

# Molecular analysis of carcinogen-induced rodent lung tumors: Involvement of microRNA expression and *Kras* or *Egfr* mutations

KEIKO YAMAKAWA, TOSHIYA KUNO, NOZOMI HASHIMOTO, MASANAO YOKOHIRA,  
SATOSHI SUZUKI, YUKO NAKANO, KOUSUKE SAOO and KATSUMI IMAIDA

Onco-Pathology, Department of Pathology and Host-Defense, Faculty of Medicine,  
Kagawa University, Kagawa 761-0793, Japan

Received August 10, 2009; Accepted September 10, 2009

DOI: 10.3892/mmr\_00000231

**Abstract.** Genetic and epigenetic alterations play a key role in lung carcinogenesis, and a high frequency of *KRAS* and epidermal growth factor receptor (*EGFR*) mutations have been observed in human lung cancers. Recent evidence indicates that the expression of specific microRNAs (miRNAs) may be involved. In rodent lung carcinogenesis models, *Kras* mutations are frequently observed, whereas genetic alteration of the *Egfr* gene is generally rare. Since little is known regarding the involvement of miRNAs in rodent lung carcinogenesis, the present study of miRNA expression levels in the liver and lung during 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)- and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-induced lung tumorigenesis in A/J mice was conducted. In addition, incidences of *Egfr* and *Kras* gene mutations in rat and mouse lung tumors induced by the chemical carcinogens NNK, MeIQx and N-bis(2-hydroxypropyl)nitrosamine (DHPN) were examined. Three miRNAs, let-7a, miR-34c and miR-125a-5p, were selected for attention. In rat lung tumors, one silent mutation was detected in the *Egfr* gene exon 20 (AAC→AAT; N772). Activating mutations of the *Kras* gene at codon 12 were detected in neoplastic lesions induced by NNK (5/6, 83%), MeIQx (1/1, 100%) and DHPN (7/15, 47%), all resulting in G/C→A/T transitions. NNK or MeIQx administration reduced the expression of miR-125a-5p (MeIQx alone group, 86.3%;

MeIQx + NNK group, 83.6%;  $p < 0.05$ , at day 15) and let-7a (MeIQx + NNK group, 56.3%;  $p < 0.001$ , at day 22) in the liver. miR-34c was up-regulated 3.5-fold with NNK treatment as compared to the control group ( $p < 0.001$ ). These findings raise the possibility that aberrant expression of miRNA is involved in lung tumorigenesis, at least in its early stages.

## Introduction

Lung cancer is one of the most common causes of cancer mortality worldwide, and non-small cell lung cancers (NSCLCs) account for approximately 80% of these cases (1,2). Rodent models of lung carcinogenesis are excellent tools for the study of the mechanisms underlying human NSCLC development and for the detection of chemopreventive agents, since the morphologies, histogenesis and molecular characteristics of the induced primary rodent lung tumors are similar to those of humans. The female A/J mouse is particularly sensitive to lung carcinogenesis (3), yielding for example a high incidence of tumors upon treatment with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine component of tobacco smoke, or 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a heterocyclic amine that is present as a mutagenic compound in fried beef and beef extracts (4,5). In the F344 rat, N-bis(2-hydroxypropyl)nitrosamine (DHPN), an alkylating agent with two propyl chains and a very potent mutagen, causes carcinomas of the lung, thyroid and kidney (6,7). Mutational activation of *Kras* is observed in these rodent tumors, induced by chemical carcinogens with smoking or non-smoking factors. Likewise, in human lung cancers, mutations of *KRAS* have been detected at a high frequency, especially in tobacco smoking patients (7-10). In human lung cancers, recent data have shown that the presence of mutations within the tyrosine kinase (TK) domain of the epidermal growth factor receptor (*EGFR*) gene is associated with sensitivity to *EGFR*-TK inhibitors. Such mutations are more frequently observed in Asian, adenocarcinoma and female patients, and in patients with a never-smoking history (9,11-15). However, in animal lung carcinogenesis models, there have been very few reports of mutations within the TK domain of *Egfr* (16,17).

---

**Correspondence to:** Dr Katsumi Imaida, Onco-Pathology, Department of Pathology and Host-Defense, Faculty of Medicine, Kagawa University, 1750-1, Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan  
E-mail: imaida@med.kagawa-u.ac.jp

**Abbreviations:** NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; DHPN, N-bis(2-hydroxypropyl)nitrosamine

**Key words:** lung carcinogenesis, epidermal growth factor receptor, *KRAS*, microRNA, animal model, gene mutation

In human NSCLC patients, survival and response to *EGFR*-TK inhibitors correlate with *EGFR* mutations, high *EGFR* copy number, overexpression of *EGFR* protein and *KRAS* mutations (13,18). However, it is unclear how mutations of *Egfr* and other molecular alterations are related to the mechanisms involved in rodent lung carcinogenesis. Not only genetic (e.g., mutations with amino acid substitutions in oncogenes or tumor suppressor genes) but also epigenetic (e.g., DNA methylation, histone modifications and RNA-mediated silencing) factors are known to be important for carcinogenesis (19).

Recently, microRNAs (miRNAs), small non-coding RNAs approximately 22 nucleotides in length, have been recognized as negative regulators through the post-transcriptional regulation of target mRNA expression or protein levels without changes in DNA sequences (20-23). Recent findings indicate that the levels of miRNAs play a key role in diverse cellular processes, including development, cell proliferation, apoptosis, differentiation and stress responses, and that altered expression of specific miRNAs may be involved in tumorigenesis (23-26). Indeed, there have been many studies on the relationship between miRNA expression in human cancer cells or tissues and prognosis, resistance to chemotherapy and invasion or metastasis (23,26-31), though little is known regarding miRNA responses to chemical carcinogen exposure during the early stages of rodent lung carcinogenesis. However, in the lungs of rats exposed to cigarette smoke, 24 miRNAs were found to be down-regulated (32). In particular, the let-7, miR-34 and miR-125 families appear to be associated with human NSCLCs (25). Let-7s have been suggested as tumor suppressors due to their ability to recognize the *KRAS* and *HMG2* oncogenes as targets for their silencing activity (33,34). The miR-34 family is directly transactivated by p53, and is thus involved in p53-dependent cell responses to DNA damage (35-38). miR-125 functions in the activation of *ERBB2*, a preferential heterodimerization partner for *EGFR* which plays an important role in lung carcinogenesis (9,39).

In the present study, using rodent lung carcinogenesis models, we examined active mutations in exon 1 of the *Kras* gene and in exons 18-21 of the *Egfr* gene, which are known to be involved in human lung cancer. Furthermore, in order to determine whether exposure to lung chemical carcinogens influences miRNA expression during the early stages of chemical lung carcinogenesis, we examined levels of let-7a, miR-34c and miR-125a-5p in the livers and lungs of rodents exposed to NNK and MeIQx.

## Materials and methods

**Animal treatment.** Experimental animals (Experiments 1 and 2) were maintained in the Kagawa University Animal Facility according to the institutional animal care guidelines. The animals were housed in polycarbonate cages with white wood chips for bedding and were given free access to drinking water and a basal CE-2 diet (CLEA Japan Inc., Tokyo, Japan) under controlled conditions of humidity (60±10%), lighting (12-h light/dark cycle) and temperature (24±2°C).

**Experiment 1. Analysis of *Kras* and *Egfr* mutations.**

**Tissue samples.** Formalin fixed paraffin embedded (FFPE) lung neoplastic lesions (hyperplasia, adenoma and adenocarcinoma)

were obtained with the following lung carcinogenesis animal models: (i) rat DHPN-induced lung carcinogenesis model (40), in which male 6-week-old F344/DuCrIj rats (Japan Charles River Inc., Kanagawa, Japan) were administered 0.1% DHPN in drinking water for 2 weeks and sacrificed at week 30; (ii) mouse NNK-induced lung carcinogenesis model (41), in which female 7-week old A/J mice (Japan SLC Inc., Sizuoka, Japan) were administered a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) or an equal volume of saline (vehicle control), then maintained without additional treatment until sacrifice at week 52; (iii) mouse MeIQx-induced lung carcinogenesis model (42), in which female 7-week old A/J mice were fed a diet containing 600 ppm MeIQx during the initial 12-week period, then maintained on a basal diet without MeIQx until sacrifice at week 32.

The protocols of the experiments were approved by the Animal Care and Use Committee of Kagawa University.

**Laser capture microdissection and DNA extraction.** DNA was extracted from one 8-μm-thick unstained paraffin section. FFPE tissue sections were dewaxed twice in xylene, washed in 100% ethanol and air-dried. Tumor lesions were selectively captured using a Laser Capture Microdissection (LCM) system (LM200; Olympus). Tumors were identified on adjacent sections stained with H&E and were clearly visible on unstained sections. Isolation of DNA from LCM specimens was performed with a Pico Pure DNA Extraction Kit (Arcturus Engineering, Mountain View, CA, USA) according to the manufacturer's protocol.

**Polymerase chain reaction and direct DNA sequencing.** For mutation analysis of *Kras* exon 1 and *Egfr* exons 18-21, lung tumor DNA was amplified by two-step polymerase chain reaction (PCR). Sequences of PCR primers for the amplification of mouse lung tumors are listed in Table I. Sequences of first step PCR primers for the amplification of rat lung tumors were as described by Kitahashi *et al* (16). The second PCR primers were appended with the sequence of the M13 plasmid (forward, 5'-GTCACGACGTTGTAAAACGAC-3'; reverse, 5'-GCGGATAACAATTTCACACAGG-3') to the 5' end of the first PCR primer. First step PCR was performed in 20 μl of reaction mixture consisting of 0.2 μM of each primer, 10X PCR Buffer, 200 μM dNTP, 0.5 U Takara Taq™ HS (Takara Shuzo, Japan) and 1 μl of template DNA. The first PCR reaction was performed under the following conditions: initial denaturation at 95°C for 30 sec; 30 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min using the Mastercycler ep gradient S thermal cycler (Eppendorf). Second step PCR was performed in 50 μl of reaction mixture consisting of 0.2 μM of each primer, 10X PCR Buffer, 200 μM dNTP, 1.25 U Takara Taq™ HS and 1 μl of the first step PCR product. The second PCR cycling parameters were the same as those of the first step PCR. The second PCR products were purified with SUPREC-02 (Takara Bio Inc., Shiga, Japan) to remove unconsumed dNTPs and primers, and then 1.5-μl aliquots were directly sequenced using a SequiTherm EXCEL™ II DNA Sequencing Kit (Epicentre Technologies, Madison, WI, USA) with sequence primers labeled with IRD800 by the second PCR (Table I) and a LI-COR 4200 DNA Analyzer (LI-COR Biosciences, Lincoln, NE, USA).

Table I. Oligonucleotide primers for PCR amplification of DNA from mouse lung tumors (Experiment 1).

Gene	Exon	Sequence (5'-3')	
First step PCR			
<i>Egfr</i>	18	F	<u>GTCACGACGTTGTAAAACGACACCAAGCCC</u> ACTTGAGGATA
		R	<u>GCGGATAACAATTT</u> CACACAGGCACTGGTTCCCAGAAGCCTA
	19	F	<u>GTCACGACGTTGTAAAACGAC</u> CTGGATCCCAGAAGGTGAGA
		R	<u>GCGGATAACAATTT</u> CACACAGGGGAAGCAAGATTGACCTTATGAA
	21	F	<u>GTCACGACGTTGTAAAACGAC</u> TTGGCAGCCAGGAATGTACT
		R	<u>GCGGATAACAATTT</u> CACACAGGGGCTGTCAGGAAAATGCTTC
<i>Kras</i>	1	F	<u>GTCACGACGTTGTAAAACGAC</u> TATAAACTTGTGGTGGTTGGCCCT
		R	<u>GCGGATAACAATTT</u> CACACAGGCCTCTATCGTAGGGTCATACT
Second step PCR and direct DNA sequencing		F	<u>CACGACGTTGTAAAACGAC</u>
		R	<u>GGATAACAATTT</u> CACACAGG

F, forward primer; R, reverse primer. Underlined is the sequence of the M13 plasmid.

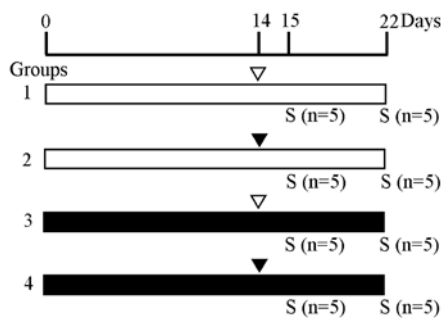


Figure 1. Experimental protocol for Experiment 2. Animals were 7-week-old female A/J mice. ■, MeIQx (600 ppm); □, basal diet (CE-2); ▼, NNK (2 mg/0.1 ml/mouse, i.p.); ▽, saline (0.1 ml/mouse, i.p.); S, sacrifice; n, number of sacrificed mice.

#### Experiment 2. Expression levels of *let-7a*, *miR-34c* and *miR-125a* in A/J mice induced by NNK and MeIQx.

**Animals and chemicals.** Female 6-week old A/J mice were purchased from Japan SLC Inc. (Shizuoka, Japan). NNK was purchased from Tronto Research Chemicals (Ontario, Canada) and MeIQx from Nard Institute (Nishinomiya, Japan).

**Experimental design.** The experimental protocol is shown in Fig. 1. A total of 40 mice were divided into 4 groups. Mice in groups 3 and 4 were fed the diet containing 600 ppm MeIQx for 22 days. At day 14, the mice in groups 2 and 4 were given a single dose of NNK (2 mg/0.1 ml saline/mouse i.p.), while those in groups 1 and 3 were given an equal volume of saline (vehicle control). At days 15 and 22 of the experiment (days 1 and 8 after NNK treatment), 5 mice in each group were sacrificed by exsanguination under deep anesthesia. The liver and lung tissues were collected, immersed in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) and stored at 4°C until RNA was extracted.

The protocol of the experiment was approved by the Animal Care and Use Committee.

**miRNA extraction and measurement.** Total RNA including miRNA was extracted using a miRNeasy Mini Kit (Qiagen). DNA digestion was performed with the RNase-Free DNase Set (Qiagen). The concentration of isolated RNA was determined spectrophotometrically by measuring the absorbance at 260 nm; purity was determined using the ratio of 260/280 nm. Total RNA samples were stored at -80°C until TaqMan analysis.

**TaqMan real-time PCR.** cDNAs were synthesized from total RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Total RNA (10 ng) from each sample was reverse transcribed (RT) with a miRNA-specific stem-loop primer supplied in a TaqMan MicroRNA Assay kit (Applied Biosystems) following the manufacturer's protocol. The RT products were diluted 5-fold in nuclease-free water and then amplified by PCR using the TaqMan MicroRNA Assay and analyzed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Real-time PCR reactions were performed in a final volume of 20 µl, containing 6 µl of diluted RT template, 3 µl of nuclease-free water, 1 µl of 20X primer/probe mix from the TaqMan MicroRNA Assay kit (Applied Biosystems) and 10 µl of 2X TaqMan Universal PCR master mix (no AmpErase UNG, Applied Biosystems). The miRNAs used in this study are listed in Table II. Relative levels of miRNA expression were quantified using the relative standard curve method with snoRNA202 as the endogenous control.

**Statistical analysis.** Relative expression levels were compared using the Kruskal-Wallis test. Differences were considered statistically significant at  $p < 0.05$ .

## Results

***Kras* and *Egfr* gene alterations in lung neoplastic lesions.** Mutation analysis of exon 1 of the *Kras* gene was performed on a total of 7 mouse lung tumors (6 NNK-induced and 1 MeIQx-induced) and 15 DHPN-induced rat lung tumors.

Table II. Target sequences of let-7a, miR-34c, miR-125a-5p and snoRNA202 using TaqMan real-time PCR (Experiment 2).

Assay name	Assay ID	Target sequence	Homology	
			Homo sapiens	Mus musculus
hsa-let-7a	000377	UGAGGUAGUAGGUUGUAUAGUU	+	+
				(mmu-let-7a)
hsa-miR-34c	000428	AGGCAGUGUAGUUAGCUGAUUGC	+	+
				(mmu-miR-34c)
hsa-miR-125a-5p	002198	UCCCUGAGACCCUUUAACCUGUGA	+	+
				(mmu-miR-125a-5p)
snoRNA202	001232	GCUGUACUGACUUGAUGAAAGUAC UUUUGAACCCUUUUCUUCUGAUG	-	+

Table III. Incidences of mutations of the *Kras* and *Egfr* genes in lung neoplastic lesions.

Animal	Treatment	Histological type	<i>Egfr</i>				<i>Kras</i>
			Exon 18	Exon 19	Exon 20	Exon 21	Exon 1
Rats	DHPN	Adenoma	0/4	0/4	0/1	0/4	1/4 (25%)
		Adenocarcinoma	0/11	0/9	1/10 (10%)	0/11	6/11 (55%)
Mice	NNK	Adenoma	0/5	0/5	-	0/4	2/2 (100%)
		Adenocarcinoma	0/4	0/3	-	0/2	3/4 (75%)
	MeIQx	Adenoma	0/3	0/3	-	0/3	1/1 (100%)
	Saline	Hyperplasia	0/1	0/1	-	0/1	-
		Adenoma	0/3	0/2	-	0/4	-
		Adenocarcinoma	0/3	0/3	-	0/2	-

-, not examined.

Table IV. Mutation patterns for the *Kras* and *Egfr* genes.

Gene	Exon	Animal	Nucleotide change	Amino acid change	Frequency
<i>Kras</i>	Exon 1	Mice	GGT→GAT	G12D	7/7
		Rats	GGT→GAT	G12D	6/6
<i>Egfr</i>	Exon 20	Rats	AAC→AAT	N772	1/1

Activating mutations of the *Kras* gene at codon 12 were detected in neoplastic lesions induced by NNK (5/6; 83%), MeIQx (1/1; 100%) and DHPN (7/15; 47%). All mutations were G/C→A/T transitions (Tables III and IV).

Mutations of the *Egfr* gene in exons 18, 19 and 21 were examined in a total of 20 mouse lung tumors (9 NNK-induced, 3 MeIQx-induced and 8 spontaneous). It was not possible to perform analysis of exon 20 of the *Egfr* gene due to insufficient lung tumor DNA. The number of samples that were analyzed by DNA sequencing were as follows: exon 18, 19 tumors; exon 19, 17 tumors; exon 21, 16 tumors. *Egfr* mutations were not detected in mouse lung tumors. On the other hand, mutation analysis of exons 18-21 of the *Egfr* gene in a total of 15 DHPN-induced rat lung tumors (number of samples analyzed

by DNA sequencing: exon 18, 15 tumors; exon 19, 13 tumors; exon 20, 11 tumors; exon 21, 15 tumors) revealed one silent mutation in exon 20 (AAC→AAT; N772) (Tables III and IV and Fig. 2).

*MiRNA expression in livers and lungs of A/J mice treated with NNK or MeIQx.* Fig. 3 shows the relative expression of let-7a, miR-34c and 125a-5p in mouse livers. In the liver, mean relative expression of miR-125a-5p was significantly reduced in the groups receiving MeIQx alone and NNK and MeIQx in combination as compared with the control group at day 15 (1 day after NNK treatment) [0.97±0.09 (86.3%),  $p<0.05$ ; 0.94±0.10 (83.6%),  $p<0.01$  vs. 1.12±0.07]. At day 22 (8 days after NNK treatment), the mean relative expression



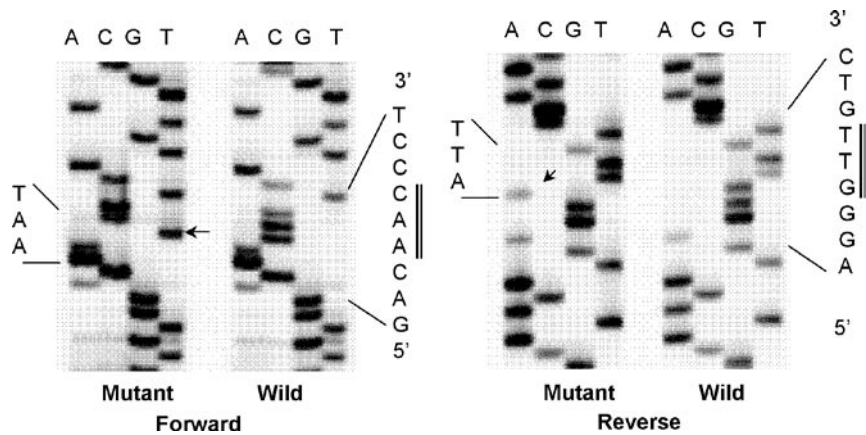


Figure 2. *Egfr* gene mutations in rat adenocarcinomas induced by DHPN. Arrows indicate mutation bands (AAC→AAT at codon 772).

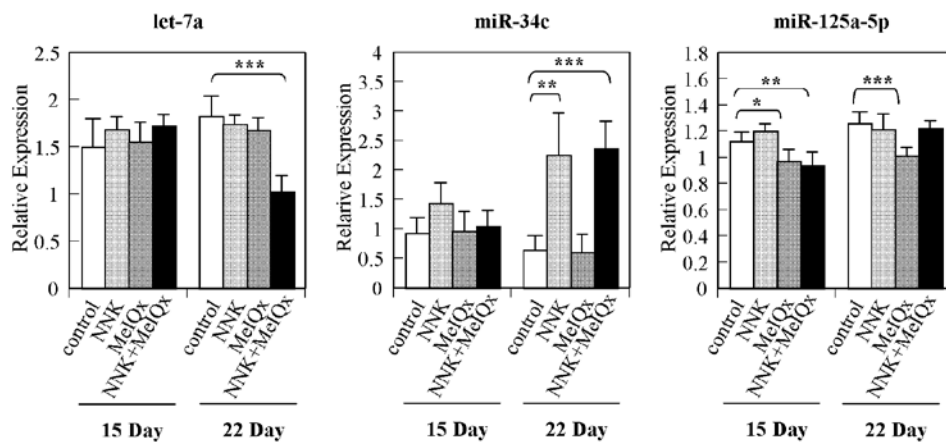


Figure 3. Relative expression levels of miRNAs (let-7a, miR-34c and miR-125a-5p) in livers at days 15 and 22 (days 1 and 8 after NNK treatment) as measured by TaqMan real-time PCR. The relative levels of miRNA expression were quantified using the relative standard curve method with snoRNA202 as the endogenous control. Shown are averages and standard deviations (error bars). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , significantly different compared to the control group.

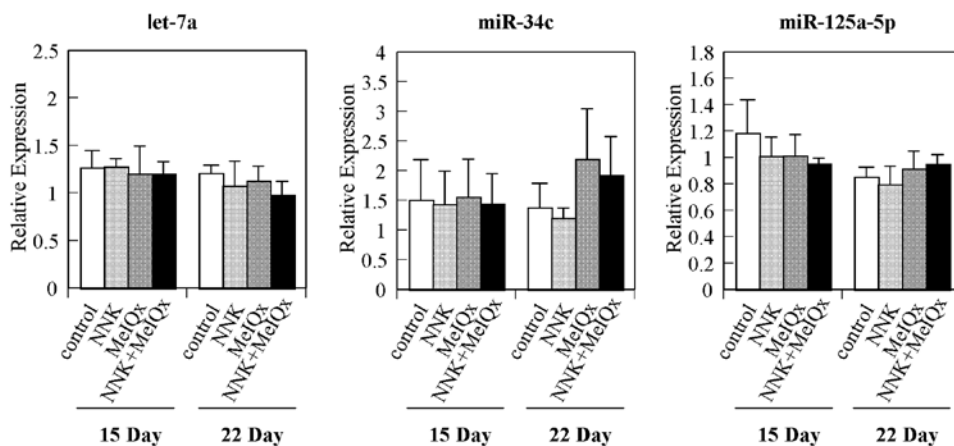


Figure 4. Relative expression levels of miRNAs (let-7a, miR-34c and miR-125a-5p) in lungs at days 15 and 22 (1 and 8 after NNK treatment) as measured by TaqMan real-time PCR. The relative levels of miRNA expression were quantified using the relative standard curve method with snoRNA202 as the endogenous control. Shown are averages and standard deviations (error bars). No significant differences were observed.

of miR-125a-5p was significantly reduced to 80% only in the group receiving MeIQx alone as compared with the control group ( $1.01 \pm 0.06$  vs.  $1.26 \pm 0.08$ ,  $p < 0.001$ ). Similarly, let-7a was significantly reduced to 56.3% in the group administered

the combination of NNK and MeIQx as compared with the control group ( $1.03 \pm 0.17$  vs.  $1.83 \pm 0.21$ ,  $p < 0.001$ ) at day 22 (8 days after NNK treatment). On the other hand, the mean relative expression of miR-34c in the liver was significantly

increased by 3.5-fold with NNK alone as compared with the control group ( $2.26 \pm 0.71$  vs.  $0.65 \pm 0.24$ ,  $p < 0.01$ ) at day 22 (8 days after NNK treatment). Similarly, with the NNK and MeIQx combination, the expression level of miR-34c was increased 3.6-fold as compared with the control group ( $2.36 \pm 0.47$  vs.  $0.65 \pm 0.24$ ,  $p < 0.001$ ). At day 15, the expression level of miR-34c in the group administered NNK alone was also up-regulated 1.5-fold, but without attaining statistical significance ( $p = 0.08$ ).

In the lung, there was no significant variation in the expression levels of these miRNAs at either day 15 or day 22 (Fig. 4).

## Discussion

In the present study, a mutation in the *Egfr* gene exon 20 was detected in one rat lung adenocarcinoma induced by DHPN, but was a silent mutation (AAC → AAT; N772). *Egfr* mutations of rodent lung carcinogenesis have only been reported under special conditions, such as X-ray irradiation (16), and in genetically engineered animals with *Ogg1* gene deficiency (17). To our knowledge, this is the first demonstration of an *Egfr* mutation in chemical lung carcinogenesis in a non-genetically engineered animal. The frequency of *EGFR* mutation in human NSCLCs is relatively high (26–40% in Asian patients and 2–12% in non-Asian patients). In one animal model, urethane-induced lung adenomas in male A/J mice were inhibited by *EGFR*-TK domain inhibitor, despite a lack of mutation in the *Egfr*-TK domain, presumably due to activated *Egfr* signaling (43). Such activation of the *Egfr* signaling pathway caused by genetic or epigenetic mechanisms without somatic mutations of *Egfr* may be involved in the growth of lung tumors in both humans and animals. Here, we detected mutations in codon 12 of the *Kras* gene (NNK, 83%; MeIQx, 100%; DHPN, 47%), with similar incidences as those previously reported (16). Mutations of the *Kras* gene exons 1 and 2 have been frequently identified in rodent lung carcinogenesis models using chemical carcinogens, as well as in human lung cancer in smokers. Recently, it was suggested that RAS oncogenic signaling is caused not only by *KRAS* activating mutations, but also by a reduction in let-7 (23,26,33), and the expression levels of let-7 family members were found to be reduced in human lung cancers (28,44). In the lungs of mice expressing a G12D activating mutation for the *Kras* gene, delivery of let-7a to the lung reduced tumor formation (45). In the present study, let-7a in the liver was down-regulated with combined administration of NNK and MeIQx at 22 days (8 days after NNK treatment). In addition, MeIQx induced the down-regulation of miR-125a-5p in the liver, while NNK treatment was without effect. In contrast, only NNK clearly induced up-regulated miR-34c in the liver, and MeIQx was without influence. These results suggest that changes in let-7a, miR-34c and miR-125a-5p expression may be involved in the early stages of tumorigenesis induced by chemicals, and with possible carcinogen specificity. This could be due to differences in the mechanisms of action of carcinogens.

There have been numerous reports pointing to a relationship between the miR-34 family and carcinogenesis. In an Fe-NTA-induced rat renal carcinogenesis model, altered expression of miR-34a was observed within a few weeks of commencing

Fe-NTA treatment (46). The lymphocytes of patients with chronic lymphomatic leukemia were found to demonstrate the up-regulation of miR-34a at 16 h after  $\gamma$ -irradiation, causing DNA damage (27). Thus, the miR-34 family may be rapidly induced by DNA-damaging agents. Recent studies suggest it plays an important role in the regulation of the p53 pathway, since: (i) the miR-34 family is up-regulated by the activation of p53 resulting in the induction of apoptosis by BCL2 and E2F3; and (ii) p53 induces cell cycle arrest through p21, and apoptosis through Bax (23,26,27,37). In our previous study, we examined the expression levels of mRNAs involved in p53 signaling pathways (cyclin G1, p21, Bax and Mdm2) in the livers and lungs of female A/J mice exposed to NNK. In the livers, up to 10-fold up-regulation was observed after 1 day. At 14 days after NNK treatment, mRNAs of cyclin G1, Bax and Mdm2 were up-regulated approximately 2-fold, and p21 mRNA was up-regulated 6-fold as compared with the control group (unpublished data). These results raise the possibility that NNK induces the activation of the p53 pathway during the early stages of tumorigenesis (during cancer initiation, before neoplastic transformation).

In human cancer, deleted chromosome regions often contain miRNA genes, causing low levels of expression (25). In animal carcinogenesis models, exposure to carcinogens may also result in a reduction in miRNA levels. In the lungs of Sprague Dawley rats exposed to cigarette smoke for 4 weeks, 24 miRNAs including members of the let-7, miR-34 and miR-125 families were found to be down-regulated by at least 3-fold (32). In the lungs of male F344 rats receiving 10 ppm of NNK in drinking water, a number of miRNAs including miR-34b were also down-regulated as compared with the control group 1 week after the initiation of treatment (47). These results suggest that a reduction in specific miRNAs is involved in the early stages of tumorigenesis. However, in this study, we did not detect any significant differences in the expression levels of let-7a, miR-125a-5p and miR-34c in the lung, which is the target organ of NNK and MeIQx. This result requires clarification.

In conclusion, the present examination of genetic and epigenetic mechanisms of rodent lung carcinogenesis revealed: (i) a high incidence of *Kras* gene mutations, but only one mutation in the *Egfr* gene exons 18–21; and (ii) changes in the miRNA expression levels of let-7a, miR-34c and miR-125a-5p during the early stages of tumorigenesis. The sequences of a large number of miRNAs are conserved between humans and rodents, and it may therefore be possible that alterations of specific miRNAs involved in human lung cancer may also be detected in a rodent lung carcinogenesis model. In order to study the participation of miRNAs in tumor initiation and progression, further studies are clearly warranted with a focus on any relationships with the activation of the *Egfr* and *Kras* genes at different stages of tumor induction.

## Acknowledgements

This work was supported by Grants-in Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan. We thank Dr. Malcolm A. Moore, a native English-speaking scientist, for his help in the preparation and critical reading of the manuscript.

## References

- Travis WD, Travis LB and Devesa SS: Lung cancer. *Cancer* 75: 191-202, 1995.
- Sun S, Schiller JH and Gazdar AF: Lung cancer in never smokers – a different disease. *Nat Rev Cancer* 7: 778-790, 2007.
- Imaida K, Yokohira M and Kuno T: Detection of carcinogenic and modifying potentials by test compounds using a mouse lung carcinogenesis bioassay. *Journal of Toxicologic Pathology* 20: 117-123, 2007.
- Peterson LA and Hecht SS: O6-methylguanine is a critical determinant of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumorigenesis in A/J mouse lung. *Cancer Res* 51: 5557-5564, 1991.
- Sugimura T, Wakabayashi K, Nakagama H and Nagao M: Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Sci* 95: 290-299, 2004.
- Konishi Y, Kondo H, Ikeda T, Kawabata A, Shoji Y and Denda A: Effect of dose on the carcinogenic activity of orally administered N-bis(2-hydroxypropyl)nitrosamine in rats. *Gann* 69: 573-577, 1978.
- Kitahori Y, Naito H, Konishi N, Ohnishi T, Shirai T and Hiasa Y: Frequent mutations of Ki-ras codon 12 in N-bis(2-hydroxypropyl)-nitrosamine-initiated thyroid, kidney and lung tumors in Wistar rats. *Cancer Lett* 96: 155-161, 1995.
- Fukuyama Y, Mitsudomi T, Sugio K, Ishida T, Akazawa K and Sugimachi K: K-ras and p53 mutations are an independent unfavourable prognostic indicator in patients with non-small cell lung cancer. *Br J Cancer* 75: 1125-1130, 1997.
- Gazdar AF, Shigematsu H, Herz J and Minna JD: Mutations and addiction to EGFR: the Achilles 'heal' of lung cancers? *Trends Mol Med* 10: 481-486, 2004.
- Ronai ZA, Gradia S, Peterson LA and Hecht SS: G to A transitions and G to T transversions in codon 12 of the Ki-ras oncogene isolated from mouse lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and related DNA methylating and pyridyloxobutylating agents. *Carcinogenesis* 14: 2419-2422, 1993.
- Lynch TJ, Bell DW, Sordella R, *et al*: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small cell lung cancer to gefitinib. *N Engl J Med* 350: 2129-2139, 2004.
- Paez JG, Janne PA, Lee JC, *et al*: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304: 1497-1500, 2004.
- Berardi R, Verdecchia L, Paolo MD, *et al*: Women and lung cancer: clinical and molecular profiling as a determinate for treatment decisions: a literature review. *Crit Rev Oncol Hematol* 69: 223-236, 2009.
- Yamamoto H, Toyooka S and Mitsudomi T: Impact of EGFR mutation analysis in non-small cell lung cancer. *Lung Cancer* 63: 315-321, 2009.
- Pao W, Miller V, Zakowski M, *et al*: EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 101: 13306-13311, 2004.
- Kitahashi T, Takahashi M, Yamada Y, *et al*: Occurrence of mutations in the epidermal growth factor receptor gene in X-ray-induced rat lung tumors. *Cancer Science* 99: 241-245, 2008.
- Igarashi M, Watanabe M, Yoshida M, *et al*: Enhancement of lung carcinogenesis initiated with 4-(N-hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone by Ogg1 gene deficiency in female, but not male, mice. *J Toxicol Sci* 34: 163-174, 2009.
- Zhang X and Chang A: Molecular predictors of EGFR-TKI sensitivity in advanced non-small cell lung cancer. *Int J Med Sci* 5: 209-217, 2008.
- Jones PA and Baylin SB: The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 3: 415-428, 2002.
- Bartel DP: MicroRNAs: genomics, biogenesis, mechanism and function. *Cell* 116: 281-297, 2004.
- Gregory RI, Chendrimada TP, Cooch N and Shiekhattar R: Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123: 631-640, 2005.
- Carthew RW: Gene regulation by microRNAs. *Curr Opin Genet Dev* 16: 203-208, 2006.
- Ryazansky SS and Gvozdev VA: Small RNAs and cancerogenesis. *Biochemistry (Mosc)* 73: 514-527, 2008.
- Lagos-Quintana M, Rauhut R, Lendeckel W and Tuschl T: Identification of novel genes coding for small expressed RNAs. *Science* 294: 853-858, 2001.
- Calin GA, Sevignani C, Dumitru CD, *et al*: Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 101: 2999-3004, 2004.
- Negrini M, Nicoloso MS and Calin GA: MicroRNAs and cancer-new paradigms in molecular oncology. *Curr Opin Cell Biol* 21: 470-479, 2009.
- Zenz T, Mohr J, Eldering E, *et al*: miR-34a as part of the resistance network in chronic lymphocytic leukemia. *Blood* 113: 3801-3808, 2009.
- Inamura K, Togashi Y, Nomura K, *et al*: let-7 microRNA expression is reduced in bronchioloalveolar carcinoma, a non-invasive carcinoma, and is not correlated with prognosis. *Lung Cancer* 58: 392-396, 2007.
- Rosell R, Wei J and Taron M: Circulating MicroRNA signatures of tumor-derived exosomes for early diagnosis of non-small cell lung cancer. *Clin Lung Cancer* 10: 8-9, 2009.
- Ma L, Teruya-Feldstein J and Weinberg RA: Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 449: 682-688, 2007.
- Yanaihara N, Caplen N, Bowman E, *et al*: Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 9: 189-198, 2006.
- Izzotti A, Calin GA, Arrigo P, Steele VE, Croce CM and De Flora S: Down-regulation of microRNA expression in the lungs of rats exposed to cigarette smoke. *FASEB J* 23: 806-812, 2009.
- Johnson SM, Grosshans H, Shingara J, *et al*: RAS is regulated by the let-7 microRNA family. *Cell* 120: 635-647, 2005.
- Lee YS and Dutta A: The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev* 21: 1025-1030, 2007.
- Tarasov V, Jung P, Verdoodt B, *et al*: Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle* 6: 1586-1593, 2007.
- Bommer GT, Gerin I, Feng Y, *et al*: p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* 17: 1298-1307, 2007.
- He L, He X, Lim LP, *et al*: A microRNA component of the p53 tumour suppressor network. *Nature* 447: 1130-1134, 2007.
- Raver-Shapira N, Marciano E, Meiri E, *et al*: Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 26: 731-743, 2007.
- Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS and Benz CC: Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. *J Biol Chem* 282: 1479-1486, 2007.
- Yokohira M, Hashimoto N, Yamakawa K, *et al*: Lung Carcinogenic Bioassay of CuO and TiO2 Nanoparticles with Intratracheal Instillation Using F344 Male Rats. *Journal of Toxicologic Pathology* 22: 71-78, 2009.
- Takeuchi H, Saoo K, Matsuda Y, *et al*: 8-Methoxypsoralen, a potent human CYP2A6 inhibitor, inhibits lung adenocarcinoma development induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in female A/J mice. *Molecular Medicine Reports* 2: 585-588, 2009.
- Matsuda Y, Takeuchi H, Yokohira M, *et al*: Enhancing effects of high fat diet 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline-induced lung tumorigenesis in female A/J mice. *Mol Med Rep* 2: 701-706, 2009.
- Zerbe LK, Dwyer-Nield LD, Fritz JM, *et al*: Inhibition by erlotinib of primary lung adenocarcinoma at an early stage in male mice. *Cancer Chemother Pharmacol* 62: 605-620, 2008.
- Takamizawa J, Konishi H, Yanagisawa K, *et al*: Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 64: 3753-3756, 2004.
- Esquela-Kerscher A, Trang P, Wiggins JF, *et al*: The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* 7: 759-764, 2008.
- Dutta KK, Zhong Y, Liu YT, *et al*: Association of microRNA-34a overexpression with proliferation is cell type-dependent. *Cancer Sci* 98: 1845-1852, 2007.
- Kalscheuer S, Zhang X, Zeng Y and Upadhyaya P: Differential expression of microRNAs in early-stage neoplastic transformation in the lungs of F344 rats chronically treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Carcinogenesis* 29: 2394-2399, 2008.