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Site-specific Mutation of the Human c-Ha-*ras* Transgene Induced by Dimethylbenzanthracene Causes Tissue-specific Tumors in Mice

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Forestomach squamous cell carcinomas, lung adenocarcinomas and spleen angiosarcomas were induced by dimethylbenzanthracene (DMBA) in the rasH2 transgenic mouse line carrying human c-Ha-*ras* genes with their own promoter, encoding the prototype p21 gene product. Fifteen out of 21 mice (71%) developed forestomach squamous cell carcinomas, while 15 out of 21 (71%) had lung adenocarcinomas and 3 out of 21 (14%) showed spleen angiosarcomas within 8 weeks after a single administration of 50 mg/kg DMBA intraperitoneally. Somatic mutation at the 61st codon of the transgenes, from CAG(Gln) to CTG(Leu), was detected in all these newly developed tumors. However, non-transgenic littermates demonstrated no tumors at all. These findings provide strong evidence that the somatic mutational activation of human c-Ha-*ras* genes is a critical event in tumorigenesis and a close relationship is therefore strongly suggested between the tissue-specific development of tumors and the somatic mutation of human c-Ha-*ras* genes in these rasH2 transgenic mice.

Key words: Somatic mutation — Dimethylbenzanthracene — Carcinoma — c-Ha-*ras* gene — Transgenic mouse

It has been reported that the *ras* genes (c-Ha-*ras*, c-N-*ras* and c-Ki-*ras*) are involved in the regulation of cellular proliferation and terminal differentiation and they have also been implicated in a wide range of human and experimental animal tumors.[1–5] Occasionally *ras* family genes are activated from their normal cellular counterparts by the acquisition of a single point mutation at codon 12, 13, 59, 61 or 117.[2] In animal model systems, there is strong evidence demonstrating that *ras* mutations are present in early stage tumors induced by chemical carcinogens.[6–9] For example, the polycyclic aromatic hydrocarbon, dimethylbenzanthracene (DMBA),[10–12] or the ethylcarbamate, urethan,[13] induce rodent epithelial tumors at a very high frequency via a specific A-T transversion mutation at the 61st codon of c-Ha-*ras* genes. Tumors which are initiated with alkylating agents such as *N*-nitroso-*N*-methylurea (MNU)[14, 15] or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)[15] have a G-A point mutation of the c-Ha-*ras* gene, as is commonly induced by alkylating agents.[16] The causal nature of these mutations in initiating carcinogenesis has been shown by the fact that tumor development in mouse skin could be reproduced after an injection of retroviruses carrying activated *ras* genes.[17, 18]

Moreover, activated *ras* genes have also recently been shown to play an important role in the tumorigenesis of transgenic mice which expressed activated *ras* genes under the control of several tissue-specific promoters and enhancer regions,[19–21] as well as under the control of their own regions.[22] It has been reported that transgenic mice (rasH2, rasH7 and rasH8 lines) carrying human hybrid c-Ha-*ras* genes with their own promoter region, encoding the prototype human p21 gene product, frequently develop tissue-specific spontaneous tumors, while they are also very susceptible to the alkylating agent MNU.[1, 23] Within 18 months spontaneous tumors were observed in nearly 50% of these transgenic mice; however, only three different tumor types were observed: spleen angiosarcomas, skin papillomas and lung adenocarcinomas.[24] The somatic point mutational activation and the expression of transgenes were detected in all these tumors but not in the normal tissues. In spleen angiosarcomas and lung adenocarcinomas, a mutation at the 61st codon from CAG(Gln) to CTG(Leu) was detected, whereas in skin papillomas the transgenes were activated by a mutation at the 12th codon from GGC(Gly) to GAC(Asp).

It was also reported that the administration of the chemical carcinogen, MNU, which has been shown to induce mutations at the 12th codon of c-Ha-*ras* genes, developed forestomach papillomas at a frequency of 100%. Somatic point mutations at the 12th codon from GGC(Gly) to GAC(Asp) were detected in almost all papillomas. In addition, no mutations at all were detected at the 12th and the 61st codon in murine c-Ha-*ras* genes.[23]

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In this study, to elucidate whether the induction of site-specific mutations could develop tissue-specific tumors or not, we administered DMBA, which has been reported to induce mutations from CAG(Gln) to CTG(Leu) at the 61st codon of c-Ha-*ras* genes preferentially,[10] to rasH2 transgenic mice. As a result, forestomach squamous cell carcinomas, lung adenocarcinomas and spleen angiosarcomas, all of which had somatic mutations at the 61st codon of the transgenes, developed at high frequencies with short latent periods.

MATERIALS AND METHODS

Transgenic mice We used a rasH2 transgenic mouse line generated from the mouse strain C57BL/6J, the characteristics of which have been reported previously.[1, 23, 24] This mouse line carries 5–6 copies of human hybrid c-Ha-*ras* genes, which encode the prototype gene product, p21, and are integrated in the mouse genome in a tandem array. Thus, they expressed human c-Ha-*ras* transgene products, p21, as well as endogenous murine c-Ha-*ras* gene products. The amount of p21 detected in various tissues was about 2 times higher in the transgenic mice than in the non-transgenic littermates. We used 85 transgenic mice (47 female mice and 38 male mice) and 106 non-transgenic littermates. All animals used were handled in accordance with the guidelines established by School of Medicine, Tokai University.

DMBA Treatment A solution of DMBA for intraperitoneal injection was prepared by dissolving dimethylbenzanthracene (Wako, Osaka) in dimethylsulfoxide and then diluting it 10-fold with steroid-suspending vehicle[25] (SSV) containing 9 mg of NaCl, 5 mg of sodium carboxymethylcellulose, 4 μl of Polysorbate 80, and 9 μl of benzyl-alcohol per ml of solution in water. This solution was administered to mice at a volume of 100 μl. For testing these chemicals, transgenic mice, rasH2 line, at 8 weeks of age were used. Within 8 weeks after administration, all mice were killed by CO_2 inhalation and both tumors and normal tissues were taken as samples. A small portion of these samples was then saved for histological examinations, and the remaining parts were frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

PCR-mediated amplification A 73-base-pair segment including the 61st codon of c-Ha-*ras* gene and a 63-base-pair segment including the 12th codon were amplified by polymerase chain reaction (PCR).[26] Oligonucleotides used as primers were synthesized on an Applied Biosystems 381A synthesizer. The sequence of primers for PCR was the same as that reported by Verlaan-de Vries *et al.*[27] and Brown *et al.*[15] Using these pairs of primers for PCR, both the murine and human c-Ha-*ras* genes were amplified. A 50 ml portion of the reaction mixture contained 0.5 mg of genomic DNA extracted from either tumors or normal tissues. Thirty cycles of amplification were performed using a Perkin-Elmer/Cetus thermal cycler, with each cycle consisting of denaturation (94°C), annealing (57°C) and polymerization (72°C) for 0.5, 0.5 and 1 min, respectively.

Detection of somatic point mutation A portion of the amplified DNA was electrophoresed on agarose gels consisting of 3% Nusieve and 1% Seakem agarose. The DNA was transferred to nylon membranes, and hybridized with each of the ^{32}P-labeled 19-base oligonucleotide probes[27]: seven probes for the 12th codon of human c-Ha-*ras* (GTC-GGC-GCC-GGC-GGT-GTG-G:Gly, CGC:Arg, AGC:Ser, TGC:Cys, GAC:Asp, GCC:Ala and GTC:Val) (Du Pont), eight probes for the 61st codon of human c-Ha-*ras* (ACC-GCC-GGC-CAG-GAG-GAG-T:Gln, CAT:His, CAC:His, AAG:Lys, GAG:Glu, CTG:Leu, CCG:Pro and CGG:Arg) (Du Pont), six probes for the 12th codon of murine c-Ha-*ras* (TG-GGC-GCT-GGA-GGC-GTG-GG:Gly, GAA:Glu, GTA:Val, GCA:Ala, CGA:Arg and AGA:Arg) and eight probes for the 61st codon of murine c-Ha-*ras* (ACA-GCA-GGT-CAA-GAA-GAG-TA:Gln, AAA:Lys, GAA:Glu, CGA:Arg, CCA:Pro, CTA:Leu, CAC:His and CAT:His) in a hybridization buffer of 3 *M* tetramethylammonium chloride (Wako).[28] The filters were washed as previously reported.[15, 27]

Histological examination Tissue samples were fixed in 10% (v/v) buffered formalin solution, embedded in paraffin, cut in sections 4 mm thick and then stained with hematoxylin and eosin by the usual method.

RESULTS

Incidence of tumors induced by the administration of DMBA In order to obtain general action of DMBA in the whole mouse body, we treated rasH2 transgenic mice with intraperitoneal injection, although topical application has been performed in many previous studies.[10–12] Since olive oil, which was used previously[29] as a solvent, proved to be toxic to rasH2 mice, as well as non-transgenic littermates, DMBAwas suspended in SSV. We treated transgenic and non-transgenic mice with a single injection of 0, 10, 20 or 50 mg/kg of DMBA per mouse (Table I). At 8 weeks after administration, all mice were examined for tumors. Although no deaths occurred immediately after the administration of DMBA, several mice died before 8 weeks after treatment. The mice that died within a short period had large tumors and a large amount of ascites. The incidence and types of tumors observed are shown in Table I. These results clearly indicated that tumor incidence by DMBA is dose-dependent. When 50 mg/kg of DMBA was administered intraperitoneally, 15 mice out of 21 (71%) developed

Table I. Tumor Types and Incidence in rasH2 Transgenic Mice Observed after the Administration of Dimethylbenzanthracene (DMBA)

DMBA dose (mg/kg)	Tg. or non-Tg.[a]	No. of mice	Forestomach SCC[b]	Lung adenocarcinoma	Spleen angiosarcoma
0	Tg.	20	0	0	0
	non-Tg.	26	0	0	0
10	Tg.	12	5 (42%)	1 (8%)	0
	non-Tg.	12	0	0	0
20	Tg.	32	19 (59%)	14 (44%)	0
	non-Tg.	32	0	0	0
50	Tg.	21	15 (71%)	15 (71%)	3 (14%)
	non-Tg.	36	0	0	0

DMBA was administered intraperitoneally only once to 8-week-old transgenic mice and their non-transgenic littermates. At 8 weeks after administration, each mouse was examined. No other tumors were detected at all.

a) Tg.: transgenic mice carrying human c-Ha-*ras* genes (rasH2 line).

b) SCC: squamous cell carcinomas.

Table II. Somatic Point Mutational Activation of Transgenes

Tumor	Number of samples tested	Point mutation at		Type of mutation
		12th codon	61st codon	
Spleen angiosarcomas	3	0	3 (100%)	CAG→CTG
Lung adenocarcinomas	16	0	16 (100%)	CAG→CTG
Forestomach SCC[a]	28	0	28 (100%)	CAG→CTG

No point mutations at the 12th or the 61st codon of the transgenes were detected in any of the normal tissue specimens.

a) SCC: squamous cell carcinomas.

forestomach squamous cell carcinomas, 15 out of 21 (71%) had lung adenocarcinomas and 3 out of 21 (14%) had spleen angiosarcomas. No tumors were found in the non-transgenic littermates.

Somatic point mutational activation of the transgenes in tumors In a previous report,[24] it was revealed that a spontaneous somatic point mutation of the transgenes, in at least one of the 5–6 copies, was detected only in tumors and not in normal tissue samples; in angiosarcomas and adenocarcinomas, a somatic point mutation was detected at the 61st codon from CAG(Gln) to CTG(Leu). Therefore, we examined the somatic mutation of the transgenes in DMBA-induced tumor cells using PCR followed by oligonucleotide hybridization.[27] Primers used for PCR were designed specifically to amplify either exon 2 or exon 3 of both human and murine c-Ha-*ras* genes. The amplified DNA fragment was probed with each oligonucleotide, comprising either the wild-type sequences or sequences containing a single mutation at the 12th codon or the 61st codon. All tumors (three spleen angiosarcomas, 16 lung adenocarcinomas and 28 forestomach squamous cell carcinomas developed after the various doses of DMBA treatment) analyzed had a specific A-T transversion at the same site, the second base of the 61st codon, of the transgene (Table II). Except for the wild-type oligomer, the only oligonucleotide to give a positive signal with DNA samples from tumors was the probe indicating the occurrence of A-T transversion at the 61st codon of at least one of the transgenes. No other changes were detected at the 12th or the 61st codon. The point mutations induced by DMBA in the transgenes were detected only in tumors and not in normal tissues at all (Fig. 1). On the other hand, no somatic mutations of murine endogenous c-Ha-*ras*, c-Ki-*ras* and c-N-*ras* genes were detected, even in tumors (data not shown).

Histological analysis Tumors that developed after DMBA treatment were subjected to histological analyses. Those in spleens were diagnosed as angiosarcomas on the basis of vascular formation and high cellularity (data not shown). Those in the lung were diagnosed as lung adenocarcinomas developed diffusely in multiple loci of the lungs (Fig. 2A, 2B). These tumors induced by DMBA showed the same appearance as spontaneous spleen angiosarcomas or lung adenocarcinomas that were previously reported.[24] In addition to these

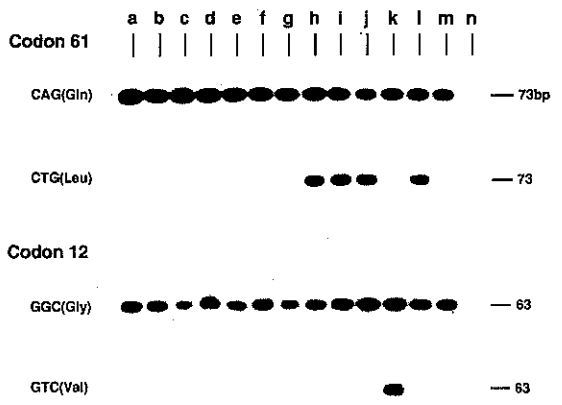

Fig. 1. Identification of somatic point mutation(s) in the transgenes of tumors induced by DMBA administration. A DNA fragment of either 73 bp including the 61st codon of the c-Ha-*ras* gene or of 63 bp including the 12th codon was amplified, and then subjected to Southern blot analysis. They were probed with oligonucleotides specific for mutations at the 12th codon and the 61st codon. The DNA samples were as follows (Lanes a–g show normal tissues in the rasH2 transgenic mouse): lane a, brain; lane b, thymus; lane c, kidney; lane d, liver; lane e, lung; lane f, spleen; lane g, forestomach; lane h, lung adenocarcinoma; lane i, spleen angiosarcoma; lane j, forestomach squamous cell carcinoma; lane k, spontaneous skin papilloma; lane l, spontaneous spleen angiosarcoma; lane m, human placenta; lane n, spleen from a non-transgenic littermate. Samples of spontaneous skin papillomas (lane k) and spontaneous spleen angiosarcomas (lane l) were from the previous experiments.[24] The activated transgenes were detected only in tumors and not in normal tissue specimens. The amplified murine endogenous c-Ha-*ras* genes were not hybridized with any of the probes used.

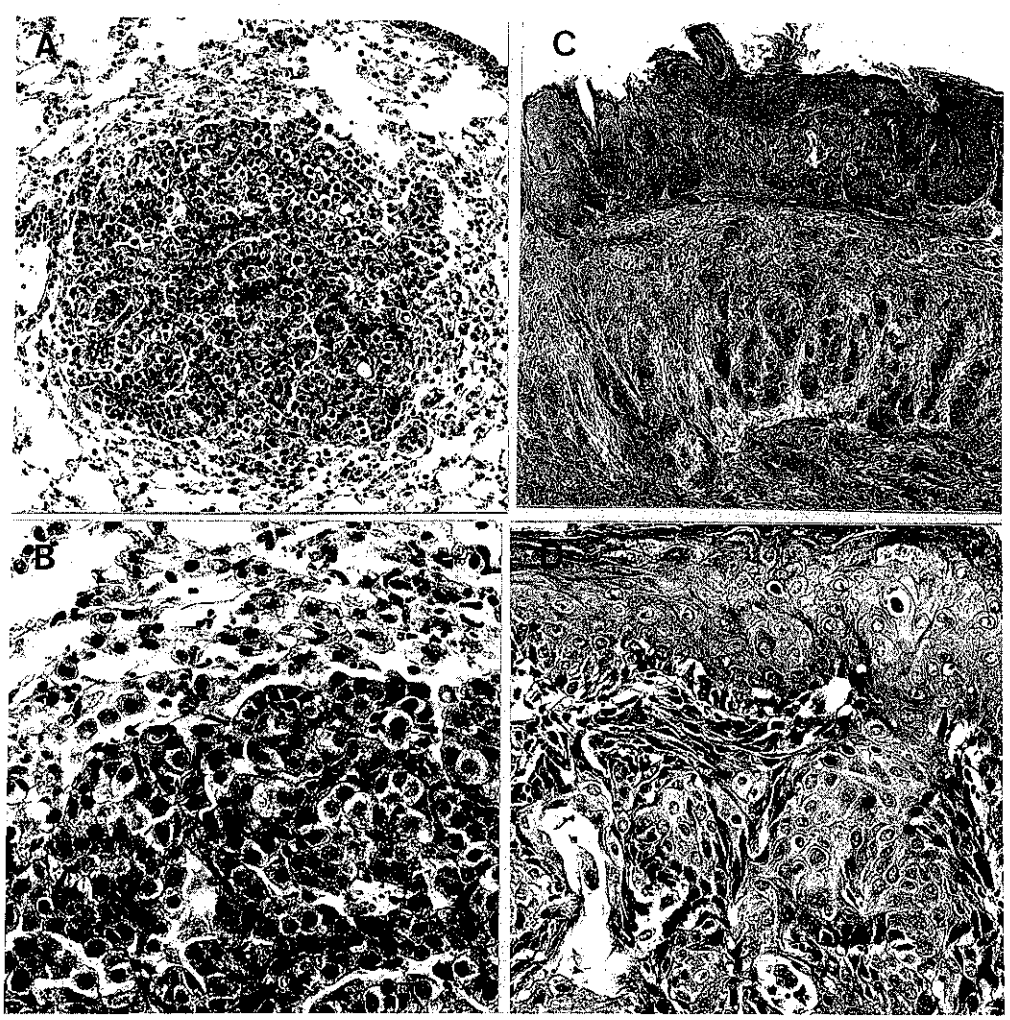

Fig. 2. The histological appearance of lung adenocarcinoma (A and B) and forestomach squamous cell carcinoma (C and D). A: ×144, B: ×360, C: ×48, D: ×240.

Table III. Mutations of Transgenes in Spontaneous and Chemically Induced Tumors

	Type of tumors	Human c-Ha-*ras* gene	
		codon 12 ···GGC·····	codon 61 ·····CAG···
DMBA-induced tumors	Angiosarcomas (spleen)	—	CTG
	Adenocarcinomas (lung)	—	CTG
	Squamous cell carcinomas (forestomach)	—	CTG
MNU-induced tumors[a)]	Papillomas (skin)	GAC	—
	Papillomas (forestomach)	GAC	—
Spontaneous tumors[b)]	Angiosarcomas (spleen)	—	CTG
	Adenocarcinomas (lung)	—	CTG
	Papillomas (skin)	GTC	—

a) Data from previous experiments.[23)]
b) Data from previous experiments.[24)]

tumors, which were also observed spontaneously in untreated animals, forestomach tumors were detected as a single, large mass in each mouse. These tumors were diagnosed as squamous cell carcinomas with a marked downgrowth of malignant squamous cells invading deep into the muscle layers with hyperkeratosis (Fig. 2C, 2D).

DISCUSSION

In rasH2 transgenic mice carrying human c-Ha-*ras* transgenes encoding the prototype p21, forestomach squamous cell carcinomas, lung adenocarcinomas and spleen angiosarcomas were induced at a very high incidence within 8 weeks after a single intraperitoneal injection of DMBA. The development of tumors by DMBA was limited to three types of tissues, whereas the papillomas induced by MNU were limited to the forestomach and skin. Since non-transgenic littermates have never developed tumors or exhibited a mutation at the 61st codon of the murine genes, it was suggested that the mutation of transgenes by DMBA activated the murine cells tissue-specifically and possibly played a causative role in tumorigenesis in these transgenic mice. On the other hand, the rasH2 line developed several tumors spontaneously with tissue-specificity, and a single point mutation was detected at the 12th codon of the transgenes in spontaneous skin papillomas and at the 61st codon of the transgenes in spontaneous spleen angiosarcomas and lung adenocarcinomas. As reported previously, skin papillomas developed after MNU treatment, with a mutation at the 12th codon of the transgenes.[23)] In the present experiment, the induced mutation at the 61st codon of the transgenes developed spleen angiosarcomas and lung adenocarcinomas, which were also observed as spontaneous tumors. Both spontaneous tumors and chemically induced tumors showed point mutation of transgenes at the same codons (Table III).

These findings strongly suggest a close relationship between the mutation at the 12th codon of transgenes and skin papillomagenesis and between the mutation at the 61st codon of transgenes and the development of spleen angiosarcomas and lung adenocarcinomas.

Since both DMBA and MNU were administered by intraperitoneal injection, all cells in the body can be assumed to have been efficiently exposed to the chemical carcinogens compared with topical application, and the mutations of c-Ha-*ras* genes might be induced in all cells. However, the development of tumors was observed in only a limited number of cell types and the mutations were detected only in the transgenes. Two questions arise from these results. One is why mutational activation was found only in the transgenes and not in the murine c-Ha-*ras* genes. It might be that the transgenes are more susceptible to MNU and DMBA than the murine c-Ha-*ras* genes. It is also possible that the tumors produced by the activated transgenes grow significantly more rapidly than those produced by the murine c-Ha-*ras* genes. Further study is needed on these points. The second question is why the tumors develop so tissue-specifically. One possibility is that the site-specific occurrence of a somatic mutation is regulated in each tissue, and therefore somatic point mutation of the transgenes occurs either specifically or preferentially only in particular tissues where the tumors tend to develop. Accumulation of DNA adducts after DMBA treatment might be tissue-specific. The second possibility is that the mutations occur at random on the chromosomes with an equivalent frequency in all cells, but cells which depend on c-Ha-*ras* gene products for proliferation, differentiation, or both, are highly susceptible to specifically activated human c-Ha-*ras* p21 during tumorigenesis. In order to solve this problem, new systems of transgenic mice, in which the mutation rate of transgenes in all tissues can be precisely detected, should be developed.

Forestomach tumors, which did not appear spontaneously, were induced both by DMBA and MNU. Eight weeks after the administration of DMBA, forestomach squamous cell carcinomas developed, whereas forestomach papillomas were induced by MNU within 12 weeks. If carcinomas develop not from pre-existing papillomas but directly from a separately initiated cell population, mutation at the 61st codon of the transgenes might cause carcinogenesis and that at the 12th codon of the transgenes might cause papillomagenesis in the forestomach in these transgenic mice. However, if carcinomas developed as a progression from existing papillomas, forestomach papillomas would be expected to be observed at less than 8 weeks after administration of DMBA and carcinomas may develop more than 12 weeks after the injection of MNU. In our preliminary experiments, forestomach papillomas were detected soon after DMBA treatment while squamous cell carcinomas were obtained more than 16 weeks after the treatment of MNU in rasH2 mice.

Since rasH2 transgenic mice developed specific tumors very frequently within a short period after treatment with DMBA and MNU, this system is expected to be useful for determining the mutagenicity of chemical agents and the efficacy of anticancer agents.

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