

Characterization of 23S rRNA gene mutation in primary and secondary clarithromycin-resistant *Helicobacter pylori* strains from East China

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Background/aims: Clarithromycin is an effective antibiotic for treating *Helicobacter pylori*; however, the development clarithromycin-resistance by multiple strains prevents the eradication of *Helicobacter pylori*. We aimed to characterize mutations in the 23S rRNA gene of primary clarithromycin-sensitive, primary clarithromycin-resistant and secondary clarithromycin-resistant *Helicobacter pylori* strains that were collected in East China and elucidate the mechanisms of clarithromycin resistance. **Materials and Methods:** The disk diffusion test and E-test method were used to determine the clarithromycin susceptibility of clinical *Helicobacter pylori* strains. The 23S rRNA gene fragments were amplified by polymerase chain reaction from 18 primary clarithromycin-resistant strains, 15 primary sensitive strains and 8 secondary clarithromycin-resistant strains. Polymerase chain reaction-products were sequenced to determine mutations of the 23S rRNA gene. **Results:** We found an A2143G (8 strains) mutation in primary clarithromycin-resistant strains, an A2143T (5 strains) mutation in secondary clarithromycin-resistant strains; but no mutations were found in position 2143 of sensitive strains. A T2182C mutation in primary clarithromycin-sensitive, primary clarithromycin-resistant and secondary clarithromycin-resistant strains was found with a prevalence of 86.7% (13 strains), 72.2% (13 strains) or 87.5% (7 strains), respectively. In addition, we found a G2254T (8 strains) and a G2172T (7 strains) mutation in secondary clarithromycin-resistant strains. These point mutations were absent in primary clarithromycin-resistant and -sensitive strains. **Conclusion:** The gene mutation in position 2143 was associated with resistance to clarithromycin, but the mutation was different between primary and secondary clarithromycin-resistant strains. The T2182C mutation was not associated with clarithromycin resistance. Two new hotspot mutations: G2254T and G2172T, in 23S rRNA were discovered in secondary clarithromycin-resistant strains.

Key words: *Helicobacter pylori*, clarithromycin, resistance, gene mutation

Doğu Çin'deki klaritromisine primer ve sekonder dirençli suşlarda 23S rRNA gen mutasyonunun karakterizasyonu

Amaç: Klaritromisin, *Helikobakter pilori* eradikasyonunda kullanılan etkin bir antibiyotiktir. Ancak, klaritromisin direnci *Helikobakter pilori* eradikasyonunu ciddi şekilde zayıflatmaktadır. Amacımız, Doğu Çin'den toplanan primer klaritromisin hassas, klaritromisin dirençli ve sekonder klaritromisin dirençli *Helikobakter pilori* suşlarındaki 23S rRNA gen mutasyonlarını karakterize etmek ve klaritromisin direnç mekanizmalarını araştırmaktır. **Gereç ve Yöntem:** Klinik *Helikobakter pilori* suşlarında klaritromisin duyarlılığını ölçmek için disk difüzyon testi ve E-Test yöntemleri kullanılmıştır. 23S rRNA gen parçacıkları primer klaritromisin dirençli, hassas ve sekonder klaritromisin dirençli suşlarından polimeraz zincir reaksiyonu ile amplifiye edilmiştir. Polimeraz zincir reaksiyonu ürünleri 23S rRNA gen mutasyon karakteristiklerinin belirlenmesi için sekanslanmıştır. **Bulgular:** Primer klaritromisin dirençli suşlarda A2143G (8/18) mutasyonu, sekonder Klaritromisin dirençli suşlardan A2143T (5/8) mutasyonu bulunmuş; ancak Klaritromisin hassas suşlarda 2143 pozisyonunda (0/15) mutasyon bulunmamıştır. T2182C mutasyonu primer klaritromisin hassas, dirençli ve sekonder klaritromisin dirençli suşlarda sırasıyla 13/15 (86.7%), 13/18 (72.2%) ve 7/8 (87.5%) bulunmuştur. Ek olarak sekonder klaritromisin dirençli suşlarda G2254T (8/8) ve G2172T (7/8) mutasyonu saptanmıştır. Bu nokta mutasyonları, primer klaritromisin rezistan ve hassas suşlarda saptanmamıştır. **Sonuç:** 2143 pozisyonundaki gen mutasyonu klaritromisin direnciyle ilişkilidir, ancak primer ve sekonder klaritromisin dirençli suşlardaki mutasyon tipi farklıdır. T2182C mutasyonu klaritromisin direnciyle ilişkili değildir. Sekonder klaritromisin dirençli suşlarda 23SrRNA'da keşfedilen iki yeni hassas nokta mutasyonu G2254T ve G2172T'dir.

Anahtar kelimeler: *Helikobakter pilori*, klaritromisin direnci, gen mutasyonu

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic, spiral bacterium that has colonized the stomachs of approximately half the world's population (1). Infection with *H. pylori* is associated with chronic gastritis and peptic ulceration and the bacterium is considered a risk factor for the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (2-5). Currently the most effective treatments for *H. pylori* combine a proton pump inhibitor with two antimicrobial agents selected from amoxicillin, metronidazole, or clarithromycin. Clarithromycin is often a key component of the standard triple therapy. However, the increasing use of clarithromycin has resulted in the development of resistance in multiple strains. The rising prevalence of clarithromycin resistance increasingly threatens to compromise the efficacy of the first-line therapy. There has been considerable research (6-9) into unraveling the factors that determine clarithromycin resistance in *H. pylori*. These studies have confirmed that clarithromycin resistance was associated with the mutations A2142G and A2143G in the 23S rRNA gene. However, most of these studies were based on primary resistant strains or strains with resistance induced *in vitro*. The aims of our study were (i) to evaluate the prevalence of primary and secondary resistance to clarithromycin and (ii) to identify the different gene mutations by gene sequencing among the primary sensitive-strains, primary resistant-strains and secondary resistant-strains.

METHODS

Patients

A total of 365 patients undergoing endoscopy for gastrointestinal symptoms at the Department of Gastroenterology, The First Affiliated Hospital of NanChang University, China, from 2002 to 2006 (345 with no history of triple therapy and 20 referred for failure of therapy) were enrolled in this study after approval by the Ethic Committee and a written informed consent was obtained from all patients before biopsies were taken. Primary clarithromycin-sensitive and clarithromycin-resistant *H. pylori* strains were obtained from patients who had not taken antibiotics, proton pump inhibitor (PPI) or nonsteroidal antiinflammatory drugs during the preceding three months. Secondary clarithromycin-resistant strains were obtained from patients who had received PPI triple the-

rapy including amoxicillin (1000 mg, bid) and clarithromycin (500 mg, bid) for one week.

H. pylori culture and antimicrobial susceptibility determination

Two gastric biopsy specimens were taken by endoscopy from patients with digestive symptoms and whose rapid urease enzyme test was positive. Each specimen was cultured on Skirrow's selective medium containing 10% sheep defibrinated blood. *H. pylori* strains were cultured under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 37°C for 3-5 days. Bacterial strains were identified according to colony morphology, Gram-staining, urease, catalase and oxidase production. The bacterial were stored at -80°C in Brucella broth supplemented with 15% glycerol and fetal calf serum (Invitrogen, USA).

Disk diffusion tests (Oxoid Ltd., England) and E-test methods (AB Biodisk, Sweden) were used to determine the susceptibility of clinical *H. pylori* strains to clarithromycin. Resistance was determined by an inhibitory zone of <30 mm for disk diffusion (10). The cut-off concentration used to define resistance by E-test method was >1 µg/mL (11). *H. pylori* NCTC11637 was included as a control clarithromycin-sensitive strain.

DNA extraction and polymerase chain reaction amplification of the 23S rRNA gene

DNA extraction was carried out using the TIAN-amp Bacteria DNA Kit (TIANGEN BIOTECH, Beijing, China) and stored at -20°C until use. The primers used for polymerase chain reaction (PCR) amplification were as follows: forward primer: 5'-CTG CAT GAA TGG CGT AAC GAG-3' (complementary to 23S rRNA gene sequence from 2047 to 2067, GenBank accession no. U27270); and reverse primer: 5'-GAG CGA CCG CCC CGA TCA AAC-3' (complementary to 23S rRNA gene sequence from 2327 to 2347, GenBank accession no. U27270), which generates a 301 bp product. Amplification was carried out in a thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer, USA.). The PCR amplification reaction mixture (50 µl) contained 25 µl 2×pfu PCR mastermix, 15 µl double distilled H₂O, 4 µl of each primer and 2 µl of DNA sample. PCR cycle conditions were 94°C for 4 min hot start then 35 cycles of 94°C for 40 s, 61.5°C for 40 s, 72°C for 1 min followed by a final extension step at 72°C for 7 min. Amplified fragments were visualized on a 2% agarose gel electrophoresis stained with ethidium bromide.

DNA sequencing and sequence analysis

PCR products were sent to Shanghai Generay Biotech Corporation (China) to conduct the DNA sequencing. Nucleotide sequences were aligned and analyzed using the DNA Star software package. Mutations in domain V of the 23S rRNA gene were determined by comparing the sequences of clarithromycin-resistant and -sensitive strains with the sequences of the reference strains U27270 and NCTC11637.

RESULT

Primary clarithromycin resistance prevalence was 9.3% (32 of 345) in 345 untreated strains. The secondary clarithromycin resistance rate was 50% (10 of 20) (Table 1). Eighteen primary clarithromycin-resistant strains, 15 clarithromycin-sensitive strains and 8 secondary clarithromycin-resistant strains were randomly selected for mutational analysis using PCR amplification of domain V of the 23S rRNA gene.

In primary clarithromycin-resistant strains, two gene mutations, A2143G and T2182C, were observed in 44.4% (8 of 18) and 72.2% (13 of 18) of strains, respectively. In secondary clarithromycin-resistant strains, the point mutation A2143G was not found. However, the gene mutation A2143T was observed in 62.5% (5 of 8) and the T2182C mutation occurred in 87.5% (7 of 8) of secondary clarithromycin-resistant strains. In primary clarithromycin-sensitive strains, no point mutations were observed at position 2143. The point mutation T2182C was observed in 86.7% (13 of 15) (Table 2).

In addition, two point mutations G2172T and G2254T were observed in 87.5% (7 of 8) and 100% (8 of 8) of secondary clarithromycin-resistant strains, while none of the primary clarithromycin-sensitive and clarithromycin-resistant strains had these mutations.

DISCUSSION

Clarithromycin is one of the most effective antibiotics for eradicating *H. pylori* infections. However, total eradication is impossible due to increasing clarithromycin resistance in *H. pylori* strains. The Maastricht-3 consensus guidelines recommend that antibiotics be chosen based on local resistance levels, therefore knowing the levels of clarithromycin resistance is essential for clinicians to develop treatment plans. The prevalence of primary and secondary clarithromycin resistance is lower in East China than Italy (12), while the prevalence is similar to that reported in Bulgaria (13). As clarithromycin is a member of the macrolide family, which has well documented cross resistance between all macrolide family members (14). Thus, the primary risk factor for clarithromycin resistance is previous consumption of macrolides (15). Accordingly, the low rate of resistance in East China and Bulgaria is likely due to relatively low macrolide consumption. Secondary clarithromycin resistance rates are significantly higher than primary clarithromycin resistance. Due to the low levels of primary clarithromycin resistance, clarithromycin is still effective as the first choice antibiotic in treating *H. pylori* infection. However, for patients with secondary clarithromycin resistance, clarithromycin should be used with caution.

Table 1. Numbers of resistant strains in untreated strains and failed eradicated strains

	No. of strains (%)		Total no. of strains
	CLAr	CLAs	
Untreated strains	32 (9.3%)	313 (90.7%)	345
Failed eradicated strains	10 (50%)	10 (50%)	20

CLAr: clarithromycin-resistant strains. CLAs: clarithromycin-sensitive strains.

Table 2. Mutations in the 23S rRNA gene in 18 primary clarithromycin-resistant (CLAr^r), 8 secondary clarithromycin-resistant (CLAr^{rr}) and 15 clarithromycin-sensitive (CLAs) strains

Genotypes of 23S rRNA mutation	No. of strains (%)		
	CLAr ^r (No. 18)	CLAr ^{rr} (No. 8)	CLAs (No. 15)
A2143G	8 (44.4%)	-	-
A2143T	-	5 (62.5%)	-
T2182C	13 (72.2%)	7 (87.5%)	13 (86.7%)
G2172T	-	7 (87.5%)	-
G2254T	-	8 (100%)	-

Gene mutations of 23S rRNA were assessed by PCR amplification and nucleotide sequencing

Clarithromycin acts by binding to ribosomes and its resistance is due to modification of ribosomal sites necessary for this interaction. In 1996, Versalovic *et al.* (5) reported that clarithromycin resistance is caused by point mutations in the peptidyl transferase domain of the 23S rRNA ribosomal subunit. They found adenine-to-guanine transition mutations at either position 2142 or 2143 in 23S rRNA were associated with clarithromycin resistance. After that, many studies (7-9,14,16) confirmed that A2143G and A2142G are the most prevalent point mutations and that these mutations play a major role in clarithromycin resistance. However, most of these studies were based on primary resistant strains or induced resistant strains *in vitro*. In the present study, no point mutation was found at position 2143 in primary clarithromycin sensitive strains. The A2143G mutation was observed in just 44.4% (8 of 18) of primary resistant strains. This result suggests that additional factors, such as the efflux pump (17), may be responsible for the primary resistance to clarithromycin. However, in secondary resistant strains, the major point mutation is A2143T instead of A2143G, and the prevalence of A2143T is high (62.5%, 5 of 8). Our study demonstrates that the point mutations at position 2143 are associated with primary and secondary resistance to clarithromycin, but the mutations at position 2143 of the primary and secondary resistant strains are different in China, which is inconsistent with that of Toracchio (12) *et al.*'s study in Italy. Their study shows that the point mutation A2143G was observed in 58% of primary resistant strains and in 60% of secondary resistant strains, but that the A2143T mutation was absent. The discrepancy between our findings and that of Toracchio *et al.* (12) may be caused by regional genetic differences. The association of the T2182C mutation and clarithromycin resistance is still controversial. Some studies (18-19) have shown that the T2182C mutation is associated with clarithromycin resistance using transformation experiments; while others (20-21) have shown that the T2182C mutation has no role in clarithromycin resistance. However,

most of these studies have not examined the point mutation at position 2182 in sensitive strains. In our study, the prevalence of the T2182C point mutation in primary clarithromycin resistant, secondary clarithromycin resistant and clarithromycin sensitive strains is high (72.2%, 87.5% and 86.7%, respectively) with no significant difference between them. The present findings indicate that the T2182C mutation is not associated with clarithromycin resistance.

Two novel point mutations, G2172T (7 of 8) and G2254T (8 of 8), are hotspot mutations in secondary resistant strains. The two point mutations were not detected in primary resistant and sensitive strains. As these mutations were not described previously, whether these mutations are associated with secondary resistance to clarithromycin remains unclear. Therefore, further study will be needed to clarify the role of these mutations in secondary resistance.

In conclusion, the prevalence of primary clarithromycin resistance is relatively low in Chinese strains, so clarithromycin still can be used as the first-line antibiotic in treating *H. pylori* infections. However, the use of clarithromycin after prior treatment failure should be limited due to the high prevalence of secondary clarithromycin resistance. Gene mutation at position 2143 in the 23S rRNA gene of *H. pylori* strains is associated with the primary and secondary resistance to clarithromycin. However, the mutation at position 2143 in the primary resistant strains (A2143G) is different from that in secondary resistant strains (A2143T). The T2182C mutation is not associated with clarithromycin resistance. Consequently, the T2182C mutation cannot be used as a marker to detect the clarithromycin resistance, at least in East China due to regional genetic differences. Finally, two novel hotspot mutations G2172T and G2254T were discovered in secondary resistant strains.

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