Differential expression of claudin-4 between intestinal and diffuse-type gastric cancer

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Abstract. Our previous microarray analysis of gastric cancer found that claudin-4 was differentially expressed between intestinal-type gastric cancer (IGC) and diffuse-type gastric cancer (DGC). Claudin-4 is a member of a large family of transmembrane proteins, claudins, essential in the formation and maintenance of tight junctions. To explore the roles of claudin-4 in the two histologically distinct types of gastric cancer, we selected 45 IGC and 48 DGC cases and then analyzed the expression of the protein using immunohistochemistry. We found that the overexpression of claudin-4 was greater in IGC than in DGC. A trend was observed between the overexpression of claudin-4 and lymph node metastasis, however, this association was not statistically significant. The results showed that the expression of claudin-4 was lower in DGC. Possibly it played a role in determining the diffuse phenotype and loose cohesion of cells in DGC in a similar manner as E-cadherin.

Introduction

Gastric cancer is the second most frequent cause of cancer death in the world after lung cancer (1). According to Lauren's classification, gastric cancer can be divided into two histologically distinct types, each of which accounts for half of the cases: Intestinal-type gastric cancer (IGC) and diffuse-type gastric cancer (DGC) (2). IGC, the predominant type of tumor in high-risk areas, has a glandular pattern and is usually accompanied by papillary formation or solid components (2). DGC, in contrast, consists of poorly cohesive cells diffusely infiltrating the gastric wall with little or no gland formation (2). A special subgroup of this type is the so-called signet ring cell carcinoma, in which the cell nucleus is pushed against the cell membrane creating a classical signet appearance due to an expanded, globoid, optically clear cytoplasm. IGC and DGC may result from the transformation of different epithelial cells or distinct molecular changes in common cell types (3).

Over the past decade, many studies have clearly demonstrated that the combination of molecular changes differs between IGC and DGC, suggesting that they have unique genetic alterations (4-6). Alterations in specific genes that play important roles in diverse cellular functions such as cell adhesion, signal transduction, differentiation, development or DNA repair have been identified (7,8). With regard to genetic alterations in tumor suppressor genes or oncogenes, gastric cancer is no exception. Inactivation due to loss of heterozygosity (LOH) and/or mutation of p53, APC and DCC have been reported in gastric cancer. Mutation of p53 was detected in ~30% of gastric cancers independent of the histological subtype (9,10). Up to 60% of the IGC cases but only 30% of DGC have a mutation/LOH of the APC gene (11). LOH of DCC has been detected in 50% of IGC cases, whereas in DGC, LOH is absent (12). The *met* proto-oncogene codes for the hepatocyte growth factor receptor which is preferentially amplified and overexpressed in DGC (13). Other growth factor and receptor signal systems that may be altered include EGFR, TGF-α and K-sam (14). Adhesion molecules such as ß-catenin have been detected in 30% of IGC mutations but are absent in DGC (15). E-cadherin is the binding partner of ß-catenin and plays a crucial role in establishing the structural integrity of epithelial tissues. E-cadherin mutations have been detected in 50% of DGC but absent in IGC (16).

Claudins are a large family of transmembrane proteins essential in the formation and maintenance of tight junctions (17). Tight junctions in epithelial cells provide a selective barrier and establish cellular polarity (18-20). These structures are typically lost in cancer and this loss may contribute to the invasive and metastatic phenotype of tumor cells (21-24). Using cDNA microarray analysis, we have previously shown that claudin-4 is differentially expressed between IGC and DGC (25). Claudin-4 was more overexpressed in IGC than in DGC. In the present study, we used 45 IGC and 48 DGC specimens to examine whether the immunohistochemical staining of claudin-4 is different between the two types of gastric cancer.

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Table I. Clinicopathological parameters of patient samples included for claudin-4 immunohistochemical analysis.

Parameters	IGC	DGC	P-value
Total	45	48	
Gender			
Male	30	26	>0.05
Female	15	22	
Age			
Mean±SD	64±9.4	57±14.2	0.00704
≤60	17	28	0.047
>60	28	20	
Depth of wall invasion			
T1	15	8	>0.05
T2	6	5	
T3	19	28	
T4	5	7	
Lymph node metastasis			
N0	19	14	>0.05
N1	15	15	
N2	7	15	
N3	4	4	
Differentiation			
Well	8	2	>0.05
Moderate	14	18	
Poor	23	28	

Patients and methods

Patients and specimens. Tissues specimens (N=93) were collected from patients with gastric cancer requiring subtotal or total gastrectomy resection in Chang Gung Memorial Hospital (CGMH) in Taiwan. All operations were performed between January 2000 and December 2001. Written informed consent was obtained before collection and this study was approved by the Institutional Review Board. Table I summarizes the clinical parameters of patients with gastric cancer. All specimens were divided into two groups according to Lauren's classification, IGC (N=45, 30 men and 15 women) and DGC (N=48, 26 men and 22 women). The mean ages of IGC and DGC patients were 64 and 57 years, respectively. Pathological TNM stages were obtained from clinical records. All tissue specimens were formalin-fixed and paraffin-embedded. Formalin-fixed tissue sections were stained with H&E and classified by a pathologist. These results were compared with the pathology record from CGMH. Final pathology was determined by consensus and reviewed if necessary.

Immunohistochemistry. The tissue block were constructed according to the method of Schraml *et al* (26) and the best representative morphological areas of the tumors were used in this study. The specimen sections were deparaffinized, treated with 3% hydrogen peroxide and microwaved after pretreatment in 10 mM citric acid to retrieve antigenicity. The sections were incubated with blocking solution containing PBS and 1% bovine serum album for 20 min at room temperature, and then incubated with anti-claudin-4 antibody (goat anti-human polyclonal antibody from Santa Cruz Biotechnology) overnight at 4˚C. After washing 4x with TBS, the sections were incubated with secondary antibody (rabbit anti-goat IgG, Santa Cruz Biotechnology). The immuno-complex was visualized by the immonoglobulin enzyme bridge technique using the Dako LSAB 2 System, HRP kit (Dako Co., Carpinteria, CA, USA) with 3, 3' diaminobenzidine tetrachloride as a substrate. The sections were lightly counterstained with hematoxylin, dehydrated with graded alcohols, cleared with xylene and mounted with a coverslip.

Scoring of the immunostaining. The immunostaining results were scored as follows according to a previous report (27). The immunostaining reaction was evaluated by subjective assessments of the median staining intensity (0, no stain; 1, weak; 2, moderate; 3, strong) and by the fraction of stained cells in percentage categories (0, 0-9%; 1, 10-49%; 2, 50-89%; and 3, ≥90%). This scoring system was previously shown to be reproducible (28). Claudin-4 immunoreactivity was classified as negative if <10% of the cells were stained. The scores of 0 to 3 were obtained as follows: Percentage categories and staining were each ranked as indicated above. The ranks for percentage and staining intensity were multiplied by each other, divided by 3, and rounded up to the nearest whole number (28). The results of immunostaining in the tumor and normal tissues were divided into three groups: strong (rank of tumor tissue >rank of normal tissue), equal (rank of tumor tissue = rank of normal tissue), and weak (rank of tumor tissue <rank of normal tissue) (Fig. 2).

Statistical analysis. Chi-square or Fisher's exact test were used to test for an association between claudin-4 expression and the clinicopathological parameters. The level of significance was set at 0.05. All reported p-values were two-sided. The data analyses were carried out using SAS statistics package (version 8.1 for windows; SAS Institute, Inc., Cary, NC).

Results

Expression of claudin-4 in IGC and DGC. The H&E staining of IGC and DGC is shown in Fig. 1A and B, respectively. IGC has a glandular pattern and is usually accompanied by papillary formation or solid components. DGC, in contrast, consists of poorly cohesive cells diffusely infiltrating the gastric wall with little or no gland formation. A special subgroup of this type is the so-called signet ring cell carcinoma, in which the cell nucleus is pushed against the cell membrane creating a classical signet appearance due to an expanded, globoid, optically clear cytoplasm. The results of immunostaining in tumor and normal tissues were divided into three groups: strong (rank of tumor tissue >rank of normal tissue) (Fig. 2A and B), equal (rank of tumor tissue = rank of normal

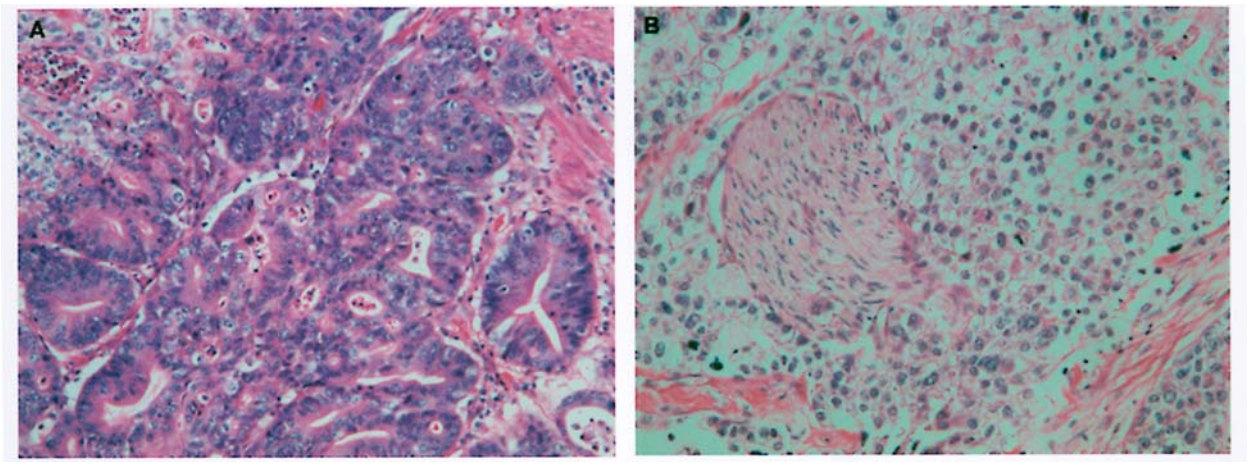

Figure 1. The H&E staining of IGC and DGC (magnification x200). (A), IGC and (B), DGC.

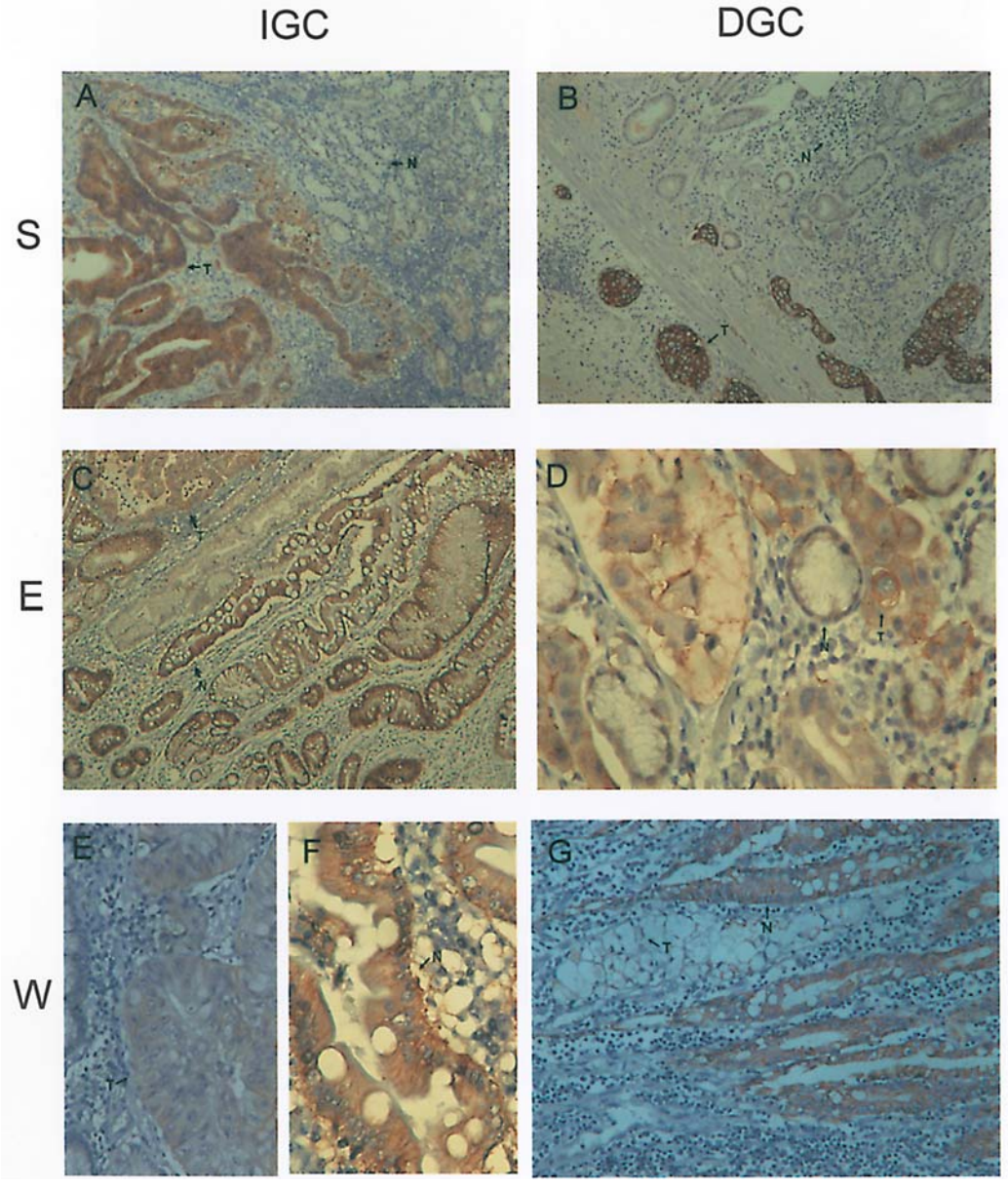

Figure 2. Immunohistochemistry of claudin-4 in IGC and DGC. The overexpression of claudin-4 in IGC and DGC greater than in normal tissues is shown in (A) (magnification x100) and (B) (magnification x100), respectively. The expression of claudin-4 in IGC and DGC equal to that in normal tissues is shown in (C) (magnification x40) and (D) (magnification x400), respectively. The expression of claudin-4 in IGC less than in normal tissues is shown in (E) (magnification x200) and (F) (magnification x400), respectively. The expression of claudin-4 in DGC less than in normal tissues is shown in (G) (magnification x200). T, tumor tissues; N, normal tissues; S, strong; E, equal; W, weak.

Table II. Correlations between claudin-4 expression in cancer and normal tissues and clinicopathological parameters.

Parameters	Claudin-4 (tumor:normal)			P-value
	Strong N (%)	Equal N (%)	Weak N (%)	
Ages				
≤60	24 (53%)	16 (36%)	5 (11%)	0.6657
>60	29 (61%)	16 (33%)	3 (6%)	
Gender				
Male	33 (59%)	16 (29%)	7 (12%)	0.1406
Female	20 (54%)	16 (43%)	1 (3%)	
Histological type				
Intestinal	34 (76%)	10 (22%)	1 (2%)	0.0011
Diffuse	19 (40%)	22 (46%)	7 (14%)	
Depth of wall invasion				
T1	12 (52%)	9 (39%)	2 (9%)	
T2	4 (36%)	5 (46%)	2 (18%)	0.6512
T3	30 (64%)	14 (30%)	3 (6%)	
T4	7 (58%)	4 (33%)	1 (9%)	
Lymph node metastasis				
-	15 (46%)	15 (46%)	3 (9%)	0.1543
+	38 (64%)	17 (28%)	5 (8%)	
Differentiation				
Well	8 (80%)	2 (20%)	0 (0%)	
Moderate	16 (50%)	13 (41%)	3 (9%)	0.6529
Poor	29 (57%)	17 (33%)	5 (10%)	

The results of immunostaining in tumor and normal tissues were divided into three groups: Strong, rank of tumor tissue >rank of normal tissue; equal, rank of tumor tissue = rank of normal tissue; weak, rank of tumor tissue <rank of normal tissue.

tissue) (Fig. 2C and D), and weak (rank of tumor tissue<rank of normal tissue) (Fig. 2E and F). The overexpression of claudin-4 in IGC and DGC greater than in normal tissues is shown in Fig. 2A and B, respectively. The expression of claudin-4 in IGC and DGC equal to that in normal tissues is shown in Fig. 2C and D, respectively. The expression of claudin-4 in IGC less than in normal tissues is shown in Fig. 2E and F, respectively. The expression of claudin-4 in DGC less than in normal tissues is shown in Fig. 2G.

Relation between claudin-4 expression and clinicopathological parameters. The relation between claudin-4 expression and the clinicopathological parameters of the patients is shown in Table II. The expression of claudin-4 in IGC is divided into three groups: strong, equal and weak (tumor: normal tissues) and their frequencies were 76%, 22%, and 2%, respectively. In comparison the frequency of claudin-4 expression in DGC was 40%, 46% and 14%, respectively. The overexpression of claudin-4 was greater in IGC than in DGC (p=0.0011). Except for histological type, the expression of claudin-4 was not associated with age, gender, depth of wall invasion, lymph node metastasis and differentiation.

Discussion

In this study, expression of claudin-4 was studied in 93 cases of gastric cancer. The comparison of claudin-4 in two histologically distinct types of gastric cancer showed that DGC had lower expression of this protein compared with IGC. Thus, the loss of claudin-4 expression may be the one phenotypic feature distinguishing the two tumors types in analogy with E-cadherin, the expression of which was also lost in DGC (29,30). The loss of claudins and other tight junction proteins in cancer has been interpreted as a mechanism for the loss of cell adhesion, an important step in the progression of cancer to metastasis. A recent study showed that expression of claudin-4 in pancreatic cancer cells reduces the invasiveness of these cells (31).

The expression of claudins has been found to be altered in several cancer types. Claudin-1 has been found to be reduced in breast cancer as well as in colon cancer (32-34). The loss

of claudin-7 is associated with a more aggressive behavior of breast carcinoma and head and neck cancer (35,36). These reports of decreased tight junction protein expression in cancer are consistent with the generally accepted idea that tumorigenesis is accompanied by a disruption of tight junctions, a process that may play an important role in the loss of cohesion, invasiveness, and the lack of differentiation observed in cancer cells. In addition to the down-regulation of protein levels, phosphorylation of tight junction proteins, including claudins, may affect tight junction function in cancer. Phosphorylation of claudin-1 by mitogen activated protein kinases (37) and protein kinase C (38), as well as phosphorylation of claudin-5 by cyclic AMP-dependent protein kinase (39,40) have been reported. Also, WNK4 kinase has been shown to phosphorylate claudin-3 and claudin-4, and decrease tight junction function (41). Phosphorylation of claudin-3 and claudin-4 in ovarian cancer cells has also been shown to disrupt tight junctions (42).

Paradoxically, other studies have shown that certain claudin proteins are up-regulated in cancer. Overexpression of claudin-3 and 4 has been shown in breast and ovarian carcinoma (35,43). In pancreatic adenocarcinoma and its precursor lesions, claudin-4 overexpression has been found to be present (31,44). Recent work has shown that, at least in the case of ovarian cells, the expression of claudin-3 and claudin-4 may lead to an increase in invasion, motility, and cell survival (45), all characteristics important for metastasis. Consistent with these *in vitro* findings is a report that claudin-4 expression in pancreatic intraductal papillary mucinous neoplasms was associated with a more invasive phenotype (46). Claudin-3 and claudin-4 are receptors for the *Clostridium perfringens* enterotoxin (CPE) (47). CPE is a single polypeptide of 35 kDa, which receptor binding causes cytolysis through its effects on membrane permeability. A high expression of claudin-3 and claudin-4 in multiple cancers may thus represent a unique opportunity for innovative therapy using CPE (48). Prostate adenocarcinoma cells expressing claudin-3 and claudin-4 have indeed been shown to be sensitive to CPE-mediated cytolysis (49).

The patterns of claudin-4 expression in the various cancers were diverse and controversial. The reasons for the up-regulation or down-regulation in tumorigenesis were unclear. We found the overexpression of claudin-4 in IGC and in general, liver metastasis was frequently seen in IGC (50). The overexpression of claudin-4 may be associated with the distal metastasis of gastric cancer. A recent report showed that claudin-4 expression was associated with increased matrix metalloproteinase-2 activity in ovarian epithelial cells and enhanced cell invasion (45). Otherwise, the less expression of claudin-4 in DGC correlated well with the general observation that peritoneal metastasis was frequently seen in DGC (50). The decreased claudin-4 expression may be associated with proximal invasion via the loss of the tight junction.

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