Effects of addition of probiotic and/or bismuth to triple therapy of *H. pylori* and analysis of genetic variation of 23S rRNA gene between patients with clarithromycin sensitivity and resistance

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Abstract: The aim of the study was to compare the effects of three treatment regimens for *H. pylori* in patients sensitive to clarithromycin and analyze the polymorphism of 23S rRNA gene between patients who were sensitive or resistant to clarithromycin in a Chinese Han population. 204 *H. pylori* sensitive cases and 45 *H. pylori* resistant Han patients were selected as subjects of the research. All *H. pylori* sensitive cases were divided into three groups based on their different therapies. The polymerase chain reaction-ligase detection reaction (PCR) was used to identify the genotype at the A2143G of the 23S rRNA gene. SPSS18.0 software was applied to analyze the data statistically. The success rate of *H. pylori* eradication in the TTP (TT + probiotic) group was higher when compared with the triple therapy (TT) group, and the difference was statistically significant. The incidence of abdominal pain, headache and diarrhea in TTP group was significantly lower than that in the TT group and the TTB (TT+ bismuth) group. Moreover, patients in the TTP group suffered less taste impairment than patients in the other two groups. In addition, there was significant difference in genotype frequency distribution between the clarithromycin-resistant group and the clarithromycin-sensitive group. It was suggested in the results of Chinese Han population that the TTP regimen was significantly superior to the other two regimens in the treatment of clarithromycin-sensitive *H.PYLORI* infection. In addition, potential genotypic differences between clarithromycin-sensitive and drug-resistant patients provided a theoretical basis for gene therapy in patients with clarithromycin resistance.

Keywords: *Helicobacter pylori*, probiotic, genetic variation.

INTRODUCTION

Human beings are the sole host of *Helicobacter pylori* (*H. pylori*). From a global perspective, *H. pylori* is widely found in the stomach of about half of the world's population, and the rate of *H. pylori* infection remains high in most countries (Malfertheiner *et al*., 2012; Eusebi *et al*., 2014). China is also a country with high rate of *H. pylori* infection. From the 90s of last century, domestic scholars have started a number of epidemiological investigations. The results of the study showed that the infection rate of *H. pylori* ranged from 41.35% to 72.3%, and the infection rate of *H. pylori* in some areas even reaches 90%, which seriously threatened human health (Xie and Lu, 2015; Huang *et al*., 2015).

It was shown in the studies that the successful eradication of *H. pylori* can promote ulcer healing, reduce the recurrence rate of ulcers, alleviate functional dyspepsia and prevent gastric cancer, thereby reducing the *H. pylori* infection and the economic burden of the sick person, as well as the suffering of the disease (Ford *et al*., 2014). The quadruple therapy of PPI combined with bismuth-containing and two antibiotics were recommended as the initial treatment protocol in the fourth consensus for the diagnosis and treatment of *H. pylori* infection in China in 2012. Probiotics are a kind of active microorganisms that are colonized on the host and beneficial to the host. They can regulate the gastrointestinal symptoms caused by antibiotics. In addition, probiotics also have the function of protecting the gastrointestinal mucosal barrier and enhancing the immunity of the body. Therefore, the microorganisms combined with probiotics plays an important role in the prevention and treatment of *H. pylori* (Zou *et al*., 2009). But so far, whether the probiotics can improve the eradication rate of *H. pylori* is still controversial at home and abroad (Mirzaee1 and Rezahosseini, 2012; Homayoun *et al*., 2013). Clarithromycin plays a key role in the eradication of *H. pylori*. The mechanism of its action is mainly binding to the 50 large subunits on the bacterial ribosome, and then acting on the polypeptide transferase loop of the 23S rRNA V region, thereby inhibiting ribosome translocation, consequently attributing to the blocking of peptide chain elongation and the inhibition of protein synthesis. This process plays a bacteriostatic role by inhibiting protein synthesis (Senbanjo *et al*., 2014; Lim *et al*., 2013; Boyanova *et al*., 2006). With the decrease of eradication rate of *H. pylori* and the increase of drug resistance rate of clarithromycin, the molecular biological mechanism of *H. pylori* resistance to clarithromycin was studied by domestic and foreign scholars. The study showed that clarithromycin resistance was related to 23S rRNA gene polymorphism (Slinger *et al*., 2009). At present, there are mutation sites such as A2142G,

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A2142C, A2143G, A2144G, T2182C, T2183C and T2245C in the 23S rRNA of clarithromycin-resistant strains, among which A2143G has the highest mutation rate (Stone *et al*., 1996), which has been found in many studies at home and abroad. However, it is unclear whether there is a mutation in the A2143G locus between the sensitive and resistant patients in the Chinese Han population.

Therefore this study was designed to compare the effects of three treatment regimens for *H. pylori* in patients sensitive to clarithromycin and analyze the polymorphism of 23S rRNA gene between patients who were sensitive or resistant to clarithromycin in a Chinese population.

MATERIALS AND METHODS

Patient information and grouping

From July 1, 2015 to December 30, 2016, *H. pylori*-positive patients who were admitted to the Department of Gastroenterology, First People's Hospital of Suqian City, PR China were collected and fecal drug susceptibility test was taken. A total of 426 patients were tested, and 249 cases of patients were enrolled, of which 204 cases were clarithromycin-sensitive and 45 cases were clarithromycin-resistant.

In addition, clarithromycin-sensitive patients were further divided into;

1. Triple therapy group (TT group) (nexium 20mg bid, clarithromycin 0.5g bid and amoxicillin1g bid for TT group);
2. Triple plus probiotic group (TTP TT + probiotic) (Nexium 20mg bid, clarithromycin 0.5g bid, amoxicillin 1g bid, Bifico 420mg tid for TTP group);
3. Triple plus bismuth-containing group (TT group) (Nexium 20 mg bid, clarithromycin 0.5g bid, amoxicillin 1g bid and colloidal bismuth pectin 200 mg bid for TTB group).

Each group of treatments was carried out for 14 days. For patients with peptic ulcers, they continued to take medicine for 4 weeks after the eradication of duodenal ulcer and continued to take medicine for 6 weeks after the eradication of gastric ulcer. The study was approved by the ethics committee of First People's Hospital of Suqian City, PR China. The reference No. was 203/IRB/2015. All patients agreed and signed informed consent before randomization.

Inclusion criteria

1. The age of the patients was 18~65 years old and the sex was not limited;
2. *H. pylori* positive patients with gastric or duodenal ulcer;
3. *H. pylori* positive patients with chronic gastritis accompanied by obvious symptoms of gastrointestinal discomfort;
4. Patients who had not previously received *H. pylori* eradication treatment;
5. Patients with negative stool susceptibility test results.

Exclusion criteria

1. Patients with antibiotics, bismuth-containing, H_2 receptor antagonists, PPI, and anticoagulant use history four weeks before treatment;
2. Patients with a history of gastrectomy and a history of gastric malignant lymphoma; 3) Patients with upper gastrointestinal active bleeding in the past week;
3. Pregnant or lactating women;
4. Patients who were allergic to the drugs used in this study;
5. Patients who were suffering from other serious diseases that influencing the study at the same time, such as severe liver disease, heart disease, kidney disease, cerebrovascular disease, malignant tumor and alcoholism;
6. Patients who needed to take a metal preparation during the treatment (Metal preparations interact with levofloxacin, except for bismuth-containing);
7. Patients who could not correctly expressed their complaints, such as psychotic patients and patients who were unable to cooperate;
8. Patients who took part in other drug studies within the first 3 months of using the research drug.

Termination test criteria

1. Patients who could not tolerate severe side effects during the trial;
2. Patients with worsening conditions or serious complications;
3. Patients with other diseases during the treatment interfered with the study;
4. Patients who were pregnant during treatment;
5. Patients who were lost to follow-up.

SNP genotyping analysis

Fecal samples were collected from each subject and DNA was extracted from the fecal suspension using the AxyPrep DNA Blood kit (Axygen Scientific Inc, Union City, CA, USA) and stored at -80°C until use. Genotyping for the SNP A2143G of 23S rRNA gene was conducted using the polymerase chain reaction-ligase (PCR) detection method. The specific steps were as follows:

- Pre-denaturation at 95°C for 2 minutes;
- Denaturation at 94^{L} for 30 seconds,
- Annealing at 57^{L} for 37 seconds,
- Extension at 72^{L} for 30 seconds, 5 cycles.
- Denaturation at 94^{L} for 15 seconds,
- Annealing at 57^{L} for 15 seconds,
- Extension at 72^{L} for 20 Seconds, 30 cycles.

In the reaction, deionized water was used as a negative control, and the *H. pylori* standard strain Sydney strain 1 (SS1) was used as a positive control.

Second round PCR

- pre-denaturation at 95^{L} for 2 minutes;
- denaturation at 94^{L} for 10 seconds,
- annealing at 63^{L} for 20 seconds,
- Extension at 72^{L} for 20 seconds, 25 cycles.
- Extension at 72^{L} for 7 minutes.

In the reaction, deionized water was used as a negative control, and the *H. pylori* standard strain Sydney strain 1 (SS1) was used as a positive control. The amplification was completed, and the results were observed under a UV lamp after electrophoresis at 100V for 15 minutes in a 1.5% agarose gel. All successful PCR amplification samples were sent to Shanghai Biotech Biotech Co., Ltd. for purification and then sequenced using the Sanger-dideoxy chain-termination method.

The method of judging H. pylori eradication

After 4 weeks of withdrawal, the patients were given C13 breath test. The negative patients were judged to be *H. pylori* eradicated, while the positive ones were *H. pylori* eradication failure. For peptic ulcer patients, the test time was after the eradication treatment, the patients with duodenal ulcer took PPI for 4 weeks, and the patients with gastric ulcer continued to take PPI for 6 weeks. The breath test was rechecked four weeks after the final withdrawal.

STATISTICAL ANALYSIS

The data was processed using SPSS18.0 statistical analysis software. Measurement data were expressed as mean ± standard deviation and analyzed by analysis of variance. While the count data were represented by percentage (%) and chi-square test was used. P<0.05 was considered to be statistically significant.

Table 1: Comparison of general information in 3 groups

Groups	N	Gender		Age	Esophagogastroduodenoscopy		
		Male	Female		Chronic Gastritis	Gastric Ulcer	Duodenal Ulcer
TT group	68	41	27	46.3 ± 12.7	3	4	10
TTB group	68	40	28	45.9 ± 13.2	4	4	11
TTP group	68	40	28	45.3 ± 14.1	3	3	9
P	---	---	---	>0.05	>0.05	>0.05	>0.05

Table 2: Adverse events in each treatment group (*P<0.05 vs TT group, $^{\#}$P<0.05 vs TTB group)

Adverse Events	n (%)		
	TT group (n=68)	TTB group (n=68)	TTP group (n=68)
Abdominal pain	47 (69.1)	43 (63.2)	18 (26.5)*$^{\#}$
Headache	23 (33.8)	18 (26.5)	3 (4.4)*$^{\#}$
Diarrhea	34 (50.0)	27 (39.7)	16 (23.5)*$^{\#}$
Vomiting	3 (4.4)	6 (8.8)	4 (5.9)
Taste impairment	51 (75.0)	20 (29.4)*	47 (69.1)
Dyspepsia	6 (8.8)	8 (11.8)	2 (2.9)
Skin rash	1 (1.5)	3 (4.4)	3 (4.4)

Table 3: Hardy-Weinberg equilibrium of 23S rRNA genotypes distribution on locus 2143A/G

Genotypes	Clarithromycin Resistant Group		Clarithromycin Sensitive Group	
	Observation Value	Expectation Value	Observation Value	Expectation Value
AA	6	9.97	115	101.65
AG	31	22.42	64	84.70
GG	8	12.61	25	17.64
p	>0.05		>0.05	

Table 4: The 2143 locus of 23S rRNA, distribution frequency of genotype in Clarithromycin Resistance and *H. pylori* Clarithromycin susceptibility

Groups	N	Frequency of Genotype (%)		
		AA	AG	GG
Clarithromycin Resistant Group	45	6 (13.33)	31 (68.89)	8 (17.78)
Clarithromycin Sensitive Group	204	115 (56.37)	64 (31.37)	25 (12.25)
p	---	<0.05		

RESULTS

Comparison of general information in each group

A total of 249 *H. pylori*-positive patients were enrolled in this study. Among these, 204 patients were sensitive to clarithromycin and were randomly divided into the triple therapy group (TT group, n=68), the triple therapy + bismuth group (TTB group, n=68) and the triple therapy + probiotic group (TTP group, n=68). Gender and age were similar in the 3 groups. There was also no significant difference in esophagogastroduodenoscopy finds among the TT group, TTB group and TTP group, as shown in table 1.

Success rate for H. pylori eradication of each group

The eradication rate was 74% (50/68) in the TT group, 82% (56/68) in the TTB group and 91% (62/68) in the TTP group. Compared with the TT group, the success rate for *H.PYLORI* eradication of the TTP group was higher and the difference was statistically significant. There was no difference between the TT group and TTB group. Nevertheless, there was a tendency between the TTB group and the TTP group, the difference was not significant in statistic (fig. 1).

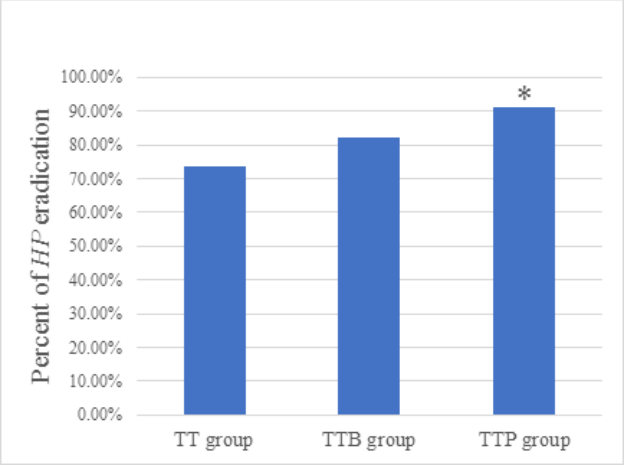

Fig. 1: Comparison of success rate for *H.PYLORI* eradication in 3 groups (*P<0.05 vs TT group).

Comparison of the occurrence of adverse events in each group

Common adverse events in the three study groups are shown in table 2. The occurrence of abdominal pain, headache and diarrhea was significantly lower in the TTP group than in the TT group and TTB group. Patients in the TTB group suffered less taste impairment than patients in the other two groups. Besides, there were no differences in the occurrence of vomiting, dyspepsia or skin rash among the 3 groups.

Genotype distribution of clarithromycin resistant group and clarithromycin sensitive group

Among 249 *H. pylori* positive patients, 204 patients were sensitive to clarithromycin and named as the clarithromycin sensitive group and the remained 45 patients were resistant to clarithromycin and named as the clarithromycin resistant group. All the subjects in the 2 groups detected two alleles of A and G at the 2143 locus of 23S rRNA. The frequencies of A in the clarithromycin resistant group and the clarithromycin sensitive group were 47.06% and 70.59% respectively, while the frequencies of G were 52.94% and 29.41% respectively. The Hardy-Weinberg equilibrium of 23S rRNA genotypes distribution on locus 2143A/G is shown in table 3. The results indicated that allele frequency reached genetic balance and had group representative ness. Among the 45 clarithromycin- resistant strains, 6 were AA genotype, 31 were AG genotype, and 8 were GG genotype, while among the 204 clarithromycin-sensitive strains, 115 were AA genotype, 64 were AG genotype, and 25 were GG genotype. There was significant difference in genotype frequency distribution between the clarithromycin resistant group and the clarithromycin sensitive group (table 4).

DISCUSSION

H. pylori is a kind of highly evolved and the only colonized bacterium that colonizes on the mucosal tissue of the human stomach, and it can colonize on the stomach in a strong acid environment. Moreover, it has its own VacA protein, CagA protein and urease activity. These unique molecular biological characteristics play an important role in its colonization (Dunn *et al*., 1997). In the treatment of *H. pylori*, PPI + amoxicillin + clarithromycin as a standard first-line protocol is mainly used in the initial treatment regimen. Although clarithromycin plays a very important role in the treatment of *H. pylori*, however, with the wide application and unreasonable application of antibiotics, the resistance rate of clarithromycin in various countries is increasing year by year (Lim *et al*., 2013). In our study, clarithromycin resistant patients in *H. pylori* infected patients reached 18.07%. Therefore, it is of great significance to select rational drugs according to drug susceptibility test and find the specific mechanism of drug resistance.

Probiotics are culturable active microorganisms. The intake of a certain dose of probiotics can inhibit the growth of harmful bacteria by producing organic acids, hydrogen peroxide, bacteriocin and so on, so that the microbiotic state of the body can be balanced and thus has a beneficial effect on the organism (Du *et al*., 2012). The studies showed that the combination of probiotics in standard triple therapy can effectively improve the eradication effect. It was also confirmed in our study that the triple drug combined with probiotics can significantly improve the eradication effect of *H. pylori* (Zou *et al*., 2009). Regardless, it has been concluded in some studies that probiotics do not further increase the efficacy of triple therapy for *H. pylori* (Mirzaee1 and Rezahosseini,

2012; Homayoun *et al*., 2013). As for this issue, we believe that there may be different types of probiotics, and the time and course of treatment are different, which makes different studies have different effects on probiotics in the treatment of *H. pylori*. It was also displayed in our study that although the success rate of *H. pylori* in the TTB group was higher than that in the TT group, the P value was still greater than 0.05. In addition, our study also showed that the incidence of abdominal pain, headache, and diarrhea in the TTP group was significantly lower than that in the TT group and the TTB group. Furthermore, patients in the TTP group had lower levels of taste loss than the other two groups. And there were no differences in the incidence of vomiting, dyspepsia and rash in the three groups. Previously it is also explored that, bismuth-containing quadruple therapies still achieve high eradication rates of *H. pylori* (Hu *et al*., 2017). Moreover, susceptibility-based therapies are alternatives because they may avoid the use of unnecessary antibiotics.

Clarithromycin clarithromycin is a 15-ring semi-synthetic macrolide, which is replaced by a methoxy group at the 6-position hydroxyl group of the lactone ring. Therefore, in the treatment of eradication of *H. pylori*, this structural modification can increase its stability to acid and its antibacterial activity. Its mechanism of action is to bind to the 50 large subunits on the bacterial ribosome, acting on the polypeptide transferase loop of the 23S rRNAV region, thereby inhibiting the ribosome translocation process and preventing the peptide chain from extending and inhibiting protein synthesis. This process acts as a bacteriostatic action by inhibiting protein synthesis (Zhou *et al*., 2008; Slinger *et al*., 2009). The production of clarithromycin resistance in *H. pylori* is closely related to the mutation of the DNA sequence of the 23S rRNA V region (Taylor *et al*., 1997). At present, there are many reports about the resistance rate of clarithromycin resistance and the genetic polymorphism of drug-resistant strains in the world, (Raymond J *et al*., 2007) in France collected 217 specimens of children infected with *H. pylori* and 50 strains of clarithromycin resistant *H. pylori* obtained by PCR-RFLP technology, of which the mutation rate of A2143G reached 90% (Raymond *et al*., 2007). According to a study by (Kim T *et al*., 2013) in Korea, the failure rate of triple elimination of PPI was 4.2% and 87.5% in patients carrying A2142G and A2143G mutation sites and concluded that there was a significant correlation between A2143G point mutation and failure of *H. pylori* eradication (Kim *et al*., 2013). Besides, there was also a significant difference in genotype frequency distribution between the clarithromycin resistant group and the clarithromycin sensitive group which was consistent with the results in the present research, and it was suggested that the G allele may be a risk gene for clarithromycin-resistant strains.

CONCLUSION

In conclusion, the study results demonstrated that the TTP regimen was significantly superior to the TTB and TT regimens in the treatment of *H. pylori*-infected patients with clarithromycin sensitivity. In addition, the polymorphism of clarithromycin resistance gene was also analyzed, indicating its potential genotypic differences, which will provide a theoretical basis for gene therapy for clarithromycin-resistant patients with *H. pylori* eradication.

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