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Napsin A is possibly useful marker to predict the tumorigenic potential of lung bronchiolo-alveolar hyperplasia in F344 rats

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ABSTRACT

There are 2 types of bronchiolo-alveolar hyperplasia found in rat lungs. One is 'inflammatory hyperplasia' with a potential to recover in future with removal of the stimulating insult and the other is 'latent tumorigenic hyperplasia' as an independent preneoplastic lesion for adenocarcinoma. In the present experiment, we focused on rat lung bronchiolo-alveolar hyperplasia induced by 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which decreases with time after induction and reverts to normal, or by N-bis(2-hydroxypropyl)nitrosamine (DHPN), with tumorigenic potential to progress to adenoma and adenocarcinoma. Though NNK is a typical carcinogen inducing lung adenocarcinoma in female A/J mice, the tumorigenic potential by NNK in rats is weak. Differences between hyperplasias induced by DHPN and by NNK were here examined immunohistochemically.

Formalin fixed paraffin embedded lung samples with hyperplastic and inflammatory lesions were obtained from rats exposed to DHPN or NNK and from lung inflammation models induced with fine particles like CuO, NiO and quartz. The 19 markers were examined immunohistochemically.

Napsin A, in the inflammatory lesions and hyperplasia induced by NNK, was positive for macrophages and secretions in the alveoli spaces but less so in the walls of the alveoli. In the proliferative lesions including hyperplasia induced by DHPN, strong positive staining for napsin A was observed in the walls of the alveoli. Thus high expression was suggested to be possibly useful for detecting tumorigenic potential of rat lung hyperplasia.

1. Introduction

Lung cancer is one of the most common cancers in the world, with cigarette smoking regarded as the predominant cause. The risk of lung cancer development remains elevated even after giving up smoking and second-hand environmental tobacco smoke from others is also considered to be a problem (Mitchell and Sanders, 2002). Therefore, identification of potential chemopreventive agents in animals models is important. In rodents, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-bis(2-hydroxypropyl)nitrosamine (DHPN) are well known lung carcinogens, but their effects differ between rats and mice. Previously, in order to establish an appropriate bioassay for detection of promoting potential to lung tumor after intratracheal instillation of fine particle in rats, sequential histopathological changes were examined after initiation of lung tumorigenesis by 3 intraperitoneal injections (IPs) of 10 mg NNK or 0.1% DHPN in drinking water for 2 weeks and intratracheal instillation (IT) to quartz, as a typical lung toxic agent (Yokohira et al., 2005, 2007, 2009b), into F344 male rats (Yokohira et al., 2009a). Lung proliferating lesions were seen by NNK and by DHPN in rats. However, whereas DHPN induced hyperplasias whose size, number and malignancy increased with time, NNK induced hyperplasia with inflammation proved reversible. Thus their multiplicity was 3.2 ± 2.6 (20 rats) at completion of treatment, but only 0.2 ± 0.4 (20 rats) and 0.8 ± 0.8 (20 rats) at 23 and 30 weeks, respectively. This is in line with the earlier finding that tumorigenic potential of NNK in rats is weak (Yokohira et al., 2009a). In contrast, NNK is well known as a strong lung carcinogen when administered to mice, especially to the A/J strain with high susceptibility to lung tumor induction (Takeuchi et al., 2003, 2006, 2009; Yokohira et al., 2008b). From our data there appear to be 2 types of bronchiolo-alveolar hyperplasia in the rat lungs. One is

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Table 1
Antibody for immunostaining in Experiments 1 and 2.

Antibody	Product code	Company	Antigen retrieval[a]	Dilution	Reaction time (min)
Cell proliferation					
PCNA(PC10)	sc-56	Santa Cruz Biotechnology, Inc., CA, USA	D	1:100	15
Ki67	NCL-Ki67p	Leica Microsystems Newcastle Ltd., Newcastle Upon Tyne, United Kingdom	A	1:2000	15
EGF-R	M 3563	Dako North America, Inc., CA, USA	C	1:200	15
Cell cycle					
Cyclin D1	413531	Nichirei Biosciences Inc., Tokyo, Japan	B	1:100	15
p27	M 7202	Dako Denmark A/S, Glostrup, Denmark	A	1:400	15
p53	M 7001	Dako Denmark A/S, Glostrup, Denmark	A	1:50	15
p16	CINtec p16	Roche mtm laboratories AG, Heidelberg, Germany	A	Fully diluted	15
Tumor producing					
CEA	A 115	Dako Denmark A/S, Glostrup, Denmark	D	1:100	15
Alveolar epithelium					
Napsin A	NCL-L-napsin A	Leica Microsystems Newcastle Ltd., Newcastle Upon Tyne, United Kingdom	D	1:100	15
TTF-1	M 3575	Dako North America, Inc., CA, USA	B	1:200	15
SP-A	M 4501	Dako Japan Co., Ltd., Kyoto, Japan	A	1:500	15
Cell membrane					
Cytokeratin 7	NCL-CK7-OVTL	Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom	A	1:500	15
Cytokeratin 20	NCL-CK20	Leica Microsystems Newcastle Ltd., Newcastle Upon Tyne, United Kingdom	A	1:75	15
Endocrine receptor					
Estrogen receptor α	NCL-ER-6F11	Leica Microsystems Newcastle Ltd., Newcastle Upon Tyne, United Kingdom	A	1:100	15
Progesterone receptor	NCL-PGR-312	Leica Microsystems Newcastle Ltd., Newcastle Upon Tyne, United Kingdom	A	1:600	15
Chromogranin A	A 0430	Dako Denmark A/S, Glostrup, Denmark	A	1:100	15
Synaptophysin	NCL-SYNAP-299	Leica Microsystems Newcastle Ltd., Newcastle Upon Tyne, United Kingdom	A	1:100	15
Squamous cell					
Cytokeratin 34βE12	M 0630	Dako Denmark A/S, Glostrup, Denmark	A	1:50	15
Cytokeratin 5/6	M 7237	Dako Denmark A/S, Glostrup, Denmark	A	1:50	15

[a] Antigen retrieval: A: heat treatment with pH7 reagent; B: heat treatment with pH 9 reagent; C: enzymatic treatment; D: non-treatment.

'inflammatory hyperplasia' with the potential for reversion to normal and the other is 'latent tumorigenic hyperplasia' considered as a preneoplastic precursor for adenocarcinoma.

In the present experiment, we focused on comparing rat lung hyperplasias induced by NNK and DHPN immunohistochemically. Experiments were conducted to find suitable marker(s) for prospective tumorigenic and malignant potential of early stage lung hyperplasia. To choose probable markers, preliminary staining of hyperplasias, adenomas and adenocarcinomas induced by DHPN after 30 weeks in F344 rats was performed (Experiment 1). Selected examples were then examined with hyperplasias induced by NNK or DHPN (Experiment 2). Additionally, expression of napsin A was examined in inflammatory lesions in lung induced by fine particles for comparison. Previously, lung toxicity of fine particles from various materials was examined in our *in vivo* bioassay using the IT method (Yokohira et al., 2007, 2008a, 2009b). With the same dose of 2 mg/rat IT, fine particles of quartz, CuO and NiO all caused severe toxicity with severe inflammatory changes featuring neutrophil infiltration and edema.

2. Materials and methods

2.1. Animals

Experimental animals (Experiments 1 and 2) were maintained in the Division of Animal Experiments, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal Experiments. All of the animals were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (60 ± 10%), lighting (12-h light/dark cycle) and temperature (24 ± 2 °C).

2.2. Experiment 1. Analysis of various markers for the lung proliferative lesions

2.2.1. Tissue samples

Formalin fixed paraffin embedded (FFPE) lung samples including neoplastic lesions (hyperplasia, adenoma and adenocarcinoma) were obtained with the rat DHPN induced lung carcinogenesis model (Yokohira et al., 2009a). Briefly, male 6-week old F344/DuCrlCrlj rats (Japan Charles River, Inc., Kanagawa, Japan) were given 0.1% DHPN (Nacalai Tesque Inc., Kyoto, Japan) in drinking water for 2 weeks, and sacrificed at week 30. At autopsy, the lungs were removed and the lungs, including the trachea and heart, were infused from the trachea with 10% phosphate buffered formalin and then rinsed in 10% phosphate buffered formalin. The lungs were immersed in 10% phosphate buffered formalin for approximately 48 h, and slices were routinely processed for embedding in paraffin for histopathological examination. The method to develop FFPE lung samples from harvested lungs was the same in Experiments 1 and 2.

2.3. Experiment 2. Validation of the selected markers from Experiment 1

2.3.1. Tissue samples

FFPE lung samples with hyperplastic lesions and inflammatory lesions were obtained with rat DHPN or NNK (Toronto Research Chemicals, ON, Canada) induced lung carcinogenesis models on week 12 and 30 (Yokohira et al., 2009a), respectively, and with