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# Mammary Carcinomas Induced in Human c-Ha-*ras* Proto-oncogene Transgenic Rats Are Estrogen-independent, but Responsive to *d*-Limonene Treatment

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**We have previously shown that transgenic rats carrying three copies of the human c-Ha-*ras* proto-oncogene (Hras128) are highly susceptible to *N*-methyl-*N*-nitrosourea (MNU) mammary carcinogenesis. All transgenic rats treated with 50 mg/kg MNU, i.v. at 50 days of age, were found to rapidly develop multiple, large mammary carcinomas within as short a period as 8 weeks. In the present study, the effects of ovariectomy and treatment with *d*-limonene, known to inhibit mammary carcinogenesis in non-transgenic female rats, were investigated in Hras128 animals treated with MNU to clarify the role of the human c-Ha-*ras* proto-oncogene and to characterize the induced mammary carcinomas. Although ovariectomy completely inhibited development of mammary carcinomas in their wild-type counterparts, it did not affect either the incidence or the multiplicity of the mammary carcinomas in the Hras128 rats. On the other hand, treatment with *d*-limonene, an inhibitor of ras protein isoprenylation, inhibited the breast tumor development. These results indicate that aberrant c-Ha-*ras* gene expression is involved in ovarian hormone-independent growth and c-Ha-ras protein isoprenylation plays an important role in mammary carcinogenesis.**

Key words: c-Ha-*ras* — Transgenic rats — Mammary carcinoma — Ovariectomy — *d*-Limonene

Growth of human breast cancers is known to be dependent on estrogen until a certain stage of progression. This property 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 such as tamoxifen. However, breast cancers frequently progress to a more aggressive estrogen-independent phenotype, non-responsive to anti-estrogen therapy. Therefore, understanding of factors that contribute to estrogen-independence of mammary tumor cells is important.

In order to investigate mammary carcinogenesis, rats are widely used because of their susceptibility to induction of mammary tumors by various chemical carcinogens without involvement of viral factors.[1)] The majority of rat mammary tumors are hormone-responsive, and ovariectomy (Ovx), hypophysectomy, and anti-estrogenic treatment have been shown to cause tumor regression.[2–5)] In *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary tumor models, up to 90% of mammary carcinomas have been reported to have mutations, G35 to A35 transition, in codon 12 of their c-Ha-*ras* gene.[6, 7)] Activation of the c-Ha-*ras* oncogene in rat mammary carcinomas does not necessarily imply acquisition of hormone-independent growth,[3)] but transfection of v-H-*ras* oncogene into MCF-7 human breast cancer cells under the transcriptional control of three tandem long terminal repeats of Ha-*MuSV*[8)] and direct insertion of an activated *ras* gene into rat mammary ductal tissue cells *in situ* with retroviral vector were shown to result in estrogen-independence.[9)] It is of interest to know whether mammary carcinogenesis of Hras128 rats is dependent on ovarian hormone.

Monoterpenes, including *d*-limonene, are known to inhibit protein isoprenylation of small G proteins, including p21 ras.[10)] Treatment with *d*-limonene has been found to inhibit development and to cause regression of chemically induced rat mammary tumors, many containing mutated *ras* oncogenes.[11–14)] One possible mechanism to explain the observed effects is inhibition of isoprenylation of the small G protein p21 ras.

Recently, we established rat lines carrying the human c-Ha-*ras* proto-oncogene with its own promoter region. One of the transgenic rats, designated as Hras128, was shown to be highly susceptible to MNU and 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis, multiple, large mammary carcinomas developing within as short a period as 8 to 16 weeks.[15, 16)] The transgenic mice which harbor the same transgene are known to be very susceptible to MNU treatment, but the treated mice developed forestomach papillomas, not mammary carcinomas.[17)]

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In the present study, in order to assess factors involved in mammary tumor growth in Hras128 rats, we investigated the effects of Ovx and *d*-limonene treatment in our model.

## MATERIALS AND METHODS

**Transgenic rat** Female Hras128 rats were raised from mating between male transgenic and non-transgenic female Sprague-Dawley animals (Clea Japan Inc., Tokyo). Heterozygotes for the transgene were identified by PCR using DNA samples from their tails as described previously.[15] Rats were housed in plastic cages on hard-wood chips, in an air-conditioned room at 22±2°C and 50% humidity with a 12h/12h light-dark cycle. They were given commercial pellets (Oriental MF; Oriental Yeast, Tokyo) and tap water *ad libitum*.

**Effects of ovariectomy** A total of 15 transgenic and 21 littermate non-transgenic female rats were injected with 50 mg/kg body weight MNU (Sigma-Aldrich Japan, Tokyo) into the tail vein at 50 days of age. Two days after the MNU injection, 7 transgenic and 11 non-transgenic rats received bilateral Ovx, performed as described previously.[2] Eight weeks after the MNU injection, all rats were killed and whole skin with mammary tissue was removed for the precise examination of all visible mammary lesions. Number of tumors per rat and the diameter of each tumor were recorded.

**Effects of *d*-limonene administration** Seventeen transgenic female rats were treated with MNU as in the Ovx study. Two days after MNU injection, 9 of them were fed on 5% *d*-limonene (Sigma-Aldrich Japan, >99% pure)-supplemented basal diet (Oriental MF, Oriental Yeast) until the end of the experiment. The remaining 8 transgenic rats were maintained on the basal diet without supplement. Diets were prepared every week. All rats were killed 8 weeks after the MNU injection for gross and histological examination of mammary tumors. Tumor volume was calculated as length × width × height/2. The effects of *d*-limonene on mammary carcinogenesis in wild-type rats have been already established,[11–14] so wild-type rats were not used for this experiment.

## RESULTS

All the transgenic rats treated with MNU developed multiple mammary adenocarcinomas histologically. Ovx did not exert significant effects on tumor incidence, multiplicity (number/rat) or diameter (Table I). On the other hand, in non-transgenic rats, Ovx completely inhibited tumor development (Table I). Representative histology of mammary tissues and tumors is shown in Fig. 1.

Treatment with *d*-limonene tended to reduce tumor multiplicity and significantly decreased tumor size and volume as compared to the control rats (Table II). No toxic effects of the *d*-limonene treatment were observed, but a slight decrease of body weight gain was seen (*d*-limonene, 242.3 g vs. control, 269.7 g).

## DISCUSSION

In the present study, growth of mammary adenocarcinomas induced by MNU injection in c-Ha-*ras* proto-oncogene transgenic rat was shown to be independent of early ovariectomy after 2 days of MNU injection, unlike the case with wild-type rats, although it should be confirmed that established tumors in Hras128 rats are not regressed by ovariectomy. It has been shown that transfection of the v-Ha-*ras* oncogene under the control of three tandem long terminal repeats of Ha-*MuSV* into the MCF-7 human breast cancer cell line results in acquisition of estrogen-independent tumor growth.[8] In contrast, mutations of the c-Ha-*ras* gene in rat mammary carcinomas induced by MNU or 7,12-dimethylbenz(*a*)anthracene were not associated with altered hormone-dependence.[3] These results may indicate that loss of regulation of the expression of the Ha-*ras* gene from the virus long terminal repeat may be responsible for the hormone-independent condition, while chemically induced tumors are regulated by their own c-Ha-*ras* promoter. Expression of the human c-Ha-*ras* proto-oncogene is regulated by the transduced human promoter. Although its structure is similar to that in rats, the two are not identical.[18, 19] Recently, an ovarian hormone-responsive transcriptional element for c-Ha-*ras* gene expression was identified in intron 1 of the human gene.[20]

Table I. Effects of Ovariectomy on Mammary Carcinogenesis in Hras128 Rats Induced by MNU

| Strain and treatment | No. of rats | No. of tumor-bearing rats | No. of tumors/rat | Tumor diameter (mm) |
|---|---|---|---|---|
| Tg+Ovx | 7 | 7 (100) | 12.4±3.21 | 15.1±23.6 |
| Tg | 8 | 8 (100) | 18.1±9.51 | 14.8±9.07 |
| Non-Tg+Ovx | 10 | 0 (0)[a)] | 0[b)] | — |
| Non-Tg | 11 | 5 (45.5) | 0.91±1.14 | 7.78±3.53 |

*a*) $\chi^2$ test at $P<0.05$.
*b*) Mann-Whitney *U* test at $P<0.05$.
Tg, Hras128; Ovx, bilateral ovariectomy; Non-Tg, non-transgenic.

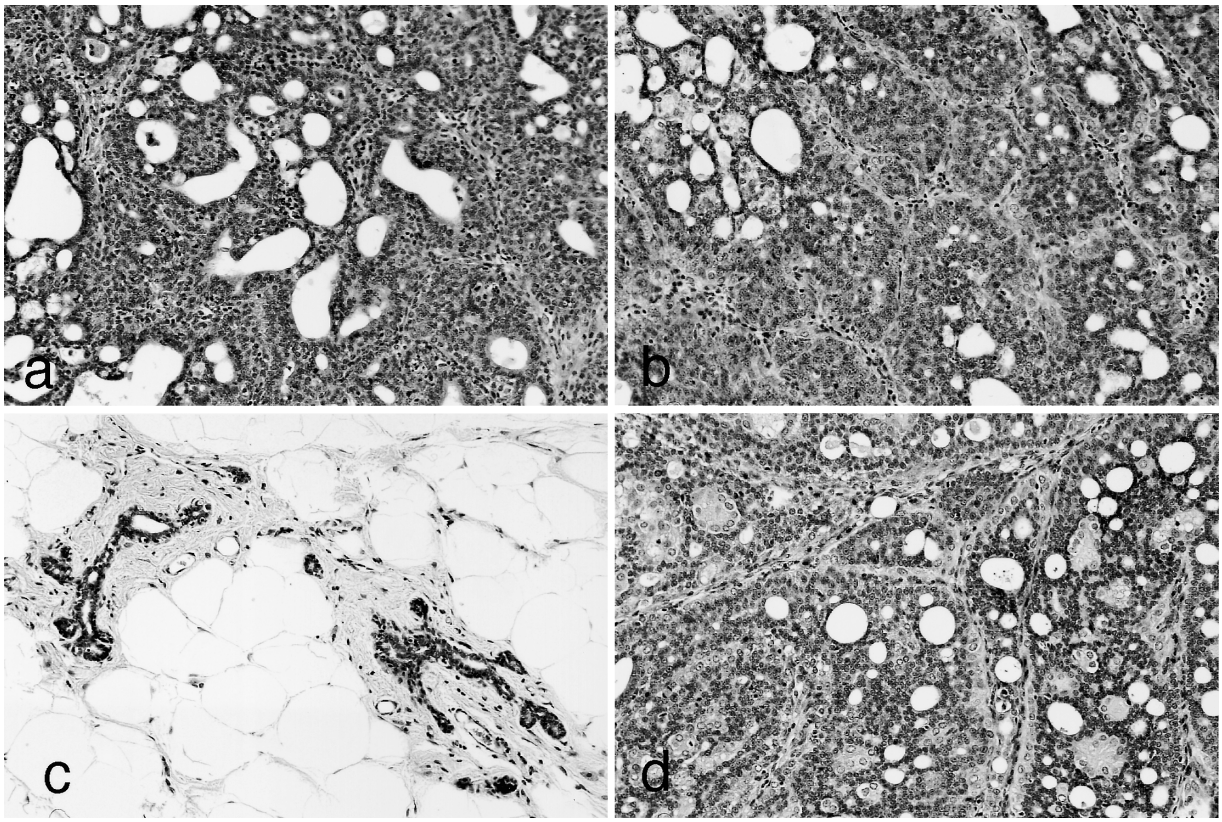


Fig. 1. Representative histological appearance of mammary adenocarcinomas induced by MNU treatment in non-transgenic rats (a), Hras128 rats (b), and ovariectomized Hras128 rats (d). In non-transgenic rats that received MNU injection and ovariectomy, only normal mammary glands were observed (c). Hematoxylin and eosin staining, ×100.

Table II. Effects of *d*-Limonene on Mammary Carcinogenesis in Hras128 Rats Treated with MNU

| Treatment | No. of rats | No. of tumors/rat | Size of tumors | |
|---|---|---|---|---|
| | | | Diameter (mm) | Volume ($mm^3$) |
| 5% *d*-limonene | 9 | 19.4±6.71 | 8.23±4.88[a)] | 269.5±735.5[b)] |
| Basal diet | 8 | 26.4±8.52 | 10.7±7.34 | 636.7±1669.3 |

Student's *t* test at $P<0.002$ (*a*) and $P<0.008$ (*b*).

Whether this operates in rats remains to be determined. Unregulated expression of the transgene may be responsible for the hormone-independency of the mammary carcinomas induced in the transgenic rats.

Post-translational modification involving cellular ras family protein isoprenylation is important for the function and subcellular localization of regulatory proteins.[21] In our study *d*-limonene, an inhibitor of isoprenylation of small G proteins including p21 ras,[10] inhibited mammary carcinogenesis in the c-Ha-*ras* transgenic rats, indicating participation of p21 ras in the tumor development, although we have no direct evidence that the *d*-limonene inhibited isoprenylation of the ras protein. Treatment with 5% *d*-limonene showed no apparent toxic effects, indicating that nutritional imbalance due to the treatment was unlikely.

Since our transgenic rat develops multiple mammary carcinomas within 8 weeks after carcinogen application,[15] this model may be useful for screening and evaluation of chemopreventive or therapeutic agents such as *d*-limonene, but not for the study of agents with anti-estrogenic actions, because of the estrogen-independent growth characteristics.

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