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Usami, Yu ; Chiba, Hideki ; Nakayama, Fumihito ; Ueda, Junya ; Matsuda, Yoshiko ; Sawada, Norimasa ; Komori, Takahide ; Ito, Akihiko ;…

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Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus[☆]

Yu Usami DDS[a, b], Hideki Chiba MD, PhD[c], Fumihito Nakayama MD[a], Junya Ueda MD[a], Yoshiko Matsuda MD[a], Norimasa Sawada MD, PhD[c], Takahide Komori DDS, PhD[b], Akihiko Ito MD, PhD[a], Hiroshi Yokozaki MD, PhD[a, *]

[a] *Division of Surgical Pathology, Department of Biomedical Informatics, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan*

[b] *Department of Oral and Maxillofacial Surgery, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan*

[c] *Department of Pathology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan*

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*Correspondence: Hiroshi Yokozaki, MD, PhD, Division of Surgical Pathology, Department of Biomedical Informatics, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Telephone, +81-78-382-5460; Fax, +81-78-382-5479;

E-mail, hyoko@med.kobe-u.ac.jp

Summary

Claudins are transmembrane proteins that seal tight junctions, bind with peripheral protein ZO-1 (zonula occludens-1), and are known to play an important role in several normal tissues and cancers. However, the role of claudin-1 and -7 expressions in esophageal squamous cell carcinoma remains to be clarified. In the present study, we confirmed the expressions of claudin-1, -7, and ZO-1 in the prickle cell layer of the normal human esophageal squamous epithelium. To clarify their role in the tumor progression, the expressions of claudin-1 and -7 at the invasive front of the esophageal squamous cell carcinoma were analyzed immunohistochemically. Reduced expression of claudin-7 at the invasive front of the esophageal cancer was significantly associated with the depth of invasion (P = 0.004), stage (P = 0.038), lymphatic vessel invasion (P = 0.001), and lymph node metastasis (P = 0.014). In contrast, significant association was not detected between claudin-1 expression and clinicopathological factors, except histological differentiation of the tumor (P = 0.0029). Comparison of claudin-7 expression at the invasive front of the primary tumor and its corresponding metastatic lymph nodes revealed significant reduction in claudin-7 expression in the metastatic lymph nodes (P = 0.007). These results suggest that the reduced expression of claudin-7 at the invasive front of esophageal squamous cell carcinoma may lead to tumor progression and subsequent metastatic events. Thus, claudin-7 can be a novel marker for the prediction of lymph node metastasis.

1. Introduction

Cell-cell adhesion plays a critical role in the establishment and maintenance of cell polarity and cell society. It has long been known that cell-cell adhesiveness is generally reduced in various human cancers. Tumor cells are dissociated throughout the tumor masses, lose their cell polarity, and infiltrate the stroma, thereby leading to subsequent metastatic events in a scattered manner. The dissociation of cancer cells from cancer nests is a crucial step, and the suppression of cell-cell adhesiveness may trigger the release of cancer cells from the primary cancer nests and confer invasive properties on a tumor [1, 2]. Previous reports have indicated a relationship between carcinomas and tight junctions [3, 4].

In simple epithelium, tight junctions are positioned at the boundary of the apical and basolateral plasma membranes, and play an important role in the paracellular barrier and cell polarity [5-8]. Several lines of evidence have revealed that the granular cell layer of stratified epithelium of the skin possesses tight junctions that are crucial for the barrier function [5, 6, 9-11]. The tight junctions consist of membrane and peripheral proteins. Occludin and claudins are known as membrane proteins and ZO (zonula occludens) -1, -2, and -3 are peripheral proteins. Claudins are composed of 4 transmembrane domains and 2 extracellular loops through which they bind to corresponding claudins in the cell-cell contact. Claudins also bind to ZO-1, -2, or -3 via their carboxyl terminal in the cytoplasm [8, 9]. Claudin-1 and -7 are two members of the 24 claudin multigene family [8, 11]. The distribution of claudin-1 and -7 has been examined in several tissues, such as lung, breast, kidney, uterine cervical epithelium and

esophageal epithelium [8, 12-18]. Loss of claudin-7 expression has been observed in ductal carcinoma of the breast and in head and neck squamous cell carcinoma [15, 16]. In contrast, increased expression of both claudin-1 and -7 has been observed in squamous intraepithelial neoplasia and invasive carcinoma of the uterine cervix [17]. Enhanced expression of claudin-1 has also been reported in colorectal cancer and esophageal squamous cell carcinoma [18, 19]. However, it remains to be clarified whether claudin-1 and -7 expressions in esophageal squamous cell carcinoma are associated with the malignant potential.

In this study, we determined the expressions of claudin-1, -7, and ZO-1 in 17 cases of normal human esophageal squamous epithelium. To determine the significance of claudins in esophageal cancer, we also analyzed the correlation of claudin-1 and -7 expressions with the clinicopathological factors of esophageal squamous cell carcinoma.

2. Materials and methods

2.1. Tissue samples

For Western blot analysis, normal human esophageal tissues were obtained from autopsies undertaken within one or two hours after death at Kobe University Hospital from 2004 to 2005, after obtaining written informed consent. Macroscopic and microscopic examinations revealed no significant postmortem changes in the esophageal tissues. The renal cortex was dissected from C57BL/6 mouse (Nippon SLC Co., Shizuoka, Japan) and used as positive control for both claudin-1 and -7 in western blotting [13, 14].

A total of 62 squamous cell carcinomas of the esophagus were studied; these carcinomas were surgically resected at Kobe University Hospital from 1997 to 2004, without any presurgical chemotherapy or radiotherapy. Informed consent was obtained from all the patients. All the specimens were fixed in 10% formalin and embedded in paraffin. Histological typing and depth of invasion were determined according to the Guide Lines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus proposed by the Japanese Society for Esophageal Diseases [20]. TNM classification was applied according to the guidelines of the International Union Against Cancer [21]. Selected blocks comprising 62 primary carcinomas, 17 adjacent normal esophageal tissue samples, and 27 metastatic lymph nodes were sliced into 4-μm-thick sections for immunohistochemical analysis.

2.2 Western blot analysis

Normal esophageal tissue and mouse renal cortex were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton-X (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 5 mM EDTA, 1% protease inhibitor, and 1% phosphatase inhibitor cocktail II (Sigma Chemical Co., Tokyo, Japan). Tissue lysates were separated by SDS-PAGE and then electrotransferred onto Immunobilon-P membrane (Millipore, Billerica, MA). Rabbit monoclonal antibodies (Abs) to claudin-1 (1:1000 dilution, Zymed Laboratories Inc., CA), rabbit polyclonal Abs to claudin-7 (1:1000 dilution, [22, 23]; Immuno-Biological Laboratories, Japan), rabbit polyclonal Abs for ZO-1 (1:1000 dilution, Zymed), and mouse monoclonal Abs to β-actin (1:1000 dilution, Sigma) were used for the primary reaction. The generation of primary rabbit polyclonal Abs against claudin-7 has been described elsewhere [22, 23]. Horseradish peroxidase-conjugated donkey anti-mouse IgG and sheep anti-rabbit IgG (Amersham Biosciences Corp., NJ) were used as secondary Abs. After washing with PBS containing 0.5% Tween-20, protein bands were visualized by enhanced chemiluminescence (ECL) method using Immunostar Reagent (Immunostar, Wako).

2.3. Immunohistochemical analysis

Immunohistochemical analysis was performed to evaluate the expression and distribution of claudin-1, -7, and ZO-1 in normal squamous epithelium samples and the expression of claudin-1 and -7 in esophageal squamous cell carcinomas. Rabbit monoclonal Abs to claudin-1 (1:100 dilution, Zymed), rabbit polyclonal Abs to claudin-7 (1:100 dilution [22, 23];

Immuno-Biological Laboratories), and rabbit polyclonal Abs for ZO-1 (1:100 dilution, Zymed) were used for the primary reaction. Deparaffinized tissue sections were immersed in 10 mM sodium citrate buffer (pH 6.0) and autoclaved for antigen retrieval. Endogenous peroxidase activity was blocked using methanol containing 0.03% H_2O_2. Following incubation with blocking buffer (0.01 M PBS containing 5% BSA [Sigma]), the sections were incubated with the primary Abs overnight at 4ºC. After gentle rinsing with 0.05 M Tris-HCl, the sections were incubated with biotinylated secondary Abs (LSAB2 kit, Dako Cytomation, CA, USA) for 30 min. Following this, the sections were again incubated with horseradish peroxidase conjugated streptavidin reagent (LSAB2 kit, Dako) for 30 min. Chromogenic fixation was performed for 5 min in a solution of 3-amino-9-ethyl carbazole substrate chromogen (Dako). The sections were counterstained with Meyer's hematoxylin.

In normal esophageal epithelium samples, the expressions of claudin-1, -7, and ZO-1 were evaluated in each of the four layers of epithelium, namely, basal cell layer, prickle cell layer, lower surface layer, and upper surface layer.

The degree of claudin-1 and claudin-7 expressions in carcinoma *in situ*, esophageal squamous cell carcinoma and metastatic lymph nodes were evaluated as follows: score 0, negative immunoreaction; score 1, spotted immunostaining on the cell membrane; score 2, strong linear immunoreactivity at cell-cell contact. Evaluation and scoring was performed at the area of the invasive front of each cancer tissue by two independent observers (YU and FN).

2.4. Statistical analysis

The statistical correlations between the results of immunohistochemical analysis and clinicopathological factors were assessed by the χ^2 –test. Staining intensities at the invasive front of the primary tumor and metastatic lymph nodes were compared using the Wilcoxon signed-ranks test. A *P* value less than 0.05 was considered as statistically significant. All statistical analyses were performed using the StatView software (SAS Institute Inc., NC).

3. Results

3.1. Expressions of claudin-1, -7, and ZO-1 in normal human esophageal epithelium

The immunohistochemical distribution of claudin-1, -7, and ZO-1 in 17 samples of normal human esophageal epithelium is summarized in Table 1. Claudin-1 and -7 were expressed at the cell-cell contact as a continuous linear pattern and had honeycomb-like appearance in the prickle cell layer (Fig.1A-D). Claudin-1 and -7 immunoreactivities were not detected in the basal cell layer. ZO-1 was expressed in the cytoplasm below the cell-cell contact of the prickle cell layer (Fig.1E, F). In contrast to the claudins, ZO-1 was expressed not only in the prickle cell layer but also in the basal cell layer in 3 samples. In 2 samples, ZO-1 was also expressed in the lower surface layer. Western blot analysis demonstrated that the Abs to claudin-1, -7, and ZO-1 detected specific single 22-kDa, 23-kDa, and 225-kDa bands, respectively, in the extracts of 4 normal esophageal tissue samples (Fig.1I).

3.2. Expression of claudin-1 and -7 in carcinoma *in situ* and squamous cell carcinoma of the esophagus

To investigate the expressions of claudin-1, and -7 in esophageal cancers, as well as their correlation with the clinicopathological factors, 60 squamous cell carcinomas and 2 cases of carcinoma *in situ* were evaluated by immunohistochemistry. In both cases of carcinoma *in situ*, no significant reduction in claudin-1 or -7 expressions was observed. In contrast to the limited expression in the prickle cell layer of the normal esophageal epithelium, both claudin-1 and -7

were expressed in the entire atypical cell layer of the carcinoma *in situ* (Fig.2A-C). In squamous cell carcinoma, claudin-1 and -7 immunoreactivities were observed at the cell-cell contact, but not in the cytoplasm or nucleus. Poorly differentiated squamous cell carcinomas demonstrated weak or loss of claudin-1 and -7 immunoreactivities (Fig.2D-F). On the other hand, well and moderately differentiated carcinomas showed strong immunoreactivities of both claudin-1 and -7 at the area of cornified differentiation (Fig.2G-I). Interestingly, the immunoreactivity of claudin-7 was heterogeneous in each cancer tissue of the esophageal cancer although the expression of claudin-1 was almost homogenous (Fig.3). Further, positive expression of claudin-7 in the upper portion of the cancerous lesion and obvious reduction or loss of claudin-7 expression at the invasive front was observed in 9 cases (Fig. 3C, F, G).

3.3. Relationships between claudin-1 and -7 expressions at the invasive front and clinicopathological factors of esophageal squamous cell carcinomas

The comparisons of claudin-1 and -7 expressions at the invasive front and clinicopathological factors in squamous cell carcinoma are summarized in Table 2. Reduction in claudin-1 and -7 expressions in esophageal squamous cell carcinoma were observed in 46 (74%) cases and 44 (71%) cases, respectively. Reduction in claudin-7 expression at the invasive front was significantly associated with the depth of invasion ($P = 0.004$), stage ($P = 0.038$), lymphatic vessel invasion ($P = 0.001$), and lymph node metastasis ($P = 0.014$). Poorly differentiated squamous cell carcinomas tended to lose claudin-7 expression in comparison

with other histological types (P = 0.076). Although the reduced expression of claudin-1 was significantly associated with histological differentiation of the tumor (P = 0.029), significant association was not detected between its expression and other clinicopathological factors.

3.4. Comparison of claudin-1 and -7 expressions in the primary esophageal tumors and lymph node metastasis

Of the 62 esophageal squamous cell carcinoma cases, 28 cases had lymph node metastasis. The immunoreactivities of claudin-1 and -7 in 27 available metastatic lymph nodes and at the invasive front of the corresponding primary tumors were compared. Reduction in claudin-1 and -7 expressions was observed in 7 and 10 cases, respectively. Despite the stable expression of claudin-1 at the both invasive fronts and in metastatic lymph nodes, reduced immunoreactivities of claudin-7 in the metastatic lymph nodes were observed in 6 cases (Fig.4A-F). When claudin-7 expression at the invasive front of the primary tumor and the corresponding metastatic lymph nodes was compared, a significant reduction in claudin-7 expression was observed in the metastatic lymph nodes (Fig.5B, P = 0.007). On the other hand, no significant reduction in claudin-1 expression was observed in metastatic lymph nodes when compared with that at the invasive front of the primary tumor (Fig.5A, P = 0.118). In 25 cases that showed reduced expression of claudin-7 at the invasive front of the cancerous lesion in comparison with that in normal epithelium, a further reduction in claudin-7 immunoreactivity was observed in the corresponding metastatic lymph nodes in 6 cases.

4. Discussion

In this study, we report the details of expression and distribution of claudin-1 and -7 in the normal esophageal stratified squamous epithelium. We detected claudin-1, -7, and ZO-1 immunoreactivities in the prickle cell layer. In the stratified squamous epithelium of the skin, the expressions of claudin-1, -3, -4, -6, -7, -8, -11, -12, and -17 have been reported [5, 6, 9, 24, 25]. Furuse et al. identified claudin-1 and -4 in the basal cell layer and granular cell layer in the skin of mouse; they reported that claudin-1-deficient mice died with wrinkled skin due to dehydration within 1 day of birth [6]. These findings indicate that tight junctions are crucial for the barrier function of the mammalian skin. The expressions of ZO-1, occludin, claudin-1, and claudin-7 have been shown in stratified squamous epithelium of the esophagus [3, 18, 26]. Our observations regarding ZO-1, claudin-1, and -7 expressions are in agreement with those of previous reports [3, 18].

It is known that cell-cell adhesiveness is generally reduced in various human cancers. The dissociation of cancer cells from the primary cancer nests is a crucial step in metastasis, and the suppression of cell-cell adhesiveness may trigger the release of cancer cells from the primary cancer nests and confer invasive properties on a tumor [2]. In this study, we discovered significant reduction or loss of claudin-7 expression at the invasive front of esophageal squamous cell carcinoma. cDNA microarray comparison between matched normal epithelium and squamous cell carcinoma cells of the head and neck cancer demonstrated down-regulation of claudin-7 gene expression [16]. Inverse correlation between loss of

claudin-7 expression and nuclear grade in ductal carcinoma *in situ* or Elston grade in invasive ductal carcinoma of the breast has been reported [15]. The present study showed that the reduced expression of claudin-7 at the invasive front was statistically correlated with the depth of invasion, stage, lymphatic vessel invasion, and lymph node metastasis of esophageal cancer; this suggests that reduction or loss of claudin-7 expression may be correlated with tumor invasiveness and metastatic potential.

The results obtained in our study are different from previous studies about claudin-1 and -7 expressions in cervical neoplasia [17] and esophageal squamous cell carcinoma [18]. Despite significant decrease in claudin-1 and -7 expressions in invasive carcinoma in comparison with intraepithelial neoplasm and carcinoma *in situ* of the uterine cervix, expressions of both claudins were still higher than those in the normal cervical epithelium, and no significant correlation was detected between T categories and claudin-7 expression [17]. Gyorffy et al. reported that the pattern and staining intensity of claudin-7 in esophageal squamous cell carcinoma did not change in comparison with those in normal squamous epithelium [18]. These discrepancies between our results and the others can be explained based on the tumor area selected for the evaluation of immunoreactivities. To investigate the relationship between claudin-1 and claudin-7 expressions and the malignant potential such as cancer cell proliferation, we observed the deepest invaded area called the invasive front [27]. On the other hand, Sobel et al. and Gyorffy et al. observed a randomly selected area [17, 18]. There was significant reduction of claudin-7 expression in metastatic lymph nodes when

compared with that at the invasive front of the corresponding primary tumors. Therefore, observations of claudin-7 expression at the invasive front may be important to evaluate its role in the esophageal cancer.

Kominsky et al. found that hypermethylation of promoter sequences was the major mechanism involved in the silencing of claudin-7 expression in breast cancer cell lines [15]. Recent studies suggested the possibility of genetic and epigenetic changes in cell adhesion molecules in cancer cells [2]. Due to heterogeneous and reduced expression of claudin-7 at the invasive front and in metastatic lymph nodes, hypermethylation of claudin-7 might have occurred at the invasive front of the esophageal squamous cell carcinoma, where the reduction or loss of claudin-7 expression was detected.

In conclusion, the present study provided evidence of reduction or loss of claudin-7 expression at the invasive front and in metastatic lymph nodes; it also clarified the correlation of claudin-7 expression with tumor invasiveness and metastatic potential in esophageal squamous cell carcinoma. We consider that reduction or absence of claudin-7 expression can be a novel marker for the prediction of metastasis. Moreover, the elucidation of biological functions of claudin-7 at the invasive front of the tumor would be valuable in clarifying the mechanism of tumor invasiveness and metastasis.

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Table legends

Table 1 Distribution of claudin-1, -7, and ZO-1 immunoreactivities in normal esophageal squamous epithelium

Expression and distribution of claudin-1, -7, and ZO-1 were evaluated in each of the four layers of epithelium; basal cell layer, prickle cell layer, lower surface layer and upper surface layer. M, male; F, female; U, including cervical esophagus and upper thoracic esophagus; M, middle thoracic esophagus; L, lower thoracic esophagus including abdominal esophagus; BL (+), expression in basal cell layer; PL (+), expression in prickle cell layer; LS (+), expression in lower surface layer.

Table 2 Relationships between claudin-1 or -7 expression and clinicopathological factors of the esophageal squamous cell carcinomas

[a] Expression of claudin-1 and -7 at the invasive front were scored as follows: score 0, negative expression; score 1, spotted staining in cell-cell contact; score 2, strong linear expression in cell-cell contact.

[b] Statistical analysis was performed by the χ^2 test with Yates' correction. *P* values less than 0.05 were considered to be statistically significant.

[c] Locations were separated as follows: Upper, including cervical esophagus and upper thoracic esophagus; Middle, middle thoracic esophagus; Lower, lower thoracic esophagus including abdominal esophagus.

[d] According to the Guide Lines for the Clinical and Pathological Studies on Carcinoma of the Esophagus proposed by the Japanese Society for Esophageal Diseases [26]. CIS, carcinoma *in situ*; well, well differentiated squamous cell carcinoma; mod, moderately differentiated squamous cell carcinoma; poor, poorly differentiated squamous cell carcinoma.

[e] According to the TNM classification by UICC [27].

Figure legends

Fig. 1 Immunohistochemistry using anti-claudin-1, anti-claudin-7 and anti-ZO-1 (zonula occludens-1) antibodies in serial sections of normal esophageal epithelium. (bar = 50 μm). I, Western blotting of claudin-1, -7, and ZO-1 in normal esophageal epithelium (Hm-Eso1-4). Mouse kidney tissues (Ms-kid) were used for positive control.

Fig. 2 Immunohistochemistry of claudin-1 and -7 in esophageal squamous cell carcinoma. Carcinoma *in situ* shows strong expression of claudin-1 (B) and -7 (C) in atypical cell layer. Poorly differentiated squamous cell carcinoma shows reduced expression of claudin-1 (E) and -7 (F). Well differentiated squamous call carcinoma with cornified differentiation shows strong expressions of claudin-1 (H) and -7 (I). Original magnification, inset: ×400, bar = 100 μm.

Fig. 3 Immunohistochemical expressions of claudin-1 and -7 in a representative case of esophageal squamous cell carcinoma. In contrast to homogenous immunoreactivity of claudin-1 (B), claudin-7 (C) immunoreactivity is decreased as the progress of invasion. Higher magnification of upper potion of cancerous lesion away from invasive front (D and F) and invasive front (E and G) of claudin-1 and claudin-7 immunostaining are presented in lower panel. Original magnification, inset: ×400, bar = 100 μm).

Fig. 4 Claudin-1 and -7 expressions at the invasive front of the primary tumor and metastatic lymph node. Despite the stable expression of claudin-1 and -7 at the invasive front (B and C) reduced immunoreactivity of claudin-7 at the metastatic lymph node were observed (F); while the expression of claudin-1 did not changed (E). B and E: claudin-1, C and F: claudin-7, A-C: invasive front of the primary tumor, D-F: metastatic lymph node, original magnification, inset: ×400, bar = 100 μm.

Fig. 5 Comparison of claudin-1 and -7 expressions at the invasive front of primary esophageal tumors and metastatic lymph nodes. No significant reduction of claudin-1 expression was observed in metastatic lymph nodes comparison with invasive front of the primary tumor (A, $P = 0.118$). Significant reduction of claudin-7 expression was observed in metastatic lymph nodes comparison with corresponding invasive front of the primary tumor (B, $P = 0.007$). Statistical analysis was performed by Wilcoxon signed-ranks test. $P < 0.05$ was considered to be statistically significant. (□: score 0, ▒: score 1, ■: score 2)

Table 1 Distribution of claudin-1, -7, and ZO-1 immunoreactivities in normal esophageal squamous epithelium

Case	Age/Gender	Location	claudin-1	claudin-7	ZO-1
1	69/M	L	PL (+)	PL (+)	PL (+)
2	73/M	M	PL (+)	PL (+)	BL (+), PL (+)
3	57/M	M	PL (+)	PL (+), LS (+)	PL (+), LS (+)
4	60/F	L	PL (+)	PL (+), LS (+)	PL (+), LS (+)
5	62/M	U	PL (+)	PL (+)	PL (+)
6	77/M	L	PL (+)	PL (+)	PL (+)
7	70/F	L	PL (+)	PL (+)	PL (+)
8	69/M	M	PL (+)	PL (+)	PL (+)
9	70/F	L	PL (+)	PL (+)	PL (+)
10	78/M	L	PL (+)	PL (+)	PL (+)
11	54/F	M	PL (+)	PL (+)	PL (+)
12	59/M	L	PL (+)	PL (+)	PL (+)
13	50/M	L	PL (+)	PL (+)	PL (+)
14	50/M	L	PL (+)	PL (+)	PL (+)
15	62/M	U	PL (+)	PL (+)	BL (+), PL (+)
16	66/M	M	PL (+)	PL (+)	BL (+), PL (+)
17	53/M	L	PL (+)	PL (+)	PL (+)

Table 2 Relationships between claudin-1 and -7 expression and clinicopathological factors of the esophageal squamous cell carcinomas

		Score of claudin-1 expression[a]				Score of claudin-7 expression[a]			
	n	0 (%)	1 (%)	2 (%)	*P* value[b]	0 (%)	1 (%)	2 (%)	*P* value[b]
Total	62	29 (47)	17 (27)	16 (26)		17 (27)	27 (44)	18 (29)	
Age									
> 65	37	21 (56)	7 (20)	9 (24)	0.108	12 (32)	14 (38)	11 (30)	0.462
65 ≥	25	8 (32)	10 (40)	7 (28)		5 (20)	13 (52)	7 (28)	
Gender									
Male	53	23 (43)	15 (28)	15 (28)	0.392	14 (26)	24 (45)	15 (29)	0.797
Female	9	6 (67)	2 (22)	1 (11)		3 (33)	3 (33)	3 (33)	
Location[c]									
Upper	13	5 (38)	4 (31)	4 (31)	0.468	5 (38)	5 (38)	3 (23)	0.835
Middle	31	17 (55)	9 (29)	5 (16)		7 (23)	15 (48)	9 (29)	
Lower	18	7 (39)	4 (22)	7 (39)		5 (28)	7 (39)	6 (33)	
Histology[d]									
CIS	2	0 (0)	0 (0)	2 (100)	0.029	0 (0)	0 (0)	2 (100)	0.076
Well	12	6 (50)	3 (25)	3 (25)		3 (25)	3 (25)	6 (50)	
Mod	37	14 (38)	14 (38)	9 (24)		9 (24)	19 (51)	9 (24)	

Poor	11	9 (82)	0 (0)	2 (18)		5 (45)	5 (45)	1 (9)	
Depth of invasion[d]									
Tis	2	0 (0)	0 (0)	2 (100)	0.223	0 (0)	0 (0)	2 (100)	0.004
T1	20	10 (50)	6 (30)	4 (20)		4 (20)	6 (30)	10 (50)	
T2	12	4 (33)	3 (25)	5 (42)		1 (8)	9 (75)	2 (17)	
T3	26	13 (50)	8 (31)	5 (19)		10 (38)	12 (46)	4 (15)	
T4	2	2 (100)	0 (0)	0 (0)		2 (100)	0 (0)	0 (0)	
Stage[e]									
0-IIa	26	13 (50)	7 (27)	6 (23)	0.890	3 (12)	10 (38)	13 (50)	0.038
IIb-IVb	36	16 (44)	10 (28)	10 (28)		14 (38)	17 (47)	5 (13)	
Vessel invasion									
Lymphatic vessels									
Negative	15	6 (40)	3 (20)	6 (40)	0.344	5 (33)	1 (6)	9 (60)	0.001
Positive	47	23 (49)	14 (30)	10 (21)		12 (25)	26 (55)	9 (19)	
Venous vessels									
Negative	24	12 (50)	5 (21)	7 (29)	0.645	5 (20)	8 (33)	11 (45)	0.068
Positive	38	17 (45)	12 (32)	9 (23)		12 (31)	19 (50)	7 (18)	
Lymph node metastasis									
Negative	34	16 (47)	9 (26)	9 (26)	0.981	8 (23)	11 (32)	15 (44)	0.014
Positive	28	13 (46)	8 (29)	7 (25)		9 (32)	16 (57)	3 (10)	

Figure 1

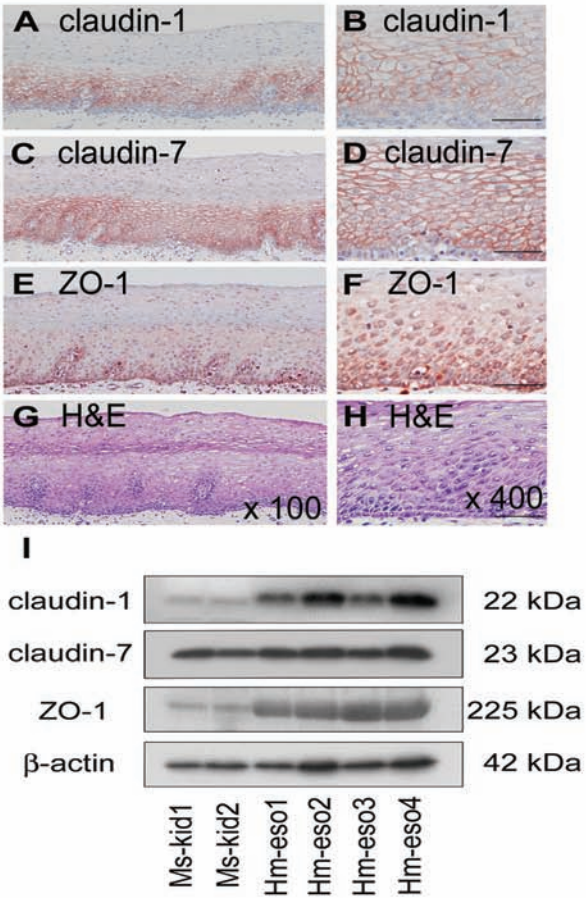

A claudin-1
B claudin-1
C claudin-7
D claudin-7
E ZO-1
F ZO-1
G H&E
x 100
H H&E
x 400
I
claudin-1
22 kDa
claudin-7
23 kDa
ZO-1
225 kDa
β-actin
42 kDa
Ms-kid1
Ms-kid2
Hm-eso1
Hm-eso2
Hm-eso3
Hm-eso4

Figure 2

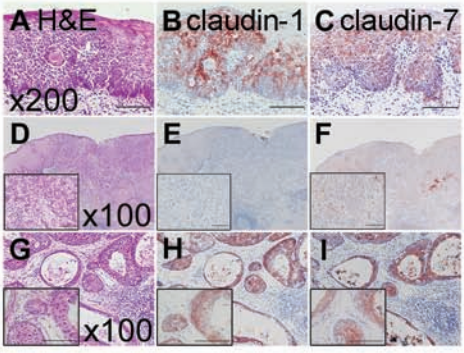


Figure 3

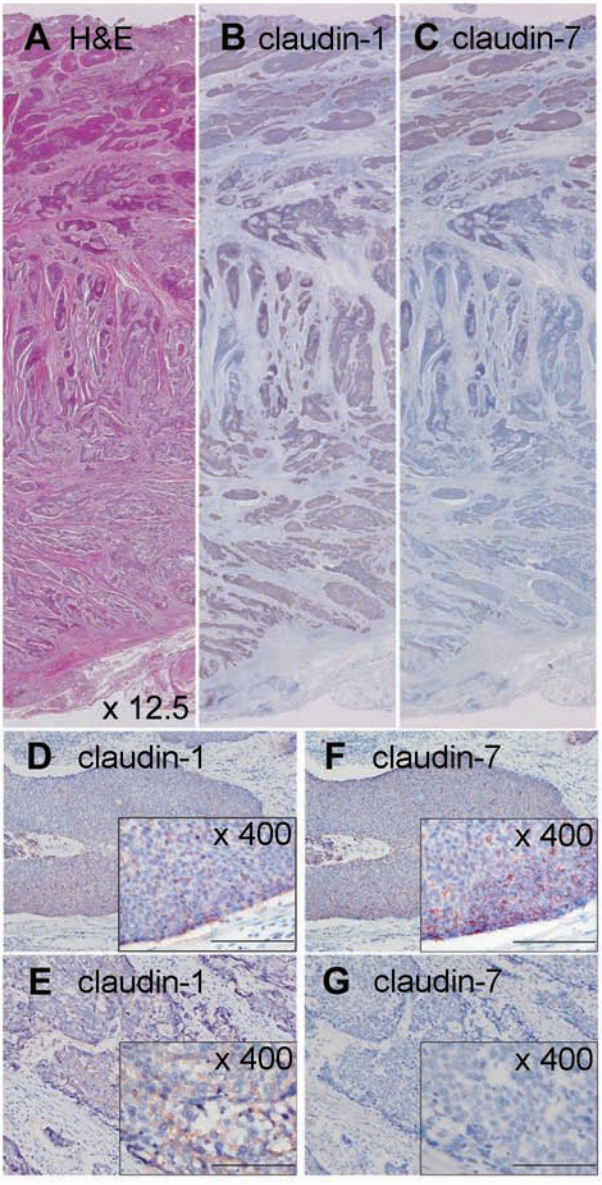

A H&E
B claudin-1
C claudin-7
x 12.5
D claudin-1
x 400
F claudin-7
x 400
E claudin-1
x 400
G claudin-7
x 400

Figure 4

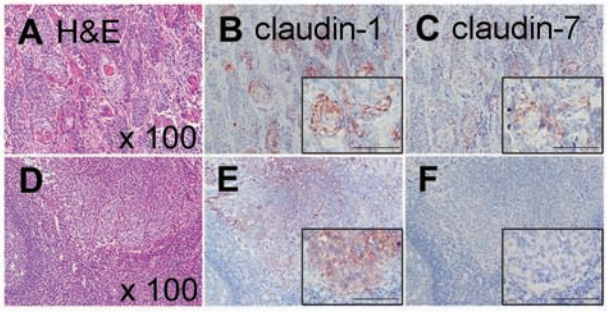
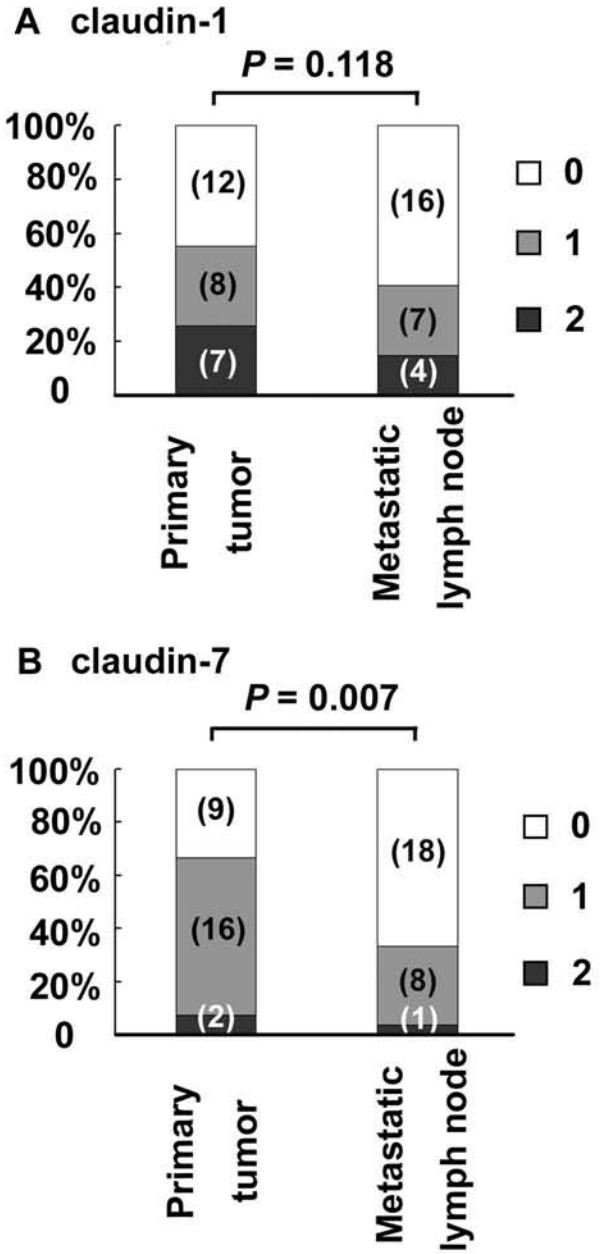


Figure 5


A claudin-1
P = 0.118
100%
80%
60%
40%
20%
0
(12)
(8)
(7)
(16)
(7)
(4)
0
1
2
Primary tumor
Metastatic lymph node
B claudin-7
P = 0.007
100%
80%
60%
40%
20%
0
(9)
(16)
(2)
(18)
(8)
(1)
0
1
2
Primary tumor
Metastatic lymph node