Characterization of Dysplastic Aberrant Crypt Foci in the Rat Colon Induced by 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine

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The multistage model of colon carcinogenesis is well established in both humans and experimental animals, and aberrant crypt foci (ACF) are generally assumed to be putative preneoplastic lesions of the colon. However, morphological analyses of ACF have suggested that they are highly heterogeneous in nature and their role in tumorigenesis is still controversial. To better understand the biological significance of ACF in carcinogenesis, morphological and genetic analyses were performed using a rat colon cancer model induced by a food-borne colon carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). ACF of different sizes were collected at weeks 6, 18, 25, and 32 after three cycles of 2-week PhIP feeding (400 ppm in diet) with 4-week intervals on a high-fat diet, and a total of 110 ACF, representing approximately three-quarters of the total ACF, were subjected to histological evaluation. Thirty (27%) were diagnosed as dysplastic ACF, based on cytological and structural abnormalities of crypts. Dysplastic ACF were detected even at week 6 (0.4 per rat), and the numbers increased slightly at later time points, being 0.8, 1.4, and 0.8 per rat at weeks 18, 25, and 32, respectively. The sizes of these dysplastic ACF varied widely from 1 to 16 crypts and 50% (15 of 30) were composed of less than 4 crypts. Immunohistochemical analysis revealed that 83% (25 of 30) of dysplastic ACF demonstrated β -catenin accumulation; 22 only in the cytoplasm and 3 in both the cytoplasm and nucleus, the latter manifesting a higher grade of dysplasia as compared with the former. Seven dysplastic ACF harbored β -catenin mutations at codon 32, 34, or 36 in exon 2, and one had an *Apc* mutation at the boundary of intron 10 and exon 11. Mutations at these sites were also commonly found in colon tumors induced by PhIP. The results of our present study indicate that dysplastic

ACF, which accounted for approximately one-fourth of the total ACF, are preneoplastic lesions of colon cancers induced by PhIP in rats. (*Am J Pathol* 2003; 163:1607-1614)

Colon cancers develop after multistep accumulation of genetic and epigenetic alterations in both humans and experimental animals.¹⁻³ The adenomatous polyposis coli gene (*APC*) serves as a gatekeeper gene for the development of colon cancers and genetic alterations in *APC* and down-regulation of *APC* mRNA expression are frequently observed in human colon cancers.^{1,3} Mutations in several other genes involved in the WNT signaling pathway, such as *CTNNB1*, *AXIN1*, and *AXIN2*, are also found in some colon cancers.⁴⁻⁶

Aberrant crypt foci (ACF) were first described by Bird⁷ and claimed to be lesions composed of enlarged crypts, slightly elevated above the surrounding mucosa and more densely stained with methylene blue than normal crypts. ACF are considered as putative preneoplastic lesions of the colon based on the following observations in both humans and experimental animals. In the case of humans, large numbers of ACF are commonly observed in familial adenomatous polyposis patients, as well as in sporadic colorectal cancer patients.⁸⁻¹¹ The number of foci decreases after treatment with nonsteroidal anti-inflammatory drugs,¹² which effectively reduce the numbers and sizes of polyps in familial adenomatous polyposis patients.¹³ In addition, increased proliferation activity¹⁰ and genetic alterations in *K-RAS*,^{11,14-17} *APC*,^{15,16} and *P53* genes,¹⁷ as well as microsatellite instability,^{18,19} have been demonstrated in ACF of humans. In the rat model, ACF are induced by various types of colon carcinogens in a dose- and time-dependent manner.^{20,21} The formation of ACF is enhanced by dietary

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Figure 1. Experimental protocol for PhIP administration. **Filled box**, AIN-93G diet containing PhIP at the concentration of 400 ppm; **open box**, high-fat diet without PhIP.

fats,²⁰ which have been demonstrated to have promoting effects on colon cancer development,²² and is suppressed by chemopreventive agents that have been demonstrated to suppress the development of colon cancers.²³

However, conflicting evidence has also been presented. For example, mutations in the K-RAS gene are relatively common in human ACF,^{11,14–17} but are detected at a relatively late stage of colon cancer development.²⁴ In rat models, although hundreds of ACF are induced per animal by azoxymethane (AOM), and K-ras mutations are frequently observed in those ACF, only a few colon tumors are observed per animal.^{25–27} Moreover, some compounds that effectively suppress the induction of ACF by AOM in rats, eg, 2-(carboxyphenyl) retinamide or genistein, enhance the development of colon cancers.^{23,28,29} This discrepancy could be caused by the heterogeneous nature of ACF and also by a wide range of biological consequences of ACF.

(2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine PhIP), one of the most abundant heterocyclic amines produced during cooking of meat and fish,³⁰ has been demonstrated to induce ACF in rats a few weeks after feeding a diet containing PhIP³¹ and colon cancers 1 or 2 years later.^{32,33} A recent report by Sinha and colleagues³⁴ indicated that PhIP could be one of the causative agents for colon carcinogenesis in humans. To elucidate the biological significance of ACF in colon carcinogenesis, ACF induced in F344 male rats by PhIP were subjected to morphological and genetic analyses.

Materials and Methods

Animal Experiments

Five-week-old F344 male rats were purchased from CLEA Japan (Tokyo, Japan) and housed in a ventilated, temperature-controlled room at 25°C with a 12-hour light/dark cycle. After a 1-week acclimatization to the housing environment on a basal diet (AIN-93G; Dyets, Bethlehem, PA), the rats were fed a basal diet (AIN-93G) containing 400 ppm of PhIP (Nard Institute, Osaka, Japan) for the first 2 weeks, followed by a high-fat diet (HF) containing hydrogenated oil PRIMEX (Dyets) for 4 weeks, as described previously, with minor modification.³⁵ Briefly, the 2-week administration of PhIP was repeated three times with 4-week intervals on the HF diet, and the HF diet without PhIP was given until the termination of the experiment at week 32 (Figure 1). Seven or 10 rats were killed at weeks 6, 18, 25, and 32 and subjected to histological and genetic analyses of ACF and colon tumors.

Detection of ACF and Colon Tumors

The colons were resected and gently flushed with 10% neutralized formalin to remove residual bowel contents, cut open longitudinally, fixed flat between filter papers, and submerged in 10% neutralized formalin overnight at 4°C. Fixed colons were stained with 0.2% methylene blue, as described by Bird,⁷ and the numbers of ACF, and total numbers of aberrant crypts (ACs) comprising ACF were counted for each rat. ACF were identified as lesions composed of enlarged crypts, with an increased pericryptal area, slightly elevated appearance above the surrounding mucosa with an oval or slit-like orifice, and higher staining intensity with 0.2% methylene blue than normal crypts. When colon tumors were detected macroscopically, tissues were embedded in paraffin and histological evaluation was performed according to the intestinal tumor classification of the rat by Pozharisski³⁶ after hematoxylin and eosin (H&E) staining.

Histological Classification of ACF

ACF of different sizes were resected along with the surrounding normal colon tissues as transverse sections with less than 2-mm width, embedded in paraffin blocks, and subjected to microscopic observation. Serial paraffin sections were prepared for each lesion at 3.5- μ m thickness, and one in every three sections was stained with H&E to evaluate the histological grade of ACF. According to their cytological and structural abnormalities, ACF were classified into two groups; nondysplastic ACF and dysplastic ACF. The former are characterized histologically as hyperplastic, and the latter as lesions with distortion of the crypt structure, decrease in goblet cells, nuclear stratification, and enlarged nuclei.

Immunohistochemical Analysis of β -Catenin

Paraffin sections of ACF and colon tumors were stained with anti- β -catenin antibody (BD Transduction Laboratories, Lexington, KY) as reported previously.³⁵ Biotinylated goat anti-mouse IgG (Kirkegaard & Perry, Inc., Gaithersburg, MD) was used as the secondary antibody at a dilution of 1:200.

Cell Proliferation

Immunohistochemical staining of Ki-67 antigen, a cell-cycle-associated antigen that serves as an endogenous marker for cell proliferation, was performed using the Vectastain ABC system (Vector Laboratories, Burlingame, CA). Tissue sections were deparaffinized and the Ki-67 antigen was activated by boiling for 15 minutes in 10 mmol/L of citrate buffer (pH 6.0). A rabbit polyclonal antibody (Novocastra Laboratories, Newcastle on Tyne, UK), raised against human Ki-67 antigen, was used as the primary antibody at a dilution of 1:1000, and biotinylated goat anti-rabbit IgG (Vector Laboratories) as the secondary antibody at a dilution of 1:200. The labeling index for the Ki-67 antigen in ACF was calculated as the

Table 1. Oligonucleotide Primers Used for PCR-SSCP Analysis

Gene	Region	Nucleotides*	Forward	Reverse	Annealing temperature (°C)	Product size (bp)	Ref.
<i>β-catenin</i>	Exon 2	172–364	5'-TGACCTCATGGAGTTGGACA-3'	5'-GCCTTGCTCCCACTCATAAA-3'	60	193	35
<i>Apc</i>	Intron 10/ exon 11	Exon/intron junction	5'-CGATTATGGATTATTGTATG-3'	5'-AAGTGAGCACCTTAGGAAGT-3'	52	300	38
	Exon 14	1791–2010	5'-GAATCAACTCTCAAAAGCGT-3'	5'-TATACCTGTGGTCTTCGTTG-3'	52	220	
	Exon 15	2492–2724	5'-CACTGGAAACATGACTGTTC-3'	5'-CTTCCATCACTTTGGCTATC-3'	54	233	
	Exon 15	4081–4351	5'-CCGCCAGGCATAAGCTGTT-3'	5'-CTTCTGCTTGGTGGCATGGT-3'	58	271	

* Nucleotide numbers are assigned according to the rat *β-catenin* and *Apc* cDNA sequences.

ratio of Ki-67-positive cells per total number of crypt cells using one representative tangential section for each ACF.

Mutation Analysis of *β-Catenin* and *Apc*

DNA extraction from ACF and colon tumors was performed as follows. Lesions were microdissected from paraffin-embedded sections using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA) according to the manufacturer's protocol. Extracted DNA was then subjected to polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis.³⁷ PCR primers for the rat *β-catenin* gene were designed to amplify the 193-bp fragment from exon 2, corresponding to functionally important phosphorylation sites and mutation hot spots in *β-catenin*. For the *Apc* gene, four primer sets were designed to amplify the 5'-GGGA-3' sites in exons 14 and 15, and the 5'-tagGGG-3' site at the boundary of intron 10 and exon 11, where the *Apc* mutations are frequently found in PhIP-induced rat colon tumors.^{35,38} Primer sequences, annealing temperatures, and expected sizes of PCR products are indicated in Table 1. PCR reactions were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems Japan, Tokyo, Japan) and SSCP analysis was performed using the GenePhor DNA separation system (Amersham Pharmacia Biotech, Little Chalfont, UK), as described previously.³⁵ Nucleotide sequences of the bands with shifted mobilities were determined by PCR-based direct sequencing using the CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, CA). Muta-

tional analyses for both ACF and colon tumors were repeated at least twice using serial paraffin sections to confirm the results.

Statistical Analysis

All statistical analyses were performed by the Kruskal-Wallis test using SPSS for Macintosh (SPSS Inc., Tokyo, Japan). Differences were considered significant when the *P* value was less than 0.05.

Results

Induction of ACF

The average numbers of ACF, total ACs, and large ACF (≥ 4 ACs) per animal observed at weeks 6, 18, 25, and 32 are summarized in Table 2. These values at week 6 after one 2-week administration of PhIP in this study were essentially equivalent to those in our previous report using the same protocol.³⁹ A slight increase of these values was observed at weeks 18 and 25 after three cycles of PhIP administration, and the values at week 32 were similar to those at week 25. Regarding the site distribution, ACF were most frequent in the mid-colon as reported previously⁴⁰ and this did not change significantly during the experimental course (data not shown).

Table 2. Number of Aberrant Crypt Foci Induced by PhIP

Exp. periods (weeks)	PhIP-exposure (weeks)	No. of rats analyzed	No. of ACF*	No. of AC	No. of large ACF (≥ 4 ACs)	Histological characterization of ACF†			
						Dysplastic ACF		Nondysplastic ACF	
						No.	No. of ACF with β -catenin accumulation	No.	No. of ACF with β -catenin accumulation
6	2	10	2.7 $\pm$ 1.5	6.3 $\pm$ 3.1	0.6 $\pm$ 0.7	0.4 $\pm$ 0.5	0.4 $\pm$ 0.5	1.5 $\pm$ 1.0	0
18	6	10	3.7 $\pm$ 2.2	11.7 $\pm$ 8.0	1.0 $\pm$ 0.9	0.8 $\pm$ 1.0	0.6 $\pm$ 0.7	1.9 $\pm$ 1.4	0
25	6	7	4.7 $\pm$ 2.0	14.6 $\pm$ 9.4	1.3 $\pm$ 1.4	1.4 $\pm$ 1.2	1.0 $\pm$ 0.8	2.3 $\pm$ 2.0	0.1 $\pm$ 0.4
32	6	10	4.7 $\pm$ 2.7	13.5 $\pm$ 11.4	1.3 $\pm$ 2.0	0.8 $\pm$ 1.0	0.7 $\pm$ 0.8	3.0 $\pm$ 1.4	0.1 $\pm$ 0.3

* All the values for ACF presented in this table are expressed as means $\pm$ SD.

† Of the total 144 ACF, 19 of 27, 27 of 37, 26 of 33, and 38 of 47 ACF at weeks 6, 18, 25, and 32, respectively, were subjected to the histological evaluation. The values listed in this column were based on the observation of these analyzed ACF, representing 70 to 80% of total ACF at respective time points, and therefore, simple summation of the numbers of dysplastic ACF and nondysplastic ones does not match the total number of ACF per rat presented in the fourth column from the left.

Figure 2. The histological features and β -catenin immunohistochemistry of nondysplastic and two types of dysplastic ACF. Representative features of nondysplastic ACF (**A** and **D**), dysplastic ACF (**B** and **E**), and high-grade dysplastic ACF (**C** and **F**) are demonstrated. **A–C:** H&E staining. **D–F:** β -Catenin immunostaining. **Arrowheads** indicate the nuclear accumulation of β -catenin.

Histological Characterization of ACF and Mode of β -Catenin Accumulation

Of 144 ACF induced in 37 rats, 110 ACF of different sizes were subjected to histological evaluation; namely 19, 27, 26, and 38 ACF at weeks 6, 18, 25, and 32, respectively (Table 2). Thirty ACF were diagnosed as dysplastic (Figure 2, B and C), and the remaining 80 as nondysplastic (Figure 2A). Dysplastic ACF were detected as early as 6 weeks and their numbers per rat increased slightly at later time points, those being 0.4, 0.8, 1.4, and 0.8 at weeks 6, 18, 25, and 32, respectively, although the differences were not statistically significant (Table 2). Immunohistochemical analysis showed β -catenin accumulation in 25 of 30 (83%) dysplastic ACF (Table 3). In most of the cases, accumulation was observed only in the cytoplasm (Figure 2E), but three of them showed accumulation in both the nucleus and the cytoplasm as illustrated in Figure 2F. The latter ones manifested a high-grade dysplasia with severe architectural abnormalities of the crypt, chromatin aggregation, loss of nuclear polarity, and almost complete loss of goblet cell differentiation. Only 3% (2 of 80) of the nondysplastic ACF showed β -catenin accumulation, this being only in the cytoplasm, and the remaining exhibited no accumulation (Table 2 and Figure 2D).

Size Distribution of ACF

Dysplastic ACF showed a wide range of size distribution, ranging from 1 to 16 ACs and 50% (15 of 30) of them were composed of less than 4 ACs (Table 3). The size distribution of nondysplastic lesions also varied widely, ranging from 1 to 10 ACs (data not shown). One of the

three high-grade dysplastic ACF was composed of three ACs and the remaining two being six and seven ACs (Table 3).

Cell Proliferation in ACF

Normal colonic epithelial cells and the majority of nondysplastic ACF showed Ki-67 antigen-positive cells restricted only to the lower halves of the crypts (Figure 3A). In contrast, most of the dysplastic ACF (85%, 22 of 26) demonstrated labeling cells extending into the upper portions of the crypts (Figure 3B). Labeling indices in dysplastic ACF were much higher compared to those of surrounding normal-like crypts, being $34 \pm 10\%$ and $17 \pm 3\%$ on average, respectively (Table 3). A small proportion of nondysplastic ACF (9%, 6 of 68) also demonstrated Ki-67-positive cells to be present in the upper portion of the crypt, similar to dysplastic ones in Figure 3B, suggesting that a subset may subsequently progress to dysplastic lesions.

Mutation Spectra of β -Catenin and Apc Genes in Dysplastic ACF

Twenty-nine dysplastic ACF were able to be subjected to PCR-SSCP analysis for both β -catenin and Apc genes. As for the β -catenin gene, seven dysplastic ACF harbored mutations, and six of them demonstrated β -catenin accumulation in the nucleus and/or cytoplasm (Table 3). All of the mutations occurred at codons 32, 34, or 36, and Gly to Val amino acid substitution at codon 34 was dominant, being observed in five cases. As for the Apc gene, only one case lacking β -catenin accumulation harbored a

Table 3. Immunohistochemical Analysis of Ki-67 Antigen and β -Catenin, and Mutation Spectrum of β -Catenin and Apc Genes in the Dysplastic ACF Induced by PhIP

Sample no.	Exp. periods (weeks)	No. of crypts in ACF	Ki-67 antigen immunostaining		β -Catenin accumulation		Mutation spectrum	
			Grade*	LI (%) [†]	Cyt. [‡]	Nuc. [§]	β -Catenin (AA substitution)	Apc
1	6	3	++	54	+	—		
2		4	++	41	+	—		
3		4	++	51	+	—	GAT → GGT(D32G)	
4		4	++	42	+	—		
5	18	3	+	17	+	—	GGA → GTT (G34V)	
6		4	++	30	+	—		
7 [¶]		6	++	32	+	+	GGA → GTT (G34V)	
8		7	++	28	—	—		
9		3	++	36	+	—		
10 [¶]		7	++	38	+	+	CAC → TAC (H36Y)	
11		16	+	20	—	—	F.A.	
12		1	++	40	+	—		
13	25	6	++	34	+	—		
14		2	N.E.**	N.E.	+	—	GGA → GTT (G34V)	
15		6	++	38	+	—		
16		3	N.E.	N.E.	+	—		
17		2	+	12	+	—		
18		1	++	29	—	—	GGA → GTT (G34V)	
19		1	N.E.	N.E.	+	—		
20		2	++	37	—	—		tagGGG → tatGGG ^{††}
21 [¶]		3	++	45	+	+		
22		8	++	25	+	—		
23	32	5	++	32	—	—		
24		8	++	39	+	—		
25		2	++	39	+	—		
26		10	+	16	+	—		
27		2	N.E.	N.E.	+	—	GGA → GTT (G34V)	F.A.
28		2	++	40	+	—		
29		4	++	31	+	—		
30		2	++	37	+	—		

* +, Ki-67 antigen-positive cells were detected only in the lower half of the crypts, the same as in normal colonic epithelial cells. ++, Ki-67 antigen-positive cells were extending into the upper portion of the crypts.

[†] The labeling index (LI) of the Ki-67 antigen was calculated as the ratio (%) of Ki-67 positive cells per total number of crypt cells.

[‡] +, The cytoplasm was weakly stained.

[§] +, The nuclei in a few cells were stained.

[¶] High grade of dysplasia.

^{||} F.A.: We failed to amplify DNA fragments by PCR.

** N.E.: Not examined.

^{††} G to T transversion at the junction of intron 10 and exon 11.

G to T transversion mutation at the boundary of intron 10 and exon 11, where the same mutation had been previously reported in a PhIP-induced colon tumor.³⁵ Of 29 nondysplastic ACF analyzed, one of the two ACF with

β -catenin accumulation, harbored GGA (Gly) to GTA (Val) mutations at codon 34. None of the 27 cases without β -catenin accumulation harbored either β -catenin or Apc mutation (data not shown).

Figure 3. Immunostaining of Ki-67 antigen. **A:** In nondysplastic ACF, crypt cells in the lower half are stained. **B:** In dysplastic ACF, cells with Ki-67 staining are extending into the upper portion of the crypt.

Table 4. Immunohistochemical Analysis of β -Catenin and Mutation Spectrum of β -Catenin Gene in Tumors Induced by PhIP

Sample no.	Type*	Exp. Periods (weeks)	β -Catenin accumulation		Mutation spectrum		
			Cytoplasm [†]	Nucleus [‡]	Codon	Type of mutation	AA substitution
1	Ad	25	+	+	34	GGA→GAA	Gly→Glu
2	AdC	25	++	++	34	GGA→GTA	Gly→Val
3	AdC	32	++	++	36	CAC→CCC	His→Pro
					38	GGT→CGT	Gly→Arg
4	AdC	32	++	++	32	GAT→AAT	Asp→Asn
5	AdC	32	++	++			
6	AdC	32	++	++	34	GGA→GTA	Gly→Val

* Ad, adenoma; AdC, adenocarcinoma.

[†] +, weakly stained; ++, densely stained.

[‡] ++, The nuclei in a few cells were stained; ++, the nuclei in most cells were stained.

Mutation Spectra of β -Catenin and Apc Genes in Colon Tumors

Two colon tumors, one adenoma and one adenocarcinoma, were detected at week 25, and four colon adenocarcinomas at week 32. As summarized in Table 4, accumulation of β -catenin was observed in these tumors in both cytoplasm and nucleus, as previously reported.³⁵ β -Catenin mutations were detected in five of six tumors, and the mutations occurred at codons 32, 34, 36, or 38 (Table 4). Mutations at codons 36 and 38, which were detected in tumor 3, have not been reported so far as mutation target sites in carcinogen-induced colon tumors, including PhIP-induced ones.^{27,35,41,42} As for the Apc gene, no mutation was observed either at 5'-GGGA-3' sites in exons 14 and 15,³⁸ or at the boundary of intron 10 and exon 11.³⁵

Discussion

ACF are characterized as lesions composed of one or multiple enlarged crypts, elevated above the surrounding mucosa and are considered as putative preneoplastic lesions of the colon. The total numbers of ACF induced by PhIP increased slightly at later experimental points, but not in direct accordance with the increasing amounts of total PhIP exposure. A subset of ACF, possibly nondysplastic ones, may become eliminated by unclarified mechanisms during the experimental course. Approximately one-fourth of ACF induced by PhIP was found to be histologically dysplastic, with the remaining being nondysplastic. The sizes of dysplastic and also of nondysplastic ACF were distributed widely, being from 1 to 16 ACs for the former and from 1 to 10 ACs for the latter. Fifty percent of dysplastic ACF were composed of one to three ACs and, moreover, one of the three high-grade dysplastic ACF was also composed of three ACs, the remaining two being six and seven ACs. Although many authors have emphasized the importance of large ACF, composed of four or more ACs,^{10,25} our results indicated that critical changes in ACF, which are essential for further development to dysplastic ACF, may also be present or occur even in small lesions.

β -Catenin accumulation was detected in most of these dysplastic ACF, and nuclear accumulation was also oc-

asionally observed. Considering that β -catenin accumulation is a common and characteristic feature of colon tumors, these dysplastic ACF, especially those with nuclear accumulation, are most likely to be preneoplastic lesions of PhIP-induced colon cancers as is also suggested for dysplastic ACF in humans.⁴³ In addition, β -catenin mutations occurred at codons 32, 34, and 36 in approximately one-fourth of dysplastic ACF with β -catenin accumulation, and one case harbored a mutation in the Apc gene at the boundary of intron 10 and exon 11. For the remaining examples with β -catenin accumulation, other genetic and/or epigenetic alterations of genes involved in the Wnt-Apc- β -catenin signaling pathway, yet to be determined, might be responsible, as with colon tumors induced by PhIP.³⁵ Our preliminary study suggests that down-regulation of Apc could be one plausible mechanism, similar to the case with AOM-induced colon tumors in mice.⁴⁴ However, further studies should be conducted in the future to clarify this point. Mutations in tumors also occurred at these sites of β -catenin and Apc genes, with that at codon 34 of β -catenin dominating. A similar preferential occurrence of β -catenin mutations at codon 34 was also recently reported in PhIP-induced ACF by Tsukamoto and colleagues.⁴² Because previous reports have also demonstrated β -catenin mutations at codons 32 and 34 to be the most common in PhIP-induced colon tumors,^{35,41,42} it is reasonable again to assume that these dysplastic ACF, and also a small portion of nondysplastic ones with β -catenin accumulation and/or mutation, may subsequently develop into colon tumors. In fact, the average number of dysplastic ACF was ~0.8 per rat in this study, which is almost equivalent to that reported so far for colon tumors induced in F344 male rats by 100 and 400 ppm of PhIP, being ranged from 0.53 to 0.66 per rat.^{32,33,35} The reason why the number of dysplastic ACF was relatively small at week 32 may possibly imply progression to adenocarcinomas.

Pretlow and colleagues⁴⁵ reported two populations of crypts as putative precancerous lesions in the colon of AOM-treated F344 rats, based on the loss of enzymatic activity of hexosaminidase; one population being morphologically aberrant, and the other morphologically normal. Yamada and colleagues^{46,47} also reported a similar type of lesion in F344 rats given AOM, which were deficient in hexosaminidase activity and could not be easily

identified as ACF by methylene blue staining, and nuclear accumulation of β -catenin and mutations around the GSK-3 β phosphorylation site in the β -catenin gene were more frequently observed in this type of lesion than in typical ACF.⁴⁶ Although we did not investigate the presence of this type of lesion and, moreover, the difference of this lesion from classic ACF is still controversial,⁴⁸ multiplicities of dysplastic ACF with β -catenin accumulation or mutations were substantial enough to account for the incidence of colon tumors induced by PhIP, being ~0.5 to 0.7 per rat. Future studies need be conducted to clarify the presence of this type of lesion by analyzing enzymatic activity of hexosaminidase⁴⁵ and by using *en face* preparations of the colon as described by Yamada and colleagues⁴⁶

Paulsen and colleagues^{49,50} recently reported the presence of flat-type ACF in *Min/+* mice (ACF_{Min}), detected as early as 2 weeks after the birth. Macroscopically, they were not elevated above the surrounding normal mucosa, but stained more strongly than the surrounding mucosa with methylene blue.^{49,50} In our current study, however, we did not detect any ACF with the flat-type appearance among 144 lesions analyzed.

In conclusion, the results of the present study indicate that progression from dysplastic ACF to colon cancer may be a major route in the development of colon cancers induced by PhIP in rats. Increase in cell proliferation and activation of the *Apc*- β -catenin signaling pathway could be causative events for the formation of dysplastic ACF.

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