# Functional promoter polymorphisms of macrophage migration inhibitory factor in peptic ulcer diseases

HISAKAZU SHIROEDA[1], TOMOMITSU TAHARA[2], TOMOYUKI SHIBATA[2], MASAKATSU NAKAMURA[2], HIDETO YAMADA[1], TOMOE NOMURA[1], RANJI HAYASHI[1], TAKASHI SAITO[1], TOMOKI FUKUYAMA[1], TOSHIMI OTSUKA[1], HIROKAZU YANO[1], KAZUAKI OZAKI[1], MUTSUMI TSUCHISHIMA[1], MIKIHIRO TSUTSUMI[1] and TOMIYASU ARISAWA[1]

[1]Department of Gastroenterology, Kanazawa Medical University; 1-1 Daigaku, Uchinada-machi, Ishikawa 920-0293; [2]Department of Gastroenterology, Fujita Health University, 1-98, Dengakugakubo, Kutsukake-cho, Toyoake 470-1192, Japan

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**Abstract.** Macrophage migration inhibitory factor (MIF) is a key proinflammatory mediator, which plays a pivotal role in inflammatory and immune diseases. We attempted to clarify the association of functional polymorphisms of MIF gene promoter with the development of gastro-duodenal ulcer. The study was performed in 471 stocked DNAs obtained from the subjects, including 93 healthy volunteers, with no evidence of gastric malignancy. We employed the PCR-SSCP method to detect gene polymorphisms. In all 471 DNAs, 92 and 43 were obtained from gastric and duodenal ulcer patients, respectively. By an unadjusted analysis, infection with *Helicobacter pylori* (*H. pylori*), male gender and non-steroidal anti-inflammatory drug (NSAID/aspirin) use were significantly associated with a risk for developing a gastric ulcer, whereas MIF promoter polymorphisms were not. On the other hand, infection with *H. pylori*, male gender and 7-CATT repeat at position -794 were significantly associated with the development of a duodenal ulcer, whereas NSAID/aspirin use was not. By the analysis after adjustment for age, gender, NSAID/aspirin use and *H. pylori* infection status, 7/7-CATT homozygote had a significantly increased risk for the development of duodenal ulcers (OR, 6.31; 95% CI, 1.50-26.6; p=0.012). No factors were significantly associated with the development of peptic ulcers in NSAID/aspirin users. Our results suggested that tetranucleotide repeat polymorphism of MIF gene promoter might be associated with the development of duodenal ulcers.

*Correspondence to*: Dr Tomiyasu Arisawa, Department of Gastroenterology, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Ishikawa 920-0293, Japan
E-mail: tarisawa@kanazawa-med.ac.jp

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## Introduction

It is well known that *Helicobacter pylori* (*H. pylori*) infection, as well as non-steroidal anti-inflammatory drug (NSAID/aspirin) use, are major contributing factors to the development of peptic ulcers (1). Infection with *H. pylori* usually leads to persistent colonization and chronic gastric inflammation. However, there are marked differences in the extent of inflammation among *H. pylori*-infected patients, so clinical consequences only develop in a small subgroup. The course of *H. pylori* infection may be influenced by bacterial virulence factors, as well as by genetic predisposition and host immunity. Inflammation induced by *H. pylori* is implicated in gastric mucosal damage and characterized by severe granulocytic and lymphocytic infiltration (2). Although the T helper cell response to *H. pylori* is considered to be dependent on type 1 helper (Th1) cells, the factors influencing this immune response to *H. pylori* infection are largely unknown. Important cytokines that are related to Th1-mediated responses and are upregulated during chronic *H. pylori* infection include interferon-γ, tumor necrosis factor, and interleukin-1ß (3-5).

Macrophage migration inhibitory factor (MIF) was originally identified as an activity isolated from T lymphocytes that is capable of inhibiting the random migration of macrophages (6,7). The human MIF cDNA was finally cloned in 1989 (8). MIF is a key proinflammatory mediator, which plays a pivotal role in inflammatory and immune diseases (9-11). It contributes toward an excessive inflammatory response both directly via an induction of proinflammatory cytokine secretion (12) and indirectly through its ability to override the anti-inflammatory activity of glucocorticoids (13). Xia *et al* hve reported that *H. pylori* infection increased MIF expression in both gastric inflammatory and epithelial cells (14), thus MIF may play an important role in *H. pylori*-related gastric inflammation and ulceration.

Polymorphisms with potential functional relevance have been identified in the MIF gene promoter, an SNP at position -173 (G to C) (15) and a tetranucleotide CATT repeat beginning at nucleotide position -794 (16) have been found to be associated with altered levels of MIF gene transcription

*in vitro*. It has been demonstrated that the functional importance of these variants includes findings of significant association with several immune-mediated inflammatory diseases (15-17). We have already reported the close association between MIF promoter polymorphisms and gastric mucosal atrophy followed by gastric carcinogenesis (18,19). However, the roles of these polymorphisms in the development of gastro-duodenal peptic ulceration remain unclear.

In the present study, we attempted to clarify the associations of G-173C and -794 CATT repeat in MIF gene promoter with the development of gastro-duodenal ulcers.

## Materials and methods

*Clinical samples*. We randomly selected 386 samples from our stocked DNA obtained from patients enrolled the Endoscopy Center of Fujita Health University Hospital between 2006-2009. All patients underwent upper gastrointestinal endoscopy and, in some patients, biopsy specimens were taken from antral mucosa. Parts of each specimen was fixed in 10% buffered formalin and embedded in paraffin, while the other part was immediately frozen and stored at -80˚C. Finally, the study population comprised 471 subjects, including 93 healthy volunteers, with no neoplastic lesions whose DNA was clearly analyzed.

*H. pylori* infection status was assessed by serology, histological examination, or the urea breath test. Patients were diagnosed as having an 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 participating subjects.

*Genotyping of polymorphisms*. Sample stocked DNAs isolated from biopsy specimens or peripheral blood were used. Polymorphism was genotyped by PCR-SSCP method as reported previously (18,19). To detect -794 CATT repeats, using the primer pair (MIFTRF, 5'-TGATCCAGTTGCTGCCTTGTC-3', and MIFTRR, 5'-TCCACTAATGGTAAACTCGGGGAC-3'), PCR was carried out in a volume of 20 $\mu$l containing 0.1 $\mu$g of genomic DNA. DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 95˚C for 30 sec, 62˚C for 40 sec, and 72˚C for 45 sec, with final extension at 72˚C for 5 min. Thereafter, 2 $\mu$l of the PCR product was denatured with 10 $\mu$l 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 (GE Healthcare, USA), after which the denatured single strand DNA bands were detected using a DNA Silver Staining Kit (GE Healthcare).

To detect G-173C polymorphism, using the primer set (MIF173F, 5'-TCTAGCCGCCAAGTGGAGAACA-3' and MIF173R, 5'-ACTGTGGTCCCGCCTTTTGTGA-3'), PCR reaction was carried out with 60˚C annealing temperature as described above. SSCP was also carried out as described above.

*Statistical analysis*. The data of age were expressed as mean ± SD. Mean ages between 2 groups were compared with Student's t-test. Allele and genotype frequencies were calculated by direct counting. The allele counts were compared by a 2x2 table using Chi-squared test. The strength of association between allele frequencies and the disease was assessed by calculating the odds ratio (OR) and 95% confidence intervals (CI) by logistic regression analysis using genotype as a variate or the number of allele as a co-variate. Adjusted ORs were calculated with the use of a regression analysis after adjustment for age, gender, NSAIDs/aspirin use and *H. pylori* infection status. For all analyses, the level of significance was set at $p<0.05$.

## Results

*Characteristics of subjects and frequencies of genotypes*. Single strand DNAs of both -794 repeat and G-173C genotypes were clearly separated by SSCP (18,19). A single strand band of 8-CATT repeat was not detected in any of the 471 subjects. 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 haplotypes.

In the 471 subjects, there were 135 with peptic ulcer (92 with gastric ulcer and 43 with duodenal ulcer, Table I). Compared with the non-ulcer group including 93 volunteers, mean age, male/female ratio and *H. pylori* positivity were higher in the ulcer group. The frequencies of n-CATT and -173C alleles were not significantly different between the 2 groups.

*Association between MIF promoter polymorphisms and peptic ulcer*. By the unadjusted analysis, male gender, *H. pylori* infection, NSAID/aspirin use and 7-CATT repeat were significantly associated with the development of peptic ulcers (Table II). Infection with *H. pylori*, male gender and NSAID/aspirin use were significantly associated with a risk for the development of gastric ulcers, whereas MIF promoter polymorphisms were not. On the other hand, infection with *H. pylori*, male gender and 7-CATT repeat were significantly associated with the development of duodenal ulcers, whereas NSAIDs/aspirin use were not.

By the analysis after adjustment for age, gender, NSAID/aspirin use and *H. pylori* infection status, 7/7-CATT homozygote had a significantly increased risk for the development of duodenal ulcers (OR, 6.31; 95%CI, 1.50-26.6; p=0.012; Table III).

*Association between MIF promoter polymorphisms and peptic ulcer in NSAID/aspirin users*. There were 47 NSAIDs/aspirin users, 24 subjects with peptic ulcer (18 with gastric ulcer and 6 with duodenal ulcer), of all 471 subjects. As shown in Table IV, male gender, infection with *H. pylori* and MIF promoter polymorphism were not significantly associated with a risk for the development of peptic ulcer disease in NSAID/aspirin users.

## Discussion

Results from our study suggested that 7-CATT repeat of MIF gene promoter at a position -794 was significantly associated with a risk for the development of peptic ulcers, especially duodenal ulcer. In addition, this polymorphism did not appear to affect the developing NSAID/aspirin-induced ulcer.

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

| | Non-ulcer | Peptic ulcer | Gastric ulcer | Duodenal ulcer |
|---|---|---|---|---|
| n | 336 | 135 | 92 | 43 |
| Mean age ± SD | 52.6±19.7 | 62.7±13.6 | 65.5±11.7 | 56.9±15.6 |
| Male:female | 180:156 | 102:33 | 70:22 | 32:11 |
| HP positive rate | 55.6% | 82.7% | 85.6% | 76.7% |
| n-CATT repeat | | | | |
| 5/5 | 53 | 17 | 11 | 6 |
| 5/6 | 112 | 41 | 28 | 13 |
| 5/7 | 47 | 14 | 11 | 3 |
| 6/6 | 61 | 24 | 17 | 7 |
| 6/7 | 56 | 30 | 20 | 10 |
| 7/7 | 7 | 9 | 5 | 4 |
| 5-CATT frequency | 39.4% | 33.0% | 33.2% | 32.6% |
| 6-CATT frequency | 43.2% | 44.1% | 44.6% | 43.0% |
| 7-CATT frequency | 17.4% | 23.0% | 22.3% | 24.4% |
| G-173C genotype | | | | |
| G/G | 207 | 77 | 53 | 24 |
| G/C | 111 | 46 | 31 | 15 |
| C/C | 18 | 12 | 8 | 4 |
| -173C frequency | 21.9% | 25.9% | 25.5% | 26.7% |

Table II. Association between gastro-duodenal ulcer and various risk factors.

| | OR (95% confidence intervals) | | |
|---|---|---|---|
| Variables | Peptic ulcer | Gastric ulcer | Duodenal ulcer |
| 5/5-CATT | 0.77 (0.43-1.38) | 0.73 (0.36-1.45) | 0.87 (0.35-2.15) |
| 7/7-CATT | 3.36 (1.22-9.21)[a] | 2.70 (0.84-8.72) | 4.82 (1.35-17.2)[c] |
| No. of 7-CATT[d] | 1.42 (1.00-2.02)[b] | 1.38 (0.91-2.08) | 1.56 (0.90-2.68) |
| -173 C/C | 1.72 (0.81-3.68) | 1.45 (0.59-3.54) | 1.81 (0.58-5.63) |
| Male gender | 2.68 (1.71-4.19) | 2.76 (1.63-4.66) | 2.52 (1.23-5.17) |
| NSAIDs/aspirin use | 2.94 (1.60-5.42) | 3.31 (1.70-6.45) | 2.21 (0.84-5.77) |
| *H. pylori* infected | 3.82 (2.28-6.40) | 4.74 (2.49-8.97) | 2.64 (1.24-5.59) |

[a]p=0.019; [b]p=0.015; [c]p=0.049 by unadjusted analysis; [d]analyses were performed using the number of 7-CATT allele as a co-variate.

Table III. Association between gastro-duodenal ulcer and MIF gene polymorphisms.

| | OR (95% confidence intervals) | | |
|---|---|---|---|
| Variables | Peptic ulcer | Gastric ulcer | Duodenal ulcer |
| 5/5-CATT | 0.93 (0.48-1.83) | 0.88 (0.40-1.94) | 0.98 (0.36-2.65) |
| 7/7-CATT | 3.06 (0.98-9.56) | 2.38 (0.63-9.01) | 6.31 (1.50-26.6)[a] |
| No. of 7-CATT[b] | 1.40 (0.94-2.09) | 1.40 (0.87-2.23) | 1.43 (0.79-2.58) |
| -173 C/C | 1.35 (0.58-3.11) | 1.21 (0.47-3.15) | 1.79 (0.53-6.05) |

Logistic regression analysis after adjustment for age, gender, NSAIDs/aspirin use and *H. pylori* infection status; [a]p=0.012; [b]analyses were performed using the number of 7-CATT allele as a co-variate.

Table IV. Association between gastro-duodenal ulcer and various risk factors in NSAIDs/aspirin users.

| | OR (95% confidence intervals) | |
|---|---|---|
| | Unadjusted OR | Adjusted OR variables[a] |
| 5/5-CATT | 0.96 (0.12-7.41) | 1.04 (0.11-10.3) |
| 7/7-CATT | 0.96 (0.12-7.41) | 1.94 (0.21-18.0) |
| No. of 7-CATT[b] | 1.29 (0.52-3.15) | 1.76 (0.64-4.84) |
| -173 C/C | 0.61 (0.092-4.01) | 0.93 (0.12-7.51) |
| Male gender | 2.60 (0.80-8.49) | - |
| *H. pylori* infected | 1.10 (0.34-3.55) | - |

[a]Adjusted for gender, age, *H. pylori* infection status; [b]analyses were performed using the number of 7-CATT allele as a co-variate.

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 (20). MIF is involved in T-cell proliferation through promoting the secretion of interleukin-2 (21) and can deliver a priming signal to neutrophils to mobilize them to produce an immediate and robust response in the presence of pathogens (22). In addition, MIF can induce the production of tumor necrosis factor (TNF) and inducible nitric oxide (iNOS) (23). Many studies have shown MIF to be a key modulator of many chronic and disabling human disorders, such as rheumatoid arthritis (24), sepsis (25), acute respiratory syndrome (26), and atopic diseases (27,28). An important role of MIF on gastric disorders has been also shown, such as gastric inflammation (14), ulcer (23) and carcinogenesis (29). However, there is no report regarding the association between MIF gene promoter functional polymorphisms and gastric disorders, although several studies have shown significant associations of these polymorphisms with cystic fibrosis (30), psoriasis (31), and atopic disorders (32).

Baugh *et al* have reported the correlation of -794 5-CATT repeat with low disease severity in rheumatoid arthritis patients (17) and Hizawa *et al* have also reported an increased risk of non -794 5-CATT carriers for atopy (32). Donn *et al* have demonstrated that -173C/-794 7-CATT haplotype is of importance in susceptibility to psoriasis (15). Thus, 5-CATT seems to correlate with low inflammation severity, whereas 7-CATT seems to be associated with an increased risk for inflammation. These findings support our results that 7-CATT repeat is associated with the development of peptic ulcers.

Promoter sequence analysis indicates that the -173C allele creates a potential activator protein 4 transcription factor binding site (15) and levels of MIF expression significantly differed among G-173C genotypes in a cell-type manner. Regarding CATT repeats, the 5-CATT allele was shown to be associated with lower basal and stimulated MIF promoter activity *in vitro* than 6-, 7- and 8-CATT alleles (16). Donn *et al* have shown that increasing CATT repeat with the -173C allele significantly increased the promoter activity in a T lymphoblast cell line (15). Thus, -173C allele and 7-CATT seemed to promote the production of MIF, subsequently 7-CATT may produce the development of peptic ulcers.

In our results, *H. pylori* infection and male gender were significantly associated with the development of both gastric and duodenal ulcers. On the other hand, MIF promoter polymorphisms were associated with the duodenal ulcer and NSAID/aspirin use was an increased risk for the gastric ulcer. After age, gender, *H. pylori* infection and NSAID/aspirin use were controlled, there was a significant association between 7/7-CATT homozygote and the presence of duodenal ulcer. This fact suggests that tetranucleotide repeats of MIF gene promoter is an independent factor in the pathogenesis of duodenal ulcer. Gastric and duodenal ulcers share many common features including pathogenesis, diagnosis, and treatment, but several factors distinguish the two. As for duodenal ulcers, the majority of gastric ulcers can be attributed to either *H. pylori*- or NSAID-induced mucosal damage. Gastric ulcers tend to occur later in life than duodenal ulcers. Duodenal ulcer has more positive correlation with antral gastritis than gastric ulcer (33). The exact mechanism of the formation of peptic ulcers is beyond the scope of this study. However, the effects of the MIF gene polymorphism on the formation of peptic ulcers were thought to depend on whether ulcers occur in the stomach or the duodenum.

Interestingly, no factors, including male gender, *H. pylori* infection and MIF gene polymorphisms, were significantly associated with the development of peptic ulcers in NSAID/aspirin users. NSAID/aspirin induce gastric mucosal damage, including bleeding, ulceration, and perforation (34). These effects are caused by the inhibition of cyclooxygenase, which catalyses the formation of prostaglandin from arachidonic acid (35). That is, the pathogenetic mechanisms of ulcer formation distinguish between *H. pylori* infection and NSAID/aspirin use. Moreover, it has been reported that NSAIDs suppressed the expression of MIF (36). Therefore, we think that MIF gene polymorphisms were not associated with the development of NSAID/aspirin-induced ulcer.

In conclusion, tetranucleotide repeat polymorphism of MIF gene promoter might be associated with the development of peptic ulcer, especially duodenal ulcer. However, there seemed to be no association between this polymorphism and NSAID/aspirin-induced ulcer.

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