Fig. 3 Overall survival of clear cell adenocarcinoma cases. The median follow-up period was 43 months (range 3–133 months). Overall survival of 16 clear cell adenocarcinoma cases with maintained CRABP1 expression was significantly better than that of 10 cases with reduced CRABP1 expression ($P = 0.049$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

Fig. 4 Disease-free survival of ovarian carcinomas. Complete remission was achieved by surgery, with/without chemotherapy, in a total of 48 ovarian carcinomas, including 35 serous and 24 clear cell carcinoma cases. The median follow-up period was 46 months (range 4–133 months). Disease-free survival of 35 carcinomas with maintained CRABP1 expression was significantly better than that of 24 cases with reduced CRABP1 expression ($P = 0.024$ by the Kaplan–Meier method). *Broken line* survival probability of maintained-CRABP1-expression cases. *Solid line* survival probability of reduced-CRABP1-expression cases

However, it occurred in 5 (50%) among 10 cases with reduced CRABP1 expression.

Association of reduction of CRABP1 expression and disease-free survival in serous and clear cell adenocarcinoma patients

Complete remission was achieved by surgery (with or without chemotherapy) in 36 cases (90%) of 40 serous adenocarcinomas and 23 cases (88%) of 26 clear cell adenocarcinomas. Disease-free survival was next analyzed in these 59 complete-remission cases. During the median follow-up period of 46 months (4–133 months), 20 cases with reduced expression of CRABP1 exhibited worse disease-free survival, compared to the other 35 cases whose CRABP1 expression was maintained, with statistical significance ($P = 0.024$ by the Kaplan–Meier method) (Fig. 4). Recurrence occurred in only 9 cases (26%) among 35 cases with maintained CRABP1 expression; however, it occurred in 11 cases (46%) among 24 cases with reduced CRABP1 expression. Statistically significant differences were not demonstrated in our analysis of each of serous and clear cell adenocarcinomas. The duration from recurrence to death did not demonstrate significant difference between histological sub-types, nor between maintained and reduced CRABP1 expression (data not shown).

Univariate and multivariate cox proportional hazards analysis for effect of alteration of CRABP1 expression on overall survival

Univariate analysis demonstrated that advanced stage (stage II, III, and IV) and reduced expression of CRABP1
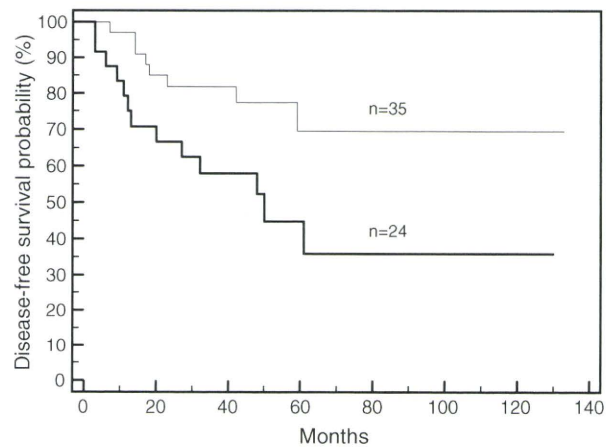
effected overall survival of the diseases. In order to further support our belief that reduced expression of CRABP1 independently relates to the prognosis of ovarian serous and clear cell adenocarcinomas, multivariate Cox proportional hazards analysis was performed (Table 1). Clear cell type, advanced stage (stage II, III, and IV), and reduced expression of CRABP1 were shown to be independent factors for overall survival of the diseases. Especially, the adjusted hazard ratio (HR) of reduced expression of CRABP1 was 8.189 (95% CI, 2.186–30.672, $P = 0.0019$).

Discussion

Although the ovarian cancer incidence rate has been slowly falling over the past 20 years, it still accounts for 3–5% of all cancers in women. In addition to its human toll, it exacts a huge financial burden; in the US about \$2.2 billion is spent annually on ovarian cancer treatments (in 2004 dollars) (www.cancer.org/docroot/cri/content/cri_2_4_1x_what_are_the_key_statistics_for_ovarian_cancer_33.asp, 2009).

Among the histological sub-types of ovarian cancers, the serous adenocarcinoma is the most frequent type. Although rarer, most serous adenocarcinoma cases have already disseminated to other pelvic tissues and the peritoneum, or have metastasized to regional lymph nodes, at the time of initial diagnosis and are thus extremely more difficult to cure (DiSaia and Creasman 2002). Cytoreductive surgery

Table 1 Multivariate cox proportional hazards analysis for effect of alteration of CRABP1 expression on overall survival

Variable	Univariate analysis	Multivariate analysis		
	P value	Adjusted HR	95% CI	P value
Age (years)	0.83			0.67
<60		1		
≥60		1.259	0.436–3.631	
Histology	0.89			0.019
Serous		1		
Clear cell		4.598	1.299–16.275	
Initial stage	0.049			0.0055
I		1		
II/III/IV		7.806	1.844–33.044	
CRABP1 expression	0.0041			0.0019
Maintained		1		
Reduced		8.189	2.186–30.672	

The adjusted HR of reduced expression of CRABP1 was 8.189 (95% CI, 2.186–30.672), compared to maintained expression CRABP1, showing statistical significance (P value was 0.0019)

HR hazard ratio

followed by combination chemotherapy using taxane and platinum improves the prognosis of some ovarian cancer patients; however, there remain serious problems in the management of this disease where additional prognostic markers would be extremely helpful.

Clear cell adenocarcinoma, which represents only 5–10% of all the ovarian carcinomas in the Caucasian-dominated United States cases (DiSaia and Creasman 2002; Kurman 1994; Berek 2002) accounts for a much larger percentage (23%) of ovarian carcinoma cases in Japan (Our unpublished data). Clear cell carcinomas are unusually resistant to standard platinum-based chemotherapy, and their prognosis is extremely poor (Sugiyama et al. 2000). Thus, clinical and basic research targeting understanding serous and clear cell adenocarcinomas has been a high priority.

In our present study, expression of CRABP1 protein as a potential prognostic marker was investigated in 100 ovarian carcinomas of various histological sub-types, including serous and clear cell adenocarcinomas. CRABP1 was demonstrated to be a useful factor for predicting the prognosis of serous and clear cell adenocarcinomas. Overall survival was significantly poorer in the cases with reduced CRABP1 expression, in both serous and clear cell adenocarcinomas, compared to the cases whose CRABP1 expression was maintained ($P = 0.0073$ and $P = 0.049$, respectively). Also, the disease-free survival of the serous and clear cell carcinoma cases with reduced CRABP1 expression was significantly poorer, compared to the cases whose CRABP1 expression was maintained ($P = 0.024$). The fact that distribution of the tumor stages was not different between the cases with reduced CRABP1 expression and those with maintained CRABP1 expression implies that the reduction of CRABP1 expression predicted the prognosis irrespective of the stage of the disease. Multivariate analysis showed

that reduced expression of CRABP1 was a significantly important prognostic factor.

Reduced expression of CRABP1 was observed by immunohistochemical analysis in 31% of 86 ovarian cancer cases. Especially in serous and clear cell adenocarcinomas, reduced expression of CRABP1 was detected in 50 and 38% of cases, respectively. However, reduced expression was found in endometrioid and mucinous adenocarcinomas in only 2 (8%) of 24 and 1 (10%) of 10 cases, respectively.

So, how it is that loss of CRABP1 expression would have an effect on ovarian tumor phenotype? Evidence that hypermethylation of the *crabp1* gene was more than just a reflection of the hypermethylation CIMP phenotype was recently shown when restoration of CRABP1 expression in esophageal carcinoma cells (ESCC) lacking the protein reduced cell growth by inducing arrest at G_0–G_1, whereas knockdown of the gene in cells expressing CRABP1 promoted cell growth (Tanaka et al. 2007). Among 113 primary ESCC tumors, the absence of immuno-reactive CRABP1 was significantly associated with de-differentiation of cancer cells and with distant lymph-node metastases in the patients (Tanaka et al. 2007). These results indicate that CRABP1 appears to have an active tumor-suppressor function in esophageal epithelium, and its epigenetic silencing may play a pivotal role during esophageal carcinogenesis. In our ovarian cancer cases, CRABP1 expression was associated with histological grade of the tumor, but not with lymph-node metastasis.

The results of Wu et al. implied that *crabp1* expression was impaired specifically in clear cell adenocarcinoma of the ovary by promoter CpG island hypermethylation. This hinted at a phenotypic preference in ovarian tumors (Wu et al. 2007; Barton et al. 2008). This also suggested that other mechanisms, including loss of heterozygosity, mutations, and post-transcriptional and post-translational alterations

that have not yet been reported, might be involved in the reduction of *crabp1* expression in other sub-types of ovarian tumors, such as serous adenocarcinomas.

CRABP1 was shown to be expressed selectively in the mesenchymal tissues at the junction of the epithelium and the mesenchyme, functioning in mesenchymal/epithelial interaction (Bhasin et al. 2003). The prognostic significance of epithelial-mesenchymal transition (EMT) was recently demonstrated in various carcinomas, including ovarian tumors (Bagnato and Rosanò 2007; Smit et al. 2009; Vasko et al. 2007; Soltermann et al. 2008; Al-Saad et al. 2008; Shim et al. 2009). In our study, CRABP1 expression was observed in the cytoplasm of the normal epithelial cells of the ovarian surface, the fallopian tube, and the adenocarcinomas.

It is possible that de-differentiation of ovarian carcinoma cells is triggered by reduction of CRABP1 and may similarly represent an epithelial-mesenchymal transition, which results in the poor prognosis. Based on these findings, it is implied that CRABP1 may normally act to induce or maintain differentiation of ovarian cells and that reduction of CRABP1 expression may lead to a failure to differentiate or a de-differentiation of tumor cells of serous and clear cell adenocarcinoma of the ovary, resulting in an early recurrence and poor overall survival.

Altered expression of CRABP1 may present as a potential target for molecular therapy in serous adenocarcinoma, which is the most frequent histological ovarian tumor type, and also for clear cell carcinoma, which often exhibits chemo-resistance. It should be noted with caution that retinoids have been proposed to have such beneficial cancer-preventive functions that they were recently used in large scale human clinical trials to reduce lung cancer incidence in high-risk individuals. However, the obtained antagonistic clinical results of RA prophylactic treatments were in direct contradiction with the previous promising in vivo and in vitro studies (Poulain et al. 2009).

Recent studies have shown that the hypermethylation of specific marker genes, including *Igfbp-3*, 18S and 28S rDNA, can act as potential prognostic markers in ovarian carcinoma (Wiley et al. 2006; Chan et al. 2005). While CIMP-related hypermethylation can be rather indiscriminant, it is thought to contribute to tumor progression by silencing important tumor-suppressor genes. The relatively frequent loss of *crabp1* gene expression in the serous and clear cell sub-types of ovarian adenocarcinomas suggests it may be such a tumor-suppressor gene, and it is reasonable to assume that loss of expression is in part due to promoter hypermethylation, but awaits experiments to demonstrate this hypothesis.

In conclusion, we have demonstrated that reduction of CRABP1 expression was observed most specifically in serous and clear cell adenocarcinomas of the ovary. The present study is the first to demonstrate that the reduced expression of CRABP1 has a potential as a prognostic marker for ovarian cancers. Further study is necessary to clarify how CRABP1 protein expression was altered and how CRABP1 affects ovarian carcinoma cells.

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Original Article

Carboplatin and paclitaxel as an initial treatment in patients with stage IVb cervical cancer: a report of 7 cases and a review of the literature

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Objective: The aim of this study is to evaluate the efficacy of carboplatin-paclitaxel (TC) as an initial treatment in patients with the International Federation of Gynecology and Obstetrics (FIGO) stage IVb cervical cancer.
Methods: We retrospectively reviewed seven patients with stage IVb cervical cancer who have been primarily treated with TC. The activity and the toxicity were evaluated. Response rate was the main endpoint.
Results: Overall, the treatment of TC was well tolerated. The overall response rate was 71.4% (2 complete response, 3 partial response). Although grade 3-4 hematologic toxicities were observed in 3 out of 7 patients (42.8%), no patients experienced grade 3-4 non-hematologic toxicities. When we combined our present results with the previous reports, the overall response rate of TC is 63.6%.
Conclusion: TC is active and well tolerated in patients FIGO stage IVb cervical cancer. This combination may be considered as an initial treatment regimen in this patient population.

Key Words: Stage IVb, Cervical cancer, Carboplatin, Paclitaxel

INTRODUCTION

Patients with the International Federation of Gynecology and Obstetrics (FIGO) stage IVb cervical cancer have a dismal prognosis.[1,2] Systemic chemotherapy and individualized radiotherapy have been proposed as initial treatments for these patients.[3] On the basis of phase III clinical trials, cisplatin-containing combination chemotherapy; i.e., cisplatin plus paclitaxel (TP), has become the standard treatment for recurrent or advanced cervical cancer.[4] Although the combination of carboplatin-paclitaxel (TC) was demonstrated to be equally effective as and less toxic than TP in ovarian cancer,[5] information on the use of TC in patients with advanced cervical cancer is limited. We herein describe our experiences with 7 cases of stage IVb cervical cancer that were primarily treated with TC.

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MATERIALS AND METHODS

Permission to proceed with data acquisition and analysis was obtained from the Osaka University Hospital's institutional review board. Seven patients with stage IVb cervical cancer that were primarily treated with TC at the Osaka University Hospital from 2007 to 2009 were identified and retrospectively reviewed. For all patients, clinical data on the following characteristics were collected: initial stage, maximal tumor diameter, cell type, performance status, primary treatment, site of recurrent disease, disease free interval (DFI), chemotherapy regimen, response, and progression free survival (PFS). PFS was measured from the start of chemotherapy to the progression of disease. The maximal tumor diameter was measured three-dimensionally based on T2-weighted magnetic resonance imaging (MRI). The longest diameter was considered valid as the maximal tumor diameter. TC was administered on a monthly basis in all patients: Carboplatin at an area under the curve (AUC) of 5 given as a 1 hour infusion, and paclitaxel at 175 mg/m^2 given as a 3 hours infusion every 28 days. The response to treatment was assessed according to the Response Evaluation Criteria in Solid Tumors after every three cycles of each regimen. A complete response (CR) was defined as the disappearance of all target and non-target lesions and no new lesions being documented after two assessments that were at least 4 weeks apart. A partial response (PR) was defined as at least a 30% decrease in the sum

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of the longest dimension of the target lesions, which was also documented in two assessments that were at least 4 weeks apart. Progressive disease (PD) was defined as a 20% increase in the longest dimension of the sum of the target lesions or the development of new lesions. Stable disease (SD) implies that none of the above applies. Performance status (PS) was graded according to the Eastern Cooperative Oncology Group performance status criteria. Toxicity related to treatment was graded according to the NCI Common Terminology Criteria for Adverse Events, ver. 3.0.

RESULTS

The patient's characteristics and clinical data are summarized in Table 1. The median age at the time of treatment was 60. Five women had squamous cell carcinoma, and two had adenocarcinoma. All patients had stage IVb disease with metastasis to distant organs including the lungs, liver, peritoneal dissemination, para-aortic lymph node, or other distant lymph node. All patients received TC as an initial treatment.

TC was administered on a monthly basis in all patients: Carboplatin at an AUC of 5 given as a 1-h infusion and paclitaxel at 175 mg/m^2 given as a 3-h infusion every 28 days. The median courses of TC administered was 6 (range, 3 to 12). As predicted, the administration of TC was generally well tolerated without any significant delays or dose reduction. Although grade 3-4 hematologic toxicities were observed in 3 out of 7 patients (42.8%), no patients developed febrile neutropenia. No patients experienced grade 3-4 non-hematologic toxicities. As shown, 2 patients showed CR, 3 showed PR, 1 showed SD, and 1 demonstrated PD. The overall response rate was 71.4%. Two patients who had achieved clinical CR to chemotherapy were further examined, and proven to be with no cytological evidence of disease in the primary site.

Three out of 5 responders and 1 non-responder received salvage radiotherapy consisting of external beam radiotherapy and high dose rate-intracavitary brachytherapy with curative intent following treatment with TC. One complete responder refused to receive additional treatment such as radiotherapy after the initial chemotherapy. Because of the rapid progression of the systemic disease, two non-responders did not receive further treatment following the treatment with TC. At the time of this study, 5 out of 7 patients were alive, and 2 of these 3 had not suffered recurrence after a median follow-up period of 21 months.

DISCUSSION

Surgery and concurrent chemoradiotherapy (CCRT) have achieved significant success in the treatment of cervical cancer both in patients with early stage cervical cancer, and in those with locally advanced cervical cancer.[6] However, in patients with stage IVb disease, no standard treatment has been established. Although systemic chemotherapy and individualized radiotherapy have been proposed as initial treatments,[2] the patients given these regimens showed a poor prognosis with a reported 5-year survival of less than 10%.[7]

Since stage IVb cervical cancer is a systemic disease, theoretically, chemotherapy is required for these patients. Based on previous phase III clinical trials, cisplatin-containing combination chemotherapy; i.e., cisplatin plus paclitaxel or topotecan, had become the standard treatment for recurrent or advanced cervical cancer.[4,8] Subsequently, a Gynecologic Oncology Group (GOG) phase III clinical trial (protocol GOG 204) comparing the efficacy of four cisplatin-based combination chemotherapies including TP, cisplatin-topotecan, cisplatin-vinorelbine, and cisplatin-gemcitabine has been conducted. In this study, although not statistically significant, TP showed the most favorable clinical activity with regard to the response rate, progression free survival, and overall survival in patients with FIGO stage IVb, recurrent, and persistent cervical cancer.[9] A recent retrospective

Table 1. Synopsis of patients with stage IVb cervical cancer treated with paclitaxel-carboplatin

Case	Age (yr)	Perfor-mance status	Histol-ogy	Maximum tumor diameter (cm)	Site of metastasis	Pelvic sidewall fixation	Course of chemo-therapy	Res-ponse	Grade 3-4 toxicity	Sub-sequent treatment	PFS (mo)	Site of recurrence	Outcome
1	50	0	SCC	6	Liver, lung, spleen, peritoneum	No	4	SD	No	Non	3	Liver, lung, spleen, peritoneal disse-mination	DOD
2	74	0	SCC	7	PALN, lung	No	3	PR	Yes	RT	9	Lung	AWD
3	60	0	SCC	6	PALN, lung	No	3	PD	Yes	RT	0	PALN, lung	AWD
4	71	0	SCC	3	Lung	No	6	CR	No	Non	12	PALN	AWD
5	37	0	SCC	4	SCLN	No	6	PR	No	RT	20	No	AWOD
6	57	0	Adeno	3.5	Liver	No	6	PR	No	Non	8	Liver	DOD
7	70	0	Adeno	2	Peritoneum, PALN	No	12	CR	Yes	RT	30	No	AWOD

PFS: progression free survival, SCC: squamous cell carcinoma, SD: stable disease, DOD: died of disease, PALN: para-aortic lymph node, PR: partial response, RT: radiotherapy, AWD: alive with disease, PD: processive disease, CR: complete response, SCLN: supraclaviclar lumph node, Adeno: adenocarcinoma, AWOD: alive without disease.

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Table 2. Paclitaxel-carboplatin as an initial treatment in patients with stage IVb cervical cancer

Article	Target disease	Dose of chemotherapy	Total no. of patients	Stage IVb patients	Response		
					No. of CR	No. of PR	Rate (%)
Piver et al.[15] (1999)	R or A	Paclitaxel: 135 m^2 Carboplatin: 300 mg/m^2 Every 4 wk	3	2	0	1	50
Tinker et al.[16] (2005)	R or A	Paclitaxel: 175 m^2 Carboplatin: AUC 5-6 Every 4 wk	25	2	0	1	50
Moore et al.[17] (2007)	R or A	NA	48	5	NA	NA	NA
Current study (2010)	A	Paclitaxel: 175 m^2 Carboplatin: AUC 5 Every 4 wk	7	7	2	3	71.4
Evaluable patients				11	2	5	63.6

CR: complete response, PR: partial response, R: recurrent cervical cancer, A: advanced-stage cervical cancer, AUC: area under the curve, NA: not available.

review of four randomized phase III GOG clinical trials suggested that advanced or recurrent cervical cancer patients who had been previously treated with radiosensitizing-cisplatin showed poorer response to platinum-based chemotherapy than those who had not.[10] With the aim to investigate the effectiveness of non-platinum containing chemotherapy in the same population, GOG has recently initiated a phase III trial (protocol 240) comparing TP versus non-platinum doublet (paclitaxel- topotecan), with or without bevacizumab.[11]

Although the combination of TC was demonstrated to be equally effective as and less toxic than TP in ovarian cancer,[5] information on the use of TC in cervical cancer is limited. Except for several case reports, to the best of our knowledge, only six retrospective studies on the value of TC in 3-48 recurrent or advanced cervical cancer patients have been reported.[12-17] Since the vast majority of patients enrolled in these studies were treated for recurrence after either radiotherapy or surgical treatment, the effectiveness of TC against stage IV disease as an initial treatment is largely unknown. Of the six retrospective studies, the total number of patients treated for stage IVb disease as an initial treatment was 9. Of these, detailed information regarding the treatment outcome was only available for 4 patients (Table 2).

We have demonstrated that TC is effective in patients with stage IVb cervical cancer. Our overall response rate of 71.4% is very similar to our previously reported response rate of 67.9% in patients with recurrent cervical carcinoma after definitive radiotherapy.[12] Moreover, when we combined our present results with the previous reports, as shown in Table 2, the overall response rate in a total of 11 patients was 63.6%.

As predicted, TC was well tolerated. Although grade 3-4 hematologic toxicities were observed in 3 out of 7 patients, no patients developed febrile neutropenia. This favorable toxicity profile of TC demonstrated in the current study may have been, at least in part, due to the treatment schedule employed in our institution: TC was administrated every 28 days as this

dosing schedule had demonstrated significant clinical activity in patients with recurrent cervical cancer.[12] However, on the basis of the concept of dose-density, we believe that we should try every 21 days administration of TC, which may result in better treatment outcome.

Although this study was retrospective and involved a relatively small number of patients, given the advantages of patient convenience and tolerance as well as the significant activity shown, we believe that TC is a reasonable treatment option for patients with stage IVb cervical cancer. To address the clinical benefit of TC compared with TP in stage IVb, persistent, or recurrent cervical cancer, the Japan Clinical Oncology Group (JCOG) is currently conducting a randomized phase III trial of their JCOG 0505 protocol.[18] In addition, since this combination chemotherapy demonstrated significant effectiveness as an initial treatment, TC may also hold promise in a CCRT or neoadjuvant chemotherapy setting in patients with cervical cancer. To establish a more effective and less toxic treatment strategy for patients with advanced cervical cancer, further studies are needed.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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Expert Opinion

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Targeting annexin A4 to counteract chemoresistance in clear cell carcinoma of the ovary

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Importance of the field: Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological malignancies in Western countries. Among the four major histological subtypes of EOC, clear cell carcinoma (CCC) of the ovary is highly resistant to platinum-based chemotherapy and is consequently associated with poor patient prognosis in advanced stages.

Areas covered in this review: An overview of the clinical characteristics of ovarian CCC; the role of annexin family proteins in tumor development and progression; the role of annexin A4 in enhancing cellular drug resistance; recent studies linking annexin A4 overexpression to chemoresistance in tumors of ovarian CCC.

What the reader will gain: Insight into the emerging role for annexin A4 in enhancing chemoresistance in ovarian CCC.

Take home message: Annexin A4 enhances cancer cell chemoresistance and is overexpressed in tumors of patients with ovarian CCC. Targeting of annexin A4 may represent a future strategy to counteract resistance to chemotherapy in ovarian CCC.

Keywords: annexin A4, chemoresistance, clear cell carcinoma of the ovary, molecular targeted therapy

1. Introduction

Epithelial ovarian cancer (EOC) is a leading cause of cancer death from gynecological malignancies in Western Europe and the USA, with approximately 204,000 new cases and 125,000 deaths reported annually worldwide [1]. EOC is associated with high morbidity and over 70% of patients present with advanced FIGO (the International Federation of Gynecology and Obstetrics) stages at diagnosis [2,3]. The 5-year survival rates for advanced stages (FIGO stage III/IV) of EOC still remain low (approximately 30% of cases), while over 90% of cases with FIGO stage I (limited to the ovary) at diagnosis can be cured [4,5].

Clear cell carcinoma (CCC) of the ovary accounts for 4 – 12% of all epithelial ovarian cancers in Western Europe [6], while in Japan over 20% of patients with EOC are classified as having ovarian CCC [7-9]. Ovarian CCC was initially termed as mesonephroid in 1939 [10], and since 1973 it was defined by the WHO as a distinct histological entity characterized by clear cells growing in solid/tubular or glandular patterns as well as hobnail cells [11]. Ovarian CCC is distinguished by clinical characteristics distinct from other histological subtypes of EOC.

Firstly, several studies support the finding that ovarian CCC tended to present at significantly earlier stages. The proportion of stage I/II tumors ranged from 59 to 71% [6,8,12]. One of the reasons for the early detection was explained by the slow growing tumor behavior [13] and frequent presentation of the tumors as relatively large pelvic masses [8,14].

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Article highlights.

- Clear cell carcinoma (CCC) of the ovary displays strong resistance to platinum-based chemotherapy and is associated with poor prognosis for advanced stages. A novel therapeutic strategy is required.
- Annexin A4 confers chemoresistance in ovarian cancer cells.
- The suggested mechanisms of annexin-A4-mediated chemoresistance are i) reduction of intracellular platinum content and ii) enhancement of NF-κB transcriptional activity via interaction of annexin A4 with NF-κB.
- Targeting of annexin A4 may represent a future strategy to counteract chemoresistance of ovarian CCC.

This box summarizes key points contained in the article.

Secondly, it is recognized that ovarian CCC has a strong association with endometriosis [15,16]. Previous studies reported that 52 – 63% of malignancies arising in ovarian endometriotic cysts were clear cell or endometrioid adenocarcinoma, whereas 20 – 30% were serous subtype [17,18]. Endometriosis is generally identified in more than half (58 – 70%) of ovarian CCC tumors [19,20]. In ovarian CCC, the underlying environment presented by endometriosis is considered to promote carcinogenesis through iron-induced persistent oxidative stress and inflammation [15,21].

Thirdly, and of particular clinical importance, ovarian CCC exhibits strong resistance to platinum-based chemotherapy [6]. The response rate of chemotherapy for CCC is approximately 11.1% with platinum-based regimens and 22 – 56% with paclitaxel plus carboplatin [7,8,22,23]. Due to the chemoresistant nature of ovarian CCC, patients with ovarian CCC at advanced stages are associated with both poor prognosis and high mortality compared with other histological subtypes of EOC [14,19,24-27].

Promising regimens with favorable and stable response for ovarian CCC have remained lacking. To develop a novel effective treatment for ovarian CCC, the identification of proteins associated with chemoresistance and the elucidation of the molecular mechanisms of this process is required.

2. Prognosis in clear cell carcinoma of the ovary

Several clinical studies have investigated the prognosis of patients with ovarian CCC compared with patients with serous adenocarcinoma (SAC) of the ovary [6,8,14,24,25,28,29]. Kennedy et al. compared 29 cases of ovarian CCC to 305 cases of non-CCC and found no survival differences for FIGO stage I/II diseases [8]. Another study by Behbakht et al. also showed that the prognosis of ovarian CCC patients in FIGO stage I/II is not significantly different to that of ovarian SAC patients [14]. Jesnison et al. showed that the survival rates for ovarian CCC were consistently lower in each of the FIGO stages compared to ovarian SAC [19]. However, this study

failed to show the poorer prognosis of ovarian CCC with statistical significance due to the relatively small number of the cases examined (44 ovarian CCC patients and 55 ovarian SAC patients).

Using a larger cohort of patients (101 CCC versus 235 SAC patients), Sugiyama et al. showed that the survival rates for stage IC patients with ovarian CCC were lower than those patients with ovarian SAC, and the 3-year and 5-year survival rates for stage III ovarian CCC patients were significantly lower compared with stage III SAC patients [6]. This study also revealed that overall response to platinum-based chemotherapy for ovarian CCC with measurable residual tumors after surgery was significantly lower than that for ovarian SAC (11.1 versus 72.5%) [6].

Recently, large population studies on prognosis of ovarian CCC at advanced stages compared with the other EOC subtypes have been reported. Chan et al. performed a study comparing 1411 cases of ovarian CCC to over 20,000 cases of other histological subtypes of EOC, and found that after adjusting for stage of disease, ovarian CCC had poorer prognosis (worse 5-year survival) compared with ovarian SAC: 85.3 versus 86.4% for stage I ($p = 0.985$), 60.3 versus 66.4% for stage II ($p = 0.314$), 31.5 versus 35.0% for stage III ($p < 0.001$), and 17.5 versus 22.2% for stage IV ($p < 0.001$), respectively [28]. Winter III et al. examined 1895 patients with FIGO stage III EOC who had undergone primary surgery followed by six cycles of intravenous platinum/paclitaxel treatment. Ovarian CCC was associated with a worse progression-free survival compared with ovarian SAC (11.4 months versus 16.9 months; $p = 0.04$) and overall survival compared with ovarian SAC (24.0 months versus 45.1 months; $p < 0.001$) [26].

In consideration of recent data obtained from large-cohort clinical studies [6,26,28], there is now an emerging consensus among gynecologic oncologists that ovarian CCC has lower sensitivity to platinum-based chemotherapy and shows poorer prognosis, compared with ovarian SAC at advanced stages.

Of the known mechanisms of chemoresistance in cancer cells, it has been suggested that chemoresistance in ovarian CCC may be in part attributed to low cell proliferation, assuming that cytotoxic drugs are primarily effective against vigorously proliferating cells [13,30]. Itamochi et al. investigated the proliferation activity and cisplatin sensitivity of 11 ovarian CCC and 5 ovarian SAC cell lines, and found that the doubling time for CCC cells was significantly longer than that for SAC cells (61.4 versus 29.8 h) [13]. Immunohistochemical analysis revealed that the expression of the cell cycle regulatory protein cyclin A was significantly lower in ovarian CCC compared with other ovarian cancers, which also suggests that ovarian CCC has low cell proliferation activity [31].

Oishi et al. demonstrated that overexpression of galectin-3 in ovarian CCC might contribute to its lower cell proliferation and lead to resistance to platinum-based chemotherapy [32]. Galectin-3 is a β-galactoside-binding protein that regulates numerous biological functions, including cell

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cycle [33,34], cell adhesion [35,36], cell proliferation [37], cell growth and anti-apoptotic activity [38]. Various studies have suggested that galectin-3 is also involved in tumor progression and chemoresistance of human malignancies of a wide variety of organs, including the ovary [32]. Oishi *et al.* performed cDNA microarray analysis and revealed that galectin-3 gene expression of ovarian CCC cell lines was over threefold higher than that of ovarian SAC cell lines and demonstrated that knockdown of galectin-3 using siRNA increased the S-phase fraction in CCC cells.

This strong resistance to chemotherapy in ovarian CCC is one of the major reasons for its poor prognosis in advanced disease, which has prompted a need to investigate the mechanism of chemoresistance in ovarian CCC as well as to find optimal chemotherapy regimens that give better response rates than a combined paclitaxel (taxol)–carboplatin (TC) regimen.

Currently, effective anti-cancer drug regimens for the treatment of ovarian CCC are lacking [39]. Thus, clarification of the molecular mechanisms of chemoresistance and the development of new therapies including molecular-targeted treatment for ovarian CCC can be expected to improve patient prognosis in this disease.

3. Candidate molecules associated with chemoresistance in ovarian CCC

To identify candidate molecules associated with chemoresistance in ovarian CCC, various research groups have investigated this issue by DNA microarray analysis and quantitative proteomic analysis, and have identified genes or proteins that are differentially expressed in ovarian CCC compared with other histological subtypes of EOC [40-45].

Among these studies, increased expression of annexin A4 in various ovarian CCC cell lines and tumors was revealed using quantitative proteomic approaches [43,44] and cDNA microarray [45]. Kim *et al.* demonstrated enhanced expression of annexin A4 in ovarian CCC cell lines compared with an ovarian SAC cell line using two dimensional fluorescence difference gel electrophoresis (2D-DIGE) [43]. In this study annexin A4 was also shown to be significantly overexpressed in ovarian CCC tumors compared with ovarian SAC tumors and endometrioid adenocarcinomas of the ovary by immunohistochemical analysis. These findings are also supported by other studies, in which elevated expression of annexin A4 was detected in ovarian CCC compared with ovarian SAC or ovarian mucinous adenocarcinoma cell lines, using cDNA microarray analysis and 2D-DIGE analysis, respectively [44,45].

Various studies have identified molecular markers in ovarian CCC other than annexin A4. Tsuchiya *et al.* reported specific expression of hepatocyte nuclear factor-1β (HNF-1β) [40]. HNF-1β is a transcription activator and regulates the promoters or enhancers of genes that are specifically expressed in liver, such as albumin and α-fetoprotein. HNF-1β is also known to regulate glucose homeostasis and HNF-1β mutations can cause early-onset diabetes mellitus.

Tsuchiya *et al.* found that the reduction of HNF-1β by RNA interference (RNAi)-induced apoptosis in ovarian CCC cell lines (TOV-21G and JHOC-5 cell lines), suggesting that HNF-1β expression was essential for the survival of CCC cells [40]. The HNF-1β-dependent pathway of ovarian CCC is considered to be associated with apoptosis and glycogen synthesis, and thus may confer resistance of ovarian CCC to anti-cancer drugs [40,46].

Yamaguchi *et al.* demonstrated that ovarian CCC gene signature contains a large (over 60 genes) gene network, including *HNF-1β, cyclin-dependent kinase inhibitor 1A (p21), HIF-1α, IL-6* and *STAT3*. They also identified the *Annexin A4* gene as being highly expressed in ovarian CCC and suggested that *Annexin A4* expression may facilitate chemoresistance [47]. HNF-1β has been reported to induce expression of annexin A4 [46], suggesting that annexin A4 mediates the observed chemoresistance functions attributed to HNF-1β.

4. Functions of annexin A4 and relevance to cancer

Annexins are a super-family of calcium-regulated, negatively charged phospholipid-membrane-binding proteins [48,49] and are classified into five groups (A – E). Annexins in group A are the human and vertebrate orthologues and group B, C, D and E contain annexins of non-vertebrate metazoans, fungi and moulds, plants and protists, respectively [50]. In vertebrates, 12 annexin subfamilies (A1 – A11 and A13) have been identified to date, each displaying tissue type-specific expression patterns. Annexins are composed of two main structural domains; the variable N-terminal region and the conserved C-terminus. They contain a characteristic motif – the annexin repeat, which is 70 amino acid residues in length. Four annexin repeats packed into an α-helical disk are contained within the C-terminal polypeptide core, which feature Ca^{2+} binding sites [49]. In annexins, the Ca^{2+}-binding sites are highly conserved and this indicates that Ca^{2+} has a central role in annexin biology, in particular, in the regulation of their intracellular activities [51]. While all annexins share this core region, the N-terminal region varies widely in sequence and length for individual subfamilies.

Annexin proteins have diverse cellular functions, including vesicle trafficking, ion transport, cell signaling, cell division and apoptosis [49,52-55]. Annexin proteins are also known to be associated with various human diseases, such as cancer (**Table 1**), cardiovascular disease, brain ischemia and Alzheimer's disease [50,56,57].

Annexin A4 is a member of the annexin protein family, which binds phospholipids in a Ca^{2+}-dependent manner and is known to self-associate on membrane surfaces and aggregate phospholipid membranes [51,58]. The overall structure of annexin A4 was first crystallized and determined by Zanotti *et al.* [59] and is highly homologous to the other annexin subfamilies, especially annexin A5. These two structurally similar annexins (annexin A4 and A5) form

Table 1. The role of annexins in tumorigenesis.

Annexin	Chromosomal location	Involvement in tumorigenesis
Annexin A1	9q12 – q21.2	Cell differentiation, proliferation, tumor invasion
Annexin A2	15q21 – q22	Tumor invasion, metastasis, angiogenesis
Annexin A3	4q13 – q22	Angiogenesis, chemoresistance
Annexin A4	2q13	Chemoresistance, tumor invasion, metastasis
Annexin A5	4q28 – q32	Apoptosis
Annexin A6	5q32 – q34	Tumor suppressor, Ras inactivation
Annexin A7	10q21.1 – q21.2	Tumor suppressor
Annexin A8	10q11.22	Tumorigenesis of pancreas
Annexin A11	10q23	Chemoresistance

Information from [50].

trigonal crystals in ordered two-dimensional arrays on membrane surfaces [51,59-61] and this trimeric complex is considered to be a functional unit (particularly well studied in annexin A5) [62-65].

Annexin A4, along with A2 and A6, has also been demonstrated to modulate plasma-mambrane Cl⁻ channels [66-68]. By western blotting analysis, Sohma *et al.* showed significantly increased expression of annexin A4 in the hippocampi of postmortem brains of alcoholic patients compared with controls [69]. In addition to this finding, the augmentation of annexin A4 expression following ethanol exposure was also demonstrated in cultured cells (rat glioma C6 cells and human adenocarcinoma A549 cells), suggesting that the expression of annexin A4 is induced by specific cellular stress [70].

In view of the increased expression of annexin A4 in ovarian CCC, the reported correlations between expression of annexin A4 and cancer are of interest. A series of studies has demonstrated that annexin A4 expression was increased in invasive renal clear cell carcinoma, colorectal cancer, pancreatic cancer and ovarian CCC (**Table 2**) [43,45,56,57,71,72]. Conversely, it has been shown that downregulated expression of annexin A4 might be involved in the progression of prostate cancer [73]. However, in this study, other annexin family proteins (annexin A1, A2, A7 and A11) were also downregulated concomitant with decreased expression of annexin A4, thus the progression of prostate cancer might be induced by proteins other than annexin A4.

Previous studies have demonstrated an association of annexin A4 with tumor dissemination and metastasis in various human cancers [57,71]. Duncan *et al.* demonstrated that enhanced expression of annexin A4 was significantly correlated with poor prognosis and lymph node metastasis in colorectal cancer [57].

Furthermore, Zimmermann *et al.* demonstrated that annexin A4 promotes tumor cell migration *in vitro,* using annexin A4-overexpressing MCF-7 breast cancer cells [71]. Zimmermann *et al.* also revealed that annexin A4 distinctly locates on the inner surface of the cell membrane in tumor cells and this was linked to loss of cell-to-cell adhesion and increased tumor cell dissemination [71]. Taken together, these studies thus highlight a role for annexin A4 in the development and progression of various cancers. Clearly further studies relating annexin A4 knockdown with chemosensitivity will be of particular interest.

5. Annexin A4-mediated chemoresistance

5.1 Inhibition of intracellular platinum accumulation

Increased expression of annexin A4 in ovarian CCC has prompted an investigation of the correlation between annexin A4 and chemoresistance in this disease. Our own studies using annexin A4-transfectant ovarian SAC (non-CCC) cells demonstrated that overexpression of annexin A4 confers chemoresistance to carboplatin in ovarian cancer cells [43]. A decrease in intracellular platinum content after carboplatin treatment in annexin A4-transfected ovarian SAC cells compared with control cells was demonstrated, suggesting that annexin A4 confers chemoresistance in part by enhancing drug efflux [43]. To date, this is the only published study concerning the mechanisms by which annexin A4 enhances chemoresistance in ovarian CCC. Conceivably, other mechanisms by which annexin A4 confers chemoresistance may exist. Thus, further studies of annexin A4 functions in this disease are required.

In agreement with these findings in ovarian cancer cells, associations of annexin A4 with chemoresistance in other human cancer cells has also been reported. Han *et al.* found that annexin A4 is overexpressed in a paclitaxel-resistant lung cancer cell line H460/T800 and it is among one of the first proteins to be induced in cells in response to cytotoxic stress, such as paclitaxel treatment [74]. They also demonstrated that transfection of annexin A4 cDNA into 293T cells results in a threefold higher resistance against paclitaxel, although the detailed mechanisms of paclitaxel resistance was not explored.

In our own preliminary studies (unpublished data), knockdown of annexin A4 using siRNA transfection was performed in uterine endometrial cancer, and significant chemosensitization was confirmed against carboplatin, paclitaxel and adriamycin. Taken together, these results suggest that annexin A4 may represent a novel target of chemoresistance in ovarian CCC.

Table 2. Expression of annexin A4 in cancer.

Cancer type	Histological type	Expression of annexin A4	Ref.
Colorectal	Adenocarcinoma	↑(associated with tumor dissemination and metastasis)	[56,57]
Renal	Clear cell adenocarcinoma	↑(associated with tumor dissemination and metastasis)	[71]
Pancreatic	Intra-epithelial neoplasia (precursor of ductal adenocarcinoma)	↑(associated with tumor progression)	[72]
Ovary	Clear cell Adenocarcinoma	↑(clear cell adenocarcinoma > serous adenocarainoma)	[43,45]
Prostate	Adenocarcinoma	↓(hormone refractory tumors < hormone sensitive tumors)	[73]

5.2 Modulation of NF-κB transcriptional activity

The NF-κB transcription factor was initially characterized as a critical component of innate and adaptive immune responses. Subsequently NF-κB was demonstrated to play key roles in cancer development and progression and in chemoresistance in a wide variety of cancer cells [75,76]. Thus NF-κB has emerged as an attractive target for new chemopreventive and chemotherapeutic strategies in cancer.

A recent study demonstrated that annexin A4 enhances NF-κB transcriptional activity in a calcium-dependent manner and induces resistance to apoptotic stimulation by the genotoxic drug etoposide. Originally, overexpression of annexin A4 in rat glioma C6 cells enhanced ethanol-induced cell lesions, accompanied by NF-κB activation [77]. Subsequently, enhanced NF-κB transcriptional activity via interaction of NF-κB with annexin A4 was demonstrated [78]. Jeon et al. first demonstrated that annexin A4 differentially modulates NF-κB transcriptional activity through interaction with the p50 subunit in a Ca^{2+}-dependent manner [78]. Using confocal microscopic and subcellular fractionation analyses, they investigated the changes of localization of p50, p65 and annexin A4 after treatment with etoposide. These results indicate that annexin A4 co-translocates to the nucleus with p50 subunit after treatment with the genotoxic drug etoposide and that high intracellular Ca^{2+} levels are critical for this co-translocation, while overexpression of annexin A4 did not affect interactions among p50, p65, and Iκ-B.

Draeger et al. demonstrated that annexins are diffusely distributed throughout the cytosol at low concentration of intracellular Ca^{2+}, while they translocate to cellular membranes after stimulation and intracellular Ca^{2+} elevation [79]. They also demonstrated that annexins have diverse Ca^{2+} sensitivities of lipid binding and the individual intracellular Ca^{2+} concentrations required for this association differ greatly. For example, the required level of free calcium for the translocation from the cytosol or the nucleus to the plasma membrane of some members of the annexin family are as follows: around 300 nM for annexin A2, 700 nM for annexin A6, 1 μM for annexin A4, and 1.5 μM for annexin A1 [79,80].

A role for modulation of intracellular calcium signaling in the mechanism of action of anti-mitotic drugs has been reported. Paclitaxel has been shown to induce a rapid cytosolic calcium response in which calcium is released from an intracellular calcium store [81]. Thus, it is conceivable that the apoptotic action of paclitaxel on ovarian CCC cells may be blocked via induction of cytosolic calcium, which in turn enhances NF-κB transcriptional activity in combination with overexpressed annexin A4. However, while a role for NF-κB in enhancing cancer cell chemoresistance has been clearly established, a synergistic role with annexin A4 in chemoresistance of ovarian CCC represents an interesting subject for future investigation. Thus, targeting of annexin A4 in ovarian CCC may have dual inhibitory effects via NF-κB, on both tumor development and tumor chemoresistance.

The suggested mechanisms of annexin A4-associated chemoresistance at present are more likely to be non-specific to a range of anti-cancer drugs rather than drug-specific. Therefore, inhibition of annexin A4 activity can be expected to sensitize cancer cells against various anti-cancer drugs, while further investigation is warranted in terms of the regulatory pathways involved in annexin A4 expression. Currently however, chemical inhibitors of annexin A4 expression/function are lacking.

6. Conclusion

Annexin A4 is overexpressed in ovarian CCC tumors and cell lines [43], and annexin A4 overexpression is associated with chemoresistance to anti-cancer drugs (carboplatin/paclitaxel/etoposide) [43,74,78]. Our own studies have suggested that annexin A4 confers chemoresistance in ovarian cancer cells in part by promoting cellular drug efflux [43]. Furthermore, a synergistic role for annexin A4 in enhancing the transcriptional activity of the NF-κB transcription factor involved in cancer development and chemoresistance has been demonstrated. Taken together, these studies highlight an emerging role for annexin A4 as a putative target of cancer development and cellular chemoresistance in ovarian CCC. Future studies are required to establish the efficacy of targeting annexin A4 as a future therapeutic strategy in patients with ovarian CCC.

7. Expert opinion

Among the four major histological subtypes of EOC, ovarian CCC is highly chemoresistant to platinum-based chemotherapy and the development of a novel treatment for ovarian CCC is of clinical importance. Increasing lines of evidence

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indicate that annexin A4 confers chemoresistance in various human cancer cells including ovarian CCC and potential mechanisms of annexin-A4-mediated chemoresistance have been reported. For these mechanisms, decreased intracellular platinum content in annexin-A4-overexpressing cells compared with those in control cells after carboplatin treatment [43], along with Ca^{2+}-dependent changes in cellular distribution of annexin A4 [79,80] and the association with NF-κB signaling pathways [78], have been considered to be involved in annexin A4-mediated chemoresistance.

Similar to the findings for annexin A4, annexin A3 was reported to be significantly overexpressed in platinum-resistant ovarian SAC cell lines and the intracellular platinum concentration and platinum–DNA binding were significantly lower in annexin-A3-overexpressing cells than those in control cells after cisplatin/carboplatin treatment [82]. Moreover, the lower cisplatin concentration in annexin-A3-overexpressing cells was accompanied by reduced induction of p53, indicating that upregulation of annexin A3 in ovarian SAC cell lines resulted in the decrease of the cisplatin-induced p53. Since in our own previous study, annexin A4 also inhibited platinum accumulation in ovarian cancer cells [43], it will be of interest to investigate if annexin A4 modulates intracellular levels of p53 in ovarian CCC.

Since in our own preliminary studies (unpublished) siRNA against annexin A4 induced chemosensitization toward carboplatin, paclitaxel and adriamycin in cancer cell lines, small-molecules that can effectively inhibit annexin A4 activity or decrease expression levels of annexin A4 may enhance sensitivity to chemotherapy, thereby decreasing the optimal doses of anti-cancer drugs. Interestingly, enforced expression of Annxin A4 in ovarian SAC induced resistance to carboplatin, suggesting that annexin A4 inhibitory therapeutics might be also adaptable to ovarian SAC tumors that are refractory or resistant to paclitaxel/carboplatin chemotherapy. Consequently, this therapeutic strategy may not be limited to ovarian CCC but also adaptable to other histological subtypes of EOC, which feature some resistance to platinum-based chemotherapy.

Clear cell adenocarcinoma is observed in a variety of organs including the ovary, the uterine endometrium, as well as the kidney and its histopathological manifestations (large cuboidal, hobnailed or flattened epithelial cells containing abundant clear cytoplasm lining tubules and cysts, and growing in solid/tubular or glandular patterns) [83] are well conserved. It has also been demonstrated that gene expression patterns of clear cell carcinomas arising in different organs are remarkably similar regardless of their origin [42] and moreover, several significant common signaling pathways observed in ovarian CCC resemble those of renal CCC [39]. Taking this into consideration, it is quite reasonable to apply treatments for renal CCC to ovarian CCC and vice versa.

Further clinical studies are required to establish annexin A4 as a biomarker of chemoresistance in patients with ovarian CCC. However, in consideration of the emerging role of annexin A4 in cancer cell chemoresistance, further studies (particularly annexin A4 knockdown) to confirm the therapeutic efficacy of targeting annexin A4 in ovarian CCC are warranted.

Declaration of interest

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Early reduction of glucose uptake after cisplatin treatment is a marker of cisplatin sensitivity in ovarian cancer

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Cisplatin is an effective chemotherapeutic agent for ovarian cancer, but the sensitivity of cancers differs in individual cases. Because cisplatin is reported to suppress glucose uptake, we investigated the correlation between glucose uptake and sensitivity to the drug. A fluorescent derivative of D-glucose, 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl) amino]-2-deoxyglucose), was used to evaluate glucose uptake. Two ovarian cancer cell lines, SKOV-3 as a relatively resistant line and OVCAR-3 as a relatively sensitive line, were analyzed. Both cell lines had a decreased number of cells accompanied by cell death 24 h after cisplatin treatment, but not at 3 h. In contrast, glucose uptake was decreased 3 h after high-dose cisplatin treatment, which correlated with the sensitivity to the drug at 24 h. The protein levels of glucose transporter 1 (GLUT1) did not change with cisplatin treatment. In contrast, the membrane localization of GLUT1 disappeared after cisplatin treatment. Other cisplatin-resistant cell lines did not show an early decrease in glucose uptake after cisplatin treatment. The early decrease in glucose uptake and later cell death also correlated in cultured cancer cells from ovarian cancer patients. Thus, the decrease in glucose uptake at an early time point after high dose cisplatin treatment reflected cisplatin chemosensitivity in ovarian cancer cells. Measuring glucose uptake might be useful as a rapid evaluation of cisplatin chemosensitivity in ovarian cancer patients. (*Cancer Sci* 2010; 101: 2171–2178)

The standard treatment for advanced ovarian cancer is surgery followed by chemotherapy.[1] First-line chemotherapy is the combination of a platinum-based agent, such as cisplatin or carboplatin, with paclitaxel or docetaxel. Although the response rate of advanced disease to first line chemotherapy is generally 70–80%, it is not effective for the remaining patients.[2–4] Even if the first treatment is effective, approximately 70% of patients relapse within 5 years.[4] In the case of a relapse, the response rate is better for patients who relapsed more than 6 months after the last platinum treatment, but the response rate is approximately 30–60%,[5] with the rest of the cases being platinum-resistant. The response rate to chemotherapy is currently determined by conventional imaging, such as computed tomography (CT) or MRI, after completing several courses of chemotherapy.[6] The development of an assay to predict chemosensitivity before the completion of treatment is imperative to avoiding ineffective chemotherapy.

In the early 1920s, Warburg *et al.*[7] reported that cancer cells exhibit an increased rate of glycolysis. Cancer cells tend to use glycolysis for energy production much more than oxidative phosphorylation, even in the presence of sufficient oxygen. In addition, malignant tumors have a wide hypoxic region where

glycolysis is more effective than oxidative phosphorylation.[8] Thus, glucose uptake is increased in cancer cells, and an increased expression of glucose transporters, especially GLUT1, is frequently observed in many types of cancers.[9] Using this characteristic of cancer cells, 2-fluoro-2-deoxy-D-glucose-positron emission tomography (FDG-PET) is widely used in the clinical diagnosis of cancer.[10] FDG-PET is reportedly also useful for predicting the response to chemotherapy,[11,12] including that of gynecological malignancies.[13–15] On the cellular level, various chemotherapeutic drugs suppress glucose uptake in cell lines, including ovarian cancer,[16] hematopoietic precursor cells,[17] gastrointestinal stromal tumor (GIST),[18] breast cancer,[19,20] gastric cancer[21] and mesothelioma.[22]

Here, we report that glucose uptake decreases over a short period of time after cisplatin treatment in not only ovarian cancer cell lines, but also primary cultured cells derived from patient specimens. The decrease in glucose uptake correlated with drug sensitivity. We propose monitoring the glucose uptake of cultured cancer cells after cisplatin treatment as a novel chemosensitivity assay.

Materials and Methods

Cell lines and cell culture. A human ovarian cancer cell line, SKOV-3, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Another ovarian cancer cell line, OVCAR-3, and uterine corpus cancer cell line, SAWANO, were purchased from RIKEN Cell Bank (Tsukuba, Japan). SKOV-3 was maintained in DMEM/F12 with 10% fetus bovine serum (FBS), OVCAR-3 in RPMI 1640 with 10% FBS, and SAWANO in minimum essential medium (MEM) with 15% FBS, all with 100 units/mL penicillin and 100 μg/mL streptomycin.

Establishment of cisplatin-resistant cells. Cisplatin-resistant cells were developed by continuous exposure to cisplatin, as described previously.[23,24] Briefly, SKOV-3 cells were initially cultured with 2 μg/mL cisplatin, and the dose was gradually increased to 3 μg/mL within 3 weeks. After switching the culture medium several times, 1 week without cisplatin and another with cisplatin, the cells began to grow in the presence of 3 μg/mL cisplatin. The cells are referred to as CisR/SKOV-3 and were cultured in cisplatin-free medium for at least 1 week before use. The growth rate of CisR/SKOV-3 was approximately the same as that of parent SKOV-3 cells (data not shown).

Reagents. The 1 mg/mL stock solution of cisplatin (Sigma-Aldrich, St Louis, MO, USA) was prepared by diluting powder

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cisplatin with distilled water before use. 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl) amino]-2-deoxyglucose (2-NBDG) was purchased from Peptide Institute (Ibaraki, Japan). The 2 mM stock solution of 2-NBDG was prepared in distilled water. Propidium iodide (PI) solution (1 mg/mL) was purchased from Invitrogen (Carlsbad, CA, USA).

Cell count and viability assay. For cell count, we applied trypan blue exclusion assay. An MTS assay (Promega, Madison, WI, USA) was performed according to the manufacturer's protocol. The details are in Appendix S1.

Apoptosis assay. The number of apoptotic cells was assessed by the Annexin V-FITC Apoptosis Detection Kit (Bio Vision, Mountain View, CA, USA). The details are in Appendix S1.

Glucose uptake assay. The glucose uptake assay was performed as previously described[25] with some modification. Cells were plated and treated with cisplatin as described in the live cell-count assay. After cisplatin treatment (3 or 24 h), the supernatant was discarded and the cells were washed with glucose-free medium. The medium was replaced with 2 mL of glucose-free DMEM and incubated at 37°C for 15 min. The 2-NBDG stock solution was added to each dish at a final concentration of 10 μM and incubated for 1 h at 37°C. The concentration of 2-NBDG was 50 μM for human samples. The incubation medium was removed and the cells washed twice with cold PBS. Cells were trypsinized and resuspended in cold PBS with PI at a final concentration of 2 μg/mL, and main-

tained at 4°C. The cells were analyzed by flow cytometry and dead cells labeled with PI eliminated from further analysis. A gate was set for the cells with a stronger signal than the frequency peak of 0 μg/mL cisplatin in the histogram and applied to cisplatin-treated cells. The 2-NBDG uptake was defined as the cell number within the gate divided by the total cell number.

Metabolic analysis. The glucose concentration was measured with Glucose C2 (Wako, Osaka, Japan). Oxygen tension was monitored using a Clark-type oxygen electrode system (Model 203; Instech Laboratories, Plymouth Meeting, PA, USA). Mito-Tracker Red CMxROS (Invitrogen) was used for measurement of the mitochondrial membrane potential. The details are in Appendix S1.

Real Time RT-PCR. Semi-quantitative RT-PCR was performed as previously described.[26] The details including primer sequence are in Appendix S1.

Immunocytochemistry. The rabbit anti-GLUT1 polyclonal antibody (Abcam, Cambridge, UK) was used as the primary antibody. Anti-rabbit IgG Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. The details are in Appendix S1.

Western blotting. Western blotting was performed as previously described.[27] The primary antibody against GLUT1 was the same that was used in the immunocytochemistry.

Clinical cancer samples. Surgically resected human ovarian cancer specimens were collected from four patients with
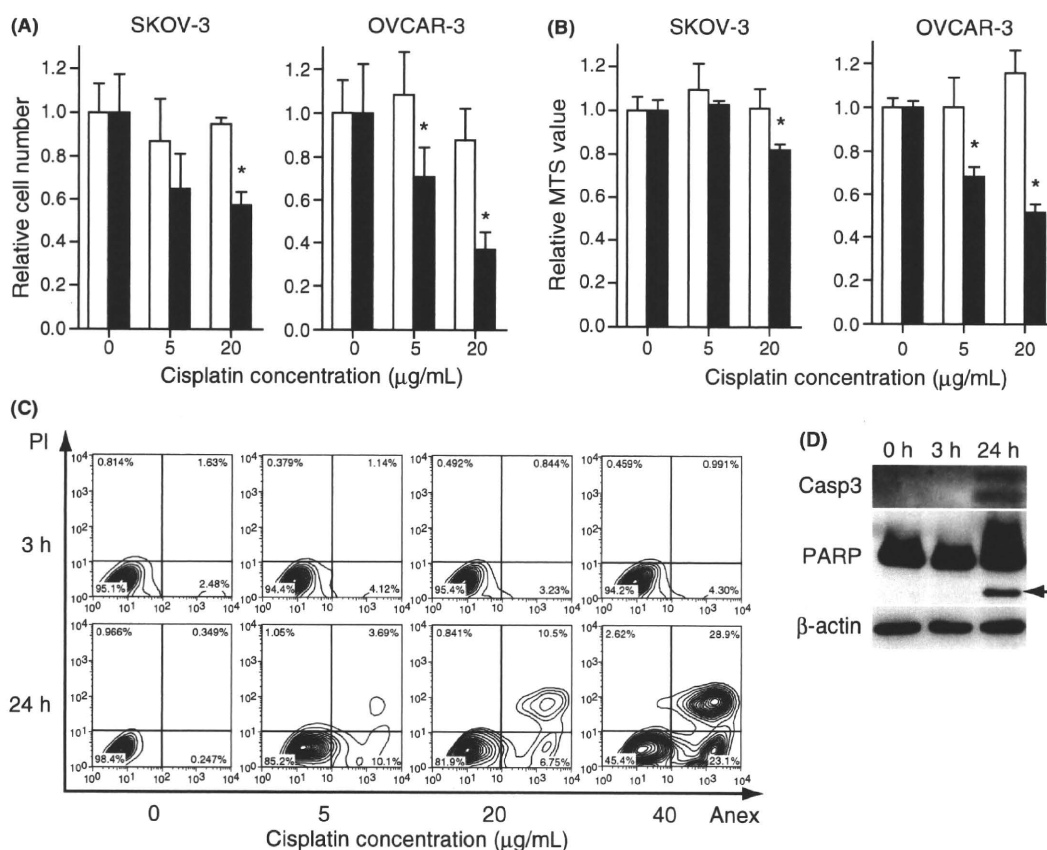

Fig. 1. SKOV-3 cells and OVCAR-3 cells were treated with cisplatin at the indicated doses. (A) Cell viability assessed by counting the number of viable cells or (B) the MTS assay. The values are relative to the untreated control group. The white bars indicate 3 h and black bars 24 h after cisplatin treatment. The results are the normalized mean values for three independent replicates. The experiments were repeated three times, and representative results are shown. *$P < 0.05$ compared with the control. (C) The apoptotic events for SKOV-3 were assessed by flow cytometry with annexin V/PI staining and (D) western blot for the cleaved form of caspase-3 and poly-ADP-ribose polymerase (PARP) (arrow). The cisplatin concentration was 20 μg/mL.

informed consent according to the ethics board of Osaka Medical Center for Cancer and Cardiovascular Diseases. Three of these specimens (#1, 2, 3) were transplanted into 4~7-week-old NOD/SCID mouse subcutaneously or into the renal capsule. The animal study was approved by the Animal Care Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. For sample #2, the patient specimen was used directly. After mechanical dissociation, the specimens were enzymatically digested at 37°C for 3 h with 2.8 U of Liberase Blendzyme 3 (Roche, Basel, Switzerland) in 20 mL of serum-free DMEM/F12. After filtering through a 40-μm cell strainer (BD), the cells were plated in DMEM/F12 with 10% FBS and cultured for several days. The cells were then treated with cisplatin for 3 h and subjected to further analysis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Significance of the results was tested with the unpaired t-test or one-way ANOVA with Tukey test. $P < 0.05$ was considered significant.

Results

Conventional cell viability assays did not predict eventual cell death after cisplatin treatment. Two ovarian cancer cell lines,

SKOV-3 and OVCAR-3, were treated with cisplatin. The effect of various doses of cisplatin was assessed at the earlier time point of 3 h, and at the later time point of 24 h. First, cell viability was assessed by trypan blue exclusion assay. A decrease in the number of viable cells was observed 24 h after cisplatin treatment at the relatively low dose of 5 μg/mL in both cell lines (Fig. 1A). The number of dead cells increased in OVCAR-3 cells at the same low dose, but it only marginally increased in SKOV-3 cells at the high dose of 20 μg/mL, indicating that cisplatin induced cell death in OVCAR-3 cells and cell-cycle arrest in SKOV-3 cells at the low dose (Fig. S1A). In contrast, no significant difference was seen in the number of viable or dead cells in these cell lines at 3 h, even at the high dose (Figs 1A and S1A).

Next, cell viability was assessed by the MTS assay, which reflects the mitochondrial reductase activity.[28] At 24 h, the value decreased marginally in SKOV-3 cells at the high dose and remarkably in OVCAR-3 cells at the low dose (Fig. 1B). Again, no difference was observed in these cell lines at 3 h, indicating the mitochondrial reductase activitiy was maintained at the time point. We further assessed cell death by annexin V staining, which is an early marker of apoptosis.[29] At 24 h, the number of early apoptotic cells (annexin V+/PI−) and late apoptotic cells (annexin V+/PI+) increased in a dose-dependent
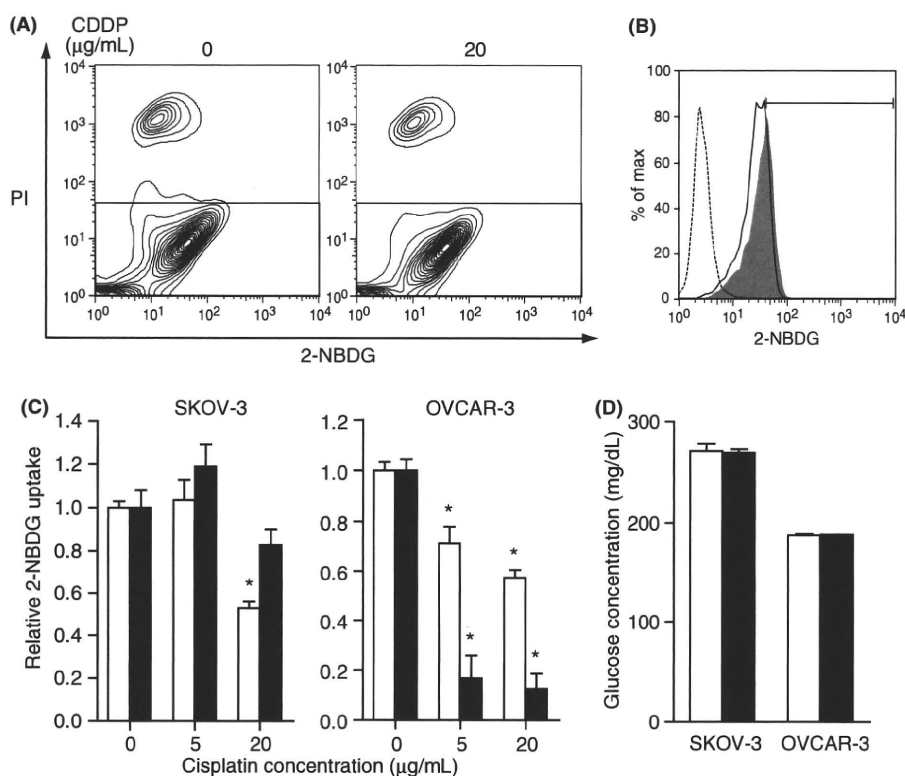

Fig. 2. Glucose uptake was evaluated by cellular 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl) amino]-2-deoxyglucose (2-NBDG) uptake was determined using flow cytometry. (A) OVCAR-3 cells were treated with cisplatin at the indicated doses. The scatter plots of the 2-NBDG and propidium iodide (PI) fluorescence intensity 3 h after treatment are shown. To exclude dead cells, the gate in the lower rectangle was applied for further analysis. (B) Histogram for the same samples in (A). The y-axis represents the percentage of maximum cell number counts (Max); the x-axis represents 2-NBDG fluorescence intensity. The dotted line indicates untreated cells, the gray shading indicates untreated cells with 2-NBDG, and the solid line indicates cisplatin (20 μg/mL)-treated cells with 2-NBDG. In the untreated cells, those showing more 2-NBDG fluorescence intensity than the histogram peak were gated to represent 2-NBDG uptake. (C) SKOV-3 cells and OVCAR-3 cells were treated with cisplatin at the indicated doses. The 2-NBDG uptake relative to the untreated group is shown. The white bars indicate 3 h and black bars 24 h after cisplatin treatment. The results are from three independent replicates. The experiments were repeated three times, and representative results are shown. *P < 0.05 compared with the control. (D) Glucose concentration of the medium. Cells were treated with 20 μg/mL cisplatin. The white bars indicate 0 h and black bars 3 h after cisplatin treatment. The results are from three independent replicates. The experiments were repeated three times, and representative results are shown.