Claudin-1, but not claudin-4, exhibits differential expression patterns between well- to moderately-differentiated and poorly-differentiated gastric adenocarcinoma

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Abstract. Claudins are members of a large family of transmembrane proteins, which are essential in the formation of tight junctions and have previously been associated with the process of tumor progression. Studies have reported the aberrant expression of claudin-1 and claudin-4 in numerous types of cancer. The present study aimed to investigate the expression of claudin-1 and claudin-4 in gastric adenocarcinoma tissue. Surgically resected gastric adenocarcinoma tissue specimens were obtained from 94 patients. Protein expression levels of claudin-1 and claudin-4 were determined using immunohistochemical staining; the association between claudin-1 or claudin-4 expression and various clinicopathological parameters were then analyzed. In gastric adenocarcinoma specimens, the expression rates of claudin-1 and claudin-4 were 43.6 and 87.2%, respectively. Claudin-1 expression demonstrated a significant correlation with histological type ($P<0.01$) and was significantly higher in well- to moderately-differentiated gastric adenocarcinomas compared with poorly-differentiated tumors. However, no correlation was observed between claudin-4 expression in adenocarcinoma and clinicopathological parameters. In conclusion, downregulation of claudin-1 expression in poorly-differentiated gastric adenocarcinoma may be involved in the biological transformation of tumors. The present findings suggested that claudin-1 may be an important protein associated with histological type and therefore may have potential for use as a prognostic marker for gastric adenocarcinoma. Further studies are required to elucidate the precise mechanism of claudin expression and its involvement in tumor progression.

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Introduction

Gastric cancer is one of the most prevalent types of malignant tumors in South America, Eastern Europe and Asian countries and adenocarcinoma is the most common form of gastric cancer (1-3). It has been well-established that the pathogenesis of gastric cancer occurs through a multistep progression from chronic gastritis to atrophic gastritis, intestinal metaplasia and dysplasia, finally resulting in cancer (4). Loss of cell polarity and disruption of intracellular adhesion are frequently observed during this process and have been reported to have a critical role in cancer progression (5,6).

Tight junctions are the most apical type of cellular junction, which function as a selective barrier and establish cellular polarity in epithelial cells (7-9). In addition, tight junctions are involved in the regulation of cell proliferation and differentiation, among other cellular functions (10). Tight junctions are typically lost in cancer, which was reported to be involved in the invasive and metastatic phenotype of tumor cells (11-13).

Claudins are a family of integral membrane proteins and are the major protein components of tight junctions. Of the numerous tight junction proteins, claudins are key functional proteins and are expressed in various types of tissues and cells. In addition, claudins were reported to have a marked impact on the biological behavior of tumor progression (14,15). Of note, the expression of claudin-1 and claudin-4 was demonstrated to be frequently altered in various tumor tissues.

The expression of claudin-1 was reported to be significantly increased in intestinal-type carcinomas compared with diffuse-type gastric carcinomas (15). However, another study demonstrated that the claudin-1 expression was significantly reduced in intestinal-type gastric carcinomas compared with the diffuse-type (16). Furthermore, transformation of claudin-1 expression was identified in gastric carcinoma (17).

Studies into the function of claudin-4 have not provided consistent results. It was reported that claudin-4 expression was significantly correlated with improved rates of patient survival in gastric cancer (16,18). However, Resnick *et al* (19) suggested that moderate to strong staining for claudin-4 in gastric cancer was associated with decreased survival rates. Soini *et al* (15) found that claudin-4 was not associated with patient survival. Overexpression of claudin-4 was demonstrated to be significantly associated with reduced invasiveness in pancreatic carcinoma (20). However, overexpression of claudin-3 and claudin-4 was reported to result in increased invasion, motility and survival of tumor cells (21).

Therefore the biological functions of claudin-1 and claudin-4 have not been clarified and studies on the role of their expression in gastric carcinomas have been limited. Further investigations are required for clarification of these controversial results and to fully elucidate the function of claudin-1 and claudin-4. The present study aimed to evaluate the clinicopathological associations of claudin-1 and claudin-4 expressions in gastric adenocarcinoma.

Patients and methods

Patients. Tissues were obtained from 94 patients with gastric adenocarcinoma who underwent surgical resection between January 2010 and April 2013 at Kagawa University Hospital (Kagawa, Japan). The patients' histological findings, along with their lymph node metastases, venous invasion and tumor, node, metastasis (TNM) stages were evaluated based on the Japanese Classification of Gastric Adenocarcinoma (22,23). All subjects provided written informed consent. The study was conducted with the approval of the Institutional Research Ethics Committee of Kagawa Prefectural University of Health Sciences (Kagawa, Japan).

Immunohistochemistry. Tissues were obtained from primary tumors (slides, 4 μm) and were deparaffinized in 99% xylene (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 15 min, then rehydrated in a graded series of ethanol (Muto Pure Chemicals Co., Ltd.), followed by antigen retrieval by microwave heating for 15 min at 2 kW in 0.01 M citrate buffer (pH 6.0) containing 38 mg/dl citric acid monohydrate and 241 mg/dl trisodium citrate dehydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (Wako Pure Chemical Industries, Ltd.). Sections were then incubated for 2 h at room temperature with the following primary antibodies: Anti-claudin-1 antibody (mouse monoclonal IgG2a; Abcam, Cambridge, UK; catalog no. ab56417; dilution, 1:100) and anti-claudin-4 antibody (rabbit polyclonal; Abcam; catalog no. ab15104; dilution, 1:200). Slides were rinsed three times with phosphate-buffered saline (PBS; Wako Pure Chemical Industries, Ltd.) and incubated for 15 min at room temperature with secondary antibodies with Histofine Simple Stain MAX PO (MULTI) (universal immuno-peroxidase polymer, anti-mouse and anti-rabbit; Nichirei Biosciences Inc., Tokyo, Japan) according to the manufacturer's instructions and stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) using a DAB substrate kit (Nichirei Biosciences Inc.). The sections were counterstained with Meyer's hematoxylin and then dehydrated, cleared with 99% xylene for 15 min and mounted in malinol (Muto Pure Chemicals, Co., Ltd.). Colon cancer samples and normal gastric mucosa samples obtained from Kagawa University Hospital were used as positive controls. The expression of claudin-1 and claudin-4 in the tissues was observed under microscope (BX53; Olympus Corporation, Tokyo, Japan) with photographs taken on a microscope camera (DP20-5; Olympus Corporation).

The classification of claudin expression was based on the criteria of Jung *et al* (14). Briefly, the immunostaining for claudin-1 or claudin-4 was assessed using the following scoring: No staining, 0; <25% cells positive and incomplete membranous staining, 1+; 25-50% cells positive and incomplete membranous staining, 2+; 50-75% cells positive and complete or incomplete membranous staining, 3+; >75% cells positive and complete membranous staining, 4+. In the evaluation, the expression of claudin-1 and claudin-4 were grouped into negative (0, 1+) and positive (2+, 3+, 4+) groups.

Table I. Clinical characteristics of 94 gastric adenocarcinoma patients.

Characteristics	Patients, n (%)
Age, years (mean ± standard deviation)	72.1±8.9
Gender	
Male	67 (71.3)
Female	27 (28.7)
Histological type	
Well to moderately differentiated	47 (50.0)
Poorly differentiated	47 (50.0)
Lymphatic invasion	
Negative	24 (25.5)
Positive	70 (74.5)
Venous invasion	
Negative	30 (31.9)
Positive	64 (68.1)
Lymph node metastasis	
N0	64 (68.1)
N1	12 (12.8)
N2	4 (4.2)
N3	14 (14.9)
Depth of tumor invasion	
T1	37 (39.4)
T2	11 (11.7)
T3	18 (19.1)
T4	28 (29.8)
Stage	
I	46 (48.9)
II	25 (26.6)
III	16 (17.0)
IV	7 (7.5)

N, degree of lymph node involvement; T, degree of tumor invasion.

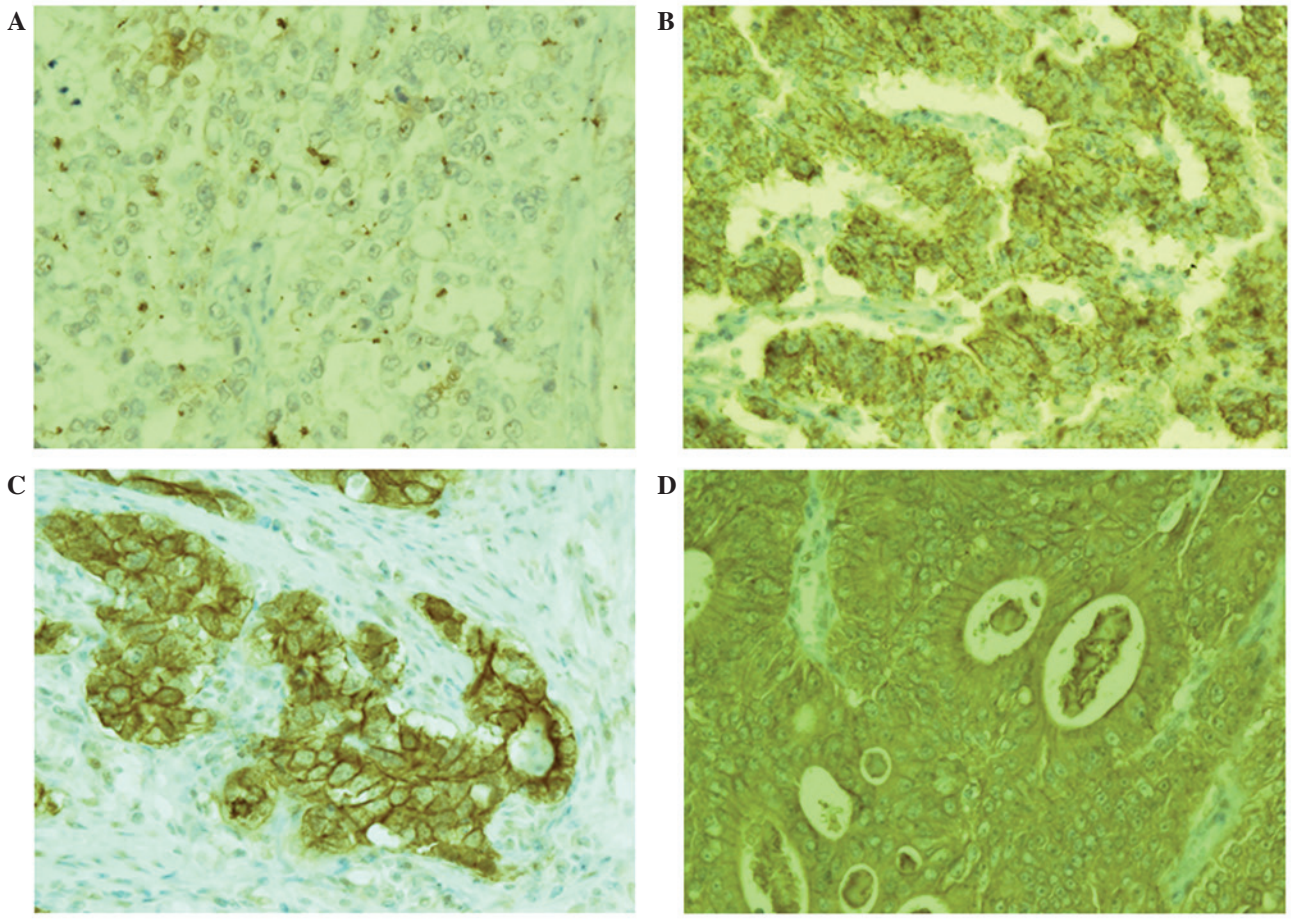

Figure 1. Immunostaining of claudin-1 and claudin-4 in gastric adenocarcinoma (magnification, x200). (A) Negative membranous expression of claudin-1 in poorly-differentiated gastric adenocarcinoma. (B) Positive membranous expression of claudin-1 in well- to moderately-differentiated gastric adenocarcinoma. Positive membranous expression for claudin-4 in (C) poorly-differentiated and (D) well- to moderately-differentiated gastric adenocarcinoma.

Table II. Immunohistochemical staining for the expression rate of claudins in 94 gastric adenocarcinoma tissue samples.

Protein	Positive expression (%)
Claudin-1	41 (43.6)
Claudin-4	82 (87.2)

Data were grouped according to immunostaining scores based on positive and incomplete membranous staining (%): Negative expression, 0 (0%) and 1+ (<25%); positive expression, 2+ (25-50%), 3+ (50-75%) and 4+ (>75%).

Statistical analysis. Univariate analysis was performed using the Chi-squared or Fisher's exact tests for categorical data. All statistical analyses were performed using SPSS 21.0 software (International Business Machines, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

Table I summarizes the clinical parameters of patients with gastric adenocarcinoma. A total of 67 (71.3%) patients were men and 27 (28.7%) patients were women, with a median age of 72-years (range, 50-91 years). Following analysis of the tumor samples, it was reported that 46 (48.9%) patients had stage I, 25 (26.6%) patients had stage II, 16 (17.0%) patients had stage III and 7 patients (7.5%) had stage IV gastric cancer.

As shown in Fig. 1, claudin-1 and claudin-4 were primarily expressed in the cell membrane of gastric adenocarcinoma cells; in addition, certain samples displayed a low level of cytoplasmic staining. The expression rate of claudin-1 was 43.6% and that of claudin-4 was 87.2% (Table II). Claudin-1 expression levels were revealed to by associated with histological type, as they were significantly higher in well- to moderately- differentiated gastric adenocarcinomas compared with poorly-differentiated adenocarcinomas ($P<0.01$) (Fig. 1, Table III). However, no significant associations were determined between the expression of claudin-1 and age, gender, lymphatic invasion, venous invasion, depth of tumor invasion, lymph node metastasis or stage of gastric cancer in patients (Table III). The expression rate of claudin-4 in poorly-differentiated gastric adenocarcinomas was comparable to that of the well- to moderately-differentiated gastric adenocarcinomas; therefore, claudin-4 was not significantly associated with any clinicopathological factors (Fig. 1, Table III).

Table III. Correlation between claudin-1 and claudin-4 expression and clinicopathologic characteristics of gastric adenocarcinoma in 94 tissue samples from patients.

	Claudin-1			Claudin-4		
Characteristics	(-)	(+)	P-value	(-)	(+)	P-value
Age, years						
≤60	8	1	0.073	3	6	0.087
>60	45	40		9	76	
Gender						
Male	35	32	0.295	9	58	1.000
Female	18	9		3	24	
Histological type						
Well to moderately differentiated	17	30	<0.001	4	43	0.354
Poorly differentiated	36	11		8	39	
Lymphatic invasion						
Negative	15	9	0.644	4	20	0.495
Positive	38	32		8	62	
Venous invasion						
Negative	13	17	0.081	4	26	1.000
Positive	40	24		8	56	
Lymph node metastasis						
N0	34	30	0.107	8	56	0.139
N1	9	3		0	12	
N2	4	0		0	4	
N3	6	8		4	10	
Depth of tumor invasion						
T1	17	20	0.345	4	33	0.612
T2	6	5		1	10	
T3	11	7		4	14	
T4	19	9		3	25	
Stage						
I	22	24	0.071	5	41	0.420
II	14	11		2	23	
III	10	6		4	12	
IV	7	0		1	6	

Data were grouped according to immunostaining scores based on positive and incomplete membranous staining: Negative expression (-), 0 (0%) and 1+ (<25%); positive expression (+), 2+ (25-50%), 3+ (50-75%) and 4+ (>75%). N, degree of lymph node involvement; T, degree of tumor invasion.

Discussion

The present study examined the expression of claudin-1 and claudin-4 in 94 patients with gastric adenocarcinoma. In order to evaluate the altered protein expression and whether it was associated with clinicopathological parameters, immunohistochemical staining was conducted using primary antibodies for claudin-1 and claudin-4. The expression of claudin-1 demonstrated a significant correlation with histological type, with significantly increased levels in well- to moderately-differentiated gastric adenocarcinomas. However, no significant correlations were observed between claudin-4 expression in gastric adenocarcinoma and clinicopathological parameters. These results may therefore provide evidence for the development of a useful molecular marker for predicting cancer progression and prognosis in gastric adenocarcinoma, as claudin-1 expression may be a phenotypic feature in well- to moderately-differentiated gastric adenocarcinoma.

Tumor cells undergo epithelial-to-mesenchymal transition (EMT) in order to execute the multi-step process of tumorigenesis and metastasis (5). Tight junction proteins, including claudins, cadherins and vimentins are essential for the process of EMT; these proteins are crucial for the preservation of the cell layer integrity and regulation of cell proliferation (24). In addition, the role of tight junction proteins in tumor progression has been associated with numerous other protein

interactions (25,26). However, numerous studies have reported that the expression of tight junction proteins was decreased in cancer cells (27,28).

Previous studies have identified the expression of claudins in several cancer types, including breast, pancreatic, liver and esophageal cancer (10,29-33). Claudin-1 expression was reported be attenuated in breast cancer as well as colon cancer (31,34,35). In addition, the expression levels of claudin-1, claudin-3, claudin-4 and claudin-5 were all significantly decreased in diffuse adenocarcinoma and were essential for determining the phenotype and loose cohesion of cells in diffuse gastric carcinoma (15). Claudin-3 expression levels were significantly depleted in advanced tumor-stage (T3 and T4) gastric adenocarcinoma cases (16); in addition, the loss of claudin-7 was correlated with increased cellular discohesion in breast carcinoma (36). Thus, the reduced expression of claudins in cancer supported the hypothesis that tumorigenesis is associated with tight junctions disruption and that this process is critical for reduced cohesion and invasiveness as well as the limited differentiation capacity of cancer cells. Decreased expression of tight junction proteins, such as claudins, in cancer results in reduced cell adhesion during the progression of cancer to metastasis (37,38). The results of the present study indicated that claudin-1 expression was reduced in poorly-differentiated gastric adenocarcinomas compared with well- to moderately-differentiated gastric adenocarcinomas; in addition, the present findings confirmed that the loss or downregulation of tight junction proteins in cancer cells was essential for tumorigenesis.

Histologically, gastric adenocarcinomas may be separated into two main categories according to their biologic behaviors: Differentiated and undifferentiated adenocarcinoma. In addition gastric adenocarcinomas may be catagorized into intestinal or diffuse type, as well as expanding or infiltrative type (39-41). In general, the intestinal type is well-differentiated with cohesive, glandular-like tumor cells, whereas the diffuse type is poorly-differentiated with infiltrating, non-cohesive tumor cells. Those tumors classed as differentiated include papillary, well-differentiated and moderately-differentiated adenocarcinomas, while undifferentiated tumors include poorly-differentiated adenocarcinomas, signet ring cell carcinomas and mucinous adenocarcinomas. Several evaluation studies of prognostic value regarding gastric carcinoma have been performed. Adachi *et al* (42) reported that the overall 5-year survival rate was increased in patients with well-differentiated gastric carcinoma compared with those patients with poorly-differentiated gastric carcinoma (76 vs. 67%, respectively). In addition, Park *et al* (43) reported that the overall cumulative 5-year survival rates for patients were 67% for well- to moderately-differentiated and 54% for poorly-differentiated gastric cancer. Therefore, patients with poorly-differentiated adenocarcinoma may have a worse prognosis compared with those with well- to moderately-differentiated types.

Immunohistochemical staining was performed for claudin-1 and claudin-4 in the present study. The frequency of claudin-1 expression was 43.6% (41/94), which was significantly decreased in poorly-differentiated gastric adenocarcinoma tissue compared with the well- to moderately-differentiated tumor tissue (23.4 vs. 63.8%); however, no significant difference was observed in other pathologic features. These results suggested that the expression of claudin-1 is associated with poorly-differentiated gastric adenocarcinoma and that the loss of claudin-1 expression may be an efficient predictive marker for tumor recurrence and survival outcome of patients.

In the present study, the expression of claudin-4 was not found to be significantly associated with the clinicopathological factors assessed. However, the correlation between claudin-4 and clinicopathological factors remains controversial; Jung *et al* (16) reported that the expression of claudin-4 was significantly lower in cases with positive lymphatic invasion in gastric cancer and Zhu *et al* (44) demonstrated that claudin-4 expression was significantly associated with tumor differentiation, gender, age and tumor location (44). By contrast, Kuo *et al* (45) reported that claudin-4 expression was not associated with age, gender, depth of wall invasion, lymph node metastasis or differentiation; these results were comparable with those of the present study.

Several studies have investigated claudin-4 expression in cancer. One study reported that claudin-4 levels were markedly lower in diffuse-type gastric cancer compared with intestinal-type gastric cancer (45). Another study demonstrated that the expression of claudin-4 was significantly reduced in patients with positive lymphatic invasion in their gastric cancer tissue (16). In addition, reduced expression of claudin-4 was reported to be correlated with glandular structure and loss of differentiation in gastric cancer (46). Furthermore, it was suggested that the expression of claudin-4 attenuated pancreatic cancer cell invasion (20). Paradoxically, overexpression of claudin-4 was observed in breast and ovarian carcinoma (38,47); in addition, claudin-4 overexpression in ovarian cells may be highly associated with features of metastasis, including invasion, motility and cell survival (21). Thus, the expression patterns of claudin-4 in various types of cancer were diverse and provided contradictory results. The mechanisms for the upregulation or downregulation of claudin-4 expression in tumorigenesis remain to be fully elucidated and these paradoxical points require further investigation.

In conclusion, downregulation of claudin-1 expression in poorly-differentiated gastric adenocarcinoma is involved in the biological transformation of tumor behavior. Based on these results, claudin-1 was suggested to be an important protein associated with histological type and may have potential for use as a prognostic marker. Further studies, with a greater number of subjects, are required in order to elucidate the association of claudin-1 expression with tumor progression and to perform a long-term clinical survival analysis.

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