Expression of claudin-4 and β-catenin in gastric premalignant lesions

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Abstract

Background and study aims : **Abnormal expression of claudin-4 and β-catenin play a role in carcinogenesis. The purpose of the present study was to examine claudin-4 and β-catenin expression in normal and precancerous gastric mucosa.**

Patients and methods : **Endoscopic biopsy specimens [normal gastric mucosa (n = 22), intestinal metaplasia (n = 24), dysplasia (n = 18), *Helicobacter pylori (H. pylori)*-associated chronic gastritis (n = 32) and remnant gastric mucosa (n = 18)] obtained from different 114 patients were examined by immunohistochemistry.**

Results : **Claudin-4 expression was present in 94.4% of dysplasia, 87.5% of intestinal metaplasia, 62.5% *H. pylori*-associated chronic gastritis, and 88.9% remnant gastric mucosa but only 18.2% of normal gastric mucosa biopsies. Decreased expression of β-catenin was present in 27.8% of dysplasia, 8.3% of intestinal metaplasia, 15.6% of *H. pylori*-associated chronic gastritis, and 22.2% of remnant gastric mucosa biopsies, but was not present in normal gastric mucosa. When compared with normal gastric mucosa, there was a significant difference in claudin-4 expression in all groups ($P < 0.05$), but a significant difference was detected in dysplasia and remnant gastric mucosa for β-catenin ($P < 0.05$).**

Conclusions : **Our results suggest that claudin-4 expression is upregulated in premalignant gastric alterations.** (Acta gastroenterol. belg., **2009**, 72, **407-412**).

Key words : claudin-4, beta-catenin, gastric epithelial dysplasia, intestinal metaplasia, *Helicobacter pylori*.

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide (1). Gastric adenocarcinoma is thought to develop in a stepwise manner. Intestinal metaplasia (IM), gastric epithelial dysplasia, *Helicobacter pylori (H. pylori)* - associated chronic gastritis, and remnant gastric mucosa are precancerous lesions. Premalignant changes of the gastric mucosa are commonly observed in routine biopsies obtained during gastroscopy. Nevertheless, clear guidelines for follow-up and treatment of these patients are lacking ; as a result, follow-up frequency and treatment of individual patients vary widely in clinical practice (2).

In recent years, DNA microarray technologies have identified many different gene expression patterns in a variety of human cancers. Claudin-4 is a member of the claudin gene family, which is essential for the formation of tight junctions (3). Claudin-4 overexpression has been shown in premalignant lesions of the stomach (4) and esophagus (5). E-cadherin and its associated cytoplasmic proteins (α, β, and γ-catenins) play an essential role in the control of epithelial differentiation (6). Abnormalities of these adhesion molecules have also been extensively studied as major factors in cancer progression and metastasis (7).

In this study, we investigated the expression pattern of claudin-4 and β-catenin in gastric epithelial dysplasia, IM, *H. pylori* - associated chronic gastritis, remnant gastric mucosa, and normal gastric mucosa, using immunohistochemical staining.

Methods

Patients and tissue samples

Endoscopic gastric biopsy specimens were obtained from 114 different patients undergoing upper gastrointestinal endoscopy to investigate dyspeptic symptoms in the Gastroenterology Clinic of Cumhuriyet University Medical School during the period January 2007 to September 2007. Eighteen of the specimens were obtained from patients with a previous partial gastrectomy. Approval of the local ethics committee was obtained prior to the study. All subjects gave informed consent to participate. All tissue specimens were formalin-fixed, paraffin-embedded, stained with hematoxylin and eosin (H&E), and classified. A total of 114 tissue microarrays were classified as normal gastric mucosa (n = 22), IM (n = 24), gastric epithelial dysplasia (n = 18), remnant gastric mucosa (n = 18), and *H. pylori*-associated chronic gastritis (n = 32). Dysplasia was diagnosed using the Padova International classification system (8). Gastric mucosal inflammation and IM were diagnosed on H&E stained sections using the Sydney classification. *H. pylori* was identified using the improved toluidine-O staining and Giemsa staining.

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Submission date : 30/03/2009
Acceptance date : 15/10/2009

Immunohistochemistry

Paraffin sections from each case were carefully selected. Sections were deparaffinized in xylene and dehydrated through graded concentrations of ethanol. After blocking endogenous peroxidase activity with 3% hydrogen peroxide for 15 min, the sections were heated in a 0.01 mol/L citrate buffer in a microwave pressure cooker for 20 min. The slides were allowed to cool to room temperature, and non-specific binding was blocked with normal horse serum for 20 min at room temperature. The sections were further incubated with the primary antibody against β-catenin (Labvision, Neomarkers, RB 9035-R7) and against claudin-4 (Lab vision, Neomarkers, RB 9266-R7) for 30 min. The sections were then stained using the avidin-biotin complex (ABC) immunoperoxidase technique, using commercially available reagent (ABC kit, Labvision, USA). To demonstrate binding sites, AEC chromogen was applied. Phosphate buffered saline was used for rinsing between each step. Finally, all sections were counterstained with Mayer's hematoxylin.

Evaluation of the immunostaining

Samples were independently examined by two experienced pathologists, and a high level of concordance (90%) was achieved. In cases of disagreement, the slides were reviewed and a consensus achieved. Staining intensity was graded semiquantitatively from 0 to 3 for claudin-4 : 0 absence of membrane staining, 1+ some membrane staining evident, 2+ strong membrane staining, 3+ very strong staining/complete membrane staining (4). An absence of staining for claudin-4 was considered normal ; an increase in the intensity of membranous staining was considered abnormal.

For β-catenin, staining intensity was graded semiquantitatively from 0 to 3 : 0 negative staining, 1+ cytoplasmic staining, 2+ heterogeneous staining, 3+ normal membranous staining. Negative staining, cytoplasmic staining, and heterogeneous staining for β-catenin were considered abnormal (9).

Statistical analysis

The proportion of positive samples was calculated in normal gastric mucosa, intestinal metaplasia, dysplasia, *H. pylori* - associated chronic gastritis, and partial gastrectomy samples. The sensitivity of the test for claudin-4 was calculated as the proportion of positive results in the groups except for the normal gastric mucosa group. Specificity for claudin-4 was calculated as the proportion of negative results in normal gastric tissue samples. Binomial exact 95% confidence intervals (95% CI) were calculated for sensitivities and specifities. Pearson chi-square or Fisher's exact test were used to test for difference between groups in the claudin-4 and β-catenin expression, using SPSS 13.0. A *P* value of less than 0.05 was considered significant.

Results

Patients

This study included 114 patients (60 males and 54 females). Median age was 61 ± 21 years. The mean time after a partial gastrectomy was 13 (10-26) years. 10 resections were performed for gastric ulcer and 8 for duodenal ulcer ; 16 patients had undergone gastrojejunostomy (Billroth II) and 2 gastroduodenostomy (Billroth I).

Immunohistochemical staining for claudin-4 and β-catenin

1. Normal gastric mucosa

There was negative expression of claudin-4 in 18/22 (82%) normal gastric mucosa samples (Fig. 1A). Abnormal staining was very weak in 2 samples, moderate in 1, and very intense in 1 (Table 1). Beta-catenin stained intensely in a membranous distribution throughout the epithelium in all slides (Table 2 ; Fig. 2A).

2. Dysplasia

There was abnormal expression of claudin-4 in 17/18 (94.4%) dysplasia samples (Fig. 1D). Abnormal staining was very weak in 5 samples, moderate in 6, and very intense in 6 (Table 1). The sensitivity of claudin-4 in distinguishing dysplasia from normal stomach was 94.4% (95% CI,73.6-98.8) and the specificity 81.8% (95% CI,53.3-93.4). There was normal membranous staining in 13/18 (72.2%) samples labeled with β-catenin and

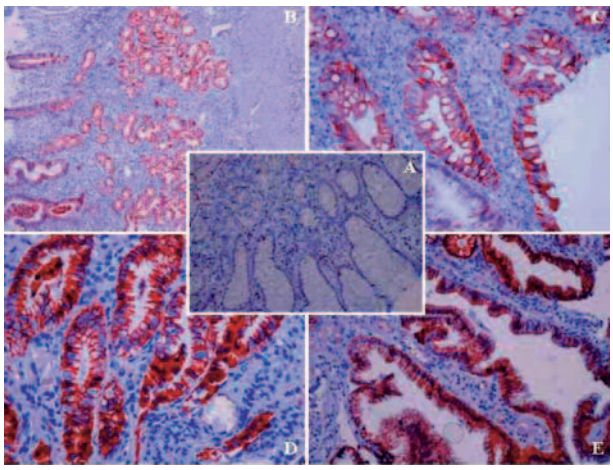

Fig. 1. — Claudin-4 immunolabeling. A. Normal gastric mucosa, nonlabeling (IHC × 20). Helicobacter pylori, intestinal metaplasia and gastric epithelial dysplasia showing 2+ expression B (IHC × 10) to D (IHC × 20). E. Remnant gastric mucosa specimen showing expression at the cell membrane and in the cytoplasm (IHC × 20) (IHC : Immunohistochemical).

Table 1. — **Claudin-4 immunostaining in gastric mucosa samples**

Pathology	Claudin-4 immunostaining				
	0	1+	2+	3+	Total abnormal staining
Normal (n = 22)	18 (81.8%)	2 (9.09%)	1 (4.55%)	1 (4.55%)	4 (18.2%)
Dysplasia (n = 18)	1 (5.56%)	5 (27.8%)	6 (33.3%)	6 (33.3%)	17 (94.4%)[a]
IM (n = 24)	3 (12.5%)	5 (20.8%)	9 (37.5%)	7 (29.2%)	21 (87.5%)[a]
H. pylori (n = 32)	12 (37.5%)	15 (46.9%)	3 (9.38%)	2 (6.25%)	20 (62.5%)[a]
RGM (n = 18)	2 (11.1%)	5 (27.8%)	6 (33.3%)	5 (27.7%)	16 (88.9%)[a]

[a] P < 0.05 compared with the normal gastric mucosa. IM : intestinal metaplasia ; *H. pylori* : *H. pylori*-associated chronic gastritis ; RGM : remnant gastric mucosa.

abnormal expression in 5/18 (27.8%) (Fig. 2B), of which 4 had heterogenous staining and 1 had cytoplasmic staining (Table 2).

When compared with normal gastric mucosa, a significant difference was detected for claudin-4 and β-catenin ($P = 0.0000015$ and $P = 0.013$, respectively).

3. Intestinal metaplasia

There was abnormal expression of claudin-4 in 21/24 (87.5%) IM samples (Fig. 1C). Abnormal staining was very weak in 5 samples, moderate in 9, and very intense in 7 (Table 1). The sensitivity of claudin-4 in distinguishing intestinal metaplasia from normal stomach was 87.5% (95% CI, 68.4-95.4) and the specificity 81.8% (95% CI, 62.6-92.5). There was normal membranous staining in 22/24 (91.7%) samples and abnormal expression in 2/24 (8.3%) labeled with β-catenin, both of which had heterogenous staining.

When compared with normal gastric mucosa, a significant difference was detected for claudin-4 ($P = 0.0000024$) but no significant difference was observed for β-catenin ($P > 0.05$).

4. *H. pylori*-associated chronic gastritis

There was abnormal expression of claudin-4 in 20/32 (62.5%) *H. pylori*-associated chronic gastritis samples. Abnormal staining was very weak in 15 samples, moderate in 3 (Fig. 1B), and very intense in 2 (Table 1). The sensitivity of claudin-4 in distinguishing *H. pylori*-associated gastritis from normal stomach was 63.5% (95% CI, 45.7-77.5) and the specificity 81.8% (95% CI, 65.5-91.6). There was normal membranous staining for β-catenin in 27/32 (84.4%) samples and abnormal staining in 5/32 (15.6%), of which 3 had heterogenous staining and 2 had cytoplasmic staining (Table 2).

When compared with normal gastric mucosa, a significant difference was detected for claudin-4 ($P = 0.00128$) but no significant difference was observed for β-catenin ($P > 0.05$).

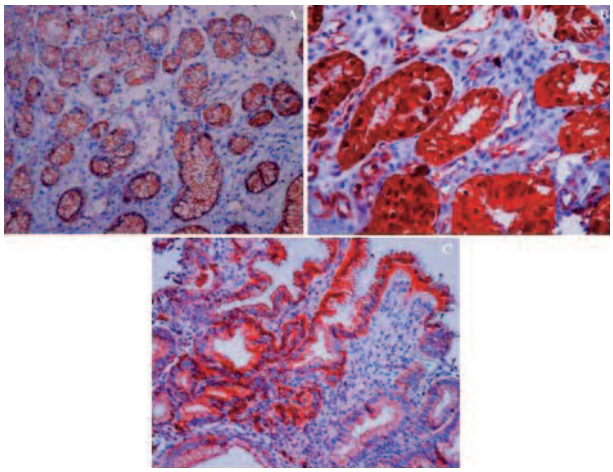

Fig. 2. — Beta-catenin immunolabeling. A. Beta-catenin expression was observed at the cell membrane of normal gastric mucosa (IHC × 20). B. Gastric epithelial dysplasia showing expression in the cytoplasm and at the cell membrane (IHC × 40). C. Remnant gastric mucosa specimen showing expression in the cytoplasm and at the cell membrane (IHC × 20) (IHC : Immunohistochemical).

5. Remnant gastric mucosa

There was abnormal expression of claudin-4 in 16/18 (89.9%) remnant gastric mucosa samples. Abnormal expression was very weak in 5 samples, moderate in 6 (Fig. 1E), and very intense in 5 (Table 1). The sensitivity of claudin-4 in distinguishing remnant gastric mucosa from normal stomach was 89.9% (95% CI, 67.3-96.9) and the specificity 81.8% (95% CI, 59.3-93.4). There was normal membranous staining for β-catenin in 14/18 (77.8%) samples and abnormal staining in 4/18 (22.2%) (Fig. 2C), of which 2 had heterogenous staining and 2 had cytoplasmic staining (Table 2).

Table 2. — **Beta-catenin immunostaining in gastric mucosa samples**

Pathology	Beta-catenin immunostaining				
	0	1+	2+	3+	Total abnormal staining
Normal (n = 22)	0	0	0	22 (100%)	0
Dysplasia (n = 18)	0	1 (5.56%)	4 (22.2%)	13 (72.2%)	5 (27.8%)[a]
IM (n = 24)	0	0	2 (8.33%)	22 (91.7%)	2 (8.33%)
H. pylori (n = 32)	0	2 (6.25%)	3 (9.38%)	27 (84.4%)	5 (15.6%)
RGM (n = 18)	0	2 (11.1%)	2 (11.1%)	14 (77.8%)	4 (22.2%)[a]

[a] $P < 0.05$ compared with the normal gastric mucosa. IM : intestinal metaplasia ; *H. pylori* : *H. pylori*-associated chronic gastritis ; RGM : remnant gastric mucosa.

When compared with normal gastric mucosa, a significant difference was detected for claudin-4 and β-catenin ($P = 0.0000086$ and $P = 0.033$, respectively).

Discussion

Claudin-4 expression was significantly higher in dysplasia (94.4%), IM (87.5%), remnant gastric mucosa (89.9%), and *H. pylori*-associated chronic gastritis (63.5%) compared to normal gastric mucosa (18.2%) ($P < 0.05$). The rate of loss of membranous staining for β-catenin was significantly higher in dysplasia (27.8%) and remnant gastric mucosa (22.2%) compared to normal gastric mucosa (0%) ($P < 0.05$). However, no significant difference was observed in IM and *H. pylori*-associated chronic gastritis ($P > 0.05$).

The claudin family consists of approximately 23 proteins that are essential for the formation of tight junctions (TJs) in epithelial and endothelial cells (10). TJs have crucial roles in the control of paracellular transport and in the maintenance of cell polarity. It is thought that various claudin family members can affect epithelial cell permeability and account for some of the selective variability of different barriers (3). Alterations in the expression levels of claudin proteins have been reported in various types of cancers (11,12). In particular, there are reports of abnormal expression of claudin-4 in intestinal-type gastric carcinomas (13,14). However, its role and significance, especially in premalignant lesions, have not been completely elucidated and studies are few. Cunningham *et al.* found that claudin-4 expression was present 100% in intestinal metaplasia lesions (n = 36) and 100% in gastric epithelial dysplasia lesions (n = 14) but only 15% in normal stomach samples (n = 109) (4). Montgomery *et al.* reported that claudin-4 was minimally expressed in normal squamous and gastric mucosa but strongly expressed in both the precursor lesion of esophageal adenocarcinoma (Barrett's esophagus, with and without dysplasia) and primary and metastatic esophageal adenocarcinoma (5). In the present study, we found claudin-4 expression to be significantly higher in dysplasia and IM compared to normal gastric mucosa ($P < 0.0000015$ and $P < 0.0000024$, respectively). Our findings are in line with above studies.

There has been little research on the relationship between *H. pylori* infection and claudin expression patterns. Fedwick *et al*. showed that the *H. pylori* strain SS1 could increase paracellular permeability by disrupting the TJ proteins claudin-4 and claudin-5 in gastric epithelial cells (15). Matsuda *et al.* reported that alteration of claudin expression may affect permeability at the TJ, possibly increasing the diffusion of nutrients and other extracellular growth factors to promote cancer cell growth and survival (16). In our study, we found that claudin-4 expression was significantly higher in *H. pylori*-associated chronic gastritis compared to the normal gastric mucosa ($P < 0.00128$). This is a striking result and suggests that *H. pylori* may play an important role in carcinogenesis.

Beta-catenin is a multifunctional protein and plays an important role in Wingless/Wnt signal transduction as well as functioning as a cell-adhesion component (17). Many studies have reported that the association of E-cadherin with catenins is crucial for cell-cell adhesion (18,19). It is postulated that changes in cell-cell and cell–matrix interactions account for the ability of cancer cells to cross normal tissue boundaries and metastasize (20). In addition, loss of cell adhesion may contribute to loss of contact inhibition and thus play a role at an earlier stage of the neoplastic process. Savas *et al.* reported that nuclear beta-catenin expression correlating with the grade of intraepithelial neoplasia in polyps and carcinomas supports its role in colorectal carcinogenesis (21). Zhou *et al.* found the incidence of abnormal β-catenin expression in gastric cancer and dysplasia to be 45% and 16%, respectively (9). They also observed normal membrane staining in intestinal metaplasia, atrophic gastritis, and control biopsy specimens. In our study, we found abnormal β-catenin expression in dysplasia compared to normal gastric mucosa ($P = 0.013$) but there was no significant difference in IM ($P > 0.05$). Our findings therefore support those of Zhou *et al.*

Beta-catenin responsive genes include *c-myc*, *cyclin D*, *MMP-7*, and *COX-2*, which affect apoptosis,

proliferation, and carcinogenesis. *H. pylori* increases the expression of each of these target genes within colonized gastric mucosa and during co-culture with gastric epithelial cells *in vitro (*22-29). *H. pylori* also increases the expression of β-catenin in cultured T84 intestinal epithelial cells (30). However, Bebb *et al.* reported that chronic infection by *H. pylori* had no effect on E-cadherin and β-catenin expression (31), although an effect during the later stages of atrophy or intestinal metaplasia could not be ruled out. Romiti *et al.* reported that *H. pylori* infection did not seem to play a direct role in β-catenin alterations, whilst it significantly increased cell proliferation (32). In our study, no significant difference was observed for β-catenin in *H. pylori*-associated chronic gastritis compared to normal gastric mucosa ($P > 0.05$). Our results therefore similarly suggest that *H. pylori* infection in the chronic gastritis phase does not affect β-catenin expression.

The remnant mucosa of the stomach resected for benign disease has an increased likelihood of chronic epithelial lesions being a precursor of cancer in relation to the almost unavoidable bile and pancreatic juice alkaline postoperative reflux following removal of the pylorus in stomach surgery (33). Furthermore, the denervation that occurs during gastrectomy may affect gastric mucosal defensive factors, such as blood flow, secretion of mucin, and renewal of cells, all of which are regulated by the nervous system and neuropeptides, and this may induce precancerous mucosal changes (34). In the present study, we detected significantly abnormal claudin-4 and β-catenin expression in remnant gastric mucosa compared to normal gastric mucosa, similar to that in dysplastic lesions. Our results therefore indicate that remnant gastric mucosa is a considerable precancerous state.

In conclusion, our results suggest that claudin-4 expression is upregulated in premalignant gastric alterations because claudin-4 expression alters in precancerous lesions such as dysplasia, IM, *H. pylori*-associated chronic gastritis, and remnant gastric mucosa. Although abnormal β-catenin expression has been detected in dysplasia and remnant gastric mucosa, it is difficult to conclude relating its role in precancerous states.

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