表 1　NAC 療法と標準治療の比較（腫瘍縮小手術における optimal surgery，生存率）

報告者（年） 治療法［症例数］	生存率の比較		腫瘍縮小手術	NAC 群の選択
Jacob（1991） 標準治療　［n＝18］ NAC 療法［n＝22］	MST 18 M 16 M NS		optimal（＜2 cm） 39%（7/18） 77%（17/22） p＝0.02	NAC 群，標準群とも他院で生検のみ施行。標準治療群は進行期，組織型，分化度，年齢を match させた control
Onnis（1996） 標準治療　［n＝284］ NAC 療法［n＝88］	3 year 31% 27% NS	5 year 21% 19% NS	optimal（＜2 cm） 29%（83/284） 42%（37/88） NA	胸水，肝転移の有無，試験開腹による切除可能性の評価により NAC 療法群を決定。NAC 療法群はより進行した症例が多い
Vergote（1998） NAC 導入前［n＝112］ NAC 導入後［n＝173］	3 year 26% 42% p＝0.0001			試験開腹，腹腔鏡による切除可能性の評価により NAC 療法群を決定
Kayikçioğlu（2001） 標準治療　［n＝158］ NAC 療法［n＝45］	5 year 24% 30% NS	MST 38 M 34 M NS	optimal（＝0） 14%（22/158） 49%（22/45） p＜0.001	胸水，肝転移，切除不能な多発転移の有無，全身状態により NAC 療法群を決定。NAC 療法群は有意に高齢（p＝0.01），PS 不良（p＜0.001）でⅣ期症例が多い（p＝0.03）
Kuhn（2001） 標準治療　［n＝32］ NAC 療法［n＝31］	MST 23 M 42 M p＝0.007		optimal（＜2 cm） 63%（20/32） 84%（26/31） p＝0.04	対象は多量の腹水（＞500 mL）を有する卵巣癌ⅢC 期に限定。臨床試験に同意が得られなかった症例に標準治療。標準治療群と NAC 療法群の背景に有意差なし
Loizzy（2005） 標準治療　［n＝30］ NAC 療法［n＝30］	MST 40 M 32 M NS	DFI 16 M 21 M NS	optimal（＜1 cm） 60%（18/30） 63%（19/30） NS	多量の胸水，腹水，全身状態，CT による切除可能性の評価により NAC 療法群を決定。標準治療群は組織型，進行期を match させた control。NAC 群は有意に高齢（p＝0.03），有意に PS 不良（p＝0.02）
Lee（2006） 標準治療　［n＝22］ NAC 療法［n＝18］	MST 55 M 53 M NS	DFI 17 M 15 M NS	optimal（＜2 cm） 46%（10/22） 78%（14/18） p＝0.04	CT，MRI により切除可能性を評価し，NAC 群を決定
Everett（2006） 標準治療　［n＝102］ NAC 療法［n＝98］	MST 42 M 33 M NS		optimal（＜1 cm） 54%（55/102） 86%（84/98） p＜0.001	肝転移，大きな上腹部転移，広範なリンパ節転移，重篤な合併症などにより NAC 群を決定。NAC 群は有意にⅣ期（p＝0.042），低分化（p＝0.025）症例が多い
Inciura（2006） 標準治療　［n＝361］ NAC 療法［n＝213］	MST 25 M 24 M NS	DFI 15 M 13 M NS	optimal（＜2 cm） 67%（242/361） 63%（134/213） NS	多量の腹水，大きな骨盤内 or 腹部腫瘍の存在により NAC 療法群を決定
Hou（2007） 標準治療　［n＝109］ NAC 療法［n＝63］	MST 47 M 46 M NS	DFI 14 M 16 M NS	optimal（＜1 cm） 71（77/109） 95（60/63） ＜0.001	重篤な合併症および画像診断で腹部を超えた進展，広範な腹腔内進展により NAC 群を決定。NAC 群で有意にⅣ期症例が多い（＜0.05），NAC 群でより高齢，より低分化腫瘍であったが有意差はなし

NA: not available，MST: median survival time，DFI: disease free interval

い場合には，診断確認のための開腹術や腹腔鏡が必須である。② 化学療法の効果が得られなければ，腫瘍縮小手術の機会を逸する，optimal surgery の達成を逸する，などの可能性がある。③ 腫瘍量の多い状態で化学療法を行うため，薬剤耐性細胞の出現数が多くなり，また血流不十分な細胞の存在により，薬剤耐性の出現の可能性も高くなる。④ 腫瘍縮小手術に際して，肉眼的に腫瘍の縮小，消失が得られているため，術式を縮小しすぎて，却って根治性を損なってしまう可能性がある。

②～④ の問題点に関しては，これらの問題がありながらも NAC 療法が標準治療と同等あるいは優る治療成績が得られるのかを，NAC 療法と標準治療の prospective な比較試験で検証する必要があると考えられる。

4．無作為比較試験による比較

retrospective study の結果を踏まえて，EORTC（European Organization for Research and Treatment of

表 2 NAC 療法と標準治療の比較（手術合併症などの比較）

報告者（年） 治療法［症例数］	手術合併症などの比較				NAC 群の選択
Vergote（1998）	手術関連死亡率				
NAC 導入前［n＝112］	6%				試験開腹，腹腔鏡による切除可能性の評価により NAC 療法群を決定
NAC 導入後［n＝173］	0%				
	NA				
Schwartz（1999）	出血量	ICU 滞在	入院期間		全身状態，合併症による手術可否の評価，CT による切除可能性の評価により NAC 療法群を決定。NAC 療法群は有意に高齢（<0.001），PS 不良（<0.001）であった
標準治療 ［n＝206］	1,000 mL	1.26 days	11 days		
NAC 療法［n＝59］	600 mL	1.03 days	7 days		
	p＝0.001	p＝0.01	p<0.001		
Kayikçioğlu（2001）	結腸切除	脾摘			胸水，肝転移，切除不能な多発転移の有無，全身状態により NAC 療法群を決定。NAC 療法群は有意に高齢（p＝0.01），PS 不良（p<0.001）で，Ⅳ期症例が多い（p＝0.03）
標準治療 ［n＝158］	16%	11%			
NAC 療法［n＝45］	2%	0%			
	p＝0.01	p＝0.02			
Morice（2003）	腸切	脾摘	重篤な合併症	輸血割合	
標準治療 ［n＝28］	61%	7%	36%	39%	試験開腹，腹腔鏡による切除可能性の評価により NAC 療法群を決定
NAC 療法［n＝57］	19%	5%	7%	21%	
	p＝0.01	NS	p＝0.01	NS	
Hegazy（2005）	出血量	ICU 滞在	入院期間		試験開腹，腹腔鏡による切除可能性の評価により NAC 療法群を決定。NAC 群は有意に高齢（p＝0.04）
標準治療 ［n＝32］	735 mL	4.4 days	15.9 days		
NAC 療法［n＝27］	420 mL	1.7 days	10.5 days		
	p＝0.02	p＝0.03	p<0.05		
Lee（2006）	出血量				CT，MRI により切除可能性を評価し NAC 群を決定
標準治療 ［n＝22］	1,061 mL				
NAC 療法［n＝18］	620 mL				
	p＝0.04				
Hou（2007）	出血量	手術時間	入院期間	輸血量	重篤な合併症，および画像診断で腹部を超えた進展，広範な腹腔内進展により NAC 群を決定。NAC 群で有意にⅣ期症例が多い（<0.05），NAC 群でより高齢，より低分化腫瘍であったが有意差はなし
標準治療 ［n＝109］	1,033 mL	276 min	8.5 days	2.4 U	
NAC 療法［n＝63］	546 mL	211 min	5.7 days	1.2 U	
	p<0.0001	p<0.0001	p<0.0001	p＝0.03	

NA: not available

Cancer）では，第Ⅲ相ランダム化比較試験として EORTC55971[15]を行っている。卵巣癌，卵管癌，腹膜癌のⅢC/Ⅳ期を対象に，診断的腹腔鏡，試験開腹，穿刺組織診のいずれかの方法で原発診断，組織診断，進行期診断の後，NAC 療法群と手術先行の標準治療群に割り付けしている。卵管癌，腹膜癌は，組織学的所見，化学療法感受性，予後が卵巣癌とほぼ同一であり，卵巣，卵管の摘出なしでは鑑別診断困難であることから対象に含めている。プロトコール治療は，NAC 療法群では 3 コースの化学療法の後，腫瘍縮小手術を行い，術後 3 コースの化学療法追加，標準治療群では PDS を行い，optimal surgery が達成できた症例では 6 コースの化学療法，suboptimal の症例では 3 コースの化学療法の後 IDS を行い，術後 3 コースの化学療法追加である。化学療法としては，白金製剤＋タキサン系薬剤のいずれの組み合わせでも可としている。この臨床試験は 2006 年 12 月で登録終了となり現在データ集積中である。

JCOG（Japan Clinical Oncology Group）の婦人科腫瘍グループでは，2003 年 1 月から，「Ⅲ/Ⅳ期卵巣癌，卵管癌，腹膜癌に対する術前化学療法の Feasibility study」（JCOG0206）[16]を行い，その結果を踏まえ，現在 EORTC と同様の第Ⅲ相比較試験「Ⅲ期/Ⅳ期卵巣癌，卵管癌，腹膜癌に対する手術先行治療 vs 化学療法先行治療のランダム化比較試験」（JCOG0602）を開始している。化学療法としては PTX と CBDCA の組み合わせの TC 療法で，NAC 群では術前 4 コース，術後 4 コースの合わせて 8 コースを行っている。これらの試験はいずれも NAC 療法が標準治療に対して，効果の点で劣らないことを検証する非劣性試験である。NAC 療法では手術に関連した侵襲の軽減（手術回数，輸血必要量など）が期待されるため，非劣性が証明されれば NAC 療法が進行卵巣癌の標準治療になると考えられる。

おわりに

retrospective studyの結果から，進行卵巣癌に対するNAC療法は治療成績およびQOLの改善が期待される治療ではあるが，診断が不正確となる可能性，手術の機会を逸する可能性，薬剤耐性の出現を助長する可能性，根治性を損なう可能性などのriskも有している。現在行われている二つのprospectiveな比較試験により，進行卵巣癌におけるNAC療法の役割が明らかとなることが期待される。

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Pilot study evaluating the efficacy and toxicity of irinotecan plus oral etoposide for platinum- and taxane-resistant epithelial ovarian cancer

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Abstract

Objectives. To evaluate the efficacy and toxicity of combination chemotherapy with intravenous irinotecan and oral etoposide in women with platinum- and taxane-resistant epithelial ovarian cancer.

Methods. Between October 2002 and September 2005, we studied 27 women with platinum- and taxane-resistant epithelial ovarian cancer. Irinotecan was administered in an intravenous dose of 70 mg/m^2 as a 90-min infusion on days 1 and 15 of a 28-day cycle, and etoposide was administered in an oral dose of 50 mg/day on days 1 to 21. For heavily pretreated patients, the initial dose of irinotecan was lowered to 60 mg/m^2. Treatment cycles were repeated until disease progression or unacceptable toxicity.

Results. All 27 patients were eligible and assessable. There were 11 partial responses and 1 complete response for an overall response rate of 44.4%. The median durations of overall response and of stable disease were 11 months and 8 months, respectively. The major toxicity was neutropenia (grade 3, 22.2%; grade 4, 37.1%). Diarrhea was infrequent and mild, and gastrointestinal toxicity was moderate and manageable. Acute myeloid leukemia (M5) developed as a secondary malignancy in 1 patient.

Conclusions. The results of our pilot study suggest that a combination of irinotecan and oral etoposide is effective and tolerable in women with platinum- and taxane-resistant epithelial ovarian cancer.

Keywords: Irinotecan; Oral etoposide; Platinum/taxane-resistant ovarian cancer

Introduction

Various agents and treatment regimens have been introduced to treat recurrent ovarian cancer resistant to platinum/taxane (PT), currently the standard first-line chemotherapy. Generally, relapse within 3 months after first-line platinum containing therapy is defined as platinum-refractory disease, relapse between 3 and 6 months after therapy is defined as platinum-resistant disease and relapse more than 6 months after therapy is defined as platinum-sensitive disease. Topotecan, gemcitabine, etoposide and liposomal doxorubicin produce response rates of 20% to 30%, but the time to progression is usually short, particularly in PT-resistant or -refractory disease [1–3]. Irinotecan is a topoisomerase-I inhibitor similar to topotecan, a drug approved by the Food and Drug Administration (FDA) for the second-line treatment of ovarian cancer [4,5]. Irinotecan has been studied in Japan for the management of ovarian cancer. In a phase II study, 55 patients received irinotecan in a dosage of 100 mg/m^2 once weekly and 150 mg/m^2 once every 2 weeks. The response rate was 23.6%. Major adverse effects were leukopenia, nausea and vomiting, diarrhea and anorexia, with incidences (grade 3 or 4 hematological toxicity and grade 2 or higher nonhematological toxicity) of 57.1%, 60.3%, 44.0% and 67.2%, respectively [6]. This compares favorably with the response to topotecan. Etoposide, a topoisomerase-II inhibitor, has high antitumor activity against various animal and human malignancies [7]. The efficacy of etoposide may be regimen-dependent, since prolonged oral administration has yielded better results than intravenous administration [8,9]. The largest

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study to date, performed by the Gynecologic Oncology Group (GOG), reported a response rate of 8.3% [10]. Long-term treatment with oral etoposide has produced better results in women with platinum-resistant ovarian carcinoma [1,11–14]. In a study by de Wit et al. [12], 50 mg/m^2 of oral etoposide was administered for 21 days every 4 weeks to 28 patients with platinum-resistant ovarian cancer, resulting in a response rate of 16.0%. Rose et al. [1] gave oral etoposide to 41 patients with platinum-sensitive or -resistant recurrent ovarian cancer and obtained response rates of 34.6% and 26.8%, respectively.

DNA topoisomerases-I and -II are nuclear enzymes that participate in various genetic processes, including transcription, replication, recombination and chromosome segregation at mitosis [4]. These two DNA topoisomerases are functionally related and act in concert. Both seem to be essential for maintaining cell viability throughout the cell cycle. Topoisomerase-I treatment induces an increase in the S-phase cell population with an increase in topoisomerase-II mRNA expression. Thus, topoisomerase-I can modulate topoisomerase-II levels to enhance the effect of topoisomerase-II inhibitors [15,16]. Therefore, combined use of topoisomerase-I- and topoisomerase-II-targeting agents could theoretically inhibit both DNA and RNA synthesis completely, resulting in synergistic cytotoxicity.

This pilot study was undertaken to evaluate the antitumor efficacy and toxicity of a combination of irinotecan, a DNA topoisomerase-I inhibitor, and oral etoposide, a DNA topoisomerase-II inhibitor, in women with platinum- and taxane-resistant epithelial ovarian cancer.

Patients and methods

Eligibility

Patients were eligible for this study if they satisfied the following criteria: (1) histologically confirmed epithelial ovarian cancer; (2) recurrent disease after previous treatment with platinum and taxanes; (3) an Eastern Cooperative Oncology Group performance (ECOG) status of <2; (4) measurable or assessable disease. Assessable disease was defined according to the following CA-125 criteria: a CA-125 level of greater than 70 U/mL at study entry; this CA-125 level must have at least doubled from the baseline level, providing evidence of disease progression while receiving a previous treatment regimen (as confirmed by at least two separate blood samples obtained >4 weeks apart) (GCIG guidelines) [17]. (5) Age <75 years; (6) adequate laboratory values (leukocyte count >4000/μL, absolute neutrophil count >1500/μL, platelet count >100,000/μL, hemoglobin level >9.5 g/dL, total bilirubin <2.0 mg/dL and serum aspartate aminotransferase or alanine aminotransferase <2 times the upper limit of normal at the center performing the test); and (7) a signed informed consent statement confirming that the subject understood the experimental nature of the study treatment.

Patients were excluded from the study if any of the following applied: (1) Previous treatment with irinotecan or topotecan; (2) concurrent active or uncontrolled infection; (3) any psychiatric disorders potentially interfering with consent or follow-up; (4) pregnant women or nursing mothers; (5) other active malignancies; (6) clinically significant comorbidity (e.g., a history of previous myocardial infarction within the past 6 months, congestive heart failure requiring therapy, a history of seizures or uncontrolled diabetes, clinically apparent metastases to the central nervous system); (7) poor oral intake due to intestinal obstruction; (8) large amounts of pleural effusion, pericardial fluid or ascitic fluid, requiring repeated drainage; (9) previous abdominal radiation therapy; (10) Apparent pulmonary fibrosis or interstitial pneumonia; and (11) watery diarrhea or other health problems that the attending physician felt would interfere with treatment. The study protocol was approved by institutional review board of each participating center.

Platinum/taxane-refractory disease was defined as tumor progression during treatment or within 3 months after the completion of therapy. Platinum/taxane-resistant disease was defined as tumor progression between 3 and 6 months after the completion of the most recent course of therapy. Any regimen that contained a platinum/taxane drug was counted as one regimen for the purpose of this study. For example, if a patient received cisplatin with paclitaxel as first-line therapy and then received weekly carboplatin and paclitaxel after recurrence, the number of regimens was considered to be two ("cisplatin with paclitaxel" and "weekly carboplatin with paclitaxel"). If the patient then received carboplatin monotherapy after progression, the number of regimens was considered to be three ("cisplatin with paclitaxel," "weekly carboplatin with paclitaxel" and "carboplatin").

Treatment schedule

Irinotecan 70 mg/m^2 was administered as a 90-min intravenous infusion on days 1 and 15 of a 28-day cycle. Etoposide 50 mg/day was given orally on an empty stomach at bedtime with metoclopramide or domperidone for 21 days starting on day 1. These starting doses were based on the results of a phase I study [18]. For patients who were heavily pretreated and received the study treatment as third- or fourth-line therapy, the starting dose of irinotecan was reduced to 60 mg/m^2. Treatment cycles were repeated until evidence of disease progression or unacceptable toxicity. A $5HT_3$-antagonist was given before the administration of irinotecan. For etoposide, premedication was left to the discretion of the attending physicians. Routine prophylactic treatment with granulocyte colony-stimulating factor (G-CSF) was not recommended. During the first course of chemotherapy, G-CSF was used to treat grade 4 neutropenia. During subsequent courses, G-CSF could be used to treat grade 3 or 4 neutropenia in accordance with published guidelines [19]. However, etoposide was withheld on days when G-CSF was administered.

Treatment with irinotecan was withheld if the patient had a leukocyte count of less than 2000/μL, a platelet count of less than 100,000/μL, or >grade 2 diarrhea, fever, or both on the day scheduled for treatment. Before the next course was started, the leukocyte count had to be at least 3000/μL, the platelet count at least 100,000/μL and the diarrhea or fever had to have completely resolved. Subsequent doses were decided on the basis of hematologic and nonhematologic toxicity. If the criteria for resuming treatment were not met for more than 6 weeks since the last dose, the patient was withdrawn from the study. The dose of irinotecan for subsequent cycles of treatment was reduced by 10 mg/m^2 if grade 4 neutropenia persisted for more than 7 days, the platelet nadir was less than 50,000/μL or >grade 3 diarrhea occurred in the preceding cycle. The minimum dose of irinotecan was set at 40 mg/m^2. Patients who had evidence of disease progression or intolerable toxicity (grade 4 diarrhea, neutropenic fever, or both for more than 7 days, or grade 2 or higher pneumonitis) were withdrawn from the study. The dose of etoposide was reduced to 25 mg/day if grade 3 or 4 (according to The National Cancer Institute [NCI] Common Terminology Criteria for Adverse Events [Version 2]; NCI-CTC ver.2) emesis occurred despite treatment with antiemetic agents.

Study evaluations

All patients underwent a complete blood count, platelet count, serum chemical analyses to measure renal and hepatic functions, electrolyte analysis, urinalysis and toxicity assessments weekly. At the end of each 4-week cycle, the CA-125 level was determined. Antitumor effects were evaluated according to the RECIST criteria [20] on the basis of computed tomographic or magnetic resonance imaging scans in patients with measurable lesions. The GCIG CA-125 response criteria proposed by Rustin et al. [17] were used to evaluate antitumor response in patients without measurable lesions. These evaluations were performed after the completion of each cycle of treatment (4 – 6 weeks). Response in patients with measurable lesions was evaluated on the basis of symptoms or imaging findings. Response in patients with non-measurable lesions was evaluated based on elevation of CA 125.

NCI-CTC Ver. 2 was used to grade organ damage [21]. Survival was calculated from the date of starting the study treatment to the date of death, or data were censored at the time of last contact.

Table 1
Patient characteristics

Characteristic	No. of patients (%)
Age, years	
Median	58
Range	34–71
No. of previous regimens	
1	5 (18.6)
2	12 (44.4)
3	9 (33.3)
≥4	1 (3.7)
Treatment-free interval, months	
<3	19 (70.3)
3–6	8 (29.7)
Performance status (PS)	
0	13 (48.2)
1	7 (25.9)
2	7 (25.9)
Measurable sites or assessable CA-125 (=70 U/mL)	
Visceral	9 (33.3)
Soft tissue	6 (22.2)
Lymph node	2 (7.4)
CA-125	10 (37.1)
Histology	
Serous	19 (70.4)
Mucinous	3 (11.1)
Endometrioid	3 (11.1)
Clear cell	2 (7.4)
No. of cycles: median	5 (range, 1–25)
CR+PR	6 (range, 1–16)
SD	5 (range, 1–25)
PD	2 (range, 2–3)

CR, complete response; PR, partial response; SD, stable Disease; PD, progressive disease.

Statistical analysis

To evaluate toxicity, time-to-event data were analyzed with the use of Kaplan–Meier survival curves. Duration of response was measured from the date an initial response was documented to the date of disease progression, relapse or death. Time to progression was calculated from the date of starting treatment with irinotecan and etoposide to the date of first documentation of tumor progression. Survival time was calculated from the date of diagnosis to the date of death or the date of the last known contact.

This study was designed to test the hypothesis that the true response rate was <0.10 versus the alternative hypothesis that it was >0.25. A two-stage sampling plan was employed, which featured accrual of 27 patients in the first stage and additional accrual of 13 patients in the second phase if at least three responses were observed in the first stage. At least 10 responses among these 40 patients were necessary to reject the null hypothesis. This design featured a size of 0.05 and a power of 0.8.

Results

Between October 2002 and September 2005 at Kurume University Hospital and Iwate Medical University Hospital, we enrolled 27 women with platinum- and taxane-resistant epithelial ovarian cancer. All were eligible for analysis. The characteristics of the subjects are shown in Table 1. The median age was 58 years (range, 34–71 years). The treatment-free interval was less than 3 months in 19 patients and 3 to 6 months in 8. Seventeen patients had measurable lesions. The most common sites of recurrent lesions were the viscera, soft tissue and lymph nodes. Ten patients lacked measurable lesions but had high CA-125 levels (>70 U/mL), with a median value of 100 U/mL (range, 75–350 U/mL) at enrollment. Five patients received the study therapy as second line, 12 as third line, 9 as fourth line and 1 as fifth line.

Response

Of the 17 patients with measurable disease, 10 (47.6%) had objective responses (1 complete response [CR] and 9 partial responses [PR]). Of the 10 patients in whom response was evaluated according to the CA-125 criteria, 6 (42.9%) had at least a 50% decrease in the level of this tumor marker. Two of these patients met the criteria for PR. Thus, the overall rate of objective response (CR+PR according to the RECIST and CA-125 criteria) in this pilot study was 44.4% (12/27) (95% confidence interval, 30.5% to 61.8%). Eleven patients (42.8%) had stable disease (SD), and the other 4 (11.4%) had progressive disease (PD). The progression-free (CR+PR+SD) rate was 85.1%.

The median duration of response in the 12 patients who had objective responses was 11 months (range, 4–18 months). The median duration of SD in the 11 patients who had SD was 8 months (range, 4–22 months). The median time to progression (TTP) in the study group as a whole was 9 months (range, 1–28 months). The median survival was 17 months (range, 3–31 months).

Toxicity and treatment received

The 27 patients received a total of 186 cycles of therapy. The initial dose of irinotecan was 70 mg/m^2 in 19 patients. The dose was subsequently reduced to 60 mg/m^2 in 4 of these patients. Among the 8 patients who initially received irinotecan 60 mg/m^2, the dose was reduced to 50 mg/m^2 in 1. There was no difference in response between 70 mg/m^2 and 60 mg/m^2. In 179 cycles, irinotecan was administered on days 1 and 15 as scheduled. In 3 cycles the dose of irinotecan scheduled for day 1 was delayed. In 4 cycles the dose of irinotecan scheduled for day 15 was skipped.

Table 2
Adverse effects (n=27)

	Grade (%)				
Adverse effect	1	2	3	4	≥3
Leukopenia	2	10	10	4	14 (51.9)
Neutropenia	4	7	6	10	16 (59.3)
Thrombocytopenia	0	1	2	0	2 (7.4)
Anemia	1	3	10	0	10 (37.1)
Nausea	13	7	3	1	4 (14.8)
Vomiting	13	2	3	1	4 (14.8)
Diarrhea	6	1	2	0	2 (7.4)
Renal	0	0	0	0	0
Neurotoxicity	0	0	0	0	0
Infection	1	0	2	1	3 (11.1)
Febrile neutropenia	0	0	2	1	3 (11.1)
Secondary malignancy	–	–	0	1[a]	1 (3.7)

[a] Acute myeloid leukemia (AML).

The dose of oral etoposide was reduced in 8 (29.6%) of the 27 patients. All patients were in-patients during first cycle of treatment; after the first cycle 24 patients (88%) were out-patients.

Table 2 lists adverse effects according to the highest grade during treatment. The nadir of the neutrophil count was usually reached around day 15, with recovery in most patients by day 18. During the first course of treatment, G-CSF was administered to 6 patients (22.2%) who had grade 4 neutropenia, and in subsequent courses a total of 7 patients (25.9%) received G-CSF. The median duration of treatment with G-CSF was 5 days (range, 2–11 days).

One 64-year-old woman had grade 4 acute myeloid leukemia (AML; karyotype of M5) as a secondary malignancy after 10 treatment cycles. The white cell count rose to $277 \times 10^2/\mu L$ after 10 cycles, and bone marrow examination confirmed AML. Despite 2 cycles of cytarabine and idarubicin, complete remission was not achieved. The patient did not respond to subsequent treatment, including 1 cycle of mitoxantrone, etoposide and cytarabine (MEC), 2 cycles of cytarabine and aclarubicin and 1 cycle of aclarubicin, vincristine and daunorubicin, She died from acute respiratory failure of unknown cause 13 months after initial treatment.

Discussion

Despite therapeutic advances during the past 5 decades, culminating in the development of cytoreductive surgery followed by PT chemotherapy, more than 60% of patients with ovarian cancer die of recurrent disease. In patients with PT-refractory or resistant disease, the response rate remains between 15% and 20%, with median survival of only 8 months [22]. In this study, all patients received the first cycle of therapy on an in-patient basis, whereas 24 (88%) received subsequent cycles as out-patients. Thus, irinotecan plus oral etoposide maintained the patients' quality of life (QOL). Such treatments must achieve a balance between antitumor effectiveness and toxicity. The results of our pilot study, performed at 2 centers, suggest that combination therapy with irinotecan and oral etoposide produces high rates of objective responses in women with recurrent ovarian cancers, especially PT-resistant disease. Our results also demonstrated that this regimen is relatively well tolerated even in heavily pretreated patients who have received multiple chemotherapeutic agents, including platinum compounds and taxanes.

In this study, the RECIST criteria [20] were used to assess response in patients with measurable disease, and the GCIG CA-125 response criteria [17] were used in patients without measurable disease. Our overall objective response rate (44.4%) was high, given that disease resistance to prior chemotherapy was higher than that in most previous trials of second-line therapy for ovarian cancer. Of note, the non-progression (CP + PR + SD) rate was 85.1%. However, this study included seven patients who were sensitive relapse in the first-line and might produce higher response rate. Actually, the response rate might be lower in general population of platinum/taxanes resistance. A study by van der Burg et al. [23] reported response rates of 46%, 91% and 92% in patients who had progression at 0–4, 4–12 and >12 months, respectively, while receiving a combination of weekly cisplatin and oral etoposide. Meyer et al. [24] reported a response rate of 46% in patients who had progression within 6 months while receiving the same regimen.

The responses to irinotecan plus oral etoposide were durable, with a median TTP of 9 months, as compared with 2.8 to 4 months in studies of single-agent irinotecan [6,25,26]. Our results suggest that irinotecan and oral etoposide may have "supra-additive" or synergistic effects against ovarian cancer, consistent with the findings of *in vitro* studies [27].

The frequency of grades 3 and 4 neutropenia with our regimen of irinotecan plus oral etoposide was slightly higher than that of hematological toxicity reported for irinotecan alone, but all reactions could be managed successfully. The frequency of severe diarrhea, a toxic effect specific to irinotecan, was less than expected. In patients with metastatic platinum-resistant or-refractory ovarian cancer, Bodurka et al. [25] found that single-agent irinotecan at a dose of 300 mg/m^2 given every 3 weeks had an overall response rate of 17.2% and caused reversible >grade 3 neutropenia and diarrhea in 36% and 33% of patients, respectively. Matsumoto et al. [26] reported a response rate of 29% and reversible >grade 3 neutropenia and diarrhea in 17.8% and 10.7% of patients, respectively, during treatment with irinotecan 100 mg/m^2 on days 1, 8 and 15 of a 28-day cycle. The frequency of severe diarrhea caused by irinotecan can thus be reduced by modifying the treatment schedule. However, 17 patients were under 60 years old. Previous studies have found that older patients with ovarian cancer are less likely to receive intensive chemotherapy regimens [28–30], and in clinical practice there is often concern about the tolerability of cytotoxic agents in older patients. Thus, our regimen might be more toxic in the general population of women with ovarian cancer.

In 1 patient AML (M5) developed as a secondary malignancy after 10 cycles of treatment. Topoisomerase-II-related AML, initially noted as a therapy-related complication of childhood leukemia [31], is characterized by lack of a myelodysplastic phase, no dysplastic changes in diagnostic bone marrow specimens, a short latency period (usually less than 3 years), balanced chromosomal translocations involving 11q23 and variable chemosensitivity [32]. Rose et al. [1] reported that AML developed in 3 of 52 patients with ovarian cancer 16, 27 and 35 months after receiving a cumulative dose of 200 mg/m^2, 1200 mg/m^2 and 2400 mg/m^2, respectively. Rose et al. [33,34] also reported that AML developed in 1 patient with ovarian cancer after 10 courses of chemotherapy with oral etoposide (total dose, 16,550 mg) and 1 patient with uterine leiomyosarcoma after 7 courses of chemotherapy with oral etoposide (total dose, 7350 mg/m^2). These leukemias are characteristically related to the cumulative dose of etoposide and have a shorter latency period (median, 24 to 30 months) than the AMLs associated with alkylating agent therapy. Le Deley et al. [35] reported that the risk of AML was related to the cumulative dose of etoposide, with a particularly high risk at dose levels exceeding 6 g/m^2. The total dose of etoposide received by our patient who had AML was 10.5 g. Because the efficacy of continuous palliative treatment with etoposide is offset by its strong leukemogenicity when the total dose exceeds

6 g/m^2, we recommend that our regimen is not given for more than 6 cycles, even if the response is sustained.

Recurrent ovarian cancer, especially PT-resistant disease, is incurable. Single-agent therapy is therefore frequently used for disease management, attempting to maximize therapeutic response while minimizing toxicity. In patients with platinum-sensitive recurrent ovarian cancer, randomized studies have shown that carboplatin-based combination therapies are more effective than carboplatin alone [36]. In contrast, survival with combination chemotherapy has not been found to be superior to that with single-agent therapy in platinum-resistant ovarian cancer. The results of our study suggest that a combination of irinotecan and oral etoposide might extend survival and maintain the QOL of patients with chemoresistant ovarian cancer. Given that our subjects had PT-resistant recurrent ovarian cancers, the median survival time of 17 months appears very promising. However, we had initially planned to enroll 43 patients, but could not because of poor accrual. Moreover, the study was done at only two centers, and secondary leukemia developed in 1 patient after ten cycles. The small size of our study and the lack of a control group preclude us from concluding that irinotecan plus oral etoposide should be the treatment of choice for PT-resistant ovarian cancer. Well-designed phase II trials are needed to confirm the efficacy and toxicity of up to 6 cycles of irinotecan plus oral etoposide or to refute our findings. A nationwide multicenter phase II study is now being considered by the Japan Clinical Oncology Group (JCOG), a large cooperative group.

In conclusion, we believe our results, although preliminary, justify further studies of irinotecan plus oral etoposide in patients with PT-resistant ovarian cancer.

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Promoter methylation status of the Cyclin D2 gene is associated with poor prognosis in human epithelial ovarian cancer

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Gene silencing associated with aberrant DNA methylation of promoter CpG islands is one mechanism through which several genes may be inactivated in human cancers. Cyclin D2, a member of the D-type cyclins, implicated in cell cycle regulation, differentiation and malignant transformation, is inactivated due to aberrant DNA methylation in several human cancers. In the present study, we examined the promoter methylation status and expression of Cyclin D2 in human epithelial ovarian cancer, and then determined the relationship between methylation status and various clinicopathological variables. Twelve ovarian cancer cell lines and 71 surgical specimens were examined by methylation-specific polymerase chain reaction and quantitative reverse transcription-polymerase chain reaction to evaluate the methylation status and expression of the Cyclin D2 gene. The relationship between methylation status and various clinicopathological variables was evaluated using statistical analysis. Aberrant methylation of Cyclin D2 was present in five of 12 ovarian cancer cell lines and 16 of 71 primary ovarian cancer tissues. In five cell lines with methylation, expression of the Cyclin D2 gene tended to be lower than in cell lines without methylation. In ovarian cancer tissues, methylation bands were detected in 16 of 71 cases. The methylation status of Cyclin D2 was associated with advanced stage and a residual tumor size (>2 cm) ($P = 0.027$ and $P = 0.031$, respectively). Based on univariate analysis, patients with aberrant methylation of the Cyclin D2 promoter had a significantly worse chance of disease-free survival than those without methylation ($P = 0.021$). Our results suggest that aberrant promoter methylation of the Cyclin D2 gene is significantly associated with patient prognosis in epithelial ovarian cancer. (*Cancer Sci* 2007; 98: 380–386)

Epithelial ovarian cancer is the most common and deadliest gynecological malignancy in developed countries. Early stages of ovarian cancer are generally asymptomatic and difficult to detect. By the time clinical diagnosis is made, most patients have widespread tumor dissemination.[1] Despite a high response rate to first-line chemotherapy, the prognosis of these women is poor, with an overall 5-year survival rate of only 10–20%.[1,2]

Epigenetic alterations, changes that affect gene expression but not the gene sequence itself, are believed to be one mechanism by which tumor suppressor genes are inactivated in human cancers.[3,4] In particular, hypermethylation of cytosine residues in CpG islands leads to heritable gene silencing via the formation of a repressive chromatin structure.[5,6] Studies of DNA hypermethylation in human ovarian cancer have identified some key genes as targets for epigenetic downregulation, including some hormone receptors,[7] cytokines, cell signaling intermediates, adhesion molecules,[8] DNA damage checkpoint genes,[9] and regulators of the cell cycle.[10] The cell cycle regulators, notably the cyclins, have the potential to function as oncogenes when regulated inappropriately.

The cyclins are a family of proteins that dictate transitions between phases of the cell cycle by regulating the activity of their downstream effectors, the cyclin-dependant kinases (cdk). The D-type cyclins, D1, D2 and D3, play a critical role in early checkpoint regulation of the G_1 phase of the cell cycle. They activate cdk4 and cdk6, leading to the phosphorylation of the retinoblastoma tumor suppressor protein (Rb). This, in turn, dissociates Rb from the transcription factor E2F, thereby permitting DNA transcription. Given the critical role of the D-type cyclins in cell cycle regulation, their abnormal or untimely expression could disrupt the normal cell cycle, resulting in cell proliferation.[11] In fact, Cyclin D1 is considered by some to be a putative protooncogene, as it is overexpressed in a number of tumor types, including breast cancer, thyroid carcinoma, stomach cancer and lymphomas.[12] Aberrant expression of Cyclin D2 has also been demonstrated in human ovarian granulose cell tumors and testicular germ cell tumor cell lines.[13]

Although well known for their proliferation-promoting activity, the D-type cyclins (notably D2) also have growth-inhibitory effects. Cyclin D2 has been shown to be dramatically upregulated under conditions of growth arrest in human and murine fibroblasts. Furthermore, transient overexpression of Cyclin D2 efficiently inhibits cell cycle progression and DNA synthesis. This suggests that an alternative role for Cyclin D2 may be to promote exiting from the cell cycle and maintenance of a non-proliferative state.[14] The expression of Cyclin D2 is frequently lost in human breast cancers, gastric cancers, lung cancers and ovarian granulose cell tumors. This loss of expression is the result of promoter hypermethylation.[10,15–18]

In the present study, we examined the promoter methylation status and gene expression of Cyclin D2 in human epithelial ovarian cancer cell lines. We also evaluated the correlation between methylation status of the Cyclin D2 promoter and various clinicopathological parameters in patients with epithelial ovarian cancer.

Materials and Methods

Cell lines. Twelve ovarian carcinoma cell lines were used. OVCAR3, SKOV3 (both adenoarcinomas), Caov3, OV90 (both serous adenocarcinoma), TOV21G, ES2 (both clear cell adenocarcinoma) and TOV112D (endometrioid adenocarcinoma) were purchased from American Type Culture Collection. JHOS2, JHOS3, HTOA (all serous adenocarcinoma), OMC3 (mucinous adenocarcinoma) and JHOC5 (clear cell adenocarcinoma) were purchased from Riken Cell Bank (Tsukuba). Cell lines were maintained in DMEM/F12 medium (Invitrogen), supplemented

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with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen), and incubated in a 5% CO_2 atmosphere at 37°C.

Surgical specimens and clinical data. The research protocol was approved by the Ethics Committee of Tohoku University Graduate School of Medicine, Sendai, Japan. We examined 71 ovarian cancer specimens obtained from patients treated between 1988 and 2002 at Tohoku University Hospital, Sendai, Japan. All specimens were retrieved from the surgical pathology files at Tohoku University Hospital. Informed consent was obtained from each patient. Specimens were fixed in 10% formalin and embedded in paraffin. Patient age, performance status on admission, histology, stage, grade, residual tumor after primary surgery, and overall survival were obtained from a chart review. The median follow-up time for patients was 59 months (range, 4–120 months). Performance status was defined according to the WHO criteria.[19] Histology, stage and grading followed the FigO criteria.[20] Residual tumor was defined as the amount of unresectable tumor left following primary volume reductive surgery. Optimal volume reduction was achieved when the residual tumor was less than 2 cm. Patients with a residual tumor greater than 2 cm were considered to have suboptimal volume reduction. Overall survival was calculated from the time of initial surgery to death or the date of the last contact. Survival times of patients still alive or lost to follow-up were censored as of December 2002.

An ovarian tissue obtained from a 50-year-old woman who had received surgical treatment for benign uterine tumor was used as a normal ovarian tissue for methylation-specific polymerase chain reaction (MSP) and reverse transcription–polymerase chain reaction (RT-PCR).

Methylation-specific polymerase chain reaction. The methylation status of the samples was assessed using MSP as described previously.[21] Genomic DNA from ovarian cancer cell lines was extracted using the AquaPure Genomic DNA kit (Bio-Rad). Genomic DNA from ovarian tumor specimens was extracted from paraffin blocks. For each tissue, the presence of carcinoma was confirmed on a H&E stained section. For DNA extraction, three 5-μm tissue sections from the same block were scraped from the slide and treated with Dexpat (Takara). The quality and integrity of the DNA were evaluated in terms of the $A_{260/280}$ ratio. Genomic DNA (1 μg) was treated with sodium bisulfite using a CpGenome DNA modification kit (Intergen) according to the manufacturer's protocol. Amplification was conducted in a 20-μL reaction volume containing 2 μL of 10× ExTaq buffer, 1.5 μL of 2.5 mM $MgCl_2$, 1 mM of each primer, 1.5 mL of 2.5 mM dNTPs, and 1 unit of Takara ExTaq polymerase (Takara). The reaction was cycled for 40 cycles, each of which consisted of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s, followed by a 7-min extension at 72°C. The primers used were 5′-AGAGTATGTGTTAGGGTTGATT-3′ and 5′-ACATCCTCACCAACCCTCCA-3′ (−1431 to −1326, 106-bp) for the unmethylated reaction (U), and 5′-GGCGGATTTTATCGTAGTCG-3′ and 5′-CTCCACGCTCGATCCTTCG-3′ (−1404 to −1304, 101-bp) for the methylated reaction (M).[18] Universal unmethylated human genomic DNA (Intergen) was used as a positive control for the unmethylated reaction. Universal methylated human male genomic DNA (Intergen) was used as a positive control for the methylated reaction. Reaction products were separated by electrophoresis on 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Quantitative RT-PCR. Total RNA was isolated from cells by phenol–chloroform extraction using Isogen reagent (Nippon Gene). RNA was treated with RNase-free DNase (Roche Diagnostics; 1 μg/μL) for 2 h at 37°C, followed by heat inactivation at 65°C for 10 min. Total RNA (5 μg) was reversed transcribed using the Superscript II first-strand synthesis system (Invitrogen) with random hexamers according to the manufacturer's protocol. Quantitative polymerase chain reaction (PCR) was carried out using an iCycler system (Bio-Rad). For the determination of Cyclin D2 cDNA content, a 25-μL reaction mixture consisting of 23 μL iQSYBR Green MasterMix, 1 μL of each primer and 1 μL of cDNA template was cycled as follows: 2-min denaturation at 90°C, 30-s annealing at either 60°C (for Cyclin D2) or 62°C (for β-actin), and 1.5-min extension at 72°C. Primers for PCR reactions were as follows: Cyclin D2-F, 5′-TACTTCAAGTGCGTGCAGAAGGAC-3′ and Cyclin D2-R, 5′-TCCCACACTTCCAGTTGCGATCAT-3′;[22] and β-actin-F, 5′-CCAACCGCGAGAAGATGAC-3′ and β-actin-R, 5′-GGAAGGAAGGCTGGAAGAGT-3′.[23] β-Actin primers were utilized as an internal positive control and Cyclin D2 expression level was calculated by dividing the quantity obtained for Cyclin D2 by the quantity obtained for β-actin. Two independent RT-PCR reactions were carried out for each sample.

5-Aza-2′-deoxycitidine and trichostatin A treatment. To confirm that epigenetic change contributed to loss of Cyclin D2 gene expression, we assessed the effect of 5-aza-2′-deoxycitidine (5azaC) (Sigma), a demethylating agent, and trichostatin A (TSA) (Sigma), a histone deacetylase inhibitor, on Cyclin D2 mRNA expression and cell growth of ovarian cancer cell lines by quantitative RT-PCR and cell count, respectively.

Ovarian cancer cell lines (OMC3, OVCAR3, JHOS2, JHOC5 and SKOV3) were cultured at a point of 70% confluence in 10-cm cell dishes. They were treated with 1.0 μM 5azaC for 3 or 5 days. They were also treated with 0.5 μM TSA.[24,25] We set up TSA treatment times of 4, 8, 16 and 32 h, and the treatments for 8 and 16 h appeared the most effective for gene expression compared to control culture (data not shown). Total RNA was prepared at each time point and the expression of Cyclin D2 mRNA was analyzed by quantitative RT-PCR. Furthermore, we investigated the effects of these chemical agents on cell growth of ovarian cancer cell lines by cell count at each time point.

Immunohistochemistry. For the purpose of investigating cell proliferation we examined the immunohistochemical expression of Ki-67 in ovarian cancer tissue. Immunohistchemical analysis was carried out with the streptavidin–biotin amplification method using the NX/ES IHC system (Ventana Medical Systems). Monoclonal antibody for Ki-67 (MIB-1) was purchased from DAKO. For antigen retrieval, the slides ware heated in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dyhydrate [pH 6.0]). The dilution of primary antibody was 1:50. Scoring of Ki-67 in carcinoma cells was counted independently by two of the authors (M. S. and J. A.), and the percentage of immnunoreactivity in at least 500 carcinoma cells (i.e. the labeling index) was determined.

Statistical analysis. Statistical analysis was carried out using Stat View 5.0 software (SAS Institute). The correlation between the Cyclin D2 mRNA expression level and methylation status was assessed using the Mann–Whitney *U*-test. The statistical significance between methylation status and various clinicopathological parameters was evaluated using Friedman's χ^2 *r*-test and the Mann–Whitney *U*-test. A univariate analysis of prognostic significance for prognostic factors was carried out using the log-rank test after each survival curve was obtained by the Kaplan–Meier method. Multivariate analysis was carried out using the Cox regression model to evaluate the predictive power of each variable independently. All patients who could be assessed were included in the intention-to-treat analysis. A result was considered significant when the *P*-value was less than 0.05.

Results

Methylation status of the Cyclin D2 gene in ovarian cancer cell lines and tissues. Bands corresponding to methylated Cyclin D2 were

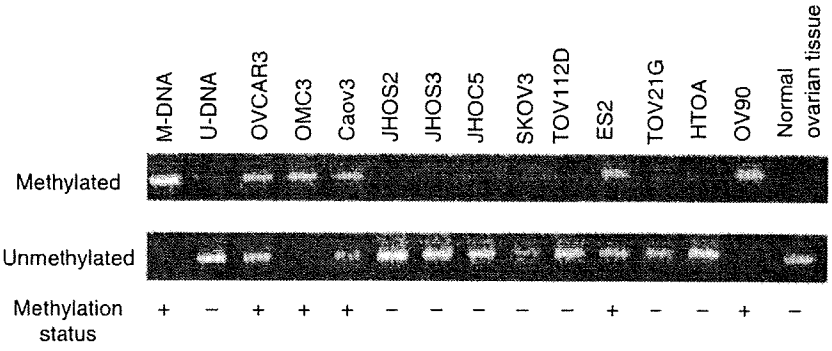

Fig. 1. Methylation status of the Cyclin D2 gene in ovarian cancer cell lines and a normal ovarian tissue. The 101-bp bands in the 'Methylated' lanes indicate the presence of methylated alleles of the Cyclin D2 gene. The 106-bp bands in the 'Unmethylated' lanes correspond to the unmethylated alleles. 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.

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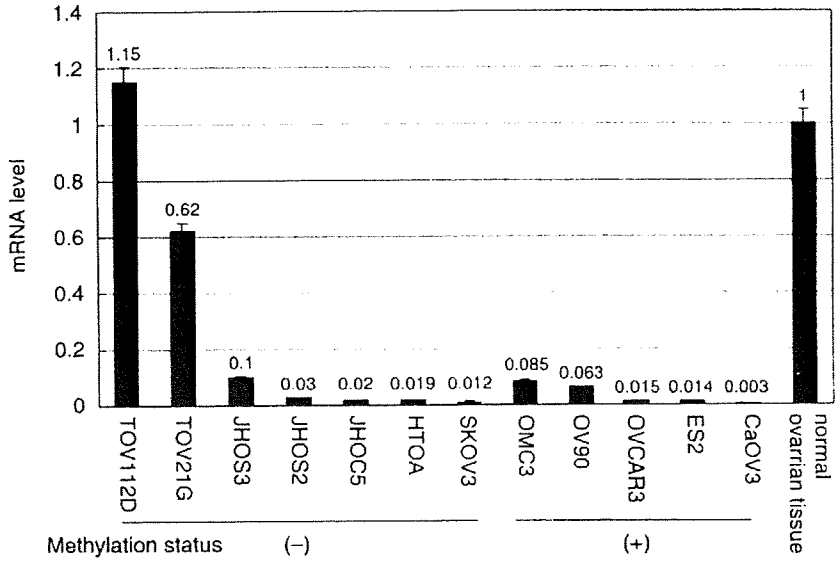

Fig. 2. Expression of the Cyclin D2 gene in ovarian cancer cell lines and normal ovarian tissue. Two independent reverse transcription–polymerase chain reactions were carried out for each sample, and the ratio of Cyclin D2: β-actin was calculated and normalized with the level of normal ovarian tissue.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

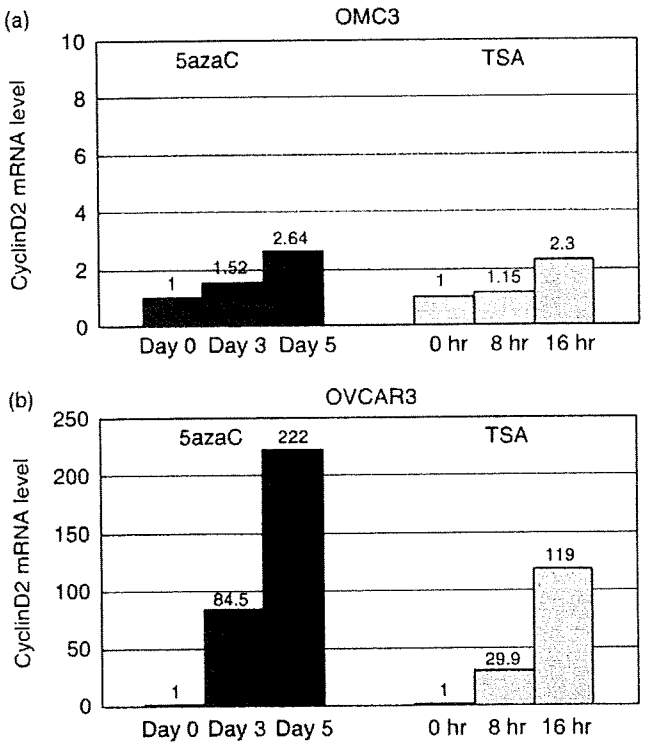

Fig. 3. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in OMC3 and OVCAR3 cells following treatment with (a) 5-aza-2′-deoxycitidine (5azaC) or (b) trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and normalized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

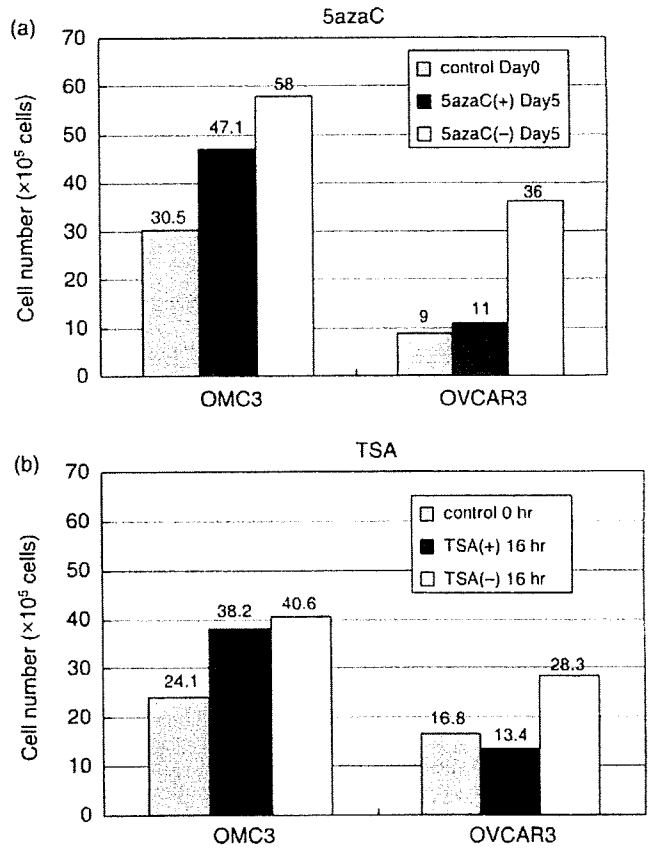

Fig. 4. Cell number of OMC and OVCAR3 cells following treatment with (a) 5-aza-2′-deoxycitidine (5azaC) or (b) trichostatin A (TSA). *Control treatment with medium alone.

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

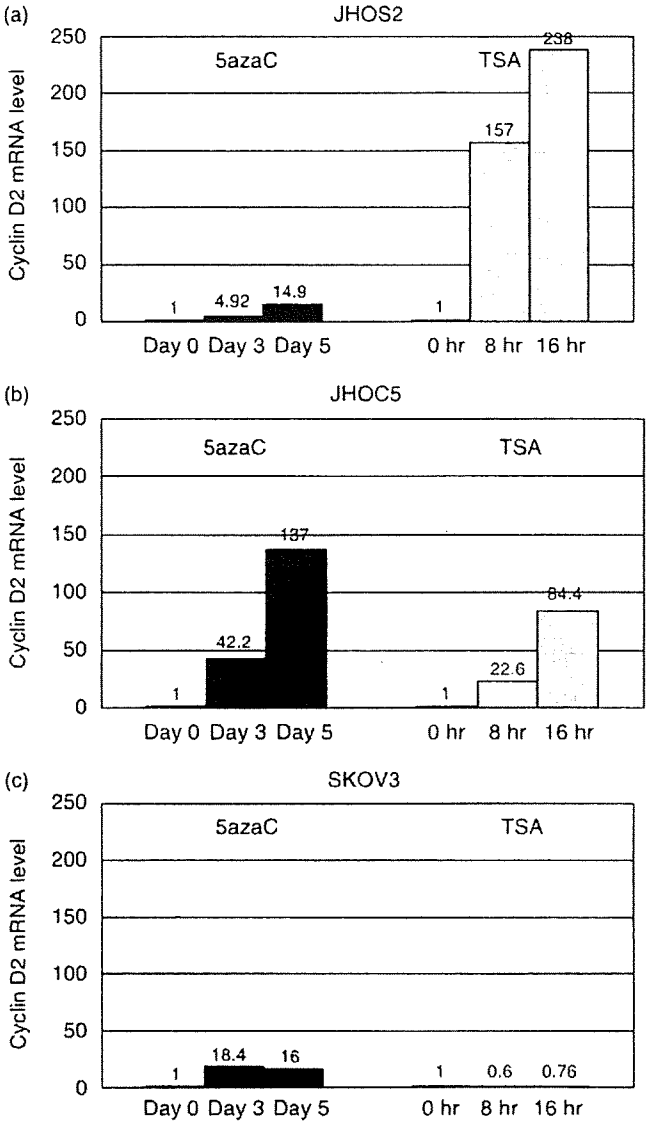

Fig. 5. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in (a) JHOS2, (b) JHOC5 and (c) SKOV3 cells following treatment with 5-aza-2′-deoxycitidine (5azaC) or trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and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

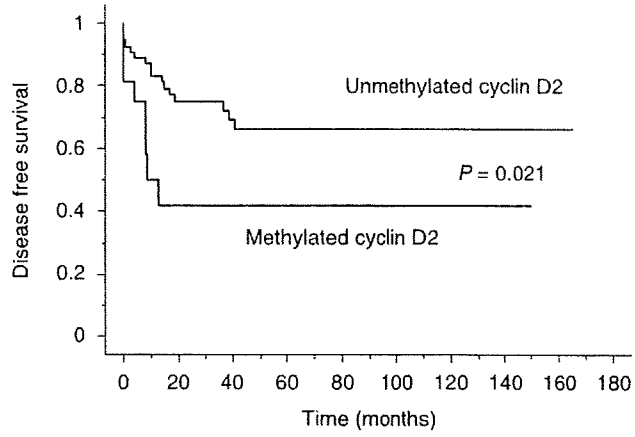

Fig. 6. Association between Cyclin D2 promoter methylation status and disease-free survival in patients with epithelial ovarian cancer.

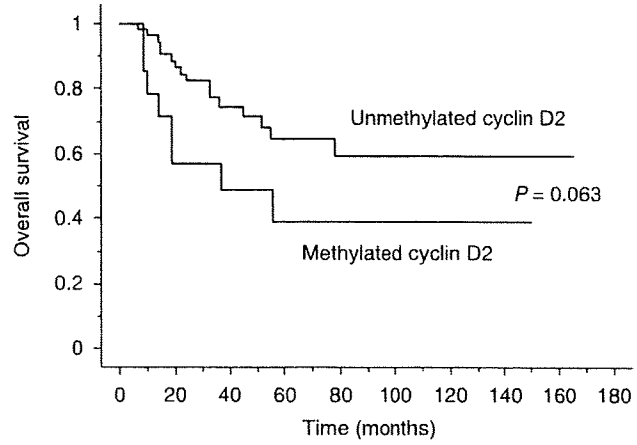

Fig. 7. Association between Cyclin D2 promoter methylation status and overall survival in patients with epithelial ovarian cancer.

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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.

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ORIGINAL ARTICLE

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Progression-free survival and overall survival of patients with clear cell carcinoma of the ovary treated with paclitaxel-carboplatin or irinotecan-cisplatin: retrospective analysis

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Abstract

Background. Irinotecan hydrochloride, a topoisomerase I inhibitor, has been preliminarily recognized as an effective agent against clear cell carcinoma of the ovary (CCC), but there are few clinical data. Our aim was to compare progression-free survival (PFS) between patients treated with irinotecan hydrochloride and cisplatin (CPT-P) and those with treated with paclitaxel and carboplatin (TC).

Methods. One hundred and seventeen patients at International Federation of Gynecology and Obstetrics (FIGO) stages Ic (ascites/malignant washing) – IV were identified by scanning the medical records of ten Japanese hospitals. After complete surgical staging procedures including lymphadenectomy, 35 patients received CPT-P and 82 patients received TC. The PFS and overall survival of the two groups were compared using the Kaplan-Meier method.

Results. There was no significant difference in median age, performance status, FIGO stage, rate of optimal cytoreduction, or follow-up period between the CPT-P and TC groups. Two-year and 5-year PFS was 48% and 40%, respectively, in the TC group and 55% and 55%, respectively, in the CPT-P group ($P = 0.31$). Multiple regression analysis revealed that only residual tumor was an independent prognostic factor for PFS ($P < 0.01$).

Conclusion. CPT-P showed a potential therapeutic effect, at least no less than that of TC therapy. Although there was no significant survival benefit in the present retrospective analysis, we recommend that the CPT-P regimen be evaluated in a larger, prospective, clinical trial.

Key words Ovarian cancer · Clear cell carcinoma · Irinotecan · Adjuvant chemotherapy · Paclitaxel · Progression-free survival

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Introduction

Clear cell carcinoma of the ovary (CCC) was initially termed "mesonephroma ovarii" by Schiller in 1939,[1] and in 1973 it was strictly defined by the World Health Organization as lesions characterized by clear cells growing in solid/tubular or glandular patterns, as well as hobnail cells.[2] Many publications have identified the distinctive behavior of CCC. The

most distinctive characteristics recognized are that patients with CCC had worse prognoses compared with those with other pathological types of epithelial ovarian carcinomas[3,4] and that CCC showed resistance to conventional platinum-based chemotherapy.[5–8]

Since the establishment of paclitaxel and carboplatin (TC) as the "gold standard" regimen for epithelial ovarian cancer,[9,10] the regimen has been widely used for all histological subtypes of ovarian tumors. But response in measurable CCC cases treated with TC was relatively low, ranging from 22% to 56%.[11–13] The survival benefit of the regimen is also controversial; one study showed superior survival benefit,[14] and another implied no survival benefit in either early or advanced cases.[15]

As irinotecan hydrochloride, a semisynthetic derivative of camptothecin, has been reported to have additive and synergistic effects in combination with cisplatin in vitro,[16–18] combination therapy with irinotecan and cisplatin (CPT-P) has been used clinically for patients with various solid tumors. Especially, a large clinical trial revealed that CPT-P had significant activity for extensive small-cell lung cancer.[19] Moreover, CPT-P has been reported to be effective in first-line and second-line chemotherapy for the treatment of CCC.[20–22] The aim of the present retrospective study was to compare the survival benefit of combination therapy with CPT-P with that of TC.

Patients and methods

A retrospective review of patients with CCC seen at ten Japanese hospitals from January 1, 1992 to December 31, 2003 was done. Of all the patients treated at these 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 para-aortic lymphadenectomy; (b) patients whose tumor specimens were confirmed as CCC by two pathologists in a central pathological review; (c) patients who were at International Federation of Gynecology and Obstetrics (FIGO) stages Ic (ascites/malignant washing), II, III, and IV; (d) patients treated with six courses of combination chemotherapy using CPT-P, or six courses of TC; (e) age 75 years or less; (f) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 2 or less; (g) pretreatment leukocyte count of 4000/mm^3 or more, platelet count of 100 000/mm^3 or more, hemoglobin, 9.0 g/dl or more, serum creatinine, less than 1.5 mg/dl, creatinine clearance, 60 ml/min or more, and GOT and GPT less than twice the upper limit of normal at the hospitals. The study was approved by the Ethics Committee at each hospital.

One cycle of the 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 irinotecan on days 1, 8, and 15, and 1 week off and it was repeated every 4 weeks. The TC regimen consisted of a drip infusion of 175–180 mg/m^2 of paclitaxel and carboplatin (AUC, 5–6).

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. The Kaplan-Meier method was used for the 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 less than 0.05 was considered statistically significant. Stat View software version 5.0 (SAS, Cary, NC, USA) was used to analyze the data.

Results

In all, 35 patients with the CPT-P regimen and 82 with the TC regimen were enrolled in the present retrospective study. The characteristics of the patients are outlined in Table 1. There was no significant difference in median age, performance status, FIGO stage, residual tumor diameter,

Table 1. Characteristics of the patients

	Irinotecan plus cisplatin	Paclitaxel plus carboplatin	P value
Patients (n)	35	82	
Median age, years (range)	52 (34–69)	54 (38–74)	0.79
Performance status			0.12
0	18 (51%)	45 (55%)	
1, 2	17 (49%)	37 (45%)	
FIGO stage			0.94
Ic (Ascites/malignant washing)	13 (37%)	28 (34%)	
II	6 (17%)	15 (18%)	
III	13 (37%)	34 (41%)	
IV	3 (9%)	5 (6%)	
Residual tumor diameter			0.62
0 cm	23 (66%)	52 (63%)	
<1 cm	5 (14%)	8 (10%)	
>1 cm	7 (20%)	22 (27%)	
Follow-up period (months)			0.28
Median	17	21	
Range	5–83	3–73	

Table 2. Multiple regression survival analysis for stage Ic (ascites/malignant washing)-IV patients with clear cell carcinoma of the ovary

Variables (number of patients)	Hazard ratio	95% Confidence interval	*P* value
Age (years)			0.35
<50 (n = 38)	1		
>51 (n = 79)	1.33	0.73; 2.42	
Performance status			0.61
0 (n = 63)	1		
1, 2 (n = 54)	1.17	0.64; 2.14	
FIGO stage			0.16
Ic (Ascites/malignant washing), II (n = 62)	1		
III, IV (n = 55)	1.70	0.81; 3.56	
Residual tumor			<0.01
None (n = 75)	1		
<1 cm (n = 13)	2.54	1.16; 5.57	
>1 cm (n = 29)	3.17	1.35; 7.40	
Chemotherapy			0.21
Irinotecan and cisplatin (n = 35)	1		
Paclitaxel and carboplatin (n = 82)	1.55	0.79; 3.03	

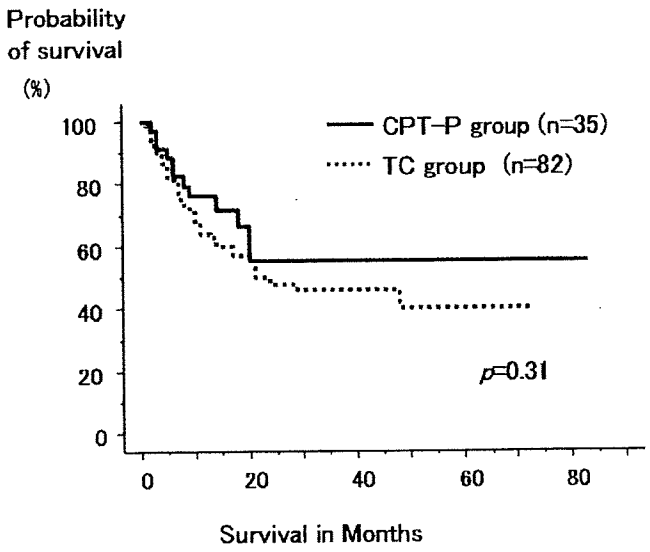

Fig. 1. Kaplan-Meier curves comparing the progression-free survival (PFS) of stage Ic (ascites/malignant washing) – IV patients according to adjuvant chemotherapy. The 2-year and 5-year PFS was 55% and 55%, respectively, in the irinotecan and cisplatin (*CPT-P*) group, and 48% and 40% in the paclitaxel and carboplatin (*TC*) group (P = 0.31)

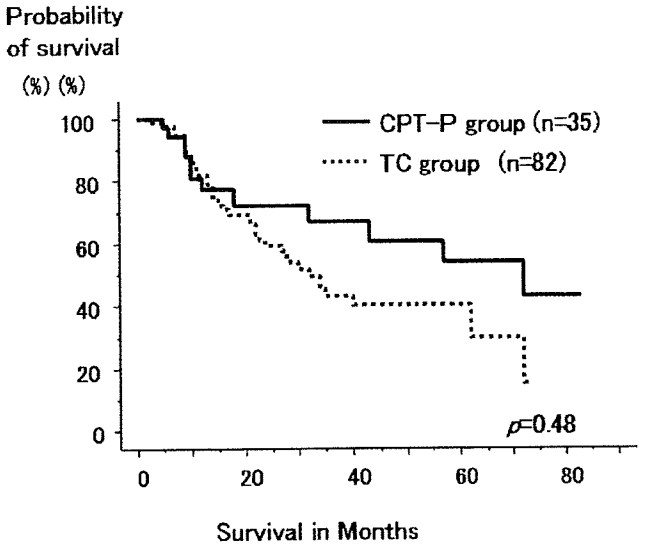

Fig. 2. Kaplan-Meier curves comparing the overall survival of all the patients treated with the combination of irinotecan and cisplatin (*CPT-P*) and those treated with paclitaxel and carboplatin (*TC*; P = 0.48). The 2-year and 5-year overall survival was 72% and 54%, respectively, in the CPT-P group and 60% and 43% in the TC group

or follow-up period between the CPT-P group and the TC group. The median age was 52 years in the CPT-P group and 54 years in the TC group. The CPT-P group included 13 patients (37%) at stage Ic (ascites/malignant washing), 6 (17%) at stage II, 13 (37%) at stage III, and 3 (9%) at stage IV. In the TC group, 28 patients (34%) were at stage Ic, 15 (18%) at stage II, 34 (41%) at stage III, and 5 (6%) at stage IV. Optimal cytoreduction (residual tumor diameter <1 cm) with the initial surgery was achieved in 80% (28/35 patients) in the CPT-P group and 73% (60/82 patients) in the TC group. In patients with tumors at FIGO stages III and IV, the rate of optimal surgery was 56% (9/16 patients) in the CPT-P group and 46% (18/39 patients) in the TC group. The median follow-up period was 17 months in the CPT-P group and 21 months in the TC group.

The 2-year and 5-year progression-free survival (PFS) rates were 55% and 55%, respectively, in the CPT-P group and 48% and 40% in the TC group (Fig. 1; P = 0.31). The 2-year and 5-year overall survival rates were 72% and 54%, respectively, in the CPT-P group and 60% and 43% in the TC group (Fig. 2; P = 0.48). Multiple regression analysis revealed that only residual tumor was an independent prognostic factor for PFS (P < 0.01; Table 2). Age, performance status, and FIGO stage were not significant prognostic factors. Additionally, chemotherapy was also not an independent factor for PFS in the CCC patients in the present

study (TC compared with CPT-P: hazard ratio, 1.55; 95% confidence interval, 0.79 to 3.03, $P = 0.21$).

Discussion

It has been well recognized that CCC has low sensitivity to conventional platinum-based chemotherapy.[3,4,7] But it is still uncertain which regimen would be the best candidate for CCC. Some reports have indicated a survival benefit of paclitaxel and platinum therapy in comparison with platinum-based chemotherapy.[10,12] A larger study implied that a combination with paclitaxel and platinum had almost the same impact on survival as conventional platinum-based chemotherapy in both early- and advanced-stage patients.[15]

The CPT-P regimen was initially introduced as a treatment for platinum-refractory ovarian cancer.[22] Since then, the regimen has been used for the treatment of CCC as first-line chemotherapy and has shown moderate activity against CCC.[20,21] The present study implies that the survival of patients treated with CPT-P might be improved compared with the survival of those treated with TC. However, our study was a limited retrospective study and failed to prove the superiority of the CPT-P regimen. The effectiveness of irinotecan as well as paclitaxel against CCC was also confirmed in vitro.[23] Combined with mitomycin C, irinotecan also showed higher activity than conventional platinum-based chemotherapy.[24] Chemotherapeutic regimens including irinotecan have been suggested to have a potential antitumor effect against CCC as first-line chemotherapy.

CCC has been reported to have distinct molecular characteristics compared with other histological subtypes. The overexpression of hepatocyte nuclear factor-1 beta[25] and that of ABCF2, a member of the ATP-binding cassette gene superfamily[26] were observed in CCC. These molecules might be another or additive target in the treatment of CCC.

Although there was no statistically significant difference in survivals between the CPT-P and TC regimens in our study, CPT-P was shown to have the same chemotherapeutic benefit in the survival of CCC patients as TC. Therefore, we recommend that the CPT-P regimen be tested in a large-scale prospective study.

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