Claudin-9 is a novel prognostic biomarker for endometrial cancer

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Abstract. The tight-junction protein claudin-9 (CLDN9) is barely distributed in normal adult tissues but is ectopically expressed in various cancer types. Although multiple databases indicated upregulation of CLDN9 in endometrial cancers at the mRNA level, its protein expression and biological roles remain obscure. In the present study, the prognostic significance of CLDN9 expression in endometrial cancer was evaluated by immunohistochemical staining and semi-quantification using formalin-fixed paraffin-embedded specimens obtained from 248 endometrial carcinoma cases. A total of 43 cases (17.3%) had high CLDN9 expression, whereas 205 cases (82.7%) exhibited low CLDN9 expression. The 5-year disease-specific survival rates in the high and low CLDN9 expression groups were 62.8 and 87.8% (P<0.001), respectively. In addition, multivariate analysis revealed that high CLDN9 expression was an independent prognostic factor (hazard ratio, 4.99; 95% CI, 1.96-12.70; P<0.001). Furthermore, CLDN9 expression was significantly correlated with the expression of CLDN6 (P<0.001), which is the closest CLDN member to CLDN9 and a poor prognostic factor for endometrial carcinoma. The 5-year disease-specific survival rate of cases with CLDN6-high/CLDN9-high, CLDN6-high/CLDN9-low and CLDN6-low/CLDN9-high status was 30.0, 37.5 and 72.7%, respectively, whereas that of CLDN6-low/CLDN9-low was 89.8% (P=0.004). In conclusion, aberrant CLDN9 expression is a predictor of poor prognosis for endometrial cancer and may be utilized in combination with CLDN6 to achieve higher sensitivity.

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Abbreviations: CI, confidence interval; CLDN, claudin; DFS, disease-free survival; DSS, disease-specific survival; ERα, estrogen receptor α; FFPE, formalin-fixed paraffin-embedded; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; IRS, immunoreactive score; LVSI, lymphovascular space involvement; mAb, monoclonal antibody; RARγ, retinoic acid receptor γ; SI, signal intensity; SFK, Src-family kinase; SD, standard deviation; YAP, Yes-associated protein

Key words: biomarker, claudin, endometrial cancer, gynecological cancer, tight junction

Introduction

Endometrial cancer is the most common gynecological malignancy in developed countries and is increasing in both incidence and associated mortality (1-3). Most patients with endometrial cancer are diagnosed in the early stages and exhibit a favorable 5-year relative survival rate (95%), if the appropriate surgical procedure is provided (4). On the other hand, patients with regional spread and distant metastasis beyond the uterus have a poor 5-year relative survival rate (69 and 17%, respectively) (4). Although radiation therapy, hormonal therapy and chemotherapy with cytotoxic agents have been used for advanced and recurrent endometrial cancer, the effectiveness of these therapies is limited. It is reported that the efficacy of the first-line chemotherapy is 30-57% and that the median progression-free survival is <1 year (5,6). Of note, the effectiveness of second-line chemotherapy is more limited (6). Therefore, novel biomarkers are required to select patients with endometrial cancer with poor prognosis at the time of biopsy or initial surgery.

Claudins (CLDNs) are tetraspan proteins with a short cytoplasmic N-terminus, two extracellular loops and a C-terminal cytoplasmic domain (7). CLDNs form tight junctions and are composed of >20 subtypes in humans. They function as a physical barrier or gate of small molecules (8-11) and as signaling platforms to coordinate diverse cellular behaviors (10-12). CLDNs are expressed in distinct patterns in different tissues and cells. Furthermore, CLDNs are useful cancer biomarkers, as they are frequently upregulated and are associated with malignant traits of cancers, such as invasion, migration, metastasis and chemoresistance (13,14).

Recent studies by our group demonstrated that aberrant CLDN6 expression is a biomarker for poor prognosis in endometrial cancer, and that abnormal CLDN6 signaling enhances malignant behaviors by AKT-dependent phosphorylation of estrogen receptor-α (ERα) through the Src-family kinases (SFK)/PI3K/AKT signaling pathway (15-17). Among CLDN subtypes, CLDN9 is the closest member to CLDN6 (18) and

their genes are located adjacent to each other on the human genome. While several experimental studies determined that CLDN9 overexpression promotes cancer malignancy (19,20), endogenous expression of CLDN9 in cancer tissues has not been indicated at the protein level and its clinicopathological significance remains obscure due to the unavailability of selective antibodies.

In the present study, it was demonstrated that high CLDN9 expression predicts poor prognosis in patients with endometrial cancer using a newly established specific monoclonal antibody (mAb) (21). In addition, it was indicated that the combination of CLDN9 and CLDN6 is beneficial for predicting poor outcome in endometrial cancer.

Materials and methods

Cell culture, expression vectors, and transfection. 293T and Ishikawa cells were gifted by Professor Suzuki, Fukushima Medical University (Fukushima, Japan) and Professor Yamada, Wakayama Medical University (Wakayama, Japan), respectively. They were grown in Dulbecco's Modified Eagle Medium (Sigma-Aldrich; Merck KGaA) with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 1% penicillin-streptomycin-amphotericin B suspension (cat. no. 161-23181; Fujifilm).

The protein-coding regions of the human *CLDN1*, *CLDN4*, *CLDN5*, *CLDN6* and *CLDN9* genes were amplified from complementary or genomic DNA extracted from 293T cells with the PrimeSTAR GXL (cat. no. R050A; Clontech) PCR kit following the manufacturer's protocol. They were cloned into the *Not*I/*Bam*HI site of the CSII-EF-MCS-IRES2-Venus plasmid (cat. no. RDB04384; RIKEN BioResource Center). For transient expression of the target genes (*CLDN1*, *CLDN4*, *CLDN5*, *CLDN6* and *CLDN9*), $5x10^6$ 293T cells were transfected with 10 μg of the indicated vectors using 30 μg of Polyethyleneimine Max (PEI Max; Cosmo Bio Co., Ltd.) eight hours after passage. The pTagRFP-laminB1 vector (cat. no. FP370; Evrogen) was co-transfected with the CLDN9-expression vector to visualize the nuclei in successfully transfected cells. The transfection efficiency was evaluated by Venus expression with a fluorescence microscope (IX71; Olympus Corporation).

Antibodies. Rat monoclonal antibodies (mAbs) against the cytoplasmic tail of human CLDN6 and CLDN9 were generated using an iliac lymph node method, as previously described (16,21). Clone #15 for CLDN6 and clone 1D1 for CLDN9 were used in the present study. A mouse anti-p53 mAb (cat. no. OP43; clone Ab-6; Calbiochem; Merck KGaA) was used for evaluation of p53 expression.

Immunoblotting. Total cell lysates were prepared using CelLytic™ MT Cell Lysis Reagent (cat. no. C3228; Sigma-Aldrich; Merck KGaA). The protein concentration of the total cell lysates was measured by Pierce™ BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) and 0.5 μg was loaded per lane for one-dimensional SDS-PAGE (12.5%). Subsequently, the protein bands were electrophoretically transferred onto an Immobilon membrane (MilliporeSigma). The membrane was blocked with PBS containing 4% skimmed milk (Morinaga) for 30 min and treated with the supernatant of the rat anti-CLDN9 hybridomaprima for 60 min at room temperature. After washing with PBS three times, the membrane was incubated with 1:2,000-fold diluted HRP-conjugated anti-rat IgG (cat. no. NA935V; GE Healthcare). The signals were detected by chemiluminescence (cat. no. WSE-7110EzWestLumi One; ATTO).

Immunofluorescence. Ishikawa cells ($5.0x10^5$) were grown on coverslips coated with Cellmatrix Type I-A (Nitta Gelatin). After 48 h, the samples were fixed in ice-cold ethanol for 10 min. After washing with PBS, they were preincubated in PBS containing 5% skimmed milk (Morinaga) at room temperature. They were subsequently incubated overnight at 4˚C with the supernatant of anti-CLDN9 hybridoma, followed by washing with PBS three times and incubation with the secondary antibody, 1:400 diluted Alexa Fluor 488 AffiniPure Donkey Anti-Rat IgG antibody (cat. no. 712-545-150; Jackson ImmunoResearch) for 60 min at room temperature. After washing with PBS, the specimens were mounted with Fluoro-Gel II with DAPI (cat. no. 17985-51; Electron Microscopy Sciences). The samples were observed and images were acquired with a fluorescent microscope (IX71; Olympus Corporation).

Tissue collection, immunostaining and histological analysis. Formalin-fixed paraffin-embedded (FFPE) tissue sections were obtained from 248 patients with endometrial cancer [age, mean ± standard deviation (SD) of 58.2±11.5 years; range, 30-83 years] who underwent hysterectomy and bilateral-salpingo-oophorectomy and/or retroperitoneal lymphadenectomy between January 2003 and March 2015 at Fukushima Medical University Hospital (FMUH; Fukushima, Japan). The subjects were limited to patients who were confirmed to have at least 5-year outcomes and those who had died due to endometrial cancer and metastasis. Detailed information, including postoperative pathology diagnosis reports, age, stage [International Federation of Gynecology and Obstetrics (FIGO) 2008] (22), histological type, histological grade, Bokhman subtype (23), lymphovascular space involvement (LVSI), lymph node metastasis, distant metastasis, recurrence status, disease-specific survival (DSS) and disease-free survival (DFS), was obtained. The staging of patients encountered between January 2003 and December 2007 was modified in accordance with the FIGO 2008 system. Distant metastasis was judged by diagnostic imaging. Normal adult tissues, including the pituitary gland, cerebrum, liver, lung, and kidney, were collected from autopsy specimens dissected at FMUH between January 2013 and December 2014. Three to four specimens among six cases (a 29-year-old male, 42-year-old female, 51-year-old female, 57-year-old male, 65-year-old male and a 71-year-old female) were examined and a representative image was presented for each organ.

For immunostaining, the FFPE tissue blocks were sliced into 5-μm-thick sections, deparaffinized with xylene and rehydrated using a graded series of ethanol. The sections were then immersed in 0.3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity.

Antigen retrieval was performed by incubating the sections in boiling 10 mM citric acid buffer (pH 5.0) using a microwave for 10 min. After blocking with 5% skimmed milk (Morinaga) at room temperature for 30 min, the sections were incubated overnight at 4˚C with supernatants of the rat anti-CLDN6 or CLDN9 hybridoma. After washing with PBS, a secondary antibody reaction was performed by using the Histofine Simple Stain mouse MAX-PO kit (cat. no. 414311; Nichirei Biosciences, Inc.) for 3',3'-diaminobenzidine as a chromogen according to the manufacturer's instructions. Immunostaining for CLDN6 was performed as previously described (16). p53 was stained following the manufacturer's protocol.

Immunostaining results were interpreted by two independent pathologists and one gynecologist using a semiquantitative scoring system. The immunostaining reactions were evaluated according to signal intensity (SI; 0, negative; 1, weak; 2, moderate; 3, strong). The receiver operating characteristic (ROC) curve was plotted and analyzed (Fig. S1) to determine the optimal cut-off values of the SI for CLDN9 expression. CLDN6 expression was assessed by the immunoreactive score (IRS) as described previously (16). p53 mutation was assessed following the most accepted criteria (24).

Statistical analysis. The χ^2 test was used to evaluate the relationship between CLDN9 expression and clinicopathological parameters such as age, stage, histological type, histological grade, Bokhman subtype, LVSI, lymph node metastasis, distant metastasis, DSS and DFS. Survival analysis was performed using the Kaplan-Meier method and differences between groups were analyzed using the log-rank test. Cox regression according to the univariate and multivariate model was used to identify predictors of survival. In addition, the expression of CLDN9 and CLDN6 was compared and statistical analysis was performed in a similar way. Two-tailed P-values of <0.05 were considered to indicate a statistically significant result. When comparing the disease-specific and disease-free survival among four groups, P<0.125 was used as the threshold for a statistically significant result to counteract the multiple comparisons problem by applying Bonferroni correction. All statistical analyses were performed using SPSS version 24.0 software (IBM Corporation).

Results

Verification of anti-CLDN9 mAb. First, the reactivity and specificity of the anti-CLDN9 mAb were tested against the C-terminal cytoplasmic region of human CLDN9 (Fig. 1A), which was recently established by our group (21). To this end, 293T cells were transiently transfected with individual CLDN expression vectors, followed by western blot and immunohistochemistry using whole-cell extracts and cell blocks, respectively. As presented in Fig. 1B and C, the anti-CLDN9 mAb appeared to specifically recognize CLDN9 but not CLDN1, CLDN4, CLDN5 or CLDN6, which are the four closest members to CLDN9 among the CLDN family. In addition, immunofluorescence staining using the anti-CLDN9 mAb revealed positive signals along cell-cell borders in the endometrial cancer cell line Ishikawa overexpressing CLDN9 (Fig. 1D). Furthermore, CLDN9 expression was detected in normal human pituitary gland (Fig. 1E), but not in cerebral, liver, lung or kidney tissue (Fig. S2).

Table I. Clinicopathological characteristics of patients with endometrial cancer (n=248).

Parameter	Value
Age, years	58.1±11.5 (30-83)
Stage	
I	182 (73.4)
II	6 (2.4)
III	40 (16.1)
IV	20 (8.1)
Histological type	
Endometrioid carcinoma	226 (91.1)
Grade 1	145 (58.5)
Grade 2	40 (16.1)
Grade 3	41 (16.5)
Serous carcinoma	7 (2.8)
Clear carcinoma	6 (2.4)
Mucinous carcinoma	2 (0.8)
Other	7 (2.8)

Values are expressed as n (%) or the mean ± standard deviation (range).

Differential expression of CLDN9 among endometrial cancer subjects. Next, the expression of CLDN9 was evaluated by immunohistochemistry in endometrial cancer tissues resected from 248 patients, whose demographic and clinicopathological characteristics are presented in Table I. CLDN9 was mainly distributed along the cell membranes of endometrial carcinoma cells, but it was diffusely localized in certain cases (Fig. 2). The expression of CLDN9 was semi-quantified by determining the SI, as the percentage of positive cells was not >10% in most cases (mean ± SD, 6.30±6.84%). The SI varied among the subjects, which was 3 in 18 subjects (7.3%), 2 in 25 (10.1%), 1 in 46 (18.5%) and 0 in 159 (64.1%) (Fig. S3). Based on the ROC analysis, the samples were divided into two groups: Low CLDN9 expression (SI <2) and high CLDN9 expression (SI ≥2; Fig. S1).

High CLDN9 expression is an independent poor prognostic marker for endometrial cancer. Kaplan-Meier plots revealed significantly shorter DSS and DFS in the high CLDN9 expression group than in the low expression group (Fig. 3A and B). The 5-year DSS rates in the low and high CLDN9 expression groups were 87.8 and 62.8%, and the DFS rates were 84.9 and 62.8%, respectively.

Among the clinicopathological factors, high CLDN9 expression was significantly associated with non-endometrioid histology (P=0.021) and lymph node metastasis (P=0.012; Table II). By contrast, younger age (P=0.370), stage III/IV (P=0.072), histological grade 3 (P=0.431), histological type II (which includes endometrioid carcinoma grade 3, serous carcinoma and clear cell carcinoma; P=0.101),

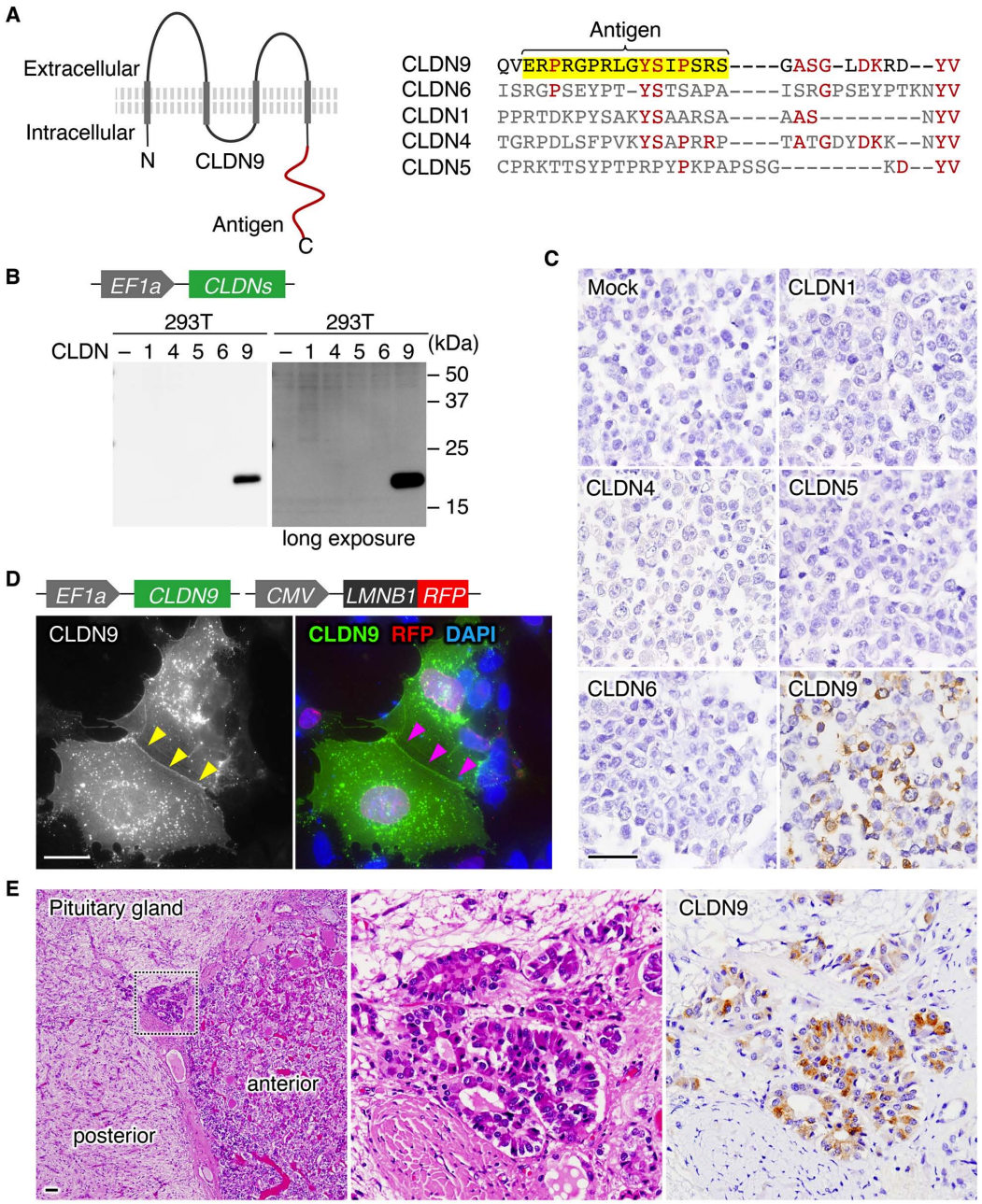

Figure 1. Establishment and characterization of a rat anti-human CLDN9 mAb. (A) Topology of CLDN9 and amino acid sequences of the C-terminal cytoplasmic domains of human CLDN9 and the corresponding regions of the closely related CLDNs. Conserved amino acids are displayed in red. The antigen region is highlighted in yellow. (B) Western blot and (C) immunohistochemical analyses indicating the specificity of the rat anti-human CLDN9 mAb. 293T cells were transfected with individual CLDN expression vectors and subjected to analyses (scale bar, 50 μm). (D) Localization of CLDN9 in Ishikawa cells co-transfected with CLDN9 and Lamin-RFP. Arrowheads indicate CLDN9-immunoreactive signals along a cell-cell boundary (scale bar, 20 μm). (E) Immunohistochemical staining of CLDN9 in the normal pituitary gland (scale bar, 200 μm). The square indicates the enlarged areas presented in the right panels. mAb, monoclonal antibody; CMV, cytomegalovirus promoter; LMNB1, Lamin B1; RFP, red fluorescent protein; CLDN, claudin; EF1a, human elongation factor 1-α promoter.

lymphovascular space involvement (LVSI; P=0.070) and distant metastasis (P=0.414) were not related to high CLDN9 expression.

In the univariate analysis, stage III/IV [hazard ratio (HR)=15.69, 95% confidence interval (CI) 7.47-32.96, P<0.001], endometrioid type [HR=0.35 (95% CI, 0.16-0.76), P=0.008], histological grade 3 [HR=4.02 (95% CI, 2.01-8.02), P<0.001], histological type II [HR=3.85 (95% CI, 2.02-7.35), P<0.001], LVSI [HR=8.99 (95% CI, 4.50-17.97), P<0.001], lymph node metastasis [HR=12.70 (95% CI, 6.37-25.32), P<0.001], distant metastasis [HR=14.25 (95% CI, 7.45-27.26), P<0.001] and high CLDN9 expression [HR=3.64 (95% CI, 1.94-6.81), P<0.001] were significant prognostic factors for DSS of patients with endometrial cancer (Table III). In addition, p53 abnormality was not associated with CLDN9 in histological type II cases (Table SI).

Subsequently, Cox multivariate analysis was performed to determine the independent predictors of survival. Among the variables analyzed, stage III/IV [HR=6.00 (95% CI, 1.94-18.56), P=0.002], LVSI [HR=3.34 (95% CI, 1.21-9.25),

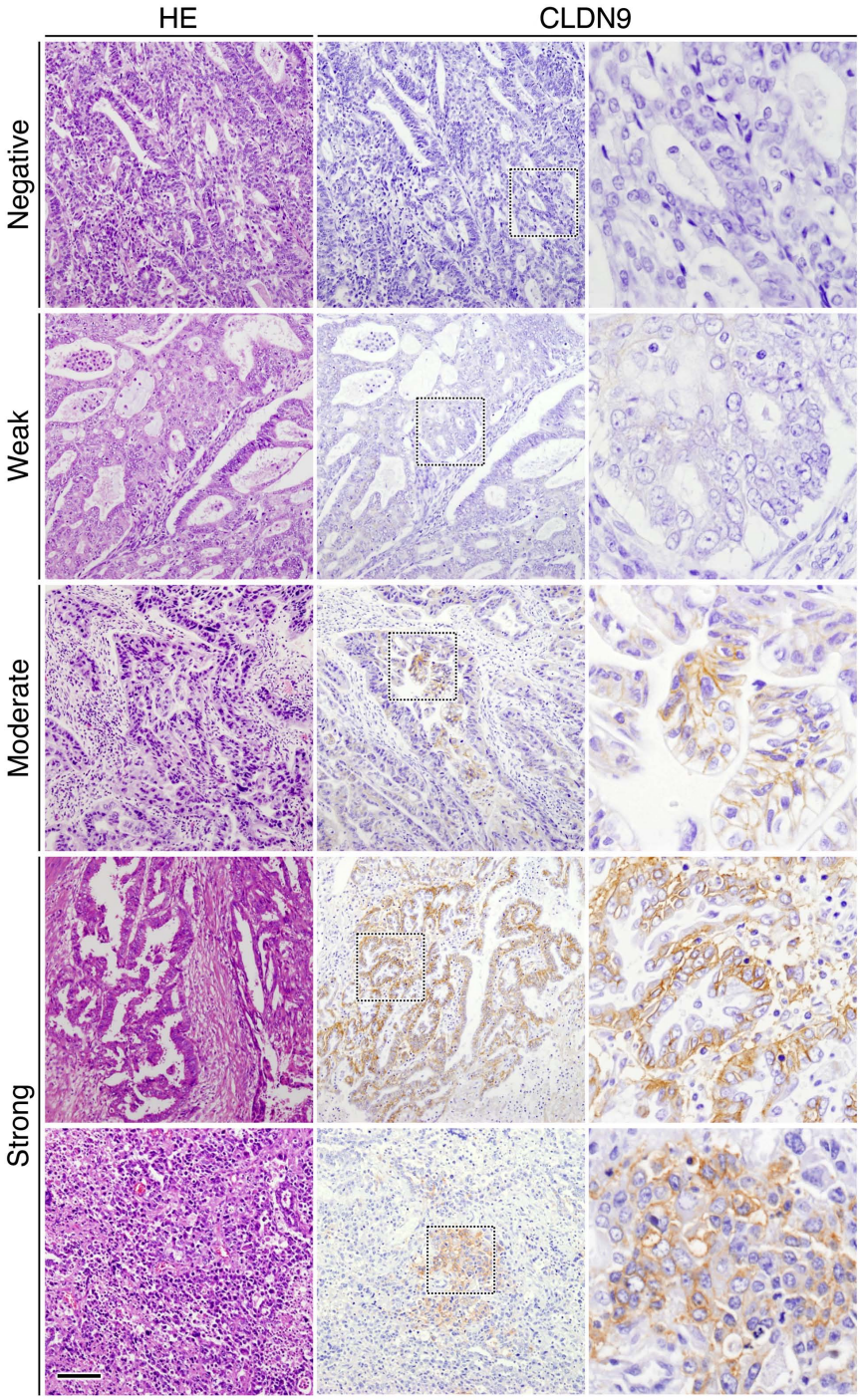

Figure 2. Immunohistochemical staining of CLDN9 in endometrial cancer tissues. Representative images show negative/weak/moderate/strong signal intensity of CLDN9 expression in endometrial cancer tissues (scale bar, 100 μm). The squares indicate the enlarged areas presented in the right panels. HE, hematoxylin-eosin; CLDN, claudin.

P=0.020], distant metastasis [HR=6.74 (95% CI, 2.32-19.57), P<0.001] and high CLDN9 expression [HR=4.99 (95% CI, 1.96-12.70), P<0.001] were independent prognostic factors for DSS of patients with endometrial cancer (Table IV). By contrast, older age, endometrioid type, histological grade 3, histological type II and lymph node metastasis were no independent prognostic variables for them.

CLDN9 expression correlates with CLDN6 expression in endometrial cancer. CLDN9 largely shares its alignment with CLDN6 (18) and its genetic locus is adjacent to CLDN6 (Fig. 4A and B). Furthermore, none of the two genes are primarily expressed in adult tissues, except for the inner ear, olfactory epithelium and anterior pituitary glands (21,25), suggesting that they are regulated by similar mechanisms. Thus, it was hypothesized that upregulation of CLDN6 and CLDN9 may be mutually correlated in endometrial cancer tissues. To compare the expression of CLDN9 and CLDN6, the 248 patients with endometrial cancer were classified into four groups according to the expression levels of CLDN9

Table II. Relationship between CLDN9 expression and clinicopathological factors in patients with endometrial cancer (n=248).

Parameter	Total	CLDN9-high (n=43)	CLDN9-low (n=205)	P-value
Age, years				0.370
<50	53 (21.4)	7 (16.3)	46 (22.4)	
≥50	195 (78.6)	36 (83.7)	159 (77.6)	
Stage				0.072
I/II	188 (75.8)	28 (65.1)	160 (78.0)	
III/IV	60 (24.2)	15 (34.9)	45 (22.0)	
Histological type				0.021[a]
Endometrioid	226 (91.1)	35 (81.4)	191 (93.1)	
Serous	7 (2.8)	1 (2.3)	6 (2.9)	
Clear	6 (2.4)	4 (9.3)	2 (1.0)	
Mucinous	2 (0.8)	0 (0.0)	2 (1.0)	
Others	7 (2.8)	3 (7.0)	4 (2.0)	
Histological grade				0.431
1/2	185 (74.6)	27 (62.8)	158 (77.1)	
3	41 (16.5)	8 (18.6)	33 (16.1)	
Other	22 (8.9)	8 (18.6)	14 (6.8)	
Histological classification				0.101
Type I	185 (74.6)	27 (62.8)	158 (77.1)	
Type II	54 (21.8)	13 (30.2)	41 (20.0)	
Other	9 (3.6)	3 (7.0)	6 (2.9)	
LVSI				0.070
(-)	178 (71.8)	26 (60.5)	152 (74.1)	
(+)	70 (28.2)	17 (39.5)	53 (25.9)	
Nodal stage				0.012
N0	203 (81.9)	30 (69.7)	173 (84.4)	
N1	34 (13.7)	11 (25.6)	23 (11.2)	
Unknown	11 (4.4)	2 (4.7)	9 (4.4)	
Metastasis stage				0.414
M0	229 (92.3)	41 (95.3)	188 (91.7)	
M1	19 (7.7)	2 (4.6)	17 (8.3)	

[a]Endometrioid vs. non-endometrioid. Values are expressed as n (%). LVSI, lymphovascular space involvement; CLDN, claudin.

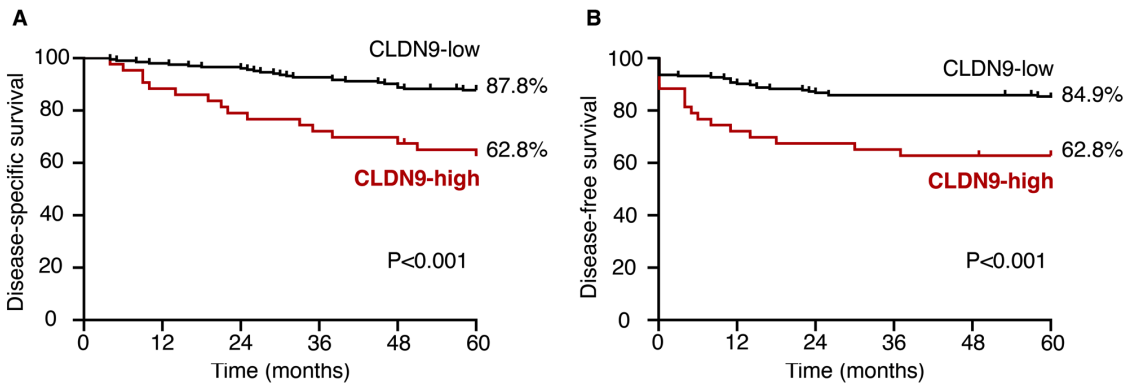

Figure 3. Association of high CLDN9 expression with poor outcomes in patients with endometrial cancer. Kaplan-Meier curves for (A) disease-specific and (B) disease-free survival for high and low expression of CLDN9 in endometrial cancer subjects. CLDN, claudin.

and CLDN6 (Fig. 4C). In the high CLDN6 expression group (18 cases), 10 (55.6%) and 8 (44.4%) had high and low CLDN9 expression, respectively, whereas in the low CLDN6 expression group (n=230), 33 (14.3%) and 197 (85.7%) cases

Table III. Univariate analysis of disease-specific survival in patients with endometrial cancer.

Variable	HR	95% CI	P-value
Age ≥50 years	2.64	0.94-7.39	0.066
Stage III/IV	15.69	7.47-32.96	<0.001
Endometrioid type	0.35	0.16-0.76	0.008
Histological grade 3	4.02	2.01-8.02	<0.001
Type II	3.85	2.02-7.35	<0.001
LVSI (+)	8.99	4.50-17.97	<0.001
N1	12.70	6.37-25.32	<0.001
M1	14.25	7.45-27.26	<0.001
CLDN9-high	3.64	1.94-6.81	<0.001

HR, hazard ratio; CI, confidence interval; LVSI, lymphovascular space involvement; N1, positive for lymph node metastasis; M1, positive for distant metastasis.

Table IV. Multivariate analysis of disease-specific survival in patients with endometrial cancer.

Variable	HR	95% CI	P-value
Stage III/IV	6.00	1.94-18.56	0.002
LVSI (+)	3.34	1.21-9.25	0.020
M1	6.74	2.32-19.57	<0.001
CLDN9-high	4.99	1.96-12.70	<0.001

HR, hazard ratio; CI, confidence interval; LVSI, lymphovascular space involvement; M1, positive for distant metastasis; CLDN, claudin.

Table V. Association between CLDN6 and CLDN9 expression in patients with endometrial cancer.

	CLDN9		
CLDN6	Low	High	P-value
Low	197 (79.4)	33 (13.3)	<0.001
High	8 (3.2)	10 (4.0)	

Values are expressed as n (%). CLDN, claudin.

had high and low CLDN9 expression, respectively. It was determined that high expression of CLDN9 in endometrial cancer cases was significantly associated with high CLDN6 expression (P<0.001; Table V), although CLDN6 and CLDN9 were principally expressed in different tumor cells within endometrial cancer tissues (Fig. 4C, panels of CLDN6-high/CLDN9-high). Of note, unlike CLDN9, p53 abnormality was positively associated with CLDN6 in histological type II cases (Table SII).

Table VI. Cox univariate analysis of disease-specific survival in patients with endometrial cancer with low CLDN6 expression.

Variable	HR	95% CI	P-value
Age ≥50 years	1.90	0.66-5.45	0.201
Stage III/IV	18.42	7.49-45.38	<0.001
Endometrioid type	0.33	0.13-0.86	0.023
Histological grade 3	4.06	1.80-9.14	<0.001
Type II	3.82	1.77-8.26	<0.001
LVSI (+)	9.70	4.29-21.93	<0.001
N1	16.47	7.08-38.29	<0.001
M1	16.51	7.71-35.37	<0.001
CLDN9-high	2.98	1.36-6.55	0.007

HR, hazard ratio; CI, confidence interval; LVSI, lymphovascular space involvement; N1, positive for lymph node metastasis; M1, positive for distant metastasis; CLDN, claudin.

Combination of CLDN6 and CLDN9 expression is advantageous in identifying patients with endometrial cancer with poor prognosis. Kaplan-Meier plots revealed that DSS and DFS in the high CLDN6 expression group were significantly lower than those in the low CLDN6 expression group regardless of the CLDN9 expression levels (Fig. 5). Furthermore, there were significant differences in both DSS and DFS between the subjects with CLDN6-low/CLDN9-low and those with CLDN6-low/CLDN9-high. Their 5-year DSS rate was 89.8% in the former and 72.7% in the latter group.

In the univariate analysis specifically for patients with endometrial cancer with low CLDN6 expression, stage III/IV [HR=18.42 (95% CI, 7.49-45.38), P<0.001], endometrioid type [HR=0.33 (95% CI, 0.13-0.86), P=0.023], histological grade 3 [HR=4.06 (95% CI, 1.80-9.14), P<0.001], histological type II [HR=3.82 (95% CI, 1.77-8.26), P<0.001], LVSI [HR=9.70 (95% CI, 4.29-21.93), P<0.001], lymph node metastasis [HR=16.47 (95% CI, 7.08-38.29), P<0.001], distant metastasis [HR=16.51 (95% CI, 7.71-35.37), P<0.001] and high CLDN9 expression [HR=2.98 (95% CI, 1.36-6.55), P=0.007] were significant prognostic variables for DSS. However, older age was not a prognostic factor for endometrial cancer with low CLDN6 expression (Table VI).

Discussion

The immunohistochemical analysis of the present study using the specific anti-CLDN9 mAb revealed that CLDN9 is differentially expressed in endometrial cancer tissues. For instance, the SI varied among the subjects with endometrial cancer and 43 in the 248 cases (17.3%) had high CLDN9 expression. In addition, there was a variation in the percentage of CLDN9-positive cells among the cases. Of note, similar heterogeneity was also observed in CLDN6 expression in endometrial cancer tissues (16).

Among the CLDN family, CLDN1 and CLDN2 are prone to be expressed in histological type I and II endometrial cancers, respectively, although the prognostic value remains to be determined (26,27). The present study demonstrated

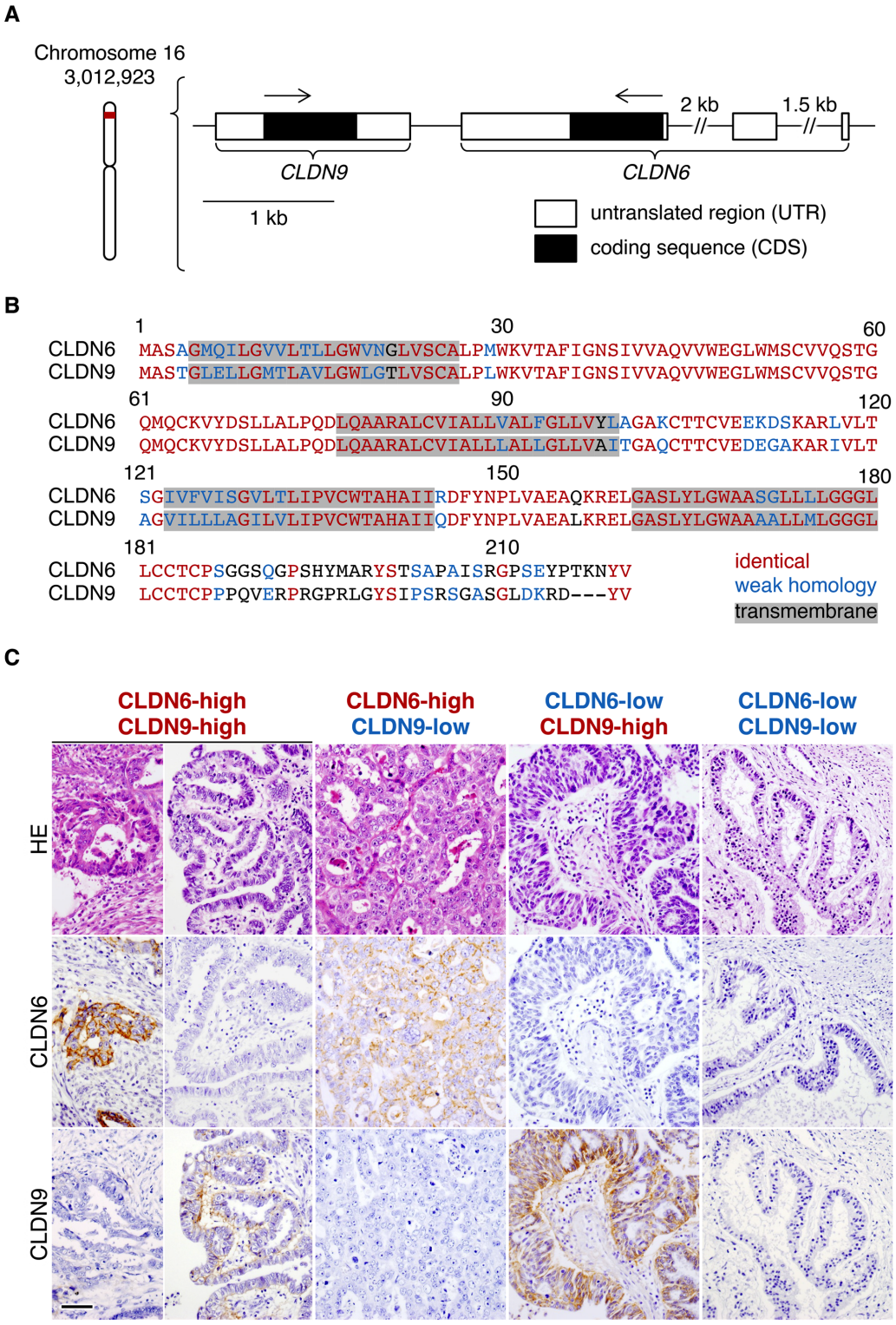

Figure 4. Association of CLDN6/9 and their expression in endometrial cancer tissues. (A) The location of *CLDN9* and *CLDN6* genes in the human genome. Their genes are located on chromosome 16 at p13.3 3014712-3020071 and p 13.3 3012923-3014505, respectively. (B) Sequence homology between human CLDN6 and CLDN9 protein. (C) Immunohistochemical staining of CLDN6 and CLDN9 in endometrial cancer tissues. A total of four representative patterns are presented with different expression patterns of CLDN6 and CLDN9 (scale bar, 100 μm). HE, hematoxylin-eosin; CLDN, claudin.

that high CLDN9 expression represents a poor prognostic marker for endometrial cancer. This conclusion was drawn from the following results: i) The DSS and DFS in the high CLDN9 expression group of endometrial cancer subjects were significantly lower compared with those in the low CLDN9 expression group; ii) high CLDN9 expression was significantly associated with adverse clinicopathological factors, including non-endometrioid type and lymph node metastasis; iii) univariate analysis indicated that high CLDN9 expression was a significant prognostic factor (HR=3.64); and iv) upon multivariate analysis, high CLDN9 expression appeared to be an independent prognostic factor for DSS

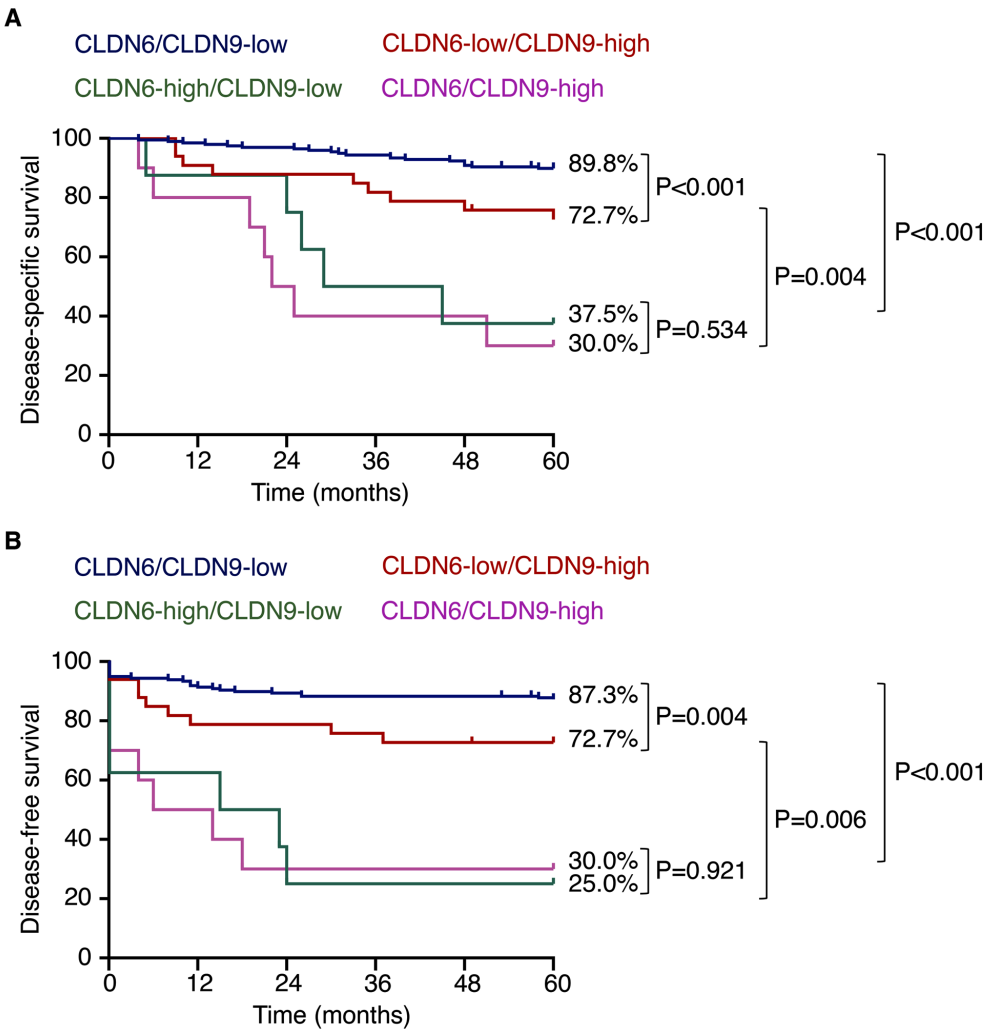

Figure 5. High CLDN9 expression is associated with poor prognosis in patients with endometrial cancer in the low CLDN6 expression group. Kaplan-Meier curves for (A) disease-specific and (B) disease-free survival are provided. CLDN, claudin.

of the endometrial cancer subjects (HR=4.99). Thus, not only aberrant CLDN6 expression (16) but also high CLDN9 expression predicts a poor outcome for endometrial cancer. The high CLDN9 expression significantly correlated with the high CLDN6 expression in endometrial cancer, further supporting this conclusion.

Recently, endometrial cancer was classified into four molecular groups based on genetic characteristics: Ultramutated, hypermutated, copy-number low and copy-number high (28,29). More recently, simplified classifications have also been proposed for broader clinical applications (30-32). However, these genetic classifications are not applicable for all cases mainly due to insufficient tumor cell content. In addition, protein expression does not necessarily correlate with gene mutations or mRNA expression (33-35). Therefore, a classification based on protein signals in FFPE specimens, which directly reflects molecular characteristics, is valuable. In the present study, it was demonstrated that the protein expression of CLDN9 and CLDN6 in FFPE specimens reflected the prognosis of patients with endometrial cancer. In more detail, the CLDN6-high group of patients with endometrial cancer exhibited markedly lower DSS and DFS irrespective of the expression levels of CLDN9. In addition, CLDN6-low/CLDN9-high cases of endometrial cancer had significantly decreased DSS and DFS compared with CLDN6-low/CLDN9-low cases. Furthermore, while the proportion of CLDN6-high cases was <10%, which was also in line with a previous study (16), nearly a quarter of cases had high expression of either CLDN6 or CLDN9. Thus, classification depending on the expression of CLDN6 and CLDN9 (CLDN6-high, CLDN6-low/CLDN9-high and CLDN6-low/CLDN9-low) would be beneficial in the aspects of easy use and broad clinical applications. In addition, abnormal expression of p53 was not associated with CLDN9-high, indicating that CLDN9-high predicts poor prognosis regardless of p53 mutation.

It remains elusive by which mechanisms high CLDN9 expression results in poor outcome for patients with endometrial cancer. However, it was previously reported that aberrant CLDN6 expression promotes endometrial cancer progression *in vitro* and *in vivo* via hijacking the CLDN6/SFK/PI3K/AKT/ERα pathway (12,17). For instance, abnormal CLDN6 signaling stimulates not only cell proliferation but also collective cell migration in the leading front of endometrial cancer cells. Since CLDN6 recruits and activates SFKs in second extracellular domain-dependent and Y196/200-dependent manners, both of which are highly

conserved in CLDN9 (12), CLDN9 may similarly act as a cancer promoter. Alternatively, since CLDN2 activates Yes-associated protein (YAP) and stimulates self-renewal of human colorectal cancer stem-like cells (36), CLDN9 may also promote YAP activity to advance endometrial cancer progression.

It is premature to discuss how the expression of CLDN6 and CLDN9 is upregulated in endometrial cancer cells. However, a previous study by our group demonstrated that the CLDN6 signaling ligand independently activates the expression of endogenous *CLDN6* gene in F9 embryonal carcinoma cells (15). Therefore, the positive loop of the CLDN6/SFK/AKT/RARγ cascade may contribute to inducing and maintaining CLDN6 expression in endometrial cancer cells. As the second extracellular domain and Y200 in CLDN6 are conserved in CLDN9 as described above, the expression of CLDN9 may be upregulated by a similar positive feedback mechanism.

As CLDNs are distributed on the cell surface, they are good druggable targets of antibody therapy. Indeed, antibody therapy against CLDN6, including CAR-T and antibody-drug conjugates, exhibits efficiency in solid tumors (37-39). Since CLDN9 has almost identical extracellular domains to those of CLDN6, it appears feasible to obtain a bispecific antibody targeting CLDN6 and CLDN9 together. Furthermore, CLDN9 and CLDN6 are rarely expressed in normal adult tissues, excluding the cochlea (25), olfactory epithelia and anterior pituitary glands (21), implying that the antibody therapy may be less toxic to non-tumor tissues. Therefore, taken together with the present findings that CLDN6 and CLDN9 are poor prognostic factors, antibody therapy targeting CLDN9 and CLDN6 would be promising for endometrial cancer treatment.

In conclusion, the present study demonstrated that upregulation of CLDN9 expression, determined using a selective mAb against CLDN9, predicted poor prognosis for patients with endometrial cancer. It is required to unveil the molecular mechanisms of CLDN9 signals in endometrial cancer to develop a new personalized medicine targeting CLDN9.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YE, KS, MakK, YK, ManK, AYH and TH performed experiments. YE, KS and HC drafted the manuscript. KS and HC conceived the study and supervised all experiments. KS, YE, YH and HC reviewed the pathological diagnosis and/or analyzed immunohistochemistry slides. SF, SS, TW and KF collected specimens and assembled the database. YH, KF and HC helped with the writing of the manuscript. YE, KS and HC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent for publication

The study was approved by the Ethics Committee of Fukushima Medical University (Fukushima, Japan; approval code, 2019-311; approval date, Mar 18, 2020). The research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards. This study was carried out by an opt-out method. Since it was conducted as a retrospective study using cases with a follow-up period of more than five years, the patients had already died or stopped visiting the hospital. The experimental protocol has been disclosed on the website and the patients or their representatives were able to decline to participate in the survey as they preferred.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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