ORIGINAL ARTICLE

Peptide Nucleic Acid Probe-Based Analysis as a New Detection Method for Clarithromycin Resistance in *Helicobacter pylori*

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Background/Aims: *Helicobacter pylori* eradication rates are decreasing because of increases in clarithromycin resistance. Thus, finding an easy and accurate method of detecting clarithromycin resistance is important. **Methods:** We evaluated 70 *H. pylori* isolates from Korean patients. Dual-labeled peptide nucleic acid (PNA) probes were designed to detect resistance associated with point mutations in 23S ribosomal ribonucleic acid gene domain V (A2142G, A2143G, and T2182C). Data were analyzed by probe-based fluorescence melting curve analysis based on probe-target dissociation temperatures and compared with Sanger sequencing. **Results:** Among 70 *H. pylori* isolates, 0, 16, and 58 isolates contained A2142G, A2143G, and T2182C mutations, respectively. PNA probe-based analysis exhibited 100.0% positive predictive values for A2142G and A2143G and a 98.3% positive predictive value for T2182C. PNA probe-based analysis results correlated with 98.6% of Sanger sequencing results (κ-value=0.990; standard error, 0.010). **Conclusions:** *H. pylori* clarithromycin resistance can be easily and accurately assessed by dual-labeled PNA probe-based melting curve analysis if probes are used based on the appropriate resistance-related mutations. This method is fast, simple, accurate, and adaptable for clinical samples. It may help clinicians choose a precise eradication regimen. **(Gut Liver 2018;12:641-647)**

Key Words: *Helicobacter pylori*; Clarithromycin; Melting array; Peptide nucleic acids

INTRODUCTION

Helicobacter pylori infection is involved in the pathogenesis of chronic gastritis, peptic ulcer disease, and mucosa-associated lymphoid tissue lymphoma and is a major risk factor for the development of gastric cancer.[1] The rate of *H. pylori* infection is higher in Korea than in other countries.[2] Traditionally, triple therapy including a proton pump inhibitor, amoxicillin, and clarithromycin has been recommended as the primary regimen for *H. pylori* eradication.[3,4] However, clarithromycin resistance is prevalent and exceeds more than 20% in Korea.[5] Therefore, increased resistance to clarithromycin has become the most important factor of treatment failure. In recent years, eradication rates with first-line therapy in South Korea have ranged from 74.6% to 75.8%.[6-8] Clarithromycin resistance is due to point mutations in the peptidyl transferase encoding region in domain V of the *H. pylori* 23S ribosomal ribonucleic acid (rRNA) gene.[9,10]

Techniques to evaluate clarithromycin resistance can be divided into two categories: standard susceptibility tests and molecular methods.[11-14] However, phenotypical tests such as the agar dilution method and Epsilometer (E)-test require complicated conditions for *H. pylori* growth and serial subcultivations for several days to determine clarithromycin resistance. Furthermore, the agar dilution method and E-test fail to give any information about point mutations.[15-17] Molecular methods such as polymerase chain reaction (PCR), real-time PCR, and fluorescence *in situ* hybridization with deoxyribonucleic acid (DNA) probes have been developed to overcome these shortcomings.[9,13,18,19]

Peptide nucleic acid (PNA) molecules are synthetic DNA mimics, with a negatively-charged sugar–phosphate backbone that confers high affinity for DNA or RNA complementary sequences.[20,21] PNA molecules are stable at higher melting temperatures and more resistant to nucleases and proteases than DNA molecules.[21] PNA probes are also usually smaller (13–18 bp) than

DNA probes (≥18 bp); therefore, they penetrate the bacterial cell wall more easily.[21,22]

In this study, we developed and validated a new genotypic method to detect clarithromycin resistance in *H. pylori*, which involved probe-based fluorescence melting curve analysis (Sea-Sun Biomaterials, Daejeon, Korea) using a set of PNA probes. Sanger sequencing was used as the reference method.

MATERIALS AND METHODS

1. Study settings

We used 70 *H. pylori* strains from clinical isolates consecutively which were collected from patients who underwent gastric biopsy at Gangnam Severance Hospital, Seoul, Korea, between March 2015 and February 2017. We performed PNA probe-based fluorescence melting curve analysis compared with the standard susceptibility culturing test (E-test; BioMerieux SA, Marcy I'Etoile, France) and PCR sequencing to assess resistance status of 70 *H. pylori* strains.

2. Isolation of *Helicobacter pylori* strains and growth conditions

One antral and one corporal biopsy specimen were placed in a transport medium. The gastric tissue specimens were seeded in egg yolk emulsion (EYE) agar plates (Yuhan LabTech, Seoul, Korea). EYE agar contained Columbia agar 43.82 μg/mL, EYE 112.36 μL/mL, IsoVitaleX 11.23 μL/mL, and 2,3,5-triphenyltetrazolium chloride 45.0 μg/mL (for colony staining). The plates were subsequently stored in a multigas incubator (microaerophilic atmosphere: 10% carbon dioxide, 5% oxygen, and 85% nitrogen at 37℃) for 3 to 7 days. If *H. pylori* was not isolated after 7 days of incubation, the plates were incubated for 3 more days. Isolation of *H. pylori* was identified on the basis of colony morphology and confirmed by matrix-assisted laser desorption/ionization-time-of-fight mass spectrometry (MALDI-TOF) using the Microflex LT system (Bruker Daltonics, Bremen, Germany), and the measured profiles were compared to a database with MALDI Biotyper 3.1 software (Bruker Daltonics). The colonies were re-identified by subculture analysis. In our institution, isolation of *H. pylori* is routinely performed from gastric biopsy specimens using the above methodology. In the present study, we used isolates of *H. pylori* that had been in storage.

3. Design of peptide nucleic acid oligonucleotide probes for the detection of clarithromycin resistance

PNA probes were designed by adapting existing DNA probes targeting three point mutations (A2142G, A2143G, and T2182C) of 23S rRNA in *H. pylori*. The three point mutations were chosen based on prior reports. 23S rRNA point mutations typically involve A2142G or A2143G; in particular, the A2143G mutation has been previously identified in approximately 70% to 90% of patients with clarithromycin-resistant *H. pylori*.[23,24]

T2182C has also been regarded as an important point mutation in clarithromycin-resistant isolates.[24] We used the U-TOP™ HPy ClaR Detection Kit (SeaSun Biomaterials) to construct the PNA probes.

4. Validation of the real-time PCR-based MeltingArray analysis

Using the three PNA probes for probe-based fluorescence melting curve analysis (SeaSun Biomaterials), real-time PCR was performed with the CFX96 Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA), according to the manufacturer's manual. Real-time PCR reactions were performed in 20-μL reaction mixtures containing 10 μL ×2 qPCR PreMix (SeaSun Biomaterials), 5 μL primer and detection PNA probe mixture, and 3 μL DNA template (15 ng/μL). The reaction conditions for amplification and melting point analysis were 95℃ for 10 minutes; 42 cycles of 95℃ for 30 seconds, 58℃ for 45 seconds, and 72℃ for 45 seconds; followed by melting point analysis. Melting point analysis was performed using a denaturation step of 95℃ for 5 minutes; 1-minute hybridization steps of 75℃, 55℃, and 45℃; and a stepwise temperature increase from 20℃ to 85℃ at 1℃ per step, with a 5-second interval between each step. The data were analyzed using Bio-Rad CFX manager v1.6 software (Bio-Rad). Mutations were distinguished by the fluorescence signal of detection probes and corresponding melting temperatures (T_m).

5. Sanger sequencing

Sanger sequencing was performed as follows. A total of five sets of primers were used to amplify genomic DNA (gDNA) regions containing the mutations. PCR was performed using 20-μL reaction mixtures containing 3 μL purified gDNA (15 ng/μL), 10 μL ×2 qPCR PreMix (SeaSun Biomaterials), and 0.5 μL each of a forward and reverse primer (10 mM). And, thermocycling was done using 40 cycles at 95℃ for 30 seconds, 58℃ for 45 seconds, and 72℃ for 45 seconds, with a final elongation step at 72℃ for 5 minutes. The PCR products were electrophoresed on a 2% agarose gel and showed with ethidium bromide staining under ultraviolet light to check their sizes and evaluate their quantities. PCR products were sequenced using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). Sequencing reaction products were electrophoresed on the ABI 3500XL genetic analyzer (Applied Biosystems). Sequence data were analyzed using the ABI 3500XL DNA Analyzer (Applied Biosystems). All gDNA samples were tested individually with each of the eight sets of primers in both directions.

6. Antimicrobial susceptibility test

The *in vitro* minimum inhibitory concentration (MIC) of clarithromycin against the *H. pylori* clinical isolates was evaluated two times using the E-test. The MIC was determined in accor-

dance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations, with the clinical breakpoint for clarithromycin defined as >0.5 mg/L.[25] The standard strain, *H. pylori* ATCC 43504 (NCTC 11637, Australia), was used as a quality control organism.

7. Statistical analysis

PNA probe-based fluorescence melting curve analysis was compared with Sanger sequencing to assess resistance status. We calculated the positive predictive value (PPV), negative predictive value (NPV), and corresponding 95% confidence intervals of PNA probe-based fluorescence melting curve analysis using Sanger sequencing results as the reference. To assess the agreement between PNA probe-based fluorescence melting curve analysis and Sanger sequencing, we calculated the κ-value. All statistical analyses were conducted using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Among 70 *H. pylori* strains, 22 strains (31.4%) were clarithromycin resistant and 48 strains (68.6%) were clarithromycin

Table 1. Minimum Inhibitory Concentrations of Clarithromycin for 70 *Helicobacter pylori* Strains

Strain no.	MIC, mg/L	Strain no.	MIC, mg/L	Strain no.	MIC, mg/L
H-1	0.032	H-34	0.032	H-67	0.016
H-2	0.032	H-35	0.094	H-68	0.016
H-3	0.064	H-36	0.064	H-69	256*
H-4	0.047	H-37	0.047	H-70	0.047
H-5	0.016	H-38	0.016	H-71	0.064
H-6	192*	H-39	0.064	H-72	0.094
H-7	256*	H-40	SF	H-73	24*
H-8	256*	H-41	0.064	H-74	256*
H-9	0.064	H-42	SF	H-75	128*
H-10	0.064	H-43	0.016		
H-11	0.125	H-44	0.064		
H-12	0.016	H-45	256*		
H-13	256*	H-46	0.032		
H-14	SF	H-47	SF		
H-15	0.19	H-48	48*		
H-16	0.047	H-49	0.125		
H-17	24*	H-50	32*		
H-18	0.032	H-51	0.016		
H-19	0.023	H-52	0.016		
H-20	0.016	H-53	32*		
H-21	SF	H-54	0.032		
H-22	0.023	H-55	64*		
H-23	3*	H-56	0.023		
H-24	48*	H-57	0.023		
H-25	0.047	H-58	64*		
H-26	0.094	H-59	0.023		
H-27	0.023	H-60	0.023		
H-28	0.094	H-61	0.064		
H-29	0.094	H-62	0.032		
H-30	256*	H-63	0.016		
H-31	256*	H-64	16*		
H-32	0.016	H-65	32*		
H-33	256*	H-66	0.064		

MIC, minimum inhibitory concentrations; H, *H. pylori* strain; SF, subculture failure.
*Resistant strains determined by the European Committee on Antimicrobial Susceptibility Testing recommendations.
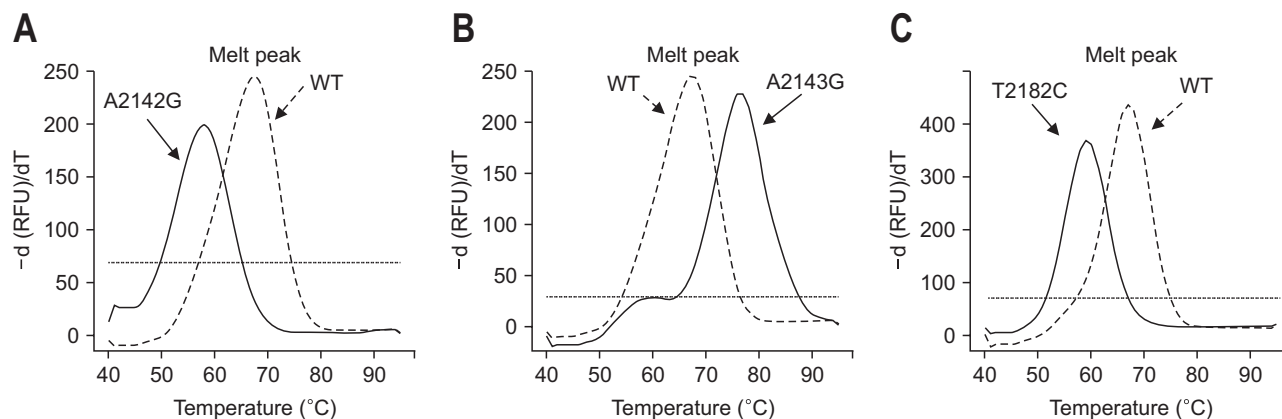
Fig. 1. Representative melting peaks of (A) A2142G, (B) A2143G, and (C) T2182C mutations. WT, wild type; RFU, relative fluorescence unit.

susceptible by E-test (Table 1). Among 22 clarithromycin resistant isolates, the A2143G mutation was observed in 16 (72.7%) isolates and the A2142G mutation was not observed in any isolate. We also conducted PNA probe-based fluorescence melting curve analysis and compared the results with those obtained using Sanger sequencing. Fig. 1 shows representative melting peaks for each mutation, as well as wild-type sequences. The PNA probe-based fluorescence melting curve analysis showed distinct melting curves specific to each allele, with T_m values for each diagnostic melting peak detected exactly as predicted.

Table 2 summarizes the results obtained by the two methods (Sanger sequencing and PNA probe-based methods). The κ-value between PNA probe-based fluorescence melting curve analysis and Sanger sequencing was 0.990 (standard error, 0.010), indicating that PNA probe-based fluorescence melting curve analysis agreed well with Sanger sequencing. When compared with Sanger sequencing, the PPV and NPV of PNA probe-based fluorescence melting curve analysis were both 100.0% for the A2142G mutation, as well as the A2143G mutation. The PPV was 98.3% and NPV was 100% for the T2182C mutation. One isolate with a T2190C mutation was recognized as a T2182C mutation in the PNA probe-based fluorescence melting curve analysis. This is likely because the melting temperatures of T2182C and T2190C overlapped. Therefore, the concordance between PNA probe-based fluorescence melting curve analysis and Sanger sequencing in our study was 98.6% (69/70).

The A2142G mutation was not observed in any of the 70 *H. pylori* isolates. However, the T2182C mutation was found in 58 isolates (82.9%), suggesting that this mutation was not associated with clarithromycin resistance (Supplementary Table 1).

DISCUSSION

New techniques using a rapid, simple, and accurate method to detect clarithromycin resistance have become an emerging issue because of increasing eradication failure of *H. pylori*. In this

Table 2. Comparison of Mutation-Detection Results Obtained with the PNA-Probe-Based Melting Curve Analysis and Sanger Sequencing

Sanger sequencing		PNA melting curve analysis kit					
		A2142G		A2143G		T2182C	
		+	–	+	–	+	–
A2142G	+	0	0				
	–	0	70				
A2143G	+			16	0		
	–			0	54		
T2182C	+					58	0
	–					1	11

PNA, peptide nucleic acid.

study, we evaluated a new diagnostic method using PNA probe-based fluorescence melting curve analysis. This method has several advantages. Firstly, this method requires only a conventional real-time PCR machine, and the procedure is performed in a single step within a closed well. Therefore, sample contamination and loss are much lower than with other methods, and the reaction and analysis procedures are much faster. Accordingly, this method can provide results promptly and accurately. The commercialized DPO-PCR (Seeplex® ClaR-*H. pylori* ACE Detection, Seegene Institute of Life Science, Seoul, Korea) is performed in two steps. DPO-PCR is a multiplex PCR and analyzed using a semi-automated system (i.e., Screen tape®). Therefore, PNA probe-based fluorescence melting curve analysis is faster and simple. In addition, PNA probes are simple to design and modify. Therefore, we can easily modify the probes and primers to change the target mutations from relatively nonspecific mutations to mutations strongly associated with clarithromycin resistance. Furthermore, PNA probe-based fluorescence melting curve analysis can detect up to four mutations in a single well. Probe-based fluorescence melting curve analysis is also a powerful tool to detect mutations.[8] PNA probes hybridize with

their complementary target DNA sequences,[26] and PNA–DNA hybridization is more sensitive to base mismatches than DNA–DNA hybridization. Because PNA probe-based fluorescence melting curve analysis is based on the temperature at which the probe-target hybridization dissociates,[27] the results are much easier to interpret than those of Sanger sequencing. However, in this study, PNA melting curve analysis was performed on DNA isolated from cultured *H. pylori*, not on gastric biopsy specimen. Therefore, the performance of PNA melting curve analysis on gastric biopsy specimens need to be verified.

In this study, we chose three specific mutations: A2142G, A2143G, and T2182C. However, the T2182C mutation, which was previously regarded as an important point mutation in clarithromycin resistance,[24,28] was detected in 58 of 70 isolates (82.9%). This result indicates that T2182C is a nonspecific mutation, which does not play an important role in clarithromycin resistance. As we can change the target mutation panel by adding or removing PNA probes and primers, we can easily replace the target mutation of T2182C to up to four different resistant target mutations.

This is the first study to report on using the MeltingArray assay based on PNA probes to assess clarithromycin resistance of *H. pylori*. The performance of PNA melting curve analysis showed almost 100% correlation with Sanger sequencing. Clinical application of our method is easy, and it should help clinicians choose appropriate treatment strategies according to the presence or absence of clarithromycin resistance. Currently, guidelines for treating *H. pylori* infection in Korea recommend an algorithm of *H. pylori* eradication according to clarithromycin resistance status.[2] Detection of clarithromycin resistance before treatment is thereby important for selecting the appropriate eradication regimen because assessing clarithromycin resistance is the first step in treating *H. pylori*. Therefore, rapid, easy, and accurate methods for detecting clarithromycin resistance are necessary, and the PNA probe-based fluorescence melting curve analysis is a good candidate method.

Clarithromycin resistance of *H. pylori* is well known. Point mutations in the 23s rRNA gene decrease the affinity between ribosomes and clarithromycin so the antibiotic is unable to inhibit protein biosynthesis.[29] Mutations A2143G, A2142C/G, and A2144G are associated with clarithromycin resistance of *H. pylori* in Asia.[30-33] In Korea, 23S rRNA gene mutations such as A2142G, A2143G, A2144G, T2182C, T2190C, A2223G, and C2195T have been reported occasionally in clarithromycin-resistant *H. pylori* strains.[24,34-36] However, these point mutations are less prevalent, indicating that a causal link between this gene and clarithromycin resistance has not been definitively proven. In this study, among 70 *H. pylori* strains, 22 (31.4%) were clarithromycin resistant and 48 (68.6%) were clarithromycin susceptible, according to the E-test (Supplementary Table 1). However, among the 22 clarithromycin-resistant isolates based on the E-test, the A2143G mutation was present in only

16 isolates (72.7%), and the A2142G mutation was not observed in any isolate. Therefore, the current commercial kit targeting A2142G and A2143G cannot detect all clarithromycin-resistant strains. However, as mentioned above, probes can be easily designed and modified for PNA probe-based fluorescence melting curve analysis. As we can change the target mutations by modifying probes and primers to identify candidate mutations associated with clarithromycin resistance, we can easily and conveniently apply this kit to clinical practice for detecting *H. pylori* resistance.

In this study, DNA sequencing was used to detect the mechanism of clarithromycin resistance. C375T, A381C, and G382A mutations that have not been previously associated with clarithromycin resistance were detected more frequently in clarithromycin-resistant isolates than in clarithromycin-susceptible isolates (Supplementary Table 2). However, the role of these three mutations remains unclear.

In the present study, PNA melting curve analysis was performed on DNA isolated from cultured *H. pylori*. However, PNA melting curve analysis can be performed on gastric biopsy specimens, as well as culture-based *H. pylori* isolates. Considering the higher rate of *H. pylori* detection by PCR of gastric biopsy specimens compared with cultures,[23,37] PNA melting curve analysis should likewise be more useful using gastric biopsy specimens than cultures because *H. pylori* is not easily isolated from cultures. Therefore, further study will be needed to validate the PNA melting curve analysis on gastric biopsy specimen.

In conclusion, although further study in a large population is necessary to confirm our results, PNA probe-based fluorescence melting curve analysis can be applied to clinical samples and may be helpful in choosing the precise *H. pylori* eradication regimen according to clarithromycin resistance. When more accurate information becomes available regarding resistance-related mutations, we will be able to promptly update our PNA probes and primers and thereby easily identify most clarithromycin-resistant strains.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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