Epigenetic Alteration of DNA in Mucosal Wash Fluid Predicts Invasiveness of Colorectal Tumors

Seiko Kamimae1, Eiichiro Yamamoto1,2, Hiro-o Yamano5, Masanori Nojima3, Hiromu Suzuki1,2, Masami Ashida1, Tomo Hatahira1, Akiko Sato1, Tomoaki Kimura6, Kenjiro Yoshikawa6, Taku Harada5, Seiko Hayashi6, Hiroyuki Takamaru2, Reo Maruyama1,2, Masahiro Kai1, Morie Nishiwaki6, Tamotsu Sugai7, Yasushi Sasaki4, Takashi Tokino4, Yasuhisa Shinomura2, Kohzoh Imai8, and Minoru Toyota1

Abstract

Although conventional colonoscopy is considered the gold standard for detecting colorectal tumors, accurate staging is often difficult because advanced histology may be present in small colorectal lesions. We collected DNA present in mucosal wash fluid from patients undergoing colonoscopy and then assessed the methylation levels of four genes frequently methylated in colorectal cancers to detect invasive tumors. We found that methylation levels in wash fluid were significantly higher in patients with invasive than those with noninvasive tumors. Cytologic and K-ras mutation analyses suggested that mucosal wash fluid from invasive tumors contained greater numbers of tumor cells than wash fluid from noninvasive tumors. Among the four genes, levels of mir-34b/c methylation had the greatest correlation with the invasion and showed the largest area under the receiver operating characteristic curve (AUC = 0.796). Using cutoff points of mir-34b/c methylation determined by efficiency considerations, the sensitivity/specificity were 0.861/0.657 for the 13.0% (high sensitivity) and 0.765/0.833 for the 17.8% (well-balanced) cutoffs. In the validation test set, the AUC was also very high (0.915), the sensitivity/specificity were 0.870/0.875 for 13.0% and 0.565/0.958 for 17.8%. Using the diagnostic tree constructed by an objective algorithm, the diagnostic accuracy of the invasiveness of colorectal cancer was 91.3% for the training set and 85.1% for the test set. Our results suggest that analysis of the methylation of DNA in mucosal wash fluid may be a good molecular marker for predicting the invasiveness of colorectal tumors. Cancer Prev Res; 4(5); 674–83. ©2011 AACR.

Introduction

Colorectal cancer is one of the most common neoplasias worldwide (1), and its early detection and accurate preoperative staging are essential for reducing the incidences of invasion and metastasis. The fecal occult blood test is widely used to screen for colorectal tumors, though its sensitivity and specificity are not high (2, 3). Conventional colonoscopy is considered the gold standard for detecting colorectal cancers and adenomas, whereas several other methods, including computed tomography, ultrasonography, and 3D magnetic resonance, have been used for staging (4, 5). Generally, tumor size is used as the marker for invasion and lymph node metastasis; however, accurate staging is often difficult because advanced histology may be present in as much as 10% of small (5–10 mm) colorectal adenomas (6–8).

Magnified endoscopy is a highly useful method for diagnosing invasive colorectal cancer (9, 10). Although conventional endoscopic examination with indigo carmine dye is not sufficient to determine whether or not a colorectal cancer is invasive, pit pattern analysis, using high-magnification observation with crystal violet, reportedly enables the diagnosis of invasive colorectal cancers. Recently, narrow-band imaging magnification endoscopy has also been used to predict the invasiveness of colorectal tumors (11). However, it has been suggested that the skills required for pit pattern analysis will limit the number of endoscopists who use the technique.

DNA methylation plays a critical role in the tumorigenesis of colorectal cancer (12, 13). For example, promoter hypermethylation is associated with the silencing of various cancer-related genes (14, 15), and aberrant methylation of the CpG islands of genes in stool and serum/plasma can be used as a molecular marker for detection of colorectal tumors (16–20). On the contrary, because DNA
methylation is an early event (21–23), it does not enable one to distinguish between premalignant lesions and invasive tumors. As yet, there is no study describing a molecular test for predicting the invasiveness of colorectal tumors.

In this study, we examined the methylation levels of 4 genes frequently methylated in colorectal cancers by using DNA obtained from mucosal wash fluid. We found that the methylation level of DNA in the wash fluid was significantly higher in patients with invasive than those with noninvasive tumors. Our results suggest that methylation of DNA in mucosal wash fluid could be a good molecular marker for predicting the invasiveness of colorectal tumors.

Materials and Methods

Specimens and sample preparation

Colorectal tumor tissues and washing fluid were collected from Japanese patients who underwent endoscopic mucosal resection (EMR) or surgical resection of colorectal tumors at Akita Red Cross Hospital. Informed consent was obtained from all patients before collection of the specimens. Approval for this study was obtained from the Institutional Review Board of Akita Red Cross Hospital and Sapporo Medical University.

We used 2 methods to obtain DNA from mucosal washing fluid. When a colorectal tumor was detected during colonoscopy, the tumor’s surface mucus was either washed away by using 20 mL of water, which was aspirated through the suction channel of the endoscope, and suspended in ThinPrep PreservCyt solution (Hologic, Inc.; method 1) or washed away with 20 mL of normal saline by using an NT tube and collected (method 2). Each sample of collected washing fluid was placed in a Non-GYN PreservCyt vial (Hologic, Inc.) for a minimum of 15 minutes, after which the solution was transferred to a disposable centrifuge tube and centrifuged at 500 × g for 20 minutes. The resultant supernatant was discarded, and the cell pellet was suspended in ThinPrep solution until DNA extraction. After collection of the washing fluid and endoscopic observation, biopsies of the colorectal tumor and corresponding normal colonic mucosa were carried out by using biopsy forceps under endoscopic guidance. For cytology analysis, a ThinPrep slide was prepared by a T2000 ThinPrep processor (Hologic, Inc.) and a nongynecologic ThinPrep Filter with 5-μm pores. The slide was then Papanicolaou stained and examined by GLab cytotecnologists and a pathologist (GeneticLab Co. Ltd.), and atypical cells were identified. Nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI) and visualized under a fluorescence microscope (Olympus) as described previously (24).

A total of 337 biopsy specimens including 150 colorectal tumors and 187 normal colonic mucosa specimens were examined. We also examined 88 samples of washing fluid from 70 colorectal tumor patients and 18 healthy patients. For testing the clinical usefulness of the study, we examined an additional 47 colorectal tumor biopsy samples as the test set, which were independently obtained several months after the collection of prior samples. On the basis of histologic examination after any type of resection, biopsy specimens and wash fluid samples from a colorectal tumor were divided into 2 groups: invasive tumors and noninvasive tumors. Invasive tumors were defined as submucosal invasive tumors.

Bisulfite pyrosequencing

DNA was extracted from biopsy specimens and washing fluid by using the standard phenol-chloroform procedure, after which 1-μg samples of genomic DNA were modified with sodium bisulfite by an EpiTect Bisulfite Kit (Qiagen). Bisulfite pyrosequencing was then carried out as described previously (25). Primers for pyrosequencing were designed by PSQ Assay Design software (Qiagen). Following PCR, the biotinylated PCR product was purified, made single-stranded, and then used as a template in a pyrosequencing reaction run according to the manufacturer’s instructions. The PCR products were bound to streptavidin Sepharose beads HP (Amersham Biosciences), after which beads with the immobilized PCR product were purified, washed, and denatured by using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was carried out by a PSQ96MA system (Qiagen) and Pyro Q-CpG software (Qiagen). The primer sequences are listed in Supplementary Table S1.

K-ras mutation analysis

Mutation of codons 12 and 13 of K-ras was examined by using direct sequencing and pyrosequencing, as described previously (26). Pyrosequencing was done by a K-ras mutation detection kit (Qiagen) as suggested by the supplier.

Statistical analysis

All statistical analyses were carried out by SPSS 15.0 (SPSS Japan Inc.). To compare differences in methylation levels or other continuous values between groups, t tests or ANOVA with a post hoc Tukey test were carried out. Fisher’s exact test was used for analysis of categorical data. To evaluate correlations between continuous values, Pearson’s correlation coefficients were calculated. Receiver operating characteristic (ROC) curves for the diagnosis of invasive tumors were constructed on the basis of methylation levels, followed by area under the curve (AUC) calculation. A diagnostic tree to discriminate invasive tumors was constructed by using the training set based on the following objective algorithm. Step 1: classify the samples based on the most efficient cutoff of tumor size. Step 2: classify the samples based on the most efficient cutoffs of methylation levels in 4 sequences under the classification of the previous step. Step 3: repeat step 2 until no additional efficacy is observed. P < 0.05 (2-sided) were considered significant.

Results

Preparation of specimens

We first compared the 2 methods used to obtain tumor cells from colonoscopy wash fluid. When tumors were
found during colonoscopy, the tumor surfaces were washed with water (method 1) or saline (method 2; Supplementary Fig. S1A and B). Both methods enabled us to obtain enough DNA for molecular analysis (Table 1), but comparison of the cytology revealed that wash fluid obtained with saline (method 2) enabled more accurate detection of anaplastic cells (Supplementary Fig. S1B; Supplementary Table 1). We therefore used method 2 for subsequent analyses.

**DNA methylation in biopsy specimens**

The clinical features of the patients examined in this study are summarized in Table 2. There was no statistically significant difference in age or gender between patients with invasive and noninvasive tumors. We selected 4 genes for analysis, mir-34b/c, SFRP1, SFRP2, and DKK2, which are frequently methylated in colorectal cancer (25, 27, 28). Methylation analysis was carried out by using bisulfite pyrosequencing with DNA from 337 specimens, including 52 invasive tumors, 98 noninvasive tumors, and 187 specimens of normal colon tissue. We found that the methylation levels of the 4 genes were significantly higher in cancerous tissue than in normal colorectal mucosa (Fig. 1); however, we found no difference in tissue methylation levels between invasive and noninvasive tumors.

**DNA methylation in mucosal washing fluid**

We next examined gene methylation in 76 samples of mucosal wash fluid from 36 patients with invasive tumors and 34 with noninvasive tumors, and from 18 patients without colorectal lesions (Fig. 1). When we compared the invasive and noninvasive tumors, we found no differences for SFRP2 and DKK2. However, mir-34b/c and SFRP1 showed higher levels of methylation in wash fluid from patients with invasive than with noninvasive tumors.

We then used ROC analysis to further compare mucosal wash fluid from invasive and noninvasive tumors (Fig. 3A and B). The odds ratios (OR) for the risk of invasion associated with the methylation levels of the 4 genes tested are shown in Table 3. High levels of methylation were significantly associated with an increased risk of invasion. Among the 4 genes, levels of mir-34b/c methylation had the greatest impact on the risk of invasion and showed the largest AUC (0.796). Using various cutoff points of mir-34b/c methylation determined on the basis of efficiency considerations for clinical use, the sensitivity/specificity were 0.861/0.657 for the 13.0% (high sensitivity) cutoff point and 0.765/0.833 for the 17.8% (well balanced) cutoff. We also subdivided the tumors on the basis of whether they were $\geq25\text{ mm}$ or $<25\text{ mm}$ in size. In tumors 25 mm or larger, mir-34b/c showed the highest AUC (0.816); its sensitivity/specificity was 0.862/0.667 and its OR was 12.5. In tumors smaller than 25 mm, SFRP1 showed the highest AUC (0.810), with a sensitivity/specificity of 0.821/0.833 and an OR of 23.0.

We further verified the utility of DNA methylation in mucosal washing, using an independent set of specimens, which was established as the test set (Figs. 2, 3C and D; Table 4). All of these ROC analyses were considered the training set. In the test set, the methylation levels of mir-34b/c showed very high AUC again (0.915), sensitivity/specificity was 0.870/0.875 for the 13.0% cutoff, and 0.565/0.958 for the 17.8% cutoff. In tumors 25 mm or larger, the AUC of the methylation levels of mir-34b/c was 0.778. In tumors smaller than 25 mm, the AUC of the methylation levels of SFRP1 was 0.695.

To make a more efficient diagnostic method suitable for clinical situations, we then constructed a diagnostic tree to classify invasive and noninvasive tumors on the basis of the combination of methylation levels detected in wash fluid (Fig. 4A). First, because most endoscopists make an endoscopic diagnosis according to the size of lesions, we defined the most efficient cutoff of tumor size as the first node of the diagnostic tree. As the next nodes, we used the most efficient cutoffs of the methylation levels of the 4 genes (mir-34, SFRP1, SFRP2, and DKK2). For example, as shown in Figure 4A, if the tumor size is more than 25 mm and the methylation level of mir-34b/c is more than 15%, this lesion is diagnosed as an invasive tumor. In the training set, the sensitivity and specificity were 0.943 (33/35) and 0.882 (30/34), respectively. The total accuracy of the diagnosis was 91.3% (63/69). For use in clinical situations, we validated this diagnostic tree by using an independent test set (n = 47). The application of the diagnostic tree to the test set is shown in Figure 4B. Although a slight reduction in

---

**Table 1. Amounts of DNA and quality of cytology obtained by using the 2 collection methods tested**

<table>
<thead>
<tr>
<th>Biopsy (n = 11)</th>
<th>Wash fluid (method 1)</th>
<th>Biopsy (n = 37)</th>
<th>Wash fluid (method 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n = 11)</td>
<td>IT (n = 8)</td>
<td>NI (n = 3)</td>
<td>Total (n = 37)</td>
</tr>
<tr>
<td>DNA, µg</td>
<td>21.28 ± 15.25</td>
<td>15.00 ± 11.12</td>
<td>17.08 ± 13.00</td>
</tr>
<tr>
<td>Cytology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnosable</td>
<td>2</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Not diagnosable</td>
<td>9</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>18.2%</td>
<td>12.5%</td>
<td>33.3%</td>
</tr>
</tbody>
</table>

Abbreviations: IT: invasive tumors, NI: noninvasive tumors.
Table 2. Clinicopathologic features of the patients

<table>
<thead>
<tr>
<th></th>
<th>Biopsy sample</th>
<th>Washing fluid</th>
<th>Biopsy sample</th>
<th>Washing fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Training set</td>
<td></td>
<td>Test set</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IT (n = 52)</td>
<td>NI (n = 98)</td>
<td>IT (n = 36)</td>
<td>NI (n = 34)</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>67.4</td>
<td>66.7</td>
<td>0.720</td>
<td>67.2</td>
</tr>
<tr>
<td>Male</td>
<td>16 (30.8%)</td>
<td>24 (24.5%)</td>
<td>126 (67.4%)</td>
<td>22 (61.1%)</td>
</tr>
<tr>
<td>Female</td>
<td>36 (69.2%)</td>
<td>74 (75.5%)</td>
<td>0.408</td>
<td>61 (32.6%)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥25 mm</td>
<td>31 (59.6%)</td>
<td>12 (12.2%)</td>
<td>27 (75%)</td>
<td>5 (14.7%)</td>
</tr>
<tr>
<td>&lt;25 mm</td>
<td>21 (40.4%)</td>
<td>86 (87.8%)</td>
<td>&lt;0.001</td>
<td>9 (25%)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>21 (40.4%)</td>
<td>44 (44.9%)</td>
<td>12 (33.3%)</td>
<td>17 (50%)</td>
</tr>
<tr>
<td>Left</td>
<td>15 (28.8%)</td>
<td>25 (25.5%)</td>
<td>14 (38.9%)</td>
<td>7 (20.6%)</td>
</tr>
<tr>
<td>Rectum</td>
<td>16 (30.8%)</td>
<td>29 (29.6%)</td>
<td>0.853</td>
<td>10 (27.8%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyper/Inflammatory</td>
<td>15 (15.3%)</td>
<td>3 (8.8%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Tubular adenoma</td>
<td>29 (29.6%)</td>
<td>10 (29.4%)</td>
<td>9 (42.9%)</td>
<td>12 (60%)</td>
</tr>
<tr>
<td>Tubulovillous adenoma</td>
<td>28 (28.6%)</td>
<td>7 (20.6%)</td>
<td>7 (33.3%)</td>
<td>7 (29.2%)</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>26 (26.5%)</td>
<td>14 (41.2%)</td>
<td>5 (23.8%)</td>
<td>5 (20.8%)</td>
</tr>
<tr>
<td>Cancer</td>
<td>52 (100.0%)</td>
<td>36 (100.0%)</td>
<td>20 (100.0%)</td>
<td>23 (100.0%)</td>
</tr>
</tbody>
</table>

Abbreviations: IT: invasive tumors, NI: noninvasive tumors.
sensitivity was observed (0.740, 17/23), the specificity was very high (0.958, 21/24). The total accuracy of the diagnosis was 85.1% (40/47) for the test set.

We also assessed the correlation between methylation levels detected in biopsy specimens and in wash fluid (Supplementary Fig. S2). We found that overall methylation levels in biopsy tissues and washing fluid were well correlated. When we divided the data for invasive and noninvasive tumors, however, only invasive tumors showed a significant correlation between methylation levels in biopsy tissue and washing fluid.

**Detection of K-ras mutation by mucosal washing fluid**

Finally, we tested for mutation of K-ras codons 12 and 13 by using DNA obtained from biopsy tissue or wash fluid (Supplementary Table S2). With invasive tumors, mutations of K-ras were found in 9 of 27 (33.3%) biopsy specimens. Among the 9 positive tumors, we were able to also detect mutations in 7 (77.8%) wash fluid samples. With noninvasive tumors, mutations were detected in 6 of 24 (25%) biopsy specimens, but in only 2 (33.3%) of the corresponding wash fluid samples. Addition of K-ras mutation did not improve the accuracy of diagnosis of invasiveness by the diagnostic tree (data not shown). Consistent with this finding, nuclear staining showed more intact nuclei in wash fluid from invasive tumors than from noninvasive tumors (Supplementary Fig. S1C). Thus, samples from invasive tumors seem to contain higher concentrations of tumor-derived DNA than samples from noninvasive tumors.

**Discussion**

Small colorectal tumors are usually removed by endoscopic mucosal dissection, but if the tumor is invasive, surgical treatment is required because of the higher risk of lymph node metastasis. Consequently, precise preoperative diagnosis is critical for appropriate treatment of colorectal tumors. Magnifying colonoscopy is a useful means of distinguishing invasive from noninvasive tumors (9, 10).
However, invasive colorectal tumors show a heterogeneous pit pattern, making it difficult to determine a therapeutic strategy based on pit pattern diagnosis alone (29). Notably, surface mucus is washed away during magnifying endoscopic analysis, so that utilization of the wash fluid could be an effective noninvasive approach to diagnosis. It has been recommended that nearly all colorectal cancer patients who receive EMR receive periodic endoscopy for early detection of relapses (30). Examination of the methylation levels in the wash fluid could provide helpful information as to how often the patient should receive the follow-up endoscopy (e.g., the lower the methylation level, the less frequently endoscopic examination may be needed). In addition, although we did not include follow-up in our study, it is possible that wash fluid analysis could help physicians detect mucosal relapse after EMR during follow-up endoscopy.

It has been reported that DNA methylation in wash fluid containing pancreatic juice, saliva, or gastric juice is useful for diagnosis and risk assessment in cancer (34–36). For example, Watanabe and colleagues reported that DNA methylation in gastric wash fluid is useful for detection of early gastric cancer (36). The unique feature of our study is that it suggests DNA methylation in colon mucosal wash fluid can be used to predict the invasiveness of tumors. Further study will be necessary to determine whether DNA methylation of colon mucosal wash might also be useful for screening or risk assessment in cancer.

Here we showed that levels of mir-34b/c gene methylation were predictive of the invasiveness of colorectal tumors (Figs. 3 and 4; Tables 3 and 4). The sensitivity (0.833) and specificity (0.765) of this approach (well balanced cutoff), as well as the ROC AUC value (0.796), suggest methylation of this gene in colonoscopic wash fluid is a good molecular marker that distinguishes invasive from noninvasive colorectal tumors. We also showed that a diagnostic tree constructed by the combination of methylation levels was highly accurate for predicting invasiveness. To avoid unneeded surgery, it is important that the prediction of invasiveness is highly specific. In this regard, the specificities of the diagnostic tree were 0.882 in the training set, and 0.958 in the test set.

There is currently no molecular test that distinguishes invasive from noninvasive colorectal tumors. DNA methylation can be used as a biomarker for detection of colorectal lesions (16–20), but genes frequently methylated in cancer are also frequently methylated in early lesions (e.g., adenomas), and even in normal colorectal mucosa from aged patients (21, 22). It is therefore difficult to distinguish invasive tumors from noninvasive ones. We previously showed that SFRP1 and SFRP2 are frequently methylated in colorectal cancer (28). However, they are also often methylated in normal colorectal mucosa in an age-related manner (34), which is consistent with our present findings. The mir-34b/c gene is a putative tumor suppressor whose expression is induced by p53 (35). We previously showed that mir-34b/c is silenced by DNA methylation in colorectal cancers and adenomas (25). In this study, we found that methylation of mir-34b/c in noninvasive tumors is as high as that in invasive tumors. By contrast, levels of mir-34b/c methylation in normal colorectal mucosa are low. Thus, given the high frequency of methylation in tumors, tumor-specific methylation of mir-34b/c may be a highly useful molecular marker for colorectal cancer.

The molecular mechanism underlying the high levels of DNA methylation in wash fluid from invasive tumors is not fully understood. Analysis of nuclear staining, DNA methylation, and K-ras mutation suggest that wash fluid–derived DNA from invasive tumors contains higher concentrations of tumor-derived DNA than wash fluid from the less frequently endoscopic examination may be needed). In addition, although we did not include follow-up in our study, it is possible that wash fluid analysis could help physicians detect mucosal relapse after EMR during follow-up endoscopy.

It has been reported that DNA methylation in wash fluid containing pancreatic juice, saliva, or gastric juice is useful for diagnosis and risk assessment in cancer (34–36). For example, Watanabe and colleagues reported that DNA methylation in gastric wash fluid is useful for detection of early gastric cancer (36). The unique feature of our study is that it suggests DNA methylation in colon mucosal wash fluid can be used to predict the invasiveness of tumors. Further study will be necessary to determine whether DNA methylation of colon mucosal wash might also be useful for screening or risk assessment in cancer.

Here we showed that levels of mir-34b/c gene methylation were predictive of the invasiveness of colorectal tumors (Figs. 3 and 4; Tables 3 and 4). The sensitivity (0.833) and specificity (0.765) of this approach (well balanced cutoff), as well as the ROC AUC value (0.796), suggest methylation of this gene in colonoscopic wash fluid is a good molecular marker that distinguishes invasive from noninvasive colorectal tumors. We also showed that a diagnostic tree constructed by the combination of methylation levels was highly accurate for predicting invasiveness. To avoid unneeded surgery, it is important that the prediction of invasiveness is highly specific. In this regard, the specificities of the diagnostic tree were 0.882 in the training set, and 0.958 in the test set.

There is currently no molecular test that distinguishes invasive from noninvasive colorectal tumors. DNA methylation can be used as a biomarker for detection of colorectal lesions (16–20), but genes frequently methylated in cancer are also frequently methylated in early lesions (e.g., adenomas), and even in normal colorectal mucosa from aged patients (21, 22). It is therefore difficult to distinguish invasive tumors from noninvasive ones. We previously showed that SFRP1 and SFRP2 are frequently methylated in colorectal cancer (28). However, they are also often methylated in normal colorectal mucosa in an age-related manner (34), which is consistent with our present findings. The mir-34b/c gene is a putative tumor suppressor whose expression is induced by p53 (35). We previously showed that mir-34b/c is silenced by DNA methylation in colorectal cancers and adenomas (25). In this study, we found that methylation of mir-34b/c in noninvasive tumors is as high as that in invasive tumors. By contrast, levels of mir-34b/c methylation in normal colorectal mucosa are low. Thus, given the high frequency of methylation in tumors, tumor-specific methylation of mir-34b/c may be a highly useful molecular marker for colorectal cancer.

The molecular mechanism underlying the high levels of DNA methylation in wash fluid from invasive tumors is not fully understood. Analysis of nuclear staining, DNA methylation, and K-ras mutation suggest that wash fluid–derived DNA from invasive tumors contains higher concentrations of tumor-derived DNA than wash fluid from...
Figure 3. ROC curve analysis. ROC curves were constructed by plotting sensitivity vs. 1-specificity. Curves are shown comparing invasive vs. noninvasive tumors. AUCs are also shown in the graphs. A and B, ROC curve analysis for the training set. Overall analysis is shown in A, and stratified analysis by tumor size (≥25 mm or <25 mm) is shown in B. C and D, the same analysis for the test set. Overall analysis is shown in C, and that stratified by tumor size (≥25 mm or <25 mm) is shown in D.

Table 4. Results of ROC analyses of the methylation levels in 4 genes in the test set.

<table>
<thead>
<tr>
<th>Test set</th>
<th>Tumor size</th>
<th>Genes</th>
<th>AUC Estimate (95% CI)</th>
<th>Cutoff (%)</th>
<th>Sensitivity Estimate (95% CI)</th>
<th>Specificity Estimate (95% CI)</th>
<th>ORs Estimate (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>miR34b/c</td>
<td>0.915 (0.839–0.997)</td>
<td>13.0</td>
<td>0.870 (0.664–0.972)</td>
<td>0.875 (0.676–0.973)</td>
<td>46.7 (8.4–258.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.8</td>
<td>0.565 (0.345–0.768)</td>
<td>0.958 (0.789–0.999)</td>
<td>29.9 (3.4–260.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21.0</td>
<td>0.348 (0.164–0.573)</td>
<td>1.000 (0.858–1.000)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;25 mm</td>
<td>SFRP1</td>
<td>0.752 (0.615–0.889)</td>
<td>45.0</td>
<td>0.348 (0.164–0.573)</td>
<td>0.875 (0.676–0.973)</td>
<td>3.7 (0.8–16.4)</td>
</tr>
<tr>
<td></td>
<td>≥25 mm</td>
<td>miR34b/c</td>
<td>0.778 (0.600–1.000)</td>
<td>15.0</td>
<td>0.667 (0.410–0.867)</td>
<td>0.667 (0.094–0.991)</td>
<td>4.0 (0.3–53.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SFRP1</td>
<td>0.695 (0.450–0.941)</td>
<td>51.0</td>
<td>0.200 (0.005–0.716)</td>
<td>1.000 (0.839–1.000)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
noninvasive tumors. It is generally accepted that colonic epithelial cells are exfoliated into the lumen, that cancer cells can be detected among stool-derived exfoliated cells, and that stool DNA/RNA derived from exfoliated cells may be useful for diagnosis (36, 37). In that context, there are several possible explanations for the higher concentration of DNA from invasive tumor cells in colonoscopy wash fluid. Resistance to apoptosis and loss of cell adhesion are characteristic features of invasive cells (38, 39), which may facilitate the survival of exfoliated cells allowing for good DNA quality. Although we did not detect high levels of methylation in wash fluid from noninvasive tumors, we did obtain relatively large amounts of DNA. The origin of the DNA remains to be determined, but it may be derived from both tumor cells and normal cells such as white blood cells.

Our findings suggest that the high levels of \( \text{mir-34b/c} \) methylation in invasive tumors could be applied to predict invasiveness by using stool DNA. To date, most diagnostic methods for detecting colorectal tumors based on DNA methylation utilize qualitative methylation analysis (16, 19, 20). Using sensitive and quantitative analysis such as BEAMing technology (18), it should be possible to predict the invasiveness of tumors by using stool DNA. Further study to optimize the threshold will be necessary, however.

In summary, high levels of DNA methylation in colorectal washing fluid were correlated with invasiveness of colorectal lesions. Combining endoscopic and DNA

Figure 4. A diagnostic tree to classify invasive and noninvasive tumors on the basis of methylation levels detected in wash fluid. A, a diagnostic tree constructed on the basis of the training set. The majority class in each leaf is the predictive class. B, the application of the diagnostic tree to the test set.
methylation analyses may facilitate accurate preoperative staging of colorectal cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
The authors thank Dr. William F. Goldman for editing the manuscript.

References

Grant Support
This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (T. Tokino, K. Imai, and M. Toyota), Grants-in-Aid for Scientific Research (S) from the Japan Society for Promotion of Science (K. Imai), a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control (M. Toyota), and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare, Japan (M. Toyota). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 25, 2010; revised January 19, 2011; accepted January 27, 2011; published online May 4, 2011.


Epigenetic Alteration of DNA in Mucosal Wash Fluid Predicts Invasiveness of Colorectal Tumors

Seiko Kamimae, Eiichiro Yamamoto, Hiro-o Yamano, et al.