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Combined DNA Methylation and Gastric Microbiome Marker Predicts *Helicobacter pylori*-Negative Gastric Cancer

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Background/Aims: While DNA methylation and gastric microbiome are each associated with gastric cancer (GC), their combined role in predicting GC remains unclear. This study investigated the potential of a combined DNA methylation and gastric microbiome signature to predict *Helicobacter pylori*-negative GC.

Methods: In this case-control study, we conducted quantitative methylation-specific polymerase chain reaction to measure the methylation levels of *DKK3*, *SFRP1*, *EMX1*, *NKX6-1*, *MIR124-3*, and *TWIST1* in the gastric mucosa from 75 *H. pylori*-negative patients, including chronic gastritis (CG), intestinal metaplasia (IM), and GC. A combined analysis of DNA methylation and gastric microbiome, using 16S rRNA gene sequencing, was performed in 30 of 75 patients.

Results: The methylation levels of *DKK3*, *SFRP1*, *EMX1*, *MIR124-3*, and *TWIST1* were significantly higher in patients with GC than in controls (all $q < 0.05$). *MIR124-3* and *TWIST1* methylation levels were higher in patients with IM than those with CG and also in those with GC than in those with IM (all $q < 0.05$). A higher methylation level of *TWIST1* was an independent predictor for *H. pylori*-negative GC after adjusting for age, sex, and atrophy (odds ratio [OR], 15.15; 95% confidence interval [CI], 1.58 to 145.46; $p = 0.018$). The combination of *TWIST1* methylation and GC microbiome index (a microbiome marker) was significantly associated with *H. pylori*-negative GC after adjusting for age, sex, and atrophy (OR, 50.00; 95% CI, 1.69 to 1,476; $p = 0.024$).

Conclusions: The combination of *TWIST1* methylation and GC microbiome index may offer potential as a biomarker for predicting *H. pylori*-negative GC. (*Gut Liver* 2024;18:611-620)

Key Words: Stomach neoplasms; DNA methylation; Gastrointestinal microbiome; RNA, ribosomal, 16S; Biomarkers

INTRODUCTION

Early detection of cancer, especially gastric cancer (GC), is crucial for increasing survival rates and maintaining quality of life. Identifying individuals at a high risk of developing GC is important for early detection. *Helicobacter pylori* infection is a major risk factor for GC.^{1,2} The presence of mucosal atrophy and intestinal metaplasia (IM) induced by chronic *H. pylori* infection also reflects an in-

creased risk of GC, even in the absence of current *H. pylori* infection.³

Aberrant DNA methylation has been suggested to be a marker of GC, particularly in *H. pylori*-negative individuals.⁴ *H. pylori* infection causes epigenetic damage, primarily aberrant DNA methylation, in the gastric mucosa.⁵ Even after the loss or eradication of *H. pylori* colonization, DNA methylation persists to some extent.⁶ DNA methylation levels of dickkopf WNT signaling pathway inhibitor



3 (*DKK3*) and secreted frizzled related protein 1 (*SFRP1*), antagonists of the Wnt signaling pathway, were elevated in patients with *H. pylori*-positive GC and remained partially persistent even after *H. pylori* eradication.^{6,7} Methylation of empty spiracles homeobox 1 (*EMX1*) and NK6 homeobox 1 (*NKX6-1*) was found to be associated with GC in patients with past *H. pylori* infection.⁸ The methylation of microRNA 124-3 (*MIR124-3*) was predictive of the risk of developing metachronous GC after *H. pylori* eradication,⁹ and hypermethylation of twist family bHLH transcription factor 1 (*TWIST1*) was also reported in GC.^{10,11}

Recent studies using 16S rRNA gene sequencing have shown differences in gastric microbiome composition between patients with and without GC.¹²⁻¹⁷ GC has been found to be associated with the enrichment of various bacterial taxa, including *Streptococcus* and *Lactobacillus*, and with the depletion of others.¹²⁻¹⁴ These differences suggest the potential of the gastric microbiome as a biomarker for GC.¹⁸ In addition, we demonstrated that the differentially abundant taxa between patients with *H. pylori*-negative GC and controls could be combined into a GC microbiome index (GCMi), which was associated with inflammatory cytokine expression in the gastric mucosa.¹⁹ Thus, the microbiome of *H. pylori*-negative patients may promote gastric carcinogenesis, specifically in the context of epigenetic changes induced by prior *H. pylori* infection or other insults. However, whether combining epigenetic markers with microbiome data can aid in predicting individual risk of GC remains unexplored.

Therefore, in this study, we aimed to investigate the role of aberrant DNA methylation in predicting *H. pylori*-negative GC compared to controls. Based on these results, we explored the potential of a combined DNA methylation and gastric microbiome signature to predict *H. pylori*-negative GC.

MATERIALS AND METHODS

1. Study participants and sample collection

This case-control study included 75 *H. pylori*-negative patients, comprising 19 patients with GC and 56 controls (chronic gastritis [CG] or IM) from a cohort established in a previous study.¹⁹ The inclusion criteria were (1) age 19 to 75 years, (2) *H. pylori*-negative status, defined as negative results by both histology with modified Giemsa staining and rapid urease test, and (3) scheduled for esophago-gastroduodenoscopy between 2020 and 2021 at Kangbuk Samsung Hospital, Seoul, Korea. The exclusion criteria were as follows: (1) intake of proton pump inhibitors, H2 receptor antagonists, muco-protective agents, antacids,

probiotics, or antibiotics within 1 month; (2) history of *H. pylori* eradication within 1 year; and (3) history of gastrectomy. All 75 patients were subject to DNA methylation analysis. Gastric microbiome data were available for analysis in 30 of the 75 patients. Therefore, we conducted a combined analysis of DNA methylation and gastric microbiome data for this microbiome subgroup. A detailed analysis of the gastric microbiome has been published previously.¹⁹ This study was approved by the Institutional Review Board of Kangbuk Samsung Hospital (IRB number: KBSMC 2020-03-027), and all patients provided written informed consent prior to enrolment.

Clinical data regarding age, sex, and body mass index were collected before endoscopy. Gastric mucosal atrophy was evaluated endoscopically by a single experienced endoscopist (H.J.Y.) and defined as pale surface, increased visibility of submucosal vessels, and diminished gastric folds.²⁰ IM was defined by histology evaluation of biopsy specimens taken from the lesser curvatures of both the antrum and corpus. CG was defined as chronic superficial gastritis or erosive gastritis without mucosal atrophy in endoscopy and chronic inflammatory cell infiltration in the gastric mucosa without IM in histology evaluation. For DNA methylation and microbiome analyses, endoscopic biopsy tissues of the gastric mucosa in the antrum of the stomach were collected during endoscopy and stored at -70°C until DNA extraction.

2. Quantitative DNA methylation analysis

Total DNA was extracted from gastric mucosal tissues using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) as previously described.¹⁹ Bisulfite conversion of genomic DNA was performed to differentiate methylated from unmethylated cytosines using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The methylation levels of target genes in bisulfite-modified DNA were quantified using quantitative methylation-specific polymerase chain reaction, as previously described.^{8,9} We selected six target genes (*DKK3*, *SFRP1*, *EMX1*, *NKX6-1*, *MIR124-3*, and *TWIST1*) based on literature review.⁶⁻¹¹ Quantitative methylation-specific polymerase chain reaction was performed using primers for methylated sequences (Supplementary Table 1) and a LightCycler 480 SYBR Green I (Roche, Welwyn Garden City, England). Mixed sex human genomic DNA (Promega Inc., Madison, WI, USA) treated with CpG methyltransferase (M.SssI) was used as a fully methylated positive control. The quantified level of each target gene was calculated as percentage methylated reference, which was the normalized quantity (target gene/ALU gene) of a sample divided by the normalized quantity of the methyl-

ated positive control and multiplied by 100.⁶

3. 16S rRNA gene sequencing and GCMI

The details of the 16S rRNA gene sequencing have been described previously.¹⁹ Briefly, the V3–V4 (337F–805R) region of the gene was amplified using universal primers, and the resulting libraries were sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Data were processed using QIIME2 (Quantitative Insights into Microbial Ecology 2, version 2021.4). Denoising, filtering, and chimera removal of demultiplexed reads were performed using the DADA2 plugin to generate amplicon sequence variants (ASVs). The mean sequencing depth after pre-processing was 44,999 (standard deviation, 21,104) reads per sample. The taxonomic classification of the ASVs was performed using the National Center for Biotechnology Information Nucleotide and Taxonomy databases (NCBI RefSeq, accessed on June 9, 2021). Further downstream analyses were performed using MetagenomeAnalyst (accessed on August 10, 2022).²¹ Singleton reads that were not assigned at the phylum level were filtered, and low-count (minimum 4), low-prevalence (minimum 10%), and low-variance (minimum 10%) ASVs were filtered out, leaving 98 ASVs for β -diversity and taxonomic analyses.²²

To understand the possible combined roles of DNA methylation and gastric microbiome data in the prediction of *H. pylori*-negative GC, we used data on the GCMI for the microbiome subgroup in this study. This index was calculated by adding the log-transformed relative abundance of *Lactocaseibacillus*, which had a positive association with *H. pylori*-negative GC, and subtracting those of *Haemophilus* and *Campylobacter*, which had a negative association.¹⁹ Relative abundance was defined as the fraction of an ASV relative to the sum of all observed ASVs in a sample.²³ These taxa were identified in a previous study using multiple differential abundance methods, including ANCOM-2 (<https://github.com/FrederickHuangLin/ANCOM>, accessed on September 23, 2022) and MaAsLin2, which were adjusted for age, sex, and body mass index.

4. Statistical analysis

Continuous variables were compared using the Mann-Whitney U test between the two groups, and categorical variables were compared using the chi-square or Fisher exact test, as appropriate. To identify potential DNA methylation marker genes for predicting *H. pylori*-negative GC, logistic regression analysis were performed. All significant factors in the univariate analysis ($p < 0.1$) were included in the multivariate analysis, and then, significant variables in the multivariate analysis were selected using forward variable selection. The identified genes were further evalu-

ated after adjusting for age, sex, and atrophy. IM was not adjusted because of multicollinearity between atrophy and IM. DNA methylation and GCMI parameters were classified as high or low based on median values. The importance of DNA methylation markers in the prediction of *H. pylori*-negative GC was ranked using the random forest (RF) model, a machine-learning ensemble method for classification. In the RF algorithm, the number of trees was set to 2000 and the number of predictors to try (mtry) was set to four. Receiver operating characteristic curves of the prediction models were plotted, and the area under the curve (AUC) was calculated and compared using the DeLong test. We additionally analyzed the association between DNA methylation and *H. pylori*-negative GC in age and sex-matched datasets. We set the nearest age matching and exact sex matching. All analyses were performed using R (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria). The threshold for statistical significance was $p < 0.05$, or false discovery rate-corrected $q < 0.05$ when comparing the DNA methylation levels of multiple genes.

RESULTS

1. Patients

The clinical characteristics of the patients included in this study are summarized in Table 1. Patients in the GC group were significantly older (median age, 65.0 years vs 51.5 years, $p < 0.001$) and more likely to be male (73.7% vs 46.4%, $p = 0.040$) than those in the control group. In addition, a higher proportion of patients with gastric mucosal atrophy (94.7% vs 53.6%, $p < 0.001$) and IM (84.2% vs 46.4%, $p = 0.004$) were found in the GC group than in the control group. The microbiome subgroup exhibited a similar distribution of clinical characteristics between the two groups (Supplementary Table 2), indicating that this subgroup represents the overall study population. In this subgroup, the median age (71 years vs 53 years, $p = 0.005$) and the proportion of atrophy (100.0% vs 39.1%, $p = 0.007$) were significantly higher in patients with GC than in controls.

2. DNA methylation levels were higher in *H. pylori*-negative GC compared to controls

The methylation levels of six candidate genes (*DKK3*, *SFRP1*, *EMX1*, *NKX6-1*, *MIR124-3*, and *TWIST1*) were measured using quantitative methylation-specific polymerase chain reaction and their levels were compared between GC and control groups (Fig. 1A). *DKK3*, *SFRP1*, *MIR124-3*, and *TWIST1* methylation levels were generally low in the control group, whereas *EMX1* and *NKX6-*

Table 1. Clinical Characteristics of Patients

Characteristic	Gastric cancer (n=19)	Control (n=56)	p-value
Age, median (IQR), yr	65.0 (57.0–72.0)	51.5 (40.0–60.8)	<0.001
Sex, No. [%]			0.040
Female	5 (26.3)	30 (53.6)	
Male	14 (73.7)	26 (46.4)	
Body mass index, No. [%]			0.178
<25 kg/m ²	10 (52.6)	39 (69.6)	
≥25 kg/m ²	9 (47.4)	17 (30.4)	
Gastric mucosal atrophy, No. [%]			0.001
Absent	1 (5.3)	26 (46.4)	
Present	18 (94.7)	30 (53.6)	
Intestinal metaplasia, No. [%]			0.004
Absent	3 (15.8)	30 (53.6)	
Present	16 (84.2)	26 (46.4)	

IQR, interquartile range.

I methylation levels were generally high. Compared to the control group, the methylation levels of *DKK3*, *SFRP1*, *EMX1*, *MIR124-3*, and *TWIST1* were significantly higher in the GC group after correction for multiple comparisons (all $q < 0.05$). When the control group was divided into patients with CG without IM and those with IM, *DKK3*, *SFRP1*, *MIR124-3* and *TWIST1* methylation levels were higher in patients with IM than in those with CG, and *MIR124-3* and *TWIST1* methylation levels were also higher in patients with GC than in those with IM (all $q < 0.05$) (Fig. 1B). In the microbiome subgroup, the differences between the GC and control groups were similar to those observed in the overall study population. The methylation levels of *MIR124-3* and *TWIST1* were significantly higher ($q = 0.025$ and $q = 0.008$, respectively) after correcting for multiple comparisons (Supplementary Fig. 1).

3. *TWIST1* methylation levels predicted *H. pylori*-negative GC

To identify marker genes that could predict *H. pylori*-negative GC, we conducted a logistic regression analysis (Table 2). In the univariate analysis, higher levels of *DKK3*, *SFRP1*, *EMX1*, and *TWIST1*, as well as age, sex, atrophy, and IM, were significantly associated with the GC group compared to the control group ($p < 0.05$). In the variable selection process of the multivariate analysis, a high methylation level of *TWIST1* remained as a single predictor significantly associated with *H. pylori*-negative GC compared to the controls (odds ratio [OR], 32.40; 95% confidence interval [CI], 4.02 to 261.05; $p = 0.001$). Even after adjusting for age, sex, and atrophy, the association between a high level of *TWIST1* methylation and *H. pylori*-negative GC remained significant (OR, 15.15; 95% CI, 1.58 to 145.46; $p = 0.018$).

The role of *TWIST1* methylation level in predicting *H. pylori*-negative GC was further evaluated using the RF model and receiver operating characteristic curve. In the RF model, the importance of potential predictive factors for *H. pylori*-negative GC was evaluated based on the mean decrease in accuracy. *TWIST1*, atrophy, and *MIR124-3* were identified as the top three important predictors (Fig. 2A), suggesting that *TWIST1* methylation level is important for differentiating between *H. pylori*-negative GC and controls. Accordingly, three receiver operating characteristic curves were compared to predict *H. pylori*-negative GC, and the AUC was calculated (Fig. 2B). The predictive performance of a high *TWIST1* methylation level, in addition to age, sex, atrophy, and metaplasia, was slightly higher (AUC, 0.833; 95% CI, 0.748 to 0.918) than that of age and sex alone (AUC, 0.741; 95% CI, 0.608 to 0.873) and age, sex, atrophy, and metaplasia without *TWIST1* (AUC, 0.792; 95% CI, 0.686 to 0.898), although the differences were not statistically significant (DeLong test, $p = 0.118$ and $p = 0.333$, respectively).

4. A combination of *TWIST1* methylation and microbiome data predicted *H. pylori*-negative GC

In the microbiome subgroup, the GCMI was significantly higher in patients with GC than in controls ($q = 0.014$) (Supplementary Fig. 2), which was consistent with the previous study.¹⁹ The relative abundance of *Lactocaseibacillus* was higher and those of *Haemophilus* and *Campylobacter* were lower in patients with GC than in controls. However, due to the small sample size, statistical significance was only reached for *Campylobacter* ($q = 0.028$). Multivariate logistic regression analysis showed that elevated levels of both *TWIST1* and GCMI were significantly associated with *H. pylori*-negative GC, even after adjusting for age, sex, and atrophy (OR, 50.00; 95% CI, 1.69 to 1,476, $p = 0.024$) (Supplementary Table 3). The RF model identified combined *TWIST1* and GCMI as the most important predictor for *H. pylori*-negative GC (Fig. 3A), further supporting the role of *TWIST1* methylation in combination with GCMI. The addition of *TWIST1* and GCMI to the clinical parameters in the RF models improved the prediction performance (Fig. 3B). The model including age, sex, atrophy, metaplasia, *TWIST1*, and GCMI showed an AUC of 0.994 (95% CI, 0.976 to 1.000), which was higher than that of the model using age and sex (AUC, 0.780; 95% CI, 0.556 to 1.000) with marginal significance ($p = 0.062$). However, there were no significant differences between the other models because of the small sample size (all $p > 0.05$).

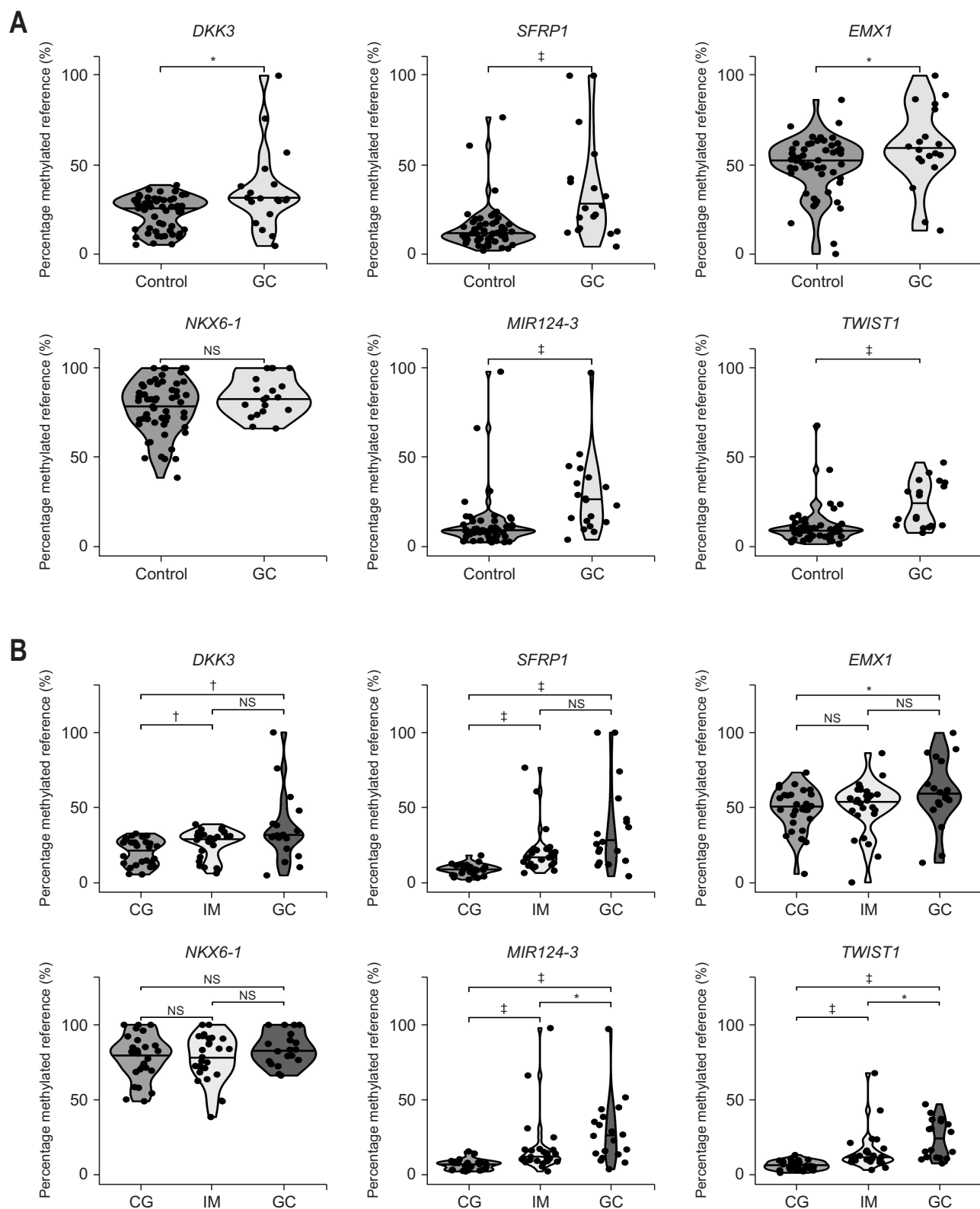


Fig. 1. High DNA methylation levels in *Helicobacter pylori*-negative gastric cancer (GC) compared to controls. DNA methylation levels in the six candidate genes, *DKK3*, *SFRP1*, *EMX1*, *NKX6-1*, *MIR124-3*, and *TWIST1* (A) between *H. pylori*-negative patients with GC and controls and (B) among the *H. pylori*-negative patients with chronic gastritis (CG), intestinal metaplasia (IM), and GC. The DNA methylation levels are presented as the median and interquartile range of the percentage methylated reference (%). The Mann-Whitney U test with correction for the false discovery rate was used to compare the two groups. *DKK3*, dickkopf WNT signaling pathway inhibitor 3; *SFRP1*, secreted frizzled related protein 1; *EMX1*, empty spiracles homeobox 1; *NKX6-1*, NK6 homeobox 1; *MIR124-3*, microRNA 124-3; *TWIST1*, twist family bHLH transcription factor 1; NS, not significant. * $q < 0.05$, † $q < 0.01$, ‡ $q < 0.001$.

Table 2. Logistic Regression Analysis of Predictive Factors for Gastric Cancer

Covariates	Univariate analysis		Multivariable analysis	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Age	1.11 (1.04–1.18)	0.001	1.03 (0.96–1.11)	0.415
Sex		0.045		0.443
Female	1		1	
Male	3.23 (1.03–10.19)		1.74 (0.42–7.14)	
Atrophy		0.010		0.506
Absent	1		1	
Present	15.60 (1.95–125.00)		2.35 (0.19–28.90)	
Intestinal metaplasia		0.008		
Absent	1			
Present	6.15 (1.61–23.51)			
<i>DKK3</i>		0.025		
Low	1			
High	3.73 (1.18–11.79)			
<i>SFRP1</i>		0.002		
Low	1			
High	8.24 (2.15–31.63)			
<i>EMX1</i>		0.079		
Low	1			
High	2.69 (0.89–8.08)			
<i>MIR124-3</i>		0.002		
Low	1			
High	8.24 (2.15–31.63)			
<i>NKX6-1</i>		0.467		
Low	1			
High	1.78 (0.52–4.22)			
<i>TWIST1</i>		0.001		0.018
Low	1		1	
High	32.40 (4.02–261.05)		15.15 (1.58–145.46)	

OR, odds ratio; CI, confidence interval; *DKK3*, dickkopf WNT signaling pathway inhibitor 3; *SFRP1*, secreted frizzled related protein 1; *EMX1*, empty spiracles homeobox 1; *NKX6-1*, NK6 homeobox 1; *MIR124-3*, microRNA 124-3; *TWIST1*, twist family bHLH transcription factor 1.

DNA methylation parameters were classified as high or low based on median values: 27.68 for *DKK3*, 13.34 for *SFRP1*, 55.10 for *EMX1*, 10.47 for *MIR124-3*, 82.18 for *NKX6-1*, and 10.92 for *TWIST1*. The high- and low-level categories of each gene included 38 and 37 patients, respectively.

5. The association of *TWIST1* methylation with *H. pylori*-negative GC in age and sex-matched analysis

We further analyzed the association between *TWIST1* methylation and *H. pylori*-negative GC in age and sex-matched datasets. In the age and sex-matched datasets of the overall cohort, a higher *TWIST1* methylation level was significantly associated with the GC group compared to the control group (OR, 13.09; 95% CI, 1.44 to 119.34; $p=0.023$). However, the association did not reach statistical significance after further adjusting for age and atrophy (OR, 9.04; 95% CI, 0.86 to 95.17; $p=0.067$) (Supplementary Table 4). In the age and sex-matched analysis of the microbiome subgroup, the elevated levels of both *TWIST1* and GCMI was significantly associated with GC (OR, 36.00; 95% CI, 1.80 to 718.68; $p=0.019$), but the significance was not maintained after adjusting for age and atrophy (OR, 34.84; 95% CI, 0.78 to 1,560; $p=0.067$) (Supplementary Table 5).

DISCUSSION

In this study, we aimed to identify a predictive marker for *H. pylori*-negative GC by evaluating aberrant DNA methylation in the gastric mucosa. Our findings showed that the methylation levels of *DKK3*, *SFRP1*, *EMX1*, *MIR124-3*, and *TWIST1* were significantly higher in *H. pylori*-negative patients with GC than in controls. Notably, after adjusting for age, sex, and gastric mucosal atrophy, *TWIST1* remained a strong predictive marker for *H. pylori*-negative GC. Furthermore, the combination of *TWIST1* and GCMI, a gastric microbiome marker, was an independent predictor for *H. pylori*-negative GC.

Epigenetic alterations, particularly DNA methylation, are considered a link between *H. pylori* and GC.^{24,25} It is believed that DNA methylation induced by *H. pylori* infection leads to epigenetic silencing of tumor suppressor genes.²⁴ Meanwhile, accumulated DNA methylation in passenger genes in normal gastric mucosa has been con-

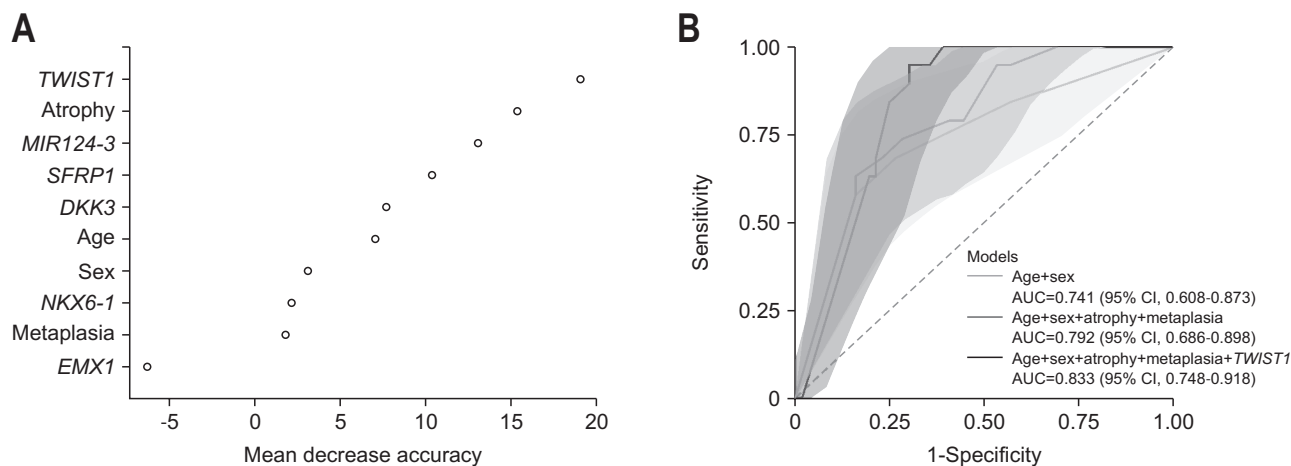


Fig. 2. *TWIST1* methylation level as a predictor of *Helicobacter pylori*-negative gastric cancer in random forest analysis. (A) Random forest analysis incorporating all candidate clinical characteristics and methylation markers was performed. The factors were presented in decreasing order of importance in discriminating between *H. pylori*-negative gastric cancer and controls. (B) Receiver operating characteristic curves with 95% confidence intervals were plotted for the three prediction models. There were no significant differences in the area under the curve (AUC) of the model with age, sex, atrophy, metaplasia, and *TWIST1* methylation compared to that with age and sex alone (DeLong test, $p=0.118$) and that with age, sex, atrophy, and metaplasia (DeLong test, $p=0.333$). *TWIST1*, twist family bHLH transcription factor 1; *MIR124-3*, microRNA 124-3; *SFRP1*, secreted frizzled related protein 1; *DKK3*, dickkopf WNT signaling pathway inhibitor 3; *NKX6-1*, NK6 homeobox 1; *EMX1*, empty spiracles homeobox 1; CI, confidence interval.

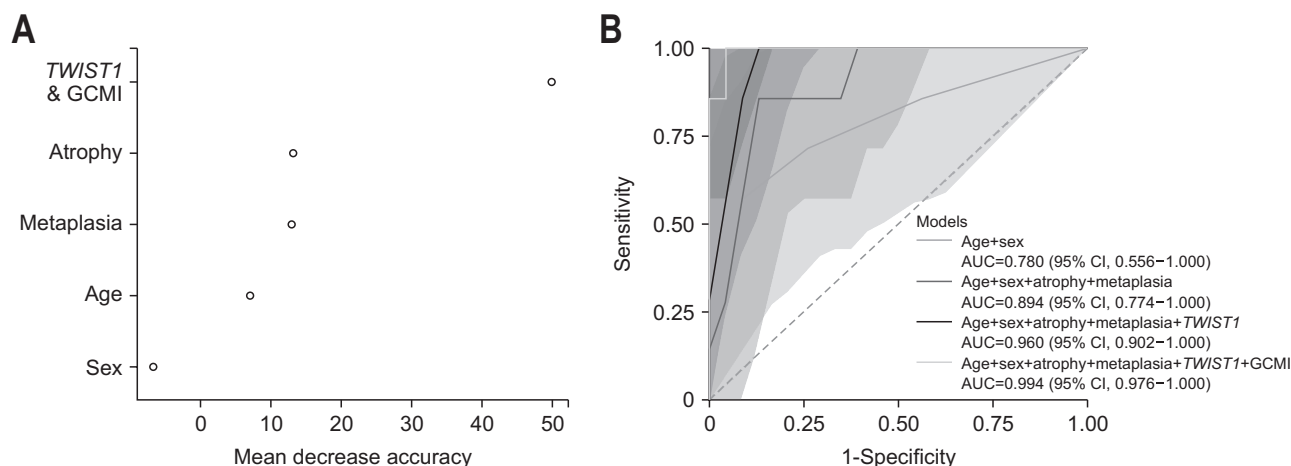


Fig. 3. A combination of *TWIST1* methylation and the gastric cancer microbiome index (GCMi) as a predictor of *Helicobacter pylori*-negative gastric cancer in random forest analysis in the microbiome subgroup. (A) Random forest analysis incorporating all candidate clinical characteristics in addition to *TWIST1* and GCMi was performed. The factors are presented in decreasing order of importance in discriminating between *H. pylori*-negative gastric cancer and controls. (B) Receiver operating characteristic curves with 95% confidence intervals were plotted for the four prediction models. The model including age, sex, atrophy, metaplasia, *TWIST1*, and GCMi showed the highest area under the curve (AUC), which was higher than that of the model using age and sex with marginal significance (DeLong test, $p=0.062$). There were no other significant differences among the models (DeLong test, all $p>0.05$). *TWIST1*, twist family bHLH transcription factor 1; CI, confidence interval.

sidered to help predict the risk of developing GC.²⁵ These methylation levels are thought to be more strongly correlated with GC in individuals who are *H. pylori*-negative rather than positive, because DNA methylation resulting from active *H. pylori* infection disappears.⁵ In our study, we found that five of the six candidate genes had higher methylation levels in *H. pylori*-negative GC than in controls. It was previously showed that the methylation levels of *DKK3* and *SFRP1* were higher in patients with *H. py-*

lori-positive GC than in *H. pylori*-negative and *H. pylori*-positive controls.⁶ Additionally, we showed that the methylation levels of these genes were increased in *H. pylori*-negative GC. We also demonstrated that the methylation levels of *EMX1* and *MIR124-3* were higher in *H. pylori*-negative GC than in controls in our study population, consistent with previous studies.^{8,26} Kim *et al.*¹⁰ reported higher levels of *TWIST1* methylation in patients with GC than in controls; our study further showed that these levels

were higher in patients with *H. pylori*-negative GC than in controls.

We identified *TWIST1* as the strongest predictive marker for *H. pylori*-negative GC. This gene was selected during the variable selection process of the multivariate logistic regression analysis and remained significant even after adjusting for age, sex, and atrophy. *TWIST1* is a member of the basic helix-loop-helix family of transcription factors that play a role in embryonic development and cancer progression.²⁷ It is also involved in epithelial-to-mesenchymal transition. Notably, *TWIST1* is frequently methylated in GC,¹¹ and hypermethylation of *TWIST1* has been associated with unfavorable outcomes in breast²⁸ and colorectal²⁹ cancers, supporting its role as a biomarker. In our study, differences in the methylation levels between GC and controls were mainly attributable to the differences between CG and GC rather than those between IM and GC. However, *TWIST1* and *MIR124-3* methylation levels were significantly different not only between CG and GC, but also between IM and GC. Hence, elevated levels of *TWIST1* methylation in the gastric mucosa of individuals without current *H. pylori* infection may be a useful biomarker for predicting GC development.

Our study revealed that a combined marker of *TWIST1* methylation and GCMI could independently predict *H. pylori*-negative GC. In the RF model, the combination of the two markers was found to be most important for classifying patients with GC and controls. Furthermore, the inclusion of these two markers improved the accuracy of predicting GC risk when combined with conventional GC risk factors, although the sample size was too small to reach statistical significance. Recently, there have been active efforts to develop microbiome biomarkers for colorectal cancer.³⁰ Moreover, Mo *et al.*³¹ showed that combining methylation and microbiome markers with fecal occult blood tests can be effective for the non-invasive detection of colorectal cancer. Regarding GC, Zhou *et al.*¹⁸ investigated the feasibility of fecal microbiome-based cancer screening. However, to the best of our knowledge, this is the first study to evaluate the effectiveness of using methylation and microbiome markers together to predict GC. Several studies have reported that the gastric microbiome is associated with GC.^{12-14,19,32} Coker *et al.*¹² reported that *Peptostreptococcus stomatis*, *Streptococcus anginosus*, *Parvimonas micra*, *Slackia exigua*, and *Dialister pneumosintes* were enriched in GC. Wang *et al.*¹³ observed an increased abundance of *Helicobacter*, *Lactobacillus*, *Streptococcus*, *Prevotella*, and *Veillonella* in GC. Similarly, Gantuya *et al.*¹⁴ found that *Carnobacterium*, *Glutamicibacter*, *Paeniglutamicibacter*, *Fusobacterium*, *Parvimonas*, and *Fir-*

micutes were associated with GC. Our previous research demonstrated that *Lactocaseibacillus* was enriched, while *Campylobacter* and *Haemophilus* were depleted, in *H. pylori*-negative GC, and that the index that merged these taxa, GCMI, was correlated with gastric mucosal *IL1B* expression.¹⁹ Because our microbiome data were specific to *H. pylori*-negative individuals, we suggest that they are suitable for combination with the methylation data. Overall, our study provides evidence that combining methylation and microbiome markers is a promising approach for predicting *H. pylori*-negative GC.

Our study had several limitations. First, the sample size was small, especially for the subgroup analysis of microbiome data. This could have caused the lack of statistical power in the predictive modeling. Second, the GC and control groups were significantly different in age, sex, atrophy, and IM. We have conducted adjusted age and sex-matched analysis for the association between DNA methylation and *H. pylori*-negative GC. GCMI was also derived from the analysis that was adjusted for age and sex and considered for the effect of atrophy and IM.¹⁹ Nevertheless, residual effects of those covariates might still have affected the outcomes. Third, our findings based on a combination of methylation and microbiome markers lack validation. The AUC values, or c-statistics of the logistic regression models including age, sex, atrophy, and metaplasia were 0.792 in the overall study population and 0.894 in the microbiome subgroup. The values appeared higher than those reported in previous studies. In a prior study, a prediction model that incorporated age, body mass index, family history, salt intake, drinking, and smoking yielded c-statistics of 0.76 for men and 0.71 for women.³³ In another study, a model including age, sex, serum pepsinogen I/II ratio, gastrin level, and *H. pylori* IgG antibody showed a c-statistics of 0.76.³⁴ The elevated AUC values in our study may be primarily due to the absence of validation, potentially leading to overfitting of the models. In addition, DNA methylation and gastric microbiome were evaluated using endoscopic biopsy tissue, which have limited clinical applicability as a biomarker because of invasiveness of endoscopy. Therefore, our findings may primarily serve a hypothesis-generating role. Further studies with larger sample sizes and improved clinical applicability are required to confirm our results.

In conclusion, our study suggests that the levels of *TWIST1* methylation in gastric mucosa may predict *H. pylori*-negative GC. Moreover, the combination of *TWIST1* methylation and gastric microbiome index may offer potential as a biomarker for predicting *H. pylori*-negative GC. Further validation studies are warranted.

CONFLICTS OF INTEREST

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

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AUTHOR CONTRIBUTIONS

Study concept and design: H.J.Y. Data acquisition: M.J.K., H.N.K., H.J.Y. Data analysis and interpretation: M.J.K., H.N.K., J.P.J., H.J.Y. Drafting of the manuscript: H.J.Y. Critical revision of the manuscript for important intellectual content: M.J.K., H.N.K., J.P.J. Statistical analysis: H.J.Y. Obtained funding: J.P.J., H.J.Y. Administrative, technical, or material support; study supervision: H.J.Y. Approval of final manuscript: all authors.

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SUPPLEMENTARY MATERIALS

Supplementary materials can be accessed at <https://doi.org/10.5009/gnl230348>.

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