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Mutational Activation of H-*ras* and K-*ras* Genes Is Absent in *N*-Nitroso-*N*-methylurea-induced Liver Tumors in Rats

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We examined mutational activation of H- and K-*ras* genes in hyperplastic nodules and hepatocellular carcinomas induced by *N*-nitroso-*N*-methylurea or diethylnitrosamine using the polymerase chain reaction, followed by dot-blot hybridization. No mutational activation was detected in these hepatic lesions. The results indicate that low incidence of the activation of H- and K-*ras* genes in rat liver tumors is due to the organ specificity rather than the nature of the carcinogens used.

Key words: *ras* genes — Mutational activation — Rat liver tumors — *N*-Nitroso-*N*-methylurea — Polymerase chain reaction

Mutational activation of *ras* gene family has been reported in a variety of spontaneous or chemically induced animal tumors. However, its incidence and location within *ras* genes are variable depending on the kinds of animals, tumors and carcinogens used.[1,2] In rats, it has been demonstrated that mammary carcinomas induced by a single dose of *N*-nitroso-*N*-methylurea (NMU)[2] during sexual development show a high frequency of point mutation of H-*ras* gene exclusively at the 2nd position of codon 12 (G to A transition).[3,4] Such mutation is considered to be derived from miscoding due to the generation of O^6-methylguanosine at that position through the methylating activity of NMU. In contrast, when dimethylbenz[*a*]anthracene was substituted for NMU to induce mammary tumors, the H-*ras* mutation was observed in lower frequency at codon 61 instead of codon 12.[4]

On the other hand, several recent reports have indicated that mutational activation of *ras* genes is rare in rat hepatic tumors induced by chemicals (aflatoxin B_1,[5] diethylnitrosamine (DEN),[6] acetoxymethylnitrosamine,[7] etc.) and in hepatocellular carcinomas of human,[8] although Sinha *et al.* demonstrated that aflatoxin B_1-induced rat liver tumors frequently contained activated N-*ras* genes.[9] However, it has not been unequivocally shown whether the low incidence of *ras* gene mutation in rat hepatic carcinogenesis is due to the nature of the chemicals used or organ specificity. Thus, in this study we examined mutational activation of H- and K-*ras* genes in preneoplastic and neoplastic lesions induced by NMU.

Male F344 rats, 4–6 weeks old, were hepatectomized and given NMU (60 mg/kg in 10 m*M* citrate buffer, pH 6.0) or DEN (30 mg/kg in phosphate-buffered saline) by a single intraperitoneal injection. All the animals were given the 0.02% 2-AAF diet for 2 weeks from the 2nd week, and a single dose of CCl_4 dissolved in corn oil (2 ml/kg body weight) at the 3rd week according to the regimen of Cayama *et al.*[10,11] The rats were killed at 5 or 32 weeks after the initiation of the experiment. The livers were fixed by perfusion with phosphate-buffered formalin for 5 min and immersed in the same fixative for another 16 h. Serial 7-μm-thick sections were made from paraffin-embedded tissue blocks. Some sections were immunostained for glutathione-*S*-transferase placental form (GST-P) by the biotin-streptoavidin method, and the others were stained with hematoxylin and eosin for routine histological examination. GST-P(+) foci were scooped out from the immunostained sections with a 27G syringe needle under a stereoscopic microscope and used as DNA templates for polymerase chain reaction (PCR).[12] The samples were suspended in sterilized distilled water, heated at 94°C for 7 min to inactivate proteases and nucleases, and then subjected to 40 cycles of PCR in a Thermal Cycler (Perkin-Elmer Cetus). The primers for PCR to amplify the sequences spanning codon 12 or 61 of H- and K-*ras* genes in rat[13,14] were produced by a DNA synthesizer (Biosearch, Cyclone) (Table I). The products of PCR were electrophoresed on a 4% NuSieve (FMC, Rockland, MD) gel to confirm the presence of amplified sequences. Then, 5–10 μl aliquots were applied to Zeta-Probe (Bio-Rad) nylon membranes. Mutations of *ras* genes were analyzed by dot-blot hybridization using synthetic oligonucleotide probes as described by Verlaan-de Vries *et al.*[15] As the control, the wild-type sequences of H- and K-*ras* were produced from normal rat liver DNA by PCR amplification. The mutated se-

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[2] Abbreviations: NMU, *N*-nitroso-*N*-methylurea; DEN, diethylnitrosamine; GST-P, glutathione-*S*-transferase placental form; PCR, polymerase chain reaction.

Table I. Oligonucleotides for Primers

Target	Primer sequence	Fragment amplified
H-*ras* 12	5′-AAGCGATGACAGAATACAAG-3′ 5′-AGCTCACCTCTATAGTGGGA-3′	123 bp
H-*ras* 61	5′-GATGGGGAGACGTGTTTACT-3′ 5′-TCCCCTGTGCGCATGTACTG-3′	89 bp
K-*ras* 12	5′-GCCTGCTGAAAATGACTGA-3′ 5′-ATTAGCTGTATCGTCAAGG-3′	82 bp
K-*ras* 61	5′-TGGAGAAACCTGTCTCTTGG-3′ 5′-CACAAAGAAAGCCCTCCCCA-3′	104 bp

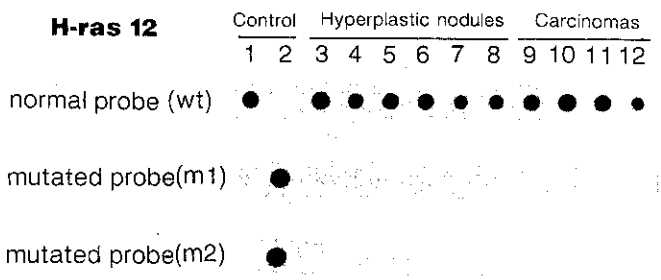

Fig. 1. Examples of dot blot analysis of liver tumors induced by NMU to detect mutations at codon 12 of the H-*ras* gene. The normal probe (wt) is the 19-mer oligonucleotide corresponding to the wild-type sequence (GGA). Mutated probes (m1, m2) are mixed oligonucleotides corresponding to the mutant sequence (m1: nGA, n=A, C; m2: GnA, n=A, C, T). Control 1 (123 bp); positive control, which was obtained by PCR amplification of normal rat genomic DNA, for normal probe. Control 2 (94 bp); positive controls, which were synthesized by PCR mutagenesis, [16] for mutated probes (for m1 probe: 5′-AGCTCACCTCTA — TCnAGCGCCCAC-3′, n=T, G; for m2 probe: 5′-AGCTCACCTCTA — TnCAGCGCCCAC-3′, n=T, G, A).

quences were also synthesized by PCR mutagenesis: that is, two primers, one of which is an oligonucleotide corresponding to the normal sequence, and the other of which is an oligonucleotide containing a single mutation in codon 12 or codon 61 of *ras* genes, were used.[16]

At the 5th week after the start of the regimen, there were many foci of GST-P(+) hyperplastic hepatocytes within the livers of either NMU- or DEN-treated rats. At 32 weeks, several hepatocellular carcinomas were present in addition to GST-P(+) nodules in both groups of animals. In all 25 GST-P(+) nodules (5 weeks) and 4 hepatocellular carcinomas (32 weeks) induced by NMU, no point mutation of H- or K-*ras* gene was detected (Fig. 1 and Table II). No point mutation of *ras* genes was found in the hepatic lesions induced by DEN (Table II).

Table II. Mutational Activation of *ras* Genes in Liver Tumors Induced by NMU or DEN

	NMU-induced tumors		DEN-induced tumors	
	Hyperplastic nodules	Carcinomas	Hyperplastic nodules	Carcinomas
H-*ras* 12	0/24	0/4	0/27	0/10
H-*ras* 61	0/21	0/4	0/27	0/9
K-*ras* 12	0/24	0/4	0/25	0/10
K-*ras* 61	0/26	0/4	0/30	0/10

These results are consistent with the previous reports of other investigators who found that point mutational activations of *ras* genes were rare in rat liver tumors induced by chemicals other than NMU.[5–7] The results of the present study clearly show that low incidence of the activation of H- and K-*ras* genes in rat liver tumors is due to the organ specificity rather than the nature of carcinogens used. Recently, it was demonstrated that neuroectodermal tumors induced by NMU in rats contained mutational activations of *neu* oncogene. This report among others indicates that mutational activations of oncogenes are different among different types of tumors, even if they are induced by the same carcinogen. It has also been reported that activation of H-*ras* gene was detected in spontaneous and chemically induced liver tumors[6, 17–19] and precancerous lesions[20] of the B6C3F1 mouse. So, it appears that the contribution of mutational activation of *ras* genes to the mechanism of carcinogenesis is different depending on the species in question.

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