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Quantitative Trait Loci Associated with Promoting Effects of Sodium L-Ascorbate on Two-stage Bladder Carcinogenesis in Rats

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In the two-stage rat bladder carcinogenesis model using *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) as an initiator and sodium L-ascorbate (SA) as a promoter, we found a notable strain difference between F344/DuCrj (F344) and WS/Shi (WS) rats in susceptibility to the promoting effect of SA. Twenty each of F344, WS and reciprocal F_1 hybrid rats were given 0.05% BBN in their drinking water for 4 weeks and then a basal diet with (BBN-SA group) or without (BBN group) a 5% SA supplement for 32 weeks. In F344 and also in reciprocal F_1 hybrids, the number of tumors per rat was significantly higher in the BBN-SA group than in the BBN group ($P<0.0001$). In contrast, WS rats were not significantly affected by either treatment ($P=0.8$). These findings indicate that F344 rats are highly susceptible to the promoter effect of SA, but WS rats are not. Linkage analysis of 108 WS×(WS×F344) F_1 backcrosses revealed that this difference was related to a quantitative trait locus mapped on rat Chr. 17 (maximum LOD score, 3.86) named *Bladder Tumor Susceptible-1* and possibly another locus on Chr. 5 (maximum LOD score, 2.39). This study has provided the first evidence that host genes influence the risk of bladder cancer development.

Key words: Bladder carcinogenesis — Rat — Sodium ascorbate — Genetic susceptibility — QTL analysis

Urinary bladder cancer is one of the commonest cancers in humans, and one for which a wide variety of chemicals, as well as smoking, has been suspected to play a pathogenetic role. Genetic factors may also be significant,[1–4] but extensive genetic analysis has not been carried out, except for a few reports on differences in tumor susceptibility among strains of laboratory rodents.[5–8]

Chemical-induced bladder carcinogenesis in rats has been shown to occur in a two-stage process. The natural history of rat bladder cancers resembles that of its human counterpart. Starting from simple hyperplasia of the urothelium, they progress to nodular hyperplasia, papilloma and ultimately carcinoma. One of the most extensively studied rat models is the initiation of bladder epithelium by treatment for a few weeks with 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN).[9–12] The threshold dose and period of BBN exposure have been well established.[9] Various agents have been shown to act as tumor promoters on BBN-initiated bladder, and to stimulate tumor growth.[13] Sodium saccharin[14,15] and sodium L-ascorbate (SA)[9] are representative promoters. Our previous study showed that the promoter effect of SA on BBN-initiated rat bladder epithelium is significantly different among rat strains.[16] In the present study, we aimed to establish the genetic mechanism determining the susceptibility to the promoter effect of SA on BBN-initiated rat bladder among rat strains. In this paper, we selected F344 as a representative susceptible strain and WS/Shi (WS), a highly inbred substrain of Wistar rats, as a resistant strain.

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MATERIALS AND METHODS

Rats Inbred male F344 rats were purchased from Charles River Japan, Inc. (Hino, Shiga). The WS is an inbred strain of rat isolated from a stock of Wistar rats and maintained for over 100 generations by brother-sister matings.[17] The WS rats were raised in Aburahi Laboratories of Shionogi Co., Ltd. (Koka, Shiga). F_1 and backcrosses were produced by appropriate matings of these two strains. All rats were obtained at 4 weeks of age and acclimatized until use. They were housed with 2–3 rats per metallic cage (RT type: Charles River Japan, Inc.) in a room maintained on 12-h light and 12-h dark cycle, at 25±1°C, with a relative humidity of 55±5%, and fed on Oriental MF (Oriental Yeast Co., Tokyo) and water *ad libitum* until 6 weeks of age. All animal experiments were carried out in Aburahi Laboratories in accordance with good laboratory practice.

Chemicals BBN was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and SA from Takeda Chemical Industries, Ltd. (Osaka).

Experimental protocols Forty male rats each of F344, WS, (WS×F344) F_1 and (F344×WS) F_1 strains were

randomly divided into 2 groups at 6 weeks of age. In the first group, rats were given 0.05% BBN for 4 weeks and thereafter maintained on a normal diet and in the second group they were given 0.05% BBN for the initial 4 weeks and then a powder diet containing 5% SA for 32 weeks. The total observation period was 36 weeks. For the linkage analysis, 111 WS×(WS×F344) F_1 backcross rats were treated as the second group. Body weight, food consumption and water intake were measured every 4 weeks. Fresh urine samples were collected by forced voiding from 9 : 00 to 11 : 00 a.m. at 12, 24 and 32 weeks after the start of the experiment from F344, WS and reciprocal F_1 hybrids. The urine samples were examined for pH and ascorbic acid (AsA) concentration with a test paper (Merck, Darmstadt, Germany), and for sodium ion concentration with an atomic absorption spectrophotometer (Varian Techtron Co., Canberra, Australia). To prepare genomic DNA, tails (approximately 2-cm long) of backcross rats in the second group were resected under ether anesthesia at 31 weeks after the start of the experiment. At the end of the observation period of 36 weeks, all the animals were killed under anesthesia. The urinary bladder was inflated by infusion of 10% neutral buffered formalin. After fixation, the urinary bladders were weighed, cut longitudinally into 12 pieces and embedded in paraffin for histopathological examination.

Histological analysis Epithelial proliferative lesions found in the bladder epithelium were classified into 3 types according to the histological criteria described previously[18]; papillary or nodular hyperplasia (PN hyperplasia), papilloma and carcinoma. After an extensive consideration of and agreement on morphological criteria, the numbers of these lesions were counted differentially under a light microscope by three independent observers.

Microsatellite analysis For linkage analysis, we employed the simple sequence repeat length polymorphism. All primers for microsatellite loci were purchased from Research Genetics, Inc. (Huntsville, AL). Among 235 marker loci, we found 70 loci polymorphic between F344 and WS. The DNAs extracted from tails were subjected to polymerase chain reaction (PCR) amplification in an "OmniGene" thermal cycler (Haybaid Ltd., Teddington, U.K.) using the following protocol: 35 cycles of 95°C for 30 s, 53°C for 40 s and 72°C for 60 s. The PCR products were electrophoresed in 4% Nusieve-agarose (Sigma Chemical Co., St. Louis, MO) gel and stained with ethidium bromide. The quality of the tail DNAs was good enough for PCR-based microsatellite analysis.

Statistical analysis and quantitative trait locus (QTL) mapping The urine analysis data were evaluated by use of the unpaired *t*-test. Differences in the number of tumors among experimental groups were analyzed by use of the Mann-Whitney test with "Statview" version 4.0 (Abacus Concepts, Berkeley, CA). To survey the loci associated with either susceptibility or resistance to the promoter action of SA, genome-wide screening with 60 polymorphic microsatellite loci was carried out in 20 rats with a larger number of tumors and 20 rats with fewer or no tumors. In this preliminary screening, the possible linkage was assessed by the χ^2 test. For chromosomes containing any loci showing possible linkage at $P < 0.01$, all 108 backcross rats were genotyped for all available polymorphic loci and a linkage map was constructed by using the MAPMAKER/EXP computer package.[19] QTL analysis was performed on the square-root transformed number of tumors using the MAPMAKER/QTL computer package as previously described.[20]

RESULTS

Tumor incidence in parental strains and reciprocal F_1 hybrids In parental strains and F_1s, all the rats given BBN or BBN-SA survived in apparent health during 36 weeks of observation. Administration of SA occasionally caused mild diarrhea in the later stage of the experiment, and the body weight in the BBN-SA group was ~10% less than that in the BBN group at the end of the experiment (Table I). Intake of BBN, food consumption and water intake (data not shown) were essentially equivalent in all groups. The SA administration caused significant elevation of pH, sodium ion concentration and ascorbic acid contents in urine, but no difference in these parameters was observed between the two parental strains. Urolithiasis was not found in any rat in this study.

We classified both papillomas and carcinomas as bladder tumors, since both tumors grew as papillary projections over the mucosal layer without showing deeper invasion, unlike mouse bladder tumors. The effects of SA on the number of bladder tumors in F344 and WS, and reciprocal F_1 hybrid rats are shown in Fig. 1. In F344, the number of tumors per rat was significantly higher in the BBN-SA group than in the BBN alone group ($P < 0.0001$). The promoter effect of SA in reciprocal F_1s was almost equivalent to that in F344, but significantly higher than that in WS ($P < 0.005$). In contrast, WS rats were not significantly affected by either treatment. Administration of BBN alone yielded equally few tumors in F344, WS and their F_1s. The reciprocal F_1 hybrid rats given BBN-SA had significantly more tumors than those given BBN alone ($P < 0.0001$). Both F_1 hybrids had significantly more tumors than the WS parents ($P < 0.005$).

Bladder tumors in backcross rats and mapping of susceptibility loci One hundred and eleven WS×(WS×F344) F_1 male rats were given BBN followed by SA. Three rats died accidentally without tumors and were excluded. Ultimately 108 rats were available for genetic analysis. A preliminary genome-wide screening was carried out using

Table I. Effects of BBN and BBN-SA on Daily Intakes of BBN, Na^+, T-AsA and Urine Properties

Strain	SA	No. of rats	Body weight (g)[a]		Daily intake (mg/kg)[a]			Properties of urine[b]		
			Start	Final	BBN	Na^+	T-AsA	pH	Na^+ (m*M*)	T-AsA (mg/100 ml)[b]
F344	+	20	146	449	69	312[c]	1,673[c]	7.8±0.2[c]	291±56[c]	932±402[c]
	−	20	147	462	68	105	1[d]	6.5±0.2	193±95	540±123
WS	+	20	178	419	82	371[c]	2,032[c]	7.3±0.4[c]	223±54[c]	1,555±567[c]
	−	20	180	479	79	112	1	6.7±0.5	132±50	268±155
(WS×F344) F_1	+	20	183	464	69	353[c]	1,951[c]	7.6±0.5[c]	268±50[c]	1,490±1,387[c]
	−	20	187	499	68	105	1	6.8±0.4	186±59	410±121
(F344×WS) F_1	+	20	196	486	73	328[c]	1,789[c]	7.7±0.4[c]	300±81[c]	1,090±410[c]
	−	20	194	520	71	101	1	6.6±0.5	166±36	405±143

a) Mean.
b) Determined at 24 weeks; mean±SD.
c) $P<0.05$, vs. the control group.
d) Estimated from the food consumption.

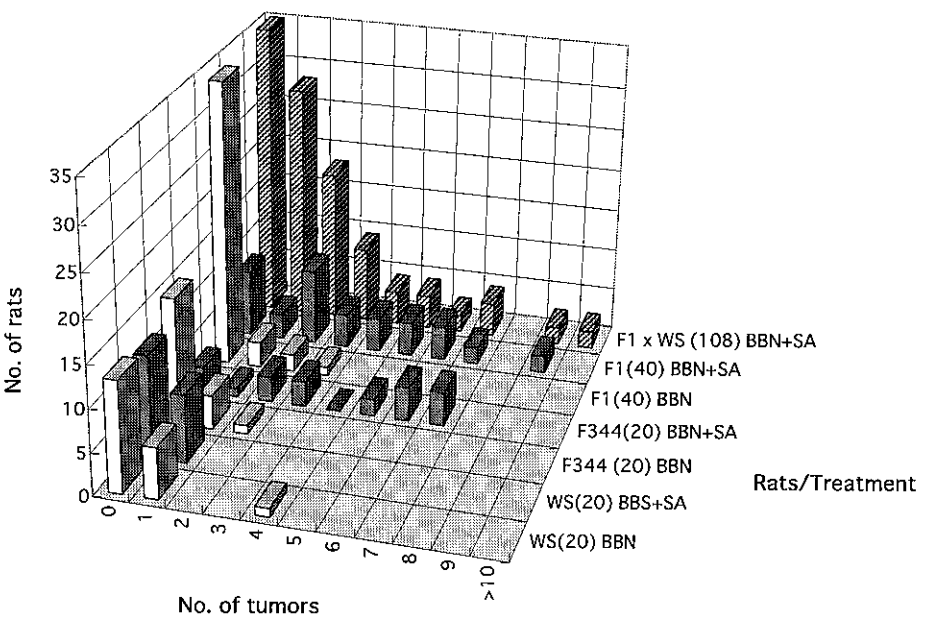

Fig. 1. Effect of SA on the number of bladder tumors. The data for reciprocal F_1s were pooled since there was no significant difference between them. The numbers of rats with given numbers of tumors (papillomas plus carcinomas) shown by open columns are those given BBN alone and by shaded column, BBN plus SA. Hatched shaded columns represents the data for backcross rats given BBN plus SA.

20 rats with more tumors and 20 rats without tumors. Genotypes were determined at 60 microsatellite marker loci polymorphic between F344 and WS. In this preliminary scan of 40 backcross progeny, we detected suggestive linkage disequilibria on chromosomes 2, 5, 9, 17 and 18, as shown in Table II. Subsequently all 108 backcross rats were genotyped for all available polymorphic marker loci on these 5 chromosomes and analyzed by QTL analysis with MAPMAKER/QTL software. Two QTLs, one on Chr. 17 and another on Chr. 5, seemed worthy of consideration as candidates influencing tumor development (Fig. 2). Other QTLs on Chr. 2, 9 and 18 were excluded because the maximum LOD scores were <2.0.[21] One QTL on Chr. 17 showed a maximum LOD score of 3.86, explaining about 15% of the variance. Its dominant allele in F344 increased the number of tumors per rat in response to SA. This locus was named *Bladder Tumor Susceptible-1* (*BTS-1*). Another QTL was mapped

Table II. Possible Linkages Found in a Preliminary Genome-wide Screening[a)]

Marker loci	Genotype of high tumor group		Genotype of low tumor or null group		P
	W/F	W/W	W/F	W/W	
D2Mgh14	11	9	9	11	0.5
D2Mgh10	15	5	7	13	0.01
D2Mgh12	14	6	9	11	0.1
D5Mgh1	8	12	13	7	0.1
D5Mit10	6	14	13	7	0.03
D5Mit4	3	17	16	4	0.00004
D5Mit13	7	13	13	7	0.06
D5Mgh14	6	14	12	8	0.06
D5Mgh9	5	15	10	10	0.1
D9Mit1	8	12	11	9	0.3
D9Mgh2	8	12	10	10	0.5
D9Mit4	5	15	13	7	0.01
D17Mgh2	13	7	7	13	0.06
D17Mit2	14	6	5	15	0.004
D17Mit3	18	2	5	15	0.00003
D17Mit4	17	3	4	16	0.00004
D18Mit1	15	5	7	13	0.01
D18Mit3	15	5	6	14	0.004
D18Mgh3	16	4	10	10	0.05

a) Data for chromosomes containing loci with linkage at $P=0.01$ or less.

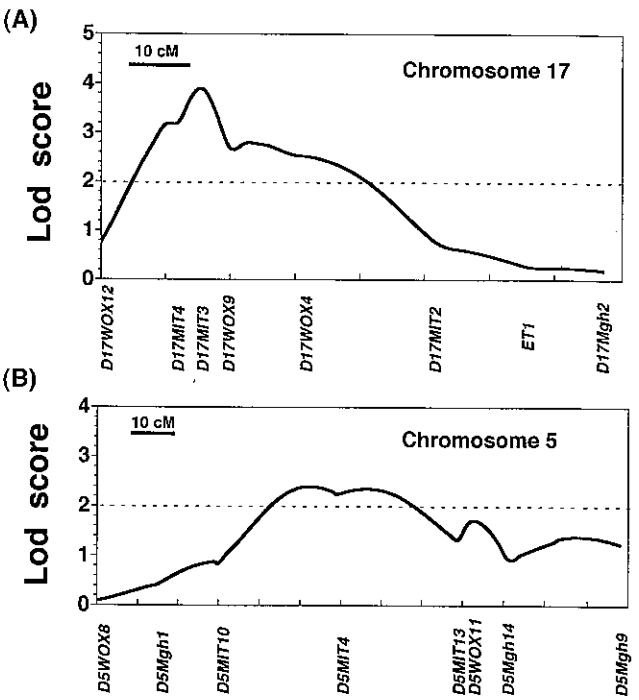

Fig. 2. Genetic linkage maps of Chr. 17 (A) and 5 (B), showing LOD scores for bladder tumor development. Orders and distances between microsatellite markers were estimated from the recombination fractions in 108 backcrosses analyzed by MAPMAKER/EXP3.0. Calculations by MAPMAKER/QTL 1.1 yielded weights for tumor development of 0.713 for *BTS-1* and −0.646 for the QTL on Chr. 5, respectively.

on Chr. 5 showing a dull peak around *D5MIT4* with a maximum LOD score of 2.39, explaining about 14% of variance. The F344-derived dominant allele at this locus seemed to suppress the tumor promotion, while the WS-derived allele seemed to favor susceptibility. These two QTLs had antagonistic effects on tumor promotion. However, the effect of the second QTL was statistically marginal, so that its significance remains to be confirmed.

DISCUSSION

Carcinogenesis is a multifactorial genetic event affected by a number of host genes, which may influence carcinogen activation or inactivation, efficiency of DNA repair, anti-tumor immunity or response to tumorigenic viruses. Host genes affect not only the final incidence of tumors, but also the length of the latent period and the types of tumors under certain conditions.[22,23] This genetic approach is useful to dissect out the critical step of carcinogenesis. In this study, we focused on the difference in genetic susceptibility to the tumor-promoting activity of SA in two-stage bladder carcinogenesis initiated by BBN in rats. Using the number of tumors, i.e., papillomas and carcinomas, as the parameter in QTL analysis, susceptibility to the tumor promoter effect of SA was studied in crosses between two inbred rat strains, susceptible F344 and resistant WS. One significant QTL, *BTS-1*, and another putative QTL with antagonistic effects on tumor development, were identified and mapped on Chr. 17 and Chr. 5, respectively. The F344-derived allele of *BTS-1* significantly favored the promoting effect of sodium ascorbate on BBN-initiated urothelium.

Rat bladder urothelium initiated with BBN provides a useful regimen to identify chemicals with tumor-promoting activity, one of which is SA.[9,13,24] However its activity as a promoter in rat bladder is intriguing. AsA itself does not show promoter activity.[11] Administration of SA induces increases in urinary pH and Na^+, whereas that of AsA does not. However, when given with an appropriate dose of $NaHCO_3$, AsA induces increases of urinary pH and Na^+, and moreover, exhibits a tumor-promoting effect. In contrast, the effects of SA on urinary properties and tumorigenesis are canceled when SA is given together with acidifying NH_4Cl.[13,25] Similar behavior has been noted with other substances, such as saccha-

rin and glutamate. These observations indicate that the tumor-promoting effect of SA and other sodium salts is essentially dependent on Na^+.[25-27] However, F344 and WS rats were equally susceptible to elevation of urinary pH and Na^+ content. Therefore, the step involved in elevation of pH and Na^+ may not be responsible for the difference in tumor promoter susceptibility.

The effects of elevated pH and Na^+ are pleiotropic. Cohen[26] proposed 5 possible mechanisms by which higher urinary pH may influence urothelial cell proliferation: (a) enhanced formation of urinary precipitates and/or crystalline material, (b) potentiation of the effects of salt on urothelium, (c) direct enhancement of urothelial cells proliferation, (d) modification of interactions between growth factors and their receptors and (e) alteration of other urinary constituents. At present, it is unclear which is the most plausible mechanism and which is the process susceptible to the genetic difference between F344 and WS rats. We have found no candidate genes on Chr. 17 or 5 or on conserved syntenic mouse or human chromosomes. As regards the effect of the second QTL on Chr. 5, although the linkage is only marginal, there are 3 points worthy of consideration. Firstly, loss of heterozygosity is frequently observed in human bladder tumors at human Chr. 9p and 9q,[28,29] a synteny conserved segment for this locus. Secondly, a_{2u}-globulin (A2G), mapped in this segment of Chr. 5, is claimed to accelerate the promoter action of sodium saccharin, forming fine crystals that damage umbrella cells.[30] However, in our previous experiment,[16] both F344 and WS rats expressed A2G at a similar level. Therefore, it seems unlikely that the level of A2G itself is responsible for the difference in SA promoter susceptibility. Thirdly, Na^+/H^+ exchanger (NHE) function may be relevant to promoter action. Szpirer *et al.* mapped the gene encoding an isoform of NHEs on rat Chr. 5.[31] In our previous experiment (Murai, unpublished data), amiloride, an NHE blocker, inhibited SA promoting activity. These candidate genes remain to be analyzed further.

In conclusion, our study has provided evidence that genetic factors influence the risk of the development of bladder tumor, although our study was confined to a one backcross. To evaluate fully the genetic effects on bladder carcinogenesis, two other crosses would be required, namely backcrosses to F344 and F_2. In this sense, the present work is preliminary. However, discovery of such genes has important implications for identification of the risk group and for the prevention of bladder tumors by avoiding possible promoter substances.

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