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 (ACG→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 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).
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 HMGA2 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/DuCrIcrj rats (Japan Charles River Inc., Kanagawa, Japan) were administered 0.1% DHPN in drinking water for 2 weeks and sacrificed at week 20; (ii) mouse NNK-induced lung carcinogenesis model (41), in which female 7-week old A/J mice (Japan SLIC 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 MelQx-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 rodent 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'-GTCAAGACGTTGTAACACGC-3'; reverse, 5'-GCGGATAACATTTCAACACAGG-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, 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 un consumed dNTPs and primers, and then 1.5-µl aliquots were directly sequenced using a SequiTTherm 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).
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 Toronto 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/mouse, i.p.); □, saline (0.1 ml/mouse, i.p.); ◻, sacrifice; n, number of sacrificed mice.

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 Step onePlus™ 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 MelIQx-induced) and 15 DHPN-induced rat lung tumors.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Sequence (5'-3')</th>
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<td>First step PCR</td>
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| Egfr | 18 | F GTCACGACGTGTTGAAAAACGACACCAAGCCACTTGGAGGATA  
R GCCGATAACAATTTCACACGGCCTGGTCCCAGAGGCTTA |
| 19 | F GTCACGACGTGTTGAAAAACGACACCTGATCCACAGAAGGAGGA  
R GCCGATAACAATTTCACACGGCCTGGTCCCAGAGGCTTA |
| 21 | F GTCACGACGTGTTGAAAAACGACCTGGGACCAGGAATGTACT  
R GCCGATAACAATTTCACACGGCCTGGTCCCAGAGGCTTA |
| Kras | 1 | F GTCACGACGTGTTGAAAAACGACACTTATAAACTTGTTGGTTTGCCCT  
R GCCGATAACAATTTCACACGGCCTGGTCCCAGAGGCTTA |
| Second step PCR and direct DNA sequencing |
| F | CACGACGTGTTGAAAAACGAC  
R GATAACAATTTCACACGG |

F, forward primer; R, reverse primer. Underlined is the sequence of the M13 plasmid.
Activating mutations of the \textit{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 \textit{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 \textit{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. \textit{Egfr} mutations were not detected in mouse lung tumors. On the other hand, mutation analysis of exons 18-21 of the \textit{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 (aa c → aa T; N772) (Tables III and IV and Fig. 2).

\textbf{MirNA expression in livers and lungs of \textit{A/J} mice treated with \textit{NNK} or MeIQx.} Fig. 3 shows the relative expression of let-7a, miR-34c and 125a-5p in mouse lungs. 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...
of miR-125a-5p was significantly reduced to 80% only in the group receiving MeIQx alone as compared with the control group (1.01±0.06 vs. 1.26±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±0.17 vs. 1.83±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±0.71 vs. 0.65±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±0.47 vs. 0.65±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 (AAAG - AAG: 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 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 mRNA 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.

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