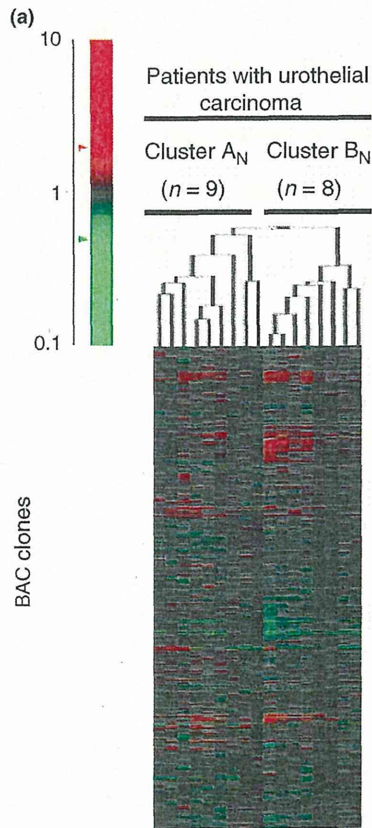

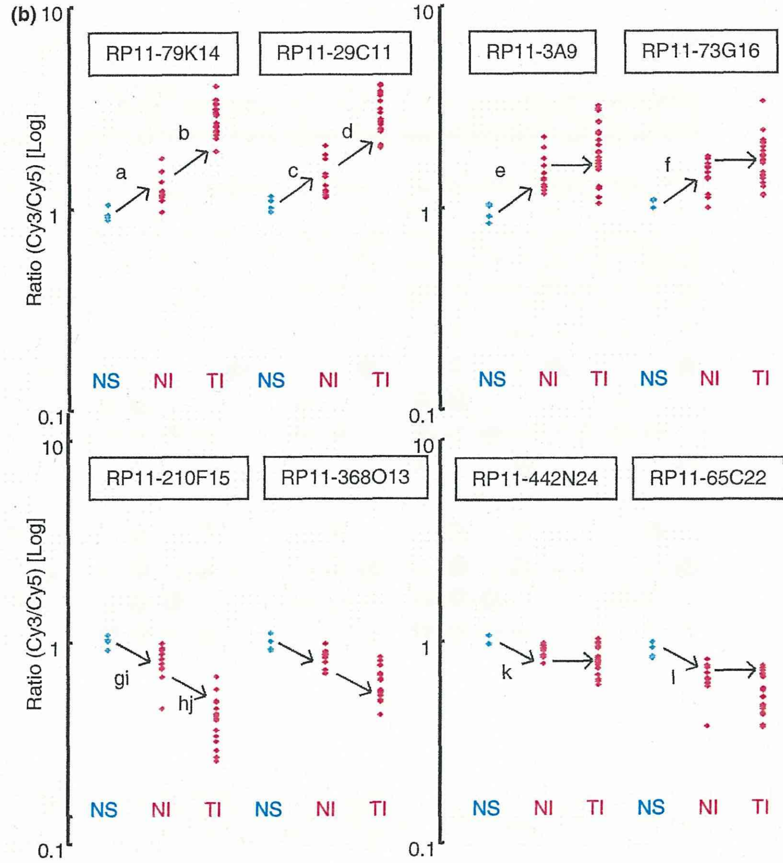

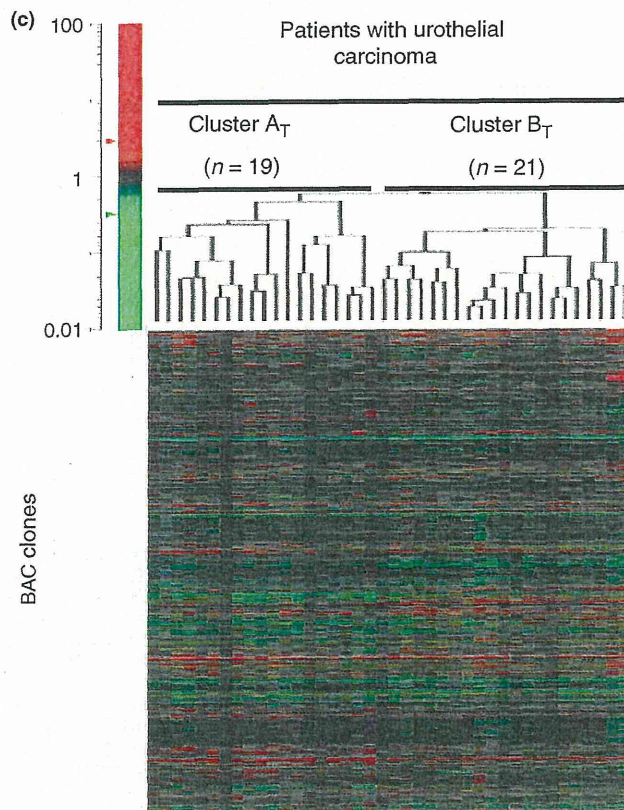

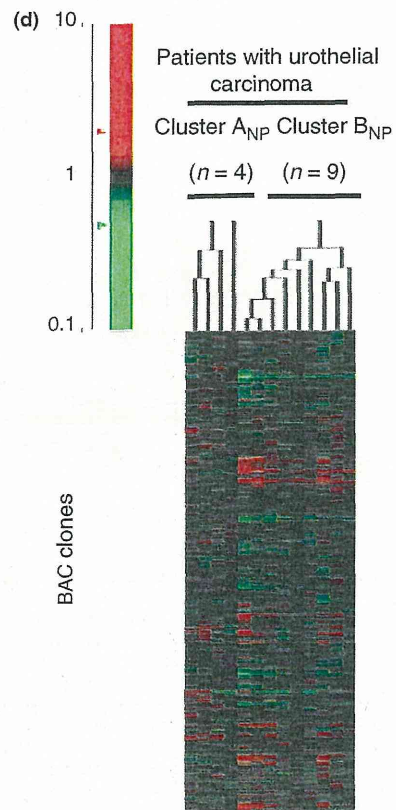

doi: 10.1111/j.1349-7006.2009.01330.x
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Fig. 2. Correlations between DNA methylation status and clinicopathological parameters. (a) Unsupervised two-dimensional hierarchical clustering analysis based on bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) data (signal ratios) in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs). The signal ratio is shown in the color range map. Seventeen patients with UCs were hierarchically clustered into two subclasses, Clusters A_N (n = 9) and B_N (n = 8). Eight patients (100%) belonging to Cluster B_N developed invasive UCs (pT2 or more), whereas five patients (55.6%) belonging to Cluster A_N did so (P = 0.0311). (b) Scattergrams of the signal ratios in tissue samples. NS, noncancerous urothelia obtained from patients with superficial UCs. NI, noncancerous urothelia obtained from patients with invasive UCs. TI, invasive UCs. If the average signal ratios in NI were significantly higher than those in NS, the average signal ratios in TI themselves were even higher than (BAC clones RP11-79K14 and RP11-29C11), or not significantly different from (BAC clones RP11-3A9 and RP11-73G16), those in NI without exception. If the average signal ratios in NI were significantly lower than those in NS, the average signal ratios in TI themselves were even lower than (BAC clones RP11-210F15 and RP11-368O13), or not significantly different from (BAC clones RP11-442N24 and RP11-65C22), those in NI without exception. ^aP = 0.001680673, ^bP = 9.23504e-7, ^cP = 0.002197802, ^dP = 3.64223e-6, ^eP = 0.000840336, ^fP = 0.007692306, ^gP = 0.004395604, ^hP = 8.31509e-6, ^iP = 0.004395604, ^jP = 1.10173e-5, ^kP = 0.005882353, ^lP = 0.001098901. (c) Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) in UCs. Forty patients with UCs were hierarchically clustered into two subclasses, Clusters A_T (n = 19) and B_T (n = 21). All four patients with recurrence belonged to Cluster B_T. (d) Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter. Thirteen patients with UCs of the renal pelvis or ureter were hierarchically clustered into two subclasses, Clusters A_{NP} (n = 4) and B_{NP} (n = 9). All four patients who developed intravesical metachronous UC belonged to Cluster B_{NP}.

scattergrams of the signal ratios in normal urothelia and noncancerous urothelia obtained from patients with UCs for representative examples of the 201 BAC clones. Using the cut-off values described in Figure 3(a), noncancerous urothelia obtained from patients with UCs were discriminated from normal urothelia with sufficient sensitivity and specificity (Fig. 3a). From the 201 BAC clones, 83 for which such discrimination was performed with a sensitivity and specificity of 75% or more than 75% were selected (Table S1). The cut-off values of the signal ratios for the 83 BAC clones, and their sensitivity and specificity, are shown in Table S1.

A histogram showing the number of BAC clones satisfying the criteria listed in Table S1 for 18 normal urothelia (C1–C18) and 17 noncancerous urothelia obtained from patients

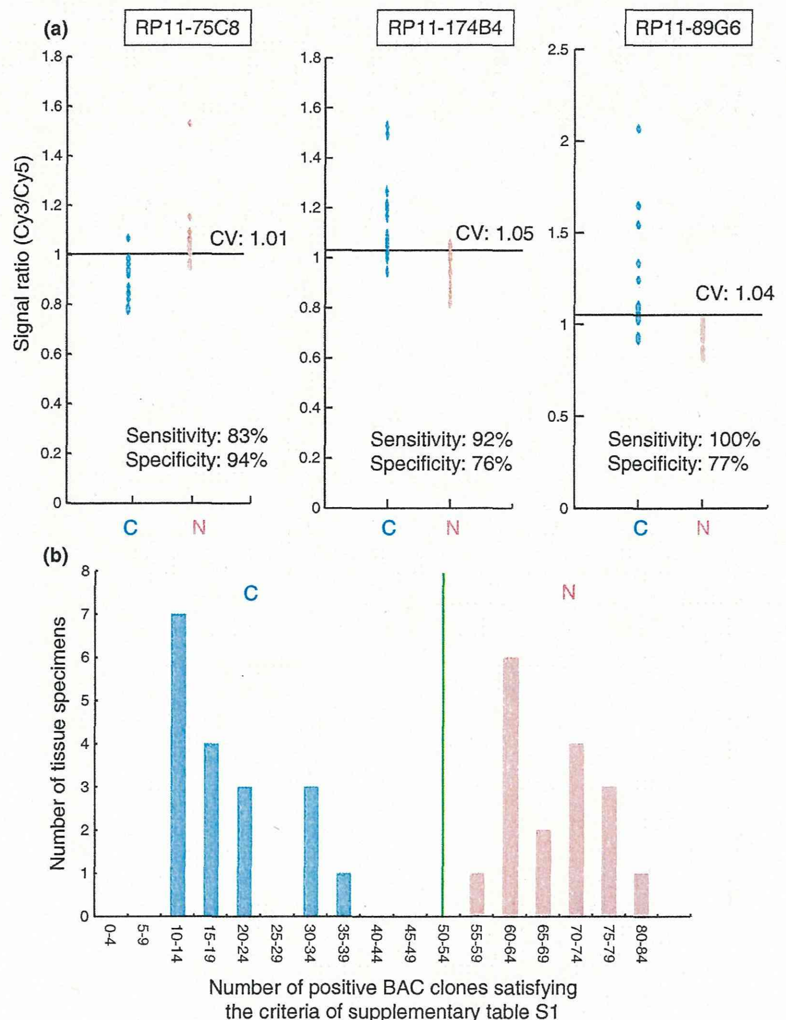

Fig. 3. DNA methylation profiles discriminating noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) (N) from normal urothelia (C). (a) Scattergrams of the signal ratios in C and N on representative bacterial artificial chromosome (BAC) clones, RP11-75C8, RP11-174B4, and RP11-89G6. Using the cut-off values (CV) described in each panel, N in this cohort were discriminated from C with sufficient sensitivity and specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S1 in samples C1–C18 and N1–N17. Based on this histogram, we established a criterion that when the noncancerous urothelia satisfied the criteria in Table S1 for 50 (green bar) or more than 50 BAC clones, they were judged to be at high risk of carcinogenesis.

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with UCs (N1–N17) is shown in Figure 3(b). Based on this figure, we finally established the following criteria: when noncancerous urothelia satisfied the criteria in Table S1 for 50 or more BAC clones (green bar in Fig. 3b), they were judged to be at high risk of carcinogenesis, and when noncancerous urothelia satisfied the criteria in Table S1 for less than 50 BAC clones, they were judged not to be at high risk of carcinogenesis. Based on these criteria, both the sensitivity and specificity for diagnosis of noncancerous urothelia obtained from patients with UCs in this cohort as being at high risk of carcinogenesis were 100%.

Association of DNA methylation profiles in UCs with recurrence. Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for UCs was able to group 40 patients into two subclasses, Clusters A_T and B_T, which contained 19 and 21 patients, respectively (Fig. 2c). Four patients (19.0%) belonging to Cluster B_T suffered recurrence after surgery (metastasis to the pelvic lymph nodes in three, and metastasis to the lung and bone in one), whereas none (0%) belonging to Cluster A_T did so ($P = 0.0449$). The mean observation period was 29.8 ± 28.0 months (mean ± SD). These data prompted us to establish criteria for predicting recurrence of UCs based on DNA methylation status.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 20 BAC clones in UCs differed significantly between the patients who suffered recurrence after surgery and patients who did not. Figure 4(a) shows scattergrams of the signal ratios in UCs obtained from patients who suffered recurrence and those in UCs obtained from patients who did not. DNA methylation status of the 20 BAC clones was able to discriminate patients who suffered recurrence from patients who did not with a sensitivity of 100% using the cut-off values shown in Figure 4(a) and Table S2. A histogram showing the number of BAC clones satisfying the criteria listed in Table S2 for all 40 UCs is shown in Figure 4(b). Satisfying the criteria in Table S2 for 17 or more BAC clones (green bar in Fig. 4b) discriminated patients who suffered recurrence from patients who did not with a sensitivity and specificity of 100%, whereas high histological grade,[21] invasive growth (pT2 or more), and vascular or lymphatic involvement were unable to achieve such complete discrimination (data not shown).

Association of DNA methylation profiles in noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter with intravesical metachronous UC development. It is well known that patients with UCs of the renal pelvis and ureter frequently suffer from metachronous UC development in the urinary bladder after nephroureterectomy.[24,25] Since such metachronous UC originates from the noncancerous urothelium of the urinary bladder, we focused on the DNA methylation status of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter. Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter was able to group 13 patients into two subclasses, Clusters A_{NP} and B_{NP}, which contained four and nine patients, respectively (Fig. 2d). Four (44%) of the patients in Cluster B_{NP} developed intravesical metachronous UCs, whereas none (0%) belonging to Cluster A_{NP} did so. These data prompted us to establish criteria that could predict the development of intravesical metachronous UC based on DNA methylation status.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 11 BAC clones in noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter differed

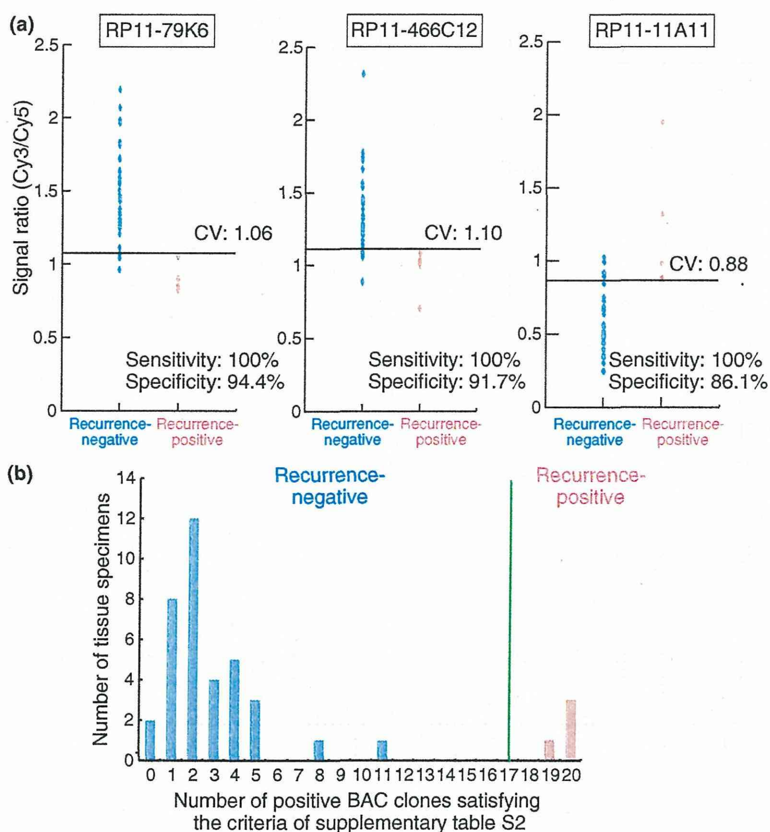

Fig. 4. DNA methylation profiles in urothelial carcinomas (UCs) associated with recurrence. (a) Scattergrams of the signal ratios in UCs from patients who did not develop recurrence ($n = 36$) and UCs from patients who developed recurrence ($n = 4$) on representative bacterial artificial chromosome (BAC), clones, RP11-79K6, RP11-466C12, and RP11-11A11. Using the cut-off values (CV) described in each panel, recurrence-positive patients were discriminated from recurrence-negative patients with 100% sensitivity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S2 in all 40 patients with UCs. Satisfying the criteria in Table S2 for 17 (green bar) or more than 17 BAC clones discriminated recurrence-positive patients from recurrence-negative patients with a sensitivity and specificity of 100%, whereas high histological grade (21), invasive growth (pT2 or more), and vascular or lymphatic involvement were unable to achieve such complete discrimination (data not shown).

doi: 10.1111/j.1349-7006.2009.01330.x
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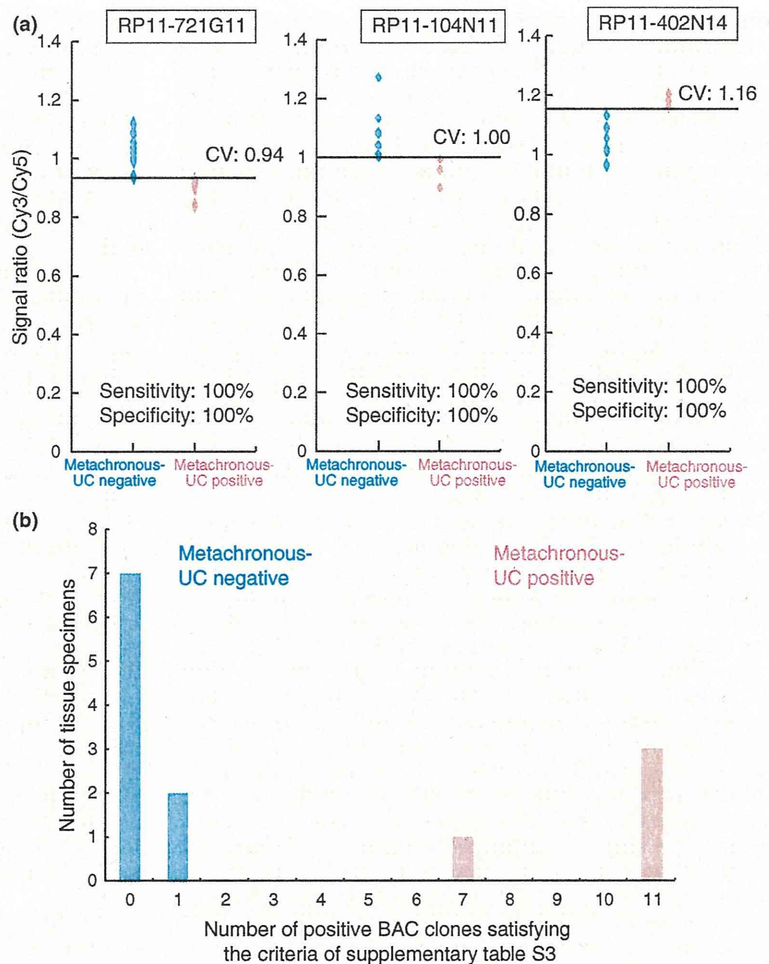

Fig. 5. DNA methylation profiles in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) of the renal pelvis or ureter associated with intravesical metachronous UC development. (a) Scattergrams of the signal ratios in noncancerous urothelia obtained from patients who did not develop intravesical metachronous UCs ($n = 9$) and noncancerous urothelia obtained from patients who developed intravesical metachronous UCs ($n = 4$) on representative bacterial artificial chromosome (BAC) clones, RP11-721G11, RP11-104N11, and RP11-402N14. Using the cut-off values (CV) described in each panel, metachronous UC-positive patients were discriminated from metachronous UC-negative patients with 100% sensitivity and specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S3 in all 13 patients with UCs of the renal pelvis or ureter from whom noncancerous urothelia were obtained. Patients who were negative and positive for metachronous UC were confirmed to show a marked difference in the DNA methylation status of the 11 BAC clones.

significantly between patients who developed intravesical metachronous UC after nephroureterectomy and patients who did not. DNA methylation status of nine of the 11 BAC clones was able to discriminate patients who suffered from intravesical metachronous UC development from patients who did not with a sensitivity and specificity of 100% using the cut-off values shown in Figure 5(a) and Table S3. A histogram showing the number of BAC clones satisfying the criteria listed in Table S3 for 13 noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter is shown in Figure 5(b).

Discussion

Urothelial carcinomas are clinically remarkable because of their multicentricity: synchronously or metachronously multifocal UCs often develop in individual patients. A possible mechanism for such multiplicity is the ''field effect,'' whereby carcinogenic agents in the urine cause malignant transformation of multiple urothelial cells.[26] Even noncancerous urothelia showing no remarkable histological features obtained from patients with UCs can be considered to be at the precancerous stage, because they may be exposed to carcinogens in the urine. On the other hand, UCs are classified as superficial papillary carcinomas or nodular invasive carcinomas according to their configuration. Superficial papillary carcinomas usually remain noninvasive, although patients need to undergo repeated urethrocystoscopic resections because of recurrences. In contrast, the clinical outcome of nodular invasive carcinomas is poor.[11,12]

In our previous study, accumulation of DNA methylation on C-type CpG islands associated with DNMT1 protein overexpression was observed even in noncancerous urothelia obtained from patients with UCs.[8,9] Aberrant DNA methylation was further increased, especially in nodular invasive carcinomas.[8–10] These previous data suggested that carcinogenetic risk estimation and prognostication of UCs based on DNA methylation status might be a promising strategy. Although optimal diagnostic indicators have never been explored using array-based genome-wide DNA methylation analysis, alterations of DNA methylation on several CpG islands in UCs have been reported separately.[27–31]

Many researchers in the field of cancer epigenetics have used promoter arrays to identify the genes that are methylated in cancer cells.[14–16] However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. DNA methylation status in genomic regions that do not directly participate in gene silencing, such as the edges of CpG islands, may be altered at the precancerous stage before the alterations of the promoter regions themselves occur.[32] Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large regions of chromosomes, which are regulated in a coordinated manner in

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human cancers due to a process of long-range epigenetic silencing, has recently attracted attention.[33] Therefore, we used a custom-made BAC array[20] that may be suitable for gaining an overview of the DNA methylation status of individual large regions among all chromosomes (Table S4), and for obtaining reproducible diagnostic indicators. In fact we have successfully obtained optimal indicators for carcinogenetic risk estimation and prognostication of renal cell carcinomas[23] and hepatocellular carcinomas[34] by BAMCA using the same array as that employed in this study. On the other hand, we must pay attention to the quantitative accuracy of BAMCA, because it is a PCR-based method differing from other genome-wide DNA methylation analyses not using PCR, such as the methylated DNA immunoprecipitation-microarray. In order to validate the results of BAMCA, we quantitatively evaluated the DNA methylation status of each Xma I/Sma I site yielding labeled products which are effective in BAMCA on representative BAC clones, by pyrosequencing. As shown in the example in Figure S1 and Table S5, pyrosequencing validated the BAMCA data on the representative BAC clone.

The present DNA methylation analysis revealed that stepwise DNA methylation alterations during urothelial carcinogenesis occurred in a genome-wide manner (Fig. 1). We then performed unsupervised hierarchical clustering analysis based on the genome-wide DNA methylation status of noncancerous urothelia, and as a result, 17 patients were subclassified into Clusters A_N and B_N. Corresponding UCs showing deeper invasion were found to be accumulated in Cluster B_N. Genome-wide DNA methylation profiles of noncancerous urothelia obtained from patients with invasive UCs were inherited by the invasive UCs themselves (Fig. 2b). DNA methylation profiles of noncancerous urothelia obtained from patients with superficial UCs were not always inherited by superficial UCs (data not shown), corresponding to the alternative malignant progression of superficial papillary carcinoma to nodular invasive carcinoma, via papillonodular carcinoma. Genome-wide DNA methylation alterations that were correlated with the development of more malignant invasive cancers were already accumulated in noncancerous urothelia, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to further accumulation of genetic and epigenetic alterations and generate more malignant cancers.

The present genome-wide analysis revealed DNA methylation profiles that were able to completely discriminate noncancerous urothelia obtained from patients with UCs from normal urothelia and diagnose them as having a high risk of urothelial carcinogenesis with a sensitivity and specificity of 100%. We are currently attempting to develop methodology for assessing the tendency for DNA methylation in the 83 BAC regions in urine samples with a view to application for screening of healthy individuals. If it proves possible to identify individuals who are at high risk of urothelial carcinogenesis, then strategies for the prevention or early detection of UCs, such as smoking cessation or repeated urine cytology examinations, might be applicable.

Even after surgery with curative intent, some UCs relapse and metastasize to lymph nodes or distant organs.[35] Recently, new systemic chemotherapy and targeted therapy have been developed for treatment for UCs.[36] In order to start adjuvant systemic chemotherapy immediately in patients who have undergone surgery and are still at high risk of recurrence and metastasis, prognostic indicators have been explored. The present genome-wide analysis revealed DNA methylation profiles that were able to discriminate patients who suffered recurrence after surgery from patients who did not with a sensitivity and specificity of 100% (Fig. 4b), whereas a high histological grade,[21] invasive growth (pT2 or more), and vascular or lymphatic involvement, which are known to have a prognostic impact,[37,38] were incapable of such complete discrimination (data not shown). Therefore, a combination of the 20 BAC clones can have significant prognostic value for patients with UCs. Since a sufficient quantity of good-quality DNA can be obtained from each surgical specimen, our array-based analysis that overviews aberrant DNA methylation of each BAC region is immediately applicable to routine laboratory examinations for prognostication after surgery. The reliability of such prognostication will need to be validated in a prospective study.

As mentioned above, UCs are remarkable because of their multicentricity. Approximately 10–30% of patients with UCs of the renal pelvis and ureter develop intravesical metachronous UCs after nephroureterectomy.[24,25] Therefore, such patients have to undergo repeated urethrocystoscopic examinations to detect intravesical metachronous UCs. To decrease the need for invasive urethrocystoscopic examinations and assist close follow-up of such patients after nephroureterectomy, indicators for intravesical metachronous UCs have been needed. All of our patients who developed intravesical metachronous UCs after nephroureterectomy belonged to Cluster B_{NP}, indicating that DNA methylation profiles of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter, which may be exposed to the same carcinogens in the urine as noncancerous urothelia from which metachronous UCs originate, are correlated with the risk of intravesical metachronous UC development. The present genome-wide analysis revealed DNA methylation profiles that were able to completely discriminate patients with UCs of the renal pelvis or ureter who developed intravesical metachronous UCs from patients who did not, in noncancerous urothelia from nephroureterectomy specimens. A combination of the present 11 BAC clones may be an optimal indicator for the development of intravesical metachronous UC. The reliability of such prognostication will again need to be validated in a prospective study.

With respect to background factors of genome-wide DNA methylation alterations during urothelial carcinogenesis, smoking history did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S6). In addition, immunohistochemically examined DNMT1 protein expression levels did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S7), indicating that expression levels of DNMT1 did not by themselves simply determine DNA methylation profiles. However, our previous study revealed remarkable protein overexpression of DNMT1 in noncancerous urothelia obtained from patients with UCs as compared to normal urothelia.[8] Therefore, undefined cofactors may recruit DNMT1 or other proteins regulating DNA methylation status to aberrant target sequences and may participate in DNA methylation alterations in noncancerous urothelia obtained from patients with UCs. Further studies are needed to elucidate molecular mechanisms of DNA methylation alterations in such noncancerous urothelia.

Moreover, when the DNA methylation status for CpG islands of *p16*, human MutL homologue 1 (*hMLH1*), thrombospondin-1 (*THBS-1*), and death-associated protein kinase (*DAPK*) genes and the methylated in tumor (MINT)-1, -2, -12, -25, and -31 clones were examined in noncancerous urothelia obtained from patients with UCs and in UCs by methylation-specific PCR and combined bisulfite restriction enzyme analysis as in our previous study,[9,39] the incidence of DNA

doi: 10.1111/j.1349-7006.2009.01330.x
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methylation on each CpG island and the average number of methylated CpG islands did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S8). Therefore, molecular mechanisms for alterations of genome-wide DNA methylation profiles may differ from those for regional DNA hypermethylation on CpG islands.

Although BAMCA mainly provides an overview of the DNA methylation status of individual large regions among all chromosomes as mentioned above, it may also be able to identify genes for which expressions are regulated by DNA methylation, since there are promoter regions of specific genes including CpG islands on BAC clones showing clinicopathologically significant DNA hypo- or hypermethylation (Table S4). Expression levels and the DNA methylation status of these genes, as well as the functions of the proteins coded by such genes, will be examined in a future investigation. If further studies identify tumor-related genes for which expression levels are regulated by DNA methylation among such candidates, these tumor-related genes may serves as targets for epigenetic prevention and therapy, along with the molecules causing alterations of genome-wide DNA methylation profiles.

Acknowledgments

This study was supported by a Grant-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan; a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan; a Grant from the New Energy and Industrial Technology Development Organization (NEDO); and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio). N. Nishiyama is an awardee of a research resident fellowship from the Foundation for Promotion of Cancer Research in Japan.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Examples of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) data validation by pyrosequencing.

Table S1. Eighty-three bacterial artificial chromosome (BAC) clones that were able to discriminate noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) (N) from normal urothelia (C) with a sensitivity and specificity of 75% or more.

Table S2. Twenty bacterial artificial chromosome (BAC) clones that were able to discriminate urothelial carcinomas (UCs) in patients who developed recurrence (Pos) from those in patients who did not (Neg).

Table S3. Eleven bacterial artificial chromosome (BAC) clones that were able to discriminate noncancerous urothelia in patients with urothelial carcinomas (UCs) of the renal pelvis or ureter who developed intravesical metachronous UC (Pos) from those in patients who did not (Neg).

Table S4. Genes, CpG islands in the promoter regions, and repeat elements of bacterial artificial chromosome (BAC) clones in Tables S1, S2, and S3.

Table S5. Primer sets for validation study by pyrosequencing.

Table S6. Correlation between smoking history and DNA methylation status in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

Table S7. Correlation between protein expression levels of DNA methyltransferase (DNMT) 1 and DNA methylation status in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

Table S8. Correlation between regional DNA hypermethylation on CpG islands and the results of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

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doi: 10.1111/j.1349-7006.2009.01330.x
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J Gastroenterol
DOI 10.1007/s00535-013-0861-7

ORIGINAL ARTICLE—Alimentary Tract

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
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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

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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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Published online: 13 August 2013

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 ($\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.

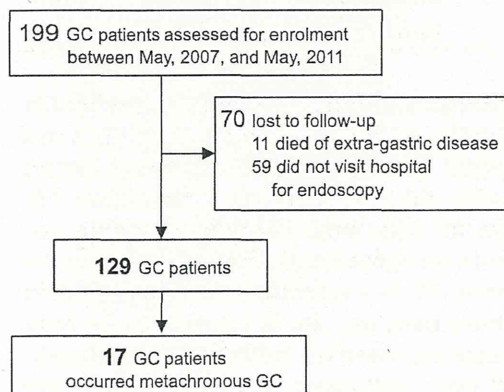

Fig. 1 Profiles of participants in this study

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).

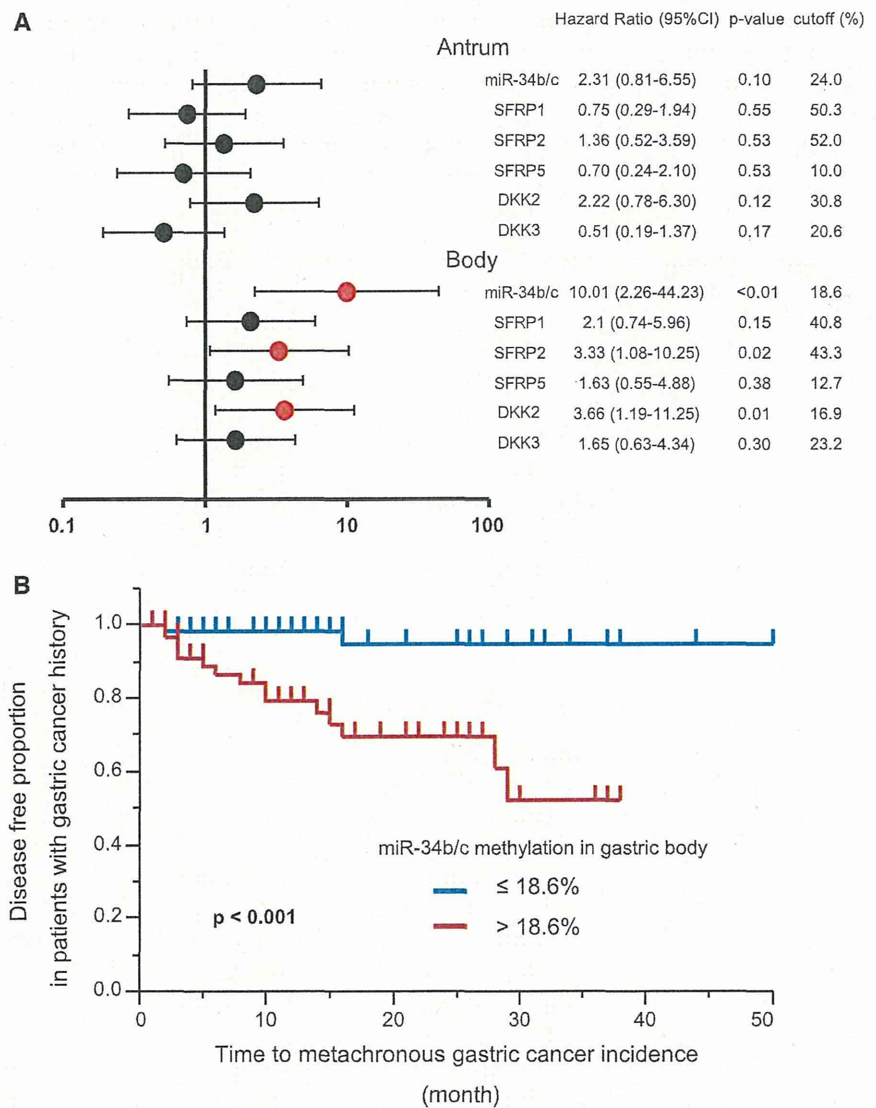

Fig. 2 Associations between DNA methylation and metachronous GC risk. **a** *Forest plot* showing hazard ratios (*closed circles*) for developing metachronous GC and 95 % confidence intervals (*bar lines*). Univariate Cox proportional hazards model analysis was performed to assess the correlations between 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

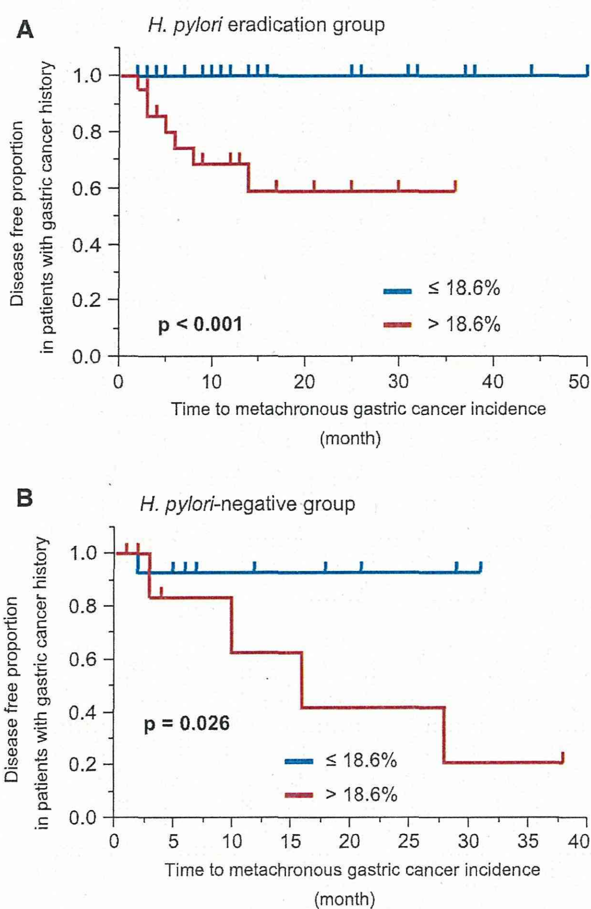

Fig. 3 Association between *miR-34b/c* methylation and metachronous GC risk in *H. pylori*-positive and -negative patients. **a** Kaplan–Meier analysis of the effect of *miR-34b/c* methylation on metachronous GC-free survival among *H. pylori*-positive patients who underwent successful eradication after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

Acknowledgments The authors thank Dr. William F. Goldman for editing the manuscript. This study was supported in part by Grants-in-Aid for Scientific Research (B) from the Japan Society for Promotion of Science (Y. Shinomura), Grants-in-Aid for Young Researchers (B) from the Japan Society for Promotion of Science (E. Yamamoto), a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control (H. Suzuki), A3 foresight program from the Japan Society for Promotion of Science (H. Suzuki) and the Project for Developing Innovative Research on Cancer Therapeutics (P-DIRECT) (H. Suzuki).

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

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GENES, CHROMOSOMES & CANCER 52:140–149 (2013)

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

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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. © 2012 Wiley Periodicals, Inc.

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

Additional Supporting Information may be found in the online version of this article.

Supported by Japan Society for Promotion of Science (Grant-in-Aid for Scientific Research (C) [H.S.]); a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control (M.T. and H.S.); Ministry of Health, Labor, and Welfare, Japan (Grant-in-Aid for Cancer Research [M.T. and H.S.)], Japan Society for Promotion of Science (A3 foresight program [H.S.)].

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Received 21 February 2012; Accepted 22 August 2012
DOI 10.1002/gcc.22013
Published online 17 October 2012 in
Wiley Online Library (wileyonlinelibrary.com).

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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