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Evaluation of the *in vivo* Mutagenicity of Nickel Subsulfide in the Lung of F344 *gpt* delta Transgenic Rats Exposed by Intratracheal Instillation: A Collaborative Study for the *gpt* delta Transgenic Rat Mutation Assay

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This study was conducted to evaluate the effectiveness of a transgenic rat mutation assay using F344 *gpt* delta rats. We investigated the mutagenic potential in the lung of nickel subsulfide (Ni_3S_2), an insoluble fine-crystalline-metallic compound and a carcinogen in the rodent and human lung. Ni_3S_2 carcinogenicity has been proposed to act via both genotoxic and non-genotoxic mechanisms. Ni_3S_2 was intratracheally instilled into male *gpt* delta rats at doses of 0.5 and 1 mg/animal once a week for four weeks; these doses of Ni_3S_2 are high enough to induce inflammation in the lung. Following a period of 28 and 90 days after the first administration, the *gpt* mutant frequencies (MFs) in lung were determined in four independent laboratories, and Spi^- selection for larger deletion mutations was done in one laboratory. The *gpt* MFs of the rats treated with Ni_3S_2 were not increased: all four laboratories obtained similar results with no statistical differences. The Spi^- MFs were also not increased by exposure to Ni_3S_2. These results indicate that intratracheally instilled Ni_3S_2 is non-mutagenic in the lung of *gpt* delta transgenic rats; however, whether Ni_3S_2 is non-mutagenic in the lung or it induces mutations which are not detectable by transgenic rodent mutation assays requires further investigation.

Key words: F344 *gpt* delta transgenic rat, nickel subsulfide, *gpt* assay, Spi^- assay

Introduction

Transgenic animals, such as Big Blue® rats and mice (1,2), Muta™ mice (3) and *gpt* delta rats and mice (4,5), are powerful tools for the detection of *in vivo* mutagenicity. The transgenic rodent mutation assay using *gpt* delta transgenic mice and rats was developed by Nohmi *et al*. (4,5). This assay system is composed of two detection methods, the 6-thioguanine (6-TG) assay for point mutations and Spi^- selection for deletion (6,7). This system is convenient for the identification of gene mutation by DNA sequencing due to small gene size (456 bp). In addition, positive selections are easier to evaluate mutant frequencies (MFs) than conventional color selections. Spontaneous MFs are comparatively lower than that of other transgenic rodent mutation assay system (7). Among the numerous substances which have been evaluated using this system are a variety of chemical compounds (8–10), radiation (11,12), and micro/nanoparticles (13), validating the usefulness of this transgenic rodent mutation assay system (7).

Recently, a protocol or guideline for the use of transgenic rodent gene mutation assays was discussed and proposed by the International Workshop on Genotoxicity Testing (IWGT) (14–16). It recommends a repeat-dose regimen with daily treatments for a period of 28 days and sampling time for 3 days following the final treatment. This protocol is being developed into an OECD Test Guideline (17). IWGT also suggests alternative treatment regimens; for example, weekly dose administration may be appropriate for some evaluations and a longer sampling time may be more appropriate if slowly proliferating tissues are of interest (16). We adopted the fundamental protocol of a collaborative

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study, which doses were a negative control group and a minimum of two dose revels (17).

The purpose of our study was to evaluate *in vivo* mutagenicity of nickel subsulfide (Ni_3S_2) by transgenic gene mutation assays in the lung as the target organ using F344 *gpt* delta rat. Ni_3S_2 is an insoluble fine-crystalline-metallic compound and a representative rodent and human lung carcinogenic metal.

Epidemiological studies of nickel refinery workers have demonstrated increased mortality from lung and nasal cancer, and it is apparent that the cancer risks have been associated with exposure to insoluble nickel compounds such as Ni_3S_2 and nickel oxide (18,19). Ni_3S_2 clearly has carcinogenic activity in the lungs of male and female F344/N rats exposed by inhalation, but it is not carcinogenic in male or female B6C3F1 mice exposed by inhalation (20). Injection of Ni_3S_2 has been shown to cause numerous carcinogenic lesions in rodents depending on the site of injection: lesions include sarcomas, rhabdomyosarcomas, fibrosarcomas, fibrous histiocytomas, mesotheliomas, renal-cell neoplasms, retinoblastomas, melanomas, and gliomas (19,21–27).

A variety of nickel compounds, including Ni_3S_2, are able to interact with molecular oxygen and generate reactive oxygen species (ROS) (27); however, like most carcinogenic metal ions, nickel compounds have weak or no mutagenic activity in bacteria (27–33), except when present at high toxic concentrations (34). In mammalian cells, carcinogenic nickel compounds are also generally poor mutagens (27,29,30,32,33,35). These compounds are, however, clastogenic in mammalian cells, inducing DNA strand breaks and accompanying chromosomal aberrations, sister-chromatid exchanges and the formation of micronuclei (29,30,32,36–39), and these clastogenicities are thought to be the mechanism by which Ni_3S_2 transforms mammalian cells *in vitro* (27,35,37,39).

Kawanishi *et al.* showed that Ni_3S_2 induced the formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) DNA adducts and DNA strand breaks, both *in vitro* and after intratracheal instillation into the lungs of Wistar rats (40). In these studies, intratracheal instillation of Ni_3S_2 also caused inflammation in the lungs of the rats. In contrast, Mayer *et al.* reported that Ni_3S_2 has no mutagenic activity in *lacZ* transgenic mice and *lacI* transgenic rats treated by single inhalation exposure for 2 h at a dose close to the maximum tolerated dose (MTD) of Ni_3S_2, even though it was mutagenic in *lacI* transgenic cells *in vitro* (41).

We assessed the *in vivo* mutagenicity of Ni_3S_2 administered into the lung by intratracheal instillation using *gpt* delta transgenic rats. The administration was limited to once a week for 4 weeks and lung sampling was at 7 days after fourth treatment due to the reduction of body weight over a few days by treatment under anesthesia, instead of 28 consecutive daily treatments and sampling time in IWGT recommendation. To manifest maximum mutation frequency, the rats were sacrificed 28 or 90 days after the first administration. The period of 90 days between the initial administration of Ni_3S_2 and sacrifice (a little less than 10 weeks after the final administration of Ni_3S_2) was chosen to allow the lung to recover from Ni_3S_2 induced inflammation.

Materials and Methods

Four laboratories (Table 1) participated in a collaborative study to evaluate the transgenic rodent gene mutation assay using *gpt* delta rats recommended by IWGT. This study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC). The animals were cared for in accordance with the Guideline for the care and use of laboratory animals in JBRC.

Chemicals: Nickel subsulfide (Ni_3S_2) (CAS.No. 12035–72–2) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The purity of the Ni_3S_2 particles (150-mesh) was 99.7%. The particle characterization of the Ni_3S_2 particle was determined using scanning electron microscopy (SU-8000, Hitachi Ltd., Tokyo Japan). The mean size was 2.9 ± 1.74 μm in diameter and more than 90% of the Ni_3S_2 particles had diameters less than 5 μm (Fig. 1). Perfluorocarbon (PF-5060) (CAS.No. 96508–42–1) was obtained from 3M (St. Paul, MN, USA).

Animals and treatment: Five-week-old male *gpt* delta rats [F344/NSlc-Tg(*gpt* delta)] were obtained from Japan SLC (Shizuoka, Japan). The inbred F344/NSlc-Tg (*gpt* delta) rat was established by backcrossing outbred *gpt* delta SD males with inbred F344 females. This strain has been backcrossed more than 15 times and is genetically homogeneous (42). The *gpt* delta transgenic rat contains approximately 5 to 10 copies of the lambda EG10 transgene in chromosome 4 and is maintained as a heterozygote (6). The animals were quarantined for one week and acclimated for 6 weeks to allow growth to a

Table 1. Participants in the collaborative study

Lab	Investigators
A	T. Kamigaito, T. Noguchi: Japan Bioassay Research Center
B	K. Narumi, R. Takashima, S. Hamada: Mitsubishi Chemical Medience Corporation
C	H. Sanada: Central Research Laboratories, Kaken Pharmaceutical Co., Ltd.
D	K. Masumura, M. Hasuko, T. Nohmi: National Institute of Health Sciences

The *gpt* MFs in all treated groups were studied by Labs A–D.
The Spi^- MFs in all treated groups were studied by Lab D.
*Animal housing, treatments to animals and tissue collection for Ni_3S_2- and ENU-treated groups were conducted by Japan Bioassay Research Center and Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd. respectively.

body weight of about 250 g. The animals were housed individually in stainless steel wire mash cages under barrier system room controlled environmental conditions (temperature of 24 ± 3°C, and relative humidity of 55 ± 15%). Fluorescent lighting was controlled automatically to provide a 12-h light-dark cycle. All animals were given basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water *ad libitum*. Twelve-week-old rats were divided by stratified randomization into 5-body weight matched groups.

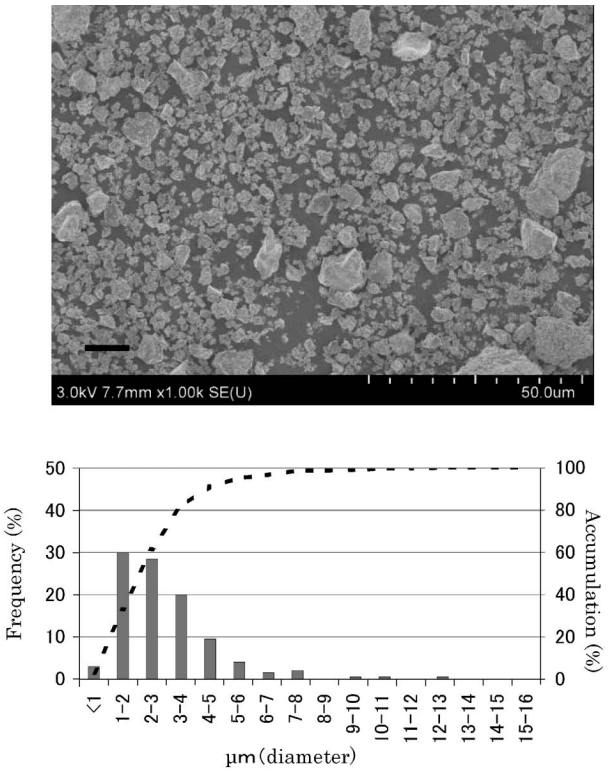

Fig. 1. Characteristics of nickel subsulfide particles in test substance. Upper panel: Scanning electron micrograph of Ni_3S_2 particles. Bar shows 10 μm. Lower panel: Frequency distribution (bar) and cumulative distribution (dotted line) in Ni_3S_2 particl.

Perfluorocarbon is a volatile organic compound (bp 56°C) which does not affect respiratory function in rodents and is an efficient vehicle for Ni_3S_2 particle delivery to pulmonary alveoli by intratracheal instillation (43). Ni_3S_2 particles homogenously suspended in perfluorocarbon were administrated into the endotrachea of the rats by intratracheal instillation, using a microspray cannula (Series 1A-1B Intratracheal Aerosolizer, PennCentury, Inc., PA, USA). Ni_3S_2 was administered at doses of 0 (vehicle control), 0.5, or 1.0 mg/animal in 0.3 mL perfluorocarbon once a week for four weeks. Each dose was administered to five rats under isoflurane anesthesia. Administration was limited to one time a week due to the harmful effects of the anesthesia. The dose of administered Ni_3S_2 was determined so as not to exceed the highest exposure dose produced only minimal interference with lung defense mechanisms based on particle clearance (40,44,45). Animals were sacrificed 28 days or 90 days after the first administration. The lung tissue was removed, weighed and a piece of the organ was fixed in 10% neutral buffered formalin and prepared and stained with hematoxylin and eosin. The remaining tissue was frozen in liquid nitrogen. Frozen lung tissue was broken, divided, packed with dry ice and sent to each of the collaborating laboratories for DNA extraction and mutation analysis (Table 1).

As a positive control, the livers of *N*-ethyl-*N*-nitrosourea (ENU) treated transgenic rats were analyzed. This treatment was conducted by Pharmaceutical

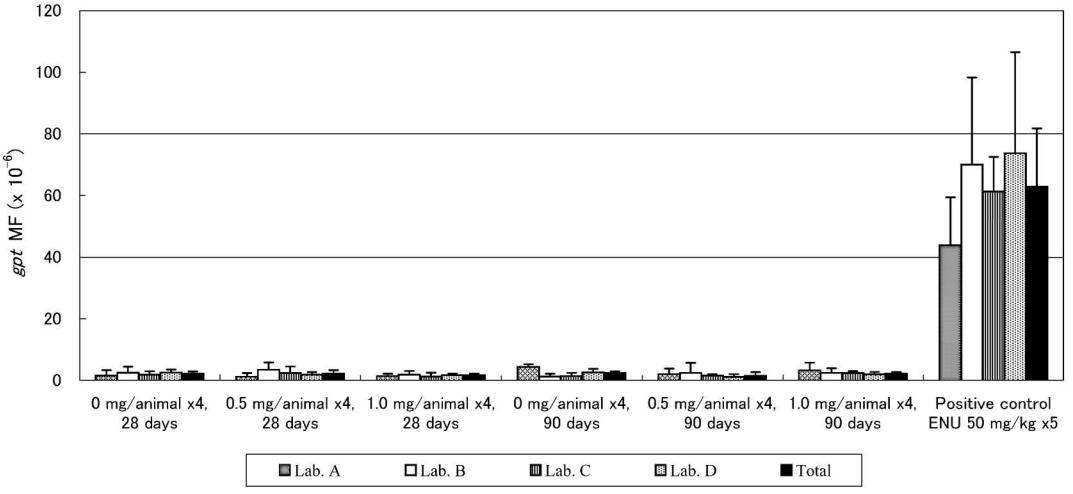

Fig. 2. *gpt* Mutant frequencies of each laboratoriy in the lung of male F344 *gpt* delta rats treated with Ni_3S_2 by intratracheal instillation and liver treated with ENU (positive control).

Research Center of Meiji Seika Pharma Co., Ltd. (Kanagawa, Japan). ENU was intraperitoneally administered to the *gpt* delta F344 male rats at a dose of 50 mg/kg once a day for five consecutive days. Twenty-six days after the final treatment the rats were killed and the livers were removed, frozen and distributed to the collaborating research laboratories (46).

DNA isolation and mutation assay: The *gpt* mutation assay was conducted at four collaborating laboratories and the Spi$^-$ assay was at a single laboratory using previously reported methods (4,6). A RecoverEase^{TM} DNA Isolation Kit (Agilent Technologies, Santa Clara, CA) was used to extract genomic DNA according to the manufacturer's instructions. The transgene was rescued from rat genomic DNA by *in vitro* lambda phage packaging using Transpack^{TM} Packaging Extract (Agilent Technologies) according to the manufacturer's instructions.

For the *gpt* assay, the *E.coli* YG6020 strain which expresses Cre recombinase was infected with the rescued lambda phage. Total colonies and 6-TG resistant mutant colonies confirmed by replating were counted to calculate the frequencies of point mutations (*gpt* assay).

For the Spi$^-$ assay, the *E.coli* XL1-Blue MRA strain and the XL1-Blue MRA(P2) strain were infected with the rescued lambda phage. Total recued plaques formed in *E.coli* XL1-Blue plates (representing total rescued lambda phage) and mutant plaques formed in the XL1-Blue MRA(P2) plates and confirmed by replating in *E.coli* WL95(P2) (representing mutant phages with deletion mutations) were counted to assess the frequencies of deletion mutations.

Statistical analysis: Bartlett's test was preliminarily used to determine whether the variances of data among control and treated groups were different or not. Body weight, organ weight and mutant frequency were analyzed by Dunnett's' multiple test because the variances of the data was not statistically different between each group. Incidences of histopathological lesions were analyzed by Fisher exact test. The level of significance was set at a p value of less than 0.05 (two-sided analysis with).

Results

Rats were exposed by intratracheal instillation to 0, 0.5 and 1.0 mg Ni_3S_2/animal once a week for four weeks. The total doses of each treated group were estimated to be 7 and 14 mg/kg, respectively. There was no difference in body weight gain between the Ni_3S_2-treated rats and the controls at 28 or 90 days after the first administration of Ni_3S_2. However, the absolute lung weight and the relative lung weight were increased in the 1.0 mg/animal group at 28 days compared to the untreated control group. No significant difference was observed at 90 days (Table 2).

Mutant frequency (MF): A summary of the *gpt* MFs determined in the four participating laboratories is shown in Table 3 and Fig. 2. There were no significant differences between the *gpt* MFs of the treated rats and the vehicle controls, at 28 days or 90 days. Under the same experimental conditions for mutation assay, ENU showed a clear positive result. The MFs obtained by the four laboratories were similar with no statistical differences between them as shown in appendix A–D (available at http:www.j-ems.org/journal/). The *gpt* MFs of the vehicle control groups in the four laboratories were 1.63 ± 1.73, 2.54 ± 1.95, 1.87 ± 1.13 and $2.60 \pm 0.98 \times 10^{-6}$ at 28 days and 4.40 ± 0.88, 1.28 ± 0.94, 1.44 ± 1.00 and $2.70 \pm 1.08 \times 10^{-6}$ at 90 days, respectively.

The *gpt* MF in the livers of *gpt* delta rats treated with ENU was $62.88 \pm 18.92 \times 10^{-6}$, dramatically increased compared to the *gpt* MFs in the lung of the vehicle control (2.28 ± 0.66 or $2.50 \pm 0.46 \times 10^{-6}$), and the spontaneous *gpt* MF in the liver of F344 *gpt* delta rat previously reported (4.4×10^{-6}) (7). Again, there was no statistical difference in the *gpt* MFs reported by the four different laboratories: 43.90 ± 15.51, 70.05 ± 28.31, 61.30 ± 11.27 and $73.74 \pm 32.81 \times 10^{-6}$.

The Spi$^-$ MFs were determined in only one of the participating laboratories (Table 4). Similar to the *gpt* MF results, there were no significant differences between the Spi$^-$ MFs of the treated rats and the vehicle controls.

Table 2. Body weight and lung weight of male F344 *gpt* delta rats treated with Ni_3S_2

Group	Sampling time (days)	No. of animals	Body weight		Lung weight	
			at first treatment (g)	at dissection (g)	Absolute (g)	Relative (%)
0 mg/animal × 4	28	5	289 ± 8	286 ± 11	1.302 ± 0.052	0.46 ± 0.02
0.5 mg/animal × 4	28	5	291 ± 11	291 ± 12	1.327 ± 0.067	0.46 ± 0.03
1.0 mg/animal × 4	28	5	288 ± 11	287 ± 7	1.420 ± 0.076*	0.49 ± 0.02*
0 mg/animal × 4	90	5	283 ± 8	372 ± 5	1.247 ± 0.063	0.34 ± 0.02
0.5 mg/animal × 4	90	5	284 ± 11	372 ± 8	1.277 ± 0.081	0.34 ± 0.02
1.0 mg/aniaml × 4	90	5	288 ± 7	374 ± 6	1.309 ± 0.042	0.35 ± 0.01

*Significant difference compared with control group (Dunnett's test, $p < 0.05$).

Table 3. Summary of *gpt* mutant frequencies in the lung of *gpt* delta rats treated with Ni_3S_2 by intratracheal administration and liver treated with ENU (positive control)

Treatment	Smpling time	Animal No.	Organ	Number of packagings	Total Population	Number of mutants	Mutant frequency ($\times 10^{-6}$)	
								Mean ± SD
0 mg/animal × 4	28 days	1001	lung	17	1,704,000	3	1.76	
		1002	lung	14	2,907,000	8	2.75	
		1003	lung	14	4,026,000	10	2.48	
		1004	lung	14	3,693,000	11	2.98	
		1005	lung	14	4,200,000	6	1.43	2.28 ± 0.66
0.5 mg/animal × 4	28 days	1101	lung	14	2,669,500	6	2.25	
		1102	lung	14	3,577,500	5	1.40	
		1103	lung	13	2,625,000	11	4.19	
		1104	lung	18	3,445,500	5	1.45	
		1105	lung	10	2,851,500	6	2.10	2.28 ± 1.13
1 mg/animal × 4	28 days	1201	lung	13	3,142,500	8	2.55	
		1202	lung	15	3,687,000	4	1.08	
		1203	lung	13	3,823,500	7	1.83	
		1204	lung	15	4,333,500	6	1.38	
		1205	lung	15	6,930,000	13	1.88	1.74 ± 0.55
0 mg/animal × 4	90 days	1011	lung	11	4,149,000	11	2.65	
		1012	lung	11	3,348,000	8	2.39	
		1013	lung	11	5,779,500	17	2.94	
		1014	lung	9	4,371,000	12	2.75	
		1015	lung	13	5,701,500	10	1.75	2.50 ± 0.46
0.5 mg/animal × 4	90 days	1111	lung	10	3,832,500	6	1.57	
		1112	lung	11	2,611,500	3	1.15	
		1113	lung	10	5,424,000	6	1.11	
		1114	lung	13	2,565,000	9	3.51	
		1115	lung	12	7,806,000	5	0.64	1.59 ± 1.12
1 mg/animal × 4	90 days	1211	lung	7	3,405,000	8	2.35	
		1212	lung	7	4,500,000	9	2.00	
		1213	lung	8	3,385,500	10	2.95	
		1214	lung	5	3,940,500	9	2.28	
		1215	lung	7	4,006,500	7	1.75	2.27 ± 0.45
ENU	31 days	51	liver	13	3,336,450	282	84.52	
50 mg/kg × 5		52	liver	11	3,780,300	152	40.21	
		53	liver	11	3,252,900	179	55.03	
		54	liver	11	3,676,500	296	80.51	
		55	liver	11	3,140,550	170	54.13	62.88 ± 18.92[†]

[†]The *gpt* MF in the livers of *gpt* delta rats treated with ENU was markedly increased compared to the spontaneous *gpt* MF in F344 *gpt* delta rat livers of 4.4×10^{-6} previously reported (7).

The Spi^- MF for vehicle controls ($6.49 \pm 3.93 \times 10^{-6}$) was higher than the spontaneous Spi^- MF in the lung reported for the *gpt* delta mouse (2.8×10^{-6}) (7). The Spi^- MF in the livers of rats treated with ENU was $16.74 \pm 9.10 \times 10^{-6}$, in the present study, increased compared to the Spi^- MF in the lung of vehicle control and compared to the spontaneous Spi^- MF in F344 *gpt* delta rat liver of 2.8×10^{-6} previously reported (7).

Histopathology: For all the groups, histopathological changes due to the intratracheal instillation of vehicle and Ni_3S_2 were observed in the lung. Table 5 summarizes the histopathological findings of rats and their severity scores at each time point. At 28 days, a slight infiltration of inflammatory cells into the lung was observed in all animals including the negative control group. Eosinophils were found in the perivascular regions. Slight to moderate infiltration of alveolar macrophages was found in three animals of 1.0 mg/animal treatment group and this incidence was significantly greater than that of the control group. Furthermore, focal fibrosis was observed in two animals in the 0.5 mg/animal treatment group and two animals in the 1.0 mg/animal treatment group. Slight to moderate infiltration of alveolar macrophages was found in three animals of 1.0 mg/animal treatment group. At 90 days, focal fibrosis was observed in only two animals, one in the 0.5 mg/animal group and the other in the 1.0 mg/animal group, and mild infiltration of inflammatory cells was seen in only a few rats in the 0.5 mg/animal and control groups. Overall, inflammation in the lung at 90 days was milder than at 28 days, indicating recovery from the intratracheal instillation procedure.

Table 4. Summary of Spi^- mutant frequencies in the lung of *gpt* delta rats treated with Ni_3S_2 by intratracheal administration and liver treated with ENU (positive control)

Treatment	Smpling time	Animal No.	Organ	Number of packagings	Total Population	Number of mutants	Mutant frequency ($\times 10^{-6}$)	
								Mean ± SD
0 mg/animal × 4	28 days	1001	lung	6	865,500	5	5.78	
		1002	lung	6	1,665,000	4	2.40	
		1003	lung	6	1,224,000	9	7.35	
		1004	lung	6	951,000	4	4.21	
		1005	lung	6	1,339,500	17	12.69	6.49 ± 3.93
0.5 mg/animal × 4	28 days	1101	lung	4	631,500	3	4.75	
		1102	lung	6	1,412,500	7	4.96	
		1103	lung	6	656,000	3	4.57	
		1104	lung	5	622,500	2	3.21	
		1105	lung	5	1,426,500	8	5.61	4.62 ± 0.88
1 mg/animal × 4	28 days	1201	lung	6	1,188,000	8	6.73	
		1202	lung	6	1,371,000	8	5.84	
		1203	lung	7	7 23,000	5	6.92	
		1204	lung	7	456,000	6	13.16	
		1205	lung	6	1,570,500	14	8.91	8.31 ± 2.93
0 mg/animal × 4	90 days	1011	lung	3	697,500	3	4.30	
		1012	lung	4	550,500	4	7.27	
		1013	lung	4	1,380,000	2	1.45	
		1014	lung	3	1,314,000	10	7.61	
		1015	lung	3	1,620,000	8	4.94	5.11 ± 2.50
0.5 mg/animal × 4	90 days	1111	lung	3	1,225,500	9	7.34	
		1112	lung	4	1,212,000	9	7.43	
		1113	lung	3	1,692,000	9	5.32	
		1114	lung	4	708,000	4	5.65	
		1115	lung	3	1,380,000	6	4.35	6.02 ± 1.34
1 mg/animal × 4	90 days	1211	lung	6	1,110,000	3	2.70	
		1212	lung	3	1,063,500	4	3.76	
		1213	lung	3	1,012,500	5	4.94	
		1214	lung	4	946,500	6	6.34	
		1215	lung	5	2,233,500	7	3.13	4.18 ± 1.47
ENU	31 days	51	liver	1	547,500	10	18.26	
50 mg/kg × 5		52	liver	1	478,500	4	8.36	
		53	liver	1	357,000	3	8.40	
		54	liver	1	219,000	4	18.26	
		55	liver	1	460,500	14	30.40	16.74 ± 9.10[†]

[†]The Spi^- MF in the livers of *gpt* delta rats treated with ENU was markedly increased compared to the spontaneous Spi^- MF in F344 *gpt* delta rat livers of 2.8×10^{-6} previously reported (7).

Table 5. Histopathological results in the lung of Ni_3S_2-treated male F344 *gpt* delta rats

Group	Sampling time	No. of animals	Infiltration of inflammatory cells (including eosinophils, perivascular)	Fibrosis (focal)	Infiltration of alveolar macrophages
Ni_3S_2 0 mg × 4	28 days	5	5 (2+)		
Ni_3S_2 0.5 mg × 4	28 days	5	5 (2+)	2 (1+)	
Ni_3S_2 1 mg × 4	28 days	5	5 (2+)	2 (1+)	3* (1(1+), 2(2+))
Ni_3S_2 0 mg × 4	90 days	5	4 (1+)		
Ni_3S_2 0.5 mg × 4	90 days	5	3 (1+)	1 (1+)	
Ni_3S_2 1 mg × 4	90 days	5	1 (1+)		

The number of the animals bearing the lesion in each exposed or control group were shown in the column. The parenthesized values indicate the number of the animals bearing the lesion with each of 4 different grades of severity, i.e., 1+: slight, 2+: moderate, 3+: marked, 4+: severe. Significant difference indicated by $*p \leqq 0.05$ by Fisher exact test compared to each control.

Discussion

The lung inflammation induced with the higher dose of Ni_3S_2 was characterized by the infiltration of alveolar macrophages and, furthermore, the fibrosis was observed in the both of Ni_3S_2-treated groups. These lung lesions indicated an inflammation and recovery from the inflammation. The histopathological results at 90-days suggested the recovery of inflammatory lesions. Similar results were obtained in lung toxicity study after 13-week inhalation exposure to Ni_3S_2 in F344 rats and mice (47). These results suggested that the treatment of conditions of the present study ensured that the rats were sufficiently challenged by Ni_3S_2 particles. And the period of sampling time was long enough to detect mutations because regeneration and/or recovery were observed in the lung tissues of the Ni_3S_2-treated groups at 28 or 90 days after the first treatment. In addition, a threshold of insoluble particles in lung clearances of F344 rats was estimated at 1–2 mg (45). The impaired lung clearance with overload of particles might induce tumor and fibrosis (48).

The *gpt* MFs in the lung were independently measured in four independent laboratories using a common standard method. The overall packaging efficiencies in each laboratory tended to be low, especially in one laboratory. One of the reasons was thought to poor DNA quality extracted. In addition, copy number of the transgene in transgenic animals may affect efficiency of recovery of the transgene. It is reasonable that *gpt* delta rat assays need more packaging than mouse assays, because copy number of the transgene par haploid is $5 \sim 10$ in rat and about 80 in mouse (4,5). Another reason might be lower activity of packaging extract used.

The *gpt* MFs obtained by the four laboratories were similar, without any statistical differences between any of the laboratories. Administration of 0.5 or 1 mg Ni_3S_2/rat once a week for four weeks did not affect *gpt* MF in the lungs of F344 *gpt* delta rats: the *gpt* MFs were all similar to the previously reported spontaneous *gpt* MF in the lungs of *gpt* delta mice (3.4×10^{-6}) (7). Further, no increase in the Spi MF was observed in the treated groups.

Ni_3S_2 is able to interact with molecular oxygen and generate reactive oxygen species (ROS) (27), and Kawanishi *et al.* (40) reported that Ni_3S_2 induced pathological inflammation and oxidative DNA damage in the lungs of rats treated with single intratracheal instillation of 0.5 or 1.0 mg/animal. Ni_3S_2 generation of DNA-damaging ROS coupled with induction of inflammation and its inhibition of DNA repair is thought to enhance its genotoxicity and tumorigenicity (27,40). This suggests that F344 *gpt* delta rats exposed by intratracheal instillation to high doses of Ni_3S_2 should acquire multiple mutations in the lung. In our study, however, although F344 *gpt* delta rats were administered Ni_3S_2 at doses high enough to induce inflammatory responses in the lung and the duration of the experiment was set as weekly dosing for four weeks with two sampling periods (28 or 90 days after the first treatment), Ni_3S_2 did not cause an increase in *gpt* or Spi^- MF in the lung. The lack of induction of mutation is in agreement with the resultsof *in vitro* mutagenesis assays (20,27–29,36–38). Moreover, administration of Ni_3S_2 by inhalation to the Muta™ Mouse and the Big Blue® rat did not induce mutations in the *lacZ* or the *lacI* genes (41). Taken together, these data suggest that *in vivo* mutagenicity of Ni_3S_2, such as induction of point mutations or small deletions, could not be observed by transgenic rodent mutation assays under those experimental conditions.

One possible reason why ROS-induced DNA damage does not lead to a detectable increase in mutations is that the damages are eliminated, either by the damage being repaired or by the cells undergoing apoptosis. Various studies have demonstrated that ROS induced by toxic metals are closely related to metal-induced apoptosis and carcinogenesis (49). Inhalation of high concentrations of amosite asbestos and bitumen fume by rats induces oxidative DNA damage (50–52). In the Big Blue® rat, a single intratracheal instillation of amosite asbestos 20–30 μm in length at a dose of 2 mg/animal resulted in a 2-fold increase in DNA mutations 16 weeks after treatment (53). In these animals, however, the amosite fibers were not eliminated from the lung and caused significant tissue damage with infiltration of neutrophils and macrophages. It is well known that long ($> 10\ \mu$m) asbestos fibers induce ROS generation by phagocytes (54–56). Therefore, ROS generation stimulated by asbestos was augmented by neutrophils and macrophages, and the production of ROS continued throughout the experiment. In contrast to asbestos fibers, bitumen fumes, which did not cause prolonged inflammation or prolonged infiltration of phagocytes, did not induce an increase in MFs in the lungs of Big Blue® rats despite the formation of DNA adducts (50).

Two other genotoxic mechanisms may contribute to the carcinogenicity of Ni_3S_2 in the rat lung. Recently, it was demonstrated that Ni_3S_2 induced silencing of the *gpt* transgene present in G12 cells, a V79-derived transgenic Chinese hamster cell line containing the *gpt* transgene, without mutation of the transgene (57). The silencing of the *gpt* gene was shown to be due to DNA methylation. If DNA hypermethylation results in aberrant silencing of a tumor suppressor gene, it could participate in carcinogenesis (27,41,57). This type of epigenetic event would not be detected by the *gpt* or Spi^- assays we used to detect Ni_3S_2 induced mutations.

Another genotoxic mechanism is the generation of chromosomal breaks leading to mutations such as DNA strand breaks which lead to chromosomal aberrations. This type of damage will be rarely detected by the *gpt* or

Spi^- assays we used to detect Ni_3S_2 induced mutations. For example, a single administration of Ni_3S_2 by nose only inhalation for 2 h at concentrations of 86 and 130 Ni_3S_2 mg/m^3 to Muta^{TM} mice and Big Blue® rats (estimated doses of 6 and 10 mg/kg) did not cause increased mutations in nasal or lung mucosa despite clear induction of DNA strand breaks (41).

Another possibility is that the *gpt* or Spi^- assays we used to detect Ni_3S_2 induced mutations were not sensitive enough to detect Ni_3S_2 generated mutations in the lung. Mutations are fixed into the genome of a cell primarily through DNA replication. Therefore, the population of stem cells in an organ is important for mutational events to occur, associating the sensitivity of an organ to a mutagen with the organ's population of stem cells and their cell division rate (16,58–60). Consequently, the primary target cells of Ni_3S_2 mutagenicity and carcinogenicity in the lung are epithelial type II cells, the lung stem cells. However, these cells make up only 12% of total alveolar cell population in 5-month-old male rats (61). In the liver, a tissue in which increases in *gpt* and Spi^- MFs were readily detected, over 80% of the mass of the organ is made up of liver stem cell hepatocytes (62). Therefore, the low sensitivity to the Ni_3S_2 mutagenicity in the lung of transgenic rodents might be due to the low population of stem cells. In addition, the lung has a well developed antioxidant system and this would decrease the mutagenicity of ROS generating genotoxic compounds (63). Dimethylarsinic acid (DMA) induces DNA damage in the lung by formation of peroxyl radical species; however, DMA was ineffective in inducing mutations in the lungs of Muta^{TM} Mice (64). The authors speculate that the mutagenicity of DMA in the lung might be too low to be detected in the Muta^{TM} Mouse. Of course, as noted above, the lack of mutagenicity may have been due to removal of cells harboring DNA damage. On the other hand, when kaolin, manufactured insoluble micro/nanoparticles which induce lung carcinogenesis in rodents, were intratracheally instilled into *gpt* delta mice, they caused 2-fold increases in *gpt* and Spi^- MFs in the lung (13). Other studies also reported the increase in *gpt* MFs in the lung of *gpt* delta mice treated with benzo[a]pyrene or 1,6-dinitropyrene by a single intratracheal instillation and diesel exhaust by inhalation for 24 weeks (65–67). Those suggest that the *gpt* and Spi^- assays using *gpt* delta transgenic animals are sensitive enough to detect an increased MF if it exists.

Briefly, possible reasons why we could not detect mutations in the lungs of F344 *gpt* delta rats exposed to the carcinogen Ni_3S_2 are (1) Ni_3S_2 exerts its effects by a non-genotoxic mechanism; (2) the type of mutation induced by Ni_3S_2 could not be detected by the *gpt* or Spi^- assays; (3) the type of mutation induced by Ni_3S_2 could be detected by the *gpt* and Spi^- assays, but these assays are not sensitive enough to detect mutations under the experimental condition we used.

Investigation of genotoxic effects in a target organ after exposure to a compound *in vivo* is critical for understanding the mechanism of carcinogenicity and for risk assessment of carcinogens. Consequently, the transgenic rodent mutation assay has become a critically important method of investigating the effects which carcinogens have on the various organs of the body. Validation of an existing system or development of a new system for determining mutagenicity in the lung is critically important to determine the risk of workplace and environmental pollutants and to properly regulate the generation of hazardous materials.

In conclusion, the results of our study indicate that Ni_3S_2 does not induce the *gpt* or Spi^- mutations in the lung of *gpt* delta rats. Therefore, this assay evaluates Ni_3S_2 as non-mutagenic in the lung of F344 *gpt* delta transgenic rats. The results obtained by four different laboratories were consistent. If the protocol is effective in measuring the mutations which occur in the target organ, it can be used to investigate the mutagenic potential of test compounds by independent laboratories. Further studies, however, are required to validate this transgenic rodent mutation assays for use in evaluating *in vivo* mutagenicity in the target organ.

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References

1 Kohler SW, Provost GS, Kretz PL, Fieck A, Sorge JA, Short JM. The use of transgenic mice for short-term, *in vivo* mutagenicity testing. Genet Anal Tech Appl. 1990; 7: 212–8.

2 Dycaico MJ, Provost GS, Kretz PL, Ransom SL, Moores JC, Short JM. The use of shuttle vectors for mutation analysis in transgenic mice and rats. Mutat Res. 1994; 307: 461–78.

3 Gossen JA, de Leeuw WJ, Tan CH, Zwarthoff EC, Berends F, Lohman PH, Knook DL, Vijg J. Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*. Proc Natl Acad Sci USA. 1989; 86: 7971–5.

4 Nohmi T, Katoh M, Suzuki H, Matsui M, Yamada M, Watanabe M, Suzuki M, Horiya N, Ueda O, Shibuya T, Ikeda H, Sofuni T. A new transgenic mouse mutagenesis test system using Spi^- and 6-thioguanine selections. Environ Mol Mutagen. 1996; 28: 465–70.

5 Hayashi H, Kondo H, Masumura K, Shindo Y, Nohmi T. Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi^- selections. Environ Mol Mutagen. 2003; 41: 253–9.

6 Nohmi T, Suzuki T, Masumura K. Recent advances in the

protocols of transgenic mouse mutation assays. Mutat Res. 2000; 455: 191–215.
7 Masumura K. Spontaneous and induced *gpt* and Spi$^-$ mutant frequencies in *gpt* delta transgenic rodents. Genes Environ. 2009; 31: 105–18.
8 Masumura K, Matsui M, Katoh M, Horiya N, Ueda O, Tanabe H, Yamada M, Suzuki H, Sofuni T, Nohmi T. Spectra of *gpt* mutations in ethylnitrosourea-treated and untreated transgenic mice. Environ Mol Mutagen. 1999; 34: 1–8.
9 Okada N, Masumura K, Nohmi T, Yajima N. Efficient detection of deletions induced by a single treatment of mitomycin C in transgenic mouse *gpt* delta using the Spi(−) selection. Environ Mol Mutagen. 1999; 34: 106–11.
10 Masumura K, Matsui K, Yamada M, Horiguchi M, Ishida K, Watanabe M, Ueda O, Suzuki H, Kanke Y, Tindall KR, Wakabayashi K, Sofuni T, Nohmi T. Mutagenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in new *gpt* delta transgenic mouse. Cancer Lett. 1999; 143: 241–4.
11 Nohmi T, Suzuki M, Masumura K, Yamada M, Matsui K, Ueda O, Suzuki H, Katoh M, Ikeda H, Sofuni T. Spi(−) selection: An efficient method to detect gamma-ray-induced deletions in transgenic mice. Environ Mol Mutagen. 1999; 34: 9–15.
12 Okudaira N, Uehara Y, Fjikawa K, Kagawa N, Ootsuyama A, Norimura T, Saeki K, Nohmi T, Masumura K, Matsumoto T, Oghiso Y, Tanaka K, Ichinohe K, Nakamura S, Tanaka S, Ono T. Radiation dose-rate effect on mutation induction in spleen and liver of *gpt* delta mice. Radiat Res. 2010; 173: 138–47.
13 Totsuka Y, Higuchi T, Imai T, Nishikawa A, Nohmi T, Kato T, Masuda S, Kinae N, Hiyoshi K, Ogo S, Kawanishi M, Yagi T, Ichinose T, Fukumori N, Watanabe M, Sugimura T. Genotoxicity of nano/microparticles in *in vivo* micronuclei, *in vivo* comet and mutation assay system. Part Fibre Toxicol. 2009; 3: 6–23.
14 Heddle JA, Dean S, Nohmi T, Boerrigter M, Casciano D, Douglas GR, Glickman BW, Gorelick NJ, Mirsalis JC, Martus H, Skopek TR, Thybaud V, Tindall KR, Yajima N. *In vivo* transgenic mutation assays. Environ Mol Mutagen. 2000; 35: 253–9.
15 Thybaud V, Dean S, Nohmi T, deBoer J, Douglas GR, Glickman BW, Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martis HJ, Mirsalis JC, Suzuki T, Yajima N. *In vivo* transgenic mutation assays. Mutat Res. 2003; 540: 141–51.
16 Lambert IB, Singer TM, Boucher SE, Douglas GR. Detailed review of transgenic rodent mutation assays. Mutat Res. 2005; 590: 1–280.
17 OECD, Detailed review paper on transgenic rodent mutation assays. Series on testing and assessment No. 103. OECD Environment, Health and Safety Publications; 2009. Available at: http://www.oecd.org/document/30/0,3746,en_2649_34377_1916638_1_1_1_1,00.html (accessed 28 September 2011).
18 IARC, IARC Monographs on the evaluation of the carcinogenic risk of chemicals to man: Cadmium, nickel, some epoxides, miscellaneous industrial chemicals and general considerations on volatile anesthetics. Vol. 11. Lyon: IARC Scientific Publications; 1976. p. 75–112.
19 IARC, IARC Monographs on the evaluation of the carcinogenic risks to human. Chromium, nickel and welding. Vol. 49. Lyon: IARC Scientific Publications; 1990.
20 National Toxicolgy Program. Toxicology and carcinogenesis studies of nickel subsulfide (CAS NO. 12035–72–2) in F344/N rats and B6C3F$_1$ mice (inhalation studies). Natl Toxicol Program Tech Rep Ser. 1996; 453: 5–122.
21 Sunderman FW Jr, Maenza RM. Comparisons of carcinogenicities of nickel compounds in rats. Res Commun Chem Pathol Pharmacol. 1976; 14: 319–30.
22 Yamashiro S, Gilman JPW, Hulland TJ, Abandowitz HM. Nickel sulphide-induced rhabdomyosarcoma in rats. Acta Pathol Jpn. 1980; 30: 9–22.
23 Jasmin G, Riopelle JL. Renal carcinomas and erythrocytosis in rats following intrarenal injection of nickel subsulfide. Lab Invest. 1976; 35: 71–8.
24 Sunderman FW Jr, Maenza RM, Hopfer SM, Michell JM, Allpass PR, Damjanov I. Induction of renal cancers in rats by intrarenal injection of nickel subsulfide. J Environ Pathol Toxicol. 1979; 21: 1511–27.
25 Damjanov I, Sunderman FW Jr, Michell JM, Allpass PR. Induction of testicular sarcomas in Fischer rats by intratesticular injection of nickel subsulfide. Cancer Res. 1978; 38: 268–76.
26 Albert DM, Gonder JR, Papale J, Craft JL, Dohlman HG, Reid MC, Sunderman FW Jr. Induction of ocular neoplasms in Fischer rats by intraocular injection of nickel subsulfide. Invest Ophthalmol Vis Sci. 1982; 22: 768–82.
27 Kasprzak KS, Sunderman FW Jr, Salnikow K. Nickel carcinogenesis. Mutat Res. 2003; 533: 67–97.
28 Marzin D, Vophi H. Study of mutagenicity of metal derivatives with *Salmonella typhimurium* TA102. Mutat Res. 1985; 155: 49–51.
29 Arrouijal FZ, Hildebrand HF, Vophi H, Marzin D. Genotoxic activity of nickel subsulphide α-Ni_3S_2. Mutagenesis. 1990; 6: 583–9.
30 Sunderman FW Jr. Recent research on nickel carcinogenesis. Environ Health Perspect. 1981; 40: 131–41.
31 Biggart NW, Costa M. Assessment of the uptake and mutagenicity of nickel chloride in *salmonella* tester strains. Mutat Res. 1986; 175: 209–15.
32 Magos L. Epidemiological and experimental aspects of metal carcinogenesis: physicochemical properties, kinetics, and the active species. Environ Health Perspect. 1991; 95: 157–89.
33 Hartwig A. Current aspects in metal genotoxicity. Biometals. 1995; 8: 3–11.
34 Pikalek P, Necasek J. The mutagenic activity of nickel in Corynebacterium sp. Folia Microbiologica. 1983; 28: 17–21.
35 Biedermann KA, Landolph JT. Induction of anchorage independence in human diploid foreskin fibroblasts by carcinogenic metal salts. Cancer Res. 1987; 47: 3815–23.
36 Sen P, Conway K, Costa M. Comparison of the localiza-

tion of chromosome damage induced by calcium chromate and nickel compounds. Cancer Res. 1987; 47: 2142–7.
37 Conway K, Costa M. Nonrandom chromosomal alterations in nickel-transformed Chinese hamster embryo cells. Cancer Res. 1989; 49: 6032–8.
38 Sen P, Costa M. Incidence and localization of sister chromatid exchanges induced by nickel and chromium compounds. Cancer Res. 1986; 7: 1527–33.
39 Costa M, Salnikow K, Cosentino S, Klein CB, Huang X, Zhuang Z. Molecular mechanisms of nickel carcinogenesis. Environ Health Perspect. 1994; 102 Suppl 3: 127–30.
40 Kawanishi S, Inoue S, Oikawa S, Yamashita N, Toyokuni S, Kawanishi M, Nishino K. Oxidative DNA damage in cultured cells and rat lungs by carcinogenic nickel compounds. Free Radic Biol Med. 2001; 31: 108–16.
41 Mayer C, Klein RG, Wesch H, Schmezer P. Nickel subsulfide is genotoxic *in vitro* but shows no mutagenic potential in respiratory tract tissues of BigBlue® rats and Muta Mouse *in vivo* after inhalation. Mutat Res. 1998; 420: 85–98.
42 Toyoda-Hokaiwado N, Inoue T, Masumura K, Hayashi H, Kawamura Y, Kurata Y, Takamune M, Yamada M, Sanada H, Umemura T, Nishikawa A, Nohmi T. Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *in vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers. Toxicol Sci. 2010; 114, 71–8.
43 Wolfson MR, Shaffer TH. Pulmonary applications of perfluorochemical liquids: ventilation and beyond. Paediatr Respir Rev. 2005; 6: 117–27.
44 Lewis TR, Morrow PE, MaaClellan RO, Raabe OG, Kennedy GL, Schwetz BA, Goehl TJ, Roycroft JH, Chhabre RS. Establishing aerosol exposure concentrations for inhalation studies. Toxicol Appl Pharmacol. 1989; 99: 377–83.
45 Marrow PE. Possible mechanisms to explain dust overloading of the lungs. Fundum Appl Toxicol. 1988; 10: 369–84.
46 Sui H, Ohta R, Shiragiku T, Akahori A, Suzuki K, Nakajima M, Hayashi H, Masumura K, Nohmi T. Evaluation of in vivo mutagenicity by 2,4-diaminotoluene and 2,6-diaminotoluene in liver of F344 *gpt* delta transgenic rat dosed for 28 days: a collaborative study of *gpt* delta transgenic rat mutation assay. Genes Environ. 2012; 34: 25–33.
47 Dunnick JK, Elwell MR, Benson JM, Hobbs CH, Hahn FF, Halt PJ, Cheng YS, Eidson FF. Lung toxicity after 13-week inhalation exposure to nickel oxide, nickel subsulfide, or nickel sulfate hexahydrate in F344/N rats and B6C3F1 mice. Fundam Appl Toxicol. 1989; 12: 584–94.
48 Oberdörster G. Lung particle overload: implications for occupational exposure to particles. Regul Toxicol Pharmacol. 1995; 21: 123–35.
49 Pulido MD, Parrish AR. Metal-induced apoptosis: Mechanisms. Mutat Res. 2003; 533: 227–41.
50 Bottin MC, Gate L, Rihn B, Micillino JC, Nathalie M, Martin A, Nunge H, Morel G, Worbel R, Ayi-Fanou L, Champmartin C, Keith G, Binet S. Genotoxic effects of bitumen fumes in Big Blue® transgenic rat lung. Mutat Res. 2006; 596: 91–105.
51 Rihn B, Coulais C, Kauffer E, Bottin M-C, Martin P, Yvon F, Vigneron JC, Binet S, Monhoven N, Steiblen G, Keith G. Inhaled crocidolite mutagenicity in lung DNA. Environ Health Perspect. 2000; 108 Suppl 4: 341–6.
52 Micillino JC, Coulais C, Binet S, Bottin M-C, Keith G, Moulin D, Rihn BH. Lack of genotoxicity of bitumen fumes in transgenic mouse lung. Toxicology. 2002; 170: 11–20.
53 Topinka J, Loli P, Georgiadis P, Dusinska M, Hurbankova M, Kovacikova Z, Volkovova K, Kazimirova A, Barancokova M, Tatrai E, Oesterle D, Wolff T, Kyrtopoulos SA. Mutagenesis by asbestos in the lung of *λ-lacI* transgenic rats. Mutat Res. 2004; 553: 67–78.
54 Donaldson K, Brown GM, Brown DM, Bolton RE, Davis JM. Inflammation generating potential of long and short fibre amosite asbestos samples. Br J Ind Med. 1989; 46: 271–6.
55 Kamp DW, Graceffa P, Pryor WA, Weitzman SA. The role of free radicals in asbestos-induced diseases. Free Radic Biol Med. 1992; 12: 293–315.
56 Kamp DW, Weitzman SA. The molecular basis of asbestos induced lung injury. Thorax. 1999; 54: 638–52.
57 Lee YW, Klein CB, Kargacin B, Salnikow K, Kitahara J, Dowjat K, Zhitkovich A, Christie NT, Costa M. Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. Mol Cell Biol. 1995; 15: 2527–57.
58 Castranova V, Rabovsky J, Tucker JH, Miles PR. The alveolar type II epitherial cell: A multifunctional pneumocytes. Toxicol Appl Pharmacol. 1988; 93: 472–83.
59 Cairns J. Somatic stem cells and the kinetics of mutagenesis and carcinogenesis. Proc Natl Acad Sci USA. 2002; 99: 10567–70.
60 Sarasin A. An overview of the mechanisms of mutagenesis and carcinogenesis. Mutat Res. 2003; 544: 99–106.
61 Pinkerton KE, Barry BE, O'neil BJ, Raub JA, Pratt PC, Crapo JD. Morphologic changes in the lung during the lifespan of Fischer 344 rats. Am J Anatomy. 1982; 164: 155–74.
62 Jones AL, Spring-Mills E. The liver and gallbladder. In: Histology: cell and tissue biology. 5th ed. Weiss L, editor. New York: Elsevier Science Ltd; 1983. p. 707–48.
63 Cross CE, Vliet A, Louie S, Thiele JJ, Halliwell B. Oxidative stress and antioxidants at biosurfaces: plants, skin, and respiratory tract surfaces. Environ Health Perspect. 1998; 106 Suppl 5: 1241–51.
64 Noda Y, Suzuki T, Kohara A, Hasegawa A, Yotsuyanagi T, Hayashi M, Sofuni T, Yamanaka K, Okada S. *In vivo* genotoxicity evaluation of dimethylarsinic acid in Muta Mouse. Mutat Res. 2002; 513: 205–12.
65 Aoki Y, Hashimoto AH, Amanuma K, Matsumoto M, Hiyoshi K, Takano H, Masumura K, Nohmi T, Yamamoto M. Enhanced spontaneous and Benzo(*a*) pyrene-induced mutations in the lung of Nrf2-deficinet *gpt* delta mice. Cance Res. 2007; 67: 5643–8.
66 Hashimoto AH, Amanuma K, Hiyoshi K, Takano H, Masumura K, Nohmi T, Aoki Y. *In vivo* mutagenesis in

the lungs of *gpt*-delta transgenic mice treated intratracheally with 1,6-dinitropyrene. Environ Mol Mutagen. 2006; 47: 277–83.
67 Hashimoto AH, Amanuma K, Masumura K, Nohmi T, Aoki Y. *In vivo* mutagenesis caused by diesel exhaust in the tests of *gpt* delta transgenic mice. Genes Environ. 2009; 31: 1–8.