Efficacy of genotypic resistance-guided sequential therapy in the third-line treatment of refractory Helicobacter pylori infection: a multicentre clinical trial

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Objectives: The efficacy of sequential therapy and the applicability of genotypic resistance to guide the selection of antibiotics in the third-line treatment of Helicobacter pylori have not been reported. We aimed to assess the efficacy of genotypic resistance-guided sequential therapy in third-line treatment.

Methods: Genotypic and phenotypic resistances were determined in patients who failed at least two eradication therapies by PCR with direct sequencing and agar dilution test, respectively. The patients were retreated with sequential therapy containing esomeprazole and amoxicillin for the first 7 days, followed by esomeprazole and metronidazole plus clarithromycin, levofloxacin or tetracycline for another 7 days (all twice daily), according to genotypic resistance determined using gastric biopsy specimens. Eradication status was determined by the 13C-urea breath test. Trial registered at clinicaltrials.gov (identifier: NCT01032655).

Results: The overall eradication rate was 80.7% (109/135, 95% CI 73.3%–86.5%) in the intention-to-treat analysis. The presence of amoxicillin resistance (OR 6.83, 95% CI 1.62–28.86, P = 0.009) and prior sequential therapy (OR 4.77, 95% CI 1.315–17.3, P = 0.017), but not tetracycline resistance (tetracycline group), were associated with treatment failure. The eradication rates in patients who received clarithromycin-, levofloxacin- and tetracycline-based sequential therapies were 78.9% (15/19), 92.2% (47/51) and 71.4% (25/35) in strains susceptible to clarithromycin, levofloxacin and tetracycline, respectively.

Conclusions: A simple molecular method guiding sequential therapy can achieve a high eradication rate in the third-line treatment of refractory H. pylori infection.

Keywords: gyrA, 23S rRNA, H. pylori, rescue

Introduction

Eradication of Helicobacter pylori, an important causal factor for peptic ulcer disease and gastric cancer, fails in 5%–10% of patients even after two eradication therapies.1–3 Susceptibility-guided therapy is recommended for patients who fail at least two treatments according to the European and American consensus reports.4,5 Determination of the MIC for H. pylori is not widely available, because it is time consuming, inconvenient and relatively expensive. Besides, the successful culture rate ranges from 75% to 90%.3,6 More importantly, only limited data are available regarding the efficacy of susceptibility-guided...
therapy in third-line treatment. Recently, point mutations in 23S ribosomal RNA (23S rRNA) and gyrase A (gyrA) were reported to be associated with clarithromycin and levofloxacin resistance, respectively. However, whether genotypic resistance is effective in guiding the choice of antibiotics in third-line treatment has not been reported.

Sequential therapy, which consists of a proton pump inhibitor plus amoxicillin for 5 days, followed by a proton pump inhibitor plus clarithromycin and tinidazole (or metronidazole) for another 5 days, has been shown to be more effective than standard triple therapy in first-line treatment. Modified sequential therapies, which used levofloxacin to replace clarithromycin, were also effective in first-line and second-line therapies. However, the efficacy of modified sequential therapies containing clarithromycin, levofloxacin or tetracycline as third-line treatment has not been reported. Therefore, we conducted this trial to assess the efficacy and tolerability of genotypic resistance-guided sequential therapy in third-line treatment.

Methods
This prospective, open-label, multicentre trial was conducted in three medical centres in Taiwan from April 2009 to April 2012 (clinicaltrials.gov identifier: NCT01032655). The study protocol was approved by the Institutional Review Board of each hospital. Written informed consent was obtained from all patients prior to enrolment. We searched published works from PubMed, MEDLINE and the Cochrane Library for the terms ‘H. pylori’, ‘sequential therapy’ and ‘genotypic resistance’ with no language or date limits. No publication of clinical trials that assessed the efficacy of genotypic resistance-guided sequential therapy in the third-line treatment of H. pylori infection was identified.

Eligibility criteria
H. pylori-infected patients who failed at least two treatments were eligible for this study. Patients were excluded from the study if any one of the following criteria was present: (i) children and teenagers aged <20 years; (ii) history of gastrectomy; (iii) non-curable malignancy; (iv) contraindication to study drugs; (v) previous allergic reaction to antibiotics (amoxicillin, clarithromycin, levofloxacin and tetracycline) and esomeprazole; (vi) pregnant or lactating women; or (vii) severe concurrent diseases.

Determination of H. pylori status
Prior to enrolment, the status of H. pylori infection was determined by either (i) the 13C-urea breath test (13C-UBT) or (ii) the rapid urease test, histology and culture. Patients with either one positive 13C-UBT or any two positive of rapid urease test, histology or culture were defined as refractory to previous treatment and were eligible for enrolment. Oesophago-gastro-duodenoscopy with biopsies from antrum (one piece for rapid urease test, two pieces for culture and two pieces for histology) and body (two pieces for histology) were performed for all patients. After third-line treatment, H. pylori status was determined by 13C-UBT ≥6 weeks after the completion of eradication therapy. Successful eradication of H. pylori was defined as a negative 13C-UBT result. All subjects were asked to stop proton pump inhibitors and histamine 2-blockers for ≥2 weeks before 13C-UBT. Positive and negative results were defined according to the results of our previous validation study as a delta value of ≥4 units and <2.5 units, respectively. Patients with inconclusive results received another 13C-UBT until the result was conclusive.

Determination of genotypic and phenotypic resistance
The biopsy specimens were cultured on plates containing Brucella chocolate agar with 7% sheep blood and incubated under microaerobic conditions (5% O2, 10% CO2 and 85% N2) for 7 days. The DNA of H. pylori was extracted from strains and gastric biopsy tissues using the same DNA extraction kit (Genta DNA Purification Kit, QIAGEN, USA) according to the manufacturer’s instructions. The H23S fragment was amplified by PCR with forward (5′-CCACACGGATTGTGCTCAG-3′) and reverse (5′-CTCATAAAGGCAAGGCC-3′) primers. The gyrA fragment was amplified by PCR with the following primers: forward, 5′-TTTRGCTTATCMATGAGCGT-3′; and reverse, 5′-GCCAGAGCTTGGTARATAA-3′. The PCR products were then purified and subjected to direct sequencing using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). The substitution of amino acids at positions 87 and 100 were considered to result in mutant gyra. Phenotypic resistance was determined by the agar dilution test if strains were available. H. pylori was inoculated onto antibiotic-containing Mueller–Hinton agar supplemented with 5% defibrinated sheep blood. H. pylori ATCC 43504 was used as the quality control strain. The MIC of each antibiotic was determined after 72 h of incubation. The breakpoints for amoxicillin, clarithromycin, levofloxacin, tetracycline and metronidazole resistance were defined as >0.5, >1, >1, >1 and >8 mg/L, respectively. The technicians who performed the agar dilution test and the genotyping were blinded to each other’s results and to the therapeutic outcomes.

Intervention and assessment of adverse effects
Patients were treated with sequential therapy containing 40 mg of esomeprazole and 1 g of amoxicillin for the first 7 days, followed by 40 mg of esomeprazole and 500 mg of metronidazole plus 500 mg of clarithromycin, 250 mg of levofloxacin or 500 mg of tetracycline for another 7 days (all drugs were given twice daily), according to the genotypic resistance determined using biopsy specimens or isolated strains. In the absence of 23S rRNA mutation, clarithromycin-based therapy would be given (see Figure 1). In the presence of the 23S rRNA mutation, levofloxacin-based therapy would be given if the strains had wild-type gyrA. In the presence of both 23S rRNA and gyrA mutations, tetracycline-based therapy would be given empirically. If the genotypic resistance could not be determined, the patients would be treated empirically according to their previous medication history to avoid the reuse of clarithromycin and levofloxacin. If the patients received sequential therapy containing either clarithromycin or levofloxacin in the previous treatment, they were retreated with tetracycline-based sequential therapy to avoid the reuse of the same regimen. The primary endpoint of the study was the overall eradication rate. Patients were informed of the common adverse effects and were asked to record their symptoms during treatment. A standardized interview and questionnaire were used to assess the adverse events and compliance at the end of treatment. Patients with low compliance, as defined by taking <80% of the pills, and those lost to follow-up were excluded from the per protocol (PP) analysis.

Genotyping of CYP2C19 and virulence factors
The pepsinogen I and II levels were determined using commercially available kits (Eiken Chemical Co., Ltd, Tokyo, Japan). The serological atrophic gastritis phenotype was defined by the levels of pepsinogen I ≤70 ng/mL and a pepsinogen I/II ratio <3. The cagA gene and the vacA signal region (s1/2) and mid-region (m1/2) mosaics were determined by PCR using DNA extracted from H. pylori strains or gastric biopsy specimens. Genomic DNA from peripheral blood lymphocytes was used to assess the CYP2C19 polymorphism using the SEQUENOM MassARRAY® System and
a matrix-assisted laser desorption/ionization–time of flight mass spectrometer at the National Genotyping Center.

Sample size estimation and statistical analysis

We assumed the estimated efficacy to be 86%, \( \varepsilon \) (the desired CIs) to be 6% and the desired confidence level to be 95%. The minimum sample size was 128 based on these assumptions. Therefore, the final size of this study should be \( \geq 135 \) patients when a 5% lost to follow-up rate was considered. Categorical data were compared using the \( \chi^2 \) test or Fisher's exact test, as appropriate. Continuous data were compared with Student's \( t \)-test and expressed as the mean (SD). The \( \kappa \) coefficient was used to assess the agreement between genotypic resistance and phenotypic resistance. Logistic regression analysis was performed to analyse factors affecting the eradication rates. All \( P \) values were two-tailed, with the level of statistical significance specified as 0.05. The statistical analyses were performed using the SPSS 12.0 statistical software for Windows.

Results

Demographic data and prevalence of antibiotic resistance

The flow of participants in this study is shown in Figure 1. \( H. \) pylori strains were available for agar dilution testing in 100 (74.1%) patients and the MIC was successfully determined in 96 (71.1%) patients. The genotypic resistance was successfully determined using gastric biopsy specimens and strains in 93.3% (83/96) and 72.6% (71/96) of patients, respectively. Of the patients with adequate information regarding their medication history, 100% (83/83) and 79.1% (71/90) of them received clarithromycin and levofloxacin in their previous eradication regimens, respectively (Table 1). The prevalence of secondary resistance to clarithromycin, levofloxacin, amoxicillin, metronidazole and tetracycline was 86.5% (83/96), 46.9% (45/96), 9.4% (9/96), 58.3% (56/96) and 3.2% (3/93), respectively (Table 1). The genotypic resistance determined using biopsy specimens correlated well with the phenotypic and genotypic resistance determined using strains for both clarithromycin and levofloxacin (Table 2). The MIC\(_{50}/\text{MIC}_{90}\) (range) of strains with AA, GA and AG at positions 2142 \( +2143 \) of 23S rRNA were 0.03/8 (0.016–16), 32/64 (16–64) and 32/64 (0.016–64) mg/L, respectively. The MIC\(_{50}/\text{MIC}_{90}\) (range) of strains without and with mutations at codon 87 of \( \text{gyrA} \) were 0.5/4 (0.125–16) and 8/16 (4–16) mg/L, respectively. The MIC\(_{50}/\text{MIC}_{90}\) (range) of strains without and with mutations at codon 91 of \( \text{gyrA} \) were 0.5/8 (0.125–16) and 8/16 (0.5–16) mg/L, respectively.
82.6% (109/132, 95% CI 75.2%–88.1%) in the PP analysis. Subgroup analysis showed that the eradication rates in patients who received clarithromycin-, levofloxacin- and tetracycline-based sequential therapy were 78.9% (15/19, 95% CI 56.3%–91.3%), 92.2% (47/51, 95% CI 81.4%–96.8%) and 71.4% (25/35, 95% CI 54.8%–83.6%) in strains susceptible to clarithromycin, levofloxacin and tetracycline, respectively. The eradication rate appeared to be higher in patients treated with levofloxacin-based therapy as compared with those treated with tetracycline-based therapy when the strains were susceptible to levofloxacin and tetracycline, respectively (mean difference 20.8%, 95% CI 4.9%–36.7%, \( P=0.011 \)).

The presence of amoxicillin resistance (OR 6.83, 95% CI 1.62–28.86, \( P=0.009 \)), \( \text{vacA} \) \( m1 \) (OR 4.5, 95% CI 0.98–20.78, \( P=0.054 \)) and prior history of sequential therapy (OR 4.77, 95% CI 1.315–17.3, \( P=0.017 \)) correlated with treatment failure in the logistic regression analyses. The eradication rates were not affected by metronidazole resistance, gastric atrophy, peptic ulcer disease, smoking, \( \text{CYP2C19} \) polymorphism and \( \text{cagA} \) positivity (Table 3).
Table 3. Factors affecting eradication rates

<table>
<thead>
<tr>
<th>Factor</th>
<th>Eradication rate</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Amoxicillin resistance</td>
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<td></td>
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<tr>
<td>no</td>
<td>84.5% (71/84)</td>
<td>0.012</td>
</tr>
<tr>
<td>yes</td>
<td>44.4% (4/9)</td>
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<tr>
<td>Metronidazole resistance</td>
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</tr>
<tr>
<td>no</td>
<td>84.2% (32/38)</td>
<td>0.469</td>
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<tr>
<td>yes</td>
<td>78.2% (43/55)</td>
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<tr>
<td>Atrophy* (serology)</td>
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<tr>
<td>no</td>
<td>84.5% (93/110)</td>
<td>0.909</td>
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<tr>
<td>yes</td>
<td>85.7% (12/14)</td>
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<tr>
<td>Peptic ulcer</td>
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<tr>
<td>no</td>
<td>82.5% (52/63)</td>
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</tr>
<tr>
<td>yes</td>
<td>82.6% (57/69)</td>
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<tr>
<td>Smoker</td>
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<td></td>
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<tr>
<td>no</td>
<td>82.9% (92/111)</td>
<td>0.762</td>
</tr>
<tr>
<td>yes</td>
<td>81.0% (17/21)</td>
<td></td>
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<tr>
<td>CYP2C19</td>
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<td></td>
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<tr>
<td>PM</td>
<td>91.7% (11/12)</td>
<td>0.692</td>
</tr>
<tr>
<td>IM/EM</td>
<td>81.2% (95/117)</td>
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<tr>
<td>cagA</td>
<td></td>
<td></td>
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<tr>
<td>negative</td>
<td>88.9% (16/18)</td>
<td>0.519</td>
</tr>
<tr>
<td>positive</td>
<td>79.8% (79/99)</td>
<td></td>
</tr>
<tr>
<td>vacA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m1</td>
<td>93.3% (28/30)</td>
<td>0.038</td>
</tr>
<tr>
<td>m2/m1+m2</td>
<td>75.7% (56/74)</td>
<td></td>
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<tr>
<td>Prior sequential therapy</td>
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<td></td>
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<tr>
<td>no</td>
<td>85.1% (103/121)</td>
<td>0.024</td>
</tr>
<tr>
<td>yes</td>
<td>54.5% (6/11)</td>
<td></td>
</tr>
</tbody>
</table>

PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; cagA, cytotoxin-associated gene A; vacA, gene encoding vacuolating cytotoxin A.

*Atrophic gastric phenotype defined as pepsinogen I ≤70 ng/mL and pepsinogen I/II ratio ≤3.

the eradication rate was not significantly affected by tetracycline resistance.

Adverse effects and compliance

No difference was observed in the tolerability of the medication or adverse effects between the three regimens. Two patients (1.5%) discontinued the drugs due to adverse effects and one (0.7%) of them failed to take ≥80% of the study drugs. Overall, 97% (131/135) of patients took the medications correctly.

Discussion

There were several novel findings in this study. This was the first study to show that genotypic resistance-guided modified sequential therapy was effective (>80%) in the third-line treatment of H. pylori infection. More importantly, we showed that readministration of clarithromycin and levofloxacin in the modified sequential therapy was effective for patients who failed prior therapies containing these antibiotics if the strains had wild-type 23S rRNA and gyrA, respectively. We further showed that the eradication rates appeared to be higher in patients treated with levofloxacin-based sequential therapy as compared with those treated with tetracycline-based sequential therapy when the strains were susceptible to levofloxacin and tetracycline, respectively. Besides, we found that the presence of amoxicillin resistance and prior treatment with sequential therapy were associated with treatment failure in patients treated with genotypic resistance-guided sequential therapy.

Although susceptibility testing was recommended before third-line treatment, the efficacies of susceptibility-guided therapy were reported only in some studies, with reported efficacies ranging from 36% to 90%. In a retrospective study, Beales' showed that the eradication rate among those treated empirically or guided by a susceptibility test was 50% (2/4) and 69% (11/16) in third-line treatment, respectively. In a prospective single-arm trial, Gomollón and colleagues showed that the eradication rates of culture-guided quadruple therapies containing omeprazole, tetracycline and bismuth with either clarithromycin or amoxicillin for 2 weeks were only 36% (5/14) and 67% (8/12) in third-line treatment, respectively. Another study showed that the eradication rate of a culture-guided quadruple therapy containing omeprazole, amoxicillin and either (i) bismuth and doxycycline or (ii) levofloxacin and clarithromycin for 1 week was 90.4% (85/94). However, the necessity of performing culture and susceptibility tests was challenged, because 95% (89/94) of the patients had strains resistant to clarithromycin and received the former treatment, which may be given empirically. In this study, we showed that 51.9% (70/135) patients could be effectively retreated with regimens containing clarithromycin and levofloxacin by the determination of genotypic resistance to these two drugs.

In this study, the treatment regimens were determined according to clarithromycin and levofloxacin resistance, but not according to tetracycline resistance. There were several reasons for the empirical use of tetracycline instead of being guided by tetracycline resistance. First, the prevalence of tetracycline resistance determined in H. pylori strains. The eradication rate of culture-guided quadruple therapies containing omeprazole, tetracycline and bismuth with either clarithromycin or amoxicillin for 2 weeks were only 36% (5/14) and 67% (8/12) in third-line treatment, respectively. Another study showed that the eradication rate of a culture-guided quadruple therapy containing omeprazole, amoxicillin and either (i) bismuth and doxycycline or (ii) levofloxacin and clarithromycin for 1 week was 90.4% (85/94). However, the necessity of performing culture and susceptibility tests was challenged, because 95% (89/94) of the patients had strains resistant to clarithromycin and received the former treatment, which may be given empirically. In this study, we showed that 51.9% (70/135) patients could be effectively retreated with regimens containing clarithromycin and levofloxacin by the determination of genotypic resistance to these two drugs.

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performing endoscopy. Some studies also showed that genotypic resistance-guided therapy using stool samples might be effective in first-line therapy. Therefore, genotypic resistance-guided therapy is a practical strategy in the treatment of refractory *H. pylori* infection in future clinical practice. The strength of this study included the relatively large sample size in third-line therapy, the pre-defined strategy to select antibiotics according to the genotyping results and the determination of genotypic resistance without the need for culture, which makes this strategy more convenient and applicable in clinical practice. However, this study had some limitations. First, this was not a randomized trial. Therefore, whether genotypic resistance-guided therapy is more effective than empirical therapy should be assessed in future studies. Nevertheless, the aim of this study was to assess the efficacy of genotypic resistance-guided sequential therapy in third-line treatment rather than to prove the superiority of genotypic resistance-guided therapy over empirical therapy. The results from this study provide important information on calculating the sample size for further randomized control trials on this issue. It is noteworthy that the prevalence of tertiary antibiotic resistance before third-line therapy should also be taken into account in the sample size calculation. In future studies, it is also important to determine the percentage of strains that remained susceptible to clarithromycin and levofloxacin after one or two cycles of treatment with these drugs, respectively. Second, metronidazole and tetracycline were given empirically without performing a susceptibility test. However, our results showed that the eradication rate of modified sequential therapy for 14 days was not affected by the presence of metronidazole and tetracycline resistance. Besides, although amoxicillin resistance was associated with treatment failure, the empirical use of amoxicillin in third-line therapy is still acceptable, because the prevalence of amoxicillin resistance remained low (9%) in patients who failed at least two eradication therapies. Therefore, the determination of genotypic resistance to clarithromycin and levofloxacin alone might be appropriate. Third, the determination of genotypic resistance using gastric biopsy specimens is expensive due to the cost of endoscopy. However, the cost could be reduced if stool specimens are used for genotyping. Fourth, the successful culture rate was only 74.1%, because it took 1–3 days to transport the biopsy specimens to the central laboratory from different hospitals. Finally, although we found the efficacy was affected by amoxicillin resistance and prior sequential therapy, the results should be validated in other studies because of the wide CIs, which indicated small case numbers for these two variables.

In conclusion, the results from this study show that a simple molecular screening method guiding sequential therapy can achieve a high eradication rate in the third-line treatment of refractory *H. pylori* infection, which deserves further validation in randomized control trials and in other countries.

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Transparency declarations
None to declare.

Author contributions
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References


