Table 3 Toxicities

	Level 1 ($n = 3$)				Level 2 ($n = 3$)				Level 3 ($n = 3$)				Level 4 ($n = 3$)			
Toxicity grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Hematological																
Leukopenia	0	3	0	0	0	2	1	0	0	2	1	0	0	2	1	0
Neutropenia	0	3	0	0	0	2	0	1	0	2	0	1	0	1	0	2
Thrombocytopenia	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0
Anemia	3	0	0	0	2	1	0	0	1	1	0	0	2	0	0	0
Nonhematological																
Mucositis	1	0	0	0	1	0	0	0	0	0	0	0	3	0	0	0
Hand foot	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0
Diarrhea	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Nausea	2	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0
Vomiting	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Appetite loss	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0

was observed. The only grade 2 or higher nonhematological toxicity was grade 2 hand-and-foot syndrome, which occurred in one patient at level 4 (Table 3).

Administration status

In total, 43 treatment courses were administered. Table 4 shows the status of postponement of the next course, dose skipping, and dose reduction in each patient. The start of the next course was postponed due to the lack of recovery from neutropenia in one patient each at levels 1, 3, and 4 and in two patients at level 2. All of these patients started the next course within 7 days without using granulocyte colony-stimulating factor. Postponement of the next course due to the lack of recovery from hand-and-foot syndrome occurred in one patient at level 4, but the next course was started within 7 days.

CPT-11 on day 15 was skipped in one patient each at level 1 and level 4, with the rate of skipping this treatment being 4.7 %. In the patient at level 1, CPT-11 administration in the second course was postponed because the neutrophil count on day 15 did not meet the criterion for administration, and the study was terminated during the second course at the discretion of the attending physician. In the patient at level 4, CPT-11 administration on day 15 in the third course was postponed, and due to the lack of recovery from leukopenia, the study was terminated at the discretion of the attending physician.

Table 4 Administration situation of CPT-11 and PLD

Patient no.	Level	CPT-11 (mg/m^2)	Stage	Cell type	Total cycles	Delay cycles (day 1)	Skip cycles (day 15)	Dose reduction
1	1	50	IIIc	SAC	4	2		
2	1	50	IIIc	SAC	4			
3	1	50	IIIc	SAC	2		1	
4	2	60	IIIc	EM	4	2		CPT-11 (−10mg/m^2), PLD (−7.5mg/m^2)
5	2	60	IIIb	SAC	4	1		
6	2	60	IIIc	CCC	4			
7	3	70	IV	SAC	4			
8	3	70	IIc	SAC	4			
9	3	70	Ic	CCC	2	1		CPT-11 (−10mg/m^2), PLD (−7.5mg/m^2)
10	4	80	Ic	CCC	4	1		
11	4	80	IIIc	SAC	3		1	CPT-11 (−10mg/m^2), PLD (−7.5mg/m^2)
12	4	80	IIIc	SAC	4			

SAC serous adenocarcinoma, *EMC* endometrioid adenocarcinoma, *CCC* clear cell carcinoma, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease

CPT-11 and PLD doses were reduced in one patient each at levels 2, 3, and 4 because of grade 4 neutropenia in the previous course. The doses were reduced in the second course in the patients at levels 2 and 3, and in the third course in the patient at level 4.

Determination of recommended dose

Three patients were assigned to each dose level, and none of them experienced DLT during the first course, precluding the determination of MTD. Therefore, the individual cases were analyzed in detail. At level 4, grade 4 leukopenia was observed in two of three patients and grade 2 leukopenia in 1. The antitumor effect at level 4 was a PR in two of the three patients and stable disease (SD) in 1. The patient with SD had clear cell adenocarcinoma. At level 3, PR was observed in one of the three patients and SD in 2. As regards treatment postponement at level 4, CPT-11 administration on day 1 was postponed in the fourth course in 1 patient, and day-1 administration in the second course and day-15 administration in the third course were postponed in 1 patient (treatment in this patient was terminated due to the lack of recovery from adverse reactions). Thus, postponement of the next course occurred in two of the three patients. In addition, in one patient at level 4, CPT-11 and PLD doses were reduced in the third course because of grade 4 neutropenia in the previous course. These results suggested that more severe neutropenia would occur if CPT-11 is increased to level 5 (90 mg), although a greater antitumor effect could be achieved. Also, it was expected that the start of the next course would be postponed, and the dose would have to be reduced in a greater number of patients. Based on the above considerations, it was concluded that the recommended CPT-11 dose should be 80 mg/m^2.

Antitumor effect

Table 5 shows antitumor effects at each level. CR was observed in one patient (8.3 %), PR in six (50.0 %), SD in two (16.7 %), and PD in three (25.0 %), with the response rate being 58.3 % and the disease control rate 75.0 %.

Table 5 Treatment response

	CR	PR	SD	PD	Overall response (%)
Level 1 (n=3)	1	1	0	1	66.7
Level 2 (n=3)	0	2	1	0	66.7
Level 3 (n=3)	0	1	0	2	33.3
Level 4 (n=3)	0	2	1	0	66.7
Total (n=12)	1	6	2	3	58.3

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

Discussion

The combined use of the topo-I inhibitor CPT-11 and the topo-II inhibitor PLD is expected to be effective, as suggested by their synergistic mechanisms of action. Also, this combination therapy allows dose reductions in each drug as compared with the monotherapy doses, thereby reducing the severity and frequency of adverse events without decreasing the antitumor effect. In light of the above, CPT-11/PLD is expected to be effective against recurrent or advanced ovarian cancer resistant to platinum or taxane agents. In the phase II clinical study on CPT-11 50 mg/m^2 (days 1, 8, 15) and doxorubicin (DXR) 40 mg/m^2 (day 3) combination therapy for recurrent ovarian cancer, Nishimura et al. [11] reported that the response rate was 23.5 % (CR, 1 patient; PR, 3 patients of 17 patients) and that the grade 3/4 adverse reactions observed were neutropenia (CPT-11, 52.9 %; DXR, 35.2 %), thrombocytopenia (5.9 %, 17.6 %), anemia (17.6 %, 0 %), and diarrhea (5.9 %, 0 %). In the present study, the rates of skipping CPT-11 on days 8 and 15 were 6.0 and 22.0 %, respectively, and that of DXR on day 3 was 4.0 %. In the phase II study on CPT-11/etoposide combination therapy for recurrent small-cell lung carcinoma, Masuda et al. [12] reported that 6 of 25 patients (24 %) received two doses of CPT-11(days 1 and 8 or days 1 or 15), and 3 of 25 patients (12 %) could receive only one dose of CPT-11. By referring to the results of the two aforementioned phase II clinical studies, we designed an administration schedule for CPT-11/PLD combination therapy. If CPT-11 was to be administered on a weekly basis, it was anticipated that the rates of skipping on days 8 and 15 would be high, resulting in 30–50 % of patients skipping day 15 administration. Therefore, it was decided to administer CPT-11 on a biweekly basis (day 1, day 15). Also, it was reported that, in giving combination therapy with a topo-I inhibiter and a topo-II inhibiter, these agents act competitively rather than synergistically if administered simultaneously [13]. By also taking account of the finding that CPT-11 is metabolized in 72 h [14], it was decided that PLD would be administered on day 3, as has been the case with combination therapy employing DXR. In past reports, the dose of PLD in the combination therapy for recurrent ovarian cancer was 25–30 mg/m^2 [15, 16]. While the recommended dose for PLD monotherapy is 50 mg/m^2, sufficient efficacy and reduced adverse reactions have also been reported at the dose of 40 mg/m^2 [17]. Therefore, in consideration of reduced adverse reactions such as hand-and-foot syndrome and mucositis, the PLD dose was fixed at 30 mg/m^2. As to CPT-11, no clinical study results are available for the combination with PLD or doxorubicin by the biweekly method and its optimal dose is unknown, and enormous variation in the dose response of CPT-11 among individuals, in general, is known [18]; therefore, the dose of CPT-11 alone was increased. A phase I clinical study was

planned to investigate the efficacy and safety of CPT-11/PLD combination therapy for recurrent ovarian cancer.

Adverse events were evaluated for each patient in the first course only. As shown in Table 3, although adverse events were observed, all were nonserious and manageable. Thus, the phase I clinical study could be conducted safely. Shoji et al. [19] reported that the rate of skipping CPT-11 on day 15 was 2.3 % with the combination therapy based on CPT-11 (60 mg/m^2) biweekly administration with etoposide oral administration for recurrent ovarian cancer. In our present study as well, CPT-11 was administered on a biweekly basis (days 1, 15), and as a result, day 15 administration was skipped in only 1 patient each at level 1 and level 4. Thus, CPT-11 on day 15 was skipped in only 2 of a total of 43 courses, with the rate of skipping treatment being just 4.7 %, suggesting biweekly administration of CPT-11 in CPT-11/PLD combination therapy to be appropriate. Since no DLT was observed at any of the levels tested, it would be feasible to increase the dose of CPT-11 to level 5 under ordinary circumstances. However, as mentioned above, as a result of detailed evaluation of grade 4 neutropenia in three patients at level 4, postponement to the next course, dose reduction, and antitumor effect, the recommended CPT-11 dose was determined to be 80 mg/m^2.

The response rate to combination therapy using CPT-11 or PLD for platinum-resistant recurrent ovarian cancer is 41.9 % with CPT-11/VP16 therapy according to Shoji et al. [19] and 44.4 % according to Nishio et al. [20], and 20 % with DTX/CPT-11 therapy according to Polyzos et al. [21]. The response rate was also reported to be 22 % with PLD/Gemcitabine therapy by Skarlos et al. [22] and 40 % [23] by Mirza et al. The response rate to CPT-11/PLD combination therapy was 58.3 %, a result not inferior to those reported previously. All 12 enrolled patients had previously been treated with taxane or platinum agents. In particular, eight of them had recurrence or recrudescence within 6 months after TC therapy. It is expected that CPT-11/PLD combination therapy will achieve a high response rate and that adverse reactions will be mild and manageable. This drug combination may thus be useful as an option for second-line chemotherapy for recurrent ovarian cancer within 6 months after TC therapy.

In the future, a phase II clinical study will be conducted to validate the usefulness of CPT-11/PLD combination therapy.

Conflict of interest The authors have no conflicts of interest to declare.

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FULL PAPER

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X-chromosome-linked inhibitor of apoptosis as a key factor for chemoresistance in clear cell carcinoma of the ovary

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Background: X-chromosome-linked inhibitor of apoptosis (XIAP) is one of the anti-apoptotic proteins leading to chemoresistance in several cancers. The aim of this study is to evaluate the impact of XIAP expression upon ovarian clear cell carcinoma (CCC) that has a platinum-resistant phenotype.

Methods: Tissue microarrays made from 90 CCC patients were analysed for immunohistochemical expression levels of XIAP, c-Met, p-Akt and Bcl-XL. In addition, CCC cell lines were evaluated whether XIAP silencing could modulate sensitivity to platinum agent *in vitro*.

Results: High XIAP expression was observed in 30 (33%) of 90 CCC cases, and was associated with c-Met (<0.01) and Bcl-XL (<0.01) expression. Cases with high XIAP expression had lower response rate to primary platinum-based chemotherapy (10% *vs* 65%, $P=0.02$). In stages II–IV tumours, high XIAP expression was related with worse progression-free survival (PFS, $P=0.02$). Furthermore, high XIAP expression was identified as an independent worse prognostic factor for PFS and overall survival. Finally, downregulation of XIAP using XIAP-specific small interfering RNA increased sensitivity to cisplatin in human cancer cells derived from CCC.

Conclusions: X-chromosome-linked inhibitor of apoptosis expression was correlated with chemoresistance of primary chemotherapy, and identified as a prognostic marker for CCC. X-chromosome-linked inhibitor of apoptosis could be a candidate for new therapeutic target in CCC.

In 1973, clear cell carcinoma (CCC) of the ovary was pathologically defined by World Health Organisation as lesions characterised by clear cells growing in solid/tubular or glandular patterns as well as hobnail cells (Serov *et al*, 1973). Since then, many publications have identified the distinctive behaviour of CCC. First, CCC showed chemoresistance to primary chemotherapy using platinum-based therapy and combination with paclitaxel and platinum (Takano *et al*, 2012). Lower response was also reported in the second-line chemotherapy (Takano *et al*, 2008), and response duration was extremely short (Takano *et al*, 2013). Second, CCC had significantly worse overall survival (OS) in advanced-staged tumours (Sugiyama *et al*, 2000; Winter *et al*, 2007). New strategy is needed for further treatment of CCC.

X-chromosome-linked inhibitor of apoptosis (XIAP) is over-expressed in several cancers including ovarian tumours (Lacasse *et al*, 1998; Tamm *et al*, 2000; Yang *et al*, 2003), and most reports showed that XIAP is a major contributor to chemoresistance to anticancer drugs. Caspase-3 had an important role for cisplatin-induced apoptosis in ovarian cancer cells, and cisplatin induced apoptosis through the decrease of XIAP protein levels and the

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increase of caspases-3 activity in cisplatin-sensitive ovarian serous cancer cells in a dose-dependent manner, but the effect was not evident in cisplatin-resistant cells (Asselin *et al*, 2001). Overexpression of XIAP by adenoviral sense XIAP complementary DNA attenuated the ability of cisplatin to induce apoptosis (Li *et al*, 2001). On the other hand, downregulation of XIAP using adenoviral anti-sense XIAP infection or small interfering RNA (siRNA) increased sensitivity to cisplatin in resistant serous ovarian cancer cells (Sasaki *et al*, 2000; Ma *et al*, 2009). Thus, XIAP was strongly associated with platinum resistance of ovarian cancer cells. However, these investigations were performed by ovarian serous cancer cells, and the roles of XIAP in CCC have not been analysed. Also, downregulation of c-Met decrease XIAP protein levels in ovarian cancer cells, suggesting that XIAP was regulated by hepatocyte growth factor (HGF) /c-Met pathway through Akt (Bu *et al*, 2011). Although c-Met has been associated with tumourigenesis in CCC (Yamamoto *et al*, 2011), the relationship of XIAP expression and c-Met has not been studied.

Herein, we assessed the immunoreactivities of XIAP, c-Met, p-Akt and Bcl-XL, and analysed the correlation of XIAP expression and clinicopathologic characteristic of CCC. Moreover, we investigated whether XIAP downregulation could sensitise ovarian CCC cells to platinum agents.

MATERIALS AND METHODS

Patients and tissue microarray. Tissue blocks from 92 patients with CCC who received primary debulking surgery at National Defense Medical College hospital between 1984 and 2008 were identified. In all, 1.5-mm cores were punched from donor blocks and inserted into a recipient block. All specimens were cut into 4-μm-thick sections. Satisfactory immunohistochemical staining was obtained in 90 cases, and these cases were enrolled in this study. No patient had received neoadjuvant chemotherapy before primary surgery. The research was approved by the Ethic Committee of National Defense Medical College, Tokorozawa, Japan.

Immunochemistry and interpretation. We used mouse monoclonal antibody for MET4 (8G6 dilution 1 : 150; Knudsen *et al*, 2009), mouse monoclonal antibody for XIAP (48; dilution 1 : 300; BD Transduction, Franklin Lakes, NJ, USA), rabbit monoclonal antibody for phospho-Akt (p-Akt; Ser473; dilution 1 : 300; Cell Signaling Technology, Danvers, MA, USA) and rabbit monoclonal antibody for Bcl-XL (54H6; dilution 1 : 300; Cell Signaling Technology), respectively. Tissue microarray slides were deparaffinised in xylene and hydrated with alcohol and boiled in an autoclave at 121 °C for 15 min in 0.01 mol l^{-1} citrate buffer (pH 6.0) and then allowed to cool at room temperature. Endogenous peroxidase was blocked by 0.3% H_2O_2/methanol. The slides were incubated at 4 °C overnight with primary antibodies and reacted with EnVision + system HRP-labelled polymer (DAKO Japan Inc., Bunkyo-ku, Tokyo) as secondary antibody for 30 min at room temperature. Specific antigen-antibody reactions were visualised with 0.2% diaminobenzine tetrahydrochloride and hydrogen peroxide, and counterstained with Mayer haematoxylin. For each antibody, negative control studies were performed without the primary antibody. No significant staining was observed in the negative control sections. As positive control, formalin-fixed paraffin-embedded tissue microarrays from 20 ovarian serous adenocarcinomas were stained. Ovarian serous adenocarcinomas were known to be stained previously by all antibodies (Bu *et al*, 2011; Yamamoto *et al*, 2011).

Immunoreactivity was scored according to intensity as negative (−), moderate (1 +) or strong (2 +). If cases had 2 + and > 50% of immunoreactive components, they were defined as high expression. Cases without high expression were defined as low expression. Two observers independently evaluated and interpreted the results of immunohistochemical staining without knowledge of the clinical data of each patient. In the interpretation of immunohistochemistry, any discrepancies between the two observers were resolved by discussion, and using a multiviewer microscope.

Cell lines and culture conditions. Ovarian cancer cell line derived from clear cell carcinoma, KK (Sasa *et al*, 1993), was used in this study. These cell lines were grown as monolayer cultures in RPMI-1640 (Life Technologies, Grand Island, NY, USA) + GlutmaxTM-I (Invitrogen Japan KK, Tokyo, Japan) medium supplemented with 10% foetal bovine serum (Invitrogen Japan KK), 100 U penicillin per ml, and 100 mg streptomycin per ml (Invitrogen Japan KK) in a humidified atmosphere of 5% CO_2 at 37 °C, and routinely tested for mycoplasma infection.

Transient transfection. Nonspecific control siRNA, XIAP-specific siRNA (Signal Science XIAP siRNA, #6446) and XIAP-specific siRNA II (Signal Science XIAP siRNA, #6550) were purchased from Cell Signaling Technology. KK cells cultured in 3.5 cm plates were transfected with 107 nM of XIAP siRNA or XIAP siRNA II and control siRNA using Lipofectamine 2000 (Invitrogen Japan KK) according to the manufacturer's specifications. X-chromosome-linked inhibitor of apoptosis knockdown was confirmed by western blot analysis in all the experiments. Names of the transfected cells using nonspecific control siRNA, XIAP-specific siRNA and XIAP siRNA II were determined as KK-C, KK-I and KK-II, respectively.

Cell proliferation and cytotoxicity assay. Cisplatin was obtained from Bristol Meier's Squib Oncology (Tokyo, Japan). KK-C, KK-I and KK-II were seeded onto 96-well plates at approximately 1×10^4 or 4×10^4 cells cm^{-2} for cytotoxicity assays after 24 h from the treatment at different cisplatin concentrations (0, 10, 20 and 30 μM). Cell viability was determined by MTT method using Tetra Color One (Seikagaku Corporation, Chiyoda-ku, Japan) according to the manufacturer's instructions.

Preparation of cell lysate for western blot analysis. Protein lysates were extracted in RIPA buffer (Wako Pure Chemical Industries Ltd, Osaka, Japan) according to the manufacturer's instructions. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis of 10 μg cytosolic fractions loaded onto Mini-PROTEIN TGXTM gel (Bio-Rad Laboratories), proteins were transferred to PVDF membranes using Trans-Blot Turbo Transfer System Transfer Pack (Bio-Rad Laboratories). Subsequently, the membranes were blocked for 1 h in 4% BSA, and incubated overnight at 4 °C in primary antibodies. The following antibodies and concentrations were used: 1 out of 2000 rabbit anti-XIAP (Cell Signaling Technology), 1 out of 1000 rabbit monoclonal antibody for phospho-Akt (p-Akt; Ser473, Cell Signaling Technology), 1 out of 1000 rabbit monoclonal antibody for Bcl-XL (54H6, Cell Signaling Technology), 1 out of 1000 CONFIRM anti-total c-MET (SP44) rabbit monoclonal antibody for C-Met (Ventana Medical Systems, Tucson, AZ, USA), 1 out of 1000 rabbit monoclonal antibody for PTEN (138G6, Cell Signaling Technology) and 1 out of 5000 rabbit β-actin (Cell Signaling Technology). After washes with TBS-T, membranes were incubated for 1 h at room temperature using horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology), and were visualised using the ECL detection system (GE Healthcare UK Ltd, Buckinghamshire, England) by a LAS-3000 imaging system (Fujifilm, Minato-ku, Tokyo). Protein expression was determined

densitometrically and normalised against β-actin expression using the software Multi Gauge version 3.1 (Fujifilm).

Statistical analysis. The Stat View software ver.5.0 (SAS Institution Inc., Cary, NC, USA) was used for statistical analysis. Progression-free survival (PFS) was defined as the interval between the primary surgery and death or the date of progression disease. Overall survival was defined as the interval between the primary surgery and death. Staging was performed according to FIGO system. Performance status (PS) was evaluated by WHO criteria. Response rate was evaluated by using Response Evaluation Criteria in Solid Tumours (RECIST) criteria (Therasse *et al*, 2000). The χ^2-test, Fisher's exact test and Mann-Whitney *U*-test were used to evaluate clinical significance of protein expression in clinicopathological parameters. Progression-free survival and OS curves were generated using the method of Kaplan–Meier. Comparisons of the survival distribution were made with log-rank test. Cox proportional hazards model was used for multivariate analysis of PFS and OS. All experiments were repeated independently at least four times. All values are presented as mean ± s.d. Statistical significance between two groups was determined by use of a two-tailed *t*-test. Statistical significance was defined as a $P<0.05$.

RESULTS

Patients' characteristics and XIAP expression. A total of 90 cases with CCC were enrolled in this study. Median age of the patients was 52 years (range 35–72). FIGO stage distribution was as follows: 46 cases (51%) in stage I, 9 cases (10%) in stage II, 31 cases (34%) in stage III and 4 cases (5%) in stage IV. Ten cases (11%) had <1 cm residual tumours and 17 cases (19%) had >1 cm residual tumours. Eighty-six cases (96%) underwent platinum-based chemotherapy as postoperative primary chemotherapy. Four cases with stage I disease refused to receive chemotherapy, despite physicians' recommendation. Distribution of XIAP immunoreactivity according to clinicopathological characteristics was shown in Table 1. X-chromosome-linked inhibitor of apoptosis expression was not influenced by age at diagnosis, WHO PS, FIGO stage and residual tumour diameter. Immunohistochemically, high expression levels of XIAP, c-Met, phospho-Akt and Bcl-XL were observed in 30 (33%), 31 (34%), 23 (26%) and 33 (37%), respectively. Representative staining of these proteins were shown in Supplementary Figure 1. The correlation of expression levels of c-Met, p-Akt and Bcl-XL in CCC tissues according to XIAP expression was also summarised in Table 1. High expression of XIAP was correlated with high expression of c-Met ($P<0.01$) and Bcl-XL ($P<0.01$). However, there was no significant association between expression levels of phospho-Akt and XIAP ($P=0.23$).

XIAP expression in stage I clear cell adenocarcinoma of the ovary. Among 46 cases with stage I disease, there were no significant differences of age, physical status, FIGO stage and delivery of chemotherapy according to XIAP expression (Supplementary Table S1). In 46 cases with stage I disease, there were no statistical differences of PFS ($P=0.22$, Supplementary Figure 2A) and OS ($P=0.99$, Supplementary Figure 2B) according to XIAP expression. In multivariate analysis for PFS and OS using variables of age, peritoneal cytology, completion of surgical staging and XIAP expression, high expression of XIAP was not identified as a prognostic factor for PFS ($P=0.29$) and OS ($P=0.99$).

XIAP expression in stages II–IV clear cell adenocarcinoma of the ovary. Expression levels of XIAP, c-Met, phospho-Akt and Bcl-XL according to response of primary chemotherapy were assessable in 27 patients who had measurable disease. All cases received platinum-based chemotherapy after primary surgery. High XIAP expression was significantly correlated with response to primary chemotherapy in CCC patients (Table 2). Significantly more cases of responders had low expression of XIAP compared with non-responders (90% *vs* 35%, $P<0.01$). Expression levels of c-Met, phospho-Akt, and Bcl-XL were not related with response to chemotherapy.

Among 44 cases with stages II–IV tumours, high XIAP expression was observed 18 cases (Supplementary Table S2). There were no statistical differences of XIAP expression according to age, WHO physical status, FIGO stage and residual tumours after primary surgery. There was a significant difference of PFS according to XIAP expression ($P=0.02$, Figure 1A), however, significant difference was not observed in OS ($P=0.07$, Figure 1B). In univariate analysis, there were no significant differences of PFS and OS according to expression of c-Met, phospho-Akt and Bcl-XL. Multivariate analysis for PFS and OS in 44 cases was shown in Table 3. In addition to residual tumour diameter, XIAP expression was identified as an independent prognostic factor for PFS (hazard ratio = 2.94, $P=0.02$) and OS (hazard ratio = 2.70, $P=0.04$).

Downregulation of XIAP by siRNA and sensitivity to cisplatin. Further, we investigated whether XIAP downregulation by siRNA could increase sensitivity to cisplatin in KK cells, which were derived from human ovarian clear cell carcinoma. First, XIAP expression ratio compared with no transfection was 73.1 ± 12.7%

Table 1. Distribution of XIAP immunoreactivity according to clinicopathological characteristics in 90 patients with clear cell carcinoma of the ovary

Clinicopathological variables	Number (%)	XIAP high	XIAP low	*P*-value
Age at diagnosis (years)				
<50	35 (39%)	15 (50%)	20 (33%)	0.13
⩾50	55 (61%)	15 (50%)	40 (67%)	
WHO performance status				
0/1	86 (96%)	28 (93%)	58 (97%)	0.47
⩾2	4 (4%)	2 (7%)	2 (3%)	
FIGO stage				
I	46 (51%)	12 (40%)	34 (57%)	0.26
II	9 (10%)	2 (7%)	7 (12%)	
III	31 (35%)	14 (46%)	17 (28%)	
IV	4 (4%)	2 (7%)	2 (3%)	
Residual tumour diameter				
None	63 (70%)	19 (63%)	44 (73%)	0.62
⩽1 cm	10 (11%)	4 (14%)	6 (10%)	
>1 cm	17 (19%)	7 (23%)	10 (17%)	
c-Met				
High	31 (34%)	16 (53%)	15 (25%)	<0.01
Low	59 (66%)	14 (47%)	45 (75%)	
p-Akt				
High	23 (26%)	10 (33%)	13 (22%)	0.23
Low	67 (74%)	20 (67%)	47 (78%)	
Bcl-XL				
High	33 (37%)	17 (57%)	16 (27%)	<0.01
Low	57 (63%)	13 (43%)	44 (73%)	

Abbreviations: FIGO = International Federation of Obstetrics and Gynecology; WHO = World Health Organisation; XIAP = X-chromosome-linked inhibitor of apoptosis.

Table 2. Expression levels of XIAP, c-Met, phospho-Akt and Bcl-XL according to response of primary chemotherapy in 27 patients with evaluable disease

Proteins	Expression level	Number of the patients	Responders[a] ($n=10$)	Non-responders[b] ($n=17$)	*P*-value
XIAP	High	12	1 (10%)	11 (65%)	0.02
	Low	15	9 (90%)	6 (35%)	
c-Met	High	11	2 (20%)	9 (53%)	0.20
	Low	16	8 (80%)	8 (47%)	
p-Akt	High	6	0 (0%)	6 (35%)	0.10
	Low	21	10 (100%)	11 (65%)	
Bcl-XL	High	10	2 (20%)	8 (47%)	0.32
	Low	17	8 (80%)	9 (53%)	

Abbreviations: CR = complete response; PD = progressive disease; PR = partial response; SD = stable disease; XIAP = X-chromosome-linked inhibitor of apoptosis.
[a]The patients with CR and PR.
[b]The patients with SD and PD.

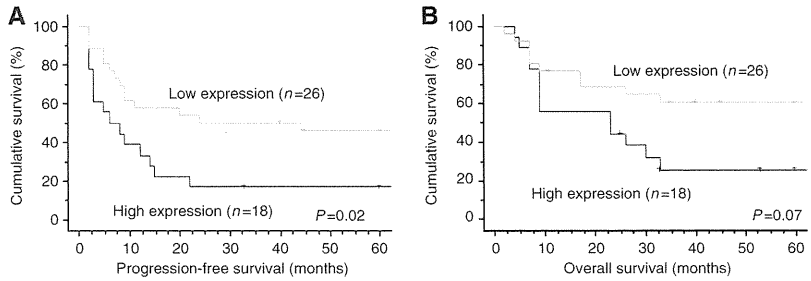

Figure 1. Progression-free survival (PFS) and overall survival (OS) curves of the patients with stages II–IV clear cell carcinoma of the ovary according to XIAP expression. (**A**) Progression-free survival curves of the patients. The patients with high expression of XIAP had significantly worse PFS ($P=0.02$). (**B**) Overall survival curves of the patients. Although high expression of XIAP was related with poor survival, significant difference was not observed in two groups ($P=0.07$).

Table 3. Multivariate analysis for progression-free survival and overall survival in 44 cases with stages II–IV ovarian clear cell carcinomas

		Progression-free survival			Overall survival		
Variables		Hazard ratio (95% CI)		*P*-value	Hazard ratio (95% CI)		*P*-value
Age	⩽50 *vs* >50	0.75	(0.32–1.76)	0.51	0.55	(0.20–1.48)	0.23
WHO performance status	0/1 *vs* ⩾2	0.74	(0.14–3.79)	0.71	0.58	(0.11–2.95)	0.51
FIGO stage	II *vs* III/IV	0.58	(0.19–1.77)	0.37	0.60	(0.16–2.22)	0.44
Residual tumour diameter	>1 cm *vs* ⩽1 cm	3.48	(1.40–8.62)	<0.01	5.35	(1.98–15.0)	<0.01
XIAP expression	High *vs* low	2.94	(1.22–7.09)	0.02	2.70	(1.02–7.16)	0.04

Abbreviations: CI = confidence interval; FIGO = International Federation of Obstetrics and Gynecology; WHO = World Health Organisation; XIAP = X-chromosome-linked inhibitor of apoptosis.

in KK-C, 20.6 ± 3.9% in KK-I and 19.5 ± 6.7% in KK-II. X-chromosome-linked inhibitor of apoptosis expression was significantly downregulated in both KK-I and KK-II in comparison with KK-C (Figure 2A). Expression levels of c-Met, Bcl-XL and PTEN were similar in those cells, however, p-Akt expression was slightly decreased in KK-I and KK-II cells compared with KK-C: 62.9 ± 11.0% in KK-I, 64.5 ± 4.6% in KK-II. (Figure 2A). Next, these cells were treated with cisplatin for 24 h at a dose of 10 μM. Expression levels of cleaved caspase-3 and cleaved PARP increased in both KK-I and KK-II cells (Figure 2B). Further, these cells were treated with cisplatin for 24 h in different dose (0, 10, 20 and 30 μM). Apoptotic ratios of KK-I and KK-II were significantly higher compared with that of KK-C at each concentration in a dose-dependent manner (Figure 2C).

DISCUSSION

Overall positive rate of XIAP in CCC was 33% in the present analysis. Previous report showed that serous adenocarcinoma of the ovary had approximately 85% positive rate of XIAP (Bu *et al*, 2011). Twenty samples used for positive control of XIAP were all judged as high expression of XIAP, implying that positive rate of XIAP in CCC was lower than that in serous ovarian cancers. Most of serous ovarian cancer had overexpression of p53; however, CCC had less involvement of p53 alteration and p53-independent mechanisms for chemoresistance in CCC was suggested (Eltabbakh *et al*, 2006). In this study, XIAP expression was not influenced by age at diagnosis, WHO PS, FIGO stage and residual tumour

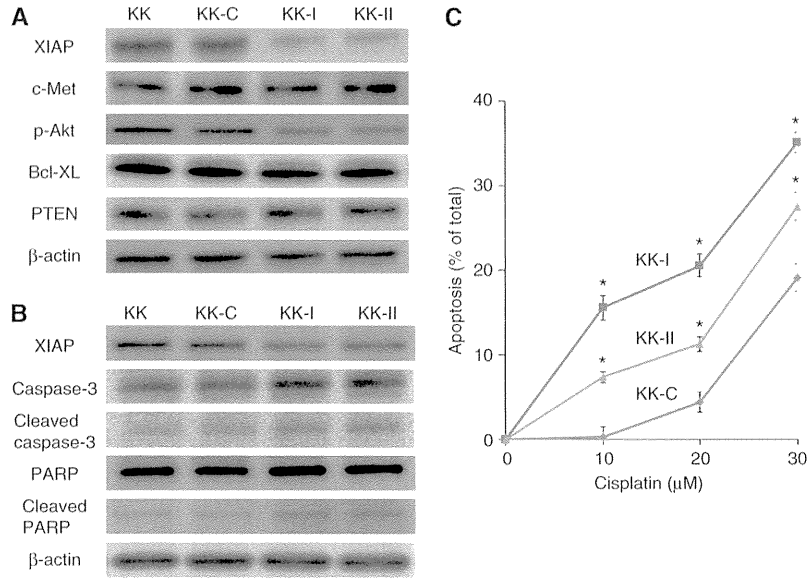

Figure 2. Downregulation of XIAP by transfection with XIAP siRNA and sensitivity to cisplatin *in vitro*. (**A**) Clear cell carcinoma cell line, KK, was transfected with nonspecific siRNA (KK-C), XIAP-specific siRNA I (KK-I) and XIAP-specific siRNA II (KK-II). Expression ratio of XIAP protein compared with KK cells without transfection was as follows: 73.14 ± 12.7% in KK-C, 20.6 ± 3.94% in KK-I and 19.54 ± 6.67% in KK-II, respectively. Phospho-Akt expression was decreased in KK-I and KK-II cells compared with KK-C: 62.9 ± 11.0% in KK-I, 64.5 ± 4.6% in KK-II. (**B**) After transfection, these cells were treated with for 24 h at a dose of 10 μM. Expression levels of cleaved caspase-3 and cleaved PARP increased in both KK-I and KK-II cells compared with KK or KK-C. (**C**) Apoptosis fractions induced by 24-h treatment of cisplatin in KK-C, KK-I and KK-II were shown. Ratio of apoptotic fraction was higher in KK-I and KK-II compared with that of KK-C at each concentration. The data represented the mean ± s.d. of at least four times. $^*P<0.01$.

diameter. However, increased XIAP expression was significantly related with resistance to platinum-based chemotherapy and worse prognosis of CCC disease. To our knowledge, there have been no reports evaluating XIAP expression in a series of consecutive cases of ovarian clear cell adenocarcinomas. X-chromosome-linked inhibitor of apoptosis expression has been recognised as a predictor of platinum resistance in several cancers (Schimmer *et al*, 2006; Yang *et al*, 2012). In addition, high XIAP expression was related with worse prognosis in human neoplasms (Tamm *et al*, 2004a, b; Mizutani *et al*, 2007). Recently, inhibition of XIAP has been recognised as a potential target of cancer therapy. Downregulation of XIAP by transfection with XIAP siRNA resulted in decreased phospho-Akt expression, and increased chemosensitivity to anticancer drugs (Jiang *et al*, 2012). X-chromosome-linked inhibitor of apoptosis has been shown to act as an E3 ubiquitin ligase for PTEN and to promote Akt activity (Van Themsche *et al*, 2009). However, PTEN expression level was not affected in the present system, suggesting another signalling pathway to activate Akt signalling, which is not related with PTEN ubiquitination. In addition, XIAP downregulation by a small-molecule inhibitor abrogated XIAP/procaspase-9 interaction, and decreased cell viability that was resistant to anticancer agents (Aird *et al*, 2010). This study revealed that XIAP overexpression resulted in chemoresistance against platinum-based chemotherapy and worse PFS of CCC patients. In the present system, downregulation of XIAP by transfection with XIAP siRNA increased sensitivity to cisplatin in CC through downregulation of p-Akt. Although further study is needed, XIAP expression is identified as one of the fundamental mechanisms as to how CCC showed intrinsic chemoresistance.

Previous immunochemical analysis for ovarian cancers mainly consisting of serous adenocarcinomas revealed that c-Met expression was correlated with expression levels of phospho-Akt-Ser 473, XIAP and Bcl-XL (Bu *et al*, 2011). Significant correlation of XIAP with c-Met expression observed in this study suggested that XIAP was regulated by HGF/c-Met in CCC. In our study, XIAP expression was not related with phospho-Akt (Ser 473). This may be simply explained by a small sample number; however, there may be another HGF/c-Met/XIAP pathway that was not through activation of phospho-Akt at Ser 473.

In a report including a large case series of advanced-staged CCC, residual tumour was the only independent prognostic factor, and adjuvant chemotherapy was not identified as a prognostic factor (Takano *et al*, 2006). Multivariate analysis in stages II–IV CCC showed that XIAP expression was an independent prognostic factor for PFS and OS in CCC, in addition to residual tumour diameter. It was revealed that high expression of XIAP was significantly correlated with resistance to primary chemotherapy, and led to worse prognosis in CCC. So, inhibition of XIAP expression using a c-Met inhibitor (Appleman, 2011) or a XIAP inhibitor (Kamsteeg *et al*, 2003) could be a novel strategy for the treatment of CCC.

The limitation of this study included a retrospective investigation and a single-institutional analysis. Also, the results obtained by this study could potentially have a bias such as selection bias, and further prospective investigation is needed to confirm the impact of XIAP expression upon prognoses of CCC patients. Nevertheless, XIAP could have an important role in chemoresistant phenotype in CCC, and high expression of XIAP was identified as an independent prognostic factor in stages II–IV CCC tumours. X-chromosome-linked inhibitor of apoptosis should be further evaluated as one of promising targets in chemotherapy for CCC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Complete remission of recurrent ovarian clear cell carcinoma by chemotherapy with bevacizumab, trabectedin and oxaliplatin

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Abstract

Clear cell carcinoma of the ovary has shown an exceedingly chemo-resistant phenotype, especially in cases that are recurrent or refractory to previous therapy. Also, progression-free survival was less than 6 months, even in the patients that achieved response when they were treated with conventional anti-cancer cytotoxic agents. We present a case with recurrent and refractory ovarian clear cell carcinoma that achieved complete remission using a combination of bevacizumab, trabectedin and oxaliplatin. The progression-free interval of the patient is over 30 months, and she is still receiving the combination therapy without toxicities of more than grade 2.

Key words: bevacizumab, clear cell carcinoma, oxaliplatin, recurrent ovarian cancer, trabectedin.

Introduction

Clear cell carcinoma of the ovary has shown an exceedingly chemo-resistant phenotype, especially in cases that are recurrent or refractory to previous therapy.[1–3] A PubMed search over the last 20 years revealed that progression-free survival was less than 6 months even in the patients that achieved response when they were treated with conventional anti-cancer cytotoxic agents.[4] The longest response duration of 14 months was obtained by weekly administration of temsirolimus in a patient among six case series.[5] So far, there is no candidate of second-line chemotherapeutic regimen for recurrent and refractory ovarian clear cell carcinomas.

A marine natural product, trabectedin, had activity against platinum-sensitive recurrent ovarian cancers in combination with pegylated liposomal doxorubicin; however, the effects were not significant in platinum-resistant cases.[6] On the other hand, trabectedin had a synergistic effect against human tumor xenografts in combination with cisplatin.[7] We hypothesized that combination with trabectedin and oxaliplatin was active against refractory ovarian cancers, because oxaliplatin had activity in cis/carboplatin+/−paclitaxel-pretreated ovarian cancers.[8] Bevacizumab, a humanized monoclonal antibody binding to vascular endothelial growth factor (VEGF), showed activity for recurrent or refractory ovarian cancer patients in two phase II studies.[9,10] Our previous investigation revealed that addition of bevacizumab enhanced the effects of a cytotoxic agent, and yielded approximately doubled response rate for platinum-resistant ovarian cancers.[11] Thus, we hypothesized that a combination of bevacizumab, trabectedin and oxaliplatin could be a candidate for refractory solid cancers. The case reported here was a recurrent and

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refractory ovarian clear cell carcinoma that showed complete response over 1 year to combination therapy with bevacizumab, trabectedin and oxaliplatin.

Methods

A 41-year-old woman, gravida 0, para 0, was referred to our clinic for the treatment of intra-abdominal recurrent disseminated tumors and massive ascites. Three years previously, the patient had undergone complete surgical staging operation for stage Ia ovarian clear cell carcinoma. After completion of six cycles of chemotherapy with paclitaxel and carboplatin, however, multiple intra-peritoneal implantations were detected by CT images. Since then, three regimens that she had received did not show response: two cycles of chemotherapy with irinotecan and mitomycin C, three cycles of combination with docetaxel and irinotecan, and three cycles of therapy with bevacizumab and pegylated liposomal doxorubicin. Next, the patient received weekly administration of temsirolimus as the fifth-line chemotherapy, and the therapy showed partial response with progression-free period of 14 months; however, metastatic lesions increased in size and intraperitoneal fluid collection was observed (Fig. 1a–c). Since then, the patient has been receiving a combination of bevacizumab, trabectedin and oxaliplatin as the sixth-line chemotherapy: a weekly administration of

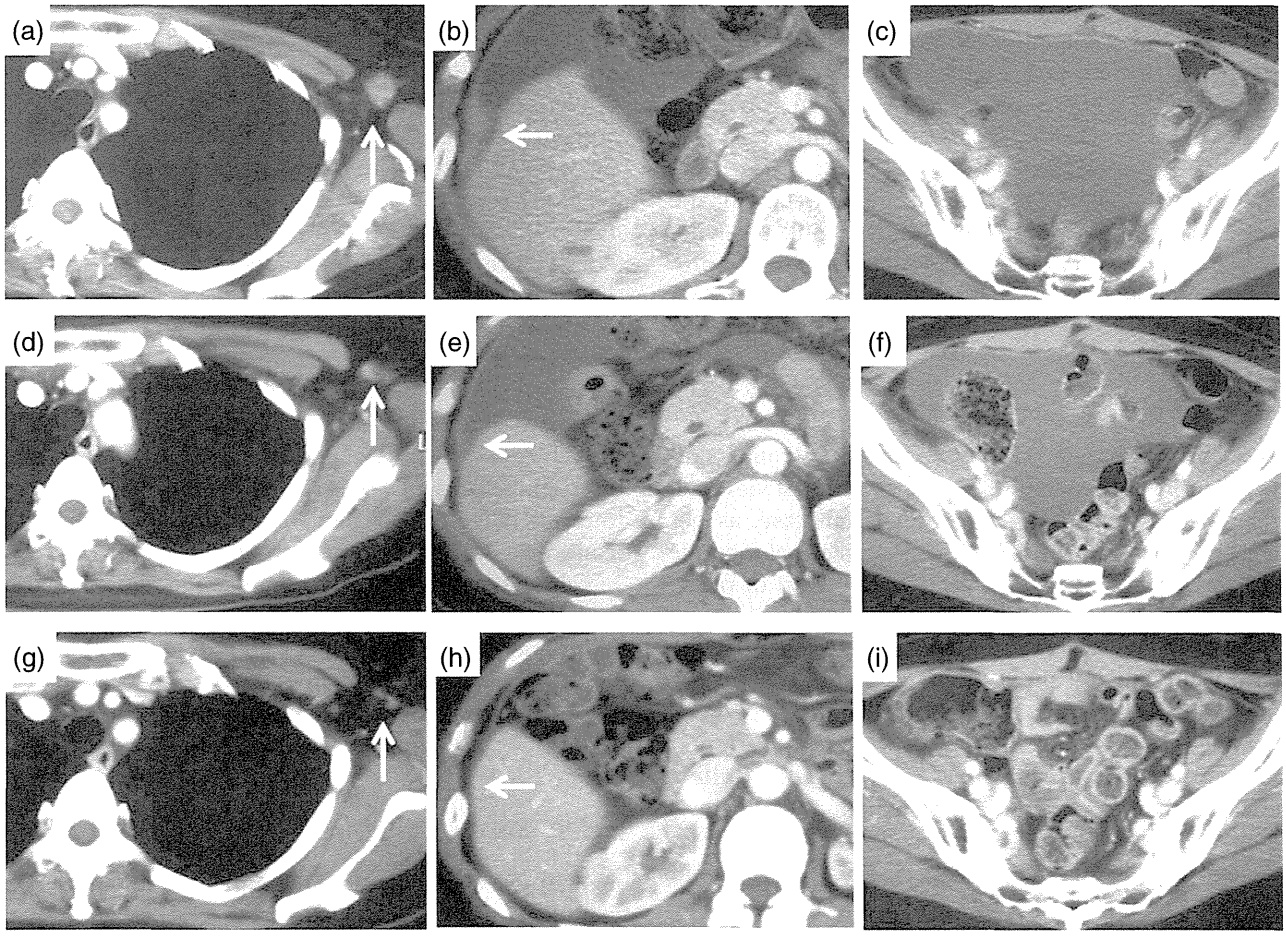

Figure 1 Computed tomography (CT) images of the patient with recurrent clear cell carcinoma of the ovary. CT images before combination therapy with bevacizumab, trabectedin and oxaliplatin showed (a) axillary lymph node metastasis, (b) intra-abdominal dissemination and (c) massive ascites. (d,e,f) Recurrent tumors showed partial response by CT images after 6 cycles of the therapy, and (g,h,i) achieved complete remission after 12 cycles of the therapy. Images a, d and g show left axillary lymph nodes (arrow), and images b, e and h show peritoneal disseminated tumor (arrow). Decrease of intraperitoneal ascites can be seen in images c, f and i.

bevacizumab at 2 mg/kg, trabectedin at 0.15 mg/m^2 and oxaliplatin at 30 mg/m^2, 3 weeks on and 1 week off, every 4 weeks. The regimen had been approved by the ethical committee of Ohki Memorial Kikuchi Cancer Clinic for Women, and the treatment was initiated after obtaining a written informed consent. As these three drugs were not approved by Japanese National Insurance for the treatment of ovarian cancer, all costs of the treatment were paid by the patient herself. During all cycles of the therapy, she occasionally developed grade 1 toxicities only: fatigue, nasal bleeding, skin pain and constipation. After six cycles of the therapy, all metastatic lesions showed shrinkage, achieving partial response according to the Response Evaluation Criteria in Solid Tumors, and massive ascites also decreased (Fig. 1d–f). Twelve months after the initiation of the combination of bevacizumab, trabectedin and oxaliplatin, the patient had neither disseminated tumor nor ascites, resulting in complete remission (Fig. 1g–i). Moreover, Eastern Cooperative Oncology Group performance status was also improved: 2 at the start of the therapy, and 0 at the 12th month. In fact, 30 months from the initial treatment, the patient is without any complaints and still receiving combination chemotherapy with no sign of tumor progression. Also, there have been no toxicities more than grade 2 through all cycles of the therapy.

Discussion

The goal of salvage therapy could be palliation and maintaining quality of life. From this point of view, the therapy was so effective in the present case, as her quality of life was improved by a decrease of massive ascites. So far, maximum response duration of recurrent and refractory ovarian clear cell carcinoma by cytotoxic agents has been almost 6 months.[4] It is not clear which one of three drugs could lead to the significant effect observed in the present case; however, complete remission over 1.5 years in a recurrent and refractory case was extremely unlikely. The additive effects of bevacizumab might be explained by the evidence that a combination of bevacizumab and a low-dose cytotoxic regimen blocks vascular repair and survival, enhancing the effects of cytotoxic drugs.[12] Also, reduction of ascites was explained by inhibition of the VEGF pathway, as bevacizumab had the potential to suspend the ascites production resulting from peritoneal dissemination in solid cancers, including ovarian cancers.[13] Recently, trabectedin showed significant antitumor activity for chemo-sensitive and chemo-resistant cell lines derived from human ovarian clear cell carcinomas.[14] A combination with these three dugs might have enhanced the effect of each drug, and possibly led to a synergistic more than an additive effect.

It is not clear whether the dose used in the present patient was completely adequate or not, as there has been no dose-finding study to determine the recommended dose. Recently, we reported a preliminary 19-case series of recurrent and refractory ovarian cancers treated with bevacizumab, gemcitabine and oxaliplatin (B-GEMOX): 2 mg/kg of bevacizumab, 300 mg/m^2 of gemcitabine and 30 mg/m^2 of oxaliplatin, 3 weeks on and 1 week off, every 4 weeks.[15] The doses of B-GEMOX combination were determined according to a previous preliminary biweekly B-GEMOX regimen: biweekly administration of 1000 mg/m^2 of gemcitabine, 65 mg/m^2 of oxaliplatin and monthly administration of 10 mg/kg of bevacizumab, every 4 weeks.[16] Weekly doses of B-GEMOX were approximately half of the biweekly regimen of the Horowitz study.[16] Weekly administration of B-GEMOX yielded a response rate of 42% and a clinical benefit rate (CR+PR+SD) of 68%; however, there were no cases that achieved response in three cases of clear cell carcinomas.[15] So, we used trabectedin instead of gemcitabine, as the effect of trabectedin was thought to be promising for ovarian clear cell carcinomas.[14] Recommended weekly dose of single-agent trabectedin was reported to be 0.58–0.61 mg/m^2 in a phase I study.[17] Approximately one-third of recommended trabectedin doses were used in the present case. Overall toxicities observed in our patient were all grade 1 and manageable, so she still continues the regimen over 2 years.

Complete remission by combination therapy with bevacizumab, trabectedin and oxaliplatin as the sixth-line chemotherapy and an extraordinarily long response duration observed in the present case suggest that the regimen could be a candidate for further treatment of ovarian clear cell carcinomas.

Disclosure

No author has any potential conflict of interest.

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Original Article

Aberrant Expression of the Mammalian Target of Rapamycin, Hypoxia-inducible Factor-1α, and Glucose Transporter 1 in the Development of Ovarian Clear-cell Adenocarcinoma

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Summary: Ovarian clear-cell adenocarcinoma (CCA) is known to be a type of cancer in humans with a high frequency of expression of the mammalian target of rapamycin (mTOR), hypoxia-inducible factor-1 (HIF-1), and glucose transporter 1 (Glut1). In this study, we aimed to determine how these alterations contribute to tumor development of CCAs. Immunohistochemical expressions of phosphorylated-mTOR (p-mTOR), HIF-1α, and Glut1 were analyzed in 36 CCAs and 60 coexistent putative precursor lesions: 19 nonatypical and 16 atypical endometriotic lesions, and 11 benign and 14 borderline clear-cell adenofibroma (CCAF) components. Twenty-one cases with solitary endometriosis were also examined. The frequencies of immunopositivity for p-mTOR (in cytoplasm or nucleus), HIF-1α (in nucleus), and Glut1 increased in accordance with higher cytological atypia in the putative precursors: 58%, 5%, and 16% in the nonatypical endometriosis; 63%, 37%, and 50% in the atypical endometriosis; 77%, 95%, and 95% in the endometriosis-associated CCAs; 27%, 0%, and 0% in the benign-CCAF components; 64%, 79%, and 43% in the borderline CCAF components; and 71%, 100%, and 93% in the CCAF-associated CCAs, respectively. p-mTOR, HIF-1α (in the nucleus), and Glut1 were positive in 10%, 5%, and 19% of the solitary endometriosis, respectively. In the putative precursor lesions coexisting with CCA, a strong correlation in the expression between p-mTOR and HIF-1α and between HIF-1α and Glut1 was identified. Expressions of p-mTOR, HIF-1α, and Glut1 have already been evident in the putative precursor lesions of CCA, and these alterations cumulatively occur in the development of ovarian CCA. **Key Words:** Ovarian clear-cell adenocarcinoma—Endometriosis—Clear-cell adenofibroma—Hypoxia-inducible factor-1—Mammalian target of rapamycin.

Among ovarian carcinomas, clear-cell adenocarcinoma (CCA) has been recognized as a distinct clinicopathological entity in view of its characteristic histology, frequent concurrence of endometriotic lesions, and highly chemoresistant nature, resulting in an extremely poor prognosis when surgical cytoreduction is insufficient (1–4). Although somatic mutations of *PIK3CA* and *ARID1A* are the most common genetic alterations identified so far in ovarian CCA (5–8), to establish a novel therapeutic strategy for ovarian CCA, it is crucial to elucidate

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molecular aberrations, which occur during tumor development for this carcinoma type.

Hypoxia-inducible factor-1 (HIF-1) is one of the transcription factors that plays a vital role in the promotion of cell survival and proliferation (9). Under hypoxic conditions, HIF-1α can avoid degradation from prolyl hydroxylase, conferring molecular stabilization in the cytoplasm (10). Stabilized HIF-1α dimerizes with HIF-1β, which moves to the nucleus, and binds to the hypoxia response element, resulting in the expression of various growth factors and cytokines (11–14). Other than hypoxia, it is also known that activation of receptor tyrosine kinase, activated signaling pathways of phosphatidylinositol 3 kinase/Akt, mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase, and dysfunction of phosphatase and tensin homolog deleted on chromosome 10 or von Hippel Lindau protein (pVHL) have the potential to stabilize HIF-1α and increase its activity (15–19). HIF-1α expression has been documented in various types of human carcinomas, including brain, bladder, breast, colon, lung, ovarian, oropharyngeal, pancreatic, prostate, and renal carcinomas (20). Moreover, HIF-1α overexpression was reported to be associated with a worse clinical outcome of patients with breast, lung, colon, and uterine cervical cancer (21–25).

Recent studies have demonstrated frequent overexpression of HIF-1α and its related factors, such as mTOR and glucose transporter 1 (Glut1) in ovarian CCAs (26–28). In ovarian carcinomas, hypoxic conditions in cancerous tissues have been shown to cause aberrant glucose metabolism, including upregulated glucose transport and glycolysis in tumor cells and angiogenesis in tumor tissue by overexpression of the Glut1, which is thought to be regulated primarily by HIF-1 (26,27). Several studies have demonstrated significant pathogenic roles of Glut1 in the development and progression of ovarian carcinomas (26,27). In addition, when compared with other histological subtypes of epithelial ovarian carcinoma, expression levels of phosphorylated-mTOR (p-mTOR) and Glut1 are more evident in CCA (27,28), suggesting that mTOR-HIF-1 signaling and subsequent Glut1 expression are significantly associated with the pathobiology of this type of carcinoma. However, it remains unclear how these alterations contribute to tumor development or progression of ovarian CCA.

In this study, we selected CCA patients with adjacent putative precursor lesions [i.e. endometriosis or clear-cell adenofibroma (CCAF)], and immunohistochemically analyzed the expressions of p-mTOR, HIF-1α, and Glut1 in both CCAs and coexisting precursor lesions. For the precursor lesions examined, the presence or absence of the cellular atypia or architectural abnormality was taken into account. These analyses will clarify whether expressions of p-mTOR, HIF-1α, and Glut1 are associated with the development of CCA as an early event in its tumorigenesis. This information would lead to a better understanding of the pathobiology of ovarian CCAs.

MATERIALS AND METHODS

Cases

According to the histopathological criteria described previously (29–31) a total of 22 CCAs with synchronous endometriosis (endometriosis-associated carcinomas) and 14 CCAs with an adjacent CCAF component (CCAF-associated carcinomas) were identified from the files of the Department of Laboratory Medicine, National Defense Medical College Hospital, Japan. These 36 patients were consecutive series, surgically resected in our hospital from 1987 to 2005, and none had undergone chemotherapy or radiotherapy before initial surgery. There was no case that overlapped between endometriosis-associated carcinoma and CCAF-associated carcinoma. Clinical staging of the disease was carried out using a criterion defined by the International Federation of Gynecology and Obstetrics. Of the 22 cases with endometriosis-associated carcinomas, 16 (73%) were Stage I, 2 (9%) were Stage II, 3 (14%) were Stage III, and I (4%) was Stage IV. Of the 14 cases with CCAF-associated carcinomas, 9 (65%) were Stage 1, 2 (14%) were Stage II, 2 (14%) were Stage III, and 1 (7%) was Stage IV. Twenty-one cases of solitary endometriosis obtained by salpingo-oophorectomy and 18 cases of non-neoplastic endometrial tissue (6 proliferative phase, 6 secretory phase, and 6 gestational phase) of a curettage specimen were also analyzed. All the specimens used in this study were formalin-fixed and paraffin-embedded tissues. The research protocol was approved by the Ethics Committee of the National Defense Medical College, Tokorozawa, Japan.

Nonatypical and Atypical Endometriosis

With reference to the histopathological criteria of "atypical endometriosis" described previously (29,31), endometriotic lesions in each endometriosis-associated case were histologically subclassified into nonatypical and atypical endometriosis (Figs. 1A, B). Briefly, the term "atypical endometriosis" has been assigned if, at most, one of the following features was histologically evident in the endometriotic epithelium: large hyperchromatic or pale nuclei with moderate to marked pleomorphism; an increased nuclear to cytoplasmic ratio; and cellular crowding with stratification or tufting. Consequently, 13 (59%) of the 22 endometriosis-associated patients had both nonatypical and atypical endometriosis, 3 (14%) had only atypical endometriosis, and 6 (27%) had only nonatypical endometriosis. Therefore, 19 nonatypical and 16 atypical endometriotic lesions and 22 endometriosis-associated invasive-carcinoma components were analyzed for immunohistochemistry.

Benign and Borderline CCAF

The histopathological features of benign CCAF (or CCAF without atypia) and borderline CCAF (or CCAF with atypia) have been described previously (29,30). In brief, CCAF is a surface epithelial–stromal tumor containing tubulocystic epithelial components, which are composed of clear, hobnail, or sometimes eosinophilic cells, embedded in a fibroma-like stroma. Subclassification of a benign or a borderline lesion was carried out by histological documentation on the basis of the cellular (i.e. nuclear pleomorphism and stratification of the epithelium) and architectural (i.e. size irregularity and crowding of the tubulocystic architecture) features of each epithelial component (29,30). According to these criteria, CCAF lesions in each

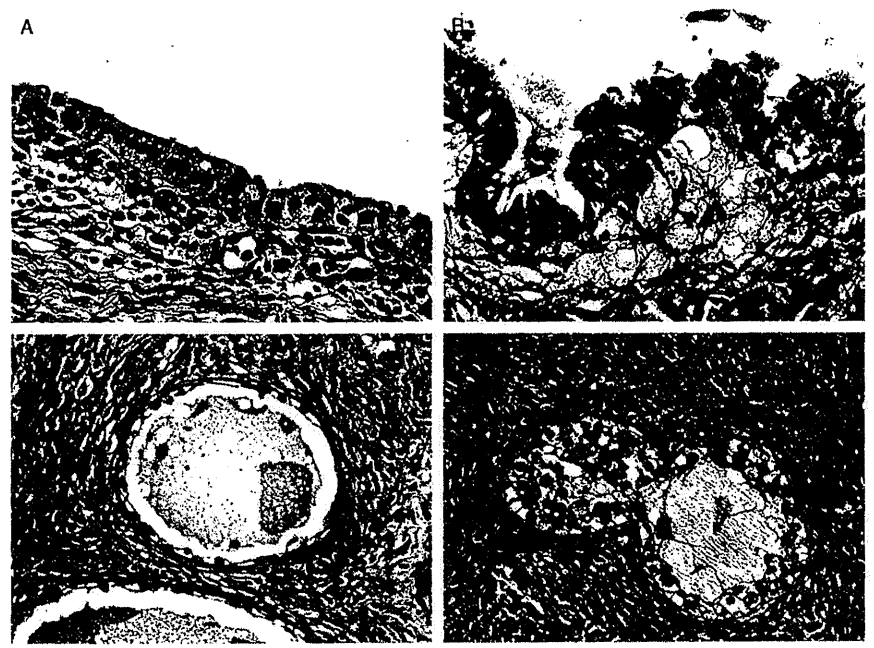

FIG. 1. Representative histological features of nonatypical and atypical endometriosis (A and B) and benign and borderline clear-cell adenofibroma (CCAF) (C and D). (A) An endometriotic lesion lacking cellular or structural atypia and (B) an endometriotic lesion showing cellular (i.e. hyperchromatic nuclei) and structural (cellular stratification) atypia. According to the previously described criteria for atypical endometriosis (29,31), lesions of (A) and (B) were defined as nonatypical and atypical endometriosis, respectively. (C) A CCAF components without cellular atypia in the epithelial component and (D) a CCAF component with cellular atypia. According to previously described criteria (29,30), lesions of (C) and (D) were defined as benign CCAF and borderline CCAF, respectively. Hematoxylin and eosin stain, original magnification, 400x for each.

CCAF-associated case were histologically subclassified into benign and borderline CCAF (Figs. 1C, D). Consequently, 11 (79%) of the 14 CCAF-associated cases had both the evaluable benign-CCAF and borderline-CCAF components, and the remaining 3 (21%) had only the borderline-CCAF component. Therefore, 11 benign-CCAF components, 14 borderline-CCAF components, and 14 CCAF-associated invasive-carcinoma components were analyzed for immunohistochemistry.

Immunohistochemistry

All the formalin-fixed and paraffin-embedded specimens selected were cut into 4-μm-thick serial sections and analyzed by immunohistochemistry. We used rabbit polyclonal antibody for p-mTOR (Ser2448; dilution 1/100; Cell Signaling Technology Inc., Bevery MA), mouse monoclonal antibody for HIF-1α (ESEE122; dilution 1/50; Novus Biologicals Inc., Littleton, CO), and rabbit polyclonal antibody for Glut1 (ab15310; dilution 1/2; Abcam plc, Cambridge, UK), respectively. Sections were deparaffinized and boiled in a microwave oven at 97°C for 20 minutes in 0.01 mol/L citrate buffer (pH 6.0) (for detection of HIF-1α and Glut1) or in an autoclave at 121°C for 15 minutes in 0.01 mol/L citrate buffer (pH 6.0) (for the detection of p-mTOR), and then allowed to cool at room temperature. Endogenous peroxidase was blocked using 5% hydrogen peroxide. The slides were incubated at 4°C overnight with primary antibodies and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase for 1 hour at room temperature. Specific antigen–antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide, and counterstaining was performed using Mayer hematoxylin. Formalin-fixed and paraffin-embedded specimens of human breast carcinomas, which were known to overexpress p-mTOR protein, served as the positive control; fibroblasts and erythrocytes in the sections of ovarian CCA patients served as built-in-controls for HIF-1α and Glut1 expression, respectively. As negative controls, sections without the primary antibody were used.

Assessment of Immunoreactions

The immunostaining was evaluated in areas with a well-preserved tissue morphology and without necrosis or artifacts. For p-mTOR detection, the presence of either 1 or a combination of nuclear or cytoplasmic immunoreaction was taken into account for assignment of immunopositivity. For Glut1 detection, a cell membranous immunoreaction was taken into account for evaluation. For HIF-1α, to assess intracellular dynamics, cytoplasmic and nuclear immunoreactivity were separately assessed for evaluation. Because the immunoreactivities of these 3 markers were found to be frequently heterogeneous staining patterns, especially in the carcinoma components, the lesions were considered as positive for each marker if 10% or more of epithelium or tumor cells in the interest area showed a moderate to strong immunoreactive intensity. The assignment of immunoreaction was performed independently by 2 observers (M.K. and S.Y.), and any discrepancies between the 2 observers were resolved by conferring over a multiviewer microscope.

Statistical Analyses

Statistical analyses were performed using State Mate IV software (ATMS, Tokyo, Japan). The frequencies of positivities for p-mTOR, HIF-1α, and Glut1 in the examined lesions were compared using the χ^2 test or the Fisher exact test. The differences at $P<0.05$ were considered to be statistically significant, and those between 0.05 and 0.10 were considered to be marginally significant.

RESULTS

The results of immunohistochemistry are summarized in Tables 1 and 2.

Expressions of p-mTOR, HIF-1α, and Glut1 in the Invasive Carcinoma Components

p-mTOR expression was judged as positive in 27 (75%) of the 36 invasive carcinoma patients enrolled (Fig. 2A): 17 (77%) of the 22 endometriosis-associated carcinomas and 10 (71%) of the 14 CCAF-associated carcinomas. Cytoplasmic expression of HIF-1α was judged as positive in all of the 36 invasive carcinomas (Fig. 2B). Nuclear expression of HIF-1α was judged as positive in 35 (97%) of the 36 carcinomas (Fig. 2B): 21 (95%) of the endometriosis-associated carcinomas and 14 (100%) of the CCAF-associated carcinomas. Glut1 expression was judged as positive in 34 (94%) of the 36 carcinomas (Fig. 2C): 21 (95%) of the endometriosis-associated carcinomas and 13 (93%) of the CCAF-associated carcinomas. There were no statistically significant differences between the 2 groups (i.e. endometriosis- and

TABLE 1. *Expressions of p-mTOR, HIF-1α, and Glut1 in ovarian clear-cell adenocarcinomas and their putative precursor lesions*

	No. Positive Cases (%)				
	Total	p-mTOR	HIF-1α (cytoplasmic)	HIF-1α (nuclear)	Glut1
Endometriosis-associated cases					
Nonatypical endometriosis	19	11 (58)	11 (58)*	1 (5)*	3 (16)
Atypical endometriosis	16	10 (63)	16 (100)	6 (38)*	8 (50)*
Clear-cell adenocarcinoma	22	17 (77)	22 (100)	21 (95)	21 (95)
CCAF-associated cases					
Benign CCAF	11	3 (27)*	1 (9)	0 (0)	0 (0)*
Borderline CCAF	14	9 (64)	14 (100)	11 (79)	6 (43)*
Clear-cell adenocarcinoma	14	10 (71)	14 (100)	14 (100)	13 (93)
Solitary endometriosis	21	2 (10)	6 (29)	1 (5)	4 (19)

*$P<0.05$.

CCAF indicates clear-cell adenofibroma; Glut1, glucose transporter 1; HIF-1α, hypoxia-inducible factor-1α; p-mTOR, phosphorylated mammalian target of rapamycin.

CCAF-associated carcinomas) with regard to the frequencies of positivity for any markers examined.

Expressions of p-mTOR, HIF-1α, and Glut1 in the Endometriosis Synchronous With CCA

p-mTOR expression was positive in 11 (58%) of the 19 nonatypical endometriosis and 10 (63%) of the 16 atypical endometriosis (Figs. 3A, D). Cytoplasmic HIF-1α expression was positive in 11 (58%) of the nonatypical endometriosis and 16 (100%) of the atypical endometriosis (Figs. 3B, E). Nuclear expression of HIF-1α was positive in 1 (5%) of the nonatypical endometriosis and 6 (38%) of the atypical endometriosis, and all of these 7 lesions showed copositivity with cytoplasmic HIF-1α expression (Fig. 3E). Glut1 was positive in 3 (16%) of the nonatypical endometriosis and 8 (50%) of the atypical endometriosis (Figs. 3C, F).

Comparison of the nonatypical and the atypical endometriosis showed statistically significant differences with regard to cytoplasmic expression of HIF-1α ($P = 0.003$) and nuclear expression of HIF-1α ($P = 0.031$). Comparison between the atypical endometriosis and the endometriosis-associated carcinoma components revealed statistically significant differences with regard to nuclear expression of HIF-1α ($P<0.001$) and Glut1 expression ($P = 0.001$). On comparing nonatypical endometriosis with endometriosis-associated carcinoma components, statistically significant differences were revealed with regard to nuclear ($P<0.001$) and cytoplasmic ($P<0.001$) expression of HIF-1α and Glut1 expression ($P<0.001$). In 18 (82%) of the 22 cases, if the expression of any markers examined were positive in nonatypical endometriosis (or in atypical endometriosis), then that expression was maintained in the more severe atypical lesions (i.e. atypical endometriosis or the invasive carcinoma components).

Expressions of p-mTOR, HIF-1α, and Glut1 in the CCAF Components Adjacent to CCA

p-mTOR was positive in 3 (27%) of the 11 benign-CCAF components and 9 (64%) of the 14 borderline-CCAF components (Figs. 3G, J). Cytoplasmic expression of HIF-1α was positive in 1 (9%) of the benign-CCAF components and 14 (100%) of the

TABLE 2. *Possible correlation in expression between p-mTOR, HIF-1α, and Glut1 in ovarian CCA development*

	No. cases (%)			
	Total	p-mTOR-positive	p-mTOR-negative	*P*
A. Correlation between nuclear HIF-1α and p-mTOR expressions in the putative precursors of CCAs				
Nuclear HIF-1α positive	18 (100)	13 (72)	5 (28)	0.079
Nuclear HIF-1α negative	42 (100)	20 (48)	22 (52)	
	No. of cases (%)			
	Total	Glut1-positive	Glut1-negative	*P*
B. Correlation between nuclear HIF-1α and Glut1 expressions in the putative precursors of CCAs				
Nuclear HIF-1α positive	18 (100)	8 (44)	10 (56)	0.069
Nuclear HIF-1α negative	42 (100)	9 (21)	33 (79)	

CCA indicates clear-cell adenocarcinoma; Glut1, glucose transporter 1; HIF-1α, hypoxia-inducible factor-1α; p-mTOR, phosphorylated mammalian target of rapamycin.

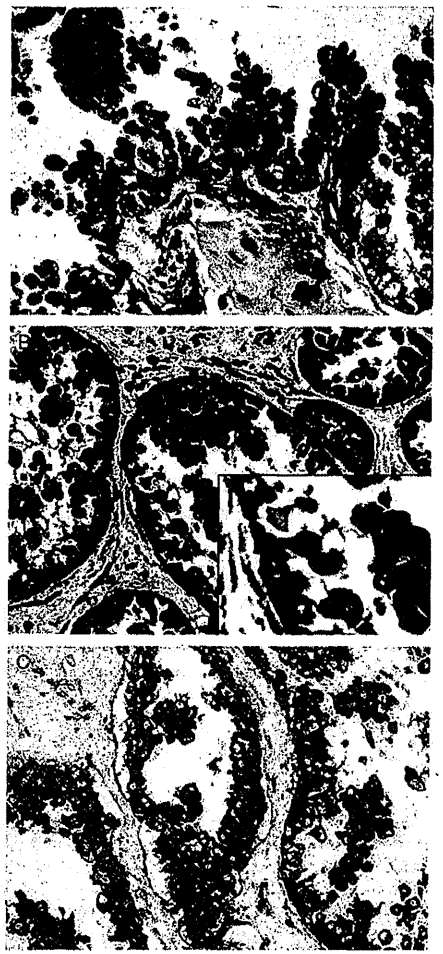

FIG. 2. Expression of phosphorylated mammalian target of rapamycin (p-mTOR) (A), hypoxia-inducible factor-1α (HIF-1α) (B), and glucose transporter 1 (Glut1) (C) in ovarian clear-cell adenocarcinoma (CCA). (A) Moderate-to-strong cytoplasmic immunoreaction for p-mTOR can be noted in this photomicrograph. (B) A CCA showing both cytoplasmic and nuclear immunoreactions for HIF-1α with strong intensities for both. (C) A CCA with a strong cell membranous immunoreaction for Glut1. Immunoperoxidase stain, original magnification, 400x for (A), (C) and inset of (B); 200x for (B).

borderline-CCAF components (Figs. 3H, K). Nuclear expression of HIF-1α was positive in none (0%) of the benign-CCAF and 11 (79%) of the borderline-CCAF components (Fig. 3K). Glut1 was positive in none (0%) of the benign-CCAF components and 6 (43%) of the borderline-CCAF components (Figs. 3I, L).

On comparing the benign-CCAFs and the borderline-CCAF components, statistically significant differences were revealed with regard to the cytoplasmic and nuclear expression of HIF-1α ($P<0.001$ and <0.001, respectively) and Glut1 expression ($P = 0.019$). Comparison of the benign-CCAFs and CCAF-associated carcinoma components revealed statistically significant differences regarding the frequencies of positivity for p-mTOR, cytoplasmic and nuclear HIF-1α, and Glut1 expressions ($P = 0.047$, <0.001, <0.001, <0.001, respectively). In 12 (86%) of the 14 cases, if the expression of any markers examined was positive in the benign-CCAF (or borderline-CCAF) component, then that expression was maintained in the more severe atypical lesions (i.e. borderline-CCAF or invasive-carcinoma component).

Expressions of p-mTOR, HIF-1α, and Glut1 in Solitary Endometriosis and Non-neoplastic Endometrial Tissues

In the 21 solitary endometriosis, p-mTOR, cytoplasmic HIF-1α, nuclear HIF-1α, and Glut1 expressions were judged as positive in 2 (10%), 6 (29%), 1 (5%), and 4 (19%) lesions, respectively. On comparing the solitary endometriosis with the nonatypical endometriosis synchronous with CCA, there was a statistically significant difference in the frequency of positivity for p-mTOR ($P = 0.001$).

All of the 18 non-neoplastic endometrial tissues were negative for p-mTOR and nuclear HIF-1α expressions, although only focal (<10%) immunoreactivity for p-mTOR and/or weak immunoreactivity for cytoplasmic HIF-1α expression were shown in some endometrial glands. Glut1 expression was judged as positive in all (100%) of the 6 gestational endometrium and 4 (67%) of the 6 secretory-phase endometrium. None of the 6 proliferative-phase endometrium showed Glut1 expression.

Correlation Among Overexpression of p-mTOR, HIF-1α, and Glut1 in the Putative Precursor Lesions of Ovarian CCAs

In a total of the 18 precursor lesions (nonatypical and atypical endometriosis and benign and borderline-CCAF) that were positive for the nuclear

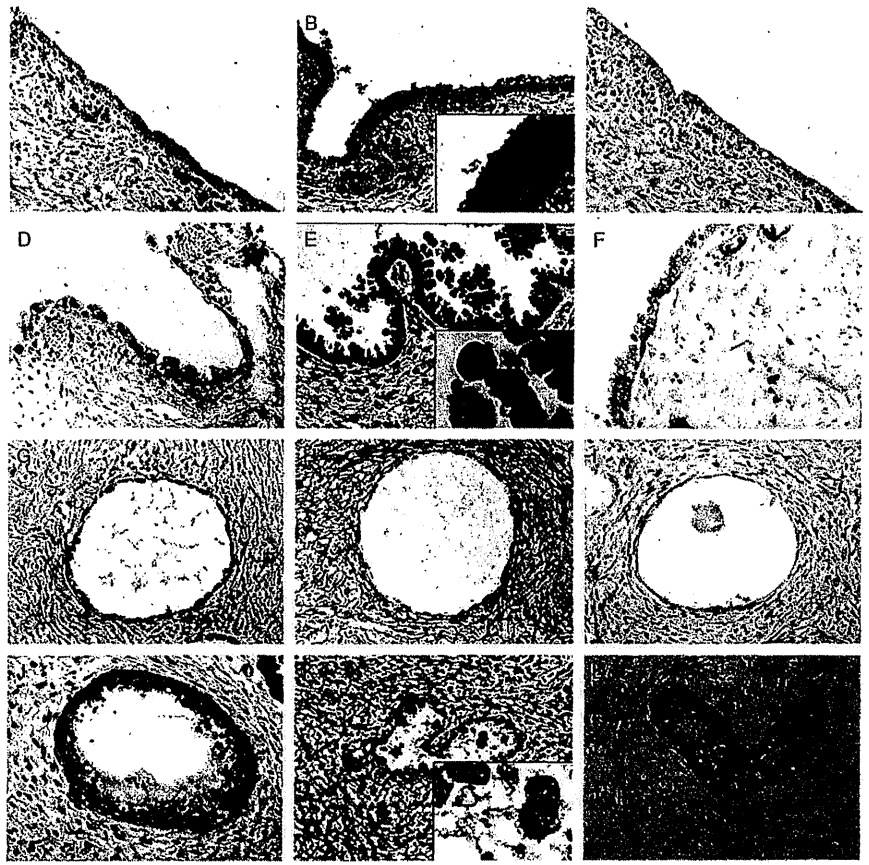

FIG. 3. Expression of phosphorylated mammalian target of rapamycin (p-mTOR), hypoxia-inducible factor-1α (HIF-1α), and glucose transporter 1 (Glut1) in the putative precursors of ovarian clear-cell adenocarcinoma. Immunoreactions for p-mTOR (A, D, G, and J), HIF-1α (B, E, H, and K), and Glut1 (C, F, I, and L) in the nonatypical endometriosis (A–C), atypical endometriosis (D–F), benign clear-cell adenofibroma (CCAF) (G–I), and borderline CCAF (J–L). Positive p-mTOR immunoreactions can be noted in the epithelial components of the nonatypical endometriosis (A), atypical endometriosis (D), benign CCAF (G), and borderline CCAF (J). Cytoplasmic immunoreactions for HIF-1α are positive in nonatypical (B) and atypical (E) endometriosis and borderline CCAF (K), but negative for benign CCAF (H). Nuclear immunoreactions for HIF-1α are positive in atypical endometriosis (E) and borderline CCAF (K). Glut1 is positive in atypical endometriosis (F) and borderline CCAF (L), but negative in nonatypical endometriosis (C) and benign CCAF (I). Immunoperoxidase stain, original magnification, 400x for (D), (F), (G), (H), (I), (L), and insets of (B), (E), and (K); 200x for (A), (B), (C), (E), (J), and (K).

expression of HIF-1α, 13 (72%) lesions were also positive for p-mTOR (Table 2A). In contrast, in a total of the 27 precursor lesions negative for p-mTOR, only 5 (19%) lesions were positive for nuclear HIF-1α expression. Consequently, there was a strong correlation in the positivity between p-mTOR and HIF-1α nuclear expressions with a statistically marginal significance ($P = 0.079$).

In a total of 17 precursor lesions positive for Glut1, 8 (47%) lesions were also positive for nuclear HIF-1α

expression (Table 2B). In contrast, in a total of 42 precursor lesions negative for nuclear HIF-1α expression, 9 (21%) lesions were positive for Glut1. Consequently, there was a strong correlation in the positivity between HIF-1α nuclear expression and Glut1 expression with a statistically marginal significance ($P = 0.069$).

DISCUSSION

Previous reports have documented the frequent overexpressions of mTOR (or p-mTOR), HIF-1α, and their related factors, such as Glut1, in ovarian CCAs; however, it had been unclear how these alterations contribute to the development of this carcinoma type (27,28). In the present study, we analyzed these protein alterations in CCA cases with adjacent putative precursor lesions.

In this study, p-mTOR, cytoplasmic and nuclear HIF-1α, and Glut1 expression were found to be very frequently positive in CCAs: 75%, 100%, 97%, and 94% of the carcinomas were judged as positive, respectively. These high frequencies were similar to or somewhat higher than those described in the previous reports: immunopositivities for p-mTOR, cytoplasmic HIF-1α, nuclear HIF-1α, and Glut1 have been reported as 60%, 86% to 100%, 50% to 75%, and 67% of the CCA cases studied, respectively (27,28,32,33). These slight discrepancies probably resulted from the difference in the primary antibodies used and the staining protocols, the definition of positivity for overexpression, and our specific patient cohort, such as CCAs with synchronous putative precursors.

When compared with the synchronous (or adjacent) putative precursor lesions lacking cytological atypia, expressions of p-mTOR, HIF-1α, and Glut1 were much more frequently involved in the invasive carcinoma components. In the putative precursor lesions, the frequencies of these alterations increased in accordance with higher cytological atypia, and there were statistically significant differences regarding the positivity for nuclear and cytoplasmic HIF-1α expression and Glut1 expression. Moreover, in most of the cases analyzed, once each protein alteration appeared in the precursor lesions without atypia (or those with atypia), the alterations were maintained in the corresponding more severe disease, that is, in the atypical precursors or invasive carcinoma components. Taken together with the very high frequent expression in invasive-carcinoma components, it has been suggested that overexpressions of p-mTOR, HIF-1α, and Glut1 are early events in ovarian clear-cell carcinogenesis, and accumulation of these alterations is highly associated with the tumor development of ovarian CCAs.

In the benign-CCAF components, although p-mTOR expression and cytoplasmic HIF-1α were found to be positive in 27 and 9% of the examined lesions, respectively; nuclear expressions of HIF-1α and Glut1 expression were not documented. However, when cytological atypia became apparent (i.e. in the borderline CCAF), more than half of the lesions were positive for p-mTOR or (cytoplasmic and nuclear) HIF-1α expression, and 43% of the lesions showed Glut1 expression. These data suggest that these protein expressions are closely related to the acquisition of cytological atypia in the CCAF, namely, progression from the benign-CCAF to borderline CCAF. However, p-mTOR and cytoplasmic HIF-1α expression was judged as positive in 58 and 58% of the nonatypical endometrioses synchronous with CCA, respectively, and these frequencies were higher than those found in the solitary endometriosis. Therefore, these data suggest that, in contrast to the CCAF-associated cases, p-mTOR and HIF-1α aberrations have already occurred at a very early stage of endometriosis-associated carcinoma development. Recently, ovarian CCAs have been believed to be composed of a heterogeneous group of tumors, some arising from an endometriosis and others from a preexisting CCAF (29,30,34). Considering the overall data available, it could be stated that the timing of p-mTOR, HIF-1α, and Glut1 alterations would be different between these 2 carcinogenic pathways.

In this study, all of the non-neoplastic endometrium examined were judged as negative for p-mTOR or nuclear HIF-1α expressions, although Glut1 was positive in all of the gestational endometrium and 4 of the 6 proliferative-phase endometrium. In contrast, some cases of the solitary endometriosis were positive for p-mTOR, nuclear HIF-1α. Moreover, previous studies have reported that some amounts of p-mTOR, HIF-1α, or Glut1 protein expression could be detected in the epithelium of solitary endometriosis (25,35–38). Oxidative stress accelerated by iron excess and Akt/mitogen-activated protein kinase signaling activated by chronic inflammation have been suggested as the possible causes of these alterations in the solitary endometriosis (37–39). In addition, it has been thought that some of the solitary endometriotic lesions are already present in the neoplastic process (40). Therefore, it may be speculated

that accumulated oxidative stress and Akt/mitogen-activated protein kinase signaling, caused by focal iron excess and chronic inflammation, would induce overexpression of p-mTOR and HIF-1α in the endometriosis and initiate its neoplastic transformation, resulting in a precancerous potential of the endometriosis. In this study, all of the lesions, including putative precursors and carcinomas, which were positive for nuclear expression of HIF-1α, were also positive for cytoplasmic HIF-1α expression, perhaps indicating the known intracellular dynamics of activated HIF-1α: its molecular stabilization and accumulation in the cytoplasm, and after translocation to the nucleus. In addition, in the putative precursor lesions, correlations in the positivity between p-mTOR expression and nuclear expression of HIF-1α and between nuclear expression of HIF-1α and Glut1 expression, were statistically supported. These findings suggest that overexpression of mTOR, HIF-1α, and Glut1 in the CCA development might be partly in the mTOR-HIF-1α-Glut1 pathway. However, in some precursor lesions, nuclear expression of HIF-1α occurred in the negative p-mTOR expression status. Other than mTOR signaling, it has been demonstrated that intracellular accumulation of HIF-1α can also be accelerated by other pathological status, such as loss of phosphatase and tensin homolog or *VHL* function (14,17,18). As frequent allelic loss of the *VHL* locus has been reported in ovarian CCAs (41), there may be an alternative pathway for the activation of HIF-1α in this carcinoma, and these issues will be analyzed in future.

What are the potential mechanisms for mTOR activation in ovarian CCAs? mTOR is one of the major downstream molecules of the receptor tyrosine kinases and subsequent phosphatidylinositol 3/Akt signaling. Of the various receptor tyrosine kinase families, platelet-derived growth factor (PDGF) receptors are frequently overexpressed in CCA cells with its ligand PDGFs, and these coexpressions are known to be highly associated with the development of CCA (29). Moreover, our recent study demonstrated that among ovarian carcinomas, copy-number alterations of the *MET* gene and its protein overexpression are characteristically and commonly involved in the CCA histology (42). Recent studies have reported a high frequency of the activating mutations of the *PIK3CA* gene in CCA, estimated as 40% to 46% of the CCAs analyzed (5,6). Therefore, interactions between the alteration of PDGF receptors/PDGF, *MET* gene or *PIK3CA* gene, and mTOR signaling in individual cases of CCA would be of interest not only for understanding the pathobiology of this tumor but also for developing new therapeutic strategies.

In summary, this study has demonstrated that expressions of p-mTOR, HIF-1α, and Glut1 may be early events in ovarian clear-cell carcinogenesis, and may be highly associated with tumor development. Although the possible correlation among mTOR, HIF-1α, and Glut1 alterations has been statistically supported in CCA tumorigenesis, alternative pathways involving HIF-1 activation and molecular mechanisms triggering mTOR activation should be analyzed in future.

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