Lack of Modifying Effects of NNK on Lung Tumorigenesis in Hamsters with Pulmonary Fibrosis Induced by MNUR

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Abstract: To investigate the modifying effects of repeated doses of NNK on the development of lung tumors in hamsters with pulmonary fibrosis, female Syrian golden hamsters received 5 subcutaneous (s.c.) injections of 0.6 mg MNUR (Groups I and II) or 0.5% ethanol solution (Groups III and IV), at 2-week intervals, and were then given distilled water containing 3 ppm NNK (Groups I and III) or water alone (Groups II and IV) for 27 weeks. Glandular metaplasia, papillary hyperplasia and bronchiolo-alveolar adenomas were induced in the lungs of all the animals of Groups I and II without any significant differences in data for multiplicity and PCNA labeling indices of these between Groups I and II. In PCR-dot blot hybridization analysis on lung proliferative lesions, point mutations of c-K-ras gene at codon 12 or 61 were seen in bronchiolo-alveolar adenomas and carcinomas from animals of Group I. The mutation incidences were 4/13 in adenomas and 3/4 in carcinomas, involving changes from GGT-to-GAT at codon 12, or CAA-to-CGA at codon 61. The results indicate that NNK treatment with the present dose regimen exerts no modifying effects on the development of lung proliferative lesions in hamsters with pulmonary fibrosis induced by MNUR. (J Toxicol Pathol 1998; 11: 169~175)

Key words: Lung tumors, Hamsters, *N*-methyl-*N*-nitrosourethane, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-1-butanone

Introduction

Idiopathic interstitial pneumonia/pulmonary fibrosis has been reported to be epidemiologically associated with lung cancers¹⁻⁶. With respect to the pathogenesis of such lung cancers, the background fibrotic lesions have been considered to play a possible causative role¹⁻⁶. It has been reported that cigarette smoking is a factor exacerbating the development of lung cancer in patients with pulmonary fibrosis^{1,3,5,6}.

In our previous studies, we established an animal model of pulmonary fibrosis, induced in Syrian golden hamsters with a total of 5-s.c. injections of 0.6 mg/animal of *N*-methyl-*N*-nitrosourethane (MNUR), at every 2-week intervals⁷⁻⁹. It was found that lung injury attributable to damage to alveolar capillaries progressed to diffuse alveolar fibrosis and then hyperplastic lesions and/or pulmonary tumors in the late phase⁷⁻⁹.

4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-1-butanone (NNK), a tobacco-specific nitrosamines, is a potent carcinogen in rats¹⁰⁻¹⁶, Syrian golden hamsters¹⁷⁻²⁰, and mice^{21,22}. It has been reported that the lung tumors induced

in these animals are topographically and morphologically similar to the human bronchiolar/alveolar adenocarcinomas²³. Because of the organ-specific carcinogenic activity of NNK, it has been used as one of the standard procedures for the induction of pulmonary tumors. Gene analysis has revealed point mutations of the Ki-ras gene, predominantly GGC-to-GAC transitions of codon 13, in NNK-induced lung tumors in hamsters²⁰.

Although lung cancer is speculated to develop from pulmonary fibrosis¹⁻³, a variety of factors including cigarette smoke and environmental carcinogens are also considered to be involved. In this context, NNK administration to hamsters with pulmonary fibrosis caused by MNUR might be appropriate for investigating their combined effects on lung carcinogenesis. The present study was therefore performed to clarify this question.

Materials and Methods

Animals and Chemicals

Female Syrian golden hamsters at 5-weeks of age were purchased from Sankyo Labo Service (Tokyo, Japan). They were individually housed in polycarbonate cages with wood chips for bedding under standard conditions (room temperature, 23±2°C; relative humidity 55±5%; 12h-light/12h-dark cycle), and given basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. A 0.2% solution of *N*-methyl-*N*-nitrosourethane (MNUR), purchased from Nakarai Tesque, Inc. (Kyoto, Japan), made up in a 0.5% solution of aqueous ethanol was freshly prepared each day for s.c. injection.

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Fig. 1 Experimental design

Experimental design

After a one week acclimatization period, female Syrian golden hamsters at 6-week old received five s.c. injections of 0.6 mg/0.2 ml/animal of MNUR, at 2-week intervals. Seven weeks after the final injection, the surviving 22-female animals were subdivided into Groups I and II, and given distilled water containing NNK (concentration of 3 ppm) or city water alone respectively, for 27 weeks (Fig. 1). In addition, 17-control female animals which had received five s.c. injections (once every 2-week) of 0.5% solution of aqueous ethanol, were subdivided into two groups (Groups III and IV), and also given NNK and water, respectively, in the same manner (Fig. 1). Animals were weighed every two weeks throughout the experimental period, and water consumption was measured every two weeks during weeks 15–42.

Light microscopic and immunohistochemical examinations

At the end of experiment, week 42, all animals in Groups I, II, III and IV were killed by ether overdose and exsanguination from the abdominal aorta. The lungs of each animal were removed, weighed and fixed by intratracheal instillation of a 10% neutral buffered formalin or a methanol-Carnoy fixative (the right posterior lobe). Five transverse sections from each lobe were embedded in paraffin, sectioned and stained with hematoxylin and eosin for light microscopic examination. Some sections from the lobe fixed with a methanol-Carnoy fixative were also subjected to immunohistochemistry using a mouse monoclonal anti- β -tubulin antibody (Sigma chemical Co., St. Louis, U.S.A., dilution 1:400) and a mouse monoclonal against the proliferating cell nuclear antigen (PCNA; DAKO Corp., Santa Barbara, CA, U.S.A., dilution 1:100). The avidin-biotin-peroxidase (ABC) method was used with a Streptavidin biotin complex peroxidase kit (DAKO Corp.). Counterstaining with hematoxylin was performed before mounting the sections.

PCR-dot blot hybridization analysis

PCR-dot blot hybridization analyses were performed on all animals in Groups I and II, the samples being 8 glandular metaplasias, 5 papillary hyperplasias, 13 adenomas and 4 carcinomas in Group I, and 7 glandular metaplasias, 4 papillary hyperplasias and 2 adenomas in Group II. For DNA amplification, 5- μ m thick sections of lung lesions, formalin-fixed and paraffin-embedded, were deparaffinized with xylene, rinsed in ethanol and hydrated with distilled water. Selected foci were separately scraped out with 28-gauge

disposable needles and collected into sterile tubes with 60 μ l of water. The samples were used directly for *in vitro* amplification without prior isolation of DNA. PCR was essentially performed according to the method of Saiki *et al.*²⁵ using a DNA thermal cycler and a GeneAmpTM DNA amplification reagent kit with AmpliTaqTM (Perkin Elmer Cetus, Newtalt, CT, U.S.A.). The primers used for DNA amplification of the region around codons 12 and 13 of K-*ras* exon 1, and codon 61 of K-*ras* exon 2 are listed in Table 1. A total volume of 100 μ l of the reaction mixture contained 10 μ l of template DNA, 1 μ M of each primer, 10 μ l of 10x buffer, 200 μ M of each dNTP (Takara Shuzo Laboratories, Kyoto, Japan), and 2.5 units of taq polymerase.

Table 1 Oligonucleotide primers and probes for gene analysis in c-K-*ras* exons 1 and 2^a

Exon 1 (codon 12, 13)			
Primer			
Sense	5'-CCTGCTCAAAATGACTGAAT-3'		
Antisense	5'-TGTTGGATCATATTCGTCCA-3'		
Probes			
1.	5'-GTTGGAGCTGGTGGCGTA-3' ^b		
2.	5'	GAT	-3' ^c
3.	5'	GTT	-3' ^c
4.	5'	GAC	-3' ^c
5.	5'	GATGAC	-3' ^c
6.	5'	GTTGAC	-3' ^c
<hr/>			
Exon 2 (codon 61)			
Primer			
Sense	5'-GTAATTGATGGAGAAACCTG-3'		
Antisense	5'-ATACACAAAGAAAGCCCTCC-3'		
Probes			
1.	5'-ACAGCAGGTCAAGAGGAGTA-3' ^b		
2.	5'	AAA	-3' ^c
3.	5'	GAA	-3' ^c
4.	5'	CGA	-3' ^c
5.	5'	CCA	-3' ^c
6.	5'	CTA	-3' ^c
7.	5'	CAT	-3' ^c
8.	5'	GTT	-3' ^c

^a: Oreffo VIC, *et al.* Carcinogenesis 1993; 14: 451–455.

^b: Normal, ^c: Mutation.

The mixture was overlaid with a drop of mineral oil (Sigma, St. Louis, MO, U.S.A.) to prevent evaporation. After denaturing at 94°C for 5 min., 30 cycles at 94°C for 1 min., 63°C for 2 min. and 72°C for 3 min. were performed. The yields of amplified products were ascertained by electrophoresis through 2% agarose gels. The probes employed to detect wild type alleles and possible point mutations at codons 12, 13, and 61 of the K-*ras* gene were synthesized by Takara Shuzo Laboratories (listed in **Table 1**) and labeled with fluorescein-dUTP by the 3'Tdt tailing reaction with an ECL 3'-oligolabelling and detection system (Amersham International, Buckinghamshire, U.K.). For the detection of *ras* gene mutations, prehybridization and hybridization were performed by the methods described by Vries *et al.*²⁶. The PCR products were denatured at 94°C for 5 min., placed immediately on ice and one-microliter aliquots were spotted onto Hyband-N membranes (Amersham International) and cross-linked by exposure to ultraviolet irradiation. The membranes were prehybridized for 1 hr at 48°C in 5x Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS) and 5x SSC with 100 µg/ml of sonicated denatured salmon sperm DNA (Sigma, St. Louis, MO, U.S.A.), and then hybridization was performed for 2 hr at 42°C in the same mixture containing 5 ng/ml of fluorescein labeled probes for each blot. After incubation, the membranes were washed 3 times in 0.2x SSC/0.1% SDS at 42°C and treated with anti-fluorescein horse-

radish peroxidase for 1 hr at room temperature. The filters were washed and, after addition of detection buffer (ECL detection reagents RPN2105, Amersham International), exposed to blue-light sensitive Kodak diagnostic films (X-OMAT™, Kodak, Rochester, NY, U.S.A.) for 15 min. and developed.

Statistical analysis

Group means and standard deviations were calculated for the data for body weights, water consumption, multiplicity and PCNA labeling indices of lung proliferative lesions. Differences in mean values were evaluated between Group I and Group II, or between Group III and IV with the Student's *t*-test. For incidences of lung proliferative lesions, the values were evaluated by the Fisher's exact test.

Results

Two animals in Group II died, in weeks 4 and 13. In Group III, decrease in body weight gain was observed in comparison with Group IV (Fig. 2). There were no differences in body weight gain between Groups I and II. No marked changes in water consumption were observed among Groups I, II, III and IV (Fig. 2).

On necropsy, consolidation of several to all lung lobes was observed as the most remarkable finding in Groups I and

Fig. 2 Body weights and water consumption data for Syrian golden hamsters treated with NNK or MNUR plus NNK

Table 2 Histological Types and Incidences of Lung Proliferative Lesions in Syrian Golden Hamsters Treated with NNK, MNUR Alone or MNUR Plus NNK

Findings	Groups	MNUR+NNK	MNUR alone	NNK	Non-treatment
	No. of animals examined	12	8	11	6
Glandular metaplasia		12 ^a (100) ^b	8 (100)	3 (27)	0
Papillary hyperplasia		12 (100)	8 (100)	0	0
Bronchiolo-alveolar adenoma		12 (100)	8 (100)	0	0
Bronchiolo-alveolar carcinoma		4 (33)	4 (50)	0	0
Bronchiolar adenocarcinoma		1 (8)	1 (13)	0	1 (17)

^a: Number of lesions.

^b: Figures in parentheses show the percentages of hamsters with lesions.

Fig. 3 Glandular metaplasia, consisting of low columnar ciliated cells in alveoli.
Group I (MNUR+NNK). HE staining. $\times 150$

Fig. 5 Bronchiolo-alveolar carcinoma, characterized by papillary/solid growth of pleomorphic tumor cells.
Group I (MNUR+NNK). HE staining. $\times 150$

Fig. 4 Bronchiolo-alveolar adenoma, characterized by papillary growth of cuboidal tumor cells with hyperchromatic nuclei.
Group II (MNUR alone). HE staining. $\times 150$

Fig. 6 Bronchiolar adenocarcinoma, consisting of columnar mucous cells with pleomorphic nuclei, and characterized by prominent intra-alveolar secretion of mucoid substances.
Group IV (Non-treatment). HE staining. $\times 150$

II. In addition, one to several white solid nodules also were detected in Groups I and II within the consolidated areas. In Group IV, only one white solid nodule was observed in one animal, and there were no lesions in Group III.

Histopathologically, all animals in Groups I and II developed inflammation and focal fibrosis identical to the lesions observed in hamsters treated with MNUR in our previous study⁷⁻⁹. Glandular metaplasia, papillary hyperplasia and bronchiolo-alveolar adenomas were also observed in all animals of Groups I and II at incidences of 100% in both cases (**Table 2**). Glandular metaplasia was characterized by proliferative foci consisting of low columnar ciliated cells within alveoli, positively stained with anti- β -tubulin antibody and with uniform round to ovoid nuclei and eosinophilic cytoplasm (Fig. 3). Papillary hyperplasia was characterized by small foci of single to double layered cuboidal cells showing papillary growth along the alveolar walls. Bronchiolo-alveolar adenoma was characterized by papillary growth of tumor cells consisting of cuboidal cells with hyperchromatic nuclei (Fig. 4). In addition, four bronchiolo-alveolar carcinomas and one bronchiolar adenocarcinoma were observed in Groups I and II (**Table 2**). The

incidences of bronchiolo-alveolar carcinomas and bronchiolar adenocarcinomas were 33% and 8% in Group I, and 50% and 13% in Group II, respectively. Bronchiolo-alveolar carcinomas were characterized by papillary/solid growth of pleomorphic tumor cells (Fig. 5). Three glandular metaplasias were observed in Group III and one bronchiolar adenocarcinoma in Group IV (**Table 2**). The bronchiolar adenocarcinoma consisted of columnar mucous cells with pleomorphic nuclei, and was characterized by prominent intra-alveolar secretion of mucoid substances (Fig. 6).

Multiplicity data for lung proliferative lesions are summarized in **Table 3**. The values for glandular metaplasia (Number of lesions, Mean $\pm$ SD) were 137 ± 46 , 135 ± 23 and 0.5 ± 1.2 in Groups I, II and III, respectively, while those of papillary hyperplasia in Groups I and II were 17.8 ± 4.8 and 21.8 ± 4.6 , respectively (**Table 3**). The total numbers (Mean $\pm$ SD) of lung tumors, were 6.4 ± 2.8 , 6.0 ± 2.6 and 0.17 ± 0.41 in Groups I, II and IV, respectively (**Table 3**). The numbers (Mean $\pm$ SD) of PCNA-positive nuclei per 100 cells in Groups I and II were 1.50 ± 0.98 and 1.63 ± 0.45 for glandular metaplasia, 7.8 ± 4.9 and 8.8 ± 2.7 for papillary hyperplasia, and 14.3 ± 4.5 and 15.8 ± 3.4 for bronchiolo-

Table 3 Multiplicities of Lung Proliferative Lesions in Syrian Golden Hamsters Treated with NNK, MNUR Alone or MNUR Plus NNK

Groups	No. of animals examined	Glandular metaplasia	Papillary hyperplasia	Adenoma ^a	Carcinoma ^b	Total ^c
MNUR+NNK	12	137±46 ^d	17.8±4.8	5.8±2.8	0.67±0.98	6.4±2.8
MNUR alone	8	135±23	21.8±4.6	5.3±2.3	0.75±0.71	6.0±2.6
NNK	11	0.5±1.2	0	0	0	0
Non-treatment	6	0	0	0	0.17±0.41	0.17±0.41

^a: Bronchiolo-alveolar adenomas.^b: Bronchiolo-alveolar carcinomas+Bronchiolar adenocarcinomas.^c: Carcinomas+Bronchiolo-alveolar adenomas.^d: Number of lesions (Mean±SD).**Table 4** PCNA Labeling Indices of Lung Proliferative Lesions in Syrian Golden Hamsters Treated with MNUR Alone or MNUR Plus NNK

Groups	No. of animals examined	Glandular metaplasia	Papillary hyperplasia	Adenoma ^a
MNUR+NNK	12	1.50±0.98 ^b	7.8±4.9	14.3±4.5
MNUR alone	8	1.63±0.45	8.8±2.7	15.8±3.4

^a: Bronchiolo-alveolar adenomas.^b: Number of PCNA-positive nuclei per 100 cells (Mean±SD).

alveolar adenomas, respectively (**Table 4**). There were no significant differences in multiplicity or PCNA labeling indices in lung proliferative lesions between the two groups.

PCR-dot blot hybridization analysis of lung proliferative lesions in Groups I (8 glandular metaplasias, 5 papillary hyperplasias, 13 adenomas and 4 carcinomas) and II (7 glandular metaplasias, 4 papillary hyperplasias and 2 adenomas) revealed point mutations in c-K-ras codons 12 and 61 limited to bronchiolo-alveolar adenomas and carcinomas. The incidences of mutation were 4/13 in adenomas and 3/4 in carcinomas. That mutation spectrum was GGT-to-GAT at codon 12, and CAA-to-CGA at codon 61 (**Table 5**).

Discussion

In the present study, glandular metaplasias, papillary hyperplasias and bronchiolo-alveolar adenomas were observed in all animals of Groups I and II, pre-treated with MNUR, along with bronchiolo-alveolar carcinomas and bronchiolar adenocarcinomas in line with our previous study⁷⁻⁹. There were no significant differences in the morphology, multiplicity or PCNA labeling indices of lung proliferative lesions between Groups I and II. Thus, there was no distinct morphological evidence that NNK treatment influenced the development of lung proliferative lesions in hamsters with pulmonary fibrosis induced by MNUR in this study. The question arises as to whether the dose of NNK applied was adequate. However, in Group III, body weight gain was depressed as compared with Group IV, suggesting an effect of NNK at 3 ppm in hamsters although no tumors were found. In rats, lung tumors were induced with oral administration (drinking water) of 0.075-0.31 mmol NNK/animal in a long term study (over 100-weeks)¹². Since the doses applied were about 1/2-2 times as compared to that in the present study, the treatment period might have been the deciding factor. Lung tumors could be induced in hamsters by the s.c. injection of NNK (19 injections of 10 mg/animal for 6.3 weeks or 75 injections of 2.5 mg/animal for 25 weeks¹⁷, a single injection of 93 mg/kg¹⁸, 32 injections of 12.5 mg/kg for 16 weeks¹⁹, 75 injections of 10 mg/kg for 25 weeks²⁰). So higher dose levels may be needed for

Table 5 Mutation Spectra at Codons 12, 13 and 61 of the c-K-ras Gene for Lung Proliferative Lesions in Syrian Golden Hamsters Treated with MNUR Alone or MNUR Plus NNK

Groups	Proliferative lesions	No of positive mutations/no. of samples tested	Codon 12	Codon 13	Codon 61
			Sequence	Sequence	Sequence
MNUR+NNK	Glandular metaplasia	0/8			
	Papillary hyperplasia	0/5			
	Bronchiolo-alveolar adenoma	4/13	4: GGT→GAT		1: CAA→CGA
	Bronchiolo-alveolar carcinoma	3/4	2: GGT→GAT		1: CAA→CGA
MNUR alone	Glandular metaplasia	0/7			
	Papillary hyperplasia	0/4			
	Bronchiolo-alveolar adenoma	0/2			

significant modifying effects of NNK on the development of lung tumors in hamsters treated with MNUR. The organ-specific carcinogenic activity of NNK can be explained by two factors; the required enzymes for metabolic activation of NNK and the bloodstream carrying the carcinogen to the sites of activation, especially to the peripheral lung and nasal mucosa^{23,24}. Degenerative changes of endothelial cells of the alveolar capillaries have been ultrastructurally observed at week 1 after the termination of treatment of MNUR in our pulmonary fibrosis model⁷ and thus, there is the possibility that vascular damages, fibrosis and/or the other lesions could have influenced the blood circulation to the lung and consequently NNK carcinogenicity.

With regard to the PCR-dot blot hybridization results, we have already reported point mutations in Ki-*ras* codons 13 or 61 in lung tumors of female Syrian golden hamsters, induced by MNUR²⁷. Similar GGC-to-GAC transitions in codon 13, have also been described for NNK-induced lung tumors in hamsters²⁰. Based on the results of our previous study and the literature, it can be concluded that mutations of the c-K-*ras* gene, mostly involving codons 12, 13, or 61, may be important keys to explain the mechanisms of induction of hamster lung tumors by chemicals. In the present study, PCR-dot blot hybridization analysis of lung proliferative lesions showed point mutations in c-K-*ras* codons 12 and 61 in bronchiolo-alveolar adenomas and carcinomas from the animals treated with MNUR+NNK, but not in MNUR alone. However, no mutations in codon 13 were detected. In our previous study, the mutation in codon 13 occurred at a low incidence in lung tumors of hamsters treated with MNUR alone (17%)²⁷. Such an absence of point mutation of c-K-*ras* in hamsters treated with MNUR alone and point mutation at codon 13 might be related to the fact that the number of tumors analyzed was insufficient in our study. Clearly, further studies are necessary to clarify the significance of the different mutations in this model.

In conclusion, the results of this study suggest that NNK treatment under the present experimental conditions does not influence lung proliferative lesions in hamsters with pulmonary fibrosis induced by MNUR. To confirm this, we are now performing a further study with subcutaneous administration of NNK to hamsters.

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