Analysis of *rdxA* and *frxA* involved in metronidazole resistance of *Helicobacter pylori*

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Directed by Professor Shin, Dong Min

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Analysis of *rdxA* and *frxA* involved in metronidazole resistance of *Helicobacter pylori*

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Metronidazole (MTZ) resistance in *Helicobacter pylori* has been found to be associated with mutations in *rdxA*, a gene encoding an oxygen-insensitive NADPH nitroreductase, and mutations in *frxA*, a gene encoding a NAD(P)H-flavin oxidoreductase. However, MTZ-resistant *H. pylori* associating with mutation of *rdxA* or *frxA* is still controversial. One of limitations in the controversial topic was the lack of the system to confirm the contribution of *rdxA* or *frxA* mutations in MTZ resistance. In this study, a novel approach of the *rdxA* replacement was performed to

distinguish resistance-associated nucleotide mutation from natural genetic diversity of *H. pylori* and hence to evaluate the contribution of *rdxA* or *frxA* mutations in MTZ resistance.

The *rdxA* of MTZ-sensitive wild-type G27 *H. pylori* was replaced with *rdxA* of clinically isolated *H. pylori*. The replacement with the inactivated *rdxA* resulted in MTZ-resistant *H. pylori* while the replacement with the missense-mutated *rdxA* containing several amino acid substitutions failed to transfer MTZ-resistant phenotype. It suggested that nonfunctional truncated RdxAs in 2 MTZ-resistant *H. pylori* isolates can induce MTZ resistance but none of RdxA with amino acid substitutions in 3 MTZ-resistant isolates was unable to cause the MTZ resistance. It is of importance that a nonsense-mutated *frxA* was found in the MTZ-sensitive *H. pylori*, indicating that the deletion of *frxA* alone is not sufficient for MTZ resistance. However, the deletion of *frxA* seemed to enhance MTZ resistance (MIC, 64 to $\geq$256 μg/ml) in the presence of nonfunctional truncated RdxA. In 2 isolates among 5 MTZ-resistant *H. pylori*, *rdxA* and *frxA* are likely functional and not responsible for the MTZ-resistant phenotype. Therefore, the MTZ resistance in *H. pylori* may arise without mutations in *rdxA* and *frxA*, clearly suggesting that other genes may be involved in MTZ resistance.

Key words : Helicobacter pylori, Metronidazole resistance, rdxA, frxA

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Ⅰ. Introduction

Since *Helicobacter pylori* was discovered from human gastric biopsies in 1982[1], it has been recognized as a contributing factor to the development of a number of gastric disorders. These include gastritis, ulcer disease and two distinct forms of gastric cancer – gastric adenocarcinoma and B-cell-mucosa-associated lymphoma[1, 2, 3, 4]. *H. pylori* is a spiral gram-negative microaerophilic bacterium[5] that chronically infects human gastric epithelial cell surfaces characterized by very acidic pH and the overlying

gastric mucin, a niche that most microbes can not survive. *H. pylori* is so well adapted to this unfriendly environment that over 50% of the world population is infected with *H. pylori* and, therefore, the infection is one of the most common infections worldwide. Metronidazole (MTZ), a synthetic nitroimidazole, was a critical ingredient of the first successful therapy for *H. pylori* and remains a major component of multidrug therapies containing a proton pump inhibitor and combination of two or more antibiotics (MTZ, clarithromycin, amoxicillin or tetracycline)[6,7]. The primary obstructions to successful *H. pylori* treatment are the presence of antibiotic resistant *H. pylori*. Resistance to MTZ is common among clinically isolated *H. pylori*, with frequencies ranging from 10 to over 90%, depending on geographic region and patient group[8, 9, 10]. This is important clinically because MTZ resistance decreases the effectiveness of popular and affordable MTZ-containing anti-*H. pylori* therapies[11, 12].

This study focused on mechanisms of susceptibility and resistance of *H. pylori* to MTZ. MTZ is also used against a wide variety of prokaryotic and eukaryotic pathogens including various anaerobic and parasitic infections[13, 14, 15]. Antimicrobial action and resistance to MTZ has been investigated using anaerobic microorganisms[13, 16, 17]. The cytotoxicity of

MTZ is due to the unstable intermediates that damage DNA, resulting in strand breakage, helix destabilization, unwinding, and cell death[18, 19]. The antimicrobial action of MTZ is dependent on reductive activation of MTZ by the redox system of the target cell. MTZ resistance has been found to be associated with mutations in *rdxA*, a chromosomal gene encoding an oxygen-insensitive NADPH nitroreductase, and mutations in *frxA*, a chromosomal gene encoding a NAD(P)H-flavin oxidoreductase. However, the association of *H. pylori* MTZ resistance with mutation of *rdxA* or *frxA* is still a debated topic. One of limitations in the research is the lack of the system to confirm the contribution of *rdxA* or *frxA* mutations in MTZ resistance. In this study, both genes, *rdxA* and *frxA*, from clinically isolated *H. pylori* were analyzed. The *rdxA* in MTZ-sensitive wild-type *H. pylori* was replaced with *rdxA* of clinical *H. pylori* isolates and MTZ MICs of *rdxA*-replaced transformants were determined to evaluate the contribution of *rdxA* mutations in the *H. pylori* isolates to MTZ resistance.

Ⅱ. Materials and Methods

1. *H. pylori* strains and culture conditions

Five MTZ-sensitive (S1 to S5) and 5 MTZ-resistant (R1 to R5) clinical *H. pylori* isolates were obtained from patients (Division of Gastroenterology, Department of Internal Medicine, College of Medicine, Catholic University, Seoul, Korea). G27 *H. pylori*[20] was obtained from Douglas Scott Merrell, Uniformed Services University of the Health Science, Bethesda, MD. These strains were cultured on columbia blood agar plates (Difco, Detroit, Mich.) containing 5% horse blood (Oxoid, UK) under 10% CO_2 at 37°C for 3 days.

2. Determination of Minimum Inhibitory Concentration (MIC)

The MIC was defined as the lowest concentration of MTZ that completely inhibited the growth of the inoculum. MICs were determined by the *E*-test method (AB Biodisk, Sweden) and agar dilution method [21]. The *E*-test was performed on columbia blood agar plates containing 5% horse blood according to the manufacturer's instructions. The agar dilution method was performed on columbia blood agar plates containing 5% horse blood as described previously[21]. Frozen bacterial stocks were

streaked on columbia blood agar and incubated for 3 days. Cells from one or a few colonies from these initial plates were then restreaked on fresh columbia blood agar plate and incubated for one more day. The resulting exponentially growing cells were suspended in phosphate-buffed saline (PBS) buffer; a series of 10-fold dilutions of these cell suspensions was prepared, and 10 μl of each dilution was spotted on freshly prepared columbia blood agar containing various concentrations (0, 0.2, 0.5, 1.5, 3, 5, 8, 16, 32, 64, or 256 μg/ml) of MTZ (Sigma Chemical Co., St. Louis, Mo.). The plates were incubated for 3 days. MTZ-resistant *H. pylori* ATCC 43504 was used as a control organism. The MIC of MTZ resistance was at ≥ 8 μg/ml.

3. Cloning of *rdxA* and *frxA genes* of clinical *H. pylori* isolates

Chromosomal DNA of clinical *H. pylori* isolates and G27 *H. pylori* was extracted from confluent cultures grown on columbia blood agar by using a wizard genomic DNA purification kit (Promega, USA). PCR amplifications for the *rdxA* and *frxA* were carried out in an DNA thermal cycler (Biometra, Göttingen, Germany) as followed: 1 cycle at 95℃ for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final elongation step at 72°C for 10 min. The oligonucleotide PCR

primers to amplify the *rdxA* gene were a forward primer (5'-aatttgagcatggggcaga-3') and a reverse primer (5'-gaaacgcttgaaaacacccct-3'). The expected PCR product was a 850 bp. A forward primer (5'-ccatcgatatggacagagaacaagtgg-3') and a reverse primer (5'-gctaacagcgtttttattcaatc-3') were employed to amplify 670 bp *frxA* gene. The amplified PCR fragments were extracted from 1% agarose gels using Qiaquick gel extraction kit (Qiagen, Hilden, Germany), ligated into the pGEM-T-easy vector (Promega, Madison, WIs) and transformed into for *Escherichia coli* DH5α, generating plasmid pRDXA-G27, -S1 to S5 and -R1 to R5, and pFRXA-G27, -S1 to S5 and -R1 to R5.

4. Nucleotide sequence analysis of *rdxA* and *frxA* of G27 and clinical *H. pylori* isolates

DNA sequences of *rdxA* and *frxA* of G27 and clinical isolates were determined for both strands of the inserts of pRDXA and pFRXA series by the Cosmogene Tec. (Cosmo, Korea) using T7 primer and SP6 primer. The resulting DNA sequences were analyzed by the vector NTI v9.1 (Invitrogen, USA) and Sequencher v4.5 (Gene code, USA) Programs.

5. Generation of *rdxA* constructs for natural transformation

A kanamycin (Km) resistance cassette (aphA-3) [22] was PCR amplified from pILL600 with a forwad primer (5'-ccaagcttggcgtatcacgaggccctttcg-3') and a reverse primer (5'-ccaagcttctaaaacaattcatccag-3'), containing *Hin*dIII restriction enzyme site. The unique *Hin*dIII restriction enzyme site in the *rdxA* fragment in pRDXA-S1 to S5 and -R1 to R5 was used to insert the Km resistance cassette. To avoid the leaky expression of *rdxA* due to the influence from the expression of Km resistance cassette, Km resistance cassette was subcloned in the reverse direction to *rdxA* gene expression. The direction was confirmed by sequencing pRDXA series with the *rdxA* forward primer. The resulting plasmids carrying an Km resistance cassette were named as pRDXA (Km) -S1 to S5 and -R1 to R5, and used for *rdxA* replacement experiment.

6. The *rdxA* replacement by natural transformation

G27 *H. pylori* strain was used for natural transformation to replace G27 *rdxA* with *rdxA* of clinical isolates. The natural transformation was performed as described previously[23] with the following modification. G27 strain was streaked, and incubated for 3 days. G27 from one or a few colonies from the initial plate was then restreaked on fresh columbia blood agar plate and incubated for 2 days. After 24 h, G27 was inoculated

as a circle form on fresh columbia blood agar plate with four 1-inch scrapes of the G27. Six h later, 2 μg DNA of pRDXA (Km) series was added on the G27 circle form. After 20 h, the whole circle was restreaked onto columbia blood agar containing kanamycin (10 μg/ml) and incubated for 3-5 days until colonies of transformants appeared.

7. PCR screening of transformants by homologous recombination

The integration of pRDXA (Km) series in the *rdxA* of G27 *H. pylori* chromosomal DNA by single cross-over homologous recombination was selected using Km resistance (Fig. 2). The *rdxA* replacement of Km resistance transformant *H. pylori* was confirmed by PCR screening with the *rdxA* primers as described above. If pRDXA (Km) series were integrated successfully in wild-type *rdxA*, two bands of 850 bp (*rdxA* gene) and 2275 bp (*rdxA* gene containing Km resistance cassette) should be observed. The 850 bp band (*rdxA* gene expressing by endogenous *rdxA* promoter) was extracted, and sequenced as described above.

Ⅲ. Results

1. Identification of MTZ-sensitive or -resistant clinical *H. pylori* isolates

To identify Metronidazole (MTZ)-sensitive or -resistant *H. pylori* strains, the MTZ MICs for G27, ATCC 43504 and clinically isolated *H. pylori* were tested by *E*-test and agar dilution method (Table 1). G27 was sensitive to MTZ (MIC, 0.75 μg/ml) while ATCC 43504 was resistant (MIC, 256 μg/ml). Five isolates (S1 to S5) sensitive to MTZ (MIC, 0.38, 0.25, 0.13, 0.19, and 0.25 μg/ml, respectively) and 5 isolates (R1 to R5) resistant to MTZ (MIC, 64, 128, and R3-5 ≥256 μg/ml, respectively) were selected (Table 1), and analyzed further.

2. Amino acid sequences of RdxA and FrxA from MTZ-sensitive and -resistant *H. pylori* isolates

To find putative resistance-associated mutations for *rdxA* and *frxA*, both genes of G27, ATCC 43504, and 10 *H. pylori* isolates were sequenced. The DNA sequences of *rdxA* and *frxA* for ATCC 43504 were identical to the sequence reported previously[24, 25]. The amino acid sequence deduced from the DNA sequences of ATCC 43504 *rdxA* and *frxA* indicated that

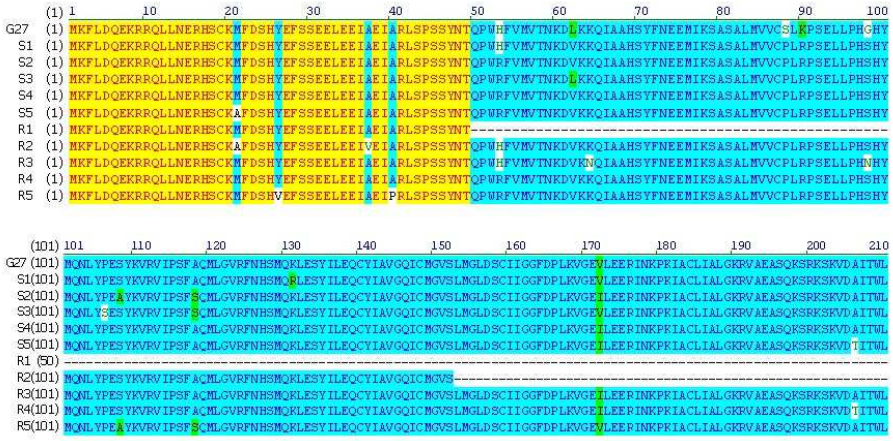

Figure 1. Comparison sequences of RdxA amino acid from 10 MTZ-sensitive and MTZ-resistant *H. pylori* isolates, including MTZ-sensitive *H. pylori* G27 (MIC, 0.75 μg/ml). The *rdxA* encodes a protein of 210 amino acid. S1-5 are MTZ-sensitive *H. pylori* isolates and R1-5 are MTZ-resistant *H. pylori* isolates. All of 6 MTZ-sensitive *H. pylori* strains contained full-length RdxA containing amino acid substitutions. R1 and R2 of 5 MTZ-resistant *H. pylori* isolates contained truncated RdxAs. The amino acid sequence of R3, R4, and R5 showed full-length RdxA containing amino acid substitutions.

ATCC 43504 produced nonfunctional truncated RdxA and FrxA[24, 25].

The RdxA amino acid sequences of G27 and 10 *H. pylori* isolates were compared to minimize the influence of the natural genetic variability on the identification of resistance-associated nucleotide transitions (Fig. 1 and Table 1). All of 6 MTZ-sensitive *H. pylori* strains contained full-length RdxA containing 5 to 8 amino acid substitutions by missense mutations. Two (R1 and R2) of 5 MTZ-resistant *H. pylori* isolates contained truncated RdxAs because of a nonsense mutation at 50 amino acid residue and frame-shift causing termination at 153 residue, respectively. The amino acid sequence of 3 MTZ-resistant isolates (R3 to R5) showed full-length RdxA containing 6 to 9 amino acid substitutions by missense mutations. By the comparison of amino acid sequence between MTZ-sensitive and -resistant *H. pylori*, substitutions of asparagine at 64 residue and asparagine at 98 residue in R3 strain, and substitutions of valine at 26 residue and proline at 40 residue in R5 might be important for the MTZ resistance. The amino acid sequence of R4 was almost identical to that of S5 except a neutral mutation (Met to Ala) at 21 residue, suggesting that the R4 RdxA is functional like S5 RdxA. Surprisingly, it was revealed by the FrxA amino acid sequence that MTZ-sensitive G27 *H. pylori* contained the truncated FrxA amino acid by a

Table 1. The changes of amino acid of RdxA in MTZ-sensitive and -resistant *H. pylori* isolates

strain	MIC (μg/mℓ)	Amino acid position and change of RdxA																	
		21	26	37	40	50	52	62	64	88	90	98	106	108	117	131	153	172	206
G27	0.75	M	Y	A	A	Q	H	L	K	S	K	G	P	S	A	K	L	V	A
S1	0.38	·[a]	·	·	·	·	·	V[b]	·	P	R	S	·	·	·	R	·	·	·
S2	0.25	·	·	·	·	·	R	V	·	P	R	S	·	A	S	·	·	I	·
S3	0.13	·	·	·	·	·	R	·	·	P	R	S	S	·	S	·	·	·	·
S4	0.19	·	·	·	·	·	R	V	·	P	R	S	·	·	·	·	·	I	·
S5	0.25	A	·	·	·	·	R	V	·	P	R	S	·	·	·	·	·	I	T
R1	64	·	·	·	·	~[c]	~	~	~	~	~	~	~	~	~	~	~	~	~
R2	128	A	·	V	·	·	·	V	·	P	R	S	·	·	·	·	~[d]	~	~
R3	256	·	·	·	·	·	·	V	N	P	R	N	·	·	·	·	·	I	·
R4	256	·	·	·	·	·	R	V	·	P	R	S	·	·	·	·	·	I	T
R5	256	·	V	·	P	·	R	V	·	P	R	S	·	A	S	·	·	·	·

[a]The amino acid is the same as that of G27 strain.

[b]The missense mutation resulted in the amino acid substitution.

[c]The nonsense mutation resulted in the stop codon at 50 residue.

[d]The frame-shift mutation resulted in the stop codon at 153 residue.

frame-shift causing termination at 21 residue. It strongly suggested that deletion of *frxA* alone can not cause MTZ resistance.

Because of the truncation of G27 FrxA, FrxA amino acid sequence of ATCC26695 as wild-type was applied to compare with those of 10 *H. pylori* isolates (Table 2). Five MTZ-sensitive *H. pylori* strains had full-length FrxA containing 6 to 9 amino acid substitutions by missense mutations. Among 5 MTZ-resistant *H. pylori* isolates, R4 contained truncated FrxA because of a nonsense mutation at 68 residue. The amino acid sequences of 4 MTZ-resistant isolates (R1, R2, R3 and R5) showed full-length FrxA containing 3 to 7 amino acid substitutions by missense mutations.

3. Replacement with *rdxA* of clinically isolated *H. pylori*

To evaluate the contribution of truncated RdxA and substituted RdxA for MTZ resistance, the *rdxA* in MTZ-sensitive wild-type *H. pylori* was replaced with *rdxA* of clinical isolates (Fig. 2). G27 was chosen for the recipient of the *rdxA* replacement because G27 was MTZ-sensitive (MIC, 0.75 μg/ml), was capable for the natural transformation, and G27 contained truncated FrxA so that it can enhance more MTZ resistance

Table 2. The changes of amino acid of FrxA in MTZ-sensitive and -resistant *H. pylori* isolates

strain	MIC (μg/mℓ)	Amino acid position and change of FrxA																									
		2	16	18	19	20	21	32	37	38	39	43	44	68	72	73	81	103	111	117	124	149	152	155	176	193	208
26695	0.75	D	A	K	Y	D	P	A	G	R	L	S	I	W	F	G	V	V	N	I	N	M	A	M	E	C	K
G27	0.75	[a]	·	[b]	R	S	[c]	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃
S1	0.38	·	T	·	·	·	·	V	E	·	·	·	V	·	·	·	I	·	·	·	S	·	·	T	·	S	N
S2	0.25	·	T	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·	M	S	·	·	T	·	S	N
S3	0.13	V	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	M	S	·	·	·	K	S	·
S4	0.19	V	T	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	K	S	·
S5	0.25	V	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	S	K	·	·	K	S	·
R1	64	·	T	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	·	M	S	·	·	·	K	S	·
R2	128	V	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	S	·	V	·	K	S	·
R3	256	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	S	·	·	·	·	·	·
R4	256	V	T	·	·	·	·	·	·	·	·	·	·	[d]	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃
R5	256	V	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	M	S	·	·	·	K	S	·

[a]The amino acid is the same as that of ATCC26695 strain.

[b]The missense mutation resulted in the amino acid substitution.

[c]The frame-shift mutation resulted in the stop codon at 21 residue.

[d]The nonsense mutation resulted in the stop codon at 68 residue.

with the replaced *rdxA* mutations than that with the *rdxA* mutations alone. Among 10 *H. pylori* isolates, *rdxA*s of S4, S5 and R1 to R5 were introduced into G27 by natural transformation with pRDXA (km)-S4, S5, and R1 to R5 (Fig 2). S4 and S5 *rdxA*s were chosen because of the most substituted amino acid residues (6 and 8 substitution, respectively) among MTZ-sensitive strains. Plasmids of pRDXA (km) series could not replicate in *H. pylori* and thus to transform Km resistance phenotype the plasmid should be integrated in the *rdxA* of *H. pylori* chromosomal DNA by single cross-over homologous recombination (Fig. 2).

In consequence, two structural *rdxA* genes found to be located in transformants but only one of the genes could be expressed by endogeneous *rdxA* promoter and the other one should be silent. After the natural transformation, *rdxA* replacement of Km resistance transformant *H. pylori* was confirmed by PCR screening with the *rdxA* primer set, which should show two bands of 850 bp (*rdxA* gene, which can be expressed with *rdxA* endogenous promoter) and 2275 bp (*rdxA* containing Km resistance cassette, which can not be expressed because of the lack of a promoter) (Fig. 2 and Fig. 3). To avoid the leaky expression of *rdxA* containing Km resistance cassette due to the influence from the expression of Km resistance cassette, Km resistance cassette was

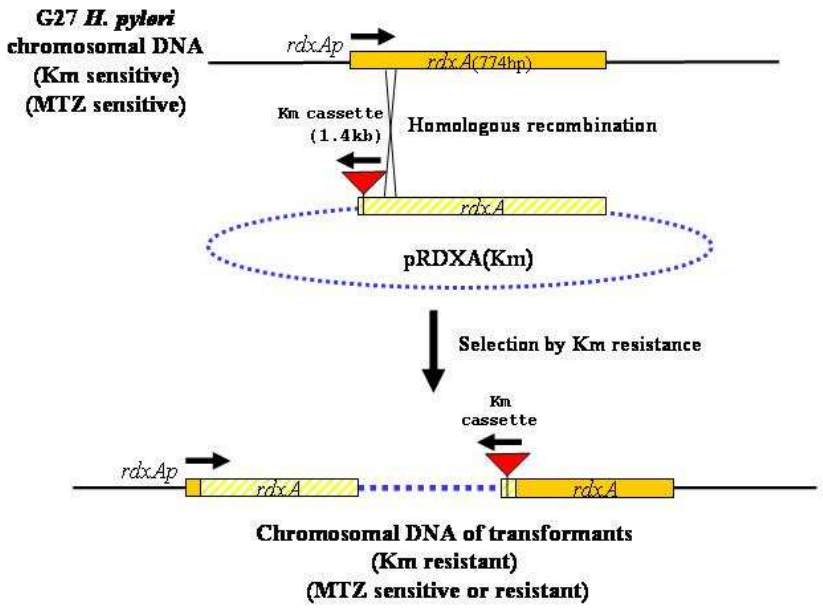

Figure 2. Schematic representation of the integration of pRDXA (Km) in the *rdxA* of G27 the chromosomal DNA by single cross-over homologous recombination. The *rdxA* in MTZ-sensitive G27 *H. pylori* was replaced with *rdxA* of clinical *H. pylori* isolates. Two structural *rdxA* genes are located in transformants but only one of the genes can be expressed by endogeneous *rdxA* promoter and the other one is silent.

The endogeneous *rdxA* promoter is indicated by *rdxAp*. The arrows indicate the direction of transcription.

subcloned in the reverse direction to *rdxA* gene expression. The expressing *rdxA* gene (small PCR fragment) was sequenced to confirm the single cross-over site and the complete replacement with the isolates *rdxA* (data not shown). To confirm the single cross-over of homologous recombination, large PCR fragment in R1 was also sequenced and the sequence showed that the single cross-over site was the same as that from sequence of small PCR fragment (data not shown). In all natural transformations, we could find transformants in which the single cross over occurred within 150 bp downstream from ATG initiation codon. Therefore, in the consequence clinical isolates *rdxA* completely replaced G27 *rdxA*. At least 3 transformants from each complete replacement were obtained and analyzed further.

4. MIC determination of *rdxA*-replaced *H. pylori* transformants

The MTZ MICs of the *rdxA*-replaced *H. pylori* transformants were determined by *E*-test and agar dilution method as shown in Table 3. In case of replacement with *rdxA* genes of MTZ-sensitive strains (S4 and S5), *H. pylori* transformants remained sensitive to MTZ (MIC, 0.75 and 0.35 μg/ml, respectively), as expected since RdxAs of MTZ-sensitive strains should be functional. The MICs were similar to that of the

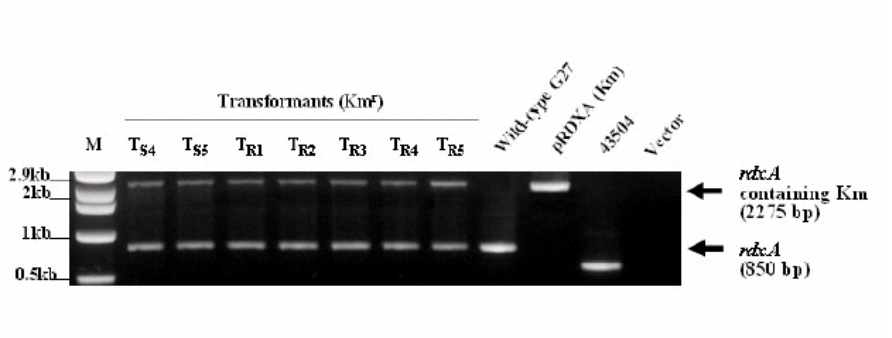

Figure 3. PCR screening for the *rdxA* replacement of transformants.

The *rdxA* replacement of Km-resistant *H. pylori* transformant was confirmed by PCR screening with *rdxA* primer set. Transformants showed two bands of 850 bp (*rdxA* gene) and 2275 bp (*rdxA* containing Km resistance cassette). MTZ-resistant ATCC43504 was 481 bp PCR fragment. The cloning vector (pGEM-T-easy vector) was used as a negative control.

recipient strain, G27 (MIC, 0.75 μg/ml). In case of replacement with *rdxA* genes from MTZ-resistant strains (R1 to R5), the results of MICs were diverse. *H. pylori* transformants by the replacement of R1 and R2 *rdxA*s became highly resistant to MTZ (MIC ≥ 256 μg/ml). The results indicated that truncated and hence nonfunctional RdxA caused MTZ resistance in *H. pylori*. Interestingly, the MICs were even higher than those of original clinical isolated *H. pylori* (R1 MIC, 64 μg/ml and R2 MIC, 128 μg/ml). In case of replacements with *rdxA* genes from MTZ-resistant strains (R3, R4, and R5), *H. pylori* transformants remained sensitive to MTZ (MIC, 0.25-0.75, 0.25-3, 0.25-3 μg/ml, respectively). Since R4 was almost identical to S5, the MTZ-sensitive MIC for R4 transformant was expected. Interesting result was MTZ sensitivity of R3 and R5 transformants. We predicted that asparagine substitution at 64 residue and asparagine at 98 residue in R3 strain, and valine at 26 residue and proline at 40 residue in R5 might be important for the MTZ resistance by the comparison of RdxA amino acid sequence between MTZ-sensitive and -resistant *H. pylori* (Table 1). However, the *rdxA* replacement showed that these changed amino acids were not significant substitutions for MTZ resistance and hence reductase function.

Table 3. MTZ MICs of transformants

The *rdxA* replacement of MTZ-sensitive G27 (MIC, 0.75 μg/ml) with *rdxA*s of clinical isolates resulted in following MTZ MICs.

Clinical isolates (MICs)	Transformants (MICs)
S4 (0.19 μg/ml)	T_{S4} (0.25-0.75 μg/ml)
S5 (0.25 μg/ml)	T_{S5} (0.25-0.35 μg/ml)
R1 (64 μg/ml)	T_{R1} (≥ 256 μg/ml)
R2 (128 μg/ml)	T_{R2} (≥ 256 μg/ml)
R3 (≥ 256 μg/ml)	T_{R3} (0.25-0.75 μg/ml)
R4 (≥ 256 μg/ml)	T_{R4} (0.25-3 μg/ml)
R5 (≥ 256 μg/ml)	T_{R5} (0.25-3 μg/ml)

Ⅳ. Discussion

This study focused on mechanisms of susceptibility and resistance of *H. pylori* to antimicrobial agent metronidazole (MTZ) and hence examined the contribution of *rdxA* and *frxA* genes to MTZ resistance. Five MTZ-sensitive and 5 MTZ-resistant *H. pylori* strains were obtained from clinical isolates. By the analysis of RdxA and FrxA amino acid sequences of isolates, various mutations in the genes were observed including premature truncations and substituted mutations. Variations in the amino acid sequence can be caused by the natural genetic diversity of *H. pylori*, which is in case of *rdxA* with 5-8%[26, 27]. Changes in the amino acid sequence in MTZ-sensitive G27 and 5 MTZ-sensitive isolates may not be important in terms of MTZ resistance and hence nitroreductase function. The replacements of MTZ-sensitive wild-type G27 *rdxA* with clinically isolated *H. pylori rdxA*s were performed to evaluate contributions of *rdxA* mutations. Our experiments showed that (i) premature truncation of *frxA* alone was not sufficient to cause MTZ resistance in *H. pylori*; but was capable to enhance MTZ resistance in *H. pylori* with a deficient *rdxA* (ii) the replacement of the inactivated *rdxA* (nonfunctional premature truncation) was sufficient enough to transform G27 *H. pylori* (normally

MTZ-sensitive) into MTZ resistance but the replacement with missense-mutated *rdxA* (6-9 amino acid substitutions) did not cause MTZ resistance; (iii) two of high MTZ-resistant isolates (MIC ≥256 μg/ml) seemed to have functional *rdxA* and *frxA*, suggesting that a MTZ-resistance phenotype may arise by other redox system in *H. pylori* without mutations in *rdxA* or *frxA*.

MTZ-resistant *H. pylori* associating with mutation of *frxA* is still one of the most controversial topics in MTZ resistance. Kwon et al. reported that *frxA* inactivation resulted in MICs (32 or 128 μg/ml) similar to those seen with *rdxA* inactivation[28] while the study of Jeong et al. showed that *frxA* inactivation enhanced MTZ resistance in *rdxA*-deficient *H. pylori* but had little effect on the MTZ susceptibility of $rdxA^+$ cells[29]. Recently, Yang et al. found the truncation of FrxA in MTZ-sensitive *H. pylori*[30]. Our result agreed with studies of Jeong and Yang groups. MTZ-sensitive G27 (MIC, 0.75 μg/ml) has the truncated FrxA by frame-shift causing termination at 21 residue. Since full length of FrxA is 217 amino acids, the truncated FrxA at 21 residue must be nonfunctional. Therefore, it suggested that the functional inactivation of FrxA alone can not induce MTZ resistance. In addition, the comparison of FrxA amino acid sequenece (Table 2) showed that no putative missense mutations were

found to be responsible for MTZ resistance since no unique missense mutation in MTZ-resistant strain as identified except a neutral mutation (Ala to Val) at 152 residue. It is worth to note that the replacement of G27 *rdxA* with the nonsense-mutated *rdxA* (premature truncation) from R1 (MIC, 64 μg/ml) and frame-shift mutated *rdxA* (premature truncation) from R2 (MIC, 128 μg/ml) resulted in higher MTZ resistance (MIC, ≥256 μg/ml) than MICs of original isolates. This result is likely due to double functional inactivations of RdxA and FrxA in the transformants because G27 contained nonfunctional FrxA while R1 and R2 isolates contained full-length and presumably functional FrxA (Table 2), even though this comparison is between *H. pylori* strains with different genetic background. In addition, ATCC 43504 contained nonfunctional RdxA and FrxA and showed high MTZ resistant (MIC, ≥256 μg/ml). It suggested that FrxA inactivation enhanced MTZ resistance in *rdxA*-deficient *H. pylori*.

Novel approach of the *rdxA* replacement was performed to evaluate *rdxA* mutation for MTZ resistance. Previously, the presumably important mutations of *rdxA* for MTZ resistance were identified by comparison with RdxA sequence between MTZ-sensitive and -resistant *H. pylori* strains. However, these mutations could not be evaluated exactly for the

contribution to MTZ resistance. In this study it was predicted by the comparison of RdxA sequences that the changes of Lys64→Asn and Gly98→Asn in R3 strain, and Tyr26→Val and Ala40→Pro in R5 strain may be responsible for MTZ resistance. However, the *rdxA* replacement proved that these substitutions are not critical change in MTZ resistance, suggesting the changes are probably in natural genetic diversity but not associated with MTZ-resistant phenotype. Recent studies revealed the RdxA amino acid changes of Arg10→Lys, Arg16→His, Met21→Ala, His53→Arg, Met56→Ile, Leu62→Val, Ala68→Val, Gly98→Ser, Gly163→Asp, and Ala206→Thr in MTZ resistant strains[31, 32, 33], suggesting that those substitutions are important in MTZ resistance. However, in this study the replacement with the missense-mutated *rdxA* containing amino acid substitutions (Met21→Ala, Leu62→Val, Gly98→Ser, and Ala206→Thr) failed to transfer MTZ-resistant phenotype, suggesting that the substitution are not important MTZ-resistance. In addition, only Paul et al. confirmed experimentally that RdxA amino acid substitutions (Cys19→Tyr and Thr49→Lys) were causative for MTZ resistance[33]. MTZ-sensitive strains were transformed with PCR products of MTZ-resistant *rdxA* and selected with MTZ. MTZ-resistant transformants were analyzed for their *rdxA*s. However, it is

possible that the selective pressure of MTZ forced other mutations to be MTZ-resistant. The PCR-transformed strains were not screened for putative resistance-associated mutations in other genes. In this study, to prevent MTZ-selective pressure, the transformants were selected with Km resistance and MTZ MICs were measured. Therefore, it will be interesting to confirm those substitutions in RdxA for the significance using the *rdxA* replacement. Two clinical isolated *H. pylori* strain (R3 and R5) out to the 5 MTZ-resistant showed highly resistant to MTZ (MIC ≥256 μg/ml) even though they have functional *rdxA* and presumably functional *frxA*. This result is in agreement with Marais et al. who suggested that a MTZ resistance phenotype may arise in *H. pylori* without mutation in *rdxA* or *frxA*[34]. It must be kept in mind that not only *rdxA* mutations, but also the regulation of gene expression may play a role in the resistance. The replaced *rdxA*s of all transformants are expressed by the same G27 endogeneous *rdxA* promoter to minimize the problem of the different gene regulation and also all transformants should have the same genetic background except *rdxA*. Several groups[28, 35, 36, 37, 38] and genome sequence annotation[1, 39, 40, 41] suggested putative other redox system for the MTZ resistance including *fdxB* (encoding a ferredoxin-like protein), *fdxA* (ferredoxin), *fldA* (flavodoxin), *oorD* (the

ɣ-subunit of 2-oxoglutarate oxidoreductase, and *porD* (the ɣ-subunit of pyruvate ferredoxin oxidoreductase). However, the research to identify other redox system except *fdxB* has been hampered because the deletion of the genes seems to be lethal for *H. pylori*[28, 29]. In summary, the finding in this study clarified some of the debating questions that the deletion of *frxA* alone can not induce MTZ resistance but enhance the resistance in a *rdxA*-deficient *H. pylori*. The novel approach of *rdxA* replacement showed that the deletion of *rdxA* induce MTZ resistance, and suggested that the substituted mutations identified previously by the sequence comparison need to be confirmed for the contribution to MTZ resistance.

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<국문요약>

*Helicobacter pylori*의 메트로니다졸 내성과 관련된 *rdxA*와 *frxA* 유전자 분석.

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*Helicobacter pylori*의 메트로니다졸 항생제에 대한 내성은 oxygen-insensitive NADPH nitroreductase를 암호화하는 *rdxA* 그리고, NAD(P)H-flavin oxidoreductase를 암호화하는 *frxA* 유전자들과 관련되어 있다고 알려져왔다. 그러나, *rdxA* 또는 *frxA* 유전자가 메트로니다졸 내성에 관련이 있는지를 확인 할 수 있는 연구방법의 한계 때문에, *rdxA* 또는 *frxA* 유전자 돌연변이와 *H. pylori*의 메트로니다졸 내성과의 관련성은 여전히 논쟁의 여지가 있다.

본 연구에서는 메트로니다졸 내성과 관련된 유전자 돌연변이와 *H. pylori*의 자연적인 유전자 다양성과의 차이점을 구별하고 분석하기 위해, 메트로니다졸에 민감한 G27 *H. pylori*의 *rdxA* 유전

자를 *H. pylori* 임상분리균의 *rdxA* 유전자로 대체하는 새로운 방법을 이용해서 *rdxA* 또는 *frxA* 유전자들이 메트로니다졸 내성에 관여하는지 평가하였다.

G27 *H. pylori*의 *rdxA* 유전자를 메트로니다졸 내성 임상분리균의 넌센스 돌연변이가 일어난 *rdxA* 유전자로 대체한 변형균은 메트로니다졸 내성을 얻었다. 그러나, 메트로니다졸 내성 임상분리균의 미스센스 돌연변이로 몇 개의 아미노산이 치환되어 있는 *rdxA* 유전자로 대체한, 변형균은 메트로니다졸 내성을 얻지 못했다. 이 결과를 볼 때, G27 변형균의 메트로니다졸 내성을 유도한 임상분리균의 넌센스 RdxA는 기능을 하지 않음을 알 수 있고, 메트로니다졸 내성을 유도하지 않은 미스센스 RdxA는 메트로니다졸 내성에 중요한 아미노산 치환을 가지고 있지 않아 기능에 영향이 없는 것을 의미한다.

그리고, 메트로니다졸에 민감한 *H. pylori*에서 전체 *rdxA* 유전자 서열을 가지고 있지만, *frxA* 유전자는 넌센스 돌연변이로 인하여 FrxA 아미노산 서열 중간이 종결된 것을 확인 할 수 있었다. 이는 *frxA* 유전자 불활성만으로는 메트로니다졸 내성을 나타내기에 충분하지 않다는 것을 의미한다. 하지만, *frxA* 불활성이 *rdxA*의 불활성과 같이 일어났을 경우에는 메트로니다졸 내성을 높여주었다 (MIC, 64 to $\geq$ 256 ㎍/㎖).

G27 *H. pylori*의 *rdxA* 유전자를 5종류 메트로니다졸 내성을 나타내는 *H. pylori* 임상분리균의 *rdxA* 유전자로 대체한 결과, 이 중 2종류 *H. pylori*의 *rdxA* 와 *frxA* 유전자들은 메트로니다졸 내성에 관련이 없는 것으로 밝혀졌다. 그러므로, *H. pylori*의 메트로니다졸 내성은 *rdxA* 와 *frxA* 유전자의 돌연변이 없이도 일어날 수 있기에 다른 유전자들이 메트로니다졸 내성에 관여함을 암시한다.

핵심되는 말 : Helicobacter pylori, 메트로니다졸 내성, rdxA 유전자, frxA 유전자