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Sequencing and Comparative Analysis of *napA* Genes from *Helicobacter pylori* Strains Associated with Iron-Deficiency Anemia

HONG, MYUNG HEE[1], YON HO CHOE[2], YANG JE CHO[3], BO YOUNG AHN[3], AND NA GYONG LEE[1]*

[1]*Department of Bioscience & Biotechnology, Sejong University, Seoul 143-747, Korea*
[2]*Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University, Seoul 135-710, Korea*
[3]*EyeGene Inc., Seoul 110-290, Korea*

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Abstract *H. pylori* is known to cause severe gastric diseases, including peptic ulcers and gastric cancers, and a link has also been suggested with iron-deficiency anemia (IDA). However, little is known about the pathogenesis of *H. pylori*-associated IDA. In the present study, to determine whether *H. pylori* strains are correlated with the prevalence of IDA, we analyzed and compared the sequences of the *napA* genes encoding a bacterioferritin-like protein in *H. pylori* strains. A total of 20 *H. pylori* strains were isolated from antral biopsies of patients with and without IDA, and the *napA* genes amplified from the genomic DNA were sequenced. A comparison of the deduced amino acid sequences for NapA revealed two sites with major variations. At residue 70, five out of the 12 non-IDA strains (41.7%) contained serine, while only one of the 8 IDA strains (12.5%) contained serine, indicating a significantly higher frequency of serine in the non-IDA strains. In addition, the NapA proteins from all 17 Western strains available on Web sites were found to contain serine residues at this position. Meanwhile, the other major variation was located at residue 73, where all eight IDA strains (100%) contained leucine, while this was only true for eight of the 12 non-IDA strains (66.7%). Therefore, these results indicated that the strains within each group were more genetically related to each other than to strains in the other group. When the expression level of the *napA* genes in the *H. pylori* strains was measured using RT-PCR, no significant difference was observed between the two groups, suggesting a similar intensity for the inflammatory responses induced by the NapA protein among the strains. Consequently, when taken together, the present data suggest that the occurrence of *H. pylori*-associated IDA may be partly determined by the infecting *H. pylori* strain, and the non-IDA strains are more closely related to Western strains than the IDA strains.

*Corresponding author
Phone: 82-2-3408-3765; Fax: 82-2-3408-3334;
E-mail: nglee@sejong.ac.kr

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Helicobacter pylori is a Gram-negative and microaerophilic bacterial pathogen. It colonizes the mucosal layer overlying the gastric epithelium of the human stomach, and *H. pylori* colonization has been associated with several gastric diseases including chronic gastritis, peptic ulcers, and gastric cancer [10]. In addition, *H. pylori* infection was recently linked to iron-deficiency anemia (IDA), particularly in adolescents and premenopausal women [3], while Choe *et al.* [5] showed a possible association of *H. pylori* infection with IDA in adolescents and demonstrated that IDA in *H. pylori*-infected patients was refractory to iron therapy and only reversed after *H. pylori* eradication, even without iron supplementation. The results of a serological study carried out by the same group also suggested a link between refractory IDA and *H. pylori* infection [6]. Several hypotheses have been proposed for the pathogenesis of *H. pylori*-related IDA, where one explanation is that gastric hypoacidity due to chronic inflammation in an *H. pylori*-infected stomach causes a poor iron uptake, and another possibility is a competition between the infecting bacteria and the host cells for iron. During the host inflammatory responses to bacterial infection, neutrophils are activated and recruited to the infection site, and then release lactoferrin, which is believed to sequester the iron on the mucous of the stomach and hinder the host uptake. As such, the ability of *H. pylori* to utilize lactoferrin as an iron source has been suggested as a possible pathogenesis mechanism for *H. pylori*-associated IDA [7, 12].

NapA was first identified in an *H. pylori* water extract and shown to have the ability to activate neutrophils [8, 11]. NapA has a dodecameric structure and is capable of binding up to 500 iron atoms [21, 22]. However, the exact

role of NapA in the iron metabolism of *H. pylori* has not been fully elucidated. Despite various reports on the possible relationship between the sequence variation of NapA and its capability to induce inflammation in the gastric epithelium [11, 13], the ability of NapA to activate neutrophils is still controversial [14].

Accordingly, this study compared the *napA* gene sequences of *H. pylori* strains isolated from patients with and without IDA, and the results suggest that the genotype of the *napA* gene may be related to the prevalence of IDA in *H. pylori*-infected adolescents.

MATERIALS AND METHODS

H. pylori Strains and Growth Conditions

The *H. pylori* clinical strains used in this study were isolated from Korean adolescents aged 10 to 18 years. The study subjects with an *H. pylori*-positive serum IgG antibody underwent gastroduodenal endoscopy, and any subjects showing evidence of a hemorrhage or ulcer in the gastric and duodenal mucosa were excluded from the study. Stool examinations for occult blood (monoclonal antibody to hemoglobin, Kit OC Haemodia; Eiken Chemical Co., Tokyo, Japan) produced negative results for all the subjects. Hematological tests were carried out, and IDA was defined as a low serum ferritin level (<12 ng/ml), low level of transferrin saturation (<15%), and low hemoglobin level (<12 g/dl) [15]. Of the 20 subjects, 8 were found to have IDA, and the remaining 12 showed normal hematological findings. The *H. pylori* isolates analyzed for the *napA* gene in this study are shown in Table 1. The NapA sequences for 3 Asian strains and 17 Western strains available on Web sites were also compared (Table 2).

Table 1. *H. pylori* strains used in this study and their genotypes.

H. pylori strain	Genotype		Characteristics
	vacA	*cagA*[a]	
G1	s1a, s1c, m1	+	non-IDA[b]
G5	s1a, s1c, m1	+	non-IDA
G7	s1a, s1c, m1	+	non-IDA
G8	s1a, s1c, m1	+	non-IDA
G9	s1a, s1c, m1	+	non-IDA
G10	s1a, s1c, m1	+	non-IDA
G11	s1a, s1c, m1	+	non-IDA
G12	s1a, s1c, m1	+	non-IDA
G14	s1a, s1c, m1	+	non-IDA
G21	s1c, m1	+	non-IDA
G22	s1a, s1c, m1	+	non-IDA
G34	s1a, s1c, m1	+	non-IDA
A15	s1a, s1c, m1	+	IDA
A16	s1a, s1c, m1	+	IDA
A18	s1a, s1c, m1	+	IDA
A19	s1a, s1c, m2	+	IDA
A23	s1a, s1c, m1	+	IDA
A30	s1a, s1c, m1	+	IDA
A31	s1a, s1c, m1	+	IDA
A38	s1a, s1c, m1	+	IDA

[a]Presence (+) or absence (-) of *cagA* as determined by PCR.
[b]IDA, iron-deficiency anemia.

Table 2. List of *H. pylori* strains compared for their NapA sequences.

Strain	Characteristics	Origin	Accession no.
1811a		China	AAF37848
MEL-HP27		China	AAQ72432
5060d		China	AAF37849
J99	PUD[a]	U.S.A.	NP_222949
8826	Gastritis	U.S.A.	AAA67928
26695	Gastritis	U.K.	NP_207041
M1 356		Ireland	AAL27479
M1 355		Ireland	AAL27478
5D		Holland	AAF37847
5A		Holland	AAF37846
2B		Holland	AAF37845
2A		Holland	AAF37844
DB2		Australia	AAF37841
SS1		Australia	AAF37840
96T/HAR	PUD	Australia	AAG28159
96T/NEW	PUD	Australia	AAG28158
96T/FRE	PUD	Australia	AAG28157
96T/ROS	PUD	Australia	AAG28156
96T/KAD	PUD	Australia	AAG28155
RHP901a		Africa	AAF37842

[a]Peptic ulcer disease.

The *H. pylori* strains were directly isolated from antral biopsy specimens and cultured on Brucella Broth (Difco, Sparks, MD, U.S.A.) agar plates supplemented with 10% horse serum (Gibco BRL, Life Technologies, Rockville, MD, U.S.A.) for three days in an incubator at 37°C under a 10% CO_2 atmosphere. For the RT-PCR analysis, bacterial cultures were collected from the agar plates, resuspended in the Brucella Broth, and adjusted to an OD_{600} of 0.1. One-hundred microliters of the bacterial suspension was then spread on a Brucella Broth agar plate supplemented with 10% newborn bovine serum (Gifco BRL) and incubated for 18 h at 37°C before being harvested. Deferoxamine mesylate (desferal; Sigma, St. Louis, MO, U.S.A.) was added to BBS10 to a final concentration of 25 mM to achieve an iron-depleted condition.

Genomic DNA Isolation and Sequencing of *napA* Genes

Bacterial cells were collected from two or three plates, and the genomic DNA extracted using a genomic DNA extraction kit, G-spin (Intron, Seoul, Korea). The *napA* genes were amplified by polymerase chain reaction (PCR) using *napA*-specific primers (forward primer, 5'-taactatcccccttaaaaatgag-

3'; reverse primer, 5'-ttaagccaaatgggcttgcagcatc-3'). The amplified 633 bp fragments were used directly for nucleotide sequencing or cloned into a pGEM-T vector using a TA cloning kit (Promega, Madison, WI, U.S.A.) and then sequenced. The nucleotide sequences of the *napA* genes were determined using an ABI PRISM Genetic Analyzer (Perkin Elmer, Norwalk, CT, U.S.A.).

vacA and *cagA* Genotyping Using PCR

The *vacA* signal and midregion of the *H. pylori* isolates were typed using PCR, as described previously [2]. The presence of *cagA* was determined by PCR using the method described by Peek *et al.* [19]. PCR was carried out in a 50 μl reaction mixture containing 1 μg of genomic DNA, 0.5 μM sense and antisense primers for the gene of interest, a 1× Taq buffer, and 1 unit of Taq DNA polymerase (Takara Korea, Seoul, Korea). PCR was performed for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, followed by 72°C for 5 min. The PCR products were then resolved on an ethidium bromide-containing 2.5% agarose gel.

Nucleotide and Amino Acid Sequence Analysis

The *napA* nucleotide sequences were translated into the corresponding protein sequences using the DNA Club software. The deduced amino acid sequences for the *H. pylori* NapA proteins obtained in this study and those available on public Web sites were then aligned using the Multalin software [9]. Fisher's exact test was used to compare the differences in the amino acid residues between the IDA and non-IDA groups. P values <0.05 were considered to be significant.

RT-PCR Analysis

The bacterial cells were harvested by centrifugation at 4°C, and the pellet washed with ice-cold PBS. The total cellular RNA was isolated using the TRIzol® Reagent according to the manufacturer's instructions (Invitrogen, Grand Island, NY, U.S.A.), and then treated with 2 units of RNase-free DNase (Promega) at 37°C for 30 min and extracted with phenol/chloroform (1:1, v/v) followed by ethanol precipitation. The purified RNA was quantified spectrophotometrically using GeneQuant Pro (Amersham Biosciences), and then resolved on an ethidium bromide-containing agarose gel to check the integrity and quantification.

For a reverse transcriptase (RT) reaction, 4 μg of the total RNA was used as a template to synthesize the cDNA. The total RNA was mixed with 4 mg of a random hexamer (Amersham Biosciences), incubated at 65°C for 10 min, and cooled on ice for 2 min. The RT reaction was then carried out in a 50 μl volume with 2 units of M-MLV RT (Invitrogen) at 42°C for 1 h, followed by heating at 95°C for 5 min to terminate the reaction. The RT reaction product was diluted to 50 μl with water, and then 5 μl was used as a DNA template for PCR that was carried out in a 50 μl reaction mixture containing the DNA template, 25 pmole of each gene-specific primer, a 1× Taq buffer, 2.5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase (Takara Korea, Seoul, Korea). The forward and reverse primers used for the PCR of the *napA* gene were 5'-gcaagcggatgcgatcgtgt-3' and 5'-cagcggtgttagagagctct-3', respectively. For the control, the level of 23S RNA was determined by a RT-PCR using oligonucleotides 5'-aacgagattccctaagtagt-3' and 5'-tcaggttctatttcactccg-3'. The PCR was performed for various cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, followed by 72°C for 5 min. The PCR products were then resolved on an ethidium bromide-containing 2.5% agarose gel, the gel was scanned with a Gel Document (BioRad) on a UV-illuminator, and the DNA bands quantitated using Quantity One software (BioRad). The experiment was repeated three times with independently grown cultures.

RESULTS

vacA and *cagA* Genotyping of *H. pylori* Isolates

The *H. pylori* strains isolated from gastric biopsies from 20 Korean adolescents with IDA and without IDA were cultured and analyzed for the presence of the *cagA* gene and types of *vacA* signal and midregion. All the isolates tested were positive for the *cagA* gene (Table 1). As regards the *vacA* signal genotype, all but one non-IDA strain, which contained the s1c genotype, presented s1a and s1c alleles irrespective of the clinical phenotypes. Meanwhile, for the *vacA* midregion, all except one IDA strain contained the m1 genotype. Therefore, these data indicate that neither the *vacA* nor *cagA* genotype of *H. pylori* strains was correlated with the prevalence of IDA in the *H. pylori*-infected patients.

Nucleotide Sequence Analysis of *napA* Genes

The nucleotide sequences of the *napA* genes in the *H. pylori* strains isolated from the patients were determined. The sequences of the *napA* coding regions in the Korean isolates, plus 3 Asian strains and 17 Western strains available on Web sites, were aligned using the computer program Multalin. The sequence comparison revealed that the nucleotide sequence similarities among the *napA* genes was at least 97.2% for the Korean IDA strains (data not shown), while the *napA* similarity was lower between the IDA strains and non-IDA strains (>95.4%) and between the IDA strains and Western strains (>92%), suggesting that the IDA strains were more genetically related to each other than the non-IDA or Western strains.

Comparison of NapA Amino Acid Sequences

To analyze the potential amino acid sequence diversity among the NapA proteins from the different *H. pylori*

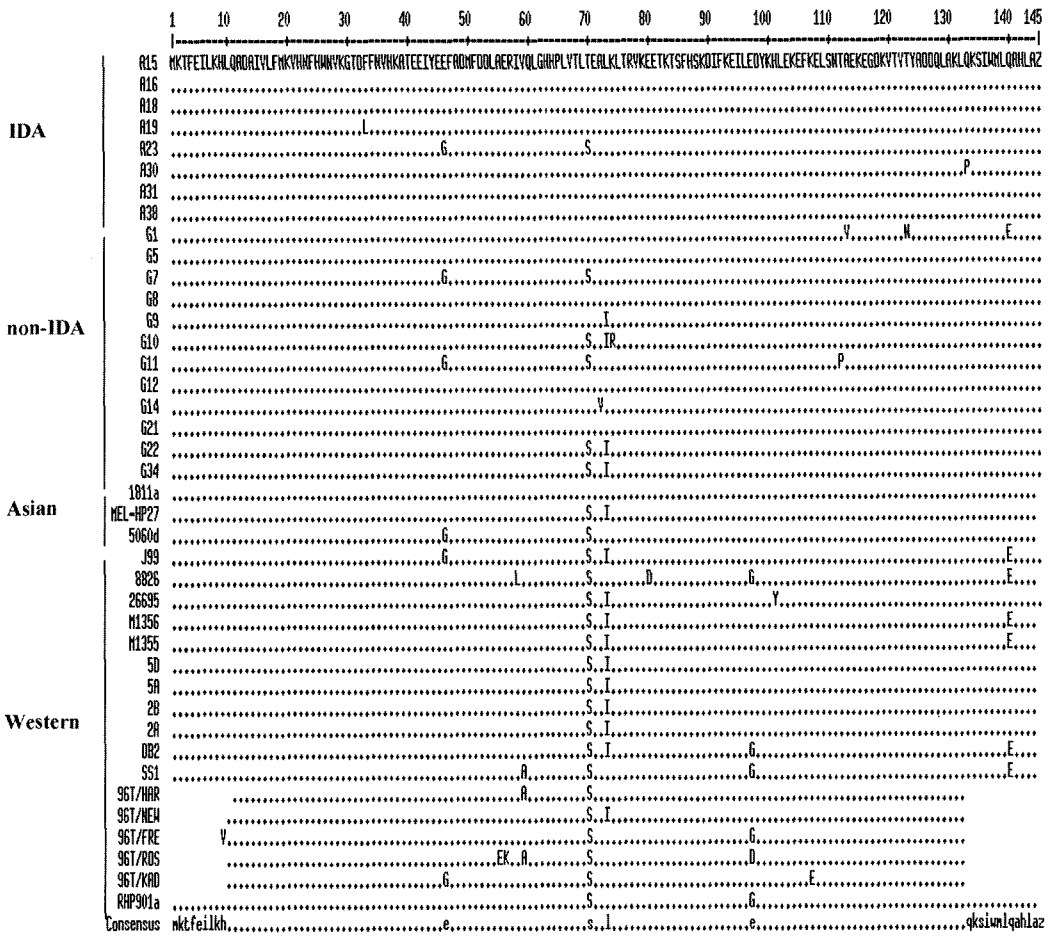

Fig. 1. Alignment of deduced amino acid sequences for *H. pylori* NapA protein.
The amino acid sequences of the NapA proteins determined in this study were aligned, along with those for 3 Asian and 17 Western strains available on Web sites. Dots denote conserved amino acid residues.

strains, the *napA* nucleotide sequences were translated and the deduced amino acid sequences aligned (Fig. 1). Amino acid polymorphisms were detected mainly in the central region of the NapA protein at five sites, corresponding to the amino acid residues 46, 70, 73, 94, and 140, while amino acid substitution was especially prominent at residues 70 and 73. Of the 8 IDA strains analyzed in this study, seven were found to contain threonine at residue 70 (87.5%) and only one contained serine (12.5%), whereas 5 out of the 12 non-IDA strains contained serine (41.7%) (Table 3), indicating a significantly higher frequency of serine at this position in the isolates from the non-IDA patients than in those from the IDA patients ($p<0.001$). The ratio of serine to threonine at residue 70 was even higher in the Asian strains (66.7%) and Western strains (100%). As for residue 73, all the IDA strains tested were found to contain leucine (100%), while 8 of the 12 non-IDA strains contained leucine (66.7%) and the remaining four contained isoleucine. The Asian strains also showed the same ratio as the non-IDA group at residue 73, whereas the Western strains had a lower ratio of leucine to isoleucine (41.2%). Therefore, these results suggest that the *napA* genotype may be related to the prevalence of *H. pylori*-related IDA, plus the non-IDA strains were more related to the Asian and Western strains than the IDA strains.

Analysis of *napA* Gene Expression in Clinical Isolates

To determine whether the IDA strains produce the NapA protein at a higher level than the non-IDA strains and how the *napA* gene expression responds to environmental iron, the mRNA expression level of the *napA* genes from the *H. pylori* strains was measured using RT-PCR. The *H. pylori* strains were cultured under iron-rich or iron-depleted conditions. RNA was purified and then converted to cDNA, which was used to amplify the *napA* genes using *napA*-specific primers. The 23S RNA level was also determined as a control for the RNA isolated from each strain and confirmed to be consistent among the strains (Fig. 2). As for the *napA* mRNA level, variations were observed among the strains, yet the differences were not

Table 3. Comparison of deduced NapA amino acid sequences from IDA and non-IDA *H. pylori* strains.

Amino acid residue	Strain		No. of strains/ total no. of strains (%)	
			threonine	serine
a.a. 70	Korean	IDA[a]	7/8 (87.5%)	1/8 (12.5%)
		non-IDA	7/12 (58.3%)	5/12 (41.7%)
		Total	14/20 (70.0%)	6/20 (30.0%)
	Asian		1/3 (33.3%)	2/3 (66.7%)
	Western		0/17 (0.0%)	17/17 (100.0%)
			leucine	isoleucine
a.a. 73	Korean	IDA[a]	8/8 (100.0%)	0/8 (0.0%)
		non-IDA	8/12 (66.7%)	4/12 (33.3%)
		Total	16/20 (80.0%)	4/20 (20.0%)
	Asian		2/3 (66.7%)	1/3 (33.3%)
	Western		7/17 (41.2%)	10/17 (58.8%)

[a] $p<0.001$ versus non-IDA group.

correlated to the clinical features of the strains. The iron concentration did not appear to affect the *napA* gene expression.

DISCUSSION

Since it was first proposed that *H. pylori* infection may be linked with IDA refractory to iron therapy, a substantial amount of supporting evidence has been accumulated [3]. However, the exact mechanism of the pathogenesis of *H. pylori*-associated IDA is not yet clearly understood, although it is presumed that both host factors and bacterial factors, like other infectious diseases, are involved in determining the final clinical outcome of *H. pylori*-associated IDA.

Accordingly, this study investigated the relationships between the *napA* genotype of *H. pylori* strains and the prevalence of *H. pylori*-associated IDA, to determine whether the *H. pylori* strain is a determining factor in the pathogenesis of IDA. As such, a comparative DNA sequence analysis of the *napA* genes from *H. pylori* strains isolated from Korean adolescents with and without IDA was conducted. It was also determined whether the *H. pylori* isolates contained the *vacA* and *cagA* genotypes, which have been reported to be highly associated with the pathogenesis of gastric ulcer and cancer in *H. pylori*-infected patients [4, 16, 20]. The results showed that most of the Korean strains analyzed in this study contained the same *vacA* genotype and all were *cagA*-positive, irrespective of the clinical features, suggesting that neither genotype has any correlation with the prevalence of IDA in *H. pylori*-infected patients. However, the *H. pylori* NapA amino acid sequences revealed a higher similarity among the IDA strains than between the IDA and non-IDA groups. In addition, the non-IDA group was more related to the Asian and Western strains than the IDA group.

The *napA* gene encodes a 17 kDa bacterioferritin-like protein, NapA [21], which forms a 150 kDa dodecamer that has been shown to be capable of binding up to 500 atoms *in vitro* and may be involved in the *in vivo* metabolism of iron [21, 22]. *H. pylori*-induced gastritis is typically associated with infiltration of the infected stomach mucosa by neutrophils, and *H. pylori* NapA is known to act as a major antigen in the human response to *H. pylori* infection and to promote neutrophils and mast cells [11, 18]. During chronic inflammation due to *H. pylori* infection, activated neutrophils are recruited to the mucus of the

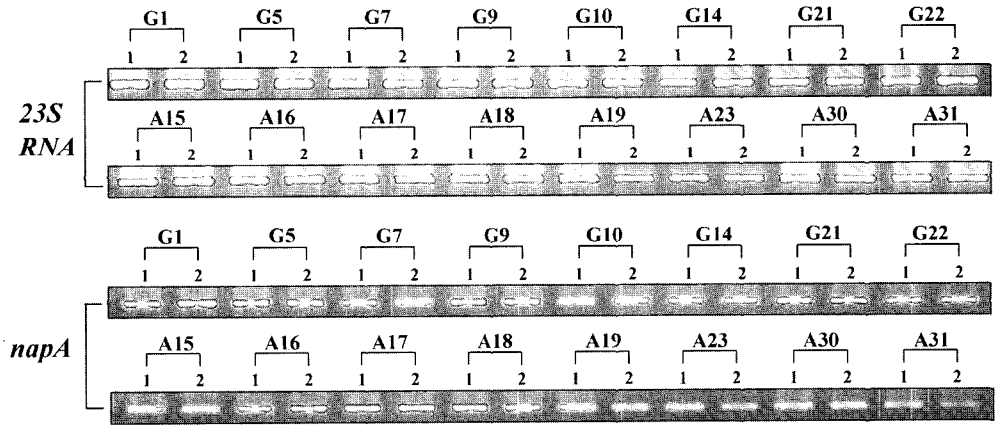

Fig. 2. Measurement of *napA* mRNA transcripts by RT-PCR analysis.
The *H. pylori* clinical strains were grown under iron-rich or iron-depleted conditions. The total RNA isolated was converted to cDNA, then analyzed for the *napA* mRNA transcript levels using RT-PCR. The RT-PCR was carried out for various cycles to obtain the optimum amplifications, and the results of 10 cycles for 23S RNA and 17 cycles for *napA* are shown. The experiments were repeated three times using independently grown cultures, and one representative set of data is shown. 1, Bacteria grown in BBS10; 2, bacteria grown in BBS10 with 25 μM desferal.

stomach and secrete lactoferrin. The lactoferrin levels have been shown to be higher in IDA patients with *H. pylori* infection than in non-IDA patients with simple gastritis or IDA patients without an *H. pylori* infection, suggesting that lactoferrin may play a role in the pathogenesis of *H. pylori*-associated IDA [7]. Thus, *H. pylori* strains producing a higher level of NapA would be expected to induce stronger inflammatory responses and subsequently inhibit iron absorption by host cells, leading to IDA. Therefore, to examine this theory, the mRNA expression levels of the *napA* gene were compared, but no significant difference was found between the IDA and non-IDA groups. The protein expression profiles of the strains on two-dimensional electrophoresis gels also indicated that the NapA protein levels for the IDA strains was similar to that for the non-IDA strains (unpublished data), corroborating that the NapA protein level produced by the strains was not the cause of *H. pylori*-associated IDA. Nonetheless, a comparison of the NapA amino acid sequences indicated that the IDA strains were more related to each other than to the non-IDA strains, suggesting that the polymorphism of the *napA* gene could be relevant to the prevalence of IDA in *H. pylori*-infected patients. However, the amino acid substitutions that distinguished the groups were only minor changes of similar amino acid residues and not likely to cause drastic changes in the protein structure or function. Therefore, although the NapA itself might not play a major role in the pathogenesis of IDA, the phylogenetic relationship of the strains based on the *napA* gene suggests that the infecting *H. pylori* strain may play an important role in determining the occurrence of IDA. In our previous study, the phylogenetic analysis of the *H. pylori* strains based on their overall protein expression profiles revealed that the IDA strains formed a separate cluster from the non-IDA strains, providing potential evidence on the clinical relevance of infecting *H. pylori* strains and their IDA phenotypes (manuscript in preparation). The results of this study revealed that the IDA strains were the most remote from the Western strains. However, there have been several reports of *H. pylori*-associated IDA in European countries [1, 17], thus it will be interesting to determine whether the strains isolated from Western IDA patients have the same *napA* genotype as Korean IDA strains.

In summary, the occurrence of *H. pylori*-associated IDA may be partly determined by the infecting *H. pylori* strain, and the non-IDA strains were found to be more closely related to Asian and Western strains than the IDA strains.

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