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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 rat 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 mice[4] and p53 knockout mice[5] have been shown to be highly susceptible to tumor 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 preneoplasias.[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 mice[11,12] due to the availability of the organotropic 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^6-methylguanine, formed due to nitrosamine attack has been detected in DNA from esophageal cancer patients in China[14,15] 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 O^6- and O^7-methylguanine adducts.[20–22] Formation of O^6-methylguanine is thought to play an important role in carcinogenesis due to its persistence, accumulation and ability to cause DNA mutations.[23,24] 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-

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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 *Bam*HI 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′-AGCCCTGTCCTCCTGCAGGAT-3′) and hHras2R (5′-GGCCAGCCTCACGGGGTTCA-3′), which amplify a 218 bp fragment of human Ha-*ras*.

Carcinogenesis protocol Six-week-old 24 Hras128 and 23 wild littermate male rats were treated with NMBA (0.5 mg/kg, body weight), 3 times weekly for 5 weeks, maintained for a further 5 weeks and then killed. The esophagus was opened longitudinally for macroscopic counting and measurement of the longest diameter of lesions. The esophagus was fixed in 10% buffered formalin then routinely processed for histological examination. For the mutation analysis of Ha-*ras* gene, tumor tissue and surrounding non-tumor mucosa from the transgenic and non-transgenic rats (5 rats each) were excised with razor blades, frozen in liquid nitrogen and stored at −80°C until use.

Mutation analysis Mutation analysis of the transgene was performed by PCR-restriction fragment length polymorphisms (RFLP) techniques. Mutations at codon 12 of the transgene were detected by the presence of a *Msp*I digestion-resistant band. This 167 bp amplified fragment with primers hHras1F and hHras1RN at an annealing temperature 60°C with 35 cycles could be clearly distinguished from the 117 and 50 bp fragments resulting from cleavage of the wild-type sequence (GGC) by this enzyme. Mutations in codon 61 were detected by the presence of an AlwNI-resistant band. An AlwNI site was generated by a mismatched primer (H61/2A2). For the first-round PCR, 218 bp fragments of exon 2 were amplified by primers hHras2F and hHras2R at an annealing temperature of 63°C for 35 cycles, and then diluted 100 times with water and used as templates for the second-round PCR using primers hHras2F and H61/2A2 at an annealing temperature of 60°C for 35 cycles. The wild-type sequence (CAG) is cleaved into 93 bp and 17 bp fragments by AlwNI. Codon 61 mutants are not cut by this enzyme and give a 110 bp band. The primer sequences used for RFLP detection of human c-Ha-*ras* point mutations are; hHras1F, (5′-GCAGGCCCCTGAGGAGCGAT-3′); hHras1RN, (5′-AGCAGCTGCTGGCACCTGGA-3′); hHras2F, (5′-AGCCCTGTCCTCCTGCAGGAT-3′); hHras2R, (5′-GGCCAGCCTCACGGGGTTCA-3′), H61/2A2, (5′-CGCATGGCGCTGTACAGCTC-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 ^{32}P end-labeled primers; hHras1FN, (5′-AGCCCTGTCCTCCTGCAGGAT-3′) for codon 12 and cHras2IF, (5′-CTGCAGGATTCCTACCGGAA-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 (GGC to GTC in codon 12)[33] (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 ^{32}P-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′-GCGATGACAGAATACAAGCT-3′); rHras1R, (5′-GAGCTCACCTCTATAGTGGG-3′) at 55°C and cHras2FI, (5′-CTGCAGGATTCCTACCGGAA-3′); cHras2IR, (5′-CACCTGTACTGGTGGATGTC-3′) at 51°C, respectively. DNAs demonstrating mutations were isolated from acrylamide gels for SSCP. Base substitutions were analyzed with a TaKaRa *Taq* Cycle Sequencing Kit (TaKaRa) under the same conditions as described above. Amplified and labeled DNAs were analyzed in 6% denaturing acrylamide gels.

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 ^{32}P-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-pan-ras antibody (kit component: Upstate Biotechnology, Inc., Boston, MA) followed by a horseradish peroxidase-conjugated goat anti-mouse antibody (Southern Biotech-

Table I. Susceptibility of c-Ha-*ras* Transgenic Rats to NMBA Esophageal Tumorigenesis

Strain	No. of rats	No. of tumor bearing rats	No. of tumors/rat	Tumor size (mm)
Tg	19	19 (100%)[a)]	11.05±7.83[b)]	2.40±1.52[c)]
Wild type	18	11 (61.1%)	1.67±2.06	1.79±1.08

a) Fisher's exact probability test, $P<0.005$.
b) Mann-Whitney *U* test, $P<0.0001$.
c) Mann-Whitney *U* test, $P<0.02$.

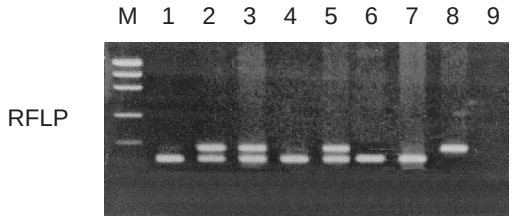

Fig. 1. Representative results of RFLP analysis of codon 12 of the transgene. Lanes 1, 2, 3, 5, and 6, esophageal tumors induced in Hras128 rats. Lanes 2, 3, and 5 exhibit mobility-shifted bands. Lanes 4 and 7, non-tumorous esophageal epithelium from Hras128 rat treated with NMBA. Lane 8, T24, positive control. Lane 9, negative control (no DNA). The sensitivity of RFLP analysis was confirmed by the dilution control as in our pervious report.[9)]

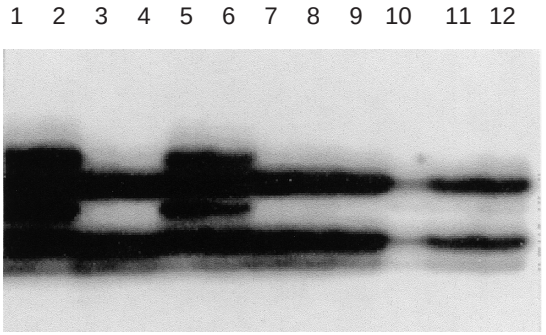

Fig. 2. Representative SSCP analysis of rat c-Ha-*ras* exon 1. Lanes 1 to 6, PCR products from esophageal tumors induced in wild-type rats. Lanes 7–12, PCR products from esophageal tumors induced in Hras128 rats. Lanes 1, 2, 5 and 6 demonstrate mobility-shifted bands, indicating the presence of mutation(s) in rat c-Ha-*ras* gene exon 1.

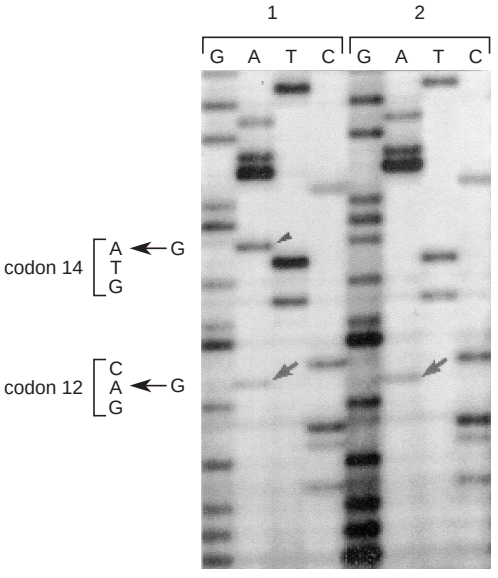

Fig. 3. Direct sequencing of PCR products from *Msp*I-resistant bands on RFLP analysis for 2 representative samples of esophageal tumors of Hras128 rats. Note the GGC to GAC mutation of rat c-Ha-*ras* gene codon 12, in addition to a codon 14 GTG to GTA mutation in sample 1.

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

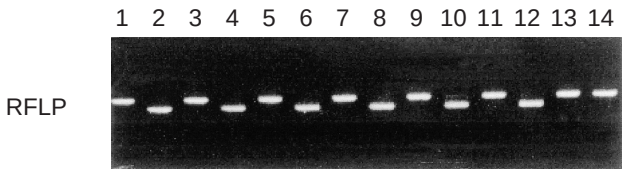

Fig. 4. Representative results of RFLP analysis for codon 61 of the transgene. Lanes 1 to 12, esophageal tumors induced in Hras128 rats. Lanes 1, 3, 5, 7, 9, 11 and 13, AlwNI undigested. Lanes 2, 4, 6, 8, 10, 12 and 14, AlwNI digested. Lanes 13 and 14, pSK-2 positive control. No mutations were detected in any of the tumors.

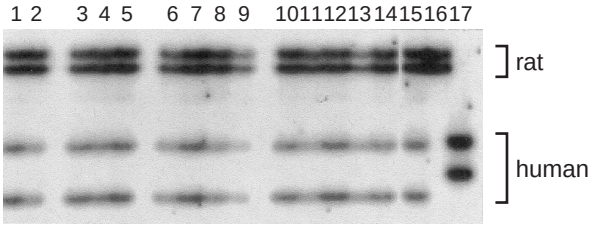

Fig. 5. Representative results of SSCP analysis for the transgene and rat endogenous c-Ha-*ras* gene exon 2. Lanes 1 to 14, PCR products from esophageal tumors of Hras128 rats, demonstrating 2 upper bands and 2 lower bands specific for rat and human, respectively. Lane 15, normal liver DNA from a Hras128 rat. Lane 16, normal liver DNA from a wild-type rat. Lane 17, pSK2 with a CAG to CTG mutation in codon 61 of the human c-Ha-*ras* proto-oncogene. No mobility shifts are apparent in any of the bands from esophageal tumors in Hras128 rats.

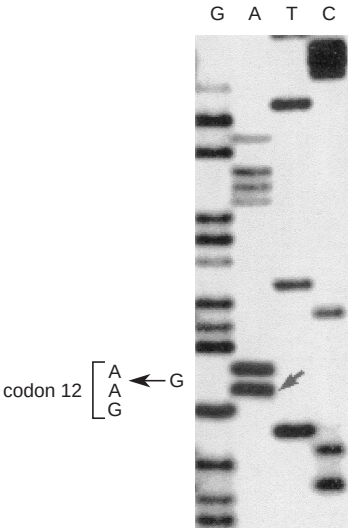

Fig. 6. Direct sequencing of the PCR product from a mobility-shifted band on a SSCP gel. Note the GGA to GAA mutation of rat c-Ha-*ras* gene codon 12.

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.

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.

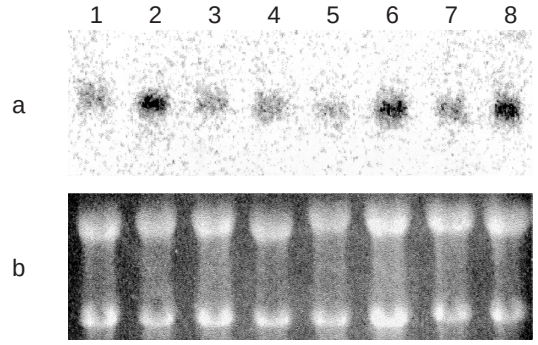

Fig. 7. Northern blot analysis for esophageal tumors and surrounding epithelial tissues treated with NMBA, and non-treated esophageal mucosa of Hras128 transgenic rats. Lanes 1, 3, and 5, esophageal tumors induced in Hras128 rats; lanes 2, 4, and 6, normal-appearing mucosa surrounding the esophageal tumors, respectively. Lanes 7 and 8, normal esophageal mucosa form untreated Hras128 transgenic rats. a, specific bands for human c-Ha-ras. b, ethidium bromide staining for total RNA to confirm equal loading of RNA.

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

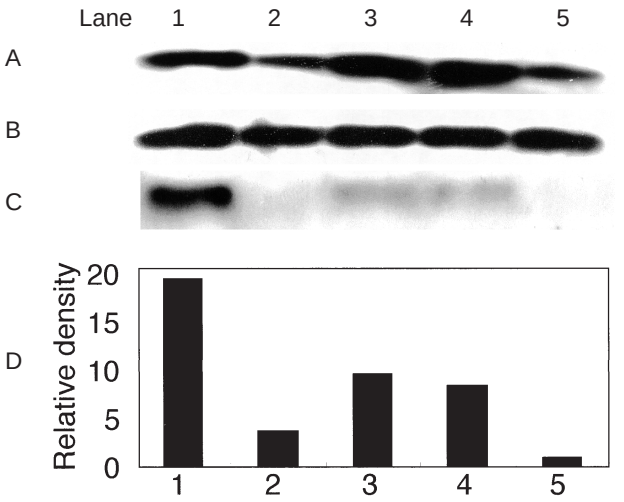

Fig. 8. Total ras and active form ras expression. (A) Pan-ras in esophagus tissue. The same amounts of proteins from tissue extracts (20 μg/lane) were applied to each lane and visualized by anti-pan-ras antibody (anti-H-, N-, and K-ras). (B) The amount of ras protein in the extracts was adjusted for each lane according to the densities of the bands from (A). (C) GTP-ras was precipitated by raf-1 agarose and detected by anti-pan-ras antibody. (D) The relative levels of signal densities were calculated from (B) and (C). Levels were expressed relative to lane 5 (taken as a value of 1). Lanes 1–4 are from NMBA-treated and lanes 4 and 5 are from untreated rats. Lane 1, tumor in a Hras128 rat; lane 2, tumor in a wild-type rat; lane 3, surrounding mucosa in a Hras128 rat; lane 4, normal mucosa in an untreated Hras128 rat; lane 5, normal mucosa in an untreated wild-type rat. Representative non-activated ras control and 100% activated ras control using the same specimens as in lane 5 were also done (not shown).

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.[1] 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.[3] 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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