

**Table 3. QTLs for prostate lesions in subgroups classified by testicular tumors and testosterone levels**

Population	Variable for linkage analysis	<i>Pcs1</i> (chromosome 19)		<i>Pcr1</i> (chromosome 2)		<i>Pcs2</i> (chromosome 20)		<i>Pcr2</i> (chromosome 1)	
		LOD scores	% Variance explained	LOD scores	% Variance explained	LOD scores	% Variance explained	LOD scores	% Variance explained
All F2 intercross rats ( <i>n</i> = 118)	Presence of any lesions	2.9	10.6	0.4	1.7	0.4	1.9	0.9	3.6
	Presence of lesions with ACI-type nucleus	5.0	17.6	0.5	2.0	1.9	8.6	0.9	3.4
	Area ratio of any lesions	0.2	0.8	5.0	20.3	0.5	2.0	0.6	2.3
	Area ratio of lesions with ACI-type nucleus	0.1	0.5	4.0	15.6	1.5	6.7	0.8	3.1
Rats with no or unilateral testicular tumors ( <i>n</i> = 51)	Presence of any lesions	0.8	7.1	0.7	6.3	1.9	17.2	1.6	17.5
	Presence of lesions with ACI-type nucleus	1.6	13.7	0.6	5.7	3.4	32.4	1.2	12.0
	Area ratio of any lesions	1.3	11.1	2.0	22.9	1.1	10.0	3.9	33.0
	Area ratio of lesions with ACI-type nucleus	0.8	6.8	1.3	11.1	1.2	10.6	4.3	35.4
Rats with bilateral testicular tumors ( <i>n</i> = 67)	Presence of any lesions	2.7	17.2	0.6	6.2	1.6	14.0	0.5	3.2
	Presence of lesions with ACI-type nucleus	3.9	24.5	1.3	11.0	1.2	10.5	1.0	6.5
	Area ratio of any lesions	0.6	3.9	3.4	22.9	1.4	9.1	0.7	6.0
	Area ratio of lesions with ACI-type nucleus	0.5	3.1	2.8	18.3	1.2	8.0	0.5	3.7

reported. Introduction of human chromosome 19p13 into both rat and human prostate cancer cells suppressed tumorigenicity in nude mice (43). As for *Pcr1*, its candidate, *Mme*, is located on human 3q25. Frequent deletion of human 13q in prostate cancer is reported (44). As for *Pcs2*, in addition to *Cdkn1a* on human 6p21, polymorphisms of *Gst11* on human 22q11 were reported to give differential risks to prostate cancers (3). As for *Pcr2*, *Srd5a1*, whose polymorphism is associated with the metastatic potential of prostate cancers (36), was located on *Pcr2*.

The genes involved in the development of testicular tumors (*Trt1*) and URA (*Ura1*) were successfully mapped on chromosomes 11 and 14, respectively. These regions correspond to human chromosome 21q21-22 and human chromosome 4q11-12, respectively. Linkage analysis is almost impossible in humans for diseases with low penetrance, such as URA, and positional information obtained here would be useful if candidate genes are obtained from their functions.

In summary, we were able to map four susceptibility and resistance genes involved in the prostate cancer susceptibility of ACI rats. A complex interaction of multiple susceptibility genes and effects of testicular tumors in prostate cancer susceptibility was shown. Combining the data with those obtained by oligonucleotide microarray, *Mme* and *Cdkn1a* were identified as candidates.

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**Table 4. Quantitative RT-PCR analyses for genes within the QTLs**

Gene	Location	Gene expression levels copy number/ $\beta$ -actin ( $\times 10^4$ , mean, <i>n</i> = 3)				
		8 wks			48 wks	
		ACI	F344	(F344 $\times$ ACI) F <sub>1</sub>	ACI	F344
<i>Mme</i>	<i>Pcr1</i> chromosome 2q31	1,413	255	976	2,608	1,328
<i>Cdkn1a</i>	<i>Pcr2</i> chromosome 20p12	9.9	15.1	16.6	8.3	37.7

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## Equivocal impact of transplacental and lactational exposure to a food-derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, on prostate and colon lesion development in F344 rats

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### Abstract

The carcinogenic potential of maternal dietary exposure to 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was investigated with the focus on the prostate and mammary glands and colons of the offspring. PhIP-DNA adducts were immunohistochemically detected in all three organs of 3-week-old animals after dams had received 200 ppm PhIP in the diet from 4 weeks before mating to weaning. The sites were essentially the same as in adult rats, suggesting that the distribution of bioactivating enzymes for PhIP do not differ greatly. Ki-67 labeling indices were increased only in the colons of the female offspring at 3-weeks of age. Development of preneoplastic and neoplastic lesions in the prostate and colon was not enhanced when the rats were maintained on PhIP-free diet until 63 weeks of age, except for significant increase in multiplicity of aberrant crypt foci in the females. The present findings indicate that PhIP exposure via placenta and breast milk may be important but that high doses and long periods may be necessary for tumor development.

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*Keywords:* 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; Maternal exposure; Prostate cancer; Rat

### 1. Introduction

Diet is considered to be a major factor determining human cancer risk, partly reflecting individual lifestyle [1]. A total of 10 carcinogenic heterocyclic amines (HCAs) have so far been found to be naturally produced during ordinary cooking of meat and fish [2].

*Abbreviations:* HCA, heterocyclic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; ACF, aberrant crypt foci; PIN, prostatic intraepithelial neoplasia.

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Among these carcinogenic HCAs, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant [3,4], and has been found to exert carcinogenicity in the colon and mammary glands of female rats and in the colon, prostate glands and lymphoid tissue of males [5–9]. It is of great interest that these types of tumors are common in the Western world where the intake of fried ground beef and fowl is relatively high [10–12]. With greater degrees of meat doneness, generating higher levels of carcinogenic HCAs, increase in risks for colorectal adenomas [13], lung [14], breast [15] and prostate cancers [16] have been observed in epidemiological studies. The available data thus suggest that PhIP might be of importance as an environmental factor in the development of human cancers.

It has recently been demonstrated that PhIP is excreted into the breast milk of rat and mouse dams treated with PhIP, resulting in adduct formation in liver DNA after both transplacental and neonatal exposure [17–19] and an elevated risk of mammary carcinoma development [20]. In consideration of the fact that PhIP or PhIP-DNA adducts can be detected in the milk of healthy women, PhIP may play a critical role in the development of human breast cancer [21,22].

In the present study, we analyzed the carcinogenic potential of a maternal dietary exposure to PhIP, in particular focusing on the prostate glands in male offspring, and the colons in both sexes.

## 2. Materials and methods

### 2.1. Chemicals and animals

PhIP hydrochloride was obtained from the Nard Institute (Osaka, Japan), with a purity of >99.9%. Male and female 6-week-old Fischer 344 rats were purchased from Charles River Japan, Inc. (Atsugi, Japan) and housed 3/cage, or 1 dam/cage with the offspring during nursing, on wood-chip bedding in an air-conditioned animal room at  $23 \pm 2$  °C and  $50 \pm 10$  % humidity. Food (Oriental MF, Oriental Yeast Co, Tokyo, Japan) and tap water were available ad libitum.

### 2.2. Experiment 1

A total of 17 virgin female rats were divided into two groups (see Fig. 1). The rats of Group 1 received 200 ppm PhIP in the diet from 4 weeks before mating with non-treated males through to weaning. Group 2 received basal diet alone and was similarly mated with non-treated males. Offspring were killed at 3 weeks of age and prostates, colons and mammary glands were removed and fixed in 10% buffered formalin or acetone. Tissue sections were stained with hematoxylin and eosin for histopathological examination and processed for immunohistochemistry.

### 2.3. Experiment 2

Experiment 2 was performed in the same manner as Experiment 1 except that rats of Group 1 received 200 ppm PhIP from the day of mating (Fig. 1). At weaning, the offspring, all which were nursed by their own dams, were divided into males and females to avoid possible sibling effects and maintained on PhIP-free diet until 63 weeks of age. At sacrifice, the rats were weighed and then killed as for Experiment 1. The prostates, colons and mammary glands were removed, and fixed in 10% buffered formalin, the large intestines being inflated with the fixative. Quantitative analyses of aberrant crypt foci (ACF) were made by a standard method.

### 2.4. Immunohistochemistry of PhIP-DNA adducts and Ki-67

Immunohistochemical analysis of PhIP-DNA adducts was performed as reported previously with minor modifications [23]. Deparaffinized sections were sequentially treated with 0.3%  $H_2O_2$  for 30 min, 250  $\mu$ g/ml RNase for 1 h at 37 °C, 2.5 N HCl for 20 min, 0.01% trypsin for 15 min at 37 °C, and then 5% skim milk for 1 h. The sections were then incubated with diluted anti-PhIP-DNA adduct antibody (1:10,000) for 2 h at room temperature and sequentially exposed to biotin-labeled goat anti-rabbit IgG and ABC (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA). The sites of peroxidase binding were visualized by diaminobenzidine with nickel enhancement. Counterstaining with hematoxylin was not performed.

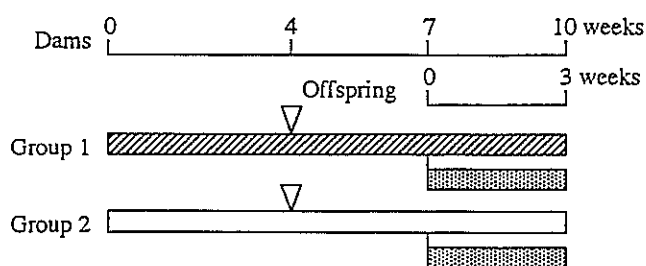
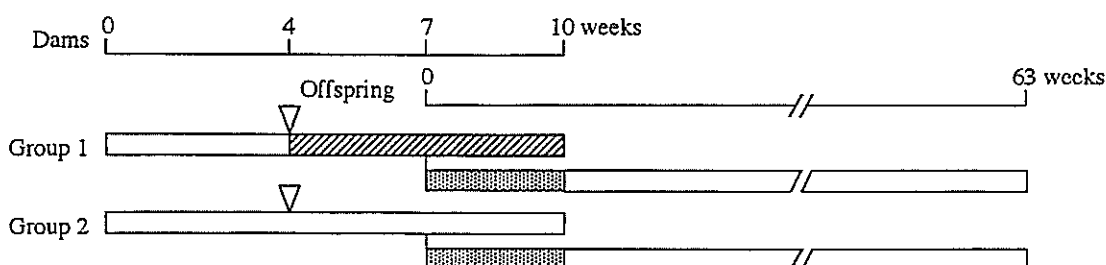
Experiment 1Experiment 2

Fig. 1. Experimental protocol. Group 1 dams were fed diet containing PhIP 200 ppm before mating (Experiment 1) during the gestation and lactation periods (Experiment 1 and Experiment 2). ▨, PhIP 200 ppm; □, basal diet; ▤, milk drinking; ▽, mating with non-treated males.

For Ki-67 immunostaining, deparaffinized sections were autoclaved for 5 min at 120 °C, and sequentially treated with 0.5% H<sub>2</sub>O<sub>2</sub> for 30 min and 5% skim milk for 1 h, before incubation with diluted mouse monoclonal Ki-67 antibody (MIB-5, 1:200, Immunotech, France) for 2 h at room temperature. They were then sequentially exposed to biotin-labeled horse anti-mouse IgG and ABC and binding was demonstrated with diaminobenzidine as the substrate, in this case without nickel enhancement. Sections were counterstained with hematoxylin for microscopic examination.

### 2.5. Statistical analysis

Statistical analysis was performed using the ANOVA test for the significance of differences between the means of treated and control groups in body weights and the data for histopathological changes and immunohistochemical parameters.

## 3. Results

### 3.1. Experiment 1

Lower mean body weights of both dams and offspring in Group 1 were observed at delivery (Table 1). However, no inter-group differences in the period of gestation or average numbers of pups were found, as reported previously [24]. Final body weights of both sexes of offspring from PhIP-treated dams were also significantly lower than those from non-treated dams at 3 weeks of age (Table 1). PhIP-DNA adducts could be immunohistochemically visualized in the prostatic epithelium of all lobes, as well as epithelial and cryptal cells of the colon and mammary gland epithelial cells in 3-week-old offspring from PhIP-treated dams (Fig. 2). There were no significant differences of the signal intensities in colon epithelial and mammary gland cells between males and females. Significant elevation of the Ki-67 labeling indices in the colons of offspring from

Table 1  
Body weight of offspring at sacrifice

Experiment	Age (week)	Sex	Maternal exposure to PhIP	No. of offspring	Body weight (g)
1	3	Male	+	29	32.2±3.5***
			–	23	41.7±3.0
		Female	+	20	32.1±5.0**
			–	26	40.1±2.7
2	63	Male	+	25	393.8±32.2***
			–	33	455.9±34.2
		Female	+	22	213.0±14.8
			–	13	220.3±11.8

\*\*,\*\*\* $P < 0.01, 0.001$ .

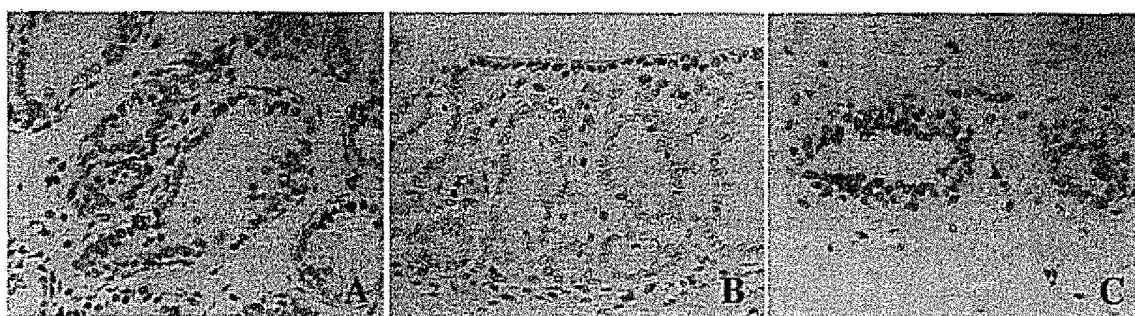


Fig. 2. Immunohistochemical demonstration of PhIP-DNA adducts in 3-week-old offspring from PhIP-treated dams (Experiment 1). A. Prostate; B. Colon; C. Mammary gland (400×).

PhIP-treated dams was also evident compared with those from control dams (Table 2). However, no such elevation was apparent in the prostate glands.

### 3.2. Experiment 2

Several animals died before the termination of the experiment. The cause of death in most cases before 30 weeks of age in Group 1 was considered to be associated with hydroencephaly. After this

the causes were unclear. Final body weights of male offspring in Group 1 were significantly decreased compared with respective control animals, but this was not the case for females (Table 1). Table 3 summarizes data for histopathological findings in the prostates of male offspring at 63 weeks of age. Preneoplastic and neoplastic lesions were found in the ventral lobe but not in dorsolateral and anterior lobes of prostate and seminal vesicles. A tendency for increase in the incidence and multiplicity of

Table 2  
Ki-67 labeling indices in the prostate glands and colons of offspring

Experiment	Age (week)	Maternal exposure to PhIP	Prostate gland (%)		Colon (%)	
			Ventral	Dorsolateral	Male	Female
1	3	+	7.3±1.3	5.7±1.8	56.9±8.5	50.7±6.5*
		–	6.1±1.9	5.0±2.1	47.1±2.8	42.0±4.6
2	63	+	1.0±0.3	1.7±1.3	41.9±5.3	47.7±5.1
		–	0.9±0.3	1.1±0.7	37.2±6.2	47.5±9.0

\* $P < 0.05$ .

Table 3  
Preneoplastic and neoplastic lesions in the ventral prostates of 63-week-old offspring

Maternal exposure to PhIP	No. of offsprings	PIN		Adenocarcinoma	
		Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)
+	25	4 (16)	0.20±0.5	3 (16)	0.24±0.7
–	33	5 (15)	0.15±0.4	1 (3)	0.03±0.2

PIN, prostatic intraepithelial neoplasia.

prostatic adenocarcinomas was found in males from dams given PhIP, but without statistical significance. The incidences of prostatic intraepithelial neoplasias (PIN) did not differ between the groups.

Colon tumor and ACF development in male offspring was not affected by maternal PhIP treatment (Table 4). On the other hand, the incidence and multiplicity of ACF larger than 4 crypts and aberrant crypts per focus in the large intestine of female offspring were significantly higher than the control values in Group 2. No mammary tumor development was observed in female offspring of either group.

#### 4. Discussion

We previously demonstrated that transplacental and trans-breast milk exposure to PhIP in rats increased risk of mammary carcinoma development when the offspring were also continuously exposed to dietary PhIP after weaning [20]. The present data demonstrated that maternal exposure to PhIP alone is not sufficient for clear increase in risk of colon and prostate cancer development in male offspring, and only of the colon in females. We encountered many

cases with congenital abnormalities (e.g. anophthalmia and hydrocephaly) in neonates exposed to 200 ppm PhIP in the present experiment in line with the previous report [24]. In fact, the duration of PhIP treatment to dams had been changed to reduce the risk of such lesions and this presumably explains the more limited carcinogenic effects. Significant increase of the incidence and multiplicity of ACF was evident, however, in the female offspring, suggesting that these are more susceptible to PhIP colon carcinogenicity during the fetal and newborn periods. An increase in susceptibility of adult females to PhIP colon carcinogenesis was earlier demonstrated when PhIP was given together with caffeine [25], contrasting with the fact that male rats preferentially develop colon tumors with normal exposure to PhIP [5]. The reason why mammary tumors did not develop and why enhancement of ACF development in colon was found only in females remains unclear. The amount of PhIP-DNA adducts in offsprings in the present study might have been very low because they were exposed to PhIP only by transplacental and trans-breast milk routes and PhIP-DNA adducts were immunohistochemically detected in 3-week-old newborn only by staining with signal enhancement. In consideration of

Table 4  
Quantitative result of aberrant crypt foci in the colon of 63-week-old offsprings

Sex	Maternal exposure to PhIP	No. of offsprings		ACF/colon				AC/focus		
		Total	Bearing cancer	<4 Crypts		≥4 Crypts		Total		
				Incidence	Multiplicity (No./rat)	Incidence	Multiplicity (No./rat)	Incidence	Multiplicity (No./rat)	
Male	+	25	1	5	0.2±0.5	4	0.2±0.5	8	0.4±0.7	1.9±3.9
	–	33	0	5	0.2±0.6	6	0.2±0.5	8	0.4±0.9	1.2±2.6
Female	+	22	0	8	0.5±0.7	13*	0.6±0.5*	15	1.1±1.0	3.1±2.9**
	–	13	0	4	0.5±0.8	1	0.1±0.3	4	0.5±1.0	0.9±1.4

\*,\*\* $P < 0.05, 0.01$ .

the fact that F344 rat strain is less susceptible to PhIP-induced mammary carcinogenesis compared with the SD rat strain [6,26], a low level of PhIP exposure could be reasons for no development of mammary tumors in the present experiment. A recent report provided evidence for a carcinogen dose threshold for ACF induction in rat colon with PhIP [27]. It may be that different thresholds may exist for the two sexes and thus the enhancement of ACF development only in females.

Cytochrome P450 1A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2), are generally regarded as bioactivating enzymes for PhIP in humans [28]. In the rat, PhIP is metabolically transformed to *N*-hydroxy derivatives by CYP1A2 or CYP1A1 in the liver, and subsequently these metabolites are *O*-acetylated by *N*-acetyltransferase in the colon [29]. Alternatively, *N*-acetoxy-PhIP formed in rat liver may enter the circulation and reach a variety of target tissues [30]. The distribution of these two enzymes in cellular levels of whole organs may account for cell type-specific formation of PhIP-DNA adducts. In the present study, immunohistochemical detection of PhIP-DNA adducts in 3-week-old offspring from PhIP-treated dams showed essentially similar results to those for adults in a previous study, in which adduct formation was observed in the pancreas, heart, mammary glands, colon, prostate glands and seminal vesicles [23]. These data provide evidence that PhIP given to dams is transferred to offspring and activated to reactive forms and suggest that the distribution of CYP1A2 and NAT2 is almost the same in both adult and newborn rats.

It has been estimated that human exposure to PhIP is the highest among the five HCAs investigated, based on a dietary survey of the United States population, and concluded that this accounts for nearly one-half of the incremental cancer risk due to HCAs [31]. Daily exposure to PhIP ranges from 5.2 to 16.6 ng/kg per day for the United States population [31–33] and from 1.0 to 2.4 ng/kg per day for Europeans [34,35], and has been estimated at 0.64 ng/kg per day for Japanese [36]. Exposure to PhIP of dams in the present Experiment 2 was equivalent to ~3–19 kg of grilled chicken per day and, therefore, very large compared to the amount ordinarily consumed by humans in daily life. However, the fact that human exposure to PhIP was practically demonstrated by its identification in

the urine of healthy volunteers on a normal diet or smokers of black tobacco [37,38] suggests that humans are continuously exposed to PhIP and that this carcinogenic HCA may be extremely important with respect to the development of cancer. Actually, several epidemiological studies have demonstrated positive correlations between PhIP intake and risk of breast and prostate cancers, and possibly also colorectal adenomas [13,15,16]. Further risk assessment studies are needed to clarify safe levels of HCA exposure under various environmental conditions in humans.

### Acknowledgements

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# Influence of atrazine administration and reduction of calorie intake on prostate carcinogenesis in probasin/SV40 T antigen transgenic rats

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Atrazine, which has been used worldwide as a pesticide, is now known to exert endocrine disrupting (antiandrogenic) effects in mammals. In this study, modifying effects of dietary feeding of 500 and 1000 p.p.m. atrazine on the development of androgen-dependent prostate cancer were investigated using male probasin/SV40 T antigen transgenic (TG) rats. As administration of atrazine has now been identified as causing a decrease in bodyweight, a dietary-restricted TG rat group was also included in order to elucidate the influence of reduction of calorie intake per se on the development of prostate cancer. At week 13, almost the entire lobes of the prostate were occupied with tumor lesions, with no clear intergroup differences in the incidences and multiplicities. Therefore, morphometrical assessment ratios of the prostate epithelial area to the whole prostate tissue area were evaluated. The ratio in the lateral lobe of the 1000 p.p.m. atrazine-treated group was significantly decreased, and there was a tendency to decrease in the ratios in the dorsal lobe of the atrazine-treated groups. However, dietary restriction itself without atrazine treatment caused the same reduction to a similar or greater extent. Testosterone levels were not affected by atrazine administration or dietary restriction. Our results indicate that the observed atrazine-related suppression of prostate carcinogenesis was probably caused by the decrease in calorie intake, rather than by atrazine-related endocrine disruption. (*Cancer Sci* 2005; 96: 221–226)

dependent, we hypothesized that exposure of atrazine to these rats would cause inhibition of testosterone production, resulting in reduced carcinoma development. To test this, we examined the prostate of animals histopathologically after dietary administration of 500 or 1000 p.p.m. atrazine for 13 weeks. We also examined serum testosterone levels at the time of necropsy. As it has been reported that administration of high doses of atrazine causes a decrease of bodyweight in rats,<sup>(8)</sup> a group of rats given a restricted amount of basal diet was included with adjustment of the mean bodyweight of this group to be similar to that of the 1000 p.p.m. atrazine treatment group. We also measured levels of atrazine and its metabolites in the serum at the end of the treatments in order to confirm absorption of the dietary administered compound.

## Materials and methods

**Animals, chemical, and diet.** Male heterozygous TG rats established in our laboratory with a Sprague-Dawley genetic background were used in the present study.<sup>(11,12)</sup> At 6 weeks of age, animals were housed two or three per cage on wood-chip bedding in an air-conditioned animal room at 23 ± 2°C and 50 ± 10% humidity and provided with powdered diet with or without atrazine and tap water ad libitum, except in the dietary-restricted group. Atrazine was from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

**Rationale for dose selection.** Friedmann<sup>(7)</sup> reported that 50 mg/kg per day atrazine given by oral gavage acted as an endocrine disrupter in male rats by inhibiting testosterone production. Therefore, atrazine concentrations in the basal diet were set at 500 and 1000 p.p.m. because mean atrazine intake in the 1000 p.p.m. group was estimated to be around 50 mg/kg/day, considering a predicted daily food consumption of TG rats.

**Experimental procedure.** A total of 40 heterozygous male TG rats were divided into four groups, each containing 10 animals. Animals in the non-treatment control group were fed basal diet (soybean-free modified NIH-07; Oriental yeast Co., Tokyo, Japan) and animals in atrazine treatment groups received the same diet containing 500 or 1000 p.p.m. atrazine. Animals in the dietary-restricted group were given an amount of basal diet restricted (15–20 g). The amount was determined on each day when bodyweights were measured, so as to adjust mean bodyweight of the dietary-restricted group to that of the 1000 p.p.m. atrazine treatment group. Mean food consumption in the dietary-restricted group was about 70% of that in the control group. Bodyweights for all the animals and food

**A** number of chemicals in the environment have the potential to disturb the endocrine system. These are classified as endocrine disruptors, and many of them are known to have an influence on reproductive potential.<sup>(1–3)</sup> Atrazine, once one of the most widely used agricultural pesticides all over the world, is now recognized to have disrupting effects on the reproductive systems of mammals.<sup>(4–6)</sup> Although agricultural use of atrazine is prohibited in many countries at present, a certain amount of the compound remains in soil and water.<sup>(9,10)</sup> Therefore, humans and wildlife are at risk of exposure and it remains important to assess the potential effects on the reproductive systems in laboratory animals. Both the female<sup>(4,5)</sup> and male<sup>(6–8)</sup> reproductive systems are affected in rats. Friedmann<sup>(7)</sup> reported that atrazine acts as an endocrine disrupter in male rats by inhibiting testosterone production in the Leydig cells directly. Therefore, we examined the effects of atrazine exposure on development of androgen dependent prostate carcinomas, using male transgenic (TG) rats having a probasin promoter/simian virus 40 (SV40) T antigen construct.<sup>(11,12)</sup> These rats, established in our laboratory, developed well-differentiated adenocarcinomas in the prostate at 100% incidence before 15 weeks of age.<sup>(11,12)</sup> Castration at 20 weeks of age caused complete regression or involution of carcinomas in these rats; and castration at 5 weeks of age completely inhibited the prostate carcinoma development.<sup>(12)</sup> Since the tumors are considered to be completely androgen

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consumptions for all the cages were measured at least once in each treatment week. At the end of the 13-week treatment period, all rats were killed by exsanguination via the thoracic and abdominal aorta under light ether anesthesia after blood collection from the abdominal aorta. The testes and the accessory sex organs were removed and fixed in 10% neutral buffered formalin except for the testes of two animals in each group, which were fixed in formalin-sucrose-acetic acid (FSA) composed of 37% formalin 5.0, 5% sucrose solution 15.0, and acetic acid 0.8 in a volume ratio, praised for its suitability in evaluation of testis morphology.<sup>(13)</sup> The testis weights were measured before fixation. The ventral prostate, the dorsolateral prostate including the urethra, and the anterior prostate/seminal vesicle complex were removed after fixation, and each was weighed. Relative organ weights were calculated as percentages of body weights at the time of necropsy.

Testosterone and atrazine levels in serum, and atrazine concentrations in diets. The collected blood samples at the time of necropsy were centrifuged at 1000 r.p.m. for 10 min to obtain serum. Testosterone levels in the serum were analyzed for every animal in each group using radioimmunoassays by a commercial laboratory (SRL, Tokyo, Japan). Serum levels of atrazine and its metabolites were measured for three animals in each group using gas chromatography-mass spectrometry according to the method of Brzezicki *et al.*<sup>(14)</sup> The concentrations of atrazine in the remaining diets at the time of necropsy were measured for each group using liquid chromatography-mass spectrometry. The measurement of atrazine in the serum and the diets was performed at Japanese Food Research Laboratories (Tokyo, Japan).

Histopathology. For histopathological observation, one transverse slice of each side of the testes, the caput, and cauda regions of each side of the epididymides, two sagittal slices from the ventral prostate, two transverse slices from the dorsolateral prostate including the urethra, and four transverse slices from each side of the seminal vesicles including the anterior prostate were embedded in paraffin, sectioned, stained with hematoxylin-eosin (HE), and examined by light microscopy. Histopathological evaluation of the prostate, including morphometry, was performed for each prostate lobe: the ventral, dorsal, lateral, and anterior lobes.

Immunohistochemistry. Immunohistochemistry for SV40 T antigens and androgen receptors (AR) were performed using avidin-biotin-peroxidase complex (ABC) method. The sections were treated with mouse anti-SV40 T antigen (Pharmingen, San Diego, CA, USA) and rabbit anti-AR (Affinity Bioreagents, Golden, CO, USA). Binding was visualized with a Vectastain Elite ABC kit (Vector Laboratory, Burlingame, CA, USA) followed by light hematoxylin counterstaining to facilitate microscopic examination.

Morphometry for prostate epithelium area. As the entire prostate lobes were occupied with tumor lesions, differences in the incidences and multiplicities of tumors were not observed clearly among the groups (see Results). Therefore, ratios of epithelial areas including tumors to whole prostate tissue areas were evaluated morphometrically. The prostate epithelial areas including prostatic intraepithelial neoplasias (PIN) and adenocarcinomas for each section were quantitatively measured using the H&E sections and an image processor for analytical pathology (IPAP) (Sumika Technos, Osaka, Japan). The whole prostate tissue area including lumen for each section was also measured in the same way to allow calculation of the ratio. All of the H&E slides were thus evaluated for the ventral, dorsal, and lateral lobes of the prostate. For the anterior lobe, the left sides only were measured.

Statistical evaluation. The data of bodyweights, serum testosterone levels, organ weights, and morphometrical ratios were analyzed statistically as follows. For the statistical evaluation between the control group and the atrazine treatment groups, first, the data were tested by Bartlett's test<sup>(15)</sup> for homogeneity of variance. When the variances were homogeneous, Williams'

test<sup>(16)</sup> was performed assuming a dose-related trend. When the variances were heterogeneous, the Shirley-Williams test<sup>(17,18)</sup> was performed assuming a dose-related trend. For the statistical evaluation between the control group and the dietary-restricted group, the data were tested by the *F*-test<sup>(15)</sup> for homogeneity of variance between the groups. When the variances were homogeneous, Student's *t*-test<sup>(15)</sup> was used, and when the variances were heterogeneous, the Aspin & Welch *t*-test<sup>(15)</sup> was performed to compare the mean in the control group with that in the dietary-restricted group. Bartlett's test was conducted at the significance level of 0.05 and the Williams and Shirley-Williams tests were conducted at the two-tailed significance level of 0.05. The *F*-test was conducted at the significance level of 0.20, and the Student's *t*-test and the Aspin & Welch *t*-test were conducted at the two-tailed significance levels of 0.05 and 0.01. The Williams test and the Shirley-Williams test were performed using the SAS function PROBMC.<sup>(19)</sup>

## Results

General observation. No deaths were observed in any group during the treatment period. Bodyweights in the atrazine treatment groups were significantly lower than in the control group, almost throughout the treatment period and with dose-dependence. Bodyweights of the dietary-restricted and 1000 p.p.m. atrazine groups were similar, and significantly lower than the control values throughout the treatment period (Fig. 1). Food consumption in the atrazine treatment groups was lower than in the control group throughout the treatment period with dose-dependence (Fig. 2). Mean atrazine intakes were calculated from

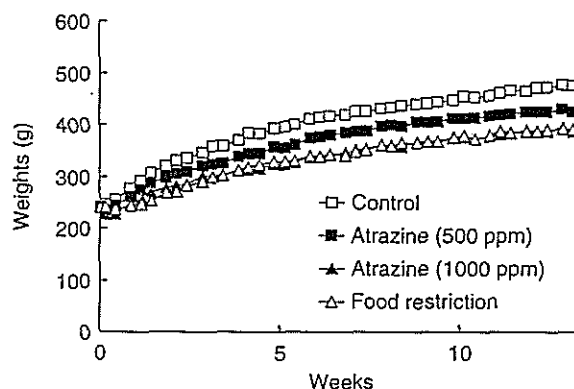


Fig. 1. Body weight curves of all four groups over the treatment period ( $n = 10$ ).

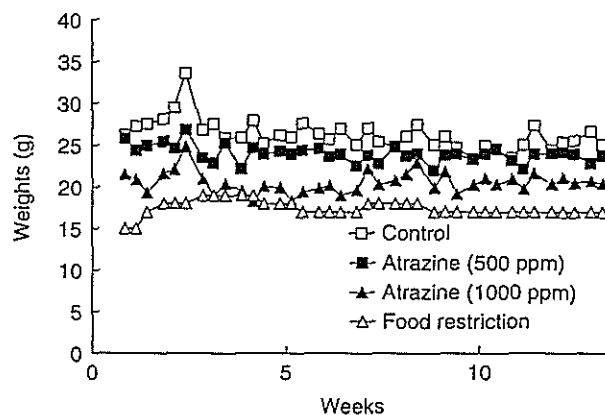


Fig. 2. Food consumption curves of all four groups over the treatment period ( $n = 10$ ).

Table 1. Final body weights and relative organ weights in TG rats (n = 10)

	Body weight (g)	Relative organ weight (%)			
		Testes	Ventral prostate	Dorsolateral prostate	Anterior prostate + Seminal vesicle
Control	475.2 ± 26.8	0.721 ± 0.031	0.076 ± 0.016	0.185 ± 0.076	0.500 ± 0.106
Atrazine (500 p.p.m.)	423.7 ± 28.6*	0.766 ± 0.226*	0.078 ± 0.014	0.165 ± 0.014	0.554 ± 0.093
Atrazine (1000 p.p.m.)	385.0 ± 22.8*	0.894 ± 0.057*	0.081 ± 0.013	0.168 ± 0.012	0.578 ± 0.081
Food restriction	389.3 ± 18.4 <sup>1</sup>	0.836 ± 0.102 <sup>1</sup>	0.057 ± 0.008 <sup>1</sup>	0.155 ± 0.017	0.432 ± 0.071

\*Significantly different from the control group,  $P < 0.05$  (Williams test), <sup>1</sup>Significantly different from the control group,  $P < 0.01$  (paired comparison).

the targeted concentrations of the substance in the prepared diets (500 p.p.m. and 1000 p.p.m.), the food consumption values, and the bodyweights. The results were 33.0 mg/kg/day for the 500 p.p.m. group and 61.6 mg/kg/day for the 1000 p.p.m. group.

**Organ weights.** A significant decrease in relative weight of the ventral prostate was noted only in the dietary-restricted group (Table 1). No other treatment-related significant change was observed in any group. Although statistically significant increases in relative weights of testes were observed in the atrazine treatment groups and in the dietary-restricted group, these were considered to be superficial changes because absolute weights did not significantly differ (data not shown). They were rather due to decrease in bodyweight gain.

**Testosterone levels in serum.** Serum testosterone levels at the end of the treatment period were not significantly different in the atrazine treatment groups and in the dietary-restricted group compared to the control group value (Fig. 3).

**Levels of atrazine in serum and concentrations of atrazine in diets.** Data for serum levels of atrazine and its metabolites in each group are summarized in Table 2. Diaminochlorotriazine (DACT) was the major metabolite, with desethylatrazine (DE-ATRA) and desisopropylatrazine (DI-ATRA) as minor metabolites. Their concentrations were dose-dependent. Atrazine and its metabolites were essentially not detectable in the serum in the control and dietary-restricted groups. Actual concentrations of atrazine in the prepared diet were 300 p.p.m. (60%) for the 500 p.p.m. group and 910 p.p.m. (91%) for the 1000 p.p.m. group.

**Histopathology and immunohistochemistry.** The histopathological lesions of the prostate were diagnosed according to the criteria described previously.<sup>(11,12)</sup> Marked epithelial proliferation was observed with formation of irregular glands and luminal bridging to give a cribriform pattern in focal populations. The nuclei demonstrated enlargement and severe atypia. These lesions appeared compatible with well-differentiated adenocarcinomas in human cases and were therefore diagnosed as adenocarcinomas (Fig. 4a,b). They were observed in the ventral, lateral, and anterior lobes of the prostate in almost all rats. In the dorsal lobe, adenocarcinomas were observed in two or three rats of each group. The incidences of adenocarcinomas in any treatment groups were not dramatically different from those of the control group (Table 3). Foci within glands with less proliferation were also observed. These exhibited crowding of stratified epithelial

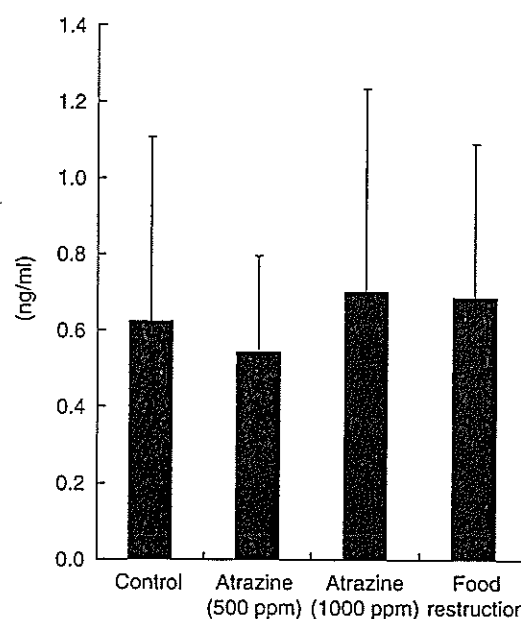


Fig. 3. Serum testosterone levels in transgenic rats at the end of the treatment period (n = 10). Data are mean ± SD. No significant difference from the control group was observed.

cells with irregular spacing and occasional luminal bridging. Although nuclear atypia were severe, basic structures of the prostate glands were mostly maintained, comparable with PIN of human cases (Fig. 4a,b). Such lesions were observed in all lobes of the prostate in all rats (Table 3) with no significant intergroup differences. SV40 Tag and androgen receptor expression were detected immunohistochemically in almost all cells of the prostate adenocarcinomas and PIN (Fig. 4c,d). No treatment-related changes in the testes, epididymides, and seminal vesicles were observed. Marked atrophy of the seminiferous tubules in the testes was observed in a rat in the 500 p.p.m. atrazine group and in another rat in the dietary-restricted group. Decreased sperm in the corresponding epididymides were observed in those

Table 2. Serum atrazine and its metabolites in TG rats (n = 3)

	ATRA* (µg/mL)	DE-ATRA <sup>1</sup> (µg/mL)	DI-ATRA <sup>1</sup> (µg/mL)	DACT <sup>1</sup> (µg/mL)
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.04
Atrazine (500 p.p.m.)	0.00 ± 0.00	0.08 ± 0.01	0.23 ± 0.04	3.63 ± 0.67
Atrazine (1000 p.p.m.)	0.02 ± 0.01	0.15 ± 0.03	0.38 ± 0.03	9.27 ± 2.05
Food restriction	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.05

\*atrazine, <sup>1</sup>desethylatrazine, <sup>1</sup>desisopropylatrazine, <sup>1</sup>diaminochlorotriazine.

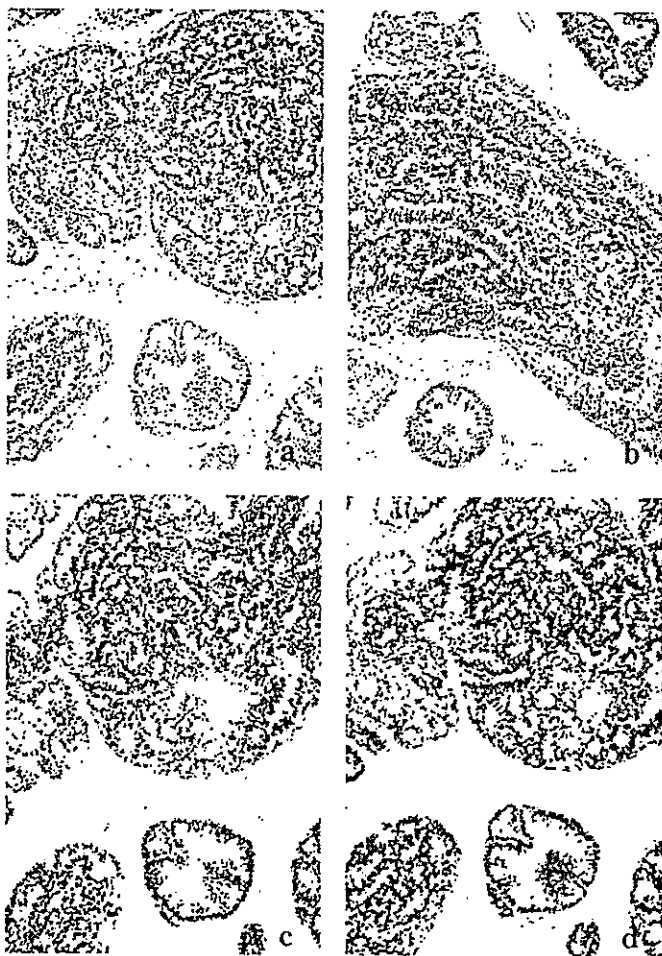


Fig. 4. Representative histopathology and immunohistochemical analysis of ventral prostates in transgenic rats. (a) Control. Atypical glands diagnosed as adenocarcinomas are evident in the upper, and prostatic intraepithelial neoplasia (PIN) (marked with \*) is also apparent. (b) 1000 p.p.m. atrazine. Adenocarcinomas are evident in the center, and PIN (marked with \*) is also apparent. (c) Most atypical cells have positive reactions for SV40 T antigen (same region as a). (d) Most atypical cells have positive reactions for androgen receptors (same region as a).

two rats. However, they were considered to be spontaneous changes, because the incidences were sporadic and the other rats showed no remarkable changes in the testes and epididymides. In addition, similar changes were often observed in non-treated rats.

Morphometry for evaluation of the prostate cancer development. Results of morphometry for the prostate epithelium including atypical and cancer cells are summarized in Fig. 5. Significant

decreases in the ratios of the epithelial areas to whole prostate tissue areas were observed in the ventral, dorsal, and lateral lobes in the dietary-restricted group, and in the lateral lobe in the 1000 p.p.m. atrazine group. The values in the dorsal lobe in the atrazine treatment groups also showed a tendency to decrease in a dose-dependent manner, albeit without statistical significance. No significant effect was observed in the anterior lobe in any treatment groups.

## Discussion

In the present study, dietary administration of atrazine for 13 weeks showed slight suppressive effects on the development of prostate cancer in the probasin-SV40 T antigen transgenic rats, even though the atrazine treatment did not affect the serum testosterone levels. The morphometrically assessed ratio of prostate epithelial lesion area to the whole tissue area was significantly decreased in the lateral lobe of the 1000 p.p.m. atrazine group. A similar tendency for decrease was also observed in the dorsal lobe. However, equal or greater suppression of prostate neoplastic lesion development was also observed in the dietary-restricted group. Therefore, the influence observed in the atrazine treated groups was probably caused by the decrease in calorie intake, rather than endocrine effects.

Bodyweights in the dietary-restricted group were similar to those in the 1000 p.p.m. atrazine group. Diet is considered to be a major and important environmental factor contributing to cancers of hormonal tissues, including the prostate.<sup>(20-26)</sup> Snyder *et al.*<sup>(23)</sup> and Pollard *et al.*<sup>(24,25)</sup> reported that dietary restriction reduced the frequency and delayed the occurrence of spontaneous prostate adenocarcinoma in Lobund-Wistar rats. Mukherjee *et al.*<sup>(26)</sup> found dietary restriction to reduce prostate cancer growth in transplantable prostate cancer models (i.e. the Dunning R3327-H adenocarcinoma in rats). Interestingly, they revealed that tumor microvessel density and vascular endothelial growth factor (VEGF) expression were reduced by dietary restriction, and they hypothesized that dietary restriction reduces prostate cancer growth by inhibiting tumor angiogenesis.

We couldn't detect histopathological differences between the control group and the treated groups in terms of mitotic figures and apoptotic body formation. In addition, even though we performed immunohistochemistry for single-stranded DNA, we couldn't find noticeable differences (data not shown). Therefore, detailed mechanisms for inhibition of prostate carcinogenesis observed in this study were not clear.

Several reports have documented the effects of atrazine on the male reproductive systems in rats.<sup>(6-8)</sup> Friedmann<sup>(7)</sup> reported that atrazine given by oral gavage acted as an endocrine disrupter in male rats by inhibiting testosterone production in the Leydig cells. However, in the present study, serum testosterone levels were not affected by dietary administration of the compound, despite the fact that serum levels of atrazine, including its metabolites, were consistent with values reported previously.<sup>(14,27)</sup> This clearly indicated that atrazine was certainly absorbed in the TG rats.

Table 3. Histopathological findings of neoplastic lesions in the prostate of TG rats

	Number of rats	Number of rats with prostatic lesions							
		Ventral		Lateral		Dorsal		Anterior	
		PIN	AC	PIN	AC	PIN	AC	PIN	AC
Control	10	10	10	10	9	10	3	10	10
Atrazine (500 p.p.m.)	10	10	10	10	9	10	3	10	10
Atrazine (1000 p.p.m.)	10	10	10	10	10	10	3	10	9
Food restriction	10	10	10	10	9	10	2	10	9

PIN, prostatic intraepithelial neoplasia; AC, adenocarcinoma.

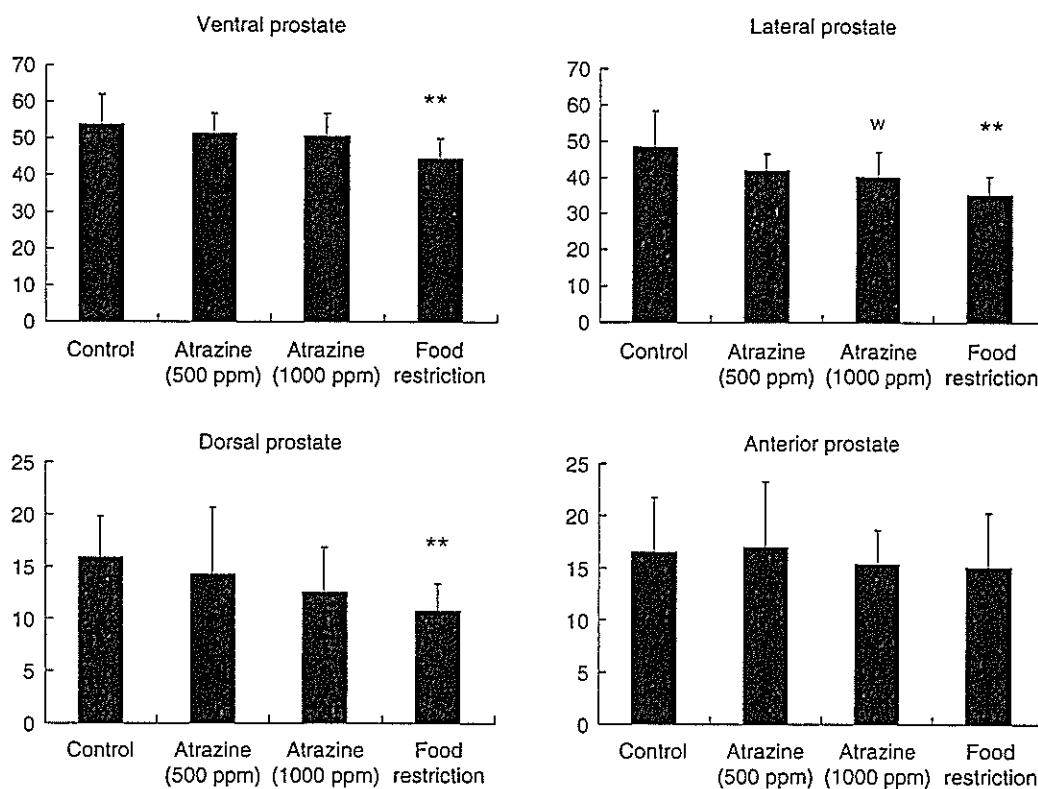


Fig. 5. Results of morphometry for the prostate epithelium in transgenic rats ( $n = 10$ ). Data are mean  $\pm$  SD. Each longitudinal axis represents a percentage ratio of the epithelial area to the whole prostate tissue area. W, significantly different from the control group,  $P < 0.05$  (Williams test). \*\*, significantly different from the control group,  $P < 0.01$  (paired comparison).

The reason for the discrepancy between the present study and the previous reports remained unclear, but gavage administration might conceivably exert different effects from dietary administration. There have been reports in which atrazine was administered by food to rats and expected effects were not observed.<sup>(28,29)</sup> Son *et al.*<sup>(28)</sup> reported that dietary administration of atrazine did not influence thyroid carcinogenesis in the rat thyroid two-stage carcinogenesis model applying N-bis(2-hydroxypropyl)nitrosamine (DHPN) as an initiator, even though atrazine given by oral gavage was documented to affect rat thyroid histopathology along with thyroid hormones in an earlier article.<sup>(30)</sup> Tanaka *et al.*<sup>(29)</sup> described dietary administration of atrazine to lack influence on the occurrence of ovarian adenocarcinoma in a rat model initiated with 7,12-dimethylbenz(a)anthracene (DMBA), even though it was reported that atrazine given by oral gavage affected rat ovaries in terms of weights and estrous cyclicity.<sup>(31)</sup>

Actual concentrations of atrazine in the prepared diets were found here to be 60–91% of the target values. The reason for the discrepancy is not clear, although homogeneity of the diet preparation or duration between the preparation and the measurement (5 or 6 months) might have affected the results.

In conclusion, our results indicate that high dose atrazine in the diet is associated with slight inhibition of prostate carcinogenesis in probasin-SV40 T antigen transgenic rats, but this effect is probably caused by suppression of calorie intake, rather than by atrazine-related endocrine disruption.

#### Acknowledgments

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# Anticancer Effect of Hyperthermia on Prostate Cancer Mediated by Magnetite Cationic Liposomes and Immune-Response Induction in Transplanted Syngeneic Rats

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**BACKGROUND.** The hyperthermic effect of magnetic particles was examined in rat prostate cancer *in vivo*. Magnetic cationic liposomes (MCLs) have a positive surface charge and generate heat in an alternating magnetic field (AMF) due to hysteresis losses.

**METHODS.** Rat prostate cancer cells (PLS 10; androgen independent) were injected subcutaneously into the flank of F344 rats. MCLs were injected into rat prostate cancer nodules that had grown to 5–6 mm in diameter, and were then exposed to an AMF. Tumor growth rates were measured. To examine whether hyperthermia caused immune induction for PLS 10, cytotoxicity assays and immunohistochemical staining for CD3, CD4, CD8, and Heat Shock Protein (HSP) 70 were performed.

**RESULT.** The tumor temperature increased to 45°C whereas the body temperature remained at around 38°C. Tumor regression was observed in the hyperthermic group. CD3, CD4, and CD8 immunocytes were present in the tumor tissues of the rats exposed to hyperthermia, but they were not detected in any of the tumor tissue of untreated rats. HSP70 also appeared in the viable area at its boundary with the necrotic area. The cytotoxic activity of tumor-transplanted rats for PLS 10 cells increased in hyperthermic-treatment rats.

**CONCLUSION.** These results suggest that hyperthermia using MCLs is an effective therapy for prostate cancer, since this treatment appears to kill the prostate cancer cells not only directly by heating but also by inducing an immune response. This therapy may cure not only the primary lesion but also metastatic lesions. *Prostate* 64: 373–381, 2005. © 2005 Wiley-Liss, Inc.

**KEY WORDS:** prostate cancer; hyperthermia; magnetite cationic liposome; immune-response induction

## INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy in Western males and its incidence is increasing rapidly in Japan [1]. This increase is believed to be attributable to longer life expectancy, growing prostate awareness, and more intense screening [2,3].

The success of early prostate cancer detection has resulted in an increased number of candidates for therapy. The main treatment options for clinically localized prostate cancer currently consist of surgical

extirpation and radiation therapy (external beam radiation therapy and/or brachytherapy) [4]. There are instances in which radical prostatectomy is not an

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option, such as when the patient is a poor risk for surgery or due to the wishes of the patient, in which cases radiation treatment becomes an option. Hormonal therapy, careful observation, or combination therapy with these methods are also options. Decisions regarding treatment must be made on an individual basis, with consideration for the patient's life expectancy and quality of life, as well as the patient's wishes [5].

Some other treatment options are less popular: cryotherapy [6–8], high-intensity focused ultrasound [9–11], and hyperthermia. The preliminary data suggest that high-intensity focused ultrasound represents a valid alternative treatment strategy for patients with localized prostate cancer who are unsuitable for surgery [9–11].

Cryosurgical ablation of the prostate is one approach to the treatment of localized prostate cancer. Third-generation cryosurgery uses gas-driven probes that enable the use of probe diameters of only 17 gauge, and this minimally invasive technique appears to be well tolerated. Cryosurgery has reemerged as an evolving technology and a minimally invasive treatment option. In 1996 the American Urological Association recognized cryoablation as a therapeutic option for prostate cancer in its position statement and removed the "investigational" label from this procedure.

Adding hyperthermia to conventional radiotherapy may also improve local control in prostate carcinoma. Hyperthermia is known to enhance the effect of radiation on prostate cancer cells *in vitro* [12]. Various hyperthermia techniques can be used to treat prostate carcinoma. Transurethral and/or transrectal hyperthermia produce a relatively uncontrollable heat distribution because of the limited heat penetration depth [13], and Algan et al. [14] reported no improvement in treatment outcome when using transrectal hyperthermia. The feasibility of interstitial [15] and regional hyperthermia for locally advanced prostate carcinoma has been reported [16–18], but these techniques require further development before they can be applied clinically.

Hyperthermia is based on the fact that tumor cells are more sensitive to heat than is healthy tissue [19]. However, it is difficult to specifically heat tumors with hyperthermia because the heating effects are influenced by various factors such as tumor size and the position of electrodes. In addition, the inevitable technical problem with hyperthermia is the difficulty of uniformly heating only the tumor region until the required temperature has been reached whilst not damaging normal tissue. Some researchers have investigated the application of submicron-sized magnetic particles for intracellular hyperthermia in order to overcome these disadvantages [20,21]. These magnetic particles generate heat under an alternating magnetic

field (AMF) due to hysteresis losses [22]. We have developed "magnetite cationic liposomes" (MCLs) for inducing intracellular hyperthermia [23,24]. MCLs have been developed to improve adsorption and accumulation in the tumor cells, and have shown a tenfold higher affinity for the tumor cells than neutrally charged magnetoliposomes [24] due to electrostatic interaction with the negatively charged cell membrane. The hyperthermic effect of MCLs against some malignant tumor cells has been demonstrated *in vivo* [25]. Our hyperthermia procedure kills tumor cells not only directly by heating but also by the induction of an immune response [26].

In the study described in the present paper, we examined the hyperthermic effect of MCLs on rat prostate cancer *in vivo*, and demonstrated that our hyperthermic protocol can induce an antitumor immune response against rat prostate cancer.

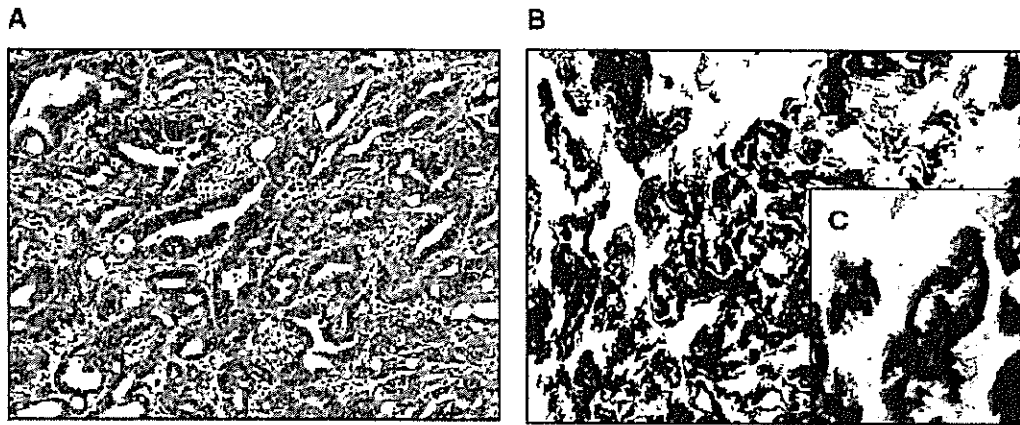
## MATERIALS AND METHODS

### Tumor Cell Type and Animal Model

Cells were obtained from the rat prostate cancer cell line PLS 10, which has been established at Department of Experimental Oncology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences [27] (Fig. 1A). This cell line has been established from 3,2'-dimethyl-4-aminobiphenyl plus testosterone-induced carcinomas in the dorsal prostate of male F344 rats. This cell line forms well-differentiated adenocarcinomas with abundant connective tissue stroma. PLS 10 cells are immunohistochemically negative for androgen receptors, and the growth of PLS 10 cells is androgen independent.

Four-week-old male F344 rats were purchased from Charles River Japan (Yokohama, Japan). To prepare tumor-bearing animals, cell suspensions including approximately  $1 \times 10^6$  PLS 10 in 100  $\mu$ l of phosphate buffer (0.05M sodium phosphate and 0.15M NaCl, pH 7.4) were injected subcutaneously into the right flank of F344 rat under short-term anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Prostate cancer nodules that had grown to a diameter of about 5–6 mm were used for the experiments. The tumor diameter was measured every 3 days. The tumor volume was determined as  $\text{Tumor volume} = 0.5 \times (\text{length} \times \text{width}^2)$ .

The experimental protocol in the present study was approved by the Animal Care Committee of Nagoya City University Medical School. Animal experiments were performed according to the principles laid down in the "Guide for the Care and Use of Laboratory Animals" prepared under the direction of the Office of the Prime Minister of Japan. In this experiment, prostate cancer derived from the prostate of F344 rats



**Fig. 1.** **A:** Histology of rat prostate cancer cell line PLS 10. The tumor is a well-differentiated adenocarcinoma. H-Estain,  $\times 200$ . **B:** Histology of a PLS 10 tumor injected with magnetic cationic liposomes (MCLs). Prostate glandular cells were stained by ferrous stain because MCLs were present in the prostate glandular cell but not on stromal tissue. Ferrous stain,  $\times 200$ . **C:** Same as B, except  $\times 400$ . [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

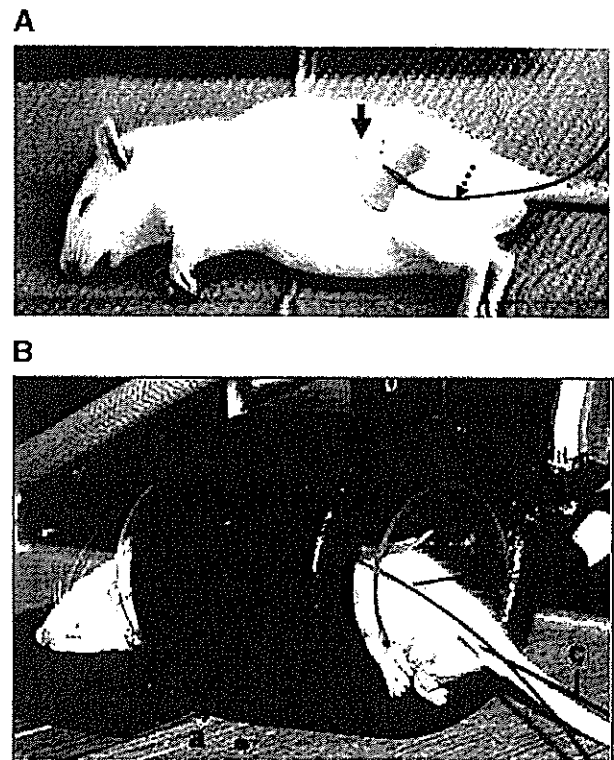
are transplanted into syngeneic rats, which differs considerably from the experiment in which human tumor tissue is transplanted into nude mice. This experiment therefore represents an excellent model for evaluating host immune responses to tumor tissue.

#### Preparation of MCLs

The magnetic particles were kindly donated by Toda Kogyo (Hiroshima, Japan), and had an average diameter of 10 nm. MCLs were prepared by sonication as described previously with some modifications [23]. One milliliter of the colloidal magnetite (net 20 mg of magnetite) was coated with a lipid membrane comprising *N*-( $\alpha$ -trimethylammonioacetyl) didodecyl-D-glutamate chloride (Sogo Pharmaceutical, Tokyo) and dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine (Sigma Chemical, St. Louis, MO) at a molar ratio of 1:2:2, respectively. The magnetite concentration was measured by the potassium thiocyanate method [28].

#### Injection of MCLs and Heat Generation in an AMF

After the prostate cancer nodules had grown to 5–6 mm in diameter, a 26-G syringe needle containing MCLs was inserted longitudinally into each prostate cancer nodule subcutaneously from the nodule edge (Fig. 2A). Two grams of MCLs was injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL) for 30 min (Fig. 1B). The rats were then separated into two groups: Group I mice were not exposed to an AMF (control group), whereas after injection of the MCLs, Group II rats were subjected once to hyperthermia for 30 min at 45°C. The AMF was created using a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05;



**Fig. 2.** **A:** Appearance of injection of MCLs with a 26-G syringe needle longitudinally into each prostate cancer nodule subcutaneously from the nodule edge. Solid arrow needle containing MCLs. **B:** Appearance of MCL-induced hyperthermia during exposure to an alternating magnetic field (AMF). The rat was placed inside the horizontal coil (a) such that the nodule was positioned at the center of the coil. Optical fiber probes were used to monitor the temperatures at the center of the tumor (b) and inside rectum (c).

Dai-ichi High Frequency, Tokyo) operating at 118 kHz. The magnetic field intensity was 30.6 kA/m (384 Oe). The rat was placed inside the coil such that the nodule was positioned at the center of the coil (Fig. 2B). Temperatures at the center of the tumor and inside the rectum (representative of the rat body temperature) during AMF were measured by optical fiber probes (FX-9020; Anritsu Meter, Tokyo). The temperature at the center of the tumor was maintained at around 45°C by controlling the magnetic field intensity.

#### Preparation of Specimens for Immunohistochemical Staining

On the 30th day after the MCL injection, tumors of the two groups were removed and specimens for immunohistochemical staining were prepared as follows. Blood was flushed out with phosphate buffer, then the tumor tissues were extracted, and fixed with Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo) at  $-20^{\circ}\text{C}$ . The frozen tumor tissues were sectioned at 10  $\mu\text{m}$ . Tissue sections were air dried for 30 min and fixed with cold acetone for 15 min. These sections were incubated with 5% normal goat serum and 1% skim milk at  $37^{\circ}\text{C}$  for 30 min to block background staining. They were then incubated at  $37^{\circ}\text{C}$  for 60 min with mouse antirat CD3 (1F4), antirat CD4 (W3/25), antirat CD8 (MRC OX-8) monoclonal antibodies (Serotec, Oxford, UK), and antirat HSP70 (W27) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200, and at  $37^{\circ}\text{C}$  for 60 min with goat antimouse monoclonal antibody-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA) at a dilution of 1:200. Each step was followed by washing with the phosphate buffer. Peroxidase activity was visualized by treatment at room temperature for 10 min with 0.02% diaminobenzidine tetrahydrochloride solution containing 0.005% hydrogen peroxidase. All sections were also stained with hematoxylin.

#### In Vitro Cytotoxicity Assay

Spleen cells were derived from the Group II rats at 1 month after the hyperthermic treatment using the Medimachine System (DAKO, Glostrup, Denmark). Naive rats that had been born at a similar time to the Group II rats were used as the controls. The spleen cells were cultured for 48 hr in RPMI 1640 supplemented with 10% fetal bovine serum and 2-mercaptoethanol ( $5 \times 10^{-5}\text{M}$ ). Mouse recombinant interleukin-2 was added once at a concentration of 5 U/ml. The cytotoxicity of spleen cells to prostate cancer cells was determined by a long-term cytotoxicity assay [29]. Rat prostate cancer cells (PLS 10,  $1 \times 10^5$  cells) were cultured in a 24-well flat plate containing DMEM.

After 24 hr, the medium was replaced with RPMI 1640. Spleen cells were added to each well. The wells were washed with phosphate buffer after 48 hr of incubation at  $37^{\circ}\text{C}$  in an atmosphere containing 5%  $\text{CO}_2$ . After incubation, 0.3 ml of RPMI 1640 and 0.03 ml of cell-counting solution (kit-8; Dojindo, Kumamoto, Japan) were added to the wells and the cells were incubated for 1 hr at  $37^{\circ}\text{C}$  in an atmosphere containing 5%  $\text{CO}_2$ . The absorbance of each well was measured at 405 nm with a spectrophotometer (V-530; Jasco, Tokyo). Cytotoxicity was calculated using the following equation: cytotoxicity (%) =  $[1 - (\text{absorbance of target cells treated with effector cells}) / (\text{absorbance of target cells only})] \times 100$ .

#### Statistical Analysis

Levels of statistical significance in the tumor-growth experiments were evaluated using the Mann-Whitney rank sum test.

## RESULTS

#### Heat Generation by MCLs in AMF

Figure 3 shows the temperature increases at the center of the tumor and inside the rectum of the rats during application of an AMF for 30 min. The temperature at the center of the tumor reached  $45^{\circ}\text{C}$  within 5 min, and was maintained at this temperature by controlling the magnetic field intensity. In contrast, the temperature inside the rectum remained below  $38^{\circ}\text{C}$ . It is noticeable that the temperature at the center of the tumor was maintained very accurately (with a small standard deviation; also for the rectal temperatures),

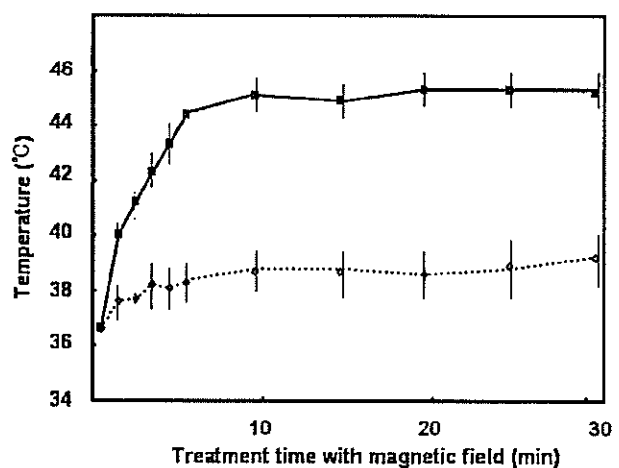


Fig. 3. Temperature increases at the center of the tumor (solid line), and after 10 min is maintained at around  $45^{\circ}\text{C}$ . Temperature inside the rectum (dotted line) increased a little but remained at around  $38^{\circ}\text{C}$ . Values shown are the mean  $\pm$  SD of five independent experiments.