Tropical Biomedicine 34(2): 346–351 (2017)

Evaluation of *frx*A and *rdx*A gene mutations in clinical metronidazole resistance *Helicobacter pylori* isolates

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Received 12 February 2016; received in revised form 19 April 2016; accepted 21 April 2016

Abstract. Metronidazole resistance is an important factor related to failure in the treatment of *Helicobacter pylori*. The mutation in the *rdx*A and *frx*A genes is the most important cause of resistance to metronidazole. Since the resistance rate of metronidazole is high in our region, we decided to assess the frequency of these mutations among *H. pylori* clinical isolates. Antral gastric biopsy specimens were cultured and minimal inhibitory concentrations (MICs) of metronidazole were determined by the E-test method. The *rdx*A and *frx*A genes were amplified in all isolates through the use of PCR with the specific primers. PCR products were purified for sequencing. The resultant sequences were compared with the wild type reference sequences to find any possible mutations. According to our findings, the rate of metronidazole resistance was 77%, with the MICs ranging from 0.25-1 µg/ml for metronidazole-sensitive group and from 16-256 µg/ml for resistance group. *H. pylori* isolates containing a single mutation in *rdx*A or *frx*A genes demonstrated a low MIC (8-16 µg/ml), while those containing mutations in both genes showed a higher MIC (32-256 µg/ml). In this study, all resistant *H. pylori* isolates contained single or multiple nucleotide substitutions in the mentioned genes. Nevertheless, no nucleotide substitutions were found in the sensitive clinical isolates. The results of our study showed that the mutations in *rdx*A are mostly related to metronidazole resistance, and mutations in *frx*A are able to enhance *H. pylori* resistance.

INTRODUCTION

Helicobacter pylori colonizes the stomach of approximately half of the world's population (Hagymasi and Tulassay, 2014). Infection is most often related with asymptomatic gastritis, but it can lead to the development of peptic ulcer disease (PUD), mucosa-associated lymphoid tissue lymphoma and gastric carcinoma (Hagymasi and Tulassay, 2014). Approximately, 16% of *H. pylori* infections develop into peptic ulcer disease (Jenks and Edwards, 2002). Therefore, curing the infection is very important as it heals gastritis, prevents ulcer recurrence and prevents progression into chronic atrophic gastritis, and finally preventing gastric cancer. Presently, the triple therapy regimens comprising two antibiotics include amoxicillin, metronidazole (Mtz) or clarithromycin and a proton pump inhibitor used to eradicate *H. pylori* infection. This treatment protocol can help in the healing of duodenal ulcers and prevents relapse of PUD. However, antibiotic resistance is a major factor contributing to treatment failure. Metronidazole is an important component of the triple therapy regimens utilized to treat infections (O'Connor *et al*., 2013). In recent years, there has been an increase in resistance to Mtz; nevertheless, this agent can be used for the treatment of infections. The

resistance rate is approximately 30% in Western Europe, but it can be as high as 80% in developing countries such as Iran (Milani *et al.*, 2012, Selgrad *et al.*, 2013, Nahaei *et al.*, 2008).

Mtz is regarded as a prodrug whose uptake and activation requires intracellular reduction, causing the production of cytotoxic radicals and other reactive species. In the cell, any redox system containing a reduction potential that is more negative than Mtz would preferentially donate its electrons to Mtz thereby leading to reductive activation. This feature makes Mtz effective against organisms such as *H. pylori* (Sobel & Sobel, 2015). Generally, Mtz resistance in *H. pylori* might be mediated by the electron carriers such as *rdx*A (NADPH nitroreductase), *frx*A (flavin oxidoreductase), *fld*A (flavodoxin), porD (ferredoxin oxidoreductase) and *oor*D (2-oxoglutarate ferredoxin oxidoreductase). Even though the molecular basis of *H. pylori* resistance to Mtz has not been completely characterized as a result of extensive research, it was found that the main causes of Mtz resistance in *H. pylori* are mutations in the *rdx*A or *frx*A genes (Marais *et al.*, 2003). Since there is a high Mtz resistance rate in Iran, (Milani *et al.*, 2012, Farshad *et al.*, 2010), and on the other hand, there are no reports of mutations in gene regulation systems in Mtz resistant isolates from Iran, this study aimed to investigate the *rdx*A and *frx*A gene deletion in Mtz resistant and sensitive *H. pylori* isolates from east Azerbaijan, Iran.

MATERIALS AND METHODS

H. pylori isolates and determination of minimal inhibitory concentrations (MICs)

A total of 170 antral gastric biopsy specimens were cultured on Brucella agar plates containing 5% sheep blood and antibiotics supplement (vancomycin 6 µg/ml, amphotericin B 2.5 µg/ml, and trimethoprim 20 µg/ml) and 75 *H. pylori* isolates were identified by using conventional methods. The plates were incubated under microaerophilic conditions (Anoxomat; Mart, Lichtenvoorde, The Netherlands) at 37°C. For all *H. pylori* strains, the MIC of Mtz was determined using the E-test method (bioMe'rieux). Bacterial suspensions were prepared in normal saline to turbidity of 3.0 McFarland units, and were spread on Mueller-Hinton agar containing 5% sheep blood. Then, E-test stripe was placed on the plates to incubate in microaerophilic conditions at 37°C for 2-3 days. Strains were classified as resistant to Mtz when the MIC was >8 µg/ml (Wikler, 2008).

DNA extraction and PCR assays

Genomic DNA was extracted as previously described (Sambrook and Russell, 2001). The *rdx*A and *frx*A genes were amplified in all isolates using PCR with the specific primers. The primers *rdx*A1 (5- TTAGGGA TTTTATTGTATGCTA -3) and *rdx*A2 (5- TCACAACCAAGTAATTGCATCAA-3) and also *frx*A1 (5- CGAATTGGATATGGCAGCCG-3) and *frx*A2 (5- TATGTGCATATCCCCTG TAGG -3) were used to amplify *rdx*A(686 bp) and *frx*A (913 bp) genes, respectively (Chisholm and Owen, 2003). The cycling program was: initial denaturation for 4 min at 94°C; 35 cycles of 94°C for 1 min, 52°C (*rdx*A) and 60°C (*frx*A) for 35 s, and 72°C for 1 min; and a final elongation for 5 min at 72°C. After PCR amplification, the amplified products were electrophoresed in 2% agarose gels and examined under UV illumination.

DNA Sequence Determination and Analysis

PCR products were purified for sequencing by purification kit (Qiagen, Victoria, Australia). The products were run duplicated on ABI automated sequencers. The resulting sequences were compared with the wild type reference sequences (*H. pylori* 26695 complete genome (using the BLASTX tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM) to find any possible mutations conferring resistance to the Mtz in the isolates.

RESULTS

In total, 75 clinical isolates of *H. pylori* were obtained from culture of 170 gastric biopsy specimens. Among the 75 isolates, 5 (6.7%)

isolates were sensitive and 70 (93.3%) isolates were resistant. The MICs ranged from 0.25-1 µg/ml in the Mtz-sensitive strains and from 16-256 µg/ml in the Mtz-resistant strains.

In order to identify defined *rdx*A and *frx*A mutations associated with Mtz resistance, we analyzed the *rdx*A and *frx*A genes in a collection of clinical *H. pylori* isolates and there was a high Mtz resistance level. Pairwise alignment between the sequences of Mtz resistant and susceptible *H. pylori* isolates indicated significant point mutations in the resistant clinical isolates. Nevertheless, no nucleotide substitutions were found in the Mtz-sensitive clinical isolates, confirming the absence of a gene mutation. Further analysis of the identified sequences was performed in order to elucidate the effect of finding nucleotide substitutions on the protein sequences of *rdx*A and *frx*A genes in the Mtz resistance strains. It's interesting to note that all resistant *H. pylori* isolates contained single or multiple nucleotide substitutions in the *rdx*A gene and/or the *frx*A gene, with resulting amino-acid substitutions. Above all, our study revealed 10 mutations in the *rdx*A gene and 7 mutations in *frx*A gene; the common mutations are shown in Table 1.

DISCUSSION

The current regimen of *H. pylori* treatment includes administration of two or more different types of antibiotics as a combinational therapy (Miftahussurur and Yamaoka, 2015). Although Mtz, as a routine choice, has been widely used in combination with other antibiotics to eradicate *H. pylori* infections, the emergence of Mtz resistance reduces its widespread use; leading to the increased cases of failure in *H. pylori* treatment (Binh *et al.*, 2015). Mtz resistance in *H. pylori* has been shown to be associated with mutations in *rdx*A and *frx*A genes. On the other hand, previous studies have shown that some Mtz-resistant strains harbored neither deletions of the *rdx*A gene nor mutations of both *rdx*A and *frx*A in clinical isolates (Kato *et al.*, 2002, Marais *et al.*, 2003). Such findings imply that the roles of *rdx*A and *frx*A in resistance are geographically variable. In this study, we found several different mutations amongst our isolates. We also indicated that mutation in *rdx*A gene was higher than *frx*A gene in our clinical setting, which is consistent with other studies (Yang *et al.*, 2004, Mirzaei *et al.*, 2016, Savari *et al.*, 2011). Nevertheless, not all Mtz-resistant strains had genomic

Table 1. Common mutations in *frx*A and *rdx*A genes

Isolates	Mutated gene	Nucleotide substitution(s)	Amino-acid changes	MIC (µg/ml)
All isolates	*rdx*A	GAT to AAT and GAC to AAC	Asp to Asn (59)	8-256
59 isolates	*rdx*A	AGA to AAA and AGG to AAG	Arg to Lys (90)	64-256
40 isolates	*rdx*A	CTT to GTT, CTC to GTC, CTA to GTA and CTG to GTG	Leu to Val (210)	32-256
36 isolates	*rdx*A	GCT to ACT, GCC to ACC, GCA to ACA and GCG to ACG	Ala to Thr (118)	128-256
36 isolates	*rdx*A	CAT to TAT and CAC to TAC	His to Tyr (97)	16-128
45 isolates	*frx*A	GAA to AAA and GAG to AAG	Glu to Lys (169)	16-128
30 isolates	*frx*A	GCT to GGT, GCC to GGC, GCA to GGA and GCG to GGG	Ala to Gly (70)	16-64
24 isolates	*frx*A	AAA to GAA and AAG to GAG	Lys to Glu (97)	8-16
24 isolates	*frx*A	GTT to ATT, GTC to ATC and GTA to ATA	Val to Ile (9)	8-16

mutation of *frx*A and interestingly, we found one Mtz-susceptible isolates with the *rdx*A gene mutation. It's interesting to note that we were able to find one Mtz-susceptible isolate with the *rdx*A gene mutation. In addition, there could be some factors other than *frx*A and *rdx*A gene mutations involving the induction of Mtz resistance (Marais *et al.*, 2003, Chisholm and Owen, 2004, Jenks and Edwards, 2002). However, our findings support that the *rdx*A gene is more important than *frx*A in contributing to the existence of high MIC of Mtz resistance in clinical *H. pylori* isolates. These results have been reported in studies from other countries (Kwon *et al.*, 2000a, Bereswill *et al.*, 2003). We also detected two Mtz-resistant strains (with a MIC ≥32 µg/ml) that did not possess *frx*A mutations but had mutations in *rdx*A. This result is in line with the results of a study which demonstrated that inactivation of both *rdx*A and *frx*A genes could result in a moderate to high-level of Mtz resistance (Kwon *et al.*, 2000b). At the protein level of *rdx*A gene, we found different kinds of amino acid substitution such as Aspartic acid to Asparagine (all strains), Arginine to Lysine (10 strains) and Histidine to Threonine (3 strains). Similar results have been reported from studies on other populations (Yang *et al.*, 2004, Han *et al.*, 2007). Results of *frx*A gene sequencing showed different substitutions which most commonly include Glutamic acid to Lysine (8 strains or isolates), Alanine to Valine (3 strains) and Alanine to Threonine (2 strains), respectively. The results were similar to findings of other studies (Han *et al.*, 2007, Jeong *et al.*, 2001). Considering these substitutions, it seems that replacing Alanine with Threonine has more effect on the function of oxygen-insensitive NAD(P)H nitroreductase. This might be as a result of the fact that Alanine is a non-polar amino acid, while the Threonine is polar amino acid. The *H. pylori* isolates which possess this substitution are associated with a high-level of Mtz resistances (with a MIC of 128-256 µg/ml) leading to increased MIC. In conclusion, we suggest that mutations in the *rdx*A gene might contribute more significantly to the resistance of Mtz than *frx*A gene in the clinical *H. pylori* isolates in East Azerbaijan.

Acknowledgements. This project was financially supported by the Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Conflict of interest: The authors declare no conflicts of interest.

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