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Tissue-specific and time-dependent clonal expansion of ENU-induced mutant cells in *gpt delta* mice

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Running title

Tissue-dependent clonal expansion in ENU-treated *gpt* mice

ABSTRACT

DNA mutations play a crucial role in the origins of cancer, and the clonal expansion of mutant cells is one of the fundamental steps in multistage carcinogenesis. In this study, we correlated tumor incidence in B6C3F1 mice during the period after exposure to *N*-ethyl-*N*-nitrosourea (ENU) with the persistence of ENU-induced mutant clones in transgenic *gpt* delta B6C3F1 mice. The induced *gpt* mutations afforded no selective advantage in the mouse cells and could be distinguished by a mutational spectrum that is characteristic of ENU treatment. The *gpt* mutations were passengers of the mutant cell of origin and its daughter cells and thus could be used as neutral markers of clones that arose and persisted in the tissues. Female B6C3F1 mice exposed for one month to 200 ppm ENU in the drinking water developed early thymic lymphomas and late liver and lung tumors. To assay *gpt* mutations, we sampled the thymus, liver, lung, and small intestine of female *gpt* delta mice at 3 days, 4 weeks, and 8 weeks after the end of ENU exposure. Our results reveal that, in all four tissues, the ENU-induced *gpt* mutations persisted for weeks after the end of mutagen exposure. Clonal expansion of mutant cells was observed in the thymus and small intestine, with the thymus showing larger clone sizes. These results indicate that the clearance of mutant cells and the potential for clonal expansion during normal tissue growth depends on tissue type and that these factors may affect the sensitivity of different tissues to carcinogenesis.

INTRODUCTION

Cancer is a multicellular disease that nonetheless evolves via genetic events that occur in single cells [Kinzler and Vogelstein 1996], which underscores the critical role of clonal expansion. For example, an acquired mutation that drives the further expansion of a mutant clone, such as the mutation of *p53* in squamous-cell carcinoma [Brennan et al. 1995] or of *Kras* in lung cancer [You et al. 1989], simultaneously increases the pool of cells susceptible to additional mutations. Assessing mutation frequency *in vivo* requires the use of a reporter system to avoid analyzing hundreds of thousands of cells in parallel by DNA sequencing or chromosomal analysis, among other techniques. Most *in vivo* mutation assay systems rely on detecting mutations that produce functional inactivation of the reporter (e.g., MutaTM mouse) [Gossen et al. 1989], which can be improved further when mutations in a repressor target gene (*lacI*) modulate gene expression in a reporter gene (*lacZ*) (e.g., Big Blue® mouse and rat) [Kohler et al. 1991]. In this scheme, a signal is produced only when the repressor gene harbors an inactivating mutation(s), thus simplifying the screening method. This use of an *ex vivo* reporter system to screen DNA from the cells of interest couples the advantages of *in vivo* responses with the ease of assessing large numbers of cells using well-established cloning techniques. *gpt* delta mice may be used to detect mutations in the *gpt* transgene, such as missense, deletion and frameshift mutations, which inactivate guanine phosphoribosyltransferase (Gpt). Selection is based on the resultant failure to incorporate the cytotoxic 6-thioguanine (6-TG) into the DNA of the host bacteria [Nohmi et al. 1996]. As the *gpt* reporter gene is only 456 bp in length, identifying mutations by sequencing is relatively easy.

The potent alkylating mutagen *N*-ethyl-*N*-nitrosourea (ENU) is representative of the *N*-nitroso compounds that are found in foodstuffs, tobacco smoke, and other environmental sources. ENU directly reacts with DNA to generate a variety of modified bases that give rise to a well-defined spectrum of mutational events (G:C to A:T transitions, A:T to G:C transitions, and A:T to T:A transversions) that become fixed into the genome by DNA replication [Ellison et al. 1989; Klein et al. 1990; Bhanot et al. 1992; Jansen et al. 1994].

Mutant frequency is determined as the fraction of **transgenes** within a tissue carrying a mutation in the reporter gene. The mutant frequency is the primary metric determined by most transgenic rodent assays to assess the potency of various mutagens, including ENU [Cosentino and Heddle 1996;

Wang et al. 2004]. Douglas et al. hypothesized that increases in mutant frequency after ENU treatment were the result of mutant stem cell expansion before eventual clearance of the cells that sustained such clones [Douglas et al. 1996]. For genotoxicity assays, clonal expansion of mutant cells after treatment can complicate comparisons of the efficacy of different agents—so much so that standardized times for the assessment of mutant frequency have been recommended [Cosentino and Heddle 2000; Hayashi et al. 2003; Thybaud et al. 2003]. Other studies have examined the correlation between mutant frequency and tumor incidence [Hakura et al. 1998; Nagao et al. 1998] and showed that, although the initial mutant frequency is increased in the target organs of carcinogenesis, the magnitude of mutant frequencies across organs does not correlate well with organ-specific tumor susceptibility. Clonal expansion of mutant cells may complicate genotoxic assays, but it is probably important to account for its effects in studies that attempt to correlate organ-specific tumor susceptibility with other parameters, such as mutation induction.

However, previous studies have rarely focused on the clonal expansion of mutant cells in the medium-to-long term after mutagen exposure. Herein, we used the *gpt* delta transgenic mouse model to detect point mutations in the *gpt* reporter gene by 6-TG selection of *Escherichia coli* transfected with the transgene DNA isolated from mouse tissues at various times after ENU treatment. Because the *gpt* mutations offer no selective advantage in mouse cells, show considerable diversity across individual events, and have a mutational spectrum characteristic of ENU treatment, they act as passengers that mark the cell of origin and its daughter clone(s) if they persist in the tissue. After a 4-week exposure of young mice to ENU, we assessed the size of mutant clones at defined intervals in the post-treatment period to determine the kinetics of clonal expansion in four tissues—thymus, small intestine, liver, and lung. In parallel, we monitored tumor incidence in these tissue types in the same mouse strain to determine the relationship between clonal expansion and organ-specific tumor susceptibility.

Tumor incidence in ENU-treated mice showed that thymic lymphomas appeared at ~110 days after exposure, whereas lung, liver, and small intestinal tumors arose after ~450 days. In all four tissues, the results of the *gpt* mutation assays revealed that the ENU-induced *gpt* mutations persisted for weeks after the end of mutagen exposure. We observed clonal expansion of mutant cells in the thymus and small intestine with larger clone sizes apparent in the thymus, consistent with the

observed sensitivity of the thymus to ENU-induced tumorigenesis.

MATERIALS AND METHODS

Mice

Female B6C3F1/Crlj mice were obtained from Charles River (Kanagawa, Japan). Female B6C3F1 *gpt* delta mice were obtained by mating male C3H/HeNCrI/Crlj mice (Charles River, Kanagawa, Japan) and female C57BL/6JJmsSlc-Tg(*gpt* delta) mice (Japan SLC, Shizuoka, Japan). Mice were housed five to a cage and maintained under controlled temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$), and lighting (12 h dark-light cycle). Mice were fed a standard laboratory diet, MB-1 (Funabashi Farm Co., Ltd., Chiba, Japan), and water *ad libitum*.

Exposure to ENU

We used mice younger than those in Standard Transgenic Rodent Mutation assay study designs (OECD Test Guideline 488; [OECD 2013]) to determine the effect of carcinogens at early ages. At age 4 weeks, mice were divided into two groups: one was exposed to 200 ppm ENU (Nacalai Tesque, Kyoto, Japan) in drinking water for four consecutive weeks (opaque bottles protected ENU from light exposure and were refreshed every third or fourth day) [Yamauchi et al. 2008], and the control group received untreated drinking water. For carcinogenesis assays, 50 ENU-treated and 50 untreated (control) B6C3F1 female mice were maintained under the experimental conditions for the remainder of their lifespan. Mice were sacrificed when they became moribund, and each underwent a detailed autopsy to assess the incidence of visible tumors. For *gpt* mutation assays, groups of five B6C3F1 *gpt* delta mice were sacrificed at 3 days, 4 weeks, and 8 weeks after the end of ENU treatment (corresponding to 8 weeks and 3 days, 12 weeks, and 16 weeks of age); the same protocol was followed for control mice. At each time point, tissues from the thymus, small intestine mucosa (scraped from the surface of the duodenum and jejunum), lung, and liver were collected and frozen immediately in liquid nitrogen before storage at -80°C . These experiments were conducted in compliance with the guidelines for animal experiments of the National Institute of Radiological Sciences concerning the care and use of laboratory animals.

***gpt* Mutation Assay**

Pieces of the frozen tissue were homogenized before isolation of high-molecular-weight DNA using the RecoverEase DNA isolation kit (Agilent Technologies, Santa Clara, CA, USA). The *gpt* assays were performed [Nohmi et al. 2000]. Briefly, for each mouse tissue sample, the lambda shuttle vector and *gpt* reporter gene DNA were incorporated into λ EG10 phages using the Transpack Packaging Extract (Agilent Technologies). The packaged sample was titrated to determine the total number of plaques as follows: 10 μ L of a 1:200 dilution (or more dilute as appropriate) of packaged sample was added to 200 μ L of *E. coli* XL1BlueMRA suspension (OD = 2.0; in duplicate) before plating on agar plates that were incubated at 37°C overnight. The packaged sample was also plated in the same quantities as above on a suspension of *E. coli* YG6020, which carries the gene for Cre recombinase. In the phage DNA, the gene for chloramphenicol resistance, linearized plasmid DNA, and *gpt* are located between two loxP sites, and they are released together by Cre-loxP recombination as a multicopy-number plasmid. *E. coli* cells harboring the *gpt*-carrying plasmid were selected based on chloramphenicol resistance by plating on agar containing M9 salts plus chloramphenicol medium. Plates were incubated at 37°C for 3 days. This colony count provided the total number of *E. coli* cells harboring *gpt* transgenes. For isolating mutant colonies, 60 μ L of undiluted packaged sample was similarly added to 200 μ L of the *E. coli* YG6020 suspension (in five replicates) except that they were plated under non-permissive conditions on plates containing M9 salts plus chloramphenicol plus 25 μ g/mL 6-TG. This resistant colony count provided the number of *E. coli* cells harboring inactivating *gpt* mutations. The mutant colonies were sampled to recover DNA from each clone.

The mutant frequency, corresponding to the number of transgenes, was calculated by dividing the number of mutant colonies by the total number of colonies. The mutation frequency was calculated by dividing the number of unique mutations by the total number of colonies (for all calculations, the respective dilution factors were taken into account). Furthermore, to focus on the kinetics of mouse cell survival after exposure to ENU, the mutant frequency per cell was calculated based on 40 copies of the transgene per cell [Masumura et al. 2015]. Each cell was assumed to contain no more than one mutant transgene in its 40 transgene cassette; thus we converted mutant transgene frequency to mutant cell frequency as follows:

Mutant cell frequency = (Number of mutants/(Total number of colonies/40)) [Fig. 1(A)],

Mutation **cell** frequency = (Number of unique mutations/(Total number of colonies/40)).

PCR and DNA Sequencing Analysis of *gpt*

A 739-bp DNA fragment containing *gpt* was amplified by PCR from the mutant colony DNA as described [Yamauchi et al. 2008]. Briefly, PCR products were processed with ExoSAP-IT PCR Product Cleanup Reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) before the cycle-sequencing reaction using Big Dye Terminator v3.1 (Applied Biosystems) and the sequencing primer (5'-ATCTCTATAATCTCGCGCAACC-3'). The analysis was carried out with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Estimating Clone Size

A clone was defined as two or more cells from a single tissue of a single mouse carrying the identical *gpt* mutation. Clone size was calculated as the ratio of clone cells to total cells in an individual mouse, as defined by the following formula:

Clone size = Mutant **cell** frequency $\times$ (Number of identical mutant cells (DNA sequenced)/Total number of mutant cells (DNA sequenced)) $\times 10^4$.

The equation gives the estimated number of clone cells per 10^4 cells [Fig. 1(B)].

Statistical Analysis

The statistical significance of the difference between the survival curves for ENU-treated and control groups was evaluated with the log-rank test. Mutant **cell** frequency data were log-transformed before statistical analysis. Statistical significance of the mutant **cell** frequency was evaluated with the Student's or Welch's *t*-test after the *F*-test. $p < 0.05$ was considered statistically significant. Differences in mutation spectra were estimated with the Adams-Skopek test [Adams and Skopek 1987; Cariello et al. 1994; Ikehata et al. 2008].

RESULTS

Tumor Incidence in B6C3F1 Mice

The overall survival curves for ENU-treated and control mice are shown in Fig. 2(A). The lifespans

of ENU-treated mice were significantly shorter compared with those of control mice ($p < 0.001$, log-rank test), and all ENU-treated mice were dead by 600 days. We examined the incidence of death from thymic lymphoma and tumors of the small intestine, lung, and liver in ENU-treated B6C3F1 mice [Fig. 2(B)]. Thymic lymphomas were observed in approximately half of the mice surviving to 600 days and appeared at 100 days whereas lung tumors appeared much later (~450 days) and were present in all mice that survived >450 days. Liver tumors appeared at approximately the same time as the lung tumors and were found in half of the mice surviving >450 days, whereas there was only a single small intestinal tumor. No tumors were observed in the control mice over the period equal to the longest lifespan in the ENU-treated group.

Mutant Cell Frequency in *gpt* delta B6C3F1 Mice

B6C3F1 mice carrying the *gpt* reporter gene were exposed to the same ENU or control conditions used in the tumor incidence experiment, but for the *gpt* mutation assays, DNA from the thymus, small intestine, lung, and liver tissues was isolated at age 8, 12, or 16 weeks. In each of the four tissues, the mean *gpt* mutant cell frequencies in control mice ranged from 0.4×10^{-4} to 4.5×10^{-4} (Fig. 3, Tables I–IV) across the three age categories tested. The thymus showed ENU-induced *gpt* mutations immediately after the end of the treatment period (mean 83.1×10^{-4}), and the mutant cell frequency decreased with age from 8 to 16 weeks ($p < 0.05$; Student's *t*-test). However, none of the ENU-treated mice had thymus mutant cell frequencies fall to less than double the highest frequency observed in the control mice. The small intestine showed higher mutant cell frequencies than the other tissues at the end of the treatment period (mean 181.5×10^{-4}), and these levels persisted in each of the mice up to 8 weeks after the end of ENU treatment. The lung and the liver showed increases in the mutant cell frequencies at the end of ENU treatment (mean 47.5×10^{-4} and 23.7×10^{-4} , respectively) that were lower than those in thymus and small intestine at the same age; these frequencies were maintained at comparable levels up to 8 weeks after the end of ENU treatment. Mutant cell frequencies of each tissue of ENU-treated mice were significantly higher than those of control mice (all four tissues, $p < 0.001$; Student's or Welch's *t*-test).

Mutation Cell frequency and Spectra of *gpt* Mutations

In three of the four tissues, there were no significant differences between the mutation cell frequencies and the mutant cell frequencies; the exception was for the thymus in the ENU-treated mice (Tables I–IV). In the thymus, the mutation cell frequency at 16 weeks of age was much lower than the mutant cell frequency because the ratio of identical mutants/total number of mutants sequenced had increased over this period. The mutation cell frequency with ENU-induced mutations are shown in Fig. 4. The frequency for A:T to T:A transversions in the thymus decreased during the period from age 8 to 16 weeks ($p < 0.05$; Student's *t*-test). The mutation cell frequency with ENU-induced mutations was maintained over a period of at least 8 weeks for the other tissues examined.

The classes and distribution of *gpt* mutations in the tissues of ENU-treated mice are shown in Tables V–VIII, Supplementary Tables I–V, and Supplementary Fig. 1. The mutations were characteristic of the transition and transversion events (G:C to A:T and A:T to G:C transitions, and A:T to T:A transversions) known to be induced by ENU. The mutation spectra were significantly different between ENU-treated and control mice (thymus, $p < 0.05$, small intestine, lung, and liver, $p < 0.001$; Adams-Skopek test) but not among the four tissues themselves in ENU-treated mice.

Estimating Clone Size

The mutant cell frequencies of each clone and clone sizes in the four tissues over time in individual mice are shown in Figs. 5 and 6. The data reveal that, in addition to having the lowest mutant cell frequencies overall, the liver and lung contained mostly unique mutations and small clones of mutant cell. The small intestine had the highest mutant cell frequency overall with two distinct classes—those that remained as single events/small clones, and those that expanded to make larger clones (2–6 times the smaller clone size). Interestingly, these clone sizes were approximately equal to each other with no dominant clone(s) emerging. In contrast, the thymus had lower mutant cell frequencies overall but was almost completely dominated by two or three large clones of mutant cells in each thymus. In some mice, the mutant clone size in the thymus had expanded to 10–20 times the size of the smallest clone [Fig. 6(B)].

DISCUSSION

In this study, we examined four organs—the thymus, small intestine, lung, and liver—for the post-treatment effects of ENU exposure on tumor incidence and the clonal expansion of *gpt* mutations in mouse strains B6C3F1 and *gpt* delta B6C3F1, respectively. ENU induced thymic lymphomas with short latency, and for those mice that survived past ~500 days of age, all succumbed to liver and lung tumors. The mutation spectra confirmed that the *gpt* mutations in ENU-treated mouse tissues were characteristic of exposure to this alkylating agent [Ellison et al. 1989; Klein et al. 1990; Bhanot et al. 1992]. The specific nature of the ENU-mutations suggested that they arose during the exposure period and persisted up to 8 weeks later, rather than arising *de novo* owing to genomic instability. Additionally, for the four organs, the mutation spectra in ENU-treated mice showed similar patterns, except the ratio of G:C to A:T transitions at non-CpG sites in the liver tended to be lower than other tissues, and nucleotide base substitutions were distributed at similar positions along the DNA sequence. Taken together, these results suggest that the DNA repair system does not differ among these organs. Because the *gpt* mutations provide no selective advantage in mouse cells, the mutations may be used as neutral markers to track the persistence and expansion of cell clones. Because *gpt* mutations occur randomly and have no function [Nohmi et al. 1996], expansion of *gpt* clones may be expected to follow normal growth kinetics within a given tissue. The clone sizes we report here are estimates and do not reflect the actual number of clone cells, and the predictive value of clone size varies depending on the proportion of mutants sequenced (a limiting factor when the mutant frequencies are very high). Nonetheless, clone size gives a relative measure with which to compare the production, expansion, and persistence of clones within and across tissues.

Several factors may influence the *gpt* mutation cell frequencies in the four tissues at the end of the exposure period. These include the relative concentrations of ENU to which the mice were exposed *in vivo*, the initial production of alkylated bases, the efficiency of faithful repair, the rate of mutation fixation, and the cellular lifespan within each tissue. The small intestine, in particular, may be expected to be exposed to greater concentrations of ENU when ENU is administered in drinking water, although the relative impact of each variable listed above was not analyzed. As opposed to the lung and liver, which showed the lowest mutant cell frequencies overall, the small intestine showed evidence of clonal expansion and the highest levels of post-exposure *gpt* mutations. These elevated

frequencies are in contrast to the lack of ENU-induced small intestinal tumors in the same mouse strain. These data illustrate that the tissue dependence of cancer risk is not limited to the initiation stage of mutation. The villus cells of the small intestine are shed into the lumen only a few days after they differentiate from stem cell progenitors and begin migrating from the base of the crypt [Cameron 1971; Tesori et al. 2013]. The persistence and likely expansion of *gpt* mutant cells over many weeks suggest that the observed mutations arose either in stem/progenitor cells or long-lived accessory cells that are not part of the high-turnover cycle of the villus. It is thus reasonable to assume that the majority of *gpt* mutations that had been induced in the migrating villus cells were already cleared before the first time point. If so, this would explain why no clearance of mutant cells was observed despite the high cell turnover of the small intestine. Furthermore, there was oligoclonal expansion in the small intestine without the establishment of dominant clones, a restriction that may be explained by the relative size of the physical niche in which the long-lived cells reside.

The risk of cancer in the post-mutation phase depends on the combined effects of a number of carcinogenic steps required to achieve malignancy in different cells types, the ability of a single cell to establish a long-lived clone in a given tissue environment, and the physiological barriers to malignancy in each organ. The results for the thymus are particularly informative. They demonstrate both the general loss of *gpt* mutants over the weeks following the end of ENU exposure and rare cases in which a putative single mutation event survives in a cell that, as part of normal tissue development, was destined to promote clonal expansion. These data are consistent with the nature of the thymus, which undergoes continual repopulation with progenitors from the bone marrow and efflux of mature T cells to the periphery [Shortman et al. 1990; Kawamoto et al. 2003]. In cases in which the mutation is randomly induced in a maturing T cell, all daughter cells are likely to be removed by normal T-cell selection processes or eventually emigrate from the thymus. When the mutation arises in an early thymic immigrant or progenitor cell, however, the mutation may undergo massive clonal expansion as part of the normal T-cell development process, providing a large pool of cells susceptible to subsequent mutagenic hits even if the initial mutation was of limited potency. Carcinogenic events in the thymus may thus 'hitchhike' along with the natural process of T-cell expansion and selection to generate a long-lived clone of sufficient size to allow the acquisition of many more mutations than may occur in another tissue such as the small intestine. Although its

incidence may be exceedingly rare, it follows that a spontaneous *gpt* mutation in a thymic stem/progenitor cell may similarly form a large persistent clone, similar to those observed after ENU exposure, and that such a clone could be found if enough mice were analyzed.

In contrast, the lung and the liver showed lower mutant cell frequencies and smaller clone sizes, and these tissues developed tumors comparatively late in life in ENU-treated mice. Because lung and liver are very low-turnover tissues, mutant clones may expand only slowly in the 8 weeks after ENU treatment. Therefore, mutant clones in such tissues would likely have to acquire a significant growth advantage to expand to form a tumor.

In conclusion, the data reported here demonstrate that even when a mutation confers no growth/survival advantage, mutated cells can survive for at least several weeks in a range of tissues, with clearance of mutant cells dependent on the tissue, and with a potential for clonal expansion in accord with normal tissue growth kinetics. Although mutant cell frequencies would not necessarily be expected to correlate with tumor susceptibility, there may be links between the probability of tumor formation and the potential for rapid clonal expansion of weakly oncogenic mutations, or even pre-functional first hits, as appears to be the case for the thymus in this study. The proposed approach of tracking a neutral marker may be useful for comparing the relative persistence kinetics of clones across tissues or within the same tissue at different ages. Finally, it must be emphasized that when a mutation confers a direct advantage that promotes clonal expansion, the kinetics of a neutral marker such as those observed in this study may be superseded by malignant forces that would be highly dependent on the nature of the tissue and the effect of the mutation.

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AUTHOR CONTRIBUTIONS

Drs. Shimada and Kakinuma designed the study. Dr. Yamauchi prepared animals. Mr. Nakayama, Ms. Masuda, Mr. Kaneko, and Drs. Sawai and Yamauchi performed the experiments and collected the data. Drs. Shimada, Tachibana, Blyth, and Kakinuma advised the people carrying out the experiments. Mr. Nakayama prepared draft figures and tables. Mr. Nakayama prepared the manuscript draft with important intellectual input from Drs. Blyth and Kakinuma. All authors approved the final manuscript. Mr. Nakayama and Dr. Kakinuma enabled access to the study data.

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FIGURE LEGENDS

Fig. 1. Diagram of the method for estimating clone size. (A) Mutant **cell** frequency from a *gpt* **delta** mouse was estimated based on the premise that one cell from a *gpt* **delta** mouse has 40 copies of the transgene. (B) Clone size in the individual mouse was estimated on the basis of mutant **cell** frequency and the results of sequence analysis of mutant clones. Details concerning the estimation of clone size are described in Materials and Methods. The estimated clone size of the smallest detectable clone (one unique sequence) in each case depends on the mutant **cell** frequency and the number of clones sequenced.

Fig. 2. (A) Kaplan-Meier survival curves that compare ENU-treated and control groups. The lifespan of ENU-treated mice was significantly shorter ($p < 0.001$, log-rank test). (B) Kaplan-Meier incidence curve for tumor deaths from four competing causes in ENU-treated mice. Mice were sacrificed when they were moribund, and tumors were diagnosed at autopsy. Censoring marks indicate when the number of mice at risk for a given tumor type was reduced by deaths attributable to a different cause. Control mice not exposed to ENU did not die of these four tumor types until after 600 days of age. Numbers in parentheses: the total number of tumors observed for each tissue type. Red tick marks: deaths within the first year attributed to factors other than the tumors under study. These mortalities were, in most cases, the result of ovarian and uterine tumors (not analyzed).

Fig. 3. Tissue specificity of *gpt* mutant **cell** frequencies in control and ENU-treated mice. The *gpt* mutant **cell** frequencies for the tissues of each mouse are plotted as points on a logarithmic scale because the frequencies in individual mouse tissues differed by >100 -fold between the ENU (gray diamonds) and control groups (white diamonds). The mutant **cell** frequency of each tissue in the ENU-treated group was significantly higher in comparison with the control group at all time points ($p < 0.01$). Large black bar: the mean of associated mutant **cell** frequencies; small black bars: 95% confidence interval.

Fig. 4. Tissue specificity in mice treated with ENU of *gpt* mutation frequencies for characteristic ENU-induced mutations. **The mutation cell frequency with ENU-induced mutations** was calculated at each time point for each tissue by subtracting the mean background frequency for the three

mutation classes from the mean frequencies in ENU-treated mice. When an identical mutation was observed multiple times in a tissue, it was assumed to be part of a clonal expansion that originated from a single mutation; thus, it was counted only once to avoid overestimating its frequency. Note that the *gpt* assay only detects inactivating mutations, and thus it does not include the frequency of silent mutations. The A:T to T:A transversions at 16 weeks of age in the thymus decreased compared with those present at 8 weeks of age ($p < 0.05$; Student's *t*-test).

Fig. 5. Specific mutant **cell** frequency of the repeatedly observed mutations (among the sequenced mutants in the same tissue sample) in individual ENU-treated mice. Each clone is represented by a differently shaded box. Box height: the relative sizes of the different clones; bar at the top of each box: the overall mutant **cell** frequency including all clones plus the single mutant sequenced. a: All mutant sequences were identical—that is, from a single clone. N.D.: no data. Mouse IDs: correspond to those listed in Tables I–IV.

Fig. 6. Tissue specificity of the number of mutations based on clone size in ENU-treated mice. (A) Clone size was estimated for each tissue of ENU-treated mice pooled across all mice for all three time points (thymus: $n = 15$, small intestine: $n = 14$, lung: $n = 15$, liver: $n = 15$). Arrows denote the locations of rare but very large clones in the thymus. (B) Data for the same thymus but with a smaller scale to compare sizes of the large clones.

Supplementary Fig. 1. Distribution of independent mutations in the *gpt* sequences of individual mice (8–16 weeks old). Letters above main sequence: nucleotide changes in ENU-treated mice; letters below main sequence: nucleotide changes in negative controls. Underline of main sequence: CpG site.

Fig. 1. Diagram of the method for estimating clone size. (A) Mutant cell frequency from a *gpt* delta mouse was estimated based on the premise that one cell from a *gpt* delta mouse has 40 copies of the transgene. (B) Clone size in the individual mouse was estimated on the basis of mutant cell frequency and the results of sequence analysis of mutant clones. Details concerning the estimation of clone size are described in Materials and Methods. The estimated clone size of the smallest detectable clone (one unique sequence) in each case depends on the mutant cell frequency and the number of clones sequenced.

100x160mm (300 x 300 DPI)

Fig. 2. (A) Kaplan-Meier survival curves that compare ENU-treated and control groups. The lifespan of ENU-treated mice was significantly shorter ($p < 0.001$, log-rank test). (B) Kaplan-Meier incidence curve for tumor deaths from four competing causes in ENU-treated mice. Mice were sacrificed when they were moribund, and tumors were diagnosed at autopsy. Censoring marks indicate when the number of mice at risk for a given tumor type was reduced by deaths attributable to a different cause. Control mice not exposed to ENU did not die of these four tumor types until after 600 days of age. Numbers in parentheses: the total number of tumors observed for each tissue type. Red tick marks: deaths within the first year attributed to factors other than the tumors under study. These mortalities were, in most cases, the result of ovarian and uterine tumors (not analyzed).

100x160mm (300 x 300 DPI)

Fig. 3. Tissue specificity of *gpt* mutant cell frequencies in control and ENU-treated mice. The *gpt* mutant cell frequencies for the tissues of each mouse are plotted as points on a logarithmic scale because the frequencies in individual mouse tissues differed by >100-fold between the ENU (gray diamonds) and control groups (white diamonds). The mutant cell frequency of each tissue in the ENU-treated group was significantly higher in comparison with the control group at all time points ($p < 0.01$). Large black bar: the mean of associated mutant cell frequencies; small black bars: 95% confidence interval.

190x75mm (300 x 300 DPI)

Accepted

Fig. 4. Tissue specificity in mice treated with ENU of *gpt* mutation frequencies for characteristic ENU-induced mutations. The mutation cell frequency with ENU-induced mutations was calculated at each time point for each tissue by subtracting the mean background frequency for the three mutation classes from the mean frequencies in ENU-treated mice. When an identical mutation was observed multiple times in a tissue, it was assumed to be part of a clonal expansion that originated from a single mutation; thus, it was counted only once to avoid overestimating its frequency. Note that the *gpt* assay only detects inactivating mutations, and thus it does not include the frequency of silent mutations. The A:T to T:A transversions at 16 weeks of age in the thymus decreased compared with those present at 8 weeks of age ($p < 0.05$; Student's t-test).

89x89mm (300 x 300 DPI)

Fig. 5. Specific mutant cell frequency of the repeatedly observed mutations (among the sequenced mutants in the same tissue sample) in individual ENU-treated mice. Each clone is represented by a differently shaded box. Box height: the relative sizes of the different clones; bar at the top of each box: the overall mutant cell frequency including all clones plus the single mutant sequenced. a: All mutant sequences were identical—that is, from a single clone. N.D.: no data. Mouse IDs: correspond to those listed in Tables I–IV.

196x209mm (300 x 300 DPI)

Fig. 6. Tissue specificity of the number of mutations based on clone size in ENU-treated mice. (A) Clone size was estimated for each tissue of ENU-treated mice pooled across all mice for all three time points (thymus: n = 15, small intestine: n = 14, lung: n = 15, liver: n = 15). Arrows denote the locations of rare but very large clones in the thymus. (B) Data for the same thymus but with a smaller scale to compare sizes of the large clones.

119x220mm (300 x 300 DPI)

Table I. Mutation Assay Results for Thymus in ENU-treated and Control Mice

Thymus															
Treatment	Mouse ID	Total No. Colonies ($\times 10^5$)	No. Mutants	Mutant Frequency ^a ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)	Mutant Cell Frequency ^b ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)	No. Mutants Sequenced	Unique Mutations ^c	Replicated Mutations ^d (%)	Mutation Frequency ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)	Mutation Cell Frequency ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)	
8W Control	C1	10.4	2	0.2		0.8		2	2	0	0.2		0.8		
	C2	N.D.	N.D.	N.D.		N.D.		N.D.	N.D.	N.D.	N.D.		N.D.		
	C3	54.2	15	0.3	0.3 $\pm$ 0.2	1.1	1.2 $\pm$ 0.6	15	13	13	0.2	0.2 $\pm$ 0.04	1.0	0.7 $\pm$ 0.2	
	C4	63.9	11	0.2		0.7		11	10	9	0.2		0.6		
	C5	26.9	14	0.5		2.1		14	4	71	0.1		0.6		
12W Control	C6	78.4	13	0.2		0.7		13	12	8	0.2		0.6		
	C7	259.4	10	0.04		0.2		10	9	10	0.03		0.1		
	C8	14.6	3	0.2	0.1 $\pm$ 0.1	0.8	0.4 $\pm$ 0.3	3	3	0	0.2	0.1 $\pm$ 0.1	0.8	0.4 $\pm$ 0.3	
	C9	183.6	13	0.1		0.3		13	11	15	0.1		0.2		
	C10	15.3	1	0.1		0.3		1	1	0	0.1		0.3		
16W Control	C11	35.4	14	0.4		1.6		12	9	25	0.3		1.2		
	C12	151.6	72	0.5		1.9		26	11	58	0.2		0.8		
	C13	49.8	14	0.3	0.4 $\pm$ 0.1	1.1	1.4 $\pm$ 0.5	13	13	0	0.3	0.3 $\pm$ 0.1	1.1	1.1 $\pm$ 0.5	
	C14	109.3	17	0.2		0.6		17	16	6	0.1		0.6		
	C15	23.4	11	0.5		1.9		10	10	0	0.5		1.9		
8W ENU	A	4.9	164	33.5		134.2		15	5	67	11.2		44.7		
	B	8.6	252	29.5		117.9		11	9	18	24.1		96.5		
	C	11.9	177	14.9	20.8 $\pm$ 10.2	59.6	83.1 $\pm$ 40.7	19	14	26	11.0	12.2 $\pm$ 7.1	43.9	48.9 $\pm$ 28.4	
	D	12.8	208	16.3		65.0		47	28	40	9.7		38.7		
	E	8.1	79	9.8		39.0		40	21	48	5.1		20.5		
12W ENU	F	19.1	538	28.2		112.8		15	5	67	9.4		37.6		
	G	12.4	19	1.5		6.1		19	17	11	1.4		5.5		
	H	12.9	326	25.3	14.4 $\pm$ 11.7	101.3	57.7 $\pm$ 46.7	16	3	81	4.7	4.9 $\pm$ 3.2	19.0	19.8 $\pm$ 13.0	
	I	29.2	265	9.1		36.3		27	20	26	6.7		26.9		
	J	23.6	188	8.0		31.9		13	4	69	2.5		9.8		
16W ENU	K	13.5	254	18.8		75.2		24	1	96	0.8		3.1		
	L	49.8	67	1.3		5.4		27	17	37	0.8		3.4		
	M	51.5	461	9.0	7.3 $\pm$ 7.1	35.8	29.1 $\pm$ 28.3	25	6	76	2.1	1.2 $\pm$ 0.6	8.6	4.8 $\pm$ 2.4	
	N	56.1	133	2.4		9.5		26	8	69	0.7		2.9		
	O	44.8	220	4.9		19.6		24	7	71	1.4		5.7		

^aThe mutant frequency was calculated by dividing the number of mutant colonies by the total number of colonies, accounting for the respective dilution factors.

^bThe mutant cell frequency, based on 40 copies of transgene per cell, was calculated as follows: Mutant cell frequency = (Number of mutants/(Total number of colonies/40))

^cUnique mutation: repeatedly observed mutations were counted as one mutation.

^dThe number of replicated mutations, which are presumed to be copies of unique events.

Table II. Mutation Assay Results for Small Intestine in ENU-treated and Control Mice

Thymus															
Treatment	Mouse ID	Total No. Colonies ($\times 10^5$)	No. Mutants	Mutant Frequency ^a ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)	Mutant Cell Frequency ^b ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)	No. Mutants Sequenced	Unique Mutations ^c	Replicated Mutations ^d (%)	Mutation Frequency ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)	Mutation Cell Frequency ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)	
8W Control	C1	N.D.	N.D.	N.D.		N.D.		N.D.	N.D.	N.D.	N.D.		N.D.		
	C2	11.6	3	0.3		1.0		3	3	0	0.3		1.0		
	C3	7.9	4	0.5	0.7 $\pm$ 0.6	2.0	2.7 $\pm$ 2.3	4	4	0	0.5	0.6 $\pm$ 0.5	2.0	2.5 $\pm$ 2.2	
	C4	11.1	17	1.5		6.1		17	16	6	1.4		5.8		
	C5	22.4	9	0.4		1.6		9	7	22	0.3		1.3		
12W Control	C6	3.3	3	0.9		3.6		3	2	33	0.6		2.4		
	C7	4.8	3	0.6		2.5		3	3	0	0.6		2.5		
	C8	31.5	13	0.4	0.6 $\pm$ 0.2	1.6	2.5 $\pm$ 0.7	13	12	8	0.4	0.5 $\pm$ 0.1	1.5	2.1 $\pm$ 0.4	
	C9	19.9	11	0.6		2.2		11	9	18	0.5		1.8		
	C10	26.7	18	0.7		2.7		18	14	22	0.5		2.1		
16W Control	C11	N.D.	N.D.	N.D.		N.D.		N.D.	N.D.	N.D.	N.D.		N.D.		
	C12	11.1	17	1.5		6.1		17	10	41	0.9		3.6		
	C13	25.4	5	0.2	1.1 $\pm$ 0.6	0.8	4.5 $\pm$ 2.5	5	3	40	0.1	0.8 $\pm$ 0.4	0.5	3.0 $\pm$ 1.8	
	C14	10.1	13	1.3		5.1		13	9	31	0.9		3.6		
	C15	9.7	14	1.4		5.8		14	11	21	1.1		4.5		
8W ENU	A	4.4	236	53.9		215.5		38	31	18	44.0		175.8		
	B	2.5	131	52.6		210.4		41	30	27	38.5		154.0		
	C	4.3	184	43.2	45.4 $\pm$ 7.6	172.8	181.5 $\pm$ 30.3	39	31	21	34.3	36.0 $\pm$ 5.4	137.3	144.2 $\pm$ 21.4	
	D	7.0	256	36.6		146.5		39	32	18	30.1		120.2		
	E	5.9	241	40.6		162.3		45	37	18	33.4		133.4		
12W ENU	F	9.5	227	24.0		96.1		38	29	24	18.3		73.3		
	G	N.D.	N.D.	N.D.		N.D.		N.D.	N.D.	N.D.	N.D.		N.D.		
	H	1.3	39	30.2	26.3 $\pm$ 7.6	120.9	105.0 $\pm$ 30.3	24	22	8	27.7	23.0 $\pm$ 7.4	110.9	91.9 $\pm$ 29.5	
	I	4.3	145	34.0		136.2		20	18	10	30.6		122.5		
	J	19.0	318	16.7		67.0		32	29	9	15.2		60.7		
16W ENU	K	4.2	205	48.3		193.3		49	43	12	42.4		169.6		
	L	2.3	97	41.5		165.8		21	19	10	37.5		150.0		
	M	1.9	103	53.6	42.6 $\pm$ 10.1	214.6	170.6 $\pm$ 40.4	25	19	24	40.8	36.0 $\pm$ 7.8	163.1	144.2 $\pm$ 31.4	
	N	44.7	1196	26.7		107.0		26	22	15	22.6		90.5		
	O	31.3	1348	43.1		172.3		28	24	14	36.9		147.7		

^aThe mutant frequency was calculated by dividing the number of mutant colonies by the total number of colonies, accounting for the respective dilution factors.

^bThe mutant cell frequency, based on 40 copies of transgene per cell, was calculated as follows: Mutant cell frequency = (Number of mutants)/(Total number of colonies/40)

^cUnique mutation: repeatedly observed mutations were counted as one mutation.

^dThe number of replicated mutations, which are presumed to be copies of unique events.

Table III. Mutation Assay Results for Lung in ENU-treated and Control Mice

Lung														
Treatment	Mouse ID	Total Colonies ($\times 10^5$)	No. Mutants	Mutant Frequency ^a ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)	Mutant Cell Frequency ^b ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)	No. Mutants Sequenced	Unique Mutations ^c	Replicated Mutations ^d (%)	Mutation Frequency ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)	Mutation Cell Frequency ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)
8W Control	C1	N.D.	N.D.	N.D.		N.D.		N.D.	N.D.	N.D.	N.D.		N.D.	
	C2	52.4	15	0.3		1.1		15	7	53	0.1		0.5	
	C3	126.8	23	0.2	0.4 $\pm$ 0.2	0.7	1.6 $\pm$ 0.9	23	14	39	0.1	0.3 $\pm$ 0.3	0.4	1.3 $\pm$ 1.0
	C4	15.6	7	0.4		1.8		7	6	14	0.4		1.5	
	C5	16.5	12	0.7		2.9		12	11	8	0.7		2.7	
12W Control	C6	6.1	8	1.3		5.3		8	7	13	1.1		4.6	
	C7	17.7	11	0.6		2.5		11	10	9	0.6		2.3	
	C8	104.6	20	0.2	0.5 $\pm$ 0.5	0.8	1.9 $\pm$ 2.1	20	14	30	0.1	0.4 $\pm$ 0.5	0.5	1.6 $\pm$ 1.8
	C9	37.1	3	0.1		0.3		3	3	0	0.1		0.3	
	C10	107.1	20	0.2		0.7		20	14	30	0.1		0.5	
16W Control	C11	14.5	22	1.5		6.1		22	16	27	1.1		4.4	
	C12	12.8	10	0.8		3.1		10	8	20	0.6		2.5	
	C13	15.6	6	0.4	0.9 $\pm$ 0.5	1.5	3.6 $\pm$ 1.9	6	6	0	0.4	0.7 $\pm$ 0.3	1.5	2.8 $\pm$ 1.2
	C14	10.9	14	1.3		5.1		14	10	29	0.9		3.7	
	C15	17.7	10	0.6		2.3		10	9	10	0.5		2.0	
8W ENU	A	3.7	18	4.8		19.4		18	16	11	4.3		17.2	
	B	10.6	27	2.5		10.2		26	23	12	2.2		9.0	
	C	1.9	28	14.8	11.9 $\pm$ 8.1	59.1	47.5 $\pm$ 32.4	25	23	8	13.6	10.4 $\pm$ 6.9	54.3	41.5 $\pm$ 27.6
	D	4.1	62	15.0		59.9		29	25	14	12.9		51.6	
	E	3.78	84	22.2		88.9		39	33	15	18.8		75.2	
12W ENU	F	1.1	20	18.4		73.5		20	18	10	16.5		66.1	
	G	1.4	26	18.0		71.8		26	22	15	15.2		60.8	
	H	6.6	44	6.6	10.9 $\pm$ 6.7	26.5	43.6 $\pm$ 26.8	28	25	11	5.9	9.7 $\pm$ 5.7	23.7	38.8 $\pm$ 22.8
	I	7.3	33	4.5		18.2		28	27	4	4.4		17.5	
	J	1.9	13	6.9		27.8		13	12	8	6.4		25.6	
16W ENU	K	3.7	29	7.9		31.8		28	22	21	6.2		25.0	
	L	2.7	42	15.7		62.9		42	36	14	13.5		53.9	
	M	0.9	10	11.3	11.5 $\pm$ 3.8	45.4	46.1 $\pm$ 15.1	10	10	0	11.3	10.0 $\pm$ 3.2	45.4	40.1 $\pm$ 12.6
	N	4.6	69	14.9		59.8		35	28	20	12.0		47.8	
	O	5.3	41	7.7		30.8		28	26	7	7.1		28.6	

^aThe mutant frequency was calculated by dividing the number of mutant colonies by the total number of colonies, accounting for the respective dilution factors.

^bThe mutant cell frequency, based on 40 copies of transgene per cell, was calculated as follows: Mutant cell frequency = (Number of mutants/(Total number of colonies/40))

^cUnique mutation: repeatedly observed mutations were counted as one mutation.

^dThe number of replicated mutations, which are presumed to be copies of unique events.

Table IV. Mutation Assay Results for Liver in ENU-treated and Control Mice

Treatment	Mouse ID	Total Colonies ($\times 10^5$)	No. Mutants	Liver														
				Mutant Frequency ^a ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)		Mutant Cell Frequency ^b ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)		No. Mutants Sequenced	Unique Mutations ^c	Replicated Mutations ^d (%)	Mutation Frequency ($\times 10^{-5}$)	Average $\pm$ S.D. ($\times 10^{-5}$)		Mutation Cell Frequency ($\times 10^{-4}$)	Average $\pm$ S.D. ($\times 10^{-4}$)	
8W Control	C1	10.5	4	0.4			1.5			4	4	0	0.4			1.5		
	C2	86.0	11	0.1			0.5			11	11	0	0.1			0.5		
	C3	53.8	12	0.2	0.4	$\pm$ 0.3	0.9	1.6	$\pm$ 1.1	12	12	0	0.2	0.3	$\pm$ 0.2	0.9	1.4	$\pm$ 0.7
	C4	15.2	13	0.9			3.4			13	9	31	0.6			2.4		
	C5	25.7	10	0.4			1.6			10	10	0	0.4			1.6		
12W Control	C6	9.1	2	0.2			0.9			2	1	50	0.1			0.4		
	C7	57.5	18	0.3			1.3			18	17	6	0.3			1.2		
	C8	100.4	20	0.2	0.2	$\pm$ 0.1	0.8	0.7	$\pm$ 0.4	20	16	20	0.2	0.1	$\pm$ 0.1	0.6	0.6	$\pm$ 0.4
	C9	138.6	12	0.1			0.3			12	8	33	0.1			0.2		
	C10	69.3	7	0.1			0.4			7	6	14	0.1			0.3		
16W Control	C11	85.3	8	0.1			0.4			8	7	13	0.1			0.3		
	C12	8.4	2	0.2			1.0			2	2	0	0.2			1.0		
	C13	50.7	20	0.4	0.4	$\pm$ 0.2	1.6	1.6	$\pm$ 1.0	20	18	10	0.4	0.3	$\pm$ 0.2	1.4	1.4	$\pm$ 0.8
	C14	17.6	11	0.6			2.5			11	8	27	0.5			1.8		
	C15	18.8	12	0.6			2.5			12	11	8	0.6			2.3		
8W ENU	A	3.0	28	9.4			37.7			28	26	7	8.8			35.0		
	B	4.7	15	3.2			12.7			15	14	7	3.0			11.9		
	C	7.2	37	5.1	5.9	$\pm$ 2.3	20.5	23.7	$\pm$ 9.3	35	29	17	4.2	5.2	$\pm$ 2.2	17.0	20.6	$\pm$ 8.9
	D	7.6	39	5.1			20.6			39	32	18	4.2			16.9		
	E	6.39	43	6.7			26.9			35	29	17	5.6			22.3		
12W ENU	F	12.1	47	3.9			15.5			38	32	16	3.3			13.1		
	G	10.8	66	6.1			24.5			40	34	15	5.2			20.8		
	H	3.7	26	7.1	7.1	$\pm$ 3.3	28.4	28.5	$\pm$ 13.3	26	24	8	6.6	6.1	$\pm$ 2.8	26.2	24.3	$\pm$ 11.1
	I	1.9	24	12.7			50.8			24	20	17	10.6			42.3		
	J	11.7	68	5.8			23.2			39	32	18	4.8			19.1		
16W ENU	K	1.4	14	9.7			38.9			14	13	7	9.0			36.1		
	L	8.4	49	5.8			23.3			39	34	13	5.1			20.3		
	M	14.9	41	2.8	5.3	$\pm$ 2.7	11.0	21.2	$\pm$ 10.8	36	31	14	2.4	4.5	$\pm$ 2.7	9.5	18.0	$\pm$ 10.9
	N	20.6	78	3.8			15.2			40	29	28	2.8			11.0		
	O	19.4	86	4.4			17.8			39	29	26	3.3			13.2		

^aThe mutant frequency was calculated by dividing the number of mutant colonies by the total number of colonies, accounting for the respective dilution factors.

^bThe mutant cell frequency, based on 40 copies of transgene per cell, was calculated as follows: Mutant cell frequency = (Number of mutants/(Total number of colonies/40))

^cUnique mutation: repeatedly observed mutations were counted as one mutation.

^dThe number of replicated mutations, which are presumed to be copies of unique events.

Table V. Classification and mutant frequency (M.F) and mutation frequency (m.f.) of *fgpt* mutations recovered from Thymus DNA from Control and ENU treated mice

	Control 8W		12W		16W		ENU 8W		ENU 12W		ENU 16W	
	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)
G:C→A:T (non-CpG)	2	0.01	2	0.01	8	0.02	6	0.02	10	0.05	10	0.05
G:C→A:T (CpG)	6	0.04	4	0.03	5	0.01	3	0.01	26	0.12	8	0.04
G:C→T:A	5	0.03	5	0.03	7	0.02	7	0.02	16	0.07	15	0.07
G:C→C:G	0	0	0	0	4	0.01	4	0.01	3	0.01	1	0.16
Total G:C	13	0.09	11	0.07	24	0.07	20	0.06	55	0.25	36	0.17
A:T→G:C	3	0.02	3	0.02	2	0.01	2	0.01	9	0.04	9	0.04
A:T→T:A	13	0.09	3	0.02	2	0.01	2	0.01	0	0	0	0
A:T→C:G	1	0.01	1	0.01	2	0.01	2	0.01	5	0.02	5	0.02
Total A:T	17	0.12	7	0.04	6	0.02	6	0.02	14	0.06	14	0.07
Base substitution	30	0.21	18	0.11	30	0.08	26	0.07	69	0.31	50	0.24
1bp deletion	6	0.04	5	0.03	7	0.02	7	0.02	5	0.02	5	0.02
1bp insertion	4	0.03	4	0.03	1	0.003	1	0.003	2	0.01	2	0.01
Total 1bp frameshift	10	0.07	9	0.06	8	0.02	8	0.02	7	0.03	7	0.03
>2 bp Frameshifts	2	0.01	2	0.01	1	0.003	1	0.003	2	0.01	2	0.01
complex total	0	0	0	0	1	0.003	1	0.003	0	0	0	0
Total	42	0.29	29	0.18	40	0.11	36	0.1	78	0.36	59	0.28

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Table VI. Classification and mutant frequency (M.F) and mutation frequency (m.f.) of *gpt* mutations recovered from Small Intestine DNA from Control and ENU treated mic

	Control 8W				12W				16W				ENU 8W				ENU12W				ENU16W			
	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)
G:C→A:T (non-CpG)	5	0.10	4	0.08	2	0.03	2	0.03	7	0.16	6	0.14	50	11.23	37	8.28	34	7.83	26	6.09	37	10.59	31	8.80
G:C→A:T (CpG)	8	0.16	7	0.15	15	0.20	9	0.12	19	0.43	9	0.21	5	1.12	3	0.67	4	0.92	3	0.70	5	1.43	4	1.14
G:C→T:A	5	0.10	5	0.10	15	0.20	15	0.19	15	0.34	12	0.28	28	6.29	24	5.37	15	3.46	14	3.28	24	6.87	22	6.24
G:C→C:G	0	0	0	0	1	0.01	1	0.01	3	0.07	1	0.02	1	0.22	1	0.22	0	0	0	0	2	0.57	2	0.57
Total G:C	18	0.37	16	0.34	33	0.44	27	0.35	44	1.00	28	0.65	84	18.87	65	14.55	53	12.21	43	10.08	68	19.46	59	16.75
A:T→G:C	3	0.06	2	0.04	4	0.05	4	0.05	0	0	0	0	31	6.96	27	6.04	20	4.61	15	3.51	22	6.30	17	4.83
A:T→T:A	5	0.10	5	0.10	3	0.04	3	0.04	1	0.02	1	0.02	74	16.62	57	12.76	26	5.99	25	5.86	43	12.31	35	9.93
A:T→C:G	3	0.06	3	0.06	1	0.01	1	0.01	1	0.02	1	0.02	10	2.25	9	2.01	11	2.53	11	2.58	14	4.01	14	3.97
Total A:T	11	0.22	10	0.21	8	0.11	8	0.10	2	0.05	2	0.05	115	25.83	93	20.82	57	13.13	51	11.95	79	22.61	66	18.73
Base substitution	29	0.59	26	0.55	41	0.54	35	0.45	46	1.05	30	0.69	199	44.70	158	35.37	110	25.34	94	22.03	147	42.08	125	35.48
1bp deletion	1	0.02	1	0.02	1	0.01	1	0.01	1	0.02	1	0.02	1	0.22	1	0.22	0	0	0	0	2	0.57	2	0.57
1bp insertion	0	0	0	0	1	0.01	1	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total 1bp frameshift	1	0.02	1	0.02	2	0.03	2	0.03	1	0.02	1	0.02	1	0.22	1	0.22	0	0	0	0	2	0.57	2	0.57
>2 bp Frameshifts	1	0.02	1	0.02	4	0.05	2	0.03	1	0.02	1	0.02	0	0	0	0	0	0	0	0	0	0	0	0
complex total	2	0.04	2	0.04	1	0.01	1	0.01	1	0.02	1	0.02	2	0.45	2	0.45	4	0.92	4	0.94	0	0	0	0
Total	33	0.67	30	0.63	48	0.63	40	0.52	49	1.11	33	0.76	202	45.38	161	36.04	114	26.26	98	22.96	149	42.65	127	36.05

Table VII. Classification and mutant frequency (M.F) and mutation frequency (m.f.) of *gprt* mutations recovered from Lung DNA from Control and ENU treated mice

	Control 8W				12W				16W				ENU 8W				ENU12W				ENU16W			
	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)
G:C→A:T (non-CpG)	8	0.06	6	0.05	11	0.09	9	0.08	10	0.15	7	0.10	36	3.12	35	3.02	14	1.33	12	1.12	31	2.48	28	2.29
G:C→A:T (CpG)	13	0.09	7	0.06	23	0.18	12	0.10	19	0.28	13	0.19	11	0.95	10	0.86	11	1.04	8	0.75	5	0.40	5	0.41
G:C→T:A	13	0.09	11	0.09	15	0.12	14	0.12	9	0.13	7	0.10	10	0.87	9	0.78	14	1.33	13	1.21	19	1.52	16	1.31
G:C→C:G	2	0.01	2	0.02	1	0.01	1	0.01	1	0.01	1	0.01	0	0	0	0	1	0.09	1	0.09	0	0	0	0
Total G:C	36	0.26	26	0.22	50	0.39	36	0.31	39	0.57	28	0.40	57	4.94	54	4.67	40	3.79	34	3.17	55	4.4	49	4.0
A:T→G:C	3	0.02	3	0.03	2	0.02	2	0.02	3	0.04	2	0.03	16	1.39	12	1.04	10	0.95	10	0.93	15	1.2	13	1.06
A:T→T:A	8	0.06	1	0.01	1	0.01	1	0.01	4	0.06	4	0.06	56	4.85	46	3.97	45	4.26	40	3.73	62	4.97	50	4.08
A:T→C:G	2	0.01	2	0.02	3	0.02	3	0.03	4	0.06	4	0.06	3	0.26	3	0.26	16	1.52	16	1.49	8	0.64	8	0.65
Total A:T	13	0.09	6	0.05	6	0.05	6	0.05	11	0.16	10	0.14	75	6.50	61	5.27	71	6.72	66	6.15	85	6.81	71	5.8
Base substitution	49	0.35	32	0.27	56	0.43	42	0.36	50	0.73	38	0.55	132	11.44	115	9.94	111	10.51	100	9.32	140	11.21	120	9.8
1bp deletion	2	0.01	2	0.02	3	0.02	3	0.03	8	0.12	7	0.10	3	0.26	3	0.26	1	0.09	1	0.09	0	0	0	0
1bp insertion	0	0	0	0	0	0	0	0	1	0.01	1	0.01	1	0.09	1	0.09	0	0	0	0	0	0	0	0
Total 1bp frameshift	2	0.01	2	0.02	3	0.02	3	0.03	9	0.13	8	0.12	4	0.35	4	0.35	1	0.09	1	0.09	0	0	0	0
>2 bp Frameshifts	5	0.04	3	0.03	2	0.02	2	0.02	2	0.03	2	0.03	1	0.09	1	0.09	2	0.19	2	0.19	3	0.24	2	0.16
complex total	1	0.01	1	0.01	1	0.01	1	0.01	1	0.01	1	0.01	0	0	0	0	1	0.09	1	0.09	0	0	0	0
Total	57	0.41	38	0.32	62	0.48	48	0.41	62	0.91	49	0.71	137	11.87	120	10.37	115	10.89	104	9.69	143	11.45	122	9.97

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Table VIII. Classification and mutant frequency (M.F) and mutation frequency (m.f.) of *gpt* mutations recovered from Liver DNA from Control and ENU treated mice

	Control 8W				12W				16W				ENU 8W				ENU12W				ENU16W			
	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)	No.	M.F ($\times 10^{-5}$)	No.	m.f ($\times 10^{-5}$)
G:C→A:T (non-CpG)	10	0.08	10	0.07	2	0.01	2	0.01	10	0.08	8	0.06	26	1.01	23	0.91	16	0.68	15	0.64	17	0.54	13	0.43
G:C→A:T (CpG)	14	0.11	10	0.07	19	0.06	10	0.03	13	0.10	9	0.07	3	0.12	3	0.12	9	0.38	6	0.26	11	0.35	6	0.20
G:C→T:A	13	0.10	13	0.10	12	0.04	11	0.03	13	0.10	12	0.09	12	0.47	12	0.48	22	0.94	21	0.90	20	0.63	17	0.56
G:C→C:G	2	0.02	2	0.01	3	0.01	3	0.01	1	0.01	1	0.01	1	0.04	1	0.04	0	0	0	0	2	0.06	2	0.07
Total G:C	39	0.31	35	0.26	36	0.11	26	0.08	37	0.28	30	0.22	42	1.64	39	1.55	47	2.00	42	1.80	50	1.58	38	1.26
A:T→G:C	2	0.02	2	0.01	7	0.02	6	0.02	1	0.01	1	0.01	30	1.17	25	0.99	30	1.28	29	1.24	24	0.76	21	0.70
A:T→T:A	2	0.02	2	0.01	4	0.01	4	0.01	2	0.02	2	0.01	68	2.65	54	2.14	75	3.20	56	2.40	86	2.72	69	2.29
A:T→C:G	1	0.01	1	0.01	0	0	0	0	3	0.02	3	0.02	8	0.31	8	0.32	7	0.30	7	0.30	5	0.16	5	0.17
Total A:T	5	0.04	5	0.04	11	0.03	10	0.03	6	0.05	6	0.04	106	4.13	87	3.45	112	4.78	92	3.94	115	3.64	95	3.15
Base substitution	44	0.35	40	0.30	47	0.15	36	0.11	43	0.32	36	0.27	148	5.76	126	4.99	159	6.78	134	5.73	165	5.22	133	4.41
1bp deletion	3	0.02	3	0.02	5	0.02	5	0.01	6	0.05	6	0.04	0	0	0	0	1	0.04	1	0.04	1	0.03	1	0.03
1bp insertion	0	0	0	0	2	0.01	2	0.01	1	0.01	1	0.01	0	0	0	0	1	0.04	1	0.04	0	0	0	0
Total 1bp frameshift	3	0.02	3	0.02	7	0.02	7	0.02	7	0.05	7	0.05	0	0	0	0	2	0.09	2	0.09	1	0.03	1	0.03
>2 bp Frameshifts	2	0.02	2	0.01	4	0.01	4	0.01	2	0.02	2	0.01	0	0	0	0	1	0.04	1	0.04	1	0.03	1	0.03
complex total	1	0.01	1	0.01	1	0.00	1	0.00	1	0.01	1	0.01	4	0.16	4	0.16	5	0.21	5	0.21	1	0.03	1	0.03
Total	50	0.40	46	0.34	59	0.18	48	0.14	53	0.40	46	0.34	152	5.92	130	5.15	167	7.12	142	6.08	168	5.31	136	4.51

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