

Gene Mutations of 23S rRNA Associated with Clarithromycin Resistance in *Helicobacter pylori* Strains Isolated from Korean Patients

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Received: December 25, 2007 / Accepted: April 6, 2008

Although resistance of *Helicobacter pylori* to clarithromycin is a major cause of failure of eradication therapies, little information is available regarding gene mutations of clarithromycin-resistant primary and secondary *H. pylori* isolates in Korea. In the present study, we examined gene mutations of *H. pylori* 23S rRNA responsible for resistance to clarithromycin. DNA sequences of the 23S rRNA gene in 21 primary clarithromycin-resistant and 64 secondary clarithromycin-resistant strains were determined by PCR amplification and nucleotide sequence analyses. Two mutations of the 23S rRNA gene, A2143G and T2182C, were observed in primary clarithromycin-resistant isolates. In secondary isolates, dual mutation of A2143G+T2182C was frequently observed. In addition, A2143G+T2182C+T2190C, A2143G+T2182C+C2195T, and A2143G+T2182C+A2223G were observed in secondary isolates. Furthermore, macrolide binding was tested on purified ribosomes isolated from T2182C or A2143C mutant strains with [¹⁴C]erythromycin. Erythromycin binding increased in a dose-dependent manner for the susceptible strain but not for the mutant strains. These results indicate that secondary isolates show a greater variety of 23S rRNA gene mutation types than primary isolates, and triple mutations of secondary isolates are associated with A2143G+T2182C in *H. pylori* isolated from Korean patients.

Keywords: Clarithromycin, gene mutations, *Helicobacter pylori*, primary isolates, resistance, secondary isolates

Helicobacter pylori infection is recognized as a causal factor of chronic gastritis, peptic ulcer, and gastric cancer. Several studies have recommended that *H. pylori* should be eradicated in patients with peptic ulceration in order to accelerate ulcer healing and prevent long-term ulcer relapse. Current treatment regimens incorporate a proton pump inhibitor (PPI) and a combination of two or more antibiotics, including amoxicillin and clarithromycin [18]. However, antibiotic resistance is a growing problem.

The frequency of resistance to antibiotics has varied widely according to geographical regions and subgroups within study populations [1, 6, 8, 15]. Primary clarithromycin resistance has been reported in 2–15% of patients infected with *H. pylori*, and it has been increasing [1, 8, 11]. In addition, resistance to clarithromycin after treatment failure (secondary resistance) has increased to 85.1% in Korean patients [14]. We recently reported that the clarithromycin resistance was associated with a significant decrease of eradication in Korean patients [15].

Clarithromycin-resistant strains frequently carry mutations in the 23S rRNA gene [17]. Several reports have demonstrated that A-to-G point mutations at positions 2142 and 2143 within domain V [24], or a T-to-C mutation at position 2717 [5] within domain VI of the 23S rRNA gene cause clarithromycin resistance [23]. Nevertheless, little information is available regarding the gene mutations of primary and secondary *H. pylori* isolates in Korea. The aim of this study was to assess mutations within domain V of the 23S rRNA gene in primary and secondary *H. pylori* isolates from Korean patients. In the studies reported here, we have demonstrated the characterization of a greater variety of 23S rRNA gene mutation types of

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clarithromycin-resistant clinical *H. pylori* isolates from Korean patients.

MATERIALS AND METHODS

Clarithromycin-Resistant *H. pylori* Strains

Twenty-one primary clarithromycin-resistant and 64 secondary clarithromycin-resistant *H. pylori* strains were collected from strains isolated at Seoul National University Hospital from 2003 to 2006 [11, 12, 14, 15]. Primary *H. pylori* strains were obtained from patients who had not taken antibiotics, PPI, or nonsteroidal anti-inflammatory drugs during the preceding three months. Secondary strains were obtained from patients who received PPI triple therapy including amoxicillin (1,000 mg, bid) and clarithromycin (500 mg, bid) for one week, as we reported previously [14]. The *H. pylori* strains were cultured under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) as previously described [12]. All stock cultures were stored at -70°C in Brucella broth supplemented with 15% glycerol. These preparations were thawed and subcultured for the present study.

Determination of Minimal Inhibitory Concentration

The MIC values of the *H. pylori* isolates to clarithromycin (Abbott Laboratories, Abbott Park, IL, U.S.A.) were examined using a serial 2-fold agar dilution method as described previously [11, 21]. Briefly, the bacteria were subcultured on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood for 48 h. The bacterial suspension, adjusted to 1×10⁷ CFU, was inoculated directly onto each antibiotic-containing agar dilution plate. After 72 h of incubation, the minimal inhibitory concentration (MIC) of clarithromycin was determined. Quality control was performed using *H. pylori* ATCC 43504. The resistance breakpoint for clarithromycin was set at >1.0 µg/ml [21].

PCR Amplification and Nucleotide Sequencing for 23S rRNA

The extraction of *H. pylori* genomic DNA was performed as reported previously [14]. To detect mutations related to clarithromycin resistance in the 23S rRNA gene, PCR amplification methods and oligonucleotide primers derived from a known sequence of the 23S rRNA gene were used (sense, 5'-CGT AAC TAT AAC GGT CCT AAG-3', positions 2365 to 2385 GenBank Accession No. U27270; antisense, 5'-TTA GCT AAC AGA AAC ATC AAG-3', positions 2635 to 2653, GenBank Accession No. U27270). Amplification was carried out in a thermal cycler (GenAmp PCR System 9600; PerkinElmer Cetus, Norwalk, CT, U.S.A.). PCR cycling conditions consisted of 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 57°C, and 1 min extension at 72°C. Sequencing was performed on the two strands of the nonrestricted amplicons, using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The updated numbering of point mutation in the *H. pylori* 23S rRNA gene sequence was designated as shown in Tayler *et al.* [22], in which position 373A in GenBank number U27270 was defined as the 5' end of the *H. pylori* 23S rRNA.

Transformation of *H. pylori*

H. pylori HP99 (0.0625 µg/ml of clarithromycin MIC), a clinical isolate, was chosen as the recipient for transformation experiments

on the basis of prior experiments that revealed it was readily transformed with DNA [13]. Transformation of *H. pylori* was accomplished as previously described with slight modification [13]. Briefly, recipient *H. pylori* cells (10⁷ per ml) were suspended in 10% horse serum BHI broth in a 50-ml Falcon tube and incubated for 6 h. The cells (200 µl) were dispensed into a 96-well microtiter plate with the addition of 1 µg of PCR-amplified DNA obtained from clarithromycin-resistant strains in a volume of 10 µl and incubated for 18 h under microaerobic conditions. Then, the cells were spread on selective BHI agar plates containing 1 µg/ml of clarithromycin and incubated for up to 5 days.

Ribosome Isolation and Erythromycin Binding Assays

Ribosomes from clarithromycin-resistant *H. pylori* isolates were purified using differential centrifugation as described by Doucet-Populaire *et al.* [4] and Goldman *et al.* [7]. Briefly, *H. pylori* cells grown on BHI agar plates (three plates) were harvested in phosphate buffer containing 137 mM NaCl and 2.7 mM KCl. The harvested cells were washed twice by centrifugation at 5,000 ×g for 5 min at 4°C. The cells were resuspended in 4 ml of buffer A (10 mM Tris-HCl containing 4 mM MgCl₂, 100 mM KCl, and 10 mM NH₄Cl, pH 7.2) and passed through a French pressure cell (Aminco, Urbana, IL, U.S.A.) at 600 lb/in². Ribosome extraction was performed by differential centrifugation, in which the cell debris was discarded by centrifugation at 30,000 ×g for 30 min at 4°C, and the supernatant was centrifuged at 100,000 ×g for 90 min at 4°C to pellet the ribosomes. Ribosomes were resuspended in buffer A and stored at -80°C until needed. The erythromycin-ribosome binding assay was commenced by the addition of [*N*-methyl-¹⁴C]erythromycin [200 nM; Perkin-Elmer (MEN), Waltham, MA, U.S.A.] to different amounts of ribosomes (at an optical density at 260 nm of 1, 2, 3, and 4) in 0.5 ml of buffer A as a reaction mixture. After 30 min, the binding reaction was stopped by diluting the reaction mixture with 3 ml of cold buffer B (10 mM Tris-HCl containing 5 mM MgCl₂ and 150 mM KCl). The ribosomes were collected on a 0.45-µm-pore-size nitrocellulose filter. After three washes with buffer B, the filters were transferred to scintillation vials containing scintillation fluid. The amount of labeled clarithromycin associated with the ribosomes was determined by scintillation counting (Beckman Instruments, Palo Alto, CA, U.S.A.).

Table 1. Mutations of the 23S rRNA gene in clarithromycin-resistant *H. pylori* isolates.^a

Genotypes of 23S rRNA mutation	No. of isolates (%)	
	Primary isolates	Secondary isolates
A2143G	15 (71.4)	5 (7.8)
T2182C	6 (28.6)	5 (7.8)
A2143G+T2182C	-	45 (70.3)
A2143G+T2182C+T2190C	-	4 (6.3)
A2143G+T2182C+C2195T	-	3 (4.7)
A2143G+T2182C+A2223G	-	2 (3.1)
Total	21 (100)	64 (100)

^aGene mutations of 23S rRNA were assessed by PCR amplification and nucleotide sequencing.

RESULTS

Mutations in 23S rRNA Genes of Primary and Secondary Clarithromycin-Resistant *H. pylori* Isolates

In primary clarithromycin-resistant isolates, two gene mutations, A2143G and T2182C, were observed in 71.4% and 28.6% of resistant isolates, respectively (Table 1). Mean MIC values of clarithromycin-resistant *H. pylori* with the A2143G mutation (13.6 $\mu\text{g/ml}$) were higher than those with T2182C (5.3 $\mu\text{g/ml}$) in primary isolates (Fig. 1), although there was no significantly statistical difference by Mann-Whitney U test (P value >0.05).

In secondary clarithromycin-resistant isolates, the single point mutations of A2143G or T2182C occurred at a lower prevalence (7.8% and 7.8%, respectively, Table 1). Dual mutation of A2143G+T2182C was frequently observed in secondary clarithromycin-resistant isolates (70.3%). Three different triple mutations of the 23S rRNA gene were also found in the secondary isolates: A2143G+T2182C+T2190C (6.3%), A2140G+T2182C+C2195T (4.7%), and A2143G+T2182C+A2223G (3.1%). In secondary isolates, mean MIC values were 40.8 $\mu\text{g/ml}$ for A2143G, 6.4 $\mu\text{g/ml}$ for T2182C, 45.0 $\mu\text{g/ml}$ for A2143G+T2182C, 52.0 $\mu\text{g/ml}$ for A2143G+T2182C+T2190C, 53.3 $\mu\text{g/ml}$ for A2143G+T2182C+C2195T, and 80.0 $\mu\text{g/ml}$ for A2143G+T2182C+A2223G (Fig. 2).

Transformation of *H. pylori* with PCR Amplimers from Resistant Strains

To confirm that the mutations detected in the amplified fragments were associated with clarithromycin resistance,

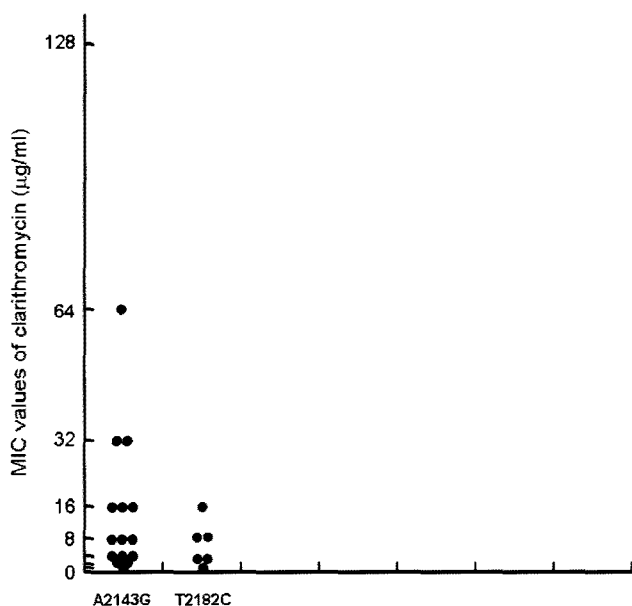


Fig. 1. The relationship between minimal inhibitory concentration (MIC) and gene mutations in primary clarithromycin-resistant *H. pylori* strains.

MICs of 21 primary isolates were determined by agar dilution. Gene mutations of 23S rRNA were assessed by PCR amplification and nucleotide sequencing.

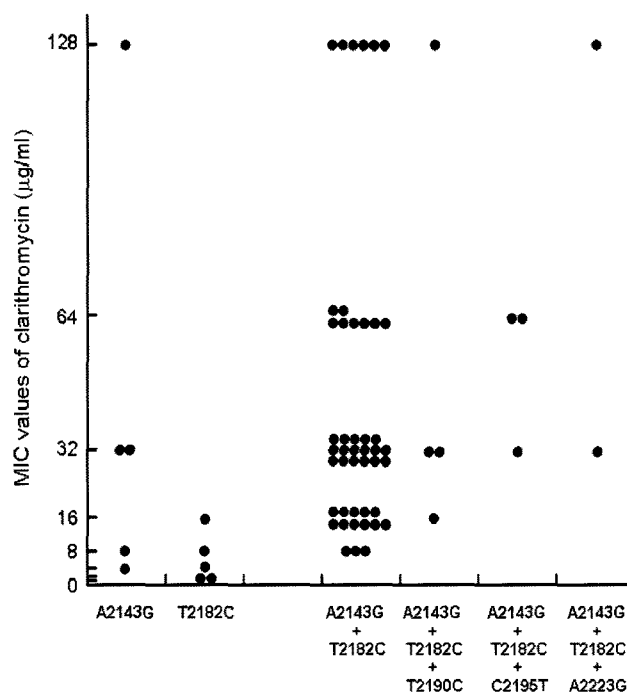


Fig. 2. The relationship between minimal inhibitory concentration (MIC) and gene mutations in secondary clarithromycin-resistant *H. pylori* strains.

MICs of 65 secondary isolates were determined by agar dilution. Gene mutations of 23S rRNA were assessed by PCR amplification and nucleotide sequencing.

the PCR products were introduced into the clarithromycin-susceptible strain *H. pylori* HP99 (0.0625 $\mu\text{g/ml}$ of clarithromycin MIC). The amplimers from resistant strains transformed the susceptible recipient to a resistant phenotype (Table 2). Nucleotide sequencing verified that the transformed cells contained the same mutation present in the donor DNA. Control experiments, in which the recipient received an aliquot of H_2O or DNase-treated DNA, did not yield any resistant colonies. These results indicate that mutations identified in the PCR amplimers may be involved in the resistance to clarithromycin.

Binding of [*N*-methyl- ^{14}C]Erythromycin to *H. pylori* Ribosomes

To further analyze the role of single-nucleotide mutation T2182C, the erythromycin-ribosome binding assay was performed. The susceptible *H. pylori* HP99 was used as the control. As shown in Fig. 3, there was an increased binding of [*N*-methyl- ^{14}C]erythromycin when the quantity of ribosomes of the susceptible parent strain increased, whereas virtually no binding was observed with ribosomes from the clarithromycin-resistant strains such as T2182C and A2143C mutants. The MIC values of the HP99, T2182C mutant, and A2143C mutant *H. pylori* to erythromycin were 0.125 $\mu\text{g/ml}$, 16 $\mu\text{g/ml}$, and 16 $\mu\text{g/ml}$, respectively.

Table 2. Changes of MIC values of clarithromycin-susceptible strains after transformation with PCR amplimers obtained from clarithromycin-resistant *H. pylori* isolates.^a

Isolates (MIC, µg/ml)	Genotypes of 23S RNA mutation	Mean MIC values of transformed cells (µg/ml) ^b
428 (16)	A2143G	12.8
542-A1 (16)	T2182C	11.2
503-A1 (32)	A2143G+T2182C	22.4
513-A1 (16)	A2143G+T2182C+T2190C	12.8
522-A2 (32)	A2143G+T2182C+C2195T	22.4
568-A1 (32)	A2143G+T2182C+A2223G	25.6

^aThe recipient strain HP99 (0.0625 µg/ml of clarithromycin MIC) was transformed with PCR-amplified DNA obtained from clarithromycin-resistant strains. The transformed cells were plated onto BHI agar containing 1 µg/ml of clarithromycin. After 5 days, five colonies from each transformation were purified and MIC values of clarithromycin evaluated.

^bData represent mean MIC values of clarithromycin from five transformed colonies.

DISCUSSION

Primary resistance of *H. pylori* to clarithromycin occurs with a prevalence ranging from 7.6% to 18.6% in Korea [10, 15, 16]. In addition, resistance to clarithromycin after treatment failure has increased to 85.1% in Korean patients [14]. This has an important clinical impact on dual and triple therapies, in which clarithromycin seems to be the better choice to achieve *H. pylori* eradication. In order to evaluate the mechanisms of clarithromycin resistance in Korean isolates, PCR amplification and nucleotide sequencing for 23S rRNA were used.

Primary clarithromycin-resistant isolates only showed a single type of point mutation such as A2143G or T2182C. In contrast, secondary isolates had a greater diversity of 23S rRNA gene mutation types, including a single mutation

of A2143G or T2182C, dual mutations of A2143G+T2182C, and triple mutations of A2143G+T2182C+T2190C, A2143G+T2182C+C2195T, or A2143G+T2182C+A2223G. Considering that dual or triple mutations were not found in primary isolates, the variety of gene mutations in secondary isolates may be a result of exposure to clarithromycin for long periods. However, a correlation between different point mutations eradication rates is necessary to be searched, since A2143G was recently reported to be associated with a very low eradication rate [3].

Although an A-to-G point or A-to-C mutation at position 2142 of domain V of the 23S rRNA gene is also known to cause clarithromycin resistance [20, 24], we did not find the A2142G point mutation in primary or secondary clarithromycin-resistant isolates. The absence of the A2142G mutation in Korean patients may be due to geographical differences.

In the present study, the single type of T2182C mutation was found in clarithromycin-resistant primary (28.6%) and secondary (7.8%) isolates. However, the role of T2182C mutation has been controversial. Thus, there are several reports of the association between T2182C and clarithromycin resistance in *H. pylori* [9, 11, 16]. In contrast, several papers have also shown that this mutation seems not to be associated with resistance to clarithromycin [2, 19, 25]. In order to confirm the role of T2182C mutation, we performed a transformation study and an erythromycin-binding study. After transformation with a PCR fragment obtained from an *H. pylori* isolate with T2182C mutation, clarithromycin MIC of a susceptible strain (MIC, 0.0625 µg/ml) showed 11.2 µg/ml. These results suggest that a single T-to-C mutation at position 2182 may be associated with clarithromycin resistance. In addition, transformation with a PCR fragment obtained from an *H. pylori* isolate with dual or triple mutations resulted in clarithromycin resistance of recipient cells. These results indicate that mutations identified in the PCR amplimers may be associated with the resistance to clarithromycin. Furthermore, in the present study, testing of erythromycin binding to free *H. pylori* ribosomes from

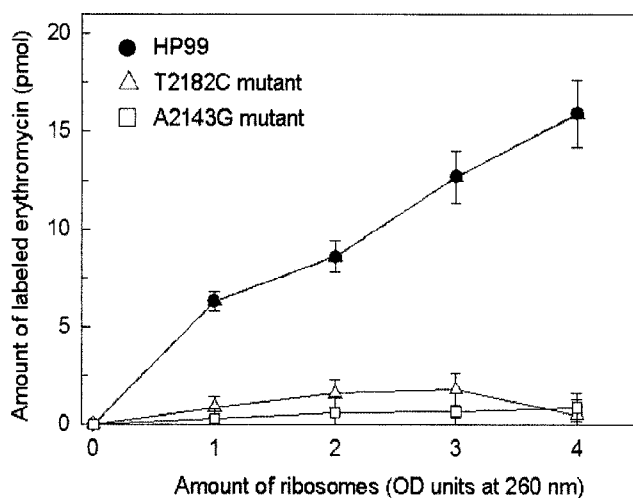


Fig. 3. Binding of [*N*-methyl-¹⁴C]erythromycin (200 nM) to increasing concentrations of 70S ribosomes (optical densities [OD], 1, 2, 3, and 4) purified from clarithromycin-susceptible (HP99) and -resistant (T2182C and A2143C mutants) *H. pylori* isolates.

Minimal inhibitory concentration (MIC) of HP99, T2182C mutant, and A2143C mutant to clarithromycin is 0.0625 µg/ml, 16 µg/ml, and 16 µg/ml, respectively. The results represent mean±SD of three independent experiments.

T2182C mutant strains was performed for the first time. Because of the cross-resistance observed between the different macrolides, labeled erythromycin was used in these experiments. The results showed the binding of labeled erythromycin to the susceptible strain and the absence of binding to T2182C and A2143C mutant strains, suggesting that the lack of binding at position 2182 in *H. pylori* is also a mechanism involved in the occurrence of clarithromycin resistance. We can therefore postulate that this lack of binding is due to a modification in the ribosomal structure following the mutation, including T2182C and A2143C.

It is interesting that all clarithromycin-resistant secondary isolates harboring dual or triple mutations have A2143G mutation. Based on this finding, it is possible that A2143G plays a major role of clarithromycin resistance but other mutations may affect accessory roles. Nevertheless, the role of the other detected genetic variations such as T2190C, C2195T, and A2223G remains unclear, although the role of T2182C point mutation was verified in the present study. Therefore, further study is needed to clarify the roles of a single mutation such as T2190C, C2195T, and A2223G.

In conclusion, A2143G and T2182C were the main point mutations in the 23S rRNA gene of both primary and secondary *H. pylori* isolates associated with clarithromycin resistance in Korean patients. However, primary clarithromycin-resistant isolates only showed single type of mutation. In contrast, triple mutations in secondary isolates always occurs in association with the A2143G+T2182C mutations, indicating that secondary isolates have a greater diversity of 23S rRNA gene mutation types than primary isolates.

Acknowledgments

We thank Dr. Sung-il Cho for statistical analysis, and Mi-Soon Kim and Han-Jin Lee for their excellent technical help. We also thank Abbott Laboratories (Abbott Park, IL, USA) for gifts of clarithromycin. This study was supported by a grant of Seoul R&BD Program and a grant of the Korea Health 21 R&D project Ministry of Health & Welfare, Republic of Korea (02-PJ3-PG6-EV08-0001).

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