NFKB1 polymorphism is associated with age-related gene methylation in *Helicobacter pylori*-infected subjects

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Abstract. CpG island aberrant methylation is shown to be an important mechanism in gene silencing. The important role of NF-κB in the inflammatory response to *H. pylori* colonization has been indicated. We investigated the influence of *NFKB1* polymorphisms, -94 ins/del (rs28362491) and -449 C>G (rs72696119), on the aberrant gene methylation under *H. pylori* infection. Gastric mucosal samples were obtained from sub-subjects without malignancies. Methylation status of genes ($p14^{ARF}$, $p16^{INK4a}$, *DAPK* and *CDH1*) was determined by methylation-specific PCR (MSP). The genotyping of *NFKB1* was performed by PCR-SSCP. There was a strong allelic association between rs28362491 and rs72696119, and all *H. pylori*-infected -94 del/del homozygotes had a -449 GG genotype. The -94 del/del homozygosity was significantly associated with risk for development of CpG island high methylation (CIHM) (two or more gene methylations), especially *DAPK* and *CDH1* methylations, and the number of methylated genes was significantly higher in -94 del/del homozygotes than in ins/del and ins/ins (ins carrier) *H. pylori*-infected elder subjects. In addition, this methylated gene number was significantly increased with age in *H. pylori*-infected del/del homozygotes, but not in infected ins carriers. Furthermore, the inflammation score was significantly higher in *H. pylori*-infected del/del homozygotes compared to ins carriers. *NFKB1* -94 ins/del ATTG polymorphism (rs28362491) was significantly associated with the increased risk for the development of age-related gene methylation in non-cancerous gastric mucosa under *H. pylori*-induced inflammation.

Introduction

Helicobacter pylori (*H. pylori*) infection first induces chronic superficial gastritis, which can progress to chronic atrophic gastritis, intestinal metaplasia, and dysplasia that leads toward gastric carcinoma (1). Lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria including *H. pylori*, is a signaling molecule for the innate immune system and is one of the main sources of inflammation (2). LPS binding to TLR4 activates signal transduction through MyD88, IRAK and TRAF6 to activate NF-κB (3). Activation of NF-κB by *H. pylori* induces nuclear translocation, which causes an increase in IL-8 messenger RNA and protein levels (4). Other NF-κB responsive genes including pro-inflammatory cytokines have also been found in elevated levels in *H. pylori*-infected gastric mucosa. In addition, the NF-κB pathway is responsible for the generation of several cell adhesion molecules including ICAM-1 whose expression is significantly correlated with an increase in *H. pylori*-induced gastritis (5). Thus, *H. pylori* is a potent activator of NF-κB in gastric epithelial cells and NF-κB is a major molecule in *H. pylori*-induced inflammation (4,6). On the other hand, NF-κB activation is known to regulate cellular growth responses, including apoptosis, and is required for the induction of inflammatory and tissue-repair genes (7). These facts suggest that NF-κB plays an important role in inflammation-associated carcinogenesis. In fact, *H. pylori* infection, activating NF-κB, is now accepted as a crucial event in the development of peptic ulcer disease and atrophic gastritis, and it is implicated in the development of gastric carcinoma, especially not located in the cardia (8-10).

Several cancers, including gastric tumors, show methylations of multiple genes (11,12). Some genes are methylated in non-neoplastic tissues with aging (13,14) and these methylations are also under the influence of chronic inflammation (15,16). In non-cancerous gastric mucosa, methylation of CpG islands was induced by *H. pylori* infection (17,18) and considered as the precancerous conditions in gastric carcinogenesis (19). Among several genes, E-cadherin (*CDH1*), death-associated protein kinase (*DAPK*) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) are frequently methylated in non-neoplastic gastric mucosa in relation to age, *H. pylori* infection, histological degree of gastritis, and gastric carcinogenesis (17,20). Therefore, there is a possibility that NF-κB activation may affect the gene methylations in *H. pylori*-induced chronic inflammation. Recently, many studies have reported the association between

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the polymorphism, -94 ins/del ATTG (rs28362491) of *NFKB1* encoding NF-κB, and various inflammatory diseases (21), as well as malignant neoplasm (22). However, these results do not always lead to the same conclusions. Furthermore, the genetic variation -449 C>G in the 5'-UTR of *NFKB1* (rs72696119) has been identified. There are no reports for the association of this polymorphism and human disorders.

Then, we attempted to clarify the association between the -94 ins/del ATTG polymorphism (rs28362491) of *NFKB1* and gene methylations in *H. pylori*-infected Japanese subjects. In addition, the -449 C>G polymorphism (rs72696119) was also investigated.

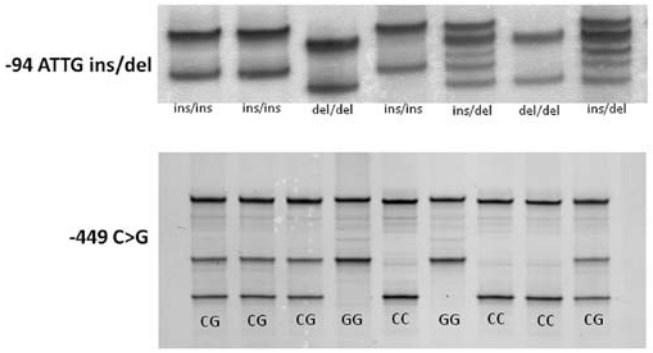

Figure 1. Images of multiplex PCR-SSCP using clinical samples. Single strand DNAs were clearly separated by SSCP.

Materials and methods

Clinical samples. The 330 *H. pylori*-infected subjects without peptic ulcers and gastric malignancies, who were enrolled at the Endoscopy Center of Fujita Health University Hospital or Kanazawa Medical University Hospital from January in 2006 to December in 2009, were selected. As a control, 205 *H. pylori*-uninfected subjects were randomly selected from our stocked DNA collected during the same period. Thus, the overall studied population comprised 535 subjects.

All subjects underwent upper endoscopy with biopsy from non-cancerous mucosa in the antrum. Parts of each specimen was fixed in 10% buffered-formalin and embedded in paraffin, while the other part was immediately frozen and stored at -85˚C. Later, genomic DNA was isolated from frozen specimens using proteinase K. The patients with severe systemic diseases, malignancies in other organs, and who had received nonsteroidal anti-inflammatory drugs, antibiotics, and *H. pylori* eradication treatment were excluded. *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 subjects with 2 or more methylations of 4 genes ($p14^{ARF}$, $p16^{INK4a}$, *DAPK* and *CDH1*) were classified into the CpG island high methylation (CIHM) group, whereas the others except the CIHM group were classified into the non-CIHM group.

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

Bisulfate modification and methylation-specific PCR (MSP). In 402 of 535 subjects (243 *H. pylori*-infected and 159 uninfected), the methylation status of 4 candidate promoter CpG islands (*p14*, *p16*, *CDH1*, and *DAPK*), which have been thought to be most susceptible for methylation in the stomach (12,19,22,23), were assessed. For the examination of DNA methylation, genomic DNA was treated with sodium bisulfite using the BislFast DNA Modification kit for methylated DNA detection (Toyobo, Co., Ltd., Osaka, Japan). Methylation status of four candidate promoter CpG islands were examined by MSP as previously described (24). The primer pairs and experimental conditions for MSP are the same as in our previous study (13,23,24). The MSP was carried out in a volume of 20 μl containing 0.1 μg of bislufite-modified DNA. The bands of MSP were detected by electrophoresis in 3.0% agarose gels stained with ethidium bromide. Hypermethylation was defined as the presence of positive methylation band, separated by electrophoresis on 2.5% agarose gels under UV illumination using an ethidium bromide staining, showing signals approximately equivalent to or greater than that of size marker (10 ng/μl: 100 bp DNA ladder; Takara Bio, Inc., Shiga, Japan), irrespective of the presence of unmethylated bands. We used DNA from the peripheral blood of a young individual without *H. pylori* infection, as the negative control (unmethylated DNA), and also used DNA being treated with *Sss*I methylase (New England Biolabs, Inc., Beverly, MA, USA), as the positive control (methylated DNA). Samples giving faint positive signals were analyzed a further two times and only those samples with consistent positive methylation band were considered as hypermethylation status.

Genotyping of polymorphisms. The DNA isolated from biopsy specimens or peripheral blood was used. The polymorphisms were genotyped by the PCR-SSCP method as previously described (25,26). To detect *NFKB1* -94 ins/del ATTG using the primer pairs (94-F, 5'-gctatggaccgcatgactctatcag-3' and 94-R, 5'-ggggctctggcttcctagcag-3'), PCR was carried out in a volume of 20 μl containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 96˚C for 15 sec, 58˚C for 40 sec, and 72˚C for 30 sec, with a final extension at 72˚C for 5 min. Thereafter, 2 μl of the 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 6˚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.).

To detect the *NFKB1* -449 C>G, using the primer pairs (449-F, 5'-cgtgtgtccgtctgtctgtatgctc-3' and 449-R, 5'-cgctggtgcacttctctctctttct-3'), PCR was carried out in a volume of 20 μl containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 95˚C for 30 sec, 57˚C for 40 sec, and 72˚C for 45 sec, with a final extension at 72˚C for 5 min. Thereafter, SSCP was carried out as described above.

Histological evaluation. In 400 of 535 subjects (256 *H. pylori*-infected and 144 uninfected subjects), the severity of chronic gastritis was classified according to the updated Sydney

Table I. Characteristics and prevalence polymorphisms and methylation status.

Characteristics	*H. pylori*-infected	*H. pylori*-uninfected	p-value[a]
Number of subjects	330	205	
Mean age ± SD	61.0±12.4	59.2±15.0	NS
Male:female	211:119	98:107	0.0003
NFKB1-94 ins/del ATTG			
ins/ins	119	79	
ins/del	172	97	
del/del	39	29	
del allele frequency	37.9%	37.8%	NS
NFKB1-449 G>C			
CC	124	83	
CG	161	91	
GG	45	31	
G allele frequency	38.0%	37.3%	NS
Methylated:unmethylated			
$p14^{ARF}$	90:153	44:115	0.053
$p16^{INK4a}$	91:152	21:138	<0.0001
CDH1	109:134	38:121	<0.0001
DAPK	139:104	58:101	<0.0001
CIHM/non-CIHM	136/107	47/112	<0.0001

[a]*H. pylori*-infected vs. *H. pylori*-uninfected. NS, not significant; CIHM, CpG island high methylation group.

system (27) by a pathologist who had no access to any clinical information.

Statistical analysis. The data were expressed as mean ± SD. The mean age among the two groups was compared by the Student's t-test. The ratios of gender and gene methylation were compared by the Fisher's extract test. The strength of association between allele frequencies and the methylation status was assessed by calculating the odds ratio (OR) and 95% confidence intervals (CI) by logistic regression analysis. Adjusted ORs were calculated after adjustment for age and gender. Each updated Sydney system score between the 2 groups were compared by the Mann-Whitney U-test. The methylation status was compared among the 2 groups by ANOVA. The relationship between age and the number of methylated genes was also assessed by ANOVA. Concerning the power of study, the β-value was calculated when setting α=0.05. For all analyses, the level of significance was set at p<0.05.

Results

Subjects and genotype. As shown in Fig. 1, single strand DNAs of each polymorphism were clearly identified by SSCP. The characteristics of the subjects are summarized in Table I. The overall distribution of -94 ins/del ATTG genotype was 198 ins/ins, 269 ins/del and 68 del/del. The distribution of -449 C>G was 207 CC, 252 CG and 76 GG. There was a strong allelic association between -94 ins/del ATTG and -449 C>G. The frequencies of distributions of both genotypes, in the Hardy-Weinberg equilibrium (p=0.12 and 1.00, respectively), were not significant difference among *H. pylori*-infected a d uninfected subjects. The male/female ratio was lower and each gene methylation ratio, except $p14^{ARF}$, was higher in *H. pylori*-infected subjects than uninfected subjects. The CIHM/non-CIHM ratio was also significantly higher in *H. pylori*-infected subjects.

Association between NFKB1 polymorphisms and CIHM. We defined the subjects with 2 or more gene methylations as the CIHM group, because the overall average number of gene methylation was 1.58. *NFKB1* -94 del/del homozygotes had an increased risk for the development of CIHM in *H. pylori*-infected over 60-year-old subjects (OR, 4.16; 95% CI, 1.14-15.3; p=0.031 and β=0.674) (Table II), although no significant risk was seen in overall infected subjects. In *H. pylori*-uninfected subjects, there was no association between -94 ins/del ATTG polymorphism and CIHM.

The association of -449 G>C with CIHM was similar to that of -94 ins/del ATTG with CIHM (Table III), because both polymorphisms were in strong linkage disequilibrium. So, in *H. pylori*-infected subjects over 60-years-old, -449 GG homozygote had an increased risk for CIHM (OR, 3.31; 95%CI, 1.04-10.6; p=0.044).

In *H. pylori*-infected over 60-year-old subjects, both -94 del/del ATTG and -449 GG homozygote had an increased risk for the development of *DAPK* methylation (OR, 5.35; 95% CI, 1.17-24.5; p=0.031 and β=0.726; and OR, 3.75; 95% CI, 1.04-13.6; p=0.044, respectively) (Table IV). In addition,

Table II. Association between *NFKB1*-94 ins/del ATTG polymorphism and CIHM.

	Genotype (n)			del/del vs. ins carrier	
	ins/ins	ins/del	del/del	OR (95% CI)	p-value
H. pylori-infected					
Overall					
Non-CIHM (n=107)	40	57	10	Reference value	-
CIHM (n=136)	50	65	21	1.58 (0.698-3.56)	0.27
60≤					
Non-CIHM (n=61)	23	35	3	Reference value	-
CIHM (n=81)	27	39	15	4.16 (1.14-15.3)	0.031
H. pylori-uninfected					
Overall					
Non-CIHM (n=112)	45	50	17	Reference value	-
CIHM (n=47)	19	20	8	1.15 (0.456-2.88)	0.77
60≤					
Non-CIHM (n=56)	21	25	10	Reference value	-
CIHM (n=27)	13	10	4	0.814 (0.228-2.91)	0.75

Logistic regression analysis after adjustment for age and gender; CIHM, CpG island high methylation group

Table III. Association between *NFKB1*-449 C>G polymorphism and CIHM.

	Genotype (n)			GG vs. C carrier	
	CC	CG	GG	OR (95% CI)	p-value
H. pylori-infected					
Overall					
Non-CIHM (n=107)	42	52	13	Reference value	-
CIHM (n=136)	48	65	23	1.30 (0.612-2.74)	0.50
60≤					
Non-CIHM (n=61)	25	32	4	Reference value	-
CIHM (n=81)	27	38	16	3.31 (1.04-10.6)	0.044
H. pylori-uninfected					
Overall					
Non-CIHM (n=112)	48	45	19	Reference value	-
CIHM (n=47)	18	20	9	1.14 (0.473-2.75)	0.77
60≤					
Non-CIHM (n=56)	23	22	11	Reference value	-
CIHM (n=27)	12	10	5	0.939 (0.287-3.07)	0.92

Logistic regression analysis after adjustment for age and gender; CIHM, CpG island high methylation group

-94 del/del homozygote had an increased risk for *CDH1*, as well as *DAPK*, methylation (OR, 2.91; 95% CI, 1.02-8.30; p=0.046). On the other hand, both polymorphisms were not associated with *CDKN2A* ($p14^{ARF}$ and $p16^{INK4a}$) methylation.

Relationship between NFKB1 polymorphisms and methylated gene number. We found strong allelic association between -94 ins/del ATTG and -449 C>G. That is, 66 of 68 del/del homozygote had -449 GG genotype. In *H. pylori*-infected subjects, all of 39 del/del homozygote had -449 GG genotype. Therefore, we investigated the association of only -94 ins/del ATTG polymorphism with gastric inflammation and gene methylation. In *H. pylori*-infected subjects over 60-years-old or more, methylated gene number was significantly higher in

Table IV. Associations between *NFKB1* polymorphisms and each gene methylation in the *H. pylori*-infected subjects older than 60-year-old.

-94 ATTG ins/del	ins/ins	ins/del	del/del	del/del vs. ins carrier; OR (95% CI)	p-value
$p14^{ARF}$-unmethylated (n=86)	35	39	12	Reference value	-
$p14^{ARF}$-methylated (n=56)	15	35	6	0.661 (0.228-1.92)	0.45
$p16^{INK4a}$-unmethylated (n=90)	29	52	9	Reference value	-
$p16^{INK4a}$-methylated (n=52)	21	22	9	1.83 (0.671-4.97)	0.24
CDH1-unmethylated (n=79)	29	44	6	Reference value	-
CDH1-methylated (n=63)	21	30	12	2.91 (1.02-8.30)	0.046
DAPK-unmethylated (n=53)	19	32	2	Reference value	-
DAPK-methylated (n=89)	31	42	16	5.35 (1.17-24.5)	0.031
-449 C>G	**CC**	**CG**	**GG**	**GG vs. C carrier; OR (95% CI)**	**p-value**
$p14^{ARF}$-unmethylated (n=86)	37	37	12	Reference value	-
$p14^{ARF}$-methylated (n=56)	15	33	8	0.925 (0.346-2.48)	0.88
$p16^{INK4a}$-unmethylated (n= 90)	31	48	11	Reference value	-
$p16^{INK4a}$-methylated (n=52)	21	22	9	1.45 (0.555-3.81)	0.45
CDH1-unmethylated (n=79)	31	40	8	Reference value	-
CDH1-methylated (n=63)	21	30	12	2.12 (0.802-5.59)	0.13
DAPK-unmethylated (n=53)	20	30	3	Reference value	-
DAPK-methylated (n=89)	32	40	17	3.75 (1.04-13.6)	0.044

Logistic regression analysis after adjustment for age and gender.

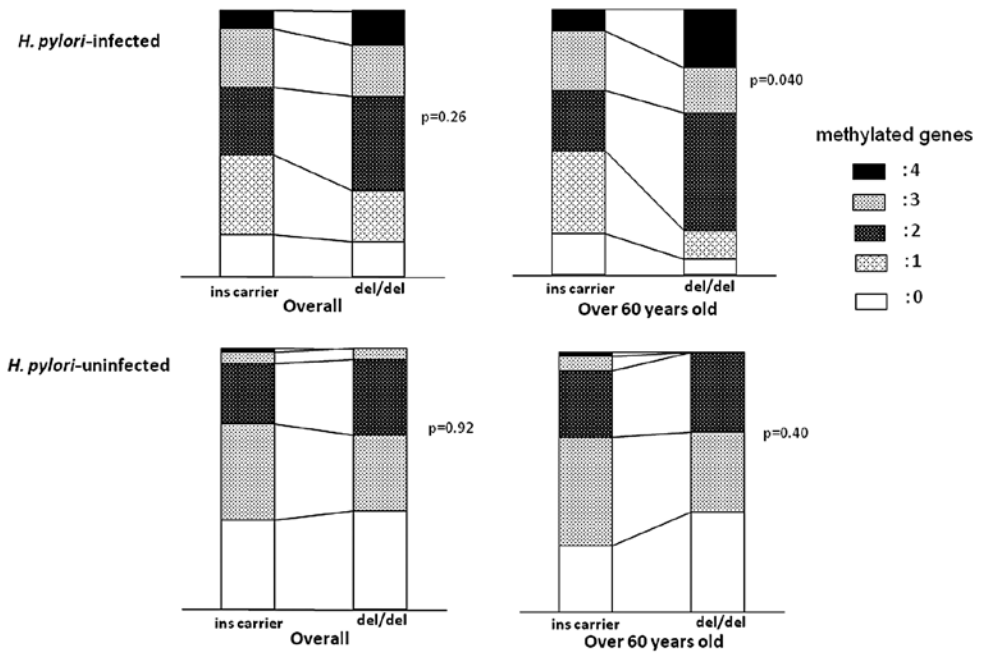

Figure 2. The number of methylated genes and *NFKB1* genotype. In *H. pylori*-infected subjects over 60-years-old, methylated gene number was significantly higher in del/del homozygote than ins carrier. A p-value was calculated by ANOVA.

del/del homozygotes than in ins carriers (p=0.040 by ANOVA) (Fig. 2), although no significant difference was seen in overall *H. pylori*-infected subjects. In *H. pylori*-uninfected subjects, there was no significant difference in the methylated gene number among two genotypes.

In *H. pylori*-infected del/del homozygote, methylated gene number was significantly correlated to age (p=0.027 by ANOVA) (Fig. 3), whereas no significant correlation was seen in the *H. pylori*-infected ins carrier and the uninfected groups.

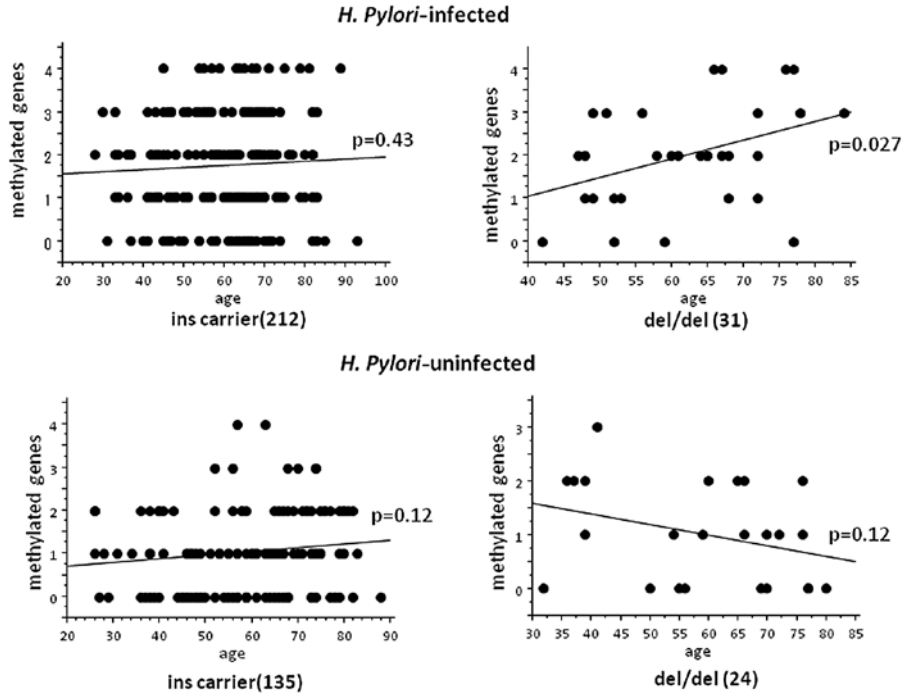

Figure 3. Correlation of the number of methylated genes to age by *H. pylori* infection status and genotype. In *H. pylori*-infected del/del homozygote, methylated gene number was significantly correlated to age. A p-value was calculated by ANOVA.

Table V. Comparison of each updated Sydney system score among del/del and ins carrier.

H. pylori-infected	del/del (n=28)	ins carrier (n=228)	p-value
Activity	0.929±0.813	0.851±0.858	NS
Inflammation	2.179±0.476	1.860±0.628	0.0091
Atrophy	1.607±0.685	1.575±0.773	NS
Metaplasia	0.786±1.067	0.982±1.015	NS
H. pylori-uninfected	del/del (n=15)	ins carrier (n=129)	p-value
Activity	0.067±0.258	0.155±0.441	NS
Inflammation	0.533±0.516	0.659±0.667	NS
Atrophy	0.133±0352	0.326±0.614	NS
Metaplasia	0	0.109±0.437	NS

Statistical anaysis was performed by the Mann-Whitney U-test. NS, not significant.

Comparison of each updated Sydney system score among del-G and non-del-G groups. In *H. pylori*-infected subjects, the inflammation score was significantly higher in del/del homozygote than ins carrier (p=0.0091 by Mann-Whitney U-test), whereas the other scores were not different among two genotypes (Table V). In *H. pylori*-uninfected subjects, there were no significant differences of all scores among two genotypes.

Discussion

Accumulation of DNA damage and aberrant methylation of various genes in gastric mucosa were induced by *H. pylori* infection and confer risk for developing gastric cancer. However, all the *H. pylori*-infected patients do not show the same hypermethylation status of genes. This suggests that some host genetic factor, such as genetic variations related to the immune response or inflammation, may be relevant to the hypermethylation of genes during gastric carcinogenesis.

Here, we evaluated the association between *NFKB1*, encoding NF-κB which plays an important role in inflammation and carcinogenesis, polymorphisms and aberrant methylation of genes in non-neoplastic gastric mucosa. *NFKB1* -94 ins/del ATTG and -449 C>G was in linkage disequilibrium and all of 39 *H. pylori*-infected -94 del/del homozygote had the -449 GG genotype. Therefore, the effects of the -94 and -449 mutant haplotype was equal to that of the -94 mutant variation. In

the present study, we demonstrated that the -94 del/del ATTG homozygotes had increased risk of aberrant methylation of *DAPK* and *CDH1* in comparatively older *H. pylori*-infected subjects. We also found that in *H. pylori*-infected del/del homozygotes, the number of methylated genes was higher in subjects over 60-years-old and was correlated to age. In addition, gastric mucosal inflammation was more severe in infected del/del homozygotes. These findings suggest that, in the *NFKB1* -94 del/del ATTG homozygote, *H. pylori* infection may accelerate severe mucosal inflammation, resulting in high age-related gene methylation. The methylation of genes is frequently observed in *H. pylori*-infected non-neoplastic mucosa (17-19), and is closely correlated with gastric cancer occurrence (12,18,20). Therefore, this epigenetic effect seems to be an early step in carcinogenesis in the stomach. Our data suggest that *NFKB1* polymorphisms may have a role in gastric carcinogenesis in the early phase via gene methylation-related pathway. In the present study, sample selection may affect the outcome, because our subjects came to hospital in order to have endoscopic examination for the compliant of abdominal discomfort, or for complete check up of gastric cancer following to barium X-ray examination in the health check, not complete healthy subjects. Moreover, the effect of type II error cannot be excluded in relatively small sample sizes. Another limitation of this study was that the male/female ratio was different among *H. pylori*-infected and uninfected subjects. However, adjustment of age and gender was performed in genotype analysis using logistic regression.

It has been reported that the *NFKB1* -94 ATTG deletion variant in the promoter region destroys a transcription factor binding site, resulting in lower expression of NF-κB (28). Due to their important role in inflammation, the lower expression of NF-κB protein seems to suppress inflammation. Furthermore, the *NFKB1* -94 deletion mutant has been associated with reduced risk for the auto-immune disorders in China (29). In stomach, Lo *et al* (30) showed that -94 deletion variant had a significantly reduced risk for the gastric carcinogenesis in China. For hepatocarcinogenesis, He *et al* (31) also showed that -94 deletion mutant had a reduced risk under the influence of hepatitis B virus infection in China. Contrary to these results, several studies have showed that -94 deletion variant is associated with increased risk for the development of inflammatory or auto-immune diseases in Caucasian (28,29,32). In colorectal carcinogenesis, Andersen *et al* (33) have showed that carriers of *NFKB1* -94 deletion were at 1.45-fold higher risk than homozygous carriers of the insertion allele. On the other hand, the lack of an association between the *NFKB1* -94 ins/del polymorphism and the inflammatory or autoimmune diseases has also been reported (34-37). These contrasting observations may be explained by differences in the genotypic composition of populations in different countries with different racial groups. In fact, the frequency of -94 deletion allele seems to be rather higher in Chinese healthy subjects (45-55%). However, in our study of Japanese subjects, the frequency was ~38%, similar to the value in Caucasians. Our study as well as the Caucasian study indicate that the -94 deletion variant may be an inflammation promoting allele.

NF-κB encompasses a number of different transcription factors that are homo- or heterodimers of p65, p50, p105, c-Rel and RelB (38). NF-κB is involved in both inflammatory and anti-inflammatory process (39). The role of NF-κB in inflammation is determined by the subunit type. *NFKB1* encodes both the subunits p105 and p50 of the transcription factor NF-κB by alternative splicing (40). As part of the p65/p50 NF-κB transcription factor complex, it is pro-inflammatory, controlling transcription of pro-inflammatory cytokines (41). Conversely, since p50 lacks this COOH-terminal transactivation domain which is necessary for the positive regulation of gene expression, p50 has anti-inflammatory properties in the p50 homodimer by repressing transcription (42). The relative abundance of p65/p50 heterodimers and p50 homodimers may determine the magnitude of inflammation by balancing the pro-inflammatory and anti-inflammatory response (38). In fact, p50-deficient mice have an increased sensitivity to lipopolysaccharide (LPS) and have increased LPS-induced inflammation (43,44). In subjects with the del/del genotype, decreased p50 synthesis may lead to decreased repressive homodimers and increased active heterodimers of the NF-κB complex. This balance may promote the *H. pylori*-induced inflammation, resulting in hypermethylation of genes.

In current study, 66 of 68 del/del homozygotes had the -449 GG genotype and, all of 39 del/del homozygotes had the -449 GG genotype in *H. pylori*-infected subjects. Therefore, we suspect that -94 ins/del ATTG polymorphism may mainly regulate the expression and function of NF-κB. From our results in this study, we could not show the role of -449 C>G polymorphism. Our results showed that, in -94 del/del homozygote, gastric inflammation was more severe and gene methylation was promoted over 60-year-old under influence of *H. pylori* infection. Because NF-κB is activated by some stimulation such as infection and stress, it is reasonable that *NFKB1* polymorphism is associated with the gastric inflammation process under *H. pylori* infection. In addition, it is also reasonable that increased gene methylation is revealed in elder subjects, because gene methylation gradually progresses with age and accumulates for a long time. Interestingly, although gastric inflammation was more severe in -94 del/del homozygote, atrophy and metaplasia scores were not different among del/del homozygote and ins carrier. This reason is unclear. The decreased p50 production may affect the action of not only the p50 homodimer but also of the p65/p50 heterodimer.

In conclusion, the *NFKB1* -94 ins/del ATTG polymorphism (rs28362491) was significantly associated with an increased risk for the development of age related-gene methylations in non-cancerous gastric mucosa under *H. pylori*-induced inflammation. The -94 del/del homozygote may have an increased risk for the development of age-related and inflammation-induced gene methylation, as a precancerous condition, in gastric mucosa.

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