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Differences in Gastric Mucosal Microbiota Profiling in Patients with Chronic Gastritis, Intestinal Metaplasia, and Gastric Cancer Using Pyrosequencing Methods

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Abstract

Background: *Helicobacter pylori* (*H. pylori*) infection plays an important role in the early stage of cancer development. However, various bacteria that promote the synthesis of reactive oxygen and nitrogen species may be involved in the later stages. We aimed to determine the microbial composition of gastric mucosa from the patients with chronic gastritis, intestinal metaplasia, and gastric cancer using 454 GS FLX Titanium.

Methods: Gastric mucosal biopsy samples were collected from 31 patients during endoscopy. After the extraction of genomic DNA, variable region V5 of the 16S rRNA gene was amplified. PCR products were sequenced using 454 high-throughput sequencer. The composition, diversity, and richness of microbial communities were compared between three groups.

Results: The composition of *H. pylori*-containing *Epsilonproteobacteria* class appeared to be the most prevalent, but the relative increase in the *Bacilli* class in the gastric cancer group was noticed, resulting in a significant difference compared with the chronic gastritis group. By analyzing the *Helicobacter*-dominant group at a family level, the relative abundance of *Helicobacteraceae* family was significantly lower in the gastric cancer group compared with chronic gastritis and intestinal metaplasia groups, while the relative abundance of *Streptococcaceae* family significantly increased. In a UPGMA clustering of *Helicobacter*-dominant group based on UniFrac distance, the chronic gastritis group and gastric cancer group were clearly separated, while the intestinal metaplasia group was distributed in between the two groups. The evenness and diversity of gastric microbiota in the gastric cancer group was increased compared with other groups.

Conclusions: In *Helicobacter* predominant patients, the microbial compositions of gastric mucosa from gastric cancer patients are significantly different to chronic gastritis and intestinal metaplasia patients. These alterations of gastric microbial composition may play an important, as-yet-undetermined role in gastric carcinogenesis of *Helicobacter* predominant patients.

Gastric cancer is a global health problem, with high prevalence in the Far East, especially in Korea and Japan. In Correa's model of gastric carcinogenesis, chronic *Helicobacter pylori* (*H. pylori*) infection of the gastric mucosa progresses through the stages of chronic active gastritis, atrophy, intestinal metaplasia, and dysplasia before the development of gastric cancer [1]. It has been well known that *H. pylori* has an important role in gastric cancer development [2,3]. However, eradication of *H. pylori* does not guarantee the prevention of gastric cancer in patients with premalignant lesions including atrophy and intestinal metaplasia [4].

Persistent colonization of *H. pylori* can induce chronic inflammation of gastric mucosa that progresses

into atrophic gastritis, a condition that predisposes the stomach to reduced gastric acid secretion and the overgrowth of non-*Helicobacter* microbiota [5,6]. It has been postulated that some microbial species colonizing the atrophic stomach may further promote *H. pylori*-initiated gastritis, genotoxic damage, and the progression to gastric cancer [7,8]. However, their identities remain largely unknown.

Characterization of the gastric microbiota has traditionally relied on cultivation of gastric juice or mucosal biopsies. However, a large fraction of the microbes residing in the stomach have not yet been cultivated, suggesting a skewed presentation from culture-based studies. Recent advances in DNA sequence-based technologies allow genetic analysis of complex microbial populations without the need for prior cultivation [9,10]. Although a number of studies using culture-independent metagenomic techniques have been performed to elucidate the relation of dysbiosis (imbalances of gastrointestinal microbiota) with diverse small bowel and colonic diseases [11–13], very little research has investigated the microbial composition of the stomach using metagenomic approaches, particularly in its relation to gastric carcinogenesis [14–16].

In this study, we aimed to determine the microbial composition of gastric mucosa from the patients with chronic gastritis, intestinal metaplasia, and gastric cancer and to analyze any difference in the microbial composition between three groups using high-throughput sequencing platform, 454 GS FLX Titanium.

Materials and Methods

Study Population and Samples Collection

Thirty-one patients including 11 noncardia gastric cancer patients, 10 intestinal metaplasia patients, and 10 chronic gastritis patients were enrolled prospectively from March 2010 to May 2011. Exclusion criteria were as follows: age under 18 years; the presence of a serious illness such as severe cardiopulmonary, renal, or metabolic disease; prior medication history of antibiotics, acid blockers (proton-pump inhibitor and H_2 receptor antagonist), anti-inflammatory agents (aspirin, nonsteroidal anti-inflammatory drugs, and steroids), or probiotics for past 6 months; prior history of any surgical gastric resection; and refusal of consent to the study. Six gastric mucosal biopsies (three for microbial sequencing, two for histopathology, one for rapid urease test) were obtained from the corpus and antrum in each of 31 patients during upper endoscopy at Hanyang University Guri Hospital. Mucosal biopsies in gastric cancer patients were performed on the noncancerous gastric mucosa adjacent to the cancer tissue. Three biopsy samples for microbial sequencing were immediately stored at −80 °C. Diagnosis of gastric cancer and intestinal metaplasia were confirmed by histopathology. Chronic gastritis was defined as chronic inflammation of gastric mucosa with no evidence of intestinal metaplasia or atrophy on histopathology based on updated Sydney system [17]. Patients were determined to be *H. pylori* positive by positive result in one or more of two following conventional tests: rapid urease test and histopathology. All patients provided their written informed consent, and the study was approved by the institutional review board of Hanyang University Guri Hospital.

Bacterial DNA Extraction

Bacterial DNA was extracted from a total of 31 gastric mucosal tissues using a phenol/chloroform extraction method combined with physical disruption of bacterial cells and a DNA clean-up kit (Qiagen DNeasy Blood and Tissue extraction kit; Qiagen, Valencia, CA, USA) as previously described [18]. Briefly, 100 mg of frozen gastric mucosal tissues was suspended in 750 μL of sterile bacterial lysis buffer (200 mmol/L NaCl, 100 mmol/L Tris [pH 8.0], 20 mmol/L EDTA, 20 mg/mL lysozyme) and incubated at 37 °C for 30 minutes. Next, 40 μL of proteinase K (20 mg/mL) and 80 μL of 10% SDS were added to the mixture and incubated at 65 °C for 30 minutes. Homogenization was accomplished by adding 300 mg of 0.1 mm zirconium beads (BioSpec Products, Bartlesville, OK, USA) and bead beating for 90 seconds at 2045 $\times g$ (TeSeE process 48; Bertin Technologies, Le Bretonneux, France). The homogenized mixture was cooled on ice and then centrifuged at 14,270 $\times g$ for 5 minutes. Bacterial DNA was extracted from supernatant by phenol/chloroform/iso-amyl alcohol (25 : 24 : 1) and then chloroform/iso-amyl alcohol (24 : 1). The supernatant was precipitated by absolute ethanol at −20 °C for 1 hour. The precipitated DNA was suspended in DNase free H_2O and then cleaned up using the DNeasy Blood and Tissue extraction kit (Qiagen) according to the manufacturer's instructions. Prior to microbial characterization, isolated DNA was stored at −80 °C.

PCR Amplification and 16S rRNA Gene Sequencing

We amplified a 16S rRNA gene fragment of 280 base pairs, which includes the V5 hypervariable region by 35-cycle PCR. The modified forward primer 784F 5′-CGTATCGCCTCCCTCGCGCCA**TCAG**-MID-*AGGATTAGATACCCTGGTA*-3′ consists of the GS FLX Titanium adapter sequence A (underlined sequence), linker nucleotides (TCAG), multiplex identifiers sequence (MID),

and the universal 16S rRNA-specific sequence (italic sequence). The reverse primer 1061R 5′-CTATGCGCCTTGCCAGCCCGC**TCAG**-MID-*CRRCACCGAGCTGACGAC*-3′ consists of the GS FLX Titanium adapter sequence B (underlined sequence), linker nucleotides (TCAG), multiplex identifiers sequence (MID), and the universal 16S rRNA-specific sequence (italic sequence). Cycling conditions were 95 °C for 5 minutes, 35 cycles of 95 °C for 40 seconds, 57 °C for 40 seconds, and 72 °C for 60 seconds, followed by 72 °C for 60 seconds. 16S rRNA PCR products were quantified, pooled, and purified for the sequencing reaction. 454 GS FLX Titanium sequencing was performed in different lanes of a PicoTiterPlate using a 454 Life Sciences Genome Sequencer FLX machine (Roche, Florence, SC, USA) according to the manufacturer's instructions.

Analysis of 16S rRNA Sequences

Massive partial reads (V5 region) of 16S rRNA generated by the 454 GS FLX Titanium sequencer were initially trimmed for quality using standard the *sff* software tools from Roche/454. Reads were preliminarily assessed and removed for possible human contaminant from the amplification process by performing a BLASTIN search against the human genome sequences. Bacterial 16S rRNA sequence data of gastric mucosal microbiota were processed through mothur pipeline [19]. PyroNoise was used for the removal of base-call error [20]. Reads were excluded from the analysis if they had low quality, contained incorrect primer sequences, or contained more than one ambiguous base. The remaining reads were sorted into chronic gastritis, intestinal metaplasia, and gastric cancer groups based on their unique nucleotide barcodes [21]. Chimeric sequences were filtered out using the UCHIME algorithm after nearest alignment space termination (NAST) based on SILVA database [22,23]. High-quality reads were taxonomically assigned using RDP classifier with 0.8 confidence threshold and used to construct the distance matrix and evaluate the abundance of observed operational taxonomic units (OTUs) into which were clustered at 3% dissimilarity in each sample [24]. The bacterial evenness and diversity indices (Shannon index) were calculated using estimators in mothur. Also, representative reads of each OTUs were used to calculate a UniFrac distance and compare samples using UniFrac program [25].

Nucleotide sequence accession number. The DNA sequences from this metagenomic project have been deposited in the NCBI Short Read Archive under the Accession No. SRP038955.

Results

Patient Characteristics and *H. pylori* Status

A total of 31 patients including 11 patients with noncardia gastric cancer, 10 intestinal metaplasia patients, and 10 chronic gastritis patients were enrolled. The average age and men to women ratio were 50.4 ± 11.5 years and 4 : 6 in the chronic gastritis group, 57.5 ± 7.3 years and 7 : 3 in the intestinal metaplasia group, and 65.7 ± 11.3 years and 6 : 5 in the gastric cancer group.

In this study, eighteen of 31 patients were positive for *H. pylori* by conventional laboratory methods including rapid urease test and histopathology. *H. pylori*-positive rates in chronic gastritis, intestinal metaplasia, and gastric cancer group were 70, 40, and 64%, respectively. On the contrary, analysis of microbial profiling based on 16S rRNA gene sequence revealed <1% of *Helicobacteraceae* family in nine of 31 patients. However, there was a significant difference in the relative abundance of *Helicobacteraceae* family between the *H. pylori*-positive patients and the *H. pylori*-negative patients by conventional methods (82.5 vs 11.3%). In four patients, the results from conventional methods and microbial profiling did not match. Interestingly, 86% (6/7) of gastric cancer patients who were positive for *H. pylori* by conventional tests showed positive result for *H. pylori* in rapid urease test, but not in histopathology. On the other hand, 100% (7/7) of chronic gastritis patients and 75% (3/4) of intestinal metaplasia patients who were positive for *H. pylori* by conventional tests showed positive results for *H. pylori* in both rapid urease test and histopathology (Table 1).

Comparison of Gastric Microbiota Composition Between Three Groups

In this study, 16S rRNA gene amplicon pyrosequencing yielded a total of 107,350 reads, which were matched to 40,328, 33,971, and 33,051 reads for 10 chronic gastritis patients, 10 intestinal metaplasia patients, and 11 patients with gastric cancer, respectively. A diverse community of 10 phyla and 220 genera (146 genera for chronic gastritis group, 136 intestinal metaplasia group, and 162 gastric cancer group) was identified from a total of 83,871 OTUs. The number of reads did not significantly differ between three groups. On average, small subunit rRNA reads were clustered into 2705 ± 1471 phylotypes per sample when reads were clustered at the 3% dissimilarity level. Good's coverage

Table 1 Demographic characteristics and *H. pylori* status for the enrolled patients

Sample	Group	Age (yrs)	Gender	Conventional test for *H. pylori*		Composition of *Helicobacteraceae* family (%)	Sample	Group	Age (yrs)	Gender	Conventional test for *H. pylori*		Composition of *Helicobacteraceae* family (%)
				RUT	Histology						RUT	Histology	
10-1	Cancer	68	F	(−)	(−)	45.63	11-1	Gastritis	51	F	(+)	(+)	94.19
10-2	Gastritis	49	M	(−)	(−)	0.75	11-2	Cancer	62	M	(+)	(+)	83.19
10-3	Cancer	75	M	(+)	(−)	87.68	11-3	Metaplasia	63	F	(+)	(+)	93.91
10-4	Gastritis	75	F	(+)	(+)	97.13	11-4	Metaplasia	47	M	(−)	(−)	87.20
10-5	Metaplasia	63	F	(+)	(+)	86.34	11-5	Cancer	51	M	(−)	(−)	0.10
10-6	Cancer	77	F	(+)	(−)	76.23	11-6	Cancer	74	M	(+)	(−)	65.86
10-7	Cancer	78	F	(−)	(−)	0.81	11-7	Cancer	71	M	(+)	(−)	18.19
10-8	Cancer	46	F	(+)	(−)	70.62	11-8	Gastritis	36	M	(+)	(+)	99.41
10-9	Metaplasia	55	M	(−)	(−)	4.55	11-9	Cancer	69	F	(+)	(−)	62.36
10-10	Gastritis	49	F	(+)	(+)	94.44	11-10	Gastritis	35	M	(+)	(+)	66.95
10-11	Metaplasia	69	M	(−)	(−)	0.80	11-11	Metaplasia	54	F	(+)	(+)	95.74
10-12	Gastritis	51	F	(+)	(+)	96.90	11-12	Gastritis	58	F	(−)	(−)	0.08
10-13	Gastritis	44	M	(+)	(+)	95.94	11-13	Gastritis	56	F	(−)	(−)	0.04
10-14	Cancer	52	M	(−)	(−)	0.68	11-14	Metaplasia	59	M	(−)	(−)	0.01
10-15	Metaplasia	48	M	(+)	(−)	99.78	11-15	Metaplasia	53	M	(−)	(−)	0.01
10-16	Metaplasia	64	M	(−)	(−)	5.77							

H. pylori, *Helicobacter pylori*; RUT, rapid urease test; M, male; F, female; yrs, years.

was ranged from 0.82 to 0.98 at all prokaryotic communities.

By analyzing the gastric microbial composition at a class level, the composition of *Epsilonproteobacteria* group that contains *H. pylori* appeared to be the most prevalent, but the relative increase in the *Bacilli* group, which contains *Streptococci* and *Lactobacilli*, in the patients with gastric cancer was also noticed, resulting in a significant difference compared with the chronic gastritis patients (Fig. 1). In subclass analysis of *Epsilonproteobacteria* into species level, *Epsilonproteobacteria* class in most of the enrolled patients (especially in *H. pylori*-positive patients by conventional methods) were predominantly composed of *H. pylori* species (see Table S1 in Supporting information).

It is well known from previous studies [26,27] that the clinical features and prognosis of gastric cancer may differ according to the *H. pylori* status; therefore, we have analyzed separately the patients with *Helicobacter* dominance. Excluding patients with the class *Epsilonproteobacteria* constituting <5% of the bacterial composition, the gastric cancer group showed a significant decrease in the relative abundances of *Epsilonproteobacteria* compared with the chronic gastritis and intestinal metaplasia group, while the relative abundances of *Bacilli* class increased significantly in the gastric cancer group (see Fig. S1 in Supporting information). By analyzing the *Helicobacter*-dominant group at a family level, it was noticed that the relative abundance of *Helicobacteraceae* family was significantly lower in the gastric cancer group compared with chronic gastritis and intestinal metaplasia group, while the relative abundance of *Streptococcaceae* family significantly increased in the gastric cancer group (see Fig. S2 in Supporting information). On the other hand, in the *Helicobacter* nondominant group, there was no significant difference in the relative microbial abundances between three groups, and the relative abundance of *Streptococcaceae* family was increased (data not shown).

By comparing the gastric microbial profiles between *H. pylori*-positive and *H. pylori*-negative chronic gastritis patients, the relative abundances of family *Bradyhizobiaceae*, *Caulobacteraceae*, *Lactobacillaceae*, and *Burkholderiaceae* were significantly higher in *H. pylori*-negative patients compared with *H. pylori*-positive patients (see Fig. S3 in Supporting information). In *H. pylori-negative* patients, diverse bacterial groups belonging to the class *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Bacilli*, *Bacteroidia*,

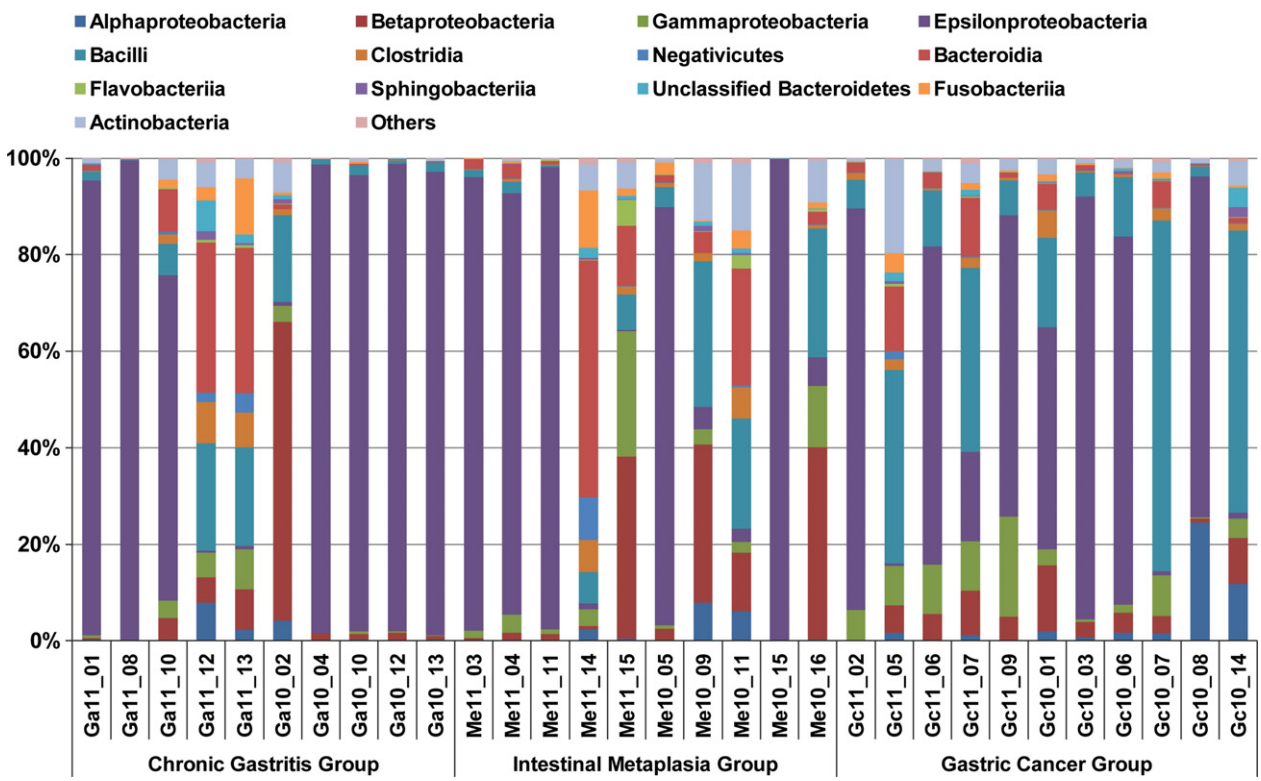

Figure 1 Relative bacterial abundance of gastric mucosa at the class level, identified by 454 pyrosequencing of the V5 16S rRNA gene region, in patients with chronic gastritis, intestinal metaplasia, and gastric cancer.

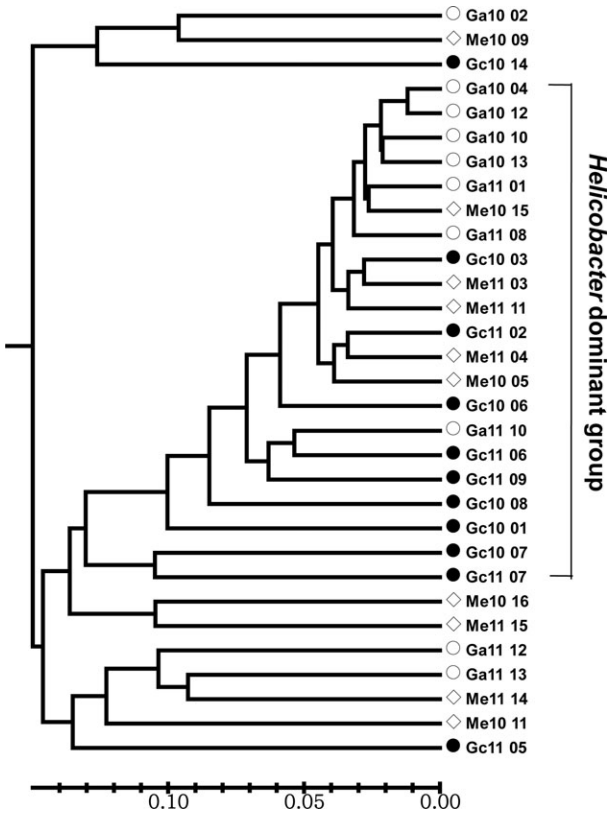

Figure 2 UPGMA clustering of gastric mucosal samples based on UniFrac distance from the patients with chronic gastritis, intestinal metaplasia, and gastric cancer. *Helicobacter*-dominant group included the patients with *Epsilonproteobacteria* class constituting <5% of the bacterial composition. Ga, chronic gastritis; Me, intestinal metaplasia; Gc, gastric cancer.

Clostridia, *Flavobacteria*, *Fusobacteria*, or *Negativicutes* were found as major taxa.

We performed a clustering based on UniFrac distance, and it was possible to perform clustering of *Helicobacter*-dominant group. In the *Helicobacter*-dominant group, the chronic gastritis group and gastric cancer group were clearly separated, while the intestinal metaplasia group was distributed in between the two groups (Fig. 2).

Comparison of Diversity of Gastric Microbiota Between Three Groups

To evaluate the bacterial evenness and diversity between the three groups, Shannon's index was compared, and the evenness and diversity of gastric microbiota in the gastric cancer group was increased compared with the chronic gastritis and intestinal metaplasia group. In addition, when looking at the *Helicobacter*-dominant group, the evenness and diversity of gastric microbiota in the gastric cancer group was distinctively increased compared with the chronic gastritis and intestinal metaplasia group (Fig. 3).

Discussion

In the present study, we investigated the differences in microbial composition of gastric mucosa from the patients with chronic gastritis, intestinal metaplasia, and gastric cancer. We identified a diverse microbial community of 10 phyla and 220 genera, demonstrating greater diversity than previously reported [14,15].

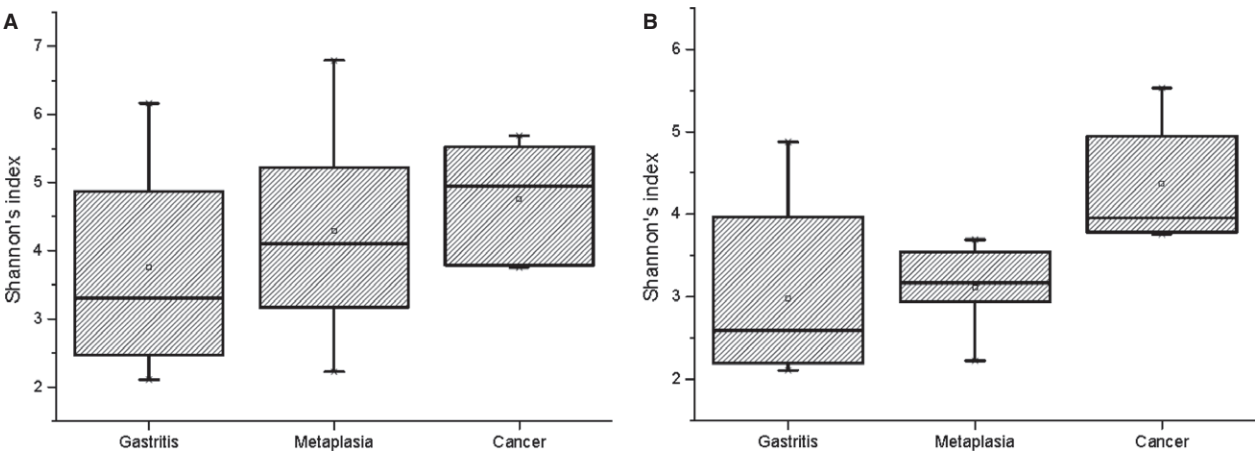

Figure 3 (*A*) Comparison of the Shannon's index of gastric mucosal samples from patients with chronic gastritis, intestinal metaplasia, and gastric cancer, based on 454 pyrosequencing data. (*B*) Comparison of the Shannon index of gastric mucosal samples from *Helicobacter*-dominant patients with chronic gastritis, intestinal metaplasia, and gastric cancer, based on 454 pyrosequencing data.

There were significant differences in the composition and diversity of gastric microbiota among patients with gastric cancer, intestinal metaplasia and chronic gastritis, especially in *Helicobacter*-dominant group.

In this study, we performed a bacterial 16S rRNA sequencing using high-throughput sequencing platform, 454 GS FLX Titanium, while previously reported two studies for molecular analysis of gastric microbiota used ABI 3730 capillary sequencer and T-RFLP with 16S rRNA sequencing method, respectively [14,15]. This so-called massively parallel sequencing technique used here has the ability to obtain far more sequence data at a short period of time [28]. We obtained a total of 107,350 reads matched into 83,871 OTUs and a diverse microbial community of 10 phyla and 220 genera were identified. These results could contain virtually all of the prevalent gastric microbial genes in our samples and would provide a basic and parallel data for future molecular studies of gastric microbial community.

It has been well known that *H. pylori* has an important role in gastric cancer development and persistent *H. pylo*ri infection of gastric mucosa progresses over decades through sequential stages of chronic inflammation, atrophy, intestinal metaplasia, dysplasia, and cancer [1–3]. Although *H. pylori* eradication significantly decreases cancer risk in patients without premalignant lesions such as atrophy and intestinal metaplasia, the benefit may be minimal to patients with established premalignant lesions [4]. In addition, *H. pylori* has been known to colonize poorly in atrophic and metaplastic stomach compared with stomach of normal gastric mucosa [29,30]. Our data showed that relative abundance of family *Helicobacteraceae* was significantly decreased in the gastric cancer group compared with the chronic gastritis group. Moreover, in a UPGMA clustering of *Helicobacter*-dominant group, the chronic gastritis group and gastric cancer group were clearly separated, while the intestinal metaplasia group was distributed in between the two groups. Taken together, these results suggest that *H. pylori* may be more important as a trigger for the development of atrophy and hypochlorhydria, a favorable environment for gastric cancer development, than as a direct inducer of gastric cancer.

In the present study, all patients (18/31) who were positive for *H. pylori* by conventional tests showed positive results for *H. pylori* (more than 1% of *Helicobacteraceae* family) in microbiome analysis. On the contrary, among 13 patients who were negative for *H. pylori* by conventional tests, four patients showed positive results for *H. pylori* in microbiome analysis, suggesting that this discrepancy might be due to patchy distribution of *H. pylori* or false-negative results of conventional tests. Interestingly, 86% (6/7) of patients with gastric cancer who were positive for *H. pylori* by conventional tests showed positive result for *H. pylori* in rapid urease test, but not in histopathology. On the other hand, 100% (7/7) of chronic gastritis patients and 75% (3/4) of intestinal metaplasia patients who were positive for *H. pylori* by conventional tests showed positive results for *H. pylori* in both rapid urease test and histopathology, suggesting that false-positive results of rapid urease test in patients with gastric cancer may be due to overgrowth of urease positive non-*Helicobacter* bacterial population rather than *H. pylori* population. In this study, the positive rate of *H. pylori* by conventional tests in chronic gastritis patients was 70%, which was lower than expected. This might be due to patchy distribution of *H. pylori*, small sample size, or decreased prevalence of *H. pylori* infection in Korean population [31].

Our study characterized the significant difference in gastric microbial composition among patients with gastric cancer, intestinal metaplasia, and chronic gastritis for the first time utilizing high-throughput molecular approach. The composition of *H. pylori*-containing *Epsilonproteobacteria* class appeared to be the most prevalent, but the relative increase in the *Bacilli* class in the gastric cancer group was noticed, resulting in a significant difference compared with the chronic gastritis group. By analyzing the *Helicobacter*-dominant group at a class and family level, the gastric cancer group showed a significant decrease in the *Epsilonproteobacteria* class and *Helicobacteraceae* family compared with the chronic gastritis and intestinal metaplasia group, while the *Bacilli* class and *Streptococcaceae* family increased significantly. On the other hand, previous study for the microbial composition of human stomach by Dicksved, et al. [15] using T-RFLP with bacterial sequencing method demonstrated that there was no significant difference in microbial composition between patients with gastric cancer and controls, while phylum *Firmicutes* and genera *Streptococcus*, *Lactobacillus*, *Veillonella,* and *Prevotella* were predominant in patients with gastric cancer . We assume that the difference in dominant species and relative abundance of gastric microbiota between our study and previous study are most likely due to the difference in the *H. pylori* infection rates and several environmental factors including diet and lifestyle between East Asian with high prevalence of *H. pylori* and western population, in addition to the difference in the molecular sequencing methods.

Although our study showed significant difference in gastric microbial composition among patients with gastric cancer, intestinal metaplasia, and chronic gastritis, it does not mean that dysbiosis of gastric microbiota

induces development of gastric cancer or plays an important role in gastric carcinogenesis. Whether these changes in microbial composition are primary or secondary events remains to be determined.

Several previous studies have suggested that a variety of non-*Helicobacter* bacteria colonize in atrophic and hypochlorhydric stomach, and some of these bacteria can lead to increased conversion of dietary nitrates into carcinogenic N-nitroso compounds and release reactive oxygen species, thus promoting gastric cancer [5–8]. Moreover, overgrowth of some bacterial species may enhance inflammatory responses accelerating atrophy, metaplasia, and cancer [32,33]. Recent animal study by Lofgren et al. [34] demonstrated that the absence of commensal flora in *H. pylori*-infected transgenic FVB/N insulin-gastrin (INS-GAS) mice delayed the development of intraepithelial neoplasia and changes in gastric microbiota composition might promote intraepithelial neoplasia in achlorhydric stomach of mice. In this study, we could not determine any significant difference in the total abundance of gastric microbiota between three groups. In addition, the evenness and diversity of gastric microbiota in the gastric cancer group was increased compared with the chronic gastritis and intestinal metaplasia group. These results suggest that the change in gastric commensal flora, not a specific pathogen, might play an important role in gastric carcinogenesis.

Interestingly, in the present study, relative abundance of *Streptococcaceae* family was higher in gastric cancer patients compared with intestinal metaplasia and chronic gastritis patients, which is in agreement with previous study by Dicksved, et al. [15] that reported the presence of 16S rRNA gene of *Streptococcus* species in gastric cancer patients. It has been demonstrated that *Streptococcus bovis* is associated with colorectal cancer and its antigens can promote cancer in animal model [35,36]. Whether these bacterial species have any potential role in gastric cancer development will require further studies to be confirmed.

The present study faces several limitations. First of all, although this study was performed prospectively, the patients were not enrolled consecutively because there was a limitation regarding the number of enrolled patients due to the extraordinary cost of pyrosequencing methods. Therefore, there is a possibility of selection bias for patient enrollment and the findings of this study can be regarded as preliminary. Further prospective study with a large population is required to confirm our data regarding such bacterial profiles of gastric mucosa. Secondly, the impact of diet on the bacterial composition of gastric mucosa was not evaluated in the present study. Diet has been known to affect the mucosal bacterial profile of stomach as well as small and large intestine [37–39]. However, considering the fact that Korean people have been exposed to a similar diet pattern for a long time, our data may reflect unique bacterial profiles of gastric mucosa from the Korean population with high prevalence of *H. pylori*.

In summary, we found for the first time that there are significant differences in composition of gastric microbiota among patients with gastric cancer, intestinal metaplasia, and chronic gastritis, especially in *Helicobacter*-dominant patients, and there are potential differences in microbial composition between East Asian and western population. In our study, when looking at the *Helicobacter*-dominant group, the gastric cancer group showed a significant decrease in the *Epsilonproteobacteria* class and *Helicobacteraceae* family compared with the chronic gastritis and intestinal metaplasia group, while the *Bacilli* class and *Streptococcaceae* family increased significantly. Further studies for functional role of these dysbiosis of gastric microbiota on gastric carcinogenesis such as studies using gnotobiotic mice with a defined group or a specific bacterium are warranted.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Relative abundances of *Epsilonproteobacteria* in gastric microbial communities of enrolled patients.

Fig. S1 Relative bacterial abundance of gastric mucosa at the class level, identified by 454 pyrosequending of the V4 16S rRNA gene region, in *Helicobacter*-dominant patients with chronic gastritis, intestinal metaplasia and gastric cancer.

Fig. S2 Average abundance of the *Helicobacteraceae* and the *Streptococcaceae* family in gastric mucosal samples from the Helicobacter-dominant patients with chronic gastritis, intestinal metaplasia and gastric cancer, based on 454 pyrosequencing data.

Fig. S3 Comparison of the gastric microbial profiles between *H. pylori* positive and *H. pylori* negative chronic gastritis patients.

