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Original Article

No carcinogenicity of poly-*trans*-[(2-carboxyethyl) germasesquioxane] (Ge-132): 26-week feeding study using rasH2 mice

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ABSTRACT — Poly-*trans*-[(2-carboxyethyl) germasesquioxane] (Ge-132) was administered at dietary levels of 0 (control), 0.3, 0.8 and 2.5% to groups of 20 male and 20 female CB6F1-Tg rasH2 (rasH2) mice for 26 weeks. As a positive control, 10 rasH2 mice of each sex received a single intraperitoneal injection of 75 mg/kg N-methyl-N-nitrosourea (MNU). There were no differences in survival between Ge-132-treated groups and the control. Loose stool, increase water intake, and dilatation of the cecum were evident in both male and female 2.5% groups; however, there were no histopathological abnormality found in the cecums of these mice. There was no significant Ge-132 treatment-related increase in the incidence of any neoplastic lesions compared to negative control. In the positive control MNU groups, malignant lymphomas and squamous cell papillomas of the forestomach frequently occurred. Thus, the experimental system employed showed clear negative results for induction of tumors due to Ge-132 administration, indicating the absence of Ge-132 carcinogenicity in mice.

Key words: Poly-*trans*-[(2-carboxyethyl) germasesquioxane], Ge-132, rasH2 mouse, 26-week carcinogenicity study, MNU

INTRODUCTION

Poly-*trans*- [(2-carboxyethyl) germasesquioxane] (Ge-132) is a water-soluble organogermanium compound which was synthesized at Asai Germanium Research Institute in 1967 (Tsutsui *et al*., 1976) and is considered to exhibit low toxicity (Sugiya *et al*., 1986a). There are several reports that Ge-132 has various physiological effects such as antitumor activity, relief of rheumatic symptoms, induction of interferon-γ production, and activation of natural killer cells and macrophages (Aso *et al*., 1989; Arimori *et al*., 1990; Aso *et al*., 1985). It has been suggested that oral intake of Ge-132 may result in adjustment of immune function and prevention of viral infection (Aso *et al*., 1985). While vacuolar degeneration of renal distal tubules and deposition of granules have been observed after oral ingestion of the inorganic compound germanium dioxide (GeO_2) in rats (Sanai *et al*., 1991a), Ge-132, which is a water-soluble organogermanium compound, shows no nephrotoxicity after oral intake (Sanai *et al*., 1991b).

In a study of Ge-132 safety in rats, no obvious toxicological changes were observed in a subacute toxicity test by oral administration, or in subacute or chronic toxicity testing by intraperitoneal administration (Sugiya *et al*., 1986a, 1986b). In addition, no obvious reproductive or developmental toxicity was observed on administration prior to or in the early stages of pregnancy, fetal organogenesis, the perinatal period, or the postnatal period in rats (Sugiya *et al*., 1986c, 1986d, 1986e). Furthermore, the safety of Ge-132 has been re-confirmed since 1990 through additional acute, subacute and chronic toxicities studies and though teratogenicity and mutagenicity (reverse mutation, chromosome aberration and rodent micronucleus assay) studies in conformity with Good Laboratory Practice (GLP) Standard (company data of Asai Germanium Research Institute Co., Ltd.). However, sufficient information to assess carcinogenesis of Ge-132 is not available.

Ge-132 is used as a raw material in health foods. Therefore, its carcinogenicity should be evaluated using two animal species, ideally conducted under Good Laboratory Practices, as advocated by IARC for evaluation of carcinogenicity in experimental animals (IARC Monographs

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on the Evaluation of Carcinogenic Risks to Humans. Preamble. 2006). The International Conference on Harmonization (ICH) in 1998 proposed that evaluation by short-term testing with transgenic mice, such as the TgH-ras2 model, could be used in conjunction with a long term study for evaluation of carcinogenicity (Federal Register, 1998). Japanese guidelines for drug testing also indicate rasH2 mice as an *in vivo* additional test system for detection of carcinogenicity (Guidelines for Toxicity Studies of Drugs, 2008). Therefore, in the current study, CByB6F1-Tg (HRAS) 2Jic (rasH2) male and female mice were used to evaluate the carcinogenicity of dietary Ge-132.

The appropriate doses of Ge-132 for use in the carcinogenicity study were determined in a preliminary study using wild type (non-Tg) mice. This was then followed by a 26-week study in which rasH2 mice were given 0, 0.3, 0.8, or 2.5% Ge-132 in their diet for 26 weeks.

MATERIALS AND METHODS

Chemicals

Poly-*trans*- [(2-carboxyethyl) germasesquioxane] (Ge-132 ($GeCH_2CH_2COOH)_2O_3$; MW, 339.41, Lot No. 012531A) was provided by Asai Germanium Research Institute Co., Ltd. (Kanagawa, Japan). Ge-132 is an acidic white powder of greater than 99% purity and was stored at room temperature avoiding light, high temperature, and humidity.

N-Methyl-N-nitrosourea (MNU, Lot No. SLBC9358V) was purchased from Sigma-Aldrich Japan Ltd. (Tokyo, Japan) and stored at 4ºC.

Preparation and analysis of feed

Ge-132 was mixed with small amounts of CRF-1 powdered basal diet (Oriental Yeast, Ltd., Tokyo, Japan) in a mortar, and added to the diet to the required level and mixed in a blender. The prepared feed was stored at room temperature and protected from light. Feed was prepared at least once every 5 weeks.

The stability, homogeneity, and concentration of Ge-132 in the prepared feed were analyzed at Asai Germanium Research Institute Co., Ltd. (Hokkaido, Japan).

Animals and husbandry

Six-week-old CByB6F1-Tg (HRAS) 2Jic (rasH2) male and female mice and CByB6F1-Tg (HRAS) 2Jic wild type (non-Tg) male and female mice were purchased from CLEA Japan Inc. (Shizuoka, Japan). The animals were acclimated for 9 days before the commencement of the experiment (exposure to dietary Ge-132 started at 7 to 8 weeks of age). Animals were housed individually in separate clear polypropylene cages (W 143 × D 293 × H 148 mm) with soft chip bedding (Hara Shouten, Co., Ltd., Aichi, Japan) in an animal facility with a temperature of 22 ± 3ºC, humidity of 55 ± 15%, ventilation frequency of at least 10 times/hour, and a 12-hr light/dark cycle (7:00 AM - 7:00 PM). Bedding was changed every week. Ichinomiya City tap water was available *ad libitum*.

This study was approved by the animal experiment committee of the DIMS Institute of Medical Science, Inc. All experimental procedures were performed in accordance with Standards for Care and Use of Laboratory Animals of the DIMS Institute of Medical Science, Inc. (May 10, 2013) based on the "Act on Welfare and Management of Animals" (Act No. 105, dated October 1973), "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice No. 88 of the Ministry of Environment, April 2006), "Basic policies for the conduct of animal experimentation in the Ministry of Health, Labor and Welfare." (Notice No. 0601005 of the Ministry of Health, Labor and Welfare, June 2006), and "Guidelines for Proper Conduct of Animal Experiments" (June 2006, Science Council of Japan).

4-week dose-finding study

The appropriate doses of Ge-132 for use in the carcinogenicity study were determined in a preliminary study using wild type (non-Tg) mice. 7 to 8-week-old male and female non-Tg mice were administered a diet containing 0, 1.25, 2.5, or 5.0% Ge-132 (5 mice of each sex per group). The general condition of the mice was observed every day; body weight was measured every week; and the two-day amount of food and water consumption was also measured every week.

Hematology testing, blood biochemical testing, gross pathological examination, and organ weight measurement were conducted at necropsy. Histopathological examination of organs and tissues throughout the body was performed for all animals.

26-week feeding carcinogenicity study

The study was conducted in compliance with principles of GLP as set forth in: Standard for conducting nonclinical laboratory studies on safety of drugs, Japanese Ministry of Health and Welfare Ordinance No. 21 (March 26, 1997), partially revised as Notification 114, Ministry of Health, Labour and Welfare (June 13, 2008).

90 male and 90 female rasH2 mice were divided into four groups of 20 animals and one group of 10 animals. Another 36 male and 36 female non-Tg mice were used

to determine plasma Ge-132 concentrations (toxicokinetics [TK] test), as detailed below. The rasH2 and wild type mice were treated identically until the end of their respective experimental periods. At the start of the experiment, body weights of the male and female rasH2 mice used for carcinogenicity testing ranged from 20.1-23.8 g and 16.9-19.6 g, respectively; and the body weights of the male and female non-Tg mice used for the TK test ranged from 23.2-25.7 g and 18.5-20.6 g, respectively.

Ge-132 was mixed with feed at concentrations of 0, 0.3, 0.8 and 2.5%, and the mixed feed was available *ad libitum* throughout the experimental period (Table 1). MNU was dissolved in citrate buffer solution (pH 4.5), and the solution was given as a single intraperitoneal administration at 75 mg/kg b.w. (10 mL/kg b.w.) to the MNU group at the start of the experiment.

The general condition of animals was observed every day. Body weight was measured at the start of the experiment and once a week until week 14, and then once every two week until the end of the experimental period. Food and water consumption were measured once a week until week 14 and then once every two weeks until the end of the experiment. Ge-132 intake during the administration period was calculated based on food consumption.

At the end of experiment, all surviving mice used for Ge-132 carcinogenicity evaluation were placed in deep isoflurane anesthesia and euthanized by exanguination from the abdominal aorta. Macroscopic pathology was made at autopsy and the findings were recorded. The weight of the brain, heart, kidney, liver, testis, ovary, and adrenal gland was measured (organ-body weight ratios were calculated based on body weight on the day of necropsy). The heart, spleen, lymph node (mandibular, mesenteric), thymus, pituitary gland, thyroid, adrenal gland, nasal cavity, trachea, lung (including bronchi), salivary gland (submandibular gland, sublingual gland), esophagus, stomach (forestomach, glandular stomach), small intestine (duodenum, jejunum, ileum), large intestine (cecum, colon, rectum), liver, gall bladder, pancreas, kidney, urinary bladder, testis, prostate, seminal vesicle, epididymis, ovary, uterus, mammary gland, vagina, brain (cerebrum, cerebellum), spinal cord, sciatic nerve, aorta, eyeball, Harderian gland, skin, bone marrow (femur, sternum), skeletal muscle, and other macroscopic lesion sites were removed, and fixed in 10% buffered formalin solution and processed for histopathological examination. Histopathological examination was performed for all organs and tissues from animal that survived to the end of the experimental period and from animals found dead or sacrificed in a moribund condition during the experimental period.

Toxicokinetics (TK) test

36 male and 36 female non-Tg mice were each divided into three groups (12 animals/group) for analysis of plasma Ge-132 concentration in mice fed 0.3, 0.8, or 2.5% Ge-132. Blood was collected from 3 TK test animals of each group at 4 weeks (at 11:00 AM and 3:00 PM) and at 26 weeks (at 11:00 AM and 3:00 PM), as shown in Table 1. Blood was collected from mice under isoflurane anesthesia using a syringe treated with EDTA-2Na. Analysis of plasma Ge-132 concentration was conducted at Hakodate Laboratory of Asai Germanium Research Institute Co., Ltd.

Statistical analysis

The hazard ratio was determined at the 5% ($P < 0.05$) or 1% ($P < 0.01$) level. Bartlett's method was used to test

Table 1. Group composition of 26-week carcinogenicity study and TK test.

Carcinogenicity		
Sex	Dose of Ge-132	No. of animals
Male	0%	20
	0.3%	20
	0.8%	20
	2.5%	20
	-a	10
Female	0%	20
	0.3%	20
	0.8%	20
	2.5%	20
	-a	10

a: MNU treatment

Toxicokinetics (TK) test				
Sex	Dose of Ge-132	Time of collected blood	No. of animals	
			4 weeks	26 weeks
Male	0.3%	11:00	3	3
		15:00	3	3
	0.8%	11:00	3	3
		15:00	3	3
	2.5%	11:00	3	3
		15:00	3	3
Female	0.3%	11:00	3	3
		15:00	3	3
	0.8%	11:00	3	3
		15:00	3	3
	2.5%	11:00	3	3
		15:00	3	3

homoscedasticity of the means of weight, food consumption, water consumption and organ weight of the Ge-132 fed and control groups; parametric Dunnett's test (two-sided) was used when the variance was homogeneous and nonparametric Steel's test (two-sided) was used when the variance was not homogeneous. An *F*-test was used to assess the means of weight, food consumption, water consumption and organ weight of the MNU and control groups; Student's *t*-test (two-sided) was used when the variance was homogeneous, and Welch's test (two-sided) was used when the variance was not homogeneous. Fisher's exact test (one-sided) was used for testing of differences in frequency data on macroscopic pathological examination and histopathological examination, and Wilcoxon's test (two-sided) was used for advanced lesions.

RESULTS

4-week dose-finding study

No dead mice were observed in either the control groups or the Ge-132 fed groups. No significance difference in food consumption was observed between the Ge-132 fed groups and the controls. There was, however, a tendency, sometimes significant, for increased water consumption in both male and female 5.0% and 2.5% Ge-132 groups. There was a decrease in the body weight of both male and female 5.0% Ge-132 mice throughout the administration period, and in some animals, this decrease was significant.

Loose stools were observed in both sexes of the 5.0% Ge-132 group. Some mice in this group showed diarrheal after day 21. Erosion around the anus was observed in both male and female 5.0% Ge-132 mice. Loose stools were also observed after day 21 in male mice and after day 24 in female mice in the 2.5% Ge-132 groups. However, neither diarrheal or erosion around the anus were observed in the 2.5% Ge-132 mice of both sexes.

Dilatation of the cecum was macroscopically observed in all animals in the 5.0% Ge-132 groups, suggesting association with the loose stools and diarrheal. A significant increase in the relative weight of the adrenal gland was observed in males in the 5.0% Ge-132 group. However, no histopathological changes were observed in the adrenal. Inflammation in the rectum close to the anus was observed in males and females of 5.0% Ge-132 groups. In the thymus, an increase in apoptotic lymphocytes was found in males and females of the 5.0% Ge-132 groups. No histopathological abnormal findings were observed in any of other organs or tissues examined in any of the Ge-132 fed groups.

In the hematology test, significant increases in reticulocyte count and neutrophil ratio and a significant decrease in lymphocyte ratio were observed in male and female mice in the 5.0% Ge-132 groups. In addition, significant decreases in the mean corpuscular volume and mean corpuscular hemoglobin, and a significant increase in the platelet count were observed in 5.0% Ge-132 females. No abnormalities were observed in the 2.5% and less Ge-132 group.

The loose stools and diarrheal in the 5.0% Ge-132 group suggest that the 5.0% dose may have caused deterioration of nutritional status. Therefore, the highest dose for carcinogenicity evaluation was set to 2.5%.

26-week feeding carcinogenicity study

The survival curves for each group are shown in Fig. 1. In males, 2 animals died at the end of the experimental period (week 27) in the 2.5% Ge-132 group, while one animal died during week 23 in the 0.3% Ge-132 group. In females, one animal died during week 8 another during week 27 in the 2.5% Ge-132 group. Survival rates at the end of the experiment in the 2.5%, 0.8% and 0.3% Ge-132 groups and the control group were 90%, 100%, 95% and 100% in males, respectively (Table 2), and 90%, 100%, 100%, and 100%, in females, respectively (Table 2). In the MNU group, one male died during week 11 and one female in week 14; animals subsequently died at rates of one or two animals every few weeks. Survival rate at the end of the experiment were 20% in males and 10% in females (Table 2).

Necropsy examination of dead mice in the Ge-132 groups disclosed a thoracic hemangiosarcoma in the 0.3% Ge-132 male and a subcutaneous hemangiosarcoma in one of the 2.5% Ge-132 males, and a malignant lymphoma was observed in one female in the 2.5% Ge-132 group. One case of bleeding in the thoracic cavity was observed in one of the 2.5% Ge-132 males and one case of pleural effusion was observed in one of the 2.5% Ge-132 female; however, these mice accompanied with no neoplastic lesion.

There were sporadic increases in food consumption by the Ge-132 fed groups compared with the control; however, no clear trend was observed. There was a clear decrease in food consumption throughout the administration period in both the male and female MNU groups. The average intake of Ge-132 per animal in the 2.5%, 0.8%, and 0.3% Ge-132 groups were 3,837 mg/kg/day, 1,199 mg/kg/day, and 436 mg/kg/day in males, respectively; and 5,442 mg/kg/day, 1,643 mg/kg/day, and 591 mg/kg/day in females, respectively (Table 2).

A significant increase in water consumption was observed in males and females in the 2.5% Ge-132 groups

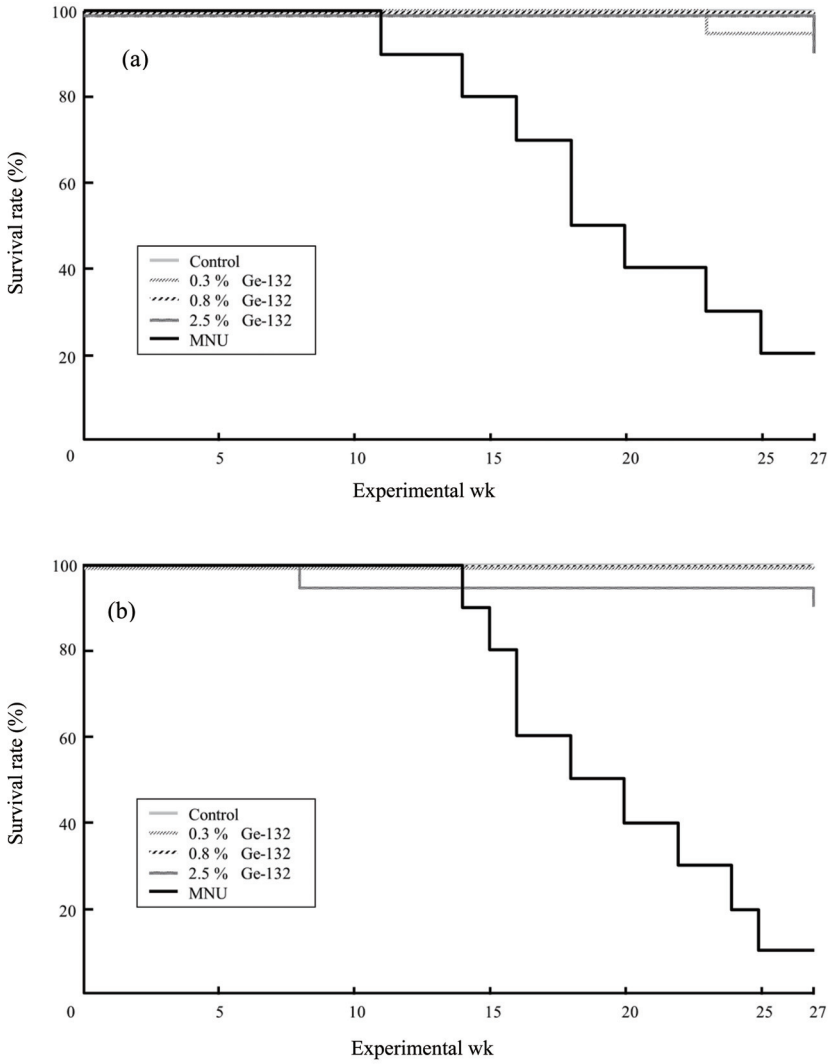

Fig. 1. Survival rates of (a) male and (b) female rasH2 mice fed a diet containing Ge-132 for 26 weeks, and received an intraperitoneal injection of MNU.

Table 2. Data for Ge-132 intake and final survival rate in rasH2 mice fed a diet containing Ge-132 for 26 weeks, and received an intraperitoneal injection of MNU.

Sex	Dose (%)	Ge-132 intake		Final no. of survivals (%)
		Total intake (mg/kg)	Average (mg/kg/day)	
Male	Control	0	0	20 (100)
	0.3% Ge-132	79417	436	19 (95)
	0.8% Ge-132	218239	1199	20 (100)
	2.5% Ge-132	698362	3837	18 (90)
	MNU	-	-	2 (20)
Female	Control	0	0	20 (100)
	0.3% Ge-132	107539	591	20 (100)
	0.8% Ge-132	298974	1643	20 (100)
	2.5% Ge-132	990516	5442	18 (90)
	MNU	-	-	1 (10)

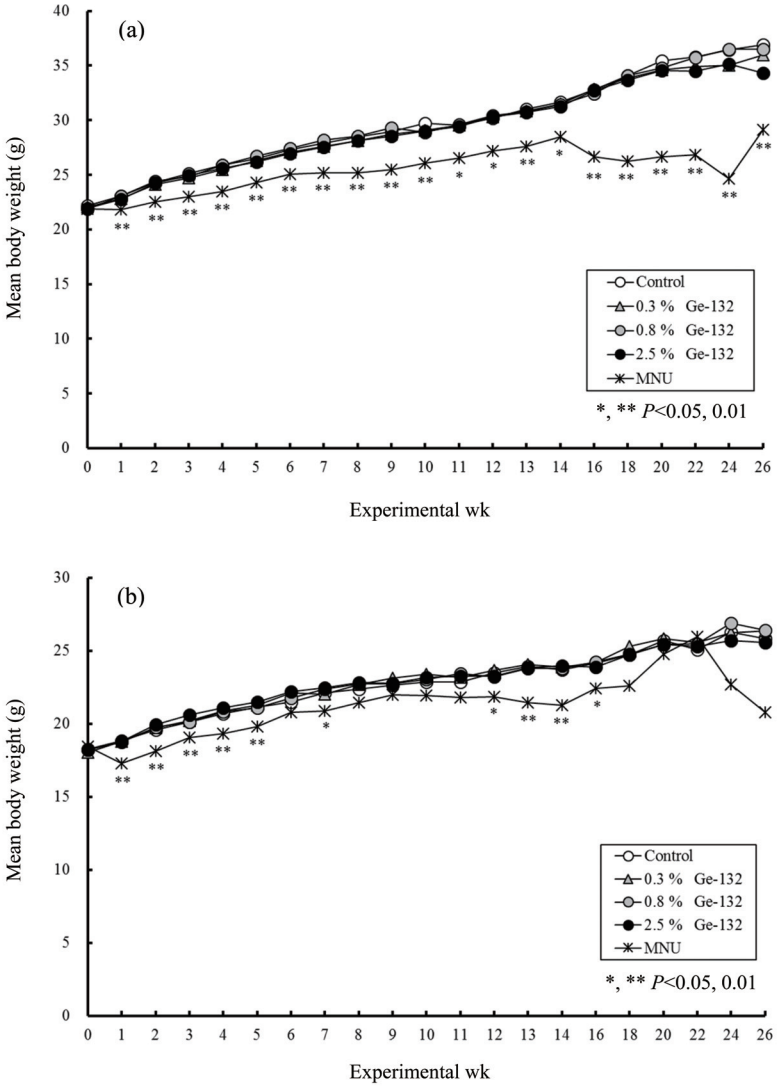

Fig. 2. Growth curves of (a) male and (b) female rasH2 mice fed a diet for 26 weeks, and received an intraperitoneal injection of MNU.

throughout the administration period. An increase in water consumption, sometimes significant, was observed in males and females in the 0.8% Ge-132 groups. There was no difference in water consumption in either the males or females in the 0.3% Ge-132 group. Males and females in the MNU group exhibited a clear decrease in water consumption.

No significant differences in body weight were observed between the Ge-132 fed groups and the control groups. However, a significant decrease of body weight was observed from week 1 until the end of the experiment in MNU males. In MNU females, a tendency of decreased body weight was seen almost continuously from week 1 until the end of the experiment, with the decreases observed from week 1 to week 5, week 7 and from week 12 to week 16 being significant (Fig. 2).

Loose stools were observed from week 4 until death or until the end of the experiment in all males and females in the 2.5% Ge-132 group. Loose stools were not observed in any animals in the 0.3 or 0.8% Ge-132 groups. Loose stools were also not observed in any animals in the MNU groups. Neither diarrheal nor erosion around the anus were observed in any of the groups.

Dilatation of the cecum was observed in 7 males and 10 females in the 2.5% Ge-132 group at terminal necropsy (including dead animals) and these increases were sig-

nificant. No histopathological abnormities were observed in the dilated cecums. Dilatation of the cecum was not observed in any animals in the 0.8% Ge-132, 0.3% Ge-132, or control groups. Dilation of the cecum was also not observed in any animals in the MNU groups.

Table 3 lists organ weights and organ-body weight ratios (relative weights) in the animals that survived to the end of the experiment. In the Ge-132 groups, a significant increase in the absolute weight of the kidney in males of the 0.8% Ge-132 group and a significant decrease of the relative weight of the kidney in females in the 0.8% Ge-132 group were observed; however, since there was no correlation of absolute or relative kidney weight with dose, this was concluded to be incidental variation. There were no significant differences in the absolute or relative weight of any other organ in any of the Ge-132 fed groups compared with the control groups.

In the MNU group, only two males survived to the end of the experiment. These males had decrease in the absolute weights of the brain, adrenal gland and testis, and increases in the relative weight of the brain, liver, and kidney. As noted above, these males also had a decrease in body weight. Only one female mouse administered MNU survived to the end of the experiment. The absolute and relative organ weights of this mouse are shown in Table 3, but no analysis was attempted.

The incidence of neoplastic lesions, including those found in mice that died before the end of the experimental period, are listed in Table 4. Hemangiomas of the bone marrow, bronchiolo-alveolar adenomas and carcinomas of the lung, squamous cell papillomas of the forestomach, hepatocellular adenomas of the liver, hemangiosarcomas of the testis, hemangiomas of the skeletal muscle, skin squamous cell papillomas and hemangiosarcoma, and thoracic hemangiosarcomas were found in males in each of the Ge-132 fed groups. No significant increases in the incidence of any neoplastic lesion in any organ or tissue were found in the male Ge-132 fed groups compared with the control groups.

Hemangiosarcomas of the spleen, thymomas, adenomas of the nasal cavity, bronchiolo-alveolar adenomas of the lung, squamous cell papillomas of the forestomach, granulosa cell tumors of the ovary, hemangiomas and endometrial stromal sarcomas of the uterus, skin squamous cell papillomas, and systemic malignant lymphomas were found in females in each Ge-132 fed groups. Granulosa cell tumors in the ovary and endometrial stromal sarcomas in the uterus ware observed in one female in the 0.3% Ge-132 group and in one female in the 0.8% Ge-132 group (frequency, 5% for each group). No significant increases in the incidence of any neoplastic lesion in any organ or tissue were found in the female Ge-132 fed groups compared with the control groups.

In the MNU groups, bronchiolo-alveolar adenomas of the lung, squamous cell carcinomas of the salivary gland, squamous cell papillomas and carcinomas of the forestomach, hemangiosarcomas of the jejunum, adenomas of the ileum, adenomas of the kidney, skin squamous cell papillomas, malignant fibrous histiocytomas, pinna squamous cell carcinomas, and systemic malignant lymphomas and myeloid leukemias were observed in males; and hemangiomas of the mesenteric lymph node, hemangiomas and hemangiosarcomas of the spleen, adenomas of the nasal cavity, bronchiolo-alveolar adenomas of the lung, squamous cell papillomas of the tongue, squamous cell carcinomas of the salivary gland, squamous cell papillomas of the esophagus, squamous cell papillomas and carcinomas of the forestomach, adenocarcinomas of the jejunum, adenocarcinomas of the ileum, adenocarcinomas of the mammary gland, hemangiomas of the ovary, adenomas and endometrial stromal polyps of the uterus, hemangiomas of the vagina, skin squamous cell papillomas, adenomas of the Harderian gland, systemic malignant lymphomas, and transitional cell papillomas of the urethra were observed in females. Squamous cell papillomas of the forestomach in both males and females and skin squamous cell papillomas in males exhibited significant increases compared with the control group.

The incidence of non-neoplastic lesions in the Ge-132 and MNU administration groups, including those found in mice that died before the end of the experimental period, are listed in Table 5. A pigmentation of the spleen was observed in 4 males in the control group, 2 in the 0.3% group, 3 in the 0.8% group, and 0 in the 2.5% Ge-132 group (Table 5). The decrease of pigmentation was statistically significant in the 2.5% group compared with the control. Pigmentations were also observed in the spleens of the three female Ge-132 groups. Squamous cell hyperplasia of the forestomach was observed in the male 0.8% Ge-132 group and the female 0.3% and 0.8% Ge-132 groups; the incidence of squamous cell hyperplasia was not significant. No histopathological changes related to Ge-132 were observed in kidneys of any of the Ge-132 groups (data not shown). No appreciable non-neoplastic lesions were observed in any other organs or tissues examined (see Methods for a complete list).

Pigmentations were also observed in the spleens of animals in the male and female MNU groups, but were not significant (Table 5). Non-neoplastic lesions present in the MNU administered groups were significant in bone marrow, lung, stomach, liver, ovary, eye, and brain. There was minimal to slight enhancement of hematopoi-

Table 3. Absolute organ and relative organ weight of rasH2 mice fed a diet containing Ge-132 for 26 weeks, and received an intraperitoneal injection of MNU.

Sex	Dose	Final body weight	Brain	Heart	Liver	Kidneys	Adrenals	Testes/Ovaries
	Organ weight (g, Mean ± S.D.)							
Male	Control	35.89 ± 3.28	0.4784 ± 0.0111	0.1831 ± 0.0168	1.6502 ± 0.2366	0.5681 ± 0.0329	0.00316 ± 0.00094	0.2578 ± 0.0209
	0.3% Ge-132	36.43 ± 3.77	0.4720 ± 0.0091	0.1826 ± 0.0166	1.6719 ± 0.1181	0.5699 ± 0.0349	0.00267 ± 0.00106	0.2591 ± 0.0193
	0.8% Ge-132	37.07 ± 3.72	0.4770 ± 0.0135	0.1903 ± 0.0202	1.7023 ± 0.1450	0.6051 ± 0.0503**	0.00279 ± 0.00065	0.2614 ± 0.0161
	2.5% Ge-132	35.97 ± 4.69	0.4727 ± 0.0176	0.1866 ± 0.0163	1.6293 ± 0.1470	0.5859 ± 0.0614	0.00258 ± 0.00083	0.2647 ± 0.0198
	MNU	28.90 ± 2.40**	0.4560 ± 0.0071*	0.1615 ± 0.0064	1.6940 ± 0.0368	0.5265 ± 0.0233	0.00150 ± 0.00071*	0.2235 ± 0.0191*
Female	Control	26.13 ± 2.93	0.5061 ± 0.0169	0.1431 ± 0.0135	1.4293 ± 0.1855	0.4298 ± 0.0370	0.00632 ± 0.00103	0.01784 ± 0.00202
	0.3% Ge-132	27.14 ± 3.01	0.5054 ± 0.0153	0.1423 ± 0.0074	1.5095 ± 0.1919	0.4214 ± 0.0270	0.00595 ± 0.00123	0.01753 ± 0.00225
	0.8% Ge-132	27.41 ± 3.06	0.5065 ± 0.0122	0.1406 ± 0.0071	1.4802 ± 0.1487	0.4199 ± 0.0255	0.00646 ± 0.00148	0.01932 ± 0.00756
	2.5% Ge-132	26.16 ± 3.16	0.4967 ± 0.0140	0.1358 ± 0.0100	1.3791 ± 0.1514	0.4211 ± 0.0330	0.00620 ± 0.00111	0.01829 ± 0.00197
	MNU	21.50 ± -[a]	0.4420 ± -[a]	0.1850 ± -[a]	1.6660 ± -[a]	0.4260 ± -[a]	0.00560 ± -[a]	0.00800 ± -[a]
	Relative organ weight (g/100g BW, Mean ± S.D.)							
Male	Control		1.3424 ± 0.1127	0.5128 ± 0.0542	4.5856 ± 0.3068	1.5904 ± 0.1114	0.00889 ± 0.00283	0.7243 ± 0.0864
	0.3% Ge-132		1.3085 ± 0.1347	0.5036 ± 0.0426	4.6131 ± 0.3288	1.5761 ± 0.1468	0.00735 ± 0.00279	0.7163 ± 0.0720
	0.8% Ge-132		1.2989 ± 0.1332	0.5144 ± 0.0352	4.6043 ± 0.2376	1.6378 ± 0.1022	0.00757 ± 0.00181	0.7100 ± 0.0667
	2.5% Ge-132		1.3326 ± 0.1618	0.5237 ± 0.0531	4.5674 ± 0.4167	1.6414 ± 0.1623	0.00731 ± 0.00249	0.7449 ± 0.0924
	MNU		1.5845 ± 0.1563*	0.5595 ± 0.0247	5.8875 ± 0.6173**	1.8250 ± 0.0707**	0.00535 ± 0.00290	0.7785 ± 0.1308
Female	Control		1.9545 ± 0.1729	0.5515 ± 0.0589	5.4632 ± 0.2450	1.6550 ± 0.1481	0.02445 ± 0.00456	0.06932 ± 0.01241
	0.3% Ge-132		1.8831 ± 0.2067	0.5293 ± 0.0526	5.5622 ± 0.3307	1.5636 ± 0.1242	0.02212 ± 0.00465	0.06531 ± 0.01042
	0.8% Ge-132		1.8668 ± 0.1862	0.5172 ± 0.0456	5.4170 ± 0.3687	1.5429 ± 0.1286*	0.02384 ± 0.00616	0.07207 ± 0.03281
	2.5% Ge-132		1.9172 ± 0.1662	0.5224 ± 0.0389	5.2880 ± 0.3841	1.6179 ± 0.1029	0.02382 ± 0.00399	0.07054 ± 0.00917
	MNU		2.0560 ± -[a]	0.8600 ± -[a]	7.7490 ± -[a]	1.9810 ± -[a]	0.02600 ± -[a]	0.03720 ± -[a]

[a] : Statistical analysis was not performed since only one animal survived.
*, ** : Significantly different from control group at $P < 0.05$, 0.01, respectively.

Table 4. Incidence of neoplastic lesions in organs of rasH2 mice fed a diet containing Ge-132 for 26 weeks, and received an intraperitoneal injection of MNU.

Organ and findings	Sex	Male					Female				
		Control	Ge-132			MNU	Control	Ge-132			MNU
	Dose		0.3%	0.8%	2.5%			0.3%	0.8%	2.5%	
	No. of animals/group	20	20	20	20	10	20	20	20	20	10
Organs	Findings										
Mesenteric lymph node	Hemangioma	0	0	0	0	0	0	0	0	0	1
Spleen	Hemangioma	0	0	0	0	0	0	0	0	0	1
	Hemangiosarcoma	1	0	0	0	0	1	2	0	2	1
Bone marrow	Hemangioma	0	1	0	0	0	0	0	0	0	0
Thymus	Thymoma	0	0	0	0	0	0	0	1	0	0
Nasal cavity	Adenoma	0	0	0	0	0	0	0	1	0	1
Lung/bronchial	Adenoma, bronchiolo-alveolar	0	0	4	0	1	0	1	3	0	1
	Carcinoma, bronchiolo-alveolar	0	1	0	0	0	0	0	0	0	0
Tongue	Papilloma, squamous cell										1[1][a]
Salivary gland	Carcinoma, squamous cell	0	0	0	0	1	0	0	0	0	1
Esophagus	Papilloma, squamous cell	0	0	0	0	0	0	0	0	0	1
Stomach	Papilloma, squamous cell	0	1	0	0	9**	0	1	0	2	8**
	Carcinoma, squamous cell	0	0	0	0	1	0	0	0	0	2
Jejunum	Adenocarcinoma	0	0	0	0	0	0	0	0	0	2
	Hemangiosarcoma	0	0	0	0	1	0	0	0	0	0
Ileum	Adenoma	0	0	0	0	1	0	0	0	0	0
	Adenocarcinoma	0	0	0	0	0	0	0	0	0	1
Liver	Adenoma, hepatocellular	1	0	1	1	0	0	0	0	0	0
Kidney	Adenoma	0	0	0	0	1	0	0	0	0	0
Testis	Hemangiosarcoma	0	1	0	0	0					
Mammary gland	Adenocarcinoma	0	0	0	0	0	0	0	0	0	1

[a] : Numbers in square bracket are for animals examined microscopically.
** : Significantly different from control group at $P < 0.01$.

Table 4. (Continued)

Organ and findings	Sex	Male					Female				
		Control	Ge-132			MNU	Control	Ge-132			MNU
	Dose		0.3%	0.8%	2.5%			0.3%	0.8%	2.5%	
	No. of animals/group	20	20	20	20	10	20	20	20	20	10
Organs	Findings										
Ovary	Granulosa cell tumor						0	1	0	0	0
	Hemangioma						0	0	0	0	1
Uterus	Adenoma						0	0	0	0	1
	Hemangioma						1	1	0	1	0
	Polyp, endometrial stromal						0	0	0	0	1
	Sarcoma, endometrial stromal						0	0	1	0	0
Vagina	Hemangioma						0	0	0	0	1
Skeletal muscle	Hemangioma	0	1	0	0	0	0	0	0	0	0
Skin/subcutis	Papilloma, squamous cell	0	0	0	1	5**	1	1	0	1	2
	Hemangiosarcoma	0	0	0	1	0	0	0	0	0	0
Harderian gland	Adenoma	0	0	0	0	0	0	0	0	0	1
Thoracic cavity	Hemangiosarcoma		1[1][a]			0[1][a]					0[4][a]
Abdominal cavity	Malignant fibrous histiocytoma					1[1][a]					0[1][a]
Pinna	Carcinoma, squamous cell					1[1][a]					
All sites	Malignant lymphoma	0[1][a]				6[7][a]				1[1][a]	8[8][a]
	Myeloid leukemia	1[1][a]				1[7][a]				0[1][a]	0[8][a]
Urethra	Papilloma, transitional cell										1[1][a]

[a] : Numbers in square bracket are for animals examined microscopically.
** : Significantly different from control group at $P < 0.01$.

Table 5. Incidence of representative non-neoplastic lesions in organs of rasH2 mice fed a diet containing Ge-132 for 26 weeks, and received an intraperitoneal injection of MNU.

Organ and findings	Sex	Male					Female				
		Control	Ge-132			MNU	Control	Ge-132			MNU
	Dose		0.3%	0.8%	2.5%			0.3%	0.8%	2.5%	
	No. of animals/group	20	20	20	20	10	20	20	20	20	10
Organs	Findings										
Spleen	Pigmentation/(1)[a]	1	0	2	0 *	0	1	3	3	3	2
	Pigmentation/(2)[a]	3	2	1	0	1	2	2	2	1	0
Bone marrow	Hematopoiesis/(1)[a]	0	0	0	0	1 *	0	0	0	0	0
	Hematopoiesis/(2)[a]	0	0	0	0	1	0	0	0	0	1
	Hypoplasia/(2)[a]	0	0	0	0	0 *	0	0	0	0	1 *
	Hypoplasia/(3)[a]	0	0	0	0	1	0	0	0	0	1
	Hypoplasia/(4)[a]	0	0	0	0	1	0	0	0	0	0
Lung/bronchial	Hemorrhage/(2)[a]	0	0	0	0	1 *	0	0	0	0	0
	Hemorrhage/(3)[a]	0	0	0	0	1	0	0	0	0	0
Stomach	Hyperplasia, squamous cell	0	0	2	0	7**	0	2	1	0	4**
Liver	Extramedullary hematopoiesis/(1)[a]	0	0	0	0	0	0	0	0	0	1 *
	Extramedullary hematopoiesis/(2)[a]	0	0	0	0	0	0	0	0	0	1
Ovary	Atrophy/(1)[a]						0	0	0	0	3*
Eye	Atrophy of retina/(1)[a]	0	0	0	0	6**	0	0	0	0	6**
Brain	Hemorrhage/(1)[a]	0	0	0	0	2*	0	0	0	0	0

[a] : Numbers in parenthesis indicate the grades of lesion : (1) Minimal (2) Slight (3) Moderate (4) Marked (5) Severe
*, ** : Significantly different from control group at $P < 0.05$, 0.01, respectively.

esis in the bone marrow in males and slight to marked hypoplasia of the bone marrow in males and females. Slight hemorrhage was observed in the lungs of 1 male and moderate hemorrhage was observed in the lungs of another male. Squamous cell hyperplasia of the forestomach in both males and females was significant. Minimal extramedullary hematopoiesis in the liver of 1 female and slight extramedullary hematopoiesis in the liver of another female was observed. Three females had minimal atrophy of the ovary. Minimal atrophy of the retina in the eye in both males and females and minimal hemorrhage in the brain in males was observed.

TK test; analysis of Ge-132 in the plasma

Plasma Ge-132 concentration was correlated with dosage after 4 weeks administration in both males and females, and there was a tendency for concentrations at 11:00 AM to be higher than those at 3:00 PM. A similar tendency was also observed after 26 weeks administration. Plasma Ge-132 concentrations were in the same range as those after 4 weeks administration (Table 6).

Analysis of Ge-132 in prepared feed

Ge-132 concentration in the prepared feeds containing 0.3% and 2.5% Ge-132 after 5 weeks were 103.5% and 99.1% of that at the start of storage, respectively. Ge-132 concentration in the prepared feeds was stable for 5 weeks.

The concentrations of first and last preparations were 99.7% and 100.7% for the 0.3% Ge-132 feed, 92.4% and 97.1% for the 0.8% Ge-132 feed, and 98.0% and 92.4% for the 2.5% Ge-132 feed.

In homogeneity tests of Ge-132 in the prepared feeds, the ratios (homogeneity) of concentrations in each upper, middle and lower layers to the mean value of the three layers were calculated. The homogeneity was 97.7-103.3% for 0.3% Ge-132 feed, 98.9-101.6% for 0.8% Ge-132 feed, and 99.6-100.8% for 2.5% Ge-132 feed. This confirmed that homogeneity of the feeds.

DISCUSSION

Overt toxicity was not observed in rats in a subacute toxicity test following oral administration of Ge-132 or in subacute and chronic toxicity testing by intraperitoneal administration of Ge-132 (Sugiya *et al*., 1986a, 1986b). However, Ge-132 is used in health foods, and therefore, information regarding its carcinogenicity is required. In this study, the carcinogenicity of ingested Ge-132 was examined using the short term rasH2 mouse model. We found that Ge-132 did not exert carcinogenic effects in the rasH2 mouse.

A four-week preliminary study using non-Tg mice was performed to ascertain the appropriate doses of Ge-132 to use in the carcinogenicity study. Loose stools, diarrheal, and erosion around the anus was observed in both sexes in the 5.0% Ge-132 group and loose stools but not diarrheal or erosion around the anus was observed in both sexes in the 2.5% Ge-132 group. Therefore, 2.5% Ge-132 was used as the highest dose in the 26-week carcinogenicity test.

Loose stools were continuously observed from around week 4 in all male and female rasH2 mice in the 2.5% Ge-132 group, but similarly to the preliminary study, diarrheal and erosion around the anus were not observed. Water consumption was increased in connection with the loose stools. Dilatation of the cecum was observed in seven males and ten females in the 2.5% Ge-132 group; however, no abnormal histopathological findings were observed in the cecum. Neither loose stools nor dilatation of the cecum were observed in males or females of the 0.8% or 0.3% Ge-132 groups. Loose stools and dilatation of the cecum showed a correlation with dosage, and they were also observed in a previous carcinogenicity study of Ge-132 in rats (Iwadate *et al*., 2017, in preparation). These results suggest the possible involvement of Ge-132 in these conditions. It is considered that such changes are caused by osmotic pressure changes in the cecum due to administration of indigestible polysaccharides, modifi-

Table 6. Plasma Ge-132 concentration in rasH2 mice fed a diet containing Ge-132 for 4 and 26 weeks.

Sex	Dose	Plasma concentration (μg/mL)							
		4 weeks				26 weeks			
		11:00		15:00		11:00		15:00	
		No. of animals	Mean concentration	No. of animals	Mean concentration	No. of animals	Mean concentration	No. of animals	Mean concentration
Male	0.3% Ge-132	3	2.27	3	1.11	3	1.64	3	1.40
	0.8% Ge-132	3	6.04	3	3.31	3	4.00	3	3.03
	2.5% Ge-132	3	12.9	3	8.63	3	10.6	3	10.5
Female	0.3% Ge-132	3	1.81	3	1.36	3	1.53	3	1.26
	0.8% Ge-132	3	5.87	3	3.00	3	4.07	3	2.43
	2.5% Ge-132	3	14.9	3	12.4	3	13.0	3	11.6

cations by enteric bacteria etc. (Newberne *et al*., 1988); therefore, it was considered that physiological property of Ge-132 might be cause these changes observed in the present study, including loose stool and diarrhea. However, it was assumed that these changes were related to lack of carcinogenicity in rasH2 mice, because no cecal lesions were observed on histopathological examination.

There is a report of renal impairment caused by intake of inorganic germanium dioxide (Sanai *et al*., 1991a); however, in this study histopathological examination of the kidney indicated no findings related to renal impairment, and no neoplastic lesions were found. These results indicated that Ge-132, which is an organogermanium compound, has no toxicological effects in the kidney.

Hemangioma and hemangiosarcoma of various tissue types including the spleen, bronchiolo-alveolar adenoma and carcinomas of the lung, squamous cell papilloma of the forestomach, hepatocellular adenoma of the liver, skin squamous cell papilloma, thymoma, adenocarcinoma of the nasal cavity, malignant lymphoma, and others are known as spontaneous tumors occurring in rasH2 in mice (Takaoka *et al*., 2003; Nambiar *et al*., 2012; Mitsumori *et al*., 1998; Mitsumori, 2003; Morton *et al*., 2002; Paranjpe *et al*., 2013; Usui *et al*., 2001). These spontaneous tumors were sporadically observed in males and females in the Ge-132 groups in this study, and no statistical differences with the control group were observed. Moreover, granulosa cell tumors of the ovary and endometrial stromal sarcoma of the uterus, which have not been reported to occur spontaneously, were observed in one of twenty females in the 0.3% Ge-132 group and in one of twenty females in the 0.8% Ge-132 group; however, this type of neoplastic lesion did not develop in the 2.5% Ge-132 group, and thus, induction of this neoplasm did not correlated with dosage. Therefore, in this study, ingestion of Ge-132 at a dose of 2.5% (3,837 mg/kg/day in males, and 5,442 mg/kg/day in females) was not carcinogenic to the rasH2 mouse.

In the MNU group, which was used as a positive control, 80% of the males and 90% of the females died due to presence of neoplastic lesions. In particular, malignant lymphomas and forestomach papillomas were observed at high frequencies. These are commonly observed tumors induced by MNU (Takaoka *et al*., 2003; Nambiar *et al*., 2012; Mitsumori *et al*., 1998; Mitsumori, 2003; Usui *et al*., 2001).

The upper limit of daily intake of Ge-132 as a health food in humans is assumed to be in the region of 800 mg/day, which is half the 1,500 mg/day dose that induced no side effects, either detected subjectively or by clinical tests, in a previous long-term administration trial to humans (Arimori and Yoshida, 1982). In the present study, the maximum amount of Ge-132 ingested was 3,837 mg/kg/day in males and 5,442 mg/kg/day in females, which is approximately 240 to 340-fold higher than the maximum daily recommended amount.

In conclusion, negative findings regarding Ge-132-mediated tumor induction were obtained in this study. Our results indicate that ingestion Ge-132 was not carcinogenic to any tissues or organs in the rasH2 mouse under the conditions used in this study, indicating the absence of Ge-132 carcinogenicity in mice.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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