

## Material and methods

### Animals, chemicals and diets

Four-week-old male *db/db* mice, *db/+* mice and *+/+* mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, and were housed in polycarbonate cages (4–5 mice/cage) with free access to drinking water and a basal diet, MF (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of relative humidity [(50 ± 10)%], lighting (12-h light/dark cycle) and temperature [(23 ± 2)°C]. AOM was purchased from the Sigma Chemical Co. (St. Louis, MO). Powdered CUSM was obtained from the Ehime Beverage Inc. (Matsuyama, Japan). The composition of CUSM (100 g) was as follows: 2.4 g moisture; 5.5 g protein; 0.3 g fat; 51 g fiber (22.0 g soluble and 29.0 g insoluble); 2.3 g ash; 26.6 g saccharide (6.1 g D-flucutose, 5.5 g glucose and 15.0 g D-sucrose); 2.2 g hesperidin and 9.7 g others that include flavonoids, carotenoids and unknown components. The experimental diets were prepared by mixing CUSM into the basal diet at a dose of 0.02%, 0.1% or 0.5% on a weekly basis.

### Experimental procedures

Male homozygous *db/db* mice (36 mice), heterozygous *db/+* mice (40 mice) and littermate controls (*+/+*) mice (40 mice) were divided into 4 groups, respectively. At 5 weeks of age, all mice were subcutaneously injected with AOM (15 mg/kg body weight) once a week for 5 weeks. Group 1 was fed the basal diet throughout the experiment. Groups 2 through 4 were fed the diets containing CUSM at dose levels of 0.02%, 0.1% and 0.5%, respectively, for 7 weeks, starting one week after the last injection of AOM. The experiment was terminated 12 weeks after the start.

All mice were provided with the experimental diets and tap water *ad libitum*, and were weighed weekly. The food intake of the animals was monitored every day. At the termination of the study (Week 12), all mice were sacrificed by an overdose of ether to analyze the number of ACF and BCACs. At autopsy, all organs, including the intestine, were carefully examined grossly, and then were examined histopathologically. The weighed liver and kidney were also submitted for histological examinations to investigate the toxicity of CUSM.

### Identification of ACF and BCACs

The presence of ACF and BCACs was determined according to the standard procedures that are routinely used in our laboratory.<sup>30,31</sup> At necropsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis and then washed with saline. They were cut, placed on the filter paper their mucosal surface up and then fixed in 10% buffered formalin for at least 24 hr. The fixed colons were stained with methylene blue (0.5% in distilled water) for 20 sec, dipped in distilled water and placed on a microscopic slide to count the ACF. After counting the ACF, the distal parts (1 cm from the anus) of the colon were cut in order to count the number of BCACs. To identify BCAC intramucosal lesions, the colon (0.58–0.87 cm<sup>2</sup>/colon) was embedded in paraffin, and then a total of 20 serial sections (4- $\mu$ m thick each) per mouse were made by an *en face* preparation.<sup>32,33</sup> For each case, 2 serial sections were used to analyze the BCACs.

### Histopathology and immunohistochemistry

Five serial sections were made from paraffin-embedded blocks. Two sections were subjected to hematoxylin and eosin (H & E) staining for histopathology and  $\beta$ -catenin immunohistochemistry to count the number of colonic BCACs,<sup>32,33</sup> and others were used for Ob-R, IGF-1R and PCNA immunohistochemistry. Immunohistochemistry for  $\beta$ -catenin was performed on 4- $\mu$ m-thick paraffin-embedded sections from the distal segments of the colons, using the labeled streptavidin-biotin method (LSAB KIT; DAKO, Glostrup, Denmark) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized

in xylene and rehydrated through graded alcohols at room temperature. A 0.05 M Tris-HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4°C with a primary antibody against  $\beta$ -catenin protein (diluted 1:1,000, Transduction Laboratories, Lexington, KY). Horseradish peroxidase activity was visualized by treatment with H<sub>2</sub>O<sub>2</sub> and diaminobenzidine for 5 min. Negative control sections were immunostained without the primary antibody. Immunoreactivity was regarded as positive if apparent staining was detected in the cytoplasm and/or nuclei to determine the BCACs.

Immunohistochemistry of Ob-R and IGF-1R was performed using a stain system kit (Zymed, South San Francisco, CA). Rabbit polyclonal antibodies against Ob-R (1:200 dilution, sc-8325, Santa Cruz Biotechnology, Santa Cruz, CA) and IGF-1R $\alpha$  (1:150 dilution, sc-7952, Santa Cruz Biotechnology) were applied overnight to the sections at 4°C according to the manufacturer's protocols. Human CRC samples were used as positive controls. The immunoreactivity cells were considered to be positive when definite cytoplasmic staining was identified. PCNA immunohistochemistry was performed on 4- $\mu$ m-thick paraffin-embedded sections from colons of the *db/db* mice group by the labeled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene and rehydrated through graded ethanol at room temperature. A 0.05 M Tris HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Incubations were performed in a humidified chamber. Cells with intensively stained nuclei were considered to be positive for PCNA, and the indices (%) were calculated in each BCAC. Calculation was done in 20 BCACs from Group 9, 15 BCACs from Group 10, 10 BCACs from Group 11 and 8 BCACs from Group 12.

### Morphometric analysis

Two serial sections from the liver of all mice were made for a morphometric analysis of liver fibrosis and fatty change. Liver sections were stained with H & E for histopathology and Sirius-red for morphometry of fibrosis. Fatty metamorphosis (% of fatty degeneration) was determined on the H & E-stained liver section, and liver fibrosis was expressed as the % of fibrosis in the area of liver section. An image analysis software, NIH Image v.1.63, was used for these calculates.

### Clinical chemistry

At sacrifice, blood to measure the serum concentrations of glucose, leptin, insulin, cholesterol and triglyceride levels was collected from 5 mice, each of genotypes *+/+*, *db/+* and *db/db*. They were starved overnight prior to blood collection for clinical chemistry. The serum glucose level was measured enzymatically using the hexokinase method. The serum triglycerides were assayed by enzymatic hydrolysis with lipase. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. Serum concentrations of leptin and insulin were measured by an enzyme immunoassay according to the manufacturer's protocol (R & D systems, Minneapolis, MN).

### Statistical evaluation

Where applicable, the data were analyzed using one-way ANOVA with Bonferroni correction or Fisher's exact probability test, with  $p < 0.05$  as the criterion considered to indicate significance.

## Results

### General observations

The carefully monitored food intake of the animals showed that the mean daily intakes of *db/db* mice (7.26 g in the AOM alone group; 7.10 g in the AOM + 0.02% CUSM group; 7.22 g in the

TABLE I—BODY, LIVER, RELATIVE LIVER, EPIDIDYMAL FAT AND PANCREAS WEIGHTS IN EACH GROUP OF MALE MICE (+/+, *db/+* AND *db/db*) THAT RECEIVED AOM AND CUSM

Group no.	Treatment	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body wt.)	Epididymal fat weight (g)
1 (+/+)	AOM alone (9)	25.3 ± 1.1	1.23 ± 0.14	4.79 ± 0.42	0.39 ± 0.05
2 (+/+)	AOM + 0.02% CUSM (9)	23.8 ± 2.7	1.02 ± 0.20	4.25 ± 0.51	0.39 ± 0.15
3 (+/+)	AOM + 0.1% CUSM (9)	24.6 ± 1.3	1.15 ± 0.10	4.68 ± 0.29	0.37 ± 0.07
4 (+/+)	AOM + 0.5% CUSM (9)	24.7 ± 1.4	1.16 ± 0.06	4.70 ± 0.25	0.40 ± 0.11
5 ( <i>db/+</i> )	AOM alone (10)	29.0 ± 1.5	1.29 ± 0.10	4.45 ± 0.26	0.65 ± 0.15
6 ( <i>db/+</i> )	AOM + 0.02% CUSM (10)	29.6 ± 1.5	1.26 ± 0.18	4.24 ± 0.58	0.75 ± 0.12
7 ( <i>db/+</i> )	AOM + 0.1% CUSM (10)	29.2 ± 1.2	1.25 ± 0.10	4.27 ± 0.28	0.74 ± 0.14
8 ( <i>db/+</i> )	AOM + 0.5% CUSM (10)	28.9 ± 1.9	1.29 ± 0.11	4.47 ± 0.32	0.61 ± 0.14
9 ( <i>db/db</i> )	AOM alone (10)	47.3 ± 5.0 <sup>1,2</sup>	2.92 ± 0.53 <sup>3,4</sup>	6.18 ± 0.73 <sup>3,4</sup>	2.21 ± 0.45 <sup>1,2</sup>
10 ( <i>db/db</i> )	AOM + 0.02% CUSM (10)	46.6 ± 3.8 <sup>3,4</sup>	2.79 ± 0.70 <sup>5,6</sup>	5.96 ± 0.80 <sup>6,7</sup>	2.28 ± 0.25 <sup>4,5</sup>
11 ( <i>db/db</i> )	AOM + 0.1% CUSM (10)	46.8 ± 7.3 <sup>5,6</sup>	2.65 ± 0.47 <sup>1,2</sup>	5.69 ± 0.92 <sup>1,2</sup>	2.16 ± 0.50 <sup>6,7</sup>
12 ( <i>db/db</i> )	AOM + 0.5% CUSM (10)	45.2 ± 5.2 <sup>8,9</sup>	2.47 ± 0.67 <sup>8,9</sup>	5.43 ± 0.96 <sup>10</sup>	2.28 ± 0.32 <sup>9,10</sup>

All values are Mean ± SD.

Statistic analysis was done by Bonferroni Multiple Comparisons Test.

Values in parentheses in Column 2 indicate the number of mice examined.

<sup>1</sup>Significantly different from Group 4 ( $p < 0.001$ ).—<sup>2</sup>Significantly different from Group 8 ( $p < 0.001$ ).—<sup>3</sup>Significantly different from Group 2 ( $p < 0.001$ ).—<sup>4</sup>Significantly different from Group 6 ( $p < 0.001$ ).—<sup>5</sup>Significantly different from Group 3 ( $p < 0.001$ ).—<sup>6</sup>Significantly different from Group 7 ( $p < 0.001$ ).—<sup>7</sup>Significantly different from Group 3 ( $p < 0.05$ ).—<sup>8</sup>Significantly different from Group 1 ( $p < 0.001$ ).—<sup>9</sup>Significantly different from Group 5 ( $p < 0.001$ ).—<sup>10</sup>Significantly different from Group 5 ( $p < 0.05$ ).

AOM + 0.1% CUSM group and 7.25 g in the AOM + 0.5% CUSM group) were 1.25–1.34 times ( $p < 0.01$  to  $p < 0.001$ ) greater than other two genotypes (+/+ and *db/+*), regardless of treatments. The average body weights at the termination of the study were high in order of the *db/db* mice, the *db/+* mice and the +/+ mice, as shown in Table I. Although the body weights of *db/db* mice were statistically higher ( $p < 0.001$ ) than those of *db/+* and +/+ mice, there was no significant difference among the treatment groups of each genotype. The liver and relative liver weights of *db/db* mice were greater than those of *db/+* and +/+ mice, but the values did not significantly differ among the treatments groups of this genotype (Table I). The epididymal fat weight was heavy in the order of *db/db*, *db/+* and +/+; the weight was insignificant among the treatment groups in each genotype (Table I). There were no significant differences regarding the mean pancreatic weight among the genotypes (data not shown). No clinical signs for the toxicity of CUSM were observed during the study.

#### Frequency of ACF and BCACs

At the end of the study, all the mice that received AOM developed colonic ACF and BCACs. Table II summarizes the data on colonic ACF formation. Regarding the mean number of ACF/colon in the AOM alone groups, the mean number of *db/db* mice was significantly higher ( $p < 0.001$ ) than that of *db/+* or +/+ mice. In comparison to the AOM alone group, the dietary administration with CUSM significantly reduced the number of ACF in all the genotypes: *db/db* mice, 53% reduction ( $p < 0.001$ ) at a dose level of 0.02% CUSM, 54% reduction ( $p < 0.001$ ) at a dose level of 0.1% CUSM and 59% reduction ( $p < 0.01$ ) at a dose level of 0.5% CUSM; *db/+* mice, 48% reduction ( $p < 0.01$ ) at a dose level of 0.1% CUSM, 38% reduction ( $p < 0.05$ ) at a dose level of 0.5% CUSM and +/+ mice, 45% reduction ( $p < 0.05$ ) at a dose level of 0.1% CUSM and 62% reduction ( $p < 0.001$ ) at a dose level of 0.5% CUSM. In addition, the percentages of ACF consisting of more than 4 aberrant crypts in all the CUSM-feeding groups in the *db/db* mice were significantly smaller (36% reduction by 0.02% CUSM,  $p < 0.01$ ; 30% reduction by 0.1% CUSM,  $p < 0.05$  and 47% reduction by 0.5% CUSM,  $p < 0.001$ ) than that of AOM alone group (Table II). Although dietary administration with CUSM reduced the percentages of ACF consisting of more than 4 aberrant crypts in the *db/+* and +/+ mice, the differences were insignificant.

BCACs also developed in the colon of all the genotypes of mice that received AOM alone, and the frequency per cm<sup>2</sup> of colonic mucosa was high in order of *db/db*, +/+ and *db/+* mice (Table III). The dietary administration with CUSM at the highest dose (0.5%) significantly reduced the number of BCACs in the +/+ (65% reduction,  $p < 0.05$ ) and *db/db* mice (74% reduction,  $p < 0.001$ ). CUSM

at a dose of 0.1% also significantly lowered the number of BCACs in *db/db* mice (53% reduction,  $p < 0.001$ ).

#### Immunohistochemical analysis of Ob-R and IGF-1R

The immunohistochemical expression of Ob-R and IGF-1R was observed in the cytoplasm and nuclei of cryptal cells. Their expression was relatively strong in the nuclei of atypical cells in BCACs, when compared with their surrounding cryptal cells. Feeding with CUSM did not influence the stainability of Ob-R and IGF-1R (data not shown).

#### PCNA-labeling index

PCNA-labeling index was determined in BCACs that developed in the *db/db* mice (Groups 9 through 12). As illustrated in Figure 1, the mean PCNA-labeling indices of Group 11 (AOM + 0.1% CUSM,  $p < 0.05$ ) and Group 12 (AOM + 0.5% CUSM,  $p < 0.005$ ) were significantly lower than that of Group 9 (AOM alone). The values of Groups 9 and 10 (AOM + 0.02% CUSM) were comparable.

#### Histopathology and morphometric analysis in the liver

A histopathological examination of the liver revealed the occurrence of fatty metamorphosis [Fig. 2A-(c)] and fibrosis [Fig. 2B-(c)] in the *db/db* mice that received AOM alone, in contrast to the +/+ [Figs. 2A-(a) and 2B-(a)] and *db/+* mice [Figs. 2A-(b) and 2B-(b)]. When the *db/db* mice were fed with 0.5% CUSM, these histopathological alterations (Fig. 3a and 3b) were inhibited ( $p < 0.001$  for fatty metamorphosis and  $p < 0.05$  for liver fibrosis).

#### Serum levels of cholesterol, triglycerides, glucose, insulin and leptin

The serum concentrations of total cholesterol, triglycerides, glucose, insulin and leptin are listed in Table IV. All the measurements in the *db/db* mice were higher than those of *db/+* and +/+ mice. The dietary administration with CUSM did not significantly affect the serum levels of total cholesterol, glucose, insulin and leptin in all the genotypes. However, the serum level of triglycerides significantly decreased in the *db/db* mice ( $p < 0.05$ ), when fed with the diet containing 0.5% CUSM (Table IV).

#### Discussion

The results of the current study confirmed the high susceptibility of AOM-induced colon carcinogenesis in the obese/diabetic *db/db* mice in our previous findings.<sup>29</sup> The high susceptibility in the *db/db* mice may be related to the increases in the body weight and the serum levels of total cholesterol, triglycerides, glucose, insulin and leptin, thus suggesting a positive association between

TABLE II - EFFECT OF CUSM ON AOM-INDUCED ACF FORMATION IN MALE MICE (+/+, db/+ AND db/db)

Group no.	Treatment	Total no. of ACF/colon	Total no. of ACS/colon	No. of ACS/focus	Total no. of 4 or more ACS/colon	% of ACF containing 4 or more ACS	No. of ACF/cm <sup>2</sup> of colon
1 (+/+)	AOM alone (9)	69 ± 12	228 ± 43	3.28 ± 0.35	23.20 ± 6.61	33.14 ± 6.57	10.78 ± 2.73
2 (+/+)	AOM + 0.02% CUSM (9)	48 ± 14 (30%) <sup>1</sup>	153 ± 60 (33%) <sup>2</sup>	3.14 ± 0.35 (4%)	15.60 ± 8.82 (33%)	30.83 ± 8.23 (7%)	8.16 ± 3.66 (24%)
3 (+/+)	AOM + 0.1% CUSM (9)	38 ± 5 (45%) <sup>1</sup>	108 ± 3 (53%) <sup>2</sup>	2.80 ± 0.33 (15%)	10.60 ± 4.04 (54%)	27.11 ± 8.02 (18%)	6.02 ± 0.79 (44%)
4 (+/+)	AOM + 0.5% CUSM (9)	26 ± 4 (62%) <sup>3</sup>	67 ± 11 (71%) <sup>3</sup>	2.61 ± 0.28 (20%) <sup>1</sup>	5.40 ± 1.82 (77%) <sup>2</sup>	20.95 ± 6.61 (37%)	4.02 ± 0.69 (63%) <sup>2</sup>
5 (db/+)	AOM alone (10)	77 ± 19	274 ± 50	3.60 ± 0.26	30.40 ± 7.44	40.00 ± 8.90 <sup>2</sup>	11.99 ± 3.13
6 (db/+)	AOM + 0.02% CUSM (10)	53 ± 13 (31%) <sup>4</sup>	160 ± 34 (42%) <sup>4</sup>	3.02 ± 0.18 (16%)	16.20 ± 5.12 (47%) <sup>5</sup>	29.95 ± 3.94 (25%)	8.04 ± 2.39 (33%) <sup>5</sup>
7 (db/+)	AOM + 0.1% CUSM (10)	40 ± 6 (48%) <sup>4</sup>	121 ± 27 (56%) <sup>6</sup>	3.00 ± 0.32 (17%) <sup>5</sup>	13.20 ± 3.03 (57%) <sup>4</sup>	32.63 ± 3.98 (18%)	6.53 ± 0.77 (46%) <sup>5</sup>
8 (db/+)	AOM + 0.5% CUSM (10)	48 ± 9 (38%) <sup>5</sup>	145 ± 23 (47%) <sup>4</sup>	2.98 ± 0.21 (17%) <sup>5</sup>	14.80 ± 4.76 (51%) <sup>5</sup>	30.51 ± 7.69 (24%)	7.62 ± 1.36 (36%)
9 (db/db)	AOM alone (10)	147 ± 23 <sup>3,6</sup>	652 ± 88 <sup>3,6</sup>	4.44 ± 0.34 <sup>3,6</sup>	76.60 ± 10.90 <sup>3,6</sup>	52.25 ± 6.02	20.83 ± 4.20 <sup>3,6</sup>
10 (db/db)	AOM + 0.02% CUSM (10)	69 ± 10 (53%) <sup>7</sup>	206 ± 28 (68%) <sup>7</sup>	2.99 ± 0.07 (33%) <sup>7</sup>	23.00 ± 5.15 (70%) <sup>7</sup>	33.56 ± 6.39 (36%) <sup>8</sup>	8.37 ± 1.09 (60%) <sup>7</sup>
11 (db/db)	AOM + 0.1% CUSM (10)	68 ± 13 (54%) <sup>7,9,10</sup>	228 ± 45 (65%) <sup>7,10,11</sup>	3.33 ± 0.16 (25%) <sup>7</sup>	25.20 ± 6.91 (67%) <sup>7,9</sup>	36.35 ± 4.72 (30%) <sup>12</sup>	8.64 ± 2.90 (59%) <sup>7</sup>
12 (db/db)	AOM + 0.5% CUSM (10)	61 ± 6 (59%) <sup>7,13</sup>	179 ± 19 (73%) <sup>7,14</sup>	2.95 ± 0.19 (34%) <sup>7</sup>	16.80 ± 1.92 (78%) <sup>7</sup>	27.85 ± 4.31 (47%) <sup>7</sup>	7.79 ± 1.30 (63%) <sup>7</sup>

All values are Mean ± SD. Statistic analysis was done by Bonferroni Multiple Comparisons Test.

Values in parentheses in Column 2 indicate the number of mice examined. Values in parentheses in Columns 3-8 indicate inhibition rate.

<sup>1</sup>Significantly different from Group 1 ( $p < 0.05$ ).<sup>2</sup>Significantly different from Group 1 ( $p < 0.01$ ).<sup>3</sup>Significantly different from Group 1 ( $p < 0.001$ ).<sup>4</sup>Significantly different from Group 5 ( $p < 0.05$ ).<sup>5</sup>Significantly different from Group 5 ( $p < 0.01$ ).<sup>6</sup>Significantly different from Group 5 ( $p < 0.001$ ).<sup>7</sup>Significantly different from Group 7 ( $p < 0.05$ ).<sup>8</sup>Significantly different from Group 7 ( $p < 0.01$ ).<sup>9</sup>Significantly different from Group 3 ( $p < 0.05$ ).<sup>10</sup>Significantly different from Group 3 ( $p < 0.01$ ).<sup>11</sup>Significantly different from Group 3 ( $p < 0.001$ ).<sup>12</sup>Significantly different from Group 9 ( $p < 0.05$ ).<sup>13</sup>Significantly different from Group 4 ( $p < 0.01$ ).<sup>14</sup>Significantly different from Group 4 ( $p < 0.05$ ).

TABLE III - EFFECT OF CUSM ON AOM-INDUCED BCAC FORMATION IN MALE MICE (+/+, db/+ AND db/db)

Group no.	Treatment	Total no. of BCAC/cm <sup>2</sup>
1 (+/+)	AOM alone (9)	24.42 ± 7.26
2 (+/+)	AOM + 0.02% CUSM (9)	16.53 ± 4.88 (32%)
3 (+/+)	AOM + 0.1% CUSM (9)	10.52 ± 4.62 (57%)
4 (+/+)	AOM + 0.5% CUSM (9)	8.62 ± 3.91 (65%) <sup>1</sup>
5 (db/+)	AOM alone (10)	23.26 ± 8.53
6 (db/+)	AOM + 0.02% CUSM (10)	17.46 ± 4.87 (25%)
7 (db/+)	AOM + 0.1% CUSM (10)	14.84 ± 8.24 (36%)
8 (db/+)	AOM + 0.5% CUSM (10)	9.32 ± 3.86 (60%)
9 (db/db)	AOM alone (10)	41.94 ± 9.06 <sup>2,3</sup>
10 (db/db)	AOM + 0.02% CUSM (10)	26.80 ± 10.52 (36%)
11 (db/db)	AOM + 0.1% CUSM (10)	19.84 ± 4.26 (53%) <sup>4</sup>
12 (db/db)	AOM + 0.5% CUSM (10)	11.10 ± 6.36 (74%) <sup>4</sup>

All values are Mean ± SD.

Statistic analysis was done by Bonferroni Multiple Comparisons Test. Values in parentheses in Column 2 indicate the number of mice examined.

Values in parentheses in Column 3 indicate inhibition rate.

<sup>1</sup>Significantly different from Group 1 ( $p < 0.05$ ).<sup>2</sup>Significantly different from Group 1 ( $p < 0.01$ ).<sup>3</sup>Significantly different from Group 5 ( $p < 0.01$ ).<sup>4</sup>Significantly different from Group 9 ( $p < 0.001$ ).

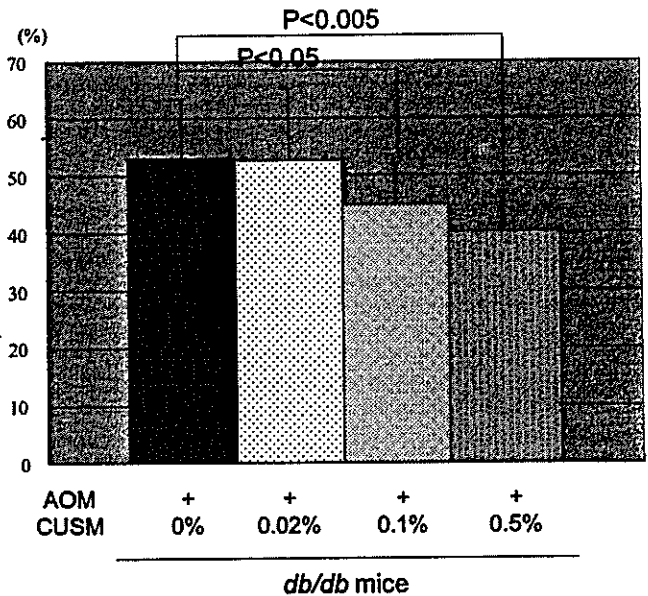


FIGURE 1 - PCNA labeling index in the BCACs that developed in the colon of db/db mice. Feeding with 0.1% ( $p < 0.05$ ) and 0.5% CUSM ( $p < 0.005$ ) significantly lowered the PCNA-labeling indices in the BCACs, but 0.02% CUSM feeding did not influence.

obesity/diabetes and colon tumorigenesis. Our findings also suggest that insulin resistance involves CRC development.<sup>34</sup> The main purpose of the current study was to investigate the effect of CUSM on the early phase of AOM-induced colon carcinogenesis in the db/db mice. Since the lesions ACF and BCACs are considered to be putative precursor lesions of colonic adenocarcinoma,<sup>28,35</sup> the results obtained clearly indicate the inhibitory effects of the dietary administration of CUSM on the development of AOM-induced ACF and BCACs in the db/db mice as well as the +/+ and db/+ mice. In the current study, all the serum measurements of total cholesterol, triglycerides, glucose, insulin and leptin were greater in the db/db mice than those of db/+ and +/+ mice, thus suggesting that these measurements may contribute to the high susceptibility of db/db mice to AOM-induced colon tumorigenesis. However, among the chemical profiles, only the triglyceride level lowered by feeding with CUSM correlated with a lower incidence of colonic preneoplastic lesions in the db/db mice. These

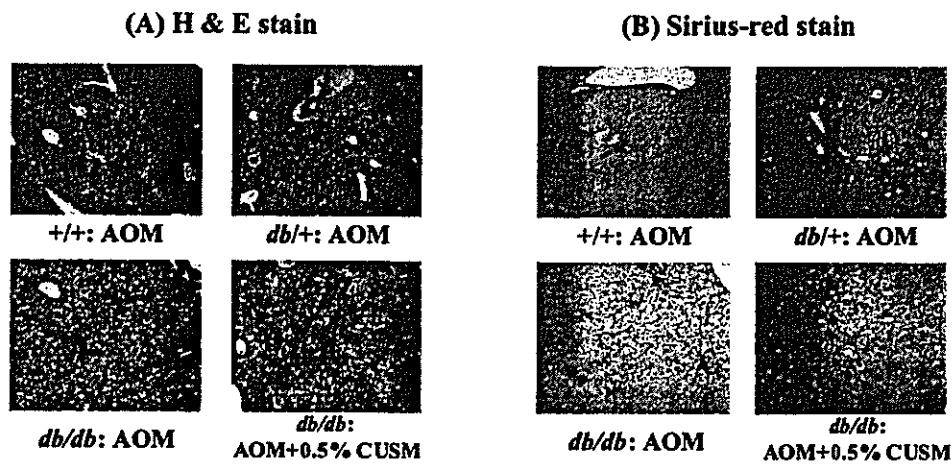


FIGURE 2 – Histopathology of liver. (A) There are numerous fat-containing vacuoles of varying size (the vacuoles are empty, the fat having dissolved in reagents) in the liver [A-(c)] of a *db/db* mouse receiving AOM, in contrast with those in the *+/+* [A-(a)] and *db/+* [A-(b)] mice treated with AOM. (B) Fibrosis (red) stained with Sirius-red is also evident in the liver [B-(c)] of a *db/db* mice that received AOM, in contrast with those in the *+/+* [B-(a)] and *db/+* [B-(b)] mice given AOM. These pathological alterations decreased after the administration of 0.5% CUSM in diet [A-(d) and B-(d)]. (A) H & E stain and (B) Sirius-red stain. Original magnification, (A) and (B)  $\times 10$ . [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

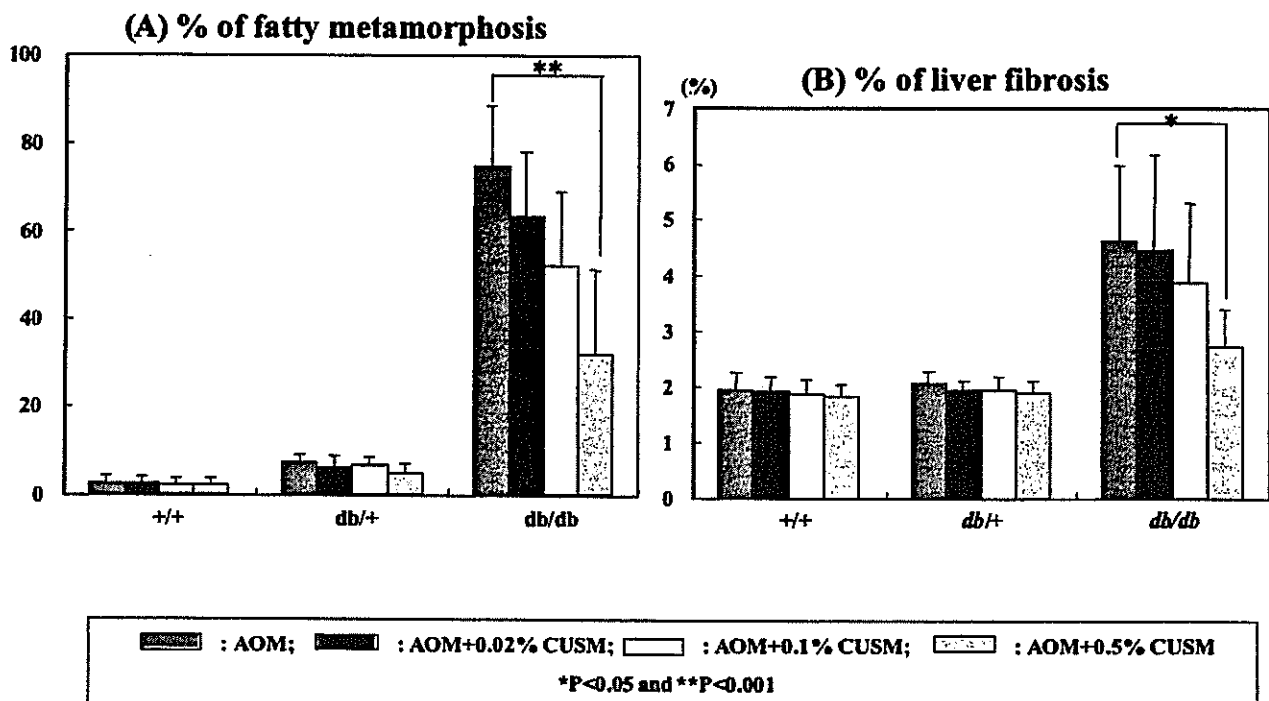


FIGURE 3 – A morphometric analysis of fatty metamorphosis and fibrosis in liver. Although the percentages of fatty metamorphosis (A) and fibrosis (B) in the liver of *+/+* and *db/+* mice were closely similar regardless of the administration of CUSM, the values were high in *db/db* mice. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

findings suggest that a high level of serum triglyceride is the most important biological effect for developing colonic tumors in *db/db* mice, and a modification (lowering) of this value may thus lead the inhibition of colon tumorigenesis. In fact, a positive association between the serum triglyceride levels and the risk of CRC development was found in humans.<sup>36</sup> This association was also suspected by the findings in animal experiments,<sup>37</sup> in which model animals for human familial adenomatous polyposis were used.<sup>38</sup>

Interestingly, feeding with a high-fat diet, which is implicated to play a role in the stimulation of colonic cryptal cell proliferation while also promoting colon carcinogenesis,<sup>39</sup> thus increases the

circulating leptin level.<sup>40</sup> In addition, dietary fiber has been reported to decrease the serum leptin concentration while reducing colon carcinogenesis by lowering the degree of cryptal cell proliferation.<sup>41</sup> However, CUSM feeding did not affect serum leptin levels in the *db/db* mice. Thus, CUSM constituents other than fiber, i.e. flavonoids may contribute to the reduction in the occurrence of putative precursor lesions, ACF and BCACs in the colon of the *db/db* mice. We suspected that hesperidin in CUSM may therefore be responsible for the inhibition of preneoplasia development in male *db/db* mice, because this chemical can inhibit chemically-induced colon carcinogenesis in rodents.<sup>19</sup>

TABLE IV - SERUM PROFILES IN EACH GROUP OF MALE MICE (+/+, *db/+* AND *db/db*) THAT RECEIVED AOM AND CUSM

Group no.	Treatment	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	Glucose (mg/dL)	Insulin (ng/mL)	Leptin (ng/mL)
1 (+/+)	AOM alone (9)	90.2 ± 12.2	173.2 ± 31.6	224.6 ± 49.8	1.38 ± 0.87	11.4 ± 4.7
2 (+/+)	AOM + 0.02% CUSM (9)	93.2 ± 2.9	122.2 ± 33.8 <sup>1</sup>	211.8 ± 19.6	1.13 ± 0.37	6.9 ± 1.5
3 (+/+)	AOM + 0.1% CUSM (9)	98.0 ± 11.5	137.6 ± 10.1	200.0 ± 30.2	1.59 ± 0.35	7.7 ± 1.6
4 (+/+)	AOM + 0.5% CUSM (9)	83.8 ± 11.0	116.4 ± 6.8 <sup>1</sup>	228.2 ± 20.0	1.11 ± 0.12	5.3 ± 2.1
5 ( <i>db/+</i> )	AOM alone (10)	108.8 ± 8.5	116.6 ± 7.0 <sup>1</sup>	197.2 ± 15.0	2.01 ± 0.37	20.3 ± 10.1
6 ( <i>db/+</i> )	AOM + 0.02% CUSM (10)	103.6 ± 4.4	134.4 ± 22.9	206.6 ± 17.6	4.49 ± 2.26	35.1 ± 7.2
7 ( <i>db/+</i> )	AOM + 0.1% CUSM (10)	113.0 ± 8.6	107.2 ± 12.4 <sup>2</sup>	211.6 ± 17.0	1.99 ± 0.38	21.7 ± 4.5
8 ( <i>db/+</i> )	AOM + 0.5% CUSM (10)	104.8 ± 5.7	114.4 ± 10.7	206.2 ± 30.9	2.21 ± 0.77	14.2 ± 5.0
9 ( <i>db/db</i> )	AOM alone (10)	149.0 ± 38.8 <sup>3</sup>	192.4 ± 35.0 <sup>4</sup>	685.0 ± 84.5 <sup>5,6</sup>	13.89 ± 8.74 <sup>1,7</sup>	244.2 ± 28.2 <sup>5,6</sup>
10 ( <i>db/db</i> )	AOM + 0.02% CUSM (10)	170.8 ± 39.0 <sup>8,9</sup>	216.2 ± 39.3 <sup>10,11</sup>	758.0 ± 71.3 <sup>8,9</sup>	18.44 ± 9.56 <sup>8,9</sup>	241.3 ± 42.8 <sup>9</sup>
11 ( <i>db/db</i> )	AOM + 0.1% CUSM (10)	180.3 ± 14.2 <sup>12,13</sup>	157.8 ± 23.1 <sup>13</sup>	641.5 ± 88.0 <sup>12,13</sup>	18.66 ± 7.44 <sup>12,13</sup>	260.0 ± 37.7 <sup>12,13</sup>
12 ( <i>db/db</i> )	AOM + 0.5% CUSM (10)	137.7 ± 33.1 <sup>14</sup>	134.8 ± 15.3 <sup>15,16</sup>	783.0 ± 84.1 <sup>17,18</sup>	11.17 ± 7.47	252.8 ± 30.8 <sup>17,18</sup>

All values are Mean ± SD.

Statistic analysis was done by Bonferroni Multiple Comparisons Test.

Values in parentheses in Column 2 indicate the number of mice examined.

<sup>1</sup>Significantly different from Group 1 ( $p < 0.05$ ).<sup>2</sup>Significantly different from Group 3 ( $p < 0.05$ ).<sup>3</sup>Significantly different from Group 1 ( $p < 0.01$ ).<sup>4</sup>Significantly different from Group 5 ( $p < 0.01$ ).<sup>5</sup>Significantly different from Group 1 ( $p < 0.001$ ).<sup>6</sup>Significantly different from Group 5 ( $p < 0.001$ ).<sup>7</sup>Significantly different from Group 5 ( $p < 0.05$ ).<sup>8</sup>Significantly different from Group 2 ( $p < 0.001$ ).<sup>9</sup>Significantly different from Group 6 ( $p < 0.001$ ).<sup>10</sup>Significantly different from Group 6 ( $p < 0.01$ ).<sup>11</sup>Significantly different from Group 2 ( $p < 0.01$ ).<sup>12</sup>Significantly different from Group 3 ( $p < 0.001$ ).<sup>13</sup>Significantly different from Group 7 ( $p < 0.001$ ).<sup>14</sup>Significantly different from Group 4 ( $p < 0.01$ ).<sup>15</sup>Significantly different from Group 9 ( $p < 0.05$ ).<sup>16</sup>Significantly different from Group 8 ( $p < 0.05$ ).<sup>17</sup>Significantly different from Group 4 ( $p < 0.001$ ).<sup>18</sup>Significantly different from Group 8 ( $p < 0.001$ ).

An association between diabetes and cancer was suggested over 100 years ago.<sup>42</sup> The increased incidence of CRC in diabetic patients, mainly in those with type 2 diabetes, has been supported by a recent prospective, population-based cohort, case-control and meta-analysis studies.<sup>5,43</sup> Thus, there is an attractive hypothesis of insulin resistance-CRC, stating that insulin resistant may thus be associated with the development of CRC,<sup>44</sup> and this malignancy may therefore become a modifiable disease.<sup>45</sup> Regarding the mechanism of action, insulin resistance is associated with hyperinsulinemia, increased levels of growth factors, including IGF-1, and alterations in nuclear factor kappa B (NF- $\kappa$ B) and peroxisome proliferator-activated receptors signaling, which may promote CRC through their effect on the colonic cryptal cell kinetics.<sup>21</sup> Among these factors, insulin and the IGF axis may be related to CRC development.<sup>46</sup> IGF-1 may be able to influence both premalignant and cancer development. Similarly, insulin stimulates growth of normal colonic cryptal and cancer cells. Recently, an interesting finding indicated that leptin may interact with IGFs to promote survival and the expansion of colonic epithelial cells that were *Apc* deficient, but not those expressing wild-type *Apc*.<sup>47</sup> In the current study, feeding with CUSM did not influence the serum level of insulin and immunoreactivities of IGF-1R and Ob-R in the BCACs in the *db/db* mice. However, the treatment reduced cell proliferation activity in the BCACs by estimating PCNA-labeling index. CUSM could reduce the occurrence or progression of BCACs through lowering the cell proliferation, although the exact mechanism(s) should be elucidated.

In the present study, *db/db* mice treated with AOM had a greater incidence and multiplicity of ACF and BCACs. In addition,

CUSM feeding inhibited fatty metamorphosis and fibrosis in the liver of *db/db* mice with hyperleptinemia treated with AOM. AOM is metabolically activated by CYP2E1.<sup>48</sup> Leptin treatment has been reported to increase the hepatic CYP2E1 expression in the *ob/ob* mutant mice.<sup>49</sup> The CYP2E1 activity that may increase due to AOM exposure and hyperleptinemia may therefore contribute to a higher incidence of putative precancerous lesions (ACF and BCACs) for CRC in the *db/db* mice. The inhibitory effects of CUSM on the development of putative precancerous lesions may be partly caused by influencing the hepatic and intestinal CYP2E1 activity,<sup>50</sup> but the content of fiber and/or pectin in the CUSM-containing diets was too low to exert their biological effects.

In summary, our data provide further evidence that *db/db* mice are susceptible to AOM-induced carcinogenesis<sup>29</sup> and such *db/db* mice can thus be an appropriate animal model for "metabolic syndrome," nonalcoholic fatty liver disease and/or nonalcoholic steatohepatitis.<sup>51</sup> Since our study focused on the effects of obesity and CUSM on the colonic premalignancies, not malignancies, further studies focusing the malignancies and NF- $\kappa$ B and I $\kappa$ B kinase<sup>52,53</sup> that can be activated through Ob-R1<sup>14,44,46</sup> and may thus play a critical role in obesity/diabetes-associated and colitis-related colon carcinogenesis in which processes the leptin involved<sup>29</sup> are needed for the prevention and treatment of the malignancies associated with these conditions.

#### Acknowledgements

We express our thanks to the staff of the Research Animal Facility.

#### References

1. Abu-Abid S, Szold A, Klausner J. Obesity and cancer. *J Med* 2002; 33:73-86.
2. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. Cancer statistics, 2005. *CA Cancer J Clin* 2005; 55:10-30.
3. Kono S. Secular trend of colon cancer incidence and mortality in relation to fat and meat intake in Japan. *Eur J Cancer Prev* 2004; 13:127-32.
4. Weiderpass E, Gridley G, Nyren O, Ekblom A, Persson I, Adami HO. Diabetes mellitus and risk of large bowel cancer. *J Natl Cancer Inst* 1997; 89:660-1.
5. La Vecchia C, Negri E, Decarli A, Franceschi S. Diabetes mellitus and colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev* 1997; 6:1007-10.
6. Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 1999; 91:916-32.
7. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JJ, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996; 379:632-5.
8. Fruhbeck G, Gomez-Ambrosi J. Rationale for the existence of additional adipostatic hormones. *FASEB J* 2001; 15:1996-2006.
9. Cusin I, Sainsbury A, Doyle P, Rohner-Jeanrenaud F, Jeanrenaud B. The ob gene and insulin. A relationship leading to clues to the understanding of obesity. *Diabetes* 1995; 44:1467-70.
10. Zhang HH, Kumar S, Barnett AH, Eggo MC. Tumour necrosis factor- $\alpha$  exerts dual effects on human adipose leptin synthesis and release. *Mol Cell Endocrinol* 2000; 159:79-88.

11. Dagogo-Jack S, Selke G, Melson AK, Newcomer JW. Robust leptin secretory responses to dexamethasone in obese subjects. *J Clin Endocrinol Metab* 1997;82:3230-3.
12. Machinal-Quelin F, Dieudonne MN, Pecquery R, Leneuve MC, Giudicelli Y. Direct in vitro effects of androgens and estrogens on ob gene expression and leptin secretion in human adipose tissue. *Endocrine* 2002;18:179-84.
13. Fain JN, Leffler CW, Bahouth SW. Eicosanoids as endogenous regulators of leptin release and lipolysis by mouse adipose tissue in primary culture. *J Lipid Res* 2000;41:1689-94.
14. Garofalo C, Surmacz E. Leptin and cancer. *J Cell Physiol* 2006;207:12-22.
15. Hardwick JC, Van Den Brink GR, Offerhaus GJ, Van Deventer SJ, Peppelenbosch MP. Leptin is a growth factor for colonic epithelial cells. *Gastroenterology* 2001;121:79-90.
16. Liu Z, Uesaka T, Watanabe H, Kato N. High fat diet enhances colonic cell proliferation and carcinogenesis in rats by elevating serum leptin. *Int J Oncol* 2001;19:1009-14.
17. Aparicio T, Guilmeau S, Goiot H, Tsocas A, Laigneau JP, Bado A, Sobhani I, Lehy T. Leptin reduces the development of the initial precancerous lesions induced by azoxymethane in the rat colonic mucosa. *Gastroenterology* 2004;126:499-510.
18. Aparicio T, Kotelevets L, Tsocas A, Laigneau JP, Sobhani I, Chastre E, Lehy T. Leptin stimulates the proliferation of human colon cancer cells in vitro but does not promote the growth of colon cancer xenografts in nude mice or intestinal tumorigenesis in *Apc<sup>Min/+</sup>* mice. *Gut* 2005;54:1136-45.
19. Tanaka T, Kohno H, Mori H. Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. *Asian Pac J Cancer Prev* 2001;2:165-77.
20. Greenwald P. Lifestyle and medical approaches to cancer prevention. *Recent Results Cancer Res* 2005;166:1-15.
21. Moore MA, Sobue T, Kuriki K, Tajima K, Tokudome S, Kono S. Comparison of Japanese, American-Whites and African-Americans—pointers to risk factors to underlying distribution of tumours in the colorectum. *Asian Pac J Cancer Prev* 2005;6:412-9.
22. Reddy BS, Mori H, Nicolais M. Effect of dietary wheat bran and dehydrated citrus fiber on azoxymethane-induced intestinal carcinogenesis in Fischer 344 rats. *J Natl Cancer Inst* 1981;66:553-7.
23. Tanaka T, Makita H, Kawabata K, Mori H, Kakumoto M, Satoh K, Hara A, Sumida T, Tanaka T, Ogawa H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis* 1997;18:957-65.
24. Suzuki R, Kohno H, Murakami A, Koshimizu K, Ohigashi H, Yano M, Tokuda H, Nishino H, Tanaka T. Citrus nobiletin inhibits azoxymethane-induced large bowel carcinogenesis in rats. *Biofactors* 2004;21:111-4.
25. Balabanski L. Weight-reducing diets. *Bibl Nutr Dieta* 1985;35:111-21.
26. Jung UJ, Lee MK, Jeong KS, Choi MS. The hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in *C57BL/KsJ-dbidb* mice. *J Nutr* 2004;134:2499-503.
27. Bird RP, Good CK. The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Toxicol Lett* 2000;112/113:395-402.
28. Yamada Y, Mori H. Pre-cancerous lesions for colorectal cancers in rodents: a new concept. *Carcinogenesis* 2003;24:1015-9.
29. Hirose Y, Hata K, Kuno T, Yoshida K, Sakata K, Yamada Y, Tanaka T, Reddy BS, Mori H. Enhancement of development of azoxymethane-induced colonic premalignant lesions in *C57BL/KsJ-dbidb* mice. *Carcinogenesis* 2004;25:821-5.
30. Suzuki R, Kohno H, Sugie S, Sasaki K, Yoshimura T, Wada K, Tanaka T. Preventive effects of extract of leaves of ginkgo (*Ginkgo biloba*) and its component bilobalide on azoxymethane-induced colonic aberrant crypt foci in rats. *Cancer Lett* 2004;210:159-69.
31. Hata K, Tanaka T, Kohno H, Suzuki R, Qiang SH, Yamada Y, Oyama T, Kuno T, Hirose Y, Hara A, Mori H.  $\beta$ -Catenin-accumulated crypts in the colonic mucosa of juvenile *Apc<sup>Min/+</sup>* mice. *Cancer Lett* 2006;239:123-8.
32. Yamada Y, Yoshimi N, Hirose Y, Kawabata K, Matsunaga K, Shimizu M, Hara A, Mori H. Frequent  $\beta$ -catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. *Cancer Res* 2000;60:3323-7.
33. Yamada Y, Yoshimi N, Hirose Y, Matsunaga K, Katayama M, Sakata K, Shimizu M, Kuno T, Mori H. Sequential analysis of morphological and biological properties of  $\beta$ -catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Res* 2001;61:1874-8.
34. Bruce WR, Wolever TM, Giacca A. Mechanisms linking diet and colorectal cancer: the possible role of insulin resistance. *Nutr Cancer* 2000;37:19-26.
35. Mori H, Hata K, Yamada Y, Kuno T, Hara A. Significance and role of early-lesions in experimental colorectal carcinogenesis. *Chem Biol Interact* 2005;155:1-9.
36. Yamada K, Araki S, Tamura M, Sakai I, Takahashi Y, Kashiwara H, Kono S. Relation of serum total cholesterol, serum triglycerides and fasting plasma glucose to colorectal carcinoma in situ. *Int J Epidemiol* 1998;27:794-8.
37. Niho N, Takahashi M, Shoji Y, Takeuchi Y, Matsubara S, Sugimura T, Wakabayashi K. Dose-dependent suppression of hyperlipidemia and intestinal polyp formation in Min mice by pioglitazone, a PPAR  $\gamma$  ligand. *Cancer Sci* 2003;94:960-4.
38. Tanaka T, Kohno H, Suzuki R, Hata K, Sugie S, Niho N, Sakano K, Takahashi M, Wakabayashi K. Dextran sodium sulfate strongly promotes colorectal carcinogenesis in *Apc<sup>Min/+</sup>* mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. *Int J Cancer* 2006;118:25-34.
39. Reddy BS. The Fourth DeWitt S. Goodman lecture. Novel approaches to the prevention of colon cancer by nutritional manipulation and chemoprevention. *Cancer Epidemiol Biomarkers Prev* 2000;9:239-47.
40. Lin X, Chavez MR, Bruch RC, Kilroy GE, Simmons LA, Lin L, Braymer HD, Bray GA, York DA. The effects of a high fat diet on leptin mRNA, serum leptin and the response to leptin are not altered in a rat strain susceptible to high fat diet-induced obesity. *J Nutr* 1998;128:1606-13.
41. Agus MS, Swain JF, Larson CL, Eckert EA, Ludwig DS. Dietary composition and physiologic adaptations to energy restriction. *Am J Clin Nutr* 2000;71:901-7.
42. Czyzyk A, Szczepanik Z. Diabetes mellitus and cancer. *Eur J Intern Med* 2000;11:245-52.
43. Will JC, Galuska DA, Vinicor F, Calle EE. Colorectal cancer: another complication of diabetes mellitus? *Am J Epidemiol* 1998;147:816-25.
44. Kominou D, Ayonote A, Richie JPI, Rigas B. Insulin resistance and its contribution to colon carcinogenesis. *Exp Biol Med* 2003;228:396-405.
45. Moore MA, Park CB, Tsuda H. Implications of the hyperinsulinemia-diabetes-cancer link for preventive efforts. *Eur J Cancer Prev* 1998;7:89-107.
46. Giovannucci E. Insulin, insulin-like growth factors and colon cancer: a review of the evidence. *J Nutr* 2001;131 (Suppl. 11):3109S-20S.
47. Fenton JJ, Hord NG, Lavigne JA, Perkins SN, Hursting SD. Leptin, insulin-like growth factor-1, and insulin-like growth factor-2 are mitogens in *Apc<sup>Min/+</sup>* but not *Apc<sup>+/+</sup>* colonic epithelial cell lines. *Cancer Epidemiol Biomarkers Prev* 2005;14:1646-52.
48. Sohn OS, Fiala ES, Requeijo SP, Weisburger JH, Gonzalez FJ. Differential effects of CYP2E1 status on the metabolic activation of the colon carcinogens azoxymethane and methylazoxymethanol. *Cancer Res* 2001;61:8435-40.
49. Leclercq IA, Field J, Enriquez A, Farrell GC, Robertson GR. Constitutive and inducible expression of hepatic CYP2E1 in leptin-deficient *ob/ob* mice. *Biochem Biophys Res Commun* 2000;268:337-44.
50. Roland N, Nugon-Baudon L, Flinois JP, Beaune P. Hepatic and intestinal cytochrome P-450, glutathione-S-transferase and UDP-glucuronosyl transferase are affected by six types of dietary fiber in rats inoculated with human whole fecal flora. *J Nutr* 1994;124:1581-7.
51. Sahai A, Malladi P, Pan X, Paul R, Melin-Aldana H, Green RM, Whittington PF. Obese and diabetic *db/db* mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G1035-G43.
52. Greten FR, Karin M. The IKK/NF- $\kappa$ B activation pathway—a target for prevention and treatment of cancer. *Cancer Lett* 2004;206:193-9.
53. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gukovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF- $\kappa$ B functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004;431:461-6.

## Dietary $\beta$ -cryptoxanthin inhibits *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in male ICR mice

KATSUHITO MIYAZAWA<sup>1</sup>, SHINGO MIYAMOTO<sup>2</sup>, RIKAKO SUZUKI<sup>2</sup>, YUMIKO YASUI<sup>2</sup>, RYOSUKE IKEDA<sup>1</sup>, HIROYUKI KOHNO<sup>2</sup>, MASAMICHI YANO<sup>3</sup>, TAKUJI TANAKA<sup>2</sup>, KAZUYA HATA<sup>4</sup> and KOJI SUZUKI<sup>1</sup>

Departments of <sup>1</sup>Urogenital Surgery and <sup>2</sup>Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293; <sup>3</sup>Department of Citrus Research, National Institute of Fruit Tree Science, 485-6 Okitsunaka-cho, Shimizu, Shizuoka 424-0292; <sup>4</sup>BMR Laboratories, Sunplanet Co., Ltd., 4388 Hagiwara, Kamiishidu, Yourou, Gifu 503-1602, Japan

Received October 9, 2006; Accepted November 10, 2006

**Abstract.** Recent epidemiological studies have indicated that high dietary consumption of fruit and vegetables results in lower risk of bladder cancer. To confirm these findings, we investigated in the current study the effects of dietary administration with  $\beta$ -cryptoxanthin extracted from *Citrus unshiu* oranges on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN)-induced urinary bladder carcinogenesis in mice. Male ICR mice were divided into 6 experimental and control groups. Groups 1 through 4 were given OH-BBN (500 ppm) in drinking water for 6 weeks to induced urinary bladder neoplasms. Mice in groups 2, 3 and 4 were fed the diets mixed with 1, 5 and 25 ppm of  $\beta$ -cryptoxanthin, respectively, starting 1 week after the cessation of OH-BBN exposure, and kept on these diets for 24 weeks until the termination of the study. Group 5 was treated with the diet containing the test compound (25 ppm) alone, and group 6 served as an untreated control. All animals were sacrificed at week 32 for histopathology and immunohistochemistry (cyclin D1). Feeding with  $\beta$ -cryptoxanthin decreased the incidence and multiplicity of preneoplastic and neoplastic lesions of urinary bladder. Notably, the highest dose (25 ppm) of the test chemical significantly lowered the occurrence of bladder carcinoma, in conjunction with reducing the cyclin D1-positive cell ratio. These findings suggest that  $\beta$ -cryptoxanthin is able to prevent OH-BBN-induced bladder carcinogenesis in mice.

### Introduction

The incidence of bladder cancer has been increasing in most industrialized countries (1). Bladder cancer is the sixth most common cancer in the United States. In 2005, 63,210 new cases were diagnosed, and 13,180 bladder cancer deaths recorded in Americans (2). In Japan, the rates per 100,000 population were 18.4 in male and 5.6 in female (3). In the light of the high incidence and recurrence rates of this malignancy, increasing attention has been focused on the prevention of bladder cancer at the earlier stage (4,5).

Chemopreventive intervention is one of the possible approaches to curb cancer incidence. Fruit, vegetables, beverages, herbs and other plants are rich sources of non-nutrients with potential to prevent the occurrence of cancers (6,7). The World Cancer Research Fund and the American Institute for Cancer Research extensively reviewed epidemiological studies and concluded that diets high in vegetables and fruits probably protect against bladder cancer (8).

Fruit and vegetables are rich source of carotenoids that have several biological functions, such as provitamin A activity, scavenging of free radicals, enhancement of gap junctions, immunomodulation, and regulation of enzyme activity involved in carcinogenesis (9). For example, we demonstrated chemopreventive ability of naturally occurring xanthophylls in animal models of carcinogenesis (10-12). A carotenoid with non-substituted  $\beta$ -ionone cycles and provitamin A property,  $\beta$ -cryptoxanthin, has also been reported to exert anti-tumor promoter action *in vitro* (13).  $\beta$ -cryptoxanthin is able to inhibit chemically-induced carcinogenesis in mouse skin (14) and rat colon (15,16). In addition, treatment with mandarin juices rich in  $\beta$ -cryptoxanthin and hesperidin also suppress rat colon (16) and mouse lung carcinogenesis (17). Furthermore, certain epidemiological studies suggest that serum level of  $\beta$ -cryptoxanthin is inversely correlated with the risk of cancer development in esophagus (18), cervix (19), lung (20,21), and the bladder (22). Thus, we hypothesized that dietary  $\beta$ -cryptoxanthin may contribute to the prevention of experimental bladder carcinogenesis.

---

**Correspondence to:** Dr Takuji Tanaka, Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan  
E-mail: takutt@kanazawa-med.ac.jp

**Key words:** chemoprevention,  $\beta$ -cryptoxanthin, urinary bladder carcinogenesis, cyclin D1

### Experimental Protocol

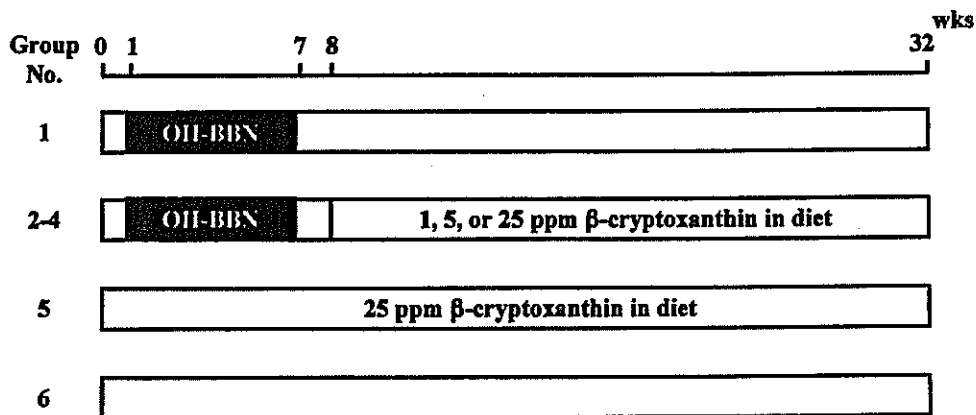


Figure 1. Experimental protocol. A total of 92 male ICR mice (5 weeks old) were used in the study. To induce urinary bladder neoplasms, mice were given OH-BBN (500 ppm) in drinking water for 6 weeks. They were fed the diet containing  $\beta$ -cryptoxanthin (1, 5 or 25 ppm). The experiment was terminated at week 32 to evaluate the chemopreventive efficacy of  $\beta$ -cryptoxanthin.

In the current study, chemopreventive effects of  $\beta$ -cryptoxanthin were evaluated by dietary exposure during the post-initiation stage of *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (OH-BBN)-induced urinary bladder carcinogenesis in male ICR mice. This animal model can be applied to detect the modifying effects of natural or synthetic xenobiotics, as we reported (10,23-26). Effects of dietary  $\beta$ -cryptoxanthin on cell cycle progression (cyclin D1) in the bladder epithelium of mice exposed to OH-BBN were also evaluated by immunohistochemical analysis, since alteration of cell cycle progression may be involved in cancer chemoprevention (25-29).

#### Materials and methods

**Animals, diet and chemicals.** A total of 92 male ICR mice were obtained from Japan SLC, Inc., Hamamatsu. OH-BBN was purchased from Tokyo Chemical Industry Co., Ltd. Tokyo, Japan. Powdered CE-2 (CLEA Japan, Inc., Tokyo) was used as a basal diet.  $\beta$ -cryptoxanthin was extracted from *Citrus unshiu* oranges by one (M.Y.) of the authors, and experimental diet mixed with the test compound at a dose of 1, 5 or 25 ppm was prepared twice a week. The drinking water containing 500 ppm of OH-BBN was prepared every other day. All animals were allowed free access to food and water. They were housed in plastic cages (3-5 per cage) with wood chips in an air-conditioned experimental animal room at  $23\pm 2^\circ\text{C}$  (SD),  $50\pm 10\%$  relative humidity, under a 12-h light/dark cycle. This experiment was approved by Kanazawa Medical University.

**Experimental procedure.** Five-week-old mice were randomly divided into 6 groups (Fig. 1). After a 1-week quarantine, animals in groups 1 (16 mice), 2 (18 mice), 3 (19 mice) and 4 (19 mice) received OH-BBN in the drinking water for 6 weeks to induce bladder carcinoma. Mice in groups 2-4 were fed the diet mixed with 1, 5 and 25 ppm of  $\beta$ -cryptoxanthin for 24 weeks, respectively, starting 1 week after of the cessation of OH-BBN treatment, and kept on this diet until the termination of the study (for 24 weeks). Group 5 (10 mice) was not exposed to the carcinogen and was fed the diet with the

test compound (25 ppm) throughout the experiment. Group 6 (10 mice) was given the basal diet without  $\beta$ -cryptoxanthin and tap water without OH-BBN throughout the experiment, serving as an untreated control. All mice were weighed once a week for the first 8 weeks, and thereafter once a month for the subsequent period. The experiment was terminated at week 32, and all animals were sacrificed under ether anesthesia. At autopsy, the urinary bladder was fully inflated with 10% buffered formalin, fixed for 5 h, and then embedded in paraffin for histopathological evaluation on hematoxylin and eosin-stained sections according to the criteria described by Fukushima *et al.* (30). Other organs including liver, kidney, lungs, stomach and intestine were also fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by conventional methods for histopathology.

**Immunohistochemical staining.** Cyclin D1 immunohistochemistry was performed according to the methods described previously with some modifications (31) for the evaluation of cell cycle activity of the transitional cell lesions. Briefly, 3- $\mu\text{m}$  paraffin-embedded sections were deparaffinized with three changes of xylene and hydrated using a graded series of alcohol. Slides were incubated in 1 mM EDTA (pH 8.0) at  $121^\circ\text{C}$  twice in an autoclave, 5 min each to effect antigen retrieval before staining, then exposed overnight to 1:100 diluted cyclin D1 mouse monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, UK), thereafter slides were developed by the avidin-biotin-peroxidase complex methods. Cells were considered positive for cyclin D1 when definite nuclear staining was identified. The positive cell ratio for cyclin D1 was determined by randomly observing 500 epithelial cells under magnification  $\times 400$  (over 50 fields) to score. Positive cell ratios were calculated as numbers per 100 cells. Over-expression of cyclin D1 in preneoplastic lesions and tumors was defined as positive when nuclear staining of  $>5\%$  of nuclei was evident (32).

**Statistical analysis.** Body weight, liver weight, relative liver weight, and cyclin D-positive cell ratios were compared among the groups using Bonferroni multiple comparisons test



Table I. Body, liver and relative liver weight at the end of the study.

Group no.	Treatment	No. of mice examined	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	OH-BBN	16	43.68±3.60*	2.23±0.28	5.13±0.75
2	OH-BBN→1 ppm β-cryptoxanthin	18	44.85±4.21	2.28±0.31	5.10±0.57
3	OH-BBN→5 ppm β-cryptoxanthin	19	45.50±6.37	2.22±0.40	4.89±0.60
4	OH-BBN→25 ppm β-cryptoxanthin	19	43.04±6.52	2.21±0.25	5.17±0.58
5	25 ppm β-cryptoxanthin	10	37.40±3.00	2.04±0.24	5.46±0.57
6	None	10	44.79±5.42	2.31±0.19	5.21±0.67

\*Mean ± SD.

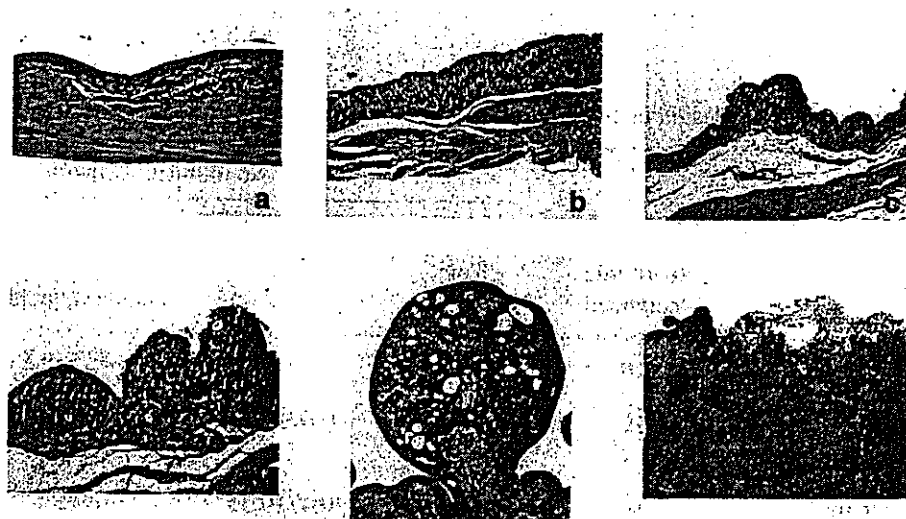


Figure 2. Histopathology of the urinary bladder lesions in mice treated with OH-BBN. (a) Non-lesional transitional epithelium, (b) simple hyperplasia, (c) papillary or nodular hyperplasia, (d) dysplasia, (e) transitional cell papilloma, and (f) transitional cell carcinoma. Hematoxylin and eosin stain, original magnification  $\times 10$ .

and Fisher's exact probability test. The results were considered statistically significant at  $P < 0.05$ .

## Results

**General observations.** There were no clinical signs of toxicity, low survival, poor condition or histological changes, suggesting no toxicity of β-cryptoxanthin in the liver, kidney and lungs caused by administration of the experiment diet. Mean body, liver and relative liver weights (g/100 g body weight) in all groups at the end of the study are shown in Table I. There were no statistical significant differences among the groups. Mean intakes of food and OH-BBN in groups 1 through 4 were comparable among the groups during the entire experiment period (data not shown).

**Multiplicity and incidence of preneoplastic lesions of urinary bladder.** The incidence and multiplicity of preneoplastic lesions (hyperplasia and dysplasia, Fig. 2b-d) in all the groups are indicated in Tables II and III. The incidence of papillary or nodular hyperplasia (Fig. 2c) of groups 2 ( $P < 0.05$ ), 3 ( $P < 0.001$ ) and 4 ( $P < 0.05$ ) were significantly lower than that of group 1 (Table II). In addition, the incidence of dysplasia (Fig. 2d) of groups 3 ( $P < 0.05$ ) and 4 ( $P < 0.05$ ) were significantly lower than that of group 1 (Table II). The multiplicities of papillary or nodular hyperplasia of groups 2 ( $P < 0.05$ ), 3 ( $P < 0.001$ ) and 4 ( $P < 0.05$ ) were significantly smaller than that of group 1 (Table III). However, there were no significant differences in the multiplicity of dysplasia noted among the groups.

Table II. Incidence of preneoplastic lesion in the urinary bladder of mice.

Group no.	Treatment	No. of mice examined	No. of mice (%) with			Dysplasia
			Hyperplasia		Papillary or nodular hyperplasia	
			Total	Simple hyperplasia		
1	OH-BBN	16	16 (100)	10 (63)	11 (69)	12 (75)
2	OH-BBN→1 ppm $\beta$ -cryptoxanthin	18	16 (89)	12 (67)	5 (28) <sup>a</sup>	9 (50)
3	OH-BBN→5 ppm $\beta$ -cryptoxanthin	19	11 (58) <sup>b</sup>	9 (47)	2 (11) <sup>c</sup>	7 (37) <sup>a</sup>
4	OH-BBN→25 ppm $\beta$ -cryptoxanthin	19	8 (42) <sup>c</sup>	5 (26) <sup>a</sup>	5 (26) <sup>a</sup>	6 (32) <sup>a</sup>
5	25 ppm $\beta$ -cryptoxanthin	10	0	0	0	0
6	None	10	0	0	0	0

<sup>a-c</sup>Significantly different from group 1 by Fisher's exact probability test (<sup>a</sup>P<0.05, <sup>b</sup>P<0.005 and <sup>c</sup>P<0.001).

Table III. Multiplicity of preneoplastic lesion in the urinary bladder of mice.

Group no.	Treatment	No. of mice examined	No. of mice (%) with			Dysplasia
			Hyperplasia		Papillary or nodular hyperplasia	
			Total	Simple hyperplasia		
1	OH-BBN	16	1.31±0.60 <sup>a</sup>	0.63±0.50	0.75±0.58	0.75±0.44
2	OH-BBN→1 ppm $\beta$ -cryptoxanthin	18	0.94±0.42	0.67±0.49	0.28±0.46 <sup>b</sup>	0.50±0.51
3	OH-BBN→5 ppm $\beta$ -cryptoxanthin	19	0.58±0.51 <sup>c</sup>	0.47±0.51	0.11±0.32 <sup>d</sup>	0.37±0.50
4	OH-BBN→25 ppm $\beta$ -cryptoxanthin	19	0.53±0.70 <sup>d</sup>	0.26±0.45	0.26±0.45 <sup>b</sup>	0.32±0.48
5	25 ppm $\beta$ -cryptoxanthin	10	0	0	0	0
6	None	10	0	0	0	0

<sup>a-d</sup>Mean ± SD. <sup>b-d</sup>Significantly different from group 1 by Bonferroni Multiple Comparisons Test (<sup>b</sup>P<0.05, <sup>c</sup>P<0.01 and <sup>d</sup>P<0.001).

*Multiplicity and incidence of tumors of urinary bladder.* The multiplicity and incidence of neoplastic lesions (Fig. 2e and f) in all the groups are listed in Tables IV and V. Feeding with the highest dose, 25 ppm  $\beta$ -cryptoxanthin significantly reduced bladder carcinoma with an incidence of 11% (2 of 19 mice, P<0.05) and a multiplicity of 0.11±0.32 (P<0.05) in comparison with the OH-BBN alone group 1 (75% incidence and

0.63±0.72 multiplicity) (Tables IV and V). A few squamous cell carcinomas were also observed in groups 1-3 (2 in group 1, 1 in group 2, and 1 in group 3), but no significant differences in the incidence of squamous cell malignancy were observed among the groups. No abnormalities were found in urinary bladder microscopically or macroscopically in mice without OH-BBN treatment (groups 5 and 6).

Table IV. Incidence of tumors in the urinary bladder of mice.

Group no.	Treatment	No. of mice examined	No. of mice (%) with				
			Total	Papilloma	Transitional cell carcinoma	Squamous cell carcinoma	Carcinoma
1	OH-BBN	16	8 (50)	1 (6)	6 (38)	2 (13)	8 (50)
2	OH-BBN→1 ppm β-cryptoxanthin	18	4 (22)	0	3 (17)	1 (6)	4 (22)
3	OH-BBN→5 ppm β-cryptoxanthin	19	4 (21)	1 (5)	3 (16)	1 (5)	4 (21)
4	OH-BBN→25 ppm β-cryptoxanthin	19	2 (11) <sup>a</sup>	1 (5)	2 (11)	0	2 (11) <sup>a</sup>
5	25 ppm β-cryptoxanthin	10	0	0	0	0	0
6	None	10	0	0	0	0	0

<sup>a</sup>Significantly different from group 1 by Fisher's exact probability test (P<0.05).

Table V. Multiplicity of tumors in the urinary bladder of mice.

Group no.	Treatment	No. of mice examined	No. of mice with				
			Total	Papilloma	Transitional cell carcinoma	Squamous cell carcinoma	Carcinoma
1	OH-BBN	16	0.69±0.79 <sup>a</sup>	0.06±0.25	0.50±0.73	0.13±0.34	0.63±0.72
2	OH-BBN→1 ppm β-cryptoxanthin	18	0.33±0.59	0	0.22±0.55	0.06±0.24	0.28±0.58
3	OH-BBN→5 ppm β-cryptoxanthin	19	0.21±0.42	0.05±0.23	0.16±0.38	0.05±0.23	0.21±0.42
4	OH-BBN→25 ppm β-cryptoxanthin	19	0.16±0.50	0.05±0.23	0.11±0.32	0	0.11±0.32 <sup>b</sup>
5	25 ppm β-cryptoxanthin	10	0	0	0	0	0
6	None	10	0	0	0	0	0

<sup>a</sup>Mean ± SD. <sup>b</sup>Significantly different from group 1 by Bonferroni Multiple Comparisons Test (P<0.05).

**Cyclin D1-positive cell ratios.** The intensity of cyclin D1-staining was generally strong among the various lesions of the urinary bladder, and was weak in non-lesional transitional epithelium (Fig. 3). As summarized in Table VI, cyclin D1 over-expression was observed in various types of lesions. Cyclin D1-positive ratios of the non-lesional areas of groups 2 (P<0.01), 3 (P<0.001) and 4 (P<0.001) and that of hyperplasia of groups 3 (P<0.05) and 4 (P<0.001) were significantly smaller than those of group 1. Furthermore, cyclin D1-positive ratios of dysplasia of groups 2 (P<0.01), 3 (P<0.001) and 4 (P<0.001) were significantly lower than that of group 1.

In the transitional cell carcinoma, cyclin D1-positive ratio of group 4 was significantly smaller than that of group 1 (P<0.05).

#### Discussion

This study clearly indicated chemopreventive efficacy of β-cryptoxanthin in diet on chemically-induced carcinogenesis in mouse urinary bladder. The potential, especially that of 25 ppm feeding in diet, was inversely correlated with cyclin D1-positive ratios in the lesions.

## Cyclin D1 immunohistochemistry

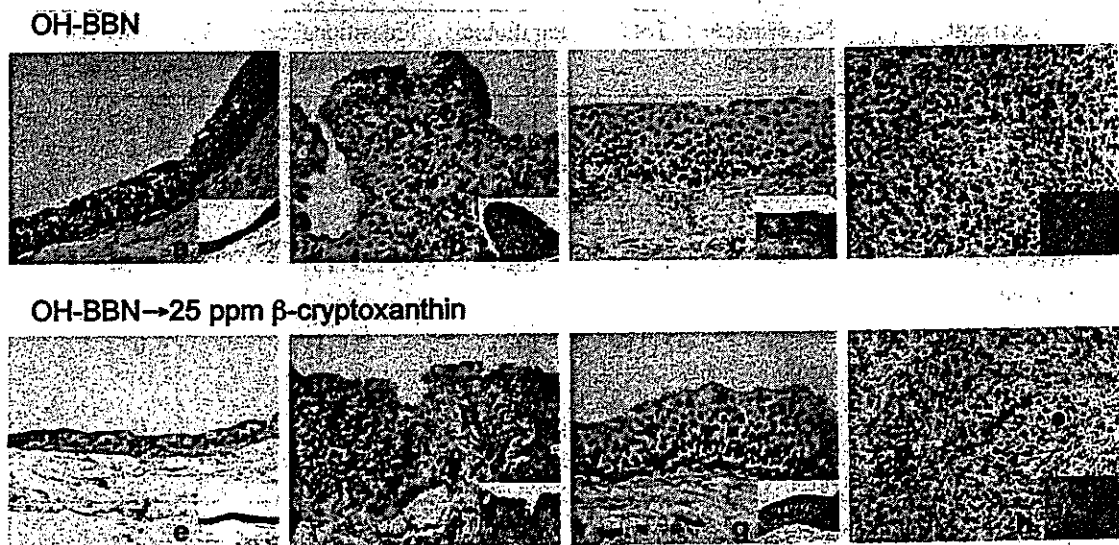


Figure 3. Cyclin D1 immunohistochemistry in the urinary bladder lesions. Inserts are their histopathology. (a-d) From mice given OH-BBN alone and (e-h) from those given OH-BBN and 25 ppm  $\beta$ -cryptoxanthin. (a) and (e), non-lesional transitional epithelium; (b) and (f), papillary or nodular hyperplasia; (c) and (g), dysplasia; and (d) and (h), transitional cell carcinoma. (a-h), Cyclin D1 immunohistochemistry and inserts, hematoxylin and eosin stain, original magnification (a-h) and inserts,  $\times 20$ .

Table VI. Cyclin D1-positive ratio in urinary bladder lesions.

Group no.	Treatment	(% of cyclin D1-positive ratio (no. of lesions or areas examined))			
		Non-lesional	Hyperplasia	Dysplasia	Carcinoma
1	OH-BBN	4.00 $\pm$ 1.46 <sup>a</sup> (16)	18.00 $\pm$ 1.86 (16)	31.50 $\pm$ 5.28 (12)	58.25 $\pm$ 10.46 (8)
2	OH-BBN $\rightarrow$ 1 ppm $\beta$ -cryptoxanthin	2.78 $\pm$ 1.11 <sup>b</sup> (18)	17.00 $\pm$ 2.50 (16)	23.67 $\pm$ 3.32 <sup>b</sup> (9)	46.25 $\pm$ 7.72 (4)
3	OH-BBN $\rightarrow$ 5 ppm $\beta$ -cryptoxanthin	2.16 $\pm$ 0.96 <sup>c</sup> (19)	15.73 $\pm$ 2.01 <sup>d</sup> (11)	17.43 $\pm$ 4.43 <sup>c,e</sup> (7)	42.75 $\pm$ 5.74 (4)
4	OH-BBN $\rightarrow$ 25 ppm $\beta$ -cryptoxanthin	1.74 $\pm$ 0.81 <sup>c,e</sup> (19)	13.00 $\pm$ 1.60 <sup>c,f,g</sup> (8)	12.50 $\pm$ 3.56 <sup>c,f</sup> (6)	35.50 $\pm$ 3.54 <sup>d</sup> (2)
5	25 ppm $\beta$ -cryptoxanthin	0.48 $\pm$ 0.18 <sup>c,f,h,i</sup> (10)	ND	ND	ND
6	None	0.30 $\pm$ 0.12 <sup>c,f,h,j</sup> (10)	ND	ND	ND

<sup>a</sup>Mean  $\pm$  SD. ND, not determined. <sup>b-d</sup>Significantly different from group 1 by Bonferroni Multiple Comparisons Test (<sup>b</sup>P<0.01, <sup>c</sup>P<0.001 and <sup>d</sup>P<0.05). <sup>e,f</sup>Significantly different from group 2 by Bonferroni Multiple Comparisons Test (<sup>e</sup>P<0.05 and <sup>f</sup>P<0.001). <sup>g,h</sup>Significantly different from group 3 by Bonferroni Multiple Comparisons Test (<sup>g</sup>P<0.05 and <sup>h</sup>P<0.001). <sup>i,j</sup>Significantly different from group 4 by Bonferroni Multiple Comparisons Test (<sup>i</sup>P<0.05 and <sup>j</sup>P<0.001).

Cyclin D1 is a member of the G1 cyclin family that is involved in regulating the transition through the G1 phase of the cell cycle (33,34). Cyclin D1 over-expression was reported

in various human malignant tumors (35) and in murine chemically-induced malignancies (36). Over-expression of cyclin D1 is suggested to be associated with BBN-induced

urinary bladder carcinogenesis, that is, over-expression was higher in the order, 'simple hyperplasia', 'papillary or nodular hyperplasia', 'papilloma' and 'carcinoma' (37). We previously reported that ceratin cancer chemopreventive agents reduced the incidence and multiplicity of epithelial malignancy in the target tissues by lowering the cyclin D1-positive ratio (16,25,26, 28,29,38,39). A significant relationship between cyclin D1 over-expression and tumor grade or stage was also reported in human transitional cell carcinomas (32). Furthermore, some investigations suggested that over-expression of cyclin D1 could be a useful marker in estimating malignancy in the early stage of urinary carcinogenesis (40). Our immunohistochemical results indicated that the intensity of cyclin D1 over-expression in mice treated with OH-BBN alone was basically in the order of 'hyperplasia', 'dysplasia' and 'carcinoma'. This is consisted with the findings in previous reports by others (41,42) and our own (25). In the current study,  $\beta$ -cryptoxanthin effectively suppressed cyclin D1-positive cell ratios of various urinary bladder lesions, implying that reduction in the incidence of tumors and preneoplastic lesions of urinary bladder is related to effectively suppressed cell cycle progression by the treatment with  $\beta$ -cryptoxanthin. In a previous study, selective cyclin D1 inhibitors, silymarin (25) and 1,4-phenylene diisothiocyanate (26), possessed chemopreventive potential on BBN-induced urinary bladder carcinogenesis in male ICR mice.

Other possible mechanisms for the anti-carcinogenic potential of plant carotenoids are proposed. They include the antioxidant functions (scavenging free radicals and quenching singlet oxygen) that are associated with lowered DNA damage, diminished membrane lipid peroxidation and inhibition of malignant transformation *in vitro* (43).  $\beta$ -cryptoxanthin is enzymatically converted to retinol, which is involved in cell differentiation, such as  $\beta$ - and  $\alpha$ -carotenes. Dietary administration with  $\beta$ -cryptoxanthin (25 ppm) significantly reduced cyclin D1-positive cell ratios in bladder lesions, and decreased the incidence of urinary bladder carcinomas as well as dysplasia. Thus, control of cell proliferation is important for cancer prevention, because cell proliferation has essential roles in carcinogenesis including the processes of initiation and promotion (44). In rodent models for carcinogenesis, certain chemopreventive agents suppress carcinogen-induced hyperproliferation of cells in the target organs when given during the initiation as well as the post-initiation phases (45). In fact, the number of abnormal mitoses in the bladder cancers of mice treated with OH-BBN and  $\beta$ -cryptoxanthin was lower than that of mice given OH-BBN alone (data not shown).

In conclusion, our study provided further evidence that dietary  $\beta$ -cryptoxanthin is able to suppress rodent carcinogenesis. The oxygenated carotenoid  $\beta$ -cryptoxanthin is one of the major carotenoids in the blood, and recent epidemiological studies revealed an inverse association between serum and diet concentrations of  $\beta$ -cryptoxanthin and the risks of cancer in various organs (46-49), including the urinary bladder (50).  $\beta$ -cryptoxanthin is non-toxic as found in this study and previous long-term *in vivo* experiments (14-17). Therefore, it is worthy to investigate the relationship between the intake of  $\beta$ -cryptoxanthin and occurrence of malignant neoplasms in

other sites. Also, detailed mechanistic studies on chemopreventive effects of this compound are warranted: such studies are underway in our laboratories.

### Acknowledgements

This study was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan; a Grant-in-Aid for the 3rd Term for a Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan; a Grants-in-Aid for Scientific Research (no. 15592007) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a Grant (H2006-6) from Kanazawa Medical University.

### References

- Coleman MP, Esteve J, Damiecki P, Arslan A and Renard H: Trends in cancer incidence and mortality. IARC Sci Publ 121: 1-806, 1993.
- Jemal A, Murray T, Ward E, *et al*: Cancer statistics, 2005. CA Cancer J Clin 55: 10-30, 2005.
- Ajiki W, Tsukuma H and Oshima A: Cancer incidence and incidence rates in Japan in 1999: estimates based on data from 11 population-based cancer registries. Jpn J Clin Oncol 34: 352-356, 2004.
- Leppert JT, Shvarts O, Kawaoka K, Lieberman R, Beldegrun AS and Pantuck AJ: Prevention of bladder cancer: a review. Eur Urol 49: 226-234, 2006.
- Grossman HB: Chemoprevention of bladder cancer. Urology 67: 19-22, 2006.
- Reddy BS, Hirose Y, Cohen LA, Simi B, Cooma I and Rao CV: Preventive potential of wheat bran fractions against experimental colon carcinogenesis: implications for human colon cancer prevention. Cancer Res 60: 4792-4797, 2000.
- Tanaka T, Kohno H and Mori H: Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. Asian Pac J Cancer Prev 2: 165-177, 2001.
- Glade MJ: Food, nutrition and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997. Nutrition 15: 523-526, 1999.
- Faure H, Fayol V, Galabert C, *et al*: Carotenoids: 1. Metabolism and physiology. Ann Biol Clin (Paris) 57: 169-183, 1999.
- Tanaka T, Morishita Y, Suzui M, Kojima T, Okumura A and Mori H: Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis 15: 15-19, 1994.
- Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K and Hara A: Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. Cancer Res 55: 4095-4064, 1995.
- Tanaka T, Kawamori T, Ohnishi M, *et al*: Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the post-initiation phase. Carcinogenesis 16: 2957-2963, 1995.
- Tsushima M, Maoka T, Katsuyama M, *et al*: Inhibitory effect of natural carotenoids on Epstein-Barr virus activation activity of a tumor promoter in Raji cells. A screening study for anti-tumor promoters. Biol Pharm Bull 18: 227-233, 1995.
- Nishino H, Tokuda H, Murakoshi M, *et al*: Cancer prevention by natural carotenoids. Biofactors 13: 89-94, 2000.
- Narisawa T, Fukaura Y, Oshima S, Inakuma T, Yano M and Nishino H: Chemoprevention by the oxygenated carotenoid  $\beta$ -cryptoxanthin of N-methylnitrosourea-induced colon carcinogenesis in F344 rats. Jpn J Cancer Res 90: 1061-1065, 1999.
- Tanaka T, Kohno H, Murakami M, *et al*: Suppression of azoxymethane-induced colon carcinogenesis in male F344 rats by mandarin juices rich in  $\beta$ -cryptoxanthin and hesperidin. Int J Cancer 88: 146-150, 2000.
- Kohno H, Taima M, Sumida T, Azuma Y, Ogawa H and Tanaka T: Inhibitory effect of mandarin juice rich in  $\beta$ -cryptoxanthin and hesperidin on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary tumorigenesis in mice. Cancer Lett 174: 141-150, 2001.

18. De Stefani E, Brennan P, Boffetta P, Ronco AL, Mendilaharsu M and Deneo-Pellegrini H: Vegetables, fruits, related dietary antioxidants, and risk of squamous cell carcinoma of the esophagus: a case-control study in Uruguay. *Nutr Cancer* 38: 23-29, 2000.
19. Goodman MT, McDuffie K, Hernandez B, *et al*: The association of plasma micronutrients with the risk of cervical atypical squamous cells of undetermined significance (ASCUS). *Asian Pac J Cancer Prev* 1: 337-345, 2000.
20. Yuan JM, Stram DO, Arakawa K, Lee HP and Yu MC: Dietary cryptoxanthin and reduced risk of lung cancer: the Singapore Chinese Health Study. *Cancer Epidemiol Biomarkers Prev* 12: 890-898, 2003.
21. Mannisto S, Smith-Warner SA, Spiegelman D, *et al*: Dietary carotenoids and risk of lung cancer in a pooled analysis of seven cohort studies. *Cancer Epidemiol Biomarkers Prev* 13: 40-48, 2004.
22. Zeegers MP, Goldbohm RA and van den Brandt PA: Are retinol, vitamin C, vitamin E, folate and carotenoids intake associated with bladder cancer risk? Results from the Netherlands Cohort Study. *Br J Cancer* 85: 977-983, 2001.
23. Hirose Y, Tanaka T, Kawamori T, *et al*: Chemoprevention of urinary bladder carcinogenesis by the natural phenolic compound protocatechuic acid in rats. *Carcinogenesis* 16: 2337-2342, 1995.
24. Yang M, Tanaka T, Hirose Y, Deguchi T, Mori H and Kawada Y: Chemopreventive effects of diosmin and hesperidin on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary-bladder carcinogenesis in male ICR mice. *Int J Cancer* 73: 719-724, 1997.
25. Vinh PQ, Sugie S, Tanaka T, *et al*: Chemopreventive effects of a flavonoid antioxidant silymarin on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in male ICR mice. *Jpn J Cancer Res* 93: 42-49, 2002.
26. Sugie S, Vinh PQ, Rahman KM, *et al*: Suppressive effect of 1,4-phenylene diisothiocyanate on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in male ICR mice. *Int J Cancer* 117: 524-530, 2005.
27. Petty WJ, Dragnev KH and Dmitrovsky E: Cyclin D1 as a target for chemoprevention. *Lung Cancer* 41 (Suppl. 1): S155-S161, 2003.
28. Kohno H, Suzuki R, Sugie S, Tsuda H and Tanaka T: Dietary supplementation with silymarin inhibits 3,2'-dimethyl-4-aminobiphenyl-induced prostate carcinogenesis in male F344 rats. *Clin Cancer Res* 11: 4962-4967, 2005.
29. Suzuki R, Kohno H, Suzui M, *et al*: An animal model for the rapid induction of tongue neoplasms in human c-Ha-ras proto-oncogene transgenic rats by 4-nitroquinoline 1-oxide: its potential use for preclinical chemoprevention studies. *Carcinogenesis* 27: 619-630, 2006.
30. Fukushima S, Murasaki G, Hirose M, Nakanishi K, Hasegawa R and Ito N: Histopathological analysis of preneoplastic changes during *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in rats. *Acta Pathol Jpn* 32: 243-250, 1982.
31. Otori K, Sugiyama K, Fukushima S and Esumi H: Expression of the *cyclin D1* gene in rat colorectal aberrant crypt foci and tumors induced by azoxymethane. *Cancer Lett* 140: 99-104, 1999.
32. Lee CC, Yamamoto S, Morimura K, *et al*: Significance of cyclin D1 over-expression in transitional cell carcinomas of the urinary bladder and its correlation with histopathologic features. *Cancer* 79: 780-789, 1997.
33. Matsushime H, Roussel MF, Ashmun RA and Sherr CJ: Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 65: 701-713, 1991.
34. Motokura T, Bloom T, Kim HG, *et al*: A novel cyclin encoded by a *bcl1*-linked candidate oncogene. *Nature* 350: 512-515, 1991.
35. Lonardo F, Rusch V, Langenfeld J, Dmitrovsky E and Klimstra DS: Over-expression of cyclins D1 and E is frequent in bronchial preneoplasia and precedes squamous cell carcinoma development. *Cancer Res* 59: 2470-2476, 1999.
36. Said TK and Medina D: Cell cyclins and cyclin-dependent kinase activities in mouse mammary tumor development. *Carcinogenesis* 16: 823-830, 1995.
37. Salim EI, Wanibuchi H, Morimura K, *et al*: Inhibitory effects of 1,3-diaminopropane, an ornithine decarboxylase inhibitor, on rat two-stage urinary bladder carcinogenesis initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Carcinogenesis* 21: 195-203, 2000.
38. Yoshida K, Hirose Y, Tanaka T, *et al*: Inhibitory effects of troglitazone, a peroxisome proliferator-activated receptor gamma ligand, in rat tongue carcinogenesis initiated with 4-nitroquinoline 1-oxide. *Cancer Sci* 94: 365-371, 2003.
39. Yoshida K, Tanaka T, Hirose Y, *et al*: Dietary garcinol inhibits 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats. *Cancer Lett* 221: 29-39, 2005.
40. Suwa Y, Takano Y, Iki M, *et al*: Cyclin D1 protein over-expression is related to tumor differentiation, but not to tumor progression or proliferative activity, in transitional cell carcinoma of the bladder. *J Urol* 160: 897-900, 1998.
41. Wanibuchi H, Yamamoto S, Chen H, *et al*: Promoting effects of dimethylarsinic acid on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in rats. *Carcinogenesis* 17: 2435-2439, 1996.
42. Jiao D, Eklind KI, Choi CI, Desai DH, Amin SG and Chung FL: Structure-activity relationships of isothiocyanates as mechanism-based inhibitors of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in *A/J* mice. *Cancer Res* 54: 4327-4333, 1994.
43. Bertram JS and Bortkiewicz H: Dietary carotenoids inhibit neoplastic transformation and modulate gene expression in mouse and human cells. *Am J Clin Nutr* 62 (Suppl. 6): S1327-S1336, 1995.
44. Tanaka T: Chemoprevention of human cancer: biology and therapy. *Crit Rev Oncol Hematol* 25: 139-174, 1997.
45. Mori H, Sugie S, Yoshimi N, Hara A and Tanaka T: Control of cell proliferation in cancer prevention. *Mutat Res* 428: 291-298, 1999.
46. Shikany JM, Witte JS, Henning SM, *et al*: Plasma carotenoids and the prevalence of adenomatous polyps of the distal colon and rectum. *Am J Epidemiol* 145: 552-557, 1997.
47. Comstock GW, Alberg AJ, Huang HY, *et al*: The risk of developing lung cancer associated with antioxidants in the blood: ascorbic acid, carotenoids,  $\alpha$ -tocopherol, selenium and total peroxyl radical absorbing capacity. *Cancer Epidemiol Biomarkers Prev* 6: 907-916, 1997.
48. Goodman MT, Kiviat N, McDuffie K, *et al*: The association of plasma micronutrients with the risk of cervical dysplasia in Hawaii. *Cancer Epidemiol Biomarkers Prev* 7: 537-544, 1998.
49. Gann PH, Ma J, Giovannucci E, *et al*: Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. *Cancer Res* 59: 1225-1230, 1999.
50. Nomura AM, Lee J, Stemmermann GN and Franke AA: Serum vitamins and the subsequent risk of bladder cancer. *J Urol* 170: 1146-1150, 2003.

# Gastric and intestinal phenotypes and histogenesis of advanced glandular stomach cancers in carcinogen-treated, *Helicobacter pylori*-infected Mongolian gerbils

Tsutomu Mizoshita,<sup>1</sup> Tetsuya Tsukamoto,<sup>1,3</sup> Yoshiharu Takenaka,<sup>1,2</sup> Xueyuan Cao,<sup>1</sup> Sosuke Kato,<sup>1</sup> Michio Kaminishi<sup>2</sup> and Masae Tatematsu<sup>1</sup>

<sup>1</sup>Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, and

<sup>2</sup>Department of Gastrointestinal Surgery, The University of Tokyo, Graduate School of Medicine, Tokyo 113-0033, Japan

(Received July 17, 2005/Revised October 4, 2005/Accepted October 24, 2005/Online publication December 18, 2005)

The *Helicobacter pylori*-infected Mongolian gerbil (MG) has been established as an appropriate animal model for studies of stomach cancer development. However, there have hitherto been no data on the phenotypic classification of glandular stomach cancers in *H. pylori*-infected and non-infected MG. We therefore examined the phenotypes of 50 and six advanced glandular stomach cancers in *H. pylori*-infected and non-infected MG, respectively, as well as adjacent non-neoplastic mucosa, using several gastrointestinal epithelial phenotypic markers. The lesions were divided phenotypically into 21 gastric, 24 gastric-and-intestinal mixed, four intestinal and one null types, with 90.0% of the lesions harboring gastric elements and 56.0% demonstrating intestinal phenotypic expression in *H. pylori*-infected MG. All six lesions were classified as gastric type in non-infected MG. There was no clear correlation with the presence of intestinal metaplasia in surrounding mucosa. In conclusion, our data suggest that most advanced adenocarcinomas retain a gastric cellular phenotype in the glandular MG stomach. Thus, it might be proposed that intestinal metaplasia is a paracancerous phenomenon rather than a premalignant condition. *H. pylori* infection may trigger intestinalization of both stomach cancers and non-neoplastic mucosa. (*Cancer Sci* 2006; 97: 38–44)

**H**istologically, human gastric cancers present as two major groups, the 'intestinal' and 'diffuse' types of Lauren,<sup>(1)</sup> which correspond approximately to the 'differentiated' and 'undifferentiated' types, respectively, of Nakamura et al.<sup>(2)</sup> and Sugano et al.<sup>(3)</sup> It has also been suggested that intestinal type carcinomas arise in intestinalized mucosa, whereas the diffuse type develops from the gastric mucosa proper.<sup>(1,2,4,5)</sup> This hypothesis is based on morphological similarities between cancers and intestinal metaplasia (IM), and on the results of comparisons of carcinomas and surrounding mucosa.<sup>(6)</sup> However, previous studies on phenotypic expression and microsatellite instability (MSI) of each IM or stomach cancer cells have pointed to several contradictions to this hypothesis.<sup>(6–17)</sup> The phenotypic expression of stomach cancer cells of each histologic type can be classified clearly into gastric and intestinal epithelial cell types by immunohistochemistry and mucin histochemistry using

gastrointestinal epithelial cell phenotypic markers such as human gastric mucin (HGM), intestinal type alkaline phosphatase (I-ALP), paradoxical concanavalin A staining (PCS), and Alcian blue–periodic acid Schiff staining (AB-PAS).<sup>(18,19)</sup>

The *Helicobacter pylori*-infected Mongolian gerbil (MG) has been established as an appropriate animal model for the study of stomach cancer development, with induction of adenocarcinomas by N-methyl-N-nitrosourea (MNU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as the carcinogens.<sup>(20–24)</sup> We previously demonstrated promoting effects on tumor development due to *H. pylori* infection in the MG model,<sup>(22–25)</sup> and several studies based on detailed histopathological assessment have shown no carcinomas in animals treated only with *H. pylori* infection, suggesting that *H. pylori* is a strong promoter of gastric carcinogenesis rather than an initiator.<sup>(21–23,26)</sup> Eradication of infection results in the curtailment of enhancing effects, particularly in the early stages of associated inflammation.<sup>(21,27,28)</sup> However, there have hitherto been no data on the phenotypic classification of glandular stomach cancers arising in *H. pylori*-infected and non-infected MG. As evaluation of the phenotype is very important with reference to histogenesis, the present study was conducted.

We here examine the phenotypes of advanced glandular stomach cancers, as well as adjacent non-neoplastic mucosa, using several gastrointestinal epithelial phenotypic markers in 50 *H. pylori*-infected and six non-infected MG treated with the carcinogen MNU. An especial focus was on the comparison of small tubular lesions less than 10 mm in diameter and the surrounding mucosa in *H. pylori*-infected and non-infected MG.

## Materials and Methods

### Samples and tissue collection

Advanced carcinomas of the glandular stomach in 50 *H. pylori*-infected and six non-infected MG treated with MNU<sup>(21,29)</sup> were classified histopathologically according to the Japanese Classification of Gastric Carcinomas.<sup>(30)</sup> Tumor

<sup>3</sup>To whom correspondence should be addressed. E-mail: ttsukamt@aichi-cc.jp

tissues and adjacent non-neoplastic mucosa were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2), Bouin's solution, 10% buffered formalin, or 95% ethanol containing 1% acetic acid,<sup>(25,28,31)</sup> sectioned at 5 µm, and stained with hematoxylin and eosin for histologic examination. All of the cancers analyzed demonstrated invasion into the muscularis propria, the subserosa, or the serosa and the peritoneal cavity, sometimes with involvement of adjacent organs.

#### Immunohistochemistry and mucin histochemistry

Immunohistochemical staining was carried out with antibodies against the following antigens: HGM (Novocastra Laboratories, Newcastle-upon-Tyne, UK); small intestinal mucinous antigen (SIMA) (Novocastra); I-ALP (kindly provided by Dr Kazuyuki Hirano, Department of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Japan); and CD10 (Novocastra) (Table 1). The precise procedures for immunohistochemical analysis were as described previously.<sup>(15,18,19,32-34)</sup> With regard to gastric phenotypic markers, we used normal gastric mucosa and normal ileum as positive and negative controls, respectively, or vice versa for intestinal phenotypic controls. Briefly, 4 µm-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% H<sub>2</sub>O<sub>2</sub>/methanol solution, antigen retrieval was achieved by heating in 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 min at 98°C. Sections were then incubated with primary antibodies. After thorough washing in PBS, they were next incubated with biotinylated secondary antibodies, and then with an avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H<sub>2</sub>O<sub>2</sub> and 0.05% 3,3'-diaminobenzidine tetrachloride. Nuclear counterstaining was accomplished using Mayer's hematoxylin.

For mucin histochemistry, we used PCS for identifying class III mucins in mucous neck and pyloric gland cells.<sup>(35,36)</sup> We also carried out AB-PAS for identifying gastric surface mucous cells, with mucin staining red and goblet cells staining blue (Table 1).<sup>(26,33)</sup>

Two independent pathologists (TM and TT) judged the histology and the immunohistochemical and mucin histochemical stainings of the phenotypic markers. With regard to the phenotypic markers, the results of immunohistochemical and mucin histochemical stainings were evaluated in terms of the percentage of positively stained cancer cells,

with 10% and above considered positive, as described previously.<sup>(15,18,19,32,34)</sup>

**Classification of cancers.** Tumors were classified phenotypically with reference to the expression patterns of the phenotypic markers (Table 1).<sup>(15,18,19,32,34)</sup> Glandular stomach cancers in which more than 10% of the section area consisted of at least one gastric or intestinal epithelial cell phenotype were classified as gastric phenotype (G type) or intestinal phenotype (I type) cancers, respectively. Those that showed both gastric and intestinal phenotypes were classified as gastric-and-intestinal mixed phenotype (GI type) cancers, whereas those showing neither gastric nor intestinal phenotype expression were grouped as null (N type).

**Comparison of phenotypic expression between adenocarcinomas and non-neoplastic surrounding mucosa**  
Non-neoplastic glandular ducts were divided histologically and phenotypically into three types: (i) gastric phenotypic glandular (G type gland), consisting of at least one gastric epithelial cell phenotype; (ii) gastric-and-intestinal mixed phenotypic glandular (GI type gland), showing both gastric and intestinal phenotypes; and (iii) intestinal phenotypic glandular (I type gland), harboring at least one intestinal counterpart.<sup>(15,18,33)</sup> Particular attention was paid to comparisons of adjacent normal glands with tubular adenocarcinomas less than 10 mm in diameter in 13 *H. pylori*-infected and five non-infected MG.

## Results

### Phenotypic expression of non-neoplastic glandular ducts in the MG glandular stomach

Typical findings for immunohistochemical and mucin histochemical staining in the non-cancerous mucosa of glandular stomach are illustrated in Fig. 1. HGM was detected in the cytoplasm of foveolar epithelial cells of the pyloric (Fig. 1A,F) and fundic glandular ducts. It was also found in GI-IM. PCS was observed in the cytoplasm of normal pyloric glands (Fig. 1A,G) and mucous neck cells. In GI-IM, the cytoplasm of columnar and goblet cells was stained red and blue, respectively, with AB-PAS (Fig. 1B,H). Regarding I-IM, the cytoplasm of goblet cells was stained blue with AB, while that of columnar cells was not stained red with PAS (Fig. 1C,I). I-ALP and CD10 were apparent at the luminal surfaces of absorptive cells in the duodenum (Fig. 1D,E,J). SIMA was evident in the cytoplasm of goblet cells in the duodenum (Fig. 1D,K).

### Phenotypic classification of advanced glandular stomach cancers in *H. pylori*-infected and non-infected MG treated with MNU

Typical findings for mucin and brush border staining in glandular stomach cancers are shown in Figs 2 and 3. We evaluated 44 differentiated and six undifferentiated glandular stomach cancers of *H. pylori*-infected MG phenotypically using several epithelial phenotypic markers. The lesions were divided phenotypically into 21 G, 24 GI, four I and one N type (Table 2). Of the 44 differentiated type cancers, 39 (88.6%) had gastric phenotypic expression, whereas 22 (50%) harbored intestinal elements. All six undifferentiated

Table 1. Phenotypic markers for gastrointestinal epithelial cells

Tissue type	Cell type	Marker
Gastric	Foveolar	HGM, PAS (mucin stained red)
	Pyloric	PCS
Intestinal	Goblet	AB (mucin stained blue), SIMA
	Absorptive	I-ALP, CD10

AB, Alcian blue staining; HGM, human gastric mucin; I-ALP, intestinal type alkaline phosphatase; PAS, periodic acid-Schiff staining; PCS, paradoxical concanavalin A staining; SIMA, small intestinal mucinous antigen.



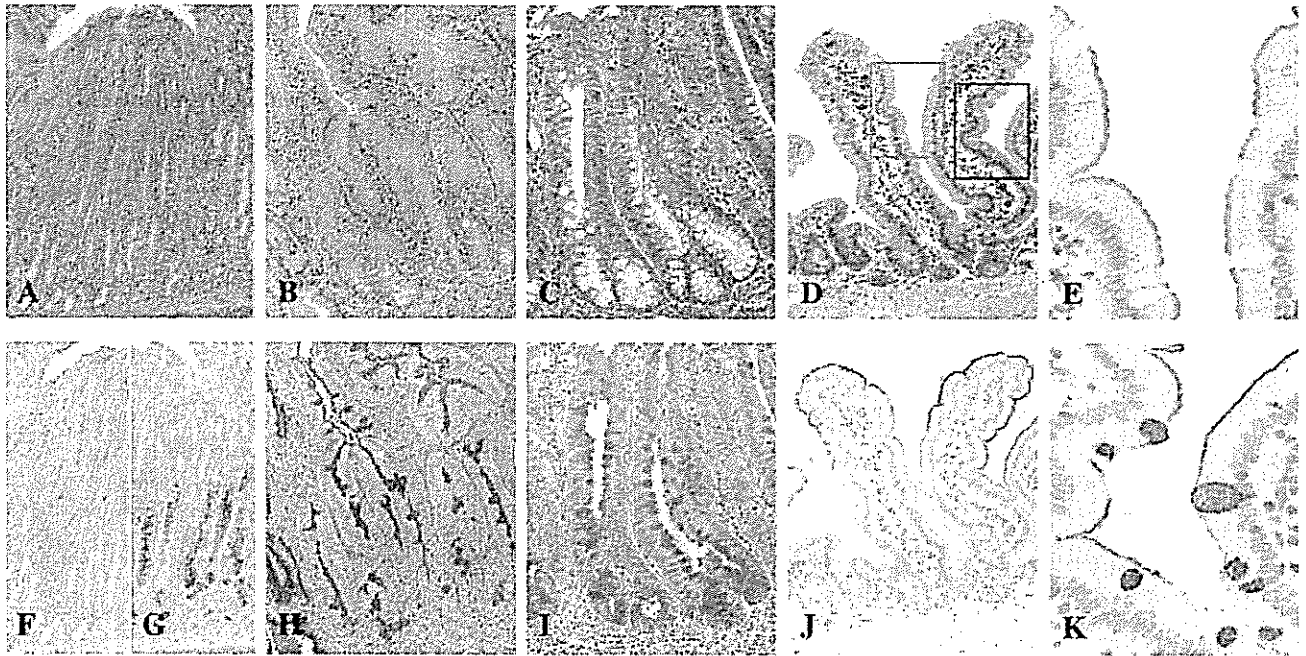


Fig. 1. Expression of gastrointestinal phenotypic markers in non-neoplastic mucosa. (A,F,G) gastric (G type) glands; (B,H) gastric-and-intestinal mixed (GI type) glands; (C,I) intestinal (I type) glands; and (D,E,J,K) normal duodenal glandular ducts. (A) H&E staining of pyloric glandular ducts. (B) H&E staining of GI-IM. (C) H&E staining of I-IM. (D) H&E staining of duodenal glandular ducts. (E) Higher magnification of the red square in part D. Note the presence of CD10 at the luminal surface of absorptive cells in the duodenum. (F) Human gastric mucin is evident in the cytoplasm of normal foveolar epithelial cells in the pyloric mucosa. (G) Paradoxical concanavalin A staining is present in the cytoplasm of normal pyloric gland cells. (H) The cytoplasm of columnar and goblet cells in the IM is stained red and blue, respectively, with AB-PAS. (I) The cytoplasm of goblet cells in IM is stained blue with AB, while that of columnar cells is not stained red with PAS. (J) Intestinal type alkaline phosphatase is present at the luminal surface of absorptive cells in duodenum. (K) Higher magnification of the blue square in part D. Small intestinal mucinous antigen is evident in the cytoplasm of goblet cells in the duodenum. Original magnification: A, F and G,  $\times 100$ ; B, D, H and J,  $\times 160$ ; C and I,  $\times 200$ ; E and K,  $\times 640$ . AB-PAS, Alcian blue-periodic acid Schiff staining; GI-IM, gastric-and-intestinal mixed phenotype IM; IM, intestinal metaplasia; I-IM, solely intestinal phenotype IM.

Table 2. Histologic and phenotypic classification in advanced carcinomas of glandular stomach in 50 *Helicobacter pylori*-infected and six non-infected Mongolian gerbils treated with *N*-methyl-*N*-nitrosourea

<i>H. pylori</i>	Histologic classification <sup>†</sup>	Phenotypic classification <sup>‡</sup>				
		G	GI	I	N	Total
Infected	Differentiated	21	18	4	1	44
	Undifferentiated	0	6	0	0	6
	Subtotal	21	24	4	1	50
Non-infected	Differentiated	5	0	0	0	5
	Undifferentiated	1	0	0	0	1
	Subtotal	6	0	0	0	6

<sup>†</sup>Classification based on the structure of elements. 'Differentiated' includes tubular types, whereas 'undifferentiated' consists of signet-ring cell and poorly differentiated types. <sup>‡</sup>The numbers of adenocarcinomas possessing intestinal phenotypic expression are 24 gastric-and-intestinal mixed (GI) and four intestinal (I) types in *H. pylori*-infected gerbils (57% among gastric [G], GI and I types), while corresponding cases do not exist in non-infected animals, the former was significantly higher (Fisher's exact test,  $P < 0.05$ ). Null (N) type is excluded from this statistical analysis.

type cancers were judged as GI type. We also evaluated five differentiated and one undifferentiated glandular stomach cancers developing in non-infected MG, all six lesions being of G type (Table 2). The lesions of *H. pylori*-infected groups

had more intestinal phenotypic expression compared with those of non-infected groups ( $P < 0.05$ ). Tumor histology was mostly homogeneous in the gerbil stomach adenocarcinomas in the current study.

#### Relationships between carcinomas of tubular structure and non-neoplastic surrounding mucosa in the glandular stomach of *H. pylori*-infected and non-infected MG from the viewpoint of phenotypic expression

The relationship between adenocarcinomas of tubular structure, which measured less than 10.0 mm in largest dimension, and adjacent non-neoplastic surrounding mucosa is shown in Table 3. Of 13 lesions, 12 (92.3%) had non-neoplastic surrounding mucosa with G type glands. One G type lesion (case 7) had adjacent non-cancerous mucosa harboring GI and I type glands, and no G type glands. The non-neoplastic surrounding mucosa had only G type glands in seven lesions of G type (cases 1, 2, 3, 4, 5, 6 and 8) (Table 3). In the remaining six cases (cases 7, 9, 10, 11, 12 and 13), the phenotypes of the adenocarcinomas of tubular structure did not coincide with those of non-neoplastic surrounding mucosa.

Regarding the glandular stomach cancers in the non-infected MG, the non-neoplastic surrounding mucosa had only G type glands in all of the five G type tubular lesions.

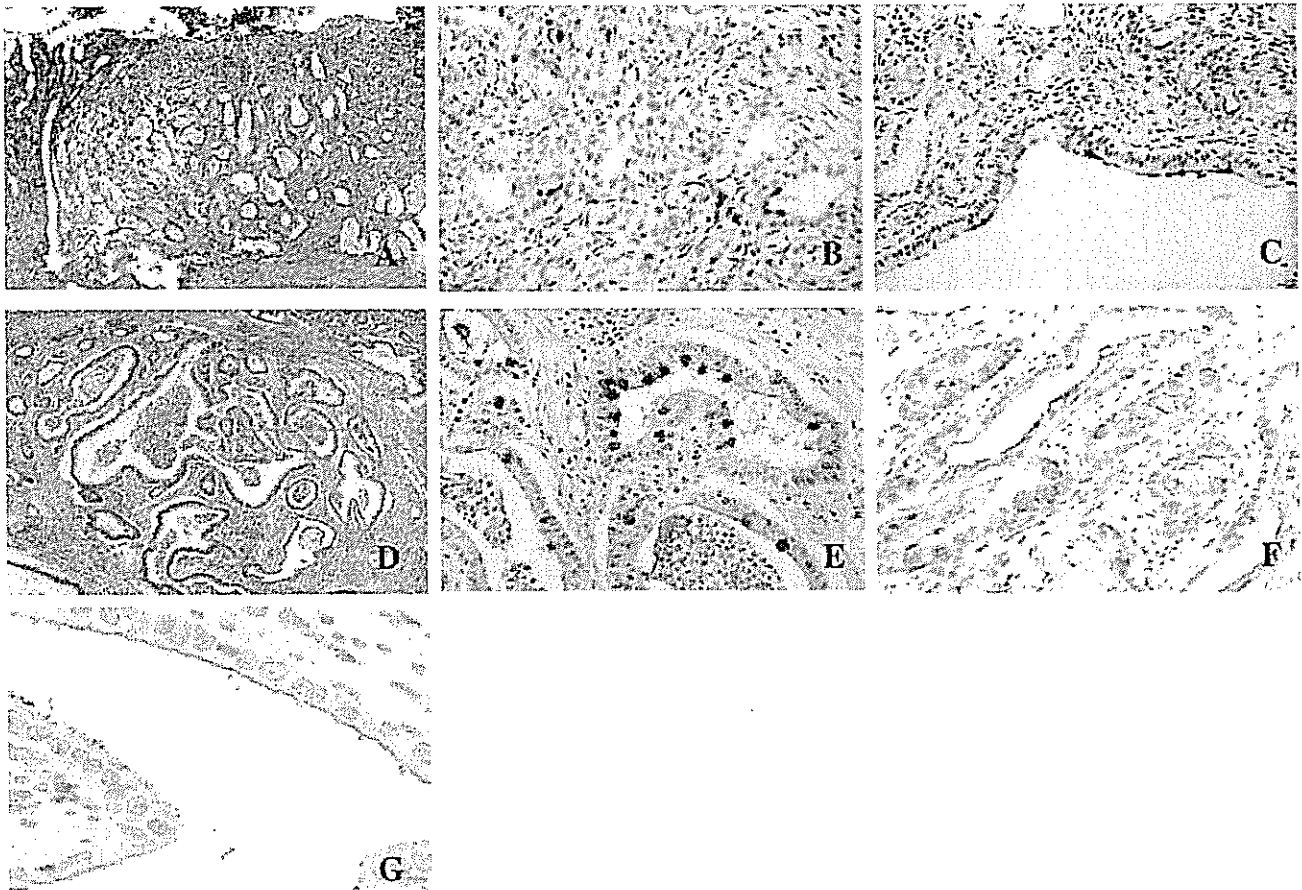


Fig. 2. Histology and phenotype of well-differentiated adenocarcinomas: (A–C) gastric (G type) and (D–G) intestinal (I type). (A) H&E staining of the G type tumor. (B) Human gastric mucin is present in the cytoplasm of tumor cells. (C) Paradoxical concanavalin A is present in the cytoplasm of cancer cells. (D) H&E staining of the I type tumor. (E) Higher magnification of the red square in part D. The cytoplasm of cancer cells is stained blue by Alcian blue-periodic acid Schiff staining. (F) Small intestinal mucinous antigen is evident in the cytoplasm of cancer cells. (G) CD10 is present at the luminal surface of cancer cells. Original magnification: A,  $\times 40$ ; D,  $\times 100$ ; B and C,  $\times 200$ ; E and F,  $\times 320$ ; G,  $\times 640$ .

Table 3. Relationships between carcinomas and adjacent non-neoplastic mucosa in the glandular stomach of *Helicobacter pylori*-infected Mongolian gerbils treated with *N*-methyl-*N*-nitrosourea

Number	Clinicopathological findings of tumors			Phenotype of adjacent non-neoplastic mucosa		
	Histologic type	Depth	Phenotype	G type glands	GI type glands	I type glands
Case 1	well	mp	G	+	-	-
Case 2	well	mp	G	+	-	-
Case 3	well	mp	G	+	-	-
Case 4	mod	mp	G	+	-	-
Case 5	well	ss	G	+	-	-
Case 6	well	ss	G	+	-	-
Case 7	well	ss	G	-	+	+
Case 8	mod	ss	G	+	-	-
Case 9	mod	ss	G	+	+	-
Case 10	well	ss	GI	+	-	-
Case 11	well	ss	GI	+	-	-
Case 12	well	mp	I	+	-	-
Case 13	well	ss	I	+	+	-

mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; well, well differentiated adenocarcinoma. Tumor types are gastric (G), intestinal (I) and gastric-and-intestinal mixed (GI).

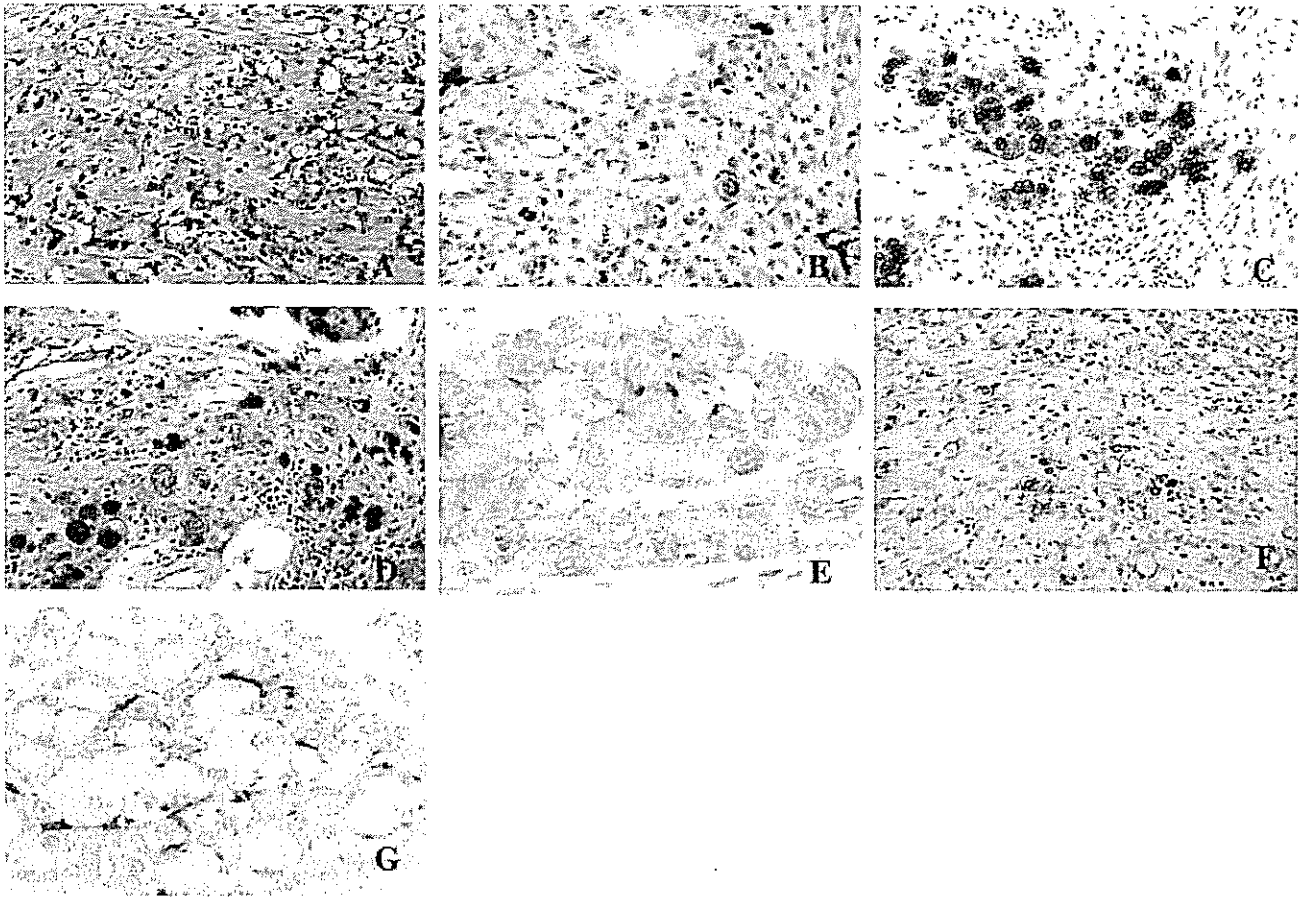


Fig. 3. Histology and phenotype of poorly differentiated adenocarcinomas: (A–C) gastric (G type) and (D–G) intestinal (I type). (A) H&E staining of the G type tumor. (B) Human gastric mucin is present in the cytoplasm of malignant cells. (C) Paradoxical concanavalin A is present in the cytoplasm of tumor cells. (D) The cytoplasm of tumor cells was stained blue by Alcian blue–periodic acid Schiff staining. (E) Small intestinal mucinous antigen is evident in the cytoplasm of cancer cells. (F) I-ALP is positive at the luminal surface of cancer cells. (G) CD10 is present at the luminal surface of cancer cells. Original magnification: A–D and F,  $\times 200$ ; E and G,  $\times 640$ .

## Discussion

Our present data provide clear evidence that most advanced adenocarcinomas of the glandular stomach in *H. pylori*-infected MG treated with MNU are characterized by gastric phenotypic expression. All of the six lesions were classified phenotypically as G type in non-infected MG treated with MNU. We have shown previously that experimentally induced adenocarcinomas in the rat glandular stomach consist mainly of gastric epithelial phenotypic cancer cells, with intestinal epithelial phenotypic cancer cells appearing later in larger tumors.<sup>(7,8,37,38)</sup> Similarly, in mice, experimentally induced adenocarcinomas in the glandular stomach<sup>(39,40)</sup> consist mainly of gastric epithelial phenotypic tumor cells.<sup>(41)</sup> We and others have also reported that the phenotypic shift from gastric to intestinal phenotypic expression occurs in accordance with increasing depth of invasion in human signet ring cell carcinomas and with progression in human differentiated gastric cancers.<sup>(42–44)</sup> Early stage papillary (papillary dominant) stomach cancers show significantly higher and more widespread high-frequency microsatellite instability (MSI-H) than other morphological types, and inactivation of human

MutL homologue 1 (hMLH1) expression by promoter hypermethylation may be an early event in carcinogenesis of this type of stomach cancer.<sup>(45,46)</sup> MSI-positive differentiated gastric cancers with gastric foveolar phenotypic expression in the early stages sometimes demonstrate intestinal phenotypic expression in advanced stages.<sup>(47)</sup> Taking into account the previous reports and our present data, we consider that the same phenotypic shift occurs in both rodents and humans, and *H. pylori* infection may trigger intestinalization of stomach cancers and non-neoplastic mucosa.

It has been suggested that differentiated gastric carcinomas arise from mucosa with IM, but that undifferentiated gastric cancers originate from mucosa without IM in view of morphological similarities between each cancer and the surrounding mucosa.<sup>(1,2,4,5)</sup> However, this has not been supported by previous studies on phenotypic expression and MSI of individual intestinal metaplastic or stomach cancer cells.<sup>(6–17)</sup> We here found seven G type tubular lesions with non-neoplastic surrounding mucosa solely of G type, but in the remaining six cases the tumor phenotype did not match that of the immediately adjacent normal glands in *H. pylori*-infected MG (Table 3). Regarding the glandular stomach

cancers in non-infected MG, the non-neoplastic surrounding mucosa had only G type glands in all five G type lesions. Heterotopic proliferative glands (HPG) frequently develop with *H. pylori* infection in the glandular stomach of infected MG, with slight dysplastic change of constituent cells.<sup>(26)</sup> They often resemble differentiated or mucinous adenocarcinomas, but do not appear to be malignant, disappearing with little evidence of persistence after eradication of bacteria. However, HPG also show a phenotypic shift from G type to GI or I type with the appearance of Paneth cells during the overall course of *H. pylori* infection.<sup>(26)</sup> Intestinalization progresses from G through GI to I type in non-cancerous and cancerous tissue independently in humans and MG.<sup>(18,48)</sup> Thus, it has been proposed that IM is important not as a pre-cancerous lesion but as a paracancerous phenomenon.<sup>(11,12)</sup> Therefore, many questions remain regarding its pathogenesis as well as the actual relationship to gastric cancers.<sup>(18,48)</sup> In the present study, of 13 lesions, 12 (92.3%) had non-neoplastic surrounding mucosa with G type glands in *H. pylori*-infected MG treated with the carcinogens. In the rat, Tatematsu et al. have proposed pepsinogen 1-altered pyloric glands, which are low in pepsinogen 1 but appear normal in the pyloric mucosa after MNNG treatment, as putative preneoplastic lesions in the glandular stomach.<sup>(49-51)</sup> Thus, a kind of G type gland may be precancerous. We therefore consider that the origin of stomach cancers might be clarified by analysis of the genetic alteration in stomach cancers and non-neoplastic G type glands in *H. pylori*-infected MG treated with the carcinogens.

Stomach cancers frequently show variable morphology within individual tumors in humans and this may reflect dif-

ferent genetic alterations. A strong relationship has been observed between early stage papillary stomach cancer and MSI-H, and promoter hypermethylation of hMLH1 may be important for the development of this type of stomach cancer.<sup>(45)</sup> Mismatch repair deficiency in MSI-positive tumors causes multiple gene inactivations through frameshift mutations in short repetitive sequences in a heterogenous way within a histologically heterogenous tumor.<sup>(52)</sup> Song et al.<sup>(46)</sup> also suggested that a modest centromere numerical abnormality might be another characteristic of stomach cancer characterized by a papillary structure. Further studies of genetic alterations in stomach cancers of MG may be needed to clarify the different carcinogenetic pathways for various types of human stomach cancers.

In conclusion, our data suggest that most advanced adenocarcinomas retain a gastric cellular phenotype in the glandular MG stomach. Thus, it might be proposed that IM is a paracancerous phenomenon rather than a premalignant condition. *H. pylori* infection may trigger intestinalization of both stomach cancers and non-neoplastic mucosa.

## Acknowledgments

The authors thank Dr Malcolm A. Moore for revision of the scientific English language and Ms Hisayo Ban for expert technical assistance. This study was supported in part by a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control, a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References

- 1 Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma: an attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; 64: 31-49.
- 2 Nakamura K, Sugano H, Takagi K. Carcinoma of the stomach in incipient phase: its histogenesis and histological appearances. *Gann* 1968; 59: 251-8.
- 3 Sugano H, Nakamura K, Kato Y. Pathological studies of human gastric cancer. *Acta Pathol Jpn* 1982; 32 (Suppl. 2): 329-47.
- 4 Correa P. A human model of gastric carcinogenesis. *Cancer Res* 1988; 48: 3554-60.
- 5 Correa P. *Helicobacter pylori* and gastric carcinogenesis. *Am J Surg Pathol* 1995; 19 (Suppl. 1): S37-43.
- 6 Kawachi H, Takizawa T, Eishi Y et al. Absence of either gastric or intestinal phenotype in microscopic differentiated gastric carcinomas. *J Pathol* 2003; 199: 436-46.
- 7 Tatematsu M, Furihata C, Katsuyama T et al. Independent induction of intestinal metaplasia and gastric cancer in rats treated with N-methyl-N'-nitro-N-nitrosoguanidine. *Cancer Res* 1983; 43: 1335-41.
- 8 Tatematsu M, Katsuyama T, Furihata C, Tsuda H, Ito N. Stable intestinal phenotypic expression of gastric and small intestinal tumor cells induced by N-methyl-N'-nitro-N-nitrosoguanidine or methylnitrosourea in rats. *Gann* 1984; 75: 957-65.
- 9 Hattori T. Development of adenocarcinomas in the stomach. *Cancer* 1986; 57: 1528-34.
- 10 Tatematsu M, Furihata C, Katsuyama T et al. Gastric and intestinal phenotypic expressions of human signet ring cell carcinomas revealed by their biochemistry, mucin histochemistry, and ultrastructure. *Cancer Res* 1986; 46: 4866-72.
- 11 Tatematsu M, Ichinose M, Miki K, Hasegawa R, Kato T, Ito N. Gastric and intestinal phenotypic expression of human stomach cancers as revealed by pepsinogen immunohistochemistry and mucin histochemistry. *Acta Pathol Jpn* 1990; 40: 494-504.
- 12 Egashira Y, Shimoda T, Ikegami M. Mucin histochemical analysis of minute gastric differentiated adenocarcinoma. *Pathol Int* 1999; 49: 55-61.
- 13 Koseki K, Takizawa T, Koike M, Ito M, Nihei Z, Sugihara K. Distinction of differentiated type early gastric carcinoma with gastric type mucin expression. *Cancer* 2000; 89: 724-32.
- 14 Saito A, Shimoda T, Nakanishi Y, Ochiai A, Toda G. Histologic heterogeneity and mucin phenotypic expression in early gastric cancer. *Pathol Int* 2001; 51: 165-71.
- 15 Mizoshita T, Tsukamoto T, Inada K et al. Immunohistochemically detectable Cdx2 is present in intestinal phenotypic elements in early gastric cancers of both differentiated and undifferentiated types, with no correlation to non-neoplastic surrounding mucosa. *Pathol Int* 2004; 54: 392-400.
- 16 Tamura G, Sakata K, Maesawa C et al. Microsatellite alterations in adenoma and differentiated adenocarcinoma of the stomach. *Cancer Res* 1995; 55: 1933-6.
- 17 Endoh Y, Sakata K, Tamura G et al. Cellular phenotypes of differentiated-type adenocarcinomas and precancerous lesions of the stomach are dependent on the genetic pathways. *J Pathol* 2000; 191: 257-63.
- 18 Tatematsu M, Tsukamoto T, Inada K. Stem cells and gastric cancer - Role of gastric and intestinal mixed intestinal metaplasia. *Cancer Sci* 2003; 94: 135-41.
- 19 Mizoshita T, Inada K, Tsukamoto T et al. Expression of the intestine-specific transcription factors, Cdx1 and Cdx2, correlates shift to an intestinal phenotype in gastric cancer cells. *J Cancer Res Clin Oncol* 2004; 130: 29-36.
- 20 Hirayama F, Takagi S, Yokoyama Y, Iwao E, Ikeda Y. Establishment of gastric *Helicobacter pylori* infection in Mongolian gerbils. *J Gastroenterol* 1996; 31 (Suppl. 9): 24-8.
- 21 Shimizu N, Ikehara Y, Inada K et al. Eradication diminishes enhancing effects of *Helicobacter pylori* infection on glandular stomach carcinogenesis in Mongolian gerbils. *Cancer Res* 2000; 60: 1512-14.
- 22 Shimizu N, Inada K, Nakanishi H et al. *Helicobacter pylori* infection enhances glandular stomach carcinogenesis in Mongolian gerbils treated with chemical carcinogens. *Carcinogenesis* 1999; 20: 669-76.