主論文

Gastric Cancer
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ORIGINAL ARTICLE

Novel epigenetic markers for gastric cancer risk stratification in individuals after *Helicobacter pylori* eradication

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Abstract

Background The risk stratification of healthy individuals after *Helicobacter pylori* eradication is an urgent issue. The assessment of aberrant DNA methylation accumulated in gastric tissues with normal appearance, which can reflect overall epigenomic damage, is a promising strategy. We aimed to establish novel epigenetic cancer risk markers for *H. pylori*-eradicated individuals.

Methods Gastric mucosa was collected from eight healthy volunteers without *H. pylori* infection (G1), 75 healthy individuals with gastric atrophy (G2), and 94 gastric cancer patients (G3) after *H. pylori* eradication. Genome-wide analysis was conducted using Infinium 450 K and differentially methylated probes were screened using large difference and iEVORA-based methods. Bisulfite pyrosequencing was used for validation.

Results Screening, using 8 G1, 12 G2, and 12 G3 samples, isolated 57 candidates unmethylated in G1 and differentially methylated in G3 compared with G2. Validation for nine candidate markers (*FLT3, LINC00643, RPRM, JAM2, ELMO1, BHLHE22, RIMS1, GUSBP5,* and *ZNF3*) in 63 G2 and 82 G3 samples showed that all of them had significantly higher methylation levels in G3 than in G2 ($P < 0.0001$). Their methylation levels were highly correlated, which indicated that they reflect overall epigenomic damage. The candidates had sufficient performance (AUC: 0.70–0. 80) and high odds ratios (5.43–23.41), some of which were superior to a previous marker, *miR-124a-3*. The methylation levels of our novel markers were not associated with gastric atrophy, gender, or age.

Conclusions Novel epigenetic markers for gastric cancer risk optimized for *H. pylori*-eradicated individuals were established.

Keywords Epigenetics · Cancer risk marker · DNA methylation · Gastric cancer · *Helicobacter pylori*

Introduction

Risk stratification is critically important for the early detection of gastric cancer, which can lead to improved curability and reduced mortality. In Japan, national health insurance approved the eradication therapy of *Helicobacter pylori* (*H. pylori*) for the indication of chronic gastritis to prevent gastric

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cancers in February 2013, based on clinical evidence [1]. This has led to an explosive increase in healthy individuals after *H. pylori* eradication [2]. However, *H. pylori* eradication does not completely inhibit gastric cancer development [1]. Therefore, periodic surveillance for all such people is currently recommended and has emerged as a burden on both the individual and medical service providers. Risk stratification can optimize the surveillance interval for individuals, which helps to manage the social burden. Therefore, the establishment of a risk stratification system for *H. pylori*-eradicated healthy individuals is an urgent issue.

To this end, epigenetic risk markers have been highlighted as a promising biomarker for cancer risk stratification. Epigenetic alterations, namely aberrant DNA methylation, are induced in gastric mucosa with normal appearance by *H. pylori* infection-triggered chronic inflammation [3–5]. After *H. pylori* eradication, methylation levels decrease, depending upon individual marker genes, and the decreased levels persist for a long time [6, 7]. Persistent methylation levels after eradication are considered to reflect methylation in stem cells [8] and are closely correlated with gastric cancer risk [9, 10]. Methylation markers are considered to be a reflection of the overall epigenomic damage in gastric mucosa that leads to gastric cancer [11]. Using previously isolated methylation markers, such as *miR-124a-3* [12, 13], we conducted a multicenter prospective cohort study for the prediction of the risk of metachronous gastric cancers and found the advocated epigenetic cancer risk diagnosis as a promising strategy [14, 15].

However, our previous markers were isolated from relatively small sample sets and by methylated DNA immunoprecipitation (MeDIP)-CpG island microarray, which is a low-resolution technique [13]. The recent development of a bisulfite-based BeadArray has enabled high-throughput epigenetic analysis with a much more comprehensive coverage of the genome. In particular, the BeadArray reaches a higher resolution using more than 450 thousand probes; additionally, the BeadArray can accurately measure DNA methylation levels through the accurate detection of slight differences. Therefore, using this new technology, it is likely that we can establish novel methylation markers with a higher accuracy than that of the previous markers.

In this study, we aimed to establish novel methylation markers for the risk stratification of gastric cancer optimized for *H. pylori*-eradicated individuals using the BeadArray technology.

Materials and methods

Tissue sample collection

A total of 232 normal or non-cancerous gastric mucosa samples collected from 169 subjects in two previous studies [14, 16]. In addition, eight normal gastric mucosa samples were collected from healthy volunteers without a history of *H. pylori* infection and three peripheral blood samples from healthy volunteers for screening. Further, four gastric mucosa samples from healthy volunteers with current *H. pylori* infection and four without a history of *H. pylori* infection were collected for expression microarray analysis. All gastric samples were endoscopically biopsied from the fixed antral region (2 cm from the pyloric ring on the lesser curvature), and stored in RNAlater (Thermo Fisher Scientific, MA, USA) at − 80 °C. For *H. pylori*-eradicated individuals, the samples were collected 6 months or more after eradication. Genomic DNA was extracted using the phenol/chloroform method.

All participants were classified into three groups (Groups 1, 2, and 3) according to their cancer risk (low, intermediate, and high, respectively) defined by the history of *H. pylori* infection and gastric cancer status (Supplementary Table 1). Group 1 (G1) consisted of four young (mean age ± SD, 28.8 ± 4.2 years) and four older (mean age ± SD, 71.0 ± 3.1 years) healthy volunteers without a history of *H. pylori* infection, and was considered a low-risk group. Group 2 (G2) consisted of 75 healthy individuals who underwent cancer screening at the Research Center for Cancer Prevention and Screening at the National Cancer Center, Japan, between April 2009 and September 2009 and experienced the successful eradication of *H. pylori* [16]. All the G2 participants were accompanied by gastric mucosal atrophy, and G2 was considered an intermediate-risk group. G2 samples were consecutively collected both before and after *H. pylori* eradication, and all the samples had sufficient quality and quantity for use in this study. Group 3 (G3) consisted of 94 gastric cancer patients who underwent the first endoscopic submucosal dissection at the National Cancer Center Hospital [14]. Such gastric cancer patients are known to have a much higher incidence of subsequent (metachronous) gastric cancer even after *H. pylori* eradication [17], reaching 3.0% per year, while *H. pylori*-eradicated healthy individuals (G2) have a low incidence of 0.23% per year [18]. All G3 patients had a history of *H. pylori* infection accompanied by gastric mucosal atrophy.

Gastric mucosal atrophy was determined before successful *H. pylori* eradication in accordance with the Kimura–Takemoto classification [19], which was correlated with the degree of histological atrophy. The extent of gastric mucosal atrophy was classified into three grades: mild (closed types I and II), moderate (closed type III and open type I), and severe (open types II and III).

All previous studies and the current study were approved by the relevant Institutional Review Boards, and written informed consent was obtained from all participants.

Preparation of a screening set and a validation set

To prepare a screening set, 12 samples were randomly selected from G2 and G3, respectively (Supplementary Table 2). In the screening set, the age and extent of gastric atrophy were not significantly different between G2 and G3 (Supplementary Table 1). In addition, G1 samples (Supplementary Table 1) were used for the selection of unmethylated probes in normal gastric mucosa. The remaining participants were used as the validation set (Supplementary Table 3). In the validation set, age distribution was comparable between G2 and G3, but G3 included significantly more males and individuals with a more severe extent of gastric atrophy than G2 (Supplementary Table 1).

Genome-wide DNA methylation analysis

A comprehensive genome-wide screening of differentially methylated CpG sites was conducted using an Infinium HumanMethylation450 BeadChip array (Illumina, CA, USA) as described previously [20]. Among the total 485,512 probes, the probes on the sex chromosomes were excluded and the remaining 473,864 probes on autosomes were analyzed. The methylation level of each probe was represented by a β value, which was in the range from 0 (completely unmethylated) to 1 (completely methylated). To adjust for probe design biases, the intra-array normalization was conducted using a peak-based correction method, beta-mixture quantile dilation (BMIQ) [21].

iEVORA-based screening

The Epigenetic Variable Outliers for Risk prediction Algorithm (iEVORA) is a novel statistical algorithm based on the hypothesis that differentially variable (DV) and methylated (DM) CpGs (DVMCs) are more likely to indicate cancer risk [22, 23]. In the iEVORA algorithm, significant DV CpGs are selected by Bartlett's test FDRs and the significant DVMCs were ranked by DM *t* statistic. The iEVORA algorithm was computed by the R script 'iEVORA.R' and probes with a Bartlett's test FDR of less than 0.001 and an unadjusted *P* value of less than 0.05, based on the *t* test, were selected.

Quantitative methylation analysis

DNA (1 µg) was modified by sodium bisulfite using an innuCONVERT Bisulfite Basic kit (Analytik Jena AG, Germany) and EZ DNA Methylation Kit (Zymo Research, CA, USA). The modified DNA was suspended in 40 µL elution buffer and a 1 µL aliquot was used for quantitative methylation analysis. Quantitative methylation-specific polymerase chain reaction (qMSP) was performed using an iCycler Thermal Cycler (Bio-Rad Laboratories, CA, USA), as previously described [12, 13]. The primer sets specific to methylated and unmethylated sequences and PCR conditions are listed in Supplementary Table 4. Standard DNAs for methylated and unmethylated sequences were prepared by cloning the PCR products from fully methylated and unmethylated control DNAs, respectively, into pGEM-T Easy vector (Promega, WI, USA). The number of molecules in a sample was determined through the comparison of its amplification with those of standard DNA with defined numbers of molecules (10^1–10^6 molecules). The methylation levels were defined as the fraction of methylated molecules in the total number of DNA molecules (the number of methylated and unmethylated molecules).

Bisulfite pyrosequencing was performed on bisulfite-converted DNA using the PSQ 96 Pyrosequencing System (Qiagen, CA, USA) as previously described [24]. Data analysis was conducted using PyroMark Q96 ID software (Qiagen, version 2.5.8). The primer sequences and PCR conditions are listed in Supplementary Table 4.

Gene expression analysis by microarray

Expression analysis was conducted with a GeneChip Human Genome U133 Plus 2.0 expression microarray (Affymetrix, CA, USA), using a pool of 4 samples from healthy individuals with current *H. pylori* infection, and another pool of 4 samples from individuals without a history of *H. pylori* infection. Data processing was conducted using GeneChip operating software (ver. 1.4). The signal intensity of each probe was normalized so that the average signal intensity of all the probes on a microarray would be 500. Mean signal intensity of all probes for a gene was used as the gene expression level. Genes were classified into those with high (> 1000), moderate (250–1000), and low (< 250) transcription, according to their signal intensities as previously described [25].

Statistical analysis

The mean methylation levels were compared by Welch's *t* test and by a paired *t* test to compare G2 samples before and after eradication. The clinical factors between G2 and G3 were compared by Welch's *t* test or the Chi-squared test. These analyses were computed using GraphPad Prism software (GraphPad Software, CA, USA). Pearson's correlation coefficients and the *P* values were calculated by Excel software. The receiver-operating characteristic (ROC) curves and AUCs (area under the curve) were computed using the R package, ROCR. The *P* values were obtained by a two-sided test and considered as significant if less than 0.05.

Table 1 Candidates for epigenetic markers

No.	IlmnID	Human genome (Hg19)		UCSC_RefGene_Name	UCSC_RefGene_Group	Relation_to_UCSC_CpG_Island
		CHR	MAPINFO			
(A) The large difference analysis						
Top 10 (TT:*P* value)						
1	cg05598562	13	28674876	*FLT3*	TSS200	Island
2	cg03401096	11	123301171			Island
3	cg15897970	14	62583962	*LINC00643*	TSS200	Island
4	cg15400238	2	154335640	*RPRM*	TSS1500	S_Shore
5	cg13769223	21	27011963	*JAM2*	1stExon	Island
6	cg07122245	7	37488428	*ELMO1*	5′UTR	Island
7	cg01257828	3	192126996	*FGF12*	TSS200	Island
8	cg04942472	16	58497239	*NDRG4*	TSS1500	Island
9	cg13282152	8	65492683	*BHLHE22*	TSS200	N_Shore
10	cg01941671	4	81952099	*BMP3*	TSS200	Island
Top 10 (Delta β value)						
1	cg03401096	11	123301171			Island
2	cg11092616	6	72596493	*RIMS1*	TSS200	S_Shore
3	cg07122245	7	37488428	*ELMO1*	5′UTR	Island
4	cg05598562	13	28674876	*FLT3*	TSS200	Island
5	cg04942472	16	58497239	*NDRG4*	TSS1500	Island
6	cg10748160	4	6202384	*JAKMIP1*	TSS200	Island
7	cg14186641	6	88876741	*CNR1*	TSS1500	Island
8	cg13769223	21	27011963	*JAM2*	1stExon	Island
9	cg13282152	8	65492683	*BHLHE22*	TSS200	N_Shore
10	cg15400238	2	154335640	*RPRM*	TSS1500	S_Shore

No.	IlmnID	Methylation levels (mean beta value)								High vs Intermediate		High-quality pyrosequencing primer
		Blood	Low-risk (G1)		Intermediate-risk (G2)	SD	High-risk (G3)	SD	Delta [High-Intermediate]	P(TT)	q(TT)	
			Young	Old								
(A) The large difference analysis												
Top 10 (TT:*P* value)												
1	cg05598562	0.009	0.014	0.013	0.099	0.110	0.426	0.106	0.327	3.97E-07	3.89E-03	Designed
2	cg03401096	0.016	0.036	0.016	0.137	0.152	0.508	0.137	0.371	4.93E-06	6.97E-03	Not designed
3	cg15897970	0.059	0.052	0.024	0.166	0.097	0.404	0.126	0.238	6.62E-05	1.27E-02	Designed
4	cg15400238	0.157	0.059	0.041	0.139	0.101	0.425	0.166	0.286	1.18E-04	1.37E-02	Designed
5	cg13769223	0.025	0.051	0.020	0.149	0.129	0.439	0.160	0.290	1.28E-04	1.38E-02	Designed

Table 1 (continued)

No.	IlmnID	Methylation levels (mean beta value)								High vs Intermediate		High-quality pyrosequencing primer
		Blood	Low-risk (G1)		Intermediate-risk (G2)	SD	High-risk (G3)	SD	Delta [High-Intermediate]	P(TT)	q(TT)	
			Young	Old								
6	cg07122245	0.103	0.038	0.035	0.139	0.145	0.469	0.185	0.330	1.41E-04	1.41E-02	Designed
7	cg01257828	0.006	0.055	0.033	0.175	0.091	0.402	0.133	0.227	1.49E-04	1.41E-02	Not designed
8	cg04942472	0.009	0.059	0.063	0.176	0.132	0.471	0.177	0.295	2.48E-04	1.51E-02	Not Designed
9	cg13282152	0.016	0.033	0.022	0.114	0.074	0.403	0.190	0.288	3.37E-04	1.57E-02	Designed
10	cg01941671	0.124	0.036	0.019	0.127	0.102	0.350	0.141	0.222	3.92E-04	1.59E-02	Not designed
Top 10 (Delta β value)												
1	cg03401096	0.016	0.036	0.016	0.137	0.152	0.508	0.137	0.371	4.93E-06	6.97E-03	Not designed
2	cg11092616	0.005	0.012	0.006	0.149	0.153	0.495	0.224	0.347	4.22E-04	1.59E-02	Designed
3	cg07122245	0.103	0.038	0.035	0.139	0.145	0.469	0.185	0.330	1.41E-04	1.41E-02	Designed
4	cg05598562	0.009	0.014	0.013	0.099	0.110	0.426	0.106	0.327	3.97E-07	3.89E-03	Designed
5	cg04942472	0.009	0.059	0.063	0.176	0.132	0.471	0.177	0.295	2.48E-04	1.51E-02	Not Designed
6	cg10748160	0.038	0.026	0.018	0.104	0.126	0.399	0.191	0.295	4.07E-04	1.59E-02	Not designed
7	cg14186641	0.005	0.030	0.020	0.207	0.177	0.501	0.153	0.294	4.14E-04	1.59E-02	Not designed
8	cg13769223	0.025	0.051	0.020	0.149	0.129	0.439	0.160	0.290	1.28E-04	1.38E-02	Designed
9	cg13282152	0.016	0.033	0.022	0.114	0.074	0.403	0.190	0.288	3.37E-04	1.57E-02	Designed
10	cg15400238	0.157	0.059	0.041	0.139	0.101	0.425	0.166	0.286	1.18E-04	1.37E-02	Designed

No.	IlmnID	Human genome (Hg19)		UCSC_RefGene_Name	UCSC_RefGene_Group	Relation_to_UCSC_CpG_Island
		CHR	MAPINFO			
(B) The iEVORA-based analysis						
The large difference						
1	cg00432059	19	20012489	*ZNF93*	Body	S_Shore
2	cg02636041	10	43698008	*RASGEF1A*	Body	Island
Three sequential probes						
1	cg07655479	4	144480551	*GUSBP5*	TSS200	Island
2	cg06123783	4	144480558	*GUSBP5*	TSS200	Island
3	cg13487949	4	144480561	*GUSBP5*	TSS200	Island
4	cg10166205	19	50316699	*FUZ*	TSS1500	S_Shore
5	cg02709432	8	120651236	*ENPP2*	TSS200	
6	cg15653282	18	11689218	*GNAL*	5′UTR	Island

Table 1 (continued)

No.	IlmnID	Methylation levels (mean beta value)							High vs Intermediate		High-quality pyrosequencing primer
		Blood	Low risk (G1)		Intermediate-risk (G2)	SD	High-risk (G3)	SD	Delta [High-Intermediate]	P(TT)	q(TT)	
			Young	Old								
(B) The iEVORA-based analysis												
The large difference												
1	cg00432059	0.069	0.008	0.019	0.034	0.025	0.271	0.204	0.236	5.03E-08	1.31E-05	Designed
2	cg02636041	0.024	0.008	0.011	0.019	0.021	0.223	0.165	0.204	5.33E-08	1.37E-05	Not designed
Three sequential probes												
1	cg07655479	0.145	0.009	0.056	0.047	0.020	0.203	0.125	0.156	8.79E-07	1.32E-04	Designed
2	cg06123783	0.044	0.011	0.029	0.032	0.014	0.166	0.113	0.134	4.20E-08	1.12E-05	Not designed
3	cg13487949	0.189	0.004	0.074	0.042	0.017	0.171	0.095	0.129	3.16E-06	3.69E-04	Not designed
4	cg10166205	0.023	0.003	0.005	0.005	0.005	0.109	0.134	0.104	6.27E-14	3.37E-10	Not designed
5	cg02709432	0.032	0.048	0.042	0.060	0.017	0.149	0.093	0.089	3.60E-06	4.04E-04	Not designed
6	cg15653282	0.007	0.002	0.004	0.009	0.007	0.074	0.092	0.065	1.49E-10	1.26E-07	Not designed

CHR chromosome number, *NA* not applicable, *TT t* test, *P P* value, *q* FDR

Results

Isolation of candidate novel markers by two algorithms

To isolate the methylation cancer risk markers, two screening algorithms (the large difference and iEVORA-based methods) were adopted (Fig. 1). In the large difference method, to minimize the influence of contamination of inflammatory cells, probes with high methylation in three peripheral blood cell samples were first removed from the 473,864 probes on autosomes to obtain 86,596 probes. Then, 28,513 probes unmethylated in G1 (healthy volunteers never infected with *H. pylori*) were selected and 555 probes with a large difference ($\Delta\beta \geq 0.2$) between G2 ($n = 12$; *H. pylori*-eradicated healthy individuals with gastric mucosal atrophy) and G3 ($n = 12$; gastric cancer patients after endoscopic treatment and *H. pylori*-eradication) were isolated. Finally, to avoid the isolation of an outlier signal in a region, 49 regions were selected in which five consecutive probes showed consistent values and the value for the central probe was used. From these 49 candidates, ten candidates selected based on their *P* values and another ten candidates (of which six overlapped) selected based on mean differences of the beta values were used for validation (Table 1).

In the iEVORA-based method, 462 probes with high variances in G3 ($n = 12$) were first selected. Then, similar to the large difference method, 446 probes unmethylated in the peripheral blood cells were selected. Among the 446 probes, two candidates were isolated based on the large mean differences between G2 ($n = 12$) and G3 ($\Delta\beta \geq 0.2$). Additionally, six regions in which three consecutive probes showed consistent values were selected and the value for the central probe was used. Collectively, eight candidates were isolated by the iEVORA-based method and used for validation (Table 1).

All candidate markers analyzed were validated by a different set

Among the 14 (large difference method) and eight (iEVORA-based method) candidates, we successfully designed pyrosequencing primers for nine regions, *FLT3*, *LINC00643, RPRM*, *JAM2*, *ELMO1*, *BHLHE22, RIMS1, GUSBP5,* and *ZNF93* (Supplementary Table 4). First, to analyze the effect of *H. pylori* eradication on their methylation levels, we analyzed 63 G2 samples taken before and after *H. pylori* eradication. Consistent with previous reports [6, 7], all candidate markers showed comparable or decreased methylation levels after eradication compared to those before eradication (Supplementary Fig. 1).

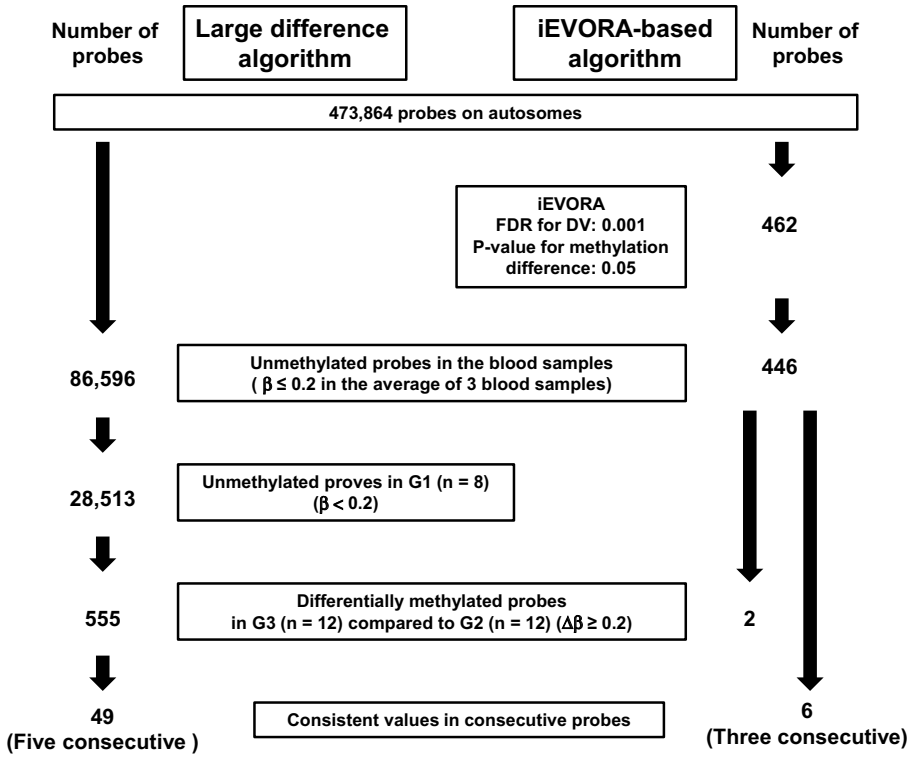

Fig. 1 The isolation of candidate novel epigenetic (DNA methylation) risk markers by two algorithms from 473,864 probes on autosomes. Left: the large difference algorithm. The probes unmethylated (β value < 0.2) in blood samples and G1 were first selected. Then, 555 probes with a large difference in methylation levels ($\Delta\beta \geq 0.2$) between G3 and G2 were selected. Finally, 49 regions in which five consecutive probes showed consistent values were obtained. Right: iEVORA-based algorithm. First, 462 probes were isolated by iEVORA. Subsequently, 446 unmethylated probes in the blood samples were selected. After consideration of both large difference and consecutive probes with consistent values, two and six probes were isolated, respectively

Next, the validation set of 63 G2 and 82 G3 samples was analyzed by the nine candidate markers and a previous marker (*miR-124a-3*) [12]. All the candidate markers and *miR-124a-3* had much higher methylation levels in G3 than in G2 ($P < 0.0001$; Fig. 2, Supplementary Table 5). Therefore, we were able to validate all the nine novel methylation markers for gastric cancer risk.

High correlations among novel methylation markers

To explore whether the novel methylation markers reflected the overall epigenomic damage that resulted from past exposure to environmental factors and host responses to them [26, 27], correlations among the markers were analyzed. As expected, high correlation coefficients ($R = 0.74$–0.97; Table 2) were observed between any two of the novel markers and *miR-124a-3*. In particular, the novel markers identified by the large difference method showed very strong correlations ($R = 0.82$–0.97) (Table 2, Supplementary Fig. 2). The known marker, *miR-124a-3*, was also highly correlated ($R = 0.66$–0.93) with the novel markers. These results suggested that our novel markers represented one shared entity, namely epigenomic damage as a result of *H. pylori* infection.

High-performance novel methylation markers

The clinical performance of the novel methylation cancer risk markers was assessed by ROC curve analysis (Fig. 3, Supplementary Table 6). All the novel markers showed high AUCs (0.70–0. 80), odds ratios (5.43–23.41), high-to-moderate sensitivity (0.59–0.94), and high-to-moderate specificity (0.59–0.83) (Supplementary Table 6). Compared with *miR-124a-3* (AUC; 0.74, odds ratio; 8.01), some novel markers had higher AUCs and better sensitivity or specificity. Thus, we were able to establish novel methylation markers for bisulfate pyrosequencing for the estimation of gastric cancer risk that was potentially superior to *miR-124a-3*.

Passenger methylation of novel marker genes in gastric carcinogenesis

To explore the possible roles of our novel marker genes in gastric carcinogenesis, we analyzed their expression levels in normal gastric mucosa using an expression microarray (Supplementary Table 7). Most of the marker genes showed very low expression levels irrespective of *H. pylori* infection statuses, which agreed with the data in the Genotype-Tissue Expression (GTEx, URL: https://www.gtexportal.org/home/) database (data not shown). This finding indicated that these genes are unlikely to play any biological roles in the stomach, and that their aberrant methylation is unlikely to be involved in gastric carcinogenesis, being passenger methylation. Nevertheless, *JAM2* and *ELMO1*, whose target probes were located in CpG sites near their transcription start sites (TSSs), showed moderate expression levels.

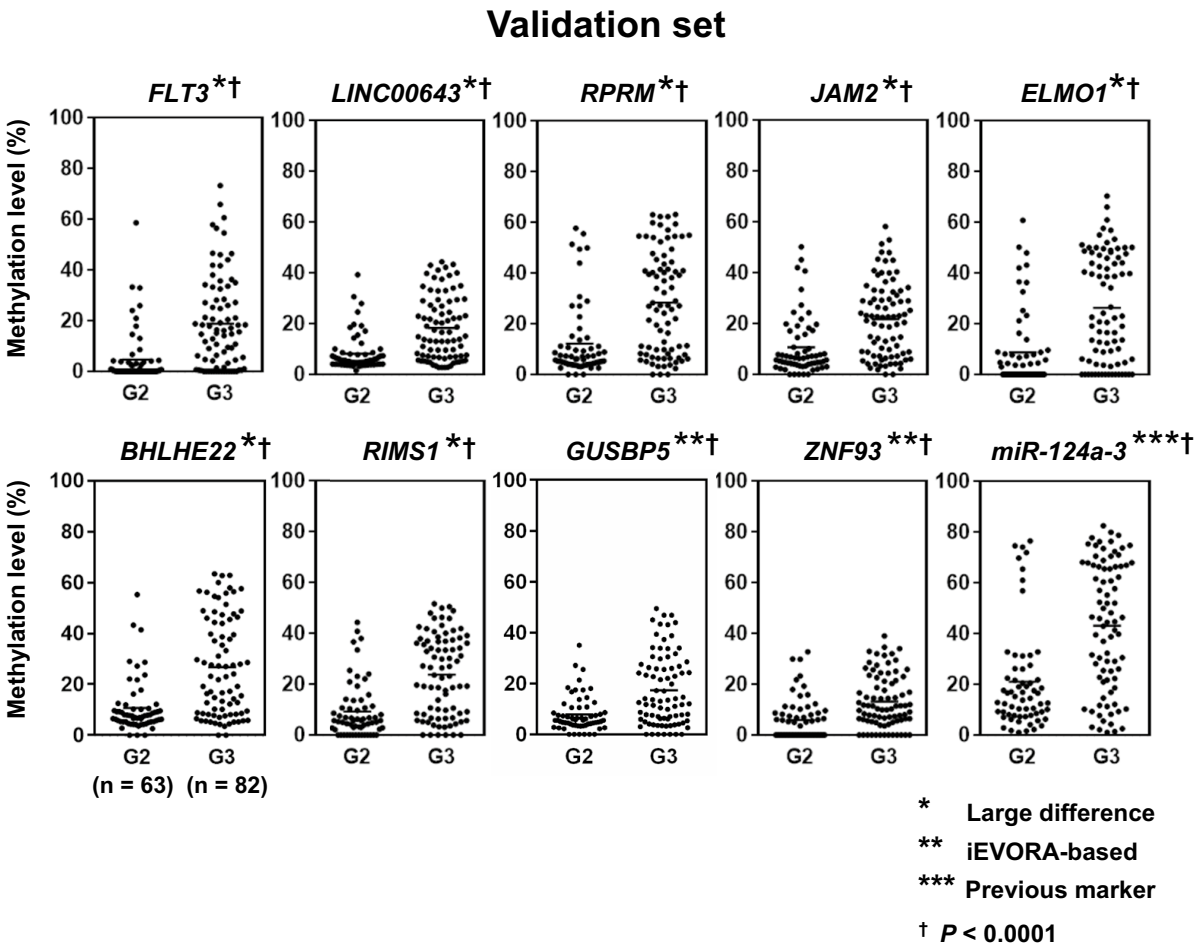

Fig. 2 The methylation levels of the nine candidate markers and a previous marker, *miR-124a-3*, in the validation set. The horizontal line represents the mean methylation level in each group. The methylation levels of all nine candidate markers, as well as *miR-124a-3*, were significantly higher in the high-risk group (G3, $n = 82$) than in the intermediate-risk group (G2, $n = 63$). *$P < 0.0001$

Relationships between methylation levels and the extent of gastric atrophy/gender/aging

Gastric atrophy and gender are also strong risk factors for gastric cancer [28, 29], and the efficacy of the novel methylation risk markers irrespective of these confounding factors was examined by subgroup analysis. First, the methylation levels of the markers did not show differences in the extent of gastric atrophy within G2 or G3 (Supplementary Fig. 3a and 3b). Among the individuals with moderate gastric atrophy, significantly higher methylation levels were observed in G3 ($n = 42$) than in G2 ($n = 26$). Significantly higher methylation levels were observed in G3 than in G2 for all novel markers and *miR-124a-3* (Supplementary Fig. 4, Supplementary Table 8a).

The subgroup analysis for men confirmed significantly higher methylation levels in G3 ($n = 73$) than in G2 (n = 41) for all markers. For women, the subgroup analysis also revealed higher methylation levels in G3 ($n = 9$) than in G2 ($n = 22$) for all markers, although some of the differences were not significant owing to the limited sample size (Supplementary Table 8b).

In addition, age-related methylation is known to contribute to predisposition to carcinogenesis by inactivating specific genes [30]. We compared methylation levels between four young and four older healthy individuals never infected with *H. pylori* (G1). No candidate novel methylation markers showed significant age-related differences (Table 1). This suggests that the novel methylation markers are unlikely to be affected by aging. On the other hand, regarding *H. pylori*-eradicated individuals (G2 and G3), the methylation levels of some markers showed weak positive correlations with age (Supplementary Fig. 5).

Taken together, these results suggested that our novel markers have the potential to be applied to individuals after *H. pylori* eradication, irrespective of the extent of gastric atrophy, gender, and age.

Table 2 Correlation coefficient between methylation markers

	Large difference							iEVORA-based		Previous marker
	Marker	*FLT3*	*LINC00643*	*RPRM*	*JAM2*	*ELMO1*	*BHLHE22*	*RIMS1*	*GUSBP5*	*ZNF93*	*miR-124a-3*
Large difference	*FLT3*	1									
	LINC00643	0.84	1								
	RPRM	0.86	0.90	1							
	JAM2	0.82	0.91	0.88	1						
	ELMO1	0.85	0.95	0.94	0.93	1					
	BHLHE22	0.84	0.88	0.88	0.87	0.90	1				
	RIMS1	0.83	0.95	0.93	0.93	0.97	0.89	1			
iEVORA-based	*GUSBP5*	0.80	0.80	0.80	0.76	0.79	0.77	0.80	1		
	ZNF93	0.74	0.83	0.76	0.78	0.77	0.78	0.80	0.88	1	
Previous marker	*miR-124a-3*	0.79	0.85	0.92	0.85	0.93	0.84	0.91	0.71	0.66	1

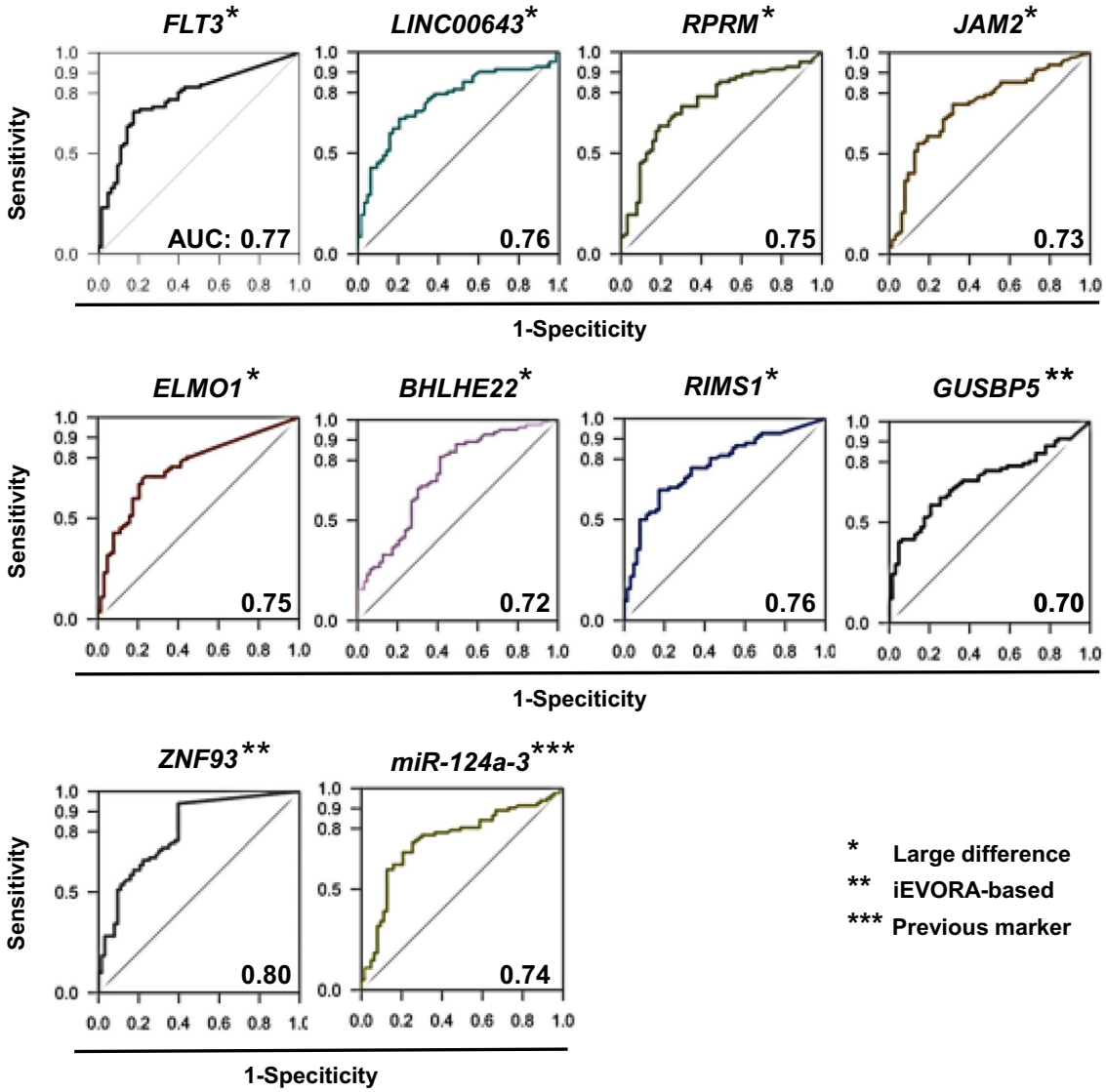

Fig. 3 The ROCs of nine novel methylation markers and *miR-124a-3*. The AUC values are shown in each box. The AUCs of some of the novel methylation markers were higher than that of *miR-124a-3*. AUC: area under the curve

Discussion

In the current study, we established nine novel epigenetic markers able to identify cancer patients even among *H. pylori*-eradicated individuals using a genome-wide screening based on two different algorithms. Our novel markers showed sufficiently high odds ratios, even among individuals with gastric atrophy. Some markers showed superior performance compared with our previous marker, *miR-124a-3*. We believe that BeadArray technology-based comprehensive methylation analysis and validation in a large number of samples provided us with promising methylation markers. Importantly, our epigenetic markers are not likely to be affected by possible contamination of blood cells in the biopsy sample because we eliminated such probes during our screening.

The novel methylation markers showed comparable or decreased methylation levels after eradication, compared to those before eradication, in agreement with previous reports [6, 7]. Our novel markers were selected as those showing very low methylation levels in blood cells. Therefore, the decrease in methylation levels after eradication is unlikely to have resulted from decreased infiltration of inflammatory blood cells after eradication, but likely resulted from the supply of new progenitor cells from unmethylated stem cells. Additionally, before eradication, a large fraction of inflammatory blood cells can reduce the fraction of epithelial cells. After eradication, an increased fraction of epithelial cells may result in a trade-off with decreased methylation levels in epithelial cells for some markers, leading to methylation levels comparable to those before eradication.

In general, genes with low expression are known to be susceptible to aberrant DNA methylation [25, 31, 32]. Indeed, seven marker genes had very low expression levels in the gastric mucosa, indicating that aberrant DNA methylation of these marker genes was passenger methylation. On the other hand, *JAM2* and *ELMO1* had moderate expression levels, and their target probes were located in CpG islands near the TSSs. Therefore, there remains a possibility that these two genes were methylation-silenced, being drivers. Methylation silencing of *JAM2*, involved in cell adhesion, and *ELMO1*, involved in cell motility, is reported in colorectal cancer [33–35]. However, more importantly, considering that the high correlations of methylation levels between the marker genes, the high susceptibility of the marker genes to aberrant methylation due to *H. pylori* infection, irrespective of their functions in gastric carcinogenesis, was considered the explanation for the good performance of these novel marker genes in risk prediction.

We also analyzed the effect of age-related methylation. However, no difference in methylation levels was observed between young and older healthy individuals never infected with *H. pylori* (G1), and the effect of aging was considered to be minimal. At the same time, in *H. pylori*-eradicated individuals (G2 and G3), some markers were slightly associated with age. Importantly, methylation levels are known to increase over time to reflect the infection period [5]; therefore, the slight increase correlated with age may be related to the infection period.

The potential limitations of our study include the sample bias collected in two studies [14, 16]. Additionally, *H. pylori*-eradicated individuals who were collected as G2 may contain a small number of cancer patients (G3). This might have resulted in a lower sensitivity and specificity in our markers than expected. In addition, the differences in prevalence of the extent of gastric atrophy and gender were observed between G3 and G2. However, the subgroup analysis suggested a high potential applicability of our methylation markers.

One of the prominent applications of the novel markers is a large-scale multicenter prospective cohort study for the risk stratification of primary gastric cancer in healthy individuals with extensive gastric atrophy after *H. pylori* eradication (UMIN000016894). This nationwide, ongoing prospective study is expected to optimize the surveillance system based on stratified individual risk, which will contribute to precision medicine.

In conclusion, we established nine novel methylation markers that identify *H. pylori*-eradicated individuals with high risk of gastric cancer.

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Compliance with ethical standards

Conflict of interest MM and TU made a joint patent application with Sysmex Corporation for identified epigenetic markers.

Ethical approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study.

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LETTERS

High impact of methylation accumulation on metachronous gastric cancer: 5-year follow-up of a multicentre prospective cohort study

We recently published in your journal a 3-year multicentre prospective cohort study demonstrating the usefulness of an epigenetic cancer risk marker for gastric metachronous cancers.[1] This study achieved the first proof of concept of epigenetic cancer risk diagnosis in any type of cancer but, due to the short follow-up period, a relatively small number of events were observed, resulting in a marginally significant difference (p=0.042). It was anticipated that a longer follow-up could lead to a clearer difference and HR with a smaller 95% CI. We now report the 5-year follow-up data, which show highly significant results.

Among the 826 enrolled patients, 795 patients received annual follow-ups by endoscopy for a median period of 5.46 years (IQR: 3.95–6.09). By the end, 133 patients had developed a metachronous gastric cancer. Among them, 116 patients developed a metachronous gastric cancer detected 1 year after the enrolment (authentic metachronous cancer).

Statistical analyses were conducted in the same manner as previously described.[1] Briefly, all the patients were categorised into quartiles (Q1: lowest to Q4: highest) according to the methylation levels of each of three genes (*miR-124a-3*, *EMX1* and *NKX6-1*). Cumulative incidences of metachronous gastric cancers were compared by a log-rank test, and HRs and 95% CIs were assessed by univariate and multivariate analyses by adjusting known risk factors and possible confounding factors, using a Cox proportional hazard regression model.

The univariate and multivariate analyses showed that Q4 (highest) of each of the three genes had significantly higher HRs than Q1 (lowest), using all and authentic metachronous gastric cancers (p<0.005) (table 1). Especially, the multivariate-adjusted HR of Q4 for *miR-124a-3* was 3.0 (95% CI 1.58 to 5.72, p=0.0017).

The Kaplan-Meier curves showed the cumulative incidences of the metachronous gastric cancers for quartiles (Q1–Q4) of methylation levels for each of the three genes (figure 1). For each gene, Q4 had a higher incidence of metachronous gastric cancer than Q1, with a p value of <0.001 by the log-rank test.

These final results based on the 5-year follow-up convincingly endorsed the proof of concept of epigenetic cancer risk diagnosis with sufficiently small p values, and provided a rationale that epigenetic markers can be used for cancer risk diagnosis. All the participants of this study once had a gastric cancer and thus originally carried a high risk of metachronous gastric cancer, as observed in Q1 (figure 1). Therefore, cancer risk stratification in this cohort was considered to be very difficult, but has been achieved. At the same time, the high risk inherent in the cohort will not allow changing the current clinical practice with annual endoscopic surveillance.

On the other hand, for asymptomatic *Helicobacter pylori*-infected individuals without a cancerous lesion, cancer risk stratification after their *H. pylori* eradication has been highly demanded because it can lead to optimisation of cancer surveillance based on an individual's risk. In order to establish precision medicine in this population, we have launched a new large-scale multicentre prospective cohort study (UMIN000016894) to predict the risk of primary gastric cancer in healthy individuals after *H. pylori* eradication.

The strong influence of methylation accumulation on gastric cancer risk was

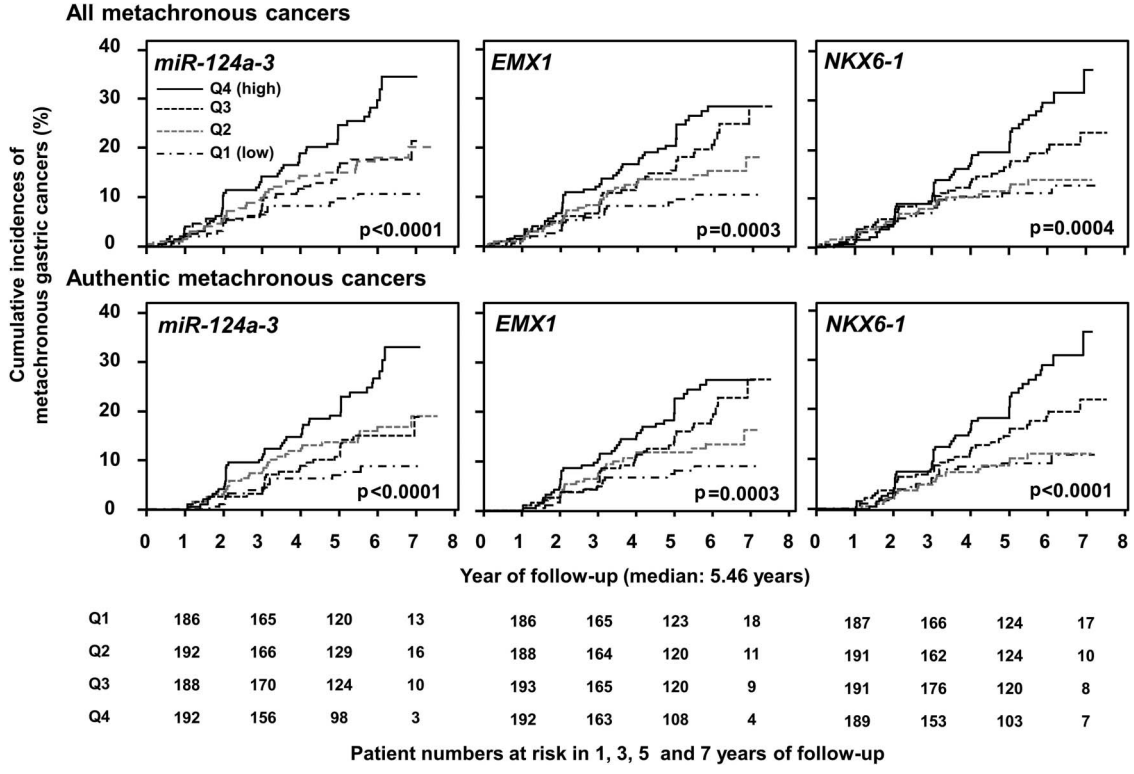

Figure 1 Cumulative incidences of metachronous gastric cancers of patients in quartiles (Q1–Q4) of methylation levels of *miR-124a-3*, *EMX1* and *NKX6-1*.

Table 1 Univariate and multivariate-adjusted HRs (95% CI) for a metachronous gastric cancer according to DNA methylation levels of the three genes

	Univariate					Multivariate*				
	Quartile of DNA methylation level					Quartile of DNA methylation level				
Variable	Q1 (lowest)	Q2	Q3	Q4 (highest)	p for trend	Q1 (lowest)	Q2	Q3	Q4 (highest)	p for trend
No of patients (795)										
miR-124a-3	198	199	199	199		198	199	199	199	
EMX1	198	199	199	199		198	199	199	199	
NKX6-1	198	199	199	199		198	199	199	199	
All metachronous gastric cancers										
miR-124a-3										
No of events	18	33	32	50		18	33	32	50	
HR (95% CI)	1	1.73 (0.97 to 3.08)	1.64 (0.91 to 2.94)	2.89 (1.66 to 5.02)	0.0002	1	1.64 (0.90 to 2.99)	1.48 (0.80 to 2.74)	2.57 (1.43 to 4.61)	0.002
EMX1										
No of events	18	28	40	47		18	28	40	47	
HR (95% CI)	1	1.47 (0.81 to 2.67)	2.09 (1.19 to 3.67)	2.63 (1.51 to 4.56)	0.0001	1	1.46 (0.79 to 2.68)	1.75 (0.98 to 3.13)	2.33 (1.31 to 4.15)	0.0027
NKX6-1										
No of events	21	24	37	51		21	24	37	51	
HR (95% CI)	1	1.13 (0.63 to 2.05)	1.65 (0.95 to 2.85)	2.42 (1.42 to 4.10)	0.0002	1	1.12 (0.61 to 2.06)	1.61 (0.91 to 2.84)	2.26 (1.30 to 3.92)	0.0008
Authentic metachronous gastric cancers										
miR-124a-3										
No of events	14	30	26	46		14	30	26	46	
HR (95% CI)	1	2.03 (1.07 to 3.85)	1.73 (0.89 to 3.33)	3.53 (1.91 to 6.53)	<0.0001	1	1.93 (1.00 to 3.75)	1.50 (0.75 to 2.97)	3.00 (1.58 to 5.72)	0.0017
EMX1										
No of events	15	24	35	42		15	24	35	42	
HR (95% CI)	1	1.52 (0.79 to 2.90)	2.21 (1.20 to 4.07)	2.87 (1.58 to 5.23)	0.0001	1	1.44 (0.74 to 2.81)	1.79 (0.95 to 3.38)	2.45 (1.31 to 4.58)	0.0028
NKX6-1										
No of events	17	18	33	48		17	18	33	48	
HR (95% CI)	1	1.07 (0.55 to 2.09)	1.86 (1.02 to 3.89)	2.92 (1.64 to 5.20)	<0.0001	1	1.03 (0.52 to 2.04)	1.74 (0.93 to 3.24)	2.63 (1.44 to 4.82)	0.0001

*Adjusted for hospital, gender and age (<50, 50–59, 60–69 or ≥70), pepsinogen index, history of endoscopic submucosal dissection (0, 1, 2 or 3 times), pack-years of smoking (0, 1–39 or ≥40) and green vegetable intake (≤2 days/week, 3–4 days/week or almost daily).

considered to be due to the major contribution of aberrant DNA methylation induced by *H. pylori* infection in gastric epithelial cells to gastric carcinogenesis, along with mutations produced by activation-induced cytidine deaminase.[2] The relatively small number of driver mutations after comprehensive mutation analyses[3 4] also supports the importance of methylation accumulation in gastric carcinogenesis.

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Contributors Study concept: TU, TN and MF. Patients' follow-up and data collection: TN, NY, TM, CY, IO, TY, MF and TG. Experiments: KA, TA and SN. Data analysis: TS. Letter concept: KA, TU and MM. Drafting of the manuscript: TU and MM.

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Patient consent Obtained.

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GUT

High impact of methylation accumulation on metachronous gastric cancer: 5-year follow-up of a multicentre prospective cohort study

Masahiro Maeda, Takeshi Nakajima, Ichiro Oda, Taichi Shimazu, Nobutake Yamamichi, Takao Maekita, Kiyoshi Asada, Chizu Yokoi, Takayuki Ando, Takeichi Yoshida, Sohachi Nanjo, Mitsuhiro Fujishiro, Takuji Gotoda, Masao Ichinose and Toshikazu Ushijima

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REVIEW ARTICLE

Mechanisms for the induction of gastric cancer by *Helicobacter pylori* infection: aberrant DNA methylation pathway

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Abstract Multiple pathogenic mechanisms by which *Helicobacter pylori* infection induces gastric cancer have been established in the last two decades. In particular, aberrant DNA methylation is induced in multiple driver genes, which inactivates them. Methylation profiles in gastric cancer are associated with specific subtypes, such as microsatellite instability. Recent comprehensive and integrated analyses showed that many cancer-related pathways are more frequently altered by aberrant DNA methylation than by mutations. Aberrant DNA methylation can even be present in noncancerous gastric mucosae, producing an "epigenetic field for cancerization." Mechanistically, *H. pylori*-induced chronic inflammation, but not *H. pylori* itself, plays a direct role in the induction of aberrant DNA methylation. The expression of three inflammation-related genes, *Il1b, Nos2, and Tnf*, is highly associated with the induction of aberrant DNA methylation. Importantly, the degree of accumulated aberrant DNA methylation is strongly correlated with gastric cancer risk. A recent multicenter prospective cohort study demonstrated the utility of epigenetic cancer risk diagnosis for metachronous gastric cancer. Suppression of aberrant DNA methylation by a demethylating agent was shown to inhibit gastric cancer development in an animal model. Induction of aberrant DNA methylation is the major pathway by which *H. pylori* infection induces gastric cancer, and this can be utilized for translational opportunities.

Keywords *Helicobacter pylori* infection · Aberrant DNA methylation · Epigenetic cancer risk diagnosis

Introduction

'How does *Helicobacter pylori* infection induce gastric cancer?' has long been a challenging question. For the last two decades, various pathogenic mechanisms of *H. pylori*-associated gastric cancer have been intensively investigated, and three major mechanisms have become clear. First, multiple signaling pathways were shown to be perturbed in gastric epithelial cells by virulence factors of *H. pylori* such as VacA and CagA [1, 2]. This mechanism is closely involved in the *H. pylori* type IV secretion machinery. Second, mutations were shown to be induced by aberrant expression of activation-induced cytidine deaminase (AID) via NFκB activation in gastric epithelial cells due to *H. pylori* infection-induced chronic inflammation [3]. Third, aberrant DNA methylation was shown to be accumulated in gastric mucosa by chronic inflammation caused by *H. pylori* infection [4].

In particular, multiple lines of evidence indicate that the accumulation of aberrant DNA methylation is very important in gastric carcinogenesis. Firstly, aberrant DNA methylation, a representative epigenetic alteration, can cause inactivation of tumor-suppressor genes. Indeed, comprehensive and integrated analyses of gastric cancer have shown that aberrant DNA methylation has a major impact [5, 6]. Secondly, the degree of accumulation of

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aberrant DNA methylation is highly correlated with gastric cancer risk [7, 8]. Furthermore, animal experiments have shown that inhibiting aberrant DNA methylation induction could prevent gastric cancer development [9].

In this review, we provide an overview of the current understanding of the mechanisms by which aberrant DNA methylation is induced by *H. pylori* infection. We also highlight potential applications of aberrant DNA methylation in precision medicine.

Deep involvement of aberrant DNA methylation in gastric cancer

Genetic and epigenetic alterations accumulate during multistep carcinogenesis through exposure to various carcinogenic factors [10]. However, few frequent driver mutations associated with gastric cancer have been identified besides *TP53* and *CDH1*. Although several new driver genes such as *ARID1A* and *RHOA* have been identified by recent exome and whole-genome sequencing [5, 11], such mutations account for less than 15 % of all gastric cancers. Indeed, more than 20 % of gastric cancers present only one or even no mutation [11].

On the other hand, a deep involvement of aberrant DNA methylation in gastric cancer has been highlighted [12]. In 1999, frequent occurrence of aberrant DNA methylation of CpG islands (CpG island methylator phenotype; CIMP) was shown to be associated with microsatellite instability in gastric cancer, as it is in colon cancer [13]. Characteristically, Epstein–Barr virus-positive gastric cancer has been known to display extreme CIMP [14, 15]. These findings were validated by The Cancer Genome Atlas (TCGA) Research Network [5]. Furthermore, a recent integrated analysis of genetic and epigenetic alterations revealed that inactivation of tumor-suppressor genes such as *p16*, *hMLH1*, and *CDH1* and activation of the WNT pathway were more frequently caused by aberrant DNA methylation than by mutations (Fig. 1) [6]. This evidence suggests that aberrant DNA methylation has as much or even more of an impact on gastric carcinogenesis than mutations.

DNA methylation level in noncancerous mucosa and gastric cancer risk

Aberrant DNA methylation can even be present in noncancerous gastric mucosa, and its levels are influenced by *H. pylori* infection (Fig. 2). The association of aberrant DNA methylation in gastric mucosa with *H. pylori* was reported by Chan et al. for the first time in 2003 [16]. However, at the same time, Kang et al. showed that there was no association between them [17]. These conflicting findings were considered to be due to nonquantitative DNA methylation analyses. Later, a quantitative methylation analysis focusing on CpG islands of passenger genes clearly demonstrated an association between high methylation levels in gastric mucosae and *H. pylori* infection [7].

At the same time, a cross-sectional study suggested that eradicating *H. pylori* leads to a decrease in DNA methylation levels [7]. Later, temporal analyses showed that eradication of *H. pylori* decreases DNA methylation levels [18–21]. Importantly, among individuals not currently infected with *H. pylori*, DNA methylation levels were much higher in cancer patients than in healthy individuals [7]. Additionally, methylation levels were higher in cases with multiple gastric cancers than in cases with a single cancer [8]. It was therefore suggested that DNA methylation levels in individuals not currently infected with *H. pylori* are closely correlated with gastric cancer risk.

Cell types and genes susceptible to aberrant DNA methylation

Gastric mucosal biopsy samples contain various types of cells in addition to epithelial cells. Therefore, the cell types that aberrant DNA methylation is induced in were unclear. This issue was addressed by observing increased methylation levels of multiple genes in gastric epithelial cells highly purified by the gland isolation technique from the stomachs of Mongolian gerbils (*Meriones unguiculatus*), a widely used animal model for *H. pylori* infection and gastric cancer [4]. Also, in a genome-wide DNA methylation analysis of human gastric mucosa, aberrant DNA methylation was still observed, even after the exclusion of CpG sites methylated in human blood cells, ruling out the possibility of increased methylation due to blood cell-specific methylation (Nanjo et al., unpublished data). These data showed that gastric epithelial cells are the real targets of aberrant DNA methylation induction. Nevertheless, there remains the possibility that aberrant DNA methylation is also induced in other types of cells, such as stromal cells, and that such epigenetic alterations may also be important for gastric cancer development.

Eradicating *H. pylori* decreases DNA methylation levels in gastric mucosae, and the decreased methylation levels persist for a long time [4, 20]. This suggests that aberrant DNA methylation consists of transient and permanent components. Mechanistically, we can speculate that the aberrant DNA methylation induced in stem cells of a gastric gland is a permanent component because methylation status in stem cells is preserved and replicated, thus determining the fraction of cells with methylation. In contrast, methylation induced only in differentiated cells will disappear when they are replaced by new cells without

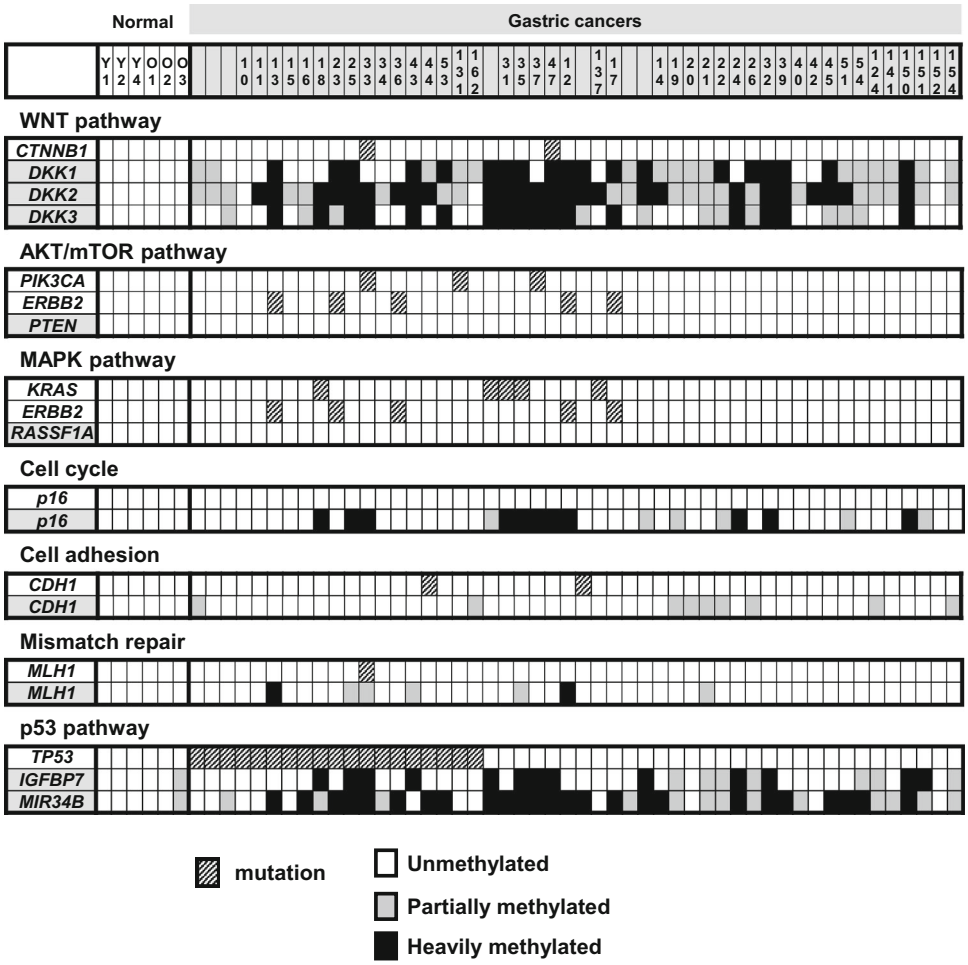

Fig. 1 Genetic and epigenetic alterations of genes in multiple signaling pathways in 6 normal gastric mucosa and 50 gastric cancer samples. Three growth-promoting and four tumor-suppressor pathways are included. Inactivation of tumor-suppressor genes such as *p16*, *hMLH1*, and *CDH1* and activation of the WNT pathway were more frequently caused by aberrant DNA methylation than by mutations. This figure was modified from [6]

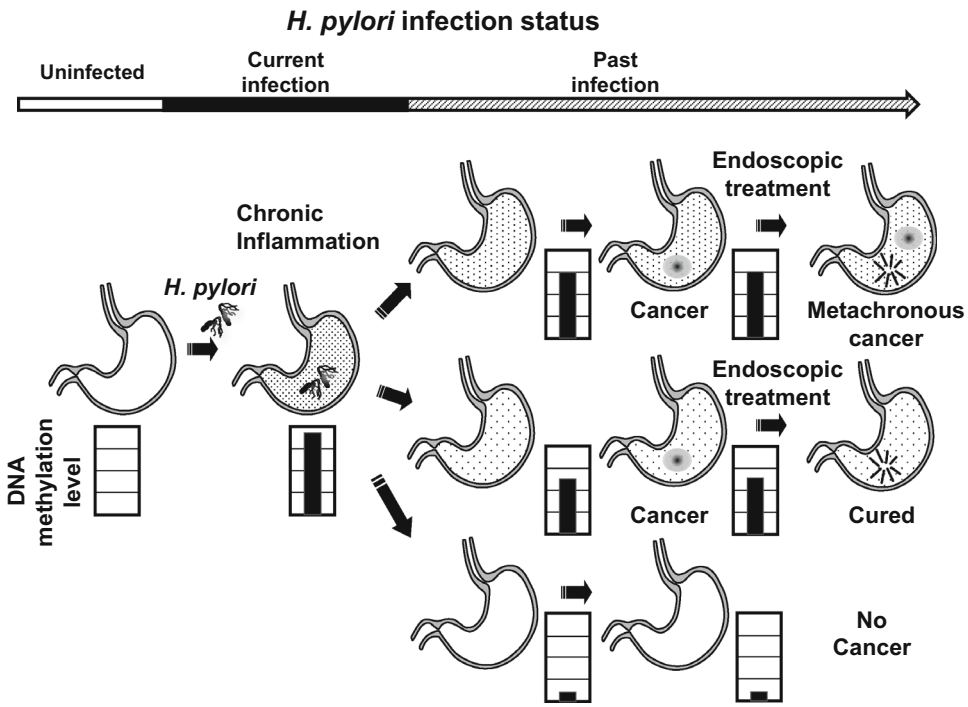

Fig. 2 *H. pylori* infection, DNA methylation induction, and gastric cancer risk. The clinical course of individuals infected with *H. pylori* is illustrated. The *H. pylori* infection occurs in childhood, causing chronic inflammation in the stomach. Chronic inflammation induces aberrant DNA methylation in gastric mucosa. Once *H. pylori* is eradicated, the DNA methylation level decreases somewhat but does not disappear completely. The degree of residual DNA methylation is strongly correlated with gastric cancer risk

methylation derived from a stem cell without methylation, meaning that this methylation induced in differentiated cells represents a transient component [22] (Fig. 3).

A large number of specific genes are aberrantly methylated by *H. pylori* infection [23]. Mechanistically, it is generally known that promoter CpG islands without active transcription of their downstream genes and with a specific histone modification, H3K27me3, are likely to become methylated [24–26]. In addition to physiological H3K27me3, aberrant H3K27me3 can be induced by environmental factors such as chronic inflammation [27]. Therefore, in gastric mucosa, genes that are not expressed naturally or those that are downregulated by *H. pylori* infection are likely to become methylated.

Such genes that are not expressed naturally are considered to play no biological role in gastric mucosae. Therefore, the methylation of such genes is likely to have no biological consequences in gastric carcinogenesis, and is thus considered a passenger event. On the other hand, although driver genes such as *p16*, *CDH1*, and *MLH1* are expressed in gastric mucosae with diverse expression levels, they are methylated in cancer cells. If we identify genes that are methylated in gastric cancer but expressed in normal gastric mucosae, they are more likely to be driver genes [28].

Mechanisms by which *H. pylori* infection induces aberrant DNA methylation

To verify that *H. pylori* infection induces aberrant DNA methylation, Mongolian gerbils were infected with *H. pylori*, and induction of aberrant DNA methylation in

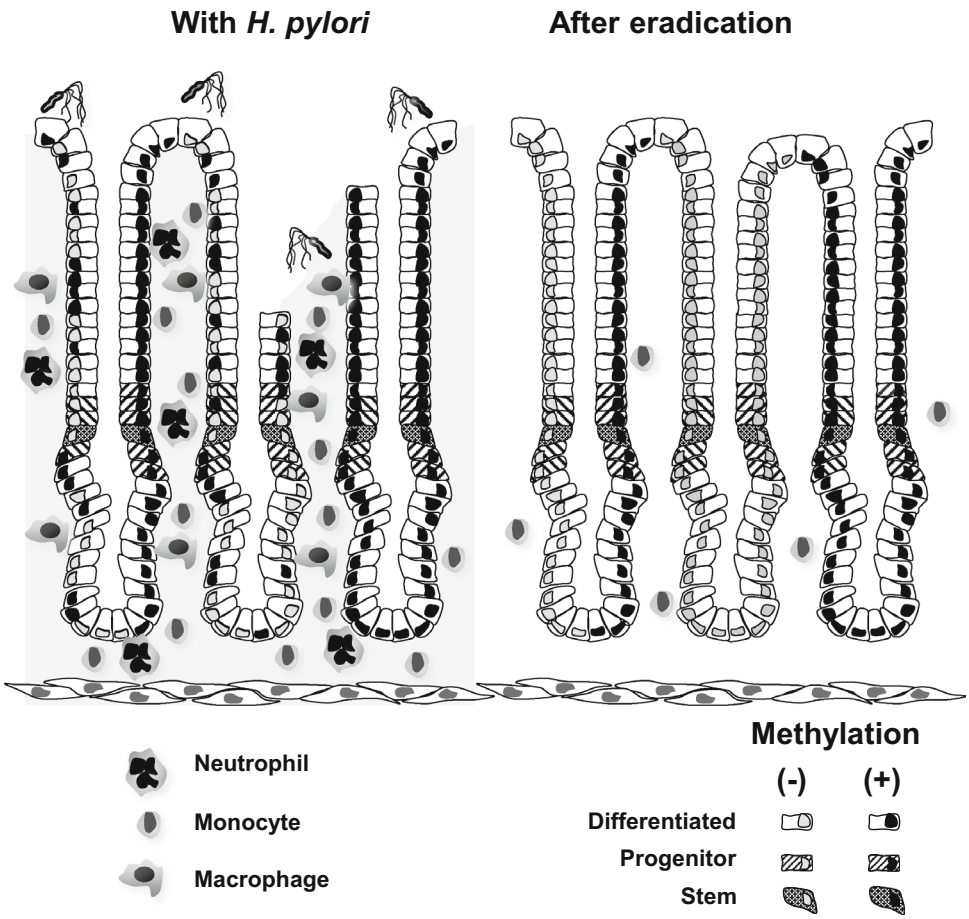

Fig. 3 Potential target cells for the induction of aberrant DNA methylation. *Left*: gastric mucosa with active *H. pylori* infection. *Right*: gastric mucosa after eradication of *H. pylori*. Chronic inflammation, characterized by infiltration of monocytes/macrophages with neutrophils, induces aberrant DNA methylation. Aberrant DNA methylation is actively induced in differentiated cells, possibly in progenitor cells (transient component), along with some stem cells. When methylation is present in a stem cell, all of the cells derived from the stem cell in a gland are methylated (permanent component). When methylation is induced in differentiated cells, heterogeneous methylation within a gland is present, and this methylation will disappear when fresh cells without methylation are derived from a stem cell. Without active induction of aberrant DNA methylation, the methylation status of a gland reflects that of its stem cell. The methylation level in the gastric mucosa is assumed to be proportional to the fraction of stem cells with methylation. This figure was modified from [22]

purified gastric gland cells was demonstrated [4]. In addition, eradicating *H. pylori* clearly decreased methylation levels, which were accompanied by diminished histological inflammatory responses (Fig. 4a). Then, to address whether *H. pylori* or the resultant chronic inflammation was responsible for inducing aberrant DNA methylation, inflammatory responses were repressed by cyclosporin A, an immunosuppressive agent, in *H. pylori*-infected gerbils. Although the amount of *H. pylori* was not reduced in the gastric mucosa, the repression completely suppressed the induction of aberrant DNA methylation [9]. Hence, it was concluded that it was not *H. pylori* itself but the inflammatory response triggered by *H. pylori* infection that was directly responsible for the induction of aberrant DNA methylation.

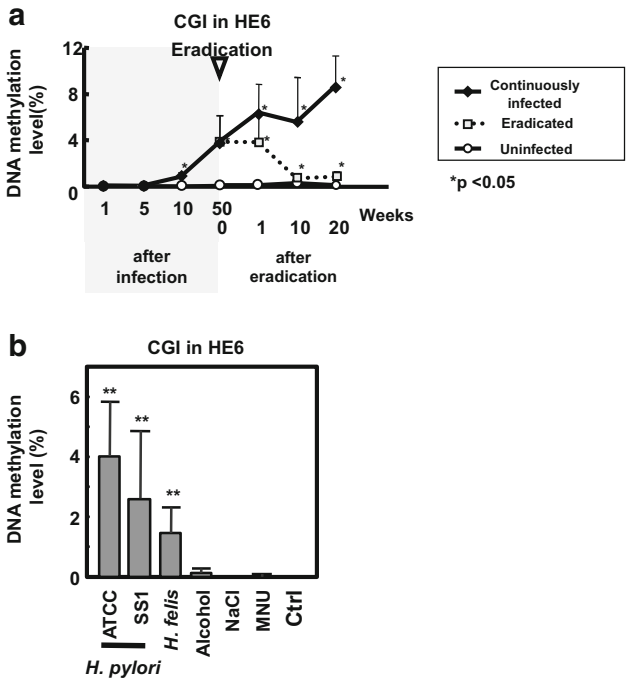

Fig. 4a, b Induction of aberrant DNA methylation by *H. pylori* infection in Mongolian gerbils and the effect of eradication. **a** After gerbils were infected with *H. pylori*, DNA methylation levels in purified gastric epithelial cells, as measured by quantitative methylation-specific PCR (qMSP), increased at ≥10 weeks of infection. After eradication, DNA methylation levels were not decreased at 1 week, but were decreased at 10 and 20 weeks. Importantly, DNA methylation levels after eradication were still higher than those in never-infected gerbils. **b** Capacities of various kinds of inflammation to induce aberrant DNA methylation. Persistent inflammation was induced by *H. pylori*, *H. pylori* strain SS1, *H. felis* infection, high concentrations of alcohol, and saturated NaCl. As controls, an MNU group and a nontreatment group were analyzed. In all eight CpG islands analyzed (methylation levels of CpG island HE6 are shown in Fig. 3b), only groups with *H. pylori*, *H. pylori* strain SS1, and *H. felis* infection showed the induction of aberrant DNA methylation. This figure was modified from [4]

The next question was whether any kind of persistent inflammation could induce aberrant DNA methylation. Mongolian gerbils were treated with alcohol or sodium chloride (NaCl), both of which are well known to be inflammation inducers. Aberrant DNA methylation was induced only by *H. pylori* and *H. felis,* but not by high concentrations of alcohol or saturated NaCl (Fig. 4b). *H. pylori* and *H. felis* triggered chronic inflammation as characterized by infiltration of monocytes/macrophages with residual neutrophils, whereas alcohol and NaCl elicited repeated acute inflammation as characterized by major infiltration of neutrophils [29].

Regarding inflammatory response genes, *Il1b*, *Nos2*, and *Tnf* were upregulated consistently in gastric mucosa of *H. pylori*- or *H. felis*-infected gerbils, and were associated with increased DNA methylation levels. Notably, *Il1b* and *Nos2* were also induced in mouse colonic mucosae with dextran sulfate sodium-induced colitis [30]. Consequently, we can conclude that aberrant DNA methylation is induced by specific types of inflammation, and is likely to be associated with the expression of *Il1b*, *Nos2*, and *Tnf*.

Application of aberrant DNA methylation induced by *H. pylori* infection

Aberrant DNA methylation in specific genes is frequent, even in noncancerous tissue, and contributes to carcinogenesis, so it could be used in a variety of applications relating to cancer risk diagnosis and chemoprevention.

Clinical study of epigenetic cancer risk diagnosis

The accumulation of aberrant DNA methylation in noncancerous tissues has been termed an "epigenetic field for cancerization" or "epigenetic field defect," especially in inflammation-associated cancers such as gastric cancer [31]. Cross-sectional studies have shown that the degree of a field defect can be assessed using appropriate cancer risk markers, as described above [32, 33]. However, cross-sectional studies inevitably include various types of biases. Recently, a multicenter prospective cohort study for predicting the risk of metachronous gastric cancer demonstrated the utility of an epigenetic cancer risk marker for the first time [34].

In this study, gastric cancer patients were enrolled after endoscopic submucosal dissection (ESD). After assessing the methylation levels of three preselected genes, annual follow-up to detect metachronous gastric cancer was conducted for 3 years by trained endoscopists who were blinded to methylation information. Multivariate analysis showed that the highest quartile of the methylation level of *miR-124a-3*, a marker gene, had a significantly higher HR

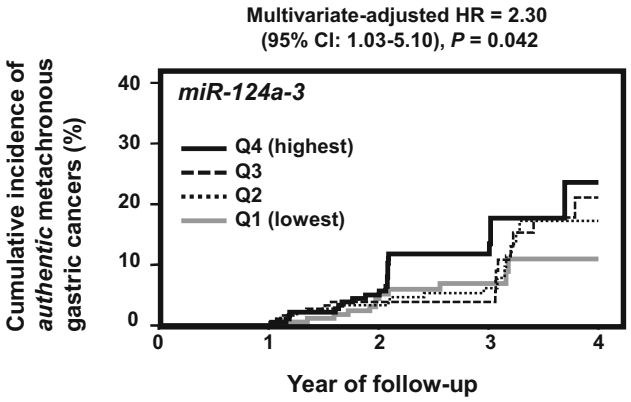

Fig. 5 Cumulative incidence of authentic metachronous gastric cancer (i.e., gastric cancer that developed after 1 year of enrollment). Patients were grouped into quartiles (Q1–Q4) based on methylation levels of *miR-124a-3*. Q4 (the highest) had a higher incidence of authentic metachronous gastric cancer than Q1 (the lowest). A multivariate analysis adjusting for hospital, gender, age, *H. pylori* infection before enrollment, pepsinogen index, past history of ER, smoking, and green vegetable intake showed that Q4 *miR-124a-3* methylation had a higher HR than Q1 methylation (95 % CI) (2.30 (1.03–5.10); $p = 0.042$). This figure was modified from [35]

of developing metachronous gastric cancer (Fig. 5) [35], showing that methylation levels can identify groups of patients at high risk for gastric cancer (Fig. 2).

That study achieved the proof-of-concept for epigenetic cancer risk diagnosis, but is unlikely to change clinical practice in relation to following up gastric cancer patients after ESD. In order to optimize a surveillance system based on individual risk, a new large-scale multicenter prospective cohort study (UMIN000016894) for predicting the risk of primary gastric cancer in healthy individuals after *H. pylori* eradication was proposed and is currently underway. The number of such healthy individuals is rapidly increasing in Japan after *H. pylori* eradication therapy was approved for chronic gastritis by the national health insurance [36].

Application to cancer prevention

Epigenetic alterations can be reversed by drug interventions and are therefore potential targets for chemoprevention. Importantly, a possible preventive effect of a DNA demethylating agent has been shown experimentally. Oral administration of a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), to *H. pylori*-infected gerbils treated with *N*-methyl-*N*-nitrosourea (MNU) reduced the incidence of gastric cancers from 55.2 to 23.3 % (Fig. 6a), which was accompanied by a decrease in methylation levels (Fig. 6b) [9]. However, currently available DNA

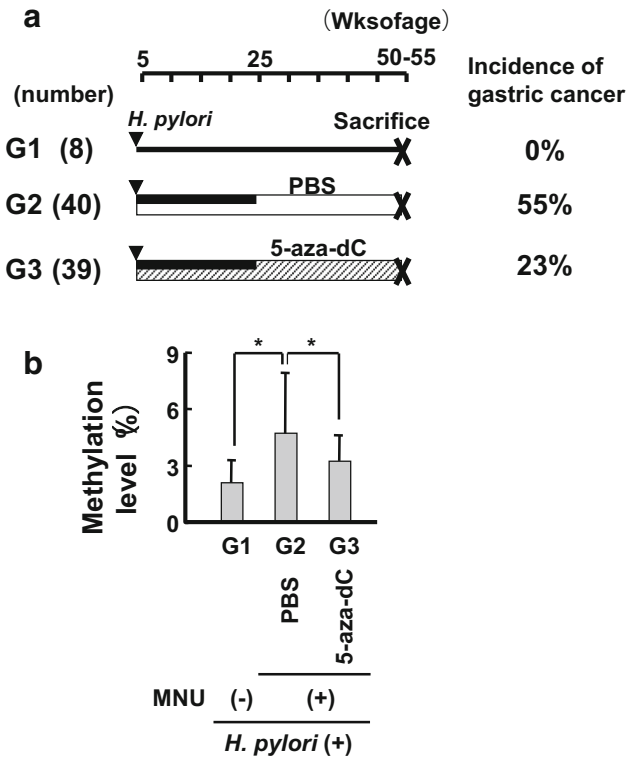

Fig. 6a, b Suppression of DNA methylation and inhibition of gastric cancers by 5-aza-dC treatment in *H. pylori*-infected gerbils treated with MNU. **a** Protocol of the carcinogenicity experiment. The incidence of gastric cancer was reduced from 55 % in group 2 of the *H. pylori*-infected gerbils with MNU to 23 % in group 3, which received 5-aza-dC ($p < 0.05$). **b** DNA methylation levels of CpG island HE6 in gastric epithelial cells (average ± SD). DNA methylation levels were significantly lower in G3 than in G2. * $p < 0.05$. This figure was modified from [9]

demethylating agents are not suitable for use in chemoprevention due to their adverse effects. Therefore, novel DNA demethylating agents with only minor adverse effects need to be developed, or intervention in an extremely high-risk population may be considered.

Conclusions

In this review, we have discussed the major impact of aberrant DNA methylation on gastric cancer and carcinogenesis, and current knowledge of the mechanisms for inducing aberrant DNA methylation. From the perspective of applying this knowledge, epigenetic cancer risk diagnosis is becoming a reality in the clinical setting. Clarification of the molecular mechanisms involved in aberrant DNA methylation induction is expected to provide a new strategy for the chemoprevention of gastric cancer.

Compliance with ethical standards

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

Ethical standards This article does not contain any studies with human or animal subjects performed by any of the authors.

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