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Clarithromycin Resistance Prevalence and *Icea* Gene Status in *Helicobacter Pylori* Clinical Isolates in Turkish Patients with Duodenal Ulcer and Functional Dyspepsia

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(Received April 16, 2006 / Accepted July 13, 2006)

Clarithromycin resistance in *Helicobacter pylori* is a principal cause of failure of eradication therapies, and its prevalence varies geographically. The *Icea* gene is a virulence factor associated with clinical outcomes. The objective of this study was to determine the current state of clarithromycin resistance prevalence, and to investigate the role of *iceA* genotypes in 87 Turkish adult patients (65 with functional dyspepsia and 22 with duodenal ulcer). A2143G and A2144G point mutations were tested by PCR-RFLP for clarithromycin resistance. Among the patients in the study, 28 patients were tested by agar dilution as well. Allelic variants of the *iceA* gene were identified by PCR. A total of 24 (27.6%) strains evidenced one of the mutations, either A2143G or A2144G. *Icea1* was found to be positive in 28 of the strains (32.2%), *iceA2* was positive in 12 (13.8%) and, both *iceA1* and *iceA2* were positive in 22 (25.3%) strains. In conclusion, we discovered no relationships between *iceA* genotypes and functional dyspepsia or duodenal ulcer, nor between clarithromycin resistance and *iceA* genotypes. Clarithromycin resistance appears to be more prevalent in Turkish patients.

Keywords: *Helicobacter pylori*, clarithromycin resistance, 23S rRNA, *iceA*

The gram-negative bacterium, *Helicobacter pylori* (*H. pylori*), colonizes the human stomach, with prevalence rates from 25% in Western countries to over 90% in developing countries (Solerman *et al.*, 2005). Persistent infection induces peptic ulcer and chronic gastritis (Sipponen, 1991; Graham *et al.*, 1992) and has also been associated with gastric cancer and mucosal-associated lymphoid tissue lymphoma (Marshall, 1994; Bayerdorffer *et al.*, 1995; Uemura *et al.*, 2001). In 1994 the IARC (World Health Organization, 1994) recognized *H. pylori* as a class 1 carcinogen.

In 1994, the National Institutes of Health Consensus (NIH) recommended that all patients with peptic ulcer disease and documented *H. pylori* infection should be treated via appropriate antibacterial therapy (NIH,

1994). *H. pylori* can be eradicated by a triple or quadruple therapy regimen, including the use of a proton-pump inhibitor (PPI) and antibiotics, primarily clarithromycin and amoxicillin. In Turkey, *H. pylori* is usually eradicated via a triple therapy which includes amoxicillin, clarithromycin, and a PPI (Guliter *et al.*, 2005). Following the *H. pylori* eradication therapy, an observed reduction in complications proves that *H. pylori* plays a role as an etiological agent in the disease. (Nomura *et al.*, 1994). Drug resistance to *H. pylori* reduces the success rate substantially (Pounder, 1997). The antibacterial activity of clarithromycin has been attributed to the inhibition of protein synthesis after binding to the 50S ribosomal subunit of the microorganism (Goldman *et al.*, 1994). Clarithromycin resistance is associated with point mutations within the peptidyltransferase region encoded in domain V of the *H. pylori* bacterial 23S rRNA gene (Versalovic *et al.*, 1996). The predominant mutations are A2143G

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and A2144G, as well as a small number of A2143C mutations (Megraud *et al.*, 1996; Alarcon *et al.*, 1999). Other rare mutations have also been reported, including G2141A, A2143T, T2183C, T2245C, A2144T, and T2717C (Versalovic *et al.*, 1996; van Doorn *et al.*, 1999; Ende *et al.*, 2001; Fontana *et al.*, 2002; Ribeiro *et al.*, 2003; Khan *et al.*, 2004; Toracchio *et al.*, 2004). The distribution of these mutations varies geographically (Dzierzanowska-Fangrat *et al.*, 2001). Most people infected with *H. pylori* harbor strains that possess the cytotoxin-associated gene A (*cagA*) and vacuolating cytotoxin gene A (*vacA*) genotypes, but nonetheless remain asymptomatic. A novel gene was discovered by Peek *et al.* in 1998, via comparisons of mRNA transcripts from an ulcer-derived and a gastritis-derived *H. pylori* strain in organisms that had adhered to human gastric cells, versus non-adherent bacteria. The expression of this gene is upregulated upon the contact of the ulcer-derived *H. pylori* strain with epithelial cells, and was designated *iceA* (induced by contact with the epithelium). *IceA* also exists in allelic variants, including *iceA1* and *iceA2*, and only *iceA1* was induced following contact with the gastric epithelium. The *IceA1* strain was associated significantly with peptic ulceration and increased mucosal IL-8 concentrations. Adherence to gastric epithelial cells *in vitro* stimulates the transcription of *iceA1* (Peek *et al.*, 1998). Some studies have reported an association of *iceA1* strains with the presence of peptic ulcers, and a higher prevalence of *iceA2* strains among patients with non-ulcer dyspepsia (van Doorn *et al.*, 1998; Figueiredo *et al.*, 2001). There are also some studies which have reported no association between *iceA* genotypes and gastric diseases (Yamaoka *et al.*, 1999; Godoy *et al.*, 2003; Han *et al.*, 2004). The prevalence of *H. pylori* clarithromycin resistance varies geographically, and changes in a dynamic manner (Megraud, 2004), and thus requires constant monitoring (Dzierzanowska-Fangrat *et al.*, 2001). The primary objective of our study was to determine the prevalence of clarithromycin resistance, and to determine whether any relationship exists between resistance and *iceA* genotypes in *H. pylori* clinical isolates in Turkish patients suffering from duodenal ulcer or functional dyspepsia.

Materials and Methods

Patients

A total of 87 adult patients, all of whom exhibited symptoms of dyspepsia, were referred and followed-up in the Department of Gastroenterology, Ankara University, School of Medicine, Cebeci Hospital, were investigated for the presence of *H. pylori* between October 2002 and December 2003. The mean ages of

the patients were 44±13 years, in a range from 23-72 years of age. Among the patients, 36 were males and 51 were females.

Culture, identification and minimal inhibitory concentration (MIC) determination

Antral gastric biopsy samples were obtained from patients. The biopsy specimens were put into sterile 20% dextrose solution, and transported immediately to the laboratory for culturing, using the standard method (Soltesz *et al.*, 1992). Brain Heart Infusion (BHI) agar (Becton Dickinson and Company, USA) with 7% defibrinated horse blood, plus 5 mg/L of amphotericin B and 10 mg/L of vancomycin was used as culture media. The bacteria were cultured under humid conditions at 37°C in an anaerobic jar (with 5% O₂, 10% CO₂, 85% N₂) manufactured by CampyGen (Oxoid Ltd., England). The plates were incubated for 3-5 days. *H. pylori* was identified by morphology, Gram staining, oxidase, catalase, and urease tests. All isolated strains were stored at -80°C in brucella broth (Sigma, Germany) medium containing 15% (v/v) glycerol.

The agar dilution method was applied in accordance with the guidelines established by the "National Committee for Laboratory Standards" (NCCLS, 1999). Bacterial suspensions were inoculated onto Mueller-Hinton agar (Lab M, UK) with 5% sheep blood, containing clarithromycin at concentrations ranging from 0.032 µg/ml to 16 µg/ml. The plates were incubated for 72 h in a microaerobic atmosphere at 37°C. *H. pylori* clarithromycin resistance was defined as MIC≥1 µg/ml. The *H. pylori* strain ATCC 43504 was used as a quality control organism.

Genomic DNA extraction

H. pylori was cultured for 3-5 days on BHI agar media containing 7% defibrinated horse blood at 37°C in a microaerobic atmosphere. We used whole biopsy samples, so that all of the *H. pylori* DNA was included. The bacteria were suspended in TE buffer and the genomic DNA was isolated via a previously described method (Lee and Megraud, 1996). DNA was dissolved in 500 µl of sterilized distilled water and prepared with 0.2 µg/ml of DNA solution.

Determination of *iceA*

The presence of *iceA* was determined by a previously described method (Yamaoka *et al.*, 1999). In brief, (detection for the *iceA1* allele), *iceA1F*; 5'-GTGTTT TTAACCAAAGTATC-3' and *iceA1R*; 5'-CTATAGC CACTCTCTTTGCA-3' primers were used to amplify a 247 bp fragment. In order to detect the *iceA2* allele, *iceA2R*; 5'-TTACCCTATTTTCTAGTAGGT-3' and *iceA2F*; 5'-GTTGGGTATATCACAATTTAT 3' primers

were used to amplify a 229 or 334 bp fragment. PCR was conducted in a 50 µl volume, containing 20 pmol/ml of primers, 2 mM dNTP, 10× buffer (750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM (NH₄)₂SO₄, 0.1% Tween20, 250 mM MgCl₂) (MBI Fermentas, Lithuania), 1 U of Taq polymerase (MBI Fermentas, Lithuania), and 0.2 µg/ml of *H. pylori* DNA. A thermal cycler (Eppendorf Mastercycler Personal, Germany) program was used for PCR, and consisted of 5 min of pre-denaturation at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 50°C for *iceA1* primers (1 min at 52°C for *iceA2* primers) and 2 min at 72°C. A final extension step was conducted for 7 min at 72°C. The amplified fragments were visualized on a 1% agarose electrophoresis gel stained with ethidium bromide.

Determination of clarithromycin resistance gene

The presence of the clarithromycin resistance gene was determined via PCR in accordance with a previously described method (Taylor *et al.*, 1997), followed by the digestion of PCR products using the *BpiI* and *BsaI* restriction enzymes (Sevin *et al.*, 1998). In order to amplify the 310 bp fragment of the 23S rRNA gene, DP1; 5'-ACGGCGGCCGTAAGTATA-3' corresponding to positions 2357 to 2374, and ZGE23; 5'-ACAGGC CAGTTAGCTA-3'; complementary to positions 2649 to 2664, primers were used (Taylor *et al.*, 1997). Primers specific for *H. pylori* were found via a BLAST search of the NCBI database. PCR was conducted under conditions identical to that of the previously described *iceA* genotyping method, except with an annealing temperature of 50°C. After the 23S rRNA PCR, we conducted RFLP in order to detect 23S rRNA mutations. We used the *BpiI* enzyme (Fermentas, Germany) to detect the A2143G mutation and *BsaI* (New England Biolabs, USA) to detect the A2144G mutation. Fifteen microliters of the amplicon and 5 U of the restriction enzyme with 5 µl buffer (10× NEBuffer3 for *BsaI*, Buffer G⁺ for *BpiI*) were incubated for 12 h at 56°C for *BsaI* and 37°C for *BpiI*. The restriction products were analyzed by electrophoresis on 1.5% agarose gel stained with

ethidium bromide.

Statistical analyses

Statistical analyses were done by Chi-square and Fisher's exact tests. A value of <0.05 was considered to be significant. If the *H. pylori* isolated from a single patient evidenced both *iceA1* and *iceA2* alleles together, the patient was considered to be harboring more than one strain. Therefore, these samples were excluded when studying the relationship between *iceA* genotypes and clinical outcome and clarithromycin resistance.

Results

Patient populations and bacterial isolates

Among the 87 patients in this study, 65 were diagnosed with functional dyspepsia (FD) and 22 with duodenal ulcer (DU). Standard microbiological tests showed that the isolates were, in fact, *H. pylori*, and those strains were subsequently verified by PCR of 310 bp 23S rRNA gene products.

Clarithromycin resistance gene and MIC results

A total of 24 (27.6%) strains evidenced mutations, thereby suggesting the presence of clarithromycin resistance (Table 1). In patients with functional dyspepsia, a total of 15 strains (23.1%) evidenced mutation. Among them, 10 (15.4%) exhibited A2144G mutations and 5 (7.7%) exhibited A2143G mutations. In bacteria isolated from DU patients, a total of 9 (40.9 %) strains manifested mutations. A2144G and A2143G mutations were present in 5 (22.7%) and 4 (18.2%) isolates, respectively. No strains were found to harbor both the A2143G and A2144G mutations.

Agar dilution was applied to 28 strains. Eighteen of them were found to harbor mutations in the 23S rRNA gene and 10 of them were wild-type. We compared the PCR-RFLP results with agar dilution (Table 2). All 18 of the mutant strains were determined to be resistant, and all 10 of the wild-type strains were susceptible, according to the results of the agar dilution studies.

Table 1. Distribution of 23S rRNA mutations among 87 *Helicobacter pylori* isolated from patients with duodenal ulcer and functional dyspepsia in Turkey. N indicates number of isolates

Mutation	Duodenal ulcer n=22	Functional dyspepsia n=65	Total n=87
A2143G	4 (18.2)*	5 (7.7)	9 (10.4)
A2144G	5 (22.7)	10 (15.4)	15 (17.2)
Total** (A2143G+A2144G)	9 (40.9)	15 (23.1)	24 (27.6)

* Percentage of the respective disease category. ** Total of A2143G and A2144G mutations shown in different strains.

IceA genotyping

Only *iceA1* was positive in 28 (FD: 21 and DU: 7) strains, *iceA2* was positive in 12 (FD: 9 and DU: 3), and both *iceA1* and *iceA2* were positive in 22 (FD: 19 and DU: 9) strains (Table 3). No significant associations were found between the *iceA* genotypes and the 23S rRNA mutations.

Discussion

Clarithromycin resistance rates vary geographically. In Europe, primary clarithromycin resistance rates were reported to occur in a range of 9.9-43.5% in two multi-centre studies (Glupczynski *et al.*, 2001; van Doorn *et al.*, 2001). Outside Europe, the prevalence of clarithromycin resistance tends to be lower (Megraud, 2004). In Canada, resistance was less than 4% (Fallone, 2000). However, the prevalence of resistance in the USA has already reached 10-15 % (Laine *et al.*, 2000; Osato *et al.*, 2001; Laine *et al.*, 2003). In the Middle East, according to surveys conducted in Iran and Israel, the prevalence occurs in a range of 8-17% (Samra *et al.*, 2002; Mohammadi *et al.*, 2003). In the far East, the prevalence is higher in Japan (11-12%) than in Hong Kong (4.5%), Korea (5-6%), and New Zealand (6.8%) (Fraser *et al.*, 1999; Kato *et al.*, 2000; Teo *et al.*, 2000; Kim *et al.*, 2001; Ling *et al.*, 2002; Perez Aldana *et al.*, 2002; Eun *et al.*, 2003; Lui *et al.*, 2003; Megraud, 2004). In Turkey, resistance rates differ, but occur in a range of 8.8-24.2 % (Sahin *et al.*, 1994; Palabiyikoglu *et al.*,

1997; Inan *et al.*, 2005; Simsek *et al.*, 2005).

We observed that 27.6% (24/87) of the isolates were clarithromycin resistant by PCR-RFLP. Although it appears that resistance rates are increasing in Turkey, to the best of our knowledge there have been no reports regarding 23S rRNA mutations in *H. pylori* clinical isolates obtained from Turkish patients. In all of the Turkish studies, resistance was detected via microbiological techniques, such as disk diffusion or E-Test.

Clarithromycin is an important macrolide due to its low MIC value, which is relatively unaffected by lowering the pH, as well as its high concentration in gastric mucosa, and the fact that it evidences the highest degree of interaction with regard to binding to *H. pylori* ribosomes (Goldman *et al.*, 1994). Macrolide consumption is a significant problem in increasing clarithromycin resistance in *H. pylori* isolates. Some studies have compared macrolide consumption and ensuing resistance in corresponding countries over the years. In Japan, clarithromycin consumption increased four-fold between 1993 and 2000, and this resulted in a fourfold increase in clarithromycin resistance (Perez *et al.*, 2002). Therefore, the prevention of unnecessary macrolide consumption can contribute to a halting of increases in clarithromycin resistance. The detection of clarithromycin resistance must, then, constitute the first step in any effective strategy for increasing resistance.

Clarithromycin resistance was higher among the duodenal ulcer patients (40.9%) than in the functional dyspepsia patients (23.1%). The majority of studies have mentioned no differences in prevalence in accordance with patient disease status, although two studies did mention such differences (Broutet *et al.*, 2003; Megraud, 2003). Strains from 5.6% of peptic ulcer patients were found to be resistant, as compared with 16.7% of strains obtained from the non-ulcer dyspepsia patients ($p=0.0005$) (Broutet *et al.*, 2003).

After the culturing of *H. pylori* from biopsy samples, we collected all of the colonies from the

Table 2. Comparison of 23S rRNA PCR-RFLP results with agar dilution results. R: resistant to clarithromycin, S: sensitive, WT: wild type, N: number of isolates

Agar dilution	23S rRNA	N
S	WT	10
R	A2143G	9
R	A2144G	9

Table 3. Distribution of *iceA* genotypes among 87 *Helicobacter pylori* isolated from patients with duodenal ulcer and functional dyspepsia in Turkey. N indicates number of isolates

Genotype	Duodenal ulcer n=22	Functional dyspepsia n=65	Total n=87
<i>iceA1</i>	7 (31.8; 25.0)*	21 (32.3; 75.0)	28 (32.2)**
<i>iceA2</i>	3 (13.6; 25.0)	9 (13.8; 75.0)	12 (13.8)
<i>iceA1+A2</i>	3 (13.6; 13.6)	19 (29.2; 86.4)	22 (25.3)
<i>iceA</i> (-)	9 (40.9; 36.0)	16 (24.6; 64.0)	25 (28.7)

*Percentages are indicated in the parenthesis. The first value is the percentage of the respective disease category, the second value expressing the percentage in respect to the total number of isolate for that genotype. **Percentage of the total number.

culture and used them to isolate *H. pylori* DNA. As infection with multiple strains is common (Hirschl *et al.*, 1994; van Doorn *et al.*, 1998), tests will appear false if only a few colonies of a culture are examined (Jorgensen *et al.*, 1996). A total of 22 patients (25.3%) who harbored both the *iceA1* and *iceA2* genotypes simultaneously were considered to have been infected with more than one strain. No significant associations were found between functional dyspepsia or duodenal ulcer and *iceA* genotypes (*iceA1* and *iceA2*). There are conflicting reports on this issue. Whereas some studies have detected a relationship between *iceA* genotypes and gastric diseases such as peptic ulcer, gastritis, and gastric cancer (Nishiya *et al.*, 2000; Ashour *et al.*, 2001; Kidd *et al.*, 2001; Wu *et al.*, 2005), some studies have reported no such relationship (Yamaoka *et al.*, 1999; Figueiredo *et al.*, 2001; Godoy *et al.*, 2003; Ribeiro *et al.*, 2003; Han *et al.*, 2004; Perng *et al.*, 2004). In this study, we observed no significant association between *iceA* genotypes and clarithromycin resistance. To the best of our knowledge, there have been no reports, except for this study, concerning *iceA* genotyping in Turkey. A few studies examining the association between virulence factors and macrolide resistance found no such association (Damaso *et al.*, 1999; Loivukene *et al.*, 2000; Godoy *et al.*, 2003). The actual biological function of the IceA protein remains unclear. Preliminary studies show that mutants of *iceA1* lacking this putative protein are unable to colonize the stomach in a monkey model, whereas the parental wild-type *iceA1* strain induces long-term colonization in monkeys (Dubois *et al.*, 1996). *IceA* is a novel candidate virulence factor, and will require more extensive studies in order to prove the association between gastric diseases and other factors, most notably resistance. Before prescribing a particular eradication therapy modality in *H. pylori*-positive patients, the detection of resistance may need to become a component of future regimens, and 23S rRNA mutation rates will clearly be important.

In conclusion, we observed a clarithromycin resistance prevalence of 27.5% in the *H. pylori* strains of Turkish patients with duodenal ulcer or functional dyspepsia. No relationship was determined to exist between *iceA* genotypes and gastrointestinal diseases. We also found no association between *iceA* genotypes and clarithromycin resistance.

Acknowledgment

We would like to acknowledge the financial support received from the Institute of Biotechnology, Ankara University, grant no 2004/134.

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