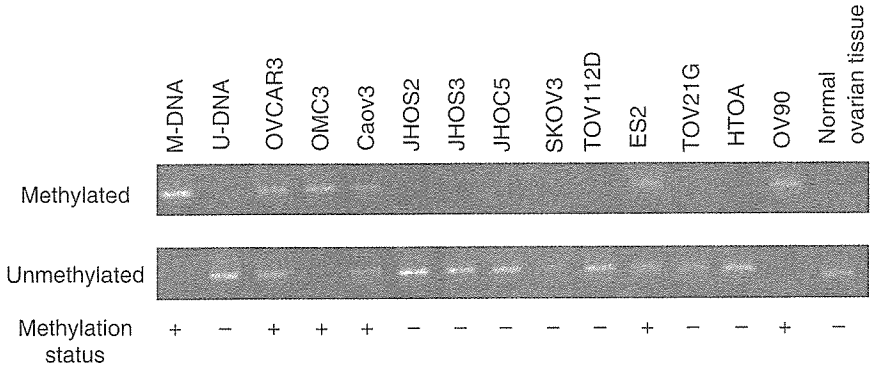

Fig. 1. Methylation status of the Cyclin D2 gene in ovarian cancer cell lines and a normal ovarian tissue. The 101-bp bands in the 'Methylated' lanes indicate the presence of methylated alleles of the Cyclin D2 gene. The 106-bp bands in the 'Unmethylated' lanes correspond to the unmethylated alleles. Methylation status is denoted as follows: +, methylated alleles with or without unmethylated alleles; −, purely unmethylated alleles. M-DNA, universal methylated human male genomic DNA, was used for positive control of methylated reaction. U-DNA, universal unmethylated fetal genomic DNA, was used for positive control of unmethylated reaction.

Table 1. Patient characteristics and cyclin D2 methylation status

Variable	*n*	Cyclin D2 methylation			*P*-value
		+	−	%	
Age (years)					
<50	29	8	21	27.6	
≥50	42	8	34	19	NS
Performance status†					
0–1	51	9	42	17.6	
2–4	19	7	12	36.8	NS
FigO stage					
I, II	35	4	31	2.9	
III, IV	36	12	24	33.3	0.027
Histological type of adenocarcinoma					
Serous	26	6	20	23.1	
Endometrioid	15	3	12	20	
Mucinous	7	3	4	75	
Clear cell	23	4	19	17.4	NS
Grade					
1	24	5	19	20.8	
2	22	7	15	31.8	
3	17	3	14	17.6	NS
Residual tumor size (cm)					
<2	47	7	40	14.9	
≥2	24	9	15	37.5	0.031
Ki-67 labeling index (median)		21.6	23.6	20.4	NS

†0, asymptomatic and fully active; 1, symptomatic, fully ambulatory, restricted in physically strenuous activity; 2, symptomatic, ambulatory, capable of self-care, more than 50% of walking hours are spent out of bed; 3, symptomatic, limited self-care, more than 50% of time is spent in bed, but not bedridden; 4, completely disabled, no self-care, bedridden.

detected in five of 12 cell lines, three of which also contained the unmethylated band, as shown in Fig. 1. The methylated band was detected in two of five cell lines derived from serous adenocarcinoma (Caov3, OV90), in one of three cell lines from clear cell carcinoma (ES2), in the one mucinous adenocarcinoma (OMC3), but not in the endometrioid adenocarcinoma. The normal ovarian tissue was negative for the methylated band. The methylated band was detected in 16 of the 71 surgical specimens (6/26 serous, 4/23 clear cell, 3/15 endometrioid and 3/7 mucinous adenocarcinoma), as shown in Table 1.

Expression of the Cyclin D2 gene in ovarian cancer cell lines and normal ovarian tissue. The expression of the Cyclin D2 gene in the cell lines is presented in Fig. 2. Quantitative RT-PCR was carried out and the ratio of Cyclin D2 to β-actin was calculated to allow for comparison among the cell lines. The median value of relative Cyclin D2 gene expression in cell lines with methylation (0.015) tended to be lower than that in cell lines without methylation (0.03), although the difference was not significant ($P = 0.19$, Mann–Whitney U-test). The expression level of the Cyclin D2 gene in normal ovarian tissue was relatively high compared with ovarian cancer cell lines.

Effects of 5azaC and TSA treatment on methylated cell lines. To confirm that promoter methylation contributed to the loss of Cyclin D2 gene expression, we assessed the effect of 5azaC, a demethylating agent, on Cyclin D2 mRNA expression by quantitative RT-PCR. OMC3 and OVCAR3 cells, which were positive for the methylated band in MSP, were treated. From MSP analysis OMC3 had only methylated alleles, but OVCAR3 had both methylated and unmethylated alleles. We also assessed the effect of TSA, a histone deacetylase inhibitor, to investigate whether another epigenetic change, histone deacetylation, contributed to the silencing of Cyclin D2 gene expression. Treatment of OMC3 cells with 5azaC for 5 days led to a 2.64-fold increase in expression (Fig. 3a). Treatment of OVCAR3 cells with 5azaC for 5 days resulted in a 222-fold increase in expression (Fig. 3b). Treatment with TSA also contributed to re-expression of the Cyclin D2 gene in OMC3 and OVCAR3 cells (2.3-fold and 119-fold, respectively) (Fig. 3). These results suggested that the decreased expression of Cyclin D2 in these cell lines was related to epigenetic change, including DNA methylation or histone deacetylation.

The effects of 5azaC and TSA on cell growth are summarized in Fig. 4. Compared with cell growth in control culture, cell growth with 5azaC or TSA treatment was suppressed in each culture. These chemical agents resulted in inhibition of cell growth in these ovarian cancer cell lines simultaneous with re-expression of the Cyclin D2 gene.

Effects of 5azaC and TSA treatment on unmethylated cell lines. In the MSP and quantitative RT-PCR analyses, expression of the Cyclin D2 gene was decreased in some cell lines without promoter methylation. We assessed the effect of 5azaC or TSA treatment in these cell lines (JHOS2, JHOC5 and SKOV3) to investigate the participation of epigenetic change in the silencing of this gene. Treatment of JHOS2 cells with TSA resulted in higher re-expression than treatment with 5azaC (Fig. 5a). Treatment of JHOC5 cells with TSA for 16 h resulted in an 84.4-fold increase in expression, and treatment with 5azaC also led to a 137-fold increase in expression (Fig. 5b). As for SKOV3 cells, treatment with TSA did not increase the expression of this gene. These results suggest that histone deacetylation may contribute to silencing of the Cyclin D2 gene in JHOS2 and JHOC5 cells, but not in SKOV3.

Correlation between clinicopathological parameters and methylation status of Cyclin D2 in epithelial ovarian cancer. The clinicopathological parameters relative to the methylation status of Cyclin D2 are presented in Table 1. Methylation status was significantly associated with advanced stage and residual tumor size >2 cm.

doi: 10.1111/j.1349-7006.2007.00394.x

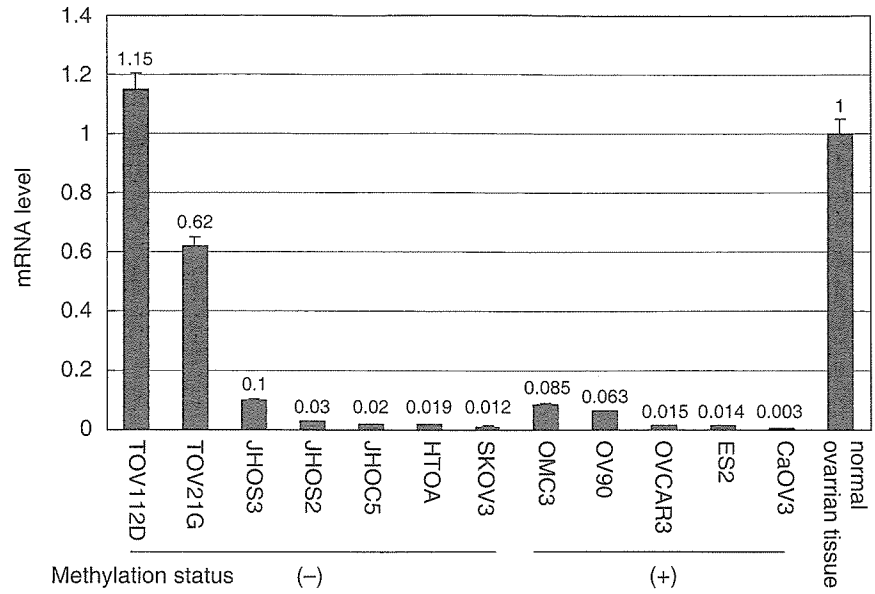

Fig. 2. Expression of the Cyclin D2 gene in ovarian cancer cell lines and normal ovarian tissue. Two independent reverse transcription–polymerase chain reactions were carried out for each sample, and the ratio of Cyclin D2: β-actin was calculated and normalized with the level of normal ovarian tissue. Methylation status is indicated in the same way as in Fig. 1.

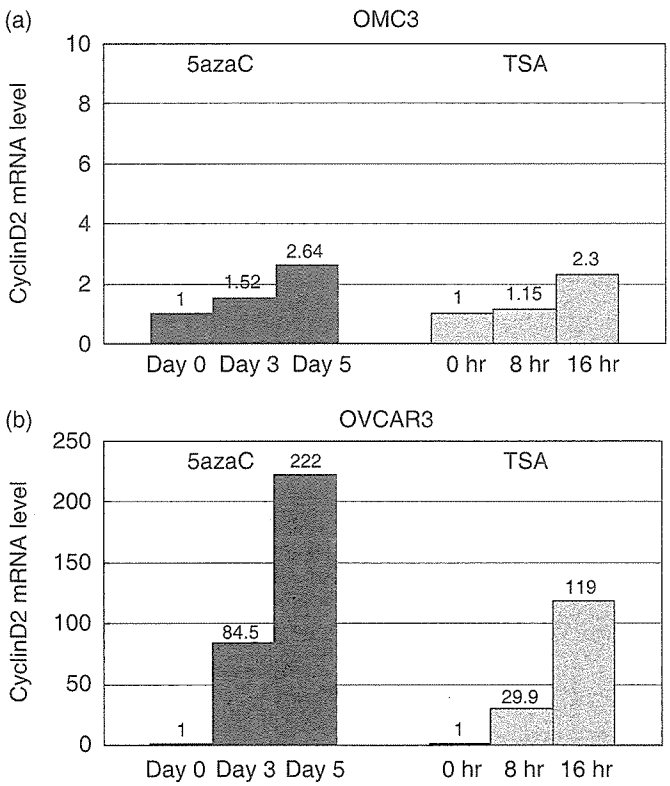

Fig. 3. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in OMC3 and OVCAR3 cells following treatment with (a) 5-aza-2′-deoxycitidine (5azaC) or (b) trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and normalized with the level before treatment.

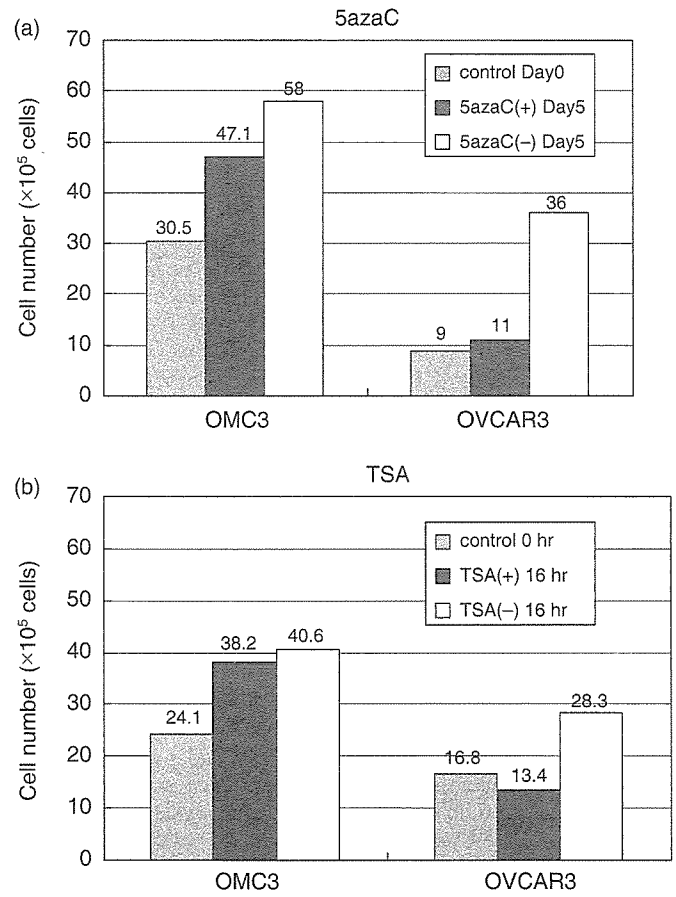

Fig. 4. Cell number of OMC and OVCAR3 cells following treatment with (a) 5-aza-2′-deoxycitidine (5azaC) or (b) trichostatin A (TSA). *Control treatment with medium alone.

There was no association between methylation status and age, performance status, histological type, histological grade or Ki-67 labeling index

The results of the univariate analysis of prognostic significance for each variable with respect to survival are summarized in Tables 2 and 3. Of the clinicopathological parameters evaluated, performance status, stage, histological grade and residual tumor size were significantly associated with disease-free and overall survival. The methylation status of Cyclin D2 was significantly associated with disease-free survival; the cases with methylation had significantly worse rates of disease-free survival than those without methylation (Fig. 6; $P = 0.021$). With

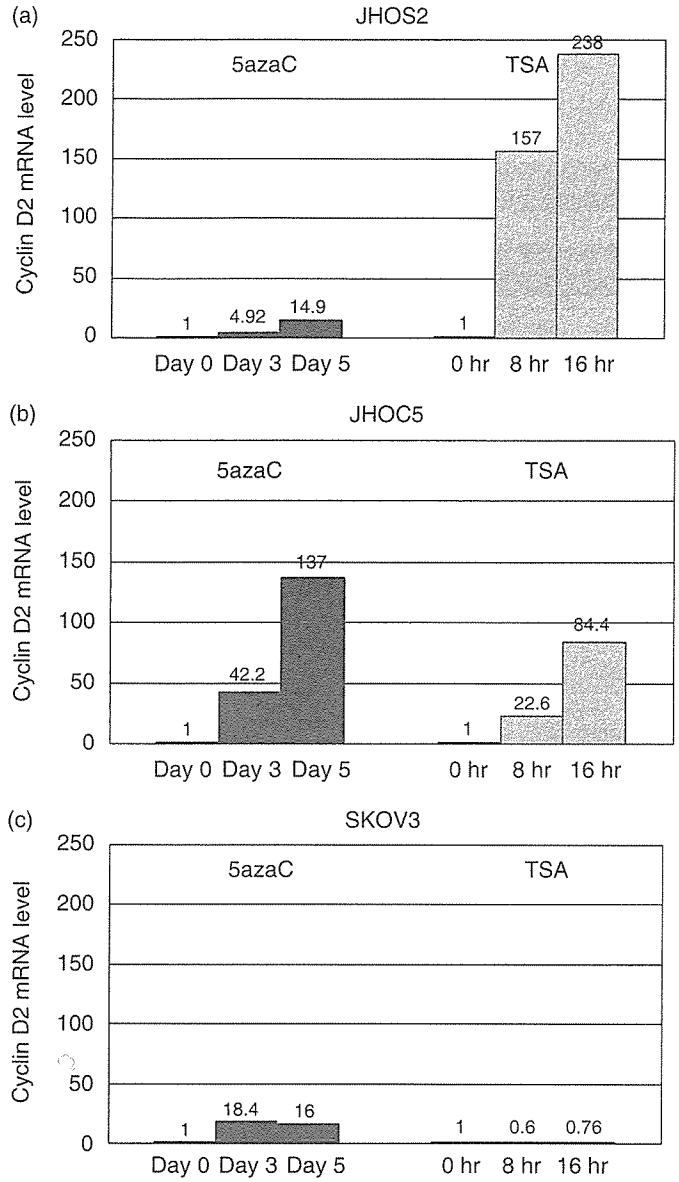

Fig. 5. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in (a) JHOS2, (b) JHOC5 and (c) SKOV3 cells following treatment with 5-aza-2′-deoxycitidine (5azaC) or trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and normalized with the level before treatment.

regard to overall survival, methylated cases had a worse prognosis than unmethylated cases, but the difference was not significant (Fig. 7; $P = 0.063$). In multivariate analysis, methylation status of cyclin D2 turned out not to be an independent prognostic factor (data not shown).

Discussion

Aberrant promoter methylation is found in many types of human cancer and is a common mechanism for transcriptional inactivation of various genes, including tumor suppressor genes, DNA repair genes, cell cycle regulatory genes and apoptosis-related genes. In the present study, we determined the Cyclin D2 promoter methylation status of several ovarian cancer cell lines and ovarian cancer surgical specimens, measured the levels of Cyclin D2 gene expression in ovarian cancer cell lines and

Table 2. Univariate analysis of disease-free survival

Variable	*P*-value
Cyclin D2 methylation status	0.0212
Age	0.6657
Performance status	<0.0001
FigO stage	0.0001
Histological type	0.4709
Grade	0.1332
Residual tumor	0.0008

Table 3. Univariate analysis of overall survival

Variable	*P*-value
Cyclin D2 methylation status	0.0625
Age	0.4195
Performance status	0.0003
FigO stage	0.0003
Histological type	0.0637
Grade	0.1983
Residual tumor	0.0016

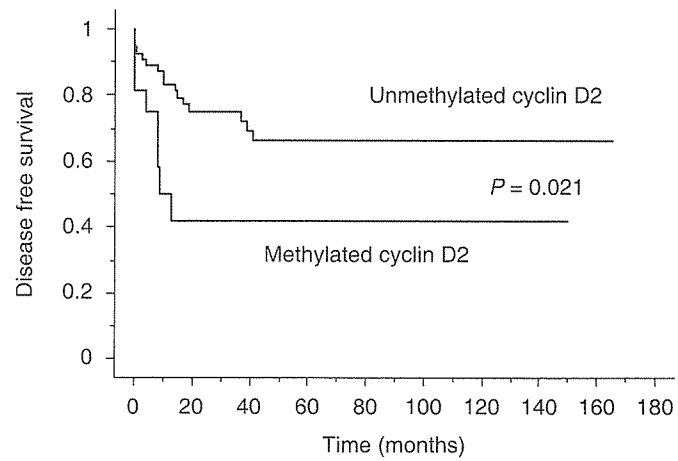

Fig. 6. Association between Cyclin D2 promoter methylation status and disease-free survival in patients with epithelial ovarian cancer.

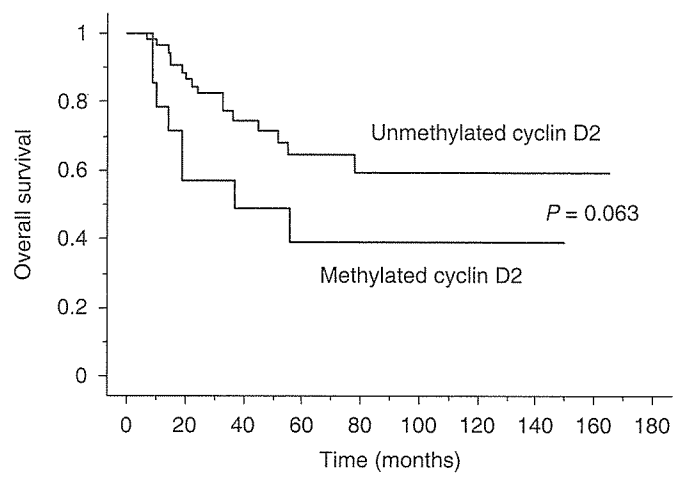

Fig. 7. Association between Cyclin D2 promoter methylation status and overall survival in patients with epithelial ovarian cancer.

doi: 10.1111/j.1349-7006.2007.00394.x

linked the methylation status of the Cyclin D2 promoter to various clinical and pathological variables in ovarian cancer patients.

From MSP and quantitative RT-PCR analysis, there was a trend towards a reduction in gene expression in the presence of hypermethylation; however, this association was not significant, and it was suggested that expression of the Cyclin D2 gene in ovarian cancer cell lines, as a whole, was considerably low in comparison with that in normal ovarian tissue. There was an increase in Cyclin D2 gene expression following the 5azaC treatment of cell lines with promoter methylation of the Cyclin D2 gene in MSP. However, TSA or 5azaC treatment of the cell lines without methylation in MSP resulted in re-expression of the Cyclin D2 gene. Together with these findings, it is suggested that some epigenetic changes, including promoter methylation or histone deacetylation, might contribute to silencing of the Cyclin D2 gene in epithelial ovarian cancer cell lines. The re-expression by treatment with 5azaC in the unmethylated cell lines JHOS2 and JHOC5 suggests that the Cyclin D2 gene may be secondary re-expressed owing to activating other suppressed gene by promoter methylation with treatment of 5azaC, or there is a possibility that aberrant methylation did exist but in a different region of the Cyclin D2 promoter to that which we analyzed. Further investigation and data regarding the acetylation status of histones, a different DNA methylation analysis to decipher the MSP results, and DNA methylation of the transcription factor of Cyclin D2 are needed to supplement our hypothesis.

Epithelial ovarian cancer cell growth following treatment with 5azaC or TSA was suppressed in OMC3 and OVCAR3 cell lines. Treatment with these chemical agents resulted in inhibition of cell growth as well as re-expression of the Cyclin D2 gene. However, another tumor suppressor gene was also re-expressed by these treatments, and these chemicals could have cell toxicity in itself[26–28]. The present data suggests that 5azaC and TSA could be therapeutic agents targeting epigenetic changes in epithelial ovarian cancer, and epigenetic gene silencing of the Cyclin D2 gene could used as a marker of tumor growth.

The D-type cyclins are early checkpoint regulators at the G_1 phase of the cell cycle. Although well known for their proliferation-promoting activity, the D-type cyclins also have growth-inhibitory effects.[14] Thus, decreased expression of Cyclin D2 could result in abnormal cell proliferation and contribute to malignant transformation. Indeed, Cyclin D2 gene silencing secondary to DNA promoter methylation has been demonstrated in several human cancers.[15–17,29] Cyclin D2 promoter hypermethylation has also been detected in nearly half of breast cancers and is associated with gene silencing. Cyclin D2 hypermethylation has also been demonstrated in small cell and non-small cell lung cancer tumor tissues and cell lines,[17] and in approximately half of gastric cancer specimens.[16] In the present study, 22.5% of the surgical specimens and 41.7% of the cell lines had aberrant Cyclin D2 promoter hypermethylation. Our results, though somewhat higher than what has been reported for ovarian granulosa cell tumors,[10] are similar to the percentages seen in several other cancers. Howver, some reports say that aberrant methylation of the Cyclin D2 promoter is an early event in tumorigenesis, as is suggested by its presence in ductal carcinoma *in situ* in breast cancer and its absence in normal ducts;[15,18,29] however, this epigenetic change was associated with advanced ovarian cancer in the present study. Our results suggest that aberrant methylation of this gene could be related to tumor progression rather than tumorigenesis of epithelial ovarian cancer.

A number of biological tumor variables, such as DNA ploidy, steroid hormone receptor status and the expression of certain oncogenes, are associated with prognosis in epithelial ovarian cancer.[30–32] The promoter methylation status of several genes, such as 14-3-3 sigma, BRCA1, hMLH1 and TMS1, has been used to predict poor survival in epithelial ovarian cancer patients.[9,24,33–35] In the present study, Cyclin D2 promoter methylation was significantly associated with advanced stage, a larger residual tumor size and poor prognosis. Because there was a trend toward the repression of gene expression in the presence of promoter hypermethylation in ovarian cancer cell lines, we presume that Cyclin D2 gene silencing might occur in primary tissues with methylation, though the levels of the Cyclin D2 gene have not been analyzed in this study. These results suggest that the aberrant promoter methylation of Cyclin D2, or decreased expression of this gene caused by methylation, may be associated with aggressive biological characteristics, and may play a significant role in disease progression in epithelial ovarian cancer.

The contribution of Cyclin D2 to the pathophysiology of epithelial ovarian cancer is not known at a rudimentary level. Though numerous studies have classified it as an oncogene, our data and that of others strongly supports the hypothesis that it functions as a tumor suppressor gene. Further studies are needed to better clarify the relationship between Cyclin D2 gene expression level and its function as either an oncogene or a tumor suppressor. A deeper understanding of the role of D-type cyclins in ovarian cancer tumor biology could provide a foundation on which to base new diagnostic tests or molecular therapies.

Acknowledgment

We thank Setsuya Aiba (Department of Dermatology, Tohoku University Graduate School of Medicine) for technical assistance related to quantitative RT-PCR analysis.

References

1 Akahira J, Yoshikawa H, Shimizu Y *et al.* Prognostic factors of stage IV epithelial ovarian cancer: a multicenter retrospective study. *Gynecol Oncol* 2001; **81**: 398–403.
2 Bonnefoi H, A'Hern RP, Fisher C *et al.* Natural history of stage IV epithelial ovarian cancer. *J Clin Oncol* 1999; **17**: 767–75.
3 Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999; **21**: 163–7.
4 Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001; **61**: 3225–9.
5 Kass SU, Pruss D, Wolffe AP. How does DNA methylation repress transcription? *Trends Genet* 1997; **13**: 444–9.
6 Razin A, Ceder H. DNA methylation and gene expression. *Microbiol Rev* 1991; **55**: 451–8.
7 O'Doherty AM, Church SW, Russell SHE *et al.* Methylation status of oestrogen receptor-α gene promoter sequences in human ovarian epithelial cell lines. *Br J Cancer* 2002; **86**: 282–4.
8 Rathi A, Virmani AK, Schorge JO *et al.* Methylation profiles of sporadic ovarian tumor and nonmalignant ovaries from high-risk women. *Clin Cancer Res* 2002; **8**: 3324–31.
9 Akahira J, Sugihashi Y, Suzuki T *et al.* Decreased expression of 14-3-3sigma is associated with advanced disease in human epithelial ovarian cancer: its correlation with aberrant DNA methylation. *Clin Cancer Res* 2004; **10**: 2687–93.
10 Dhillon VS, Shahid M, Husain SA. CpG methylation of the FHIT, FANCF, cyclin-D2, BRCA2 and RUNX3 genes in granulose cell tumors (GCTs) of ovarian origin. *Mol Cancer* 2004; **3**: 33.
11 Messague J. G1 cell-cycle control and cancer. *Nature* 2004; **432**: 298–306.
12 Zhang P. The cell cycle and development: redundant roles of cell cycle regulators. *Curr Opin Cell Biol* 1999; **11**: 655–62.
13 Sicinski P, Donaher JL, Geng Y *et al.* Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 1996; **384**: 470–4.
14 Meyyappan M, Wong H, Hull C, Raibowol KT. Increased expression of cyclin D2 during multiple status of growth arrest in primary established cells. *Mol Cell Biol* 1998; **18**: 3163–72.

15 Evron E, Umbricht CB, Korz D *et al.* Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. *Cancer Res* 2001; **61**: 2782–7.
16 Yu J, Leung WK, Ebert MPA *et al.* Absence of cyclin D2 expression is associated with promoter hypermethylation in gastric cancer. *Br J Cancer* 2003; **88**: 1560–5.
17 Virmani A, Rathi A, Heda S *et al.* Aberrant methylation of the cyclin D2 promoter in primary small cell, nonsmall cell lung and breast cancers. *Int J Cancer* 2003; **107**: 341–5.
18 Fackler MJ, McVeigh M, Evron E *et al.* DNA methylation of RASSF1A, HIN-1, RAR-b, Cyclin D2 and Twist in *in situ* and invasive lobular breast carcinoma. *Int J Cancer* 2003; **107**: 970–5.
19 WHO. *Handbook for Reporting Results of Cancer Treatment.* WHO Publication No. 48. Geneva: WHO, 1979.
20 Shimizu Y, Kamoi H, Amada S *et al.* Toward the developing of a universal grading system for ovarian epithelial carcinoma. Prognostic significance of histopathologic features – problems involved in the architectural grading system. *Gynecol Oncol* 1998; **70**: 2–12.
21 Herman JG, Graff JR, Myohanen S *et al.* Methylation-specific PCR: a novel PCR assay for methylation status of CpG islamds. *Proc Natl Acad Sci USA* 1996; **93**: 9821–6.
22 Choi DS, Yoon S, Lee EY *et al.* Characterization of cyclin D2 expression in human endometrium. *Gynecol Invest* 2002; **9**: 41–6.
23 Akahira J, Suzuki T, Ito K *et al.* Differential expression of progesterone receptor isoform A and B in the normal ovary, and in benign, borderline, and malignant ovarian tumors. *Jpn J Cancer Res* 2002; **93**: 807–15.
24 Akahira J, Sugihashi Y, Ito K *et al.* Promoter methylation status and expression of *TMS1* gene in human epithelial ovarian cancer. *Cancer Sci* 2004; **95**: 40–3.
25 Kamikihara T, Arima T, Kato K *et al.* Epigenetic silencing of the imprinted gene *ZAC* by DNA methylation is an early event in the progression of human ovarian cancer. *Int J Cancer* 2005; **115**: 690–700.
26 Schwartsmann G, Fernandes MS, Schaan MD *et al.* Decitabine (5-Aza-2′-deoxycytidine; DAC) plus daunorubicin as a first line treatment in patients with acute myeloid leukemia: preliminary observations. *Leukemia* 1997; **11**: S28–31.
27 Bender CM, Pao MM, Jones PA. Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res* 1998; **58**: 95–101.
28 Juttermann R, Li E, Jaenisch R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci USA* 1994; **91**: 11 797–801.
29 Evron E, Dooley WC, Umbricht CB *et al.* Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. *Lancet* 2001; **357**: 1335–6.
30 Silvestrini R, Daidone MG, Veneroni S *et al.* The clinical predictivity of biomarkers of stage III–IV epithelial ovarian cancer in a prospective randomized treatment protocol. *Cancer* 1998; **82**: 159–67.
31 Akahira J, Inoue T, Suzuki T *et al.* Progesterone receptor isoforms A and B in human epithelial ovarian carcinoma: immunohistochemical and RT-PCR studies. *Br J Cancer* 2000; **83**: 1488–94.
32 Berchuck A, Kamel A, Whitaker R *et al.* Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res* 1990; **50**: 4087–91.
33 Chiang JW, Karlan BY, Cass L, Baldwin RL. BRCA1 promoter methylation predicts adverse ovarian cancer prognosis. *Gynecol Oncol* 2006; **101**: 403–10.
34 Gifford G, Paul J, Vasey PA *et al.* The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. *Clin Cancer Res* 2004; **10**: 4420–6.
35 Terasawa K, SagaeS, Toyota M *et al.* Epigenetic inactivation of TMS1/ASC in ovarian cancer. *Clin Cancer Res* 2004; **10**: 2000–6.

doi: 10.1111/j.1349-7006.2007.00394.x

Int J Clin Oncol (2006) 115:454–460
DOI 10.1007/s10147-006-0610-5

ORIGINAL ARTICLE

Takashi Yoshioka · Masato Sakayori · Shunsuke Kato
Natsuko Chiba · Shukichi Miyazaki · Kenji Nemoto
Hiroyuki Shibata · Hideki Shimodaira · Kazunori Ohtsuka
Yuichi Kakudo · Yuh Sakata · Chikashi Ishioka

Dose escalation study of docetaxel and nedaplatin in patients with relapsed or refractory squamous cell carcinoma of the esophagus pretreated using cisplatin, 5-fluorouracil, and radiation

Received: May 16, 2005 / Accepted: August 8, 2006

Abstract
Background. Definitive chemoradiation with cisplatin (CDDP) and 5-fluorouracil (5FU) has been playing an important role in the treatment of esophageal cancer, but some patients are not curable or have recurrent lesions. However, few chemotherapeutic regimens are available for such patients. Docetaxel and nedaplatin are active for esophageal cancer. We conducted a dose-escalation study of docetaxel and nedaplatin as second line-chemotherapy after definitive chemoradiation in patients with relapsed or refractory squamous cell carcinoma of the esophagus after chemoradiation.
Methods. Nedaplatin was administered on day 1 and docetaxel was administered on days 1 and 15, every 4 weeks. Dose escalation was based on the dose-limiting toxicity (DLT) observed during the first cycle.
Results. Twelve patients were enrolled. At a docetaxel dose of 30 mg/m^2 and a nedaplatin dose of 80 mg/m^2, one grade 4 neutropenia occurred and caused one treatment break longer than 2 weeks, but there were few DLTs. At doses of 35 and 80 mg/m^2, respectively, two grade 4 neutropenias and one grade 2 thrombopenia occurred and caused three treatment breaks longer than 2 weeks. Therefore, the maximum tolerated dose was established at this dose level. Two grade 3 anorexias and one grade 3 nausea occurred, but other non-hematological toxicities were generally mild. Responses were seen in one-fourth of the 12 patients, including one complete remission.
Conclusion. The recommended doses of docetaxel and nedaplatin were 30 and 80 mg/m^2, respectively. This combination could be a potential second-line treatment for this target population.

Key words Docetaxel · Nedaplatin · Esophageal cancer · Definitive chemoradiation · Dose escalation study

T. Yoshioka (✉) · M. Sakayori · S. Kato · N. Chiba · H. Shibata · H. Shimodaira · K. Ohtsuka · Y. Kakudo · C. Ishioka
Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
Tel. +81-22-717-8547; Fax +81-22-717-8548
e-mail: ytakashi@idac.tohoku.ac.jp

S. Miyazaki
Division of Advanced Surgery and Technology, Tohoku University Graduate School of Medicine, Sendai, Japan

K. Nemoto
Department of Radiation Oncology, Tohoku University Graduate School of Medicine, Sendai, Japan

Y. Sakata
Misawa City Hospital, Misawa, Japan

Introduction

Carcinoma of the esophagus is a highly aggressive neoplasm. Surgical resection has improved the survival of patients with esophageal cancer during the past two decades, but the survival remains relatively poor, with 5-year survival rates of 20%–40%.[1,2] Since the results of an intergroup randomized controlled trial (Radiation Therapy Oncology Group 85-01), which compared chemoradiation (CRT) with radiation alone, were reported, CRT for esophageal cancer has been revealing promising results.[3,4] Recently, several reports have shown curability by using definitive CRT[5,6] and almost equal survival compared to surgical resection.[7] Preoperative CRT and surgery has also been compared with surgery alone, but the survival benefit is not yet clear.[8–10]

The standard chemotherapy regimen in CRT for esophageal cancer has been a combination of cisplatin (CDDP) and 5-fluorouracil (5FU). It has shown the best clinical outcome, not only because of the synergism of the two agents[11] but also because of the radiosensitizing effects.[12] However, it was reported that the complete remission rates were 33%–75% after definitive CRT[5,6,13] and the incidences of recurrences were also high after preoperative CRT and surgery.[8–10] It is clear that there is an urgent need for active and tolerable chemotherapeutic regimens that can be available to patients with relapsed or refractory lesions after CRT involving CDDP and 5FU. However, there have been few trials of second-line treatment[14–16] and their results have been poor.

Docetaxel (TXT; Taxotere; Sanofi-Aventis, Paris, France) is a novel semisynthetic taxoid obtained from 10-deacetylbaccatin III, a precursor extracted from the needles of the European yew, *Taxus baccata*. It works as an antimitotic agent, enhancing microtubule assembly and inhibiting the depolymerization of tubulin, resulting in the inability of cells to divide.[17] A phase II study of TXT in advanced or recurrent esophageal cancer was conducted in Japan.[16] The overall response rate for the 49 eligible patients was 20.4%. Of the 36 patients previously treated with chemotherapy or CRT, 16.6% responded, compared with 36.4% of the 11 untreated patients. The incidences of grade 3/4 toxicities were: neutropenia, 87.8%; leucopenia, 73.5%; febrile neutropenia, 18.4%; anorexia, 18.4%; infection, 16.3%; anemia, 12.2%; and general fatigue, 12.2%, but there was no treatment-related death.[16]

Nedaplatin (cis-diammine-glycolatoplatinum; CDGP; Shionogi Pharma, Osaka, Japan) is a second-generation platinum derivative that was developed with the aim of decreasing the renal and gastrointestinal toxicities but maintaining the effectiveness of CDDP.[18] A phase II study of CDGP in 29 patients with esophageal cancer showed a response rate of 51.7%, involving 2 responders of 4 patients who were resistant to CDDP-based regimens.[19] The incidences of grade 3/4 toxicities were: thrombocytopenia, 27.9%; leucopenia, 19.7%; anemia, 19.7%; nausea and vomiting, 11.5%; and anorexia, 4.9%, but there was no severe renal toxicity and no treatment-related death.[19]

We considered a combination chemotherapy with TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus after definitive CRT with CDDP and 5FU. TXT was administered on days 1 and 15, and CDGP was administered on day 1, every 4 weeks. The rationale for this combination is that the drugs have different action mechanisms and safety profiles.[16,19] The biweekly administration of TXT might make it possible to reduce neutropenia, which is the dose-limiting toxicity (DLT) of TXT.[20] CDGP may be used safely in patients who were treated with CDDP, because it has lower renal toxicity than CDDP.[18] Moreover, in the phase II study noted above,[19] CDGP was effective in patients with esophageal cancer who were resistant to a CDDP-based regimen, although the number of patients was small.

We performed a dose-escalation study of TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus who had received definitive CRT using CDDP and 5FU. The objectives of this study were to assess the safety and toxicity profiles of this regimen and to determine the maximum tolerated dose (MTD), DLT, and recommended dose (RD) for a phase II study.

Patients and methods

Patient selection

Patients with histologically confirmed squamous cell carcinomas of the esophagus were enrolled at our institutes. Eligible patients with metastatic, or locally recurrent, or residual disease not curable with surgery had been previously treated with CDDP, 5FU, and radiation, with total dosages of more than 160 mg/m^2 of CDDP, more than 8 g/m^2 of 5FU, and more than 50 gray (Gy) of radiation. Other eligibility criteria included an Eastern Clinical Oncology Group (ECOG) scale performance status of 2 or less; age between 20 and 79 years; life expectancy of at least 3 months; provision of written informed consent in accordance with government and institutional guidelines; and adequate organ functions, with a WBC count of more than 3000/mm^3; absolute neutrophil count of more than 1500/mm^3; platelet count of more than 10×10^4/mm^3; aspartate aminotransferase (AST) and alanine aminotransferase (ALT levels) within three times the upper limit of normal (ULN) or five times the ULN in the presence of liver metastasis; total bilirubin less than 1.5 mg/dl; serum creatinine less than 1.5 mg/dl and/or creatine clearance more than 50 ml/min. Exclusion criteria included the following: concomitant uncontrolled, nonmalignant disease (malignant hypertension; cardiac, pulmonary, renal, or hepatic disease; active infection), neuropathy of more than grade 2, active double cancer, pregnant women, brain metastases with any symptoms, or a prior history of treatment for psychiatric diseases. Patients with active interstitial pneumonitis or severe pulmonary fibrosis on chest X-rays or computed tomography (CT) were also excluded. The protocols were approved by the ethics committee of our institution.

Study treatment

CDGP was dissolved in 500 ml of saline and administered as a 2-h IV infusion, followed by the administration of TXT, on day 1. Antiemetic therapy with dexamethasone and 5-hydroxy-tryptamine-3 receptor antagonists was administered as a 30-min IV infusion before the administration of CDGP. TXT was diluted in 250 ml of 5% glucose and administered as a 90-min IV infusion on days 1 and 15. The protocol of this study included the criteria for starting and continuing this treatment. To start the first course of this treatment, the eligibility criteria had to be fulfilled. To receive TXT on day 15, patients were required to maintain a WBC count of more than 2000/mm^3; absolute neutrophil count of more than 1000/mm^3; platelet count of more than 7.5×10^4/mm^3; and serum creatinine of less than 1.6 mg/dl. To start the next course, patients were required to maintain a WBC count of more than 3000/mm^3; absolute neutrophil count of more than 1500/mm^3; platelet count of more than 10×10^4/mm^3; and serum creatinine of less than 1.5 mg/dl. If these conditions were not fulfilled, TXT on day 15 and the next course was administered after recovery from these toxicities. If patients did not recover within 2 weeks, they were withdrawn from the study.

Five escalating dose levels of TXT/CDGP were prepared (Table 1). Level 1 was the starting dosage level, but level 0 was also prepared, because level 1 could have been the MTD. The starting doses of TXT and CDGP were 30 mg/m^2 and 80 mg/m^2, respectively. The initial dose of TXT was half

Table 1. Dose escalation scheme

Dosage level	TXT (mg/m^2)	CDGP (mg/m^2)	No. of enrolled patients
0	30	70	none
1	30	80	6
2	35	80	6
3	35	90	none
4	40	90	none

Five dose levels of docetaxel (TXT) / nedaplatin (CDGP) were prepared. Level 1 was the starting dosage level, but level 0 was also prepared, because level 1 may have been the MTD

the dose approved in Japan. The initial dose of CDGP was based on the lower limit of the dose recommended by the Japanese Government Health Care insurance. Individual drug escalations were alternated, and at least three new patients were entered at each level. Dose escalation was not allowed in individual patients.

Dose-limiting toxicity (DLT)

All toxicities were graded according to the Japanese version of the National Cancer Institute common toxicity criteria (NCI-CTC).[21] DLT was defined as follows: (1) grade 4 neutropenia for 3 days or more; (2) grade 3 febrile neutropenia; (3) grade 4 thrombocytopenia or anemia; (4) more than grade 2 renal toxicity; (5) grade 3 or 4 non-hematologic toxicity, except for alopecia; and (6) delay of more than 14 days in carrying out any treatment or in initiating the second cycle of therapy and discontinuation of this protocol treatment due to hematologic adverse effects. For purposes of determining the MTD, only DLTs occurring during the first cycle of therapy were considered. If one patient at a dose level experienced DLT, then three additional patients were treated at the same dose level. The MTD was defined as the dose level that resulted in two of six patients developing the DLTs. The recommended dose was to be the dose immediately below the MTD.

Pretreatment and follow-up studies

Pretreatment evaluation included complete patient histories, physical examinations, complete blood cell counts, biochemistry involving liver and renal functions, urinalysis, tumor markers (e.g., squamous cell carcinoma; [SCC]), electrocardiogram, esophagogastro-endoscopy, and radiologic studies (roentgenograms, CT scans and magnetic resonance imaging [MRI]). Bone scintigraphy was performed if serum alkaline phosphatase was elevated, and audiography was performed if clinically indicated.

While the patients were receiving the treatment course, complete blood cell counts, biochemistry involving liver and renal function, and urinalysis were performed weekly. If necessary, other appropriate examinations were added. CT scans were performed after every course to assess tumor response, although that was not the purpose of this study. Esophagogastro-endoscopy and the measurement of tumor markers were also performed if considered necessary. Tumor responses of measurable or assessable lesions were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[22] Recurrences or residues in the esophagus were also evaluated by the criteria of the Japanese Society for Esophageal Diseases.[23]

Table 2. Patient characteristics

Characteristic	No. of patients
Patients enrolled	12
Male	11
Female	1
Age (years)	
Median	70
Range	54–75
Clinical stage prior to CRT	
II	4
III	7
IVa	1
ECOG performance score	
0	5
1	5
2	2
Prior treatment except for CRT	2
Salvage surgery	1
Bypass surgery	1
Sites of lesions	
Esophagus	5
Lymph node	8
Cervix	1
Mediastinum	5
Abdomen	2
Lung	3
Liver	1
Pl C	1

Baseline characteristics of all 12 patients are listed. All patients had received definitive chemoradiation
ECOG, Eastern Clinical Oncology Group; CRT, chemoradiotherapy

Results

Twelve patients were enrolled in this study and patient characteristics are listed in Table 2. The patients were 11 men and one woman, and the median age was 70 years (range, 54 to 75 years). All patients had histologically confirmed squamous cell carcinomas of the esophagus before the definitive CRT, and 4, 7, and 1 patients, respectively, had stages II, III, and IVa prior to CRT. Five, 5, and 2 patients showed performance status 0, 1 and 2, respectively. All patients had previously received treatment with CDDP, 5FU, and radiation, for which the total dosages were more than 160 mg/m^2 of CDDP, more than 8 g/m^2 of 5FU, and more than 50 Gy of radiation. One patient had received a salvage operation because of a locally relapsed lesion in the esophagus after the definitive CRT, but metastases occurred in the cervical lymph nodes 6 months after the operation. One patient received a bypass operation upon demand, because the primary site was refractory to the definitive CRT and did not alleviate dysphagia. All patients

Table 3. Hematological adverse reactions after the first cycle

Toxicity	Dosage level	No. of patients	Grade of adverse reaction				Incidence of grade 4 (%)
			1	2	3	4	
Leukopenia	1	6	1	1	1	0	0
	2	6	1	2	2	0	0
Neutropenia	1	6	0	1	0	1	16.7
	2	6	1	1	1	2	33.3
Febrile neutropenia	1	6	0	0	0	0	0
	2	6	0	0	0	0	0
Thrombocytopenia	1	6	2	0	0	0	0
	2	6	0	3	1	0	0
Anemia	1	6	0	1	1	0	0
	2	6	0	3	0	0	0

All hematological adverse reactions after the first cycle are listed. Grade 4 was observed only for neutropenia without fever, and others were mild
Grade is according to the NCI-CTC (National Cancer Institute common toxicity criteria)

Table 4. Nonhematological adverse reactions after the first cycle

Toxicity	Dosage level	No. of patients	Grade of adverse reaction				Incidence of grade 3 or 4 (%)
			1	2	3	4	
Anorexia	1	6	0	1	1	0	16.7
	2	6	0	0	0	0	0
Nausea	1	6	0	0	1	0	16.7
	2	6	0	0	0	0	0
Fatigue	1	6	1	0	0	0	0
	2	6	0	0	0	0	0
Creatinine	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
Edema	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
SGOT	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
SGPT	1	6	0	0	0	0	0
	2	6	0	1	0	0	0
Injection site reaction	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
Alopecia	1	6	1	0	0	0	0
	2	6	0	0	0	0	0
Neuropathy (sensory)	1	6	0	0	0	0	0
	2	6	0	0	0	0	0

All nonhematological adverse reactions after the first cycle are listed. Grade 3 adverse reactions were observed for anorexia and nausea, but others were mild
Grade is according to the NCI-CTC (National Cancer Institute common toxicity criteria)
SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase

had relapsed or refractory lesions, and the sites of the lesions were: five esophageal recurrences, eight lymph nodes (one cervical, five mediastinal, and two abdominal), three lung metastases, one liver metastasis and one pleuritis carcinomatosa.

All patients were assessable for toxicity. Hematological and non-hematological adverse events and DLTs are shown in Tables 3, 4, and 5, respectively. The only grade 4 hematological adverse event observed in this study was neutropenia without fever, and other adverse events were mild. Grade 3 non-hematological adverse events were anorexia and nausea. Fatigue, renal and liver toxicities, edema, injection-site reaction, and alopecia were also observed, but were mild. There was no treatment-related death in this study.

At dose level 1, one of the six patients had DLT. This patient had grade 4 neutropenia and grade 3 anorexia and nausea. Both toxicities began after the administration of TXT on day 15, and the anorexia and nausea resolved 4 days after their onset. But the recovery from neutropenia was delayed for 4 weeks, resulting in the patient's withdrawal from this study. One patient had grade 2 nausea and anorexia, which occurred after the administration of TXT on day 15. The anorexia resolved after 5 days, but the patient rejected continuation of the protocol. The other four patients had no DLT and continued the treatment, and the dose escalation was allowed.

At dose level 2, three of the six patients had DLTs. Two patients had grade 4 neutropenia after the administration of TXT on day 15, and showed a delay of 3 weeks until starting

Table 5. DLT after the first cycle

DLT	Dosage level (No. of patients)	1 (6)	2 (6)
Neutropenia grade 4 for more than 3 days		1	2
Febrile neutropenia grade 3/4		0	0
Thrombopenia grade 4		0	0
Anemia grade 4		0	0
Non-hematologic toxicity grade 3/4		2	0
Anorexia		1	0
Nausea		1	0
Treatment break		1	3

From these results, the MTD of docetaxel/nedaplatin was determined to be 35/80 mg/m^2
Treatment break, break of more than than 2 weeks in therapy

Table 6. Objective responses according to RECIST

Dosage level	No. of patients	No. of patients with each clinical response					Response rate (%)
		CR	PR	SD	PD	NE	
1	6	1	0	2	1	2	16.6
2	6	0	2	3	1	0	33.3
Overall	12	1	2	5	2	2	25

At level 1, two patients were not evaluable because they received only one cycle of the treatment. This study showed a response rate of 25%, including one complete remission

Table 7. Responses of lesions in the esophagus

Dosage level	No. of patients	No. of patients with each clinical response					Response rate (%)
		CR	PR	SD	PD	NE	
1	4	0	1	1	0	2	25
2	1	0	0	1	0	0	0
Overall	5	0	1	2	0	2	20

Recurrences or residues in the esophagus were evaluated by the criteria of the Japanese Society for Esophageal Diseases. The response rate of these lesions was 20%

the second cycle of treatment. One patient had grade 2 thrombocytopenia, and it took 3 weeks to recover to baseline. These three patients were treated at level 1 from the second cycle, and more than two cycles could have been continued without DLTs. The other three patients had no DLT in the first cycle, but all of them had grade 4 neutropenia in the second or third cycle and dosages had to be reduced to dose level 1 to continue the treatment.

From these results, the MTD and RD of TXT/CDGP were determined to be 35/80 mg/m^2 and 30/80 mg/m^2 , respectively.

In this dose-escalation study, the efficacy of the therapy was also evaluated according to RECIST, and the results are shown in Table 6. At dose level 1, two patients were not evaluable because they received only one cycle of the treatment. One patient showed complete remission at dose level 1; this patient had multiple lung and mediastinal lymph node metastases and received nine cycles of the therapy. Two patients, who both had multiple lung and mediastinal lymph node metastases, achieved partial responses at dose level 2. In this study, the overall response rate was 25%. Recurrences or residues in the esophagus were also evaluated by the criteria of the Japanese Society for Esophageal Diseases,[23] and the results are shown in Table 7. One patient, who received five cycles of the therapy, achieved a partial response at dose level 1. The response rate of the lesions in the esophagus was 20%.

Discussion

There is no effective recommended treatment for patients with relapsed or refractory esophageal cancer after definitive CRT using CDDP and 5FU. Lordick et al.[15] reported a phase II trial of irinotecan and TXT in CDDP-pretreated relapsed or refractory esophageal cancer and indicated a small likelihood of major remission in this population of patients, but more investigations are essential.

We considered that a combination of TXT and CDGP might be suitable for the treatment of patients resistant to CRT. TXT is a mitotic spindle poison that promotes tubulin polymerization and inhibits the depolymerization of microtubules,[16] and its action mechanisms are quite different from

those of CDDP, CDGP, and 5FU. CDGP has shown superior antitumor activity and less renal and gastrointestinal toxicity as compared with CDDP in some studies.[18,19,24–29] Because there is a possibility that the renal background capacity is decreased in the CDDP-pretreated patient, it may be safe to use CDGP in such patients. It is unclear whether CDGP is cross-resistant to CDDP. It was shown that human leukemia cells resistant to CDDP were sensitive to CDGP,[30] but, on the other hand, CDGP showed cross-resistance to CDDP in lung,[31] ovarian,[32] and cervical[33] cell lines in in vitro studies. However, CDGP showed efficacy in some patients who showed resistance to CDDP-based therapy in the phase II trial we have previously noted[19] and in our preliminary study,[24] and this encouraged us to use CDGP in the treatment of patients resistant to CRT. The DLTs of TXT and CDGP are neutropenia and thrombocytopenia, respectively.[17,19] The toxicity profiles of these two drugs are different, but neutropenia is severe in a 3-week schedule of TXT as the standard regimen. Berstein et al.[20] reported that the administration of TXT in a weekly schedule was effective and well tolerated in women with metastatic breast cancer. Their data showed that decreasing the amount of the drug and shortening the interval between administrations made it possible to maintain efficacy but avoid neutropenia. For these reasons, bi-weekly administration of TXT was used in our study.

The objectives of the present dose escalation study were to determine the MTD, DLT, and RD of combination chemotherapy with TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus after treatment with CDDP, 5FU, and radiation. The MTD was determined to be 35/80 mg/m^2 of TXT/CDGP and the RD was determined to be 30/80 mg/m^2 of TXT/CDGP. DLTs related to the MTD were two grade 4 neutropenias and three treatment breaks longer than 2 weeks due to delays in recovery from these neutropenias, and grade 2 thrombocytopenia. Grade 3 anorexia and nausea and grade 1 renal toxicity were observed, but these non-hematological adverse effects were not related to the DLTs.

Another advantage of this combination therapy was that the treatment was manageable in an outpatient setting. Because CDGP has low renal toxicity and does not require hydration,[18] it takes only 2 or 4 h to complete the administration of TXT and/or CDGP. Most of the adverse events observed in this study were moderate and manageable.

In this study, one-fourth of the patients were responders, including one complete remission lasting for about 8 months. A Japanese phase II study of TXT in advanced or recurrent esophageal cancer reported a response rate of 15.8% in patients previously treated with chemotherapy or CRT.[17] A phase II trial of vinorelbine in metastatic squamous cell esophageal carcinoma reported a response rate of 6% in patients who had prior chemotherapy.[14] A phase II trial of irinotecan plus TXT in CDDP-pretreated relapsed or refractory esophageal cancer reported a response rate of 12.5%.[15] Although our study was a dose-escalation trial and the number of patients was very small, TXT and CDGP showed a response rate of 25% and might be promising agents in the target population.

In conclusion, we conducted a dose-escalation study of combination chemotherapy with TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus pretreated with CDDP, 5FU, and radiation. The RD was determined to be TXT 30 mg/m^2 days 1 and 15 and CDGP 80 mg/m^2 day 1, every 4 weeks. This combination could be a potential second-line treatment for the target population. A phase II study of this combination is ongoing.

Acknowledgments We thank Mr. Akira Nagai and Mr. Yasushi Nakamura for their secretarial assistance.

References

1. Muller JM, Erasmi H, Stelzner M, et al. (1990) Surgical therapy of oesophageal carcinoma. Br J Surg 77:845–857
2. Ando N, Ozawa S, Kitagawa Y, et al. (2000) Improvement in the results of surgical treatment of advanced squamous esophageal carcinoma during 15 consecutive years. Ann Surg 232:225–232
3. Herskovic A, Martz K, Al-Sarraf M, et al. (1992) Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. N Engl J Med 326:1593–1598
4. Cooper JS, Guo MD, Herskovic A, et al. (1999) Chemoradiotherapy of locally advanced esophageal cancer. Long-term follow-up of a prospective randomized trial (RTOG 85-01). JAMA 281:1623–1627
5. Ohtsu A, Boku N, Muro K, et al. (1999) Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. J Clin Oncol 17:2915–2921
6. Chan A, Wong A (1999) Is combined chemotherapy and radiation therapy equally effective as surgical resection in localized esophageal carcinoma? Int J Radiat Oncol Biol Phys 45:265–270
7. Hironaka S, Ohtsu A, Boku N, et al. (2003) Nonrandomized comparison between definitive chemoradiotherapy and radical surgery in patients with T2-3NanyM0 squamous cell carcinoma of the esophagus. Int J Radiat Oncol Biol Phys 57:425–433
8. Walsh TN, Noonan N, Hollywood D, et al. (1996) A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. N Engl J Med 335:462–467
9. Bosset JF, Gignoux M, Triboulet JP, et al. (1997) Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the esophagus. N Engl J Med 337:161–167
10. Urba SG, Orringer MB, Turrisi A, et al. (2001) Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional esophageal carcinoma. J Clin Oncol 19:305–313
11. Scanlon KJ, Newman EM, Lu Y, et al. (1986) Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. Proc Natl Acad Sci USA 83:8923–8925
12. Byfield JE (1990) Combined modality infusional chemotherapy with radiation. In: Lokich JJ (ed) Cancer Chemotherapy by infusion (2nd edn). Percepta, Chicago, pp 521–551
13. Araujo CMM, Souhami L, Gil RA, et al. (1991) A randomized trial comparing radiation therapy versus concomitant radiation therapy and chemotherapy in carcinoma of the thoracic esophagus. Cancer 67:2258–2261
14. Conroy T, Etienne PL, Adenis A, et al. (1996) Phase II trial of vinorelbine in metastatic squamous cell esophageal carcinoma. J Clin Oncol 14:164–170
15. Lordick F, von Schilling C, Bernhard H, et al. (2003) Phase II trial of irinotecan plus docetaxel in cisplatin-pretreated relapsed or refractory oesophageal cancer. Br J Cancer 89:630–633
16. Muro K, Hamaguchi T, Ohtsu A, et al. (2004) A phase II study of single-agent docetaxel in patients with metastatic esophageal cancer. Ann Oncol 15:955–959
17. Ringel I, Horwitz SB (1991) Studies with RP56976 (Taxotere): a semisynthetic analogue of Taxol. J Natl Cancer Inst 83:288–291

18. Totani T, Aono K, Komura M (1986) Synthesis of (glycolato-o, o') diammine platinum (II) and its related complexes. Chem Lett 429–432
19. Taguchi T, Wakui A, Nabeya K, et al. (1992) A phase II clinical study of cis-diammine glycolato platinum, 254-S, for gastrointestinal cancers. Jpn J Cancer Chemother 19:483–488
20. Burstein HJ, Manola J, Younger J, et al. (2000) Docetacel administered on a weekly basis for metastatic breast cancer. J Clin Oncol 18:1212–1219
21. Fukuda H, Saijyo N (2001) National Cancer Institute – Common Toxicity Criteria (NCI-CTC version 2.0, April 30, 1999) – The second version translated into Japanese by Japan Clinical Oncology Group. Jpn J Cancer Chemother 28:1993–2027
22. Therasse P, Arbuck SG, Eisenhauer EA, et al. (2000) New guidlines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 92:205–216
23. Japanese Society for Esophageal Diseases (1992) Guidelines for the clinical and pathologic studies on carcinoma of the esophagus (8th edn). Kanehara Syuppan, Tokyo
24. Yoshioka T, Gamoh M, Shineha R, et al. (1999) A new combination chemotherapy with cis-diammine-glycolatoplatinum (nedaplatin) and 5-fluorouracil for advanced esophageal cancers. Internal Medicine 38:844–848
25. Muro K, Shirao K, Shimada Y, et al. (1999) A phase I-II study of nedaplatin and 5-fluorouracil in patients with advanced esophageal cancer. Proc ASCO 18: 258a
26. Shibata S, Kawasaki H, Nakai M, et al. (2002) Chemoradiotherapy using platinum analogs/5-FU for advanced esophageal cancer. Jpn J Cancer Chemother 29:2209–2212
27. Nakamura T, Ide H, Eguchi R, et al. (2003) Nedaplatin and 5-fluorouracil combined with radiotherapy for advanced esophageal cancer. Jpn J Chemoter 30:803–807
28. Nemoto K, Matsushita H, Ogawa Y, et al. (2003) Radiation therapy combined with cis-diammine-glycolatoplatinum (nedaplatin) and 5-fluorouracil for untreated and recurrent esophageal cancer. Am J Clin Oncol 26:46–49
29. Kato H, Fukuchi M, Manda R, et al. (2003) Efficacy and toxicity of nedaplatin and 5-FU with radiation for advanced esophageal carcinomas. Anticancer Res 23:3493–3498
30. Kobayashi H, Takemura Y, Miyachi H, Ogawa T (1991) Antitumor activities of new platinum compounds, DWA2114R, NK121 and 254-S, against human leukemia cells sensitive or resistant to cisplatin. Invest New Drugs 9:313–319
31. Fukuda M, Ohe Y, Kanazawa F, et al. (1995) Evaluation of novel platinum complexes, inhibitors of topoisomerase I and II in non-small cell lung cancer (NSCLC) sublines resistant to cisplatin. Anticancer Res 15:393–398
32. Alberts DS, Fanta PT, Running KL, et al. (1997) In vitro phase II comparison of the cytotoxicity of a novel platinum analog, nedaplatin (254-S), with that of cisplatin and carboplatin against fresh, human ovarian cancers. Cancer Chemother Pharmacol 39:493–497
33. Monk BJ, Alberts DS, Burger RA, et al. (1998) In vitro phase II comparison of the cytotoxicity of a novel platinum analog, nedaplatin (254-S), with that of cisplatin and carboplatin against fresh, human cervical cancers. Gynecol Oncol 71:308–312

Adjuvant chemotherapy with irinotecan hydrochloride and cisplatin for clear cell carcinoma of the ovary

MASASHI TAKANO[1,13], YOSHIHIRO KIKUCHI[1], NOBUO YAEGASHI[2], MITSUAKI SUZUKI[3], HIROSHI TSUDA[4], SATORU SAGAE[5], YASUHIRO UDAGAWA[6], KAZUO KUZUYA[7], JUNZO KIGAWA[8], SATOSHI TAKEUCHI[9], HITOSHI TSUDA[10], TAKUYA MORIYA[11] and TORU SUGIYAMA[12]

[1]Department of Obstetrics and Gynecology, National Defense Medical College, Tokorozawa, Saitama 359-8513; [2]Department of Obstetrics and Gynecology, Tohoku University, Sendai, Miyagi 980-8574; [3]Department of Obstetrics and Gynecology, Jichi Medical College, Kawachi-gun, Tochigi 329-0498; [4]Department of Obstetrics and Gynecology, Osaka City General Hospital, Osaka, Osaka 534-0021; [5]Department of Obstetrics and Gynecology, Sapporo Medical University, Sapporo, Hokkaido 060-8556; [6]Department of Obstetrics and Gynecology, Fujita Health University, Toyoake, Aichi 470-1192; [7]Department of Gynecology, Aichi Cancer Center Hospital, Nagoya, Aichi 464-8681; [8]Department of Obstetrics and Gynecology, Tottori University, Yonago, Tottori 683-8504; [9]Department of Obstetrics and Gynecology, Kobe National Hospital, Kobe, Hyogo 554-0155; [10]Department of Pathology II, National Defense Medical College, Tokorozawa, Saitama 359-8513; [11]Pathology Laboratory of Central Clinical Facilities, Tohoku University, Sendai, Miyagi 980-8574; [12]Department of Obstetrics and Gynecology, Iwate Medical University, Morioka, Iwate 020-8505, Japan

Received July 4, 2006; Accepted August 14, 2006

Abstract. Clear cell carcinoma (CCC) of the ovary has distinct characteristics showing resistance to conventional platinum-based regimen. Our aim was to evaluate the effects of combination therapy with irinotecan hydrochloride and cisplatin (CPT-P), comparing to regimen with paclitaxel and platinum (TP). We retrospective reviewed 172 patients with complete surgical staging procedures including lymphadenenctomy. Forty-six patients received CPT-P and 126 patients were treated with TP. Survival of the two groups was compared. Between CPT-P group and TP group, there was no significant difference in median age, performance status, FIGO stage, rate of optimal cytoreduction, and follow-up period. There was no significant difference in progression-free survival of patients with stage I tumors (p=0.95) and suboptimally debulked stage II-IV tumors (p=0.92). Although there was no significant difference of overall survival, progression-free survival of CPT-P group was significantly better than that of TP group in optimally debulked stage II-IV (p=0.03). Multiple regression survival analysis revealed CPT-P regimen (p=0.02) and residual tumor diameter (p<0.01) were both independent prognostic factors in stage II-IV tumors. The combination of CPT-P was shown to have a potential therapeutic benefit for advanced CCC of the ovary, especially for cases with optimal debulking surgery. However, this is a limited retrospective study, therefore we recommend that the CPT-P regimen be evaluated in a larger prospective study.

Correspondence to: Dr Masashi Takano, [13]*Present address*: Institute of Reproductive and Developmental Biology (IRDB), Imperial College of London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
E-mail: m.takano@imperial.ac.uk

Key words: ovarian cancer, clear cell carcinoma, irinotecan hydrochloride, adjuvant chemotherapy, paclitaxel, multicentre, retrospective

Introduction

Clear cell carcinoma (CCC) was initially termed as mesonephroid by Schiller in 1939 (1), and since 1973 it was strictly defined by World Health Organization as lesions characterized by clear cells growing in solid/tubular or glandular patterns as well as hobnail cells (2). Since then, many studies have identified the distinctive behavior of the tumors. The most distinctive difference is that patients with CCC of the ovary have poorer prognosis compared with those with other pathological types of epithelial ovarian carcinomas (3,4). Additionally, CCC of the ovary was implied to show resistance to conventional platinum-based chemotherapy (5,6). Recent studies confirmed the evidence in the analysis of measurable CCC patients: response was observed in 11-45% with conventional platinum-based regimen, whereas patients with serous subtype showed a significantly higher response rate of 73-81% (7,8).

Combination with paclitaxel and platinum (TP), recognized as 'Gold standard' regimen for ovarian cancer, is now used to treat patients with all subtypes of ovarian neoplasms, but in measurable CCC cases treated with TP regimen, response was relatively low, ranging from 22 to 56% (9,10; Enomoto T, *et al*, Proc Am Soc Clin Oncol 22: 447, abs. 1797, 2003). One

report showed survival benefit of conventional chemotherapy with paclitaxel and platinum after complete surgery in CCC patients (11). However, the result from large series of CCC patients treated with paclitaxel and platinum showed no survival benefit compared with conventional platinum-based chemotherapy in both early and advanced cases (12).

Irinotecan hydrochloride, a semi-synthetic derivative of camptothecin, has additive and synergic effects in combination with cisplatin *in vitro* (13-15). The combination therapy with irinotecan hydrochloride and cisplatin (CPT-P) was reported to be effective for patients with various solid tumors. Especially, a large clinical trial revealed that CPT-P had significant activity for extensive small-cell lung cancer (16). Additionally, CPT-P has been reported to be effective in first-line and second-line chemotherapy for the treatment of CCC of ovary (17,18). The aim of the present study was to evaluate the therapeutic activity of CPT-P and TP in a retrospective analysis.

Patients and methods

A retrospective review of the patients seen at ten Japanese hospitals from 1 January 1992 to 31 December 2003 was done. Of all the patients treated in those hospitals, the following patients were selected: a) patients who underwent complete surgical staging procedures including hysterectomy, bilateral salpingo-oophorectomy, peritoneal washing, omentectomy, pelvic lymphadenectomy and paraaortic lymphadenenctomy; b) patients whose tumor specimens were confirmed as CCC of the ovary by two pathologists in central pathologic review; c) patients treated with six courses of combination chemotherapy using irinotecan hydrochloride and cisplatin (CPT-P) or six courses of paclitaxel and platinum combination (TP); d) age ≤75 years; e) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤2; f) pretreatment leukocyte count ≥4000/mm^3, platelet count ≥100000/mm^3, hemoglobin ≥9.0 g/dl, serum creatinine <1.5 mg/dl, creatinine clearance ≥60 ml/min, and GOT and GPT less than twice upper limit of normal at the hospitals; g) the study approved by the Committee on Ethics of each hospital.

One cycle of CPT-P regimen consisted of a drip infusion of 50-60 mg/m^2 of cisplatin on day 1 and 50-60 mg/m^2 of CPT-11 on days 1, 8 and 15, and one week off and it was repeated every 4 weeks. TP regimen consisted of a drip infusion of 175-180 mg/m^2 of paclitaxel and 50-75 mg/m^2 of cisplatin or carboplatin (AUC = 5-6).

Response was evaluated with CT or MR images at least every 2 cycles of chemotherapy in the patients with measurable disease. A complete response (CR) was defined as the complete disappearance of all detectable disease for at least 4 weeks. A partial response (PR) was defined as a >50% decrease in tumor size for at least 4 weeks. Stable disease (SD) was defined as the absence of any significant change in measurable lesions for at least 4 weeks. Progressive disease (PD) was defined as the appearance of a new lesion or a >25% increase in tumor size. Serum levels of tumor markers including CA125 were not used for response evaluation of chemotherapy in the present study.

The time to progression was defined as the interval from the date of primary surgery until the date of recurrence or tumor progression. Survival duration was determined as the time from the date of primary surgery until death or the date of last follow-up contact. Kaplan-Meier method was used for calculation of patient survival distribution. The significance of the survival distribution in each group was tested by a generalized Wilcoxon test and the log-rank test. The χ^2 test and Student's t-test for unpaired data were used for statistical analysis. A p-value of <0.05 was considered statistically significant. The Stat View software ver. 5.0 (SAS Institution Inc., Cary, NC, USA) was used to analyze the data.

Results

In total, 46 cases with CPT-P regimen and 126 cases with TP regimen were enrolled in the present retrospective study. TP group included 118 cases treated with paclitaxel and carboplatin and 8 cases treated with paclitaxel and cisplatin. The characteristics of the patients are outlined in Table I. There was no significant difference in median age, performance status, FIGO stage, rate of optimal cytoreduction, and follow-up period between CPT-P group and TP group. Median age of all cases was 53 years (range, 27-75 years). CPT-P group included 24 patients (52%) of stage I, 6 (2%) in stage II, 13 (28%) in stage III and 3 (6%) in stage IV. In TP group, 72 cases (56%) were in stage I, 15 (12%) in stage II, 34 (27%) in stage III and 5 (4%) in stage IV. Optimal cytoreduction (residual tumor diameter <1 cm) with their initial surgery was achieved in 83% (39/46) in CPT-P group and 83% (104/126) in TP group, respectively. The patients with optimal cytoreductive surgery included 6 stage II, 7 stage III and 2 stage IV tumors in CPT-P group and 14 stage II, 16 stage III and 2 stage IV tumors in TP group. Median follow-up period was 28 months in CPT-P group and 27 months in TP group.

All patients who received CPT-P regimen were evaluable for toxicity. In 46 cases who received CPT-P regimen, 35 cases were treated with 50 mg/m^2 of cisplatin and 60 mg/m^2 of CPT-11, 7 cases with 60 mg/m^2 of cisplatin and 60 mg/m^2 of CPT-11, and 4 cases with 50 mg/m^2 of cisplatin and 50 mg/m^2 of CPT-11, respectively. Hematological and non-hematological toxicities in all cases with CPT-P regimen are shown in Table II. The major toxicities were neutropenia and diarrhea. The incidences of grade 3 and 4 neutropenia were 22 and 7%, respectively. Grade 3 nausea and grade 3 diarrhea was observed in 4 and 9% of all cases, respectively. CPT-11 was omitted in 12 (26%) patients on days 8 or 15 because of toxicities, and 20% dose reduction of CPT-11 was documented in 10 (22%) patients.

Response evaluation was assessed in the patients with suboptimal reduction; 22 cases of PT regimen and 7 cases of CPT-P regimen. The patients treated with PT included 1 (5%) CR, 6 (27%) PR, 3 (14%) SD and 12 (55%) PD. CPT-P group consisted of 3 (43%) PR, 2 (29%) SD and 2 (29%) PD, respectively. Response was observed in 7 (32%) of 22 cases in TP group and 3 (43%) of 7 cases in CPT-P group. The rates of non-PD patients were 71% (5 of 7 cases) in CPT-P group and 45% (10 of 22 cases) in TP group, respectively (p=0.047).

Two-year and progression-free and overall survival in stage I tumors was 77 and 92% in CPT-P group and 78 and 94% in TP group, respectively. There were no significant survival differences in progression-free survival (Fig. 1A, p=0.95) and overall survival (p=0.40) in stage I tumors.

Table I. Characteristics of the patients.

	Irinotecan plus cisplatin (%)	Paclitaxel plus platinum (%)	P-value
Patients	46	126	
Median age (years) (range)	52 (32-69)	53 (27-75)	0.85
Performance status			0.17
0	26 (57)	85 (67)	
1, 2	20 (43)	41 (33)	
FIGO stage			0.88
I	24 (52)	72 (57)	
II	6 (13)	15 (12)	
III	13 (28)	34 (27)	
IV	3 (7)	5 (4)	
Primary surgery			0.32
Optimal reduction	39 (85)	104 (83)	
Suboptimal reduction	7 (15)	22 (17)	
Follow-up period (months)			0.43
Median	28	27	
Range	6-83	3-73	

Table II. Hematological and non-hematological toxicity of combination chemotherapy of irinotecan hydrochloride and cisplatin for clear cell carcinoma of ovary.

	Grade of toxicities				
	0 n (%)	1 n (%)	2 n (%)	3 n (%)	4 n (%)
Hematological					
Neutropenia	4 (9)	18 (39)	11 (24)	10 (22)	3 (7)
Anemia	14 (30)	20 (43)	10 (22)	1 (2)	1 (2)
Thrombocytopenia	41 (89)	2 (4)	1 (2)	1 (2)	1 (2)
Non-hematological					
Nausea	1 (2)	23 (50)	20 (43)	2 (4)	-
Fatigue	25 (54)	14 (30)	7 (15)	0 (0)	0 (0)
Diarrhea	12 (26)	21 (46)	9 (20)	4 (9)	0 (0)

Patients with suboptimal cytoreduction surgery included 7 cases of CPT-P group and 22 cases of TP group. In these patients with suboptimal reduction, median survival was 10 months in CPT-P group and 12 months in TP group, and there were no significant differences in progression-free survival (Fig. 1B, p=0.92) and overall survival (p=0.82).

In stage II-IV tumors, optimal reduction at their initial surgery was achieved in 15 (68%) of 22 patients in CPT-P group and 32 (59%) of 54 patients in TP group. Among optimally debulked patients, CPT-P group included 10 cases (67%) of no residual tumor and 5 (33%) cases of tumor diameter <1 cm, and TP group consisted of 24 cases (75%) of no residual tumor and 8 cases (25%) of tumor diameter <1 cm. There was no significant distribution difference of residual tumor diameter in optimally debulked patients between CPT-P and TP group (p=0.55).

Progression-free survival of CPT-P group was significantly better than that of TP group in the group with stage II-IV optimal cytoreduction (Fig. 2, p=0.03). Two-year progression-free survival was 86% in CPT-P group and 44% in TP group, respectively. Median progression-free survival of TP group was 15 months. There was no significant difference in overall

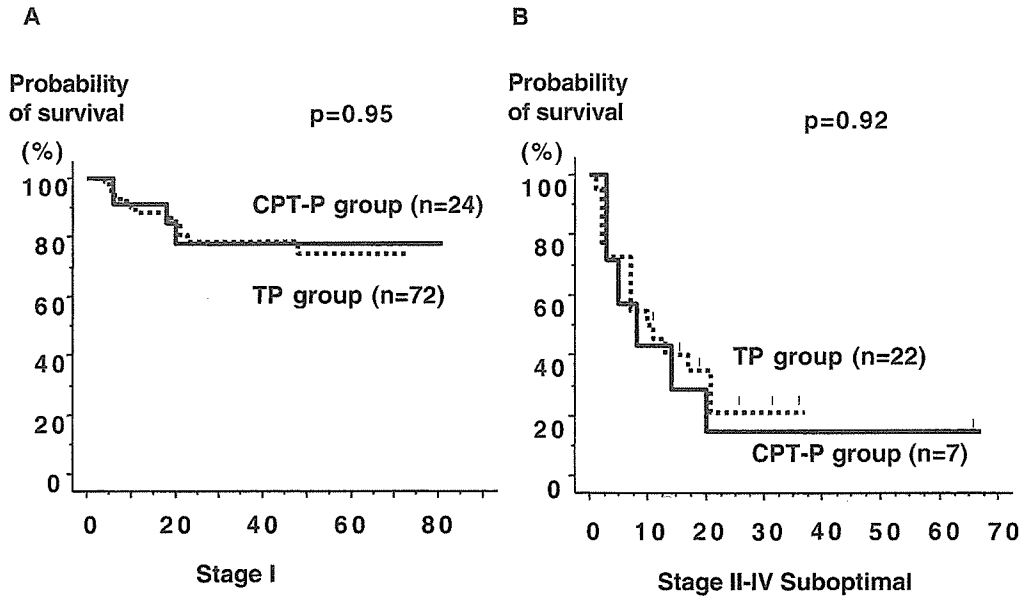

Figure 1. Kaplan-Meier curve comparing the progression-free survival of stage I patients (A) and stage II-IV patients with suboptimal debulking surgery (B) according to adjuvant chemotherapy. No significant difference was observed in patients with stage I (p=0.95), and those with stage II-IV suboptimal debulking patients (p=0.92).

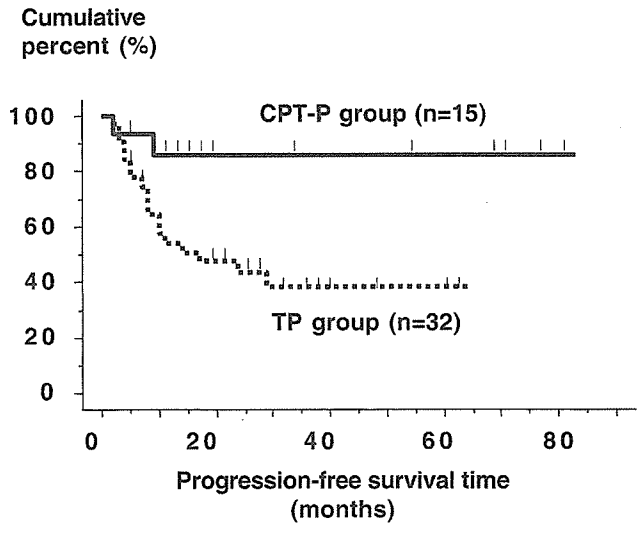

Figure 2. Kaplan-Meier curve comparing the progression-free survival of the stage II-IV patients with optimal debulking surgery according to adjuvant chemotherapy. The survival of the patients with combination with irinotecan hydrochloride and cisplatin (CPT-P) was significantly better than that with paclitaxel and platinum (TP) (p=0.03). Two-year progression-free survival was 86% in CPT-P group and 44% in TP group and median progression-free survival time was 13 months in CPT-P group and 11 months in TP group, respectively.

survival of optimally debulked stage II-IV patients according to two regimens (p=0.14).

In stage II-IV tumors with optimal reduction, two parameters were independent prognostic factors for progression-free survival as determined by multivariate analysis: residual tumor diameter <1 cm (hazard ratio, 3.77; p<0.01); paclitaxel and platinum (hazard ratio, 6.454; p=0.02) (Table III). Age, performance status, and FIGO stage were not significant prognostic factors.

Discussion

In a previous study with CPT-P regimen for ovarian cancer using 70 mg/m^2 of cisplatin on day 1 and 60 mg/m^2 of irinotecan hydrochloride on days 1, 8 and 15, toxicities more than grade 3 was observed in 52-70% for neutropenia and 4-10% for diarrhea (17,19). Japanese large clinical trial using 60 mg/m^2 of cisplatin and 60 mg/m^2 of irinotecan hydrochloride for patients with lung cancer (16) showed high abundance of grade 3-4 toxicities compared with the present study. The lower rates of grade 3-4 toxicities might be explained by a large proportion of patients who received a lower dose (50 mg/m^2) of irinotecan hydrochloride. A variety of single nucleotide polymorphisms (SNPs) in human UDP-glucuronosyltransferase could cause a different toxicity profile in patients treated with irinotecan hydrochloride (20,21). A variety of SNPs in specific genes might cause the disease-specific toxicity profiles. In the present study, the most common grade 3-4 adverse effect was neutropenia observed in 28% of patients, but it was reversible in all cases. Diarrhea more than grade 3 was observed in 4 (9%) patients and it was reversible with conservative treatment. It was suggested that CPT-P regimen was relatively safe and well-tolerated in first-line chemotherapy in CCC patients.

CCC of the ovary has been suggested to lack sensitivity compared to conventional platinum-based chemotherapy (3,4,7). As combination chemotherapy of TP regimen is now established as 'Gold standard' regimen for epithelial ovarian cancer (22,23), the regimen is widely used for all histological sub-types of ovarian tumors. Some reports indicated survival benefit of TP therapy compared with platinum-based chemotherapy in stage I CCC disease combined with complete surgical staging procedures (11) and stage III, IV tumors (9). However, a recent study including 254 patients with complete surgical staging revealed that there was no significant survival

Table III. Multiple regression survival analysis for stage II-IV patients with optimally debulked clear cell carcinoma of the ovary.

Variables	Hazard ratio	95% confidence interval	P-value
Age (years)			0.43
<50	1		
>51	1.55	0.53; 4.41	
Performance status			0.88
0	1		
1, 2	1.08	0.40; 2.90	
FIGO stage			0.33
II	1		
III	1.60	0.32; 8.00	
IV	3.04	0.55; 16.67	
Residual tumor			<0.01
None	1		
<1 cm	3.77	1.42; 9.98	
Chemotherapy			0.02
Irinotecan hydrochloride and cisplatin	1		
Paclitaxel and platinum	6.454	1.40; 29.86	

benefit with TP regimen compared with conventional platinum-based chemotherapy in either early or advance staged patients (12). So far, no anti-cancer agents have been established as a standard regimen for CCC. From view of molecular profiling, CCC is recognized as a completely different category from tumors of other histological subtypes (24,25). These distinct molecular characteristics might support the necessity of another approach for the treatment of CCC of the ovary.

CPT-P regimen, initially introduced as a treatment of platinum-refractory ovarian cancer (19), showed moderate activity for first-line chemotherapy of CCC (17). *In vitro* study suggested that irinotecan hydrochloride as well as paclitaxel was the candidate for anti-neoplastic agents for CCC (26). The present study shows that response rate of CPT-P was almost the same as TP. High abundance of SD patients in CPT-P regimen, acting as 'tumor-dormancy' effects, could possibly lead to better progression-free survival in patients with stage II-IV optimal cytoreductive surgery. The significance of CPT-P regimen was also identified as a favorable prognostic factor with multivariate analysis as well as absence of residual tumor. In addition to the toxicity profile, the efficacy of CPT-P was considered to be satisfactory for the treatment for CCC of the ovary.

Irinotecan hydrochloride was shown to have higher activity than conventional platinum-based regimen for adjuvant setting in combination with mitomycin C (27). Combination chemotherapy including irinotecan hydrochloride was suggested to have a potential anti-tumor effect against CCC of the ovary. CCC of ovary has been reported to have a distinct molecular characteristics as well as a distinctive clinical behavior. Targeting therapy for CCC-specific molecular markers such as hepatocyte nuclear factor-1 ß (28) or ABCF2, a member of ATP-binding cassette gene superfamily (29), could possibly be another strategy for the treatment of CCC of the ovary. Although CPT-P could be a candidate regimen showing some efficacy, a large-scale prospective trial is needed to confirm these observations.

Acknowledgements

We are indebted to Drs T. Kita (National Defense Medical College), M. Sakuma (Tohoku University), Y. Saga (Jichi Medical College), M. Sugimura (Sapporo Medical University), K. Hasegawa (Fujita Health University), M. Shimada (Tottori University), A. Yoshizaki (Iwate Medical University) who allowed us to review the patients' medical charts.

References

1. Schiller W: Mesonephroma ovarii. Am J Cancer 35: 1-21, 1939.
2. Serov SF, Scully RE and Sobin LH: International histologic classification of tumors. In: Histologic Typing of Ovarian Tumors. World Health Organization, Geneva, No. 9, 1973.
3. O'Brien ME, Schofield JB, Tan S, Fryatt I, Fisher C and Wiltshaw E: Clear cell epithelial ovarian carcinoma cancer (mesonephroid): bad prognosis only in early stages. Gynecol Oncol 49: 250-254, 1993.
4. Omura GA, Brady MF, Homesley HD, Yordan E, Major FJ, Buchsbaum HJ and Park RC: Long-term follow-up prognostic factor analysis in advanced ovarian carcinoma: the Gynecologic Oncology Group experiences. J Clin Oncol 9: 1138-1150, 1991.
5. Goff BA, Sainz de la Cuesta R, Muntz HG, Fleischhacker D, Ek M, Rice LW, Nikrui N, Tamimi HK, Cain JM, Greer BE and Fuller AF Jr: Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. Gynecol Oncol 60: 412-417, 1996.

6. Recio FO, Piver MS, Hempling RE and Driscoll DL: Lack of improved survival plus increase in thromboembolic complications in patients with clear cell carcinoma of the ovary treated with platinum versus non-platinum-based chemotherapy. Cancer 78: 2157-2163, 1996.
7. 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. Cancer 88: 2584-2589, 2000.
8. Pectasides D, Fountzilas G, Aravantinos G, Kalofonos C, Efstathiou H, Farmakis D, Skarlos D, Pavlidis N, Economopoulos T and Dimopoulos MA: Advanced stage clear-cell epithelial ovarian cancer: the Hellenic cooperative oncology group experience. Gynecol Oncol (In press).
9. Ho CM, Huang YJ, Chen TC, Huang SH, Liu FS, Chang Chien CC, Yu MH, Mao TL, Wang TY and Hsieh CY: Pure-type clear cell carcinoma of the ovary as a distinct histological type and improved survival in patients treated with paclitaxel-platinum-based chemotherapy in pure-type advanced disease. Gynecol Oncol 94: 197-203, 2004.
10. Utsunomiya H, Akahira J, Tanno S, Moriya T, Toyoshima M, Niikura H, Ito K, Morimura Y, Watanabe Y and Yaegashi N: Paclitaxel-platinum combination chemotherapy for advanced or recurrent ovarian clear cell adenocarcinoma: a multicenter trial. Int J Gynecol Cancer 16: 52-56, 2006.
11. Ho CM, Chien TY, Shih BY and Huang SH: Evaluation of complete surgical staging with pelvic and para-aortic lymphadenectomy and paclitaxel plus carboplatin chemotherapy for improvement of survival in stage I ovarian clear cell carcinoma. Gynecol Oncol 88: 394-399, 2003.
12. Takano M, Kikuchi Y, Yaegashi N, Kuzuya K, Ueki M, Tsuda H, Suzuki M, Kigawa J, Takeuchi S, Tshuda H, Moriya T and Sugiyama T: Clear cell carcinoma of the ovary: a retrospective multicentre experience of 254 patients with complete surgical staging. Br J Cancer 94: 1369-1374, 2006.
13. Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, Sakamoto S and Miura Y: Effects of CPT-11 in combination with other anticancer agents in culture. Int J Cancer 50: 604-610, 1992.
14. Minagawa Y, Kigawa J, Ishihara H, Itamochi H and Terakawa N: Synergistic enhancement of cisplatin cytotoxicity by SN-38, an active metabolite of CPT-11, for cisplatin-resistant HeLa cells. Jpn J Cancer Res 85: 966-971, 1994.
15. Fukuda M, Nishio K, Kanzawa F, Ogasawara H, Ishida T, Arioka H, Bojanowski K, Oka M and Saijo N: Synergistic enhancement of cisplatin cytotoxicity by SN-38, an active metabolite of CPT-11, for cisplatin-resistant HeLa cells. Cancer Res 56: 789-793, 1996.
16. Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, Fukuoka M, Mori K, Watanabe K, Tamura T, Yamamoto S and Saijo N; Japan Clinical Oncology Group: Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Eng J Med 346: 85-91, 2002.
17. Adachi S, Ogasawara T, Yamasaki N, Shibahara H, Kanazawa R, Tsuji Y, Takemura T and Koyama K: A pilot study of CPT-11 and cisplatin for ovarian clear cell adenocarcinoma. Jpn J Clin Oncol 29: 434-437, 1999.
18. Kita T, Kikuchi Y, Kudoh K, Takano M, Goto T, Hirata J, Tode T and Nagata I: Exploratory study of effective chemotherapy to clear cell carcinoma of the ovary. Oncol Rep 7: 327-331, 2000.
19. Sugiyama T, Yakushiji M, Nishida T, Ushijima K, Okura N, Kigawa J and Terakawa N: Irinotecan (CPT-11) combined with cisplatin in patients with refractory or recurrent ovarian cancer. Cancer Lett 128: 211-218, 1998.
20. Jinno H, Saeki M, Saito Y, Tanaka-Kagawa T, Hanioka N, Sai K, Kaniwa N, Ando M, Shirao K, Minami H, Ohtsu A, Yoshida T, Saijo N, Ozawa S and Sawada J: Functional characterization of human UDP-glucuronosyltransferase 1A9 variant, D256N, found in Japanese cancer patients. J Pharmacol Exp Ther 306: 688-693, 2003.
21. Sai K, Saeki M, Saito Y, Ozawa S, Katori N, Jinno H, Hasegawa R, Kaniwa N, Sawada J, Komamura K, Ueno K, Kamakura S, Kitakaze M, Kitamura Y, Kamatani N, Minami H, Ohtsu A, Shirao K, Yoshida T and Saijo N: UGT1A1 haplotypes associated with reduced glucuronidation and increased serum bilirubin in irinotecan-administered Japanese patients with cancer. Clin Pharmacol Ther 75: 501-515, 2004.
22. McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL and Davidson M: Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Eng J Med 334: 1-6, 1996.
23. Bookman MA, Greer BE and Ozols RF: Optimal therapy of advanced ovarian cancer: carboplatin and paclitaxel vs. cisplatin and paclitaxel (GOG158) and an update on GOG0182-ICON5. Int J Gynecol Cancer 13: 735-740, 2003.
24. Zorn KK, Bonome T, Gangi L, Chandramouli GV, Awtrey CS, Gardner GJ, Barrett JC, Boyd J and Birrer MJ: Gene expression profiles of serous, endometrioid and clear cell subtypes of ovarian and endometrial cancer. Clin Cancer Res 15: 6422-6430, 2005.
25. Marquez RT, Baggerly KA, Patterson AP, Liu J, Broaddus R, Frumovitz M, Atkinson EN, Smith DI, Hartmann L, Fishman D, Berchuck A, Whitaker R, Gershenson DM, Mills GB, Bast RC Jr and Lu KH: Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon. Clin Cancer Res 11: 6116-6126, 2005.
26. Itamochi H, Kigawa J, Sultana H, Iba T, Akeshima R, Kamazawa S, Kanamori Y and Terakawa N: Sensitivity to anticancer agents and resistance mechanisms in clear cell carcinoma of the ovary. Jpn J Cancer Res 93: 723-728, 2002.
27. 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 carcinoma. Gynecol Oncol 97: 893-897, 2005.
28. Tsuchiya A, Sakamoto M, Yasuda J, Chuma M, Ohta T, Ohki M, Yasugi T, Taketani Y and Hirohashi S: Expression profiling in ovarian clear cell carcinoma: identification of hepatocyte nuclear factor-1 beta as a molecular marker and a possible molecular target for therapy of ovarian clear cell carcinoma. Am J Pathol 163: 2503-2512, 2003.
29. Tsuda H, Ito YM, Ohashi Y, Wong KK, Hashiguchi Y, Welch WR, Berkowitz RS, Birrer MJ and Mok SC: Identification of overexpression and amplification of ABCF2 in clear cell ovarian adenocarcinoma by cDNA microarray analysis. Clin Cancer Res 11: 6880-6888, 2005.

Synthesis and biological analysis of new curcumin analogues bearing an enhanced potential for the medicinal treatment of cancer

Hisatsugu Ohori,[1,2] Hiroyuki Yamakoshi,[3] Masaki Tomizawa,[3] Masatoshi Shibuya,[3] Yuichi Kakudo,[1,2] Atsuko Takahashi,[1] Shin Takahashi,[1] Satoshi Kato,[4] Takao Suzuki,[4] Chikashi Ishioka,[1,2] Yoshiharu Iwabuchi,[3] and Hiroyuki Shibata[1,2]

[1]Department of Clinical Oncology, Institute of Development, Aging and Cancer, [2]Tohoku University Hospital, and [3]Department of Organic Chemistry, Graduate School of Pharmaceutical Science, Tohoku University; and [4]Department of Clinical Oncology, Sendai Medical Center, National Hospital Organization, Sendai, Japan

Abstract

Curcumin (diferuloylmethane) is a dietary phytochemical with low toxicity that exhibits growth-suppressive activity against a variety of cancer cells and possesses certain chemopreventive properties. Curcumin has already been the subject of several clinical trials for use as a treatment in human cancers. Synthetic chemical modifications of curcumin have been studied intensively in an attempt to find a molecule with similar but enhanced properties of curcumin. In this study, a series of novel curcumin analogues were synthesized and screened for anticancer activity. New analogues that exhibit growth-suppressive activity 30 times that of curcumin and other commonly used anticancer drugs were identified. Structurally, the new analogues are symmetrical 1,5-diarylpentadienone whose aromatic rings possess an alkoxy substitution at each of the positions 3 and 5. Analysis of the effects of the analogues on the expression of cancer-related genes usually affected by curcumin indicated that some induced the down-regulation of β-catenin, Ki-ras, cyclin D1, c-Myc, and ErbB-2 at as low as one eighth the concentration at which curcumin normally has an effect. The analogues, however, exhibited neither harmful nor growth-suppressive effects on normal hepatocytes where oncogene products are not activated. They also exhibited no toxicities *in vivo* that they may provide effective alternative therapies for the prevention and treatment of some human cancers. [Mol Cancer Ther 2006;5(10):2563–71]

Introduction

Many anticancer therapies currently in use are inadequate not only in terms of their therapeutic efficacy but also because they have undesirable side effects. On the other hand, certain dietary constituents known as phytochemicals have been shown to exhibit growth-suppressive activity and chemopreventive properties against various types of cancers (1) without the adverse side effects normally associated with current chemotherapies. Curcumin is one of the most widely characterized of the phytochemicals, exhibiting both growth-suppressive potential in a wide variety of tumor cells and a chemopreventive effect in certain types of cancer, such as colon and skin cancers (2, 3).

The mechanism of action of curcumin has been extensively studied at the molecular level (4). It is known that curcumin interferes with the transcriptional activation induced by transcription factors, such as nuclear factor-κB (NF-κB; ref. 5) and activator protein-1 (6), resulting in the negative regulation of various cell cycle control genes and oncogenes, such as *c-Myc, cyclin D1, Bcl-2*, and *Bcl-XL* (4). Cyclooxygenase-2, which is overexpressed in colorectal cancers through NF-κB or activator protein-1 transactivation, is also suppressed by curcumin (7), and other cyclooxygenase-2 inhibitors are known to have chemopreventive and antiangiogenic properties (4). Curcumin has been shown to arrest the cell cycle at G_0-G_1 or G_2-M through up-regulation of the cyclin-dependent kinase inhibitors p21 and p27 and down-regulation of Cdc2 and cyclin B1 (8). Curcumin blocks growth factor signaling via inhibition of tyrosine kinase activity or depletion of ErbB-2 (9). More recently, it has been shown that curcumin causes the cleavage of β-catenin, resulting in apoptosis in a colon cancer–derived cell line (10, 11). Loss of function of the *APC* tumor suppressor gene, which is mutated in most colon cancers, inhibits β-catenin degradation (12) and the resulting accumulation of β-catenin in the cytosol that is translocated to the nucleus causing transactivation of oncogenes, including *c-Myc* and *cyclin D1* (13). The loss of the *APC* gene function and subsequent accumulation of β-catenin is therefore believed to be the initiation event of colorectal carcinogenesis (14, 15). Hence, the ability of curcumin to target β-catenin for degradation is considered to be the basis of the chemopreventive effect of curcumin in colorectal cancer. It was found that curcumin treatment

Received 3/29/06; revised 6/28/06; accepted 8/16/06.

Grant support: Grant-in-Aid for Scientific Research (Category C, 16590571) from the Japanese Society for the Promotion of Science (S. Kato, Y. Iwabuchi, and H. Shibata) and Miyagi Health Service Association grant (C. Ishioka).

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.

Note: Y. Iwabuchi and H. Shibata contributed equally to this work.

Requests for reprints: Hiroyuki Shibata, Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan. Phone: 81-22-717-8547; Fax: 81-22-717-8548. E-mail: hiroyuki@idac.tohoku.ac.jp

doi:10.1158/1535-7163.MCT-06-0174

reduced the incidence of adenoma formation in the familial adenomatous polyposis mouse model to 40% of control (2).

However, in clinical trials of oral administration of curcumin to human cancer patients, the systemic availability of curcumin was found to be negligible, especially outside the gut, due to poor absorption of the compound (16, 17). We synthesized and tested the growth-suppressive ability of >50 synthetic analogues of curcumin to increase the potentials of curcumin and circumvent the low bioavailability while keeping its low toxicity. Several derivatives showed an enhanced ability to induce apoptosis in different cancer cell lines. These derivatives also decreased the expression levels of oncoproteins, including β-catenin, Ki-ras, cyclin D1, and ErbB-2, at concentrations much lower than those normally used for curcumin.

Materials and Methods

Compounds

Chemical synthesis, physical properties, and molecular formulas of the new derivative compounds are published as supporting information (Supplementary Fig. S1).[5] Curcumin (Sigma-Aldrich, Inc., St. Louis, MO) and its analogues were dissolved in DMSO at 50 mmol/L as stock solution. Caspase-3/caspase-8 inhibitor N-CBZ-ASP-GLU-VAL-ASP fluoromethyl ketone (Z-DEVD-fmk) was purchased from Sigma-Aldrich.

Cell Lines and Culture Conditions

All cell lines, except below, were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). HCT116 carrying wild-type p53 [HCT116 cells ($p53^{+/+}$)] and HCT116 lacking wild-type p53 [HCT116 cells ($p53^{-/-}$)] were a kind gift from Dr. B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD; ref. 18). Normal human primary hepatocytes (hNHeps) were purchased from Cambrex Bio Science Walkersville, Inc. (East Rutherford, NJ).

Cell Growth Suppression Analysis

Growth-suppressive effects of the derivative compounds were measured in different cancer cell lines for 96 hours. Cell viability was assayed by quantitation of the uptake and digestion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan) by 96-well plate reader, MPR-A4i (Tosoh Corp., Tokyo, Japan). The percentage cell growth of the control, which was treated with 1% DMSO alone, was calculated and plotted, and then the mean growth-inhibitory concentration (IC_{50}) value was determined.

Cell Cycle Analysis

Cell cycle phase was determined by fluorescence-activated cell sorting analysis. Cells from the cell line HCT116 were inoculated into six-well plates at a concentration of 5×10^5 per well, exposed to the derivative compounds at their various concentrations, cultured for 30 hours, collected, and sorted using a FACScan flow cytometer (Beckman Coulter, Inc., Fullerton, CA) as described previously (19). The percentage of each cell fraction corresponding to the sub-G_1, G_0-G_1, S, and G_2-M phases was calculated using MacCycler (Phoenix Flow Systems, San Diego, CA).

Caspase-3-Like Activity

The induction of caspase-3-like activity was measured by fluorescence as described previously (20). In brief, cells were treated with curcumin analogues for 24 hours, washed with PBS, lysed in a buffer containing 0.5% NP40, 10 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 5 μg/mL leupeptin, and then spun. The caspase-3 substrate N-acetyl-ASP-GLU-VAL-ASP-7-amido-4-methylcoumarin (50 μmol/L; Calbiochem, La Jolla, CA) was incubated with the supernatant containing 250 μg of total protein at 37°C for 30 minutes. Fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Caspase-3-like activity was represented as fluorescence units per minute per milligram of protein.

NF-κB Transactivation

NF-κB transactivation was measured by ELISA using a NF-κB p50 kit (Stressgen Bioreagents, Victoria, British Columbia, Canada) according to the manufacturer's instructions. Shortly, cells were treated with curcumin analogues for 8 hours, washed with PBS, and lysed in the buffer containing radioimmunoprecipitation assay extraction reagents. Whole-cell extracts containing 25 μg protein were applied to the assay kit. The chemiluminescence derived from the active form of NF-κB p50 was measured using a CCD camera (Las-1000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

Expression Profile

Total RNA was extracted from HCT116 treated with compounds using an RNeasy mini kit (Qiagen, Inc., Chatsworth, CA). Total RNA (500 ng) was amplified and labeled using low RNA input linear amplification and a labeling kit according to the manufacturer's protocol (Agilent Technologies, Inc., Palo Alto, CA). For cRNA labeling, cyanin 3-CTP (Cy3) and cyanin 5-CTP (Cy5; Perkin-Elmer, Inc., Wellesley, MA) were used. Except for the dye swap experiment, controls were labeled with Cy3 and samples were labeled with Cy5. The integrity of the labeled cRNA and labeling index of the dye were confirmed by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Labeled cRNA (1 μg) was hybridized with the microarray with 41,058 human cDNA oligonucleotides (Human Whole Genome Oligo Microarray kit with SuperPrint Technology, Agilent Technologies) according to the manufacturer's protocol. After washing, the array slide was scanned using ScanArray 5000 (GSI Group, Inc., Wilmington, MA). Images were processed and signals were quantitated using ArrayVision version 8 (Amersham Biosciences Corp., Piscataway, NJ). The obtained data were normalized using

[5] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).