Expression of macrophage migration-inhibitory factor in duodenal ulcer and its relation to *Helicobacter pylori* infection

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ABSTRACT. The aim of this study was to examine the expression of macrophage migration-inhibitory factor (MIF) in duodenal ulcer epithelial cells and its relation to *Helicobacter pylori* (Hp) infection, and to discuss the pathogenic roles of MIF expression and Hp infection in duodenal ulcer. MIF protein and mRNA expression was examined in samples from patients with duodenal ulcer with and without Hp infection (N = 40 each, experimental group), and in normal duodenal bulb mucosal tissue (N = 40, control group) using immunohistochemistry and *in situ* hybridization. Patients without Hp infection received routine treatment, and treatment was provided to the patients positive for Hp to eradicate Hp infection. Hp and MIF expression levels before treatment and after the ulcer had been cured were compared. The positive rates of MIF protein and mRNA in patients with Hp infection before treatment were 67.5 and 65%, respectively, and were 18.9 and 21.6% in the 37 patients from whom Hp was eliminated. These were statistically different both before and after treatment compared with controls ($P < 0.05$). In the patients without Hp infection, the positive rates of MIF protein and

mRNA expression before (45 and 47.5%, respectively) and after (32.5 and 30%) treatment were not significantly different ($P > 0.05$). The results of this study suggested that MIF is related to the development of duodenal ulcer, and that the presence of Hp is closely related with the expression of MIF in the duodenal mucosa and the development of duodenal ulcer.

Key words: Macrophage migration-inhibitory factor (MIF); *Helicobacter pylori*; Duodenal ulcer; MIF expression

INTRODUCTION

Macrophage migration-inhibitory factor (MIF) is a protein containing 115 amino acids with a molecular weight of approximately 12.5 kDa (Sun et al., 1996). It exists in the normal tissues of a variety of organs, and might contribute to the pathogenic process of various diseases. *Helicobacter pylori* (Hp) is an important pathogenic factor of chronic gastritis and peptic ulcers. Infection by Hp can cause gastric and duodenal mucosa to excrete a variety of cellular factors, including MIF, which might contribute to the occurrence and development of chronic gastritis, gastric ulcer, and gastric carcinoma. In recent years, there have been reports indicating that the expression of MIF is connected to chronic gastritis and peptic ulcers. However, there have been no studies that linked both Hp infection and MIF expression to the formation of chronic peptic ulcers. This study examined MIF protein and mRNA expression among patients with duodenal ulcer with or without Hp infection, as well as their changes after successful treatment, in order to discuss the relationship between Hp infection and the formation of duodenal ulcer.

MATERIAL AND METHODS

Clinical data

Duodenal bulb ulcerous tissue samples were collected during endoscopic examination by experienced endoscopists. Immediately subsequently, Hp infection was quantified and histopathological diagnosis was established. Hp infection was excluded in 40 patients (28 men and 12 women, ages ranging from 19 to 70 years with a mean of 44.5 years) and confirmed in another 40 patients (22 to 68 years with a mean of 45 years). Duodenal mucosa tissue samples from 40 healthy adults with no active inflammation (by histopathological examination) and without Hp infection were used as the control group. None of the participants had taken proton pump inhibitors, colloid roxatidine bismuth citrate, antibiotics, or other drugs in the 2 weeks prior to examination. Liver and kidney function tests were normal. Three pieces of ulcerative tissue (approximately 3.0 x 3.0 x 3.0 mm in size) were taken from the edge of the duodenal bulb ulcer. One of the three pieces was used immediately for a rapid urease test, and the other two pieces were directly fixed in 10% formaldehyde.

Reagents

Urease test paper was obtained from Beisiqi Reagent Co. Ltd. (Guangzhou, China).

Urea [14C] breath test kits were purchased from Nuclear Haidewei Biological Technology Co. Ltd. (Shenzhen, China). Rat anti-human monoclonal MIF antibodies (1:100) were purchased from ABCAM Biotechnology Company (Cambridge, UK). Goat anti-mouse antibody IgG (H + L) tagged with horseradish peroxidase was purchased from KPL Biotech Company (Gaithersburg, MD, USA) at a working concentration of 1:300. An MIF *in situ* hybridization detection kit was purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China). The MIF mRNA probe sequences were: 1) 5'-TTC CTC TCC GAG CTC ACC CAG CAG CTG GGG CAG GC-3'; and 2) 5'-TCT ACA TCA ACT ATT ACG ACA TGA ACG CGG CCA AT-3'.

Experimental methods

Rapid urease test

To test for urease, the biopsy specimen was quickly transferred to rapid urease test paper at room temperature. A test color change from light yellow to red was considered to be a positive result.

Warthin-Starry silver staining

After conventional elution with wax and water, the slices were placed in 1% $AgNO_2$ and dip-dyed for 60 min in the dark at 60°C. The slices were then dipped in silver liquid, which had been prepared before, and rinsed with distilled water. Development working solution was immediately (within 1 min) poured into a glass container containing the slice for 2-4 min in the dark at room temperature. When the biopsy tissue became dark-brown, the developer was removed and the developing process was stopped by rinsing with water. The slice was then subjected to conventional dehydration until becoming transparent, and sealed with optical resins for visual assessment. Under a light microscope, Hp was visible as black corynebacterium parvum or S-shaped stain against the background of golden tissue that was dyed before. Hp infection was considered present when the results of both the urease test and silver-staining assays were positive.

14C urea breath test (14C-UBT)

Breath gas was collected with CO_2 set cylinders from fasting subjects 25 min after oral ingestion of urea capsules and tested on a HUBT-01 liquid scintillation counter system, which is from Jiusheng Medical Instrument Company (Chongqing, China.) 14C-UBT ≥ 100 dpm/mM CO_2 was considered a positive result.

Design and production of tissue chips

Design and generation of the 6*5 point tissue matrix proceeded as follows: blank receptor wax blocks were produced by filling the embedding box with wax to approximately 2.0 mm thickness, which was allowed to solidify for 5 min at room temperature. A homemade hollow tube (diameter = 2.0 mm) was used to drill a matrix of holes separated by 0.3 mm to the bottom of the embedding box. The holes were labeled 1 to 6 in the horizontal orientation

and A to F longitudinally. A blank hole at the end was used as a number marker. Dehydrated and wax embedded tissue specimens were placed into the holes in sequence and left in a 60°C incubator for 1-2 min until they became soft; liquid wax was then poured into the holes and allowed to fuse with specimens for 30 min at 60°C. After cooling, 4-μm thick slices were made from the blocks and mounted onto siliconized slides for examination.

Immunohistochemistry for MIF detection

Phosphate-buffered saline was used in place of the first antibody as a negative control, and a confirmed lung adenocarcinoma specimen was used as the positive control. Dealing slices with conventional elution with wax and water and restoration of the tissue clip slices with high pressure, 10% H_2O_2 was added to the tissue chip slices to inactivate endogenous peroxidase. Subsequently, a normal goat serum working solution (1:300, 100 μL) was added as a blocking agent for 30 min at 37°C, followed by the first antibody (1:400), goat anti-rabbit IgG (1:400, 100 μL) labeled with biotin for 30 min at 37°C, and the chain mildew avidin working solution (1:300, 100 μL) labeled with horseradish peroxidase for 15 min at 37°C. The preparation was then developed using DAB and counterstained with hematoxylin. The presence of a claybank color was considered a positive result.

In situ hybridization for MIF mRNA detection

After a conventional elution with wax and water, the tissue chip slice was put in 3% H_2O_2 for 10 min in order to inactivate endogenous peroxidases. Then, pepsin diluted to 3% with fresh citric acid was added to expose the mRNA fragments. Following washing the non-specific staning sufficiently, then added blocking solution (1:300, 100 μL), biotinylated rat anti-digoxin antibody (1-2 ng/μL, 100 μL), streptavidin-biotin complex, and biotinylated peroxidase (1:300, 100 μL) was added consecutively. Slides were processed with DAB color development for assessment of MIF mRNA hybridization; claybank was considered as a positive result.

Interpretation of results

Two pathologists read the slides at high magnification (400X) independently by using criteria recommended by Axiotis et al. (1991). Ten randomly selected fields (or actual fields available for assessment on a slide if less than 10) were examined on each section and the percentage of positive cells among the total number of cells of the same type and staining intensity were scored. Scores for staining were as follows: no color = 0, yellow = 1, clay = 2, and tawny = 3; scores for positive cells: <10% = 0; 10-40% = 1; 41-70% = 2; and >70% = 3. The final rating was negative (-) if the combined staining and positive cell score was less than 2, mildly positive (+) if the score was 2, positive (++) for a score of 3-4, and strongly positive (+++) for a score of 5-6. Two positive results among the urease, Warthin-Starry silver-staining test and 14C-UBT were considered to indicate Hp infection.

Statistical analysis

All data were processed using the SAS 9.1 statistics software (SAS was invited by

A.J. Barr and J.H. Goodnight who work for the University of North Crolina State), including Pearson chi-square and Pearson segmentation chi-square tests. $P < 0.05$ was considered to be statistically significant.

RESULTS

The positive rates of MIF protein and mRNA expression in the 40 patients with duodenal ulcers who were Hp positive were 67.5 and 65% before treatment, whereas they were 18.9 and 21.6% in the 37 patients in whom Hp had been eradicated (Table 1). MIF contents in sera of patients with duodenal ulcers before and after being cured of the ulcer and eradicated of Hp are shown in Table 2. Comparisons of before and after treatment, and of before treatment and the healthy control group, found statistically significant differences (the χ^2 of before and after treatment was 18.3967, the χ^2 of before treatment and the control group was 25.2083; all P values <0.05). When the group with eradicated Hp was compared with the control group, the χ^2 was 0.6020 ($P > 0.05$), which suggested that the expression rates of MIF protein and mRNA decreased significantly in patients with duodenal ulcers after Hp infection was eradicated. The Hp infections of the remaining three patients were not eradicated. The expression of MIF protein and mRNA was positive in patients with duodenal ulcer who were Hp positive before treatment (Figure 1A and B); after the eradication of Hp, expression of MIF protein and mRNA was negative (Figure 2A and B). The positive rates of MIF protein and mRNA in the 40 patients with duodenal ulcer who were Hp negative were 45 and 47.5%, respectively; the rates were 32.5 and 30% after treatment. No statistically significant differences were found between the rates before and after treatment ($\chi^2 = 1.3167$, $P > 0.05$).

Table 1. Expression of MIF in duodenal ulcers.

Group	Time	N	MIF expression + score		(%)	MIF mRNA + score		(%)	P value
			≤2	>2		≤2	>2		
Control		40	35	5	12.5	35	5	12.5	
Hp+	Before Hp elimination	40	13	27	67.5	14	26	56.0	<0.05
	After Hp elimination	37	30	7	18.9	29	8	21.6	
Hp-	Before treatment	40	22	18	45.0	21	19	47.5	
	After treatment	40	27	13	32.5	28	12	30.0	

MIF = migration-inhibitory factor; Hp = *Helicobacter pylori*.

Table 2. Chi-square test results of the analysis of MIF expression in duodenal ulcers before and after Hp elimination.

	χ	P
Before and after Hp elimination	18.3967	<0.0001
Before and after treatment in Hp-negative subjects	1.3167	0.2512
Control and pre-Hp elimination	25.2083	<0.0001
Control and post-Hp elimination	0.6020	0.4378
Control and before treatment in Hp-negative subjects	10.3127	0.0013
Control and after treatment in Hp-negative subjects	4.5878	0.0322

MIF = migration-inhibitory factor; Hp = *Helicobacter pylori.*

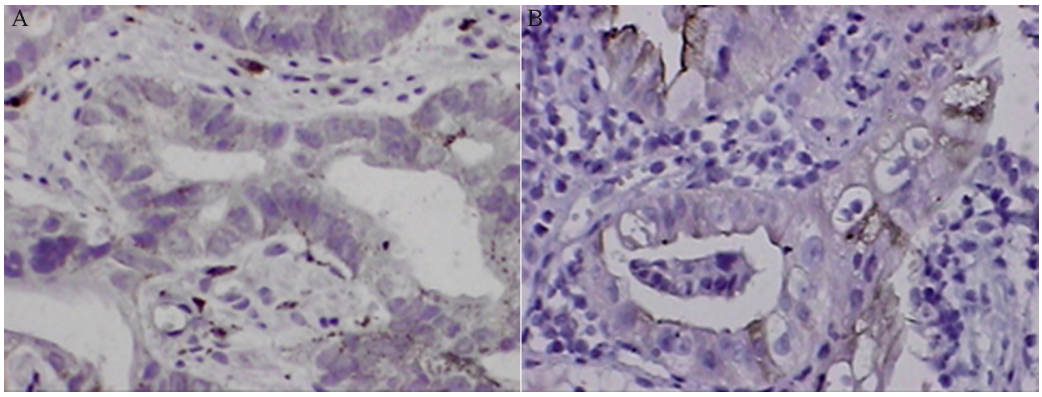

Figure 1. Negative MIF expression in normal bulb mucosa (200X). **A.** MIF; **B.** MIF mRNA. MIF, migration-inhibitory factor.

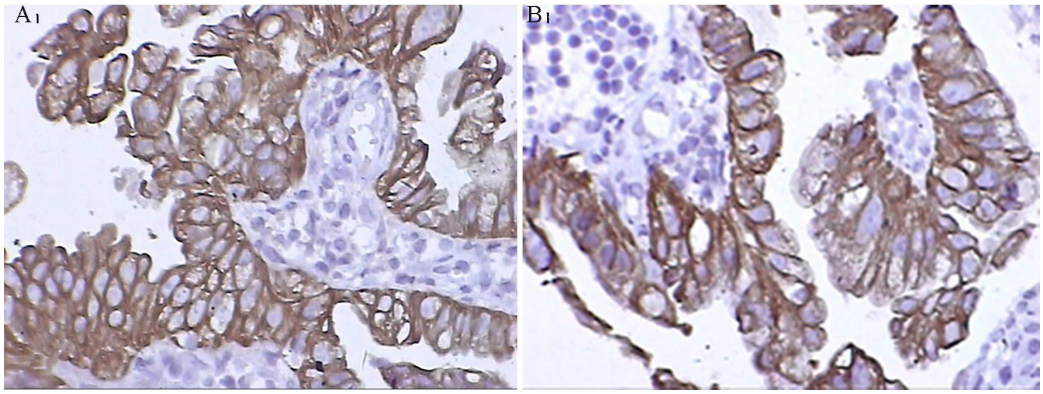

Figure 2. Positive MIF mRNA expression in normal bulb mucosa (200X). **A1.** MIF; **B1.** MIF mRNA. MIF, migration-inhibitory factor.

Few claybank cells could be seen, the combined staining and positive cell score was less than 2, so the final rating was negative.

Much mRNA staining was presenting claybank or tawny color. The combined staining and positive cell score was more than 2, so the final rating was positive.

DISCUSSION

The discovery of Hp greatly promoted the pathogenesis, diagnosis, and treatment of chronic gastritis and peptic ulcer. Exogenous Hp infection in human gastric and duodenal mucosa membrane epithelia causes the expression and release of related cytokines, including MIF (Shiroeda et al., 2010; Fehlings et al., 2012). Excessive expression of inflammation-promoting factors in gastric and duodenal mucosal epithelial cells aggravates the injury to the gastric and duodenal mucosa, leading to the occurrence of chronic gastritis and peptic ulcers (Gong et al., 2010; Bohumil, 2011). Wu et al. (2006) incubated *H. pylori* ATCC26695 with

THP-1 mononuclear cells and found that the total number of cells, including MIF-positive T cells, total macrophages, MIF-positive macrophages, and MIF mRNA-positive cells in the gastric antrum and body mucosa in 62 patients with chronic gastritis were significantly higher in the 42 patients positive for Hp infection than in the other 20 patients without Hp infection. In addition, MIF expression was shown to increase along with the severity of gastritis in the 42 patients with Hp infection. They suggested that increased expression of MIF in inflammatory cells in the gastric mucosa infected with Hp might play an important role in the formation of inflammation. Wong et al. (2009) also proposed that the expression of MIF played a very important role in the development of chronic gastritis with Hp infection. Xia et al. (2005) showed that Hp might stimulate MIF expression, further promoting the proliferation of gastric epithelial cells. However, Lebiedz et al. (2006) showed that MIF expression was not related to gastritis mucosa Hp infection and concluded that it had yet to be determined whether Hp infection would stimulate MIF expression and promote gastric mucosal inflammation. Nishihira (2012) suggested that MIF played a significant role in disease and that MIF knockdown could significantly decrease the pathological damage in inflammatory bowel disease. Abating MIF expression using anti-MIF antibodies has been proposed as a potential treatment approach for inflammatory bowel disease. Zhang et al. (2009) first reported decreased MIF over-expression in gastric mucosa after eliminating Hp infection, and proposed that this might prove to be a significant factor in the prevention and mitigation of gastric carcinoma. Kebapcilar et al. (2010) also indicated that eliminating Hp infection could reduce the expression of MIF and C-reactive protein while increasing the expression of the anti-inflammatory factor fetal globulin-A. Our study demonstrated that the expression of MIF mRNA decreased significantly after Hp elimination as compared with both before Hp eradication and with normal controls ($P < 0.05$). In contrast, the expression of MIF mRNA and MIF protein before and after treatment in duodenal ulcers without Hp infection was not significantly different ($P > 0.05$). We, therefore, believe that Hp infection is an external factor in the pathogenesis of duodenal ulcer, while excessive MIF expression stimulated by the presence of Hp is most likely an innate factor for the development of duodenal ulcers.

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Conflicts of interest

The authors declare no conflict of interest.

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