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

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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 ß-cryptoxanthin extracted from *Citras 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 ß-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 ß-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 ß-cryptoxanthin is able to prevent OH-BBN-induced bladder carcinogenesis in mice.

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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 ß-ionone cycles and provitamin A property, ß-cryptoxanthin, has also been reported to exert anti-tumor promoter action *in vitro* (13). ß-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 ß-cryptoxanthin and hesperidin also suppress rat colon (16) and mouse lung carcinogenesis (17). Furthermore, certain epidemiological studies suggest that serum level of ß-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 ß-cryptoxanthin may contribute to the prevention of experimental bladder carcinogenesis.

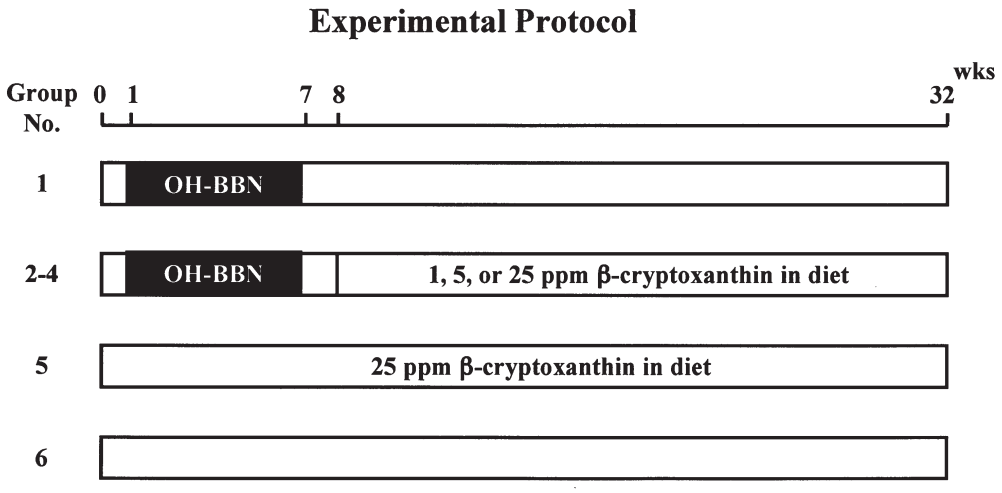

Figure 1. Experimental protocol. A total of 92 male ICR mice (5 weeks old) were used in the study. To induce urinary bladder neoplasms, mice were given OH-BBN (500 ppm) in drinking water for 6 weeks. They were fed the diet containing ß-cryptoxanthin (1, 5 or 25 ppm). The experiment was terminated at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

In the current study, chemopreventive effects of ß-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 ß-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. ß-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±2˚C (SD), 50±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 carinoma. Mice in groups 2-4 were fed the diet mixed with 1, 5 and 25 ppm of ß-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 ß-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-μm 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˚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 x400 (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[a]	2.23±0.28	5.13±0.75
2	OH-BBN→1 ppm ß-cryptoxanthin	18	44.85±4.21	2.28±0.31	5.10±0.57
3	OH-BBN→5 ppm ß-cryptoxanthin	19	45.50±6.37	2.22±0.40	4.89±0.60
4	OH-BBN→25 ppm ß-cryptoxanthin	19	43.04±6.52	2.21±0.25	5.17±0.58
5	25 ppm ß-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

[a]Mean ± SD.

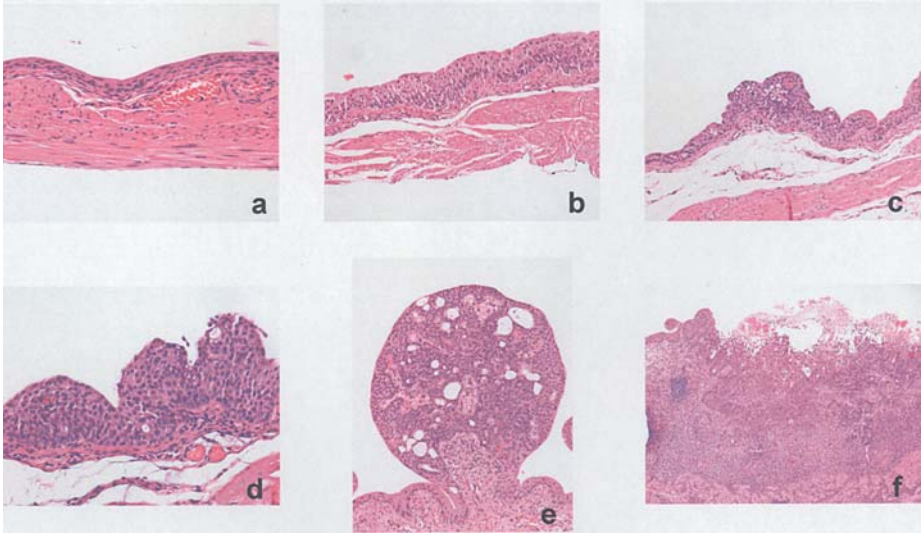

Figure 2. Histopathology of the urinary bladder lesions in mice treated with OH-BBN. (a) Non-lesional transitional epithelium, (b) simple hyperplasia, (c) papillary or nodular hyperplasia, (d) dysplasia, (e) transitional cell papilloma, and (f) transitional cell carcinoma. Hematoxylin and eosin stain, original magnification x10.

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 ß-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			
			Hyperplasia			Dysplasia
			Total	Simple hyperplasia	Papillary or nodular hyperplasia	
1	OH-BBN	16	16 (100)	10 (63)	11 (69)	12 (75)
2	OH-BBN→1 ppm ß-cryptoxanthin	18	16 (89)	12 (67)	5 (28)[a]	9 (50)
3	OH-BBN→5 ppm ß-cryptoxanthin	19	11 (58)[b]	9 (47)	2 (11)[c]	7 (37)[a]
4	OH-BBN→25 ppm ß-cryptoxanthin	19	8 (42)[c]	5 (26)[a]	5 (26)[a]	6 (32)[a]
5	25 ppm ß-cryptoxanthin	10	0	0	0	0
6	None	10	0	0	0	0

[a-c]Significantly different from group 1 by Fisher's exact probability test ([a]P<0.05, [b]P<0.005 and [c]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			
			Hyperplasia			Dysplasia
			Total	Simple hyperplasia	Papillary or nodular hyperplasia	
1	OH-BBN	16	1.31±0.60[a]	0.63±0.50	0.75±0.58	0.75±0.44
2	OH-BBN→1 ppm ß-cryptoxanthin	18	0.94±0.42	0.67±0.49	0.28±0.46[b]	0.50±0.51
3	OH-BBN→5 ppm ß-cryptoxanthin	19	0.58±0.51[c]	0.47±0.51	0.11±0.32[d]	0.37±0.50
4	OH-BBN→25 ppm ß-cryptoxanthin	19	0.53±0.70[d]	0.26±0.45	0.26±0.45[b]	0.32±0.48
5	25 ppm ß-cryptoxanthin	10	0	0	0	0
6	None	10	0	0	0	0

[a]Mean ± SD. [b-d]Significantly different from group 1 by Bonferroni Multiple Comparisons Test ([b]P<0.05, [c]P<0.01 and [d]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 ß-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 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 ß-cryptoxanthin	18	4 (22)	0	3 (17)	1 (6)	4 (22)
3	OH-BBN→5 ppm ß-cryptoxanthin	19	4 (21)	1 (5)	3 (16)	1 (5)	4 (21)
4	OH-BBN→25 ppm ß-cryptoxanthin	19	2 (11)[a]	1 (5)	2 (11)	0	2 (11)[a]
5	25 ppm ß-cryptoxanthin	10	0	0	0	0	0
6	None	10	0	0	0	0	0

[a]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[a]	0.06±0.25	0.50±0.73	0.13±0.34	0.63±0.72
2	OH-BBN→1 ppm ß-cryptoxanthin	18	0.33±0.59	0	0.22±0.55	0.06±0.24	0.28±0.58
3	OH-BBN→5 ppm ß-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 ß-cryptoxanthin	19	0.16±0.50	0.05±0.23	0.11±0.32	0	0.11±0.32[b]
5	25 ppm ß-cryptoxanthin	10	0	0	0	0	0
6	None	10	0	0	0	0	0

[a]Mean ± SD. [b]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-legional 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 ß-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.

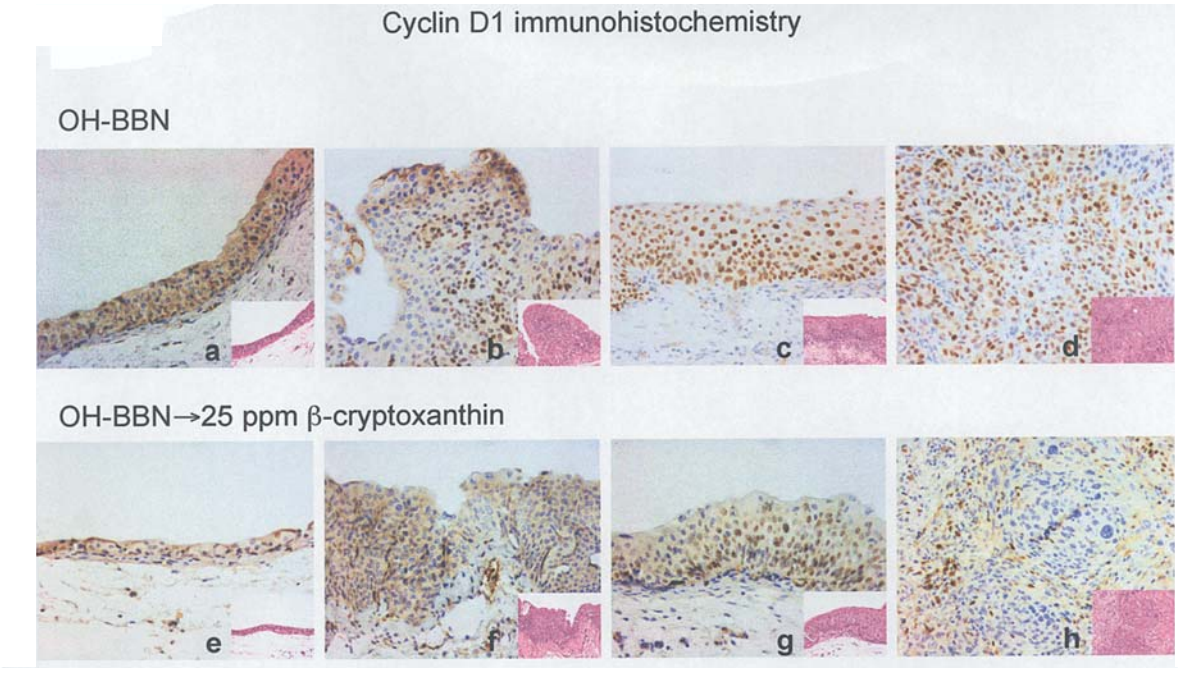

Figure 3. Cyclin D1 immunohistochemistry in the urinary bladder lesions. Inserts are their histopathology. (a-d) From mice given OH-BBN alone and (e-h) from those given OH-BBN and 25 ppm ß-cryptoxanthin. (a) and (e), non-lesional transitional epithelium; (b) and (f), papillary or nodular hyperplasia; (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, x20.

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±1.46[a] (16)	18.00±1.86 (16)	31.50±5.28 (12)	58.25±10.46 (8)
2	OH-BBN→1 ppm ß-cryptoxanthin	2.78±1.11[b] (18)	17.00±2.50 (16)	23.67±3.32[b] (9)	46.25±7.72 (4)
3	OH-BBN→5 ppm ß-cryptoxanthin	2.16±0.96[c] (19)	15.73±2.01[d] (11)	17.43±4.43[c,e] (7)	42.75±5.74 (4)
4	OH-BBN→25 ppm ß-cryptoxanthin	1.74±0.81[c,e] (19)	13.00±1.60[c,f,g] (8)	12.50±3.56[c,f] (6)	35.50±3.54[d] (2)
5	25 ppm ß-cryptoxanthin	0.48±0.18[c,f,h,i] (10)	ND	ND	ND
6	None	0.30±0.12[c,f,h,j] (10)	ND	ND	ND

[a]Mean ± SD. ND, not determined. [b-d]Significantly different from group 1 by Bonferroni Multiple Comparisons Test ([b]P<0.01, [c]P<0.001 and [d]P<0.05). [e,f]Significantly different from group 2 by Bonferroni Multiple Comparisons Test ([e]P<0.05 and [f]P<0.001). [g,h]Significantly different from group 3 by Bonferroni Multiple Comparisons Test ([g]P<0.05 and [h]P<0.001). [i,j]Significantly different from group 4 by Bonferroni Multiple Comparisons Test ([i]P<0.05 and [j]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, ß-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 ß-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). ß-cryptoxanthin is enzymatically converted to retinol, which is involved in cell differentiation, such as ß- and α-carotenes. Dietary administration with ß-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 ß-cryptoxanthin was lower than that of mice given OH-BBN alone (data not shown).

In conclusion, our study provided further evidence that dietary ß-cryptoxanthin is able to suppress rodent carcinogenesis. The oxygenated carotenoid ß-cryptoxanthin is one of the major carotenoids in the blood, and recent epidemiological studies revealed an inverse association between serum and diet concentrations of ß-cryptoxanthin and the risks of cancer in various organs (46-49), including the urinary bladder (50). ß-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 ß-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

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