Promising aberrant DNA methylation marker to predict gastric cancer development in individuals with family history and long-term effects of *H. pylori* eradication on DNA methylation

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Abstract

Objective It remains unknown whether individuals with a family history (FH) of gastric cancer (GC) are associated with aberrant DNA methylation. The aim of this study was to investigate the association between aberrant DNA methylation and FH of GC.

Design Using quantitative MethyLight assay, *MOS*, *miR124a-3*, *NKX6-1*, *EMX1*, *CDH1*, and *TWIST1* methylation levels in the noncancerous gastric mucosa was compared between subjects with and without FH based on GC and *Helicobacter pylori* (Hp) infection. Changes in the methylation levels were evaluated over time after Hp eradication.

Results In Hp-positive GC patients, *MOS* ($P < 0.001$), *CDH1* ($P < 0.001$), and *TWIST1* ($P = 0.004$) methylation were decreased in subjects with FH ($n = 64$) than in those without FH ($n = 58$). In Hp-positive controls, *MOS* methylation was lower in subjects with FH ($n = 73$) than in those without FH ($n = 50$) ($P = 0.042$), while *miR124a-3* ($P = 0.006$), *NKX6-1* ($P < 0.001$), and *CDH1* ($P < 0.001$) methylation were higher in subjects with FH. *CDH1* methylation constantly decreased from 2 years in GC patients and 3–4 years in controls after Hp eradication (all $P < 0.001$). A persistent decrease in methylation over time was not observed in other genes after eradication.

Conclusion The methylation of *MOS* and *CDH1* provided an association between aberrant DNA methylation and gastric carcinogenesis in FH of GC, a useful marker for GC risk in individuals with FH. Furthermore, *CDH1* methylation decreased after Hp eradication.

Keywords DNA methylation · Gastric cancer · Family history · *Helicobacter pylori*

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Introduction

Aberrant DNA methylation is a major epigenetic mechanism and is profoundly involved in gastric carcinogenesis [1]. Gene silencing of most tumor suppressor genes and tumor-related genes, mediated by regional hypermethylation in promoter regions, is almost exclusively associated with CpG islands and has been widely studied in gastric cancer (GC) [2]. Additionally, aberrant DNA methylation has been observed in the noncancerous mucosa of patients with GC and non-neoplastic gastric mucosa of subjects without GC, at levels correlating with the risk of cancer development, producing a so-called ‘epigenetic field for cancerization’ [1, 3]. *Helicobacter pylori* (Hp)-induced chronic inflammation plays a direct role in the induction of aberrant DNA methylation [4–7], and this methylation may partially reverse in certain genes with the eradication of infection [8, 9]. Conversely, residual methylation in gastric mucosa after Hp

eradication might be associated with the risk of developing GC [10].

In GC, family history (FH) is a well-established risk factor [11]. Most GCs are sporadic, with approximately 10% of GCs attributed to familial clustering; however, nearly 1–3% of GCs arise from inherited GC predisposition syndromes, such as hereditary diffuse gastric carcinoma and familial adenomatous polyposis [12]. The risk of GC in individuals with FH is approximately threefold higher than in those without FH [11]. A Korean study has shown that in women with both FH and Hp infection the risk of GC increased by fivefold when compared with those without these predisposing factors [13]. Indeed, family members share various factors, from exposure to the same carcinogens (i.e., nitrogen, cigarette smoke, and alcohol consumption) to similar levels of hygiene, dietary habits (salty and spicy food, smoked food), bacterial virulence, and genetic susceptibility [14]. Single nucleotide polymorphisms related to cytokines such as transforming growth factor- β 1 and interleukin-1 have been investigated to establish genetic predisposition in GC patients with FH [14, 15]. Additionally, GC patients with FH present a lower cancer stage (I–II) at diagnosis than those without FH, but overall survival was not different between patients with and without FH [16].

Although associations between GC risk and aberrant DNA methylation have been previously reported, evidence indicating the association of FH of GC with DNA methylation levels in GC patients and healthy individuals remains limited. If FH of GC is associated with DNA methylation levels even in healthy individuals, methylation levels could serve as a potential marker of molecular changes in the multistep process of GC development. In this study, we hypothesized that aberrant DNA methylation of GC-related genes is associated with GC development in subjects with FH. Thus, the aim of this study was to investigate the methylation of these genes in noncancerous gastric mucosa from patients with GC and controls, considering FH of GC and Hp infection. Furthermore, the long-term effects of Hp eradication on epigenetic molecular alterations were investigated in these patients. In this study, six genes, *MOS*, *miR124a-3*, *NKX6-1*, *EMX1*, *CDH1*, and *TWIST1* were selected. The methylation level of *MOS* was significantly elevated in patients with metachronous gastric neoplasm (gastric dysplasia and early GC) compared patients without that in our previous researches [17]. In addition, decrease of *MOS* methylation level following *H. pylori* eradication was significant among controls without intestinal metaplasia (IM) (P value for slope < 0.05) [18]. *miR124a-3*, *NKX6-1* and *EMX1* methylation were an informative marker for predicting the risk of metachronous GC in patients after endoscopic resection in Japanese multicenter prospective cohort study [19]. *CDH1* is a tumor suppressor gene and has been reported frequently hypermethylated in GC and also suggested marker of GC

development [20, 21]. *TWIST1* encodes a basic-helix-loop-helix transcription factor called Twist1 that regulates metastasis and epithelial–mesenchymal transition [22]. Although Twist1 protein may have tumor or metastasis-promoting effects, paradoxically, hypermethylated *TWIST1* has been reported in GC and other cancer [22].

Material and methods

Study subjects

The study subjects who had gastrointestinal symptoms such as epigastric discomfort, nausea, vomiting or presented for regular check-up for surveillance of GC at the Seoul National University Bundang Hospital from February 2006 to September 2019 were consecutively enrolled. The subjects with a previous history of Hp eradication or gastrectomy, and past Hp infection were excluded. In total, 328 subjects including 181 healthy controls and 147 patients with GC were selected (Fig. 1). The controls showed no evidence of GC, dysplasia, mucosa-associated lymphoid tissue lymphoma, esophageal cancer, or peptic ulcer. All subjects, who provided informed consent, were asked to complete a questionnaire, requesting data regarding age, sex, smoking and drinking habits, history of Hp eradication, and FH of GC. A “positive family history” was defined as having any first-degree relatives (parent, sibling, or offspring) diagnosed with GC. Hp-positive subjects were treated with a standard 7-day triple therapy (esomeprazole 40 mg b.i.d., clarithromycin 500 mg b.i.d., and amoxicillin 1000 mg b.i.d.) for Hp eradication. To evaluate Hp eradication, ^{13}C -Urea breath testing was performed at least 4 weeks after completion of the eradication therapy. The study protocol was approved by the Ethical Committee at Seoul National University Bundang Hospital (IRB number: B-1012/117-013).

H. pylori testing and histology

During endoscopy, 10 biopsy specimens were obtained to determine the Hp infection status, as well as histological evaluation. Among these specimens, two from the greater curvature side of the antrum and two from the body were fixed in formalin to assess the presence of Hp using modified Giemsa staining, and to examine the degree of inflammatory cell infiltration, atrophy, and IM, determined by hematoxylin and eosin staining. The histologic features of the gastric mucosa were recorded using the updated Sydney scoring system. One specimen from each, the lesser curvature of the antrum and the body, was used for rapid urease testing (CLOtest, Delta West, Bentley, Australia). Subsequently, two specimens from the antrum and the body were used for culture, and organisms present were

Fig. 1 Proposed study algorithm for the inclusion and classification of subjects. Hp, *H. pylori*; FH, family history

identified as Hp by Gram staining, colony morphology, and positive oxidase, catalase, and urease reactions. The remaining biopsy specimens and GC tissues were immediately frozen at -70 °C until DNA extraction.

Positivity for CLOtest, Giemsa staining, and culture was defined as current Hp infection. If results obtained from three biopsy-based tests were negative, ¹³C-urea breath test (UBiTkkit; Otsuka Pharmaceutical, Tokyo, Japan) and serum immunoglobulin G antibodies for Hp detected by an enzyme-linked immunosorbent assay (Genedia Hp ELISA; Green Cross Medical Science Corp., Eumseong, South Korea) were performed. Hp bacteria are spontaneously disappeared in severely atrophic mucosa and advanced gastric diseases once they have caused gastric atrophy and IM after long-time colonization, and the spontaneous seroconversion of anti-Hp antibodies often occurs [23]. Therefore, among the patients with negative results from all the above clinical tests and no prior history of Hp eradication, the true negative Hp infection was diagnosed as follows: PG I/II ratio > 3 and no or mild atrophy and IM either in the antrum and body. Positivity for the Hp serology test and/or an Hp eradication history indicated a past Hp infection; individuals with a past Hp infection were excluded from this analysis.

DNA extraction, bisulfite modification, and MethyLight assay

Genomic DNA was extracted directly from noncancerous antral biopsy specimens using sodium bisulfite. Briefly, specimens were homogenized in proteinase K solution [20 mmol/L Tris-HCl (pH 8.0), 10 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate, and 10 mg/mL proteinase K] using a sterile micropestle, followed by incubation for 3 h at 52 °C. DNA was isolated from homogenates using phenol/chloroform extraction and ethanol precipitation. Genomic DNA (1 µg) was bisulfite modified using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) by following the manufacturer's instructions. The methylation status of six genes (*MOS*, *miR124a-3*, *NKX6-1*, *EMX1*, *CDH1*, and *TWIST1*) from bisulfite-modified DNA samples was quantified using real-time polymerase chain reaction (PCR)-based MethyLight technology [24]. MethyLight, as a sensitive, high-throughput methylation assay, allows the highly specific detection of methylation using probes that cover methylation sites, as well as methylation-specific primers. The primer and probe sequences used in the reaction are shown in Supplementary Table S1. The quantified level of each gene was reported as a percentage

of methylated reference (PMR), which is the relative methylation ratio of the target gene to the ALU gene of a sample, divided by the ratio of the target gene to the ALU gene of sodium bisulfite and CpG methyltransferase (M.SssI)-treated sperm DNA, multiplied by 100 [24].

Statistical analysis

Comparisons of demographic and clinicopathologic variables were performed using the Student *t* test or Chi-square test (Fisher's exact test) for continuous and categorical variables, respectively. The Mann–Whitney *U* test was used to compare medians when the sample size was less than 30.

All statistical analyses were performed using SPSS (version 21.0; SPSS, Chicago, IL, USA). A *P* value < 0.05 was considered significant.

Results

Subjects characteristics

The clinicopathological characteristics of the study subjects are summarized in Table 1. Overall, 328 patients, including 147 patients with GC and 181 healthy controls, were enrolled in this study. No significant differences were

Table 1 Baseline characteristics of study subjects

	Gastric cancer (<i>n</i> = 147)			Controls (<i>n</i> = 181)		
	Without FH (<i>n</i> = 71)	With FH (<i>n</i> = 76)	<i>P</i> value	Without FH (<i>n</i> = 73)	With FH (<i>n</i> = 108)	<i>P</i> value
Male	42 (59.2)	45 (59.2)	0.995	30 (41.1)	43 (39.8)	0.863
Age (mean ± SD, years)	59.29 ± 10.87	60.09 ± 11.24	0.663	54.27 ± 11.32	49.45 ± 12.14	0.008
Current/ex-smoker	38 (53.5)	40 (52.6)	0.914	19 (26.0)	40 (37.0)	0.121
Alcohol drinker	49 (69.0)	51 (67.1)	0.804	44 (60.3)	66 (61.1)	0.910
Positive <i>H. pylori</i> infection	58 (81.7)	64 (84.2)	0.684	50 (68.5)	73 (67.6)	0.899
BMI (mean ± SD, m ² /kg)	24.05 ± 2.98	23.10 ± 2.94	0.054	23.61 ± 3.51	23.65 ± 2.74	0.941
Intestinal metaplasia	45 (63.4)	51 (67.1)	0.635	27 (37.0)	49 (45.4)	0.262
Serum pepsinogen						
I	67.72 ± 49.25	61.67 ± 39.75	0.419	54.65 ± 39.85	56.34 ± 40.52	0.786
II	22.52 ± 14.63	20.22 ± 10.79	0.285	20.27 ± 20.17	16.09 ± 10.55	0.075
I/II ratio	3.36 ± 2.01	3.68 ± 4.40	0.583	4.02 ± 3.97	4.17 ± 1.94	0.766
Moderate/severe neutrophil infiltration at antrum	32 (45.1)	32 (42.1)	0.717	33 (45.2)	56 (51.9)	0.668
Moderate/severe neutrophil infiltration at body	55 (77.5)	56 (73.7)	0.594	44 (60.3)	55 (50.9)	0.215
Moderate/severe monocyte infiltration at antrum	51 (71.8)	55 (72.4)	0.942	44 (60.3)	72 (66.7)	0.446
Moderate/severe monocyte infiltration at body	54 (76.1)	55 (72.4)	0.610	53 (72.6)	65 (60.2)	0.085
Lauren classification ^a						
Intestinal	42 (64.6)	38 (57.6)	0.409			
Diffuse	23 (35.4)	28 (42.4)				
Stage ^a						
I	65 (92.9)	50 (69.4)	0.005			
II	3 (4.3)	11 (15.3)				
III	2 (2.9)	9 (12.5)				
IV	0 (0.0)	2 (2.8)				
Number of affective relatives						
1		61 (80.3)			84 (77.8)	
2		14 (18.4)			22 (20.4)	
≥ 3		1 (1.3)			2 (1.9)	

Numbers in parentheses are percentages. Values presented in bold indicate statistically significant differences

SD standard deviation, BMI body mass index

^aSome data were missing

observed in sex, smoking, drinking, current Hp infection, IM, body mass index, serum pepsinogen (PG) I/II ratio, and the degree of neutrophil and monocyte infiltration between subjects with and without FH in GC patients and controls. The proportion of stage I patients was significantly lower in GC patients with FH than in those without FH (69.4% vs. 92.9%, $P < 0.001$). Furthermore, controls with FH were younger than those without FH (49.45 ± 12.14 vs. 54.27 ± 11.32 , $P = 0.008$) (Table 1).

Aberrant DNA methylation according to Hp infection status and the presence of GC

Methylation levels in six genes were compared according to the presence of GC (GC vs. controls) and Hp infection status (positive vs. negative). For *MOS*, *miR124a-3*, *NKX6-1*, *EMX1*, *CDH1*, and *TWIST1*, methylation levels were higher in patients with GC than in controls. In control subjects, methylation levels for *EMX1*, *CDH1*, and *TWIST1* were extremely low, demonstrating median PMR values less than 10.

Compared with Hp-negative patients with GC, methylation levels of *MOS*, *miR124a-3*, *CDH1*, and *TWIST1* were elevated in Hp-positive GC patients. Among controls, methylation levels of *MOS*, *miR124a-3*, *EMX1*, and *CDH1* were significantly elevated in Hp-positive controls compared to levels observed in Hp-negative subjects.

Aberrant DNA methylation according to FH in Hp-positive subjects

In GC patients with FH, methylation levels of *MOS*, *CDH1*, and *TWIST1* were lower than those observed in patients without FH (*MOS*, 29.98 ± 13.62 vs. 50.98 ± 21.23 , $P < 0.001$; *CDH1*, 13.21 ± 18.00 vs. 33.94 ± 28.17 , $P < 0.001$; *TWIST1*, 12.77 ± 17.34 vs. 24.00 ± 23.83 , $P = 0.004$) (Fig. 2). In controls with FH, methylation level of *MOS* were lower than those observed in subjects without FH (32.79 ± 14.24 vs. 39.56 ± 21.76 , $P = 0.042$); however, methylation levels of *miR124a-3*, *NKX6-1*, and *CDH1* were elevated in controls with FH (*miR124a-3*, 23.91 ± 23.91 vs. 14.35 ± 13.73 , $P = 0.006$; *NKX6-1*, 30.15 ± 13.78 vs. 19.25 ± 11.85 , $P < 0.001$; *CDH1*, 8.51 ± 6.15 vs. 2.45 ± 4.28 , $P < 0.001$) (Fig. 2).

Subgroup analysis based on the presence of IM demonstrated similar results. In IM-positive GC patients, methylation levels of *MOS*, *CDH1*, and *TWIST1* were decreased in subjects with FH compared to those without FH (*MOS*, 31.03 ± 14.10 vs. 51.64 ± 20.71 , $P < 0.001$; *CDH1*, 11.68 ± 16.11 vs. 27.63 ± 26.01 , $P = 0.001$; *TWIST1*, 15.31 ± 18.78 vs. 26.55 ± 25.68 , $P = 0.025$), as well as in IM-negative patient with GC (*MOS*, 27.08 ± 12.11 vs. 49.47 ± 22.96 , $p = 0.001$; *CDH1*, 17.46 ± 22.44

vs. 47.95 ± 28.40 , $P = 0.001$; *TWIST1*, 5.73 ± 9.89 vs. 18.33 ± 18.50 , $P = 0.018$) (Figs. 3 and 4). Among IM-positive controls, *miR124a-3*, *NKX6-1*, and *CDH1* were differentially methylated in the presence of FH (*miR124a-3*, 28.84 ± 27.73 vs. 11.40 ± 10.53 , $P < 0.001$; *NKX6-1*, 30.85 ± 14.18 vs. 17.30 ± 8.20 , $P < 0.001$; *CDH1*, 9.46 ± 6.14 vs. 3.12 ± 5.05 , $P < 0.001$) (Fig. 3). In the case of IM-negative controls, *NKX6-1* and *CDH1* were differentially methylated in the presence of FH (*NKX6-1*, 29.02 ± 13.29 vs. 21.04 ± 14.36 , $P = 0.039$; *CDH1*, 6.99 ± 5.95 vs. 1.84 ± 3.40 , $P < 0.001$) (Fig. 4).

In subgroup analysis by histology, methylation levels of *MOS*, *CDH1*, and *TWIST1* were decreased in subjects with FH than those without FH among intestinal-type GC (*MOS*, 29.97 ± 13.50 vs. 48.32 ± 20.31 , $P < 0.001$; *CDH1*, 11.83 ± 17.37 vs. 33.77 ± 29.09 , $P < 0.001$; *TWIST1*, 12.46 ± 16.97 vs. 24.81 ± 25.62 , $P = 0.018$) (Fig. 5). Methylation levels of *MOS* and *CDH1* were lower in subjects with FH than in those without FH among diffuse-type GC (*MOS*, 30.43 ± 15.34 vs. 55.82 ± 19.33 , $P < 0.001$; *CDH1*, 13.74 ± 18.47 vs. 36.17 ± 27.32 , $P = 0.006$) (Fig. 5).

Aberrant DNA methylation according to FH in Hp-negative subjects

In Hp-negative GC and controls, *MOS*, *miR124a-3*, *NKX6-1*, *EMX1*, *CDH1*, and *TWIST1* were not differentially methylated between subjects with and without FH (Fig. 2). In subgroup analysis by IM and histology, no significant differences were found.

Changes in DNA methylation levels over time

Figure 6 reveals changes in the levels of DNA methylation over time in Hp-eradicated subjects. It presented as the mean values of collected data at each interval. The average follow-up duration was 57.2 months (range 2–150 months). In patients with GC, a significant decrease in *CDH1* methylation was observed 2 years after Hp eradication (2 years, $P < 0.001$; 3–4 years, $P < 0.001$; ≥ 5 years, $P = 0.014$); in controls, *CDH1* methylation was significantly decreased 3–4 years after eradication (3–4 years, $P = 0.025$; ≥ 5 years, $P = 0.01$) (Fig. 6). The levels of *MOS*, *miR124a-3*, *NKX6-1*, *EMX1*, and *TWIST1* did not significantly change after Hp eradication in patients with GC, as well as controls (Fig. 6).

Following subgroup analysis according to the presence of FH, *CDH1* methylation levels demonstrated similar trends. *CDH1* methylation levels were significantly decreased at 2 ($P < 0.001$), 3–4 ($P = 0.002$), and ≥ 5 years ($P = 0.001$) after Hp eradication observed in GC patients without FH, and 2 ($P = 0.04$) and 3–4 years ($P = 0.002$) after eradication in GC patients with FH, and 2 years ($P = 0.035$) after eradication in controls without FH, and 3–4 ($P < 0.001$)

Fig. 2 Comparison of *MOS* (a), *miR124a-3* (b), *NKX6-1* (c), *EMX1* (d), *CDH1* (e), and *TWIST1* (f) methylation levels between gastric cancer (GC) patients and controls with and without family history (FH) according to *H. pylori* (Hp) infection. Methylation levels in *MOS*, *CDH1*, and *TWIST1* are significantly decreased in Hp-positive GC patients with FH when compared with those without FH. *MOS* methylation levels are significantly decreased in Hp-positive controls; however, methylation levels in *miR124a-3*, *NKX6-1*, and *CDH1* are significantly increased in Hp-positive controls with FH when compared with those without FH. Bar represents mean of DNA methylation levels. Hp, *H. pylori*; FH, family history; GC, gastric cancer

and ≥ 5 years ($P=0.001$) after eradication in controls with FH when compared with baseline levels (Fig. 6). Additionally, methylation levels of *miR124a-3* were significantly decreased at 2 ($P=0.002$) and ≥ 5 years ($P<0.001$) after eradication in GC patients with FH when compared with baseline levels (Fig. 6).

When subjects with FH and without FH were compared, significant differences in the methylation levels of *MOS* were constantly observed at 1 ($P=0.023$), 3–4 ($P=0.005$), and ≥ 5 years ($P=0.001$) after eradication in GC patients, and 2 ($P=0.035$), 3–4 ($P=0.014$), and ≥ 5 years ($P=0.048$) after eradication in controls (Fig. 6). However, significant differences in the methylation levels of *CDH1* and *TWIST1* in GC patients disappeared from 1 year after Hp eradication (Fig. 6). The difference in *miR124a-3*, *NKX6-1* and *CDH1*

methylation levels between controls with and without FH were not observed from 1 year after Hp eradication (Fig. 6).

Discussion

In the present study, the methylation status was compared between subjects with and without FH to identify specific genes associated with GC susceptibility in the presence of FH. We observed that methylation levels of *MOS*, *CDH1*, and *TWIST1* decreased in Hp-positive GC patients with FH when compared with those without FH. In Hp-positive healthy controls, *MOS*, *miR124a-3*, *NKX6-1*, and *CDH1* were differentially methylated between subjects with and without FH. Additionally, *CDH1* methylation levels

Fig. 3 Comparison of *MOS* (a), *miR124a-3* (b), *NKX6-1* (c), *EMX1* (d), *CDH1* (e), and *TWIST1* (f) methylation levels between intestinal metaplasia-positive gastric cancer (GC) patients and controls with and without family history (FH) according to *H. pylori* (Hp) infection. Among Hp-positive GC patients, methylation levels in *MOS*, *CDH1*, and *TWIST1* are significantly decreased in patients with FH when compared with those without FH. Among Hp-positive controls, methylation levels in *miR124a-3*, *NKX6-1*, and *CDH1* are significantly increased in controls with FH when compared with those without FH. Bar represents mean of DNA methylation levels. Hp, *H. pylori*; FH, family history; GC, gastric cancer

decreased to certain levels following Hp eradication in both GC patients with and without FH.

Regarding the six genes examined in this study, it has been previously demonstrated that hypermethylation of these genes in noncancerous gastric mucosa is associated with GC occurrence [3, 25–28]. Additionally, these genes are linked to Hp-induced DNA methylation in GC development [3, 25]. However, to date, evidence demonstrating the relationship between aberrant DNA methylation and FH of GC remains scarce. Kim et al. have reported that methylation of helicase-like transcription factor (HLTF), determined by methylation-specific PCR, is significantly associated with early GC patients with FH, suggesting that HLTF methylation could serve as a susceptible marker to assess the risk of GC in individuals with FH [29]. Chang et al. have revealed

that *ROBO2* gene methylation, as measured by methylation-specific PCR, is significantly lower in patients with FH of GC than in patients without FH [30]. We observed a decrease in methylation levels of *MOS*, *CDH1*, and *TWIST1* in GC patients with FH when compared with those without FH. Our results suggest that *MOS*, *CDH1*, and *TWIST1* gene methylations may be associated with sporadic gastric tumorigenesis, and epigenetic silencing of *MOS*, *CDH1*, and *TWIST1* may emerge after the development of GC rather than before GC development, or during the formation of an epigenetic field.

In the present study, subjects with and without FH demonstrated similar inflammation-associated characteristics, such as the presence of IM, the degree of neutrophil and monocyte infiltration, and serum PG I/II ratio. Despite

Fig. 4 Comparison of *MOS* (a), *miR124a-3* (b), *NKX6-1* (c), *EMX1* (d), *CDH1* (e), and *TWIST1* (f) methylation levels between intestinal metaplasia-negative gastric cancer (GC) patients and controls with and without family history (FH) according to *H. pylori* infection. Among Hp-positive GC patients, methylation levels in *MOS*, *CDH1*, and *TWIST1* are significantly decreased in patients with FH when compared with those without FH. Among Hp-positive controls, methylation levels in *NKX6-1* and *CDH1* are significantly increased in controls with FH when compared with those without FH. Bar represents mean of DNA methylation levels. Hp, *H. pylori*; FH, family history; GC, gastric cancer

previous findings suggesting that IM is associated with aberrant DNA methylation levels [31], our findings demonstrating decreased methylation levels of *MOS*, *CDH1*, and *TWIST1* in GC patients with FH persisted after adjustment for IM. Additionally, analyses of *NKX6-1* and *CDH1* revealed significant differences between controls with and without FH after adjustment for IM. However, we did not detect any association of FH with genetic DNA methylation levels among those without current Hp infections. The small number of subjects with true negative Hp infection could be insufficient to prove significance. The negative Hp infection could have hindered the detection of an association with aberrant DNA methylation.

We demonstrated that, compared with baseline levels, methylation levels of *CDH1* decreased constantly 2 years

after Hp eradication in patients with GC and 3–4 years after eradication in controls. Among patients with GC, *CDH1* methylation levels did not completely disappear for more than 5 years, and remained persistently higher than levels of control. Similar trends in changes of *CDH1* methylation levels were found in subjects with and without FH. In GC patients with FH, methylation levels of *MOS*, *miR124a-3*, and *TWIST1* decreased 2 and/or ≥ 5 years after eradication, suggesting that the changes in methylation levels could differ according to the presence of FH. Although our study did not demonstrate that methylation levels of *miR124a-3*, *NKX6-1*, *EMX1*, and *TWIST1* decreased constantly beginning 1 year after Hp eradication, it should be stated that these levels did not significantly increase when compared with baseline levels at any interval. Notably, methylation levels of *MOS* in

Fig. 5 Comparison of *MOS* (a), *miR124a-3* (b), *NKX6-1* (c), *EMX1* (d), *CDH1* (e), and *TWIST1* (f) methylation levels between gastric cancer (GC) patients and controls with and without family history (FH) according to *H. pylori* infection and histology (intestinal vs diffuse). Among intestinal type GC patients with Hp infection, methylation levels in *MOS*, *CDH1*, and *TWIST1* are significantly decreased in patients with FH when compared with those without FH. Among diffuse type GC patients with Hp infection, *MOS* and *CDH1* methylation levels are significantly decreased in patients with FH compared with those without FH. Bar represents mean of DNA methylation levels. Hp, *H. pylori*; FH, family history; GC, gastric cancer

the Hp-eradicated GC patients and controls remained persistently higher than baseline levels. Additionally, the significant difference in the *miR124a-3*, *NKX6-1*, *CDH1* and *TWIST1* methylation levels between subjects with and without FH disappeared after Hp eradication though *MOS* methylation levels in subjects with FH were constantly decreased than those in subjects without FH after eradication. Based on our findings, as well as previous studies, Hp eradication could prevent GC, which could be attributed to the amelioration of chronic inflammation and atrophy, as well as to the prevention of further progression of an epigenetic field for cancerization and restoration of epigenetic alterations. Residual methylation in the gastric mucosa after eradication might be relevant for GC development after successful Hp eradication. Furthermore, these findings might support

one of the molecular mechanisms explaining the results of previous studies reporting that Hp eradication reduces the incidence of metachronous GC [32].

Although several studies have demonstrated changes in DNA methylation after Hp eradication, most studies on aberrant methylation were followed up within 1 year of treatment [9, 10]. A previous report has shown that *CDH1* methylation levels decreased at 6 weeks [10] and 1 year after Hp eradication [9]. Recently, a 5-year follow-up study has indicated that the *CDH1* methylation rate decreased gradually over time after Hp eradication in both non-IM and IM, but only 78 patients including 27 GC patients were enrolled [33]. Our study included total 382 subjects and followed 60 GC patients and 72 controls over 5 years. Furthermore, several previous studies have utilized methylation-specific

Fig. 6 Changes in DNA methylation levels over time in the *MOS* (a), *miR124a-3* (b), *NKX6-1* (c), *EMX1* (d), *CDH1* (e), and *TWIST1* (f) genes in *Helicobacter pylori* (Hp)-eradicated gastric cancer (GC) patients and controls according to the presence of family history (FH). The *CDH1* methylation level is significantly decreased in GC patients at 2 years after Hp eradication (2 years, $P < 0.001$; 3–4 years, $P < 0.001$; ≥ 5 years, $P = 0.014$) and in controls at 3–4 years after eradication (3–4 years, $P = 0.025$; ≥ 5 years, $P = 0.01$) when compared with baseline levels. Methylation levels of *CDH1* were significantly decreased from 2 years after Hp eradication (2 years, $P = 0.001$; 3–4 years, $P = 0.001$; ≥ 5 years, $P = 0.032$) after Hp eradication than baseline levels in GC patients without FH, and 2 ($P = 0.030$) and 3–4 years ($P < 0.001$) in GC patients with FH, 2 years ($P = 0.022$) in controls without FH, and 3–4 ($P = 0.001$), and ≥ 5 years ($P = 0.001$) in controls without FH. Significant decreases in methylation levels of *MOS*, *miR124a-3*, and *TWIST1* were observed at 2 years (*miR124a-3*, $P = 0.008$; *TWIST1*, $P = 0.008$) and ≥ 5 years (*MOS*, $P = 0.008$; *miR124a-3*, $P = 0.005$) after eradication in GC patients with FH. DNA methylation results are presented as mean $\pm$ standard error. Hp, *H. pylori*; FH, family history; GC, gastric cancer

PCR, but it does possess some limitations, including several false-positive results and provides a qualitative analysis [34]. In this study, the strengths included the adoption of quantitative methods, MethyLight in the evaluation of DNA methylation, and enrollment of both Hp-negative and Hp-positive subjects, as well as analysis of subjects considering the presence of IM and histology.

In summary, our results suggest that *MOS* and *CDH1* methylation can provide a link between aberrant DNA methylation and GC with FH, and might be a useful marker for the risk of GC development. Hp eradication was associated with a significant decrease in *CDH1* in GC patients and controls, and significant difference of *CDH1*

methylation levels among subjects with and without FH disappeared in Hp-eradicated GC patients and controls. Further prospective and long-term follow-up studies with larger patient groups are required to confirm the difference of these gene methylation levels among subjects with and without FH, and the direct effect of Hp eradication on the methylation changes in various genes in gastric epithelial cells.

Author contributions HJK analyzed the data and drafted the article; NK designed the protocol, enrolled participants, collected data, and edited the manuscript; HWK analyzed data; JHP performed the

molecular biological study; CMS and DHL interpreted the data and edited the manuscript. All authors have read and approved the final draft of this paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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