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Antitumor-promoting effect of Kampo formulations on rat urinary bladder carcinogenesis in a short-term test with concanavalin A

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Abstract

We examined the effect of 15 Kampo formulations in a short-term test of bladder carcinogenesis, using agglutinability of isolated bladder epithelial cells with concanavalin A (Con A). Rats were given 0.01% N-butyl-N-(4-hydroxybutyl)nitrosamine (BHBN) in drinking water for one week, and then 5% sodium saccharin (SS) in a powdered diet alone or 5% SS in a powdered diet and 5-fold the usual daily dose of Kampo formulations was administered orally for 3 weeks. Treatment with Chorei-to (270 mg/kg/day), Gorei-san (350 mg/kg/day), Hochu-ekki-to (800 mg/kg/day), Hachimi-jio-gan (600 mg/kg/day), Toki-shakuyaku-san (500 mg/kg/day), Keishi-ka-ryukotsu-borei-to (500 mg/kg/day) or Saiko-ka-ryukotsu-borei-to (600 mg/kg/day) reduced the increase in the number of Con A-dependent aggregates of bladder epithelial cells induced by SS by 100, 85, 85, 85, 77, 77, and 62%, respectively. Among these formulations, Chorei-to exhibited the strongest inhibitory effect, and its ED_{50} was 12.8 mg/kg. Chorei-to also significantly reduced the Con A-dependent agglutinability of bladder epithelial cells from rats treated with the bladder tumor promoters 3% DL-tryptophan, 2% butylated hydroxyanisole, 3% uracil or 0.01% BHBN for 3 weeks. These findings suggest that Chorei-to has a suppressive effect on rat bladder carcinogenesis.

Key words urinary bladder carcinogenesis, Chorei-to, N-butyl-N-(4-hydroxybutyl)nitrosamine, sodium saccharin, butylated hydroxyanisole.

Abbreviations BHA, butylated hydroxyanisole ; BHBN, N-butyl-N-(4-hydroxybutyl)nitrosamine ; Chorei-to (Zhu-Ling-Tang), 猪苓湯 ; Con A, concanavalin A ; Dai-saiko-to (Da-Chai-Hu-Tang), 大柴胡湯 ; ED_{50}, 50% effective dose ; EDTA, ethylenediaminetetraacetic acid ; Eppi-ka-jutsu-to (Yue-Bi-Jia-Shu-Tang), 越婢加朮湯 ; Gorei-san (Wu-Ling-San), 五苓散 ; Hachimi-jio-gan (Ba-Wei-Di-Huang-Wan), 八味地黄丸 ; Hochu-ekki-to (Bu-Zhong-Yi-Qi-Tang), 補中益気湯 ; Juzen-taiho-to (Shi-Quan-Da-Bu-Tang), 十全大補湯 ; Keishi-ka-ryukotsu-borei-to (Gui-Zhi-Jia-Long-Gu-Mu-Li-Tang), 桂枝加竜骨牡蛎湯 ; α-MM, α-methyl mannoside ; Oren-gedoku-to (Huang-Lian-Jie-Du-Tang), 黄連解毒湯 ; PBS, phosphate buffered saline ; PGE_2, prostaglandin E_2 ; Ryutan-shakan-to (Long-Dan-Xie-Gan-Tang), 竜胆瀉肝湯 ; Saiko-ka-ryukotsu-borei-to (Chai-Hu-Jia-Long-Gu-Mu-Li-Tang), 柴胡加竜骨牡蛎湯 ; Seishin-renshi-in (Qing-Xin-Lian-Zi-Yin), 清心蓮子飲 ; Sho-saiko-to (Xiao-Chai-Hu-Tang), 小柴胡湯 ; Sho-seiryu-to (Xiao-Qing-Long-Tang), 小青竜湯 ; SS, sodium saccharin ; Toki-shakuyaku-san (Dang-Gui-Shao-Yao-San), 当帰芍薬散 ; Trp, DL-tryptophan.

Introduction

About 70 % of human urinary bladder cancers are of the papillary superficial type, which are characterized by frequent multiple intravesical ectopic tumor growth after transurethral resection.[1] Bladder tumor promoters are suggested to play an important role in

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the new tumor growth.[2] Kakizoe *et al.*[3-7] established a new short-term bioassay for detection of urinary bladder tumor promoters using agglutinability of isolated bladder cells with concanavalin A (Con A) as the endpoint. N-butyl-N-(4-hydroxybutyl)nitrosamine (BHBN),[3-5] sodium saccharin (SS),[6] DL-tryptophan (Trp),[7] L-isoleucine,[7] and L-leucine[7] were found to have a tumor promoting effect by this method, and were later proved to be bladder tumor promoters in a long-term carcinogenicity test.[8-14] Moreover, Kakizoe *et al.*[15,16] screened various compounds for antitumor promoting activity in bladder carcinogenesis in rats with a view to using them clinically to inhibit postoperative intravesical ectopic tumor growth of superficial papillary bladder cancer by modifying this method. Aspirin, α-difluoromethylornithine, and all-trans aromatic retinoid were shown to have antiumor promoting activity by this method, and were later found to inhibit the bladder carcinogenesis in a long-term carcinogenicity test.[17-19] Thus, this assay is very useful for screening not only bladder carcinogens and bladder tumor promoters but also antipromoters on bladder carcinogenesis.

Kampo formulations have been recently utilized for the treatment of cancer patients, with a view toward improving the patient's immunological function.[20-22] The chemopreventive and antitumor promoting effects of Kampo formulations have been also reported.[23-25] Oka *et al.*[23] found that Sho-saiko-to prevented or delayed the occurrence of latent hepatocellular carcinoma in patients with liver cirrhosis. Tatsuta *et al.*[24] demonstrated that Sho-saiko-to inhibited the development of hepatic foci.

By using this method, we examined the antipromoting effects of 15 Kampo formulations, which have been used traditionally as a remedy for urinary system disorders, inflammation, anemia, and fatigue,[26] on bladder tumor promoters, SS, Trp, BHA,[27] BHBN and uracil.[28]

Materials and Methods

Animals : Five-weeks-old, male, Wistar rats were obtained from Japan SLC, Inc., Hamamatsu, Japan, and kept in rooms with controlled temperature (23±1°C), humidity (50±5 %) and 12-h light 12-h dark cycles. They were fed commercial rat chow (MF: Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*, and were used after one week of acclimation (average weight, 180 g).

Chemicals : The following chemicals were used : BHBN (Tokyo Kasei Organic Chemicals, Co., Ltd., Tokyo) ; SS (Wako Pure Chemical Industries, Ltd., Tokyo) ; Trp (Takara Kohsan, Co., Ltd., Tokyo) ; Con A, α-MM, and uracil (Sigma Chemical, Co., St. Louis, MO) ; Krestin (Sankyo, Co., Ltd., Tokyo).

Kampo Medicines : Raw herbal medicines for Kampo formulations were purchased from Yamamoto Yakuhin Kogyo, Co., Ltd., Tokyo. They were blended according to the formula,[28] and then extracted with boiling water for 60 min. After cooling, the extracts were filtered, and then lyophilized. The lyophilized materials were dissolved in water immediately before use.

Treatment of Animals : The BHBN solution was freshly prepared every three days in water at a concentration of 0.01 % and given to rats using a shading bottle. SS, Trp, BHA, and uracil were given to rats at a concentration of 5 % ,3 %, 2 %, and 3 %, respectively, in the MF powdered diet. Kampo formulations, Krestin, and furosemide were administered orally (*p. o.*) once a day at 11 in the morning during the exposure period.

The rats were randomly divided into 3 groups consisting of 6 rats each as shown in Fig. 1. The rats in Groups 1 to 3 were given 0.01 % BHBN in their drinking water, and basal powder diet for one week. For the next three weeks, rats in Group 2 were given water and the powdered diet containing the promoters, SS, Trp, BHA, and uracil, and those in Group

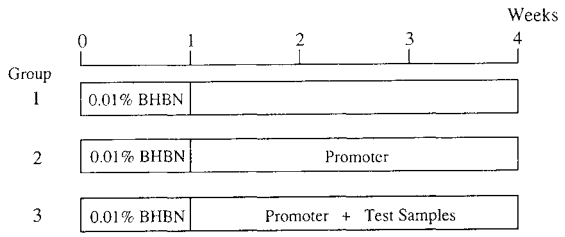

Fig. 1 Experimental design for examining the effect of Kampo formulations on agglutinability of bladder cells with Con A.
BHBN, N-butyl-N-(4-hydroxybutyl)nitrosamine.

3 were given water, the powdered diet containing the promoters, and Kampo formulations or Krestin or furosemide. Rats in Groups E2 and 3 were given drinking water containing 0.01 % BHBN for an additional 3 weeks.

Agglutination Assay : All rats were killed in week four and the urinary bladder was excised. The Con A agglutination assay was performed as reported previously.[16] Briefly the urinary bladder was washed with physiological saline, everted and incubated in 0.15 M NaCl containing 5 mM EDTA. Epithelial cells were separated by sonicating and squashing the bladder mucosa. In each group, the epithelial cells from 2 animals were combined and collected by centrifugation. The cell suspension (4 to 6×10^6 cells/ml) was incubated with Con A (3.2 mg/ml) in a total volume of 40 μl of PBS (pH 7.4) with or without α-MM (6.4 mg/ml), a specific inhibitor of Con A binding, for 30 min at 37°C. Then the number of aggregates of more than four cells per 200 total cells was measured using a hemocytometer after staining with trypan blue. The number of Con A-dependent aggregates was calculated as the difference in numbers with or without α-MM. Three assays, of each composite from 2 rats, were performed in each group of 6 animals. The statistical significance of differences of agglutination values between Kampo formulation-treated and -untreated groups was examined by using Student's t test.

The inhibitory rate was calculated by the following formula : Inhibitory rate (%)= $\{1-(Y-Z)/(X-Z)\}\times100$, where X, Y and Z are the mean number of Con A-dependent aggregates of Groups 2, 3 and 1, respectively.

The ED_{50} of Chorei-to was determined using the equation obtained by the least squares method for 4 dose levels (270, 54, 5.4 and 0.54 mg/kg).

Results

Effect of Kampo formulations on SS-induced bladder tumor promotion

Table I summarizes the effect of Kampo formulations on Con A-dependent agglutinability of isolated bladder epithelial cells. No significant increase in the number of Con A-dependent aggregates was observed by treatment with BHBN for one week (Group A1). However, after treatment with SS for 3 weeks (Group A2), the number of Con A-dependent aggregates increased markedly (14±1 aggregates per 200 cells). Additional treatment with 5-fold the usual daily dose of Chorei-to (270 mg/kg), Gorei-san (350 mg/kg), Hochu-ekki-to (800 mg/kg), Hachimi-jio-gan (600 mg/kg), Toki-shakuyaku-san (500 mg/kg), Keish-ka-ryukotsu-borei-to (500 mg/kg) or Saiko-ka-ryukotsu-borei-to (600 mg/kg)(Groups A3-1 to 3-7) suppressed significantly this increase of aggregates by 100, 85, 85, 85, 77, 77, and 62 %, respectively. The inhibitory effects of these Kampo formulations were higher than that of Krestin used as a positive control, in which the highest inhibitory effect was about 60 % at doses of more than 50 mg/kg (Group A3-16). Diuretic furosemide (600 and 150 mg/kg) had a slight inhibitory effect on the increase of Con A dependent-aggregates by SS (Group A3-17).

The average food intake of rats given Kampo formulations was greater than that for rats fed SS alone (Group A2) in most groups. The final body weight of rats in Group A2 fed SS was 9.8 % less than that of untreated rats, Group A1, but that of the rats in Groups A3-1 to 3-15, given Kampo formulations was similar to the average body weight of rats in Group A2.

Effect of Chorei-to on bladder tumor promotion

Chorei-to, which showed the strongest inhibitory effect on the SS-induced bladder tumor promotion in the Kampo formulations tested, dose-dependently inhibited the increase of aggregates by SS, and showed a significant inhibitory effect at a dose of more than the usual daily dose (54 mg/kg/day, *p.o.*), and the ED_{50} of Chorei-to was 12.8 mg/kg/day (Table II).

Table III summarizes the inhibitory effect of Chorei-to on the tumor promotion induced by Trp, BHA, uracil, and BHBN. Treatment with Trp, BHA, uracil, or BHBN after BHBN increased the number of Con A-dependent aggregates to 13, 13, 14, or 15 per 200 cells, respectively. Chorei-to inhibited significantly the increase of aggregates by Trp, BHA, and BHBN at doses of 5- and 10-fold the usual daily dose, and the increase of aggregates by uracil was suppressed significantly in the presence of 10- and 20-fold the

Table I Inhibitory effect of Kampo formulations on saccharin sodium-induced bladder tumor promotion.

Group	Treatment	Dose of test sample (mg/kg/day)	No. of Con A-dependent aggregates[a]	Inhibition (%)	Intake (g/day) Water	Intake (g/day) Diet	Final body weight (g)
A1	0.01% BHBN alone	0	1±1	—	21.6	18.6	253.6
A2	0.01% BHBN+5%SS	0	14±1[b]	—	25.4	15.8	228.8
A3-1	0.01% BHBN+5%SS+Chorei-to	270	1±1[c]	100	31.5	18.4	219.8
2	+Gorei-san	350	3±1[c]	85	31.4	18.1	220.9
3	+Hochu-ekki-to	800	3±1[c]	85	27.9	16.1	225.9
4	+Hachimi-jio-gan	600	3±1[c]	85	27.4	15.3	222.8
5	+Toki-shakuyaku-san	500	4±1[d]	77	32.9	17.1	204.5
6	+Keishi-ka-ryukotsu-borei-to	500	4±1[d]	77	31.0	16.5	226.7
7	+Saiko-ka-ryukotsu-borei-to	600	6±1[d]	62	29.2	18.4	235.7
8	+Sho-saiko-to	550	8±4	46	31.2	18.2	235.7
9	+Sho-seiryu-to	450	9±1	38	35.3	15.3	223.0
10	+Juzen-taiho-to	850	10±0	31	31.0	14.8	227.0
11	+Eppi-ka-jutsu-to	580	10±0	31	28.9	16.4	217.2
12	+Dai-saiko-to	550	13±2	8	31.1	16.4	219.6
13	+Ryutan-shakan-to	800	14±3	0	32.0	18.6	239.4
14	+Oren-gedoku-to	100	14±3	0	29.6	15.8	215.3
15	+Seishin-renshi-in	550	14±0	0	27.4	16.5	229.8
16	+Krestin	200	6±1[d]	62	29.4	16.9	221.7
		50	7±1[d]	54	35.9	17.9	226.1
17	+Furosemide	600	12±1	15	32.0	18.1	233.6
		150	10±1	31	31.6	17.6	229.3

All the rats in each group were given 0.01 % BHBN in drinking water for one week, and then rats in each group were given a 5 % SS-containing diet for 3 weeks. Kampo formulations were also injected p.o. at a dose of 5-fold the usual daily dose once a day for 3 weeks.
Fig. 1 shows the experimental conditions for each group. Agglutination of urinary bladder cells in each group was induced by 3.2 mg/ml Con A, with or without 6.4 mg/ml α-methyl mannoside (α-MM). Three assays, each a composite cell suspension from 2 rats, were carried out in each group of 6 rats.
a) The number of Con A-dependent aggregates was calculated as the difference between the numbers with or without α-MM (Mean±S.D., n=3).
b) $p<0.01$ compared with BHBN alone group.
c) $p<0.01$ compared with BHBN+5% SS treated group.
d) $p<0.05$ compared with BHBN+5% SS treated group.

Table II Inhibitory effect of Chorei-to on saccharin sodium-induced bladder tumor promotion.

Treatment	Dose of Chorei-to (mg/kg/day)	No. of Con A-dependent aggregates[a]	Inhibition (%)	Final body weight (g)
0.01% BHBN	0	1±1	—	253.6
0.01% BHBN+5% SS	0	14±1[b]	—	228.8
0.01% BHBN+5% SS+Chorei-to	540	1±1[c]	100	231.0
	270	1±1[c]	100	229.8
	54[d]	5±1[e]	69	236.4
	5.4	9±2	38	243.0
	0.54	14±4	0	233.0

Experimental conditions for each group are shown in Fig. 1. Chorei-to was administered *p.o.* to rats once a day for 3 weeks.
a) Mean±S.D.(n=3).
b) $p<0.01$ compared with 0.01% BHBN alone group.
c) $p<0.01$ compared with 0.01% BHBN+5% SS treated group.
d) The usual daily dose of Chorei-to.
e) $p<0.05$ compared with 0.01% BHBN+5% SS treated group.

Table III Inhibitory effect of Chorei-to on Trp-, BHA-, uracil-, and BHBN-induced bladder tumor promotion.

Group	Treatment	Dose of Chorei-to (mg/kg/day)	No. of Con A-dependent aggregates[a]	Inhibition (%)	Intake (g/day) Water	Intake (g/day) Diet	Final body weight (g)
B1	0.01% BHBN alone	0	1±1		21.2	12.9	243.4
2	0.01% BHBN+3% Trp	0	13±2[b]		22.1	12.2	240.0
3-1	0.01% BHBN+3% Trp+Chorei-to	540[c]	2±2[d]	92	28.4	13.8	223.3
3-2	0.01% BHBN+3% Trp+Chorei-to	270	5±1[e]	67	21.8	12.2	220.0
C1	0.01% BHBN alone	0	1±1		23.1	17.1	270.2
2	0.01% BHBN+2% BHA	0	13±1[b]		15.4	8.8	199.4
3-1	0.01% BHBN+2% BHA+Chorei-to	540	2±1[d]	92	20.2	12.6	234.0
3-2	0.01% BHBN+2% BHA+Chorei-to	270	3±2[e]	83	21.6	14.5	228.9
D1	0.01% BHBN alone	0	1±1		26.7	18.9	261.1
2	0.01% BHBN+3% uracil	0	14±3[b]		33.6	9.5	186.9
3-1	0.01% BHBN+3% uracil+Chorei-to	1080	4±1[d]	77	36.0	9.5	181.4
3-2	0.01% BHBN+3% uracil+Chorei-to	540	6±2[e]	62	35.2	10.8	186.4
E1	0.01% BHBN alone	0	1±2		23.3	13.9	254.7
2	0.01% BHBN+0.01% BHBN	0	15±3[b]		30.0	15.1	266.4
3-1	0.01% BHBN+0.01% BHBN+Chorei-to	540	1±1[d]	100	28.7	15.1	267.7
3-2	0.01% BHBN+0.01% BHBN+Chorei-to	270	8±2[e]	50	29.8	15.4	266.1

All the rats in each group were given 0.01 % BHBN in their drinking water for one week, and then rats in Groups B2 to 3-2, C2 to 3-2, D2 to 3-2 and E2 to 3-2 were given 3% Trp-, 2% BHA- and 3% uracil-containing diet and 0.01% BHBN-containing water for 3 weeks, respectively. Chorei-to was also injected *p.o.* at doses of 5-, 10-, or 20-fold the usual daily dose once a day for 3 weeks. Trp, DL-tryptophan ; BHA, butylated hydroxyanisole and BHBN, N-butyl-N-(4-hydroxybutyl)nitrosamine.

a) Mean±S.D. (n=3).

b) $p<0.01$ compared with each BHBN alone group.

c) Ten-fold the usual daily dose of Chorei-to.

d) $p<0.01$ compared with each BHBN+promoter treated group.

e) $p<0.05$ compared with each BHBN+promoter treated group.

usual daily doses of Chorei-to.

The average final body weight and food intake of rats in Group C2 fed BHA were, respectively, 26.2 % and 48.5 % less than those in the untreated rats (Group C1), and those Group D2 fed uracil 28.4 % and 49.7 % less. Treatment with Chorei-to in Groups C3-1 and 3-2 caused an increase in body weight and food intake, although there were no differences in Groups D3-1 and 3-2.

Discussion

In the present study, oral treatment with Chorei-to resulted in significant inhibition of the rat bladder tumor promotion by SS, Trp, BHA, uracil, and BHBN in a short-term test with Con A.

We examined the effects of 15 Kampo formulations on the SS-induced bladder tumor promotion at a dose of 5-fold the usual daily dose. The average food intake of rats given Kampo formulations was greater than that of rats fed SS alone, regardless of the inhibitory effect against the SS-induced tumor promotion. Moreover, the final body weight of rats given Kampo formulations was similar to that of rats fed SS alone. These suggest that the toxicity of 5-fold the usual daily dose of Kampo formulations is very low and Kampo formulations possess an aperitive effect.

Herbal medicines are known to contain a variety of bioactive polysaccharides.[29-31] The polysaccharide preparation, Krestin[32,33] has been reported to have antitumor activity mediated by their ability to stimulate immunologic host defense mechanisms and is used clinically. Fujita *et al.*[34] found that Krestin had an inhibitory effect on the development of malignancy in bladder tumors in a long-term test. Therefore, we used Krestin as a positive control. Krestin showed an antipromoting effect and the highest inhibition rate was about 60 % at doses of over 50 mg/kg/day. Of the 15 Kampo formulations tested, 7 showed a higher inhibitory effect than that of Krestin. Among these formulations, Chorei-to, Gorei-san, Hachimi-jio-gan, and Toki-shakuyaku-san have been used traditionally as a remedy for urinary system disorders, and have diuretic effects.[35-37] We considered at first that

the antipromoting effects of these formulations might be caused by the diuretic activity of these formulations, because the promoter SS may be diluted with increased urine volume in the target organ urinary bladder. However, the diuretic furosemide had only a slight inhibitory effect, suggesting that the antipromoting effect of these formulations is produced by some other mechanisms.

Several studies have shown that dietary calcium is negatively associated with the risk of colon cancer.[39, 40] Therefore, we examined whether calcium-containing formulations, Keishi-ka-ryukotsu-borei-to and Saiko-ka-ryukotsu-borei-to, could inhibit bladder tumor promotion by SS. Both of the formulations exhibited the antitumor promoting effect, suggesting that calcium contained in Osterae Testa (oyster shell) and Fossilia Ossis Mastodi (fossilized animal bone)[41] is probably important in the inhibition of bladder tumor promotion. In fact, calcium phosphate, Ca $(H_2PO_4)_2$, inhibited significantly bladder tumor promotion by SS and BHBN at doses of more than 50 mg/kg/day (data not shown).

Hochu-ekki-to and Juzen-taiho-to have been used as a remedy for patients with anemia, anorexia or fatigue[28] and are known to enhance antitumor effects.[42, 43] However in this study, only Hochu-ekki-to showed a significant antipromoting effect. Further study is required to evaluate the difference in activity between the two, such as administration doses of these formulations.

Recently Tomita *et al.*[44] found that Sho-saiko-to prevented bladder carcinogenesis by BHBN in a long-term test. Moreover, a recent study has shown that Sho-saiko-to inhibits the proliferation of carcinoma cells due partly to induction of apoptosis.[45] In this short-term test, Sho-saiko-to inhibited bladder tumor promotion by 46 %. This suggests that the formulations Chorei-to, Gorei-san, Hochu-ekki-to, Hachimi-jio-gan, Toki-shakuyaku-san, Keishi-ka-ryukotsu-borei-to and Saiko-ka-ryukotsu-borei-to, have a higher inhibitory rate than Sho-saiko-to on rat bladder carcinogenesis.

Chorei-to exhibited the highest inhibitory effect on bladder tumor promotion by SS in the formulations tested, and the ED_{50} (12.8 mg/kg/day) was less than the usual daily dose (54 mg/kg/day). We examined whether treatment with Chorei-to could inhibit any other bladder tumor promoters, *i.e.*, Trp, BHA, uracil and BHBN. Chorei-to showed a significant antipromoting activity and the effect was dose-dependent. Many studies have been done to demonstrate the mechanism (s) of bladder tumor promoters including SS, Trp, BHA, uracil and BHBN, but the actual mechanism (s) is still unknown. Only the following findings have been reported ; a) high doses of SS led to the formation of silicate-containing precipitate and microcrystals in the urine of male rats,[46] b) DL-tryptophan had promoting activity on urinary bladder carcinogenesis in male rats induced by BHBN,[13] but L-tryptophan, which is biologically the more important form, did not exert any significant promoting effect,[47] c) BHA increased in levels of PGE_2, cAMP and ascorbic acid along with elevated DNA synthesis in bladder tissues,[48, 49] d) uracil induced urinary bladder stones,[27] e)N-butyl-N-(3-carboxypropyl)nitrosamine, the principal urinary metabolite of BHBN in rats, selectively induced the bladder carcinoma in rats.[50] These findings suggest differences in the underlying mechanisms of these bladder tumor promoters. Therefore, Chorei-to could be an effective chemopreventive formulation for urinary bladder cancer. A test of the effect of Chorei-to in a long-term experiment on bladder carcinogenesis in rats is now in progress in our laboratory.

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和文抄録

15種類の漢方方剤の膀胱発癌プロモーターに対する抑制効果を，膀胱上皮細胞の concanavalin A (Con A) 依存性凝集活性を指標とする短期試験法を用いて検討した。本研究では Wistar ラットに 0.01 % の N-butyl-N-(4-hydroxybutyl)nitrosamine (BHBN) を飲料水として1週間自由摂取させた後，5 % sodium saccharin (SS) (混餌) を単独，または常用量の5倍の量の漢方方

剤（経口投与）と共に3週間与え，漢方方剤の膀胱発癌プロモーション抑制効果を検討した。

その結果，猪苓湯(270 mg/kg)，五苓散(350 mg/kg)，補中益気湯（800 mg/kg)，八味地黄丸（600 mg/kg)，当帰芍薬散（500 mg/kg)，桂枝加竜骨牡蛎湯（500 mg/kg)，柴胡加竜骨牡蛎湯（600 mg/kg）が，SSによる膀胱上皮細胞のCon A依存性凝集数の増加をそれぞれ100, 85, 85, 85, 77, 77および62％抑制することがわかった。これらのうち猪苓湯は最も強い抑制効果を示し，そのED_{50}は12.8 mg/kgであった。さらに猪苓湯は膀胱発癌プロモーターである3％ DL-tryptophan, 2％ butylated hydroxyanisole, 3％ uracilおよび0.01％ BHBNを3週間投与したラットの膀胱上皮細胞のCon A依存性凝集活性を強く抑制することが明かになった。

本知見より，猪苓湯がラット膀胱発癌を抑制する可能性が示唆された。

References

1) Yamamura, Y. and Sugimura, T.: "Clinical Oncology No. 4, Urinary Bladder Cancer", Medical View Co., Ltd., Tokyo, 1986.
2) Niijima, T., Umeda, T., Kakizoe, T., Suzuki, T. and Akaza, H.: "Progress in Cancer Research '84" Nakayama Press, Tokyo, pp. 909-920, 1984.
3) Kakizoe, T., Kawachi, T. and Okada, M.: Concanavalin A agglutination of bladder cells of rats treated with bladder carcinogens; a rapid new test to detect bladder carcinogens. *Cancer Lett.* **5**, 285-290, 1978.
4) Kakizoe, T., Kawachi, T. and Sugimura, T.: Agglutination of bladder cells by concanavalin A during the early phase of treatment of rats with N-butyl-N-(4-hydroxybutyl)nitrosamine. *Cancer Res.* **39**, 3353-3356, 1979.
5) Kakizoe, T., Komatsu, H., Niijima, T., Kawachi, T. and Sugimura, T.: Increased agglutinability of bladder cells by concanavalin A after administration of carcinogens. *Cancer Res.* **40**, 2006-2009, 1980.
6) Kakizoe, T., Komatsu, H., Niijima, T., Kawachi, T. and Sugimura, T.: Maintenance by saccharin of membrane alterations of rat bladder cells induced by subcarcinogenic treatment with bladder carcinogens. *Cancer Res.* **41**, 4702-4705, 1981.
7) Kakizoe, T., Nishio, Y., Ohtani, M., Niijima, T., Sato, S. and Sugimura, T.: Correlation of results of agglutination assays with concanavalin A and carcinogenesis experiments on promoters of bladder cancer. *Jpn. J. cancer Res.* **76**, 930-936, 1985.
8) Ito, N. and Fukushima, S.: Promotion of urinary bladder carcinogenesis in expermental animals. *Exp. Pathol.* **36**, 1-15, 1989.
9) Ito, N., Arai, M., Sugihara, S., Hirao, K., Makiura, S., Matayoshi, K. and Denda, A.: Experimental urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nitrosamine. *GANN Monograph on Cancer Res.* **17**, 367-381, 1975.
10) Ito, N.: Early changes caused by N-butyl-N-(4-hydroxbutyl)-nitrosamine in the bladder epithelium of different animal species. *Cancer Res.* **36**, 2528-2531, 1976.
11) Cohen, S.M., Ellwein, L.B. Okamura, T., Masui, T., Johansson, S. L., Smith, R.A., Wehner, J.M., Khachab, M., Chappel, C.I., Schoenig, G.P., Emerson, J.L. and Garland, E.M.: Comparative bladder tumor promoting activity of sodium saccharin, sodium ascorbate, related acids, and calcium salts in rats. *Cancer Res.* **51**, 1766-1777, 1991.
12) Sakata, T., Shirai, T., Fukushima, S., Hasegawa, R. and Ito, N.: Summation and synergism in the promotion of urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)-nitrosamine in F344 rats. *Gann* **75**, 950-956, 1984.
13) Fukushima, S., Hagiwara, A., Ogiso, T., Shibata, M. and Ito, N.: Promoting effects of various chemicals in rat urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)-nitrosamine. *Fd. Chem. Toxic.* **21**, 59-68, 1983.
14) Nishio, Y., Kakizoe, T., Ohatani, M., Sato, S., Sugimura, T. and Fukushima, S.: L-isoleucine and L-leucine: tumor promoters of bladder cancer in rats. *Science* **231**, 843-845, 1986.
15) Kakizoe, T., Takai, K., Tobisu, K., Ohtani, M. and Sato, S.: Validity of short-term examination for antipromoters of bladder carcinogenesis. *Jpn. J. Cancer Res.* **79**, 235-239, 1988.
16) Suzuki, M., Wakabayashi, K., Sone, H., Kushida, H., Sugiyama, K., Kakizoe, T., Nagao, M. and Sugimura, T.: Inhibitory effect of 2-*O*-octadecylascorbic acid in agglutination assay with concanavalin A; short-term examination of rat urinary bladder carcinogenesis. *Jpn. J. Cancer Res.* **82**, 386-389, 1991.
17) Uchida, K., Seidenfeld, J., Rademaker, A. and Oyasu, R.: Inhibitory action of α-difluoromethylornithine on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced rat urinary bladder carcinogenesis. *Cancer Res.* **49**, 5249-5253, 1989.
18) Sakata, T., Hasegawa, R., Johansson, S.L., Zenser, T.V. and Cohen, S.M.: Inhibition by aspirin of N-[4-(5-nitro-3-furyl)-2-thiazoil]formamide initiation and sodium saccharin promotion of urinary bladder carcinogenesis in male F344 rats. *Cancer Res.* **46**, 3903-3906, 1986.
19) Miyata, Y., Nakatsuka, T., Arai, M., Murasaki, G., Nakanishi, K. and Ito, N.: Inhibition by an aromatic retinoid of DNA damage induced by the bladder carcinogen N-butyl-N-(3-carboxypropyl)-nitrosamine. *Gann* **71**, 341-348, 1980.
20) Yamada, T.: Clinical study of Juzen-taiho-to administration for postoperative esophageal carcinoma, gastric carcinoma and colorectal carcinoma. *J. Med. Pharm. Soc. WAKAN-YAKU* **9**, 157-164, 1992.
21) Adachi, I. and Watanabe, T.: Role of supporting therapy of Juzen-taiho-to (JTT) in advanced breast cancer patients. *Jpn. J. Cancer Chemother.* **16**, 1538-1543, 1989.
22) Kato, K., Hamano, K., Takiguchi, S. and Horie, Y.: Interleukine II activity and nutritional state after administration of traditional Chinese medicine for post-operative patients of gastric cancer. *Biotherapy* **3**, 774-781, 1989.
23) Oka, H., Yamamoto, S., Kanno, T., Kuroki, T., Mizoguchi, Y., Kobayashi, K., Nakao, M., Harihara, S., Marumo, T., Kobayashi, Y. and Monna, T.: Controlled prospective evaluation of Sho-saiko-to in prevention of hepatocellular carcinoma in patients with cirrhosis of the liver. *J. Med. Pharm. Soc. WAKAN-YAKU* **6**, 40-44, 1989.
24) Tatsuta, M., Iishi, H., Baba, M., Nakaizumi, A. and Uehara, H.: Inhibition by Xiao-chai-hu-tang (TJ-9) of development of hepatic foci induced by N-nitrosomorpholine in Sprague-Dawley rats. *Jpn. J. Cancer Res.* **82**, 987-992, 1991.
25) Konoshima, T., Takasaki, M., Kozuka, M. and Tokuda, H.: Anti-tumor promoting activities of Kampo prescriptions. II. Inhibitory effects of Shouseiryu-to on two-stage carcinogenesis of

mouse skin tumors and mouse pulmonary tumors. *YAKUGAKU ZASSHI* **114**, 248-256, 1994.
26) Kobe Research Association of Chinese Medicine, "Explanation of prescriptions in Chinese Medicine", Ishiyaku Press. Tokyo, 1985.
27) Imaida, K., Fukushima, S., Shirai, T., Ohtani, M., Nakanishi, K. and Ito, N.: Promoting activities of butylated hydroxyanisole and butylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of γ-glutamyl transpeptidase-positive foci development in the liver of rats. *Carcinogenesis* **4**, 895-899, 1983.
28) Shirai, T., Ikawa, E., Fukushima, S., Masui, T. and Ito, N.: Uracil-induced urolithiasis and the development of reversible papillomatosis in urinary bladder of F3444 rats. *Cancer Res.* **46**, 2062-2067, 1986.
29) Chihara, G., Hamuro, J., Maeda, Y.Y., Arai, Y. and Fukuoka, F.: Fractionation and purification of the polysaccharides with marked antitumor activity, especially Lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Res.* **30**, 2776-2781, 1970.
30) Ueno, Y., Okamoto, Y., Yamauchi, R. and Kato, K.: An antitumor activity of the alkali-soluble polysaccharide (and its derivatives) obtained from the sclerotia of *Grifora umbellata* (Fr.) PILAT. *Carbohydrate Research* **101**, 160-167, 1982.
31) Kiyohara, H., Takemoto, N., Komatsu, Y., Kawamura, H., Hosoya, E. and Yamada, H.: Characterization of mitogenic pectic polysaccharides from Kampo (Japanese herbal) medicine "Juzen-Taiho-To". *Planta Med.* **57**, 254-259, 1991.
32) Yoshikumi, C., Nomoto, K., Matsunaga, K., Fujii, T. and Takeya, K.: Effect of PS-K on recovery of immunologic responsiveness after administration of anti-tumor drugs or X-irradiation. *Jpn. J. Cancer Chemother.* **2**, 853-858, 1975.
33) Itoh, I.: Krestin (PS-K). *Jpn. J. Cancer Chemother.* **4**, 425-430, 1977.
34) Fujita, K., Fujita, H., Suzuki, K., Tajima, A. and Aso, Y.: Effect of Krestin on bladder tumor induction in rats by N-butyl-N-(4-hydroxybutyl)nitrosamine. *Acta Urol. Jap.* **25**, 917-920, 1979.
35) Haranaka, R., Watabe, Y., Mochizuki, N., Ohwada, S., Kosoto, Y. and Kobayashi, S.: Pharmacological effects of Kampo medicines, Gorei-san, Chorei-to, Sairei-to, and Hachimi-jio-gan, in animal. *Kampo-igaku* **5**, 9-13, 1981.
36) Tanaka, S., Akira, T. and Tabata, M.: Pharmacological analysis of the traditional Chinese prescription "Goreisan-ryo". *YAKUGAKU-ZASSHI* **104**, 601-606, 1984.
37) Seki, M., Fujioka, M., Hatano, T. and Ikeda, H.: Analysis of regulatory effects of Goreisan-ryo on circulatory, metabolic and diuretic function. *Jpn. J. Oriental Medicine* **42**, 313-322, 1992.
38) Timmerman, R.J., Springman, F.R. and Thoms, R.K.: Estimation of a new diuretic furosemide. *Curr. Ther. Res.* **6**, 88-91, 1964.
39) Sorenson, A.W., Slattery, M.L., and Ford, M.H.: Calcium and colon cancer: a review. *Nutr. Cancer.* **11**, 135-145, 1988.
40) Lapré, J.A., De Vries, H.T., Termont, D.S.M.L., Kleibeuker, J.H., De Vries, E.G.E. and Van der Meer, R.: Mechanism of the protective effect of supplemental dietary calcium on cytolytic activity of fecal water. *Cancer Res.* **53**, 248-253, 1993.
41) Namba, T.: "The encyclopedia of Wakan-Yaku". Hoikusha publishing Co., Ltd., Osaka, 1993.
42) Haranaka, R., Hasegawa, R., Nakagawa, S., Sakurai, A., Satomi, N. and Haranaka, K.: Antitumor activity of combination therapy with traditional Chinese medicine and OK432 or MMC. *J. Biol. Response Modifiers* **7**, 77-90, 1988.
43) Ito, H.: Study of anti-tumor effect of Japanese Kampo medicine.: *Biotherapy* **3**, 760-766, 1989.
44) Tomita, K., Kameyama, S., Matsuki, K., Matsushima, H., Honma, Y. and Aso, Y.: Proceedings of the Japanese Cancer Association, 51 th Annual Meeting, p.29, 1992.
45) Yano, H., Mizoguchi, A., Fukuda, K., Haramaki, M., Ogasawara, S., Momosaki, S. and Kojiro, M.: The herbal medicine Sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G_0/G_1 phase. *Cancer Res.* **54**, 448-454, 1994.
46) Cohen, S.M., Cano, M., Earl, R.A., Carson, S.D. and Garland, E.M.: A proposed role for silicates and protein in the proliferative effects of saccharin on the male rat urothelium. *Carcinogenesis* **12**, 1551-1555, 1991.
47) Fukushima, S., Friedell, G., H., Jacobs, J.B. and Cohen, S.M.: Effect of L-tryptophan and sodium saccharin on urinary tract carcinogenesis initiated by N-[4-(5-nitro-3-furyl)-2-thiazoil] formamide. *Cancer Res.* **41**, 3100-3103, 1981.
48) Shibata, M., Yamada, M., Asakawa, E., Hagiwara, A. and Fukushima, S.: Response of rat urine and urothelium to bladder tumor promoters: possible roles of prostaglandin E_2 and ascorbic acid synthesis in bladder carcinogenesis. *Carcinogenesis* **10**, 1651-1656, 1989.
49) Shibata, M., Yamada, M., Tanaka, H., Kagawa, M. and Fukushima, S.: Changes in urine composition, bladder epithelial morphology, and DNA synthesis in male F344 rats in response to ingestion of bladder promoters. *Toxicol. Appl. Pharmacol.* **99**, 37-49, 1989.
50) Okada, M., Suzuki, E., Aoki, J., Iiyoshi, M. and Hashimoto, Y.: Metabolism and carcinogenicity of N-butyl-N-(4-hydroxybutyl)-nitrosamine and related compounds, with special reference to induction of urinary bladder tumors. *GANN Monograph on Cancer Res.* **17**, 161-176, 1975.