SPANDIDOS PUBLICATIONS

The relationship between *Helicobacter pylori* infection and promoter polymorphism of the Nrf2 gene in chronic gastritis

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Abstract. The transcription factor Nrf2 regulates the expression of detoxifying and antioxidant genes. Three polymorphisms of the Nrf2 gene have been reported. We attempted to clarify the relationship between Nrf2 gene polymorphism and chronic gastritis in a Japanese population. The study was performed in 159 patients with no evidence of gastric malignancy on upper gastrointestinal endoscopy (mean age, 62.03 years; male:female ratio, 102:57; peptic ulcer diseases in 69 patients, and *Helicobacter pylori (H. pylori)* positivity in 73.0%). We employed the PCR-SSCP method to detect gene polymorphisms using DNA extracted from peripheral blood cells or from antral biopsy specimens obtained by endoscopy. The severity of the histological chronic gastritis in antral biopsy specimens was classified according to the updated Sydney system. Although the frequencies of the SNP(-686) and SNP(-650) A alleles were decreased in subjects with peptic ulcers or severe mucosal atrophy, no significant differences were seen. However, the number of -686 G alleles was correlated with both neutrophil activity and mononuclear cell infiltration (p=0.036 and p=0.010, respectively), while the -650 C/C genotype was an independent risk factor for mononuclear cell infiltration (p=0.021 by ANOVA). In addition, both the number of -686 G alleles and the -650 C/C genotype showed an interaction with *H. pylori* infection to promote the infiltration of mononuclear cells (p=0.037 by ANCOVA and p=0.041 by ANOVA, respectively). Nrf2 promoter polymorphisms are significantly associated with the development of gastric mucosal inflammation, either independently or by interacting with *H. pylori* infection.

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Introduction

Infection with *Helicobacter pylori* (*H. pylori*) usually leads to persistent colonization and chronic gastric inflammation. Infected patients can develop multifocal atrophic gastritis, intestinal metaplasia, dysplasia, and distal gastric carcinoma (1-3). However, there are marked differences in the extent of inflammation among *H. pylori*-infected patients, so clinical consequences only develop in a small subgroup. The course of *H. pylori* infection is influenced by bacterial virulence factors, as well as by genetic predisposition and host immunity. That is, in addition to bacterial factors, unknown host factors seem to influence the inflammatory response and the development of severe gastritis. Inflammation induced by *H. pylori* is implicated in gastric mucosal damage and is characterized by severe granulocytic and lymphocytic infiltration (4,5). Although the T helper cell response to *H. pylori* is considered to be dependent on type 1 helper (Th1) cells, the factors influencing this immune response to *H. pylori* infection are largely unknown. Important cytokines that are related to Th1-mediated responses and are upregulated during chronic *H. pylori* infection include interferon-γ, tumor necrosis factor, and interleukin-1ß (6-9). It has been reported that the genes encoding cytokines have various polymorphisms, which are considered to alter gene transcription and thereby influence the inflammatory response (10,11). In fact, there have been several reports about the association between cytokine gene polymorphisms and gastric inflammation during *H. pylori* infection (12-14).

Another important factor that influences *H. pylori*-induced gastric inflammation is oxidative stress (15). Reactive oxygen species (ROS) are believed to be involved in promoting inflammation and in regulating the expression of oncogenes (16). Enhanced ROS production has been demonstrated in endoscopic biopsy samples from the duodenum and stomach of *H. pylori*-infected patients (17,18). Thus, there seems to be no doubt that ROS have an important role in the development of gastric inflammation induced by *H. pylori* infection. On the other hand, studies have suggested that nuclear factor-erythroid 2-related factor 2 (Nrf2) is an important regulator of genes induced by oxidative stress, such as heme oxygenase-1 and peroxiredoxin 1 (19). In addition, susceptibility to hyperoxia is tightly linked to the Nrf2 locus (20). Furthermore, Nrf2 null mice were found to

be highly susceptible to hyperoxic lung injury (21), and it was also reported that the impaired defenses against the oxidative stress of these mice showed a substantially decreased clearance of ROS (22). More recently, three polymorphisms of the promoter region (positions, -686, -684, and -650) of the human Nrf2 gene were identified (23). This study did not reveal a close connection between the risk of inflammatory diseases and these polymorphisms, yet further examination of the link between Nrf2 polymorphisms and oxidative stress-related diseases is important.

In the present study, we investigated the association between Nrf2 polymorphisms and chronic gastric inflammation in a Japanese population, as well as the interaction between these polymorphisms and *H. pylori* infection.

Materials and methods

Clinical samples and extraction of DNA. The study population comprised 159 patients with no neoplastic lesions, who were enrolled at the Endoscopy Center of Fujita Health University Hospital. All of the patients underwent upper gastrointestinal endoscopy with biopsies being taken from the antral mucosa. One part of each specimen was fixed in 10% buffered formalin and embedded in paraffin, while the other part was immediately frozen and stored at -80˚C.

All histological diagnoses were made at the Division of Pathology of our hospital. The severity of chronic gastritis was also classified according to the updated Sydney system (24) by a pathologist who had no access to clinical information. According to the severity of gastric mucosal atrophy, the subjects were divided into the following 3 groups: the non-atrophy (NA) group (atrophy score = 0 and metaplasia score = 0), the severe atrophy (SA) group (atrophy score ≥ 2 or metaplasia score ≥ 2), and the mild atrophy (MA) group (all others). Patients with severe systemic diseases were excluded.

Genomic DNA was isolated from the frozen specimens by digestion using proteinase K. The *H. pylori* infection status was assessed by serology, histological examination, or the urea breath test. Patients were diagnosed as having infection when at least one of the diagnostic tests was positive.

The Ethics Committee of Fujita Health University School of Medicine approved the protocol, and written informed consent was obtained from all of the participating subjects.

Genotyping of Nrf2 polymorphisms. Nrf2 polymorphisms were genotyped by PCR-SSCP. We employed the nested PCR reaction because the quality of PCR-SSCP is dependent on the purity of the PCR reactants. The primer sequences are shown in Table I. The first PCR was carried out using the NRF2F and NRF2R primer pair in a volume of 20 μl containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 5 min, followed by 35 cycles at 95˚C for 30 sec, 62˚C for 40 sec, and 72˚C for 60 sec, with final extension at 72˚C for 5 min. The second PCR was carried out in a volume of 20 μl containing 2 μl of the first PCR product diluted 100-fold with distilled water as the sample and two primer pairs (NRF2-AF, -AR and NRF2-BF, -BR for -686, -684 and -650, respectively). After denaturation at 95˚C for 5 min, 35 PCR cycles were performed (95˚C for 15 sec, 62˚C for 30 sec, and 72˚C for 45 sec), followed by a final extension at 72˚C for 5 min. In the second PCR, other reverse primers (NRF2-AAG, -AAA, -AGG, and -AGA) and forward primers (NRF2-BA and -BC) were also used to create a positive control DNA fragment. Both PCR reactions were performed using EX Taq (Takara Bio Inc., Shiga, Japan).

Then 2 μl of the second PCR product was denatured with 10 μl of formamide (Sigma-Aldrich Co., St. Louis, MO, USA) at 90˚C for 5 min. SSCP was carried out at 5˚C or 18˚C using a GenePhor DNA separation system with GeneGel Excel 12.5/24 (Amersham Biosciences Corp., USA), after which the denatured single-strand DNA bands were detected using a DNA silver staining kit (Amersham Biosciences Corp.).

Statistical analysis. Associations were analyzed by the Mann-Whitney U test for age and the Chi-square test for gender and *H. pylori* positivity. The odds ratio (OR) and 95% confidence intervals (CI) were estimated by logistic regression analysis using the number of -686 G alleles or -650 C alleles as a covariate after adjustment for gender and *H. pylori* infection status. The interactions between *H. pylori* infection

Table I. Primer sequences for PCRs.

1st PCR		NRF2F	forward	5'-AAACGATTACAGCATGTTGTGGT-3'
		NRF2R	reverse	5'-TGATTTGGAGTTGCAGAACCTT-3'
2nd PCR	-686-684	NRF2-AF	forward	5'-GCTCTGGGTGGGCAATACTG-3'
		NRF2-AR	reverse	5'-CGCAGTCACCCTGAACGC-3'
	-650	NRF2-BF	forward	5'-TGACTGCGAACACGAGCTG-3'
		NRF2-BR	reverse	5'-GGCTAAAGATTTGGACCCAGAC-3'
Positive control		NRF2-AAG	reverse	5'-CGCAGTCACCCTGAACGC**C**C**T**CC-3'
		NRF2-AAA	reverse	5'-CGCAGTCACCCTGAACGC**T**C**T**CC-3'
		NRF2-AGG	reverse	5'-CGCAGTCACCCTGAACGC**C**C**C**CC-3'
		NRF2-AGA	reverse	5'-CGCAGTCACCCTGAACGC**T**C**C**CC-3'
		NRF2-BA	forward	5'-TGACTGCGAACACGAGCTGCCGG**A**G-3'
		NRF2-BC	forward	5'-TGACTGCGAACACGAGCTGCCGG**C**G-3'

Bold characters, mismatched bases in position -686, -684, and -650.

Characteristics of subjects.

	n	Mean age ± SD	Male:Female	HP-positive rate
Total	159	62.03±13.03	102:57	73.0%
Non-ulcer	90	61.17±12.29	44:46	61.1%
Peptic ulcer	69	63.16±13.95	58:11[a]	88.4%[b]
NA	43	58.81±14.00[c]	20:23[d]	25.6%[f]
MA	60	60.88±14.40	0:25[e]	83.3%[g]
SA	56	65.73± 9.60	47:9	98.2%

NA, non-atrophy; MA, mild atrophy; SA, severe atrophy. [a]p<0.0001, [b]p=0.0001 vs non-ulcer, [c]p<0.0001 vs SA, [d]p<0.0001, [e]p=0.0008 vs SA, [f]p<0.0001 vs MA or SA and [g]p=0.0063 vs SA.

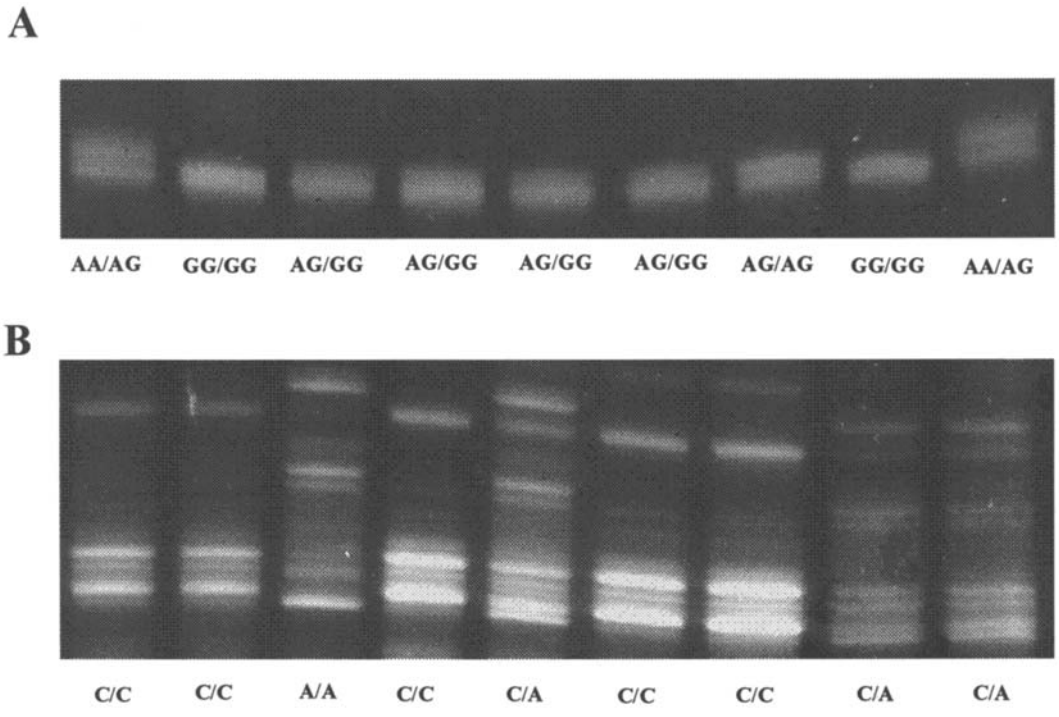

Figure 1. PCR-SSCP images. (A) Identification to bases in positions -686 and -684 using clinical samples. AA, -686A and -684A; AG, -686A and -684G; GG, -686G and -684G. There was no GA allele in all cases. (B) Identification to base in position -650.

status and Nrf2 polymorphisms with respect to the activity and inflammation scores were analyzed by ANOVA or ANCOVA using the number of -686G alleles as a covariate. The Mann-Whitney U test and linear regression analysis were employed to assess the association between Nrf2 polymorphisms and the updated Sydney system scores. For all analyses, the level of significance was set at $p<0.05$.

Results

Subjects. A total of 159 subjects participated in this study. Their characteristics are summarized in Table II. Sixty-nine of the 159 subjects had gastric or duodenal ulcers. There was no significant difference of age between the non-ulcer and peptic ulcer subjects, whereas the male/female ratio and the *H. pylori*-positive rate were significantly higher in the peptic ulcer than in the non-ulcer subjects.

With respect to gastric mucosal atrophy, 43, 60, and 56 of the subjects were classified into the NA, MA, and SA groups, respectively. The mean age of the SA group was significantly higher than that of the NA group. The male/female ratio was also higher in the SA group than in the other groups. Regarding the *H. pylori*-positive rate, there were significant differences among the 3 groups (SA>MA>NA).

The frequencies of Nrf2 polymorphism and gastric mucosal changes. The results of electrophoresis using clinical samples are shown in Fig. 1. The -686-684 haplotypes and the -650 alleles were clearly separated. The frequency of each Nrf2 promoter polymorphism in our subjects is shown in Table III. Strong allelic associations were recognized among 3 polymorphisms [estimated D' =1, 0.95, and 1 for SNP(-686) and SNP(-684), SNP(-686) and SNP(-650), and SNP(-684) and SNP(-650), respectively]. Since a strong allelic association

Table III. The frequencies of genotypes.

Position		Genotype				OR	95% CI	p value
		G/G	G/A	A/A	A allele (%)			
-686	Total	53	71	29	42.2			
	Non-ulcer	29	36	20	44.7	reference		
	Peptic ulcer	24	35	9	39.0	1.049	0.634-1.735	0.85
	NA	12	18	10	47.5	reference		
	MA	20	26	12	43.1			
	SA	21	27	7	37.3	1.370	0.404-4.649	0.61
-684	Total	141	12	0	3.9			
-650	Total	86	68	5	24.5			
	Non-ulcer	47	38	5	26.7	reference		
	Peptic ulcer	39	30	0	21.7	1.196	0.629-2.273	0.58
	NA	21	20	2	27.9	reference		
	MA	34	23	3	24.2			
	SA	31	25	0	22.3	2.250	0.521-9.705	0.28

There were no significant differences between all genotypes. Allelic associations were seen among three polymorphisms (D'=1, 0.95, and 1; -686:-684, -686:-650, and -684:-650, respectively).

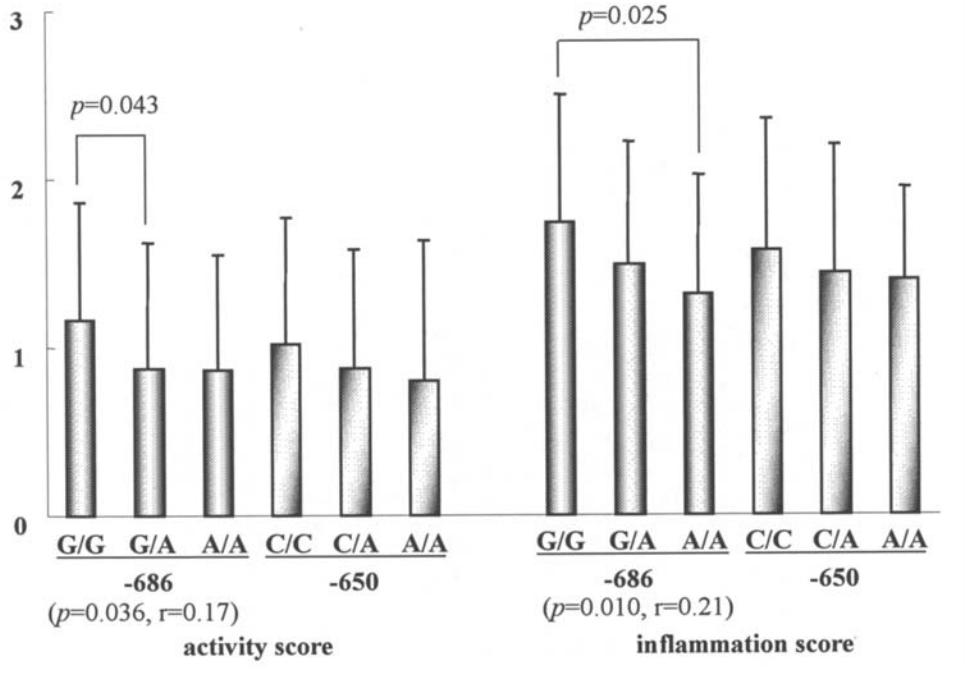

Figure 2. The association between SNP(-686) or (-650) and the activity or inflammation score. The comparison among two groups was performed by the Mann-Whitney U test and the associations between SNP(-686) and each score were assessed by linear regression analysis using the number of -686G alleles as a covariate.

was seen between SNP(-686) and SNP(-684), and the -684 A allele was quite rare, the association between the diseases and -684 polymorphism was not analyzed. Although the frequencies of SNP(-686) and SNP(-650) A alleles were decreased in the subjects with peptic ulcers or severe mucosal atrophy, no significant differences were seen.

Nrf2 polymorphism and inflammatory cell infiltration. The associations between SNP(-686) or SNP(-650) and the activity and inflammation scores are shown in Fig. 2. Using the number of G alleles as the covariate, both the activity and inflammation scores were significantly correlated with SNP(-686) positivity by linear regression analysis.

Interaction between *H. pylori* infection and SNP(-686) or SNP(-650).

	-686			-650		
	G/G	G/A	A/A	C/C	C/A	A/A
Activity score						
0	7 (2)	24 (10)	9 (1)	20 (6)	22 (8)	2 (0)
1	32 (29)	33 (30)	15 (11)	47 (42)	33 (29)	2 (1)
2	12 (12)	13 (12)	5 (3)	16 (15)	13 (12)	1 (0)
3	2 (2)	1 (1)	0	3 (3)	0	0
Inflammation score						
0	4 (0)	8 (0)	4 (0)	9 (1)	9 (0)	0
1	12 (8)	22 (15)	12 (4)	25 (14)	22 (14)	3 (1)
2	31 (31)	39 (36)	13 (11)	46 (45)	35 (33)	2 (0)
3	6 (6)	2 (2)	0	6 (6)	2 (2)	0

( ); *H. pylori*-infected patient number. Activity score: SNP(-686), p=0.385; HP(+), **p=0.014**; SNP(-686)*HP(+), p=0.135; SNP(-650), p=0.470; HP(+), **p=0.015**; SNP(-650)*HP(+), p=0.664. Inflammation score: SNP(-686), p=0.164; HP(+), **p<0.0001**; SNP(-686)*HP(+), **p=0.037**; SNP(-650), **p=0.021**; HP(+), **p=0.009**; SNP(-650)*HP(+), **p=0.041**.

Table IV shows the distributions of SNP(-686) and SNP(-650) in patients with or without *H. pylori* infection for each activity and inflammation score. *H. pylori* infection showed a significant independent association with higher activity and inflammation scores by ANOVA or ANCOVA. An increase in the inflammation score and SNP(-650) also showed a significant independent relation. In addition, there was a significant interaction between SNP(-650) and *H. pylori* infection in relation to an increase in the inflammation score by ANOVA. SNP(-686) did not show an independent association, but an interaction with *H. pylori* infection was also recognized in relation to an increase in the inflammation score by ANCOVA using the number of G alleles as covariate. On the other hand, there was no significant relationship between any of these SNPs and an increase in the activity score.

Discussion

In this study, we investigated the relationship between three Nrf2 polymorphisms (all genotypes of which were in the Hardy-Weinberg equilibrium) and chronic gastritis. The subjects were patients with upper gastroesophageal symptoms who underwent gastroduodenal endoscopy. Among the participants, 37.8% had moderate gastric mucosal atrophy and 31.4% had severe atrophy. The overall *H. pylori*-positive rate of these subjects was comparatively high (73.0%). A previous study demonstrated that the A allele frequencies for SNPs at position -686, -684, and -650 were 42.6%, 4.3%, and 31.5%, respectively. The reason why we observed a comparatively lower SNP(-650) A allele frequency may be that this study was not performed in a healthy population.

There have been few previous studies of the human Nrf2 gene, despite many reports on studies performed using rodents or *in vitro*. Therefore, it has not been clarified how polymorphisms influence the activity and expression of Nrf2, and we also obtained no evidence. However, it has been demonstrated that Nrf2-deficient mice display various pathological features, some of which are similar or related to human disorders (25-28). In addition, Nrf2 is reported to be an important regulator of genes induced by oxidative stress (19). Thus, there is no doubt that Nrf2 plays an important role in the elimination of ROS. In C57BL/6J mice, a strain sensitive to hyperoxic stress, an SNP was detected in the promoter region of the Nrf2 gene (21). Although there has only been one report of an association between the Nrf2 -650 C/C genotype and chronic obstructive pulmonary disease in humans (p=0.089) (23), Nrf2 promoter polymorphisms may influence inflammatory diseases by altering the activity of the gene product.

We excluded SNP(-684) from analysis because of the low allele frequency of this polymorphism and its very strong allelic association (D'=1, -686 and -684). In -650C/C carriers, we observed an increase in mononuclear cells infiltrating the gastric mucosa. In addition, neutrophil activity and the infiltration of mononuclear cells were correlated with the number of -686 G alleles. Interactions between *H. pylori* infection and the -650 C/C genotype or the number of -686 G alleles with mononuclear cell infiltration were also observed. There was a strong allelic association between positions -686 and -650 (D' =0.95), so that 97% of -686 G was estimated to be -650 C. Therefore, the investigation of the relationship between chronic gastritis and the G-C haplotype (-686--650) may clarify the importance of Nrf2.

There were no significant associations between Nrf2 polymorphism and gastric mucosal atrophy or metaplasia, although there was a significant relation between these polymorphisms and inflammatory cell infiltration. Factors released by inflammatory cells may be involved in the development of gastric mucosal atrophy or metaplasia. However, since gastritis can occur without atrophy or metaplasia in patients with marked inflammatory cell infiltration, the development of mucosal atrophy or metaplasia seems to be influenced by various factors, such as regulating

tissue remodeling or cell differentiation. Thus, Nrf2 polymorphism may be associated with the promotion of inflammatory cell infiltration, but additional factors may be needed to cause mucosal atrophy or metaplasia.

H. pylori infection is a powerful pathogenic factor and was also a significant risk factor for an increase in the updated Sydney system scores in our study. Since an interaction between *H. pylori* infection and Nrf2 polymorphism was noted in relation to inflammatory cell infiltration, Nrf2 may generally influence inflammation in the stomach.

In conclusion, the number of -686 G alleles was correlated with neutrophil activity and mononuclear cell infiltration, and the -650 C/C genotype was an independent risk factor for the mononuclear cell infiltration. In addition, both of these factors interacted with *H. pylori* infection to promote the infiltration of mononuclear cells. Thus, Nrf2 promoter polymorphisms are significantly associated with the development of gastric mucosal inflammation, either independently or by interacting with *H. pylori* infection.

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