Transgenic Rats Carrying Human c-Ha-ras Proto-oncogene Are Highly Susceptible to N-Nitrosomethylbenzylamine Induction of Esophageal Tumorigenesis

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A transgenic line carrying three copies of the human c-Ha-ras proto-oncogene, including its own promoter region, has been established in our laboratory (Hras128 rats), and shown to be highly susceptible to induction of mammary and urinary bladder tumors. Mutation analysis of induced lesions indicated the majority to contain some but not all cells with transgene mutation. In the present study, the susceptibility of Hras128 rats to N-nitrosomethylbenzylamine (NMBA) induction of esophageal tumors was examined with a similar mutation analysis of the transgenes. Male 6-week-old Hras128 and wild littermate rats were treated with NMBA, 0.5 mg/kg subcutaneously, 3 times a week for 5 weeks and then maintained for 5 weeks without any further treatment. Multiple esophageal tumors, squamous cell papillomas and carcinomas, rapidly developed within this 10-week experimental period in Hras128 rats (11.05±±±± 7.83/rat). In contrast, wild-type littermates had only small numbers of mostly benign tumors (1.67±±±± 2.06/rat). The Hras128 rats had no other tumors or abnormalities. In their esophageal lesions, codon 12 GGC to GAC mutations of the transgene were frequently detected by restriction fragment length polymorphisms (RFLP) and subsequent direct sequencing analysis (19/25, 76%). In the endogenous rat c-Ha-ras gene they were less frequent (2/25, 8%), than in wild-type rats (8/14, 57.1%). The densities of mutated bands in the RFLP analysis indicated that mutated cells were major populations in tumors, in contrast to the case with mammary and urinary bladder lesions. Furthermore, activated ras protein, detected by binding to raf protein, was clearly increased in tumors as compared to surrounding epithelium or the normal esophagus of untreated rats. The results show that Hras128 rats are highly susceptible to NMBA esophageal carcinogenesis, as well as induction of mammary and urinary bladder tumors, but that tissue-specific characteristics exist for the roles of transgene ras mutations.

Key words: Transgenic rats — c-Ha-ras — Esophageal tumors — N-Nitrosomethylbenzylamine

Transgenic mice are widely used for analysis of gene function and as animal models for various diseases. In the field of chemical carcinogenesis, transgenic mice harboring a human c-Ha-ras proto-oncogene,1, 2 v-Ha-ras transgenic mice (Tg.AC mice),3 pim-1 transgenic mice4 and p53 knockout mice5 have been shown to be highly susceptible to induction by certain carcinogens, with indication that the transgene is actively involved. However, for studies of chemical carcinogenesis,6 rats have been more frequently used, so that abundant information has accumulated regarding various biological characteristics of pre-neoplasias.7, 8 Therefore the Hras128 rats generated in our laboratory have great potential for studies of neoplasia.9, 10 For esophageal tumors, rats are preferable to mice11, 12 due to the availability of the organotrophic carcinogens and the larger size of the organ.

Human esophageal squamous cell carcinomas are presumed to be caused by exposure to nitrate and nitrite, precursors of nitrosamines.13 The promutagenic adduct, O'-methylguanine, formed due to nitrosamine attack has been detected in DNA from esophageal cancer patients in China14, 15 and N-nitrosomethylbenzylamine (NMBA) is known to be a potent esophageal carcinogen in rats.16–19 Its mechanism of action is through microsomal activation to the methyldiazonium ion, an electrophilic metabolite which methylates DNA, producing O6- and O7-methylguanine adducts.20–22 Formation of O6-methylguanine is thought to play an important role in carcinogenesis due to its persistence, accumulation and ability to cause DNA mutations.21, 24 In line with this hypothesis, G to A mutations in codon 12 of c-Ha-ras are frequent in tumors induced by NMBA in rats.25–28 Mammary carcinomas induced by N-methyl-N-nitrosourea (MNU) in rats also often demonstrate c-Ha-ras mutations.29 We have reported the Hras128 rats carrying the same human c-Ha-ras proto-oncogene to be highly susceptible to mammary carcinogenesis induced by MNU, and we showed that the induced carcinomas contain minor popula-
tions of cells with mutation in the transgene.9,30) Similar results were observed for urinary bladder carcinogenesis caused by N-butyl-N-(4-hydroxybutyl)nitrosamine. To determine whether a similar situation might exist for NMBA induction of esophageal tumors, the present study was performed, including an assessment of transgene mutations and activation of the ras protein.

MATERIALS AND METHODS

Transgenic rat A rat line carrying the human Ha-ras proto-oncogene (Hras128) was established and an initial characterization was performed.9 Briefly, Sprague-Dawley rats (Clea Japan, Inc., Tokyo) were used. The DNA construct utilized for the Hras128 rats has been previously described.31) A 6.8 kb BamH1 fragment of the human c-Ha-ras proto-oncogene with its own promoter region was injected into pronuclei of rat embryos collected from superovulated female rats.32) The Hras128 rats contain 3 copies of the transgene and give rise to transgenic offspring according to Mendelian genetics. For screening, the polymerase chain reaction (PCR) was used with DNA samples from rat tails obtained by the proteinase K phenol-chloroform method. PCR reactions were performed using Ampli Taq Gold (Perkin Elmer, Branchburg, NJ) and human Ha-ras exon 2 specific primers, hHras2F (5′-AGC-CCTGTCTCCTGACTGAGAT-3′) and hHras2R (5′-GCCATGGCGCTGATCAGCTC-3′). Digested samples of each reaction product were electrophoresed on 2% or 4% agarose gels. When an enzyme-resistant band was visualized, the responsible fragments were amplified using hHras1F and hHras1R for codon 12 and hHras2F and H61/2A2 for codon 61, and then directly sequenced using 3P end-labeled primers; hHras1FN, (5′-AGCCCTGTCTCCTGACTGAGAT-3′) for codon 12 and cHras2IF (5′-CTGCAGATTCCCTACGAGGAA-3′) for codon 61, and a TaKaRa Taq Cycle Sequencing Kit (TaKaRa, Otsu).

As a positive control for detection of mutations in human Ha-ras codons 12 and 61, DNA from T24 cells (GHC to GTC in codon 12)31) (JCRB0711, obtained from Health Science Research Resources Bank, Japan Health Sciences Foundation, Tokyo) and a pSK2 plasmid (CTG to CAG in codon 61)31) (CO001, obtained from Health Science Research Resources Bank, Japan Health Sciences Foundation) was used.

Density analysis was performed using dilution control of the mutated DNA taken from T24. The analysis was performed in conjunction with work documented previously.9) Mutation analysis of the endogenous c-Ha-ras gene was performed by PCR-single-strand conformation polymorphism analysis (SSCP) using Ampli Taq Gold and primers labeled with γ 3P-ATP and T4 polynucleotide kinase (TaKaRa). Amplified and labeled DNAs were analyzed in 5% non-denaturing acrylamide gels (49:1) with 5% glycerol at 4 or 20°C. The sequences of the primers applied and the annealing temperatures for exon 1 of rat and exon 2 of human and rat c-Ha-ras are: rHras1F, (5′-GGATGACAGAATACAAAGT-3′); rHras1R, (5′-GAGCCTCACCTCTAGTGGG-3′) at 55°C and cHras2F, (5′-CTGCAGATTCCCTACTGGGAA-3′); cHras2R, (5′-CACCTCTGACTGAGGTGTC-3′) at 51°C, respectively. DNAs demonstrating mutations were isolated from acrylamide gels for SSCP. Base substitutions were analyzed with a DNA sequencer (Takara Bio).

Northern blot analysis Total RNAs were extracted by...
the acid guanidium thiocyanate phenol/chloroform method from epithelium of the esophagus of transgenic and non-transgenic rats and 10 µg aliquots were loaded onto 1% agarose gels, electrophoresed and transferred to nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Buckinghamshire, England). A human Ha-ras mRNA-specific oligonucleotide probe (5'-GGG GTT CCG GTG GCA TTT GG-3') was labeled with γ 32P-ATP and T4 polynucleotide kinase (TaKaRa), and hybridized to the membrane.

**Western blot and ras activation analysis** Activated ras in tissues was measured with a ras Activation Assay Kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. The method is based on the selective binding of Ras-GTP to Raf-1. Therefore, Ras-GTP in the tissue was detected by Raf-1-agarose precipitation followed by western blotting for Ras. Briefly, proteins were extracted from samples of normal epithelial mucosa and tumors of the esophagus from 3 or 4 transgenic and wild-type rats subjected to the same protocol as in the tumor incidence and mutation assays. The protein contents of the extracts were measured with a BCA protein assay kit (Pierce, Rockford, IL), and 20 µg aliquots were applied to 5–20% polyacrylamide gels (Bio-Rad Lab., Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The total ras in the tissues was detected with a mouse monoclonal anti-ras antibody (kit component: Upstate Biotechnology, Inc., Boston, MA) followed by a horseradish peroxidase-conjugated goat anti-mouse antibody (Southern Biotech-
nology Associates, Inc., Birmingham, AL) and ECL-Plus detection reagents (Amersham Pharmacia Biotech). For quantitative analysis of activated ras protein, the sample volume was adjusted to contain the same amount of total ras proteins according to the band densities. The concentration was confirmed before mixing with Raf-1-agarose. Raf-1-bound ras (GTP-Ras) was detected by western blotting as detailed above. In order to activate Ras in vitro as a positive control, GTP-Ras was mixed with the extracts and as a negative control, GDP was added before loading onto raf-1-agarose.

RESULTS

Susceptibility to NMBA induction of esophageal tumors  By the end of week 10, Hras128 rats proved to be highly susceptible to NMBA induction of esophageal tumors, exhibiting larger and greater numbers of squamous cell lesions than the wild-type rats. Data for tumor incidence and mean tumor size (largest diameter) are summarized in Table I. From histopathological diagnosis, most were squamous cell papillomas with a few carcinomas. No other macroscopic or microscopic lesions, including dysplasia, were detected in either Hras128 or wild-type rats. A long-term study to determine spontaneous tumor incidence in the Hras128 rats revealed no development of esophageal tumors up to week 78 after birth (our unpublished data).

Mutation analysis of the transgene of the esophageal tumors  Out of 25 tumors taken from 19 Hras128 rats treated with NMBA, 19 (76%) had mutations in codon 12 of the transgene (Fig. 1), and 2 (8%) in codon 12 of the endogenous c-Ha-ras gene (Fig. 2). Direct sequencing of mutated bands of the transgene revealed that among the 19 tumors with mutations in codon 12, 17 tumors had GGC to GAC, 1 had two mutations, codon 12 GGC to GAC and codon 14 GTG to GTA, and 1 had codon 12 GGC to TGC mutations (Fig. 3). In the Hras128 rats, 2 tumors had codon 12 GGA to GAA mutations in the endogenous c-Ha-ras gene. No mutations were detected in codon 61 or exon 2 of the Hras128 or the endogenous c-Ha-ras proto-oncogenes (Figs. 4 and 5). In contrast, a relatively high frequency of codon 12 GAA to GGA mutations of the endogenous Ha-ras gene was detected, in 8 of 14 (57.1%) tumors of wild rats (Fig. 6). These results are summarized in Table II.

Level of mRNA expression  Transgene expression was only detected in tissues from transgenic rats. Endogenous c-Ha-ras gene expression was not detectable. The expression level of the transgene did not show any significant
difference between tumors and normal surrounding mucosa. NMBA treatment did not cause any difference in the transgene mRNA expression level (Fig. 7).

c-Ha-ras protein expression  Western blot analysis of the proteins extracted from tumors and normal surrounding epithelium from transgenic and wild-type rats revealed slightly higher expression in tumor and normal tissues from transgenic rats than in their wild-type counterparts. However, no difference was observed between tumors and normal tissues, in agreement with the results of northern blotting. In the wild-type rats, ras protein was detected by an antibody that recognizes all members of the ras family (Fig. 8A).

The percentages of the active form in the total ras protein calculated from the band densities (Fig. 8C) divided by total ras protein loaded (Fig. 8B) are given in Fig. 8D. High binding of ras to raf-1-agarose was detected only in tumors from transgenic rats. In tumors from wild-type rats, binding of raf-1 to Ras was not observed.

### DISCUSSION

The present study revealed that Hras128 rats carrying copies of the human c-Ha-ras transgene, that encodes prototype p21, respond to injection of NMBA by development of multiple, large-sized esophageal tumors at a 100% incidence within 10 weeks, while wild-type littermates

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**Table II. Ha-ras Mutations in NMBA-induced Esophageal Tumors**

| Strain    | No. of tumors | Human Ha-ras | Rat Ha-ras |
|-----------|---------------|-------------|------------|
|           |               | Codon 12    | Codon 61   | Codon 12 | Codon 61 |
| Tg        | 25            | 19 (76%)\(^a\) | 0          | 2 (8%)\(^b\) | 0        |
| Wild type | 14            | —           | —          | 8 (57.1%)\(^b\) | 0        |

\(^a\) Seventeen tumors had codon 12 GGC to GAC mutations and one tumor had a codon 14 GTG to GTA mutation in addition. The other tumors had a codon 12 GGC to TGC mutation.

\(^b\) GGA to GAA mutation.
have much fewer and smaller lesions. The results thus indicate that expression of the transgene is associated with enhanced susceptibility. Furthermore, the relatively high frequency of transgene mutation in tumors of the Hras128 rats suggests that expression of mutant-type c-Ha-ras may be directly involved in the tumor development.

Rat mammary and esophageal tumors caused by MNU and NMBA, respectively, are known to have high incidences of c-Ha-ras gene codon 12 mutations (GGA to GAA).\(^{26–28}\) In contrast, mutations in the c-Ha-ras gene are rare in bladder or liver tumors induced by carcinogens in rats.\(^{34–36}\) We have found that our Hras128 strain is highly susceptible to MNU-induced mammary,\(^{9}\) N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder\(^{37}\) and, in the present work, NMBA-induced esophageal carcinogenesis. The results indicate that mutations of c-Ha-ras can play important roles in neoplasia in the esophagus, in contrast to the breast and urinary bladder.

PCR-RFLP analysis and direct sequencing of codons 12 and 61 of the transduced human c-Ha-ras proto-oncogene in MNU-induced mammary tumors indicated that while the majority of tumors contained cells with mutations at codon 12 in exon 1, the signal densities of the mutated bands revealed these to be not a major sub-population.\(^{11}\) Similarly, the transgene mutation rate in urinary bladder lesions induced by another nitrosamine, N-butyl-N-(4-hydroxybutyl)nitrosamine, was not predominant.\(^{37}\) In contrast, the mutated band densities observed in esophageal tumors in the current study indicated the tumors to contain cells with mutated transgene as the major population (Fig. 1). The reason for the differences in constituent subpopulations of cells with transgene mutations in the 3 different organs, despite the similar high susceptibility to chemical carcinogens, is not clear. However, since activated ras protein is clearly increased in esophageal (Fig. 7) and mammary carcinomas (our unpublished observation) and the mutation rates in either the transgene or the endogenous c-Ha-ras gene are high (21 tumors out of 25), possible involvement of transgene mutation may be suggested. However, an alternative pathway without involvement of H-ras mutation may also exist in some cases. In this regard, further studies using activated ras transgenes gene would certainly be of interest. With regard to the observed differences in RFLP band density in the present series of tumors, their fusiform structure, comprising mutated and non-mutated cells, might be important.

Transgenic mice carrying the human c-Ha-ras proto-oncogene, the same gene as introduced into the Hras128 rats, are reported to be highly susceptible to induction of forestomach and skin papillomas by MNU, with almost all of the tumors exhibiting mutations of the transgene in codon 12, GGC to GAC.\(^{35}\) However, since population analysis of mutated cells was not performed in the mouse case, it is impossible to reach any conclusion regarding the significance of the mutations in these cases. Our findings suggest that the transgene is a target of NMBA, and mutations may play crucial roles in esophageal tumor induction.

As with mammary tumors, mRNA expression levels were not different between tumors and surrounding tissues, suggesting that this parameter is not directly relevant to carcinogenesis in the transgenic rat. It should be noted, however, that while total ras protein levels were not different between tumors and non-tumor mucosa, levels of the activated form were clearly increased in tumor tissue in transgenic rats. Endogenous c-Ha-ras mRNA expression could not be detected in tumors of wild-type rats, which lacked activated ras protein.

In conclusion, Hras 128 rats are highly susceptible to NMBA-induction of esophageal tumors, in which transgene mutations associated with ras activation play important roles.

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