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## 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 *vacAs1* 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 rDNA 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)

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## 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, 36.1% 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 *iceA* 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 *iceA*, *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 stain 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 MgCl<sub>2</sub>, 100 nM 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'-CTTTTAG-GAGCGACCGCCCC-3') (GenBank accession number U27270). The amplified 696-bp DNA fragments were digested with *Mbo*II and *Bsa*I (New England Biolabs, Inc.), allowing for detection of the A2142G and A2143G mutations, respectively. The restriction products were analyzed by electrophoresis in 2% agarose gel. In addition, amplicons were purified with a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan) and the same primers for the PCR reaction were used for sequencing. Sequencing was performed on both strands with an automated DNA sequencer (ABI PRISM 3730, Applied BioSystems, Darmstadt, Germany).

#### 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  $\chi^2$  test and Fisher's exact test. A *p* value of  $\leq 0.05$  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  $\pm$  14.9 years; 55 men). Among these patients, 50 (23 men and 27 women; mean age, 56  $\pm$  12 years) had peptic ulcer disease (PU, 37.6%), and 83 (32 men and 51 women; mean age, 49  $\pm$  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 *vacA*s1 allele, 59 cases had m1 allele (44.4%; PU, 24; CG, 35), and 74 isolates had m2 (55.6%; PU, 25; CG, 49) (Table 1).

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

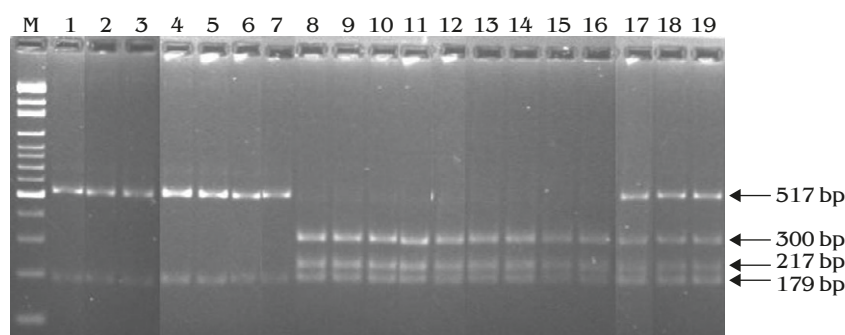
Among the 133 isolates, 18 (13.5%) showed clarithromycin resistance. The distribution of minimal inhibitory concentrations (MIC) in the 18 clarithromycin-resistant strains was 1  $\mu$ g/mL in one of the isolates, 2  $\mu$ g/mL in two, 3  $\mu$ g/mL in one, 8  $\mu$ g/mL in two, 16  $\mu$ g/mL in one, 48  $\mu$ g/mL in one, and  $\geq 256$   $\mu$ g/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 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: a 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 ( $\geq 256$   $\mu$ g/mL), except for one isolate (1  $\mu$ g/mL), and those of the A2143G single mutants ranged from 2 to 48  $\mu$ g/mL. However, MICs of the T2182C and A2143G double mutants were from 3 to  $\geq 256$   $\mu$ g/mL (Table 2).

**Table 1 — Distribution of genotypes among the 133 *Helicobacter pylori* isolates from patients in Eastern Taiwan**

Genotype	From patients with chronic gastritis (n=84)	From patients with peptic ulcer (n=49)	Total (n=133)
<i>cagA</i>			
Positive	79	48	127
Negative	5	1	6
<i>vacAs1</i>			
m1	35	24	59
m2	49	25	74
<i>iceA</i>			
1	58	31	89
2	5	5	10
1+2	21	13	34

n = number of isolates.



**Fig. 1 — Representative electrophoretogram of *Bsal*-restriction profiles of the 696-bp 23S rDNA fragments which were amplified by PCR from clarithromycin-resistant *H. pylori* isolates. Lane M: DNA size markers. Lanes 1–7: with one 517-bp and one 179-bp product which means lack of a restriction site at nt 2143. Lanes 8–16: each with a 300-, 217- and 179-bp product indicating an A to G mutation at position 2143. Lanes 17–19: with 4 fragments (517, 300, 217 and 179 bp) which represent a heterozygous condition where one 23S rDNA allele is of the wild-type genotype (A2143) and the other has the mutant genotype (A2143G).**

**Table 2 — Minimal inhibitory concentrations (MIC) and mutation profiles of the clarithromycin-resistant *Helicobacter pylori* isolates**

Isolate	Clarithromycin MIC ( $\mu\text{g}/\text{mL}$ )	Mutation sites
HP39	$\geq 256$	T2182C
HP55	$\geq 256$	T2182C
HP157	$\geq 256$	T2182C
HP181	$\geq 256$	A2143G, T2182C
HP187	1	T2182C
HP260	12	A2143G, T2182C
HP263	$\geq 256$	A2143G*, A2223G
HP272	$\geq 256$	T2182C
HP322	2	A2143G
HP324	$\geq 256$	A2143G, T2182C
HP355	48	A2143G
HP368	3	A2143G, T2182C
A444	2	A2143G*
A472	$\geq 256$	A2143G*, T2182C
A480	8	A2143G, T2182C
A504	8	A2143G, T2182C
A546	$\geq 256$	C2227T
A1349	$\geq 256$	T2182C

\*With a heterozygous condition where one 23S rDNA allele was of the wild-type genotype (A2143) and the other was of the mutant genotype (A2143G), as revealed by PCR-RFLP and sequence analysis.

### 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 *vacAm1* 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 *H. 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 rDNA (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: 48–53% of isolates had an A2142G mutation, 39–45% an A2143G mutation, and up to 7% an A2142C mutation in the USA [38,39]; 23–33% 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 ( $\geq 256 \mu\text{g/mL}$ ). 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 rDNA 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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