Jpn. J. Cancer Res. 86, 802–810, September 1995

Role of Point Mutation of the K-*ras* Gene in Tumorigenesis of B6C3F$_1$ Mouse Lung Lesions Induced by Urethane

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In order to elucidate the role of point mutation of the K-*ras* gene in the tumorigenetic process of lung tumors, an experimental model of lung lesions in mice induced by the administration of urethane was used. A total of 135 B6C3F$_1$ male mice, 6 weeks old, were given urethane in the drinking water at 0, 6, 60, 600 or 1200 ppm, and were then killed after varying periods of time. The lung lesions were histologically characterized as hyperplasia, adenoma and adenocarcinoma. Point mutations in codons 12 and 61 of the K-*ras* gene were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and confirmed by using dideoxy sequencing analysis. K-*ras* gene mutation was identified in 9 (23.7%) of 38 lesions classified as hyperplasia, 31 (46.3%) of 67 adenomas, and 3 (50%) of 6 adenocarcinomas. The most frequent mutation was an AT-to-TA transversion at the second base of codon 61 and this pattern accounted for 65% of the three mutant forms observed. These results suggest that the point mutation of K-*ras* gene is involved in all stages of mouse lung tumorigenesis, i.e., activation of this gene can also influence the later stages of lung lesions.

Key words: Urethane — Ethyl carbamate — K-*ras* gene — B6C3F$_1$ mouse — Lung tumor

Animal model systems have led to major advances in our understanding of tumorigenesis in humans and furthermore have recently been used to elucidate whether activation of various oncogenes and functional loss of tumor suppressor genes are correlated with the initiation and progression of tumors.[1,2] In particular, activation of the *ras* oncogene has been identified in various tumors including skin papillomas, murine lung adenoma, pancreatic ductal hyperplasia of the hamster, and others.[2–18] Moreover, mutations of H- and K-*ras* genes detectable before the histological onset of tumors have been reported, and they have been identified in normal rat mammary cells within 12 days after carcinogen treatment.[4] This indicates that *ras* oncogene activation arises in the early stage of tumorigenesis, and suggests that these genes play an important initial role in the multistep tumorigenetic process.[1,4,6,16,17,19,20]

Point mutations in the *ras* gene family, H-, K-, and N-*ras*, have been observed at codons 12, 13, and 61 in rodent tumors induced by chemical carcinogens with mutagenic activity. It is supposed that these mutations initiate transformation in pre-neoplastic or pre-malignant cells, which then progress to more malignant stages, possibly acting in collaboration with additional promotional factors such as other genetic or epigenetic events.[1,4] In mouse lung, the K-*ras* gene (a member of the *ras* family) is known to have a high rate of mutation in tumors induced by chemical carcinogens and in those arising spontaneously. There are several reports of mouse lung lesions induced by chemical carcinogens including ethyl carbamate (urethane), benzopyrene, tetranitromethane and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK).[2,5,8,12,15] These carcinogens induce a specific type of mutation in the K-*ras* gene, probably via carcinogen-specific DNA adduct formation.[11,15,21–23] In the case of urethane, it was demonstrated that activating mutations occurred in codon 61 of the K-*ras* gene, affecting the second base in codon 61 and resulting in the substitution of either arginine (CGA)(AT-to-GC transition) or leucine (CTA)(AT-to-TA transversion) for the wild-type glutamine (CAA).[2,5,8,24] However, it is still unclear at which stage in the tumorigenetic process the mutation occurs.

Therefore, in order to establish when mutation of the K-*ras* gene occurs, we attempted to examine the frequency of such mutations at each stage of the tumorigenetic process, using a mouse lung tumor model induced by urethane. In addition, the influences of dose and duration of administration of urethane on K-*ras* gene mutation were examined.

MATERIALS AND METHODS

Experiments and tissue preparations One hundred and thirty-five 6-week-old C57BL/6J×C3H/HeJ (B6C3F$_1$) male mice were purchased from Charles River Japan, Inc. The mice were given urethane dissolved in saline in place of drinking water, and were subdivided into 14 groups according to the urethane concentration, time and period of treatment as shown in Fig. 1. At the end of each experiment, the lungs were removed from the killed mice, and fixed with 70% ethyl alcohol at 4°C followed

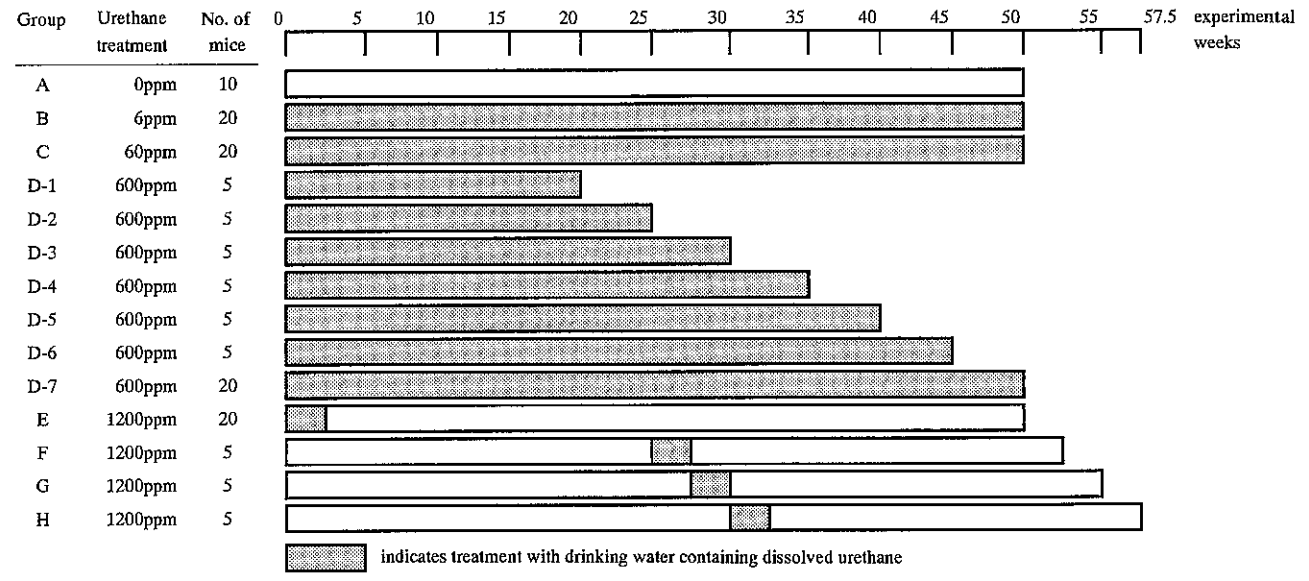

Fig. 1. Experimental design.

by buffered paraformaldehyde for 12–24 h. Sections 4 μm-thick were cut and stained with hematoxylin-eosin (H & E) for morphological observations.

Histological classification Lung lesions were classified into 3 categories: alveolar/bronchiolar hyperplasia, adenoma, and adenocarcinoma. Hyperplasia was defined as the presence of mildly atypical cells with no significant destruction of the original histological structure and without expansive growth (Fig. 2A). Adenoma was defined as the presence of moderately atypical cells or those with nuclear pleomorphism and was characterized by evident compression of adjacent alveolar tissue (Fig. 2B). Adenocarcinoma involved markedly atypical tumor cells with occasional abnormal mitoses and invasive growth into adjacent alveolar tissue, bronchioles or vessels (Fig. 2C).

Isolation of tissue (microdissection method) and DNA isolation Ten 10-μm thick sections were cut from a paraffin block, and then deparaffinized. The lesions were scraped from each slide using a 27 gauge needle observed under the microscope, taking special care to limit the amount of surrounding tissue removed. The tissue samples were placed in 100% ethanol, centrifuged for about 2 min to deposit the tissue at the bottom of the tubes, and then dried in a vacuum. Digestion was performed using proteinase K for 12–24 h at 37°C, followed by incubation at 95°C for 10 min to inactivate the enzyme. Phenol-chloroform extraction was carried out three times. The precipitates were dissolved in 100–150 μl of TE buffer (template DNA) for use in the polymerase chain reaction (PCR).

PCR DNA was amplified by the PCR method of Saiki *et al.*[25] The amplification primers were prepared to amplify the 1st and 2nd exons of mouse K-*ras* gene as reported by Belinsky *et al.*[15]: 1st exon, 5′-primer 5′-ATGACTGAGTATAAACTTGT-3′, 3′-primer 5′-TCGTACTCATCCTCAAAGTG-3′; 2nd exon, 5′-primer 5′-TACAGGAAACAAGTAGTAATTGATGGAGAA-3′, 3′-primer 5′-ATAATGGTGAATATCTTCAAATGATTTAGT-3′. PCR conditions were 95°C for 1 min, 51–52°C for 2 min, and 72°C for 2 min. The process included 45 (exon 1) and 40 (exon 2) cycles of amplification on an MJ research thermal cycler, using Taq DNA polymerase (Takara). Exon 1 generated a fragment of 98 base pairs and exon 2 one of 171 base pairs. Amplified DNA products of the PCR were electrophoresed in a 4% agarose gel (Nusieve), followed by ethidium bromide staining.

Restriction fragment length polymorphism (PCR-RFLP) method A PCR-RFLP method was used to identify predominant mutations in the mouse K-*ras* gene, because this method has the advantage that many samples can be processed simultaneously. The specific patterns of K-*ras* gene mutations in codons 12 and 61 treated with various chemical carcinogens have been reported.[2, 5, 8, 24] The predominant K-*ras* gene mutation in codon 12 was GGT (wild-type sequence) to GAT transition, and therefore, the restriction enzyme *Hph* I [5′-GGTGA(N)$_8$*-3′ (* indicates the restriction site) obtained from New England Biolaboratory (NEB Inc.)] was chosen to ascertain the point mutation by using a mismatch primer containing a G mismatch instead of a C in the second

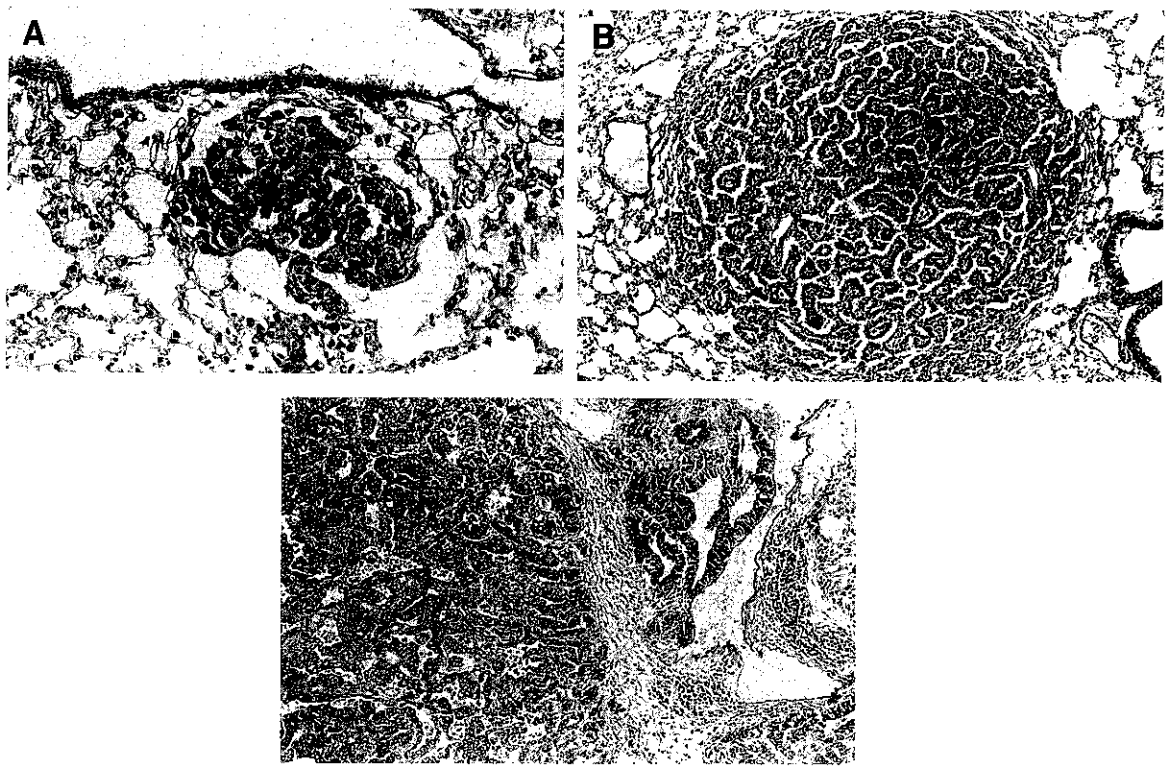

Fig. 2. Microscopical views of lung lesions. A. Alveolar/bronchiolar hyperplasia (H & E staining, ×130). B. Alveolar/bronchiolar adenoma (H & E staining, ×65). C. Alveolar/bronchiolar adenocarcinoma (H & E staining, ×100).

Table I. Incidence of Lung Lesions Induced by Urethane in Mice

Group	Urethane dose (ppm)	Weeks of treatment (range)	No. of effective (initial) mice	No. of mice (%) with lung lesions	Mean no. of lung lesions per mouse	No. of lung lesions		
						Hyperplasia	Adenoma	Adeno-carcinoma
A	0	—	9 (10)	2 (22)	0.6	3	2	0
B	6	50	20 (20)	2 (10)	0.1	1	1	0
C	60	50	19 (20)	10 (53)	0.6	2	8	1
D-1	600	20	5 (5)	0 (0)	1.0	5	0	0
D-2	〃	25	5 (5)	4 (80)	3.2	14	2	0
D-3	〃	30	5 (5)	5 (100)	4.6	18	5	0
D-4	〃	35	3 (5)	3 (100)	6.3	8	11	0
D-5	〃	40	4 (5)	4 (100)	13.0	22	30	1
D-6	〃	45	5 (5)	5 (100)	6.6	8	25	0
D-7	〃	50	13 (20)	13 (100)	7.6	29	66	4
E	1200	2.5 (0–2.5)	13 (20)	13 (100)	2.3	9	16	5
F	〃	2.5 (25–27.5)	3 (5)	0 (0)	0	0	0	0
G	〃	2.5 (27.5–30)	3 (5)	0 (0)	0	0	0	0
H	〃	2.5 (30–32.5)	2 (5)	1 (50)	1.0	1	1	0
			Total number of lung lesions			120	167	11

base from the 3′ end. The sequence of the mismatch primer was 5′-AACTTGTGGTAGTTGGAGGT-3′ and the antisense primer was 5′-TTACCTCTATCGTAGGGTCGTACTCATCCA-3′. PCR amplification using mismatch primers generated a fragment of 102 base pairs. The mismatch nucleotide at the site of the point

mutation is part of the recognition sequence of a restriction enzyme used in RFLP analysis. PCR products generated using this strategy will contain the enzyme cleavage site only if they are derived from the mutated locus. Amplified DNA products were therefore digested with *Hph* I at 37°C for 2–3 h followed by the NEB reaction protocol.

On the other hand, in codon 61 of exon 2, the restriction enzyme *Xba* I [5′-T*CTAGA-3′ (* indicates the restriction site); NEB] was chosen to recognize a CAA (wild-type sequence) to CTA transversion, and the enzyme *Taq* I [5′-T*CGA-3′ (* indicates the restriction site); NEB] to recognize a CAA-to-CGA transition. Both enzymes recognize a 2nd base substitution in codon 61 as a single point mutation in the K-*ras* gene. The conditions of these enzyme reactions were as follows: digestion with *Xba* I for 2–3 h at 37°C and with *Taq* I for 2–3 h at 65°C. After the reactions, 10 μl of the PCR-RFLP products was electrophoresed in 4–6% agarose and/or 8% polyacrylamide gels. The former was run for 30 min at 100 V, and the latter for 3–4 h at 200 V; digested DNA bands were visualized by ethidium bromide staining. The method was based on previous reports.[15, 26]

Dideoxy sequencing analysis Sequencing primers (1–2 pmol) for exons 1 and 2 were the same as those described above. These primers, end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (double-stranded DNA cycle sequencing kit; GIBCO BRL), were added to the reaction mixture and the labeled primer-DNA mixture was added to 4 tubes containing dideoxy-ATP, dideoxy-CTP, dideoxy-GTP, or dideoxy-TTP. Thermal cycle programs were then carried out and terminated with 5 μl of stop solution. Samples were heated at 90°C for 5 min and subjected to electrophoresis on an 8% denaturing polyacrylamide gel. The gel was dried and then exposed to X-ray film for 18–24 h.

RESULTS

Incidences of mice with lung lesions The incidence of mice with lung lesions is shown in Table I. In group B, the incidence was lower than that of group A, but in group C, it was higher than that of group A. In group D, all mice with more than 30 weeks of treatment showed a gradually increasing number of lung lesions in proportion to the period of treatment. The mice in group E showed a high incidence, but in the other groups (F, G and H) lung lesions were rare. The total numbers of lesions were 120 hyperplasias, 167 adenomas, and 11 adenocarcinomas.

***Identification of K-ras* mutations in lung lesions** When the PCR products derived from DNA with the mutated locus in codon 12 or codon 61 as described in "Materials and Methods" are digested with these restriction enzymes, DNA fragments are cleaved by *Hph* I to yield subfragments of 72 and 30 base pairs, by *Xba* I to yield subfragments of 108 and 63 base pairs, and by *Taq* I to yield subfragments of 108, 50, and 13 base pairs. The PCR product derived from the normal allele was also cut with *Taq* I enzyme, producing subfragments of 121 and 50 base pairs. These results of the PCR-RFLP technique

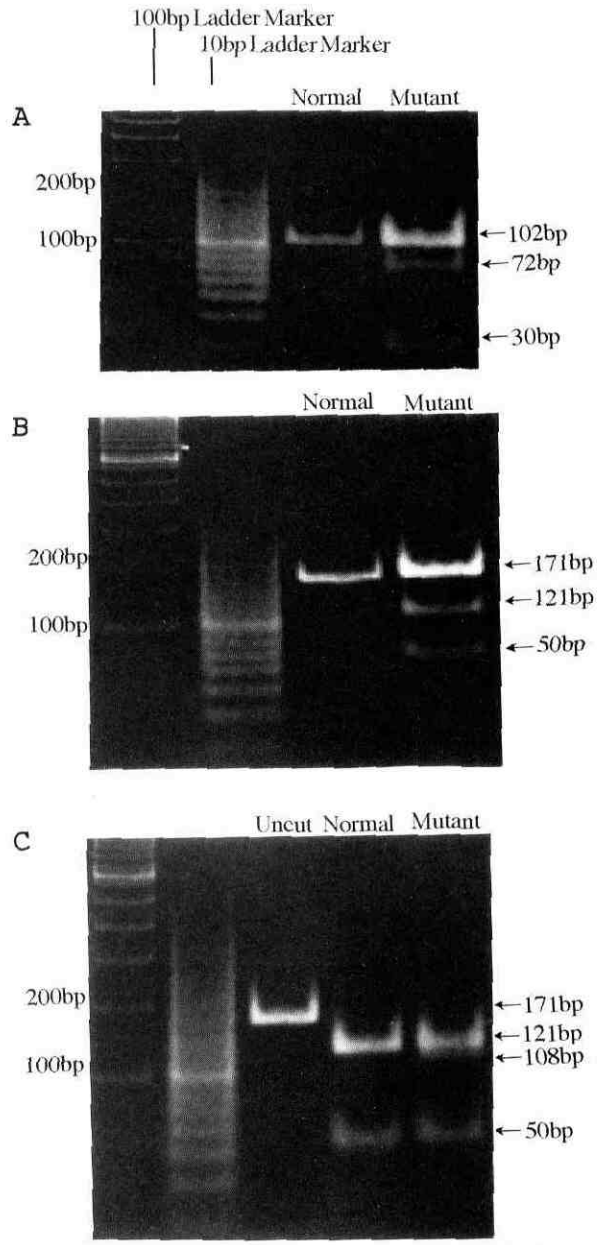

Fig. 3. Result of PCR-RFLP analysis using restriction enzymes. A. Detection of GGT-to-GAT mutation in codon 12 by using *Hph* I enzyme. B. Detection of CAA-to-CTA mutation in codon 61 by using *Xba* I enzyme. C. Detection of CAA-to-CGA mutation in codon 61 by using *Taq* I enzyme.

Table II. PCR-RFLP Analysis of Mutations in K-*ras* Gene from Lung Lesions Induced by Urethane in Mice

Lung lesion	No. of lesions examined	No. of lesions with activated K-*ras* (%)	No. of lesions with codon 12 (GGT, Gly) mutations	No. of lesions with codon 61 (CAA, Gln) mutations	
			GAT (Asp)	CTA (Leu)	CGA (Arg)
Hyperplasia	38	9 (23.7)	1	7	1
Adenoma	67	31 (46.3)	6	19	6
Adenocarcinoma	6	3 (50.0)	0	2	1
Total	111	43 (38.7)	7	28	8

Table III. Comparison of Frequencies of K-*ras* Mutation in Adenomas between Experimental Groups

Group (ppm)	Weeks of treatment	No. of lesions examined	No. of lesions with activated K-*ras* (%)	No. of lesions with codon 12 (GGT) mutations	No. of lesions with codon 61 (CAA) mutations	
				GAT	CTA	CGA
A (0)	–	2	0 (0)	0	0	0
B (6)	50	1	0 (0)	0	0	0
C (60)	50	5	2 (40.0)	1	0	1
D-1 (600)	20	0	–	–	–	–
D-2 (〃)	25	0	–	–	–	–
D-3 (〃)	30	3	1 (33.3)	1	0	0
D-4 (〃)	35	3	0 (0)	0	0	0
D-5 (〃)	40	8	5 (62.5)	2	3	0
D-6 (〃)	45	4	3 (75.0)	0	3	0
D-7 (〃)	50	25	17 (68.0)	2	15	0
E (1200)	2.5	15	1 (6.7)	0	0	1

are shown in Fig. 3. One hundred and eleven lung lesions were examined by this method, and K-*ras* gene mutation was detected in 9 (23.7%) of 38 hyperplastic lesions, 31 (46.3%) of 67 adenomas, and 3 (50.0%) of 6 adenocarcinomas (Table II). The incidence of K-*ras* gene mutation in lung lesions increased in proportion to the biological grade as defined by histological classification. The most frequent K-*ras* gene mutation was an AT-to-TA transversion at the second base of codon 61 and this pattern accounted for 65% of the three forms of mutation. There was no difference in the pattern of mutation among the various grades of histological classification of lung lesions.

The K-*ras* gene mutation was looked for in 9 spontaneous lung adenomas in B6C3F$_1$ mice which had not received any treatment (including 7 tumors examined in the previous study in our laboratory), but no mutation was identified in these tumors.

Table III shows a comparison of the incidence of K-*ras* gene mutation in adenomas among the groups. As the treatment period was longer in group D, the frequency of mutation was higher. In group E, K-*ras* gene mutation was rare. No lung lesion was found with both codon 12 and 61 mutations in the K-*ras* gene. When 24 samples of normal lung tissue from urethane-treated mice were examined, no K-*ras* gene mutation was identified in either codon 12 of exon 1 or codon 61 of exon 2. The presence of these mutations detected by the PCR-RFLP method was confirmed by dideoxy sequencing analysis (Fig. 4).

DISCUSSION

Urethane has been shown previously to be a multipotential carcinogen with initiating and promotive abilities, and it has been reported that the lung is one of the organs susceptible to tumorigenesis initiated by urethane.[21, 22, 27–32] In general, carbamates induce the same spectrum of tumors in mice and rats, and vinyl carbamate, a structural analog of urethane, has a greater carcinogenic activity than ethyl carbamate. These carbamates are metabolized to vinyl carbamate epoxide, which is thought to be the ultimate carcinogenic metabolite. It has been reported that these carbamates and their metabolites may share common steps in tumorigenesis, but the mechanisms of genetic susceptibility and the induction of mutations by these metabolites, as well as the nature and the structure of DNA adducts formed in the lung, have not yet been elucidated.[21, 22, 28, 32, 33]

Susceptibility to urethane-induced pulmonary tumorigenesis varies among inbred strains of mice. B6C3F$_1$ mice

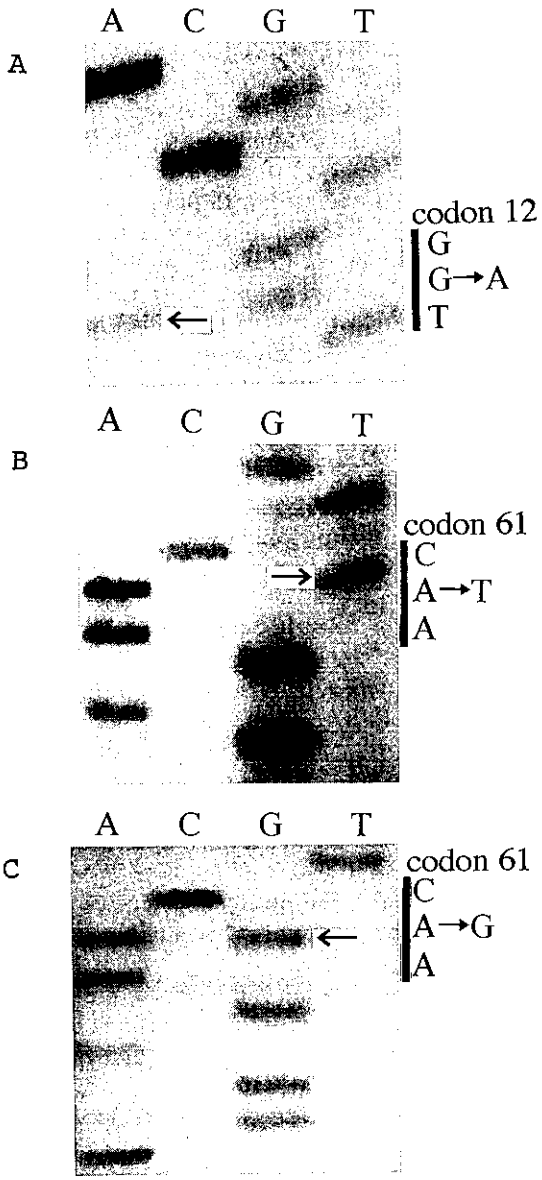

Fig. 4. Identification of point mutations in the K-*ras* gene by using dideoxy sequencing analysis. A. Dideoxy sequencing analysis demonstrates GGT-to-GAT mutation in codon 12, indicated by the arrow. B. CAA-to-CTA mutation in codon 61. C. CAA-to-CGA mutation in codon 61.

have low susceptibility to urethane in comparison with strain A/J or BALB/cByJ mice, and it has been reported that the incidence of spontaneous lung tumors is approximately 10–20%.[8, 24, 30, 31, 33] In the present experiment, the incidence of lung lesions in the control group was similar to those reported in previous studies.[31] In most of the previous reports,[2, 5] lung lesions were classified into lung adenomas and adenocarcinomas, but in the present experiment adenomas were further divided into two groups, hyperplasia and adenoma, because the aim of this study was to examine pre-neoplastic lesions more precisely. According to this histological classification, adenomas arising in hyperplasia or adenocarcinomas arising in adenoma were occasionally identified in the present study, and therefore a sequential development, hyperplasia-adenoma-carcinoma, of the lung lesions induced by urethane was established.

The incidence and multiplicity of lung lesions were correlated with the concentration of urethane and the duration of treatment, and the present results confirmed the tumorigenicity of urethane. Interestingly, the incidence of lung lesions in the 6 ppm group was lower than that in the control group. Our previous study showed the same tendency.[31] In addition, the incidence of lung lesions in the 1200 ppm group was lower than that in the 600 ppm groups. These results may indicate the importance of duration of treatment in tumorigenesis. Furthermore, from the results in the 1200 ppm groups, beginning the administration of urethane while the mice were young appeared to induce a higher incidence of lung lesions as well as increasing the extent of progression to carcinoma. These observations are compatible with tumorigenesis by chemical carcinogens in general.

The *ras* oncogene has been found to be activated in a variety of chemically induced animal tumors. Activation of the K-*ras* gene family (H, K, and N-*ras*) has been detected in lung tumors, including pre-neoplastic lesions. The most frequent activating sites in the K-*ras* gene, so-called "hot spots," have been reported as codon 12 of exon 1 and codon 61 in exon 2, and furthermore a second base change in these codons has been commonly demonstrated. Also, the base substitution that results from exposure to certain chemicals is specific.[7, 8, 10–12, 15–17, 24, 33, 34]

The selectivity of mutation in the K-*ras* gene induced by urethane has been studied by several investigators.[2, 5] In strain A mice, K-*ras* gene activation was detected in 10 of 11 lung tumors induced by urethane, and the mutations were identified as follows: 7 tumors showed an AT-to-TA transversion (CTA) in the second base of codon 61 and 2 tumors showed an AT-to-GC transition (CGA), detected by NIH3T3 transfection assay.[8] On the other hand, 6 of 10 spontaneous lung tumors in strain A mice showed a point mutation in codon 12. However, there has been a report that K-*ras* gene mutation was identified in only 1 of 10 spontaneous lung tumors occurring in B6C3F$_1$ mice. Furthermore, lung adenoma and adenocarcinoma induced by urethane were reported to show different patterns of mutant K-*ras* gene expression, that is, very small adenomas primarily expressed AT-to-TA transversion, while adenocarcinoma and transformed lung cell lines primarily expressed AT-to-GC transition in the second base of codon 61.[2] The

frequency of K-*ras* gene mutation in mouse lung tumors induced by urethane has also been investigated in relation to tumor size. Preferential activation of the K-*ras* gene in codon 61 occurred in most lung tumors, but there was no difference in the mutation pattern between small and large tumors.

On the basis of the results of previous work and the present study, urethane is a potent inducer of mouse lung tumors,[31] and the predominant mutation in the K-*ras* gene induced by urethane is AT-to-TA transversion in the second base of codon 61, which was found in 65% of the lung lesions in which mutation had occurred in our study. However, there was no clear difference in the predominant pattern of K-*ras* gene mutation related to the histological appearance, although the frequency of mutation increased as the lesions progressed from hyperplasia to adenoma or adenocarcinoma.

Certainly, the findings in the present study are consistent with the hypothesis that such an activated K-*ras* gene is seen in the early stage of tumorigenesis and is concomitant with initiation of neoplasia. However, as the frequencies of mutation were 46.3% in adenoma and 50% in adenocarcinoma, it appears that K-*ras* gene mutation did not necessarily occur only in the early stage of tumorigenesis, but could also influence the later stages. In other words, it appears that K-*ras* gene mutation is involved in all stages of tumorigenesis.

The lower frequency of mutation in hyperplasia than in adenoma and adenocarcinoma throws doubt on the hypothesis that K-*ras* gene mutation occurs in the early stage, but several explanations for the lower frequency are possible. Firstly, it is possible that K-*ras* gene mutation occurs in only very few cells in a lesion and therefore, may not be detected; mutated cells might subsequently have a selective advantage.[16] Secondly, other types of K-*ras* gene mutation may exist, which can not be detected by the PCR-RFLP method used in the present study. Thirdly, the lesions diagnosed as hyperplasia on the basis of histological findings may include reactive or reversible lesions induced by chronic irritation or inflammation. Therefore, the frequency of mutation in hyperplasia has possibly been underestimated.

When the frequencies of K-*ras* gene mutation in adenoma after different periods of carcinogen administration were compared, it was found that the frequency increased in proportion to the length of the administration period. In addition, K-*ras* gene mutation was identified in only 1 of 16 adenomas occurring in the group given 1200 ppm while they were young. Taking these facts into consideration, we suggest that the occurrence of *ras* gene mutation may be associated with long-term carcinogen exposure, and *ras* gene mutation associated with short-term exposure may be reversible. Some lesions of hyperplasia will occur without mutation of the K-*ras* gene. Using only histological observation, it is not possible to distinguish whether the lesion involves K-*ras* gene mutation or not. It is known that, when normal cells are induced to proliferate clonally, and then acquire malignant potential, there is an accumulation of many gene alterations. For instance, *ras* gene activation by itself is insufficient for full expression of the tumorigenic phenotype, and other alterations including activation of other oncogenes or deletion of tumor suppressor genes are required.[1, 4, 20, 35–37] Therefore, further analysis is necessary to elucidate the role of K-*ras* gene mutation in lung tumorigenesis and the possible association with other oncogenes or with deletion of tumor suppressor genes.

The function of the *ras* gene may be related to the signal transduction system; if so, mutation of the *ras* gene may change the normal cellular proliferation program to one of uncontrolled growth, if the mutated *ras* protein loses its ability to become inactivated and thus stimulates growth or differentiation autonomously.[1, 4, 20, 35, 36] However, the numerical value of labeling indices of bromodeoxy-uridine and proliferative cell nuclear antigen did not increase in those lung lesions in which K-*ras* gene mutation was identified, and there was no positive correlation between the presence of K-*ras* gene mutation and the proliferative activity of these lesions (data not shown). Consequently, the precise function of the mutated *ras* gene remains unclear; further studies are necessary.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

(Received April 3, 1995/Accepted June 13, 1995)

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