

## Sessile serrated adenoma with early neoplastic progression : A clinicopathologic and molecular study

Fujita, Kohei

Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University |  
Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University

Yamamoto, Hidetaka

Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University

Matsumoto, Takayuki

Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University

Hirahashi, minako

Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University

他

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Sessile serrated adenoma with early neoplastic progression: A clinicopathological and molecular study

Running title: Adenocarcinoma arising in SSA

Kohei Fujita, MD<sup>1,2</sup>, Hidetaka Yamamoto, MD, PhD<sup>1</sup>, Takayuki Matsumoto, MD, PhD<sup>2</sup>,  
Minako Hirahashi, MD, PhD<sup>1</sup>, Masaki Gushima, MD, PhD<sup>1,2</sup>, Junji Kishimoto, PhD<sup>3</sup>,  
Ken-ichi Nishiyama, MD, PhD<sup>4</sup>, Tomoaki Taguchi, MD, PhD<sup>5</sup>, Takashi Yao, MD, PhD<sup>6</sup>,  
Yoshinao Oda, MD, PhD<sup>1</sup>

<sup>1</sup>Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

<sup>2</sup>Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

<sup>3</sup>Department of Digital Medicine Initiative, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

<sup>4</sup>Department of Pathology, Institute for Clinical Research, National Kyushu Cancer Center, Fukuoka, Japan

<sup>5</sup>Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

<sup>6</sup>Department of Human Pathology, Juntendo University School of Medicine, Tokyo, Japan

Corresponding author: Hidetaka Yamamoto, MD, PhD

Department of Anatomic Pathology, Graduate School of Medical Sciences

Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Phone +81-92-642-6061; Fax +81-92-642-5968

E-mail: [hidetaka@surgpath.med.kyushu-u.ac.jp](mailto:hidetaka@surgpath.med.kyushu-u.ac.jp)

Address reprint requests to:

Yoshinao Oda, MD, PhD

Department of Anatomic Pathology, Graduate School of Medical Sciences

Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

## Abstract

Sessile serrated adenoma (SSA), also referred to as sessile serrated polyp (SSP), has been proposed as a precursor lesion to microsatellite unstable carcinoma. However, the mechanism of stepwise progression from SSA to early invasive carcinoma has been unclear. The purpose of this study was to elucidate the histological characteristics and possible role of p53,  $\beta$ -catenin, BRAF, KRAS, and PIK3CA in the development and progression of SSA. We analyzed 12 cases of SSA with neoplastic progression (SSAN), including 7 cases of intraepithelial high-grade dysplasia (HGD) and 5 cases of submucosal invasive carcinoma, and compared them with 53 SSAs and 66 hyperplastic polyps (HPs) by immunohistochemistry and gene mutation analysis. Histologically, 75% (9/12) of SSANs showed tubular or tubulovillous growth patterns rather than serrated ones in the HGD/intramucosal carcinoma component. All 5 SSANs with invasive carcinoma lost their serrated structure and developed increased extracellular mucin in their submucosal carcinoma component, a consistent feature of mucinous adenocarcinoma. Nuclear accumulations of  $\beta$ -catenin and p53 were observed in 50% (6/12) and 41.7% (5/12) of SSANs, respectively, and were exclusively present in HGD/carcinoma areas. By contrast, neither nuclear  $\beta$ -catenin nor p53 expressions were seen in HPs or SSAs ( $p < 0.0001$ ). BRAF mutations (V600E) were observed in 45.8% (11/24) of HPs, 60.9% (14/23) of SSAs, and 63.6% (7/11) of SSANs, and were equally found in both SSA and carcinoma/HGD areas of the individual SSANs. KRAS exon1 mutations were uncommon in all 3 groups (4.2%, 4.4%, and 0%, respectively). No mutations of PIK3CA exon9 or exon20 were found in any cases examined. These findings suggest that BRAF mutations may be associated with the pathogenesis of SSA, but progression to HGD or early invasive carcinoma may be associated with other

factors such as alterations of p53 and  $\beta$ -catenin. In addition, our histological observations suggest a possible close association between SSAN and mucinous adenocarcinoma.

**Key words:** sessile serrated adenoma, adenocarcinoma, serrated neoplasia pathway, BRAF, KRAS, PIK3CA

## Introduction

Most colorectal cancers develop through a conventional adenoma-carcinoma sequence characterized by chromosomal instability and stepwise accumulation of multiple genetic mutations such as APC/ $\beta$ -catenin, KRAS, and p53.<sup>38</sup> Recent increasing evidence suggests that 10% to 20% of colorectal cancers develop via another pathway, known as the “serrated polyp-neoplasia pathway.” Such carcinomas may originate from serrated polyps. The endpoint carcinomas of this pathway are assumed to be associated with BRAF mutation, DNA hypermethylation, and microsatellite instability (MSI).<sup>16, 25, 40</sup> Serrated polyps are histologically classified into hyperplastic polyp (HP), traditional serrated adenoma (TSA), sessile serrated adenoma (SSA), and mixed hyperplastic/adenomatous polyp.<sup>34</sup> In particular, there are many clinicopathological and genetic similarities between SSA and MSI-high colorectal cancers; e.g., right-sided predilection, decreased expression of DNA mismatch repair protein hMLH1, frequent BRAF mutation, and infrequent KRAS mutation.<sup>7, 10, 28, 36</sup> Therefore, SSA can be considered as the precursor to some populations of MSI-high carcinomas of the proximal colon.<sup>34</sup> Thus, there is a growing consensus that SSA has a significant risk of malignant change and that SSA should be carefully treated despite a lack of overt cytological atypia. However, the details of the molecular mechanism of stepwise progression of SSA remain unclear. In fact, carcinomas actually arising from SSAs are rarely encountered in routine pathological practice. Therefore, investigation of SSA with early neoplastic progression may give insights into the understanding and prevention of the progression of the serrated neoplasia pathway, a clinically important subject.

In colorectal cancer, KRAS, BRAF, and PIK3CA mutations have been considered to play an important role in tumorigenesis by activating the RAS-RAF-MAPK or

PI3K-PIP3-AKT signaling pathways.<sup>6, 21, 31</sup> Recently, mutations in the PIK3CA gene, which encodes the p110 $\alpha$  catalytic subunit of phosphatidylinositol 3-kinases (PI3Ks), have been found in 10% to 30% of colorectal cancer cases. Because oncogenic RAS also activates PI3Ks, the PI3K signaling pathway via RAS mutation or PIK3CA mutation is considered to play an important role in colorectal carcinogenesis.<sup>3, 21</sup> In fact, the presence of PIK3CA mutation is reported to be associated with KRAS mutation, MSI, and worse prognosis of colon carcinomas.<sup>1, 3, 26, 31</sup> However, the prevalence and clinicopathological significance of PIK3CA mutation in serrated pathway-derived carcinomas are unclear.

The aims of this study were to elucidate the clinicopathological and morphologic features of SSA with histological progression and investigate the potential roles of p53,  $\beta$ -catenin, BRAF, KRAS, and PIK3CA mutations.

## **Materials and Methods**

### *Cases and histological evaluation*

We retrospectively reassessed 362 cases of polypectomized serrated polyp specimens between 1993 and 2009, and 335 cases of surgically resected colorectal carcinoma specimens between 2004 and 2009 at our institution.

The clinical records of the patients were reviewed with regard to patient age, sex, clinical diagnosis, family history, and colonoscopic findings. Lesions found in patients with established diagnoses of familial adenomatous polyposis, hereditary nonpolyposis colorectal carcinoma (HNPCC), inflammatory bowel diseases, or hyperplastic/serrated polyposis syndrome were not included in this study.

Histologic findings of the serrated polyps were reviewed independently by 3

pathologists (K.F., H.Y., and T.Y.) without the clinical information. In this study, we used only the cases in which a diagnostic consensus among the 3 pathologists was obtained. The serrated polyp, which includes HP, SSA, and SSA with neoplastic progression (SSAN), was defined as a polypoid lesion that demonstrated a serrated or “saw-tooth” architecture due to infolding of the crypt epithelium.

HPs can be further classified into 3 subtypes based on the amount of mucin and cell type (microvesicular, goblet cell, or mucin-poor)<sup>36</sup>; however, we used only microvesicular type HPs because this type is closest to SSA in terms of clinicopathological and histological features.

The diagnosis of SSA was based on the presence of irregular, dilated glands; serration at the base of crypts; and horizontally and/or laterally branched crypt bases.<sup>8, 34</sup>

<sup>36</sup> Polyps with mixed features of SSA and TSA were not included in this study.

In this study, SSAN was defined as a tumor in which intraepithelial high-grade dysplasia or early invasive adenocarcinoma (adenocarcinoma with submucosal invasion) areas were continuously surrounded by SSA areas at both oral and distal sides of the individual tumor. This was to avoid the possibility of a collision tumor, in which sporadic high-grade dysplasia or carcinoma inadvertently involved a nearby SSA. Both high-grade dysplasia and early invasive carcinoma in SSAN correspond to Category 4 (noninvasive high-grade neoplasia) or Category 5 (invasive neoplasia), respectively, according to the Vienna classification of gastrointestinal epithelial neoplasia.<sup>32</sup>

Finally, 12 SSAN cases, including 7 polypectomized cases and 5 surgically resected cases, were obtained. As controls, 66 HPs and 53 SSAs, all of which were polypectomized specimens, were retrieved. The location of each lesion was classified as proximal or distal to the splenic flexure. Macroscopic types were divided into



protruded-type and sessile-type. This study was approved by the Institutional Review Board of Kyushu University, Japan (no. 22-12).

### *Immunohistochemistry*

Immunohistochemical staining was performed using monoclonal antibodies to  $\beta$ -catenin (clone 14; Transduction Laboratories, Lexington, KY, USA, diluted 1:200) and to p53 (clone PAb1801; Oncogene Research Products, Cambridge, MA, USA, diluted 1:100). Sections were cut into thicknesses of 4  $\mu$ m, deparaffinized in xylene, and dehydrated in descending dilutions of ethanol. For antigen retrieval, each slide was treated by microwave heating in citrate buffer (pH 6.0) for 10 min (p53) or 20 min ( $\beta$ -catenin). Endogenous peroxidase activity was blocked by 30 min of incubation with 0.3% hydrogen peroxidase in absolute methanol. Background staining was minimized by incubation with 1% normal rabbit serum for 10 min. Sections were incubated with a primary antibody overnight at 4°C, followed by testing with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). Diaminobenzidine tetrahydrochloride was used as the chromogen. Finally, sections were counterstained with Mayer's hematoxylin.

$\beta$ -catenin labeling was evaluated with respect to membranous and/or nuclear expression in the epithelial cells with the exception of crypt bases because nuclear staining of  $\beta$ -catenin is usually observed there in normal colorectal mucosa.

Membranous labeling without nuclear expression was considered normal. Nuclear  $\beta$ -catenin accumulation (moderate to strong expression in  $> 5\%$  of the epithelium) and loss of membranous  $\beta$ -catenin expression were considered to be abnormal reactivities. p53 staining was interpreted as either positive (moderate to strong expression in  $> 50\%$  of the epithelium) or negative (negative, weak staining, or moderate or strong expression in  $< 50\%$  of the epithelium).

#### *Detection of mutations of BRAF, KRAS, and PIK3CA genes*

Genomic DNA was extracted from formalin-fixed, paraffin-embedded specimens using a macrodissection technique for 24 HPs and 23 SSAs and a microdissection technique for 11 SSANs. For the macrodissection method, nontumorous tissue was macroscopically removed from the slides using a 20-gauge needle, and remaining specimens (predominant tumor tissue) on slides were then retrieved in the tubes for DNA extraction. For the microdissection method, we used the Leica LMD6000 laser microdissection system (Leica Microsystems, Tokyo, Japan) to obtain at least 1000 tumor cells per sample independently from the dysplastic/carcinomatous and nondysplastic epithelium. Subsequently, genomic DNA was extracted from the samples using a DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan) or QIAamp DNA Micro Kit (Qiagen, Tokyo, Japan), respectively, in accordance with the manufacturer's protocols. Polymerase chain reaction (PCR) amplifications were performed using the following primers: 5'CTTCATGAAGACCTCACAGT3' (BRAF-exon15-forward); 5'CATCCACAAAATGGATCCAG3' (BRAF-exon15-reverse); 5'GGCCTGCTGAAAATGACTGA3' (KRAS-exon1-forward); 5'GTTGGATCATATTCGTCCAC3' (KRAS-exon1-reverse);

5'GCTAGAGACAATGAATTAAGGGAAA3' (PIK3CA-exon9-forward);

5'AGCACTTACCTGTGACTCCA3' (PIK3CA-exon9-reverse);

5'AACTGAGCAAGAGGCTTTGG3' (PIK3CA-exon20-forward);

5'CTTTTCAGTTCAATGCATGCTG3' (PIK3CA-exon20-reverse). The amplified PCR

products were then purified using Montage centrifugal filters (Millipore, Bedford, MA,

USA). After purification, direct sequencing was carried out using a Perkin Elmer ABI

PRISM 310 sequence analyzer (Applied Biosystems, Foster City, CA, USA). If the

quality of DNA or level of PCR amplification was insufficient for mutation analysis, the

cases were excluded from the molecular study.

### **Statistical Analysis**

We performed the  $\chi^2$ -test, Fisher's exact test, and Student's t-test using JMP Statistical Discovery Software (version 8.0J; SAS Inc., Cary, NC, USA). Results were considered statistically significant if  $p < 0.05$ .

## Results

### *Clinicopathological findings*

The comparison of clinicopathological findings among 66 HPs, 53 SSAs, and 12 SSANs are summarized in Table 1. The detailed clinicopathological findings of 12 SSANs are also listed in Table 2. The ages of patients with SSAN (mean, 70.9 years) were greater than those with HP (mean, 57.1 years) ( $p < 0.0001$ ) or those with SSA (mean, 62.7 years) ( $p = 0.0156$ ). SSANs (mean, 11.3 mm) were larger in size than HPs (mean, 7.2 mm) ( $p < 0.0001$ ), while there was no significant difference in size between SSANs and SSAs (mean, 10.9 mm) ( $p = 0.7505$ ). Patients with SSANs showed a female predominance compared with HPs and SSAs ( $p < 0.0001$  and  $p = 0.0007$ , respectively). SSANs and SSAs were preferentially located in the right colon compared with HPs ( $p < 0.0001$ ).

### *Histological findings*

Microvesicular type HPs showed crypts that were predominantly serrated on the surface, but predominantly tapered and narrow at the deeper mucosa (Fig. 1A). Most crypt epithelium contained abundant microvesicular mucin, and goblet cells were less frequent.

SSAs showed crypts that were irregularly dilated or serrated from the surface to the deeper mucosa, and horizontal branching or budding at the crypt bases (Fig. 1B). Focal nuclear enlargement and pseudostratification were also noted.

Among 12 cases of SSANs, 7 (58.3%) contained areas of high-grade dysplasia (HGD) and 5 (41.7%) contained areas of adenocarcinoma invading the submucosa (Table 2). The histological features of the HGD area (Fig. 1C-E) and intramucosal

carcinoma area were assessed by their predominant growth pattern of severely atypical epithelial cells: a tubular, tubulovillous, or fused glandular pattern, mimicking conventional adenomatous high-grade dysplasia; or a serrated glandular pattern, preserving the serrated or saw-toothed structure with infolding of the crypt epithelium. A tubular or tubulovillous growth pattern was observed in 75% (9/12) of HGD/intramucosal carcinoma areas. A serrated growth pattern was observed in 58.3% (7/12) of HGD/intramucosal carcinoma areas; of the 7 cases, however, a serrated growth pattern was predominant in only 3, and the remaining 4 cases showed a predominantly tubular or tubulovillous growth pattern. In the serrated growth pattern area, the enlarged nuclei of tumor cells were round to oval rather than elongated or pseudostratified. The cells also had more eosinophilic cytoplasm and more prominent nucleoli compared with cells in the tubular or tubulovillous growth pattern area.

Of 5 cases of invasive adenocarcinoma (Fig. 1F-H), 3 showed a serrated growth pattern in the intramucosal carcinoma area (predominantly in 2 cases and focally in 1 case). However, no cases retained the serrated growth pattern in the submucosal invasive area; alternatively, all 5 invasive carcinomas showed a tubular or tubulovillous pattern of adenocarcinoma in the submucosa. No cases showed poorly differentiated component in both intramucosal and invasive carcinoma areas. All 5 cases with invasive adenocarcinoma also contained increased extracellular mucinous components of 20% to 50% of the entire tumor area. Mucin pools lined by well-differentiated adenocarcinoma cells or floating carcinoma clusters disrupted by the presence of abundant, extruded intraluminal mucin were observed in those cases. Two of the 7 cases of SSAN with HGD also contained focal extracellular mucin.

Lymphatic vessel invasion was observed in one case of adenocarcinoma (Case

no. 12). Blood vessel invasion of carcinoma cells was not present in any case of SSAN. In addition, lymph node metastasis was found in one case of adenocarcinoma (Case no. 10).

#### *Immunohistochemical findings*

The results of immunohistochemical staining are summarized in Tables 3 and 5. Aberrant p53 nuclear accumulation was observed in 41.7% (5/12) of SSANs. All cases of HP and SSA were negative for p53 nuclear expression (Fig. 2). The incidence of aberrant p53 expression was higher in SSAN than in HP and SSA (each  $p < 0.0001$ ).

Nuclear  $\beta$ -catenin accumulation was observed in 50% (6/12) of SSANs, but not at all in HPs or SSAs (each  $p < 0.0001$ ) (Fig. 3). Partial loss of membranous  $\beta$ -catenin expression was observed in 10.6% (7/66) of HPs and in 20.8% (11/53) of SSAs, but nuclear accumulation of this protein did not accompany those cases. Among 12 SSAN cases, HGDs showed nuclear accumulation of  $\beta$ -catenin and p53 in 43% (3/7) of cases, and invasive carcinoma showed nuclear accumulation of  $\beta$ -catenin and p53 in 60% (3/5) and 40% (2/5) of cases, respectively. As for the 5 cases of invasive adenocarcinoma, there was no different expression pattern of p53 and  $\beta$ -catenin between intramucosal and invasive carcinoma components.

#### *Mutation analysis*

The results of mutation analysis of exon 15 of BRAF, exon 1 of KRAS, and exons 9 and 20 of PIK3CA are summarized in Table 4. The V600E (T to A transversion) BRAF mutations were observed in 45.8% (11/24) of HPs, 60.9% (14/23) of SSAs, and 63.6% (7/11) of SSANs (Fig. 4). As for SSAN, both SSA and HGD/carcinoma

components showed essentially the same mutation pattern (see below). There was no significant difference in the prevalence of BRAF mutation among the 3 groups. In all 3 groups, the presence of BRAF mutation did not correlate with clinicopathological features, such as anatomic location, polyp size, and gender.

KRAS mutations were uncommon in all 3 groups; only each single case of HP and SSA had KRAS mutations in codon 12 (G12S) and codon 13 (G13D), respectively. BRAF mutations and KRAS mutations were mutually exclusive; the case with BRAF mutation had no KRAS mutation, and vice versa. No PIK3CA mutations were found in any case of HP, SSA, or SSAN.

#### *Comparison between adjacent SSA area and HGD/carcinoma area in SSAN*

The results of immunohistochemical staining and molecular analysis using microdissection methods in SSAN is summarized in Table 5. In all but one patient, both SSA and HGD/carcinoma components had the same V600E mutations. HGD/carcinoma components showed frequent nuclear p53 and  $\beta$ -catenin nuclear accumulation, whereas the adjacent SSA areas showed no nuclear accumulation of these proteins. There was no significant correlation between the presence of BRAF mutation and  $\beta$ -catenin expression or p53 nuclear expression in HGD/carcinoma components of SSAN.

#### *Comparison between morphological growth patterns of the HGD/carcinoma area in SSAN (tubular/tubulovillous type vs. serrated type)*

As for the HGD/intramucosal carcinoma component of 12 cases of SSAN, there was no significant difference of the prevalence of BRAF mutation,  $\beta$ -catenin expression, or p53 nuclear expression between the tubular/tubulovillous type (n = 9) and

serrated type (n = 3) (data not shown).



## Discussion

With the increasing evidence of malignant progression of SSA in molecular and epidemiological backgrounds, SSA has been considered as a precursor of right-sided, MSI-high colon carcinoma. However, the details of stepwise progression of SSA have been unclear, especially from a histological point of view. One possible reason is the rarity of cases with dysplasia and carcinoma actually arising in SSA. Although it has been estimated that transformation from SSA to invasive adenocarcinoma occurs via low- to high-grade dysplasia and intramucosal adenocarcinoma, we rarely encountered these lesions in daily pathological practice. Another possible reason is the confusing terminology of serrated lesions. Since the original definition for SSA was lacking overt dysplasia, SSAs with a discrete dysplastic area (adenomatous epithelium) were prone to be expediently categorized as mixed hyperplastic/adenomatous polyps or TSAs, and the etiology of such lesions has hardly been discussed.<sup>17, 20, 27, 37</sup> Therefore, we critically defined sessile serrated adenoma with neoplastic progression (SSAN) as only cases with high-grade dysplastic or invasive carcinoma areas surrounded by nondysplastic SSA circumferentially. As a result, we have demonstrated that high-grade dysplastic or carcinoma components arising from SSAs were not always serrated structure, but predominantly tubular or tubulovillous structure. The results were consistent with previous reports by Goldstein et al.<sup>9</sup> Additionally, we found that tubular adenomatous high-grade dysplastic areas and SSAs shared the same BRAF mutation pattern. This is contrary to the fact that sporadic conventional tubular adenoma hardly harbored BRAF mutation.<sup>18, 35</sup> These findings support the fact that SSAN in this study was not a collision but a stepwise transitional lesion. Iino et al. also reported similar molecular changes in both HPs and tubular adenomatous components in the study of MSI status of

12 cases of “mixed polyps.”<sup>13</sup>

It is also noteworthy that 83% (10/12) of SSANs were located in the proximal colon, and 58% (7/12) of SSANs, including all 5 cases of invasive carcinoma, showed increased extracellular mucin production. Sheridan et al. had also noticed extracellular mucin and “signet-type” mucinous carcinoma in their series of SSAN; however, the description was only in the figure legends, and the details of prevalence and clinicopathological associations had not been documented.<sup>33</sup> Lu et al. recently described that subsequent colon carcinomas in patients with prior SSA diagnosed by biopsy exhibited the features of mucinous or medullary carcinoma; these phenotypes are usually associated with MSI.<sup>19</sup> In our series, we specifically demonstrated the close association or overlap between SSAN and mucinous “colloid-type” adenocarcinoma. In fact, right-sided mucinous carcinoma is also associated with the MSI-H phenotype and expression of MUC5AC mucin core proteins, as in SSA.<sup>2, 8, 12, 30</sup>

In the current study, most HPs and SSAs had BRAF mutation, whereas KRAS mutation was rarely present in such serrated polyps. This result is consistent with several previous reports showing that BRAF, rather than KRAS, mutation is the predominant genotype in SSAs.<sup>14, 25, 40</sup> Likewise, both benign and high-grade components of SSA-derived dysplasia/carcinoma harbored frequent BRAF mutation but no KRAS mutation in our series. This result suggests that BRAF, rather than KRAS, mutation may play an important role in the early steps of SSA formation, and both genes may have no major role in the malignant progression of SSA.

In this investigation, we found that aberrant nuclear p53 expression was present in 43% (3/7) of high-grade dysplastic areas and 40% (2/5) of invasive carcinoma areas of SSANs, but not in nondysplastic areas of SSANs, HPs, or SSAs. Parfitt et al. also

reported a greater degree of p53 expression in high-grade dysplastic areas than in nondysplastic or low-grade dysplastic areas in a study of 11 cases of SSA with dysplasia.<sup>29</sup> This phenomenon is similar to that seen in the conventional tubular adenoma-carcinoma sequence. As we did not examine p53 gene mutation in this study, the interpretation of p53 protein immunostaining might be debatable concerning the discrimination between wild- and mutant-type protein expression. However, it has been reported that nuclear p53 expression in more than 50% of tumor cells is closely associated with the presence of p53 mutation in the malignant component of the tubular adenoma-carcinoma sequence.<sup>5</sup> In our SSAN series, the vast majority of high-grade cells showed p53 immunoreactivity when a nuclear accumulation of p53 was present. Therefore, the inactivation of p53 due to its gene mutation might play an important role in the malignant progression of SSA.

The presence of BRAF mutation in benign serrated polyps (HP and SSA) and the subsequent occurrence of p53 nuclear accumulation in association with malignant progression lead us to hypothesize that this phenomenon may be similar to the oncogene-induced senescence and escape from senescence checkpoint seen in the transition from cutaneous nevus to malignant melanoma. This idea is essentially consistent with the proposal by Minoo and Jass.<sup>23</sup> Oncogene-induced senescence is a cellular response for protection against the activation of oncogenes such as KRAS and BRAF. Melanocytic nevi harbor frequent BRAF mutations,<sup>4, 11, 22</sup> but this oncogenic BRAF signaling is usually attenuated by induction of tumor suppressors, such as p16<sup>INK4a</sup> and p53, resulting in cell cycle arrest. However, the subsequent inactivation of such tumor suppressor genes may again allow the mutant BRAF to exert a tumorigenic effect that leads to the development of malignant melanoma. To date, it is unclear

whether the concept of oncogene-induced senescence and evasion from it can be exactly applied to the serrated neoplasia pathway because we have not examined the expression of senescence-associated  $\beta$ -galactosidase in serrated polyps and related carcinomas. However, the introduction of such a novel oncogenic concept might help to establish diagnostic and therapeutic strategies in the serrated neoplasia pathway.

Some investigators have reported that abnormal nuclear  $\beta$ -catenin expression was observed in 42.8% to 100% of SSAs with low-grade dysplasia.<sup>15, 39</sup> In the current series, aberrant nuclear accumulation of  $\beta$ -catenin was also observed in half of SSANs, and it was present exclusively in the high-grade dysplastic component but not in non-dysplastic SSA. Cumulative data and our results suggest that nuclear accumulation of  $\beta$ -catenin may be closely related to early progression of SSA.  $\beta$ -catenin is one of the target genes of the Wnt pathway, and gene alteration of  $\beta$ -catenin itself and its regulators, such as APC, is well studied in conventional tubular adenoma and related carcinomas. We cannot yet draw a conclusion about the molecular basis of nuclear accumulation of  $\beta$ -catenin in SSAN. However, the presence of nuclear  $\beta$ -catenin accumulation might evoke the possibility of a common mechanism between the adenoma-carcinoma sequence and serrated neoplasia pathway, proposed as the “fusion pathway” by Jass et al.<sup>14</sup>

To the best of our knowledge, this study was the first to investigate the prevalence of PIK3CA mutations in SSA and related high-grade dysplasia and carcinomas. As a result, no PIK3CA mutations were detected in any case of benign or malignant serrated lesions. This result suggests that the PIK3CA mutation is a quite rare event in the early phase of the serrated neoplasia pathway, although there remains a possibility that this gene mutation may emerge in the latter stages of carcinogenesis, similar to the

adenoma-carcinoma sequence.<sup>24</sup>

In conclusion, although the number of cases analyzed in this study is limited, we demonstrated that glandular serration was lost and tubular/tubulovillous high-grade cells with abundant extracellular mucin became predominant during SSA progression, suggesting a close association between SSAN and mucinous adenocarcinoma. Our results also suggested that BRAF mutations, rather than KRAS or PIK3CA mutations, may play an important role in the pathogenesis of SSA, and that progression to high-grade dysplasia or early invasive carcinoma may be associated with other factors such as alterations of p53 and  $\beta$ -catenin in some population of SSA. These findings might fit the concept of oncogene-induced senescence and escape from it. Further study with larger cohorts is needed to elucidate the mechanism of progression of SSA.

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### **Conflict of interest statement**

We declare that we have no conflicts of interest.

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**Figure legends**

**Figure 1.** Comparative illustrations of hyperplastic polyp (HP), sessile serrated adenoma (SSA), and sessile serrated adenoma with neoplastic progression (SSAN).

(A). Hyperplastic polyps (HPs) have elongated crypts with serrated architecture in the upper half of the crypts and tapered, narrow bases. There is no cytological atypia or architectural dysplasia.

(B). Sessile serrated adenomas (SSAs) contain dilated or laterally branched crypts without overt cytological atypia. Exaggerated luminal serration in the lower crypts is more prominent compared with that in HP.

(C). Low-power view of SSAN with high-grade dysplasia (Case no. 4). An adjacent SSA area is seen at the right portion of the image.

(D). High-power view of the tubular pattern of high-grade dysplasia in a SSAN (same case as Figure 1C). High-grade dysplastic glands with pseudostratified nuclear elongation, crowding, and loss of goblet cells are arranged in a cribriform pattern, mimicking conventional adenomatous high-grade dysplasia.

(E). High-power view of the serrated pattern of high-grade dysplasia in a SSAN (Case no. 3). The serrated structure is retained in the high-grade dysplastic area.

(F). Colonoscopic view of invasive adenocarcinoma arising in SSA, showing a reddish protrusion (arrow), surrounded by a whitish, flat elevated area (arrow heads) (Case no. 11). The former component corresponds to invasive adenocarcinoma and the latter one corresponds to SSA.

(G). Low-power view of invasive adenocarcinoma arising in SSA (same case as Figure 1F). Extracellular mucin production is prominent in the invasive front.

(H). High-power view of an invasive adenocarcinoma area (same case as Figure 1F).

Tumor cells float within the pools of mucin. The tumor cells show nuclear crowding and eosinophilic cytoplasm. A serrated growth pattern was not observed in the submucosal invasive area.

**Figure 2.** Immunohistochemical staining for p53.

(A). No nuclear expression of p53 is detected in HPs.

(B). No nuclear expression of p53 is detected in SSAs with the exception of a few immunoreactive cells in the crypt basal regions.

(C). Diffuse nuclear expression of p53 is seen in the high-grade dysplastic area (left field) in a SSAN (Case no. 3). In contrast, only a few cells show nuclear immunoreactivity in the SSA area (right field).

**Figure 3.** Immunohistochemical staining for  $\beta$ -catenin.

(A). No nuclear expression of  $\beta$ -catenin is detected in HP. Membranous expression is retained in this case (inset).

(B). No nuclear expression of  $\beta$ -catenin is detected in SSA. Focal loss of membranous expression is seen in this case (inset).

(C). Widespread nuclear expression of  $\beta$ -catenin is exclusively present in the high-grade dysplastic area in SSAN (Case no. 3). In contrast, few cells show nuclear immunoreactivity in the SSA area.

**Figure 4.** Representative example of sequence results for BRAF in a SSAN. There is a GTG-to-GAG change (V600E) at codon 600.

