

## Original Article

# Expression of miR-204 and MMP-9 in Helicobacter pylori-associated gastric ulcer

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**Abstract:** Aims: This study is to analyze the expression pattern of matrix metalloprotein 9 (MMP-9) in patients with Helicobacter pylori-associated gastritis and to investigate the molecular mechanism responsible for its regulation in H. pylori-associated gastritis. Methods: Ulcer tissues, blood and saliva samples were collected from 46 patients with H. pylori-associated gastritis. Normal stomach mucosa, blood and saliva in 29 healthy people confirmed by endoscopy biopsy were collected as control group. The levels of MMP-9 mRNA expression were measured by quantitative Real-time PCR, and MMP-9 protein levels were analyzed by Western Blot and ELISA. MiRNAs that regulate MMP-9 expression was predicted by bioinformatics analysis, and the expression of predicted miRNA was measured by quantitative Real-time PCR. Results: MMP-9 was significantly upregulated at both mRNA and protein levels in ulcer tissues, blood and saliva samples from patients with H. pylori-associated gastritis compared with that from healthy control. MiR-204 was predicted to regulate MMP-9 expression, and miR-204 level was significantly downregulated in samples from patients with H. pylori-associated gastritis. Conclusion: The miR-204 expression is decreased whereas the MMP-9 expression is increased in H. pylori-associated gastritis. MiR-204 might affect the healing of H. pylori-associated gastritis by regulating the expression of MMP-9, and therefore this miRNA could serve as a potential biomarker in the diagnosis of H. pylori-induced gastritis.

**Keywords:** miR-204, matrix metalloproteinase-9 (MMP-9), helicobacter pylori, gastritis

## Introduction

Peptic ulcer (PU), a disease that often occurs on stomach and duodenum gastrointestinal mucosa, refers to the mucosal inflammation and necrotic lesion that extends deeply to the muscularis mucosae under the influence of multiple pathogenic factors [1]. Recent investigation demonstrated that GU is an universal disease with about 10% of population suffered GU at some point of in their life, and older people as well as men are more sensitive to GU [2]. Common causes of GU include Helicobacter pylori infection, intake of non-steroidal anti-inflammatory drugs (NSAIDs) and the self-digestion of gastric mucous by gastric acid/pepsin, among which H. pylori infection could be detected in 70-80% of GU patients, a percentage significantly higher than that in normal population [3, 4].

Previous studies found that the occurrence of GU is related to multiple signaling pathways, in

which many genes are involved and play their own roles. MiRNAs were also reported to be associated with PU as well as gastric cancer [5, 6]. At present it is acknowledged that the ulcerogenesis is related to H. pylori is the outcome of the collaboration of multiple mechanisms [7].

During the healing of ulcer, a class of zinc ion and calcium dependent matrix metalloproteinases (MMPs) play a crucial role to delay intestinal epithelial wound healing, whose major functions include digesting the active components of ECM, regulating cell adhesion and participating in inflammatory processes either directly or indirectly [8]. Another study showed that intestinal permeability was increased in patients with ulcerative colitis [9]. Schmitz et al. found that the inflamed colonic mucosa in ulcerative colitis has an impaired barrier function because of altered tight junction structure [10]. Other study also demonstrated that overexpression of MMPs or increase activity of

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**Table 1.** Association between H. pylori-associated gastric and the presence of oral diseases

Parameters	Control	Patients	$\chi^2$	*P
Age range (median)	15-52 (36.5)	16-55 (38.6)	0.379	0.944
Gender				
Male	16	26	0.013	0.909
Female	13	20		
Oral disease <sup>#</sup>				
Yes	5	39	33.46	0.00
No	24	7		

Note: \*Analyzed by Chi Square test, <sup>#</sup>Oral diseases include caries, pulpitis, periapical periodontitis, gingivitis and periodontal disease.

**Table 2.** Primers used for Real-time PCR

Primers	Primer sequence (5'-3')
MMP-9_Forward	5'-GCTGGCAGAGGAATACCTGTAC-3'
MMP-9_Backward	5'-CAGGGACAGTTGCTTCTGGA-3'
$\beta$ -actin_Forward	5'-CTGGAACGGTGAAGGTGACA-3'
$\beta$ -actin_Backward	5'-AAGGGACTTCCTGTAACAACGCA-3'
U6_Forward	5'-CTCGCTTCGGCAGCACA-3'
U6_Backward	5'-AACGCTTCACGAATTTGCGT-3'
miR-204_Forward	5'-ACACTCCAGCTGGGTTCCCTTTGTCATCCTAT-3'
miR-204_Backward	5'-CTCAACTGGTGTCTGGA-3'

MMPs leads to the delayed rate of closure and an inflammatory environment in gastrointestinal surgery [11], and MMP-9 was reported to be ectopic expressed in gastric ulcer tissues [12]. These studies indicate that MMP-9 is one of the key factors in the regeneration of the epithelium. Overexpression of MMP-9 results in defects in epithelium regeneration, reduces the integrity of joint complex and finally influences the quality of ulcer healing. However, little is known about genes that regulate MMP-9 expression upstream in GU.

In this study, we predicted a miRNA, miR-204, which binds to the 3' UTR of MMP-9 by bioinformatics analysis. The expression of MMP-9 and miR-204 was analyzed by quantitative Real-time PCR, Western blot and ELISA. By analyzing the expression of MMP-9 and miR-204, we investigated the regulatory mechanisms for GU involved miR-204 and MMP-9.

### Material and methods

#### Patients and samples

A total of 46 patients in the active state of primary GU and 29 healthy controls from the People's hospital of Laiwu between March,

2013 and May, 2015 were enrolled in the study. All healthy controls were diagnosed as normal by endoscopic biopsy of gastric mucosa. Data of sex and age is shown in **Table 1**, and no smoking history was reported by patients. No non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitor, antibiotics, or bismuthizing agents were taken by these patients during the two-week period before diagnose as confirmed by pathologists from Laiwu People's hospital. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Laiwu People's hospital.

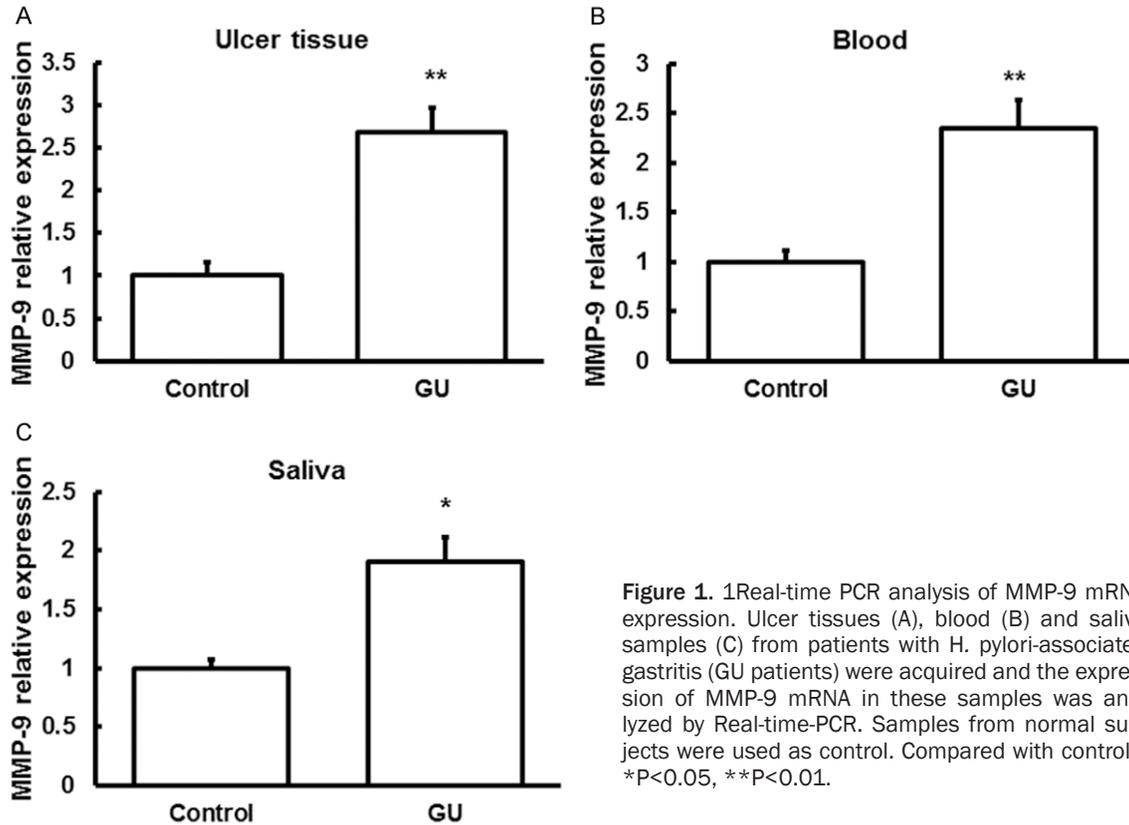
Gastric mucosa specimens were taken from pre-pylor and stored in liquid nitrogen. Peripheral blood and whole unstimulated saliva samples were collected at the day of endoscopic examination by the standard method. Briefly, sterile cotton swabs were soaked by 2% citric acid solution and put on the posterolateral of one side of tough for about 5 s and saliva were collected, followed by another side of tough. This procedure was repeated for several times until 5 mL saliva was collected. Subjects were instructed to not drink, eat or smoke and perform oral hygiene procedures at least 2 hours before the collection.

#### Helicobacter pylori infection detection

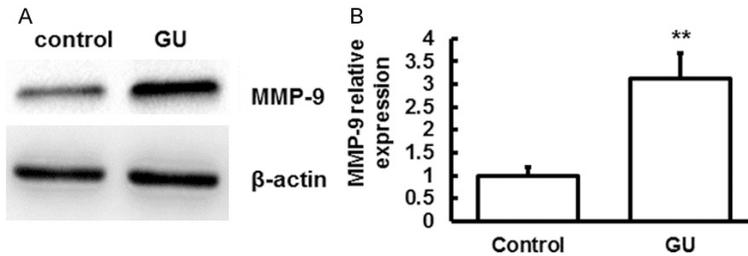
After endoscopic biopsy of gastric mucosa in patients, Helicobacter pylori (H. pylori) infection was confirmed by positive results for at least two of three diagnostic tests, including <sup>13</sup>C-urea breathe test, identification of the distal stomach on tissue sections by Giemsa stain and rapid urea enzyme test. Absence of infection was defined by a negative result in all three tests or only one positive result. Cases satisfying at least two test results were defined as positive for infection.

#### RNA isolation and quantitative real-time PCR

Total RNA was extracted by Trizol Reagent (Yusheng Biotech, Shanghai, China). miRNAs were extracted by miRcute miRNA isolation kit (Tiangen, Beijing, China) from tissues and by



**Figure 1.** Real-time PCR analysis of MMP-9 mRNA expression. Ulcer tissues (A), blood (B) and saliva samples (C) from patients with *H. pylori*-associated gastritis (GU patients) were acquired and the expression of MMP-9 mRNA in these samples was analyzed by Real-time-PCR. Samples from normal subjects were used as control. Compared with controls, \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 2.** Western blot analysis of MMP-9 protein expression in ulcer tissues. A. Ulcer tissues from patients with *H. pylori*-associated gastritis (GU patients) were acquired and the expression of MMP-9 protein was analyzed by Western blot. Gastric mucosa from normal subjects was used as control. B. Quantification of Western blot was shown. Compared with controls, \*\* $P < 0.01$ .

miRNeasy Serum/Plasma Kit (Jianlun Biotech, Guangzhou, China) from saliva and serum. Following gel electrophoresis verification of RNA integrity and quantification using UV spectrophotometer, mRNA was reverse transcribed by TIANScriptII cDNA kit (Tiangen, Beijing, China), and miRNA was reverse transcribed by miRcute miRNA cDNA kit (Tiangen, Beijing, China).

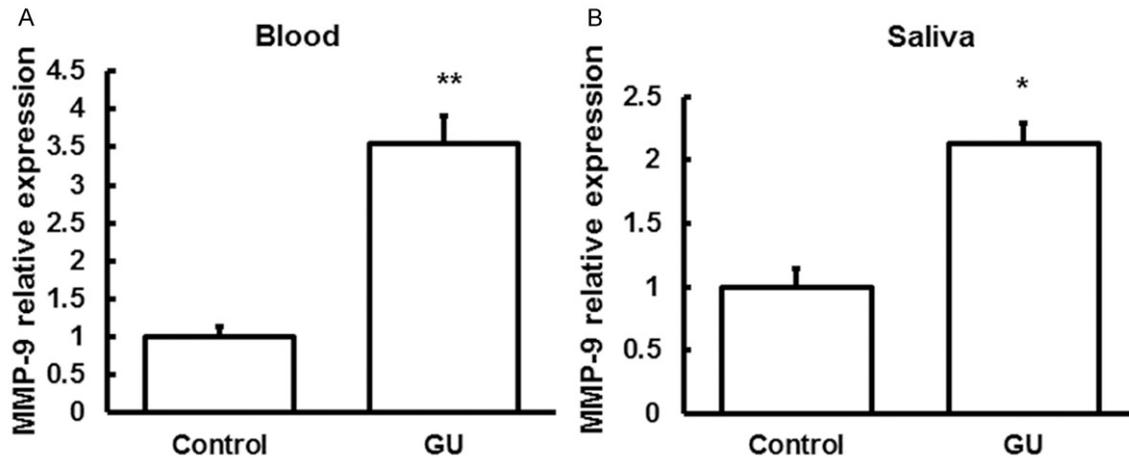
Quantitative real-time PCR was performed using a miRcute miRNA qPCR detection kit

(Tiangen, Beijing, China) for miRNAs and SuperReal Pre-Mix (SYBR Green) (Tiangen, Beijing, China) for mRNA on IQ-5 (Bio-Rad, Hercules, California, USA). For mRNA quantification, the reaction mixture was incubated for 1 cycle at 94°C for 5 min, followed by 40 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s. For miRNA, the reaction mixture was incubated for 1 cycle at 94°C for 2 min, followed by 40 cycles at 94°C

for 2 min, 62°C for 15 s, and 72°C for 32 s. Primers used for detection was shown in **Table 2**. The relative expression levels were evaluated using the  $2^{-\Delta\Delta Ct}$  method, and the expression of  $\beta$ -actin or U6 snRNA was used as internal control for mRNA and miRNA, respectively.

*Western blot*

Proteins were extracted by protein extract kit (BestBio, Shanghai, China) and determined by BCA assay kit (Zhongruiketai, Beijing, China). A



**Figure 3.** ELISA analysis of MMP-9 protein expression in blood and saliva. Blood (A) and saliva samples (B) from patients with *H. pylori*-associated gastritis (GU patients) were acquired and the expression of MMP-9 protein in these samples was analyzed by ELISA. Samples from normal subjects were used as control. Compared with controls, \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 4.** Bioinformatics prediction of MMP-9 as a target of miR-204. The results of bioinformatics software showing the binding of miR-204 to the 3' UTR of MMP-9 mRNA were shown.

total of 20 ug proteins were separated on 10% SDS-Page and transferred to polyvinylidene difluoride membranes (PVDF). After blocking with 5% skimmed milk, the membranes were probed with the following antibodies: rabbit anti-MMP9 polyclonal antibody (1:1000, Abcam, Cambridge, UK) and rabbit anti- $\beta$ -actin monoclonal antibody (1:5000, Abcam). For detection, goat anti-rabbit (1:2500, Abcam) secondary antibodies conjugated to HRP (Abcam) were used. Signal detection was performed using chemiluminescence reaction (ECL) (ab65623, Abcam, GA, USA). The acquired images were analyzed by Image lab 3.0 (Bio-Rad, Hercules, California, USA) and the relative protein expression was expressed as the densitometric value ratio of MMP-9 band to  $\beta$ -actin band.

#### ELISA

Serum was obtained by centrifugation at 3000 rpm for 10 min. MMP-9 protein levels were measured by MMP-9 ELISA kit (Bolingkewei, Beijing, China). In brief, 1:4 dilution of serum or saliva samples and eight serial dilutions of

standard substrate were incubated overnight at 4°C. After antibody incubations, the chromogenic substrate solution was added. The reaction was stopped with  $H_2SO_4$  and read at 450 nm. The amount of MMP-9 was calculated from the parallel standard curves.

#### Statistical analysis

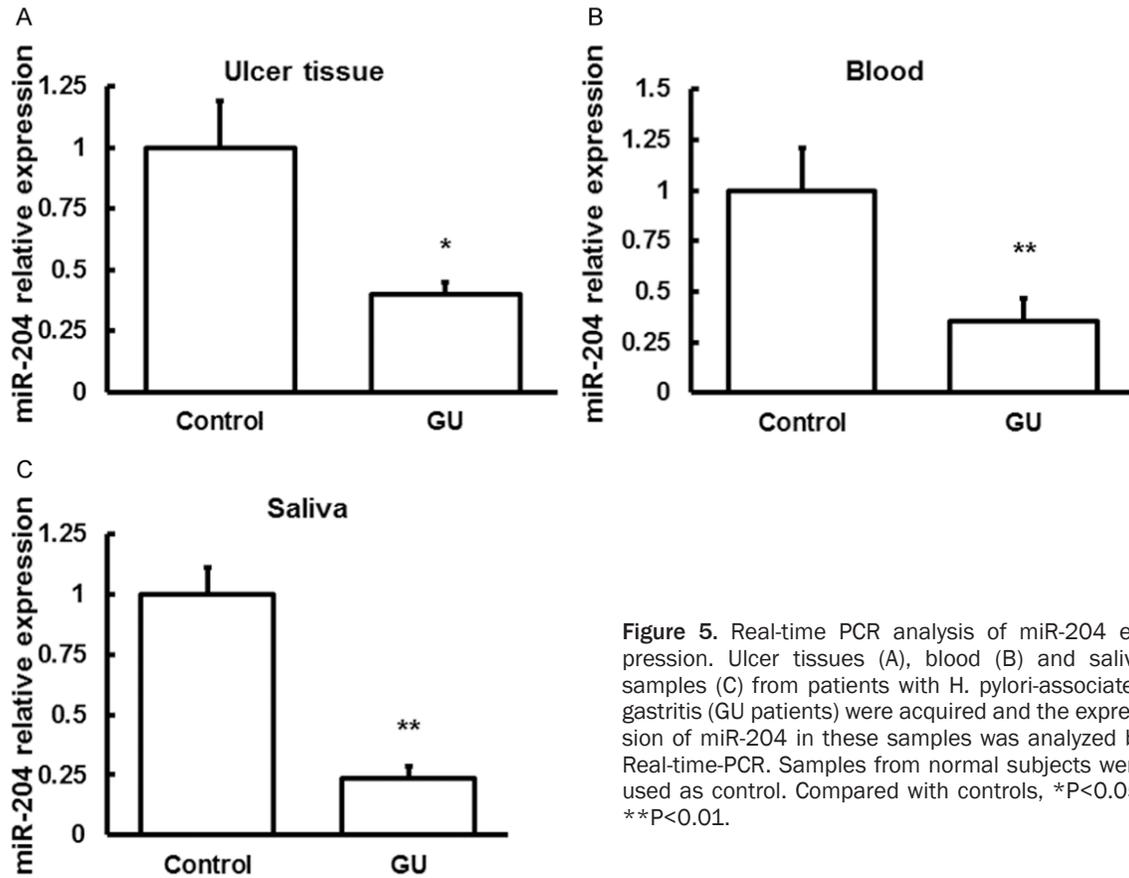
Data analysis was carried out using the SPSS software (IBM Corp, Chicago, IL, USA) and expressed as mean  $\pm$  SD. Normal test was performed. Chi Square test was applied on the changes in oral diseases between healthy controls and patients. Differences between groups were evaluated for significance using one-way ANOVA. LSD or SNK methods were used when variances were equal, and Tamhane's T2 or Dunnett's T3 methods were used when variances were unequal.  $P < 0.05$  was considered as significant.

#### Results

##### Expressions of MMP-9 mRNA in *H. pylori*-associated gastritis samples

Ulcer tissues, blood and saliva samples from patients with *H. pylori*-associated gastritis were collected and the expression of MMP-9 mRNA in these samples were analyzed by quantitative Real-time PCR. Samples from normal subjects were used as control. Compared with that in normal controls, MMP-9 mRNA expression in the three ulcer samples was significantly upregulated (**Figure 1**), suggesting a regulatory role

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**Figure 5.** Real-time PCR analysis of miR-204 expression. Ulcer tissues (A), blood (B) and saliva samples (C) from patients with *H. pylori*-associated gastritis (GU patients) were acquired and the expression of miR-204 in these samples was analyzed by Real-time-PCR. Samples from normal subjects were used as control. Compared with controls, \* $P < 0.05$ , \*\* $P < 0.01$ .

of MMP-9 in the development of *H. pylori*-associated gastritis.

### *Expressions of MMP-9 protein in H. pylori-associated gastritis samples*

Next the expression profile of MMP-9 mRNA was confirmed by Western blot at the protein level in ulcer tissues, and we found that the MMP-9 protein level was also upregulated in tissues from *H. pylori*-associated gastritis patients compared with healthy controls (Figure 2). We further investigated the expression of MMP-9 protein in saliva and blood by ELISA and found that the changes of MMP-9 protein expression is consistent with its mRNA expression in these samples (Figure 3), indicating that upregulated MMP-9 in both mRNA and protein level may function in *H. pylori*-associated gastritis.

### *MMP-9 is a predicted target of miR-204*

To investigate the mechanism that regulating MMP-9 expression, five bioinformatics soft-

ware (miRanda, TargetScan, PicTar, MiRanda and BibiServ) were used for a reliable prediction of miRNAs that may target MMP-9 mRNA. MiR-204 was predicted to directly bind to the 3' UTR of MMP-9 (Figure 4) and selected for further study since this miRNA was reported to target MMP-9 in gastric cancer. Real-time PCR analysis showed that miR-204 was significantly downregulated in ulcer tissues, blood and saliva samples from *H. pylori*-associated gastritis patients compared with that in healthy controls (Figure 5). These results indicated that miR-204 may contribute to the regulation of *H. pylori*-associated gastritis by targeting MMP-9 mRNA and the resulted downregulation of MMP-9 protein.

### *Correlation between H. pylori-associated gastritis and oral diseases*

Analyzed by chi-square test, we found that there is a significant difference concerning oral diseases between patients with *H. pylori*-associated gastritis and healthy controls ( $P < 0.05$ , Table 1), suggesting the correlation between *H.*

pylori-associated gastric and the presence of oral diseases.

### Discussion

In this study, we analyzed the expression of MMP-9 mRNA and protein as well as the expression of miR-204 in ulcer tissues, blood and saliva in patients with *H. pylori*-associated gastritis and healthy controls. Mir-204 was predicted as a regulator upstream of MMP-9 which can bind to the 3' UTR of MMP-9 mRNA. Therefore, the regulatory mechanisms underlying the development of GU involved miR-204 and MMP-9 was preliminarily discussed.

GU is a common clinical disease. In 1983, Barry J, Marshal and J Robin Warren first isolated *H. pylori* from patients with gastritis [13]. In 1984, they published a Lancet paper to report that *H. pylori* could be identified in most of patients with active chronic gastritis and PU and to show that inflammation of the stomach, and stomach ulcers are resulted from an infection of the stomach caused by *H. pylori* bacteria [14]. The use of proton pump inhibitor (PPI) drugs and eradication of *H. pylori* have become routine treatment in recent years, which increased the rate of ulcer healing greatly, but relapse of ulcer on former ulcer sites is still difficult to control [15]. Studies on experimental gastric ulcer healing found that so called healed ulcers remain histologically and ultra-structurally abnormal, which was considered as the basis of gastric ulcer relapse [16-18]. Moreover, *H. pylori*-associated gastric ulcer is positively linked to stomach cancer because the damage of gastric mucosa [19]. Therefore, it is of great value to investigate the genes involved in the formation of gastric ulcer.

Matrix metalloproteinase 9 (mmp-9) gene is 7.7 kb in length and located on chromosome 20q11.2-13.1, which includes 13 exons ranging from 280 bp to 104 bp. This gene was first identified by Sopata and Danczewicz from human neutrophils with the function to degrade collagens [20]. Later, Murphy [21] and Wilhelm [22] separated an enzyme that could degrade types IV and V collagens from rabbit bone culture medium and SV40-transformed human lung fibroblasts respectively, and they named this enzyme MMP-9. MMP-9 has been found to be associated with numerous pathological processes, and its role in many cancers, such as

colon carcinoma, rectum carcinoma, breast cancer and kidney cancer has been thoroughly investigated [23-26]. Akihito et al. found that MMP-9 is related to the initial steps of gastric cancer invasion. Patients with T1 disease had a higher positivity rate for and mean value of MMP-9 than patients with intraepithelial tumor (Tis) disease. In T1 tumors, MMP-9 expression was greater in cells in cancer stroma than in cells in noncancerous stroma [27]. Another study also demonstrated that expression of MMP-9 was significantly increased along with the development of stomach cancer, from normal gastric mucosa, ectopic proliferated mucosa to cancer tissues [28]. Until now many evidences have indicated that MMP-9 is ectopically expressed in most mucosa lesions, although the mechanism underlying this is still not fully understood. The significant elevation of MMP-9 in gastric ulcer tissues suggested that MMP-9 may regulate mucosa lesion in GU by degrading collagens and creating lesions as we expected. Moreover, this elevation was also found in body fluid samples such as blood and saliva. As unhealthy dental cavity is an ideal growth environment for *H. pylori* [29], there could be correlation between upregulated MMP-9 expression in saliva and *H. pylori* growth.

Small non-coding RNAs (microRNAs) play important roles in the regulation of many diseases by binding the 3' UTR of target mRNAs and repress their expression [30]. Therefore, expression of many mRNAs was downregulated or upregulated under the complex regulation of miRNAs [31]. To further investigate mechanisms that regulate MMP-9 expression, miRNAs that directly regulate MMP-9 in the upstream were predicted by bioinformatics analysis, and miR-204 was found to be one of these miRNAs that may bind MMP-9 mRNA and repress its expression, which may lead to repression of MMP9 expression [32]. Literature search found that MMP9 was a target of miR-204 in the development of preeclampsia and miR-204 inhibited trophoblastic invasion [32]. MiR-204 has been shown to play an important role in human diseases. Conte I et al. found that miR-204 maybe associated with inherited retinal dystrophy [33]. An J et al found that downregulation of miR-204 appears to promote human corneal epithelial cell proliferation and migration and this miRNA could be used as a

biomarker for corneal injury healing [34]. The researches on miR-204 were mainly focused on gastric cancer when related to gastric diseases. MiR-204 is downregulated in gastric cancer, and a decreased microRNA expression levels in *H. pylori* dependent gastric cancer compared with that in *H. pylori* independent gastric cancer was reported [35, 36]. MiR-204 could inhibit gastric cancer cell proliferation and invasion, and revert epithelial-mesenchymal transition (EMT) by targeting its identified target genes, including USP47, RAB22A, SOX4 and SIRT1 [35, 37]. Overall, miR-204 is closely related to *H. pylori* associated gastric diseases, especially the metastasis of mucosa cells. Our study also showed that miR-204 was significantly downregulated in tissues from patients with *H. pylori* associated gastric ulcer, which indicated that MMP-9 may be directly targeted by miR-204. The downregulation of miR-204 attenuated its repression of MMP-9 mRNA expression, resulted in the development and relapse of gastric ulcer. Expression of miR-204 was also downregulated in blood and saliva, suggesting its role in the oral infection of *H. pylori*. Theoretically, miR-204 is more stable in blood and saliva and results of our study demonstrated the downregulation of miR-204 in gastric ulcer, suggesting that this miRNA is a potential biomarker for the diagnosis of *H. pylori* associated gastric diseases and some oral diseases.

It is worth noting that saliva samples were also included in this study in addition to blood and mucosa tissues, which is due on the characteristic of *H. pylori* and the environment of gastric ulcer. Our study on miR-204 and MMP-9 expression in saliva indicated that saliva has great application prospect in the diagnosis of *H. pylori* associated gastric ulcer. The merits of saliva examination such as no-invasive acquisition and certain sensitivity and specificity make it an alternative for conventional clinical examination [38]. Further studies on the biological function of miR-204 in *H. pylori* associated gastric ulcer on the cellular level are still needed to prove the direct regulation of MMP-9 by miR-204, and to provide theoretical basis for the prevention, diagnosis and treatment of gastric ulcer.

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### Disclosure of conflict of interest

None.

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