研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Original Article

Effects of co-treatment of dextran sulfate sodium and MeIQx on genotoxicity and possible carcinogenicity in the colon of *p53*-deficient mice

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ABSTRACT — To investigate the effects of dextran sulfate sodium (DSS) and/or 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) on *in vivo* genotoxicity in the colon, male C57BL/6 *p53* (+/+), *p53* (+/-) or *p53* (-/-) *gpt* delta mice were twice given 1-week treatment with DSS, 2 weeks apart, and then sacrificed after 2 and 14 weeks. Although colon length was significantly shortened after DSS treatment in all genotypes at each time point, no significant difference in *gpt* mutant frequency (MF) and tumorigenicity was found between DSS and control groups regardless of genotype. Then, male $B6C3F_1$ *p53* (+/+) or *p53* (+/-) *gpt* delta mice were given DSS as described above and/or fed 300 ppm MeIQx for 7 weeks. Colon length was significantly shortened with DSS in either genotype at weeks 7 and 26, but no effects of co-treatment with MeIQx or *p53* deficiency were evident. MeIQx showed a tendency to increase *gpt* MF in the colon of mice with either genotype, but co-treatment with DSS did not affect these increments. Appreciable incidences of colonic aberrant crypt foci (ACFs) were reported in DSS as well as co-treatment groups of each genotype. Colonic adenomas were observed in co-treatment groups of both genotypes as well as the DSS-only group of *p53* (+/+). No effects of the combination of DSS and MeIQx on colon pre- and neoplastic lesions were reported. Our results indicate that MeIQx may take more than 7 weeks to induce genotoxicity in the colon and that the co-treatment of mice did not enhance colon tumorigenicity even in *p53*-deficient mice.

Key words: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, Dextran sulfate sodium, *In vivo* mutagenicity, *gpt* delta mouse, *p53*

INTRODUCTION

Chronic inflammation is regarded as a risk contributing to cancer development. Inflammatory bowel diseases, including ulcerative colitis (UC) and Crohn's disease are believed to be triggers for the development of colorectal cancer. Dextran sulfate sodium (DSS) is known to cause colitis in experimental animals in a manner similar to human UC. This has facilitated the use of the DSS-induced colitis model in the study of colitis-associated colorectal tumorigenesis in rodents (Okayasu *et al.*, 1990; Cooper *et al.*, 2000; Tanaka *et al.*, 2003).

The tumor suppressor protein, p53, plays a key molecular role in several pathways including inflammatory responses (Hussain and Harris, 2006). Inflammatory stress activates p53, and DNA-damaged cells are repaired

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or removed through pathways such as cell cycle arrest, DNA repair and apoptosis induced after DNA damage. It is suggested that lack of p53 function is an early event in the development of colitis-associated cancer in patients with UC (Hussain *et al.*, 2000). However, the role of p53 remains unclear in the DSS-induced colitis model. Recent studies have shown the enhancing effects of *p53* deficiency in inflammation-related *in vivo* mutagenicity or tumorigenicity (Fujii *et al.*, 2004; Chang *et al.*, 2007; Jenks *et al.*, 2003). Fujii *et al.* (2004) and Chang *et al.* (2007) reported that DSS significantly increased the incidence and multiplicity of colon tumors and dysplasias in *p53* homozygous deficient [*p53* (-/-)] mice than in *p53* heterozygous deficient [*p53* (+/-)] and *p53* proficient [*p53* (+/+)] mice. Therefore, p53 is believed to play the key role in this model as well as in human colorectal cancer.

Gpt transfected in rodents as a reporter gene is a useful tool in the assessment of the *in vivo* genotoxicity of environmental mutagens and in estimation of the carcinogenic risk of environmental chemicals (Gorelick *et al.*, 1996; Nohmi *et al.*, 2000; Nishikawa *et al.*, 2001). In this model, point mutations are positively identified using 6-thioguanine (6-TG) selection (Nohmi *et al.*, 2000).

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is a heterocyclic amine (HCAs) produced in cooked meat, inducing tumors in rats (liver, Zymbal gland, clitoral glands and skin), and mice (liver, lungs, hematopoietic system and colon) following DSS (Sugimura *et al.*, 2004; Nishikawa *et al.*, 2005). *In vivo* mutagenicity of MeIQx in transgenic mice has also been reported in the colon (Itoh *et al.*, 2000; Masumura *et al.*, 2003). In the human, correlations between MeIQx intake and risk of colon cancer were demonstrated in epidemiological studies (Sinha, 2002; Marchand *et al.*, 2002). In the current study, to investigate whether DSS-induced colitis affected *in vivo* mutagenicity and carcinogenicity induced by MeIQx, we examined *in vivo* mutagenicity and carcinogenicity in the colons of *p53*-proficient and -deficient *gpt* delta mice co-treated with DSS.

MATERIALS AND METHODS

Chemicals

DSS with a molecular weight of 36,000-50,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH, USA). MeIQx was purchased from Toronto Research Chemicals (North York, Canada).

Animals and treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences.

Experiment I: *p53* (+/-) C57BL/6 *gpt* delta mice were raised by matting C57BL/6 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in haploid genome and *p53* (-/-) mice with a C57BL/6 background established by Tsukada *et al.* (1993). *p53* (+/-) C57BL/6 *gpt* delta mice were enclosed with *p53* (+/-) C57BL/6 *gpt* delta mice; then, homozygous, heterozygous, and wild-type animals obtained from the F1 generation were genotyped by polymerase chain reaction (PCR) on tail DNA. As shown in Fig. 1, 50 of *p53* (+/+), 53 of *p53* (+/-) and 48 of *p53* (-/-) male C57BL/6 *gpt* delta mice were randomized by weight into 2 groups for each genotype. The animals were housed in a room with a barrier system, and maintained under the following constant conditions: temperature of 23 ± 2°C, relative humidity of 55 ± 5%, ventilation frequency of 18 times/hr, and a 12 hr light-dark cycle with free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Starting at 5 weeks of age the mice were treated with two cycles of DSS dissolved in the drinking water which was given *ad libitum*, 2 weeks apart. Based on the results of a preliminary study, the dose of DSS was selected to be 2% (data not shown). The frequency of DSS cycles and experimental period were determined as previously reported by Fujii *et al.* (2004). At week 6, 8 mice per each group were sacrificed by exsanguination under ether anesthesia, the colonic mucosa were collected for *in vivo* mutation assays, and the collected epithelium samples were frozen immediately in liquid nitrogen and stored at -80°C until further assays. Some mice from each group were housed for 12 weeks to investigate carcinogenicity in the colon. Eighteen weeks after starting the experiment, all mice were sacrificed in the same manner as interim necropsy. At necropsy, body weight and colon length, as a colitis-related disease marker (Okayasu *et al.*, 1990), were measured. The colons were removed and fixed in 10% buffered formalin solution and routinely processed for paraffin blocks for histopathological examination. Hematoxylin and eosin (H-E)-stained tissue preparations cut from the blocks were examined under a microscope.

Experiment II: Male B6C3F$_1$ *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in haploid genome were raised by mating C57BL/6 *gpt* delta and non-transgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Heterozygous *p53*-deficient mice with a C57BL/6 background established by Tsukada *et al.* (1993) were crossed with C57BL/6 mice (Charles River Japan Inc., Kanagawa, Japan), then, heterozygous *p53*-deficient and wild-type mice obtained from the F1 generation were genotyped by PCR on tail DNA. As shown in Fig. 1, 64

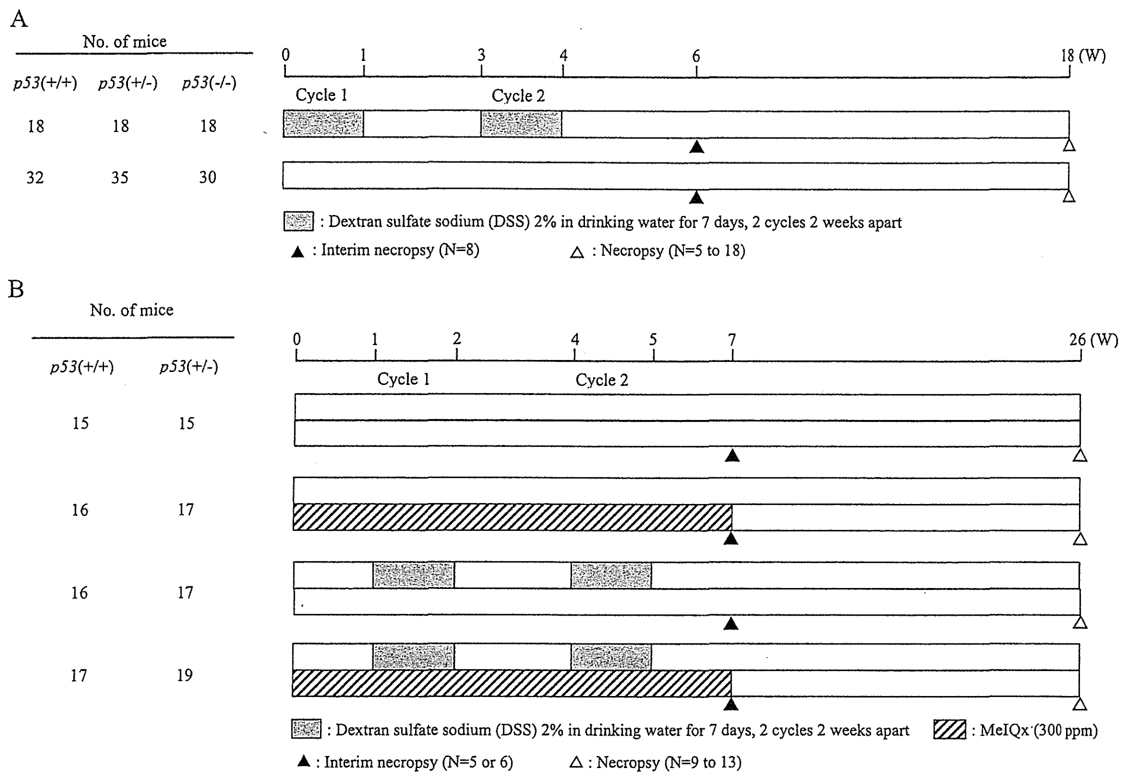

Fig. 1. Experimental designs. (A) Experimental design in Experiment I for investigating *in vivo* mutation and carcinogenesis in the colon of C57BL/6 *gpt* delta mice given DSS. (B) Experimental design in Experiment II for investigating the effect of co-treatment with DSS and MeIQx on *in vivo* mutation and carcinogenesis in the colon of B6C3F$_1$ *gpt* delta mice given DSS.

of *p53* (+/+) and 68 of *p53* (+/-) male B6C3F$_1$ *gpt* delta mice were randomized by weight into 4 groups. These animals were housed under the same conditions described above.

Starting at 8 weeks of age the mice were fed a diet containing 300 ppm of MeIQx or maintained as non-treatment controls for 7 weeks, and/or were given water containing 2% DSS. The dose of MeIQx was selected based on previous studies that MeIQx induced genotoxicity in the colon of *gpt* delta mice when given for 12 weeks, and tumorigenicity in the colon of mice received for 12 weeks following DSS (Nishikawa *et al.*, 2001; Masumura *et al.*, 2003). At week 7, 5 or 6 mice per each group were sacrificed by exsanguination under ether anesthesia, the colonic mucosa were collected for *in vivo* mutation assays, and the collected epithelium samples were frozen immediately in liquid nitrogen and stored at -80°C until further assays. Some mice of each group were housed for 19 weeks to investigate carcinogenicity in the colon. Twenty-six weeks after initiating the experiment, all mice were sacrificed in the same manner as interim necropsy. At necropsy, the body weight and colon length were measured. The colons were cut longitudinally and examined for the presence of aberrant crypt foci (ACFs) as reported previously (Suzuki *et al.*, 2007). The colons were then fixed in 10% buffered formalin solution and routinely processed for paraffin blocks for histopathological examination. H-E-stained tissue preparations cut from the blocks were examined under a microscope.

In vivo mutation assays

The 6-TG selections were performed as previously described (Nohmi *et al.*, 2000). Briefly, genomic DNA was extracted from the colon and lambda EG10 DNA (48 kb) was rescued as the lambda phage by *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with *E. coli* YG6020, which expresses Cre recombinase, and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, 9,000-fold

diluted phages were used to infect YG6020, and were poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37°C for the selection of 6-TG-registant colonies. Positively selected colonies were counted on day 3 and collected on day 4. The mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

Statistical evaluation

For statistical analysis, the Tukey test was used to compare body weight, colon length and MFs between groups. Incidence data were evaluated by Fisher's exact probability test, and multiplicity data were analyzed by Student's *t*-test or the Welch's *t*-test.

RESULTS

Experiment I: The survival ratios of *p53* (+/+), *p53* (+/-) and *p53* (-/-) mice with DSS treatment (during two cycles of DSS treatment) were 75% (24/32), 69% (24/35) and 83% (25/30), respectively. In these animals hemorrhage was observed on the colon mucosa at necropsy. After two cycles of DSS treatment, the survival ratios of *p53* (-/-) non-treated and DSS-treated mice were 50% (5/10) and 65% (10/15) respectively, but no dead animals were found among *p53* (+/+) and *p53* (+/-) mice. The dead animals showed enlargement of the thymus and/or spleen. It was suggested that lymphoma might have been the cause of death, since it is known that the malignant lymphoma is caused in *p53* knockout mice (Donehower, *et al.*, 1992). Data for final body weight and colon length are shown in Table 1. At the interim necropsy, a statistically significant decrease in body weight was observed in DSS-treated *p53* (+/+) and *p53* (+/-) mice as compared with control mice. DSS-treated mice showed shortening of colon length with or without significant differences as compared with control mice of each genotype. No significant changes were observed in body weight and colon length between the genotypes. At the necropsy of week 18, statistically significant decreases in body weight were observed in DSS-treated *p53* (+/+) mice as compared with control mice. DSS-treated mice showed shortening of colon length as compared with control mice of each genotype. No significant changes in body weight and colon length between genotypes were also observed. In the histopathological examination of the colon, adenocarcinoma developed only in 1 animal among the DSS-treated *p53* (-/-) mice (Table 2). Statistically significant differences in incidences were not observed as compared to any group.

The data for *gpt* MFs analyzed by 6-TG selection are summarized in Table 3. There were no significant increases of *gpt* MFs in the colon mucosa DNA of the DSS-treated mice as compared to the non-treatment control value. Similarly, no significant value of MFs was observed among any *p53* genotypes.

Experiment II: Only one *p53* (+/-) animal treated with MeIQx and DSS was found dead during the DSS treatment period. In the animal that died, marked hemorrhage was observed on the colon at necropsy. No death was observed in the other groups studied. Data for final body weight and colon length are shown in Table 4. At the interim necropsy, a statistically significant shortening of colon length was observed in DSS-treated *p53* (+/+) and *p53* (+/-) mice as compared with control or MeIQx-fed mice. No significant changes were observed in body weight and colon length between the genotypes. At the week 26 necropsy, a statistically significant decrease in body weight was observed in DSS-only treated *p53* (+/+) mice as compared with control mice. DSS-treated *p53* (+/+) mice showed shortening of colon length as compared with control mice. In the DSS-only treated animals, the colon length was shorter in *p53* (+/-) mice in comparison to that of the *p53* (+/+) mice.

The data for *gpt* MFs in the colon of *p53* (+/+) mice analyzed by 6-TG selection are summarized in Table 5. The MF was increased in MeIQx-treated mice as compared to the control mice albeit without any statistical significance. In the combined treatment group, no comparative increase in MF was observed. As shown in Table 6, in the *p53* (+/-) mice, the MF in mice fed with MeIQx showed a tendency to increase, but this was not statistically significant. As in the *p53* (+/+) mice, the combined treatment with DSS and MeIQx did not affect the increase of MF. Upon necropsy after 26 weeks, the incidence of ACFs was significantly increased in the distal colon of DSS-treated mice. Likewise, there was an increase in the number of mice displaying ACFs. However, the combined treatment of mice with MeIQx and DSS did not affect these changes (Table 7). In the histopathological examination of the colon, adenomas were reported to develop at incidences of 2, 1 and 1 in DSS-treated *p53* (+/+), combined treatment of *p53* (+/+) and *p53* (+/-), respectively (Table 8). Statistically significant differences in incidences were not observed as compared to any group.

DISCUSSION

In the present study, the effects of DSS-induced colitis on *in vivo* mutation were not clear in C57BL/6 *gpt* delta mice in any genotype. Likewise, tumorigenicity of the colon was not affected by *p53*-deficiency in mice with DSS-induced colitis. Fujii *et al.* (2004) and Chang *et al.*

Table 1. Final body weight and colon length in C57BL/6 *gpt* delta mice (Exp. I)

Stage	Genotype	Treatment	No. of mice	Survival ratio (%) [a]	Final body weight (g)	Colon length (cm)
6-week	*p53* (+/+)	DW	8	100	26.3 ± 1.6	7.7 ± 1.3
		DSS	8	75	24.7 ± 1.3**	7.0 ± 0.9
	p53 (+/-)	DW	8	100	27.4 ± 1.8	8.7 ± 0.6
		DSS	8	69	25.3 ± 1.3**	6.9 ± 0.8**
	p53 (-/-)	DW	8	100	26.2 ± 3.2	8.6 ± 0.4
		DSS	8	83	25.4 ± 1.1	7.0 ± 0.7**
18-week	*p53* (+/+)	DW	10	100	32.7 ± 3.9	9.4 ± 0.8
		DSS	16	100	30.0 ± 2.6**	8.4 ± 0.7**
	p53 (+/-)	DW	10	100	30.9 ± 2.8	9.3 ± 0.5
		DSS	18	100	31.5 ± 3.5	8.7 ± 0.8**
	p53 (-/-)	DW	5	50	30.8 ± 2.5	9.8 ± 0.9
		DSS	10	65	29.7 ± 2.5	8.3 ± 0.9**

** ; Significantly different from the DW-treatment group for each genotype at $p < 0.01$.
[a]: Survival ratio of 6-week or 18-week shows the survival rate during week 1 to 6 or week 7 to18, respectively.

Table 2. Incidence of colon adenoma/adenocarcinoma in C57BL/6 *gpt* delta mice given DSS (Exp. I, 18W)

Genotype	Treatment	No. of mice	Incidence (%)	
			Adenoma	Adenocarcinoma
p53 (+/+)	DW	10	0	0
	DSS	16	0	0
p53 (+/-)	DW	10	0	0
	DSS	18	0	0
p53 (-/-)	DW	5	0	0
	DSS	10	0	1 (10)

No significant difference among any groups.

(2007) reported that DSS-treated *p53* (-/-) mice showed a significantly increased incidence and multiplicity of cancer than DSS-treated *p53* (+/+) and *p53* (+/-) mice. However, in the present study, we report that only one out of ten *p53* (-/-) C57BL/6 mice showed an increase in incidence and multiplicity of cancer. A number of animals were found dead after a preliminary study using 4% DSS for 2 or 3 cycles as reported by Fujii *et al.* (2004) and Chang *et al.* (2007) (data not shown). This necessitated a change in the present study, whereby the animals were administered 2% DSS for 2 cycles. The different condition of DSS dosing might be responsible for the incompatible results.

Although an increased incidence of tumors in the colon was not noted, the MFs in the colon of DSS-treated mice were increased by approximately two-fold as compared to the control in *p53* (+/-) $B6C3F_1$ *gpt* delta mice. Although DSS is not in itself a genotoxic agent (Mori *et al.*, 1984),

Table 3. *gpt* MFs in the colon of *p53*-proficient and deficient C57BL/6 *gpt* delta mice (Exp. I, 6W)

Genotype	Treatment	Animal No.	Cm^R colonies (x 10^5)	6-TG^R and Cm^R colonies	MF (x 10^{-5})	Mean ± S.D.
p53 (+/+)	DW	1	7.3	10	1.36	0.82 ± 0.43
		2	16.9	6	0.35	
		3	9.3	11	1.18	
		4	10.6	6	0.57	
		5	9.6	6	0.62	
	DSS	6	10.7	6	0.56	0.83 ± 0.80
		7	11.1	25	2.26	
		8	8.0	4	0.50	
		9	11.1	4	0.36	
		10	10.3	5	0.49	
p53 (+/-)	DW	11	2.6	2	0.77	0.76 ± 0.50
		12	9.4	5	0.53	
		13	6.2	10	1.62	
		14	11.2	5	0.45	
		15	11.3	5	0.44	
	DSS	16	13.1	6	0.46	0.40 ± 0.12
		17	9.9	4	0.40	
		18	13.1	3	0.23	
		19	10.8	6	0.55	
		20	11.1	4	0.36	
p53 (-/-)	DW	21	10.1	10	0.99	0.64 ± 0.29
		22	11.1	6	0.54	
		23	6.6	6	0.91	
		24	18.6	6	0.32	
		25	9.2	4	0.44	
	DSS	26	14.1	12	0.85	0.72 ± 0.16
		27	8.9	8	0.90	
		28	9.5	5	0.53	
		29	9.7	6	0.62	
		30	8.4	6	0.72	

No significant difference among any groups.

long-term repeated DSS treatment induced colorectal tumors in wild-type mice (Seril *et al.*, 2002). The risk of cancer is increased under chronic inflammatory conditions including UC. It is known that reactive oxygen and nitrogen species are produced at sites of chronic inflammation, and may cause genomic stress and induce mutations. After DSS treatment, the levels of εA, εC and 8-OHdG were increased on colonic epithelial DNA of alkyladenine DNA glycosylase (Aag) deficient mice, which cannot remove base lesions formed by reactive oxygen and

Table 4. Final body weight and colon length in B6C3F$_1$ *gpt* delta mice (Exp. II)

Stage	Genotype	Treatment	No. of mice	Final body weight (g)	Colon length (cm)
7-week	*p53* (+/+)	Control	6	30.4 ± 2.2	9.7 ± 0.5
		MeIQx	6	29.8 ± 3.1	9.7 ± 0.7
		DSS	6	27.9 ± 2.6	8.0 ± 0.7**
		MeIQx + DSS	5	32.2 ± 3.9	8.7 ± 0.6*,#
	p53 (+/-)	Control	6	31.6 ± 3.2	10.0 ± 0.7
		MeIQx	6	29.4 ± 3.8	9.4 ± 0.8
		DSS	6	29.7 ± 1.1	8.1 ± 1.3*
		MeIQx + DSS	6	29.0 ± 2.3	9.1 ± 0.6*
26-week	*p53* (+/+)	Control	9	43.2 ± 3.3	11.9 ± 0.7
		MeIQx	10	40.3 ± 4.6	11.4 ± 0.8
		DSS	10	39.7 ± 3.1*	10.8 ± 0.7*
		MeIQx + DSS	12	39.6 ± 4.5	11.1 ± 0.9*
	p53 (+/-)	Control	9	41.6 ± 3.5	11.5 ± 0.9
		MeIQx	11	43.2 ± 1.6	12.0 ± 0.8
		DSS	10	38.5 ± 3.6	11.9 ± 0.4[ss]
		MeIQx + DSS	13	41.4 ± 1.6	11.4 ± 1.0

*, ** ; Significantly different from the control group for each genotype at $p < 0.05$, 0.01.
[#]; Significantly different from the MeIQx alone group for each genotype at $p < 0.05$.
[ss] ; Significantly different from the DSS-treated *p53* (+/+) mice at $p < 0.01$.
One animal dead in the DSS treated *p53* (+/-) group at week 3.

nitrogen species by deficient of Aag (Meira *et al.*, 2008). Infection by *Helicobacter pylori* is considered to be an important risk factor for gastric cancer. Jenks *et al.* (2003) reported the MF in *p53*-deficient mice infected with *H. felis* was increased by approximately two-fold compared with *H. felis*-infected *p53*-proficient mice. However, the effects of DSS-induced colitis on *in vivo* mutation were not clear in C57BL/6 *gpt* delta mice in any genotype in the present study.

It is well documented that mutated adenomatous polyposis coli causes accumulation of β-catenin in colon epithelial cells and a subsequent intranuclear shift of mutated β-catenin itself, both of which activate the Wnt signaling pathway and consequently drive abnormal cell proliferation (Roh *et al.*, 2001). It is also well known that *p53* is closely associated with degradation of β-catenin (Sadot *et al.*, 2001). In carcinogenesis in *p53*-deficient mice, factors other than repair dysfunction for DNA damaged cells derived from loss of p53 function may be involved.

Although the MFs were increased in MeIQx-treated *p53* (+/+) and *p53* (+/-) B6C3F$_1$ mice for 7 weeks as compared to the control mice, these results were not statistically significant. The combined treatment group did not show an increase in MF in comparison to any groups in both genotypes. We have reported that treatments with single gavage or 4-week feeding of MeIQx failed to induce genotoxicity in the colon of reporter gene transgenic mice, but the 12-week feedings induced genotoxicity (Itoh *et al.*, 2000; Nishikawa *et al.*, 2001; Masumura *et al.*, 2003). It was also documented that treatment of mice with 300 ppm MeIQx followed by 1.5% DSS induced colon tumors in mice (Nishikawa *et al.*, 2005). In the present study, the administration period of MeIQx might have been relatively short to clearly demonstrate the influence of inflammation since the increase of *in vivo* mutagenicity of MeIQx in B6C3F$_1$

Table 5. *gpt* MFs in the colon of *p53* (+/+) B6C3F$_1$ *gpt* delta mice (Exp. II, 7W)

Treatment	Animal No.	Cm^R colonies (x 10^5)	6-TG^R and Cm^R colonies	MF (x 10^{-5})	Mean ± S.D.
Control	11	17.1	3	0.18	0.79 ± 0.83
	12	9.0	4	0.45	
	13	2.9	5	1.74	
MeIQx	21	2.5	16	6.35	2.63 ± 2.60
	22	11.2	27	2.42	
	23	15.2	7	0.46	
	24	14.6	19	1.30	
DSS	31	15.3	8	0.52	0.74 ± 0.20
	32	11.2	9	0.80	
	33	12.3	11	0.91	
MeIQx + DSS	41	11.3	16	1.42	1.17 ± 0.55
	42	9.5	9	0.95	
	43	3.8	2	0.52	
	44	19.0	34	1.79	

No significant difference among any groups.

Table 6. *gpt* MFs in the colon of *p53* (+/-) B6C3F$_1$ *gpt* delta mice (Exp. II, 7W)

Treatment	Animal No.	Cm^R colonies (x 10^5)	6-TG^R and Cm^R colonies	MF (x 10^{-5})	Mean ± S.D.
Control	51	10.4	5	0.48	0.53 ± 0.15
	52	8.5	3	0.35	
	53	7.1	5	0.71	
	54	14.2	8	0.56	
MeIQx	61	5.2	8	1.55	1.12 ± 0.48
	62	12.4	16	1.29	
	63	13.8	6	0.44	
	64	7.4	9	1.21	
DSS	71	8.8	14	1.59	1.11 ± 0.51
	72	8.7	5	0.58	
	73	3.4	4	1.19	
MeIQx + DSS	81	10.5	14	1.33	1.44 ± 0.88
	82	11.5	7	0.61	
	83	3.8	9	2.35	

No significant difference among any groups.

Table 7. Incidence and multiplicity of colonic ACFs in B6C3F$_1$ *gpt* delta mice given MeIQx and/or DSS (Exp. II, 26W)

Genotype	Treatment	No. of mice	Proximal		Middle		Distal	
			Incidence (%)	No. of ACFs/ mouse	Incidence (%)	No. of ACFs/ mouse	Incidence (%)	No. of ACFs/ mouse
p53 (+/+)	Control	9	0 (0)	0	0 (0)	0	0 (0)	0
	MeIQx	10	0 (0)	0	1 (10)	0.10	0 (0)	0
	DSS	8	0 (0)	0	0 (0)	0	4 (50)**	1.91
	MeIQx + DSS	11	0 (0)	0	3 (27)	0.27	4 (36)	1.25
p53 (+/-)	Control	9	0 (0)	0	1 (11)	0.11	0 (0)	0
	MeIQx	10	0 (0)	0	1 (10)	0.10	0 (0)	0
	DSS	8	0 (0)	0	0 (0)	0	4 (50)**	1.91
	MeIQx + DSS	11	0 (0)	0	1 (9)	0.09	7 (64)**	2.27

** ; Significantly different from the control group for each genotype at $p < 0.01$.

Table 8. Incidence and multiplicity of colon adenoma in B6C3F$_1$ *gpt* delta mice given MeIQx and/or DSS (Exp. II, 26W)

Genotype	Treatment	No. of mice	Incidence (%)	Multiplicity (mean)
p53 (+/+)	Control	9	0	0
	MeIQx	10	0	0
	DSS	10	2 (20)	0.20
	MeIQx + DSS	12	1 (8)	0.08
p53 (+/-)	Control	9	0	0
	MeIQx	10	0	0
	DSS	10	0	0
	MeIQx + DSS	12	1 (8)	0.08

No significant difference among any groups.

mice was not observed when the mice were treated with MeIQx alone. Alternatively, to investigate whether DSS-induced colitis affected *in vivo* mutagenicity and carcinogenicity induced by MeIQx, mice were treated with DSS and MeIQx at the same time. The fact that DSS-induced colitis at the early stage is characterized by inflammatory cell infiltration with erosion of mucosal epithelium allows for the speculation that MeIQx-initiated cells did not fully receive the effects of the inflammatory factors due to enhanced cell turnover.

It is known that mouse strains differ in their susceptibility to DSS induced colitis (Mähler *et al.*, 1998; Melgar *et al.*, 2005). In the Experiment I, we used C57BL/6 *gpt* delta mice referred to the previous study (Fujii *et al.*, 2004), and high mortality was seen during DSS treatment period. Thus, we selected that B6C3F$_1$ *gpt* delta mice expected that receptivity was lower than C57BL/6 *gpt* delta mice from result of the preliminary study (data not shown) in the experiment II. Although DSS-treated mice in both strains were observed shortening of colon length

which is known caused by DSS-induced colitis (Okayasu *et al.*, 1990), only one mouse was found dead in the $B6C3F_1$ *gpt* delta mice. The difference was seen by susceptibility to DSS-induced colitis between 2 strains, but a clear difference was not observed in DSS-induced colitis associated carcinogenesis.

In conclusion, 7 weeks of exposure of mice to MeIQx at a dose of 300 ppm might have been insufficient in inducing genotoxicity in the colon. Although a close link between colitis and colon tumorigenesis has previously been noted, under the present experimental conditions, co-treatment of MeIQx with DSS demonstrated no influence on the *in vivo* mutagenicity of MeIQx and carcinogenicity even in *p53*-deficient mice.

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Original Article

Enhancing effects of carbon tetrachloride on *in vivo* mutagenicity in the liver of mice fed 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)

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ABSTRACT — Chronic stimulus subsequent to cell injury plays an important role in cancer development, but the precise mechanisms remain unknown partly because appropriate animal models are lacking. In the present study, the effects of hepatotoxicant carbon tetrachloride (CCl_4) on *in vivo* mutagenicity were investigated using *gpt* delta mice with or without p53. Female B6C3F$_1$ *p53*-proficient or -deficient *gpt* delta mice were given a diet containing 300 ppm of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) for 13 weeks, concurrently with intraperitoneal injection of 1 ml/kg CCl_4 solution once a week. Mutant frequencies of *gpt* and *red/gam* in *p53*-proficient mice fed MeIQx were both significantly elevated by CCl_4 co-treatment. Enhancing effects of CCl_4 treatment were also noted in *p53*-deficient mice. In the mutation spectra analysis of *gpt* mutant colonies, G:C to T:A transversions were predominantly observed regardless of CCl_4 injection, and clonal expansion of *gpt* colonies were increased in the co-treated group as compared with MeIQx alone group. The present data showing no significant changes in mRNA expression levels of *CYP1A2* and *GSTa4* between MeIQx-treated groups with and without CCl_4. In the Western blotting analysis, CYP1A2 protein levels were significantly decreased in the co-treated group as compared to MeIQx alone group, and GSTα protein levels were not changed among any groups. It is suggested that the mutant frequency by co-treatment with CCl_4 might result from some factors other than *p53* or MeIQx metabolism/excretion. Thus, our data clearly demonstrate that this model could be a powerful tool for identifying the mechanisms underlying combinatorial effects on carcinogenesis.

Key words: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, Carbon tetrachloride, *In vivo* mutagenicity, *gpt* delta mouse

INTRODUCTION

It has been widely recognized that chronic stimulus might play an important role in cancer development. For example, infection by the hepatitis C virus (HCV) is considered a trigger for development of hepatocellular carcinoma, in which the modes of action might involve cell injury and subsequent regenerative cell proliferation by HCV infection (Hayashi *et al.*, 1999; Kato *et al.*, 2003). Alternatively, several cytokines released from inflammatory cells supposedly take part in carcinogenesis (Lu *et al.*, 2006). In the liver, TNF-α and IL-6 released from activated Kupffer cells are promising candidate contributing factors (Roberts *et al.*, 2007), because they could be

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key molecules that trigger a cascade causing cell proliferation in liver regeneration (Montes *et al.*, 2006). However, it remains uncertain as to which chronic stimulus related factors actually affect carcinogenesis at the molecular level, partly because of the absence of suitable animal models for simultaneously investigating the correlation between the two events.

Recently, rodents carrying reporter genes have attracted attention as useful tools to assess *in vivo* genotoxic as well as carcinogenic risk of environmental chemicals (Gorelick and Mirsalis, 1996; Nohmi *et al.*, 2000; Nishikawa *et al.*, 2001). Such rodent bioassays conducted with similar protocols to the repeated dose toxicity study enable us to investigate the precise mechanisms underlying carcinogenesis. Up to now, along with the reporter gene mutation assay, we have shown that concurrent measurements of several parameters such as glutathione *S*-transferase placental form (GST-P) immunohistochemistry (Kanki *et al.*, 2005), bromodeoxyuridine (BrdU) labeling index (Kuroiwa *et al.*, 2007), and levels of thiobarbituric acid-reactive substances and 8-hydroxydeoxyguanosine (Umemura *et al.*, 2007) provide crucial information on the modes of action. Therefore, it is likely that the effects of alterations in the tissue microenvironment such as chronic inflammation on *in vivo* mutagenicity can be demonstrated by application of conventional transgenic rodents.

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is one of the heterocyclic amines (HCAs) produced in cooked meat, inducing tumors in the liver, Zymbal gland, clitoral glands, and skin in rats, and in the liver, lung, hematopoietic system, and colon of mice (Ohgaki *et al.*, 1987; Sugimura *et al.*, 2004; Nishikawa *et al.*, 2005). *In vivo* mutagenicity of MeIQx in transgenic mice has also been reported in the liver (Itoh *et al.*, 2000; Masumura *et al.*, 2003). Carbon tetrachloride (CCl_4) is a potent hepatotoxicant in rodents, and single administration at higher doses causes extensive hepatocellular necrosis with inflammatory cell infiltration (Kim *et al.*, 2009).

In the present study, for developing an animal model using reporter transgenic mice, we examined the effects of CCl_4-induced hepatic injury on *in vivo* mutagenicity of MeIQx in the liver of *gpt* delta mice. In addition, considering the enhancing effects of *p53* deficiency in *in vivo* mutagenicity and tumorigenicity on tissue damage by dextran sulfate sodium-induced colitis model or *helicobacter felis*-infected mice model (Levine, 1997; Fujii *et al.*, 2004; Chang *et al.*, 2007; Jenks *et al.*, 2003), the same protocol was also performed in *p53*-deficient *gpt* delta mice.

MATERIALS AND METHODS

Chemicals

MeIQx and CCl_4 were purchased from Toronto Research Chemicals (North York, Canada) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Animals and treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Female B6C3F$_1$ *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in haploid genome were raised by mating C57BL/6 *gpt* delta and non-transgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Heterozygous *p53*-deficient mice with a C57BL/6 background established by Tsukada *et al.* (1993) were crossed with C57BL/6 mice (Charles River Japan Inc., Kanagawa, Japan); then, heterozygous *p53*-deficient and wild-type mice were obtained from the F1 generation and genotyped by the polymerase chain reaction (PCR) on tail DNA. As shown in Fig. 1, twenty female B6C3F$_1$ *gpt* delta mice were randomized by weight into 4 groups. Animals were housed in a room with a barrier system, and maintained under the following constant conditions: 23 ± 2°C, 55 ± 5% relative humidity, ventilation frequency of 18 times/hr, and a 12 hr light-dark cycle with free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Starting at 8-wk of age the *gpt* delta transgenic mice were fed a diet containing 300 ppm MeIQx or maintained as non-treatment controls for 13 weeks. To induce hepatic injury, mice were intraperitoneally injected with 1 ml/kg CCl_4 solution (20 v/v% CCl_4 in corn oil) once a week from week 2. The dose and interval of CCl_4 administration were selected based on a study by Kato *et al.* (2003), as conditions to recover from increased levels of serum alanine aminotransferase (ALT) to basal levels. All mice were sacrificed and a part of the left lateral lobe of the liver was preserved with an ISOGEN kit (Nippon Gene, Tokyo, Japan) and stored at -80°C until used to isolate total RNA. The remaining liver was also stored at -80°C for subsequent *in vivo* mutation assays. At autopsy, the body and liver weights were measured.

In vivo mutation assays

The 6-TG and Spi$^-$ selections were performed as previously described (Nohmi *et al.*, 2000). Briefly, genomic DNA was extracted from the liver, and lambda EG10 DNA (48 kb) was rescued as lambda phage by *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with *E. coli* YG6020, which expresses Cre

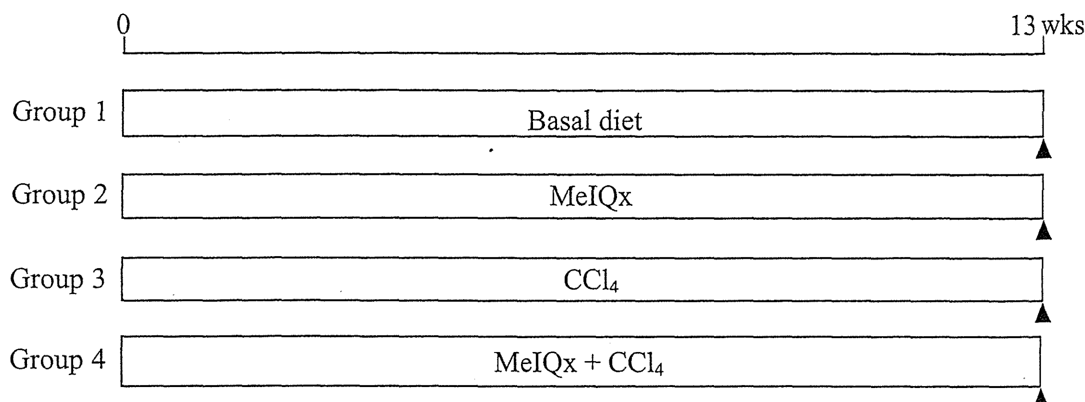

Fig. 1. Experimental design. Twenty female *p53*-proficient or -deficient *gpt* delta mice with $B6C3F_1$ background were divided into 4 groups, each consisting 5 animals. Group 1 was fed a basal diet as non-treatment control for 13 weeks. Group 2 was fed a diet containing 300 ppm MeIQx for 13 weeks. Group 3 received CCl_4 solution (20 v/v% CCl_4 in corn oil) by i.p. injection once a week at a dose of 1 ml/kg from week 2. Group 4 was fed a diet containing MeIQx and injected CCl_4 in the same manner with group 2 or 3. All mice were sacrificed at the closed triangle time point.

recombinase, and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, 9,000-fold diluted phages were used to infect YG6020, and were poured on five plates containing chloramphenicol without 6-TG. Plates were incubated at 37°C for selecting 6-TG-registant colonies. Positively selected colonies were counted on day 3 and collected on day 4 from all plates. The mutant frequency was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For Spi- selection, packaged phage was incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase soft agar and poured onto lambda-trypticase agar plates. The next day, plaques (Spi- candidates) were punched out with sterilized glass pipettes and the agar plugs were suspended in SM buffer. To confirm the Spi- phenotype, suspensions were spotted on three plate types where XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. Real Spi- mutants, which made clear plaques on every plate, were counted.

For characterizing the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of *gpt* was amplified by PCR as described previously (Nohmi *et al.*, 2000). DNA sequencing was carried out at the Dragon Genomics Center of Takara Bio (Mie, Japan). On the MeIQx alone and co-treatment groups, *gpt* mutants were analyzed only on one selected plate based on the assumption that the colony number on the selected plate was closest to the average number of five plates.

Quantitative real time RT-PCR

Total RNA was isolated by using the ISOGEN kit according to the manufacturer's instructions. RNA reverse transcription with random hexamers was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). PCR was performed on an Applied Biosystems 7900HT FAST Real-Time PCR Systems (Applied Biosystems) with TaqMan® Fast Universal PCR Master Mix (Applied Biosystems), TaqMan® Gene Expression Assays (Applied Biosystems) and TaqMan® Rodent GAPDH control reagents (Applied Biosystems). The assayed genes included cytochrome P450 family 1, subfamily A, polypeptide 2 (*CYP1A2*) and glutathione S-transferase alpha 4 (*GSTα4*). Rodent glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. The expression levels of the target genes *CYP1A2* and *GSTα4* were evaluated by a relative standard curve method.

Preparation of liver microsome and cytosol

For preparation of hepatic cytosol solutions and microsomal suspensions, liver samples were thawed on ice and homogenized in ice-cold 67 mM phosphate buffer (pH 7.5) containing 1.15% (w/v) KCl. Hepatic homogenates were centrifuged at 9,000 g for 20 min at 4°C and supernatants were centrifuged at 105,000 g for 60 min at 4°C. The supernatants were aliquoted and stored at -80°C until use. The precipitates were homogenized with the phos-

phate buffer and centrifuged at 105,000 g for 60 min at 4°C. The precipitates were suspended with phosphate buffer and stored at -80°C until use.

Western blot analysis

Protein levels of CYP1A2 and GST were analyzed by Western blotting. CYP1A2 and GST proteins were measured in liver microsomes and cytosols. Rabbit anti-rat polyclonal antibody for CYP1A2 (Millipore Co., Billerica, MA, USA) and goat anti-rat polyclonal antibody for GST (Abcam, Cambridge, UK) were used as primary antibodies. Mouse monoclonal anti β-actin antibody (Sigma, St. Louis, MO, USA) was used as loading control. The liver microsomes and cytosols were separated by 10 or 12% Mini PROTEAN TGX gel (Bio-Rad Laboratories, Hercules, CA, USA), respectively, and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked with 0.5% casein in PBS for 1 hr at room temperature. Primary antibodies diluted in 0.5% casein in PBS were hybridized for 1 hr at room temperature. After four washes in the wash buffer, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. After four washes in wash buffer and twice in PBS, protein were detected by ECL PLUS Western Blotting Detection Reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and band intensity was measured by ChemiDoc™ XRS+ system (Bio-Rad Laboratories).

Statistical evaluation

The Tukey test was used to compare liver and body weights, quantitative real-time RT-PCR, MFs and Western blotting analysis between groups.

RESULTS

Body and liver weights

There were no significant changes in final body and liver weights among all genotype groups (Table 1). However, significant increase of body weight in the MeIQx alone group, and significant decrease of relative liver weight in the MeIQx alone and MeIQx plus CCl_4 groups of *p53*-deficient mice were noted as compared with each treatment group in *p53*-proficient mice. The absolute liver weights were comparable between groups.

gpt and Spi⁻ MFs in the liver

Data for *gpt* MFs in the liver of *p53*-proficient mice analyzed by 6-TG selection are summarized in Table 2. In the *gpt* mutation assay, MF (8.39 ± 2.63) in mice fed MeIQx was significantly increased as compared to the basal diet control (0.20 ± 0.03). Also, co-treatment with MeIQx and CCl_4 caused marked elevation of MF (15.69 ± 2.69), which was 1.9-fold significantly higher than that in the MeIQx alone group. In the Spi$^-$ mutation assay, MFs of *red/gam* in mice fed MeIQx (MeIQx alone; 1.54 ± 0.48, MeIQx plus CCl_4; 4.16 ± 1.28) were significantly elevated as compared to the control (0.37 ± 0.13), the combined treatment 2.7-fold enhancing the increase of MF with sta-

Table 1. Final body, absolute and relative liver weights of *gpt* delta mice

Genotype	Treatment	No. of mice	Final body weight (g)	Liver weight	
				Absolute (g)	Relative (g/100 g B.W.)
p53 (+/+)	Control	5	28.3 ± 3.8[a]	1.34 ± 0.15	4.80 ± 0.90
	MeIQx	5	26.3 ± 1.6	1.18 ± 0.06	4.48 ± 0.12
	CCl_4	5	25.9 ± 2.8	1.17 ± 0.15	4.52 ± 0.17
	MeIQx + CCl_4	5	25.9 ± 2.7	1.18 ± 0.06	4.57 ± 0.38
p53 (+/-)	Control	5	28.2 ± 1.6	1.24 ± 0.13	4.37 ± 0.29
	MeIQx	5	29.8 ± 2.9*	1.24 ± 0.14	4.14 ± 0.15**
	CCl_4	5	27.4 ± 1.2	1.12 ± 0.06	4.08 ± 0.30*
	MeIQx + CCl_4	5	25.4 ± 1.3	1.15 ± 0.10	4.53 ± 0.32

*,** ; Significantly different from the wild type mice at $p < 0.05$ and $p < 0.01$, respectively.
[a] ; Mean ± S.D.

Table 2. *gpt* MFs in *p53* (+/+) *gpt* delta mice livers

Treatment	Animal No.	Cm^R colonies (x 10^5)	$6\text{-}TG^R$ and Cm^R colonies	Mutant frequency (x 10^{-5})	Mean ± S.D.
Control	11	11.8	3	0.25	0.20 ± 0.03
	12	43.2	8	0.19	
	13	20.1	4	0.20	
	14	24.1	4	0.17	
MeIQx	21	9.8	111	11.31	8.39 ± 2.63*
	22	8.7	85	9.69	
	23	15.6	84	5.41	
	24	14.2	101	7.13	
CCl_4	31	31.9	12	0.38	0.28 ± 0.12
	32	58.4	6	0.10	
	33	16.6	5	0.30	
	34	23.7	8	0.34	
MeIQx + CCl_4	41	16.8	301	17.88	15.69 ± 2.69*,#,$
	42	16.1	190	11.79	
	43	13.2	223	16.91	
	44	15.7	253	16.16	

* ; Significantly different from the control group at $p < 0.01$.
# ; Significantly different from the MeIQx alone group at $p < 0.01$.
$; Significantly different from the CCl_4 alone group at $p < 0.01$.

tistical significance (Table 3).

In *p53*-deficient mice, *gpt* and *red/gam* MFs showed a tendency similar to the *p53*-proficient mice (Tables 4 and 5). The *gpt* MFs in *p53*-deficient mice fed MeIQx (MeIQx alone; 7.40 ± 2.40, MeIQx plus CCl_4; 23.28 ± 8.19) were significantly increased as compared to the basal diet control (0.79 ± 0.36), 3.1-fold increase being observed in the co-treatment with MeIQx plus CCl_4 group as compared with MeIQx alone group. MFs of *red/gam* in mice fed MeIQ (MeIQx alone; 0.63 ± 0.49, MeIQx plus CCl_4; 1.75 ± 0.66) were elevated without or with statistical significance as compared to the control (0.38 ± 0.29). The co-treatment with MeIQx and CCl_4 caused marked elevation of MF, which was 2.8-fold significantly higher than that in the MeIQx alone group. No difference of these MFs was seen between genotypes.

gpt mutation spectra analysis

To characterize *gpt* mutations in the liver of *p53*-proficient mice, DNA sequencing was performed (Table 6). In control mice, G:C to A:T transitions predominated (5/15 = 33.3%). In the CCl_4 alone group, G:C to T:A transversions (8/21 = 38.1%) and G:C to A:T transitions (9/21 = 42.9%) were observed at about the same frequency. On the other hand, the predominant spectra of colonies with *gpt* mutation were G:C to T:A transversions in mice treated with MeIQx regardless of CCl_4 treatment (MeIQx alone group: 34/58 = 58.6%, MeIQx plus CCl_4 group: 54/111 = 48.6%). In addition, clonal expansions of *gpt* mutant colonies more frequently occurred in the MeIQx plus CCl_4 treated group compared to the MeIQx alone group. In the co-administered group, 119/182 of mutant colonies with G:C to T:A transitions were identified; however, 53/81 were identified in the MeIQx alone group (unpublished data). Spectra analysis was not done

Table 3. Spi⁻MFs in *p53* (+/+) *gpt* delta mice livers

Treatment	Animal No.	Total population (x 10^5)	Total Spi⁻ mutants	Mutant frequency (x 10^{-5})	Mean ± S.D.
Control	11	9.3	2	0.21	0.37 ± 0.13
	12	9.9	4	0.41	
	13	8.8	3	0.34	
	14	11.3	6	0.53	
MeIQx	21	12.0	10	0.84	1.54 ± 0.48*
	22	7.2	12	1.68	
	23	7.9	14	1.78	
	24	9.1	17	1.87	
CCl_4	31	12.7	5	0.39	0.46 ± 0.08
	32	10.0	4	0.40	
	33	8.9	5	0.56	
	34	10.5	5	0.47	
MeIQx + CCl_4	41	15.1	60	3.97	4.16 ± 1.28*,#,$
	42	4.5	16	3.52	
	43	5.2	26	4.98	
	44	6.3	16	2.52	
	45	4.0	23	5.81	

* ; Significantly different from the control group at $p < 0.01$.
# ; Significantly different from the MeIQx alone group at $p < 0.01$.
$; Significantly different from the CCl_4 alone group at $p < 0.01$.

in the *p53*-deficient mice.

Expression levels of *CYP1A2* and *GSTα4* mRNA

Expression levels of *CYP1A2* and *GSTα4* in the liver of *p53*-proficient mice are shown in Fig. 2. Expression levels of *CYP1A2* were significantly increased in MeIQx alone and co-treatment groups as compared to basal control or CCl_4 injected groups. There were no significant changes in *CYP1A2* levels among MeIQx alone and co-treatment groups ($p < 0.01$). Significant changes in *GSTα4* expression levels were not observed among any groups ($p < 0.01$).

Expression levels of CYP1A2 and GSTα protein

Expression level of CYP1A2 and GSTα protein in the liver of *p53*-proficient mice are shown in Fig. 3. Expression levels of CYP1A2 were significantly decreased in the co-treated group as compared to MeIQx alone group ($p < 0.05$). Also, CYP1A2 levels in the co-treatment groups were significantly decreased as compared to basal control group ($p < 0.05$). Although GSTα protein expression levels were decreased in CCl_4 alone group as compared to other groups, no statistical significances were observed among any groups.

DISCUSSION

In the present study, 13-week feeding of MeIQx caused significant elevation of *gpt* MFs in the livers of *p53*-proficient *gpt* delta mice. In the mutation spectra analysis of *gpt* mutant colonies, G:C to T:A transversions were predominantly observed, which is in line with a previous