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Expression profile of tight junction protein claudin 3 and claudin 4 in ovarian serous adenocarcinoma with prognostic correlation

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Summary. Tight junction proteins claudin 3 (CLDN3) and claudin 4 (CLDN4) are frequently altered in several human cancers, including ovarian carcinomas. Here, we examined the gene expression of *CLDN3* and *CLDN4* in various tumors, including 19 normal ovaries and 47 ovarian carcinomas by analyzing Affymetrix HG-U133 array data. Furthermore, a total of 114 ovarian serous tumors, including 10 adenomas, 20 borderline tumors and 84 carcinomas, were analyzed immunohistochemically to confirm the expression of two proteins and we assessed the association of their expression with the clinicopathological characteristics and survival of the patients. The microarray experiment revealed *CLDN3* and *CLDN4* transcripts were significantly up-regulated by 5-fold or more in most subtypes of ovarian epithelial carcinomas while the immunohistochemical analyses indicated that each protein was expressed in 68 (81.0%) and 72 (85.7%) of 84 serous adenocarcinomas, respectively. Borderline serous tumors and adenomas showed significantly lower expression of these proteins than the adenocarcinomas. Kaplan-Meier survival analysis showed that serous adenocarcinoma patients with high CLDN3 expression had substantially shorter survival (P=0.027). Multivariate analysis demonstrated that CLDN3 overexpression is an independent negative prognostic factor. Our findings suggest that CLDN3 overexpression can be used as a prognostic indicator in ovarian serous carcinomas. Moreover, CLDN3 may be a promising target for antibody-based therapy of ovarian carcinomas.

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Introduction

Ovarian cancer is responsible for more deaths than any other cancer of the female genital tract due to difficulties in detecting it early and diagnosing and treating it. Most ovarian cancers are serous carcinomas arising from the ovarian surface epithelium (Singer et al., 2002; Heinzelmann-Schwarz et al., 2004; Shih Ie and Kurman, 2004). Although biomarkers such as CA-125 are now available, their usefulness in the initial diagnosis is limited as they generally show low specificity and sensitivity (Heinzelmann-Schwarz et al., 2004). Many recent studies have been performed to identify new molecular markers for ovarian cancer that will aid early diagnosis, act as prognostic indicators, or serve as therapeutic targets (Hough et al., 2000; Heinzelmann-Schwarz et al., 2004; Hibbs et al., 2004; Lu et al., 2004; Santin et al., 2004). Many of these studies have used large scale gene expression measurement techniques to identify the differentially expressed genes in ovarian carcinoma compared to normal ovarian cells. These techniques include Serial Analysis of Gene Expression (SAGE) and microarray analysis and their use has led to the identification of several candidate genes that are expressed at higher levels in ovarian cancer compared to normal ovaries. These include apolipoprotein J (*APOJ*), ß8 integrin subunit, *CD24*, claudin 3 (*CLDN3*), claudin 4 (*CLDN4*), discoidin domain receptor 1 (*DDR1*), epithelial cell adhesion molecule (*Ep-CAM*) and *S100A1* (Hough et al., 2000; Heinzelmann-Schwarz et al., 2004; Hibbs et al.,

2004; Santin et al., 2004).

The genes encoding the tight junction proteins CLDN3 and CLDN4 have been consistently identified in several studies as being highly up-regulated in ovarian carcinoma (Hough et al., 2000; Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004; Hibbs et al., 2004; Lu et al., 2004; Santin et al., 2004; Zhu et al., 2006). Their high level of expression at the protein level has also been confirmed by immunohistochemical staining (Hough et al., 2000; Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004; Hibbs et al., 2004; Lu et al., 2004; Zhu et al., 2006). Claudins (CLDNs) are major integral membrane proteins that form the tight junction strands that are crucial for the maintenance of cell polarity and paracellular transport in epithelia and endothelia (Tsukita et al., 2001). To date, 23 members of the claudin proteins have been identified in humans (Morin, 2005). The above described functions of CLDN3 and CLDN4 increase the possibility that these two proteins may be useful tumor markers for the detection and diagnosis of ovarian cancer as well as being potential targets for antibody-based therapy. However, how they become overexpressed in cancer and the role they play in ovarian tumorigenesis remain unclear. In addition, how the expression of these two proteins correlates with clinicopathological characteristics, including patient survival, has not yet been investigated.

This study was designed to evaluate the association of CLDN3 or CLDN4 expression with the clinicopathological parameters and survival of patients with ovarian cancers.

Materials and methods

Gene expression analysis

Expression values of tumor and normal tissue biopsies were obtained from the GeneExpress Oncology Datasuite™ of Gene Logic Inc., based on the Affymetrix Human Genome U133 array set. Briefly, RNA was obtained from 281 normal tissues, including 19 normal ovaries, and 472 various cancers, including 47 ovarian carcinomas (Table 1). Outliers were detected by Principal Component Analysis using the MatLab program (The MathWorks, Inc.), and excluded from further analysis. We analyzed the expression profiles of the normal and cancer tissue sets listed in Table 1. The primer sets used for *CLDN3* and *CLDN4* analysis are 203954_x_at and 201428_x_at, respectively. The ratio of the geometric means of expression intensities in cancer tissues to normal tissues (fold change) was computed and the *P*-values regarding the fold change were also calculated by using t-tests. Differences were considered significant if the *P*-value was <0.05.

Patients and tissue samples

114 patients with ovarian serous tumor were retrospectively identified from the surgical pathology files of the Department of Pathology at Samsung Medical Center and their archival tissues were obtained. These samples consisted of 10 serous cystadenomas, 20 serous cystadenomas of borderline malignancy, and 84 serous adenocarcinomas. The inclusion criteria were histopathological diagnosis of serous ovarian tumor,

Table 1. List of the 17 tissue types and 32 cancer types in the HG-U133 array that were analyzed for *CLDN3* and *CLDN4* expression in this study.

Sample/ Tissue Site	Pathology/Morphology	Number of tissues
Breast	N : Normal	27
	IDC : Infiltrating duct carcinoma	55
	IDLC : Infiltrating duct and Lobular carcinoma	9
	ILC : Infiltrating Lobular carcinoma	14
Cervix	N : Normal	5
	SCC : Squamous cell carcinoma	10
Colon	N : Normal	26
	AC : Adenocarcinoma	37
	MAC : Mucinous Adenocarcinoma	7
Duodenum	N : Normal	10
	AC : Adenocarcinoma	5
Endometrium	N : Normal	9
	AC : Adenocarcinoma	9
	MMT : Mullerian Mixed Tumor	5
Esophagus	N : Normal	14
	AC : Adenocarcinoma	12
Kidney	N : Normal	29
	CCA : Clear Cell Adenocarcinoma	10
	OC : Oncocytoma	6
	RCC : Renal Cell Carcinoma	16
Liver	N : Normal	21
	HCC : Hepatocellular Carcinoma	23
Lung	N : Normal	32
	AC : Adenocarcinoma	20
	SCC : Squamous Cell carcinoma	24
Lymph Node	N : Normal	5
	HD : Hodgkin's Disease	4
	ML : Malignant Lymphoma	16
Myometrium	N : Normal	5
	LM : Leiomyoma	4
Ovary	N : Normal	19
	AC : Adenocarcinoma	6
	CCA : Clear Cell Adenocarcinoma	6
	MCA : Mucinous Cyst Adenocarcinoma	6
	PSA : Papillary Serous Adenocarcinoma	22
	SCA : Serous Cyst Adenocarcinoma	7
Pancreas	N : Normal	19
	AC : Adenocarcinoma	30
Prostate	N : Normal	15
	AC : Adenocarcinoma	28
Rectum	N : Normal	18
	AC : Adenocarcinoma	21
Skin	N : Normal	5
	BCC : Basal Cell Carcinoma	5
	MM : Malignant Melanoma	5
	SCC : Squamous Cell Carcinoma	6
Stomach	N : Normal	22
	AC : Adenocarcinoma	38
	SRCC : Signet Ring Cell Carcinoma	6

availability of clinical follow-up data, and availability of paraffin-embedded tissue specimens. All samples were collected anonymously according to Institutional Review Board guidelines. All patients had undergone a surgical operation and had received neither chemotherapy nor radiotherapy before surgical resection. All cases were reevaluated and classified according to the classification recently accepted by the World Health Organization. TNM staging according to the staging system of the American Joint Committee on Cancer (AJCC) was used. Five cases of normal ovaries from hysterectomy specimens resected for non-ovarian disease were also added.

Tissue microarray of serous adenocarcinomas

We used tissue microarray slides for immunohistochemical analysis of 84 serous adenocarcinomas. To prepare these slides, we punched tissue columns (2 or 3 mm in diameter) from the original blocks and inserted them into new paraffin blocks (each containing 30 holes to accept the tissue columns). This yielded serially-sectioned slides. Since each tissue microarray slide (1x3 inches) could hold at most 60 or 30 specimens, we could simultaneously analyze 60 or 30 specimens with minimum variation during the staining process. Each specimen had a round shape and was 2 or 3 mm in diameter, which is sufficient for histopathological analysis. 4-μm sections were prepared on silane-coated slides (Sigma, St Louis, MO, USA).

Immunohistochemistry

The tissue microarray sections were used only for the immunohistochemistry of serous ovarian adenocarcinomas, and whole sections were utilized for that of normal ovary, serous cystadenomas, and serous cystadenomas of borderline malignancy because the cores from them may not contain enough area for representative lesion. The tissue sections in the microslides were deparaffinized with xylene, hydrated by using a diluted alcohol series, and immersed in 3% H_2O_2 in methanol to quench the endogenous peroxidase activity. The sections were then microwaved in 10 mM citrate buffer (pH 6.0) and 50 mM borate buffer (pH 8.0) for CLDN3 and CLDN4, respectively, for 15 min for antigen retrieval. To reduce non-specific staining, each section was treated with 4% bovine serum albumin in PBS with 0.1% Tween 20 (PBST) for 30 min. 10mg/L dextran (Sigma, St Louis, MO, USA) was added for CLDN3 staining. The sections were then incubated with rabbit anti-CLDN3 polyclonal antibody (dilution: 1:40, Zymed Laboratories Inc.,CA, USA) or anti-CLDN4 monoclonal antibody (dilution: 1:50, clone 3E2C1, Zymed Laboratories Inc.) in PBST containing 3 mg/mL goat globulin (Sigma) for 60 min at room temperature, followed by three successive rinses with a washing buffer. Sections stained for CLDN4 were incubated with biotinylated goat anti-mouse IgG for 30 min at room temperature, and then incubated with a streptavidin-peroxidase conjugate for 30 min at room temperature. Sections stained for CLDN3 were incubated with an anti-rabbit polymer kit (Dako, Carpinteria, CA, USA) for 30 min at room temperature. The chromogen used was 3,3'-diaminobenzidine (Dako). Sections were counterstained with Meyer's hematoxylin. Negative controls with normal mouse and rabbit serum were processed in parallel, and no positive staining was observed.

Evaluation of immunohistochemical staining

Each lesion was examined and scored separately by two pathologists (Y-L.C. and J.K), who were unaware of the diagnosis or outcome of the cases, and cases with discrepant scores were discussed until unity was achieved. Cases with less than 10% staining in tumor cells were considered negative for expression. In normal, adenoma and borderline cases, the positive cases were classified on the basis of the intensity of protein expression in the membrane of the tumor cells. In serous adenocarcinoma cases, CLDN3 and CLDN4 were expressed both in the cytoplasm and the membrane and the expression of these proteins was classified on the basis of the intensity and area of staining, which were measured by a semiquantitative method: 0, less than 10% of the cells; +1, weak in more 10% or moderate in 10-25%; +2, moderate in more than 25% or intense in 25-50%; and +3, intense in more than 50%. A sample was regarded as being positive for CLDN3/CLDN4 staining when it was classified as +1, +2, or +3. The cases with 0 and +1 staining were considered as lower expressors, while the cases with +2 and +3 staining were considered as higher expressors.

Statistical methods

SPSS software version 11.5 (SPSS, Chicago, USA) was used for statistical evaluation. Mann-Whitney test was used to compare the expression between groups, epithelia of normal ovary, cystadenoma, borderline tumor and adenocarcinoma. The correlation between CLDN3 or CLDN4 expression levels and clinicopathological parameters was assessed by Fisher's exact test and Pearson's χ^2 test. With regard to survival analysis, we analyzed 84 patients with invasive ovarian carcinoma by Kaplan-Meier analysis. Log rank test was used to compare the survival curves between groups. Univariate and multivariate survival analyses were then conducted using the Cox regression model. $P<0.05$ was regarded as statistically significant.

Results

CLDN3 and CLDN4 transcript levels in normal tissues and malignant tumors

The gene expression of *CLDN3* and *CLDN4* in various normal and cancerous tissues (Table 1) was determined by using Affymetrix HG-U133 (Fig. 1). The

detailed expression data in all tissues, including fold changes and their associated *P*-values, are indicated in Table 2. Notably, *CLDN3* and *CLDN4* were detected at markedly higher levels in normal colon and rectum than in other normal tissues.

Several types of epithelial cancers, namely, cancers of the esophagus, ovary, prostate and stomach, showed 2 or more fold greater *CLDN3* transcript level when compared to their normal tissues. In contrast, *CLDN3* transcript level was decreased by 2 or more fold in duodenal adenocarcinoma, Hodgkin's disease, malignant lymphoma, and pancreatic adenocarcinoma. With regard to *CLDN4* transcript level, it was increased by 2 fold or more in cancers of the ovary, pancreas and stomach but decreased by over 2 fold in Hodgkin's disease, malignant lymphoma, and basal cell carcinoma and malignant melanoma in the skin. The most striking observation of the oligonucleotide array analysis is that, of all the tumors examined, the cancers of the ovary showed the most striking change (up-regulation) in *CLDN3* and *CLDN4* expression (Fig. 1). Ovarian adenocarcinomas (AC) showed particularly marked up-regulation (>10-fold change; $P<0.05$), as did papillary serous adenocarcinoma (PSA) (>9-fold change; $P<0.05$). Ovarian clear cell carcinoma (CCA) and serous cystadenocarcinoma (SCA) both also showed >5-fold changes in claudin expression ($P<0.05$) relative to the low expression in the normal ovary (Fig. 1).

Another interesting observation was that *CLDN3* expression was down-regulated in pancreatic adenocarcinoma by 2 fold while *CLDN4* was up-regulated. In contrast, in other cancerous tissues, either the two *CLDNs* were both up- (or down-) regulated or only one showed a change in expression.

Ovarian serous adenocarcinomas show higher CLDN3 and CLDN4 expression than ovarian adenomas and borderline tumors

To validate the oligonucleotide array results and

Table 2. Expression of *CLDN3* and *CLDN4* in several types of cancer tissues (C) compared to the normal tissue (N) counterpart as determined by analysis of the HG-U133 Affymetrix array.

	CLDN3				CLDN4			
Tissue	Mean(N)	Mean(C)	FC[a]	*P*-value	Mean (N)	Mean(C)	FC[a]	*P*-value
Breast_N_IDC	113.96	154.13	1.35	0.042	143.76	143.18	-1	0.970
Breast_N_IDLC	113.96	135.85	1.19	0.421	143.76	118.79	-1.21	0.292
Breast_N_ILC	113.96	132.5	1.16	0.512	143.76	172.35	1.2	0.219
Cervix_N_SCC	34.23	23.27	-1.47	0.329	80.87	74.99	-1.08	0.857
Colon_N_AC	549.39	422.17	-1.3	0.023	328.54	324.14	-1.01	0.896
Colon_N_MAC	549.39	366.35	-1.5	0.157	328.54	251.49	-1.31	0.177
Duodenum_N_AC	297.42	109.52	-2.72*	0.075	166.92	204.41	1.22	0.187
Endometrium_N_AC	100.53	158.03	1.57	0.191	81.87	108.45	1.32	0.440
Endometrium_N_MMT	100.53	77.51	-1.3	0.680	81.87	62.22	-1.32	0.610
Esophagus_N_AC	20.75	66.12	3.19*	0.007	122.89	161.33	1.31	0.262
Kidney_N_CCA	80.2	82.4	1.03	0.911	86.7	80.5	-1.08	0.727
Kidney_N_OC	80.2	43.3	-1.85	0.192	86.7	52.1	-1.66	0.062
Kidney_N_RCC	80.2	118	1.47	0.228	86.7	94.2	1.09	0.732
Liver_N_HCC	45.17	62.32	1.38	0.088	20.71	28.53	1.38	0.014
Lung_N_AC	53.67	76.1	1.42	0.210	99.05	127.29	1.29	0.122
Lung_N_SCC	53.67	29.72	-1.81	0.004	99.05	80.67	-1.23	0.257
LymphNode_N_HD	84	20	-4.2*	0.071	93.87	20	-4.69*	0.026
LymphNode_N_ML	84	20.27	-4.14*	0.073	93.87	21.84	-4.3*	0.030
Myometrium_N_LM	25.03	26.24	1.05	0.892	21.7	20	-1.09	0.374
Ovary_N_AC	26.96	535.4	19.86*	<0.001	27.41	294.99	10.76*	<0.001
Ovary_N_CCA	26.96	277.43	10.29*	0.011	27.41	158.22	5.77*	0.011
Ovary_N_MCA	26.96	118.51	4.4*	0.052	27.41	205.62	7.5*	<0.001
Ovary_N_PSA	26.96	417.61	15.49*	<0.001	27.41	262.06	9.56*	<0.001
Ovary_N_SCA	26.96	242.21	8.98*	0.003	27.41	140.27	5.12*	0.002
Pancreas_N_AC	124	47.9	-2.58*	<0.001	62.1	185	2.98*	<0.001
Prostate_N_AC	138.76	445.92	3.21*	<0.001	154.62	227.45	1.47	0.006
Rectum_N_AC	559.51	450.21	-1.24	0.127	335.78	312.96	-1.07	0.532
Skin_N_BCC	20	20	-1	1.00	51.46	22.54	-2.28*	0.043
Skin_N_MM	20	21.34	1.07	0.374	51.46	23.17	-2.22*	0.048
Skin_N_SCC	20	20	-1	1.00	51.46	46.08	-1.12	0.776
Stomach_N_AC	35.6	106.09	2.98*	<0.001	35.97	173.74	4.83*	<0.001
Stomach_N_SRCC	35.6	140.61	3.95*	0.056	35.97	212.96	5.92*	0.004

[a]: Fold change was calculated by dividing the expression in cancer tissues by the one in normal tissues; *: 2-fold greater and statistically significant ($P<0.05$); **: For abbreviations, see Fig. 1.

assess whether *CLDN3* and *CLDN4* are also expressed at higher levels in ovarian cancer at the protein level, we performed immunohistochemical analysis. Five samples of normal ovarian surface epithelium showed no immunoreactivity for CLDN3 and CLDN4. Table 3 details the immunohistochemical findings of CLDN3 and CLDN4 expression in serous tumors. With regard to the ten cases of cystadenoma examined, all either did not or only weakly expressed the two proteins in the membrane of the tumor cells. Of the 20 cases of serous borderline tumors examined, four and two moderately expressed CLDN3 and CLDN4 in the membrane of the tumor cells, respectively, while the remainder either did not (2 and 3 cases, respectively) or only weakly expressed (14 and 15 cases, respectively) these claudins. In the cystadenomas and serous borderline tumors,

Table 3. Expression of CLDN3 and CLDN4 in normal ovarian epithelium, serous adenoma, serous borderline tumor and serous carcinoma.

Diagnosis	Expression of CLDN3 (Staining intensity)			
	0	+1	+2	+3
Normal (n=5)	5 (100%)	-	-	-
Adenoma (n=10)	6 (60%)	4 (40%)	-	-
Borderline (n=20)	2 (10%)	14 (70%)	4 (20%)	-
Adenocarcinoma (n=84)	16 (19.0%)	22 (26.2%)	21 (25.0%)	25 (29.8%)
Diagnosis	**Expression of CLDN4 (Staining intensity)**			
	0	+1	+2	+3
Normal (n=5)	5 (100%)	-	-	-
Adenoma (n=10)	5 (50%)	5 (50%)	-	-
Borderline (n=20)	3 (15%)	15 (75%)	2 (10%)	-
Adenocarcinoma (n=84)	12 (14.3%)	21 (25.0%)	30 (35.7%)	21 (25.0%)

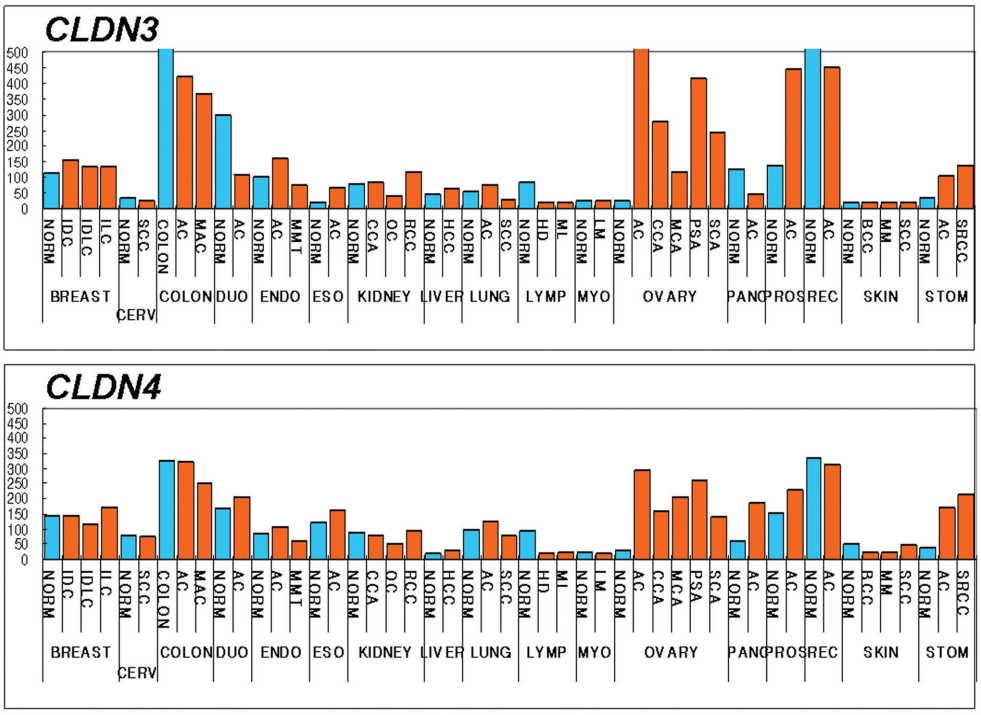


Fig. 1. Affymetrix U133 microarray analysis to determine *CLDN3* and *CLDN4* expression in various cancers. Each bar represents the expression level of *CLDN3* (top) and *CLDN4* (bottom) in 17 normal tissues (blue) and 32 tumor tissues (orange). The Y-axis represents the geometric means of the expression intensities of *CLDN3* and *CLDN4* gene fragments. The tissue types were abbreviated as follows: BREAST, breast; CERV, cervix; COLON, colon; DUO, duodenum; ENDO, endometrium; ESO, esophagus; KIDNEY, kidney; LIVER, liver; LUNG, lung; LYMP, lymph node; MYO, myometrium; OVARY, ovary; PANC, pancreas; PROS, prostate; REC, rectum; SKIN , skin; STOM, stomach. Each tissue set included normal (N) tissue and primary tumors of various subtypes: IDC, infiltrating duct carcinoma; IDLC, infiltrating duct and lobular carcinoma; ILC, infiltrating lobular carcinoma; SCC, squamous cell carcinoma; AC, adenocarcinoma; MA, mucinous adenocarcinoma; MMT, mullerian mixed tumor; CCA, clear cell adenocarcinoma; OC, oncocytoma; RCC, renal cell carcinoma; HCC, hepatocellular carcinoma; HD, Hodgkin's disease; ML, malignant lymphoma; LM, leiomyoma; MCA, mucinous cystadenocarcinoma; PSA, papillary serous adenocarcinoma; SCA, serous cystadenocarcinoma; BCC, basal cell carcinoma; MM, malignant melanoma; SRCC, signet ring cell carcinoma.

CLDN3 and CLDN4 positivity was restricted to the lateral border of the ovarian epithelium (Fig. 2a,b).

Unlike the infrequent and low staining of the adenomas and borderline tumors, many of the 84 cases of serous adenocarcinoma examined were positive for CLDN3 and CLDN4 expression (68/84 and 72/84; 81.0% and 85.7%, respectively). Of these, 25 and 21 cases (29.8% and 25.0%) showed +3 staining of CLDN3 and CLDN4, respectively, while 21 and 30 cases (25.0% and 35.7%) showed +2 staining and 22 and 21 cases

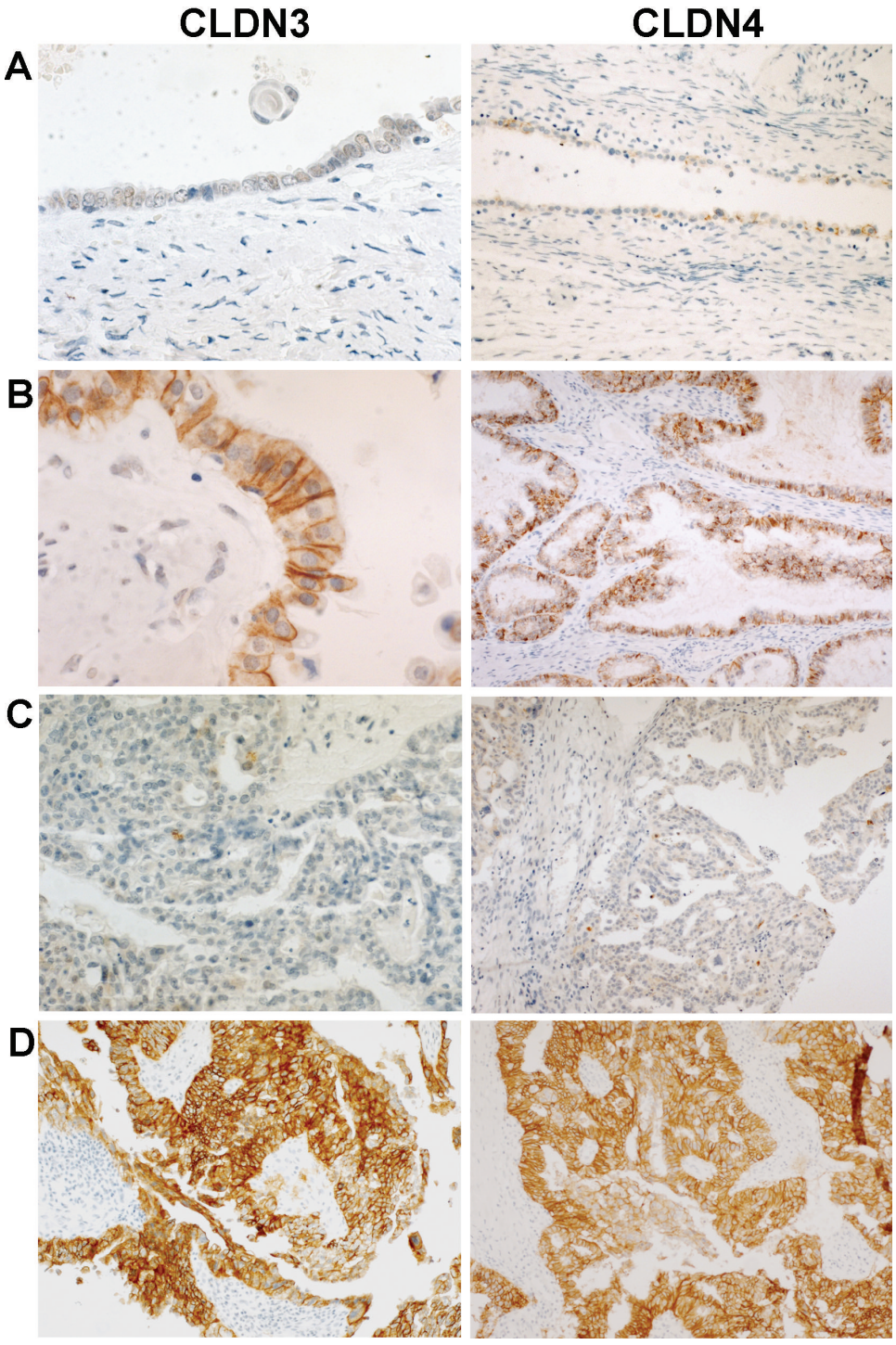


Fig. 2. Representative immunohistochemical staining of serous cystadenoma, borderline tumor and adenocarcinoma with anti-CLDN3 and anti-CLDN4 antibodies. **a.** Equivocal staining in the membrane of tumor cells in a cystadenoma sample (CLDN3: x 200, CLDN4: x 100). **b.** Moderate expression in the membrane of tumor cells in a borderline tumor (CLDN3: x 400, CLDN4: x 100). **c.** A serous adenocarcinoma sample that does not show CLDN3 or CLDN4 expression (CLDN3: x 200, CLDN4: x 100). **d.** An adenocarcinoma sample that shows pronounced staining at the membrane and in the cytoplasm (x 100).

(26.2% and 25.0%) showed +1 staining, respectively. Staining intensity was significantly increased in ovarian carcinomas compared to that of cystadenomas and borderline tumors (Table 3, Table 4, $P<0.05$). The expression of CLDN3 and CLDN4 were also significantly up-regulated in borderline tumors and adenocarcinomas compared to normal ovaries (Table 4, $P<0.05$). Notably, many of the adenocarcinoma cases that were strongly positive for CLDN3 and CLDN4 expression exhibited diffuse cytoplasmic staining with an accentuation in the membranous area while some adenocarcinoma cases did not express two proteins (Fig. 2c,d).

CLDN3 up-regulation in ovarian cancer is associated with shorter survival

To assess the potential value of CLDN3 and/or CLDN4 expression as a prognostic indicator for ovarian cancer, we examined how the expression levels of the 84 serous adenocarcinoma samples correlated with the clinicopathological characteristics and outcome of the patients (Table 5). Most of the serous ovarian carcinoma patients presented with high grade (grade III = 77%) and advance stage (stage III or IV = 89%) cancers. The intensity of CLDN3 and CLDN4 staining did not correlate statistically significantly with patient age, tumor grade, advanced stage or the presence of ascites (Table 5). However, intense CLDN4 staining did tend to correlate with higher grade cancers, although this trend did not reach statistical significance ($P = 0.068$; Table

Table 4. Comparison of expression of CLDN3 and CLDN4 in normal ovary epithelium, serous adenoma, serous borderline tumor and serous adenocarcinoma.

	P-value*	
	CLDN3	CLDN4
Normal- cystadenoma	0.111	0.061
Normal-borderline tumor	0.001	0.001
Normal-adenocarcinoma	0.002	0.001
Cystadenoma-borderline tumor	0.003	0.034
Cystadenoma-adenocarcinoma	0.001	<0.001
Borderline tumor-adenocarcinoma	0.031	0.001

* Mann-Whitney test

Table 6 Univariate analysis of the overall survival of ovarian cancer patients using Kaplan-Meier survival analysis.

		Overall survival (%)		
Variables	No. of patients	1-year	3-year	*P*-value
Age				0.273
< 50	31	96.67	78.36	
≥ 50	53	96.23	81.91	
Tumor stage				0.162
I – II	9	100	100	
III-IV	75	95.98	78.92	
Tumor Grade				0.743
I-II	19	100	84.42	
III	65	95.36	79.48	
Ascites				0.095
Absent	6	100	100	
Present	41	97.50	76.92	
CLDN3 expression**				0.027*
Low (0/+1)	38	97.37	94.42	
High (+2/+3)	46	95.60	67.57	
CLDN4 expression**				0.474
Low (0/+1)	33	100	78.87	
High (+2/+3)	51	94.08	81.70	

*: Statistically significant; **: Expression of CLDN3 and CLDN4 were measured by immunohistochemistry

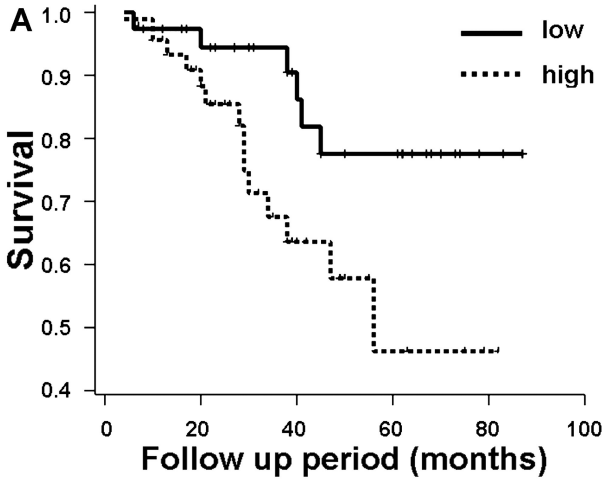
Table 5. Correlation of CLDN3 and CLDN4 expression in serous ovarian cancer with clinicopathological features.

Variables	Expression of CLDN3			Expression of CLDN4		
	Low (0/+1)	High (+2/+3)	*P*-value	Low (0/+1)	High (+2/+3)	*P*-value
Age			0.657[a]			0.934[a]
0-49	15/31	16/31		12/31	19/31	
+50	23/53	30/53		21/53	32/53	
Tumor grade			0.295[b]			0.068[b]
I-II	11/19	8/19		11/19	8/19	
III	27/65	38/65		22/65	43/65	
Stage			0.503[b]			0.733[b]
I-II	3/9	6/9		4/9	5/9	
III-IV	35/75	40/75		29/75	46/75	
Ascites			1.00[b]			0.204[b]
Absent	4/6	2/6		5/6	1/6	
Present	23/41	18/41		20/41	21/41	

[a]: Pearson's χ^2 test (asymptotic significance, two-sided); [b]: Fisher's exact test

5).

We then analyzed how the expression levels of CLDN3 and CLDN4 in the ovarian serous adenocarcinomas correlate with patient prognosis by calculating survival curves according to the Kaplan-Meier method (Fig. 3a, Table 6). The patients that showed low expression (0, +1) of CLDN3 protein (38 cases) had a mean survival time of 75 months (95% confidence interval: 67-84). In contrast, the patients with high expression (+2, +3) of CLDN3 protein (46 cases)



B

Predictors	Univariate RR	Univariate *P*-value	Multivariate RR	Multivariate *P*-value
Age	1.746	0.280	1.780	0.265
Stage	23.19	0.365	771325	0.979
Grade	1.140	0.757	1.241	0.701
CLDN3	2.850	0.034*	3.203	0.019*

RR: relative risk
*Statistically significant.

Fig. 3. Survival analysis of 84 serous adenocarcinoma patients whose tumors show low or high CLDN3 expression. **a.** Kaplan-Meier survival curves based on CLDN3 expression intensity. Higher CLDN3 expression is a poor prognostic factor for survival (*P*=0.027). **b.** Univariate and multivariate analyses of the overall survival of ovarian serous adenocarcinoma patients by using Cox-proportional hazards regression.

Table 7. CLDN3 and CLDN4 expression patterns in various cancers that have been reported in the literature.

CLDN / Cancer	Microarray results in this study	Expression in other studies[a]	References
CLDN3			
Barrett's esophagus/ adenocarcinoma	Up*	Up	(Gyorffy et al., 2005; Montgomery et al., 2006)
Breast carcinoma	Not changed	Up	
		Not changed	(Kominsky et al., 2004; Tokes et al., 2005)
Colorectal carcinoma	Not changed	Up	(de Oliveira et al., 2005)
Epithelial ovarian caner	Up*	Up	(Hough et al., 2000; Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004; Lu et al., 2004; Santin et al., 2004; Zhu et al., 2006)
Gastric cancer	Up*	Up	(Resnick et al., 2005)
Prostate cancer	Up*	Persistent high level	(Long et al., 2001)
Uterine serous papillary cancer (endometrium)	Not tested	Up	(Santin et al., 2005)
CLDN4			
Barrett's esophagus/ adenocarcinoma	Not changed	Up	(Gyorffy et al., 2005; Montgomery et al., 2006)
Bile tract cancer		Up	(Lodi et al., 2006)
Breast carcinoma	Not changed	Up	
		not changed or down**	(Kominsky et al., 2004; Tokes et al., 2005)
Cervical carcinoma***	Not changed	Up	(Sobel et al., 2005)
Colorectal carcinoma	Not changed	Up	(de Oliveira et al., 2005)
Epidermis, squamous cell carcinoma	Not changed	Down	(Morita et al., 2004)
Epithelial ovarian cancer	Up*	Up	(Hough et al., 2000; Rangel et al., 2003; Hibbs et al., 2004; Santin et al., 2004; Zhu et al., 2006)
Gastric cancer	Up*	Up	(Resnick et al., 2005)
Intraductal papillary-mucinous tumors of pancreas	Up*	Up	(Terris et al., 2002; Sato et al., 2004)
Pancreatic cancer		Up	(Michl et al., 2001; Nichols et al., 2004)
Uterine serous papillary cancer (endometrium)	Not tested	Up	(Santin et al., 2005)

[a]: Expression was confirmed at protein level by immunohistochemical analysis; *: Up- or down-regulated by 2 fold compared to normal tissues; **: Down-regulation in ductal carcinoma grade 1, special types of breast carcinoma (mucinous, papillary, tubular), and apocrine metaplasia; ***: Includes cervical intraepithelial neoplasia grade 1, 2, and 3, carcinoma *in situ*, and invasive squamous cell carcinoma

had a survival time of 56 months (95% confidence interval: 46-66). Thus, high expression of CLDN3 correlates strongly with poor 3-year overall survival (P=0.027, Table 6). Univariate and multivariate analysis using the Cox proportional hazards model were also performed to assess the independent predictive value of high CLDN3 expression. The classic prognostic variables, namely, patient age at diagnosis, stage, and grade, were also included in the model. High CLDN3 expression remained an independent prognostic variable after both the univariate and multivariate analyses (univariate, P=0.034; multivariate, P=0.019, Fig. 3b). In contrast, CLDN4 expression levels did not correlate statistically significantly with patient survival (P=0.474, Table 6). In conclusion, Kaplan-Meier analysis and the Cox regression model demonstrate that patients with high CLDN3 expression have substantially shorter survival.

Discussion

Several human cancers have been found to aberrantly up- or down-regulate the expression of CLDNs, which are the major transmembrane proteins that form tight junction. In particular, CLDN3 (Hough et al., 2000; Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004; Lu et al., 2004; Santin et al., 2004; Zhu et al., 2006) and CLDN4 (Hough et al., 2000; Rangel et al., 2003; Hibbs et al., 2004; Santin et al., 2004; Zhu et al., 2006) have been found to be overexpressed in ovarian cancers. In addition, elevated expression of CLDN4 has been observed in pancreatic cancer (Michl et al., 2001; Nichols et al., 2004), while prostate cancer is associated with high CLDN3 expression (Long et al., 2001). The published data on the expression of CLDN3 and CLDN4 in several types of cancers are summarized in Table 7.

Our microarray data also showed that *CLDN3* expression is elevated in several other epithelial cancers, namely, those of the esophagus, prostate and stomach. This is consistent with previous works indicating that CLDN3 is up-regulated in esophageal adenocarcinoma (Gyorffy et al., 2005) and gastric adenocarinomas (Resnick et al., 2005). The up-regulation of *CLDN3* in cancers of the esophagus, prostate and stomach suggests that CLDN3 may also be a useful diagnostic biomarker or therapeutic target for these cancers. With regard to *CLDN4*, our microarray data showed up-regulated expression in pancreatic cancers, which is consistent with what has been observed previously (Michl et al., 2001; Terris et al., 2002; Nichols et al., 2004; Sato et al., 2004). Moreover, CLDN4 up-regulation in gastric adenocarinomas has also been reported in a previous study (Resnick et al., 2005) in accordance with our data. Resnick et al (Resnick et al., 2005) have shown that CLDN4 expression was increased in gastric cancers in comparison to the normal gastric epithelium and increased CLDN4 expression was associated with decreased survival in Cox multivariate analysis.

In the present study, we confirmed that ovarian tumors show marked up-regulation of CLDN3 and CLDN4 at both the protein and transcript levels. Moreover, our immunohistochemical analysis of the CLDN3 and CLDN4 expression levels in normal ovarian tissue and benign, borderline and malignant serous ovarian tumors revealed that many of the serous adenocarcinoma cases were positive for CLDN3 and CLDN4 (81% and 85.7%, respectively) and showed significantly more intense staining than the borderline tumors. The borderline tumors in turn expressed the two claudins at higher levels and more frequently than the cystadenomas, which showed no or only weak staining at the membrane of the tumor cells. Normal ovarian tissue did not express CLDN3 and CLDN4. These results confirmed the previous finding (Zhu et al., 2006) that CLDN3 and CLDN4 were significantly increased in ovarian adenocarcinomas compared to benign and borderline tumors and support those of a previous study (Rangel et al., 2003) that suggest the expression of these proteins may be related to malignancy. However, the mechanism of CLDN3 and CLDN4 up-regulation and the biological role it plays in ovarian carcinogenesis remains unclear. It may be that these proteins are expressed at higher levels by malignant cells to compensate for the loss of cell adhesion that occurs in cancer progression and which disrupts the tight junctions (Heinzelmann-Schwarz et al., 2004). Alternatively, or in addition, the increased expression of these proteins may be a downstream effect of the intracellular signaling that is associated with tumor progression (Christofori, 2003; Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004). Supporting this notion is that the transforming growth factor ß (TGFß) and Ras/Raf/MAP kinase pathways have been reported to inversely regulate CLDN4 expression in pancreatic cancer cells (Michl et al., 2003). In addition, claudins modulate the activation of matrix metalloproteinase-2 (MMP-2) (Miyamori et al., 2001) and the expression of CLDN3 and CLDN4 in ovarian epithelial cells is associated with increased MMP-2 activity (Agarwal et al., 2005). Thus, CLDN up-regulation could promote ovarian tumor progression.

High CLDN3 expression in serous adenocarcinoma was significantly associated with shorter patient survival (P=0.027; Table 6) but did not correlate significantly with patient age, tumor grade, advanced stage or the presence of ascites (Table 5). Multivariable Cox regression analysis also indicated that the level of CLDN3 expression is an independent factor for predicting the disease outcome. In contrast, the CLDN4 expression levels did not correlate significantly with either the survival rate or clinicopathological features of the patients, although intense CLDN4 expression did tend to occur more frequently in higher grade tumors (P=0.068; Table 5). This is the first report that reveals that high CLDN3 expression in malignant ovarian tumors is associated with poor prognosis. This suggests that CLDN3 may be useful as a prognostic indicator in ovarian cancer. Notably, our observations seem at first glance to contrast with those from the study of

Heinzelmann-Schwarz et al. (2004), which found poor ovarian cancer patient outcome tended to be associated with low CLDN expression. However, Heinzelmann-Schwarz et al. (2004) only analyzed the association between patient survival and the membrane expression of CLDN3. In contrast, we analyzed the overall expression, including cytoplasmic staining, as we found CLDN3 and CLDN4 can be expressed in malignant ovarian tumors both at the membrane and in the cytoplasm. This is consistent with findings in other reports (Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004). The cytoplasmic expression of CLDN3 and CLDN4 is likely to be the predominant expression pattern in malignant ovarian tumors, with fewer cases showing membrane expression. In fact, the cytoplasmic overexpression of various membrane proteins reflects membranous overexpression in malignant tumors.

That poor patient survival was only associated with CLDN3 expression and not with CLDN4 expression as well was unexpected. Thus, while increased CLDN3 expression seems to be involved in patient survival, high expression of CLDN4 does not contribute to disease outcome. The expression of both proteins in ovarian epithelial cells has been reported to increase tumor cell invasion, which suggests that these proteins promote ovarian tumorigenesis and metastasis (Agarwal et al., 2005). On the other hand, CLDN4 overexpression in pancreatic cancer has been found to be associated with decreased invasiveness *in vitro* and *in vivo* (Michl et al., 2003). This suggests that the CLDNs may have different functions depending on the tissue in which they are expressed. However, these disparate results may also reflect the well-known fact that *in vitro* observations showing specific proteins increase invasion or metastatic potential often extrapolate poorly to the clinical situation. Further investigation is required to elucidate the mechanism by which CLDN3 overexpression could cause or is associated with poor outcome.

That particular tumors show up-regulated CLDN3 and CLDN4 expression relative to the surrounding tissue, including malignant ovarian tumors, suggests that either or both of these molecules could be useful for diagnosing these cancers or as targets of novel therapeutic strategies including antibody-mediated cancer therapy. Further supporting this possibility is that chicken polyclonal antibodies against peptides containing two extracellular loops of CLDN3 and CLDN4 have been shown to bind CLDNs on the cell surface (Offner et al., 2005).

In conclusion, we have shown here that CLDN3 and CLDN4 are frequently expressed in ovarian serous adenocarcinoma at high levels compared to adenoma and borderline tumors. High CLDN3 expression is significantly associated with the poor survival of serous adenocarcinoma patients and thus could be used to predict the disease prognosis. Although how CLDN3 contributes to the generation, progression and outcome of ovarian tumors remains unclear, our findings also suggest that CLDN3 may be a promising target for the treatment of ovarian cancer by specific therapeutic monoclonal antibodies.

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