Analysis of Resistance to Clarithromycin and Virulence Markers in *Helicobacter pylori* Clinical Isolates from Eastern Taiwan

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**Abstract**

**Objective:** Little information is available concerning the relationships between clarithromycin resistance and virulence marker genes (*iceA*, *cagA* and *vacA*) in *Helicobacter pylori* isolated in Taiwan. The aim of this study was to evaluate the possible association between clarithromycin resistance and genotypes of the virulence markers on clarithromycin-resistant *H. pylori* isolates obtained in eastern Taiwan.

**Materials and Methods:** The genotypes of the virulence marker genes (*iceA*, *cagA* and *vacA*) were analyzed by PCR, and the 23S rDNA region from 18 clarithromycin-resistant clinical isolates of *H. pylori* was amplified by PCR and sequenced.

**Results:** Point mutations were found to occur in all isolates. Two isolates had A2143G, six had T2182C, one had C2227T, six had A2143G plus T2182C, and three had heterozygous alleles. The latter included a wild-type allele (A2143) plus (i) an A2143G, (ii) an A2143G plus an A2223G, and (iii) an A2143G plus a T2182C. The prevalence of the marker genes *cagA*, *iceA1*, *iceA2*, and both *iceA1* and *iceA2*, in the isolates was 95.5%, 66.9%, 7.5%, and 25.6%, respectively. The *vacA*s1 allele was detected in all isolates, whereas the m1 and m2 alleles were found in 44.4% and 55.6% of the isolates, respectively.

**Conclusion:** There were no significant associations between clarithromycin resistance and the presence of the *cagA* gene, *vacA* allele mosaicism, and the *iceA* genotypes. The most notable finding of our study was that the C2227T single mutation in 23S rRNA could also be related to the high clarithromycin minimal inhibitory concentrations in clinical isolates from eastern Taiwan. *(Tzu Chi Med J 2009;21(2):123–128)*
1. Introduction

_Helicobacter pylori_ may be the most common infection in the world. The prevalence of this infection is higher in developing countries than in the USA and Western Europe [1, 2]. Infection by this organism has been documented as an etiologic factor in chronic gastritis, peptic ulcer disease, gastric carcinoma, and lymphoma [3–7]. Successful treatment of _H. pylori_ infection not only results in eradication of the pathogen, but may also cure and prevent the associated diseases. However, drug resistance to this organism reduces the success rates substantially.

The prevalence rate of antimicrobial resistance to _H. pylori_ varies with geographical regions. In Taiwan, varied rates of resistance have been reported including 13.5% to clarithromycin, 56.1% to amoxicillin, and 52% versus 32% to metronidazole in the eastern and western parts of the country, while almost all _H. pylori_ strains are susceptible to tetracycline [8]. Therefore, the most widely used primary regimen for _H. pylori_ eradication in eastern Taiwan is triple therapy with clarithromycin, tetracycline, and a proton-pump inhibitor. Unfortunately, the clarithromycin resistance rate has begun to increase recently in eastern Taiwan.

Clarithromycin resistance has been associated with point mutations in the peptidyltransferase region encoded in domain V of the _H. pylori_ 23S ribosomal DNA (rDNA) gene, conferring an altered binding target [9, 10]. _H. pylori_ contains two copies of the 23S rDNA gene, and the two most common mutations are A→G transitions at positions 2142 or 2143 in the gene [9–11]. Other less frequent mutations, such as A2115G, G2141A, A2142C, A2144T, T2182C and T2717C, have also been reported [10, 12–15]. Distribution of these mutations varies geographically. In addition, the genetic diversity has clinical significance because there are strains with markers of enhanced virulence.

Patients carrying strains containing _cagA_ or having the gene that is induced on contact (_iceA1_) and _vacAs1m1_ alleles are at an increased risk of gastric-related diseases [16, 17]. _CagA_ is the marker for presence of the pathogenicity island (Cag PAI), which is the most studied putative virulence factor [18]. _VacA_ encodes a vacuolating toxin that causes target cell degeneration by interfering with intracellular membrane fusion. Allelic variation of the signal sequence region (s1, s2) and of the mid-region (m1, m2) mosaic combination is related to the production of vacuolating cytotoxin [19]. The _iceA1_ gene, induced by contact with gastric epithelial cells, has two main allelic variants (_iceA1_ and _iceA2_) and only the _iceA1_-carrying strain has been reported to be associated with a more severe clinical outcome [16, 17]. A strong association between clarithromycin resistance and presence of _iceA1_, _cagA_ and the _vacAs1m2_ allele has been reported [20–23]. However, molecular basis has not been established for the association between antibiotic resistance and bacterial genetic patterns. In this study, we analyzed the _iceA1_, _cagA_ and _vacA_ status and evaluated the possible association between clarithromycin resistance and genotypes of the virulence markers on 18 clarithromycin-resistant _H. pylori_ isolates obtained in eastern Taiwan.

2. Materials and methods

2.1. Bacterial strains

The 133 clinical _H. pylori_ isolates obtained from gastric biopsy specimens from patients before treatment have been described [8]. They were stored at −80°C in brain heart infusion broth containing 30% glycerol, recovered from frozen stocks and used in this study. The clarithromycin-sensitive _H. pylori_ NCTC 11637 (originally from the American Type Culture Collection as ATCC 43504) was used as the reference strain.

2.2. DNA extraction

Genomic DNA was isolated from _H. pylori_ isolates by phenol/chloroform extraction and ethanol precipitation. Genomic DNA were resuspended in an appropriate volume of sterile deionized water and stored at −20°C until used.

2.3. PCR amplification of _lspA-glmM_ genes and restriction fragment length polymorphism (RFLP)

For strain verification, we performed PCR-RFLP for _lspA-glmM_, a conserved gene formerly known as _ureCD_. Primers used for PCR amplification had the same nucleotide sequences as previously published ones, 5'-'TGGGACTGATGGCGTGAGGG-3' and 5'-ATCATGACATCAGCGAAGTTAAAAATGG-3', which amplified a 1720-bp product [24]. Amplifications were performed in a model 2700 Perkin-Elmer thermal cycler (Perkin-Elmer Corp., Norwalk, CO, USA) using 10 ng of _H. pylori_ DNA, 0.5 units of Taq DNA polymerase, 1X PCR buffer (Roche Molecular Biochemicals, Indianapolis, IN, USA), 1.5 mM MgCl2, 100 mM of each primer, and 200 μM of each dNTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The cycling program included an initial denaturation step at 94°C for 2 minutes, followed by 35 cycles with the following profiles: 1 minute at 94°C, 1 minute at 50°C, and 2 minutes at 72°C. The PCR products were electrophoresed in agarose gel, followed by _HhaI_ digestion.
(3 hours at 37°C) in the buffer recommended by the supplier (New England Biolabs, Inc., Beverly, MA, USA). The digests were electrophoresed in 5% polyacrylamide gel, stained with ethidium bromide and photographed. Each *H. pylori* isolate was thus characterized by the banding patterns.

### 2.4. Analysis of mutations associated with clarithromycin resistance

To detect resistance to clarithromycin in *H. pylori* isolates, point mutations of the 23S rDNA gene were examined by PCR-RFLP as described previously (25). Briefly, DNA extracted from *H. pylori* isolates was subjected to PCR with primers extending from position 2031 to 2050 (forward, 5’-ATCGCTGATACCGTCGTGCC-3’) and from 2726 to 2706 (reverse, 5’-CTTTTTTAGGACCGACCGCCC-3’) (GenBank accession number U27270). The amplified 696-bp DNA fragments were digested with *Mbo*II and *Bsa*I (New England Biolabs, Beverly, MA, USA). Digestion was performed (3 hours at 37°C) in the buffer recommended by the supplier (New England Biolabs, Inc., Beverly, MA, USA).

#### 2.5. Detection of cagA, iceA, and vacA genotypes by PCR

The genotypes of the cagA, iceA, and vacA genes were determined via PCR according to a previously described method (17).

#### 2.6. Statistical analyses

Statistical analyses were performed using the Pearson χ² test and Fisher’s exact test. A p value of ≤ 0.05 was accepted as statistically significant.

### 3. Results

#### 3.1. PCR-RFLP fingerprinting

The analysis of the DNA fingerprinting obtained through the PCR-RFLP for *lspA-glmM*, a conserved gene formerly known as *ureCD*, confirmed the total heterogeneity among these isolates from different individuals (data not shown).

#### 3.2. Distribution of the cagA gene, iceA genotype and vacA mosaicism

The cagA, iceA, and vacA (s and m region) genotypes were assessed in the 133 *H. pylori* infected patients (mean age, 51.2 ± 14.9 years; 55 men). Among these patients, 50 (23 men and 27 women; mean age, 56 ± 12 years) had peptic ulcer disease (PU, 37.6%), and 83 (52 men and 51 women; mean age, 49 ± 16 years) had chronic gastritis (CG, 62.4%). Among these 133 isolates, 127 were found to have cagA (95.5%; PU, 48; CG, 79), only 89 had iceA1 (66.9%; PU, 31; CG, 58), 10 had iceA2 (7.5%; PU, 5; CG, 5), 34 possessed both iceA1 and iceA2 (25.6%; PU, 13; CG, 21), whereas all of the isolates had the vacAs1 allele. 59 cases had m1 allele (44.4%; PU, 24; CG, 55), and 74 isolates had m2 (55.6%; PU, 25; CG, 49) (Table 1).

#### 3.3. Mutation sites associated with clarithromycin resistance

Among the 133 isolates, 18 (13.5%) showed clarithromycin resistance. The distribution of minimal inhibitory concentrations (MIC) in the 18 clarithromycin-resistant strains was 1 μg/mL in one of the isolates, 2 μg/mL in two, 3 μg/mL in one, 8 μg/mL in two, 16 μg/mL in one, 48 μg/mL in one, and ≥ 256 μg/mL in 10, as described previously (8). Analysis of the 23S rDNA gene mutation by PCR-RFLP was performed for all 18 isolates, which were judged to be resistant to clarithromycin by the E-test (AB Biodisk, Solna, Sweden). A2143G mutations were detected in eight (44.4%) resistant isolates; however, the restriction cleavage was incomplete in three amplicons (Fig. 1). None of these resistant isolates was cleaved by *Mbo*II, indicating that there was no A2142G mutation type in these strains. On sequencing the PCR amplicons of these 18 strains, two were shown to have a point mutation of A to G at position 2143 (A2143G), six had a T to C mutation at position 2182 (T2182C), one had a C to T mutation at position 2227 (C2227T), and six had an A2143G plus A2223G transition and an additional T2182C mutation. Furthermore, sequencing of the fragments from individual colonies showed that three of the isolates with an incomplete pattern of *Bsa*I digestion had heterozygous genotypes: wild-type allele (A2143) plus (i) an A2143G, (ii) an A2143G plus an A2223G, and (iii) an A2143G plus a T2182C (Table 2). None of the fragments from the sensitive isolates were cleaved by either the *Bsa*I or *Mbo*II enzyme.

MIC values of the T2182C mutants were relatively high (≥ 256 μg/mL), except for one isolate (1 μg/mL), and those of the A2143G single mutants ranged from 2 to 48 μg/mL. However, MICs of the T2182C and A2143G double mutants were from 2 to ≥ 256 μg/mL (Table 2).
3.4. Genotype variations and clarithromycin resistance

Analysis of genotypes of the virulence factors (cagA status, iceA and vacA alleles) related to clarithromycin resistance revealed that all of the clarithromycin resistant isolates were cagA positive, and most of them were of the iceA1 genotype (13/18; 72.2%). The prevalence of both the vacA m1 allele (41.7% vs. 61.1%; \( p = 0.119 \)) and m2 allele (58.3% vs. 38.9%; \( p = 0.125 \)) did not significantly differ between susceptible and resistant strains.

4. Discussion

Clarithromycin is the second most widely used antibiotic in Helicobacter pylori-infected patients, so resistance is a prime concern for physicians. The resistance rates vary geographically. For example, occurrence ranges from 9.9% to 43.5% in Europe (26), and is less than 4% in Canada (27), 10–15% in the USA (28), and 8–17% in Iran and Israel (29,30). However, the
prevalence rates in the far East are higher in Japan (11–12%) [31] and Taiwan (13.5% in the east, 18% in the northwest, 6% in the southwest) [8,32,33] than in Hong Kong (4.5%) [34] and Korea (5–6%) [35]. The mechanism of _H. pylori_ resistance to clarithromycin was first shown to be caused by a single point mutation within domain V of 23S rRNA (A to G transition) [9]. Other mutation points were also reported, such as A2115G, G2141A, A2142C or G or T, A2143C, T2182C, G2224A, C2245T, and T2289C [14,36,37]. It is also known that the prevalence of mutations in 23S rDNA associated with clarithromycin resistance varies in different parts of the world as follows: 48–55% of isolates had an A2142G mutation, 39–45% an A2143G mutation, and up to 7% an A2142C mutation in the USA [38,39]; 23–35% had an A2142G mutation, 44–67% an A2143G mutation, and 2–10% an A2142C mutation in Europe [40,41]; while the A2142C mutation was not detected, and more than 90% of the mutant strains had an A2143G mutation in Japan [42]; the mutation A2143G occurred in 100% of isolates in China [43]. In this study, we have characterized the 23S rRNA gene mutations of all 18 clarithromycin-resistant isolates from Buddhist Tzu Chi General Hospital in Hualien. The most prevalent mutations were the T2182C (6/18; 33.3%) single mutation, which was identified in Korea [14], as well as the T2182C plus A2143G double point mutations (6/18; 33.3%). However, the dominant mutation is A2144G in western Taiwan [32]. Moreover, these mutations seem to be associated with high levels of clarithromycin resistance (≥256 μg/mL). In contrast, the A2143G single mutation, the major type of mutation in Europe, the USA, Japan and China, exhibits a low prevalence (2/18; 11.1%) in our area [38–43]. Furthermore, there is evidence to suggest that a heterozygous condition exists in clarithromycin-resistant _H. pylori_, where one 23S rRNA allele is of the wild-type genotype while the other copy is of the mutant genotype [9]. Stone et al indicated that two of 40 clarithromycin-resistant isolates, one with a mutation at position 2143 and the other with a mutation at position 2144 in the 23S rDNA gene, were heterozygous [39]. In this study, we also found that three of the eastern Taiwan isolates had heterozygous genotypes. The most notable finding in our study is the C2227T single mutation, which is related to high clarithromycin MICs and has not been reported elsewhere. The reason could be that the C2227T transition, which falls in a highly conserved region of the 23S rRNA associated with the functional site, domain V, has a strong effect on the secondary structure of the 23S RNA and with its interaction with macrolide [44]—suggesting that the T-to-C transition at position 2717 may be responsible for clarithromycin resistance.

A strong association between clarithromycin resistance and the presence of _iceA1_, _cagA_ and the _vacAs1m2_ allele has previously been reported [20–23]. However, we observed no significant association between clarithromycin resistance and the _cagA_ gene, _vacA_ allele mosaicism, and the _iceA_ genotypes in this study. To the best of our knowledge, there have been no other reports concerning the mutation sites in clarithromycin-resistant strains and the association between drug resistance and the genotypes of virulence factors in Taiwan.

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**References**


