Reduced Claudin-12 Expression Predicts Poor Prognosis in Cervical Cancer

学 位 論 文

Reduced Claudin-12 Expression Predicts Poor Prognosis in Cervical Cancer

(Claudin-12 タンパク質の発現低下は子宮頸がんの予後不良因子である)

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概要

細胞間接着分子クローディン・ファミリーは上皮細胞の間をつなぐ糊の役割を果たしている。クローディン（CLDN）は CLDN-1 から CLDN-27 まで 20 種類以上存在し、皮膚では CLDN-1/-4/-7 が、腸では CLDN-3/-4/-7 が、血管では CLDN-5 が主として発現するなど、臓器ごとに特有の分布を示す。がんになるとこれら臓器特異的な CLDN の発現パターンが変化し、正常では発現しない CLDN のがん組織における発現や、正常で発現する CLDN のがん組織における発現欠失が知られている。よって、かねてから CLDN をがんの診断マーカーとして利用する試みが活発に行われてきた。

我々のグループは以前、CLDN-12 が腸管のカルシウム吸収に重要であることを解明した（Fujita *et al., Mol Biol Cell*, 2008）。しかしながら CLDN-12 の発現を評価できる病理診断に有用な特異抗体が長らく開発されておらず、人体における CLDN-12 タンパク質の発現は未解明である。そこで我々はまず CLDN-12 を特異的に認識するモノクローナル抗体を開発し、子宮頸がん患者の手術標本における CLDN-12 の発現量を免疫組織化学的に解析した。その結果、CLDN-12 タンパク質が高発現する患者 112 例、低発現する患者 26 症例を見出した。CLDN-12 タンパク質の発現と年齢やステージなどの臨床病理学的因子との関連を検討したところ、CLDN-12 タンパク質が低発現する患者では有意に再発する患者が多いことが明らかとなった。さらに予後との関連を検討したところ、CLDN-12 の発現が高い患者では 10 年後の生存率が 80 %以上であった一方、CLDN-12 の発現が低い患者では 60 %以下であった。よって、今後は CLDN-12 の発現量に応じて治療の強度を変えるなど、CLDN-12 を「予後予測マーカー」として活用できる可能性が期待される。全世界で子宮頸がん患者は 50 万人以上と報告されており、本研究は患者ひとりひとりに合った個別化治療戦略医療を推進する上で有用な知見を提供するものである。また CLDN-12 は卵巣がんや大腸がんなど様々な臓器のがんで発現していることから、本研究で得られた抗体はさらに多くの患者の診断や治療に役立つ可能性が示唆される。

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1. Introduction

Cervical cancer is the fourth leading cause of cancer-related deaths in women [1, 2]. In 2018, it was estimated that more than half a million patients were diagnosed with cervical cancer, and that over 300,000 deaths occurred worldwide [3]. The most and second-most histological types of cervical cancers are squamous cell carcinoma (SCC) and adenocarcinoma (ADCA), which account for 70–80% and 15–25% of all cases, respectively [4, 5]. In addition, the major risk factor for cervical intraepithelial neoplasia (CIN) and cervical cancer is persistent infection with high-risk human papillomavirus (HPV) strains, largely HPV16 and HPV18 [6]. The incidence and mortality of cervical cancer have been reduced in developed countries, due to organized screening programs [7]. Nevertheless, the 5-year overall survival (OS) for women with locally advanced cervical cancer is about 70% following chemoradiotherapy, and the prognosis for patients with recurrent or metastatic disease remains poor [2]. Along these lines, biomarkers that can distinguish cervical cancer cases with good and poor outcomes are required for patients to avoid both overtreatment and undertreatment, as well as to improve the quality of care. Several molecules including Villin 1 [8], C-C chemokine receptor type 7 [9], and FAM83A [10] have been reported as prognostic biomarkers for cervical cancer, but none of them have yet been clinically applied.

The claudin (CLDN) family of tight-junction proteins functions not only as paracellular barriers or pores for selective ions and solutes, but also as signaling platforms to coordinate diverse cellular behaviors [11-16]. Concerning the signaling properties of CLDNs, our lab recently demonstrated that the CLDN6-adhesion signal regulates transcription factor activity [17]. In summary, our lab identified that CLDN6 couples with Src-family kinase (SFKs) in

its extracellular loop 2 (EC2)- and C-terminal cytoplasmic Y196/200-dependent manners. They also showed that the CLDN6/SFK/PI3K/AKT cascade targets the AKT-phosphorylation sites in mouse retinoic acid receptor γ and human estrogen receptor α (ERα), and stimulates their activities.

CLDNs frequently show aberrant expression and/or subcellular localization in a wide variety of cancers, resulting in either promotion or repression of tumor progression [18-25], hypothetically due to dysregulated CLDN signaling. Our lab previously reported that high CLDN6 expression in endometrial cancer is an independent prognostic factor that is significantly associated with several clinicopathological variables [26]. In a subsequent study, our lab uncovered that aberrant CLDN6 expression promotes endometrial cancer progression by hijacking the CLDN6–ERα axis [27]. Our lab also found that aberrant CLDN6–ERα signaling contributes not only to cellular proliferation, but also to collective cell migration in the leading front of endometrial cancer cells.

Among CLDN subtypes, CLDN12 belongs to nonclassical CLDN members. CLDN12 is critical for vitamin D-dependent paracellular Ca^{2+} transport in the intestine and kidney [28-31]. On the other hand, upon searching the Cancer Genome Atlas (TCGA) database [32, 33], *CLDN12* mRNA is highly overexpressed in diverse histological types of human cancer tissues, such as SCC and ADCA in a range of organs. However, the available anti-CLND12 antibodies, including our lab established one [28, 34], hamper the verification of the protein expression and function in normal and pathological tissues due to the insufficient specificities and applications [35]. Hence, an additional anti-CLND12 antibody with high selectivity and titer is absolutely prerequested to further study the nature of CLDN12.

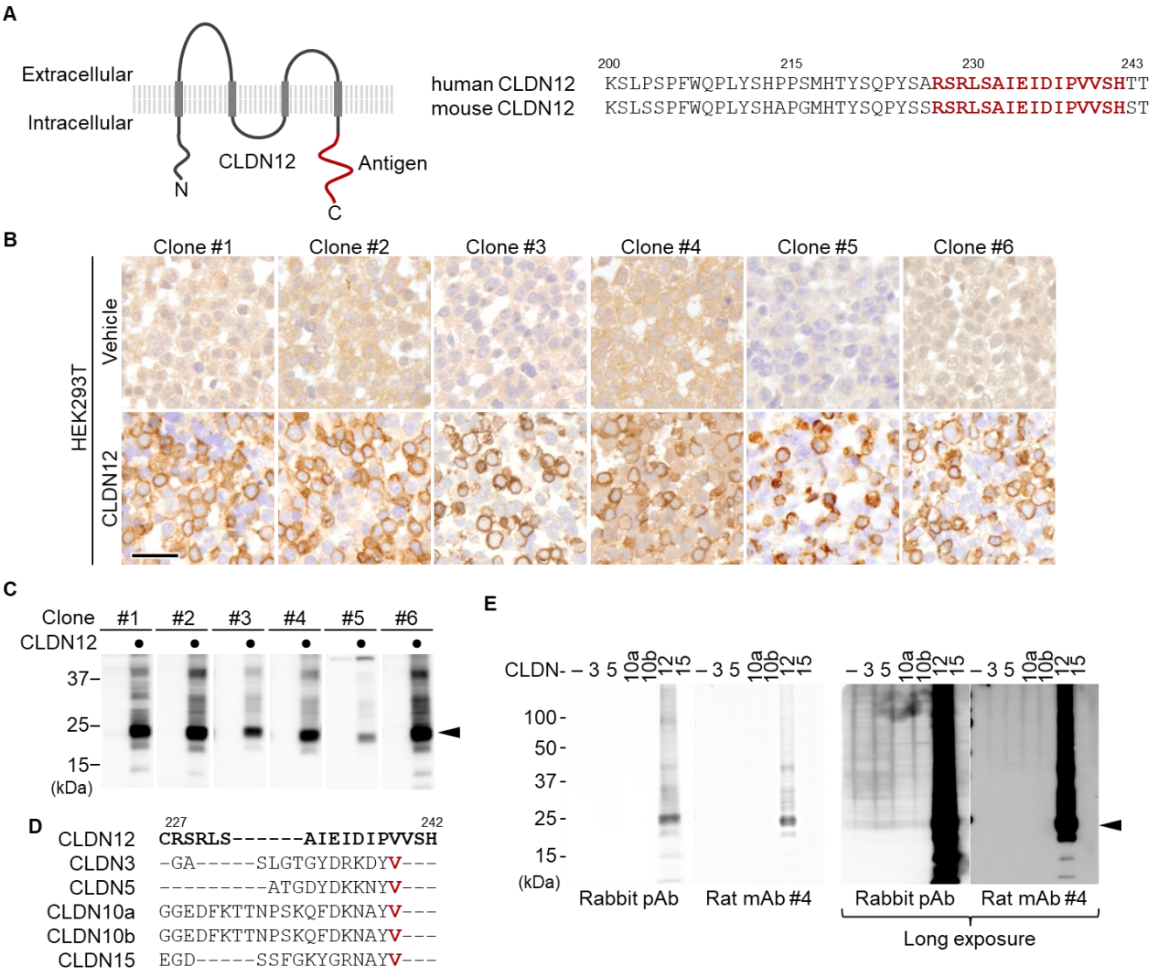
In the present study, I developed a novel monoclonal antibody (mAb) that selectively recognizes human CLDN12 and works for immunohistochemistry of formalin-fixed paraffin-embedded (FFPE) tissues. Using this specific mAb, I show that the diminished CLDN12 expression is a poor prognostic biomarker for cervical cancer.

2. Results

2.1. Establishment of an Anti-Human/Mouse CLDN12 mAb

I first generated a novel mAb against the same C-terminal cytoplasmic region between human and mouse CLDN12 (Figure 1A) using the medial iliac lymph-node method [36]. Among 202 hybridomas, 48 clones were selected by ELISA, six (clones #1/2/3/4/5/6) of which were able to detect positive signals by immunohistochemistry using cell block of CLDN12-expressing HEK293T cells (Figure 1B). Western blot analysis also revealed that these six clones reacted with human CLDN12 in HEK293T cells (Figure 1C).

To check the specificity of the rat anti-CLDN12 mAb (clone #4) and the formerly established rabbit anti-CLDN12 polyclonal antibody (pAb) [28, 34], HEK293T cells were transiently transfected with distinct human CLDN expression vectors, followed by Western blot analysis. Both Abs selectively recognized CLDN12, but not CLDN3, CLDN5, CLDN10a/b or CLDN15, which are closely related to CLDN12 within the CLDN family (Figure 1D, E). I confirmed CLDN-3, -5, -10a, -10b, and -15 overexpression by western blot (Figure 2).


A
Extracellular
Intracellular
CLDN12
Antigen
N
C
human CLDN12 KSLPSPFWQPLYSHPPSMHTYSQPYSARSRLSAIEIDIPVVSHTT
mouse CLDN12 KSLSSPFWQPLYSHAPGMHTYSQPYSSRSRLSAIEIDIPVVSHST
B
Clone #1
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Clone #4
Clone #5
Clone #6
HEK293T
Vehicle
CLDN12
C
Clone #1 #2 #3 #4 #5 #6
CLDN12
37–
25–
15–
(kDa)
D
CLDN12 CRSRLS------AIEIDIPVVSH
CLDN3 -GA-----SLGTGYDRKDYV---
CLDN5 ---------ATGDYDKKNYV---
CLDN10a GGEDFKTTNPSKQFDKNAYV---
CLDN10b GGEDFKTTNPSKQFDKNAYV---
CLDN15 EGD-----SSFGKYGRNAYV---
E
CLDN- – 3 5 10a 10b 12 15
100-
50-
37-
25-
15-
(kDa)
Rabbit pAb
Rat mAb #4
Long exposure
F
Rabbit pAb
Rat mAb #4

Figure 1. Generation of rat monoclonal antibodies (mAbs) against human/mouse claudin-12 (CLDN12). (**A**) Topology of CLDN12 (left) and amino acid sequences of the C-terminal cytoplasmic domains of human and mouse CLDN12 (right). The C-terminal region that correspond to an antigenic polypeptide is indicated in red. (**B**,**C**) HEK293T cells were transfected with the CLDN12 or empty expression vector, and cell blocks were subjected to immunohistochemical and Western blot analyses using the indicated anti-CLDN12 mAb clones. (**D**) Amino acid sequences of the antigenic peptide of the C-terminal cytoplasmic domain of human CLDN12 and the corresponding regions of the closely related CLDNs. Conserved amino acids are shown in red. (**E**) HEK293T cells were transfected with individual CLDN expression vector, and subjected to Western blot analysis using the indicated anti-CLDN12 Abs. (**F**) Normal human liver tissues were immunohistochemically stained with the indicated anti-CLDN12 Abs. Arrows and arrowheads reveal cytoplasmic and plasma membrane signals, respectively. Scale bars, 100 μm.

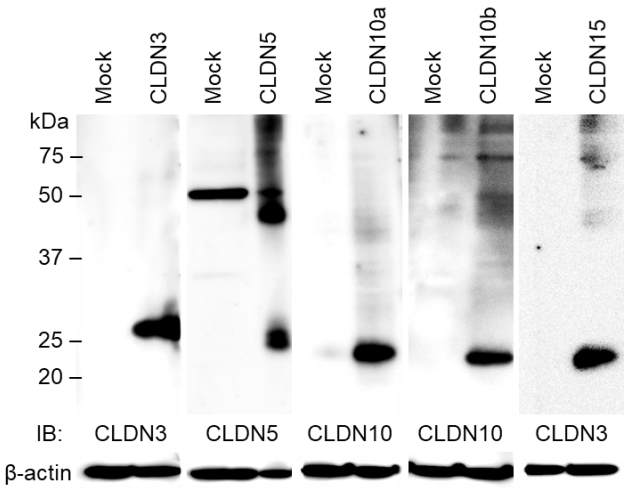


Figure 2. Western blot conformed successful overexpression of CLDN-3, -5, -10a, -10b, and -15 in transfected HEK293T cells.

Based on analysis using TCGA database, *CLDN12* mRNA is most abundantly overexpressed in colorectal cancer. Therefore, I next validated the above-mentioned anti-CLDN12 mAbs by immunohistochemistry of colorectal cancer tissues, and selected clone #4 for further analyses. (Figure 3).

Immunohistochemical analysis using clone #4 revealed that plasma membrane and cytoplasmic CLDN12 signals appeared to be detected in hepatocytes of normal human liver tissues without lobular gradient (Figure 1F). In addition, CLDN12 is strongly expressed in portal cholangiocytes. Weak cytoplasmic CLDN12 signals were also observed in vascular smooth muscle cells, in good agreement with a previous report using CLDN12-lacZ-knockin mice [35]. Furthermore, CLDN12 was expressed in colorectal cancer tissues (Figure 3B). In marked contrast, by immunohistochemistry, the anti-CLDN12 pAb did not detect any specific signal in normal liver tissues or colorectal cancer tissues.

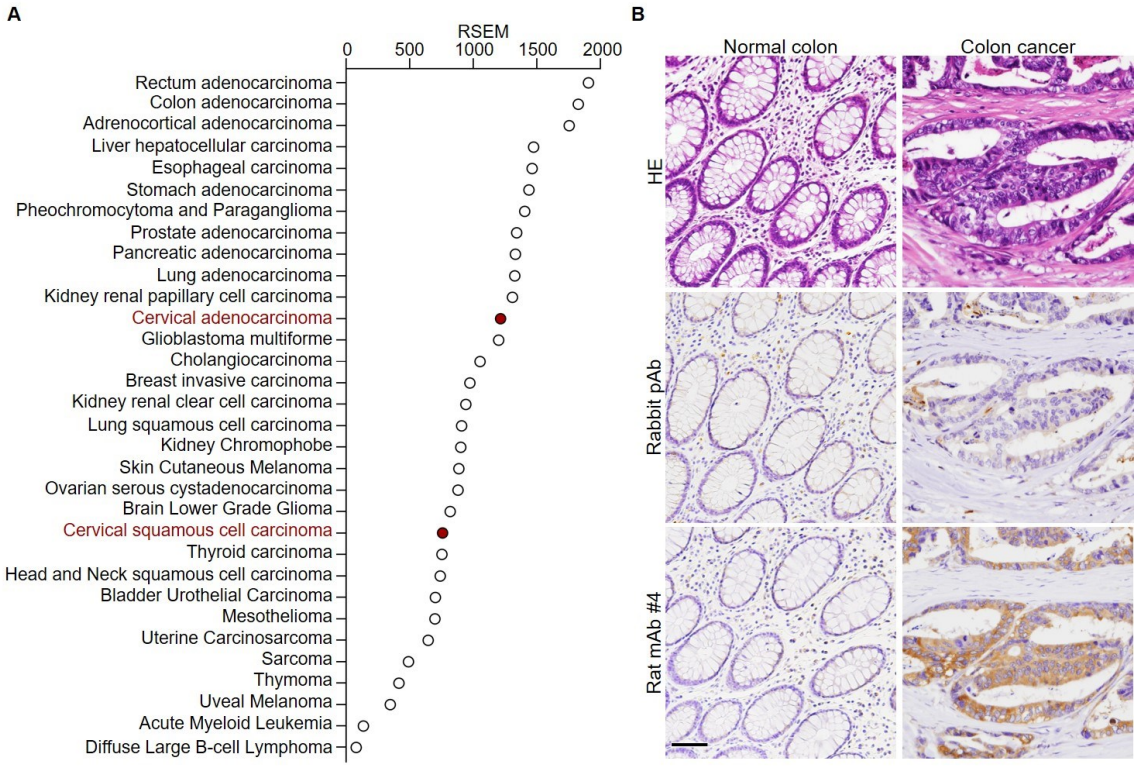


Figure 3. The *CLDN12* mRNA expression in various cancer tissues and the CLDN12 protein expression in normal and malignant colon tissues. (**A**) Expression levels of *CLDN12* mRNA in the indicated cancer tissues were analyzed using TCGA database. RSEM, RNA-Seq by Expectation-Maximization. (**B**) Normal and cancer tissues of the colon were immunohistochemically stained with the anti-CLDN12 pAb and the anti-CLDN12 mAb. HE, hematoxylin-eosin. Scale bar, 100 μm.

2.2. Expression of CLDN12 Protein in Normal, Premalignant and Malignant Tissues of the Uterine Cervix

I next determined by immunohistochemistry the CLDN12 expression in normal, premalignant, and malignant epithelial tissues of the uterine cervix. As shown in Figure 4A, CLDN12 was distributed in cell-cell borders of normal cervical gland epithelia; on the other hand, it was not detected in squamous epithelial cells of the normal uterine cervix. Weak CLDN12 immunoreactivity was also observed in normal vascular and nonvascular smooth muscle cells in the uterus.

Unlike normal squamous epithelial cells of the uterine cervix, CLDN12 was expressed throughout the cytoplasm of both low-grade squamous intraepithelial legion (LSIL) and high-grade SIL (HSIL) tissues (Figure 4B). CLDN12-immunoreactive signals were also observed in the cytoplasm of cervical SCC tissues, but the signal intensity (SI) appeared to be varied among the subjects (Figure 5A). By contrast, both intracellular and plasma membrane signals for CLDN12 were detected in cervical ADCA tissues with the different SI (Figure 5B and 5D). By semiquantification of the CLDN12 expression in cervical cancer tissues, 26 of the 138 cases (18.8%) showed low CLDN12 expression, and the remaining 112 cases (81.2%) exhibited high CLDN12 expression (Table 1).

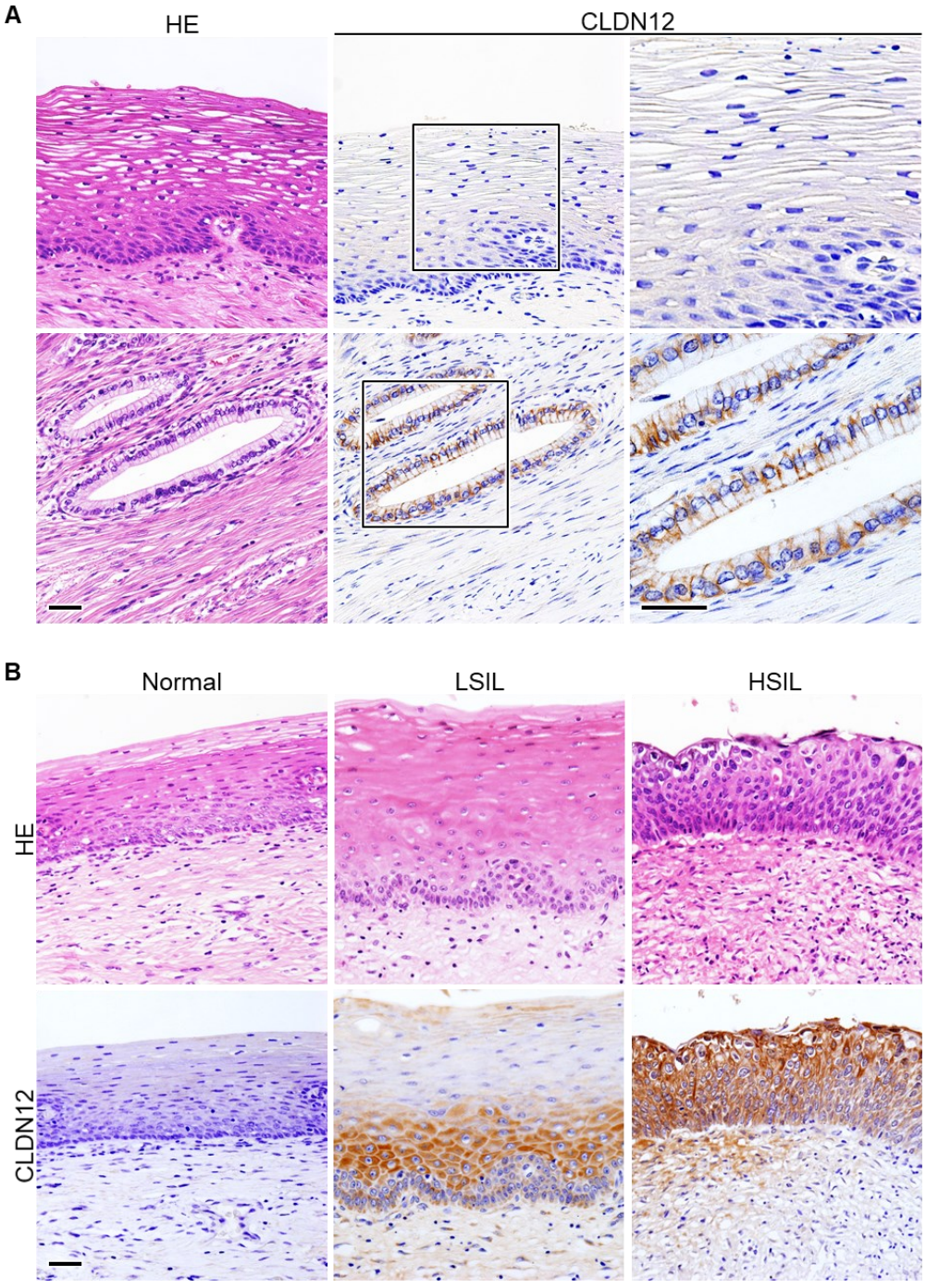


Figure 4. Expression of CLDN12 protein in normal and premalignant epithelial tissues of the uterine cervix. (**A**) Normal human cervical tissues and (**B**) squamous intraepithelial lesion (SIL) tissues were immunohistochemically stained with the anti-CLDN12 mAb. HE, hematoxylin-eosin; LSIL, low-grade SIL; HSIL, high-grade SIL. Scale bars, 100 μm.

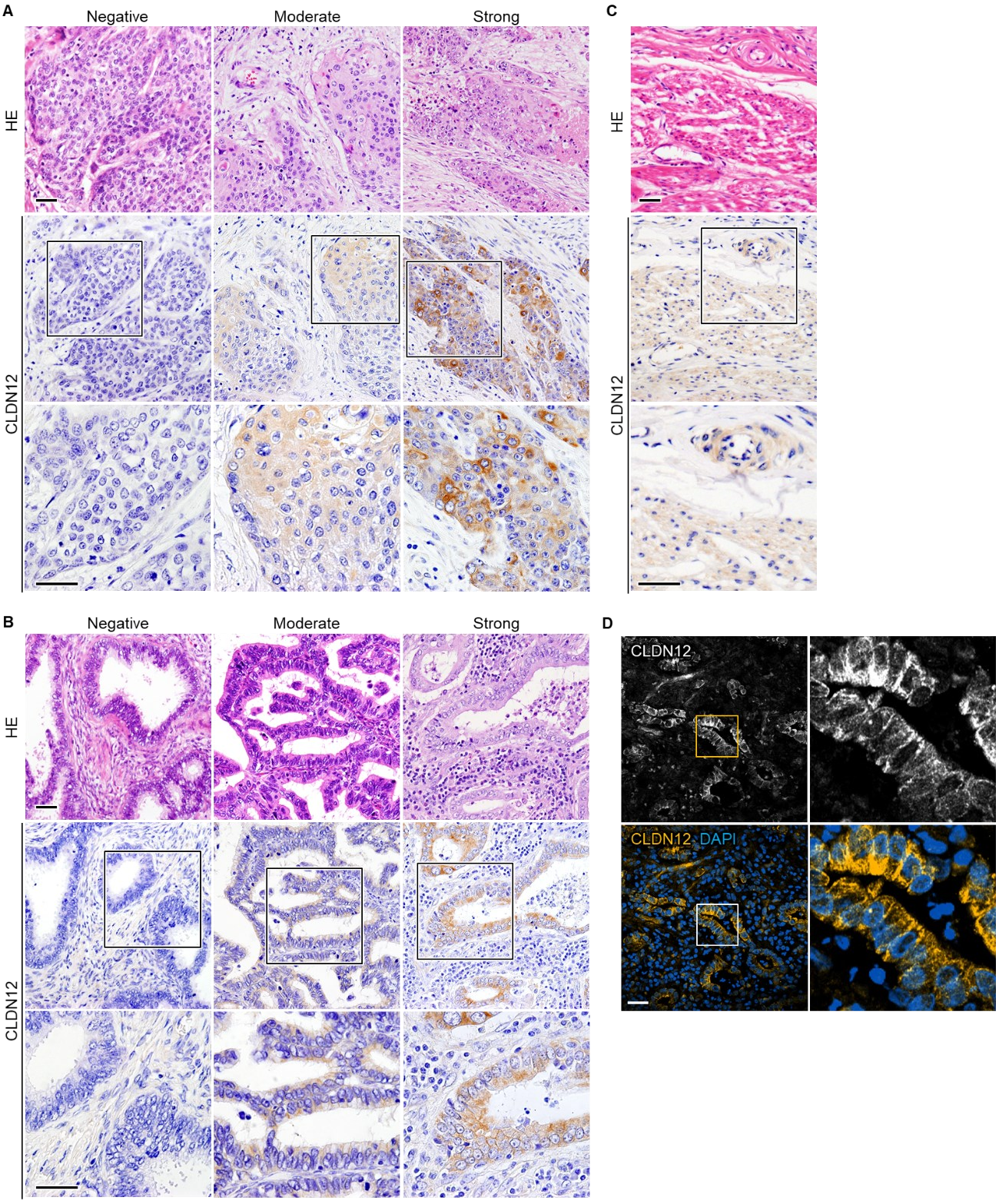

A
Negative
Moderate
Strong
HE
CLDN12
C
HE
CLDN12
B
Negative
Moderate
Strong
HE
CLDN12
D
CLDN12
CLDN12 DAPI

Figure 5. CLDN12 protein expression in cervical cancer tissues. (A) Squamous cell carcinoma (SCC) and (B) adenocarcinoma (ADCA) tissues were immunohistochemically stained with the anti-CLDN12 mAb. Smooth muscle was used as internal control for staining (C). Immunofluorescence staining of frozen ADCA tissue (D). HE, hematoxylin-eosin. Scale bars, 100 μm.

2.3. Low Expression of CLDN12 Correlates with Poor Prognosis and Recurrence in Cervical Cancer

Kaplan−Meier plots revealed significant differences in disease-specific survival (DSS) and recurrence-free survival (RFS) between the low and high CLDN12 expression groups (Figure 6A, B). The twelve-year DSS rates in the low and high CLDN12 groups were 57.4% and 82.8%, respectively.

Among the clinicopathological factors, the low CLDN12 expression was significantly associated with the recurrence of cervical cancer ($p = 0.0385$), but not with age, histological type, FIGO stage, tumor size, vascular involvement, lymphatic involvement, lymph node metastasis, distant metastasis or chemoradiotherapy (Table 1).

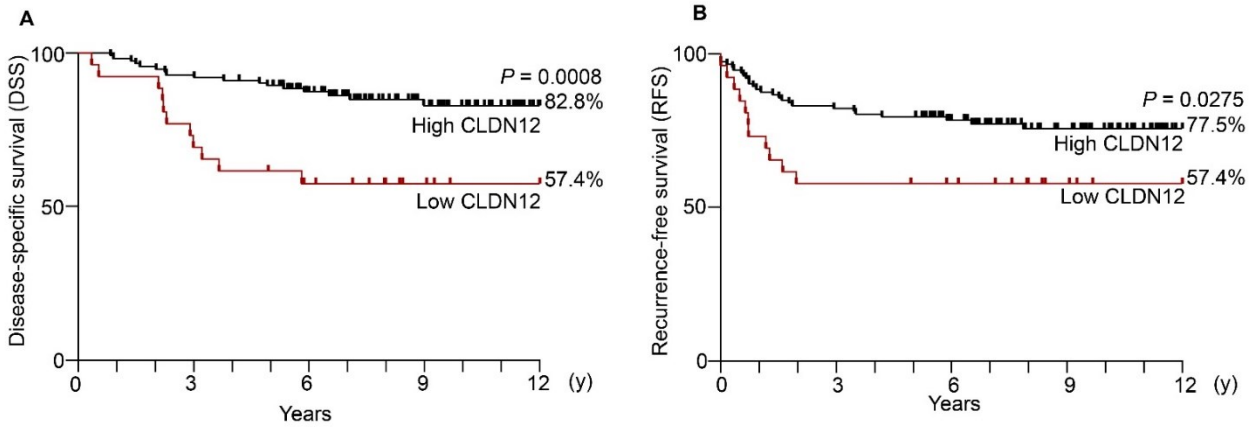


Figure 6. Low CLDN12 expression is associated with poor outcome in cervical cancer patients. (**A**) The disease-specific and (**B**) recurrence-free survival for low and high expression of CLDN12 protein in cervical cancer subjects are indicated.

Table 1. Relationship between CLDN12 expression and clinicopathological factors in cervical cancer.

Characteristics		Total (n= 138)		*P*-value
		High CLDN12 (n = 112)	Low CLDN12 (n = 26)	
Age				
	<50	74 (66%)	15 (58%)	0.4964
	≥50	38 (34%)	11 (42%)	
Histological type				
	Adenocarcinoma	19 (17%)	1 (4%)	
	Adenosquamous carcinoma	14 (13%)	4 (15%)	SCC vs non-SCC
	Squamous cell carcinoma	73 (65%)	20 (77%)	0.3533
	Other	6 (5%)	1 (4%)	
FIGO Stage				
	I	71 (63%)	13 (50%)	
	II	37 (33%)	12 (46%)	I vs II/III/IV
	III	0 (0%)	0 (0%)	0.2653
	IV	4 (4%)	1 (4%)	
Tumor size				
	1	68 (61%)	12 (46%)	
	2	43 (38%)	13 (50%)	pT1 vs pT2/3/4
	3	0 (0%)	0 (0%)	0.1921
	4	0 (0%)	1 (4%)	
	Unknown	1 (1%)	0 (0%)	
Vascular involvement				
	No	60 (54%)	12 (46%)	
	Yes	47 (42%)	13 (50%)	0.5088
	Unknown	5 (4%)	1 (4%)	
Lymphatic involvement				
	No	51 (46%)	10 (38%)	
	Yes	57 (51%)	15 (58%)	0.6567
	Unknown	4 (4%)	1 (4%)	
Lymph node metastasis				
	N0	72 (64%)	17 (65%)	
	N1	32 (29%)	7 (27%)	1.0000
	NX	8 (7%)	2 (8%)	
Distant metastasis				
	M0	105 (94%)	25 (96%)	
	M1	4 (4%)	0 (0%)	1.0000
	MX	3 (3%)	1 (4%)	
Chemoradiotherapy				
	Chemotherapy	16 (14%)	4 (15%)	
	Radiotherapy	29 (26%)	7 (27%)	
	Concurrent chemoradiotherapy	39 (35%)	10 (38%)	no vs yes
	Other	1 (1%)	0 (0%)	0.7971
	None	27 (24%)	5 (19%)	
Recurrence				
	No	91 (81%)	16 (62%)	0.0385*
	Yes	21 (19%)	10 (38%)	

*P<0.05

2.4. Reduced CLDN12 Represents an Independent Poor Prognostic Marker for Cervical Cancer

In the univariable analysis, the low CLDN12 expression (hazard ratio [HR] 3.412, $p = 0.002$), FIGO stage IIb/III/IV (HR 4.866, $p < 0.001$), tumor size (HR 3.945, $p < 0.001$), vascular involvement (HR 3.509, $p = 0.005$), lymphatic involvement (HR 2.973, $p = 0.019$), distant metastasis (HR 5.915, $p = 0.004$), chemoradiotherapy (HR 4.495, $p = 0.041$), and recurrence (HR 19.787, $p < 0.001$), showed significant prognostic variables for the DSS of cervical cancer patients (Table 2). By contrast, age, histological type, or lymph node metastasis were not prognostic markers for cervical cancer.

I subsequently performed multivariable analysis of the twelve-year DSS in cervical cancer subjects. Among the analyzed variables, the low CLDN12 expression (HR 2.615, $p = 0.029$), FIGO stage IIb/III/IV (HR 4.075, $p = 0.002$), and recurrence (HR 14.852, $p < 0.001$) were independent prognostic factors for the DSS of cervical cancer patients (Table 3).

Table 2. Univariable analysis of disease-specific survival in cervical cancer patients.

Univariate analysis				
Variable		*P* -value	Hazard ratio	95% CI
CLDN12	Low	0.00156**	3.412	1.595 - 7.300
Age	≥ 50	0.603	0.810	0.366 - 1.792
Histological type	SCC	0.336	0.689	0.323 - 1.472
FIGO Stage	≥ 2b	<0.001***	4.866	2.277 - 10.400
Tumor size	> 4cm	<0.001***	3.945	1.862 - 8.360
Vascular involvement	Yes	0.00486**	3.509	1.465 - 8.409
Lymphatic involvement	Yes	0.01933*	2.973	1.193 - 7.408
Lymph node metastasis	Yes	0.355	1.452	0.659 - 3.199
Distant metastasis	Yes	0.00372**	5.915	1.780 - 19.66
Chemoradiotherapy	Yes	0.04065*	4.495	1.066 - 18.949
Recurrence	Yes	<0.001***	19.787	7.969 - 49.135

*P<0.05; **P<0.01; ***P<0.001

Table 3. Multivariable analysis of disease-specific survival in cervical cancer patients.

Multivariate analysis				
Variable		*P* -value	Hazard ratio	95% CI
CLDN12	Low	0.02943*	2.615	1.101 - 6.214
FIGO Stage	≥ 2b	0.0019**	4.075	1.679 - 9.891
Recurrence	Yes	<0.001***	14.852	5.847 - 37.726

*P<0.05; **P<0.01; ***P<0.001

3. Discussion

In the present study, I developed a novel rat anti-CLDN12 mAb (clone #4) that specifically reacted with CLDN12. My immunohistochemical analysis of normal human liver FFPE tissues revealed that the anti-CLDN12 mAb was able to detect strong, moderate, and weak signals in portal cholangiocytes, hepatocytes and vascular smooth muscle cells, respectively. These results are in good agreement with previous findings using CLDN12-lacZ-knockin mice [35], ensuring a high reliability of our anti-CLDN12 mAb. When normal human uterus FFPE tissues were stained with the anti-CLDN12 mAb, CLDN12 immunoreactivity was observed in cervical gland epithelial cells. Taken together with the prominent selectivity of the novel anti-CLDN12 mAb, it could provide a powerful tool to determine expression and function of CLDN12 in a variety of normal and pathological tissues.

On Western blot and immunofluorescent analyses, the formerly generated anti-CLDN12 pAb selectively recognized mouse and human CLDN12 ([28, 34]; this study). In the current study, however, I showed by immunohistochemistry that the anti-CLDN12 pAb, unlike the anti-CLDN12 mAb, detected no specific signal in normal liver or colorectal cancer FFPE tissues. As FFPE samples are the mainstay of tissue archiving with well over 400 million FFPE tissue samples assembled in biorepositories [37], anti-CLDN12 mAb which was established in this study would be useful for multi-center based retrospective study to validate prognostic performance not only in cervical cancer but also in other gynecologic cancers. In addition, our lab previously established anti-CLDN6 pAb [38] and anti-CLDN6 mAb [26]. On both Western blot and immunohistochemical analyses, the anti-CLDN6 mAb selectively recognized CLDN6, whereas the anti-CLDN6 pAb reacted not only with CLDN6, but also

with overexpressed CLDN4 and CLDN5. Furthermore, it is known that certain anti-CLDN5 pAb recognizes both CLDN5 and CLDN6 [39]. Thus, it is of particular importance to verify the validity and cross-reactivity of the used anti-CLDN Abs depending on the applications.

Another issue that should be discussed is the subcellular localization of the CLDN12 protein in the normal and cancer tissues analyzed. The plasma membrane CLDN12 signals appeared in uterine cervical epithelial cells, while the cytoplasmic signals were observed in portal cholangiocytes and smooth muscle cells. In hepatocytes, CLDN12 was distributed along cell membranes and throughout the cytoplasm. Both plasma membrane and cytoplasmic CLDN12 signals were also observed in uterine cervical ADCA tissues. Moreover, in colorectal cancer and uterine cervical SCC tissues, CLDN12 was localized in the cytoplasm. Although it is unknown why CLDN12 possesses distinct subcellular localization as described above, it has been reported that the CLDN1 localization is altered from cell membranes to cytoplasm at the invasion front of tongue SCC tissues [40]. Therefore, mislocalization of CLDN12 from cell-cell junction to cytoplasm might have an adverse effect in cancer progression.

Expression of CLDN1/2/4/7 proteins has been reported to be increased in cervical cancer tissues [41-47], but the clinicopathological significance has yet to be defined. I demonstrated in the current study that reduced CLDN12 expression predicts poor outcome in patients with cervical cancer. This conclusion was drawn from the following results: (1) the DSS and RFS in the low CLDN12 expression group of the cervical cancer subjects were significantly decreased compared with those in the high expression group; (2) the low CLDN12 expression was significantly associated with recurrence of cervical cancer; (3) upon univariable analysis,

the low CLDN12 expression was found to be a significant prognostic variable for DSS of cervical cancer patients (HR 3.412, $p = 0.002$); (4) multivariable analysis revealed that the low CLDN12 expression was an independent prognostic factor for the DSS of cervical cancer subjects (HR 2.615, $p = 0.029$). Analysis of a larger number of cases would be required to obtain more solid conclusions about the clinicopathological relevance of the low CLDN12 expression in patients with cervical cancer.

It is unknown how the low CLDN12 expression contributes to poor prognosis in cervical cancer subjects. However, our lab has recently reported that aberrant CLDN6 signaling advances endometrial cancer progression via hijacking of the CLDN6–ERα pathway [27]. On the other hand, CLDN18 deficiency promotes tumorigenesis and progression of lung and gastric ADCA [48-51] through activating YES-associated protein (YAP) signaling. Along this line, the diminished CLDN12 expression may stimulate certain intracellular signaling pathways, such as YAP, resulting in cervical cancer progression.

4. Materials and Methods

4.1. Generation of Antibodies

A rabbit pAb against CLDN12 was generated in cooperation with Immuno-Biological Laboratories (#18801, Fujioka, Japan) as described previously [34].

Rat mAbs against CLDN12 were established using the iliac lymph node method [36]. In brief, a polypeptide, (C)RSRLSAIEIDIPVVSH, corresponding to the cytoplasmic domain of human and mouse CLDN12, was coupled via the cysteine to Imject Maleimide-Activated

mcKLH (Thermo Fisher Scientific, Waltham, MA, USA). The conjugated peptide was intracutaneously injected with Imject Freund's Complete Adjuvant (Thermo Fisher Scientific) into the footpads of anesthetized eight-week-old female rats. All animal experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Committee of Fukushima Medical University (FMU) (approval code, 2019-001; approval date, 1 April 2019). The animals were sacrificed 14 days after immunization, and the median iliac lymph nodes were collected, followed by extraction of lymphocytes by mincing. The extracted lymphocytes were fused with cells of the SP2 mouse myeloma cell line using polyethylene glycol. Hybridoma clones were maintained in GIT medium (Wako, Osaka, Japan) with supplementation of 10% BM-Condimed (Sigma–Aldrich, St. Louis, MO, USA). The supernatants were screened by enzyme-linked immunosorbent assay (ELISA).

4.2. Cell Culture, Expression Vectors and Transfection

HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Glendale, AZ, USA) with 10% fetal bovine serum (FBS; Sigma–Aldrich) and 1% penicillin–streptomycin mixture (Gibco, Waltham, MA, USA).

The protein coding regions of human *CLDN3*, *CLDN5*, *CLDN10a*, *CLDN10b, CLDN12* and *CLDN15* were cloned into the *Bam*HI/*Not*I site of the CSII-EF-MCS-IRES2-Venus (RIKEN, RDB04384, Wako, Japan) plasmid.

For transient expression of the above-mentioned target genes, 5×10^6 HEK293T cells were transfected with 10 μg of the indicated vectors using 30 μg of Polyethylenimine Max (PEI Max, Cosmo Bio, Carlsbad, CA, USA) 8 h after passage. Transfection efficiency was evaluated by Venus expression, with a fluorescent microscope (IX71, Olympus, Tokyo, Japan).

4.3. Immunoblotting

Total cell lysates were collected with CelLytic MT Cell Lysis Reagent (Sigma), followed by one-dimensional SDS-PAGE, and were electrophoretically transferred onto a piece of Immobilon (Millipore, Burlington, MA, USA). The membrane was saturated with PBS containing 4% skimmed milk and treated with primary antibodies. Supernatants of rat anti-CLDN12 hybridoma, Rabbit anti-CLDN3 polyclonal antibody (1:1,000, Cat# 34-1700, Invitrogen), Rabbit anti-CLDN5 polyclonal antibody (1:1,000, Cat# 18855, IBL), Rabbit anti-CLDN15 polyclonal antibody (1:1,000, Cat# 18805, IBL), and Rat-anti CLDN10 monoclonal antibody (in-house, unpublished) used as primary antibodies, and rabbit anti-CLDN12 pAb was diluted at 1:2,000 in PBS. The signal was detected by chemiluminescence using 1:2,000-times diluted HRP-conjugated anti-rat IgG (NA935V, GE Health Care, Chicago, IL, USA) or anti-rabbit IgG (NA934V, GE Health Care).

4.4. Cell Blocks

Cells were centrifuged at 1200 rpm for 10 min and fixed with 10% formalin for 16 h at 4 °C. Fixed cell pellets were mixed with 1% sodium alginate followed by 1 M calcium

chloride and embedded in paraffin (Tissue-Tek VIP 5 Jr, Sakura Finetek Japan, Tokyo, Japan).

4.5. Tissue Collection, Immunostaining and Analysis

FFPE tissue sections were obtained from: 138 patients with uterine cervical cancer (age, 29–79 years; average ± SD = 46.4 ± 12.0) who underwent hysterectomy alone or together with bilateral salpingo-oophorectomy and/or lymphadenectomy between 2005 and 2015 at Fukushima Medical University Hospital (FMUH); and 37 patients with SIL subjects of the cervix; and four autopsy cases. Informed consent was obtained from all the patients or the next of kin for each subject. The cervical cancer subjects were limited to patients who were confirmed to have at least 5-year outcomes and those who had died due to cervical cancer and metastasis. Detailed information, including postoperative pathology diagnosis reports, age, histological type, stage (FIGO 2008), tumor size, vascular involvement, lymphatic involvement, lymph node metastasis, distant metastasis, chemoradiotherapy, recurrence status, DSS, and RFS, was obtained. The staging of patients between 2005 and 2007 was modified in accordance with the FIGO 2008 system. Distant metastasis was judged by diagnostic imaging. Colorectal cancer and normal liver tissues were also obtained at FMUH. The study was approved by the Ethics Committee of FMUH (approval code, 2019-311; approval date, 18 March 2020).

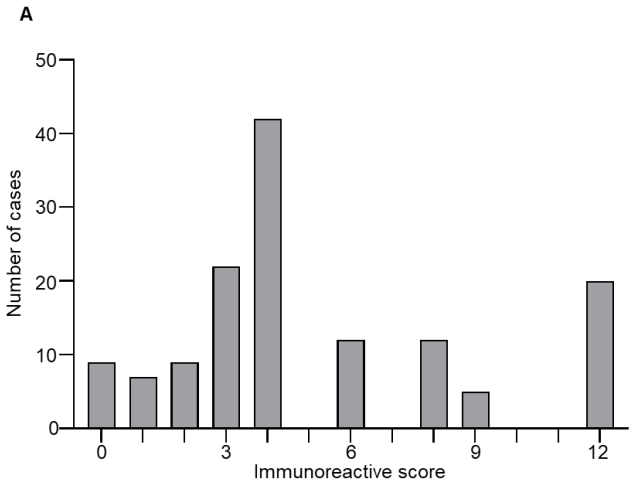
For immunostaining, the 10% FFPE tissue blocks were sliced into 5-μm-thick sections, deparaffinized with xylene, and rehydrated using a graduated series of ethanol. The sections

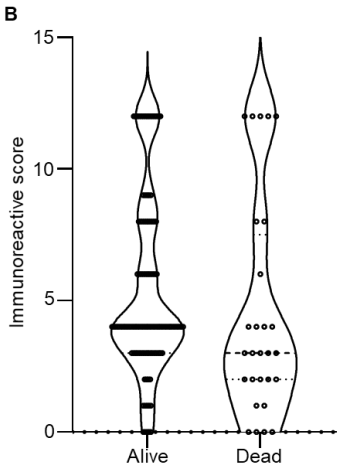
were then immersed in 0.3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was performed by incubating the sections in boiling 10 mM citric acid buffer (pH 5.0) using a microwave. After blocking with 5% skimmed milk at room temperature for 30 min, the sections were incubated overnight at 4 °C with supernatants of the rat anti-CLDN12 hybridoma (1:4, clone #4) or the rabbit anti-CLDN12 pAb (1:100). After washing with PBS, a secondary antibody reaction was performed by using the Histofine mouse PO-Rat secondary antibody (Nichirei, Tokyo, Japan) or Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei) with 3′,3′-diaminobenzidine (DAB) as a chromogen according to the manufacturer's instructions.

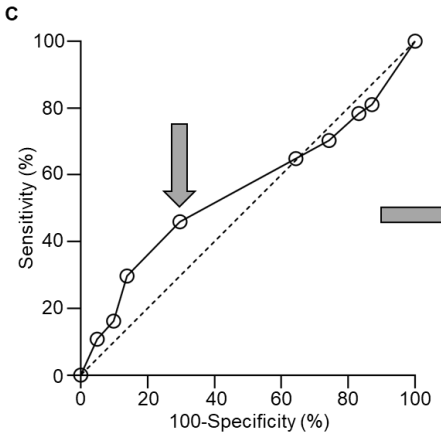
Immunostaining results were interpreted by two independent pathologists and one gynecologist using a semiquantitative scoring system (immunoreactive score; IRS) [52]. The immunostaining reactions were evaluated according to SI (0, no stain; 1, weak; 2, moderate; 3, strong) and percentage of positive cells (PP: 0, <1%; 1, 1–10%; 2, 11–30%; 3, 31–50%; and 4, >50%). The SI and PP were then multiplied to generate the IRS for each case. To determine the optimal cut-off values of IRS for CLDN12 expression, first, I consider distribution of IRS in all cases which I used in this study (Figure 7A), second, I split patient into 2-groupes based-on vital status (Figure 7B), and third, the receiver operating characteristic (ROC) curve was plotted (Figure 7C). I selected the point that is maximize both of sensitivity and specificity, i.e., low expression group (IRS < 3) and high expression group (IRS ≥3).

For Immunofluorescent staining, cervical cancer frozen tissue was sliced into 5-μm-thick sections and fixed with 4% Paraformaldehyde solution for 10 min at room temperature. After

membrane permeabilization with 0.1% TritonX-100/PBS for 10 min at room temperature and block nonspecific antibody reactivity using 1% Bovine serum albumin/PBS for 30 min at room temperature, the sections were incubated overnight at 4 °C with supernatants of the rat anti-CLDN12 hybridoma (1:4, clone #4) and incubated with 1:500-times diluted Alexa-488 conjugated Donky anti-rat IgG secondary antibody for 1 hr at room temperature. Fluorescent signal was observed using FV-1000 fluorescent microscope (Olympus, Tokyo, Japan)







IRS cut off	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 0.5000	10.81	4.285% to 24.71%	95.05	88.93% to 97.87%	2.184
< 1.500	16.22	7.651% to 31.14%	90.1	82.73% to 94.53%	1.638
< 2.500	29.73	17.49% to 45.78%	86.14	78.07% to 91.56%	2.145
< 3.500	45.95	31.04% to 61.62%	70.3	60.77% to 78.33%	1.547
< 5.000	64.86	48.76% to 78.17%	35.64	26.99% to 45.35%	1.008
< 7.000	70.27	54.22% to 82.51%	25.74	18.22% to 35.05%	0.9463
< 8.500	78.38	62.80% to 88.61%	16.83	10.78% to 25.31%	0.9424
< 10.50	81.08	65.80% to 90.52%	12.87	7.679% to 20.78%	0.9306

Figure 7. Distribution of Immunoreactive score (IRS) in cervical cancer cases. (A) Histogram showed IRS distribution in each case. (B) IRS distribution stratified by patient vital status. (C) The receiver operating characteristic (ROC) curves for disease specific survival using cutoff score of CLDN12-immunoreactive score (IRS) ≧ 3. CI, confidence interval.

4.6. Statistical Analysis

The chi-squared test was used to evaluate the relationship between CLDN12 expression and various clinicopathological parameters. Survival analysis was performed using the Kaplan–Meier method, and differences between the groups were analyzed using the log-rank test. The Cox regression multivariable model was used to detect the independent predictors of survival. Two-tailed *p*-values < 0.05 were considered to indicate a statistically significant result. All statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA) and StatFlex ver.7 (Artech, Osaka, Japan).

5. Conclusions

In summary, I here established an mAb that selectively recognized CLDN12. I also demonstrated that the reduced CLDN12 expression was an independent prognostic variable for cervical cancer. The novel anti-CLDN12 mAb would be extremely valuable to determine the biological relevance of the CLDN12 expression in diverse diseases, including various types of cancer.

Acknowledgments:

I thank Professor Hideki Chiba, head of Department of Basic Pathology, Fukushima Medical University, for his generosity and guidance.

I also express my gratitude to Dr. Kotaro Sugimoto and Dr. Makoto Kobayashi for their supervised directions and immense contribution to this research.

I thank Dr. Yuta Endo, Dr. Manabu Kojima, Dr. Shigenori Furukawa, and Dr. Takafumi Watanabe for their enormous assistance in this project and maintenance of patient related prerequisites.

Additionally, I pay homage to Professor Shu Soeda, Professor Yuko Hashimoto, and Professor Keiya Fujimori for their kind collaboration from the respective departments.

Finally, I thank Seiko Watanabe, Ai Kubo, and Keiko Watari for their skillful technical assistance.

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