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SHORT COMMUNICATION

K-*ras* mutations are frequent in pulmonary squamous cell carcinomas but not in adenocarcinomas of WBN/Kob rats induced by *N*-nitrosobis(2-oxopropyl)amine

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Pulmonary carcinomas induced by *N*-nitrosobis(2-oxopropyl) amine (BOP) in WBN/Kob rats were screened for point mutations in the K-*ras* protooncogene. Exons 1 and 2 were polymerase chain reaction amplified from paraffin-embedded sections, followed by direct DNA sequencing. G → A transition mutations in the second base of codon 12 of the K-*ras* gene were found in 6/24 (25%) rat lung tumors induced by BOP. The incidence of point mutations was significantly higher ($P < 0.005$) in squamous cell carcinomas (5/7; 71%) than in adenocarcinomas (1/17; 6%), suggesting that the mutational activation of K-*ras* is associated with a differential growth advantage in these two histologically distinct types of lung tumors in rats. No mutations were found in codons 13, 61 or adjacent regions of these codons.

Altered expression or mutational activation of the *ras* protooncogene family are the most common genetic alterations in human and animal neoplasms (1–5). Activation of *ras* genes generally occurs by the introduction of a point mutation in codons 12, 13 or 61 (1–5). The members of the *ras* family of protooncogenes, i.e. H-*ras*, K-*ras* and N-*ras*, appear to be activated with a certain organ preference. In the lung, mutational activation of the K-*ras* protooncogene seems to prevail and has been identified as one of the major steps leading to malignant transformation both in humans and rodents (1,2,6–9).

There is increasing evidence that several factors, i.e. causative agents, cell types and host factors, modify the mutational activation of the K-*ras* gene in lung carcinogenesis. In human lung cancer, K-*ras* mutations, mostly G → T transversions in codon 12, have been detected in ~30% of adenocarcinomas from smokers (8,9), but they were rarely or never found in adenocarcinomas from non-smokers (9) or in the other histologic types of lung cancer including small cell carcinomas, large cell carcinomas and squamous cell carcinomas (8,10). In the development of murine lung tumors, the incidence of K-*ras* mutations, the *ras* codon affected (12, 13 or 61), and mutational pattern largely depend on the type of chemical carcinogen by which the respective neoplasm was induced (11–18). The incidence and pattern of K-*ras* mutations are also influenced by host factors, e.g. genetic susceptibility (19,20). Here we present evidence that in nitrosamine-induced lung tumors of the rat, the involvement of K-*ras* mutations depends on the histological tumor type.

Forty male 5 week old WBN/Kob rats (Japan SLC, Inc., Shizuoka, Japan) were treated with *N*-nitrosobis(2-oxopropyl) amine (BOP*) by s.c. injection of 20 mg BOP/kg body wt once a day for 5 days. Rats were killed after 52 weeks and lung tumors were fixed with buffered formalin, and embedded in paraffin. These lung tumors were histologically classified into two types, adenocarcinomas and squamous cell carcinomas. Adenocarcinomas were alveolar tumors with glandular or papillary structures. Squamous cell carcinomas were well differentiated and keratin pearl formation was prominent in most cases (21). In the present study, 17 adenocarcinomas from eight rats and seven squamous cell carcinomas from five rats were analyzed for K-*ras* mutations as shown in Table I.

DNA from lung tumors was extracted from paraffin-embedded sections as described previously (22). A mixture of DNA solution (5 µl), 12.5 pmol of each primer, 200 µM dNTPs, 10 mM Tris (pH 8.8), 50 mM KCl, 1 mM $MgCl_2$, and 2.5 U Taq polymerase in a total volume of 50 µl was subjected to 40 amplification cycles using a Perkin–Elmer Cetus thermal cycler (DNA denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and extension at 72°C for 1.5 min). Sequences of primers for amplification of the K-*ras* gene were 5′-GCCTGCTGAAAATGACTGAG and 5′-CTCTATCGTAGGATCATATT for exon 1 and 5′-GACTCCTACAGGAAACAAGT and 5′-AGAAAGCCCTCCCCAGTTCT for exon 2. Negative control samples for polymerase chain reaction (PCR) without DNA showed no amplification in each reaction. After amplification, 40 µl of the PCR reaction was electrophoresed on a 6% polyacrylamide gel. The amplified bands were cut out, eluted in 0.5 M ammonium acetate and 1 mM EDTA at 37°C overnight and precipitated with ethanol. Dried DNA was resuspended in 10 µl of TE buffer.

Sanger dideoxynucleotide sequencing was performed using [α-^{32}P]dATP and sequencing primer (5′-CATCCACAAAGTGATTCTG for exon 1 and 5′-GTAATTGATGGAGAAACCTG for exon 2). The template–primer mixture (4 µl DNA solution and 10 pmol primer) in 10 µl reaction buffer (10% DMSO, 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 25 mM NaCl) was heated at 95°C for 5 min and immediately placed in liquid nitrogen. After adding 0.1 M dithiothreitol, 0.5 µCi [α-^{32}P]-dATP and 2 U of Sequenase version 2.0 (USB), samples were divided into four wells containing each termination mixture and incubated at 37°C for 10 min. Samples were mixed with 4 µl stop solution, heated at 80°C for 2 min and immediately loaded onto a 6% polyacrylamide/7 urea gel. Gels were dried and autoradiographed for 1–5 days. Samples that showed mutations were repeatedly confirmed by independent PCR and sequencing. Normal rat liver DNA was amplified and sequenced in each reaction.

*Abbreviations: BOP, *N*-nitrosobis(2-oxopropyl)amine; PCR, polymerase chain reaction; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

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Table I. Mutations in codon 12 of the K-*ras* gene in lung tumors induced by BOP in WBN/Kob rats

Rat	Tumor	Histology	Mutation in K-*ras* codon 12 (GGT → GAT)
SP5	a	adenocarcinoma	−
	b	adenocarcinoma	−
SP6	a	adenocarcinoma	−
	b	adenocarcinoma	+
	c	adenocarcinoma	−
SP7	a	adenocarcinoma	−
	b	squamous cell carcinoma	+
	c	squamous cell carcinoma	+
SP9	a	adenocarcinoma	−
SP11	a	adenocarcinoma	−
	b	adenocarcinoma	−
	c	adenocarcinoma	−
	d	adenocarcinoma	−
	e	adenocarcinoma	−
	f	adenocarcinoma	−
SP13	a	adenocarcinoma	−
SP14	a	squamous cell carcinoma	−
SP16	a	squamous cell carcinoma	+
SP22	a	squamous cell carcinoma	+
	b	squamous cell carcinoma	+
SP28	a	squamous cell carcinoma	−
SP31	a	adenocarcinoma	−
SP39	a	adenocarcinoma	−
	b	adenocarcinoma	−

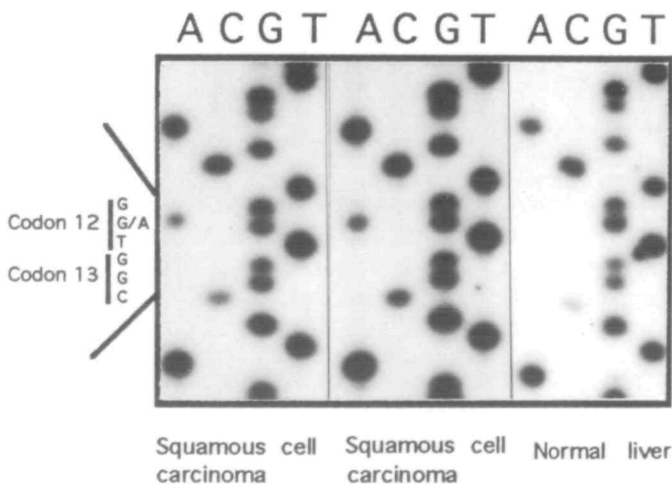

Fig. 1. DNA sequencing autoradiographs of the K-*ras* protooncogene in rat lung tumors induced by BOP. The additional bands A are shown at the second base (G) of codon 12 of the K-*ras* gene in a squamous cell carcinoma in rat no. SP7 (left) and a squamous cell carcinoma in rat no. SP22 (middle). Normal rat liver DNA shows the wild-type sequence (right).

Transition mutations in the second base of codon 12 of the K-*ras* gene (GGT → GAT) were identified in 6/24 (25%) of rat lung tumors induced by BOP (Table I). In all cases, wild-type base was detected together with mutated base. Typical DNA sequencing autoradiographs are shown in Figure 1. The incidence of point mutations was significantly higher ($P < 0.005$) in squamous cell carcinomas (5/7; 71%) than in adenocarcinomas (1/17; 6%). This tendency is exemplified by rat SP7 in which two pulmonary squamous cell carcinomas contained K-*ras* mutations, whereas the simultaneously induced adenocarcinoma did not (Table I). No mutation was observed in codons 13 or 61, or in the surrounding regions of these codons.

While murine lung tumors are almost exclusively adenomas or adenocarcinomas, rats develop squamous cell carcinomas as well as adenocarcinomas, though the former are usually less frequent. The present study was carried out on tumors that developed in inbred Wistar rats treated with BOP (21). BOP is a *N*-nitroso compound which is enzymically converted to a methylating agent that reacts with various cell constituents, including nucleic acids. Among the promutagenic DNA bases produced by this class of methylating *N*-nitroso compounds, O^6-methyldeoxyguanosine causes G → A transition mutations during DNA replication. This type of mutation was first shown to be present in rat mammary tumors induced by NMU which consistently contain a G → A transition mutation in codon 12 of the H-*ras* gene (23). Similarly, it has been reported that pancreatic duct carcinomas in hamsters induced by BOP contain a high incidence of G → A mutations in the second base of codon 12 of the K-*ras* gene (24−26). The presence of the same mutation in rat lung tumors induced by BOP (Table I) suggests that these mutations, too, are directly produced by this carcinogen, most likely through the formation of O^6-methyldeoxyguanosine.

The present study showed a mutational activation of the K-*ras* gene in >70% of BOP-induced squamous cell carcinomas, whereas adenocarcinomas induced in rats by the same carcinogen in the same experiment contained K-*ras* mutations 10 times less frequently (6%). This suggests that in the lung of this rat strain K-*ras* mutations in codon 12 confer a cell-type-specific growth advantage. K-*ras* mutations have been also observed in 3/4 (75%) pulmonary squamous cell carcinomas induced by tetranitromethane (27) and in 11/33 (33%) of these tumors induced by plutonium-239 (28). At high doses, the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces squamous cell carcinomas (29) in rat lung, but there are no reports on the presence of *ras* mutations in this histological subtype.

In rat pulmonary adenomas and adenocarcinomas, the involvement of K-*ras* mutations appears to be controversial. Stowers *et al.* (27) reported that 9/12 (75%) adenocarcinomas induced by tetranitromethane in F344 rats contained point mutations in the second base of codon 12 of the K-*ras* gene. Similarly, adenomas and adenocarcinomas induced by plutonium-239 in F344 rats contained mutations in the first or second base of codon 12 of the K-*ras* gene at an incidence of 46% (28), whereas Belinsky *et al.* (30,31) reported the complete absence of K-*ras* mutations in lung adenomas and adenocarcinomas induced by NNK in F344 rats. The biological basis of this discrepancy is not fully elucidated. It should, however, be noted that BOP and NNK require cytochrome P450-mediated bioactivation, which often occurs in a cell-specific mode (29−31) and that both carcinogens exert their adverse effects through the formation of methyl cations as the ultimate carcinogen. Tetranitromethane utilizes different enzymatic pathways and its ultimate reactive form is not known. Plutonium is assumed to act randomly without preference for specific cell types and produces DNA damage different from that by methylating agents. Belinsky *et al.* (29−31) reported that after treatment with NNK, the level of O^6-methylguanine in bronchiolar Clara cells of the rat lung was up to 30-fold higher than that in alveolar type II cells. Although the level of O^6-methylguanine in Clara cell DNAs and the tumor incidence correlated well in a dose−response manner, the neoplasms

induced by NNK in rat lung may actually be derived from alveolar type II cells (29–31). The infrequent mutational activation of the K-*ras* gene in NNK- and BOP-induced adenocarcinomas may indicate that these two *N*-nitroso compounds share the same target (type II cells) for malignant transformation and that in this cell type, the acquisition of K-*ras* mutations does not confer a significant growth advantage.

Since BOP induces both adenocarcinomas and squamous cell carcinomas in rats, this experimental model may prove useful for the study of molecular mechanisms involved in the development of these two different histologic types of lung tumors.

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