MOLECULAR CARCINOGENESIS 42:121–126 (2005)

Phenotypic Characteristics of Mouse Lung Adenoma Induced by 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone

Chigusa Okubo,[1] Yukio Morishita,[2] Yuko Minami,[1] Tadashi Ishiyama,[1] Junko Kano,[1] Tatsuo Iijima,[1] and Masayuki Noguchi[1]*

[1]*Department of Pathology, Institute of Basic Medical Sciences, University of Tsukuba, Tennodai, Tsukuba-shi, Ibaraki, Japan*
[2]*Department of Clinical Pathology, Institute of Clinical Medicine, University of Tsukuba, Tennodai, Tsukuba-shi, Ibaraki, Japan*

The expression profile of adenoma induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in A/J mice was compared with that of normal lung tissue by suppression subtractive hybridization (SSH). The mRNAs of surfactant-associated protein A (SP-A) and lysozyme showed characteristically higher transcription in the adenoma tissue than in normal lung. High expression of both SP-A and lysozyme in tumor cells was confirmed by in situ hybridization (ISH). In normal lung, alveolar type II pneumocytes were positive for both SP-A and lysozyme, indicating that tumor cells retained the phenotypic characteristics of the murine alveolar type II pneumocytes. Previous studies of human adenocarcinomas have shown that the two proteins are expressed reciprocally; SP-A and lysozyme are differential markers of atypical adenomatous hyperplasia (AAH) and non-goblet cell type adenocarcinoma, and of goblet cell type adenocarcinoma, respectively. Thus, the present results indicate that the phenotype of NNK-induced A/J mouse adenoma differs from that of AAH, which is thought to be a preinvasive lesion of human adenocarcinoma.

Key words: carcinogenesis; A/J mouse; microdissection; lysozyme; surfactant apoprotein

INTRODUCTION

The histogenesis and carcinogenesis of peripheral type adenocarcinoma of the lung have been investigated with various animal models. Fischer 344 rats, Syrian golden hamsters, A/J mice, CD-1 mice, C3H/He mice, and SENCAR mice [1–6] are usually used to examine the tumorigenic activities of various chemical carcinogens. Among these animals, A/J mice are highly sensitive to carcinogens that are present in tobacco and tobacco smoke [7,8]. Two of the nicotine-derived nitrosamines, *N*′-nitrosonornicotine [9] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [10], are known to be strong carcinogens in laboratory animals [5]. Cells of lung tumors induced by NNK in A/J mice show growth that replaces the original alveolar structure [11] and are morphologically very similar to those of atypical adenomatous hyperplasia (AAH) and/or localized bronchioloalveolar carcinoma (LBAC) in humans. AAH is a diagnostic criterion newly included in the World Health Organization classification (3rd edition) as a pre-invasive lesion of peripheral type adenocarcinoma. LBAC is an in situ adenocarcinoma that shows a pure bronchioloalveolar growth pattern replacing the original alveolar pneumocytes. LBAC is further subclassified into non-mucinous, mucinous, and mixed mucinous, and non-mucinous or indeterminate cell types. Most cases of AAH and LBAC are composed of non-mucinous-type tumor cells [12,13].

Many previous studies of the lung tumors induced by NNK in A/J mice have demonstrated a high frequency of K-*ras* mutation [14–16]. Morphological examinations have shown that the cytoplasm of the tumor cells contains lamellar inclusion bodies that are one of the major hallmarks of type II pneumocytes [3]. On the other hand, the biological characteristics of AAH and LBAC have been extensively examined by immunohistochemistry. Mizutani et al.

Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AAH, atypical adenomatous hyperplasia; LBAC, localized bronchioloalveolar carcinoma; SP-A, surfactant-associated protein A, surfactant apoprotein A; TTF-1, thyroid transcription factor-1; BAC, bronchioloalveolar carcinoma; SSH, suppression subtractive hybridization; TALPAT, T7 RNA polymerase promoter-attached, adaptor ligation-mediated, and PCR amplification followed by in vitro T7-transcription; ISH, in situ hybridization.

*Correspondence to: Department of Pathology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki 3058575, Japan.

Received 10 February 2004; Accepted 6 October 2004
DOI 10.1002/mc.20070

[1] and Maeshima et al. [17] immunostained various human lung carcinomas with monoclonal antibodies against surfactant apoprotein A (SP-A) and thyroid transcription factor-1 (TTF-1), which are specific differential markers of type II pneumocytes. Most tumor cells of AAH and non-mucinous-type bronchioloalveolar carcinoma (BAC) are positive for SP-A and TTF-1, and therefore they are considered to be of type II pneumocyte origin. In contrast, an immunohistochemical study of mucinous-type adenocarcinoma by Maeshima et al. [17] detected the expression of lysozyme in 73% of cases, whereas no such expression was detected in non-mucinous-type adenocarcinomas.

In this study, we examined the characteristics of lung tumors induced by NNK in A/J mice using suppression subtraction hybridization (SSH) [18], and found several phenotypic differences between mouse adenoma cells and human AAH and BAC.

MATERIALS AND METHODS

Animals and Treatment

Female A/J mice were purchased at 5 wk of age from Japan SLC, Inc. (Shizuoka, Japan). After 2 wk of acclimation—at 7 wk of age—the mice were given a single i.p. dose of 2 mg NNK in saline. All the mice were killed at 20 wk after the carcinogen treatment. The lungs were immediately excised and embedded in Tissue-Tek™ O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), frozen in dry ice-acetone, and stored at −80°C until analysis.

Transmission Electron Microscopy

Small pieces of lung tumor tissue were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, for 3 d at 4°C, postfixed with 2% osmium tetroxide in the buffer, and embedded in Epon 812. Semithin and ultrathin sections were cut on a Bromma 2008 ultratome V (LKB, Schweden, Germany). The simithin sections were stained with 1% troluidine blue and examined with a light microscope. The adjascent thin sections were stained with uranyl acetate followed by lead citrate and examined at 60 kV with a H7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Laser Microdissection and RNA Extraction

Frozen serial 20 μm thick sections were made and mounted on glass slides covered with PEN foil (Leica Microsystems K.K., Wetzlar, Germany) which were precoated with poly-L-lysine solution (Sigma-Aldrich Co., St. Louis, MO). They were fixed with an ethanol/acetic acid (19: 1) mixture at −20°C for 3 min, and gently washed with diethylpyrocarbonate-treated water. The sections were then stained with 0.05% toluidine blue solution. Subsequently, the toluidine blue solution was rinsed out with diethylpyrocarbonate-treated water, and the sections were air-dried.

The tumor tissue and normal pulmonary epithelial tissue were selectively laser-microdissected with an AS LMD system (Leica Microsystems K.K.) [19], and the total RNA was extracted from each tissue type with 500 μL TRIzol reagent (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions.

mRNA Amplification by T7 RNA Polymerase Promoter-Attached, Adaptor Ligation-Mediated, and PCR Amplification Followed by In Vitro T7-Transcription (TALPAT)

Separate mRNAs extracted from tumor tissues and normal pulmonary tissues of three mice were amplified by the TALPAT method [20], which enables very small amounts of mRNA to be amplified with faithful maintenance of the relative levels of mRNA expression. Briefly, TALPAT consists of five enzymatic reaction steps: step 1, cDNA synthesis with an oligo(dT)-T7 promoter primer (5′-pGGCC-AGTGAATTGTAATACGACTCACTATAGGGAGGC-GGTTTTTTTTTTTTTTTTTTTTTTTT-3′); step 2, cRNA amplification by in vitro transcription using T7 RNA polymerase; step 3, cDNA synthesis with $pd(N)_6$ random hexamer (Amersham Biosciences Corp., Piscataway, NJ) for first-strand cDNA and oligo(dT)-T7 promoter primer for second-strand cDNA; step 4, adaptor ligation-mediated RCR; and step 5, cRNA amplification by in vitro transcription using T7 RNA polymerase.

SSH and Differential Screening

SSH [18] between the TALPAT samples of tumor tissue and normal pulmonary tissue and subsequent differential screening were performed with a Superscript™ Choice System (Invitrogen Corp.), a PCR-Select™ cDNA Subtraction Kit and a PCR-Select™ Differential Screening Kit (both from BD Bioscience Clontech, Palo Alto, CA), with modification. In brief, after preparation of the double-stranded cDNAs from each TALPAT sample, the cDNA was digested with *Rsa* I and ligated to adaptors supplied with the PCR-Select™ cDNA Subtraction Kit. Two-directional (forward and reverse) subtractive hybridizations and unsubtractive hybridizations were performed between tumor tissue and normal pulmonary epithelial tissue, and the subtractive hybridization products were amplified by suppression PCR, according to the manufacturer's instructions.

The overexpressed cDNA pool from the tumor tissue (forward subtracted cDNA) was cloned into the PCR 2.1 vector (Invitrogen Corp.). One thousand bacterial colonies were picked up and their inserted cDNAs were amplified by PCR. They were blotted onto nylon membranes and hybridized with ^{32}P-labeled forward- and reverse-subtracted cDNA probes and unsubtracted cDNA probes for both. Then, the PCR-Select™ Differential Screening Kit (BD Bioscience Clontech) was used to screen differ-

entially expressed clones, according to the manufacturer's instructions.

Semi-Quantitative Screening of the Subtracted cDNA Libraries

The differentially screened clones were re-blotted onto nylon membranes, and re-screened with probes that were the ^{32}P-labeled TALPAT products of tumor tissue and normal pulmonary tissue. The hybridized membranes were exposed to X-ray films and relatively evaluated against the intensity of glyceraldehyde-3-phosphate dehydrogenase with an imaging densitometer (Bio-Rad Laboratories, Hercules, CA).

Sequence Analysis

The PCR products of the insert fragments were sequenced with a BigDye terminator v3.0 cycle sequencing ready reaction kit and an ABI PRISM 310 genetic analyzer (both from Applied Biosystems Japan Ltd., Tokyo, Japan).

In Situ Hybridization (ISH)

The overexpressed cDNAs, which had been subcloned into plasmids, were amplified by PCR with T7 RNA polymerase promoter-attached primers. Then the PCR products were transcribed to antisense or sense cRNA probes with T7 RNA polymerase.

The tissue sections for ISH were deparaffinized, rehydrated, and washed with 0.1M phosphate buffer, pH 7.4. After treatment with proteinase K, 4% paraformaldehyde, and 0.2N HCl, the sections were acetylated with 0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0. The sections were then washed with phosphate buffer, dehydrated in a graded ethanol series, and air-dried. The hybridization mixture contained 50% deionized formamide, 10 mM Tris-HCl (pH 7.6), 200 μg/mL yeast tRNA, 1× Denhardt's solution, 10% dextran sulfate, 10% NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0), and approximately 1.0 μg/mL digoxigenin-labeled cRNA probe. Fifty microliters of the mixture was applied to each section, covered with parafilm, and hybridized in a humid chamber for 18 h at 50°C. Immunodetection of the ISH signal was performed with a 400× alkaline phosphatase-labeled antidigoxigenin antibody (DakoCytomation Co. Ltd., Kyoto, Japan). For the color reaction, the slides were incubated in a solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma-Aldrich Co.). After washing in water, the slides were air-dried and mounted in Pristin Mount.

RESULTS

All of the mice treated with NNK developed adenomas within 20 wk. Histologically, tumor cells were polygonal and mimicing type II pneumocytes (Figure 1A). By transmission electron microscopy, they contained lamellar inclusion bodies and lysosomes but few Clara granules (Figure 2).

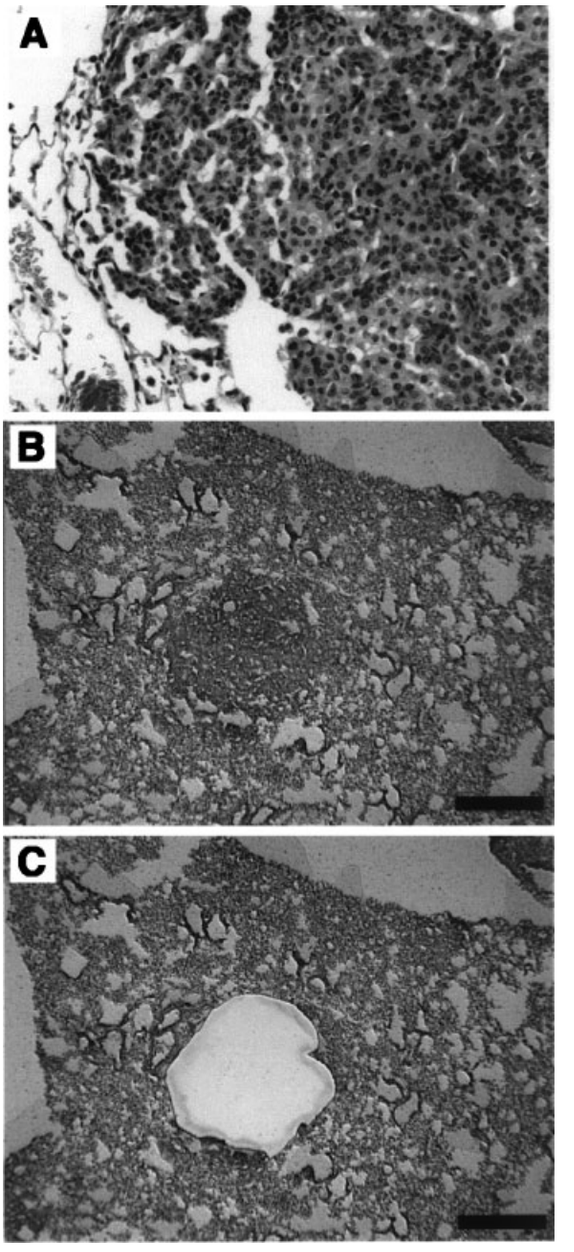

Figure 1. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumor in an A/J mouse. Histology of the tumor (H.E., ×400) (A). Histology of frozen section before (B) and after (C) laser microdissection (toluidine blue, ×40; bars 500 μm).

Tumor and normal pulmonary tissue from three mice were individually microdissected from frozen sections (Figure 1B and C). Subsequently, the total RNA from each tissue was extracted and 100 ng of each total RNA was amplified to over 35 mg cRNA.

Following SSH analysis, a total of 1056 clones were randomly chosen from the forward subtracted library of the first mouse tissue pair (tumor tissue vs. normal pulmonary epithelial tissue) and blotted

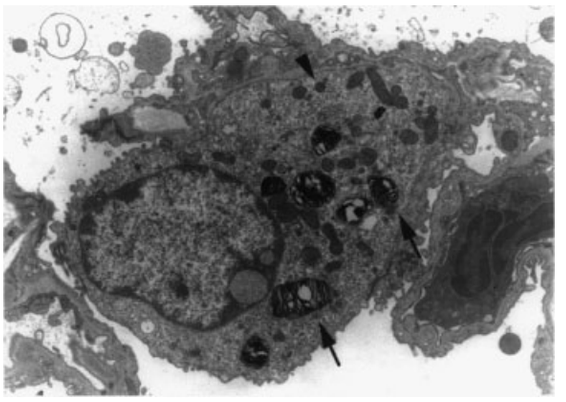

Figure 2. Transmission electron micrograph of NNK-induced lung tumor in an A/J mouse. Tumor cells contained characteristic lamellar inclusion bodies (arrows) and lysosomes (arrowhead). ×5000.

onto nylon membranes. Positive clones were selected from those showing a conspicuous signal with the forward subtracted cDNA probe, and furthermore those showing hardly any detectable signal with the reverse subtracted cDNA probe and weak or no signals with the two kinds of unsubtracted cDNA probes. Positive clones that were differentially screened with the probes for the first mouse tissue pair were further screened with those for the second pair, followed by the third pair. Consequently, 50 differentially expressed clones were obtained in common. Subsequent semi-quantitative screening with the TALPAT products from the three mice revealed 20 clones that were highly expressed in tumor tissue, compared with normal-pulmonary tissue.

As Table 1 shows, the 20 positive cDNA clones were revealed after sequencing. Fourteen of these cDNAs encoded lysozyme and one encoded surfactant-associated protein A (SP-A). Four other clones were chimera fragments between lysozyme and others such as interferon-inducible protein 203, brain MY042 protein, alcohol dehydrogenase PAN2, and catenin. The remaining clone was also a chimera fragment between secretory protein SEC8 and ESTs.

Table 1. Summary of cDNA Clones That Were Highly Expressed in Tumor Cells

Description	Number of clones
Lysozyme	14
Surfactant-associated protein A	1
Interferon-inducible protein 203/lysozyme	1
Brain MY042 protein/EST/lysozyme	1
Weakly similar to alcohol dehydrogenase PAN2/lysozyme	1
Catenin src/lysozyme	1
EST/EST/secretory protein SEC8	1

Expression of mRNAs for lysozyme and SP-A was confirmed by ISH with formalin-fixed, paraffin-embedded A/J mice lung tissues that contained tumors induced by NNK. Signals for lysozyme were detected only in the cytoplasm of tumor cells, and that of type II pneumocytes and histiocytes in normal tissue (Figure 3). SP-A was detected in the cytoplasm of tumor cells and that of type II pneumocytes in normal tissue (Figure 4).

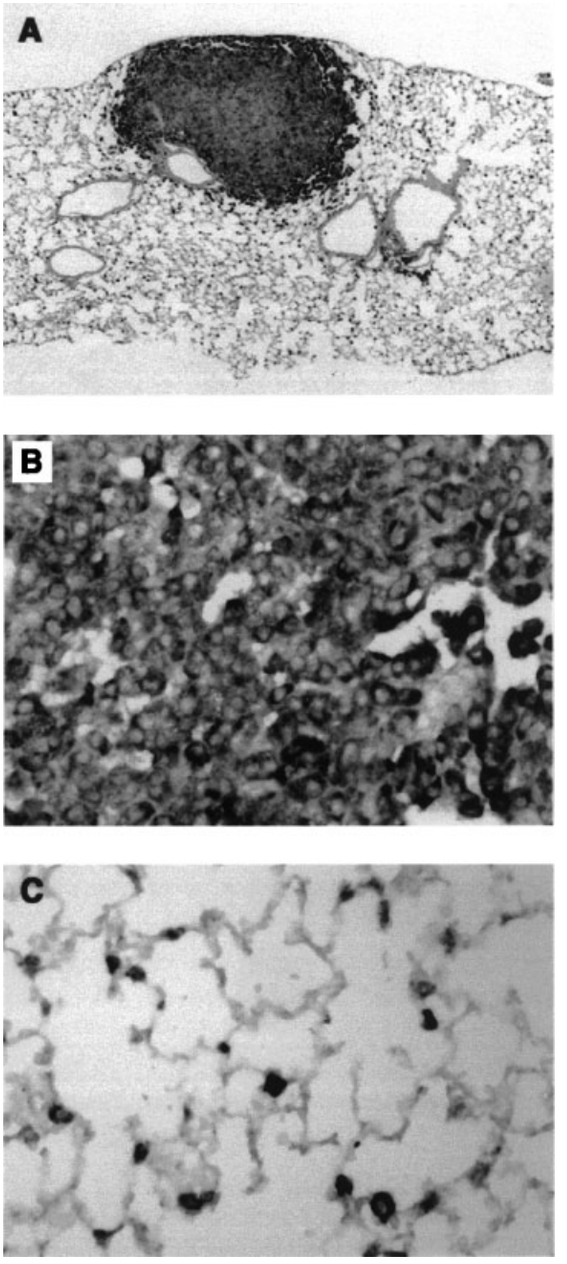

Figure 3. In situ hybridization (ISH) of lysozyme. Tumor cells are strongly positive for lysozyme (×40; A, ×400; B). Type II pneumocytes are also positive for lysozome (×40; A, ×400; C).

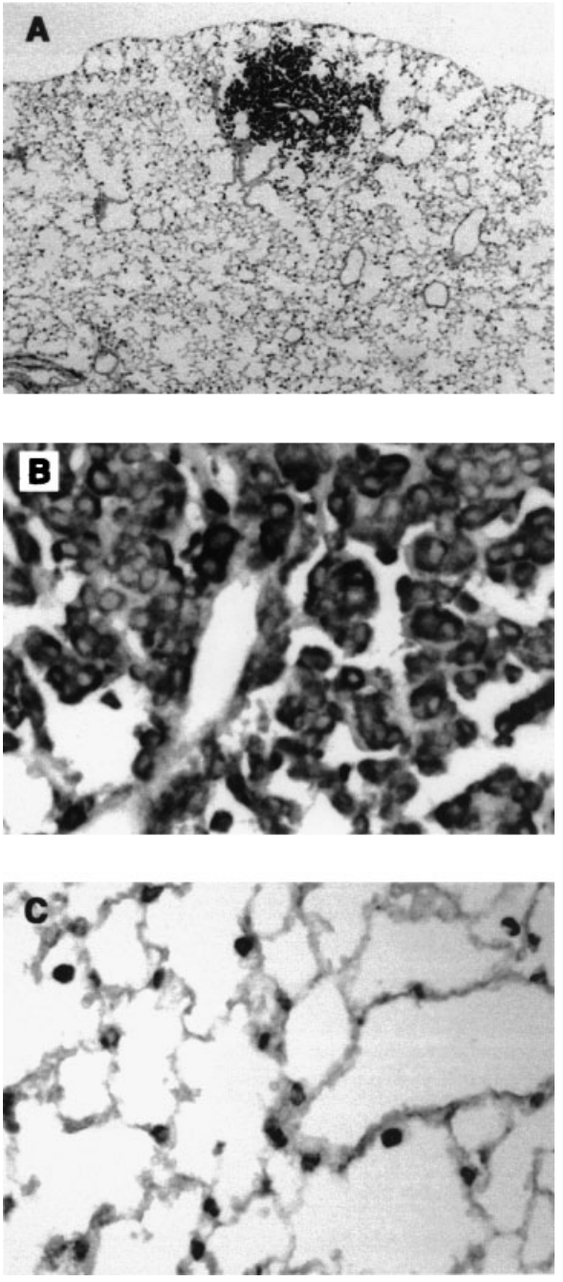

Figure 4. ISH of surfactant-associated protein A (SP-A). Tumor cells are strongly positive for SP-A (×40; A, ×400; B). Type II pneumocytes are also positive for SP-A (×40; A, ×400; C).

DISCUSSION

In this study, we were able to select 20 clones that were highly expressed in adenomas compared to normal mouse lung. Fourteen of them were mRNAs for lysozyme and one was mRNA for SP-A. Four other clones were chimera fragments between lysozyme and other mRNAs encoding products such as interferon-inducible protein 203, brain MY042 protein, alcohol dehydrogenase PAN2, and catenin. We speculated that the chimera fragments had been artificially constructed during the SSH analysis, and selected because they contained the mRNA for lysozyme. Expression of both genes in the tumor cells was confirmed by ISH. Thus, we found two major highly expressed genes–those of lysozyme and SP-A—in NNK-induced adenoma of A/J mice.

The biological characteristics of carcinogen-induced animal lung tumors have been extensively examined. Rehm et al. [3] studied antigens in lung tumors induced by *N*-nitrosoethylurea in C3H/He mice, and found that they were positive for SP-A and negative for lysozyme. On this basis, they concluded that *N*-nitrosoethylurea-induced lung tumors were derived from type II pneumocytes. NNK-induced lung tumors in A/J mice have also been examined. Belinsky et al. [11] measured the concentration of DNA adducts in the tumor cells and concluded that the cells were derived from Clara cells and/or type II pneumocytes. They also examined the tumor cells with electron microscopy and reported that the proliferating cells in hyperplasia resembled type II pneumocytes. Other studies of rat tissues have shown that SP-A and lysozyme are both present in type II pneumocytes [21–23]. These reports indicate that carcinogen-induced animal lung tumors commonly express SP-A and have the characteristics of type II pneumocytes. On the other hand, Schuller et al. [24] found Clara granules in the cytoplasms of the tumor cells of NNK-induced hamster lung adenomas and Rehm et al. [25] immunohistochemically showed that Clara cells are the progenitor cells of hamster lung carcinoma induced by *N*-nitrosodiethylamine. In this study, we proved that NNK-induced tumor cells in A/J mice clearly expressed both SP-A and lysozyme. And we could not find any expressions specifically related with Clara cell. Electron microscopically, most of tumor cells contained lamellar inclusion bodies and lysosomes but few characteristic Clara granules. On the basis of these results, the progenitor cells of NNK-induced lung tumors in A/J mice are speculated to be type II pneumocytes.

In human lung tissue, type II pneumocytes are also thought to be the progenitor cells of AAH and BAC of the nongoblet cell type, and are positive for SP-A but negative for lysozyme. The two proteins are reciprocally expressed in human pulmonary adenocarcinomas, and very few cells express both [1,26]. Most cases of AAH and BAC are nonmucinous-type carcinoma and positive for SP-A and TTF-1, but negative for lysozyme. In contrast, goblet cell type adenocarcinoma is positive for lysozyme but negative for SP-A and TTF-1. Furthermore, the frequency of K-*ras* gene mutation in nonmucinous-type adenocarcinoma is very low, but the mutation is frequently detected in mucinous-type adenocarcinoma [17,27]. Although NNK-induced tumors in A/J mice closely resemble human AAH and BAC histologically, their

immunohistochemical characteristics differ. Therefore, care is needed when extrapolating the results of experimental studies with A/J mice to the mechanisms of human carcinogenesis.

SSH is a very powerful method for examining differences between expressed mRNAs. In this study, we examined the differences between NNK-induced tumors and normal lung tissue in A/J mice and detected two mRNAs (those for SP-A and lysozyme) that were characteristically expressed in NNK-induced adenoma. However, these two proteins are both phenotypic markers and are not key proteins that are related to proliferation, invasion, or metastasis. In order to clarify the mechanisms responsible for the proliferation and/or tumorigenicity of pulmonary adenocarcinoma, detailed comparison of expression profiles between tumor cells and their progenitor cells (type II pneumocytes) is considered to be more important.

REFERENCES

1. Mizutani Y, Nakajima T, Morinaga S, et al. Immunohistochemical localization of pulmonary surfactant apoproteins in various lung tumors. Special reference to nonmucus producing lung adenocarcinomas. Cancer 1988;61:532–537.
2. Morse MA, Eklind KI, Toussaint M, Amin SG, Chung FL. Characterization of a glucuronide metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its dose-dependent excretion in the urine of mice and rats. Carcinogenesis 1990;11:1819–1823.
3. Rehm S, Devor DE, Henneman JR, Ward JM. Origin of spontaneous and transplacentally induced mouse lung tumors from alveolar type II cells. Exp Lung Res 1991;17: 181–195.
4. Hoffmann D, Hecht SS. Nicotine-derived *N*-nitrosamines and tobacco-related cancer: Current status and future directions. Cancer Res 1985;45:935–944.
5. Hecht SS, Hoffmann D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 1988;9:875–884.
6. Anderson LM, Hecht SS, Dixon DE, et al. Evaluation of the transplacental tumorigenicity of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in mice. Cancer Res 1989;49:3770–3775.
7. Castonguay A, Lin D, Stoner GD, et al. Comparative carcinogenicity in A/J mice and metabolism by cultured mouse peripheral lung of *N*′-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and their analogues. Cancer Res 1983;43:1223–1229.
8. Hecht SS, Abbaspour A, Hoffman D. A study of tobacco carcinogenesis. XLII. Bioassay in A/J mice of some structural analogues of tobacco-specific nitrosamines. Cancer Lett 1988;42:141–145.
9. Hoffmann D, Hecht SS, Ornaf RM, Wynder EL. *N*′-nitrosonornicotine in tobacco. Science 1974;186:265–267.
10. Hecht SS, Chen CB, Hirota N, Ornaf RM, Tso TC, Hoffmann D. Tobacco-specific nitrosamines: Formation from nicotine in vitro and during tobacco curing and carcinogenicity in strain A mice. J Natl Cancer Inst 1978;60:819–824.
11. Belinsky SA, Devereux TR, Foley JF, Maronpot RR, Anderson MW. Role of the alveolar type II cell in the development and progression of pulmonary tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the A/J mouse. Cancer Res 1992;52:3164–3173.
12. Noguchi M, Morikawa A, Kawasaki M, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer 1995;75:2844–2852.
13. Kitamura H, Kameda Y, Ito T, Hayashi H. Atypical adenomatous hyperplasia of the lung. Implications for the pathogenesis of peripheral lung adenocarcinoma. Am J Clin Pathol 1999;111:610–622.
14. Matzinger SA, Crist KA, Stoner GD, et al. K-ras mutations in lung tumors from A/J and A/J × TSG-p53 F1 mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and phenethyl isothiocyanate. Carcinogenesis 1995;16:2487–2492.
15. Kawano R, Takeshima Y, Inai K. 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. Jpn J Cancer Res 1996;87:44–50.
16. Belinsky SA, Devereux TR, White CM, Foley JF, Maronpot RR, Anderson MW. Role of Clara cells and type II cells in the development of pulmonary tumors in rats and mice following exposure to a tobacco-specific nitrosamine. Exp Lung Res 1991;17:263–278.
17. Maeshima A, Sakamoto M, Hirohashi S. Mixed mucinous-type and non-mucinous-type adenocarcinoma of the lung: Immunohistochemical examination and K-ras gene mutation. Virchows Arch 2002;440:598–603.
18. Diatchenko L, Lau YF, Campbell AP, et al. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 1996;93:6025–6030.
19. Kolble K. The LEICA microdissection system: Design and applications. J Mol Med 2000;78:B24–B25.
20. Aoyagi K, Tatsuta T, Nishigaki M, et al. A faithful method for PCR-mediated global mRNA amplification and its integration into microarray analysis on laser-captured cells. Biochem Biophys Res Commun 2003;300:915–920.
21. Haller EM, Shelley SA, Montgomery MR, Balis JU. Immunocytochemical localization of lysozyme and surfactant protein A in rat type II cells and extracellular surfactant forms. J Histochem Cytochem 1992;40:1491–1500.
22. Gibson KF, Phadke S. Intracellular distribution of lysozyme in rat alveolar type II epithelial cells. Exp Lung Res 1994;20: 595–611.
23. Gibson KF, Widnell CC. The relationship between lamellar bodies and lysosomes in type II pneumocytes. Am J Respir Cell Mol Biol 1991;4:504–513.
24. Schuller HM, Witschi HP, Nylen E, Joshi PA, Correa E, Becker KL. Pathobiology of lung tumors induced in hamsters by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the modulating effect of hyperoxia. Cancer Res 1990;50:1960–1965.
25. Rehm S, Takahashi M, Ward JM, Singh G, Katyal SL, Henneman JR. Immunohistochemical demonstration of Clara cell antigen in lung tumors of bronchiolar origin induced by *N*-nitrosodiethylamine in Syrian golden hamsters. Am J Pathol 1989;134:79–87.
26. Komatsu H, Tamura A, Yoneda R. Immunohistochemical findings in resected lung cancer. Nihon Kyobu Shikkan Gakkai Zasshi 1991;29:1234–1238.
27. Gemma A, Noguchi M, Hirohashi S, et al. Clinicopathologic and immunohistochemical characteristics of goblet cell type adenocarcinoma of the lung. Acta Pathol Jpn 1991; 41:737–743.