• GASTRIC CANCER •

Helicobacter pylori infection generated gastric cancer through p53-Rb tumor-suppressor system mutation and telomerase reactivation

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Abstract

AIM: To investigate the relationship between *Helicobacter pylori* (*H.pylori*) infection and the expressions of the p53, Rb, c-myc, bcl-2 and hTERT mRNA in a series of diseases from chronic gastritis (CG), intestinal metaplasia type I or II (IMI-II), intestinal metaplasia type III (IMIII), mild or modest dysplasia (DysI-II), severe dysplasia (DysIII) to gastric cancer (GC) and to elucidate the mechanism of gastric carcinogenesis relating to *H.pylori* infection.

METHODS: 272 cases between 1998 and 2001 were available for the study including 42 cases of CG, 46 cases of IMI-II, 25 cases of IMIII, 48 cases of DysI-II, 27 cases of DysIII, 84 cases of GC. *H.pylori* infection and the expressions of p53, Rb, c-myc, bcl-2 were detected by means of streptavidin-peroxidase (SP) immunohistochemical method. HTERT mRNA was detected by *in situ* hybridization(ISH).

RESULTS: The expressions of p53, Rb, c-myc, hTERT mRNA and bcl-2 were higher in the GC than in CG, IM, Dys. The expression of c-myc was higher in IMIII with *H.pylori* infection (10/16) than that without infection (1/9) and the positive rate in DysI-II and DysIII with *H.pylori* infection was 18/30 and 13/17, respectively, higher than that without infection (4/18 and 3/10, respectively). In our experiment mutated p53 had no association with *H.pylori* infection, the expression of Rb was associated with *H.pylori* infection in GC, but the p53-Rb tumor-suppressor system abnormal in DysI-II cases, DysIII and GC cases with *H.pylori* infection was 21/30, 15/17 and 48/48 respecively, higher than non-infection groups (4/18, 3/10, 28/36). Furthermore the level of hTERT mRNA in GC with *H.pylori* infection (47/48) was higher than that without infection (30/36), however the relationship between bcl-2 and *H.pylori* was only in IMIII. C-myc had a close association with hTERT mRNA in DysIII and GC (*P*=0.0 253,0.0 305 respectively).

CONCLUSION: In the gastric carcinogenesis, *H.pylori* might cause the severe imbalance of proliferation and apoptosis in the precancerous lesions (IMIII and GysIII) first, leading to p53-Rb tumor-suppressor system mutation and telomerase reactivation, and finally causes gastric cancer.

INTRODUCTION

Gastric cancer is one of the most fatal malignancies and in the world about 628 000 persons die of it every year. A close association between *Helicobacter pylori* (*H.pylori*) and gastric cancer has been found[1-14], mainly on the basis of seroepidemiological data. Recently the animal models that developed gastric cancer owing to *H.pylori* infection provided powerful evidence[15]. Although *H.pylori* has been classified as a type I carcinogen for gastric cancer by the International Agency for Research on Cancer (IARC)[16], the exact nature and strength of the association with gastric cancer has remained indistinct.

A two-stage pattern has been introduced to explain the escape from senescence of the cultured human cells[17,18]. The mortality stage 1 (M1) mechanism causes senescence, where normal human diploid cells become incapable of further division. However this cessation of cellular division can be overcome by the inactivation of human tumor suppressor genes p53 and Rb in fibroblasts[18,19] that are mutated in a variety human neoplasm. In non-neoplastic cells, wild type p53 modulates cell proliferation and differentiation by regulating the transcription of several gene products, inducing $p21^{waf1}$ expression which acts as a regulator of the cell cycle at the G1 checkpoint[20,21], and plays a crucial role in repairing damaged DNA through inducing GADD45 (growth arrest and DNA damage)[22]. Moreover p53 induces the Bax gene, which is followed by apoptosis, in contrast to bcl-2[23]. The cells which have overcame the M1 mechanism are able to divide until crisis occurs, which is caused by the mortality stage2 (M2) mechanism. It is difficult for human cells to escape from this crisis, but in rare instances some populations can achieve immortality by overcoming this M2 mechanism through the reactivation of the enzyme telomerase[24-29]. Telomerase is a unique ribonucleoprotein enzyme that is responsible for adding the telomic repeats onto the 3' end of chromosomes and composed of a catalytic protein submit (hTERT, for human telomerase reverse transcription) and a template RNA (TR), and hTERT is the rate-limiting enzyme in the telomerase complex[30-34].

From all the above it is apparent that the understanding of the association of *H.pylori* infection with some genes (for example p53, Rb) as well as telomerase can contribute to the elucidation of the mechanisms that regulate the development of gastric cancer. The aim of the present study, therefore, was to examine this association by detecting the expressions of *H.pylori*, hTERT, p53, Rb, c-myc and bcl-2 in a series of gastric diseases.

MATERIALS AND METHODS

Patients

Two hundred seventy-two patients (174 men, 98 women, ranging in age from 21 to 80 years, mean 54.22 years) underwent endoscopy (noncancerous patients) or curative gastrectomy (gastric cancer patients) in our hospital between 1998 to 2001, including 42 cases of CG, 46 cases of IMI-II, 25 cases of IMIII, 48 cases of DysI-II, 27 cases of DysIII, 84 cases of GC diagnosed according to the updated Sydney System[35] and the Japanese Research Society for Gastric Cancer[36]. No patients had received chemotherapy or radiation therapy before surgery.

All specimens were fixed in 10 % buffered neutral formalin and embedded in paraffin and serial sections (4 μm thick).

Histochemical staining
Hematoxylin-eosin (HE) staining was used for the histopathological diagnosis, evaluation and grading of gastritis, atrophy, intestinal metaplasia, dysplasia and cancer. High iron diamine (HID)-alcian blue (AB) (PH 2.5)-periodic acid schiff (PAS) method was used to distinguish sulphates, neutral and acid mucus, which were stained brown-black, red and blue respectively. Using both morphological and histochemical criteria, the cases of intestinal metaplasia were classified into three types[37].

Immunohistochemistry
Was performed using the streptavidin-peroxidase (sp) method. The following primary antibodies and the kit were used: monoclonal antibodies against p53, Rb, c-myc, bcl-2 and the kit (Maixin-Bio, Fujian), polyclonal antibodies against *H.pylori* (antibody, diagnostica Inc. USA). Dewaxed sections were heated in a microwave oven (700W) for 12 min to retrieve the antigens and cooled to room temperature. Endogenous peroxide was blocked by 3 % hydrogen peroxide (H_2O_2) for 15 min in methanol. After being washed with phosphate-buffered-saline (PBS, 0.01M), the sections were further blocked by 10 % rabbit serum for 15 min to reduce nonspecific antibody binding and then incubated with the primary antibodies of p53, Rb, c-myc, bcl-2 or *H.pylori* (1:60 dilution) at 4 ℃ overnight. After being washed with PBS for 2×5 min, the sections were incubated with the secondary anti-mouse immunoglobulin (Ig) conjugated with biotin at room temperature for 15 min, washed again with PBS, followed by incubation with streptavin-peroxidase complex for 15 min. The reaction products of peroxidase were visualized by incubation with 0.05 M Tris-HCL buffer (PH7.6) containing 20 mg 3,3'-diaminobendizine (Maixin-Bio, Fujian) and 100 μl 5 % hydrogen peroxide per 100 ml. Finally, the sections were counterstained for nuclei by hematoxilin solution. To examine the specificity of immunostaining, PBS was used to replace the primary antibodies as the control. The assessment of all the samples was conducted by an observer who did not know any details of this study by calculating the average ratio of positive cells (the nuclei or the plasma, staining brown-yellow) under ten 400× microscopes. If the ratio was more than 10 %, this sample was considered positive. However the *H.pylori* immunostaining was assessed positive as long as the brown-black dotish, stake or bend material was stained on the surface of mucosa or in the gland's cave.

In situ hybridization
(ISH) was used to detect hTERT (human tolomerase reverse transcriptase) mRNA. The probe (5'-CCCAG GCGCC GCACG AACGT GGCCA GCGGC-3') and the kit were bought from Boster Bio (Wuhan). Dewaxed sections were incubated with 3 % hydrogen peroxide for 30 min to reduce the non-specific binding and then with 1 μg/ml pepsin for 5-8 min to improve the penetration of the probe. The prehybridization was performed at 40 ℃ for 3 h to enhance hybridization efficiency, and the hybridization was conducted in a 42 ℃ water bath with each section covered with a coverslip, then the thorough washing procedure was followed: 2×SSC (sodium chloride and sodium citrate) at 37 ℃ for 15 min, 0.5 ×SSC for 15 min, 0.2×SSC for 15 min. Then the sections were visualized according to the kit manufacturer's instructions. We calculated the positive cells ratio to assess the positive sample by calculating the average ratio of positive cells (the plasma was stained brown-yellow) under ten 400× microscopes. If the ratio was more than 10 %, this sample was considered positive.

Statistics
The chi-square test and the Fisher's exact probability test were used to compute the frequencies by SPSS 10.0 for Windows. $P < 0.05$ was considered to be statistically significant.

RESULTS
With the development of the diseases from CG, IM, Dys to GC, the positive expressions of p53, Rb, c-myc, bcl-2 and hTERT mRNA were augmented significantly (Table 1). In particular, the frequencies of Rb, c-myc and hTERT mRNA in IMIII (8/25, 11/25, 4/25, respectively) were statistically higher than those in IMI-II (4/46, 8/46, 1/46, respectively) ($P < 0.05$). The expressions of all variables in GC were statistically higher than in DysIII and IMIII, higher in DysIII e than in IMIII ($P < 0.05$). In general the frequencies of them in *H.pylori* infection groups were higher than those in non-infection groups. The expression of c-myc was 10/10, 18/30,

Table 1 Expressions of c-myc, bcl-2,p53, Rb and hTERTmRNA in the different gastric diseases (%)

	GC	IMI-II	IMIII	DysI-II	DysIII	GC
c-myc	5(11.90)	8(17.39)	11(44.00)	22(45.83)	16(59.26)	74(88.09)
bcl-2	3(7.14)	17(36.96)	10(40.00)	24(50.00)	18(66.67)	55(65.48)
p53	0(0.00)	3(6.52)	5(20.00)	14(29.17)	10(37.04)	64(76.19)
Rb	3(7.14)	4(8.70)	8(32.00)	14(29.17)	12(44.44)	61(72.62)
HTERTmRNA	0(0.00)	1(2.17)	4(16.00)	10(16.67)	12(44.44)	78(92.86)

Table 2 The correlations between p53, Rb, c-myc, hTERT, bcl-2 and *H.pylori* in benign diseases and gastric cancer

H.pylori	CG		IMI-II		IMIII		DysI-II		DysIII		GC	
	+(31)	-(11)	+(32)	-(14)	+(16)	-(9)	+(30)	-(18)	+(17)	-(10)	+(48)	-(36)
c-myc	5	0	7	1	10[a]	1	18[a]	4	13[a]	3	44	30
bcl-2	3	0	12	5	9[a]	1	17	7	13	5	38	17
p53	0	0	2	1	3	2	12[a]	2	6	4	39	25
Rb	3	0	4	0	6	2	11	3	9	3	40[a]	21
P53-Rb	3	0	4	1	6	3	21[a]	4	15[a]	3	48[a]	28
hTERT	0	0	1	0	4	0	8	2	9	3	47[a]	30

[a]$P < 0.05$ *vs* the non-infected group.

13/17 in IMIII, DysI-II and DysIII with *H.pylori* infection respectively, higher than non-infection group (1/9, 4/18, 3/10, respectively). In our experiment mutated p53 had no association with *H.pylori* infection, the expression of Rb was associated with *H.pylori* infection in GC, but the p53-Rb tumor-suppressor system abnormal in DysI-II cases, DysIII and GC cases with *H.pylori* infection was 21/30, 15/17 and 48/48, respectively, higher than that in non-infection groups (4/18, 3/10, 28/36, respectively). The association between bcl-2 and *H.pylori* only existed in IMIII (9/16, 1/9). The expression of hTERT mRNA in GC with *H.pylori* infection was 47/48, higher than non-infection group (30/36) (Table 2). We also found the association between c-myc and hTERT mRNA in GC and DysIII ($P<0.05$) (Table 3).

Table 3 Correlation between c-myc and hTERT in the gastric benign diseases and gastric cancer

c-myc	CG		IMI-II		IMIII		DysI-II		DysIII		GC	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
hTERT(+)	0	0	1	0	3	1	7	3	10[a]	2	70[a]	7
(-)	5	37	7	39	8	13	15	23	6	9	4	3

[a]$P<0.05$ *vs* the negative group.

DISCUSSION

Gastric cancer occurs after a multi-step process of alterations in oncogenes, tumor-suppressor genes, cell-adhesion molecules, telomerase as well as genetic instability at several microsatellite loci. Studies have demonatrated that *H.pylori* infection is closely associated with these abnormal alterations. Konturek' s study[38] showed that *H.pylori* induced apoptosis in gastric mucosa through upregulation of Bax and bcl-2 expression. The bcl-2 gene family plays an important role in regulating apoptosis. In our studies, *H.pylori* enhanced the expression of c-myc and bcl-2 significantly in IMIII ($P<0.05$). The c-myc protein is a critical component for the control of normal cell growth, but the altered c-myc activity by translocation, amplification, overexpression, and mutation is widespread in tumor cells and important for multi-step carcinogenesis[39,40]. C-myc is a strong inducer of proliferation and it is believed to be critical for the oncogenic properties. Some studies showed that the abnormal c-myc expression derived cells inappropriately through the cell cycle, leading to uncontrolled proliferation, a characteristic of neoplastic cells. Form our study we found that *H.pylori* infection caused a higher proliferation and a lower apoptosis in IMIII than in CG and IMI-II and accelerated cell proliferation, leaving a good chance for all kinds of genes and proteins to mutate or overexpress, presumably heightening the genetic instability consistent with the development of carcinoma[41,42].

Mutations in the tumor suppressor genes p53 and Rb are common events in human cancers that exert their control on the cell cycle at the G1-S phase transition through independent but interconnected pathways[43-50]. Williams' s studies showed that germ-line mutation in p53 and Rb might have cooperative tumorigenic effects in mice[51]. It has been generally accepted that p53 and Rb tumor-suppressor system, including p53, Rb, p16, p15, p14 and p21waf1[52-60], play an important role in carcinogenesis. Some studies observed this phenomenon that NO (nitric oxide) generated by *H.pylori* caused p53 mutation at the spot C:G to A:T and at the same time p53 was found mutated at the same spot in IM, Dys and GC, thus *H.pylori* probably caused p53 mutation. In our study, we found that the expressions of p53-Rb were continually enhanced from chronic gastritis (3/42) to gastric cancer (76/84) and in the DysIII and

GC which had a close association with *H.pylori* ($P<0.05$). Chen[61] reported that the mRNA levels of p53-Rb in gastric cancer were significantly lower than those in their non-cancerous tissues using quantitative analysis method. Some studies favored the view that c-myc drove initial proliferation and subsequent differentiation, concomitant with the activation of the p53 G2 checkpoint and also demonstrated that inactivation of the p53-Rb pathway is required for immortalization through overrepression of Myc[62,63]. Although c-myc and p53-Rb had no direct association in our study, *H.pylori* infection might initially provoke the c-myc and bcl-2 more than in non-infection group in IMIII and then inactivate the p53-Rb tumor-suppress system in Dys and they collaborated in GC.

Telomerase activity has been found in 85-90 % of all human cancers but not in adjacent normal cells[27]. It has thus been hypothesized that for a cancer cell to undergo sustained proliferation beyond the limits of cell senescence, it must reactivate telomerase or an alternative mechanism in order to maintain telomeres. This makes telomerase a target not only for the novel etiology agent but also a mark for cancer diagnosis. Many studies showed that telomerase activity was higher in cancers than in non-cancerous tissues and higher in IM than in CG[64-69], which was similar to our results. The association between *H.pylori* infection and telomerase activity is still controversial. Suzuki' study[67] indicated that hTERT mRNA which was expressed in precancerous lesions and gastric cancer could be induced at an early stage of gastric carcinogenesis, but it was not correlated with *H.pylori*. Kuniyasu[70] found that *H.pylori* evidently caused the release of reactive oxygen species (ROMs) and reactive nitrogen species (NO) which might be strong triggers for "stem cell" hyperplasic in IM, followed by telomere reduction and increased telomerase activity as well as hTERT overexpression. We found in GC hTERT expression was significantly higher in infection group (47/48) than in non-infection group (30/36) but had no association in Dys or IM, maybe because there exist two different genetic pathways to two histological types: intestinal-type and diffuse-type gastric cancer. Some studies showed c-myc could stimulate expression of hTERT and thereby enhance telomerase activity which was an important step in carcinogenesis[71-80]. In our study c-myc had an association with hTERT in Dys and GC, which suggests that *H.pylori* stimulates telomerase directly or indirectly by the overexpression of c-myc. We did not find the association between hTERT and p53-Rb or bcl-2.

There maybe exit this mechanism of gastric caicinogenesis relating to chronic *H.pylori* infection, which leads to imbalance of proliferation and apoptosis in the early stage, and furthermore p53-Rb tumor-suppressor system mutation, telomerase reactivation and finally gastric cancer generation. Hence this molecular pathology mechanism should be applied in routine diagnostic procedures, classification systems, disease monitoring, and even prognostic assessment.

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