Genetic and epigenetic profiling in early colorectal tumors and prediction of invasive potential in pT1 (early invasive) colorectal cancers

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Introduction

Morphologically, early colorectal tumors are divided into two groups, protruded-type tumors and flat-type tumors. Although some studies have shown genetic alterations in protruded-type tumors, little is known about genetic and epigenetic alterations in flat-type tumors, as well as pT1 (early invasive) colorectal cancers (CRCs). In the current study, we compared the frequencies of genetic and epigenetic alterations of the RAS–RAF and Wnt signaling pathways in flat-type and protruded-type tumors. In addition, we investigated the relationship between those alterations and invasive potential of pT1 CRCs. Methylation of RASSF2, O-6-methylguanine-DNA methyltransferase (MGMT), Wnt inhibitory factor-1 (WIF-1), EPHB2, CDKN2A and MLH1 were detected in 44.3, 30.3, 81.4, 7.5, 43.6 and 13.4% of the 307 early colorectal tumors, respectively. Mutations of KRAS, BRAF, catalytic subunit alpha of phosphatidylinositol 3'-kinase (PIK3CA) and beta-catenin were detected in 25.4, 4.6, 1.6 and 9.4% of those tumors, respectively. Methylation of MGMT, WIF-1 and CDKN2A were detected in significantly higher percentages of protruded-type tumors than in flat-type tumors. Mutation of at least one gene was detected in a significantly higher percentage of flat-type tumors than in protruded-type tumors. RASSF2 methylation was correlated significantly with KRAS, BRAF or PIK3CA mutation. Multiple logistic analysis showed that lymphatic invasion and RASSF2 methylation with KRAS, BRAF or PIK3CA mutation were independent risk factors for venous invasion in pT1 CRCs. In conclusion, since genetic alterations of these pathways have frequently occurred in flat-type tumors, flat-type tumors seem to have a distinct genetic profile different from that of protruded-type tumors. RASSF2 methylation with oncogenic activation is a promising biomarker for predicting invasive potential of pT1 CRCs.

Abbreviations: CRC, colorectal cancer; MGMT, O-6-methylguanine-DNA methyltransferase; MSI-H, high-frequency microsatellite instability; MSI, microsatellite instability; MSP, methylation-specific polymerase chain reaction; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3'-kinase; PIK3CA, catalytic subunit alpha of phosphatidylinositol 3'-kinase; WIF-1, Wnt inhibitory factor-1.
These tumor samples consisted of 27 hyperplastic polyps, 5 serrated adenomas, 71 adenomas with low-grade dysplasia, 31 adenomas with high-grade dysplasia, 70 intra-mucosal carcinomas and carcinoma in situ and 103 pT1 (early invasive carcinomas) CRCs (pT1 in the TNM classification of the Union International Against Cancer). Tumor samples were carefully microdissected by expert pathologists. In case of pT1 CRCs, tumor samples were taken from the microscopically visible deepest invading part of the tumor. All samples of pT1 CRCs were obtained by surgical treatment to analyze the presence or absence of lymph node metastasis. No patients fulfilled the criteria for hereditary CRC.

Locations of the tumors were divided into proximal colon (cecum, ascending and transverse colon) and distal colon (descending and sigmoid colon and rectum). Macroscopic types were divided into protruded type (height of tumor ≥2.5 mm) and flat type (height of tumor <2.5 mm) according to the Paris classification (1). It was difficult to divide pT1 CRCs into protruded-type or flat-type tumors because colorectal tumors become thick when they have invaded the submucosal layer. Therefore, macroscopic type was classified only in early colorectal tumors other than pT1 CRCs. Informed consent was obtained from each subject and the institutional review committee approved this study.

Detection of KRAS, BRAF, PIK3CA and β-catenin exon 3 mutations
KRAS mutations at codon 12 and codon 13, BRAF mutations at codon 600 and PIK3CA mutations in exon 9 and exon 20 were analyzed by direct DNA sequencing as described previously (8,11,12). Exon 3 of β-catenin was amplified by PCR using the following primer pair: forward, 5′-GAACCAGACA-GAAAGCGGCTG-3′ and reverse, 5′-ACTCATACGGACTTGGAGG-3′. Products were purified and then sequenced in both directions using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The sequence reactions were run and analyzed on an ABI 3100 Genetic Analyzer (Applied Biosystems).

Quantitative real-time PCR to measure DNA methylation (MethyLight)
Sodium bisulfite treatment of genomic DNA and MethyLight were performed as described previously (25–28). We used an ABI 7000 (Applied Biosystems) for quantitative real-time PCR (Figure 1A). Using seven sets of primers and probes, we amplified promoters of six genes of interest (RASSF2, MGMT, WIP-1, EPHB2, CDKN2A and MLH1) and β-actin (ACTB) to normalize for the amount of input bisulfite-converted DNA. These primers and probes were designed specifically for bisulfite-converted DNA. The sequences of primers and probes and PCR conditions are available upon request.

Immunohistochemistry of MGMT
Immunohistochemistry with an anti-human MGMT mouse monoclonal antibody (MAB16200, 1:100 dilution; Chemicon International) was done as described previously (29). The sections were examined microscopically by two
well-trained pathologists who were blinded to the clinicopathological characteristics. Normal-appearing epithelium and stromal cells in each section provided positive internal controls for binding of the primary antibody. MGMT expression in nuclei was scored as present or absent (14).

Microsatellite instability analysis
Early colorectal tumors were analyzed for microsatellite instability (MSI) by using five microsatellite markers (BAT-25, BAT-26, D2S123, D5S346 and D17S250) as described previously (30).

Statistical analysis
Alteration of each target gene was assessed for associations with clinicopathological characteristics (age, size, gender, location and invasive potential) and the genetic and epigenetic alterations for the unmatched data were examined using unconditional logistic regression analysis.

Results
Methylation of RASSF2, MGMT, WIF-1, EPHB2, CDKN2A and MLH1
Methylations of RASSF2, MGMT, WIF-1, EPHB2, CDKN2A and MLH1 were detected in 136 (44.3%), 93 (30.3%), 250 (81.4%), 23 (7.5%), 134 (43.6%) and 41 (13.4%) of the 307 early colorectal tumors, respectively. Methylations of RASSF2 and WIF-1 were correlated significantly with tumors of larger size (P = 0.0055) and pT1 CRCs (P = 0.0297). Regarding BRAF mutations, all the tumors positive for mutations demonstrated missense mutations at codon 600. All the PIK3CA mutations occurred in exon 9 (codon 545) or exon 20 (codons 1047 and 1049). Neither BRAF nor PIK3CA mutation was correlated significantly with any clinicopathological characteristics. There was a mutually exclusive relationship between KRAS, BRAF and PIK3CA mutations. In other words, no KRAS mutation was detected in tumors with BRAF or PIK3CA mutation, and vice versa. Exon 3 β-catenin mutations were single-base substitutions that were located within the critical serine/threonine codons for glycogen synthase kinase-3β phosphorylation of β-catenin (codons 29–48), β-catenin mutation was correlated significantly with tumors of larger size (P = 0.0034).

Relationships between morphology and genetic and epigenetic alterations
Methylations of MGMT, WIF-1 and CDKN2A were detected in significantly higher percentages of protruded-type tumors (37.0, 91.8 and 60.3% of 73 tumors) than in flat-type tumors (23.7, 76.3 and 42.0% of 131 tumors; P = 0.0432, 0.0061 and 0.0122, respectively) (Table I). KRAS mutation was detected in a significantly higher percentage of flat-type tumors (26.7% of 131 tumors) than in protruded-type tumors (12.3% of 73 tumors; P = 0.0166) (Table II). KRAS mutation in G-to-A transitions was detected in a significantly higher percentage of flat-type tumors (20.6%) than in protruded-type tumors (8.2%; P = 0.0212). Mutation of at least one gene was detected in a significantly higher percentage of flat-type tumors (40.5% of 131 tumors) than in protruded-type tumors (19.2% of 73 tumors; P = 0.0019).

Table I. Epigenetic alterations and clinicopathological characteristics in early colorectal tumors

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RASSF2 methylation</th>
<th>MGMT methylation</th>
<th>WIF-1 methylation</th>
<th>EPHB2 methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years ± SD)</td>
<td>65.01 ± 10.7</td>
<td>63.9 ± 10.5</td>
<td>63.9 ± 10.9</td>
<td>64.6 ± 10.5</td>
</tr>
<tr>
<td>Mean size (mm ± SD)</td>
<td>19.3 ± 13.0</td>
<td>19.0 ± 15.1</td>
<td>20.0 ± 15.5</td>
<td>15.4 ± 8.1</td>
</tr>
<tr>
<td>Methylations</td>
<td>Positive: 136 (44.3%)</td>
<td>Negative: 171 (55.7%)</td>
<td>Positive: 93 (30.3%)</td>
<td>Negative: 214 (69.7%)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male: 94 (46.6%)</td>
<td>Female: 42 (39.6%)</td>
<td>Male: 60 (33.1%)</td>
<td>Female: 33 (40.2%)</td>
</tr>
<tr>
<td>Location</td>
<td>Proximal: 68 (46.3%)</td>
<td>Distal: 48 (42.0%)</td>
<td>Proximal: 30 (20.7%)</td>
<td>Distal: 63 (38.9%)</td>
</tr>
<tr>
<td>Histopathology</td>
<td>Non-pT1 cancer: 89 (43.6%)</td>
<td>pT1 cancer: 47 (45.6%)</td>
<td>Non-pT1 cancer: 38 (28.4%)</td>
<td>pT1 cancer: 54 (38.0%)</td>
</tr>
</tbody>
</table>

*Non-pT1 cancer was defined as a hyperplastic polyp, serrated adenoma, adenoma, intramucosal carcinomas and carcinoma in situ.
Table II. Genetic alterations and clinicopathological characteristics in early colorectal tumors

<table>
<thead>
<tr>
<th></th>
<th>KRAS mutation</th>
<th>BRAF mutation</th>
<th>PIK3CA mutation</th>
<th>β-catenin mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>P</td>
<td>Positive</td>
</tr>
<tr>
<td>Age (years ± SD)</td>
<td>(n = 78)</td>
<td>(n = 229)</td>
<td>0.00252</td>
<td>(n = 14)</td>
</tr>
<tr>
<td>Mean size (mm ± SD)</td>
<td>66.7 ± 10.7</td>
<td>63.6 ± 10.5</td>
<td>&lt;0.0001</td>
<td>64.4 ± 12.5</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>41 (20.4%)</td>
<td>160</td>
<td>0.0055</td>
<td>7 (3.5%)</td>
</tr>
<tr>
<td>Female</td>
<td>37 (34.9%)</td>
<td>69</td>
<td></td>
<td>7 (6.6%)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>41 (28.3%)</td>
<td>104</td>
<td>0.2747</td>
<td>7 (4.8%)</td>
</tr>
<tr>
<td>Distant</td>
<td>37 (22.8%)</td>
<td>125</td>
<td></td>
<td>7 (4.3%)</td>
</tr>
<tr>
<td>Histopathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-pT1 cancer*</td>
<td>44 (21.6%)</td>
<td>160</td>
<td>0.0297</td>
<td>12 (5.9%)</td>
</tr>
<tr>
<td>pT1 cancer</td>
<td>34 (33.0%)</td>
<td>69</td>
<td></td>
<td>2 (1.9%)</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td>35 (26.7%)</td>
<td>96</td>
<td>0.0166</td>
<td>10 (7.6%)</td>
</tr>
<tr>
<td>Proltered</td>
<td>9 (12.3%)</td>
<td>64</td>
<td></td>
<td>2 (2.7%)</td>
</tr>
</tbody>
</table>

*N=28 cancer was defined as a hyperplastic polyp, serrated adenoma, adenoma, intramucosal carcinomas and carcinoma in situ.

Discussion

In the current study, we compared the frequencies of genetic and epigenetic alterations of the RAS–RAF and Wnt signaling pathways in flat-type and protruded-type colorectal tumors. In addition, we investigated the relationship between those alterations and invasive potential of pT1 CRCs. As a result, we have shown that genetic alterations frequently occur in flat-type colorectal tumors and that RASSF2 methylation with oncogenic activation is correlated significantly with invasive potential.

We analyzed methylations of RASSF2, MGMT, WIF-1, EPHB2, CDKN2A and MLH1 by using MethyLight. A methylation-specific polymerase chain reaction (MSP) has been used in most previous studies. However, since MSP is not able to distinguish low levels of methylation from high levels of methylation, the frequency of DNA hypermethylation might be overestimated. Therefore, quantitative measurement is needed to clarify the frequency of DNA hypermethylation.

In a previous study using combined bisulfite restriction analysis, RASSF2 methylation was detected in 21 (43%) of 49 colorectal adenomas and in 51 (42%) of 122 advanced CRCs (16). The frequency of RASSF2 methylation in the present study was similar to those in that report. In addition, RASSF2 methylation was correlated significantly with KRAS, BRAF or PIK3CA mutation.

It has been reported that down-regulation of WIF-1 mRNA expression was observed in 32 (72.7%) of 44 colorectal adenomas and 18 (78.2%) of 23 pT1 CRCs (21). The methylation status determined by using MSP was significantly correlated with down-regulation of WIF-1 expression. The frequency of WIF-1 expression in early colorectal tumors in the present study was similar to the frequencies reported previously. It has been reported that EPHB2 methylation was observed in 54 (53.5%) of 101 advanced CRCs by using MSP (24). Battie et al. (31) showed that loss of EPHB2 expression strongly correlates with late stage of CRCs. Therefore, the low frequency of EPHB2 methylation in the present study might be due to the fact that our samples were early colorectal tumors and/or the fact that different methods were used for analyzing EPHB2 methylation.

Ogino et al. (25) showed by using MethyLight that methylations of MGMT, CDKN2A and MLH1 occurred in 41, 32 and 15% of advanced CRCs, respectively. Although the frequency of CDKN2A methylation in pT1 CRCs in the present study was similar to that found in the previous study, the frequencies of MGMT and MLH1 were significantly lower.
methylation in the present study were lower than those reported previously. This discrepancy might be due to the fact that our samples were early colorectal tumors and/or the fact that different primers were used for analyzing methylation.

Since MSI-H due to defective DNA mismatch repair occurs in 10–15% of sporadic CRCs, we analyzed 307 early colorectal tumor tissues for MSI. However, no tumor tissue was classified as MSI-H. Previous studies have demonstrated that sporadic colorectal adenomas and early invasive cancers that show MSI-H are very rare (4,32). Thus, our results might be due to the fact that all tumor samples were early colorectal tumors.

The percentage of KRAS mutation in G-to-A transitions was significantly lower in protruded-type tumors than in flat-type tumors and pT1 CRCs. Maltzman et al. (33) reported that KRAS mutation in G-to-A transitions was detected in 58 (7.9%) of 738 colorectal adenomas.
This result is consistent with our data on mutational patterns in pro-
truded-type tumors. Therefore, most of the adenomas analyzed in that
previous study might be protruded-type tumors. On the other hand, it
was reported that the mutational patterns of KRAS in 51 (21.8%) of
234 sporadic advanced CRCs were transitions from G-to-A at codon 12
and codon 13 (12). This is consistent with our data on mutational pat-
tterns in flat-type tumors and pT1 CRCs. Therefore, CRCs with KRAS
mutation in G-to-A transitions could largely arise from early flat-type
colorectal tumors. Further analysis is needed to clarify this issue.

Although methylations of MGMT, WIF-1 and CDKN2A were de-
tected in a significantly higher percentage of protruded-type tumors
than in flat-type tumors, mutation of at least one gene was detected in
a significantly higher percentage of flat-type tumors than in protruded-
type tumors. Therefore, genetic alterations of the RAS–RAF and Wnt
signaling pathways might frequently occur in flat-type tumors. Since
early detection of flat-type tumors is sometimes difficult compared with
early detection of protruded-type tumors, analysis of these alter-
ations seems to be applicable to a new diagnostic strategy, such as
fecal DNA examination for flat-type tumors.

To date, therapy for pT1 CRCs has varied from endoscopic to de-
finitive surgery. The overall frequency of lymph node metastasis in pT1
CRCs may be as low as 10% (34,35), and endoscopic local resection is
an effective therapy for most pT1 CRCs without lymph node meta-
stasy. Therefore, it is very important to develop a method to preopera-
tively predict the metastatic potential of pT1 CRCs. Kurokawa et al.
(35) showed by using multiple logistic analysis that tumor matrrixlin
expression and venous invasion were independent risk factors for
lymph node metastasis in pT1 CRCs. In addition, lymphatic invasion
and tumor matrinxlin expression were shown to be stage-independent
risk factors for lymph node metastasis. Ueno et al. (36) showed that
the absence of an unfavorable tumor grade, vascular invasion and tumor
budding would be the most informative combination of criteria for
selecting patients with a low risk of recurrence of pT1 CRCs.

On the other hand, Ahnen et al. (37) showed that KRAS mutations
could have a higher rate of subclinical lymphatic involvement and are
associated with a poor prognosis in stage II (pt3-T4 N0 M0) CRCs.
Thus, not only lymph node metastasis but also lymphatic and venous
invasions are thought to be significant risk factors in the early stage of
CRCs. In the present study, KRAS mutation and methylations of
MGMT and WIF-1 were found to be correlated significantly with
invasive potential. In addition, RASSF2 methylation with KRAS, BRF,
or PIK3CA mutation was found to be correlated more strongly with
invasive potential.

MGMT methylation was correlated reversely with venous invasion.
Although we found a significant correlation between KRAS mutation
in G-to-A transitions and reduced MGMT protein expression, reduced
MGMT protein expression was not correlated significantly with in-
vasive potential. Kohonen-Corish et al. (38) have shown that MGMT
defect is unlikely to contribute to the poor prognosis of low-level MSI
CRCs. Therefore, our results are consistent with this previous report.

Multiple logistic analysis showed that lymphatic invasion and
RASSF2 methylation with KRAS, BRAF or PIK3CA mutation were
independent risk factors for venous invasion in pT1 CRCs. These
results suggest that accumulation of genetic and epigenetic alterations
in the RAS–RAF signaling pathway might play an important role in the
aggressive biological behaviors of pT1 CRCs.

In summary, combined analysis of genetic and epigenetic alterations
is needed to clarify the mechanisms of development and pro-
gression of early colorectal tumors. Since genetic alterations of the
RAS–RAF and Wnt signaling pathways have frequently occurred in flat-
type tumors, flat-type tumors seem to have a distinct genetic pro-
file different from that of protruded-type tumors. In addition, RASSF2
methylation with oncogenic activation is a promising biomarker for
predicting invasive potential of pT1 CRCs.

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Conflicts of Interest Statement: None declared.

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