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Analysis of gastric microbiota by next generation sequencing: A key role of *Helicobacter pylori* in the gastric carcinogenesis

차세대 시퀀싱을 이용한 위내 세균과 위암 발병의 관련성에 관한 연구

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February 2016

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Abstract

Analysis of gastric microbiota by next generation sequencing: A key role of *Helicobacter pylori* in the gastric carcinogenesis

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Introduction: Little is known about the role of gastric microbiota except for *Helicobacter pylori* (HP) in human health and disease. We compared the differences of human gastric microbiota according to gastric cancer or control and HP infection status and assessed the role of bacteria other than HP.

Materials and methods: Gastric microbiota of 63 antral mucosa, 18 corpus mucosa samples were analyzed by barcoded 454-pyrosequencing of the 16S rRNA gene. Antral samples were divided into the four subgroups based on HP positivity in pyrosequencing and presence of cancer. The analysis was focused on bacteria other than HP, especially, nitrosating or nitrate reducing bacteria (NB). The changes of NB in antral mucosa of 16 subjects were followed-up.

Results: The number of NB other than HP (non-HP-NB) was two times higher in the cancer groups than control groups but it did not reach statistical significance. The number of non-HP-

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NB tends to increase over time, but this phenomenon was prevented by HP eradication in the HP-positive control group, but not in the HP-positive cancer group.

Discussion: We found out HP plays more important role than other bacteria in the gastric carcinogenesis.

Keywords: Gastric microbiota, *Helicobacter pylori*, gastric cancer, pyrosequencing, nitrate reducing bacteria, nitrosating bacteria

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List of Abbreviations

HP, *Helicobacter pylori*

IM, intestinal metaplasia

rRNA, ribosomal RNA

NOC, N-nitroso compounds

AG, atrophic gastritis

PPI, proton-pump inhibitor

PCR, polymerase chain reaction

ESD, endoscopic submucosal dissection

IgG, immunoglobulin G

OTUs, operational taxonomic units

gDNA, genomic DNA

ACE, abundance-based coverage estimator

UPGMA, unweighted pair group method with arithmetic mean

RA, relative abundance

ROC, receiver operator characteristic

Non-HP-NB, nitrosating bacteria other than HP

NGS, next generation sequencing

1. Introduction

Human gut, populated by complex communities of microorganisms, plays central roles in the digestion and the absorption of nutrients (1), the stimulation of intestinal epithelial renewal (2), and immune responses (3). Keeping these communities in balance with host is crucial for health maintenance and disease prevention (4). Human stomach as an ecological niche for bacteria received attention after the discovery of *Helicobacter pylori* (HP) in the 1980s. Before the discovery of HP, human stomach environment thought to be sterile due to acidic gastric environment suppressing the microorganisms from the oral cavity.

HP infection is a major cause of gastric cancer worldwide. The interaction of bacterial factors, environmental factors, and the host immune response initiate and progress the carcinogenesis; mucosal atrophy, intestinal metaplasia (IM), and dysplasia toward gastric cancer (5). Bacteria other than HP that lead to endogenous formation of N-nitroso compounds (NOC) might also have a role in gastric carcinogenesis (6). NOC can be formed from nitrite and secondary amines by nitrosating bacteria of stomach, which have nitrosating enzyme such as cytochrome cd_1-nitrite reductases (7). Various bacteria including *Clostridium, Veillonella, Haemophilus, Staphylococcus,* and *Neisseria* etc. play a role in the formation of NOC (8-10). This formation of NOC has been suggested to increase the risk of cancer in patients with hypochlorhydria due to HP-associated atrophic gastritis (AG) (8). There were efforts to demonstrate the existence of non-HP organisms in the achlorhydric or proton-pump inhibitor (PPI)-induced hypochlorhydric stomach (8, 10). However, culture-dependent method was the only available approach before the introduction of polymerase chain reaction (PCR)-based methods for the detection and identification of microorganisms. Little is known about stomach microbiota with the cultivation methods.

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Recent development of molecular methods provided more detailed insights into the human microbiota in various organs including skin, gut, and so on (11-14). Analysis of the 16S ribosomal RNA (rRNA) gene contents of microbial samples after amplification by PCR has revolutionized the characterization of microbial communities. There have been a few studies on stomach microbes since the first gastric microbiota in healthy controls was described (15-17). Human gastric microbiota has been characterized by the presence of HP, the major organism in the stomach. Interestingly, HP sequence was observed even in the biopsy samples of subjects who were determined to be HP-negative by a combination of conventional methods (15, 18). There have been studies focused on non-HP microbiota in the human stomach (17, 19). Bacteria having genes encoding nitrate and nitrite reductases have been found in the oral cavity and stomach by molecular methods (17). There have been a study which focused on gastric microbiota of patients who have gastritis without HP infection to evaluate other bacterial contribution with gastritis development (19). However, our understanding about gastric microbiota is still in the early stages, including the composition of microbiota and the factors that influence gastric microbiota (20).

Our group has conducted research on gastric microbiota focusing on the relationship between HP and bacteria other than HP. During our study, we encountered circumstances wherein low abundance gastric HP sequences were detected by pyrosequencing in stomach samples that were found to be HP-negative by a combination of conventional HP tests including serology. Based on these circumstances, we aimed to determine the cut-off value of HP sequence percentage for HP colonization in gastric mucosa samples by pyrosequencing. Eventually, we aimed to compare the differences in microbiota according to HP infection status and presence or absence of gastric cancer with a focus on the alleged nitrosating or nitrate reducing bacteria and to assess the role of bacteria other than HP by pyrosequencing method.

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2. Materials and Methods

2.1. Subjects

Subjects were enrolled in the study at Seoul National University Bundang Hospital from January 2004 to March 2014. All subjects were of Korean origin. They underwent upper gastrointestinal endoscopy with blood sampling for HP serology. Most subjects underwent standard endoscopy as part of a screening program for premalignant gastric mucosal lesions or gastric cancer. Diagnosis of gastric cancer was based on histological data of surgical or endoscopic submucosal dissection (ESD) specimens (21). Subjects who underwent endoscopy for screening or complained of mild dyspepsia and agreed to undergo multiple biopsies for HP study in advance during the same study period but did not show any evidence of gastric cancer or dysplasia, mucosa-associated lymphoid tissue lymphoma, or esophageal cancer, were assigned to the control group. All subjects received endoscopy by the same gastroenterologist (N. K.), and a total of 10 biopsies per subject were obtained. Each participant completed a questionnaire on family history about first-degree relative with gastric cancer, smoking, alcohol consumption habits, antibiotics use, and history of HP eradication under the supervision of a well-trained interviewer before the endoscopy. Patients with a previous history of gastric surgery or HP eradication treatment, antibiotics use or a systemic disease requiring chronic medication were excluded from the study. All subjects provided their informed consents. The study protocol was approved by the Institutional Review Board of Seoul National University Bundang Hospital.

2.2. Determination of HP infection status

To determine the presence of current HP infection according to conventional tests, 8 of 10 biopsy specimens per subjects were obtained to perform three types of HP testing (histology,

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rapid urease test, and culture). Two biopsy specimens were taken each from the greater curvature of the antrum and the body of the stomach. The other specimens were taken from the lesser curvature of the antrum and the body of the stomach. These 4 biopsy specimens were assessed for the presence of HP (by modified Giemsa staining) and for the degree of inflammatory cell infiltration, AG, and IM (hematoxylin and eosin staining). Histological features of gastric mucosa were recorded using the updated Sydney scoring system (i.e., 0 = none, 1 = slight, 2 = moderate, 3 = marked) (22). Another 2 of these 8 specimens were used for culturing of HP. The remaining 2 specimens from the lesser curvature of the antrum and the stomach body were used for rapid urease test (CLO test; Delta West, Bentley, WA, Australia). Detailed protocols used for the three biopsy-based tests have been described previously (23).

To avoid contamination, the gastroscope was washed and disinfected by immersing in a detergent solution containing 7% proteolytic enzymes and 2% glutaraldehyde (24), and sterilized endoscopy forceps were used while taking another biopsy from the same patient. The biopsies were stored at 80 ^{o}C.

By conventional methods, current HP infection was defined as a positive result from any of the 3 tests; histology, rapid urease test, and culture. To distinguish a past infection from a current infection, the following two methods described previously were used: enzyme-linked immunosorbent assay to screen for immunoglobulin G (IgG) specific for HP in the serum (Genedia HP ELISA; Green Cross Medical Science Corp, Eumsung, South Korea) and history of HP eradication (25). If, all the 3 tests were negative and have history of HP eradication or positive IgG for HP, the subject was counted as past infection. If all the 5 tests were negative, the subject was categorized into the conventional HP-negative group.

2.3. Bacterial DNA extraction and construction of 16S rRNA clone libraries

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The 63 antral mucosal samples, 18 corpus mucosal samples were subjected to pyrosequencing. Seventeen of 18 subjects provided corpus mucosa with antral mucosa samples together. In addition, 16 of 63 subjects who provide antral mucosal samples were followed up.

Two pieces of gastric mucosa were excised and subjected to bacterial genomic DNA (gDNA) extraction using a commercial kit (iNtRON Biotechnology, Gyeonggi, Korea) which does not include beadbeating. Briefly, tissues were treated with lysozyme at 37°C for 15 min followed by cell lysis with a buffer containing proteinase K and RNase A at 65°C for 15 min. Cell lysates were subsequently mixed with binding buffer. The gDNA was purified with resin columns.

2.4. Amplification of 16S rRNA gene and sequencing

Extracted gDNA was amplified using primers targeting the V1 to V3 regions of the 16S rRNA gene. For bacterial gDNA amplification, barcoded primers of 9F (5'-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-AGAGTTTGATCMTGGCTCAG-3'; underlining indicates the primer for the target region) and 541R (5'-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-ATTACCGCGGCTGCTGG-3'; 'X' indicates the unique barcode for each subject) (http://www.ezbiocloud.net/resource/M1001) were used. The amplifications were carried out at the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 45 sec, and extension at 72°C for 1 min 30 sec, with a final elongation at 72°C for 5 min. PCR product was analyzed by 2% agarose gel electrophoresis and visualized with a Gel Doc system (BioRad, Hercules, CA, USA). PCR products were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified PCR products were quantified using a Nanodrop and equal amounts were pooled together and purified with Ampure beads kit (Agencourt Bioscience, Beverly, MA, USA). The quality and product size were assessed with Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using DNA 7500 chip. Emulsion PCR was

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conducted using mixed amplicons. The mixed amplicons were deposited on picotiter plates. Sequencing was carried out at Chunlab, Inc. (Seoul, Korea) with GS Junior Sequencing system, the modified laboratory benchtop form of 454 sequencing systems (Roche, Branford, CT, USA) according to the manufacturer's instructions. The pyrosequencing was performed from the reverse primer end (541R).

2.5. Analysis and taxonomic assignment of individual sequencing reads

Pyrosequencing analysis was conducted using published methods (26, 27). Reads obtained from different samples were sorted by their unique barcodes of each PCR product. After detecting the target region in barcoded primers (9F or 541R), all linked sequences including adapter, barcode, and linker were removed. Any reads containing two or more ambiguous nucleotides, reads with low quality score (average score < 25), and reads shorter than 300 base pairs were discarded. Potential chimeric sequences were detected by Bellerophon method by comparing BLASTN search results of the forward half and the reverse half sequences (28). After removing chimeric sequences, taxonomic classification of each read was assigned against EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (29). The EzTaxon-e database contains 16S rRNA gene sequence of type strains that have valid published names and representative species level phylotypes of either cultured or uncultured entries in GenBank public database with complete hierarchical taxonomic classification from the phylum to the species. Then, we binned reads into operational taxonomic units (OTUs) based on 97% similarity (3% cut-off value). Phylogenetic trees were not created as we assigned reads into OTU according to BLAST results. The raw unprocessed 16S rRNA gene sequence which had been generated by our study was deposited in NCBI's SRA (GSE61493).

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2.6. Calculation of species richness and diversity indices

The species richness of samples was determined by abundance-based coverage estimator (ACE), Chao1 estimator, and Jackknife estimator. In addition, Simpson diversity index, Shannon diversity index, and Good's library coverage were calculated in the CLcommunity program (Chunlab Inc., Seoul, Korea). Random subsampling was conducted to equalize the read size of samples to compare different read sizes among samples. To compare OTUs between samples, shared OTUs were obtained with XOR analysis of the CLcommunity program (Chunlab Inc., Seoul, Korea). UniFrac-based unweighted pair group method with arithmetic mean (UPGMA) dendrogram and principal coordinates analysis were used to compare the microbial communities of sub-groups.

2.7. Sub-group analysis and follow-up sample

HP-positive or -negative groups were re-determined by the pyrosequencing result based on the cut-off value of 1% relative abundance (RA) of HP (30). If RA of HP was equal to or lower than 1%, the sample re-determined as HP-negative group. Finally, subjects were classified to 4 sub-groups according to disease status (cancer or control) and HP infection or not as follows: HP-negative control group [HP (-) control, n = 13], HP-positive control group [HP (+) control, n = 16], HP-negative cancer group [HP (-) cancer, n = 19], and HP-positive cancer group [HP (+) cancer, n = 15].

One or more years after first upper endoscopy, subjects underwent a second upper endoscopy with the combination of aforementioned conventional tests. The antral mucosal samples of 16 cases followed up, were additionally analyzed by pyrosequencing [HP (-) control, n = 3; HP (+) control, n = 4; HP (-) cancer, n = 5; HP (+) cancer, n = 4]. Six of eight HP-positive subjects were treated with the standard 7-day triple therapy (esomeprazole 40 mg b.i.d., clarithromycin 500 mg b.i.d., and amoxicillin 1000 mg b.i.d.) after the first endoscopy exam. In four subjects (each

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two subjects in the HP (+) control and HP (+) cancer groups), HP was successfully eradicated. The rRNA sequence data were intensively analyzed with a focus on the bacteria that have the ability to nitrosate or reduce nitrate such as *Clostridia, Veillonellae, Haemophili, Staphylococci*, and *Neisseriae*, which were identified in previous studies (7, 8, 10). Table 1 shows the specific species included in the analysis.

2.8. Statistics

Descriptive statistics were reported as mean ± standard deviation. The average of the percent of HP sequence in each sample was described here instead of using total HP sequences per total reads. Confidence intervals were computed as two-tailed using 95% coverage. Categorical variables were reported as frequencies and proportions. Comparisons between continuous parameters were performed by t-test and Kruskal-Wallis test. Comparison between categorical variables was performed by Fisher's exact test. Logistic regression and receiver operator characteristic (ROC) curve analyses were performed to determine the threshold of HP sequence. Statistical analyses were performed using PASW 18.0 (IBM, Somers NY, USA). Statistical difference was considered when p-value was less than 0.05.

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Table 1. The species of nitrosating bacteria and nitrate reducing bacteria

Helicobacter pylori	*Haemophilus influenzae*
Bacteroides fragilis	*Haemophilus parainfluenzae*
Bacteroides plebeius	*Klebsiella pneumoniae*
Campylobacter uc	*Lactobacillus fermentum*
Citrobacter freundii	*Lactobacillus gasseri*
Clostridium bartlettii	*Neisseria bacilliformis*
Clostridium bifermentans	*Neisseria cinerea*
Clostridium g4_uc	*Neisseria elongata*
Clostridium lituseburense	*Neisseria flava*
Clostridium perfringens	*Neisseria flavescens*
Clostridium sordellii	*Neisseria macacae*
Corynebacterium accolens	*Neisseria mucosa*
Corynebacterium afermentans	*Neisseria oralis*
Corynebacterium amycolatum	*Neisseria perflava*
Corynebacterium argentoratense	*Neisseria sicca*
Corynebacterium durum	*Neisseria sicca group*
Corynebacterium glucuronolyticum	*Neisseria subflava*
Corynebacterium kroppenstedtii	*Neisseria uc*
Corynebacterium macginleyi	*Pseudomonas aeruginosa*
Corynebacterium matruchotii	*Pseudomonas stutzeri*
Corynebacterium mucifaciens	*Staphylococcus aureus*
Corynebacterium mycetoides	*Staphylococcus epidermidis*
Corynebacterium pilbarense	*Staphylococcus hominis*
Corynebacterium pseudogenitalium	*Staphylococcus aprophyticus*
Corynebacterium singulare	*Stenotrophomonas maltophilia*
Corynebacterium suicordis	*Veillonella atypica*
Corynebacterium thomssenii	*Veillonella denticariosi*
Corynebacterium tuberculostearicum_group	*Veillonella dispar*
Corynebacterium uc	*Veillonella parvula*
Corynebacterium ureicelerivorans	*Veillonella rodentium*
Corynebacterium variabile	*Veillonella rogosae*
Corynebacterium vitaeruminis	*Veillonella tobetsuensis*
Corynebacterium xerosis	*Veillonella uc*
Escherichia coli	

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3. Results

3.1. Baseline characteristics according to HP status by conventional methods

A total of 63 subjects were enrolled in this study, including 34 cancer patients and 29 controls. There were 35 HP-negative and 28 HP-positive subjects based on conventional diagnosis. There was no significant difference ($p = 0.956$, t-test) in the proportion of cancer patients between the two groups (Table 2). The mean age was significantly different between the HP-negative and HP-positive groups ($p = 0.048$, t-test). The grade of neutrophils and monocytes infiltration was significantly lower in the HP-negative group compared to that in the HP-positive group ($p < 0.001$ each, by t-test) (Table 2). However, the grades of AG and IM were not significantly different between these two groups (Table 2).

3.2. Relative abundance at phylum and genus level based on pyrosequencing

In total, 256,679 reads were obtained from 63 samples of gastric antral mucosa. The entire dataset was clustered into 1,371 species and 21 phyla with maximum within cluster dissimilarity of 3%. The most representative phylum was *Proteobacteria* (65.0%), followed by *Firmicutes* (20.0%), *Actinobacteria* (7.8%), *Bacteroidetes* (3.7%), and *Fusobacteria* (0.7%). In the phylum *Proteobacteria*, *Helicobacter, Stenotrophomonas, Ralstonia, Citrobacter*, and *Haemophilus* were the 5 major genera. *Firmicutes* were mainly composed of the genera *Streptococcus* and *Veillonella.* In addition*, Propionibacterium, Actinomyces*, and *Rothia* were the major genera of phylum *Actinobacteria,* while *Prevotella* was the major genus in the phylum *Bacteroidetes*.

The pyrosequencing method detected HP sequences in 60% (21/35) of the subjects who were originally identified as HP-negative based on the combination of conventional methods as described previously. We divided the subjects into the following three groups based on both

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Table 2. Baseline characteristics of patients according to the presence of *H. pylori* by conventional methods

	HP-negative (n = 35)	HP-positive (n = 28)	*p*-value
Cancer patients (No., %)	19 (54.3%)	15 (53.6%)	.956
Mean age (years)	60.97 ± 11.43	55.04 ± 11.80	.048*
Sex (No., male %)	22 (62.9%)	14 (50.0%)	.121
Neutrophil infiltration (mean ± SD)	0.06 ± 0.24	1.79 ± 0.97	< .001*
Monocyte infiltration (mean ± SD)	1.26 ± 0.44	2.07 ± 0.63	< .001*
Atrophic gastritis (mean ± SD)	0.66 ± 0.80	0.75 ± 0.93	.672
Intestinal metaplasia (mean ± SD)	1.11 ± 1.05	0.93 ± 0.86	.453
Number of follow-up visit	2.82	3.57	.250

HP, *H. pylori*

* $p < 0.05$, t-test

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conventional and pyrosequencing methods: group A, HP-negative based on both conventional and pyrosequencing; group B, HP-negative based on the conventional method and HP-positive based on the pyrosequencing method; group C, HP-positive based on both methods. The relative abundance at phylum level in group A to C was shown in Fig. 1a. The proportion of *Proteobacteria* in groups A, B, and C was 65.5%, 48.3%, and 77.4% respectively, with significant (p = 0.007, Kruskal-Wallis test) difference between group B and C. In addition, the proportion of *Actinobacteria* was significantly (p = 0.004, Kruskal-Wallis test) different between group B (13.9%) and group A (4.8%) or C (4.6%) (Fig.1a). However, if HP sequences were eliminated from the analysis, there was no difference in the proportion of *Proteobacteria* or *Actinobacteria* among the three groups.

The 10 most abundant genera were in the following order: *Helicobacter, Stenotrophomonas, Streptococcus, Ralstonia, Propionibacterium, Citrobacter, Prevotella, Haemophilus, Veillonella,* and *Actinomyces*. In group A, *Stenotrophomonas* (34.83%), *Streptococcus* (13.90%), *Pseudomonas* (8.40%), *Ralstonia* (6.46%), and *Enhydrobacter* (5.15%) were the five most abundant genera. In group B, five major genera were *Streptococcus* (22.31%), *Stenotrophomonas* (15.21%), *Propionibacterium* (10.05%), *Ralstonia* (9.19%), and *Enterobacter* (4.31%). *Helicobacter* (58.64%) was the most abundant genus in group C, followed by *Streptococcus* (9.94%), *Stenotrophomonas* (4.87%), *Citrobacter* (3.47%), and *Propionibacterium* (3.15%) (Fig. 1b).

3.3. Sequence diversity and library coverage estimations among 3 groups

The average number of reads per specimen was 4124.4 ± 2849.8. HP sequences were detected in all samples from the HP-positive group and 21 of 35 HP-negative samples based on conventional methods (Table 3). The mean percentage of HP sequences per each samples in group A, B, and C was 0%, 0.67%, and 58.51%, respectively. The overall Good's coverage

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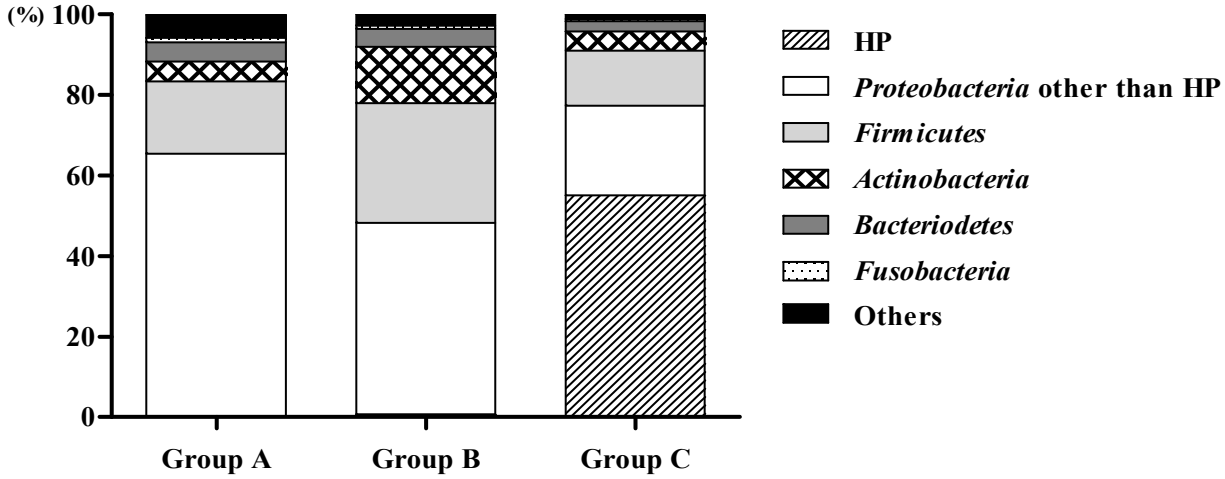
Figure 1 Relative abundance of the phylum and genus level (% similarity) in gastric mucosa samples by pyrosequencing. The most abundant phylum was *Proteobacteria* (65.0%), followed by *Firmicutes* (20.0%), *Actinobacteria* (7.8%), *Bacteroidetes* (4.0%), and *Fusobacteria* (0.78%).

a. Group A: HP-negative by both conventional and pyrosequencing methods, Group B: HP-negative by the conventional methods and HP-positive by the pyrosequencing method, Group C: HP-positive by both the methods. The proportion of *Proteobacteria* and *Actinobacteria* was significantly different among group B and A/C ($p = 0.049$, $p = 0.039$, respectively by Kruskal-Wallis test).



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b. Groups displayed are identical to those in Figure 1a. Relative abundance of the genus level (% similarity) in gastric mucosa samples by pyrosequencing. The 5 most abundant genera were *Helicobacter, Stenotrophomonas, Streptococcus, Ralstonia*, and *Propionibacterium*.

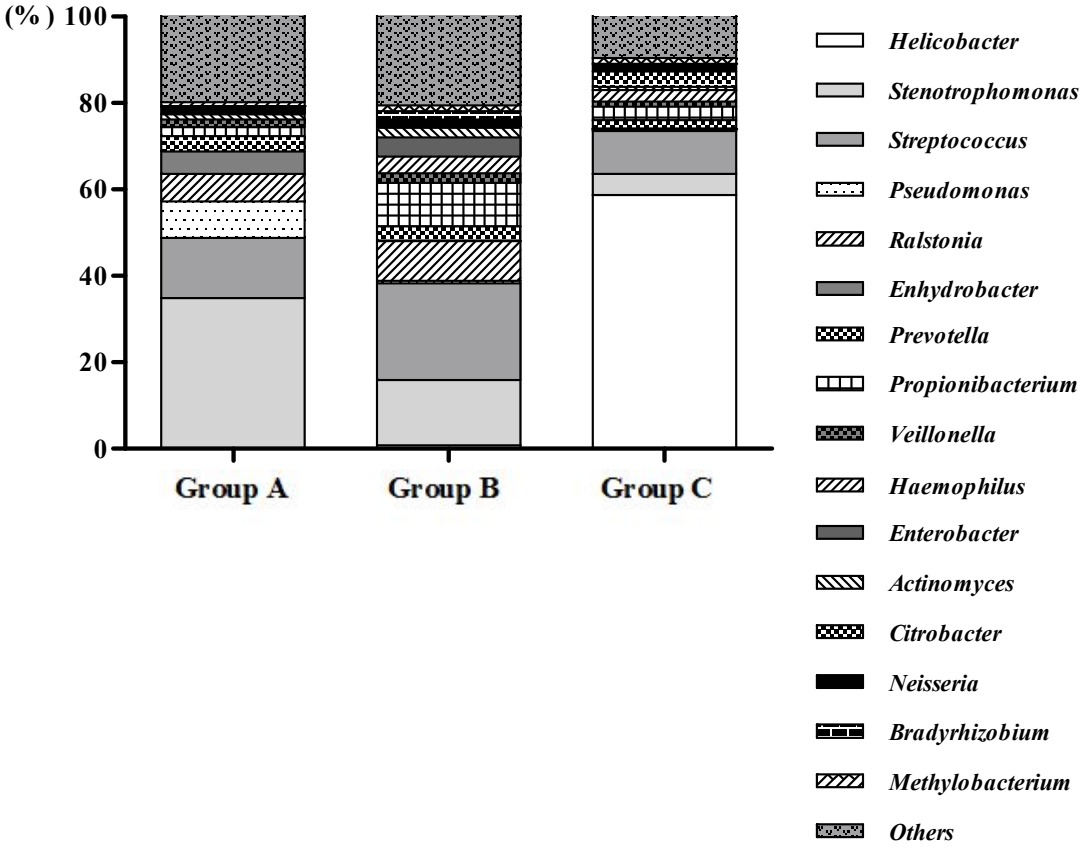

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Table 3. Sequence diversity and library coverage estimations among the three groups

	Group A (n = 14)	Group B (n = 21)	Group C (n = 28)	*p*-value
HP status by conventional tests	-	-	+	
Average number of reads	2133.7 ± 1139.08	4067 ± 2 911.07	5162.5 ± 2927.82	.009*
Average number of OTUs	96.7 ± 51.86	195.6 ± 130.26	150.7 ± 120.25	.009*
HP sequences, %	0	0.67 ± 1.09	58.51 ± 37.22	<.001*
ACE diversity	157.8 ± 88.96	331.3 ± 307.66	316.9 ± 344.97	.187
Chao diversity	138.1 ±74.15	284.5 ± 221.52	248.9 ± 231.55	.114
Jackknife	145.9 ± 81.30	326.3 ± 279.32	285.9 ± 281.21	.110
Shannon's index for diversity	2.27 ± 1.273	3.16 ± 1.161	2.06 ± 1.260	.009*
Simpson's index for diversity	0.35 ± 0.298	0.16 ± 0.186	0.40 ± 0.266	.007*

HP, *H. pylori*
ACE, Abundance-based coverage estimator
Group A, conventional test (-) and HP sequence (-); Group B, conventional test (-) and HP sequence (+); Group C, conventional test (+) and HP sequence (+)
Diversity and richness estimators were calculated using the CLcommunity program (Chunlab Inc., Seoul, Korea).
* $p < 0.05$, Kruskal Wallis test

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was 98%, indicating that two more phylotypes would be present in 100 additional sequenced clones. Species richness indicators, such as ACE, Chao1, and Jackknife, were calculated by the CL community program (Chunlab Inc., Seoul, Korea). Average number of OTUs was significantly (p = 0.009, Kruskal Wallis test) higher in group B than that of group A (Table 3). In addition, there was no significant difference in ACE, Chao1, or Jackknife among the three groups. The value for the Shannon diversity index in group B was significantly (p = 0.009, Kruskal Wallis test) higher than that in group C. Simpson index in group B was significantly (p = 0.007, Kruskal Wallis test) more closer to 0 than that in group C, indicating that there were more diverse samples in group B than in group C (Table 3).

3.4. ROC curve analysis for the cut-off value for HP colonization

The logistic regression model and ROC curve analysis confirmed that the threshold of HP-colonization by pyrosequencing and conventional status was in good correlation. The regression line had a significantly good discriminatory power (AUC = 0.957; $p < 0.001$) (Fig. 2). The best cutoff value of HP sequence percentage for HP colonization was 1.22%. The overall sensitivity and specificity was 92.9% and 88.6%, respectively.

3.5. Comparison of antral samples among 4 groups according to 1% cut-off by pyrosequencing

HP-positive or -negative groups were re-determined by the pyrosequencing result based on the cut-off value of 1% relative abundance (RA) of HP (30). Baseline characteristics of clinical and pyrosequencing results of gastric antral mucosal samples were shown in Table 4. The mean age of patients was higher in the HP-negative groups than in the HP-positive groups (p = 0.017). However, there was no significant difference among 4 groups (p = 0.120, Table 4).

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Figure 2 ROC curve of HP sequences in HP-positive or HP-negative individuals. HP sequences in HP-positive (n = 28) and HP-negative individuals (n = 35) were plotted to create a ROC curve with corresponding coordinates of the curve and statistics for the area under the curve (bold line). A threshold of 1.22% (arrow) showed high sensitivity and specificity (the overall sensitivity and specificity was 92.9% and 88.6%, respectively).

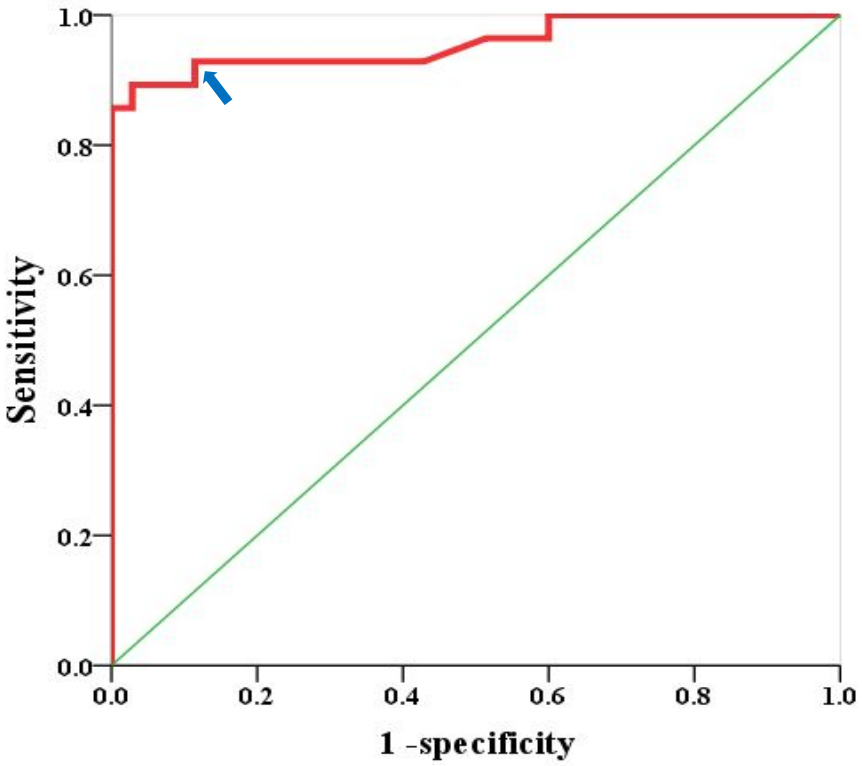

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Table 4 Baseline characteristics of clinical and pyrosequencing results of gastric antral mucosal samples

	HP (-) control (n = 13)	HP (-) cancer (n = 19)	HP (+) control (n = 16)	HP (+) cancer (n = 15)	*p*- value
Age (mean ± SD, yr)	62.3 ± 13.61	61.8 ± 10.92	55.9 ± 10.97	54.1 ± 12.50	0.120
Male (n, %)[a]	4 (30.8)	14 (73.7)	8 (50)	10 (66.7)	0.084
PG I/II ratio (mean ± SD)	4.1 ± 1.68	4.7 ± 2.08	2.9 ± 2.12	3.3 ± 1.47	0.023*
Atrophy (n, %)[a, b]					
No	7 (54)	11 (58)	9 (56)	9 (60)	
Mild	6 (46)	5 (26)	6 (38)	2 (13)	0.217
Moderate	0	0	1 (6)	3 (20)	
Marked	0	3 (16)	0	1 (7)	
IM (n, %)[a, b]					
No	5 (39)	6 (32)	8 (50)	6 (40)	
Mild	2 (15)	4 (21)	6 (38)	2 (13)	0.325
Moderate	5 (39)	7 (37)	2 (13)	7 (47)	
Marked	1 (8)	2 (11)	0	0	
Neutrophil infiltration (n, %)[a, b]					
No	7 (54)	11 (58)	9 (56)	9 (60)	
Mild	6 (46)	5 (26)	6 (38)	2 (13)	<0.001
Moderate	0	0	1 (6)	3 (20)	
Marked	0	3 (16)	0	1 (7)	
Monocyte infiltration (n, %)[a, b]					
No	5 (39)	6 (32)	8 (50)	6 (40)	
Mild	2 (15)	4 (21)	6 (38)	2 (13)	0.002
Moderate	5 (39)	7 (37)	2 (13)	7 (47)	
Marked	1 (8)	2 (11)	0	0	
Total reads	45,586	71,148	80,595	59,350	-
Target reads (mean ± SD)	3,507 ± 2674.7	3,745 ± 3067.8	5,037 ± 3268.2	3,957 ± 2321.9	0.285
Read other than HP (mean ± SD)	3,504 ± 2672.3	3,742 ± 3067.6	1,417 ± 1185.2	1,731 ± 1804.7	0.003*
OTUs (mean ± SD)	154.8 ± 149.13	138,8 ± 106.12	150.1 ± 99.84	155.1 ± 129.36	0.947
Diversity index (mean ± SD)					
ACE	287.7 ± 353.16	244.0 ± 200.91	317.1 ± 321.86	315. ± 345.10	0.716
Chao 1	239.2 ± 251.98	213.6 ± 160.46	252.0 ± 212.25	252.3 ±232.03	0.897
Shannon index	2.64 ± 1.365	2.49 ± 1.414	2.04 ± 1.245	2.55 ± 1.250	0.545
Simpson index	0.27 ± 0.260	0.30 ± 0.286	0.41 ±0.280	0.29 ± 0.241	0.553
Goods library coverage	0.98 ± 0.015	0.98 ± 0.014	0.98 ± 0.012	0.98 ± 0.011	0.667

HP, *H. pylori*; IM, intestinal metaplasia; OTUs, operational taxonomic units; PG ratio, pepsinogen I/II ratio; a, Fisher's exact test; b, pathologic finding. NA, not assessed, *$p < 0.05$, Kruskal-Wallis test

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Pepsinogen I/II ratio reflecting gastric atrophy was higher in the HP (-) groups compared to the HP (+) groups ($p = 0.023$). In terms of degree of atrophy or intestinal metaplasia, there was no significant differences between cancer and control groups in HP (-) or HP (+) groups (Table 4). There were significant difference in neutrophil and monocyte infiltrations among 4 groups and it was due to HP status ($p < 0.001$, $p = 0.002$, respectively, Fisher's exact test). Average number of OTUs was not significantly different among 4 groups (Table 4). In addition, diversity indices, such as ACE, Chao1, Shannon diversity index, and Simpson diversity index were not different among 4 groups.

The composition of phylum in gastric antral mucosa was shown in Fig. 3. There was no significant difference in composition of microbiota among 4 groups. The proportion of *Proteobacteria* was lower in the cancer groups than in the control groups without statistical significance (60.6% vs. 70.2%, $p = 0.235$). Instead, the proportion of *Actinobacteria* was higher in the cancer groups (10.5% vs. 4.6%, $p = 0.043$).

The analysis of genus level is presented in Table 5. *Stenotrophomonas* genus was the most abundant in both HP (-) control and HP (-) cancer group, and the major species of the genus *Stenotrophomonas* was *Stenotrophomonas maltophilia*. In all 4 sub-groups, *Streptococcus* genus was ranked second. The unweighted UniFrac analysis indicated that there was very little separation between control and cancer groups under the identical HP infection status (Fig. 4).

3.6. Gastric antrum vs. corpus

Baseline characteristics of clinical and pyrosequencing results of 18 corpus samples are shown in Table 6. We analyzed the paired mucosa samples of the antrum and corpus (n = 17). Chao1 which reflect species richness was higher in corpus samples than that of antrum samples (530.1

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Figure 3 The composition of phylum in gastric antral mucosa. The microbiota composition is not significantly different among 4 groups by pyrosequencing method. The proportion of *Actinobacteria* was higher in the cancer groups (p = 0.043, Mann Whitney test). HP, *H. pylori*; P1 = HP (-) control, P2 = HP (+) control, P3 = HP (-) cancer, P4 = HP (+) cancer.

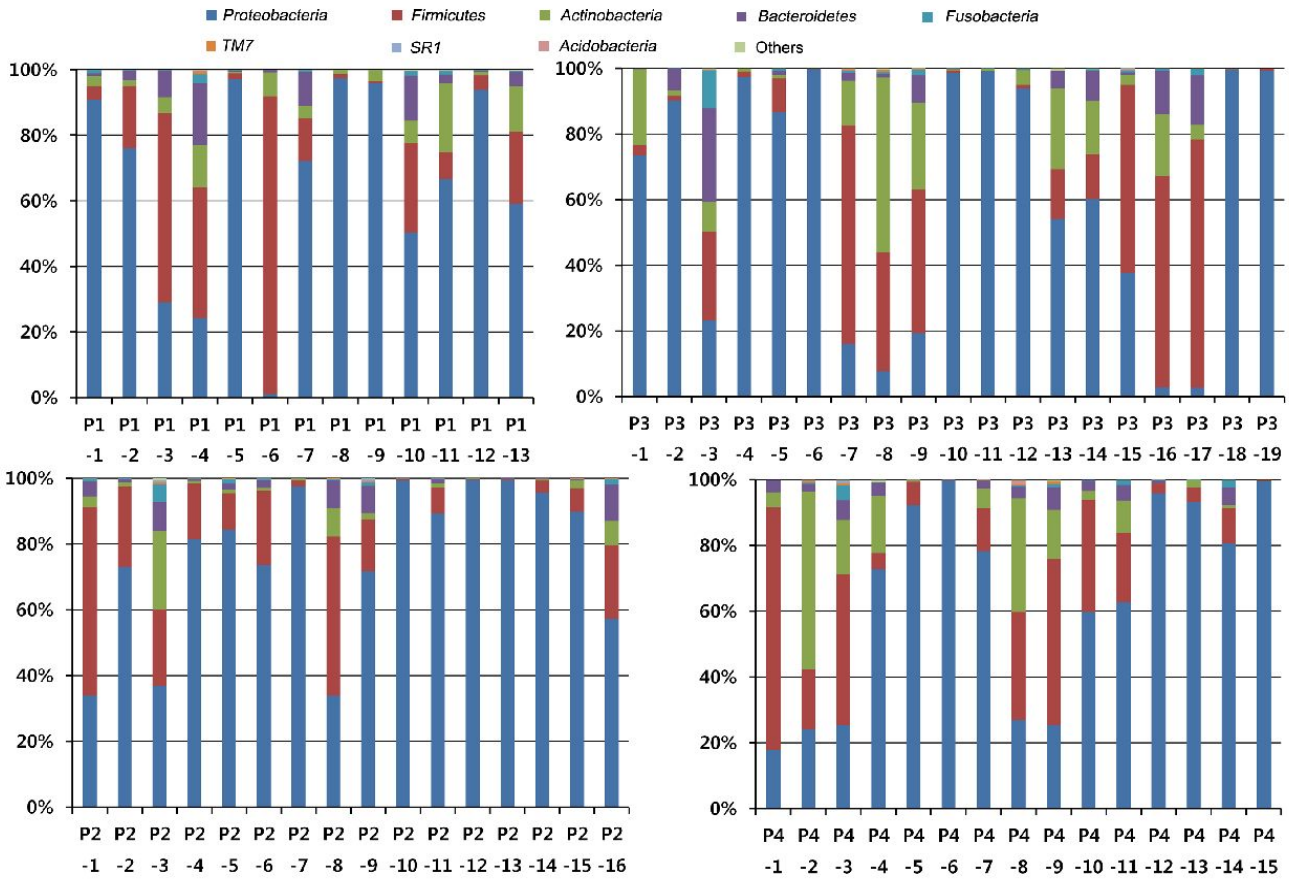

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Table 5. The order of genus frequency of gastric antral mucosal samples

Order of genus	HP (-) control (n = 13)	mean (%)	HP (-) cancer (n = 19)	mean (%)	HP (+) control (n = 16)	mean (%)	HP (+) cancer (n = 15)	mean (%)
1	*Stenotrophomonas*	25.91	*Stenotrophomonas*	26.25	*Helicobacter*	61.21	*Helicobacter*	44.95
2	*Streptococcus*	16.62	*Streptococcus*	15.52	*Streptococcus*	12.55	*Streptococcus*	15.30
3	*Ralstonia*	14.04	*Propionibacterium*	6.77	*Stenotrophomonas*	5.16	*Propionibacterium*	8.73
4	*Pseudomonas*	8.95	*Ralstonia*	5.28	*Haemophilus*	3.07	*Haemophilus*	3.89
5	*Prevotella*	3.32	*Citrobacter*	5.12	*Prevotella*	2.24	*Neisseria*	2.53
6	*Propionibacterium*	2.76	*Enterobacter*	4.72	*Sphingomonas*	1.99	*Methylobacterium*	2.11
7	*Schlegelella*	2.74	*Enhydrobacter*	4.03	*Propionibacterium*	1.95	*Prevotella*	2.09
8	*Veillonella*	2.11	*Prevotella*	3.17	*Bradyrhizobium*	1.46	*Veillonella*	1.72
9	*Haemophilus*	1.94	*Acidovorax*	2.22	*Veillonella*	1.20	*Stenotrophomonas*	1.67
10	*Actinomyces*	1.48	*Actinomyces*	2.18	*Neisseria*	1.08	*Actinomyces*	1.04
11	*Rothia*	1.23	*Veillonella*	1.83	*Actinomyces*	0.88	*Caulobacter*	0.87
12	*Neisseria*	1.21	*Methylobacterium*	1.61	*Gemella*	0.70	*Acinetobacter*	0.85
13	*Acinetobacter*	1.19	*Haemophilus*	1.53	*Fusobacterium*	0.61	*Bradyrhizobium_g1*	0.79
14	*Bradyrhizobium*	1.10	*Neisseria*	1.45	*Porphyromonas*	0.56	*Gemella*	0.74
15	*Caulobacter*	0.85	*Caulobacter*	1.39	*Granulicatella*	0.28	*Granulicatella*	0.64
16	*Pleomonas*	0.79	*Bradyrhizobium_g1*	1.02	*Methylobacterium*	0.25	*Escherichia*	0.60
17	*Methylobacterium*	0.64	*Pleomonas*	0.85	*GQ130066_g*	0.24	*Staphylococcus*	0.59
18	*Bradyrhizobium_g1*	0.58	*Gemella*	0.82	*Acinetobacter*	0.20	*Pseudomonas*	0.52
19	*Sphingomonas*	0.57	*Granulicatella*	0.78	*Rothia*	0.19	*Fusobacterium*	0.49
20	*Granulicatella*	0.52	*Pseudomonas*	0.73	*Atopobium*	0.16	*Klebsiella*	0.38

HP, *H. pylori.*

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Figure 4 Unweighted UniFrac-based principal coordinates analysis of gastric antral microbial communities. This analysis is based on taxa clustered at 97% sequence 16S rRNA gene identity. Samples obtained from patients of HP (-) control, HP (-) cancer, HP (+) control, and HP (+) cancer are represented by blue, red, green, and purple circles, respectively.

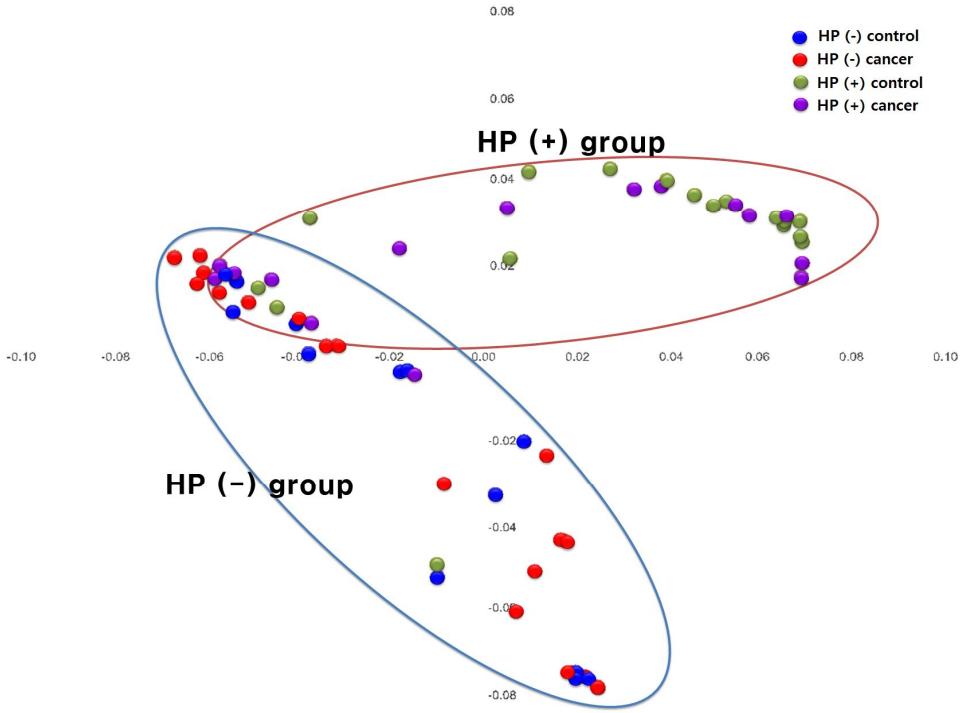

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Table 6. Baseline characteristics of clinical and pyrosequencing results of gastric corpus samples

	HP (-) control (n = 4)	HP (-) camcer (n = 2)	HP (+) control (n = 5)	HP (+) cancer (n = 7)	*p*-value
Age (mean ± SD, yr)	53.3 ± 13.72	48.8 ± 7.36	68.0 ± 9.90	53.6 ± 10.81	0.303
Male (n, %)[a]	2 (50.0)	1 (50.0)	2 (40.0)	4 (57.1)	1.000
PG I/II ratio (mean ± SD)	5.4 ± 1.06	2.4 ± 0.63	1.9 ± 2.04	3.1 ± 1.64	0.059
Atrophy (n, %)[a, b]					
No	2 (33.3)	1 (100)	3 (60)	2 (50)	
Mild	1 (66.7)	0	1 (20)	2 (50)	1.000
Moderate	0	0	1 (20)	0	
Marked	0	0	0	0	
IM (n, %)[a, b]					
No	3 (25)	0	5 (100)	4 (57.1)	
Mild	1 (75)	1 (50)	0	3 (42.9)	0.083
Moderate	0	1 (50)	0	0	
Marked	0	0	0	0	
Neutrophil infiltration (n, %)[a, b]					
No	4 (100)	1 (50)	1 (20)	1 (14.3)	
Mild	0	1 (50)	1 (20)	0	0.007
Moderate	0	0	2 (40)	6 (85.7)	
Marked	0	0	1 (20)	0	
Monocyte infiltration (n, %)[a, b]					
No	0	0	0	0	
Mild	3 (75)	1 (50)	1 (20)	2 (28.6)	0.812
Moderate	1 (25)	1 (50)	1 (20)	3 (42.8)	
Marked	0	0	3 (60)	2 (28.6)	
Total reads	25,619	10,643	47,793	56,546	-
Target reads (mean ± SD)	6,404 ± 4941.7	5,322 ± 999.1	9,559 ± 5536.2	8,078 ± 4042.5	0.567
OTUs (mean ± SD)	760.5 ± 479.62	582.5 ± 53.03	299.0 ± 294.87	218.6 ± 186.14	0.134
Diversity index (mean ± SD)					
ACE	2384 ± 1444.9	1541 ± 123.0	672 ± 627.6	617± 563.0	0.105
Chao 1	1590 ± 974.0	1095 ± 147.4	538 ± 520.7	442 ± 384.2	0.151
Shannon index	4.33 ± 0.910	4.45 ± 0.781	1.63 ± 1.460	1.74 ± 1.644	0.036*
Simpson index	0.06 ± 0.053	0.41 ± 0.018	0.59 ± 0.325	0.53 ± 0.361	0.047*
Goods library coverage	0.93 ± 0.020	0.94 ± 0.007	0.98 ± 0.014	0.98 ± 0.025	0.028*

HP, *H. pylori*; IM, intestinal metaplasia; OTUs, operational taxonomic units; PG ratio, pepsinogen I/II ratio; a, Fisher's exact test; b, pathologic finding. NA, not assessed * $p < 0.05$, Kruskal-Wallis test

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vs. 1245.5, p = 0.019, Mann-Whitney test). However, mean OTUs and other diversity indices including Shannon diversity index and Simpson diversity index were not significantly different between antrum and corpus samples (data not shown). In corpus samples, neutrophil infiltration was significant higher in HP (+) groups (p = 0.007, Fisher's exact test, Table 6). Shannon diversity index was higher and Simpson diversity index was closer to 0 in HP (-) groups which means more diverse than HP (+) groups (Table 6). The UPGMA dendrogram which clusters total antrum (a) and corpus (b) samples according to Fast UniFrac distance matrix. It was more clustered by HP infection status than by site; antrum or corpus. Antrum and corpus samples of B1, C1, C2, and D5 were closer than any other samples (Fig. 5).

3. 7. The relationship between intestinal metaplasia (IM) and microbiota

IM were classified to low degree IM group (grade 0 or 1) and high degree IM group (grade 2 or 3). HP was more abundant in the low degree IM group compared to the high degree IM group. In contrast, the proportion of the genus *Streptococcus* was increased in the high degree IM group. These differences were more pronounced in the control groups (Fig. 6).

3.8. Nitrosating bacteria and nitrate reducing bacteria

There was no statistical difference in the mean proportion of non-HP-NB among 4 groups (Table 7). However, the mean proportion of *Staphylococcus epidermidis* was higher in the cancer groups than in the control groups (0.191% vs. 0.088%, p =0.020, Mann Whitney test). The number of non-HP-NB was about two times higher in the cancer groups than in the control groups in the same HP status, there was no statistical significance (Table 7).

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Figure 5 The unweighted pair group method with arithmetic mean (UPGMA) dendrogram of antrum (a) and corpus (b) samples. HP (-) control, A1-A4; HP (-) cancer, B1-B2; HP (+) control, C1-C5; HP (+) cancer, D1-D6

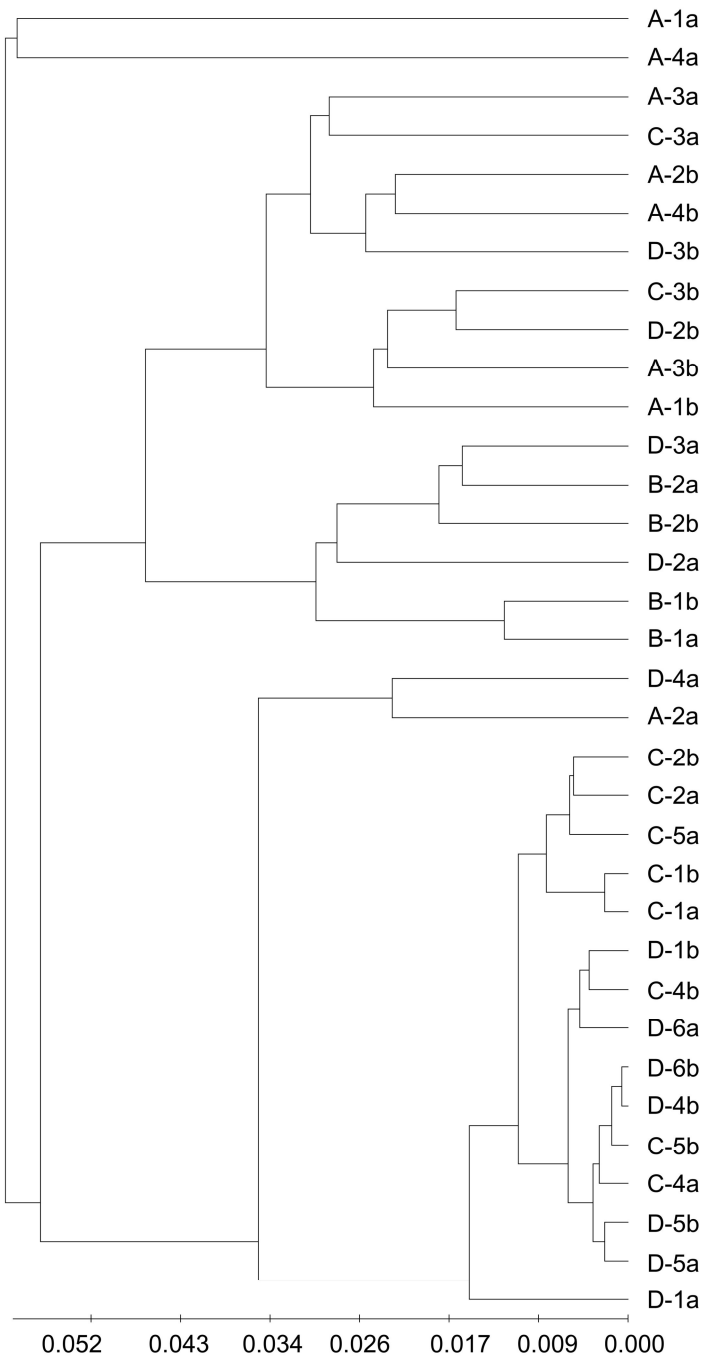

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Figure 6 The proportion of *H. pylori* and genus *Streptococcus* according to the degree of intestinal metaplasia (IM).

a. *H. pylori* was more abundant in the low degree IM group. b. Genus *Streptococcus* was increased in the high degree IM group. Grade 0 (none) or grade 1 (slight) IM were classified to low degree IM group and grade 2 (moderate) and grade 3 (marked) IM were classified into high degree IM group.

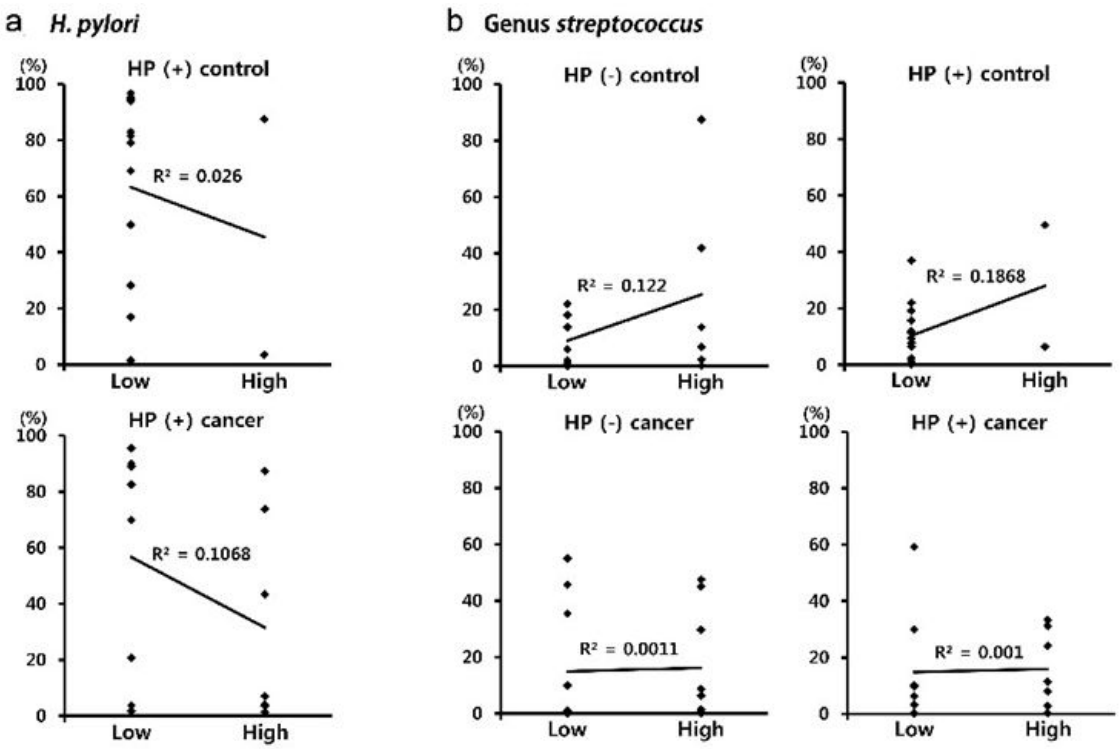

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Table 7. The mean proportion of nitrosating bacteria and nitrate reducing bacteria of gastric antral mucosal samples

	HP (-) control (n = 13)	HP (-) cancer (n = 19)	HP (+) control (n = 16)	HP (+) cancer (n = 15)	*p*-value
Total NB[a]	5.138	10.387	64.948	52.033	<0.001*
Non-HP-NB[b]	5.081	10.287	3.901	7.162	0.730
Helicobacter pylori	0.057	0.100	61.047	44.871	<0.001*
Citrobacter frueindi	0.000	3.257	0.057	0.000	0.650
Escherichia coli	0.158	0.197	0.023	0.524	0.400
Haeophilus influenzae	0.014	0.050	0.528	0.018	0.482
Haeophilus parainfluenzae	1.422	0.651	0.784	0.758	0.193
Kelbsiella pneumoniae	0017	0.176	0.000	0.364	0.192
Neisseria mucosa	0.038	0.102	0.114	0.145	0.668
Neisseria subflava	0.729	0.214	0.480	1.295	0.418
Pseudomonas stutzeri	0.018	0.000	0.000	0.005	0.429
Staphylococcus aureus	0.000	0.000	0.002	0.000	0.541
Staphylococcus epidermidis	0.008	0.118	0.095	0.284	0.121
Staphylococcus hominis	0.002	0.017	0.001	0.070	0.436
Veillonella atypica	0.928	0.8845	0.685	0.513	0.809
Veillonella dispar	0.998	0.772	0.391	1.063	0.805
Veillonella parvula	0.025	0.050	0.014	0.007	0.879
Xanthomonas campestris	0.019	0.000	0.028	0.016	0.138

HP, *H. pylori*; NB, nitrosating bacteria and nitrate reducing bacteria; a, Total nitrosating/nitrate reducing bacteria contain 67 species that have ability to reduce nitrate to nitrite or to form N-nitroso compounds. (The specific species are shown in the Table 1.); b, Non-HP-NB means all nitrosating/nitrate reducing bacteria other than HP.
*$p < 0.05$, Kruskal-Wallis test

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3.9. Changes in microbiota at follow-up

The mean duration between first and follow-up sample of 16 subjects was 52.7 months (range, 12-101 months). In the HP (-) group, the sum of non-HP-NB showed an increasing pattern at time of follow-up except one case (C-5) of HP (-) cancer group (Fig. 7a and 7c). That case was the only case that HP sequences increased above the cut-off value of 1%.

On the other hand, in the HP (+) control group, the sum of non-HP-NB decreased in the two HP-eradicated samples (B-3 and B-4, Fig. 7b), while it increased in the samples where HP eradication not tried (B-1) or failed (B-2, Fig. 7b). In the HP (+) cancer group, there was no reduction in the sum of non-HP-NB in cases where HP eradication failed (D-1, Fig. 7d), and even in cases where HP was successfully eradicated by therapy (Fig. 7d; D-3 and D-4).

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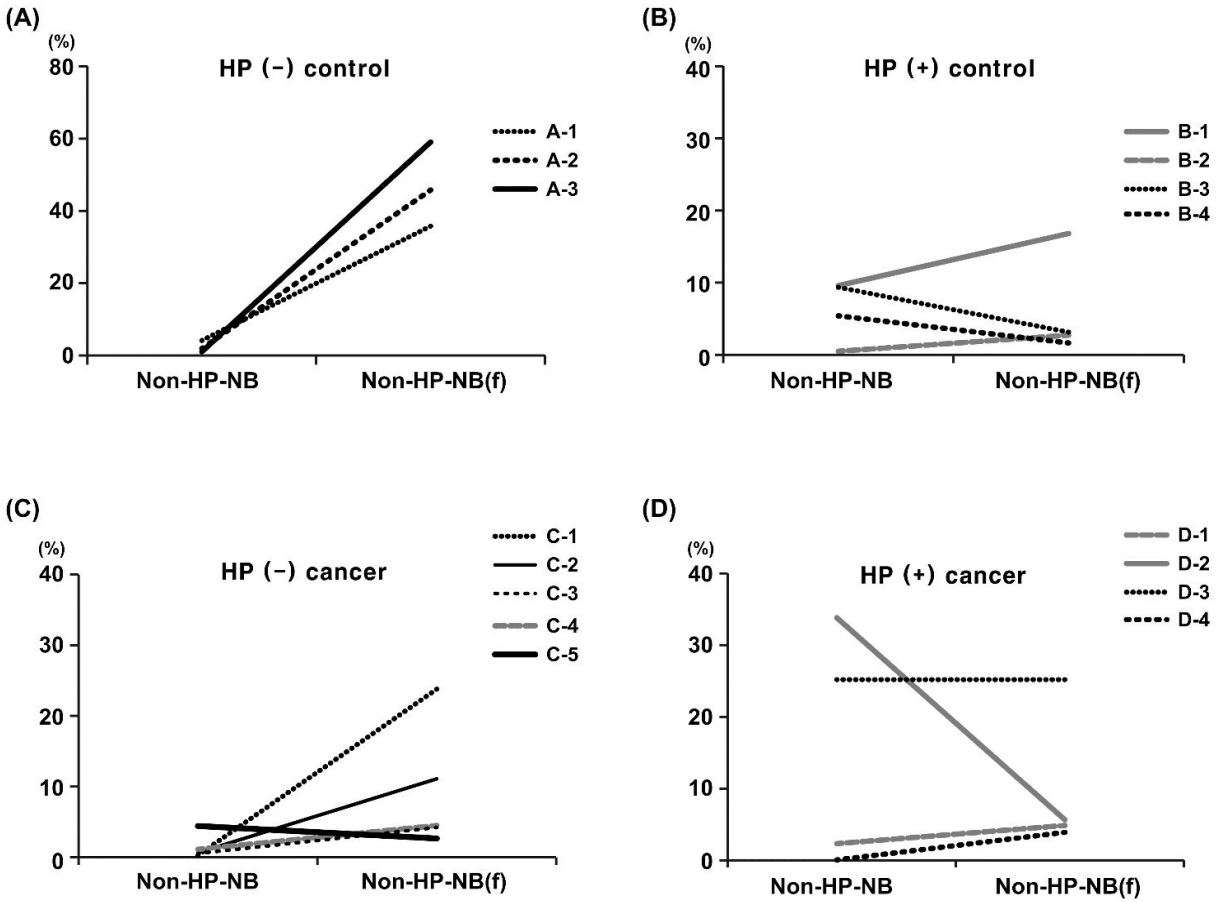
Figure 7 The changes of microbiota at follow-up.

a, c Usually, in the HP (-) groups, the sum of non-HP-NB was increased at follow-up. b In the HP (+) control group, the sum of non-HP-NB decreased in cases that received HP eradication therapy. d However, in the HP (+) cancer group, there was no reduction in the sum of non-HP-NB even in the cases that were treated with HP eradication therapy.

HP, *H. pylori*; Non-HP-NB, nitrosating bacteria or nitrate reducing bacteria other than HP of initial stage; non-HP-NB(f), nitrosating bacteria or nitrate reducing bacteria other than HP at follow-up stage.



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4. Discussion

Our group was interested in gastric microbiota with focus on relationship between HP and bacteria other than HP. We encountered difficulties in defining HP-negative samples because the pyrosequencing method frequently detected HP in those samples that were HP-negative based on conventional methods. There have been a growing number of studies on gastric microbiota (15-17, 20, 24, 31, 32). The first study reported that the gastric microbiota in healthy controls was more diverse than had been predicted (15). Another study compared human gut microbiota depending on location and HP status based on culture method (16). However, there was no specific mention on low abundance of HP in conventionally HP-negative samples (16). A study focused on gastric microbiota comparison between cancer patients and healthy controls reported only one HP-positive subject by both conventional methods and pyrosequencing (17). Some studies covered only HP-negative subjects by both the rapid urease test and 16S rRNA sequencing (19, 31).

The gastric microbiota in the present study was diverse and dominated by phyla *Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria,* and *Fusobacteria*, which is consistent with findings of previous studies (15-17, 19). *Helicobacter* was dominant genus in the conventionally HP-positive samples (15). *Streptococcus* or *Propionibacterium* have been reported as the major genera in conventionally HP-negative stomach (15-17, 19, 24). However, in contrast to previous studies (15, 18) *Lactobacillus* was found in only one sample in this study. This difference could be due to different dietary habits considering the fact that microbiota composition can be affected by host physiology as well as external environmental factors, including diet (20, 31). Additionally, different results in overall bacterial composition and the presence of specific bacteria could be influenced by methodological differences such as specific experimental design (31).

First, we have to determine the cut-off value of HP sequence percentage for HP colonization by

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pyrosequencing in HP-negative subjects based on conventional methods. HP sequences were detected in 60% of conventionally HP-negative subjects and the average percentage of HP sequences per sample was 0.67% compare to 58.51% of the conventionally HP-positive subjects. Our results correspond well with two earlier studies that have reported HP sequences in HP-negative subjects strictly determined by the combination of conventional methods (15, 18). In a temporal temperature gradient gel electrophoresis based study, HP-specific bands were detected in 2 of 5 conventionally HP-negative samples (18). Although HP sequences could not be precisely quantified, the band size was much smaller in conventionally HP-negative subjects (18). Another study detected HP from the 16S rRNA clone libraries of 7 of 11 conventionally HP-negative subjects (15). The average percent of HP reads was 11%, and it was lower than 72.3% of conventionally HP-positive subjects (15). The lower level of the mean percentage of HP sequences in our study comparing to that of the previous study might be attributed to different study population. Those previous studies did not include subjects with gastric cancer, while the HP sequence percentages were lower in cancer group. Technological advances in pre-data analysis or alignment method, as well as the mass sequencing technique itself might reduce the false-positivity. In addition, the possibility of cross-contamination with HP DNA during tissue processing might be lower in the present study.

The average percent of HP sequence in each sample was 0.67 % in subjects conventionally HP-negative and HP-positive by the pyrosequencing method. By ROC curve, HP sequences above 1.22% could be a threshold for HP-colonization. The percentage of HP sequence was between 1.00% and 1.22% in only one of the HP-negative subjects. Therefore, 1% could be used as the cut-off value for HP-positivity in ongoing studies on gut microbiota to determine HP status.

Then, we assessed to evaluate the differences in gastric microbiota depending on the HP infection status and the presence of cancer in 63 subjects with considering nitrosating or nitrate

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reducing bacteria to assess the role of bacteria other than HP by pyrosequencing method.

The phylotype analysis in the present study showed the presence of major phyla in the following order; *Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes*, and *Fusobacteria*. The pattern of phylum composition was identical in all of the four groups including HP (-) groups. This finding was quite different from the previous studies which reported that *Actinobacteria, Firmicutes*, and *Bacteroidetes* were the most abundant phyla in the HP-negative stomachs (15, 16, 19). Other studies showed that *Propionibacterium* and *Lactobacillus* accounted for the majority of cultivable bacteria in human stomach (24, 33).

In the culture-based study, the number of *Bifidobacteria, Lactobacilli, Veillonella*, and *Streptococcus* was increased in gastric cancer patients (34, 35). However, recent culture-independent studies did not show any difference between the bacterial community of cancer patients and that of the controls (17, 36, 37), except one study which shows relative abundance of *Streptococcaceae* family in HP-dominant gastric cancer groups than in controls (33). Similarly, there was no significant difference of microbial composition between cancer and control groups under the same HP infection status in the present study. We could find increased proportion of *Actinobacteria* in the cancer groups than control groups regardless of HP status.

The vast majority of human gastric microbiota was not different between the two anatomical sites (19, 20) . The microbiota diversity in the antrum showed no significant difference according to the HP infection status, whereas in the analysis using the corpus tissue (data not shown), stomach displayed a diverse microbiota when HP was absent or in low-abundance, which was similar to the previous studies (15, 16). These differences in microbiota may be related to different ambient pH in the corpus and antrum. HP-infection induces gastric atrophy and reduces the parietal cell mass, thus elevating the gastric pH, which predisposes to colonization by environmental microbiota (38). Accordingly, the pH of the corpus would be

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more influenced by HP infection than the pH of antrum, and this might explain the difference in microbial diversity between the antrum and corpus.

The formation of NOC has been suggested to increase the risk of gastric cancer (8, 17). Also, some bacteria of stomach have been known to be involved in the formation of the NOC (8, 10, 17). Based on this background, we re-analyzed the pyrosequencing results with a focus on the alleged nitrosating or nitrate reducing bacteria. In the HP (+) groups, *Staphylococcus epidermidis, Klebsiella pneumonia*, and *Neisseria flava* accounted for a larger part of gastric microbiota in the cancer group compared to the control group. Especially, it was interesting to observe that the genus *Enterobacter*, known as nitrosating bacteria (39), was detected only in the HP (-) cancer group (data not shown). Also, *Citrobacter freundii* with an ability to reduce nitrate and nitrite (10) was observed in one sample of the HP (-) cancer group with approximately 62% of all sequences in gastric flora. This finding might support the claim that not only HP but also other nitrosating or nitrate reducing bacteria attribute to gastric carcinogenesis. However, our present study did not show the significant role of bacteria other than HP in gastric cancer, although the total number of non-HP-NB was higher in the cancer groups than in the control groups. Interestingly, in the stomach cancer groups, there was a higher proportion of *Staphylococcus epidermidis* compared to control groups. As *Staphylococcus* was the most cultivable genera with *Propionibacterium, Lactobacillus*, and *Streptococcus*, and lower in sequencing studies (24), to prove the role of *S. epidermidis* in gastric carcinogenesis need further studies.

Furthermore, non-HP-NB showed an increasing trend at follow-up. In the HP (+) control group, the proportion of non-HP-NB was affected by HP eradication treatment. The number of non-HP-NB was decreased in the two HP-eradicated samples, while it was increased in other two HP-non-eradicated samples. However, this change was not consistently observed in the HP (+)

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cancer group.

The present study has some limitations. Although next generation sequencing (NGS) molecular approach is powerful and provides a complete view of the overall microbiota, it is unable to distinguish between living and dead bacteria (24, 39, 40). For a better understanding, conventional culturing would provide complementary results (24). However, the rapid growth bacteria covered the plate over the slowly growing bacteria in culturing method, and results were fluctuated according to culture environment that make difficult to prove it is causative bacteria which provoke disease or not. From this reason, we converted to NGS method from the culture based study. Secondly, long duration of storage may influence the some samples. However, we have controlled the other conditions such as DNA extraction, amplification, and pyrosequencing and processing. The third limitation is contamination of the biopsy channel with throat bacteria could not be completely ruled out. Even biopsy forcep has been sterilized in each endoscopy to prevent this contamination in the present study; still it is difficult to overcome this problem with the currently available clinical methods (31). The last limitation is that it is difficult to set the true HP (-) cancer group in the study. Detection rates of HP decrease with the progression of gastric atrophy and intestinal metaplasia (25). To overcome this problem, serologic (41, 42) and multiple biopsies (43) were performed here. In addition, considering false negative of conventional tests we apply 1% cut-off of pyrosequencing method to determine HP (-) group. Nevertheless, these processes could not make sure the true HP (-) cancer group. In addition, there was no significant difference in atrophy grade according to HP status on cancer group in this study.

In spite of these limitations, this study is valuable because we assessed the differences in microbiota composition from various aspects using a large number of samples. In conclusion, there was no significant difference of microbial composition between cancer group and control

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group, although non-HP-NB tends to increase in the cancer group. Bacteria other than HP, including non HP-NB may have a weak role compared to HP in gastric carcinogenesis.

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국문초록

서론: 16S rRNA 시퀀싱 방법의 발달로 위내 미세환경을 분석하는 방법이 개선되었다. 헬리코박터 파일로리는 위암의 위험인자로 잘 알려져 있으나, 그 외의 위내 세균의 역할에 대해서는 알려진 바가 적다. 본 연구에서는 차세대 시퀀싱 방법을 이용하여 헬리코박터균의 유무 및 위암의 유무에 따른 위내 세균 조성의 차이에 대해 알아보고자 하였다.

방법: 위암 환자와 대조군에서 얻은 위 전정부 조직 검체 63개와 위 체부 조직 검체 18개에서 454-파이로시퀀싱을 이용해 16S rRNA 분석을 시행하였다. 454-파이로시퀀싱 방법에서 헬리코박터균의 유무는 ROC 곡선을 통해 결정하였는데, 이 때 혈청 검사, 요소분해효소 검사, 배양 검사 및 조직학적 검사의 조합을 표준 검사로 이용하였다. 위 전정부 검체는 헬리코박터균의 유무 및 위암의 유무에 따라 4개의 그룹으로 나누어 분석하였다. 본 연구에서는 헬리코박터균과 그 이외의 세균, 특히 니트로화 물질을 발생시키는 세균에 주목해 분석하였다. 또한 16명의 환자는 1-8년 후 추적 내시경을 시행하여 혈청 검사, 요소분해효소 검사, 배양 검사, 조직 검사 및 파이로시퀀싱 검사를 통해 변화 추이를 분석하였다

결과: 454-파이로시퀀싱 방법에서 헬리코박터균의 존재 여부를 정하는 기준은 1%로 확인되었다. 위 전정부 검체는 이 기준에 따른 헬리코박터균의 유무 및 위암의 유무에 따라 4개의 그룹으로 나누어 분석하였다. 위 전정부의 세균 조성은 4개의 그룹 간에 통계적으로 유의한 차이가 없었다. 하지만 위암 환자와 대조군으로 나누어 분석하였을 때 방선균(*Actinobacteria*)이 대조군(4.6%)에 비해 위암

환자(10.5%)에서 높은 것을 확인할 수 있었다(p = 0.043). 위 전정부와 체부의 세균 조성에는 유의한 차이가 없었으며, 일부 검체를 제외하면 위내 세균은 위치보다는 헬리코박터균의 감염 여부에 따라 군집하는 경향을 보였다. 헬리코박터균 외에 니토로화 물질을 생성하는 세균은 4개의 그룹 간에 뚜렷한 차이가 없었으며, 대조군에 비해 위암 환자에서 더 많은 경향을 보였으나 통계적으로 유의한 차이는 없었다. 그 중에서 표피포도상구균(*Staphylococcus epidermidis*)은 위암 환자(0.191%)에서 대조군(0.088%)에 비해 더 많은 경향을 보였으며(p = 0.020, Mann Whitney test), 이에 대해서는 추가 연구가 필요하다. 추적 관찰에서 헬리코박터균 외에 니트로화 물질을 생성하는 세균은 증가하는 추세를 보였으나, 헬리코박터균 양성 대조군에서 헬리코박터 제균을 시행하면 이러한 추세를 예방할 수 있었다. 하지만 이러한 효과는 헬리코박터 양성 위암 환자에서는 확인할 수 없었다.

결론: 본 연구에서는 헬리코박터균과 비교해 다른 위내 세균이 위암의 발생 과정에서 의미 있는 역할을 하는 것을 확인하지 못하였다.

주요어: 위내세균조성, 헬리코박터 파일로리, 위암, 파이로시퀀싱, 차세대 시퀀싱

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