examined whether these two mutations occurred in the same allele or not. After PCR amplification using specific primer sets for amplifying the fragment simultaneously covering the region of the two mutations, the amplified fragments were cloned into plasmid vectors. The sequence analyses of these plasmid clones revealed that no clones simultaneously carried both two mutations, thus demonstrating that these tumors were compound-heterozygotes in *Apc* alleles.

Somatic *Ctnnb1* mutations in the small intestinal tumors

β-catenin is reported to have 4 putative GSK3β-phosphorylation sites. Phosphorylation of β-catenin by GSK3β in a complex with Axin and Apc is required to target β-catenin for degradation by the proteasome. Therefore, the mutations at the phosphorylation sites of β-catenin are considered to be related to its stability that would lead to the accumulation of β-catenin in nuclei, thereby up-regulating the expression of the target genes. In addition, the region of mouse (but not human) *Ctnnb1* encoding GSK3 β-phospharylation sites of β-catenin contains AGAA sequences. Therefore, we analyzed mutations in exon 3 of *Ctnnb1* encoding GSK3β-phospharylation sites of β-catenin. Among 62 tumors from 4 *Mutyh*-deficient mice and 11 tumors from 10 wild-type mice, 36 out of 62 tumors (58.1%) from *Mutyh*-deficient mice and 3 out of 11 tumors (27.3%) from wild-type mice showed a mutation in the region corresponding to the GSK3β-phosphorylation sites (Table 3). All the mutations in *Mutyh*-deficient mice were base substitution mutations, in which 35 mutations (97.2%) were identified as G:C to T:A transversions and 1 mutation (2.8%) as A:T to T:A transversion. Remarkably, 34 G:C to T:A transversions occurred at the AGAA sequences associated with either Ser33 or Ser37, the putative phosphorylation sites, changing them to tyrosine. Two mutations found on either side of codon for Ser33, which converted the original amino acids (Asp32 or Gly34) to Valine. No tumor carried mutations in both *Apc* and *Ctnnb1* genes, simultaneously. In wild-type mice, 2 base substitution mutations and a 24-bp-deletion were identified. Both base substitution mutations were G:C to A:T transitions, not related to 8-oxoG-induced transversions. One of them occurred at Ser41 changing it to Isoleucine, where no mutation was found in *Mutyh*-deficient mice.

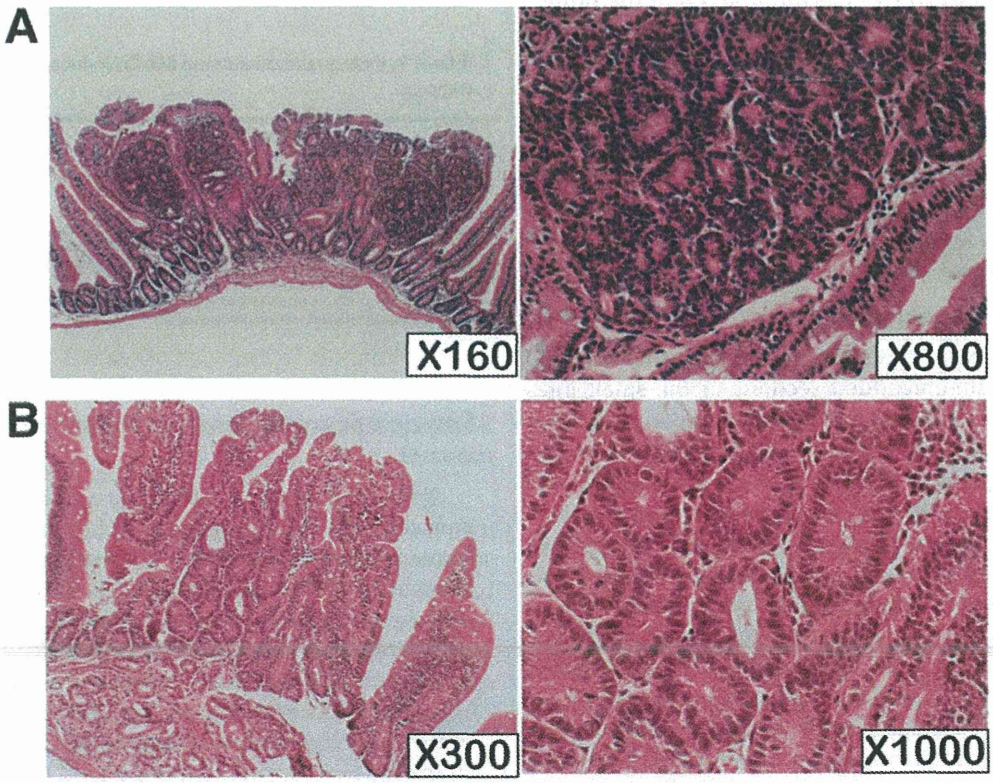

Figure 1. H.E. staining of the $KBrO_3$-induced small intestinal tumors developed in *Mutyh*-deficient and wild type mouse. (**A**) One of the typical tumors from *Mutyh*-deficient mouse; classified as category 4. (**B**) An exceptional case classified as category 3 developed in wild-type mouse. Magnifications are indicated in lower right of the photos.

Table 2. Somatic *Apc* mutations in the small intestinal tumors

Mouse ID[1]	Tumor ID[2]	Nucleic acid change[3]	Amino acid change[4]	Secuence context[5]
44f	1	93828 G→T	E1396X	TGAG
44f	5	92805 G→T	E1055X	AGAA
44f	6	93226 C→A	S1195X	*TGAA*
44f	6	93405 G→T	E1255X	AGAA
44f	9	92607 G→T	E989X	TGAA
44f	9	93481 C→A	S1280X	*TGAT*
44f	10	93141 G→T	E1167X	TGAA
44f	13	93343 C→A	S1234X	*TGAA*
50m	22	93220 C→A	S1193X	*TGAT*
50m	24	92694 G→T	E1018X	AGAA
50m	24	93708 G→T	G1356X	AGGA
50m	25	92550 G→T	G970X	TGGA
50m	28	92805 G→T	E1055X	AGAA
50m	28	93667 C→A	S1342X	*TGAA*
50m	29	92805 G→T	E1055X	AGAA
50m	29	92238 G→T	E853X	AGAG
50m	30	93042 G→T	E1134X	TGAA
50m	30	93099 G→T	E1153X	AGAA
56f	33	93099 G→T	E1153X	AGAA
56f	46	93492 G→T	E1284X	TGAA
56f	48	92826 G→T	E1062X	CGAG
56f	48	93102 G→T	E1154X	AGAA
124m	49	92811 G→T	E1057X	TGAA
124m	54	93498 G→T	G1286X	AGGA
124m	60	92199 G→T	E853X	AGAG

NOTE: 62 tumors from 4 *Mutyh*-deficient mice were analyzed for *Apc* mutation.

[1] The 4 *Mutyh*-deficient mice consisting of two males and two females were analyzed.

[2] ID number of tumors carrying mutations in *Apc*. Sixteen tumors from each mouse were analyzed, except for 124m mouse from which 14 tumors were analyzed.

[3] The number indicates a position of a mutation site. The numbers correspond to nucleotide sequence of *APC* deposited in GenBank (Accession: NC_000084).

[4] The number indicates a position of a changed amino acid. X represents a nonsense codon. The numbers correspond to amino acid numbers of APC deposited in GenBank (Accession: NP_031488).

[5] Sequence context surrounding G:C to T:A mutations. G shown in bold indicates the mutation site. GAA sequence is underlined. Sequence of non-transcribed strand is italicized.

Table 3. Mutation analysis for *Ctnnb1*

Genotype	Amino acid change[1]	Nucleic acid change[2]	Sequence context[3]	Number of mutations
Wild type	37S→F	17219C→T	*AGAA*	1
	41T→I	17231C→T	ACCA	1
	del (31-39) [4]	24-bp deletion	-	1
Mutyh-deficient	32D→V	17204A→T	GATT	1
	33S→Y	17207C→A	*AGAA*	26
	34G→V	17210G→T	GGAA	1
	37S→Y	17219C→A	*AGAA*	8

[1] The number indicates a position of a changed amino acid. The numbers correspond to amino acid numbers of β-catenin deposited in GenBank (Accession: NP_001159374).

[2] The number indicates a position of a mutation site. The numbers correspond to nucleotide sequence of *Ctnnb1* deposited in GenBank (Accession: NC_000075).

[3] Sequence context surrounding G:C to T:A mutations. Letters shown in bold indicate the mutation site. GAA sequence is underlined. Sequence of non-transcribed strand is italicized.

[4] Nine amino acids from no.31 to 39 were replaced by newly arisen one serine resulted from the 24-bp deletion.

No tumors had mutations in either Kras or Trp53

It has been shown that *KRAS* mutations were common in tumors developed in MAP patients, and all the mutations were G:C to T:A transversions at first G of codon12 (37)(38). In addition, *TP53* muations were also detected in tumors from MAP patients, albeit at relatively low frequency (37). Thus, we further searched for the mutations in exon 2 of *Kras* (containing codon 12 and 13) and in exon 5 to 8 of *Trp53* (conserved region) in tumors from $KBrO_3$-treated *Mutyh* mice. Neither *Kras* nor *Trp53* mutations was found in the regions analyzed.

Immunohistochemistry

Mutations occurred both in *Apc* and in *Ctnnb1* would result in the accumulation of β-catenin in the nucleus. To verify this possibility, immunohistochemistry for β-catenin was performed with 71 small intestinal tumors developed in the 4 *Mutyh*-deficient mice treated with $KBrO_3$. The tumors with more than 5% of nuclear stained cells were counted for positive. Nuclear staining was identified in 41 tumors (57.7%), indicating the accumulation of β-catenin in the nuclei (Figure 2). These findings support the results of mutation analysis in *Apc* and *Ctnnb1*.

Discussion

We previously reported that *Mutyh*-deficient mice showed the susceptibility to tumorigenesis, especially adenoma/carcinoma in the intestine, hemangioma in the liver and angiosarcoma in the spleen (17). The intestinal tumor susceptibility of *Mutyh*-deficient mice was further enhanced by treatment with $KBrO_3$, a known oxidative renal carcinogen associated with 8-oxoG accumulations. The multiple tumor-formation in the mutant mice is consistent with the malignant capacity of multiple colorectal adenomatous polyposis in MAP patients, suggesting that *Mutyh*-deficient mouse is an animal model for investigating the pathogenesis of MAP. Thus, we performed the characterization of tumors induced in *Mutyh*-deficient mice treated with $KBrO_3$.

The small intestinal tumors analyzed in this study were exclusively classified in category 4 according to the Vienna classification of gastrointestinal neoplasia (Table 1). Only one tumor developed in a wild-type mouse with $KBrO_3$-treatment was classified in category 3. Even the tiny tumors were also classified in category 4. These results suggested that the $KBrO_3$-induced small intestinal tumors show significantly dysplastic change in the early stage of tumor-development. This gives rise to a possibility that the chronic exposures of $KBrO_3$ to intestinal mucosa in *Mutyh*-deficient mice might evoke concurrent mutations in multiple genes, although we could find any mutations neither in *Kras* nor in *Trp53*.

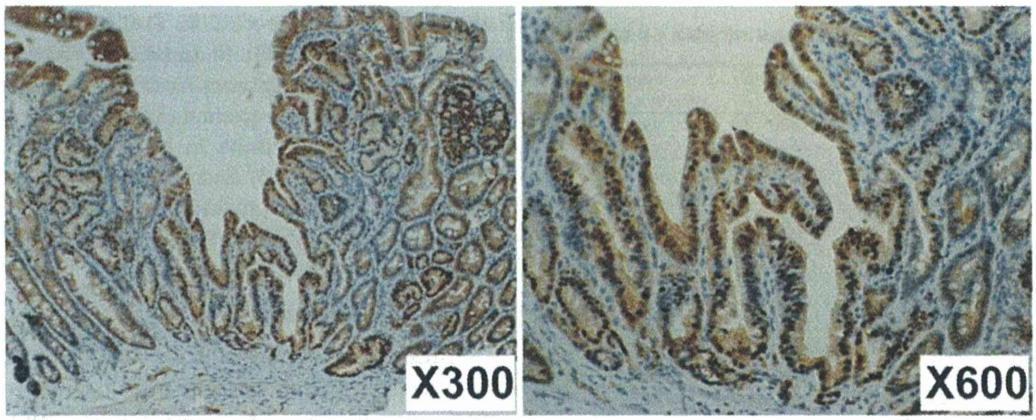

Figure 2. Immunohistochemistry for β-catenin. Many nuclear steins are observed in tumor cells but not in normal cells. The junctions of epithelial cells are also well stained. Magnifications are indicated in lower right of the photos.

Tumors from MAP patients exhibit a significantly increased incidence of G:C to T:A somatic mutations in *APC*. These mutations exclusively occurred in AGAA or TGAA sequences. We examined mutations in the region of *Apc* in which many somatic mutations were identified in tumors from MAP patients, and found 25 somatic mutations in 18 out of 62 tumors. All the mutations were characterized as G:C to T:A transversions, and 68.0% (17 out of 25) of the mutations were found at either AGAA or TGAA sequence. These results are highly consistent with the findings in the tumors developed in MAP patients. We also analyzed *Ctnnb1* for incidence of mutations because *CTNNB1* mutations were found in about a half of colorectal cancers lacking *APC* mutation in humans (25)(29)(30). We identified 36 mutations (35 G:C to T:A transversions and 1 T:A to A:T transversion) in *Ctnnb1* among 62 tumors. All the G:C to T:A transversions detected in *Ctnnb1* occurred at the GAA sequence, 34 in AGAA and one in second G of GGAA sequences. Fifty-four out of 62 (87.1%) tumors developed in $KBrO_3$-treated *Mutyh*-deficient mice carried mutations either in *Apc* or *Ctnnb1*. The immunohistochemistry confirmed β-catenin accumulation in the nuclei of the tumors harboring either *Apc* or *Ctnnb1* mutations. These observations suggest the association between the defect in the Wnt-signaling pathway and multi-tumor formation in *Mutyh*-deficient mice treated with $KBrO_3$. On the other hand, we could not detect any *Kras* mutations in the $KBrO_3$-induced tumors, although *KRAS* mutations in codon12 were frequently observed in the cancers (64%) and adenomas (43%) of MAP patients (37, 38). These differences may be attributed to a short period (16 weeks) for tumor-formation under the consecutive and enhanced oxidative stress in *Mutyh*-deficient mice. It is also possible that *Kras* mutation may occur at a relatively late stage in multistep carcinogenesis, so that the mutation, if any, could not be detected with the methods we applied in this study.

We also examined for *Ctnnb1* mutations in the small intestinal tumors developed in wild-type mice. None of the mutations was G:C to T:A transversion; two were G:C to A:T transitions and one was a 24-bp deletion. Thus, the mutations found in the $KBrO_3$-treated wild-type mice would not result from the mutagenic effects of 8-oxoG. These results indicates that Mutyh can sufficiently suppress the appearance of premalignant cells containing G:C to T:A transversions in wild-type mouse, even under the condition of enhanced oxidative stress.

The small intestinal tumors induced by $KBrO_3$-treatment displayed more mutations in *Ctnnb1* than in *Apc*, although *APC* mutation is mostly common in human colorectal cancer and MAP polyps. GSK3β-phosphoryration sites and their surroundings of β-catenin are highly conserved; there are no difference of amino acid sequence in the region between human and mouse. However, there are some differences in the DNA sequence corresponding to this region. In this study, *Ctnnb1* mutations were frequently found in the codon for Ser33 and the corresponding DNA sequence is TCT in both human and mouse. There is a TTC sequence spanning codon 32 and 33 in mouse *Ctnnb1* but not in human *CTNNB1* because of the difference in codon-usage for Asp32; GAT in mouse but GAC in human. Because AGAA sequences (and its reverse-complementary sequence TTCT) appear to be the target sites for oxidative stress-induced mutagenesis in *MUTYH*-deficient genetic background, it is conceivable that this difference would be a main reason why *Ctnnb1* mutations were predominantly observed in the tumors from *Mutyh*-deficient mice.

The observed high incidence of mutations either in *Apc* or *Ctnnb1* imply that the initial event of tumor-formation would be a dysfunction of Wnt-signal pathway in intestinal epithelial cells of *Mutyh*-deficient mice treated with $KBrO_3$. Our study further demonstrated that in agreement with the findings in MAP, G:C to T:A transversions were detected in the genes controlling Wnt-signal pathway with high frequency, and the mutations predominantly occurred at AGAA or TGAA sequences. Thus, in addition to the multiple tumor-formation in the mutant mice, specificity with respect to the genetic defects and its mutational spectra and specificity are also considerably match to the observations in the multiple colorectal adenomatous polyposis in MAP patients. We, therefore, consider that $KBrO_3$-induced tumorigenesis using *Mutyh*-deficient mice is a useful experimental system for studying on molecular processes in the early development of adenoma and carcinoma in MAP, as well as for examining the preventive and therapeutic approaches for the oxidative stress-induced intestinal tumors (40).

Abbreviations

8-oxoG: 8-oxoguanine; MAP: MUTYH-associated polyposis; GSK3β: glycogen synthase kinase 3 beta

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Competing Interests

The authors have declared that no competing interest exists.

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Genotoxicity of phenacetin in the kidney and liver of Sprague-Dawley *gpt* delta transgenic rats in 26-week and 52-week repeated-dose studies

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ABSTRACT

Transgenic rat mutation assays can be used to assess genotoxic properties of chemicals in target organs for carcinogenicity. Mutations in transgenes are genetically neutral and accumulate during a treatment period; thus, assays are suitable for assessing the genotoxic risk of chemicals using a repeated-dose treatment paradigm. However, only a limited number of such studies have been conducted. To examine the utility of transgenic rat assays in repeated-dose studies, we fed male and female Sprague-Dawley *gpt* delta rats with a 0.5% phenacetin-containing diet for 26 and 52 weeks. A long-term feeding of phenacetin is known to induce renal cancer in rats. Phenacetin administration for 52 weeks in males significantly increased *gpt* (point mutations) mutant frequency (MF) in the kidney, the target organ of carcinogenesis. In the liver, the nontarget organ of carcinogenesis, *gpt* MFs were significantly elevated in phenacetin treatment groups of both genders during 26- and 52-week treatments. Furthermore, sensitive to P2 interference (Spi⁻deletions) MF increased in the liver of both genders following 52-week treatment. MFs were higher after treatment for 52 weeks than after treatment for 26 weeks. Frequencies of phenacetin-induced mutations were higher in the liver than in the kidney, suggesting that the intensity of genotoxicity does not necessarily correlate with the induction of tumor formation. Results from *gpt* delta rat assays of repeated-dose treatments are extremely useful to elucidate the relationship between gene mutations and carcinogenesis in the target organ induced by cancer-causing agents.

1. Introduction

Transgenic mutation assays are a highly valuable tool for assessing in vivo genotoxicity (Nohmi et al., 2000; Nohmi and Masumura, 2005). The technique uses experimental methods that ensure mutations in reporter genes integrated into chromosomes of transgenic rodents can be identified in organs and tissues. Reporter genes are identified following extraction from rodent cells and transfer into bacterial cells via a phage vector. Since mutations can be analyzed at various treatment time points (Lambert et al., 2005) and mutations in reporter genes accumulate over time with repeated treatment (de Vries et al., 1997; Suzuki et al., 1996), transgenic mutation assays provide an ideal technique for assessing the potential genotoxic risk of chemicals dosed repeatedly.

The International Conference on Harmonisation (ICH) guidance S2 (R1) proposes 2 equally suitable approaches to test the genotoxicity of small molecules (ICH, 2011). Option 1 includes the Ames test, an in vitro mammalian cell assay, and in vivo micronuclei assay. Option 2 calls for an additional in vivo test instead of an in vitro study using cultured cells, since the latter

Abbreviations: 6-TG, 6-thioguanine; Cm, chloramphenicol; ICH, International Conference on Harmonisation; MF, mutant frequency; OECD, Organisation for Economic Co-operation and Development; CDER, Center for Drug Evaluation and Research.

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exhibits a comparatively high false-positive rate (Kirkland et al., 2005). One of the promising candidates for the second in vivo test is a transgenic mutation assay (Toyoda-Hokaiwado et al., 2010; Kawamura et al., 2013). Conditions permitting, the ICH guidance recommends that the in vivo genotoxicity assay be integrated into a 28-day repeated-dose toxicity study, similar to the Organisation for Economic Co-operation and Development (OECD) TG488 guideline (Heddle et al., 2000; OECD, 2011; Thybaud et al., 2003), thus reducing the number of animals needed to assess the safety of chemicals. In a genotoxicity assessment of 90 carcinogens, transgenic mutation assays were shown to have a high sensitivity and good positive predictability (Lambert et al., 2005). However, the majority of the 90 carcinogens assessed were strong mutagens that could be used as positive controls in genotoxicity studies, and nearly 70% of the studies had been conducted using a single- or repeated-dose regimen for up to a maximum of 5 days (Lambert et al., 2005). Therefore, it remains unclear how repeat dosing for 4 weeks or longer affects the results of transgenic mutation assays. Indeed, potassium bromate-induced mutations in rat kidney, the target organ for carcinogenesis, were not detected until after 5 weeks of treatment (Umemura et al., 2006).

The *gpt* delta rat is an established transgenic strain that possesses reporter genes for in vivo mutations (Hayashi et al., 2003; Toyoda-Hokaiwado et al., 2010; Kawamura et al., 2013). In the transgenic rodent mutation assay, about 5–10 copies of lambda EG10 DNA carrying the *gpt* (guanine phosphoribosyltransferase) gene of *Escherichia coli* and the *red/gam* genes of lambda phage are integrated per haploid genome of Sprague Dawley rats. After recovery of lambda EG10 phage, point mutations in the *gpt* gene and deletions in the *red/gam* genes are identified by 6-thioguanine and Spi$^-$ (sensitive to P2 interference) selection, respectively (Hayashi et al., 2003). The aim of this study was to determine the relevance of gene mutations in carcinogenicity induced by long-term treatment of phenacetin. To achieve this, we used Sprague-Dawley *gpt* delta rats to detect in vivo mutations of phenacetin in target (kidney) and nontarget (liver) organs for carcinogenesis following repeat-dose treatment for 26 and 52 weeks (Isaka et al., 1979).

2. Materials and methods

2.1. Sprague-Dawley gpt delta rats

All animals were bred at Japan SLC, Inc. (Shizuoka, Japan). The original Sprague-Dawley *gpt* delta transgenic rat strain was developed by Hayashi et al. (2003). The *gpt* delta rat contains approximately 5–10 copies of lambda EG10 transgene in chromosome 4 as a heterozygote (Hayashi et al., 2003).

2.2. Chemicals

Phenacetin (CAS# 62-44-2, purity 98%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Diet containing phenacetin was prepared by mixing phenacetin with CRF-1 powder diet (Oriental Yeast Co., Ltd. Tokyo, Japan).

2.3. Animals and treatment

All animal studies were conducted under the approval of the Animal Care and Utilization Committee of Meiji Seika Pharma Co., Ltd. (study no. S01723). Seven-week-old male and female Sprague-Dawley *gpt* delta rats were acclimated for 7 days before dosing. Rats were individually housed in stainless steel cages, with free access to tap water and CRF-1 diet. The animal rooms were maintained at a temperature of 23 ± 2 °C, a relative humidity of 55 ± 10%, and a light–dark cycle of 12:12 h. Phenacetin-treated *gpt* delta rats (n = 6–9 per group) were fed a diet containing 0.5% phenacetin for 26 and 52 weeks. Control groups (n = 3–5 per group) were fed a diet without phenacetin. The mean daily dose of phenacetin in male and female rats in the present study was estimated to be 202 and 246 mg/(kg · day), respectively. The dose of phenacetin used was similar to the dose used in 2-year cancer bioassays in which renal cell carcinoma or urothelial hyperplasia in kidney pelvis was noted (Isaka et al., 1979; Johansson and Angervall, 1976). At the end of the dosing period, rats were euthanized and necropsied for collection of the kidney, liver, spleen, and urinary bladder. A portion of the collected kidney and liver was immediately frozen in liquid nitrogen and stored at −80 °C. The remaining parts and other organs were fixed in formalin solution for histopathology. The following parameters were monitored: clinical signs, body weight, food intake, hematology, blood chemistry, autopsy findings, organ weight, and histopathology.

2.4. Detection of mutations

The *gpt* and Spi$^-$ assays were conducted in accordance with previously published methods (Nohmi et al., 1996, 2000). Genomic DNA was extracted from liver or kidney using the RecoverEase™ DNA Isolation Kit (Agilent Technologies, Santa Clara, CA), and lambda EG10 phages were recovered from genomic DNA with Transpack® Lambda Packaging Extract (Agilent Technologies). In order to detect point mutations with the *gpt* assay, *Escherichia coli* YG6020 was infected with the phage, spread onto M9 salt plates containing chloramphenicol (Cm) and 6-thioguanine (6-TG), and then incubated for 72 h at 37 °C for selection of the colonies harboring plasmids carrying the Cm acetyltransferase gene and the mutated *gpt* gene. Mutant frequencies (MFs) of the *gpt* gene in the liver and kidney were calculated by dividing the number of confirmed 6-TG resistant colonies by the number of rescued plasmids. In order to detect several types of deletion with the Spi$^-$ assay, rescued phages were incubated with *E. coli* XL-1 Blue MRA for survival titration or with *E. coli* XL-1 Blue MRA P2 for mutant selection. Spi$^-$ MFs in the liver and kidney were calculated by dividing the number of confirmed Spi$^-$ mutant plaques by the number of rescued phages.

2.5. Characterization of gpt mutation spectra

The *gpt* mutant colonies detected using the methods described in Section 2.4 were used for polymerase chain reaction and subsequent DNA sequencing analysis used to identify mutations (Nohmi et al., 1999). The *gpt* mutations were categorized into 4 classes: transition, transversion, deletion, and insertion. Number of the independent mutations was estimated by counting the same mutation from the same animal as one mutation. The entire sequence of lambda EG10 is available at http://www.nihs.go.jp/dgm/dgm3/gptdeltainfo.html

2.6. Statistical analysis

Continuous data expressed as mean ± standard deviation were analyzed by type F analysis. Homogeneity of variance among groups was first tested by Bartlett's test (significance level: 0.05). When variances were homogeneous ($p \geq 0.05$), Student's t test was used to compare control- and treatment-group means (significance level: 0.05). When variances were heterogeneous ($p < 0.05$), Aspin–Welch's t test (significance level: 0.05) was used to compare means after the data were log transformed. EXSAS software version 8.0 was used for statistical comparisons (CAC EXICARE Corporation, Tokyo, Japan). Additionally, statistical comparisons of the mutational spectra were performed using the Adams and Skopek test (significance level: 0.05) (Cariello et al., 1994).

3. Results

3.1. gpt and Spi⁻ mutations induced by phenacetin in the kidney

Phenacetin administered in the diet for 26 weeks had no significant effects on *gpt* and Spi⁻ MF in the kidney compared to controls (Table 1). In male rats administered phenacetin for 52 weeks, *gpt* MF in the kidney increased 3.5-fold compared with control ($p < 0.05$) (Table 2). However, no significant difference was observed in female rats treated for this length of time (Table 2). In the 52-week study, no change occurred in Spi⁻ MF in the kidney of rats of either gender (Table 2).

3.2. gpt and Spi⁻ mutations induced by phenacetin in the liver

In rats treated with phenacetin for 26 weeks, *gpt* MF in the liver increased 11.9- and 4.3-fold in male and female rats, respectively, compared with controls ($p < 0.01$ for both genders) (Table 3). Similarly, *gpt* MF in the 52-week phenacetin treatment groups were elevated 17.6- and 5.0-fold in male and female rats, respectively, compared with controls ($p < 0.01$ for both genders) (Table 4).

Treatment with phenacetin for 26 weeks had no significant effects on Spi⁻ MF in the liver compared with the control groups in either male or female rats (Table 3). However, in rats treated with phenacetin for 52 weeks, Spi⁻ MFs were elevated 5.8- and 2.3-fold compared with controls in male ($p < 0.01$) and female ($p < 0.001$) rats, respectively (Table 4).

3.3. Characterization of gpt mutation spectra

We identified mutations by DNA sequencing analysis to characterize the spectra of *gpt* mutations in the kidney and liver of rats treated with phenacetin for 26 and 52 weeks (Tables 5 and 6). Since the gender difference was not noted in the characteristics of phenacetin-induced mutations, number of mutations was calculated by adding values of male and female rats. In the statistical analysis to compare the mutation spectra between control groups and phenacetin-treated groups, differences in the pattern of mutations were observed in the liver, but not in the kidney, in the 26- and 52-week studies (Supplementary Table 1 and 2). The predominant base substitutions were G:C to A:T transitions in the kidney and liver of phenacetin-treated rats. The G:C to A:T transitions at guanine base pairs 26 and 416 of the *gpt* gene were the most frequently observed mutations in the liver of phenacetin-treated rats (Supplementary Table 2). Transitions at A:T base pairs were also induced in phenacetin-treated groups compared with their respective control groups.

3.4. Evaluation of the toxicity of phenacetin in gpt delta rats

A summary of the toxicity data for rats treated with dietary phenacetin for 26 and 52 weeks is shown in Supplementary Tables 3 and 4. No mortalities or abnormal clinical signs were noted during either dosing periods. No significant changes in body weight, food intake, blood chemistry, and organ weight were noted between phenacetin-treated rats and their respective control groups. White blood cell and neutrophilic leukocyte counts in female rats treated with phenacetin for 26 weeks were slightly reduced. In female rats treated with phenacetin for 52 weeks, no significant change in blood biochemistry was observed compared with the control group. In histopathology assessments, chronic nephropathy was noted in both male and female rats treated with phenacetin for 52 weeks. However, the same finding was also noted in the control group. Bile duct proliferation in the liver and yellow–brown pigment deposition in the spleen was noted in most rats regardless of treatment. No significant changes were observed in the urinary bladder of phenacetin- or control-treated *gpt* delta transgenic rats.

4. Discussion

The aim of the present study was to assess the utility of *gpt* delta transgenic rats in assessing the genotoxicity risk of

Table 1
Mutant frequencies in the kidney of *gpt* delta rats given dietary administration of phenacetin for 26 weeks.

Gender			*gpt*					Spi⁻				
					Mutant frequency (X 10^{-6})					Mutant frequency (X 10^{-6})		
	Treatment (dose)	Animal no.	Total population	No. of mutants		Mean	SD	Total population	No. of mutants		Mean	SD
Male	Control	1	1,311,000	2	1.53			1,047,000	1	0.96		
	(Normal diet)	2	1,021,500	1	0.98			885,000	1	1.13		
		3	1,248,000	0	0.00	0.83	0.77	2,110,500	6	2.84	1.64	1.04
	Phenacetin	4	954,000	0	0.00			1,330,500	2	1.50		
	(0.5%)	5	903,000	1	1.11			1,045,500	2	1.91		
		6	1,203,000	0	0.00			2,076,000	11	5.30		
		7	984,000	1	1.02			990,000	2	2.02		
		8	1,473,000	2	1.36			705,000	1	1.42		
		9	1,093,500	1	0.91			1,108,500	4	3.61		
		10	882,000	3	3.40	1.11	1.14	1,060,500	8	7.54	3.33	2.32
Female	Control	11	807,000	2	2.48			724,500	1	1.38		
	(Normal diet)	12	997,500	2	2.01			1,936,500	2	1.03		
		13	933,000	0	0.00			744,000	1	1.34		
		14	802,500	0	0.00	1.12	1.31	1,203,000	2	1.66	1.35	0.26
	Phenacetin	15	652,500	0	0.00			982,500	1	1.02		
	(0.5%)	16	1,150,500	1	0.87			1,812,000	0	0.00		
		17	1,378,500	3	2.18			915,000	1	1.09		
		18	786,000	4	5.09			723,000	1	1.38		
		19	463,500	2	4.31			507,000	4	7.89		
		20	564,000	1	1.77			891,000	2	2.24		
		21	771,000	1	1.30			942,000	1	1.06		
		22	646,500	2	3.09			1,093,500	5	4.57		
		23	511,500	1	1.96	2.29	1.63	937,500	4	4.27	2.61	2.50

Table 2
Mutant frequencies in the kidney of *gpt* delta rats given dietary administration of phenacetin for 52 weeks.

Gender			*gpt*					Spi$^-$				
					Mutant frequency (X 10^{-6})					Mutant frequency (X 10^{-6})		
	Treatment (Dose)	Animal No.	Total population	No. of mutants		Mean	SD	Total population	No. of mutants		Mean	SD
Male	Control	101	1,146,000	4	3.49			1,879,500	9	4.79		
	(Normal diet)	102	1,696,500	2	1.18			1,311,000	4	3.05		
		103	1,249,500	1	0.80	1.82	1.46	1,672,500	6	3.59	3.81	0.89
	Phenacetin	104	970,500	10	10.30			1,546,500	10	6.47		
	(0.5%)	105	1,455,000	9	6.19			1,695,000	2	1.18		
		106	1,444,500	4	2.77			1,762,500	24	13.62		
		107	1,657,500	12	7.24			1,536,000	6	3.91		
		108	1,039,500	7	6.73			1,558,500	12	7.70		
		109	1,153,500	6	5.20	6.41*	2.48	1,741,500	8	4.59	6.24	4.25
Female	Control	110	1,509,000	7	4.64			1,866,000	8	4.29		
	(Normal diet)	111	1,678,500	4	2.38			2,212,500	5	2.26		
		112	1,314,000	6	4.57			2,172,000	6	2.76		
		113	924,000	1	1.08			1,411,500	7	4.96		
		114	775,500	2	2.58	3.05	1.53	1,239,000	6	4.84	3.82	1.24
	Phenacetin	115	1,542,000	4	2.59			2,211,000	12	5.43		
	(0.5%)	116	1,437,000	4	2.78			1,065,000	14	13.15		
		117	1,113,000	11	9.88			1,752,000	9	5.14		
		118	960,000	6	6.25			1,206,000	11	9.12		
		119	1,041,000	4	3.84			2,212,500	5	2.26		
		120	1,273,500	14	10.99			2,269,500	10	4.41		
		121	1,047,000	5	4.78			1,582,500	5	3.16		
		122	1,158,000	2	1.73			1,912,500	6	3.14		
		123	1,077,000	3	2.79	5.07	3.34	1,708,500	8	4.68	5.61	3.45

* $p < 0.05$ (Student *t*-test).

phenacetin in 2 separate repeated-dose studies. Our key findings were that, compared with controls, 52-week phenacetin treatment induced an increase in *gpt* MF in the target organ (kidney) of carcinogenicity in male, but not in female rats. However, no significant change in *gpt* MF was observed in kidney of rats of either gender in the 26-week study. In contrast, phenacetin caused an increase in *gpt* MF in the liver, a nontarget organ of carcinogenesis, in both genders and both studies. Furthermore, Spi$^-$MF increased in the liver of both genders following 52-week treatment. There was a tendency toward higher *gpt* and Spi$^-$ MFs in the 52-week compared with 26-week treatment groups.

Table 3
Mutant frequencies in the liver of *gpt* delta rats given dietary administration of phenacetin for 26 weeks.

Gender			*gpt*					Spi$^-$				
					Mutant frequency (X 10^{-6})					Mutant frequency (X 10^{-6})		
	Treatment (dose)	Animal no.	Total population	No. of mutants		Mean	SD	Total population	No. of mutants		Mean	SD
Male	Control	1	963,000	5	5.19			964,500	4	4.15		
	(Normal diet)	2	828,000	1	1.21			940,500	7	7.44		
		3	1,462,500	0	0.00	2.13	2.72	2,035,500	4	1.97	4.52	2.76
	Phenacetin	4	1,003,500	18	17.94			1,171,500	10	8.54		
	(0.5%)	5	927,000	27	29.13			1,104,000	8	7.25		
		6	907,500	32	35.26			1,054,500	8	7.59		
		7	1,015,500	14	13.79			1,926,000	11	5.71		
		8	1,534,500	31	20.20			2,179,500	16	7.34		
		9	832,500	36	43.24			1,774,500	13	7.33		
		10	1,323,000	24	18.14	25.39**	10.80	2,265,000	8	3.53	6.75	1.65
Female	Control	11	924,000	2	2.16			1,308,000	4	3.06		
	(Normal diet)	12	847,500	4	4.72			871,500	6	6.88		
		13	1,125,000	1	0.89			1,906,500	4	2.10		
		14	1,111,500	3	2.70	2.62	1.59	2,251,500	4	1.78	3.45	2.35
	Phenacetin	15	1,041,000	12	11.53			1,374,000	12	8.73		
	(0.5%)	16	913,500	12	13.14			1,329,000	9	6.77		
		17	984,000	11	11.18			1,417,500	9	6.35		
		18	1,075,500	12	11.16			1,407,000	8	5.69		
		19	1,008,000	8	7.94			1,252,500	8	6.39		
		20	1,074,000	12	11.17			1,813,500	15	8.27		
		21	1,201,500	3	2.50			1,831,500	4	2.18		
		22	856,500	18	21.02			1,867,500	4	2.14		
		23	1,107,000	14	12.65	11.36**	4.85	2,110,500	6	2.84	5.49	2.52

** $p < 0.01$ (Student *t*-test).

Table 4
Mutant frequencies in the liver of *gpt* delta rats given dietary administration of phenacetin for 52 weeks.

Gender			*gpt*					Spi$^-$				
					Mutant frequency (X 10^{-6})					Mutant frequency (X 10^{-6})		
	Treatment (dose)	Animal no.	Total population	No. of mutants		Mean	SD	Total population	No. of mutants		Mean	SD
Male	Control (Normal diet)	101	898,500	2	2.23			1,548,000	1	0.65		
		102	972,000	1	1.03			1,032,000	2	1.94		
		103	1,146,000	5	4.36	2.54	1.69	1,132,500	3	2.65	1.74	1.02
	Phenacetin (0.5%)	104	1,212,000	26	21.45			1,821,000	19	10.43		
		105	411,000	12	29.20			420,000	7	16.67		
		106	990,000	23	23.23			1,486,500	14	9.42		
		107	1,026,000	84	81.87			1,584,000	14	8.84		
		108	907,500	52	57.30			1,294,500	13	10.04		
		109	955,500	52	54.42	44.58**	23.99	1,305,000	6	4.60	10.00*	3.89
Female	Control (Normal diet)	110	936,000	7	7.48			1,008,000	4	3.97		
		111	1,015,500	7	6.89			855,000	2	2.34		
		112	1,131,000	4	3.54			1,761,000	4	2.27		
		113	858,000	3	3.50			1,224,000	4	3.27		
		114	1,146,000	3	2.62	4.80	2.21	1,704,000	6	3.52	3.07	0.75
	Phenacetin (0.5%)	115	1,071,000	13	12.14			826,500	5	6.05		
		116	1,227,000	8	6.52			739,500	3	4.06		
		117	933,000	18	19.29			534,000	3	5.62		
		118	789,000	23	29.15			2,076,000	15	7.23		
		119	976,500	23	23.55			903,000	7	7.75		
		120	1,321,500	21	15.89			1,444,500	6	4.15		
		121	1,057,500	44	41.61			946,500	11	11.62		
		122	1,023,000	27	26.39			1,375,500	12	8.72		
		123	1,051,500	44	41.84	24.04**	12.22	1,033,500	9	8.71	7.10***	2.43

* $p < 0.01$ (Student t-test).
** $p < 0.01$ (Aspin–Welch t-test).
*** $p < 0.001$ (Aspin–Welch t-test).

Phenacetin is an analgesic, widely used until 1980s. Its use has declined due to its potential to induce kidney damage. Additionally, it is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals (National Toxicology Program, 2011). In a carcinogenicity study, 18-month dietary administration of 2.5% phenacetin induced renal cell carcinoma of the kidney pelvis and transitional cell carcinoma of the urinary bladder in rats. Furthermore, tumor incidence was greater in males than in females (Isaka et al., 1979). In another long-term carcinogenicity study, dietary phenacetin administered for 117 weeks at 0.535% induced renal pelvic tumors in male rats (Johansson, 1981). Proliferative lesions of the renal pelvis were observed, but tumors were not noted in female rats (Johansson and Angervall, 1976). In the present study, increases in *gpt* MF in the kidney were only observed in male rats (Table 2). This apparent gender difference in susceptibility to genotoxicity may be associated with that of phenacetin-induced carcinogenicity. Two overlapping hypotheses for the mechanisms of renal cancer induced by phenacetin have been proposed (Stewart et al., 1999). The first is that weak genotoxicity plays an important role and the second is that renal papillary injury leads to pelvic tumors. In humans, there are numerous case reports of renal failure among patients who had consumed large amounts of phenacetin (Grimlund, 1963; Carro-Ciampi, 1978; Inglis, 1966). Most patients

Table 5
Classification of the independent *gpt* mutations in the kidney of *gpt* delta rats administered dietrary phenacetin for 26 and 52 weeks.

Type of mutation	Number of mutations[a]							
	26 weeks				52 weeks			
	Control		0.5% phenacetin		Control		0.5% phenacetin	
	No.	%	No.	%	No.	%	No.	%
Base substitutions								
Transitions								
G:C → A:T	3	50	12	63.2	4	22.2	21	67.7
A:T → G:C	0	0.0	2	10.5	0	0.0	2	6.5
Transversions								
G:C → T:A	1	16.7	1	5.3	7	38.9	3	9.7
G:C → C:G	0	0.0	1	5.3	1	5.6	1	3.2
A:T → T:A	0	0.0	1	5.3	1	5.6	1	3.2
A:T → C:G	0	0.0	1	5.3	1	5.6	2	6.5
Deletions								
−1 bp	1	16.7	0	0.0	2	11.1	0	0.0
>2 bps	1	16.7	1	5.3	1	5.6	0	0.0
Insertions	0	0.0	0	0.0	1	5.6	1	3.2
Total	6	100.0	19	100.0	18	100.0	31	100.0

[a] Number of mutations was calculated by adding the values of male and female rats.