- 現在放射線療法/化学療法と放射線療法の第Ⅲ相試験が進行中である（PORTEC-3, GOG258）[16,17]。
- Ⅲ/Ⅳ期術後症例の放射線療法後に施行したAP療法，早期高リスク子宮体がんにおける放射線療法/化学療法と，放射線療法の第Ⅲ相試験（NSGO-EC-9501/EORTC 55991）では放射腺療法/化学療法群において PFS が良好である[18]。
- また，先に述べた通りⅠ～Ⅱ期の中/高リスク群における化学療法の役割は依然課題である。子宮に限局しリスクを有する症例（Grade 3，深い筋層浸潤，脈管侵襲，漿液性腺癌，明細胞腺癌）は，放射線療法だけでは予後不良であり，補助化学療法が考慮される。これらの中/高リスクに対する臨床試験が進行中である[19]。

当院の取り組み

- 現在進行中である術後初回療法を対象にしたAP療法，TC療法，DP療法を比較する第Ⅲ相ランダム化比較試験であるJGOG 2043の勝者が，本邦における子宮体がんに対する術後補助療法の標準レジメンとなる可能性がある。しかし標準治療決定後もさらなる成績の向上と認容性にすぐれたレジメンの探索を行うことが求められる。
- 本邦の各地の臨床試験グループで行われた過去の臨床試験にて TEC 療法，TAC 療法，ddTC 療法は子宮体がんに対する有望な化学療法と考えられているが，いずれも単アームで行われた研究であった[20~22]。そこで，今後最終的には，JGOG2043で決定される標準療法と比較することを念頭に，TEC 療法・TAC 療法・ddTC 療法のランダム化第Ⅱ相比較試験を開始した。
- この研究は,国内臨床試験グループ(GOGO, GOTIC, JKTB, KCOG, SGSG, TGCU, WJGOG)（アルファベット順）が共同で行う intergroup study であり，大阪大学医学部附属病院を含むGOGO(Gynecologic Oncology Group of Osaka) が研究事務局を務める。各臨床試験グループに参加されていない施設からの参加も可能であり，GOGO ホームページ（http : //osaka-gogo. com/）を参照されたい。

おわりに

- 近年増加傾向にある子宮体がんでは，早期がんの再発予防が肝要である。それには，臨床試験を通してエビデンスを確立し，リスク群を明確に判別し適切な補助化学療法を決定することが必要である。現在までの臨床試験から早期例は再発リスク分類をさらに細分化する必要があると思われる。
- また進行例には今後集学的治療が開発されていくと思われるが，問題点である認容性をクリアし子宮体がんの予後が改善されることを期待する。

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ORIGINAL ARTICLE

Taxane-sensitivity of ovarian carcinomas previously treated with paclitaxel and carboplatin

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Abstract

Purpose The aim of the present study was to investigate, in ovarian carcinoma cases, the predictive association between the treatment-free interval (TFI) after an initial paclitaxel plus carboplatin (TC) therapy and the subsequent effectiveness of a second-line taxane-containing chemotherapy.
Methods Patients with a TFI < 6 months from the first-line TC therapy were treated with a combination chemotherapy using docetaxel and irinotecan; patients with a TFI ≥ 6 months were retreated with the same regimen as the initial TC therapy. The clinical data of these patients were retrospectively analyzed for this study.
Results The response rate of those with a TFI equal to 6–12 months was greater than that of those with a TFI < 6 months ($p = 0.014$) and less than that of those with a TFI > 12 months ($p = 0.012$). The progression-free survival of the cases with TFI equal to 6–12 months was longer than that of those with TFI < 6 months ($p = 0.012$) and shorter than that of those with TFI > 12 months ($p = 0.0011$). Overall survival of cases with a TFI equal to 6–12 months was longer than that of those with TFI < 6 months ($p = 0.012$) and shorter than that of those with TFI > 12 months ($p = 0.0005$).
Conclusions The effectiveness of using a second-line taxane-containing chemotherapy was shown to be predictable by the TFI after the first-line taxane-containing chemotherapy, implying that the theory of 'taxane-sensitivity'
may be applied for second-line chemotherapy in the same way as that of 'platinum-sensitivity'.

Abbreviations
CR   Complete response
OS   Overall survival
PD   Progressive disease
PFS   Progression-free survival
PR   Partial response
RR   Responsive rate
SD   Stable disease
TFI   Treatment-free interval
TC   Paclitaxel and carboplatin

Introduction

Ovarian cancer is the ninth most common cancer in US women, yet causes more deaths than any other cancer of the female reproductive system. The preferred primary management of ovarian carcinoma is surgical debulking followed by adjuvant TC (paclitaxel and carboplatin) therapy. Ovarian carcinomas respond well to first-line TC therapy. However, even though the initial response rate to platinum-based chemotherapy can be 70–80 %, most of those patients with advanced disease will eventually relapse and die of a chemo-resistant disease [1–3].

For a relapsed disease, a second-line chemotherapy is usually attempted. The probability of a response to the second-line chemotherapy can be estimated by the treatment-free interval (TFI) after the initial platinum-based chemotherapy. The second-line platinum chemotherapy

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was considered to be more likely to be efficacious the longer the TFI was. Relapsing cases with a TFI < 6 months after the first-line platinum-based chemotherapy were considered likely to be 'platinum-resistant'; on the other hand, those cases with a TFI ≥ 6 months were considered likely to still be 'platinum-sensitive' [4–6]. Regimens other than repeating the initial chemotherapy were recommended for the quickly relapsing cases; however, patients with a TFI ≥ 6 months had a better chance of responding well either to a rechallenge with the initial platinum-based first-line treatment (such as TC therapy) or to certain other drugs. In the 'platinum-sensitive' cases, a combination chemotherapy using liposomal doxorubicin and carboplatin was demonstrated to be more effective than TC therapy [7]. Gemcitabine plus carboplatin was also shown to be more effective for 'platinum-sensitive' cases than carboplatin alone [8].

Among the 'platinum-sensitive' cases, those with a TFI of 6–12 months still exhibited a relatively worse response to second-line chemotherapy using carboplatin after a standard first-line TC therapy than those with a TFI of ≥12 months and were considered to be 'partially sensitive' cases [6]. For those ovarian carcinoma patients with 6–12 months of TFI after the first-line TC therapy, a combination chemotherapy of liposomal doxorubicin and carboplatin was shown to provide a better prognosis [9].

These findings were based on the theory of 'platinum-sensitivity'. To our knowledge, there has been little similar discussion related to relapse and 'taxane-sensitivity'. In the present study, the effectiveness of a combination chemotherapy using taxane with irinotecan or carboplatin as a second-line therapy after initial TC therapy was investigated to provide evidence for predicting 'taxane-sensitivity' in relapsing tumors.

Materials and methods

Patients

During the 7-year study period of 2002–2009, we conducted a prospective phase I/II study of a combination chemotherapy using docetaxel and irinotecan for TC-refractory or TC-resistant ovarian carcinoma cases (GOGO-OV2) (to be described in detail elsewhere). In brief, docetaxel and irinotecan were administered on day 1 and day 8, every 3 weeks, for the patients whose TFI was shorter than 6 months from the first-line TC therapy (175 mg/m^2 for paclitaxel and AUC 5 for carboplatin, every 3 weeks). In a phase I component, the recommended dose was determined to be 30 mg/m^2 (day 1 and day 8) for docetaxel and 50 mg/m^2 (day 1 and day 8) for irinotecan. On the other hand, the patients whose TFI was equal to or

longer than 6 months from the first-line TC therapy were again treated with the same regimen as the initial TC therapy.

In the present study, the cases with a TFI < 6 months that were treated with a combination chemotherapy using docetaxel and irinotecan in a phase II component and the patients with a TFI ≥ 6 months who were treated with TC therapy were retrospectively analyzed.

Methods

In order to evaluate the therapeutic effect of the second-line chemotherapy, we used the previously described standard criteria from the World Health Organization [10] and others [11–13]. The tumors were assessed with a CT scan and/or MRI at baseline and every three treatment courses thereafter. A complete response (CR) was defined as the disappearance of all known disease, determined by two observations no less than 4 weeks apart. Partial response (PR) was defined as a 50 % or more reduction in the summed products of the two largest perpendicular dimensions of bidimensionally measurable lesions, for at least 4 weeks. Stable disease (SD) was defined as a less than 50 % decrease, or a less than 25 % increase, in tumor size, with no new detectable lesions. Progressive disease (PD) was defined as a greater than 25 % increase in tumor size, or as the appearance of new lesions.

Progression-free survival (PFS) was measured from the date of the last administration of chemotherapy to the date of the radiologic or pathologic denoted relapse, or to the date of the last follow-up. Overall survival (OS) was defined as the period from the start of chemotherapy to the patient's death, or to the date of the last follow-up, as previously described. TFI was defined as the period between the last administration of first-line chemotherapy and the initiation of the second-line chemotherapy, as previously described [14].

Statistical analysis of effect of second-line chemotherapy

Associations between the TFI and the patients' characteristics, including age, histology and initial stage, were analyzed by Pearson's Chi-square test. Association between sensitivity to second-line chemotherapy and TFI was analyzed by Fisher's exact test. PFS and OS curves determined by TFI were constructed using the Kaplan–Meier method and were evaluated for statistical significance by the log-rank test. The Bonferroni correction was used to assess differences among the three groups, and a value of $p < 0.017$ was considered statistically significant.

Results

Clinical characteristics of the study cases

During the 7-year study period, 145 patients underwent a second-line chemotherapy against a refractory or resistant disease, after having first received an adjuvant or salvage first-line chemotherapy using a TC regimen. The clinico-pathological characteristics of these patients are shown in Table 1. Sixty-two patients with a TFI < 6 months received a combination chemotherapy of docetaxel and irinotecan; 36 patients with TFI = 6–12 months and 47 patients with TFI > 12 months were treated with this TC regimen.

Outcome of the patients after second-line chemotherapy

Only nine (15 %) of 62 patients whose TFI was shorter than 6 months exhibited sensitivity to a second-line chemotherapy using docetaxel and irinotecan; however, 13 (36 %) of 36 patients whose TFI was 6–12 months and 30 (64 %) of 47 cases >12 months responded to second-line TC therapy (Table 2).

The longer the TFI was, the higher the response rate was. The response rate of the cases with TFI = 6–12 months was significantly longer than that of those with TFI < 6 months, and that of those with TFI > 12 months

was longer than that of those with TFI = 6–12 months ($p = 0.014$ and $p = 0.012$, respectively). These associations were statistically significant (Fisher's exact test with Bonferroni's correction).

PFS and OS after second-line chemotherapy, by TFI

Differences by TFI in effectiveness of second-line chemotherapy regimens were investigated. The median PFS was 5 months (2–17 months) for 62 patients with TFI < 6 months, 8 months (1–65 months) for 36 patients with TFI = 6–12 months and 13 months (3–83 months) for 47 patients with TFI > 12 months. The longer the TFI was, the longer the PFS rate was. These associations were statistically significant ($p = 0.012$ and $p = 0.0011$, respectively) (log-rank test with Bonferroni's correction) (Fig. 1).

The median OS was 15 months (3–50 months) for 62 patients with TFI < 6 months, was 24 months (3–65 months) for 36 patients with TFI = 6–12 months and was 37 months (8–83 months) for 47 patients with TFI > 12 months. The longer the TFI was, the longer the PFS rate was. These associations were statistically significant ($p = 0.012$ and $p = 0.0005$, respectively) (log-rank test with Bonferroni's correction) (Fig. 2).

Conclusions

Chemotherapy plays an extremely important role in the treatment for ovarian carcinoma. Platinum has long been a key drug for ovarian carcinoma, and now a combination chemotherapy of platinum and taxane, especially TC therapy, is the gold standard for first-line regimens. For relapsed diseases, a second-line chemotherapy is usually performed. The effectiveness of this second-line chemotherapy was known to be associated with the TFI from the platinum-based first-line chemotherapy. The early relapsing cases, those with a TFI less than 6 months, were considered likely to be 'platinum-resistant', those with a TFI of 6–12 months were considered as 'partially sensitive' to platinum, and those with TFI ≥ 12 months were considered to be 'platinum-sensitive' [4–6].

Because platinum has been effectively used in the first-line chemotherapy, 'platinum-sensitivity' has often been used as the most important predictive factor of efficacy of second-line chemotherapy. However, taxane drugs, including paclitaxel and docetaxel, were also shown to be effective for ovarian carcinoma [15–17], and a combination TC therapy is currently regarded as a standard therapy. Under the specific circumstance that a TC therapy is used as the first-line chemotherapy, sensitivity not only to platinum but also to taxane may be a predictive factor for efficacy of the second-line chemotherapy.

Table 1 Clinical characteristics of the cases

TFI	<6–12 months	6–12 months	>12 months	p Value
Number	62	36	47	–
Age (years)				0.39
<60	39	20	33	
≥60	3	16	14	
Histology				0.08
Serous	40	26	34	
Endometrioid	7	5	6	
Clear	8	1	5	
Mucinous	6	0	1	
Others	1	4	1	
Initial stage				0.42
I/II	12	5	12	
III/IV	50	31	35	

Clinical characteristics of the cases with a TFI < 6 months after first-line TC therapy that were treated with a combination chemotherapy using docetaxel and irinotecan, the patients with a TFI = 6–12 months and those with a TFI ≥ 12 months, who were treated with TC therapy again, are shown. Association between TFI and the patients' characteristics, including age, histology and initial stage, was analyzed by Pearson's Chi-square test

TFI treatment-free interval

Table 2 Association between TFI and effectiveness of a second-line chemotherapy using taxane

Second-line chemotherapy	Docetaxel + irinotecan	Paclitaxel + carboplatin	
TFI	<6 months	6–12 months	>12 months
CR	1	6	4
PR	8	7	16
SD	15	10	8
PD	38	13	9
Response rate (%)	15	36	64

The response rate of the cases with a TFI = 6–12 months was significantly better than that of those with TFI < 6 months, and that of those with TFI > 12 months was better than that of those with a TFI = 6–12 months ($p = 0.014$ and $p = 0.012$, respectively). These associations were statistically significant (Fisher's exact test with Bonferroni's correction)

TFI treatment-free interval, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease
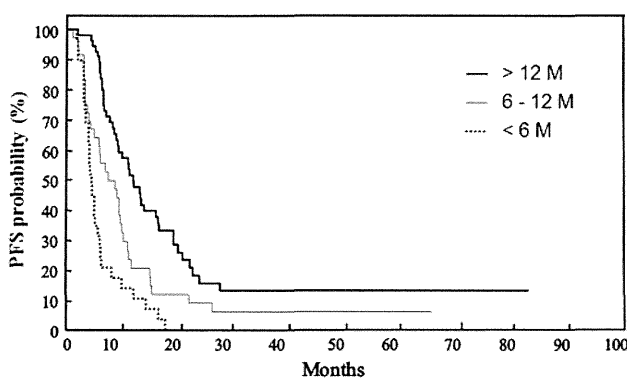
Fig. 1 PFS after second-line chemotherapy by TFI. The PFS of 62 patients with a TFI < 6 months was shorter than that of 36 patients with a TFI = 6–12 months, which was shorter than that of 47 patients with a TFI > 12 months. These associations were statistically significant ($p = 0.012$ and $p = 0.0011$, respectively) (log-rank test, with Bonferroni's correction) *PFS* progression-free survival
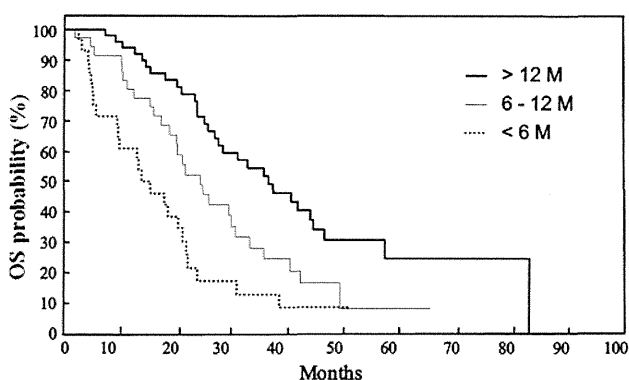
Fig. 2 OS after second-line chemotherapy by TFI. The OS of 62 patients with a TFI < 6 months was shorter than that of 36 patients with a TFI = 6–12 months, which was shorter than that of 47 patients with a TFI > 12 months. These associations were statistically significant ($p = 0.012$ and $p = 0.0005$, respectively) (log-rank test, with Bonferroni's correction) *OS* overall survival

Gronlund et al. [3] showed that retreatment with a TC regimen in the patients with TFI ≥ 6 months yielded a high response rate. There was a relative increase in response rates comparing TFI = 6–9 months ($n = 9$), TFI = 9–12 months ($n = 6$) and TFI > 12 months ($n = 22$), but the differences were not statistically significant, probably due to the small sample sizes.

Docetaxel exhibits a response rate of 22.4 % for those diseases which progressed either while undergoing therapy or within 6 months of completing therapy with paclitaxel and platinum [18]. Aravantinos et al. [19] reported that a response rate of 26.8 % was observed by treatment with docetaxel plus vinorelbine in 41 platinum-resistant and paclitaxel-pretreated patients who had a TFI < 6 months. Recently, Fu et al. [20] demonstrated that a PR was obtained by perifosine plus docetaxel in one (5 %) of 21 platinum- and- taxane-resistant or platinum-and-taxane-refractory high-grade ovarian carcinoma cases. Ushijima et al. [21] showed that a combination chemotherapy of docetaxel and irinotecan (the same regimen as in our study) exhibited a response rate of 6.3 % in the ovarian carcinoma cases with a TFI < 6 months (refractory or resistant) from a first-line chemotherapy, with at least two cycles of platinum and/or taxane. Polyzos et al. [22] also showed that six (20 %) of 30 paclitaxel-pretreated patients with likely platinum-resistant (TFI < 6 months) recurrences exhibited complete or partial response to a second-line docetaxel plus irinotecan regimen.

To our knowledge, the effectiveness of using a taxane-containing second-line chemotherapy in those patients previously treated with taxane-containing chemotherapy, especially the highly used gold standard TC therapy, has never been systematically investigated. In the present study, the effectiveness of a second-line combination chemotherapy using taxane with another drug (after a first-line TC therapy) was analyzed to redress that gap in our knowledge.

In the present study, 62 patients with a TFI < 6 months received a combination chemotherapy of docetaxel and irinotecan, and 36 patients with a TFI = 6–12 months and 47 patients with a TFI > 12 months were treated with a TC regimen. We were clearly able to demonstrate a significant association between the TFI after a first-line TC therapy and the response to a second-line chemotherapy containing taxane. The response rate of the cases with TFI = 6–12 months was significantly better than that of those with a TFI < 6 months, and the response rate of those with a TFI > 12 months was better yet than that of those with a TFI = 6–12 months ($p = 0.014$ and $p = 0.012$, respectively).

Moreover, a significant association between TFI after first-line TC therapy and the survival effect of the second-line chemotherapy using taxane with irinotecan, or carboplatin, was also demonstrated. The PFS of the cases with a TFI = 6–12 months was significantly longer than that of those with a TFI < 6 months, and that of those with a TFI > 12 months was longer than that of those with a TFI = 6–12 months ($p = 0.012$ and $p = 0.0011$, respectively). The OS of the cases with a TFI = 6–12 months was significantly longer than that of those with a TFI < 6 months, and that of those with a TFI > 12 months was longer than that of those with a TFI = 6–12 months ($p = 0.012$ and $p = 0.0005$, respectively).

These results imply that effectiveness of second-line taxane-containing chemotherapy is predictable by the TFI after first-line taxane-containing chemotherapy. Second-line regimens might thus be intelligently selected based on the likely 'taxane-sensitivity' of the relapsing tumor.

Paclitaxel and carboplatin therapy is currently used for ovarian carcinoma cases as a standard first-line chemotherapy all over the world. If the theory of 'taxane-sensitivity' can be applied for second-line chemotherapy in the same way as that of 'platinum-sensitivity', a combination chemotherapy of taxane with platinum, and other drugs, including liposomal doxorubicin and gemcitabine, might be effective. Markman et al. [23] showed a 25 % response of weekly paclitaxel even in TC-resistant cases. Weekly administration of taxane may be effective for some 'taxane-resistant' cases.

Our present study provides, for the first time, good evidence that the longer the TFI is after first-line taxane-containing chemotherapy, the more effective the second-line taxane-containing chemotherapy is likely to be, implying the model of 'taxane-sensitivity' may be applied for the second-line chemotherapy in the same way as that of 'platinum-sensitivity'. However, in our study, all the patients received platinum combined with taxane as the first-line chemotherapy, and those with late relapse (>6 months) were treated with a chemotherapy using platinum (carboplatin) combined with taxane. These data

may reflect, in some part, platinum-sensitivity phenomenon. Further investigation is still required to establish an idea of 'taxane-sensitivity' and an efficacious strategy for second-line chemotherapy for advanced or recurrent ovarian cancer.

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ORIGINAL ARTICLE

Salvage chemotherapy for recurrent or persistent clear cell carcinoma of the ovary: a single-institution experience for a series of 20 patients

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Abstract

Background Recurrent or persistent clear cell carcinoma (CCC) of the ovary is particularly chemotherapy resistant. The purpose of this study was to review our extensive institutional experiences with recurrent or persistent CCC with the aim of finding a more effective chemotherapy regimen.

Methods The medical records of 67 patients treated for CCC of the ovary were retrospectively reviewed to select patients subsequently treated for recurrence or persistence of the disease.

Results The review identified 20 patients treated for recurrent or persistent CCC. For these 20 patients, 9 chemotherapeutic regimens, with 125 cycles, were administered. Gemcitabine monotherapy showed the best response rate [1 partial response (20%) and 2 stable diseases out of 5 patients so treated]. A partial response was observed with a combination of docetaxel plus irinotecan in 1 of 11 patients (9%). Stable disease was observed in 1 of 9 cases on a paclitaxel/carboplatin doublet and in 1 case on a docetaxel/carboplatin doublet. The median overall survival time was 8 months (range, 2–52). One group of patients who received gemcitabine therapy showed significantly better survival ($n = 5$, median 18 months) compared with a group who did not ($n = 15$, median 7 months) ($P = 0.0108$, by univariate analysis). In addition, multivariate Cox proportional hazards analysis revealed that gemcitabine administration was a significant factor for survival (hazard ratio: 13.0, 95% CI: 1.4727–115.2255, $P = 0.02$).

Conclusion Although most chemotherapeutic regimens for recurrent or persistent CCC have little or no effect, gemcitabine showed modest activity and is the most effective agent we have tested to date.

Keywords Chemotherapy · Clear cell carcinoma · Gemcitabine · Ovarian cancer · Persistence · Recurrence

Introduction

Epithelial ovarian cancer (EOC) is the second most lethal of the gynecological malignancies (after cervical cancer), causing approximately 125,000 deaths annually worldwide [1]. Standard therapy for EOC includes maximal surgical debulking followed by chemotherapy with platinum and taxane drugs. Despite an initial response rate to this primary therapy of approximately 80%, most EOC patients suffer subsequent recurrence and mortality.

Clear cell carcinoma (CCC) is a subtype of EOC that is relatively uncommon in western countries, including the USA, where CCC comprises only 5–10% of ovarian tumors. In contrast, in Japan, CCC has a higher incidence rate, at 20–25% of all EOCs. The reason behind this significantly higher incidence is not yet fully understood [2].

CCC has distinct biological activities relative to other histological types of ovarian cancer. Sugiyama et al. [3] have reviewed the distinct chemo-resistance and poorer prognosis of CCC. Enomoto et al. [4] showed that this problem continues, even with our best current standard regimen of a paclitaxel/carboplatin doublet.

For recurrent EOC, the treatment strategy depends on the tumor's response to the primary chemotherapy. When

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recurrence occurs more than 12 months after the completion of the initial therapy, re-administration of the same chemotherapy can be effective in many cases, resulting in extended survival times. However, if the recurrence occurs before 6 months have passed, most chemotherapeutic agents are usually no longer effective [5, 6].

Recurrences of the CCC subtype of EOC tend to be highly chemoresistant to any previous chemotherapy regimen, no matter when they reoccur. Some medical groups have attempted to overcome this resistance by a number of different strategies. Irinotecan (CPT-11) combined with cisplatin (CPT-P) was introduced as an efficacious regimen for refractory or recurrent general EOC, and has been used specifically for CCC. A retrospective Japanese multi-center study reported that a CPT-P group showed significantly better progression-free survival than a group receiving standard TP (taxane plus platinum) [7]. In another strategy, postoperative whole-abdominal radiotherapy (WAR) was carried out. The 5-year overall and disease-free survival in the WAR group was significantly better than that for the standard platinum-based chemotherapy group. However, the adverse effects in the bowel were occasionally severe, causing some patients to require surgery [8].

Clinical trials using novel agents specifically for CCC are ongoing. For persistent or recurrent disease, sunitinib is being evaluated in a phase II study by GOG (NCT 00979992, http://www.clinicaltrials.gov). Another phase II study is evaluating temsirolimus in combination with a paclitaxel/carboplatin doublet followed by temsirolimus consolidation as a first-line therapy in the treatment of stage III–IV CCC (NCT 01196429, http://www.clinicaltrials.gov). The results of these studies should give us a clue as to how to overcome CCC.

In this review, we recount our past experiences with recurrent and persistent CCC, seeking clues for overcoming the scourge that is CCC of the ovary.

Patients and methods

During the period of 1998–2009, 67 cases of CCC of the ovary (all of Japanese descent) underwent cytoreductive surgery within the Department of Obstetrics and Gynecology at the Osaka University Hospital, Osaka, Japan. The medical records of the patients were reviewed, revealing that the FIGO (International Federation of Gynecology and Obstetrics) staging of these cases was distributed as follows: stage I in 46 cases (Ia; 16 cases, Ib; 1 case, and Ic; 29 cases), stage II in 5 cases (IIc for all), stage III in 14 cases (IIIb; 3 cases, and IIIc; 11 cases), and stage IV in 2 cases.

Study inclusion eligibility criteria for those patients who were treated for recurrent or persistent disease included the following: (1) pathological diagnosis of CCC of the ovary at the initial surgery, (2) subsequent measurable recurrent or persistent disease, (3) treatment for the recurrent or persistent disease with one or more systemic chemoregimens, and (4) availability of adequate clinical information. The following patient information was abstracted from their medical records: age; date of primary surgery; residual disease; stage of disease based on FIGO criteria; date of completion of the primary chemotherapy; date of first detected recurrence or progression; regimens of each systemic agent administered; date of start and completion of each treatment; number of cycles of each systemic agent; response to each systemic agent administered; status at the last patient contact; and the date of last contact or death. Responses to the systemic agents were recorded according to version 1.0 of the Response Evaluation Criteria in Solid Tumors (RECIST) criteria.

Statistical analyses were performed using MedCalc for Windows (version 11.3.3.0, MedCalc Software, Mariakerke, Belgium). Treatment-free interval (TFI) was defined as the time (months) from the completion of initial therapy to recurrence with a radiological confirmation. For recurrent disease, overall survival time (OS) was calculated from the date of first recurrence to either the date of death or date of last contact. For persistent disease, OS was calculated from the date of primary surgery to either the date of death or date of last contact. A multivariate Cox proportional hazards analysis with selected variables was used to determine the significantly important factors for survivals. The Kaplan–Meier statistical method was used to calculate the overall survival times. Statistical significance was analyzed by the log-rank test. We considered the results to be significant when the P value was less than 0.05.

Results

After reviewing the medical records of 67 patients with CCC of the ovary, 21 patients were identified as having subsequently had a recurrence or a persistent disease. Of these 21 patients, 1 patient refused to receive any systemic agents and was therefore excluded from this study. A total of 20 patients received systemic agents, thereby meeting the eligibility requirements for this study, and were subsequently analyzed.

The characteristics of these 20 patients are shown in Table 1. The median age was 53 years; ages ranged from 35 to 65. In stage I patients, recurrence occurred in 4 cases with stage Ic (recurrence rate 9% for stage I overall, 14% specifically for stage Ic). There was also a single case of stage IIc, which thus showed a recurrence of 20%. Thirteen of 14 cases (93%) with stage III showed recurrence or persistent disease. Both cases with stage IV had persistent

disease (100%). Ten of the patients were known to still have residual disease after the initial debulking surgery (50%); the remaining 10 patients were classified as having had recurrent disease (50%). Retroperitoneal (pelvic and para-aortic) lymphadenectomy was performed in 12 cases in their initial surgeries (60%).

In our hospital, until 2003, postoperative chemotherapy with paclitaxel/carboplatin doublet (TC) was administered as the standard regimen for all EOC, regardless of histological subtype. Five of our 20-patient pool underwent this

Table 1 Characteristics of patients with recurrent or persistent clear cell carcinoma of the ovary

Characteristics	$n = 20$	%
Age		
Median	53	
Range	35–65	
FIGO stage		
I	4	20
II	1	5
III	13	65
IV	2	10
Residual disease		
No	10	50
Yes	10	50
Postoperative chemotherapy		
None	1	5
Paclitaxel/Carboplatin	5	25
Docetaxel/Irinotecan	14	70
Disease status		
Recurrence	10	
TFI: <6 months	4	20
TFI: ≥6 months	6	30
Persistent disease	10	50

FIGO International Federation of Gynecology and Obstetrics, *TFI* treatment-free interval

regimen. Thereafter, starting in 2003, because of the low response rate of this TC regimen, a docetaxel plus irinotecan (DIr) regimen was used for postoperative chemotherapy for advanced stages of CCC, and of these, 14 study patients received this regimen [4]. Among the 10 patients who had no detectable residual disease after initial surgery, but thereafter showed recurrence, 6 had equal to or more than 6 months of TFI, and the remaining 4 had less than 6 months of TFI.

As shown in Table 2, 9 treatment regimens were administered. Paclitaxel/carboplatin doublet (TC) was administered to 9 patients, with a total of 28 cycles, where 1 cycle consisted of paclitaxel (175 mg/m^2) plus carboplatin (AUC = 5) every 3 weeks. A docetaxel/carboplatin doublet (DC) was administered for 1 patient, for a total of 3 cycles, where 1 cycle consisted of docetaxel (70 mg/m^2) plus carboplatin (AUC = 5) every 3 weeks. A weekly treatment of a paclitaxel/carboplatin doublet (wTC) was administered to 3 patients, for a total of 8 cycles, where 1 cycle consisted of paclitaxel (80 mg/m^2 on days 1, 8 and 15) plus carboplatin (AUC = 2 on days 1, 8, and 15) every 4 weeks. DIr was administered to 11 patients, for a total of 41 cycles, where 1 cycle consisted of docetaxel (30 mg/m^2 on days 1 and 8) plus irinotecan (60 mg/m^2 on days 1 and 8) every 3 weeks. The single-agent gemcitabine (GEM) was administered to 5 patients, for a total of 18 cycles, where 1 cycle consisted of gemcitabine (800 mg/m^2 on days 1, 8, and 15) every 4 weeks. The single-agent carboplatin was administered to 1 patient as a single cycle/ single dose of AUC = 5. Oral etoposide was administered to 1 patient, for a total of 2 cycles, where 1 cycle consisted of oral etoposide (50 mg/day for 21 days) every 4 weeks. Pegylated liposomal doxorubicin (PLD) was administered to 1 patient for 2 cycles. One cycle consisted of PLD (40 mg/m^2 on day 1) once every 4 weeks. Wilms' tumor 1 vaccine (WT1) was administered to 2 patients, for a total of 22 cycles, where 1 cycle consisted of intradermal injections of an HLA-A*2402-restricted, modified 9-mer WT1

Table 2 Regimens and maximum responses for recurrent or persistent clear cell carcinoma of the ovary

Regimens	No. of patients	Total cycles	Median cycles	No. of maximum responses, duration
Docetaxel + Irinotecan	11	41	3	1 PR, 6 m
Paclitaxel + Carboplatin	9	28	3	1 SD, 7 m
Gemcitabine	5	18	4	1 PR, 6 m; 2 SD, 4 and 5 m
Paclitaxel + Carboplatin (weekly)	3	8	3	PD
WT1 vaccine	2	22	6	PD
Docetaxel + Carboplatin	1	3	3	1 SD, 4 m
Carboplatin	1	1	1	PD
Pegylated liposomal doxorubicin	1	2	2	PD
Oral etoposide	1	2	2	PD

PR partial response, *SD* stable disease, *PD* progressive disease

Table 3 Details of responders who showed more than stable disease with recurrent or persistent clear cell carcinoma of the ovary

Case	Age	Stage	Residual tumor (sites)	First-line regimen, cycles	TFI (when recurrent) or response (when persistent)	Second-line regimen, cycles	Response, duration	Third-line regimen, cycles	Response, duration	Fourth-line regimen, cycles	Response, duration	Fifth-line regimen, cycles	Response, duration	Status
1	65	Ic(2)	No	TC ×6	TFI; 31 m	DC ×3	SD, 4 m							DOD
2	42	IIc	Yes (om, pnm, msty)	DIr ×6	PD	wTC ×3	PD	GEM ×3	PR, 6 m					DOD
3	54	IIIb	Yes (om, pnm)	DIr ×6	PD	TC ×3	PD	GEM ×10	SD, 5 m					DOD
4	51	IIc(2)	No	DIr ×6	TFI; 7 m	TC ×6	SD, 7 m	WT1 ×6	PD	GEM ×4	SD, 4 m	PLD ×2	PD	AWD
5	56	IIIc	No	DIr ×6, T ×12	TFI; 5 m	DIr ×6	PR, 6 m							AWD

Ic(2) and IIc(2) positive cytology of ascites, *om* omentum, *pnm* peritoneum, *msty* mesentery, *TFI* treatment-free interval (months), *DC* docetaxel + carboplatin, *TC* paclitaxel + carboplatin, *T* paclitaxel (consolidation), *wTC* weekly paclitaxel + carboplatin, *DIr* docetaxel + irinotecan, *GEM* gemcitabine, *WT1* Wilms' tumor 1 vaccine, *PLD* pegylated liposomal doxorubicin, *PR* partial response, *SD* stable disease, *PD* progressive disease, *DOD* dead of disease, *AWD* alive with disease

peptide every week [9]. Dose reduction was performed in response to toxicity to the patient's hematological status.

The majority of these administered regimens did not show significant responsiveness. A few showed some modest clinical activity. For example, gemcitabine represented the best response rate, in 1 of 5 patients (20%) it gave a partial response as a third-line treatment, and in 2 stable diseases it gave a response as a third- or fourth-line treatment. A partial response was also observed with DIr in one of 11 patients (9%) when used as a second-line treatment. Stable disease was observed in 1 of 9 cases treated with TC and in 1 case treated with DC, both as second-line efforts. Details of the responders who showed equal to, or more than, stable disease are shown in Table 3.

The median overall survival time of the recurrent or persistent patients was 8 months (range, 2–52), as shown in Fig. 1. Using univariate analysis, a group of patients who received gemcitabine therapy ($n = 5$) showed significantly better survival (median 18 months) compared with a group who did not receive it ($n = 15$, median 7 months) ($P = 0.0108$). A multivariate Cox proportional hazards analysis with selected variables (age, stage, postoperative chemotherapy, TFI, chemotherapy for recurrence or persistent disease) was used to determine the significantly important factors in survival. The analysis revealed that use of DIr for postoperative chemotherapy ($P = 0.02$) and use of gemcitabine for recurrence or persistent disease ($P = 0.02$) were significant factors in survival, as shown in Table 4.

Discussion

Clear cell carcinoma (CCC) of the ovary is relatively rare in the USA and Europe; however, its incidence in Japan
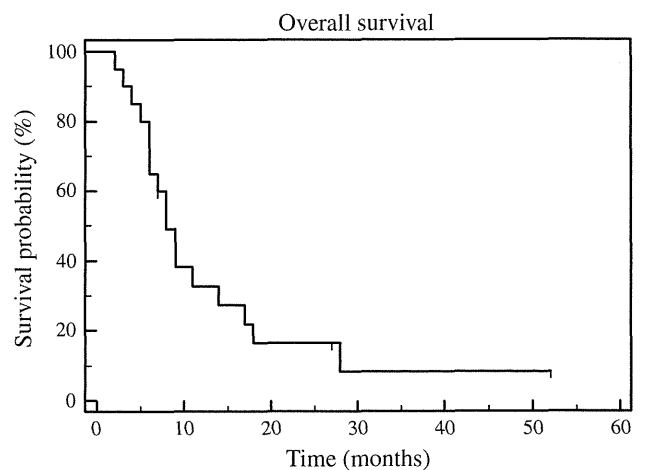
Fig. 1 Kaplan–Meier curve showing overall survival time of 20 patients with recurrent or persistent CCC. The median survival time was 8 months

Table 4 Multivariate Cox proportional hazards analysis for recurrent or persistent clear cell carcinoma of the ovary

Variables	Hazard ratio	95% CI	P value
Age			
<53 (n = 9)	1		0.18
≥53 (n = 11)	0.37	0.0898–1.6041	
Stage			
I/II (n = 5)	1		0.18
III/IV (n = 15)	6.16	0.4298–88.5383	
Postoperative chemotherapy			
TC (n = 5)	1		0.02
DIr (n = 14)	0.05	0.004–0.5792	
TFI			
<6 months (n = 14)	5.39	0.8834–32.9883	0.06
≥6 months (n = 6)	1		
Chemotherapy for recurrent or persistent disease			
DIr administration (n = 11)	1		0.23
Without (n = 9)	2.54	0.5407–11.9450	
TC administration (n = 9)	1		0.99
Without (n = 11)	0.99	0.2205–4.5342	
Gemcitabine administration (n = 5)	1		0.02
Without (n = 15)	13.0	1.4727–115.2255	

TFI treatment-free interval, *DIr* docetaxel/irinotecan, *TC* paclitaxel/carboplatin

accounts for roughly 20% of all EOC. We treated a total of 67 primary cases of CCC, along with 20 examples of recurrence or persistent CCC disease, during the 10-year study period from 1998 to 2009.

As previous reports have described, we also found that recurrent and persistent CCC was extremely chemoresistant. We noted that among the 9 different chemotherapy regimens we attempted, gemcitabine monotherapy showed the better response rate. Our patients who received gemcitabine therapy showed significantly better survival compared with a group who did not receive it. Furthermore, multivariate Cox proportional hazards analysis revealed that gemcitabine administration was a significant factor for survival (hazard ratio: 13.0, 95% CI: 1.4727–115.2255, $P = 0.02$). Therefore, we propose that gemcitabine may be an active chemotherapeutic agent for recurrent or persistent CCC.

Gemcitabine (2′,2′-difluorodeoxycytidine), a synthetic nucleoside analog of cytidine, has already been demonstrated to be an active agent for various other solid tumors, such as non-small-cell lung, pancreatic, genitourinary, and breast cancers [10]. As described in pioneering work from the Plunkett laboratory, gemcitabine is a prodrug that is metabolized to gemcitabine diphosphate and triphosphate, whose incorporation into DNA results in chain termination by inhibiting DNA polymerase activity [11]. Consequently, tumor cells are blocked in the G1 phase of the cell cycle. Gemcitabine triphosphate metabolite can be also incorporated into RNA, thus inhibiting RNA production [12].

Gemcitabine was studied for the first time as a single-agent treatment for recurrent EOC at a dose of 800 mg/m^2

on days 1, 8, and 15 every 28 days, thereafter, in a population of platinum-resistant ovarian cancers that included all histological subtypes [13]. In a review by Lorusso et al. [14], the results from a total of 411 patients treated by the single-agent gemcitabine were combined from 12 reports. The combined and re-analyzed data showed a mean gemcitabine response rate of 19%.

Recently, several large randomized control studies have been performed using gemcitabine in ovarian cancer patients. Mutch et al. have shown the safety and efficacy of gemcitabine monotherapy compared with PLD in their phase III trial in patients with platinum-resistant (Pt-R) recurrent ovarian cancer. In their report, gemcitabine and PLD seem to have comparable therapeutic indices, indicating that single-agent gemcitabine may be an acceptable alternative to PLD for patients with Pt-R recurrent disease [15]. For platinum-sensitive (Pt-S) recurrent disease, Pfisterer et al. reported that the addition of gemcitabine to carboplatin significantly improved progression-free survival and response rate compared with carboplatin alone without worsening quality of life in their phase III study [16]. Thus, gemcitabine is recognized as an active agent for both Pt-R and Pt-S recurrent ovarian cancer.

In most reports, gemcitabine's adverse effects and toxicity were easily manageable, transitory, noncumulative, and rarely represented a cause for dose reduction or treatment interruption. Gemcitabine has a well-proven activity in platinum and/or paclitaxel-resistant ovarian cancer patients, and seems to cause no cross-resistance with platinum compounds. However, it should be noted that

most of these studies represented treatments for mainly serous adenocarcinomas, with CCCs accounting for less than 5% of the cases. Therefore, the efficacy of gemcitabine for CCC is still largely unknown.

There are reports which suggest that gemcitabine may have a beneficial clinically active effect for CCC. Crotzer et al. [17] analyzed 51 patients treated for recurrent CCC. Their series received a total of 105 regimens with 344 cycles. In the platinum-sensitive setting, a partial response was observed in only 9% of cases, much lower than the response rates of 50–90% reported for platinum-sensitive disease in all cell types of EOC combined [18]. Among patients with platinum-resistant disease, only 1 patient had a partial response to gemcitabine and 1 patient had stable disease in response to 2 different regimens, paclitaxel and gemcitabine. Generally, second-line chemotherapy for platinum-resistant disease gives response rates of 15–20% when using an active agent.

Komiyama et al. [19] reported successful control with gemcitabine of a single case of peritonitis carcinomatosa presenting with massive ascites in a patient with a heavily pretreated recurrent CCC. Ferrandina et al. described a case of multi-drug-resistant CCC of the ovary showing a selective susceptibility to gemcitabine at first administration and again at re-challenge. Moreover, they showed that the tumor expressed a certain molecular profile that likely made it highly sensitive to gemcitabine [20]. Their finding points out that, although most reports of chemotherapy for CCC are highly disappointing, case-by-case molecular targeting therapy may be the key to combating this difficult to treat disease.

In conclusion, gemcitabine may be a key chemotherapeutic agent for the treatment of aggressive CCCs of the ovary. Additional adjunct molecular targeting therapy should also be considered.

Conflict of interest The authors declare that there are no potential conflicts of interest.

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Plasma membrane proteomics identifies bone marrow stromal antigen 2 as a potential therapeutic target in endometrial cancer

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This report utilizes a novel proteomic method for discovering potential therapeutic targets in endometrial cancer. We used a biotinylation-based approach for cell-surface protein enrichment combined with isobaric tags for relative and absolute quantitation (iTRAQ) technology using nano liquid chromatography–tandem mass spectrometry analysis to identify specifically overexpressed proteins in endometrial cancer cells compared with normal endometrial cells. We identified a total of 272 proteins, including 11 plasma membrane proteins, whose expression increased more than twofold in at least four of seven endometrial cancer cell lines compared with a normal endometrial cell line. Overexpression of bone marrow stromal antigen 2 (BST2) was detected and the observation was supported by immunohistochemical analysis using clinical samples. The expression of BST2 was more characteristic of 118 endometrial cancer tissues compared with 59 normal endometrial tissues ($p < 0.0001$). The therapeutic effect of an anti-BST2 antibody was studied both *in vitro* and *in vivo*. An anti-BST2 monoclonal antibody showed *in vitro* cytotoxicity in BST2-positive endometrial cancer cells *via* antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. In an *in vivo* xenograft model, anti-BST2 antibody treatment significantly inhibited tumor growth of BST2-positive endometrial cancer cells in an NK cell-dependent manner. The anti-BST2 antibody had a potent antitumor effect against endometrial cancer both *in vitro* and *in vivo*, indicating a strong potential for clinical use of anti-BST2 antibody for endometrial cancer treatment. The combination of biotinylation-based enrichment of cell-surface proteins and iTRAQ analysis should be a useful screening method for future discovery of potential therapeutic targets.

Anticancer monoclonal antibodies are a growing family of novel agents applied in the treatment regimens for hematopoietic and solid tumors. Antibody-based therapeutic agents against CD20 or Her2 have been successfully clinically developed and have significant therapeutic effects.[1,2] Tumor-associated antigens which are easily accessible from the tumor neovasculature are particularly attractive for intravenously-administered antibody-based therapeutic agents. During the last decade, several new technologies for high-throughput screening have identified many potential therapeutic targets. Thus far, no single approach or combination of methods has emerged as the preferred paradigm. It is clear that new tools and strategies are needed so that tumor-associated antigens can be screened efficiently.

Proteomic methods can now be tailored to search directly for targetable cell-surface proteins that distinguish cancer cells from normal cells. The complexity and concentration of individual proteins in the sample are crucial when performing proteomic analyses because abundant proteins, such as cytoskeletal proteins, may hinder the detection of low abundance proteins, such as plasma membrane proteins.[3] One way to enrich the potentially accessible cell-surface proteins is by whole cell protein tagging followed by affinity purification. A method for enrichment of such cell-surface proteins

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What's new?

In this study, we have used a biotinylation-based approach for cell-surface protein enrichment combined with iTRAQ technology to identify and quantify membrane proteins which might represent potential therapeutic targets of endometrial cancer. A monoclonal antibody targeting BST2, one of the proteins identified in the iTRAQ analysis, have a potent antitumor effect against endometrial cancer both *in vitro* and *in vivo*, indicating a strong potential for clinical use of anti-BST2 antibody for endometrial cancer treatment.

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via their biotinylation and affinity purification has been reported.[4,5] In most cases, concentrated cell-surface proteins are separated by SDS-PAGE and the enzymatically digested peptides are analyzed by mass spectrometry, while highly accurate quantitative data cannot be obtained by using this method. To acquire more quantitative information, stable isotope labeling using amino acids in cell culture (SILAC) based quantitative proteomics has been used, with high quantitative accuracy; however, the SILAC approach has the limitation that only a maximum of three samples can run in any single analysis.[6,7] Compared with SILAC, the more recently developed isobaric tags for relative and absolute quantitation (iTRAQ) technology has a distinct advantage regarding sample number handling capability in a single analysis, because iTRAQ can compare up to eight samples simultaneously.[7,8]

Endometrial cancer is the most common malignant tumor of the female genital tract. Its incidence varies among regions; it is overall the fourth most common malignancy in North America.[9] In general, the prognosis of these patients is excellent as the majority present with early-stage disease that is confined to the uterus at the time of diagnosis, which is followed by simple hysterectomy, leading to a 5-year survival rate of 84%.[9] Unfortunately, those women who present with recurrent or advanced-stage disease have a much poorer prognosis, with a median survival of less than a year.[10] To date, combination chemotherapy of cisplatin, doxorubicin, and paclitaxel has demonstrated the greatest efficacy.[10-12] However, these cytotoxic agents are associated with intolerable side effects and infrequent sustainable remission.[11,12] Thus, new and more effective targeted therapies for endometrial cancer are urgently needed. However, thus far the search for agents effective in the treatment of either recurrent or advanced endometrial cancer has been disappointing.[12]

Aiming for the identification of surface-accessible tumor antigens best suitable for antibody-based therapeutic intervention, it is important to analyze plasma membrane proteins known to be involved in endometrial cancer. For this purpose, we have utilized a novel proteomic technology by combining biotinylation-based approach for cell membrane enrichment and iTRAQ technology using nano liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. In this study, one normal endometrial cell line (EM-E6/E7/TERT cells, immortalized normal endometrial cells) and seven endometrial cancer cell lines were used as a comparative model for studying the plasma membrane proteins related to endometrial cancer. Among 272 proteins identified

by iTRAQ analysis, bone marrow stromal antigen 2 (BST2) was investigated in more detail. By immunohistochemical analysis using actual clinical specimens, we found that the expression level of BST2 was significantly higher in endometrial cancer tissues compared with normal endometrial tissues. An anti-BST2 antibody showed potent antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against BST2-positive endometrial cancer cells *in vitro*. In an *in vivo* xenograft model, anti-BST2 antibody treatment significantly inhibited tumor growth.

Taken together, our strategy of screening cell-surface tumor-specific antigens might be useful for identifying new therapeutic targets.

Material and Methods

Cell lines and cultures

We previously established an immortalized normal endometrial cell line (EM-E6/E7/TERT cells).[13,14] Nine human endometrial cancer cell lines (HEC-1, HEC-1A, HEC-6, HEC-88nu, HEC-108, HEC-116, HEC-251, SNG-II, and SNG-M cells) were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), where they were tested and authenticated on June 30, 2011. The method used for testing was multiplexed PCR amplification of eight short tandem repeat loci (TH01, D5S818, D13S317, D7S820, D16S539, CSF1PO, vWA, and TPOX) and amelogenin was performed using the PowerPlex™16 System (Promega, Madison, WI). PCR-amplified fragments were analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Then the fragments were typed based on allelic ladders. EM-E6/E7/TERT cells were maintained in a 1:1 mixture of DMEM and Ham's F12 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS (HyClone Laboratories, Logan, UT) and 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C under a humidified atmosphere of 5% CO_2. HEC-1, HEC-1A, HEC-6, HEC-88nu, HEC-108, HEC-116, and HEC-251 cells were maintained and propagated in DMEM (Wako Pure Chemical Industries) supplemented with 10% FBS and 1% penicillin-streptomycin. SNG-II and SNG-M cells were maintained in Ham's F12 (Invitrogen, Carlsbad, CA) with 10% FBS and 1% penicillin-streptomycin.

Biotinylation of bovine serum albumin (BSA)

BSA (30 µM) was biotinylated with a 100-fold molar excess of sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate

(sulfo-NHS-SS-biotin; Pierce, Rockford, IL) and desalted as described previously.[15]

Capture of cell-surface proteins

To isolate cell-surface proteins, the normal endometrial cell line (EM-E6/E7/TERT cells) and seven endometrial cancer cell lines (HEC-1, HEC-1A, HEC-6, HEC-108, HEC-116, HEC-251, and SNG-II cells) were grown to approaching confluency (up to 90%) in three 15 cm dishes. Cells were washed three times with prewarmed PBS and then the cell-surface proteins were biotinylated for 15 min at room temperature with 15 ml of 500 µM sulfo-NHS-SS-biotin solution dissolved in PBS. The residual biotinylation reagent was quenched with 5 mM lysine for 5 min at room temperature. After biotinylation, the cells were washed with PBS twice, harvested by scraping, and collected by centrifugation (1,500 rpm, 4°C, 5 min). Detailed methods of extraction and purification of biotinylated cell-surface proteins are described in the Supporting Information Materials and Methods section.

iTRAQ labeling

Trypsin-digested peptides were dissolved in 5 µl of 9.8 M urea and 20 µl of 1M TEAB. Samples were labeled with the iTRAQ reagent according to the manufacturer's protocol (Applied Biosystems). EM-E6/E7/TERT cells were labeled with iTRAQ reagent 113, HEC-1 cells with 114, HEC-1A cells with 115, HEC-6 cells with 116, HEC-108 cells with 117, HEC-116 cells with 118, HEC-251 cells with 119, and SNG-II cells with 121. The labeled peptide samples were then pooled and desalted with Sep-Pak Light C18 Cartridges (Waters, Manchester, UK) and peptides were dried in a centrifugal concentrator (Micro Vac MV-100, Tomy, Tokyo, Japan) before strong cation exchange (SCX) fractionation.

SCX fractionation

In order to remove excess unreacted iTRAQ reagent and to simplify the complexity of the peptide mixture, the labeled peptide mixtures were purified and fractionated using SCX column (SCX, PolySulfoethyl A column, 2.1 × 150 mm, 5 µm, 300 Å) on an Agilent 1200 HPLC system. Detailed information is provided in the Supporting Information Materials and Methods section.

Mass spectrometric analysis

Nano LC-MS/MS analyses were performed on an LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA) equipped with a nano-ESI source and coupled to a Paradigm MG4 pump (Michrom Bioresources, Auburn, CA) and autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland). Detailed information is provided in the Supporting Information Materials and Methods section.

iTRAQ data analysis

Protein identification and quantification for iTRAQ analysis was carried out using Proteome Discoverer software (v. 1.1)

(Thermo Fisher Scientific) against Swiss Prot human protein database (SwissProt_2011_11, 533,049 entries). Taxonomy was set to *Homo sapiens* (20,326 entries) or mammalian (65,656 entries). Search parameters for peptide and MS/MS mass tolerance were 10 ppm and 0.8 Da, respectively, with allowance for two missed cleavages made from the trypsin digest. Carbamidomethylation (Cys) and iTRAQ8plex (Lys, N-terminal) were specified as static modifications, whereas CAMthiopropanoyl (Lys, N-terminal), iTRAQ8plex (Tyr), and oxidation (Met) were specified as variable modifications in the database search. The false discovery rate of 1% was calculated by Proteome Discoverer based on a search against a corresponding randomized database. Relative protein abundances were calculated using the ratio of iTRAQ reporter ion in the MS/MS scan. For subcellular localization, all the proteins identified in this analysis were analyzed using the UniprotKB (available at: http://www.uniprot.org/) and Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA).

Quantitative reverse transcription-PCR (qRT-PCR) analysis

To confirm the altered expression of BST2 in endometrial cancer, the normal endometrial cell line (EM-E6/E7/TERT cells) and nine endometrial cancer cell lines (HEC-1, HEC-1A, HEC-6, HEC-88nu, HEC-108, HEC-116, HEC-251, SNG-II, and SNG-M cells) were subjected to qRT-PCR. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNAs were synthesized with a QuantiTect Reverse Transcription Kit (Qiagen), all according to the manufacturers' instructions. qRT-PCR was performed using SYBR Premix Ex taq (Takara Bio, Shiga, Japan) and an ABI 7900HT real-time PCR instrument (Applied Biosystems). β-Actin was used as a housekeeping gene for normalization of quantitative real-time PCR analysis. The primer sequences and the expected sizes of PCR products were as follows: BST2, forward primer 5′-GGAGGAGCTTGAGGGAGAG-3′ and reverse primer 5′-CTCAGTCGCTCCACCTCTG-3′, 75 bp; β-actin, forward primer 5′-AGCCTCGCCTTTGCCGA-3′ and reverse primer 5′-CTGGTGCCTGGGGCG-3′, 174 bp. Relative quantitation of gene expression was performed using the standard curve method as outlined by Applied Biosystems. Experimental conditions were tested in triplicate and three independent experiments were performed.

Fluorescence activated cell sorting (FACS) analysis

Cells were washed twice in PBS (Nacalai Tesque) and detached with 0.02% EDTA solution (Nacalai Tesque). Cells were washed twice with cold FACS buffer (PBS supplemented with 1% FBS and 0.1% sodium azide) and then incubated with mouse anti-human BST2 antibody (Biolegend, San Diego, CA) at a 1:100 dilution and labeled with Alexa Fluor 488-labeled donkey anti-mouse IgG antibody (Invitrogen). Stained cells were analyzed using a FACS Canto cytometer (Becton Dickinson, Mountain View, CA) and the results were analyzed using FlowJo software (Tree Star, Stanford, CA).

Patients and tissue samples

The formalin-fixed, paraffin-embedded tissue sections of 59 cases of normal endometrium and 118 cases of endometrial cancer were obtained from 177 patients who underwent surgical resections at Osaka University Hospital, Japan, between 1998 and 2007. Cases of normal endometrium were obtained from 59 patients who underwent simple hysterectomy for benign indications such as leiomyoma and uterine prolapse. Histological features of the tissues were reviewed by board-certified pathologists. The degree of histological differentiation and surgical pathological staging of 118 cases of endometrial cancer were assigned according to the 1988 recommendations of International Federation of Gynecology and Obstetrics. A summary of clinicopathological information for these patients is shown in Supporting Information Table S1. Written informed consent was obtained for all the cases and the experimental protocol was approved by the ethics committees of Osaka University and National Institute of Biomedical Innovation.

Immunohistochemistry

Sections were prepared from formalin-fixed, paraffin-embedded tissue specimens, deparaffinized, and rehydrated in graded alcohols. Immunohistochemical staining for BST2 was performed using the avidin-biotin-peroxidase complex (ABC) method using a rabbit polyclonal anti-BST2 antibody (Sigma-Aldrich, St. Louis, MO) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Immunostained sections were photographed with an Olympus FSX100 (Olympus, Tokyo, Japan). Detailed information is provided in the Supporting Information Materials and Methods section.

Evaluation of immunohistostaining

Immunostainings were scored according to the intensity of the staining (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) and the extent of stained cells (0–9% = 0, 10–40% = 1, 41–70% = 2, 71–100% = 3). The final immunohistochemistry (IHC) score was determined by multiplying the intensity score (0, 1, 2, or 3) with the positivity score (0, 1, 2, or 3), resulting in a maximum score of 9. Three independent gynecologic oncologists (Y.U., K.Y., and M.F.), blinded to the histological data, analyzed the stained sections using an Olympus BH2 microscope (Olympus). In case of disagreement, the staining results were re-evaluated by careful discussion until a consensus was reached.

Cell proliferation assay

Endometrial cancer cells plated in 96-well plates (1,000 cells per well) were grown in their respective media for 24, 48, or 72 hr after the addition of antibody or small interfering RNA (siRNA) transfection. At each time point, cell proliferation was assessed by a WST-8 assay according to the manufacturer's protocol (Nacalai Tesque). Detailed information for these assays can be found in the Supporting Information Materials and Methods section.

ADCC assay

ADCC was measured by calcein-acetoxymethyl ester (calcein-AM) release assay, with sensitivity similar to the traditional ^{51}Cr release assay.[16,17] Detailed information for this assay can be found in the Supporting Information Materials and Methods section.

CDC assay

CDC was evaluated using a ^{51}Cr release assay.[18] Detailed information for this assay can be found in the Supporting Information and Methods section.

Tumor xenograft and antibody therapy

Healthy female severe combined immunodeficient (SCID) and nonobese diabetic (NOD)/SCID mice at 8 weeks of age were obtained from Charles River Japan (Yokohama, Japan) and maintained in a specific pathogen-free facility. For subcutaneous xenograft experiments, SCID mice were inoculated subcutaneously with 5×10^6 HEC-88nu or SNG-II cells in a total volume of 50 μl of 1/1 (v/v) PBS/Matrigel (Becton Dickinson) into the abdomen. NOD/SCID mice were inoculated with 5×10^6 HEC-88nu cells. PBS, isotype control (mouse IgG2aκ, Sigma-Aldrich), or mouse anti-human BST2 antibody (clone 1B4: Chugai Pharmaceutical) was administered intraperitoneally at a dose of 5 mg/kg (SNG-II) or 10 mg/kg (HEC-88nu) in 400 μl of PBS. Six mice were used per group. The first dose was given on day 4 (SNG-II) or 9 (HEC-88nu) and continued twice weekly for 4 weeks. Tumors were measured twice weekly from days 4 (SNG-II) or 9 (HEC-88nu) using vernier calipers throughout the study. Tumor volumes were calculated using the following formula: tumor volume (mm^3) = length × width × height. After 8 (HEC-88nu) or 12 (SNG-II) weeks, tumors were resected and weighted. All animal experiments were conducted according to the institutional ethical guidelines for animal experimentation of the National Institute of Biomedical Innovation.

Statistical analysis

For immunohistochemistry, statistical significance of difference between normal endometrium and endometrial cancer was analyzed by the nonparametric Mann–Whitney U test. Differences in the *in vitro* cytotoxic assay were determined by using the Kruskal–Wallis test followed by the Steel procedure. For all subcutaneous tumor comparisons, groups were analyzed using the Kruskal–Wallis test followed by the Steel-Dwass procedure.

Results

Protein expression profiles in normal endometrium and endometrial cancer

To identify potential therapeutic targets of endometrial cancer, we performed comparative protein expression profiling between normal endometrium (EM-E6/E7/TERT cells) and

endometrial cancer (HEC-1, HEC-1A, HEC-6, HEC-108, HEC-116, HEC-251, and SNG-II cells) at the cell surface level. We identified a total of 272 proteins by a biotinylation-based approach for cell membrane enrichment combined with iTRAQ technology using nano LC-MS/MS analysis. The complete list of all the proteins identified is shown in Supporting Information Table S2. The list of proteins identified with single peptide is provided in Supporting Information Table S3. MS/MS spectra of all single-peptide-based assignments with masses detected as well as fragment assignments are presented in Supporting Information Table S4. The raw MS data of this analysis is publicly available for download from PeptideAtlas (available at: http://www.peptideatlas.org/PASS/PASS00032). To correct the error of quantitation during chromatographic procedures, we added the equivalent moles of the sulfo-NHS-SS-biotin labeled BSA into the each sample as an internal standard. The iTRAQ ratio of BSA (0.873 to 1.131, Supporting Information Table S3) was used for the correction of quantitation information accurately. According to the annotation from UniprotKB and Ingenuity Pathway Analysis, 139 proteins (51% of the identified proteins) were located in the plasma membrane (Fig. 1a). Among these 139 plasma membrane proteins identified, 11 proteins were increased more than twofold in at least four of seven endometrial cancer cell lines compared with the normal endometrial cell line (Table 1). As expected, neural cell adhesion molecule L1, a plasma membrane protein previously known to be overexpressed in endometrial cancer, was identified again. Interestingly, BST2 was found to show one of the most significant differences in expression between normal endometrial cells and endometrial cancer cells, making it a prime target.

Confirmatory studies by qRT-PCR and FACS

To confirm the altered expression of BST2 in endometrial cancer, we first evaluated its transcripts by qRT-PCR in the normal endometrial cell line (EM-E6/E7/TERT cells) and nine endometrial cancer cell lines (HEC-1, HEC-1A, HEC-6, HEC-88nu, HEC-108, HEC-116, HEC-251, SNG-II, and SNG-M cells). BST2 mRNA expression was clearly detected in seven of the nine endometrial cancer cell lines, while the normal endometrial cell line showed no detectable expression of BST2 transcripts (Fig. 1b).

We then evaluated the expression of BST2 at the protein level and confirmed the surface localization of BST2 by FACS analysis. Protein expression of BST2 was very weak in EM-E6/E7/TERT cells. In contrast, a considerably higher level of BST2 protein expression was detected in six of the nine endometrial cancer cell lines on the cell surface (Fig. 1c). Together our data demonstrate that BST2 was overexpressed in endometrial cancer cells at both the mRNA and protein level; this was consistent with our iTRAQ analysis.
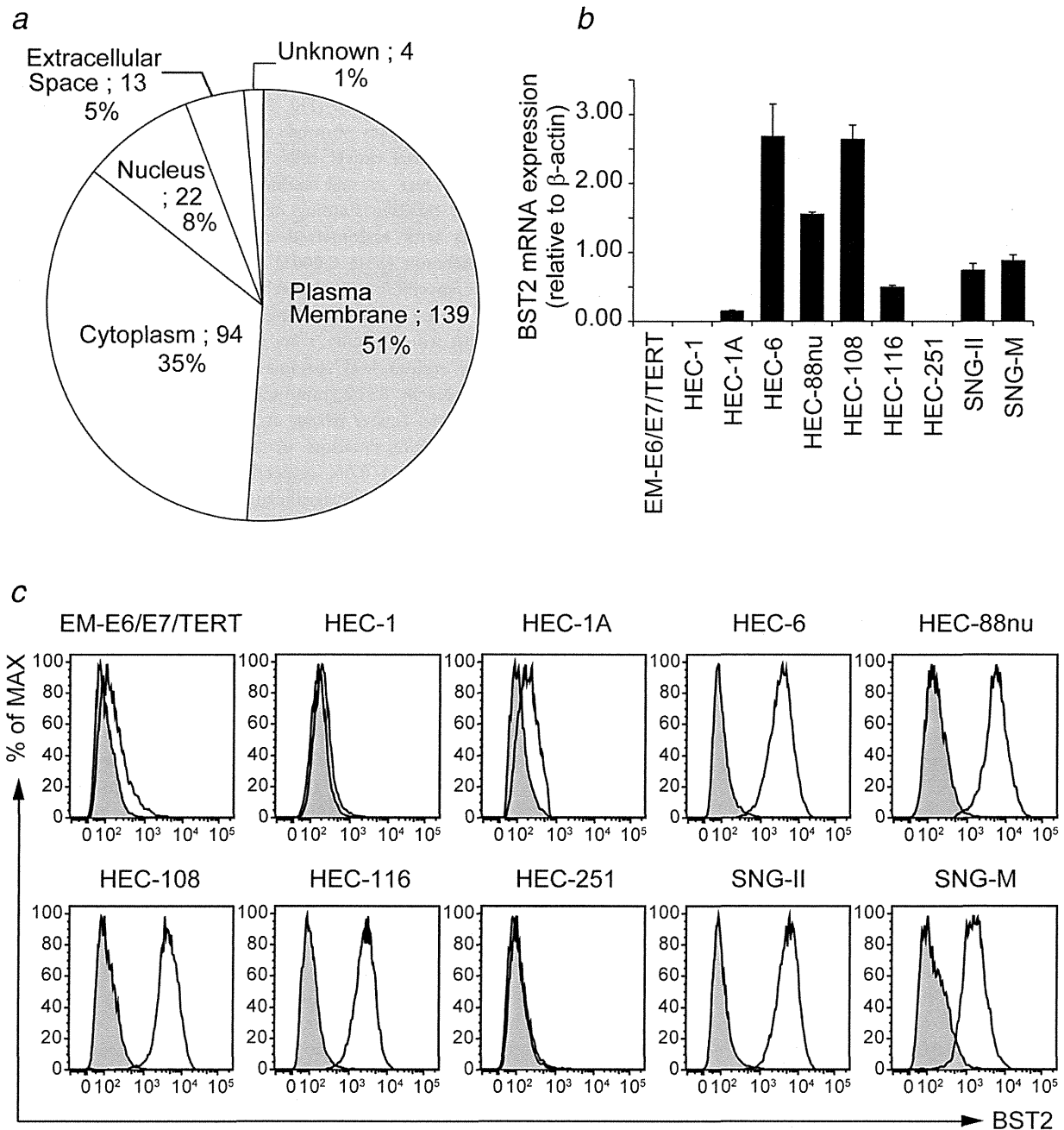
Validation study by IHC

As a validation study, immunohistochemical analyses were performed by examination of the BST2 expression pattern

in paraffin-embedded tissue samples (Supporting Information Table S1). Representative immunohistochemical staining of BST2 in tissue sections from patients revealed intense BST2 staining in endometrial cancer compared with normal endometrium (Fig. 2a). In addition, immunohistochemical analyses showed membranous immunoreactivity in endometrial cancer cells, indicating that the localization of BST2 was at cell surface. We observed significantly stronger positive staining of BST2 in tissue sections from patients with endometrial cancer compared with normal endometrium ($p < 0.0001$) (Fig. 2b). In 118 endometrial cancer specimens, moderately to strongly positive staining (IHC score = 3–9) was detected in 71.2% of specimens (84 of 118), whereas only 1.7% (1 of 59) were positive in the normal endometrial specimens. There were no significant differences in BST2 immunohistochemical staining among endometrial cancer tissues according to their degree of histological differentiation or surgical pathological staging ($p = 0.77$ and 0.06, respectively, by the Kruskal–Wallis test). There were no significant differences in BST2 staining among normal proliferative-phase, secretory-phase, and atrophic endometrium ($p = 0.82$ by the Kruskal–Wallis test). These results indicate that BST2 was overexpressed on the cell surface of endometrial cancer tissues much more frequently than in normal endometrium, raising the possibility that BST2 might represent a potential therapeutic target.

BST2-siRNA and anti-BST2 antibody treatment *in vitro*

To examine whether the BST2 expression contributes to cell proliferation of endometrial cancer cells, the effect of BST2-siRNA treatment in four of the endometrial cancer cell lines expressing BST2 (HEC-6, HEC-88nu, HEC-116, and SNG-II cells) was evaluated using the WST-8 assay. To ensure silencing efficiency, BST2 expression was analyzed by FACS analysis after 48 hr of siRNA transfection. The two siRNAs targeting BST2 (Hs_BST2_1 and Hs_BST2_5) had a similar silencing effect on the protein level (Supporting Information Fig. S1). There were no significant differences in cell proliferation among BST2-siRNA and control-siRNA treated cells (Fig. 3a). Similarly, anti-BST2 antibody treatment did not affect *in vitro* cell proliferation (Fig. 3b).

We subsequently examined whether an anti-BST2 antibody can induce ADCC among endometrial cancer cells using the calcein-AM release assay. To study the specificity of anti-BST2 antibody-mediated ADCC against BST2-expressing target cells, an ADCC assay was performed using a BST2-expressing endometrial cancer cell line (HEC-88nu cells) and a BST2-negative cell line (HEC-1 cells). As shown in Figure 3c, HEC-88nu cells treated with the anti-BST2 antibody showed specific lysis *via* ADCC ($p = 0.045$), whereas the anti-BST2 antibody showed no lytic activity against HEC-1 cells.

a Extracellular Space ; 13 5% / Unknown ; 4 1% / Nucleus ; 22 8% / Cytoplasm ; 94 35% / Plasma Membrane ; 139 51%

b

c EM-E6/E7/TERT, HEC-1, HEC-1A, HEC-6, HEC-88nu, HEC-108, HEC-116, HEC-251, SNG-II, SNG-M

Cancer Therapy

Figure 1. (*a*) Subcellular localization of the identified 272 proteins analyzed by UniprotKB and Ingenuity Pathway Analysis. (*b*) Confirmation of iTRAQ results by qRT-PCR. qRT-PCR was used to quantify BST2 mRNA; β-actin was used as the internal control. Data are mean ± SEM of three independent experiments, each performed in triplicate. BST2 mRNA expression was not detected in the normal endometrial cell line (EM-E6/E7/TERT cells), but seven of nine endometrial cancer cell lines exhibited positive expression of BST2 mRNA. (*c*) Confirmation of iTRAQ results by FACS analysis. The shaded histogram profile indicates the isotype control, and the open histogram indicates the anti-BST2 antibody staining results.

We also examined CDC exhibited by the anti-BST2 antibody. Figure 3*d* shows that the BST2-expressing endometrial cancer cell line (HEC-88nu cells), but not the BST2-negative cell line (HEC-1 cells), was sensitive to CDC ($p = 0.045$).

Therapeutic effect of the anti-BST2 antibody *in vivo*
To evaluate the therapeutic efficacy of anti-BST2 antibody therapy, *in vivo* studies were performed using an endometrial cancer xenograft model. SCID mice injected with either