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Progressive methylation during the serrated neoplasia pathway of the colorectum

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Serrated adenoma is a recently described entity characterized by having combined architectural features of hyperplastic polyps and classical adenoma. To understand the role of gene regulation in the progression of the serrated neoplasia pathway, we examined the methylation profiles of the promoter regions of 19 genes, DNA ploidy, and mutator phenotype status. In all, 40 sporadic, classical serrated adenomas were pathologically reviewed and divided into four pathologic groups according to their histologic grades. Methylation-specific PCR was performed using primers for *p16*, *hMLH1*, *RASSF1A*, *APC*, *HIC-1*, *DAPK*, *MGMT*, *SLC5A8*, *RB1*, *H-Cadherin*, *E-Cadherin*, *TIMP3*, *PTEN*, *THBS1*, *LKB1*, *p14*, *p15*, *FHIT*, and *VHL*. Dual flow-cytometric analyses using cytokeratin and DAPI and MSI studies using BAT26 were also performed. Methylation was observed in 2.5–82.5% (mean 33.9%) of the CpG islands in the promoter regions of 16 genes. The tumors with higher histologic grades, including carcinomas, showed more extensive methylation compared to those with lower grades, and serrated adenomas in the right colon showed more frequent methylation than those in the left (P<0.05). Tumor-specific promoter methylation of *SLC5A8* was observed in 33 (82.5%) of the serrated adenomas. Aneuploidization with near-diploid DNA indices was detected in four out of 28 cases examined (14.3%); two were low-grade serrated adenomas and two were carcinomas in the left colon. The high mutator phenotype was not observed in any of the cases examined. Our results indicate that: (1) aberrant, widespread methylation of CpG islands increases with the histological progression of serrated adenomas; (2) methylation of *SLC5A8* is an early event; and (3) additional methylation of the *p16*, *p14*, *MGMT*, *TIMP3*, and *FHIT* genes are important tumorigenic steps in the serrated neoplasia pathway.
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Serrated adenoma is a recently defined colorectal neoplasm with a prevalence ranging from 0.6 to 1.3%.[1,2] The serrated neoplasia pathway suggests that dysplasia can arise within hyperplastic colonic polyps, resulting in the formation of a serrated adenoma and potentially the development of a colorectal carcinoma.[2–5] The process, presumably driven by a progressive accumulation of genetic changes within the clonal cell mass, is phenotypically characterized by increasing dysplasia in an epithelium that retains a distinctive serrated architecture.[6] The polyps of this pathway differ morphologically and genetically from those of the traditional adenoma–carcinoma sequence.[7] Genetically, serrated adenomas are differentiated from sporadic tubular adenomas by a high frequency of the low mutator phenotype,[3,8] *p53* mutations,[9] *KRAS* mutations,[9,10] *BRAF* mutations,[11] and a low frequency of *APC* gene mutations.[12] However, the prevalence of the high mutator phenotype and its significance during tumorigenesis are controversial and the chromosomal status of serrated adenoma has not been studied.

Methylation of CpG islands is a mechanism for the suppression of gene transcription in physiological and pathological settings, including neoplasia.[13] The recently described CpG island methylator phenotype is an alternative tumorigenesis pathway characterized by methylation of multiple promoter regions of tumor suppressor genes harboring CpG islands. Recent studies have shown that CpG island methylator phenotype is very common in sporadic serrated adenomas, and especially in the hyperplastic polyps of hyperplastic polyposis.[14,15] However, the methylation profiles of tumor-related genes in

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the serrated neoplasia pathway during the progression of serrated adenomas remains to be studied.

In the present study, serrated adenomas selected according to strict criteria were histologically divided into low and high grades with or without carcinomas and their methylation status was studied using primers related to tumor suppressor genes, genes related to DNA repair or carcinogen detoxification, cell cycle regulators, an angiogenesis inhibitor, and invasion or metastasis suppressors. The methylation status of these genes was compared with the chromosomal aberration status and high mutator phenotype of the tumors.

Materials and methods

Materials

In all, 40 sporadic serrated adenomas were collected by polypectomy from patients at Our Lady of Mercy Hospital, The Catholic University of Korea, in Inchon, and Samsung Medical Center, Sungkyunkwan University, Seoul, from 1998 to 2003. The 40 patients included 14 females and 26 males with a mean age of 54.7 (range 35–86). In the present study, strict and objective histologic criteria were used for the diagnosis of serrated adenoma: serration in $\geq$20% of the lesions, frequent pseudostratification, nuclear atypia, and micropapillations of the surface epithelium with eosinophilic cytoplasm.[7] All of our cases were the traditional type of serrated adenomas according to the provisional classification of serrated polyps by Torlakovic *et al*.[16] The serrated adenomas were divided into four pathologic groups according to the presence of hyperplastic polyp components described by Longacre and Fenoglio-Preiser[1] and epithelial atypia based on nuclear atypia (nuclear enlargement, hyperchromasia, prominent nucleoli, and presence of vesicular nuclei) and archictectural atypia (cribriform pattern and partial loss of polarity). The four groups were serrated adenomas with low histologic grade and hyperplastic area ($N=5$), serrated adenomas with low-grade atypism ($N=23$), serrated adenomas with high-grade atypism ($N=6$), and serrated adenomas with carcinomas ($N=6$). When there was low- and high-grade atypism within one polyp, it was regarded as high grade. Tumors larger than 0.5 cm were selected to ensure a sufficient amount of DNA for analysis. In 15 cases, normal colon tissue was obtained from the histologically normal portion of the polypectomy specimens (seven cases) or from patients without tumors (eight cases).

Methylation-Specific PCR

Methylation-specific PCR was performed using the primers described in Table 1. Briefly, DNA was extracted from 10 μm sections stained with 0.1% methylene blue or hematoxylin & eosin by manual microdissection under microscopy using 20-gauge needles and proteinase K solution as previously described.[17] More than 40 000 cells were procured from each sample and more than 80% of these were tumor cells. After a bisulfite modification, β-actin was analyzed to standardize the DNA amount of each sample. PCR reactions were performed in a 25 μl reaction volume with 1.25 U of Ex*Taq* polymerase (Takara Shuzo Co., Kyoto, Japan) as previously described.[18] As a positive control, *Sss*-I methylase (New England Biolabs Inc, Beverly, MA, USA) was used to methylate 100 μg of DNA. The PCR products were electrophoresed in a 6% polyacrylamide gel, stained with SYBR Gold (Molecular Probes, Eugene, OR, USA), and then visualized under UV illumination.

The promoters of 19 genes were examined for their methylation status. These genes included tumor suppressor genes (*p15*^{INK4b}, *p16*^{INK4a}, *HIC-1*, *APC*, *SLC5A8*, *PTEN*, *RB*, *FHIT*, *RASSF1A*, *VHL*, *LKB1*), genes involved in DNA repair and the detoxification of carcinogens (*hMLH1*, O^6-methylguanine-DNA-methyltransferase (*MGMT*)), cell cycle genes (*PTEN*, *p14*^{ARF}), an angiogenesis inhibitor (*TIMP3*), and invasion or metastasis suppressors (*E-cadherin*, *TIMP3*, *H-cadherin*).

Flow Cytometry, Analysis of MSI, and Immunohistochemistry

Dual flow cytometric analyses using anti-cytokeratin (CAM5.2, Becton-Dickinson, CA, USA) and DAPI were performed as previously described.[19] The ploidy level of cytokeratin-positive tumor cells was compared with benign, cytokeratin-negative diploid cells to create a DNA index. To allow for instrument error of 5%, the diploid tumors have DNA index of 0.95–1.05. All nondiploid tumors were analyzed twice.

To examine the microsatellite instability (MSI) status, we examined the tumor samples using the BAT26 primer set, which is a very sensitive marker of tumors with high MSI.[20,21] PCR analyses were performed using a DNA autosequencer (Applied Biosystems 373A sequencer; Applied Biosystems, CA, USA). The mobility shift of PCR products from the tumor DNA was compared to that of corresponding normal colonic mucosa. Immunohistochemistry was carried out on 4 μm paraffin sections with antibodies against *hMLH1* (clone G168-15; 1:20 dilution) from BD Pharmingen (San Diego, CA, USA), and *MGMT* (clone MT3.1;1:50 dilution) and *p16*^{INK4a} (*p16*^{INK4a} Research Kit clone E6H4;1:30 Dilution) from DakoCytomation (Denmark). Sections were deparaffinized in xylene and rehydrated in graded series of alcohol to distilled water. Subsequently, antigen retreival by heating microwave oven in 10 mM citrate buffer (pH 6.0) was performed for 10 min. The detection was performed using a streptavidin-biotinylated horseradish peroxidase

Table 1 Primer sequences and methylation-specific PCR conditions

Primer name		*Forward primer (5′–3′)*	*Reverse primer (5′–3′)*	*Product size (bp)*	*Annealing temp (°C)*
p16	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65
	U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60
hMLH1	M	TATATCGTTCGTAGTATTCGTGT	TCCGACCCGAATAAACCCAA	153	60
	U	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60
RASSF1A	M	GTGTTAACGCGTTGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	93	60
	U	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA	105	60
APC	M	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	98	55
	U	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	108	60
HIC-1	M	TCGGTTTTCGCGTTTTGTTCGT	AACCGAAAACTATCAACCCTCG	95	62
	U	TTGGGTTTGGTTTTTGTGTTTTG	CACCCTAACACCACCCTAAC	118	62
DAPK	M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60
	U	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	98	60
MGMT	M	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	59
	U	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	59
SLC5A8	M	TCGAACGTATTTCGAGGC	ACAACGAATCGATTTTCCG	108	56
	U	TTGAATGTATTTTGAGGTG	TCAATTTTCCAAAATCCC	100	56
14-3-3 Sigma	M	TGGTAGTTTTTATGAAAGGCGTC	CCTCTAACCGCCCACCACG	105	56
	U	ATGGTAGTTTTTATGAAAGGTGTT	CCCTCTAACCACCCACCACA	106	56
RB1	M	GGGAGTTTCGCGGACGTGAC	ACGTCGAAACACGCCCCG	172	60
	U	GGGAGTTTTGTGGATGTGAT	ACATCAAAACACACCCCA	172	60
H-Cad	M	TCGCGGGGTTCGTTTTTCGC	GACGTTTTCATTCATACACGCG	243	60
	U	TTGTGGGGTTGTTTTTTGT	AACTTTTCATTCATACACACA	243	60
E-Cad	M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	115	57
	U	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97	53
TIMP3	M	CGTTTCGTTATTTTTTGTTTTCGGTTTTC	CCGAAAACCCCGCCTCG	116	59
	U	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCCAAAAACCCCACCTCA	122	59
PTEN	M	TTTTTTTTCGGTTTTTCGAGGC	CAATCGCGTCCCAACGCCG	134	59
	U	TTTTGAGGTGTTTGGGTTTTTGGT	ACACAATCACATCCCAACACCA	124	59
THBS1	M	TGCGAGCGTTTTTTTAAATGC	TAAACTCGCAAACCAACTCG	74	62
	U	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	115	62
LKB1	M	CGATCGAGCGGATTTTTCG	CGCTCGAACAAACGTTTACG	64	56
	U	AATGTTTTGTTGTGGATGATTG	CAACAACCACCTTAAAAATCAC	60	56
p14	M	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122	60
	U	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132	60
p15	M	GCGTTCGTATTTTGCGGTT148	CGTACAATAACCGAACGACCGA	147	65
	U	TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	154	61
FHIT	M	TTGGGGCGCGGGTTTGGGTTTTTACGC	CGTAAACGACGCCGACCCCACTA	100	65
	U	TTGGGGTGTGGGTTTGGGTTTTTATG	CATAAACAACACCAACCCCACTA	100	65
VHL	M	TGGAGGATTTTTTTGCGTACG	GAACCGAACGCCGCGAA	158	60
	U	GTTGGAGGATTTTTTTGTGTATGT	CCCAAACCAAACACCACAAA	165	60

complex detection system (DakoCytomation, Denmark) according to the manufacturer's instructions and visualized with 3,3′-diaminobenzidine as a substrate. The sections were counterstained with Mayer's hematoxylin.

Statistics

Statistical comparisons of the clinical, histologic, flow cytometry, mutator phenotype, immunohistochemistry, and methylation results were performed with the χ^2 test and ANOVA using the statistical software SPSS (version 10.0, Chicago, IL, USA).

Results

Clinicopathologic Findings, MSI and Ploidy Status

The serrated adenomas in this study met strict pathologic criteria and were polypoid and nodular in shape, with sizes ranging from 0.5 to 2.5 cm (mean 1.1 cm). Six cases occurred in the right colon and 34 occurred in the left colon. Of these 40 serrated adenomas, a low histologic grade of atypia was found in 28 cases, with five showing a hyperplastic area in the neck portion of the polyp (Figure 1). Representative examples of low- and high-grade atypism are depicted in Figure 2. Carcinomas arising from serrated adenomas were found in six cases (12.5%), including five intramucosal carcinomas and one deeply invasive carcinoma showing an invasion into pericolic adipose tissue by well-preserved serrated glands with a mucin pool (Figure 3). All carcinomas in the serrated adenomas were observed in the head portion of the polyp. In four of the 28 analyzed cases (14.3%), aneuploidization was detected using very sensitive, dual flow cytometry (Figure 4). All aneuploid tumors showed near-diploid DNA indices ranging 1.1–1.2. The four near-diploid tumors included two low-grade serrated adenomas and two carcinomas within serrated adenomas in the left colon. The high mutator phenotype was not observed in any of the cases (Figure 5).

Methylation Analyses of 19 Tumor-Related Genes

Methylation within serrated adenomas was observed in 16 promoter regions ranging from 2.5 to 82.5%. The overall methylation status are described in Table 2. In the normal colonic mucosa, methylation was observed in 13 promoter regions and ranged from 6.6 to 26.6%. Within the 15 samples of normal colonic mucosa, methylation was found with the indicated frequency in the promoters of *p15* (4), *SLC5A8* (2), *H-cadherin* (2), *E-cadherin* (2), *TIMP3* (2), *FHIT* (2), *DAPK* (2), *p14* (2), *THBS* (2), *p16* (1), *RASSF1A* (1), *APC* (1), *MGMT* (1), *RB1* (0), *LKB1* (0), *hMLH1* (0), *HIC-1* (0), *PTEN* (0), and *VHL* (0). All promoter regions related to tumor suppressors were methylated significantly more in the tumors than in normal tissues.

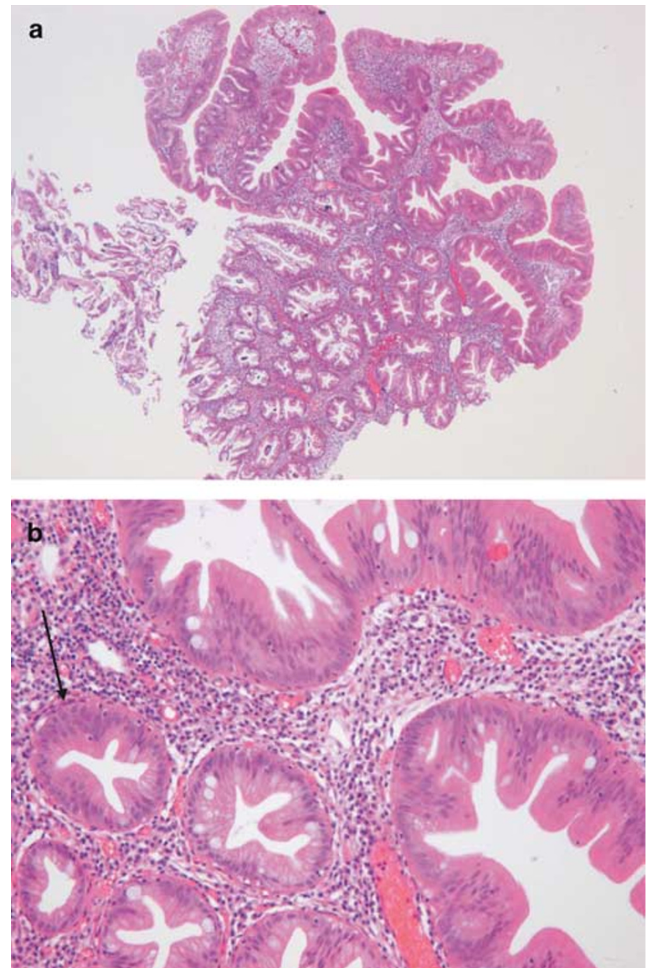

Figure 1 Serrated adenoma with hyperplastic area in the neck portion of the polyp (**a**). At higher power (**b**), a transitional gland (arrow) having features of both a hyperplastic polyp (lower left) and serrated adenoma (upper right) can be seen.

No methylation was observed in the *hMLH1*, *RB1*, and *VHL* promoter regions from either the tumors or normal tissues. When the average number of genes methylated (percent methylation) was compared in each pathologic group, a stepwise increase was observed during the progression of the serrated neoplasia pathway ($P<0.05$) (Table 3). There were statistically significant differences in the methylation of serrated adenomas in the 'low' and 'carcinoma' groups and the 'serrated adenomas' 'carcinoma' groups ($P<0.05$). The methylation of the *p16, MGMT, TIMP3, p14*, and *FHIT* genes was also correlated with the progression of histologic grades (Table 3). Neither the percent methylation nor the methylation of individual genes was related to the patients' gender, age, size of tumors, or ploidy status. The right colon showed a higher frequency of methylation for *the p16*, *p14*, *TIMP3,* and *FHIT*

Figure 2 Representative examples of serrated adenomas with low-(**a**) and high-(**b**) grade atypism.

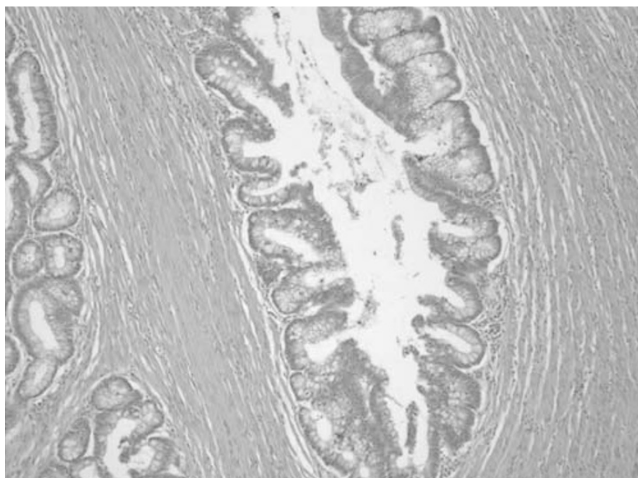

Figure 3 Photomicrograph of deeply infiltrating carcinoma glands showing well-preserved serration.

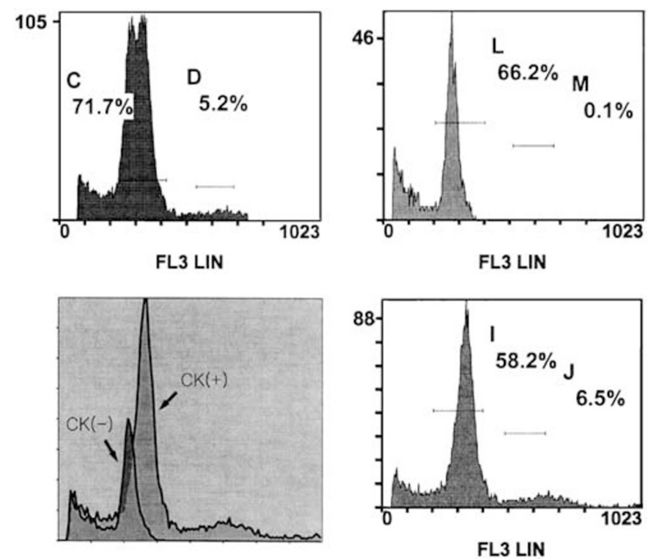

Figure 4 Near-diploid tumor cells evaluated with dual flow cytometry using DAPI and cytokeratin. Upper left: total cell counts, Upper right: gating of cytokeratin-negative nontumorous cells, Lower right: gating of cytokeratin-positive tumor cells, Lower left: overlay of cytokeratin-positive and -negative cells.

genes ($P<0.05$). Also, the overall percent methylation was higher in the right colon (36.8%) than the left (27.1%).

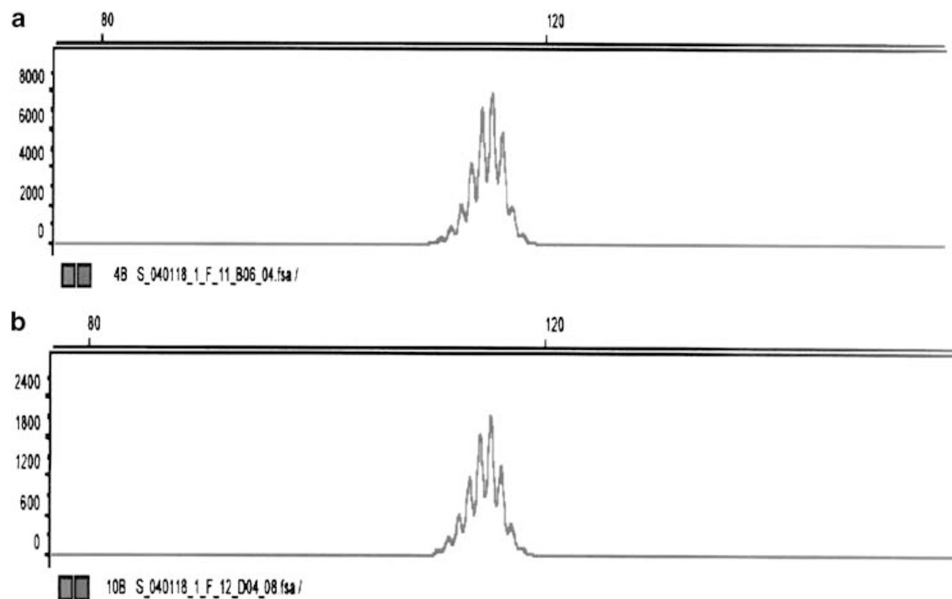

Figure 5 Microsatellite instability analyses using BAT26 show no shift between a normal control (**a**) and serrated adenoma sample (**b**).

A high percentage of promoter methylation of *SLC5A8* was detected in 33 serrated adenomas (82.5%) (Figure 6). Only two of the 15 (13.3%) normal colon tissue samples showed methylation of *SLC5A8*. A review of the colonic control samples with methylated *SLC5A8* showed relatively hyperplastic crypts in the superficial portion of the mucosae. All four aneuploid tumors and two serrated adenomas in the right colons of female patients showed methylation of *SLC5A8* gene. To evaluate the role of methylation in the pathogenesis of the serrated neoplasia pathway, 10 sporadic hyperplastic polyps were further evaluated for the presence of *SLC5A8* methylation. Seven cases of sporadic hyperplastic polyps showed methylation of *SLC5A8*. *p16* methylation was observed in nine of the 40 serrated adenomas (22.5%) including five with high-grade and four with low-grade atypism. Notably, methylation of *p*16 was found in seven cases with *SLC5A8* methylation (Figure 6) and *p16* methylation was significantly correlated with the methylation status of the *HIC-1*, *TIMP3*, *p14*, and *FHIT* genes ($P<0.05$). To evaluate the importance of methylation during tumorigenesis, immunohistochemistry was performed using available antibodies against *hMLH1*, *MGMT*, and *p16*. The promoter methylation status completely or partially matched the expression of these proteins: there was no loss of *hMLH1* and partial losses of *MGMT* and *p16*.

Discussion

In this study, we examined the promoter regions of 19 tumor-related genes. We found that the methylation of the promoter regions of *p16*, *MGMT*, *TIMP3*, *p14*, and *FHIT* genes is related to the histologic progression of serrated adenomas, and the methylation of the *SLC5A8* gene occurs early in the serrated neoplasia pathway. *SLC5A8* has recently been identified as a candidate tumor-suppressor gene that is involved in sodium transport. The silencing of *SLC5A8* by methylation confers a specific growth advantage in the subset of colon cancers in which this locus is inactivated.[22] It is methylated in 59% of primary colonic carcinomas and classical tubular adenomas but is rarely methylated in normal colonic mucosae. A high frequency of *SLC5A8* methylation was identified in the serrated adenomas (82.5%) regardless of the histologic grade or clinicopathologic parameters. Hypermethylation of *SLC5A8* was observed in all cases of serrated adenomas with hyperplastic areas and in 70% of hyperplastic polyps. In previous methylation studies using sporadic hyperplastic polyps, Chan *et al*[15] did not detect any methylation within five promoter regions, including those of *p16*, *MINT1*, *MINT2*, *MINT31*, and *hMLH1*, while Bariol *et al*[23] found methylation of these genes using fresh DNA from hyperplastic polyps. The tumor-specific methylation of *SLC5A8* found in hyperplastic polyps and serrated adenomas with low-grade atypism suggests that methylation of CpG islands occurs early during the serrated neoplasia pathway. Although the promoters of 16 of the tumor-related genes were methylated, neither *hMLH1* methylation nor a high mutator phenotype was found in this study. This low frequency of MSI may be due to the use of a single microsatellite marker, the predominantly left location of the cases used (85%), or the very low frequency of MSI-high colorectal carcinoma and *hMLH1* methylation among elderly subjects in

Table 2 Frequency of methylation observed in 19 promoter regions according to their clinical, pathological, and flow cytometric findings

	SLC5A8		*HIC-1*		*THBS1*		*TIMP3*		*DAPK*		*p14*		*RASSF1A*		*p15*		*H-cad*		*APC*		*MGMT*		*p16*		*E-cad*		*FHIT*		*LKB1*		*PTEN*		*Total*
	M	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	*M*	*U*	
Age																																	
≤60	23	7	19	11	19	11	13	17	12	18	9	21	7	23	10	20	9	21	7	23	7	23	6	24	5	25	6	24	4	26	1	29	30
>60	10	0	6	4	4	6	6	4	5	5	5	5	5	5	2	8	3	7	3	7	2	8	3	7	4	6	2	8	0	10	0	10	10
Sex																																	
Female	13	1	7	7	7	7	8	6	5	9	6	8	3	11	3	11	5	9	3	11	5	9	3	11	2	12	3	11	3	11	0	14	14
Male	20	6	18	8	16	10	11	15	12	14	8	18	9	17	9	17	7	19	7	19	4	22	6	20	7	19	5	21	1	25	1	25	26
Localization																																	
Right side	5	1	6	0	4	2	5	1	2	4	5	1	2	4	2	4	2	4	1	5	2	4	3	3	0	6	3	3	0	6	0	6	6
Left side	28	6	19	15	19	15	14	20	15	19	9	25	10	24	10	24	10	24	9	25	7	27	6	28	9	25	5	29	4	30	1	33	34
Size																																	
≤1.1	24	3	17	10	15	12	12	15	10	17	7	20	8	19	7	20	7	20	8	19	7	20	6	21	5	22	6	21	3	24	1	26	27
>1.1	9	4	8	5	8	5	7	6	7	6	7	6	4	9	5	8	5	8	2	11	2	11	3	10	4	9	2	11	1	12	0	13	13
Histologic grade																																	
Low+HP	5	0	3	2	3	2	1	4	2	3	0	5	1	4	2	3	2	3	0	5	0	5	0	5	1	4	0	5	0	5	0	5	5
Low	17	6	14	9	15	8	10	13	10	13	6	17	8	15	6	17	6	17	7	16	5	18	4	19	5	18	3	20	2	21	1	22	23
High	6	0	4	2	2	4	3	3	4	2	3	3	0	6	1	5	2	4	1	5	1	5	3	3	1	5	3	3	1	5	0	6	6
Carcinoma	5	1	4	2	3	3	5	1	1	5	5	1	3	3	3	3	2	4	2	4	3	3	2	4	2	4	2	4	1	5	0	6	6
Flow cytometry																																	
Aneuploid	4	0	1	3	2	2	2	2	0	4	2	2	2	2	2	2	2	2	0	4	1	3	0	4	0	4	0	4	1	3	0	4	4
Diploid	19	5	17	7	15	9	13	11	13	11	8	16	8	16	8	16	6	18	6	18	4	20	5	19	6	18	6	18	1	23	1	23	24
NA	10	2	7	5	6	6	4	8	4	8	4	8	2	10	2	10	4	8	4	8	4	8	4	8	3	9	2	10	2	10	0	12	12
Total	33	7	25	15	23	17	19	21	17	23	14	26	12	28	12	28	12	18	10	30	9	31	9	31	9	31	8	32	4	36	1	39	40

NA: not available, M: methylated by MS-PCR, U: unmethylated by MS-PCR, Low+HP: serrated adenoma with low-grade atypism and hyperplastic polyp.
Promoters marked with blue background indicate progressive increase of methylation according to histologic progression.

Table 3 Frequency of methylation in different histologic grades of serrated adenomas

	Low+HP	*Low*	*High*	*Carcinoma*	*P-value*
*p*14	0.0% (0/5)	26.1% (6/23)	50.0% (3/6)	83.3% (5/6)	0.001
TIMP3	20.0% (1/5)	43.5% (10/23)	50.0% (3/6)	83.3% (5/6)	0.03
FHIT	0.0% (0/5)	13.0% (3/23)	50.0% (3/6)	33.3% (2/6)	0.04
MGMT	0.0% (0/5)	21.7% (5/23)	16.7% (1/6)	50.0% (3/6)	0.08
*p*16	0.0% (0/5)	17.4% (4/23)	50.0% (3/6)	33.3% (2/6)	0.08
% methylation	21.05%	27.23%	30.70%	37.72%	0.04

Low+HP: serrated adenomas with low-grade atypism and hyperplastic area, Low: serrated adenomas with low-grade atypism, High: serrated adenomas with high-grade atypism, Carcinoma: serrated adenomas with carcinomas, % methylation: the average percent of genes methylated.

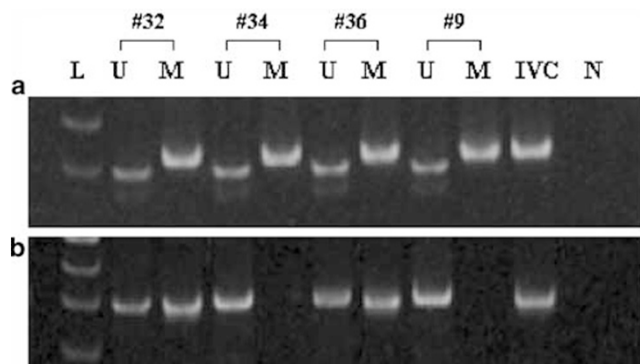

Figure 6 Representative examples of methylation-specific PCR analysis of SLC5A8 (**a**) and *p*16 (**b**) for low-grade serrated adenomas (#32 and #34), a high-grade serrated adenoma (#36), and a carcinoma with serrated adenoma (#9). L: Marker, U: unmethylated, M: methylated, IVC: *in vitro* methylated positive control, N: negative control.

Korea.[24] Our result is compatible with previous results reporting that high MSI and *hMLH1* methylation are uncommon in serrated adenomas. Most high-MSI tumors are admixed hyperplastic and adenomatous polyps rather than serrated polyps.[3,4,8,11,14,15] In our study, the right colon showed a higher percentage of methylation and more frequent methylation of the *p16*, *p14*, *TIMP3*, and *FHIT* genes than the left colon. These results are comparable with the results reported by Burri *et al*[25] using colonic carcinoma samples. It is believed that serrated adenomas and hyperplastic polyps occurring in the proximal colon in elderly females are at particular risk of progression via the serrated neoplasia pathway.[26] Both of the female patients with serrated adenomas in the right colon showed a high percentage of methylation, suggesting a close relation between methylation and a higher possibility of malignant transformation in this situation.

To date, methylation studies on serrated adenomas have been limited due to the rare occurrence of serrated adenomas and the large amounts of DNA that are needed for methylation studies.[14,15] Serrated adenomas larger than 0.5 cm were selected in this study, with 21 larger than 1 cm in diameter. As a result, a large-scale methylation study has been achieved using these serrated adenomas.

Although Iwabuchi *et al*[27] first reported aneuploidy in two serrated adenoma cases using an image analysis, DNA ploidy studies in serrated adenomas using flow cytometry have not been reported previously. We found that the frequencies of aneuploidization within serrated adenomas are similar to the results from conventional classical adenomas,[28] as detected with a highly sensitive crypt isolation technique and cytometric analysis. However, the serrated adenomas exclusively showed near-diploid DNA indices rather than the high aneuploid indices reported in traditional tubular or villous adenomas. Genetic instability of tumors is proportional to their degree of aneuploidy,[29,30] so the near-diploid DNA indices observed in our four aneuploid serrated adenomas and carcinomas suggest that subtle chromosomal changes may underlie the serrated neoplasia pathway. Aneuploidy observed in serrated adenomas with both low-grade atypism and carcinomas also suggests that aneuploidization occurs early during tumor progression. When examining the results obtained from our methylation study and the flow cytometric analyses, it seems clear that the widespread hypermethylation of promoters and the subtle chromosomal aberrations are early events in the progression of the serrated neoplasia pathway.

Here, we have reported frequent, tumor-specific methylation of *SLC5A8*; methylation of the *p16*, *MGMT*, *TIMP3*, *p14*, and *FHIT* genes associated with histologic progression; and no *hMLH1* methylation in serrated adenomas. The correlation of aberrant, progressive, widespread CpG-island methylation with the histologic progression and aneuploidization that occurs in low-grade serrated adenomas suggests these events occur early and are stable during the progression of the serrated neoplasia pathway.

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