Expression of claudin-2 in the multistage process of gastric carcinogenesis

X. Song¹, X. Li², Y. Tang¹, H. Chen¹, B. Wong³, J. Wang¹ and M. Chen¹
¹Department of Gastroenterology, the First Affiliated Hospital, Sun-Yat Sen University, Guangzhou, China, ²Key Laboratory of Department of Nephrology of National Key Subject, Key Laboratory of Nephrology of Ministry of Education and Key laboratory of Nephrology of Ministry of Health, the First Affiliated Hospital, Sun-Yat Sen University, Guangzhou, China and ³Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong

Summary. Objective: Claudin-2 is an important component of the tight junction (TJ) of epithelial cells, and its protein expression between many tumours is significantly different. It is unclear if Claudin-2 overexpression is a trigger or a consequence during carcinogenesis. The multistage tissues of gastric carcinogenesis provides us a valuable model for determining tumourigenicity. Methods: This paper investigated, for the first time, claudin-2 expression in the pathological paraffin tissues of sinus ventriculi from gastroscopic biopsy. To determine claudin-2 expression in gastric carcinogenesis by immunochemical ABC technique. Results: Altogether, 108 chronic superficial gastritis, 55 chronic atrophic gastritis, 109 intestinal-type metaplasia, 93 dysplasia and 52 gastric intestinal-type adenocarcinoma samples were analyzed. Results indicated that the percentage of claudin-2-positive cases was 0% for chronic superficial gastritis (0/108), 0% for chronic atrophic gastritis (0/55), 0% for intestinal-type metaplasia (0/109), 35.87% for dysplasia (33/92), and 73.47% for gastric intestinal-type adenocarcinoma (36/49) respectively, primarily in the cell membrane, and gradually increased in the multistage process of gastric carcinogenesis (P<0.001). Conclusions: This suggests that claudin-2 protein overexpression may be closely correlated to gastric carcinogenesis.

Key words: Claudin-2, Immunohistochemistry, Gastric cancer, Expression, Tight junction

Introduction

Claudins are small proteins of 20-24 kDa, with two extracellular domains, four transmembrane domains, and cytoplasmic N- and C-termini (Itoh et al., 1999). To date, 24 subtypes of claudin have been identified (Tsukita et al., 2001), which are expressed in an organ-specific manner and regulate the tissue-specific physiological properties of tight junctions (Tsukita and Furuse, 2000; Tsukita et al., 2001). Claudins form the backbone of tight-junction strands (Sasaki et al., 2003), which are involved in both paracellular sealing and membrane domain differentiation (Michl et al., 2003). Thus, claudins have clearly been demonstrated to be key structural, as well as functional, components of TJ strands (Furuse et al., 1999; Tsukita and Furuse, 2000). Deficiency, mutation or aberrant expression of distinct claudins has been reported to be associated with various pathological conditions, including inflammation and tumorigenesis (Simon et al., 1999; Tsukita et al., 2001; Wilcox et al., 2001; Morin, 2005).

Claudin-2 is a structural and functional component of TJ in many tissues (Sakaguchi et al., 2002). Claudin-2 is considered to form ion-selective channels in tight junctions of epithelial cells (Amasheh et al., 2002), and claudin-2 plays a crucial role in the paracellular barrier function by opening pores for small cations. But, in the expression of claudin-2 there exist significant, even contrary differences in different tissues. In Madin-Darby canine kidney II cells, the addition of claudin-2 markedly decreases the tightness of individual TJ strands (Kiuchi-Saishin et al., 2002). The presence of claudin-2 destroys the formation of cation-selective channels (Amasheh et al., 2002). Claudin-2 is selectively

Abbreviations: TJ, tight junction; APES, 3–Amnopropyltriethoxy Silane; DAB, 3,3’-diaminobenzidine; IHC, immunohistochemistry; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin; Cdx, caudal-related homeobox.
expressed in the proximal nephron in mouse kidney, and it may be responsible for their uniquely leaky permeability properties (Enck et al., 2001). In contrast, removal of claudin-2 from the tight junction complexes results in increased paracellular flux, as well as decreased transepithelial electrical resistance (Furuse et al., 1998).

Some studies demonstrate that claudin-2 has the potential of directing the variability of paracellular transport and barrier functions within gastro-intestinal organs (Tsukita et al., 2001). Altered expressions of claudin-2 are associated with colon cancer (Miyoshi and Takai, 2005). In Soini’s study (Soini et al., 2006), expression of claudins 1, 3, 4 and 5 can be seen significantly in gastric carcinomas of intestinal type, and their expression is significantly associated with each other, while strong expression of claudin-5 was associated with higher cell proliferation and apoptosis. Cunningham’s findings (Cunningham et al., 2006) indicate that Claudin-4 expression is present in 100% of intestinal metaplasia lesions and 100% of gastric epithelial dysplasia lesions, but in only 15% of normal stomach samples. Thus, Claudin-4 is considered to be useful in the diagnosis and therapeutic targeting of gastric adenocarcinoma precursor lesions.

It is generally accepted that intestinal-type gastric adenocarcinoma arises through a multistep process originating with chronic gastritis, progressing through stages of atrophy, intestinal metaplasia and dysplasia, and finally invasive carcinoma (Correa, 1996). With the stimulation of all kinds of factor, enhanced rates of apoptosis could potentially accelerate progression to atrophic gastritis with a concomitant increase in the risk of distal gastric adenocarcinoma. In contrast, reduced rates of cell loss, especially when accompanied by hyperproliferation, could lead to a heightened retention of mutagenized cells, which might also predispose certain colonized individuals towards development of gastric cancer (Richard et al., 2002). This sequential multifactorial oncogenetic process is known as the "Correa cascade" (Zivny et al., 2003). Until now, Claudin-2 has not been studied in multistage gastric carcinogenesis. Therefore, the aim of this paper is to investigate Claudin-2 protein expression in the multistage process of gastric carcinogenesis. Immunological analysis could provide some experimental basis for determining gastric pathogenesis.

### Materials and methods

**Patients and specimens**

This study comprised 417 patients (272 males and 145 females; mean age, 50.42±13.86; age range, 17–85 years). Cases with gastritis, atrophy, intestinal metaplasia, dysplasia and intestinal-type gastric carcinoma were 108, 55, 109, 93 and 52 respectively (Table 1). Biopsy specimens were collected from the antrum of the stomach by gastroscopy between 2000 and 2004 from the archives of the Department of Pathology, the First Affiliated Hospital, Sun-Yat Sen University. All samples had been fixed in neutral buffered formalin and embedded in paraffin. Serial 5 µm sections were stained with hematoxylin-eosin. Chronic, active, or chronic-active gastritis, atrophy (loss of proper glands) and intestinal metaplasia were classified according to the Updated Sydney System (Dixon et al., 1996), and dysplasia (low or high grade) was evaluated according to previously published criteria (Rugge et al., 2000). Types of intestinal metaplasia were classified into complete and incomplete, according to the findings of the absorptive cells, brush border, Paneth cells and columnar mucous cells (Jass, 1980; Craanen et al., 1991).

Early gastric cancer was pathologically diagnosed by the growth of tumour confined to the mucosa and submucosa of the stomach as described previously (Kodama et al., 1983). Gastric carcinoma was categorized pathologically as either intestinal type or diffuse type according to the classifications of Laurén (Laurén, 1965). When diffuse and intestinal patterns coexisted, the gastric carcinoma histotype was assessed according to the most-represented phenotype. Clinical data were reviewed to ensure that they were indeed gastric primaries.

Patients were excluded if they had received anti-ulcer agents or antibiotics during the two months before the examination, or had any known histories of gastric tumours, gastric or duodenal ulcers, and gastric surgery. A (neo-)adjuvant radio-or chemotherapy was not performed.

**Claudin-2 antibody**

Mouse monoclonal IgG2b antibody via C-terminus of the human Claudin-2 protein (clone 12H12) used for

### Table 1. Composition of age and gender of cases.

<table>
<thead>
<tr>
<th>group</th>
<th>cases</th>
<th>age ($\bar{\text{x}}\pm S$)</th>
<th>gender</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(<strong>male</strong>)</td>
<td>(<strong>female</strong>)</td>
</tr>
<tr>
<td>chronic superficial gastritis</td>
<td>108</td>
<td>45.56±14.32</td>
<td>74 (68.52)</td>
</tr>
<tr>
<td>chronic atrophy gastritis</td>
<td>55</td>
<td>50.15±12.43</td>
<td>34 (61.82)</td>
</tr>
<tr>
<td>intestine-type metaplasia</td>
<td>109</td>
<td>51.68±12.91</td>
<td>60 (55.05)</td>
</tr>
<tr>
<td>dysplasia</td>
<td>93</td>
<td>49.33±13.41</td>
<td>69 (74.19)</td>
</tr>
<tr>
<td>intestine-type gastric cancer</td>
<td>52</td>
<td>60.10±12.05</td>
<td>35 (67.31)</td>
</tr>
<tr>
<td>sum</td>
<td>417</td>
<td>50.42±13.86</td>
<td>272 (65.23)</td>
</tr>
</tbody>
</table>
immunohistochemistry was purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA) and used in formalin-fixed paraffin-embedded tissues.

**Immunohistochemistry**

Samples with chronic gastritis, atrophic gastritis, intestinal-type metaplasia, atypical hyperplasia, and gastric carcinoma during carcinogenesis multistage were processed for immunohistochemical analyses to determine claudin-2 levels and distribution patterns. Paraffin-embedded samples were cut to 4 µm thickness in a cryostat (CM 3000, Leica, Bensheim), and mounted onto APES (Sigma)-coated slides. Sections were stained with hematoxylin-eosin for morphological analysis. Tissue sections were also stained for the presence of human gastric epithelia using a mouse anti-Claudin-2 (zymed) antibody. Tissues that were stained with anti-Claudin-2 antibodies used the Novostain super ABC kit (Novocastra Laboratories, Newcastle, UK), according to the manufacturer’s instructions. All tissue sections were baked in an oven at 60°C for one hour to melt the paraffin. Samples were then deparaffinized and hydrated by incubation in xylene and a graded alcohol series. Endogenous peroxidases were destroyed by immersion in 0.3% hydrogen peroxide in methanol for 30 minutes. After, the sections were heated in a microwave oven in 10 mM citrate buffer (pH 6.0) for 10 min at 800 W for antigen retrieval. Then samples were blocked with 10% horse serum for 20 minutes at room temperature. Primary antibodies were diluted in PBS according to the manufacturers’ recommendations (1:100 for anti-Claudin 2) and a total volume of 150 µl was added to each section overnight. All subsequent steps were performed at room temperature. After two 5-minute washes with PBS, tissue sections were incubated with biotinylated anti-mouse (Novostain super ABC kit) secondary antibody for 30 minutes. Samples were again washed twice with PBS for 5 minutes each. Streptavidin-peroxidase conjugate incubations were then performed for 30 minutes. Color visualization of the complex was achieved by incubating tissue sections with a chromogenic substrate 3,3′-Diaminobenzidine (DAB, Dako Corporation, Carpinteria, CA, USA) for 5 minutes. All tissue sections were briefly rinsed in water and counterstained with Mayer’s hematoxylin (Sigma, Catalog # MHS-16) and mounted with Eukitt (Kindler, Freiburg, Germany). Normal colon tissue served as positive controls. Negative controls were achieved by substituting preimmune mouse IgG for the primary antibodies. Normal gastric mucosae were used as an internal negative control. All sections were examined using an Olympus B·51/ DP70 microscope.

**Assessment of tissue staining**

All of the hematoxylin-eosin-stained sections and immunohistochemical studies were independently and blindly reviewed by two pathologists. Staining was evaluated in columnar epithelial or tumor cells. The percentage of positively stained cells was an average after counting the intensely stained and the total number of cells from at least 10 high-power fields. When the evaluations differed, a consensus interpretation was reached for all slides using a two headed microscope.

The immunoreactivity was assessed as follows: −, no immunostaining present; +, <25% of cells positive; ++, 25–50% of cells positive; +++; 50–75% of cells positive; ++++, 75–100% of cells positive. In the evaluation, expression of claudin-2 of more than 50% of tumor cells is regarded as positive immunoreactivity throughout this paper. Both membrane-bound and cytoplasmic positivity was considered significant (Soini, 2005).

**Statistical analyses**

Comparison between groups in terms of claudin-2 expression was performed by means of the Chi-Square test. The statistical software used was SPSS11.0 (SPSS Inc. Chicago). Statistical tests were two-sided and P < 0.05 was considered statistically significant.

**Ethical approval**

The study was approved by the Ethics Committee of the First Affiliated Hospital, Sun-Yat Sen University. Written informed consent was obtained from all patients.

**Results**

**Expression of claudin-2 in the multistage process of gastric carcinogenesis**

Cases with gastritis, atrophy, intestinal metaplasia, dysplasia and intestinal-type gastric carcinoma were 108, 55, 109, 93 and 52, respectively (Figs. 2-4). But, one of 93 cases with dysplasia was excluded because of observing no pathological change of dysplasia under a microscope in the hematoxylin-eosin-stained sections during the experiment, perhaps owing to excessive section cut from tissue sample embedded by paraffin wax. Two of 52 cases with intestinal-type gastric

![Fig. 1. Percentage of Claudin-2-positive cases in the multistage process of gastric carcinogenesis.](image-url)
carcinoma were excluded because of shedding from the glass slide during the experiment, and one of 52 was excluded due to nonspecific stain by IHC.

Claudin-2 protein was primarily expressed in the cell membrane, and partly in the cytoplasm, stained with yellow, brown yellow or dark brown by DAB (Figs. 5, 6). The percentage of claudin-2-positive cases of chronic superficial gastritis, chronic atrophic gastritis, intestine-type metaplasia, dysplasia and gastric intestine-type adenocarcinoma was respectively 0 (0/108), 0 (0/55), 0

Fig. 2. Histological features and immunohistochemistry for claudin-2 in the chronic superficial gastritis. A and B. The superficial lamina propria, into which gastric glands protrude, includes plasma cells with abundant eosinophilic cytoplasm without loss of gastric glands. Haematoxylin-eosin staining. Pathological tissue section number: 35808. C-E. No immunohistochemical staining; ABC staining method/DAB. Counterstained with haematoxylin. Scale bars: A, D, 20 µm; B, E, 50 µm; C, 10 µm.
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Claudin-2 protein expression was negative in the chronic superficial gastritis, chronic atrophic gastritis, intestine-type metaplasia, and increased gradually in the dysplasia and gastric intestine-type adenocarcinoma (P<0.001). The results suggest that Claudin-2 protein overexpression was correlated to gastric carcinogenesis (Table 2).

Fig. 3. Histological features and immunohistochemistry for claudin-2 in the chronic atrophic gastritis. **A and B.** Loss of glandular tissue in multiple foci, leading to thinning of the mucosa. Haematoxylin-eosin staining. Pathological tissue section number: 54470. **C-E.** No immunohistochemical staining; ABC staining method/DAB. Counterstained with haematoxylin. Scale bars: A, D, 20 µm; B, E, 50 µm; C, 10 µm.
Discussion

Our present results indicate that claudin-2 protein is not expressed in the chronic superficial gastritis, chronic atrophic gastritis, intestine-type metaplasia, but significantly in the dysplasia (35.87%) and gastric intestine-type adenocarcinoma (73.47%) (Table 2, Fig. 1). This suggests that claudin-2 expression is related to the development of gastric cancer.

**Fig. 4.** Histological features and immunohistochemistry for claudin-2 in the gastric foveolar epithelium with intestinal metaplasia. **A and B.** The foveolar and granular epithelium can be recognized morphologically by the presence of scattering goblet cells, absorptive cells, and cells resembling colonocytes; a well-demarcated focus of intestinalized and dilated glands, irregular in shape and size, shows back-to-back architecture. Haematoxylin-eosin staining. Pathological tissue section number: 35970. **C-E.** No immunohistochemical staining; ABC staining method/DAB. Counterstained with haematoxylin. Scale bars: A, D, 20 µm; B, E, 50 µm; C, 10 µm.
closely to gastric carcinogenesis. But Soini et al. (2005) also analyzed claudin-2 expression in a small number of gastric carcinoma by using the monoclonal anticlaudin-2 antibody and reported that 12 of 13 cases with gastric carcinoma (92%) were positive for claudin-2. In our study, the percentage of claudin-2-positive cases only in intestine-type gastric adenocarcinoma is a little lower when compared to Soini’s studies in gastric carcinoma.

Fig. 5. Histological features and immunohistochemistry for claudin-2 in the gastric foveolar epithelium with dysplasia (black arrows). A and B. The foveolae with architectural distortion; the tubular structures are irregular in shape, branching and folding. The dysplastic glands are lined by crowded elongated cells with large, pseudostratified and hyperchromatic nuclei. The dysplastic cells extend to the surface epithelium. There are areas of loss of polarity. Haematoxylin-eosin staining. Pathological tissue section number: 19384. C-E. Membrane-bound and cytoplasmic marked staining for claudin-2 in the gastric epithelium with dysplasia (black arrows), partly in the focal area of the gastric foveolar epithelium in the aberrant hyperplasia (white arrows); no staining in the normal gastric epithelium (swallow-tailed arrows); ABC staining method/DAB. Counterstained with haematoxylin. Scale bars: A, D, 20 µm; B, E, 50 µm; C, 10 µm.
partly due to claudin-2 expression both in intestine-type and diffuse-type gastric adenocarcinoma and/or much less samples in Soini’s studies. However it seems that the explanation cannot get the support from another report (Soini et al., 2006), showing significant expression of claudins 1, 3, 4 and 5 in gastric carcinomas.

Fig. 6. Histological features and immunohistochemistry for claudin-2 in the gastric intestine-type adenocarcinoma. **A and B.** Neoplastic glands, irregular in shape and size, invading the muscular tissue. Haematoxylin-eosin staining. Pathological tissue section number: 24443. **C-E.** Membrane-bound moderate staining of claudin-2 in a gastric intestine-type adenocarcinoma; ABC staining method/DAB. Counterstained with haematoxylin. Scale bars: A, D, 20 μm; B, E, 50 μm; C, 10 μm.
of intestinal type, but lower or none in diffuse-type gastric carcinomas.

Some studies (Lauren, 1991; Yuasa, 2003) suggest intestine-type and diffuse-type may be of different histogenesis, etiology and pathogenesis. Intestine-type gastric cancers are thought to develop from intestinal metaplasia, while diffuse type ones mainly develop from the normal mucosa. Thus, we think that investigation of gastric adenocarcinoma in the multistage process of gastric carcinogenesis should mainly be correlated to intestine-type.

Even more confusing, Aung et al. (2006) investigated claudin-2 expression in normal stomach and in gastric carcinoma cases, and of the 146 cases of gastric carcinoma, only 3 (2.1%) were positive for claudin-2, without obvious staining of claudin-2 found in normal stomachs. There is no significantly different expression between gastric carcinoma and normal stomach. Compared to our results, there exist significant differences, but it is difficult to explain the discrepancy between them only according to sample size and/or antibody sensibility. Thus, it is maybe more reasonable to investigate claudin-2 expression respectively in intestine-type, and diffuse-type gastric carcinoma.

It is quite intriguing that this investigation indicated that claudin-2 was not expressed in gastric metaplasia, significantly in dysplasia and intestinal adenocarcinoma, whereas claudins 1, 3, 4, 5 are expressed in gastric intestinal metaplasia (Soini et al., 2006). It has also been reported that claudin-2 is consistently undetectable in normal esophageal epithelium, but detectable at low-to-moderate levels in 2 of 8 Barrett’s biopsies (metaplasia) (Rendon-Huerta et al., 2003). Results above suggest that claudin-2 expression may be a mid-late event in the multistage process of gastric carcinogenesis.

Accumulating evidence demonstrated that claudin-2 is modulated by many transcriptional factors. The human claudin-2 promoter contains two sites for the intestine-specific homeodomain protein family Cdx (Suh et al., 1994), designated CdxA and CdxB. In addition, the promoter also has binding sites for HNF-1α and HNF-3β (Cereghini, 1996), as well as putative AP-1 (Wasylko et al., 1989), NF-κB (Gilmore, 1990), and GATA-binding sites (Orkin, 1992). Furthermore, cotransfection experiments showed that the claudin-2 promoter is activated by HNF-1 and GATA-4 in a cooperative manner. Forced GATA-4 expression in Caco-2 cells enhances maintenance of claudin-2 expression during differentiation (Escaffit et al., 2005). More recently, claudin-2, which is involved in cell-cell adhesion, has been identified as a novel transcriptional target of Cdx2, which is crucial for the development of gastric atrophy and for intestinal transdifferentiation processes (Sakaguchi et al., 2002; Faller and Kirchner, 2005). Also, the changes of connexin32 and claudin-2 may be controlled at the transcriptional level via NF-κB, HNF-1α, and Cdx2 (Yamamoto et al., 2004). This indicates that these transcriptional factors mentioned above may regulate claudin-2 expression. Also, our previous study has concluded that Cdx2 expression increased gradually in the multistage tissue of gastric carcinogenesis (Chen et al., 2006). The results above suggest that claudin-2 is probably regulated by several kinds of transcriptional factors.

Further studies are needed to explore the exact mechanism of claudin-2 involvement in gastric adenocarcinoma pathology.

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