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Original article

Expression of tight-junction-associated proteins in human gastric cancer: downregulation of claudin-4 correlates with tumor aggressiveness and survival

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Abstract

Background. **Claudin, occludin, and zonula occludens (ZO)-1 are known as tight-junction-associated proteins. The aim of this study was to examine the expression of these proteins in gastric carcinoma.**

Methods. **Gastric cancer tissues ($n = 124$) were obtained from 124 patients who underwent gastrectomy at our hospital between January 2000 and December 2004. The expression of the above tight-junction-associated proteins in carcinoma, normal mucosa, and metaplastic epithelium was examined using immunohistochemistry. In addition, the expression of claudin-4 mRNA was examined in fresh frozen tissue obtained from 34 patients.**

Results. **Significant correlations were seen between the expression of claudin-4, occludin, and ZO-1. In regard to claudin-4, significant correlations were seen between the expression of claudin-4 evaluated by immunohistochemistry and the expression of claudin-4 mRNA. Claudin-4 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma, advanced T stage, lymph node metastasis, and peritoneal metastasis. Occludin and ZO-1 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma. Overall survival was significantly shorter in patients with low claudin-4 expression. Cox multivariate analysis revealed that low claudin-4 expression was independently associated with significantly decreased overall survival.**

Conclusion. **Tight-junction-associated proteins, particularly claudin-4, may play important roles in determining invasiveness, metastatic potential, and survival in gastric cancer.**

Key words Occludin · Zonula occludens-1 protein · Metastasis

Introduction

Tight junctions, adherens junctions, gap junctions, and desmosomes are the known cell membrane structures that participate in cell-to-cell adhesion. Tight junctions are present in epithelial and endothelial cell membranes, forming a component of intercellular junctional complexes and playing important roles in barrier function, cell polarity, and cell signaling pathways [1].

Claudins are major tight-junction constituents and display four transmembrane domains. This multiple gene family is expressed in a tissue-specific pattern. To date, 24 members of the claudin family have been identified [1].

Occludin is another constituent of tight junctions [1, 2], and again has four transmembrane domains. To date, no occludin isotypes have been identified in any species [1]. Occludin-deficient epithelial cells demonstrate a well-developed network of tight junction strands [3], suggesting that occludin is an accessory protein in terms of tight-junction strand formation [1].

Zonula occludens (ZO)-1, -2 and -3 are membrane-associated proteins that connect tight junctions to the cytoskeleton [2]. ZO-1 establishes a link between occludin and the actin cytoskeleton [3], and is part of a signaling pathway linking tight junctions to the regulation of gene expression [4].

In normal gastric mucosa, positive immunostaining has been detected for claudin-18, but not for claudin-4 [5]. We have previously revealed, using an oligonucleotide microarray, that claudin-4 is upregulated in gastric cancer [6], and another study using an oligonucleotide microarray has reported that claudin-4 is upregulated in intestinal-type gastric cancer [7]. Moreover, claudin-4 is reportedly highly expressed in gastric intestinal-type adenocarcinoma [5, 8, 9]. To date, several studies have been reported regarding the biological functions of claudin-4 in gastric cancer. For example, it has been reported that *Helicobacter pylori* was able to increase paracellular permeability by disrupting occludin, claudin-4, and claudin-5 [10], and it has been shown that Cdx2 plays an important role in the regulation of intestinal claudin expression, not only

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in gastric mucosa with intestinal metaplasia but also in gastric carcinoma [11].

Considering the circumstances mentioned above, claudin-4 may play an important role in gastric carcinogenesis. However, the exact function of claudin-4 in gastric cancer is still unclear. As previously reported, it is probably true that claudin-4 expression is decreased in diffuse-type adenocarcinoma [5, 7, 8, 12]. In terms of the aggressiveness of gastric carcinoma, a few studies have reported about it. For example, a trend was observed between the overexpression of claudin-4 and lymph node metastasis [13], and a trend was observed between reduced claudin-4 expression and advanced T stage [12]. For patient survival, Lee et al. [12] reported that reduced expression of claudin-4 showed an associative tendency with a high cumulative recurrence rate in patients with gastric carcinoma, whereas Resnick et al. [8] reported a significant association between claudin-4 expression and poor survival.

To sum up, the correlation between claudin-4 expression and the aggressiveness of gastric cancer, shown by such features as invasion, metastasis, and survival, is still controversial.

Therefore, the aim of this study was to examine the expression of claudin-4 in gastric carcinoma, normal mucosa, and metaplastic epithelium, and to correlate the expression of this protein with features of the aggressiveness of gastric cancer such as invasion, metastasis and survival. In addition to the expression of claudin-4, the expression of occludin and ZO-1 is reportedly reduced in poorly differentiated gastric adenocarcinoma [14], and there is very little combined analysis of claudin-4, occludin, and ZO-1 expression. We therefore also examined the expression of occludin and ZO-1, as known components of tight junctions, in gastric carcinoma to provide some insights into gastric carcinogenesis.

Patients, materials and methods

Tissue samples

A total of 124 gastric cancer tissues were obtained from 124 patients who underwent gastrectomy at Fukushima Medical University between January 2000 and December 2004.

Written informed consent was obtained from all patients and ethical approval of this study was obtained from the ethics committee of Fukushima Medical University. Patient characteristics are shown in Table 1. The mean age of the patients (89 men and 35 women) was 66.8 years (range, 18–86 years). Follow-up time ranged from 31 to 1931 days (median value was 666.5 days). There were 57 cases of total gastrectomy, 54 cases of distal gastrectomy, 5 cases of proximal gastrectomy, and 7 cases of pylorus-preserving gastrectomy. One hundred and nine patients received curative operations and 15 received noncurative operations.

According to the *Japanese classification of gastric carcinoma* (2nd English edition; Table 1) [15], there were 52 cases of stage IA, 27 cases of stage IB, 11 cases of stage II, 9 cases of stage IIIA, 4 cases of stage IIIB, and 21 cases of stage IV. Histological type was classified as: (a) differentiated-type adenocarcinoma, including papillary adenocarcinoma and tubular adenocarcinoma; and (b) undifferentiated-type adenocarcinoma, including poorly differentiated adenocarcinoma, mucinous adenocarcinoma, and signet ring cell carcinoma. According to this classification, there were 70 cases of differentiated-type adenocarcinoma and 54 cases of undifferentiated adenocarcinoma. Five-year survival stratified according to pT and pN is shown in Table 2.

Immunohistochemistry

Resected specimens were fixed with 10% buffered formalin and embedded in paraffin. Tissue blocks were then sliced into 4-μm sections and mounted on glass slides. The tissue sections were dewaxed and rehydrated through graduated changes of xylene and graded alcohol, then washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by incubating the sections with 0.3% hydrogen peroxide for 10 min, and then the sections were washed with PBS. The sections were pretreated in 10-mM citrate buffer (pH 6), using a microwave-based antigen retrieval method for 15 min. After being washed with PBS, to block nonspecific background reactions, the sections were exposed to normal bovine serum for 10 min, and then incubated with monoclonal antihuman claudin-4, occludin, ZO-1 antibody (Zymed Laboratories, South San Francisco, CA, USA) overnight at a dilution of 1: 100. After being washed with PBS, the sections were incubated with biotinylated secondary antibody for 10 min. After a further wash with PBS, the sections were incubated with peroxidase-conjugated streptavidin for 5 min. The peroxidase reaction was carried out in a solution of hydrogen peroxide as a substrate and 3, 3′ diaminobenzidine tetrahydrochloride as a chromogen. Positive controls were colonic mucosa and negative controls were the same specimens in which the primary antibody was replaced with nonreactive antibodies. We used a Histofine SAB-PO kit (Nichirei, Tokyo, Japan), which contains blocking antibody, biotinylated secondary antibody, and peroxidase-conjugated streptavidin.

Table 1. Patient characteristics

Sex		Lymph node metastasis	
Male	89	pN0	80
Female	35	pN1	26
Age (years)	18–86 (Mean, 66.8)	pN2	12
Type of surgery		pN3	6
TG	57	Histological type	
DG	54	pap	5
PG	5	tub1	40
PPG	7	tub2	25
Other	1	por1	10
Peritoneal metastasis		por2	18
P0	112	sig	18
P1	12	muc	7
Hepatic metastasis		Other	1
H0	118	Stage	
H1	6	IA	52
Depth of invasion		IB	27
pT1	59	II	11
pT2	35	IIIA	9
pT3	23	IIIB	4
pT4	7	IV	21
		Curability	
		A	85
		B	24
		C	15

A total of 124 patients who underwent gastrectomy at Fukushima Medical University from 2000 to 2004 were included. Patient characteristics were classified according to the *Japanese classification of gastric carcinoma* (2nd English edition) [15].
pap, papillary adenocarcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; por1, poorly differentiated adenocarcinoma (solid type); por2, poorly differentiated adenocarcinoma (non-solid type); sig, signet ring cell carcinoma; muc, mucinous adenocarcinoma; TG, total gastrectomy; DG, distal gastrectomy; PG, proximal gastrectomy; PPG, pylorus-preserving gastrectomy

Table 2. Relationship of pT, pN and patient survival

	5-Year survival (%)
pN1	78.4
pN2	85.3
pN3	27.2
pT1	78.5
pT2	68.9
pT3	41.6
pT4	—

RNA extraction and cDNA synthesis

RNA was extracted from fresh frozen cancer tissue obtained from 34 patients, using an RNeasy Mini Kit and QIA shredder (Qiagen, Hilden, Germany). We extracted the samples from the invasive front of cancer without necrosis. Total RNA (1 µg) was reverse-transcribed using random hexamer, SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 10 M dNTP (Sigma-Aldrich, St. Louis, MO, USA). A reverse transcriptase-polymerase chain reaction (RT-PCR) assay was performed using the Gene Amp PCR System (Applied Biosystems, Foster City, CA, USA). Integrity of the isolated RNA was established by RT-PCR analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Real-time quantitative polymerase chain reaction (RQ-PCR)

An RQ-PCR assay was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR reactions proceeded in a mixture (50 µl) containing 2.5 µl of TaqMan gene expression assays (Applied Biosystems) including claudin-4-specific oligonucleotide primers (assay ID Hs00533616 s1), 25 µl of Taq Man Universal PCR Master Mix (Applied Biosystems), and 2 µl of cDNA sample. Amplification was performed for 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and then claudin-4 mRNA expression level was normalized against quantified GAPDH mRNA expression. Non-template control was used as a negative control. We confirmed that there was no decomposition of RNA using electrophoresis (data not shown).

Interpretation of immunohistochemistry and RQ-PCR

In immunohistochemistry, staining was scored by a coauthor without knowledge of clinical factors. Carcinoma, adjacent normal fundic mucosa without gastritis or metaplasia, and metaplastic epithelium in the same slides were reviewed when present. Whole mucosa was evaluated, not only surface epithelia. In accordance with previously published criteria, the incidence of positively stained cells was graded as follows: 0, less than 10%; 1, 10%-50%; 2, 50%-90%; 3, more than 90% [12, 14], and for the purpose of data analysis, the incidence of positively stained cells was graded into two groups: low expression, fewer than 50% of cells stained (incidence score 0 or 1); and high expression, more than 50% of cells stained (incidence score 2 or 3) [12]. Representative staining patterns of tight-junction-associated-proteins in normal gastric mucosa, metaplastic epithelium, and carcinoma are shown in Fig. 1. In immunohistochemistry, as the staining patterns of normal mucosa and metaplastic epithelia are similar to that of carcinoma, we scored the expression of the tight-junction-associated proteins in normal mucosa and metaplastic epithelia in the same way as the carcinoma.

We then examined correlations between the expression of tight-junction-associated proteins and clinicopathological factors such as depth of tumor invasion, lymph node metastasis, hepatic metastasis, peritoneal metastasis, and overall survival.

In RQ-PCR, we examined the expression of claudin-4 mRNA to confirm claudin-4 expression estimated by immunohistochemistry.

Statistical analysis

Statistical analyses were performed using Dr. SPSS II for Windows (SPSS, Chicago, IL, USA). The χ^2 test for independence was used to test the correlations of claudin-4, occludin, and ZO-1 expression with clinicopathological factors, and this test was also used for testing correlations between the expression of claudin-4 as evaluated by immunohistochemistry and the expression of claudin-4 mRNA.

Survival time was estimated using the Kaplan-Meier method, and the log-rank test was used for testing differences in survival time between groups. The multivariate Cox proportional hazard model was applied to detect independent predictors of survival. Values of $P < 0.05$ were considered statistically significant.

Results

All specimens had normal fundic mucosa, and 93 specimens had metastatic epithelia. In immunohistochemistry, as previously reported [5, 8, 12], the staining patterns for claudin-4 and ZO-1 in carcinoma, metaplastic epithelia, and normal mucosa were membranous, and nucleus and cytoplasm were faintly stained. For occludin, membranous staining was also observed in carcinoma, metaplastic epithelia, and normal mucosa (Fig. 1).

Claudin-4 was highly expressed in carcinoma and metaplastic epithelium. In contrast, the expression of claudin-4 was low in normal mucosa. Occludin and ZO-1 were highly expressed in normal mucosa as well as in carcinoma and metaplastic epithelium (Fig. 2).

Significant correlations were identified between the expression of claudin-4, occludin, and ZO-1 (Table 3).

For claudin-4, a significant correlation was seen between expression of claudin-4 as estimated by immunohistochemistry and the expression of claudin-4 mRNA ($P = 0.0030$; Fig. 3). Claudin-4 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), advanced T stage ($P = 0.0012$), lymph node metastasis ($P < 0.0001$), and peritoneal metastasis ($P < 0.0001$; Table 4).

Occludin expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), lymph node metastasis ($P = 0.0470$), and peritoneal metastasis ($P = 0.0232$; Table 4).

ZO-1 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P = 0.0030$) and advanced T stage ($P = 0.0049$; Table 4).

Relapse occurred in nine patients who received curative surgery, and recurrence patterns were peritoneal

Table 3. Correlation between expression of claudin-4, occludin and ZO-1

	Claudin-4				Claudin-4				Occludin		
	Low	High	*P*		Low	High	*P*		Low	High	*P*
Occludin			<0.0001	ZO-1			0.0046	ZO-1			0.0001
Low	28	9		Low	18	8		Low	18	8	
High	27	59		High	37	60		High	19	79	

A significant correlation was identified between the expression of claudin-4, occludin, and ZO-1. The χ^2 test for independence was used for statistical analysis

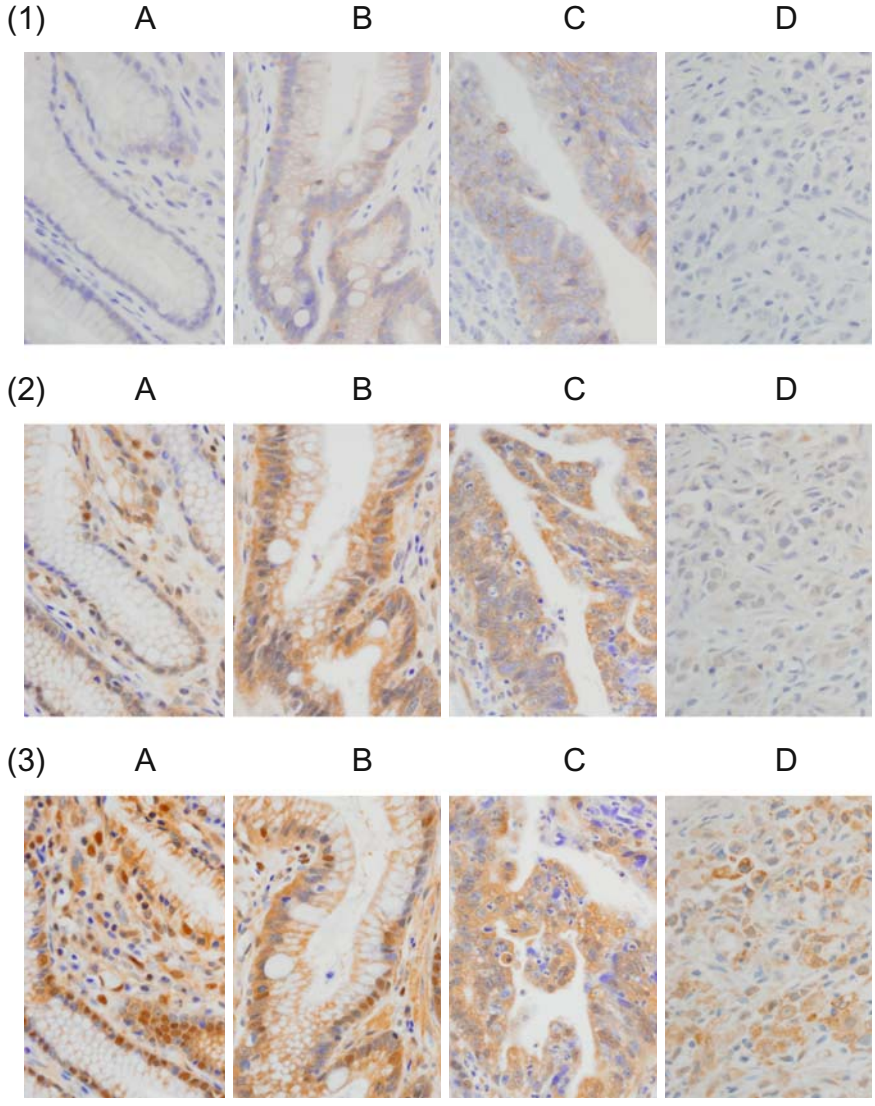

Fig. 1. Immunohistochemistry for claudin-4 (*1*), occludin (*2*), and zonula occludens-1 (ZO-1) (*3*). *A* Normal epithelium. *B* Metaplastic epithelium. *C* Well-differentiated adenocarcinoma (tub1). *D* Poorly differentiated adenocarcinoma (por2). × 200

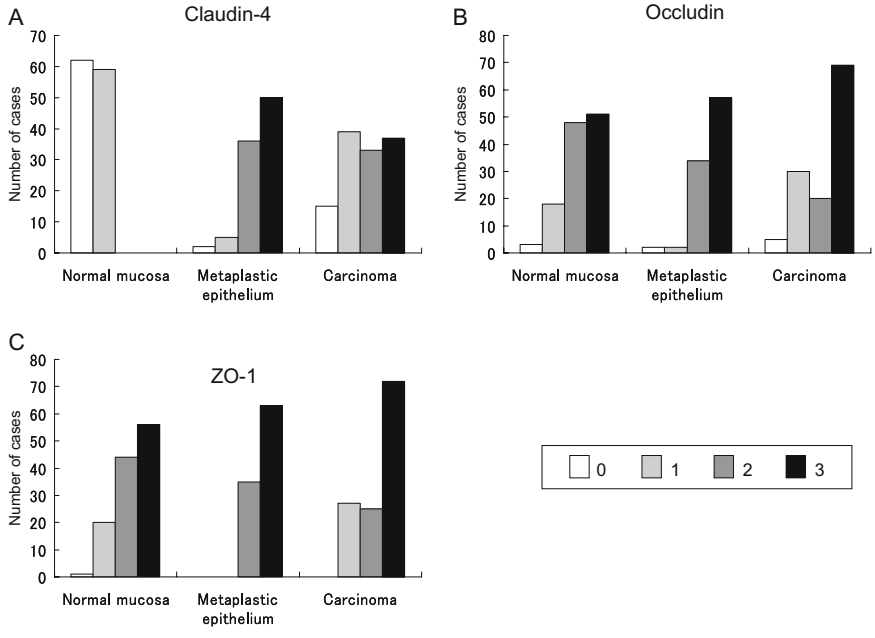

Fig. 2A–C. Expression of claudin-4, occludin, and ZO-1 in normal mucosa, metaplastic epithelium, and carcinoma. **A** Claudin-4 was highly expressed in carcinoma and metaplastic epithelium. In contrast, expression of claudin-4 was low in normal mucosa. **B**, **C** Occludin and ZO-1 were highly expressed in normal mucosa, as well as in carcinoma and metaplastic epithelium. *0*, less than 10%; *1*, 10%–50%; *2*, 50%–90% *3*, more than 90%

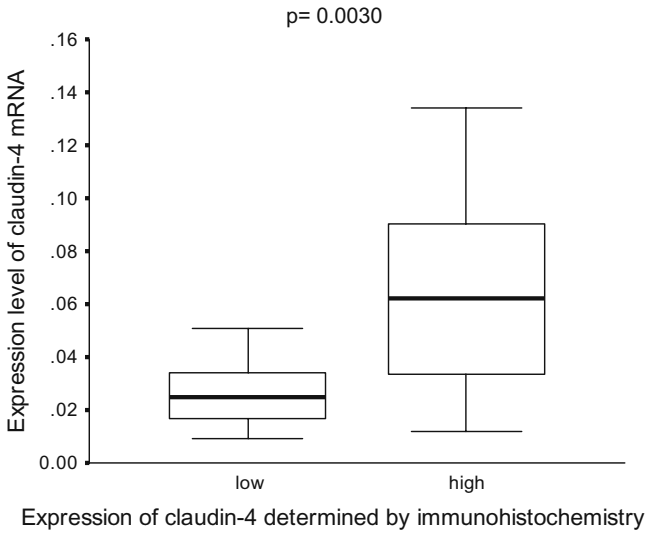

Fig. 3. Correlation between expression of claudin-4 as evaluated by immunohistochemistry and expression of claudin-4 mRNA. A significant correlation was seen between the expression of claudin-4 as determined by immunohistochemistry and the expression level of claudin-4 mRNA ($P = 0.0030$)

metastasis ($n = 5$), hepatic metastasis ($n = 3$), and lymph node metastasis ($n = 1$). Causes of death in all patients (including patients with noncurative operations) were peritoneal metastasis ($n = 9$), lymph node metastasis ($n = 4$), hepatic metastasis ($n = 4$), and other diseases ($n = 11$). Patients who underwent noncurative surgery received postoperative chemotherapy using S-1, cisplatin, paclitaxel, or irinotecan according to the choice of the physician.

Overall survival according to claudin-4 expression was significantly shorter in patients with low claudin-4 expression than in patients with high expression ($P = 0.0091$). No significant differences were seen according to the expression of occludin or ZO-1 ($P = 0.4031$ and $P = 0.3142$, respectively; Fig. 4).

Cox multivariate analysis for overall survival revealed that undifferentiated-type adenocarcinoma, lymph node metastasis, peritoneal metastasis, and low expression of claudin-4 were independently associated with significantly decreased survival ($P = 0.0089$, $P = 0.0377$, $P = 0.0290$ and $P = 0.0070$, respectively; Table 5).

Table 4. Correlation between expression of tight-junction-associated proteins and clinicopathological factors

	Claudin-4			Occludin			ZO-1		
	Low	High	*P*	Low	High	*P*	Low	High	*P*
Differentiated	4	65	<0.0001	9	61	<0.0001	8	62	0.0030
Undifferentiated	51	3		28	26		18	36	
T1	17	41	0.0012	13	46	0.0703	6	53	0.0049
T2/3/4	38	27		24	41		20	45	
N0	25	54	<0.0001	19	61	0.0470	13	67	0.0818
N1/2/3	30	14		18	26		13	31	
P0	43	68	<0.0001	30	82	0.0232	23	89	0.7181
P1	12	0		7	5		3	9	
H0	50	67	0.0511	36	82	0.4698	24	94	0.4456
H1	5	1		1	5		2	4	

Expression of tight-junction-associated proteins was classified as low expression (incidence of positively stained cells <50%) or high expression (incidence of positively stained cells >50%). The χ^2 test for independence was used for statistical analysis.
Claudin-4 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), advanced T stage ($P = 0.0012$), lymph node metastasis ($P < 0.0001$), and peritoneal metastasis ($P < 0.0001$). Occludin expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P < 0.0001$), lymph node metastasis ($P = 0.0470$), and peritoneal metastasis ($P = 0.0232$). ZO-1 expression was significantly decreased in tumors with undifferentiated-type adenocarcinoma ($P = 0.0030$) and advanced T stage ($P = 0.0049$)

Table 5. Multivariate overall survival analysis (Cox proportional hazard model)

Variable	β	SE	*P*	HR	95% CI
Differentiated vs undifferentiated	2.0556	0.7861	0.0089	7.8112	1.6734–36.4611
T1 vs T2/3/4	–0.0814	0.5660	0.8856	0.9218	0.3039–2.7954
N0 vs N1/2/3	1.1265	0.5422	0.0377	3.0849	1.0660–8.9273
H0 vs H1	0.0410	0.6775	0.9517	1.0418	0.2761–3.9310
P0 vs P1	1.2811	0.5866	0.0290	3.6004	1.1402–11.3687
Claudin-4 expression; high vs low	2.0121	0.7461	0.0070	7.4787	1.7327–32.2791
Occludin expression; high vs low	0.0619	0.5745	0.9142	1.0639	0.3450–3.2802
ZO-1 expression; high vs low	0.1336	0.5228	0.7983	1.1429	0.4102–3.1842

Cox multivariate analysis revealed that undifferentiated adenocarcinoma, lymph node metastasis, peritoneal metastasis, and low expression of claudin-4 were independently associated with significantly decreased overall survival ($P = 0.0089$, $P = 0.0377$, $P = 0.0290$, and $P = 0.0070$, respectively)
SE, standard error; HR, hazard ratio; CI, confidence interval

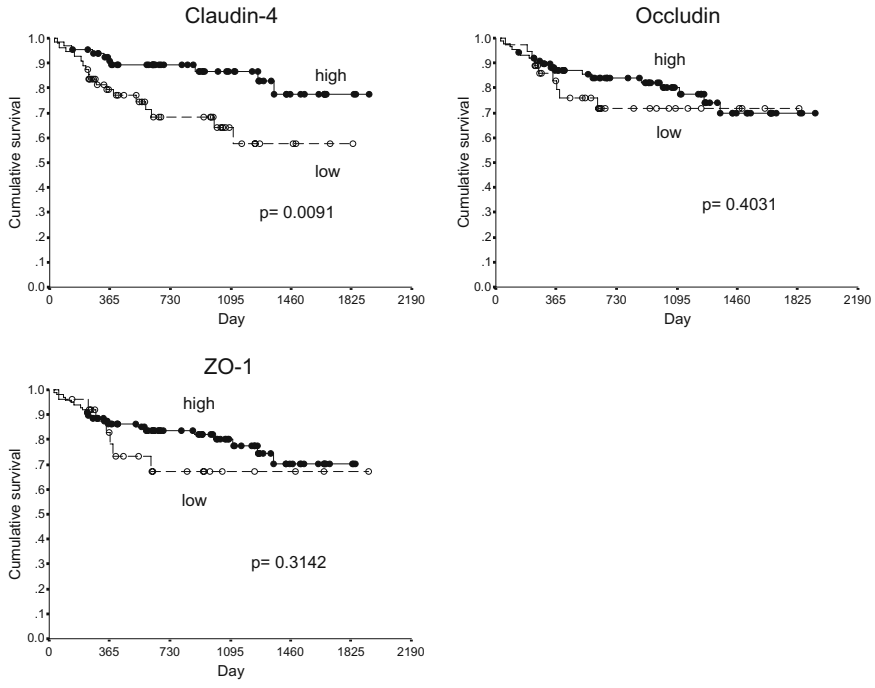

Fig. 4. Overall survival analysis according to tight-junction-associated proteins. Low claudin-4 expression was significantly associated with decreased overall survival ($P = 0.0091$). No significant correlation existed between the expression of occludin or ZO-1 and overall survival ($P = 0.4031$, $P = 0.3142$, respectively)

Discussion

Several studies have been reported about the relationship between tight-junction-associated proteins and gastric carcinogenesis. For example, it has been reported that *H. pylori* was able to increase paracellular permeability by disrupting occludin, claudin-4, and claudin-5 [10], and Cdx2 was shown to play an important role in the regulation of intestinal claudin expression not only in gastric mucosa with intestinal metaplasia but also in gastric carcinoma [11]. Moreover, ALL-1 fusion partner from chromosome 6 (AF-6), which is a Ras target, interacts with ZO-1 [16, 17]. As previously described, tight junctions play important roles in barrier function, cell polarity, and cell signaling pathways [1]. Therefore, disruption of the tight junction can cause the loss of cell polarity, resulting in an abnormal influx of growth factors, which could provide autocrine and paracrine stimulation to tumorigenic epithelial cells [5]. However, the exact roles of tight-junction-associated proteins in gastric cancer remain unclear [18].

In the present study, claudin-4 was highly expressed in differentiated adenocarcinoma and metaplastic epithelium, but was expressed very little in normal epithelium, as evaluated by immunohistochemistry. In gastric cancer, this result was confirmed by the result that a significant correlation was seen between the expression of claudin-4 estimated by immunohistochemistry and the expression of claudin-4 mRNA ($P = 0.0030$; Fig. 3). Claudin-4 is reportedly highly expressed in metaplastic epithelium and carcinoma, but little expressed in normal epithelium [5, 8, 9]. These results may be related to the fact that intestinal-type adenocarcinomas are derived from metaplastic epithelia [8].

Our study revealed that occludin and ZO-1 were expressed in almost all carcinoma, metaplastic epithelium, and normal mucosa specimens. Similar to our findings, ZO-1 was shown to be expressed in almost all carcinoma, metaplastic epithelium, and normal epithelium [8].

Claudin-4 is reportedly highly expressed in gastric intestinal-type adenocarcinoma [5, 7, 8, 12], and the expression of occludin and ZO-1 is reduced in poorly differentiated adenocarcinoma [14]. These results may indicate that tight junctions are important for the construction of gland structure [12].

As previously described, a few studies have reported on the correlation between claudin-4 expression and the aggressiveness of gastric cancer. Similar to our findings, a trend was observed between the reduced expression of claudin-4 and advanced T stage [12], but, contrasting with our results, a trend was observed between the overexpression of claudin-4 and lymph node metastasis [13]. However, there have been no reports about the relationship between occludin expression and the aggressiveness of gastric cancer. In regard to ZO-1, it has been reported that there was no apparent correlation between ZO-1 expression and advanced T stage or lymph node metastasis [12]. Our results suggest that decreased claudin-4 expression is related to undifferentiated-type adenocarcinoma, advanced T stage, lymph node metastasis, peritoneal metastasis, and poor survival. Regard-

ing occludin, we found that decreased expression of occludin was related to undifferentiated-type adenocarcinoma, lymph node metastasis, and peritoneal metastasis, and decreased occludin expression showed a tendency to be associated with advanced T stage and poor survival. Regarding ZO-1, decreased ZO-1 expression was related to undifferentiated-type adenocarcinoma and advanced T stage, and decreased ZO-1 expression showed a tendency to be associated with lymph node metastasis and poor survival. In addition, we showed significant correlations between the expression of claudin-4, occludin, and ZO-1. These results suggest that, of the tight-junction-associated proteins, not only claudin-4 but also occludin and ZO-1 may be related to the aggressiveness of gastric carcinoma.

Survival analysis showed that overall survival was significantly worse in patients with low expression of claudin-4. Cox multivariate analysis also revealed that low expression of claudin-4 was independently associated with significantly decreased overall survival. Contrasting with our results, a strong trend was detected between high claudin-4 expression and poor survival [8], but, similar to our findings, reduced expression of claudin-4 tended to be associated with a high cumulative recurrence rate [12], and the reduced expression of claudin-4 was found to be correlated with poor survival [5].

During the invasion process, loss of intercellular adhesion is one of the early critical steps toward metastasis [19]. If cellular polarity is maintained in cancer cells, cells adhere to each other at the adherens junction and form a basement membrane, then tight junctions at the apical borders are closed by tight junction proteins, and tubular gland structures are subsequently formed [12]. E-cadherin is known as the principal constituent of the adherens junction, and impairment of either the expression or the function of E-cadherin has been reported in cancer cell lines [20, 21]. Some human cancer cells may display impaired E-cadherin-mediated cell adhesiveness through the downregulation of α-catenin expression [22]. Adherens junctions are important for the adhesion of cell-to-cell junctions. In addition, the function of tight junctions may also be important for the construction of tubular gland structures and cell-to-cell adhesion [12]. As for the relationship between claudin-4 and E-cadherin, it has been reported that the reduced expression of claudin-4 and E-cadherin correlates with the disruption of glandular structure and loss of differentiation [12].

In tumor cells derived from rat mammary carcinoma, tight junctions were observed between weakly metastatic tumor cells and normal fibroblasts [23]. In pancreatic carcinoma, claudin-4 is overexpressed and this is associated with decreased invasiveness both in vitro and in vivo [24]. These findings, as well as the present results, suggest that reduced cell-to-cell adhesions formed by tight junctions lead to the dissociation of cancer cells from the original tumor, thus facilitating tumor invasiveness and metastatic potential [24].

In light of these observations, assuming that downregulated claudin-4 correlates with poor survival appears reasonable.

Gastric carcinogenesis may be related not only to claudin-4 but also to other tight-junction-associated proteins, because our study identified significant correlations between the expression of claudin-4, occludin, and ZO-1. The reason that only claudin-4 was associated with invasiveness, metastatic potential, and survival in our study may be related to the fact that claudin is major structural components of tight junction strands [2].

A limitation of this study is that we evaluated the expression of tight-junction-associated proteins using immunohistochemistry. Therefore, we confirmed the quality of immunohistochemistry by RQ-PCR, but we evaluated only claudin-4 because we had no data available about occludin and ZO-1.

Conclusions

We showed that the downregulation of tight-junction-associated proteins, especially of claudin-4, was associated with loss of differentiation in gastric carcinoma and tumor aggressiveness. Survival analysis revealed that the downregulation of claudin-4 was associated with poor survival by the multivariate Cox proportional hazard model as well as by the Kaplan-Meier method. In conclusion, the investigation of tight-junction-associated proteins, especially claudin-4 expression, in gastric carcinoma could be useful for predicting tumor aggressiveness, particularly for determining patient prognosis.

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