needle-shaped crystals. Finally, a total of 8 g of tricin was prepared from 40,000 kg of the leaves and was used in this study.

AOM was purchased from Sigma-Aldrich. DSS with a molecular weight of 36,000 to 50,000 was obtained from MP Biomedicals, LLC. DSS 1.5% (*w/v*) was prepared shortly before use to induce colitis.

Animals and diets

Five-week-old male Crj: CD-1 (ICR) mice were purchased from Charles River Laboratories, Inc. All animals were housed in plastic cages (three or four mice/cage) and had free access to tap water and a basal diet, Charles River Formula-1 (Oriental Yeast, Co., Ltd.). The animals were kept in an experimental animal room under controlled conditions of humidity (50 ± 10%), light (12/12-h light/dark cycle) and temperature (23 ± 2°C). After 1 wk of quarantine, animals were divided into six experimental groups and one control group. Experimental diets were prepared by mixing tricin in powdered basal diet at two dose levels, 50 and 250 ppm. The highest dose was one eighth of the dose used by Cai et al. (23) because we investigated the potential clinical application of low doses of tricin.

Animal experiment

The experimental and study design were approved by the Committee of Kanazawa Medical University Animal Facility under the Institutional Animal Care guideline. All handling and procedures were carried out in accordance with the appropriate Institutional Animal Care Guidelines.

A total of 95 male ICR mice were divided into six experimental groups and one control group. Mice in groups 1 ($n = 20$), 2 ($n = 20$), and 3 ($n = 19$) were given a single i.p. injection of AOM (10 mg/kg body weight). Beginning 7 d after the AOM injection, they also received 1.5% (*w/v*) DSS in drinking water for 7 d. Beginning 1 wk following the final DSS exposure, the mice in groups 2 were fed an experimental diet containing tricin at the rate of 50 ppm and the mice in group 3 were fed an experimental diet containing 250 ppm tricin. Both groups received the experimental diets for 15 wk. The mice in groups 4 ($n = 9$) received only the 250 ppm tricin-containing diet. The mice in group 5 ($n = 9$) received only AOM, and the mice in group 6 ($n = 9$) received only 1.5% DSS in drinking water. The mice of group 7 ($n = 9$) served as untreated controls.

At week 8, four mice each from groups 1, 2, and 3 and three mice each from groups 4, 5, 6, and 7 were randomly selected and sacrificed to measure mRNA expression of target inflammatory enzymes and cytokines in the colonic mucosa by quantitative reverse transcription-PCR (RT-PCR). At sacrifice, the large bowel of each animal was removed, the contents (feces) were washed out by physiologic saline, and the length from the ileocecal junction to the anal verge were measured. After the large bowels were cut open longitudinally along the main axis and gently washed with saline, scraped colonic mucosa tissue was dipped into the RNAlater solution (Applied Biosystems/Ambion).

At week 18, all of the remaining animals were euthanized by exsanguinations through the abdominal aorta under diethylether anesthesia and subjected to a complete gross necropsy examination to determine the incidence and multiplicity of tumors in the large bowel. At sacrifice, the large bowel was removed and the length was measured. Each large bowel was cut open longitudinally along the main axis and gently washed with saline, then examined manually to determine the incidence and multiplicity of tumors. The colon was fixed in 10% buffered formalin for at least 24 h. Histopathologic examination was done on H&E-stained sections made from paraffin-embedded blocks. Colonic tumors were diagnosed according to criteria established in a prior study (34). The number and density of mucosal ulcers on H&E-stained sections was also recorded.

Immunohistochemistry of proliferating cell nuclear antigen

Immunohistochemical analysis for the proliferating cell nuclear antigen (PCNA) in the colon with or without tumors was done on 4-μm-thick paraffin-embedded sections by the labeled avidin-biotin-peroxidase complex method using a Vectastain ABC kit (Vector Laboratories), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene, and rehydrated with ethanol at room temperature. PBS (pH 7.4; 0.01 mol/L) as used to prepare the solutions and for washes between the preparation steps. Incubations were done in a humidified chamber. The sections were treated for 40 min at room temperature with mouse IgG blocking reagent (Vector Laboratories), and incubated overnight at 4°C with the primary antibody (1:300 dilution; DAKO Japan, Co., Ltd.). The antibody was applied to the sections according to the manufacturer's protocol. Horseradish peroxidase activity was visualized by treatment with H_2O_2 (DAKO Japan, Co., Ltd.) and 3,3′-diaminobenzidine (DAKO Japan) for 5 min. In the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck). For each examination, negative controls were done on serial sections. The numbers of nuclei with positive reactivity for PCNA-immunohistochemistry were counted by two observers (T.O. and T.T.) who were unaware of the treatment groups to which the slides belonged. The positive rates were evaluated in >100 cancer cells each of 15 different areas of the adenocarcinomas and 10 different crypts of the "normal"-appearing colonic mucosa from five mice each from groups 1 to 3 and expressed as percentage (mean ± SD).

Mitotic index and anaphase bridging index of adenocarcinoma cells

To examine the effects of dietary tricin on chromosomal instability (41) in adenocarcinoma cells, the anaphase bridging index (ABI) was determined on H&E-stained sections. The numbers of mitoses and anaphase bridging were counted in >100 cancer cells from five adenocarcinomas each from groups 1 through 3. The mitotic index (MI; number of mitoses per cancer cells) and ABI (number of anaphases with bridging per mitoses) were expressed as percentages (mean ± SD).

Quantitative RT-PCR

The normal-appearing colonic mucosa of mice from groups 1 through 3 were assayed for mRNA expression of COX-2, iNOS, TNF-α, NF-κB, IκBα, and IKKβ by RT-PCR. RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen Co.). Real-time PCR was done in a LightCycler (Roche Diagnostics Co.) with SYBR Premix Ex Taq (TAKARA BIO, INC.). The expression level of each gene was normalized to the β-actin expression level using the standard curve method. Each assay was done six times and the average was calculated. The primers used for amplifications are listed in Supplementary Table S1.

Statistical analysis

Where applicable, data were analyzed using one-way ANOVA with Tukey-Kramer Multiple Comparisons Test or Bonferroni (GraphPad Instat version 3.05, GraphPad Software) with $P < 0.05$ as the limit for statistical significance. Fisher's Exact Probability test or the χ^2 test were used for comparison of the incidence of lesions between the two groups. Data on mRNA expression (mean ± SEM) were analyzed by Mann-Whitney *U* test.

Results

General observation

All animals remained healthy throughout the experimental period. Food consumption (grams/day/mouse) did not differ significantly among the groups (data not shown). The body weight gains by mice in all of the seven groups were similar during the study (Fig. 1B). The mean body weight of group 2 (AOM/DSS/50 ppm tricin) was significantly lower than that of group 1 ($P < 0.01$; Supplementary Table S2). The mean colon length of group 1 was significantly shorter than the mean

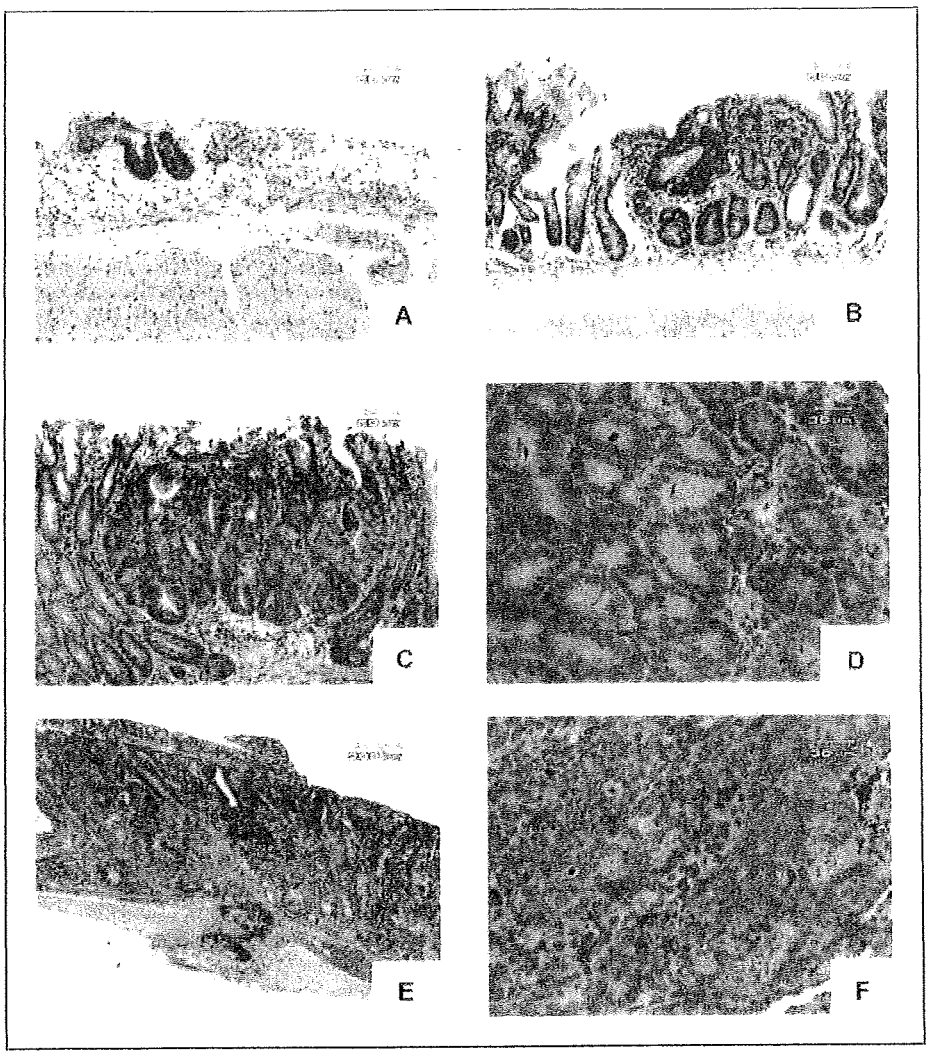

Fig. 2. Representative histopathology of the colonic lesions in group 1 (AOM/DSS). *A*, mucosal ulcer; *B*, dysplastic crypts; *C* and *D*, tubular adenomas; *E* and *F*, moderately differentiated tubular adenocarcinomas.

colon length of group 7 (no treatment; $P < 0.01$; Supplementary Table S2).

Incidence and multiplicity of colonic lesions

The incidence of macroscopic colonic lesions, including tumors and small ulcerations, were seen in the mice in group 1, 2, 3, and 6 (Fig. 1C-E). All mice in groups 1 through 3, which were treated with AOM/DSS with or without tricin, developed colonic tumors (adenoma and/or adenocarcinoma). The mice of group 4, 5, and 7 did not develop colonic tumors.

Microscopic examinations revealed various pathologic colonic lesions in mice from groups 1, 2, 3, and 6. The lesions included mucosal ulcers (Fig. 2A), dysplastic crypts (Fig. 2B), tubular adenomas (Fig. 2C and D), and tubular adenocarcinomas (Fig. 2E and F). Some of the adenocarcinomas that developed in the group 1 mice invaded the subserosa of the colon (Fig. 2E). Table 1 summarizes the microscopic data on the incidence and multiplicity of colonic lesions. The dietary administration of 50 ppm tricin (group 2) significantly reduced the incidence ($P = 0.0117$) and multiplicity ($P < 0.05$) of adenomas and the number of total tumors (adenoma + adenocarcinoma, $P < 0.05$) when compared with group 1. Feeding with 250 ppm tricin (group 3) also significantly lowered the numbers of adenocarcinomas and total tumors when compared with group 1 ($P < 0.05$ for each comparison). The mean numbers of dysplastic crypts in groups 2 ($P < 0.05$) and 3 ($P < 0.01$) were significantly lower than that of dysplastic crypts in group 1. The mean numbers of mucosal ulcers in group 2 ($P < 0.05$) and 3 ($P < 0.001$) were also significantly smaller than that of group 1.

PCNA labeling indices of the normal-appearing crypts and adenocarcinomas

The data on the proliferative kinetics in the normal-appearing crypts and colonic adenocarcinomas by estimating the PCNA labeling indices are shown in Fig. 3. The dietary administration of tricin significantly lowered the PCNA labeling index of the normal-appearing crypts in group 2 (38 ± 11, $P < 0.05$) and group 3 (36 ± 12, $P < 0.05$) when compared with group 1 (48 ± 11). The PCNA labeling indices for colonic adenocarcinomas in groups 2 (74 ± 6, $P < 0.05$) and 3 (71 ± 4, $P < 0.001$) were significantly lower than in group 1 (80 ± 8).

Table 1. Incidence and multiplicity of colonic lesions

Group no.	Treatment (no. of mice examined)	Mucosal ulcer	Dysplasia (high grade)	Adenoma	Adenocarcinoma	Total tumors (AD+ADC)
1	AOM/1.5% DSS (16)	100% (2.69 ± 0.95)*	100% (5.00 ± 3.79)	88% (4.19 ± 4.22)	94% (4.63 ± 3.74)	94% (8.81 ± 6.21)
2	AOM/1.5% DSS/50 ppm tricin (16)	94% (1.81 ± 1.11)	80% (2.56 ± 1.79)†,‡	44%§ (1.44 ± 1.79)†,‡	75% (3.19 ± 2.64)	75% (4.63 ± 4.05)†,‡
3	AOM/1.5% DSS/250 ppm tricin (15)	60% (0.87 ± 0.83)‖	73% (1.53 ± 1.13)¶	67% (1.87 ± 1.73)	67% (1.80 ± 2.04)†,‡	80% (3.67 ±3.37)†,‡
4	250 ppm tricin (6)	0%	0%	0%	0%	0%
5	AOM (6)	0%	0%	0%	0%	0%
6	1.5% DSS (6)	33% (0.33 ± 0.52)	0%	0%	0%	0%
7	None (6)	0%	0%	0%	0%	0%

Abbreviations: AD, adenoma; ADC, adenocarcinoma.
*Mean ± SD.
†Significantly different from group 1 by one-way ANOVA, and Tukey-Kramer Multiple Comparisons test.
‡$P < 0.05$.
§Significantly different from group 1 by Fisher's exact probability test ($P = 0.0117$).
‖$P < 0.001$.
¶$P < 0.01$.

The effects of tricin on the MI and ABI

Dietary administration with tricin affected the number of mitosis (Fig. 4A) and anaphase bridging (Fig. 4B) in adenocarcinomas. As illustrated in Fig. 4C, dietary feeding with tricin significantly decreased the MI in group 2 (17.4 ± 0.9, $P < 0.05$) and group 3 (12.7 ± 2.0, $P < 0.001$) compared with group 1 (20.8 ± 2.4). The treatment also lowered the ABI in group 2 (0.50 ± 0.24) and group 3 (0.29 ± 0.10, $P < 0.05$) compared with group 1 (1.10 ± 0.57).

Expressions of inflammatory enzyme and cytokine genes in colonic mucosa

At week 8, we assayed mRNA levels of COX-2, iNOS, TNF-α, NF-κB, IκBα, and IKKβ in the nonlesional colonic mucosa of

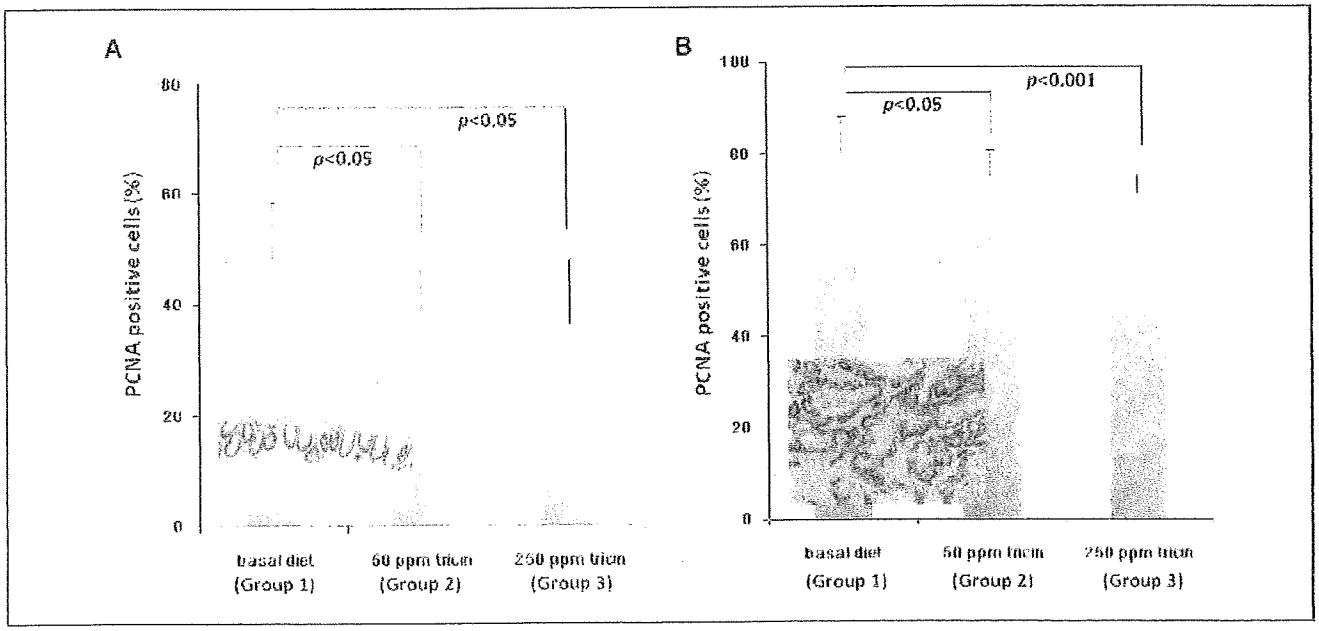

Fig. 3. The PCNA labeling indices of the normal-appearing crypts (*A*) and adenocarcinomas (*B*). Feeding with tricin (groups 2 and 3) significantly lowered the PCNA labeling indices of the normal-appearing crypts ($P < 0.05$ for each comparison) and adenocarcinomas (group 2, $P < 0.05$; and group 3, $P < 0.001$) compared with group 1.

mice in groups 1 through 3 by semiquantitative real-time RT-PCR (Fig. 5). The TNF-α expression significantly decreased in group 3 compared with group 1 ($P < 0.05$; Fig. 5A). Feeding with tricin did not significantly affect the expression of COX-2 (Fig. 5B), iNOS (Fig. 5C), NF-κB (Fig. 5D), IκBα (Fig. 5E), and IKKβ (Fig. 5F).

Discussion

The results described herein clearly indicate that dietary administration with tricin at two dose levels (50 and 250 ppm) significantly inhibited AOM/DSS-induced colonic tumorigenesis in male ICR mice. The high dose (250 ppm) of tricin significantly inhibited development of adenocarcinomas induced by AOM followed by DSS in mice. The dietary administration with tricin also significantly affected the expression of TNF-α in the colonic mucosa at week 8. The treatment resulted in the reduction of the PCNA labeling index, MI, and ABI in the colonic epithelial malignancies at week 18.

The antitumor and chemoprevention activities of tricin have been reported in both *in vitro* and *in vivo* studies. *In vivo* experiments included transplanted human breast cancer cell lines in nude mice (21). In addition, Cai et al. reported that 0.2% tricin in diet effectively inhibited the number of adenomas in the small intestine of $Apc^{Min/+}$ mice (42). They did not, however, observe inhibition of the development of colonic tumors (42). In the current study, we observed the cancer chemopreventive activity of dietary tricin in carcinogenesis in the inflamed colon. In addition, feeding with tricin lowered the occurrence of mucosal ulcers and preneoplasms (dysplastic crypts).

We can point several mechanisms by which tricin may suppress AOM/DSS-induced colon carcinogenesis in this study. Our findings that dietary tricin lowered the PCNA labeling index, MI, and ABI of colonic adenocarcinomas may suggest an antigrowth effect of tricin on colonic malignancy. The findings are in agreement with the reports by Cai et al. (21) that showed tricin or tricin-containing extracts of brown rice inhibited the growth of the colon and mammary cells *in vitro* and *in vivo* (22). In addition, the results that dietary tricin lowered the ABI of adenocarcinoma cells suggest that tricin affects the chromosomal instability of cancer cells and possibly their telomerase activity (41). Tricin may exert chemopreventive activity through inhibition of COX-1 and 2 enzymes and prostaglandin E_2 production in human colon cancer cell lines (HT-29 and HCA-7) and the small intestine of $Apc^{Min/+}$ mice (23, 24). Unexpectedly, dietary tricin did not significantly alter the expression of COX-2 or iNOS at week 8. The suppression of NF-κB–signaling pathway by dietary administration with tricin was insignificant. However,

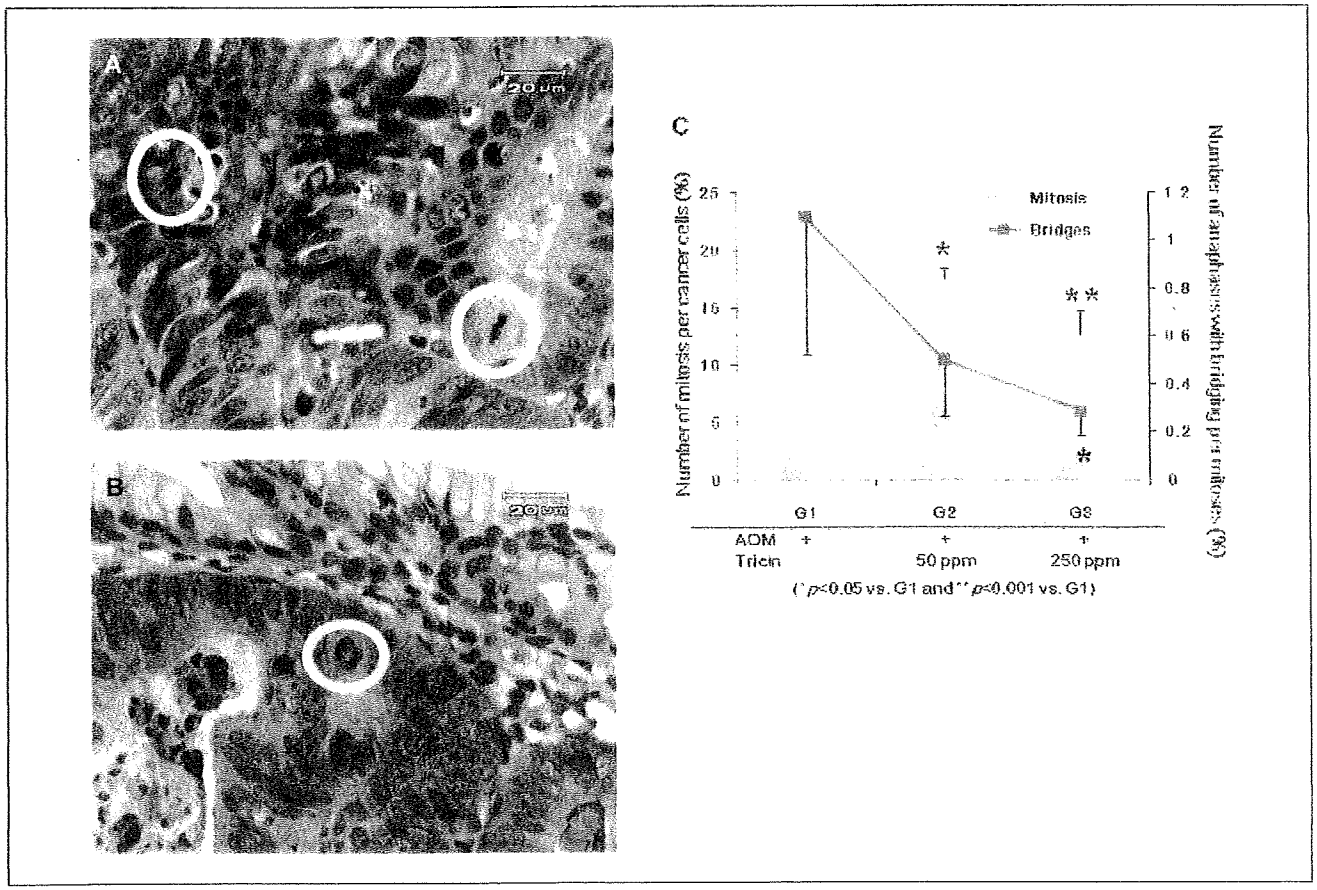

Fig. 4. The effects of dietary tricin on the MI and ABI. *A*, representative mitotic figures (*left circle*, anaphase; *right circle*, metaphase) in an adenocarcinoma, (*B*) representative anaphase bridging (*circle*) in an adenocarcinoma, and (*C*) MI (*columns*) and ABI (*lines*). Dietary administration of tricin significantly reduced the MI (50 ppm tricin, $P < 0.05$; and 250 ppm tricin, $P < 0.001$) and ABI (250 ppm tricin, $P < 0.05$). *G1*, group1; *G2*, group2; and *G3*, group 3.

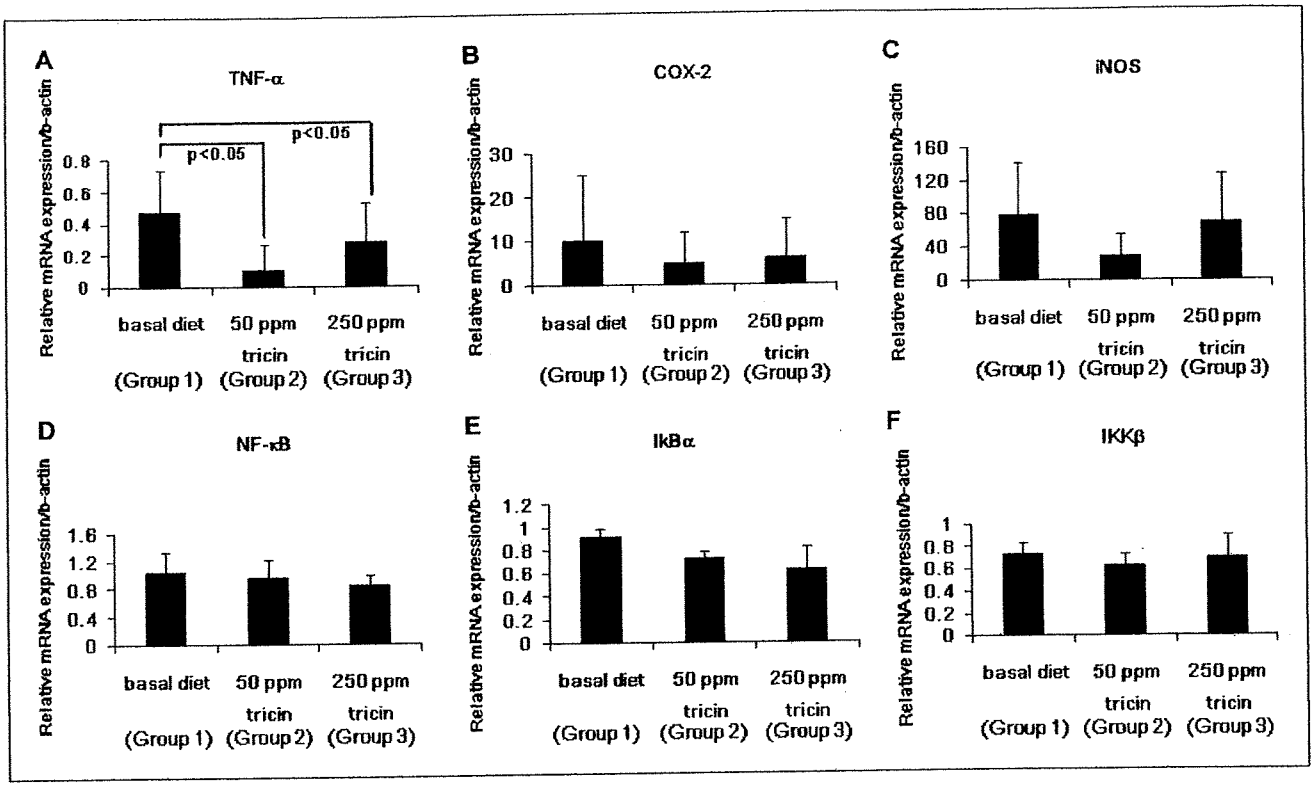

Fig. 5. The expression of (*A*) TNF-α (*B*) COX-2, (*C*) iNOS, (*D*) NF-κB, (*E*) IκBα, and (*F*) IKKβ in the normal-appearing colonic mucosa of groups 1 to 3 that were assessed by semiquantitative real-time RT-PCR. The expression of TNF-α was significantly inhibited by feeding with tricin (groups 2 and 3, $P < 0.05$ for each comparison). Feeding with tricin lowered the expression of COX-2, iNOS, and the NF-κB signaling pathway, but the reduction did not reach statistical significance. The expression was normalized to β-actin mRNA expression. Samples were analyzed in triplicate. *Columns*, mean of three independent experiments; *bars*, SEM; $n = 12$, Statistical analysis was done by the Mann-Whitney *U* test.

we observed that dietary tricin significantly inhibited the expression of TNF-α in the nonlesions colonic mucosa. Such effects are of interest because TNF-α acts as a master switch to establish an intricate link between inflammation and cancer (39, 40).

In conclusion, the dietary administration with tricin effectively suppressed AOM/DSS-induced colon carcinogenesis by suppressing the expression of TNF-α in the early phase and MI and ABI in the later phase. The effects of tricin on TNF-α expression are also important in the chemopreventive activity of tricin in inflammation-associated colorectal carcinogenesis. The safety of tricin was reported by Verschoyle et al. (43). A natural flavonoid tricin is present in edible plants, including rice, oats, barley, and wheat (10). In the current study, we isolated tricin from the dried leaves of *Sasa albo-marginata* that contain a large amount (0.2 ppm) of tricin than rice (Oryza sativa L.; 0.066 ppm). Tricin is thus a candidate for clinical use for fighting colorectal cancer development in patients without colitis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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CLINICAL REVIEW

Association of digestive organ disease with metabolic syndrome: role of adipocytokine and its molecular mechanisms

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Abstract Recently, lifestyle-related disease due to excess nutrition and insufficient physical exercise has been increasing in developed countries, including Japan. Metabolic syndrome is related to visceral fat accumulation in individuals with upper body obesity. Adipose tissue is an endocrine organ that secretes adipocytokines such as adiponectin, leptin, and TNF-a. Obesity alters the secretion of adipocytokines, leading to insulin resistance and various other metabolic disorders. Little is known about how altered regulation of adipocytokines is related to the development and progression of digestive organ disease. Clarification of the mechanisms whereby such altered adipocytokine secretion participates in pathophysiology of digestive organ disease could lead to the development of preventive and therapeutic measures.

Keywords Metabolic syndrome · Obesity · Adiponectin · NASH · Colorectal cancer · AMPK · Tumor suppressor gene · LKB1 · p53

Introduction

Obesity is increasing worldwide in developed countries due to excess nutrition and insufficient physical exercise. Metabolic syndrome, a condition related to visceral fat accumulation, has not been noted to play a role in the development of type-2 diabetes and cardiovascular disease [1], and also implicated in the development and progression of certain digestive organ disease, although the mechanisms responsible remain obscure [2–8].

Although Japanese have lower incidence of obesity than Caucasians, metabolic syndrome is becoming more common in Japan, and it has been observed that Japanese individuals have a high prevalence of visceral fat accumulation, even if their body mass index (BMI) is less than 25 [1]. This has led to a hypothesis that BMI 25 in Japanese is equivalent to BMI 30 in Caucasians.

Several lines of evidence suggest that adipose tissue is not merely a fat (energy)-storing tissue, but also an endocrine organ secreting various bioactive substances (so-called "adipocytokines") such as adiponectin [1]. The altered secretion from adipose tissue, especially visceral fat, in individuals with metabolic syndrome is related to various pathophysiological conditions, such as insulin resistance, hyperlipidemia, and hypertension.

Investigation of digestive organ disease based on altered secretion of adipocytokines resulting from metabolic syndrome would be an important approach for understanding the mechanisms whereby such pathophsiological conditions develop. Such efforts could lead to the development of preventive procedures for diseases linked to metabolic syndrome, for example, NAFLD (nonalcoholic fatty liver disease)/NASH (nonalcoholic steatohepatitis) [2–5] and colorectal cancer [6–8].

Pathophysiological conditions in digestive organs based on visceral fat accumulation

Fatty liver and cholelithiasis are known to be digestive organ diseases that develop in a background of obesity. Recently, it has been reported that visceral fat accumulation plays an important role in the development of NAFLD [2–5].

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It has been considered that BMI is closely associated with the development of NAFLD. However, NAFLD with neither obesity nor diabetes has been recognized and accounts for about 20% of all cases of NAFLD. Clinical hepatologists often encounter young adult male patients with this type of NAFLD, who show a weight gain of only 2–3 kg due to a change in lifestyle, for example, marriage, although they still have a BMI ≤25 and appear slim. CT scan shows that some of them have visceral fat accumulation.

Obesity is associated with the development of GERD (gastro-esophageal reflux disease). Also, it is well-known that colorectal adenoma and cancer are associated with upper body obesity and insulin resistance in both western countries [6, 7] and Japan [8]. However, the associations with visceral fat accumulation have not been studied.

Visceral fat accumulation, rather than high BMI, is an essential feature of metabolic syndrome

Recent studies have shown that visceral fat accumulation, rather than a high BMI, is an essential feature of metabolic syndrome. This appears to be true for the development of digestive organ disease due to excess nutrition and inadequate physical exercise. Differences in adipose tissue distribution appear to be related to morbidity. Matsuzawa et al. [1] have shown that visceral fat accumulation is more closely associated with risk factors for diabetes, hyperlipidemia, and hypertension than is subcutaneous fat accumulation. Therefore, metabolic syndrome is based on visceral fat accumulation rather than BMI per se as an index of whole body fat.

Visceral adipose tissue is metabolically and endocrinologically more active than subcutaneous adipose tissue. Visceral adipocytes produce and secrete adipocyotokines, such as adiponectin, leptin, angiotensinogen, and TNF-a (Fig.1) [1]. Altered regulation of the adipocytokines is thought to be related to various clinical manifestations in metabolic syndrome. For example, increased TNF-a and decreased adiponectin levels are related to insulin resistance, and an increased angiotensinogen level is relates to hypertension.

There is another reason why visceral fat accumulation is relatively prevalent in Japanese. So-called thrifty genes, which act to save energy and accumulate fat in adipose tissue, seem to be clustered in Japanese. Japanese tend to be more resistant to starvation than Caucasians through the function of the thrifty genes, but under conditions of excess nutrition, they easily develop metabolic syndrome because of visceral fat accumulation. This genetic background explains why the incidence of diabetes is relatively high in Japanese, even though the proportion of the population with BMI ≥30 is much less than in Caucasian populations.

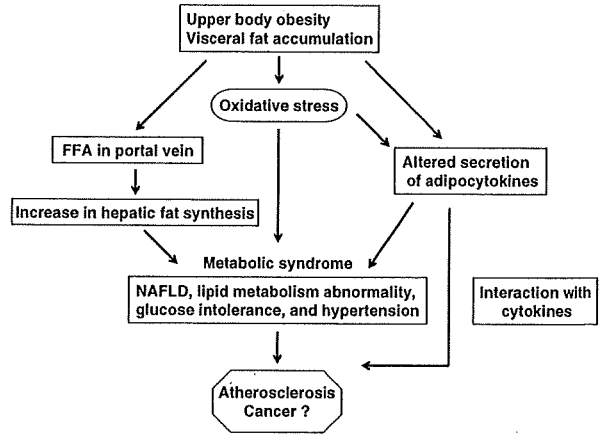

Fig. 1 Role of visceral fat accumulation and adipocytokine in metabolic syndrome

Role of adipocytokines in development of NAFLD and NASH

Increased influx of free fatty acids into the liver from visceral adipose tissue via the portal vein stimulates triglyceride (TG) synthesis in hepatocytes, which in turn lead to the development of fatty liver. In addition, secretion of very low density lipoprotein (VLDL) is accelerated by an increase in the synthesis of apolipoprotein B (apo B) and activity of microsomal triglyceride transfer protein (MTP). This can partly explain the hypertriglyceridemia in metabolic syndrome.

NASH is a severe form of NAFLD that can progress to hepatic cirrhosis and hepatocellular carcinoma [9, 10]. Visceral fat area assessed by CT scan is increased in patients with NASH [11]. Adiponectin is a peptide hormone secreted specifically from adipocytes, and its plasma level is inversely decreased when visceral fat accumulates [12]. The plasma adiponectin level is decreased in NAFLD/NASH [13–16] and is correlated positively with the grade of hepatic steatosis and negatively with HOMA-IR as an index of hepatic insulin resistance, implying that a decreased adiponectin level due to accumulation of visceral fat results in hepatic steatosis and insulin resistance [14]. Although our preliminary data have shown that the plasma adiponectin level is negatively correlated with visceral fat area in patients with NASH, it is noteworthy that some patients have a normal adiponectin level and no accumulation of visceral fat.

Recently, an experimental study using adiponectin knockout mice demonstrated a role of adiponectin depletion in the development of NASH [17]. Intake of a choline-deficient L-amino acid-defined (CDAA) diet for 1 week induced hepatic steatosis in adiponectin knockout mice, but only slight fat infiltration in wild mice, whereas intake of

this diet for 4 weeks induced marked steatosis in both groups of mice. However, transfection of the adiponectin gene using an adenovirus expression vector restored hepatic fat accumulation. Furthermore, intake of the CDAA diet for 24 weeks induced lobular inflammation and pericellular fibrosis similar to the histology of NASH in the knockout mice. This observation that adiponectin deficiency induces not only steatosis, but also lobular inflammation and fibrosis [18] implies an association of the adipose-specific hormone with NASH development. In addition to inflammation and fibrosis, 6 out of 14 knockout mice developed liver cirrhosis and hepatic tumors after 24 weeks, whereas the 15 wild-type mice showed only simple steatosis [17].

Role of adipocytokines in development of colorectal adenoma and cancer

Are insulin resistance and adiponectin related to colorectal adenoma and cancer? Upper body obesity and hyperinsulinemia based on insulin resistance are known to be risk factors for colorectal cancer [5–7] via the IGF (insulin-like growth factor)-1-axis [19, 20]. Under conditions of insulin resistance, the free fraction of IGF-1 is relatively increased because of suppressed expression of IGF-1-binding proteins, resulting in an enhanced cell growth and attenuated apoptosis. This may lead to the development of colorectal carcinogenesis as IGF-1 is a potent growth factor.

Another explanation is the function of insulin as a growth factor. Hyperinsulinemia due to insulin resistance seems to be related to enhanced cell growth and suppressed apoptosis. Recently, it was reported that patients with type-2 diabetes treated with insulin injection have a two-fold risk of colorectal cancer, possibly due to the role of insulin per se as a growth factor. One explanation is that, among the intracellular signals from insulin receptor, the growth signal via the MAPK pathway is not blocked, unlike metabolic signals, such as glucose uptake and utility, in a background of insulin resistance. Further investigation is needed to understand the role of insulin in the development and growth of cancers [22].

Altered secretion of adipocytokines, particularly a decrease in the level of plasma adiponectin, is reported to be associated with colorectal cancer [23]. A recent report from our laboratory [23] showed that colorectal adenoma was associated with visceral accumulation and a lower level of plasma adiponectin. Visceral fat area assessed by CT scan was increased in 51 patients with colorectal adenoma in comparison with 52 control subjects without colorectal tumors ($P < 0.001$), who had undergone total colonoscopy for survey of colorectal cancer (Fig. 2). The plasma level of adiponectin was decreased in the patients with colorectal adenoma ($P < 0.001$) (Fig. 2). However, it is of interest that colorectal adenoma appears to have no association with BMI.

Furthermore, a prospective cohort study (health professional follow-up study) has demonstrated that a low level of plasma adiponectin is associated with colorectal cancer [24]. The subjects (18,225 men) enrolled in this cohort were followed up for about 8 years, during which time 179 of them developed colorectal cancer. The risk for colorectal cancer in the highest quintile (Q5) was 0.42 in comparison with that in the lowest quintile (Q1).

The mechanisms whereby a reduced adiponectin level is related to colon carcinogenesis still remains unclear. Recently, obesity has been considered to be a low-grade systemic inflammatory disease. Cross-talk is known to

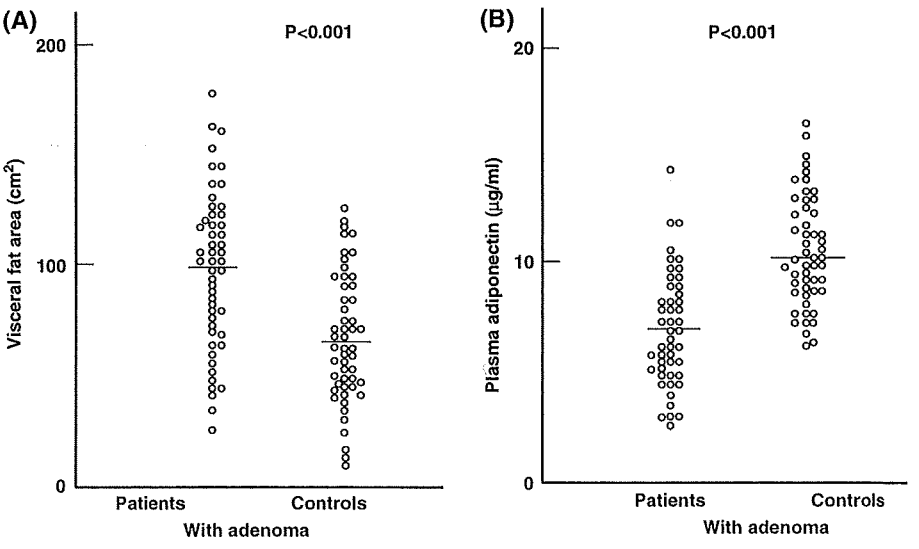

Fig. 2 Visceral fat area and plasma adiponectin in patients with colon adenoma (Wilcoxon rank test) (cited from [23])

exist between adiponectin and proinflammatory cytokines (IL-6 and TNF-a) and anti-inflammatory (IL-10) cytokines. Furthermore, adiponectin is reported to inhibit the NF-kB signal. A reduced adiponectin level could lead to inflammation and suppress apoptosis, possibly triggering carcinogenesis. In addition to colorectal cancer, reduced adiponectin is associated with endometrial and breast cancer [25–27]. Further study is needed to clarify the roles of adiponectin in carcinogenesis.

An increased level of serum leptin is also associated with colorectal cancer [28], although the mechanism whereby leptin participates in colorectal carcinogenesis is not known.

Participation of tumor suppressor genes in energy homeostasis

Recently, it has been noted that tumor suppressor genes participate in energy homeostasis [29]. Such new evidence implies a link between energy homeostasis and carcinogenesis based on metabolic syndrome. Shaw et al. [29] demonstrated that the LKB1 gene responsible for Peutz-Jeghers syndrome is located upstream of AMPK (AMP-activated protein kinase), which is an energy sensor involved in regulation of cellular energy homeostasis. The concept that a so-called tumor suppressor gene could control both cell growth (cancer) and energy homeostasis (metabolic syndrome and diabetes) as an upstream master gene had a very strong impact.

In 1998, LKB1 was recognized as the causative gene of Peutz-Jeghers syndrome coding serine/threonine protein kinase [30]. This protein kinase is activated by phosphorylation by AMPK, the energy sensor, preventing metabolic diseases such as diabetes. AMPK is switched on by metabolic stress (depletion of ATP), including hypoglycemia, hypoxia, and physical exercise [31]. When the intracellular energy level is depleted, resulting in an increase in the AMP/ATP ratio, the increased AMP activates AMPK. Therefore, AMPK is a key player in the maintenance of energy homeostasis.

Once AMPK is activated, anabolic pathways are accelerated, while simultaneously catabolic pathways are suppressed (Fig.3). AMPK phosphorylates the rate-limiting enzymes of the anabolic pathways, for example, in lipid metabolism, HMG-CoA reductase and acetyl-CoA carboxylase are phosphorylated. Also, protein synthesis is suppressed via suppression of mTOR (mammalian target-of-rapamycin) by AMPK. Interestingly, activation of AMPK inhibits entry into the S phase of the cell cycle via G1 arrest, in collaboration with the tumor suppressor gene p53 [32].

It is also noteworthy that p53 has been reported to regulate both cell death and energy homeostasis [33], functioning as a switch for mitochondrial respiration and glycolysis (Fig. 4). p53 is the gene in which mutations are recognized most frequently in human cancers. Loss of p53 protein shifts the pathway of ATP synthesis from the mitochondrial electron transport system to glycolysis. The target molecule for the shift is known to be synthesis of cytochrome c oxidase 2 (SCO2) to regulate the cytochrome c oxidase complex (COX) in mitochondria.

Cancer is a gene-based disease that induces abnormalities of the cellular systems that control cell growth and death [34]. Otto Warburg won the Nobel Prize in 1931 for his discovery that aerobic respiration in mitochondria is suppressed in cancer cells and that ATP synthesis is dependent on anaerobic glycolysis. Although the molecular basis on Warburg's phenomenon remained unknown for many years, part of the puzzle was solved 75 years ago when he received the Nobel Prize.

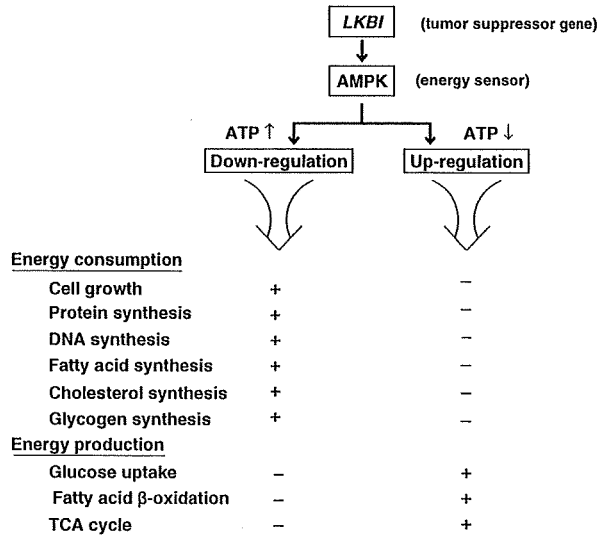

Fig. 3 Tumor suppressor gene LKB1 and AMPK cascade for controls of energy homeostasis and cell growth

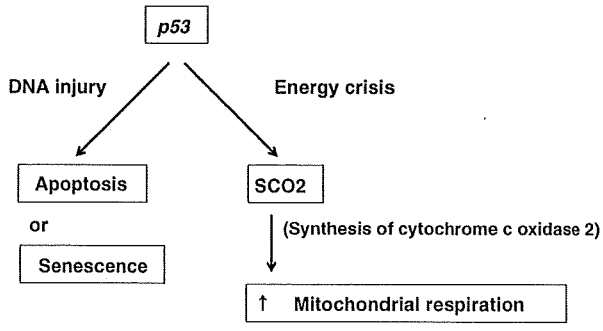

Fig. 4 A novel role of tumor suppressor gene p53 in energy homeostasis

An allelic mutation of p53 induces oxygen independency in the cells and enables for the cells to survive in a hypoxic environment, possibly leading to carcinogenesis. The relationship between p53 function and oxidative stress and aging could be a focus of interest in the future [35]. In addition to p53, another tumor suppressor gene, VHL (von Hippel-Lindau), is reported to participate in mitochondrial respiratory chain biogenesis [36].

Possible role of the adipocytokine-AMPK axis in carcinogenesis

AMPK controls body energy balance via regulation by hormones and cytokines, especially by adipocytokines, such as adiponectin [37] and leptin [38], which stimulate the uptake and utility of glucose and fatty acid b-oxidation. On the other hand, the adipocytokines suppress gluconeogenesis and lipid biosynthesis and inhibit cell growth, possibly contributing to suppression of carcinogenic potentials.

Insulin, LKB1, and mTOR pathways are responsible for the fundamental control of cell growth and its regulation by nutrients. The LKB1–AMPK axis regulates the cell cycle by G1 arrest. Mutation of LKB1 accelerates CREB-dependent and SREBP-1-dependent transcription, resulting in carcinogenesis. Several lines of evidence suggest that constitutive activation of CREB induces carcinogenesis in cells [39]. The LKB1–AMPK axis also activates the product of tumor suppressor gene, tuberous sclerosis complex 2 (TSC2), and subsequently suppresses protein synthesis by inhibition of mTOR. In fact, the mTOR signal is enhanced in intestinal tumor developing in LKB1 knockout mice [29]. The molecular mechanisms whereby LKB1–AMPK-axis participates in carcinogenesis still remain unknown.

Cancer prevention and energy homeostasis

Many studies have indicated that an appropriate level of physical exercise and calorie restriction are effective for cancer prevention [40]. The IARC (the International Agency for Research on Cancer) reported that one-fourth or one-third of human cancers is associated with excess nutrition and inadequate physical exercise, respectively [41].

For example, a meta-analysis has demonstrated that physical activity significantly decreases the risk of cancer [40]. Linkage between physical activity and cancer prevention is well recognized epidemiologically. However, as the molecular linkage has remained obscure, the issue of physical exercise in cancer prevention has not been considered necessarily important in clinical fields. However, recent studies have begun to focus on this aspect, and it is likely to become an interesting new field of cancer research. The concept that prevention and therapy of metabolic syndrome may also be effective for cancer prevention will doubtless become a focus of interest, and a large-scale prospective cohort study is expected to yield valuable data.

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Aberrant Crypt Foci as Precursors of the Dysplasia-Carcinoma Sequence in Patients with Ulcerative Colitis

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Abstract **Purpose:** Long-standing ulcerative colitis (UC) predisposes patients to the development of colorectal cancer, but surveillance of colitis-associated cancer by detecting the precancerous lesion dysplasia is often difficult because of its rare occurrence and normal-looking appearance. In sporadic colorectal cancer, aberrant crypt foci (ACF) have been reported by many investigators to be precursor lesions of the adenoma-carcinoma sequence. In the present study, we analyzed the genetic background of ACF to determine whether they could be precursors for dysplasia, and we examined the usefulness of endoscopic examination of ACF as a surrogate marker for surveillance of colitis-associated cancer.

Experimental Design: ACF were examined in 28 UC patients (19 patients with UC alone and 9 patients with UC and dysplasia; 2 of those patients with dysplasia also had cancer) using magnifying endoscopy. K-ras, APC, and p53 mutations were analyzed by two-step PCR RFLP, *in vitro* – synthesized protein assay, and single-strand conformation polymorphism, respectively. Methylation of p16 was analyzed by methylation-specific PCR.

Results: ACF that appeared distinct endoscopically and histologically were identified in 27 out of 28 UC patients. They were negative for K-ras, APC, and p53 mutations but were frequently positive for p16 methylation (8 of 11; 73%). In dysplasia, K-ras and APC mutations were negative but p53 mutation (3 of 5; 60%) and p16 methylation (3 of 5; 60%) were positive. There was a significant stepwise increase in the number of ACF from patients with UC alone to patients with dysplasia and to patients with cancer. Univariate and multivariate analyses showed significant correlations between ACF and dysplasia.

Conclusions: We have disclosed an ACF-dysplasia-cancer sequence in colitis-associated carcinogenesis similar to the ACF-adenoma-carcinoma sequence in sporadic colon carcinogenesis. This study suggests the use of ACF instead of dysplasia for the surveillance of colitis cancer and warrants further evaluation of ACF as a surveillance marker in large-scale studies.

It is commonly recognized that long-standing ulcerative colitis (UC) predisposes patients to the development of colorectal cancer (1). However, the detection of early colorectal cancer is often difficult in patients with UC because there is inflammation in the background mucosa and it predominantly represents flat-type ill-delineated lesions (2, 3). Therefore, colitis-associated cancer is often detected at an advanced stage and is characterized by a very poor prognosis. One approach to overcome this difficulty is to use dysplasia, which is considered to be a precancerous lesion in colitis-associated cancer, as a surrogate marker for early detection of colitis-associated cancer (4, 5). However, identification of dysplasia by endoscopy requires greater skill than detection of cancer itself because of its rare occurrence and apparently normal-looking appearance (6).

We previously succeeded in identifying aberrant crypt foci (ACF) in non-UC subjects using magnifying endoscopy (7) and showed that the number of ACF increased in the order of normal subjects, patients with adenomas and then patients with cancer, and proposed an ACF-adenoma-carcinoma sequence for sporadic colon carcinogenesis. Adler et al. also observed ACF using magnifying endoscopy and found that the number of rectal ACF in patients with colorectal cancer was significantly higher than in normal subjects (8). The increased number of ACF was further observed in patients with flat adenoma and cancer (9). Seike et al. showed that ACF could be a predictive factor for advanced rectal cancer by multivariate analysis (10). Thus, magnifying endoscopy has become a

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common methodology to observe ACF. Regarding the genetic abnormality of ACF, we found a highly frequent K-ras mutation and GSTP1-1 overexpression and also showed that GSTP1-1 endows ACF with resistance to bile salt–induced apoptosis (11–13).

With regard to the genetic abnormality of colitis-associated cancer and dysplasia, mutations of *K-ras* and *APC* are relatively rare (14–17), and in contrast, *p53* mutation is frequently positive (16, 18, 19). Moreover, hypermethylation of genes such as the *p16* gene has recently been detected (20, 21). On the basis of these previous reports, we first attempted to define ACF as precursors for colitis-associated dysplasia by analyzing their genetic background, including mutation of *K-ras*, *APC*, *p53*, and hypermethylation of p16, and then examined the feasibility of using ACF as surrogate markers for the surveillance of colitis-associated cancer.

Materials and Methods

Subjects. This study was approved by the ethics committee of Sapporo Medical University. Fifty-six subjects were enrolled after obtaining written informed consent. The subjects were comprised of 28 UC patients (19 patients with UC alone and 9 patients with UC and dysplasia; 2 of those patients with dysplasia also had cancer), 24 healthy subjects, and 4 patients with Crohn's disease as a control. Average ages and male/female ratios in these groups were as follows: UC, 38.3 ± 6.7 years and 1/1; healthy subjects, 41.5 ± 7.8 years and 3/5; Crohn's disease patients, 30.3 ± 7.4 years and 2/2. The diagnosis of UC was made according to established criteria, including clinical symptoms, radiological findings, blood examination, endoscopy, and pathologic observation of inflamed intestinal mucosa.

Magnifying endoscopy. UC patients in remission underwent magnifying endoscopy, which was done as described previously (7, 11). In order to improve the visualization of ACF (i.e., accurate evaluation of ACF number), plenty of polyethyleneglycol was administered before examination. All patients underwent total colonoscopy. After observation of the entire colorectum, the lower rectum from the middle Houston valve to the dentate line was washed with plenty of water, stained with 0.2% methylene blue, and again washed with sufficient water for identification of ACF. Biopsies were taken under magnifying endoscopy as described previously (7, 11). In this particular study, to avoid laborious and lengthy procedures considering the future application of ACF as a surveillance marker, the observation of ACF was limited to the lower rectum on the basis of our previous report; the number of ACF in the lower rectum correlated with that in entire colorectum (7). All procedures were recorded on videotape and evaluated by two independent observers who were unaware of the subjects' clinical histories.

ACF criteria. ACF were defined as lesions in which crypts were more darkly stained with methylene blue than normal ones and had larger diameters, often with oval or slit-like lumens and thicker epithelial linings (22, 23).

Two-step PCR and RFLP for detection of K-ras codon 12 mutations. Cellular DNA was extracted from the biopsy specimens and used as a template for PCR. The PCR products were amplified using mismatched primers and analyzed by RFLP to detect point mutations in codon 12 of the *K-ras* gene, as described previously (12, 24).

In vitro–synthesized protein assay for detection of mutations in APC. *In vitro*–synthesized protein assays were performed according to a method described previously (25). In brief, primer pairs were prepared for segments 3 (codons 686-1022) and 4 (codons 996-1693) of the *APC* gene, which include the whole mutation cluster region. These primer pairs were specially designed to place the necessary transcriptional and translational regulatory sequences at the 5'-ends of the PCR products. Genomic DNA were extracted from ACF and dysplasia tissue samples were obtained by microdissection. After amplification of the *APC* gene, the PCR products were used directly, without cloning, as templates in coupled transcription and translation reactions (Promega Corp.) in a mixture containing 10 μCi of ^{35}S-methionine. The proteins thus synthesized were analyzed on 10% to 20% gradient SDS polyacrylamide gels and visualized by autoradiography.

Single-strand conformation polymorphism analysis of p53. Four primer pairs for exons 5, 6, 7, and 8/9 (Takara), which include the hotspot region of *p53* mutation were used. Single-strand conformation polymorphism analyses were done according to the method described previously (26). In brief, genomic DNA were amplified using each primer pair. Aliquots of the PCR product were denatured for 5 min at 80°C in a sample buffer containing 98% formamide, and then cooled quickly on ice. Each sample was electrophoresed on a 15% polyacrylamide gel, which was stained using a silver staining kit (Bio-Rad).

Analysis of hypermethylation of p16. Bisulfite treatment of DNA was done as described previously (27). Briefly, 2 μg of genomic DNA were denatured in 0.2 mol/L of NaOH at 37°C for 20 min, followed by incubation with 3 mol/L of sodium bisulfite (Sigma Chemical Co.); hydroquinone (Sigma Chemical Co.) was added at a final concentration of 0.5 mmol/L. The reaction was done at 55°C for 16 h. After treatment, modified DNA was purified using a Wizard DNA Clean-Up kit (Promega) as recommended by the manufacturer, and resuspended in 30 μL of distilled water. Two microliters of the bisulfite-incubated DNA were used as a template for each bisulfite-PCR, and primer pairs were used as described previously (27). After amplification, each PCR sample was electrophoresed on 10% to 20% polyacrylamide gels, stained with ethidium bromide and directly visualized under UV illumination.

RNA extraction and reverse transcription-PCR. Total RNA was isolated from the frozen samples of ACF and normal mucosa of UC patients using the total RNA isolation system (Promega). Reverse transcription-PCR (RT-PCR) was done as previously described (28). Briefly, the reverse transcription reactions were achieved using avian myeloblastosis virus reverse transriptases. Then, the $p16^{INK4A}$-specific exon 1 was amplified with primers 5'-ATGGAGCCTTCGGCTGACTGG-3' and 5'-GATCGGCCTCCGACCGTAAC-3'. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard.

Statistical analysis. The number of ACF in relation to potential risk factors for colitis-associated cancer were compared by Mann-Whitney *U* test. Multivariate analysis was carried out by multiple logistic regression analysis using SAS software (SAS Institute Japan).

Results

Endoscopic appearance and histology of ACF in UC patients. An endoscopic view of dysplasia, which is often difficult to identify by standard endoscopy in a patient with UC is shown in Fig. 1A. Histologically, the nuclei of goblet cells are hyperchromatic, have lost their normal polarity, and show some nuclear stratification. These characteristics are consistent with low-grade dysplasia (29). Figure 1D shows a representative endoscopic view of ACF in a patient with UC (colitis ACF) in comparison with typical ACF in non-UC patients (non-UC ACF), which we reported previously (Fig. 1G). Both types of ACF could be identified as a focus consisting of large crypts darkly stained with methylene blue. However, in comparison to the typical non-UC ACF, the lining of each crypt of the colitis ACF was obscure and the boundaries of individual crypts were unclear. Moreover, most of the colitis ACF showed distorted shapes in contrast to round or oval shapes of the non-UC ACF in the majority. Histologically, the

colitis ACF specimens showed marked infiltration of lymphocytes in the stroma (Fig. 1E) as compared with non-UC ACF (Fig. 1H). They also showed a more diverse range of crypt sizes, enlarged nuclei in epithelial cells, and increased chromatin staining.

We identified a total of 164 ACF in 27 out of 28 patients with UC by using methylene blue in magnifying endoscopy, and no side effects were noted. Of these, 147 (91.3%) showed the typical appearance of colitic ACF as illustrated in Fig. 1D. Only 17 ACF (9.7%) in these patients showed the typical appearance of non-UC ACF. Therefore, we confined further examinations to the colitis ACF.

Colitis-associated colorectal cancer is reported to have a higher incidence of histologically mucinous type tumors than sporadic colorectal cancer. It has also been reported that goblet cell hyperplasia is frequently observed in dysplasia from patients with UC and that the frequency of goblet cell hyperplasia is positively correlated with the duration of UC (30). Therefore, goblet cells in nine colitis ACF, nine non-UC ACF, seven dysplasia, and seven normal rectal epithelial specimens were examined by Alcian blue staining. The frequencies of goblet cells observed in colitis ACF and dysplasia tissues were 49.5 ± 9.7% and 52.7 ± 14.2%, respectively. They were significantly greater than those in the normal background mucosa of ACF tissues (32.3 ± 5.9%) and in non-UC ACF (35.7 ± 15.4%), suggesting the existence of goblet cell hyperplasia in colitis ACF as well as dysplasia. These results are also consistent with the hypothesis that ACF are precursor lesions of dysplasia in patients with UC.

Analysis of K-ras mutation in colitis ACF and dysplasia. Because *K-ras* mutations have frequently been detected in ACF from non-UC patients (11, 31, 32), we screened colitis ACF specimens and dysplasia tissue from UC patients for *K-ras* codon 12 mutations by a two-step PCR-RFLP method. *K-ras* mutations were found in 20% (2 of 10) and 0% (0 of 5) of colitis ACF and dysplasia specimens, respectively, from UC

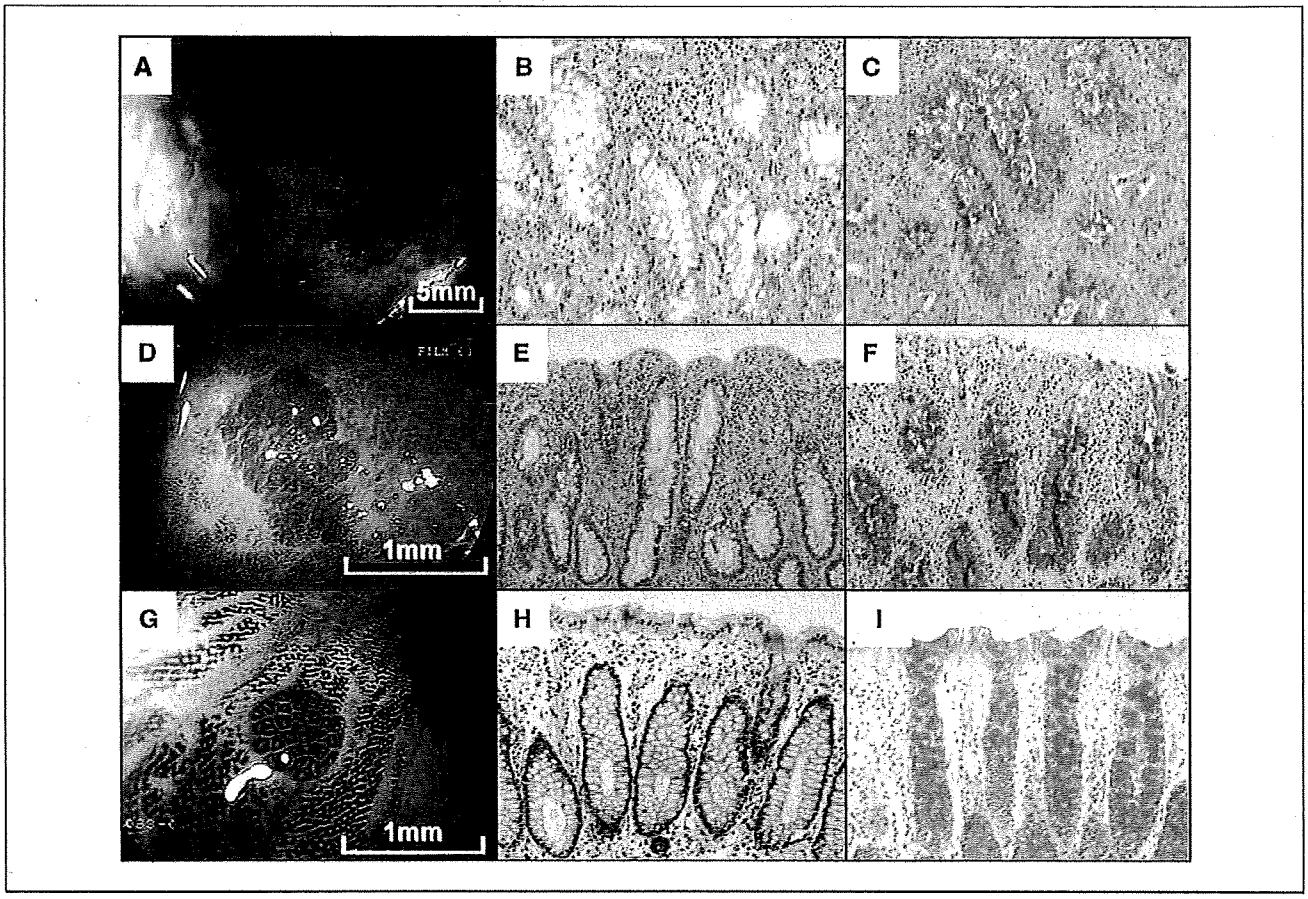

Fig. 1. Endoscopic and histologic features of dysplasia (*A-C*), ACF in a patient with UC (*D-F*), and ACF in a non-UC patient (*G-I*). *A*, dysplasia in a UC patient, which was not visible unless chromoendoscopy was done. *B*, the crypt was lined by columnar epithelia with some nuclear stratification, hyperchromatic nuclei, and loss of normal polarity (H&E; magnification, ×150). *C*, increased numbers of goblet cells were seen in the dysplasia of UC patients. Some dystrophic goblet cells were observed (Alcian blue; magnification, ×150). *D*, a representative ACF in a UC patient, which was characterized by darker staining with methylene blue and larger crypts with thicker epithelial lining than the background mucosa. The lining of each crypt was obscure and the boundaries of individual crypts were more unclear than in non-UC ACF (*G*). *E*, the colitis ACF showed marked infiltration of lymphocytes in the stroma, a more diverse range of crypt sizes, enlarged nuclei in epithelial cells, and increased chromatin staining compared with non-UC ACF (H&E; magnification, ×120). *F*, an increase in the number of goblet cells was seen in colitis ACF, similar to dysplasia in UC patients. Some dystrophic goblet cells were also identified (Alcian blue; magnification, ×120). *G*, a representative non-UC ACF consisting of crypts with round and oval lumens and with a wide pericryptal space. *H*, non-UC ACF showed slight enlargement, irregularity, and elongation of the ducts (H&E; magnification, ×150). *I*, the number of goblet cells in non-UC ACF was apparently fewer than that in colitis ACF (Alcian blue; magnification, ×150).

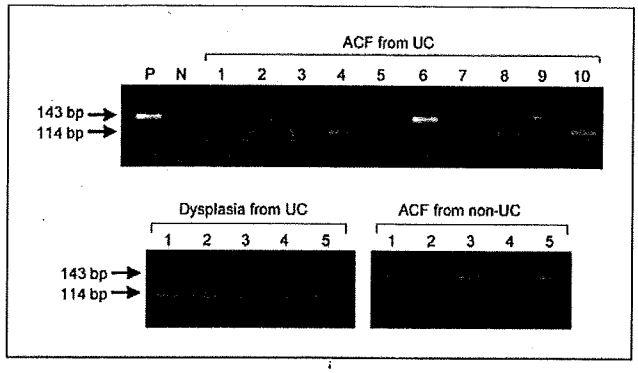

Fig. 2. Analysis for *K-ras* mutation in colitis ACF, dysplasia from UC patients, and non-UC ACF by two-step PCR and RFLP. A pancreatic cancer cell line, APSC (ATCC CRL1862; American Tissue Culture Collection), which is known to have a K-ras point mutation, was used as a positive control (*P*). A normal colonic mucosa was used as a negative control. *K-ras* mutations were found in 20% (2 of 10) and 0% (0 of 5), respectively, of colitis ACF and dysplasia from UC patients. In contrast, mutations were detected in four of five patients with (80%) non-UC ACF.

patients. In contrast, mutations were detected in 4 of 5 (80%) non-UC ACF specimens (Fig. 2), consistent with our previous reports and those of other laboratories (7, 11, 14, 15). Thus, the frequency of *K-ras* mutations in colitis ACF was relatively low compared with that of non-UC ACF.

Analysis of APC mutation in colitis ACF and dysplasia. Because *APC* mutation is an early genetic event in colorectal carcinogenesis in non-UC patients (32–34), we examined *APC* mutations in colitis ACF and dysplasia specimens. Segments 3 and 4 of the *APC* gene, which include the entire mutation cluster region, were analyzed by an *in vitro*–synthesized protein assay in 11 colitis ACF and 2 dysplasia tissue specimens. No *APC* mutations were detected in any of the 11 colitis ACF (0 of 11, 0%) or the 2 dysplasia specimens (0 of 2, 0%; Fig. 3). Likewise, no *APC* mutations were detected in any of the 7 ACF specimens from non-UC patients (data not shown), consistent with our previous report (11).

Analysis of p53 mutation in colitis ACF and dysplasia. It has been reported that p53 mutations are frequently detected in dysplasia and cancer tissues from patients with UC (16, 18, 19). Therefore, we investigated for *p53* mutations in the hotspot region (exons 5-9) employing nonradioisotopic single-strand conformation polymorphism in 11 colitis ACF and 5 dysplasia specimens from patients with UC. No mutations were detected in any of the exons 5, 6, 7, or 8/9 in the 11 ACF specimens, whereas mutations were detected in exons 5, 6, or 8/9 of the 5 dysplasia specimens (Fig. 4). Overall, for exons 5 to 9, *p53* mutations were found in 3 of the 5 dysplasia specimens (60%) but in none of the 11 ACF lesions (0%) from patients with UC.

Hypermethylation of the* p16 *gene promoter in colitis ACF and dysplasia. Recently, it was reported that the *p16* gene promoter is often hypermethylated in dysplasia and cancer from UC patients (20, 21). Therefore, we examined the methylation status of the promoter region of the *p16* gene in 11 ACF, 5 dysplasia, and 4 normal epithelia specimens from 4 UC patients using methylation-specific PCR. No methylation of the *p16* gene promoter, which was represented by a 152 bp band, was detected in any of the 4 normal epithelia specimens. However, it was found in 8 of the 11 ACF (73%) and in 3 of the 5 dysplasia specimens (60%; Fig. 5A).

We then determined the expression of $p16^{INK4A}$ mRNA employing RT-PCR in eight ACF, two dysplasia, and four normal epithelia specimens from the other four UC patient groups. The reason we dealt with specimens from other UC groups (four patients) than the group (four patients) for methylation analyses, was that analyses of methylation and mRNA on the same small specimens was technically difficult. Nevertheless, the $p16^{INK4A}$ mRNA was readily detected in all four specimens of normal epithelia. Although it was detectable in only two of eight (25%), very faintly detectable in one of eight (12.5%), and undetectable in five of eight (63%) ACF specimens and was undetectable in two of two dysplasia specimens (Fig. 5B). These results suggested that $p16^{INK4A}$ expression is suppressed by the methylation of its promoter.

The number of colitis ACF in UC patients with or without dysplasia. If ACF are indeed precursor lesions of dysplasia, it would be expected that UC patients with dysplasia would have more ACF than those without dysplasia. Therefore, we investigated the number of ACF in UC patients with and without dysplasia and compared them. The number of ACF in the dysplasia-positive group (8.7 ± 4.5) was significantly higher than that in the dysplasia-negative group (3.5 ± 2.6; $P = 0.0112$). In particular, the number of ACF in the two patients with both dysplasia and cancer were 17 and 13, respectively, which represents very high numbers even for the dysplasia-positive group. All cases in the dysplasia-positive

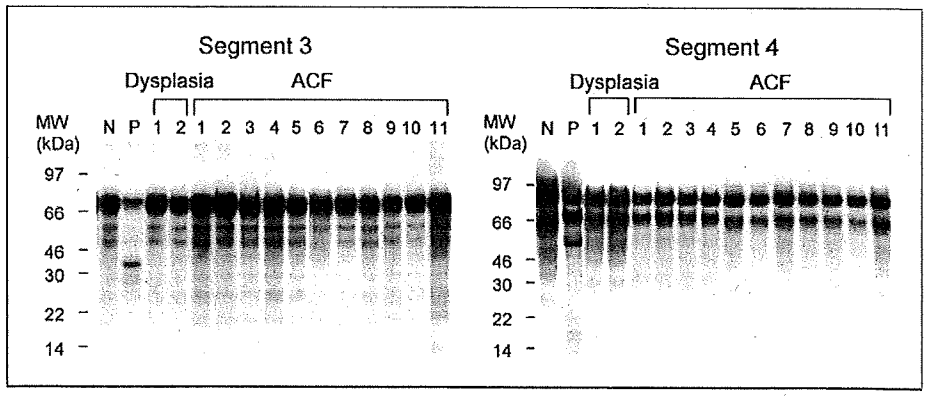

Fig. 3. Analysis for *APC* mutations in colitis ACF, dysplasia from UC patients, and non-UC ACF by *in vitro*–synthesized protein assay. Segments 3 and 4 of the *APC* gene, which include the whole mutation cluster region, were analyzed. A colonic adenoma was used as a positive control and normal colonic mucosa was used as a negative control. No *APC* mutations were detected in any of the 11 colitis ACF samples (0 of 11, 0%) or in any of the two dysplasia specimens (0 of 2, 0%).

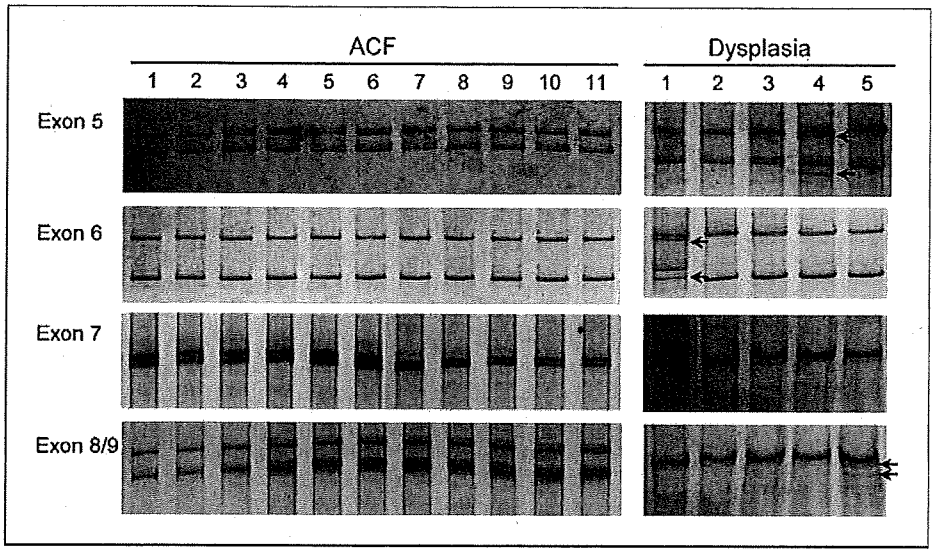

Fig. 4. Analysis for *p53* mutations in colitis ACF and dysplasia from UC patients by nonradioisotopic single-strand conformation polymorphism assay. The hotspot regions (exons 5-9) of p53 mutations were analyzed. No mutations were detected in any of the exons 5, 6, 7, or 8/9 in the 11 ACF specimens (0 of 11, 0%), whereas mutations were detected in exons 5, 6, or 8/9 of the dysplasia specimens (3 of 5, 60%).

group had five or more ACF. The mean number of ACF in age-matched healthy subjects (1.0 ± 1.7) was apparently smaller than that in either the dysplasia-positive or dysplasia-negative group of UC patients. The mean number of ACF in the four patients with Crohn's disease, an inflammatory bowel disease from which cancer develops at a low rate, was only 0.3 ± 0.6 (Fig. 6).

The number of colitis ACF in relation to potential risk factors for colitis cancer. We analyzed the relationship between the number of ACF and potential risk factors for colitis cancer such as gender, age at onset, the extent of lesions, duration of disease, and the existence of dysplasia. Univariate analyses showed significant correlations between ACF numbers and the existence of dysplasia (P = 0.0112) or duration of disease (P = 0.0306). There were no significant correlations between the number of ACF and gender, age, or extent of lesions (Table 1). Multiple logistic regression analysis of the relationship between dysplasia and various background factors showed significant correlations between the presence of dysplasia and the number of ACF (P = 0.0189) or disease duration (P = 0.0492; Table 2).

Discussion

In this study, we successfully and to our knowledge, for the first time, identified colitis ACF employing magnifying

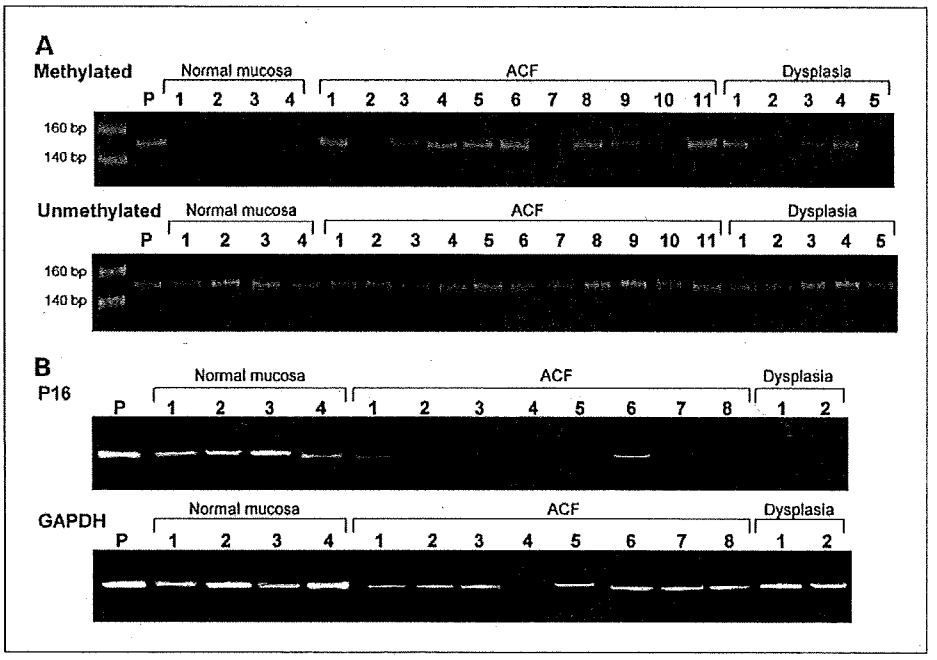

Fig. 5. *A*, analysis for *p16* methylation in colitis ACF and dysplasia specimens from UC patients by methylation-specific PCR. A colonic cancer specimen from a non-UC patient was used as a positive control. Methylation of the *p16* gene promoter, which was represented by a 152 bp band, was found in 8 of the 11 colitis ACF specimens (73%) and in 3 of the 5 dysplasia specimens (60%). *B*, expression of $p16^{INK4A}$ mRNA in colitis ACF and dysplasia specimens from UC patients analyzed by RT-PCR. A colonic cancer specimen from a non-UC patient was used as a positive control. $p16^{INK4A}$ mRNA was clearly detected in only 2 of 8 (25%), very faintly detected in 1 of 8 (12.5%), and was not detected in 5 of 8 (63%) ACF specimens. It was absent in two of the dysplasia specimens.

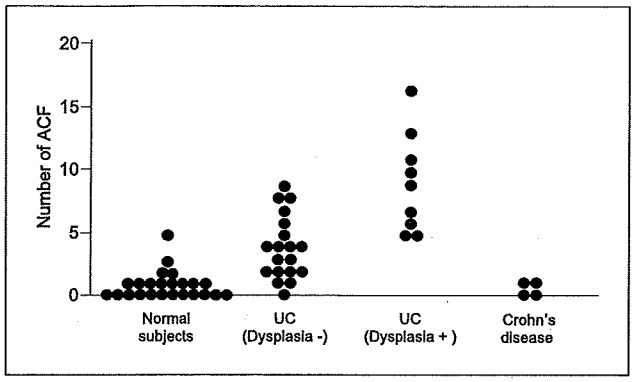

Fig. 6. The number of ACF in healthy volunteers and patients with UC or Crohn's disease. The number of ACF differed significantly ($P = 0.0112$) between the dysplasia-positive group (8.7 ± 4.5) and the dysplasia-negative group (3.5 ± 2.6). The mean number of ACF in the four cases of Crohn's disease was only 0.3 ± 0.6.

endoscopy, which were more darkly stained with methylene blue than normal crypts and had larger diameters with oval or slit-like lumens and thicker epithelial linings (7, 11). The appearance of colitis ACF was distinct from sporadic ACF. Endoscopically, the boundaries of individual crypts in colitis ACF were obscure in contrast to the clear lining of each crypt in sporadic ACF, and most of the colitis ACF showed distorted shapes in contrast to round or oval shapes of sporadic ACF. Histologically, much more lymphocyte infiltration was seen in colitis ACF than in sporadic ACF.

Differences were also evident with respect to genetic background. Sporadic ACF were frequently positive for *K-ras* mutation and p16 overexpression, as previously reported by us and others (11, 12, 28, 35), whereas colitis ACF were essentially negative for *K-ras* mutation and also negative for p16 expression due to hypermethylation of the gene. These results suggest that the etiology of ACF may be different in UC patients from that in sporadic ACF subjects. In this context, it is intriguing that in other types of inflammation-associated carcinogenesis, such as hepatitis C–associated hepatocellular carcinoma and chronic gastritis-associated gastric cancer, silencing of the *p16* gene (p16 hypermethylation) is frequently observed (36–38).

With regard to the relationship between colitis ACF and dysplasia, it is highly plausible that the former are precursor lesions of the latter because the gene abnormalities of both lesions were similar in terms of negativity for *APC* and *K-ras* and positivity for *p16* hypermethylation. Multivariate analysis showed a close correlation between the number of ACF and occurrence of dysplasia, which also strongly supported the precursor theory of ACF. The close correlation between lesions and the fact that ACF were readily detectable in higher numbers than dysplasia, which requires total chromoendoscopy spraying methylene blue on the entire colorectum for detection, suggests that ACF are a more appropriate surveillance marker than dysplasia for colitis-associated cancer.

Incidentally, the ACF found in UC patients were not all colitis ACF but were mixed with typical sporadic (non-UC) ACF, as far as endoscopic appearance was concerned. However, the incidence was very low (only 9.7%). This may be explained by the fact that the prevalence of sporadic ACF sharply increases after the age of 40 to 50 years (7), whereas the mean age of UC patients in this study was 38.3 ± 6.7 years. Nevertheless, because of the low incidence, sporadic ACF contamination with colitis ACF would not hamper the usefulness of colitis ACF as a surveillance marker.

A possible obstacle to using colitis ACF as a surveillance marker is that patients are obliged to undergo endoscopic examination, which itself may aggravate UC activity. However, this is unlikely to be a significant obstacle as long as endoscopy is done only when UC is in an inactive state and the survey of ACF is limited to the rectum, spending only 10 to 15 min on the whole procedure on the basis of our previous finding that the number of ACF in the rectum correlated with that in the entire colorectum (7). In this study, indeed, no particular aggravation of UC activity was observed in any of the patients with the evidential results of univariate and multivariate analyses supporting the validity of the use of rectal ACF in place of entire colorectal ACF as a surveillance marker.

In conclusion, in this study, we disclosed an ACF-dysplasia-cancer sequence in colitis-associated carcinogenesis similar to the ACF-adenoma-carcinoma sequence in sporadic colon carcinogenesis. We then proposed the feasibility of using ACF instead of dysplasia for the surveillance of colitis-associated cancer. Further evaluation of ACF as a surveillance marker in large-scale studies is warranted.

Table 1. Univariate association of ACF with potential risk factors for colorectal cancer in UC patients

Risk factor		Number of ACF-like lesions	P
Gender	Male	5.0 ± 3.9	0.3166
	Female	6.7 ± 4.9	
Age at onset (y)	<40	5.7 ± 4.7	0.8830
	*R*40	6.0 ± 4.3	
Extension	Total colon	7.0 ± 4.6	0.1179
	Left side colon	4.3 ± 3.8	
Duration (y)	<8	3.6 ± 2.2	0.0306
	*R*8	7.3 ± 4.9	
Dysplasia	Positive	8.7 ± 4.5	0.0112
	Negative	3.5 ± 2.6	

Acknowledgments

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Table 2. Multiple logistic regression analysis of risk factors associated with dysplasia in patients with UC

Risk factor	Odds ratio (95% confidence interval)	P
Gender	3.274 (0.709-25.195)	0.1645
Age	0.955 (0.801-1.085)	0.5138
Extension	1.424 (0.649-3.347)	0.1793
Duration	1.398 (0.785-1.603)	0.0492
ACF	1.533 (1.073-2.191)	0.0189

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Treatment of Hepatocellular Carcinoma by AdAFPep/Rep, AdAFPep/p53, and 5-Fluorouracil in Mice

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Although conditionally replicable adenovirus (CRAd) has been used in the clinical treatment of hepatocellular carcinoma (HCC), it suffers from the inherent drawback of having relatively low antitumor activity. Here, we have sought to overcome this drawback. First, we combined CRAd (AdAFPep/Rep) driven by -fetoprotein enhancer/promoter (AFPep) with a replication-incompetent adenovirus carrying a p53 transgene that is also driven by AFPep. The synergism of this combination produced a significantly improved tumoricidal effect on the human HCC cell line Hep3B, which has a relatively short doubling time in comparison with other human HCC cell lines, through the transactivation of p53 by early region 1A transcribed by AdAFPep/Rep. This synergistic interaction was augmented by the addition of a subtumoricidal dose (0.5 g/mL) of 5-fluorouracil (5-FU), which enhanced p53 expression and facilitated the release of virions from tumor cells. When relatively large (10-mm-diameter) Hep3B tumors grown in nude mice were injected with the two viruses in combination, they showed significantly impaired growth in comparison with those treated with each virus separately. The growth suppression effect of the virus combination was enhanced by a low dose (600 g) of 5-FU. Survival of the tumor-bearing mice treated with these three agents was significantly longer than that of control mice. Moreover, the tumor completely disappeared with the repeated injection of these agents. *Conclusion:* This combination strategy holds promise for the treatment of relatively large and rapidly growing HCCs that may be encountered clinically. (HEPATOLOGY 2008;48:828-840.)

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Despite tremendous efforts, the incidence of HCC-related mortality is still increasing, and except for a recent study in which sorafenib improved the survival of patients with advanced HCC, truly efficient treatments with respect to mortality have been scarcely reported.[1]

Various genetic defects have been found to be associated with HCC, and this has led to attempts to develop a gene therapy–based treatment for this cancer. The initial gene therapy approach for HCC was to use replication-incompetent viruses as vectors to carry therapeutic transgenes such as p53 and herpes simplex virus thymidine kinase.[2-5] However, because of difficulties in ensuring that the vector reaches all the cancer cells composing the tumor nodule and in achieving sufficient expression of the transgenes, the efficacy of this approach has proved to be limited.[6] An alternative approach utilizing conditionally replicable adenovirus (CRAd) was subsequently developed.[7] Habib et al.[8] treated patients with primary and secondary liver tumors with an intratumoral, intra-arterial, or intravenous administration of E1B55-kDa–deleted CRAd that was designed to replicate only in cells with nonfunctioning p53. They found that this treatment regime had some clinical efficacy with no severe side effects. However, this type of first-generation CRAd was subsequently found to replicate not only in tumor cells but also in normal diploid cells undergoing proliferation.[9-13] Several attempts, such as those using oncofetal

Abbreviations: 5-FU, 5-fluorouracil; Adp53, adenoviral p53; AFP, -fetoprotein; AFPep, -fetoprotein enhancer/promoter; BAI-1, brain-specific angiogenesis inhibitor 1; CI, combination index; CMVp, cytomegalovirus promoter; CRAd, conditionally replicable adenovirus; DAPI, 4 ,6-diamidino-2-phenylindole; E1A, early region 1A; FBS, fetal bovine serum; Hc, primary human hepatocytes; HCC, hepatocellular carcinoma; MOI, multiplicity of infection; P5, PLC/PRF/5; PBS, phosphate-buffered saline; RDAd, replication-defective adenovirus.

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