Histol Histopathol (2016) 31: 921-931

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Analysis of the expression and localization of tight junction transmembrane proteins, claudin-1, -4, -7, occludin and JAM-A, in human cervical adenocarcinoma

Taishi Akimoto[1], Akira Takasawa[2], Masaki Murata[2], Yui Kojima[2], Kumi Takasawa[2], Masanori Nojima[4], Tomoyuki Aoyama[2], Yutaro Hiratsuka[2], Yusuke Ono[2], Satoshi Tanaka[2], Makoto Osanai[2], Tadashi Hasegawa[3], Tsuyoshi Saito[1] and Norimasa Sawada[2]

Departments of [1]Obstetrics and Gynecology, [2]Pathology and [3]Surgical Pathology, Sapporo Medical University School of Medicine, Sapporo and [4]Division of Advanced Medicine Promotion, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo,Tokyo, Japan

Summary. Objective. Tight junction proteins have recently been reported to be useful for distinguishing between neoplastic and non-neoplastic tissues. In this study, we evaluated the expression and localization of tight junction transmembrane proteins in human cervical adenocarcinoma and adenocarcinoma *in situ* (AIS), and we determined whether their expression patterns could distinguish cervical adenocarcinoma from non-neoplastic cervical glands. Methods. Fifty-five patients with cervical adenocarcinoma or AIS were included in this study. Surgical specimens were immunohistochemically stained for claudin (CLDN) -1, -4, -7, occludin, and JAM-A. Results. Significantly higher expression levels of CLDNs and JAM-A were found in cervical AIS and adenocarcinoma than in non-neoplastic glands. In cervical AIS and adenocarcinoma, localization of CLDN-1 and JAM-A was extended throughout the whole cell membranes, whereas they were predominantly expressed at the most apical cell-cell junction in non-neoplastic glands. ROC curve analysis revealed that immunoreactivities of CLDN-1 or JAM-A successfully distinguished neoplasms from non-neoplastic cervical glands with high specificity (CLDN-1, 79.1%; JAM-A, 79.1%) and high sensitivity (CLDN-1, 84.1%; JAM-A, 95.5%). Conclusions. As expected, there were immunohistochemical differences between cervical adenocarcinoma and non-neoplastic cervical glands by using antibodies against tight junction transmembrane proteins. These results suggest that CLDN-1 and JAM-A are potential biomarkers for cervical adenocarcinoma.

Key words: Tight junction transmembrane protein, Claudin, JAM-A, Immunohistochemistry, Cervical adenocarcinoma, Molecular marker

Offprint requests to: Akira Takasawa, Nishi 16-chome, Minami 1-jo, Chuo-ku, Sapporo-shi, Hokkaido 060-8543, Japan. e-mail: atakasawa@sapmed.ac.jp

DOI: 10.14670/HH-11-729

Introduction

The incidence and mortality rate of carcinomas of the uterine cervix have declined in the USA, Western Europe and other developed countries. The decline is mainly due to implementation of cytologic screening and detection of premalignant conditions. However, previous research showed increasing incidences of adenocarcinoma compared with the incidences of squamous cell carcinoma (SCC). Between the 1950s and 1960s, approximately 95% of cervical cancers were SCC, and adenocarcinoma accounted for only 5% of cervical cancer in the United States and Western Europe (Heplar et al., 1952; Mikuta and Celebre, 1969). Now, SCC accounts for 75-80% of invasive cervical cancers, whereas adenocarcinoma accounts for 20-25%. The incidence of cervical adenocarcinoma has shown a tendency to increase in young women (Smith et al., 2000; Sasieni and Adams, 2001; Wang et al., 2004; Bray et al., 2005; Buld et al., 2005; Gien et al., 2010).

Although some studies showed no difference between survival of patients with SCC and patients with adenocarcinoma, numerous studies have shown that adenocarcinoma has a worse prognosis than that of SCC. Accurate initial diagnosis has important implications related to appropriate triage of high-risk patients, but many studies have shown that diagnosis of a cervical glandular lesion is more difficult than that of a squamous cell lesion (Krane et al., 2001; Costa et al., 2007; Jordan et al., 2013). Accordingly, new useful biomarkers for diagnosis are needed to improve the poor outcome of the disease. In recent years, tight junction proteins have received attention as diagnostic biomarkers for several types of malignant tumors (Soini et al., 2012; Keira et al., 2015).

The claudin (CLDN) family, which consists of at least 27 trans-membrane protein members, is solely responsible for the formation of tight junction strands and for the regulation of tight junction barrier and fence functions (Scneeberger and Lynch, 1992; Mineta et al., 2011). CLDNs regulate the permeability of epithelial and endothelial cell layers, and they also contribute to determination of cell polarity and segregation of apical parts of the cell membrane from basal areas. Thus, they create compartments on surfaces of polarized epithelial cells (Krause et al., 2008; Sawada, 2013). Junctional adhesion molecule-A (JAM-A), another transmembrane protein of the tight junction, associates with the tight junction components cingulin and occludin and with the PDZ domain-containing protein ZO-1, which can act as a scaffold for larger complexes (Bazzoni et al., 2000). It has been suggested that JAM-A may participate in regulation of the tight junctions and maintenance of paracellular permeability (Williams et al., 1999). The role and expression of JAM-A in inflammation has been documented in detail. JAM-A plays a role in regulation of leukocyte transendothelial migration and angiogenesis (Severson and Parkos, 2009). More recently, it has been shown that JAM-A overexpression was significantly associated with clinical stage and prognosis of several cancers (McSherry et al., 2009; Zhang et al., 2013).

In recent years, some researchers have reported that CLDNs are expressed in a tissue-specific manner. Furthermore, unique claudin composition is also characteristic of cancerous tissues, which exhibit tumor type-specific and even differentiation-specific up- or down-regulation of certain CLDNs (Soini, 2005; Szabo et al., 2009; Sawada, 2013). These specific patterns can even be exploited as differential diagnostic or prognostic tools (Soini, 2005; Lodi et al., 2006; Choi et al., 2007; Szabo et al., 2009; Szasz et al., 2011). Some authors have also previously suggested that CLDNs may be of use as markers of the initial stage in cervical cancer and cervical intraepithelial neoplasia (Lee et al., 2005; Sobel et al., 2005; Cunniffe et al., 2012). In addition, JAM-A is abnormally regulated and therefore represents a promising molecular target for therapy (Murakami et al., 2011; Goetsch et al., 2012). However, little is known about the relationship between tight junction proteins and cervical adenocarcinoma.

In this study, we examined the immunohistochemical expression and localization of CLDNs (1, 4, and 7), occludin, and JAM-A, transmembrane proteins in the tight junction barrier, in adenocarcinoma of the uterine cervix and AIS and analyzed the diagnostic usefulness of these markers for diagnosis of cervical adenocarcinoma.

Materials and methods

Case selection of surgical specimens

Specimens of 61 cases of cervical adenocarcinoma (33 usual type of endocervical adenocarcinoma "MuE", 4 intestinal type of mucinous carcinoma "MuI", 3 gastric type of mucinous carcinoma (or minimal deviation type) "MuM", 2 signet-ring cell type of mucinous carcinoma "MuS", 3 villoglandular type "MuV", and 16 adenocarcinoma *in situ* "AIS") obtained by surgical resections from 55 patients during the period from 2004 to 2012 were retrieved from the pathology file of Sapporo Medical University Hospital, Sapporo, Japan. The tissues of the 55 patients contained (i) adenocarcinoma only (39 cases), (ii) adenocarcinoma and AIS (6 cases), and (iii) AIS only (10 cases). As controls, adjacent non-neoplastic regions were examined as normal tissues (n=55). The histological type was based on WHO classification of tumors of the uterine cervix (4th edition). Tumor staging was based on both UICC classification (7th edition) and International Federation of Gynecology and Obstetrics (2008). All slides were independently evaluated by two pathologists (TA and MM). Discordant cases were discussed, and a consensus was reached.

Immunohistochemical staging of surgical specimens

The H&E slides of all cases were reviewed to select representative sections. New sections from paraffin blocks were obtained and stained immunocytochemically by the labeled polymer method. Sections were dewaxed, rehydrated and moistened with phosphate-buttered saline (PBS) (pH 7.4) and then pretreated in an autoclave at 121°C for 5 min in 10 mmol/l citrate buffer (pH 6.0), followed by 30-min incubation with antibodies to the following antigens in an automated immunostaining system (Dako Autostainer; Dako, Carpinteria, California, USA): CLDN-1 (Invitrogen, x 100), CLDN-4 (IBL, x 100), CLDN-7 (IBL, x 100), occludin (Invitrogen, x100), and JAM-A (abcam, x 200). Evaluation of immunoreactivity was classified as cytoplasmic (C), membranous (M), and tight junctional (TJ). We summarized the percentage and type of localization of tight junction proteins in each histological type. If immunocytochemical expression was observed in two or more cellular compartments, we evaluated the stronger staining intensity as the main localization. The intensity of staining was assessed as strong (3), moderate (2), weak (1) or negative (0). The

proportions of positively stained tumor cells were recorded as 0 (no staining), 1 (1-10%), 2 (11-50%), 3 (51-80%) and 4 (81-100%). Because neoplasm heterogeneity caused varying degrees of immunoreactivity in the slides, we used an immunoreactive score (IRS) (i.e., intensity 3 x proportion 4=immunoreactive score 12, scale of 0 to 12) for improvement in accuracy (Remmele et al., 1986).

Statistics

Continuous variables between groups were compared using ANOVA with post hoc Dunnett's test. The reference group for Dunnett's comparison was the non-neoplastic group. Each cutoff value was calculated for the IRS of CLDN-1, CLDN-4, CLDN-7, occludin, and JAM-A so that it could distinguish neoplasms (adenocarcinoma / AIS) from non-neoplastic glands. The score with the highest sensitivity and specificity was used to define the receiver operator characteristic (ROC) curve. From the ROC curve, the area under the receiver operator characteristic curve (AUC) was calculated. The 95% confidence intervals were used to test the hypothesis that AUC is 0.5. Kruskal-Wallis test was applied to evaluate statistical differences of localization status between neoplastic groups (adenocarcinoma or AIS) and non-neoplastic group.

All statistical calculations were performed with the use of SPSS statistics ver. 20.

Results

Patient characteristics and clinicopathological characteristics

Patient characteristics and clinicopathological characteristics are summarized in Table 1. The study population consisted of 55 patients with an age range of 25 to 79 years. The median age of the patients was 42 years. The clinical stage status was determined and the numbers of patients were: AIS=10, IA=4, IB=33, IIA=3, and IIB=5 (by FIGO classification), and 0=10, IA=6, IB=28, IIA=4, IIB=1, and IIIB=6 (by UICC classification). Endocervical type adenocarcinoma (MuE) was the most represented histological type. The frequencies of lymphovascular space invasion (LVSI) and lymph node metastasis were 27.3% and 10.9%, respectively. It should be noted that partially represented AIS lesions in tissues of cervical adenocarcinoma patients were also included in the AIS of histological type.

Immunohistochemistry of CLDNs, occludin, and JAM-A

To examine expression patterns of tight junction proteins in non-neoplastic and neoplastic cervical gland tissues, we conducted immunohistochemistry on the surgical specimens from patients with cervical mucinous adenocarcinoma and adenocarcinoma *in situ* (AIS). In the present study, we examined five tight junction-associated integral membrane proteins, CLDNs (1, 4, and 7), occludin, and JAM-A.

Notably, four of the five antibodies revealed prominent immunoreactivities in adenocarcinoma including AIS, characterized by the preservation of normal glandular architecture with an abrupt transition between normal and cytologically atypical cells in H&E staining (Figs. 1, 2). As shown in Fig. 1, strong immunostaining of CLDN-1 was observed along whole cell membranes of AIS cells, forming a linear staining pattern. On the other hand, CLDN-1 immunostaining was undetectable in most of the non-neoplastic cervical glands. In some non-neoplastic glands, positive staining was very weakly visible as dots, which represent the most apical cell-to-cell junction known as a tight junction. The different staining patterns of CLDN-1 allowed us to clearly distinguish AIS from non-neoplastic regions. Most types of adenocarcinoma also showed a staining pattern similar to that of AIS

Table 1. Clinicopathological features of cervical adenocarcinomas.

Patients (n=55)	
Age (range, median)	27-79, 42
Histological type	
Adenocarcinoma	45
Endocervical type (MuE)	33
Intestinal type (MuI)	4
Signet-ring cell type (MuS)	2
Minimal deviation type (MuM)	3
Villoglandular type (MuV)	3
AIS (Adenocarcinoma *in situ*)	16
Tumor stage (FIGO)	
AIS	10
IA	4
IB	33
IIA	3
IIB	5
Tumor stage (UICC)	
0	10
IA	6
IB	28
IIA	4
IIB	1
IIIA	0
IIIB	6
Tumor size	
AIS only	10
≤7mm	8
≤40mm	24
>40mm	13
Lymph node metastasis	
Negative	49
Positive	6
Lymphovascular infiltration	
Negative	40
Positive	15

throughout the cell membrane (Fig. 2), though we did not observe a linear membranous staining pattern of CLDN-1 in signet-ring cell type adenocarcinoma (MuS).

Staining patterns of CLDN-4 and -7 were basically similar to that of CLDN-1 in both AIS and adenocarcinoma, although immunoreactivities of CLDN-4 and -7 were not completely concentrated at tight junctions in non-neoplastic regions (Figs. 1, 2): CLDN-7 immunoreactivity was preferentially observed as a linear pattern throughout the cell membrane even in non-neoplastic cervical glands, and CLDN-4 was partially spread to the whole cell membrane beyond the tight junction region. Among the three molecules, anti-CLDN-1 antibody demonstrated the most obvious difference in staining pattern between non-neoplastic and neoplastic regions. As in the case of CLDN-1, strong immunoreactivity of JAM-A was observed on the whole cell membrane in adenocarcinoma and AIS, whereas immunoreactivity of JAM-A was restricted to tight junctions as dot-like staining in non-neoplastic cervical glands. In most adenocarcinoma cases, CLDNs and JAM-A were observed on the whole cell membrane, but CLDN-4 in a villoglandular type case and JAM-A in an endocervical type (Fig. 2) and a villoglandular type (data not shown) case were restricted to tight junctions. Immunostaining of occludin showed a dotted pattern at apical cell-to-cell junctions along membranes in non-neoplastic cervical glands and AIS (Fig. 1). In adenocarcinoma, various patterns were observed. A dotted pattern along the cell borders was found in half of the endocervical type cases, and intracellular glandular spotty positivity was observed in about 20% of the cases.

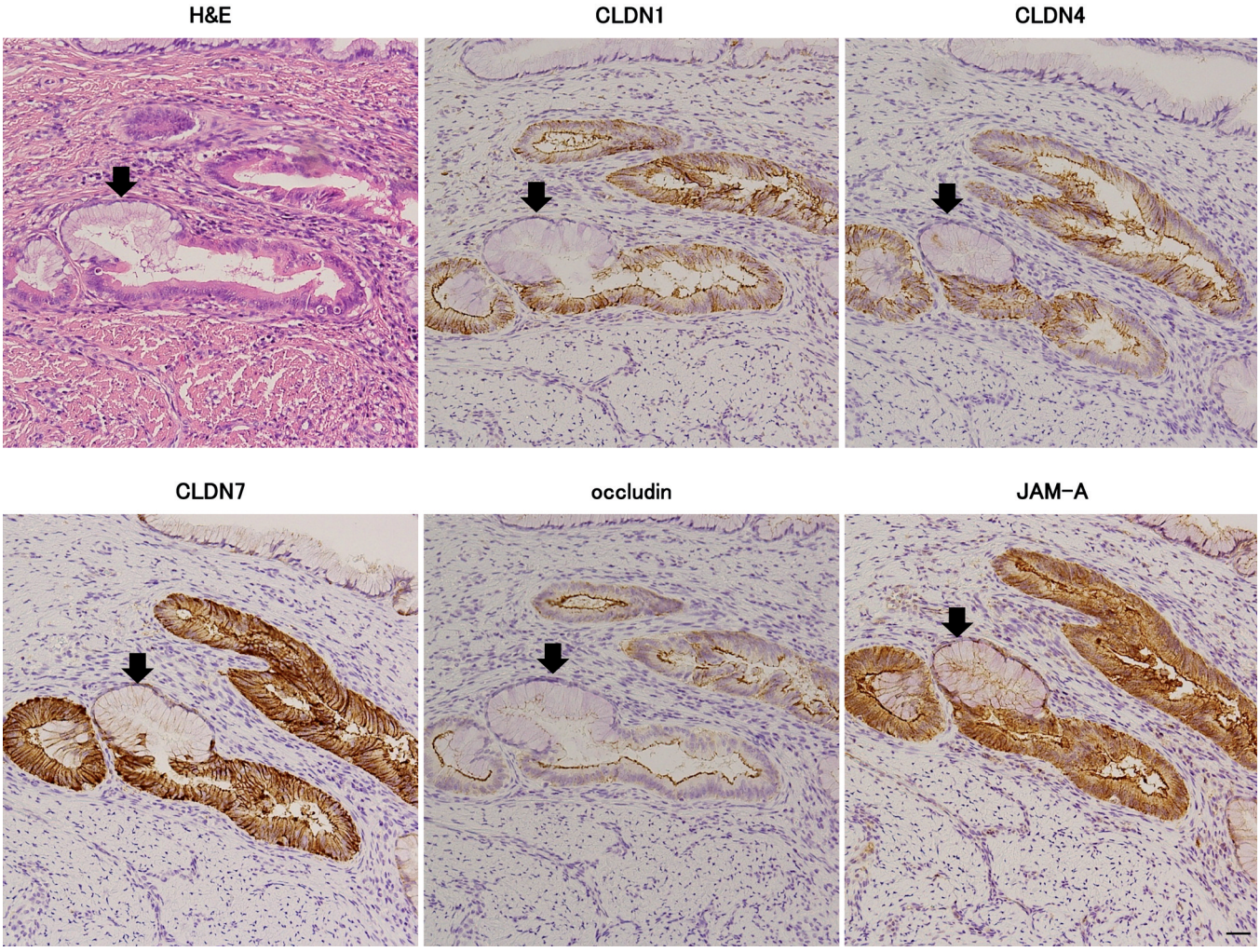

Fig. 1. Expression of tight junction proteins in surgical specimens of non-neoplastic cervical glands and AIS. Claudin (CLDN) -1, -4, -7 and JAM-A were strongly expressed throughout the whole cell membrane of AIS cells, allowing us to clearly distinguish AIS from non-neoplastic regions (arrows). Scale bar: 50 μm.

Increased expression of CLDNs and JAM-A in cervical adenocarcinoma and AIS

These results suggested that immunoreactivity of tight junction proteins could effectively differentiate precursor and malignant lesions from non-neoplastic cervical glands. To maintain high levels of reproducibility and accuracy of immunohistochemical evaluation, we defined a parameter, designated as immunoreactive score (IRS), which was calculated by multiplying intensity (4 grades) and proportion (5 grades) of immunoreactivity (Remmele et al., 1986). Mean ± SD values of IRS of CLDN-1, -4, -7, occludin and JAM-A in non-neoplastic cervical glands/AIS were 0.9±0.2/6±0.9, 1.6±0.44/4.1±1.27, 2.7±0.5/7.1±1.1, 1.8±0.4/4.7±1.2 and 1.9±0.3/8.9±0.9, respectively (Fig. 3). CLDN-1, -4, -7 and JAM-A were expressed at significantly higher levels in AIS than in non-neoplastic cervical glands.

Since adenocarcinoma also showed significantly higher expression levels of CLDN-1, -4, and -7 and JAM-A than the levels in non-neoplastic cervical glands (Fig. 3), we further examined the IRS with respect to each histological type. CLDN-1 expression was significantly higher in endocervical type (7.4±0.6), intestinal type (8.3±1.4) and minimal deviation type (9.0±0) than in non-neoplastic cervical glands (0.9±0.2). CLDN-4 expression was significantly higher in endocervical type (5.5±0.84) than in non-neoplastic cervical glands (1.6±0.44). CLDN-7 was more intensely expressed in endocervical type (9.2±0.6) and intestinal type (11±0.8) than in non-neoplastic cervical glands (2.7±0.5). Only JAM-A demonstrated significantly higher expression levels in all cervical adenocarcinomas including signet-ring cell type. IRS of occludin was not significantly increased in any of the histological types. We found that IRS for each molecule differed depending on the histological types, and endocervical type, the most common type of adenocarcinoma, had significantly high IRS for all of the molecules except for occludin. Furthermore, JAM-A had the highest IRS for each type of neoplasm, including AIS, among the examined tight junction proteins.

To distinguish neoplasms from non-neoplastic cervical glands in the surgical specimens from patients with cervical adenocarcinoma and AIS, we calculated the areas under the receiver operator characteristic curves (AUCs) for CLDN-1, -4, -7, occludin and JAM-A. The AUC of CLDN-1 was 0.944 [95% CI, 0.903 to

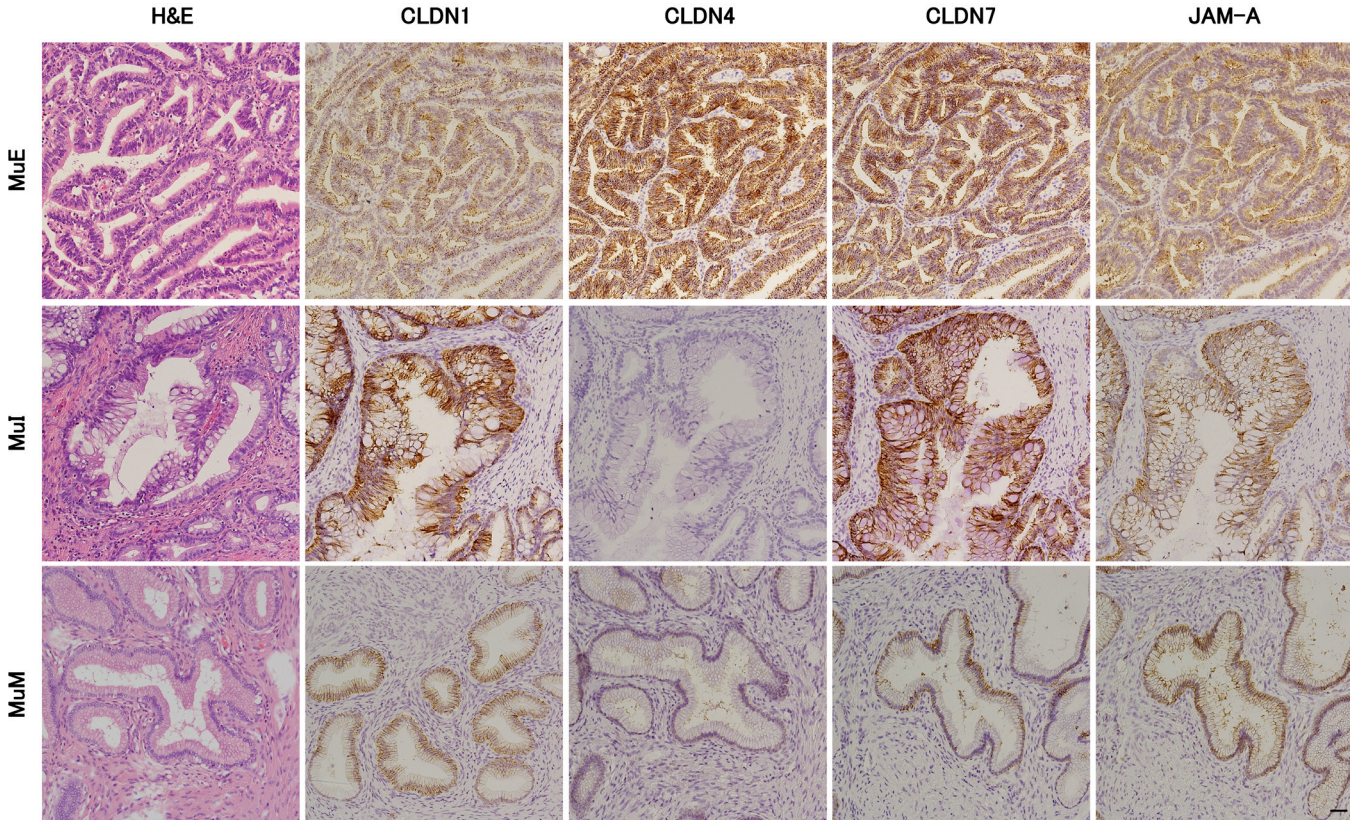

Fig. 2. Expression of tight junction proteins in different types of cervical adenocarcinoma. CLDN-1 and -7 and JAM-A were strongly expressed on the whole cell membrane in all three types. CLDN-4 was strongly expressed in MuE, the most common type of adenocarcinoma in this study. MuE: endocervical type, MuI: intestinal type, MuM: minimal deviation type. Scale bar: 50 μm.

0.984] and the highest sensitivity (86.9%) and specificity (90.9%) were obtained at a cutoff value of 1. As shown in Table 2, at a cutoff value of 1, 53 of 61 (86.9%) specimens with adenocarcinoma / AIS (38 of 45 with adenocarcinoma and 15 of 16 with AIS) were detected as neoplastic. Among specimens with non-neoplastic epithelium, 50 of 55 specimens were detected as non-neoplastic. The AUC of JAM-A was 0.988 [95% CI, 0.973 to 1.0] and the highest sensitivity (98.4%) and specificity (96.4%) were obtained at a cutoff value of 5. At that cutoff value, 57 of 61 (98.4%) specimens with adenocarcinoma / AIS (44 of 45 with adenocarcinoma and 13 of 16 with AIS) were detected as neoplastic. Among specimens with non-neoplastic epithelium, 53 of 55 specimens were detected as non-neoplastic. The AUC of CLDN-4 was 0.636 [95% CI, 0.660 to 0.817], and the

Table 2. Expression and localization of CLDN-1 and JAM-A in surgical specimens of non-neoplastic cervical glands, adenocarcinoma *in situ*, and adenocarcinoma.

	CLDN-1				JAM-A			
	+		-		+		-	
	M	TJ		p value	M	TJ		p value
Non-T	0	5	50		0	2	53	
Tumor	52	1	4	<.001	54	3	4	<.001
AIS	14	1	1	<.001	12	1	3	<.001
Adeno	38	0	3	<.001	42	2	1	<.001

Abbreviations: +, positive for cutoff value; -, negative for cutoff value; M, cell membrane; TJ, tight junction; Non-T, non-neoplastic glands; AIS, adenocarcinoma *in situ*; Adeno, adenocarcinoma. P value calculated vs. Non-T.

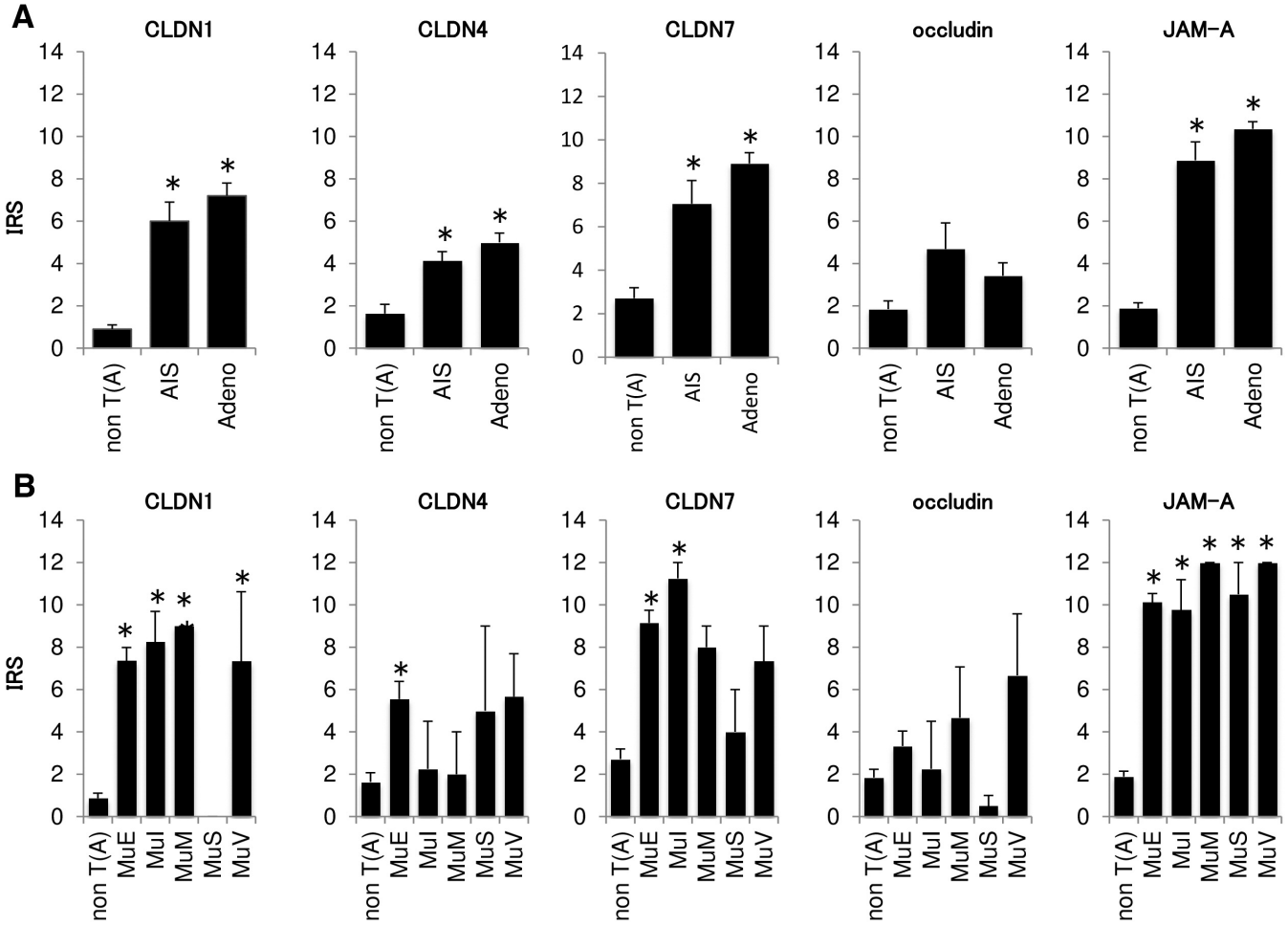

Fig. 3. Expression levels of CLDNs and JAM-A are elevated in cervical adenocarcinoma and AIS. **A and B.** Immunoreactive scores of tight junction proteins were quantified as described in Methods. Briefly, the score was calculated by multiplying intensity (4 grades) by proportion (5 grades) of immunoreactivity. **A.** The scores of CLDN-1, -4 and -7 and JAM-A were significantly higher in both AIS and Adeno than in non T(A). **B.** Immunoreactive scores of tight junction proteins in different histological types of adenocarcinoma. Data are represented as means ± SD. *p<0.001 vs. non T(A). IRS: immunoreactive score, non T(A): non-neoplastic gland, Adeno: adenocarcinoma, MuE: endocervical type, MuI: intestinal type, MuM: minimal deviation type, MuS: signet-ring cell type, MuV: villoglandular type.

highest sensitivity and specificity were 63.6% and 67.2%, respectively. The AUC of CLDN-7 was 0.914 [95% CI, 0.861 to 0.966], and the highest sensitivity and specificity were 83.6% and 85.5%, respectively. The AUC of occludin was 0.525 [95% CI, 0.562 to 0.746], and the highest sensitivity and specificity were 52.5% and 72.7%, respectively. ROC curve analysis revealed that the IRS of CLDN-1 and that of JAM-A can differentiate non-neoplastic cervical glands from adenocarcinomas with high levels of specificity and sensitivity.

Adenocarcinoma-associated translocation of CLDNs and JAM-A

In addition to increased expression of tight junction proteins, we found that the majority of adenocarcinoma cells showed diffuse membranous staining for proteins that were basically restricted to tight junction in non-neoplastic cells. These observations prompted us to examine whether the localization would help us to differentiate neoplasms from non-neoplastic cervical glands. To discuss the subcellular localization based on an objective evaluation, we took note of the subcellular staining localization pattern (tight junction, cell membrane and cytoplasm) for the tight junction proteins in each of the cases (see Methods for details). By this process, we successfully detected significant localization changes ($p<0.001$) of CLDN-1, -4 and JAM-A from tight junctions (non-neoplastic glands) to the whole cell membrane (AIS and adenocarcinoma) even though there was some heterogeneity in immunohistochemical staining in each case (Fig. 4).

It should be noted that all of the five specimens of non-neoplastic glands positive for CLDN-1 showed exclusive staining in the tight junction area, whereas almost all of the CLDN-1-positive specimens of AIS and adenocarcinoma were assigned to membranous staining at the best cutoff value of 1 (98.1%, 52/53, Table 2, Fig. 5). The same was the case for JAM-A: both of the JAM-A-positive specimens with non-neoplastic glands showed tight junction staining, whereas most of the JAM-A-positive specimens of AIS and adenocarcinoma were assigned to membranous staining at the best cutoff value of 5 (94.5%, 54/57). No significant difference in CLDN-1 and JAM-A localization was detected between AIS and invasive adenocarcinoma (Table 2).

Discussion

Patients with adenocarcinoma of the uterine cervix had a worse prognosis with 10-20% difference in the 5-year survival rates than those for patients with SCC. Clinical stage and lymph node metastasis are the most important prognostic factors in cervical adenocarcinoma (Wang et al., 2004), and early diagnosis is therefore critical. Thus, molecular profiling is crucial for a better understanding of cervical adenocarcinoma and may contribute to a better therapeutic approach to these tumors in the future. Moreover, these profiles carry great differential diagnostic value. This study was undertaken to analyze the expression of tight junction-associated integral membrane proteins, CLDNs (1, 4, and 7), occludin and JAM-A, in AIS and invasive adenocarcinoma of the uterine cervix. In the present study, we demonstrated a new approach for the diagnosis of AIS and invasive cervical adenocarcinoma by using immunohistochemistry of CLDNs (1, 4, and 7), occludin and JAM-A on surgical specimens. In cervical adenocarcinoma, this study is the first immunohistochemical investigation of tight junction proteins on surgical specimens.

We found that the expression levels of CLDN-1, -4, -7 and JAM-A were greatly increased in AIS and cervical adenocarcinoma tissues compared to their levels in non-neoplastic cervical glands. ROC curve analysis revealed that immunoreactivity of JAM-A and CLDN-1 can differentiate non-neoplastic cervical glands from

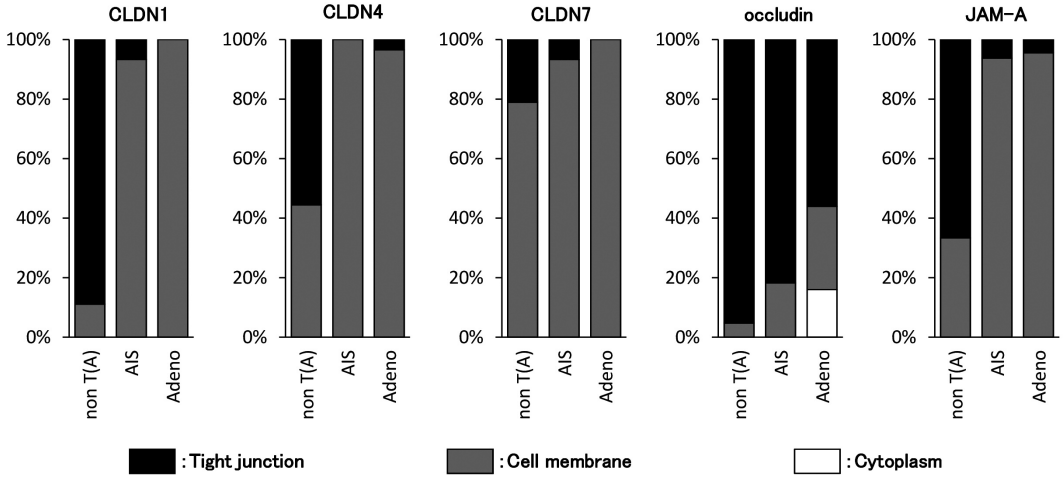

Fig. 4. Altered localization of tight junction proteins in cervical adenocarcinoma and AIS. Most CLDNs and JAM-A are extensively expressed throughout the whole cell membrane in AIS and adenocarcinomas, whereas their localizations are relatively limited to tight junctions in non T(A).non T(A): non-neoplastic gland, Adeno: adenocarcinoma.

adenocarcinomas with high levels of specificity and sensitivity, suggesting that they are useful diagnostic markers.

Furthermore, we demonstrated significant immunoreactivity localization changes of CLDN-1, -4, -7 and JAM-A from tight junctions (non-neoplastic glands) to the whole cell membrane (AIS and adenocarcinoma). Translocation of tight junction proteins provides additional evidence to distinguish neoplastic regions from non-neoplastic glands. Actually,

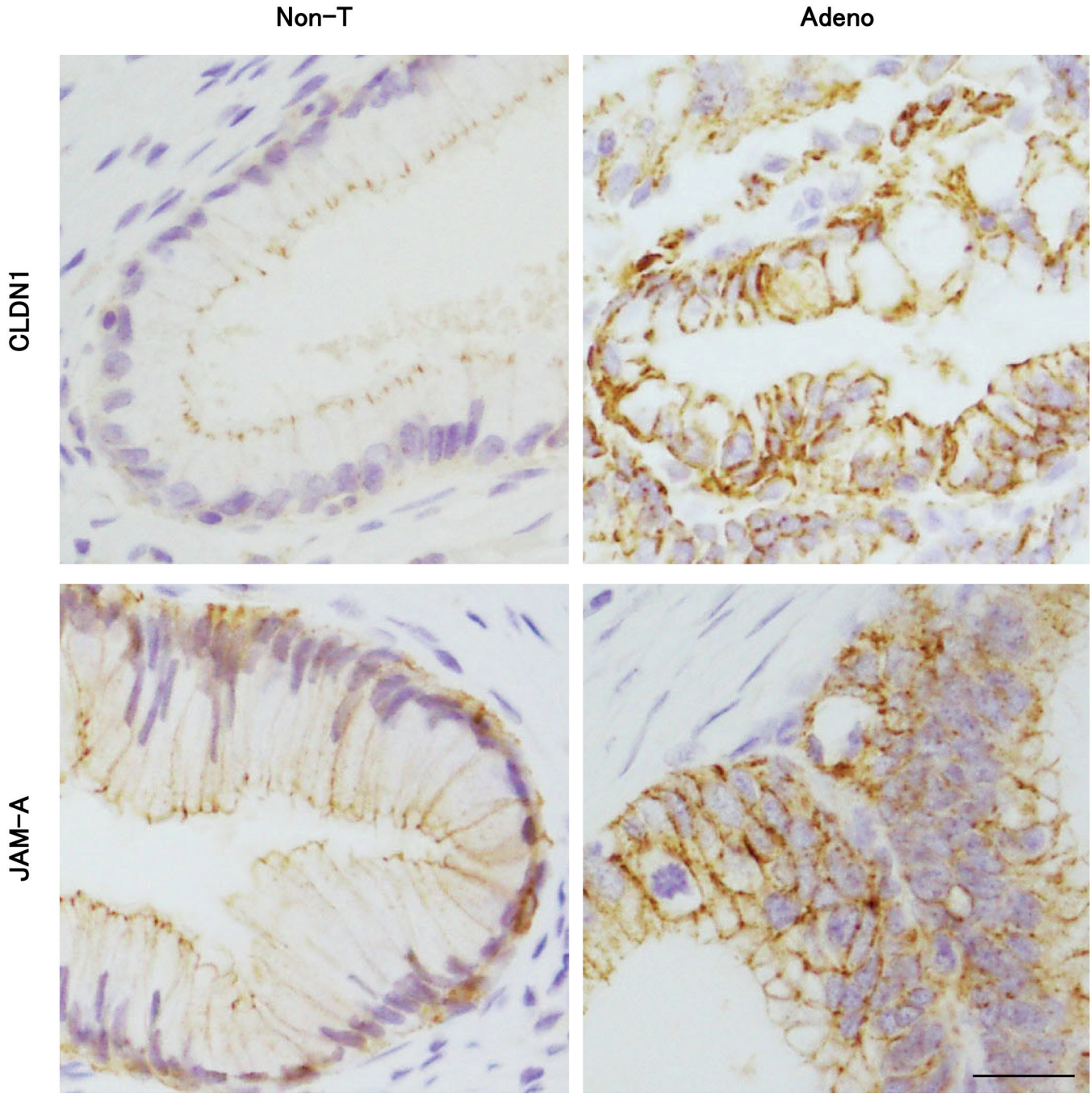

Fig. 5. Altered localization of CLDN-1 and JAM-A in cervical adenocarcinoma. At the best cutoff value, localization of CLDN-1 and JAM-A was restricted to tight junctions in positively judged non-neoplastic glands (Non-T), whereas their localization was throughout the membrane in positively judged adenocarcinomas (Adeno). Scale bar: 50 μm.

all of the specimens with the membranous staining pattern belonged to AIS or adenocarcinoma (CLDN-1 and JAM-A in Table 2, Fig. 5). We also found that all of the CLDN-1-positive specimens from samples with non-neoplastic glands showed a tight junction localization pattern. Considering the tight junction-restricted staining pattern, 100% (55/55) of the specimens with non-neoplastic glands could be detected as non-neoplastic even if a specimen had been judged as being positive solely on the basis of IRS (Table 2 and Fig. 5). According to our findings, the localization of CLDN-1 and JAM-A could rescue cases with non-neoplastic glands that had formerly been judged as positive for malignancy. In AIS and adenocarcinoma, since almost all of the specimens showed membranous localization, aberrant membranous localization is an important sign to identify adenocarcinomas of the uterine cervix.

In cancer cell lines, membranous localization of tight junction-associated proteins is frequently observed by PKC activation (Kyuno et al., 2011), stimulations of epidermal growth factor (EGF) signaling (Ogawa et al., 2012), and transforming growth factor-beta signaling (Kurose et al., 2007), all of which play critical roles in the invasion, migration, and epithelial-mesenchymal transition of tumor cells. Multiple mechanisms may be involved in changes of tight junction protein localization. Thus, the association between translocation of tight junction proteins and malignant transformation in the uterine cervix found in this study might be explained by impairment of fence function via various stimuli.

Among cervical adenocarcinomas, minimal deviation adenocarcinoma of the uterine cervix (MDA), otherwise known as adenoma malignum, is a rare variant and its deceptively benign microscopic appearance makes a definitive diagnosis difficult. In the present study, we demonstrated that CLDN-1, -7 and JAM-A were increased in MDA (MuM in this study) and that their subcellular localization was extended from the tight junction region to the whole cell membrane region. Our results suggest that the tight junction proteins may be helpful for immunohistochemical diagnosis of MDA. Recent immunohistochemical studies on CLDNs indicated that CLDNs may be useful markers for differentiating adenocarcinoma from normal epithelium. Soini et al. reported that CLDN-7 and CLDN-18 were more frequently expressed in pancreatic ductal carcinoma than in non-neoplastic pancreatic ducts (Soini et al., 2012). We also reported that the CLDN-18 staining pattern permits us to distinguish biliary tract cancers and biliary intraepithelial neoplasia from normal epithelium (Keira et al., 2015).

Overexpression of CLDN-4 in the cell membrane of adenocarcinoma cells suggested that targeted therapy using clostridium perfringens enterotoxin (CPE) might be effective for cervical adenocarcinomas. CPE, a 35-kDa single polypeptide comprised of 319 amino acids, is a major source of food poisoning and is known as a ligand for CLDN-3 and -4. CPE binds to CLDN-3 and -4 with resultant pore formation on the cell membrane, causing cell death via influx of Ca^{2+} into the cell (Smedley et al., 2007). Hence, CLDN-3 and CLDN-4 are expected to be promising candidates for targeted therapy using CPE against carcinomas over-expressing them, such as prostate, breast, and ovarian cancers (Kominsky et al., 2004; Morin, 2005; Santin et al., 2005, 2007; Maeda et al., 2012). When CLDN-4 was located in the membrane region, cytotoxicity of CPE was observed (Maeda et al., 2012). In our investigation, CLDN-4 was observed in the most apical region in almost all of the non-neoplastic cervical glands, but CLDN-4 was redistributed to the whole cell membrane region in AIS and cervical adenocarcinoma. These findings suggest that the use of CPE might be an effective therapeutic method targeting CLDN-4 in cervical adenocarcinoma.

Overexpression of JAM-A in tumor cells has previously been described, but the role of JAM-A in tumor proliferation and dissemination is controversial. Naik et al. initially reported that JAM-A expression on breast cancer cell lines inversely correlated with invasiveness and motility potential. Additionally, loss of JAM-A was associated with tumor progression in breast cancer patients (Naik et al., 2008). On the other hand, more recently, a significant association was found between JAM-A overexpression and poor prognosis in several large independent cohorts of breast and lung cancer patients (McSherry et al., 2009; Murakami et al., 2011; Zhang et al., 2013). In those reports, JAM-A was referred to as a therapeutic target. Recently, an anti-JAM-A monoclonal antibody has been demonstrated to be effective in breast cancer and lung cancer in an animal model. Xenograft tumors significantly shrank in the mice with an anti-JAM-A antibody treatment compared with those in the untreated mice (Goetsch et al., 2012). In the present study, JAM-A was found to be overexpressed in AIS and cervical adenocarcinoma compared to the expression level in non-neoplastic cervical glands, and the anti-JAM-A monoclonal antibody might therefore be effective for these tumors.

In cervical adenocarcinoma, CLDN-1 and JAM-A were strongly expressed and distributed throughout the whole cell membranes, whereas they were stained faintly and predominantly at apical cell-to-cell junctions known as tight junctions in non-neoplastic glands. Statistical analysis revealed that immunohistochemistry of CLDN-1 or JAM-A successfully distinguished neoplasms from non-neoplastic cervical glands with high specificity and high sensitivity. The results suggest that CLDN-1 and JAM-A are potential biomarkers for cervical adenocarcinoma.

Acknowledgements. This work was supported by JSPS Grants-in-Aid for Scientific Research Grant Number 26460421.

Conflict of interest. The authors declare that they have no conflict of interest.

References

Bazzoni G., Maria O., Estrada M., Orsenigo F., Cordenonsi M., Citi S. and Dejana E. (2000). Interaction of junction adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. J. Biol. Chem. 275, 20520-20526.

Bray F., Carstensen B., Moller H., Zappa M., Zakelj M.P., Lawrence G., Hakama M. and Weiderpass E. (2005). Incidence trends of adenocarcinoma of the cervix in 13 European countries. Cancer Epidemiol. Biomarkers Prev. 14, 2191-2199.

Buld S., Visser O., Rozendaal L., Verheijen R.H.M. and Meijer C.J.L.M. (2005). Cervical cancer in the Netherlands 1989-1998: Decrease of squamous cell carcinoma in older women, increase of adenocarcinoma in younger women. Int. J. Cancer 113, 1005-1009.

Choi Y.L., Kim J., Kwon M.J., Choi J.S., Bae D.S., Koh S.S., In Y.H. Park Y.W., Kim S.H., Ahn G. and Shin Y.K. (2007). Expression profile of tight junction protein claudin 3 and claudin 4 in ovarian serous adenocarcinoma with prognostic correlation. Histol. Histopathol. 22, 1185-1195.

Costa S., Negri G., Sideri M., Santini D., Martinelli G., Venturoli S., Pelusi C., Syrjanen S., Syrjanen K. and Pelusi G. (2007). Human papillomavirus (HPV) test and PAP smear as predictors of outcome in conservatively treated adenocarcinoma *in situ* (AIS) of uterine cervix. Gynecol. Oncol. 106, 170-176.

Cunniffe C., Ryan F., Lambkin H. and Brankin B. (2012). Expression of tight and adherens junction proteins in cervical neoplasia. Br. J. Biomed. Sci. 69, 147-153.

Gien L.T., Beauchmin M.C. and Thomas G. (2010). Adenocarcinoma: Aunique cervical cancer. Gynecol. Oncol. 116, 140-146.

Goetsch L., Haeuw J.F., Bear-Larvor C., Gonzalez A., Zanna L., Malissard M., Lepecquet A.M., Robert A., Bailly C., Broussas M. and Corvaia N. (2012). A novel role for junctional adhesion molecule-A in tumor proliferation: modulation by an anti-JAM-A monoclonal antibody. Int. J. Cancer 132, 1463-1474.

Heplar T.K., Dockerty M.B., Tnadall L.M. and Minn R. (1952). Primary adenocarcinoma of the cervix. Am. J. Obstet. Gynecol. 63, 800-808.

Jordan S.M., Chase D.M., Watanabe T., Osann K. Monk B.J. and Rutgers J.K.L. (2013). High pathologic misdiagnosis of cervical adenocarcinoma *in situ*. Eur. J. Gynaecol. Oncol. 34, 446-449.

Keira Y., Takasawa A., Murata M., Nojima M., Takasawa K., Ogino J., Higashiura Y., Sasaki A., Kimura Y., Mizuguchi T., Tanaka S., Hirata K., Sawada N. and Hasegawa T. (2015). An immunohistochemical marker panel including claudin-18, mapsin, and p53 improves diagnosis accuracy of bile duct neoplasms in surgical and presurgical biopsy specimens. Virchows Arch. 466, 265-277.

Kominsky S.L., Vali M., Korz D., Gabig T.G., Weitzman S.A., Argani P. and Sukumar S. (2004). Clostridium perfringens enterotoxin elicits rapid and specific cytolysis of breast carcinoma cells mediated through tight junction proteins claudin 3 and 4. Am. J. Pathol. 164, 1627-1633.

Krane J.F., Granter S.R., Trask C.E., Hogan C.L. and Lee K.R. (2001). Papanicolaou smear sensitivity for the detection of adenocarcinoma of the cervix: a study of 49 cases. Cancer 93, 8-15.

Krause G., Winkler L., Mueller S.L., Haseloff R.F., Piontek J. and Blasig I.E. (2008). Structure and function of claudins. Biochim. Biophys. Acta 1778, 631-645.

Kurose M., Kojima T., Koizumi J., Kamekura R., Ninomiya T., Murata M., Ichimiya S., Osanai M., Chiba H., Himi T. and Sawada N. (2007). Induction of claudins in passaged hTERT-transfected human nasal epithelial cells with an extended life span. Cell Tissue Res. 330, 63-74.

Kyuno D., Kojima T., Ito T., Yamaguchi H., Tsujiwaki M., Takasawa T., Murata M., Tanaka S., Hirata K., Sawada N. (2011). Protein kinase Cα inhibitor enhances the sensitivity of human pancreatic cancer HPAC cells to Clostridium perfringens enterotoxin via claudin-4. Cell Tissue Res. 346, 369-381.

Lee J.W., Lee S.J., Seo J.W., Seo J.W., Song S.Y., Ahn G., Park C.S., Lee J.H. Kim B.G. and Bae D.S. (2005). Increased expressions of claudin-1 and claudin-7 during the progression of cervical neoplasia. Gynecol. Oncol. 97, 53-59.

Lodi C., Szabo E., Holczbauer A., Batmunkh E., Szijarto A., Kupcsulik P., Kovalszky I., Paku S., Illyes G., Kiss A. and Schaff Z. (2006). Claudin-4 differentiates biliary tract cancers from hepatocellular carcinomas. Mod. Pathol. 19, 460-469.

Maeda T., Murata M., Chiba H., Takasawa A., Tanaka S., Kojima T., Masumori N., Tsukamoto T. and Sawada N. (2012). Claudin-4-targeted therapy using Clostridium perfringens enterotoxin for prostate cacner. Prostate 72, 351-360.

McSherry E.A., McGee S.F., Jirstrom K., Doyle E.M., Brennan D.J., Landberg G., Dervan P.A., Hopkins A.M. and Gallagher W.M. (2009). JAM-A expression positively correlates with poor prognosis in breast cancer patients. Int. J. Cancer 125, 1343-1351.

Mikuta J.J. and Celebre J.A. (1969). Adenocarcinoma of the cervix. Obstet. Gynecol. 33, 753-756.

Mineta K., Yamamoto Y., Yamazaki Y., Tanaka H., Tada Y., Saito K., Tamura A., Igarashi M., Endo T., Takeuchi K. and Tsukita S. (2011). Predicted expansion of the claudin multigene family. FEBS Lett. 585, 606-612.

Morin P.J. (2005). Claudin proteins in human cancer; promising new targets for diagnosis and therapy. Cancer Res. 65, 9603-9606.

Murakami M., Giampietro C., Giannotta M., Corada M., Trselli I., Orsenig F., Cocito A., d'Ario G., Mazzarol G., Confalonieri S., Di Fiore P.P. and Dejana E. (2011). Abrogation of junctional adhesion molecule-A expression induces cell apoptosis and reduces breast cancer progression. Plos One 6, e21242.

Naik M.U., Naik T.U., Suckow A.T., Duncan M.K. and Naik U.P. (2008). Attenuation of junctional adhesion molecule-A is a contributing factio for breast cancer cell invasion. Cancer Res. 68, 2194-2203.

Ogawa M., Kojima T., Someya M., Nomura K., Takasawa A., Murata M., Tanaka S., Saito T. and Sawada N. (2012). Epidermal growth factor modulates claudins and tight junctional functions in ovarian cancer cell lines. Histochem. Cell Biol. 138, 323-328.

Remmele W., Hidebrand U., Hienz H.A., Klein P.J., Vierbuchen M., Behnken L.J. Heicke B. and Scheidt E. (1986). Comparative histological, histochemical, immunohistochemical and biochemical studies on estrogen receptors, lectin receptors, and Barr bodies in human breast cancer. Virchows Arch. A 409, 127-147.

Santin A.D., Cane S., Bellone S., Palmieri M., Siegel E.R., Thomas M., Roman J.J., Burnett A., Cannon M.J. and Pecorelli S. (2005). Treatment of chemotherapy-resistant human ovarian cancer xenografts in C.B-17/SCID mice by intraperitoneal administration of Clostridium perfringens enterotoxin. Cancer Res. 65, 4334-4342.

Santin A.D., Bellone S., Siegel E.R., McKenney J.K., Thomas M., Roman J.J., Burnett A., Tognon G., Bandiera E. and Pecorelli S. (2007). Overexpression of *Clostridium perfringens* enterotoxin receptors claudin-3 and claudin-4 in uterine carcinosarcomas. Clin. Cancer Res. 13, 3339-3346.

Sasieni P. and Adams J. (2001). Changing rates of adenocarcinoma

and adenosquamous carcinoma of the cervix in England. Lancet 357, 1490-1493.

Sawada N. (2013). Tight junction-related human diseases. Pathol. Int. 63, 1-12.

Scneeberger E.E. and Lynch R.D. (1992). Structure, function, and regulation of cellular tight junctions. Am. J. Physiol. 262, L647-L661.

Severson E.A. and Parkos C. (2009). Structural determinants of junctional adhesion molecule A (JAM-A) function and mechanisms of intracellular signaling. Curr. Opin. Cell Biol. 21, 701-707.

Smedley J.G. 3rd, Uzal F.A. and McClane B.A. (2007). Identification of a prepore large-complex stage in the mechanism of action of Clostridium perfringens enterotoxin. Infect. Immun. 75, 2381-2390.

Smith H.O., Tiffany M.F., Qualls C.R. and Key C.R. (2000). The rising incidence of adenocarcinoma relative to squamous cell carcinoma of the uterine cervix in the United States-A 24-year population-based study. Gynecol. Oncol. 78, 97-105.

Sobel G., Paska C., Szabo I., Kiss A., Kadar A. and Schaff Z. (2005). Increased expression of claudins in cervical intraepithelial neoplasia and invasive carcinoma. Hum. Pathol. 36, 162-169.

Soini Y. (2005). Expression of claudins 1, 2, 3, 4, 5 and 7 in various types of tumors. Histopathology 46, 551-560.

Soini Y., Takasawa A., Eskelinen M., Juvonen P., Karja V., Hasegawa T., Murata M., Tanaka S., Kojima T. and Sawada N. (2012). Expression of claudins 7 and 18 in pancreatic ductal adenocarcinoma: association with features of differentiation. J. Clin. Pathol. 65, 431-436.

Szabo I., Kiss A., Schaff Z. and Sobel G. (2009). Claudins as diagnostic and prognostic markers in gynecological cancer. Histol. Histopathol. 24, 1607-1615.

Szasz A.M., Nemeth Z., Gyorffy B., Micsinai M., Krenacs T., Baranyai Z., Harsanyi L., Kiss A., Schaff Z., Tokes A.M. and Kulka J. (2011). Identification of a claudin-4 and E-cadherin score to predict prognosis in breast cancer. Cancer Sci. 102, 2248-2254.

Wang S.S., Sherman M.E., HIdesheim A., Lacey Jr. J.V. and Devesa S. (2004). Cervical adenocarcinoma and squamous cell carcinoma incidence trends among white women and black women in the United States for 1975-2000. Cancer 100, 1035-1044.

Williams L.A., Martin-Padura I., Dejana E., Hogg N. and Simmons D.L. (1999). Identification and characterization of human junctional adhesion molecule (JAM). Mol. Immunol. 36, 1175-1188.

Zhang M., Luo W., Huang B., Liu Z., Sun L., Zhang Q., Qui X, Xu K. and Wang E. (2013). Overexpression of JAM-A in Non-Small Cell Lung Cancer correlates with tumor progression. Plos One 11, e79173.

Accepted February 5, 2016