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In Vivo Genotoxicity of *Ginkgo Biloba* Extract in *gpt* Delta Mice and Constitutive Androstane Receptor Knockout Mice

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The National Toxicology Program study of *Ginkgo biloba* extract (GBE), a herbal supplement, reported concerns regarding genotoxicity and clear evidence of hepatocarcinogenicity and liver hypertrophy in mice. To clarify the genotoxicity of GBE *in vivo*, we performed reporter gene mutation assay using *gpt* delta mice. We also used a combined liver comet assay and bone marrow micronucleus assay using C3H-derived constitutive androstane receptor knockout (CARKO) and wild-type mice. No remarkable increases in *gpt* or Spi$^-$ mutation frequencies were observed in DNA extracted from the livers of *gpt* delta mice that had been exposed to GBE up to 2000 mg/kg bw/day. In the comet and micronucleus assays, no statistically significant increases in positive cells were observed at doses up to 2000 mg/kg bw/day of GBE in either mouse genotype. The present study provides clear evidence that GBE is not genotoxic *in vivo*. Our results indicate that GBE-induced hepatocarcinogenesis in mice occurs through a non-genotoxic mode of action.

Key words: **genotoxicity; *Ginkgo biloba* extract (GBE); comet assay; micronucleus assay; gene mutation assay; constitutive androstane receptor (CAR).**

Ginkgo biloba extract (GBE) has antioxidant and neuroprotective properties, and it has been used for a variety of medicinal purposes (Chan *et al.*, 2007). In Japan and the United States, GBE has also been used as a herbal supplement. Although GBE has been widely used, there have been insufficient studies regarding its potential for genotoxicity and carcinogenicity. Recently, a National Toxicology Program (NTP) study reported the results of two genotoxic assays *in vitro* and *in vivo*. In a bacterial gene mutation assay (Ames assay), GBE was mutagenic in *Salmonella typhimurium* strains, TA98 and TA100, and the *Escherichia coli* strain, Wp2 *uvr*A/pKM101, with and without S9 mix (NTP, 2013). In a peripheral blood micronucleus assay in male and female mice administered GBE for 13 weeks, GBE was negative in males but judged to be equivocal in females because of positive results in a significant trend test (NTP, 2013). Based on these results, GBE was suspected to have genotoxic potential. Furthermore, the NTP 2-year study of GBE reported significant increases in the incidence of hepatocellular adenoma/carcinoma and hepatoblastomas in B6C3F1 mice of both sexes at the lowest dose tested (200 mg/kg bw/day) and showed dose dependency, indicating clear evidence of hepatocarcinogenicity in mice (NTP, 2013). Consequently, GBE is suspected to be a hepatocarcinogen with genotoxicity. Theoretically, genotoxic carcinogens have no dose threshold for their carcinogenic potential. Therefore, clarification of *in vivo* genotoxicity of GBE is a critical issue for human cancer risk assessment.

Other treatment-related changes based on short- and long-term studies in the NTP study showed that GBE increased liver weights and induced centrilobular hepatocyte hypertrophy in rats and mice (NTP, 2013). The pattern of GBE-mediated liver hypertrophy indicated that the mode of action might involve the constitutive androstane receptor (CAR). CAR is a nuclear receptor that plays critical roles in regulating enzymes involved in xenobiotic metabolism and has a key role in liver tumor formation in rodents (Ueda *et al.*, 2002; Wei *et al.*, 2000; Yamamoto *et al.*, 2004). Therefore, we investigated CAR involvement in liver carcinogenesis by GBE using C3H-derived CAR knockout (CARKO) mice. However, it is unknown whether the sensitivity to GBE genotoxicity differs between CARKO and C3H (wild-type) mice. Hence, using both wild-type mice and CARKO mice should provide useful information regarding the genotoxicity of GBE.

In the present study, we investigated the *in vivo* genotoxicity of GBE using several methods. To clarify the genomic mutation potential in the liver, we performed gene mutation assays using *gpt* delta mice treated with GBE for 90 days. To evaluate the potential induction of DNA damage and chromosomal aberration, we carried out a combined liver comet assay and bone marrow micronucleus assay using wild-type and CARKO mice administrated GBE for 3 days. We used GBE of the same lot used in the NTP 2-year study. A dose level of 2000 mg/kg/day was se-

lected as the maximal dose because it induced liver tumors in the 2-year study of mice.

MATERIALS AND METHODS

Chemicals and Dose Volumes

The GBE used in the present study was from the same lot used for the NTP 2-year study. It was a gift provided by the National Institute of Environmental Health Sciences (North Carolina, USA). This GBE was produced by Shanghai Xing Ling Science and Technology Pharmaceutical Company, Ltd (Shanghai, China). A positive control compound used in the comet assay and the micronucleus assay, ethyl methanesulfonate (EMS; CAS 62-50-10), was purchased from Invitrogen. EMS was diluted in saline at the day of administration. Test agents were dosed at a volume of 10 ml/kg body weight, based on body weight at the day of the administration.

Animals

Male B6C3F1 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome were raised by mating C57BL/6 *gpt* delta and nontransgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). C3H/HeNCrl background CARKO mice were generously provided by Dr M. Negishi, National Institute of Environmental Health Sciences, NC (Kodama *et al.* 2004; Yamamoto *et al.* 2004). C3H/HeNCrlCrlj mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and used as wild-type mice in the experiment 2 study. All protocols were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, and all studies followed the guidelines for the use of laboratory animals of the National Institute of Health Sciences.

Experiment 1: 90-Day-Repeated Dose Test Using B6C3F1 gpt Delta Mice

Study design. Six-week-old male B6C3F1 mice were divided into four groups consisting of 10 mice per group. GBE was administered daily for 13 weeks at doses of 0 (corn oil alone), 20, 200, and 2000 mg/kg by gavage. At the termination, all mice were euthanized with isoflurane (Mylan Inc., Tokyo, Japan) anesthesia. After necropsy, the livers were weighed and dissected. Five mice per group were selected for *in vivo* reporter gene mutation assays.

Reporter gene mutation assays. The protocol for the *in vivo* reporter gene mutation assay followed the OECD guideline for Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays (OECD, 2011). 6-thioguanine (6-TG) and Spi$^-$ selections were performed using the method of Nohmi *et al.* (2000). We used DNA sample extracted from the liver of an animal that had been treated with 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) as a positive control for confirmation of this methodology.

Experiment 2: 3-Day-Repeated Dose Test Using C3H-Derived CARKO and Wild-Type Mice

Study design. Seven-week-old male and female wild-type and CARKO mice were divided into five groups consisting of five mice per group for each genotype and sex. GBE was administered for 3 days (48, 24, and 3 h before the animals were terminated) at doses of 0 (corn oil alone), 500, 1000, and 2000 mg/kg by gavage to both sexes of both genotypes. As a positive control for the micronucleus assay and the comet assay, EMS was administered for 2 days (24 and 3 h before the animals were terminated) at 200 mg/kg by gavage to both sexes of both genotypes. Three hours after the final dosing, all mice were euthanized under isoflurane anesthesia. After complete necropsy, the livers were weighed and dissected for the comet assay and the bone marrows were excised for the micronucleus assay.

Comet assay in the liver. The experimental procedure basically conformed to the JaCVAM protocol for international validation study (JaCVAM, 2009). Cell suspensions from the livers were mixed with 0.5% low-melting point agarose (NuSieve GTG Agarose, Lonza Rockland Inc., Basel, Switzerland) and deposited on Matsunami adhesive silane (MAS)-coated slide glass (Matsunami Glass Ind. Ltd, Osaka, Japan). After electrophoresis, we stained the cells with SYBR gold (Life Technologies, Carlsbad, CA) and analyzed using a fluorescent microscope connected to a comet image analyzer (comet assay IV, Perceptive Instruments Ltd, Suffolk, UK). For each sample, 100 cells (50 cells/slide) were analyzed to calculate the percentage of DNA in the tail (% Tail DNA). Heavily damaged cells, commonly referred to as "hedgehogs" (showing small or nonexistent head and largely diffused tail) were excluded from the comet data collection. Hedgehog cells were separately scored for 100 cells (50 cells/slide), and the frequency of hedgehogs was calculated.

Micronucleus assay in the bone marrow. Cells were collected from the bone marrow using fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and were smeared on slide glass. After drying, the cells were fixed in methanol, and the slides were stored at room temperature. The cells were stained with acridine orange solution and immediately observed by fluorescence microscopy. Micronucleated polychromatic erythrocytes (MNPCEs) were recorded based on the observation of 2000 polychromatic erythrocytes (PCEs), and the MNPCE/PCE ratio was calculated. In addition, 200 total erythrocytes (PCEs + normochromatic erythrocytes) were scored for PCE frequency (PCE%).

Pathological Examination of the Liver

The remaining liver tissue samples obtained from experiments 1 and 2 were fixed in 10% buffered formaldehyde solution, embedded in paraffin wax, and stained with hematoxylin-eosin for histopathological examination.

TABLE 1
Final body and liver weights and histopathological findings of livers in B6C3F1*gpt* delta, wild-type and CARKO mice treated with GBE

Genotype	Sex	Compound	Dose (mg/kg)	Periods	Body weight (g)	Absolute liver weight (g)	Relative liver weight (mg/g)	Histopathological findings of livers
B6C3F1 *gpt* delta	Male	Corn oil	0	90 days	32.4 ± 2.6	1.34 ± 0.13	41.4 ± 1.8	-
		GBE	20		32.4 ± 3.3	1.32 ± 0.13	40.7 ± 1.5	-
			200		33.9 ± 3.2	1.43 ± 0.15	42.2 ± 0.8	-
			2000		30.7 ± 2.2	1.63 ± 0.19**	52.9 ± 3.4**	Hypertrophy[a] (3.0)[b], necrosis (0.5)
C3H (Wild-type)	Male	Corn oil	0	3 days	23.6 ± 0.7	1.12 ± 0.05	47.7 ± 1.3	-
		GBE	500		23.7 ± 1.1	1.29 ± 0.08*	54.2 ± 2.6**	Hypertrophy (2.0), vacuolation[c] (0.6)
			1000		23.4 ± 1.2	1.37 ± 0.10**	58.6 ± 2.9**	Hypertrophy (2.0), vacuolation (0.8)
			2000		23.2 ± 1.2	1.48 ± 0.08**	63.9 ± 1.6**	Hypertrophy (2.4), vacuolation (1.4)
		EMS	200		23.2 ± 1.5	1.10 ± 0.09	47.6 ± 1.7	-
	Female	Corn oil	0	3 days	19.4 ± 0.6	0.92 ± 0.03	47.4 ± 1.6	-
		GBE	500		19.2 ± 1.4	1.04 ± 0.12	53.8 ± 2.9**	Hypertrophy (1.0), vacuolation (1.2)
			1000		19.4 ± 1.2	1.09 ± 0.07*	55.8 ± 1.2**	Hypertrophy (2.0), vacuolation (1.4)
			2000		19.9 ± 0.8	1.25 ± 0.07**	62.5 ± 1.4**	Hypertrophy (2.0), vacuolation (1.8)
		EMS	200		18.6 ± 2.6	0.84 ± 0.15	45.0 ± 2.9	-
CARKO	Male	Corn oil	0	3 days	25.6 ± 1.1	1.17 ± 0.08	45.6 ± 2.8	-
		GBE	500		26.3 ± 1.6	1.31 ± 0.05*	49.9 ± 3.0	-
			1000		24.8 ± 1.1	1.19 ± 0.07	48.0 ± 1.8	-
			2000		25.5 ± 0.7	1.21 ± 0.07	47.6 ± 3.3	Hypertrophy (0.2)
		EMS	200		25.0 ± 2.0	1.07 ± 0.04#	42.8 ± 2.1	-
	Female	Corn oil	0	3 days	21.2 ± 0.6	1.03 ± 0.02	48.6 ± 0.9	-
		GBE	500		21.4 ± 1.5	1.06 ± 0.10	49.7 ± 2.3	-
			1000		21.4 ± 1.2	0.98 ± 0.11	45.8 ± 2.5	-
			2000		21.0 ± 0.9	1.05 ± 0.07	50.2 ± 2.2	-
		EMS	200		20.9 ± 2.7	0.91 ± 0.13	43.6 ± 2.6##	-

Note. CARKO, CAR knockout; GBE, *Ginkgo biloba* extract; EMS, ethyl methanesulfonate.
The data shown are mean ± SD.
Significantly different from the control group (0 mg/kg): (*$p < 0.05$, **$p < 0.01$: Dunnett's test; #$p < 0.05$, ##$p < 0.01$: Student and Welch test).
[a]Hepatocellular hypertrophy was found at centrilobular area.
[b]Average of severity grade based on 0–4 grading scale (0: none; 1: slight; 2: mild; 3: moderate; 4: severe).
[c]Vacuolation was found at midzonal to periportal area.

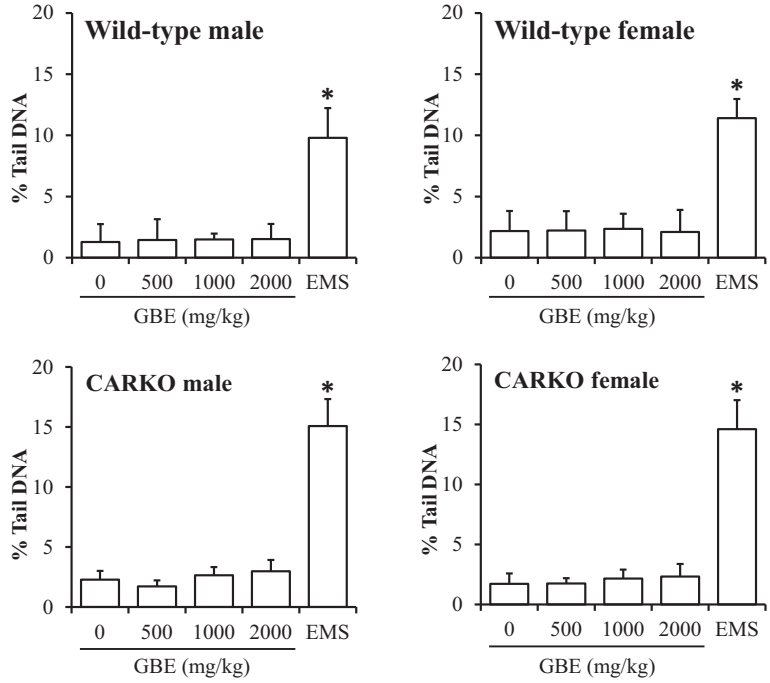

FIG. 1. Liver comet assay in wild-type and CARKO mice treated with GBE or EMS. Each datum represents the mean group value ± SD of % Tail DNA. Significantly different from the control group (0 mg/kg): (*$p < 0.05$: Student test).

Statistical Analysis

All data were expressed as means ± SD per group. The data for body and liver weights, *gpt* and Spi$^-$ mutant frequencies (MFs), and parameters in comet assays were analyzed with Dunnett's multiple comparison test. The significance of differences for positive control data was analyzed with the Student-Welch test. The data for PCE ratios were analyzed with the Wilcoxon's rank sum test. The data for MNPCE/PCE ratios were analyzed with a conditional binomial test using Kastenbaum and Bowman's table (Kastenbaum and Bowman, 1970).

RESULTS

Experiment 1

Clinical signs, body weight, liver weight, and histology of hepatocytes. No treatment-related clinical signs or deaths were detected during the 90-day-repeated administration study. Final body weights, liver weights, and histopathology in the liver are summarized in Table 1. There were no effects on body weight or food consumption (data not shown) in animals dosed with GBE at any of the dosage levels. No macroscopic abnormalities were noted in any animals at necropsy. Relative liver weights were significantly increased in the 2000 mg/kg group. Hepatocellular hypertrophy in the centrilobular area and slight focal necrosis were observed in the 2000 mg/kg group.

Gene mutation assays in the liver. Data for *gpt* MFs analyzed by 6-TG selection are shown in Table 2. No statistically significant increase of *gpt* MFs was detected in the liver DNA of the GBE-treated *gpt* delta mice compared with those in the control group. In the analysis of Spi$^-$ selection assessing deletion mutations (Table 3), there was no statistically significant variation in Spi$^-$ MFs values between GBE-treated and control mice.

Experiment 2

Clinical signs, body weight, liver weight, and histology of hepatocytes. In the 3-day-repeated administration study, final body weights, liver weights, and histopathological findings of livers are shown in Table 1. There were no abnormal clinical signs and no treatment-related effects on body weight in any of the GBE-treated groups. Relative liver weight was significantly increased in male and female wild-type mice at all doses of GBE and the effect was dose dependent. In contrast, in CARKO mice of both sexes at all doses, liver weights in all the GBE-treated groups were comparable with that in the control group. Microscopically, wild-type mice in all the GBE-treated groups showed slight-to-moderate hepatocellular hypertrophy in the centrilobular area and the effect was dose dependent. In contrast, hepatocellular hypertrophy was not observed in CARKO mice, with the exception of a single animal that showed slight hepatocellular hypertrophy at the highest dose. There were no histopathological findings suggestive of cytotoxicity in the liver in any of the GBE-treated groups.

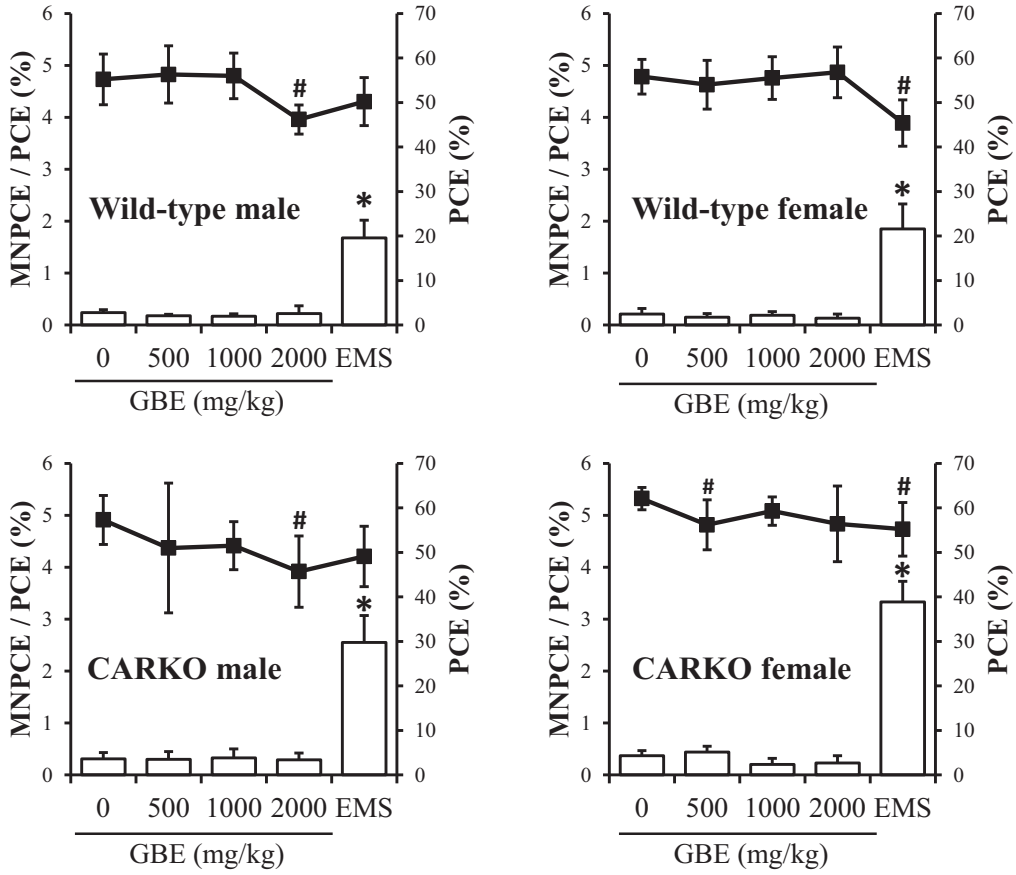

FIG. 2. Bone marrow micronucleus assay in wild-type and CARKO mice treated with GBE or EMS. Each datum represents the mean group value ± SD of proportion of MNPCE (bar graphs, left) and ratio of PCE (line graphs, right). Significantly different from the control group (0 mg/kg): (*$p < 0.05$: Kastenbaum bowman test; #$p < 0.05$: Wilcoxon's rank sum test).

Comet assay in the liver. The results of the liver comet assays are graphically summarized in Figure 1. There was no significant difference in % Tail DNA in any of the GBE-treated groups. In the positive control group, the mean value of % Tail DNA was significantly higher than that in the control group (0 mg/kg). Hedgehogs, indicative of cytotoxic effects such as necrosis or apoptosis, were not detected in any of the animals (data not shown).

Micronucleus assay in the bone marrow. The results of the bone marrow micronucleus assays are shown in Figure 2. There was no statistically significant change in the MNPCE/PCE ratio in any of the GBE-treated groups. In females, no significant alteration in the percentages of PCEs was seen in either genotype. In contrast, a significant decrease in the percentage of PCEs was observed in males in both genotypes, suggesting that GBE induced bone marrow toxicity in males. In the positive control group, the MNPCE/PCE ratio was significantly higher than that in the control group (0 mg/kg).

DISCUSSION

In the present study, we used several methods to investigate the *in vivo* genotoxicity of GBE. Using *in vivo* reporter gene mutation assays, no remarkable increases in *gpt* or Spi$^-$ MFs were observed in DNA extracted from the livers of *gpt* delta mice that had been exposed to GBE up to 2000 mg/kg bw/day. These results suggest that genomic mutations were not induced. Also, in the comet assay using liver, no significant increases in the % Tail DNA were detected at doses up to 2000 mg/kg, suggesting that GBE did not have the potential for inducing DNA damage. Furthermore, in the micronucleus assay, no changes in the MNPCE/PCE ratio were seen, indicating that chromosomal aberrations were not induced. In addition, GBE did not induce DNA damage or chromosomal aberrations in CARKO mice as in wild-type mice. In all of these studies, except for CARKO mice, clear liver hypertrophy was detected at the maximal dose, indicating that GBE did indeed reach the liver. In the positive control group, *gpt* and Spi$^-$ MFs, the % Tail DNA and the MNPCE/PCE ratio were significantly higher than those in the control group (0 mg/kg). Thus, all the exper-

TABLE 2
***gpt* MFs in the livers of B6C3F1*gpt* delta mice treated with GBE for 90 days**

Compound	Dose (mg/kg)	Animal no.	Cm^R colonies ($x10^5$)	6-TG^R and Cm^R colonies	MFs ($x10^{-5}$)	Mean ± SD
Control	0	1	14.3	1	0.07	0.69 ± 0.43
		2	8.1	5	0.61	
		3	7.4	6	0.81	
		4	7.1	5	0.71	
		5	4.7	6	1.27	
GBE	20	11	4.8	4	0.84	0.55 ± 0.29
		12	17.1	3	0.18	
		13	23.0	11	0.48	
		14	19.4	8	0.41	
		15	8.2	7	0.85	
	200	21	10.2	7	0.69	0.74 ± 0.18
		22	13.2	11	0.83	
		23	15.3	10	0.65	
		24	16.8	9	0.54	
		25	11.9	12	1.01	
	2000	31	18.2	14	0.77	0.39 ± 0.22
		32	29.3	11	0.37	
		33	12.4	3	0.24	
		34	12.2	3	0.25	
		35	26.3	8	0.30	
IQ		P1	5.3	210	39.89	39.89

Note. GBE, *Ginkgo biloba* extract; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; Cm^R, chloramphenicol-resistant; 6-TG^R, 6-thioguanine-resistant; MFs, mutant frequencies.

imental trials clearly provided evidence that the present studies were conducted under appropriate conditions and that GBE was not genotoxic *in vivo*.

GBE is composed of a number of ingredients and constituent concentrations vary widely in commercially available GBE products (Kressmann *et al.*, 2002). Several ingredients in GBE are known *in vitro* mutagens. Quercetin, one of the principal components of GBE, was a strong mutagen in *S. typhimurium* with and without S9 mix (Bjeldanes and Chang, 1977; MacGregor and Jurd, 1978; Nagao *et al.*, 1981; Resende *et al.*, 2012). Therefore, quercetin is considered to be one of the factors producing a positive result in the Ames assay in the NTP study of GBE. Importantly, quercetin has generally produced negative results in many *in vivo* studies (Aeschbacher *et al.*, 1982; Caria *et al.*, 1995; NTP, 1992). Furthermore, no evidence of liver carcinogenicity was observed in a 2-year study (NTP, 1992). From these results, the International Agency for Research on Cancer (IARC) concluded that quercetin is not classifiable as to its carcinogenicity to humans (IARC, 1999). Additionally, quercetin is allowed for use as a food additive in Japan (Ministry of Health and Welfare, 1996). On the basis of these international or national assessments of quercetin, concerns about the genotoxicity

TABLE 3
Spi⁻ MFs in the livers of B6C3F1*gpt* delta mice treated with GBE for 90 days

Compound	Dose (mg/kg)	Animal no.	Plaques within XL-1 blue MRA ($x10^5$)	Plaques within WL95 (P2)	MFs ($x10^{-5}$)	Mean ± SD
Control	0	1	4.3	1	0.23	0.51 ± 0.29
		2	5.4	5	0.93	
		3	14.7	7	0.48	
		4	7.4	2	0.27	
		5	7.6	5	0.66	
GBE	20	11	3.8	1	0.26	0.59 ± 0.37
		12	7.4	9	1.22	
		13	11.3	5	0.44	
		14	16.0	7	0.44	
		15	8.3	5	0.60	
	200	21	16.1	6	0.37	0.51 ± 0.30
		22	7.3	6	0.82	
		23	9.5	8	0.84	
		24	13.4	3	0.22	
		25	6.7	2	0.30	
	2000	31	28.8	15	0.52	0.39 ± 0.11
		32	12.2	4	0.33	
		33	6.0	3	0.50	
		34	10.1	3	0.30	
		35	12.2	4	0.33	
IQ		P1	7.4	35	4.74	4.74

Note. GBE, *Ginkgo biloba* extract; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MFs, mutant frequencies.

quercetin in GBE could be excluded. As for other compounds in GBE causing genotoxicity, ginkgolic acids were reported to induce DNA damage as measured by the comet assay in primary rat hepatocytes *in vitro* (Westendorf and Regan, 2000). The standard level of GBE used in prescriptions in Germany and France is designed to limit the amount of ginkgolic acids to 5 ppm or less, due to their mutagenic, cytotoxic, and allergenic potential (Kressmann *et al.*, 2002). GBE used for our study contained 10.45 ppm ginkgolic acids, a concentration higher than recommended (NTP, 2013). Nevertheless, this level of GBE showed no genotoxic effects in the present study. Our results suggest that commercially available GBE products within the recommended level of ginkgolic acid do not have the potential for genotoxicity *in vivo*.

Three-day treatment showed a clear relationship between CAR and GBE. In wild-type mice, GBE induced liver weight increases and hepatocellular hypertrophy. In CARKO mice, hepatocellular hypertrophy was not detected in most GBE-treated animals. These results indicate that CAR was involved in GBE-induced liver hypertrophy. GBE is a known inducer of xenobiotic metabolizing enzymes through activation of CAR, pregnane X receptor (PXR), and aryl hydrocarbon receptor (AHR) in

human HepG2 cells (Li *et al.*, 2008) and mice (Umegaki *et al.*, 2007). In the present study, GBE induced slight hepatocellular hypertrophy in one animal in the 2000 mg/kg group in CARKO male mice, suggesting the involvement of other nuclear receptors such as PXR or AHR.

For nongenotoxic hepatocarcinogens, several carcinogenic modes of action (MOA) have been identified that act through nuclear receptors-mediated mechanism (Budinsky *et al.*, 2014; Corton *et al.*, 2014; Elcombe *et al.*, 2014). Among them, a MOA for CAR-mediated liver tumor formation has been well studied. Phenobarbital (PB) is a nongenotoxic indirect CAR activator and induces liver tumors in mice and rats (Holsapple *et al.*, 2006; Whysner *et al.*, 1996). The key events in the PB-induced liver tumor MOA comprise activation of CAR, increased cell proliferation and formation of altered hepatic foci, and eventually the development of liver tumors (Elcombe *et al.*, 2014; Holsapple *et al.*, 2006). Although PB induces liver tumors in rodents, epidemiologic studies of patients undergoing long-term PB therapy showed no increase in the incidence of hepatic cancer in humans (IARC, 2001; Lamminpää *et al.*, 2002). Hence, it is commonly believed that CAR-mediated carcinogenic MOA similar to PB are not likely relevant to humans (Holsapple *et al.*, 2006). For that reason, we have undertaken a hepatocarcinogenesis study using CARKO mice to clarify whether CAR is involved in liver carcinogenesis by GBE. If GBE-induced liver carcinogenesis is CAR mediated in this study, then it can be concluded that its hepatocarcinogenic potential is not relevant to humans.

In conclusion, the present study demonstrates that GBE does not have the potential for genotoxicity *in vivo*, indicating that GBE-induced hepatocarcinogenesis in mice occurs through a non-genotoxic MOA.

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