Functional promoter polymorphisms of the macrophage migration inhibitory factor gene in gastric carcinogenesis

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Abstract. The macrophage migration inhibitory factor (MIF) is a key proinflammatory mediator. Two functional polymorphisms have been identified in the promoter region of the MIF gene. We attempted to clarify the associations of these polymorphisms with the development of gastric cancer. The study was performed in 229 patients with gastric cancer and 428 subjects with no evidence of gastric malignancies on the upper gastro-duodenal endoscopy. The severity of histological chronic gastritis was classified according to the updated Sydney system. Overall, the 5-CATT carriers had a reduced risk of developing gastric cancer (OR, 0.67; 96% CI, 0.48-0.93; p=0.015), especially the diffuse type cancer. In subjects >60 years, the adjusted risk for gastric cancer among individuals who were -173C carriers was 1.71 (range, 1.03-2.84; p=0.038) compared to the G/G homozygous genotype. The number of 7-CATT alleles was also positively correlated with the development of intestinal type gastric cancer (adjusted OR, 1.70; 95% CI, 1.02-2.58; p=0.043). In subjects <60 years, the 7/7-CATT homozygous genotype was linked with a risk for the progression of atrophic gastritis (adjusted OR, 8.74; 95% CI, 1.31-58.6; p=0.026). In addition, the number of 7-CATT alleles was significantly correlated with the activity and inflammation scores (p=0.010 and 0.030, respectively). Our results suggested that functional promoter polymorphisms of the MIF gene are associated with the progression of gastric mucosal inflammation and the development of mucosal atrophy at an early stage in life and these genotypes may increase the risk for the subsequent development of gastric cancer, especially the intestinal type, in older subjects.

Introduction

Gastric cancer is one of the most common cancers worldwide, but the etiology of this tumor remains unclear. Infection with *Helicobacter pylori* (*H. pylori*) leads to persistent colonization and chronic inflammation of the gastric mucosa, thereby increasing the risk of developing peptic ulceration and gastric adenocarcinoma (1). However, there are marked interindividual differences in the extent of inflammation among persons with the *H. pylori* infection, so clinical consequences only develop in a small subgroup. Although bacterial virulence factors are associated with enhanced inflammation and cancer development (2,3), it is unclear why a considerable proportion of patients infected with cagA+ *H. pylori* strains do not develop severe pathologies throughout life. In addition to bacterial factors, mostly unknown host factors, such as genetic predisposition and host immunity, seem to influence the inflammatory response and the development of a more severe pathology. *H. pylori*-induced inflammation is implicated in the development of mucosal damage and is characterized by strong granulocytic and lymphocytic infiltration (4,5). The T helper cell response to *H. pylori* is considered to be dependent on type 1 helper (Th1) cells, but the factors influencing this immune response to the *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 (6-8).

The macrophage migration inhibitory factor (MIF) was originally identified as an activity isolated from T lymphocytes that was capable of inhibiting the random migration of macrophages (9,10). The human MIF cDNA was finally cloned in 1989 (11). MIF is a key proinflammatory mediator that plays a pivotal role in inflammatory and immune diseases (12). It contributes toward an excessive inflammatory response directly, via an induction of proinflammatory cytokine secretion (13), and indirectly through its ability to override the antiinflammatory activity of glucocorticoids (14). These

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mechanisms suggest critical roles for MIF in various cancers (15). Recently, Xia *et al* reported that the *H. pylori* infection increased the MIF expression in both gastric inflammatory and epithelial cells (16), thus MIF may play an important role in *H. pylori* related gastric inflammation.

Polymorphisms with potential functional relevance have been identified in the MIF gene promoter. Moreover, an SNP at the nucleotide position -173 (G to C) (17) and a tetra-nucleotide CATT repeat beginning at position -794 (18) have been found to be associated with altered levels of the MIF gene transcription *in vitro*. Although it has been demonstrated that the functional importance of these variants includes findings of significant association with several immune-mediated inflammatory diseases (17-19), the roles of these polymorphisms in the development of gastric disorders remain unclear.

In the present study, we attempted to clarify the associations of G-173C and -794 tetranucleotide repeats in the MIF gene promoter with gastric carcinogenesis.

Materials and methods

Clinical samples. As a gastric cancer group, 232 patients with gastric cancer (GC group), who were enrolled at the Endoscopy Center of Fujita Health University Hospital in 2006, were selected. As a control group, 430 subjects without malignant neoplasm were randomly selected from our stock DNA collected during the same period (non-GC group). The studied population comprised of 657 subjects whose polymorphisms could be clearly analyzed, including 229 in the GC group and 428 in the non-GC group.

All patients underwent upper gastrointestinal endoscopy and, in some of them, biopsy specimens were taken from antral mucosa. Parts of each specimen were fixed in 10% buffered formalin and embedded in paraffin, while the other parts were immediately frozen and stored at -80˚C. All histological diagnoses were conducted at the Division of Pathology at our hospital. The severity of chronic gastritis was also classified according to the updated Sydney system by a pathologist who had no access to any clinical information. According to the severity of gastric mucosal atrophy, the subjects were divided into the following 2 groups: the atrophic gastritis group (atrophy score ≥2) and the non-atrophy group (atrophy score ≤1). 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 the polymorphisms. The stock DNAs isolated from biopsy specimens or peripheral blood were used. Polymorphism was genotyped by the PCR-SSCP method as previously reported (20,21). To detect the -794 tetranucleotide repeat using the primer pair (MIFTRF: 5'-TGATCCAGTTG CTGCCTTGTC-3' and MIFTRR: 5'-TCCACTAATGGT AAACTCGGGGAC-3'), PCR was carried out in a volume of 20 ml containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 95˚C

Table I. Characteristics of the subjects and frequencies of genotypes.

	non-GC group	GC group
No. of subjects	428	229
Mean age ± SD	54.72±18.84	62.99±10.73
Male : female	253:175	163:66
HP-positive rate	66.0%	90.7%
n-CATT repeat		
5/5	66	36
5/6	143	54
5/7	59	30
5/8	0	1
6/6	76	50
6/7	75	46
6/8	0	1
7/7	9	11
5-CATT frequency	39.0%	34.3%
6-CATT frequency	43.2%	43.9%
7-CATT frequency	17.8%	21.4%
8-CATT frequency	0%	0.44%
G-173C genotype		
G/G	261	123
G/C	144	94
C/C	23	12
-173C frequency	22.2%	25.8%

for 30 sec, 62˚C for 40 sec and 72˚C for 45 sec, with a final extension at 72˚C for 5 min. Thereafter, 2 ml of the PCR product was denatured with 10 ml of formamide (Sigma-Aldrich Co., St. Louis, 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 G-173C polymorphism using the primer set (MIF173F: 5'-TCTAGCCGCCAAGTGGAGAACA-3' and MIF173R: 5'-ACTGTGGTCCCGCCTTTTGTGA-3'), the PCR reaction was carried out with 60˚C annealing temperature as described above. SSCP was also carried out as described above.

Statistical analysis. The strength of association between the allele frequencies and the disease was assessed by calculating the odds ratio (OR) and 95% confidence intervals (CI) by logistic regression analysis using a genotype as a variate or the number of alleles as a co-variate. Adjusted ORs were calculated with the use of logistic regression analysis after adjustment for age, gender and the *H. pylori* infection status. The Mann-Whitney U test and linear regression analysis were employed to test the association between MIF promoter polymorphisms and the updated Sydney system score. For all analyses, the level of significance was set at p<0.05.

Table II. Association between the MIF polymorphisms and gastric cancer.

	5-CATT cancer vs the others		The number of 7-CATT alleles[a]		-173C carrier vs the others	
	Unadjusted OR	Adjusted OR[b]	Unadjusted OR	Adjusted OR[b]	Unadjusted OR	Adjusted OR[b]
Overall						
Gastric cancer	0.67 (0.48-0.93)[c]	0.83 (0.56-1.23)	1.31 (0.98-1.75)	1.04 (0.72-1.48)	1.35 (0.97-1.86)	1.22 (0.82-1.80)
Intestinal type	0.70 (0.47-1.03)	0.79 (0.49-1.27)	1.38 (0.98-1.95)	1.23 (0.80-1.87)	1.47 (0.99-2.18)	1.59 (0.99-2.54)
Diffuse type	0.64 (0.41-0.99)[d]	0.96 (0.56-1.66)	1.22 (0.81-1.83)	0.74 (0.44-1.26)	1.19 (0.76-1.87)	0.79 (0.45-1.38)
≤60 years						
Gastric cancer	0.86 (0.52-1.42)	0.81 (0.41-1.64)	0.89 (0.56-1.42)	0.70 (0.37-1.33)	1.03 (0.63-1.69)	0.76 (0.40-1.47)
Intestinal type	1.19 (0.59-2.42)	0.94 (0.36-2.43)	0.82 (0.43-1.57)	0.79 (0.32-1.93)	1.13 (0.59-2.17)	1.01 (0.43-2.37)
Diffuse type	0.66 (0.35-1.23)	0.78 (0.35-1.77)	0.95 (0.53-1.72)	0.64 (0.29-1.39)	0.96 (0.51-1.79)	0.63 (0.28-1.42)
>60 years						
Gastric cancer	0.59 (0.38-0.92)[e]	0.71 (0.43-1.18)	1.65 (1.12-2.43)[g]	1.47 (0.93-2.34)	1.65 (1.07-2.56)[j]	111.71 (1.03-2.84)[l]
Intestinal type	0.59 (0.36-0.98)[f]	0.63 (0.35-1.11)	1.71 (1.10-2.64)[h]	1.70 (1.02-2.85)[i]	1.73 (1.05-2.85)[k]	2.10 (1.17-3.75)[m]
Diffuse type	0.60 (0.31-1.13)	0.98 (0.45-2.12)	1.58 (0.89-2.78)	0.98 (0.47-2.05)	1.51 (0.80-2.87)	1.06 (0.48-2.31)

[a]The number of 7-CATT alleles was used as a co-variate. [b]Adjusted for gender, age and the *H. pylori* infection status. Data are shown as OR (95% confidence intervals); [c]p=0.015, [d]p=0.045, [e]p=0.019, [f]p=0.040, [g]p=0.011, [h]p=0.017, [i]p=0.043, [j]p=0.025, [k]p=0.033.[l]p=0.038 and [m]p=0.012.
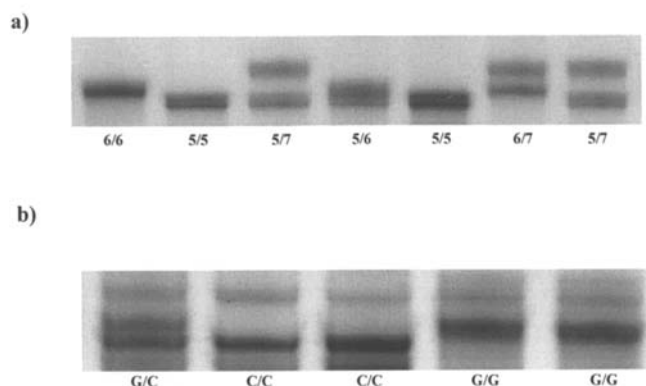

Figure 1. The images of PCR-SSCP using clinical samples. (a) Identification of the tetranucleotide (CATT) repeats (5/7) at position -794. (b) Identification of nucleotide (G/C) at position -173.

Results

The characteristics of subjects and the frequencies of genotypes. As shown in Fig. 1, single-strand DNAs of both -794 tetranucleotide repeats and G-173C genotypes were clearly separated by SSCP. The 8-CATT allele was found in only two patients with gastric cancer. These polymorphisms were in significant linkage disequilibrium, with the -173C allele strongly associated with the 7-CATT repeat allele. The most frequent haplotypes were -173G/5-CATT, -173G/6-CATT and -173C/7-CATT, which constituted ~90% of the haplotypes.

The characteristics and the frequencies of alleles in all 657 subjects are summarized in Table I. Compared to the non-GC group, the mean age, male/female ratio and *H. pylori* positivity were higher in the GC group.

The association between MIF promoter polymorphisms and gastric cancer. By using the unadjusted analysis, the 5-CATT allele carriers had a significantly reduced risk of developing gastric cancer (OR, 0.67; 95% CI, 0.48-0.93; p=0.015), especially the diffuse type (Table II). In subjects >60 years old, the -173C allele carriers had a significantly increased risk of developing gastric cancer, especially the intestinal type of gastric cancer, by using unadjusted and adjusted analyses (OR, 1.73; 95% CI, 1.05-2.85 and OR, 2.10; 95% CI, 1.17-3.75, respectively). The number of 7-CATT alleles was also positively correlated with the development of the intestinal type gastric cancer (OR, 1.70; 95% CI, 1.02-2.58; p=0.043). On the other hand, there were no significant associations between the MIF promoter polymorphisms and the development of gastric cancer in subjects <60 years old.

The association between the MIF promoter polymorphisms and clinicopathological features of gastric cancer. The frequency of the 7/7-CATT homozygous genotype in the lymph invasion-positive or venous invasion-positive cases was significantly higher compared to the non-GC group (Table III). In addition, the 5-CATT carriers had a reduced risk and the -173C carriers had an increased risk for the peritoneal dissemination of cancer cells.

The association between the MIF promoter polymorphisms and chronic gastritis. The severity of histological gastritis was assessed in 420 out of 657 subjects. The frequency of the 7/7-CATT homozygous genotype in the atrophic gastritis group was significantly higher than that in the non-atrophy group. By using the adjusted analysis, 7/7-CATT homozygous genotype also had an increased risk of developing gastric mucosal atrophy in subjects <60 years old (OR, 8.74; 95% CI, 1.31-58.6; p=0.026). In addition, the number of 7-CATT alleles was positively correlated with the activity and

Table III. Association between the MIF polymorphisms and clinicopathological features of gastric cancer.

	5-CATT carrier vs the others		7/7-CATT vs the others		-173C carrier vs the others	
	Unadjusted OR	Adjusted OR[a]	Unadjusted OR	Adjusted OR[a]	Unadjusted OR	Adjusted OR[a]
Cancer stage						
≤TI	0.83 (0.52-1.30)	0.73 (0.44-1.20)	1.02 (0.22-4.82)	0.77 (0.15-3.79)	1.13 (0.72-1.78)	1.17 (0.72-1.92)
≥T2	0.88 (0.54-1.43)	0.92 (0.55-1.55)	1.84 (0.49-6.95)	1.72 (0.42-7.01)	1.12 (0.69-1.83)	1.15 (0.69-1.93)
Lymph invasion						
Positive	0.98 (0.59-1.63)	0.96 (0.56-1.66)	3.37 (1.10-10.4)[c]	2.76 (0.84-9.09)	1.19 (0.72-1.96)	1.22 (0.72-2.07)
Negative	0.82 (0.49-1.38)	0.73 (0.42-1.27)	NC	NC	0.95 (0.56-1.60)	0.95 (0.54-1.67)
Venous invasion						
Positive	1.19 (0.56-2.53)	1.31 (0.59-2.88)	4.66 (1.20-18.1)[d]	4.64 (1.07-20.1)[e]	1.66 (0.82-3.38)	1.83 (0.87-3.85)
Negative	0.83 (0.54-1.27)	0.75 (0.47-1.19)	0.86 (0.18-4.05)	0.61 (0.12-3.03)	0.93 (0.60-1.43)	0.91 (0.57-1.47)
Lymph node metastasis						
Positive	0.84 (0.50-1.39)	0.86 (0.50-1.48)	2.02 (0.54-7.66)	2.17 (0.52-9.12)	1.12 (0.67-1.85)	1.18 (0.69-2.02)
Negative	0.86 (0.55-1.34)	0.78 (0.48-1.26)	0.95 (0.20-4.47)	0.69 (0.14-3.38)	1.13 (0.73-1.76)	1.16 (0.72-1.88)
Peritoneal dissemination						
Positive	0.43 (0.19-0.98)[b]	0.43 (0.18-1.02)	NC	NC	2.19 (0.95-5.04)	2.37 (1.00-5.60)[f]
Negative	0.95 (0.65-1.40)	0.91 (0.60-1.39)	1.63 (0.54-4.94)	1.31 (0.40-4.21)	1.01 (0.69-1.48)	1.03 (0.68-1.57)

[a]Adjusted for gender, age and the *H. pylori* infection status. Data are shown as OR (95% confidence intervals). NC, not calculated; [b]p=0.045, [c]p=0.034, [d]p=0.027, [e]p=0.041 and [f]p=0.050.
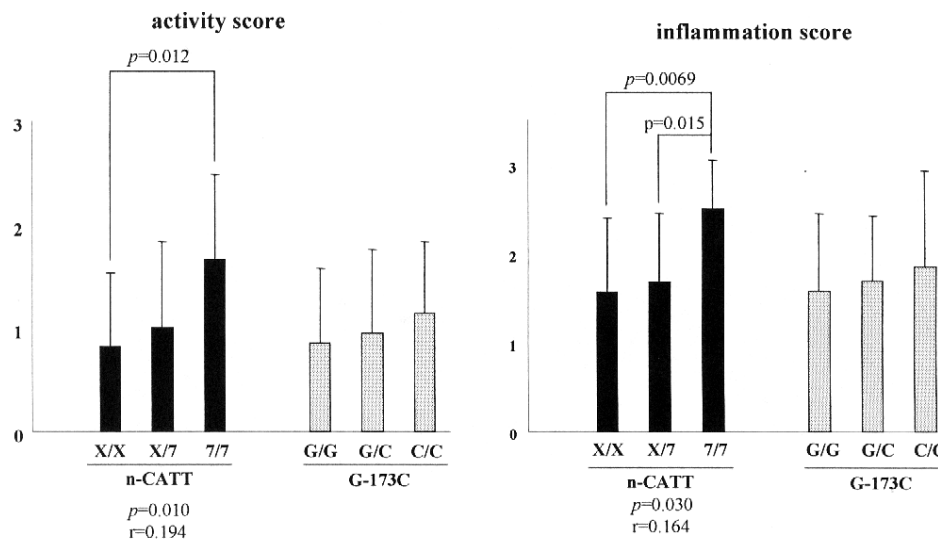

Figure 2. The association between promoter polymorphisms of the MIF gene and the activity or inflammation score in subjects <60 years old. The comparison between the two groups was performed by the Mann-Whitney U test and the associations between the number of 7-CATT alleles and each score was assessed by a linear regression analysis.

inflammation scores in subjects <60 years old (p=0.010 and 0.030, respectively, Fig. 2), whereas no correlations were found in subjects >60 years old.

Discussion

Results from our study suggested that the 7-CATT repeat of the MIF gene promoter at position -794 was significantly associated with a risk for the severity of gastric mucosal inflammation in younger subjects and for the subsequent development of gastric mucosal atrophy. Furthermore, both the -173C allele and 7-CATT together were found to confer a higher risk of developing gastric cancer, especially the intestinal type of gastric cancer, in older (>60 years old) subjects. Since the -173C allele was strongly associated with the 7-CATT repeats, the -173C/7-CATT haplotype may be

Table IV. Association between the MIF polymorphisms and gastric mucosal atrophy.

	5-CATT carrier vs the others		7/7-CATT vs the others		-173C carrier vs the others	
	Unadjusted OR	Adjusted OR[a]	Unadjusted OR	Adjusted OR[a]	Unadjusted OR	Adjusted OR[a]
Overall subjects	1.00 (0.67-1.51)	0.89 (0.56-1.42)	3.12 (1.00-9.71)[b]	3.47 (0.96-12.6)	0.98 (0.65-1.47)	0.98 (0.62-1.55)
≤60years	1.21 (0.61-2.41)	0.85 (0.40-1.85)	5.08 (0.90-28.7)	8.74 (1.31-58.6)[c]	0.90 (0.46-1.74)	0.90 (0.43-1.86)
>60years	0.94 (0.56-1.58)	0.88 (0.49-1.61)	2.18 (0.48-9.96)	2.01 (0.35-11.4)	1.03 (0.61-1.72)	1.14 (0.63-2.09)

The frequency of genotype was compared between the atrophic gastritis group (atrophy score ≥2) and non-atrophy group (atrophy score ≤1). [a]Adjusted for gender, age and the *H. pylori* infection status. Data are shown as OR (95% confidence intervals); [b]p=0.050 and [c]p=0.026.

associated with gastric carcinogenesis. In addition, the 5-CATT allele carriers had a reduced risk for developing gastric cancer, especially the diffuse type cancer.

It is generally believed that MIF functions as a cytokine to promote the recruitment of neutrophils and macrophages and the migration of these cells to the site of inflammation (22). MIF is involved in T cell proliferation by promoting the secretion of interleukin-2 (23) and can deliver a priming signal to neutrophils to mobilize them into producing an immediate and robust response in the presence of pathogens (24). In addition, MIF can induce production of the tumor necrosis factor and inducible nitric oxide (25). Through these effects on inflammation, an important role of MIF on gastric disorders has been shown, such as gastric inflammation (15), ulcer (24) and carcinogenesis (26). However, there is no report regarding the association between functional promoter polymorphisms of the MIF gene and gastric disorders, although several studies have shown significant associations of these polymorphisms with cystic fibrosis (27), psoriasis (28), atopic disorders (29) and inflammatory bowel disease (30).

Baugh *et al* have reported the correlation of the -794 5-CATT repeat with low disease severity in rheumatoid arthritis patients (18) and Hizawa *et al* have also reported an increased risk of non-794 5-CATT carriers for atopy (28). Donn *et al* have demonstrated that the -173C/7-CATT haplotype is of importance in the susceptibility to psoriasis (16). Thus, 5-CATT seems to correlate with low inflammation severity, whereas 7-CATT seems to be associated with an increased risk for inflammation. These results support our results that 7-CATT was associated with the severity of gastric inflammation and with the development of gastric mucosal atrophy.

The promoter sequence analysis indicates that the -173C allele creates a potential activator protein-4 transcription-factor binding site (16) and levels of the MIF expression significantly differed among the G-173C genotypes in a cell-type manner. Regarding the CATT repeats, the 5-CATT allele was shown to be associated with a lower basal and stimulated MIF promoter activity *in vitro* than 6-, 7- and 8-CATT alleles. Donn *et al* have shown that an increasing CATT repeat with the -173C allele significantly increased the promoter activity in a T lymphoblast cell line (17). Thus, the -173C and 7-CATT haplotype together seemed to promote the maximal production of MIF and the 7-CATT allele may thus promote the development of gastric inflammation and mucosal atrophy.

Since we found significant interactions between the age and the number of -173C and 7-CATT alleles by ANCOVA (p=0.037 and 0.019, respectively), the age-specific analysis was performed. Then, a significant relationship between the number of 7-CATT alleles and the inflammatory cell infiltrations was seen only in subjects <60 years old. It has been shown that long-term exposure to *H. pylori* is a significant risk factor for the progression of atrophic and metaplastic gastritis (31,32). In older subjects, gastric mucosal inflammation may decrease because of the progression of gastric atrophy and metaplasia. In addition, a lower serum MIF level was exhibited in older rats (33). Therefore, MIF may promote the gastric mucosal inflammation at an early stage in life, whereas it may not in older subjects. As a result, gastric mucosal atrophy may develop at an earlier age in the 7-CATT carriers.

According to the Lauren classification (34), there are two histologically distinct types of gastric cancer. The intestinal type consists of gland-like structures that mimic the intestinal glands and a series of precancerous lesions are recognized. The diffuse type of gastric cancer lacks any glandular structures and arises closer to the advancing edge of the gastric mucosal inflammation without any identifiable histological precursor lesion (35). The former develops in the stomach affected by chronic inflammation and passes through the intermediate steps of atrophic gastritis or intestinal metaplasia (36). On the other hand, the severity of mucosal inflammation and various host features may directly induce mutagenetic events that ultimately lead to the onset of the latter. Our findings suggested that the -173C/7-CATT haplotype may be related to the severity of gastric mucosal atrophy at a younger age because of the more strongly activated MIF gene expression and may increase the risk for the development of gastric mucosal atrophy-related disorders, such as the intestinal type of gastric cancer, at an older age. The 5-CATT allele may suppress the gastric mucosal inflammation that leads to the progression of the diffuse type gastric cancer.

In conclusion, the present study demonstrated that the 7-CATT repeat and -173C allele of the MIF gene promoter was associated with the progression of gastric mucosal inflammation and the development of gastric mucosal atrophy in younger subjects and these allele carriers may have an increased risk for the subsequent development of gastric cancer, especially the intestinal type. The 5-CATT repeat may reduce the risk for the development of diffuse type gastric cancer via decreased gastric inflammation.

Acknowledgements

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