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# Distinct WNT/β-catenin signaling activation in the serrated neoplasia pathway and the adenoma-carcinoma sequence of the colorectum

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**Sessile serrated adenoma/polyp (SSA/P) is considered as an early precursor in the serrated neoplasia pathway leading to colorectal cancer development. The conventional adenoma-carcinoma sequence is associated with activation of the WNT signaling pathway, although its role in serrated lesions is still controversial. To clarify differences in WNT signaling activation in association with *MLH1* methylation or *BRAF*/*KRAS* mutations between serrated and conventional routes, we performed β-catenin immunostaining, methylation-specific PCR for *MLH1* and WNT signaling associated genes such as *AXIN2*, *APC*, and *MCC* and *secreted frizzled-related proteins (SFRPs)*, and direct sequencing of *BRAF*/*KRAS* in 27 SSA/Ps, 14 SSA/Ps with high-grade dysplasia and 9 SSA/Ps with submucosal carcinoma, as well as 19 conventional adenomas, 26 adenomas with high-grade dysplasia and 25 adenomas with submucosal carcinoma. Nuclear β-catenin labelings were significantly lower in the serrated series than in their adenoma counterparts, and a significant increment in those labelings was found from SSA/Ps to those with high-grade dysplasia or submucosal carcinoma. The frequency of *MLH1* and *SFRP4* methylation was significantly higher in SSA/P series, as compared with corresponding adenoma series. *AXIN2* and *MCC* were more frequently methylated in SSA/Ps with high-grade dysplasia and those with submucosal carcinoma than in adenoma counterparts. Stepwise increment of *AXIN2* and *MCC* methylation was identified from SSA/Ps through those with high-grade dysplasia to those with submucosal carcinoma. A significant correlation was seen between nuclear β-catenin expression and methylation of *AXIN2* or *MCC* in the SSA/P series. *BRAF* mutation was more frequent, whereas *KRAS* mutation was less frequent in the SSA/P series as compared with the adenoma series. There was an inverse association of *BRAF* mutation with *AXIN2* methylation in SSA/P series. In conclusion, WNT/β-catenin signal activation mediated by the methylation of *SFRP4*, *MCC*, and *AXIN2* may make different contributions to colorectal neoplasia between the serrated and conventional routes.**

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Torlakovic *et al.*[1] reported evidence of abnormal proliferation in colorectal serrated polyps that superficially resembled hyperplastic polyps but that could be distinguished histologically on the basis of their abnormal architectural features, introducing the terms 'sessile serrated polyp' and 'sessile serrated adenoma.' Currently, this category is designated as sessile serrated adenoma/polyp according to the recommendations of the World Health Organization.[2] Sessile serrated adenoma/polyp (SSA/P) is considered as an early precursor lesion in the serrated neoplasia pathway, which results in colorectal carcinomas with high levels of microsatellite instability.[3–5] Recent studies have

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shown associations of SSA/P and those with dysplasia or carcinoma with methylation or loss of protein expression for DNA repair genes, ie, *MLH1*,[1,4,6–9] a CpG island methylator phenotype,[3,4,6,8] *BRAF* mutations,[3,4,6–14] and a lack of genetic alterations in *CTNNB1* (the gene coding for β-catenin protein).[14] This pathway is thought to be distinct from the conventional adenoma-carcinoma pathway, where adenomas progress to invasive colorectal carcinomas through the influence of a series of genetic alterations including *adenomatous polyposis coli* (*APC*) and *KRAS* mutations.[4,6,10,11,15,16]

The WNT signaling pathway has a vital role in embryogenesis,[17] and its deregulation is also implicated in colorectal carcinogenesis.[18] β-Catenin in the resting state is degraded by proteasomes resulting from its phosphorylation by a multiprotein complex containing APC, AXIN, and glycogen synthase kinase 3β (GSK3β). When WNT binds to the cell surface receptor Frizzled and activates disheveled, GSK3β is dissociated from this complex. As a result, free β-catenin accumulates and translocates into the nucleus and subsequently binds to the T-cell factor/lymphoid enhancer factor initiating transcription of target genes such as *c-myc*.[17] β-Catenin is also regulated by various other components such as *mutated in colorectal cancer* (*MCC*) and *secreted frizzled-related proteins* (*SFRPs*);[17] the functions of *MCC* or *SFRPs* as negative regulators of WNT/β-catenin signaling may have important implications in genesis of colorectal carcinomas[19–21] as well as SSA/P.[22] *AXIN2* has been found to be silenced, apparently as a result of methylation of its promoter region, specifically in colorectal carcinomas with high levels of microsatellite instability.[23] An association of *CTNNB1* mutations with microsatellite instability status was previously suggested in colorectal carcinomas.[24] Although the conventional adenoma-carcinoma pathway is associated with activation of the WNT/β-catenin signaling pathway,[15,16,18,25] any contribution to serrated neoplasia remains controversial.[13,14,22,25,26]

The aim of this study was thus to elucidate the potential roles of WNT/β-catenin signaling in association with *MLH1* methylation or *BRAF*/*KRAS* mutations in the serrated neoplasia pathway, in comparison with the conventional adenoma-carcinoma sequence.

## Materials and methods

### Patients and Materials

The materials for our study were 120 colorectal polyps (from 120 patients) resected endoscopically or surgically at Juntendo University Hospital and our affiliated hospitals between 2006 and 2012. These comprised 27 SSA/Ps, 14 SSA/Ps with high-grade dysplasia, 9 SSA/Ps with submucosal carcinoma, 19 conventional tubular adenomas, 26 tubular adenomas with high-grade dysplasia, and 25 tubular adenomas with submucosal carcinoma. The histologic features of the high-grade dysplasia were assessed according to the previous description[2,13] as follows: a tubular, tubulovillous, or fused glandular pattern, mimicking conventional adenomatous high-grade dysplasia or a serrated glandular pattern, preserving the serrated or sawtoothed structure with infolding of the crypt epithelium, which consisted of cuboidal and eosinophilic dysplastic cells with substantially larger nuclei, irregular thickening of the nuclear membrane (so-called 'serrated type' high-grade dysplasia). All samples were reviewed independently by two experienced gastrointestinal pathologists (HM and TY) applying the criteria for sessile serrated adenomas of Torlakovic *et al*.[1] Interobserver variation was resolved by re-evaluation and discussion to reach consensus. Data for clinicopathological features of polyps studied, including patient age, sex, location (proximal colon was classified as proximal to the splenic flexure and the remaining region was defined as distal), macroscopic type, and size of tumor, are summarized in Table 1. This study was approved by the Institutional Review Board and the ethical committee of our hospital (registration #2012015).

### Immunohistochemistry

Four μm-thick serial tissue sections prepared from formalin-fixed and paraffin-embedded tissues were subjected to immunohistochemistry. Monoclonal antibodies used in the present study were against β-catenin (clone 14, 1:200 dilution, BD Bioscience, San Diego, CA, USA). Antigen retrieval was executed by heating in an autoclave in Tris-EDTA buffer (pH 6.0). The sections were incubated at 4 °C for overnight by reaction with primary antibodies. Immunohistochemical staining was performed using an Envision Kit (Dako, Grostrup, Denmark) with substrate-chromogen solution.

For topological evaluation of the nuclear β-catenin labeling index (%), crypts in the lamina propria were separated into three equal zones (upper, middle, and lower thirds), and the number of immunoreactive nuclei per ~300 tumor cells were counted in each zone (for a total of ~1000 cells in whole crypts). Results are expressed as median percentages with interquartile ranges. The total nuclear β-catenin labeling index was additionally classified as follows: <5%, low expresser; 5–14%, intermediate expresser; ≥15%, high expresser. Slides were scored by two of the authors (TM and HM) independently, without previous knowledge of clinicopathological data or the genetic status of each polyp. Discrepancies were resolved by re-evaluation to reach consensus.

### Methylation Analysis of *MLH1*, *AXIN2*, *APC*, *MCC*, and *SFRPs*

Genomic DNA was extracted from five 10-μm-thick formalin-fixed paraffin-embedded sections using a

**Table 1** Clinicopathological characteristics of the colorectal polyps studied

| *Variable* | *SSA/P* (n = *27*) | *SSA/P with high-grade dysplasia* (n = *14*) | *SSA/P with submucosal carcinoma* (n = *9*) | *Conventional adenoma* (n = *19*) | *Adenoma with high-grade dysplasia* (n = *26*) | *Adenoma with submucosal carcinoma* (n = *25*) |
|---|---|---|---|---|---|---|
| Age (years) | 63 ± 11 (39–81) | 67 ± 10 (54–84) | 71 ± 10 (55–84) | 68 ± 9 (51–88) | 71 ± 10 (44–88) | 66 ± 8 (51–79) |
| *Sex* | | | | | | |
| Male | 14 | 9 | 3 | 14 | 14 | 18 |
| Female | 13 | 5 | 6 | 5 | 12 | 7 |
| *Location* | | | | | | |
| Proximal colon | 22 | 12 | 9 | 12 | 9 | 2 |
| Distal colon | 5 | 2 | 0 | 7 | 17 | 23 |
| *Macroscopic type* | | | | | | |
| Sessile | 27 | 14 | 8 | 12 | 17 | 14 |
| Semipedunculated | 0 | 0 | 1 | 6 | 1 | 3 |
| Pedunculated | 0 | 0 | 0 | 1 | 8 | 8 |
| Size of tumor (mm) | 13 ± 5 (3–25) | 12 ± 9 (5–36) | 10 ± 3 (6–15) | 11 ± 6 (4–24) | 17 ± 6 (10–32) | 17 ± 7 (8–30) |

Abbreviation: SSA/P, sessile serrated adenoma/polyp.
Age and tumor size are presented as mean ± s.d. (range) values.

**Table 2** Primers used for the methylation-specific PCR analysis

| *Gene* | *Forward primers (5′–3′)* | *Reverse primers (5′–3′)* | *Tm (°C )* | *Product size (bp)* | *PCR cycles* |
|---|---|---|---|---|---|
| *MLH1* | M: TTACGGGTAAGTCGTTTTGAC | M: CGCCACTACGAAACTAAACA | 58 | 100 | 35 |
| | UM: GGTTATGGGTAAGTTGTTTTGAT | UM: CACCACTACAAAACTAAACACA | 58 | 100 | 35 |
| *AXIN2* | M: ATATAGTTTAGCGGTTGGGAGTGC | M: CACTCGACCAAAACGCACG | 68 | 113 | 35 |
| | UM: ATAGTTTAGTGGTTGGGAGTGT | UM: CCACTCAACCAAAACACACA | 58 | 112 | 35 |
| *APC* | M: TATTGCGGAGTGCGGGTC | M: TCGACGAACTCCCGACGA | 64 | 98 | 35 |
| | UM: GTGTTTTATTGTGGAGTGTGGGTT | UM: CCAATCAACAAACTCCCAACAA | 61 | 108 | 35 |
| *MCC* | M: TATTGTTTCGGAACGGGGCGT | M: CAAAAAACTCGATAACGCGACG | 58 | 94 | 40 |
| | UM: GGTATTGTTTTGGAATGGGGTG | UM: CTCAATAACACAACACACTCAC | 58 | 99 | 40 |
| *SFRP1* | M: GGGGATTGCGTTTTTTGTTTTC | M: CATACCGACTCTACGCCCTA | 62 | 109 | 35 |
| | UM: GTTTTTTGTTTGTTGGGGTT | UM: ATAAAAATACACACCACCTC | 58 | 109 | 35 |
| *SFRP2* | M: GGGTTTGTAGCGTTTCGTTC | M: ACCCGCTCTCTTCGCTAAAT | 62 | 113 | 35 |
| | UM: GGGTTTGTAGTGTTTTGTT | UM: ACCCACTCTCTTCACTAAAT | 58 | 113 | 35 |
| *SFRP4* | M: GTTTTTTGTTTGTCGGGGTC | M: ATAAAAATACGCACCGCCTC | 62 | 133 | 35 |
| | UM: GTTTTTTGTTTGTTGGGGTT | UM: ATAAAAATACACACCACCTC | 58 | 133 | 35 |

Abbreviations: M, methylated DNA; Tm, annealing temperature; UM, unmethylated DNA.

QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Sections were stained lightly with hematoxylin, and areas of normal mucosa were excluded by modified microdissection with observation of the tissue directly under a light microscope. The quality and integrity of the DNA were checked spectrophotometrically.

Sensitive methylation-specific PCR was used to detect promoter methylation. Bisulfite modification was conducted using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). The bisulfate-treated DNA was then amplified using specifically designed primers for methylated and unmethylated alleles. Sequences of the primers, annealing temperature, and product size are listed in Table 2. After amplification, products were electrophoresed using 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

### Mutation Analysis of *BRAF* and *KRAS*

Mutation analyses for *BRAF* and *KRAS* were performed using genomic DNA derived from formalin-fixed paraffin-embedded tissue. Mutations were examined in exon 15 of *BRAF* and exon 2 of *KRAS* by PCR followed by direct sequencing. The primer sequences in this study were as previously

described.[27] Purified PCR products were sequenced with dideoxynucleotides (BigDye Terminator v3.1, Applied Biosystems, Foster City, CA, USA) and specific primers, purified using a BigDye X Terminator Purification Kit (Applied Biosystems), and then analyzed with a capillary sequencing machine (3730xl Genetic Analyzer, Applied Biosystems). Sequences were then examined with Sequencing Analysis V3.5.1 software (Applied Biosystems). Mutations were concluded if the height of the mutated peak reached 20% of the height of the normal peak.[28]

### Statistical Analysis

All statistical analyses were carried out using StatView for Windows Version 5.0 (SAS Institute Inc., Cary, NC, USA). Continuous data were compared with the Mann–Whitney *U*-test. Categorical analysis of variables was performed using either the $\chi^2$-test (with Yates' correction) or the Fisher's exact test, as appropriate. A *P*-value $<0.05$ was considered statistically significant.

## Results

### Expression of Nuclear β-Catenin

Total nuclear β-catenin labeling indices (Figure 1a) were significantly lower in SSA/Ps (median 2%; interquartile ranges 0–4%) than conventional tubular adenomas (22%; 14–37%, $P<0.001$), and a similar trend was observed between SSA/Ps with high-grade dysplasia (8%; 2–15%) and tubular adenomas with high-grade dysplasia (19%; 8–33%, $P=0.025$). The labeling indices tended to be lower in SSA/Ps with submucosal carcinoma (8%, 4–22%) than tubular adenomas with submucosal carcinoma (27%; 6–41%, $P=0.133$). Differences in the labeling indices between the two polyp groups were observed in each crypt zone (Figure 1b) but were largest in the upper crypt zone; the values being for SSA/Ps (0%; 0–1%) *vs* conventional tubular adenomas (22%; 8–43%, $P<0.001$), SSA/Ps with high-grade dysplasia (0%; 0–7%) *vs* tubular adenomas with high-grade dysplasia (19%; 3–39%; $P=0.006$), and SSA/Ps with submucosal carcinoma (6%; 4–20%) *vs* tubular adenomas with submucosal carcinoma (31%; 10–52%; $P=0.032$). Interestingly, a significant increment in nuclear β-catenin labeling indices was noted from SSA/Ps to those with high-grade dysplasia (SSA/Ps *vs* SSA/Ps with high-grade dysplasia, $P=0.026$) or SSA/Ps with submucosal carcinoma (SSA/Ps *vs* SSA/Ps with submucosal carcinoma, $P=0.001$), without differences between the latter two ($P=0.378$). In addition, their labelings were most prominent in the lower crypt zone in all SSA/P categories, but they did not differ in adenoma series. Low nuclear β-catenin expressers were most frequent in SSA/Ps, whereas high expressers were most prominent in conventional tubular adenomas ($P<0.001$). Similar tendencies were found between SSA/Ps with high-grade dysplasia and tubular adenomas with high-grade dysplasia or SSA/Ps with submucosal carcinoma and tubular adenomas with submucosal carcinoma, without statistical significance. High expressers were more frequent in SSA/Ps with high-grade dysplasia ($P=0.006$) and SSA/Ps with submucosal carcinoma ($P=0.003$) than SSA/Ps (Table 3). Typical morphology of the SSA/P series studied, and their expression of β-catenin in representative cases are illustrated in Figure 2.

### Methylation Analysis of *MLH1*, *AXIN2*, *APC*, *MCC* and *SFRPs*

Methylation-specific PCR products were successfully obtained in all samples. In normal mucosa, methylation of the genes was undetectable. Representative results of methylation-specific PCR analysis are illustrated in Figure 3, and frequencies of methylation for different lesions are summarized in Table 4. *MLH1* was methylated in 20 out of 27 (74%) SSA/Ps, 13 of 14 (93%) SSA/Ps with high-grade dysplasia, and 8 of 9 (89%) SSA/Ps with submucosal carcinoma, as opposed to 1 of 19 conventional tubular adenomas (5%; $P<0.001$), 3 of 26 tubular adenomas with high-grade dysplasia (12%; $P<0.001$), and 3 of 25 tubular adenomas with submucosal carcinoma (12%; $P<0.001$), respectively. Similar trends were found in the frequency of *SFRP4* methylation (SSA/Ps *vs* conventional tubular adenomas; SSA/Ps with high-grade dysplasia *vs* tubular adenomas with high-grade dysplasia; SSA/Ps with submucosal carcinoma *vs* tubular adenomas with submucosal carcinoma, $P=0.001$–0.006). *AXIN2* and *MCC* showed a highly frequency of methylation in SSA/Ps with high-grade dysplasia and those with submucosal carcinoma, as compared with tubular adenomas with high-grade dysplasia and those with submucosal carcinoma, respectively ($P\leq0.001$). Interestingly, stepwise increment of *AXIN2* and *MCC* methylation was identified from SSA/Ps through those with high-grade dysplasia to those with submucosal carcinoma ($P\leq0.001$).

### Mutation Analysis of *BRAF* and *KRAS*

Frequencies of *BRAF* and *KRAS* mutations in the polyps studied are summarized in Table 5. All of serrated groups had *BRAF,* but not *KRAS* mutations, whereas all of adenoma groups except for one tubular adenoma with high-grade dysplasia harbored *KRAS,* but not *BRAF* mutations (SSA/P groups *vs* adenoma groups, $P\leq0.003$). *BRAF* and *KRAS* mutations were mutually exclusive. All *BRAF* mutations were V600E (c.1799 T>A). With *KRAS* mutations for conventional tubular adenomas, four of five were G13D (c.38 G>A) and one was G12V (c.35 G>T), while three of six for tubular adenomas

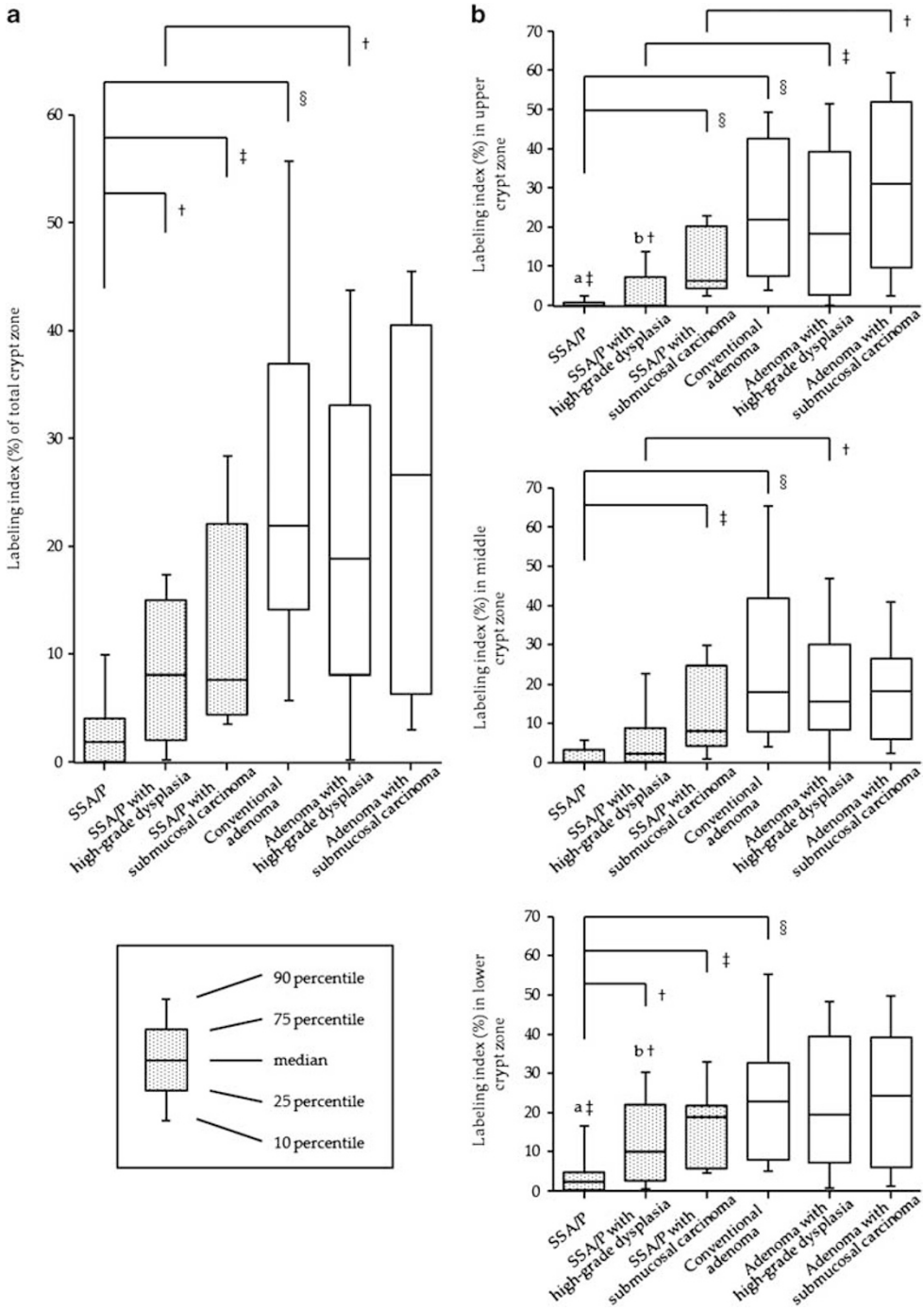



**Figure 1** Nuclear β-catenin labeling indices for the total crypt zone (**a**) and in the each (upper/middle/lower) crypt zone (**b**). Data are expressed as median percentages with interquartile ranges. †$P<0.05$; ‡$P<0.01$; §$P<0.001$; SSA/P, sessile serrated adenoma/polyp.

with high-grade dysplasia were G12D (c.35 G>A), two were G12V and one was G13D. With *KRAS* mutations for tubular adenomas with submucosal carcinoma, three each of seven were G12V and G13D and one was G12D.

A schematic depiction of β-catenin expression, in association with the results of methylation-specific PCR analyses and *BRAF*/*KRAS* mutations in each polyp type studied is shown diagrammatically in Figure 4.

### Associations of Nuclear β-Catenin Expresssion with Methylation of *MLH1* and *WNT* Signaling Associated Genes in Serrated Lesions

We further analyzed the correlation between the nuclear β-catenin expression with methylation status of *MLH1* and WNT signaling pathway genes including *AXIN2*, APC, *MCC*, and *SFRPs (1,2 and 4)* in SSA/P groups. Six out of 31 (19%) serrated

polyps with low nuclear β-catenin expression have demonstrated *AXIN2* methylation, while 5 of 7 (71%) high expressers were methylated ($P=0.013$). A similar trend was apparent between nuclear β-catenin expresser and *MCC* methylation status ($P=0.020$; Table 6).

### Associations of Nuclear β-Catenin Expression with BRAF Gene Mutations in Serrated lesions

We further analyzed the nuclear β-catenin expression and *BRAF* mutations in the SSA/P group ($n=50$), but there were no significant associations.

### Associations of Methylation of WNT Signaling Associated Genes with Mutation of *BRAF* Gene in Serrated Lesions

There was an inverse association of *BRAF* mutations with methylation of *AXIN2* in the SSA/P group ($P=0.021$; Table 7).

A schematic depiction of differences in nuclear β-catenin expression and methylation of WNT signaling associated genes between SSA/P and adenoma groups is shown diagrammatically in Figure 5.

## Discussion

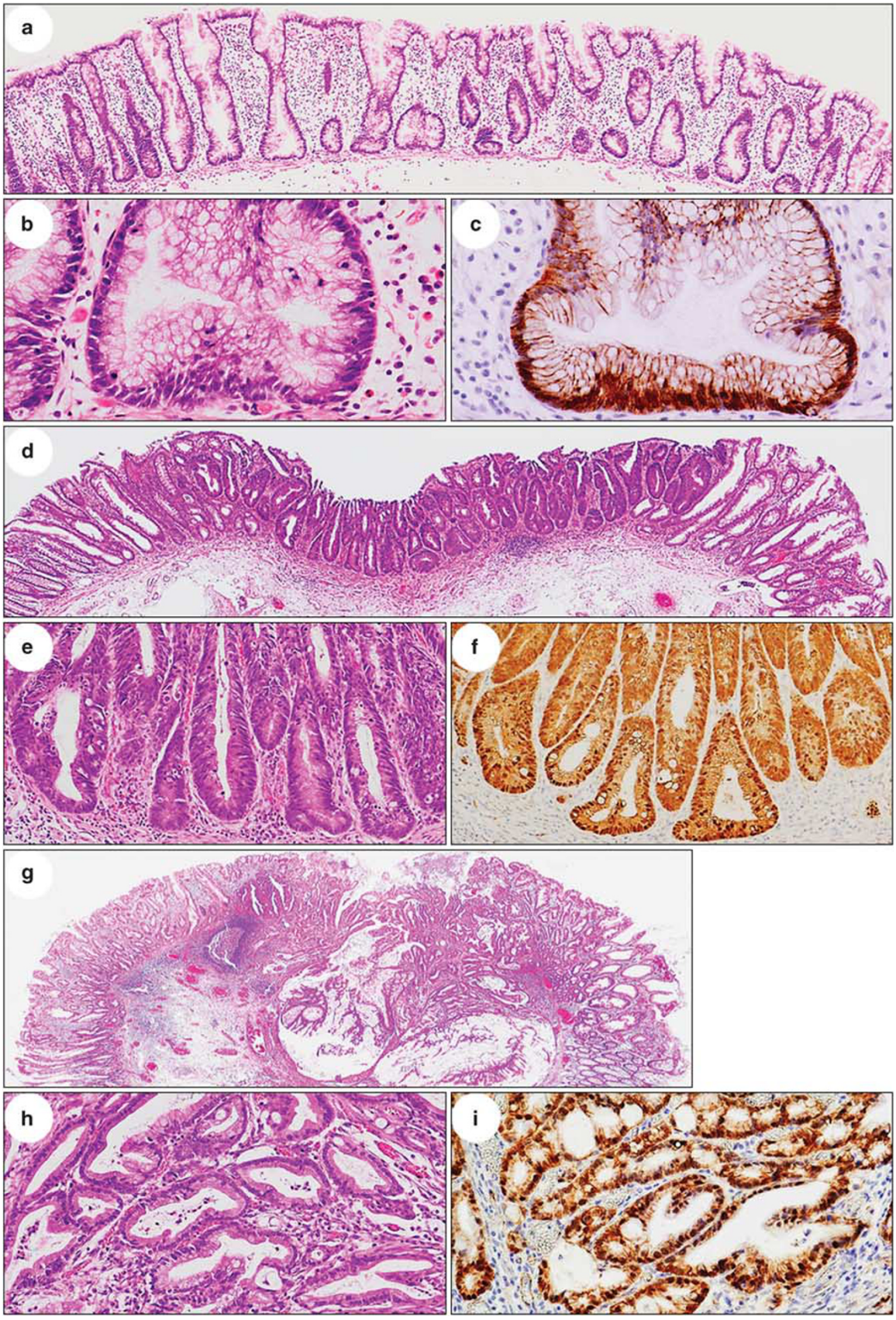
It is well established that the WNT signaling pathway involving β-catenin has a crucial role in the development of colorectal carcinomas through the conventional adenoma-carcinoma sequence.[18,25] However, the role of WNT/β-catenin signaling in the tumorigenesis of SSA/Ps is still controversial.[13,14,22,25,26] In previous reports, 18–100% of adenomas and 78% of adenocarcinomas of the colorectum displayed nuclear β-catenin immunoreactivity.[14,22,25] Various investigators have reported that nuclear β-catenin expression was observed in 0–60% of SSA/Ps,[7,9,13,14,22,25,26] 43–100% of SSA/Ps with high-grade dysplasia,[9,13,14] and 60% of SSA/Ps with submucosal carcinoma.[13] Nuclear β-catenin labeling indices in our study were significantly lower in the SSA/P series than the adenoma series, suggesting that levels of WNT/β-catenin signaling activation may be different between the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence. Interestingly, we found that nuclear β-catenin labeling indices were significantly increased with progression from SSA/Ps to those with high-grade dysplasia and with submucosal carcinoma, and that high expressers were more frequent in SSA/Ps with high-grade dysplasia and those with submucosal carcinoma than SSA/Ps. In addition, their labelings were most prominent in the lower crypt zone in all SSA/P categories. In this context, an earlier report of nuclear β-catenin expression in the lower crypt zone, but not in the upper or middle zone of SSA/P, is of interest.[26] However, no significant differences in their labelings were observed in each crypt zone of all adenoma groups. In normal colonic crypts, endocrine cells and Paneth cells exist in proliferative and intermediate regions, and goblet cells are present only in the intermediate region; however, numerous goblet cells are identified at the base of the crypts (proliferative region) as well as in the intermediate region in SSA/Ps.[1] In SSA/Ps, there are abnormalities in the location of the various compartments (especially in the lower crypt zone), a feature that Torlakovic *et al.* [1] described as abnormal proliferation or dysmaturation of crypt cells. It was conceivable that their histological features were associated with upward expression of nuclear β-catenin from the base of the crypts. In a recent report, nuclear β-catenin expression in SSA/P was detected solely by an N-terminus antibody, whereas its nuclear expression in adenoma was almost entirely detected by a C-terminus antibody.[22] This could explain at least some of the discrepancies in β-catenin immunoreactivity.

Sequencing of genomic DNA extracted from a subset of SSA/Ps and examples with dysplasia earlier failed to identify any *CTNNB1* mutation to account for abnormal β-catenin nuclear labeling.[14] Therefore, we conducted the current study and for the first time comparatively analyzed methylation of the WNT/β-catenin signaling associated genes such

**Table 3** Nuclear β-catenin expresser in the colorectal polyps studied

| | *SSA/P* (n = *27*) | *SSA/P with high-grade dysplasia* (n = *14*) | *SSA/P with submucosal carcinoma* (n = *9*) | *Conventional adenoma* (n = *19*) | *Adenoma with high-grade dysplasia* (n = *26*) | *Adenoma with submucosal carcinoma* (n = *25*) |
|---|---|---|---|---|---|---|
| Low expresser | 22 (81 %) | 6 (42 %) | 3 (33 %) | 2 (10 %) | 6 (23 %) | 6 (24 %) |
| Intermediate expresser | 5 (19 %) | 4 (29 %) | 3 (33 %) | 3 (16 %) | 4 (15 %) | 3 (12 %) |
| High expresser | 0 (0 %) | 4 (29 %) | 3 (33 %) | 14 (74 %) | 16 (62 %) | 16 (64 %) |

Abbreviation: SSA/P, sessile serrated adenoma/polyp.
Total nuclear β-catenin labeling index was classified as follows: <5%, low expresser; 5–14%, intermediate expresser; ≥15%, high expresser; SSA/P *vs* SSA/P with high-grade dysplasia, $P=0.006$; SSA/P *vs* SSA/P with submucosal carcinoma, $P=0.003$; SSA/P *vs* conventional adenoma, $P<0.001$.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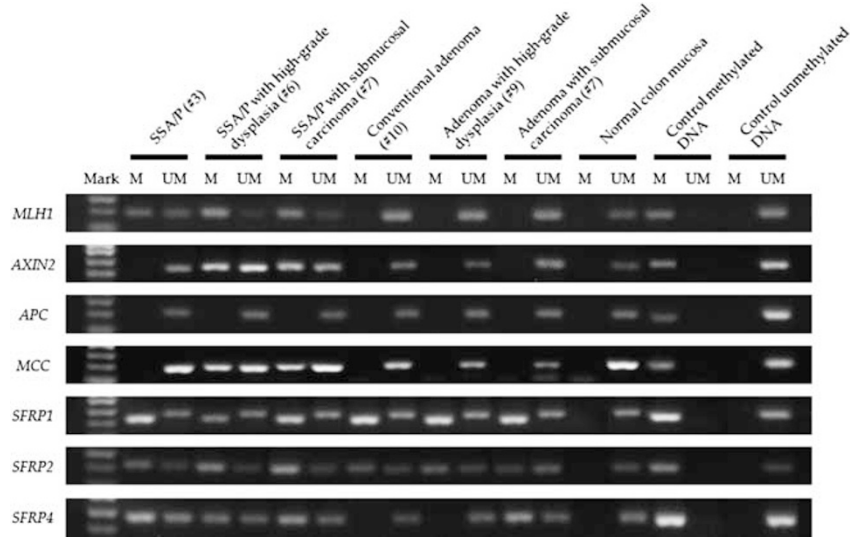


**Figure 3** Representative results of methylation-specific PCR in single cases of each groups and normal colon mucosa. Each lane contains products generated from separate PCR reactions using probes specific for methylated (M) or unmethylated (UM) DNA templates. Commercially available CpGs for completely methylated DNA and unmethylated DNA (methylated and unmethlyated EpiTect Control DNA, Qiagen) were used as controls. Blank controls without DNA template were included (not shown), and a 50-bp ladder was applied for molecular weight markers (*Mark*). SSA/P, sessile serrated adenoma/polyp.

**Table 4** Frequency of methylation of *MLH1*, *AXIN2*, *APC*, *MCC*, and *SFRPs* in the colorectal polyps studied

| | *SSA/P* (n = *27*) | *SSA/P with high-grade dysplasia* (n = *14*) | *SSA/P with submucosal carcinoma* (n = *9*) | *Conventional adenoma* (n = *19*) | *Adenoma with high-grade dysplasia* (n = *26*) | *Adenoma with submucosal carcinoma* (n = *25*) |
|---|---|---|---|---|---|---|
| *MLH1*[a] | 20 (74 %) | 13 (93 %) | 8 (89 %) | 1 (5 %) | 3 (12 %) | 3 (12 %) |
| *AXIN2*[b] | 1 (4 %) | 9 (64 %) | 7 (78 %) | 1 (5 %) | 3 (12 %) | 1 (4 %) |
| *APC* | 0 (0 %) | 0 (0 %) | 0 (0 %) | 2 (11 %) | 0 (0 %) | 1 (4 %) |
| *MCC*[c] | 4 (15 %) | 14 (100 %) | 9 (100 %) | 2 (11 %) | 4 (15 %) | 4 (16 %) |
| *SFRP1* | 25 (93 %) | 14 (100 %) | 9 (100 %) | 18 (95 %) | 22 (85 %) | 22 (88 %) |
| *SFRP2* | 26 (96 %) | 14 (100 %) | 9 (100 %) | 17 (89 %) | 23 (88 %) | 22 (88 %) |
| *SFRP4*[d] | 22 (81 %) | 14 (100 %) | 9 (100 %) | 7 (37 %) | 13 (50 %) | 12 (48 %) |

Abbreviation: SSA/P, sessile serrated adenoma/polyp.
[a]SSA/P *vs* conventional adenoma, $P < 0.001$; SSA/P with high-grade dysplasia *vs* adenoma with high-grade dysplasia, $P < 0.001$; SSA/P with submucosal carcinoma *vs* adenoma with submucosal carcinoma, $P < 0.001$.
[b]SSA/P *vs* SSA/P with high-grade dysplasia or SSA/P with submucosal carcinoma, $P < 0.001$; SSA/P with high-grade dysplasia *vs* adenoma with high-grade dysplasia, $P = 0.001$; SSA/P with submucosal carcinoma *vs* adenoma with submucosal carcinoma, $P < 0.001$.
[c]SSA/P *vs* SSA/P with high-grade dysplasia or SSA/P with submucosal carcinoma, $P < 0.001$; SSA/P with high-grade dysplasia *vs* adenoma with high-grade dysplasia, $P < 0.001$; SSA/P with submucosal carcinoma *vs* adenoma with submucosal carcinoma, $P < 0.001$.
[d]SSA/P *vs* conventional adenoma, $P = 0.005$; SSA/P with high-grade dysplasia *vs* adenoma with high-grade dysplasia, $P = 0.001$; SSA/P with submucosal carcinoma *vs* adenoma with submucosal carcinoma, $P = 0.006$.

**Figure 2** Typical morphology of the SSA/P series studied and expression of β-catenin in representative cases; (**a–c**) SSA/P (#22). (**a**) Low power view. SSA/P shows dilated crypts with horizontal growth along the muscularis mucosae and deep serration. (**b**) High power view of Figure 2a: SSA/P featuring goblet cell hyperplasia at the crypt base. (**c**) Immunostaining of β-catenin in same portion as Figure 2b. Nuclear staining of β-catenin (labeling index = 1.7%) is seen only at the crypt base. (**d–f**) SSA/P with high-grade dysplasia (#3). (**d**) High-grade dysplasia demonstrating cytologic atypia and architectural dysplasia without submucosal invasion. Adjacent SSA/P areas are seen at both ends of the lesion. (**e**) Dysplastic crypts with pseudostratified nuclei and loss of goblet cells mimicking conventional high-grade adenoma (high-grade dysplasia area of Figure 2d). (**f**) Expression of nuclear β-catenin is increased from the lower crypt zone, through the middle to upper zone in an area of high-grade dysplasia (labeling index = 16.9%). (**g–i**) SSA/P with submucosal carcinoma (#2). (**g**) SSA/P with submucosal carcinoma has architectural dysplasia with submucosal invasion and adjacent SSA/P. (**h**) High-grade cellular atypia is apparent in the submucosal invasive carcinoma. (**i**) β-Catenin is strongly expressed in almost all nuclei of invasive carcinoma cells (labeling index = 22.0%).

**Table 5** Frequency of *BRAF* and *KRAS* mutations in the colorectal polyps studied

| | *SSA/P* (n = *27*) | *SSA/P with high-grade dysplasia* (n = *14*) | *SSA/P with submucosal carcinoma* (n = *9*) | *Conventional adenoma* (n = *19*) | *Adenoma with high-grade dysplasia* (n = *26*) | *Adenoma with submucosal carcinoma* (n = *25*) |
|---|---|---|---|---|---|---|
| *BRAF*[a] | 22 (81%) | 9 (64%) | 4 (44%) | 0 (0%) | 1 (4%) | 0 (0%) |
| *KRAS*[b] | 0 (0%) | 0 (0%) | 0 (0%) | 5 (26%) | 6 (23%) | 7 (28%) |

Abbreviation: SSA/P, sessile serrated adenoma/polyp.
[a]SSA/P *vs* conventional adenoma, $P<0.001$; SSA/P with high-grade dysplasia *vs* adenoma with high-grade dysplasia, $P<0.001$; SSA/P with submucosal carcinoma *vs* adenoma with submucosal carcinoma, $P=0.003$.
[b]SSA/P *vs* conventional adenoma, $P=0.009$.

as *AXIN2*, *APC*, *MCC*, and *SFRPs* between the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence. In our study, *AXIN2*, *MCC*, and *SFRP4* were more frequently methylated in SSA/P series than in the corresponding adenoma counterparts. In addition, there was a progressive increase in the frequency of methylation from SSA/Ps through those with high-grade dysplasia to those with submucosal carcinoma, but not in the corresponding adenoma-carcinoma progression. In fact, there was a progressive increase in the number of genes methylated from SSA/Ps to those with high-grade dysplasia.[9]

*SFRP1* and *2* were earlier reported to be methylated in 90–100% of SSA/Ps and those with dysplasia[9] as well as in 80–90% of conventional tubular adenomas and adenocarcinomas.[19] By contrast, *SFRP4* was highly methylated in SSA/Ps (85%) and those with high-grade dysplasia (83%),[9] whereas its methylation was relatively low in conventional adenomas (24%) and adenocarcinomas (36%).[19] We also confirmed that *SFRP1*, *2*, and *4* were methylated in most (82–100%) of the SSA/P series, but figures for *SFRP4* were relatively low (37–50%) in the adenoma series. Consequently, silencing of *SFRP* genes, especially *SERF4*, induced by promoter methylation might have a more central role in the serrated neoplasia pathway than the conventional adenoma-carcinoma sequence.

Koinuma *et al.*[23] noted that *AXIN2* was frequently methylated in microsatellite instability associated colorectal carcinomas. In our study, *AXIN2* was more highly methylated in SSA/Ps with high-grade dysplasia and those with submucosal carcinoma, as compared with tubular adenomas with high-grade dysplasia and those with submucosal carcinoma. Interestingly, stepwise increment of *AXIN2* methylation was identified from SSA/Ps (4%) through those with high-grade dysplasia (64%) to those with submucosal carcinoma (78%), indicating that *AXIN2* methylation has an important role in the serrated neoplasia pathway as well as microsatellite instability associated colorectal carcinomas, some of which has been considered to be the end point of progression in the serrated pathway.[3–5]

Loss of APC function by gene mutation or methylation is the reason for β-catenin translocation into the nucleus in the conventional adenoma-carcinoma sequence.[18,25] The majority (60–91%) of conventional tubular adenomas or adenocarcinomas have *APC* mutations,[15,16] but to our knowledge no mutational study of *APC* has been conducted in SSA/Ps, although methylation of *APC* has been reported to be more frequent in tubular adenomas (56–65%) than SSA/Ps (22–25%).[8,20] In the present work, *APC* was not methylated in our SSA/P series, suggesting that methylation of *APC* is not responsible for nuclear translocation of β-catenin. In immunohistochemical studies, strong *APC* expression was observed in most SSA/Ps, whereas *MCC* expression was reported to be frequently lost.[21,22] *MCC* methylation is more common in SSA/Ps (89%) than in adenomas (35%).[20] Our study showed that *MCC* was methylated in 15% of SSA/Ps, and in all of SSA/Ps with high-grade dysplasia and those with submucosal carcinoma, but only 11–16% of the adenoma series. In our SSA/P series, a fairly strong correlation was evident between nuclear β-catenin expression and methylation of *AXIN2* or *MCC*. Historical data and our results therefore suggest that the WNT/β-catenin signal activation mediated by methylation of *SFRP4*, *MCC*, and *AXIN2,* but not *APC,* may differently contribute between the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence (Figure 5).

*MLH1* methylation has been reported to be present in 14–75% of SSA/Ps,[4,6–9] 73% of SSA/Ps with dysplasia, and 50% of adenocarcinomas arising in SSA/Ps.[4,8] In our study, *MLH1* was more frequently methylated not only in SSA/Ps (74%) but also in those with high-grade dysplasia (93%) and those with submucosal carcinoma (89%), compared with the corresponding adenoma groups (tubular adenomas, 5%; those with high-grade dysplasia, 12%; those with submucosal carcinoma, 12%). The wide range in the rates may be due to variation in the primers or methodology used. In a recent study in which two separate experiments were conducted using different primers, the frequency of *MLH1*

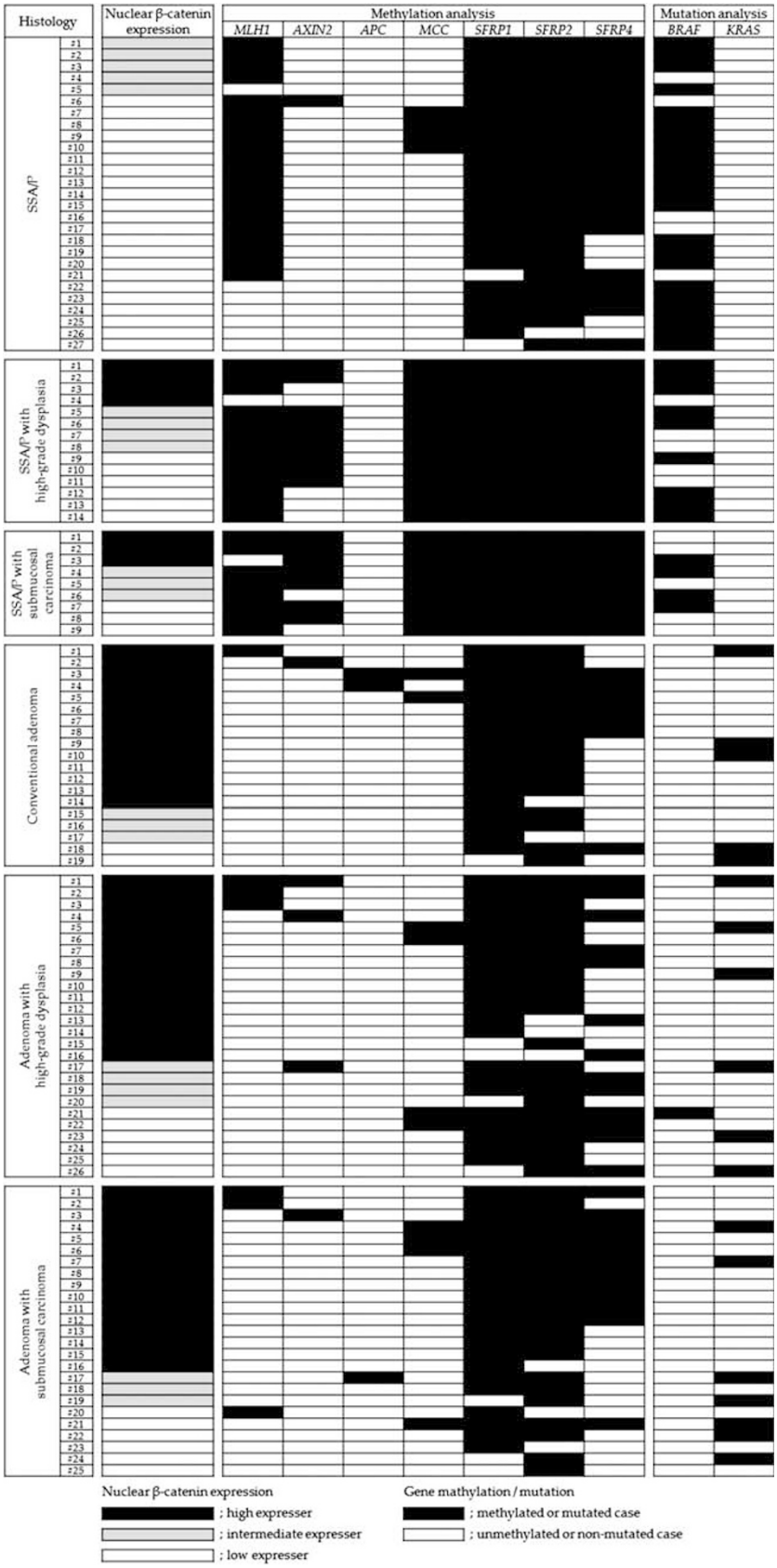


**Figure 4** Schematic depiction of β-catenin expresser, methylations of *MLH1* or WNT signaling associated genes and *BRAF*/*KRAS* mutations in each polyp studied. SSA/P, sessile serrated adenoma/polyp.

**Table 6** Associations of nuclear β-catenin expresser with gene methylation in serrated lesions

| | | *Nuclear β-catenin expresser* | | | |
|---|---|---|---|---|---|
| | *Total* | *Low* | *Intermediate* | *High* | P-*value* |
| *MLH1* methylation | | | | | |
| Yes | 41 (82%) | 25 (81%) | 11 (92%) | 5 (71%) | NS |
| No | 9 (18%) | 6 (19%) | 1 (8%) | 2 (29%) | |
| *AXIN2* methylation | | | | | |
| Yes | 17 (34%) | 6 (19%) | 6 (50%) | 5 (71%) | 0.013 |
| No | 33 (66%) | 25 (81%) | 6 (50%) | 2 (29%) | |
| *APC* methylation | | | | | |
| Yes | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | NS |
| No | 50 (100%) | 31 (100%) | 12 (100%) | 7 (100%) | |
| *MCC* methylation | | | | | |
| Yes | 27 (54%) | 13 (42%) | 7 (58%) | 7 (100%) | 0.02 |
| No | 23 (46%) | 18 (58%) | 5 (42%) | 0 (0%) | |
| *SFRP1* methylation | | | | | |
| Yes | 48 (96%) | 29 (94%) | 12 (100%) | 7 (100%) | NS |
| No | 2 (4%) | 2 (6%) | 0 (0%) | 0 (0%) | |
| *SFRP2* methylation | | | | | |
| Yes | 49 (98%) | 30 (97%) | 12 (100%) | 7 (100%) | NS |
| No | 1 (2%) | 1 (3%) | 0 (0%) | 0 (0%) | |
| *SFRP4* methylation | | | | | |
| Yes | 45 (90%) | 26 (84%) | 12 (100%) | 7 (100%) | NS |
| No | 5 (10%) | 5 (16%) | 0 (0%) | 0 (0%) | |

Abbreviation: NS, not significant.

**Table 7** Associations of gene methylation with *BRAF* mutations in serrated lesions

| | | *BRAF mutation* | | |
|---|---|---|---|---|
| | *Total* | *Yes* | *No* | P-*value* |
| *MLH1* methylation | | | | |
| Yes | 41 (82%) | 27 (77%) | 14 (93%) | NS |
| No | 9 (18%) | 8 (23%) | 1 (7%) | |
| *AXIN2* methylation | | | | |
| Yes | 17 (34%) | 8 (23%) | 9 (60%) | 0.021 |
| No | 33 (66%) | 27 (77%) | 6 (40%) | |
| *APC* methylation | | | | |
| Yes | 0 (0%) | 0 (0%) | 0 (0%) | NS |
| No | 50 (100%) | 35 (100%) | 15 (100%) | |
| *MCC* methylation | | | | |
| Yes | 27 (54%) | 17 (49%) | 10 (67%) | NS |
| No | 23 (46%) | 18 (51%) | 5 (33%) | |
| *SFRP1* methylation | | | | |
| Yes | 48 (96%) | 34 (97%) | 14 (93%) | NS |
| No | 2 (4%) | 1 (3%) | 1 (7%) | |
| *SFRP2* methylation | | | | |
| Yes | 49 (98%) | 34 (97%) | 15 (100%) | NS |
| No | 1 (2%) | 1 (3%) | 0 (0%) | |
| *SFRP4* methylation | | | | |
| Yes | 45 (90%) | 30 (86%) | 15 (100%) | NS |
| No | 5 (10%) | 5 (14%) | 0 (0%) | |

Abbreviation: NS, not significant.

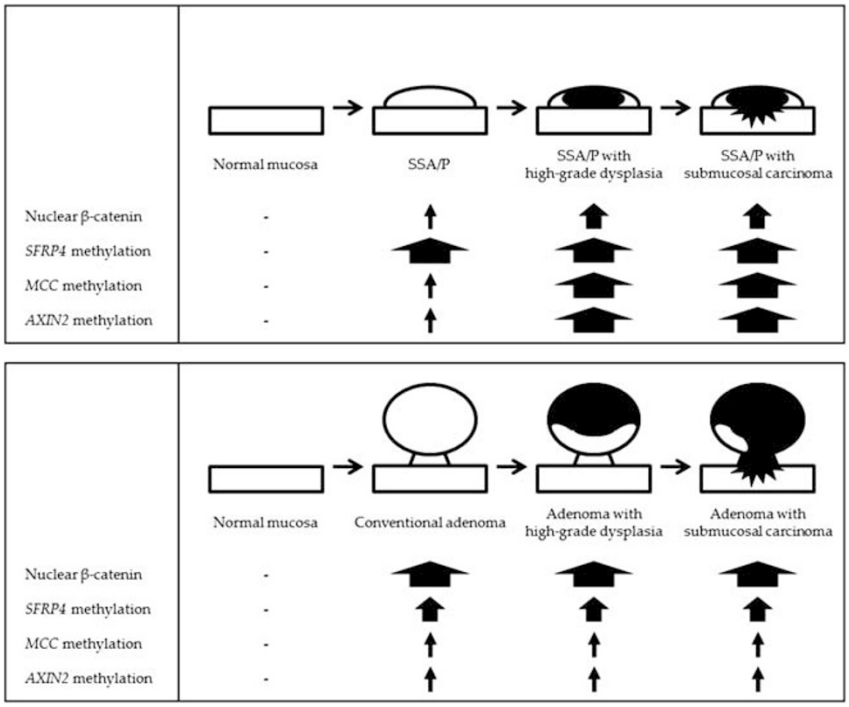


**Figure 5** Differences in nuclear β-catenin expression and methylation of WNT signaling associated genes in the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence. Nuclear β-catenin immunoreactivity: −, none; small arrow, low expresser; medium sized arrow, intermediate expresser; large arrow, high expresser; frequency of methylation: −, none; small arrow, 1–20%; medium sized arrow, 20–50%; large arrow, ≥51%; SSA/P, sessile serrated adenoma/polyp.

methylation was 73% and 23% in SSA/Ps.[8] We noted no significant associations of nuclear β-catenin expression with *MLH1* methylation, in line with the finding that colorectal carcinomas with *MLH1* methylation showed no *CTNNB1* mutation.[24]

Rare occurrence of *BRAF* mutations has been documented for conventional adenomas (0–5%), although they are frequent in SSA/Ps (50–90%).[4,6–13] In contrast, *KRAS* mutations have shown to be rare in SSA/Ps (0–8%), but more common in conventional tubular adenomas (5–37%).[4,6,10,11,13] In our study, *BRAF* mutations were frequent (82%), whereas *KRAS* mutation was not detected in SSA/Ps, with clearly contrasting results for tubular adenomas (*BRAF* mutation, 0%, *KRAS* mutation, 26%). Similar trends were found in SSA/Ps with high-grade dysplasia *vs* tubular adenomas with high-grade dysplasia and SSA/Ps with submucosal carcinoma *vs* tubular adenomas with submucosal carcinoma. Any association between activation of the RAS-RAF-MAPK pathway and WNT/β-catenin signaling activation in the serrated neoplasia pathway is clearly of interest. In the present study, *BRAF* mutation resulting in activation of the RAS-RAF-MAPK pathway was inversely correlated with *AXIN2* methylation as indicated by WNT/β-catenin signaling activation in SSA/P series. These findings support the hypothesis that activation of those signal pathways is mutually exclusive in the serrated neoplasia pathway. In contrast, colorectal carcinomas with *BRAF* mutation more frequently harbored *AXIN2* methylation than those without.[23]

In conclusion, we here obtained evidence pointing to different mechanisms of WNT/β-catenin signal activation, ie, methylation of *SFRP4*, *MCC*, and *AXIN,* between the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence. SSA/Ps may grow into subsequent SSA/Ps with high-grade dysplasia or those with submucosal carcinoma more rapidly at least in some patients.[29] *SFRP1* methylation in stool DNA has already shown to be useful in early detection of colorectal carcinomas.[30] With this approach, *SFRP4* would appear to be a good candidate for screening for precursors in the serrated neoplasia pathway. Further study is needed to elucidate WNT/β-catenin signal activation in this pathway in more detail and to confirm clinical utility of such markers because the number of cases of SSA/P with dysplastic (malignant) transformation was limited in the present study.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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