Association between the presence and genotype of *Helicobacter pylori* and periodontitis

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Abstract. Whether *Helicobacter pylori* (*H. pylori*) infection is associated with periodontitis has been contested for decades. The relationship between *H. pylori* genotypes and periodontitis has not been clarified either. The present study provides a novel perspective to better understand the role of *H. pylori* in the pathogenesis of periodontitis. A total of 53 volunteers were recruited and divided into 3 groups in this cross-sectional study, namely the periodontally healthy group (15 participants), the stage I/II periodontitis group (20 participants) and the stage III/IV periodontitis group (18 participants). DNA from the subgingival plaque of all participants was extracted and PCR was performed using specific primers for the urease C gene and cytotoxin-associated gene A (*cagA*)/vacuolating cytotoxin gene A (*vacA*) to detect the presence and genotype of *H. pylori*. A χ^2 test and one-way ANOVA were performed on the data. There was no significant difference in sex, age or body mass index between the groups. The detection rate of *H. pylori* was 39.62% in the total population and increased with the deepening of probing depth and clinical attachment loss. There were significant differences in the detection rate of *H. pylori* among the three groups, with 13.33, 40.00 and 61.11% in the periodontally healthy, stage I/II periodontitis and stage III/IV periodontitis groups, respectively (χ^2=8.760, P<0.001). The *cagA*$^-$/*vacA*s2m2 genotype was most commonly detected in the periodontally healthy group (100%). In the periodontitis group, *cagA*$^+$/*vacA*s1m2 was the most commonly detected genotype in the stage I/II periodontitis group (37.5%) and *cagA*$^+$/*vacA*s1m1 in the stage III/IV periodontitis group (36.3%). The results of the present study suggest that the detection rates and genotypes of *H. pylori* in the subgingival plaque are associated with the status of periodontitis. *cagA*$^+$/*vacA*s1m1 and *cagA*$^+$/*vacA*s1m2 may be considered virulence markers of periodontitis. However, given the small sample size and lack of correlation analysis of the study, further larger scale and high-quality clinical trials are required to confirm these findings.

Introduction

Helicobacter pylori (*H. pylori*) is a spiral-shaped Gram-negative bacterium that was first isolated from the gastric mucosal biopsy of patients with chronic gastritis by Australian scholars in 1982 (1). Since then, numerous studies have shown that *H. pylori* is an important pathogen associated with the etiology of human chronic gastritis, gastric ulcers and mucosa-associated lymphoid tissue lymphoma (2-4). More recently, it has been designated as a Group 1 carcinogen by the International Agency for Research on Cancer (5). In addition to gastrointestinal diseases, the latest data indicate that this microorganism may be related to certain oral diseases, including halitosis (6), caries (7), recurrent oral ulcers (8), chronic gingivitis (9) and periodontitis (10).

Periodontitis is one of the most common diseases of the human oral cavity. A total of >50% of the global population suffers from periodontitis (11). Dental plaque biofilm is the initiating factor of periodontitis. A wide variety of bacteria are attached to dental plaque. The existence and interactions of these bacteria cause the occurrence and development of periodontitis. In 1989, Krajden *et al* (12) isolated and cultured *H. pylori* from dental plaque. In 1993, Ferguson *et al* (13) obtained viable *H. pylori* from saliva. The relationship between *H. pylori* infection and periodontitis gradually garnered increased attention. Studies have since indicated that *H. pylori* infection is related to periodontitis, and the detection rate of *H. pylori* in patients with periodontitis is higher than that in periodontally healthy individuals (14,15). However, by contrast, Salehi *et al* (16) used PCR to detect *H. pylori* in the gingival crevicular fluid of periodontally healthy individuals

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and patients with periodontitis, and the results showed that periodontitis was not associated with *H. pylori* infection. Al-Ahmad *et al* (17) also failed to detect the presence of *H. pylori* in the saliva, supragingival plaque, subgingival plaque and tongue mucosa swabs of 15 patients with periodontitis. These studies suggested that *H. pylori* infection was not associated with periodontitis. Considering the inconsistency of the previous results, the primary aim of the present study was to explore the relationship between the presence of *H. pylori* and the periodontal condition.

Whether *H. pylori* can cause disease depends on the virulence factors of different *H. pylori* strains, and the strength of virulence in association with the expression of virulence genes has been previously established (10). The virulence genes of *H. pylori* primarily include vacuolating cytotoxin gene A (*vacA*) and cytotoxin-associated gene A (*cagA*) (18). *VacA* encoded by the *vacA* gene enters the cell by binding to the receptor on the target cell, causing damage to the lysosome and endoplasmic reticulum, resulting in vacuolar degeneration. There is a mosaic structure of alleles in the *vacA* gene sequence, which includes two important variant regions: The signal peptide region (s region) and the middle region (m region). According to the combination of different s and m regions, the virulence genes of *vacA* can be divided into 4 subtypes: s1m1, s1m2, s2m1 and s2m2. The differences in the combinations of these signal sequence regions affects the vacuolar cytotoxic activity of *H. pylori*, which is closely related to its pathogenicity. A previous study showed that the virulence of *H. pylori* is s1m1 > s1m2 > s2m1 > s2m2 from strong to weak (19). *CagA* encoded by the *cagA* gene is highly immunogenic; it enters the host cell through the type IV secretion system and undergoes phosphorylation, which interferes with the EMT-associated signaling pathway, miRNA-584, miRNA-1290 and other gene expression levels to causes inflammation in the host cell; there are no subtypes of *cagA* (20).

To date, studies have shown that the expression of *vacA* and *cagA* genotypes is associated with the severity of gastrointestinal diseases (21,22). However, the relationship between genotype of *H. pylori* and periodontal status remains unclear. Therefore, the second aim of the present study was to investigate the association between periodontal conditions and *H. pylori* genotypes.

Materials and methods

Study population. The present study consisted of a cohort of individuals who visited the Department of Periodontics, Stomatological Hospital of China Medical University (Shenyang, China) between April 2020 and May 2021. A total of 53 participants were selected. The exclusion criteria were as follows: i) <20 natural teeth, ii) symptoms of dyspepsia, iii) systemic disease, iv) current history of antibiotic usage or use during the previous 2 months, and v) smokers (23). The demographic information of the participants was recorded, including sex, age, height and weight. The present study was approved by the Ethics Committee of the Affiliated Stomatological Hospital of China Medical University (approval no. 2018-30). Prior to the examination, the subjects were informed of the purpose of the study, and written informed consent was obtained.

Measurement of periodontitis. Periodontal examinations were performed by a periodontist who was not involved in the study, and κ tests were performed to ensure consistency (κ>0.75). Probing depth (PD) and clinical attachment loss (CAL) of all the teeth were recorded from 6 points (mesiobuccal, midbuccal, distobuccal and the corresponding points lingually). The mean values of PD and CAL in every subject were calculated and recorded. Periodontitis was diagnosed when the clinical examination met observed ≥2 non-adjacent teeth with CAL; or ≥2 teeth with buccal or lingual CAL ≥3 mm and simultaneous PD of ≥3 mm (24). Patients with periodontitis were graded according to the 2017 AAP/EFP classification (25).

Subgingival plaque collection. The subgingival plaque was collected. Before periodontal treatment, the subgingival plaque was obtained from the index teeth (16/11/26/31/36/46) of each participant (26) and frozen at -80˚C for subsequent use. PD and CAL of the index teeth were recorded from 6 points. For each sextant only the highest score was recorded (27). The PD and CAL according to the 2017 new classification were divided into healthy group, stage I/II periodontitis group and stage III/IV periodontitis group to compare the difference in *H. pylori* detection rate in subgingival plaque at the tooth level (25).

DNA extraction and PCR amplification. DNA was extracted from subgingival samples using the Magnetic Bead Micro Genomic DNA Extraction Kit [cat. no. B518749; Sangon Biotech (Shanghai) Co., Ltd.] according to the manufacturer's protocol.

To detect the presence of *H. pylori*, PCR was performed utilizing the primers for the ureC gene (28). All *H. pylori*-positive samples in the same individual were mixed for further genotyping at the individual level using specific PCR to detect the *cagA* gene and *vacA* alleles (s1, s2, m1 and m2), respectively. The sequences of the primers used in this study are shown in Table I. PCR was conducted using a Takara Ex Taq DNA polymerase (Takara Bio, Inc.). The thermocycling conditions were: Initial denaturation at 95˚C for 3 min, followed by 30 cycles of denaturation at 98˚C for 10 sec, annealing at 58˚C for 30 min and extension at 72˚C for 1 min, and then a final extension step at 72˚C for 10 min. The amplified products were analyzed by electrophoresis in a 1% (w/v) agarose gel (Sheng gong Biological Company) and stained with ethidium bromide (0.5 μg/ml). Stained amplicons were visualized on a UV transilluminator at 260 nm, and imaged (Bio-Rad Laboratories, Inc.). *H. pylori* standard strain ATCC 43504 (gifted by the laboratory of Guizhou University of Traditional Chinese Medicine) was used as a positive control. A PBS solution was used as a negative control. As long as one or more samples from each subject were positive for *ureC*, the subject was marked as positive for *H. pylori*.

Statistical analysis. SPSS version 21 (IBM Corp.) was used for statistical analysis. A one-way ANOVA followed by Tukey's post-hoc test to compare age, BIM, PD and CAL. A χ^2 or Fisher's exact test was used to analyze the categorical data such as the presence of *H. pylori*, as well as the frequency of each genotype in the different groups. All tests were two-tailed tests, and P<0.05 was considered to indicate a statistically significant difference.

Table I. Sequences of primers used in the present study.

Target gene	Sequence	Product size, bp	(Refs.)
ureC		296	(28)
Forward	5'-GGATAAGCTTTTAGGGGTGTTAGGGG-3'		
Reverse	5'-GCTTACTTTCTAACACTAACGCGC-3'		
cagA		400	(29)
Forward	5'-AATACACCAACGCCTCCAAG-3'		
Reverse	5'-TTGTTGCCGCTTTTGCTCTC-3'		
vacA s1		258	(30)
Forward	5'-ATGGAAATACAACAAACACAC-3'		
Reverse	5'-CTGCTTGAATGCGCCAAAC-3'		
vacA s2		199	(31)
Forward	5'-GCTAACACGCCAAATGATGC-3'		
Reverse	5'-CTGCTTGAATGCGCCAAAC-3'		
vacA m1		570	(30)
Forward	5'-CAATCTGTCCAATCAAGCGAG-3'		
Reverse	5'-GCGTCTAAATAATTCCAAGG-3'		
vacA m2		352	(32)
Forward	5'-GGAGCCCCAGGAAACATTG-3'		
Reverse	5'-CATAACTAGCGCCTTGCAC-3'		

ureC, urease C; *cagA*, cytotoxin-associated gene A; *vacA*, vacuolating cytotoxin gene A.

Table II. Clinicopathological characteristics of participants in the present study.

Group	Male/female patients, n	Age, years[a]	BMI, kg/m^2[,a]	PD, mm[a]	CAL, mm[a]
Periodontally healthy (n)=15	5/10	34.530±7.41	23.05±2.98	2.30±0.72	0.47±0.70
Stage I/II periodontitis (n=20)	6/14	32.85±7.74	22.46±1.18	3.49±0.36	2.50±1.10
Stage III/IV periodontitis (n=18)	10/8	37.50±8.71	22.64±1.23	5.60±0.50	5.44±1.07
χ^2/F-value	2.932	1.533	0.426	155.650	98.030
P-value	0.231[b]	0.226[c]	0.656[c]	<0.0001[c]	0.0001[c]

[a]Mean ± SD; [b]Fisher's exact test; [c]one-way ANOVA. PD, probing depth; CAL, clinical attachment loss; BMI, body mass index.

Results

Clinicopathological characteristics of the patients. Among the 53 subjects, there were 20 individuals with stage I/II periodontitis (6 men and 14 women), with a mean age of 32.85±7.74 years (16-48), and 18 individuals with stage III/IV periodontitis (10 men and 8 women), with a mean age 37.50±8.71 years (28~59). The 15 periodontally healthy individuals (5 men and 10 women) had a mean age of 34.53±7.41 years (27~55). There was no significant difference in terms of sex (χ^2=2.932, P=0.231), age (F=1.533, P=0.226) or body mass index (F=0.426, P=0.656) among the groups (Table II).

H. pylori detection rates in periodontal states. Among the 53 subjects, 21 individuals were positive for *H. pylori* infection, accounting for 39.62% of all subjects. The number of *H. pylori*-positive individuals in the periodontally healthy group, stage I/II periodontitis group and stage III/IV periodontitis group was 2 (13.33%), 8 (40.00%) and 11 (61.11%), respectively (Table III). The difference between the three groups was significant (χ^2=8.760, P<0.0001). The electrophoresis detection results of the ureC gene are shown in Fig. 1. The results in Table IV show that the detection rate of *H. pylori* in subgingival plaque of index teeth increased with the deepening of PD and CAL. The differences between PD (χ^2=41.909, P<0.0001) and CAL (χ^2=41.521, P<0.0001) among the three groups were significant.

Association between the genotype of H. pylori and periodontal conditions. The genotype of *H. pylori*-positive individuals is characterized using PCR by detecting *cagA* and *vacA* alleles (Fig. 2). In the periodontally healthy group, 2 individuals tested positive for *H. pylori* and both of these were negative for *cagA*. The detection

Table III. *H. pylori* presence in individuals with different periodontal states.

H. pylori detection	Periodontally healthy (n=15)	Stage I/II periodontitis (n=20)	Stage III/IV periodontitis (n=18)	Total (n=53)	χ^2-value	P-value
H. pylori-positive, n	2	8	11	21		
H. pylori-positive, %	13.33	40.00	61.11	39.62	8.76	<0.0001

H. pylori, *Helicobacter pylori*.

Table IV. *H. pylori* presence in subgingival plaque with different periodontal conditions.

Variable	PD ≤3 (n=114)	3< PD ≤5 (n=120)	PD >5 (n=84)	CAL <1 (n=73)	1≤ CAL <5 (n=174)	CAL ≥5 (n=71)
H. pylori-positive, n	9	37	41	6	42	39
H. pylori-positive, %	7.89	30.83	48.81[a]	8.22	24.14	54.93[a]
χ^2 value			41.91			41.521
P-value			<0.0001			<0.0001

[a]Subgingival plaque was divided according to the PD and CAL values, and there were significant differences in the detection rate of *H. pylori* in the plaque among the three groups. *H. pylori*, *Helicobacter pylori*; PD, probing depth; CAL, clinical attachment loss.

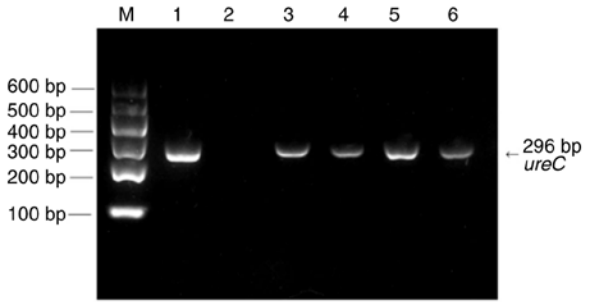

Figure 1. PCR was performed using specific primers targeting ureC gene to detect the presence of *Helicobacter pylori*. Lane M, molecular weight marker 0.1 to 0.6 kb DNA marker; lane 1, positive control; lane 2, negative control (without DNA); lanes 3-6, *ureC*-positive samples (DNA from samples). *ureC*, urease C.

rates of *cagA* in the stage I/II periodontitis group and the stage III/IV periodontitis group were 87.50 and 90.91%, respectively (Table V). The positive rate of the *cagA* genotype in the periodontitis group was markedly higher than that in the healthy group.

The presence of *cagA* and *vacA* combination genotypes varied with periodontal status. The *cagA*$^-$/*vacA*s2m2 genotype was detected in 100% of *H. pylori*-positive individuals without periodontitis. The *cagA*$^+$/*vacA*s1m2 (37.5%) and *cagA*$^+$/*vacA*s1m1 (36.3%) were the most frequently detected allele combinations in the stage I/II periodontitis and stage III/IV periodontitis groups, respectively (Table V).

Discussion

Whether *H. pylori*, colonized in subgingival plaque, influences the occurrence and development of periodontitis has been contested for decades. The present study showed that as the degree of aggravation of periodontal inflammation increased, the positive rate of *H. pylori* detection increased significantly. This suggested that the severity of periodontitis may be aggravated by the colonization of *H. pylori*. This is consistent with the results of studies by Anand *et al* (14) and Zheng and Zhou (33). Conversely, successful eradication of *H. pylori* can reduce the risk of developing periodontitis (34). This suggests that in addition to conventional treatments for periodontitis, a periodontist should be cognizant of *H. pylori* infections. Patients with periodontitis plus *H. pylori* infection should be treated with the aim of *H. pylori* eradication to reduce the risk of recurrence and aggravation of periodontitis.

Studies have shown that *H. pylori* in dyspeptic diseases may enter the mouth through acid reflux and colonize in the oral cavity (35-37). This may affect the accuracy of the detection rate of oral *H. pylori*. Therefore, participants with symptoms of dyspepsia were excluded. However, the relationship between subgingival plaque and gastric *H. pylori* is still contested. Several studies reported that there was a close relationship between gastroesophageal disease and oral status (37,38). Conversely, other studies found that the occurrence of *H. pylori* in the oral cavity was not correlated with an infected stomach or with the oral dental status of patients (15,17). Based on the aforementioned contrasting results, it is suggested that the genotype of *H. pylori* should be considered when assessing the differences between subgingival plaque and gastric *H. pylori*.

The results of the present study were consistent with those in the study by Falsafi *et al* (39), which found that *H. pylori* was detectable in the oral cavity of 45% of the population, but that only a minority of affected individuals demonstrated symptoms of the disease. It was speculated that this may be attributed to differences in virulence among different *H. pylori* strains.

Table V. Expression of each genotype in *H. pylori*-positive individuals.

H.pylori genotype	Periodontally healthy (n=2)	Stage I/II periodontitis (n=8)	Stage III/IV Periodontitis (n=11)	Total (n=21)
cagA, n (%)				
Positive	0 (0.0)	7 (87.5)	10 (90.9)	17 (81.0)
Negative	2 (100.0)	1 (12.5)	1 (9.09)	4 (19.0)
vacA, n (%)				
s1[a]	0 (0.0)	4 (50.0)	7 (63.6)	11 (52.4)
s2[a]	2 (100.0)	1 (12.5)	1 (9.1)	4 (19.0)
s1s2[a]	0 (0.0)	3 (37.5)	3 (27.3)	6 (28.6)
m[1]a	0 (0.0)	4 (50.0)	8 (72.7)	12 (57.1)
m2[a]	2 (100.0)	3 (37.5)	3 (27.3)	8 (38.1)
m (-)[a]	0 (0.0)	1 (12.5)	0 (0.0)	1 (4.8)
s1m1[b]	0 (0.0)	1 (12.5)	4 (36.3)	5 (23.8)
s1m2[b]	0 (0.0)	3 (37.5)	3 (27.3)	6 (28.6)
s2m1[b]	0 (0.0)	1 (12.5)	1 (9.1)	2 (9.5)
s2m2[b]	2 (100.0)	0 (0.0)	0 (0.0)	2 (9.5)
s1s2m1[b]	0 (0.0)	2 (25.0)	3 (27.3)	5 (23.8)
s1s2m(-)[b]	0 (0.0)	1 (12.5)	0 (0.0)	1 (4.8)
cagA$^+$/*vacA* s[1]m1c	0 (0.0)	1 (12.5)	4 (36.3)	5 (23.8)
cagA$^+$/*vacA* s[1]m2c	0 (0.0)	3 (37.5)	3 (27.3)	6 (28.6)
cagA$^+$/*vacA* s2m[1]c	0 (0.0)	1 (12.5)	1 (9.1)	2 (9.5)
cag$^+$/*vacA* s1s2m1[c]	0 (0.0)	1 (12.5)	2 (18.2)	3 (14.3)
cag$^+$/*vacA* s1s2m(-)[c]	0 (0.0)	1 (12.5)	0 (0.0)	1 (4.8)
cagA$^-$/*vacA* s2[m]2c	2 (100.0)	0 (0.0)	0 (0.0)	2 (9.5)
cagA$^-$/*vacA* s1s2m1[c]	0 (0.0)	1 (12.5)	1 (9.1)	2 (9.5)

[a]Alleles of *vacA*; [b]genotypes of *vacA*; [c]combination of two virulence genotypes of *cagA* and *vacA*. The types of gene combinations not shown in the table were all undetected.

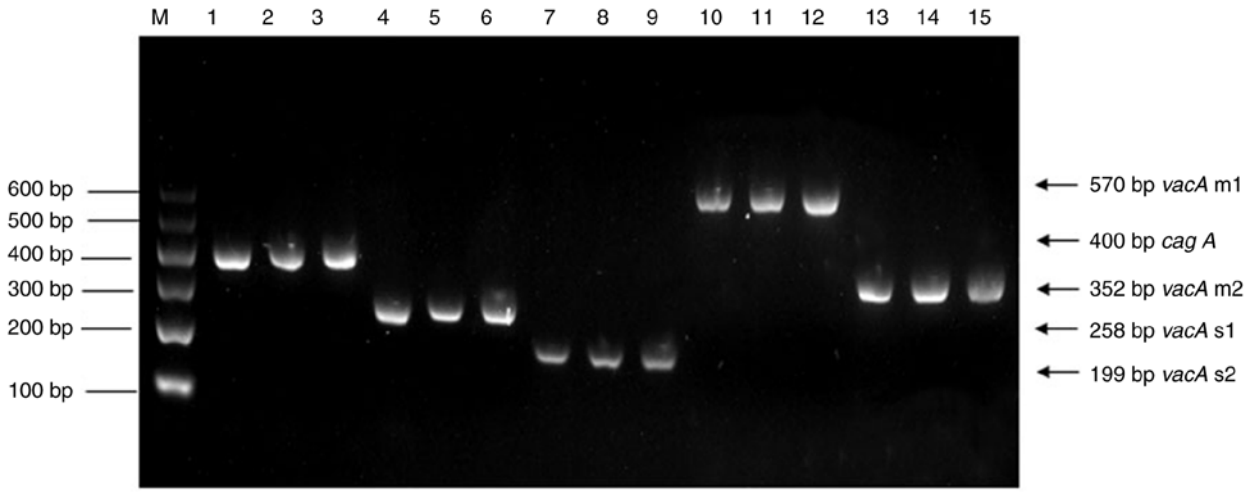

Figure 2. Genotypes of *Helicobacter pylori*-positive individuals were characterized using PCR by detecting the presence of the *cagA* gene and *vacA* alleles. Lane M, molecular weight marker 0.1 to 0.6 kb DNA marker; lanes 1-3, *cagA*-positive samples (DNA from samples); lanes 4-6, s1 allele-positive samples (DNA from samples); lanes 7-9, s2 allele-positive samples (DNA from samples); lanes 10-12, m1 allele-positive samples (DNA from samples); lanes 13-15, m2 allele-positive samples (DNA from samples); *cagA*, cytotoxin-associated gene A; *vacA*, vacuolating cytotoxin gene A.

A study by Miehlke *et al* (40) showed that there was a robust association between the *vacA*s1m1 and *cagA* genotypes of *H. pylori* in patients with gastric cancer. It was shown that the detection of virulence genes may assist in identifying patients with an increased risk of gastric cancer from the population. Research by Hu *et al* (41) found that individuals infected with

the *vacA*s1m2 strain had a significantly increased risk of peptic ulcers, while the *vacA*s1m1 genotype significantly increased the risk of active gastritis. Based on the aforementioned findings, we hypothesized that different diseases may be associated with *H. pylori* strains with different dominant genes and different genotypes, and thus different clinical outcomes.

In the *H. pylori*-positive samples in the present study, the detection rate of *cagA* was 80.95%, which meant that most of the *H. pylori* strains carried the *cagA* gene. This was consistent with the results of the studies by Link *et al* (42) and Falsafi *et al* (39). In another study, a biopsy of stomach tissues was performed. The results showed that the positive rate of *H. pylori cagA* was associated with the degree of inflammation of the gastric mucosa (43). Ferreira *et al* (44) found that after infection of the stomach mucosa with *cagA*$^+$ *H. pylori*, the concentration between IL-8 and neutrophils increased. Mendoza-Cantú *et al* (45) assessed 100 samples of supragingival plaque from patients with chronic gingivitis and showed that the detection rate of *cagA* was 16.7 and 80.8% in the healthy and chronic gingivitis groups, respectively. It was suggested that the detection rate of the *cagA* gene was related to gingival inflammation. The results of the present study indicated that the detection rate of the *cagA* gene was related to the extent of periodontal inflammation. In general, a *cagA*$^-$positive strain of *H. pylori* in the stomach tissues, gingiva or subgingival plaque is more likely to be associated with a state of disease.

In the present study, the most frequently detected genotype was *cagA*$^-$/*vacA*s2m2 in the periodontally healthy group, present in 100% of patients in this group, whereas the *cagA*$^+$/*vacA*s1m2 genotype was the predominant genotype in stage I/II patients, and the *cagA*$^+$/*vacA*s1m1 was most common in patients with stage III/IV periodontitis, accounting for 37.5 and 36.3%, respectively. The aforementioned results illustrated that the *cagA*$^-$/*vacA*s2m2 genotype is the dominant genotype in the periodontally healthy individuals, while the *cagA*$^+$/*vacA*s1m1 and *cagA*$^+$/*vacA*s1m2 genotypes are dominant in those with periodontitis. This further confirmed the theory that different genotypes of *H. pylori* strains are associated with different periodontal conditions. Furthermore, a previous study showed that the majority of *H. pylori* strains carrying the *cagA*$^+$/*vacA*s1m1 genotype could be isolated from patients with severe gastric diseases, including duodenal and gastric ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (46). Therefore, it is safe to assume that different diseases may be associated with different dominant genes. Periodontologists should pay more attention to the *cagA*$^+$/*vacA*s1m1 and *cagA*$^+$/*vacA*s1m2 genotypes, which were frequently detected in the periodontitis groups in the present study. In addition, the results showed that the detection rates of the *cagA*$^+$/*vacA*s1m1 genotype also increased with the aggravation of periodontal inflammation. However, further studies are needed to further confirm this speculation to provide a clinical reference for the treatment of oral *H. pylori* infection and periodontitis.

In conclusion, the detection rate and genotypes of *H. pylori* in subgingival plaques are associated periodontal conditions. *H. pylori* infection with the genotype of *cagA*$^+$/*vacA*s1m1 and *cagA*$^+$/*vacA*s1m2 may be predictive of a worse periodontal status. However, due to the small sample size in the present study, a larger scale and high-quality clinical study is required to confirm the findings.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

RL, DZ and YP designed the study. YL, QD and JP acquired and analyzed the data. YY and YM obtained the clinical samples and revised the manuscript. RL and JP wrote and revised the manuscript. All authors have read and approved the final manuscript. JP and DZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Stomatological Hospital of China Medical University (Shenyang, China; approval no. 2018-30). Prior to the examination, the subjects were informed of the purpose of the study, and written informed consent was obtained.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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