

## Claudin-4: A Potential Therapeutic Target in Chemotherapy-resistant Ovarian Cancer

HIROYUKI YOSHIDA, TOSHIYUKI SUMI, XU ZHI, TOMOYO YASUI, KEN-ICHI HONDA and OSAMU ISHIKO

*Department of Obstetrics and Gynaecology,  
Osaka City University Graduate School of Medicine, Abeno, Osaka, 545-8585, Japan*

**Abstract.** *Background: Claudin-4, a component of the tight junction, plays an important role in tumorigenesis and metastasis of ovarian cancer, but its role in platinum resistance has not been elucidated. Materials and Methods: Claudin-4 expression in ovarian cancer cells was inhibited and the changes in cisplatin sensitivity were examined. Fluorescence-labeled cisplatin was used to examine whether inhibition of claudin-4 changed the cellular accumulation of cisplatin. Claudin-4 expression in ovarian cancer tissue resected from the patients surgically was evaluated immunohistochemically. Results: Suppression of claudin-4 resulted in a significant increase of cisplatin sensitivity and cellular accumulation of fluorescence-labeled cisplatin. Claudin-4 expression was significantly greater in ovarian cancer tissue from chemoresistant patients compared to chemosensitive patients. The overall survival was significantly shorter for claudin-4-positive than claudin-4-negative cases. Conclusion: These data suggest that claudin-4 contributes to platinum resistance in ovarian cancer and may be a potential target in the treatment of platinum-resistant tumors.*

Ovarian cancer is known to have the worst prognosis among gynaecological malignancies (1). Since there is a lack of characteristic symptoms in the early stage and effective screening methods have not been established, about 70% of patients with ovarian cancer are diagnosed in an advanced stage (1). Following cytoreductive surgery, treatment with platinum and paclitaxel has been recommended for initial chemotherapy (2). First-line chemotherapy with platinum and paclitaxel yields a response rate of >80%. However, despite an initial high response rate, nearly all patients

experience relapse (3). The treatment for recurrent ovarian cancer is often difficult due to resistance to chemotherapeutic agents (4), and the 5-year survival rate of patients with advanced ovarian cancer is only less than 30% (1). Therefore, novel strategies for overcoming chemotherapy resistance are needed.

The platinum drugs cisplatin and carboplatin are widely used in ovarian cancer treatment. However, ovarian cancer exhibits resistance to platinum drugs, as well as to other chemotherapeutic agents. The precise mechanisms that account for such drug resistance have not been fully identified. Decreased cellular accumulation of drugs, enhanced detoxification capability, an aberrant apoptosis pathway, and increased DNA repair ability have been proposed (5-12).

The tight junction (TJ) is part of the apical junctional complex and is involved in both paracellular permeability and cell polarity (13, 14). The claudins are the main constituents of TJs and comprise 24 closely related transmembrane proteins (15, 16) that control the ability of TJs to regulate the paracellular transport (17). Furthermore, a recent study suggests that claudins play a role in a wide variety of processes, including cell signaling, proliferation, differentiation, and motility (16). In addition, recent evidence suggests that the expression of claudins is altered in various types of malignant tumors (18). For example, in ovarian cancer, several studies have reported that claudin-3 and claudin-4 are overexpressed (18-21), and claudin-4 was shown to be overexpressed in serous ovarian cancer more than in adenoma or borderline ovarian tumors (22). Claudin-3 and claudin-4 expression have been shown to be associated with an increase in invasion, motility, and cell survival of ovarian cancer cells (23). It has been reported that by administering small-interfering RNA (siRNA) or monoclonal antibodies that inhibit claudin-3 or claudin-4 to mice, the growth of ovarian cancer cells transplanted into mice was suppressed (24, 25). Based on these reports, claudin-3 and claudin-4 are considered to play important roles in tumorigenesis, metastasis, and survival of ovarian cancer cells. Therefore, claudin-3 and claudin-4 are likely to be useful targets in diagnosis and treatment of ovarian cancer.

*Correspondence to:* Hiroyuki Yoshida, MD, Ph.D. Department of Obstetrics and Gynaecology, Osaka City University Graduate School of Medicine. 1-4-3 Asahimachi Abeno, Osaka, 545-8585, Japan. Tel: +81666453862, e-mail: hirogynecol@aol.com

*Key Words:* Ovarian cancer, claudin-4, chemotherapy resistance.

Overcoming chemotherapy resistance is very important for improving the prognosis of patients with ovarian cancer. However, understanding the mechanism of the chemotherapy resistance and overcoming it are still not sufficient. As mentioned above, claudins play a variety of important roles in ovarian cancer. However, there are only a few reports available regarding the involvement of claudins in chemotherapy resistance of ovarian cancer (26, 27), and most aspects of the involvement have not been elucidated. Therefore, in this study we investigated the relationship between platinum resistance in ovarian cancer and claudin.

## Materials and Methods

**Cell culture.** The human serous ovarian cancer cell line OVCAR-3 (Riken BioResource Center, Ibaraki, Japan) was maintained in RPMI-1640 culture medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL). The human serous ovarian cancer cell line Caov-3 (American Type Culture Collection, Manassas, VA, USA) was grown in Dulbecco's modified Eagle's medium (Gibco BRL) with 10% FBS. Both cell lines were cultured in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C.

**Transfection of ovarian cancer cells with siRNA.** The sequence of siRNA oligomer (synthesized by Qiagen, Tokyo, Japan) to inhibit claudin-4 expression was as follows: 5'-GAGUGGAUGGACGGG UUUAd(TT)-3'. Ovarian cancer cells were transfected with oligomers by using HiPerFect Transfection Reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. After 48 h, cells were harvested, and cell lysates were prepared to measure protein levels of claudin-4 using Western blot analysis, as described below. Blots were also probed by using actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As controls, ovarian cancer cells were mock-transfected (without siRNA) or transfected with negative control siRNA (Qiagen).

**Western blot analysis.** Equal amounts of protein from cell lysates were separated by SDS-PAGE. Proteins were then transferred to PVDF membranes (ATTO, Tokyo, Japan). Following Western transfer, membranes were probed with anti-claudin-4 antibody (1 µg/ml; Invitrogen, Tokyo, Japan) and anti-β-actin antibody (2 µg/ml; Santa Cruz Biotechnology). Peroxidase-conjugated antibody was used at 0.05 µg/ml, and binding was revealed using Chemi-Lumi One L (Nakarai, Kyoto, Japan).

**Chemosensitivity assay.** The sensitivity of cells to cisplatin (Bristol-Myers Squibb, Tokyo, Japan) was determined using the tetrazolium compound WST-8 (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). Cells were diluted with culture medium to the seeding density (1×10<sup>5</sup> cells/ml for OVCAR-3 and 5×10<sup>4</sup> cells/ml for Caov-3), suspended in 96-well tissue culture plates (150 µl/well) and treated with siRNA for 48 h. Cells were then treated with a series of cisplatin concentrations for 48 h (0.3-20 µg/ml) to obtain a dose-response curve. Subsequently, 10 µl of WST-8 reagent was added, and incubation was continued for 4 h. Absorbance at 450 nm was measured with a microplate reader (Corona Electric, Ibaraki, Japan). Dose-response curves were plotted as the percentage of viable cells compared with the control cells, which was obtained

from the no-cisplatin-exposure sample. The IC<sub>50</sub> value, defined as the cisplatin concentration required to reduce cell survival by 50%, was determined graphically from the concentration-response curves. The assays were performed in quadruplicate for each cell line.

**Immunofluorescence detection of Alexa Fluor 546-labeled cisplatin.** To assess the intracellular cisplatin accumulation by confocal microscopy, cells were stained by Alexa Fluor 546-labeled cisplatin (AF-CDDP) (Molecular Probes, Eugene, OR, USA) (28-30). Cells were seeded in 8-well glass Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) and treated with siRNA for 48 h. Cells were then incubated with AF-CDDP at a final concentration of 200 units/ml (1 unit is defined as the reagent solution required to label 25 ng of DNA *in vitro*) for 1 h at 37°C. The slides were fixed with ice-cold 70% ethanol for 15 min at -20°C and counter-stained with 4', 6-diamino-2-phenylindole (DAPI) for staining of nuclei. Immunofluorescence images were observed under a fluorescence microscope (BX50; Olympus Corporation, Tokyo, Japan). Semi-quantitative analysis of intensities of AF-CDDP in cells was performed using NIH ImageJ 1.43 software (National Institutes of Health, Bethesda, MD, USA). The intensities of AF-CDDP in cells were measured at ×400 magnification and divided by the cell number to calculate the intensity of each cell. The intensity of AF-CDDP was evaluated in five separate fields.

**Immunohistochemical staining.** The 43 cases of ovarian cancer patients that had surgery as their initial treatment at the Osaka City University Medical School Hospital between 2002 and 2009 were reviewed (Table I). Informed consent was obtained from all patients, and the Ethics Committee of Osaka City University Hospital approved this study. All patients underwent platinum-based chemotherapy after surgery. In this study, only patients with measurable lesions that could be evaluated before and after chemotherapy were included. Tumor responses to chemotherapy were determined using the Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.0) (31). Patients were staged according to the International Federation of Obstetrics and Gynaecology (FIGO) classification. Ovarian cancer tissues were fixed with 10% buffered formalin and embedded in paraffin. Five-micrometer sections were deparaffinized, hydrated, and stained according to the Dako Envision protocol (Dako, Kyoto, Japan) using a 1:50 dilution of claudin-4 mouse monoclonal antibody (Zymed Laboratories, South San Francisco, CA, USA). To determine the specificity of the reaction, the primary antibody was replaced with commercially available negative control reagent containing mouse immunoglobulins (Dako). Colon cancer tissue was also included as a positive control for claudin-4 (32). The percentage of claudin-4-positive cells was calculated in three separate fields at ×200 magnification. For quantitative analysis of claudin-4, the immunostaining was scored as follows: 0: no immunostaining, 1: fewer than 25% of cells positive, 2: 26-50% of cells positive, 3: 51-75% of cells positive, 4: 76-100% of cells positive. The claudin-4 expression score of 0 was defined as negative expression and those ≥1 as positive expression.

**Statistical analysis.** The statistical significance of differences in the IC<sub>50</sub> and the intensities of AF-CDDP between cells transfected with specific, non-specific, or no siRNA was calculated by Student's *t*-test, and the significance of differences in claudin-4 expression between chemoresistant patients and chemosensitive patients was

Table I. Relationships between the expression of claudin-4 and clinicopathological parameters of ovarian cancer patients.

	Total cases (n=43)	Expression of claudin-4		<i>p</i> -Value <sup>a</sup>
		Negative (n=10)	Positive (n=33)	
		n (%)	n (%)	
Age				
<60 years	29	7 (24.1)	22 (75.9)	0.999
≥60 years	14	3 (21.4)	11 (78.6)	
Histology				
Serous	22	8 (36.4)	14 (63.6)	0.659
Endometrioid	5	0 (0)	5 (100)	
Mucinous	3	0 (0)	3 (100)	
Clear cell	10	1 (10)	9 (90)	
Undifferentiated	3	1 (33.3)	2 (66.7)	
Stage				
I + II	5	1 (20)	4 (80)	0.999
III + IV	38	9 (23.7)	29 (76.3)	

<sup>a</sup>Chi-square test between claudin-4 negative and positive groups.

calculated by the Mann–Whitney *U*-test. The significance evaluation of the relationships between expression of claudin-4 and clinicopathological parameters was performed using the Chi-square test. Survival curves were generated using the Kaplan–Meier method, and the significance of differences in survival curves were calculated using the generalized Wilcoxon test. *P*-values less than 0.05 were considered statistically significant.

## Results

**Inhibition of claudin-4 enhances the cisplatin sensitivity of ovarian cancer cells.** The expression of claudin-4 protein in two types of ovarian cancer cell lines, OVCAR-3 and Caov-3, was examined by Western blot analysis. Claudin-4 protein was expressed in both types of cell (Figure 1A). Claudin-4 siRNA was transfected into both ovarian cancer cell lines, and the effect of claudin-4 inhibition was examined by Western blot analysis 48 h later. Compared to the mock-transfected cells and negative control siRNA-transfected cells, claudin-4 siRNA-transfected cells showed a decrease in claudin-4 expression (Figure 1A). Therefore, we concluded that claudin-4 siRNA used in this study effectively inhibited claudin-4 expression in OVCAR-3 and Caov-3 cells. We studied whether the sensitivity of ovarian cancer cells to cisplatin would change due to claudin-4 inhibition. When claudin-4 expression in OVCAR-3 was inhibited by claudin-4 siRNA, the IC<sub>50</sub> for cisplatin was 0.92±0.43 µg/ml, which was significantly reduced compared to that of the mock-transfected cells (IC<sub>50</sub>=1.78±0.43 µg/ml) and of the negative control siRNA-transfected cells (IC<sub>50</sub>=2.13±0.67 µg/ml). Similarly, the IC<sub>50</sub> for cisplatin in claudin-4 siRNA transfected Caov-3 cells

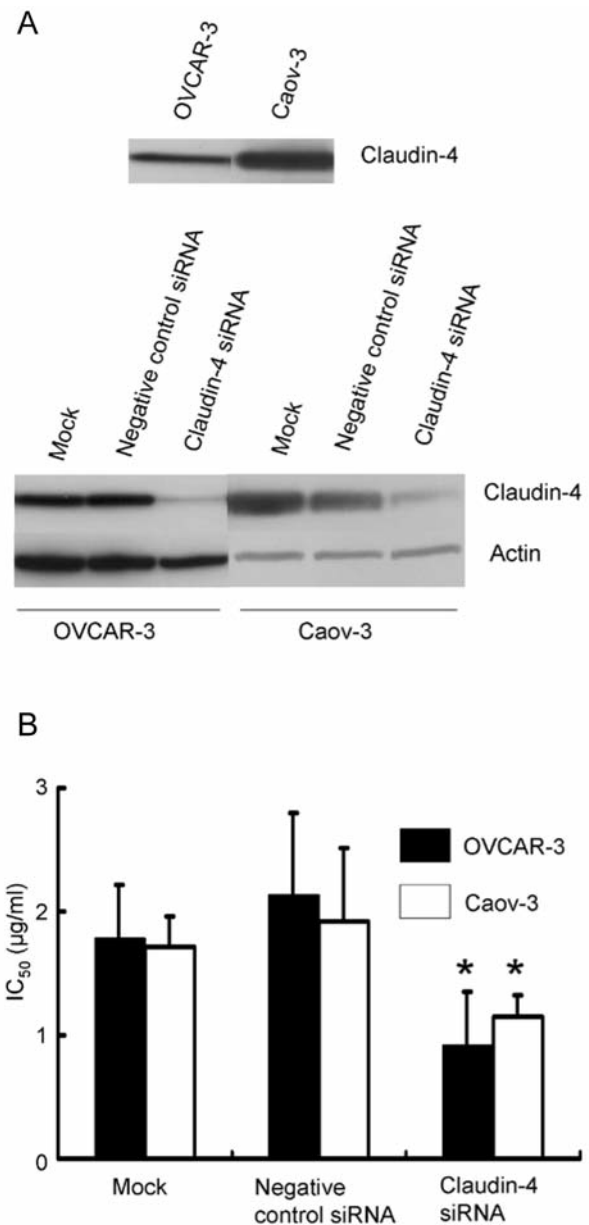


Figure 1. Effect of claudin-4 silencing on cisplatin sensitivity in ovarian cancer cell lines. A: Western blot analysis showing an expression of claudin-4 in ovarian cancer cell lines. Claudin-4 was expressed in the ovarian cancer cell lines OVCAR-3 and Caov-3. Claudin-4 siRNA transfected cells showed significant reduction of claudin-4 expression compared with cells treated with transfection reagent without siRNA (mock-transfected) and negative control siRNA-treated cells.  $\beta$ -Actin was used as loading control. B: Effect of claudin-4 silencing on cisplatin sensitivity in ovarian cancer cell lines. OVCAR-3 and Caov-3 cells were treated with transfection reagent without siRNA (mock-transfected) or transfected with claudin-4 or negative control siRNA. After 48 h, cells were treated with cisplatin (0.3–20 µg/ml). Following 48 h of cisplatin exposure, the WST-8 assay was performed and IC<sub>50</sub> values (dose causing 50% cell survival) were calculated. Mean values for the IC<sub>50</sub> of four independent experiments are shown; bars=SD. \**p*<0.05, compared with the mock-transfected and the negative control siRNA-transfected cells (Student's *t*-test).

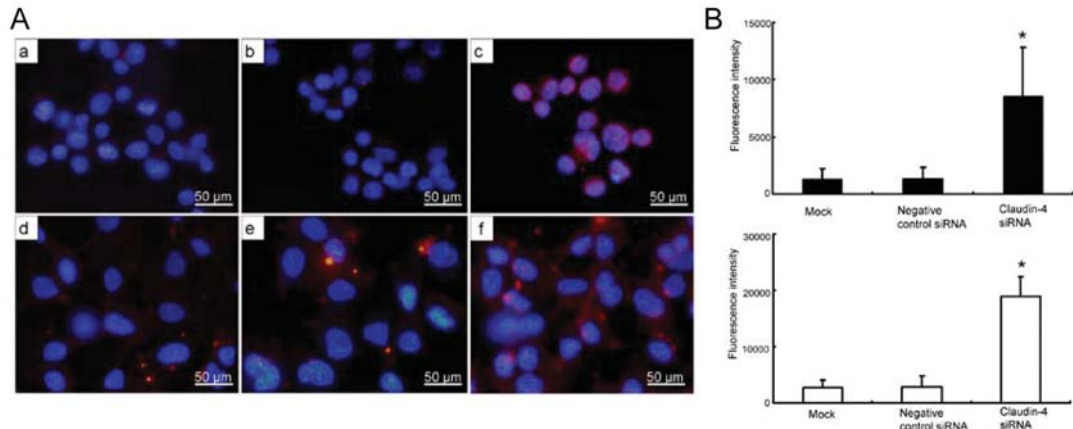


Figure 2. A: Effect of claudin-4 silencing on fluorescence-labeled cisplatin accumulation in ovarian cancer cell lines. OVCAR-3 (a-c) and Caov-3 (d-f) were treated with transfection reagent without siRNA (mock-transfected, a and d) or transfected with claudin-4 (c and f) or negative control (b and e) siRNA. After 48 h, cells were treated with Alexa Fluor 546-labeled cisplatin (AF-CDDP, red) and counterstained with DAPI for staining of nuclei (blue). B: A semi-quantitative analysis of intensities of AF-CDDP in cells was performed using NIH ImageJ 1.43 software. The intensities of AF-CDDP in cells were measured at  $\times 400$  magnification in five separate fields. The accumulation of AF-CDDP in OVCAR-3 (upper panel) and Caov-3 (lower panel) cells is significantly increased in claudin-4-siRNA-transfected cells. Mean values for the fluorescence intensity are shown; bars=SD. \* $p < 0.05$ , compared with the mock-transfected and the negative control siRNA-transfected cells (Student's *t*-test).

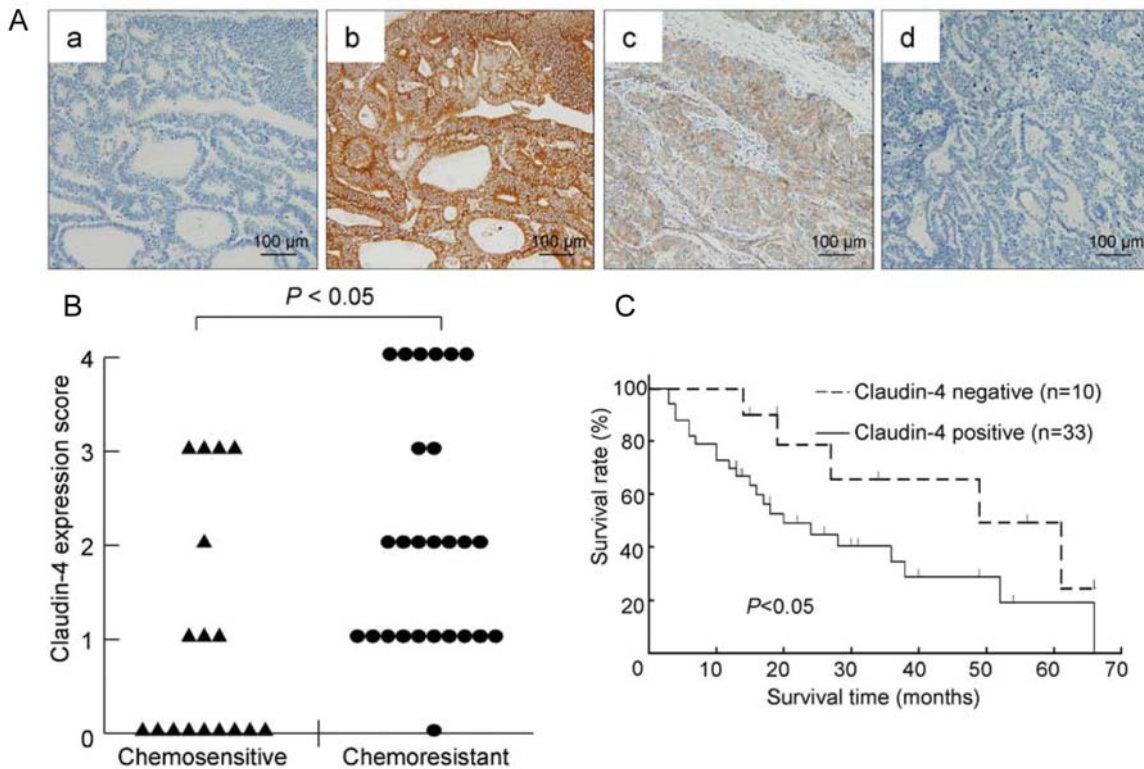


Figure 3. A: Immunoreactivity of claudin-4 observed in ovarian cancer tissue. (a) Stage IIc endometrioid cystadenocarcinoma was stained in the absence of primary antibody to act as a negative control. (b) Stage IIc endometrioid cystadenocarcinoma (same tissue sample as that in negative control). Positive expression of claudin-4 can be observed in tumor cells. (c) Stage IIIc serous adenocarcinoma. Claudin-4 expression can be seen in tumor cells. (d) Stage IIc serous papillary adenocarcinoma. No expression of claudin-4 was observed (original magnification,  $\times 200$ ). B: Claudin-4 expression in 43 ovarian cancer tissues treated with platinum-based chemotherapy. Claudin-4 expression was significantly higher in the chemoresistant group (n=26) compared to the chemosensitive group (n=17) (Mann-Whitney U-test,  $p < 0.05$ ). C: The overall survival of 43 ovarian cancer patients according to immunoexpression of claudin-4. Kaplan-Meier survival analysis showing that positive claudin-4 expression (n=33) was associated with a shorter overall survival than negative claudin-4 expression in ovarian cancer patients (n=10;  $p < 0.05$ ; generalized Wilcoxon test).



( $IC_{50}=1.15\pm0.17$   $\mu\text{g/ml}$ ) was significantly reduced compared to that of the mock-transfected cells ( $IC_{50}=1.72\pm0.24$   $\mu\text{g/ml}$ ) and of the negative control siRNA-transfected cells ( $IC_{50}=1.92\pm0.59$   $\mu\text{g/ml}$ ) (Figure 1B).

*Inhibition of claudin-4 increases AF-CDDP accumulation in ovarian cancer cells.* We studied whether the intracellular accumulation of cisplatin in ovarian cancer cells changes due to claudin-4 inhibition. AF-CDDP was used to evaluate the intracellular accumulation of the cisplatin. We found that AF-CDDP (red fluorescence) was overexpressed in claudin-4 siRNA-transfected ovarian cancer cells compared to the mock-transfected and the negative control siRNA-transfected cells (Figure 2A). Figure 2B shows that the accumulation of AF-CDDP was significantly increased in claudin-4 siRNA-transfected ovarian cancer cells.

*Claudin-4 expression in ovarian cancer cells correlates with chemotherapy response rate and survival period.* Immunoreactivity for claudin-4 was observed in the cytoplasm and cell membrane of the ovarian cancer cells (Figure 3A). Thirty-three out of 43 (76.7%) patients with ovarian cancer examined in this study were positive for claudin-4 expression (Table I). The effect of platinum-based chemotherapy was evaluated according to the RECIST guidelines. The patients who presented with complete response or partial response were grouped as the chemosensitive group. The patients with stable disease or progressive disease were grouped as the chemoresistant group. When claudin-4 expression was compared between the two groups, claudin-4 expression was significantly increased in the chemoresistant group compared to the chemosensitive group (Figure 3B). Claudin-4 expression in ovarian cancer tissue and the patients' prognoses were examined. Compared to the claudin-4 expression-negative group, the positive group showed a significantly poorer prognosis (Figure 3C). No clear correlation was seen between other clinicopathological factors and claudin-4 expression (Table I).

## Discussion

In this study, we have shown that inhibiting claudin-4 leads to increased sensitivity to cisplatin and increased accumulation of AF-CDDP in ovarian cancer cells. In addition, we have also shown that claudin-4 expression in ovarian cancer tissue was increased in patients who showed resistance to platinum-based chemotherapy, which supports the results obtained from ovarian cancer cell lines. Moreover, the patients who were positive for claudin-4 expression had significantly poorer prognoses than the patients who were negative for claudin-4. These findings indicate that claudin-4 has an important role in platinum resistance in ovarian cancer and is a potential prognostic factor in ovarian cancer patients.

Only a few reports have been published on the relation between chemotherapy resistance in ovarian cancer and claudins. It has been reported that chemotherapy-resistant ovarian cancer showed increased expression of claudin-3 and claudin-4 compared to chemotherapy-sensitive ovarian cancer (26). On the other hand, another report stated that there is no relation between the effectiveness of chemotherapy in ovarian cancer and claudin-4 expression (27). Thus it is controversial whether or not claudin is related to chemotherapy resistance. However, in this study, we have confirmed that claudin-4 contributes to platinum resistance in ovarian cancer cells.

Several studies have provided evidence that decreased cellular accumulation of cisplatin causes cisplatin resistance in various cancer cells (33-37). In this study, we have confirmed an increased cellular accumulation of fluorescence-labeled cisplatin in claudin-4 siRNA-transfected ovarian cancer cells. This result suggests that claudin-4 contributes to the cisplatin resistance of ovarian cancer cells by reducing the cellular accumulation of cisplatin. Given that claudin is a transmembrane protein and is associated with membrane permeability for molecules, it is conceivable that claudin-4 may affect the transmembrane transportation of cisplatin. However, it remains unknown whether claudin-4 plays a role as an influx or efflux transporter of cisplatin. Further examination is needed to elucidate the details of the mechanism involved in this event. In this study, we used fluorescence-labeled cisplatin, which is primarily used for labeling DNA in cells. It is possible that fluorescence-labeled cisplatin did not reflect the precise behavior of cisplatin, however, several studies have reported the validity of using fluorescence-labeled cisplatin to examine the cellular accumulation of cisplatin (28-30).

Meanwhile, TJs have a role as a barrier and regulator of the passage of a variety of ions and molecules between cells. Claudins, as major components of TJs, control the intercellular permeability. The possibility has been suggested that by inhibiting claudin expression in tumor cells, intercellular permeability would be increased, resulting in increased penetration of chemotherapeutic agents into tumor tissue and greater effectiveness of the chemotherapeutic agents (38). In this study, we have confirmed that increased expression of claudin-4 was associated with chemotherapy resistance in ovarian cancer patients. Claudin-4 overexpression may inhibit the penetration of chemotherapeutic agents into ovarian cancer tissue, and, as a result, induce chemotherapy resistance. Thus, in addition to promoting the aforementioned resistance to chemotherapeutic agents at the cellular level, claudin-4 may be involved in chemotherapy resistance at the tumor tissue level.

It has been reported that claudin-4 is associated with a poor prognosis in gastric cancer and endometrial cancer (39, 40), and in ovarian cancer claudin-3 was found to be an independent negative prognostic factor (22). In this

study, patients who were positive for claudin-4 expression had significantly poorer prognoses compared to patients who were negative for claudin-4 expression. This result suggests that claudin-4 could serve as a prognostic factor, and that chemotherapy resistance caused by claudin-4 may contribute to a worse prognosis for ovarian cancer patients.

The prognosis for ovarian cancer patients has shown a tendency towards improvement with the development of novel treatments. However, the long-term prognosis is still poor. With further research on claudin-4, it is expected that new strategies for diagnosis and treatment of ovarian cancer will emerge.

## Acknowledgements

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

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Received January 30, 2011

Revised March 21, 2011

Accepted March 22, 2011