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Rare Activation of the Human c-Ha-*ras* Transgene of Mice in Hemangioendothelial Sarcomas and Liver Tumors Induced by Glu-P-1

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A transgenic mouse (Tg), having the human c-Ha-*ras* proto-oncogene, has been demonstrated to develop hemangioendothelial sarcomas (HESs) which are associated with the transgene mutation, but not to develop liver tumors. In this study, we examined the effects of 2-amino-6-methyldipyrido [1,2-*a*:3′,2′-*d*] imidazole (Glu-P-1), a food-borne carcinogen, which has been demonstrated to induce HESs and liver tumors in CDF_1 mice, on Tg mice. Chronic administration of 0.05% Glu-P-1 in the diet induced HESs in Tg (7/17), but not in 18 non-transgenic mice (N-Tg). With basal diet, two out of 17 Tg but none of 22 N-Tg, developed HESs. In contrast, Glu-P-1 administration induced liver tumors, both in Tg and in N-Tg; 16/17 in Tg and 13/18 in N-Tg. The incidence of hepatocellular carcinomas in Tg was higher than that in N-Tg (8/17 versus 3/18). With basal diet, only one out of 17 Tg and none of 22 N-Tg developed liver tumors. The Ha-*ras* mutation in tumors developed by the groups administered Glu-P-1, was examined. No mutations were detected in the transgene and mouse c-Ha-*ras* genes in all three HESs examined. In contrast, when 29 liver tumors taken from Tg were examined, two mutations of the transgene were detected in two HCCs. No mouse c-Ha-*ras* gene mutations were detected in any of the 47 liver tumors examined, which had developed in Tg and N-Tg mice. These results suggest that the transgene plays a role in the development of HESs induced by Glu-P-1, but not as a result of its mutation. Futher, the transgene plays no significant role in the development of liver tumors induced by Glu-P-1, but does play a role in the malignant conversion of some liver tumors, as a result of its mutation.

Key words: Ha-*ras* Tg mouse — c-Ha-*ras* mutation — Glu-P-1 — Liver tumor — Hemangioendothelial sarcoma

Transgenic mice (Tg) carrying the human c-Ha-*ras* proto-oncogene with its own promoter were generated.[1)] These mice, with a genetic background of C57BL/6 × Balb/c F_2 or its backcross to C57BL/6, spontaneously developed angiosarcomas [now diagnosed as hemangioendothelial sarcomas (HESs)], lung adenocarcinomas, skin papillomas, lymphoma and Harderian gland adenocarcinomas by the age of 540 days. Interestingly, no liver tumors developed spontaneously in Tg.[1)]

More than half of these spontaneous tumors had mutations in the transgene, but not in the intrinsic c-Ha-*ras* gene. Interestingly, all HESs had a mutation exclusively at codon 61 of the transgene.[1)] Thus, it was considered that the transgene is prone to mutation, although the mechanisms involved are not known. Further, it was demonstrated that Tg are very sensitive to some types of carcinogen, such as *N*-methyl-*N*-nitrosourea, and 7,12-dimethylbenz[*a*]anthracene. Tumors induced by treatment with these carcinogens had mutations in the transgene.[2,3)]

2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) is one of the heterocyclic amines produced by heating glutamic acid. Glu-P-1 in the diet at 0.05% induced HESs in brown fat tissue located between the scapulas, and liver tumors in CDF_1 mice by 685 days.[4)] The Ha-*ras* transgene in Tg contributes to keeping the Ha-*ras* expression level high and endows a proneness to Ha-*ras* mutation, but its role seems to be different in the development of HESs and liver tumors. Consequently we were curious about the effect of Glu-P-1 on Tg. We therefore examined its carcinogenicity in Tg. We further analyzed Ha-*ras* mutations in the tumors induced by Glu-P-1, in order to clarify the role of the transgene and the intrinsic Ha-*ras* gene mutation in carcinogenesis.

MATERIALS AND METHODS

Carcinogenicity test Tg carrying the human c-Ha-*ras* proto-oncogene under the control of its own promoter[1)] and non-transgenic mice (N-Tg) were provided by DNARD Inc. (Kawasaki). The genetic background of these mice was C57BL/6. Synthetic Glu-P-1 was pur-

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chased from Katsura Chemical Co. (Tokyo), and its purity was confirmed to be 99% by HPLC-A260 analysis.

Male mice at the age of seven weeks and female mice at eight weeks were housed in wire cages in an air-conditioned room at 25°C and 55% humidity. Administration of a diet (CE-2, CLEA Japan, Tokyo) containing 0.05% Glu-P-1 was started at the age of eight weeks for male mice and nine weeks for female mice. Each group consisted of 8–13 mice. Autopsies were performed when the mice died or became moribund, and the surviving animals were killed at the end of the experiment (Glu-P-1 groups, at age 43 weeks for males and 54 weeks for females; basal diet groups, at age 80 weeks for males and females).

Histological analysis All organs were carefully examined for the presence of tumors and then fixed in 10% neutralized formalin at 4°C, embedded in paraffin, processed, and stained with hematoxylin and eosin (HE). Routine histological analyses were performed on the brain, tongue, salivary glands, esophagus, stomach, small and large intestines, liver, pancreas, kidney, urinary bladder, testis, epididymis, seminal vesicle, ovary, uterus, lung, heart, spleen, skin, pituitary gland, thyroid and parathyroid glands, adrenal gland, Harderian gland and the interscapular brown adipose tissue. Organs which showed macroscopic abnormalities were included.

Statistical analysis The χ^2 test was used for the statistical analysis of differences in tumor incidences.

Tissue samples and preparation of genomic DNA Tumors and corresponding normal samples were removed. One half of a tumor sample was stored at −80°C and the other half was fixed with neutralized formalin for histological examination. DNA was extracted from the frozen samples by proteinase K treatment followed by the phenol/chloroform method.[5]

Southern blot hybridization Ten micro gram of DNA was digested with *Sac*I (Toyobo, Tokyo). Digests were electrophoresed in 0.9% agarose gel and capillary-transferred to a nylon filter membrane (Hybond N: Amersham, UK). The pSK2 plasmid,[6] which contained a single copy of the human c-Ha-*ras* gene, was labeled with a random hexamer and [α-^{32}P]dCTP using Multiprime (Amersham), and used as a probe in the hybridization solution (0.65 *M* NaCl, 0.1 *M* PIPES, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA and 50% formamide) at 42°C for 8 h. After having been washed in the wash solution (2× SSC and 0.1% SDS) four times at 50°C, the filter was exposed to XAR film (Kodak, New York, NY) at −80°C for 12 h.

The intensity of the band in Tg DNA was 1.5-fold higher than that of human DNA. Thus, we estimated that the transgene contains three copies of c-Ha-*ras* in one Tg diploid cell (Fig. 1). This was estimated to be five copies in the previous report.[1] At present it is not clear whether this difference is due to technical problems, or to the loss of the transgene during breeding of Tg animals.

Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis A total of 5 μl of

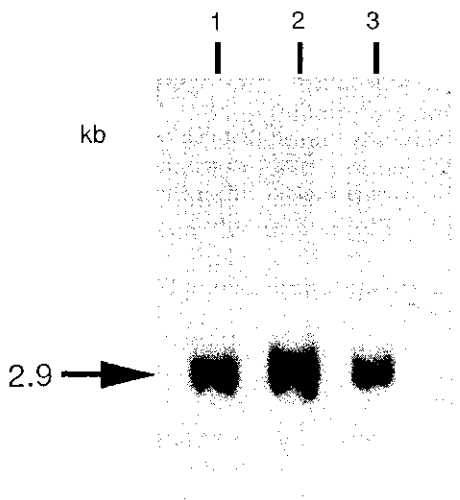

Fig. 1. Southern blot hybridization. Ten micro gram of human lymphocyte DNA (lane 1) or 10 μg (lane 2) or 5 μg (lane 3) of Tg mouse liver DNA was hybridized with pSK2 plasmid, which contains human c-Ha-*ras* gene. The intensity of the band (2.9 kb) from Tg mouse DNA was about 1.5-fold higher than that of human DNA from diploid cells.

Table I. Primer Sequences for PCR-SSCP Analysis

Region	Sense	Antisense
Human		
exon 1	5′-TGAGGAGCGATGACGGAATA-3′	5′-TCACCTCTATAGTGGGGTC-3′
exon 2	5′-AGCCCTGTCCTCCTGCAGGA-3′	5′-GTTCACCTGTACTGGTGGAT-3′
Mouse		
exon 1	5′-AGGAGCTCCTGGATTGGCAGCCGCT-3′	5′-CTCTATAGTGGGATCATACTCGTCC-3′
exon 2	5′-GGTGGTCATTGATGGGGAGA-3′	5′-CATGTACTGGTCCCGCATGG-3′

reaction mixture, containing 100 ng of genomic DNA, 0.4 pmol of each primer end-labeled with [γ-^{32}P]ATP (ICN Radiochemicals, Irvine, CA), 100 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin and 0.5 unit of recombinant Taq polymerase (Takara, Osaka), was used for PCR amplification. The primers were synthesized in a 329 RNA/DNA synthesizer (Applied Biosystems Japan, Tokyo). The mouse and human specific primers were designed to analyze separately mutations in exons 1 and 2 of the mouse and human Ha-*ras* genes (Table I). Conditions for PCR were; initial denaturation at 94°C for 3 min followed by 40 cycles of the reaction at 94°C for 0.5 min, and at 60°C for 2 min. Five μl of PCR products was diluted with 45 μl of SSCP-dilution buffer (0.1% SDS and 10 mM EDTA). Equal volumes of diluted products and formamide dye (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 20 mM EDTA) were mixed. After denaturation by heating at 95°C, one μl of the mixture was electrophoresed using 5% non-denaturing polyacrylamide gel, with or without 5% glycerol at 40W at 14°C. As positive controls, pKY1 and pSK2,[6,7] provided by the Japanese Cancer Research Resources Bank (JCRB), were used. They contain the c-Ha-*ras* gene having mutations at codons 12 and 61, respectively.

Cloning and sequencing When shifted bands were detected on SSCP gel, those bands were excised and the eluate from the gel was used as a template for PCR with phosphorylated primers. The PCR products were cloned into pBluescript II vectors (Toyobo), which had been digested with *Eco*RV and treated with calf intestinal alkaline phosphatase. After cloning, plasmid DNA was sequenced by the chain-termination DNA sequencing method using Sequenase version 2.0 (United States Biochemical, Cleveland, OH).

RESULTS

Effect of the transgene and Glu-P-1 on tumor induction There was no significant difference between the diet intake of the Tg and N-Tg experimental groups (2.9±0.4 and 2.7±0.1 for male Tg and N-Tg, and 2.9±0.7 and 3.4 ±0.1 for female Tg and N-Tg, respectively). The values of tumor incidence for Tg and N-Tg administered Glu-P-1 or basal diet are summarized in Table II.

HESs were induced in Tg in the Glu-P-1 groups; 2/8 (25%) in males and 5/9 (56%) in females. They devel-

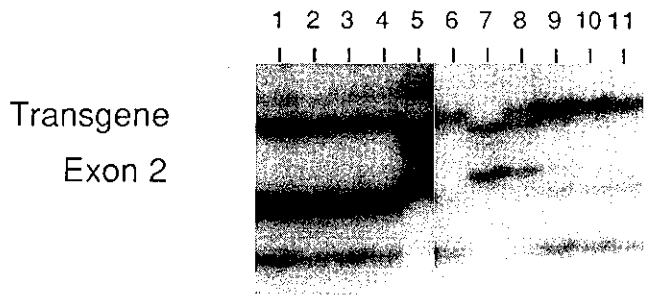

Fig. 2. PCR-SSCP analysis of the transgene in HESs induced by Glu-P-1. Lanes 1 and 2; HESs from the Tg mouse spleen. Lane 3; HES from the Tg mouse ovary. Lane 4; normal tissue from Tg mice. Lane 5; pSK2 plasmid DNA, which contains the human c-Ha-*ras* gene with a mutation at the second base in codon 61 (A-to-T transition). Lanes 6 to 11; mixture of wild-type and mutant Ha-*ras* DNA fragments. The ratio of mutant DNA fragments was 100% (lane 7), 50% (lane 8), 10% (lane 9), 5% (lane 10), 1% (lane 11) and 0% (lane 6). No mobility shifts were detected in the bands in any of 5 tumors. It was possible to detect the mutation when at least 5% of the sample DNA was mutant DNA.

Table II. Tumor Incidence in Tg and N-Tg Mice Fed Glu-P-1-containing and Basal Diet

Diet	Sex	Group	Effective no.	No. of mice with tumor (%)						
				Blood vessels	Liver			Lung		
				HES	Adenoma	HCC	Total	Adenoma	Adeno-carcinoma	Total
Glu-P-1	M	Tg	8	2 (25)	4 (50)	3 (38)*,**	7 (87)**	2 (25)	0	2 (25)
diet		N-Tg	10	0	5 (50)	0	5 (50)**	1 (10)	0	1 (10)
	F	Tg	9	5 (56)*	4 (44)	5 (56)**	9 (100)**	1 (11)	0	1 (11)
		N-Tg	8	0	5 (63)	3 (37)**	8 (100)**	0	0	0
Basal	M	Tg	9	0	0	0	0	4 (44)	1 (11)	5 (55)
diet		N-Tg	9	0	0	0	0	2 (22)	0	2 (22)
	F	Tg	8	2 (25)	1 (13)	0	1(13)	1 (13)	0	1 (13)
		N-Tg	13	0	0	0	0	3 (23)	0	3 (23)

* Significantly different at $P<0.05$ when Tg and N-Tg groups are compared.
** Significantly different at $P<0.05$ when Glu-P-1 and basal diet groups are compared.

Table III. Ha-*ras* Gene Mutations in Tumors of Mice Administered Glu-P-1

Samples	No. of tumors analyzed	No. of tumors having mutation in Ha-*ras*		Type of mutation
		Intrinsic	Transgene	
Liver tumors				
Tg mice				
Adenoma	13 (M 5, F 8)[a)]	0	0	
HCC	16 (M 6, F 10)	0	2 (M 1, F 1)	codon 12 GGC→TGC (Gly to Cys) codon 12 GGC→GTC (Gly to Val)
N-Tg mice				
Adenoma	13 (M 6, F 7)	0	–	
HCC	5 (M 0, F 5)	0	–	
Blood vessel tumors				
Tg mice				
Hemangio-endothelial sarcoma	3 (M 2, F 1)	0	0	

a) Number of tumors obtained from male (M) and female (F) mice.

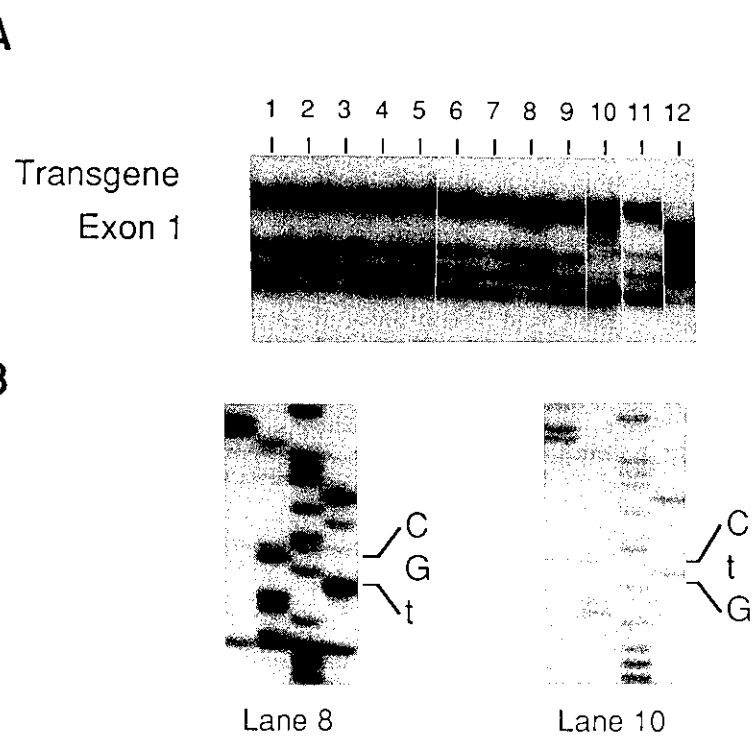

Fig. 3. Analysis of the transgene in Glu-P-1-induced liver tumors. A: PCR-SSCP analysis. Lanes 1 to 5; adenomas, lanes 6 to 10; HCCs. Lane 11; normal tissue from a Tg mouse. Lane 12; pKY1 plasmid DNA, which contained a fragment of the human c-Ha-*ras* gene with a mutation at the second base in codon 12 (G-to-T transversion). HCC samples in lanes 8 and 10 showed additional shifted bands. B: Sequencing of the shifted bands indicated G-to-T transversion at the first base (left) and at the second base (right) of codon 12.

oped in the spleen (3 cases), ovary (2 cases), uterus (1 case) and liver (1 case). No HESs were observed in N-Tg (10 males and 8 females). In the basal diet groups, two female Tg developed one HES each, in the uterus and forestomach. No mice in the other basal diet groups developed HESs.

Liver tumors [adenoma or hepatocellular carcinoma (HCC)] were induced, in contrast with HESs, in both Tg and N-Tg in the Glu-P-1 groups. There was no significant difference between the incidences of liver tumors in Tg and N-Tg; 87% versus 50% in males and 100% in both groups for females. However, the incidence of HCCs was significantly higher in male Tg than in male N-Tg (38% versus 0%), and a similar tendency was observed in females (56% versus 37%), although the difference was not significant. In the basal diet groups, no macroscopic

liver tumors were detected and only one adenoma was detected in a female Tg by microscopic examination.

No increase in lung tumor formation was observed with Glu-P-1 administration.

Ha-*ras* mutations in HESs and liver tumors in Glu-P-1-administered mice The sensitivity of our PCR-SSCP analysis was estimated since the population of mutant alleles in DNA samples was expected to be small in tumor specimens. By serially diluting mutant DNA in normal Tg DNA, it was confirmed that we could detect mutations when a mutant allele was present in at least 5% of cases (Fig. 2); this is similar to the sensitivity of the oligohybridization method used in previous reports.[1] Since the number of copies of the transgene was 3 in a diploid cell (Fig. 1), mutations could be detected when at least 15% of the cells in the sample had a mutation in one of the 3 transgene copies.

Three HESs from two male and one female Tg in the Glu-P-1 groups (two from the spleen and the other from the ovary), which were large enough to dissect macroscopically for mutation analysis, had no mutations in the transgene or the intrinsic Ha-*ras* gene (Fig. 2). However, an HES which had developed in the uterus of a female Tg in the basal diet group had a transgene mutation at codon 61. This HES was smaller than the Glu-P-1-induced HESs examined.

DNA obtained from 47 liver tumors developed in Tg and N-Tg in the Glu-P-1 groups was examined for Ha-*ras* mutations. The samples from Tg consisted of 16 HCCs and 13 adenomas, while those from N-Tg consisted of 5 HCCs and 13 adenomas (Table III). Mutational activation of the transgene was detected in two HCCs (Fig. 3A and 3B); G-to-C transversions at the first base (Gly to Cys) and second base of codon 12 (Gly to Val) were detected. No mutations in the intrinsic Ha-*ras* gene were detected in any of the 47 liver tumors.

DISCUSSION

In this study, we examined the carcinogenicity of Glu-P-1 in transgenic mice having the human c-Ha-*ras* gene that spontaneously develops HESs, but not liver tumors. Tg developed HESs at a higher incidence with Glu-P-1 administration (7/17) than without (2/17). No HESs were observed in N-Tg even after Glu-P-1 administration. Although HESs were reported to be induced by Glu-P-1 administration in CDF_1 mice, the experimental period was much longer.[4] Thus, it is suggested that the tumorigenesis of HESs is more enhanced by the transgene than by Glu-P-1 treatment, although both are cooperative.

Although the HESs induced by Glu-P-1 in CDF_1 mice developed exclusively in the brown fat tissue, those induced in Tg by Glu-P-1 were in a variety of organs, including spleen, but not in brown fat tissue. Since the genetic background of Tg used in this study was C57BL/6, this difference in organ-specific HES development between CDF_1 and Tg may be due to a difference in genetic background.

A previous report showed that all HESs that developed spontaneously in Tg had a specific type of somatic mutation at codon 61 of the c-Ha-*ras* transgene.[1]. Although the same result was obtained in this study, we could not detect any mutation in either the transgene or the intrinsic Ha-*ras* gene in the HESs of Tg induced by Glu-P-1. Our results suggest that the transgene and the intrinsic Ha-*ras* gene are not target genes for Glu-P-1-induced HESs, in contrast to spontaneously developed HESs. Glu-P-1 might induce mutation(s) in gene(s) other than Ha-*ras* and increase HES incidence. Since the level of expression of the transgene was reported to be higher than that of the intrinsic Ha-*ras* gene,[1] the total level of Ha-*ras* expression should be much higher in Tg than in N-Tg. This overexpression of Ha-*ras* may favor HES development in Tg, as reported in carbon tetrachloride-induced liver tumors in Tg.[8]

In contrast to HES, liver tumorigenesis was enhanced by Glu-P-1, but was enhanced only to a small extent by the transgene. The same line of transgenic mice also failed to show high susceptibility to hepatocarcinogenesis induced with *N*,*N*-diethylnitrosamine (DEN).[9] Involvement of the Ha-*ras* transgene appeared to be minor in the early stage of hepatocarcinogenesis, at least in that induced by Glu-P-1 or DEN. However, the incidence of HCCs in males and females was higher in Tg than in N-Tg, the difference being significant between male Tg and N-Tg. Since mutations of the c-Ha-*ras* transgene were observed in two of these HCCs in Tg, mutation of the transgene was suggested to play a role in the malignant conversion of some liver tumors.

Tg and N-Tg in the basal diet groups showed higher (although not significant) incidence of lung tumors than those in the Glu-P-1 groups. As reported previously,[1] the rate of c-Ha-*ras* transgene mutation was low in spontaneous lung tumors in Tg, suggesting a less important role of the transgene in lung carcinogenesis. The longer experimental period of the basal diet groups (80 weeks) as compared with the Glu-P-1 groups (43–54 weeks) may have resulted in the increase of lung tumors in which the c-Ha-*ras* transgene plays little role.

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