



Published in final edited form as:

*Lung Cancer*. 2013 December ; 82(3): 390–396.

## Development of ferret as a human lung cancer model by injecting 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

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### Abstract

**Objective**—Development of new animal lung cancer models that are relevant to human lung carcinogenesis is important for lung cancer research. Previously we have shown the induction of lung tumor in ferrets (*Mustela putorius furo*) exposed to both tobacco smoke and a tobacco carcinogen (4-(*N*-Methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, NNK). In the present study, we investigated whether NNK treatment alone induces both preneoplastic and neoplastic lesions in the lungs of ferrets.

**Methods**—We exposed ferrets to NNK by i.p. injection of NNK (50 mg/kg BW) once a month for four consecutive months and then followed up for 24, 26 and 32 weeks. The incidences of pulmonary preneoplastic and neoplastic lesions were assessed by histopathological examination. The expressions of  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$  nAChR, which has been shown to promote lung carcinogenesis) and its related molecular biomarkers in lungs were examined by immunohistochemistry and/or Western blotting analysis.

**Results**—Ferrets exposed to NNK alone developed both preneoplastic lesions (squamous metaplasia, dysplasia and atypical adenomatous hyperplasia) and tumors (squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma), which are commonly seen in humans. The incidence of tumor induced by NNK was time-dependent in the ferrets (16.7%, 40.0% and 66.7% for 24, 26 and 32 weeks, respectively).  $\alpha 7$  nAChR is highly expressed in the ferret bronchial/bronchiolar epithelial cells, and alveolar macrophages in ferrets exposed to NNK,

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### Conflict of Interest Statement

None declared.

and in both squamous cell carcinoma and adenocarcinoma of the ferrets. In addition, we observed the tendency for an increase in phospho-ERK and cyclin D1 protein levels ( $p = 0.081$  and  $0.080$ , respectively) in the lungs of ferrets exposed to NNK.

**Conclusion**—The development of both preneoplastic and neoplastic lesions in ferret lungs by injecting NNK alone provides a simple and highly relevant non-rodent model for studying biomarkers/molecular targets for the prevention, detection and treatment of lung carcinogenesis in humans.

## Keywords

ferret; tobacco carcinogen; NNK; lung cancer;  $\alpha 7$  nicotinic acetylcholine receptor

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## 1. Introduction

Lung cancer is the leading cause of cancer death in the United States and worldwide. While several factors increase the risk for developing lung cancer, cigarette smoking is the single most important factor associated with 90% of lung cancer cases [1-3]. As cigarette smoke contains more than 60 carcinogens (particularly nitrosamines), the tobacco-specific nitrosamines 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*<sup>7</sup>-nitrosonornicotine (NNN) are by far the most prevalent carcinogens in tobacco products [2, 3], and have been shown to induce lung carcinogenesis *in vivo* [4, 5]. Various animal models for lung cancer induced by cigarette smoke and/or carcinogens in cigarette smoke have been reported [5-7].

While rodent models are easier to use, the lung structure and bronchial branching of mice is different from humans [6, 8-10]. Further, there are limitations with regard to the relevance of tumors that the rodents develop compared with human tumors. For example, mice typically develop hyperplasia of alveolar epithelium in response to carcinogens, whereas squamous differentiation is more common in the human lung [6]; the most common subtypes of lung cancer in humans are squamous cell carcinomas and adenocarcinomas [11]. By contrast, the most common neoplastic subtypes resulting from carcinogen induction in mice are adenomas and, to a lesser extent, adenocarcinomas [8]. Although it is rare to observe a combination of different histological types in a single mouse tumor, such observations occur in 30-60% of human lung tumors [6, 8]. Furthermore, mice treated with carcinogens tend to develop multiple tumors which rarely metastasize; in humans, multiple tumors are uncommon and lung cancer metastasis is frequent [6, 8]. Given the need to develop better detection and treatment options for lung cancer, it is essential to develop animal models that are more representative of lung carcinogenesis in humans.

The ferret (*Mustela putorius furo*) has been shown to be a useful model for pulmonary research studies since the pulmonary structure and airways of ferrets are similar to humans [12]. The ferret is commonly used for studies of infectious lung disease since it is susceptible to infection from a large number of pathogens [13]. We previously reported that the ferrets exposed to both cigarette smoke and NNK develop adenocarcinomas and squamous cell carcinomas, the most common lung tumors in humans [14]. The morphological characteristics of ferret lung tumors are very similar to those of humans,

further suggesting that the ferret may be a relevant model to study lung carcinogenesis [14]. However, the requirement for a smoking exposure device for ferrets has limited the wide use of the ferret model for lung carcinogenesis, and it is unknown whether both preneoplastic and neoplastic lesions could be induced by simple NNK injections in ferrets.

In the present study, we investigated the appropriateness of the ferret as a model for human lung cancer. We describe here the incidence of preneoplastic and neoplastic lesions and histopathological types of lung tumors in ferrets exposed to NNK alone for 24 to 32 weeks.

## 2. Materials and Methods

### 2.1. Animals, study group and carcinogen treatment

Male ferrets (1.1 – 1.7 kg, aged between 3 – 5 months old) were obtained from Marshall Farms (North Rose, NY). In order to avoid potential variation due to the dietary components and to make it easier for the future dietary/chemopreventive intervention study, the animals were fed a semi-purified ferret diet (Research Diets, Inc, New Brunswick, NJ: D90001 Diets contains the following components: Casein 34.5%; L-Arginine 0.5%; L-Methionine 0.3%; Maltodextrin 7.5%; Corn starch 26.5%; Cellulose 5.0%; Corn oil 10.5%; Lard 10.5%; Salt mix (S90002) 3.5%; Vitamin mix (V90002) 1.0%; Choline bitartrate 0.2%), and had access to food and water *ad libitum* throughout all experiment periods. After acclimation, 22 ferrets were given intraperitoneal (i.p.) injections of NNK (Toronto Research Chemicals, Ontario, Canada) at a dose of 50 mg/kg BW, once a month for four consecutive months and then maintained for 24 weeks (n=6), 26 weeks (n=10) and 32 weeks (n=6). Control animals (n=8) received a sham injection of normal saline and observed for 26 weeks. At the end of each experimental period, ferrets were killed by terminal exsanguination under deep isoflurane (Isothesia, Butler Schein, Dublin, OH) anesthesia. Tissues and plasma were collected and stored at –80 °C until analyzed. This study protocol was approved by the Animal Care and Use Committee at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

### 2.2. Histopathology

The upper right lobe of each ferret lung was inflated post-mortem and fixed by an intratracheal instillation of 10% formalin, as previously described [15]. The lungs were embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological examination. The incidence of preneoplastic lesions (squamous metaplasia and dysplasia and atypical adenomatous hyperplasia) and neoplastic lesions (adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, and lymphoma) were assessed. Histology slides were examined at both 10X and 40X magnification and observations were confirmed by two independent investigators who were blinded to the treatment groups.

### 2.3. Immunohistochemistry

The localization of  $\alpha 7$  nicotinic acetylcholine receptor (nAChR) expression in lung, pulmonary cancerous bodies and macrophages was examined by immunohistochemistry using the avidin-biotin complex immunoperoxidase methods according to previously

described method [16, 17]. Briefly, four  $\mu\text{m}$  thick sections from formalin-fixed, paraffin-embedded lung tissue blocks were immunostained for  $\alpha 7$  nAChR (AChR $\alpha 7$  (319): sc-58607, Santa Cruz Biotechnology, Santa Cruz, CA) with dilutions of 1:50. After then, the sections were incubated with biotinylated second goat anti-rat antibody (Vector Laboratories, Inc., Burlingame, CA) diluted 1:250, and further incubated with VECTASTAIN Elite ABC reagent (Vector Laboratories Inc., Burlingame, CA). The sections were subsequently processed with peroxidase substrate solution (Vector Laboratories Inc., Burlingame, CA). The sections were then counterstained with hematoxylin.

#### 2.4. Western blots analysis

Western blotting was performed with whole cell lysate of lung tissues by using a previously described method [18]. Antibodies against  $\alpha 7$  nAChR and cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total-Akt, phospho-Akt, total-ERK1/2 and phospho-ERK1/2 were purchased from Cell Signaling (Beverly, MA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Millipore (Milford, MA) was used as a loading control. Results were quantified using a densitometer and expressed as fold change in the treatment group vs. the control group. The dilutions of each antibody were as follows:  $\alpha 7$  nAChR 1:300; cyclin D1 1:1000; total-Akt 1:2000; phospho-Akt (Ser473) 1:500; total-ERK1/2 1:1000; phospho-ERK1/2 1:500; GAPDH 1:20000.

#### 2.5. Statistical analysis

All results are expressed as means  $\pm$  SEM. Comparisons of data between two groups were made using unpaired Student's *t*-test. Results were considered statistically significant at  $P < 0.05$ .

### 3. Results

#### 3.1. Mortality of ferrets exposed to NNK

NNK has been widely used as a carcinogen for inducing lung tumorigenesis. NNK is also a toxic chemical and the LD<sub>50</sub> has been reported to be 1,000 mg/kg BW by the i.p. injection to mice [19]. In the present study, 50 mg/kg BW of NNK was given i.p. at 4-week intervals for a total of four doses over 4 months so as to minimize the toxicity of NNK. This total dose of NNK (200 mg) used in the ferrets was based on that the mink, receiving a single of dose (150 mg) of tobacco-carcinogen N-nitrosoamine (NNN), did not show any toxic effects [20]. Three ferrets died in the NNK treated group during the experiment period (between 11 – 14 weeks after the first injection of NNK) without any obvious pathology. Although it was unlikely, we cannot exclude the possibility that the death of these ferrets was due to the NNK injection. There were no significant differences in the body weight between the control ( $1.57 \pm 0.14$  kg) and the NNK treated ( $1.72 \pm 0.35$  kg) groups at 24 weeks.

#### 3.2. Incidence of lung preneoplastic lesions, neoplastic lesions and lung pathologic types in the lungs of ferrets exposed to NNK

We observed 83.3 to 100% incidences of preneoplastic lesions in ferrets exposed to NNK after 24 to 32 weeks. NNK resulted in various histopathological types of preneoplastic

lesions, including squamous metaplasia, squamous dysplasia and atypical adenomatous hyperplasia in ferret lungs (Table 1, Fig. 1). In addition, we observed a 16.7% incidence of neoplastic pulmonary tumors (squamous cell carcinoma, adenosquamous carcinoma and adenocarcinoma) in ferrets exposed to NNK for 24 weeks; the incidence of such tumors increased to 40.0% or 66.7% in ferrets treated with NNK and maintained for 26 or 32 weeks, respectively (Table 2, Fig. 1). Although there were no statistically significant differences in the incidence between the different time points because of the small sample size, these data suggested that NNK could induce lung tumors in a time dependent manner. NNK treatment alone, for different periods of time, predominantly induced adenocarcinomas and, to a smaller extent, resulted in the induction of squamous cell carcinomas and adenosquamous carcinomas. We also observed a lymphoma in a ferret treated with NNK alone for only 32 weeks. Therefore, it appears that NNK treatment by itself can induce various tumor types normally seen with exposure to both NNK and cigarette smoke.

### 3.3. $\alpha 7$ nAChR protein expressions in ferret lung exposed to NNK by immunohistochemistry and western blotting

Components of cigarette smoke, including nicotine and NNK, can induce cell proliferation, angiogenesis and apoptosis resistance by exerting its cellular functions through nAChRs [21]. Among the nAChRs,  $\alpha 7$  nAChR plays an important role in the development and progression of lung cancer [21-23]. Since NNK is known to be a high-affinity ligand for  $\alpha 7$  nAChR [24], we examined whether NNK induces protein levels of  $\alpha 7$  nAChR, which mediates the tumor growth signaling pathway, in the lungs of ferrets. Since we only had the control group at 26 weeks, we assessed the protein expressions of  $\alpha 7$  nAChR in the lung of ferrets with or without NNK exposure for 26 weeks. As the result, NNK exposure significantly increased  $\alpha 7$  nAChR protein levels in whole lung homogenates (Fig. 2A). In addition, the  $\alpha 7$  nAChR was highly expressed in the ferret bronchial/bronchiolar epithelial cells, exposed to NNK exposure (Fig. 3B), as compared with that of the ferrets without NNK injection (Fig. 3A). In addition,  $\alpha 7$  nAChR was greatly expressed in both adenocarcinoma and squamous cell carcinoma of the ferrets (Fig. 3D, 3E) as well as in alveolar macrophages and tumor associated macrophages (Fig. 3C, 3F). Akt protein levels were not significantly changed (Fig. 2B) between the groups. However, we observed the tendency for an increase in phospho-ERK and cyclin D1 protein levels ( $p = 0.081$  and  $0.080$ , respectively; Fig. 2C, 2D), which were mainly involved in activation of cell proliferation. Since we observed two bands in cyclin D1 (Fig. 2D) in ferrets which were almost in the same location of cyclin D1 protein as that of human BEAS-2B and were strongly dose-dependent (correlation factor:  $R^2 > 0.85$ ) (data not shown), we concluded that both bands were proteins of cyclin D1.

## 4. Discussion

In the present study, we demonstrated that the ferret developed both preneoplastic and neoplastic lesions in the lungs by intraperitoneal injecting NNK alone, indicating that the ferret was a highly relevant non-rodent model of lung carcinogenesis to humans. The preneoplastic lesions of ferrets exposed to NNK alone included squamous metaplasia, squamous dysplasia and atypical adenomatous hyperplasia (Table 1). These preneoplastic

lesions morphologically closely resembled squamous carcinoma or adenocarcinoma, supporting the notion that ferret's lung carcinomas derived from squamous metaplasia/dysplasia and atypical adenomatous hyperplasia. As we further evaluated the type of lung tumors that ferrets developed after NNK exposure, we detected adenocarcinomas, squamous cell carcinomas and adenosquamous carcinomas in the lungs of ferrets exposed to NNK (Table 2). As previously discussed, the most common types of human lung cancer are squamous cell carcinomas and adenocarcinomas [11], which are rarely seen in mouse models, emphasizing the relevance of the ferret models to human tumors. Furthermore, NNK treatment induced adenosquamous carcinomas, containing the combined phenotypes of both glandular and squamous differentiation. These combinations of different phenotypes are common in human lung tumors, but are not seen in mouse models of lung cancer [6]. We also observed a lymphoma in the lungs of a ferret exposed to NNK for 32 weeks (Table 2). Ferrets can spontaneously develop lymphomas [25, 26]. However, there was a previous study indicating that NNK treatment induces lymphomas *in vivo* [27]. Since the lymphoma observed in this study occurred in the lungs and occupied almost two-third of the right side of lung, it seems that this lymphoma would be induced by the NNK treatment.

Cigarette smoking is a major risk factor for the development of lung cancer in both men and women. There is no information regarding sex predilection on cancer development of ferret [28, 29]. We have previously reported that the induction of lung tumor exposed to both tobacco smoke and NNK in male ferrets (Table 3). In order to compare with the effect of NNK treatment with or without smoke-exposure, we used the same male ferrets to investigate whether NNK treatment alone induce lung tumor in the present study. We found that NNK induced lung tumors in a time-dependent manner, with the highest incidence (66.7%) after 32 weeks subsequent to NNK treatment. Interestingly, when we compared the incidence of lung preneoplastic and neoplastic lesions in ferrets treated with NNK and/or exposed to cigarette smoke from previous and current studies (Table 3), we observed that ferrets exposed to NNK alone for 24 weeks had a 16.7% incidence of lung tumors; the ferrets treated with the same dose of NNK and exposed to two doses of cigarette smoke (equivalent to 1/4 or 1.0 consumer packs of cigarettes/day) in our previous studies had an increased incidence of lung tumors (25% and 50%, for 22 and 24 weeks, respectively) [14, 30]. Ferrets treated with the higher dose of smoke-exposure alone (equivalent to 2.0 consumer packs of cigarettes/day) had precancerous lesions without incidence of lung tumors at 24 weeks post-treatment [31, 32]. In our previous ferret studies exposed to both smoke and NNK [14, 17, 30], there was an increased inflammatory response suggested by the clinical signs including cough, shortness of breath, excessive salivation and unhealthy fur in the ferrets. We did not observed these clinical signs in the ferrets with NNK alone in the present study. There were no significantly differences in the body weights between the control and the NNK treated groups at 24 weeks, however, we observed that ferrets exposed to NNK alone had less incidences of lung tumors, as compared with the ferrets treated with the same dose of NNK and exposed to cigarette smoke. In addition, we observed the presence of pathological lesions (including inflammatory cell infiltration and obstructive bronchiolitis) in the lungs of ferrets exposed to both smoke and NNK. Therefore, we believe that the smoke-induced inflammatory responses promote the progression of tumors after initiation by NNK in the ferret.

One of the interesting observations in this study was that the  $\alpha 7$  nAChR is highly expressed on the membranes of bronchial/bronchiolar epithelial cells, and alveolar macrophages in ferrets exposed to NNK (Fig. 3). These observations consisted with the previous reports indicating that nAChRs were localized on the membrane of cells [33]. In addition,  $\alpha 7$  nAChR was expressed in both squamous cell carcinoma and adenocarcinoma of the ferrets (Fig. 3). As we pointed above, NNK is known to be a high-affinity ligand for  $\alpha 7$  nAChR [24], we believe that NNK can initiate lung carcinogenesis and also promote lung tumor development by activating  $\alpha 7$  nAChR. This observation was supported by previous reports that  $\alpha 7$  nAChR expression was higher in smoking patients with squamous carcinoma than those with adenocarcinoma [34], the demonstration that administration of nicotine (either by i.p. injection or through dermal patches) could promote tumor growth and metastasis in immunocompetent mice [35], and that the increased expression of  $\alpha 7$  nAChR has been described in the lung Type-II and epithelial cells by NNK treatment in A/J mice [36].

Although the ferret model exposed to NNK alone could be applicable to the assay of the expression of  $\alpha 7$  nAChR, we only observed the tendency to increase  $\alpha 7$  nAChR's downstream protein levels such as phospho-ERK and cyclin D1 (Fig. 2). Since we have previously shown that NNK and cigarette smoke treatment resulted in increased ERK activation [37] and proliferating cellular nuclear antigen [31, 38-40], this could be due to the limited sample size or NNK treatment alone (without smoke exposure) in the present study. It has been reported that the expression of  $\alpha 7$  nAChR was related to the major activation of the Rb-Raf-1/phospho-ERK pathway and was associated with proliferation of lung cancer cells [34, 41]. Meanwhile, nicotine induced an increase in cell proliferation and a decrease of apoptosis in cancer cell lines, mediated by  $\alpha 7$  nAChR and their down-stream products, not only ERK but also those of the PI3K/AKT pathways [42].

In summary, ferrets injected with NNK alone developed morphological characteristics of pulmonary neoplasia and biomarkers related to carcinogenesis which are typically seen in smokers and lung cancer patients. Additionally, ferrets provide ample plasma samples to monitor biomarkers and the concentration of chemopreventive agents, both during the experiment and at its end. Ferrets also provide ample tissue quantities with which one can investigate molecular mechanisms at the protein level. Furthermore, the ferret is an excellent animal model for studying effects of micronutrient such as carotenoids, because of the similarities between ferrets and human in their absorption and accumulation [43]. In our previous studies [17, 30-32, 37-40], we have investigated the chemopreventive effects and potential biological mechanisms by dietary antioxidants supplementation, such as  $\beta$ -carotene, lycopene,  $\beta$ -cryptoxanthin, and both vitamins E and C, on cigarette-smoke and NNK exposure induced lung carcinogenesis using ferrets. Since this is the first report to establish the ferret as a model of pulmonary carcinogenesis by NNK exposure alone, we are currently investigating potential chemopreventive effects of carotenoids using this ferret model. Recently, the first ferret *CFTR*-knockout model of cystic fibrosis demonstrated many of the characteristics of human cystic fibrosis and provided a useful genetically-engineered ferret model for studying cystic fibrosis [44]. The ferret genome sequencing project is ongoing and has recently identified close to 16,000 partial ferret genes [45]. Therefore, the

ferret could prove to be a valuable model to identify biomarkers/molecular targets for the prevention, detection and treatment of lung carcinogenesis.

## Acknowledgments

This study was supported by the NIH/NCI CA104932 grant, and partially supported by the fund from Kagome Co., Ltd. (Japan). Any opinions, findings, conclusions, and recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the sponsors.

## The abbreviations used are

<b>BW</b>	body weight
<b>ERK</b>	extracellular signal-regulated kinase
<b>GAPDH</b>	glyceraldehyde 3-phosphate dehydrogenase
<b>LD<sub>50</sub></b>	lethal dose 50
<b>nAChR</b>	nicotinic acetylcholine receptor
<b>NNK</b>	4-( <i>N</i> -methyl- <i>N</i> -nitrosamino)-1-(3-pyridyl)-1-butanone
<b>NNN</b>	<i>N</i> '-nitrosornicotine
<b>PI3K</b>	Phosphoinositide 3-kinase

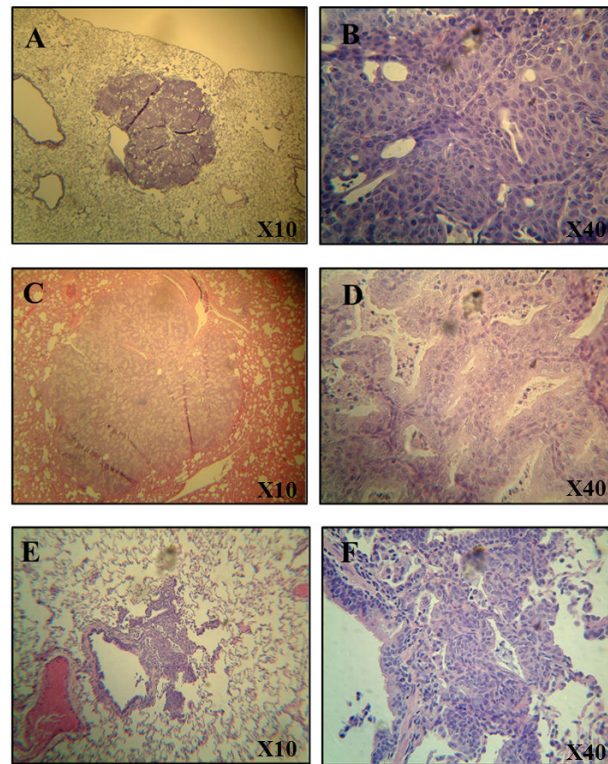
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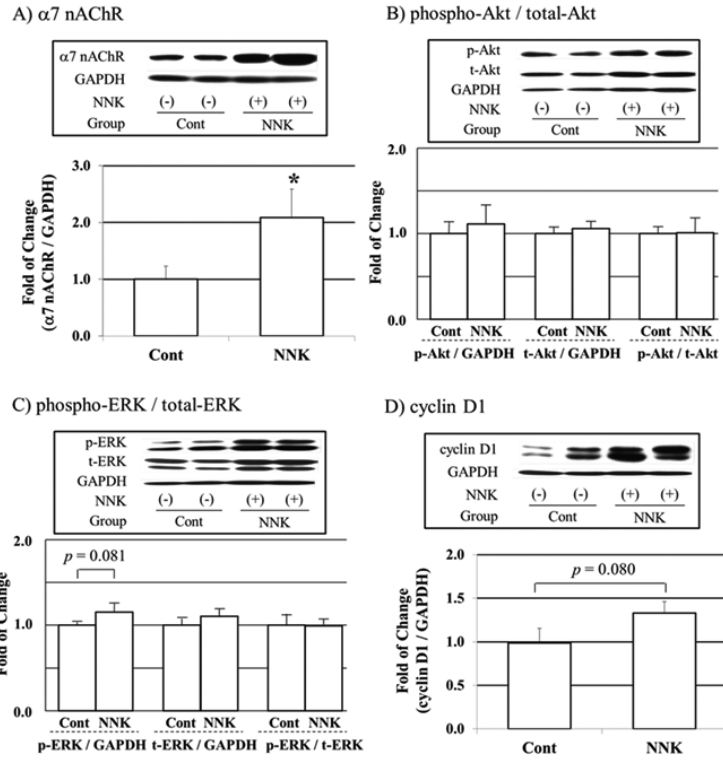


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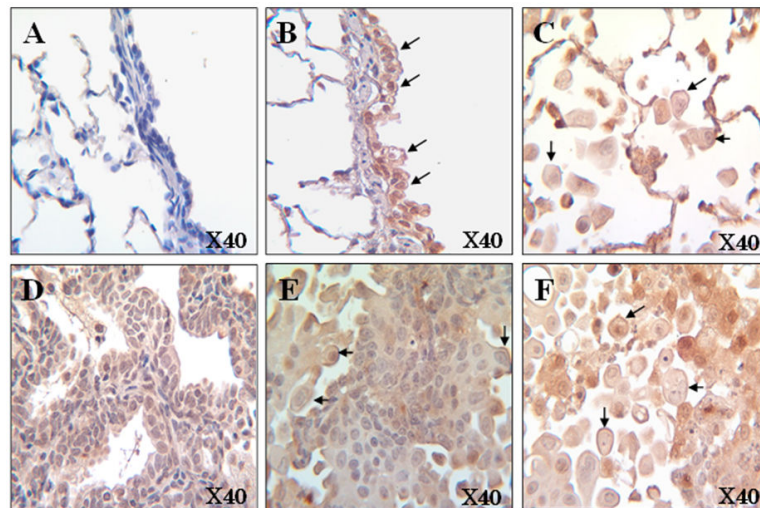
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**Fig. 1.** Representative image of squamous cell carcinoma (Panels A and B), adenocarcinoma (Panels C and D) and preneoplastic lesions (Panels E and F) at 10X and 40X magnifications in the lung of ferrets exposed to NNK for 24 - 32 weeks.



**Fig. 2.** The expression of  $\alpha 7$  nAChR (A), phospho-Akt/total-Akt (B), phospho-ERK/total-ERK (C) and cyclin D1 (D) protein levels in the lung of ferrets that were non-exposed (Cont) and exposed to NNK (NNK) for 26 weeks, measured by western blotting from whole lung homogenate. GAPDH was chosen as internal control for protein equal loading. Values are expressed Mean  $\pm$  SEM. \* $p < 0.05$ .



**Fig. 3.** Representative image of  $\alpha 7$  nAChR immunohistochemistry in the lung of ferrets that were non-exposed (Panel A) and exposed to NNK for 26 weeks [bronchiolar epithelial cells (Panel B), alveolar macrophage (Panel C), adenocarcinoma (Panel D), squamous cell carcinoma (Panel E), and tumor associated macrophage (Panel F)] at 40X magnifications. The arrows indicated the expression of  $\alpha 7$  nAChR (stained in reddish-purple) on the cell membranes.

**Table 1**

Incidences of preneoplastic lesions with pathological subtypes in the lungs of ferrets with or without NNK exposure for 24 - 32 weeks<sup>a)</sup>.

Group	NNK (mg/kg BW)	Time (weeks)	n	Preneoplastic lesions		No. of preneoplastic lesion subtypes		
				Positive animal	% Incidence	SM <sup>b)</sup>	SD <sup>c)</sup>	AAH <sup>d)</sup>
1	None	26	8	0	0	0	0	0
2	200	24	6	6	100	2	3	4
3	200	26	10	10	100	4	8	6
4	200	32	6	5	83.3	1	1	3

<sup>a)</sup>Including the data of dead animals during the experiment period. Some ferrets developed more than one type of pathologic lesions.

<sup>b)</sup>SM: squamous metaplasia.

<sup>c)</sup>SD: squamous dysplasia.

<sup>d)</sup>AAH: atypical adenomatous hyperplasia.

**Table 2**

Incidences of neoplastic lesions with pathological subtypes in the lungs of ferrets with or without NNK exposure for 24 - 32 weeks<sup>a)</sup>.

Group	NNK (mg/kg BW)	Time (weeks)	n	Neoplastic lesions		No. of neoplastic lesion subtypes			
				Positive animal	% Incidence	ADC <sup>b)</sup>	SQC <sup>c)</sup>	ADSQC <sup>d)</sup>	L <sup>e)</sup>
1	None	26	8	0	0	0	0	0	0
2	200	24	6	1	16.7	1	0	0	0
3	200	26	10	4	40.0	3	1	1	0
4	200	32	6	4	66.7	2	1	0	1

<sup>a)</sup>Including the data of dead animals during the experiment period. Some ferrets developed more than one type of pathologic lesions.

<sup>b)</sup>ADC: Adenocarcinoma.

<sup>c)</sup>SQC: Squamous cell carcinoma.

<sup>d)</sup>ADSQC: Adenosquamous carcinoma.

<sup>e)</sup>L: Lymphoma.

**Table 3**

Comparison of the incidence of lung preneoplastic and neoplastic lesions in ferrets treated with NNK and/or exposed to cigarette smoke by present and previous studies.

Treatment		Time (weeks)	n	Preneoplastic Lesions <sup>a)</sup> (% Incidence)	Neoplastic Lesions <sup>b)</sup> (% Incidence)	Ref.
NNK (mg/kg BW)	Smoking Exposure (min/d)					
200	None	24	6	100 %	16.7 %	Present study
200	None	26	10	100 %	40.0 %	Present study
200	None	32	6	83.3 %	66.7 %	Present study
200	15	22	16	87.5 %	25.0 %	30
200	60	24	12	83.3 %	50.0 %	14
None	60	6	9	44.4 %	ND <sup>c)</sup>	31
None	120	24	6	33.3 %	ND <sup>c)</sup>	31
None	120	24	8	62.5 %	ND <sup>c)</sup>	32
None	120	9	6	100 %	ND <sup>c)</sup>	17, 38

<sup>a)</sup>Including squamous dysplasia, squamous metaplasia and atypical adenomatous hyperplasia.

<sup>b)</sup>Including adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma and lymphoma.

<sup>c)</sup>ND: not detected.