

Host *TNF- α* –1031 and –863 Promoter Single Nucleotide Polymorphisms Determine the Risk of Benign Ulceration after *H. pylori* Infection

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- OBJECTIVES:** This study tested whether host genotypes of the tumor necrosis factor-alpha (*TNF- α*) promoter single nucleotide polymorphism (SNP) could determine clinical and histological outcomes after *Helicobacter pylori* infection.
- METHODS:** A total of 524 dyspeptic patients, 424 with and 100 without *H. pylori* infection, were checked for *TNF- α* promoter SNP over the locus on –1031(T/C), –863(C/A), –857(C/T), –806(C/T), and –308(G/A) by sequence-specific oligonucleotide probe. Each patient received panendoscopy to take gastric biopsy to detect *H. pylori* infection and its related histology using the updated Sydney's system. Gastric *TNF- α* expressions were stained by immunohistochemistry.
- RESULTS:** In *H. pylori*-infected patients, –1031C or –863A carriers of *TNF- α* promoter had more severe gastric neutrophil infiltration and *TNF- α* gastric staining than individuals with –1031TT or –863CC genotype, respectively ($p < 0.05$). The multivariate logistic regression verified both –1031C and –863A carriers were independent risk factors to have duodenal ulcers and gastric ulcer without IM in the *H. pylori*-infected hosts ($p < 0.05$). As compared to –863CC and –1031TT genotype combinations, the ulcer risk after *H. pylori* infection was 2.46 (95% CI: 1.32–4.59, $p \leq 0.00001$) for the carriers with either –1031C or –863A allele, and even elevated to 6.06 (95% CI: 3.57–10.21, $p \leq 0.00001$) for the individuals harboring both –863A and –1031C alleles. For patients with gastric ulcer, the 863CC genotype had a higher rate to have intestinal metaplasia than –863A carrier ($p \leq 0.005$).
- CONCLUSIONS:** *TNF- α* –1031 and –863 promoter SNP should be novel host factors to determine the gastric inflammation and risk of peptic ulceration upon *H. pylori* infection.

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INTRODUCTION

Helicobacter pylori infection is now recognized as closely related to peptic ulcer diseases and even gastric carcinogenesis (1, 2). Despite positive clinical evidence illustrating how the eradication of *H. pylori* improves ulcer healing and diminishes ulcer relapse (3, 4), the exact pathophysiology of *H. pylori* in ulcerogenesis warrants further investigation. While *H. pylori* infection often leads to chronic gastric inflammation, only some of those infected develop peptic ulcers. Therefore, there appear to be differences in the bacterial virulence factors in those with and those without peptic ulceration after *H. pylori* infection.

A more virulent triple-positive strain of *H. pylori*—positive for *cagA*, *vacAs1*, and *babA2*—increases the risk of ulcers and cancer in western countries (5–7). In Asian countries with a high prevalence of triple-positive *H. pylori*, bacterial factors

have limited value for determining disease outcomes as either peptic ulcers or cancers (8–12). Therefore, it is worth determining whether there could be any host genetic predispositions or consequences of immunological responses related to the different outcomes after *H. pylori* infection in Taiwan, where nearly 100% of the *H. pylori* isolates have been triple-positive (8–10).

A hallmark of *H. pylori*-triggered mucosal inflammation is the continuous recruitment of neutrophils and mononuclear cells to the gastric lamina propria (1–3). This process is thought to be induced by inflammatory cytokines, tumor necrosis factor-alpha (*TNF- α*), for example, after *H. pylori* infection (13, 14). *TNF- α* may then play a crucial role in the host immunological defense against the infection, because a high concentration of *TNF- α* is also closely related to the damage of gastric epithelium (13–15). The production of *TNF- α* may be regulated at the transcriptional,

Table 1. Oligonucleotide Probes and Competitors Used in SSOP Experiments

SNP Loci	Probes	Competitors
-1031 loci		
T allele:	5'-TGAGAAGAT <u>T</u> GAAGGAAA	5'-TGAGAAGAC <u>C</u> GAAGGAAA
C allele:	5'-TGAGAAGA <u>C</u> GAAGGAAA	5'-TGAGAAGAT <u>T</u> GAAGGAAA
-863 loci		
C allele:	5'-TGGGGACCCCC <u>C</u> CTTAA	5'-TGGGGACCCCC <u>A</u> CTTAA
A allele:	5'-TGGGGACCCCC <u>A</u> CTTAA	5'-TGGGGACCCCC <u>C</u> CTTAA
-857 loci		
C allele:	5'-CTTAAC <u>C</u> GAAGACAGGGC	5'-CTTAAT <u>T</u> GAAGACAGGGC
T allele:	5'-CTTAAT <u>T</u> GAAGACAGGGC	5'-CTTAAC <u>C</u> GAAGACAGGGC
-806 loci		
C allele:	5'-TCCAGGAC <u>C</u> TCCAGGTA	5'-TCCAGGAC <u>T</u> TCCAGGTA
T allele:	5'-TCCAGGAC <u>T</u> TCCAGGTA	5'-TCCAGGAC <u>C</u> TCCAGGTA
-308 loci		
G allele:	5'-AGGGGCATG <u>G</u> GGACGGG	5'-AGGGGCATG <u>A</u> GGACGGG
A allele:	5'-AGGGGCATG <u>A</u> GGACGGG	5'-AGGGGCATG <u>G</u> GGACGGG

The underlined bold capital letters in the nucleotide sequence indicated the specific site as having single nucleotide polymorphism (SNP) on the different loci of the *TNF-α* promoter.

posttranscriptional, and translational levels (16–19). Several biallelic polymorphisms have been described within the *TNF-α* promoter region upstream of the transcriptional start site: positions -238, -308, -857, -863, and -1031 (20–22). There are striking individual differences in the ability to produce cytokines with genetic polymorphisms within the regulatory regions (19, 23, 24). It is thus logical to see whether polymorphisms in the *TNF-α* promoter region affect *TNF-α* levels in gastric tissue or affect the gastric histopathological change upon *H. pylori* infection.

Unlike the -238 locus, the *TNF-α* -308 promoter polymorphism is closely related to *cagA*-positive *H. pylori* infection, and to an increase in the risk of atrophic gastritis, peptic ulceration, and even gastric cancer (20, 25). The role of the other novel positions of *TNF-α* promoter should be promising tested in the patients with *H. pylori* infection. Moreover, there is no available data that directly show the difference of tissue *TNF-α* levels in gastric mucosa epithelium between patients with different polymorphisms in the *TNF-α* promoter region after *H. pylori* infection. In this study, therefore, we enrolled dyspeptic patients to examine whether the genotypes defined by *TNF-α* promoter single nucleotide polymorphism

(SNP) at -308, -806, -857, -863, and -1031 determine the histopathologic changes and clinical course of *H. pylori* infection in Taiwan. This study is, to the best of our knowledge, the first one to demonstrate novel *TNF-α* promoter SNP, other than -308 locus, and is closely associated with the risk of peptic ulceration in an endemic area with nearly 100% of *H. pylori* infections triple-positive for *cagA*, *vacA* *sl*, and *babA2*.

MATERIALS AND METHODS

Patients and Study Design

After obtaining informed consent, each of the 524 enrolled dyspeptic subjects underwent panendoscopy to obtain biopsy tissue for histology studies and *H. pylori* cultures. Five gastric biopsy specimens, two from the antrum, two from the corpus, and one from the cardia, were obtained during endoscopy (26). The specimen from each of the three sites was stained with hematoxylin-eosin and modified Giemsa stains. The remaining antrum and corpus specimens were used for *H. pylori* cultures (8, 10). *H. pylori* infection was defined as a positive result of histology or culture. None of the 524 subjects was

Table 2. Demographic Background, Endoscopic Diagnosis, and Genotype Distribution of *TNF-α* Promoter SNP in the Study Patients

Parameters	Without <i>H. pylori</i> Infection (N = 100)	With <i>H. pylori</i> Infection (N = 424)	<i>p</i> -Value
Age (yr)	46.8 ± 15.1	46.9 ± 16.4	NS
Gender (Female:Male)	46:54	209:215	NS
Endoscopic diagnosis (n)			
DU:GU:Gastritis	5:6:89	90:70:264	<0.0001
<i>TNF-α</i> ; genotypes n (%)			
308 loci (GG:GA:AA) ^a	84 (84):15 (15):1 (1)	351 (85):59 (14.3):3 (0.7)	NS
806 loci (CC:CT:TT) ^b	98 (98): 2 (2): 0 (0)	404 (97.8): 9 (2.1): 0 (0)	NS
857 loci (CC:CT:TT) ^c	70 (70):26 (26):4 (4)	315 (74.8):100 (23.8):6 (1.4)	NS
863 loci (CC:CA:AA) ^d	74 (74):23 (23):3 (3)	293 (69.3):118 (27.9):12 (2.8)	NS
1031 loci (TT: TC:CC) ^e	78 (78):20 (20):2 (2)	309 (73.4):107 (25.4):5 (1.2)	NS

DU: duodenal ulcer; GU: gastric ulcer. Gastritis indicates absence of ulcer during panendoscopy. *p*-Value indicates difference between patients with and without *H. pylori* infection. NS = not significant.

^{a,b,c,d,e} indicated there were 11, 11, 3, 1, and 3 DNA samples of patients with *H. pylori* infection that could not be amplified for the genotypes of -308, -806, -857, -863, and -1031 loci of *TNF-α* promoter SNP, respectively.

Table 3. Association of *TNF- α* Promoter SNP to the *H. pylori*-related Histology of the 264 Patients without Peptic Ulcer

<i>TNF-α</i> Genotypes	Dense AIG (%)	Severe CIG (%)	AT (%)	IM (%)
-308GG (n = 216)	67 (31)	109 (50.5)	112 (51.8)	29 (13.4)
A carrier (n = 44)	20 (45.5)	28 (63.6)	20 (45.5)	9 (20.5)
OR [95% CI]	1.85 [0.96–3.59]	1.72 [0.88–3.36]	0.92 [0.65–2.41]	1.54 [0.71–3.31]
-806CC (n = 253)	85 (33.6)	132 (52.2)	123 (48.6)	47 (18.6)
T carrier (n = 7)	2 (28.6)	5 (71.4)	4 (5.7)	2 (28.6)
OR [95% CI]	0.79 [0.15–4.16]	2.29 [0.44–12.03]	1.41 [0.31–6.43]	1.73 [0.33–9.18]
-857CC5 (n = 188)	65 (34.6)	98 (52.1)	97 (51.6)	37 (19.7)
T carrier (n = 73)	22 (30.1)	39 (53.4)	30 (41.1)	12 (16.4)
OR [95% CI]	0.82 [0.46–1.46]	1.05 [0.61–1.81]	0.66 [0.38–1.13]	0.81 [0.39–1.65]
-863CC (n = 214)	64 (29.9)	115 (53.7)	111 (51.9)	40 (18.7)
A carrier (n = 47)	23 (48.9)	22 (46.8)	20 (42.5)	9 (19.1)
OR [95% CI]	2.25 [1.18–4.27]*	0.76 [0.41–1.43]	0.73 [0.36–1.96]	1.08 [0.48–2.42]
-1031TT (n = 218)	67 (30.7)	115 (52.8)	113 (51.8)	39 (17.9)
C carrier (n = 42)	20 (47.6)	22 (52.4)	19 (45.2)	10 (23.8)
OR [95% CI]	2.05 [1.05–4.01]*	0.99 [0.51–1.91]	0.86 [0.61–1.74]	1.52 [0.69–3.77]

Dense AIG: total acute inflammation score ≥ 6 ; severe CIG: total chronic inflammation score ≥ 6 ; AT: antral atrophy; IM: intestinal metaplasia. OR: the relative risk of the carrier, compared to the major corresponding homozygote of *TNF- α* promoter, to have such a histologic feature or ulcer.

* $p < 0.05$. There were 4, 4, 3, 3, and 4 patients with either DNA could not be amplified for the genotypes of -308, -806, -857, -863, and -1031 loci of *TNF- α* promoter SNP or lacking the pathology data, respectively.

long-term aspirin or NSAID users. As well, none of the 524 enrolled patients had previous history of receiving anti-*H. pylori* therapy. Subjects should refrain from taking aspirin and NSAID medications at least 2 wk before gastroscopy. Subjects who were identified with malignant gastric ulcer or other gastric malignancy were excluded. The blood sample of each patient was also checked for the *TNF- α* promoter SNP over the locus on -1031 (T/C), -863 (C/A), -857 (C/T), -806 (C/T), and -308 (G/A) by sequence-specific oligonucleotide probe (SSOP). For some selected patients with different genotypes of the *TNF- α* promoter, the antral and corpus specimens were immunohistochemically graded for *TNF- α* expression.

Genotyping of *TNF- α* Promoter SNP by SSOP

The DNA was extracted from the peripheral blood mononuclear cell (27). Some modifications were made, including the isolated nuclei digested with proteinase K, and extracted with a commercial blood and tissue genomic DNA extraction miniprep system according to the manufacturer's directions (Viogene, Taiwan). The SNPs at nucleotide loci on -1031

(T/C), -863 (C/A), -857 (C/T), -806 (C/T), and -308 (G/A) were determined by SSOP, modified from the procedures described previously (23). A 1279-bp DNA fragment for the 5'-flanking region of the *TNF- α* gene at positions -1094 to -185 was amplified by PCR, using a sense primer (5' ₋₁₀₉₄GATGGACTCACCAGGTGAG₋₁₀₇₆) and antisense primer (5' ₁₈₅CTCATGGTGTCTTTCCAGG₁₆₆). One volume of PCR product was denatured at 25°C by adding five volumes of 0.4 N NaOH, 25 mM EDTA for 10 min. Subsequently, 50 μ l of PCR product was spotted on a positively charged nylon membrane, Hybond-N⁺ (Amersham Pharmacia Biotech, Buckinghamshire, England) using a 96-well blotter (SRC 96D Minifold I; Schleicher & Schull, Dassel, Germany). The DNA was fixed to the membrane by UV-cross linking using a UV Stratalinker 1800 (Stratagene CA, USA). Each membrane was incubated for 30 min with 10 ml of blocking solution A (4 \times SSPE, 0.1% lauroylsarcosine, and 1% blocking solution) (GmbH Roche Molecular Biochemicals, Mannheim, Germany) before prehybridization in 10 ml of DIG EASY HYB solution (GmbH Roche) for 1 h at 42°C. Each membrane was hybridized for 2 h at 42°C in DIG

Table 4. The Distributions of the Different *TNF- α* Promoter SNP among Patients with Duodenal Ulcer, Gastric Ulcer, and Non-Ulcer Patients with *H. pylori* Infection

N (%) <i>TNF-α</i> Genotypes	Duodenal Ulcer (N = 90)	Gastric Ulcer (N = 70)	Non-ulcer Patients (N = 264)	p^{\dagger} Value
-308GG:A carrier ^a	71 (82.6):15 (17.4)	60 (89.5) : 7 (10.5)	220 (84.6) : 40 (15.4)	NS
-806CC:T carrier ^b	84 (98.8) : 1 (1.2)	67 (98.5) : 1 (1.5)	253 (96.6) : 7 (3.4)	NS
-857CC:T carrier ^c	65 (71.9) : 25 (28.1)	59 (86.4) : 11 (15.6)	188 (72) : 73 (28)	NS
-863CC:A carrier ^d	51 (56.7) : 39 (43.3)	28 (40) : 42 (60)	216 (82.1) : 47 (17.9)	<0.001
-1031TT:C carrier ^e	54 (60) : 36 (40)	36 (51.4) : 34 (48.6)	219 (84) : 42 (16)	<0.001

^{a,b,c,d,e}Indicate there were 11 (4 in duodenal ulcer, 3 in gastric ulcer, and 4 in non-ulcer patients), 11 (5 in duodenal ulcer, 3 in gastric ulcer, and 4 in non-ulcer patients), 3 (3 in non-ulcer patients), 1 (non-ulcer patient), 4 (non-ulcer patients) DNA samples of patients with *H. pylori* infection that could not be amplified for the genotypes of -308, -806, -857, -863, and -1031 loci of *TNF- α* promoter SNP, respectively.

[†]Indicates significant difference of the distribution of *TNF- α* genotypes among the patients with duodenal ulcer, gastric ulcer, and non-ulcer (by χ^2 test). Moreover, the "gray area" indicated there was a significantly higher frequency of -863A carrier in patients with gastric ulcer than in duodenal ulcer (60% vs 43.3%, $p < 0.05$ by 2-tailed Fisher's exact test, odds ratio [95% confidence interval, CI]: 1.96 [1.04–3.69]).

Table 5. The Demographic Background, Histological Features, and Association with -1031C Carrier of the *H. pylori*-positive Gastric Ulcer with Different -863 Genotypes of *TNF-α* Promoter SNP

<i>TNF-α</i> genotype Parameter	Gastric Ulcer			Duodenal Ulcer		
	-863CC (n = 28)	-863A carrier (n = 42)	<i>p</i> -Value	-863CC (n = 51)	-863A carrier (n = 39)	<i>p</i> -Value
Mean age (yr)	44.1	46.5	NS	47.4	48.2	NS
Sex (F:M)	13:15	26:16	NS	24:27	22:17	NS
Location of ulcer (Antrum:Corpus)	17:11	40:2	<0.001	–	–	–
Dense AIG N(%)	5 (17.9)*	30 (71.4)	<0.005	25 (49.1)	27 (69.3)	<0.05
Severe CIG N(%)	14 (50)	30 (71.4)	<0.01	29 (56.9)	23 (59)	NS
AT (%)	21 (75)*	18 (42.9)	<0.05	21 (41.8)	14 (35.9)	NS
IM (%)	14 (50)*	6 (14.2)	<0.005	6 (12)	5 (12.8)	NS
-1031C carrier (%)	4 (13.3)	30 (71.4)	<0.001	4 (7.8)	32 (82.1)	<0.001

Dense AIG: total acute inflammation score ≥6; severe CIG: total chronic inflammation score ≥6; AT: antral atrophy; IM: intestinal metaplasia.

*This indicated such parameters (including dense AIG, AT, and IM) of the gastric ulcer patients with -863CC of *TNF-α* genotype were significantly different from those of the duodenal ulcer patients with both -863CC and -863A carrier (*p* < 0.05, by χ^2 test).

EASY HYB solution containing 2 pmol/ml of DIG-labeled SSO probes (Table 1) and a predetermined concentration of competitor. After hybridization, the membrane was washed twice in 2 × SSPE-0.1% SDS at room temperature for 5 min and then washed in 10 ml washing solution (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 2 mM EDTA, 3 M TMACl, and 5 × Denhardt’s solution) at 46°C for 15 min. The subsequent immunological and enzymatic detection was the same as previously described (28). The genotypes of promoter polymorphism in each site were confirmed by direct sequencing of PCR products from 37 randomly selected patients using the ABI Prism 377 automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

Analysis of *H. pylori*-Related Histology

The same pathologist, blinded to the endoscopic findings and host genotypes of *TNF-α* promoter SNP, examined the gastric histology. The following features were scored using the updated Sydney System: acute inflammation (range, 0–3), chronic inflammation (range, 0–3), atrophic change (absence: 0; presence: 1), and intestinal metaplasia (IM; absence: 0,

presence: 1) (29). The total acute (AIS) and chronic (CIS) inflammation scores were a sum of the scores from the antrum and corpus (range: 0–6). Based on histology AIS, the patients were divided into either a dense (AIS ≥ 4) or loose (AIS < 4) neutrophil infiltration group (AIG). Also, by selecting a cut-off of 6 for CIS, patients were defined into a mild (CIS < 4) or severe (CIS ≥ 4) chronic inflammation group (CIG). Antral atrophy (AT) was defined as the presence of atrophy only in the antrum (score 1 in the antrum, but 0 in the corpus and cardia).

Immunohistochemistry for the Gastric Expression of *TNF-α*

Tissue immunohistochemical staining was performed using monoclonal antibodies of human *TNF-α* (mouse IgG1κ; BMS154; Bender MedSystem Diagnostics, Vienna, Austria) and an HRP polymer detection method (PicTure-Plus Kit (Mouse-DAB); Zymed Laboratories, Inc., South San Francisco, CA). Formalin-fixed paraffin-embedded tissues were deparaffinized and immersed in 3% peroxide in methanol to stop the endogenous peroxidase activity. Nonspecific binding

Table 6. Multivariate Logistic Regression for the Independent Factors Relevant to the Presence of Duodenal Ulcer or Gastric Ulcer in the Patients with *H. pylori* Infection

Parameters	Coefficient	Standard Error	<i>p</i> -Value	95% Confidence Interval
Duodenal and gastric ulcer without IM				
<i>TNF-α</i> -863A carrier	0.352	0.118	0.003*	(0.124–0.589)
<i>TNF-α</i> -1031C carrier	0.231	0.117	0.045*	(0.089–0.331)
Age	-0.001	0.003	0.845	(-0.007–0.005)
Sex as female	0.078	0.092	0.401	(-0.103–0.259)
Dense AIG	-0.042	0.099	0.789	(-0.198–0.151)
Gastric ulcer with IM				
<i>TNF-α</i> -863A carrier	0.012	0.049	0.807	(-0.085–0.111)
<i>TNF-α</i> -1031C carrier	0.121	0.059	0.016*	(0.023–0.219)
Age	-0.001	0.001	0.331	(-0.003–0.001)
Sex as female	0.068	0.081	0.627	(-0.053–0.226)
Dense AIG	0.024	0.089	0.556	(-0.048–0.089)

IM = intestinal metaplasia; dense AIG = total acute inflammation score ≥6.

*Indicates significant difference. The multiple logistic regression was conducted to determine the independent factors to get peptic ulcer, and the *H. pylori*-infected non-ulcer patients were served as controls.

Table 7. The Odds Ratio of the Different Genotype Combinations in the -863 and -1031 of *TNF- α* Promoter SNP to Have Duodenal Ulcer or Gastric Ulcer without Intestinal Metaplasia After *H. pylori* Infection

<i>TNF-α</i> Halotypes (n)	-1031 TT and -863 CC	-1031C or -863A Carrier	-1031 C and -863A carrier
DU and GU without IM (N = 140)	62	22	56
Non-ulcer control (N = 260)*	201	29	30
Odds ratio (95% CI)	1	2.46 (1.32–4.59)	6.06 (3.57–10.21)

DU = duodenal ulcer; GU = gastric ulcer; IM = intestinal metaplasia.

*There were 4 non-ulcer patients lacking of DNA samples to be amplified for the genotypes of either -863 or -1031 loci of *TNF- α* promoter SNP, and thus only 260 patients without ulcer can be analyzed for the genotype combination.

sites were saturated with 0.3% bovine serum albumin. Tissue sections were treated with primary antibody against TNF- α at a dilution of 1:40 at room temperature for 1 h. HRP/Fab polymer conjugate (PicTure-Plus Kit (Mouse-DAB); Zymed Laboratories) was adapted for staining by the manufacturer's instructions. 3-Amino-9-ethylcarbazole was selected as the chromogen. Sections were counterstained with hematoxylin. Non-immune mouse IgG was substituted for the primary antibody to serve as a negative control. The TNF- α^+ cells were calculated by direct counting under a 40 \times objective lens for the 1 mM scheduled mucosal area. Epithelial cells and mononuclear cells of the lamina propria were counted and recorded separately. The TNF- α^+ grades were as follows: grade 0, ≤ 30 cells; grade 1, 31–100 cells, grade 2, 101–300 cells; grade 3, 301–500 cells; and grade 4, >500 cells with positive staining. The other investigator, blinded to the host genotypes of *TNF- α* promoter, graded the TNF- α expression in gastric tissue.

Statistics

Hardy-Weinberg equilibrium was tested among the non-*H. pylori*-infected controls and among the group of *H. pylori*-infected subjects without peptic ulcer to confirm the control as suitable. The Student's *t*-test and a one-way analysis of variance (ANOVA) model with Bonferroni corrections were applied for parametric differences as appropriate. Pearson's χ^2 test was used for nonparametric differences. The difference of the TNF- α genotypes among patients with duodenal ulcer, gastric ulcer, and non-ulcer controls was also assessed by Pearson's χ^2 test (Table 3). Fisher's exact test was used to verify whether different TNF- α genotypes have different histological parameters (Table 3) and whether different TNF- α genotype combinations varied in the peptic ulcer rates (Table 7). The odds ratio (OR) and 95% confidence interval (95% CI) were calculated. The multiple stepwise logistic regressions were applied to determine the independent risk factor related with the presence of gastroduodenal ulcers with enrolling age and sex as adjustment to prevent confounding bias. All tests were 2-tailed with statistical significance setting at the level of $p < 0.05$.

RESULTS

H. pylori Infection among Different Genotypes of *TNF- α* Promoter SNP

A total of 524 dyspeptic patients, 424 with and 100 without *H. pylori* infections, were analyzed. There were no differences

in the age, sex, or genotype frequency distribution of *TNF- α* promoter SNP between patients with and without *H. pylori* infection (Table 2). The rate of the benign gastroduodenal ulcers was significantly higher in the patients with *H. pylori*-infection than in the patients without *H. pylori*-infection ($p < 0.0001$).

Specific *H. pylori*-related Histology Associated with *TNF- α* Promoter SNP

In Table 3, the correlation between *TNF- α* promoter SNP and specific *H. pylori*-related histological pattern was listed for those 264 patients without peptic ulceration. No loci of *TNF- α* promoter SNP were related to the CIG, AT, or IM in *H. pylori*-infected patients (Table 3). Two loci (-1031 and -863) of *TNF- α* promoter SNP were correlated with a high rate of dense neutrophil infiltration (dense AIG). Patients who were C carriers of the -1031 locus had a significantly higher rate of dense AIG than those with the -1031 TT genotype ($p < 0.05$, OR: 2.05; 95% CI: 1.05–4.01). In addition, patients who were A carrier at the -863 locus had a higher rate of dense AIG than those homozygous for the -863C allele ($p < 0.05$, OR: 2.25; 95% CI: 1.18–4.27).

TNF- α Promoter SNP and Peptic Ulcer Risk

In this study, there were 90 *H. pylori*-infected patients with duodenal ulcers and 70 with gastric ulcer. Excluding those patients whose DNA could not be determined, the distribution of genotypes at the -308, -806, and -857 loci of *TNF- α* promoter were similar among the *H. pylori*-infected patients with duodenal ulcer, gastric ulcer, and the non-ulcer gastritis control (Table 4). In contrast, also in Table 4, there were significant higher genotype frequencies of -863A carrier and -1031C carrier in the patients with either duodenal ulcer or gastric ulcer than in the non-ulcer patients ($p < 0.001$). Moreover, the patients with gastric ulcer had a higher genotype frequency of -863A carrier than the patients with duodenal ulcer (60% vs 43.3%, $p < 0.05$).

Based on the difference in the -863 genotype of *TNF- α* , the difference of demographic and histological features of gastric ulcer and duodenal ulcer was compared in Table 5. For both duodenal and gastric ulcer patients, the -863 A carrier had a higher rate of dense AIG ($p < 0.05$) than those of patients with genotype as -863CC. Moreover, the -863 A allele had a linkage disequilibrium with -1031 C allele ($p < 0.001$). Also in Table 5, the gastric ulcer patients with -863CC genotype had a higher rate of AT ($p < 0.05$) and IM

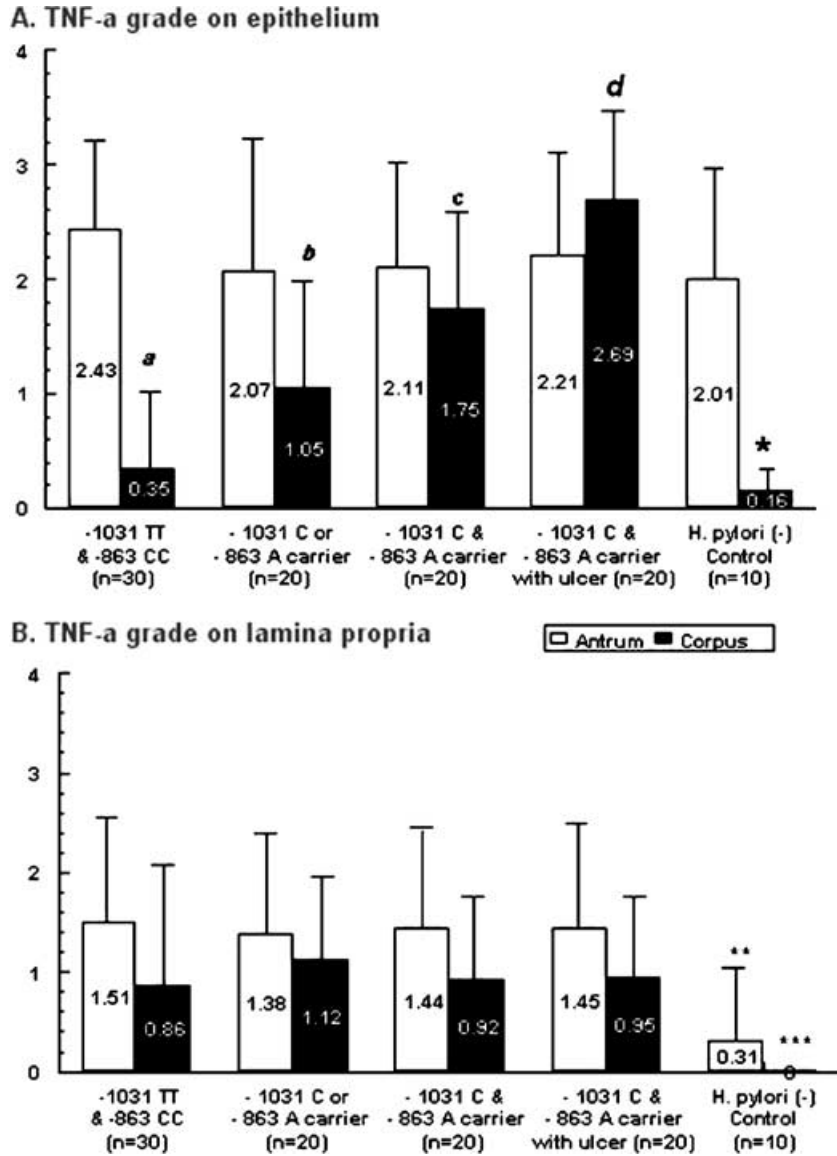


Figure 1. The mean grades of α of the 90 selected *H. pylori*-infected (70 without and 20 with ulcer) and the 10 *H. pylori*-negative non-ulcer controls are shown over the antrum (white column) and the corpus (black column) in A (for epithelium) and B (for lamina propria). For *over-the-corpus epithelium, **over-the-antrum lamina propria, and ***over-the-corpus lamina propria, there was a lower grade of *TNF-α* in the 20 *H. pylori*-negative controls than in the *H. pylori*-positive patients ($p < 0.001$). ^{a,b,c} indicate an upward trend of *TNF-α* grade over-the-corpus epithelium in the non-ulcer *H. pylori*-infected patients whose genotype ranking was -1031TT and -863CC, -1031C or -863A carrier, -1031C and -863A carrier ($p < 0.05$, one-way ANOVA). ^d indicates that among *H. pylori*-infected patients with -863 A and -1031 C alleles, those currently with ulcer had a higher *TNF-α* grade on the corpus epithelium than did those without ulcer ($p < 0.05$).

($p < 0.005$) than the -863A carriers. Moreover, such gastric ulcer patients with -863CC genotype had fewer dense AIG, but had a higher rate of AT and IM than that of the duodenal ulcer patients with both -863CC and -863A carrier ($p < 0.05$).

As gastric ulcer with IM carries a higher risk of gastric malignancy than those without IM, we divided the patients with gastric ulcer into either with or without IM. In Table 6, the multivariate logistic regression verified both -1031C and -863A carriers were two independent risk factors to have duodenal ulcers and gastric ulcer without IM in the *H. pylori*-

infected hosts ($p < 0.05$). In contrast, the independent risk factor to have gastric ulcer with IM was only -1031C carrier, different from that of getting duodenal ulcer and gastric ulcer without IM.

There is genetic linkage between -863A and -1031C allele of *TNF-α* among the *H. pylori*-infected patients ($\chi^2 = 147.4, p \leq 0.0001$) and non-infected patients ($\chi^2 = 46.3, p \leq 0.0001$). In Table 7, the risk of getting duodenal ulcer and/or gastric ulcer without IM were listed for the different genotype combinations of -863 and -1031 loci of *TNF-α* promoter. As compared to individuals with -863CC and -1031TT,

the ulcer risk was elevated to 2.46 (95% CI: 1.32–4.59, $p < 0.00001$) for the individuals carrying either –1031C or –863A allele. For those harboring both –863A and –1031C alleles, the odd ratio was further increased to 6.06 (95% CI: 3.57–10.21, $p < 0.00001$).

Gastric TNF- α Level by Different TNF- α Promoter SNP on –1031 and –863 Loci

The gastric biopsy of 90 *H. pylori*-infected patients included 30 non-ulcer patients with –1031TT and –863CC, 20 non-ulcer patients with either –1031C or –863A carrier, 20 non-ulcer patients with both –1031C and –863A carriers, and 20 duodenal ulcer patients with both –1031C and –863A carriers. The mean grades of TNF- α over the epithelium and the lamina propria of the corpus were significantly higher in the 90 selected *H. pylori*-infected patients than in the 10 *H. pylori*-negative non-ulcer controls ($p < 0.001$) (Fig. 1).

In *H. pylori*-infected non-ulcer patients, the grade of TNF- α on the corpus epithelium was lower in those homozygotes with both –863C and –1031T alleles than in those who were carriers of either –863A or –1031C ($p < 0.05$, one-way ANOVA model) (Fig. 1A). There was an upward trend of TNF- α grade over-the-corpus epithelium in the non-ulcer *H. pylori*-infected patients whose genotype ranking was –1031TT and –863CC, –1031C or –863A carrier, –1031C and –863A carrier ($p < 0.05$, one-way ANOVA). Furthermore, for *H. pylori*-infected patients, carriers of both –863A and –1031C who also had ulcers had an even higher TNF- α grade on the corpus epithelium than those without ulcers (2.69 vs 1.75, $p < 0.05$). In contrast, the TNF- α grade of the lamina propria over the antrum or corpus was similar in all patients irrespective of their genotypes ($p > 0.05$) (Fig. 1B).

DISCUSSION

This study disclosed that, in addition to the –308 loci, the other loci of the TNF- α promoter, including –1031, –863, –857, and –806, were not related to the increase of the prevalence of *H. pylori* infection. Our data indicated that TNF- α promoter polymorphism would not be a key factor in determining the success of *H. pylori* colonization and persistence. This study, however, also showed that individuals manifesting different gastric histological pictures or immune responses after *H. pylori* infection are associated not with the widely studied –308 locus but with different genotypes of TNF- α promoter at two novel loci, –1031 and –863. Dense infiltration of PMN was the only histological feature significantly related to the genotypes of TNF- α promoter over –1031 and –863 loci. The dense PMN infiltration disclosed in the patients with –1031C and –863A alleles addressed several issues. First, the data illustrated that host factors such as TNF- α played a significant role in dictating the extent of gastric PMN infiltration after *H. pylori* infection. Second, different genotypes classified by these two loci of TNF- α promoter SNP

may induce different levels of gastric immune responses after *H. pylori* infection. Last, it once again indicated that host genetic composition might determine the gastric pathological outcome in *H. pylori* infection (20–22).

Both the –863A and the –863C alleles can bind NF- κ B (p65-p50). The –863C allele, however, has a higher affinity for p50-p50, which inhibits transcription activation of p65-p50 and subsequently express less TNF- α than the –863A allele upon LPS induction (30). These facts may help account for the observations that –863A carriers expressed high TNF- α in gastric tissue and had dense PMN infiltration, and thus had nearly a 4-fold increase of ulcer risk after *H. pylori* infection. Nevertheless, up to now, there was no available molecular genetic mechanism to explain why an individual with the –1031C allele may have more tissue TNF- α than an individual homozygous for the –1031T allele. Further study is thus anticipated to test whether the presence of *H. pylori* may enhance nuclear protein binding to –1031C allele and thus leads to higher TNF- α expression over the gastric epithelium.

H. pylori infection increased TNF- α expression over the epithelium of the antrum and over the lamina propria of both the antrum and the corpus (Fig. 1). In addition, for those with *H. pylori* infection but without ulceration, significantly higher TNF- α levels over the epithelium of corpus was observed in –863A or –1031C carriers, or both, than in those with both 863CC and 1031TT genotypes. Because TNF- α is a strong chemoattractant for PMN and monocytes, such high gastric levels of TNF- α expression after *H. pylori* infection in patients different at the –863 and –1031 loci were consistent with the observation that dense infiltration of PMN was more readily detected in –863A and/or –1031C carriers.

In non-ulcer *H. pylori*-infected patients, TNF- α expression over the epithelium of the corpus was further increased in those harboring both the –863A and –1031C alleles than in those carrying either the –863A or the –1031C allele alone. In addition, multivariate regression analysis confirmed that –1031 and –863 polymorphisms are two independent risk factors to get peptic ulceration in the *H. pylori*-infected host. In Table 7, the genotype combination analysis confirmed that the ulcer risk is getting higher to 2.46 for the carrier of either –1031C or –863A, and for individuals with both –863A the –1031C allele, the risk was even increased to 6.06 ($p < 0.00001$). Although there is genetic linkage between –863A and –1031C alleles, our data support there could be synergistic effect to enhance the gastric tissue level of TNF- α (Fig. 1) and thus to carry a higher risk of peptic ulceration after *H. pylori* infection.

It is interesting to disclose the –863 genotypes as A carrier of TNF- α promoter was significantly higher in the patients with gastric ulcer than in patients with duodenal ulcer (60% vs 43.3%, $p < 0.05$) (Table 4). As gastric ulcer is disclosed with an increased risk as compared to duodenal ulcer to get carcinogenesis, it is thus important to test whether there were any specific features between gastric ulcer patients with –863CC genotype and –863A carrier. As shown in Table 5,

the gastric ulcer patients with -863CC genotype had fewer dense AIG, but had a higher rate of AT and IM than that of -863A carrier ($p \leq 0.05$). We agree it is particularly impressed by the fact that there are differences between gastric ulcer with the -863CC genotype and gastric ulcer with the -863CA or -863AA genotypes. These data strongly suggest that gastric ulcer patients may include two entities: The first one expressed as -863CC and had a higher rate of IM, which may predispose to a higher risk of carcinogenesis. The second one expressed as -863A carrier and has similar features to the duodenal ulcer patients, which may thus carry less risk of gastric cancer. In Table 6, the independent risk factor of gastric ulcer with IM is only -1031C carrier of TNF- α promoter SNP, crucially different to both -863A and -1031C carriers for gastric ulcer without IM or duodenal ulcer. Such a genomic evidence further supports that there should be different pathophysiology between gastric ulcer with and without IM. Moreover, it may imply the genomic analysis to host -863 loci will be promising to clarify the risk of gastric carcinogenesis among patients with gastric ulcers.

In contrast to PMN infiltration, the other histological features, such as chronic inflammation, atrophy, and intestinal metaplasia were not different between patients with different genotypes of any loci of TNF- α promoters (Table 3). This data could indirectly support to Machado *et al.*, disclosing patients infected by *cagA* (+) strains had an increased risk of getting gastric atrophy and cancers than those infected with *cagA* (-) strains, despite their TNF- α -308 polymorphism to be either A allele carriers or GG genotype (21). As our study was conducted in Taiwan, where nearly 100% of *H. pylori* infections are *cagA* (+) (8-10), it was thus reasonable to see genotyping of the -308 locus only is inadequate for predicting the risk of such precancerous lesions after *H. pylori* infection. In this study, we also disclosed similar -308 distributions between patients with and without ulcer, which was quite different from the previous studies (31, 32). We supposed the nearly 100% *cagA*-positive infection in Taiwan would be also accounted for the difference of such finding between oriental and western countries.

This study denoted that TNF- α promoter SNP as -1031C and -863A carriers had a higher risk of benign peptic ulceration after *H. pylori* infection. Nevertheless, as shown in Table 3, there remained many patients with peptic ulcer without expressing as either -1031C or -863A carriers. That is to say, for certain patients, none of these polymorphisms is of functional significance and the true culprit for the peptic ulcer association is an as yet unidentified variant in linkage disequilibrium with these promoter polymorphisms.

In summary, TNF- α promoter SNP on the -1031 and -863 loci should be novel host factors related with the severity of gastric inflammation, TNF- α expression, and the peptic ulcer risk after *H. pylori* infection. Applying such host factors to select a subset of non-ulcer dyspeptic patients for anti-*H. pylori* therapy would be promising to control the development of peptic ulcers in health care and clinical practice.

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