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Flat-type colorectal advanced adenomas (laterally spreading tumors) have different genetic and epigenetic alterations from protruded-type advanced adenomas

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Morphologically, colorectal adenomas can be divided into two groups, protruded-type and flat-type. However, the accurate frequencies of genetic and epigenetic alterations in flat-type colorectal advanced adenomas (laterally spreading tumors) have remained largely unknown. In the current study, we investigated genetic and epigenetic alterations in 101 flat-type colorectal advanced adenomas and 68 protruded-type colorectal advanced adenomas by using direct DNA sequencing and quantitative real-time PCR (MethyLight), respectively. *KRAS* mutation was detected in a significantly higher percentage of flat-type adenomas (35%) than in protruded-type adenomas (13%). When the samples were limited to the tumors in the distal colon, the difference of *KRAS* mutation was still significant. *KRAS* mutation in G-to-A transitions at codons 12 and 13 was detected in a significantly higher percentage of flat-type adenomas (26%) than in protruded-type adenomas (9%). *BRAF* and *β-catenin* mutations were detected in 3 and 8% of the 101 flat-type adenomas, respectively. No significant difference was found between frequencies of those mutations in flat-type adenomas and protruded-type adenomas. Methylations of *MGMT*, *CDKN2A* (*p16*) and *MLH1* were detected in 28, 33 and 9% of the 101 flat-type adenomas, respectively. *CDKN2A* methylation was detected in a significantly lower percentage of flat-type adenomas than in protruded-type adenomas (63%). Methylation of at least one gene was detected in a significantly lower percentage of flat-type adenomas (54%) than in protruded-type adenomas (78%). In conclusion, *KRAS* mutation was frequently detected in flat-type advanced adenomas and the mutational patterns in most of them with *KRAS* mutations were a transition from G-to-A. Therefore, these genetic alterations seem to play an important role in the development of flat-type advanced adenomas, especially in the distal colon. Epigenetic alterations infrequently occurred in flat-type advanced adenomas, suggesting that they have different genetic and epigenetic alterations from those of protruded-type advanced adenomas.
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Morphologically, colorectal adenomas can be divided into two groups, protruded-type and flat-type. As early detection of flat-type adenomas is difficult compared with protruded-type adenomas, they are sometimes overlooked and tend to be found at a late stage.[1] Flat-type colorectal adenomas may be endoscopically defined by a height of less than half of their diameter or histologically defined by thickness of the lesion of less than twice that of the adjacent normal colonic mucosa.[2] The Paris classification compares the height of the lesion to that of the height of closed cups of a biopsy forceps (2.5 mm).[3,4] Lesions protruding above the level of the closed jaws of the biopsy forceps are classified as protruded-type, whereas those that protrude below this level are classified as flat-type.

Large flat-type colorectal tumors are often labeled as 'carpet lesions' in the United States and 'laterally spreading tumors' in Japan.[5–10] Laterally spreading

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tumors are superficial neoplasms that spread laterally over the mucosa, and these have recently received significant attention from gastroenterologists. However, the accurate frequencies of these genetic and epigenetic alterations in flat-type colorectal tumors have remained largely unknouwn.

Recently, we reported a case of laterally spreading tumor with interstitial deletion, including *β-catenin* exon 3.[11] The β-catenin protein has two major functions. First, it acts as a cell–cell adhesion regulatory protein that binds cadherin.[12] Second, it is thought to act as a downstream transcriptional activator in the Wnt signaling pathway. However, the frequency of *β-catenin* mutations in flat-type colorectal tumors has not been reported.

On the other hand, the RAS-RAF signaling pathway mediates cellular responses to growth signals not only in colorectal cancers but also in colorectal adenomas. *KRAS* mutation has been shown to be associated with epigenetic silencing of *O6-methylguanine-DNA methyltransferase* (*MGMT*), which is known to encode a DNA repair protein that removes potentially carcinogenic and cytotoxic alkyl adducts from the O6 position of guanine.[13–17] Alterations in the *MGMT* gene impair the ability of the MGMT protein to remove alkyl groups from the O6 position of guanine. Therefore, alterations in the *MGMT* gene are thought to increase the mutational rate and the risk of cancer.[13,16] Epigenetic silencing of *MGMT* has been shown to be associated with the appearance of G-to-A transitions in *KRAS* mutations during colorectal tumorigenesis.[15,16] On the other hand, *BRAF* mutations have also been reported in hyperplastic polyps and serrated adenomas.[18] Subsequently, *BRAF* mutations have been shown to be associated with the epigenetic silencing of *MLH1* but not with germline mutation of mismatch repair genes.[19] Regarding epigenetic alterations in flat-type colorectal tumors, Sakamoto *et al*[20] reported that *RASSF1A* promoter hypermethylation was detected in 81% of such tumors.

In the current study, we investigated genetic and epigenetic alterations in 101 flat-type colorectal advanced adenomas, 68 protruded-type colorectal advanced adenomas, and 35 colorectal cancers by using direct DNA sequencing and quantitative real-time PCR to measure DNA methylation (MethyLight), respectively. To our knowledge, this is the first report presenting results of genetic and epigenetic analysis of over 100 flat-type colorectal advanced adenoma tissues.

Materials and methods

Patients and Tissue Samples

Formalin-fixed paraffin-embedded tissues of 169 colorectal adenomas and 35 colorectal cancers were obtained from patients who had undergone polypectomy or surgical treatment. All of the adenoma samples were limited to advanced adenomas to match the histopathology of flat-type and protruded-type adenomas. Advanced adenoma was defined as an adenoma of 10 mm or more in diameter, an adenoma with high-grade dysplasia. Intramucosal carcinoma and carcinoma *in situ* were classified as adenoma with high-grade dysplasia.[21,22] The subjects were classified according to the most advanced lesion identified. These advanced adenoma samples consisted of four serrated adenomas, 49 adenomas with low-grade dysplasia, 29 adenomas with high-grade dysplasia, and 87 intramucosal carcinomas and carcinoma *in situ*. None of the patients reported a family history of colorectal cancers in interviews.

Table 1 Clinicopathological characteristics of colorectal advanced adenomas

Characteristics	*Morphology*	
	Flat-type (n = *101*)	*Protruded-type* (n = *68*)
Age (years ± s.d.)*	65.6 ± 9.5	62.1 ± 9.4
Mean size (mm ± s.d.)	22.8 ± 15.0	20.5 ± 12.0
*Gender***		
Male	59	52
Female	42	16
*Location****		
Proximal	67	31
Distal	34	37
Histopathology		
Serrated adenoma	3	1
Adenoma with low-grade dysplasia	32	17
Adenoma with high-grade dysplasia	13	16
Intramucosal carcinoma and carcinoma *in situ*	53	34

*Flat *vs* protruded, $P = 0.0193$; **flat *vs* protruded, $P = 0.0153$; ***flat *vs* protruded, $P = 0.0074$.

Locations of the colorectal 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). The clinicopathological characteristics of 101 flat-type adenomas and 68 protruded-type adenomas are shown in Table 1. Informed consent was obtained from each subject and the institutional review committee approved this study.

Detection of *KRAS* Codon 12 and Codon 13, *BRAF* Codon 600 and *β-catenin* Exon 3 Mutations

Mutations of *KRAS* (codon 12 and codon 13), *BRAF* (codon 600) and *β-catenin* exon 3 were detected by direct DNA sequencing. *KRAS* was amplified by

PCR using primer pair: forward, 5′-AAAATGACTGA ATATAAACTTGTGG-3′ and reverse, 5′-CTCTATTG TTGGATCATATTCGTC-3′. *BRAF* was amplified by PCR using primer pair: forward, 5′-CTTCATGAAGA CCTCACAGT-3′ and reverse, 5′-CATCCACAAAATG GATCCAG-3′. *β-catenin* exon 3 was amplified by PCR using primer pair: forward, 5′-GAACCAGACAG AAAAGCGGCTG-3′ and reverse, 5′-ACTCATACAG GACTTGGGAGG-3′. Products were purified and then sequenced in both directions using Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). 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 conversion of genomic DNA was performed as described previously.[23,24] Quantitative real-time PCR to measure DNA methylation (MethyLight) was performed as previously.[23] We used an ABI 7000 (Applied Biosystems, Foster City, CA, USA) for quantitative real-time PCR (Figure 1). Primers and probes specific for methylated DNA and used for MethyLight reactions are listed in Table 2. The percentage of methylated reference (PMR) at a specific locus was calculated by dividing the *GENE*: *ACTB* ratio of a sample by the *GENE*: *ACTB* ratio of Universal Methylated DNA (fully methylated) (Chemicon International, Temecula, CA, USA) and multiplying by 100. We used PMR cutoff of four to distinguish methylation positive (PMR > 4) from negative (PMR ≤ 4). The PMR cutoff of 4 was previously validated.[24]

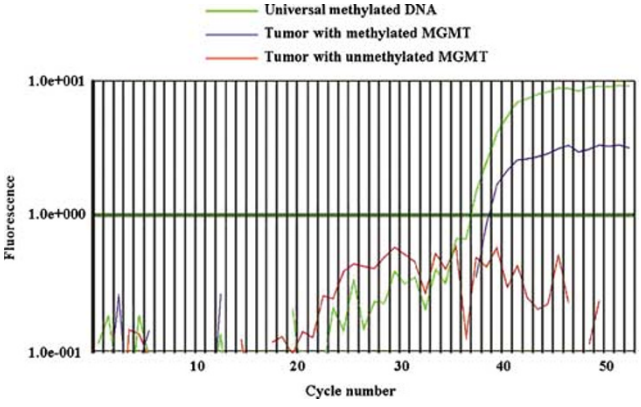

Figure 1 A quantitative real-time PCR to measure DNA methylation (MethyLight) showing the analysis of MGMT methylation. Bisulfite-converted universal methylated DNA was used for PMR calculation. The fluorescence is plotted *vs* the PCR cycle number for reaction and each sample is indicated.

Immunohistochemistry

Immunohistochemistry with an anti-human MGMT mouse monoclonal antibody (MAB16200, 1:100 dilution; Chemicon, Temecula, CA, USA) was carried out as described previously.[25] 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. The immunoreactivity of MGMT protein was evaluated semiquantitatively, and a positive reactivity of <5% of nuclei of tumor cells was regarded as negative.[26]

Statistical Analysis

Alteration of each target gene was assessed for associations with clinicopathological characteristics using the following statistical tests: Mann–Whitney *U*-test for age and size, and the χ^2 two-tailed test or Fisher's exact test for the remaining parameters.

Results

Clinicopathological Characteristics of Flat-Type Adenoma and Protruded-Type Adenoma Tissues

The mean age of flat-type adenomas was significantly larger that of protruded-type adenomas ($P = 0.0193$) (Table 1). Regarding gender, flat-type adenomas were more frequently found in females than were protruded-type adenomas ($P = 0.0153$). Regarding tumor location, flat-type adenomas were more frequently located in the proximal colon than were protruded-type adenomas ($P = 0.0074$).

Table 2 MethyLight primer and probe sequences

HUGO gene name	*Forward primer sequences (5′–3′)* *Reverse primer sequences (5′–3′)*	*Probe sequences (5′–3′)*
MGMT	GCGTTTCGACGTTCGTAGGT CACTCTTCCGAAAACGAAACG	6FAM-CGCAAACGATACGCACCGCGA-BHQ-1
CDKN2A	TGGAATTTTCGGTTGATTGGTT AACAACGTCCGCACCTCCT	6FAM-ACCCGACCCCGAACCGCG-BHQ-1
MLH1	AGGAAGAGCGGATAGCGATTT TCTTCGTCCCTCCCTAAAACG	6FAM-CCCGCTACCTAAAAAAATATACGCTTACGCG-BHQ-1

KRAS Codon 12 and Codon 13 Mutations

KRAS mutation was detected in a significantly higher percentage of flat-type adenoma tissues (35% of 101 tumors) than in protruded-type adenoma tissues (13% of 68 tumors; $P=0.0019$) (Table 3). When the samples were limited to the tumors in the distal colon, the difference of *KRAS* mutation was still significant ($P=0.0007$). On the other hand, no significant difference was found in the proximal colon. *KRAS* codon 12 mutation, codon 13 mutation and both mutations were detected in 20, 14 and 1 of the 35 flat-type adenomas, respectively (Figure 2a). In 15 of the 35 flat-type adenomas with *KRAS* mutations, the mutational patterns were 13 GGC-to-GAC (G13D). In 11 cases, they were 12 GGT-to-GAT (G12D). Various mutational patterns were found in the remaining cases: 12 GGT-to-GCT (G12A), 12 GGT-to-GTT (G12V), 12 GGT-to-AGT (G12S), and 12 GGT-to-TGT (G12C) (Table 4). *KRAS* mutation in G-to-A transitions at codon 12 and codon 13 was detected in a significantly higher percentage of flat-type adenoma tissues (26% of 101 tumors) than in protruded-type adenoma tissues (9% of 68 tumors; $P=0.0059$). When the samples were limited to the tumors in the distal colon, the difference of *KRAS* mutation in G-to-A transitions was still significant ($P=0.0240$).

In flat-type adenomas, *KRAS* mutation was not correlated significantly with any clinicopathological characteristics. On the other hand, in protruded-type adenoma tissues, *KRAS* mutation was correlated significantly with size ($P=0.0003$) and gender (female > male) ($P=0.0011$). On the other hand, *KRAS* mutation was detected in 13 (37%) of the 35 colorectal cancer tissues. *KRAS* mutation in G-to-A transitions at codon 12 and codon 13 was detected in nine (26%) of the 35 colorectal cancers.

BRAF Codon 600 (V600E) Mutation

BRAF mutation was detected in three (3%) of the 101 flat-type adenoma tissues. All of the tumors

Table 3 Genetic and epigenetic alterations of colorectal advanced adenomas

	Morphology		P-*value*
	Flat-type n = *101 (%)*	*Protruded-type* n = *68 (%)*	
Mutation			
KRAS	35 (34.7)	9 (13.2)	0.0019
BRAF	3 (3.0)	2 (2.9)	>0.9999
β-catenin	8 (7.9)	4 (5.9)	0.6129
Methylation			
MGMT	28 (27.7)	24 (35.3)	0.2957
CDKN2A	33 (32.7)	43 (63.2)	<0.0001
MLH1	9 (8.9)	11 (16.2)	0.1516

Table 4 Spectrum of KRAS mutations in 101 flat-type colorectal advanced adenomas

Mutation (amino acid change)	*Number of FCAA patients (%)*
12 GGT-to-GAT (G12D)	10 (9.9)
12 GGT-to-GCT (G12A)	4 (4.0)
12 GGT-to-GTT (G12V)	4 (4.0)
12 GGT-to-AGT (G12S)	1 (1.0)
12 GGT-to-TGT (G12C)	1 (1.0)
13 GGC-to-GAC (G13D)	14 (13.9)
12 GGT-to-GAT (G12D) and 13 GGC-to-GAC (G13D)	1 (1.0)

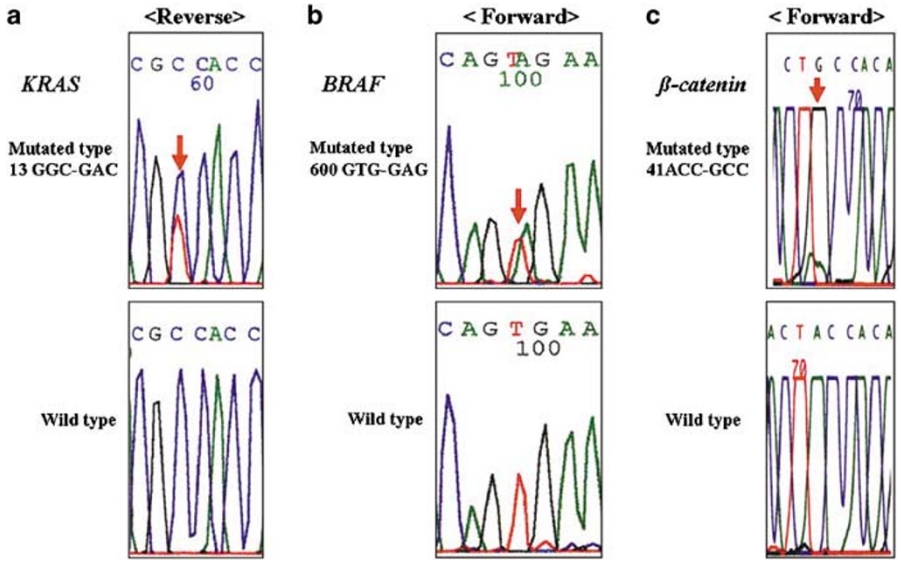

Figure 2 DNA direct sequencing of colorectal adenomas with *KRAS*, *BRAF* or *β-catenin* exon 3 mutation. Wild-type, sequence of a wild case; mutated type, sequence of a mutated case. (**a**) A GGC-to-GAC change (G13D) at KRAS codon 13. (**b**) A GTG-to-GAG change (V600E) at BRAF codon 600. (**c**) An ACC-to-GCC (T41A) change at β-catenin codon 41.

demonstrated missense mutations at codon 600 (V600E) (Figure 2b). There was a mutually exclusive relationship between *KRAS* and *BRAF* mutations. In flat-type adenoma tissues, *BRAF* mutation was detected in two (67%) of the three serrated adenomas. No significant difference was found between frequencies of those mutations in flat-type adenomas and protruded-type adenomas (Table 3). In flat-type adenoma and protruded-type adenoma tissues, *BRAF* mutation was not correlated significantly with any clinicopathological characteristics. *BRAF* mutation was detected in three (9%) of the 35 colorectal cancer tissues.

β-catenin Exon 3 Mutation

β-catenin exon 3 mutation was detected in eight (8%) of the 101 flat-type adenoma tissues. These mutations were single-base substitutions that were located within the critical serine/threonine codons for GSK-3*β* phosphorylation of *β-catenin* (codons 29–48) (Figure 2c). All sites of *β-catenin* exon 3 missense mutation have been reported previously.[27–31] Interstitial deletions of *β-catenin* exon 3 were detected in two flat-type adenoma tissues. No significant difference was found between frequencies of those mutations in flat-type adenomas and protruded-type adenomas (Table 3). In flat-type adenoma and protruded-type adenoma tissues, *β-catenin* exon 3 mutation was not correlated significantly with any clinicopathological characteristics. On the other hand, *β-catenin* exon 3 mutation was detected in three (9%) of the 35 colorectal cancer tissues.

Methylation of *MGMT*, *CDKN2A* (*p16*) and *MLH1*

The frequencies of methylation of *MGMT*, *CDKN2A* and *MLH1* in flat-type adenoma and protruded-type adenoma tissues are summarized in Table 3. Methylations of *MGMT*, *CDKN2A* and *MLH1* were detected in 28 (28%), 33 (33%) and 9 (9%) of the 101 flat-type adenoma tissues, respectively.

CDKN2A methylation was detected in a significantly lower percentage of flat-type adenoma tissues than in protruded-type adenoma tissues (63% of 68 tumors; $P<0.0001$). When the samples were limited to the tumors in the proximal colon and the distal colon, respectively, the difference of *CDKN2A* methylation was still significant (data not shown). On the other hand, a significant difference was not found between frequencies of any other methylations in flat-type adenoma and protruded-type adenoma tissues. Methylation of at least one gene was detected in a significantly lower percentage ($P=0.0012$) of flat-type adenoma tissues (54% of 101 tumors) than in protruded-type adenoma tissues (78% of 68 tumors). When the samples were limited to the tumors in the proximal colon and the distal colon, respectively, the differences were still significant (data not shown).

In flat-type adenoma tissues, *MGMT* methylation was correlated significantly with age ($P=0.0316$) and location (distal > proximal) ($P=0.0087$). On the other hand, in protruded-type adenoma tissues, *CDKN2A* was correlated significantly with size ($P=0.0088$) and *MGMT* methylation was correlated significantly with age ($P=0.0178$) and location (distal > proximal) ($P=0.0025$). Although *MGMT* methylation was detected in a higher percentage of flat-type adenomas with *KRAS* mutation in G-to-A transition, no significant relationship was found between the mutation and the methylation. On the other hand, methylation of *MGMT*, *CDKN2A* and *MLH1* was detected in 11 (31%), 14 (40%) and 4 (11%) of the 35 colorectal cancers, respectively.

Immunohistochemical Staining of MGMT in Flat-Type Adenomas with or without *MGMT* Methylation

The expression of MGMT was analyzed immunohistochemically in 30 flat-type adenoma tissues (Figure 3). Expression of MGMT protein was negative in six (20%) of the 30 tumors. Expression of MGMT protein was negative in four (40%) of the 10 tumors with *MGMT* methylation and in two (10%) of the 20 tumors without *MGMT* methylation (Figure 2a and b). Although *MGMT* methylation was detected in a higher percentage of flat-type adenomas with reduced protein expression, no significant correlation was found between them.

We further examined the expression of MGMT in 30 protruded-type adenomas and 20 colorectal cancers by immunohistochemistry. MGMT expression was negative in 27% of protruded-type adenomas and 25% of colorectal cancers. No significant difference was found between frequencies of the MGMT negativity in adenomas (flat-type and protruded-type) and colorectal cancers.

Discussion

In the current study, *KRAS* mutation was detected in a significantly higher percentage of flat-type advanced adenomas than in protruded-type advanced adenomas. The mutational patterns in most of the flat-type adenomas with *KRAS* mutations were a transition from G-to-A. Mutations of *BRAF* and *β-catenin* were detected in some flat-type adenomas. On the other hand, methylation of at least one gene was detected in a significantly lower percentage of flat-type adenomas than in protruded-type adenomas. These results suggest flat-type advanced adenomas have distinct genetic and epigenetic features different from those of protruded-type advanced adenomas (Figure 4).

Although more than 10 years have passed since Kudo[6] advocated the new category 'laterally spreading tumor', only a few genetic alterations of laterally spreading tumors such as *KRAS*, *β-catenin* and *p53* mutations,[5,8,10,11] and overexpression of COX-2[7] and

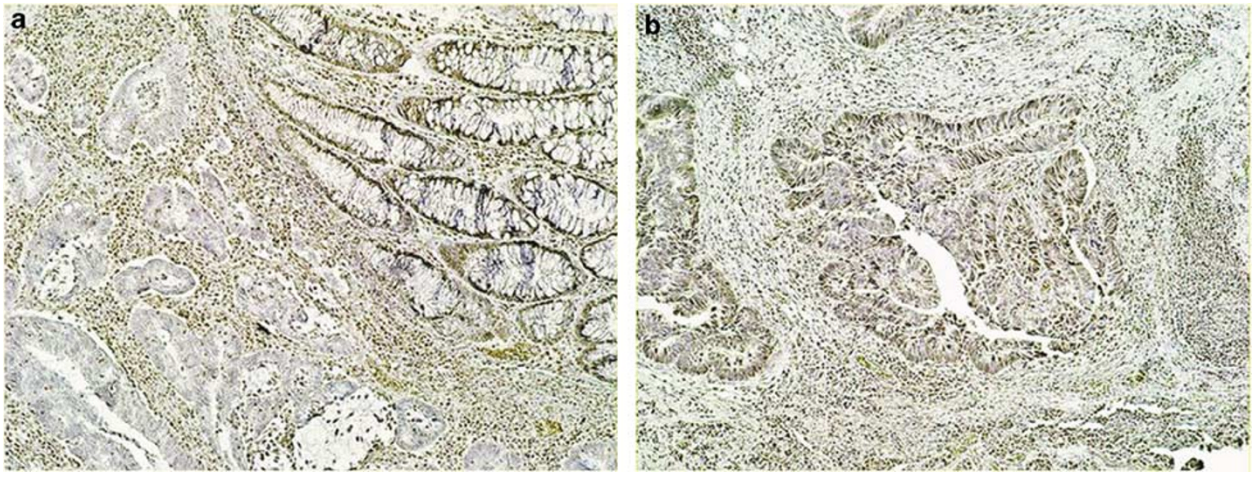

Figure 3 Immunohistochemical staining of MGMT protein in colorectal adenomas. (**a**) A tumor with MGMT methylation showed no staining in tumor cells but clear staining in the nuclei of non-tumor cells (× 100). (**b**) A tumor without MGMT methylation showed staining for the MGMT protein in tumor cell nuclei (× 100).

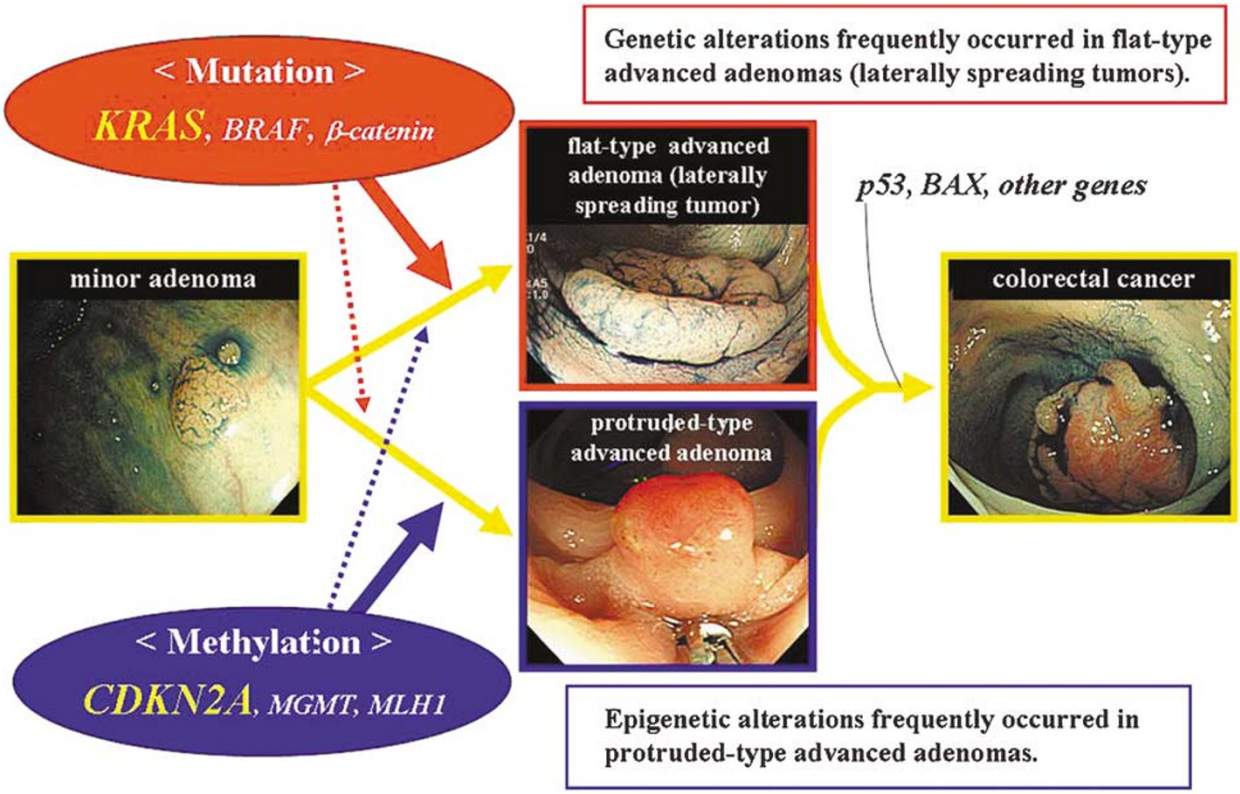

Figure 4 A diagram to illustrate the genetic pathways of flat-type advanced adenoma (laterally spreading tumor) and protruded-type advanced adenoma leading to colorectal cancers. Flat-type advanced adenomas seems to have different genetic and epigenetic alterations from those of protruded-type advanced adenomas.

gastrin protein[8] have been reported. laterally spreading tumors are defined as lesions >10 mm in diameter with a low vertical axis that extend laterally along the luminal wall,[6] and most of them remain as adenoma or early invasive cancer.[7] As the criteria of laterally spreading tumor are almost consistent with those of flat-type advanced adenoma, we compared the frequencies of *KRAS* mutations in flat-type advanced adenomas in the current study and in laterally spreading tumors in previous studies.

Kusaka *et al*[10] reported that *KRAS* codon 12 mutation was detected in nine (50%) of 18 laterally spreading tumor tissues. With one exception, these

tumors had the same mutational pattern: 12 GGT-to-GTT (G12V). Mukawa *et al*[8] reported that *KRAS* mutation was detected in 18 (50%) of 36 laterally spreading tumor tissues and in 13 (50%) of 26 protruded-type tumor tissues. In eight of the 21 tumors with *KRAS* mutations, the mutational patterns were 12 GGT-to-GTT (G12V). In further eight cases, they were 12 GGT-to-GAT (G12D). Noro *et al*[5] reported that *KRAS* mutation was detected in six (21%) of 28 laterally spreading tumor tissues and in six (26%) of 23 protruded-type tumor tissues. Frequent mutational patterns were 12 GGT-to-TGT (G12C) in laterally spreading tumors and 12 GGT-to-GAT (G12D) in protruded-type tumors. No *KRAS* mutations within codon 13 were detected in any of the tumors.

The discrepancy in these mutational patterns may be because of the small numbers of samples analyzed in previous studies and/or differences in the methods of measurement. On the other hand, *KRAS* mutation was detected in a smaller percentage of flat-type adenomas in the current study than in laterally spreading tumors in previous studies. The reason for the low frequency of *KRAS* mutations in flat-type adenoma tissues is not known. It might be due to the fact that depressed areas were seen in the surfaces of some flat-type adenoma tissues. Umetani *et al*[32] reported that the frequency of *KRAS* mutations in flat-type tumors with depressed areas was significantly lower than that in flat-type tumors without depressed areas.

In the current study, when the samples were limited to the tumors in the distal colon, *KRAS* mutation in G-to-A transitions was detected in a significantly higher percentage of flat-type adenomas than in protruded-type adenomas. This result suggests that these genetic alterations play an important role in the development and/or progression of flat-type advanced adenomas (laterally spreading tumors), especially in the distal colon. In addition, Nagasaka *et al*[13] reported that *KRAS* mutation in G-to-A transitions at codon 12 and codon 13 was detected in 51 (22%) of 234 colorectal cancers. This is consistent with our data on mutational patterns in flat-type advanced adenomas and colorectal cancers. Therefore, colorectal cancers with *KRAS* mutation in G-to-A transitions seem to largely arise from flat-type advanced adenomas. Further analysis is needed to clarify this issue.

Promoter hypermethylation of CpG islands provides an epigenetic mechanism for transcriptional repression of genes such as *MGMT*, *CDKN2A* and *MLH1*.[13–16,18] In addition, aberrant methylation of these genes in association with other genetic alterations has been shown to be an important mechanism of colorectal carcinogenesis.[13,15,16,18,19] Petko *et al*[14] found by using methylation-specific polymerase chain reaction (MSP) that methylation of *MGMT*, *CDKN2A* and *MLH1* occurred in 49, 34 and 7% of adenomas, respectively, and that they are more common in histopathologically advanced adenomas. The frequencies of methylation of *CDKN2A* (45%; 76/169) and *MLH1* (12%; 20/169) in advanced adenomas in the current study are similar to these previously reported frequencies. However, *MGMT* methylation was detected in a smaller percentage of advanced adenomas (31%; 52/169) and colorectal cancers (31%; 11/35) in the current study than in colorectal adenomas in that previous study.[14] The discrepancy may be because of the differences in the methods of measurement using MethyLight and MSP.

In the current study, not only *CDKN2A* methylation but also methylation of at least one gene was detected in a significantly lower percentage of flat-type adenomas than in protruded-type adenomas. Therefore, epigenetic alterations might infrequently occur in flat-type adenomas.

MGMT methylation was not correlated significantly with *KRAS* mutation in G-to-A transition in flat-type adenoma tissues, despite their high rates of *KRAS* mutations. Therefore, some other mismatch repair genes for *KRAS* mutation might contribute to the mutational patterns, which were a transition from G-to-A, in flat-type adenoma tissues. In addition, no significant correlation was found between the presence of *MGMT* methylation and reduced MGMT protein expression. Six tumors with *MGMT* methylation expressed MGMT protein. This might be due to a low level of methylation, which reflects methylation of only a few tumor cells, or methylation of one allele but absence of methylation of the other allele of the gene. On the other hand, expression of MGMT protein was reduced in two tumors without *MGMT* methylation. It has been reported that *MGMT* mutations are infrequently found in colorectal cancers and contribute to reduction of their protein function.[17] Therefore, in these cases, reduced expression of MGMT protein might have arisen from genetic and/or other epigenetic abnormalities.

In flat-type adenoma tissues, *BRAF* mutation was frequently detected in serrated adenomas. Therefore, *BRAF* mutations are thought to be associated with the hyperplastic polyp-serrated adenoma-carcinoma pathway not only in protruded-type adenomas but also in flat-type adenomas. *β-catenin* exon 3 mutation was detected in 8% of the flat-type adenomas in the current study. As *β-catenin* is involved in cell adhesion, abnormalities of *β-catenin* might play an important role not only in the tumorigenesis but also in the morphological features of a subset of flat-type adenomas (laterally spreading tumors).[11,12]

In summary, *KRAS* mutation was frequently detected in flat-type advanced adenoma tissues and the mutational patterns in most of them with *KRAS* mutations were a transition from G-to-A. Therefore, these genetic alterations seem to play an important role in the development of flat-type advanced adenomas (laterally spreading tumors), especially in the distal colon. Epigenetic alterations infrequently occurred in flat-type advanced

adenomas, suggesting that they have different genetic and epigenetic alterations from those of protruded-type advanced adenomas.

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Ethics approval statement

An informed consent was obtained from each patient and the institutional review committee approved this study.

Conflict of interest statement

None decleared.

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