• *H pylori* •

Increased oxidative DNA damage, inducible nitric oxide synthase, nuclear factor κB expression and enhanced antiapoptosis-related proteins in *Helicobacter pylori*-infected non-cardiac gastric adenocarcinoma

Chi-Sen Chang, Wei-Na Chen, Hui-Hsuan Lin, Cheng-Chung Wu, Chau-Jong Wang

Chi-Sen Chang, Wei-Na Chen, Division of Gastroenterology, Chung Shan Medical University, Taichung, Taiwan, china
Chi-Sen Chang, Wei-Na Chen, Division of Gastroenterology, Taichung Veterans General Hospital, Taichung, Taiwan, china
Cheng-Chung Wu, Division of General Surgery, Taichung Veterans General Hospital, Taichung, Taiwan, china
Chi-Sen Chang, Wei-Na Chen, Hui-Hsuan Lin, Chau-Jong Wang, Institute of Biochemistry, Chung Shan Medical University, Taichung, Taiwan, china
Cheng-Chung Wu, National Young-Min Medical University, Taipei, Taiwan, china
Correspondence to: Chau-Jong Wang, Institute of Biochemistry, College of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Chien Kuo North Road, 402, Taichung, Taiwan, china. wcj@csmu.edu.tw
Telephone: +886-4-24730022   **Fax:** +886-4-23248167
Received: 2004-03-15   **Accepted:** 2004-04-09

Abstract

AIM: Several epidemiological studies have demonstrated a close association between *Helicobacter pylori* (*H Pylori*) infection and non-cardiac carcinoma of the stomach. *H pylori* infection induces active inflammation with neutrophilic infiltrations as well as production of oxygen free radicals that can cause DNA damage. The DNA damage induced by oxygen free radicals could have very harmful consequences, leading to gene modifications that are potentially mutagenic and/or carcinogenic. The aims of the present study were to assess the effect of *H pylori* infection on the expression of inducible nitric oxidative synthase (iNOS) and the production of 8-hydroxy-deoxyguanosine (8-OHdG), a sensitive marker of oxidative DNA injury in human gastric mucosa with and without tumor lesions, and to assess the possible factors affecting cell death signaling due to oxidative DNA damage.

METHODS: In this study, 40 gastric carcinoma specimens and adjacent specimens were obtained from surgical resection. We determined the level of 8-OHdG formation by HPLC-ECD, and the expression of iNOS and mechanism of cell death signaling [including nuclear factor-κB(NFκB), MEKK-1, Caspase 3, B Cell lymphomal leukemia-2 (Bcl-2), inhibitor of apoptosis protein (IAP) and myeloid cell leukemia-1 (Mcl-1)] by Western-blot assay.

RESULTS: The concentrations of 8-OHdG, iNOS, NFκB, Mcl-1 and IAP were significantly higher in cancer tissues than in adjacent non-cancer tissues. In addition, significantly higher concentrations of 8-OHdG, iNOS, NFκB, Mcl-1 and IAP were detected in patients infected with *H pylori* compared with patients who were not infected with *H pylori*. Furthermore, 8-OHdG, iNOS, NFκB, Mcl-1 and IAP concentrations were significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.

CONCLUSION: Chronic *H pylori* infection induces iNOS expression and subsequent DNA damage as well as enhances anti-apoptosis signal transduction. This sequence of events supports the hypothesis that oxygen-free radical-mediated damage due to *H pylori* plays a pivotal role in the development of gastric carcinoma in patients with chronic gastritis.

INTRODUCTION

Helicobacter pylori (*H pylori*) infection is associated with an increased risk of both peptic ulcer and gastric cancer[1] Chronic gastritis induced by *H pylori* significantly increases the risk for non-cardiac gastric cancer[2,3], and host responses that may affect the threshold for carcinogenesis include alteration of epithelial cell proliferation and apoptosis. Although the linkage between *H pylori* infection and gastric cancer is convincing, the molecular mechanism or mechanisms responsible are unclear, and both bacterial and host factors are implicated. One possible factor is that increased inflammation generates reactive oxygen and nitrogen intermediates that have been shown to damage DNA directly[4].

Mucosal hyperproliferation has been reproducibly demonstrated in *H pylori*-infected human[5-7] and rodent gastric tissue[8-10], and proliferation scores normalize following successful eradication in humans[5-7]. However, maintenance of tissue integrity requires that enhanced cell production is accompanied with increased rates of cell loss consequently, studies have also examined the effect of *H pylori* on apoptosis. In contrast to hyperproliferation, *H pylori* has been associated with increased[11-14], unchanged[15], or even decreased[16] levels of apoptosis *in vivo*, and within a particular population, substantial heterogeneity exists among apoptosis scores[11-14]. These observations suggest that increases in proliferation that are not balanced by concordant increases in apoptosis over years of colonization may heighten the retention of mutated cells, ultimately enhancing the risk for gastric malignancy in certain populations. Alteration of epithelial cell proliferation and apoptosis is a manifestation of *H pylori*-induced gastritis. However, the precise mechanisms underlying these effects remain incompletely clarified. In this study, we aimed to assess the effect *H pylori* infection on the expression of iNOS and the production of 8-OHdG, a sensitive marker of oxidative DNA injury in human gastric mucosa with and without tumor lesions, as well as the possible factors affecting cell death signaling due to oxidative DNA damage.

MATERIALS AND METHODS

Patients

In this study, 40 patients (aged 68.3 ± 11.4 years, range 39-84 years, M/F: 26/14) with non-cardiac gastric adenocarcinoma were included. Specimens from tumor site and adjacent non-tumor site were obtained from surgical resection. By TNM staging according to the criteria described by the International Union Against Cancer[17], there were 2 cases in stage 1, 6 cases in stage 2, 8 cases in stage 3 and 24 cases in stage 4 (Table 1). *H pylori* infection was assessed by the following three methods: histology (haematoxylin and eosin staining or Giemsa staining, rapid urease test (CLO test, Delta West Pty Ltd, Perth, Australia) and serum *H pylori* ELISA IgG assays (Trinity Biotech USA, Jamestown, NY). A patient was considered *H pylori* positive if one or more of the diagnostic methods applied were positive, and *H pylori* negative if all methods were negative. There were 29 patients (72.5%) with *H pylori* infection and 11 patients (27.5%) without *H pylori* infection. We determined the level of 8-OHdG formation by HPLC-ECD, and the expression of iNOS and cell death signaling, including NFκB, MEKK1, Caspase 3, Bcl-2, Mcl-1 and IAP by Western-blot assay.

Table 1 Characteristics of all patients

Case No	Stage	*H pylori* infection	Case No	Stage	*H pylori* infection
1	II	+	21	III	+
2	IV	+	22	IV	+
3	IV	+	23	IV	+
4	III	+	24	IV	+
5	IV	+	25	IV	-
6	IV	+	26	IV	+
7	IV	+	27	I	+
8	III	-	28	I	-
9	III	+	29	IV	-
10	III	+	30	II	+
11	IV	+	31	IV	+
12	IV	-	32	IV	+
13	IV	-	33	IV	+
14	IV	+	34	III	+
15	IV	+	35	II	+
16	II	+	36	III	+
17	III	-	37	IV	-
18	IV	+	38	IV	+
19	IV	+	39	IV	+
20	II	-	40	II	-

DNA extraction and digestion

DNA was extracted with Dneasy tissue kit (QIAGEN) according to the protocol of Dahlhaus and Apple[18] with minor modifications as described previously[19]. Briefly, one volume of nuclear fraction obtained from surgical tissue was mixed with ten volumes of extraction buffer (1 mol/L NaCl, 10 mmol/L Tris–HCl, 1 mmol/L EDTA, 5 g/L SDS, pH 7.4) and one volume of chloroform:isoamylalcohol (12:1 v/v). After vigorous shaking, the aqueous phase was separated by centrifugation. This step was repeated three times before DNA was precipitated with absolute ethanol (-70). DNA pellet was dissolved in TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 7.4), and then incubated with RNase A to remove RNA. After incubation, DNA was extracted with chloroform:isoamylalcohol, and precipitated with absolute ethanol again. The DNA pellet was dissolved in 10 mmol/L Tris–HCl (pH 7.0) and hydrolyzed according to the previous procedure[20]. Briefly, 200 μg DNA in 200 μL 10 mmol/L Tris-HCl (pH 7.0) was first denatured at 95 for 3 min. The digestion was carried out at 37 for 1 h with 100 units DNase I (from bovine pancreas, Sigma) in a buffer containing 10 mmol/L $MgCl_2$, followed by incubation with 5 units nuclease P_1 (from penicillium citrinum, Sigma) in

100 μmol/L $ZnCl_2$ for an additional hour. The mixture was incubated finally with 3 units alkaline phosphatase (from *Escherichia coli*, Sigoma) at 37 for 1 h. The incubation was terminated with acetone (HPLC grade, Merck, Germany) to precipitate proteins. After 12 000 *g* centrifugation for 15 min, the supernatant was dried under vacuum in a rotary evaporator (Eyela, Tokyo, Japan) and DNA was dissolved in distilled H_2O.

8-OHdG assay

8-OHdG levels were determined by HPLC/ECD consisting of a BAS PM-80 pump (Bioanalytical Systems, West Lafayette, IN, USA), a CMA-200 microautosampler (CAM/Microdialysis, Stockholm, Sweden), a BAS-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), a microbore reversed-phase column (Inertsil-2, 5-μm ODS, 1.0×150 mm I.D. G.L. Sciences, Tokyo, Japan) Beckman I/O 406 interface, and Beckman system gold data analysis software (Beckman Instruments). A potential of +0.6 V was selected for the glassy carbon working electrode which corresponded to a silver/silver chloride reference electrode. The mobile phase consisted of 12.5 mmol/L citric acid, 30 mmol/L NaOH, 25 mmol/L sodium acetate, 10 mmol/L acetic acid glacial and 50 mL/L methanol (pH 5.3). The flow rate was set at 1 mL/min. Under these conditions, the retention time of 8-OHdG was 8.49 min with a significantly higher signal than background noise.

Immunoblot analysis

Cancerous and non-cancerous tissue samples were homogenized with ten volumes of homogenization buffer (50 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L sodium chloride, 10 g/L Nonidet P-401 g/L SDS, 5 g/L deoxycholic acid, 1 mmol/L sodium orthovanadate, 170 μg/mL leupeptin, 100 μg/mL phenylsulfonyl fluoride; pH 7.5). After 30 min of mixing at 4 , the mixture was centrifuged at 800 r/min for 10 min, and the supernatant was collected. The protein content of the samples was determined with the Bio-Rad protein assay reagent using BSA as a standard. For Western blotting analysis, 50 μg protein from both cancerous and non-cancerous tissue samples was resolved on 100 g/L SDS-PAGE gels along with prestained protein molecular weight standards (Bio-Rad). The separated proteins were then blotted onto NC membrane (0.45 μm, Bio-Rad) and reacted with primary antibodies (against iNOS, NFκB, MEKK1, Caspase 3, Bcl-2, Mcl-1 and IAP). After washed, the blots were incubated with peroxidase-conjugated goat anti-mouse antibody. Immunodetection was carried out using the enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Corp., UK). Relative protein expression levels were quantified by densitometric measurement of ECL reaction bands and normalized with values of tubulin. Finally, enhanced chemiluminescence (ECL) was used to detect the membrane, and then the membrane was exposed to X-ray film to observe results. All the immunoblot results were determined by Alpha Imager 2000 documentation & analysis system (Alpha Innotech Corporation).

Statistical analysis

Each parameter expressed by fold of increase of the data from cancer tissue was compared to the adjacent non-cancer tissue. The results were reported as mean±SE from individual magnitudes. Data were analyzed with Student's *t*-test. Statistical differences were considered significant when $P<0.05$.

RESULTS

To assess the effect of *H pylori* infection on each determined parameter, the results were expressed by fold of increase comparing the data of cancer tissue with those of the adjacent non-cancer tissue. The concentrations of 8-OHdG were analyzed by HPLC/ECD. There were significantly higher concentrations
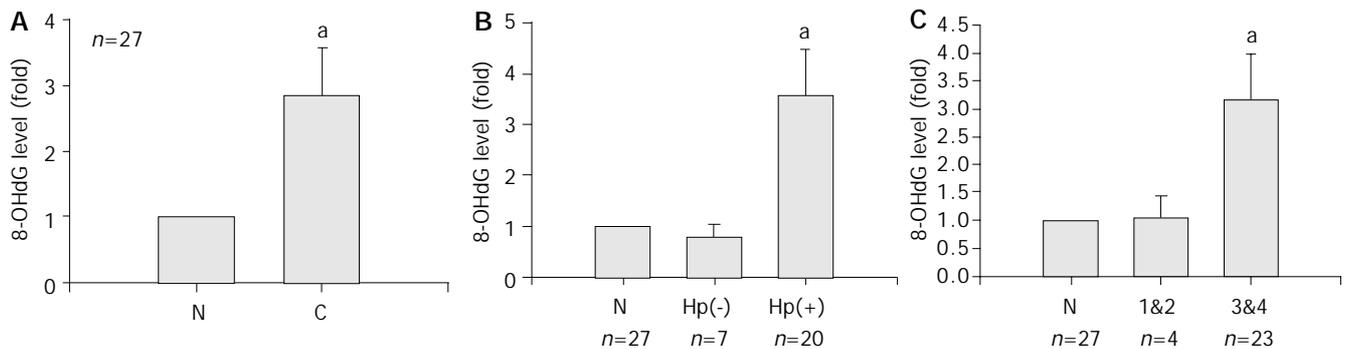
Figure 1 Concentrations of 8-OHdG. A: Higher concentration of 8-OhdG in cancer tissues compared with adjacent non-cancer tissues. B: Higher concentration of 8-OhdG in cancer tissues of patients with and without *H pylori* infection. C: Significantly higher F8-OHdG concentrations in stage 3 and 4 patients [a]*P*<0.05, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4.

of 8-OHdG in cancer tissues than in adjacent non-cancer tissues (Figure 1). In addition, significantly higher concentrations of 8-OHdG were detected in patients infected with *H pylori* than in those without *H pylori* infection. 8-OHdG levels were also significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.

Immunoblot analysis of several factors affecting cell death signaling related to oxidative DNA damage showed increased concentrations of iNOS and NFκB in patients infected with *H pylori* compared to patients who were tested negative for *H pylori* (Figure 2A-B). Furthermore, iNOS and NFκB concentrations were significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.

Although there were some increases in the concentrations of MEKK1 and Caspase-3 in the cancer tissue (Figure 2C-D), these changes were not statistically significant. In addition, no significant increases of MEKK1 and Caspase-3 in patients infected with *H pylori* were noted. The concentrations of Caspase-3 in stage 1 and 2 patients were significantly lower than those in stage 3 and 4 patients.

Among the anti-apoptosis-related proteins, there were some increases in the concentrations of Bcl-2 in the cancer tissue (Figure 2E). However, the changes were not statistically significant. In addition, no significant increase of Bcl-2 in patients infected with *H pylori* was noted. The concentrations of Bcl-2 in stage 1 and 2 patients were significantly lower than those in stage 3 and 4 patients. Mcl-1 and IAP concentrations were significantly higher in the cancer tissues than in the adjacent non-cancer tissues (Figure 2F-G). In addition, Mcl-1 and IAP concentrations were significantly higher in *H pylori*-positive patients than in *H pylori*-negative patients. Stage 3 and 4 patients had significantly higher concentrations of Mcl-1 and IAP than stage 1 and 2 patients.

Table 2 8-oHdG, iNOS, NFκB, Mel-1 and IAP Concentrations in cancer and adjacent non-cancer tissues

	Cancer tissue	*H pylori* (+)	Stages I & II	Stages III & IV
8-OHdG	a	a	-	a
iNOS	b	b	-	b
NF-κB	a	a	-	a
MEKK1	-	-	-	-
Caspase 3	-	-	b	-
Bcl-2	-	-	b	-
Mcl-1	a	a	-	a
IAP	a	a	-	a

 : increase,   : decrease, -: no significant change, [a]*P*<0.05, [b]*P*<0.01 compared with control, by one sample *t*-test and independent *t*-test, respectively.

In summary, 8-OHdG, iNOS, NFκB, Mcl-1 and IAP concentrations were significantly higher in cancer tissues than in adjacent non-caner tissues (Table 2). In addition, significantly higher concentrations of 8-OHdG, iNOS, NFκB, Mcl-1 and IAP were detected in patients infected with *H pylori* compared to patients who were not infected with *H pylori*.

Furthermore, 8-OHdG, iNOS, NFκB, Mcl-1 and IAP concentrations were significantly higher in stage 3 and 4 patients than in stage 1 and 2 patients.
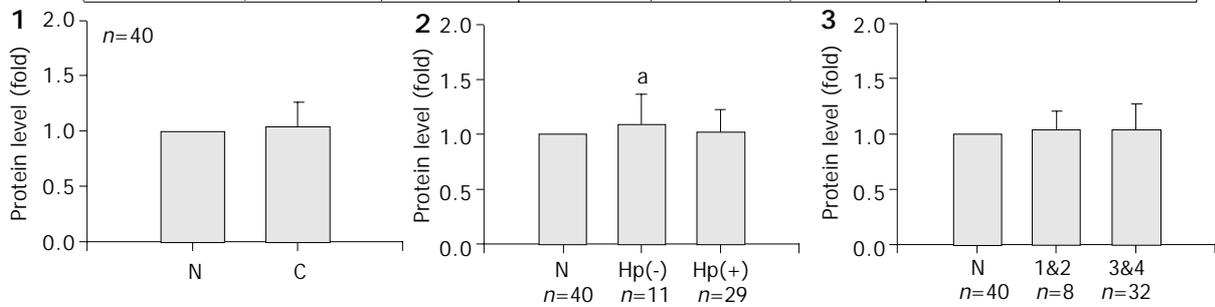
DISCUSSION

H pylori infection may influence the progression from chronic gastritis to adenocarcinoma of the distal stomach by a multifactorial, multistep process, eas which chronic inflammation plays a major role. Free radical mediated oxidative DNA damage was involved in this process[21-23]. The DNA damage provoked by oxygen free radicals can have very harmful consequences, leading to gene modifications that are potentially mutagenic and/or carcinogenic. Recently, 8-OHdG has been accepted as a sensitive marker for oxidative DNA damage in affected organs. Chronic gastritis due to *H pylori* infection is characterized by the accumulation of oxidative DNA damage measured by concentrations of 8-OHdG, indicating mutagenic and carcinogenic potential[24]. In this study, we demonstrated an increase in the concentrations of 8-OHdG in cancer tissues. Furthermore, the increase was significantly higher in patients infected with *H pylori* as well as in patients with advanced gastric cancer. The increased level of oxidative DNA damage suggested a mechanistic link between *H pylori* infection and gastric carcinoma[25,26].

H pylori-associated inflammation related to DNA damage is indicated by increased levels of oxidative DNA damage, increased occurrence of apoptosis, as well as increased expression of iNOS, which seemed to provide the mechanistic links between *H pylori* infection and gastric carcinogenesis[27,28]. Upregulation of iNOS expression might contribute to the oxidative DNA damage observed during *H pylori* infection[25]. Recent studies have shown that nitric oxide (production of iNOS) reacts rapidly with superoxide anions produced by *H pylori*-infected gastric tissues to form peroxynitrite, which could induce oxidative damage[29]. This may increase the mutation rate in infected hyperproliferative gastric mucosa. In addition, the expressions of iNOS are closely related to tumor angiogenesis, and involved in progression of the disease as well as lymph node metastasis. Thus the expressions of iNOS might serve as indexes for evaluating staging of gastric carcinoma. In this study, we observed significantly higher increases in the concentrations of iNOS in patients infected with *H pylori* and in patients with advanced gastric cancer, compared with *H pylori*-negative patients and low stage gastric cancer patients, respectively.
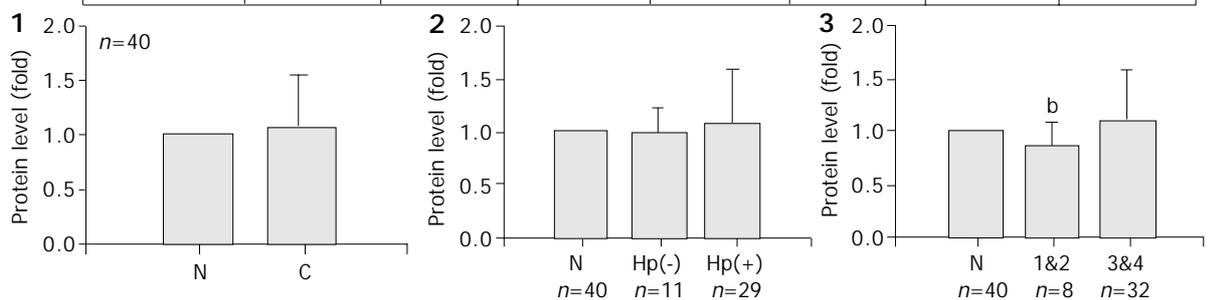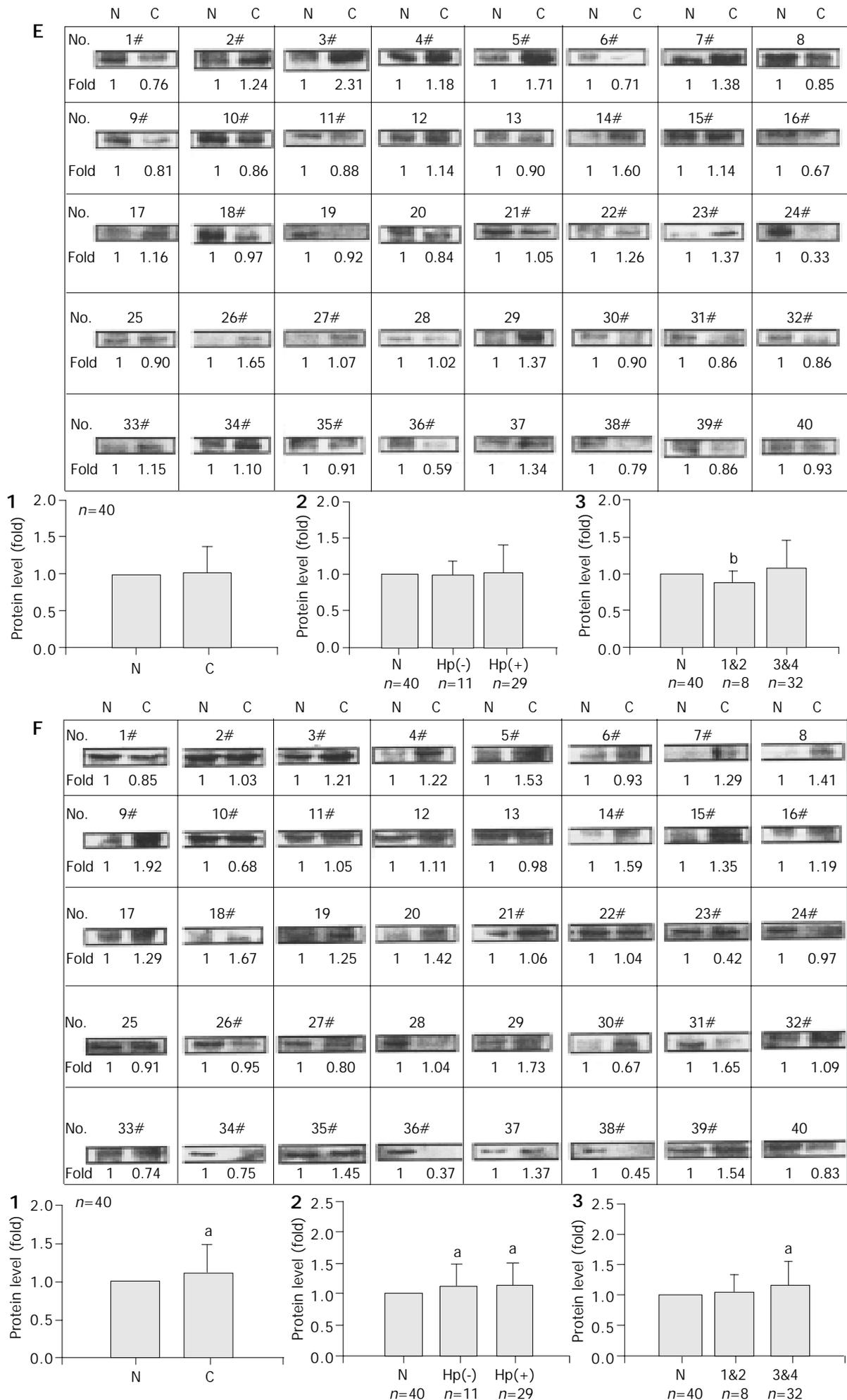
C
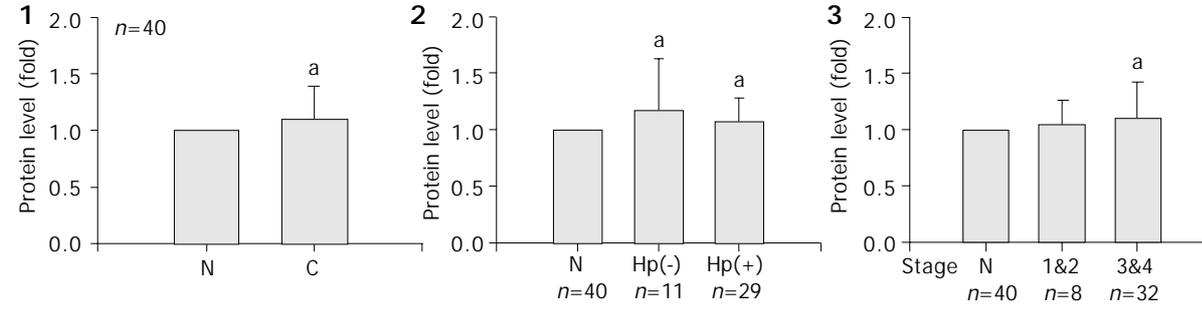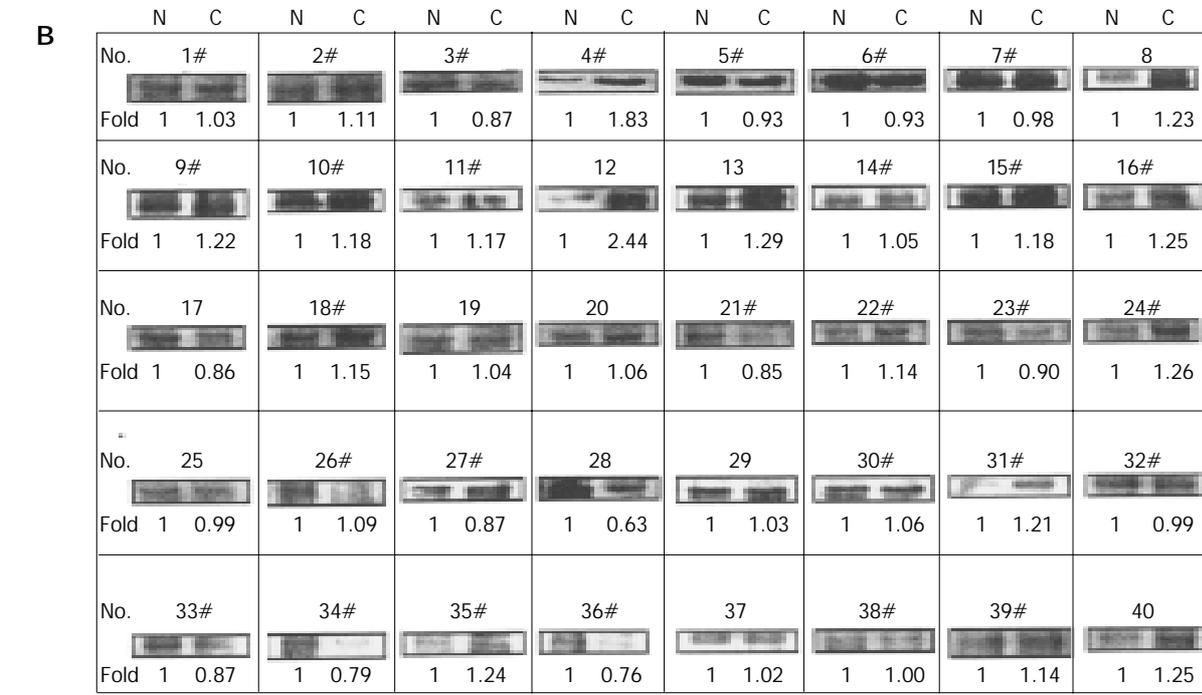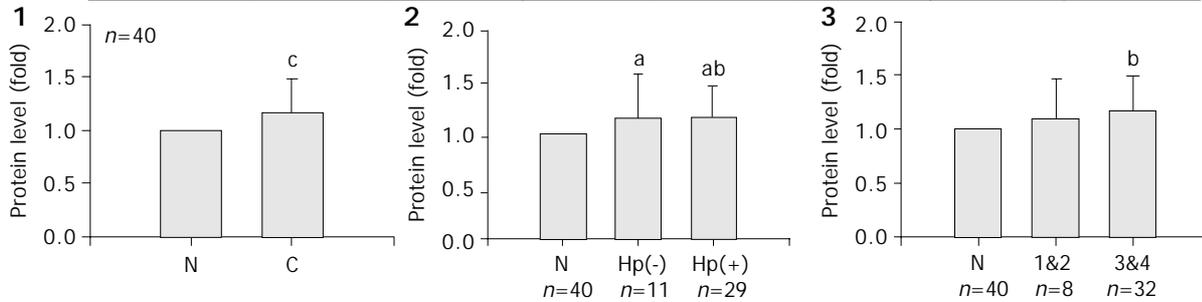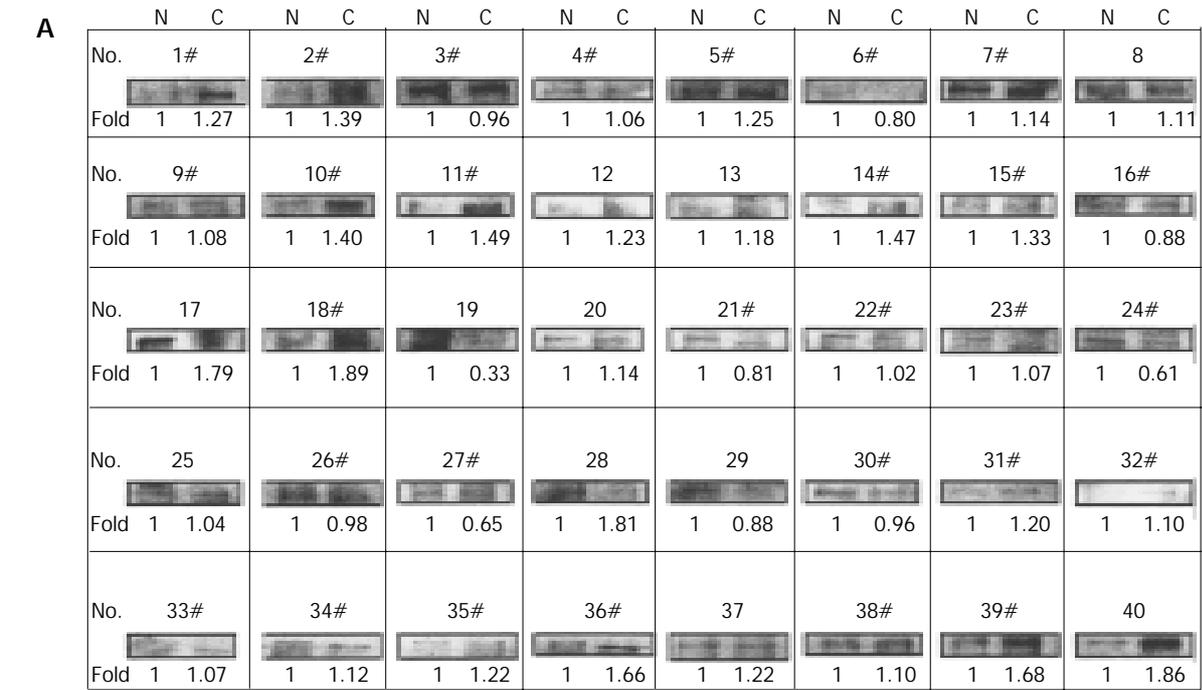
	N C	N C	N C	N C	N C	N C	N C	N C
No.	1#	2#	3#	4#	5#	6#	7#	8
Fold	1 0.94	1 0.97	1 1.01	1 1.19	1 0.94	1 1.01	1 0.78	1 1.10
No.	9#	10#	11#	12	13	14#	15#	16#
Fold	1 0.90	1 1.03	1 1.11	1 1.21	1 0.80	1 1.03	1 0.96	1 0.81
No.	17	18#	19	20	21#	22#	23#	24#
Fold	1 1.03	1 1.05	1 0.90	1 1.20	1 0.75	1 1.26	1 0.65	1 1.16
No.	25	26#	27#	28	29	30#	31#	32#
Fold	1 1.64	1 1.63	1 1.21	1 0.95	1 1.37	1 0.88	1 1.19	1 1.24
No.	33#	34#	35#	36#	37	38#	39#	40
Fold	1 0.89	1 0.98	1 1.09	1 0.92	1 0.75	1 1.04	1 1.19	1 1.24

1 Protein level (fold) — $n=40$ — N, C

2 Protein level (fold) — a — N ($n=40$), Hp(-) ($n=11$), Hp(+) ($n=29$)

3 Protein level (fold) — N ($n=40$), 1&2 ($n=8$), 3&4 ($n=32$)

D

	N C	N C	N C	N C	N C	N C	N C	N C
No.	1#	2#	3#	4#	5#	6#	7#	8
Fold	1 0.76	1 1.26	1 2.00	1 1.28	1 1.35	1 0.61	1 1.51	1 1.25
No.	9#	10#	11#	12	13	14#	15#	16#
Fold	1 1.72	1 0.63	1 0.87	1 1.12	1 0.99	1 1.08	1 0.98	1 0.62
No.	17	18#	19	20	21#	22#	23#	24#
Fold	1 1.12	1 0.59	1 0.53	1 1.28	1 0.94	1 2.94	1 0.75	1 1.28
No.	25	26#	27#	28	29	30#	31#	32#
Fold	1 0.84	1 0.79	1 0.96	1 0.93	1 1.06	1 0.83	1 1.45	1 1.06
No.	33#	34#	35#	36#	37	38#	39#	40
Fold	1 0.74	1 0.68	1 0.85	1 0.38	1 0.98	1 1.06	1 0.89	1 0.58

1 Protein level (fold) — $n=40$ — N, C

2 Protein level (fold) — N ($n=40$), Hp(-) ($n=11$), Hp(+) ($n=29$)

3 Protein level (fold) — b — N ($n=40$), 1&2 ($n=8$), 3&4 ($n=32$)

E

No.	1#	2#	3#	4#	5#	6#	7#	8
Fold	1 0.76	1 1.24	1 2.31	1 1.18	1 1.71	1 0.71	1 1.38	1 0.85
No.	9#	10#	11#	12	13	14#	15#	16#
Fold	1 0.81	1 0.86	1 0.88	1 1.14	1 0.90	1 1.60	1 1.14	1 0.67
No.	17	18#	19	20	21#	22#	23#	24#
Fold	1 1.16	1 0.97	1 0.92	1 0.84	1 1.05	1 1.26	1 1.37	1 0.33
No.	25	26#	27#	28	29	30#	31#	32#
Fold	1 0.90	1 1.65	1 1.07	1 1.02	1 1.37	1 0.90	1 0.86	1 0.86
No.	33#	34#	35#	36#	37	38#	39#	40
Fold	1 1.15	1 1.10	1 0.91	1 0.59	1 1.34	1 0.79	1 0.86	1 0.93

F

No.	1#	2#	3#	4#	5#	6#	7#	8
Fold	1 0.85	1 1.03	1 1.21	1 1.22	1 1.53	1 0.93	1 1.29	1 1.41
No.	9#	10#	11#	12	13	14#	15#	16#
Fold	1 1.92	1 0.68	1 1.05	1 1.11	1 0.98	1 1.59	1 1.35	1 1.19
No.	17	18#	19	20	21#	22#	23#	24#
Fold	1 1.29	1 1.67	1 1.25	1 1.42	1 1.06	1 1.04	1 0.42	1 0.97
No.	25	26#	27#	28	29	30#	31#	32#
Fold	1 0.91	1 0.95	1 0.80	1 1.04	1 1.73	1 0.67	1 1.65	1 1.09
No.	33#	34#	35#	36#	37	38#	39#	40
Fold	1 0.74	1 0.75	1 1.45	1 0.37	1 1.37	1 0.45	1 1.54	1 0.83

Figure 2 Immunoblot analysis of factors affecting cell death. A: Immunoblot analysis of iNOS. 1: Significantly higher concentrations of iNOS in cancer tissues compared with adjacent non-cancer tissues. 2: Significantly higher concentrations of iNOS in cancer tissues of patients with and without *H pylori* infection. 3: Significantly higher iNOS concentrations in stage 3 and 4 patients. #=*H pylori* infected, [a]*P*<0.05, [b]*P*<0.01, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. B: Immunoblot analysis of NFκB. 1: Significantly higher concentrations of NFκB in cancer tissues in comparison with adjacent non-cancer tissues. 2: Significantly higher concentrations of NFκB in cancer tissues of patients with and without *H pylori* infection. 3: Significantly higher NFκB concentrations in stage 3 and 4 patients #=*H pylori* infected, *P*<0.05, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. C: Immunoblot analysis of MEKK1. 1: Concentrations of MEKK1 in cancer tissues and adjacent non-cancer tissues. 2: Significantly higher concentrations of MEKK1 in cancer tissues of patients without *H pylori* infection. 3: No differences in MEKK1 concentrations in cancer tissues with different stages. #= *H pylori* infected, [a]*P*<0.05, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. D: Immunoblot analysis of Caspase 3. 1: Similar concentrations of Caspase 3 in cancer tissues and adjacent non-cancer tissues. 2: Similar concentrations of Caspase 3 in cancer tissues of patients with and without *H pylori* infection. 3: Significantly lower Caspase 3 concentrations in stage 1 and 2 patients. #=*H pylori* infected, [b]*P*<0.01, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. E: Immunoblot analysis of Bcl-2. 1: Similar concentrations of Bcl-2 in cancer tissues and adjacent non-caner tissues. 2: No differences in concentrations of Bcl-2 in cancer tissues of patients with and without *H pylori* infection. 3: Significantly higher Bcl-2 concentrations in stage 1 and 2 patients. #=*H pylori* infected, [b]*P*<0.01, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. F: Immunoblot analysis of Mcl-1. 1: Significantly higher concentrations of Mcl-1 in cancer tissues in comparison with adjacent non-cancer tissues. 2: Significantly higher concentration of Mcl-1 in cancer tissues of patients with and without *H pylori* infection. 3: Significant higher Mcl-1 concentrations in stage 3 and 4 patients than #=*H pylori* infected, [a]*P*<0.05, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4. G: Immunoblot analysis of IAP. 1: Significantly higher concentrations of IAP in cancer tissues compared with adjacent non-cancer tissues, 2: Significantly higher concentrations of IAP in cancer tissues with *H pylori* infection, 3: Significantly higher IAP concentrations in stage 3 and 4 patients #=*H pylori* infected, [a]*P*<0.05, N: non-cancer tissue, C: cancer tissue, Hp(-): without *H pylori* infection, Hp(+): with *H pylori* infection, 1&2: gastric cancer stages 1 and 2, 3&4: gastric cancer stages 3 and 4.

NFκB has been found to be a critical regulator of genes involved in inflammation, cell proliferation, and apoptosis[30]. Recent studies suggested that NFκB might play a critical role in protecting cells against apoptosis[31,32]. The magnitude of the stimulus and the cell type involved may determine whether NFκB leads to cell survival or cell death. Maeda *et al*. reported cag PAI positive *H pylori* was capable of inducing apoptotic effects mainly through the mitochondrial pathway. Antiapoptotic effects mediated by NFκB activation were also observed by Maeda *et al*[33]. In this study, we revealed an

increase in the concentrations of NFκB in cancer tissues. In addition, the increase was significantly higher in patients infected with *H pylori* as well as in patients with advanced gastric cancer compared with *H pylori*-negative patients and low stage gastric cancer patients, respectively.

Recent investigations implicated mitogen-activated protein kinases (MAPK) as additional mediators of *H pylori*-dependent NFκB activation and IL-8 expression. Studies have demonstrated the presence of cross-talk between the MAPK and NFκB pathways[21-34]. MAPK cascades are signal transduction networks that target transcription factors and thus participate in a diverse array of cellular functions including cytokine production. In this study, there was an increase in the concentrations of MEKK1, a member of the MAPK signaling cascade in cancer tissues. However, the increase was not significant in all the studied patient groups.

Kanai *et al*. noted that transforming growth factor-α played an anti-apoptotic role in gastric mucosal cells by enhancing the expression of Bcl-2 family proteins via an NFκB-dependent pathway[35] NFκB is now known to upregulate transcription of several anti-apoptotic genes, including a cellular inhibitor of apoptosis, a Bcl-2 homolog, and cyclooxygenase-2, as well as pro-apoptotic genes, such as bax and p53. Tumor cells can have abnormal expression levels of factors such as Bcl-2 family proteins that slow the progression of apoptosis and elevate NFκB-regulated transcription, which can inhibit TNF-α-induced apoptosis. However, in this study, no significant changes in the concentrations of Bcl-2 in cancer tissues or patients infected with *H pylori* were noted. Furthermore, upregulation of anti-apoptotic gene mcl-1 might play a role in interleukin-6-mediated protection of gastric cancer cells from the apoptosis induced by hydrogen peroxide[36]. The anti-apoptotic role played by NFκB also involves the ability of this transcriptional factor to induce expression of genes that promote cell survival, such as the genes coding for TRAF1, TRAF2, and the cellular inhibitors of apoptosis 1 and 2 (c-IAP1, c-IAP2)[37]. In this study, we demonstrated an increase in the concentrations of IAP and Mcl-1, anti-apoptosis related proteins in cancer tissues. Furthermore, the increase was significantly higher in patients infected with *H pylori* as well as in patients with advanced gastric cancer, compared with *H pylori*-negative and non-advanced gastric patients, respectively. Therefore, enhanced antiapoptosis-related protein expression may contribute to disease progression.

Caspase3 is a member of the cell death effector (CED)-3 family, which is involved in the induction of apoptosis. Hoshi *et al*. reported Caspase 3 was involved in the development or regulation of apoptotic cell death in cell turnover of normal and neoplastic mucosa of the human stomach[38]. These results indicate that gastric adenocarcinoma is associated with an inhibition of apoptosis and the augmentation of proliferative activity of tumor cells when compared to non-neoplastic gastric mucosa. However, in this study, there was no difference of increase in Caspase 3 between patients with or without *H pylori* infection or patients with or without advanced gastric cancer.

In conclusion, chronic *H pylori*-induced iNOS expression and subsequent DNA damage as well as signal transduction appear to make an important contribution to *H pylori*-related gastric carcinogenesis. This sequence of events found in this study supports the hypothesis that oxygen-free radical-mediated damage induced by *H pylori* plays a vital role in the development of gastric carcinoma in patients with chronic gastritis.

ACKNOWLEDGEMENT

REFERENCES

1 **Wang KX,** Wang XF, Peng JL, Cui YB, Wang J, Li CP. Detection of serum anti-Helicobacter pylori immunoglobulin G in patients with different digestive malignant tumors. *World J Gastroenterol* 2003; **9**: 2501-2504

2 **Nomura A,** Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* 1991; **325**: 1132-1136

3 **Parsonnet J,** Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131

4 **Van Antwerp DJ,** Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-α induced apoptosis by NF-κB. *Science* 1996; **274**: 787–789

5 **Correa P,** Ruiz B, Shi TY, Janney A, Sobhan M, Torrado J, Hunter F. *Helicobacter pylori* and nucleolar organizer regions in the gastric antral mucosa. *Am J Clin Pathol* 1994; **101**: 656-660

6 **Fraser AG,** Sim R, Sankey EA. Effect of eradication of *Helicobacter pylori* on gastric epithelial cell proliferation. *Aliment Pharmacol Ther* 1994; **8**: 167-173

7 **Lynch DA,** Mapstone NP, Clarke AM, Sobala GM, Jackson P, Morrison L, Dixon MF, Quirke P, Axon AT. Cell proliferation in *Helicobacter pylori* associated gastritis and the effect of eradication therapy. *Gut* 1995; **36**: 346-350

8 **Fox JG,** Dangler CA, Taylor NS, King A, Koh TJ, Wang TC. High-salt diet induces gastric epithelial hyperplasia and parietal cell loss, and enhances *Helicobacter pylori* colonization in C57BL/6 mice. *Cancer Res* 1999; **59**: 4823-4828

9 **Peek R,** Wirth HP, Moss SF, Yang M, Abdalla AM, Tham KT, Zhang T, Tang LH, Modlin IM, Blaser MJ. *Helicobacter pylori* alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils. *Gastroenterology* 2000; **118**: 48-59

10 **Israel DA,** Salama N, Arnold CN, Moss SF, Ando T, Wirth HP, Tham KT, Camorlinga M, Blaser MJ, Falkow S, Peek RM Jr. *Helicobacter pylori* strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. *J Clin Invest* 2001; **107**: 611-620

11 **Moss SF,** Calam J, Agarwal B, Wang S, Holt PR. Induction of gastric epithelial apoptosis by *Helicobacter pylori*. *Gut* 1996; **38**: 498-501

12 **Mannick EE,** Bravo LE, Zarama G, Realpe JL, Zhang XJ, Ruiz B, Fontham ET, Mera R, Miller MJ, Correa P. Inducible nitric oxide synthase, nitrotyrosine and apoptosis in *Helicobacter pylori* gastritis: effects of antibiotics and antioxidants. *Cancer Res* 1996; **56**: 3238-3243

13 **Jones NL,** Shannon PT, Cutz E, Yeger H, Sherman PM. Increase in proliferation and apoptosis of gastric epithelial cells early in the natural history of *Helicobacter pylori* infection. *Am J Pathol* 1997; **151**: 1695-1703

14 **Rudi J,** Kuck D, Strand S, von Herbay A, Mariani SM, Krammer PH, Galle PR, Stremmel W. Involvement of the CD95 (APO-1/Fas) Receptor and Ligand System in *Helicobacter pylori*-induced Gastric Epithelial Apoptosis. *J Clin Invest* 1998; **102**: 1506-1514

15 **Anti M,** Armuzzi A, Iascone 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. *Ita J Gastroenterol Hepatol* 1998; **30**: 153-159

16 **Hirasawa R,** Tatsuta M, Iishi H, Yano H, Baba M, Uedo N, Sakai N. Increase in apoptosis and decrease in ornithine decarboxylase activity of the gastric mucosa in patients with atrophic gastritis and gastric ulcer after successful eradication of *Helicobacter pylori*. *Am J Gastroenterol* 1999; **94**: 2398-2402

17 International Union Against Cancer; Sobin L and Wittekind C, eds. TNM Classification of Malignant Tumors. 6th ed. New York, USA: *Wiley-Liss* 2002: 65-68

18 **Dahlhaus M,** Appel KE. N-Nitrosodimethylamine, N-nitrosodiethylamine, and N-nitrosomorpholine fail to generate 8-hydroxy-2'-deoxyguanosine in liver DNA of male F344 rats. *Mutat Res* 1993; **285**: 295–302

19 **Shen HM,** Ong CN, Lee BL, Shi CY. Aflatoxin B1-induced 8-hydroxydeoxyguanosine formation in rat hepatic DNA. *Car-*

cinogenesis 1995; **16**: 419-422

20 **Yermilov V,** Rubio J, Becchi M, Friesen MD, Pignatelli B, Ohshima H. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro. Carcinogenesis* 1995; **16**: 2045–2050

21 **Mercurio F,** Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NFκB activation. *Science* 1997; **278**: 860–866

22 **Yu J,** Russell RM, Salomon RN, Murphy JC, Palley LS, Fox JG. Effect of *Helicobacter mustelae* infection on ferret gastric epithelial cell proliferation. *Carcinogenesis* 1995; **16**: 1927-1931

23 **Peek RM Jr**, Moss SF, Tham KT, Perez-Perez GI, Wang S, Miller GG, Atherton JC, Holt PR, Blaser MJ. *Helicobacter pylori* cagA[+] strains and dissociation of gastric epithelial cells proliferation from apoptosis. *J Natl Cancer Inst* 1997; **89**: 863-868

24 **Farinati F,** Cardin R, Degan P, Rugge M, Mario FD, Bonvicini P, Naccarato R. Oxidative DNA damage accumulation in gastric carcinogenesis. *Gut* 1998; **42**: 351-356

25 **Baik SC,** Youn HS, Chung MH, Lee WK, Cho MJ, Ko GH, Park CK, Kasai H, Rhee KH. Increased oxidative DNA damage in *Helicobacter pylori*-infected human gastric mucosa. *Cancer Res* 1996; **56**: 1279-1282

26 **Papa A,** Danese S, Sgambato A, Ardito R, Zannoni G, Rinelli A, Vecchio FM, Gentiloni-Silveri N, Cittadini A, Gasbarrini G, Gasbarrini A. Role of *Helicobacter pylori* CagA[+] infection in determining oxidative DNA damage in gastric mucosa. *Scand J Gastroenterol* 2002; **37**: 409-413

27 **Hahm KB,** Lee KJ, Kim JH, Cho SW, Chung MH. *Helicobacter pylori* infection, oxidative DNA damage, gastric carcinogenesis, and reversibility by rebamipide. *Dig Dis Sci* 1998; **43**(Suppl 9): 72S-77S

28 **Koh E,** Noh SH, Lee YD, Lee HY, Han JW, Lee HW, Hong S. Differential expression of nitric oxide synthase in human stomach cancer. *Cancer Lett* 1999; **146**: 173-180

29 **Tamir S,** Tannenbaum S. The role of nitric oxide in the carcinogenic process. *Biochim Biophys Acta* 1996; **1288**: F31-36

30 **Baldwin AS Jr**. The NF-κB and I κB proteins: new discoveries and insights. *Annu Rev Immunol* 1996; **14**: 649-683

31 **Wang CY,** Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science* 1996; **274**: 784-787

32 **Beg AA,** Baltimore D. An essential role for NF-κB in preventing TNFa-induced cell death. *Science* 1996; **274**: 782-784

33 **Maeda S,** Yoshida H, Mitsuno Y, Hirata Y, Ogura K, Shiratori Y, Omata M. Analysis of apoptotic and antiapoptotic signaling pathways induced by *Helicobacter pylori. Gut* 2002; **50**: 771-778

34 **Malinin NL,** Boldin MP, Kovalenko AV, Wallach D. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature* 1997; **385**: 540–544

35 **Dumont A,** Hehner SP, Hofmann TG, Ueffing M, Droge W, Schmitz ML. Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-κB. *Oncogene* 1999; **18**: 747-757

36 **Lin MT,** Juan CY, Chang KJ, Chen WJ, Kuo ML. IL-6 inhibits apoptosis and retains oxidative DNA lesions in human gastric cancer AGS cells through up-regulation of anti-apoptotic gene mcl-1. *Carcinogenesis* 2001; **22**: 1947-1953

37 **Wang CY,** Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998; **281**: 1680-1683

38 **Hoshi T,** Sasano H, Kato K, Yabuki N, Ohara S, Konno R, Asaki S, Toyota T, Tateno H, Nagura H. Immunohistochemistry of Caspase3/CPP32 in human stomach and its correlation with cell proliferation and apoptosis. *Anticancer Res* 1998; **18**: 4347-4353

Edited by Wang XL **Proofread by** Xu FM