surgical staging system of the International Federation of Gynecology and Obstetrics (1988) and WHO (1994). All tumors were diagnosed histologically as endometrioid carcinomas of the uterine corpus and included 94 endometrioid adenocarcinomas, 10 adenoacanthomas, and 2 adenosquamous carcinomas. No patient received preoperative neoadjuvant therapy. Normal endometrium was obtained from 27 patients with leiomyoma who underwent hysterectomy. The study was approved by the review board of Yamaguchi University.

Microdissection and DNA extraction. Routine frozen resection specimens were fixed in 99.5% ethanol followed by H&E staining. DNA was prepared from 5-μm microdissected histopathologic sections as described previously (16).

Hypermethylation of genes. Hypermethylation of *CDH13* and *Rassf1* is frequent in endometrial cancer (17). However, association of methylation of *CDH13* and *Rassf1* with clinicopathologic and cytogenetic features in endometrial cancer has not been clarified. This challenged us to investigate the relations. Furthermore, some of endometrial cancers and colon cancers have a common molecular feature such as microsatellite instability via *hMLH1* methylation (18-20). This promoted us to study *hMLH1* methylation status in endometrial cancer followed by methylation of other genes, including *p16, SFRP1, SFRP2, SFRP4, SFRP5, WIF1, APC, ATM,* and *MGMT*, which are methylated frequently in colon cancer (21-26).

The methylation status of *Rassf1, SFRP1, SFRP2, SFRP4, SFRP5, p16, hMLH1, MGMT, ATM,* and *APC* was determined by bisulfite treatment of DNA followed by methylation-specific PCR or combined bisulfite restriction analysis as described previously (21-25, 27).

WIF1 methylation-specific PCR primers for the methylation reaction were 5′-CGTTTTATTGGGCGTATC-3′ (forward) and 5′-CGAAACCAACAATCAACG-3′ (reverse) and for the unmethylation reaction were 5′-GGGTGTTTTATTGGGTGTATT-3′ (forward) and 5′-CTAACAAAACCAACAATCAACA-3′ (reverse; Supplementary Fig. S1).

Methylation status of *CDH13* was assessed by combined bisulfite restriction analysis assay. Primer sequences were 5′-TTTAAAGAAGTAAATGGGATGTT-3′ and 5′-CCAAAACCAATAACTTTACAAA-3′. The PCR product was digested with *Nru*I (TaKaRa). The digested PCR products were separated by electrophoresis on 3% agarose gels. The digested fragments, which represent methylated DNA, were quantitated by densitometry.

DNA from normal lymphocytes was used as the control for unmethylated genes and placental DNA treated with *Sss*I (CpG) methylase (New England Biolabs) was used as the positive control for methylated genes. Each sample was analyzed in duplicate. The criterion for the presence of hypermethylation was detection of a methylated band in both independent methylation-specific PCR assays. In combined bisulfite restriction analysis assay, hypermethylation was defined as ≥14% of methylation for *CDH13* and ≥9% of methylation for *Rassf1* on the bases of upper 95% of mean methylation level of each gene in normal endometrium.

Assay for p53 *and* CDC4 *mutations.* DNA sequencing was used to screen for mutations in the *p53* and *CDC4* genes. For *p53*, four sets of oligonucleotide primers were used to amplify exons 5 to 8 as described previously (Supplementary Table S1; ref. 28).

For *CDC4*, 10 sets of oligonucleotide primers were used to amplify exons 2 to 11 (Supplementary Table S1). PCR products were purified with shrimp alkaline phosphatase and exonuclease I (GE Healthcare) per the manufacturer's instructions. Purified PCR products were sequenced on an ABI Prism 3100 DNA Analyzer with the ABI Prism BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems). Primers used for amplification were also used for sequencing. Sequencing results were analyzed with DNA Sequencing Analysis Software version 5.1 (Applied Biosystems) and Mutation Surveyor (SoftGenetics).

Fluorescence in situ *hybridization analysis.* Fluorescence *in situ* hybridization was carried out on touch smear specimens on cell array glass slides with chromosome-specific centromeric probes for chromosomes 7, 8, 10, 11, and 17 (Abbott Molecular) as described previously (29). Briefly, the DNA probe mixture and 10 μg of Cot-1 DNA (Abbott Molecular) were dissolved in hybridization buffer (Abbott Molecular). The probe mixture was denatured at 73°C for 5 min, applied to the denatured touch smears, and incubated in a moist chamber at 37°C overnight. After the slide was rinsed, nuclei were counterstained with 4′,6-diamidino-2-phenylindole II (Abbott Molecular). The number of nuclear hybridization signals was determined for 100 nuclei from each sample. The variant fraction was defined as the fraction of cells for which the chromosome number differed from the modal chromosome number (30). Unstable chromosomes were tentatively defined as >20% of the average variant fractions of chromosomes 7, 8, 10, 11, and 17, with reference to Lengauer et al. (31) and Yamamoto et al. (32). A tumor was considered to have chromosomal instability (CIN) if the tumor was unstable for more than three chromosomes as described previously (29).

Laser scanning cytometry. LSC was carried out to determine DNA ploidy as described previously (29). Briefly, touch smears fixed with 95% ethanol were stained in 25 μg/mL propidium iodide solution containing 0.1% RNase. A coverslip was put on the slide and sealed with nail polish. DNA content was measured with a laser scanning cytometer (LSC101, Olympus). A DNA histogram was generated, and DNA ploidy was determined. The DNA index was calculated according to published principles (33). Tumors with a DNA index ≤1.2 were categorized as diploid tumors, and those with a DNA index >1.2 were classified as aneuploid tumors (29).

Statistical analysis. Statistical analysis was done with StatView statistical software (SAS). To compare variables, Fisher's exact test, Student's *t* test, and logistic regression method were used. A P value of <0.05 was considered statistically significant. To identify potential distinct subgroups among endometrial cancer patients, we applied an unsupervised hierarchical cluster analysis based on methylation profiling of *CDH13* and *Rassf1*, aneuploidy, and CIN using Euclidean distances and average linkage algorithm (Clustered Image Map program package, CIMminer,[6] ref. 34).

Results

Methylation and mutation profile. Clinicopathologic features, gene hypermethylation, gene mutation, aneuploidy, and CIN profiles in 106 endometrial cancers are shown in Fig. 1. The frequencies of gene hypermethylation in normal

[6] http://discover.nci.nih.gov

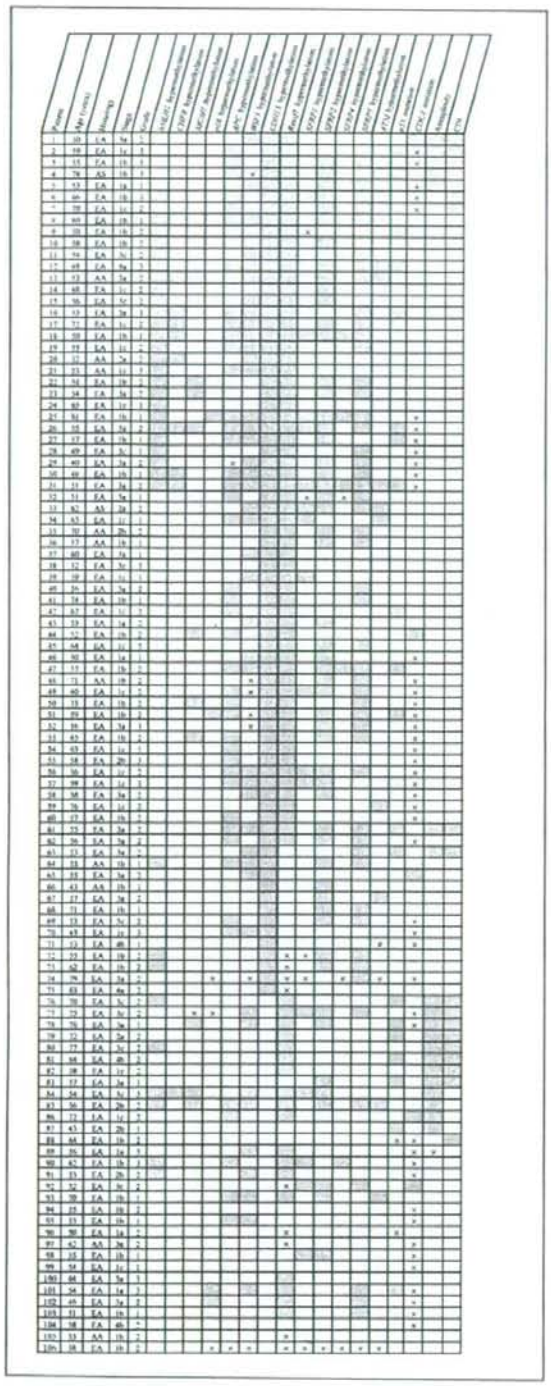

Fig. 1. Diagram of clinicopathologic, epigenetic, and genetic alterations in 106 endometrial cancers. Shading indicates the presence of a molecular alteration, × indicates unavailable data, and an open cell indicates absence of the alteration.

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

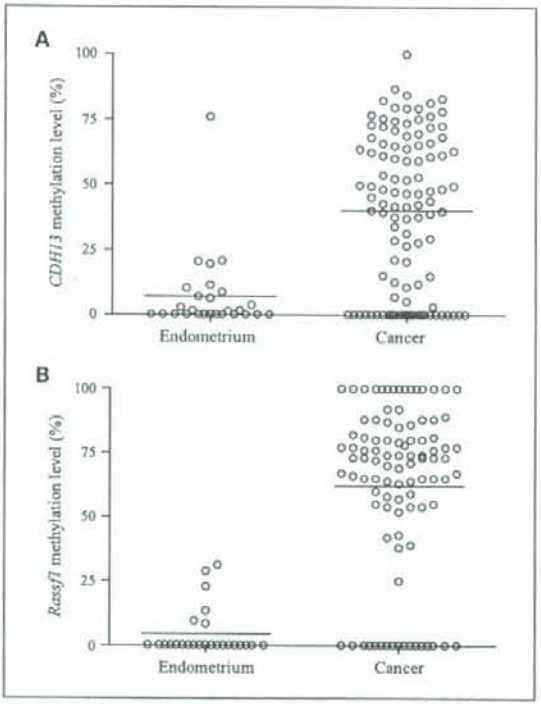

Fig. 2. Distribution of *CDH13* and *Rassf1* methylation (%) in normal endometrium and endometrial cancer. The horizontal lines represent the mean methylation level in each group.

($P < 0.0001$). 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.

References

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer 1999;80:827–41.
2. Aoki D, Hirasawa A, Susumu N, Nozawa S. Outline of treatment for endometrial cancer (in Japanese). Nippon Rinsho 2004;62 Suppl 10:324–8.
3. Chang SC, Lacey JV, Jr., Brinton LA, et al. Lifetime weight history and endometrial cancer risk by type of menopausal hormone use in the NIH-AARP diet and health study. Cancer Epidemiol Biomarkers Prev 2007;16:723–30.
4. Scholten AN, Smit VT, Beerman H, van Putten WL, Creutzberg CL. Prognostic significance and interobserver variability of histologic grading systems for endometrial carcinoma. Cancer 2004;100:764–72.
5. van der Putten HW, Baak JP, Koenders TJ, Kurver PH, Stolk HG, Stolte LA. Prognostic value of quantitative pathologic features and DNA content in individual patients with stage I endometrial adenocarcinoma. Cancer 1989;63:1378–87.
6. Podratz KC, Wilson TO, Gaffey TA, Cha SS, Katzmann JA. Deoxyribonucleic acid analysis facilitates the pretreatment identification of high-risk endometrial cancer patients. Am J Obstet Gynecol 1993;168:1206–13.
7. Susini T, Rapi S, Savino L, Boddi V, Berti P, Massi G. Prognostic value of flow cytometric deoxyribonucleic acid index in endometrial carcinoma: comparison with other clinical-pathologic parameters. Am J Obstet Gynecol 1994;170:527–34.
8. Zaino RJ, Davis AT, Ohlsson-Wilhelm BM, Brunetto VL. DNA content is an independent prognostic indicator in endometrial adenocarcinoma. A Gynecologic Oncology Group study. Int J Gynecol Pathol 1998;17:312–9.
9. Susini T, Amunni G, Molino C, et al. Ten-year results of a prospective study on the prognostic role of ploidy in endometrial carcinoma: DNA aneuploidy identifies high-risk cases among the so-called 'low-risk' patients with well and moderately differentiated tumors. Cancer 2007;109:882–90.
10. Terada K, Mattson D, Goo D, Shimizu D. DNA aneuploidy is associated with increased mortality for stage I endometrial cancer. Gynecol Oncol 2004;95:483–7.
11. Sasaki K, Kurose A, Miura Y, Sato T, Ikeda E. DNA ploidy analysis by laser scanning cytometry (LSC) in colorectal cancers and comparison with flow cytometry. Cytometry 1996;23:106–9.
12. Kops GJ, Weaver BA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. Nat Rev Cancer 2005;5:773–85.
13. Michel LS, Liberal V, Chatterjee A, et al. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. Nature 2001;409:355–9.
14. Dai W, Wang Q, Liu T, et al. Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. Cancer Res 2004;64:440–5.
15. Cahill DP, Lengauer C, Yu J, et al. Mutations of mitotic checkpoint genes in human cancers. Nature 1998;392:300–3.
16. Suehiro Y, Umayahara K, Ogata H, et al. Genetic aberrations detected by comparative genomic hybridization predict outcome in patients with endometrioid carcinoma. Genes Chromosomes Cancer 2000;29:75–82.
17. Fiegl H, Gattringer C, Widschwendter A, et al. Methylated DNA collected by tampons—a new tool to detect endometrial cancer. Cancer Epidemiol Biomarkers Prev 2004;13:882–8.
18. Esteller M, Catasus L, Matias-Guiu X, et al. hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. Am J Pathol 1999;155:1767–72.
19. Esteller M, Levine R, Baylin SB, Ellenson LH, Herman JG. MLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. Oncogene 1998;17:2413–7.
20. Cunningham JM, Christensen ER, Tester DJ, et al. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res 1998;58:3455–60.
21. Akino K, Toyota M, Suzuki H, et al. The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. Gastroenterology 2005;129:156–69.
22. Suzuki H, Watkins DN, Jair KW, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet 2004;36:417–22.
23. Rashid A, Shen L, Morris JS, Issa JP, Hamilton SR. CpG island methylation in colorectal adenomas. Am J Pathol 2001;159:1129–35.
24. Kondo Y, Shen L, Issa JP. Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer. Mol Cell Biol 2003;23:206–15.
25. Esteller M, Sparks A, Toyota M, et al. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res 2000;60:4366–71.
26. Bai AH, Tong JH, To KF, et al. Promoter hypermethylation of tumor-related genes in the progression of colorectal neoplasia. Int J Cancer 2004;112:846–53.
27. Roy K, Wang L, Makrigiorgos GM, Price BD. Methylation of the ATM promoter in glioma cells alters ionizing radiation sensitivity. Biochem Biophys Res Commun 2006;344:821–6.
28. Kim SG, Chan AO, Wu TT, Issa JP, Hamilton SR, Rashid A. Epigenetic and genetic alterations in duodenal carcinomas are distinct from biliary and ampullary carcinomas. Gastroenterology 2003;124:1300–10.
29. Kawasaki K, Suehiro Y, Umayahara K, et al. 11q23-24 loss is associated with chromosomal instability in endometrial cancer. Int J Mol Med 2003;12:727–31.
30. Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. Nature 1997;386:623–7.
31. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature 1998;396:643–9.
32. Yamamoto Y, Matsuyama H, Kawauchi S, et al. Biological characteristics in bladder cancer depend on the type of genetic instability. Clin Cancer Res 2006;12:2752–8.
33. Hiddemann W, Schumann J, Andreef M, et al. Convention on nomenclature for DNA cytometry. Committee on Nomenclature, Society for Analytical Cytology. Cancer Genet Cytogenet 1984;13:181–3.
34. Shen L, Toyota M, Kondo Y, et al. Integrated genetic and epigenetic analysis identifies three different

subclasses of colon cancer. Proc Natl Acad Sci U S A 2007;104:18654–9.

35. Goel A, Nagasaka T, Arnold CN, et al. The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. Gastroenterology 2007;132:127–38.

36. Hedley DW, Clark GM, Cornelisse CJ, Killander D, Kute T, Merkel D. Consensus review of the clinical utility of DNA cytometry in carcinoma of the breast. Report of the DNA Cytometry Consensus Conference. Cytometry 1993;14:482–5.

37. Fattorossi A, Battaglia A, Maggiano N, et al. Laser scanning cytometry can complement the flow cytometric DNA analysis in paraffin-embedded cancer samples: a paradigmatic case. Cytometry 2000;42:123–5.

38. Riou P, Saffroy R, Chenailler C, et al. Expression of T-cadherin in tumor cells influences invasive potential of human hepatocellular carcinoma. FASEB J 2006;20:2291–301.

39. Sato M, Mori Y, Sakurada A, Fujimura S, Horii A. The H-cadherin (CDH13) gene is inactivated in human lung cancer. Hum Genet 1998;103:96–101.

40. Toyooka S, Toyooka KO, Harada K, et al. Aberrant methylation of the CDH13 (H-cadherin) promoter region in colorectal cancers and adenomas. Cancer Res 2002;62:3382–6.

41. Vestal DJ, Ranscht B. Glycosyl phosphatidylinositol-anchored T-cadherin mediates calcium-dependent, homophilic cell adhesion. J Cell Biol 1992;119:451–61.

42. Ivanov D, Philippova M, Tkachuk V, Erne P, Resink T. Cell adhesion molecule T-cadherin regulates vascular cell adhesion, phenotype and motility. Exp Cell Res 2004;293:207–18.

43. Ivanov DB, Philippova MP, Tkachuk VA. Structure and functions of classical cadherins. Biochemistry Mosc 2001;66:1174–86.

44. Weaver BA, Cleveland DW. Does aneuploidy cause cancer? Curr Opin Cell Biol 2006;18:658–67.

45. Jefford CE, Irminger-Finger I. Mechanisms of chromosome instability in cancers. Crit Rev Oncol Hematol 2006;59:1–14.

46. Harada K, Nishizaki T, Ozaki S, Kubota H, Ito H, Sasaki K. Intratumoral cytogenetic heterogeneity detected by comparative genomic hybridization and laser scanning cytometry in human gliomas. Cancer Res 1998;58:4694–700.

47. Qu GZ, Grundy PE, Narayan A, Ehrlich M. Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. Cancer Genet Cytogenet 1999;109:34–9.

48. Yeh A, Wei M, Golub SB, Yamashiro DJ, Murty VV, Tycko B. Chromosome arm 16q in Wilms tumors: unbalanced chromosomal translocations, loss of heterozygosity, and assessment of the CTCF gene. Genes Chromosomes Cancer 2002;35:156–63.

49. Ehrlich M, Woods CB, Yu MC, et al. Quantitative analysis of associations between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. Oncogene 2006;25:2636–45.

50. Widschwendter M, Jiang G, Woods C, et al. DNA hypomethylation and ovarian cancer biology. Cancer Res 2004;64:4472–80.

51. Zhivotovsky B, Kroemer G. Apoptosis and genomic instability. Nat Rev Mol Cell Biol 2004;5:752–62.

52. Duensing A, Duensing S. Guilt by association? p53 and the development of aneuploidy in cancer. Biochem Biophys Res Commun 2005;331:694–700.

53. Lu X, Magrane G, Yin C, Louis DN, Gray J, Van Dyke T. Selective inactivation of p53 facilitates mouse epithelial tumor progression without chromosomal instability. Mol Cell Biol 2001;21:6017–30.

54. Schmitt CA, Fridman JS, Yang M, Baranov E, Hoffman RM, Lowe SW. Dissecting p53 tumor suppressor functions *in vivo*. Cancer Cell 2002;1:289–98.

55. Rajagopalan H, Nowak MA, Vogelstein B, Lengauer C. The significance of unstable chromosomes in colorectal cancer. Nat Rev Cancer 2003;3:695–701.

56. Hubalek MM, Widschwendter A, Erdel M, et al. Cyclin E dysregulation and chromosomal instability in endometrial cancer. Oncogene 2004;23:4187–92.

57. Kemp Z, Rowan A, Chambers W, et al. CDC4 mutations occur in a subset of colorectal cancers but are not predicted to cause loss of function and are not associated with chromosomal instability. Cancer Res 2005;65:11361–6.

ANTICANCER RESEARCH *28*: 2933-2940 (2008)

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

KATSUJI KOKAWA[1], NAOHIKO UMESAKI[1], KAICHIRO YAMAMOTO[2], KEN TAKIZAWA[3], IKUO KONISHI[4] and KAZUO HASEGAWA[5]; JAPAN GYNECOLOGIC ONCOLOGY GROUP

[1]*Department of Obstetrics and Gynecology, Wakayama Medical University, Wakayama;*
[2]*Department of Gynecology, Kinki University Medical School, Sakai;*
[3]*Department of Obstetrics and Gynecology, Cancer Institute Hospital, Tokyo;*
[4]*Department of Obstetrics and Gynecology, Shinshu University Graduate School of Medicine, Matsumoto;*
[5]*Inamino Hospital, Hyogo, Japan*

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).

Correspondence to: Naohiko Umesaki, MD, Department of Obstetrics and Gynecology, Wakayama Medical University, 811-1, Kimiidera, Wakayama 641-0012, Japan. Tel: +81 734410631, Fax: +81 734451161, e-mail: umesaki@wakayama-med.ac.jp

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

0250-7005/2008 $2.00+.40

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.

Acknowledgements

The following institutions where cervical cancer committee members of JGOG participated in this study are acknowledged as follows: Wakayama Medical University, Kinki University Medical School, Mitsui Memorial Hospital, and Shinshu University Graduate School of Medicine.

References

1 Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, Suzuki M, Sato I and Taguchi K: Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. Cancer *88*: 2584-2589, 2000.

2 Enomoto T, Kuragaki C, Yamasaki M, Sugita N, Otsuki Y, Ikegami H, Matsuzaki N, Yamada T, Wakimoto A and Murata Y; for the Gynecologic Oncology Group of Osaka: Is clear cell carcinoma and mucinous carcinoma of the ovary sensitive to combination chemotherapy with paclitaxel and carboplatin? Proc Am Soc Clin Oncol *22*: 444 (#1797), 2003.

3 Ozols RF, Bundy BN, Greer BE, Fowley JM, Clarke-Pearson D, Burger RA, Mannel RS, DeGeest K, Hartenbach EM and Baergen R: Gynecologic Oncology Group: Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group Study. J Clin Oncol *21*: 3194-3200, 2003.

4 du Bois A, Lück HJ, Meier W, Adams HP, Möbus V, Costa S, Bauknecht T, Richter B, Warm M, Schröder W, Olbricht S, Nitz U, Jackisch C, Emons G, Wagner U, Kuhn W and Pfisterer J: Arbeitsgemeinschaft Gynäkologische Onkologie Ovarian Cancer Study Group: A randomized clinical trial of cisplatin/paclitaxel *versus* carboplatin/paclitaxel as first-line treatment of ovarian cancer. J Natl Cancer Inst *95*: 1320-1329, 2003.

5 Shimizu Y, Umezawa S and Hasumi K: A phase II study of combined CPT-11 and mitomycin-C in platinum refractory clear cell and mucinous ovarian cancer. Ann Acad Med Singapore *27*: 650-656, 1998.

6 Smith HO, Tiffany MF, Qualls CR and Key CR: The rising incidence of adenocarcinoma relative to squamous cell carcinoma of the uterine cervix in the United States: a 24-year population-based study. Gynecol Oncol *78*: 97-105, 2000.

7 Keys HM, Bundy BN, Stehman FB, Muderspach LI, Chafe WE, Suggs CL III, Walker JL and Gersell D: Cisplatin, radiation, and adjuvant hysterectomy compared with radiation and adjuvant hysterectomy for bulky stage IB cervical carcinoma. N Engl J Med *340*: 1154-1161, 1999.

8 Green JA, Kirwan JM, Tierney JF, Symonds P, Fresco L, Collingwood M and Williams CJ: Survival and recurrence after concominant chemotherapy and radiotherapy for cancer of the uterine cervix: a systematic review and meta-analysis. Lancet *358*: 781-786, 2001.

9 Lukka H, Hirte H, Fyles A, Thomas G, Elit L, Johnston M, Fung MF and Browman G: Cancer Care Ontario Practice Guidelines Initiative Gynecology Disease Site Group: Concurrent cisplatin-based chemotherapy plus radiotherapy for cervical cancer - a meta-analysis. Clin Oncol *14*: 203-212, 2002.

10 Berek JS, Castaldo TW, Hacker NF, Petrilli ES, Lagasse LD and Moore JG: Adenocarcinoma of the uterine cervix. Cancer *48*: 2734-2741, 1981.

11 Eifel PJ, Burke TW, Morris M and Smith TL: Adenocarcinoma as an independent risk factor for disease recurrence in patients with stage IB cervical carcinoma. Gynecol Oncol *59*: 38-44, 1995.

12 Burke TW, Hoskins WJ, Heller PB, Shen MC, Weiser EB and Park RC: Clinical patterns of tumor recurrence after radical hysterectomy in stage IB cervical carcinoma. Obstet Gynecol *69*: 382-385, 1987.

13 Shingleton HM, Bell MC, Fremgen A, Chmiel JS, Russell AH, Jones WB, Winchester DP and Clive RE: Is there really a difference in survival of women with squamous cell carcinoma, adenocarcinoma, and adenosquamous cell carcinoma of the cervix? Cancer *76*: 1948-1955, 1995.

14 Peters WA III, Liu PY, Barrett RJ II, Stock RJ, Monk BJ, Berek JS, Souhami L, Grigsby P, Gordon W Jr and Alberts DS: Concurrent chemotherapy and pelvic radiation therapy compared with pelvic radiation therapy alone as adjuvant therapy after radical surgery in high-risk early-stage cancer of the cervix. J Clin Oncol *18*: 1606-1613, 2000.

15 Huang HJ, Chang TC, Hong JH, Tseng CJ, Chou HH, Huang KG and Lai CH: Prognostic value of age and histologic type in neoadjuvant chemotherapy plus radical surgery for bulky (> 4 cm) stage IB and IIA cervical carcinoma. Int J Gynecol Cancer *13*: 204-211, 2003.

16 Umesaki N, Izumi R, Fushiki H, Hasegawa K, Kono I, Nishida M, Noguchi H, Okuda H, Sugimori H, Takizawa K, Udagawa Y, Yamamoto K, Tanaka T and Noda K: Cervical adenocarcinoma, a novel combination chemotherapy with mitomycin C, etoposide, and cisplatin for advanced or recurrent disease. Gynecol Oncol *75*: 142-144, 1999.

17 Lissoni A, Gabriele A, Gorga G, Tumolo S, Landoni F, Mangioni C and Sessa C: Cisplatin-, epirubicin- and paclitaxel-containing chemotherapy in uterine adenocarcinoma. Ann Oncol *8*: 969-972, 1997.

18 Thigpen JT, Blessing JA, Fowler WC Jr and Hatch K: Phase II trials of cisplatin and piperazinedione as single agents in the treatment of advanced or recurrent non-squamous cell carcinoma of the cervix: a Gynecologic Oncology Group study. Cancer Treat Rep *70*: 1097-1100, 1986.

19 Papadimitriou CA, Sarris K, Moulopoulos LA, Fountzilas G, Anagnostopoulos A, Voulgaris Z, Gika D, Giannakoulis N, Diakomanolis E and Dimopoulos MA: Phase II trial of paclitaxel and cisplatin in metastatic and recurrent carcinoma of the uterine cervix. J Clin Oncol *17*: 761-766, 1999.

20 Wall ME, Wani MC, Cook CE and Palmar KH: Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. J Am Chem Soc *88*: 3888-3890, 1996.

21 Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka T and Mutai M: Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-10-piperidino]carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin, against murine tumors. Cancer Res *47*: 5944-5947, 1987.

22 Matsuzaki T, Yokokura T, Mutai M and Tsuruo T: Inhibition of spontaneous and experimental metastasis by a new derivative of camptothecin, CPT-11, in mice. Cancer Chemother Pharmacol *21*: 308-312, 1988.

23 Irvin WP, Price FV, Bailey H, Gelder M, Rosenbluth R, Durivage HJ and Potkul RK: A phase II study of irinotecan (CPT-11) in patients with advanced squamous cell carcinoma of the cervix. Cancer *82*: 328-333, 1998.

24 Sugiyama T, Yakushiji M, Noda K, Ikeda M, Kudoh R, Yajima A, Tomoda Y, Terashima Y, Takeuchi S, Hiura M, Saji F, Takahashi T, Umesaki N, Sato S, Hatae M and Ohashi Y: Phase II study of irinotecan and cisplatin as first-line chemotherapy in advanced or recurrent cervical cancer. Oncology *58*: 31-37, 2000.

25 Umesaki U, Fujii T, Nishimura R, Tanaka T, Nishida M, Fushiki H, Takizawa K, Yamamoto K, Hasegawa K and Izumi R: Phase II study of irinotecan combined with mitomycin-C for advanced or recurrent squamous cell carcinoma of the uterine cervix: the JGOG study. Gynecol Oncol *95*: 127-132, 2004.

26 Kokawa K, Nishimura R, Fujii T and Umesaki N: Neoadjuvant chemotherapy with irinotecan and mitomycin-C for locally advanced squamous cell carcinoma of the uterine cervix. Anticancer Res *27*: 2721-2728, 2007.

27 Sugiyama T, Yakushiji M, Kamura T, Ikeda M, Umesaki N, Hasegawa K, Ishikawa M, Saji F, Hiura M, Takahashi T, Sato S, Ochiai K, Kikkawa F, Takeuchi S, Ohashi Y and Noda K for the Japan CPT-11 Study Group: Irinotecan (CPT-11) and cisplatin as first-line chemotherapy for advanced ovarian cancer. Oncology *63*: 16-22, 2002.

28 Nishino K, Aoki Y, Amikura T, Obata H, Sekine M, Yahata T, Fujita K and Tanaka K: Irinotecan hydrochloride (CPT-11) and mitomycin C as the first-line chemotherapy for ovarian clear cell adenocarcinoma. Gynecol Oncol *97*: 893-897, 2005.

29 Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N, Elfring GL and Miller LL: Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med *343*: 905-914, 2000

30 Saltz LB, Douillard JY, Pirotta N, Alakl M, Gruia G, Awad L, Elfring GL, Locker PK and Miller LL: Irinotecan plus fluorouracil/leucovorin for metastatic colorectal cancer: a new survival standard. Oncologist *6*: 81-91, 2001.

31 Fuchs CS, Marshall J, Mitchell E, Wierzbicki R, Ganju V, Jeffery M, Schulz J, Richards D, Soufi-Mahjoubi R, Wang B and Barrueco J: Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: results from the BICC-C Study. J Clin Oncol *25*: 4779-4786, 2007.

32 Look KY, Blessing JA, Valea FA, McGehee R, Manetta A, Webster KD and Andersen WA: Phase II trial of 5-fluorouracil and high-dose leucovorin in recurrent adenocarcinoma of the cervix: a Gynecologic Oncology Group study. Gynecol Oncol *67*: 255-258, 1997.

33 Hara K, Iwasaka T, Matsuo N, Nakao Y, Yokoyama M, Yamasaki F, Miura M and Sugimori H: Effects of single and combined application of anti-cancer drugs on cervical adenocarcinoma. I. Antitumor activity *in vitro*. Acta Obstet Gynecol Scand *74*: 330-335, 1995.

34 Villalona-Calero MA and Kolesar JM: Mitomycin as a modulator of irinotecan anticancer activity. Oncology *16*: 21-25, 2002.

35 Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET and Carbone PP: Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol *5*: 649-655, 1982.

36 Tobina K, Kohno A, Shimada Y, Watanabe T, Tamura T, Takeyama K, Narabayashi M, Fukutomi T, Kondo H, Shimoyama M and Suematu K; members of the Clinical Trial Review Committee of the Japan Clinical Oncology Group: Toxicity grading criteria of the Japan Clinical Oncology Group. JPN J Clin Oncol *23*: 250-257, 1993.

37 Pamela G, Hai-Yen Sung, Sawaya GF. Changes in cervical incidence after three decades of screening US women less than 30 years old. Obstet Gynecol *102*: 765-773, 2003.

38 Bulk S, Visser O, Rozendaal L, Verheijen RHM, Meijer CJLM. Cervical cancer in the Netherlands 1989-1998: Decrease of squamous cell carcinoma in older women, increase of adenocarcinoma in younger women. Int J Cancer *113*: 1005-1009, 2005.

Received April 22, 2008
Revised June 24, 2008
Accepted July 1, 2008

Available online at www.sciencedirect.com
ScienceDirect
Gynecologic Oncology 108 (2008) 326–331
Gynecologic Oncology
www.elsevier.com/locate/ygyno

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

Msatoshi Yokoyama [a,*], Mitsuyo Noguchi [a], Yoshifumi Nakao [a], Makio Ysunaga [a], Fumio Yamasaki [b], Tsuyoshi Iwasaka [a]

[a] *Department of Obstetrics and Gynecology, Faculty of Medicine, Saga University, 5-1-1, Nabeshima, Saga, 849-8501, Japan*
[b] *Department of Pathology, Saga Medical School Hospital, Saga, Japan*

Received 18 August 2007
Available online 26 November 2007

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.

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

* Corresponding author. Fax: +81 0952 34 2057.
E-mail address: yokoyam1@cc.saga-u.ac.jp (M. Yokoyama).

0090-8258/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygyno.2007.10.013

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*	Endocervix	HPV-18	(−)
HeLa	Cx, adenocarcinoma	HPV-18	(+)
TMCC-1	Cx, adenocarcinoma	HPV-18	(+)

HEN=human endocervical cells; −=negative; +=positive.
* 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

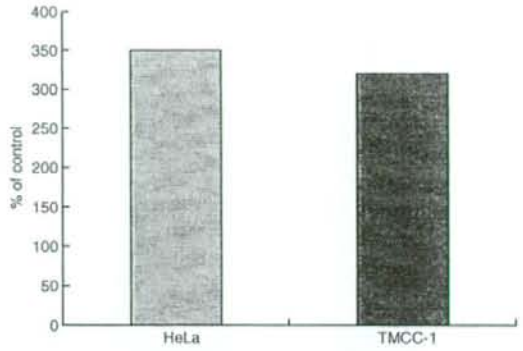

Fig. 2. Effect of epigallocatechin gallate (EGCG) and/or retinoic acid (RA) on caspase-3 activity in HeLa and TMCC-1.

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

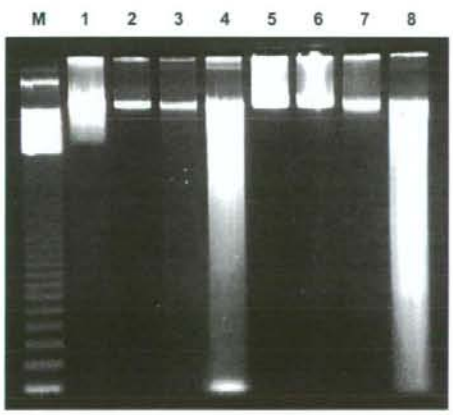

Fig. 1. Effect of epigallocatechin gallate (EGCG) and/or retinoic acid (RA) on apoptosis in TMCC-1 and HeLa. Apoptosis was assayed by DNA ladder formation analysis. TMCC-1 (lanes 1, 2, 3 and 4) and HeLa (lanes 5, 6, 7 and 8) were cultured in medium with EGCG and/or RA. M: 123 bp ladder marker; lanes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

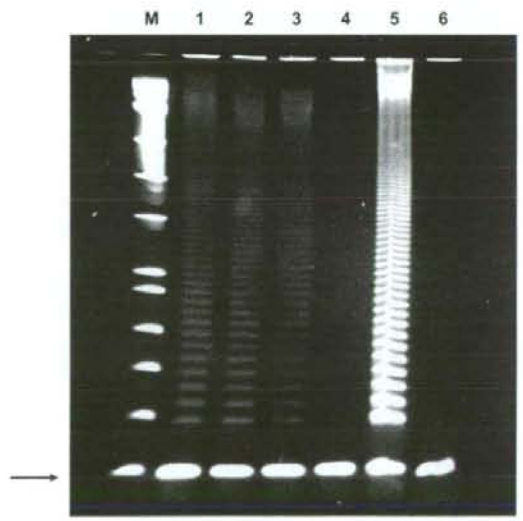

Fig. 3. Effect of epigallocatechin gallate (EGCG) and/or retinoic acid (RA) on telomerase activity in HeLa. Semiquantitative telomerase activity was assayed by stretch PCR assay method. HeLa was cultured in medium with 1 μM of RA and/or 100 μM of EGCG M:φX174/Hinf I marker; lane 1: control medium; lane 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).