WNT pathway gene mutations are associated with the presence of dysplasia in colorectal sessile serrated adenoma/polyps

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Abstract

Sessile serrated adenoma/polyps (SSA/Ps) are believed to be the major precursor of serrated-pathway derived colorectal carcinomas. To better characterize the process of progression from SSA/Ps to carcinomas, we analyzed 46 SSA/Ps with dysplasia and 45 SSA/Ps without dysplasia using targeted next-generation sequencing and immunohistochemistry. Among the WNT pathway genes analyzed, protein-truncating mutations of *RNF43*, *APC*, and *ZNRF3* were identified in 23 (50%), 4 (9%), and 3 (7%) SSA/Ps with dysplasia, respectively. In contrast, SSA/Ps without dysplasia rarely had WNT pathway gene mutations, except for three lesions with *RNF43* mutations (7%). None of the SSA/Ps had *CTNNB1* mutations or *RSPO* fusions. Thus, WNT pathway gene mutations were more common in SSA/Ps with dysplasia than in SSA/Ps without dysplasia ($p = 3.0 \times 10^{-8}$). Consistently, nuclear β-catenin accumulation and MYC overexpression, indicative of active WNT signaling, were present in most of the SSA/Ps with dysplasia, but were rare in those without dysplasia. *BRAF* (86%) or *KRAS* mutations (7%) were identified in the majority of SSA/Ps, regardless of the presence or absence of dysplasia. MLH1 expression was lost in 14 SSA/Ps with dysplasia (30%). The majority of MLH1-deficient SSA/Ps with dysplasia had *RNF43* mutations (86%), most of which were frameshift mutations involving mononucleotide repeats. In contrast, MLH1-retained lesions had less frequent *RNF43* mutations with no hot spots (34%), and four had *APC* mutations (13%). These results suggest that WNT pathway gene mutations are involved in the development of dysplasia in SSA/Ps and that MLH1-deficient and MLH1-retained SSA/Ps with dysplasia exhibit distinct mutation profiles of WNT pathway genes.

Introduction

There are two major pathways of colorectal carcinogenesis: the conventional and serrated pathways. Each pathway is associated with different types of precursor lesions and involves a distinct series of genetic aberrations.[1-3] The conventional pathway, also referred to as the adenoma-carcinoma sequence, constitutes the predominant pathway.[4,5] Alternatively, approximately 25% of sporadic colorectal carcinomas arise via the serrated pathway, which represents tumorigenesis from serrated lesions, including hyperplastic polyps, sessile serrated adenoma/polyps (SSA/Ps), and traditional serrated adenomas (TSAs).[1-3,6,7] These lesions harbor *BRAF* or, less frequently, *KRAS* mutations and usually lack *APC* mutations.[8-11] Given that among colorectal polyps, *BRAF* mutations are exclusive to serrated lesions, colorectal cancers with *BRAF* mutations are believed to develop via the serrated pathway.[11-15] *BRAF*-mutated cancers are located predominantly in the proximal colon and show distinct clinical features depending on the mismatch repair (MMR) status. *BRAF*-mutated MMR-deficient colorectal carcinomas exhibit indolent clinical behavior and are associated with a favorable prognosis.[16-18] In contrast, *BRAF*-mutated MMR-proficient tumors are regarded as the most aggressive molecular subtypes of colorectal carcinoma.[17-21]

SSA/Ps constitute approximately 15–25% of all serrated polyps, mostly occurring in the proximal colon.[22] Histologically, these lesions are characterized by distorted crypts with prominent serrations.[23] The majority of SSA/Ps are cytologically bland, but a subset of them show dysplastic changes, which are believed to herald the progression to carcinoma.[24,25] Because of their typical proximal localization and the frequent presence of *BRAF* mutations, SSA/Ps are believed to be the major

precursor of serrated-pathway derived colorectal carcinomas.[1-3,26,27] However, the detailed process of progression from SSA/Ps to carcinomas remains elusive.

Several studies have reported that nuclear β-catenin accumulation is common in SSA/Ps with dysplasia, but not in those without dysplasia.[28-32] This observation implies that the activation of WNT signaling is involved in the development of dysplasia in SSA/Ps. However, the mechanisms underlying the WNT activation in SSA/Ps remain unclear. Although *APC* and *CTNNB1* mutations are major causes of WNT signaling activation in conventional-type adenomas, these genetic alterations are absent in SSA/Ps.[15,28] Accordingly, it is postulated that SSA/Ps with dysplasia have a mechanism of WNT activation that is distinct from that of the conventional pathway. Recently, several studies reported that a minor subset of SSA/Ps have *RNF43* mutations.[15,33,34] RNF43 is a transmembrane ubiquitin E3 ligase that downregulates WNT receptors;[35] therefore, loss-of-function mutations of *RNF43* enhance WNT signaling, as observed in several types of tumors.[36-42] In colorectal cancers, *RNF43* mutations are frequently found in MMR-deficient and MMR-proficient adenocarcinomas with *BRAF* mutations.[33,42,43] These data suggest that *RNF43* mutations are specifically involved in serrated-pathway tumorigenesis. However, it is not clear at what point during the multistep progression of SSA/P-derived tumorigenesis the mutation occurs.

In the present study, we conducted a targeted mutational analysis of colorectal SSA/Ps with and without dysplasia, focusing on alterations in WNT pathway genes to clarify the roles and underlying mechanisms of the WNT pathway activation in the carcinogenesis via SSA/Ps.

Materials and methods

Tissue samples and inclusion criteria

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. We analyzed 46 SSA/Ps with dysplasia and 45 without dysplasia. All tissue samples were obtained endoscopically at the National Cancer Center Hospital, Tokyo, Japan from January 2010 to December 2016. All specimens were fixed in 10% formalin and embedded in paraffin. We adopted the strict histological criteria used in the study by Bettington *et al*[32] for the diagnosis of SSA/Ps with dysplasia to ensure that the cohort consists of homogeneous lesions without contamination by other types of polyps. The diagnosis of SSA/Ps with dysplasia requires (1) a component of ordinary SSA/P at the edge of the lesion, (2) abrupt transition from ordinary SSA/P to overt cytological dysplasia, and (3) exclusion of cases representing TSA arising in an SSA/P (Figure 1).[44] Three pathologists (TH, HY and SS), blinded to the results of the mutational studies, reviewed the sections based on the criteria above and the discrepancies were discussed to reach a consensus.

Clinicopathological data collection

Polyp location was divided as proximal side (cecum, ascending and transverse colon) and distal side (descending, sigmoid colon, and rectum). The size of the polyps and the dysplastic components was measured on the glass slides. Low-grade and high-grade dysplasia were classified based on both cytological changes and architectural complexity.[45]

Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed paraffin-embedded specimens. Antigen retrieval was performed by autoclaving the samples in 10 mM citrate buffer (pH 6.0) for 10 min. Anti-β-catenin (14; 1:200 dilution; BD Biosciences, Franklin Lakes, NJ, USA), anti-MYC (Y69; 1:200 dilution; Abcam, Cambridge, UK) and anti-MLH1 (ES05; 1:200 dilution; Dako, Glostrup, Denmark) antibodies were used as the primary antibodies. For staining, we used an automated stainer (Dako) according to the manufacturer's protocol. ChemMate EnVision (Dako) methods were used for detection. The staining results were evaluated by two pathologists (TH and SS). For β-catenin staining, nuclear expression in ≥50% of cells was considered positive for nuclear accumulation. For MYC staining, if MYC expression, which is normally confined to the base of the crypts,[46] extended to the upper half of the glands in ≥50% of the lesion, it was judged as overexpression. For MLH1 staining, lesions devoid of nuclear staining were considered MLH1-expression-deficient, using lymphocytes and endothelial cells as internal positive controls.

DNA and RNA extraction

Deparaffinized 10-μm-thick sections from each paraffin block were microdissected under a microscope using sterilized toothpicks. In the lesions of SSA/Ps with dysplasia, only the dysplastic areas were microdissected. The microdissected samples were subjected to DNA and RNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and Recover All Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA), respectively.

Targeted next-generation sequencing

Amplicon libraries of the target regions were prepared using the Ion AmpliSeq Custom Panel (ThermoFisher Scientific) with 40 ng genomic DNA. This panel targets frequently mutated regions of *APC*, *CTNNB1*, *BRAF*, *KRAS*, *NRAS*, and *GNAS* and the entire coding regions of *RNF43* and *ZNRF3* (Supplementary Table 1).[15] Sequencing was performed using an Ion Proton Sequencer (ThermoFisher Scientific) and PI chip (ThermoFisher Scientific) with an Ion PI Hi-Q Sequencing 200 Kit (ThermoFisher Scientific) according to the manufacturer's protocol. The resulting sequences were mapped onto the human reference genome hg19, and sequence variations were analyzed on a CLC Genomics Workbench 8.5 (CLC bio, Aarhus, Denmark). Sequence variations with minimum coverages of more than 100 reads, minimum coverages on either strand of more than 5 reads, and variant frequencies of more than 10% for single-nucleotide variants and more than 15% for insertions/deletions were identified as candidate mutations. Synonymous mutations and common single nucleotide polymorphisms, based on the Single Nucleotide Polymorphism Database build 137, were excluded. All resulting mutation candidates were subjected to Sanger sequencing for confirmation.

Sanger sequencing

Mutation candidates identified by next-generation sequencing and low-coverage regions in next-generation sequencing were further analyzed by Sanger sequencing. Polymerase chain reactions (PCRs) were performed using standard protocols (Supplementary Table 2). The PCR products were purified and sequenced using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Reverse transcription-PCR

Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). PCR conditions for previously reported *RSPO* fusions, *EIF3E-RSPO2* and *PTPRK-RSPO3*, were described previously.[15] Amplification of *ACTB* was used as a positive control; if it was unsuccessful, the samples were regarded to be of insufficient quality and excluded from the fusion gene analysis.

Statistical analysis

Categorical and continuous variables were compared using Fisher's exact test and Welch's t test, respectively. A p value of ≤0.05 was considered significant.

Results

Clinicopathological features

The mean age of patients with SSA/Ps with dysplasia was significantly higher than that of patients with SSA/Ps without dysplasia (Table 1). Of the 46 SSA/Ps with dysplasia, 26 (57%) were in women, 39 (85%) were proximal, and the mean polyp size was 8.5 mm. The mean size of the dysplastic component was 4.7 mm. Four SSA/Ps with dysplasia showed high-grade dysplasia (9%).

Genetic alterations

Targeted next-generation sequencing identified 138 potential non-synonymous genetic alterations. Verification using Sanger sequencing confirmed 133 mutations in a total of 91 samples (Supplementary Table 3). Supplementary Sanger

sequencing, targeting low-coverage regions, identified no additional mutations. Among the identified mutations, the biological significance of missense mutations in tumor suppressor genes, including one *APC* mutation and nine *RNF43* mutations, was difficult to determine conclusively, and because of the unavailability of non-neoplastic tissue samples, the possibility of rare germline polymorphisms could not be excluded. Thus, only protein-truncating mutations in *APC* and *RNF43* were considered for the following analyses.

Protein-truncating mutations of *RNF43*, *APC* and *ZNRF3* were identified in 23 (50%), 4 (9%), and 3 (7%) SSA/Ps with dysplasia, respectively (Figure 2, Supplementary Figure, Table 1, and Supplementary Table 4). *CTNNB1* mutations or *RSPO* fusions were not observed in any of the lesions, although the *RSPO* fusion analysis was unsuccessful in one lesion. *APC* mutations and the other WNT pathway gene mutations were mutually exclusive, but two lesions concurrently had *RNF43* and *ZNRF3* mutations. Collectively, 28 SSA/Ps with dysplasia (61%) harbored WNT pathway gene mutations. On the other hand, among SSA/Ps without dysplasia, *RNF43* mutations were detected in three lesions (7%), but no other WNT pathway gene mutations were detected. Comparing the two groups, lesions with WNT pathway gene mutations were more frequent in SSA/Ps with dysplasia than in those without dysplasia.

BRAF mutations were identified in the majority of SSA/Ps (86%), regardless of the presence or absence of dysplasia. *KRAS* mutations were identified in four SSA/Ps with dysplasia (9%) and two SSA/P without dysplasia (4%). *BRAF* and *KRAS* mutations were mutually exclusive, except for in one lesion harboring an atypical

BRAF mutation (c.1780G>A). *GNAS* mutations were found in two SSA/Ps with dysplasia (4%).

β-Catenin and MYC expression

We next examined the expression of β-catenin and MYC by immunohistochemistry. Nuclear β-catenin accumulation was more prevalent in SSA/Ps with dysplasia (35 lesions, 76%) than in those without dysplasia (5 lesions, 11%) (Figure 3A, B). Overexpression of MYC, a downstream target of the WNT signaling pathway, was observed in 42 lesions (91%) of SSA/Ps with dysplasia (Figure 3C, D). In contrast, MYC overexpression was detected only in four lesions among the SSA/Ps without dysplasia (9%). Nuclear β-catenin expression and MYC overexpression were significantly correlated with the presence of WNT pathway gene mutations.

Expression of MLH1 and its clinicopathological significance in SSA/Ps with dysplasia

Loss of MLH1 expression was observed in 14 SSA/Ps with dysplasia (30%; Figure 4), but in none of the SSA/Ps without dysplasia. The mean age of patients with MLH1-deficient SSA/Ps with dysplasia was significantly higher than that in patients with MLH1-retained SSA/Ps with dysplasia (Table 2). MLH1-deficient SSA/Ps with dysplasia were more common in female patients in contrast to MLH1-retained SSA/Ps with dysplasia (79% vs. 47%), but the difference did not reach statistical significance. Although all the MLH1-deficient SSA/Ps were located in the proximal colon, seven MLH1-retained SSA/Ps with dysplasia (22%) occurred in the distal colon. Most of MLH1-deficient SSA/Ps with dysplasia harbored *BRAF* mutations, except for two lesions with *KRAS* mutations (14%). The majority of MLH1-deficient

SSA/Ps with dysplasia had *RNF43* mutations (12 of 14 lesions, 86%), most of which were frameshift mutations affecting two mononucleotide repeats within the coding region (Figure 5). In contrast, *RNF43* mutations were less frequent in MLH1-retained SSA/Ps with dysplasia (11 of 32 lesions, 34%) and were preferentially localized to the first half of the coding region. All the four lesions with *APC* mutations retained MLH1 expression.

Discussion

The present study showed that SSA/Ps with dysplasia had clinical features similar to those generally recognized in SSA/Ps,[22,47] including proximal location and predominance in female patients. Interestingly, SSA/Ps with dysplasia tended to occur in older individuals but were similar in size to those without dysplasia. In agreement with our observations, Bettington *et al* recently analyzed 137 SSA/Ps with dysplasia and reported that the mean age of patients with SSA/Ps with dysplasia was 17 years older than that of patients with SSA/Ps without dysplasia and that the size of the lesions was similar regardless of the presence of dysplasia.[32]

BRAF mutations were identified in most SSA/Ps with or without dysplasia. This supports the notion that *BRAF* mutation is an early event in the serrated pathway of tumorigenesis. In contrast, WNT pathway gene mutations were common in SSA/Ps with dysplasia but were rare in those without dysplasia, suggesting a role in the development of dysplasia. Unlike conventional-type adenomas, which predominantly harbor *APC* or *CTNNB1* mutations, *RNF43* mutations were the most prevalent WNT pathway gene mutations in SSA/Ps with dysplasia. Loss-of-function

RNF43 mutations enhance ligand-dependent WNT pathway activation,[35] in contrast to *APC* or *CTNNB1* mutations, which lead to cell autonomous, ligand-independent WNT pathway activation.[48] Thus, although WNT pathway gene alterations are common in both SSA/Ps with dysplasia and conventional-type adenomas, the preferential mode of pathway activation appears to be distinct. Two preceding studies reported the prevalence of *RNF43* mutations in SSA/Ps as 6% and 10%, respectively.[15,33] Considering the rarity of SSA/Ps with dysplasia, these results likely reflect the prevalence of *RNF43* mutations in non-dysplastic SSA/Ps.

The majority of SSA/Ps with dysplasia exhibited nuclear accumulation of β-catenin and overexpression of MYC, indicative of active WNT signaling, in agreement with previous studies.[28-32] Notably, a significant proportion of SSA/Ps with dysplasia without detectable WNT pathway gene mutations also showed nuclear β-catenin expression and/or MYC overexpression, implying the existence of other mechanisms leading to WNT pathway activation. In contrast, mutations of WNT pathway genes, nuclear β-catenin accumulation, and MYC overexpression were rarely identified in SSA/Ps without dysplasia. Along with mutational analysis results, these findings indicate that activation of the WNT signaling pathway does not play a major role in the development of SSA/Ps, but is associated with dysplastic changes.

We did not find any SSA/Ps with *RSPO* fusions, another cause of WNT pathway activation in colorectal cancers.[49] Our previous study showed *PTPRK-RSPO3* fusions in 31% of TSAs, but these were not identified in SSA/Ps.[15] However, this observation is conflicting with a recent study by Yan *et al*,[34] which reported *RSPO*

fusions in 4 of 34 sporadic SSA/Ps. We expect that this discrepancy is attributable to the use of different histological criteria to diagnose TSAs. Yan *et al* classified a polyp as TSA only if it displayed predominantly complex villiform growth and exuberant ectopic crypt formation; however, these findings are generally regarded as characteristic but not consistent histological features of TSAs.[3,23,44,50-53] The use of their restrictive criteria might result in the misclassification of a subset of TSAs as SSA/Ps. Although further confirmation is desirable, our present and previous studies suggest that among colorectal polyps, *PTPRK-RSPO3* fusions are absent in SSA/Ps and exclusive to TSAs.

A subset of SSA/Ps with dysplasia is reported to show loss of MLH1 in association with *MLH1* promoter methylation.[30,32,54] In the present study, SSA/Ps with dysplasia showed clinicopathologically and molecularly different features depending on the MLH1 expression statuses. MLH1-deficient SSA/Ps with dysplasia tended to develop in older patients, occurred exclusively in the proximal colon, and showed female predominance. In contrast, MLH1-retained SSA/Ps with dysplasia occurred in younger patients, could also be located in the distal colon, and showed almost equal gender distribution. The difference in location and gender did not reach the statistical significance, but this might have been due to the relatively limited number of subjects in the present study. Genetically, MLH1-deficient SSA/Ps with dysplasia mostly had frameshift *RNF43* mutations involving the mononucleotide repeats, p.Arg117fs and p.Gly659fs. These findings suggest that *RNF43* mutations are consequences of MMR deficiency and are preceded by loss of MLH1 expression. However, MLH1-retained SSA/Ps with dysplasia had less frequent *RNF43* mutations, which did not show apparent hot spots, and several of them had *APC*

mutations. Of note, these clinicopathological features and *RNF43* mutation profiles of MLH1-deficient and MLH1-retained SSA/Ps with dysplasia were shared by MMR-deficient and MMR-proficient colorectal carcinomas with *BRAF* mutations, respectively,[18-21,42,43] supporting their histogenetic relationship, as previously suggested (Figure 6).

It is worth noting that two *KRAS*-mutated SSA/Ps with dysplasia were MLH1-deficient. This observation suggests that *KRAS*-mutated SSA/Ps represent previously underrecognized precursors of MMR-deficient colorectal carcinomas. Despite previous studies indicating the presence of *KRAS*-mutated SSA/Ps,[29,55,56] further studies involving a larger number of lesions are required to better characterize these lesions.

In summary, genetic alterations leading to the WNT signaling pathway activation are common in SSA/Ps with dysplasia but rare in those without dysplasia, suggesting their roles in the progression from non-dysplastic to dysplastic SSA/Ps. Unlike conventional-type adenomas, *RNF43* is the predominant WNT pathway gene mutated in the SSA/P-related tumorigenesis and shows distinct mutation spectra depending on the MLH1 expression status.

Disclosure/conflict of interest

The authors declare no conflicts of interest.

FIGURES
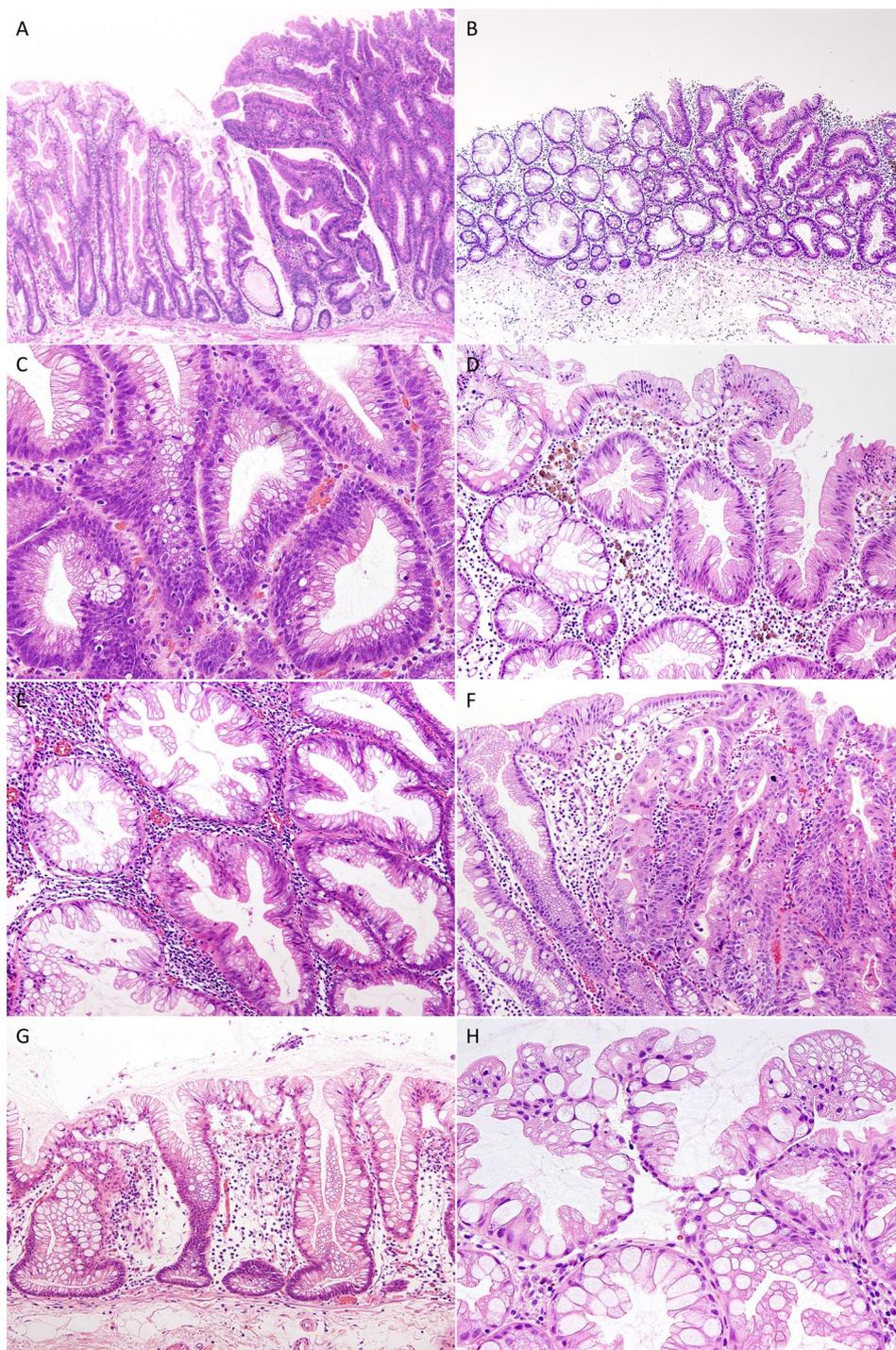
Figure 1 Representative histology of sessile serrated adenoma/polyps (SSA/Ps) with and without dysplasia.

(**A**, **B**) SSA/Ps with dysplasia. Non-dysplastic components (left) are sharply demarcated from dysplastic areas (right). (**C**) High-power view of the dysplastic

area. In this lesion, dysplastic glands resemble those of conventional adenoma; the glands are non-serrated, and lined by columnar cells with pseudostratified, hyperchromatic nuclei. (**D, E**) Abrupt transition from non-dysplastic (left) to dysplastic components (right). Both non-dysplastic and dysplastic glands exhibit serrated structure in these lesions. (**F**) SSA/P with high-grade dysplasia. Dysplastic glands show complex architecture and the cells have notable nuclear atypia. Non-dysplastic component is associated (left). (**G**) An SSA/P without dysplasia shows abnormal crypt architecture with broad bases, forming a "boot"-like appearance. (**H**) High-power view of an SSA/P without dysplasia. Small nuclei locate orderly at the basal side of the cells.
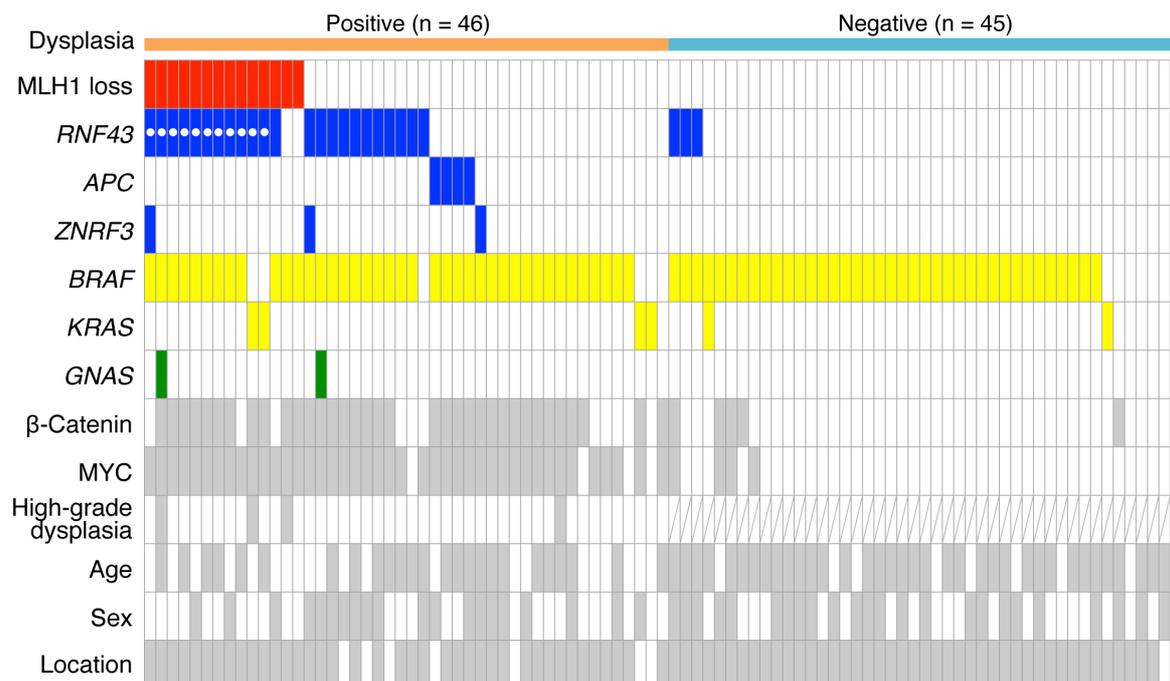
Figure 2 Genetic alterations and clinicopathological features of sessile serrated adenoma/polyps (SSA/Ps). White circles in *RNF43* indicate frameshift mutations involving mononucleotide repeats. Gray bars in clinicopathological features indicate: β-catenin, positive nuclear β-catenin accumulation; MYC, MYC overexpression; high-grade dysplasia, SSA/Ps with high-grade dysplasia; age, <70 years old; sex, male; location, proximal.
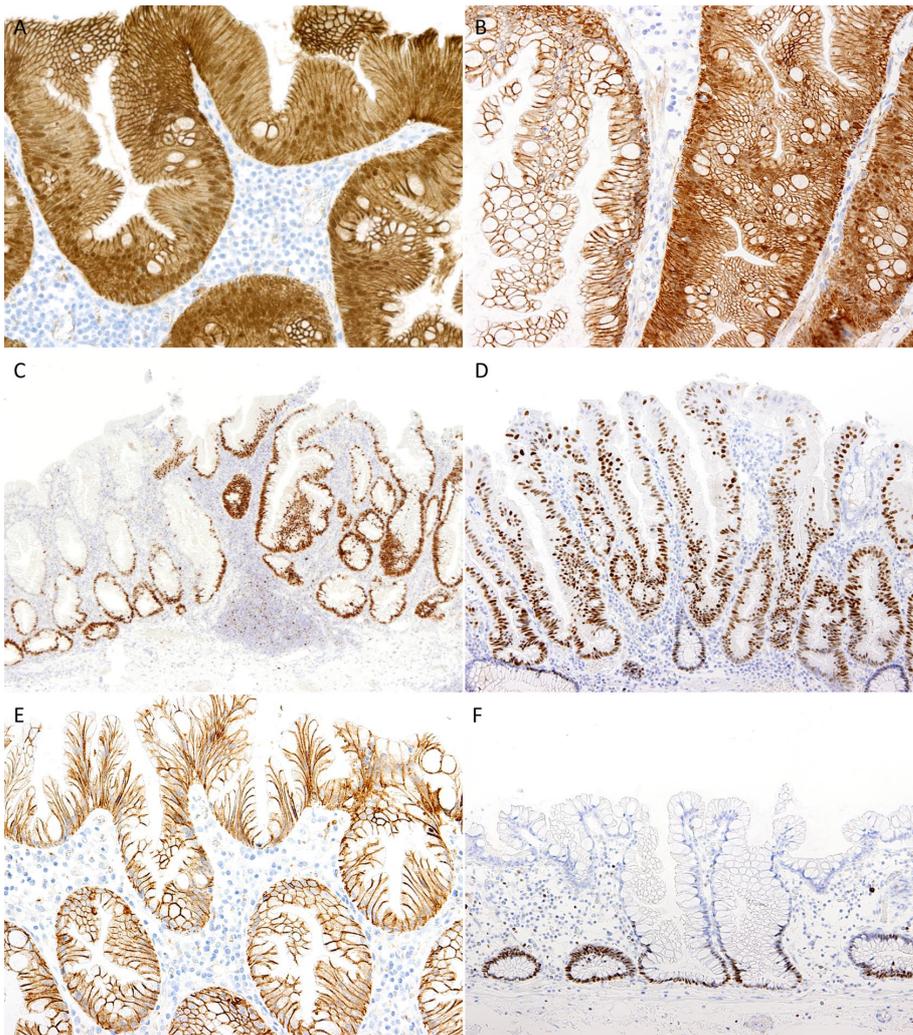
Figure 3 Immunohistochemistry for β-catenin and MYC in sessile serrated adenoma/polyps (SSA/Ps). (**A**) Diffuse nuclear, in addition to membranous, expression of β-catenin in an SSA/P with dysplasia. (**B**) An SSA/P with dysplasia shows membranous localization of β-catenin in the non-dysplastic area (left) and nuclear and membranous β-catenin expression in the dysplastic area (right). (**C**, **D**) MYC overexpression in an SSA/P with dysplasia. MYC expression is limited to the bottom of the glands in the non-dysplastic area (C, left), whereas MYC is diffusely expressed in the dysplastic area (C, right, and D). (**E**) Exclusively membranous localization of β-catenin in an SSA/P without dysplasia. (**F**) MYC expression is limited to the bottom of the glands in an SSA/P without dysplasia.
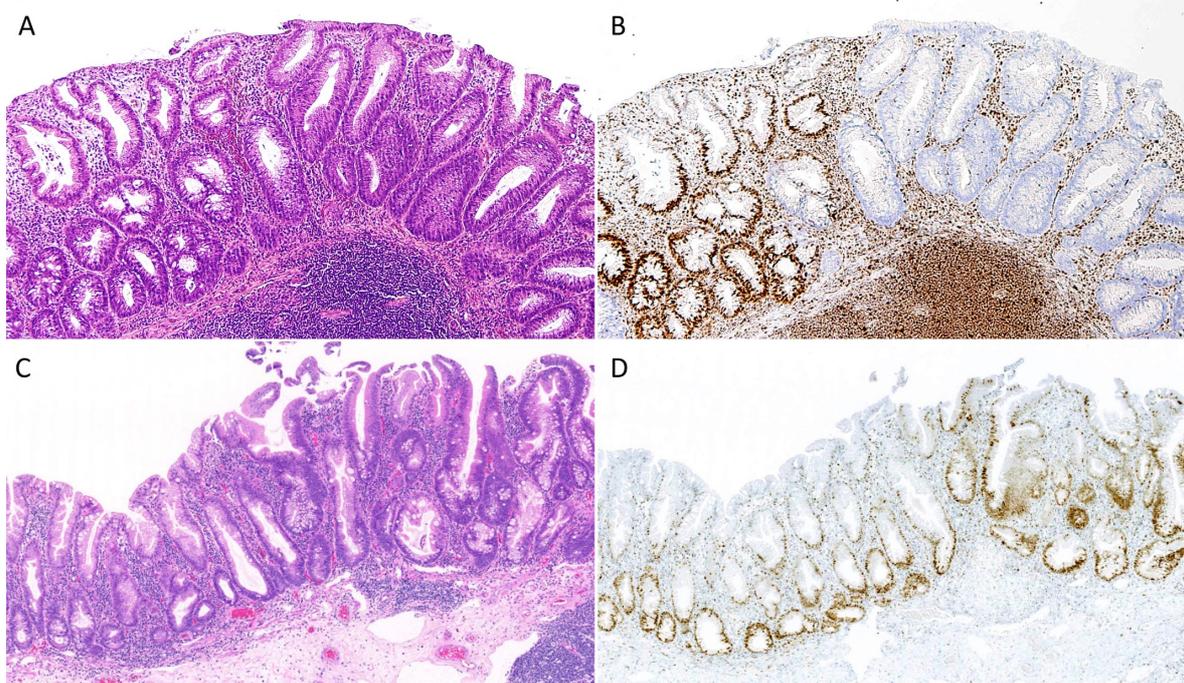
Figure 4 MLH1 expressions in sessile serrated adenoma/polyps (SSA/Ps) with dysplasia. (**A**, **B**) MLH1-deficient SSA/P with dysplasia. A non-dysplastic SSA/P component (left) and dysplastic glands (right) coexist side-by-side (A). MLH1 expression is lost in dysplastic glands (B). (**C**, **D**) MLH1-retained SSA/P with dysplasia. Retained MLH1 expression in the dysplastic area (D).
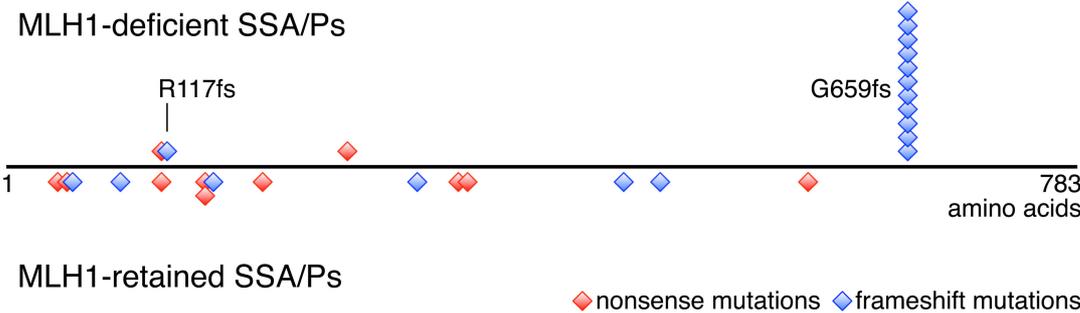
Figure 5 Distributions of protein-truncating *RNF43* mutations in sessile serrated adenoma/polyps (SSA/Ps). In MLH1-deficient SSA/Ps, *RNF43* mutations are accumulated in two mononucleotide repeats. In contrast, *RNF43* mutations in MLH1-retained SSA/Ps do not show an apparent hotspot and are predominantly localized to the first half of the coding region.
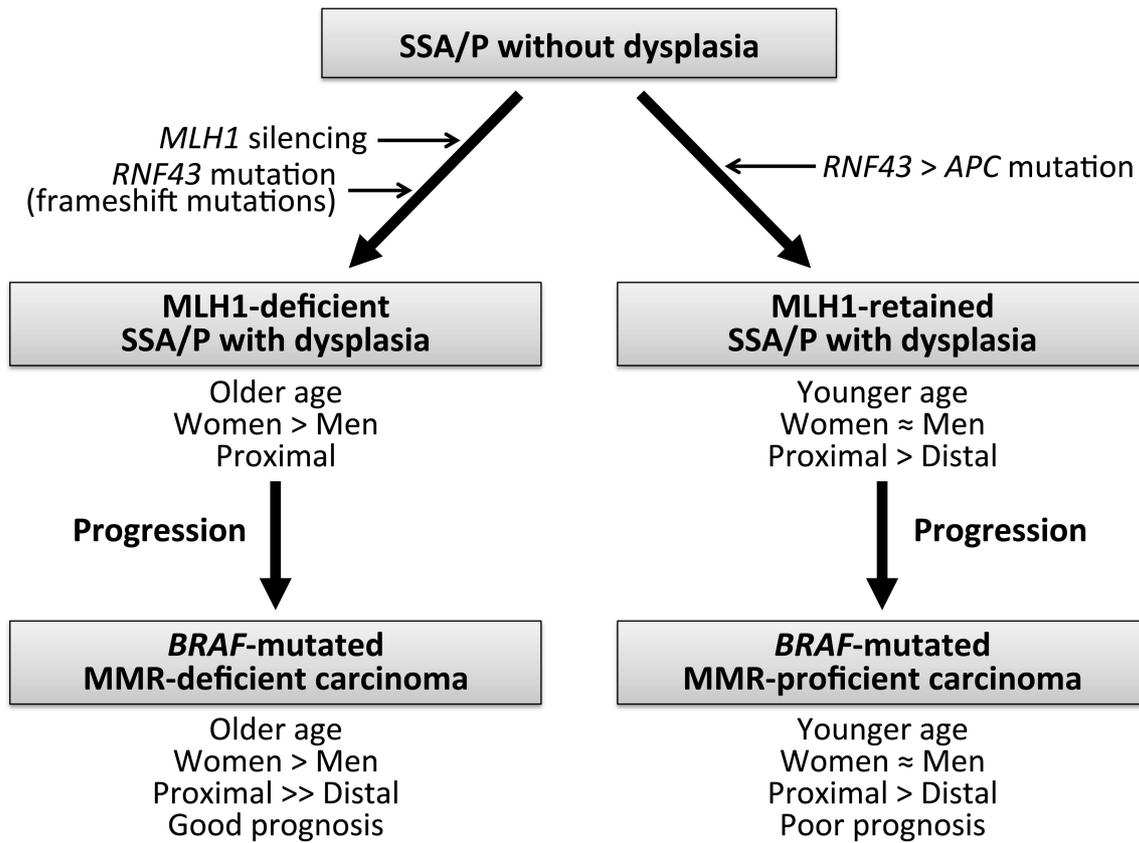
Figure 6 Supposed pathways of colorectal carcinogenesis from sessile serrated adenoma/polyps (SSA/Ps). SSA/Ps without dysplasia progress to MLH1-deficient SSA/Ps with dysplasia by *MLH1* silencing and subsequent frameshift *RNF43* mutations. In contrast, an *RNF43* or *APC* mutation is involved in the progression to MLH1-retained SSA/Ps with dysplasia. MLH1-deficient and MLH1-retained SSA/Ps with dysplasia show distinct clinicopathological features that are shared by MMR-deficient and MMR-proficient carcinomas with *BRAF* mutations, respectively.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. Target regions of next-generation sequencing and minimum coverage of each amplicon.

Supplementary Table 2. Primers used for Sanger sequencing.

Supplementary Table 3. Mutations detected in sessile serrated adenoma/polyps.

Supplementary Table 4. Clinicopathological and molecular features of sessile serrated adenoma/polyps.

Supplementary Figure. *RNF43* **and** *APC* **mutations in sessile serrated adenoma/polyps (SSA/Ps) with dysplasia.**

TABLES

Table 1 Clinicopathological and molecular features of sessile serrated adenoma/polyps with and without dysplasia

	Dysplasia		p Value
	Positive (n = 46)	Negative (n = 45)	
Age (years), mean (range)	68.1 (42–85)	59.7 (40–77)	1.4×10^{-4}
Male/Female	20/26	24/21	0.40
Proximal/Distal	39/7	44/1	0.059
Polyp size (mm), mean (range)	8.5 (4–18)	8.6 (4–22)	0.86
Dysplastic component (mm), mean (range)	4.7 (1–12)	–	
WNT pathway gene mutations	28 (61%)	3 (7%)	3.0×10^{-8}
RNF43	23 (50%)	3 (7%)	
APC	4 (9%)	0	
ZNRF3	3 (7%)	0	
CTNNB1	0	0	
RSPO fusion	0*	0	
MAPK pathway gene mutations	44 (96%)	39 (87%)	0.16
BRAF	40 (87%)	38 (84%)	
KRAS	4 (9%)	2 (4%)	
NRAS	0	0	
GNAS	2 (4%)	0	
Nuclear β-catenin	35 (76%)	5 (11%)	2.0×10^{-10}
MYC overexpression	42 (91%)	4 (9%)	1.3×10^{-16}

*RT-PCR was unsuccessful in one lesion.

Table 2 Comparison of MLH1-deficient and MLH1-retained sessile serrated adenoma/polyps with dysplasia

	MLH1 expression		p Value
	Deficient (n = 14)	Retained (n = 32)	
Age (years), mean (range)	71.9 (63–82)	66.4 (42–85)	0.047
Male/Female	3/11	17/15	0.059
Proximal/Distal	14/0	25/7	0.083
Polyp size (mm), mean (range)	8.2 (4–16)	8.6 (4–18)	0.72
Dysplastic component (mm), mean (range)	3.8 (1–6)	4.9 (2–12)	0.048
WNT pathway gene mutations	12 (86%)	16 (50%)	0.047
RNF43	12 (86%)	11 (34%)	
APC	0	4 (13%)	
ZNRF3	1 (7%)	2 (6%)	

REFERENCES

1.   Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology.* 2007;50:113-130.

2.   Leggett B, Whitehall V. Role of the serrated pathway in colorectal cancer pathogenesis. *Gastroenterology.* 2010;138:2088-2100.

3.   Bettington M, Walker N, Clouston A, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. *Histopathology.* 2013;62:367-386.

4.   Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med.* 1988;319:525-532.

5.   Rajagopalan H, Nowak MA, Vogelstein B, et al. The significance of unstable chromosomes in colorectal cancer. *Nat Rev Cancer.* 2003;3:695-701.

6.   Snover DC. Update on the serrated pathway to colorectal carcinoma. *Hum Pathol.* 2011;42:1-10.

7.   O'Brien MJ, Zhao Q, Yang S. Colorectal serrated pathway cancers and precursors. *Histopathology.* 2015;66:49-65.

8.   Dehari R. Infrequent APC mutations in serrated adenoma. *Tohoku J Exp Med.* 2001;193:181-186.

9.   Sawyer EJ, Cerar A, Hanby AM, et al. Molecular characteristics of serrated adenomas of the colorectum. *Gut.* 2002;51:200-206.

10.   Yamamoto T, Konishi K, Yamochi T, et al. No major tumorigenic role for beta-catenin in serrated as opposed to conventional colorectal adenomas. *Br J Cancer.* 2003;89:152-157.

11.   Yang S, Farraye FA, Mack C, et al. BRAF and KRAS Mutations in hyperplastic polyps and serrated adenomas of the colorectum: relationship to

histology and CpG island methylation status. *Am J Surg Pathol.* 2004;28:1452-1459.

12.   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;53:1137-1144.

13.   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;30:1491-1501.

14.   Kakar S, Deng G, Cun L, et al. CpG island methylation is frequently present in tubulovillous and villous adenomas and correlates with size, site, and villous component. *Hum Pathol.* 2008;39:30-36.

15.   Sekine S, Yamashita S, Tanabe T, et al. Frequent PTPRK-RSPO3 fusions and RNF43 mutations in colorectal traditional serrated adenoma. *J Pathol.* 2016;239:133-138.

16.   Ogino S, Shima K, Meyerhardt JA, et al. Predictive and prognostic roles of BRAF mutation in stage III colon cancer: results from intergroup trial CALGB 89803. *Clin Cancer Res.* 2012;18:890-900.

17.   Lochhead P, Kuchiba A, Imamura Y, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. *J Natl Cancer Inst.* 2013;105:1151-1156.

18.   Seppala TT, Bohm JP, Friman M, et al. Combination of microsatellite instability and BRAF mutation status for subtyping colorectal cancer. *Br J Cancer.* 2015;112:1966-1975.

19.   Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with

the BRAF V600E mutation in microsatellite-stable colon cancers. *Cancer Res.* 2005;65:6063-6069.

20.	Pai RK, Jayachandran P, Koong AC, et al. BRAF-mutated, microsatellite-stable adenocarcinoma of the proximal colon: an aggressive adenocarcinoma with poor survival, mucinous differentiation, and adverse morphologic features. *Am J Surg Pathol.* 2012;36:744-752.

21.	Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. *Gastroenterology.* 2015;148:77-87 e2.

22.	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;131:1400-1407.

23.	Torlakovic E, Skovlund E, Snover DC, et al. Morphologic reappraisal of serrated colorectal polyps. *Am J Surg Pathol.* 2003;27:65-81.

24.	Sheridan TB, Fenton H, Lewin MR, et al. Sessile serrated adenomas with low- and high-grade dysplasia and early carcinomas: an immunohistochemical study of serrated lesions "caught in the act". *Am J Clin Pathol.* 2006;126:564-571.

25.	Goldstein NS. Small colonic microsatellite unstable adenocarcinomas and high-grade epithelial dysplasias in sessile serrated adenoma polypectomy specimens: a study of eight cases. *Am J Clin Pathol.* 2006;125:132-145.

26.	Rosty C, Hewett DG, Brown IS, et al. Serrated polyps of the large intestine: current understanding of diagnosis, pathogenesis, and clinical management. *J Gastroenterol.* 2013;48:287-302.

27.	Yang HM, Mitchell JM, Sepulveda JL, et al. Molecular and histologic considerations in the assessment of serrated polyps. *Arch Pathol Lab Med.*

2015;139:730-741.

28.   Yachida S, Mudali S, Martin SA, et al. Beta-catenin nuclear labeling is a common feature of sessile serrated adenomas and correlates with early neoplastic progression after BRAF activation. *Am J Surg Pathol.* 2009;33:1823-1832.

29.   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.

30.   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;129:1889-1898.

31.   Murakami T, Mitomi H, Saito T, et al. Distinct WNT/beta-catenin signaling activation in the serrated neoplasia pathway and the adenoma-carcinoma sequence of the colorectum. *Mod Pathol.* 2015;28:146-158.

32.   Bettington M, Walker N, Rosty C, et al. Clinicopathological and molecular features of sessile serrated adenomas with dysplasia or carcinoma. *Gut.* 2017;66:97-106.

33.   Tsai JH, Liau JY, Yuan CT, et al. RNF43 Is an Early and Specific Mutated Gene in the Serrated Pathway, With Increased Frequency in Traditional Serrated Adenoma and Its Associated Malignancy. *Am J Surg Pathol.* 2016;40:1352-1359.

34.   Yan HH, Lai JC, Ho SL, et al. RNF43 germline and somatic mutation in serrated neoplasia pathway and its association with BRAF mutation. *Gut.* 2016. [Epub ahead of print].

35.   Koo BK, Spit M, Jordens I, et al. Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature.* 2012;488:665-669.

36.   Furukawa T, Kuboki Y, Tanji E, et al. Whole-exome sequencing uncovers

frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. *Sci Rep.* 2011;1:161.

37.   Wu J, Jiao Y, Dal Molin M, et al. Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. *Proc Natl Acad Sci U S A.* 2011;108:21188-21193.

38.   Ong CK, Subimerb C, Pairojkul C, et al. Exome sequencing of liver fluke-associated cholangiocarcinoma. *Nat Genet.* 2012;44:690-693.

39.   Jiang X, Hao HX, Growney JD, et al. Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. *Proc Natl Acad Sci U S A.* 2013;110:12649-12654.

40.   Ryland GL, Hunter SM, Doyle MA, et al. RNF43 is a tumour suppressor gene mutated in mucinous tumours of the ovary. *J Pathol.* 2013;229:469-476.

41.   Wang K, Yuen ST, Xu J, et al. Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. *Nat Genet.* 2014;46:573-582.

42.   Giannakis M, Hodis E, Jasmine Mu X, et al. RNF43 is frequently mutated in colorectal and endometrial cancers. *Nat Genet.* 2014;46:1264-1266.

43.   Bond CE, McKeone DM, Kalimutho M, et al. RNF43 and ZNRF3 are commonly altered in serrated pathway colorectal tumorigenesis. *Oncotarget.* 2016;7:70589-70600.

44.   Bettington ML, Walker NI, Rosty C, et al. A clinicopathological and molecular analysis of 200 traditional serrated adenomas. *Mod Pathol.* 2015;28:414-427.

45.   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, 4th ed.* Lyon, France: IARC, 2010;160–165.

46.   Baker AM, Van Noorden S, Rodriguez-Justo M, et al. Distribution of the c-MYC gene product in colorectal neoplasia. *Histopathology.* 2016;69:222-229.

47.   Bettington M, Walker N, Rosty C, et al. Critical appraisal of the diagnosis of the sessile serrated adenoma. *Am J Surg Pathol.* 2014;38:158-166.

48.   Barker N, Ridgway RA, van Es JH, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature.* 2009;457:608-611.

49.   Seshagiri S, Stawiski EW, Durinck S, et al. Recurrent R-spondin fusions in colon cancer. *Nature.* 2012;488:660-664.

50.   Oka S, Tanaka S, Hiyama T, et al. Clinicopathologic and endoscopic features of colorectal serrated adenoma: differences between polypoid and superficial types. *Gastrointest Endosc.* 2004;59:213-219.

51.   Kim MJ, Lee EJ, Suh JP, et al. Traditional serrated adenoma of the colorectum: clinicopathologic implications and endoscopic findings of the precursor lesions. *Am J Clin Pathol.* 2013;140:898-911.

52.   Bettington ML, Chetty R. Traditional serrated adenoma: an update. *Hum Pathol.* 2015;46:933-938.

53.   Chetty R. Traditional serrated adenoma (TSA): morphological questions, queries and quandaries. *J Clin Pathol.* 2016;69:6-11.

54.   Lee EJ, Chun SM, Kim MJ, et al. Reappraisal of hMLH1 promoter methylation and protein expression status in the serrated neoplasia pathway. *Histopathology.* 2016;69:198-210.

55.   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;35:1274-1286.

56.   Inoue A, Okamoto K, Fujino Y, et al. B-RAF mutation and accumulated gene methylation in aberrant crypt foci (ACF), sessile serrated adenoma/polyp (SSA/P) and cancer in SSA/P. *Br J Cancer.* 2015;112:403-412.