Original Article

Prebiotic treatment reduced preneoplastic lesions through the downregulation of toll like receptor 4 in a chemo-induced carcinogenic model

Masanobu Fukuda,[1,#] Yutaka Komiyama,[2,#] Keiichi Mitsuyama,[3] Akira Andoh,[4] Takahiko Aoyama,[1] Yoshiaki Matsumoto[1] and Osamu Kanauchi[1,2,4,*]

[1]College of Pharmacy, Department of Clinical Pharmacokinetics, Nihon University, 7-7-1, Narashinodai, Funabashi, Chiba 274-8555, Japan
[2]Central Laboratories for Frontier Technology, Kirin Holdings Co., Ltd. 1-13-5, Fukuura Kanzawa-ku, Yokohama, Kanagawa 236-0004, Japan
[3]Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan
[4]Division of Mucosal Immunology, Graduate School of Medicine, Shiga University of Medical Science, Tsukinowa, Seta, Otsu, Shiga 520-2192, Japan

(Received 14 September, 2010; Accepted 27 November, 2010; Published online 14 May, 2011)

Germinated barley foodstuff contains prebiotics which are reported to have anti-cancerous effects in colorectal cancer model, but the detailed mechanism remains unclear. Recent studies revealed that the role of microbiota was strongly related to the regulation of incidence and progression of colorectal cancer. The aim of this study was to examine the anti-neoplastic mechanism by prebiotics. Azoxymethane treated F344 rats were used as the sporadic cancerous model. After azoxymethane injection, either a control or germinated barley foodstuff diet was administered to the rats for another 5 weeks, and the number of abberant crypt foci, toll like receptor 4, Kirsten rat sarcoma viral oncogene homolog, adenomatous polyposis coli tumor suppressor gene and cyclooxygenase 2 mRNA expression of colonic mucosa and cecal short chain fatty acids were examined. The germinated barley food stuff significantly attenuated the number of abberant crypt focis and the expression of toll like receptor 4 and cyclooxygenase 2 mRNA, compared to the control group. In addition, the cecal butyrate production in the germinated barley foodstuff group was significantly higher than that in the control. In conclusion, this prebiotic treatment for colorectal cancer may be useful without causing the adverse effects seen in either anti-cancer drugs or anti-inflammatory drugs.

***Key Words*:** **toll like receptor 4, cyclooxygenase 2, butyrate, prebiotics, microbiota**

The colon is different from the other digestive organs, because it harbors an enormous number of coexisting microbiota.[1] Dietary fiber has been shown to prevent colorectal cancer (CRC) by diluting or adsorbing fecal carcinorgens (i.e. endogenous bile acid metabolite, food-derived carcinogen), thus reducing the colonic transit time, alternating the bile acids metabolism, increasing short chain fatty acids (SCFA) accompanying the change in microbiota,[2] although this is also a controversial issue.[3] Germinated barley foodstuff (GBF) is a heterogeneous mixture of insoluble protein and dietary fiber that functions as a prebiotic in the intestine, and is effective in patients with active ulcerative colitis.[4] GBF has a potent effect and it increases the number of microbiotic metabolites, including SCFA and butyrate, which is an anti-inflammatory reagent and can also induce apoptosis in cancer cells.[5,6] Because butyrate produced by microbiota following prebiotic treatment is stably maintained at a physiological concentration in the entire colon, this prebiotic treatment may therefore be a useful medical option for reducing the risk of CRC.[7]

Aberrant crypt foci (ACF) are lesions consisting of a large and thick crypt in methylene blue stained colonic mucosa, which showed a loss of polarity, and the stratification of the nuclei in the crypt epithelium histologically. ACF have been reported to be precursors of adenoma and cancer, because patients with colon cancer have a higher number of ACF than control subjects.[8] Azoxymethane (AOM) is commonly used to mimic human sporadic CRC in rodents, and pre-neoplastic lesions are detected as ACF.[9,10] However, the detailed mechanism for the anti-carcinogenic effects of GBF still remains poorly understood.

Recent studies have revealed that toll like receptor 4 (TLR4) or one of the sodium-coupled monocarboxylate transporters (SLC5A8 also known as SMCT-1) was strongly related to the regulation of incidence and progression of CRC.[11,12] Both parameters were related to the microbiota (TLR4) or its metabolites (SLC5A8), respectively. TLR4 has been identified as one of the regulators of the mucosal immune system to lipopolysaccharide (LPS), the pathogenic product of gram negative microbiota in the colon, and TLR4 were recently reported to show an increased expression in colon cancers, compared to the normal mucosa.[13] Interestingly, the microbiota metabolite butyrate resulted in the downregulated TLR4 expression and IL-8 production in HT29 cells,[14] and also stimulated the expression of SLC5A8, which induced anti-carcinogenic effects in the colon.[12]

In the present study, we evaluated the anti-cancerous mechanism of prebiotic GBF on the sporadic AOM-induced CRC model, particularly in the modulation of the microbiota and the changes in its metabolites, to examine the role of TLR4, a key regulator of the mucosal barrier functions.

Materials and Methods

Chemical composition and physiological characteristics of GBF. GBF consists of the aleurone and scutellum fractions of germinated barley, and is made by milling and sieving the residue of brewers' spent grain. The dietary fiber fraction of GBF is primarily composed of low-lignified hemicellulose, which accumulates in the scutellum and aleurone fractions, as the roots and shoots of barley are produced during germination. During germination, GBF exhibits a conspicuously higher water-holding capacity than other insoluble dietary fiber types.[15] As described in our previous study, GBF contains approximately 46% protein and 30% dietary fiber by weight.[16]

*To whom correspondence should be addressed.
E-mail: kanauchio@kirin.co.jp
#Contributed equally to this work.

doi: 10.3164/jcbn.10-114

Table 1. Composition of the experimental diets

	Control	GBF*
	(g/kg diet)	
Casein	146	100
Vitamin Mix**	10	10
Mineral Mix**	35	35
Choline chloride	2	2
Cellulose	30	0
GBF	0	100
Corn oil	50	50
Corn starch	727	703

*Germinated Barley Foodstuff (GBF) (Protein; 46.3%, Dietary fiber; 34.0%).
** According to AIN 93G formula.

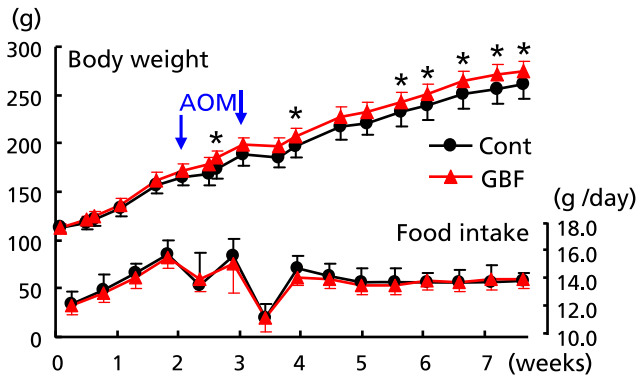

Fig. 1. Changes in body weight and food intake in rats fed either the control (Cont) or the germinated barley foodstuff (GBF) diet. The arrows show the time of the injection of azoxymethane (AOM) subcutaneously. Data are expressed as the mean changes ± SD. *Represents significant differences between the Cont and GBF groups (p<0.05).

Animals and treatments. All animal experiments were approved by the Kirin Holdings animal experiments ethical committee and the Nihon University animal experiments ethical committee. Rats were housed individually in cages in a room kept at 20 to 25°C with 40 to 70% relative humidity on a 12-h lighting cycle (conventional conditions). The animals had free access to both food and drinking water.

Sporadic colon cancer model induced by AOM. Twenty 5-week-old male Fischer 344 rats were purchased from Charles River Japan (Kanagawa, Japan). First, the 20 rats were fed laboratory chow for 1 week during the acclimatization period. We started the experiments, and the rats were divided into two groups (n = 10/group). One group received a control diet, and the other group received the GBF diet. The total volume of protein and dietary fiber in both diets was adjusted to 14.6% protein and 3.0% dietary fiber, respectively (Table 1). After two weeks of pre-feeding using the respective experimental diets, the rats were given two subcutaneous injections of AOM at a dose of 15 mg/kg body weight once per week for two continuous weeks.[17] After the second AOM injection, the rats were administered the respective diets for more 5 weeks *ad libitum*, and were euthanized thereafter. Changes in the body weight and food intake are shown in Fig. 1. On the final day, rats were anesthetized with pentobarbital (i.p.), and the serum, colon and cecal contents were collected.

Histological examination. The entire colon was obtained for the histological observation of ACF. The rectum segment approximately 5 cm from the pectinate line was fixed in 4% buffered paraformaldehyde (Wako Pure Chemicals Ind., Osaka, Japan) and stained with 0.2% methylene blue. In accordance with the methods carried out in a previous report, the ACF were scored under a light microscope (Carl Zeiss Axioskop 40 and AxioVision, Japan) with an area of 1 cm^2 of colon being quantified at 100× magnification.[17] ACF were distinguished from normal crypts by their increased size, more prominent epithelial cells, and increased pericryptal area, and representative ACF images are shown in Fig. 2.

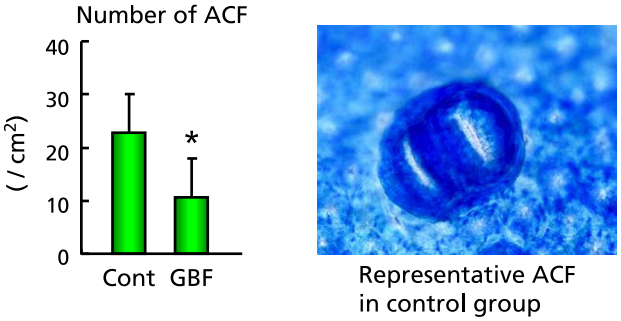

Fig. 2. The number of aberrant crypt foci (ACF) were counted under a light microscope (Carl Zeiss Axioskop40 and AxioVision, Japan) per 1 cm^2 of the distal colon at 100× magnification. The data are expressed as the mean changes ± SD. ACF in the germinated barley foodstuff (GBF) group were significantly lower than in the control (Cont) group. *p<0.05 for the differences between the Cont and the GBF. Representative ACF in the colon are shown (100×). Aberrant crypts appeared larger and had a thicker epithelial lining compared to normal crypts, and were gathered into a focus consisting of several crypts. *Represents significant different between Cont and GBF groups (p<0.05).

Reverse transcription-polymerase chain reaction (RT-PCR) to examine the mucosal expression of mRNA. Residual colonic mucosal specimens were evaluated for the mucosal expression of TLR4, Kirsten rat sarcoma viral oncogene homolog (KRAS),[18] adenomatous polyposis coli tumor suppressor gene (APC) and cyclooxygenase 2 (COX2) mRNA using reverse transcription-polymerase chain reaction (RT-PCR).[19,20] according to the methods used in our previous study.[21] Briefly, total mucosal RNA was isolated using the Trizol reagent (Invitrogen Japan, Tokyo). First-strand cDNA was synthesized using an oligo (dT) primer and Superscript II reverse transcriptase (GIBCO BRL, Rockville, MD) and PCR was performed thereafter (10 X Taq Buffer and Taq Gold polymerase, thermal cycler; GeneAmp 2400; Perkin Elmer Cetus Co., Norwalk, CT). The PCR products were subjected to electrophoresis on 1.5% agarose gels and were stained with ethidium bromide. Primers specific for TLR4, APC, KRAS, COX2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as housekeeping gene) were constructed according to the open software program for designing PCR primers (http://frodo.wi.mit.edu/primer3/): (Shown in Table 2).

Organic acids analysis of cecal content. An organic acid analysis of the cecal contents in rats fed the respective diets was performed. Briefly, 0.2 g of the cecal contents were weighed, then 1.0 ml of Milli-Q water was added and the mixture was incubated at 4°C for 30 min. After centrifugation at 12,000 rpm at 4°C, for 10 min, the supernatant was obtained, and continuously filtered using a 0.22 μm filter. The organic acids were separated with the Shim-pack SPR-H 250L (Shimadzu Co. Ltd., Kyoto, Japan). The mobile phase was 4 mM of p-toluene sulfonic acid, and the detector was electric conductivity (Shimadzu CDD-6A, Kyoto, Japan).[16]

Statistical analysis. All values are presented as the mean ± SD. Comparisons of data were made using Student's t test or the χ^2 test. Differences were considered to be statistically significant for p values of less than 0.05.

doi: 10.3164/jcbn.10-114

Table 2. Primer sequences used to analyze the host response by RT-PCR

Target	Direction	Sequence
TLR4	Fw	AGCCATTGCTGCCAACATCATCCAG
	Rv	TGCTGCCTCAGCAAGGACTTCTCCA
APC	Fw	TGCGGAATTTGTCTTGGCGAGCAG
	Rv	AAATGCCAGTGCCCCGTCCACA
KRAS	Fw	GGAGCTGGTGGCGTAGGCAAGA
	Rv	CAAAGAAAGCCCTCCCCAGTTCTCA
COX2	Fw	ATCCCGCCCTGCTGGTGGAAAA
	Rv	GGCTGCGGGTCTTGCACATTGAA
GAPDH (as housekeeping gene)	Fw	TGGCATGGCCTTCCGTGTTCCT
	Rv	TGCCAGCCCCAGCATCAAAGGT

Primer was constructed according to the open software program for designing PCR primers (http://frodo.wi.mit.edu/primer3/).

Table 3. Cecal organic acid contents

	Control	GBF*
	(mM/g content)	
Succinic acid	5.23 + 1.27	2.23 + 0.73
Acetic acid	45.05 + 2.92	43.41 + 2.45
Propionic acid	9.32 + 0.49	8.47 + 0.48
iso-Butyric acid	3.32 + 0.17	2.99 + 0.10
Butyric acid	7.77 + 1.04	15.86 + 1.14**

* GBF means germinated barley foodstuff.
** represents significant difference between groups ($p<0.05$).

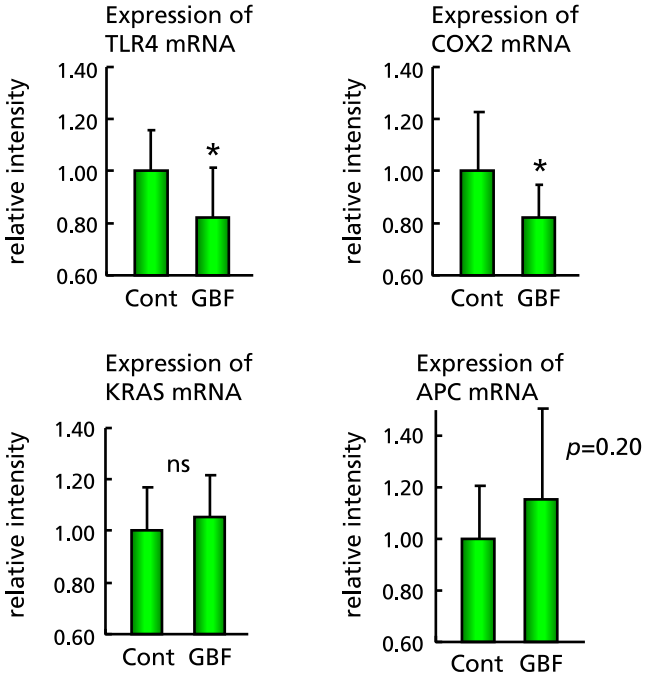

Fig. 3. The expression levels of toll like receptor 4 (TLR4), cyclooxygenase 2 (COX2), Kirsten rat sarcoma viral oncogene homolog (KRAS) and adenomatous polyposis coli tumor suppressor (APC) genes in the colonic mucosa were normalized to the expression levels of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase. The levels of TLR4 and COX2 mRNA in the germinated barley foodstuff (GBF) group were significantly lower than in the control (Cont) group. The data are expressed as the mean changes ± SD. *$p<0.05$ for differences between the Cont and the GBF groups.

Results

Fig. 1 shows the changes in the body weight, food intake and the schedule of AOM administrations. The control group had significant lower body weights than the GBF group, although there were no significant differences in the food intake between the groups. The detailed reason for this observation is still unknown; GBF may have the ability to attenuate the damage induced by carcinogenic AOM. The number of ACF in 1 cm^2 section of the colon is shown in Fig. 2. After GBF treatment, the number of ACF was significantly lower than in the control group. The data clearly showed that GBF prevented the onset of neoplastic changes in the early stages of carcinogenic sequence.[22] In addition, the macroscopic observations revealed that adenomatous polyposis, inflammatory signs, including ulcer and erosion, and squamous metaplasia were not observed in both groups.

Table 3 indicates the changes in the cecal organic acid content of both groups. The GBF treatment group had a significant increase in the production of butyrate compared to the control group. Although our previous study demonstrated that GBF significantly decreased succinate production, this trend did not reach statistical significance between the groups ($p = 0.057$). These data suggested that the composition of microbiota, especially anaerobic microbiota, were reorganized by prebiotic treatment, accompanied by the changes in metabolite SCFAs. Fig. 3 shows the changes in the colonic mucosal mRNA expression of TLR4, APC, KRAS and COX2. Following GBF treatment, the mucosal TLR4 and COX2 mRNA was significantly decreased compared to the control group. TLR4 was reported to play a pivotal role in the development of colitis-associated neoplasia, and the inhibition of TLR4 signaling may prevent dysplasia.[13] In addition, the increase of COX2 mRNA in colorectal adenocarcinomas was observed, compared with normal colonic mucosa in the same patients.[23]

The expression of KRAS mRNA was not significantly different between the groups. The APC gene is considered to be a gatekeeper gene which maintains the stability of the colonic epithelium. It was also reported that carcinogenic treatment in rats resulted in decreased APC expression.[22] APC mutations were present at a relatively low level, and APC mutations did not contribute to the initiation of ACF.[24] Although not statistically significant, the expression of APC mRNA in the GBF group tended to be higher than in the control group.

Discussion

It has previously been reported that sporadic colorectal carcinoma has an adenoma-carcinoma sequence, in which genomic instability and the loss of key tumor suppressor genes such as APC result in the loss of p53 gene function, thereby driving the adenoma to a carcinoma.[25] ACF have been detected in the colonic mucosa in a rodent model as well as in human colon cancers, and ACF, particularly large and dysplastic appearance foci, were reported to be useful and identifiable precursors of adenoma and carcinoma.[8] The number of ACF was significantly inhibited in the GBF group compared to the control group. Prebiotic GBF had anti-cancer effects in the present rodent model. In addition, patients with colon cancer were found to have a greater number of ACF than patients with noncancerous lesions, which was accompanied by a high rate of KRAS mutations.[8,26] However, we found that the change in KRAS expression, instead of KRAS mutations, was less important in the present study, and we were unable to detect any differences in KRAS expression between the two groups.

The APC gene is considered to be one of the key component of the β-catenin signaling pathway, which plays an important role

in colon carcinogenesis. APC mutations are reported to be responsible for the accumulation of β-catenin in the nucleus, and the constitutive activating transcription of T-cell factor or lymphoid enhancer factor. Therefore, APC mutations activated the oncogene β-catenin signaling pathway.[27,28] However, APC mutations have been reported to be present at relatively low levels, and the APC mutations did not contribute to the development of ACF.[24] GBF may therefore reinforce the role of the gatekeeper genes in the present experiment, because carcinogen-treated rats have been reported to cause a decrease in the APC mRNA expression.[22] In the present study, the expression of APC gene was slightly higher in the GBF group than in the control group, although this trend did not reach statistical significance ($p = 0.20$). In the future, we will evaluate the detailed mutation profiles of the APC and KRAS genes in this colon cancer model to confirm the anti-carcinogenic mechanism of GBF treatment.

Interestingly, the level of COX2 mRNA of colonic mucosa in patients with colorectal cancer has been reported to be markedly elevated, compared with that of healthy controls[23,29] and thus reducing the COX2 expression by green tea may contribute to the attenuation of colonic neoplastic lesions in rats.[10] In our experiment, the colonic mucosal mRNA expression of COX2 was significantly lower in the prebiotic GBF group than in the control.

The present study suggested the modulation of microbiota by the prebiotic GBF, which was comprised of an increase in butyrate in the lower intestinal tract. Butyrate has been reported to have anti-neoplastic effects under physiological concentrations (2–5 mM) due to cell cycle regulation (increased apoptosis in colonic tumor cell lines).[30] Interestingly, butyrate, a metabolite of microbiota induced by anaerobic fermentation in the lower intestine, has been shown to suppress TLR4 gene expression.[31] The ligand of TLR4 is well-known to be LPS, and LPS induces the degradation of IκBα, which results in the transmigration of nuclear factor kappa B (NF-κB) to the nucleus, where it binds to DNA and induces the production of pro-inflammatory cytokines.[31] Although the detailed mechanism remains unclear, LPS stimulated the induction of IL-8 in the HT-29 cell line, and butyrate has been reported to attenuate LPS-induced IL-8 production, and this anti-inflammatory action is considered to cause a downregulation of the TLR4 expression.[14] In addition, TLR4 was upregulated in ulcerative colitis patients, and TLR4 was reported to detect the gram negative microbiota and to activate the innate immune system.[32] Furthermore, a recent study revealed that TLR4 had the ability to regulate the COX2 expression in chronic colon cancer model via NF-κB expression.[32,33] In the present study, GBF decreased the TLR4 expression through an increase in butyrate production and decrease of COX2 expression, compared with that of the control group. In our previous study, butyrate and short chain fatty acids also dramatically attenuated the proinflammatory cytokine IL-6 production as well as the TLR4 mRNA expression in the T84 cell line.[34]

Increased butyrate in the present study may therefore have contributed to the increased tumor suppressor action, as described by other reviews.[35] Coleman *et al.*[36] reported the inverse correlation between butyrate concentration and the number of ACF to suggest a pivotal mechanism of protection against neoplasia. These actions have been reported and include the upregulation of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1; increase of anoikis), the death receptor DR5 (increase of sensitivity to TNF-related apoptosis-inducing ligand) and Fas-mediated cytotoxicity, and the downregulation of COX2 and glutathione (increased resistance to oxidative stress). Anti-inflammatory agents, including NSAIDs, are also known to have anti-tumorigenic effects due to the inhibition of NFκB activation.[37] In particular, butyrate has been reported to have anti-inflammatory effects and it has thus been used for the treatment of patients with chronic inflammatory bowel disease, including ulcerative colitis, as an enema solution.[38] GBF has also been reported to have an attenuated NFκB activity in a colitis model,[16] and such anti-inflammatory effects may therefore play a pivotal role in the progression of colon cancer. Prebiotics, and GBF in particular, may deliver the natural anti-inflammatory agent butyrate to the entire colon at physiological concentrations without any adverse effects. These may play an important role in the inhibition of CRC to control mild or undetectable inflammation, as well as to monitor the invasion of pathogenic microbiota by TLR signals in the colon.

Considering the above factors, the appropriate improvement of the microbiota in the AOM-induced sporadic colon cancer model by prebiotics may contribute to the attenuation of low-grade inflammation, and the reduced incidence of CRC. In conclusion, GBF demonstrated a preventive effect on tumorigenicity in the AOM-induced CRC model. Although more detailed GBF studies concerning the mechanism of the onset of CRC, particularly regarding the changes in the microbiota and its metabolites on mucosal barrier function, are still required, the nutraceutical preventative treatments of GBF for CRC may be useful without causing any of the adverse effects seen in treatments with either anti-cancer drugs or anti-inflammatory drugs.

Acknowledgments

We are most grateful to Fumi Yoshinaka, Megumi Andoh, Kyoko Tazumi (Kirin Holdings Co., Ltd.), and Kimiko Oikawa, Masanori Kihara, Naoko Sato, Kaori Ito (Nihon University) for their excellent technical assistance.

Abbreviations

ACF	aberrant crypt foci
AOM	azoxymethane
APC	adenomatous polyposis coli tumor suppressor gene
COX2	cyclooxygenase 2
CRC	colorectal cancer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBF	germinated barley foodstuff
KRAS	kirsten rat sarcoma viral oncogene homolog
LPS	lipopolysaccharide
RT-PCR	reverse transcription-polymerase chain reaction
SCFA	short chain fatty acids
SLC5A8 or SMCT-1	sodium-coupled monocarboxylate transporters
TLR4	toll like receptor 4

References

1 Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 2004; **126**: 1620–1633.

2 Fuchs CS, Giovannucci EL, Colditz GA, and *et al*. Dietary fiber and the risk of colorectal cancer and adenoma in women. *N Engl J Med* 1999; **340**: 169–176.

3 Alberts DS, Martinez ME, Roe DJ, and *et al*. Lack of effect of a high-fiber cereal supplement on the recurrence of colorectal adenomas. Phoenix Colon Cancer Prevention Physicians' Network. *N Engl J Med* 2000; **342**: 1156–1162.

4 Mitsuyama K, Saiki T, Kanauchi O, and *et al*. Treatment of ulcerative colitis with germinated barley foodstuff feeding: a pilot study. *Aliment Pharmacol Ther* 1998; **12**: 1225–1230.

5 Comalada M, Bailón E, de Haro O, and *et al*. The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype. *J Cancer Res Clin Oncol* 2006; **132**: 487–497.

6 Hinnebusch BF, Meng S, Wu JT, Archer SY, Hodin RA. The effects of short-

doi: 10.3164/jcbn.10-114

chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J Nutr* 2002; **132**: 1012–1017.
7 D'Argenio G, Cosenza V, Delle Cave M, and *et al*. Butyrate enemas in experimental colitis and protection against large bowel cancer in a rat model. *Gastroenterology* 1996; **110**: 1727–1734.
8 Takayama T, Katsuki S, Takahashi Y, and *et al*. Aberrant crypt foci of the colon as precursors of adenoma and cancer. *N Engl J Med* 1998; **339**: 1277–1284.
9 Caderni G, Dolara P, Fazi M, and *et al*. Cell cycle variations in azoxymethane-induced rat colorectal carcinogenesis studied by flow cytometry. *Oncol Rep* 1999; **6**: 1417–1420.
10 Chen J, Huang XF. The signal pathways in azoxymethane-induced colon cancer and preventive implications. *Cancer Biol Ther* 2009; **8**: 1313–1317.
11 Fukata M, Chen A, Vamadevan AS, and *et al*. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. *Gastroenterology* 2007; **133**: 1869–1881.
12 Paroder V, Spencer SR, Paroder M, and *et al*. Na(+)/monocarboxylate transport (SMCT) protein expression correlates with survival in colon cancer: molecular characterization of SMCT. *Proc Natl Acad Sci USA* 2006; **103**: 7270–7275.
13 Fukata M, Hernandez Y, Conduah D, and *et al*. Innate immune signaling by Toll-like receptor-4 (TLR4) shapes the inflammatory microenvironment in colitis-associated tumors. *Inflamm Bowel Dis* 2009; **15**: 997–1006.
14 Lee SK, Il Kim T, Kim YK, and *et al*. Cellular differentiation-induced attenuation of LPS response in HT-29 cells is related to the down-regulation of TLR4 expression. *Biochem Biophys Res Commun* 2005; **337**: 457–463.
15 Bamba T, Kanauchi O, Andoh A, Fujiyama Y. A new prebiotic from germinated barley for nutraceutical treatment of ulcerative colitis. *J Gastroenterol Hepatol* 2002; **17**: 818–824.
16 Kanauchi O, Serizawa I, Araki Y, and *et al*. Germinated barley foodstuff, a prebiotic product, ameliorates inflammation of colitis through modulation of the enteric environment. *J Gastroenterol* 2003; **38**: 134–141.
17 Nozawa H, Yoshida A, Tajima O, and *et al*. Intake of beer inhibits azoxymethane-induced colonic carcinogenesis in male Fischer 344 rats. *Int J Cancer* 2004; **108**: 404–411.
18 Weijenberg MP, Lüchtenborg M, de Goeij AF, and *et al*. Dietary fat and risk of colon and rectal cancer with aberrant MLH1 expression, APC or KRAS genes. *Cancer Causes Control* 2007; **18**: 865–879.
19 Kishimoto Y, Yashima K, Morisawa T, and *et al*. Effects of long-term administration of sulindac on APC mRNA and apoptosis in colons of rats treated with azoxymethane. *J Cancer Res Clin Oncol* 2002; **128**: 589–595.
20 Kishimoto Y, Yashima K, Morisawa T, Shiota G, Kawasaki H, Hasegawa J. Effects of cyclooxygenase-2 inhibitor NS-398 on APC and c-myc expression in rat colon carcinogenesis induced by azoxymethane. *J Gastroenterol* 2002; **37**: 186–193.
21 Kanauchi O, Oshima T, Andoh A, Shioya M, Mitsuyama K. Germinated barley foodstuff ameliorates inflammation in mice with colitis through modulation of mucosal immune system. *Scand J Gastroenterol* 2008; **43**: 1346–1352.
22 Cheng L, Lai MD. Aberrant crypt foci as microscopic precursors of colorectal cancer. *World J Gastroenterol* 2003; **9**: 2642–2649.
23 Eberhart CE, Dubois RN. Eicosanoids and the gastrointestinal tract. *Gastroenterology* 1995; **109**: 285–301.
24 Furukawa F, Nishikawa A, Kitahori Y, Tanakamaru Z, Hirose M. Spontaneous development of aberrant crypt foci in F344 rats. *J Exp Clin Cancer Res* 2002; **21**: 197–201.
25 Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G7–G17.
26 Pretlow TP, Barrow BJ, Ashton WS, and *et al*. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. *Cancer Res* 1991; **51**: 1564–1567.
27 Yamada Y, Mori H. Pre-cancerous lesions for colorectal cancers in rodents: a new concept. *Carcinogenesis* 2003; **24**: 1015–1019.
28 Femia AP, Dolara P, Giannini A, Salvadori M, Biggeri A, Caderni G. Frequent mutation of Apc gene in rat colon tumors and mucin-depleted foci, preneoplastic lesions in experimental colon carcinogenesis. *Cancer Res* 2007; **67**: 445–449.
29 Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; **107**: 1183–1188.
30 Russo GL, Della Pietra V, Mercurio C, and *et al*. Protective effects of butyric acid in colon cancer. *Adv Exp Med Biol* 1999; **472**: 131–147.
31 Bocker U, Yezerskyy O, Feick P, and *et al*. Responsiveness of intestinal epithelial cell lines to lipopolysaccharide is correlated with Toll-like receptor 4 but not Toll-like receptor 2 or CD14 expression. *Int J Colorectal Dis* 2003; **18**: 25–32.
32 Fukata M, Michelsen KS, Eri R, and *et al*. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G1055–G1065.
33 Hernandez Y, Sotolongo J, Breglio K, and *et al*. The role of prostaglandin E2 (PGE 2) in toll-like receptor 4 (TLR4)-mediated colitis-associated neoplasia. *BMC Gastroenterol* 2010; **10**: 82.
34 Kanauchi O, Fukuda M, Matsumoto Y, and *et al*. Eubacterium limosum ameliorates experimental colitis and metabolite of microbe attenuates colonic inflammatory action with increase of mucosal integrity. *World J Gastroenterol* 2006; **12**: 1071–1077.
35 Gupta N, Martin PM, Prasad PD, Ganapathy V. SLC5A8 (SMCT1)-mediated transport of butyrate forms the basis for the tumor suppressive function of the transporter. *Life Sci* 2006; **78**: 2419–2425.
36 Coleman LJ, Landstrom EK, Royle PJ, Bird AR, McIntosh GH. A diet containing alpha-cellulose and fish oil reduces aberrant crypt foci formation and modulates other possible markers for colon cancer risk in azoxymethane-treated rats. *J Nutr* 2002; **132**: 2312–2318.
37 Carothers AM, Davids JS, Damas BC, Bertagnolli MM. Persistent cyclooxygenase-2 inhibition downregulates NF-{kappa}B, resulting in chronic intestinal inflammation in the min/+ mouse model of colon tumorigenesis. *Cancer Res* **70**: 4433–4442.
38 Venkatraman A, Ramakrishna BS, Shaji RV, Kumar NS, Pulimood A, Patra S. Amelioration of dextran sulfate colitis by butyrate: role of heat shock protein 70 and NF-kappaB. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G177–G184.