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High claudin-7 expression is associated with a poor response to platinum-based chemotherapy in epithelial ovarian carcinoma

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ABSTRACT

Background: Claudin-7 (CLDN-7) is a tight junction protein that has been shown overexpressed in several human cancers. We investigated prognostic significance of CLDN-7 overexpression in patients with epithelial ovarian carcinoma (EOC) and its functional role on cell proliferation in ovarian carcinoma cell lines.

Patients and methods: CLDN-7 expression was evaluated by real-time RT-PCR and immunohistochemical analysis in 71 patients with EOC. We assessed the association of CLDN-7 expressions with prognosis of the patients including sensitivity to platinum-based chemotherapy. *In vitro* experiment was performed with and without inhibition of CLDN-7 by its siRNA to evaluate the sensitivity of the human ovarian cancer cells to cisplatin chemotherapy.

Results: CLDN-7 transcripts in EOCs were significantly up-regulated compared with normal ovarian tissues ($P < 0.001$). The expression of CLDN-7 protein was observed in majority (69/71, 97.1%) of the EOCs but not in normal ovarian tissues ($P < 0.001$). High CLDN-7 expression in primary tumour correlated with shorter progression-free survival (PFS) of the patients ($P = 0.005$) and poor sensitivity to platinum-based chemotherapy ($P = 0.024$). Moreover, CLDN-7 was highly expressed in 2774 and HeyA8 human ovarian cancer cells and inhibition of CLDN-7 by its siRNA significantly enhanced the sensitivity of 2774 and HeyA8 cells to cisplatin treatment.

Conclusion: These findings suggest CLDN-7 expression is an independent prognostic factor for PFS and it may play a role in regulating response to platinum-based chemotherapy in the treatment of EOC.

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1. Introduction

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynaecological cancers in women.[1] Most of EOC cases are advanced at the time of diagnosis and the majority of these patients develop recurrent disease, despite an initial response to primary treatment consisting of surgical debulking and chemotherapy.[2] Although EOC is highly responsive to initial platinum-based combination chemotherapy, successful management of advanced or recurrent gynaecologic malignancies is often difficult due to both intrinsic and acquired resistance of cancer cells to cytotoxic drugs.[3,4] Therefore, novel strategies that may be effective in enhancing sensitivity or reversing resistance to chemotherapy are urgently needed for those patients with EOCs who either do not respond to initial therapy or develop recurrent disease.

Claudins (CLDNs) are a family of tight junction (TJ) – specific integral membrane proteins which were discovered in 1998.[5] Since that time, at least 23 human CLDNs have been identified.[6] The patterns of expression of CLDNs in normal tissue, benign tumours and cancer are complex and appear to be organ-dependent.[7] Their abnormal expression has recently been shown to alter cell polarity, improve cell survival, motility and invasion.[8] Whereas expressions of CLDNs have been found to be decreased in gastric cancer,[9–11] it is highly elevated in breast, prostate and cervical cancer.[11–13] The relationship between altered CLDN expression and cancer initiation or progression has not been completely understood yet.

The expression of CLDN-7 has been reported to be altered in several human cancers including cervical, endometrial, and ovarian cancers.[11,14–19] Overexpression of CLDN-7 was observed in EOC and it was suggested that CLDN-7 might represent a potential marker for ovarian carcinoma.[18–20] However, functional significance of CLDN-7 overexpression in EOC is not known yet. Therefore, we investigated the prognostic significance of CLDN-7 overexpression in patients with EOC and its functional role on cell proliferation in ovarian carcinoma cell lines.

2. Materials and methods

2.1. Tumour samples and cell lines

Fresh frozen tumour specimens were obtained from patients with EOC ($n = 44$) at the time of surgery. Also, normal ovarian tissues ($n = 11$) were obtained as controls at the time of hysterectomy for benign disease including uterine myoma or pelvic organ prolapse. Surgery was performed at the Department of Obstetrics and Gynecology, Samsung Medical Center between October 2003 and November 2005. In addition, a total of 83 paraffin-embedded, formalin-fixed specimens (71 EOCs and 12 normal ovaries) were obtained from the Department of Pathology. This study was approved by the Institutional Review Board of our hospital (IRB No. 2005-09-032). All patients underwent primary maximum cytoreductive surgery followed by intravenous paclitaxel (175 mg/m^2) or docetaxel (75 mg/m^2) plus carboplatin (AUC 5) combination chemotherapy every 3 weeks for 6–8 cycles. Patients were divided into two groups according to the sensitivity for first-line platinum-based combination chemotherapy: platinum-resistant is defined as platinum-free interval < 6 months; platinum-sensitive, platinum-free interval $\geqslant$ 6 months.[21]

Human ovarian carcinoma cell lines 2774, HeyA8, SK-OV-3, PA-1 and OVCAR-3 were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). 2774 and OVCAR-3 were grown in RPMI1640, HeyA8 and SK-OV-3, were in McCoy's 5A and PA-1 was in MEM. All cells were grown at 37 °C in 5% CO_2 and supplemented with 10% foetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml).

2.2. Isolation of total RNA, cDNA synthesis, semi-quantitative PCR and real-time quantitative PCR

Total RNA was extracted from the EOCs and NOTs using a mirVana™ total RNA isolation Kit (Ambion, TX, USA) according to the manufacturer's protocol. The concentration was quantified using the NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, USA). The cDNA synthesis was performed with a High Capacity cDNA Archive kit (4368813, Applied Biosystems, Foster City, CA, USA) following the protocol supplied. About 1 μl of cDNA was amplified by PCR using TOP DNA polymerase PCR Kit (E-3101-2, BIONEER, Daejeon, South Korea) and CLDN-7 primers. β-Actin was used as the housekeeping gene control. The sequences of primers for semi-quantitative PCR were as follows: for CLDN-7, 5′-AATGTACGACTCGGTGCTCG-3′ (forward) and 5′-ATTCCCA GGACAGGAACAGG-3′ (reverse), for β-actin, 5′-GATGCAGAA GGAGATCACTG-3′ (forward) and 5′-AGTCATAGTCCGCCTAGA AG-3′ (reverse). PCR was carried out for initial denaturation at 95 °C for 5 min, followed by 30 cycles for CLDN-7 and 28 cycles for β-actin of denaturation (95 °C, 50 s), annealing (58 °C for CLDN-7, 56 °C for β-actin, 50 s) and extension (72 °C, 50 s). This was followed by a final extension step of 72 °C for 5 min. Amplification products were electrophoresed on 1% agarose gel and visualised by ethidium bromide staining under ultraviolet transillumination.

TaqMan PCR was done with an ABI PRISM 7900HT Fast-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR primers and probes for CLDN-7 (Hs00600772) and GAPDH (4310884E) were purchased from Applied Biosystems. The relative expression of CLDN-7 mRNA was normalised to the amount of GAPDH in the same cDNA by using the ΔΔCt method described by the manufacturer.[22]

2.3. Immunohistochemical analysis

Immunohistochemical staining was performed with the standard peroxidase/DAB method (DakoCytomation) on formalin-fixed, paraffin-embedded tissue sections. CLDN-7 protein expression was detected by primary mouse monoclonal claudin-7 antibody (Cat. No. 37-4800, Invitrogen Corporation, Camarillo, CA, USA). Immunohistochemical procedures were done as described previously.[14] Antigen antibody complexes were detected with the Dako REAL DAB/Chromogen (K5007,

DakoCytomation) according to the manufacturer's instructions. Tissue sections were lightly counterstained with haematoxylin and then examined by light microscopy. Anti-mouse IgG (AI-2000, Vector Laboratories, Burlingame, CA) was used in place of the primary antibody as a negative control. Two pathologists (SYS, COS) blindly reviewed slides and evaluated the immunohistochemical staining. The staining extent was scored semi-quantitatively on a scale from 0 to 4+, as follows: 0, no staining; 1+, staining of 1–5%; 2+, 6–25%; 3+, 26–75%; 4+, 76–100% of tumour cells.[20]

2.4. Transfection of CLDN-7 siRNA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay

The validated form of CLDN-7 siRNA (Sl00348040, Sl00348047) and its negative control (1027280) were obtained from QIAGEN (Germantown, Maryland, USA). Transfection of each oligonucleotide was used with HiPerFect Transfection reagent (QIAGEN, Maryland, USA) according to the manufacturer's protocol. Human ovarian carcinoma cell lines 2774, HeyA8, SK-OV-3, PA-1 and OVCAR-3 obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) were used for the knockdown of CLDN-7. Cell viability was determined by MTT assay. For the MTT assay, 5000 cells in 200 μl media per well were seeded in a 96 well plate. After overnight incubation to allow cells to attach to the plate, the media was removed by suction. About 1 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) solution (1 ml of 10 mg/ml MTT in PBS added to 9 ml serum free medium) was added to each well. The cells were then incubated for 3 h at 37 °C to allow MTT to be metabolised. The media was dumped off, and then 100 μl of dimethyl sulfoxide (DMSO) per well was added to resuspended the MTT metabolic product (pupple formazan). The plate was placed on the shaking table for 10–20 min at room temperature to thoroughly mix the formazan into DMSO. The absorbance was read at 540 nm.

2.5. Western blot

Cells were lysed in RIPA buffer [50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 1% NP40, 0.1% SDS and 10 mM sodiumdeoxycholate]. Cell lysates were separated in SDS–PAGE and transferred to membrane. Membrane was blocked in 25 mM Tris–Cl (pH 8.0) containing 125 mM NaCl, 0.1% Tween 20, and 5% dry milk. Protein bands were probed with CLDN-7 antibody (Invitrogen Corporation) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and labelled with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Amersham, Piscataway, NJ, USA). Bands were visualised by enhanced chemoluminescence using the ECL kit (Amersham) according to the manufacturer's protocol.

2.6. Treatment of CLDN-7 siRNA and cisplatin in human ovarian cancer cells

Cells were treated with described concentrations of cisplatin (Sigma–Aldrich, St. Louis, MO, USA). For co-treatment of siRNA and cisplatin, 5000 cells per well in a 96 well plate were first transfected with siRNA at the described concentration. After 24 h, medium overlaid the cells was removed, and the cisplatin in serum-free medium at the described concentration was added to the cells transfected with siRNA. After 48 h of treatment, the cell viabilities were estimated by the MTT assay as described above.

2.7. Statistical analysis

Statistical analysis was performed with the SPSS for window version 15.0 (SPSS, Inc.). Each experiment was carried out at least three times in triplicate. The Pearson's chi-square and Fisher's exact tests were used to assess the statistical significance of the association between CLDN-7 expression and clinicopathological parameters. The comparisons of CLDN-7 expression between normal ovarian tissues and EOCs were performed using the Mann–Whitney test (for real-time RT-PCR analysis and immunohistochemistry). Univariate survival analysis was carried out according to Kaplan–Meier method with Log rank test. Differences in cell survival rates as measured by the MTT assay were assessed by a two-sample *t*-test or two-way of ANOVA test. *P*-value < 0.05 was considered significant.

3. Results

3.1. CLDN-7 mRNA expression

As shown in Fig. 1, CLDN-7 transcripts were quantified in 44 EOCs and in 11 normal ovarian tissues. A statistically significant difference between CLDN-7 mRNA expressions was noted between EOCs and normal ovarian tissues ($P < 0.001$). We found an increased CLDN-7 expression in EOCs, irrespective of the histological subtype, but not in normal ovarian tissues.

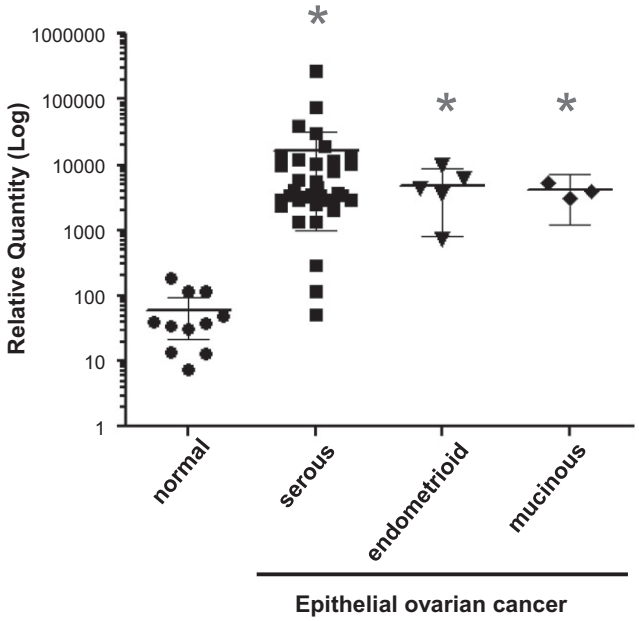

Fig. 1 – Claudin-7 (CLDN-7) mRNA expression in normal ovarian tissues and epithelial ovarian carcinomas using real-time quantitative RT-PCR. Bars, SE. *P < 0.001.

3.2. *Immunohistochemical staining of CLDN-7*

Immunohistochemical analysis also demonstrated significant different staining of CLDN-7 between EOCs and normal ovarian tissues ($P < 0.001$) (Fig. 2 and Table 1). CLDN-7 expression was found in the majority of the EOC tumour cells proper (69/71, 97%), but not in stromal tissues adjacent to tumour (Fig. 2 and Table 1). The CLDN-7 immunostaining was observed mainly on the cell membrane and occasionally within cytoplasm as a punctated staining (Fig. 2). But CLDN-7 was not expressed in all of normal ovarian tissues which were evaluated (Fig. 2D).

3.3. *CLDN-7 expression and prognosis of EOC*

When expression categories according to the staining extent were divided into two groups as low expression (stain $\leqslant$ 25% of cancer cell; 0–2+) and high expression (stain > 25% of cancer cell; 3+/4+)[20], high CLDN-7 expression was significantly correlated with poor progression-free survival (PFS) of the patients (Fig. 3, $P = 0.012$). Univariate analysis revealed that advanced stage, larger residual tumour volume and high expression of CLDN-7 predicted PFS. Further multivariate analysis showed high expression of CLDN-7 was an independent prognostic factor for progression-free survival of these patients regardless of initial tumour stage and residual tumour volume after surgery (Table 2). Interestingly, high CLDN-7 expression was significantly correlated with poor sensitivity to first-line platinum-based combination chemotherapy after surgery (Table 3, $P = 0.024$). Patients with high CLDN-7 expression were found more frequently in platinum-resistant group than in platinum sensitive group (90.5% versus 64.0%). There was no significant correlation between the staining intensity of CLDN-7 and clinicopathological parameters, such as age, grade, FIGO stage and histological type (Table 3).

3.4. *CLDN-7 down regulation and sensitivity to cisplatin in ovarian carcinoma cell lines*

We assessed the basal expression of CLDN-7 in human ovarian carcinoma cell lines including 2774, OVCAR-3, PA-1, Hey A8 and SK-OV-3 cells by Western blot. The CLDN-7 expressions in cells examined varied from weak to strong (Fig. 4A). Based on this result, we decided to use HeyA8 and 2774 cells with high CLDN-7 expressions to evaluate the efficacy of CLDN-7 siRNA on cell survival. Proliferation assay as shown in Fig. 4B with CLDN-7 siRNA showed growth-inhibiting effect

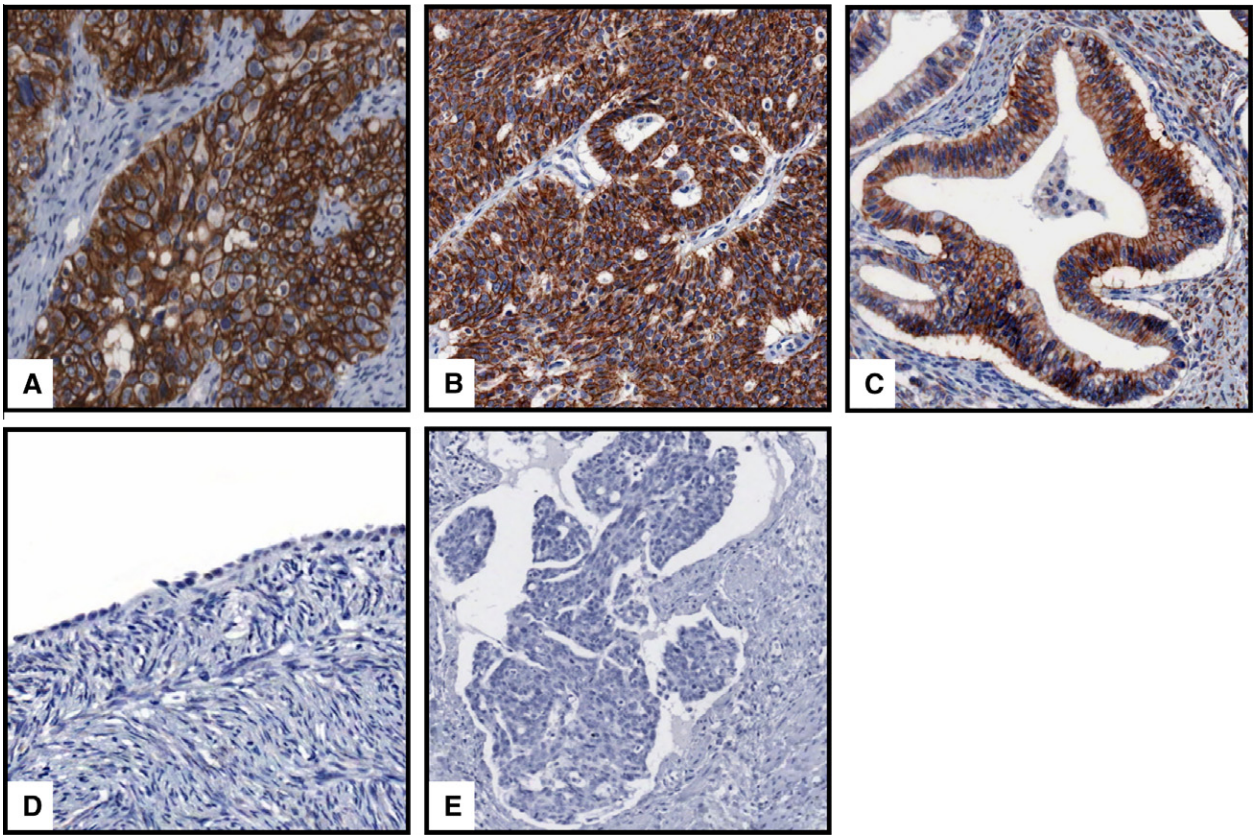

Fig. 2 – Immunohistochemical evaluation of CLDN-7 in the ovarian tissues. Representative staining with anti-CLDN-7 antibodies of primary ovarian carcinomas including serous (A), endometrioid (B) and mucinous (C) subtype is shown. A membrane-bound positivity was generally observed and occasionally associated to a punctated staining pattern within cytoplasm. No staining is observed in normal ovarian surface epithelium and stroma (D). Negative control (E). Tissues section was incubated with only secondary antibody. A–D photographs were taken at original magnification ×200. E photograph was taken at original magnification ×100.

Table 1 – Summary of CLDN-7 expression in epithelial ovarian carcinomas using immunohistochemistry.

Cell type	Staining extent (%)				
	0	1+	2+	3+	4+
Normal	12 (100)	–	–	–	–
Serous	2 (4)	7 (13)	9 (17)	18 (33)	18 (33)
Endometrioid	–	2 (15)	–	6 (46)	5 (39)
Mucinous	–	1 (20)	–	2 (60)	1 (20)

Staining extent was scored on a scale from 0 to 4: 0, no staining; 1, staining of 1–5%; 2, staining of 6–25%; 3, staining of 26–75%; and 4, staining of 76–100%.

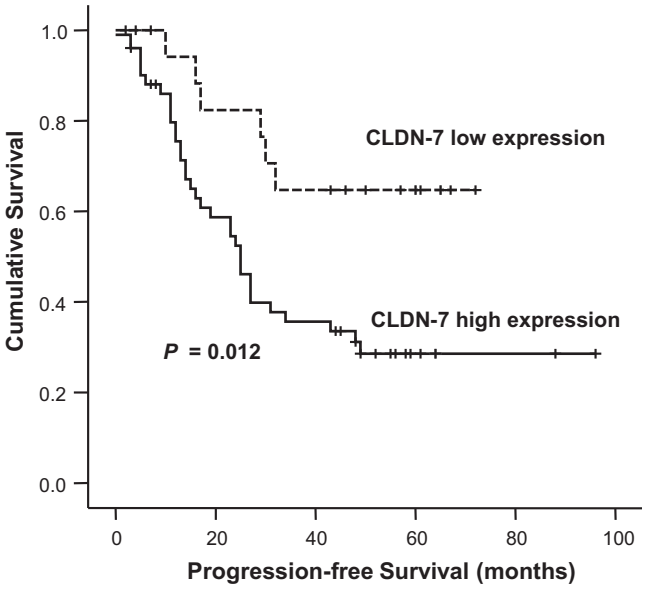

Fig. 3 – Kaplan–Meier survival curves. A significant correlation between progression-free survival (PFS) and the level of CLDN-7 expression. Patient with high CLDN-7 expression (solid line, *n* = 51) had a shorter PFS than those with low CLDN-7 expression (dotted line, *n* = 20).

at 3 or 4 d after transfection in both cells. In particular, CLDN-7 siRNA inhibited the cell growth with dose-dependent manner in HeyA8 cells. Moreover, co-treatment of CLDN-7 siRNA and cisplatin showed a significant reduction in cell survival compared to cisplatin alone in both cell lines (Fig. 4C, both $P < 0.05$). These results suggested that CLDN-7 siRNA could enhance the sensitivity to cisplatin treatment in these cells.

4. Discussion

In this study, we found that the expression of CLDN-7 was significantly higher in primary EOC tissues compared with normal ovarian ones (Fig. 1), and high CLDN-7 expression in EOCs was associated with shorter progression-free survival in multivariate analysis. Interestingly, high CLDN-7 expression correlated with poor response to first-line platinum-based combination chemotherapy (Fig. 3 and Table 3) and the inhibition of CLDN-7 using specific siRNA enhanced the sensitivity to cisplatin in human ovarian carcinoma cells (Fig. 4).

Our results of high CLDN-7 expression at mRNA and protein levels regardless of histologic subtype of EOCs are in agreement with previous studies.[18–20] Tassi and colleagues reported that high CLDN-7 expression was observed in all histological types of EOCs, ascites and pleural effusion, and suggested that it might be practical for diagnostic and potential therapeutic applications.[19] Kleinberg and colleagues reported significantly higher expression of CLDN-7 in serous effusion compared with primary and metastatic EOCs. And they suggested that higher expression of CLDN-7 in effusions correlates with poor progression-free survival and overall survival and it may serve as novel prognostic markers in EOCs.[20] In this study, we also found correlation between high CLDN-7 expression and short progression-free survival in EOCs but not overall survival. The sample size of this study might not be sufficient to find association with overall survival. In addition, our data are different from their study in two aspects. One is that the association between high CLDN-7 expression

Table 2 – Univariate and multivariate analysis for progression-free survival (PFS) of individual parameters.

	Univariate analysis		Multivarate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age	1.03 (0.99–1.05)	0.088	1.02 (0.99–1.06)	0.243
Histology (serous versus non-serous)	0.55 (0.24–1.24)	0.151	0.76 (0.28–2.12)	0.605
Grade (I/II versus III)	0.87 (0.38–1.99)	0.732	0.64 (0.27–1.49)	0.299
Stage (I/II versus III/IV)	6.40 (2.47–16.57)	0.000	2.40 (0.66–8.75)	0.186
Optimal debulking (<1 cm versus ⩾1 cm)	2.71 (1.45–5.08)	0.002	0.75 (0.32–1.74)	0.499
CLDN-7 expression (0–2+ versus 3+/4+)	2.90 (1.22–6.93)	0.016	3.36 (1.15–9.83)	0.027

HR = Hazard Ratio, CI = Confidence Interval, Cox proportional-hazards model was used for the analyses.

Table 3 – Correlation of CLDN-7 in epithelial ovarian carcinomas (EOCs) with clinicopathological features.

		Low (0–2+) N = 20	High (3+ – 4+) N = 51	P-value
Age	<50	7 (35.0%)	23 (45.1%)	0.438[b]
	⩾50	13 (65.0%)	28 (54.9%)	
Histology	Seours	17 (85.0%)	37 (72.5%)	0.361[b]
	Non-serous	3 (15.0%)	14 (27.5%)	
Grade	I–II	2 (15.4%)	9 (22.0%)	0.715[b]
	III	11 (84.6%)	32 (78.0%)	
	Unknown	7	10	
Stage	I–II	8 (40.0%)	15 (29.4%)	0.391[b]
	III–IV	12 (60.0%)	36 (70.6%)	
Optimal debulking	<1 cm	16 (80.0%)	29 (56.9%)	0.094[b]
	⩾1 cm	4 (20.0%)	22 (43.1%)	
Chemosensitivity[a]	Sensitive	18 (36.0%)	32 (64.0%)	0.024[b]
	Resistant	2 (9.5%)	19 (90.5%)	
Mean PFS time, mo		54.3 (2–71.7)	25.1 (0–95.8)	0.012[c]

[a] Adjuvant platinum-based combination chemotherapy after surgery: platinum-resistant, platinum-free interval ⩽ 6 months; platinum-sensitive, platinum-free interval > 6 months. PFS = progression-free survival.
[b] Fisher's exact test or Pearson chi-square.
[c] Kaplan–Meier survival analysis.

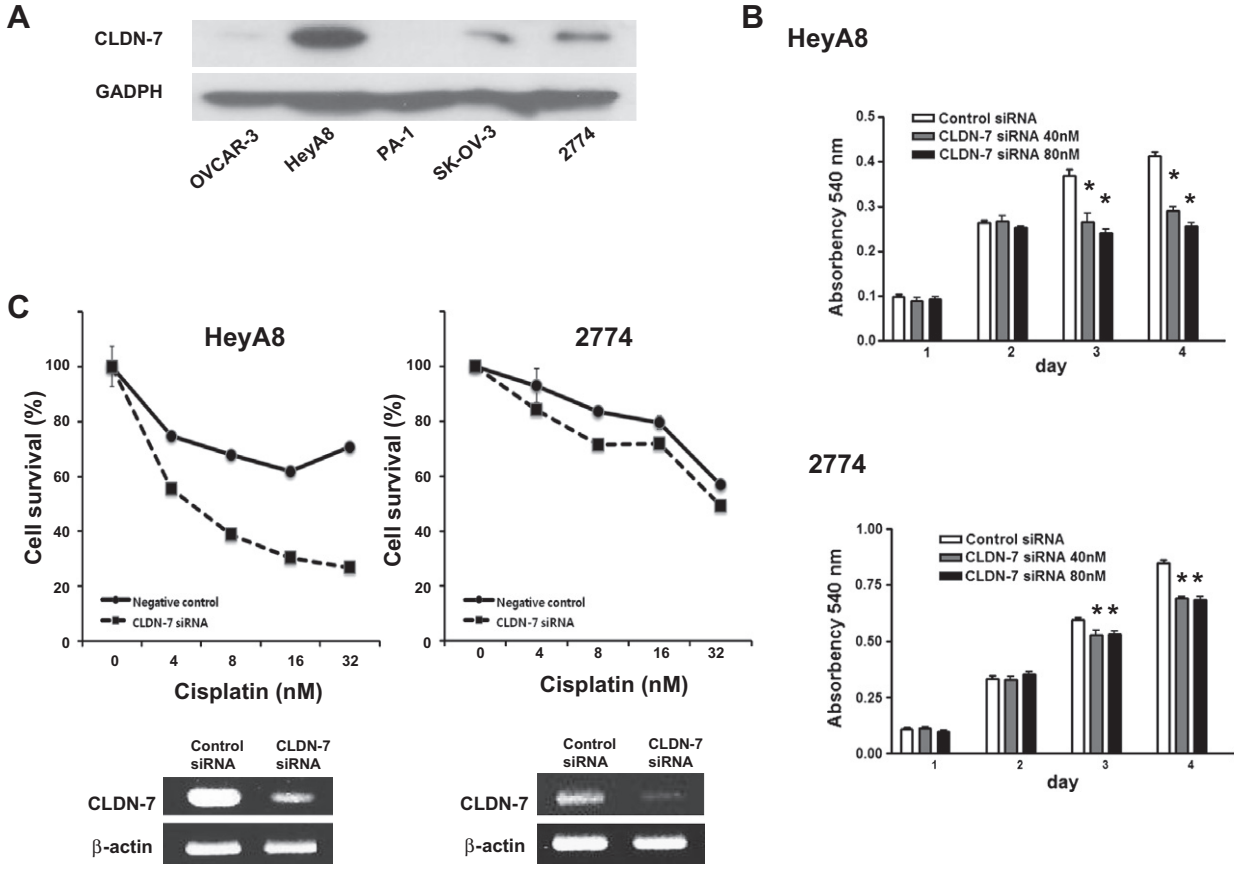

Fig. 4 – The basal expression of CLDN-7 in ovarian carcinoma cell lines and effect of CLDN-7 siRNA on ovarian carcinoma cell survival. HeyA8 and 2774 cells have relatively high expression of CLDN-7 assessed by Western blot (A). Proliferation assay with CLDN-7 siRNA showed that cell proliferation was significantly decreased 3 or 4 d later after transfection in both cells (*P < 0.001). Moreover, the combined effect of CLDN-7 siRNA (40 nm) and cisplatin showed a significant reduction in cell survival in these cells (C, both P < 0.05). Negative control (NC) means control siRNA.

and poor progression-free survival in our study was explored in primary ovarian tumours, not in serous effusion. The other is that the response to platinum-based chemotherapy was correlated with high CLDN-7 expression in primary tumour of EOC in our study, but they reported that the CLDN-7 expression in serous effusion was not associated with chemotherapy response. CLDN-7 may have different functional role in primary ovarian tumour and in its serous effusion.

CLDNs have been selected as a candidate marker for prognostic evaluation in several human cancers including colon,[23] liver[24] and stomach.[25] There were two reports in EOCs in which CLDN-3 overexpression was associated with poor survival.[26,27] And recent study suggested that CLDN-3 and -7 expression in serous effusion of ovarian carcinoma patients independently predict poor survival.[20] Poor survival with high expression of CLDN-7 may be partly explained by the poor response to platinum-based chemotherapy shown from the current study. To our knowledge, this is the first report demonstrating that high CLDN-7 expression in primary tumour of EOCs was associated with poor response to platinum based combination chemotherapy and its sensitivity to cisplatin could be enhanced by downregulating CLDN-7 in ovarian cancer cell lines. These results suggest that CLDN-7 may play a role in regulating response to platinum based chemotherapy and may be used in choosing an alternative chemotherapeutic regimen for those individuals having high CLDN-7 expression.

Although the function of CLDNs in cancer cells is not completely known, recent findings suggest the involvement of CLDNs in cancer cell survival, migration and invasion.[28,29] Several studies have been conducted using CLDNs as possible therapeutic targets. One of the approaches to targeting CLDNs for cancer therapy involves the use of *Clostridium perfringens* enterotoxin (CPE). CPE binding to the second extracellular domain of CLDN-3 or -4 triggers the formation of a large multiprotein membrane–pore complex, which disrupts cellular osmotic equilibrium, causing cell lysis.[30] Another is the use of anti-CLDN antibody-mediated therapy which has gained favour in recent years owing to the potential for increased tumours specificity and lower toxicity profiles.[31] Other is gene silencing with CLDNs siRNA in human carcinoma. Huang and colleagues reported that lipidoid-formulated CLDN-3 siRNA via intraperitoneal injection suppresses tumour growth in ovarian cancer mice model.[32] CLDN-7 could be expected as another potential therapeutic target for the treatment of EOC because CLDN-7 expression was found in the majority of the EOC and the inhibition of CLDN-7 with siRNA showed a significant reduction in cell survival on cisplatin treatment in ovarian cancer cell lines. However, CLDN-7 expression in functional vital organs, such as the kidney, colon and lung, might represent a significant limitation to this approach by systemic therapy.

In conclusion, our study has demonstrated that CLDN-7 is highly expressed in EOCs and its high expression is associated with shorter progression-free survival and poor sensitivity to platinum-based chemotherapy. In addition, treatment with CLDN-7 siRNA enhanced the sensitivity of epithelial ovarian cancer cells to cisplatin treatment. These results suggest that CLDN-7 may play a role in regulating response to platinum based chemotherapy in the treatment of EOC. Further studies are needed to evaluate the molecular mechanism of CLDN-7 activity and the potential role of CLDN-7 as a therapeutic target in EOC.

Conflict of interest statement

None declared.

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