Expression of the E-cadherin/β-catenin/tcf-4 Pathway in Gastric Diseases with Relation to Helicobacter pylori Infection: Clinical and Pathological Implications

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Abstract

Objective: To determine the expression of E-cadherin, β-catenin, and transcription factor 4 (TCF4) proteins in gastric diseases with relation to Helicobacter pylori infection. Methods: A total of 309 patients including 60 with superficial gastritis (SG), 57 with atrophic gastritis (AG) and 192 with gastric cancer (GC), were enrolled. The expression of E-cadherin, β-catenin, TCF4 proteins in the gastric mucosa was detected by immunohistochemistry and H. pylori infection by immunohistochemistry and PCR. Results: The expression rates of E-cadherin were significantly higher in SG and AG than in GC (P<0.01), while those of β-catenin in the nucleus were significantly lower in SG and AG than in GC (P<0.05). In GC cases, the expression rates of E-cadherin, β-catenin and TCF4 were significantly higher in the intestinal type than in the diffuse type (P<0.05). In GC patients, the expression rate of E-cadherin was significantly higher in the presence of H. pylori than in the absence of infection (P=0.011). Moreover, the expression level of TCF4 and β-catenin protein was significantly higher in the nucleus and cytoplasm in H. pylori positive than in H. pylori negative GC patients, especially in those with the intestinal type (all P < 0.05). Conclusion: The expression of E-cadherin and β-catenin progressively decreases during the process of GC tumorigenesis, while overexpression of TCF4 occurs. H. pylori infection is associated with a significant increase in the expression of E-cadherin and β-catenin in the cytoplasm and nucleus in GC patients, especially those with the intestinal type.

Keywords: Gastric cancer - H. pylori - E-cadherin - β-catenin - transcription factor 4

Introduction

Gastric cancer (GC) is the most common gastrointestinal malignancy. The development of GC is a complex, multistage, and multifactorial process involving abnormal regulation of multiple genes (Kim et al., 2010). Helicobacter pylori infection is closely associated with the development of gastric carcinoma, and has been classified as a Group I carcinogen by the World Health Organization (WHO) (Watanabe et al., 2010). It has been reported that H. pylori infection induces GC by changing host gene and protein expression through several cell signaling pathways (Kim et al., 2010; Watanabe et al., 2010).

The Wnt pathway is confirmed to involve a large number of proteins that can regulate the production of Wnt signaling molecules, including Wnt proteins, frizzled, β-catenin and transcription factors (TCF). These molecules play important roles in cell differentiation, migration and proliferation. Among these molecules, β-catenin is the key factor in the Wnt pathway. Normally, β-catenin is integrated with E-Cadherin in the membrane, and they form a complex that mediates homocellular adherence. However, aberrant expression of proteins in the complex has been reported in some epithelial cancers (Chelidonis et al., 2009; Pagaki et al., 2010). It is postulated that changes of the proteins in the complex may inhibit glycogen synthase kinase 3 (GSK-3)-mediated phosphorylation of β-catenin, allowing it to translocate from the membrane to the nucleus where it combines with TCF/LEF (Lymphoid enhancer-binding factor), interacts with transcription factors, regulates gene transcription, and consequently induces cell proliferation and even tumor formation (Bienz et al., 2000; Prasad et al., 2009; Wang et al., 2010b). Recently, E-Cadherin, Wnt, β-catenin and transcription factor 4 (TCF4) have been implicated in gastric carcinogenesis (Ding et al., 2010; Wu et al., 2010).

However, the effects of H. pylori infection on the expression of proteins in the Wnt/β-catenin pathway and the interactions between H. pylori infection and the Wnt/β-catenin pathway in gastric carcinogenesis have not been fully elucidated. Therefore, this study was carried out to determine the expression of E-cadherin, β-catenin, and TCF4 proteins in different stages of gastric carcinogenesis in relation to H. pylori infection.

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Materials and Methods

Patients and Study design

The study protocol was approved by the Ethics Committee of China Medical University. A total of 309 patients (including 194 males and 115 females, with mean age 59.15 ranged from 30-79), from 1992 to 2007 participating in a health check program for gastric cancer screening in Zhuanghe of Liaoning Province, China, were included in the study, where the gastric cancer screening were done by China Medical University. They were histologically diagnosed with superficial gastritis (SG) (n=60), atrophic gastritis (AG) (n=57) and gastric cancer (GC) (n=192). Classification and grading of gastritis was based on Updated Sydney System (Dixon MF, et al., 1996; Stolte M, et al., 2001) . According to Lauren’s classification (Lauren, 1965), 97 of the 192 GC cases were the intestinal type and 95 were the diffuse type. Gastric specimens that were taken from gastric sites with apparent lesions or from resected gastric tumors were fixed in 95% ethanol, embedded in paraffin, and cut into 5 μm slices. These specimens were used for histological examinations with hematoxylin and eosin staining as described above, and for immunohistochemical assessment of the proteins as describe below.

Immunohistochemistry for detection of E-cadherin, β-catenin and TCF4 protein expression

Immunohistochemical methods were adopted for the expression of E-cadherin, β-catenin and TCF4 proteins. The primary antibodies were rabbit anti-human E-cadherin (H-108, dilution 1:100, Santa Cruz, California, USA), β-catenin (E5, dilution 1:200, Santa Cruz), and TCF4 (EP2033Y, dilution 1:200 Epitomics, Burlingame, USA) monoclonal IgG antibodies. The amplification system that was used in the reaction was the streptavidin-peroxidase kit (Maixin Biotechnology Co. Ltd, Fujian, China). Sections were dewaxed, and incubated with methanol containing 3% H2O2 for 20 min to block endogenous peroxidase activity. To enhance antigen retrieval, sections were treated in a microwave oven. Briefly, sections were immersed in 0.01 mol/L citrate buffer (pH 6.0) and heated in a microwave oven at 100°C for 20 min. Subsequently, they were washed three times with distilled water, and then blocked with 1% bovine serum albumin for 30 min. Sections were incubated overnight at 4°C with rabbit polyclonal IgG of H. pylori, E-cadherin, β-catenin and TCF4. A subsequent reaction was carried out using second antibodies (Maixin Biotechnology Co. Ltd, Fujian, China) for 15 min at 37°C. Sections were washed three times with phosphate buffered saline (PBS) buffer and subsequently displayed color with the usage of diaminobenzidine (Zhongshan Goldenbridge Biotechnology Co. Ltd, Beijing, China) for about 5 min. Nuclei were lightly counterstained with hematoxylin. Immunohistochemical staining for E-cadherin, β-catenin and TCF4 in the H. pylori negative GC tissue was used as a positive control for the expression of these proteins. Staining using PBS, instead of the primary antibody, was used as the negative control.

A comprehensive assessment (overall scoring) system (Yu et al., 2009) was used to determine the expression of E-Cadherin staining in the cell membrane, TCF4 in the nucleus, and β-catenin in the cell membrane, cytoplasm and nucleus. Briefly, five visual fields were randomly selected under a light microscope (×400) to assess the number of positively immunostained cells in a total of 100 cells per visual field and the intensity of immunostaining. Then, a comprehensive assessment was performed according to the ratio of positive cells and staining intensity (Yu et al., 2009). The positive cell ratio score was classified semi-quantitatively according to the following criteria: 0, < 5% of cells discretely expressed; 1, ≥5 and < 25% of cells discretely expressed; 2, ≥26% and <50% of cells discretely expressed; and 3, ≥ 50% of cells discretely expressed. The staining intensity score was classified according to the following criteria: 0, no brown granules in the cells; 1, light brown granules in the cells; 2, obvious brown granules in the cells; and 3, strong brown or brown-yellow granules in the cells. The overall score was calculated by the positive cell ratio score × the staining intensity score. A patient with the final score of 1 or more was defined as positive for the expression of the protein.

Detection of H. pylori infection

H. pylori infection was detected by immunohistochemistry and polymerase chain reaction (PCR) method. The immunohistochemistry was carried out as described above, except that the primary antibody was H. pylori-IgG (DAKO, Denmark A/S, 1:25), and the secondary antibody was rabbit polyclonal IgG of H. pylori. In addition, immunohistochemical staining of a smear of cultured H. pylori bacteria was used as a positive control for the presence of H. pylori infection. The presence of H. pylori bacterial cells were observed on the surface of gastric epithelium, in the gastric pits or lumens of the glands within the gastric mucosa. H. pylori infection was considered positive if yellow or brownish yellow stained short rod or curved shape bacilli in immunohistochemistry staining sections were observed. The presence and the density of H. pylori infection were determined by the mean number of immunostained bacteria of two visual fields under the light microscope (×1000) (Liu et al., 2008): 0, no bacteria; 1, ≥ 1 and <9 bacteria; 2, ≥10 and <29 bacteria; 3, ≥30 and <99 bacteria; and 4, ≥100 bacteria. A specimen with a density score of 1 or more was defined positive for H. pylori.

For PCR, DNA samples were extracted from the paraffin fixed gastric specimens using WaxFREETMDNA Kit (Quick DNA preparation for FFPE, TrimGen, Cat. No. WF-100). H. pylori 16s rRNA and glmM (formally ureC) genes were detected using polymerase chain reaction (PCR) method, as previously described (Lu et al., 1999; Riggio et al., 2000). The primer sequences for PCR were as follows: 16s rRNA, forward primer, 5’-CGTTAGCTGCAATTACTGGAGA-3’, and reverse primer 5’-GAGGCGGTAGGCGGATGTC-3’, glmM, forward primer, 5’-AAGCTTTAGGGGTGTTAGGGGT TT-3’, and reverse primer 5’-AAGCTTTACTTTCATACAC TAAACGC-3’. The expected amplification products were 295 bp and 294bp, respectively. PCR cycling conditions were 94°C for 5 min, followed by 25 cycles for 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, and a final extension step for 5 min at 72°C.
### Results

**The expression of E-cadherin, β-catenin and TCF4 proteins in patients with SG, AG and GC**

E-cadherin protein expression was located in the cell membrane (Figures 1A). The positive rate of E-cadherin protein expression was significantly higher in SG (100%) and AG (100%) than in GC (81.3%, P<0.01, respectively), while there was no significant difference between SG and AG. In GC patients, E-cadherin protein expression rate was higher in the intestinal type GC than in the diffuse type GC (95.9% vs. 66.3%, P<0.001, Table 1).

β-catenin protein expression was also located in the cell membrane, cytoplasm and/or nucleus (Figures 1B). The positive rate of β-catenin expression was higher, albeit not statistically significantly, in SG (78.3%) than in AG (66.7%), and in GC (63.5%) (SG vs. AG, χ²=3.191, P=0.075; AG vs. GC, χ²=4.319, P=0.037). In the cell membrane, the positive rate of β-catenin expression was higher in the intestinal type GC (C4) than in AG (66.7%) and in SG (63.5%). In the cell cytoplasm, the positive rate of β-catenin expression was decreased gradually from SG to AG and then to GC, (56.7%, 52.6% and 56.7%, respectively) (SG vs. AG, χ²=1.587, P=0.210; AG vs. GC, χ²=4.528, P=0.035). In the cell nucleus, the positive rate of β-catenin expression was significantly higher in the intestinal type than in the diffuse type in the cell membrane (77.3% vs. 49.5%, χ²=16.064, P=0.000, cytoplasm (68.0% vs. 36.8%, χ²=18.738, P=0.000) or nucleus (23.7% vs. 6.3%, χ²=11.326, P=0.001) (Tables 1 & 2).

TCF4 protein expressed in the nucleus (Figures 1C), and there was no significant difference in the expression rate of the protein among SG, AG and GC. However, in GC cases, the positive rate of TCF4 protein expression was higher in the intestinal type than in the diffuse type (93.8% vs. 78.9%, χ²=9.061, P=0.003) (Table 1).

### Association between H. pylori infection and the expression of E-cadherin, β-catenin and TCF4 proteins in patients with SG, AG and GC

Under the high-magnification microscope, H. pylori bacterial cells were presented in distinct mucosal surface mucus, on the surface epithelium of gastric pits, or in the glandular cavity in many cases with SG, AG and GC (Figure 2). Detection of H. pylori 16s rRNA and glmM genes is shown in Figure 3. Overall H. pylori infection was present in SG (85.0%, 51/60), AG (87.7%, 50/57) and GC (60.4%, 116/192) of patients; the rates were significantly higher in SG and AG than in GC (SG vs. GC, χ²=12.359, P=0.000; and AG vs. GC, χ²=14.743, P=0.000, Table 2).
Table 3. Association Between H. pylori (Hp) Infection and the Expression of E-Cadherin, β-Catenin and TCF4 Proteins in Superficial Gastritis (SG), Atrophic Gastritis (AG) and Gastric Cancer (GC)

<table>
<thead>
<tr>
<th></th>
<th>Superficial gastritis</th>
<th>Atrophic gastritis</th>
<th>Gastric cancer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hp negative</td>
<td>Hp positive</td>
<td>Hp negative</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>9 (100.0)</td>
<td>51 (100.0)</td>
<td>7 (100.0)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>3 (100.0)</td>
<td>38 (74.5)</td>
<td>6 (85.7)</td>
</tr>
<tr>
<td>Membrane</td>
<td>3 (100.0)</td>
<td>38 (74.5)</td>
<td>8 (11.4)</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>6 (66.7)</td>
<td>26 (54.9)</td>
<td>4 (57.1)</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0 (0.0)</td>
<td>69 (138.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>TCF4</td>
<td>2 (6.7)</td>
<td>84 (168.0)</td>
<td>2 (4.0)</td>
</tr>
</tbody>
</table>

Compared to the Hp negative group: *P=0.011; **P=0.018; and ***P=0.024

Table 4. Association Between H. pylori (Hp) Infection and E-Cadherin, β-Catenin and TCF4 Protein Expression in Different Histological Type of GC

<table>
<thead>
<tr>
<th>Intestinal type</th>
<th>Diffuse type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp negative</td>
<td>Hp positive</td>
</tr>
<tr>
<td>n</td>
<td>64</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>29 (93.5)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>24 (77.4)</td>
</tr>
<tr>
<td>Membrane</td>
<td>25 (80.6)</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>14 (45.2)</td>
</tr>
<tr>
<td>Nucleus</td>
<td>3 (9.7)</td>
</tr>
<tr>
<td>TCF4</td>
<td>26 (83.9)</td>
</tr>
</tbody>
</table>

Compared with Hp negative group: *P=0.001, and **P=0.026, ***P=0.012

Discussion

The development and progression of GC involves a number of genetic and epigenetic abnormalities. At present, although the roles of H. pylori infection and Wnt/β-catenin in the development and metastasis of GC have been independently studied (Macleod RJ, 2012; Neal JT, et al., 2013), the association between H. pylori infection and Wnt/β-catenin in the development and progression of gastric carcinoma remains to be clarified (Ilyas, 2005; McMillan et al., 2005). Thus, it is essential to simultaneously examine H. pylori infection and the expression of E-cadherin, β-catenin and TCF4 proteins, and their association in various gastric mucosal lesions, thereby providing an additional theoretical basis on GC tumorigenesis and progression.

E-cadherin is a calcium-dependent cell adhesion molecule binding of β-catenin in normal epithelial cells (Aoki et al., 2007). When E-cadherin expression declines, the cell adhesion among tumor cells in the primary tumor is weakened; hence, tumor cells invasion and metastasis would easily occur. In our study, we found that E-cadherin expression was decreased in the progress from gastritis to GC, and the intestinal type of GC had a higher expression compared with the diffuse type of GC. Tamura et al. reported that E-cadherin expression was decreased in diffuse GC due to methylation, and that decreased expression of E-cadherin reduced the content of combined β-catenin in the complex (Tamura, 2008). β-catenin is a multifunctional protein found in three cell compartments: the plasma membrane, the cytoplasm, and the nucleus. The cell has developed elaborate ways of regulating the level and localization of β-catenin to
assure its specific function in each compartment. In the normal tissue, the Wnt signaling pathway is turned off, and β-catenin mainly locates in the cell membrane at a low level. However, either β-catenin expression increases or ectopic expression of β-catenin occurs in the malignant tumors (Ogasawara N, et al., 2006; Pandurangan AK, 2013). In our study, β-catenin membrane expression was decreased following the progression from SG to GC, while the expression in the nucleus was increased. In addition, the positive rate of β-catenin expression in the intestinal type of GC was significantly higher than that in the diffuse type of GC, regardless of the location (i.e. the membrane, cytoplasm and nucleus). These findings suggest that ectopic expression of β-catenin is more closely related to the intestinal type of GC than the diffuse type of GC. There was a report based on the similar topic (Ogasawara N, et al., 2006). In a retrospective study of 157 gastric carcinomas using immunohistochemistry and molecular genetics, Nabais et al. concluded that the pattern of β-catenin expression is closely related to the histological types of gastric cancer (Nabais et al., 2003).

TCF4 is the downstream protein of the Wnt/β-catenin signaling pathway and plays the role of a molecular switch in the pathway (Thevenod et al., 2010). When the Wnt/β-catenin signaling pathway is activated, β-catenin protein competitively binds TCF after entering the nucleus; thereby the transcription of target genes is enhanced (Thevenod et al., 2010). In the present study, TCF4 protein expression was increased with the aggravation of gastric mucosal lesions, and the positive rate of the expression in intestinal type GC was higher than that in diffuse GC. Following the aggravation of gastric mucosal lesions, E-cadherin expression was reduced, resulting in the ectopic expression of β-catenin protein in the nucleus, where β-catenin acts as a TCF binding factor and an activator for transcription by the displacement of Groucho-HDAC co-repressors (Kavak et al., 2010; Wang et al., 2010a).

H. pylori infection is closely associated with the development of gastric carcinoma, but the specific mechanism is still unclear (Ding et al., 2010; Osman et al., 2013). It has been shown that H. pylori induces cell proliferation through MAPK and NF-kappaB, thereby contributing to GC (Sibony et al., 2012). On the other hand, it is acknowledged that the Wnt/β-catenin signaling pathway is the most important pathway in the process of gastrointestinal tract tumor tumorigenesis and development. Therefore, a question of whether or not there was an association between H. pylori infection and the Wnt/β-catenin signaling pathway in GC tumorigenesis was raised. Kirikoshi et al. suggested that up-regulation of WNT10A induced by TNF-alpha and H. pylori infection might play key roles in human GC through activation of the Wnt/β-catenin signaling pathway (Kirikoshi et al., 2001). The sequence of events in H. pylori-associated gastric carcinogenesis is probably as follows: normal gastric mucosa, SG, AG, IM, dysplasia and GC (Correa et al., 2007; Correa et al., 2012). In our study, the rate of H. pylori infection was lower in GC cases (60.4%), compared with those in SG (85.0%) and AG (87.7%) cases. Our findings were similar to those obtained in a Chinese study which showed that whereas the rates were 63.3% and 80.8%, respectively, in gastric erosion and gastric ulcer, the overall rate was 52.4% in patients with early gastric cancer, with the rates being 35.0%, 50.7%, 34.6%, respectively, in the antrum, corpus, and incisura of stomach (Zhang et al., 2005). Indeed, it has been shown that development of precancerous and cancerous lesions may create a hostile environment to H. pylori, and thus H. pylori infection may disappear following the development of these lesions (Genta et al., 1993). In GC cases, the E-cadherin expression level was higher in H. pylori positive than in H. pylori negative cases, and the β-catenin protein expression level in the cytoplasm and nucleus was higher in H. pylori positive than in H. pylori negative cases. Kurashima et al. reported that infection with H. pylori cagA-positive strains caused gastritis and peptic ulceration, and was associated with gastric adenocarcinoma (Kurashima et al., 2008). CagA also destabilizes the E-cadherin/β-catenin complex to elicit aberrant activation of the β-catenin signal that underlies intestine metaplasia (Correa et al., 2007; Correa et al., 2011; Correa et al., 2012).

At present, most researchers believe that H. pylori and the Wnt/β-catenin signaling pathway play an important part in the intestinal type of GC tumorigenesis (Jin et al., 2008; Tuynman et al., 2008). However, the correlation between H. pylori infection and the Wnt/β-catenin signaling pathway in GC tumorigenesis is unclear. The present study showed that β-catenin protein expression level in the cytoplasm and the nucleus was higher in H. pylori positive than in H. pylori negative patients with GC, especially those with the intestinal type, in addition the similar phenomenon was also observed in TCF4 protein, which indicates that H. pylori infection can cause an E-cadherin/β-catenin complex fracture, so that β-catenin could enter the nucleus and bind to TCF4, resulting in increased TCF4 protein expression, which then subsequently contributes to the occurrence of intestinal-type GC.

In conclusion, the expression of E-cadherin and β-catenin is progressively decreased following the process of GC tumorigenesis, with β-catenin expression being translocated from the cytoplasm to the nucleus, contrarily, overexpression of TCF4 was observed in GC carcinogenesis process. H. pylori infection is associated with a significant increase in the expression of E-Cadherin and β-catenin in the cytoplasm and nucleus in patients with gastric cancer. In intestinal-type GC group, higher TCF4 and β-catenin in the cytoplasm and nucleus present in H. pylori infection cases.

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