The influence of promoter polymorphism of nuclear factor-erythroid 2-related factor 2 gene on the aberrant DNA methylation in gastric epithelium

TOMIYASU ARISAWA, TOMOMITSU TAHARA, TOMOYUKI SHIBATA, MITSUO NAGASAKA, MASAKATSU NAKAMURA, YOSHIO KAMIYA, HIROSHI FUJITA, DAISUKE YOSHIOKA, YUKO ARIMA, MASAAKI OKUBO, ICHIRO HIRATA and HIROSHI NAKANO

Department of Gatroenterology, Fujita Health University, School of Medicine, 1-98, Dengakugakubo, Kutsukake-cho, Toyoake 470-1192, Japan

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Abstract. Aberrant promoter methylation is an important mechanism for gene silencing. Inflammation-related reactive oxygens contribute to this CpG island methylation. The nuclear factor-erythroid 2-related factor 2 gene (Nrf2) is known to regulate the expression of detoxifying and antioxidant genes. We investigated the relationship between promoter polymorphisms of Nrf2 gene and the CpG island methylation in non-cancerous gastric mucosa. The study was performed in 85 subjects (46 without gastric malignancies, non-GC group, and 39 with gastric cancer, GC group). The promoter methylation status of p14(ARF), p16(INK4a) and p21(Waf1) genes was determined by methylation-specific-polymerase chain reaction. The Nrf2 gene genotypes were determined by the PCR-SSCP method. In the 85 subjects, CpG island methylation was found in 25.9% for p14, 15.3% for p16, none for p21. The frequency of the methylated genes was significantly higher in GC group than non-GC group (OR, 2.67; 95% CI, 1.10-6.49; p=0.029). In particular, the frequency of p16 gene methylation was much higher in GC group (p=0.0023). The Nrf2 -686/-684 G/G haplotype was positively associated and A/G haplotype was inversely associated with the development of CpG island methylation, especially p14 gene methylation (OR, 3.28; 95% CI, 1.26-8.59; p=0.015, and OR, 0.38; 95% CI, 0.15-0.96; p=0.040, respectively). In *Helicobacter pylori* (*H. pylori*) infected subjects, the number of -686/-684 G/G allele was positively correlated and that of A/G allele was inversely correlated to the methylation status, especially p14 methylation, by the adjusted analysis (OR, 2.90; 95% CI, 1.14-7.36; p=0.026, and OR, 0.33; 95% CI, 0.13-0.88; p=0.027, respectively). Our results suggested that the promoter polymorphisms of Nrf2 gene may affect the methylation status of tumor-related genes, especially the p14 gene, under the influence of *H. pylori*-induced gastric inflammation.

Correspondence to: Dr Tomiyasu Arisawa, Department of Gatroenterology, Fujita Health University, School of Medicine, 1-98, Dengakugakubo, Kutsukake-cho, Toyoake 470-1192, Japan
E-mail: tarisawa@fujita-hu.ac.jp

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Introduction

DNA methylation has been shown to be an important mechanism in gene silencing. Such CpG island methylation is commonly detected in human cancers (1,2). Meanwhile, some genes are also methylated in non-neoplastic tissues with aging, and this alteration is known as age-related methylation (3,4). It has also shown that gene methylation may be present in non-neoplastic colorectal mucosa in patients with inflammatory bowel desease (5,6), esophageal mucosa in patients with Barrett's esophagitis (7,8), and liver tissues in chronic hepatitis (9). In gastric carcinogenesis, DNA methylation of tumor-related genes has been shown to occur in early stage and it increases in parallel (10,11). In addition, it was indicated that methylation of CpG island was induced by *Helicobacter pylori* (*H. pylori*) infection in non-cancerous mucosa (12,13). These findings suggest that gene methylation could be a result of chronic inflammation, may be an early event in tumorigenesis, and the gene methylation would be expected to indicate an increased risk of tumor formation.

On the other hand, reactive oxygen species (ROS) produced by inflammatory cells are strongly involved in the carcinogenesis process. Involvement of inflammation-related ROS has been suggested in the etiology of human lung cancer (14). Weitzman *et al* reported that free radical injury may explain some of the altered methylation observed during carcinogenesis (15). Thus, ROS may influence the carcinogenesis during the chronic inflammation via inducing the aberrant DNA methylation. Recent studies have indicated that nuclear factor-erythroid 2-related factor 2 (Nrf2) is an important regulator of the genes induced by oxidative stress, such as heme oxygenase-1 and peroxiredoxin 1 (16). In addition, susceptibility to hyperoxia is tightly linked to the nrf2 locus (17) and nrf2 null mice show impaired defenses against oxidative stress with substantially decreased clearance of ROS (18). The promoter region of the human Nrf2 gene

has three known polymorphisms (positions, -686, -684 and -650) (19). We have already revealed that these polymorphisms were significantly associated with the development of gastric mucosal inflammation, as well as ulcerative colitis (20,21). Then, we hypothesized the possibility that the Nrf2 polymorphisms influence the aberrant methylation of tumor suppressor genes, such as p14, p16 and p21 genes, in *H. pylori*-related chronic gastritis.

In the present study, we investigated the relationship between promoter polymorphisms of the Nrf2 gene and the aberrant DNA methylation in non-cancerous gastric epithelium.

Materials and methods

Tissue samples, DNA extraction and Helicobacter Pylori infection status. The studied population comprised 85 subjects (46 with no malignant tumors and 39 with gastric cancer) recruited at the Endoscopy Center of Fujita Health University Hospital. All subjects underwent upper endoscopy with biopsy from non-cancerous mucosa in antrum. The specimens were immediately frozen and stored at -80˚C. The patients with systemic severe diseases were excluded. Genomic DNA was isolated from frozen specimens using proteinase K. *H. pylori* infection status was assessed by serologic, histological analysis or urea breath test. Patients were diagnosed as infected when at least one of the diagnostic tests was positive.

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

Bisulfate modification and Methylation-Specific PCR (MSP). To examine DNA methylation, genomic DNA was treated with sodium bisulfite using BislFast DNA Modification Kit for methylated DNA detection (Toyobo, Co., Ltd., Osaka, Japan). MSP were carried out with primers as follows: p14(ARF) methylated: forward, 5'-tgagtttggttttggaggtgg-3'; reverse, 5'-aaaaccacaacgacgaacg-3'. p14(ARF) unmethylated: forward, 5'-gagtttggttttggaggtgg-3'; reverse, 5'-aaccacaacaa caaacacccct-3'. p16(INK4a) methylated: forward, 5'-ttattagag ggtggggcggatcgc-3'; reverse, 5'-accccgaaccgcgaccgtaa-3'. p16(INK4a) unmethylated: forward, 5'-ttattagagggtgggg tggattgt-3'; reverse, 5'-caaccccaaaccacaaccataa-3'. p21(Waf1) methylated: forward, 5'-tgtagtacgcgaggtttcg-3'; reverse, 5'-tca actaacgcaactcaacg-3'. p21(Waf1) unmethylated: forward, 5'-tt tttgtagtatgtgaggttttgg-3'; reverse, 5'-aacacaactcaacacaacccta-3'.

The annealing temperature and times were determined using DNA from peripheral blood of a young individual without *H. pylori* infection and DNA methylated with SssI methylase (New England BioLabs Inc., Beverly, MA). The MSP was carried out in a volume of 20 μl containing 0.1 μg of bisulfite-modified DNA. The DNA was denatured at 95˚C for 5 min, followed by 33 cycles at 95˚C for 30 sec, 64-68˚C according to primers for 1 min, and 72˚C for 1 min with a final extension at 72˚C for 5 min. MSP reactions were done using EX Taq HS (Takara Bio Inc., Shiga, Japan). The bands of MSP were detected by electrophoresis in 3.0% agarose gels stained with ethdium bromide. With regard to the assessment for p16 gene methylation, the value of fluorescence intensities of methylated and unmethylated bands were measured by a digital densitometer. The methylation ratio, calculated as the ratio of this value of methylated band to methylated plus unmethylated bands, over 50% was estimated as significantly methylated. The subjects were divided into the following 2 groups: the methylated group (either p14 or p16 gene was methylated) and the unmethylated group (neither p14 nor p16 genes were not methylated).

Genotyping of Nrf2 polymorphisms. Nrf2 polymorphisms were genotyped by PCR-SSCP as reported previously (20,21). We employed the nested PCR reaction because the quality of the PCR-SSCP depends on the purity of the reactants. Primer sequences for the PCRs are as follows: 1st PCR forward, 5'-aaacgattacagcatgttgtggt-3' (NRF2F); reverse, 5'-tgatttggagttg cagaacctt-3' (NRF2R). 2nd PCR for -686/-684: forward, 5'-gctctgggtgggcaatactg-3' (NRF2-AF); reverse, 5'-cgcagtcaccct gaacgc-3' (NRF2-AR) and for -650: forward, 5'-tgactgcgaaca cgagctg-3' (NRF2-BF); reverse, 5'-ggctaaagatttggacccagac-3' (NRF2-BR).

The first PCR was carried out using a pair of primers (NRF2F and NRF2R) 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 of 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 a sample using two pairs of primers (NRF2-AF, -AR and NRF2-BF, -BR for bases -686/-684 and -650, respectively). The DNA was denatured at 95˚C for 5 min, followed by 35 cycles of 95˚C for 15 sec, 62˚C for 30 sec and 72˚C for 45 sec, with final extension at 72˚C for 5 min.

Then 2 μl of the 2nd PCR product was denatured with 10 μl of formamide (Sigma-Aldrich Co., St. Louis, USA) for 5 min at 90˚C. SSCP was carried out at 6 or 18˚C using a GenePhor DNA separation system with GeneGel Excel 12.5/24 (Amersham Biosciences Corp., USA). The denatured single-stranded DNA bands were detected using a DNA Silver Staining Kit (Amersham Biosciences Corp.).

Statistical analysis. Mean ages between two groups were compared with Student's t-test. The ratio of gender, *H. pylori* positivity and DNA methylation were compared using χ^2 test. The allele counts were also compared between the methylated group and the unmethylated group by a 2x2 table using χ^2 test. The strength of association between allele frequencies and the disease was assessed by calculating the odds ratio (OR) and 95% confidence intervals (CI). Adjusted ORs were calculated with the use of a regression analysis after adjustment for age, gender and *H. pylori* infection status. For all analyses, the level of significance was set at $p<0.05$.

Results

Subjects. A total of 46 non-cancer patients (non-GC) and 39 gastric cancer patients (GC) participated in this study. The characteristics are summarized in Table I. There was no significant difference between the two groups in the distribution of age and sex, while *H. pylori* positivity was significantly higher in GC group (p=0.0007).

Table I. Characteristics of the subjects and frequencies of gene methylation.

	Overall	Non-GC	GC	OR (95% CI)	P-value
No. of subjects	85	46	39		
Mean age ± SD	65.6±11.3	65.2±12.9	66.2±9.7	NS	
Male/Female	59/26	32/14	27/12	NS	
H. pylori positivity	61/85	26/46	35/39	6.73 (2.05-22.1)	0.0007
p14 or p16 gene[a]	35/85	14/46	21/39	2.67 (1.10-6.49)	0.029
p14 gene[a]	22/85	12/46	10/39	NS	
p16 gene[a]	13/85	2/46	11/39	8.64 (1.78-41.9)	0.0023

[a]The ratio of subjects with methylated gene/total subjects. NS, not significant.

Table II. The frequencies of genotypes among methylated and unmethylated groups.

	Unmethylated (%)	Methylated (%)	OR (95% CI)	P-value
No. of subjects	50	35		
Mean age ± SD	64.3±11.9	67.6±10.6	NS	
Male/Female	35/15	24/11	NS	
H. pylori positivity	31/50	30/35	3.68 (1.22-11.1)	0.017
Non-GC/GC	32/18	14/21	2.67 (1.10-6.49)	0.029
-686/-684 haplotype				
GG/GG	10	16		
GG/AG	24	12		
GG/AA	3	1		
AG/AG	7	6		
AG/AA	5	0		
-686A allele frequency	52.0	35.7	0.51 (0.27-0.96)	0.036
-684A allele frequency	8.2	1.4	NS	
-650 genotype				
C/C	24	20		
C/A	24	13		
A/A	2	2		
-650A allele frequency	28.0	24.3	NS	

NS, not significant. One case in the unmethylated group could not be genotyped at positions -686/-684.

The methylation of p21 gene was not detected in all subjects. CpG island methylation of tumor-related genes was found in 41.2% (25.9% for p14 and 15.3% for p16). The frequency of CpG methylation was significantly higher in GC group than non-GC group (p=0.029). The higher frequency of p16 gene was found in GC group compared with non-GC group, whereas there was no significant difference of the methylation frequency of p14 gene among the GC and non-GC groups.

The frequencies of genotypes of Nrf2 gene among the methylated and unmethylated groups. There were no significant differences between the methylated and unmethylated groups in the distribution of age and sex, while *H. pylori* positivity was significantly higher in the methylated group than the unmethylated group (p=0.017, Table II). The frequency of the patients with gastric cancer was also significantly higher in the methylated group (p=0.029).

The frequency of Nrf2 gene -686A allele was significantly higher in the unmethylated group than the methylated group (p=0.036), whereas the frequencies of -684A and -650A alleles were not significant different between the groups.

The influence of the Nrf2 gene polymorphisms on the CpG island methylation. By unadjusted analyses, the number of -686/-684 G/G allele was positively correlated to the CpG

Table III. The risk of the Nrf2 gene polymorphisms for CpG island methylation.

	Overall methylation		p14 gene methylation		p16 gene methylation	
	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]
-686/-684 G/G allele[b]	1.94 (1.02-3.68)[c]	1.59 (0.80-3.17)	1.85 (0.90-3.80)	1.71 (0.80-3.66)	1.66 (0.70-3.95)	1.19 (0.43-3.29)
-686/-684 A/G allele[b]	0.66 (0.35-1.26)	0.84 (0.42-1.67)	0.62 (0.29-1.30)	0.66 (0.31-1.43)	0.76 (0.32-1.85)	1.26 (0.47-3.43)
-686/-684 G/G homozygote	3.28 (1.26-8.59)[d]	1.04 (0.32-3.41)	3.13 (1.13-8.67)[f]	2.87 (0.97-8.47)	2.19 (0.65-7.31)	1.76 (0.45-6.80)
-686/-684 A/G carrier	0.38 (0.15-0.96)[e]	0.52 (0.20-1.38)	0.34 (0.13-0.93)[g]	0.37 (0.13-1.08)	0.60 (0.18-1.97)	1.02 (0.27-3.84)
-650C/C homozygote	1.44 (0.61-3.45)	1.19 (0.47-3.02)	1.16 (0.44-3.08)	1.01 (0.37-2.78)	1.60 (0.48-5.36)	1.31 (0.35-4.95)

[a]Adjusted for the age, gender and *H. pylori* infection status. [b]Analysis using the number of each allele as a co-variate. P-value: [c]0.044, [d]0.015, [e]0.040, [f]0.028, [g]0.035.

island methylation status (OR, 1.94; 95% CI, 1.02-2.68; p=0.044). In addition, -686/-684 G/G homozygous genotype had an increased risk (OR, 3.28; 95% CI, 1.26-8.59; p=0.015) and -686/-684 A/G carriers had a reduced risk (OR, 0.38; 95% CI, 0.15-0.96; p=0.040) for the development of CpG island methylation, especially p14 gene methylation. However, there were no significant associations between the Nrf2 gene polymorphisms and the CpG island methylation by the adjusted analyses (Table III).

No significant relationship was found between C-650A polymorphism of Nrf2 gene and CpG island methylation status.

The risk of Nrf2 gene polymorphisms for CpG island methylation in H. pylori positive subjects. Because a significant interactions between *H. pylori* infection and -686/-684 genotype (G/G allele, p=0.050 and A/G allele, p=0.012) by Ancova using the number of each allele as co-variate, the analysis was performed in *H. pylori* infected subjects. As shown in Table IV, the number of -686/-684 G/G allele was positively correlated to the degree of the CpG island methylation. In addition, -686/-684 G/G homozygous genotype had an increased risk for the development of CpG island methylation by unadjusted and adjusted analyses (OR, 3.29; 95% CI, 1.12-9.66; p=0.031, and OR, 3.24; 95% CI, 1.09-9.61; p=0.034, respectively).

There were no significant associations between the Nrf2 gene polymorphisms and p16 gene methylation. However, the number of -686/-684 G/G allele was positively correlated and that of A/G allele was inversely correlated to the development of p14 gene methylation (OR, 2.90; 95% CI, 1.14-7.36; p=0.026, and OR, 0.33; 95% CI, 0.13-0.88; p=0.027, respectively). Furthermore, -686/-684 G/G homozygous genotype had an increased risk and A/G carriers had a reduced risk for the development of p14 gene methylation (OR, 3.66; 95% CI, 1.14-11.8; p=0.030, and OR, 0.25; 95% CI, 0.075-0.83; p=0.023, respectively). There were no significant associations between the Nrf2 C-650A polymorphism and the CpG island methylation status.

Discussion

In the present study, we investigated the association between the Nrf2 gene polymorphisms and the degree of CpG island methylation in non-cancerous gastric mucosa. We found that -686/-684 G/G genotype was positively associated and A/G genotype was inversely associated with the development of CpG island methylation, especially p14 gene methylation in *H. pylori* infected subjects.

The p16(INK4a) and p14(ARF) are involved in the negative cell cycle regulation via the pRb and p53 pathways, respectively. These two proteins have an independent first exon (exon 1α and 1ß, respectively) but share exon 2 and 3 (22,23). Aberrant promoter methylation is an important mechanism for gene silencing. Methylation of p16 and p14 genes has been shown to be present in gastric cancer as well as premalignant lesions (24,25). The p21(Waf1) is also involved in cell cycle arrest (26), which is up-regulated in *H. pylori* infection (27,28). These genes may play crucial roles in cell cycle control, apoptosis and DNA repair in the stomach and its disorder may be closely associated with gastric carcinogenesis. In our study, the frequency of the CpG island methylation in non-cancerous gastric mucosa was significantly higher in the subjects with gastric cancer compared with the subjects without gastric cancer. In particular, the frequency of p16 gene methylation was much higher (p=0.0023) and p21 gene methylation was not detected. A previous study suggested that tumor suppressor genes showed a gene type-specific methylation profile and that aberrant CpG island methylation tended to accumulate

Table IV. The risk of the Nrf2 gene polymorphisms for CpG island methylation in *H. pylori* infected subjects.

	Overall methylation		p14 gene methylation		p16 gene methylation	
	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]
-686/-684 G/G allele[b]	2.25 (1.03-4.93)[c]	2.20 (1.00-4.87)	2.83 (1.11-7.20)[f]	2.90 (1.14-7.36)[j]	1.26 (0.50-3.21)	1.09 (0.39-3.08)
-686/-684 A/G allele[b]	0.51 (0.24-1.11)	0.53 (0.24-1.17)	0.36 (0.14-0.93)[g]	0.33 (0.13-0.88)[k]	0.94 (0.38-2.37)	1.27 (0.45-3.56)
-686/-684 G/G homozygote	3.29 (1.12-9.66)[d]	3.24 (1.09-9.61)[e]	3.63 (1.15-11.4)[h]	3.66 (1.14-11.8)[l]	1.72 (0.48-6.15)	1.77 (0.46-6.83)
-686/-684 A/G carrier	0.36 (0.13-1.03)	0.38 (0.13-1.11)	0.27 (0.084-0.86)[i]	0.25 (0.075-0.83)[m]	0.75 (0.21-2.66)	0.92 (0.24-3.55)
-650C/C homozygote	1.88 (0.67-5.28)	1.92 (0.67-5.48)	1.58 (0.50-5.00)	1.64 (0.51-5.28)	1.50 (0.40-5.66)	1.54 (0.38-6.23)

[a]Adjustment for the age and gender. [b]Analysis using the number of each allele as a co-variate. P-value: [c]0.043, [d]0.031, [e]0.034, [f]0.029, [g]0.035, [h]0.028, [i]0.027, [j]0.026, [k]0.027, [l]0.030, [m]0.023.

along the pathway of multistep carcinogenesis (11). Our results also suggested that CpG island methylation was associated with gastric carcino-genesis (OR, 2.67; 95% CI, 1.09-9.61; p=0.029).

Some genes are methylated with age (29) and chronic inflammation (30) in colorectal epithelium. In the stomach, aberrant CpG island hypermethylation of chronic gastritis is related with age, gender, intestinal metaplasia, and chronic inflammation (31). The stomach is one of the organs that shows frequent methylation of CpG islands of genes in non-cancerous epithelial cells (32,33). The mechanisms of age- or inflammation-related methylation are unknown. Several factors may contribute to this methylation, such as exogenous carcinogens, generated reactive oxygens, and host genetic differences (29). Endogenously generated reactive oxygen species, such as peroxides and oxygen-free radicals, may play an important role in carcinogenesis (34). Reactive oxygen species (ROS) are believed to be involved in the process of inflammation and the expression of oncogenes (35). *H. pylori*-induced expression of oncogenes and cell-cycle regulators may be mediated by ROS-induced activation of oxidant-sensitive transcription factors in gastric epithelial cells (36,37). These oxidants also produce a variety of effects characteristic of carcinogens, including altered cytosine methylation (38). Weitzman *et al* reported that methylation of cytosine residues in DNA can be greatly influenced by hydroxyl-free radical adducts on adjacent guanine residues (15). Nrf2 is an important regulator of the genes induced by oxidative stress, such as heme oxygenase-1 and peroxiredoxin 1, and induces phase II detoxifying enzymes and antioxidant enzymes via Nrf2 acting on the antioxidant response elements (ARE) located in the 5'-flanking region of these enzyme genes. Therefore, it is entirely possible that polymorphisms of the Nrf2 gene affect the alteration of CpG island methylation.

We previously reported that -686/-684 G/G haplotype was associated with the increased inflammation severity (20,21). In the present study, the frequency of CpG island methylation was higher in -686/-684 G/G genotype and significantly lower in A/G genotype. This finding was more distinct in *H. pylori* infected subjects. One of the most important factors causing gastric inflammation is *H. pylori* infection (39,40), under which gastric mucosa is suffering from various oxidative stress (41,42). In *H. pylori* infected subjects, the effect of Nrf2 may be more apparent because of ROS produced abundantly by *H. pylori* infection.

In our study, Nrf2 promoter polymorphisms correlated to p14 gene methylation. On the other hand, gastric carcinogenesis was associated with p16 gene methylation, not p14 gene. Therefore, the alteration of methylation status affected by Nrf2 genotype may not directly contribute to gastric carcinogenesis. It is unknown why Nrf2 polymorphisms affect only p14 gene methylation, not p16 gene methylation. However, Iida *et al* revealed that p14(ARF) alterations might be involved in diffuse-type gastric carcinogenesis (43). With regard to this point, further studies will be needed. *H. pylori* infection has been reported to potently induce the methylation of CpG islands to various degrees (12). That is, there are marked interindividual differences in the degree of CpG island methylation among persons with *H. pylori* infection. Nrf2 polymorphisms may contribute to these interindividual differences and the subsequent development of gastric cancer.

In conclusions, the promoter polymorphisms of Nrf2 gene affect the methylation status of tumor-related genes, especially p14 gene, under the influence of *H. pylori*-induced gastric inflammation. Further studies are needed to clarify whether this influence of Nrf2 gene polymorphisms on the CpG island methylation lead to the gastric carcinogenesis or not.

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