HELICOBACTER PYLORI AND THE ROLE OF GENETIC MUTATIONS IN ANTIBIOTIC RESISTANCE

Jinfeng Dai[#], Xuan Huang[#], LiJun Cai , Bin Lv , Jing Zhao[*]
Department of Gastroenterology, The First Affiliated Hospital, Zhejiang Chinese Medical University, 54 Youdian Road, Shangcheng District, Hangzhou City 310006, Zhejiang Province, People's Republic of China
[#]*These authors contributed equally to this work as co-first author*

ABSTRACT

Background: *High prevalence and low eradication rates of Helicobacter pylori have become major challenges to clinicians. The growing antibiotic resistance rate via mutational changes is the primary reason for the sharp decline in the H. pylori eradication rate.*

Aim: To study the role of genetic mutations in the antibiotic resistance of H. pylori.

Methods: *H. pylori strains were isolated between 2017 and 2018 from patients with H. pylori infection. Antimicrobial susceptibility testing was performed for amoxicillin, fura-zolidone, clarithromycin, metronidazole, levofloxacin and tetracycline using the E-test method. Gene mutations were investigated by PCR-RDB assay for the antibiotic-resistant strains.*

Results: *In total, 118 patients with H. pylori infection were enrolled, including 21 subjects from whom strains failed to be isolated. Only 6 strains were sensitive to all of the antibiot-ics tested. Isolates were characterized by no resistance to amoxicillin (0%), furazolidone (0%), and tetracycline (0%); a concerning resistance rate to clarithromycin (27.8%) and levofloxacin (29.9%); and a high resistance rate to metronidazole (88.7%). Of all the clarithromycin-resistant strains, 88.0% had the A2143G mutation in 23S rRNA, while the A2142G mutation was not detected. N87K, D91G, D91N and D91Y were four common mutations in the levofloxacin resistance-determining region of gyrA. The G616A muta-tion (30.2%) was not prevalent in metronidazole-resistant strains.*

Conclusions: *Metronidazole should not be considered when eradicating H. pylori in adults in Zhejiang, China. The first-line empirical regimen for H. pylori eradication should include amoxicillin if the patient is not allergic to it. Gene mutations play an important role in the antibiotic resistance of H. pylori, especially clarithromycin and levofloxacin.*

Keywords: *Helicobacter pylori, antibiotic resistance, gene mutation, metronidazole, amoxicillin, tet-racycline, clarithromycin, furazolidone, levofloxacin.*

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Introduction

Barry J. Marshall and J. Robin Warren won the Nobel Prize in Medicine in 2005 for their landmark discovery of Helicobacter pylori (H. pylori). Their research not only offered a key to healing peptic ulcers, but also provided new perspective on how to study gastric cancer. In 2015, the Kyoto global consensus report on H. pylori gastritis stressed the im-portance of eradicating H. pylori[1]. However, high prevalence and low eradication rates remain major challenges to physicians[2-3]. As we reviewed before, the growing antibi-otic resistance rate through mutational changes is the main reason for the sharp decline in the H. pylori eradication rate. Point mutations in the 23S rRNA gene and alterations in the 16S rRNA gene and in rdxA, frxA, pbp1, gyrA and gyrB, result in resistance to clar-ithromycin, metronidazole, amoxicillin, levofloxacin and tetracycline, respectively[4]. Nevertheless, most of these data were from Western countries, and H. pylori is highly di-verse among various populations[5, 6].

In Zhejiang, there is both a high rate of gastric cancer morbidity and a high prevalence of the associated H. pylori infection, suggesting the need for more effective therapies to eradicate H. pylori. Therefore, this study explored the role of genetic mutations in the antibiotic resistance of H. pylori in Zhejiang, China, which may shed light on the diagnosis and treatment of H. pylori.

Material and methods

Study design

This was a non-randomized, single-centred, non-inferiority, open-label study of the mo-lecular mechanism of antibiotic resistance of H. pylori. H. pylori strains were isolated be-tween December 2017 and December 2018 from infected patients in the First Affiliated Hospital of Zhejiang Chinese Medical University.

Antimicrobial susceptibility testing was performed for amoxicillin, furazolidone, clarithromycin, metronidazole, levofloxacin and tetracycline using the E-test method. Mutations in rdx (G616A), 23S rDNA (A2142G, A2143G) and gyrA (N87K, D91G, D91N, and D91Y) were investigated through polymerase chain reaction reverse dot blot hybridization (PCR-RDB) for the re-lated antibiotic-resistant strains.

Genomic DNA extraction

5 ml EDTA-Na2 anticoagulant was obtained, nucleated cells were separated with lympho-cyte separation liquid, and genomic DNA was extracted using a genomic DNA extraction kit (Yaneng Bio-Sciences, centrifugal column type).

Amplification of PCR-RDB

Probes were designed using Primer 5 software (Shanghai SANGON Biotechnology Com-pany). Case and serial numbers were printed on the hybridization filter.

Then, the filter was put into 10% 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), washed with distilled water several times, and dried on filter paper. ASO (1 μl) was added to the dried filter. After 15 min, the filter was put into 0.1 mol/L NaOH and then dried.

Polymerase chain reaction (PCR) amplification

The primers were designed with Primer 5 software (Shanghai SANGON Biotechnology Company) (Table 1).

The 5' end of each primer was labelled with biotin. Final volume of the PCR was 50 μl, with 8.0 pmol/L primers, 1 μl DNA, 2 U LA Taq enzyme, (Invitrogen) and distilled water. PCR amplification was performed under the following conditions: 10 min 50°C reaction with UDG enzyme; 10 min 95°C initial denaturation; followed by 45 cycles at 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min.

Category	Points	Mark	Sequence (5'-3')
Experimental Probes	23S rRNA (point 2142/2143)	23S W*	ACGGAAAGACCCCGTG
	gyrA (point 87)	87 W	GGCGATAATGCGGTTT
	gyrA (point 91)	91W	TTTATGATGCACTAGTGAG
	(point 616)	616W	AAGTTGATGCAATTACTTG
	23S rRNA (point 2142)	A2142G	GACGGGAAGACCC
	23S rRNA (point 2143)	A2143G	AGACGGAGAGACCCC
	gyrA (point 87)	N87K	GCGATAARGCGGTTT
	gyrA (point 91)	D91G	ATGGTGCGYTAGTGAGA
		D91Y	TTATTATGCGYTAGTGAG
		D91N	TTATAATGCGCTAGTGAG
	rdxA (point 616)	G616A	CAAAAGTTGATACAATTACTT
Control	β-globin	IC	CCTCTTATCTTCCTCCCAC

Table 1: Sequence of probes used in study.
W: wild type.

RDB hybridization

The filter combining specific ASO probes was immersed in hybridization solution. Then, three tubes of PCR products were added to the solution. After denaturation for 10 min in a 100°C water bath, the mixed solution was put in a hybridization oven (Robbins Scien-tific) for 1.5 h at 48°C. The filter was then washed to remove the unhybridized PCR products. Finally, the filter was left in 20 ml of developing solution for 5 to 15 min at room temperature. Blue-purple spots were recorded as positive results.

Results

In total, 118 patients with H. pylori infection were enrolled. There was no significant dif-ference in sex (male:female = 1.03:1, p>0.05). The mean age of these patients was 46 years old. Twenty-one strains failed to be cultured in vitro; among the remaining 97 strains, 13 strains failed to be detected by PCR, and 9 strains had failed eradication histo-ry. Of the 9 strains with failed eradication history, 8 were resistant only to metronidazole, and 1 was resistant to clarithromycin, levofloxacin and metronidazole.

To our surprise, only 6 strains were sensitive to all antibiotics. The remaining isolates were characterized by no resistance to amoxicillin (0%), furazolidone (0%) and tetracycline (0%); a concern-ing resistance rate to clarithromycin (27.8%) and levofloxacin (29.9%); and a high re-sistance rate to metronidazole (88.7%) (Table 2). The dual drug resistance rate and triple drug resistance rate were 22.7% and 15.5%, respectively.

Drug name	Resistance rate	Mutated gene and its positive rate
Amoxicillin	0	-
Furazolidone	0	-
Tetracycline	0	-
Clarithromycin	27.8%	A2143G (23S rRNA) 88%
Levofloxacin	29.9%	N87K (31%); D91N (20.7%); D91G (10.3%); D91Y (6.9%)
Metronidazole	88.7%	G616A (rdxA) 30.2%

Table 2: The drug-resistance rate and mutated genes.

Resistance gene for clarithromycin

Twenty-seven strains were resistant to clarithromycin, and all but 2 of these had dual drug resistance or triple drug resistance. Two strains failed to be detected by PCR. Among the remaining 25 strains, 22 strains (88.0%) had A2143G mutations in 23S rRNA, and no strains harboured A2142G mutations in 23S rRNA (Table 2).

Resistance gene for levofloxacin

Twenty-nine strains were resistant to levofloxacin, and among these strains, only 1 had single drug resistance. Mutations in the levofloxacin resistance gene (the most common point mutations being N87K, D91G, D91Y and D91N in gyrA) were examined. The N87K mutation (31%) was the most common mutation in the levofloxacin resistance-determining region of gyrA.

Mutations at point 91 varied among D91G (10.3%), D91N (20.7%) and D91Y (6.9%) (Table 2). The sensitivity of PCR-RDB in levofloxacin re-sistance examination was 75.9%.

Resistance gene for metronidazole

Eighty-six strains were resistant to metronidazole, and 36 strains were resistant to 2 or more drugs, including metronidazole.

The most common point mutation, G616A, in rdxA was examined via PCR-RDB. The G616A mutation was present in only 30.2% of the metronidazole-resistant isolates (Table 2).

Discussion

H. pylori was considered an independent risk factor in World Cancer Report 2014, pub-lished by WHO Press. According to this report, the prevalence of H. pylori was relatively high in east Asia, especially in China and Japan[7]. A large survey including 26341 peo-ple from 39 centres in 19 Chinese provinces conducted between 2001-2004 by the Chinese Medical Association showed that 59% of Chinese people were infected with H. pylori[8]. The eradication therapy improved every several years because of the increasing anti-biotic-resistance rates. Among the antibiotics recommended by the latest guidelines, the prevalence of H. pylori resistance to metronidazole was as high as 60%~70%, while the prevalence of resistance to clarithromycin and levofloxacin was 20%~38% and 30%~38%, respectively. Fortunately, this study also showed low resistance rates of H. py-lori to amoxicillin, furazolidone and tetracycline, ranging from 1% to 5%[9, 10]. It should be noted however, that tetracycline is rarely used in China due to its potential ad-verse effects.

Extensive and unreasonable application of antibiotics is a main contributor to develop-ment of antibiotic resistance[11]. As we reviewed previously, many mechanisms contrib-ute to H. pylori antibiotic resistance, including gene mutations, increased oxygen radical scavenger system activity, and increased DNA repair enzyme activity. Among these mech-anisms, point mutations in various genes play an important role[4]. An increase in clar-ithromycin resistance has been observed in many countries, including Japan, Korea, and the United States[12, 13]. Clarithromycin resistance is associated with a mutation in 23S rDNA (i.e., A2143G and A2142G) in the 50S ribosome, and its conferring mutations have geographic differences. Ubhayawardana NL et al. found that the dominant point mutation was at the A2142G site in 23S rDNA, while the A2143G mutation was not de-tected[14]. However, studies from China and Japan showed that A2143G was the primary mutation in clarithromycin-resistant strains; our study found similar results[15, 16].

There is likewise a high rate of levofloxacin resistance. Studies have found 4 genes relat-ed to fluoroquinolone resistance, including gyrA, gyrB, parC and parE. In Neisseria gon-orrhoeae, gyrA and parC mutations account for most fluoroquinolone resistances. How-ever, parC and parE were not found in H. pylor[17, 18]. Therefore, gyrA mutations are primarily responsible for fluoroquinolone resistance in H. pylori. Studies showed that mutations at codons 87 (N87K) and 91 (D91G, D91N, D91A, or D91Y) of the gyrA gene are the most frequent[19-22]. In this study, we only examined mutations in the gy-rA gene. N87K was the most common mutation, followed by D91N; sensitivity was 75.9%. Geographic differences were also present in fluoroquinolone-resistant genes. Whereas we observed mutations in N87K, the main gyrA gene mutation for fluoroquino-lone

resistance in Brazil (11.3%) was found in N87I[23]. In Asia, 66.6% (8/12) of the metronidazole resistance of H. pylori was caused by rdxA inactivation, while 33.3% (4/12) of the remaining resistance was caused by both rdxA and frxA inactivation[24]. Similarly, in Malaysia, metronidazole resistance rates were 89.1% for insertions/deletions in rdxA and/or frxA[25]. However, in the same study, 10.8% of strains resistant to metronidazole exhibited no change in either rdxA or frxA. Jeong et al. found that metronidazole resistance development in Canada was mostly asso-ciated with inactivation in rdxA with or without frxA37. Their study also showed that the development of metronidazole resistance in H. pylori rarely requires the inactivation of frxA alone[26].

In our study, only the G616A mutation in rdxA was detected in isolates. Less than 32% of the metronidazole-resistant strain had G616A mutations in rdxA, which calls to mind the complex mutations in rdxA[25]. In addition, other mechanisms, includ-ing increased oxygen radical scavenger system activity, increased DNA repair enzyme ac-tivity, reduced uptake and increased efflux, also contribute to metronidazole resistance in H. pylori[4].

According to the new Toronto Consensus[27], first-line therapy should consider regional antibiotic resistance patterns and eradication rates. According to this study, H. pylori in Zhejiang, China, has a high resistance rate to metronidazole, clarithromycin and levofloxacin. Empirically chosen treatments with amoxicillin and furazolidone should be considered as the first-line treatment. Meta-analysis showed that tailoring therapy via E-test and PCR, the two most popular methods to confirm the antimicrobial susceptibility of H. pylori, is a better alternative for H. pylori eradication compared with empirically chosen treatments[28]. In Zhejiang, China, the E-test remains the first choice, while hospitals rarely perform PCR to diagnose H. pylori or screen its antibiotic resistance. In this study, an E-test demonstrated that 8 previously uneradicated strains were only resistant to met-ronidazole, and sensitive to amoxicillin, furazolidone, clarithromycin, levofloxacin and tetracycline. According to our questionnaire, no one used metronidazole during first-line eradication. This phenomenon might be due to the different environments in vivo and in vitro. The E-test takes an average of one week, and patients may not wish to wait for treatment. In our study, PCR-RBD only took 1 day, offering much quicker results than the E-test. Giorgio F et al. found that compared to tissue molecular analysis, it was feasible to use stool RT-PCR analysis as a non-invasive tool for detection of H. pylori DNA se-quences and antibiotic resistance point mutations[29]. Therefore, PCR, especially stool PCR analysis, might be a potential method to confirm the antimicrobial susceptibility of H. pylori.

Conclusions

Metronidazole should not be considered in the eradication of H. pylori in adults in Zhejiang, China. The first-line empirical regimen for H. pylori eradication should include amoxicillin (if no allergy) and furazolidone. Gene mutations play an important role in the antibiotic resistance of H. pylori, especially for clarithromycin and levofloxacin. Gene mutations detected by PCR could be an alternative to the E-test to screen antibiotic re-sistance of H. pylori when the first line fails or if patients are allergic to amoxicillin.

References

1) Liu WZ, Xie Y, Lu H, Cheng H, Zeng ZR, et al. Fifth Chinese National Consensus Re-port on the management of Helicobacter pylori infection. Helicobacter 2018; 23: 12475.
2) Atkinson NS, Braden B. Helicobacter pylori infection: diagnostic strategies in primary diagnosis and after therapy. Dig Dis Sci 2016; 61: 19-24.
3) Idowu A, Mzukwa A, Harrison U, Palamides P, Haas R, et al. Detection of Helicobac-ter pylori and its virulence genes (cag A, dup A, and vac A) among patients with gastroduodenal diseases in Chris Hani Baragwanath Academic Hospital, South Africa. BMC Gastroenterol 2019; 19: 73.
4) Hu Y, Zhang M, Lv B Dai JF. Helicobacter pylori and Antibiotic Resistance, A Contin-uing and Intractable Problem. Helicobacter 2016; 21: 349-363.
5) Waskito LA, Yih-Wu J, Yamaoka Y. The role of integrating conjugative elements in Helicobacter pylori: a review. J Biomed Sci 2018; 25: 86.
6) Hu Y, Zhu Y, Lu NH. Primary Antibiotic Resistance of Helicobacter pylori in China. Dig Dis Sci 2017; 62: 1146-1154.
7) Maumy L, Harrissart G, Dewaele P, Aljaber A, Bonneau C, et al. [Impact of nutrition on breast cancer mortality and risk of recurrence, a review of the evidence]. Bull Cancer 2019; 19: 30306.
8) Nagy P, Johansson S, Molloy-Bland M. Systematic review of time trends in the preva-lence of Helicobacter pylori infection in China and the USA. Gut Pathog 2016; 8: 8.

9) Song Z, Zhang J, He L, Chen M, Hou X, et al. Prospective multi-region study on primary antibiotic resistance of Helicobacter pylori strains isolated from Chinese patients. Dig Liver Dis 2014; 46: 1077-1081.
10) Miftahussurur M, Cruz M, Subsomwong P, Jiménez Abreu JA, Hosking C, et al. Clar-ithromycin-Based Triple Therapy is Still Useful as an Initial Treatment for Helicobac-ter pylori Infection in the Dominican Republic. Am J Trop Med Hyg 2017; 96: 1050-1059.
11) Megraud F, Coenen S, Versporten A, Kist M, Lopez-Brea M, et al. Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption. Gut 2013; 62: 34-42.
12) Mori H1, Suzuki H, Omata F, Masaoka T, Asaoka D, Kawakami K, et al. Current sta-tus of first- and second-line Helicobacter pylori eradication therapy in the metropoli-tan area: a multicenter study with a large number of patients. Therap Adv Gastroen-terol 2019; 12: 1756.
13) Shiota S, Reddy R, Alsarraj A, El-Serag HB, Graham DY. Antibiotic Resistance of Hel-icobacter pylori Among Male United States Veterans. Clin Gastroenterol Hepatol 2015; 13: 1616-1624.
14) Ubhayawardana NL, Weerasekera MM, Weerasekera D, Samarasinghe K, Gunasekera CP, et al. Detection of clarithromycin-resistant Helicobacter pylori strains in a dyspep-tic patient population in Sri Lanka by polymerase chain reaction-restriction fragment length polymorphism. Indian J Med Microbiol 2015; 33: 374-377.
15) Kageyama C, Sato M, Sakae H, Obayashi Y, Kawahara Y, et al. Increase in antibiotic resistant Helicobacter pylori in a University Hospital in Japan. Infect Drug Resist 2019; 12: 597-602.
16) Matsumoto H, Shiotani A, Katsumata R, Fujita M, Nakato R, et al. Helicobacter pylori eradication with proton pump inhibitors or potassium-competitive acid blockers: the effect of clarithromycin resistance. Dig Dis Sci 2016; 61: 3215-3220.
17) Ruiz J, Goñi P, Marco F, Gallardo F, Mirelis B, et al. Increased Resistance to Quin-olones in Campylobacter jejuni: A Genetic Analysis of gyrA Gene Mutations in Quinolone-Resistant Clinical Isolates. Microbiol & Immunol 2013; 42: 223-226.
18) El LM, Aubert S, Galtier E, Schmitt C, Anger K, et al. The Sole DEAD-Box RNA Hel-icase of the Gastric Pathogen Helicobacter pylori Is Essential for Colonization. Mbio 2018; 9: 2071.
19) Faramarzi M, Azadfallah P, Book HE, abatabaei KR, Taheri H, et al. A randomized controlled trial of brief psychoanalytic psychotherapy in patients with functional dys- pepsia. Asian J Psychiatr 2013; 6: 228-234.
20) Park KS, Yang HS, Nam YS, Lee HJ. Mutations in DNA Gyrase and Topoisomerase IV in Ciprofloxacin-Non-susceptible Extended-Spectrum-Lactamase-Producing Escherichia coli and Klebsiella pneumoniae. Clin Lab 2017; 63: 535-541.
21) An Y, Wang Y, Wu S, Wang YH, Qian X, et al. Fourth-generation quinolones in the treatment of Helicobacter pylori infection: A meta-analysis. World J Gastroenterol 2018; 24: 3302-3312.
22) Hanafi A, Lee WC, Loke MF, Teh X, Shaari A, et al. Molecular and Proteomic Analy-sis of Levofloxacin and Metronidazole Resistant Helicobacter pylori. Front Microbiol 2016; 7: 2015.
23) Sanches BS, Martins GM, Lima K, Cota B, Moretzsohn LD, et al. Detection of Heli-cobacter pylori resistance to clarithromycin and fluoroquinolones in Brazil: a national survey. World J Gastroenterol 2016; 22: 7587-7594.
24) Pourakbari B, Mahmoudi S, Parhiz J, Sadeghi RH, Monajemzadeh M, et al. High fre-quency of metronidazole and clarithromycin-resistant Helicobacter pylori in forma-lin-fixed, paraffin-embedded gastric biopsies. Br J Biomed Sci 2018; 75: 61-65.
25) Teh X, Khosravi Y, Ching Lee W, Leow AH, Loke MF, et al. Functional and Molecu-lar Surveillance of Helicobacter pylori Antibiotic Resistance in Kuala Lumpur. PLOS ONE 2014; 9: 101481.
26) Williams K, Colquhoun A, Munday R, Goodman KJ. Antibiotic dispensation rates among participants in community-driven health research projects in Arctic Canada. BMC Public Health 2019; 19: 949.
27) Chen H, Dang Y, Zhou X Liu B, Liu S, et al. Tailored Therapy Versus Empiric Cho-sen Treatment for Helicobacter pylori Eradication: A Meta-Analysis. Medicine (Balti-more) 2016; 95: 2750.
28) Ierardi E, Giorgio F, Iannone A, Losurdo G, Principi M, et al. Noninvasive molecular analysis of Helicobacter pylori: Is it time for tailored first-line therapy? World J Gas-troenterol 2017; 23: 2453-2458.
29) Boltin D, Schmilovitz-Weiss H, Gingold-Belfer R, Leibovitzh H, Snir Y, et al. Tempo-ral Trends in Helicobacter pylori Eradication Success in a Test-and-Treat Population. Digestion 2018; 98: 169-174.

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Jinfeng Dai analyzed the data and wrote the paper. Bin Lu designed and coordinated the research; Jing Zhao performed the experiments; Xuan Huang and Lijun Cai collected the mucosal samples.

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This research project had been reviewed and approved by the [The First Affiliated Hospi-tal of Zhejiang Chinese Medical University] Institutional Review Board.

Corresponding Author:
Jing Zhao
Email: x2xkrz@163.com
(China)