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

Original article

Title : Distinct Wnt/β-catenin signalling activation in the serrated neoplasia pathway and the adenoma-carcinoma sequence of the colorectum

Takashi Murakami [1,2], Hiroyuki Mitomi [3], Tsuyoshi Saito [1], Michiko Takahashi [1], Naoto Sakamoto [2], Naoshi Fukui [4], Takashi Yao [1], Sumio Watanabe [2]

[1]Department of Human Pathology, Juntendo University School of Medicine, Tokyo, Japan; [2]Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan; [3]Department of Surgical and Molecular Pathology, Dokkyo Medical University School of Medicine, Tochigi, Japan; [4]Clinical Research Center, National Hospital Organization Sagamihara Hospital, Kanagawa, Japan

Takashi Murakami, M.D., Departments of Gastroenterology and of Human Pathology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. t-murakm@juntendo.ac.jp

Hiroyuki Mitomi, M.D. Ph.D., Department of Surgical and Molecular Pathology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Shimotsuga, Tochigi, 321-0293, Japan. E-mail: hmitomi@dokkyomed.ac.jp

Tsuyoshi Saito, M.D. Ph.D., Department of Human Pathology, Juntendo University School of Medicine, 1-1-19 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. tysaitou@juntendo.ac.jp

Michiko Takahashi, B.Sc., Department of Human Pathology, Juntendo University School of Medicine, 1-1-19 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. mctakaha@juntendo.ac.jp

Naoto Sakamoto, M.D. Ph.D., Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. sakamoto@juntendo.ac.jp

Naoshi Fukui, M.D. Ph.D., Clinical Research Center, National Hospital Organization Sagamihara Hospital, 18-1 Sakuradai, Sagamihara, Kanagawa 228-8522, Japan. n-fukui@sagamihara-hosp.gr.jp

Takashi Yao, M.D. Ph.D., Department of Human Pathology, Juntendo University School of Medicine, 1-1-19 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. tyao@juntendo.ac.jp

Sumio Watanabe, M.D. Ph.D., Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. sumio@juntendo.ac.jp

Corresponding author: Hiroyuki Mitomi, M.D. Ph.D.

Department of Surgical and Molecular Pathology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Shimotsuga, Tochigi 321-0293, Japan. Phone: 81-282-87-2130; Fax: 81-282-86-1681

Phone: 81-282-87-2130; Fax: 81-282-86-1681; E-mail: hmitomi@dokkyomed.ac.jp

Running title : Wnt/β-catenin and serrated pathway

Abstract :

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, though its role in serrated lesions is still controversial. To clarify differences in Wnt signaling activation in association with *hMLH* methylation or *BRAF*/*Kras* mutations between serrated and conventional routes, we performed β-catenin immunostaining, methylation specific PCR for *hMLH1* or Wnt signaling associated genes such as *AXIN2*, *APC*, *MCC* and *secreted frizzled-related proteins (SFRPs)*, and direct sequencing of *BRAF*/*Kras* in 27 SSA/Ps, 14 SSA/Ps with high grade dysplasia (SSA/P-HDs) and 9 SSA/Ps with submucosal carcinoma (SSA/P-CAs), as well as 19 conventional tubular adenomas (ADs), 26 ADs with high grade dysplasia (AD-HDs) and 25 ADs with submucosal carcinoma (AD-CAs). Nuclear β-catenin labeling was significantly lower in the SSA/P series than in their AD counterparts whereas a significant increment was found from SSA/Ps to SSA/P-HDs or SSA/P-CAs. The frequency of *hMLH1* and *SFRP4* methylation was significantly higher in SSA/Ps, SSA/P-HDs and SSA/P-CAs, as compared to corresponding AD series. Although *APC* was only rarely methylated in all groups, *AXIN2* and *MCC* were more frequently methylated in SSA/P-HDs and SSA/P-CAs than in AD counterparts. Stepwise increment of *AXIN2* and *MCC* methylation was identified from SSA/Ps through SSA/P-HDs to SSA/P-CAs. 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, while *Kras* mutation was less frequent in the SSA/P as compared to the AD series. There was an inverse association of *BRAF* mutation with *AXIN2* methylation in SSA/P lesions. In conclusion, Wnt/β-catenin signal activation mediated by methylation of *SFRP4*, *MCC* and *AXIN2* may make different contributions to colorectal neoplasia between the serrated and conventional routes.

Key Words : sessile serrated adenoma/polyp, serrated neoplasia pathway, Wnt/β-catenin signaling, conventional adenoma-carcinoma sequence, secreted frizzled-related protein

Introduction

Torlakovic et al. 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 [1]." Currently, this category is designated as sessile serrated adenoma/polyp (SSA/P) according to the recommendations of the World Health Organization [2]. SSA/P is considered as an early precursor lesion in the serrated neoplasia pathway, which results in colorectal carcinomas (CRCs) with high levels of microsatellite instability (MSI) [3-5]. Recent studies have shown associations of SSA/Ps and those with dysplasia or carcinoma with methylation or loss of protein expression for DNA repair genes, *i.e.*, *hMLH1* [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 CRCs 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/Wingless signaling pathway plays 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 CRCs [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 CRCs with MSI [23]. An association of *CTNNB1* mutations with MSI status was previously suggested in CRCs [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 *hMLH* 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 sessile serrated adenomas/polyps (SSA/Ps), 14 SSA/Ps with high grade dysplasia (SSA/P-HDs), 9 SSA/Ps with submucosal carcinoma (SSA/P-CAs), 19 conventional tubular adenomas (ADs), 26 ADs with high grade dysplasia (AD-HDs), and 25 ADs with submucosal carcinoma (AD-CAs). 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 reevaluation 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 supplementary 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 (LI; %), tumor glands in the lamina propria were separated into three equal zones (upper, middle and lower thirds), and the number of immunoreactive nuclei per approximately 300 tumor cells were counted in each zone (for a total of approximately 1,000 cells in whole glands). Results are expressed as median percentages with interquartile ranges (IQRs). The total nuclear β-catenin LI was additionally classified as follows: < 5%, low expresser; 5 - 14%, intermediate expresser; ≥ 15%, high expresser. Slides were scored by 2 of the authors (T.M. and H.M.) independently, without prior knowledge of clinicopathological data or the genetic status of each polyp. Discrepancies were resolved by re-evaluation to reach consensus.

Methylation analysis of hMLH1, AXIN2, APC, MCC, *and* SFRPs

Genomic DNA was extracted from five 10-μm-thick formalin-fixed paraffin-embedded sections using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH , Hilden, Germany), according to the manufacturer's instructions. Sections were stained lightly with hematoxylin and areas of tumor were separated 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 (MSP) 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 supplementary 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-squared 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 LIs (Fig. 1A) were significantly lower in SSA/Ps (median 1.9%; IQRs 0.2 - 4.1%) than ADs (21.9%; 14.1 - 36.9%, $P < 0.001$) and a similar trend was observed between SSA/P-HDs (8.1%; 2.1 - 15.0%) and AD-HDs (18.9%; 8.1 - 33.1%, $P = 0.025$). The LIs tended to be lower in SSA/P-CAs (7.6%, 4.4 - 22.0%) than AD-CAs (26.7%; 6.4 - 40.5%, $P = 0.133$). Differences in the LIs between the two polyp groups were observed in each crypt zone (Fig. 1B), but were largest in the upper crypt zone; the values being for SSA/Ps (0%; 0 - 0.6%) vs. ADs (22.0%; 7.6 - 42.7%, $P < 0.001$), SSA/P-HDs (0.2%; 0 - 7.1%) vs. AD-HDs (18.5%; 2.6 - 39.2%; $P = 0.006$), and SSA/P-CAs (6.1%; 4.4 - 20.3%) vs. AD-CAs (31.1%; 9.7 - 51.9% ; $P = 0.032$). Interestingly, a significant increment in nuclear β-catenin LIs was noted from SSA/Ps to SSA/P-HDs (SSA/Ps vs. SSA/P-HDs, $P = 0.026$) or SSA/P-CAs (SSA/Ps vs. SSA/P-CAs, $P = 0.001$), without differences between the latter two ($P = 0.378$). Low nuclear β-catenin expressers were most frequent in SSA/Ps whereas high expressers were most prominent in ADs ($P < 0.001$). Similar tendencies were found between SSA/P-HDs and AD-HDs or SSA/P-CAs and AD-CAs, without statistical significance. High expressers were more frequent in SSA/P-HDs ($P = 0.006$) and SSA/P-CAs ($P = 0.003$) than SSA/Ps (Table 1). Typical morphology of the SSA/P series studied and their expression of β-catenin in representative cases are illustrated in Fig. 2.

Methylation analysis of hMLH1, AXIN2, APC, MCC *and* SFRPs

MSP products were successfully obtained in all samples. In normal mucosa, methylation of

the genes was undetectable. Representative results of MSP analysis are illustrated in supplementary Fig. 1, and frequencies of methylation for different lesions are summarized in Table 2. *hMLH1* was methylated in 20 out of 27 (74.1%) SSA/Ps, 13 of 14 (92.9%) SSA/P-HDs and 8 of 9 (88.9%) SSA/P-CAs, as opposed to 1 of 19 ADs (5.3%; $P < 0.001$), 3 of 26 AD-HDs (11.5%; $P < 0.001$) and 3 of 25 AD-CAs (12.0%; $P < 0.001$), respectively. Similar trends were found in the frequency of *SFRP4* methylation (SSA/Ps *vs.* ADs; SSA/P-HDs *vs.* AD-HDs; SSA/P-CAs *vs.* AD-CAs, $P = 0.001$ - 0.006). *AXIN2* and *MCC* showed a highly frequency of methylation in SSA/P-HDs and SSA/P-CAs, as compared with AD-HDs and AD-CAs, respectively ($P \leq 0.001$). Interestingly, stepwise increment of *AXIN2* and *MCC* methylation was identified from SSA/Ps through SSA/P-HDs to SSA/P-CAs ($P \leq 0.001$).

Mutation analysis of BRAF *and* Kras

A schematic representation of *BRAF* and *Kras* mutational patterns is shown in supplementary Fig. 2, and frequencies of *BRAF* and *Kras* mutations in the polyps studied are summarized in Table 3.

All of SSA/P groups had *BRAF,* but not *Kras* mutations whereas all of AD groups except for one AD-HD harbored *Kras,* but not *BRAF* mutations (SSA/P groups *vs.* AD groups, $P \leq 0.003$). *BRAF* and *Kras* mutations were mutually exclusive. All *BRAF* mutations were V600E (c.1799 T>A). With *Kras* mutations for ADs, four of five were G13D (c.38 G>A) and one was G12V (c.35 G>T), while three of six for AD-HDs were G12D (c.35 G>A), two were G12V and one was G13D. With *Kras* mutations for AD-CAs, 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 MSP analyses and *BRAF*/*Kras* mutations in each polyp type studied is shown diagrammatically in supplementary Fig. 3.

Associations of nuclear β-catenin expresssion with methylation of hMLH1 *and Wnt signaling associated genes in serrated lesions*

We further analyzed the correlation between the nuclear β-catenin expression with methylation status of Wnt signaling pathway genes including *hMLH1*, *AXIN2*, APC, *MCC* and *SFRPs (1,2 and 4)* in SSA/P groups. Six out of 31 (19.4%) serrated polyps with low nuclear β-catenin expression have demonstrated *AXIN2* methylation while 5 of 7 (71.4%) high expressors were methylated ($P = 0.013$). A similar trend was apparent between nuclear β-catenin expressor and *MCC* methylation status ($P = 0.020$; Table 4).

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 SSA/P groups ($n = 50$), 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 5).

A schematic depiction of differences in nuclear β-catenin expression and methylation of Wnt signaling associated genes between SSA/P and AD groups is shown diagrammatically in Fig 3.

Discussion

It is well established that the Wnt signaling pathway involving β-catenin plays 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 ADs and 78% of CRCs 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/P-HDs [9, 13, 14] and 60% of SSA/P-CAs [13]. Nuclear β-catenin LIs in our study were significantly lower in the SSA/P series than the AD series, suggesting that levels of Wnt/β-catenin signalling activation may be different between the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence. Interestingly, we found that nuclear β-catenin LIs were significantly increased with progression from SSA/Ps to SSA/P-HDs and SSA/P-CAs, and that high expressers were more frequent in SSA/P-HDs and SSA/P-CAs than SSA/Ps. In addition, labeling was most prominent in the lower crypt zone in all 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 nuclear β-catenin LIs were observed among AD groups, as well as in each crypt zones of AD groups. In a recent report, nuclear β-catenin expression in SSA/P was detected solely by an N-terminus antibody whereas its nuclear expression in AD 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 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 AD counterparts. In addition, there was a progressive increase in frequency of methylation from SSA/Ps through SSA/P-HDs to SSA/P-CAs, but not from ADs through AD-HDs to AD-CAs. In fact, there was a progressive increase in the number of genes methylated from SSA/Ps to SSA/P-HDs [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 adenomas and CRCs [19]. By contrast, *SFRP4* was highly methylated in SSA/Ps (85%) and SSA/P-HDs (83%) [9], whereas its methylation was relatively low in adenomas (24%) and CRCs (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 AD series. Consequently, silencing of *SFRP* genes, especially *SERF4*, induced by promoter methylation might play a more central role in the serrated neoplasia pathway than the conventional adenoma-carcinoma sequence.

Koinuma et al noted that *AXIN2* was frequently methylated in MSI associated CRCs [23]. In our study, *AXIN2* was more highly methylated in SSA/P-HDs and SSA/P-CAs, as compared to AD-HDs and AD-CAs. Interestingly, stepwise increment of *AXIN2* methylation was identified from SSA/Ps (4%) through SSA/P-HDs (64%) to SSA/P-CAs (78%), indicating that *AXIN2* methylation plays an important role in serrated neoplasia pathway as well as MSI associated CRCs, some of which has been considered to be the endpoint of SSA/P progression [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 ADs or CRCs have *APC* mutations [15, 16], but to our knowledge no mutational study of *APC* has been conducted in SSA/P, although methylation of *APC* has been reported to be more frequent in ADs (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 ADs (35%) [20]. Our study showed that *MCC* was methylated in 15% of SSA/Ps, and in all of SSA/P-HDs and SSA/P-CAs, but only 11 - 16% of the AD 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 (Fig. 3).

hMLH1 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, *hMLH1* was more frequently methylated not only in SSA/Ps (74%), but also in SSA/P-HDs (93%) and SSA/P-CAs (89%), compared to the corresponding AD groups (ADs, 5%; AD-HDs, 12%; AD-CAs, 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 *hMLH1* methylation was 73% and 23% in SSA/Ps [8]. We noted no significant associations of nuclear β-catenin expression with *hMLH1* methylation, in line with the finding that CRCs with *hMLH* methylation showed no β-catenin 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]. On the other hand, *Kras* mutations have shown to be rare in SSA/Ps (0 - 8%), but more common in ADs (5 - 37%) [4, 6, 10, 11, 13]. In our study, *BRAF* mutations were frequent (82%), while *Kras* mutation was not detected in SSA/Ps, with clearly contrasting results for ADs (*BRAF* mutation, 0%, *Kras* mutation, 26%). Similar trends were found in SSA/P-HDs *vs.* AD-HDs and SSA/P-CAs *vs.* AD-CAs. 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, CRCs 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, *i.e.,* methylation of *SFRP4*, *MCC* and *AXIN*, between the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence. SSA/Ps may grow into subsequent SSA-HD or SSA/P-CA more rapidly at least in some patients [29]. *SFRP1* methylation in stool DNA has already shown to be useful in early detection of CRCs [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.

Disclosure/conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Dr. Seiji Igarashi (Division of Pathology, Tochigi Cancer Center), Dr. Shin-ich Ban (Department of Pathology, Saiseikai Kawaguchi General Hospital), Dr. Minako Hirahashi (Department of Anatomic Pathology, Pathological Sciences, Graduate School of

Medical Sciences, Kyushu University), and Dr. Yumi Oshiro (Department of Pathology, Matsuyama Red Cross Hospital) for kindly providing samples and clinical information. The authors also are grateful to Mrs. Keiko Mitani for her histological assistance. The work was supported in part by a Grant-in-Aid from the Japan Society for the Promotion of Science (#24590429 to H. Mitomi and #23590434 to T. Saito).

References

1. Torlakovic E, Skovlund E, Snover DC, Torlakovic G, Nesland JM. Morphologic Reappraisal of Serrated Colorectal Polyps. Am J Surg Pathol 2003;27:65-81.
2. Snover DC, Ahnen DJ, Burt RW, Odze RD. Serrated polyps of the colon and rectum and serrated polyposis, In: Bosman FT, Carneiro F, Hruban RH, Theise ND, (eds). WHO Classification of Tumours of the Digestive System. IARC Press: Lyon, France; 2010, pp 160-165.
3. Kambara T, Simms LA, Whitehall VL, et al. *BRAF* mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004;53:1137-44.
4. O'Brien MJ, Yang S, Mack C, et al. Comparison of Microsatellite Instability, CpG Island Methylation Phenotype, BRAF and KRAS Status in Serrated Polyps and Traditional Adenomas Indicates Separate Pathways to Distinct Colorectal Carcinoma End Points. Am J Surg Pathol 2006;30:1491-501.
5. Patil DT, Shadrach BL, Rybicki LA, Leach BH, Pai RK. Proximal colon cancers and the serrated pathway: a systematic analysis of precursor histology and BRAF mutation status. Mod Pathol 2012;25:1423-31.
6. Kim YH, Kakar S, Cun L, Deng G, Kim YS. Distinct CpG island methylation profiles and *BRAF* mutation status in serrated and adenomatous colorectal polyps. Int J Cancer 2008;123:2587−93.
7. Sandmeier D, Benhattar J, Martin P, Bouzourene H. Serrated polyps of the large intestine: a molecular study comparing sessile serrated adenomas and hyperplastic polyps. Histopathology 2009;55:206-13.
8. Kim KM, Lee EJ, Ha S, et al. Molecular Features of Colorectal Hyperplastic Polyps and Sessile Serrated Adenoma/Polyps From Korea. Am J Surg Pathol 2011;35:1274-86.
9. Dhir M, Yachida S, Van Neste L, et al. Sessile serrated adenomas and classical adenomas: an epigenetic perspective on premalignant neoplastic lesions of the gastrointestinal tract. Int J Cancer 2011;129:1889-98.

10. Jass JR, Baker K, Zlobec I, et al. Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a 'fusion' pathway to colorectal cancer. Histopathology 2006;49:121-31.
11. Spring KJ, Zhao ZZ, Karamatic R, et al. High Prevalence of Sessile Serrated Adenomas With *BRAF* Mutations: A Prospective Study of Patients Undergoing Colonoscopy. Gastroenterology 2006;131:1400-7.
12. Carr NJ, Mahajan H, Tan KL, Hawkins NJ, Ward RL. Serrated and non-serrated polyps of the colorectum: their prevalence in an unselected case series and correlation of *BRAF* mutation analysis with the diagnosis of sessile serrated adenoma. J Clin Pathol 2009;62:516-8.
13. Fujita K, Yamamoto H, Matsumoto T, et al. Sessile Serrated Adenoma With Early Neoplastic Progression: A Clinicopathologic and Molecular Study. Am J Surg Pathol 2011;35:295-304.
14. Yachida S, Mudali S, Martin SA, Montgomery EA, Iacobuzio-Donahue CA. Beta-Catenin Nuclear Labeling is a Common Feature of Sessile Serrated Adenomas and Correlates with Early Neoplastic Progression Following *BRAF* Activation. Am J Surg Pathol 2009;33:1823-32.
15. Powell SM, Zilz N, Beazer-Barclay Y, et al. *APC* mutations occur early during colorectal tumorigenesis. Nature 1992;359:235-7.
16. Miyoshi Y, Nagase H, Ando H, et al. Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. Hum Mol Genet 1992;1:229-33.
17. Willert K, Nusse R. β-catenin: a key mediator of Wnt signaling. Curr Opin Genet Dev 1998;8:95-102.
18. Morin PJ, Sparks AB, Korinek V, et al. Activation of β-Catenin-Tcf Signaling in Colon Cancer by Mutations in β-Catenin or APC. Science 1997;275:1787-90.
19. Qi J, Zhu YQ, Luo J, Tao WH. Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor. World J Gastroenterol 2006;12:7113-7.
20. Kohonen-Corish MR, Sigglekow ND, Susanto J, et al. Promoter methylation of the

mutated in colorectal cancer gene is a frequent early event in colorectal cancer. Oncogene 2007;26:4435-41.

21. Fukuyama R, Niculaita R, Ng KP, et al. Mutated in colorectal cancer, a putative tumor suppressor for serrated colorectal cancer, selectively represses β-catenin-dependent transcription. Oncogene 2008;27:6044-55.
22. Li L, Fu X, Zhang W, et al. Wnt signaling pathway is activated in right colon serrated polyps correlating to specific molecular form of β-catenin. Hum Pathol 2013;44:1079-88.
23. Koinuma K, Yamashita Y, Liu W, et al. Epigenetic silencing of *AXIN2* in colorectal carcinoma with microsatellite instability. Oncogene 2006;25:139-46.
24. Koinuma K, Shitoh K, Miyakura Y, et al. MUTATIONS OF *BRAF* ARE ASSOCIATED WITH EXTENSIVE *hMLH1* PROMOTER METHYLATION IN SPORADIC COLORECTAL CARCINOMAS. Int J Cancer 2004;108:237-42.
25. Joo M, Shahsafaei A, Odze RD. Paneth cell differentiation in colonic epithelial neoplasms: evidence for the role of the Apc/β-catenin/Tcf pathway. Hum Pathol 2009;40:872-80.
26. Wu JM, Montgomery EA, Iacobuzio-Donahue CA. Frequent β-Catenin Nuclear Labeling in Sessile Serrated Polyps of the Colorectum With Neoplastic Potential. Am J Clin Pathol 2008;129:416-23.
27. Imamhasan A, Mitomi H, Saito T, Arakawa A, Yao T. Clear cell variant of squamous cell carcinoma originating in the esophagus: Report of a case with immunohistochemical and oncogenetic analyses. Pathol Int 2012;62:137-43.
28. Manié E, Vincent-Salomon A, Lehmann-Che J, et al. High Frequency of *TP53* Mutation in *BRCA1* and Sporadic Basal-like Carcinomas but not in *BRCA1* Luminal Breast Tumors. Cancer Res 2009;69:663-71.
29. Lazarus R, Junttila OE, Karttunen TJ, Mäkinen MJ. The Risk of Metachronous Neoplasia in Patients With Serrated Adenoma. Am J Clin Pathol 2005;123:349-59.
30. Zhang W, Bauer M, Croner RS, et al. DNA Stool Test for Colorectal Cancer: Hypermethylation of the Secreted Frizzled-Related Protein-1 Gene. Dis Colon Rectum

2007;50:1618-26.

Figure legends

Fig. 1 Nuclear β-catenin labeling indices (LIs) 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 (IQRs). †, $P < 0.05$; ‡, $P < 0.01$; §, $P < 0.001$; SSA/P, sessile serrated adenoma / polyp; SSA/P-HD, SSA/P with high grade dysplasia; SSA/P-CA, SSA/P with submucosal invasive carcinoma; AD, conventional adenoma; AD-HD, AD with high grade dysplasia; AD-CA, AD with submucosal invasive carcinoma.

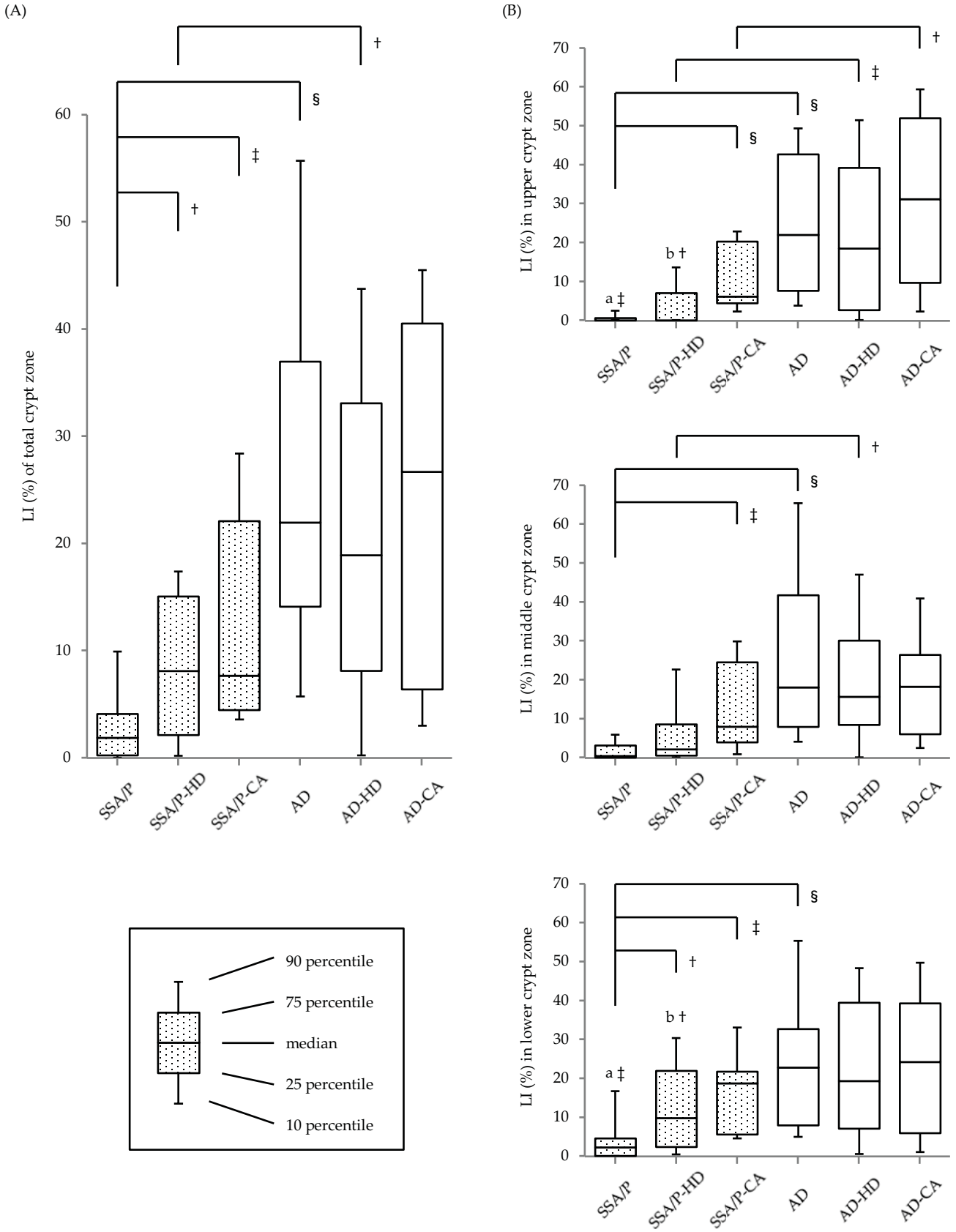
Fig. 2 Typical morphology of the SSA/P series studied and expression of β-catenin in representative cases; A-C, SSA/P (#22). A, Low power view of SSA/P. SSA/P shows dilated crypts with horizontal growth along the muscularis mucosae and deep serration. B, High power view of Fig. 2A: SSA/P featuring goblet cell hyperplasia at the crypt base. C, Immunostaining of β-catenin in same portion as (B). Nuclear staining of β-catenin (LI = 1.7%) is seen only at the crypt base. D-F, SSA/P-HD (#3). D, SSA/P-HD demonstrating cytologic atypia and architectural dysplasia without submucosal invasion. Adjacent SSA/P areas are seen at both ends of the lesion. E, Dysplastic glands with pseudostratified nuclei and loss of goblet cells mimicking conventional high-grade adenoma (HD area of Fig. 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 (LI = 16.9%). G-I, SSA/P-CA (#2). G, SSA/P-CA 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 (LI = 22.0%). SSA/P, sessile serrated adenoma / polyp; SSA/P-HD, SSA/P with high grade dysplasia; SSA/P-CA, SSA/P with submucosal invasive carcinoma; LI, labeling index.

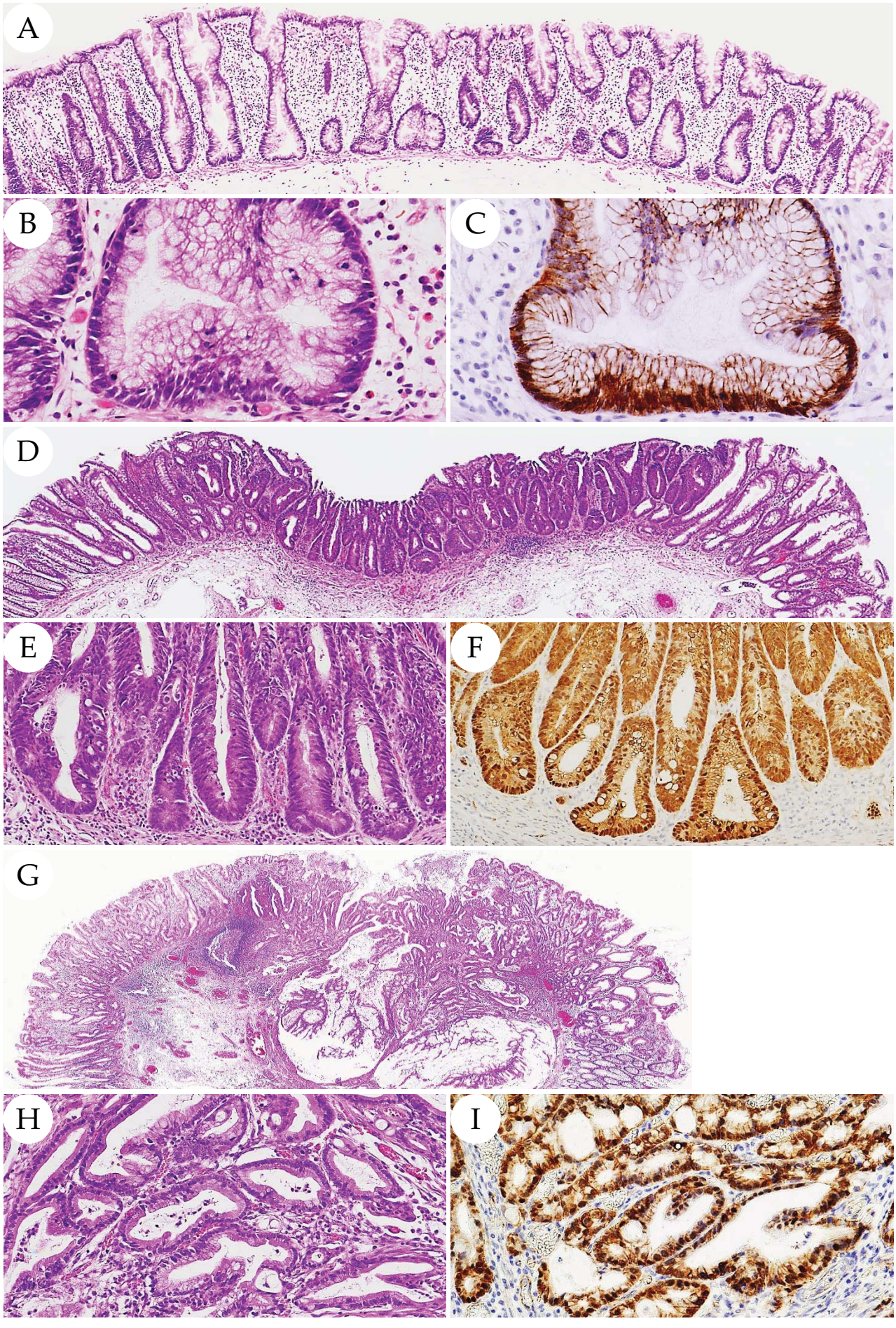
Fig. 3 Differences in nuclear β-catenin expression and methylation of Wnt signaling associated genes in the serrated neoplasia pathway and the conventional adenoma-carcinoma sequence. Nuclear β-catenin immunoreactivity: -, none; small arrow, low expresser; medium sized arrow, intermediate expresser; 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; SSA/P-HD, SSA/P with high grade dysplasia; SSA/P-CA, SSA/P with submucosal invasive carcinoma; AD, conventional adenoma; AD-HD, AD with high grade dysplasia; AD-CA, AD with submucosal invasive carcinoma.

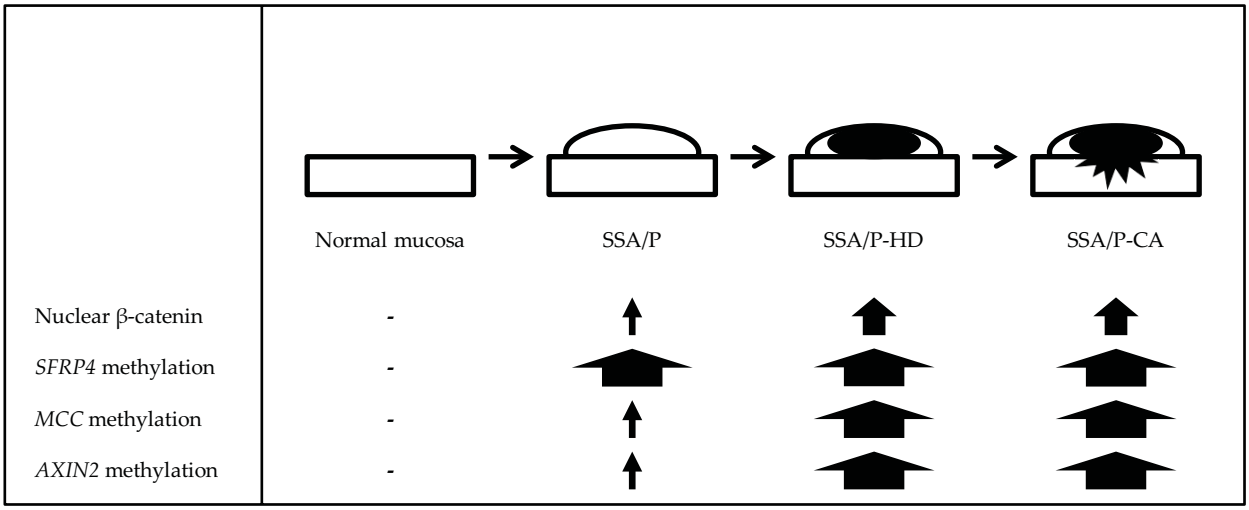
Supplementary Fig. 1 Representative results of MSP in single cases of SSA/Ps, SSA/P-HDs, SSA/P-CAs, ADs, AD-HDs, AD-CAs and normal colon mucosa (*N*). Each lane contains products generated from separate PCR reactions using probes specific for methylated (*M*) or unmethylated (*U*) DNA templates. Commercially available CpGs for completely methylated DNA (*C*+) and unmethylated DNA (*C*-) (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*).

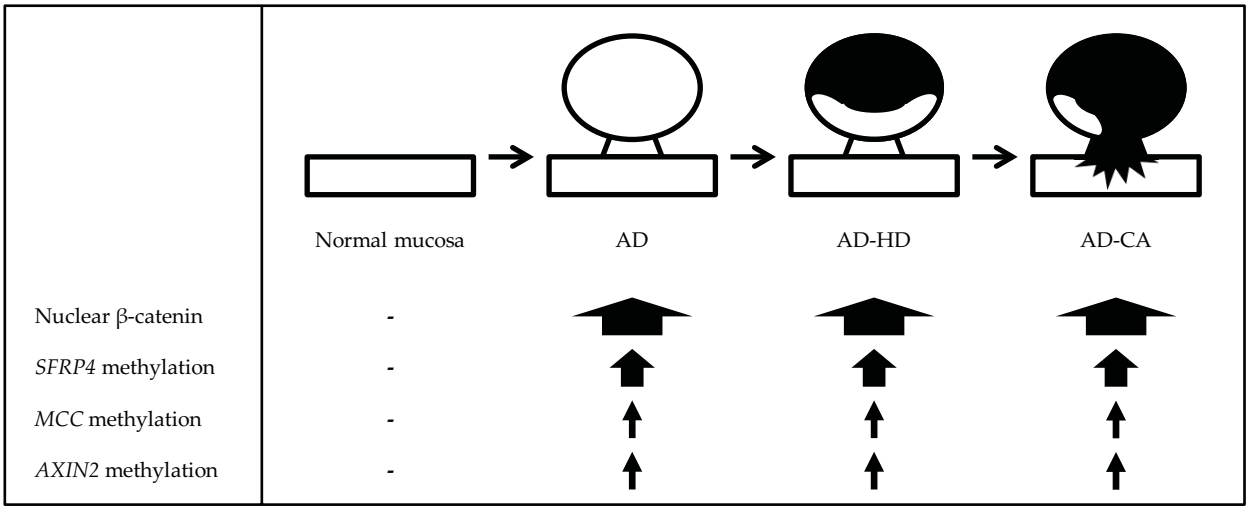
Supplementary Fig. 2 Schematic representation of *BRAF* and *Kras* mutational patterns in the colorectal polyps studied. In *A*, *BRAF* mutation (*arrow*) presenting as c. 1799 T>A in a case of SSA/P-HD *(#6*). In *B*, *Kras* mutation (*arrow*) presenting as c. 38 G>A in a case of AD-CA *(#12*).

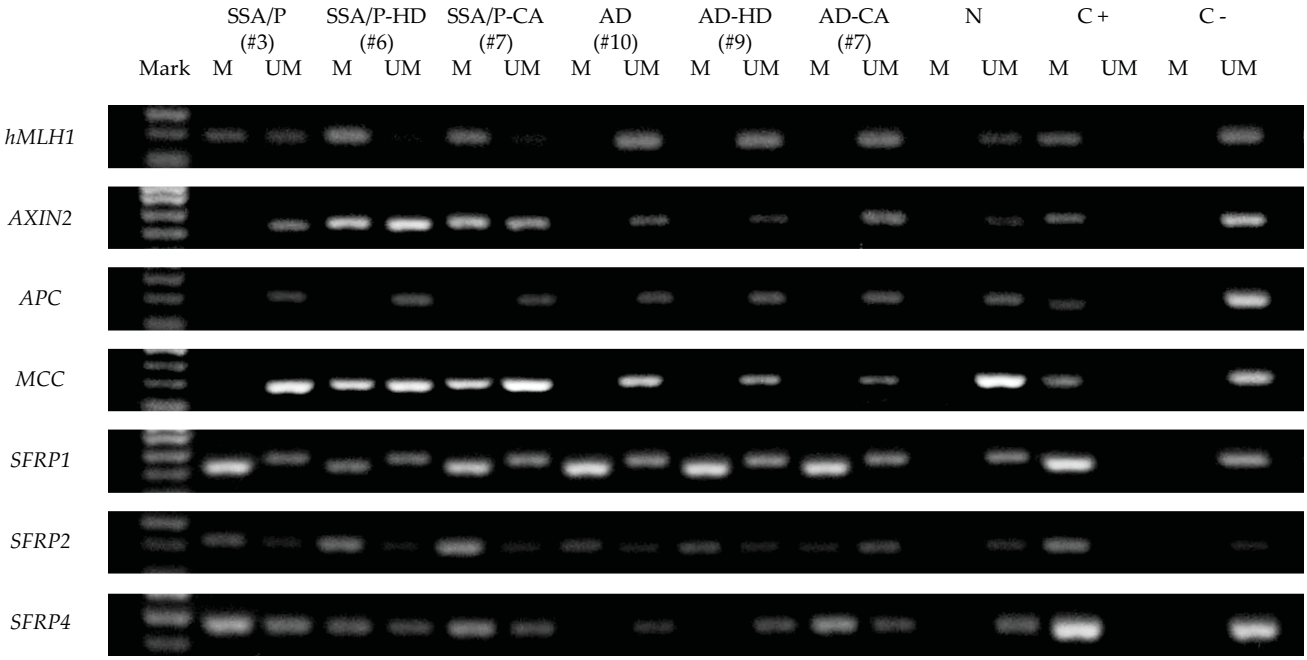
Supplementary Fig. 3 Schematic depiction of β-catenin expresser, methylations of *hMLH1* or Wnt signaling associated genes and *BRAF*/*Kras* mutations in each polyp studied.


(A)
LI (%) of total crypt zone
0
10
20
30
40
50
60
†
‡
§
SSA/P
SSA/P-HD
SSA/P-CA
AD
AD-HD
AD-CA
90 percentile
75 percentile
median
25 percentile
10 percentile
(B)
LI (%) in upper crypt zone
LI (%) in middle crypt zone
LI (%) in lower crypt zone
0
10
20
30
40
50
60
70
a ‡
b †
†
‡
§
SSA/P
SSA/P-HD
SSA/P-CA
AD
AD-HD
AD-CA

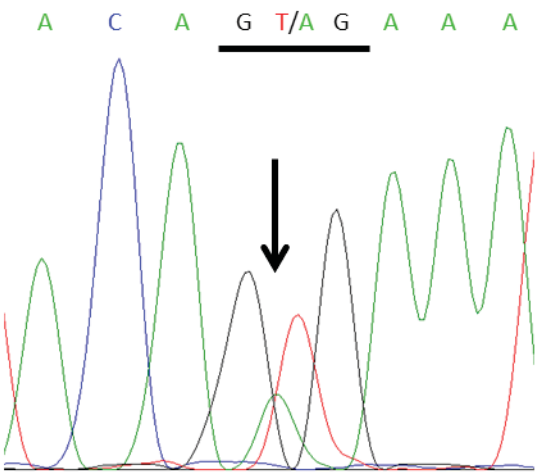

A
B
C
D
E
F
G
H
I


Normal mucosa
SSA/P
SSA/P-HD
SSA/P-CA
Nuclear β-catenin
SFRP4 methylation
MCC methylation
AXIN2 methylation
-
-
-
-


Normal mucosa
AD
AD-HD
AD-CA
Nuclear β-catenin
SFRP4 methylation
MCC methylation
AXIN2 methylation
-
-
-
-

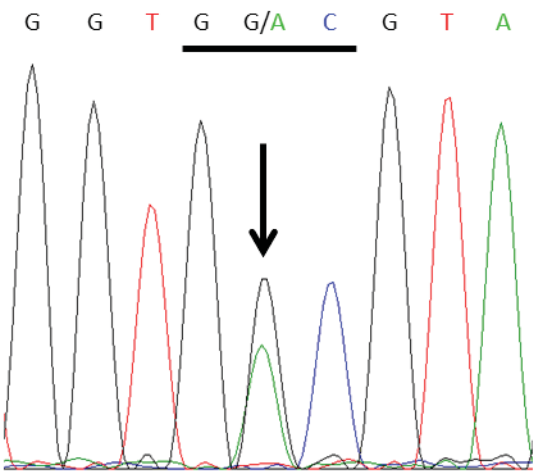

SSA/P (#3)
SSA/P-HD (#6)
SSA/P-CA (#7)
AD (#10)
AD-HD (#9)
AD-CA (#7)
N
C +
C -
Mark
M
UM
hMLH1
AXIN2
APC
MCC
SFRP1
SFRP2
SFRP4

A



BRAF c. 1799 T>A
(SSA/P-HD *#12*)

B



Kras c. 38 G>A
(AD-CA *#21*)

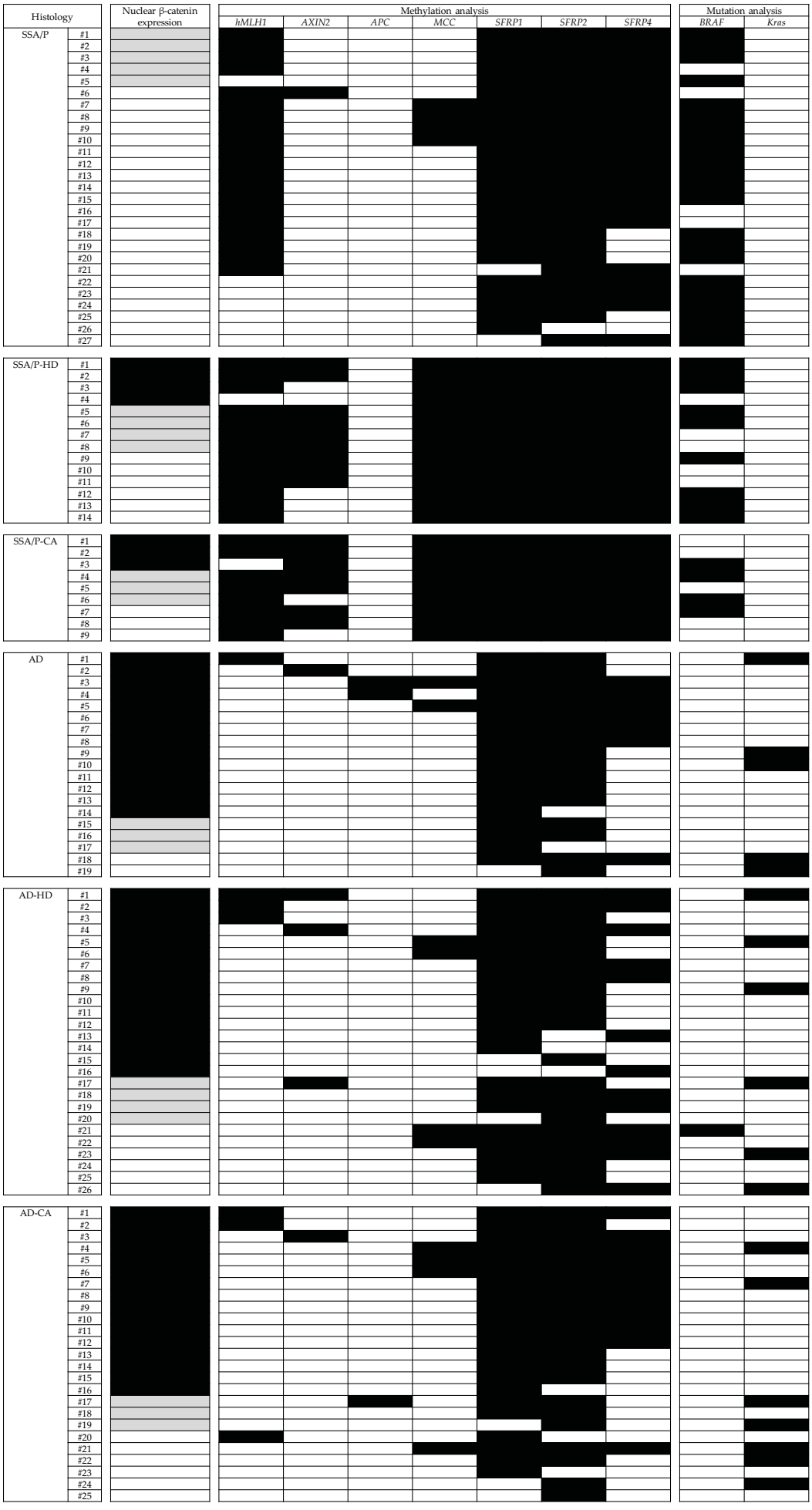

Histology
Nuclear β-catenin expression
Methylation analysis
hMLH1
AXIN2
APC
MCC
SFRP1
SFRP2
SFRP4
Mutation analysis
BRAF
Kras
SSA/P
SSA/P-HD
SSA/P-CA
AD
AD-HD
AD-CA
Nuclear β-catenin expression
; high expressor
; intermediate expressor
; low expressor
Gene mathylation / mutation
; methylated or mutated case
; unmethylated or non-mutated case

Table 1. Nuclear β-catenin expressor in the colorectal polyps studied

	SSA/P (n = 27)	SSA/P-HD (n = 14)	SSA/P-CA (n = 9)	AD (n = 19)	AD-HD (n = 26)	AD-CA (n = 25)
low expressor	22 (81.5 %)	6 (42.9 %)	3 (33.3 %)	2 (10.5 %)	6 (23.1 %)	6 (24.0 %)
intermediate expressor	5 (18.5 %)	4 (28.6 %)	3 (33.3 %)	3 (15.8 %)	4 (15.4 %)	3 (12.0 %)
high expressor	0 (0 %)	4 (28.6 %)	3 (33.3 %)	14 (73.7 %)	16 (61.5 %)	16 (64.0 %)

Total nuclear β-catenin LI was classified as follows: < 5%, low expressor; 5 - 14%, intermediate expressor; ≥ 15%, high expressor. SSA/P, sessile serrated adenoma / polyp; SSA/P-HD, SSA/P with high grade dysplasia; SSA/P-CA, SSA/P with submucosal invasive carcinoma; AD, conventional adenoma; AD-HD, AD with high grade dysplasia; AD-CA, AD with submucosal invasive carcinoma.

SSA/P vs. SSA/P-HD, $P = 0.006$; SSA/P vs. SSA/P-CA, $P = 0.003$; SSA/P vs. AD, $P < 0.001$

Table 2. Frequency of methylation of *hMLH1* , *AXIN2* , *APC* , *MCC* and *SFRPs* in the colorectal polyps studied

	SSA/P (n = 27)	SSA/P-HD (n = 14)	SSA/P-CA (n = 9)	AD (n = 19)	AD-HD (n = 26)	AD-CA (n = 25)
hMLH1 [a]	20 (74.1 %)	13 (92.9 %)	8 (88.9 %)	1 (5.3 %)	3 (11.5 %)	3 (12.0 %)
AXIN2 [b]	1 (3.7 %)	9 (64.3 %)	7 (77.8 %)	1 (5.3 %)	3 (11.5 %)	1 (4.0 %)
APC	0 (0 %)	0 (0 %)	0 (0 %)	2 (10.5 %)	0 (0 %)	1 (4.0 %)
MCC [c]	4 (14.8 %)	14 (100 %)	9 (100 %)	2 (10.5 %)	4 (15.4 %)	4 (16.0 %)
SFRP1	25 (92.6 %)	14 (100 %)	9 (100 %)	18 (94.7 %)	22 (84.6 %)	22 (88.0 %)
SFRP2	26 (96.3 %)	14 (100 %)	9 (100 %)	17 (89.5 %)	23 (88.5 %)	22 (88.0 %)
SFRP4 [d]	22 (81.5 %)	14 (100 %)	9 (100 %)	7 (36.8 %)	13 (50.0 %)	12 (48.0 %)

SSA/P, sessile serrated adenoma / polyp; SSA/P-HD, SSA/P with high grade dysplasia; SSA/P-CA, SSA/P with submucosal invasive carcinoma; AD, conventional adenoma; AD-HD, AD with high grade dysplasia; AD-CA, AD with submucosal invasive carcinoma.

a: SSA/P vs. AD, $P < 0.001$; SSA/P-HD vs. AD-HD, $P < 0.001$; SSA/P-CA vs. AD-CA, $P < 0.001$

b: SSA/P vs. SSA/P-HD or SSA/P-CA, $P < 0.001$; SSA/P-HD vs. AD-HD, $P = 0.001$; SSA/P-CA vs. AD-CA, $P < 0.001$

c: SSA/P vs. SSA/P-HD or SSA/P-CA, $P < 0.001$; SSA/P-HD vs. AD-HD, $P < 0.001$; SSA/P-CA vs. AD-CA, $P < 0.001$

d: SSA/P vs. AD, $P = 0.005$; SSA/P-HD vs. AD-HD, $P = 0.001$; SSA/P-CA vs. AD-CA, $P = 0.006$

Table 3. Frequency of *BRAF* and *Kras* mutations in the colorectal polyps studied

	SSA/P (n = 27)	SSA/P-HD (n = 14)	SSA/P-CA (n = 9)	AD (n = 19)	AD-HD (n = 26)	AD-CA (n = 25)
BRAF [a]	22 (81.5 %)	9 (64.3 %)	4 (44.4 %)	0 (0 %)	1 (3.8 %)	0 (0 %)
Kras [b]	0 (0 %)	0 (0 %)	0 (0 %)	5 (26.3 %)	6 (23.0 %)	7 (28.0 %)

SSA/P, sessile serrated adenoma / polyp; SSA/P-HD, SSA/P with high grade dysplasia; SSA/P-CA, SSA/P with submucosal invasive carcinoma; AD, conventional adenoma; AD-HD, AD with high grade dysplasia; AD-CA, AD with submucosal invasive carcinoma

a: SSA/P vs. AD, $P < 0.001$; SSA/P-HD vs. AD-HD, $P < 0.001$; SSA/P-CA vs. AD-CA, $P = 0.003$

b: SSA/P vs. AD, $P = 0.009$

Table 4. Associations of nuclear β-catenin expressor with gene methylation in serrated lesions

	Total	nuclear β-catenin expressor			*P*
		low	intermediate	high	
hMLH1 methylation					
Yes	41	25	11	5	N.S.
No	9	6	1	2	
AXIN2 methylation					
Yes	17	6	6	5	0.013
No	33	25	6	2	
APC methylation					
Yes	0	0	0	0	N.S.
No	50	31	12	7	
MCC methylation					
Yes	27	13	7	7	0.02
No	23	18	5	0	
SFRP1 methylation					
Yes	48	29	12	7	N.S.
No	2	2	0	0	
SFRP2 methylation					
Yes	49	30	12	7	N.S.
No	1	1	0	0	
SFRP4 methylation					
Yes	45	26	12	7	N.S.
No	5	5	0	0	

N.S. : not significant

Table 5. Associations of gene methylation with *BRAF* mutations in serrated lesions

	Total	*BRAF* mutation		*P*
		Yes	No	
hMLH1 methylation				
Yes	41	27	14	N.S.
No	9	8	1	
AXIN2 methylation				
Yes	17	8	9	0.021
No	33	27	6	
APC methylation				
Yes	0	0	0	N.S.
No	50	35	15	
MCC methylation				
Yes	27	17	10	N.S.
No	23	18	5	
SFRP1 methylation				
Yes	48	34	14	N.S.
No	2	1	1	
SFRP2 methylation				
Yes	49	34	15	N.S.
No	1	1	0	
SFRP4 methylation				
Yes	45	30	15	N.S.
No	5	5	0	

N.S. : not significant

Supplementary Table 1. Clinicopathological characteristics of the colorectal polyps studied

Variable	SSA/P (n = 27)	SSA/P-HD (n = 14)	SSA/P-CA (n = 9)	AD (n = 19)	AD-HD (n = 26)	AD-CA (n = 25)
Age (years)	63.0 ± 11.0 (39 - 81)	67.1 ± 9.8 (54 - 84)	70.8 ± 10.1 (55 - 84)	67.6 ± 9.3 (51 - 88)	70.8 ± 10.2 (44 - 88)	65.5 ± 8.4 (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)	12.8 ± 5.3 (3 - 25)	12.1 ± 9.0 (5 - 36)	10.2 ± 2.9 (6 - 15)	10.8 ± 5.9 (4 - 24)	17.1 ± 5.8 (10 - 32)	17.4 ± 6.8 (8 - 30)

Age and tumor size are presented as mean ± SD (range) values; SSA/P, sessile serrated adenoma / polyp; SSA/P-HD, SSA/P with high grade dysplasia; SSA/P-CA, SSA/P with submucosal invasive carcinoma; AD, conventional adenoma; AD-HD, AD with high grade dysplasia; AD-CA, AD with submucosal invasive carcinoma

Supplementary Table 2. Primers used for the MSP analysis

Gene	Forward primers (5' - 3')	Reverse primers (5' - 3')	Tm (°C)	Product size (bp)	PCR cycles
hMLH1	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

M, methylated DNA; U, unmethylated DNA; Tm , annealing temperature