

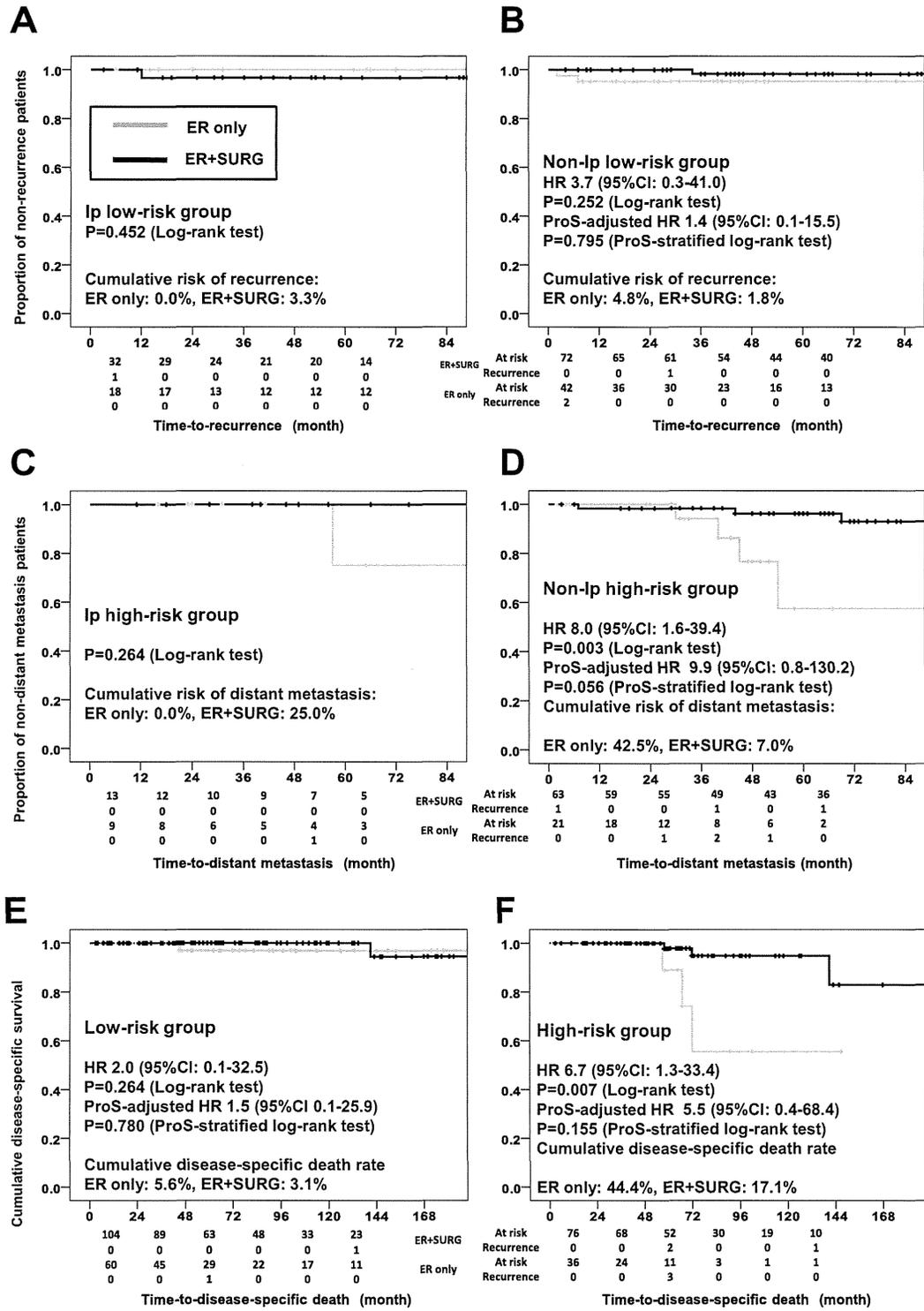
## Supplementary Material

### *Patients and Methods*

**Clinical data collection.** Patient data regarding age, sex, body mass index, Eastern Cooperative Oncology Group performance status, and comorbidities were obtained from physician notes, nursing notes, and discharge summaries until the date of ER. Data regarding the lesion size (maximum diameter), location, configuration, and ER method (en bloc or piecemeal) were also obtained.

**Evaluation of histologic findings.** Vertical margins were regarded as positive when the carcinoma was exposed at the submucosal margin of the resected specimen. Submucosal invasion depth was measured

from the muscularis mucosae to the deepest invasive front, regardless of the macroscopic type, in cases where it was possible to estimate the muscularis mucosae. In cases where an estimate was not possible, the depth was measured from the surface of the lesion. For pedunculated lesions with tangled muscularis mucosae, we measured the depth from the neck of the polyp. A submucosal invasion depth of  $<1000 \mu\text{m}$  was classified as superficial invasion, whereas a depth of  $\geq 1000 \mu\text{m}$  was classified as deep submucosal invasion. Budding was defined as an isolated single cancer cell or a cluster of  $<5$  cancer cells at the invasive margin of the lesion and graded under a microscopic field at  $\times 200$  magnification as follows: low-grade (0–4 buds) and high-grade ( $\geq 5$  buds) budding.<sup>8</sup>



**Supplementary Figure 1.** Kaplan–Meier curves for recurrence and distant metastasis with a post hoc log-rank test stratified by the risk groups. CRR in the Ip low-risk patients (A) and in the non-Ip low-risk patients (B). The cumulative risk of distant metastasis after ER in the Ip high-risk (C) and non-Ip high-risk patients (D). The cumulative disease-specific survival after ER in the low-risk (E) and high-risk patients (F). HR, hazard ratio.

**Supplementary Table 1.** Cumulative Risk and Cox Regression Analysis for Efficacy of Subsequent Surgery

Risk	Treatment	N		Cumulative recurrence Rate, % (95% CI) <sup>a</sup>	Cox regression		Log-rank
		At risk	Event		Crude HR <sup>a</sup> (95% CI)	ProS-adjusted HR <sup>a</sup> (95% CI)	ProS-adjusted P value
<b>Distant metastasis</b>							
Low-risk	ER only	60	1	1.7 (0-5.0)	0.9 (0.1-10.1)	0.7 (0.5-8.3)	.739
	ER+SURG	104	2	2.3 (0-5.4)	Reference	Reference	
High-risk	ER only	36	5	37.7 (9.5-65.9)	7.6 (1.7-32.8)	5.6 (0.8-39.3)	.070
	ER+SURG	76	3	6.1 (0-13.1)	Reference	Reference	
<b>Local recurrence</b>							
Low-risk	ER only	60	1	1.7 (0-5.0)	N/A	N/A	.414
	ER+SURG	104	0	0.0 (0-3.4) <sup>b</sup>	Reference	Reference	
High-risk	ER only	36	10	54.5 (23.1-85.9)	36.8 (4.6-294)	20.4 (2.4-173)	.001
	ER+SURG	76	1	1.5 (0-4.4)	Reference	Reference	
<b>Disease-specific survival</b>							
Low-risk	ER only	60	1	3.1 (0-9.2)	2.0 (0.1-32.4)	1.5 (0.8-25.9)	.780
	ER+SURG	104	1	5.6 (0-16.2)	Reference	Reference	
High-risk	ER only	36	3	44.4 (5.0-83.8)	6.7 (1.3-33.4)	5.5 (0.4-68)	.155
	ER+SURG	76	3	17.1 (0-39.6)			

<sup>a</sup>Calculated by Greenwood's formula.<sup>b</sup>Calculated by the binomial exact method.

## Aberrant methylation of microRNA-34b/c is a predictive marker of metachronous gastric cancer risk

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Received: 8 April 2013 / Accepted: 25 July 2013 / Published online: 13 August 2013  
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### Abstract

**Background** Metachronous gastric cancer (GC) can develop after endoscopic resection of GC and cannot be predicted based on clinical signature. Aberrant DNA methylation in noncancerous gastric mucosa is strongly implicated in gastric carcinogenesis and could be a useful biomarker of GC risk. We evaluated the clinical utility of DNA methylation as a biomarker of metachronous GC risk. **Method** We carried out scheduled follow-up endoscopy in 129 patients after curative endoscopic resection of GC. Biopsy specimens were collected from noncancerous mucosa in the gastric antrum and body, after which quantitative methylation analysis of *miR-34b/c*, *SFRP1*, *SFRP2*, *SFRP5*, *DKK2* and *DKK3* was carried out using bisulfite pyrosequencing. The utility of the methylation for predicting the risk of metachronous GC development was

assessed using Kaplan–Meier and Cox proportional hazards model analyses.

**Results** During the follow-up period, 17 patients (13 %) developed metachronous GCs. The cumulative incidence of metachronous GC was significantly higher among patients with elevated *miR-34b/c*, *SFRP2* and *DKK2* methylation in their gastric body. *MiR-34b/c* showed the strongest association with the risk of metachronous GC, and the cumulative incidence of metachronous GC was much higher in the high-*miR-34b/c*-methylation group than the low-methylation group. Multivariate analysis adjusted for age, sex, *H. pylori* status and pathological findings showed *miR-34b/c* methylation in gastric body to be an independent predictor of metachronous GC risk.

**Conclusion** Our results suggest that methylation of *miR-34b/c* in the mucosa of the noncancerous gastric body may be a useful biomarker for predicting the risk of metachronous GC.

R. Suzuki, E. Yamamoto contributed equally to this work.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00535-013-0861-7) contains supplementary material, which is available to authorized users.

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**Keywords** Metachronous gastric cancer · DNA methylation · Biomarker · Risk assessment

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## Introduction

Gastric cancer (GC) is a major cause of cancer mortality worldwide, and its early detection and endoscopic resection are essential for reducing the incidences of invasion and metastasis and improving survival. Endoscopic submucosal dissection (ESD) enables en bloc and histologically complete resection with no restriction on lesion size [1–4]. Although this approach minimizes the recurrence rate and preserves the entire stomach and the patient's quality of life, metachronous GC develops in the remnant stomach in about 10–20 % of patients after curative ESD [5–7]. Consequently, assessing the risk of metachronous GC after ESD is extremely important for early detection of subsequent GC and reduction of mortality.

*Helicobacter pylori* (*H. pylori*) infection plays an important role in gastric carcinogenesis [8]. GCs are thought to arise from *H. pylori*-related gastritis, and the severe mucosal atrophy and intestinal metaplasia it causes are associated with the development of metachronous GC. Correspondingly, eradicating *H. pylori* after ESD reduces the likelihood of metachronous GC [9]. However, the individuals at high risk of developing metachronous GC cannot be predicted based on clinicopathological findings, including *H. pylori* status. Thus, identification of a molecular marker useful for predicting the risk of metachronous GC would be highly desirable.

Epigenetic alterations such as DNA methylation play a key role during gastric carcinogenesis [10, 11]. For example, DNA methylation is known to silence a variety of genes involved in cell-cycle control, apoptosis, cell signaling and DNA repair in GC [10, 12]. Earlier reports have shown that *H. pylori* infection induces methylation of tumor suppressor and other tumor-related genes in the noncancerous gastric mucosa, suggesting aberrant DNA methylation is an early event during gastric carcinogenesis [13]. We and others previously demonstrated that the level of DNA methylation of tumor suppressor genes is increased in cases of gastritis that are at epidemiologically high risk for developing GC and in the background noncancerous gastric mucosa in GC [14–16]. In addition, we previously reported that the level of *miR-34b/c* gene methylation is significantly higher in noncancerous gastric mucosa from patients with multiple GCs than in those with a single GC or in *H. pylori*-positive healthy individuals [17]. These results suggest DNA methylation in noncancerous gastric mucosa may be a useful biomarker for evaluating the risk of metachronous GC after ESD.

Our aim in the present study was to assess the clinical utility of DNA methylation in the noncancerous gastric mucosa as a marker of the risk of metachronous GC. Our approach was to perform a prospective study in a cohort of GC patients who underwent curative ESD.

## Materials and methods

### Study population

This study included 129 patients who received curative endoscopic resection of early GC at Akita Red Cross Hospital between May 2007 and May 2011. Surveillance endoscopy was regularly performed every 6–12 months. At the time of enrollment (first detection of GC), biopsy specimens of noncancerous gastric mucosa were obtained from the gastric body and antrum for histological examination and extraction of genomic DNA. After enrollment, patients were hospitalized and their tumors were treated with ESD. The follow-up period was defined as being between the first biopsy and the detection of metachronous GC, the censoring of the patients or the end of this study. The updated Sydney system was used to estimate the degree of gastritis [18]. Genomic DNA was extracted using the standard phenol–chloroform procedure. *H. pylori* infection was assessed using the rapid urease test, the serum antibody test and the urea breath test. If any one of these assays was positive, the patients were considered to be *H. pylori*-positive. All individuals had no history of *H. pylori* eradication therapy. After endoscopic treatment, 49 *H. pylori*-positive GC patients received *H. pylori* eradication treatment, and successful eradication was confirmed. Metachronous GC was defined as new GC developing after curative resection of GC. Written informed consent was obtained from all patients before collection of the specimens. Approval of this study was obtained from the Institutional Review Board of Akita Red Cross Hospital and Sapporo Medical University.

### Methylation analysis by bisulfite-pyrosequencing

Genomic DNA (1 µg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (QIAGEN, Hilden, Germany), after which bisulfite sequencing and pyrosequencing were carried out as described previously [19]. For bisulfite pyrosequencing, the biotinylated PCR product was purified, made single-stranded and used as the template in a pyrosequencing reaction run according to the manufacturer's instructions. The pyrosequencing reaction was carried out using a PSQ96 system with a PyroGold reagent Kit (QIAGEN), and the results were analyzed using Q-CpG software (QIAGEN). Sequence information for primers is shown in Supplementary Table 1.

### Statistical analysis

The cumulative incidences of metachronous GC were calculated using the Kaplan–Meier method. Comparisons between groups were made using the log-rank test.

Univariate and multivariate analyses were performed using Cox proportional hazard models to assess the risk factors for metachronous GC. Fisher's exact test or Pearson's chi-squared test was used for analysis of categorical data. Values of  $p < 0.05$  (two-sided) were considered statistically significant. All statistical analyses were performed using SPSS Statistics 18 (IBM Corporation, Somers, NY, USA) and JMP ver. 10 (SAS Institute Inc., Cary, NC, USA).

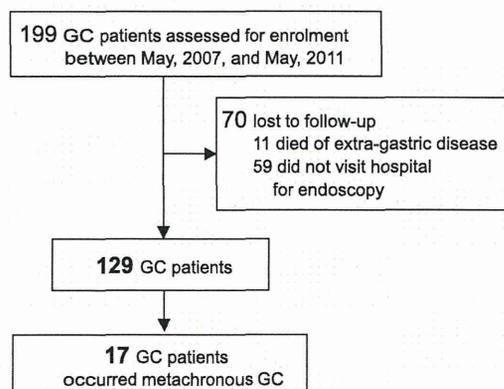
## Results

### Patient characteristics

The profiles of the study participants are shown in Fig. 1. After excluding patients who were lost to follow-up or died of extragastric disease, 129 GC patients were enrolled in the study. The median follow-up period for the 129 GC patients was 1.5 (range 0.1–6.1) years. A total of 86 patients exhibited *H. pylori* infection; of those, 51 underwent eradication therapy after endoscopic resection of their GC, and the *H. pylori* was successfully eradicated in 49 (96 %). Twenty-six patients were *H. pylori*-negative, although the majority of those were presumed to have been infected in the past, as they exhibited intestinal metaplasia or gastric mucosal atrophy (data not shown). *H. pylori* status was not available for 17 patients. During the follow-up period, 17 patients (13 %) developed metachronous GCs. This included 12 *H. pylori*-positive patients, among whom five were successfully treated for *H. pylori* after endoscopic resection of their GC (Table 1).

### Association between *miR-34b/c* methylation and the occurrence of metachronous GC

The primary endpoint of this study was the occurrence of metachronous GC during the follow-up period. At the time



**Fig. 1** Profiles of participants in this study

each patient entered the study, biopsy specimens of non-cancerous gastric mucosa were collected from the antrum and body of the stomach, after which methylation levels of *miR-34b/c*, *SFRP1*, *SFRP2*, *SFRP5*, *DKK2* and *DKK3* were determined using quantitative bisulfite pyrosequencing. These six genes are known to be frequently methylated in *H. pylori*-related gastritis as well as in GC [17, 20, 21]. Based on the results, patients were categorized into a high-methylation or low-methylation group, relative to the median baseline methylation levels in the gastric body and antrum among the 129 GC patients. Thereafter, we assessed the correlations between methylation of the respective genes and the incidence of metachronous GC. Using a univariate Cox proportional hazards model, the greatest hazard ratio (HR) was obtained for patients showing higher levels of *miR-34b/c* methylation in the gastric body when a cutoff of 18.6 % was employed (HR, 10.01; 95 % CI, 2.26–44.23,  $p = 0.002$ ). Similarly, we found an elevated risk of metachronous GC in patients with higher levels of *SFRP2* (cutoff, 43.3 %; HR, 3.33; 95 % CI, 1.08–10.25,  $p = 0.02$ ) and *DKK2* (cutoff, 16.9 %; HR, 3.66; 95 % CI, 1.19–11.25,  $p = 0.01$ ) methylation in the gastric body (Fig. 2a). In addition, Kaplan–Meier analysis revealed that greater methylation of these three genes in the gastric body was significantly associated with shorter metachronous GC-free survival (*miR-34b/c*,  $p \leq 0.001$ ; *SFRP2*,  $p = 0.025$ ; *DKK2*,  $p = 0.015$  by log-lank test) (Fig. 2b, Supplementary Figure 1). By contrast, we found no significant correlation between the incidence of metachronous GC and methylation in the antral mucosa (Supplementary Figure 2).

The results summarized above prompted us to select *miR-34b/c* methylation in the gastric body for further analysis (Supplementary Figure 3). The 2-year cumulative incidence of metachronous GC in the high-methylation group ( $\geq 18.6$  %) was 30.4 % (95 % CI, 15.9–44.9), while that for the low-methylation group ( $\leq 18.6$  %) was only 3.8 % (95 % CI, 0–12.6) (Table 2). When we then divided the 129 GC patients into four quartiles based on the level of *miR-34b/c* methylation in the gastric body, we found that the cumulative incidence of metachronous GC significantly differed among the four groups ( $p \leq 0.001$  by log-lank test) (Supplementary Figure 4). These results suggest that levels of *miR-34b/c* methylation in the gastric body mucosa could be a predictive marker of the risk of metachronous GC.

### Clinicopathological features of the high and low methylation groups

The associations between the clinicopathological features and *miR-34b/c* methylation are summarized in Table 3. Among the GC patients, *miR-34b/c* methylation was associated with older age and a greater degree of

**Table 1** Clinicopathological features of the patients enrolled in this study

	Total ( <i>N</i> = 129)	Incidence of metachronous GC		<i>p</i> value
		No ( <i>N</i> = 112)	Yes ( <i>N</i> = 17)	
Age (means)	69.2 (35–89)	68.6 (35–86)	72.8 (56–89)	0.104
Sex				
Male	92 (71.3 %)	77 (68.7 %)	15 (88.2 %)	0.149
Female	37 (28.6 %)	35 (31.2 %)	2 (11.7 %)	
Follow-up years (means)	1.5 (0.1–6.1)	1.4 (0.1–6.1)	2.6 (0.3–6.1)	0.039
<i>H. pylori</i> infection				
Positive	86 (66.6 %)	74 (66.0 %)	12 (70.5 %)	0.538
Negative	26 (20.1 %)	21 (18.7 %)	5 (29.4 %)	
Unknown	17 (13.1 %)	17 (15.1 %)	0 (0.0 %)	
Pathological findings of noncancerous gastric mucosa ( <i>N</i> = 122)				
Antrum				
Inflammation				
Severe–moderate	23 (18.8 %)	22 (20.7 %)	1 (6.2 %)	0.301
Mild–none	99 (81.1 %)	84 (79.2 %)	15 (93.7 %)	
Activity				
Severe–moderate	19 (15.5 %)	18 (16.9 %)	1 (6.2 %)	0.462
Mild–none	103 (84.4 %)	88 (83.0 %)	15 (93.7 %)	
Atrophy				
Severe–moderate	101 (82.7 %)	87 (82.0 %)	14 (87.5 %)	0.736
Mild–none	21 (17.2 %)	19 (17.9 %)	2 (12.5 %)	
Metaplasia				
Severe–moderate	71 (58.2 %)	60 (56.6 %)	11 (68.7 %)	0.424
Mild–none	51 (41.8 %)	46 (43.4 %)	5 (31.2 %)	
Body				
Inflammation				
Severe–moderate	78 (63.9 %)	66 (62.2 %)	12 (75.0 %)	0.409
Mild–none	44 (36.0 %)	40 (37.7 %)	4 (25.0 %)	
Activity				
Severe–moderate	68 (55.7 %)	60 (56.6 %)	8 (50.0 %)	0.788
Mild–none	54 (44.2 %)	46 (43.4 %)	8 (50.0 %)	
Atrophy				
Severe–moderate	69 (56.5 %)	56 (52.8 %)	13 (81.2 %)	0.056
Mild–none	53 (43.4 %)	50 (47.1 %)	3 (18.7 %)	
Metaplasia				
Severe–moderate	30 (24.5 %)	24 (22.6 %)	6 (37.5 %)	0.219
Mild–none	92 (75.4 %)	82 (77.3 %)	10 (62.5 %)	

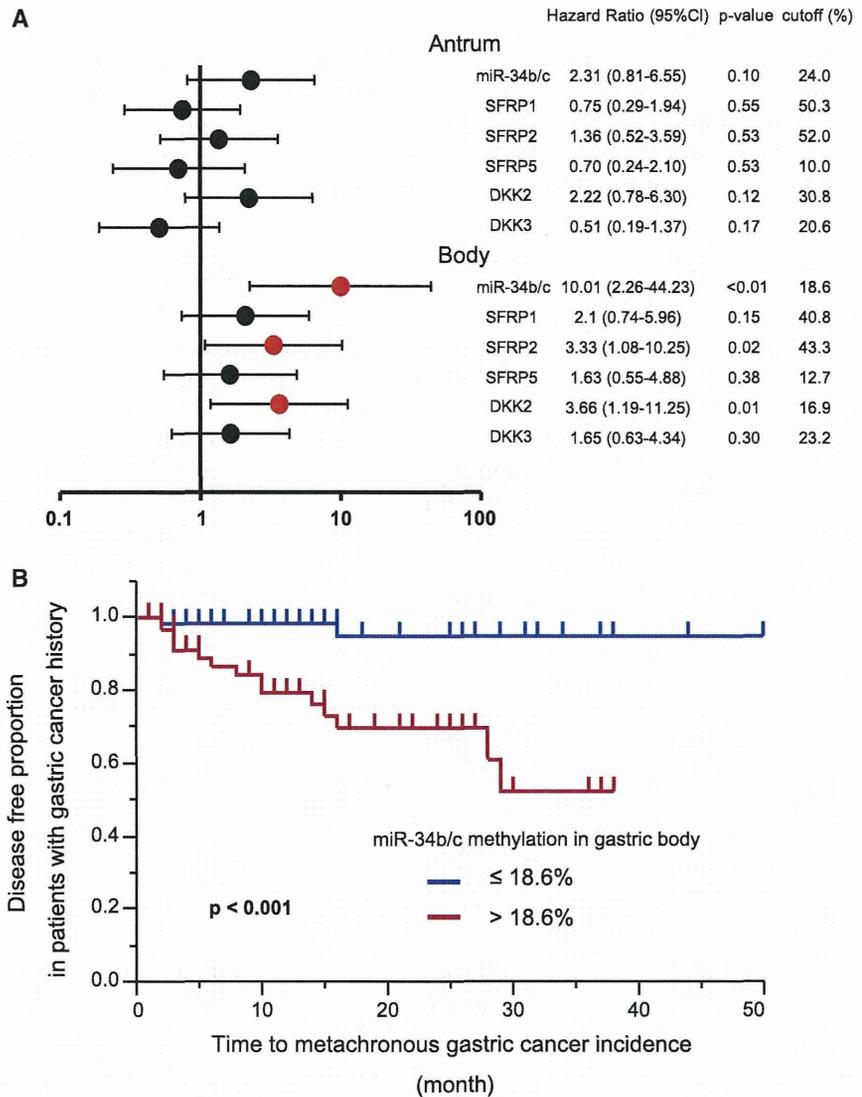
inflammation, activity, atrophy and metaplasia in the gastric body (Table 3). By contrast, no significant differences were found with respect to sex, *H. pylori* infection or pathological findings in the antral mucosa (Table 3).

Evaluating the risk of metachronous GC with clinical predictors

We next used univariate analysis and multivariate Cox proportional-hazards analysis to evaluate the utility of the *miR-34b/c* methylation level in the mucosa of the noncancerous

gastric body as a predictive marker of metachronous GC risk. In the univariate analysis, *miR-34b/c* methylation and mucosal atrophy in the gastric body were significantly associated with the occurrence of metachronous GC (Table 4). On the other hand, inflammation, activity and metaplasia were not significant predictors of the occurrence of metachronous GC. In a multivariate analysis adjusted for *miR-34b/c* methylation, age, sex, *H. pylori* status and pathological findings (inflammation, activity, atrophy and metaplasia), *miR-34b/c* methylation and inflammation were independently associated with metachronous GC (Table 4).

**Fig. 2** Associations between DNA methylation and metachronous GC risk. **a** Forest plot showing hazard ratios (closed circles) for developing metachronous GC and 95 % confidence intervals (bar lines). Univariate Cox proportional hazards model analysis was performed to assess the correlations between methylation of the six indicated genes and the incidence of metachronous GC. **b** Kaplan–Meier analysis of the effect of *miR-34b/c* methylation in the gastric body on metachronous GC-free survival ( $n = 129$ )



**Table 2** Methylation of *miR-34b/c* in noncancerous gastric body mucosa and its association with metachronous gastric cancer

Methylation (%)	Periods	Total	Non MGC	MGC	Incidence rate (%)	95 % CI	
						Lower (%)	Upper (%)
≤18.6	1 Year	65	64	1	1.7	0.0	5.0
	2 Years	31	30	1	3.8	0.0	12.6
	3 Years	24	24	0	3.8	0.0	12.6
>18.6	1 Year	64	58	10	20.7	9.1	32.3
	2 Years	24	21	3	30.4	15.9	44.9
	3 Years	12	10	2	47.8	24.3	71.3

CI confidence interval, MGC metachronous gastric cancer

*MiR-34b/c* methylation, *H. pylori* status and the incidence of metachronous GC

Recent studies demonstrated that eradicating *H. pylori* after endoscopic resection of early GC reduces the risk of

metachronous GC [9]. To assess the relationship between *miR-34b/c* methylation and *H. pylori* status, we compared GC patients who had undergone successful eradication therapy after enrolling in this study ( $n = 49$ ) with those who were *H. pylori*-negative at the start of the study but

were presumed to have had a past infection ( $n = 26$ ). Kaplan–Meier analysis indicated that *miR-34b/c* methylation in the noncancerous gastric body was significantly associated with a risk of metachronous GC in patients successfully treated for *H. pylori* ( $p \leq 0.001$ ) (Fig. 3a). Moreover, we also found a significant correlation

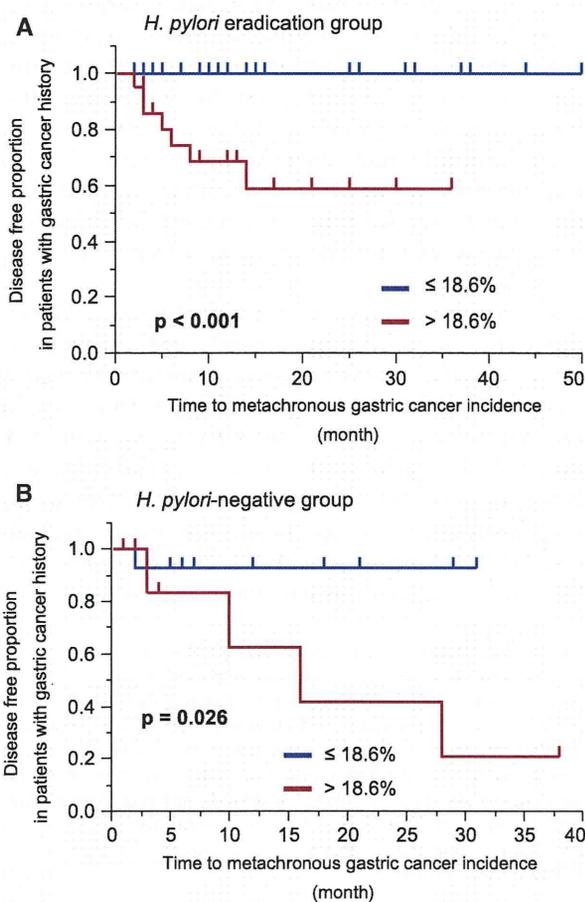
between methylation and metachronous GC in *H. pylori*-negative patients ( $p = 0.026$ ) (Fig. 3b). These results suggest that *miR-34b/c* methylation in the mucosa of the gastric body may be a useful marker for predicting metachronous GC risk, irrespective of the presence of *H. pylori*.

**Table 3** Association between clinicopathological features and *miR-34b/c* methylation

	Methylation-low ( $\leq 18.6\%$ ) $N = 64$	Methylation-high ( $>18.6\%$ ) $N = 65$	$p$ value
Age (mean $\pm$ SD)	66.4 ( $\pm 11.2$ )	72.0 ( $\pm 7.0$ )	0.004
Sex			
Male	41 (64.0 %)	51 (78.5 %)	0.071
Female	23 (35.9 %)	14 (21.5 %)	
<i>H. pylori</i> infection			
Positive	38 (59.3 %)	49 (75.4 %)	0.152
Negative	16 (25.0 %)	10 (15.4 %)	
Unknown	10 (15.6 %)	6 (9.2 %)	
	Methylation-low ( $\leq 18.6\%$ ) $N = 62$	Methylation-high ( $>18.6\%$ ) $N = 60$	$p$ value
Pathological findings of noncancerous gastric mucosa			
Antrum			
Inflammation			
Severe–moderate	13 (21.0 %)	10 (16.7 %)	0.544
Mild–none	49 (79.0 %)	50 (83.3 %)	
Activity			
Severe–moderate	12 (19.4 %)	7 (11.7 %)	0.242
Mild–none	50 (80.6 %)	53 (88.3 %)	
Atrophy			
Severe–moderate	53 (85.5 %)	48 (80.0 %)	0.423
Mild–none	9 (14.5 %)	12 (20.0 %)	
Metaplasia			
Severe–moderate	39 (62.9 %)	32 (53.3 %)	0.284
Mild–none	23 (37.1 %)	28 (46.7 %)	
Body			
Inflammation			
Severe–moderate	32 (51.6 %)	46 (76.7 %)	0.004
Mild–none	30 (48.4 %)	14 (23.3 %)	
Activity			
Severe–moderate	29 (46.8 %)	39 (65.0 %)	0.043
Mild–none	33 (53.2 %)	21 (35.0 %)	
Atrophy			
Severe–moderate	24 (38.7 %)	45 (75.0 %)	<0.001
Mild–none	38 (61.3 %)	15 (25.0 %)	
Metaplasia			
Severe–moderate	10 (16.1 %)	20 (33.3 %)	0.027
Mild–none	52 (83.9 %)	40 (66.7 %)	

**Table 4** Risk factors for development of metachronous GC analyzed by univariate and multivariate analysis

	Univariate analysis				Multivariate analysis			
	p value	HR	95 % CI		p value	HR	95 % CI	
			Lower	Upper			Lower	Upper
<i>miR-34b/c</i> methylation (>18.6 versus ≤18.6 %)	0.002	10.01	2.26	44.23	0.014	9.00	1.56	52.01
Age (>71 yr versus ≤71)	0.057	2.64	0.97	7.16	0.283	1.84	0.61	5.56
Male sex (versus female)	0.22	2.53	0.58	11.09	0.638	1.48	0.29	7.72
<i>H. pylori</i> infection	0.263	0.55	0.19	1.57	0.046	0.21	0.04	0.97
Pathological findings in gastric body								
Inflammation	0.167	2.23	0.72	6.92	0.007	15.71	2.11	117.09
Activity	0.863	0.92	0.34	2.45	0.076	0.31	0.09	1.13
Atrophy	0.020	4.48	1.27	15.76	0.309	2.12	0.50	9.02
Metaplasia	0.125	2.21	0.80	6.08	0.322	1.81	0.56	5.82



**Fig. 3** Association between *miR-34b/c* methylation and metachronous GC risk in *H. pylori*-positive and -negative patients. **a** Kaplan–Meier analysis of the effect of *miR-34b/c* methylation on metachronous GC-free survival among *H. pylori*-positive patients who underwent successful eradication after endoscopic treatment of their initial GC ( $n = 49$ ). **b** Kaplan–Meier analysis of the effect of *miR-34b/c* methylation on metachronous GC-free survival among *H. pylori*-negative patients ( $n = 26$ )

**Discussion**

Surveillance of patients after ESD has important implications for early detection and treatment of metachronous GC. Although eradication of *H. pylori* can reduce the risk of metachronous GC after endoscopic treatment of the initial GC, it does not ensure complete prevention in all patients [7, 9]. Moreover, current diagnostic tools show patients with past *H. pylori* infections to be *H. pylori*-negative, and they do not receive eradication therapy. Thus, periodic endoscopy is recommended for GC patients after ESD [7]. In an effort to establish a more effective surveillance strategy, we assessed DNA methylation in a set of candidate marker genes in noncancerous gastric mucosa, and then carried out scheduled endoscopic surveillance. This is the first prospective cohort study designed to assess the utility of DNA methylation as a predictive marker of metachronous GC risk.

The evidence collected in various studies to date shows a strong relationship between aberrant methylation of cancer-related genes in noncancerous gastric mucosa and GC risk [16, 22–25]. In addition, genome-wide analyses of DNA methylation using microarray technology has shown that a larger number of genes are methylated in noncancerous gastric mucosa from GC patients than in gastric mucosa from *H. pylori*-positive healthy individuals [26, 27]. In the present study, we focused on *miR-34b/c*, *SFRP* and *DKK* family genes, because of the high frequency of their methylation in both GC tissue and adjacent gastric mucosa [17, 20, 21]. That *SFRP* and *DKK* family genes are frequently methylated in both GC and adjacent gastric mucosa suggests the involvement of an epigenetic field defect [20, 21]. *miR-34b/c* is a putative tumor suppressor gene that acts as a downstream effector of p53 and is frequently silenced in association with CpG island hypermethylation in various malignancies [28]. We previously