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Inhibitory effects of soy isoflavones on rat prostate carcinogenesis induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)

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Intake of isoflavones derived from soybean products may impact on prostate cancer risk. Here we evaluated the effects of Fujiflavone, a commercial isoflavone supplement, on rat prostate carcinogenesis induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most abundant heterocyclic amine in cooked meat. F344 male rats were given intragastric administrations of PhIP at the dose of 200 mg/kg twice weekly for 10 weeks. The rats subsequently fed a diet containing 0.25% Fujiflavone showed a significantly lower incidence of prostate carcinomas than those fed a soy-free diet. Interestingly fewer carcinomas but more foci of prostatic intra-epithelial neoplasia (PIN) were observed in the Fujiflavone group although the sum of the two lesions was not altered by Fujiflavone treatment. cDNA array analyses confirmed by semi-quantitative reverse transcription polymerase chain reactions (RT–PCR) revealed Fujiflavone to alter gene expression of ornithine decarboxylase (ODC), prothymosin alpha (PTA) in the rat prostate. No modification of PhIP-induced colon carcinogenesis was evident, except for increased multiplicity of aberrant crypt foci >4 crypts in size. These results indicate that a commercial isoflavone supplement can inhibit PhIP-induced rat prostate carcinogenesis without any adverse effects, possibly by inhibiting progression of PIN to carcinoma, and that down-regulation of ODC and PTA could be related to the underlying mechanisms. Thus, intake of dietary isoflavones can be promising for prevention of human prostate cancer.

Introduction

Prostate cancer is one of the chief causes of male cancer death in North America and Europe, but is much less frequent in Asia (1). The reason might be partly explained by differences of dietary habits because Japanese emigrants to America who adopt a Western diet demonstrate higher risk of prostate cancer than native Asians (2).

Western people tend to consume a large amount of cooked meat (3–5), from which a number of heterocyclic amines (HCAs), known to be highly mutagenic carcinogens, have been isolated (6,7). Among them we have found previously 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most abundant HCA (8), to be a carcinogen in the rat prostate (9). PhIP also causes mammary and colon cancers in the rat (10), both of which are common in Western countries as well as prostate cancer (1). Therefore, this HCA is a candidate for human carcinogen.

Asians consume a great deal of soy products containing abundant isoflavones (3). Many biological actions of isoflavones related to tumor growth suppression and cancer prevention have been reported (11) and epidemiological studies have revealed that a high intake of isoflavones is associated with low breast, prostate and colon cancer risk (12), suggesting that isoflavones might be promising candidates for chemoprevention of these cancers.

Many previous studies on the anticancer effects of isoflavones were focused mainly on genistein, a major isoflavone (11). However, single genistein for chemoprevention is not practical because of difficulties and expense in purification. In addition, promoting effects of pure genistein on colon carcinogenesis were found in a previous investigation (13). In the present study we evaluated the effects of Fujiflavone, a commercial isoflavone supplement containing relatively low genistein derivatives, on prostate carcinogenesis induced by PhIP. Alterations of gene expression in the rat prostate after the treatment with Fujiflavone were also examined.

Materials and methods

Animals

Five-week-old F344 male rats were purchased from Charles River Japan (Atsugi, Japan) and housed 2 or 3/plastic cage with wooden chips for bedding in an air-conditioned room at 22 ± 2 and 55 ± 5% humidity with a 12 h light/dark cycle. Tap water and food were available *ad libitum*.

Chemicals

PhIP hydrochloride with purity >99.9% was purchased from the NARD institute (Osaka, Japan). Fujiflavone P40, an enriched isoflavone extract from soy germ, was kindly provided by the manufacturer (Fujicco Co., Kobe, Japan). Manufacturing procedures and characteristics of Fujiflavone were described in a previous report (14) and are available on the website (http://www.fujicco.co.jp/english). Isoflavone components in Fujiflavone are listed in Table I.

Animal experiment 1

PhIP suspended in corn oil was administered to 6-week-old F344 male rats intragastrically at the dose of 200 mg/kg twice a week for 10 weeks. The rats in one group were fed powdered soy-free diet (soybean-free modified NIH-07; Oriental yeast Co., Tokyo, Japan) and those in the other were given the same diet containing Fujiflavone at a concentration of 0.25% for the next 50 weeks (Figure 1). The animal weights were recorded once or twice a week until the end of the experiment. In addition, food consumption was monitored twice a month during the latter half of the experimental course. At the age of 66 weeks, all rats were killed under ether anesthesia and their prostates, kidneys, livers and colons were excised. Kidneys and livers were weighed and prostates and colons were processed for histological examination. Blood was collected from five rats of each group for determination of serum hormone levels.

Abbreviations: ACF, aberrant crypt foci; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; ODC, ornithine decarboxylase; PIN, prostatic intra-epithelial neoplasia; PTA, prothymosin alpha.

Tissue processing

Prostates and colons were fixed in 10% buffered formalin. Prostates were then divided into ventral lobes, dorso-lateral lobes and seminal vesicles with anterior lobes. Each lobe was weighed and the whole ventral lobe, two transverse slices of the dorso-lateral lobe and four transverse slices of the seminal vesicle with the anterior lobe from each side were embedded in paraffin for histological examination. Colons were stained with 0.5% methylene blue for counts of aberrant crypt foci (ACF), followed by paraffin embedding of macroscopically visible tumors for histological diagnoses.

Histological and histomorphometric examinations

Routine histological examination was performed with hematoxylin and eosin staining. Multiplicity of the prostate neoplastic lesions was determined as the number of neoplastic foci per cm^2 of each section. Areas of each prostate lobe were measured with an image processor (Image Processor for Analytical Pathology; Sumika Technoservice, Osaka, Japan). ACF were counted with a stereoscopic microscope (SZH10; Olympus Optical, Tokyo, Japan).

Table I. Contents of isoflavones in Fujiflavone P40[a]

Isoflavones	Contents (g/100 g)
Daizin	22.72
Glycitin	18.17
Genistin	5.69
Malonyldaizin	0.18
Malonylgycitin	0.06
Malonylgenistin	0
Acetyldaizin	1.23
Acetylglycitin	0.88
Acetylgenistin	0.43
Diazein	0.31
Glycitein	0.58
Genistein	0.08
Total	50.33

[a]Analysis by the manufacturer.

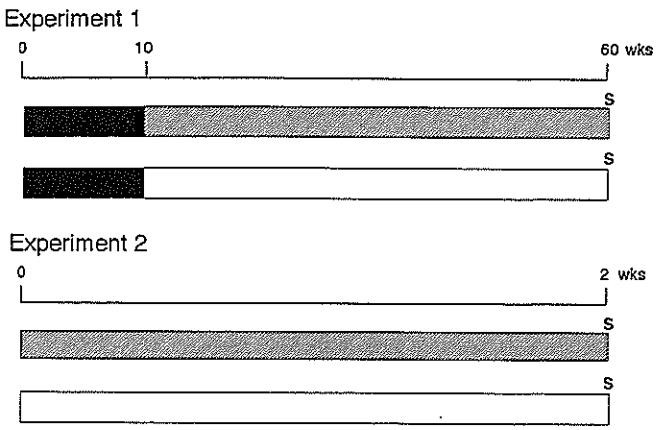

Fig. 1. Experimental protocols. Animals were F344 male rats 6 weeks old at commencement. Closed box, PhIP 200 mg/kg body weight intragastric administration twice weekly. Hatched box, 0.25% Fujiflavone administered in soy-free diet. Open box, Soy-free diet only. S, sacrifice.

Animal experiment 2

Six-week-old F344 male rats were divided into two groups. One group of five rats were fed the soy-free diet and the other of five rats were fed the same diet with 0.25% Fujiflavone for 2 weeks (Figure 1). At the age of 8 weeks, all rats were killed under ether anesthesia and their prostates were excised, frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Extraction of total RNA and cDNA array analysis

Total RNA extraction from the rats of 'experiment 2' followed by DNase treatment were performed with the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Fifty micrograms of total RNA from rats in each group were pooled, respectively, and used for probe synthesis of cDNA arrays. Poly(A)+ RNA enrichment and ^{32}P labeling were performed according to the manufacturer's instructions (Atlas Pure Total RNA Labeling System; Clontech). The Atlas Rat Stress/Toxicology Array (Clontech) was used to compare gene expression profiles of the rat prostate in the Fujiflavone group with those in the soy-free group. Signal spots for each gene were detected and analyzed with an image analyzer (FLA-3000G; Fujifilm, Tokyo, Japan) using Array Gauge software (Fujifilm). The expression ratio of each gene was calculated from the average signal intensity determined by duplicate analyses. More than 2-fold increase or decrease (ratio over 2 or under 0.5) was regarded as a significant change.

Semi-quantitative reverse transcription-polymerase chain reactions

One microgram of the RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (Takara, Otsu, Japan) in 20 μl of reaction mixture. Aliquots of 2 μl of cDNA samples were subjected to quantitative PCR in 20 μl reactions using FastStart DNA Master SYBR Green I and a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany). Primers are listed in Table II. Initial denaturation at 95°C for 10 min was followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 56–60°C for 5 s and elongation at 72°C for 30 s. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of formed PCR product, was monitored at the end of each elongation step. mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to normalize the sample cDNA content.

Statistics

Mann–Whitney's *U*-test was employed for statistical analyses of body and organ weight, tumor multiplicity and gene expression levels. Tumor incidence was analyzed with Fisher's exact probability test.

Results

Body and relative organ weights

Body weight gain of the rats did not differ between the Fujiflavone and soy-free diet groups through the experiment. Relative weights of the kidney and the liver were not affected by administration of Fujiflavone (Table III) but that of the prostate was smaller in the Fujiflavone group due to decrease in weight of the seminal vesicle with the anterior lobe (Table IV). The weight of the ventral lobe, where neoplastic lesions are usually caused by PhIP (9), demonstrated no significant difference between the two groups (Table IV).

Table II. Primers used for semi-quantitative RT-PCR

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
PTA	CGCCAGAGTCCTCGAAACTC	CATTCTCTGCCTCCTCCACAA
ODC	GTTGGTTTTGCGGATTGCC	TGCCCGTTCCAAGAGAAGC
Microsomal glutatione *S*-transferase 1	TCGTTCCCTTTCTCGGTATCG	TGAGCAGCCTGTAAGCCATTG
Glutathione peroxidase 1	AGTGCGCATACAGCAGGC	CCGTCATGGAAAAACCTCCC
Glutathione *S*-transferase, mu type 2	GAGAGGATTCGTGTGGACGTT	GCCCAGGAATTCGGAGTAAA
Bcl-2 associated death agonist	AGAGTTTGAGCCGAGTGAGC	TGACTGTTATTGGCTGCCTG
Cyclin D1	GCCCTCCGTTTCTTACTTC	AGACCTCCTCTTCGCACTTC
Cyclin-dependent kinase 4	TTCGTGAGGTGGCCTTGTTA	GCGCATCAGATCCTTAATGGT
GAPDH	TGATTCTACCCACGGCAAGTTC	TTCACACCCATCACAAACATGG

Table III. Final body weights and relative organ weights

Group	Treatment	No. of rats	Final body weight (g)	Liver (%)	Kidneys (%)	Prostate (%)
1	PhIP + Fujiflavone	14	359.8 ± 22.8	2.70 ± 0.14	0.56 ± 0.03	0.44 ± 0.07[a]
2	PhIP + soy-free diet	16	347.4 ± 18.5	2.74 ± 0.07	0.57 ± 0.04	0.49 ± 0.08

Data are mean ± SD values.
[a]$P < 0.01$ *vs* group 2.

Table IV. Relative weight of each lobe of the prostate

Group	Treatment	No. of rats	Prostate (%)			
			Ventral	Dorsolateral	Anterior-SV	Total
1	PhIP + Fujiflavone	14	0.07 ± 0.02	0.13 ± 0.02	0.24 ± 0.05[a]	0.44 ± 0.07[a]
2	PhIP + soy-free diet	16	0.07 ± 0.01	0.14 ± 0.03	0.29 ± 0.05	0.49 ± 0.08

Data are mean ± SD values. SV, seminal vesicle.
[a]$P < 0.01$ *vs* group 2.

Table V. Incidence and multiplicity of neoplastic lesions in the ventral prostate

Group	Treatment	No. of rats	Incidence (%)		Multiplicity[a]		
			PIN	CA	PIN	CA	PIN + CA
1	PhIP + Fujiflavone	14	14 (100)	3 (21.4)[b]	3.47 ± 1.14[b]	0.31 ± 0.62[b]	3.78 ± 1.46
2	PhIP + soy-free diet	16	15 (93.8)	10 (62.5)	2.26 ± 1.39	0.90 ± 0.84	3.16 ± 2.09

[a]Expressed by the number of foci per cm^2 of one section. Data are mean ± SD values.
[b]$P < 0.05$ *vs* group 2.
CA, carcinoma.

Table VI. Incidence and multiplicity of neoplastic lesions of the colon

Group	Treatment	No. of rats	Carcinoma		ACF				
			Incidence (%)	Multiplicity (%)	Incidence (%)	Multiplicity[b]			
						Foci/rat	Crypts/rat	Crypts/focus	ACF with ≥4 crypts/rat
1	PhIP + Fujiflavone	14	5 (35.7)	1.00 ± 0.00	14 (100)	11.57 ± 6.35	39.07 ± 27.21	3.28 ± 0.59	4.57 ± 2.87[c]
2	PhIP + soy-free diet	16	4 (25.0)	1.00 ± 0.00	16 (100)	8.25 ± 4.17	27.25 ± 14.01	3.43 ± 1.42	2.31 ± 1.82

[a]No. of tumors per tumor-bearing rat.
[b]Data are mean ± SD values.
[c]$P < 0.05$ *vs* group 2.

Incidence and multiplicity of neoplastic lesions in the prostate

Prostatic intra-epithelial neoplasia (PIN), a putative precancerous lesion of the rat prostate (15,16) as in the human prostate (17–19) was found in anterior and ventral lobes of almost all rats in both groups. As reported previously (9), carcinomas were observed only in the ventral lobe, all of which were non-invasive. They were observed in 62.5% of rats in the soy-free group, while 21.4% of those in the Fujiflavone group, showing statistical difference with $P < 0.05$ (Table V). PIN and dysplasia were observed in the anterior lobe and the seminal vesicle of most rats in both groups, respectively. No neoplastic lesions were found in the dorsolateral lobe (data not shown).

Concerning multiplicity of PIN and carcinoma in the ventral lobe, more PIN foci and less carcinoma foci were observed in the Fujiflavone group than in the soy-free group with significant difference ($P < 0.05$). However, the sums of the two lesions were about equal in each group (Table V).

Effects of Fujiflavone on PhIP-induced colon carcinogenesis

The incidence of colon carcinomas was 35.7% in the Fujiflavone group and 25% in the soy-free group showing no significant difference. All carcinomas were non-invasive and every tumor-bearing rat had only single lesions (Table VI). As to ACF, putative carcinoma-related lesions (20,21), no significant difference was shown on comparison of incidence.

Multiplicity of ACF with ⩾4 crypts was statistically higher in the Fujiflavone group (Table VI).

Change of gene expression profile by treatment with Fujiflavone

cDNA array analyses detected seven up-regulated genes and nine down-regulated genes with >2-fold expression change (Table VII). Confirmation of the data using semi-quantitative reverse transcription–polymerase chain reactions (RT–PCRs) showed more considerable expression changes of the down-regulated genes by Fujiflavone treatment than those of the up-regulated genes, especially down-regulation of prothymosin alpha (PTA), ornithine decarboxylase (ODC) were evident with 0.36- and 0.52-fold expression, respectively (Figure 2).

Discussion

In the present study significant inhibitory effects of Fujiflavone, a commercial isoflavone supplement, on rat prostate carcinogenesis were clearly shown, consistent with previous studies with various types of isoflavones (22–27). We applied PhIP as a prostate carcinogen as it might be more relevant to the human situation than other carcinogens (28–30), considering its target organs (9,10) and possible daily intake by people with

Table VII. Up- or down-regulated genes with Fujiflavone treatment in the rat prostate on cDNA array analyses

	GenBank accession no.	Gene name
Up-regulated (>×2.0)	J02592	Glutathione *S*-transferase, mu type 2 (Yb2)
	J03752	Microsomal gluthione *S*-transferase 1
	L11007	Cyclin-dependent kinase 4
	M69249	Serine (or cysteine) proteinase inhibitor, clade H, member 1
	M86389	Heat shock 27 kDa protein
	X02904	Glutathione *S*-transferase, pi2
	X12367	Glutathione peroxidase 1
Down-regulated (<×0.5)	AF003523	Bcl-2 associated death agonist
	D13374	Expressed in non-metastatic cells 1
	D14014	Cyclin D1
	D16309	Cyclin D3
	J04792	ODC 1
	M20035	PTA
	S78556	Mitochondrial stress-70 protein precursor (MTHSP70)
	U41853	Oxygen regulated (150 kDa)

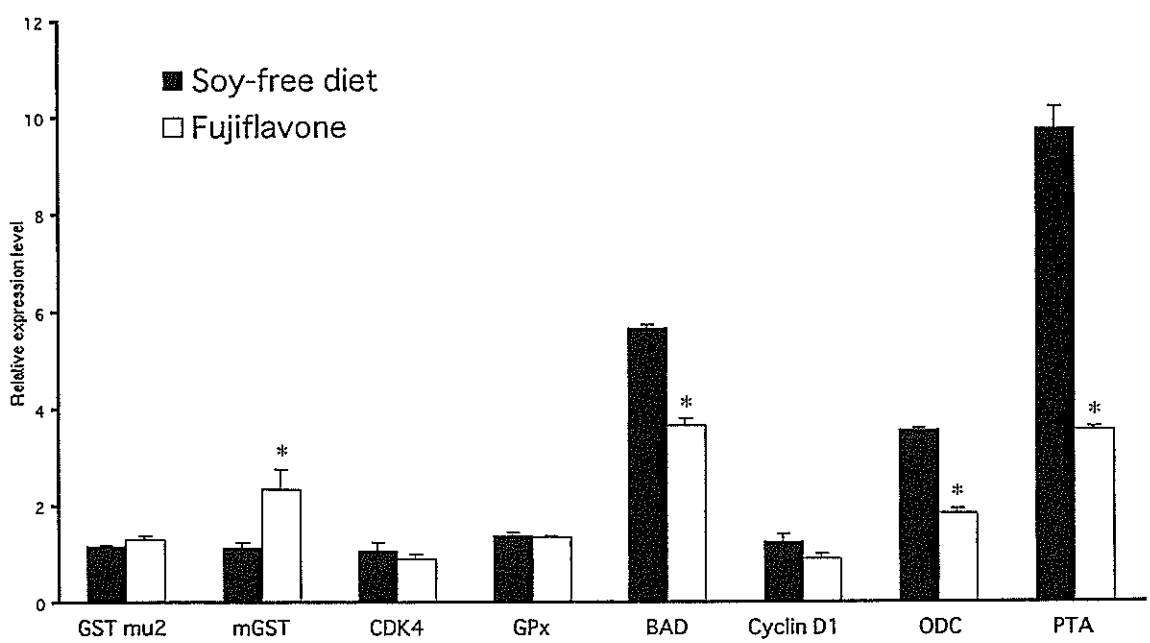

Fig. 2. Expression levels of genes detected by cDNA array analyses confirmed with semi-quantitative RT-PCR. Expression levels are presented as ratios to GAPDH expression level. Values are means of triplicate experiments ±SD. GST mu2, glutathione *S*-transferase, mu type 2; mGST, microsomal glutathione *S*-transferase; CDK, cyclin-dependent kinase; GPx, glutathione peroxidase; BAD, Bcl-2 associated death agonist; ODC, ornithine decarboxylase; PTA, prothymosin alpha. *$P < 0.05$.

Western-style diet (31). Anticancer effects of isoflavones have been explained by referring to various possible mechanisms including inhibition of tyrosine kinases (32,33), topoisomerase II (34), angiogenesis (35) and growth factor-induced c-fos expression (36). Antioxidant properties (37,38) and promotion of apoptosis (39) have also been reported. PhIP-induced rat prostate carcinomas are androgen receptor-positive and show androgen-dependent growth, like human prostate cancers (unpublished data). Although isoflavones have weak estrogenic properties (40) and a recent report has shown genistein is likely to inhibit 5-alpha reductase activity resulting in a reduction of dihydrotestosterone (DHT)—active form of testosterone—in several tissues including the prostate (25), serum luteinizing hormone, testosterone and DHT levels and DHT/TES ratio was not modified by Fujiflavone treatment (data not shown). Besides weights of each prostate lobe did not differ with the Fujiflavone treatment and microscopically prostate glands showed no involution (data not shown). These results suggest that anti-androgenic effects were not significantly involved in the mechanism of Fujiflavone to inhibit prostate carcinogenesis. Interestingly fewer carcinomas but more PIN foci were observed in the Fujiflavone group although the sum of the two lesions was not altered by Fujiflavone treatment. This might indicate Fujiflavone inhibits progression of PIN to carcinomas.

To evaluate the effects of Fujiflavone on gene expressions in the prostate tissue, cDNA array analysis of the rat prostate with Fujiflavone treatment was performed without PhIP pretreatment in the present study. This protocol was used since PhIP is likely to alter gene expressions in the prostate by itself. For example, PhIP induces cytochromes P450, and is mutagenic and forms DNA adducts (41,42). Such changes could modify or mask the alterations that Fujiflavone might bring, and obscure which chemical is responsible for the observed effects. PTA, one of the down-regulated genes confirmed by semi-quantitative RT–PCR in the present study, is a small acidic nuclear protein ubiquitously expressed in various types of cells. Its physiological role remains unclear but several studies have indicated its involvement with cell proliferation including processes leading to malignant transformation (43,44) and poor prognosis in some clinical cancers (45,46). ODC 1, another down-regulated gene in the Fujiflavone-treated prostate, codes the key enzyme for synthesis of polyamines known as cell proliferation factors. High activity of ODC in cancer cells or cancerous tissues including the prostate has been reported and over-expression of ODC can cause cellular transformation like a proto-oncogene product (47). Development of ODC inhibitors as anticancer agents is under way (48,49), also targeting prostate cancer (50,51). As described above, less PIN lesions progressed into carcinomas in the Fujiflavone-treated prostate in the present study. These findings indicated that down-regulation of PTA and ODC might be related to the mechanism of Fujiflavone to inhibit prostate carcinogenesis. It is probable that down-regulation of PTA and ODC by Fujiflavone is specific to the prostate because their expression changes were not detected at least on cDNA array analyses of the Fujiflavone-treated colon, forestomach and liver (data not shown). Therefore, further studies for involvement of these genes in prostate cancer might be necessary to reach unique preventive or therapeutic options against prostate cancer.

Most previous studies on anticancer effects of isoflavones focused mainly on genistein. Although soybean products generally contain genistein derivatives as major isoflavones, other isoflavone components such as daizein and glycitein cannot be negligible (52). A recent epidemiological study has shown a correlation between daizein-metabolizing ability and prostate cancer progression (53). Although Fujiflavone used in the present study contains more daizein and glycitein derivatives than other isoflavone mixtures used in previous reports (23–25) and relatively low amounts of genistein derivatives, inhibitory effects on rat prostate carcinogenesis were apparent and not less than seen in previous studies using similar protocols. Some reports described adverse effects of pure genistein including the promotion of mammary and colon cancer (13,54). Here the incidence of ACF with $\geq$4 crypts was significantly elevated by Fujiflavone, whereas the final incidence and multiplicity of colon tumors were almost identical in the two groups, indicating no promotion of tumorigenesis. These findings do not accord with the reported correlation between ACF with $\geq$4 crypts and final colon tumor outcome (55). The present colon tumor results suggest that isoflavone mixture might have a less adverse effect than purified genistein and be more suitable as a cancer-preventive agent. And it would be important to study all critical aspects of each isoflavone component including their interactions.

In the present study each rat fed Fujiflavone consumed ~80 mg/kg/day of total isoflavones, estimated by food consumption monitoring (data not shown). Such an isoflavone intake is very high compared with the Japanese daily isoflavone intake (52,56). As to toxicity, 868 mg/day has been found safe in a human trial (57) and a single administration of 5000 mg/kg demonstrated no toxicity in mice (T.Toda, personal communication). Previous studies with relatively high dose of isoflavones have not revealed any toxic findings (22–26). Considering the data, together with body weight gain during the experimental course and the final organ weights in the present study, Fujiflavone seems to have no toxicity even at high dose.

In conclusion, Fujiflavone, a commercial isoflavone mixture supplement, showed inhibitory effects on rat prostate carcinogenesis induced by PhIP, an environmental human carcinogen candidate, possibly by inhibiting progression of PIN to carcinoma with no evident adverse effects. And the down-regulation of PTA and ODC could be related to the mechanism. These results suggest that intake of dietary isoflavones is promising for prevention of human prostate cancer.

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References

1. Jemal,A., Thomas,A., Murray,T. and Thun,M. (2002) Cancer statistics, 2002. *CA Cancer J. Clin.*, 52, 23–47.
2. Shimizu,H., Ross,R.K., Bernstein,L., Yatani,R., Henderson,B.E. and Mack,T.M. (1991) Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br. J. Cancer*, 63, 963–966.
3. Menotti,A., Kromhout,D., Blackburn,H., Fidanza,F., Buzina,R. and Nissinen,A. (1999) Food intake patterns and 25-year mortality from

coronary heart disease: cross-cultural correlations in the Seven Countries Study. The Seven Countries Study Research Group. *Eur. J. Epidemiol.*, **15**, 507–515.
4. Rose,D.P. (1993) Diet, hormones and cancer. *Annu. Rev. Public Health*, **14**, 1–17.
5. Schiffman,M.H. and Felton,J.S. (1990) Re: 'Fried foods and the risk of colon cancer'. *Am. J. Epidemiol.*, **131**, 376–378.
6. Wakabayashi,K., Nagao,M., Esumi,H. and Sugimura,T. (1992) Food-derived mutagens and carcinogens. *Cancer Res.*, **52**, 2092s–2098s.
7. Keating,G.A., Layton,D.W. and Felton,J.S. (1999) Factors determining dietary intakes of heterocyclic amines in cooked foods. *Mutat. Res.*, **443**, 149–156.
8. Felton,J.S. and Knize,M.G. (1991) Occurrence, identification and bacterial mutagenicity of heterocyclic amines in cooked food. *Mutat. Res.*, **259**, 205–217.
9. Shirai,T., Sano,M., Tamano,S. *et al.* (1997) The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) derived from cooked foods. *Cancer Res.*, **57**, 195–198.
10. Ito,N., Hasegawa,R., Sano,M., Tamano,S., Esumi,H., Takayama,S. and Sugimura,T. (1991) A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Carcinogenesis*, **12**, 1503–1506.
11. Messina,M.J., Persky,V., Setchell,K.D. and Barnes,S. (1994) Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. *Nutr. Cancer*, **21**, 113–131.
12. Adlercreutz,H. (1998) Epidemiology of phytoestrogens. *Baillieres Clin. Endocrinol. Metab.*, **12**, 605–623.
13. Rao,C.V., Wang,C.X., Simi,B., Lubet,R., Kelloff,G., Steele,V. and Reddy,B.S. (1997) Enhancement of experimental colon cancer by genistein. *Cancer Res.*, **57**, 3717–3722.
14. Uesugi,T., Fukui,Y. and Yamori,Y. (2002) Beneficial effects of soybean isoflavone supplementation on bone metabolism and serum lipids in postmenopausal japanese women: a four-week study. *J. Am. Coll. Nutr.*, **21**, 97–102.
15. Slayter,M.V., Anzano,M.A., Kadomatsu,K., Smith,J.M. and Sporn,M.B. (1994) Histogenesis of induced prostate and seminal vesicle carcinoma in Lobund-Wistar rats: a system for histological scoring and grading. *Cancer Res.*, **54**, 1440–1445.
16. Xie,W., Wong,Y.C., Tsao,S.W. and Wong,N.S. (2000) Expression of a kallikrein-like protein in prostatic intraepithelial neoplasia in ventral prostate of the noble rat. *Prostate*, **42**, 8–17.
17. Bostwick,D.G. (1992) Prostatic intraepithelial neoplasia (PIN): current concepts. *J. Cell Biochem. Suppl.*, **16H**, 10–19.
18. Qian,J., Jenkins,R.B. and Bostwick,D.G. (1996) Potential markers of aggressiveness in prostatic intraepithelial neoplasia detected by fluorescence *in situ* hybridization. *Eur. Urol.*, **30**, 177–184.
19. Bostwick,D.G. (1996) Progression of prostatic intraepithelial neoplasia to early invasive adenocarcinoma. *Eur. Urol.*, **30**, 145–152.
20. Bird,R.P. (1987) Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151.
21. Nakagama,H., Ochiai,M., Ubagai,T., Tajima,R., Fujiwara,K., Sugimura,T. and Nagao,M. (2002) A rat colon cancer model induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, PhIP. *Mutat. Res.*, **506–507**, 137–144.
22. Kato,K., Takahashi,S., Cui,L., Toda,T., Suzuki,S., Futakuchi,M., Sugiura,S. and Shirai,T. (2000) Suppressive effects of dietary genistin and daidzin on rat prostate carcinogenesis. *Jpn. J. Cancer Res.*, **91**, 786–791.
23. Onozawa,M., Kawamori,T., Baba,M., Fukuda,K., Toda,T., Sato,H., Ohtani,M., Akaza,H., Sugimura,T. and Wakabayashi,K. (1999) Effects of a soybean isoflavone mixture on carcinogenesis in prostate and seminal vesicles of F344 rats. *Jpn. J. Cancer Res.*, **90**, 393–398.
24. Pollard,M. and Wolter,W. (2000) Prevention of spontaneous prostate-related cancer in Lobund-Wistar rats by a soy protein isolate/isoflavone diet. *Prostate*, **45**, 101–105.
25. Zhou,J.R., Yu,L., Zhong,Y., Nassr,R.L., Franke,A.A., Gaston,S.M. and Blackburn,G.L. (2002) Inhibition of orthotopic growth and metastasis of androgen-sensitive human prostate tumors in mice by bioactive soybean components. *Prostate*, **53**, 143–153.
26. Mentor-Marcel,R., Lamartiniere,C.A., Eltoum,I.E., Greenberg,N.M. and Elgavish,A. (2001) Genistein in the diet reduces the incidence of poorly differentiated prostatic adenocarcinoma in transgenic mice (TRAMP). *Cancer Res.*, **61**, 6777–6782.
27. Wang,J., Eltoum,I.E. and Lamartiniere,C.A. (2002) Dietary genistein suppresses chemically induced prostate cancer in Lobund-Wistar rats. *Cancer Lett.*, **186**, 11–18.
28. Katayama,S., Fiala,E., Reddy,B.S., Rivenson,A., Silverman,J., Williams,G.M. and Weisburger,J.H. (1982) Prostate adenocarcinoma in rats: induction by 3,2′-dimethyl-4-aminobiphenyl. *J. Natl Cancer Inst.*, **68**, 867–873.
29. Pour,P.M. (1983) Prostatic cancer induced in MRC rats by *N*-nitrosobis (2-oxopropyl)-amine and *N*-nitrosobis (2-hydroxypropyl)amine. *Carcinogenesis*, **4**, 49–55.
30. Bosland,M.C. and Prinsen,M.K. (1990) Induction of dorsolateral prostate adenocarcinomas and other accessory sex gland lesions in male Wistar rats by a single administration of *N*-methyl-*N*-nitrosourea, 7,12-dimethylbenz(a)anthracene and 3,2′-dimethyl-4-aminobiphenyl after sequential treatment with cyproterone acetate and testosterone propionate. *Cancer Res.*, **50**, 691–699.
31. Bogen,K.T. and Keating,G.A. (2001) U.S. dietary exposures to heterocyclic amines. *J. Expo. Anal. Environ. Epidemiol.*, **11**, 155–168.
32. Akiyama,T., Ishida,J., Nakagawa,S., Ogawara,H., Watanabe,S., Itoh,N., Shibuya,M. and Fukami,Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, **262**, 5592–5595.
33. Trevillyan,J.M., Lu,Y.L., Atluru,D., Phillips,C.A. and Bjorndahl,J.M. (1990) Differential inhibition of T cell receptor signal transduction and early activation events by a selective inhibitor of protein-tyrosine kinase. *J. Immunol.*, **145**, 3223–3230.
34. Okura,A., Arakawa,H., Oka,H., Yoshinari,T. and Monden,Y. (1988) Effect of genistein on topoisomerase activity and on the growth of [Val 12]-Ha-ras-transformed NIH 3T3 cells. *Biochem. Biophys. Res. Commun.*, **157**, 183–189.
35. Fotsis,T., Pepper,M.S., Montesano,R., Aktas,E., Breit,S., Schweigerer,L., Rasku,S., Wahala,K. and Adlercreutz,H. (1998) Phytoestrogens and inhibition of angiogenesis. *Baillieres Clin. Endocrinol. Metab.*, **12**, 649–666.
36. Tripathi,Y.B., Lim,R.W., Fernandez-Gallardo,S., Kandala,J.C., Guntaka,R.V. and Shukla,S.D. (1992) Involvement of tyrosine kinase and protein kinase C in platelet-activating-factor-induced c-fos gene expression in A-431 cells. *Biochem. J.*, **286** (Pt 2), 527–533.
37. Wei,H., Wei,L., Frenkel,K., Bowen,R. and Barnes,S. (1993) Inhibition of tumor promoter-induced hydrogen peroxide formation *in vitro* and *in vivo* by genistein. *Nutr Cancer*, **20**, 1–12.
38. Messina,M., Barnes,S. and Setchell,K.D. (1997) Phyto-oestrogens and breast cancer. *Lancet*, **350**, 971–972.
39. Stephens,F.O. (1997) Phytoestrogens and prostate cancer: possible preventive role. *Med. J. Aust.*, **167**, 138–140.
40. Morito,K., Hirose,T., Kinjo,J., Hirakawa,T., Okawa,M., Nohara,T., Ogawa,S., Inoue,S., Muramatsu,M. and Masamune,Y. (2001) Interaction of phytoestrogens with estrogen receptors alpha and beta. *Biol. Pharm. Bull.*, **24**, 351–356.
41. Degawa,M., Kobayashi,K., Miura,S., Arai,H., Esumi,H., Sugimura,T. and Hashimoto,Y. (1992) Species difference among experimental rodents in induction of P450IA family enzymes by 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine. *Jpn. J. Cancer Res.*, **83**, 1047–1051.
42. Schut,H.A. and Snyderwine,E.G. (1999) DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis*, **20**, 353–368.
43. Pineiro,A., Cordero,O.J. and Nogueira,M. (2000) Fifteen years of prothymosin alpha: contradictory past and new horizons. *Peptides*, **21**, 1433–1446.
44. Orre,R.S., Cotter,M.A.,2nd, Subramanian,C. and Robertson,E.S. (2001) Prothymosin alpha functions as a cellular oncoprotein by inducing transformation of rodent fibroblasts *in vitro*. *J. Biol. Chem.*, **276**, 1794–1799.
45. Magdalena,C., Dominguez,F., Loidi,L. and Puente,J.L. (2000) Tumour prothymosin alpha content, a potential prognostic marker for primary breast cancer. *Br. J. Cancer*, **82**, 584–590.
46. Sasaki,H., Nonaka,M., Fujii,Y., Yamakawa,Y., Fukai,I., Kiriyama,M. and Sasaki,M. (2001) Expression of the prothymosin-a gene as a prognostic factor in lung cancer. *Surg. Today*, **31**, 936–938.
47. Schipper,R.G., Romijn,J.C., Cuijpers,V.M. and Verhofstad,A.A. (2003) Polyamines and prostatic cancer. *Biochem. Soc. Trans.*, **31**, 375–380.
48. Meyskens,F.L.,Jr and Gerner,E.W. (1999) Development of difluoromethylornithine (DFMO) as a chemoprevention agent. *Clin. Cancer Res.*, **5**, 945–951.
49. Bachrach,U. and Wang,Y.C. (2002) Cancer therapy and prevention by green tea: role of ornithine decarboxylase. *Amino Acids*, **22**, 1–13.
50. Esmat,A.Y., Refaie,F.M., Shaheen,M.H. and Said,M.M. (2002) Chemoprevention of prostate carcinogenesis by DFMO and/or finasteride treatment in male Wistar rats. *Tumori*, **88**, 513–521.
51. Devens,B.H., Weeks,R.S., Burns,M.R., Carlson,C.L. and Brawer,M.K. (2000) Polyamine depletion therapy in prostate cancer. *Prostate Cancer Prostatic Dis.*, **3**, 275–279.

52. Toda,T., Tamura,J. and Okuhira,T. (1997) Isoflavone content in commercial soybean foods. *Foods Food Ingredients J. Jpn*, **172**, 83–88.
53. Akaza,H., Miyanaga,N., Takashima,N., Naito,S., Hirao,Y., Tsukamoto,T. and Mori,M. (2002) Is daidzein non-metabolizer a high risk for prostate cancer? A case-controlled study of serum soybean isoflavone concentration. *Jpn. J. Clin. Oncol.*, **32**, 296–300.
54. Allred,C.D., Ju,Y.H., Allred,K.F., Chang,J. and Helferich,W.G. (2001) Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. *Carcinogenesis*, **22**, 1667–1673.
55. Dashwood,R.H., Xu,M., Hernaez,J.F., Hasaniya,N., Youn,K. and Razzuk,A. (1999) Cancer chemopreventive mechanisms of tea against heterocyclic amine mutagens from cooked meat. *Proc. Soc. Exp. Biol. Med.*, **220**, 239–243.
56. Kimira,M., Arai,Y., Shimoi,K. and Watanabe,S. (1998) Japanese intake of flavonoids and isoflavonoids from foods. *J. Epidemiol.*, **8**, 168–175.
57. Fukui,Y., Nasada,M., Toda,T., Okuhira,T. and Hayashi,A. (1999) Influence of ingestible soy isoflavones in Japanese men. *J. Nutri. Food*, **2**, 63–71.

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p-Nonylphenol pretreatment during the late neonatal period has no effect on 3,2′-dimethyl-4-aminobiphenyl-induced prostate carcinogenesis in male F344 rats

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Abstract

The modifying effects of late neonatal administration of *p*-nonylphenol (NP), a suspected xenoestrogen, on 3,2′-dimethyl-4-aminobiphenyl (DMAB)-induced prostatic carcinogenesis were investigated in male F344 rats. Three-week-old rats received 25, 250 or 2000 ppm of NP in the diet for 3 weeks prior to DMAB treatment and were sacrificed at 67 weeks of age for histopathological assessment of lesions and Ki-67 immunohistochemical analysis of cell cycle kinetics. Dietary administration of NP during the sexually immature period had no effects on maturation of male sex organs. Incidence, multiplicity and areas of neoplastic lesions in the prostate and seminal vesicles, and Ki-67 labeling indices in normal-looking epithelium were not significantly different among the experimental groups. These results indicate that late neonatal treatment with NP has no modulating effects on DMAB-induced rat prostatic carcinogenesis.

Keywords: *p*-Nonylphenol; Endocrine disrupting chemical; 3,2′-Dimethyl-4-aminobiphenyl; Rat; Prostate carcinogenesis

1. Introduction

Recent studies have demonstrated hormone-like activities of environmental agents and considerable attention has been focused on endocrine disrupting chemicals. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) under the US Environmental Protection Agency (EPA) has recommended screening of compounds for their potential to act as agonists or antagonists of estrogen

Abbreviations: DMAB, 3,2′-dimethyl-4-aminobiphenyl; NP, *p*-nonylphenol; OP, *p*-octhylphenol; SV, seminal vesicles; PIN, prostatic intraepithelial neoplasia; ER, estrogen receptor; AR, androgen receptor; CYP, cytochrome P450 enzymes; NOAEL, 'no observed adverse effect' level; DHPN, *N*-bis(2-hydroxypropyl)nitrosamine.

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or androgen receptors, as well as their ability to modulate thyroid functions.

Environmental estrogen-mimicking chemicals are classified into three major types: (1) phytoestrogens, (2) mycoestrogens and (3) xenoestrogens. The first two are naturally existing and the last are man-made, synthesized for commercial use or formed as byproducts of manufacturing processes and combustion of waste. Xenoestrogens include bisphenols [1], polychlorinated bisphenols [2], phthalates [3], and alkylphenol ethoxylates [4,5]. Alkylphenols are derived from biodegradation of alkylphenolpolyethoxylate and are mainly *p*-nonylphenol (NP), the most abundant product accounting for 80% of the alkylphenol, and *p*-octhylphenol (OP). In the environment, NP accumulation is due to commercial sources, and human exposure may occur through contaminated water, food, and contact with the skin, continuously at a dose of around 5–10 μg/day/kg [6,7]. For infants exclusively fed with breast milk or infant formulas daily intakes of 0.2 and 1.4 μg/day NP, respectively, could be estimated in Germany [8].

Estrogenic activity of alkylphenols was first reported in 1936 [9]. Soto et al. [10] reported estrogenic activity of NP in 1991 on establishing the E-SCREEN in vitro bioassay [11]. There have also been experiments conducted to assess the modifying effects of NP using rodent carcinogenic models [12–14]. However, to our knowledge, there has been no report of effects of pretreatment of NP on 3,2′-dimethyl-4-aminobiphenyl (DMAB)-induced rat prostate carcinogenesis. In the present study, we therefore assessed the influence of exposure to NP during the late neonatal period (3–6 week old) on maturation of male sex organs, as well as risks for prostate cancer with DMAB-induced prostate cancer model in F344 rats.

2. Materials and methods

2.1. Chemicals, animals and diets

Male 3-week-old F344 (F344/DuCrj) rats were purchased from Charles River Japan, Inc. (Atsugi) and housed 3/cage on wood-chip bedding in an air-conditioned animal room at 23 ± 2 °C and 50 ± 10% humidity. DMAB was obtained from the Nard Institute (Osaka), with a purity of >98.0%. NP was from Tokyo Kasei Kogyo Co., Ltd (Tokyo). The experimental diet was prepared by adding NP at doses of 25, 250 and 2000 ppm (W/W) to soy-protein-free CRF-1 diet (Oriental Yeast Co, Tokyo) to avoid the effects of the phytoestrogens included in the ordinary CRF-1 diet. Food and tap water were available ad libitum.

2.2. Experimental procedure

The 95 rats were divided into five groups. Twenty rats each in groups 1–4 received the diet containing NP at doses of 0, 25, 250 and 2000 ppm from 3 weeks of age for 3 weeks. Although it is well known that the first 2 weeks of postnatal life are very sensitive to estrogens and androgens including endocrine disrupting chemicals, we started the experiment after weaning for oral administration of NP. Diet administration of NP at the dose of 250 ppm corresponds to approximately 11–44 mg/kg/day in rats, near the 'no observed adverse effect' level (NOAEL) of NP (50 mg/kg/day) [15]. This dose is estimated approximately 1/10 of the maximum tolerated dose (MTD). After administration of NP, they were treated with subcutaneous injection of DMAB at 50 mg/kg body weight every other week for a total of 10 injections from 7 weeks of age. The 15 rats in group 5 were given NP in the same manner as for group 4 without administration of DMAB. The experiment was terminated at week 64 and the animals were subjected to complete autopsy. All organs were carefully inspected and fixed in 10% buffered formalin. After fixation, all grossly abnormal lesions and accessory sex organs were embedded in paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin for histopathological examination. The numbers and areas of histological lesions of prostate and seminal vesicles (SV) were quantitatively measured with an Image processor for Analytical Pathology (IPAP) (Sumika Technos Co., Osaka). Serum testosterone levels were analyzed using a radioimmunoassay by a commercial laboratory (SRL, Tokyo).

2.3. Immunohistochemistry of Ki-67

For Ki-67 immunostaining, deparaffinized sections were autoclaved for 5 min at 120 °C, and sequentially

Table 1
Intakes of food and test chemicals

Group	Treatment		Dietary intake		Total intake of NP (mg/rat)
	NP (ppm)	DMAB	Food (g/day/rat)	NP (mg/day/rat)	
1	0	+	11.11 ± 0.44[a]	–	–
2	25	+	11.58 ± 0.63	0.29	6.1
3	250	+	11.36 ± 0.34	2.84	59.7
4	2000	+	10.84 ± 0.49	21.68	455.2
5	2000	–	11.05 ± 0.88	22.10	464.2

[a] Mean ± SD.

treated with 0.5% H_2O_2 for 30 min and 1% skimmed milk for 30 min. The sections were incubated with diluted rabbit polyclonal Ki-67 antibody (NCL-Ki67p, 1:3000, Novocastra Laboratories Ltd, Newcastle, UK) for 2 h at room temperature and sequentially exposed to biotin-labeled horse anti-mouse IgG and ABC (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA). Diaminobenzidine was used for visualizing the sites of peroxidase binding. Ki-67 labeling indices were generated by counting over 1000 normal-looking prostate epithelial cells in each lobe under a microscope at high magnification and were expressed as numbers of Ki-67-positive cells per 100 prostate epithelial cells.

2.4. Statistical analysis

Statistical analysis was performed using the post-hoc test (Bonferroni/Dunn) with StatView (version 5.0) software (SAS Institute Inc., Cary, NC) for the significance of differences between the treatment groups in body weights and the data for histopathological lesions and immunohistochemical analysis.

3. Results

3.1. Food intake

Food consumption (g/day/rat) did not significantly differ among the groups (Table 1). Estimated intakes of NP were well correlated with doses applied (Table 1).

3.2. Survival rate and final body and relative organ weights

The survival curves of rats with NP/DMAB treatment demonstrated groups 2 and 4 to have the best rates followed by groups 1 and 3 (Fig. 1). The cause of death in the majority of cases was due to the development of tumors of the Zymbal glands and malignant schwannomas of soft tissue. No obvious effects of NP administration were detected on tumor induction on Zymbal gland and soft tissue. The final body and relative organ weights of rats at week 64 are shown in Table 2. The mean relative weights of muscle levator ani, a valuable model tissue for studies of hormone action [16] of group 2 were significantly smaller than those of group 3 ($P < 0.01$).

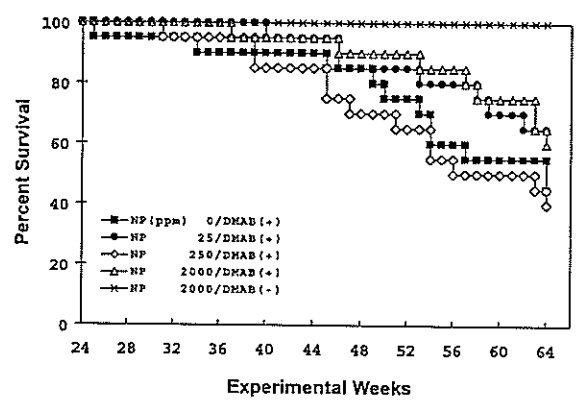

Fig. 1. Survival curves of NP/DMAB treated rats.

Table 2
Final body and relative organ weights of rats given NP and then DMAB

Group	Treatment		No. of rats	Body weight (g)	Relative organ weight					
	NP (ppm)	DMAB			Testis (%)	Epididymis (%)	Muscli levator ani (%)	Prostate		
								Ventral (‰)	Dorsolateral (‰)	Anterior + SV (‰)
1	0	+	9	395.6 ± 45.3[a]	0.72 ± 0.08[a]	0.20 ± 0.02[a]	0.19 ± 0.03[a]	0.93 ± 0.23[a]	1.30 ± 0.17[a]	2.32 ± 0.25[a]
2	25	+	13	413.0 ± 28.0	0.82 ± 0.19	0.21 ± 0.02	0.18 ± 0.02	0.87 ± 0.16	1.27 ± 0.21	1.97 ± 0.58
3	250	+	8	402.9 ± 36.1	0.79 ± 0.12	0.23 ± 0.10	0.21 ± 0.02[b]	0.86 ± 0.29	1.41 ± 0.26	2.21 ± 0.75
4	2000	+	12	403.4 ± 33.9	0.74 ± 0.08	0.21 ± 0.02	0.18 ± 0.02	0.91 ± 0.22	1.26 ± 0.13	2.07 ± 0.36
5	2000	-	15	431.1 ± 30.7	0.74 ± 0.03	0.21 ± 0.05	0.19 ± 0.01	0.90 ± 0.14	1.37 ± 0.18	2.37 ± 0.44

[a] Mean ± SD.
[b] $P < 0.01$ vs. Group 2.

3.3. Lesions in sex accessory organs

Animals that survived longer than 52 weeks were included in the effective numbers. Tables 3–5 summarize the data on neoplastic lesions developed in sex accessory organs. In the ventral prostates, almost all DMAB treated rats developed prostatic intraepithelial neoplasia (PIN). No significant differences were observed in the numbers and areas of either PINs or adenocarcinomas among the groups. In the anterior prostate, no adenocarcinomas were detected and no significant differences were found in PIN development. In the dorsolateral prostate, the number and size of PINs were very small and no adenocarcinomas developed in any of the groups. In the SV, dysplasias were observed in almost all animals. From the macro and microscopic analysis, there were no obvious effects of NP treatment on maturation of the male sex organs.

3.4. Sex hormone levels in serum

No significant differences were observed in the serum testosterone levels among the experimental groups (Table 6).

3.5. Ki-67 labeling indices

Ki-67 labeling indices in normal-looking epithelium of the prostates and SV are given in Table 7. No significant differences were observed among the groups in any of the sites examined.

4. Discussion

The present study demonstrated that dietary administration of NP at late neonatal period (for 3 weeks starting from 3-weeks of age) did not affect

Table 3
Neoplastic lesions of ventral prostate of rats given NP and then DMAB

Group	Treatment		Effective no. of rats	PIN			Adenocarcinoma		
	NP (ppm)	DMAB		Incidence	Multiplicity (/cm^2)	Area (mm^2/cm^2)	Incidence	Multiplicity (/cm^2)	Area (mm^2/cm^2)
1	0	+	14	14 (100)	2.75 ± 1.24[a]	0.18 ± 0.13[a]	4 (29)	0.28 ± 0.52[a]	0.05 ± 0.08[a]
2	25	+	17	16 (94)	1.98 ± 1.28	0.10 ± 0.10	5 (29)	0.31 ± 0.56	0.15 ± 0.31
3	250	+	12	10 (83)	2.14 ± 2.01	0.10 ± 0.12	5 (42)	0.35 ± 0.47	0.17 ± 0.33
4	2000	+	16	14 (88)	1.78 ± 1.16	0.14 ± 0.16	5 (31)	0.33 ± 0.59	0.10 ± 0.20
5	2000	–	15	1 (7)	0.04 ± 0.14	0.00 ± 0.00	0 (0)	0	0

[a] Mean ± SD.

Table 4
Neoplastic lesions of dorsolateral and anterior prostates of rats given NP and then DMAB

Group	Treatment		Effective no. of rats	PIN					
	NP (ppm)	DMAB		Dorsolateral			Anterior		
				Incidence	Multiplicity (/cm^2)	Area ($\times 10^{-2}$ mm^2/cm^2)	Incidence	Multiplicity (/cm^2)	Area (mm^2/cm^2)
1	0	+	14	3 (21)	0.17 ± 0.34[a]	0.19 ± 0.41[a]	6 (43)	1.37 ± 1.65[a]	0.17 ± 0.22[a]
2	25	+	17	2 (12)	0.10 ± 0.28	0.28 ± 0.85	4 (24)	0.90 ± 1.67	0.14 ± 0.36
3	250	+	12	1 (8)	0.08 ± 0.27	0.42 ± 1.46	5 (42)	1.55 ± 2.13	0.41 ± 0.70
4	2000	+	16	1 (6)	0.05 ± 0.18	0.15 ± 0.59	4 (25)	0.81 ± 1.45	0.11 ± 0.25
5	2000	−	15	2 (13)	0.09 ± 0.25	0.20 ± 0.55	2 (13)	0.37 ± 0.98	0.05 ± 0.16

[a] Mean ± SD.

the subsequent DMAB-induced prostate carcinogenesis in male F344 rats. To our knowledge, this is the first report that the endocrine-modulating chemical NP has no effect on chemically induced prostate carcinogenesis. We also demonstrated that dietary treatment of NP during a sexually immature period did not persistently affect the maturation of the male sex organs and their cell cycle kinetic activity. From the viewpoint of mean relative weights of muscli levator ani, although significant differences were found between groups 2 and 3, we could not determine the effect of NP because of the lack of obvious dose dependency.

It has been reported that the commercial alkylphenol NP, one of the xenoestrogens, possesses weak estrogenic activity [10]. It was shown that NP stimulated the proliferation of MCF-7 cells, an estrogen-dependent human breast cancer cell line [10]. It was also demonstrated that NP possessed a stimulating effect on epithelial cells of the Noble rat mammary gland [17], and uterotropic effects in mice [10] and rats [18]. It was considered that alkylphenols interferes with estrogen binding to its receptor [19] and that the action of alkylphenols are mediated by the estrogen receptor, as their effects depended on its presence and was blocked by estrogen antagonists [5]. Moreover, it is likely that they interact with a similar region of the hormone-binding domain as 17β-estradiol, because the mutant receptor G-525R, which is defective in estrogen binding, is also insensitive to alkylphenols [5]. A recent study demonstrated that not only the estrogen receptor, but also the androgen receptor could be a target of NP [20]. NP appears to affect multiple steps in the activation and function of the androgen receptor as an antagonist [20].

The cytochrome P450 enzymes (CYP) responsible for the metabolism of xenobiotic chemicals and steroids comprise a superfamily. It is widely recognized that specific agents, many of which are also substrates themselves, can modulate their expression levels or catalytic activities in vivo. In rats, NP is known to be metabolized by CYP2B2 isozyme,

Table 5
Neoplastic lesions of seminal vesicles of rats given NP and then DMAB

Group	Treatment		Effective no. of rats	Dysplasia		
	NP (ppm)	DMAB		Incidence	Multiplicity (/cm^2)	Area (mm^2/cm^2)
1	0	+	14	14 (100)	3.82 ± 1.67[a]	0.62 ± 0.31[a]
2	25	+	17	17 (100)	6.25 ± 5.34	1.20 ± 0.74
3	250	+	12	12 (100)	6.55 ± 5.22	1.03 ± 0.82
4	2000	+	16	16 (100)	4.32 ± 2.21	0.82 ± 0.39
5	2000	−	15	0 (0)	0	0

[a] Mean ± SD.

Table 6
Serum testosterone levels

Group	Treatment		No. of rats	Testosterone (ng/ml)
	NP (ppm)	DMAB		
1	0	+	5	0.24 ± 0.07[a]
2	25	+	5	0.24 ± 0.13
3	250	+	5	0.14 ± 0.04
4	2000	+	5	0.11 ± 0.03
5	2000	−	5	0.27 ± 0.16

[a] Mean ± SD.

a homologue of human CYP2B6 [21], and after first-pass metabolism, the predominant metabolites are glucuronide conjugates in the serum [22]. Lee et al. [23] also reported that treatment with NP reduces the 7-ethoxyresorufin O-deethylase activity of CYP1A, owing to the suppression of CYP1A2 production at the mRNA and protein levels. Since CYP1A2 was shown to contribute as a catalyst to DMAB metabolism [24], the pretreatment with NP might have been expected to affect the induction of neoplastic lesions by modulating the metabolic activation of DMAB. However, in the present experiment, no effect of pretreatment with NP was observed in the incidence, numbers and area of neoplastic lesions. This could be due to DMAB administration was carried out for long after NP treatment, at which NP effects on modification of metabolism had not been remained.

The NOAEL of NP has been estimated as 50 mg/kg/day [15]. Fourie et al. [25] investigated this dose using biochemical markers for epididymal function and concluded that it is borderline for effects of NP in adult male rats. In our study, the oral administration of NP at 250 and 2000 ppm corresponds to approximately 35–45 and 200–280 mg/kg/day, respectively. Histopathological analysis, however, demonstrated no significant effects on the maturation of male sex accessory organs even at the highest dose. Furthermore, the Ki-67 labeling index, a biological marker for cell cycle kinetics, did not significantly differ among the treatment groups, in line with the absence of modulating effects on male sex organs. While the possibility remains that longer treatment with NP, or a shorter evaluation period after NP administration might have revealed effects, the present results point to a lack of influence.

The present DMAB prostate cancer model, in which microscopic, androgen-dependent adenocarcinomas develop in the ventral prostates of F344 rats, was established in our laboratory [26–29], and it has been used to identify promoters and chemopreventive agents [30,31]. Investigation of modifying effects of the phytoestrogens, genistin and daidzin, in this model demonstrated anti-cancer effects on relatively early stages of prostate cancer development [32]. Concerning xenoestrogens, although the period of test chemical treatment and the DMAB administration manner were different from our experiment, Kohno et al. [33] assessed the modifying effects of OP, possessing 10–20-fold more estrogenic potency than NP [5], in the DMAB prostate carcinogenesis model. They concluded that OP had no modifying effects on DMAB induced prostatic tumorigenesis. In other models, Son et al. [13,14] demonstrated no modifying effects of NP on *N*-bis(2-hydroxypropyl)nitrosamine (DHPN) induced thyroid carcinogenesis in rats, while Seike et al. [12] reported that dietary NP may act as a promoter in the lung, causing increase in cell proliferation, in a F344 rat multiorgan carcinogenesis model. Effects might thus be organ dependent and are

Table 7
Ki-67 labeling indices on normal looking epithelium of prostate and seminal vesicles

Group	Treatment		No. of rats	Prostate			Seminal vesicles
	NP (ppm)	DMAB		Ventral	Dorsolateral	Anterior	
1	0	+	5	1.1 ± 0.3[a]	1.0 ± 0.2[a]	0.9 ± 0.3[a]	1.8 ± 0.5[a]
2	25	+	5	1.0 ± 0.4	0.9 ± 0.3	0.8 ± 0.2	1.7 ± 0.8
3	250	+	5	0.8 ± 0.2	1.1 ± 0.4	0.8 ± 0.3	1.6 ± 0.5
4	2000	+	5	0.9 ± 0.3	1.0 ± 0.3	0.8 ± 0.4	1.5 ± 1.0
5	2000	−	5	0.9 ± 0.3	1.0 ± 0.4	0.9 ± 0.2	1.5 ± 0.5

[a] Mean ± SD.

also dependent on the age and/or exposureperiod and dose levels.

From our experimental data, dietary feeding of NP during the late neonatal period did not act as a modulator of accessory sex organ maturation and it also had no effects on the prostate cancer induction on DMAB rat prostatic carcinogenesis. In conclusion, extrapolating to the human situation, we consider that the risks are quite low with the paraneonatal period exposure to NP, because the prevailing levels of NP in the environment are much lower than the experimental levels.

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References

[1] A.V. Krishnan, P. Stathis, S.F. Permuth, L. Tokes, D. Feldman, Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving, Endocrinology 132 (1993) 2279–2286.

[2] J.M. Bergeron, D. Crews, J.A. McLachlan, PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination, Environ. Health Perspect. 102 (1994) 780–781.

[3] S. Jobling, T. Reynolds, R. White, M.G. Parker, J.P. Sumpter, A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic, Environ, Health Perspect. 103 (1995) 582–587.

[4] S. Jobling, D. Sheahan, J.A. Osborne, P. Matthiessen, J.P. Sumpter, Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals, Environ. Toxicol. Chem. 15 (1996) 194–202.

[5] R. White, S. Jobling, S.A. Hoare, J.P. Sumpter, M.G. Parker, Environmentally persistent alkylphenolic compounds are estrogenic, Endocrinology 135 (1994) 175–182.

[6] A.C. Nimrod, W.H. Benson, Environmental estrogenic effects of alkylphenol ethoxylates, Crit. Rev. Toxicol. 26 (1996) 335–364.

[7] J. Toppari, J.C. Larsen, P. Christiansen, A. Giwercman, P. Grandjean, L.J. Guillette Jr., et al., Male reproductive health and environmental xenoestrogens, Environ. Health Perspect. 104 (suppl. 4) (1996) 741–803.

[8] K. Guenther, V. Heinke, B. Thiele, E. Kleist, H. Prast, T. Raecker, Endocrine disrupting nonylphenols are ubiquitous in food, Environ. Sci. Technol. 36 (2002) 1676–1680.

[9] E.C. Dodds, W. Lawson, Synthetic estrogenic agents without the phenanthrene nucleus, Nature 137 (1936) 996.

[10] A.M. Soto, H. Justicia, J.W. Wray, C. Sonnenschein, p-Nonylphenol: an estrogenic xenobiotic released from modified polystyrene, Environ. Health Perspect. 92 (1991) 167–173.

[11] A.M. Soto, C. Sonnenschein, K.L. Chung, M.F. Fernandez, N. Olea, F.O. Serrano, The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants, Environ. Health Perspect 103 (suppl. 7) (1995) 113–122.

[12] N. Seike, H. Wanibuchi, K. Morimura, M. Wei, T. Nishikawa, K. Hirata, J. Yoshikawa, S. Fukushima, Enhancement of lung carcinogenesis by nonylphenol and genistein in a F344 rat multiorgan carcinogenesis model, Cancer Lett. 192 (2003) 25–36.

[13] H.Y. Son, A. Nishikawa, T. Ikeda, F. Furukawa, M. Hirose, Lack of modification by environmental estrogenic compounds of thyroid carcinogenesis in ovariectomized rats pretreated with *N*1-bis(2-hydroxypropyl)nitrosamine (DHPN), Jpn. J. Cancer Res. 91 (2000) 966–972.

[14] H.Y. Son, A. Nishikawa, T. Ikeda, H. Nakamura, M. Miyauchi, T. Imazawa, et al., Lack of modifying effects of environmental estrogenic compounds on the development of thyroid proliferative lesions in male rats pretreated with *N*-bis(2-hydroxypropyl)nitrosamine (DHPN), Jpn. J. Cancer Res. 91 (2000) 899–905.

[15] H.C. Cunny, B.A. Mayes, K.A. Rosica, J.A. Trutter, P. Van Miller, Subchronic toxicity (90-day) study with para-nonylphenol in rats, Regul. Toxicol. Pharmacol. 26 (1997) 172–178.

[16] S.R. Max, J.F. Knudsen, Effect of sex hormones on glucose-6-phosphate dehydrogenase in rat levator ani muscle, Mol. Cell Endocrinol. 17 (1980) 111–118.

[17] J.B. Coleramgle, D. Roy, Exposure of environmental estrogenic compound nonylphenol to Noble rats alters cell-cycle kinetics in mammary gland, Endocrine (1996) 112–115.

[18] R.L. Divi, H.C. Chang, D.R. Doerge, Anti-thyroid isoflavones from soybean: isolation, characterization, and mechanisms of action, Biochem. Pharmacol. 54 (1997) 1087–1096.

[19] B.J. Danzo, Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins, Environ. Health Perspect. 105 (1997) 294–301.

[20] H.J. Lee, S. Chattopadhyay, E.Y. Gong, R.S. Ahn, K. Lee, Anti-androgenic effects of bisphenol A nad nonylphenol on the function of androgen receptor, Toxicol. Sci. 75 (2003) 40–46.

[21] P.C. Lee, M. Marquardt, J.J. Lech, Metabolism of nonylphenol by rat and human microsomes, Toxicol. Lett. 99 (1998) 117–126.

[22] D.R. Doerge, N.C. Twaddle, M.I. Churchwell, H.C. Chang, R.R. Newbold, K.B. Delclos, Mass spectrometric determination of *p*-nonylphenol metabolism and disposition following oral administration to Sprague–Dawley rats, Reprod. Toxicol. 16 (2002) 45–56.

[23] P.C. Lee, S.C. Patra, C.T. Stelloh, W. Lee, M. Struve, Interaction of nonylphenol and hepatic CYP1A in rats, Biochem. Pharmacol. 52 (1996) 885–889.
[24] M.A. Butler, M. Iwasaki, F.P. Guengerich, F.F. Kadlubar, Human cytochrome P-450PA (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines, Proc. Natl Acad. Sci. USA 86 (1989) 7696–7700.
[25] M.H. Fourie, M.S. Bornman, E. Viljoen, Epididymal markers in rats exposed to the xenoestrogen *p*-nonylphenol: no biochemical effects at low dosages, Andrologia 33 (2001) 305–310.
[26] N. Ito, T. Shirai, Y. Tagawa, A. Nakamura, S. Fukushima, Variation in tumor yield in the prostate and other target organs of the rat in response to varied dosage and duration of administration of 3,2′-dimethyl-4-aminobiphenyl, Cancer Res. 48 (1988) 4629–4632.
[27] T. Shirai, S. Fukushima, E. Ikawa, Y. Tagawa, N. Ito, Induction of prostate carcinoma in situ at high incidence in F344 rats by a combination of 3,2′-dimethyl-4-aminobiphenyl and ethinyl estradiol, Cancer Res. 46 (1986) 6423–6426.
[28] T. Shirai, M. Sano, K. Imaida, S. Takahashi, T. Mori, N. Ito, Duration dependent induction of invasive prostatic carcinomas with pharmacological dose of testosterone propionate in rats pretreated with 3,2′-dimethyl-4-aminobiphenyl and development of androgen-independent carcinomas after castration, Cancer Lett. 83 (1994) 111–116.
[29] T. Shirai, S. Takahashi, L. Cui, M. Futakuchi, K. Kato, S. Tamano, K. Imaida, Experimental prostate carcinogenesis—rodent models, Mutat. Res. 462 (2000) 219–226.
[30] T. Shirai, S. Iwasaki, T. Masui, T. Mori, T. Kato, N. Ito, Enhancing effect of cadmium on rat ventral prostate carcinogenesis induced by 3,2′-dimethyl-4-aminobiphenyl, Jpn. J. Cancer Res. 84 (1993) 1023–1030.
[31] K. Imaida, S. Tamano, K. Kato, Y. Ikeda, M. Asamoto, S. Takahashi, et al., Lack of chemopreventive effects of lycopene and curcumin on experimental rat prostate carcinogenesis, Carcinogenesis 22 (2001) 467–472.
[32] K. Kato, S. Takahashi, L. Cui, T. Toda, S. Suzuki, M. Futakuchi, et al., Suppressive effects of dietary genistin and daidzin on rat prostate carcinogenesis, Jpn. J. Cancer Res. 91 (2000) 786–791.
[33] H. Kohno, Y. Tsukio, Y. Yanaida, M. Tanino, T. Tanaka, Lack of modifying effects of 4-*n*-octylphenol on 3,2′-dimethyl-4-aminobiphenyl-induced prostate carcinogenesis in rats, Ecotoxicol. Environ. Saf. 51 (2002) 210–215.

Research Article

Linkage and Microarray Analyses of Susceptibility Genes in ACI/Seg Rats: A Model for Prostate Cancers in the Aged

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Abstract

ACI/Seg (ACI) rats develop prostate cancers spontaneously with aging, similar to humans. Here, to identify genes involved in prostate cancer susceptibility, we did linkage analysis and oligonucleotide microarray analysis. Linkage analysis was done using 118 effective rats, and *prostate cancer susceptibility 1* (*Pcs1*), whose ACI allele dominantly induced prostate cancers, was mapped on chromosome 19 [logarithm of odds (LOD) score of 5.0]. *PC resistance 1* (*Pcr1*), whose ACI allele dominantly and paradoxically suppressed the size of prostate cancers, was mapped on chromosome 2 (LOD score of 5.0). When linkage analysis was done in 51 rats with single or no macroscopic testicular tumors, which had larger prostates and higher testosterone levels than those with bilateral testicular tumors, *Pcs2* and *Pcr2* were mapped on chromosomes 20 and 1, respectively. By oligonucleotide microarray analysis with 8,800 probe sets and confirmation by quantitative reverse transcription-PCR, only two genes within these four loci were found to be differentially expressed >1.8-fold. *Membrane metalloendopeptidase* (*Mme*), known to inhibit androgen-independent growth of prostate cancers, on *Pcr1* was expressed 2.0- to 5.5-fold higher in the ACI prostate, in accordance with its paradoxical effect. *Cdkn1a* on *Pcs2* was expressed 1.5- to 4.5-fold lower in the ACI prostate. Additionally, genes responsible for testicular tumors and unilateral renal agenesis were mapped on chromosomes 11 and 14, respectively. These results showed that prostate cancer susceptibility of ACI rats involves at least four loci, and suggested *Mme* and *Cdkn1a* as candidates for *Pcr1* and *Pcs2*. (Cancer Res 2005; 65(7): 2610-6)

Introduction

Prostate cancer is one of the leading causes of cancer-related death in men in most developed countries, and its incidence is increasing in Japan (1). A variety of genetic and environmental factors are considered involved in the initiation and progression of human prostate cancers (2, 3), and clarification of these factors is urgently required. Genes that are likely to confer dominant susceptibility to prostate cancers have been mapped by linkage analysis of familial prostate cancers at 1q24-25 (4), 1q42-43 (5), 1p36 (6), Xq27-28 (7), 20q13 (8), 17p (9), and 8p22-23 (10). Three putative prostate cancer susceptibility genes, *RNASEL/HPC1* at 1q25 (11), *MSR1* at 8p22 (10), and *ELAC2/HPC2* at 17p11 (9), have been recently identified. In addition, polymorphisms of the *prostate-specific antigen*, isoforms of cytochrome *P450*, *androgen receptor* (12), *vitamin D receptor* (13), and *steroid 5α-reductase 2* (*SRD5A2*; ref. 14) have been proposed to be related to prostate cancer risks. In spite of the efforts and expertise in the field, identification of a responsible gene in each locus and clarification of the interaction among the genes are still facing difficulties due to the complex interactions among these genetic factors in individuals who have been exposed to different levels of various environmental factors.

Experimental animal models are useful because a population of animals can be exposed to the same homogeneous environmental factors and the number of genes involved is limited. As for prostate cancers, a number of rodent models are reported (15). Among these, ACI/Seg (ACI) rats are unique in that they spontaneously develop a high incidence of microscopic cancers of the ventral prostate along with aging (16–18). By 33 months of age, 95% to 100% of the rats develop intra-alveolar dysplasia in the ventral lobe, and 35% to 40% develop invasive carcinomas (17). The high incidence of microscopic cancers and the lower incidence of macroscopic cancers are considered to mimic the natural history of human prostate cancers, where the incidence of microscopic cancers is very high and that of clinical diseases is much lower (19, 20). The ACI model was used to show that a high-fat diet had a promotional effect on the early stage of prostate carcinogenesis (21), that 5α-reductase inhibitor suppresses prostate carcinogenesis (22), and that long-term feeding of a 1% cholesterol diet promotes prostate carcinogenesis, possibly by increasing tissue oxidative stress (23). However, no information is available for the genetic loci or genes responsible for the predisposition of ACI rats to prostate cancers.

In this study, we did linkage and oligonucleotide microarray analyses to identify candidate gene(s) involved in the genetic predisposition of ACI rats to prostate cancers. Linkage analysis was done in F_2 intercross rats produced by crossing ACI rats with F344 rats, which were less susceptible to prostate cancers (24) but more susceptible to spontaneous testicular tumors (25). Oligonucleotide microarray analysis was done considering that if genes differentially expressed in the prostates of ACI and F344 rats are present in the mapped loci, they are good candidates for the responsible genes. Additionally, genes responsible for testicular tumors and unilateral renal agenesis (URA) were mapped.

Materials and Methods

Animals and carcinogenicity test. ACI/SegHgd (ACI) and F344/Jcl (F344) rats were purchased from Harlan (Indianapolis, IN) and CLEA Japan, Inc. (Tokyo, Japan), respectively. Male ACI and female F344 rats were mated to produce F_1 progeny. F_1 rats were intercrossed to produce F_2 intercross

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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Table 1. Results of the carcinogenicity test

	ACI/Seg (n = 12)	F344/Jcl (n = 8)	(F344 × ACI)F_1 (n = 14)	(F344 × ACI)F_2 (n = 118)
Body and organ weights				
Body weight (g)	419.6 ± 65.8 (309.2–506.9)	350.7 ± 29.7 (309.3–391.6)	509.5 ± 67.5 (315.2–583.3)	418.9 ± 77.5 (195.9–652.9)
Prostate weight (g)	1.54 ± 0.83 (0.61–3.00)	0.92 ± 0.19 (0.67–1.26)	1.61 ± 0.75 (0.86–2.91)	1.64 ± 1.58 (0.38–11.75)
Ventral prostate weight (g)	0.32 ± 0.14 (0.13–0.58)	0.27 ± 0.07 (0.19–0.42)	0.36 ± 0.12 (0.22–0.59)	0.35 ± 0.32 (0.07–2.20)
Testes weight (g)	2.81 ± 0.50 (2.04–3.59)	6.62 ± 2.51 (2.48–9.84)	7.73 ± 9.10 (2.57–33.93)	4.20 ± 2.50 (0.67–21.16)
Liver weight (g)	11.3 ± 1.4 (8.5–12.7)	12.7 ± 3.4 (9.6–19.0)	15.0 ± 4.8 (1.5–20.3)	13.2 ± 2.9 (4.7–31.3)
Spleen weight (g)	0.80 ± 0.14 (0.57–1.06)	2.34 ± 1.74 (1.11–5.68)	1.10 ± 0.23 (0.82–1.63)	1.31 ± 0.79 (0.34–5.57)
Any lesions in prostate				
Presence (%)	100	50	100	89
Number	5.3 ± 2.1	1.5 ± 1.9	4.5 ± 1.9	3.8 ± 3.3
Area (mm^2)	1.78 ± 1.22	0.22 ± 0.30	1.03 ± 0.80	1.11 ± 1.50
Area ratio (%)	1.61 ± 1.29	0.25 ± 0.33	0.84 ± 0.59	1.31 ± 1.75
Lesions with a solid structure				
Presence (%)	92	25	93	59
Number	3.0 ± 2.6	0.4 ± 0.7	2.0 ± 1.3	1.3 ± 1.6
Area (mm^2)	1.17 ± 1.25	0.06 ± 0.14	0.40 ± 0.35	0.40 ± 1.07
Area ratio (%)	1.09 ± 1.31	0.07 ± 0.15	0.38 ± 0.37	0.48 ± 1.27
Lesions with ACI-type nuclei				
Presence (%)	100	38	100	81
Number	5.1 ± 2.4	1.3 ± 1.8	3.3 ± 1.4	3.1 ± 3.0
Area (mm^2)	1.73 ± 1.24	0.22 ± 0.30	0.89 ± 0.74	0.90 ± 1.41
Area ratio (%)	1.57 ± 1.31	0.24 ± 0.33	0.73 ± 0.56	1.08 ± 1.68

rats. A total of 21 male ACI rats, 22 male F344 rats, 21 male F_1 rats, and 219 male F_2 intercross rats were fed an Oriental MF (Oriental Yeast Co., Tokyo, Japan) diet and housed three per plastic cage on woodchip bedding in an air-conditioned animal room at a temperature of 21°C to 25°C and a humidity of 40% to 60% with a 12-hour light 12-hour dark cycle. Moribund rats were sacrificed to clarify the cause of death, and the remaining rats were sacrificed at the age of 30 months. The animal experiments followed the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Medical School and were approved by the Institutional Animal Care and Use Committee.

Histologic and serum analysis. The male sex organs and bladder were resected *en bloc* and were examined for gross abnormalities and fixed in 10% buffered formalin. After fixation, the ventral prostate was removed from the base of the bladder neck and the entire organ weighed. One sagittal slice through the ventral prostate was embedded in paraffin, and three 4-μm-thick sections were stained with H&E. The areas of ventral lobe and lesions were quantitatively measured by an Image Processor for Analytical Pathology (IPAP-WIN, Sumika Technoservice, Takarazuka, Japan). The area of lacuna with secretions was included as the area of the prostate. Hyperplastic and tumorous lesions were classified according to their structural and nuclear atypism as described in Results. The area ratio was calculated as the percentage of the area of a lesion in the total area of the ventral lobe.

Testicular tumors were diagnosed by histologic examination of H&E-stained samples. Leukemia was diagnosed by hypertrophy of the spleen and the liver with infiltration of leukemic cells. Unilateral renal agenesis was diagnosed by the lack of one kidney. All histologic diagnoses were made by experienced pathologists (To.S and S.S.) and a urologist (Y.H.).

Serum testosterone levels are known to be at their peaks between 9:00 am and 1:00 pm (26), and blood samples were collected between 10:00 am and 12:00 noon. Concentrations were measured by RIA at SRL, Inc. (Tokyo, Japan).

Genotyping and linkage analysis. Genotyping was done using 201 markers (157 microsatellite markers and 44 AP-RDA markers; Supplementary Table S1) and genomic DNA extracted from the tails (27–30). Linkage maps were constructed by MAPMAKER/EXP (version 3.0b) software (31), and quantitative trait locus (QTL) analysis was done using MAPMAKER/QTL (version 1.1b) software. The presence of a lesion(s) and absence of any lesions were scored as "1" and "0," respectively, and treated like a QTL. The number of lesions was square root transformed to obtain improved normality. The area and area ratio of lesions were treated as actual values. Association of a quantitative trait with an allele was evaluated by a logarithm of odds (LOD) score. Threshold LOD scores at 2.8 and 4.3 were considered as "suggestive" and "significant" linkages, respectively (32).

Oligonucleotide microarray analysis. Equal amounts of RNA were pooled from the whole prostates of three F344 and three ACI rats at two ages 8 and 48 weeks. Oligonucleotide microarray analysis was done using GeneChip Rat Genome U34A (Affymetrix, Santa Clara, CA) with 8,800 probe sets as in our previous studies (33–35). The signal intensities were normalized so that the average of all the genes on a GeneChip would be equal, and the data were processed using Affymetrix Microarray Suite version 5.0 at the default variable. The annotation was according to RG_U34A Annotations (June 2004; Affymetrix).

Quantitative reverse transcription-PCR. cDNA was synthesized from 2 μg of total RNA with oligo $(dT)_{12\text{-}18}$ primer. Real-time PCR analysis was done separately for the three rats in each group using an iCycler iQ detection system (Bio-Rad Laboratories, Hercules, CA) with SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA). Primers with the following sequences were used, *Mme* upper 5′- CCTACCGGCCAGAG-TATGCAG -3′ and lower 5′- TATGAGTTCTTGCGGCAATGA -3′, *Cdkn1a* upper 5′- CCTTCTTCTGCTGTGGGTCA -3′ and lower 5′- AAGACACAC-TGAATGAAGGCTA -3′. To quantify the number of molecules of a specific gene in a sample, a standard curve was generated using templates that contained 10^1 to 10^6 copies of the gene and was normalized to that of β-actin.

Results

Incidences and sizes of prostate lesions in inbred and crossed rats. Twelve of 21 ACI, 8 of 22 F344, 14 of 21 F_1, and 118 of 219 F_2 intercross rats survived to 30 months of age,

premature deaths being caused by renal failure due to URA and leukemia. Hyperplastic and/or tumorous lesions in the ventral prostate were observed in 100%, 50%, 100%, and 89% of ACI, F344, F_1 and F_2 intercross rats, respectively (Table 1). The lesions in ACI and F344 rats had distinct morphologies (Fig. 1). Lesions in ACI rats showed a solid structure, and round, irregular (ACI type) nuclei whereas those in F344 had a papillary or cribriform structure, and angled nuclei with regular sizes. The lesions in ACI rats were much larger (1.8 versus 0.2 mm^2, $P = 0.001$), often associated with inflammatory infiltrates, and occasionally invaded into the adjacent alveoli. In contrast, lesions in F344 rats never developed into macroscopic cancers. Based on the structural and nuclear atypisms and sizes of tumors, ACI ($n = 12$) and F344 ($n = 8$) rats could be distinguished with 95% accuracy in a blinded manner.

Therefore, all the lesions were classified according to their structural and nuclear atypisms (Table 1). A solid structure was present in 92%, 25%, 93%, and 59% of ACI, F344, F_1 and F_2 intercross rats, respectively. ACI-type nuclei were present in 100%, 38%, 100%, and 81%, respectively. In ACI and F_2 intercross rats, 100% and 99%, respectively, of lesions with a solid structure had ACI-type nuclei.

Testicular tumors, unilateral renal agenesis, leukemia, and subcutaneous tumors. Testicular tumors, histologically diagnosed as Leydig cell tumors, were observed in 92%, 100%, 100%, and 92% of ACI, F344, F_1 and F_2 intercross rats, respectively. Bilateral macroscopic testicular tumors were observed in 25%, 88%, 100%, and 57%, respectively. URA was observed in 24%, 0%, 0%, and 11%, respectively, at 30 months. Leukemia was observed in 0%, 25%, 0%, and 9%, respectively, at 30 months. S.c. tumors were observed in 0%, 38%, 0%, and 10%, respectively.

Linkage mapping of a gene that affects the development and the size of prostate cancers. Linkage analysis with the presence of (i) any lesions, (ii) lesions with a solid structure, and (iii) lesions with ACI-type nuclei was done in the 118 F_2 intercross rats, and one QTL was identified in the vicinity of *D19Rat75* (LOD scores of 2.9, 3.0 and 5.0, respectively; Table 2). The same QTL was mapped using the numbers of these three kinds of lesions. When F_2 intercross rats were classified by the genotypes at *D19Rat75*, lesions with ACI-type nuclei were observed in 85% (22 of 26), 92% (60 of 65), and 52% (14 of 27) of rats with the ACI/ACI, ACI/F344, and F344/F344 genotypes, respectively. This locus, *prostate cancer susceptibility 1* (*Pcs1*), therefore conferred ACI-dominant prostate cancer susceptibility (Fig. 2*A*). This region (*D19Rat75-D19Rat46*) corresponded to rat 19q11-12.

Table 2. Results of QTL analysis in F_2 intercross rats

Lesions used for linkage analysis	*Pcs1* (chromosome 19)		*Pcr1* (chromosome 2)	
	LOD scores	% Variance explained	LOD scores	% Variance explained
Any lesions in prostate				
Presence	2.9	10.6	0.4	1.7
Number	3.2	12.2	1.9	7.6
Area (mm^2)	0.4	1.5	3.7	16.2
Area ratio (%)	0.2	0.8	5.0	20.3
Lesions with a solid structure				
Presence	3.0	11.4	0.7	2.7
Number	2.8	10.7	1.1	4.2
Area (mm^2)	0.1	0.4	2.3	9.0
Area ratio (%)	0.2	0.7	2.9	11.4
Lesions with ACI-type nuclei				
Presence	5.0	17.6	0.5	2.0
Number	3.7	13.5	1.3	5.2
Area (mm^2)	0.4	1.7	2.7	11.4
Area ratio (%)	0.1	0.5	4.0	15.6

Linkage analysis was done using areas containing the three kinds of lesions, and a QTL was identified in the vicinity of *D2Rat161* (LOD scores of 3.6, 2.3, and 2.7, respectively; Table 2). The same QTL was mapped using the area ratio. Notably, this locus conferred ACI-dominant resistance to an increase in size, and the effect was clearer using areas with any lesion than with using areas with lesions characteristic to ACI rats. F_2 intercross rats with the ACI/ACI, ACI/F344, and F344/F344 genotypes at *D2Rat161* had area ratios of 0.8 ± 1.2% (mean ± SD), 0.9 ± 1.0%, and 2.5 ± 2.6%, respectively (Fig. 3). This locus, *prostate cancer resistance 1* (*Pcr1*), had a paradoxical effect on the size of prostate cancers (Fig. 2*B*). This region (*D2Rat26-D2Rat40*) corresponded to rat 2q23-q32.

Effect of bilateral testicular tumors on prostate cancers. The size of the ventral prostate was significantly smaller in F_2 intercross rats with bilateral macroscopic testicular tumors (0.24 ± 0.13 g, $n = 67$) than in those with single or no tumors (0.49 ± 0.42 g, $n = 51$, $P < 0.005$). However, the incidences and mean areas of tumorous lesions in the ventral prostate were not different (60 of 67 and 1.0 ± 1.4 mm^2 in the former rats, and 45 of 51 and 1.3 ± 1.6 mm^2 in the latter rats). The mean serum testosterone level at 30 months was 0.1 ± 0.2 ng/mL in rats with bilateral tumors and 1.3 ± 5.6 ng/mL (0.3 ± 0.4 ng/mL when two extremely high values were excluded) in rats without bilateral tumors. Although the presence of bilateral testicular tumors did not affect the incidence or area of tumorous/hyperplastic lesions in the prostate, two new QTLs were mapped when linkage analysis was done in rats without bilateral testicular tumors (Table 3). One QTL in the vicinity of *D20Rat3*, *Pcs2*, conferred an ACI-dominant increase in the incidence of lesions

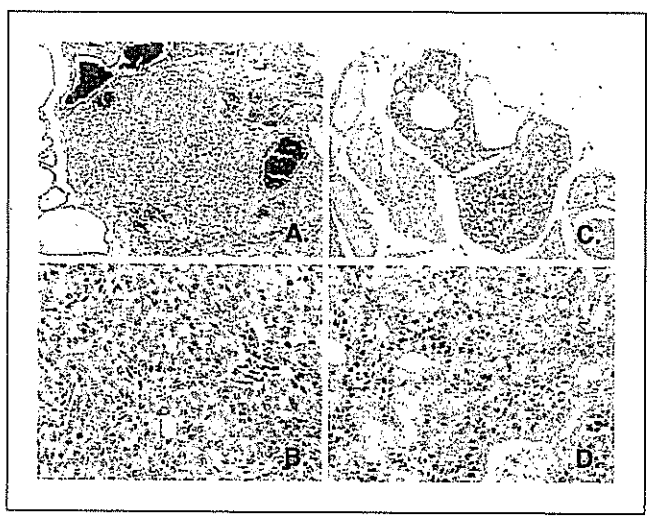

Figure 1. Distinct histological features of prostate cancers in ACI and F344 rats. Lesions of ACI rats (*A* and *B*) were characterized by high incidences of solid structures (*A*) and round, irregular nuclei (*B*). Lesions of F344 rats (*C* and *D*) were characterized by a papillary or cribriform pattern (*C*) and nuclei with angles and relatively regular sizes (*D*). Lesions in ACI rats were larger and invaded into flanking lobules showing their more malignant characteristics.

(LOD scores of 1.9-3.3; Fig. 2*C*). Another QTL in the vicinity of *D1Rat211*, *Pcr2*, conferred a paradoxical effect, ACI-dominant suppression of the area ratio (LOD scores of 3.9-4.3; Fig. 2*D*). *Pcs2* (*D20Rat41-D20Rat5*) and *Pcr2* (*D1Rat121-D15Rat45*) corresponded to rat 20p12-11 and 1p12-q11, respectively.

Oligonucleotide microarray analysis of genes within the loci. By oligonucleotide microarray analysis using the prostates of ACI and F344 rats at 8 weeks, 219 and 143 probes were found to be expressed higher and lower, respectively, at ≥1.8-fold in the ACI prostates. At 48 weeks, 371 and 154 probes were expressed higher and lower, respectively, in the ACI prostates. Forty-eight and 34 genes were expressed higher and lower both at 8 and 48 weeks (Supplementary Table S2). Among these genes, those located within the *Pcs1*, *Pcr1*, *Pcs2*, and *Pcr2* regions were RT1 class II (locus *DMa*; 2.1- to 3.0-fold), *membrane metalloendopeptidase* (*Mme*; 1.9- to 3.7- fold), and *Gstt2* (3.0- to 6.1-fold).

RT1 and *Gstt2* in *Pcs2* were found to have sequence polymorphisms in their oligonucleotide probes, which caused apparent expression difference in the microarray analysis (35). In contrast,

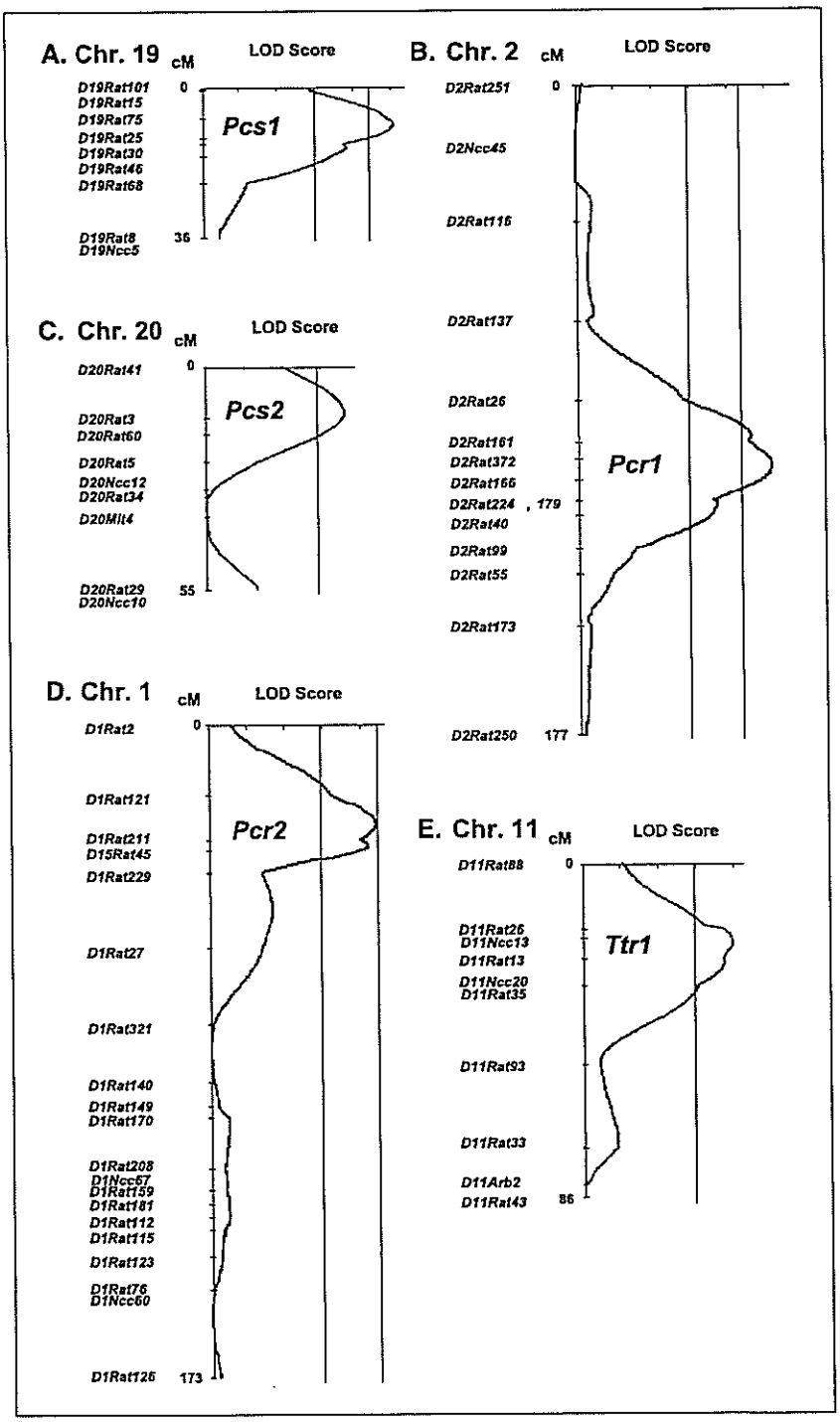

Figure 2. LOD score obtained for linkage with prostate lesions and testicular tumors. *A*, LOD score obtained by the presence of lesions with ACI-type nuclei in the 118 F_2 intercross rats. ACI allele in this locus dominantly promoted induction of the lesions. *B*, LOD score obtained by the area ratio of any lesions in the 118 F_2 intercross rats. ACI allele in this locus dominantly and paradoxically suppressed the growth of the lesions. *C*, LOD score obtained by the presence of lesions with ACI-type nuclei in the F_2 intercross rats with single or no testicular tumors. ACI allele in this locus dominantly promoted the growth of the lesions in this subgroup of rats. *D*, LOD score obtained by the area ratio of any lesions in the F_2 intercross rats with single or no testicular tumors. ACI allele in this locus dominantly and paradoxically suppressed the growth of the lesions in this subgroup. *E*, LOD score obtained by development of testicular tumors. The F344 allele in this locus promoted development of testicular tumors. *Dashed line*, Lander-Kruglark threshold for "significance" (LOD score of 4.3 in intercross), and "suggestive" (LOD score of 2.8 in intercross) linkage (32).

Mme in *Pcr1* was confirmed to be expressed at higher levels in the ACI prostate (2.0- to 5.5-fold) by quantitative reverse transcription-PCR (RT-PCR; Table 4). The difference and its location in the *Pcr1* region suggested *Mme* as a candidate for *Pcr1*. In addition, we previously found that *Cdkn1a* (*p21*) on *Pcs2* was differentially expressed in the prostates of various rat strains,[4] and its high expression in F344 rats was confirmed by quantitative RT-PCR (1.5- to 4.5-fold, Table 4). Although the *Cdkn1a* difference could not be identified by the oligonucleotide microarray analysis, the difference and its location in the *Pcs2* region suggested *Cdkn1a* as a candidate for *Pcs2*.

Mapping for the other QTLs. Linkage analysis with the presence of testicular tumors was done in the 118 F_2 intercross rats, and one QTL was identified in the vicinity of *D11Ncc20*. An *F344* allele of this locus dominantly promoted the development of testicular tumors (LOD score of 3.9; Fig. 2*E*). The incidences were 71% (17 of 24) in rats with the ACI/ACI genotype and 94% (91 of 94) in rats with the ACI/F344 and F344/F344 genotypes. The QTL, *testicular tumor resistance 1* (*Ttr1*), explained 14% of the total variance.

One QTL, whose ACI allele semidominantly induced URA, was mapped in the vicinity of *D14Rat65* (LOD score of 2.9). URA was observed in 20% (10 of 49), 9% (7 of 77), 0% (0 of 56) of rats with ACI/ACI, ACI/F344 and F344/F344 genotypes, respectively. The QTL, *Ura1*, explained 13% of the total variance. No QTLs were mapped for the serum testosterone levels, the development of leukemia and that of s.c. tumors at 30 months.

Discussion

Prostate cancers in ACI rats occasionally progressed into invasive cancers, which were considered to model human clinically significant prostate cancers. In contrast, prostate cancers in F344 rats stayed as small lesions, if present, which was considered to model human indolent prostate cancers. By using these strains, two prostate cancer susceptibility and two resistance genes were mapped using F_2 intercross progeny of ACI and F344 rats. Dominant ACI alleles of *Pcs1* (chromosome 19) and *Pcs2* (chromosome 20) increased the incidence and the number of prostate lesions, and these genes were considered to be involved in the development of prostate cancers. In contrast, dominant ACI alleles of *Pcr1* (chromosome 2) and *Pcr2* (chromosome 1) paradoxically decreased the area and area ratio of lesions, and these genes were considered to suppress the growth of prostate cancers. A similar example that an allele of a susceptible strain confers a resistance has been reported (27). Even when these four genes were combined, these did not fully explain the genetic predisposition of ACI rats. It was therefore shown that susceptibility of ACI rats to prostate cancers is determined by the four genes mapped here and many other weak genes.

The effect of *Pcs2* and *Pcr2* was clearly observed in rats without bilateral macroscopic testicular tumors and masked in rats with such tumors. Testicular tumors of rats are known to be associated with a tendency towards feminization (37). Although serum testosterone levels are known to be highly variable (26), 30-month-old rats with bilateral macroscopic testicular tumors had lower testosterone levels in this study. Therefore, as a

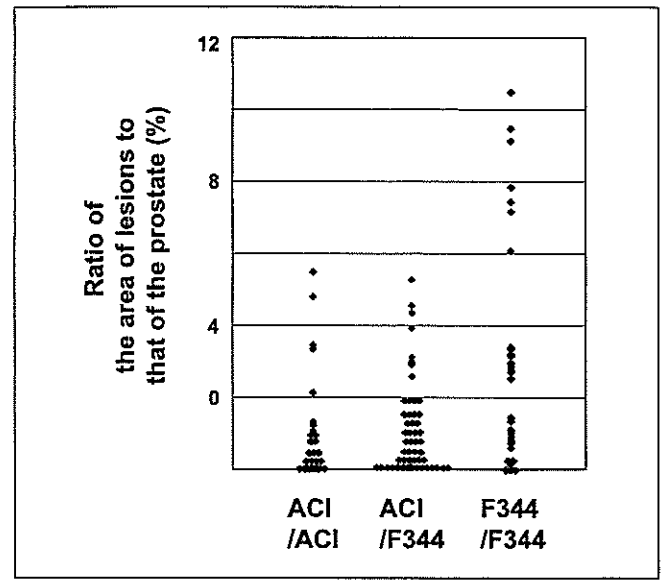

Figure 3. Effect of *Pcr1* locus on the area ratio of lesions. When F_2 intercross rats were classified by their genotype at *D2Rat161*, rats with the ACI allele(s) showed smaller area ratios.

possible mechanism for the differential effects of *Pcs2* and *Pcr2*, involvement of different molecular pathways in the development of prostate cancers depending upon testosterone levels was considered. Because *Pcs2* promotes and *Pcr2* suppresses prostate cancers and many other unmapped genes are potentially involved, the incidence and area of prostate cancers were not different between rats with and without bilateral macroscopic testicular tumors. Temporal analysis on the development of prostate cancers, that of testicular tumors, and testosterone levels is necessary to clarify possible involvement.

By combining positional information obtained by linkage analysis and expression information obtained by oligonucleotide microarray analysis (38), two candidate genes were identified. GeneChip "Rat Genome U34A" covered 39 of 158 genes within the region of *Pcs1*, 77 of 308 in *Pcr1*, 86 of 191 in *Pcs2*, and 39 of 115 in *Pcr2*, accounting for 31% of the genes within the mapped loci. In addition, the responsible genes in the loci are not necessarily differentially expressed. Therefore, the combination strategy is effective only when candidate genes are obtained, and no good candidates were found for *Pcs1* and *Pcs2* in this study. Nevertheless, *Mme* (also called *Neutral endopeptidase*, *Cd10*), which showed higher expression in ACI rats, was considered as a good candidate for *Pcr1* that had a paradoxical effect. *Mme* expression was known to suppress growth of androgen-independent prostate cancer cells and was decreased in androgen-independent prostate cancers (39). Therefore, its higher expression in ACI was in accordance with its paradoxical effect. *Cdkn1a*, which showed lower expression in ACI rats, was a good candidate for *Pcs2*. *Cdkn1a* is a well-established negative regulator of the cell cycle, and a structural abnormality in its 5′ of promoter region has been identified.[4]

Chromosomal regions around *Pcs1*, *Pcr1*, *Pcs2*, and *Pcr2* corresponded to human 19p13, 4q28-31, and 16q21-22; 3q26-27, 13q12-14, 3q21-26, and 4q32; 6p21, 21q22, 22q11, and 10q21; and 6q23-25 and 5p15, respectively. As for *Pcs1*, linkage of Swedish familial prostate cancers to 19p13 (40) and frequent loss of heterozygosity in human prostate cancers (16q22; refs. 41, 42) are

[4] Yamashita et al., submitted for publication.