Observational Study

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Clinical relevance of point mutations in the 23S rRNA gene in *Helicobacter pylori* eradication

A prospective, observational study

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Abstract

Clarithromycin-based triple therapy is prescribed worldwide for *Helicobacter pylori* eradication. However, increases in the clarithromycin resistance of *H pylori* are thought to be responsible for eradication failure. Here, we studied whether point mutations in domain V of the 23S rRNA gene can affect *H pylori* eradication failure in a prospective, open-label, observational study. Of the 755 enrolled patients, 299 patients (39.6%) had positive *Campylobacter*-like organism (CLO) tests. DNA sequencing analysis of *H pylori* 23S rRNA in 295 patients revealed that 2143G was the most frequent point mutation (24.7% of patients), followed by the 2182T mutation (11.5%). The overall eradication failure rate was 20.9% (42/201) in clarithromycin-based triple therapy. Patients with the 2143G had an approximately 60% eradication failure rate, which suggested that 2143G was a high-risk genotype for eradication failure. Patients with the 2182C genotype without 2143G had an 8.7% failure rate, and patients without 2143G or 2182C had only a 4.3% failure rate. The presence of 2143G, which was associated with previous eradication history and female sex, was an independent risk factor for eradication failure. In conclusion, the 2143G point mutation in the 23S rRNA of *H pylori* was an independent risk factor for eradication failure in clarithromycin-based triple therapy. Personalized tailored therapy based on the genotypes of 23S rRNA can increase eradication success rates in *H pylori* infections.

Abbreviations: CI = confidence interval, CLO = *Campylobacter*-like organism, *H pylori* = *Helicobacter pylori*, PCR = polymerase chain reaction, RR = relative risk, rRNA = ribosomal ribonucleic acid, UBT = urea breath test.

Keywords: 23S rRNA, clarithromycin resistance, *Helicobacter pylori*, PCR genotyping

1. Introduction

Helicobacter pylori is a gram-negative rod bacterium found in the gastric mucosal lining and is virtually always associated with chronic active gastritis, which predisposes the host to the development of gastric ulcer, gastric atrophy, and ultimately gastric carcinoma.[1] The prevalence of *H pylori* reaches 80% in developing countries, 20% to 50% in industrialized countries and approximately 50% in the global population.[2] Patients with *Campylobacter*-like organism (CLO) test-positive peptic ulcers should be treated for *H pylori* to prevent ulcer recurrence.[1,3] Clarithromycin-based triple therapy is prescribed worldwide as the first-line eradication regimen for *H pylori*.[1,4] However, the clarithromycin resistance rate of *H pylori* is reported to be 8% to 15% in Korea and 13% in the United States, and clarithromycin-resistant strains are the most common cause of treatment failure in compliant patients.[5–9] Although *H pylori* culture and antimicrobial sensitivity tests are ideal ways to predict eradication outcomes, they are time consuming and require specific micro-aerobic culture conditions.[10] As a result, it is hard to routinely examine *H pylori* cultures and sensitivity tests for antibiotics in a clinical setting.

Clarithromycin belongs to a family of macrolide antibiotics that binds to the 23S rRNA of bacterial ribosomes, resulting in the inhibition of bacterial peptide translation.[11] The resistance of *H pylori* to clarithromycin is mostly due to point mutations affecting domain V of the 23S rRNA gene, which is involved in the peptidyl transferase reaction, a critical step in translation.[11] Ideally, mutations in the 23S rRNA of *H pylori* are examined prior to treatment, and treatment regimens are tailored to the

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mutation to reduce eradication failure. However, few studies have investigated the clinical relevance of point mutations in *H pylori* eradication.[12]

In this study, we investigated the point mutations of *H pylori* via full DNA sequencing of 23S rRNA domain V. Then, we studied the association between these point mutations and the eradication failure of clarithromycin-based regimens.

2. Patients and methods

2.1. Patients and outcomes

This prospective, open-label, observational study was conducted in Daegu Fatima Hospital from June 2016 to November 2017. The study protocol was approved by the Institutional Review Board of Daegu Fatima Hospital (study number: DFH16DRIS026). This study was registered in the Clinical Research Information Service (CRIS) (study number: KCT0002668). Patients aged ≥ 18 years who were willing to perform an upper gastrointestinal endoscopic examination were eligible for enrollment. Patients who had a history of gastrectomy, were pregnant or lactating, who had severe concurrent disease or disability or were unable to understand the consent were excluded. Informed consent was obtained from all patients before enrollment. Patients were enrolled from June 2016 to May 2017, and the last follow-up visit was in November 2017.

The primary end point of this study was to clarify the effect of point mutations in the 23S rRNA of *H pylori* in the eradication failure of clarithromycin-based triple therapy. The secondary end point was to determine the eradication efficacy of a secondary metronidazole-based regimen and to assess whether the point mutations affected the eradication efficacy of the second-line regimen.

2.2. H pylori detection and eradication treatment

H pylori infection status was determined by a rapid urease test (CLO test) from antrum tissue biopsy using a Proton Dry NEW kit (Gastrex, France). Patients with positive CLO tests were prescribed a 7-day course of the clarithromycin-based triple regimen, which consisted of 500 mg of clarithromycin twice daily, 1000 mg of amoxicillin twice daily and 30 mg of lansoprazole twice daily. Posttreatment *H pylori* status was determined by in vitro ^{13}C urea breath test (UBT) at least 6 weeks after the completion of treatment using the HUBT-20 *H pylori* breath analysis system (Headway, China).

When the UBT was positive, patients were treated with second-line metronidazole-based quadruple therapy, which was composed of 250 mg of metronidazole four times daily, 300 mg of bismuth subcitrate four times daily, 500 mg of tetracycline four times daily and 20 mg of omeprazole twice daily for 14 days, and 6 weeks later, the UBT was repeated to confirm *H pylori* eradication.

2.3. DNA extraction and direct sequencing of the 23S rRNA gene of H pylori

If the CLO test was positive, whole DNA was extracted from additional biopsy tissue using a QIAamp DNA Mini Kit (QIAGEN, #51306) according to the manufacturer's protocol. The extracted DNA concentration was assessed with NanoDrop spectrophotometers (Thermo Scientific, Waltham, MA), and patients whose DNA extraction concentration was <50 ng/mL were excluded from sequencing analysis. We examined the nucleotide sequence of domain V in the 23S rRNA gene of *H pylori* by amplifying a segment of approximately 330 bp in seven *H pylori* strains using PCR primers 23S F (5′-TGA ATG TAA CGA GAT G-3′, corresponding to *H pylori* 23S rRNA positions 2052–2070) and 23S R (5′-GCC AAA GCC CTT ACT TCA-3′, positions 2216–2233). Nucleotide sequencing of the amplified DNA was performed by a commercial expert agency (www.solgent.com, Daejeon, South Korea).

2.4. Sample size estimation and statistical analysis

We assumed that the prevalence of *H pylori* was 45% and that the eradication success rate of the clarithromycin-based triple regimen was 80% based on references.[13,14] The prevalence of the 2143G and 2182T point mutations in 23S rRNA was approximately 14% and 12% in the literature, respectively.[15–19] Based on this assumption, we calculated that a total 750 patients needed to be included to obtain 65 patients with a 2143G or 2182T point mutation considering a 15% dropout during eradication treatment and UBT.

Data were analyzed using SPSS software (version 18.0). Baseline continuous data were expressed as the means and standard deviations, and categorical data were presented as numbers and percentages. Continuous data were compared using Student *t*-tests, and categorical data were compared by Chi-squared tests. Binary logistic regression analysis was performed to calculate the independent relative risk and corresponding 95% confidence intervals for *H pylori* eradication failure with a clarithromycin-based triple regimen. For all tests, $P < .05$ indicated statistical significance.

3. Results

3.1. Participant flow and demographic data

Participant selection and drop out are presented in a flow chart in Figure 1. A total of 755 patients were enrolled for upper gastrointestinal endoscopy and completed CLO testing. The CLO results were positive in 299 patients (39.6%); *H pylori* 23S rRNA sequencing was conducted in 295 of these patients, but it could not be performed in 4 patients due to low DNA concentrations (<50 ng/mL). First-line clarithromycin-based eradication therapy for *H pylori* was prescribed in 253 patients. Then, UBT was conducted in 201 patients at least 6 weeks later, and 42 patients (20.9%) had a positive UBT result, suggesting eradication failure. Second-line metronidazole-based quadruple therapy was prescribed to 32 patients, and a second UBT was conducted 6 weeks later, resulting in only 1 case of treatment failure (Fig. 1).

Baseline demographic data showed that patients with positive CLO results were older than patients in with negative results ($P = .027$). Other parameters, such as gender, body mass index, the incidence of gastric or duodenal ulcer, and previous *H pylori* eradication history, failed to show any differences between the CLO-positive and CLO-negative groups (Table 1).

3.2. Sequencing data for the H pylori 23S rRNA gene in 295 patients

We detected 5 single-nucleotide substitution mutations in domain V of the *H pylori* 23S rRNA gene in 295 Korean participants (Table 2). The 2143A>G point mutation was the most frequent (approximately 25% of patients) and was followed by the 2182C>T mutation (11.5%). The other three mutations had a

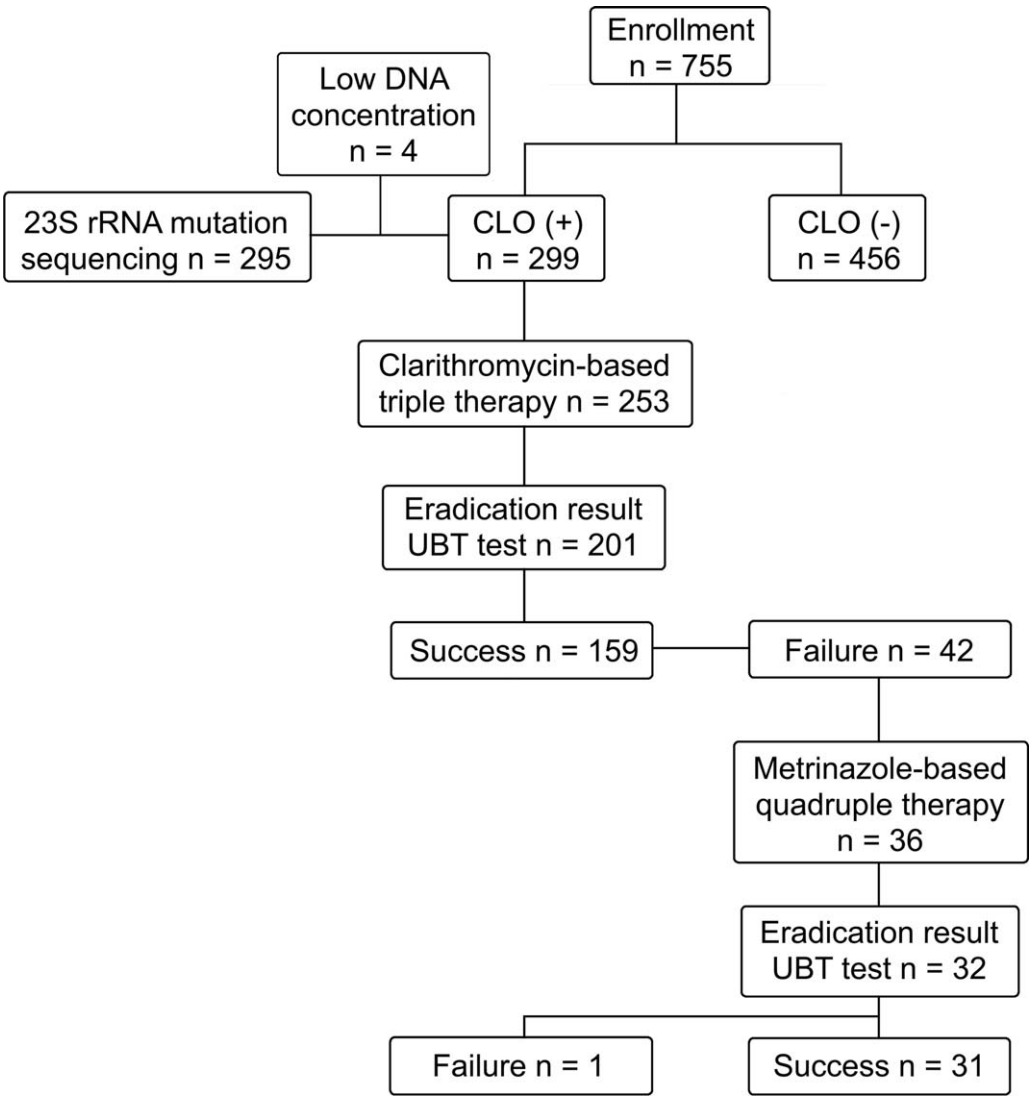

Figure 1. Flow diagram of participants in this study.

lower incidence: 1.7% for 2142A>G and 1.4% for 2190T>C and 2195C>T. None of our patients had mutations at nucleotide positions 2115, 2144, 2711, 2223, or 2244, which had been reported to confer clarithromycine resistance in *H pylori*.[17] In terms of combined genotype structures, 2143A-2182C was most frequent in 64.5% of patients, which was followed by 2143G-2182C in 23.6% of patients, and 2143A-2182T in 10.5% of patients. Patients with 2143G-2182T were in the minority (1.0%) (Table 2).

Table 1

Demographic data according to *Campylobacter*-like organism (CLO) test results (n = 755).

	CLO (+) (n = 299)	CLO (−) (n = 456)	*P*
Age, years	58.57 ± 11.75	60.63 ± 13.57	.027
Sex, male	153 (51.2%)	230 (50.4%)	.844
BMI, kg/m^2	23.96 ± 4.38	23.90 ± 4.54	.603
Gastric ulcer	71 (23.7%)	89 (19.5%)	.164
Gastric carcinoma	18 (6.0%)	23 (5.0%)	.563
Duodenal ulcer	266 (89.0%)	424 (93.0%)	.054
Eradication history	51 (17.1%)	80 (17.5%)	.863

CLO test was positive in 39.6% of the examined individuals.
BMI = body mass index, CLO = *Campylobacter*-like organism.

3.3. Eradication failure and point mutations in the H pylori 23S rRNA gene

We investigated the role of point mutations in the *H pylori* 23S rRNA gene in the eradication failure of clarithromycine-based triple therapy by clarithro the failure rate according to the most frequent mutations of 2143A>G and 2182C>T (Table 3). The overall eradication failure rate of the clarithromycine-based regimen was 20.9% (42/201). The presence of the 2143G genotype in patients with 2143G-2182C and 2143G-2182T increased the eradication failure rate to 57.7%, suggesting that it was a high-risk genotype for eradication failure in the clarithromycine-based regimen. Interestingly, the presence of the 2182T without 2143G reduced the treatment failure rate to approximately 4%, meaning that 2143A-2182T was the low-risk genotype for predicting eradication success. The most frequent

Table 2

Genetic mutation analysis of 23S rRNA domain V in *H pylori*.

	Direct sequencing (n = 295)				
Genotype	**2142A>G**	**2143A>G**	**2182C>T**	**2190T>C**	**2195C>T**
Dominant	289 (98.0%)	222 (75.3%)	261 (88.5%)	291 (98.6%)	291 (98.6%)
Rare	5 (1.7%)	73 (24.7%)	34 (11.5%)	4 (1.4%)	4 (1.4%)
Not determined	1 (0.3%)	0	0	0	0
Combined genotype	**2143A-2182C**	**2143G-2182C**	**2143A-2182T**		**2143G-2182T**
	191 (64.5%)	70 (23.6%)	31 (10.5%)		3 (1.0%)

Table 3

Eradication failure rate based on urea breath test (UBT) according to the 2143A>G and 2182 C>T genotypes of 23S rRNA in *H pylori*.

Genotype	2143A	2143G	2182C	2182T	
Failure/Total	12/149 (8.1%)	30/52 (57.7%)	39/175 (22.3%)	3/26 (11.5%)	
Combined genotype	2143A-2182C	2143A-2182T	2143G-2182C	2143G-2182T	Total
Failure/Total	11/126 (8.7%)	1/23 (4.3%)	28/49 (57.1%)	2/3 (66.7%)	42/201 (20.9%)

combined genotype, 2143A-2182C, had an eradication failure rate of 8.7%.

We then conducted risk factor analysis for eradication failure in the clarithromycine-based regimen (Table 4). In univariate analyses, older age was related to eradication failure ($P = .040$), whereas male sex, the presence of gastric or duodenal ulcer, and eradication history failed to show statistical significance. The presence of the 2143G and the 2143G-2182C in 23S rRNA was significantly associated with eradication failure in first-line therapy ($P < .001$), and the presence of the 2143A-2182T was sensitive to eradication treatment in univariate analysis ($P = .038$). We conducted logistic regression analyses to elucidate whether the point mutations in *H pylori* 23S rRNA are independent risk factors for eradication failure. When we consider interactions with other risk factors, such as age and female sex, the presence of the 2143G genotype and the 2143G-2182C contributes to a more than 16-fold and 13-fold increase in the risk of eradication failure in multivariate model 1 and 2, respectively (Table 4). However, the presence of 2143A-2182T combined genotype failed to show an independent role in eradication success, which may have resulted from low statistical power due to the low incidence of the 2182T mutation (data not shown). When we clarithr factors associated with the presence of 2143G, previous eradication history and female sex were independent risk factors for the presence of 2143G, the risk genotype for eradication failure (Table 5).

3.4. Eradication rate of the second-line metronidazole-based regimen

Among the 42 patients with positive UBT, 34 were treated with second-line metronidazole-based quadruple therapy for 14 days. Surprisingly, the second UBT assessed 6 weeks later revealed only one case of eradication failure. This result suggested that the empirical use of a metronidazole-tetracycline-bismuth-PPI regimen in 2143G-positive patients could profoundly reduce the eradication failure rate (Fig. 1).

4. Discussion

The most interesting finding of this study was that the presence of the 2143G point mutation in the 23S rRNA of *H pylori* independently contributed to a 16-fold increase in the eradication failure risk of the clarithromycine-based triple regimen. The eradication failure rate in patients with 2143G reached approximately 60%. In addition, patients with the 2143A-2182T were susceptible to the clarithromycine-based regimen; only one patient failed to eradicate *H pylori* (1/23; 4.3%)

Table 4

Univariate and multivariate analyses of factors influencing *H pylori* eradication failure.

	Univariate				Multivariate model 1		Multivariate model 2	
	Success (n = 159)	**Failure (n = 42)**	**RR (95% CI)**	***P***	**RR (95% CI)**	***P***	**RR (95% CI)**	***P***
Age, years	57.40 ± 11.67	61.50 ± 10.52	1.033 (1.001–1.066)	.040	1.036 (0.998–1.075)	.064	1.034 (0.997–1.071)	.070
Sex, female	71 (44.7%)	25 (59.5%)	1.823 (0.913–3.637)	.086	1.051 (0.454–2.434)	.908	1.044 (0.453–2.403)	.921
Gastric ulcer	42 (26.4%)	9 (21.4%)	0.760 (0.336–1.720)	.509				
Duodenal ulcer	140 (88.1%)	39 (92.9%)	1.764 (0.496–6.272)	.375				
Eradication history	21 (13.7%)	8 (19.0%)	0.647 (0.264–1.585)	.338				
2143G	22 (13.8%)	30 (71.4%)	15.568 (6.948–34.882)	<.001	15.873 (6.803–37.037)	<.001		
2182C	23 (14.5%)	3 (7.1%)	0.455 (0.130–1.595)	.208				
2143G/2182C	21 (13.2%)	28 (66.7%)	13.143 (5.971–28.927)	<.001			13.395 (5.830–30.778)	<.001
2143A/2182T	22 (13.8%)	1 (4.3%)	0.152 (0.020–1.161)	.038				

CI = confidence interval, RR = relative risk.

Table 5

Logistic regression analysis of factors associated with the presence of 2143G in the *H pylori* 23S rRNA gene.

	Univariate			Multivariate	
	2143G (n = 73)	2143A (n = 222)	*P*	RR (95% CI)	*P*
Age, years	59.64 ± 11.49	58.27 ± 11.77	.383		
Sex (female)	46 (63.0%)	94 (42.3%)	.002	0.432 (0.237–0.785)	.006
BMI, kg/m^2	24.0 ± 3.01	24.13 ± 4.80	.841		
Gastric ulcer	12 (16.4%)	60 (27.0%)	.068	0.985 (0.483–2.006)	.966
Duodenal ulcer	68 (93.2%)	197 (88.7%)	.279		
Eradication history	27 (37.0%)	19 (8.6%)	<.001	3.127 (3.127–12.544)	<.001

BMI = body mass index, CI = confidence interval, RR = relative risk.

(Table 3). Based on our results, 2143G can be categorized as a high-risk genotype with an eradication failure rate of approximately 60%, 2182C without 2143G can be categorized as a medium-risk genotype with an approximately 10% failure rate, and the absence of 2143G and 2182C can be categorized as susceptible to clarithromycin-based triple therapy. These results suggest the possibility of a personalized eradication regimen for the 2143A>G and 2182C>T genotypes that can dramatically improve the eradication success rate. If patients with 2143G were treated with a second-line regimen from the beginning, the total eradication failure rate would be reduced to less than 10%.

This study also found important preconditions for the clinical application of 23S rRNA point mutations in predicting the clarithromycin resistance of *H pylori*. First, 23S rRNA DNA sequencing was possible in 99% of patients (295/299, Fig. 1). This finding suggests that we can obtain a sufficient amount of DNA (more than 50 ng/mL) for DNA sequencing analysis from one piece of a gastric antral biopsy specimen. Considering that the sensitivity of diagnostic *H pylori* cultures by experienced laboratories has been reported as 50–92%,[20–22] 23S rRNA DNA mutation analysis has excellent sensitivity compared to *H pylori* cultures. Second, DNA sequencing of domain V in the 23S rRNA gene revealed only 2 major point mutations, 2143A>G and 2182C>T. Other point mutations, such as 2142A>G, 2190T>C and 2195C>T, showed low mutation rates (below 2%), and mutations at nucleotide positions 2115, 2144, 2711, 2223, and 2244, which were reported to confer clarithromycin resistance, were not found in our participants.[17] If multiple point mutations affecting clarithromycin resistance exist and form complex interactions, it can be difficult to estimate clarithromycin resistance via a point mutation test. However, point mutations can have patterns that vary by region or country, and further investigation of the regional differences in point mutation patterns in the 23S rRNA of *H pylori* are needed.

When the 2182T is combined with the 2143A allele, the resulting phenotype is susceptible to the clarithromycin-based regimen. Microbiologic evidence revealed that transduction of the 2182C genotype into a susceptible wild-type *H pylori* strain failed to result in the clarithromycin-resistant phenotype, which mainly depends on the presence of the 2143G allele.[23] Considering this evidence, the protective phenotype of the 2182T and 2143A alleles can result from the status of linkage disequilibrium between 2143A>G and 2182C>T. This observation suggests that the 2182C>T genotype is coupled with the 2143A>G as they are located close to one another, and therefore, the effect of the 2182 genotype on clarithromycin resistance depends on the presence of 2143G.[24] Approximately 90% of patients with 2182T had the 2143A genotype, representing a susceptible genotype (Table 3).

In this study, we discovered an association between previous eradication history and the presence of the 2143G clarithromycine-resistant allele, which was expected (Table 5). In the absence of antibiotics, the wild-type *H pylori* 23S rRNA gene has the 2143A genotype, which results in a phenotype susceptible to clarithromycine, and a small fraction of mutated *H pylori* has the 2143G genotype. In the presence of antibiotic pressure, these mutated bacteria with 2143G, which are resistant to clarithromycine, can selectively accumulate to form the majority of the population.[25] If genotype analysis for 2143A>G and 2182C>T is selectively available due to its cost, it may be reasonable to perform genotype tests in patients with a previous eradication history.

Female patients showed a lower eradication rate than male patients in univariate analysis, even though this trend failed to obtain a statistical significance (Table 3). In addition, female patients had a higher incidence of the 2143G clarithromycine-resistant genotype than male patients, although the positive rate of *H pylori* infection was not different between sexes (Table 5). Several lines of evidence revealed that females showed a higher prevalence of clarithromycine or metronidazole resistance than males, and this difference led to lower eradication rates in females than in males.[26–28] Pathologically, females with *H pylori* infection showed a lower degree of inflammation and lower activity scores in the antrum than males, which were associated with lower interleukin-8 production in the gastric mucosal samples of females than of males.[29] It is well known that both microbial and host factors are critical for the clinical manifestation of *H pylori* infection and eradication failure. The sex-based difference in the inflammatory response may influence the eradication rate and accumulation of mutated *H pylori*.

In conclusion, this study demonstrated that the presence of the 2143G point mutation in the 23S rRNA of *H pylori* has an approximately 60% eradication failure rate and was an independent risk factor for eradication failure in a clarithromycine-based regimen. The prevalence of 2143G was 24.7%, and this mutation was independently associated with previous eradication history and female sex. These observations may suggest a personalized treatment strategy for *H pylori* infection according to 23S rRNA genotype.

Author contributions

Author contributions: Concept and design: CP, HJ, and SH; analysis and interpretation of the data: CP, SK, and SH; data collection and experimental procedures: SK, CP, and EL; drafting

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