RAPID COMMUNICATION Jpn. J. Cancer Res. 83, 933–936, September 1992

Absence of *ras* Family Point Mutations at Codons 12, 13 and 61 in N-Ethyl-N-hydroxyethylnitrosamine- or N-Nitrosomorpholine-induced Renal Cell Tumors in Rats

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The prevalence of Ki-*ras*, Ha-*ras* and N-*ras* point mutation within exons 1 and 2 was studied in 17 cases of renal cell tumors (8 carcinomas and 9 adenomas) induced by N-ethyl-N-hydroxyethylnitrosamine or N-nitrosomorpholine. DNA samples prepared from acetone-fixed, paraffin-embedded tissues were amplified by means of the polymerase chain reaction, and point mutations at codons 12, 13 and 61 were analyzed by direct sequence methods with oligonucleotide primers. No mutations were detected in any of the renal tumors. The results thus indicated that *ras* family point mutation is not necessary for kidney tumor development in rats, supporting the view that *ras* mutations may not be generally relevant to neoplastic development in various organs in different species.

Key words: *ras* Point mutation — Rat renal cell tumor — Nitrosamine-induced tumor — Polymerase chain reaction

Point mutations of the *ras* gene family have been reported to occur in a variety of spontaneous and chemically induced animal tumors and have also been found in surgically resected human tumors from different organs.[1-10] However, the incidence and location of mutations within *ras* genes are variable, depending on species and organs. In rats, it has been demonstrated that mammary carcinomas induced by a single dose of N-nitroso-N-methylurea (NMU) applied during the appropriate gestation stage of development show a high frequency of point mutation at Ha-*ras* codon 12.[2] On the other hand, point mutation at Ha-*ras* codon 13 was detected more frequently than that at codon 12 or 61 in Zymbal's gland tumors induced by heterocyclic amines.[11] Recently, intensive studies regarding *ras* point mutation have revealed such alterations in several chemically induced tumors in rats. This includes, for example, Ha-*ras* at codon 12 in esophageal papillomas induced by N-methyl-N-benzylnitrosamine,[12] Ha-*ras* at codon 12 and/or 61 in urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nitrosamine,[13] Ki-*ras* at codon 12 in prostatic carcinomas and sarcomas induced by NMU,[14] Ki-*ras* at codon 12, 13 or 59 in colon tumors induced by 1,2-dimethylhydrazine,[15] and Ki-*ras* codon 12 in kidney tumors.[16]

Therefore, it would appear that *ras* point mutation might be involved to some extent to tumor development in the rat. For the purpose of clarifying the relevance of such mutations in rat tumors, it is important to investigate their occurrence in various tumor types induced by different carcinogens. The present study was performed to examine the incidence of *ras* family oncogene mutation in chemically induced renal cell tumors in the rat.

Male Wistar and F344 rats (Charles River Japan Inc., Atsugi) were purchased at 6 weeks of age and given 0.1% N-ethyl-N-hydroxyethylnitrosamine (EHEN) (Tokyo Kasei Kogyo Co. Ltd., Tokyo) in drinking water for the initial 4 weeks, then maintained for up to 40 weeks until renal cell tumors developed. Male SD rats (Charles River Japan Inc.) received 0.02% N-nitrosomorpholine (NNM) (Tokyo Kasei Kogyo Co. Ltd.) in drinking water for 2 weeks and were then maintained for up to 60 weeks. Resected renal tumor tissues were immediately fixed in acetone at 0°C, embedded in paraffin and stained with hematoxylin and eosin (H&E) for histological examination. A total of 17 primary renal tumors (8 carcinomas and 9 adenomas) were serially sectioned at 4 μm thickness and mounted on a slide glass. Tumor areas uncontaminated with surrounding tissue were carefully selected then taken by the use of a dissecting microscope. DNA samples were prepared by incubation in poly-

Abbreviations: EHEN, N-ethyl-N-hydroxyethylnitrosamine; NNM, N-nitrosomorpholine; NMU, N-nitroso-N-methylurea; PCR, polymerase chain reaction.

Table I. Primer Sequences Used for PCR and Nucleotide Sequencing

	PCR and third primer	Length of amplified fragment
Ki-*ras* 12, 13	5′-GCCTGCTGAAAATGACTGAG-3′	120 bp
	5′-CTATCGTAGGATCATATTCA-3′	
Third primer	5′-AGTGATTCTGAATTAGCTGT-3′	
Ki-*ras* 61	5′-GACTCCTACAGGAAACAAGT-3′	130 bp
	5′-CACAAAGAAAGCCCTCCCCA-3′	
Third primer	5′-GTAATTGATGGAGAAACCTG-3′	
Ha-*ras* 12, 13	5′-CTGTAGAAGCGATGACAGAA-3′	103 bp
	5′-TCGTCCACAAAATGGTTCTG-3′	
Third primer	5′-AAGCGATGACAGAATACAAG-3′	
Ha-*ras* 61	5′-TTTGCAGGACTCCTACCGGA-3′	172 bp
	5′-GGTCACCTGTACTGATGGAT-3′	
Third primer	5′-TGCAGGACTCCTACCGGAAA-3′	
N-*ras* 12, 13	5′-TCGTAATTGCTGCTTTCC-3′	160 bp
	5′-ATAAGGACCAGGCAGTGG-3′	
Third primer	5′-CCTACAGATTTTTGCAGGTG-3′	
N-*ras* 61	5′-AATAAAGTTTGGGGCTGT-3′	267 bp
	5′-GGTGTTTCAGAAAACATT-3′	
Third primer	5′-GATGGCAAACACACAGAGGA-3′	

merase chain reaction (PCR) buffer supplemented with 0.45% Tween 20 (Nihon Bio-Rad Co. Ltd., Tokyo), 0.45% NP 40 (Sigma Chemical Co., St. Louis, MO), and 10 mg/ml proteinase K, at 55°C for 1 h, and then at 90°C for 10 min to inactivate proteinase K, after deparaffinization with xylene.[17] Oligonucleotide primers were synthesized using a DNA synthesizer (Model 391, Applied Biosystems, Foster City, CA). Sequences of the oligonucleotide primers for PCR and the oligonucleotide third primers for direct sequencing are shown in Table I. Approximately 100 ng of DNA from each tumor was amplified by PCR with 2.5 units of *Taq* polymerase (Takara Biochemicals Co. Ltd., Kyoto) using 50 and 5 pmol of forward and reverse primers to detect point mutation at codons 12, 13 and 61 for each of Ki-*ras*, Ha-*ras* and N-*ras*. The relevant primer sequences were based on rat Ki-*ras*, Ha-*ras* and N-*ras* genes, respectively.[18–20] A total of 100 μl of reaction mixture was subjected to 50 cycles of reaction, consisting of a 45-s denaturing step at 94°C, followed by a 1-min annealing step at 55°C and then a 2-min polymerization step at 72°C, using a programmed temperature control system (ASTEC Co. Ltd., Fukuoka). After purification of the product by phenol/chloroform extraction and ethanol precipitation, unincorporated nucleotides and primers were removed by filtration through a polysulfone filter with a cut-off MW of 10,000 (Ultrafree C3GC, Millipore Ltd. Japan, Tokyo). The third primers were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and sequencing reactions were performed with Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions. In none of the 8 carcinomas and 9 adenomas investigated could point mutations of Ki-, Ha- and N-*ras* genes be detected (Table II). Examples to illustrate the lack of point mutations in Ki-*ras* codons 12 and 13 in representative DNA samples from benign and malignant lesion are shown in Fig. 1. The plasmid into which a rat lung carcinoma Ki-*ras* sequence was ligated (unpublished results by Ushijima *et al.*), used as the control, showed mutation at the second position of Ki-*ras* codon 12 (GGT to GAT).

Table II. Incidences of Mutational Activation of *ras* Genes in Renal Cell Tumors Induced by EHEN or NNM

Codon	Carcinomas	Adenomas
Ki-*ras* 12, 13	0/8	0/9
Ki-*ras* 61	0/8	0/9
Ha-*ras* 12, 13	0/8	0/9
Ha-*ras* 61	0/8	0/9
N-*ras* 12, 13	0/8	0/9
N-*ras* 61	0/8	0/9

The present results do not support the reported high incidence of point mutation in Ki-*ras* codon 12 in renal epithelial tumors induced by dimethylnitrosamine.[16]

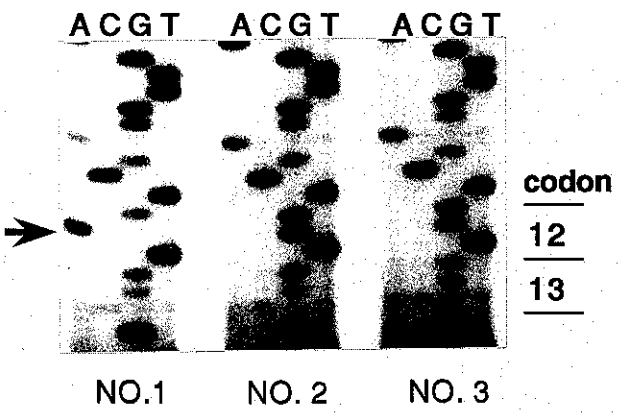

Fig. 1. Results of direct sequencing around codons 12 and 13 of Ki-*ras*. Sample numbers 1, 2 and 3 correspond to positive control, adenoma and carcinoma, respectively. The second position at codon 12 of sample No. 1 was mutated from G to A (arrow), however, no point mutations at codon 12 or 13 are evident in samples 2 and 3.

However, our results are generally in line with previous reports of other investigators indicating that point mutations of *ras* genes are rare in rat liver tumors induced by different chemical carcinogens except aflatoxin B_1.[21-24] These variable incidences could indicate that the mutations may be linked with the carcinogens given. Although activation of Ki-*ras* and N-*ras* assessed by the NIH/3T3 transfection assay was reported in renal mesenchymal tumors induced by administration of methyl(methoxymethyl)nitrosamine to newborn Fischer 344 rats,[25] this is clearly not the case for kidney tumors of epithelial origin. Further comparative investigations regarding such gene mutations proposed to be relevant to tumor development should give a better understanding of whether or not they actually have an essential role to play. Although we have not examined *ras* mutations other than in codons 12, 13 and 61 by means of single strand conformation polymorphism (SSCP) analysis, it appears that *ras* point mutation may not be involved in the development of chemically induced renal cell tumors in rats.

This research was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and from the Ministry of Health and Welfare, and by a Research Grant from Fujita Health University. The authors would like to express their gratitude to Katsumi Imaida, Nagoya City University Medical School, for providing rat lung carcinomas for use as the positive control.

(Received May 6, 1992/Accepted June 24, 1992)

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