

Clarithromycin-Resistant Genotypes and Eradication of *Helicobacter pylori*

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Background: Three point mutations (A2143G, A2142G, and A2142C) have been involved in *Helicobacter pylori* clarithromycin resistance.

Objective: To compare the eradication rates among the different point mutations and the efficacy of triple therapy and a sequential regimen according to genotypic resistance.

Design: Post hoc subgroup study from a multicenter, randomized trial.

Setting: Two hospitals in central and southern Italy between January and December 2001.

Patients: 156 patients with *H. pylori* infection.

Measurements: Real-time polymerase chain reaction for assessing clarithromycin resistance; histology, rapid urease test, and ¹³C-urea breath test at entry and after 4 to 6 weeks.

Intervention: 7-day triple therapy (20 mg of rabeprazole, 500 mg of clarithromycin, and 1 g of amoxicillin) in 75 patients or a 10-day sequential regimen (20 mg of rabeprazole plus 1 g of amoxicillin for

5 days and 20 mg of rabeprazole, 500 mg of clarithromycin, and 500 mg of tinidazole for the remaining 5 days) in 81 patients. All drugs were given twice daily.

Results: *Helicobacter pylori* infection was eradicated in 11 of 23 patients (48%) with the A2143G mutation and in 14 of 15 patients (93%) with either A2142G or A2142C strains (difference, 45 percentage points [95% CI, 15 to 65 percentage points]; $P = 0.004$). The sequential regimen achieved a higher cure rate than triple therapy in A2143G mutate strains (difference, 49 percentage points [CI, 8 to 72 percentage points]; $P = 0.024$).

Limitations: The post hoc substudy design may require further confirmation. Other limitations are the accessibility to the tool and the cost of investigations (€70 per patient).

Conclusions: The A2143G mutation seemed to be associated with a very low eradication rate. The sequential regimen achieved a higher cure rate than standard therapy even in patients with these strains.

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H*elicobacter pylori* infection plays a major role in peptic ulcer disease, low-grade mucosa-associated lymphoid tissue lymphoma, and gastric cancer (1), and its eradication dramatically affects the natural history of both peptic ulcer and gastric lymphoma (2). European and U.S. guidelines advised the use of triple therapies (proton-pump inhibitor, clarithromycin plus amoxicillin, or metronidazole) for 7 to 14 days to cure this infection (3, 4). However, *H. pylori* resistance against clarithromycin is increasing worldwide, reducing the success rate of standard triple therapies to mean values as low as 18% to 44% (5–7).

Novel culture-free polymerase chain reaction (PCR)-based assays have allowed the detection of the genetic mutations that are involved in the mechanisms of clarithromycin resistance (8, 9). In detail, A2143G and A2142G transitions are the most prevalent point mutations in Europe and the United States (10, 11), while the A2144G mutation is more frequent in Asia (12, 13). Although such genetic mutations have been associated with different degrees of bacterial resistance in vitro, data are still conflicting (7, 14). Moreover, no study has assessed the role of these different mutations on *H. pylori* treatment outcome.

In a recent multicenter study, a novel sequential regimen, consisting of a simple dual therapy given for the first 5 days followed by a triple therapy for the remaining 5 days, achieved a very high cure rate as compared with standard triple therapy (92% vs. 74%) (15). Whether such

a high cure rate may depend on increased efficacy of the sequential regimen against the clarithromycin-resistant strains is unknown.

We wanted to evaluate the role of different point mutations in the success of eradication therapy and to compare the efficacy of standard triple therapy and the sequential regimen for these mutations.

METHODS

Study Design

To assess the role of primary clarithromycin resistance in therapeutic outcome, we designed a post hoc subgroup analysis of a previous study involving 8 Italian centers (15). In detail, we selected patients from those who were previously enrolled by our 2 centers to participate in a multicenter study between January and December 2001 (Fig-

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ure). Demographic and clinical characteristics of patients enrolled in our substudy were similar to those of patients enrolled in the original multicenter study. Briefly, in the original study, Zullo and colleagues (15) allocated patients who were never treated for *H. pylori* infection, according to a computer-generated randomization list drawn in each center, to receive standard 7-day treatment (20 mg of rabeprazole, 500 mg of clarithromycin, and 1 g of amoxicillin twice daily) or 10-day sequential therapy (20 mg of rabeprazole plus 1 g of amoxicillin twice daily for the first 5 days followed by 20 mg of rabeprazole, 500 mg of clarithromycin, and 500 mg of tinidazole twice daily for the remaining 5 days). To assess *H. pylori* status, the investigators performed upper endoscopy with several gastric biopsies for histology (Giemsa staining), a rapid urease test, and a standard ^{13}C -urea breath test at entry and at 4 to 6 weeks after therapy. Investigators considered infections to be eradicated when all 3 test results were negative and considered treatment to have failed when at least 1 test result was positive. The local ethics committee approved the protocol, and all participants gave written informed consent.

For our current study, we selected 75 of 192 patients who were treated with standard triple therapy and 81 of 185 patients who were treated with the sequential regimen in the 2 participating centers. We recruited patients consecutively from the randomization lists of the previous study, independent of the eradication status. The final study sample included 58 of 140 patients whose infections were eradicated and 17 of 52 patients whose infections were not eradicated after standard triple therapy and 76 of 177 patients whose infections were eradicated and 5 of 8 whose infections were not eradicated after the sequential regimen.

Clarithromycin Resistance Assessment

We assessed the 3 point mutations (A2142C, A2142G, and A2143G) of clarithromycin resistance by using a validated real-time PCR, as reported elsewhere (16). Briefly, we extracted the DNA by using NucleoSpin Tissue (Macherey-Nagel GmbH & Co., Düren, Germany), according to the manufacturer's instructions, applied on paraffin-embedded sections. We applied the same procedure to homogeneous bacterial cultures of *H. pylori* (positive and negative controls), for which clarithromycin resistance had been previously assessed with Etest (AB BIODISK, Solna, Sweden). We estimated final DNA concentrations by ultraviolet absorbance at 260 nm.

Preparation of the Probes and Primers

We designed TaqMan minor groove binder (MGB) probes and primers to hybridize with wild-type and mutant DNA by using the Primer Express program and Custom TaqMan SNP Genotyping Assay service (Applied Biosystems, Foster City, California) that synthesized the primers and probes for each mutation.

Context

Point mutations in the peptidyltransferase region of the 23S ribosomal RNA gene may be responsible for *Helicobacter pylori* clarithromycin resistance.

Contribution

This study related mutations to eradication rates in 156 adults treated with clarithromycin regimens for *H. pylori* infection. Eradication was successful in 14 of 15 patients with either A2142G or A2142C point mutations but in only 11 of 23 patients with the A2143G point mutation.

Cautions

This was a post hoc subgroup study of selected participants in a multicenter randomized trial.

Implications

The A2143G point mutation may be associated with a low eradication rate of *H. pylori* infection.

—The Editors

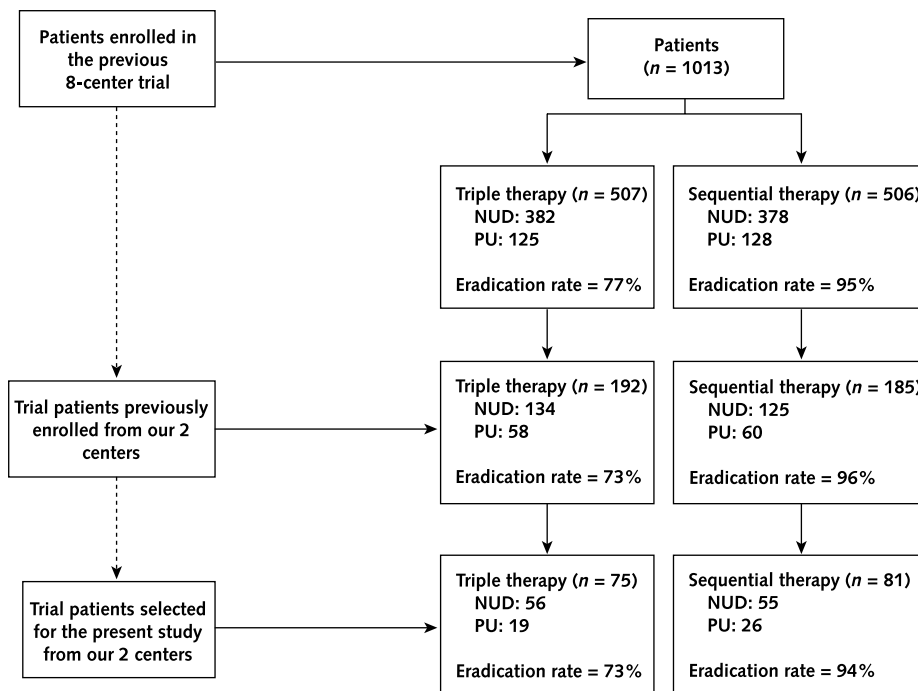
Genotyping Assay

The assay reagents for the genotyping single nucleotide mutation from the Assays-by-Design service (Applied Biosystems) consisted of a 40X mix of unlabeled PCR primers and TaqMan MGB probes (FAM and VIC fluorochrome dye-labeled). These assays were designed for the genotyping of specific mutations. Each assay enables scoring of both genotypes in a single well. Since a recent study showed that the conjugation of MGB to oligonucleotides stabilizes nucleic acid duplexes, causing a dramatic increase in oligonucleotide melting temperature (17, 18), we used an attachment of the MGB, which enables the use of shorter fluorogenic probes, thus resulting in improved mismatch discrimination. Our probes were distinguished by being labeled with different fluorescent reporter dyes (FAM dye and VIC dye). A substantial increase in FAM or VIC dye fluorescence indicated homozygosity for the FAM- or VIC-specific allele, while an increase in both signals indicated heterozygosity (19).

Real-Time PCR Assay and Allelic Discrimination

We performed the real-time PCR procedure according to the method of Wada and colleagues (20). We enclosed positive and negative controls in each assay. We analyzed fluorescence of hybridized probes by multicomponent graphics, where we examined dye-labeled (FAM and VIC), background, and passive control (ROX fluorochrome dye-labeled) fluorescence and expressed them as normalized reporter signal (ΔRn). We clustered all samples by using the maximum likelihood algorithm based on the ratio of normalized reporter dye signal. The result of the analysis yields 3 major clusters corresponding to the 3 genotypic constituents: wild-type homozygous, mutated-type homozygous, and heterozygous.

Figure. Flow chart showing data of patients recruited in this substudy from the previous multicenter study.



NUD = nonulcer dyspepsia; PU = peptic ulcer.

Characterization of Positive and Negative Controls by Amplification and Sequencing of the *Hp23S* Fragment

We obtained the *Hp23S* fragment by PCR amplification of *H. pylori* extracted DNA from homogeneous bacterial cultures (strains with and without clarithromycin resistance, previously assessed by Etest) by using primer Hp23-F (5'-CCACAGCGAT GTG GTCTCAG-3') and Hp23-R (5'-CTCCATAAGAGCCAAAGCCC-3') according to conventional PCR assay (21).

Before sequencing, we purified the PCR products by using the Wizard PCR preps (Promega, Madison, Wisconsin). We performed the sequencing reaction with the same primers for PCR, as described by Sanger and colleagues (22), by using the Dye Terminator 3.1 Ready Reaction Kit (Applied Biosystems) as indicated by the manufacturer. We performed sequencing on the 2 strands of each PCR product with the automated ABI Prism 377 DNA Sequencer (Applied Biosystems) and aligned the resulting nucleotide sequence by using the Sequence Navigator software package (Applied Biosystems).

Statistical Analysis

We determined sample size before the start of the study on the basis of the available data in the literature. In detail, an eradication rate ranging from 18% to 44% was reported after standard triple therapy in patients with primary clarithromycin-resistant strains (5–7), whereas the sequential regimen eradicated the infection in 79% of such

patients (15). Assuming a high eradication rate for the triple therapy (45%) and a relatively poor success rate for the sequential regimen (70%) in patients with primary clarithromycin-resistant strains, we calculated that at least 68 patients per group were needed to detect a statistically significant difference with 0.8 power and an α level of 0.05 (2-sided). After the study was completed, we realized that our sample size estimate provided the necessary number of clarithromycin-resistant patients and should have been inflated, on the basis of a presumed overall rate of clarithromycin resistance, to provide an estimate of total sample size. We compared eradication rates by *H. pylori* clarithromycin-resistant strain mutation (A2142C, A2142G, and A2143G) by using the Fisher exact test or chi-square test, as appropriate. We determined point mutation groupings after reviewing eradication rates by individual mutation. We compared clinical characteristics among the different groups by using the Student *t*-test for unpaired data (age) and the chi-square test (sex and gastroduodenal disease distribution and eradication rates). We fit multivariable logistic models by using therapeutic outcome as the dependent variable and by including the different point mutation groups, treatment regimen, gastroduodenal disease, age, and sex as possible covariates. We considered all differences to be statistically significant at a 5% probability level. We performed statistical analysis by using StatSoft 6.0 program for Windows 98.00 (StatSoft Inc., Tulsa, Oklahoma).

Table 1. Demographic and Clinical Characteristics of Patients

Characteristic	Triple Therapy Group (n = 75)	Sequential Therapy Group (n = 81)
Mean (SD) age, y	46.4 (14.8)	48.8 (13.7)
Men/women, n/n	30/45	42/39
Patients with nonulcer dyspepsia/peptic ulcer, n/n	56/19	55/26
Clarithromycin-susceptible strains, n (%)	59 (78.7)	59 (72.8)
Clarithromycin-resistant strains, n (%)	16 (21.3)	22 (27.2)

Role of the Funding Sources

The University of Foggia, Foggia, Italy, provided funding for reagents, and the University of Bari, Bari, Italy, provided the instruments for the real-time PCR analysis. The authors were responsible for the final version of the manuscript. All but 1 investigator had full access to the data files. The investigator who performed the real-time PCR for genotyping resistance was blinded to all demographic and clinical characteristics of patients. The funding sources had no role in the design, conduct, or reporting of the study or in the decision to submit the manuscript for publication.

RESULTS

Genotypic Clarithromycin Resistance Distribution

We assessed clarithromycin resistance in biopsy specimens obtained before therapy in all 156 enrolled patients, and we detected primary clarithromycin resistance in 38 patients (24%). Tables 1 and 2 provide the baseline characteristics of patients and the distribution of genotypic pattern of clarithromycin resistance. The prevalence of the 3 point mutations did not statistically differ between men and women (25% vs. 20%) and between patients with nonulcer dyspepsia and patients with peptic ulcer (24% vs. 20%).

Clarithromycin Resistance and Therapeutic Outcome

After treatment, *H. pylori* was eradicated in 134 patients, while treatment failed in 22 cases. The infection was cured in 109 patients (92%) with clarithromycin-susceptible strains and in 25 patients (66%) with clarithromycin-resistant strains (difference, 26 percentage points [95% CI, 12 to 43 percentage points]; $P < 0.001$). In subsequent analyses, we combined A2142G and A2142C point mutations because of similar eradication rates (90% and 100%,

respectively) and we pooled the 2 individuals with double mutation with the A2143G group because we assumed a dominant mode of inheritance. As shown in Table 3, the presence of A2143G mutation (9 alone plus 2 combined) was associated with a lower cure rate compared with the other 2 mutations (A2142G or A2142C) cumulatively computed (11 of 23 patients [48%] vs. 14 of 15 patients [93%]; difference, 45 percentage points [CI, 15 to 65 percentage points]; $P = 0.004$).

After the standard triple therapy and the sequential regimen, *H. pylori* infection was eradicated in 86% and 98% of patients with clarithromycin-susceptible strains, respectively, and in 44% and 82% of those with clarithromycin-resistant strains, respectively. The sequential regimen was more effective than triple therapy in both clarithromycin-susceptible strains (98% vs. 86%; difference, 12 percentage points [CI, 2 to 23 percentage points]; $P = 0.0353$) and clarithromycin-resistant strains (82% vs. 44%; difference, 38 percentage points [CI, 7 to 61 percentage points]; $P < 0.0155$).

The eradication rate achieved with the sequential regimen was statistically significantly higher than that achieved with triple therapy in patients with either A2143G or double mutation strains (69% vs. 20%; difference, 49 percentage points [CI, 8 to 72 percentage points]; $P = 0.024$), while the rate did not statistically significantly differ in those patients with either A2142G or A2142C mutate strains (100% vs. 83%; difference, 17 percentage points [CI, -16 to 56 percentage points]; $P = 0.40$).

Sample size limited our ability to examine the relationship between therapeutic failure and other covariates within 1 multivariable logistic model. In a logistic model with terms for treatment and A2143G group, both terms were independently related to therapeutic failure ($P < 0.010$ for each). When we added terms for age, sex, and gastroduodenal disease individually to this model, the relationships between therapeutic failure and treatment and A2143G group remained.

Table 2. Genotypic Clarithromycin Resistance Distribution

Therapy	Patients with A2143G, n (%)	Patients with A2142G, n (%)	Patients with A2142C, n (%)	Patients with Double Mutation, n (%)
Triple (n = 75)	10 (13)	2 (3)	4 (5)	0 (0)
Sequential (n = 81)	11 (14)	8 (10)	1 (1)	2 (2)
Overall (n = 156)	21 (13)	10 (6)	5 (3)	2 (1)

DISCUSSION

Clarithromycin is the most powerful antibiotic that is currently available for *H. pylori* infection, and its use in first-line therapy regimens is endorsed by European and U.S. guidelines (3, 4). *Helicobacter pylori* clarithromycin resistance is the main factor hampering the efficacy of stan-

Table 3. Eradication Rates after Standard and Sequential Treatment Regimens in Clarithromycin-Susceptible and Clarithromycin-Resistant Strains*

Therapy	Patients with Susceptible Strains, n/n (%)†	Patients with A2143G or Double Mutation, n/n (%)†	Patients with A2142G or A2142C Mutation, n/n (%)†
Triple	51/59 (86)	2/10 (20)	5/6 (83)
Sequential	58/59 (98)	9/13 (69)	9/9 (100)
Total	109/118 (92)	11/23 (48)	14/15 (93)

* $P < 0.050$ for all comparisons between standard and sequential regimens.

† Data are patients/total (percentage).

standard triple therapies, and it is increasing worldwide (7). Indeed, the overall prevalence of primary clarithromycin resistance that we found (24%) would seem higher than that computed in previous Italian studies (range, 1.8% to 14.0%) (23–25) and is similar to that detected in another recent study (23%) (26). We also confirm that the probability of success of a standard therapy regimen is markedly reduced by clarithromycin resistance to values less than 45%, which is similar to 2 large pooled data analyses (5, 6) that calculated a mean eradication rate ranging from 18% to 44%.

In order to assess *H. pylori* susceptibility to clarithromycin, clinical trials have commonly used a bacterial culture. However, *H. pylori* is a rather “fastidious” bacterium to be isolated, and bacterial culture has several limitations (27). In the past decade, novel culture-free PCR-based clarithromycin resistance assays have been proposed, showing very high accurateness, even on paraffin-embedded biopsy specimens (9). Moreover, different point mutations in the peptidyltransferase region encoded in domain V of the *H. pylori* 23S ribosomal RNA gene have been shown to prevent the clarithromycin binding to the bacterial ribosome (8–11). In particular, A2143G and A2142G are the most prevalent point mutations in western countries, and studies have claimed that these mutations play a major role in clarithromycin resistance (10–14). The prevalence distribution of A2143G (55%) and A2142G (26%) in our patients is similar to those of patients in an Italian study and other western studies (9–11, 28).

Previous microbiological observations have shown that such different mutations are associated with different minimal inhibitory concentration values in vitro, but the few available data have yielded conflicting results. Two studies (11, 14) associated the A2143G mutation with high minimal inhibitory concentration values in vitro, while 2 further studies (9, 10) found high minimal inhibitory concentration values associated with the A2142G mutation. Moreover, the clinical relevance of such point mutations has not yet been clarified (7). Our study showed that the outcome of *H. pylori* therapy is markedly affected by the A2143G point mutation occurring in the 23S ribosomal

RNA gene. In detail, a very low eradication rate (48%) occurred in the A2143G mutate strains, while the rate was higher than 90% in the absence of this mutation. On the other hand, clarithromycin resistance associated with the A2142G mutation seems to be clinically meaningless because the eradication rates are higher than 90% in both mutated-type and wild-type strains. On the basis of a similarly high prevalence of the A2143G point mutation in Europe and the United States (9–11, 14, 28), analogous results may be expected in other western countries. The different therapeutic outcomes observed among different point mutations could be due to diverse 3-dimensional alterations on the link site, leading to different loss of affinity for clarithromycin (14).

We also aimed to compare the efficacy of standard triple therapy and the sequential regimen according to primary bacterial resistance. The overall higher success rates for sequential therapy seem to be related not only to its higher efficacy for clarithromycin-susceptible strains but also to a clearly higher success rate on clarithromycin-resistance strains. Indeed, although the A2143G mutation reduced the eradication rate after the sequential regimen, the cure rate increased more than 3-fold (69%) with this regimen compared with standard triple therapy (20%). The use of a therapy with a higher efficacy for such resistant strains would markedly reduce the overall cost associated with clarithromycin resistance management in clinical practice.

Although the reasons why the sequential regimen is more effective for resistant strains need to be clarified, some hypotheses may be put forward. An inducible synthesis of adenosine triphosphatase-dependent pumps on the bacterial membrane has been shown to cause a rapid efflux of the antibiotics in resistant strains, contrasting with their bactericidal action (29, 30), and has been regarded as an alternative mechanism of clarithromycin resistance (31). The ability of amoxicillin to destroy the bacterial wall could impair such a transmembrane efflux system, resulting in an intracellular entrapment of the macrolide. The highest intracellular macrolide concentration—due to both a higher access and a lower efflux through an already riddled membrane—could overcome the rescue attempt engineered by the bacterium to prevent the fatal clarithromycin-ribosomal binding. Therefore, the first course of sequential regimen based on amoxicillin administration could act as an “induction” therapeutic phase, favoring the efficacy of the clarithromycin-tinidazole therapy that immediately follows, even in some clarithromycin-resistant strains.

Our results will clearly affect future research on *H. pylori* resistance, and genotypic evaluation is tempting for potential use in clinical practice. Genotypic evaluation could presumably be regarded as the most accurate test to assess the prevalence of resistant *H. pylori* strains that affect the therapeutic outcome. Moreover, genotypic evaluation seems useful in comparing further therapy regimens against

clarithromycin-resistant strains. From a clinical point of view, genotypic assay may be proposed after failure of second-line regimens similar to bacterial culture, according to international guidelines, especially when paraffin-embedded biopsy specimens are the only available samples (3).

Among the possible limitations of our study are its post hoc design and relatively small sample size. Additional larger multicenter studies may confirm these data. Moreover, the limited availability of the PCR tool, time-consuming assessment, and need for dedicated expertise may prevent widespread use of this technique. Finally, the cost of PCR assessment (approximately €70 in Italy) is an obvious concern when dealing with a very common infection.

In conclusion, we believe that our study is the first to show that A2143G is the main genetic mutation involved in *H. pylori* clarithromycin resistance in vivo and the first to determine differences in outcome with 2 eradication regimens. The sequential regimen had statistically significantly higher efficacy than standard therapy, even in A2143G mutate strains. This, as well as its higher eradication rate in smokers, patients with nonulcer dyspepsia, and patients with cytotoxin-associated gene A (*CagA*)–negative strains (32–35), may suggest its use as first-line therapy in *H. pylori* treatment.

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