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ORIGINAL PAPER

# Identification of potential serum markers for endometrial cancer using protein expression profiling

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**Abstract**
*Objectives* Screening method of endometrial cancer (EC) has not been established yet. Our study was to explore serum biomarkers of EC patients using surface-enhanced laser desorption and ionization-time-of-flight mass spectrometry (SELDI-TOF MS).
*Methods* Serum samples from 65 EC patients and 40 controls were analyzed by SELDI-TOF MS (training set). Single- and multi-variant analyses were performed to compare protein profiles in serum of EC patients and healthy controls. Subsequently, blind test set including 40 EC patients and 40 controls were analyzed for validation.
*Results* A panel of four biomarker candidates were selected in training set analysis. These markers could also distinguish stage I patients from controls. Among them, two biomarkers were purified and identified as apolipoprotein A1 and a modified form of apolipoprotein C1. Screening for blind test set using dual-biomarker analysis yielded a sensitivity of 82% and a specificity of 86%.
*Conclusions* Involvement of apolipoproteins with EC is first suggested in this study. In addition to possibility of screening method for EC, findings of these new biomarkers might be related with carcinogenesis or predisposition to EC.

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## Introduction

Endometrial cancer (EC) is the most common malignancy of the female reproductive tract and its incidence is increasing in North America and Europe (Amant et al. 2006; Shang 2006). There are numerous reports regarding screening methods for EC. Elevation of CA125 has been detected in a number of gynecologic diseases including EC (Jacobs and Bast 1987). However, raised serum levels of CA125 (>35 U/ml) have been reported in only 11–33.9% of the patients of this disease, and elevation of CA125 was closely related with only advanced disease (Gadducci et al. 2004). Additionally, elevation of other tumor-associated markers, such as CA19-9, CEA, and CA15-3, were detected in only one-fourth of the patients (Cherchi et al. 1999). Although the utilities of ultrasound or hysteroscopy in combination with biopsy and cytology significantly improved the screening efficacy of EC (Emoto et al. 2002; Minagawa et al. 2005; Tabor et al. 2002; Mutter et al. 2000), some cases would still be missed with false-positive test results.

Surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS) is a proteomic technique which enable to identify multiple differentially expressed proteins in a large set of samples. The ProteinChip SELDI system (Bio-Rad Laboratories, Hercules, CA) has been applied for the discovery of new biomarkers in many diseases. In the present study, we have attempted to find out novel biomarkers for EC. Through the proteomic analysis of ProteinChip SELDI system, two biomarkers related with metabolism of lipid, were discovered. In addition to application for the detection of EC, these findings may be closely related with the carcinogenesis of the tumors.

## Materials and methods

### Patient and serum sample preparation

Serum samples from pathologically confirmed endometrial cancer (EC) patients were obtained after written informed consent. The study was performed after approval of each institutional review board. Between April of 2005 and October of 2006, all patients were treated in collaborating hospitals in Japan; National defense medical college hospital, Ohki memorial Kikuchi cancer clinic for women, Teikyo university hospital, Tottori university hospital, Hokkaido university hospital, Sasaki research institute Kyoundo hospital, Iwate medical university hospital, Tohoku university hospital, Osaka city general hospital, Seto hospital. Further, blood samples from age-matched healthy controls were obtained with written informed consent. For the training set, we analyzed serum samples of 40 healthy controls and 65 EC cases; 50 stage I, 7 stage II, and 8 stage III tumors. Next, 40 endometrial cancers and 40 healthy controls ware analyzed for the validation study for blind test set analysis (Table 1). Endometrial cancer group of training set included 49 cases of endometrioid grade 1 (stage I 40, stage II 5, stage III 4), 11 cases of endometrioid grade 2 (stage I 7, stage II 2, stage III 2), and 5 cases of endometrioid grade 3 or other carcinoma (stage I 3, stage III 2). Endometrial cancer group of blind set group included 28 cases of endometrioid grade 1 (stage I 20, stage II 3, stage III 5), 9 cases of endometrioid grade 2 (stage I 6, stage II 1, stage III 2), and 3 cases of endometrioid grade 3 or other carcinoma (stage I 2, stage III 1). Approximately 8 ml of blood was drawn by venipuncture and placed on ice. The samples were centrifuged at 3,000 rpm for 20 min within 2 h and serum was aliquoted and stored at −80° until use.

**Table 1** Number of the patients in training set and blind test set

| Group | Training set | Blind test set |
|---|---|---|
| Control group | 40 | 40 |
| Endometrial cancer group | 65 | 40 |
| FIGO stage | | |
| Stage I | 50 | 28 |
| Stage II | 7 | 4 |
| Stage III | 8 | 8 |
| Histology | | |
| Endometrioid, grade 1 | 49 | 28 |
| Endometrioid, grade 2 | 11 | 9 |
| Endometrioid, grade 3 or others | 5 | 3 |

*FIGO* The International Federation of Gynecology and Obstetrics

### SELDI ProteinChip array

Serum samples endometrial cancer patients and controls were denatured by 9 M urea, 2% CHAPS, 50 mM Tris–HCl, pH 9 and were pre-fractionated into six fractions using strong anion exchange resin by reducing of buffer pH. Obtained fractions were incubated in three different chip conditions; CM10, H50, and IMAC30 arrays (Bio-Rad Laboratories). Pretreatment with binding and washing buffers was performed according to the manufacture's instruction. All sample pre-fractionation and chip profiling process was performed by Biomek2000 robot (Beckman Coulter, Inc., Fullerton, CA). Briefly, arrays were incubated with 150 µl binding buffer [100 mM Sodium Acetate (pH4) and 50 mM HEPES (pH7) for CM10 chip, 50 mM HEPES (pH7) for H50 chip, and 100 mM Sodium Phosphate (pH7)/ 0.5 M NaCl for Copper immobilized IMAC30 chip] for 5 min followed by application of 10 µl of pre-fractionated

serum samples and 90 μl binding buffer to each spot. After incubation for 30 min at room temperature, spots were washed three times with binding buffer for 5 min and rinsed with distilled water. After air drying, 0.5 μl of a saturated solution of sinapinic acid or of a 50% saturated solution of α-cyano-4-hydroxycinnamic acid)in 0.5% trifluoroacetic acid and 50% acetonitrile was applied onto each bait surface of arrays. Following a final air drying, protein profiles were read using SELDI-TOF MS (ProteinChip SELDI System, Bio-Rad Laboratories) on the same day. All samples were run in duplicate to confirm the consistency of assay.

Statistical analysis and SELDI-TOF mass spectra

All of the duplicated spectra were complied, and the protein peak intensities were normalized using ProteinChip Data Manager Software (Bio-Rad Laboratories). The mass range from 3,000 to 10,000 *m/z* was measured as a low mass range, and the mass range of 10,000 to 30,000 *m/z* was measured as a high mass range. Maximum measured mass range was 200,000 *m/z*. Next, peak clustering was generated, and single- and multi-marker analyses were performed to compare protein profiles in serum of endometrial cancer patients and healthy controls. Receiver operating characteristics (ROC) curves was constructed to evaluate the predictive power for each peak, and Mann–Whitney test were used for statistical analysis. A $P$ value of $<0.05$ was considered significant. Further multi-marker analysis such as classification analysis were performed by ProteinChip Pattern Analysis Software (Bio-Rad Laboratories).

Protein Identification

After purification of the marker candidates, they were excised from the polyacrylamide gel stained with Colloidal Blue Staining Kit (Invitrogen Japan K.K., Tokyo, Japan). The extracts were applied to NP20 ProteinChip arrays and reanalyzed with the ProteinChip Reader to confirm *m/z* values of the excised/extracted proteins. After treatment with trypsin, peptide identification was performed using the tandem mass spectrometer equipped with a PCI-1000 ProteinChip Interface (Ciphergen Biosystems, Inc.). MS/MS spectra were submitted to the database mining tool (Mascot; Matrix Sciences) for identification.

## Results

Discovery of endometrial cancer-specific serum biomarker in training set

Initially, a total of 9,175 peaks were detected in SELDI-TOF MS analysis in the *m/z* region of 1,000–10,000.

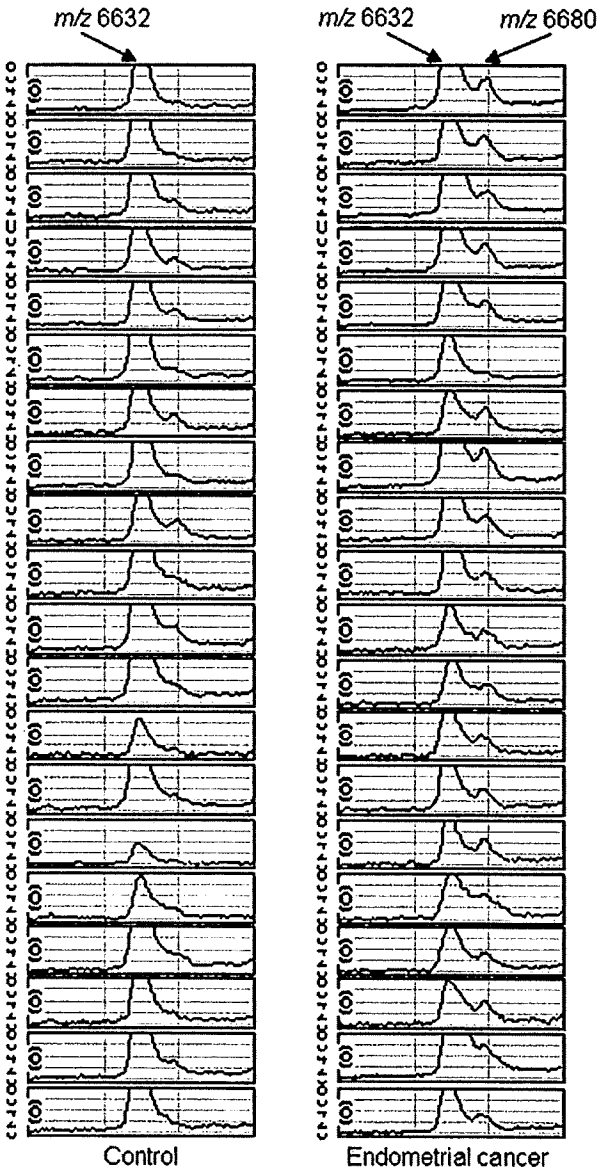


**Fig. 1** Representative differential SELDI-TOF mass spectra of serum samples of endometrial cancer patients and healthy control. The mass spectrographic profile reveals upregulation of *m/z* 6,680 peak in endometrial cancer patients

Among these peaks, the spectra generated from control group and endometrial cancer group were analyzed using ProteinChip Pattern Analysis Software. In these peaks, eight peaks were discovered for the discrimination of endometrial cancer and non-cancer control. Finally, four markers were selected as candidates after reproducibility check. These four peaks corresponded to *m/z* of 3,340, 6,680, 9,300, 28,000 (Table 2). The peaks at *m/z* ratio of 3,340, 6,680 were upregulated, and the peaks at *m/z* ratio of 9,300, 28,000 were downregulated in the cancer group. Peak intensity of the candidate at *m/z* of 6,680 was increased in

**Table 2** Peak intensity and area under the curve (AUC) of receiver operating characteristics (ROC) analysis for biomarker candidates

| Biomarker candidate (*m/z*) | Peak intensity | EC/control | AUC | *P* value |
|---|---|---|---|---|
| 6,680 | 9.37 ± 1.83 | 1.13 ± 0.22 | 0.88 | 0.00001 |
| 3,340 | 7.43 ± 1.17 | 1.16 ± 0.18 | 0.92 | 0.00000017 |
| 28,000 | 0.21 ± 0.05 | 0.72 ± 0.17 | 0.86 | 0.000056 |
| 9,300 | 0.49 ± 0.09 | 0.88 ± 0.16 | 0.88 | 0.000015 |

*EC* Endometrial cancer

endometrial cancer patients compared with control (Fig. 1), and the differences was also significant for the stage I patients only (mean value; 9.57 vs. 8.31, $P = 0.00003$). Serum level of the candidate at *m/z* of 28,000 was significantly decreased in endometrial cancer patients compared with control, and the difference was still significant when the cases were limited in the stage I patients (mean value; 0.20 vs. 0.29, $P = 0.0003$). ROC curves of the biomarker candidates at 6,680 *m/z* and at 28,000 *m/z* are shown in Fig. 2. The AUC of the ROC plot was 0.88 at *m/z* of 6,680, and 0.86 at *m/z* of 28,000, respectively.

There were no significant differences of mean peak intensities of four biomarkers between grade 1/2 endometrioid adenocarcinoma cases and grade 3 endometrioid adenocarcinoma/other carcinomas (data not shown).

## Identification of the candidates

The *m/z* 28,000 peak was purified and identified as apolipoprotein A1. All five tryptic digested peptides were sequenced by tandem mass spectrometry and confirmed this finding. The *m/z* 9,300 peak was identified triple charge of *m/z* 28,000 peak, apolipoprotein A1.

Peaks at *m/z* ratio of 6,632 were neighboring on the peaks at *m/z* 6,680, and higher than the peaks. There was no statistical significant difference of *m/z* 6,632 peak intensity between control and EC group (data not shown). The molecular weight (MW) of the 6,680 Da peptide and neighboring peptide (6,632 Da) were searched against SWISS-PROT using TagIdent that predicts protein/peptide by MW and pI. The neighboring peptide at 6,632 Da was predicted as apolipoprotein C1. To confirm *m/z* 6,680 is apolipoprotein C1 related protein or not, the interaction assay was established by using PS10 Chip (Bio-Rad Laboratories) attaching anti-human antibody of apolipoprotein C-1 (abcam plc., Cambridge, UK) or control antibody were prepared for interaction analysis. The *m/z* 6,632 peak located close to the present candidate at *m/z* 6,680 was identified apolipoprotein C-1 through SELDI-TOF MS interaction study. Target peak at *m/z* 6,680 as well as peak at *m/z* 6,632 (apolipoprotein C-1) was detected through the ProteinChip attaching anti-apolipoprotein C-1 antibody, but the peak was not detected by the ProteinChip attaching control immunoglobulin G (Fig. 3). This candidate protein was identified as a modified form of apolipoprotein C1. The peak at *m/z* ratio of 3,340 was identified as double charge of candidate protein showing peak at *m/z* 6,680.

## Validation analysis for screening of endometrial cancer in blind test set

The efficacy of these biomarkers for detection of EC was summarized in Table 3. From the efficacy of the training set analysis, cut off point of peak intensity level was determined as 0.2 for *m/z* 28,000, and 9.8 for *m/z* 6,680, respectively. In training set analysis, dual-marker analysis of these two biomarkers yielded a sensitivity of 78% and a specificity of 90%. In the analysis for blind test set, dual-marker analysis of two peaks yielded a sensitivity of 82% and a specificity of 86% (Table 3).

## Discussion

Using ProteinChip techniques, many studies have isolated serum biomarkers of malignancy for the detection of early stage tumors. However, there have been few reports analyzing

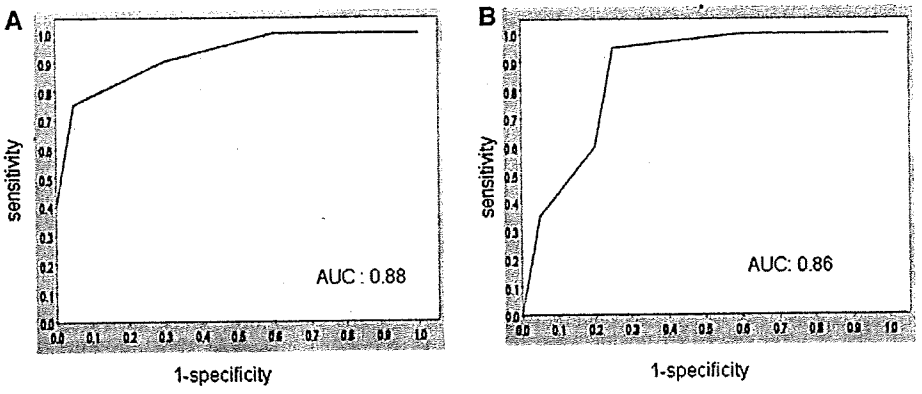


**Fig. 2** Receiver operating characteristics (ROC) curves of the biomarker candidates at 6,680 *m/z* (**a**) and at 28,000 *m/z* (**b**). The area under the curve (AUC) of the ROC curve was 0.88 at 6,680 *m/z* and 0.86 at 28,000 *m/z*

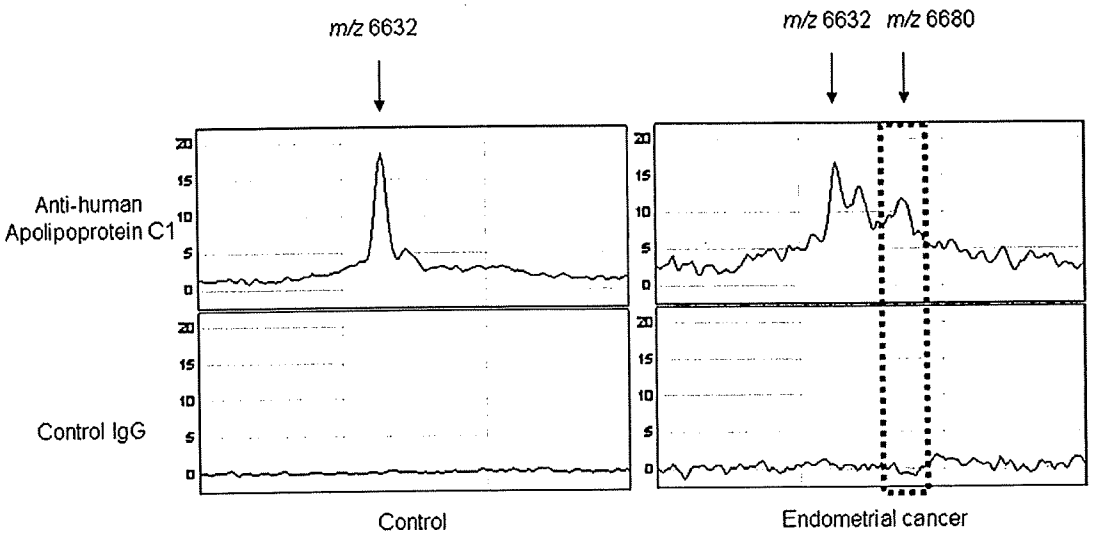


**Fig. 3** Representative SELDI-TOF MS profiles of interaction study for serum samples of endometrial cancer patients and controls. Target peak at *m/z* 6,680 as well as peak at *m/z* 6,632 (apolipoprotein C-1) was detected through the ProteinChip attaching anti-apolipoprotein C-1 antibody, but the peak was not detected by the ProteinChip attaching control immunoglobulin G

**Table 3** Efficacy of candidate biomarkers for detection of endometrial cancer

| Group of analysis | Single marker analysis (28,000 *m/z*) | | | Dual-marker analysis |
|---|---|---|---|---|
| | Cut off of average peak intensity | | | 28,000* and 6,680 *m/z* |
| | 0.2 | 0.235 | 0.25 | |
| Training set | | | | |
| Sensitivity (%) | 48 | 58 | 69 | 78 |
| Specificity (%) | 95 | 70 | 60 | 90 |
| Blind test set | | | | |
| Sensitivity (%) | 55 | 68 | 75 | 82 |
| Specificity (%) | 90 | 75 | 68 | 86 |

*Cut off of average peak intensity was 0.2 of 28,000 *m/z*

serum biomarkers for endometrial cancer (EC). In the present study, two candidate serum markers were discovered using this proteomic analysis. One marker was downregulated, and the other was upregulated in patients with EC.

Many authors have described on the pathogenesis of EC. Alteration of *PTEN* appeared to be the earliest and most fundamental genetic change, and observed in up to 80% of EC tumors (Mutter et al. 2000, 2001; Kanamori et al. 2001, 2002; Salvesen et al. 2004). Mutations of *K-ras* were observed in up to 30% of the EC tumors (Lagarda et al. 2001; Lax et al. 2000; Enomoto et al. 1995). Alterations of *p53* were not commonly observed in tumors of ECs, whereas grade 3 EC tumors harbored the mutations of *p53* (Lax et al. 2000; Kolasa et al. 2006; Feng et al. 2005). Recently, the extracellular-regulated kinase and PI3K pathway had been reported as another important pathway related to carcinogenesis and prognosis of EC patients (Mori et al. 2007; Mizumoto et al. 2007; Velasco et al. 2006). Through the global expression analysis by proteomics, other distinct biomarkers have been discovered. Using SELDI-TOF MS technology, a study has shown increased expression of chaperonin 10, a member of heat shock protein, in tissue samples of EC (Yang et al. 2004). Further, the authors have identified calgranulin A as another biomarker for EC. Calgranulin A is a member of S100 family of calcium binding protein and might be involved in inflammation caused by malignant tumors (Guo et al. 2005). Yoshizaki et al. (2005) have investigated that EC tumors showed increased peak level at *m/z* 9,600 and decreased expression level at *m/z* 11,300 by SELDI-TOF MS analysis. These biomarkers discovered by proteomic analysis have not been clearly implicated in EC; however, the findings would facilitate the pathogenic analysis of EC and be expected for new clinical markers of screening or monitoring EC.

Two biomarkers discovered in the present study belong to apolipoproteins. Downregulation of serum apolipoprotein A1 has been observed in several cancers including ovary

(Zhang et al. 2004; Gadomska et al. 2005; Kozak et al. 2005; Moore et al. 2006), breast (Huang et al. 2006; Chang et al. 2007), pancreas (Ehmann et al. 2007). Apolipoprotein A1 is the major lipoprotein found in high density lipoprotein (HDL), and has been found to have potent anti-inflammatory and antioxidant properties (Navab et al. 2007). Considering the concept that inflammation is a critical component for tumor progression has been widely accepted (Coussens and Werb 2002), it is possible that apolipoprotein A1 might be related with predisposition of the host to EC.

Recently, the clinical and pathological effects of apolipoprotein C-1 upon malignancy have been elucidated (Takano et al. 2008). Apolipoprotein C-1 was highly expressed in pancreas cancer cells, and is also detected in the culture medium of the pancreatic cancer cell line in vitro, suggesting that cancer cells secrete apolipoprotein C-1. Also, apolipoprotein C-1 is related with cell proliferation and cell apoptosis in vitro, and with the aggressiveness of pancreatic cancer in vivo. Furthermore, RELN pathway through signaling via the very low density lipoprotein (VLDL) receptor, to which apolipoprotein C-1 is known to bind, influences cell motility in pancreatic cancer (Sato et al. 2006). The *m/z* 6,680 peak detected in the present study was identified as a modified form of apolipoprotein C-1, but the modification of the protein has not been elucidated yet. The identification of the modification would facilitate further understanding of the pathogenesis or host reaction to EC tumors.

In the blind test set analysis for EC screening, the sensitivity of single marker test using apolipoprotein A-1 (*m/z* 28,000) yielded up to 75%, and dual-marker analysis showed a sensitivity of 82%. However, the specificity was 68–90% in single marker test with apolipoprotein A-1 and 86% in dual-marker analysis. The cut off points for cancer screening should be selected to maximize sensitivity, but that would lead to lower specificity. Further discovery of new biomarkers or new imaging technology might contribute to maximal screening effects in combination with the present biomarkers. For the successful application of these biomarkers, apolipoprotein A-1 and a modified form of apolipoprotein C-1, additional work to assure consistency of measured peak intensities without institutional bias and sample bias is needed and the investigation should be confirmed in the prospective analysis for EC.

**Conflict of interest statement** We declare that we have no conflict of interest.

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# The Impact of Complete Surgical Staging Upon Survival in Early-Stage Ovarian Clear Cell Carcinoma

## *A Multi-institutional Retrospective Study*

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**Abstract:** Pure-type clear cell carcinoma (CCC) has been recognized as a distinct subtype of ovarian cancer, showing resistance to conventional platinum-based chemotherapy and resulting in poor prognosis. The aim of the study was to evaluate the effects of complete surgical staging procedures for early-stage CCC patients in a retrospective multi-institutional analysis. During the period 1992 to 2002, a total of 199 patients with pT1 M0 CCC were identified. Survival analysis was estimated by Kaplan-Meier methods, and prognostic factors were evaluated using a Cox regression model. Among pT1 M0 tumors, retroperitoneal lymph node status was negative in 125 cases (pN0, 63%), positive in 10 cases (pN1, 5%), and unknown in 64 cases (pNx, 32%). Progression-free survival of pN1 was significantly worse than that of pN0 ($P < 0.05$), whereas there was no significant difference between pN1 and pNx. There was no significant difference of overall survival (OS) among the 3 groups. Multivariate analysis revealed that peritoneal cytology status was the only independent prognostic factor for progression-free survival ($P = 0.04$), but completion of surgical staging procedures was not a prognostic factor. There was no significant prognostic factor for OS. Our study implied that complete surgical staging enabled us to distinguish a high-risk group of recurrence in pT1 M0 CCC; however, the procedure could not improve OS. Although the study was a limited retrospective study, the impact of peritoneal cytology status was more important than complete surgical staging procedure in CCC patients. More effective treatment modality was warranted, especially for CCC cases positive for malignant peritoneal cytology.

**Key Words:** Ovarian cancer, Clear cell carcinoma, Surgical staging, Lymphadenectomy, Washing cytology

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Despite recent improvement of treatment modality including chemotherapy, ovarian carcinoma is the leading cause of death in all gynecologic malignancy in the most developed countries.[1,2] In several histological subtypes of ovarian cancers, clear cell carcinoma (CCC) is comparatively resistant to most of anticancer drugs including conventional platinum-based chemotherapy[3–6]

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TABLE 1. Characteristics of the patients with ovarian CCC confined to the ovary (pT1 M0)

| | pN0 | pN1 | pNx | P |
|---|---|---|---|---|
| Total | 124 | 10 | 65 | |
| Age, y | | | | 0.97 |
| <54 | 78 (63%) | 6 (60%) | 40 (62%) | |
| >55 | 46 (37%) | 4 (40%) | 25 (38%) | |
| Performance status | | | | 0.15 |
| 0 | 117 (94%) | 8 (80%) | 62 (95%) | |
| 1, 2 | 7 (6%) | 2 (20%) | 3 (5%) | |
| pT status | | | | 0.61 |
| pT1a | 32 (26%) | 3 (30%) | 13 (20%) | |
| pT1c | 92 (74%) | 7 (70%) | 52 (80%) | |
| Ascites/ malignant washing | | | | 0.98 |
| Negative | 77 (62%) | 6 (60%) | 41 (63%) | |
| Positive | 47 (38%) | 4 (40%) | 24 (37%) | |
| Postoperative chemotherapy | | | | 0.39 |
| Paclitaxel + platinum | 51 (41%) | 6 (60%) | 20 (31%) | |
| Others* | 64 (52%) | 4 (40%) | 38 (58%) | |
| None | 9 (7%) | 0 (0%) | 6 (9%) | |

*CAP, cyclophosphamide + doxorubicin + cisplatin; CP, cyclophosphamide + doxorubicin + cisplatin; CPT-P, cisplatin + irinotecan hydrochloride.

and paclitaxel-based regimen,[7,8] resulting in poor prognosis, especially in advanced cases. On the other hand, approximately half of CCC tumors were diagnosed as stage I tumor.[9] Surgical staging including retroperitoneal lymphadenectomy was inevitable for the FIGO (International Federation of Gynecology and Obstetrics) staging system.[10] There have been several reports regarding the survival of stage I CCC patients[11–15]; however, it still remains unclear whether complete surgical staging procedure is clinically beneficial for survival of early-stage CCC. The aim of the present study was to evaluate the effect of complete surgical staging procedures upon survival of CCC tumors confined to the ovary (pT1 M0) in a large retrospective multi-intuitional analysis.

## METHODS

### Patients and Tumors

Between 1992 and 2002, 334 patients with CCC of the ovary were identified by scanning the medical records of the collaborating institutions and central pathological review by 2 independent pathologists with no knowledge of the patients' clinical data. Tumors were diagnosed as CCC if typical clear or hobnail cells growing in a papillary, solid, or tubulocystic pattern are present in more than 90% of all pathological specimens. Patients received initial treatment and follow-up at 9 institutions belonging to Japan Clear Cell Carcinoma Study Group; National Defense Medical College Hospital, Tohoku University Hospital, Jichi Medical University Hospital, Osaka City General Hospital, Sapporo Medical University, Fujita Health University Hospital, Aichi Cancer Center Hospital, Tottori University Hospital, Kobe National Hospital, and Iwate Medical University Hospital. In those patients, 135 patients were excluded because of advanced FIGO stages more than stage II. The remaining 199 patients (60%) were enrolled on the present study.

Among enrolled patients, 134 patients underwent complete surgical staging procedures including hysterectomy, bilateral salpingo-oophorectomy, peritoneal washing, omentectomy, pelvic lymphadenectomy, and paraaortic lymphadenectomy. Pelvic lymphadenectomy was done from the common, external and internal iliac node, and obturator vessel to the femoral ring. Paraaortic lymphadenectomy was done from the bottom of the left renal vessel including the left infrarenal lymph nodes to the bifurcation of the aorta. Cases that underwent hysterectomy and bilateral salpingo-oophorectomy with only lymph node exploration or sampling were regarded as pNx tumors. Selection of the surgical procedure was determined by the physicians' preference. The resected lymph node counts were not considered for the completion of the lymphadenectomy. A pN1 case was determined as having 1 or more lymph node metastases in pelvic or paraaortic lymph nodes.

### Chemotherapy

One hundred eighty-four patients (92%) received 3 to 6 cycles of postoperative chemotherapy after initial surgery. The regimen of the chemotherapy was as follows: paclitaxel and platinum in 77 cases, other platinum-based regimens in 106 cases, and no chemotherapy in 15 cases. Paclitaxel and platinum regimen consisted of an infusion of 175 to 180 mg/m$^2$ of paclitaxel and 50 to 75 mg/m$^2$ of cisplatin or carboplatin (area under concentration curve = 5–6).

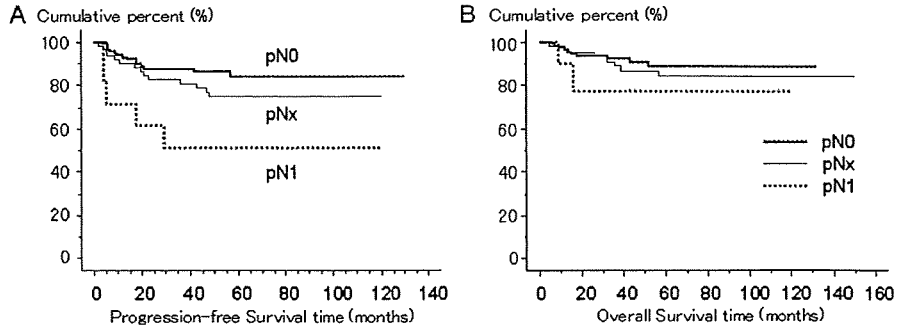


FIGURE 1. A, Progression-free survival time according to lymph node status. There is no significant prognostic difference between pN0 group and pNx group ($P = 0.16$) and between pNx group and pN1 group ($P = 0.06$). The patients with pN0 had significantly better PFS than those with pN1 ($P = 0.001$). Five-year PFS rate was 84% in pN0 group, 75% in pNx group, and 51% in pN1 group. B, Overall survival time according to lymph node status. There are no statistical differences among 3 groups; pN0 versus pNx, $P = 0.52$; pNx versus pN1, $P = 0.46$; pN0 versus pN1, $P = 0.22$. Five-year OS rate was 88% in pN0 group, 84% in pNx group, and 79% in pN1 group.

TABLE 2. Multiple regression analysis for the PFS of patients with ovarian CCC confined to the ovary (pT1 M0)

| Variables | Hazard Ratio | 95% Confidence Interval | P |
|---|---|---|---|
| Age, y | | | 0.92 |
| <54 | 1 | | |
| >55 | 1.04 | 0.50–2.14 | |
| Performance status | | | 0.83 |
| 0 | 1 | | |
| 1, 2 | 1.08 | 0.54–2.15 | |
| pT status | | | 0.39 |
| Stage Ia | 1 | | |
| Stage Ic | 1.67 | 0.52–5.37 | |
| pN status | | | 0.05 |
| pN0 | 1 | | |
| pNx | 1.62 | 0.20–3.33 | |
| pN1 | 3.75 | 0.44–31.93 | |
| Ascites/malignant washing | | | 0.04 |
| Negative | 1 | | |
| Positive | 2.07 | 1.03–4.16 | |
| Postoperative chemotherapy | | | 0.17 |
| Paclitaxel + platinum | 1 | | |
| Other regimens* | 0.45 | 0.16–1.24 | |
| None | 0.86 | 0.42–2.38 | |

*CAP, cyclophosphamide + doxorubicin + cisplatin; CP, cyclophosphamide + doxorubicin + cisplatin; CPT-P, cisplatin + irinotecan hydrochloride.

Other regimens included CAP (cyclophosphamide [500 mg/m$^2$], doxorubicin [50 mg/m$^2$], and cisplatin [50–70 mg/m$^2$], every 3–4 weeks), CP (cyclophosphamide [500 mg/m$^2$] and cisplatin [50–70 mg/m$^2$], every 3–4 weeks), and CPT-P (irinotecan hydrochloride [50–60 mg/m$^2$, on days 1, 8, and 15] and cisplatin [50–60 mg/m$^2$, on day 1], every 4 weeks).

Progressive disease was defined as the appearance of a new lesion evaluated by computed tomography of chest/abdomen or pelvic magnetic resonance images. Serum levels of tumor markers including CA-125 were not used for survival analysis in the present study. The time to progression was defined as the interval from the date of primary surgery until the date of progressive disease. Survival duration was determined as the time from the date of primary surgery until death or the date of last follow-up contact.

## Statistical Methods

Kaplan-Meier method was used for calculation of patient survival distribution. The significance of the survival distribution in each group was tested using the log-rank test. The $\chi^2$ test and Student $t$ test for unpaired data were used for statistical analysis. $P < 0.05$ was considered statistically significant. The StatView software version 5.0 (SAS Institute Inc, Cary, NC) was used to analyze the date.

## RESULTS

A total of 199 pT1 M0 CCC patients were enrolled on the study. Among them, retroperitoneal lymph node status was negative in 125 cases (pN0, 63%), positive in 10 cases (pN1, 5%), and unknown in 64 cases (pNx, 32%). Median follow-up time was 48 months (range, 6–130 months) in pN0 group, 50 months (range, 5–83 months) in pN1 group, and 57 months (range, 5–150 months) in pNx group. Characteristics of the patients are summarized in Table 1. There were no statistical differences among 3 groups according to age, performance status, pT status, ascites/malignant washing, and postoperative chemotherapy.

Five-year progression-free survival (PFS) rate was 84% in pN0 group, 75% in pNx group, and 51% in pN1 group. Progression-free survival of pN1 was significantly worse than that of pN0 (Fig. 1A, $P = 0.001$), whereas there was no significant difference between pN1 and pNx ($P = 0.06$). There is no significant prognostic difference between pN0 group and pNx group ($P = 0.16$). Five-year overall survival (OS) rate was 88% in pN0 group, 84% in pNx group, and 79% in pN1 group (Fig. 1B). There were no statistical differences of OS among the 3 groups: pN0 versus pNx, $P = 0.52$; pNx versus pN1, $P = 0.46$; pN0 versus pN1, $P = 0.22$.

Multiple regression analysis was performed to detect prognostic factors for PFS of stage I CCC. Values of age, performance status, pT and pN status, ascites or peritoneal washing status, and chemotherapy were compared. As a result, ascites or peritoneal cytology status was the only independent prognostic factor for PFS of CCC tumors confined to the ovary (Table 2, $P = 0.04$). Although pN status showed a marginal difference ($P = 0.05$), it was not an independent prognostic factor. Other variables were not prognostic factors for PFS including pT status and chemotherapeutic regimen. In addition, the same variables as described in Table 2 were analyzed for OS of CCC patients; however, there were no significant prognostic factors for OS (data not shown). Progression-free survival and OS curves according to peritoneal cytology status are

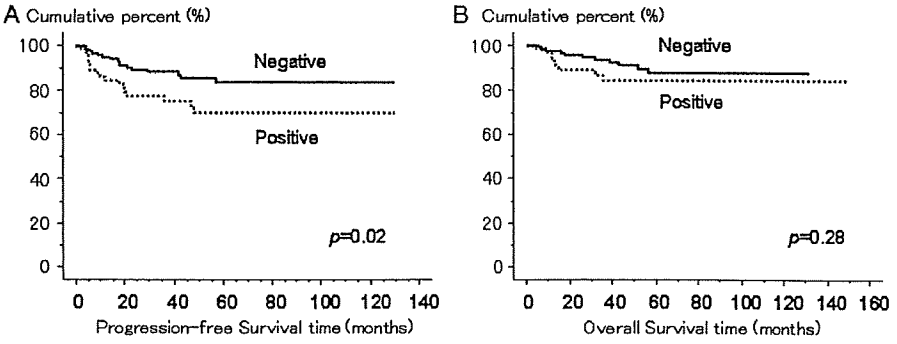


FIGURE 2. Progression-free survival time (A) and OS time (B) according to malignant ascites/washing peritoneal cytology status. Progression-free survival of the patients with malignant ascites/positive peritoneal washing (positive group) was significantly worse than those with negative cytology (negative group) ($P = 0.02$). Overall survival time was similar in the 2 groups ($P = 0.28$).

shown in Figure 2. Progression-free survival of the patients with malignant ascites/positive peritoneal washing was significantly worse than those with negative cytology ($P = 0.02$), but there was no difference in OS ($P = 0.28$).

## DISCUSSION

It has been reported that the incidence of lymph node metastasis in stage I (pT1) ovarian cancer was approximately 20%.[16–19] Previous reports demonstrated that serous tumors had a higher incidence of lymph node metastasis than nonserous tumors including CCC tumors.[20,21] The incidence of lymph node involvement of CCC disease was 9.1% in pT1a and 7.1% in pT1c tumors in a large retrospective case series.[9] Lower incidence of lymphatic involvement in CCC has been suggested in comparison with other histological subtypes, especially serous tumors.

In early-stage ovarian cancer, clinical significance of complete surgical staging procedures upon survival was not determined. Some reports showed a positive relationship between node status and survival,[18,22–24] whereas others showed survival similarity between node positive and negative tumors.[25,26] However, the results in these reports were derived from retrospective analysis with relatively small numbers of patients. A prospective randomized clinical trial to assess the value of systematic lymphadenectomy in comparison with lymph node sampling was conducted by Maggioni et al,[27] enrolling a total of 268 cases. The incidence of positive nodes was significantly higher in the lymphadenectomy group: 22% versus 9%, $P = 0.007$; however, there were no significant differences in 5-year OS and PFS between both groups. In our present analysis, pN status showed only a marginal significance upon PFS and no significance upon OS. Together, it is possible that there are only small advantages in systemic lymphadenectomy upon survival of early-stage ovarian cancers including CCC. However, sentinel node detection methods have not been established yet[18]; comprehensive lymphadenectomy is still recommended as an essential procedure for accurate staging and prediction of recurrence in early-stage ovarian cancers.

In the present analysis, malignant peritoneal cytology was an independent prognostic factor for increased risk of recurrence for CCC tumors confined to the ovary (pT1 M0). Previous studies have demonstrated that positive peritoneal washing was associated with a poorer prognosis in patients with ovarian cancers.[28,29] The significant impact of peritoneal cytology upon survival was observed in the cases with CCC. Because CCC tumors generally showed resistance to conventional chemotherapy including platinums and taxans, these observations suggested the existence of intra-abdominal microdissemination, which included chemoresistant clones when the patients were positive for peritoneal washing. Further studies are needed to establish more effective regimens for CCC of the ovary.

In the aspects of molecular biologic characteristics as well as clinical behavior, it is hypothesized that CCC belongs to a different entity from other histological subtypes of ovarian carcinoma. There are many publications supporting this hypothesis, showing that the expression pattern was completely different in several molecular markers such as HNF-1β,[30,31] WT1,[32] ABCF2.[33] These molecules might be candidates used for targeting therapy for CCC in the near future.

The present study was, to our knowledge, 1 of the largest study including CCC tumors confined to the ovary, although it was retrospectively analyzed. The results implied that complete surgical staging enabled us to distinguish a high-risk group of recurrence in pT1 M0 CCC; however, we could not draw the conclusion that the procedures could improve OS of these patients.

For further evaluation, the study using the same chemotherapeutic regimen would be needed to eliminate the chemotherapy bias. In addition, definition of lymphadenectomy should be determined for adequate lymph node assessment. Approximately 81 lymph nodes (range, 49–128 lymph nodes) were reported to be located between the pelvis and the aortic area: 50 at the pelvis and 31 at the aorta.[34] Removal of 25 pelvic lymph nodes and 13 aortic lymph nodes was recommended for adequate detection of nodal involvement in epithelial ovarian cancer.[35] The definition like this would be extremely helpful not only for comparing retrospective analyses but also for designing prospective studies.

Although the study was a limited retrospective study, it was suggested that the impact of peritoneal cytology is more important than that of complete surgical staging procedure in CCC patients. More effective treatment modality was warranted, especially for cases positive for malignant peritoneal cytology. In addition, further efforts to elucidate the therapeutic effects of comprehensive staging procedures are needed in a large prospective study.

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# Differential Expression of Hypoxia-Inducible Protein 2 Among Different Histological Types of Epithelial Ovarian Cancer and in Clear Cell Adenocarcinomas

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**Objectives:** Epithelial ovarian cancer (EOC) can be classified into 5 major histological types. Among them, clear cell adenocarcinoma (CCC) has a poor response to chemotherapy and poor prognosis compared with other histological types. Previously, we reported that the hypoxia-inducible protein 2 (HIG2) gene might be a new biomarker for CCCs, based on its expression profile. In this study, we generated a polyclonal antiserum to HIG2 to explore the use of HIG2 as a predictive biomarker in EOC. In addition, HIG2 expression was evaluated in uterine endometrial and renal CCCs.

**Methods:** Hypoxia-inducible protein 2 expression was analyzed by immunohistochemistry in formalin-fixed surgical samples from 254 EOC, 17 endometrial, and 29 renal CCC patients.

**Results:** Hypoxia-inducible protein 2 is expressed in 175 of 254 ovarian cancer cases. Cytoplasmic HIG2 expression is significantly more frequent in ovarian CCC (83.1%) than in serous (54.9%, $P = 0.0001$), mucinous (40%, $P = 0.00002$), or endometrioid (58.1%, $P = 0.003$) adenocarcinoma. The chemoresponse rate was higher in 24 ovarian CCC patients with cytoplasmic HIG2 expression than in 6 CCC patients without HIG2 expression (62.5% [15/24] vs 0% [0/6], $P = 0.02$). In contrast, there was no relationship between nuclear HIG2 expression and chemoresponse. Cytoplasmic and nuclear HIG2 expressions are significantly more frequent in ovarian and uterine than renal CCC ($P = 0.04$).

**Conclusions:** Hypoxia-inducible protein 2 may be used as a marker for early detection of ovarian CCCs or for prediction of response to chemotherapy, but HIG2 expression does not predict survival of patients with CCC.

**Key Words:** Ovary, Clear cell adenocarcinoma, Kidney, Endometrium

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The authors have no conflicts of interest to declare.

Although epithelial ovarian cancer (EOC) has the worst prognosis of all gynecologic malignancies, the prognosis has improved dramatically. The survival rate of patients with EOC was greatly improved after introduction of platinum-taxane combination chemotherapy. Epithelial ovarian cancer can be subdivided into 5 major histological types (serous, mucinous, endometrioid, clear cell, and poorly differentiated), and among them, clear cell adenocarcinoma (CCC), which constitutes 10% to 20% of EOCs, differs from other histological types with respect to its clinical characteristics.[1,2] Clear cell adenocarcinoma is usually more resistant to systemic chemotherapy than other types and has a worse prognosis.[3,4] We have previously reported that because of chemotherapy resistance, successful primary cytoreductive surgery leaving no residual tumor and establishment of new chemotherapy regimens are important for improving the prognosis of CCC.[5,6]

We have also reported that ABCF2 (ATP-binding cassette superfamily F) protein expression is higher in CCCs than in other types, and its expression correlates with chemoresponse in patients with CCC.[7,8] Clear cell adenocarcinoma develops in different organs such as the ovary, the uterine corpus, and the kidney, and the prognosis of these cancers is usually poor.[3,4,9–11] We have previously reported that ovarian and uterine CCCs showed significantly higher levels of ABCF2 expression compared with renal CCC,[8] perhaps related to their differing origins, because ovarian and uterine CCCs are müllerian in origin, whereas renal CCC originates from the wolffian duct.[12] In addition, we have shown that hypoxia-inducible protein 2 (HIG2) expression is higher in CCCs than in the serous type, based on microarray and reverse transcriptase polymerase chain reaction (PCR) analysis.[7] Togashi et al[13] reported that HIG2 protein is highly expressed in renal CCC and plays an essential role in proliferation of these cells, acting through an autocrine mechanism. In this study, we generated an antiserum to HIG2 protein and evaluated HIG2 expression in 5 major histological types of EOC, as well as uterine and renal CCCs, and analyzed the relationship between HIG2 expression and clinical factors or prognosis.

## MATERIALS AND METHODS

### Clinical Samples

Two hundred fifty-four EOC, 17 uterine endometrial CCC, and 29 renal CCC samples were included in this study. The median age of ovarian cancer cases was 54 years (range, 22–80 years). Among the EOCs, there were 51 serous adenocarcinomas (18 stage I/II, 33 stage III/IV; histological grade: 36 good/moderate, 15 poor), 25 mucinous adenocarcinomas (17 stage I/II, 8 stage III/IV; histological grade: 24 good/moderate, 1 poor), 130 CCCs (85 stage I/II, 45 stage III/IV), 31 endometrioid adenocarcinomas (11 stage I/II, 20 stage III/IV; histological grade: 17 good/moderate, 14 poor), and 17 undifferentiated carcinomas (1 stage I/II, 15 stage III/IV). There were 17 uterine endometrial CCCs (8 stage I/II, 9 stage III/IV) and renal CCCs (4 stage I/II, 25 stage III/IV). The median age was 62 years (range, 52–83 years) and 63 years (range, 17–86 years), respectively. All patient-derived paraffin sections were collected and archived under protocols approved by the institutional review boards of the parent institutions.

### Generation and Validation of a Polyclonal Anti-HIG2 Antibody

A polyclonal anti-HIG2 antiserum was generated by injecting the purified HIG2 fusion protein into 2 rabbits. The specificity of the antiserum was determined by Western blot and immunocytochemical analyses on human embryonic kidney cells (293T) transfected with empty expression vector pcDNA3.1, with a vector containing a full-length HIG2 coding sequence (OriGene Technologies, Rockville, Md) or a full-length HIG2 coding sequence with *myc* and His tags. In brief, the open reading frame encoding the human HIG2 gene was amplified from the pET28a (+) plasmid containing HIG2, using the forward primer 5′-AATAGGATCCACCA TGCCCTCCGACCTGGC-3′ and the reverse primer 5′-AATAACTAGTCACGTTGTGGGTCCTCTTGG-3′. The PCR product was ligated in frame into the *Bam*HI and *Spe*I sites of the mammalian expression vector pcDNA3.1/*myc*-His A (Invitrogen, Carlsbad, Calif), which encodes a c-terminal *myc* epitope and 6 His amino acids. To exclude the His tag, a reverse primer 5′-AATAACTAGTTCACACGTT GTGGGTCCTCTTG-3′ was designed that included a stop codon in front of the *Spe*I site. In addition, PCR-amplified IRES-EGFP from pIRES2-EGFP (Takara Bio, Shiga, Japan) was inserted into the *Pme*I site of the pcDNA3.1 vector to allow evaluation of the transduction efficiency in 293T cells by detection of green fluorescent protein. The pcDNA3.1 vector alone or vectors containing the HIG2 sequence (pcDNA3.1/HIG2) and the *myc*-His–tagged HIG2 sequence (pcDNA3.1/HIG2mH) were then transiently transfected into human embryonic kidney cells (293T) grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 1 U/mL penicillin, and 1 μg/mL streptomycin using Lipofect AMINE (Invitrogen). After 3 days, cells were lyzed. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 12.5% polyacrylamide gel. Electrophoresed proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass). After blocking with phosphate-buffered saline containing 5% bovine serum albumin and 0.1% $NaN_3$, the membrane was incubated with rabbit anti–human HIG2 polyclonal antiserum or a mouse monoclonal anti-*myc* antibody (Medical & Biological Laboratories, Nagoya, Japan) for 1 hour at room temperature. Peroxidase-conjugated anti–rabbit IgG or peroxidase-conjugated anti–mouse IgG (Medical & Biological Laboratories) was then added and incubated for 1 hour at room temperature. Enzyme activity was detected using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

### Immunohistochemistry

In brief, formalin-fixed histological sections (4 μm) were affixed to glass slides, dewaxed, and rehydrated. The sections were incubated in 3% hydrogen peroxide for 10 minutes at room temperature to quench endogenous peroxidase activity. The sections were incubated with the anti-HIG2 antiserum

(diluted 1:5000) at 4°C overnight. Peroxidase activity was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxide for 2 to 10 minutes at room temperature. The sections were then counterstained with hematoxylin. The slides were read by 2 independent pathologists who were blind to the clinical background of the patients. At least 1000 tumor cells were scored for nuclear HIG2 protein. Nuclear HIG2 expression level was indicated as labeling index (LI), which was the percentage of tumor cells with nuclear staining. Cytoplasmic HIG2 expression was scored for staining intensity, as related to a positive control (0, no staining; 1, weak; 2, moderate; 3, strong), and finally, it was categorized as positive(1+, 2+, 3+) or negative(0).

## Chemotherapy Response or OS in Patients With CCC

Chemoresponse data from a total of 30 patients with CCCs of the ovary were available. All these cases had at least 1 measurable tumor lesion documented radiographically after primary surgery. The 2 largest perpendicular tumor diameters were measured, and tumor response was evaluated according to the World Health Organization criteria following calculation of the product of the 2 measured diameters. A complete response was defined as the disappearance of all detectable lesions with no new lesions appearing for at least 4 weeks; a partial response was defined as ≧50% reduction of the sum of the products of measurable lesions for at least 4 weeks. Progressive disease was defined as a ≧25% increase in the sum of the products of all measurable lesions, reappearance of any lesion that had disappeared, or appearance of a new lesion. No change was defined as any outcome that did not qualify as response or progression. Both complete-response and partial-response patients were defined as responders.

## Statistical Analysis

The relationship between HIG2 expression, age, clinical stage, and histological grade was analyzed using the *t* test and $\chi^2$ test. Differences in cytoplasmic HIG2 expression between the histological groups of EOC were analyzed by the Ryan method. Differences in HIG2 nuclear expression between the histological groups of EOC were examined by 1-way analysis of variance followed by Steel-Dwass tests. To determine whether any significant effects could be explained by other variables, multivariate logistic regression or multivariate regression was performed with the following covariates in the model: age (in years), clinical stage, and histological type. Histological grade was excluded because there is no universal system for grading ovarian clear cell carcinomas.

Overall survival (OS) distribution was calculated using the Kaplan-Meier method. Differences in HIG2 expression in the nucleus and cytoplasm of ovarian, endometrial, and renal CCCs were evaluated using the *t* test and $\chi^2$ test. $P < 0.05$ was considered statistically significant.

# RESULTS

## Immunolocalization of HIG2

To confirm the specificity of the anti-HIG2 antiserum, Western blot analysis was performed on cell lysates prepared from 293T cells transfected with pcDNA3.1 vector alone, or with vector containing the HIG2 sequence (pcDNA3.1/HIG2) and the *myc*-His–tagged HIG2 sequence (pcDNA3.1/HIG2mH). The results showed that the anti-HIG2 antiserum recognized a single 10- to 15-kd protein band in 293T cells transfected with pcDNA3.1/HIG2 and a band with a slightly higher molecular weight in cells transfected with pcDNA3.1/HIG2mH. Furthermore, when the anti-*myc* antibody was used, a protein band was observed only in cells transfected with pcDNA3.1/HIG2mH (Fig. 1A). Immunohistochemical analysis with the anti-HIG2 antiserum demonstrated strong cytoplasmic HIG2 staining and weak nuclear staining in cells transfected with the pcDNA3.1/HIG2 or pcDNA3.1/HIG2mH constructs, compared with the mock transfectants. Furthermore, strong cytoplasmic *myc* staining, as well as weak nuclear staining, was observed only in cells transfected with pcDNA3.1/HIG2mH using the anti-*myc* antibody (Fig. 1B). These data strongly suggest that the anti-HIG2 antiserum is specific for the HIG2 protein, which is predominantly located in the cytoplasm of transfected cells.

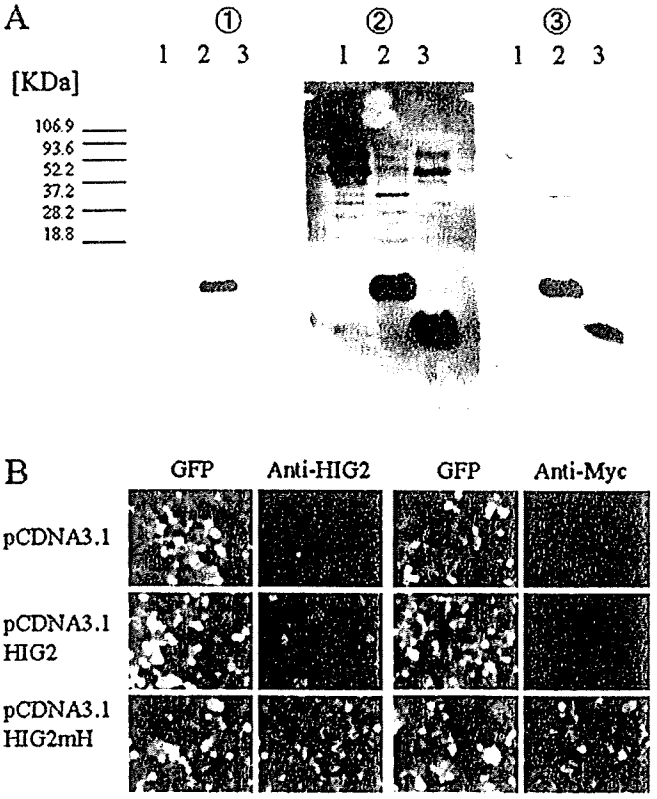


FIGURE 1. A, Western blot analysis of cell lysates prepared from 293T wild type (lane1) or from 293T transfected by pcDNA3.1 expressing *myc*-His–tagged HIG2 (lane2) or HIG2 (lane3) using an anti-HIG2 polyclonal antiserum or an anti-*myc* tag monoclonal antibody. ①anti-*myc*-tag, ②HIG2 antiserum (5 μg/mL), ③HIG2 antiserum (1 μg/mL). B, Immunolocalization of HIG2 and *myc* proteins in 293T cells transfected with pcDNA3.1 vector alone or with vectors containing HIG2 or *myc*-His–tagged HIG2. All vectors also express GFP.

TABLE 1. HIG2 expression in EOC

| | HIG2 (Cytoplasm) | | | HIG2 (Nuclear) | |
|---|---|---|---|---|---|
| Parameter | Positive | Negative | *P* | LI (95% CI) | *P* |
| Age | | | | | |
| <Median | 90 | 37 | NS* | 6.97 (4.23–9.71) | NS† |
| >Median | 85 | 42 | | 4.61 (2.48–6.73) | |
| FIGO stage | | | | | |
| I+II | 98 | 35 | NS* | 7.14 (4.35–9.94) | NS† |
| III+IV | 77 | 44 | | 4.30 (2.36–6.24) | |
| Histological type | | | | | |
| Clear | 108 | 22 | | 8.19 (5.35–11.04) | |
| Serous | 28 | 23 | 0.0001‡ | 4.22 (0.32–8.12) | NS§ |
| Mucinous | 10 | 15 | 0.00002‡ | 1.20 (−1.28 to 3.68) | 0.009§ |
| Endometrioid | 18 | 13 | 0.002‡ | 3.55 (0.95–6.15) | NS§ |
| Undifferentiated | 11 | 6 | NS | 2.95 (−1.42 to 7.31) | NS§ |
| Histological grade | | | | | |
| Low (G1+G2) | 87 | 46 | NS* | 2.73 (0.31–5.16) | NS† |
| High (G3) | 35 | 20 | | 3.94 (0.91–6.97) | |

*$\chi^2$ Test.
†Independent *t* test.
‡Multiple comparison by Ryan.
§Multiple comparison by Steel-Dwass.
CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; NS, not statistically significant.

## HIG2 Expression in Ovarian Cancer

There was no effect of age, clinical stage, or histological grade on HIG2 expression in the nucleus or cytoplasm of EOC (Table 1). The frequency of HIG2 expression in the cytoplasm was significantly higher in CCC than in serous, mucinous, and endometrioid adenocarcinomas. The HIG2 nuclear LI was higher in CCC than in mucinous adenocarcinoma, but there was no difference in HIG2 nuclear expression between CCC and serous, endometrioid, or undifferentiated adenocarcinoma. Multivariate logistic regression showed that CCC was associated with HIG2 expression in the cytoplasm and nucleus (Table 2). Normal ovarian epithelium was weakly stained. Figure 2 shows representative images of HIG2 staining in EOC and the normal ovary.

## Relationship Between HIG2 Expression, Response to Chemotherapy, and OS in CCC of the Ovary

Tumor response was evaluated in 30 cases with CCC and in 50 cases with non-CCC. These 80 cases received platinum-based chemotherapy.

In CCC cases, the overall response rate was 50% (15/30). The frequency of HIG2 expression in the cytoplasm was significantly higher in 15 responders than 15 nonresponders

TABLE 2. Multivariable predictors of HIG2 expression

| | HIG2 (Cytoplasm) | | | HIG2 (Nuclear) | | |
|---|---|---|---|---|---|---|
| Parameter | OR | 95% CI | *P* | OR | 95% CI | *P* |
| Age* | 0.7 | 0.387–1.210 | NS | 0.8 | 0.429–1.439 | NS |
| FIGO stage† | 0.9 | 0.520–1.683 | NS | 1.1 | 0.562–1.976 | NS |
| Histological type‡ | 0.2 | 0.133–0.443 | 0.0001 | 0.4 | 0.229–0.823 | 0.01 |

*Greater than median versus less than median.
†Stage III+IV versus I+II.
‡Clear cell versus non–clear cell.
OR, Odds ratio.

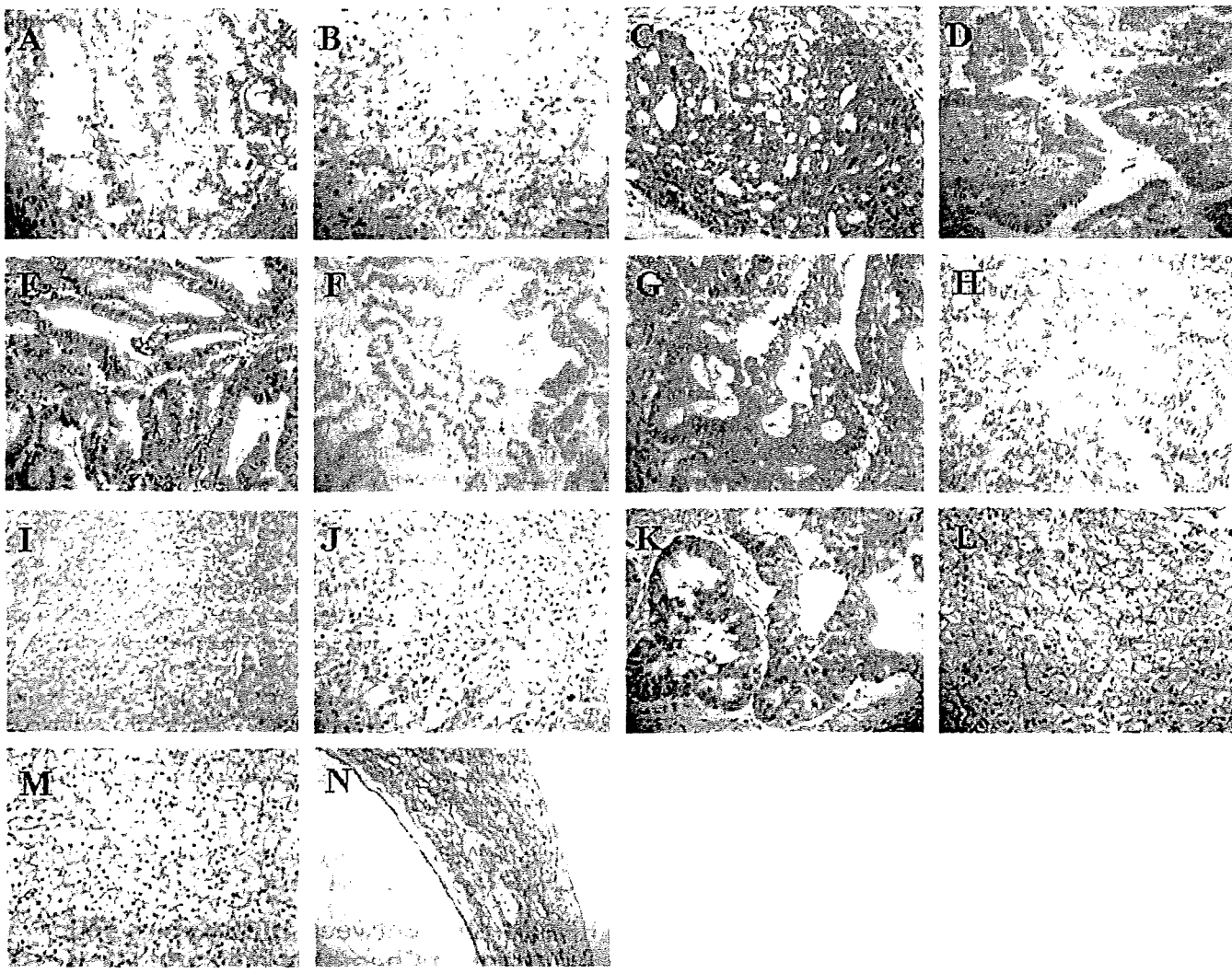


FIGURE 2. Typical image of HIG2-positive and HIG2-negative cases of each histological type of EOC. A, HIG2-positive ovarian CCC, (B) HIG2-negative ovarian CCC, (C) HIG2-positive serous cystadenocarcinoma, (D) HIG2-negative serous cystadenocarcinoma, (E) HIG2-positive mucinous cystadenocarcinoma, (F) HIG2-negative mucinous cystadenocarcinoma, (G) HIG2-positive endometrioid adenocarcinoma, (H) HIG2-negative endometrioid adenocarcinoma, (I) HIG2-positive undifferentiated adenocarcinoma, (J) HIG2-negative undifferentiated adenocarcinoma, (K) HIG2-positive endometrial CCC, (L) HIG2-positive renal CCC, and (M) HIG2-negative renal CCC. N, HIG2 staining of normal ovary.

(100% vs 60%, $P = 0.02$). However, there was no difference in the nuclear HIG2 LI between responders and nonresponders (3.01 vs 2.67, $P = 0.9$). The chemotherapy response rate was higher in 24 ovarian CCCs showing cytoplasmic HIG2 expression than in 6 CCCs with no cytoplasmic HIG2 (Table 3). However, during a median follow-up period of 39 months (range, 3–186 months), 7 of 22 cytoplasmic HIG2-negative cases and 31 of 108 cytoplasmic HIG2 positive cases died.

TABLE 3. Relationship between HIG2 expression and chemotherapy response

| Histology | HIG2 (Cytoplasm) | n | Response Rate | *P* | HIG2 (Nuclear) | Response Rate | *P* |
|---|---|---|---|---|---|---|---|
| Clear cell | Positive | 24 | 62.5% (15/24) | 0.02 | Positive | 50.0% (12/24) | NS |
| | Negative | 6 | 0% (0/6) | | Negative | 50.0% (3/6) | |
| Non–clear cell | Positive | 24 | 66.7% (16/24) | NS | Positive | 83.3% (5/6) | NS |
| | Negative | 26 | 57.7% (15/26) | | Negative | 59.1% (26/44) | |

TABLE 4. HIG2 expression in CCC of each site

| | Cytoplasmic Staining | P | Nuclear Staining | P |
|---|---|---|---|---|
| Ovary | 83.1% (108/130) | | 8.19 (5.35–11.04) | |
| Uterine corpus | 100% (17/17) | NS | 4.71 (−1.36 to 10.77) | NS |
| Kidney | 65.5% (19/29) | 0.04 | 1.04 (−0.44 to 2.51) | 0.04 |

There was thus no significant difference in OS between HIG2-positive and HIG2-negative cases (median survival time, 42 vs 50 months; $P = 0.4$).

In non-CCC cases, the response rate was 62% (31/50). There was no significant relationship between HIG2 expression in the cytoplasm or nucleus and response to chemotherapy (Table 3). In addition, there was no significant difference in OS between HIG2-positive and HIG2-negative cases (median survival time, 26 vs 22 months; $P = 0.4$).

## HIG2 Expression in CCC of the Ovary, Uterine Corpus, and Kidney

There were no differences in HIG2 nuclear or cytoplasmic expression between ovarian and endometrial CCCs. In contrast, cytoplasmic and nuclear HIG2 expression was significantly higher in ovarian and endometrial than renal CCC (Table 4). Figure 2 shows representative images of HIG2 staining in endometrial and renal CCCs.

## DISCUSSION

Early diagnosis of EOC is essential especially for CCCs, which has a worse prognosis than other histological types, particularly when it presents at an advanced stage.[3,4] CA125, a glycoprotein recognized by the monoclonal antibody OC125, is the most reliable marker for ovarian cancer and is elevated in more than 80% of cases[14,15] but, unfortunately, less frequently in CCCs.[16,17] In contrast, HIG2 is expressed in 83.1% of ovarian CCCs overall and is detectable in 73.7% of early-stage disease samples. In addition, HIG2 is a secreted molecule, and an enzyme-linked immunosorbent assay for HIG2 protein has been developed.[13] Togashi et al[13] reported that HIG2 protein was highly expressed in renal CCCs, and in agreement with this, we found the HIG2 expression rate to be 65.5% in renal CCC. Accordingly, HIG2 may be used as a marker for early detection of CCCs.

Togashi et al[13] evaluated the function of HIG2 protein and suggested that it plays an essential autocrine role in proliferation of renal CCC cells, based on the following observations: (1) addition of HIG2 protein to the culture medium enhances cell growth, (2) the growth-promoting effect of HIG2 is neutralized by addition of anti-HIG2 antibody to the culture medium, (3) significant growth suppression of renal CCC cells occurs when endogenous expression is reduced by HIG2-specific RNAi, and (4) treatment with anti-HIG2 antibody induces apoptosis of renal CCC cells. A critical role of the HIG2 autocrine oncogenic pathway, in the development and progression of renal CCC as well as proliferation, is also indicated by (1) HIG2 activation, (2) HIG2-FZD10 interaction, (3) activation of the Wnt signaling pathway, (4) β-catenin accumulation and stabilization, (5) transcriptional activation of the β-catenin/TCF complex, and (6) transactivation of HIG2. Furthermore, Denko et al[18] reported that, in cultured cells, HIG2 expression is induced by hypoxia and by glucose deprivation, and tumor xenografts derived from human cervical cancer cells display increased expression of HIG2 when they are deprived of oxygen. They concluded that there is a coordinated transcriptional response of eukaryotic cells to microenvironmental stresses found in solid tumors. It has been shown also that the Ki-67 LI in ovarian CCC is significantly lower than in serous cystadenocarcinoma and among ovarian CCCs in nonresponders than responders to chemotherapy.[19] These findings suggest that low proliferation activity may contribute to chemoresistance in ovarian CCCs and that there may be a relationship between HIG2 expression, proliferation, and chemoresponse. In the present study, the chemotherapy response rate was higher in 24 ovarian CCCs with cytoplasmic HIG2 expression than in 6 CCCs without (62.5% vs 0%), demonstrating that HIG2 expression is related to chemoresponse. These findings support the idea that low proliferative activity may contribute to chemoresistance in ovarian CCC lacking HIG2 expression. On the other hand, we failed to show a relation between HIG2 expression and chemotherapy response in non-CCC cases, so the resistance mechanisms may differ among histological types.

Morphologically similar CCCs can arise from the ovary, the uterine corpus, and the kidney. In this study, HIG2 expression was higher in CCCs of the ovary and uterine corpus than in renal CCC. The differential expression patterns may be explained by the fact that both endometrial and ovarian CCC are müllerian in origin, in contrast to the wolffian duct origin of renal CCC.

Although further studies are necessary to determine the reason for different expression patterns of HIG2, it is a candidate marker for early detection of ovarian CCCs and for prediction of response to chemotherapy. We plan to test its utility by measuring serum HIG2 protein in ovarian CCC in future studies.

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# Phase II Study of Intraperitoneal Carboplatin With Intravenous Paclitaxel in Patients With Suboptimal Residual Epithelial Ovarian or Primary Peritoneal Cancer

## *A Sankai Gynecology Cancer Study Group Study*

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**Purpose:** To assess the antitumor efficacy and safety of 2 treatment modalities: intraperitoneal carboplatin combined with intravenous (IV) paclitaxel.

**Patients and Methods:** Eligible patients were those with epithelial ovarian carcinoma or primary peritoneal carcinoma stages II to IV who underwent initial surgery and had a residual tumor size of 2 cm or larger. Patients received IV paclitaxel 175 mg/m$^2$ followed by intraperitoneal carboplatin AUC6. The primary end point was a response. Secondary end points were toxicity, progression-free survival, and overall survival.

**Results:** Twenty-six patients were enrolled, and 24 patients were eligible for assessment. The response rate was 83.3% (95% CI, 62.6%–95.3%; Table 4). The median progression-free survival was 25 months. The median overall survival had not been reached. Incidences of grade (G) 3/4 hematological toxicities were absolute neutrophil count, 96%; hemoglobin, 29%; and thrombocytopenia, 16%. Nonhematological toxicities included G2 liver function, 4%; G3 sensory neuropathy, 8%; and G3 myalgia and arthralgia, 4%.

**Conclusions:** Intraperitoneal administration of carboplatin combined with IV paclitaxel was well tolerated and showed satisfactory response in the patients with bulky residual tumor. Large-scale phase III trial comparing with IV carboplatin is warranted in this patient population.

**Key Words:** Intraperitoneal chemotherapy, Carboplatin, Ovarian cancer, Suboptimal residual disease, Phase II study

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Despite the development of new anticancer agents such as platinum and taxane, ovarian cancer remains the most lethal of gynecologic malignancies. One strategy to treat ovarian cancer is intraperitoneal (IP) chemotherapy, and because the most distinct characteristic of ovarian or peritoneal cancer is early intra-abdominal dissemination of the disease, this seems to be a reasonable approach. The IP modality has been investigated for years, including several phase I and II studies using various anticancer agents.[1] Intraperitoneal cisplatin and IP paclitaxel are now considered the choice of treatments based on a series of randomized phase III trials conducted in the United States that evaluated the survival benefit of IP over intravenous (IV) administration of these agents. Three randomized trials[2–4] showed that IP cisplatin-based chemotherapy significantly improved survival compared with cisplatin chemotherapy administered intravenously. A meta-analysis showed a 22% reduction of hazards ratio to death, prompting the US National Cancer Institute to