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p21$^{WAF1/CIP1}$ and Ki-67 Expression of Serrated Adenoma of the Colorectum: An Investigation of Cell Differentiation/Maturation and its Relationship with Cell Proliferation

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Summary. **The immunohistochemical expression of p21$^{WAF1/CIP1}$ and Ki-67 of serrated adenoma (SA) (n=19) of the colorectum was evaluated in order to elucidate its cell differentiation/maturation status and its relationship with cell proliferation. This was then compared with those of a hyperplastic polyp (HP) (n=18), traditional tubular adenoma (TA) (n=21), and normal colonic mucosa (n=11). Longitudinally sectioned hemicrypts were selected from each sample, and the percentage of the p21$^{WAF1/CIP1}$(+) portion and the proliferative zone (PZ) per entire hemicrypt length were determined. In SA, although the topographic regulation of cell differentiation/maturation and proliferation was maintained, the percentage of PZ (26.5±1.8%) decreased and that of the p21$^{WAF1/CIP1}$(+) portion (61.4±2.1%) increased compared with those of normal colonic mucosa (31.8±1.3% and 30.6±1.4%) ($p<0.05$, respectively), indicating that the cell kinetic balance of SA was inclined toward cell differentiation/maturation. A similar cell kinetic imbalance was seen in HP, whereas the cell kinetic balance was inclined toward cell proliferation in TA. These findings suggest that, unlike TA, SA is a disorder of cell differentiation/maturation rather than cell proliferation, and the elongation of the cell life span and the subsequent reduction of exfoliation, which are the known fundamental cell kinetics of HP, may contribute to its neoplastic growth.**

Key words—serrated adenoma, p21, Ki-67, cell maturation, cell proliferation.

INTRODUCTION

Serrated adenoma (SA) is a recently proposed subtype of colorectal adenoma characterized by a serrated glandular structure similar to a hyperplastic polyp (HP) and neoplastic cytological atypia[1]. The concept of SA has been generally accepted, and various studies have demonstrated its unique genetic and cellular phenotypic features as well as its histology. APC and K-ras mutations, which are common genetic events in the morphogenesis of colorectal adenomas[2,3], were reported to be infrequent[4-7], whereas microsatellite instability was found more frequently than in traditional colorectal adenoma[8,9]. Immunohistochemical studies of mucin core protein expression revealed that SA is characterized by the up regulation of MUC5AC[10,11], which is normally limited to the gastric foveolar epithelium[12].

Neoplasms are characterized by a disorder of cell kinetics induced by known and/or unknown genetic alterations, and the cell kinetics comprises cell prolif-

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Abbreviations—APC, adenomatous polyposis coli; CDK, cyclin-dependent kinase; HP, hyperplastic polyp; SA, serrated adenoma; TA, tubular adenoma; PZ, proliferative zone; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine-triphosphate nick-end labeling.

eration, loss, cell differentiation, and maturation. Most cell kinetics studies of SA have focused on cell proliferation[13-17] in order to confirm its neoplastic nature. Recently, cell kinetics studies have been extended to the field of cell loss evaluated by apoptosis[18,19]. Previously, we found that cell loss by apoptosis in SA was far less than that in traditional colorectal adenoma[19]. However, studies on cell differentiation/maturation of SA are still limited[20], and the cell kinetics of SA in this field remain to be clarified.

$p21^{WAF1/CIP1}$ is a universal inhibitor of cyclin-dependent kinases (CDKs) and is known to play an important role in maintaining growth arrest in terminally differentiated cells[21-23]. Its immunohistochemical expression is thought to be a useful morphological marker of cell differentiation/maturation[24-27]. In this study, we examined the immunohistochemical expression of $p21^{WAF1/CIP1}$ in SA in order to elucidate its cell differentiation/maturation status, and investigated its relationship with cell proliferation as evaluated by Ki-67 immunohistochemistry, which is a marker of cell proliferation[28]. The results were compared with those from HP and traditional tubular adenomas (TA) of the colorectum.

MATERIALS AND METHODS

Specimens and histological diagnosis

Formalin-fixed, paraffin-embedded tissues of 19 SA (mean±SD, 9.7±4.3 mm), 18 HP (mean±SD, 5.9±2.1 mm), 21 TA (mean±SD, 8.2±4.2 mm), and 11 normal colonic mucosas resected endoscopically were studied. SA was defined as a polyp composed of serrated glands similar to HP, and the following neoplastic cytological features: pseudostratification of enlarged and/or elongated spindle-shaped nuclei, loss of mature goblet cells, a dark eosinophilic cytoplasm, lack of surface maturation, and lack of a thickened collagen table at the free surface[1]. SA containing traditional adenomatous elements (mixed polyp) or an adenocarcinoma was excluded from the study. The histological grade of the atypia of adenomas was classified as low and high[29], which was respectively, equivalent to mild or moderate dysplasia, according to WHO classification[30]. All SA and TA samples in this study were classified as low-grade atypia. Three-μm-thick serial sections were prepared for hematoxylin and eosin (HE) staining as well as $p21^{WAF1/CIP1}$ and Ki-67 immunostaining.

Immunohistochemical staining

Immunohistochemical staining was performed by the streptavidin-biotin immunoperoxidase method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan)[31] using an anti- $p21^{WAF1/CIP1}$ monoclonal antibody (clone EA10, Oncogene Research Products, Calbiochem, MA, USA) and anti-Ki-67 monoclonal antibody (clone MIB-1, Immunotech, Marseille, France). The sections were lightly counterstained with hematoxylin. Specimens containing lymphoid follicles with germinal centers (proliferative zone) were used as positive controls for Ki-67 immunostaining. Negative controls for each immunostaining were prepared by omitting the application of the primary antibodies in the staining procedure. In each staining, a brownish nuclear staining, regardless of its intensity, was considered positive.

Evaluation of immunostainings

For the evaluation of $p21^{WAF1/CIP1}$ and Ki-67 staining, longitudinally sectioned hemicrypts, of which the entire lengths were visible, were selected from each sample, and the lengths of the proliferative zone (PZ) and crypt portion including $p21^{WAF1/CIP1}$-positive cells ($p21^{WAF1/CIP1}(+)$ portion) were measured. PZ of the crypt was determined semi-quantatively as the portion in which the Ki-67 labeling index appeared to be more than 30%[32]. In each hemicrypt, the percentages of the $p21^{WAF1/CIP1}(+)$ portion and the percentage of the PZ per entire hemicrypt length were determined.

Statistical analysis

Statistical differences were assessed by the Kruskal-Wallis test and Mann-Whitney's U test and the differences were considered significant at p less than 0.05.

RESULTS

In total, 116, 221, 165, and 67 hemicrypts were evaluated for SA, HP, TA, and normal colonic mucosa, respectively (Table 1). The average numbers of hemicrypts per lesion were 6.0, 10.8, 7.9, and 6.1, respectively.

$p21^{WAF1/CIP1}(+)$ cells were observed in all hemicrypts of normal mucosa (Fig. 1), HP (Fig. 2), and SA (Fig. 3), and in 93.9% (155/165) of TA (Fig. 4) hemicrypts. $p21^{WAF1/CIP1}(+)$ cells were distributed in the upper portion of the hemicrypts in normal mucosa and TA (Figs. 1b and 4b, respectively), and in the

Table 1. Percentages of the $p21^{WAF1/CIP1}(+)$ portion and PZ per hemicrypt (mean±SE)

	No. of hemicrypts	Percentage of p21(+)	Percentage of PZ	p21(+)/PZ ratio
SA (n : 19)	116	61.4 ± 2.1^a	26.5 ± 1.8^a	3.5 ± 0.2^a
HP (n : 18)	221	57.8 ± 1.1^b	32.3 ± 1.0^b	2.1 ± 0.1^b
TA (n : 21)	165	32.9 ± 2.0^c	69.0 ± 1.9^c	0.5 ± 0.1^c
Norm (n : 11)	67	30.6 ± 1.4^d	31.8 ± 1.3^d	1.0 ± 0.1^d

PZ, proliferative zone; SA, serrated adenoma; HP, hyperplastic polyp; TA, tubular adenoma; Norm, normal colonic mucosa. Statistically significant ($p<0.05$ by Kruskal-Wallis test and Mann-Whitney's U test) percentage of p21(+): a vs. c, d; b vs. c, d, percentage of PZ: a vs. b, c, d; b vs. c; c vs. d, p21(+)/PZ ratio: a vs. b, c, d; b vs. c, d; c vs. d.

upper to the middle portion in HP (Fig. 2b) and SA hemicrypts (Fig. 3b). The percentage of the $p21^{WAF1/CIP1}(+)$ portion per entire hemicrypt length in SA was 61.4±2.1%, which was significantly higher than those in TA (32.9±2.0%) and normal colonic mucosa (30.6±1.4%), whereas it showed no significant difference from that in HP (57.8±1.1%). Ki-67-positive cells were distributed mainly in the basal portion of the hemicrypts in normal colonic mucosa (Fig. 1c), HP (Fig. 2c), and SA (Fig. 3c), whereas those in TA hemicrypt (Fig. 4c) were mainly found in the upper to the middle portion, and occasionally in the entire length of the hemicrypts. The percentage of PZ per entire length of the hemicrypt in SA was 26.5±1.8%, which was significantly lower than those in the other three epithelial conditions (32.3±1.0, 69.0±1.9, and 31.8±1.3% for HP, TA, and normal colonic mucosa, respectively).

The ratio of the $p21^{WAF1/CIP1}(+)$ portion to PZ was approximately equal to 1.0 in normal colonic mucosa (p21(+)/PZ: 1.0±0.1), larger than 1.0 in SA (3.5±0.2) and HP (2.1±0.1), and smaller than 1.0 in TA (0.5±0.1). The ratio in SA was significantly larger than those in the other three epithelial conditions. The relation between the percentage of PZ per entire hemicrypt length (PZ/hemicrypt) and the percentage of $p21^{WAF1/CIP1}(+)$ portion per entire hemicrypt length (p21(+)/hemicrypt) for each evaluated hemicrypt is depicted in the scatter graph (Fig. 5).

In normal colonic mucosa, there was no topographic overlapping of the $p21^{WAF1/CIP1}(+)$ portion and the PZ. In SA and HP, the $p21^{WAF1/CIP1}(+)$ portion in the hemicrypt reached the PZ in 35.3% and 13.0% of evaluated hemicryts, respectively (data not shown). In TA, the $p21^{WAF1/CIP1}(+)$ portion totally overlapped with the PZ.

There was no correlation among the results of immunostainings and the size and macroscopic type of the samples (data not shown).

DISCUSSION

The cell kinetics of the normal colonic crypt is balanced by the regulation of cell proliferation and that of cell differentiation/maturation. Renewal of epithelial cells takes place in the basal to the middle portion of the crypt, and the cells migrate upward within the crypt with differentiation and maturation, are terminally differentiated in the upper portion of the crypt, and are finally exfoliated to the lumen[33]. The present results of the immunohistochemical staining of $p21^{WAF1/CIP1}$ and Ki-67 in normal colonic crypts were consistent with the established knowledge of their cell kinetics. $p21^{WAF1/CIP1}$-positive cells were distributed in the upper portion of the crypts, as was already shown in early studies[24,25,27], and were completely separated from the PZ that was located in the basal portion of the crypt, as revealed by Ki-67 immunostaining. The percentages of the $p21^{WAF1/CIP1}(+)$ portion and PZ per entire hemicrypt length were 30.6% and 31.8%, respectively, and the p21(+)/PZ ratio, which indicates the ratio of the terminally differentiated compartment to PZ in the hemicrypt, showed a precise balance, being almost equal to one (1.0±0.1) (Table 1 and Fig. 5a).

So far, the investigation of $p21^{WAF1/CIP1}$ expression and its relationship with cell proliferation in SA is limited to the study of Rashid et al.[20], which focused on hyperplastic polyposis, and the number of SA samples examined was small (6 lesions). In the present study, the immunostaining patterns of $p21^{WAF1/CIP1}$ and Ki-67 were similar in SA and HP in that the $p21^{WAF1/CIP1}(+)$ portion and the PZ in their hemicrypts were located in the upper to the middle portion and in the basal portion, respectively. These results indicate that the topographic regulation of cell differentiation/maturation and proliferation is basically maintained in SA and HP. The disruption of cell kinetics involved in SA and HP was the extension of

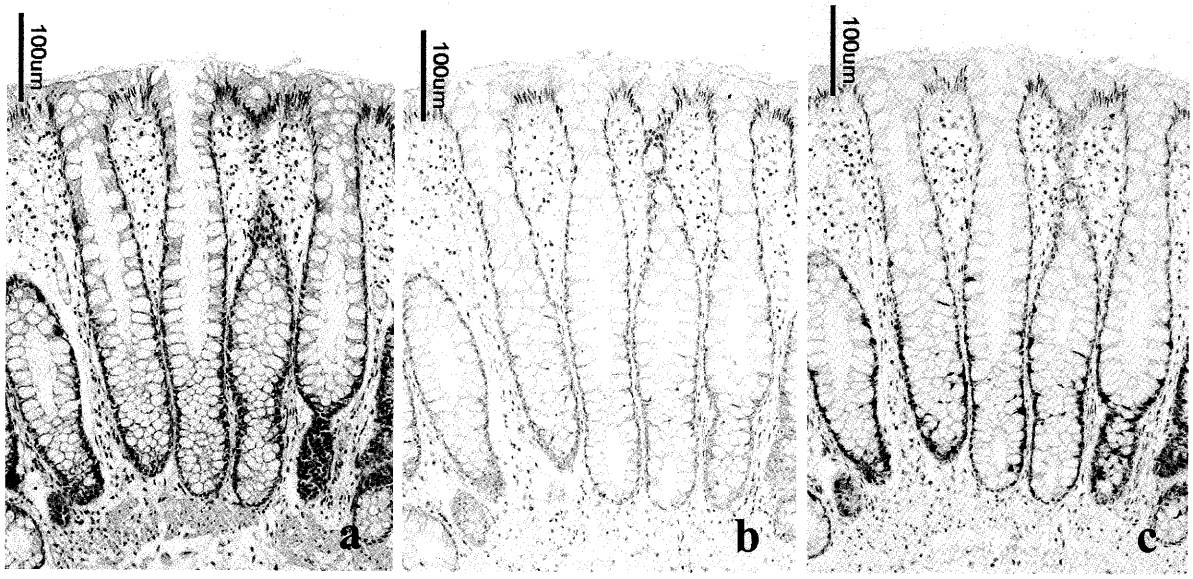

Fig. 1. Normal colonic mucosa. **a.** HE, **b.** $p21^{WAF1/CIP1}$ immunostaining, **c.** Ki-67 immunostaining. $p21^{WAF1/CIP1}$ -positive cells are distributed in the upper portion of the crypts, and the PZ detected by Ki-67 immunostaining are located in the basal portion of the crypts. There is no topographic overlapping of the $p21^{WAF1/CIP1}(+)$ portion and the PZ.

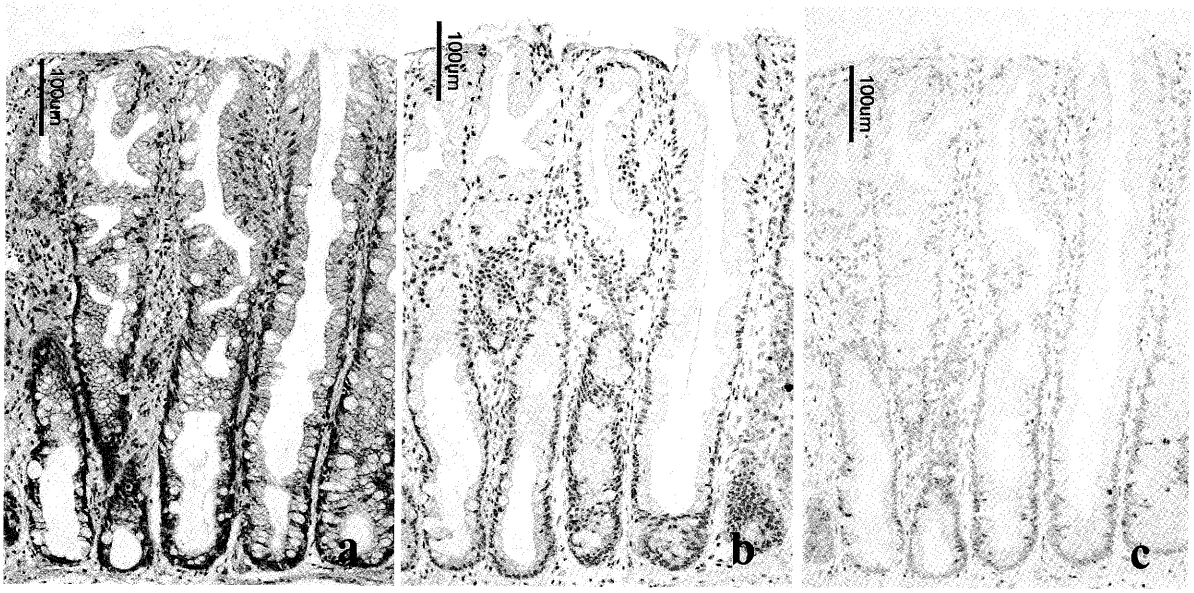

Fig. 2. Hyperplastic polyp (HP). **a.** HE, **b.** $p21^{WAF1/CIP1}$ immunostaining, **c.** Ki-67 immunostaining. $p21^{WAF1/CIP1}$ -positive cells are distributed in the upper to the middle portion of the crypts, and the PZ detected by Ki-67 immunostaining are located in the basal portion of the crypts.

the $p21^{WAF1/CIP1}(+)$ portion compared with that of the normal colonic crypt (61.4% and 57.8% vs. 30.6%). The percentage of PZ per entire hemicrypt length was similar (HP) or lower (SA) than that of normal colonic crypt, which resulted in a significant increase in the p21(+)/PZ ratio compared with that of normal colonic crypts (3.5 and 2.1 vs. 1.0). These results imply that the cell kinetic balance between cell differentiation/maturation and proliferation is inclined toward the former in SA and HP (Fig. 5b and c).

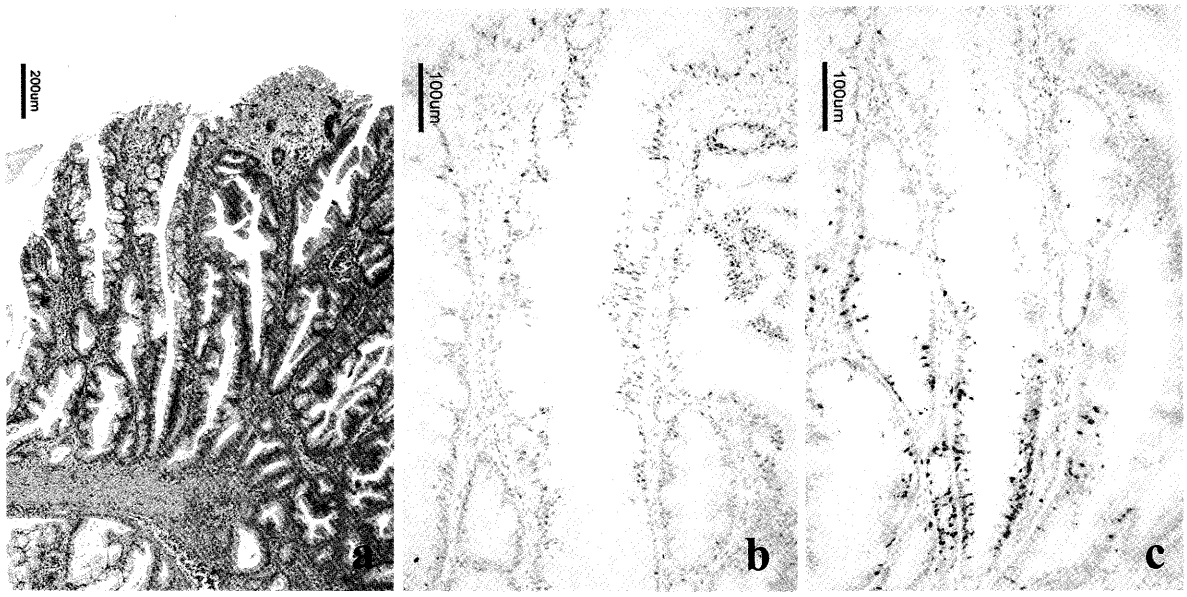

Fig. 3. Serrated adenoma (SA). **a.** HE, **b.** $p21^{WAF1/CIP1}$ immunostaining, **c.** Ki-67 immunostaining. $p21^{WAF1/CIP1}$ -positive cells are distributed in the upper to the middle portion of the crypts, and the PZ detected by Ki-67 immunostaining are located in the basal portion of the crypts.

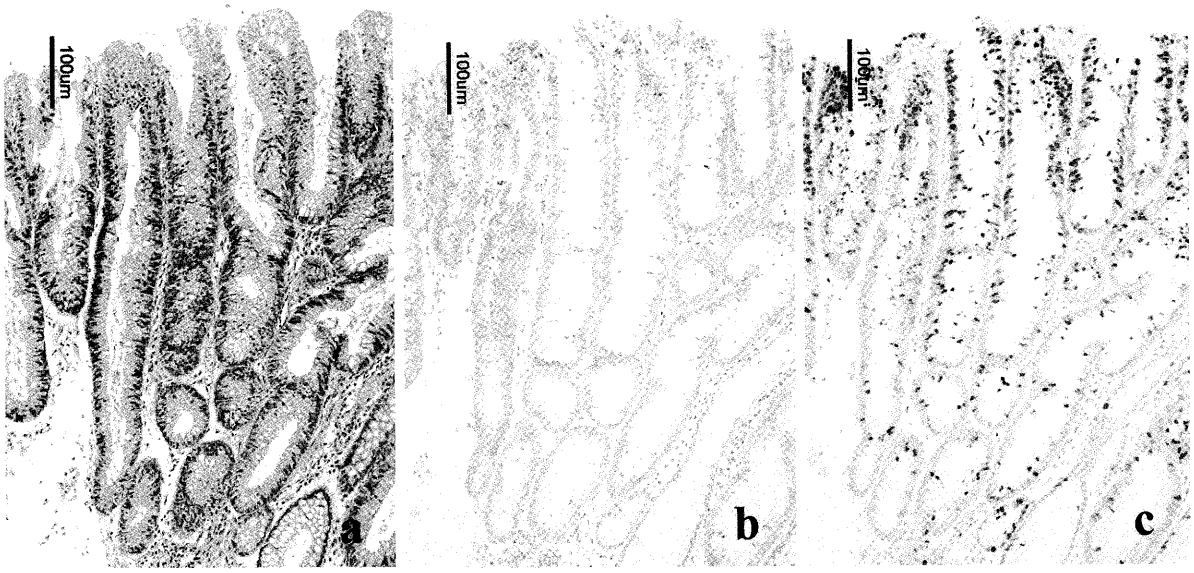

Fig. 4. Tubular adenoma (TA). **a.** HE, **b.** $p21^{WAF1/CIP1}$ immunostaining, **c.** Ki-67 immunostaining. $p21^{WAF1/CIP1}$ -positive cells are distributed in the upper portion of the crypts, and the PZ detected by Ki-67 immunostaining are located in the upper to the middle portion of the crypts. $p21^{WAF1/CIP1}$(+) portion totally overlapped with the PZ.

The known fundamental cell kinetic abnormality of HP, which was demonstrated by an in vivo [3H]-thymidine uptake study[34], is the delayed upward epithelial cell migration within the crypt and consequent elongation of the cell life span. In HP, epithelial cells, which should have been exfoliated from the surface of the mucosa, are retained within the crypt because of their life span elongation; this causes the increase in crypt cell population, and the excessive number of cells results in its luminal serra-

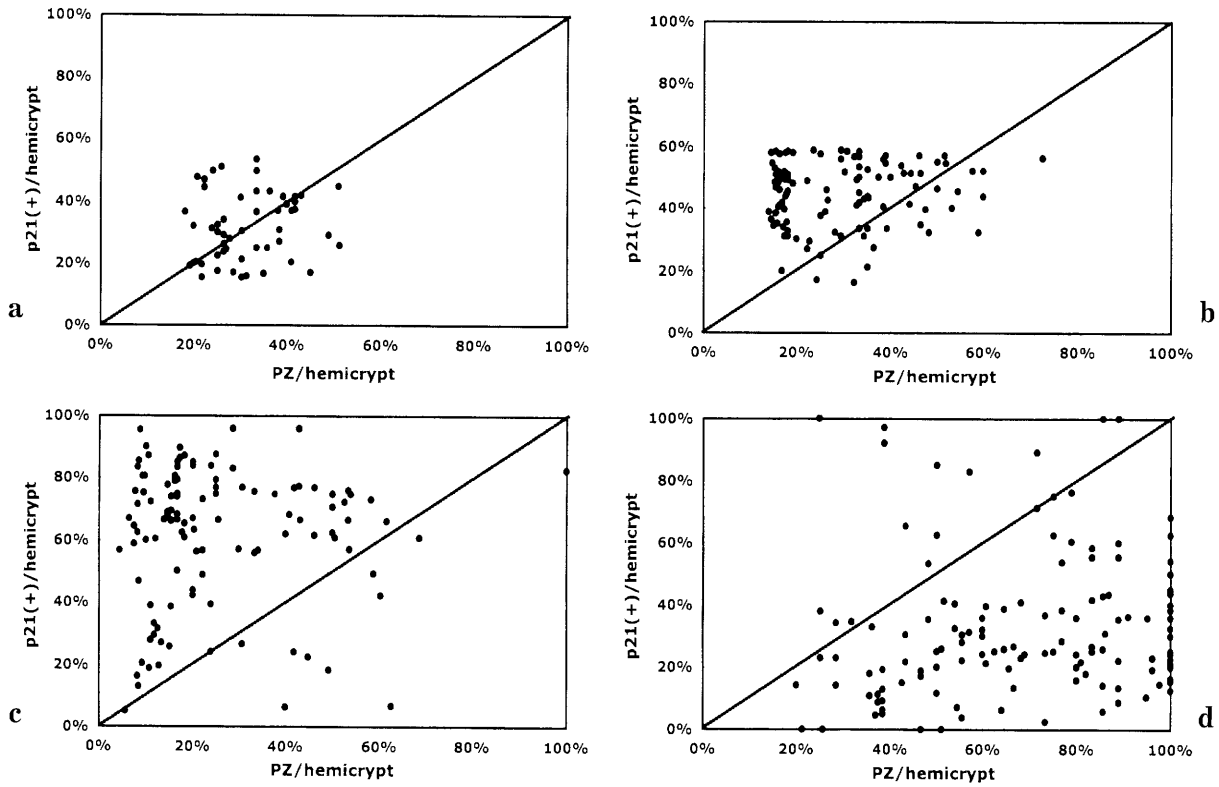

Fig. 5. Scatter graph showing the relation between the percentage of PZ per entire hemicrypt length (PZ/hemicrypt) and the percentage of the $p21^{WAF1/CIP1}(+)$ portion per entire hemicrypt length (p21(+)/hemicrypt) for each evaluated hemicrypt. **a.** normal colonic mucosa (n=67), **b.** HP (n=221), **c.** SA (n=116), **d.** TA (n=165).

tion[34]. It is suggested that the extension of the $p21^{WAF1/CIP1}(+)$ portion in HP demonstrated in the present immunohistochemical study is a morphological representation of the cell life span elongation. It is suggested that this cell life span elongation also occurs in SA, which shows similar $p21^{WAF1/CIP1}$ and Ki-67 immunostaining patterns and the histological features of luminal serration similar to those of HP.

In contrast, cell kinetic balance between cell differentiation/maturation and proliferation is thought to be inclined toward the latter in TA (Fig. 5d). The PZ of TA was significantly extended compared with that of normal colonic crypts (69.0% vs. 31.8%) and the $p21^{WAF1/CIP1}(+)$ portion was within the normal range, which resulted in the reduction of the p21(+)/PZ ratio to 0.5 (Table 1). Furthermore, topographic dysregulation of $p21^{WAF1/CIP1}$ expression and proliferation was observed in TA. The PZ of TA was translocated to the upper portion of the crypt, and it overlapped with the hemicrypt portion with $p21^{WAF1/CIP1}$ expression. Although both SA and TA are classified as benign colorectal epithelial neoplasias (adenomas), their cell kinetics in terms of the balance between cell differentiation/maturation and proliferation are completely different.

The disorder of cell proliferation is thought to be central to the neoplasm, and the upregulation of its proliferative activity enables the tumor to gain an advantage over the surrounding normal tissue in terms of growth. In fact, colorectal neoplasms have been demonstrated to result from a series of genetic alterations that disrupts the normal regulatory system of cell proliferation[2,3]. However, we speculate that the disorder of cell proliferation is not the main feature of the neoplastic process of SA. Most of the genetic studies of SA failed to detect K-ras mutation[4-6], which is known to upregulate the mitogenic signal transduction pathway[35], thereby resulting in the growth of sporadic adenomas[2,3]. In our recent study[19], the growth fraction estimated by Ki-67 immunostaining and the apoptotic index determined by the TUNEL method were significantly lower than those of traditional TA, implying that SA is characterized by the disorder of cell loss rather than cell proliferation. The present study supports our specula-

tion that unlike traditional TA, the cell kinetic balance of SA is not inclined toward cell proliferation, and suggests that the elongation of cell life span and the subsequent reduction of exfoliation may contribute to the neoplastic growth of SA as well as the reduction of cell loss by apoptosis.

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