Lack of effect of human *c-Ha-ras* proto-oncogene overexpression on prostate carcinogenesis in *probasin/SV40 T antigen* transgenic rats

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We have previously reported that transgenic (Tg) rats bearing the SV40 T antigen under probasin promoter control (PB/SV40T) develop prostate carcinomas at 100% incidence, showing their prostate carcinoma growth to be completely androgen-dependent. Transgenic rats carrying three copies of the human *c-Ha-ras* proto-oncogene (Hras128) are also highly susceptible to carcinogen induction of multiple mammary carcinomas, in this case estrogen-independent, since ovariectomy does not affect mammary tumor formation. A relationship between ras/mitogen-activated protein kinase signaling and androgen responsiveness of prostate cancer cells has been reported. Therefore it is of interest to investigate whether expression of human *c-Ha-ras* affects the androgen-dependence of prostate carcinomas developing in the PB/SV40T Tg rat. For this purpose, we established double transgenic (rasTag) rats bearing both PB/SV40T and Hras128. In prostate tissues of the rasTag rats, expression of both human *c-Ha-ras* and *SV40T* was confirmed, but the prostate tumor incidence and growth were not significantly affected. Castration at 15 weeks of age induced complete tumor involution in the rasTag rats. These results indicate that the human *c-Ha-ras* proto-oncogene product does not influence the androgen-dependence of prostate carcinogenesis due to the probasin-mediated SV40 T antigen, despite the estrogen-independence of mammary carcinogenesis in Hras128 rats. (Cancer Sci 2003; 94: 1042–1045)

Prostate cancer is one of the most common forms of neoplasia in many parts of the developed world.[1,2] Recently, its prevalence has also been increasing in Japanese men.[3,4] Most prostate cancers initially respond to androgen ablation therapy because their growth is hormone-dependent, but within 1–2 years, androgen-independent growth ability generally appears.[5,6] Recently, we have established transgenic (Tg) rats, PB/SV40T, bearing a probasin promoter/simian virus 40 T antigen (SV40 Tag),[7–9] which develops non-invasive prostate adenocarcinomas in all lobes at 15 weeks of age. These are completely androgen-dependent and castration at 5 or 20 weeks of age results in atrophic glands without tumors at the age of 25 weeks.[7]

Ras has been implicated in control of cell proliferation, differentiation and apoptosis, and *ras* gene mutations can be found in a variety of tumor types, although the incidence varies greatly. The highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%); in thyroid tumors (50%); and in myeloid leukemia (30%).[10] However, the incidence of *Ras* mutations appears to be low in prostate carcinomas,[11–13] although immunohistochemical studies have demonstrated strong epithelial staining for Ras oncoprotein in a majority of cases, with a significant difference in the expression level from that in normal epithelial cells.[14,15] These observations suggest that functional activation of wild-type Ras might contribute to the prostate tumorigenesis, and it was recently reported that ras signaling might play important roles in determining androgen responsiveness or sensitivity of prostate cancer cells.[16,17]

Mammary tumors are also known to be hormone-dependent. This characteristic has been utilized as the basis for therapeutic strategies aimed at reducing levels of circulating estrogen by either ovariectomy or treatment with anti-estrogenic drugs. Transgenic rats carrying three copies of the human *c-Ha-ras* proto-oncogene (Hras128) without any mutations are highly susceptible to *N*-methyl-*N*-nitrosourea (MNU) mammary carcinogenesis,[18] MNU administration causing rapid development of multiple, large mammary carcinomas in 100% of Hras128 Tg rats. In contrast, wild-type littermates had no or only small tumors.[18] In addition, while ovariectomy completely inhibited the development of mammary carcinomas in wild-type animals, it did not affect either the incidence or the multiplicity of mammary carcinomas in the Hras128 Tg rats.[19] The data thus indicate that overexpression of human *c-Ha-ras* proto-oncogene may endow mammary tumors with hormone-independence. Therefore, it is of interest to determine the effects of overexpression of *c-Ha-ras* on prostate carcinogenesis, especially with regard to androgen-dependent lesions. To study the effect of ras on the prostate carcinogenesis, we here generated double transgenic rats by interbreeding the Hras128 Tg and PB/SV40T Tg strains.

Materials and Methods

Animals. The rasTag rats were generated by crossing PB/SV40T Tg rats[7] on an SD background with Hras128 Tg rats. Male Hras128 Tg rats maintained in Clea Japan, Inc. (Tokyo) were mated with heterozygous PB/SV40T Tg female rats.[18] Pups were randomly divided into groups of three animals per plastic cage with hard wood chips as bedding in an air-conditioned room at 22±2°C and 55±5% humidity with a 12 h light/dark cycle. Food (Oriental MF, Oriental Yeast Co., Tokyo) and tap water were available *ad libitum*.

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University Medical School.

Screening for transgenic rats. DNA samples were obtained from rat tails by the proteinase K/phenol/chloroform method. PCR was performed using *Taq* DNA polymerase and provided buffers (TaKaRa, Otsu). Primers used for the PB/SV40T transgene were 5′-AGCCCTGTCCTCCTGCAGGAT-3′ and 5′-

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The abbreviations used are: PB/SV40T, probasin promoter/simian virus 40 T antigen; Hras128, three copies of the non-mutated human c-Ha-ras proto-oncogene; rasTag, double transgenic bearing both PB/SV40T and Hras128; Tg, Transgenic; AR, androgen receptor.

GGCCAGCCTCACGGGGTTCA-3′; for the human *c-Ha-ras* (transgene) they were 5′-TGTGGCCTGAAGCGGTCT-3′ and 5′-ACCAGCTGCCAACCTCGTCC-3′. The annealing temperature in both cases was 62°C.

Experimentation. Fifteen rats were divided into 2 groups for each strain (rasTag, PB/SV40T Tg, Hras128 Tg, Wild). In group 1, six rats were maintained until 20 weeks old, when they were killed. In group 2, nine rats were castrated at 15 weeks of age and kept until 20 weeks old, when they were killed.

Preparation and analysis of tissues. One-half of each prostate collected at necropsy was routinely fixed in 10% phosphate-buffered formalin for 48 h and then processed for embedding in paraffin. Five-micrometer-thick sections were cut and stained with H&E. Immunohistochemical analyses of SV40 Tag, and androgen receptor (AR) were performed using mouse anti-simian virus 40 large T antigen monoclonal antibody (PharMingen, San Diego, CA), and rabbit polyclonal anti-androgen receptor antibody (Affinity Bioreagents, Golden, CO). Binding was visualized with a Vectastain Elite ABC kit (Vector Lab, Burlingame, CA) and light hematoxylin counterstaining was conducted to facilitate microscopic examination. Photographs were taken with a digital camera (DP11, Olympus, Tokyo) and printed out using a digital printer (Pictrography 3000, Fujifilm, Tokyo). The other half of each prostate was frozen in liquid nitrogen for extraction of RNA.

Extraction of total RNA and quantitative RT-PCR. Extraction of total RNA from ventral lobes of prostate was performed according to an ISOGEN protocol (Nippon Gene, Toyama) with DNase treatment using DNase I and the provided buffer (CLONTECH). One microgram of the RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (TaKaRa) in 20 μl of reaction mixture. Aliquots of 2 μl of

Table 1. Means of prostate weights and histpathological findings of neoplastic lesion

Treatment	Strain	No. of rats	Body (g)	Prostate (g)		No. of rats with adenocarcinomas (%)		
				Whole	Ventral	Ventral	Dorso-lateral	Anterior
Castration	rasTag	9	477.67±37.85	0.446±0.043	0.056±0.005	0 (0)[3)]	0 (0)	0 (0)
	PB/SV40T	9	475.38±48.04	0.426±0.049	0.053±0.010	0 (0)	0 (0)	0 (0)
	Hras128	9	492.26±31.65	0.421±0.061	0.069±0.015	0 (0)	0 (0)	0 (0)
	Wild	9	471.88±20.63	0.394±0.039	0.061±0.009	0 (0)	0 (0)	0 (0)
Non Treatment	rasTag	6	529.32±29.61	3.907±0.664[2)]	0.415±0.100[1)]	6 (100)	6 (100)	6 (100)
	PB/SV40T	6	494.80±64.38	4.343±0.775[1)]	0.383±0.048[1)]	6 (100)	6 (100)	6 (100)
	Hras128	6	462.26±43.59	3.588±0.737	0.740±0.124[1)]	0 (0)	0 (0)	0 (0)
	Wild	6	520.95±46.61	3.082±0.313	0.575±0.044	0 (0)	0 (0)	0 (0)

Values are means±SD.
P values were examined by Bonferroni/Dunn's posthoc test.
1) $P<0.0001$ compared with Wild.
2) $P<0.001$ compared with Wild.
3) Percentages of tumor incidence in parentheses.

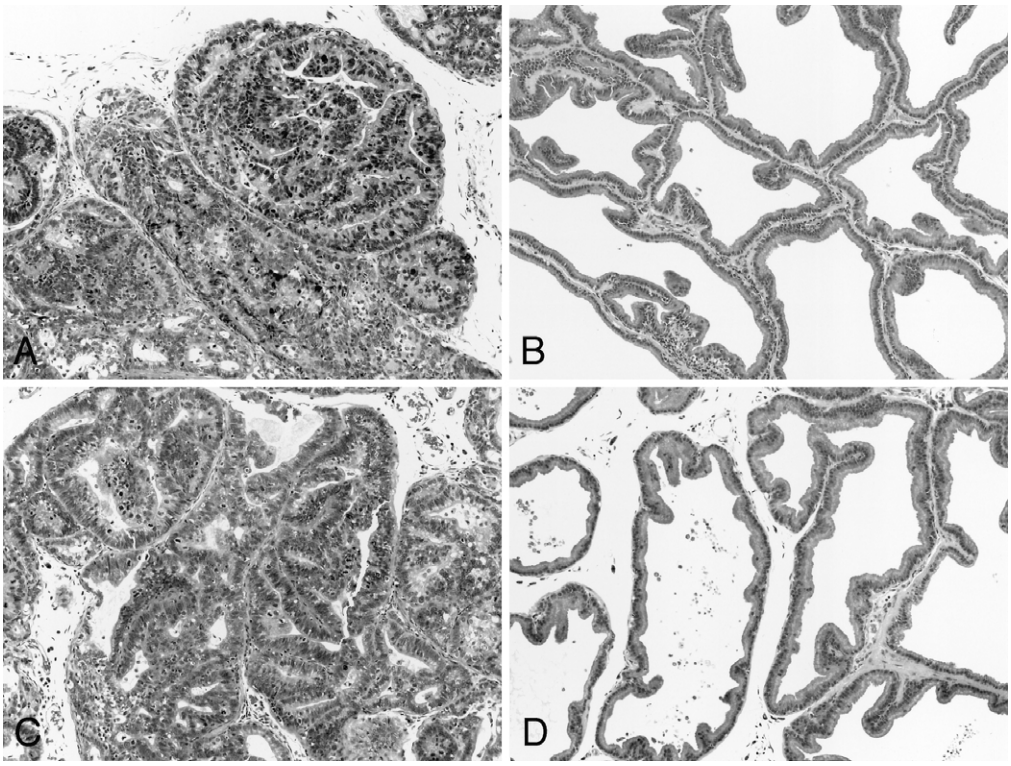

Fig. 1. Histology of ventral prostates of double transgenic (rasTag) (A), Hras128 (B), PB/SV40T (C), and wild-type (D) rats. A and C, Adenocarcinomas. B and D, Normal ventral prostate glands. There are no apparent histopathological differences between A and C, or B and D.

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 used for *SV40T* were 5′-GTCAGCAGTAGCCTCATCAT-3′ and 5′-GGTTGATTGCTACTGCTTCG-3′; for *AR*, 5′-GACTATTACTTCCCACCCCAG-3′ and 5′-ACATTTCCGGAGACGACACGA-3′; for human *c-Ha-ras*, 5′-CGCCCAGCACAAGCTC-3′ and 5′-CGGTGGCATTTGGGATGTTC-3′; and for rat *c-Ha-ras*, 5′-AGGAGGTGCTGTCGGAAGG-3′ and 5′-GTGGCAACGGGATAGTTCAT-3′. Initial denaturation at 95°C for 10 min was followed by 30 to 35 cycles with denaturation at 95°C for 15 s, annealing at 55°C (except at 45°C for *SV40T*, at 52°C for *AR*,and at 59°C for human *c-Ha-ras*) 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.

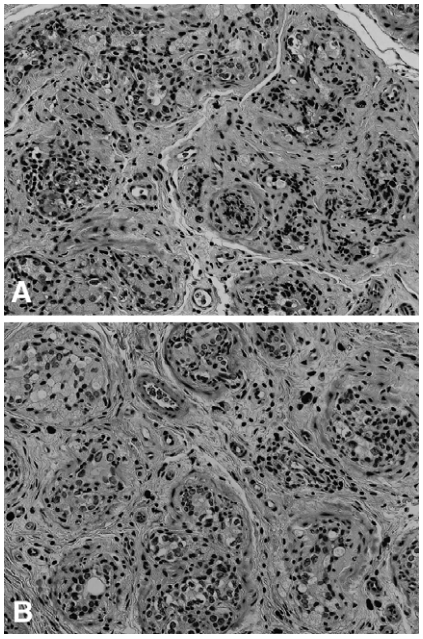

Fig. 2. Ventral prostates from castrated rasTag(A) and PB/SV40T(B) rats. Atrophic glands and fibrosis are evident in both cases, with no appreciable difference.

Results

Prostate adenocarcinomas in the ventral, dorsolateral, and anterior lobes were observed at 100% incidence in PB/SV40T Tg and rasTag rats at 20 weeks of age. There were no obvious differences between PB/SV40T Tg and rasTag rats in the prostate weights, and incidences (Table 1), macroscopic appearance and microscopic morphology of the prostate carcinomas (Fig. 1). Their prostate weights were increased as compared to wild-type values. While Hras128 Tg rats had histologically normal glands in all lobes, prostate weights were increased because of elevated secretion.

There was no difference in expression of AR detected immunohistochemically in nuclei at prostate epithelial cells among the strains. SV40 Tag expression also did not differ immunohistochemically in the 2 strains (PB/SV40T Tg and rasTag) carrying the SV40T transgene.

Castration at 15 weeks of age completely suppressed tumor development and expression of AR and SV40 Tag in the rasTag rats, similar to the PB/SV40T Tg rats as reported previously.[7] They had only atrophic glands without atypia surrounded by abundant fibrous connective tissue (Fig. 2). In Hras128 Tg rats, castration caused atrophy of prostate glands, as in wild-type rats.

Using material from non-castrated animals, mRNA expression of the transgenes, endogenous rat Hras and AR was investigated by quantitative RT-PCR. We confirmed that the transgenes were specifically expressed in the transgenic strains. In rasTag, both human *c-Ha-ras* and *SV40T* were expressed. The expression levels of *SV40T* and human *c-Ha-ras* in rasTag rats were lower and higher, respectively, than those in each single transgenic strain. The rat endogenous *c-Ha-ras* and *AR* were up-regulated in the prostate tumors in both PB/SV40T Tg and rasTag rats (Fig. 3).

Discussion

Prostate cancer can be treated effectively by androgen ablation therapy in the early stages, but in many cases recurrence of the tumor occurs after the therapy.[5, 6] Clinical androgen ablation therapy reduces, but does not eliminate, androgen from the circulation in men and residual steroids may create conditions favorable for selection of cancer cells having the ability to proliferate with reduced levels of androgen.[20] Mechanisms that allow growth at castrate levels of androgen are not fully understood, but overexpression or mutation of the androgen

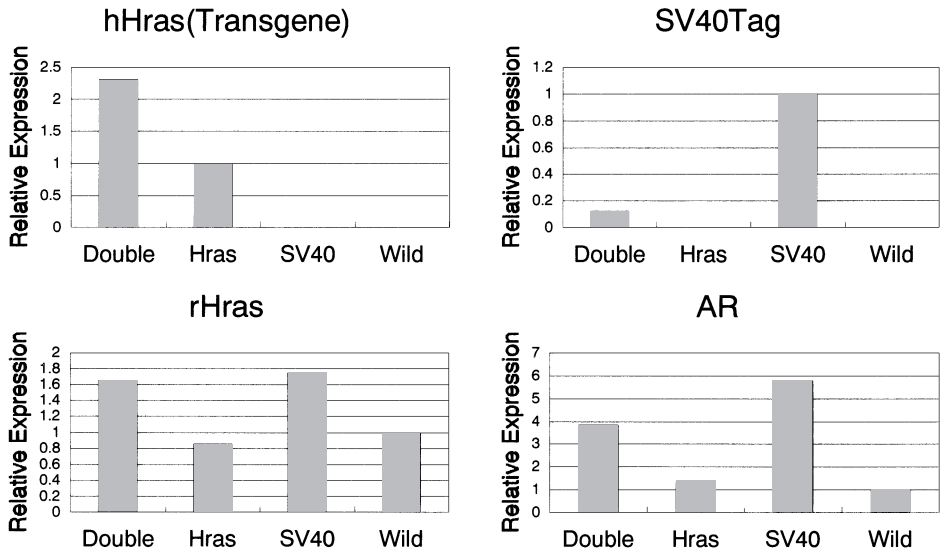

Fig. 3. Relative expression levels of rat c-Ha-ras (rHras), human c-Ha-ras (hHras), androgen-receptor (AR) and SV40T antigen (SV40Tag) in rasTag (Double), Hras128 (Hras), PB/SV40T (SV40) and wild-type (Wild) rats, as revealed by quantitative RT-PCR.

receptor,[21, 22] overexpression of transcriptional coregulators,[23, 24] and activation of Ras signaling[16, 17, 25] may be involved. It is known that stable expression of Ras effector-loop mutants that activate the Ras/MAP kinase pathway is sufficient to reduce the androgen requirement of LNCaP prostate cancer cells for growth.[17] Attenuation of Ras signaling by a dominant negative mutant of Ras was also found to restore androgen sensitivity to hormone-refractory prostate cancer cells.[16]

In the present study, we demonstrated that expression of the human *c-Ha-ras* proto-oncogene does not appreciably affect prostate carcinogenesis in PB/SV40T Tg rats. Furthermore, castration caused complete involution of prostate tumors in rasTag rats, indicating that *c-Ha-ras* overexpression did not change their androgen-hormone dependence or sensitivity. A number of explanations are possible. After castration in rats, despite loss of prostate tissue, serum androgen derived from adrenal glands can be detected and the serum levels of the active form of testosterone, 5 α-dihydrotestosterone, decrease to only 50% of the intact value within 12 h postcastration and remain at more than 50% of the intact control level even long-term (for up to 20 weeks) following castration.[26] Furthermore, castration plus adrenalectomy produces an almost complete androgen disappearance, but does not induce any further reduction in prostate weight.[26] We also examined the effects of a 5 α-reductase inhibitor, Finasteride, on the progression of prostate lesions in PB/SV40T Tg rats. Finasteride (10 mg/kg/day, 5 days a week) was administered to 10-week-old Tg rats orally for up to 7 weeks. Although there were no significant changes in serum testosterone concentration, relative prostate weight was obviously reduced by Finasteride administration, and the development of prostate adenocarcinomas was significantly suppressed (unpublished data). These results indicate that quite a high concentration of DHT is required to maintain prostate cell growth in rats. Therefore, overexpression of *c-Ha-ras* may not be sufficient to endow the cells with any capacity for growth under castrated conditions. Another possible explanation is that expression of *c-Ha-ras* in the rat prostate does not activate the Ras/Map kinase signaling pathway, in contrast to the case in mammary tissue in transgenic rats overexpressing *c-Ha-ras* (Hras128),[18] whose mammary tumors are estrogen-independent.[19]

In male PB/SV40T Tg rats, mammary gland tumors which are androgen-receptor- and SV40 Tag-positive develop at low frequency (unpublished data). Therefore, in a preliminary study, we investigated the susceptibility of female double transgenic rats to MNU-induced mammary carcinogenesis. However, the incidence of lesions did not differ significantly from that in Hras128 Tg rats, and the tumors did not express the SV40 Tag protein as judged from immunohistochemical staining (unpublished findings). This may be the reason why the susceptibility of rasTag female rats is the same as that of Hras128 Tg rats. Mutations in the exogenous human Hras codon 12 were detected in almost all tumors of rasTag, but the involved populations appeared small, in line with earlier reports.[18]

In summary, in the present study, human *c-Ha-ras* proto-oncogene overexpression did not affect PB/SV40T Tg prostate carcinogenesis or its androgen-dependence.

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