Author Contributions

Conceived and designed the experiments: EA YK. Performed the experiments: TS EA TK. Analyzed the data: TS EA KS TB YK. Contributed reagents/materials/analysis tools: EA KT SW YK. Wrote the paper: TS EA YK.

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ORIGINAL ARTICLE—Alimentary Tract

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

Ryo Suzuki · Eiichiro Yamamoto · Masanori Nojima · Reo Maruyama · Hiro-o Yamano · Kenjiro Yoshikawa · Tomoaki Kimura · Taku Harada · Masami Ashida · Takeshi Niinuma · Akiko Sato · Katsuhiko Nosho · Hiroyuki Yamamoto · Masahiro Kai · Tamotsu Sugai · Kohzoh Imai · Hiromu Suzuki · Yasuhisa Shinomura

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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.

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

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

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R. Suzuki · E. Yamamoto · R. Maruyama · T. Niinuma · K. Nosho · H. Yamamoto · Y. Shinomura (✉)
Department of Gastroenterology, Rheumatology and Clinical Immunology, Sapporo Medical University, S1, W16, Chuo-Ku, Sapporo 060-8543, Japan
e-mail: shinomura@sapmed.ac.jp

E. Yamamoto · R. Maruyama · T. Harada · M. Ashida · T. Niinuma · A. Sato · M. Kai · H. Suzuki (✉)
Department of Molecular Biology, Sapporo Medical University, S1, W17, Chuo-Ku, Sapporo 060-8556, Japan
e-mail: hsuzuki@sapmed.ac.jp

M. Nojima
The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

H. Yamano · K. Yoshikawa · T. Kimura
Department of Gastroenterology, Akita Red Cross Hospital, Akita, Japan

T. Sugai
Department of Pathology, Iwate Medical University, Morioka, Japan

K. Imai
The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

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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 Pearsons' 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 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 (≥18.6 %) was 30.4 % (95 % CI, 15.9–44.9), while that for the low-methylation group (≤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.

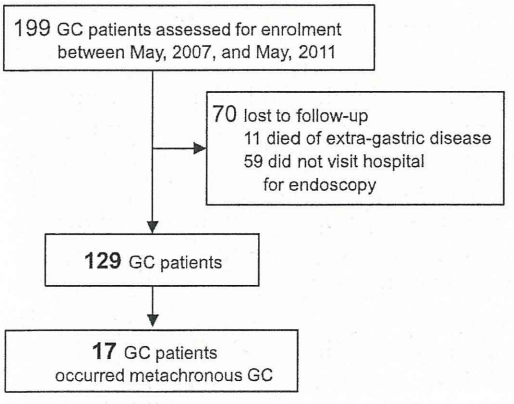

Fig. 1 Profiles of participants in this study

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 ($N = 129$)	Incidence of metachronous GC		p value
		No ($N = 112$)	Yes ($N = 17$)	
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
H. pylori 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 ($N = 122$)				
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). By contrast, no significant differences were found with respect to sex, *H. pylori* infection or pathological findings in the antral mucosa (Table).

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). 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).

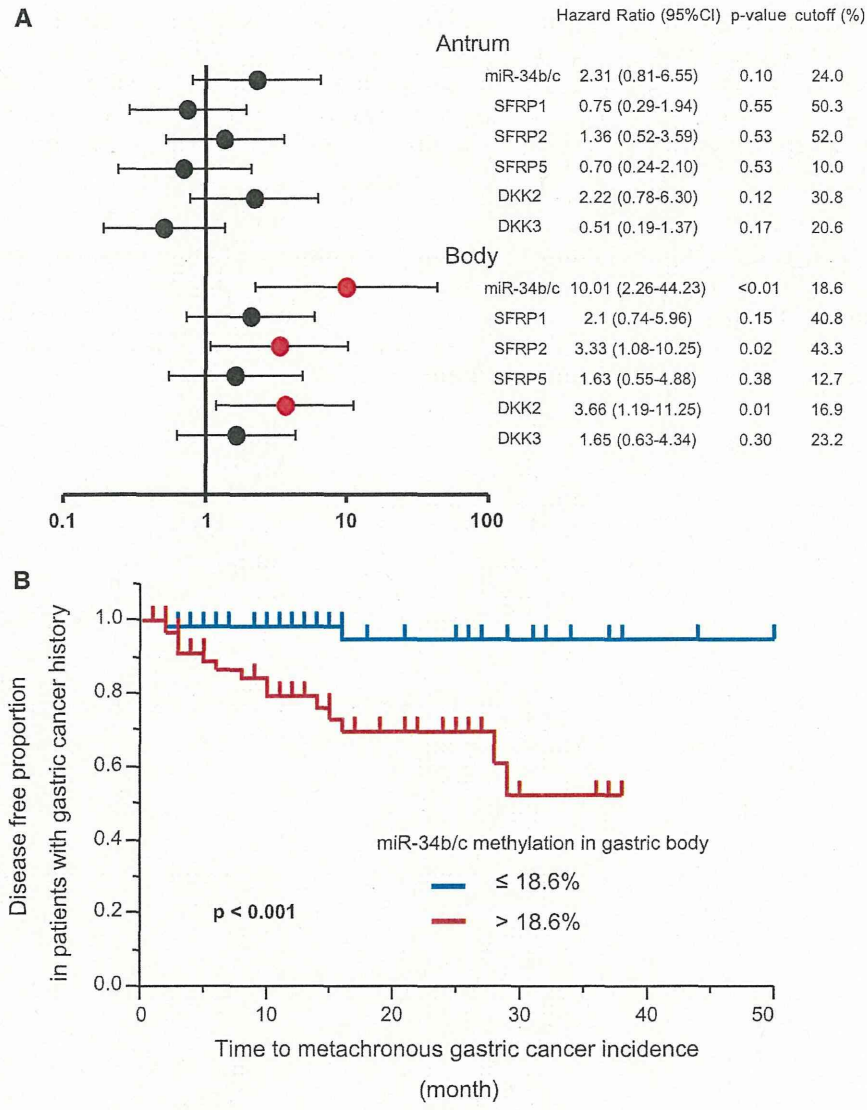

Fig. 2 Associations between DNA methylation and metachronous GC risk. **a** *Forest plot* showing hazard ratios (*closed circles*) for developing metachronous GC and 95 % confidence intervals (*bar lines*). Univariate Cox proportional hazards model analysis was performed to assess the correlations between 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. a). Moreover, we also found a significant correlation between methylation and metachronous GC in *H. pylori*-negative patients ($p = 0.026$) (Fig. b). 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 (≤18.6 %) $N = 64$	Methylation-high (>18.6 %) $N = 65$	p value
Age (mean ± SD)	66.4 (±11.2)	72.0 (±7.0)	0.004
Sex			
Male	41 (64.0 %)	51 (78.5 %)	0.071
Female	23 (35.9 %)	14 (21.5 %)	
H. pylori 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 (≤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
miR-34b/c 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
H. pylori 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

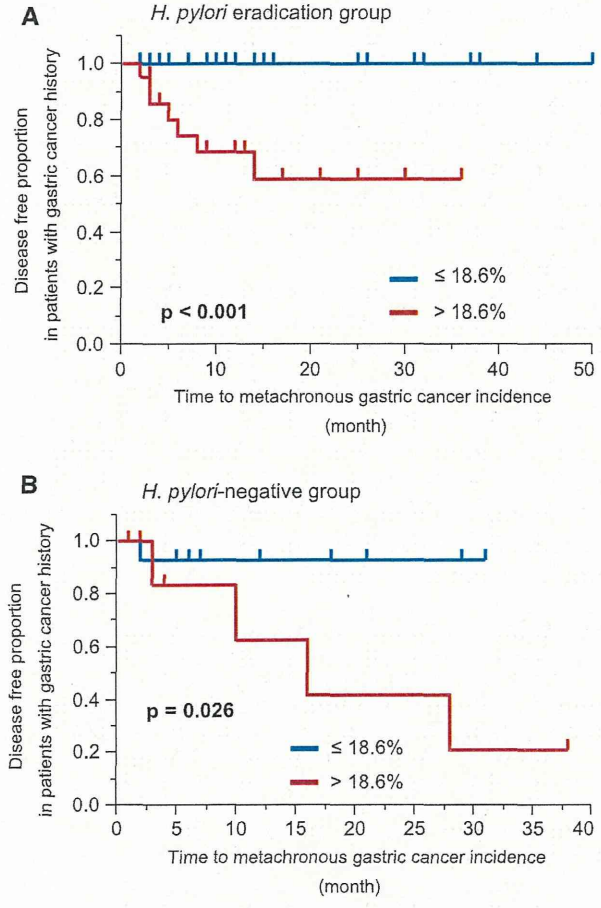

Fig. 3 Association between *miR-34b/c* methylation and metachronous GC risk in *H. pylori*-positive and -negative patients. **a** Kaplan–Meier analysis of the effect of *miR-34b/c* methylation on metachronous GC-free survival among *H. pylori*-positive patients who underwent successful eradication after 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

showed that levels of *miR-34b/c* methylation in noncancerous gastric mucosa from patients with multiple GCs were significantly higher than in noncancerous gastric mucosa from patients with single GCs or from healthy individuals [17].

We found that methylation of *miR-34b/c*, *SFRP2* and *DKK2* in the gastric body mucosa was strongly associated with a risk for metachronous GC, and *miR-34b/c* showed the greatest potential to serve as a predictive marker. Multivariate analysis adjusted for age, sex, *H. pylori* status and pathological findings revealed that *miR-34b/c* methylation and inflammation are independently associated with the development of metachronous GC. A number of studies have shown that chronic inflammation is strongly associated with aberrant DNA methylation, and one recent study also showed that the inflammatory response, not *H. pylori* itself, is responsible for the altered DNA methylation in the infected stomach [29, 30]. Consistent with those earlier findings, our study confirms the tight correlation between inflammation and aberrant DNA methylation, and shows that both inflammation and aberrant DNA methylation are independent risk factors of metachronous GC.

It also remains unclear why methylation in the gastric body strongly correlates with increased metachronous GC risk but methylation in the antrum does not. In gastritis patients, the antral mucosa generally exhibits more advanced histological features (e.g., metaplasia and atrophy) than the gastric body mucosa, though inflammation and activity are usually less severe in the antrum [31, 32]. In this study, we observed that methylation of a number of genes was higher in the antrum than in the body (Fig. 2a, Supplementary Figures 1 and 2). In addition, pathological findings in the gastric body mucosa are more likely to reflect the degree and extent of the inflammation and activity of the gastritis than those in the antral mucosa with severe atrophy or metaplasia. An earlier study also showed that individuals with active inflammation in the gastric body (e.g., pangastritis or corpus-predominant gastritis) are at higher risk of developing GC [8]. It is thus conceivable that aberrant methylation in the gastric body is associated an increased risk of metachronous GC.

Interestingly, we found that *miR-34b/c* methylation was also associated with metachronous GC risk in patients who underwent successful *H. pylori* eradication after treatment of their GC. Similarly, while eradication of *H. pylori* after endoscopic resection of early GC can reduce the risk of metachronous GC risk [9], eradication in patients without a precancerous lesion more effectively reduces the risk of developing GC [33, 34]. One possible reason for the development of metachronous GC, even after *H. pylori* eradication, is the presence of malignant cells that cannot be detected through endoscopic examination. In addition, our results suggest that a certain amount of aberrant DNA methylation may not be reversed by eradication, and individuals with high levels of *miR-34b/c* methylation may remain at a high risk of metachronous GC.

There are several limitations to this study. First, patient samples were collected at a single institution, and the follow-up period was relatively short (average 18 months). The cumulative incidence rate for metachronous GCs increases linearly with time [7], and we think the incidence of metachronous GCs in our study population would likely increase with a longer follow-up period. Second, the association between *miR-34b/c* methylation and GC risk in healthy individuals remains unclear because we focused on early GC patients who underwent endoscopic treatment. Thus, our findings should be validated in an independent long-term, multicenter study that includes a larger number of patients. Third, our study did not include patients who underwent *H. pylori* eradication therapy prior to their diagnosis. Earlier studies suggest that levels of aberrant methylation in *H. pylori*-infected noncancerous mucosa can be reduced by eradication therapy, but it is unclear how long the effect persists [35–38]. Further studies will be needed to define the relation between *miR-34b/c* methylation and GC risk in patients with history of *H. pylori* eradication therapy.

In summary, we observed that the level of *miR-34b/c* methylation in noncancerous gastric body mucosa is a useful biomarker that is predictive of the risk for metachronous GC risk after endoscopic resection. Thus, for early GC patients with elevated *miR-34b/c* methylation in the gastric body, more intensive and frequent follow-up endoscopy may be recommended after ESD. Our findings may greatly improve of the surveillance strategy used after ESD and contribute to the early detection of metachronous GC and a reduction in its mortality.

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

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Association Between Genomic Alterations and Metastatic Behavior of Colorectal Cancer Identified by Array-Based Comparative Genomic Hybridization

Takeshi Sawada,[1,2] Eiichiro Yamamoto,[3] Hiromu Suzuki,[1]* Masanori Nojima,[4] Reo Maruyama,[1] Yoshihiro Shioi,[5] Risaburo Akasaka,[5] Seiko Kamimae,[1] Taku Harada,[1] Masami Ashida,[1] Masahiro Kai,[1] Yasushi Adachi,[3] Hiroyuki Yamamoto,[3] Kohzoh Imai,[6] Minoru Toyota,[1] Fumio Itoh,[2] and Tamotsu Sugai[5]*

[1]Department of Molecular Biology, Sapporo Medical University, Sapporo, Japan
[2]Division of Gastroenterology and Hepatology, St Marianna University School of Medicine, Kawasaki, Japan
[3]First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan
[4]Department of Public Health, Sapporo Medical University, Sapporo, Japan
[5]Division of Molecular Diagnostic Pathology, Department of Pathology, School of Medicine, Iwate Medical University, Morioka, Japan
[6]Division of Novel Therapy for Cancer, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Colorectal cancers (CRCs) exhibit multiple genetic alterations, including allelic imbalances (copy number alterations, CNAs) at various chromosomal loci. In addition to genetic aberrations, DNA methylation also plays important roles in the development of CRC. To better understand the clinical relevance of these genetic and epigenetic abnormalities in CRC, we performed an integrative analysis of copy number changes on a genome-wide scale and assessed mutations of *TP53*, *KRAS*, *BRAF*, and *PIK3CA* and DNA methylation of six marker genes in single glands isolated from 39 primary tumors. Array-based comparative genomic hybridization (array-CGH) analysis revealed that genomic losses commonly occurred at 3q26.1, 4q13.2, 6q21.32, 7q34, 8p12-23.3, 15qcen and 18, while gains were commonly found at 1q21.3-23.1, 7p22.3-q34, 13q12.11-14.11, and 20. The total numbers and lengths of the CNAs were significantly associated with the aberrant DNA methylation and Dukes' stages. Moreover, hierarchical clustering analysis of the array-CGH data suggested that tumors could be categorized into four subgroups. Tumors with frequent DNA methylation were most strongly enriched in subgroups with infrequent CNAs. Importantly, Dukes' D tumors were enriched in the subgroup showing the greatest genomic losses, whereas Dukes' C tumors were enriched in the subgroup with the greatest genomic gains. Our data suggest an inverse relationship between chromosomal instability and aberrant methylation and a positive association between genomic losses and distant metastasis and between genomic gains and lymph node metastasis in CRC. Therefore, DNA copy number profiles may be predictive of the metastatic behavior of CRCs.

INTRODUCTION

Colorectal cancers (CRCs) develop through multiple genetic alterations, including allelic losses at chromosomal loci (e.g., 5q, 17p, and 18q) (Fearon and Vogelstein, 1990). In addition, epigenetic changes, including aberrant DNA methylation and histone modifications, are also strongly implicated in the pathogenesis of CRC, and a subset of CRCs show concurrent hypermethylation in multiple loci, which is now classified as the CpG island methylator phenotype (CIMP) (Toyota et al., 1999). Recent studies have shown that there are two types of CRCs with distinct genomic abnormalities: chromosomal instability (CIN), which accounts for 80–85% of sporadic CRCs and was originally characterized based on the presence of aneuploid/polyploid karyotypes, and microsatellite instability (MSI), also termed MIN, which accounts for 15–20% of sporadic CRCs and is characterized by mismatch repair defects and a near-diploid karyotype (Grady and Carethers, 2008). CIN cancers exhibit gains and losses at multiple chromosomal loci (copy number alterations; CNAs) (Rajagopalan and Lengauer, 2004), whereas MSI cancers show considerable

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*Correspondence to: Tamotsu Sugai, Division of Molecular Diagnostic Pathology, Department of Pathology, School of Medicine, Iwate Medical University, 19-1, Morioka 020-8505, Japan. E-mail: tsugai@cocoa.ocn.ne.jp or Hiromu Suzuki, Department of Molecular Biology, Sapporo Medical University, S1, W17, Chuo-ku, Sapporo 060-8556, Japan. E-mail: hsuzuki@sapmed.ac.jp

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overlap with CIMP cancers (Toyota et al., 1999; Ogino et al., 2006; Weisenberger et al., 2006).

In addition to the commonly observed CRC-related allelic losses on chromosome arms 5q, 17p, and 18q, gains and losses on many other chromosomes have been identified using conventional comparative genomic hybridization (CGH) analysis (Ried et al., 1996; Meijer et al., 1998). Diep et al. (2006) conducted a meta-analysis of the chromosomal changes in a series of 859 CRC specimens identified using CGH and reported that specific CNAs are associated with each step during the progression of CRC. Still, conventional CGH has limited resolution and can only detect CNAs of ~10 Mb or greater in length. On the other hand, array-based CGH (array-CGH) can detect genetic changes with a resolution of 1 Mb or less, making it a powerful tool with which to analyze genomic alterations (Douglas et al., 2004; Jones et al., 2005).

From a clinical viewpoint, previous studies have shown that CRCs can be categorized into distinct subgroups based on the characteristics of their CNAs (Hermsen et al., 2002), and such subtyping has predictive value with respect to prognosis (Poulogiannis et al., 2010) and the response to chemotherapy (Postma et al., 2009). Similarly, a number of studies have shown that epigenetic alterations, especially CIMP, are strongly associated with the clinical behavior of CRCs (Shen et al., 2007a; Jover et al., 2011). However, although it is recognized that CRCs develop via multiple molecular pathways, including CIN, MSI, and CIMP (Jass, 2007; Shen et al., 2007b; Issa, 2008; Hinoue et al., 2012), the associations between genetic and epigenetic abnormalities are still not fully understood. In this study, we performed an integrative analysis of copy number changes on genome-wide scale and assessed genetic mutation of *TP53*, *KRAS*, *BRAF*, and *PIK3CA* and DNA methylation of six marker genes within crypts isolated from surgically resected CRCs, and assessed their relevance to the clinicopathological characteristics.

MATERIALS AND METHODS

Patients and Tissue Samples

A total of 39 primary CRCs and corresponding normal tissue specimens were obtained from consecutive patients at the Iwate Medical University Hospital. Informed consent was obtained from all patients before collection of the specimens, and approval of this study was obtained from the Institutional Review Board of Iwate Medical University. The clinicopathological features of the patients are summarized in Table 1. Pathological diagnosis and staging were performed using a combination of the Japanese classification (Japanese Society for Cancer of the Colon and Rectum, 1997) and modified Dukes' classification (Turnbull et al., 1967). Tumor locations were classified as left- or right-sided and rectal.

TABLE 1. Clinicopathological Features of the CRC Samples Used in this Study

Age (years, median ± SD)	69 ± 11.7
Sex	
Male	24 (62%)
Female	15 (38%)
Location	
Right	14 (36%)
Left	5 (13%)
Rectum	20 (51%)
Histology	
Mod	29 (74%)
Well	7 (17%)
Pap	1 (3%)
Por	1 (3%)
Muc	1 (3%)
Dukes' stage	
A	7 (18%)
B	5 (13%)
C	13 (33%)
D	14 (36%)
Lymph node metastasis	
Positive	23 (59%)
Negative	16 (41%)

Isolation of Glands and Genomic DNA Extraction

Glands were isolated from the tumors and normal mucosae as described previously (Arai and Kino, 1989; Nakamura et al., 1994). The isolated glands were routinely processed to confirm their nature using paraffin-embedded histological sections. Contamination by other materials such as interstitial cells was not evident in the samples examined, which is consistent with previous reports (Sugai et al., 2000; Sugai et al., 2005). Genomic DNA was extracted using the standard phenol-chloroform procedure.

Analysis of *TP53*, *BRAF*, *KRAS*, and *PIK3CA* Mutations

Exons 5–8 of *TP53* were PCR amplified and then analyzed using single-strand conformational polymorphism (SSCP). PCR amplification, PCR-SSCP, and the sequencing of *TP53* were performed as described previously (Dix et al., 1994; Habano et al., 1996; Sugai et al., 2000). In addition,

mutation of codon 600 of *BRAF* and codons 12 and 13 of *KRAS* was examined by pyrosequencing using *BRAF* and *KRAS* pyro kits (Qiagen) according to the manufacturer's instructions, and exons 9 and 20 of *PIK3CA* were directly sequenced as described previously (Jhawer et al., 2008).

DNA Methylation Analysis

CpG island methylation was analyzed as described previously (Toyota et al., 2008). Briefly, genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen). Pyrosequencing was carried out using a PSQ 96MA system (Qiagen) with a Pyro Gold Reagent Kit (Qiagen), and the results were analyzed using Pyro Q-CpG software (Qiagen). A cutoff value of 15% was used to define genes as methylation-positive. Tumors were defined as "tumors with frequent DNA methylation" when methylation was detected in three or more loci out of six markers (*MINT1*, *MINT2*, *MINT12*, *MINT31*, *CDKN2A*, and *MLH1*).

Array-Based CGH

Array CGH analysis was performed as described previously (Igarashi et al., 2010). Briefly, 500 ng of genomic DNA and gender-matched reference DNA (Promega) were digested with *Alu*I and *Rsa*I before labeling and hybridization. Using a Genomic DNA Enzymatic Labeling Kit (Agilent Technologies), tumor DNA and reference DNA were, respectively, labeled with Cy5 and Cy3 after being hybridized to a Human Genome CGH Microarray Kit 105A (G4412A; Agilent Technologies), which contains approximately 99,000 probes annotated against National Center for Biotechnology Information Build 36. The ADM-2 algorithm included in the Genomic Workbench software ver. 5 (Agilent Technologies) was used to identify DNA copy number aberrations. A copy number loss was defined as a log2 ratio <-0.5, and a copy number gain was defined as a log2 ratio >0.5. All genomic positions were defined according to the University of California Santa Cruz Human version hg 18. Unsupervised hierarchical analysis was performed on the log2 Cy5/Cy3 fluorescence ratio data using the Ward's linkage algorithm (JMP version 8, SAS Institute, Cary, NC).

Statistical Analysis

Continuous data was analyzed using *t* tests (for two groups) or ANOVA with a post hoc Tukey's HSD test (for more than two groups). To detect specific differences within groups, adjusted standardized residuals were calculated for the categorical data. If the absolute values of the residuals were more than 1.96, we considered them significantly different from a random distribution. *P* values <0.05 were considered significant. All statistical analyses were performed using SPSS 20 (IBM Corporation, Somers, NY) and Prism 5 (GraphPad Software, La Jolla, CA).

RESULTS

Overview of Array-CGH Analysis

The results of our array-CGH analysis of crypts obtained from 39 CRC tumors are summarized in Figure 1. Genomic losses were commonly observed at several loci, including 3q26.1 (75%), 4q13.2 (80%), 6q21.32 (83%), 7q34 (58%), 8p12-23.3 (55%), 15qcen (50%), and 18 (80%), while gains were commonly observed at 1q21.3-23.1 (41%), 7p22.3-q34 (48%), 13q12.11-14.11 (50%), and 20 (75%). Large genomic losses (>10 Mb in length) were frequently seen at 8p (54%), 18p (59%), and 18q (77%), and large gains (>10 Mb in length) were seen at 7 (41%), 13q (46%), 20p (44%), and 20q (74%) (Fig. 1, Supporting Information Fig. 1). These findings are mostly consistent with earlier results obtained using conventional CGH and array-CGH (Ried et al., 1996; Meijer et al., 1998; Douglas et al., 2004; Jones et al., 2005; Diep et al., 2006).

Mutation and Methylation Analysis

Among the 39 CRC specimens tested, *TP53* and *KRAS* mutations were found in 21 (54%) and 15 (38%), respectively, which is also consistent with earlier findings (Supporting Information Fig. 1 and Table 1) (Dix et al., 1994; Smith et al., 2002; Baldus et al., 2010). However, the frequency of samples with mutations in both *TP53* and *KRAS* (15%) was higher than previously reported (9%) (Smith et al., 2002), probably because our study included tumors at more advanced stages. By contrast, *PIK3CA* mutation was found in only four (10%) specimens, which is less frequent than previously reported (Samuels et al., 2004; Baldus et al., 2010). *BRAF* mutation was not detected in any samples.

Bisulfite-pyrosequencing analysis revealed that 9 of the 39 tumors (23%) exhibited methylation at 3 or more loci, although none showed methylation of *MLH1*. *KRAS* mutation was more prevalent among tumors with frequent DNA methylation (6/9, 67%) than among those without frequent methylation (9/30, 30%). Previous studies demonstrated that

Figure 1. Summary of chromosomal aberrations and their frequencies in 39 CRC specimens determined using array-CGH analysis. Losses (green bars) are displayed on the left, and gains (red bars) are on the right. The chromosome ideogram was generated using Genomic Workbench software.

BRAF mutation and MSI are significantly more prevalent among CIMP-high (CIMP-H or CIMP1) CRCs, whereas *KRAS* mutation is more prevalent among CIMP-low (CIMP-L or CIMP2) tumors (Ogino et al., 2006; Shen et al., 2007b; Hinoue et al., 2012).

Inverse Correlation Between DNA Methylation and Chromosomal Alterations

To quantitatively evaluate copy number aberrations on a genome-wide scale, we calculated the total numbers and lengths of CNAs (losses + gains) identified by the array-CGH analysis. We observed a strong correlation between the total numbers of CNAs in the CRC samples tested and the total lengths of the CNAs (Supporting Information Fig. 2). We therefore used the total CNA length as an index representing the degree of chromosomal alteration and assessed the relationship between total CNA length and methylation status. We found that total CNA lengths were smaller in CRCs with frequent DNA methylation than in those without frequent methylation ($P = 0.033$, Fig. 2C). Interestingly, when we analyzed genomic losses and gains separately, we again observed a significant difference in the magnitude of the losses between frequent methylation-positive and -negative tumors, whereas no such difference was found for gains (Figs. 2A and 2B). We also analyzed the relationship between CNAs and mutation of *TP53* or *KRAS* but found no statistically significant correlations (Supporting Information Fig. 3).

Chromosomal Alterations and Their Association with Clinical Stage in CRC

To determine whether chromosomal alterations accumulate during the progression of CRCs, we assessed the CNA status of tumors at each Dukes' stage. When genomic gains and losses were analyzed separately, we found a tendency toward greater genomic losses in tumors at higher Dukes' stages, but the trend was not statistically significant (Fig. 3A). By contrast, Dukes' C tumors showed the greatest genomic gains, whereas Dukes' D tumors exhibited unexpectedly small gains (Fig. 3B). The total CNA lengths were greatest in Dukes' C tumors, and again Dukes' D tumors showed less chromosomal alteration than Dukes' C tumors (Fig. 3C).

We found that losses at several loci, including 3p24.3, 4p13-15.31, 5qcen-11.2, 8p11-q11, 9p21.3-21.1, 17q24.2-24.3, and 22q13.31, were prevalent in tumors with distant metastasis (Dukes' D) (Supporting Information Table 2), while gains at 11q13.1-13.2, 17q12, and 17q21.2 were prevalent among Dukes' D tumors (Supporting Information Table 3). In addition, losses at 4q21-34, 5q12.1, 5q35.3, and 11qcen-12.1 and gains at 2p15-16.1, 2p13.3, 5p13, 5p35.3, 6p21, 8q12.1-12.3, and 19q13.31 were commonly observed in tumors with lymph node metastasis (Supporting Information Tables 4, 5).

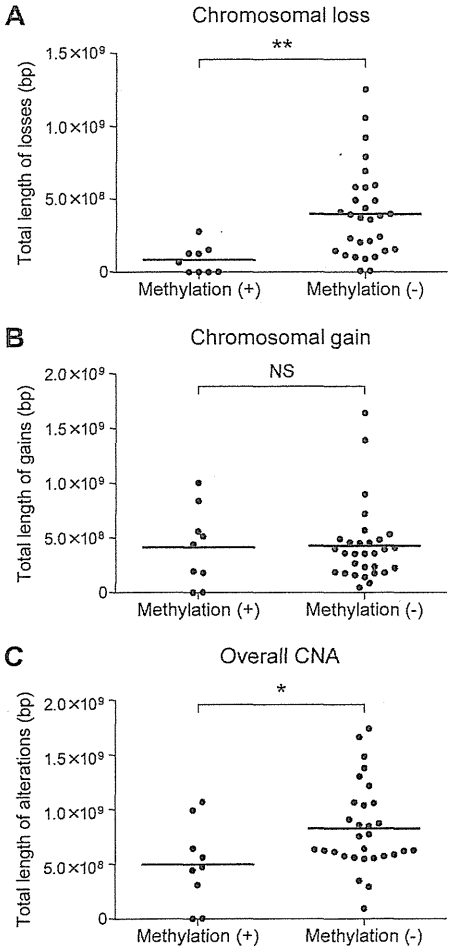

Figure 2. Association between CNAs and DNA methylation status. Total lengths of genomic losses (A) and gains (B) and overall CNAs (C) in tumors with and without frequent DNA methylation are shown. Each dot represents a single tumor. Methylation (+), tumors with frequent DNA methylation; Methylation (−), tumors without frequent DNA methylation; $*P < 0.05$; $**P < 0.01$; NS, not significant.

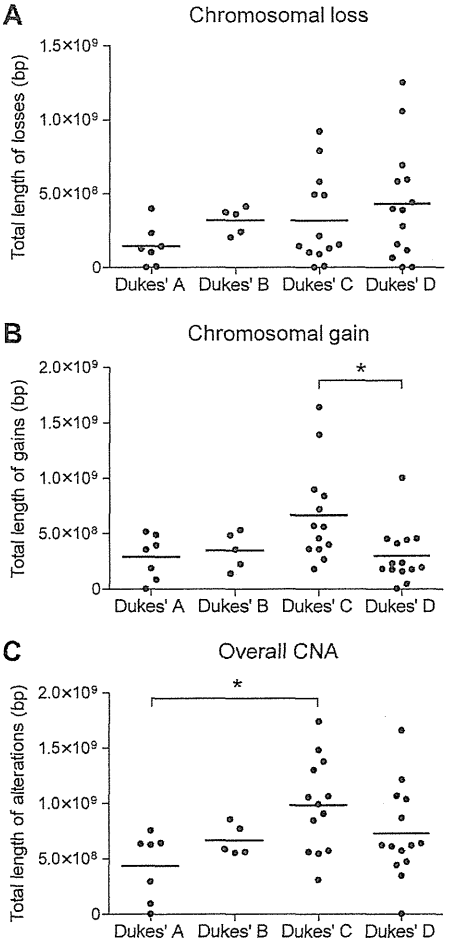

Figure 3. Association between CNAs and Dukes' stages. Total lengths of genomic losses (A), gains (B), and overall CNAs (C) in CRCs at each Dukes' stage are shown. $*P < 0.05$.

Clustering Analysis of CNAs and Their Association with Clinical Stage in CRC

Previous studies have shown that categorization of CRCs according to their chromosomal aberrations has strong relevance to their clinical behavior (Hermsen et al., 2002; Postma et al., 2009; Poulogiannis et al., 2010). For that reason, we carried out unsupervised clustering analysis using our array-CGH data (excluding the sex chromosomes) and then compared the results with genetic mutations and epigenetic alterations (Fig. 4A). We found that CRCs could be subcategorized into at least four clusters based on their CNAs. Gene mutations, DNA methylation status, and genomic alterations on representative chromosomes in each cluster are summarized in Table 2.

Tumors in cluster 1 are characterized by infrequent genomic losses and gains (Figs. 4A–4C). Losses were most prevalent among tumors in cluster 2, while gains were most prevalent among tumors in cluster 4 (Figs. 4A–4C). The total CNA lengths were greater in tumors in clusters 2 and 4 than in clusters 1 and 3 (Fig. 4D). Tumors with frequent DNA methylation were most strongly enriched in cluster 1 (5 of 9, 56%). Tumors in cluster 1 were also characterized by frequent *KRAS* mutation (6 of 10, 60%) and infrequent *p53* mutation (3 of 10, 30%), whereas *p53* mutation was most prevalent in cluster 3 tumors (10 of 15, 67%), although the difference was not statistically significant. Importantly, Dukes' D tumors were highly enriched in cluster 2, within which tumors showed the greatest genomic losses. By contrast, Dukes' C tumors were enriched in cluster 4 and

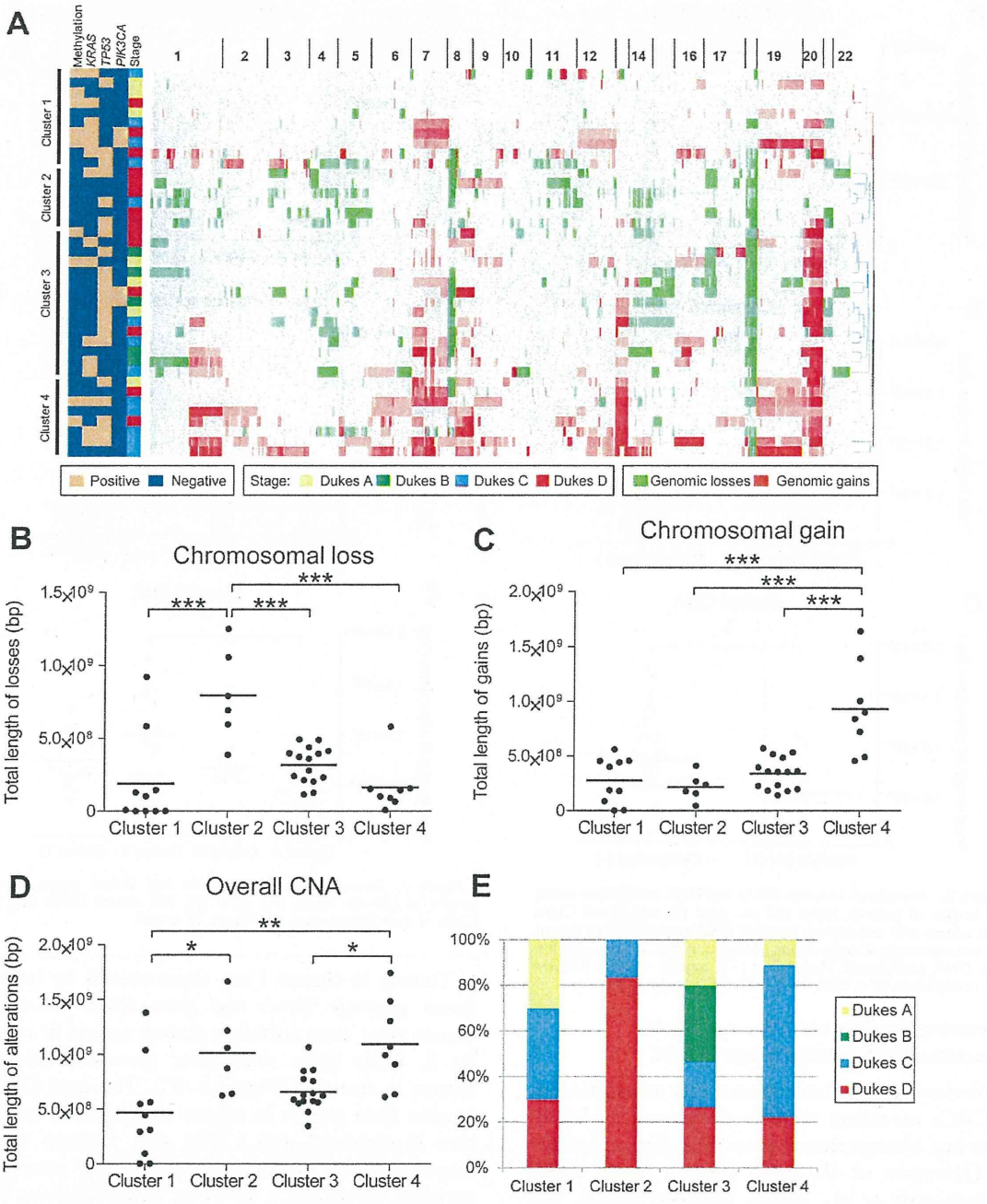

Figure 4. (A) Summarized results of unsupervised hierarchical analysis using the array-CGH data obtained from the 39 CRCs. Tumors were categorized into four clusters as indicated on the left. Tumors with frequent DNA methylation, *KRAS* mutation, *TP53* mutation, *PIK3CA* mutation, and Dukes' stages are also indicated on the left. (B–D) Total lengths of genomic losses and gains and overall CNAs in tumors within each cluster are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (E) Percentages of tumors at the respective Dukes' stages in each cluster are shown.

showed the greatest genomic gains. These results suggest that there is an inverse relationship between CIN and aberrant DNA methylation, and that there is a possible association between genomic losses and distant metastasis and between genomic gains and lymph node metastasis in CRC.

To determine whether a similar clustering pattern could be seen in an independent set of CRC samples, we carried out the same unsupervised

TABLE 2. Mean DNA Copy Number Alterations Within the Four Groups Obtained from the Unsupervised Clustering Analysis

Cluster	Cluster 1	Cluster 2	Cluster 3	Cluster 4	*P* value
Sample	10	6	15	8	
Age (mean ± SD)	66±14.7	69±11.2	69±10.1	63±15.2	
Sex					
Male	4	3	10	7	
Female	6	3	5	1	
Methylation	5/10*	0/6	2/15	2/8	
KRAS	6/10	1/6	5/15	3/8	
TP53	3/10	3/6	10/15	5/8	
PIK3CA	2/10	0/6	2/15	0/8	
18q loss	4/10*	6/6	15/15*	5/8	
20q gain	4/10*	3/6	14/15*	8/8	
18p loss	3/10*	4/6	13/15*	3/8	
8p loss	2/10*	4/6	11/15	4/8	
13q gain	1/10*	2/6	8/15	8/8*	
20p gain	2/10	0/6*	9/15	6/8*	
7p gain	4/10	1/6	5/15	6/8*	
7q gain	4/10	0/6*	7/15	5/8	
Loss (Mb)	189.4	795.8	318.5	162.6	<0.001
Gain (Mb)	277.4	216.3	339.1	930.3	0.001
Total CNA (Mb)	466.8	1012.1	657.6	1092.9	<0.001

Figures are the mean area of the CNAs per genome in each group, which were compared using one-way ANOVA. The total CNA is the sum of the losses and gains. Methylation, tumors with frequent DNA methylation.
*Significantly different from a random distribution, determined from the absolute value of the adjusted standardized residuals >1.96.

hierarchical clustering analysis using the publicly available data set (n = 121) reported by Nakao et al. (2004). We found that the CRC samples were subcategorized into four clusters, among which group 1 showed the highest levels of copy number gains, while group 3 showed the greatest genomic losses (Supporting Information Fig. 4).

DISCUSSION

CIN, which is inferred from DNA ploidy patterns, is the most common form of genomic instability in CRC and is defined by the presence of multiple structural or numerical chromosome changes (Rajagopalan and Lengauer, 2004; Grady and Carethers, 2008). Because mutations in mitotic checkpoint regulators are found in only a small population of CIN CRCs, the mechanism underlying CIN is largely unknown (Grady and Carethers, 2008). Moreover, no method for accurately evaluating the extent of CIN has yet been established. In this study, we used the total CNA length as a surrogate for CIN and analyzed its association with clinical and molecular variables. As described in an earlier study (Gaasenbeek et al., 2006), losses of heterozygosity (LOH) without accompanying copy number changes (e.g., somatic uniparental disomies) were not included as CNAs due to CGH array limitations. It is well established that estimates of CIN prevalence and mutation detection are influenced by the method used for analysis and the purity of the samples (Nakamura et al., 1994; Habano et al., 1996; Sugai et al., 2000; Cardoso et al., 2004; Issa, 2008). We therefore used a crypt isolation technique to avoid contamination by nontumorous cells. The high levels of marker gene methylation detected by quantitative pyrosequencing reflect the purity of the cancer cells in the isolated gland specimens and are indicative of the advantage of using the crypt isolation technique.

Several groups have reported an inverse relationship between CIMP and CIN (Goel et al., 2007; Cheng et al., 2008). Goel et al. (2007) found that CIN status measured as the LOH for eight microsatellite markers was inversely correlated with the methylation frequency at CIMP-related markers in sporadic CRCs, even those without MSI. In addition, Cheng et al. (2008) found that CIN measured as the number of chromosomal arms with gains/losses or LOH was inversely associated with CIMP status. In this study, we also observed an inverse relationship between concurrent methylation at multiple loci (which may represent CIMP) and CIN, although none of the tumors in this study exhibited *MLH1* methylation. Interestingly, we detected no significant difference in the magnitude of the chromosomal gains between tumors with frequent methylation and those without it. This suggests gains at certain genomic loci may be commonly involved in the pathogenesis of CRCs, irrespective of the methylation status. The presence of tumors with a high degree of chromosomal aberration in a subset of CIMP tumors may support this idea (Cheng et al., 2008).

There have been few studies in which the extent of chromosomal aberration during the progression of CRCs was analyzed as we have done in the present study. Hermsen et al. (2002) used conventional CGH to show that the progression of adenoma to carcinoma is associated with an increase in chromosomal aberration. A meta-analysis of 31 conventional CGH studies also showed that primary metastatic CRCs had significantly greater genomic alterations than non-metastatic CRCs (Diep et al., 2006). In addition, the results

of our array-CGH analysis suggest that accumulation of chromosomal losses may play an important role in the progression of CRCs from Dukes' A to Dukes' D. Unexpectedly, however, the overall level of CNAs was highest in Dukes' C tumors, with Dukes' D tumors showing somewhat less chromosomal alteration, which is probably attributable to the greater genomic gains in the Dukes' C group. Assuming that chromosomal gains are irreversible during CRC progression, at least a subset of Dukes' D tumors may have originated as Dukes' A or B tumors, not Dukes' C tumors with their larger chromosomal gains. Our results thus suggest that genomic losses may have a more significant impact on the metastatic properties of tumor cells than genomic gains, and that tumor cells that acquire losses at early stages in regions that are critical for metastasis may have a greater chance to metastasize to distant organs.

We also assessed the association between CNAs and tumor progression, including lymph node and distant metastasis. A previous meta-analysis of conventional CGH data suggests that losses at 4p are associated with the progression from Dukes' A tumors to Dukes' B–D tumors, and losses at 8p and gains at 7p and 17q correlate with liver metastasis (Diep et al., 2006). Our present findings that Dukes' D tumors frequently show losses at 4p and 8p and gains at 17q are consistent with those earlier findings. Importantly, our results seem to reflect the recent findings that losses at 4p and 8p are indicators of liver metastasis and a poor prognosis in CRC (Sheffer et al., 2009).

From an epigenetic viewpoint, Ju et al. (2011) reported that larger numbers of genes were methylated in stage I-III CRCs than in stage IV samples, and that CRCs at stages I-III exhibited methylation profiles that distinctly differed from the profiles of stage IV tumors. Together with the observation that even early stage tumors show intratumor heterogeneities, including ploidy pattern variation (Miyazaki et al., 1999), allelic imbalances (Boland et al., 1995; Sugai et al., 2005), and gene mutations (Baldus et al., 2010), tumor cells with metastatic potential may arise through early genetic and epigenetic lesions, as proposed in the "initiation" (Threadgill, 2005) or "parallel evolution" models (Gray, 2003).

In CRCs with distant metastasis, we frequently observed losses at 3q13.11, where *ACVR2B*, encoding activin A receptor type IIB, is located. Activins are growth and differentiation factors; they belong to the TGF-β superfamily and regulate cell differentiation, proliferation, and apoptosis in a variety of cancer cell types (Chen et al., 2006). Mutation of *ACVR2A*, which is 69% identical to *ACVR2B*, results in loss of its expression and is frequently found in MSI-positive CRCs (Jung et al., 2004). Furthermore, restoration of *ACVR2A* suppresses CRC cell growth, suggesting that it acts as a tumor suppressor (Jung et al., 2007). Together with an earlier report that expression of ACVR2B is weaker in CRC tissues than in normal colon mucosa (Babel et al., 2009), our findings suggest genomic loss of the *ACVR2B* locus may be involved in carcinogenesis and distant metastases in a subset of CRCs.

Consistent with the aforementioned meta-analysis of CGH data (Diep et al., 2006), we commonly observed genomic gains at 17q12, which encompasses a region encoding *ERBB2* and is frequently amplified and/or overexpressed in various types of cancer. One recent study showed that amplification of *ERBB2* leads to persistent activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, which in turn leads to resistance to treatment with cetuximab (Yonesaka et al., 2011). It has also been reported that the median overall survival was significantly longer for CRC patients without *ERBB2* amplification than for those with it (Yonesaka et al., 2011). Thus assessment of chromosomal aberrations at functionally important loci in CRCs could provide useful information for predicting distant metastasis and prognosis, as well as for the development of therapeutic strategies.

There have been a number of studies evaluating the utility of genomic instabilities, including CIN and CNAs, as prognostic markers in CRC (Pritchard and Grady, 2011). A recent meta-analysis confirmed that CIN (measured flow cytometrically as the presence of aneuploidy/polyploidy) is associated with an unfavorable prognosis (Walther et al., 2008). Our array-CGH analysis revealed an increase in copy number losses during the progression of tumor stage, suggesting that the total magnitude of genomic losses could potentially serve as a surrogate marker for genomic instabilities and a prognostic marker in CRC. Recently, Poulogiannis et al. (2010) carried out a hierarchical clustering analysis of array-CGH data obtained from 109 primary CRCs and showed that tumors could be categorized into four groups, in which tumors in groups I and II exhibited CNAs only infrequently, while those in groups III and IV exhibited an abundance of CNAs. CRCs in group I were characterized by a lack of CNAs and frequent MSI-positivity. This appears to be consistent with cluster 1 in our study, which was

enriched in tumors with frequent DNA methylation, and may suggest that the copy number profiles of CRCs with MSI are similar to those with frequent methylation but without *BRAF* mutation. They also reported that CRCs in group II, in which CNAs were somewhat more prevalent than in group I, were associated with a lack of lymph-node metastasis and a better prognosis, irrespective of MSI status (Poulogiannis et al., 2010). Thus group II may correspond to cluster 3 in our study, in which Dukes' B (lymph-node negative) tumors were significantly enriched. By contrast, most patients in our cluster 2 were staged as Dukes' D, with significantly greater genomic losses than tumors in other clusters. Furthermore, Dukes' C tumors were enriched in cluster 4, within which tumors exhibited the greatest genomic gains. Although the proportions of Dukes' C and D tumors in the study by Poulogiannis et al. differ from those in our study, the highest number of Dukes' D tumors were seen in group III and were characterized by significant genomic losses, while the majority of the Dukes' C tumors were in group IV and exhibited significant genomic gains. Although this distribution did not reach statistical significance, the tendency is consistent with our results. These findings suggest that cancers with gains are likely to metastasize to lymph nodes or unlikely to metastasize to distant sites. Thus a tumor's CNA pattern may define its metastatic behavior.

This study has several limitations, including a small sample size, the absence of tumors with *BRAF* mutation and/or *MLH1* methylation, and a lack of survival information; nonetheless, our results indicate several important findings. First, there is an inverse relationship between methylation status and the extent of the CNAs, particularly chromosomal losses. Second, tumor progression from Dukes' A to Dukes' C may be associated with the accumulation of CNAs, whereas a subset of Dukes' D tumors with smaller chromosomal gains may have been derived from Dukes' A or B tumors, but not Dukes' C tumors, which show significant genomic gains. Third, the different CNA patterns revealed by our hierarchical clustering analysis may be associated with different tumor behaviors, including local and distant metastasis, which suggests the presence of distinct molecular pathways in the development of CRC. Further study to clarify the differences between these subclasses will likely provide new insight into the molecular mechanisms that determine prognosis and the responses of CRCs to therapy.

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