SPANDIDOS PUBLICATIONS

Association between IL-17A, -17F and MIF polymorphisms predispose to CpG island hyper-methylation in gastric cancer

TOMOMITSU TAHARA[1], TOMOYUKI SHIBATA[1], MASAKATSU NAKAMURA[1], HIROMI YAMASHITA[1], DAISUKE YOSHIOKA[1], MASAAKI OKUBO[1], JOH YONEMURA[1], YOSHITERU MAEDA[1], NAOKO MARUYAMA[1], TOSHIAKI KAMANO[1], YOSHIO KAMIYA[1], HIROSHI FUJITA[1], YOSHIHITO NAKAGAWA[1], MITSUO NAGASAKA[1], MASAMI IWATA[1], ICHIRO HIRATA[1] and TOMIYASU ARISAWA[2]

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

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Abstract. CpG island hyper-methylation (CIHM) is one of the major events in the gastric carcinogenesis. IL-17A, -17F and MIF have a crucial role in the gastric inflammation and carcinogenesis. Recently, we showed that the genetic polymorphisms of MIF-794-CATT repeat are associated with CIHM status in the non-neoplastic gastric mucosa. Consequently, the CIHM status in the gastric cancer tissue, in relation to IL-17A (-197G>A), -17F (7488T>C), and MIF (-173G>C and -794 tetranucleotide repeats) polymorphisms was investigated. Gastric cancer tissues were obtained from 102 patients. CIHM of p14, p16, DAP-kinase and CDH1 genes were determined by methylation-specific polymerase chain reaction (MSP). CIHM high was defined as three or all CpG islands methylated. We employed the PCR-SSCP (multiplex PCR for IL-17A and -17F) method to detect the gene polymorphisms. We did not find significant association between CIHM status and IL-17F (7488T>C) and MIF (-173G>C) polymorphisms. However, concerning the IL-17A (-197G>A) polymorphism, we found that IL-17A G carrier (GG+GA) held a significantly higher risk of CIHM of p16 (OR=11.22, 95% CI=1.38-91.17, p=0.024) and CIHM high (OR=3.51, 95% CI=1.15-10.68, p=0.027). An association was also found between the 7-CATT repeat carrier (5/7 + 6/7 + 7/7) of the MIF polymorphism (-794-CATT) and reduced risk of CIHM of CDH1 (OR=0.36, 95% CI=0.14-0.92, p=0.032). No association was found between CHIM status and homozygote genotypes of each repeat (-794-CATT 5/5, 6/6, and 7/7). The present results provided evidence that the genetic polymorphisms of IL-17A, and MIF-794-CATT repeat are associated with CIHM status in the gastric cancer. Genetic polymorphisms of IL-17A, and MIF-794-CATT repeat may be involved in methylation-related carcinogenesis in the stomach.

Correspondence to: Dr Tomomitsu Tahara, Department of Gastroenterology, Fujita Health University School of Medicine, 1-98 Dengakugakubo Kutsukake-cho, Toyoake, Aichi, 470-1192, Japan
E-mail: tomomiccyu@yahoo.co.jp

Abbreviations: CIHM, CpG island hyper-methylation; MIF, macrophage migration inhibitory factor; *H. pylori*, *Helicobacter pylori*; MSP, methylation-specifc PCR

Key words: CpG island hyper-methylation, stomach, cancer, interleukin-17, macrophage migration inhibitory factor, genetic polymorphism

Introduction

CpG island hyper-methylation (CIHM) has been shown to be an important mechanism in gene silencing. In many kinds of cancer, several genes acquire CIHM, 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 (1,2). Methylation of p16 and p14 has been shown to be present in gastric cancer as well as premalignant lesion (3,4).

E-cadherin (CDH1) is an adhesion molecule involved in tumour invasion/metastasis. Silencing of E-cadherin by promoter CpG methylation has also been shown in gastric cancer (5). Death-associated protein kinase (DAPK) is a calcium/calmodulin-dependent serine/threonine kinase, and participates in various apoptosis systems. Methylation of DAPK has been reported in many cancers (6) including gastric cancer (7,8).

CIHM of the above 4 genes frequently occurs in gastric cancer tissue as well as premalignant lesions (3-8). Therefore, they may be susceptible candidate genes for CIHM in gastric cancer.

As the possible mechanisms of CIHM, exogenous carcinogens, generated reactive oxygen, and host genetic differences may influence its status (9). One of the most

important factors causing CIHM in the stomach is *H. pylori* infection (5,10,11), which induces chronic inflammation, causing oxidative stress to the gastric epithelium (9,12). However not all of *H. pylori*-infected patients show the same CIHM status, suggesting that some inter-individual genetic difference may contribute to the susceptibility to methylation-related gastric carcinogenesis.

Interleukin-17 (IL-17) is a relatively newly described cytokine which bridges the adaptive and innate immune systems. IL-17 family members belong to a distinct category of cytokines and play a role in coordinating local tissue inflammation by inducing the release of proinflammatory and neutrophil-mobilizing cytokines (13). In addition, IL-17A and -17F share similar functions in terms of their ability to induce chemokines that are important in neutrophil recruitment and activation. Kawaguchi *et al* reported that the IL-17F 7488T/C (rs763780), which causes a His-to-Arg substitution at amino acid 161 (H161R) variant, influences the risk of asthma and is a natural IL-17F antagonist in the known polymorphisms of the IL-17 gene (14).

Macrophage migration inhibitory factor (MIF) was originally identified as an activity isolated from T lymphocytes which was capable of inhibiting the random migration of macrophages (15). MIF contributes toward an excessive inflammatory response both directly and via an induction of proinflammatory cytokine secretion (16). Polymorphisms with potential functional relevance have been identified in the MIF gene promoter; an SNP at position -173 (G to C) (17) and a tetranucleotide CATT repeat beginning at nucleotide position -794 (18) were found to be associated with altered levels of MIF gene transcription *in vitro*.

Recently, we also showed the potential roles of IL-17A, -17F and MIF polymorphisms in gastric inflammation (19,20) and carcinogenesis (21,22). Furthermore, we showed that the genetic polymorphisms of MIF-794-CATT repeat are associated with CIHM status in the non-neoplastic gastric mucosa in cancer-free subjects (23). Our data suggest that genetic polymorphisms of MIF-794-CATT repeat may be involved in methylation-related carcinogenesis in the stomach.

Consequently, this study was performed to clarify whether the polymorphisms of IL-17A (-197G>A, rs2275913), -17F (7488T>C, rs763780), and MIF (-173C>G and -794 tetranucleotide repeats) influence the CIHM status in gastric cancer tissue.

In this study, we investigated the prevalence of CIHM status of 4 candidate genes, p14, p16, CDH1 and DAP-kinase, in gastric cancer samples, and its relation to IL-17A, IL-17F and MIF polymorphisms.

Materials and methods

Patients, tissue samples, DNA extraction, and H. pylori infection status. The study population comprised of 102 gastric cancer patients, attending the Endoscopy Center of Fujita Health University Hospital from January 2005 to May 2008. All gastric cancers were diagnosed histologically and were classified according to Lauren's classification (24). All patients underwent an upper endoscopy with a biopsy from cancer lesions and the biopsy specimens were immediately frozen and stored at -80˚C. Genomic DNA was isolated from frozen specimens using proteinase K. *H. pylori* infection the status was assessed by serologic or 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 written informed consent was obtained from all participating subjects.

Bisulfite modification and methylation-specifc PCR (MSP). To detect CIHM, we chose four candidate promoter CpG islands (3-8): p14, p16, DAP-kinase and CDH1. For the examination of DNA methylation, genomic DNA from the cancer lesion 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 methylated forward (p14 MF); 5'-gtgttaaag ggcggcgtagc-3', p14 methylated reverse (p14 MR); 5'-aaa accctcactcgcgacga-3', which amplify 122-bp product, p14 unmethylated forward (p14 UF); 5'-tttttggtgttaaagggtggtgt agt-3', p14 unmethylated reverse (p14 UR); 5'-cacaaaaacc ctcactcacaacaa-3' which amplify 132-bp product (25), p16 methylated forward (p16 MF); 5'-ttattagagggtggggcggatcgc-3', p16 methylated reverse (p16 MR); 5'-gaccccgaaccgcgaccg taa-3', which amplify 150-bp product, p16 unmethylated forward (p16 UF); 5'-ttattagagggtggggtggattgt-3', p16 unmethylated reverse (p16 UR); 5'-caaccccaaaccacaaccataa-3', which amplify 151-bp product (26), CDH1 methylated forward (CDH1 MF); 5'-ttaggttagagggttatcgcgt-3', CDH1 methylated reverse (CDH1 MR); 5'-taactaaaaattcacctaccgac-3', which amplify 115-bp product, CDH1 unmethylated forward (CDH1 UF); 5'-taattttaggttagagggttattgt-3', CDH1 unmethylated reverse (CDH1 UR); 5'-cacaaccaatcaacaacaca-3', which amplify 97-bp product (26), DAP-kinase methylated forward (DAP-kinase MF); 5'-ggatagtcggatcgagttaacgtc-3', DAP-kinase methylated reverse (DAP-kinase MR); 5'-ccctcccaaacgccga-3', which amplify 98-bp product, DAP-kinase unmethylated forward (DAP-kinase UF); 5'-ggaggatagttggattgagttaatgtt-3', DAP-kinase unmethylated reverse (DAP-kinase UR); 5'-caaa tccctcccaaacaccaa-3', which amplify 106-bp product (27).

An 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-modificated DNA. The DNA was denatured at 95˚C for 5 min, followed by 33-35 cycles at 95˚C for 30 sec and 57-69˚C according to primers for 1 min, and 72˚C for 1 min with a final extension at 72 for 5 min. MSP reactions were done using EX Taq HS (Takara Bio Inc., Shiga, Japan). PCR products (10 μl) were separated by electrophoresis in 2.5% agarose gels, and visualized by UV illumination using ethdium bromide staining. CIHM was defined as the presence of a positive methylation band, showing signals approximately equivalent to or greater than that of size marker (10 ng/μl: 100 bp DNA Ladder, Takara Bio Inc.), irrespective of the presence of unmethylated bands. Samples giving faint positive signals were analyzed a further two times and only those samples with consistent positive methylation band were considered as CIHM. In addition, we measured the fluor-

Clinicopathological characteristics of 102 gastric

Variable (n)	
Mean age ± SD (years)	63.0± 11.5
Gender (Male:Female)	77:25
Lauren's histologic subtype	
Intestinal type	56
Diffuse type	46
H. pylori infection status	
H. pylori (+)	83
H. pylori (-)	19
Stage	
Early cancer	54
Advanced cancer	48

escence intensities of methylated bands for randomly selected 50 CHIM samples using a digital densitometer (Lane Analyzer, ATTO, Tokyo, Japan), and confirmed that the fluorescence intensities of all 50 methylated bands were approximately equivalent to or greater than that of size marker (data not shown). CIHM high was also defined as three or more methylated CpG islands.

Genotyping of polymorphisms. Polymorphism was genotyped by the PCR-SSCP method as previously described (19-23). To detect the IL-17A and -17F polymorphisms, using the primer pairs (IL-17AF, 5'-aacaagtaagaatgaaaagaggac atggt-3'; IL-17AR, 5'-cccccaatgaggtcatagaagaatc-3'; IL-17FF, 5'-gtgtaggaacttgggctgcatcaat-3'; and IL-17FR: 5'-agtggatat gcacctcttactgcaca-3', respectively), one-tube multiplex PCR was carried out in a volume of 20 μl containing 0.1 μg of genomic DNA. The DNA was denatured at 96˚C for 90 sec, followed by 35 cycles at 96˚C for 15 sec, 58˚C for 30 sec, and 72˚C for 45 sec, with a final extension at 72˚C for 3 min. Thereafter, 2 μl of the PCR product was denatured with 10 μl of formamide (Sigma-Aldrich Co., St. Louis, USA) at 90˚C for 5 min. SSCP was carried out at 6 or 12˚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 MIF -794 CATT repeats and the G-173C polymorphism, using the primer pairs (MIFTRF, 5'-tga tccagttgctgccttgtc-3'; MIFTRR, 5'-tccactaatggtaaactcgg ggac-3'; MIF173F, 5'-tctagccgccaagtggagaaca-3'; and MIF173R, 5'-actgtggtcccgccttttgtga-3', respectively), PCR was carried out in a volume of 20 μl containing 0.1 μg of genomic DNA. The DNA was denatured at 95˚C for 3 min, followed by 35 cycles at 95˚C for 30 sec, 60 or 62˚C for 40 sec, and 72˚C for 45 sec, with a final extension at 72˚C for 5 min. Thereafter, SSCP was carried out in a similar manner as described above.

Statistical analysis. Statistical analysis was done with χ^2 test for the comparison of CIHM in different gender and, *H. pylori* infection status. Student t-test was used for the association between CIHM and age. Logistic regression analysis with adjustment for sex, age and *H. pylori* infection status were used for the association between CIHM and two groups of different genotypes. $p<0.05$ was considered statistically significant.

Results

Characteristics of subjects. A total of 102 gastric cancer patients participated in this study. The characteristics of subjects are shown in Table I.

Association between CIHM and age, gender and H. pylori infection status. All 102 gastric cancer samples were available for MSP analysis. CIHM was found in 44 (43.1%) for p14, 22 (21.6%) for p16, 73 subjects (71.6%) for CDH1 and 86 (84.3%) for DAP-kinase. CIHM high was also found in 41 subjects (40.2%). CIHM of p14 was weakly correlated with the female gender. In addition, CIHM of CDH1 was significantly associated with the female gender. On the other hand, CIHM of p14 was significantly associated with *H. pylori* negative gastric cancer. In addition, CIHM high was weakly correlated with *H. pylori* negative gastric cancer (Table II). No association was found between CIHM status and different staging and Lauren's subtypes (data not shown).

Multivariate logistic regression analysis for relationship between CHIM and IL-17A, IL-17F and MIF polymorphisms. IL-17A (-197G>A) and IL-17F (7488T>C) polymorphisms were successfully genotyped for 98 and 97 patients, respectively, while MIF (-173G>C) and MIF (-794-CATT repeat) polymorphisms were successfully genotyped for all patients. The prevalence of IL-17A, IL-17F and MIF genotypes are shown in Table III. We did not find significant association between CIHM status and IL-17F (7488T>C) and MIF (-173G>C) polymorphisms. However, concerning the IL-17A (-197G>A) polymorphism, we found that IL-17A G carrier (GG+GA) held a significantly higher risk of CIHM of p16 (OR=11.22, 95% CI=1.38-91.17, p=0.024) and CIHM high (OR=3.51, 95% CI=1.15-10.68, p=0.027). An association was also found between the 7-CATT repeat carrier (5/7 + 6/7 + 7/7) of the MIF polymorphism (-794-CATT) and reduced risk of CIHM of CDH1 (OR=0.36, 95% CI=0.14-0.92, p=0.032). No association was found between CHIM status and homozygote genotypes of each repeat (-794-CATT 5/5, 6/6, and 7/7) (data not shown).

Discussion

Our present results showed that CIHM of p14 was weakly correlated with the female gender. In addition, CIHM of CDH1 was significantly associated with the same subjects. On the other hand, CIHM of p14 was significantly associated with *H. pylori* negative gastric cancer. In addition, CIHM high was weakly correlated with the same subjects. While no-association was found between CIHM and aging and other clinicopathological factors, such as stage and Lauren's classification (data not shown).

Table II. CIHM status of 4 promoter CpG islands in gastric mucosa, in relation to gender, age and *H. pylori* infection status.

Variables (n)	Age (mean ± SD)	Gender[a] Male	Gender[a] Female	*H. pylori* infection status[b] *H. pylori* (-)	*H. pylori* infection status[b] *H. pylori* (+)
p14					
Unmethylated (58)	62.9±12.0	48	10	6	52
Methylated (44)	63.2±11.1	29	15	13	31
p16					
Unmethylated (80)	62.9±11.8	61	19	16	64
Methylated (22)	63.5±11.2	16	6	3	19
CDH1					
Unmethylated (29)	65.2±11.5	26	3	7	22
Methylated (73)	62.2±11.5	51	22	12	61
DAP-kinase					
Unmethylated (16)	62.2±10.7	11	5	3	13
Methylated (86)	67.3±14.8	66	20	16	70
CIMH high					
CIMH high (-) (61)	63.3±12.2	48	13	8	53
CIMH high (+) (41)	62.6±10.7	29	12	11	30

CIHM high was defined as three or more methylated CpG islands. [a]p14; p=0.054, CDH1; p=0.046: χ^2 test. [b]p14; p=0.02, CIHM high; p=0.09: χ^2 test.

In non-neoplastic gastric mucosa, it has been shown that CIHM of tumor suppressor genes are generally associated with aging, the male gender (28), and *H. pylori* infection (5,10,11).

Discrepancies of our result of correlation between CIHM of p14, CDH1 and female gender, CIHM high and *H. pylori* negative gastric cancer may be partly due to the difference of CIHM status between cancerous and non-cancerous tissues. In addition, the CpG islands, examined in this study are frequently methylated in non-neoplastic gastric epithelium (11), suggesting that CIHM of these CpG islands may be a very early step in gastric carcinogenesis. Thus, they may not influence the biological behavior, such as stage and Lauren's classification.

Concerning the possible mechanisms of CIHM, exogenous carcinogens, generated reactive oxygen, and host genetic differences may influence its status (9). One of the most important factors causing CIHM in the stomach is *H. pylori* infection (5,10,11), which induces chronic inflammation, causing oxidative stress to the gastric epithelium (9,12). The inter-individual difference in the susceptibility to methylation-related carcinogenesis may be influenced by genetic differences of related genes. Since important roles of both IL-17 and MIF in the *H. pylori*-related inflammatory response have been demonstrated (29,30), we speculated that genetic polymorphisms in IL-17 and MIF genes may modify the susceptibility to methylation-related gastric carcinogenesis.

In our study, IL-17A G carrier (GG + GA) held a significantly higher risk of CIHM of p16 (OR=11.22, 95% CI=1.38-91.17, p=0.024) and CIHM high (OR=3.51, 95% CI=1.15-10.68, p=0.027). An association was also found between the 7-CATT repeat carrier (5/7 + 6/7 + 7/7) of the MIF polymorphism (-794-CATT) and reduced risk of CIHM of CDH1 (OR=0.36, 95% CI=0.14-0.92, p=0.032).

Kawaguchi *et al* revealed functional consequences of the H161R substitution examined by using recombinant wild-type and mutant IL-17F proteins (13), so the expression and/or activity of IL-17F may be suppressed in IF-17F/7488C allele carriers. On the other hand, there has been no study on the influence of rs2275913 (IL-17A/-197G/A) on the expression of gene products. Therefore, it has not been clarified how this polymorphism influences the activity and expression of IL-17A, and we did not obtain any evidence either. Our data, however, showed that IL-17A G carrier, but not IL-17F polymorphism was an independent risk factor for the CIHM of p16 and CIHM high. While the IL-17A A allele, but not the G allele, was significantly associated with the development of gastric cancer, especially intestinal-type (22). Regarding this point, further studies will be needed, although we suspect that the IL-17A promoter polymorphism (rs2275913) may influence CIHM status in gastric cancer by altering the activity of the gene product.

As for the MIF gene, promoter sequence analysis indicated that the -173C allele creates a potential activator protein 4 transcription factor binding site (17), and levels of MIF expression significantly differed among -173G>C 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 (18). Donn *et al* showed that increasing CATT

Table III. Association between IL-17A, IL-17F, MIF polymorphisms and methylation status of 4 promoter CpG islands in gastric cancer.

	IL-17A (-197G>A)[a]			IL-17F (7488T>C)			MIF (-173G>C)			MIF (-794-CATT repeat)[b]							
	Genotype (n)			Genotype (n)			Genotype (n)			Genotype (n)							
Variables (n)	GG	GA	AA	TT	TC	CC	GG	GC	CC	5/5	5/6	5/7	5/8	6/6	6/7	6/8	7/7
p14																	
Unmethylated	18	23	16	48	7	1	28	27	3	11	12	9	1	11	11	1	2
Methylated	14	17	10	30	10	1	23	18	3	6	5	4	0	12	14	0	3
p16																	
Unmethylated	23	28	25	58	16	1	40	35	4	13	12	11	1	20	18	1	3
Methylated	9	12	1	20	1	1	11	10	2	4	5	2	0	3	7	0	2
CDH1																	
Unmethylated	6	10	12	21	6	1	12	14	3	3	1	7	0	7	9	0	2
Methylated	26	30	14	57	11	1	39	31	3	14	16	6	1	16	16	1	3
DAP-kinase																	
Unmethylated	5	6	5	13	3	0	6	10	0	2	4	2	0	3	4	1	0
Methylated	27	34	21	65	14	2	45	35	6	15	13	11	1	20	21	0	5
CIMH high																	
CIMH high (-)	18	22	20	48	10	1	29	28	4	9	9	9	1	15	14	1	3
CIMH high (+)	14	18	6	30	7	1	22	17	2	8	8	4	0	8	11	0	2

[a]IL-17A -197G carrier (GG+GA) vs. others; p16 methylated: OR (95% CI) p=11.22 (1.38-91.17) 0.024, CIHM high: OR (95% CI) p=3.51 (1.15-10.68) 0.027. [b]Seven repeat carrier (5/7 + 6/7 + 77) vs. others; CDH1 methylated: OR (95% CI) p=0.36 (0.14-0.92) 0.032. All data were adjusted for age, gender and *H. pylori* infection status. CIHM high was defined as three or more CpG islands methylated. IL-17A and IL-17F polymorphisms could not be genotyped for four and five subjects, respectively.

repeats with the -173C allele significantly increased the promoter activity in a T lymphoblast cell line (17). Thus, the -173C allele and 7-CATT seemed to promote the production of MIF, although there is no clear relationship between these polymorphisms and the transcriptional regulation of the MIF gene. Baugh *et al* reported the correlation of the 5/5-CATT repeat with low disease severity in rheumatoid arthritis patients (31), and Hizawa *et al* also reported an increased risk of non-5-CATT carriers for atopy (32). Donn *et al* demonstrated that the -173C·7-CATT haplotype is of importance in the susceptibility to psoriasis (33). We recently showed that both the 7/7-CATT repeat and the -173 C/C genotypes, as well as *H. pylori* infection and older age, were significantly associated with the development of gastric mucosal atrophy (19). In addition, we showed that the 5-CATT carriers had a reduced risk of gastric cancer, especially the diffuse type cancer, and -173C carriers and the number of 7-CATT alleles were also positively correlated with the risk of gastric cancer in older subjects and intestinal type histopathology (21). However, our present data showed that 7-CATT repeat carrier, which is associated with higher stimulated MIF promoter activity, and higher risk of gastric cancer, was associated with reduced risk of CIHM of CDH1. Furthermore, no association was found between MIF -173G>C polymorphism and CIHM status. Although, our data suggest that MIF 7-CATT allele is also associated with mathylation-related carcinogenesis, detailed mechanisms of association between 7-CATT repeat carrier and reduced risk of CIHM remains to be unexplained.

In conclusion, we showed that IL-17A -197G carrier is associated with a higher risk of CIHM of p16 and CIHM high. Furthermore, the 7-CATT repeat carrier of the MIF gene (-794-CATT) is associated with a reduced risk of CIHM of CDH1. The present results provided evidence that the genetic polymorphisms of IL-17A and MIF MIF-794-CATT repeat polymorphisms may be involved in CIHM in gastric cancer. However, our data did not provide the detailed mechanisms of these polymorphisms in CIHM. In addition, why the interplay between IL-17A and MIF-794-CATT repeat polymorphisms and CIHM is different in different genes is still unexplained. Furthermore, the effect of MIF and IL-17 polymorphisms on CIHM status is different among gastric cancer tissue and non-neoplastic tissue (23). Only a more extensive understanding of the regulation of methylation in relation to gene expression and carcinogenesis will allow us to fully interpret our findings.

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