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Effects of K-*ras* Gene Mutations in the Development of Lung Lesions Induced by 4-(*N*-Methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone in A/J Mice

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The relationship between the development of peripheral lung lesions induced by tobacco-specific 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and K-*ras* gene mutation in A/J mice, and the correlations between histological alterations and the course of lung lesion development after NNK treatment and K-*ras* gene mutation were investigated. The acquisition of a selective growth advantage by the lung lesions with mutations was also examined using immunohistochemical labeling with bromodeoxyuridine. Thirty female 5 weeks old A/J mice were each injected intraperitoneally with a single dose of NNK (100 mg/kg body weight) and subdivided into 6 groups according to the time after NNK treatment. The lung lesions were characterized histologically as alveolar/bronchiolar hyperplasia, adenoma and adenocarcinoma, and point mutations in codons 12 and 61 of the K-*ras* gene were detected by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) and dideoxy sequencing methods. K-*ras* gene mutations were identified in 7 (58.3%) of 12 hyperplasias, 42 (75.0%) of 56 adenomas and 3 (75.0%) of 4 adenocarcinomas. The most frequent K-*ras* gene mutation was a G-to-A transition at the second base of codon 12 and this accounted for 86.5% of all the mutations detected. Neither the frequency of activation of this gene nor the specific mutation was affected by the time after NNK treatment and there was no positive correlation between the proliferative activity of lung lesions and the presence of K-*ras* gene mutations. Thus, K-*ras* gene mutation is closely associated with the development of NNK-induced peripheral lung lesions in A/J mice, but it plays no role in the selective growth advantage of these lesions.

Key words: 4-(*N*-Methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) — K-*ras* gene — A/J mouse — Lung tumor

Recently, the incidence of lung carcinoma in humans has been found to be increasing dramatically. Furthermore, the ratio of the incidence of adenocarcinoma relative to that of squamous cell carcinoma has increased rapidly during the past two decades. Therefore, it is important to understand the carcinogenic process of lung adenocarcinoma.[1] However, in contrast to squamous cell carcinoma, little is known about the preneoplastic and/or premalignant lesions preceding adenocarcinoma of the human lung.[2] Experimental models of lung adenocarcinomas induced by various chemical carcinogens in small rodents have yielded information on carcinogenic processes, and they appear to be useful for analyzing genetic abnormalities involved in these processes.

Epidemiological studies over the past several decades have provided a considerable body of evidence linking lung carcinoma to chemicals in cigarette smoke.[1,3] It is well known that tobacco and its smoke contain many mutagenic and carcinogenic compounds, including a variety of nitrosamines.[4,5] Of these compounds, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a major tobacco-specific nitrosamine, present in both mainstream and sidestream smoke, as well as in unburned tobacco.[5] The results of experiments on small rodents have identified NNK as a potent carcinogen of various organs, such as the lung, nasal cavity, liver and pancreas.[6–8] In such experiments, sequential development of hyperplasia-adenoma-adenocarcinoma has been observed in the peripheral lung of small rodents.[9–11] Furthermore, several workers have reported high K-*ras* gene mutation frequencies in these lesions, a specific mutational spectrum related to DNA adduct formation was observed[12–18] and other studies showed that the nature of the K-*ras* gene mutation differed according to the histological type of the lesion.[19]

Peripheral adenocarcinoma of the lung is generally considered to show a less definite relationship than squamous cell carcinoma to cigarette smoking in humans.[3] Interestingly, however, the relatively steep increase in the incidence of adenocarcinoma has been hypothesized to be associated with the higher NNK content of modern, compared with previous, cigarette smoke.[1] On the basis of the above findings, knowledge of a causal relationship between NNK exposure and lung tumorigenesis, including genetic abnormalities, could be important for preventing human lung adenocarcinomas.

Therefore, we examined the K-*ras* gene mutation frequency in NNK-induced lung lesions in A/J mice in

order to elucidate whether specific mutations of the K-*ras* gene correlate with the histological alterations and developmental course of these lung lesions.

MATERIALS AND METHODS

Experimental design and tissue preparation NNK was obtained from Chemsyn Science Laboratories (Lenexa, KS). Forty female 4 weeks old A/J mice were purchased from Charles River Japan Inc. (Tokyo). When they were 5 weeks old, 30 were each injected intraperitoneally with a single dose of NNK (100 mg/kg body weight) dissolved in 0.9% NaCl and subdivided into 6 groups according to the time after treatment, as shown in Fig. 1; the remaining 10 were untreated controls. At the end of each experiment, 20 mg/kg body weight bromodeoxyuridine (BrdU) (Sigma Chemical, St. Louis, MO) was injected intraperitoneally, in order to evaluate the proliferative activity of the lung lesions, and 1 h later, the mice were killed and their lungs were removed and fixed with 70% ethyl alcohol at 4°C, followed by buffered paraformaldehyde for 12–24 h. Four-μm-thick sections were cut and stained with hematoxylin-eosin for morphological observations. The lung lesions were classified into 3 categories: alveolar/bronchiolar hyperplasia, adenoma and adenocarcinoma. The histological criteria and morphological appearances of these lesions were the same as those described in our previous report.[20]

Isolation of tissue (microdissection method) and DNA isolation Ten 10-μm-thick sections were cut from each paraffin block and deparaffinized. Under microscopic observation, the lesions were scraped from the slides, taking particular 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. The samples were digested with proteinase K for 12–24 h at 37°C, then incubated at 95°C for 10 min to inactivate the enzyme and extracted three times using the phenol-chloroform method. The resulting precipitates were dissolved in 100–150 μl of water for use in the polymerase chain reaction (PCR).

PCR and restriction fragment length polymorphism (RFLP) method In order to ascertain whether point mutations at the 1st or 2nd base of codon 12 of exon 1 had occurred, the PCR was performed using a mismatch primer containing a C mismatch instead of a T in the second base from the 3′ end. The mismatch primer sequence was 5′-AACTTGTGGTGGTTGGAGCCG-3′ and the antisense primer was 5′-TTACCTCTATCGTAGGGTCGTACTCATCCA-3′. The following 5′- and 3′-amplification primers of the 2nd exon of the K-*ras* gene were used: 5′-TACAGGAAACAAGTAGTAATTGATGGAGAA-3′, and 5′-ATAATGGTGAATATCTTCAAATGATTTAGT-3′. The PCR conditions were 95°C for 1 min, 55°C (exon 1) or 51–52°C (exon 2) for 2 min and 72°C for 2 min. The process involved 45 (exon 1) or 40 (exon 2) amplification cycles, which generated 131- and 171-base-pair fragments of exons 1 and 2, respectively. The predominant point mutations of codon 12 of the K-*ras* gene are 1st or 2nd base changes, and, therefore, the restriction enzyme *Msp* I (5′-C*CGG-3′, * indicates the restriction site; obtained from New England Biolaboratory Inc. [NEB, Beverly, MA]) was chosen. The mismatch nucleotide at the site of the point mutation is part of the recognition sequence of the restriction enzyme used in the RFLP analysis. The PCR products generated using this strategy contain the enzyme cleavage site only if they are derived from the mutated locus. Therefore, the amplified DNA products were digested with *Msp* I at 37°C for 2–3 h and then subjected to the NEB reaction protocol. When the PCR products containing mutations in the 1st or 2nd base of codon 12 of exon 1 were digested with *Msp* I, the diagnostic 131-base-pair fragments were not cleaved, whereas the PCR products derived from the normal codon were cleaved to yield 112- and 19-base-pair fragments. In codon 61 of exon 2, the restriction enzyme *Xba*

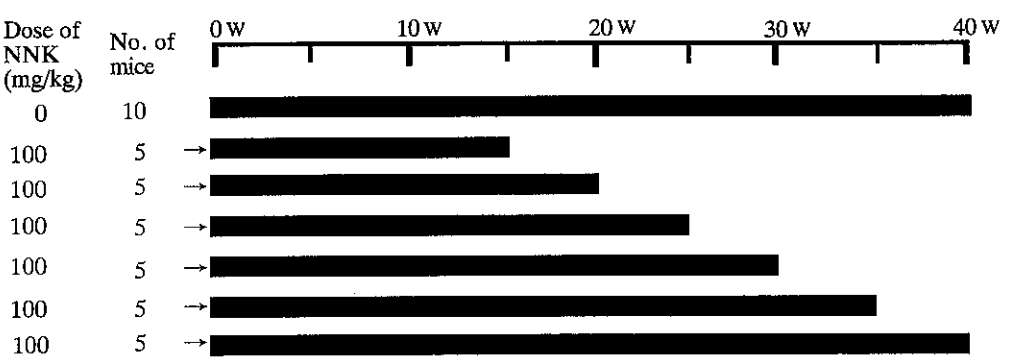

Fig. 1. Experimental design. →, intraabdominal injection.

I (5′-T*CTAGA-3′; NEB) was chosen to recognize a CAA (wild-type sequence)-to-CTA transversion, and the enzyme *Taq* I (5′-T*CGA-3′; NEB) to recognize a CAA-to-CGA transition. The method was described previously.[20] On completion of these reactions, 10-μl aliquots of the PCR-RFLP products were electrophoresed using 4–6% agarose and/or 8% polyacrylamide gels.

Dideoxy sequencing analysis All the sample DNAs with codon 12 mutations detected by the PCR-RFLP method were reamplified by PCR in order to determine the sequence, using the following 5′- and 3′-amplification primers of the 1st exon of the K-*ras* gene: 5′-ATGACTGAGTATAAACTTGT-3′ and 5′-CACTTTGTGGATGAGTACGA, respectively. The sequencing primers (1–2 pmol) for exon 1 were the same as those described above. Sequencing of codon 61 of exon 2 was performed on some samples to confirm the PCR-RFLP results using a Sequenase PCR product sequencing kit (USB, Cleveland, OH). The PCR products were treated with exonuclease I and shrimp alkaline phosphatase at 37°C for 15 min and 80°C for 15 min to remove unwanted material. The sequencing primers (5 pmol/μl) described above were added to the annealing mixture and denatured by heating at 100°C for 2–3 min. The annealed DNA mixture was added to the reaction buffer, which contained dithiothreitol solution, diluted labeling mix, ^{35}S and the Sequenase DNA polymerase mixture, and incubated at room temperature for 5 min. Portions of this labeling mixture were transferred to each of four termination tubes (containing 7-deaza-dATP, -dCTP, -dGTP,

Table I. Incidence of NNK-induced Lung Lesions in A/J Mice

Dose of NNK (mg/kg)	Time of killing after NNK treatment (wk)	No. of effective (initial) mice	No. of mice (%) with lung lesions	Mean no. of lung lesions per mouse	No. of lung lesions		
					Hyperplasia	Adenoma	Carcinoma
0	—	10 (10)	3 (30)	0.5	3	2	0
100	15	5 (5)	2 (40)	0.8	1	3	0
100	20	5 (5)	4 (80)	2.8	7	7	0
100	25	5 (5)	4 (80)	4.2	5	16	0
100	30	4 (5)	4 (100)	3.5	4	10	0
100	35	5 (5)	5 (100)	7.4	13	23	1
100	40	5 (5)	5 (100)	7.8	12	24	3
			Total no. of lung lesions		45	85	4

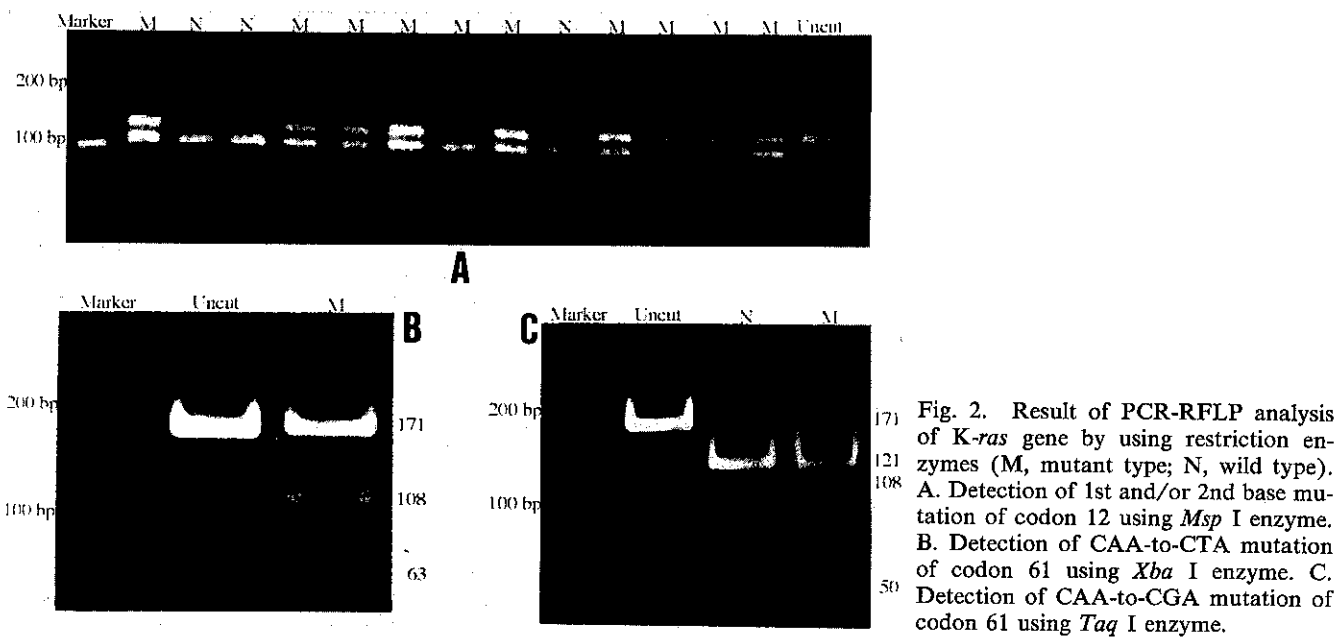

Fig. 2. Result of PCR-RFLP analysis of K-*ras* gene by using restriction enzymes (M, mutant type; N, wild type). A. Detection of 1st and/or 2nd base mutation of codon 12 using *Msp* I enzyme. B. Detection of CAA-to-CTA mutation of codon 61 using *Xba* I enzyme. C. Detection of CAA-to-CGA mutation of codon 61 using *Taq* I enzyme.

and -dTTP) and incubated at 37°C for 5–10 min. The termination reactions were ended by adding stop solution and the material was denatured at 95°C for 5 min, then electrophoresed on 8% acrylamide gel containing urea, after which the gels were fixed with 10% acetic acid and 10% methanol, dried and autoradiographed using X-ray film at room temperature for 1–2 days.

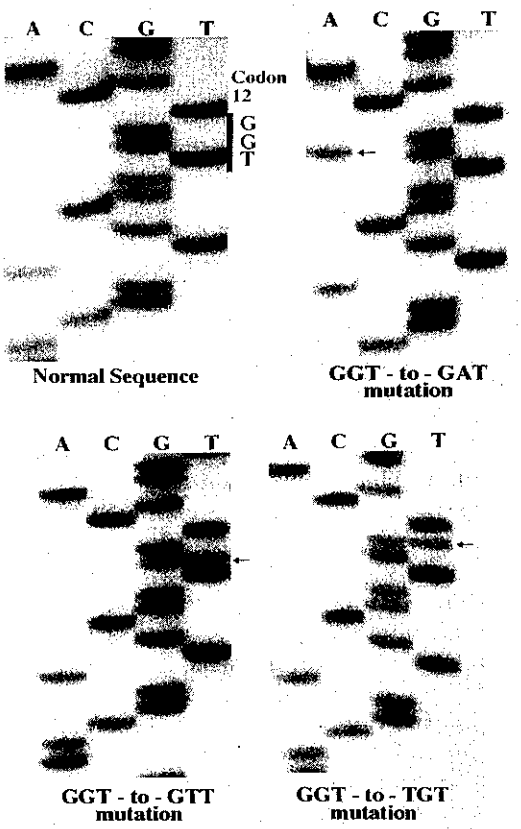

Fig. 3. Identification of codon 12 mutations in the K-*ras* gene by using dideoxy sequencing analysis. Base substitution is indicated by the arrow.

BrdU immunohistochemistry Tissue sections for immunohistochemical staining were cut from 70% buffered, alcohol-fixed, paraffin-embedded tissue blocks, which were deparaffinized, treated with 40% HCl for 20 min and neutralized with boric acid and borate solution. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in absolute methanol and serum, the sections were stained immunohistochemically with an anti-BrdU monoclonal antibody (diluted 1:20; Dakopatts, Copenhagen) by a standard avidin-biotin complex technique using an ABC kit (Nichirei, Tokyo) and the nuclei were counterstained with hematoxylin. The number of cells with positive immunoreactivity was counted (at least 300 cells) in serial sections of each lung lesion, and the labeling index (LI) of each lesion was determined. The statistical analysis was performed by the least-squares method.

RESULTS

Lung lesion incidences The lung lesion incidences are shown in Table I. Three (30%) of the 10 control mice had a total of 5 lung lesions, whereas in the NNK-treated groups, the number of mice with lung lesions and the mean number of lung lesions per mouse increased gradually in proportion to the time after treatment. The total

Table III. Comparison of Frequency of K-*ras* Gene Mutation among Experimental Groups

Time of killing (wk)	No. of lesions examined	No. of lesions with activated K-*ras* gene (%)	No. of lesions with GGT-to-GAT mutation (%)
15	3	1 (33.3)	1 (100)
20	8	5 (62.5)	4 (80.0)
25	14	11 (78.6)	11 (100)
30	11	4 (36.4)	3 (75.0)
35	25	22 (88.0)	20 (90.9)
40	11	9 (81.8)	5 (55.6)

Table II. Frequency and Nature of Mutations in Codons 12 and 61 of K-*ras* Gene in NNK-induced and Spontaneous Mouse Lung Lesions in A/J Mice

	No. of lesions examined	No. of lesions with activated K-*ras* gene (%)	No. of lesions with codon 12 mutations (wild, GGT)				No. of lesions with codon 61 mutations (wild, CAA)	
			GAT	GTT	TGT	CGT	CGA	CTA
Hyperplasia	12	7 (58.3)	6	0	1	0	0	0
Adenoma	56	42 (75.0)	36	1	1	1	2	1
Carcinoma	4	3 (75.0)	3	0	0	0	0	0
Total	72	52 (72.2)	45	1	2	1	2	1
Spontaneous lung lesions	5	3 (60.0)	1	0	2	0	0	0

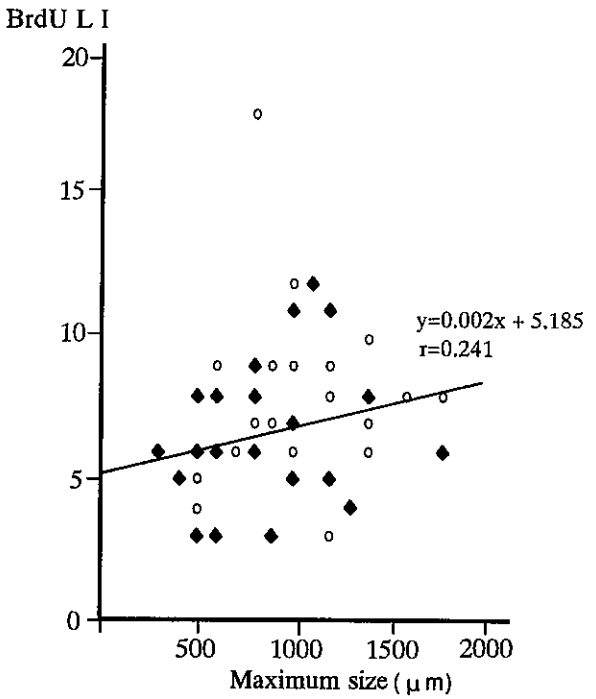

Fig. 4. Correlation between the labeling index of BrdU, the size of lung lesions, and the K-*ras* gene mutation in the lesions. The regression lines were obtained by the least-squares method and r is the correlation coefficient. Circles and diamonds indicate lesions with K-*ras* gene mutation and lesions without K-*ras* gene mutation, respectively.

number of lung lesions was 134, of which 45 were hyperplasias, 85 adenomas and 4 adenocarcinomas.

Identification of K-*ras* gene mutation by PCR-RFLP and dideoxy sequencing analysis The K-*ras* gene mutation results are shown in Figs. 2 and 3. Seventy-two NNK-induced lung lesions were examined by these methods and K-*ras* gene mutations were detected in 7 (58.3%) of 12 hyperplasias, 42 (75.0%) of 56 adenomas and 3 (75.0%) of 4 adenocarcinomas (Table II). The predominant mutation, which accounted for 86.5% of all those examined, was a G-to-A transition at the 2nd base of codon 12. There was no difference between the mutation patterns of lung lesions of different histological types. K-*ras* gene mutations were identified in 3 (60%) of the 5 lung lesions in the control mice: one was GGT to GAT and the other two were GGT to TGT. The K-*ras* gene mutation frequencies in the groups killed at different times after NNK treatment are compared in Table III. There was no clear correlation between the time after NNK treatment and the mutation frequency or specific mutational pattern. Numerous samples of normal lung tissue from NNK-treated mice were examined and no K-*ras* gene mutation was identified in either codon 12 of exon 1 or codon 61 of exon 2.

Proliferative activity of lung lesions determined by BrdU immunohistochemistry The correlations between the LI, lung lesion size and K-*ras* gene mutations in each lesion are depicted in Fig. 4. The LI increased in direct proportion to the lung lesion size (statistically significant, $r=0.241$), but there was no positive correlation between the LI and the presence of K-*ras* gene mutations.

DISCUSSION

Wynder and Hoffmann proposed that the reason for the prevailing increase of adenocarcinoma is that low-yield cigarette smoking has brought about deeper inhalation and a substantially changed profile of smoke constituents compared with high-yield cigarette smoking in the past: the benzo[*a*]pyrene yield has been reduced by over 50%, whereas the relative dose of NNK has increased by about 45%.[1] Therefore, NNK is an important chemical that should be considered when evaluating the occurrence of lung adenocarcinoma in humans.

NNK is a potent mutagen and carcinogen and two metabolic pathways that may activate it have been suggested: (1) α-hydroxylation, which results in the formation of O^6-methylguanine adducts,[21] and (2) hydroxylation at the *N*-methyl carbon to yield, upon hydrolysis, a pyridyloxobutyldiazohydroxide, which reacts with DNA to form a bulky adduct.[22] Several studies have indicated that the formation of the O^6-methylguanine adduct via α-hydroxylation is the more important of the two pathways involved in the carcinogenicity of NNK in small rodents.[9, 15] Moreover, adduct formation in target tissues or cells has been suggested to be closely related to a specific mutation based on base mispairing in DNA.[14–16] In the present study, K-*ras* gene mutations were identified in 72.2% of all the NNK-induced lung lesions examined and the major mutation was a G-to-A transition at the 2nd base of codon 12, which clearly differed from the mutational pattern in untreated control mice. In view of these findings, we assumed that K-*ras* gene activation in NNK-induced lung lesions plays an important role in the development of lung lesions, and the specific mutation of a G-to-A transition at the 2nd base of codon 12 provides evidence of mutagenic activity as a result of O^6-methylguanine DNA adduct formation.

These results differed from the K-*ras* gene mutation frequency and pattern in mouse lung lesions induced by urethane (ethyl carbamate) that we observed in our previous study.[20, 23, 24] The average K-*ras* gene mutation frequency in all the urethane-induced lung lesions examined was 38.7% and the predominant mutation was an A-to-T transversion at the 2nd base of codon 61.[20] Furthermore, in another study, K-*ras* gene activation

was detected in 6 of 9 B6C3F$_1$ mouse lung tumors induced by 1,3-butadiene and all the activating mutations were G-to-C transversions at the 1st base of codon 13.[25] Therefore, the frequency and pattern of K-*ras* gene mutations in mouse lung lesions appear to depend directly on the nature of the chemical carcinogen.

However, K-*ras* gene activation is not necessarily involved in the lung lesion development process, because lesions without mutations exist. Therefore, the possibility that other genetic events are involved in the occurrence of such lesions can not be discounted[26, 27] and it was suggested by Cha *et al.* that "carcinogen-induced" tumors might arise from cells with preexisting *ras* mutations.[28] Furthermore, not only G-to-A mutation caused by O^6-methylguanine adduct formation, but also other types of mutation were present. A possible explanation for this is the formation of other minor specific DNA adducts, such as O^4-methylthymine or other species arising from a pyridyloxobutylating agent involved in the metabolic process of NNK. Another possible reason is dose-dependent differences in K-*ras* gene mutation. Chen *et al.* demonstrated that the K-*ras* gene mutations of lung tumors induced by low-dose NNK were more consistent with the formation of O^6-methylguanine adducts, a GGT-to-GAT transition, unlike those induced by high-dose NNK, and concluded that other factors might be involved in tumor induction in rodents given higher doses.[29] Thus, the various mutations observed in the present study may have been attributable to the high dose of NNK administered.

In a previous study on mice, we showed that the K-*ras* gene mutational event depended not only on the urethane dose, but also on the duration of exposure, when continuous oral treatment was administered.[20] However, no clear relationship between the mutation frequency and time after NNK treatment was identified in our present study. These results suggest that different mechanisms are involved in the mutations induced as a result of one hit by a carcinogen and continuous oral treatment. Further studies comparing the dose- and time-dependent differences in the profile of *ras* mutations induced by chemical carcinogens are needed.[29, 30]

Cell kinetic analysis is helpful for understanding the biological behavior of a tumor. In general, it is assumed that the proportion of cells at the S-phase is higher in a rapidly growing than in a slowly growing tumor.[31, 32] Our present results imply that the NNK-induced lung lesions were composed of cells with high growth activity relative to their size and thus were likely to progress to more advanced stages. Furthermore, the size of a lung lesion has been suggested to reflect its morphological type. In other words, carcinoma cells grow more rapidly than cells in hyperplastic or adenomatous lesions. Mutation of the *ras* gene changes the normal cellular proliferation program to one of uncontrolled growth; the mutated *ras* protein loses its ability to become inactivated and thus stimulates growth or differentiation autonomously.[26, 27] However, K-*ras* gene mutation had no influence on the proliferative activity of NNK-induced lung lesions. Therefore, other factors, including genetic events, appear to be concerned with the acquisition of the growth advantage of NNK-induced lung lesions.

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