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Sessile Serrated Polyps Without Dysplasia Thrives With Asymmetric Relocation of Cell Proliferation-domains

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Abstract. *Background/Aim: Sessile serrated polyps without dysplasia (SSPND) are characterized by crypts with serrated epithelium, albeit with irregular, corrupted shapes (CCS). Patients and Methods: Cell proliferation was explored in the CCS from 60 SSPND and in the crypts from 12 normal colons. Sections were immuno-stained with the Ki-67 proliferation-cell (PC) marker, and with the p53 tumour-suppressor gene. Results: Three predominant PC-phenotypes were found in the CCS from the 60 SSPND: 44 (73.3%) exhibited ectopic, asymmetric, randomly distributed PC-clusters, 12 (20.0%), continuous PC in one or in both slopes of the crypts, and in the remaining 4 (6.7%), single, randomly distributed PC were recorded. In contrast, the scrutiny of more than 200,000 normal colon crypts (controls) showed symmetrically aligned PC, restricted to the lower third of the crypts. p53-up-regulation in CCS was recorded in 11(18.3%) of the 60 NDSSP, but in none of the normal crypts in the 12 controls. Conclusion: The non-dysplastic epithelium that lines CCS in SSPND coexists with an asymmetric relocation of the PC-domains. In addition, the CCS in nearly one-fifth of the SSPND exhibited p53-up-regulated cells. Taken together, the non-dysplastic CCS epithelium in SSPND thrives with somatic mutations. The accretion of putative mutated non-dysplastic CCS might be a crucial event in the evolution of colonic SSPND towards sessile serrated adenomas.*

Progression of colorectal adenoma to carcinoma is one of the most intensively investigated trails in human malignancies (1-3). In later years, colorectal serrated polyps, comprising hyperplastic polyps (HP), sessile serrated adenomas/polyps (SSA/P), and traditional serrated adenomas (TSA), have received much attention (4-6). Although the most common of all serrated polyps -namely HP- is considered innocuous, patients with Hyperplastic polyposis coli syndrome (HPCS) are at a high risk to develop a colorectal cancer (7-9). Sporadic, solitary SSA/P and TSA are also prone to progress to invasive carcinoma (10-12). About 30% of all colorectal carcinomas in humans, progress via the serrated pathway (10).

At endoscopic examination, SSA/P appears as a flat or minimally elevated, pale lesion, with indistinct borders and loss of the vascular pattern (13). However, SSA/P are often concealed by a mucus cap or debris (13, 14). Overlooked SSA/P seem to account for a significant proportion of interval-cancers evolving between two surveillance colonoscopic examinations (14). At histologic examination, the normal colon mucosa is composed of a single epithelial cell layer with tubes-like invaginations, called crypts. In histological sections cut perpendicular to the mucosal surface, the crypts are aligned as "row of test tubes" (15-17). The epithelial lining is built of mucus-secreting goblet cells and columnar absorptive cells. Goblet cells are found practically in the entire crypt, chiefly in the distal colon (18). In contrast, SSA/P are epitomized by epithelial serrations often reaching the lower third of the crypts and by crypts with bottle-shaped basal dilatations or with lateral extensions, running parallel to the *muscularis mucosae* (crypt horizontalization). Crypts with lateral extensions have been described as resembling a boot, an L or an inverted T (19-22). According to some authors, the presence of a single boot-like crypt, a single L-like crypt or a single inverted T-shaped crypt, is indicative of SSA/P (22). Occasionally, herniation through the *muscularis mucosae* (pseudo-invasion) may occur (19-22).

In 2003, Torlacovic *et al.* found abnormal cell proliferation in up to 18% of 51 serrated polyps (23) and in 2008, Torlacovic *et al.* reported either expanded, reduced or absent cell proliferation in the various crypts from 28 SSA (24), but the phenomenon was not further elaborated. In

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2005, Higuchi *et al*. found a significant decrease in Ki-67-positive cells in the full length of the crypts in 27 SSA/P (25). Subsequently, Fujimori *et al*. recorded a high percentage of asymmetrically distributed proliferating cells in 24 SSA/P, but the phenomenon was not further elaborated (26). More recently, Hisamatzu *et al*. found in 42 SSA/P, that the proliferative zone was mainly basal (27), and Fortuna *et al*. that MCM2 (a protein involved in DNA replication) demonstrated expansion of the proliferative compartments in 100% of 58 SSA; some crypts exhibited full MCM2 staining. SSA with dysplasia showed consistent diffuse polyp staining (28). Hence, Higuchi, Hisamatzu and Fortuna (25, 27, 28) found no asymmetrical cell proliferation in SSA/P. Whether the asymmetrical cell proliferation remained unnoticed, unexplored or it was not present, remains unknown.

In previous publications, we found below the neoplastic tissue of sporadic conventional polypoid adenomas (29) and of non-polypoid adenomas (30), colon crypts with normal epithelium displaying corrupted shapes (CCS). Subsequent studies of sections immuno-stained with the proliferation marker Ki-67, and with the tumour-suppressor p53, revealed CCS with disparate distributions of proliferating cells (PC) and p53-mutated cells, both in conventional polypoid adenomas and in non-polypoid adenomas (29, 30). These findings strongly suggested that the CCS beneath the neoplastic tissue of both conventional polypoid adenomas and non-polypoid adenomas, harbored somatic mutations.

The purpose of the present investigation was to assess the distribution of PC in the CCS from a cohort of sessile serrated colonic polyps without dysplasia (SSNDP).

Patients and Methods

Sections (4 μm thick) from 60 endoscopically-removed SSPND were retrieved from the archives of the Department of Pathology, Karolinska Institute. Sections were immunoassayed with the proliferation marker Ki-67 (batch MIB1, DAKO Automation, Denmark). Additional sections were also immuno-stained with the primary mouse monoclonal antibody (IgG1, kappa) against the human p53 protein (antip53, DO-7; Ventana Medical System, Inc., Roche, Basel, Switzerland).

PC in CCS from SSPND. The Ki-67 antibody labels all transit amplifying daughter cells (TADs) (26-30). By using 10× oculars and 4× Apo objective (aperture number 0.20), the FOV was 5 mm in diameter. At that magnification, all PC in CCS are easily identified.

p53 up-regulated cells in CCS from SSPND. The p53 transcription factor (encoded by the human gene *TP53*) is a central tumor-suppressor that regulates numerous signaling pathways in carcinogenesis. Given the laboratory resources needed for *TP53* gene sequencing, most researchers use p53 immuno-histochemistry as a surrogate, assuming that p53 overexpression is associated with a mutation, and that the absence of expression is indicative of wild-type p53. In this work, only cells exhibiting p53 immune-reactivity, of the same strong intensity as the neoplastic cells of the neoplastic tissue from a colon adenoma included in positive-run controls, were regarded as overexpressing the p53 protein. To highlight up-regulated cells in 24/60 SSNDP, the immunostaining in some cases was performed without Harris-hematoxylin counterstain.

Table I. *The frequency of predominant PC phenotypes in crypts with normal epithelial lining albeit with corrupted shapes (CCS) found underneath the neoplastic canopy in 60 sessile serrated non-dysplastic polyps (SSNDP).*

Predominant PC phenotype	No. cases (%)
Haphazardly distributed PC clusters*	44 (73.3%)
Continuous PC-domain	12 (20.0%)
Haphazardly-distributed single PC	4 (6.7%)
Total	60 (100%)

*≥two consecutive PC.

Cell proliferation in normal colon crypts. Sections from 12 normal colonic segments proximal or distal to surgically removed colonic adenocarcinoma were immuno-stained with Ki-67 (batch MIB1) and p53 immunostain.

Statistical analysis. The non-parametric Kruskal–Wallis test was applied to compare difference between groups. Statistical significance was defined as $p<0.05$. The Regional Ethical Review Board in Stockholm (no. 2018/688-32 and 2018/2024-32), approved this study.

Results

PC phenotypes in CCS from SSPND. The PC-distribution in CCS could vary in individual SSPND. Therefore, the predominant CCS-PC-phenotype was selected to epitomize each lesion. Three predominant PC-phenotypes were found in the CCS from the 60 SSPND: i) Random, asymmetrically distributed PC clusters (≥two consecutive PC), ii) Continuous PC-domains (in one or in both sides of the crypts), and iii) Random, asymmetrically distributed single PC (Figures 1 and 2).

Random, asymmetrically distributed PC clusters were found in 44 (73.3%), continuous PC-domains in 12 (20.0%), and random, asymmetrically distributed single PC in the remaining 4 (6.7%) (Table I). The difference between SSPND having CCS with random, asymmetrically distributed PC-clusters and the other two groups in Table I, was significant ($p<0.05$). Even crypts with less corrupted shapes within SSPND exhibited derangement in PC-domains.

p53 up-regulated cells in CCS from SSPND. In CCS, haphazardly-asymmetrically distributed p53-up-regulated cells, often as single cells were recorded in 18.3% (11/60) of the SSPND (Figure 3). In the remaining 81.7% (49/60) SSPND, no p53-up-regulated cells in CCS could be demonstrated.

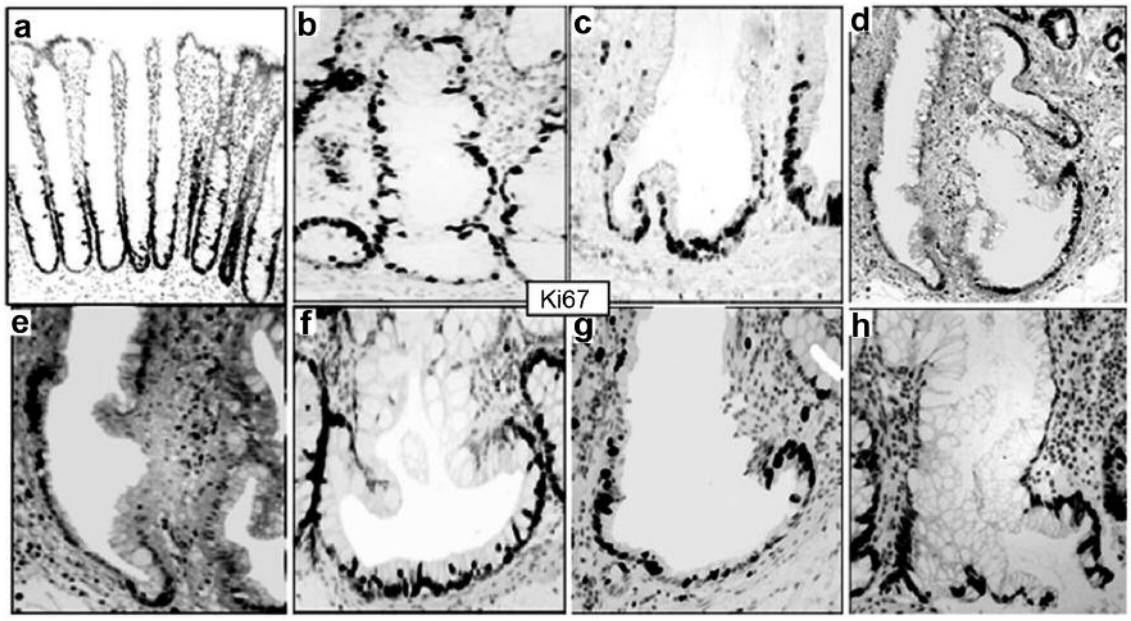

Figure 1. *Cell proliferation in normal colon crypts and in crypts with corrupted shapes in sessile serrated non-dysplastic polyps of the colon. a: Normal colonic crypts showing symmetrically aligned proliferating cells (PC) in "Indian files", limited to the lower third of the crypts (Normal colon, Ki-67 original ×4), b to h: Crypts with corrupted shapes (CCS) in sessile serrated non-dysplastic polyps (SSNDP) of the colon, exhibiting anomalous PC-domains; b: CCS with discontinuous PC at the base of a crypt, but with continuous PC in two-thirds of the CCS (Ki-67 immunostain, original ×10), c to h: CCS in SSNDP showing asymmetrically-haphazardly distributed PC (c to e: original ×20, f to h: original ×40).*

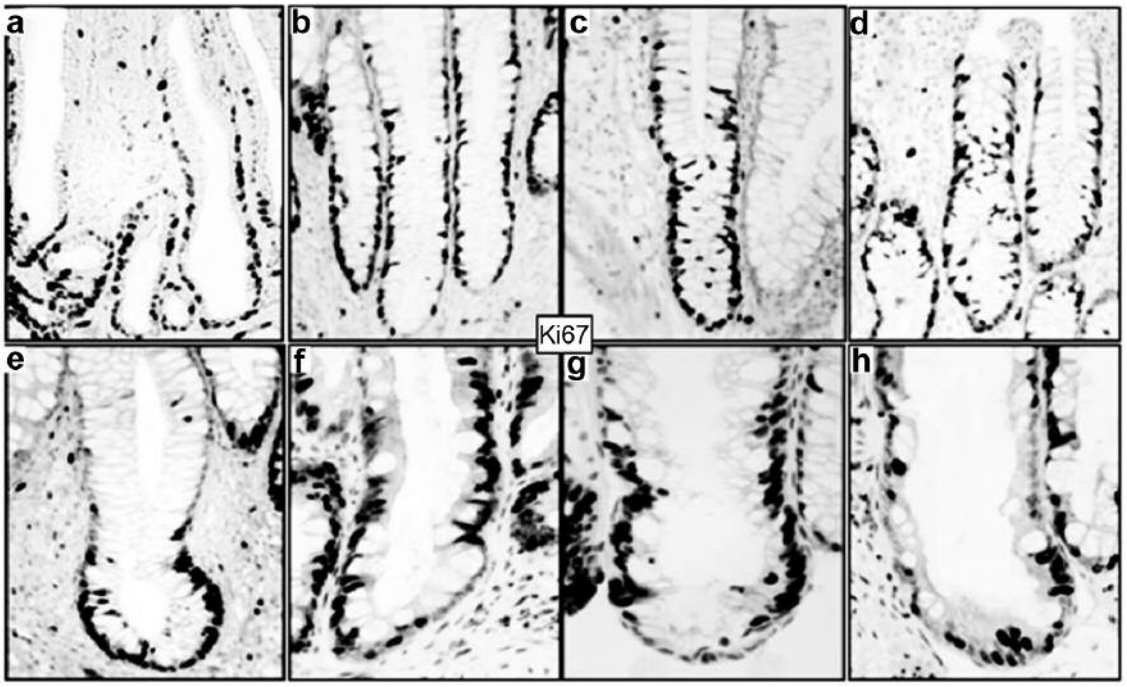

Figure 2. *Cell proliferation in crypts with less corrupted shapes in sessile serrated non-dysplastic polyps of the colon. a, b, c, d, e, and f: Crypts with less corrupted shapes showing atypical PC-distribution, including luminal PC expansion within SSNDP, e and g: Crypts with less corrupted shapes, showing quasi-symmetrical PC-domains in the lower thirds of the crypts, h: Crypt from a SSNDP showing asymmetrically, haphazardly distributed PC domains (Ki-67 immunostain, a to d: original ×20, e to h: original ×40).*

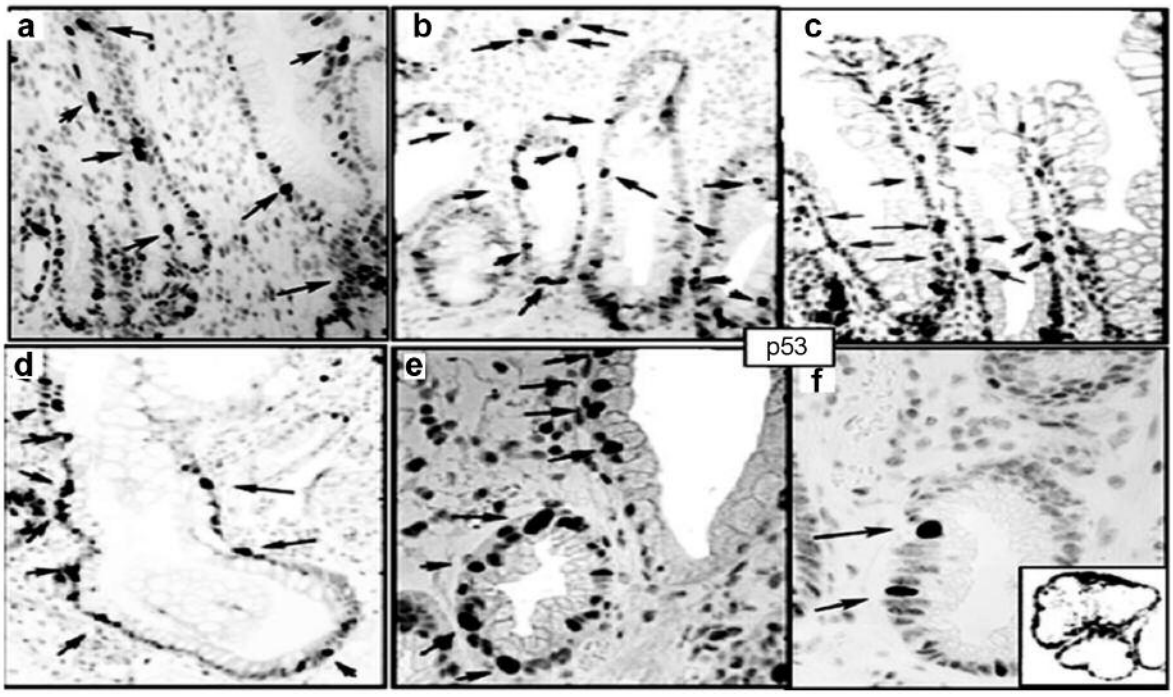

Figure 3. *p53-up-regulation in crypts with corrupted shapes in sessile serrated non-dysplastic polyps of the colon. a to f: Crypts in SSNDP with p53 up-regulated cells (at arrows). The immunoreactivity is of the same intensity (+++) as that in f (insert) from a parallel-run positive control (adenomatous tissue from a conventional adenoma), (p53 immunostain, a: original ×4, b, c and d: original ×10, e and f: original ×20).*

Table II. *The frequency of p-53 up-regulation in sessile serrated non-dysplastic polyps (SSNDP) and in sessile serrated adenoma/polyps (SSA/P) in the literature.*

Author, year (reference)	SSNDP	% p53 labelled cells in SSNDP	SSA/P with dysplasia	% p53-labelled cells or cases in SSAP with dysplasia
Bettington 2017 (11)	137	0 in ordinary SSA component	137	19/137 (14%)
Jass 2006 (45)	0	0	15	13%
Parfitt 2007 (46)	0	0	7	Generally low >15%
Shrivasta 2008 (47)	3	0	2 with cancer	2/2 (100%)
Wu 2008 (48)	22	0	0	--
Sandmaier 2009 (49)	0	0	16	6/16 (38%)
Ngo 2010 (50)	19	19 (100%)	0	--
Fujita 2011 (51)	12	0	12 with neoplastic progression	5 (42%) exclusively in HGD/carcinoma
Tsai 2014 (52)	0	0	16 (TSA)	7/16 (44%)
Ban 2014 (53)	8	0	8 with cancer	6/8 (75%)
Tamoto 2017 (54)	26	0	0	--

PC and p53 in controls. The review of more than 200,000 crypts found in sections from 12 normal colonic portions proximal or distal to the surgically removed colonic adenocarcinoma, showed symmetrically aligned PC in "Indian files", restricted to the lower third of the crypts. None of the crypts showed p53-up-regulation.

Discussion

This study showed that the epithelium lining the CCS of SSPND thrives with atypical, *i.e.* random, asymmetrically, haphazardly distributed PC-domains. This contrasted with the colonic crypts in controls, showing symmetrically

aligned PC restricted to the lower third of the crypts (29). Moreover, CCS in 18% of the SSPND disclosed p53-up-regulated cell-domains, while p53-up-regulated cells were not found in any of the normal crypts in the 12 colon segments.

The stem cells in the normal colon, situated at the base of crypts, synchronize the repopulation of the crypts by virtue of their transient amplifying daughter (TAD) progenitor cells (31-35). These progenitor cells [according to Testa (36), between 120 and 150 TAD cells/crypt *et al.*] comprise the bulk of the PC in the crypts. Since PC-domains are generated by stem cells in normal crypts (33-37), the existence of multiple PC-clusters in TAD from CCS rationally implies the presence of several stem cells in the CCS of SSPND. This assumption is in concert with Baker *et al.* (38), and Tóth *et al.* (39) studies in humans. These authors calculated that up to 6 (32), to 8 (33) stem cells, respectively, exist in each normal colon crypt. Thus, contrasting with the standard position of the stem cells at the base of normal crypts (31-37), the stem cells (that fuel proliferating TAD cells) seem to have been relocated in the CCS from SSPND. The relocation of the normal position of the PC-domain in the CCS from SSPND, often as random, asymmetrically located PC-clusters, and less frequently, as continuous PC in one of the slopes of the crypts or as haphazardly distributed single cells, supports the perception that the serrated epithelium of the CCS in SSPND might harbor somatic mutations, a notion substantiated by the finding of up-regulated p53 cell-domains (Figure 3).

Following a single injection of the colonotropic carcinogen azoxymethane, Whetstone and Gold (40) quantified stem cell mutations in the colon of C57Bl/6 mice. The authors found stem cell mutations in the treated group, but not in any of the >100,000 inspected crypts in the control group (40). Obviously, stem cell mutations ensue in the histologically normal crypt epithelium of the colon in carcinogen-treated C57Bl/6 mice.

There are up to 15 times more mitotic figures in an entire colonic crypt than in the 4 μm thick histological sections according to Goodblad *et al.* (41). Considering that the number of proliferating cells per crypt is much higher than the number of mitotic cells/crypt, it is possible that the CCS in SSNDP with multiple single PC, or with one or more PC-domains, contain a higher number of DNA-synthetizing cells or clusters elsewhere, in other areas from the same serrated crypt. The same might be valid for p53-up-regulated cells in the CCS of SSPND. In the present study, we also used 4 μm thick sections to assess the distribution of PC-domains and p53-up-regulated cells.

Boman and Fields (42) postulated that histological abnormalities occur only in dysplastic crypts. The present demonstration that the non-dysplastic serrated epithelium of CCS in SSPND displayed random, asymmetrically distributed PC-domains, as well as p53-up-regulated cell-domains, strongly suggests that morphological (H&E) changes in the crypts of SSPND might thrive with profound biological alterations in the regulation of both cell proliferation and tumor suppression.

The crucial question is: Which are the morphogenic signals that induce colonic crypts lined with serrated non-dysplastic epithelium, to assume corrupted shapes in SSPND? Morphogenesis is the ability of a system to change its form (43). These authors found that colorectal crypt formation is regulated by a protein encoded by the *PTEN* gene (phosphatase and tensin homologue deleted on chromosome 10, PTEN). In addition, three-dimensional studies of human colon glands demonstrated that the Na^+/H^+ exchanger regulatory factor (NHERF1 protein) controls gland morphogenesis (44). Accordingly, the CCS-phenotypes found in SSNDP in the present study, might had been generated by alterations in NHERF1 and PTEN morphogenetic-signals.

The haphazard distribution of PC, and the putative relocation of stem cells in CCS, raises the question as to whether the natural equilibrium between interdependent elements coordinating stem cells-crypt homeostasis, has been severely altered in the CCS from SSPND.

Crypts with corrupted shapes have not been reported in the normal human colon (15-19). However, a recent review of the normal mucosa in 22 colon segments proximal or distal to surgically removed colon cancer at this department showed occasional CCS (mean=3.7 CCS, range=2-5) (23). The remnant crypts in the 22 colonic segments showed crypts with normal shapes. Hence, the normal colonic mucosa (15-19) and the vast majority of the normal mucosa of patients having synchronously a colon cancer (23), display crypts with normal, regular shapes.

Several reports deal with the expression of p53 in SSP without dysplasia and in SSA/P (11, 45-54). The results of those studies are condensed in Table II. It can be observed that out of the seven studies on SSPND (*i.e.* SSA/P without dysplasia) (11, 47, 48, 50, 51, 53, 54), only one reported p53 immunoreactivity (50). However, the immunoreactivity in that report was described and illustrated as low and diffuse, affecting the cells of the lower third of the crypts (base) (50). In the present work, p53-up-regulated cells were regarded as those having the same degree of intense staining as in a parallel-run positive control (adenomatous epithelium from a conventional adenoma) (Figure 3, insert). Cells with low and diffuse p53 immunoreactivity were disregarded. Thus, the accretion of CCS limited area of a SSPND, exhibiting atypical PC and p53-up-regulated cell-domains in nearly one-fifth of the SSPND investigated, emerges as a remarkable finding, substantiating the notion of putative somatic mutations in SSPND.

As a corollary, SSPND are characterized by crypts with corrupted shapes exhibiting asymmetric distribution of PC-domains. Since the Ki-67 proliferation marker labels only

progenitor TAD daughter cells generated by stem cells, each Ki-67-labelled cell domain in CCS must have been generated by a stem cell, rationally suggesting that CCS house several ectopic stem cells (34-38). In addition, haphazardly distributed p53-up-regulated cells, often as single cells, were found in nearly one-fifth of the SSPND investigated. Taken together, the asymmetric rearrangement of the PC-domains as well as the presence of p53-up-regulated cells, suggest that SSPND might harbor somatic mutations. The accretion of putative mutated CCS appears to be a crucial event in the evolution of colonic SSPND towards sessile serrated adenomas.

Conflicts of Interest

The Authors declare that they have no competing interests related to this study.

Authors' Contributions

CAR: Designed the experiment, performed procedures, data analysis and wrote the manuscript; PTS harvested endoscopically the lesions, introduced suggestions and approved the final manuscript.

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