Effect of Helicobacter pylori infection on expressions of Bcl-2 family members in gastric adenocarcinoma

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INTRODUCTION

Helicobacter pylori (H pylori) infection is the most common chronic infection in humans and is the major cause of gastritis worldwide. This infection is also accepted as the etiological factor of the majority of peptic ulcers. It has been implicated as a significant contributing factor in the development of gastric malignancy, both gastric MALT lymphoma and gastric adenocarcinoma[1-14], and H pylori was classified as a group 1 carcinogen in gastric cancer in 1994 by the WHO and International Agency for Research on Cancer (IARC)[15]. The role of H pylori infection in the gastric carcinogenesis is not clear. It might be involved in imbalance between apoptosis and proliferation[16-33]. Bcl-2 family members have been closely related to apoptosis, which could either promote cell survival (Bcl-2, Bcl-xL, A1, Mcl-1, and Bcl-w) or promote cell death (Bax, Bak, Bcl-xS, Bad, Bid, Bik, Hrk, Bok)[34-36]. In the present study, we investigated the effect of H pylori infection on the expressions of Bcl-2 family members in gastric adenocarcinoma and resection margin tissues.

MATERIALS AND METHODS

Tissue specimens

Specimens of gastric adenocarcinoma of 95 patients (72 males and 23 females, age range 31 to 84 years, mean 56 years), who had undergone resectional surgery at the Southwest Hospital in Chongqing and had not taken anti-H pylori drugs before operation, were collected from 2001 to 2002. Gastric adenocarcinoma tissues were examined microscopically. Resection margin tissues were also examined to verify that they did not contain malignant cells. The histological diagnosis was confirmed by a professional pathologist. The remaining specimens were snap-frozen and stored at -80 °C until assayed. Warthin-Starry silver staining and polymerase chain reaction (PCR) analysis for H pylori urease gene A (ureA) were performed to detect H pylori infection. PCR analysis for H pylori cagA gene was performed to verify cagA+ H pylori infection. Fifty-eight patients whose both Warthin-Starry staining and PCR for ureA showed positive results were diagnosed as suffering from H pylori infection and 37 were cagA- H pylori.

RT-PCR analysis of Bid, Bax and Bcl-2 mRNA

According to references[37-38], primers were designed for β-actin (GenBank accession No.BC013380), 5'-GGG GGC CCG CCC AGG CAC CA-3' (sense) and 5'-CTG CTT AAT GTC ACG CAC GAT TTC-3' (antisense), 540 bp product; for Bid (GenBank accession No.AF087891), 5'-ATG GAC TGT TGA GGT CAA CAA C-3' (sense) and 5'-TCA GTC CAT CCC ATT TCT GGC T-3' (antisense), 588 bp product; for Bax (GenBank accession No.AY217036), 5'-ACC AAG AAG CGT AGG GAG TGT C-3' (sense) and 5'-ACA AAG ATG GTG ACG GTC TGC C-3' (antisense), 332 bp product; and for Bcl-2 (GenBank accession No.M13994), 5'-TGG ACC TGA CGC CCT TCA C-3' (sense), 5'-AGA CAG CCA GGA GAA ATC AAA CAG-3' (antisense), 293 bp product. Total RNA was prepared from gastric adenocarcinoma
tissues and resection margin tissues by using TriPure isolation reagent (Roche) according to the manufacturer’s protocol. Reverse transcription (RT) was performed for first-strand cDNA by using 2 µg of total RNA and 1 µl of oligo(dT)18 primer in the presence of 5 unit AMV reverse transcriptase (Promega). 20 unit RNase inhibitor, 0.5 mmol/L of each dNTP and 1×buffer in 20 µl for 60 min at 42 °C. Then 2 µ reverse transcription products were used for PCR. In a total of 20 µl reaction mixture, 5 pmol/L sense primer and 5 pmol/L antisense primer, 0.25 mmol/L of each dNTP, 1×reaction buffer and 1.5 unit Taq polymerase were mixed. The reaction was run for 33 cycles, and each consisted of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s, extension at 72 °C for 60 s and final extension prolonged for 7 min at 72 °C. PCR-amplified products (8 µl each) were analyzed on 1.5% agarose gels after reactive mixture, 5 pmol/L sense primer and 5 pmol/L antisense primer. 20 unit Taq polymerase were mixed. The reaction was run for 33 cycles, and each consisted of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s, extension at 72 °C for 60 s and final extension prolonged for 7 min at 72 °C. PCR-amplified products (8 µl each) were analyzed on 1.5% agarose gels after being stained with ethidium bromide. Expression levels of Bid, Bax and Bcl-2 were quantitated using Quantity One quantitation software (Bio-Rad Laboratories) and were reported to be normalized to β-actin levels.

Statistical analysis

All data were presented as means ± standard error. Differences in means were examined by ANOVA, and correlations were analyzed by using Spearman’s rank correlation coefficient (SPSS 10.0 for Windows). A P value < 0.05 was considered significant.

RESULTS

Effect of H. pylori on expressions of Bid, Bax and Bcl-2 mRNA

In gastric adenocarcinoma tissues, expressions of Bid and Bax mRNA in H. pylori negative group, cagA+ H. pylori infection group and cagA- H. pylori infection group increased in an ascending pattern, respectively (P < 0.05). Expression of Bcl-2 in H. pylori negative group was significantly lower than that in H. pylori infection group (P < 0.05). Levels of Bcl-2 mRNA between cagA+ H. pylori infection group and cagA- H. pylori infection group did not show any significant difference.

In resection margin tissues, expressions of Bid, Bax and Bcl-2 mRNA in H. pylori negative group, cagA+ H. pylori infection group and cagA- H. pylori infection group increased in turn (P < 0.05) (Figure 1, Tables 1-3).

| Table 1 | Effect of H. pylori infection on expression of Bid |
|---------|-----------------------------------------------|
|         | Adenocarcinoma | Resection margin |
| H. pylori (-) | 0.30±0.113 | 0.37±0.119 |
| H. pylori (+) | 0.42±0.149* | 0.68±0.285* |
| cagA (-) | 0.85±0.305* | 0.99±0.383* |
| cagA (+) | 0.65±0.393* | 0.64±0.327* |
| cagA (±) | 0.97±0.375* | 1.00±0.361* |

*P < 0.05, vs H. pylori negative group, †P < 0.05, vs cagA+ H. pylori infection group.

| Table 2 | Effect of H. pylori infection on expression of Bax |
|---------|-----------------------------------------------|
|         | Adenocarcinoma | Resection margin |
| H. pylori (-) | 0.30±0.123 | 0.35±0.139 |
| H. pylori (+) | 0.65±0.319* | 0.64±0.327* |
| cagA (-) | 0.97±0.375* | 1.00±0.361* |
| cagA (±) | 0.84±0.352* | 0.61±0.243* |

*P < 0.05, vs H. pylori negative group, †P < 0.05, vs cagA+ H. pylori infection group.

| Table 3 | Effect of H. pylori infection on expression of Bcl-2 |
|---------|-----------------------------------------------|
|         | Adenocarcinoma | Resection margin |
| H. pylori (-) | 0.41±0.132 | 0.37±0.153 |
| H. pylori (+) | 0.69±0.319* | 0.48±0.241* |
| cagA (-) | 0.84±0.352* | 0.61±0.243* |

*P < 0.05, vs H. pylori negative group, †P < 0.05, vs cagA+ H. pylori infection group.

Correlation among levels of Bid, Bax and Bcl-2 mRNA

In H. pylori negative group, levels of Bid and Bax mRNA correlated negatively with that of Bcl-2 in gastric adenocarcinoma tissues (Bid vs Bcl-2, r=-0.409, P < 0.05; Bax vs Bcl-2, r=-0.451, P < 0.05). In H. pylori positive group, expressions of Bid and Bax did not correlate with that of Bcl-2 in adenocarcinoma tissues (Bid vs Bcl-2, r=0.187, P > 0.05; Bax vs Bcl-2, r=0.201, P > 0.05), but correlated positively with that of Bcl-2 respectively in resection margin tissues (Bid vs Bcl-2, r=0.331, P < 0.05; Bax vs Bcl-2, r=0.295, P < 0.05).

Figure 1: Effect of H. pylori infection on mRNA expressions of Bcl-2 family members in gastric adenocarcinoma and resection margin tissues. A: Expression of Bid in gastric adenocarcinoma, B: Expression of Bid in resection margin, C: Expression of Bax in gastric adenocarcinoma, D: Expression of Bax in resection margin, E: Expression of Bcl-2 in gastric adenocarcinoma, F: Expression of Bcl-2 in resection margin, 1: H. pylori negative group, 2: cagA- H. pylori group, 3: cagA+ H. pylori group.
DISCUSSION
Bid, Bax and Bcl-2 are representative members of Bcl-2 family. Bid and Bax are pro-apoptosis members. Bcl-2 is an anti-apoptosis member. In the mechanism of regulating apoptosis, Bid as a BH3 domain protein, is one of the initiators to apoptosis and Bax is the key member. Either Bid or Bcl-2 must rely on Bax to induce or inhibit apoptosis.[39-43]

Studies have shown that *H pylori* infection could induce Fas antigen (Fas Ag) expression in gastric epithelial cells.[44] In addition, *H pylori* infection was also associated with increased mucosal inflammatory cytokines, including TNF-α[45] and IFN-γ.[46] The cytokines generated during the immune response to *H pylori* also increased expression of Fas Ag in gastric cell lines.[47] Fas Ag, after binding specifically to its ligand (Fas L), trimerizes and activates Caspase-8. Activation of Caspase-8 could result in the cleavage of cytosolic Bid to truncate tBid, which could translocate to mitochondria and initiate apoptosis.[48]

Shibayama et al.[49] found that *H pylori* infection induced the activation of Caspase-8 and the expression of Bid in human gastric epithelial cells, and inhibition of Caspase-8 suppressed the expression of Bid. In the present study, we found that *H pylori* infection upregulated expression of Bid mRNA in both gastric adenocarcinoma and resection margin tissues. That might be due to the upregulation of Fas and the activation of Caspase-8.

Bax is a cytosolic protein and translocates from the cytosol to the mitochondria for integration into the membrane following a proapoptotic stimulus. This action then results in cytochrome C release and initiates apoptosis. We have previously demonstrated that *H pylori* infection can promote Bax protein expression in chronic gastritis and premalignant lesions. Expression of Bax correlated positively with apoptotic index. The apoptotic index in Bax expression positive group in intestinal metaplasia, gastric dysplasia and gastric carcinoma was significantly higher than that in Bax negative group. In the present study, we found *H pylori* infection also increased levels of Bax mRNA in both gastric adenocarcinoma and resection margin tissues, and the effect was stronger in CagA* H pylori* group than cagA− *H pylori* group. These results showed *H pylori* infection might promote apoptosis in gastric adenocarcinoma and its resection margin tissues. Bcl-2 is an important anti-apoptosis protein. We found that Bcl-2 mRNA levels in *H pylori* negative group in gastric adenocarcinoma tissues were lower than in *H pylori* positive group. In resection margin tissues, Bcl-2 mRNA levels in *H pylori* negative group, cagA* H pylori* infection group and cagA+ *H pylori* infection group respectively increased in turn, suggesting that *H pylori* might promote expression of Bcl-2 in both gastric adenocarcinoma and resection margin tissues. Although *H pylori* could promote expressions of Bid, Bax and Bcl-2, the correlations among them are still unclear. In the present study, it showed that levels of Bid and Bax mRNA were correlated negatively with that of Bcl-2 in gastric adenocarcinoma tissues without *H pylori* infection. This result is correspondent with the findings that apoptosis decreases in tumor tissues. In *H pylori* positive group, levels of Bid, Bax and Bcl-2 mRNA in gastric adenocarcinoma all increased. Besides, levels of Bid, Bax, and Bcl-2 did not correlate with each other. In the resection margin tissues, levels of Bid and Bax mRNA were correlated positively with that of Bcl-2. These results indicated that although *H pylori* could promote expressions of Bid, Bax and Bcl-2, it might play a different role in the development of gastric adenocarcinoma. In benign gastric lesions, *H pylori* infection might mainly upregulate expressions of pro-apoptotic genes such as Bid and Bax, and this effect might be stronger than its upregulatory effect on Bcl-2, which is consistent with the phenomena that *H pylori* infection increases apoptosis in atrophic gastritis and gastric ulcer. During development of gastric adenocarcinoma, upregulatory effect of *H pylori* on anti-apoptotic genes, for example Bcl-2, might increase gradually and counteract pro-apoptosis effect of Bid and Bax, which may induce or worsen deregulation of apoptosis-associated genes expressions during the course of the formation of gastric adenocarcinoma.

Recently, it was found that not only excessive proliferation played an important role in gastric adenocarcinoma, but also reduction of apoptosis contributed to the carcinogenesis of gastric mucosa. The abnormal expressions of Bid, Bax and Bcl-2 induced by *H pylori* might result in inhibition of apoptosis, which may play an important role during development of gastric adenocarcinoma induced by *H pylori*.[40]. The detailed mechanism still remains to be studied.

REFERENCES

1. Jones RG, Trowbridge DB, Go MF. *Helicobacter pylori* infection in peptic ulcer disease and gastric malignant. Front Bioso 2001; 6: E213-226
2. Parsonnet J, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, Oreinreth N, Vogelman JH, Friedman GD. *Helicobacter pylori* infection and gastric lymphoma. N Engl J Med 1994; 330: 1267-1271
3. Huang JQ, Sridhar S, Chen Y, Hunt RH. Meta-analysis of the relationship between *Helicobacter pylori* seropositivity and gastric cancer. Gastroenterology 1998; 114: 1169-1179
4. Matsubura N. Relation between *Helicobacter pylori* pylori and diseases: knowledge for clinician. J Nippon M e Sch 2002; 69: 200-204
5. Miwa H, Go MF, Sato N. *H pylori* and gastric cancer: the Asian enigma. Am J Gastroenterol 2002; 97: 1106-1112
6. Lan J, Xiong YY, Lin YX, Wang BC, Gong LL, Xu HS, Guo GS. *Helicobacter pylori* infection generated gastric cancer through p53-Rb tumor-suppressor system mutation and telomerase reactivation. World J Gastroenterol 2003; 9: 54-58
7. Gao HJ, Yu LZ, Baj MF, Peng YS, Sun G, Zhao HL, Miu K, Lu XZ, Zhang XY, Zhao QZ. Multiple genetic alterations and behavior of cellular biology in gastric cancer and other gastric mucosal lesions: *H pylori* infection, histological types and staging. World J Gastroenterol 2000; 6: 840-854
8. Wang RT, Wang T, Chen K, Wang JY, Zhang JP, Lin SR, Zhu YM, Zhang WM, Cao YX, Zhu CW, Yu H, Cong YJ, Zheng S, Wu BQ. *Helicobacter pylori* infection and gastric cancer: evidence from a retrospective cohort study and nested case-control study in China. World J Gastroenterol 2002; 8: 1103-1107
9. Xue FB, Xu YY, Wan Y, Pan BB, Ren J, Fan DM. Association of *H pylori* infection with gastric carcinoma: a Meta analysis. World J Gastroenterol 2001; 7: 801-804
10. Cai L, Yu SZ, Zhang ZF. *Helicobacter pylori* infection and risk of gastric cancer in Changhe County,Fujian Province,China. World J Gastroenterol 2000; 6: 374-376
11. Zhang ZW, Farthing MJ. Molecular mechanisms of *H pylori* associated gastric carcinogenesis. World J Gastroenterol 1999; 5: 369-374
12. Hu GY, Yu BP, Dong WG, Li MQ, Yu JP, Luo HS, Rang ZX. Expression of TFF2 and *Helicobacter pylori* infection in carcinogenesis of gastric mucosa. World J Gastroenterol 2003; 9: 910-914
13. Yang YL, Xu B, Song YG, Zhang WD. Overexpression of c-fos in *Helicobacter pylori*-induced gastric precancerous of Mongolian gerbil. World J Gastroenterol 2003; 9: 521-524
14. Guo XL, Wang LE, Du SY, Fan CL, Li L, Wang P, Yuan Y. Association of cyclooxygenase-2 expression with *H pylori* infection in gastric cancer. World J Gastroenterol 2003; 9: 245-249
15. Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Lyon, 7-14 June 1994. IARC Monogr Eval Carcinog Risks Hum 1994; 61: 1-241
16. Jang TJ, Kim JR. Proliferation and apoptosis in gastric epithelial cells of patients infected with *Helicobacter pylori*. J Gastroenterol 2000; 35: 265-271
17. Chan AO, Wong BC, Lam SK. Gastric cancer: past, present and future. Can J Gastroenterol 2001; 15: 469-474
18. Liu HF, Liu WW, Fang DC, Men RP. Expression and significance of proapoptotic gene Bax in gastric carcinoma. World J Gastroenterol 1999; 5: 15-17
Hepatol 2001; 7: 764-765

Kodama M, Fujitaka T, Kodama R, Takahashi K, Kubota T, Murakami K, Nasu M. p53 expression in gastric mucosa with Helicobacter pylori infection. J Gastroenterol Hepatol 1999; 14: 215-219

Zhu GH, Yang XL, Lai KC, Ching CK, Wong BC, Yuen ST, Ho J, Lam SK. Nonsteroidal antiinflammatory drugs could reverse Helicobacter pylori-induced apoptosis and proliferation in gastric epithelial cells. Dig Dis Sci 1998; 43: 1957-1963

Kohda K, Tanaka K, Aiba Y, Yasuda M, Miwa T, Koga Y. Role of apoptosis induced by Helicobacter pylori infection in the development of duodenal ulcer. Gut 1999; 44: 456-462

Konturek PC, Pierzchalski P, Konturek SJ, Meixner H, Faller G, Kirchner T, Hahn EG. Helicobacter pylori induces apoptosis in gastric mucosa through an upregulation of Bax expression in humans. Scand J Gastroenterol 1999; 34: 375-383

Fan X, Crowe SE, Behar S, Gunsanosa H, Ye G, Haeberle H, Van Houten N, Gourley WK, Ernst PB, Reyes VE. The effect of class II cyclooxygenases. J Gastroenterol Hepatol 1998; 13: 1659-1669

Anti M, Armuzzi A, Iacone E, Valenti A, Lippi ME, Covino M, Vecchio FM, Pierconti F, Buzzi A, Pignataro G, Bonvicini F, Gasbarrini G. Epithelial-cell apoptosis and proliferation in Helicobacter pylori-related chronic gastritis. Ital J Gastroenterol Hepatol 1998; 30: 153-159

Watanabe S, Takagi A, Koga Y, Kaniya S, Miwa T. Helicobacter pylori induces apoptosis in gastric epithelial cells through inducible nitric oxide. J Gastroenterol Hepatol 2000; 15: 168-174

Takagi A, Watanabe S, Ishizawa H, Kishi H, Hamamura H, Doi Y, Shibata N, Yagi T, Nada T, Iinuma Y, Shibayama K. Helicobacter pylori infection: implications in gastric carcinogenesis. Am J Gastroenterol 2001; 96: 16-26

Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of B3-only proteins. Cell Death Differ 2002; 9: 505-512

Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998; 281: 1322-1326

Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999; 13: 1899-1911

Lee EH, Wan KH, Song J, Kang JJ, Cho JW, Seo KY, Lee JH. Lens epithelial cell death and reduction of anti-apoptotic protein Bcl-2 in human anterior polar cataracts. Mol Vis 2002; 8: 235-240

Tafani M, Karpinich NO, Hurster KA, Pastorino G, Schneider T, Russo MA, Farber JL. Cytochrome c release upon Fas receptor activation depends on translocation of full-length bid and the induction of the mitochondrial permeability transition. J Biol Chem 2002; 277: 10073-10082

Zong WX, Lindsten T, Ross Aj, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 2001; 15: 1481-1486

Letia A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutic. Cancer Cell 2002; 2: 183-192

Theodorakis P, Lomonossova E, Chinnadurai G. Critical requirement of BAX for manifestation of apoptosis induced by multiple stimuli in human epithelial cancer cells. Cancer Res 2002; 62: 3373-3376

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ. BCL-2, BCL-X(L) sequester BAX domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 2001; 8: 705-711

Shangary S, Johnson DE. Peptides derived from BH3 domains of Bcl-2 family members: a comparative analysis of inhibition of Bcl-2, Bcl-X(L), and Bax oligomerization, induction of cytochrome c release, and activation of cell death. Biochemistry 2002; 41: 9465-9495

Houghton J, Macara-Bloch LS, Harrison L, Kim KH, Korah RM. Nuclear tumour factor alpha and interleukin 1beta up-regulate gastric mucosal Fas antigen expression in Helicobacter pylori infection. Infect Immun 2000; 68: 1189-1195

D’Eliaos MM, Maghetti M, De Carli M, Costa F, Baldari CT, Burrini D, Telford JL, Romagnani S, Del Prete G. T helper 1 effector cells specific for Helicobacter pylori in the gastric antrum of patients with peptic ulcer disease. J Immunol 1997; 158: 962-967

Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology 1996; 111: 419-425

Houghton J, Korah RM, Condon MR, Kim KH. Apoptosis in Helicobacter pylori-associated gastric and duodenal ulcer disease is mediated via the Fas antigen pathway. Dig Dis Sci 1999; 44: 465-478

Wei MC, Lindsten T, Moorthy VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ. IBD, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 2000; 14: 2060-2071

Konturek PC, Konturek SJ, Pierzchalski P, Bielawska W, Duda A, Marlicz K, Starzyńska T, Hahn EG. Cancerogenesis in Helicobacter pylori infected stomach—role of growth factors, apoptosis and cyclooxygenases. M ed Sci M onit 2003; 7: 1092-1107

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