A single-institution study of gemcitabine plus docetaxel yielded high objective response rates among patients with advanced LMS in both the second-line [31] and first-line settings [32]. Recently, gemcitabine plus docetaxel has been shown to yield higher response rates, and longer progression-free and overall survivals than single-agent gemcitabine in a randomized trial for patients with soft tissue sarcoma who had received up to three prior regimens [30]. In a Gynecologic Oncology Group (GOG) phase II trial for women with advanced leiomyosarcoma who had received one prior cytotoxic regimen, gemcitabine plus docetaxel achieved objective responses in 28 % of patients, with an additional 50 % having stable disease (SD). The high dose of docetaxel (100 mg/m$^{2)}$ in this study, however, produced profound myelosuppression necessitating the use of growth factor support [33].

A prospective study of gemcitabine plus docetaxel has been eagerly anticipated in Japan. However, such studies have not been conducted because the GOG regimen, as either prophylactic G-CSF at a dose of 150 μg/m^2 or docetaxel at a dose of 100 mg/m^2, is not approved in Japan. The maximum approved dose of docetaxel in Japan is 70 mg/m^2.

Therefore, the aim of this single-institution study was to evaluate the efficacy and toxicity of a regimen of gemcitabine 900 mg/m^2 plus dose-reduced docetaxel 70 mg/m^2 without prophylactic G-CSF support in Japanese patients with advanced or recurrent LMS and UES.

Patients and methods

Patients

Women with measurable advanced or recurrent LMS and UES with non-resectable disease were eligible. All tumors were histologically confirmed. Patients were permitted to have had prior chemotherapy and pelvic radiotherapy; however, patients previously treated with either docetaxel or gemcitabine were excluded. Patients were required to have an ECOG performance status of 0–2, and adequate bone marrow function [absolute neutrophil count (ANC) greater than or equal to 1500/μl, and platelets greater than or equal to 100,000/μl]; renal function (creatinine less than or equal to 1.5 × the institutional upper limit of normal); hepatic function (bilirubin less than or equal to 1.5 × the institutional upper limit of normal, and serum glutamic oxaloacetic transaminase [sGOT] and alkaline phosphatase less than or equal to 2.5 × the institutional upper limit of normal); and neurological function [baseline neuropathy, sensory and motor, less than or equal to National Cancer Institution Common Toxicity Criteria version 3.0 (CTC 3.0) grade 1]. Patients with a history of another invasive malignancy within the past 5 years were not eligible. All patients provided written, informed consent. The protocol and consent were reviewed and approved annually by Institutional Review Boards of Tohoku University Hospital.

Treatment

All participants had baseline imaging with a computed tomography (CT) scan of the chest, abdomen, and pelvis, within 4 weeks of starting therapy. CT imaging was repeated following every other cycle of treatment to assess response. A history was taken, and a physical examination and assessment of toxicities were performed at each cycle. Complete blood counts and comprehensive metabolic panels were monitored weekly. Participants received gemcitabine 900 mg/m^2 on days 1 and 8 intravenously infused over 90 min, followed by docetaxel 70 mg/m^2 on day 8 intravenously infused over 60 min. Treatment cycles were repeated approximately every 3 weeks, and patients continued on the study treatment until disease progression, achievement of discontinuation criteria as defined in the study protocol, or at the discretion of the investigator. Recommended pre-medication for the docetaxel was dexamethasone 8 mg orally twice a day starting the day prior to docetaxel. Early intervention with diuretics was encouraged for signs of docetaxel-related fluid retention. Patients received the day 1 treatment of each cycle provided the ANC was greater than or equal to 1500/μl and the platelet count was greater than or equal to 100,000/μl. Patients received full-dose day 8 treatment provided the ANC was greater than or equal to 1000/μl and platelet count greater than or equal to 100,000/μl. Seventy-five percent of the planned day-eight dose was given if the ANC was between 500 and 1000/μl or the platelet count was between 50,000 and 100,000/μl, and provided bilirubin levels from day 1 or after were within institutional normal limits. Day-8 treatment with docetaxel was omitted if the bilirubin remained above normal on day 8. Day-8 gemcitabine and docetaxel were both omitted if the day-8 ANC was under 500/μl or the platelet count was less than 50,000/μl. Patients were given therapeutic and second-line prophylactic G-CSF if they had grade 4 neutropenia. Doses of both docetaxel and gemcitabine were reduced by 25 % in subsequent cycles if a patient experienced grade 3 elevations in sGOT, serum glutamic pyruvic transaminase (sGPT), or alkaline phosphatase, and treatment was not resumed until such grade 3 elevations had resolved to grade 1 or less. Patients who experienced grade 2 or worse neurotoxicity had treatment held for a maximum of 2 weeks and could resume treatment at 75 % of the prior docetaxel dose if the neuropathy had improved. Other non-hematological toxicities with an impact on organ function of grade 2 (or greater) required 25 % dose reduction and delay in subsequent therapy for a maximum of 2 weeks until it recovered to no worse than grade 1.

Assessment of response and toxicity

All patients who received at least 1 cycle of study treatment were considered assessable for response. Response was assessed by the Response Evaluation Criteria in Solid Tumors (RECIST). Responses according to these criteria are defined as follows: Complete response (CR) is the disappearance of all target and non-target lesions and no evidence of new lesions documented by 2 disease assessments at least 4 weeks apart. Partial response (PR) is at least a 30 % decrease in the sum of the longest dimensions (LD) of all target measurable lesions taking as the reference the baseline sum of LD. There could be no unequivocal progression of non-target lesions and no new lesions. Documentation by 2 disease assessments at least 4 weeks apart is required. In the case where the only target lesion is a solitary pelvic mass measured by physical examination, and which is not radiographically measurable, a 50 % decrease in the LD is required. Progression of disease (PD) requires at least a 20 % increase in the sum of LD of target lesions taking as references the smallest sum of LD, the appearance of new lesions, death due to disease or global deterioration due to disease. SD is any condition not meeting the above criteria. All 11 patients enrolled in the study were included in the assessment of response, apart from 1 patient who was not treated because of ileus. The primary endpoint was the overall response rate (RR: CR + PR), and secondary endpoints were progression-free survival (PFS), overall survival (OS), and adverse events. Time to treatment failure (TTF) was defined as the time from enrollment to treatment discontinuation for any reason, including disease progression, treatment toxicity, patient preference, or death. Adding to PFS, TTF is generally not accepted as a valid endpoint, but was also included as an endpoint in this study because 3 SD patients electively opted to change chemotherapy. Toxicities were graded according to CTC 3.0.

Results

Patient characteristics

Between February 2009 and June 2011, 11 women were enrolled in this phase II study. One patient (No. 8) underwent and was diagnosed by intrauterine cytology and curettage. One patient (No. 11) developed a prolonged postoperative ileus shortly after enrollment and was not included in the analysis. The remaining cases were included in the calculation of the objective response rate (Table 2). The median age of the cohort was 60.1 years (range 50–74 years). Nine patients had an ECOG performance status of 0 or 1, one had a performance status of 2. Eight of 10 patients had confirmed LMS, and 2 had UES. Nine of 10 patients had undergone a total abdominal hysterectomy plus bilateral salpingo-oophorectomy. Five of 6 recurrent patients had received 1 or more prior cytotoxic regimens, and in the majority, the prior therapy had been doxorubicin and ifosfamide-based. Three IVB stage patients were enrolled for first-line treatment. The main target regions were lung (40 %), pelvis (40 %), liver (10 %), and omentum (10 %). After 3 cycles, 3 SD patients (Nos. 4, 6, and 7) requested to be switched to other chemotherapies, and 1 patient (No. 5) refused further treatment. One patient (No. 3) desired surgical resection of the downsized pelvic tumor. Nine of 10 (90 %) received three or more cycles of study treatment. The median number of cycles of study treatment delivered per patient was five (range 2–18 cycles).

Response and survival

The RECIST-measured objective RR was observed in 3 of the 10 patients enrolled (30 %). One patient had CR (10 %), 2 had confirmed PR (20 %), and 4 (40 %) had SD (Table 2). The disease control rate (DCR; CR + PR + SD) was 70 %. Three of 10 (30 %) had PD. Mean PFS was 5.4 months (range 1.3–24.8 months), and mean TTF was 3.1 months (range 2.4–15.4 months). Mean OS was 14 months (range 5.3–38.4 months). Among 3 objective responses, the median response duration was 19.7 months (range 5.9–28.3 months).

Adverse events

Among the total of 50 cycles, the median number of cycles per patient was 5 (range 2–18 cycles); 22 cycles (44 %, median 5 times/cycle: range 3–7 times) were for 4 patients who required G-CSF at a dose of 75 $\mu g/m^2$ (half the dose used in the GOG trials). Myelosuppression was the major toxicity: neutropenia grade 3 in 20 %, grade 4 in 50 %; anemia grade 3 in 10 %, grade 4 in 10 %; thrombocytopenia grade 3 in 10 %, grade 4 in 20 %. There were no cases of grade 4 febrile neutropenia. One patient had grade 3 liver toxicity (Table 3). No grade 3/4 pulmonary toxicity was observed.

Discussion

Efficacy

In Japan, prophylactic G-CSF at a dose of 150 $\mu g/m^2$ and docetaxel at a dose of 100 mg/m^2 are not approved for use. For this reason, we performed the current feasibility study of gemcitabine 900 mg/m^2 plus dose-reduced docetaxel

Table 2 Patient characteristics and results

No.	Age (years)	PS	Stage	Hist.	Preprotocol treatments	Target lesion	Cycles	BR	Reason for discontinuation	Post treatments		Status
										Surgery	Chemo./ irradiation	
1	51	0	IVB	LMS	TAH + BSO	Omentum	6	CR	NA	None	None	NED
2	66	0	Rec.	LMS	TAH + BSO IAP × 3, TC × 3	Lung	18	PR	PD	None	Irradiation	DOD
3	53	0	Rec.	LMS	TAH + BSO IAP × 6	Pelvis	6	PR	Change strategy	Lt. pelvic tumor resection	GD × 2	DOD
4	59	0	IVB	UES	TAH + BSO	Lung	3	SD	Patient preference	None	IP × 3	DOD
5	74	0	Rec.	LMS	TAH + BSO IAP × 3	Liver	3	SD	Patient's reason	None	None	DOD
6	51	0	Rec.	UES	TAH + BSO IAP × 3	Pelvis	3	SD	Patient preference	None	TC × 2	DOD
7	50	0	Rec.	LMS	TAH + BSO	Lung	3	SD	Patient preference	None	IA × 3	DOD
8	55	1	IVB	LMS	None	Uterus Pelvic LN	2	PD	PD	None	Irradiation	DOD
9	40	1	Rec.	LMS	TAH + BSO	Lung	3	PD	PD	Lt. lower lobectomy	None	DOD
10	74	1	Rec.	LMS	TAH + BSO, CPT11 × 8, AP × 3	Pelvic LN	3	PD	PD	None	None	DOD
11[a]	60	2	Rec.	LMS	TAH + BSO	Lung	0	NA	NA	None	None	DOD

PS Performance status, *Rec.* recurrence, Hist., histology, *LMS* leiomyosarcoma, *UES* undifferentiated endometrial sarcoma, *TAH* total abdominal hysterectomy, *BSO* bilateral salpingo-oophorectomy, *IAP* ifosfamide + doxorubicin + cisplatin, *TC* paclitaxel + carboplatin, *CPT-11* irinotecan, *AP* doxorubicin + cisplatin, *IP* ifosfamide + cisplatin, *IA* ifosfamide + doxorubicin, *GD* gemcitabine + docetaxel, *BR* best response, *NA* not applicable, *NED* no evidence of disease, *DOD* dead of disease, *CR* complete response, *SD* stable disease, *Lt.* left, *PD* progression of disease, *LN* lymph node

[a] Patient No. 11 developed a prolonged postoperative ileus shortly after enrollment and was not treated with gemcitabine and docetaxel

70 mg/m^2 without prophylactic G-CSF support in Japanese patients with advanced or recurrent LMS and UES.

The GOG conducted a phase II trial for women with advanced, unresectable LMS whose disease had progressed after one previous cytotoxic regimen (gemcitabine–docetaxel as second-line therapy) [33]. This study enrolled 51 patients, of whom 48 were evaluable for response. Ninety percent of the patients had received previous doxorubicin-based therapy. Patients were treated with gemcitabine 900 mg/m^2 on days 1 and 8 over 90 min, and docetaxel 100 mg/m^2 on day 8 of a 21-day cycle with G-CSF support. Patients who had received previous pelvic radiation were given 25 % lower doses. Three of 48 patients (6.3 %) achieved CR, and 10 (20.8 %) achieved PR for an overall objective RR of 27 %. An additional 50 % of women had SD lasting a median duration of 5.4 months. The median number of cycles per patient was 5.5 (range 1–22 cycles). The PFS rate at 12 weeks was 73 %, and at 24 weeks was 52 %. Median PFS was 5.6+ months (range 0.7–27+ months). The median duration of objective response exceeded 9 months (range 3.9–24.5+ months). The GOG has conducted a prospective phase II trial to assess the efficacy of first-line, fixed-dose-rate gemcitabine plus docetaxel in women with advanced LMS [34]. The doses and schedule are the same as in their previously reported second-line treatment study. Objective responses were observed in 35.8 % of patients, CR in 4.8 % and PR in 31 %. An additional 26.2 % had SD. Half of the patients received 6 or more cycles of study treatment. The median PFS was 4.4 months (range 0.4–37.2+ months). Among the patients with an objective response, the median response duration was 6 months (range 2.1–33.4+ months). Median OS exceeded 16 months (range 0.4–41.3 months). The RR (30 %, 27.1 % [33], 35.8 % [34]), PFS (5.4 months), DCR (70 %), OS (14 months), and duration of objective response (19.7 months) in our study nearly equaled those of the 2 prior GOG trials (RR: 27.1 % [33], 35.8 % [34]; PFS: 5.6+ [33], 4.4 months

Table 3 Adverse events compared with GOG first-line [32] and second-line [33] studies, all grades, by number of patients experiencing the event

Adverse event	Grade by National Cancer Institution Common Toxicity Criteria version 3.0					
	0	1	2	3	4	3/4 (%)
Neutropenia						
This study	0	0	3	2	5	70.0
GOG first-line	27	2	6	2	5	16.7
GOG second-line	19	9	10	6	4	20.8
Anemia						
This study	5	1	2	1	1	20.0
GOG first-line	0	7	25	10	0	23.8
GOG second-line	4	6	26	10	2	25.0
Thrombocytopenia						
This study	5	1	1	1	2	30.0
GOG first-line	9	22	5	4	2	14.3
GOG second-line	8	11	10	14	5	39.6
RBC transfusion						
This study	10	0	0	0	0	0.0
GOG second-line	24	0	0	24	0	50.0
Platelet transfusion						
This study	10	0	0	0	0	0.0
GOG second-line	42	0	0	6	0	12.5
Nausea/vomiting						
This study	3	7	0	0	0	0.0
GOG second-line	29	12	6	0	1	2.1
Anorexia						
This study	3	7	0	0	0	0.0
GOG first-line	12	12	12	5	1	14.3
GOG second-line	18	15	12	2	1	6.3
Liver dysfunction						
This study	5	3	1	1	0	10.0
GOG first-line	35	7	0	0	0	0.0
GOG second-line	38	6	3	1	0	2.1
Pulmonary						
This study	10	0	0	0	0	0.0
GOG first-line	32	6	3	0	1	2.4
GOG second-line	36	4	4	3	1	8.3
Fatigue						
This study	3	3	4	0	0	0.0
GOG first-line	11	15	9	7	0	16.7
GOG second-line	40	2	5	1	0	2.1
Alopecia						
This study	6	4	0	0	0	0.0
GOG second-line	21	1	26	0	0	0.0
Infection						
This study	9	0	0	1	0	10.0
GOG first-line	30	3	8	1	0	2.4
GOG second-line	43	2	1	2	0	4.2
Genitourinary						
This study	9	0	0	1	0	10.0
GOG first-line	36	3	3	0	0	0.0
GOG second-line	45	2	1	0	0	0.0

Table 3 continued

Adverse event	Grade by National Cancer Institution Common Toxicity Criteria version 3.0					
	0	1	2	3	4	3/4 (%)
Neurotoxicity						
This study	10	0	0	0	0	0.0
GOG first-line	32	7	2	1	0	2.4
GOG second-line	26	15	7	0	0	0.0
Allergic reaction						
This study	10	0	0	0	0	0.0
GOG first-line	33	5	3	1	0	2.4
GOG second-line	46	0	2	0	0	0.0

RBC red blood cell, *GOG* Gyencologic Oncology Group

[34]; DCR: 77 % [33], 62 % [34]; OS: 14.7 [33], 16.1 months [34]; and durations of objective response: 9+ [33], 6 months [34]). Thus, we conclude that 900 mg/m^2 gemcitabine plus dose-reduced docetaxel (70 mg/m^2)was highly efficacious in treated and untreated Japanese patients with advanced or recurrent LMS and UES (Table 1).

Toxicity

The toxicities associated with treatment were mainly bone marrow suppression: neutropenia grade 3 in 20 %, grade 4 in 50 %; anemia grade 3 in 10 %, grade 4 in 10 %; thrombocytopenia grade 3 in 10 %, grade 4 in 20 %. In the GOG second-line study, which employed G-CSF for 7 days, the toxicities associated with treatment were mainly uncomplicated myelosuppression: thrombocytopenia grade 3 (29 %), grade 4 (10.4 %); neutropenia grade 3 (12.5 %), grade 4 (8.3 %); and anemia grade 3 (20.8 %), grade 4 (4.2 %) [33]. Although neutropenia (grade 3 in 12.5 %, grade 4 in 8.3 %) was less frequent than that in this study (grade 3 in 20 %, grade 4 in 50 %), we had no episodes of life-threatening neutropenia. In the GOG first-line study, grade 3/4 myelosuppression was less frequent than that in the second-line study, with neutropenia grade 3 in 5 %, grade 4 in 12 %; anemia grade 3 in 24 %; and thrombocytopenia grade 3 in 9.5 %, grade 4 in 5 % [34]. In the GOG second-line study, the median number of cycles was 5.5, with a range extending up to 22 cycles [33] and in the first-line study, half of patients received more than 6 cycles of therapy [34]. In our study, among the total 50 cycles, 22 cycles (44 %) were for 4 patients who required the use of G-CSF (half the dose of and shorter term than the GOG trials). No grade 4 febrile neutropenia was observed. The median number of treatment cycles per patient was 5 (range 2–18 cycles), fewer than in the GOG second-line (5.5) [33] and first-line (6+) [34] studies. This was expected because 3 SD patients in the present study elected to change the chemotherapeutic regimen after the third cycle. These data support the suggestion that gemcitabine plus docetaxel without prophylactic G-CSF support is a tolerable regimen, and should be considered as a treatment option for advanced or recurrent LMS and UES in Japanese patients.

Active study

Further research is required to assess whether molecularly targeted therapies are effective in LMS and UES. In a phase I study in which gemcitabine, docetaxel, and bevacizumab (5 mg/kg) were all given concurrently every 2 weeks to patients with previously untreated soft tissue sarcoma (LMS, 5 patients; angiosarcoma, 3 patients; other histologies, 19 patients), 11 of 25 assessable patients had objective responses, including three with a complete remission [35]. The results of a randomized phase III trial of docetaxel and gemcitabine plus G-CSF with bevacizumab versus docetaxel and gemcitabine plus G-CSF with placebo in the treatment of advanced LMS (GOG0250) are awaited.

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Conflict of interest The authors declare that they have no conflict of interest.

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Small Cell Carcinoma of the Uterine Cervix: Clinical Outcome of Concurrent Chemoradiotherapy with a Multidrug Regimen

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Small cell carcinoma of the uterine cervix (SCCC) is a rare subtype of cervical cancer with an aggressive behavior. Although SCCC has a worse prognosis than other histological types of uterine cervical cancer such as squamous cell carcinoma or adenocarcinoma, standard therapy for SCCC remains to be established due to its rarity. The purpose of this study was to evaluate the efficacy and toxicity of concurrent chemoradiotherapy (CCRT) using a regimen consisting of vincristine, adriamycin, and cyclophosphamide alternating with cisplatin and etoposide (VAC/PE). We analyzed a series of 9 patients with SCCC. Five patients with stage IB disease underwent radical hysterectomy followed by CCRT. Four patients with advanced stage disease received CCRT primarily. With a median follow-up duration of 47.4 months (range, 10.5 to 86.4 months), 4 out of 5 patients with stage IB disease were alive without recurrence. In 4 patients with advanced stage disease, the response rate was 75% (complete response, 1; partial response, 2; progressive disease, 1). One patient with stage IVB disease remained without recurrence for 89.5 months. At 5 years, overall survival (OS) and progression-free survival for all patients was 52% and 56%, respectively. Patients with early-stage disease had an 80% 5-year OS rate compared to 25% for patients with advanced stage disease. Although all patients developed grade 3-4 neutropenia, CCRT using VAC/PE is feasible in both the primary and adjuvant settings for SCCC. In particular, this combined modality therapy may improve both local control and survival as postoperative treatment in patients with early-stage disease.

Keywords: concurrent chemoradiotherapy; neuroendocrine carcinoma; small cell carcinoma of the uterine cervix; uterine cervical cancer; VAC/PE

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Cervical cancer is the 5th most common cancer among women in Japan; the mortality from cervical cancer in 2010 was 4.1 per 100,000 female population (Matsuda et al. 2011). Cervical cancer encompasses several histologic types, of which squamous cell carcinoma (SCC) is the most common histologic subtype accounting for 70-80% of invasive carcinomas. Adenocarcinoma (AC) and adenosquamous carcinoma comprise 10-15% of all cases (Kurman et al. 2011).

Cervical neuroendocrine tumors are uncommon and four categories include: (1) typical carcinoid tumor, (2) atypical carcinoid tumor, (3) large cell neuroendocrine carcinoma, and (4) small cell carcinoma. Small cell carcinoma of the uterine cervix (SCCC), which was first described in 1957, accounts for only 1% of uterine cervical cancers and is an extremely aggressive subtype of uterine cervical cancer (Tsunoda et al. 2005; Crowder and Tuller 2007; Agarwal et al. 2011). Compared to the more common squamous cell carcinoma of the cervix, SCCC is more likely to show lymphovascular invasion, metastasize to the lymph nodes, and recur. Looking at the prognosis by histological subtype, 5-year overall survivals (OS) of SCC and AC are 70.5% and 68.7%, respectively (Quinn et al. 2006). The 5-year survival rate for SCCC ranges from 32% (n = 25) to 36.8% (n = 135), even for patients with early-stage disease (Chan et al. 2003; Cohen et al. 2010). Survival following radical hysterectomy without adjuvant therapy is worse than that of non-SCCC of comparable stage (5-year disease-free survival: SCCC [n = 11], 36% vs. non-SCCC [n = 301], 71%) (Sevin et al. 1996). Given the poor prognosis, it is important to establish standard, effective therapy for SCCC.

Small cell carcinoma occurs most frequently in the lung, and the clinical and biologic characteristics of small cell lung cancer (SCLC) are distinct from those of non-

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SCLC. SCLC exhibits aggressive behavior, with rapid growth, early spread to distant sites, and sensitivity to chemotherapy and radiation. Because SCCC is rare, the therapeutic strategies used for SCLC can offer valuable information that may contribute to the establishment of standard therapy for SCCC. Although irradiation improves both the local control and survival rates in SCLC patients (Warde and Payne 1992), chemotherapy has historically been the cornerstone of SCLC management. Chang et al. attempted to treat SCCC in a fashion similar to that used in SCLC, and reported a 5-year overall survival (OS) of 68% (n = 28) for patients treated with vincristine, adriamycin, and cyclophosphamide alternating with cisplatin and etoposide (VAC/PE) compared to 33% (n = 12) for patients who received a regimen consisting of cisplatin, vinblastine, and bleomycin (Chang et al. 1998). In another small phase II trial of alternating VAC/PE in early-stage SCCC before hysterectomy, 6 of 7 patients experienced a clinical complete response (CR) (Chang et al. 1999). Hoskins et al. (1995) reported that while radiotherapy with concurrent chemotherapy using the PE regimen was effective for locally advanced disease (n = 11; 3-year OS, 28%), it was inadequate to control systemic disease. We therefore adopted a protocol of CCRT using the alternating VAC/PE regimen in an effort to improve outcomes at our center.

In this study, we evaluated the efficacy and toxicity of CCRT using the alternating VAC/PE regimen and analyzed progression-free survival (PFS) and overall survival in patients with SCCC.

Materials and Methods

Patients

Eligible patients were required to have histologically confirmed small cell carcinoma in the cervix. Histopathologic diagnosis was based on morphologic criteria following hematoxylin-eosin (H-E) staining (Albores-Saavedra et al. 1997; Ishida et al. 2004; Katahira et al. 2004). The morphologic criteria included the presence of small cells with hyperchromatic nuclei and scanty cytoplasm, absent or inconspicuous nucleoli, and numerous mitotic figures and extensive necrosis. Nine patients received the diagnoses of SCCC from January 2001 to December 2009. Of the nine patients, five patients were diagnosed with immunohistochemistry as shown in Table 1.

All tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) 2008 classification system for cervical cancer, based on physical examination. Early stage was defined as Stages I-IIA and advanced as Stages IIB-IVB. Five patients with FIGO stage IB1 or IB2 underwent hysterectomy and pelvic lymphadenectomy. Four patients with advanced stage disease underwent CCRT. All patients had neither previous chemotherapy nor irradiation. Patients also had adequate hematological and hepatic function parameters, and an Eastern Cooperative Oncology Group performance status of two or less. All of the patients were informed that there was no standard chemotherapy regimen for SCCC and that VAC/PE was a favorable regimen for SCCC based on the reports by Chang et al (Chang et al. 1999). This study to analyze the case series was approved as a retrospective study by our institutional review board.

Treatment

As primary treatment, stage IB-IIA patients underwent radical hysterectomy (type III or type II) and pelvic lymphadenectomy. Subsequently, external beam pelvic radiotherapy (EBRT) was initiated within six weeks of surgery. EBRT was delivered to a total dose of 45-50 Gy in 23-25 daily fractions over five to six weeks. Tumor-directed radiation was utilized in one patient in whom a metastatic lymph node was not resected (Patient 8). Concurrent chemotherapy was administered within a week after initiating radiotherapy, with 3 cycles every 6 weeks as follows: intravenous infusion of 1 mg/m^2 of vincristine, 40 mg/m^2 of adriamycin and 1,000 mg/m^2 of cyclophosphamide, day 1; intravenous infusion of 100 mg/m^2 of cisplatin, day 22; 100mg/m^2 of etoposide, day 22-24. Cycles were delayed for adverse events as defined by the Common Toxicity Criteria (CTC) grade 2 or greater, neutropenia < 1,500 per ul or thrombocytopenia < 100,000 per ul. The dose of each drug was reduced by 25% of the previous dose in the case of grade 3/4 toxicities.

For advanced-stage disease, the initial 20 Gy was delivered to the

Table 1. Patient Characteristics.

Patient no.	Age	Type of SCCC	IHC					FIGO stage	T	N	M	Tumor size (cm)	Site of metastasis	PS
			NSE	CGA	Synaptophysin	NCAM	CD56							
1	28	pure	N/D	N/D	N/D	N/D	N/D	IVb	3b	1	1	5	lung	0
2	44	pure	N/D	N/D	N/D	N/D	N/D	IVb	3b	1	1	5	lt. infraclavilular LN	0
3	34	pure	N/D	N/D	N/D	N/D	N/D	IIb	2b	1	0	8	none	0
4	61	pure	positive	positive	positive	N/D	positive	IIb	2b	0	0	6	none	0
5	31	pure	N/D	positive	N/D	positive	N/D	Ib1	1b1	1	0	2	none	0
6	39	mixed	positive	negative	negative	positive	N/D	Ib2	1b2	1	0	8	none	0
7	44	pure	N/D	N/D	N/D	N/D	N/D	Ib2	1b2	1	0	6	none	0
8	59	mixed	positive	negative	positive	N/D	positive	Ib1	1b1	0	0	3	none	0
9	40	pure	positive	positive	positive	positive	N/D	Ib1	1b1	0	0	2	none	0

mean 42

SCCC, small cell carcinoma of the uterine cervix; IHC, immunohistochemistry; NSE, Neuron-specific enolase; CGA, Chromogranin A; NCAM, Neural cell adhesion molecule; N/D, not done; FIGO, International Federation of Gynecology and Obstetrics.

whole pelvis. After that, 30 Gy was administered through the same whole-pelvis field with a midline block. The first high dose-rate intracavitary brachytherapy session (6 Gy per week, 4-5 fractions) with chemotherapy was performed within 10 days after the initial 20 Gy of E□RT. The VAC/PE chemotherapy was administered concurrently with the same dose for early stage disease within a week of the initial radiotherapy. A paraaortic boost (50 Gy) was added when paraaortic lymph node metastasis was recognized by computed tomography (CT). □ysterectomy was added for residual disease by biopsy or on magnetic resonance imaging one month following the completion of primary treatment.

Clinical and pathological variables analyzed are shown in Table 1. The primary end point was any cancer-related death. All end points were calculated from the date of the start of primary treatment to death, or censored at last follow-up. The date of death was obtained from the medical records. All end points were updated in December 2011.

Efficacy assessment

Pretreatment evaluation included past medical history and physical examination, complete blood cell count with differentials, chemistry, CT scan of chest, abdomen and pelvis, chest x-ray and any other diagnostic procedures as clinically indicated. During treatment, physical examination including toxicity assessment, complete blood cell count, and chemistry were performed every week before each cycle. Appropriate imaging studies including a CT scan were used to evaluate treatment response or to document disease progression. A CT scan confirmed responses four to six weeks after the initial response documentation. Patients were assessed every month with imaging for disease progression following the completion of the chemotherapy. Responses were classified according to the RECIST criteria (Therasse et al. 2000). Progression free survival (PFS) was calculated from the first day of treatment to the date on which disease progression was first documented or the date of the last follow-up. Overall survival (OS) was calculated from the first day of treatment to the date of death or last follow-up. Toxicity was monitored according to the National Cancer □nstitute (NC□) CTC scale version 3.0.

Statistical Analysis

Statistical analysis was performed using JMP Pro 9.0.2 (SAS □nstitute □nc. North Carolina, USA). The impact of clinical and pathologic risk factors on the survival of patients with SCCC was evaluated using the Kaplan-Meier life table analyses and log-rank tests. All tests were two-tailed with P values < 0.05 were considered significant.

Results

The mean patient age at diagnosis was 42 years (range: 28-61) and all of the patients were diagnosed with SCCC by a punch biopsy. Patient characteristics are listed in Table 1. The □□GO stage distribution was as follows: three were stage □□1, two were □□2, two were stage □□□, and two were stage □V□. Three patients had a non-bulky primary tumor, with the remaining presenting with bulky disease. The therapeutic regimens and clinical outcomes for all nine patients are shown in Table 2. The patients with stage □□ disease underwent a type □□or type □□□radical hysterectomy followed by CCRT. □our patients with advanced stage disease received CCRT primarily. Patients □2 and □3 had partial responses and underwent hysterectomy after CCRT. □oth of them had remains of disease at the cervix and cardinal ligament. Eight of nine patients completed three cycles of VAC/PE and radiation. Of these, three patients (□3, □5, and □8) had dose reductions of both cisplatin and etoposide at the third cycle for grade 4 bone marrow suppression. One patient (patient □7) with severe fatigue and grade 4 neutropenia received two cycles of paclitaxel and carboplatin therapy after the first cycle of VAC/PE therapy. Treatment toxicities are listed in Table 3. The highest level of toxicity at each cycle is listed. All patients had severe neutropenia (grade 3 or 4) and two patients had febrile neutropenia (22.2%). The incidence of grade 3 and 4 toxicity was as follows: anemia, 44.4%; neutropenia, 100%; thrombocytopenia, 55.5%; nausea, 11.1%. No treatment-related death occurred during therapy.

□our patients with primary CCRT were evaluable for an objective response because five patients who underwent surgery primarily had no residual tumor after surgery. □ ith

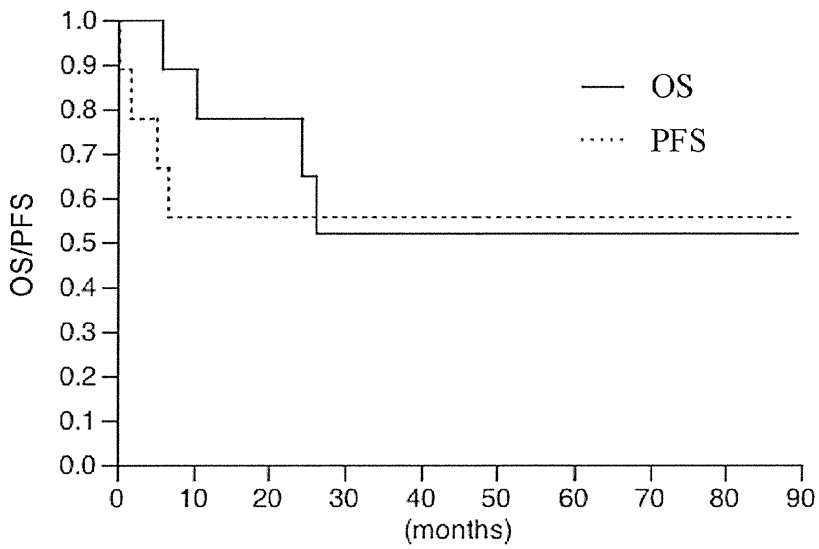
Table 2. Therapy and outcomes.

Patient no.	Primary therapy	Radiation (Gy)	Chemo regimen	REC□ST	Site of recurrence	P□S (Mo)	Outcome	OS (Mo)
1	CCRT	□ P 50 □RALS 24	VAC/PE	CR	none	89.5	alive	89.5
2	CCRT→EH+BSO	□ P 50 □PAN 50 □ RALS 30 □neck LN 60	VAC/PE	PR	stump	6.8	death	26.3
3	CCRT→RH+BSO+PLA	□ P 50 □RALS 30	VAC/PE	PR	bone marrow	1.8	death	6.0
4	CCRT	□ P 50 □RALS 24	VAC/PE	PD	lung	0.4	death	24.4
5	EH+BSO+PLA→CCRT	□ P 50	VAC/PE	none	none	47.3	alive	59.6
6	RH+BSO+PLA→CCRT	□ P 45	VAC/PE	none	none	39.9	alive	47.4
7	RH+BSO+PLA→CCRT	□ P 50	VAC/PE→TC	none	none	78.4	alive	86.4
8	RH+BSO+PLA→CCRT	□ P 50 □rt. □liac LN 10	VAC/PE	none	bone, lung, LN	5.3	death	10.5
9	RH+BSO+PLA→CCRT	□ P 50	VAC/PE	none	none	17.7	alive	19.4

CCRT, concurrent chemoradiotherapy; E□, extended hysterectomy; R□, radical hhysterectomy; □SO, bilateral salpingoophorectomy; PLA, pelvic lymphadenectomy; □ P, whole pelvis; RALS, Remote After loading System; LN, lymph node; P□S, progressive free survival; OS, overall survival.

Table 3. Adverse effects by treatment (NC□CTC).

	No. of patients (n = 9)				
Grade	0	1	2	3	4
Anemia	1	0	4	3	1
Neutropenia	0	0	0	2	7
Thrombocytopenia	1	3	0	2	3
Diarrhea	2	4	3	0	0
Nausea	0	2	6	1	0
Malaise	0	2	7	0	0
□ebrile neutropenia	0	0	0	2	0
Renal Insufficiency	6	3	0	0	0



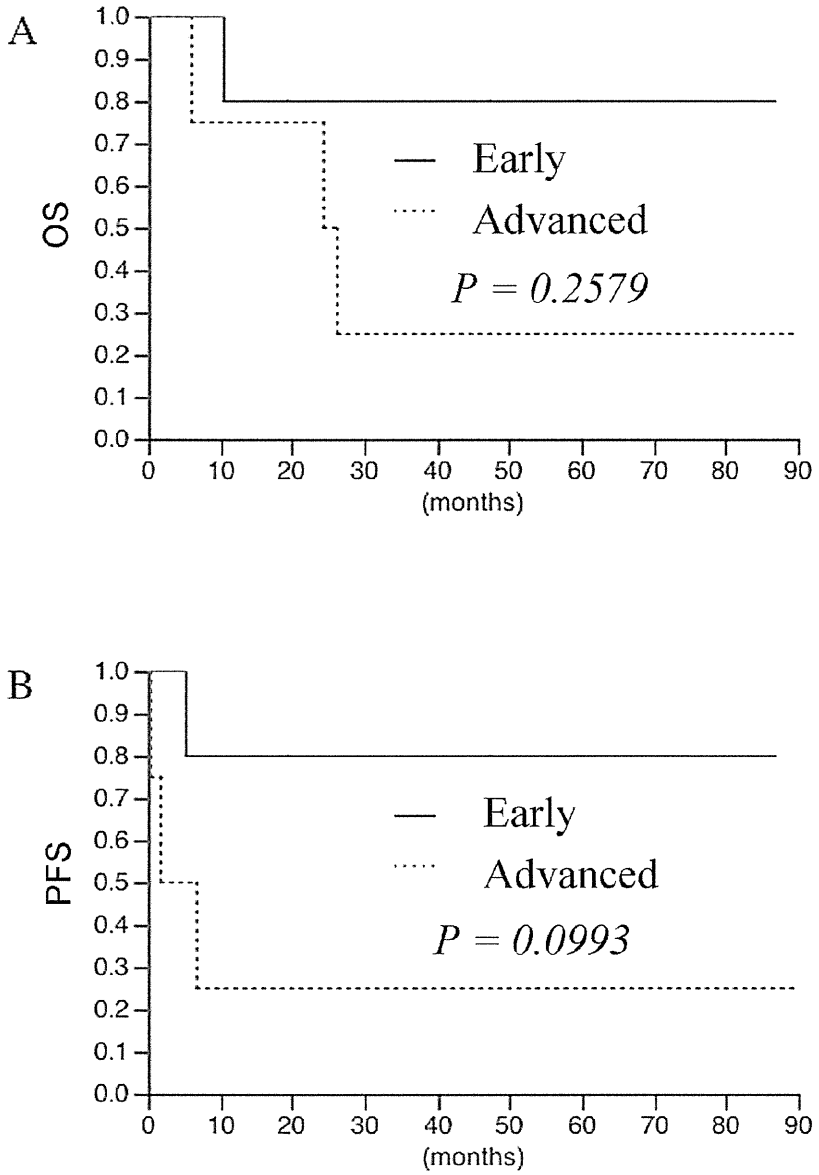
□ig. 1. Survival rates of all patients.
Overall survival rates and Progression free survival rates of all patients (n = 9), plotted by the Kaplan-Meier method.

a median follow-up duration of 47.4 months (range from 10.5 to 86.4), four out of five stage IB patients were alive without recurrence at the time of analysis. The response rate of CCRT as primary therapy was 75% (CR: 1, PR: 2, PD: 1). Remarkably, one patient with stage □V□ disease was alive without recurrence at 89.5 months.

The median OS periods for those who survived and died during the evaluation period were 59.6 and 17.5 months, respectively. The OS and P□S rates for all patients at five years were 52% and 56%, respectively (Fig. 1). The patients with early stage disease had an 80% three-year OS compared to 25% for patients with advanced stage disease. There was no significant difference between the early stage group and the advanced stage group (□ig. 2A, □). Relapse sites included the vaginal cuff (n = 1), bone marrow (n = 1), lung (n = 2), bone (n = 1), and lymph node (n = 1). All patients with relapse were dead within three years of the first treatment.

□iscussion

SCCC is a rare and carries a poor prognosis, primarily due to its propensity for early hematogenous and lymphatic spread. Many authors have recommended adjuvant chemotherapy due to the aggressive behavior of this tumor (Chang et al. 1998; Boruta et al. 2001); however, it is difficult to perform a large scale randomized controlled study given the rarity of the condition. The Gynecologic Oncology Group attempted to study small cell cervical carcinoma in protocol 66 between 1982 and 1986, but failed to recruit sufficient numbers of patients. Consequently, treatments for SCCC have been largely extrapolated from the experience with SCLC. SCLC is highly responsive to multiple chemotherapeutic drugs, and chemotherapy dramatically prolongs survival compared to best supportive care (Agra et al. 2003). □n multiple randomized trials, the PE regimen appears to be at least as effective as older regimens such as vincristine, doxorubicin, and cyclophosphamide (VAC) and has less toxicity for SCLC (□ukuoka et al. 1991; Sundstrom et al.



□ig. 2. Comparison between early and advanced stage.
(A) Overall survival rates. (□) Progression free survival rates of early stage ($n = 5$) and advanced stage ($n = 4$), plotted by the Kaplan-Meier method.

2002). Recently, as with SCLC, early stage patients with SCCC who received post-operative PE regimen with or without radiation experienced an 83% three-year recurrence-free survival, compared to 0% for those who did not receive adjuvant chemotherapy (□ivanovic et al. 2009). □n addition to chemotherapy, there is a significant role for radiation therapy in the treatment of limited stage SCLC (Gaspar et al. 2005). □n general, for patients with limited stage-SCLC, the PE regimen is used in conjunction with thoracic radiation because the PE regimen has little mucosal toxicity and modest hematologic toxicity.

To establish the appropriate therapy for SCCC, both the standard treatment for uterine cervical cancer, as well as the similarities in biologic behavior between SCLC and SCCC should be considered. □n 1999, NC□issued a clinical alert that CCRT should be considered the standard of care over radiotherapy alone for women with cervical cancer, and concurrent cisplatin (40 mg/m2 weekly for five weeks) with external beam RT (50 Gy) is now widely used in both the primary and adjuvant settings. According to the clinical guidelines for cervical cancer treatment in Japan, postoperative CCRT using weekly cisplatin is recommended for patients at high risk of recurrence (Nagase et al. 2010). □owever, there is a room for discussion whether this setting is suitable for histological subtypes other than SCC. The Society of Gynecologic Oncology (SGO) also proposed that combination chemotherapy with radiation should be considered for non-surgical candidates with neuroendocrine carcinoma of the uterine cervix, with emphasis on individualized treatment (Gardner et al. 2011). Despite the lack of prospective data,

the use of postoperative CCRT against SCCC has become widely accepted.

In this study we analyzed a series of 9 patients with SCCC. Of them, five patients received surgery followed by CCRT using the VAC/PE regimen. Notably, all five patients with early stage SCCC including two cases with bulky tumors had no pelvic recurrence, a median survival of 47.4 months and an OS rate at five years of 80%. Although limited by the small number of patients included in this analysis, we did show improvement in the overall survival rate over the previously reported five-year survival of 31.6-46.6% for stage I-IIA patients (Chan et al. 2003; Lee et al. 2008; Cohen et al. 2010). In addition, Cohen et al. reported that radical hysterectomy was an independent prognostic factor for survival in a multivariate analysis of 188 patients with SCCC (Cohen et al. 2010). These results suggest that CCRT using the VAC/PE regimen in addition to surgical resection may improve both local control and survival in patients with early stage disease.

We applied the combination VAC/PE regimen with radiation to the patients with advanced stage disease, with post-treatment hysterectomy reserved for those patients with partial responses. While one patient (patient 2) with advanced disease entered long term remission following an interval hysterectomy, two patients treated with hysterectomy after CCRT died of their disease (patient 2, 3). These results suggest that hysterectomy after CCRT may confer little benefit in the setting of advanced stage SCCC; although some studies report that extrafascial hysterectomy after CCRT is a reasonable option if there are histological factors suggesting poor prognosis (Motton et al. 2010).

Moskins et al. reported that stage III/IV patients with SCCC had a 38% three-year recurrence-free survival if treated with combination chemotherapy (PE) in addition to concurrent radiation (Moskins et al. 2003). We experienced two stage IV patients treated with CCRT using the VAC/PE regimen, one of whom (patient 1) had prolonged survival without recurrence. When radiotherapy and chemotherapy are given together, the evaluation of toxicities becomes important. We adopted the VAC/PE regimen instead of the PE regimen, with a similar toxicity profile to that reported by Moskins et al. There were no treatment-related deaths in either protocol. Although there are no randomized trials comparing chemotherapy alone versus CCRT, either as a primary therapy, or in the adjuvant setting following radical hysterectomy, CCRT using the VAC/PE regimen is feasible and has the potential to cure some SCCC patients with metastatic disease.

Limitations of this study include its small size and that all patients were treated at a single institute. A strength, however, is that the treatment strategies (radical hysterectomy with adjuvant CCRT using VAC/PE regimen for early stage patients, and definitive radiation therapy with VAC/PE chemotherapy for advanced stage patients) were uniform across the study period. In conclusion, combination chemotherapy (VAC/PE) in addition to concurrent radiation is feasible in both the primary and adjuvant settings. Postoperative adjuvant CCRT using the VAC/PE regimen may improve both local control and prognosis for patients with early stage disease. Given the rarity of SCCC, multi-institutional clinical trials are required to attain sufficient power to develop standardized treatment protocols.

Conflict of Interest

The authors report no conflict interest.

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ORIGINAL RESEARCH

Analysis of the antitumor activity of gemcitabine and carboplatin against ovarian clear-cell carcinoma using the DNA damage marker γH2AX

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Background: Differences in the incidence and type of DNA damage induced by antitumor agents for ovarian clear-cell carcinoma (CCC) were determined in two CCC cell lines, using γH2AX.

Materials and methods: The antitumor activity of gemcitabine (GEM) and carboplatin (CBDCA) were examined using cultured cell lines of CCC (OVISE and RMG-I). Each cell line was treated with GEM and CBDCA, the cells were collected, fixed, and then reacted with anti-γH2AX antibody. γH2AX and nuclear DNA were then simultaneously detected by flow cytometry using fluorescein isothiocyanate and propidium iodide, respectively, to determine the amounts of γH2AX formed in each cell-cycle phase.

Results: After administration of GEM, both cell lines showed DNA damage and cell-cycle arrest in the S and G_2/M phases, and increased apoptosis. Similarly, with CBDCA, OVISE showed S- and G_2/M-phase arrest, while RMG-I showed G_2/M-phase arrest.

Conclusion: The mechanism of action of GEM and CBDCA in CCC cell lines was elucidated using γH2AX as a DNA damage marker. Our findings suggested that concomitant use of GEM plus CBDCA may be effective in the treatment of CCC.

Keywords: γH2AX, clear-cell carcinoma, ovarian cancer, DNA damage, apoptosis, gemcitabine, carboplatin

Introduction

Ovarian clear-cell carcinoma (CCC), a subtype of epithelial ovarian cancer, is relatively less sensitive to chemotherapy, and is therefore classified as a refractory ovarian cancer.[1] It has been shown that a combination of carboplatin (CBDCA) and paclitaxel (PTX), a standard therapy for ovarian cancer,[2,3] is effective against serous adenocarcinoma and endometrioid adenocarcinoma, with a response rate of approximately 75%, while CCC shows lower response rates, ranging from 18% to 50%.[4] The incidence of CCC has been increasing and is now estimated to be 23% in Japan, while that in Europe is reported to be 5%–6%. No treatment has been established yet for this histological subtype of ovarian cancer. Histopathology remains the gold standard for classifying epithelial ovarian cancer into subgroups; however, there is emerging evidence indicating differences in the genetic and molecular profiles among these cancers. On the other hand, there is no international consensus regarding the necessity of establishing treatment strategies based on the histological subtype. Current chemotherapeutic options for ovarian cancer include drugs inducing DNA damage (eg, cisplatin and CBDCA), microtubule inhibitors (eg, PTX), topoisomerase inhibitors (eg, polyethylene glycolated liposomal

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doxorubicin, topotecan, irinotecan), and antimetabolites (eg, gemcitabine [GEM] and 5-fluorouracil).

Recently, it has become apparent that phosphorylation of histone H2AX, one of the variants of the nucleosome core histone H2A, can serve as a sensitive and reliable marker of DNA damage (Figure 1A). More specifically, DNA damage, particularly that involving the formation of DNA double-strand breaks, induces phosphorylation of histone H2AX on Ser-139; phosphorylated H2AX is termed γH2AX (Figure 1B).[5] Dot γH2AX, detectable using γH2AX-specific antibody, is considered to be a specific marker of DNA damage. Therefore, DNA damage can be detected by immunocytochemistry.[6]

We reported previously that γH2AX is a useful marker for the evaluation of DNA damage and apoptosis.[7] In this study, we focused on γH2AX as a marker of DNA damage to examine the cellular effects of GEM and CBDCA on CCC in terms of cell-cycle arrest, DNA damage, and induction of apoptosis. In addition, chemotherapeutic regimens that are likely to be effective in the treatment of CCC are discussed.

Materials and methods

Cell culture

We used two CCC cell lines (OVISE and RMG-I) obtained from the Health Science Research Resources Bank (Osaka, Japan). OVISE was established from a patient with metastatic disease after completion of six cycles of a platinum-based combination therapy, and was cultured in dishes (BD, Franklin Lakes, NJ, USA) containing Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum. RMG-I was established from a chemotherapy-naïve patient with ascites, and was reported to show primary platinum resistance.[8] RMG-I was grown in dishes (BD) in Ham F-12 medium supplemented with 10% fetal bovine serum. For both cell lines, the medium was supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Meiji Seika, Tokyo, Japan). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Drug

GEM was dissolved in dimethyl sulfoxide (Sigma-Aldrich); the final concentration of dimethyl sulfoxide in the culture medium never exceeded 0.1% (w/v). CBDCA was dissolved in phosphate-buffered saline (PBS). The concentrations of GEM and CBDCA were set to correspond to the blood concentration at a standard clinical dose. Clinical maximum drug concentration and minimum effective concentrations of GEM and CBDCA are 25 μg/mL and 5 ng/mL, and 55 μg/mL and 10 μg/mL, respectively.

Immunohistochemistry

Both the cells floating in the medium and the cells that remained attached after trypsinization were collected and fixed with 1% methanol-free formaldehyde (Polysciences,

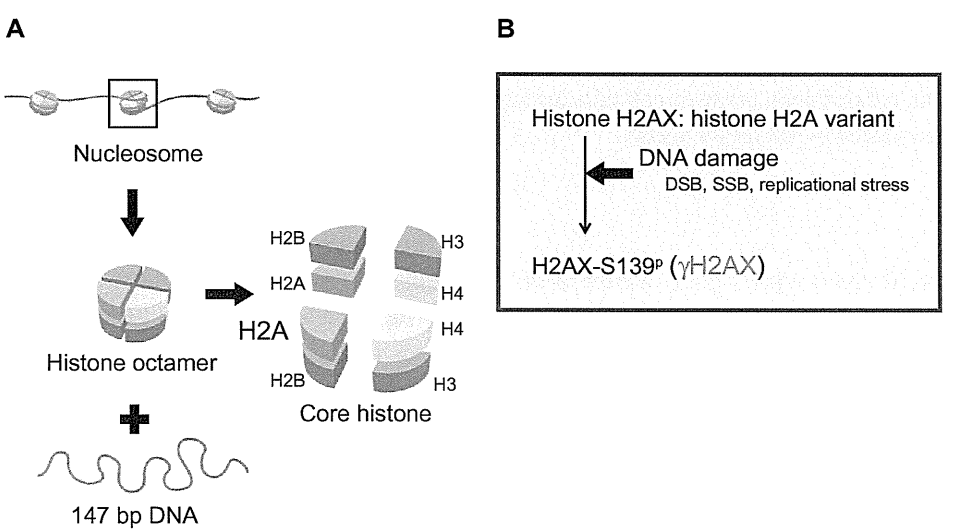

Figure 1 (**A**) Nucleosomes, units of chromatin, consist of core histones wrapped in 146 bp of DNA and linker DNA. Core histones are octamers designated as H2A, H2B, H3, and H4. Not all nucleosomes include typical histone octamers containing H2A, H2B, H3, and H4. In some parts of the chromosome, specific histones are replaced by histone variants that are slightly different histones involved in the local chromosome function. Histone H2AX is a variant of histone H2A. H2AX is known to be highly concentrated in areas of DNA damage. (**B**) When DNA damage occurs, serine 139 of histone H2AX in the chromatin on both sides of the damaged site is phosphorylated. Phosphorylated histone H2AX is referred to as γH2AX.
Abbreviations: DNA, deoxyribonucleic acid; DSB, double strand break; SSB, single strand break.

Warrington, PA, USA) in PBS at 0°C for 15 minutes and postfixed with 80% ethanol for at least 2 hours at –20°C. The fixed cells were washed twice in PBS and suspended in a 1% (w/v) solution of bovine serum albumin (Sigma-Aldrich) in PBS, to suppress nonspecific antibody binding. The cells were then incubated in 100 μL of 1% bovine serum albumin containing 1:100 diluted antiphosphohistone H2AX (Ser-139) monoclonal antibody (Upstate, Lake Placid, NY, USA) for 2 hours at room temperature, washed twice with PBS, and resuspended in 100 μL of 1:20 diluted fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of goat antimouse immunoglobulin (Dako, Glostrup, Denmark) for 30 minutes at room temperature in the dark. The cells were then counterstained with 5 μg/mL propidium iodide (Sigma-Aldrich) in the presence of 100 μL of ribonuclease A (Sigma-Aldrich) for 30 minutes.

Fluorescence measurements by flow cytometry

The fluorescein isothiocyanate (green) and propidium iodide (red) fluorescences of individual cells in suspension induced by excitation with a 488 nm argon ion laser were measured using a FACScan flow cytometer (BD). The green and red fluorescences from each cell were separated and quantified using standard optics and CellQuest software (BD). Ten thousand cells were measured per sample. All experiments were repeated at least three times.

After γH2AX and DNA staining, the DNA content and γH2AX content determined by flow cytometry were represented on the *x* and *y* axes, respectively. The γH2AX content in each cell cycle was determined, so as to allow examination of the relationships between cell kinetics and the DNA damage induced by antitumor agents.

Results

GEM

In the OVISE cells, treatment with 5 ng/mL or more of GEM mainly caused DNA damage in cells of the early S-phase. After exposure to 100 ng/mL or more of GEM, the S-phase cells showing DNA damage underwent apoptosis. Similarly, in the RMG-I cells, DNA damage was primarily seen in the early S-phase cells following exposure to 5 ng/mL or more of GEM. Treatment with 100 ng/mL or 1 μg/mL GEM induced DNA damage not only in S-phase cells but also in G_2/M-phase cells. These cells, however, did not undergo apoptosis (Figure 2A). To investigate the time course of the changes, both cell lines were treated with GEM at the minimum concentration causing DNA damage (5 ng/mL) for different periods of time. In the OVISE cells, DNA damage was mainly confined to S-phase cells after exposure to GEM for 24 hours or more. However, after exposure for 48 hours or more, DNA damage also extended to the cells of the G_2/M phase. The S-phase cells with DNA damage underwent apoptosis after exposure to GEM for 48 hours or more, while the number of cells in the G_1 phase gradually decreased and there was S-phase arrest. Moreover, G_2/M-phase cells showing DNA damage remained viable without undergoing apoptosis. In RMG-I cells, marked DNA damage was observed in the S-phase cells after 24 hours of exposure to GEM, although the cells underwent apoptosis after 72 hours' exposure to GEM. Similar to the OVISE cells, the gradual decreases in the number of cells in the G_1 phase and S-phase arrest were also noted in RMG-I cells. G_2/M-phase cells showing DNA damage remained viable without apoptosis even after 120 hours of exposure to GEM, and G_2/M-phase arrest was induced (Figure 2B).

CBDCA

DNA damage in the S phase was seen gradually after exposure to CBDCA for 24 hours in the OVISE and RMG-I lines at 1 μg/mL and 10 μg/mL, respectively (Figure 3A). Subsequently, cells with damaged DNA underwent apoptosis. Gradually, both cell lines showed DNA damage in the G_2/M phase and underwent apoptosis. OVISE showed S- and G_2/M-phase arrest, while RMG-I showed G_2/M-phase arrest (Figure 3B).[9]

Discussion

Numerous distinct dots of γH2AX are usually observed when cells are pretreated with antitumor agents and immunohistochemically stained using γH2AX antibodies. Each of these dots is considered to correspond to a site of DNA damage.[6] In apoptotic cells, because of DNA fragmentation, nuclear fragments showing strong staining of γH2AX are commonly observed. Thus, DNA damage and apoptosis can be visualized using γH2AX as an indicator. In a cell, all chromosomal DNA is replicated and the amount of DNA doubles during the S phase; then cell division occurs during the M phase to produce two daughter cells that initiate a new cell cycle. After immunofluorescence staining of γH2AX and counterstaining of DNA, histograms were constructed by plotting the amount of DNA and amount of γH2AX in the cells, determined by flow cytometry, on the *x* and *y* axes, respectively, to detect the amount of γH2AX formed in each cell-cycle phase; this allowed a visual estimation of

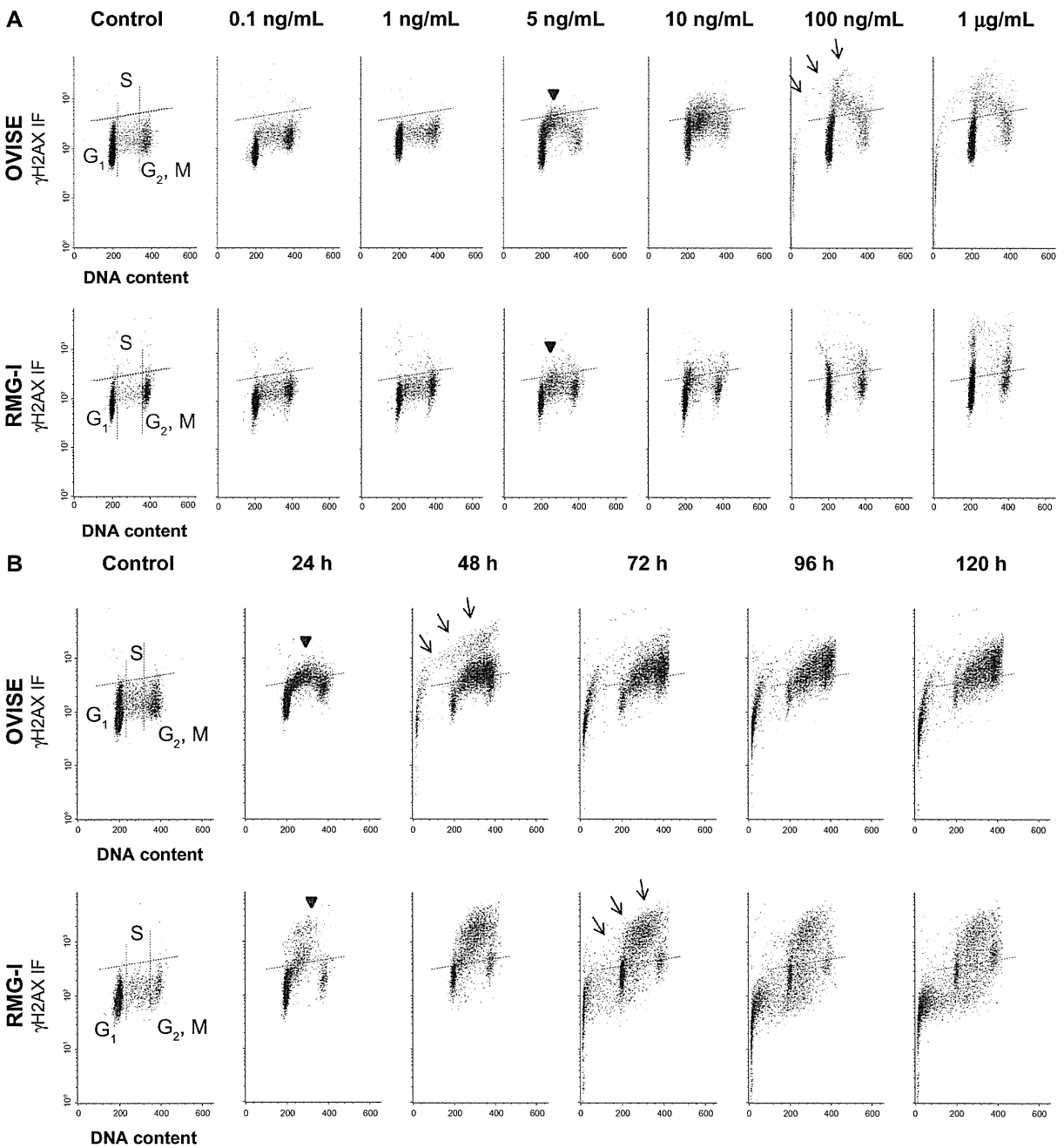

Figure 2 (**A** and **B**) Bivariate distributions (DNA content vs γH2AX) in the clear-cell carcinoma cell lines OVISE and RMG-I, treated with gemcitabine. The dotted lines indicate the upper level of γH2AX immunofluorescence in 95% of cells in the untreated (control) culture. Arrowheads indicate elevation in the immunofluorescence intensity of γH2AX, suggestive of DNA damage. Arrows indicate apoptotic cell populations with marked increase in the amount of γH2AX and gradual decrease in DNA. (**A**) Both cell lines were treated with various concentrations of gemcitabine for 24 hours. Both cell lines exhibited DNA damage in S-phase cells at the minimum concentration of 5 ng/mL. In the OVISE line, apoptosis was induced at concentrations of 100 ng/mL or higher. RMG-I cells remained with DNA damage. (**B**) Both cell lines were treated with 5 ng/mL, calculated as the minimum concentration inducing DNA damage, for various reaction times. S-phase cells of OVISE showing DNA damage underwent apoptosis after 48 hours. In addition, S- and G_2/M-phase arrest was observed. DNA damage was induced in the S-phase cells of RMG-I after 24 hours, and the cells showing DNA damage underwent apoptosis after 72 hours. Furthermore, cell-cycle arrest occurred in the S- and G_2/M phases.
Abbreviations: DNA, deoxyribonucleic acid; S, synthesis phase; G_1, Gap 1 phase; G_2, Gap 2 phase; M, mitotic phase; h, hours.

the extent of DNA damage caused by antitumor agents and examination of changes in the cellular kinetics. In this immunohistochemical γH2AX-detection method, DNA damage can be detected with high sensitivity at much lower concentrations of the necessary agents than in the comet assay, and the extent of DNA damage can be correlated with the phase of the cell cycle.[10]

Combination chemotherapy with PTX and CBDCA is established as the gold standard for ovarian cancer. This therapy, however, is not sufficiently effective for CCC, and it

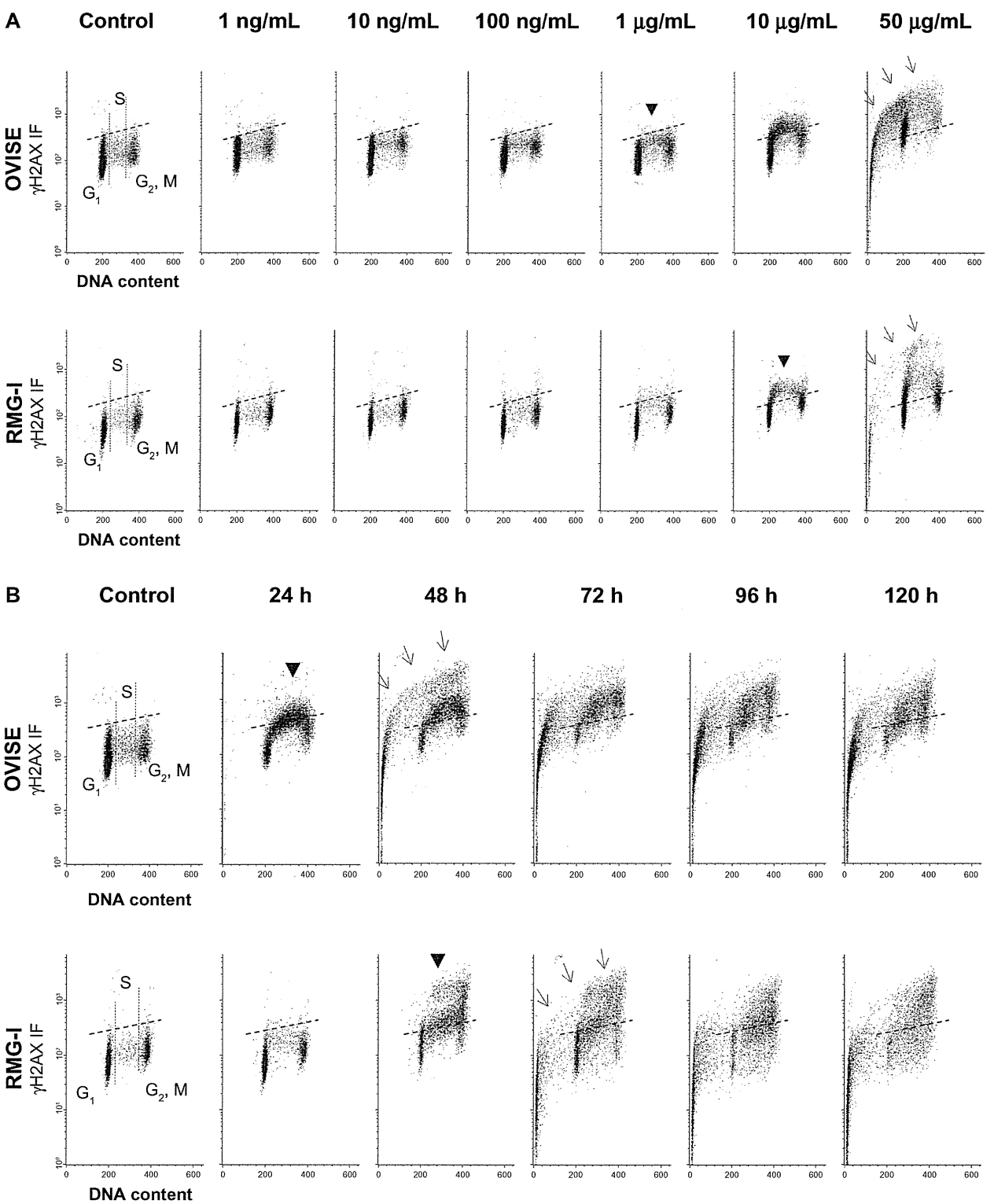

Figure 3 (**A** and **B**) Bivariate distributions (DNA content vs γH2AX) of OVISE and RMG-1 cell lines, treated with carboplatin. The dotted lines indicate control. Arrowheads and arrows indicate DNA damage and apoptosis, respectively. (**A**) Both cell lines were treated with various concentrations of carboplatin for 24 hours. DNA damage was observed in the S-phase cells at 1 µg/mL and 10 µg/mL concentrations in OVISE and RMG-1, respectively. DNA damage was found in both cell lines at every cell cycle as the concentration increased, and apoptosis occurred at a concentration of 50 µg/mL. More cells remained free of DNA damage in RMG-1 than in OVISE. (**B**) Both cell lines were treated with 1 µg/mL, the minimum concentration inducing DNA damage in either cell line, for various reaction times. In OVISE, S-phase cells with DNA damage progressed to apoptosis after 48 hours. DNA damage was also found in G_2/M-phase cells after 48 hours, but most did not progress to apoptosis. S- and G_2/M-phase arrests were observed. DNA damage was found in S- and G_2/M-phase cells after 48 hours in RMG-1. The S-phase cells with DNA damage progressed to apoptosis 72 hours later, but G_2/M-phase cells showing DNA damage remained. S- and G_2/M-phase arrests were observed.

Abbreviations: DNA, deoxyribonucleic acid; S, synthesis phase; G_1, Gap 1 phase; G_2, Gap 2 phase; M, mitotic phase; h, hours.

has been pointed out that individualization of chemotherapy based on the histological subtypes is needed for this type of cancer.[4] GEM has proved to be effective in patients with ovarian cancer,[11,12] and a publication-based application of GEM was submitted in September 2010 in Japan. In this study, we attempted to demonstrate the efficacy of GEM and CBDCA, and examine the possibility of expanding the treatment options for patients with CCC, for whom the current treatment options are limited.

GEM is an antimetabolite used to treat recurrent ovarian cancer that is known to exert its antitumor activity via becoming incorporated into the cellular DNA. In both the CCC cell lines used in this study, GEM induced marked DNA damage and cell-cycle arrest in the S phase, probably as a result of the GEM-induced stalled replication forks. However, many cells with DNA damage remained viable even after exposure to GEM for 120 hours, indicating that GEM had only a weak cytocidal effect on the CCC cells.

The results of the study showed that GEM exerted a weaker antitumor effect on the RMG-I cells than on the OVISE cells. Possible reasons for this include the higher percentage of G_0/G_1 cells and lower percentage of S-phase cells in the RMG-I cell line. A relatively low proportion of cells in the S phase is generally observed in CCCs, and may account for the insufficient antitumor effect of GEM monotherapy in patients with this type of ovarian cancer. Unlike in human myelogenous leukemia cell lines,[13] GEM caused cell-cycle arrest not only in the S phase but also in the G_2/M phase in the CCC cell lines. In addition, cells arrested in the G_2/M phase of the cell cycle also showed DNA damage. Taking into account the GEM concentrations and the time course of the cellular changes, this may be attributable to the cell-cycle arrest induced at the G_2/M checkpoint after progression of the S-phase cells showing DNA damage to the G_2 phase. Although the factors involved in the phosphorylation of ataxia telangiectasia and Rad3-related protein after recognition of DNA damage are not yet clearly defined, it is considered that BRCA1, a human tumor-suppressor gene product, may play a role in the process and is responsible for G_2/M checkpoint regulation in response to DNA damage. A recent study has shown *BRCA1* mutations involved in CCC.[14] Thus, *BRCA1* gene mutations in CCC cell lines may be involved in the cell-cycle arrest at the G_2/M phase observed in this study.

After CBDCA administration, DNA damage was seen in the S and G_2/M phases in both cell lines. OVISE contained a remarkable cell population rescued from apoptosis and surviving with DNA damage. On the other hand, most RMG-I cells with DNA damage underwent apoptosis. These results suggest that cell lines respond differently to platinum agents, ie, RMG-I was cisplatin-resistant but responded to CBDCA.

This study infers that for residual cells in which the cell cycle remains arrested due to DNA damage caused by GEM, effective cytocidal action can theoretically be obtained by additionally or concomitantly administering CBDCA, which exerts effects on any cells in cell-cycle arrest (Table 1). These mechanisms of action for both drugs have already been elucidated in many types of carcinomas other than ovarian CCC. In this paper, the mechanisms of action in CCC are reported. The results obtained suggest that combination therapy with GEM plus CBDCA might be useful in the treatment of CCC. This conclusion was derived from our own study method using flow cytometry with γH2AX as a marker. In order to establish GEM-plus-CBDCA therapy, which is currently being administered in clinical trials, we considered it to be essential to demonstrate its usefulness not only for other types of carcinomas but also for CCC in basic studies. Moreover, another study is currently being conducted to assess whether there are synergistic effects of GEM plus CBDCA.

Currently, a randomized clinical trial (iPLAS) is ongoing as an intergroup study in Japan to compare the efficacy and safety of GEM plus CBDCA with those of polyethylene glycolated liposomal doxorubicin plus CBDCA in patients with platinum-sensitive, recurrent ovarian cancer. Molecular-targeted agents have come to be increasingly used in chemotherapy for ovarian cancer around the world. However, it is still impossible to use such drugs in clinical settings other than physician-initiated clinical trials in Japan. Therefore, effective treatment of CCC needs to be developed using antitumor drugs covered by health insurance. In this study, we used γH2AX as an indicator to examine the antitumor effects of GEM and CBDCA on OVISE and RMG-I cells, and the results suggested that combination chemotherapy with GEM plus CBDCA may be effective for CCC. The method employed in this study is convenient and very

Table 1 Cell kinetics of CCC cells treated with anticancer drugs

	DNA damage	Apoptosis	Cell cycle arrest
GEM			
OVISE	S	+	S, G_2/M
RMG-I	S, G_2/M	+	S, G_2/M
CBDCA			
OVISE	S, G_2/M	+	S, G_2/M
RMG-I	S, G_2/M	+	G_2/M

Abbreviations: CCC, clear cell carcinoma; CBDCA, carboplatin; GEM, gemcitabine; S, synthesis phase; G_2, Gap 2 phase; M, mitotic phase.