Occurrence of mutations in the epidermal growth factor receptor gene in X-ray-induced rat lung tumors

Tsukasa Kitahashi,[1] Mami Takahashi,[1] Yutaka Yamada,[2] Yoichi Oghiso,[3] Masanao Yokohira,[4] Katsumi Imaida,[4] Masahiro Tsutsumi,[5] Nobuo Takasuka,[1] Takashi Sugimura[1] and Keiji Wakabayashi[1,6]

[1]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045; [2]Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, Anagawa, 4-9-1, Inage-ku, Chiba 263-8555; [3]Department of Radiobiology, Institute for Environmental Sciences, 1-7 Ienomae, Obuchi, Rokkasho-mura, Kamikita-gun, Aomori 039-3212; [4]Department of Onco-Pathology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793; [5]Department of Pathology, Saiseikai Chuwa Hospital, 323 Abe, Sakurai, Nara 633-0054, Japan

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Epidermal growth factor receptor (*EGFR*) gene alterations have been found in human lung cancers. However, there is no information on the factors inducing *EGFR* mutations. In rodents, K-*ras* mutations are frequently found in many lung carcinogenesis models, but hitherto, *Egfr* mutations have not been reported. Their presence was therefore investigated in representative lung carcinogenesis models with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosobis(2-hydroxypropyl)amine (BHP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and ethyl carbamate (urethane), as well as X-ray irradiation. With the chemical carcinogenesis models, no mutations were detected in *Egfr*, which is in clear contrast to the high rates observed in either codon 12 or 61 of K-*ras* (21/23 of the lung tumors induced with NNK, 4/5 with MeIQx, 1/4 with urethane and 7/18 with BHP). However, in the X-ray-induced lung tumors, *Egfr* mutations with amino acid substitution were observed in exons 18 and 21 (4/12, 33%), but no activating mutation of K-*ras* was detected. In addition, one and four silent mutations were identified in K-*ras* (exon 1) and *Egfr* (exons 18, 20 and 21), respectively. Most mutations in both *Egfr* and K-*ras* were G/C→A/T transitions (7/8, 88% and 31/34, 91%, respectively). Although, the mutational patterns in equivalent human lesions were not completely coincident, this first report of *Egfr* mutations in an experimental lung tumor model suggests that X-rays or other factors producing oxygen radicals could cause *EGFR* mutations in some proportion of lung cancers in humans. (*Cancer Sci* 2008; 99: 241–245)

Lung cancer is the major cause of death in both sexes in Japan and many parts of the world[1,2] so analysis of causative factors and development of preventive methods is important, in addition to advances in diagnostic and therapeutic methods. Genetic alterations (*KRAS*, *TP53* etc.) in lung cancers have been studied in this context.[3]

Epidemiologic studies of lung cancers have pointed to many risk factors including tobacco smoking, air pollution, occupational environments, and ionizing radiations including radon.[4] Interestingly, dietary habits have also been demonstrated to influence the risk of lung cancer, with well-cooked red meat consumption demonstrated as a risk factor.[5,6] Tobacco smoking appears to be correlated with *KRAS* mutations.[3]

Recently, somatic mutations of the epidermal growth factor receptor (*EGFR*) gene, a tyrosine kinase of the ErbB family, have been reported to be frequent in human lung adenomas and adenocarcinomas, especially in Asians, women, and non-smokers.[7–10] However, factors inducing *EGFR* mutations are quite unclear. To prevent the presently increasing rates of lung adenocarcinomas, this question demands our urgent attention.

Experimental animal models of lung carcinogenesis have been established to elucidate mechanisms and to allow screening for enhancing and suppressing factors. Representative carcinogens inducing high incidences of lung cancers include: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), found in tobacco smoke; *N*-nitrosobis(2-hydroxypropyl)amine (BHP), a synthesized carcinogen not existing in nature; 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a heterocyclic amine having mutagenicity and carcinogenicity, which exists in cooked meat and fish;[5,6,11] ethyl carbamate (urethane) and X-rays. Mutations of K-*ras* have been reported in mouse and rat tumors, including NNK- and BHP-induced lung adenomas and adenocarcinomas.[12,13] However, alterations of *Egfr* have hitherto not been identified.

In the present study, to assess possible mutational factors impacting on *Egfr*, we investigated genetic alterations in *Egfr* exons 18–21, frequently found in human lung cancer[7–10] in a series of animal lung neoplasms induced by NNK, BHP, MeIQx, urethane and X-rays. For comparison, K-*ras* exons 1 and 2 were also analyzed.

Materials and Methods

Chemicals. NNK and urethane were purchased from Sigma (St Louis, MO, USA), MeIQx from Nard Institute (Nishinomiya, Japan), and BHP from Nakarai Tesuque (Kyoto, Japan).

Animal treatments. Experimental animals were purchased from Japan SLC, Inc. (Shizuoka, Japan) and each experimental treatment started after adaptation for a week.

To obtain animal lung tumor samples, animal experiments with five lung carcinogenesis models were carried out as follows.

For NNK-induced lung tumors, 7-week-old female A/J mice were given a single dose of NNK (2 mg/0.1 mL saline/mouse, i.p.), and then maintained without additional treatment until sacrificed at week 52. This experiment was conducted by M. Yokohira and K. Imaida.

For MeIQx-induced lung tumors, 7-week-old female A/J mice were given 600 p.p.m. MeIQx in a basal diet for 12 weeks, and were then maintained on the basal diet without MeIQx until sacrificed at week 32. This experiment was also conducted by M. Yokohira and K. Imaida.

For the urethane-induced lung tumors, 8-week-old female A/J mice were given a single dose of urethane (250 mg/kg, i.p.), and then maintained without additional treatment until sacrifice at week 50. This experiment was conducted by N. Takasuka.

The protocol for BHP-induced lung tumors was based on previous reports[14] and the experiment was conducted by M. Tsutsumi.

The protocol for X-ray-induced lung tumors was conducted by Y. Yamada and Y. Oghiso.[15] Briefly, for local thoracic X-irradiation, female Wistar (W/M) strain rats at the ages of 100–120 days were exposed to 3.0 Gy of X-rays and then maintained without additional treatment until sacrifice when moribund, dead, or at 24 months.

[6]To whom correspondence should be addressed. E-mail: kwakabay@gan2.res.ncc.go.jp

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Table 1. Oligonucleotide primers for PCR amplification

Gene	Animal	Treatment	Exon	Primer sequence (5′-3′)		Target codons†	Annealing temperature
K-*ras*	Mouse	MeIQx, NNK	1	F; ACTGAGTATAAACTTGTGGT	R; CCTCTATCGTAGGGTCGTAC	9–30	53°C
			2	F; AAGTAGTAATTGATGGAGAA	R; TTATGGCAAATACACAAAGA	50–77	53°C
		Urethane	1	F; AGGCCTGCTGAAAATGACTG	R; CCTCTATCGTAGGGTCGTAC	4–30	55°C
			2	F; AAGTAGTAATTGATGGAGAA	R; TGGTGAATATCTTCAAATGATTTAGT	50–86	53°C
	Rat	BHP	1	F; AGGCCTGCTGAAAATGACTG	R; GCAGCATTTACCTCTATCGT	4–33	53°C
			2	F; AAGTAGTAATTGATGGAGAA	R; TGGTGAATATCTTCAAATGATTTAGT	50–86	53°C
		X-ray	1	F; TGACTGAGTATAAACTTGTGGTAGTTG	R; TCGTAGGATCATATCATTCCACAAAG	11–26	57°C
			2	F; AAGTAGTAATTGATGGAGAA	R; GGCAAATACACAAAGAAAGC	50–76	53°C
Egfr	Mouse	MeIQx, NNK	18	F; CTCCCTTCTTCACAGCTCG	R; TCTCCAGGATGTTACCTTATAC	692–727	55°C
			19	F; TTCTTAATCTCAGGGTCTCT	R; GAAAACTCACGTCAAGGAT	734–760	55°C
			20	F; GTCCTTACCTTGTAGGAAGC	R; TCCCAACGTGCTTACCTTTG	766–823	55°C
			21	F; GGGCATGAACTACCTGGAAG	R; AGGACTTACTTTGCCCCCCTC	833–873	55°C
		Urethane	18	F; CTCGTGGAACCTCTCACACC	R; ATGTTACCTTATACACTGTGCCAAATG	697–723	55°C
			19	F; CAAGTTAATGTCAGCCCTCTTC	R; TAAAAGAAAACTCACGTCAAGGATTTC	731–759	55°C
			20	F; AGGAAGCCTATGTGATGGCTA	R; GACGTAGTCCAGGAGGCAAC	771–797	60°C
			21	F; CCTCTGTATTTCAGGGCATG	R; ACTCCCAGGACTTACTTTGC	828–875	55°C
	Rat	BHP	18	F; TGGTGCTAGCATCTCTGGTC	R; AGTCCAGACCTGTCTCCAGG	689–729	55°C
			19	F; CAGGTTAATGTCAGCCCTCTTC	R; GGAAACCGTGGTTAGCAAGA	730–762	55°C
			20	F; CCCATCAGCCAAGAAACAAT	R; GTACTCCAGGGGGCAGACCT	763–824	55°C
			21	F; GGGCATGAACTACCTGGAAG	R; AGGACTTACTTTGCCCCCCTC	832–872	55°C
		X-ray	18	F1; TGGTGCTAGCATCTCTGGTC	R1; CTCCTGAACCCAGAACTTTGA	689–715	55°C
				F2; GGAGAAGCTCCGAACCAAG	R2; AGTCCAGACCTGTCTCCAGG	704–729	55°C
			19	F; CAGCCCTCTTCTTAATCTCAGG	R; GCAAGACATAAAAGGAAACTCACA	731–762	55°C
			20	F1; CACATGTGTTGTCCTTACCTTG	R1; AACCATAGGGCATGAGTTGTG	763–790	55°C
				F2; ACCTCCACTGTCCAGCTCAT	R2; GCAGACCTTCCAATGTGCTTA	791–824	55°C
			21	F1; TGAAGCGTCTTCTGTGTTTCA	R1; TTGGCCAGTCCAAAATCTGT	825–854	55°C
				F2; TACTGGTAAAGACACCACAGCA	R2; GCTTCCTGACTTATTCTCAGGACT	852–876	55°C

†Corresponding to mouse and rat codons. BHP, *N*-nitrosobis(2-hydroxypropyl)amine; *Egrf*, epidermal growth factor receptor; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PCR, polymerase chain reaction.

Table 2. Incidences of mutations with amino acid substitution and silent mutations of the K-*ras* and *Egfr* genes in animal lung neoplasms

Treatment	Animal	K-*ras*		Egfr			
		Exon 1	Exon 2	Exon 18	Exon 19	Exon 20	Exon 21
Mutations with amino acid substitution							
NNK	Mice	21/23 (91%)	0/22	0/23	0/22	0/15	0/14
MeIQx	Mice	4/5 (80%)	0/5	0/5	0/4	0/4	0/5
Urethane	Mice	0/8	1/4 (25%)	0/4	0/4	0/5	0/3
BHP	Rats	7/18 (39%)	0/14	0/18	0/18	0/17	0/18
X-ray	Rats	0/12	0/11	3/12 (25%)	0/11	0/12	1/11 (9%)
Silent mutations							
X-ray	Rats	1/12 (8%)	0/11	1/12 (8%)	0/11	2/12 (17%)	1/11 (9%)

BHP, *N*-nitrosobis(2-hydroxypropyl)amine; *Egrf*, epidermal growth factor receptor; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

All the studies were conducted according to the Guidelines for Animal Experiments in the respective facilities.

Tissue preparation and DNA extraction. Upon sacrifice, the lungs were immediately excised and portions were fixed in neutrally buffered 10% formalin and embedded in paraffin. Two serial thin sections were made, one of 3 μm thickness to be stained with hematoxylin and eosin for histological examination, and the other of 8 μm thickness for DNA extraction.

For analysis of K-*ras* and *Egfr* mutations, paraffin-embedded lung neoplastic lesions (alveolar hyperplasia [AH], adenoma [Ad] and adenocarcinoma [AC]) from each animal model were used. Neoplastic lesions were scraped off from paraffin sections using needles and DNA was extracted using DEXPAT (TaKaRa Shuzo, Shiga, Japan).

Polymerase chain reaction (PCR). K-*ras* and *Egfr* gene fragments were amplified by PCR from lung DNA samples. PCR primers were synthesized at Operon Biotechnologies Inc. (Tokyo, Japan) with oligonucleotide purification cartridge grade. The sequences and target codons are listed in Table 1. Different primer sets were used according to each sample quality. In some cases, one exon was analyzed with two short PCR products overlapping partially.

PCR for analysis of the gene alterations was performed in 50 μL of reaction mixture consisting of 0.5 μM of each primer, 10 × PCR buffer (Applied Biosystems, Foster City, CA, USA), 200 μM each dNTP, 2.5 U AmpliTaq Gold (Applied Biosystems) and 0.5–5 μL of template DNA. The mixture was heated at 94°C for 9 min and subjected to 50 cycles of denaturation (94°C, 30 s), annealing (at each temperature in Table 2, 30 s)

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and extension (72°C, 1 min) using a thermal cycler, DNA Engine PTC-200 (Bio-Rad Laboratories Inc. Hercules, CA, USA).

Single strand conformation polymorphism (SSCP) analysis. SSCP analysis was conducted by the method of Orita *et al*. with modifications.[16] PCR products were treated using ExoSAP-IT (USB Corp., Cleveland, OH, USA) before application to SSCP analysis. Four and a half μL of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol were added to 0.5 μL PCR products treated by ExoSAP-IT, heated at 90°C for 3 min, cooled at 4°C for 1 min and then applied to 5–20% gradient polyacrylamide gel (e-PAGEL, ATTO corporation, Tokyo, Japan).

Electrophoresis was carried out at 300 V for 1.5 h at 4°C and the gels were soaked in 10% trichloroacetate and in 50% methanol for 10 min each. DNA bands were detected by silver staining using 2D Silver Staining Solution II (Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan). Detected shifted bands were homogenated, heated and centrifuged with DEXPAT to extract DNA and again applied to PCR and direct sequencing for verification of the mutation.

Direct DNA sequencing. With 2 μL of the ExoSAP-IT-treated PCR products and 5′ or 3′ of each PCR primer (Table 1), cycle sequencing reactions were carried out using a DYEnamic ET terminator cycle sequencing kit (GE Healthcare UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, England) and the sequences were determined with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Results

Histological findings. Lung neoplastic lesions induced by each treatment mostly originated from alveolar type II cells or bronchiolar Clara cells. Almost all histopathological types of lung neoplasms with each treatment were epithelial types, being classified into AH, Ad, AC, adenosquamous carcinoma and squamous cell carcinoma categories. A total of 66 lesions, classified into 4 AHs, 7 Ads, and 12 ACs from NNK, 2 AHs and 3 Ads from MeIQx, 8 Ads from urethane, 15 Ads and 3 ACs from BHP, 4 Ads and 8 ACs from X-ray-treated animal lungs, respectively, were used in the present mutational analyses.

K-*ras* alterations in lung neoplastic lesions. Activating mutations of the K-*ras* gene at codons 12 and 61 were detected in neoplastic lesions induced by NNK (21/23; 91%), MeIQx (4/5; 80%), urethane (1/4; 25%) and BHP (7/18; 39%), respectively, but not in X-ray-induced tumors (Table 2). Histological classifications in neoplasms with K-*ras* mutations were 3 AHs, 7 Ads and 11 ACs from NNK, 1 AH and 3 Ads from MeIQx, 1 Ad from urethane, 5 Ads and 2 ACs from BHP-induced neoplasms. Only one silent mutation was detected in a X-ray-induced Ad (1/12; 8% in Table 2). Mutations were mostly G/C→A/T transitions. In one neoplasm, each in the MeIQx, urethane and X-ray models, G/C→C/G (AH), A/T→T/A (Ad) and A/T→T/A (Ad) transversions, respectively, were observed (Table 3). Particular histological differences were not observed with each mutation pattern.

Table 3. Mutation patterns for the K-*ras* and *Egfr* genes in lung neoplasms

Treatment	Animal	Gene	Exon	Nucleotide alteration	Amino acid substitution†	Frequency
NNK	Mice	K-*ras*	1	GGT→GAT	G12D	21/21
MeIQx	Mice	K-*ras*	1	GGT→GAT	G12D	3/4
				GGT→GCT	G12A	1/4
Urethane	Mice	K-*ras*	2	CAA→CTA	Q61L	1/1
BHP	Rats	K-*ras*	1	GGT→GAT	G12D	7/7
X-ray	Rats	K-*ras*	1	GGA→GGT	G10	1/1
		Egfr	18	CCC→CTC	P695L	1/8
				GGA→GAA	G697E	1/8
				GTT→GTC	V718	1/8
				GGT→GAT	G720D	1/8
			20	ACC→ACT	T784	1/8
				CAG→CAA	Q788	1/8
			21	CAC→CAT	H836	1/8
				GGT→GAT	G864D	1/8

†Corresponding to mouse and rat codons. BHP, *N*-nitrosobis(2-hydroxypropyl)amine; *Egrf*, epidermal growth factor receptor; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

***Egfr* gene alterations in lung neoplastic lesions.** Lung neoplastic lesions induced by NNK, MeIQx, urethane and BHP were found to harbor activating K-*ras* mutations, but not *Egfr* mutations. On the other hand, *Egfr* mutations with amino acid substitution were detected in X-ray-induced tumors (4/12; 33% in Table 2). Representative mutation charts are shown in Fig. 1.

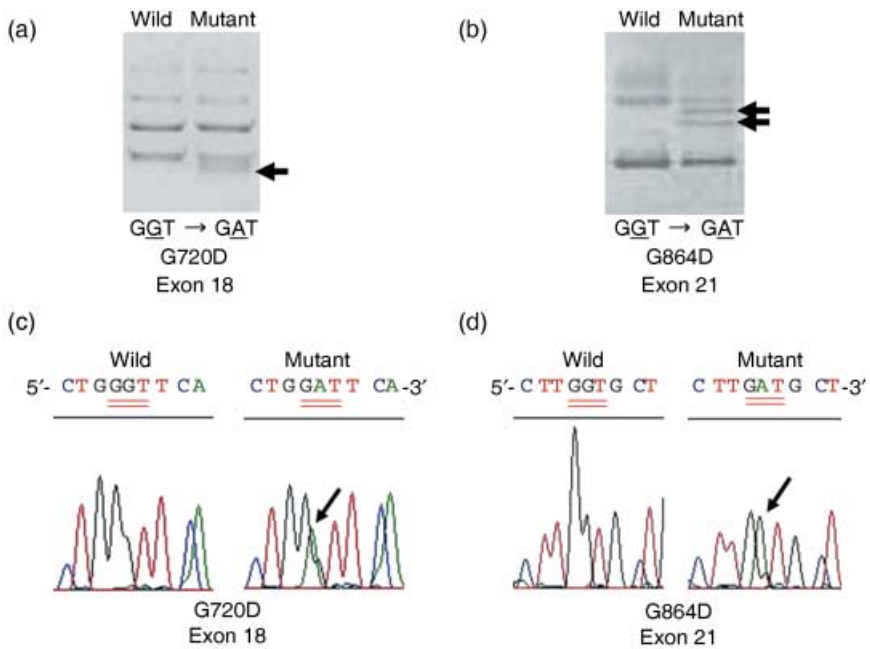

Fig. 1. Representative examples of single strand conformation polymorphism analysis (a, b) and charts of *Egfr* mutations confirmed by direct sequencing (c, d). Arrows in panels (a) and (b) indicate shifted bands associated with mutations. Panels a, c and b, d show G/C→A/T mutations found at codon 720 in exon 18 and at codon 864 in exon 21, respectively. Both arrows in panel (b) indicate the same mutation pattern of GGT to GAT at codon 864.

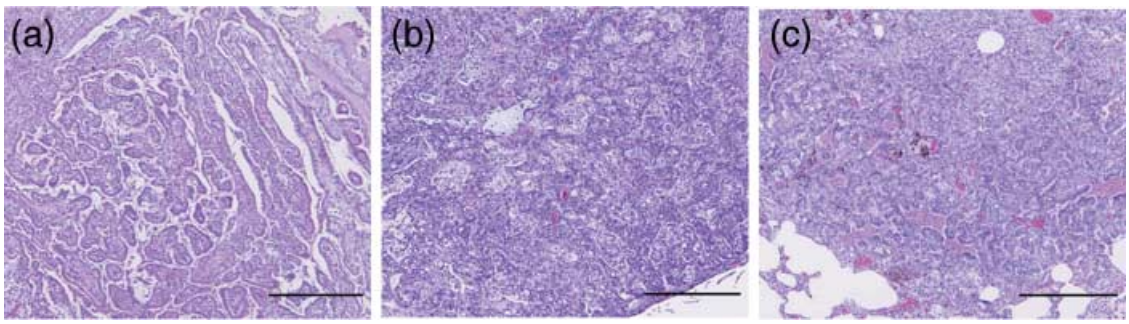

Fig. 2. Typical histological subtypes of X-ray-induced rat lung tumors with and without the *Egfr* mutations. (a) Papillary type adenocarcinoma. (b) Solid type adenocaricnoma. (c) Acinar type adenocarcinoma. The tumors of (a) and (b) exhibit the *Egfr* mutations and (c) is without the *Egfr* mutation. Histological classifications refer to our previous study.[15,20] Scale bars, 500 μm.

All the *Egfr* mutations were reconfirmed by sequencing of independent PCR products derived from the original template DNA. Mutation sites were mostly found in exon 18 (3/4) and the remainder in exon 21 (1/4). In addition to the mutations with amino acid substitution, silent mutations were also detected (4/12, 33%) in exons 18 (1/4), 20 (2/4) and 21 (1/4), respectively, two of the four silent mutations overlapping with mutations causing amino acid substitution. None of the tumors with *Egfr* mutations had K-*ras* mutations. *Egfr* alteration sites were not located in specific codons, but distributed over exons 18–21 (except 19) (Table 3). Tumors harboring *Egfr* mutations with amino acid substitution were histologically classified as 1 Ad and 3 ACs all with G/C→A/T patterns. Histological classifications of tumors with silent mutations of *Egfr* were 1 Ad with G/C→A/T, 3 ACs with 2 G/C→A/T and T/A→C/G patterns, respectively. Among these tumors, two ACs exhibited both missense and silent mutations, 1 AC with G/C→ A/T at codon 697 in exon 18 and G/C→A/T at codon 784 in exon 20, and 1 AC with G/C→A/T at codon 720 in exon 18 and T/A→C/G at codon 718 in exon 18, respectively. In addition, we further classified histological subtypes of the tumors with or without the *Egfr* mutations. Typical histological subtypes of X-ray-induced tumors used in the present study are shown in Fig. 2. Out of six tumors with the *Egfr* mutations, three were papillary type and the other three were solid type, while one papillary, one solid, three acinar and one bronchiolo-alveolar types were included in six tumors without the *Egfr* mutation. The papillary and solid types were frequent in the tumors with the *Egfr* mutations compared to the tumors without the *Egfr* mutation in the X-ray model.

Discussion

In the present study of mutation profiles of K-*ras* and *Egfr* in chemical and X-ray-induced lung carcinogenesis animal models, *Egfr* alterations were detected in 4 of 12 (33%) X-ray-induced tumors. To our knowledge, this is the first demonstration of mutations in *Egfr* in an animal lung carcinogenesis model. These mutations detected in this study are thought to be somatic, because experimental animals used for X-ray lung carcinogenesis were inbred and no mutations were detected in non-neoplastic parts of lung sections used for extracting DNA samples. The *Egfr* mutation sites and patterns found in X-ray-induced tumors have no codon specificity and were scattered over exons 18–21 (except 19). An amino acid substitution at codon 720 is reported as a mutation site in human lung cancer (corresponding to codon 719 in human EGFR).[10] This similarity suggests that the rat lung carcinogenesis model induced by X-rays may reflect, in part, human lung carcinogenesis with *EGFR* mutation. However, the other mutations differed from the most frequent mutations in human cases.

Yuan *et al*. reported that T/A→G/C substitutions, a change frequently detected in *EGFR* of human lung cancer, were induced by a 5.0 Gy dose of X-ray irradiation in mouse cells.[17] They speculated that this mismatch substitution resulted from repair activity of polymerase-β. However, in the present study, T/A→G/C substitutions were not detected in rat lung tumors induced *in vivo* by 3.0 Gy of X-ray irradiation. The differences between this study's data and Yuan's data could be due to differences in irradiation doses and species dependence regarding metabolism and repair systems.

In X-ray-induced tumors, silent mutations in *Egfr* and K-*ras* were also observed (Table 3). Generally, these have been believed to not affect events of protein levels, but a recent study demonstrated that silent mutations may also alter the conformation and activity of a protein.[18] Although half of the detected silent mutations (2/4) in *Egfr* overlapped with mutations generating amino acid substitution, some role in lung carcinogenesis could not be ruled out, at least in the other two cases.

In the histological evaluation, *Egfr* mutations were detected in both Ad and AC induced by X-rays in the lung, suggesting a possible involvement in an early stage of the neoplastic process, as with activating K-*ras* mutations. In the present study, the papillary type was frequently observed in the tumors with the *Egfr* mutations compared to the tumors without the *Egfr* mutations in the X-ray model. In humans, the papillary type was also predominant in lung cancers with *EGFR* mutations.[19] There are some similarities of the histological subtype between X-ray-induced lung tumors and human lung cancers with *EGFR* mutations.

In our previous study, immunohistochemical staining for surfactant apoprotein A and Clara cell 10 kDa protein have revealed that most of the lung tumors induced by X-rays originated from either type II alveolar or Clara cells.[20] On the bases of our previous data, we compared the expression of SP-A and CC-10 between the tumors with and without the EGFR mutations in the present study. However, no specificity of cell differentiation phenotypes was observed between tumors with and without the *Egfr* mutations.

In the present study, we conducted immunostaining for the Egfr downstream molecules of phospholyrated ERK (pERK) and Akt (pAkt). However, pERK and pAkt were mostly negative in both X-ray-induced tumors with and without the *Egfr* mutations (data not shown). No specificity of the expression patterns of pERK and pAkt were observed between tumors with and without the *Egfr* mutations. In the human lung cancer, Ikeda *et al*. reported that pAkt expression was significantly associated with the codon 858 mutation in the exon 21, but not in the exon 19 deletions, while pERK did not have any correlation.[21] The *Egfr* mutation patterns and sites detected in the present study were different from the above two mutations of human lung cancer. Moreover, to investigate the influence of the

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Egfr mutations detected in the present study on cell proliferation activity, we also conducted immunostaining for proliferating cell nuclear antigen (PCNA). The ratio of PCNA positive cells tended to be higher in X-ray-induced lung tumors with the *Egfr* mutations (22.1 ± 6.9% [mean ± standard deviation]) than those without the *Egfr* mutation (13.9 ± 3.7%). Further analysis such as transfection study in cell culture system is warranted to clarify the biological effect of the *Egfr* mutations, found in the present study.

The nitroso compounds, NNK and BHP, are well known to frequently induce K-*ras* mutations with G/C→A/T transitions in animal models[12,13] as confirmed in our present study. Lung tumors induced by MeIQx and urethane, non-smoking factors, were also found to harbor K-*ras*, but none of them featured any *Egfr* mutations. These data suggest that mutation hotspots might differ between chemical and X-ray mutagenesis.

Most chemical carcinogens modify DNA bases by forming adducts, whereas X-rays are known to cause genomic DNA damage, mostly having indirect effects, by producing oxygen radicals derived mainly from O_2 and H_2O molecules *in vivo*. These different mechanisms of DNA damages may contribute to different gene targets. Indeed, the coexistence of both K-*ras* and *Egfr* mutations was not observed in the present study as observed in human cases. In addition to X-rays, microparticles, a factor of air pollutions derived from various industrial activities, might therefore also be likely to induce *EGFR* mutations because of their ability to produce oxygen radicals.[22]

Recently, the proportion of lung adenocarcinomas is increasing in our country. The prevalence of lowtar filter cigarettes makes smokers inhale more deeply and this is suggested to be a cause for recent increases. However, the reason for the increases of lung adenocarcinomas, especially with the *EGFR* mutations among non-smokers in Asia, is unclear. Ashakumary *et al*. reported that administration of a high-fat diet increases the concentration of lipid peroxides in rat lung tissue.[23] Recent changes of lifestyle may contribute to the increases of lung adenocarcinomas. The fact that endogenous oxygen radicals are produced by chronic inflammation is of interest in this context.[24] Clearly, the causative factors for *EGFR* mutations warrant further attention.

As an animal model for human lung adenocarcinomas, transgenic mice expressing mutant *EGFR* of human patterns in alveolar epithelium have been established.[25] That model is considered to be useful for developing therapeutic methods for human lung adenocarcinomas bearing *EGFR* mutations. The present model of X-ray-induced rat lung adenocarcinoma with the *Egfr* mutations may also be useful for studying lung carcinogenesis processes and developing therapeutic methods.

In conclusion, though the mechanisms of X-ray lung carcinogenesis have yet to be fully elucidated at the molecular level, in the present study we predominantly detected *Egfr* mutations in X-ray-induced lung tumors of rats. X-ray irradiation or endogenous factors producing oxygen radicals may thus cause *EGFR* mutations in some proportion of human lung cancers.

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