MAFK Polymorphisms Located in 3'-UTR are Associated with Severity of Atrophy and *CDKN2A* Methylation Status in the Gastric Mucosa

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Objective: This study was designed to clarify the association of the *MAFK* polymorphisms (rs4268033, rs3735656, and rs10226620) with the degree of gastric mucosal atrophy and *CDKN2A* CpG methylation status.

Methods: A total of 491 subjects were enrolled in this study. Genotypes and methylation status were determined by polymerase chain reaction (PCR)-single-stranded conformation polymorphism and methylation-specific PCR (Fujita Health University, HM18-094).

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Results: Both rs3735656 and rs10226620, located in the 3'-UTR of *MAFK*, were significantly associated with the severity of gastric mucosal atrophy using a dominant genetic model (odds ratio [OR], 2.10; $p=0.0012$, and OR, 1.98; $p=0.0027$, respectively). Under a recessive genetic model, no significant association was found between three polymorphisms and gastric mucosal atrophy. The serum pepsinogen I/II ratio was significantly lower in subjects with minor alleles of rs3735656 and rs10226620 than in subjects that were homozygous WT ($p=0.018$ and 0.013 , respectively). In a subgroup including 400 of the 491 subjects, the CpG of *p14*^{ARF} and *p16*^{INK4a} were methylated in 132 and 112 subjects, respectively. Fifty subjects had both CpG methylations and 206 subjects had neither methylation. When comparing the groups with both and neither methylations, there were no significant associations between any of the three polymorphisms and *CDKN2A* methylation under a dominant genetic model. However, all polymorphisms investigated in this study (rs4268033, rs3735656, and rs10226620) were significantly associated with *CDKN2A* methylation in a recessive genetic model (OR, 3.58; $p=0.0071$, OR, 4.49; $p=0.0004$, and OR, 3.45; $p=0.0027$, respectively).

Conclusions: Our results indicate that possession of either of the minor allele *MAFK* polymorphisms that are located in the 3'-UTR increase the risk of severe gastric mucosal atrophy; furthermore *CDKN2A* CpG methylation may develop in subjects who are homozygous for the minor alleles of the these *MAFK* 3'-UTR SNPs.

Keywords: *MAFK*, polymorphisms, gastric mucosal atrophy, *CDKN2A*, CpG hypermethylation

Introduction

IN THE FISCAL year 2018, an estimated 1 million people were diagnosed with gastric cancer worldwide, and 783,000 people died from the disease (Bray *et al.*, 2018). In Japan alone, ~29,000 people died of gastric cancer in the fiscal year 2018, suggesting that it is a disease of global concern. Previous studies have shown that *Helicobacter pylori* infection is involved in gastric cancer (Plummer *et al.*, 2016), which develops through the atrophic gastritis triggered by the infection (Watanabe *et al.*, 1997; Ozasa *et al.*, 1999). In addition, severe atrophy, corpus-dominant gastritis, and intestinal

metaplasia have been reported as risk factors for the development of gastric cancer (Uemura *et al.*, 2001).

DNA methylation is closely associated with gene inactivation; correspondingly, CpG island hypermethylation (CIHM) of tumor suppressor genes has been identified in several human malignancies (Jones and Baylin, 2002; Li and Chen, 2013). Such epigenetic alterations have been observed in gastric cancer (Tan and Yeoh, 2015). This aberrant methylation has previously been observed in the precancerous nontumorous mucosa, suggesting an association with inflammation caused by *H. pylori* infection (Niwa *et al.*, 2010). Both *p14*^{ARF} and *p16*^{INK4a}, which are tumor suppressor

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genes produced from the alternative splicing of *CDKN2A*, are relatively frequently methylated (Maekita *et al.*, 2006; Tahara *et al.*, 2007).

Oxidative stress is one of the most essential factors affecting inflammation in *H. pylori* infection (Naito and Yoshikawa, 2002). Nuclear factor-erythroid 2-related factor 2 (Nrf2) plays an important role in eliminating reactive oxygen species (ROS) produced by oxidative stress (Ishii *et al.*, 2000). Nrf2 alone was demonstrated to be unable to induce antioxidant enzymes such as heme oxygenase-1 (HO-1) and peroxiredoxin 1; moreover, it exerted transcriptional activity by forming a heterodimer with small musculoaponeurotic fibrosarcoma (Maf; Itoh *et al.*, 1997). We have previously found a genetic polymorphism of *NFE2L2* that encodes Nrf2 is associated with the degree of gastric mucosal atrophy and the CpG methylation of *CDKN2A* (Arisawa *et al.*, 2007, 2008). We also clarified that there is a significant association between a genetic polymorphism of *MAFK* that codes for MafK, a small Maf, and the susceptibility of ulcerative colitis (Arisawa *et al.*, 2017).

This study was aimed at elucidating whether a genetic polymorphism of *MAFK* is associated with gastric mucosal atrophy, a precancerous condition, and aberrant *CDKN2A* methylation.

Methods

Clinical samples

The samples were selected at random among subjects attending the Endoscopic Center of Fujita Health University Hospital and registered from January 2006 to December 2012. All study subjects underwent upper gastrointestinal endoscopy as a part of a health checkup, as a secondary examination following barium X-ray gastrography, or due to symptoms of abdominal discomfort. Subjects with severe systemic diseases or malignancies of the stomach or other organs, as well as those with a history of abdominal surgery or *H. pylori* eradication were excluded. Among all registered subjects, we randomly selected 400 subjects from 514 subjects stocked in our database with the assessment of histological degree of atrophy and CIHM status of *CDKN2A* in the antral mucosa, and 200 subjects with the assessment of histological degree of gastric mucosal atrophy and the measurement of serum pepsinogen (PG) I and II levels; consequently, the study population comprised 491 subjects, because 109 subjects were overlapped. In 1000 genomes database, the frequency of rs4268033 minor allele was ~40%. We assume that a 30% decrease in the prevalence of an allelic frequency would be of clinical relevance. Assuming an alpha value = 0.05 and a power = 0.80, at least 243 subjects in each group would be sufficient to identify a clinically relevant difference. Accordingly, we thought that this sample size could be evaluated. The protocol for this study was approved by the Ethics Committee of Fujita Health University (HM18-094), and written consent was obtained in all cases.

At the time of endoscopy, a biopsy was performed from the antrum. A portion of the biopsy tissue was frozen and stored at -80°C until used for the detection of methylation. Meanwhile, another portion of the biopsy tissue was hematoxylin eosin stained and provided to the pathologist, who had not been informed of the background information, for grading the extent of histological gastritis according to the updated

Sydney system (Dixon *et al.*, 1996). The subjects were divided into two groups based on the degree of histological atrophy, as follows:

NA (nonatrophy, $n = 288$) group: atrophy score = 0 or 1 and metaplasia score = 0. AG (atrophic gastritis, $n = 203$) group: remaining subjects. Serum PG I and PG II levels were measured in 200 subjects through a radioimmunoassay. When any one of the results of the rapid urease test, urea breath test, histological test, or serum antibody test was positive, the patient was determined to have *H. pylori* infection.

Selection of single nucleotide polymorphisms of *MAFK*

In *MAFK* genome region, there are two linkage disequilibrium blocks above 0.05 of Hardy-Weinberg equilibrium (HWE) p -value and above 0.05 of minor allele frequency. At first, we selected rs3735656, located in 3'-UTR, among two Tag single nucleotide polymorphisms (SNPs) of each block. Next, within 2000 bp around *MAFK*, two large linkage blocks are found with the same conditions described above. Then, we selected a Tag SNP (rs4268033), which is not in linkage disequilibrium with rs3735656. Finally, the other SNP rs10226620, which is located in 3'-UTR where several microRNA bind to, was also selected.

Genotyping

Genomic DNA was extracted from a part of the cryopreserved specimens using proteinase K. In some subjects, genomic DNA was extracted from the peripheral blood collected at the time of endoscopy and stored at -80°C. Genotyping was performed by the polymerase chain reaction (PCR)-single-stranded conformation polymorphism (SSCP), which we previously reported (Arisawa *et al.*, 2017). The used primer sets are shown in Table 1. PCR was performed using EX Taq HS (Takara Bio, Shiga, Japan) with the addition of 0.1 µg of genetic DNA to 20 µL of a buffer and under the following conditions: denaturing at 95°C for 3 min, followed by 35 cycles of 15 s at 96°C, 30 s at 60°C, and 30 s at 72°C; and a final extension for 5 min at 72°C for rs4268033 and rs10226620. The same PCR conditions were used for rs3735656, except the 35 repeating cycles were replaced by those of 15 s at 96°C and 40 s at 54°C. Then, 2 µL of the PCR product was treated in 10 µL of formamide for 5 min at 90°C and denatured to a single strand. The SSCP analysis was performed using the Gene Phor DNA separation system with the Gene Gel Excel 12.5/24 kit (GE Health Care Bio-Sciences AB, Stockholm, Sweden) at a constant temperature of 18°C, and the denatured bands were detected with a DNA silver staining kit (GE Health Care Bio-Sciences AB).

Bisulfite reaction and methylation-specific PCR methods

To detect DNA methylation, genomic DNA extracted from the stocked frozen biopsy tissues was treated with sodium bisulfite using the BisFast DNA Modification Kit for Methylated DNA Detection (Toyobo, Osaka, Japan). The methylation-specific PCR was performed as previously described (Tahara *et al.*, 2007, 2009). The used primer sets are shown in Table 1. Using EX Taq HS (Takara Bio), the PCR was performed with the addition of 0.1 µg of bisulfite-modified DNA in 20 µL of a buffer. The PCR conditions were

TABLE 1. PRIMER SETS USED IN THIS STUDY

	Forward	Reverse
Primer sets for MAFK		
rs4268033	5'-taatcccaactgcagcatctgtgt-3'	5'-gggtctgacttagctgggaaagtgc-3'
rs3735656	5'-atctcagcggacacaggcagga-3'	5'-ctgcactgaccacagttggtgagaa-3'
rs10226620	5'-gtccctctgtgactgggtctct-3'	5'-aggcaccaccttgaggcttatgt-3'
Primer sets for MSP		
p14 unmethylated	5'-gagtttggtttggaggtgg-3'	5'-aaccacaacaacaacaccct-3'
p14 methylated	5'-tgagtttggtttggaggtgg-3'	5'-aaaaccacaacgacgaacg-3'
p16 unmethylated	5'-ttattagaggggtgggtgattgt-3'	5'-caaccccaaacacaaccataa-3'
p16 methylated	5'-ttattagaggggtgggcggtatgc-3'	5'-accccgaccgcgaccgtaa-3'

MSP, methylation-specific polymerase chain reaction.

those previously reported (Tahara *et al.*, 2007, 2009). Electrophoresis using 2.5% agarose gel was performed with 10 μ L of the PCR products, which were stained with ethidium bromide and visualized by UV illumination. The presence of CIHM was determined when the signal of the positively methylated band was stronger compared with the size maker (10 ng/ μ L: 100 bp DNA Ladder; Takara Bio), regardless of the presence of unmethylated bands. The validity of judgment for CIHM status was confirmed in our previous study (Tahara *et al.*, 2011). In addition, in each experiment, the validity of the results was confirmed using both negative control (DNA from the peripheral blood of a young individual without *H. pylori* infection) and positive control (DNA, used negative control, treated with SssI methylase, New England BioLabs, Inc., Beverly, MA, USA). Samples giving faint positive signals were analyzed a further two times and only those samples with consistent positive methylation band were considered CIHM.

Statistical analysis

Deviations from HWE were confirmed by the χ^2 test. The mean age was expressed as the mean $\pm$ standard deviation

and assessed by the Student's *t*-test. The *H. pylori* infection rate and sex ratio were assessed by Fisher's exact test. The genotype distributions and allele frequencies were also assessed by Fisher's exact test. The odds ratios and 95% confidence intervals for the strength of genotype involvement in CIHM were calculated using a logistic regression analysis adjusted for sex (male = 1), age, and the presence of *H. pylori* infection (infected = 1). The serum PG I/II ratio was assessed by the Student's *t*-test. All analyses were considered significant when $p < 0.05$. Stata software (version 13; StataCorp LP, College Station, TX, USA) was used for the statistical processing.

Results

The clinical background and genotype distribution of the 491 subjects are shown in Table 2. The distributions of the MAFK polymorphisms rs4268033, rs3735656, and rs10226620 were all in HWE ($p = 0.91$, 0.76 , and 0.09 , respectively). There were 288 subjects in the NA group and 203 in the AG group. Compared with the NA group, the mean age, sex ratio (males to females), and *H. pylori* infection rate were significantly higher in the AG group. There was no significant

TABLE 2. SUBJECT CHARACTERISTICS AND GENOTYPE FREQUENCIES

	Total	NA group	AG group	p ^a
No. of subjects	491	288	203	
Mean age $\pm$ SD	59.7 $\pm$ 13.3	57.5 $\pm$ 13.8	62.9 $\pm$ 11.9	<0.0001
Male:female	290:201	152:136	138:65	0.0008
<i>Helicobacter pylori</i> positive rate	309/491	119/288	190/203	<0.0001
rs4268033 G > A				
GG	257	161	96	NS
GA	196	104	92	
AA	38	23	15	NS
A allele frequency	27.7%	26.0%	30.0%	NS
rs3735656 T > C				
TT	229	155	74	0.0002
TC	210	107	103	
CC	52	26	26	NS
C allele frequency	32.0%	27.6%	38.2%	0.0005
rs10226620 C > T				
CC	241	160	81	0.0007
CT	194	96	98	
TT	56	32	24	NS
T allele frequency	31.2%	27.8%	36.0%	0.0078

^aNA group versus AG group.

AG, atrophic gastritis; NA, nonatrophic; NS, not significant; SD, standard deviation.

TABLE 3. ASSOCIATION BETWEEN *MAFK* POLYMORPHISMS AND GASTRIC MUCOSAL ATROPHY

	Genotype			OR (95% CI); p	OR (95% CI); p
rs4268033 G > A	GG	GA	AA	GG versus GA + AA	GG + GA versus AA
NA group (n = 288)	161	104	23	Reference	Reference
AG group (n = 203)	96	92	15	1.50 (0.966–2.34); 0.071	1.34 (0.606–2.96); 0.47
rs3735656 T > C	TT	TC	CC	TT versus TC + CC	TT + TC versus CC
NA group (n = 288)	155	107	26	Reference	Reference
AG group (n = 203)	74	103	26	2.10 (1.34–3.29); 0.0012	0.752 (0.379–1.49); 0.42
rs10226620 C > T	CC	CT	TT	CC versus CT + TT	CC + CT versus TT
NA group (n = 288)	160	96	32	Reference	Reference
AG group (n = 203)	81	98	24	1.98 (1.27–3.09); 0.0027	1.10 (0.563–2.14); 0.78

By logistic regression analysis after adjustment for age, gender (male = 1), and *Helicobacter pylori* infection status (infected = 1). CI, 95% confidence interval; OR, odds ratio.

difference in the distribution of the rs4268033 polymorphism between the AG and NA groups. Conversely, for the rs3735656 and rs10226620 polymorphisms, the wild homozygous frequencies were significantly lower and the minor allele frequencies were significantly higher in the AG group compared with the NA group.

The association of minor alleles of *MAFK* gene polymorphisms with gastric mucosal atrophy was examined in a regression analysis adjusted for sex, age, and *H. pylori* infection status (Table 3). In a dominant genetic model, carrying the minor allele of rs4268033 showed a tendency to be associated with the degree of gastric mucosal atrophy, but this association was not significant. By contrast, carrying the minor alleles of rs3735656 and rs10226620 displayed significant positive associations in the dominant genetic model.

The serum PG levels of 200 of the 491 enrolled subjects were evaluated. A comparison of the serum PG I/II ratios in the groups with wild homozygous and minor allele carriers for the *MAFK* gene polymorphisms is shown in Figure 1. No significant difference in the serum PG I/II ratio was found between the subjects with wild homozygous alleles and those with minor alleles for the rs4268033 polymorphism; conversely, for the rs3735656 and rs10226620 polymorphisms, the serum PG I/II ratios were significantly higher in the subjects with the wild homozygous alleles.

The clinical background of the 400 subjects who were evaluated for the degree of CpG methylation of *CDKN2A* is shown in Table 4. The distributions of the genotypes of rs4268033, rs3735656, and rs10226620 all met HWE ($p = 0.36$, 0.67 , and 0.91 , respectively). CIHM of $p14^{ARF}$ and $p16^{INK4a}$ was observed in 132 and 112 of the 400 subjects, respectively. The subjects with genes corresponding to either methylation had a higher mean age and *H. pylori* infection rate, and more of those subjects were classified in the AG group. A total of 50 and 206 subjects had CIHM of both genes and neither of the genes, respectively. The mean age, *H. pylori* infection rate, and AG/NA ratio were significantly higher in the both-methylated group than in the neither-methylated group (Table 4). The ratios of minor allele homozygosity for the three polymorphisms were significantly higher in both-methylated group compared with neither-methylated group. In addition, the minor allele frequencies of rs3735656 and rs10226620 were also higher in the both-methylated group.

For the *MAFK* polymorphism rs4268033, we found that the frequency of minor allele homozygosity showed a tendency, although nonsignificant, to be positively associated with $p14^{ARF}$ and $p16^{INK4a}$ methylations in a recessive genetic model (Table 5). The trend in the association was significant when comparing the both-methylated and neither-methylated groups.

FIG. 1. Comparison of serum PG I/II ratio among *MAFK* genotypes. There is no significant difference between wild homozygous and minor allele carriers of rs4268033 (3.85 ± 3.05 vs. 4.22 ± 3.41). Whereas, of both rs3735656 and rs10226620, serum PG I/II ratio was significantly lower in minor allele carriers than in wild homozygous (3.76 ± 3.10 vs. 4.36 ± 3.26 and 3.73 ± 3.18 vs. 4.37 ± 3.15 , respectively). PG, pepsinogen.

TABLE 4. CHARACTERISTICS AND PREVALENCE OF POLYMORPHISMS AND METHYLATION STATUS

	Overall	<i>p14</i> ^{ARF} -M	<i>p16</i> ^{INK4a} -M	Both methylated	Neither methylated	p ^a
The no. of subjects	400	132	112	50	206	
Mean age ± SD	60.2 ± 13.6	62.8 ± 13.6	61.5 ± 11.9	63.8 ± 12.3	58.7 ± 13.9	0.018
Male:female	230:170	69:53	61:51	25:25	125:81	NS
<i>Helicobacter pylori</i> -positive ratio	239/400	89/132	88/112	39/50	101/206	0.0002
NA:AG	229:171	57:75	42:70	20:30	150:56	<0.0001
rs4268033 G > A						
GG	181	57	53	23	94	NS
GA	183	58	44	17	98	
AA	36	17	15	10	14	0.016
A allele frequency	31.9%	34.8%	33.0%	37.0%	30.6%	NS
rs3735656 T > C						
TT	157	51	44	18	80	NS
TC	193	57	44	17	109	
CC	50	24	24	15	17	0.0001
C allele frequency	36.6%	39.8%	50.0%	47.0%	34.7%	0.028
rs10226620 C > T						
CC	164	51	44	18	87	NS
CT	183	58	44	17	98	
TT	53	23	24	15	21	0.0010
T allele frequency	36.1%	39.4%	50.0%	47.0%	34.0%	0.020

^aBoth methylated versus neither methylated.
M, methylated.

For the rs3735656, we found that the frequency of minor allele homozygosity was significantly associated with *p14*^{ARF} and *p16*^{INK4a} methylations using the recessive genetic model (Table 5). This association was stronger when comparing the both-methylated and neither-methylated groups.

For the rs10226620, we found that the frequency of minor allele homozygosity was not significantly associated with *p14*^{ARF} methylation, but was significantly associated with *p16*^{INK4a} methylation (Table 5). The frequency of minor

allele homozygosity was significantly associated with *p14*^{ARF} and *p16*^{INK4a} methylation when comparing the both-methylated and neither-methylated groups.

Discussion

Currently, three major types of human small Maf proteins are recognized (Kataoka *et al.*, 1995). These small Maf proteins have high homology with one another and

TABLE 5. ASSOCIATION BETWEEN *MAFK* POLYMORPHISMS AND *CDKN2A* METHYLATIONS

	Genotype (n)			OR (95% CI); p	OR (95% CI); p
rs4268033	GG	GA	AA	GG versus GA + AA	GG + GA versus AA
<i>p14</i> ^{ARF} -UM (268)	124	125	19	Reference	Reference
<i>p14</i> ^{ARF} -M (132)	57	58	17	1.17 (0.762–1.80); 0.47	1.92 (0.953–3.88); 0.068
<i>p16</i> ^{INK4a} -UM (288)	128	139	21	Reference	Reference
<i>p16</i> ^{INK4a} -M (112)	53	44	15	0.856 (0.542–1.35); 0.50	1.98 (0.922–3.95); 0.082
Neither methylated (n = 206)	94	98	14	Reference	Reference
Both methylated (n = 50)	23	17	10	1.05 (0.545–2.04); 0.88	3.58 (1.41–9.06); 0.0071
rs3735656	TT	TC	CC	TT versus TC + CC	TT + TC versus CC
<i>p14</i> ^{ARF} -UM (268)	106	136	26	Reference	Reference
<i>p14</i> ^{ARF} -M (132)	51	57	24	1.02 (0.654–1.58); 0.94	1.98 (1.07–3.66); 0.029
<i>p16</i> ^{INK4a} -UM (288)	113	149	26	Reference	Reference
<i>p16</i> ^{INK4a} -M (112)	44	44	24	0.870 (0.545–1.39); 0.56	2.50 (1.33–4.68); 0.0042
Unmethylated (n = 206)	80	109	17	Reference	Reference
Both methylated (n = 50)	18	17	15	1.05 (0.529–2.09); 0.89	4.49 (1.95–10.4); 0.0004
rs10226620	CC	CT	TT	CC versus CT + TT	CC + CT versus TT
<i>p14</i> ^{ARF} -UM (268)	113	125	30	Reference	Reference
<i>p14</i> ^{ARF} -M (132)	51	58	23	1.14 (0.738–1.77); 0.55	1.60 (0.875–2.91); 0.13
<i>p16</i> ^{INK4a} -UM (288)	120	139	29	Reference	Reference
<i>p16</i> ^{INK4a} -M (112)	44	44	24	1.00 (0.628–1.59); 1.00	2.45 (1.21–4.15); 0.0099
Unmethylated (n = 206)	87	98	21	Reference	Reference
Both methylated (n = 50)	18	17	15	1.32 (0.668–2.61); 0.42	3.45 (1.54–7.72); 0.0027

By logistic regression analysis after adjustment for age, gender (male = 1), and *H. pylori* infection status (infected = 1).
UM, unmethylated; M, methylated.

transcriptional activity through the formation of a homodimer or heterodimer with other small Maf proteins or with other basic leucine zipper (bZIP) transcription factors (Igarashi *et al.*, 1994). Previous studies using knockout mutant mice have shown that MafF^{-/-}/G^{-/-} mice and MafF^{-/-}/K^{-/-} mice are viable, whereas MafG^{-/-}/K^{-/-} was lethal in mice (Onodera *et al.*, 2000; Motohashi *et al.*, 2004). Other studies revealed that induction of the expression of the antioxidant enzyme HO-1 is controlled by the MafK-Bach1 (Broad-Complex, Tramtrack and Bric a brac and Cap'n'collar [CNC] homology 1) system and that the Nrf2-MafK heterodimer upregulates HO-1 expression (Sun *et al.*, 2002; Zhang *et al.*, 2006). Thus, MafK appears to play an important role in eliminating the ROS. Moreover, the HapMap-JPT population revealed a large number of SNP linkage blocks in the vicinity of the *MAFK* gene, whereas fewer SNP linkage blocks were found near the *MAFG* gene. Therefore, we thought that genetic polymorphisms of *MAFK* might play an essential role in human inflammatory diseases.

All three genetic polymorphisms studied in the current population of 491 subjects met HWE. Moreover, the distributions of the gene polymorphisms rs4268033 and rs10226620 did not deviate from the distributions of genotypes in the HapMap-CHB + JPT population ($p=0.84$ and 0.55 , respectively), and the distribution of rs3635656 did not deviate from the distribution in the HapMap-JPT population ($p=0.11$). Therefore, it can safely be stated that the genetic polymorphisms studied in this study are representative of the genetic polymorphisms found in the average Japanese population. We have already reported the association of these three *MAFK* gene polymorphisms with susceptibility to ulcerative colitis (UC) (Arisawa *et al.*, 2017). In that report, because the presence of homozygous minor alleles of the polymorphisms rs4368033 and rs3735656, which is a tag SNP with two large linkage blocks, was shown to be significantly associated with susceptibility to UC, we thought that the minor allele variants in the *MAFK* gene polymorphism are of proinflammatory individuals. In this study, the minor alleles of rs3735656 and rs10226620 were significantly associated with the severity of gastric mucosal atrophy according to the results of the dominant genetic model; they were also associated with the CpG methylation status of *CDKN2A* according to the recessive genetic model results. These data are in general agreement with our previous results.

Few studies have described associations between *MAFK* polymorphisms and various disorders, and most of those that have been published concerned associations with drug-induced hepatic injury (Nanashima *et al.*, 2012; Martínez-Hernández *et al.*, 2014; Chen *et al.*, 2019). Nanashima *et al.* (2012) reported that the minor allele of rs4720833 in the same linkage block as rs4268033 increased susceptibility to drug-induced liver injury based on a dominant genetic model, but the minor allele of rs3808337 in the same block as rs3735656 did not increase susceptibility. They also reported that minor alleles of *MAFK* polymorphisms in this linkage block promoted inflammation. As for gastric carcinogenesis, there is thought to be a sequence of steps from *H. pylori* infection to chronic inflammation of the stomach, gastric mucosal atrophy, accumulation of aberrant genetic CpG methylation, and finally carcinogenesis (Correa, 1992). Our results indicate that the minor alleles of *MAFK* are associated with the degree of gastric mucosal atrophy based on the dominant genetic model. The serum PG I/II ratio was also

reduced in carriers of minor alleles, indicating that minor alleles were significantly associated not only histologically but also with the extent of atrophic mucosa, because the degree of the decrease in PG I/II ratio serves as an indicator of the extent of gastric mucosal atrophy (Sipponen *et al.*, 2002). In addition, minor alleles were associated with increased methylation of *CDKN2A* based on the recessive genetic model. This might indicate that homozygous mutations have a greater effect on the MafK protein than heterozygous mutations, making subjects with homozygous mutations more likely to advance to the next step of disease development. The strong involvement of polymorphisms at the 3'-UTR of *MAFK* in this study suggests that these SNPs promote structural changes in 3'-UTR, which subsequently affect the interaction with certain microRNAs.

The mechanism of the involvement of *MAFK* gene polymorphisms in disease is unclear. One hypothesis is that changes in MafK expression lead to a reduction in the response to ROS or other toxic substances. The small Maf proteins reside in the nucleus and form a dimer with CNC-bZIP family proteins such as Bach1 (Fujiwara *et al.*, 1993). Under oxidative stress, the small Maf-Bach1 heterodimer leaves the antioxidant-responsive element and is replaced by the Maf-Nrf2 heterodimer, which binds and induces a group of antioxidant enzymes. Nanashima *et al.* (2012) speculated that polymorphisms in the 3'-UTR of *MAFK* induce the expression of MafK that dimerizes with Nrf2 and/or reduce the expression of MafK that dimerizes with Bach1. This could be the reason polymorphisms in *MAFK* 3'-UTR appeared to be associated with the severity of atrophic gastritis and CIHM of *CDKN2A* in our study. Another possibility is the result of the effects of *MAFK* on inflammation and immunity. A previous report showed that overexpression of the MafK protein in T cells inhibits T cell proliferation and causes the decreased secretion of interleukin-2 (IL-2; Yoh *et al.*, 2001). Hence, reduced MafK expression might stimulate IL-2 secretion and continued inflammation. Conversely, it has recently been revealed that MafK positively regulates nuclear factor- κ B (NF- κ B) expression (Hwang *et al.*, 2013), suggesting that the overexpression of MafK might induce inflammation through increased NF- κ B expression. In any case, further studies on the protein expression of *MAFK* gene polymorphisms are necessary.

In this study, we demonstrated a significant association between *MAFK* polymorphisms and the CIHM status. Both *p14^{ARF}* and *p16^{INK4a}* regulate the cell cycle negatively through the p53 and retinoblastoma protein pathways, respectively (Rizos *et al.*, 2000; Tannapfel *et al.*, 2001). Therefore, the possibility that the CpG methylation of both these genes might be directly involved in the development of gastric cancer cannot be excluded. Conversely, CpG methylation of *CDKN2A* has been reported relatively early in chronic inflammatory mucosa in non-neoplastic areas (Maekita *et al.*, 2006; Tahara *et al.*, 2007; Niwa *et al.*, 2010). CIHM and non-CpG hypomethylation have been observed in human cancers, which are known as changes in global DNA methylation patterns (Li and Chen, 2013). It is possible that the CpG methylation of *CDKN2A* investigated in this study is only part of this early change in global DNA methylation patterns. At any rate, the involvement of *MAFK* gene polymorphisms in gastric cancer development is of interest and is a subject for future study.

Conclusion

In conclusion, carrying the minor alleles of various *MAFK* polymorphisms, especially those located in the 3'-UTR, is associated with a higher risk of severe gastric mucosal atrophy, and CpG methylation of *CDKN2A* may occur in homozygous minor alleles of these polymorphisms.

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References

- Arisawa T, Nakamura M, Otsuka T, *et al.* (2017) Genetic polymorphisms of *MAFK*, encoding a small Maf protein, are associated with susceptibility to ulcerative colitis in Japan. *World J Gastroenterol* 23:5364–5370.
- Arisawa T, Tahara T, Shibata T, *et al.* (2007) The relationship between *Helicobacter pylori* infection and promoter polymorphism of the Nrf2 gene in chronic gastritis. *Int J Mol Med* 19:143–148.
- Arisawa T, Tahara T, Shibata T, *et al.* (2008) The influence of promoter polymorphism of nuclear factor-erythroid 2-related factor 2 gene on the aberrant DNA methylation in gastric epithelium. *Oncol Rep* 19:211–216.
- Bray F, Ferlay J, Soerjomataram I, *et al.* (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68:394–424.
- Chen S, Pan H, Chen Y, *et al.* (2019) Association between genetic polymorphisms of NRF2, KEAP1, MAFF, *MAFK* and anti-tuberculosis drug-induced liver injury: a nested case-control study. *Sci Rep* 9:14311.
- Correa P (1992) Human gastric carcinogenesis: a multistep and multifactorial process—First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 52: 6735–6740.
- Dixon MF, Genta RM, Yardley JH *et al.* (1996) Classification and grading of gastritis. The updated Sydney system. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 20:1161–1181.
- Fujiwara KT, Kataoka K, Nishizawa M (1993) Two new members of the maf oncogene family, mafK and mafF, encode nuclear b-Zip proteins lacking putative trans-activator domain. *Oncogene* 8:2371–2380.
- Hwang YJ, Lee EW, Song J, *et al.* (2013) MafK positively regulates NF- κ B activity by enhancing CBP-mediated p65 acetylation. *Sci Rep* 3:3242.
- Igarashi K, Kataoka K, Itoh K, *et al.* (1994) Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* 367:568–572.
- Ishii T, Itoh K, Takahashi S, *et al.* (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 275:16023–16029.
- Itoh K, Chiba T, Takahashi S, *et al.* (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 236:313–322.
- Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 3:415–428.
- Kataoka K, Igarashi K, Itoh K, *et al.* (1995) Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor. *Mol Cell Biol* 15: 2180–2190.
- Li W, Chen BF (2013) Aberrant DNA methylation in human cancers. *J Huazhong Univ Sci Technol Med Sci* 33: 798–804.
- Maekita T, Nakazawa K, Mihara M, *et al.* (2006) High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. *Clin Cancer Res* 12 Pt 1:989–995.
- Martínez-Hernández A, Gutierrez-Malacatt H, Carrillo-Sánchez K, *et al.* (2014) Small MAF genes variants and chronic myeloid leukemia. *Eur J Haematol* 92:35–41.
- Motohashi H, Katsuoka F, Engel JD, *et al.* (2004) Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc Natl Acad Sci U S A* 101:6379–6384.
- Naito Y, Yoshikawa T (2002) Molecular and cellular mechanisms involved in *Helicobacter pylori*-induced inflammation and oxidative stress. *Free Radic Biol Med* 33 323–336.
- Nanashima K, Mawatari T, Tahara N, *et al.* (2012) Genetic variants in antioxidant pathway: risk factors for hepatotoxicity in tuberculosis patients. *Tuberculosis (Edinb)* 92:253–259.
- Niwa T, Tsukamoto T, Toyoda T, *et al.* (2010) Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. *Cancer Res* 70:1430–1440.
- Onodera K, Shavit JA, Motohashi H, *et al.* (2000) Perinatal synthetic lethality and hematopoietic defects in compound mafG::mafK mutant mice. *EMBO J* 19:1335–1345.
- Ozasa K, Kurata JH, Higashi A, *et al.* (1999) *Helicobacter pylori* infection and atrophic gastritis: a nested case-control study in a rural town in Japan. *Dig Dis Sci* 44:253–256.
- Plummer M, de Martel C, Vignat J, *et al.* (2016) Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health* 4:e609–e616.
- Rizos H, Darmanian AP, Mann GJ, *et al.* (2000) Two arginine rich domains in the p14ARF tumour suppressor mediate nucleolar localization. *Oncogene* 19:2978–2985.
- Sipponen P, Härkönen M, Alanko A, *et al.* (2002) Diagnosis of atrophic gastritis from a serum sample. *Clin Lab* 48:505–515.
- Sun J, Hoshino H, Takaku K, *et al.* (2002) Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J* 21:5216–5224.
- Tahara T, Arisawa T, Shibata T, *et al.* (2007) Risk prediction of gastric cancer by analysis of aberrant DNA methylation in non-neoplastic gastric epithelium. *Digestion* 75:54–61.
- Tahara T, Arisawa T, Shibata T, *et al.* (2009) Increased number of methylated CpG islands correlates with *Helicobacter pylori* infection, histological and serological severity of chronic gastritis. *Eur J Gastroenterol Hepatol* 21:613–619.
- Tahara T, Shibata T, Nakamura M, *et al.* (2011) Polymorphisms of DNA repair and xenobiotic genes predispose to CpG island methylation in non-neoplastic gastric mucosa. *Helicobacter* 16:99–106.

- Tan P, Yeoh KG (2015) Genetics and molecular pathogenesis of gastric adenocarcinoma. *Gastroenterology* 149:1153–1162.
- Tannapfel A, Busse C, Weinans L, *et al.* (2001) INK4a-ARF alterations and p53 mutations in hepatocellular carcinomas. *Oncogene* 20:7104–7109.
- Uemura N, Okamoto S, Yamamoto S, *et al.* (2001) *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* 345:784–789.
- Watanabe Y, Kurata JH, Mizuno S, *et al.* (1997) *Helicobacter pylori* infection and gastric cancer. A nested case-control study in a rural area of Japan. *Dig Dis Sci* 42:1383–1387.
- Yoh K, Sugawara T, Motohashi H, *et al.* (2001) Transgenic over-expression of MafK suppresses T cell proliferation and function in vivo. *Genes Cells* 6:1055–1066.
- Zhang J, Ohta T, Maruyama A, *et al.* (2006) BRG1 interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative stress. *Mol Cell Biol* 26:7942–7952.

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