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Original Article

Microbial Profile of the Stomach: Comparison between Normal Mucosa and Cancer Tissue in the Same Patient

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Gastric cancer is the third most common cancer and the third most frequent cause of cancer mortality in Asia. It is predicted that gastric cancer will remain an important cause of death at least during the next half century because of the increasing number of new cases in an aging population. However, little has been revealed about the role of gastric microbes and their reaction to gastric cancer. In this study, we identified differences in the microbial communities between gastric cancer and normal gastric mucosa by comparing the microbiomes of tissues from the same patients. The clustering analysis results showed different bacterial communities between normal gastric mucosa and gastric cancer. A comparison of bacterial communities at the species level revealed that *Helicobacter pylori* was significantly reduced in cancer tissue compared to that in normal gastric mucosa in the same patient. A comparison at the genus level showed that *Propionibacterium* spp., *Staphylococcus* spp., and *Corynebacterium* spp. had significantly reduced populations in cancer tissue, whereas *Clostridium* spp. and *Prevotella* spp. had significantly increased populations in cancer tissue.

Key Words: Gastric cancer, *Helicobacter pylori*, Microbiome

INTRODUCTION

The stomach was considered sterile for a long time because of the acidic environment until *Helicobacter pylori* was found in the 1980s (1). Since then, *H. pylori* have been identified as a major causative agent of gastric adenocarcinoma (2, 3). In addition, the presence of microorganisms besides *H. pylori* has been demonstrated based on culture-dependent methods. New non-culturable microbes have been discovered through DNA-based approaches (4, 5). However, we have limited knowledge about the stomach microbiome, as these organisms are highly diverse between patients and among experimental studies (6). Contamination of ingested microorganisms from the mouth and throat also makes study difficult (7).

Gastric cancer is the third most common cancer and the third most frequent cause of cancer mortality in Asia (8). Many reports are available about the mechanism by which *H. pylori* induces gastric adenocarcinoma (9), but little is known about the role of other microorganisms in stomach cancer incidence (10). Moreover, not much is known about changes in the microbial communities including *H. pylori* after the occurrence of gastric cancer. Many studies have focused on changes in the microbiome due to increased gastric pH from proton-pump inhibiting drugs (11, 12) or atrophic gastritis as a result of *H. pylori* infection (13). Dicksved *et al*. investigated gastric microbiota from 10

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patients with gastric cancer and compared them with gastric microbiota from five dyspeptic control patients who had normal gastric mucosa morphology (14). However, because of the high diversity in the gastric microbiomes between individuals, it was difficult to assess the effect of gastric cancer on the microbiome. Therefore, in this study, we identified differences in microbial communities between gastric cancer and normal gastric mucosa by comparing them from the same patients using a culture-independent method.

MATERIALS AND METHODS

Data source

Thirty-two RNA sequencing data of gastric cancer and adjacent normal gastric mucosa from 16 patients in Korea were obtained from NCBI Sequence Read Archive with accession number SRP014574. Detailed information about the patients and samples were referenced from the publication by Yoon (15), and described in Tables 1 and 2. From the analysis of normal gastric mucosa in 16 patients, 11 were considered *H. pylori* carriers whose *H. pylori* bit score accounted for more than 10% of the total bit score; thus, the sequence analysis results of those 11 patients were used for further statistical testing.

Bacterial sequence detection from the raw data

The computational subtraction method was applied to detect the pathogen from sequence data (16, 17) with a modification. Raw data sequences were aligned to the hg19 human reference genome (NCBI) using TopHat (18). A total of 62~88% of all reads was mapped to the human

Table 1. Clinical information for the samples used in the analysis.

Patient ID[a]	Sex[a]	Age[a]	Location[a]	Size (cm)[a]	Histologic subtype[a] (WHO classification)	Lauren[a]	Stage[a]	HP (%)[b]	Select[c]
43	M	51	U	7.0	WD	Gastric	IIIb	2.7	
80	M	56	L	5.0	PD	Diffuse	IIIb	1.3	
87	M	53	U	8.0	MD	Diffuse	IIIb	0	
95	M	65	L	6.0	WD	Intestinal	IIIa	31.1	O
119	M	48	L	10.0	WD	Intestinal	IIIa	11.7	O
130	M	57	L	6.5	MD	Intestinal	IIb	0	
134	M	57	L	8.5	WD	Intestinal	IIIb	31.8	O
135	M	37	L	6.0	Mucinous	Intestinal	IIb	92.0	O
136	M	55	L	4.5	MD	Diffuse	IIIa	17.5	O
195	M	67	U	6.0	Papillary	Intestinal	IIIa	30.4	O
236	F	72	L	6.5	MD	Mixed	IIa	68.1	O
849	M	64	L	5.4	PD	Intestinal	Ib	63.8	O
859	F	60	L	5.0	MD	Intestinal	Ib	74.8	O
882	F	75	L	3.5	MD	Intestinal	Ia	57.4	O
889	F	66	L	8.7	MD	Intestinal	IIa	56.6	O
917	M	73	L	7.9	MD	Intestinal	IIa	6.5	

Information marked with '[a]' were obtained from 'Yoon *et al*., Comprehensive genome- and transcriptome-wide analyses of mutations associated with microsatellite instability in Korean gastric cancers, Genome Res 23 (2013) 1109-1117. HP(%)[b], percentage of *Helicobacter pylori* population from entire blast results; Select[c], selected patients for analysis accroding to the presence of *Helibacter pylori* in the normal gastric mucosa; U, upper third; L, lower third; WD, tubular adenocarcinoma and well differentiated; MD, tubular adenocarcinoma and moderately differentiated; PD, tubular adenocarcinoma and poorly differentiated.

Table 2. Summary of the sequence statistics.

SRA Run[a]	Patient ID[b]	Sample[b]	Total reads[c]	Unmapped with hg19[d]	Unmapped read (%)[e]	Contigs[f]	Matching with bacteria[g]	Above threshold[h]
SRR546237	43	Normal	52,088,890	13,775,741	26.4	98,994	1,722	246
SRR546236	43	Tumor	52,088,890	12,407,493	23.8	33,734	388	49
SRR825140	80	Normal	43,269,450	9,637,598	22.3	41,315	514	172
SRR546238	80	Tumor	42,996,076	12,289,307	28.6	69,175	5,567	532
SRR546239	87	Normal	44,471,834	14,136,479	31.8	93,583	1,227	190
SRR546240	87	Tumor	49,814,912	12,612,740	25.3	70,767	3,171	227
SRR825143	95	Normal	49,357,002	10,892,291	22.1	52,590	613	295
SRR825141	95	Tumor	46,801,172	8,781,017	18.8	39,856	138	69
SRR546226	119	Normal	52,088,890	14,958,865	28.7	62,186	2,739	327
SRR546225	119	Tumor	55,022,224	16,085,525	29.2	35,455	135	58
SRR825136	130	Normal	46,515,690	9,967,511	21.4	32,930	142	75
SRR825135	130	Tumor	49,181,346	10,449,965	21.2	34,317	117	58
SRR546227	134	Normal	52,400,004	12,290,105	23.5	31,281	500	97
SRR546228	134	Tumor	55,022,224	13,180,350	24.0	34,657	252	91
SRR546230	135	Normal	52,088,890	11,573,353	22.2	68,630	1,766	997
SRR546229	135	Tumor	52,266,668	10,269,450	19.6	47,252	166	43
SRR546232	136	Normal	54,755,556	14,774,615	27.0	66,951	845	177
SRR546231	136	Tumor	53,511,112	10,454,368	19.5	26,586	166	66
SRR546233	195	Normal	53,511,112	12,381,166	23.1	55,855	305	107
SRR546234	195	Tumor	52,088,890	14,704,878	28.2	47,228	499	94
SRR825139	236	Normal	41,204,902	9,277,569	22.5	44,692	857	498
SRR825137	236	Tumor	43,956,792	6,135,987	14.0	20,651	192	89
SRR801424	849	Normal	58,124,992	10,104,003	17.4	139,959	216	54
SRR801425	849	Tumor	63,880,118	7,752,423	12.1	27,819	90	6
SRR801427	859	Normal	59,019,350	10,010,433	17.0	114,625	356	104
SRR801426	859	Tumor	56,227,424	6,982,330	12.4	86,906	188	57
SRR801428	882	Normal	63,549,430	9,720,473	15.3	119,616	355	150
SRR801429	882	Tumor	59,102,148	7,322,912	12.4	75,218	354	137
SRR801430	889	Normal	44,498,444	7,450,469	16.7	88,745	412	122
SRR801431	889	Tumor	59,655,378	7,622,162	12.8	25,708	881	134
SRR801432	917	Normal	62,755,892	7,906,885	12.6	74,454	382	131
SRR801433	917	Tumor	63,306,020	8,839,289	14.0	131,114	413	162

SRA Run[a], Run number of each sample in the NCBI Sequence Read Archive; Information marked with '[b]' were obtained from 'Yoon *et al*., Comprehensive genome- and transcriptome-wide analyses of mutations associated with microsatellite instability in Korean gastric cancers, Genome Res 23 (2013) 1109-1117. Total reads[c], Total number of sequenced reads; Unmapped with hg19[d], Number of unmapped reads after aligning with hg19 human reference genome using TopHat; Unmapped read (%)[e], Percentage of unmapped reads to total sequenced reads; Contigs[f], Number of contigs *de novo* assembled by Vetvet; Matching with bacteria[g], Number of contigs which were matched with bacterial reference genomes; Above threshold[h], Number of contigs with E-value$\leq 1 \times E^{-40}$ and identity$\geq$95% to the reference sequence among matched contigs with bacterial reference genomes.

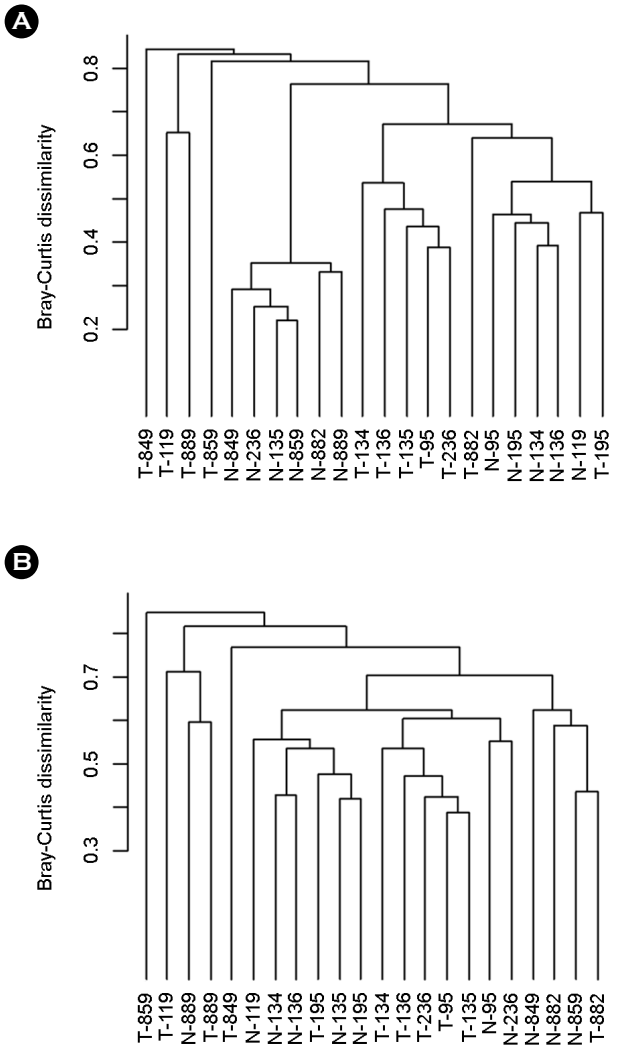

Figure 1. Clustering of the samples based on bacterial composition at the species level. Bacterial composition is different between normal gastric mucosa and gastric cancer. Y-axis represents the Bray-Curtis dissimilarity. (A) Whole bacterial species were used for analysis including *Helicobacter pylori*. (B) Clustering analysis result after excluding *H. pylori*.

genome from each sample (Table 2). The unmapped reads were assembled by Velvet (19) to produce a longer sequence that allowed a strict match with the bacterial genome. The assembly produced 20,651~139,959 contigs from each sample. A bacterial reference database was built using 2,646 sets of the bacterial reference genome (NCBI) to match the bacterial sequences from the assembled reads. Then, the contigs were aligned to the bacterial sequences using NCBI's nucleotide megablast against the bacterial reference database. Only one query with the lowest *E*-value and highest bit score was extracted from each matched contig to the reference. Among the extracted queries, hits with *E*-values > $1 \times E^{-40}$ and identity < 95% to the reference sequence were removed. The total bit score was calculated and used in the analysis by summing all bit scores of the remaining hits according to genus and species.

Statistical analysis

Bray-Curtis dissimilarity was used with R statistical software and the Vegan package (20) for the cluster analysis. The Wilcoxon signed-rank test was performed by pairing results of the cancer and normal samples from the same patient to analyze differences in the microbiomes between gastric cancer and normal gastric mucosa. $P < 0.05$ was considered significant.

RESULTS

Twenty-two RNA-sequencing samples were used for statistical analysis to find differences in the microbiomes between gastric cancer and adjacent normal gastric mucosa from 11 patients who had a high *H. pylori* sequence number in normal mucosa from the results of *de novo* assembly among 16 patients. A total of 350 bacterial species were detected from all 32 samples (data not shown). The clustering analysis results for the bacterial populations obtained by BLASTing the assembled non-host sequences from each sample showed differences in the bacterial communities between normal gastric mucosa and that of gastric cancer (Fig. 1). A similar clustering result was observed when the *H. pylori* sequences were removed from the blast results to exclude statistical error because *H. pylori* was the dominant bacterium in the 11 selected patient samples.

Comparing bacterial communities at the species level, *H. pylori* decreased significantly in cancer tissue compared to that in normal gastric mucosa from the same patient (Table 3, Fig. 2). Comparison at the genus level after removing the *H. pylori* sequences from the blast results showed that *Propionibacteirum* spp., *Staphylococcus* spp., and *Corynebacterium* spp. decreased significantly in cancer tissue

Table 3. Microbiota with different populations between normal gastric mucosa and that of gastric cancer.

Genera	*p*-value
Helicobacter pylori	0.000977
Propionibacterium spp.	0.013672
Staphylococcus spp.	0.014433
Clostridium spp.	0.034611
Prevotella spp.	0.036032
Corynebacterium spp.	0.044011

Significantly different microbiota in the population by comparing normal gastric mucosa and gastric cancer from the same patient. *p*-value for *Helicobacter pylori* was analyzed by comparing all strains at the species level. Other bacterial genera were analyzed at the genus level after removing all *H. pylori* sequences from the blast results. The Wilcoxon signed-rank test was applied for statistical analysis.

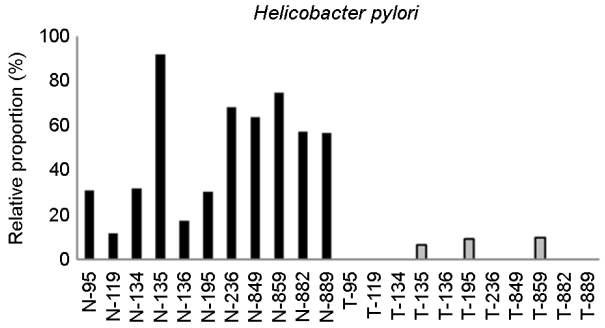

Figure 2. Relative abundances of *Helicobacter pylori* in normal gastric mucosa and that of gastric cancer. Normal gastric mucosa has a higher abundance of *Helicobacter pylori* than that of gastric cancer. Y-axis represents the relative proportion of *H. pylori* to all bacterial species from each sample. 'N' denotes normal gastric mucosa, 'T' denotes gastric cancer. Numbers are identification codes for each sample; the same identification code means they were obtained from the same patient.

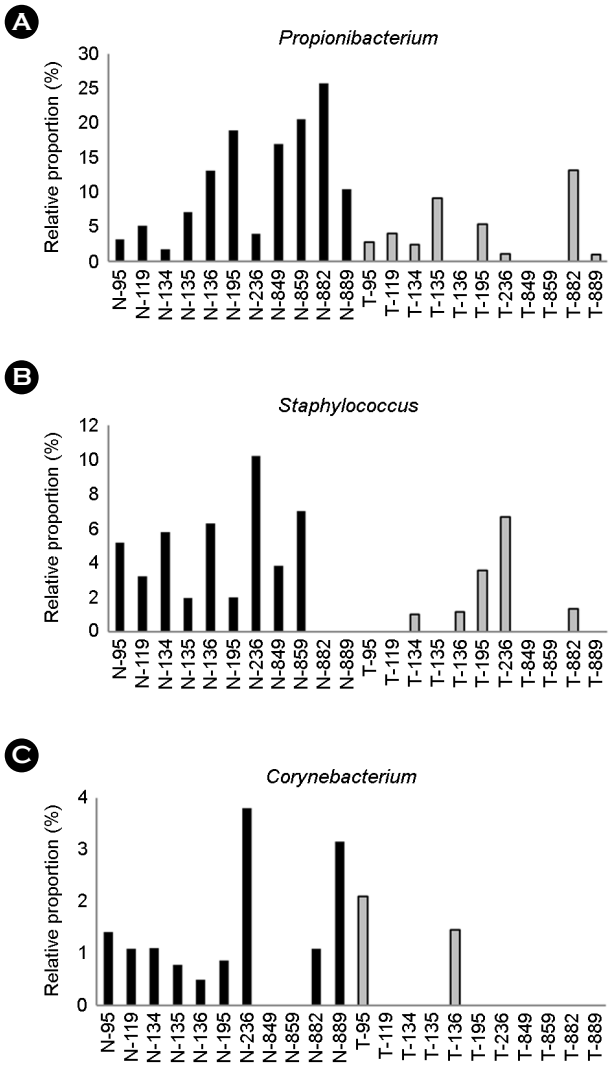

Figure 3. More abundant bacterial genera in normal gastric mucosa than that of gastric cancer. Presence of (A) *Propionibacterium* spp., (B) *Staphylococcus* spp., and (C) *Corynebacterium* spp. in normal gastric mucosa and that of gastric cancer. Y-axis represents the relative proportion of each bacterial genus to all bacterial genera from each sample. 'N' denotes normal gastric mucosa, 'T' denotes gastric cancer. Numbers are identification codes for each sample; the same identification code means they were obtained from the same patient.

mucosa, whereas *Clostridium* spp. and *Prevotella* spp. increased significantly in cancer tissue mucosa (Fig. 3 and 4).

DISCUSSION

The incidence and mortality of gastric cancer continues to decrease worldwide, but it is predicted that gastric cancer will remain an important cause of death at least during the next half century because of the increasing number of new cases in an aging population (21, 22). The role of *H. pylori* in gastric cancer tumorigenesis has been studied extensively. In contrast, little has been revealed about the role and reaction of other gastric microbes in patients with gastric cancer. Information about the stomach microbiome has been accumulating continuously. The increased use of

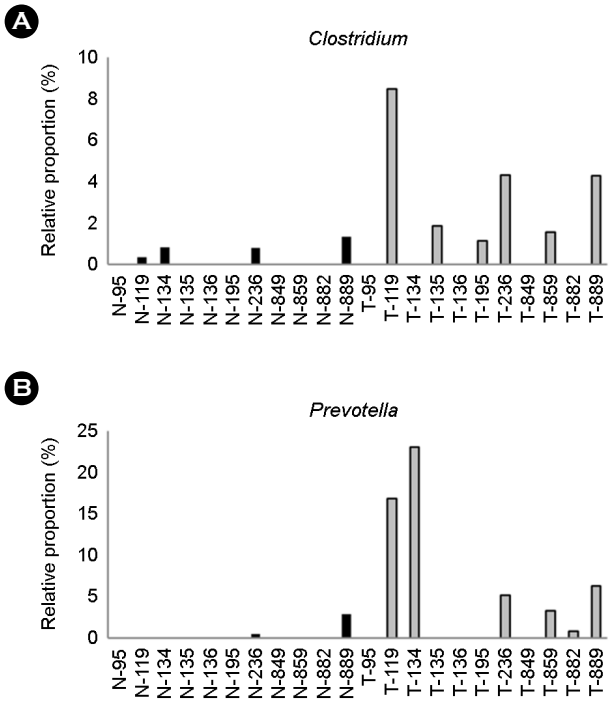

Figure 4. More abundant bacterial genera in gastric cancer than those in normal gastric mucosa. Presence of (A) *Clostridium* spp. and (B) *Prevotella* spp. in normal gastric mucosa and that of gastric cancer. Y-axis represents the relative proportion of each bacterial genus to all bacterial genera from each sample. 'N' denotes normal gastric mucosa, 'T' denotes gastric cancer. Numbers are identification codes for each sample; the same identification code means they were obtained from the same person.

gastroendoscopy for diagnostic and therapeutic purposes and advances in high-throughput sequencing methods have enabled a considerable amount of genetic information to be obtained in a short time (1, 23). Despite these advances, information about the gastric cancer microbiome has been insufficient until now. Therefore, in this study, we investigated the microbiome by matching non-host sequences from sequencing data of the human stomach to the bacterial reference genomes to elucidate differences in the microbiomes between normal gastric mucosa and that of gastric cancer.

The samples we used were obtained by gastrectomy with immediate harvest of specimens under an aseptic condition (15). Thus, they were free from contamination through the biopsy channel of the throat and mouth (1). Furthermore, we included all non-culturable bacteria for investigation because the analysis was performed based on bacterial sequences. However, the purpose of sample preparation and sequencing was not aimed at assessing the bacterial sequence. Nevertheless, several studies have demonstrated that this approach is valid to study microorganisms (16, 17, 24, 25). Moreover, this is the first study that has considered differences in the microbiomes between gastric cancer and normal mucosa of the stomach from the same patient using a culture-independent method rather than analyzing samples obtained from different patients with gastric cancer and healthy volunteers. Thus, we provide valuable insight about changes in the gastric cancer microbiome.

We only included samples from *H. pylori* carriers who had the *H. pylori* sequence in their normal gastric mucosa to focus on the relationships between *H. pylori* and other microbiota in the mucosa. *H. pylori* was absent or was significantly reduced in cancer tissue mucosa (Fig. 2). This result is consistent with a study showing a decrease in the *H. pylori* population in cancer tissue by measuring the relative abundance of terminal restriction fragments (14). Changes in the mucosal environment makes it difficult for *H. pylori* to colonize cancer tissue because gastric adenocarcinoma originates from the mucosa and becomes hypochlorhydric (26), whereas *H. pylori* colonizes gastric mucosa due to chemotaxis driven by pH (27, 28).

According to the clustering results of Fig. 1A, after removing *H. pylori* (Fig. 1B), the normal mucosa samples were clustered with normal mucosa samples, and gastric cancer samples were clustered with gastric cancer samples rather than normal to cancer in the same patient. Normal gastric mucosa had larger populations of *Propionibacterium* spp., *Staphylcoccus* spp., and *Corynebacterium* spp. than those of gastric cancer (Fig. 3). *Propionibacterium* inhabiting the stomach should be highly resistant to acidic conditions (29), but growth of *Staphylococcus* and *Corynebacterium* is suppressed in an acidic environment (30). Some evidence indicates that elevated gastric pH due to *H. pylori*-induced inflammation predisposes colonization by environmental microbiota (31). Moreover, ammonia and bicarbonate produced by *H. pylori* from urea can be used

as substrates by other bacteria (32). In contrast, *Clostridium* and *Prevotella* were denser in gastric cancer mucosa than that in normal mucosa of the stomach (Fig. 4). This result was consistent with previous reports showing that patients with gastric carcinoma harbor higher numbers of *Clostridium* (32) and *Prevotella* (14). *Clostridium* increases significantly as gastric pH increases (11), and *Clostridium* preferentially accumulates in tumors (33). Furthermore, *Prevotella* can be affected by the presence of *H. pylori* because *Prevotella* spp. are found only in *H. pylori*-negative gerbils (13). Therefore, we concluded that the changes in the mucosal layer due to gastric cancer such as increased pH are a major factor involved in the changes of the gastric cancer microbiome. Further investigation is needed to determine whether the existence of *H. pylori* between normal gastric mucosa and gastric cancer affects composition of the gastric microbiome. The influence of *H. pylori* on the composition of stomach microbiome remains controversial (4, 34, 35).

The microbial species obtained from the 32 samples seemed to contain normal flora of the mouth and throat, bacteria from the environment and food, and major stomach bacteria, as discussed above. Further research on the gastric cancer microbiome will be needed to identify the involvement of microorganisms in suppressing or inducing gastric cancer. This may help in the prevention and treatment of gastric cancer by comparing the gastric cancer microbiome with that of normal gastric mucosa.

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