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Age-dependent sensitivity of Big Blue transgenic mice to the mutagenicity of *N*-ethyl-*N*-nitrosourea (ENU) in liver☆

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Abstract

The incidence of childhood cancer is increasing and recent evidence suggests an association between childhood cancer and environmental exposure to genotoxins. In the present study, the Big Blue transgenic mouse model was used to determine whether specific periods in early life represent windows of vulnerability to mutation induction by genotoxins in mouse liver. Groups of mice were treated with single doses of 120 mg *N*-ethyl-*N*-nitrosourea (ENU)/kg body weight or the vehicle either transplacentally to the 18-day-old fetus or at postnatal days (PNDs) 1,8, 15, 42 or 126; the animals were sacrificed 6 weeks after their treatment. The *cII* mutation assay was performed to determine the mutant frequencies (MFs) in the livers of the mice. Liver *cII* MFs for both sexes were dependent on the age at which the animals were treated. Perinatal treatment with ENU (either transplacental treatment to the 18-day-old fetus or i.p. injection at PND 1) induced relatively high MFs. However, ENU treatment at PNDs 8 and 15 resulted in the highest mutation induction. The lowest mutation induction occurred in those animals treated as adults (PND 126). For instance, the *cII* MF for the PND 8 female group was 646×10^{-6} while the MF for female adults was only 145×10^{-6}, a more than 4-fold difference. Molecular analysis of the mutants found that A:T → T:A transversions and A:T → G:C transitions characterized the pattern of mutations induced by ENU in both the neonate and adult mice, while the predominate type of mutation in the controls was G:C → A:T. The results indicate that mouse liver is most sensitive to ENU-induced mutation during infancy. This period correlates well with the age-dependent sensitivity to carcinogenicity in mouse liver, suggesting that mutation is an important rate-limiting factor for age-related carcinogenesis.

Keywords

Ethylnitrosourea; Age; Neonate; Mutagenicity; Transgenic mice; Sensitivity

1 Introduction

The protection of children from environmental toxins is a major challenge to modern society because children can be more vulnerable due to their unique exposures and susceptibilities. Childhood exposure to environmental chemicals may contribute to or exacerbate certain

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chronic and disabling diseases like cancer [1]. Although the causes of cancer in newborns and infants remain unknown, environmental exposure to genotoxins during the perinatal period and susceptibility to these toxins during early development are strong possibilities [2]. Data from the National Cancer Institute's Surveillance Epidemiology and End Results program suggest that the annual incidence of childhood cancers is increasing and about 12% of the cancer cases were tumors acquired during infancy [3–5].

The mouse model has been used to evaluate factors modifying the carcinogenic response of various tissues to different types of chemical carcinogens. The age at which animals are exposed is the most effective modulator of carcinogenesis in liver and lung, as well as some other tissues. Infancy appears to be the most susceptible period for carcinogenesis in a great variety of tissues [6]. Exposure of newborn (≤24-h-old) and neonatal (≤3-week-old) mice to only a few doses of any one of a number of genotoxic chemical carcinogens results in tumors approximately one year after the treatment. The primary sites of tumor induction are the liver and the lung. For example, polycyclic aromatic hydrocarbons usually do not induce liver cancer when administered to young adult mice, but do so when given to newborn and neonatal animals [7].

Mutations play an important role in the etiology of cancer. Newborn and neonatal mice are highly sensitive to carcinogens that exert tumorigenicity through a genotoxic mechanism [8–11]. Therefore, it is reasonable to suspect that these animals also possess higher sensitivity to the mutagenicity of genotoxins than do adult animals. However, studies on age-related chemical mutagenicity are rare because mutagenesis studies are often conducted using in vitro tests or in vivo tests in which endogenous genes are utilized. Assays that test mutations in endogenous genes often require testing rapidly dividing tissues like lymphocytes, and may not have the capability to detect the effect of age because cells in these tissues are rapidly dividing regardless of age [12]. The recently developed Big Blue transgenic mutation models represent a novel approach for studying mutant frequencies (MFs) and types of mutations in nearly all tissues, thus permitting the direct comparison of cancer incidence with MF [13–15]. In these systems, the chromosomally-integrated λLIZ*/lacI* or *cII* gene is used as the target for mutation. Following recovery of the transgene from the tissue(s) of choice, the target sequence is packaged into phage particles, bacteria are infected, and mutants are identified and quantified. In present study, we treated different age Big Blue transgenic mice with *N*-ethyl-*N*-nitrosourea (ENU) using a carcinogenesis protocol to explore the age-related mutagenic sensitivity in liver and its relationship to car-cinogenesis.

2 Materials and methods

2.1 Animals and treatments

The recommendations set forth by our Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and sacrifice of the animals were followed. Pregnant female Big Blue C57BL/6 transgenic mice were purchased from Taconic Laboratories (Germantown, NY). These mice had been bred with non-transgenic C3H male mice by the supplier. The B6C3F_1 offspring were separated according to sex, pooled, and then distributed to individual litters during the first week following birth. Six to eight same-sex pups were assigned to each foster mother. The ENU was purchased from Sigma (St. Louis,

MO) and administered in dimethylsulfoxide (DMSO). Animals were given a single i.p. injection of 120 mg ENU/kg body weight or vehicle control in a volume of 2 ml/kg body weight at various times during their development. For the prenatal treatment groups, five pregnant mice were treated at 18 days of gestation, and at birth the pups were pooled and assigned randomly to the mothers. For the postnatal treatment groups, the pups were pooled and distributed randomly into five groups at birth. Male and female mice received treatment at postnatal days (PNDs) 1, 8, 15, 42, or 126. For male mice, there were 3 concurrent controls. Control 1 was administered DMSO at PND 8 to serve as a control for the prenatal, PNDs 1, 8 and 15 treatment groups. Controls 2 and 3 were treated with DMSO at PNDs 42 and 126 to serve as concurrent controls for those treatment groups. Since we found no significant difference among the male controls, only one group of female mice was treated with DMSO at PND 8 and served as a control for all of the female treatment groups. The animals were sacrificed six weeks after their treatment. The livers were isolated, frozen quickly by using liquid nitrogen, and stored at –80 °C.

2.2 cII Mutant assay

High-molecular-weight genomic DNA was extracted from the livers using a RecoverEase DNA Isolation Kit (Stratagene, La Jolla, CA) and stored at 4 °C until DNA packaging was performed. The packaging of the phage, plating of the packaged DNA samples, and determination of MF were carried out following Stratagene's procedure fort he λ select-*cII* mutation detection system for Big Blue rodents. The shuttle vector containing the *cII* target gene was rescued from total genomic DNA with phage packaging extract (Transpack; Stratagene). The plating was performed with the Escherichia coli host strain G1250. To determine the total titer of packaged phages, G1250 bacteria were mixed with a 1:3000 dilution of phage, plated on TB1 plates, and incubated overnight at 37 °C (nonselective conditions). For mutant selection, the packaged phages were mixed with G1250, plated on TB1 plates and incubated at 24 °C for about 42 h (conditions for *cII*- selection). At 24 °C, phages with wild-type *cII* genes undergo lysogenization and infected bacteria become part of the developing lawn, whereas phages with mutated *cII* genes undergo lytic growth and give rise to plaques. When incubated at 37 °C, phages with wild-type *cII* genes also undergo a lytic cycle, resulting in plaque formation. The *cII* MF was calculated as the ratio of the total number of mutant plaques (determined at 24 °C) to the total number of plaques screened (determined at 37 °C). The packaging was repeated until a minimum of approximately 2×10^5 plaque-forming units (pfus) from each liver sample was screened for mutations.

2.3 Sequence analysis of the cII mutants

cII Mutant plaques were selected at random from different animals and replated at low density to verify the mutant phenotype. Single, well-isolated plaques were selected from these plates and transferred to a microcentrifuge tube containing 100 μl of autoclaved distilled water. The tubes were placed in a thermocycler at 99.9 °C for 5min and centrifuged at 1500 g for 5min immediately after the heating. The *cII* target DNA was amplified by PCR with primers 5′-AAAAAGGGCATCAAATTAACC-3′ and 5′-CCGAAGTTGAGTATTTTTGCTG-3′. For PCR amplification, 10 μl of the supernatant and 0.03 μl of each primer (148 μM) were added to 10 μl of 2× PCR Master Mix (Promega, Madison, WI). The PCR reaction was carried out with the following cycling parameters: a 3

min denaturation at 95 °C; followed by 30 cycles of 30 s at 95 °C, 1 min at 60 °C, and 1 min at 72 °C; with a final extension of 10 min at 72 °C. The PCR products were purified using PCR purification kits (Qiagen, Chatsworth, CA). The *cII* mutant DNA was sequenced and analyzedwith a CEQDTCS-Quick Start Kit and a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. The primers for *cII* mutation sequencing were the same as those used for the PCR.

2.4 Statistical analyses

Statistical analyses of MFs were performed using SigmaStat (SPSS Science, Chicago, IL). All MF data are expressed as the mean ± standard error (SE) from different animals. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test for comparison of multiple treatment groups. Since the variance increased with the magnitude of the MF, the data were log-transformed before conducting the analysis. Mutational spectra were compared using the computer program written by Cariello [16] forthe Monte Carlo analysis developed by Adams and Skopek [17].

3 Results

3.1 Determination of appropriate MF sampling time for Big Blue mice treated transplacentally with ENU

A time–course study was conducted to determine the proper sampling time. This time–course study was designed to determine whether the *cII* transgene is genetically neutral when animals were treated perinatally. Pregnant female mice were treated with a single dose of ENU by i.p. injection 3 days prior to giving birth (18-day-old fetus). Their male pups were sacrificed at different time points. The MFs in the liver *cII* gene at 6, 12 and 24 weeks after the treatment were 279 ± 55, 271 ± 25, and 290 ± 41 × 10^{-6}, respectively, while the MF in the concurrent control mice were 45 ± 6, 36 ± 13 and 42 ± 6 × 10^{-6} (Fig. 1). ENU increased MFs in all the time points over the controls (p <0.001), whereas no significant difference was found among the treated animals sampled at the different times ($p \geq 0.79$).

3.2 Effect of age at treatment on the cII MFs induced by ENU in the liver

Big Blue mice were treated with 120 mg ENU/kg body weight at different ages and sacrificed 6 weeks after the treatment. The livers from the animals of both sexes were assayed for *cII* gene mutants (Tables 1 and2). Although ENU significantly increased the MFs over the controls at every age (p <0.01 for male and p < 0.001 for female), the level of the MF induction was not the same among the treatment groups. For both sexes, MFs were the highest for mice treated at PNDs 8 and 15 (neonate) and the lowest for mice treated at PND 126 (adult) (p <0.05 for male and p <0.01 for female). ENU also induced higher *cII* MFs in neonatal mice than at PND 42 (young adult), the difference being significant in males (p<0.05) but not in females (p = 0.18). For the ENU-treated female mice, there were significant differences for the MFs between the neonate and PND 1 (newborn) (p <0.001), as well as between young adult and adult mice (p <0.01). Although the MF for each age group of females was higher than males (Tables 1 and 2), the differences were not significant (p >0.05).

3.3 Mutational spectra in the liver cII gene from mice treated with ENU as neonates and adults

ENU-induced *cII* mutations were evaluated by DNA sequence analysis of 90 mutants from 12 male mice treated as neonates (PNDs 8 and 15), and 47 mutants from the 4 adult male mice from the PND 126 ENU treatment group (Table 3). The average MFs were 427 ± 50 for the neonate mice and 136 ± 30 for the adult mice. Mutations that were found more than once in the mutants isolated from a single animal were assumed to be siblings resulting from a single independent mutation. Accordingly, a total of 89 independent mutations from neonate and 41 from adult mice were identified. Table 4 summarizes the types of mutations in the liver *cII* gene from the ENU-treated neonate and adult male mice, along with the types of spontaneous mutation in the liver *cII* gene from neonate and adult male mice reported previously [18]. Among the independent mutations from ENU-treated animals, 97% for neonates and 86% for adults were base pair substitutions. A:T → T:A transversion was the major type of mutation (33% in neonate and 29% in adult versus 0% in both the neonate and adult controls). No significant difference was found between the mutation spectra for ENU-treated neonate and adult mice (p =0.17). The overall patterns of mutations in ENU-treated neonate or adult groups, however, were significantly different from those in their control groups (p <0.0001 for both the neonate and adult comparisons). G:C → A:T transition (50% in neonate control and 41% in adult control) and frameshifts (13% in neonate control and 22% adult control) were the predominant types of spontaneous mutations, and both types of mutations were found at lower frequencies in ENU-treated mice.

4 Discussion

The results for the time-course study are consistent with the liver *cII* gene being genetically neutral. In mice treated perinatally with ENU, the *cII* MF seems to reach a maximum at or before 6 weeks after the treatment and the MF remained relatively constant thereafter. Using ENU-treated Muta mice, Douglas et al. [19] showed that the peak MF in the liver *lacZ* gene was achieved at 5 weeks after treatment and did not significantly change from 5 to 8 weeks. The evidence for the neutrality of the *cII* gene is also supported by *cII* MF analysis of the small intestine in Muta mice treated with ENU. The MFs were similar at 10 and 70 days after treatment [20]. Since the *cII* gene is non-functional in the mouse, mutants in this transgene in vivo would confer no selective growth advantage or disadvantage to the cells. Therefore, mutational data resulting from the *cII* mutagenesis assay might be a reasonable estimate of mutations occurring throughout the mouse genome and at different periods during the life span of the mouse.

A review of carcinogenicity studies using benzo(*a*)pyrene, dibenz(*a,h*)anthracene, diethylnitrosamine (DEN), dimethylbenzanthracene, ENU, 3-methylchloanthrene, urethane and X-rays indicates that juvenile animals are 3- to 10-fold more sensitive to tumor induction than adult animals for a wide range of tissues including liver, lung, kidney, mammary tissue, the hematopoeitic system and nervous tissues [20]. Also, the neonatal mouse model, in which mice are treated at various time-points between PNDs 1 and 15, has been used experimentally since 1959 [22]. While the strain of mouse can greatly influence tumor incidence, in general, this model is very sensitive to carcinogens that operate via a

genotoxic mode of action [11]. Mutations are thought to be involved in carcinogenesis because the transition from a normal somatic cell to a cancer cell is due to mutations in protooncogenes, tumor suppressor genes or genes that function in the maintenance of genomic stability [23,24]. Therefore, it is reasonable to hypothesize that the neonatal animals also possess greater sensitivity to the mutagenicity of genetoxic carcinogens than adult animals. To test this hypothesis, we treated Big Blue mice with ENU using a treatment protocol similar to those used in carcinogenesis studies [25,26]. Both the carcinogenicity and mutagenicity studies were conducted in mice with a B6C3F1 background, thus avoiding strain-specific factors that might specially affect mutagenicity or carcinogenicity. ENU was chosen because it is a direct-acting mutagen, which avoids complications from chemical bioactivation. The 18th day of gestation has been identified as a sensitive time point for the carcinogenicity of ENU during the prenatal period [25]. The PNDs 1, 8, 15 and 42 treatment time points were based on a protocol used for tumorigenicity assays [26]. Mice at PNDs 42 and 126 are in the young adult and adult stages of life, respectively.

Our MF data (Tables 1 and 2) show that the age at the time of ENU exposure is an effective modulator of mutation induction. In both male and female mice, prenatal and PND 1 treatments with ENU induced relatively high MFs. ENU treatment at PNDs 8 and 15, however, resulted in the highest mutation induction. The lowest induced MFs occurred in those animals treated as adults (PND 126). The MFs in the PND 8 treatment groups were about 2- to 4-fold higher than those in the PND 126 treatment groups. This age-dependent pattern of mutation induction is comparable to that for tumor induction. A comparison between the mutant frequencies in the liver *cII* gene and the incidence of liver tumors in the Big Blue or generic B6C3F1 mice treated with 120 mg ENU/kg body weight is given in Table 5. In the carcinogenesis studies [25,26], development of liver tumors was modified significantly by the age at which ENU was given. The tumor incidence was 11% in untreated male animals and increased following ENU treatment to 67% for the males treated as 18-day-old faetuses. The cancer incidence reached a maximum of 94% for the males treated at PND 15 and then dropped to 35% for the mice treated at PND 42. The pattern of tumor responses to ENU in female mice was similar to that observed in males. Females, however, showed a consistently lower incidence of liver tumors than males treated at the same age. This apparent comparability between the mutagenicity and carcinogenicity of ENU is consistent with mutation induction being the rate-limiting factor in the carcinogenicity of ENU. Also the high sensitivity of neonatal mice to mutation induction by ENU indicates that treatments during this period may result in a general increase in the sensitivity of in vivo mutagenesis assays.

We sequenced the *cII* DNA of mutants from neonate and adult mice treated with ENU to observe if there were any differences between neonatal and adult mice in terms of clonal expansion or mutational spectra. High rates of cell proliferation can increase the probability that the initiated clone will expand before cell death [21]. If an initially mutated clone was disproportionately expanded and resulted in a higher MF, the number of sibling mutants in the animal would increase. Such events, however, were rare. The sequencing data gave no evidence for clonal expansion playing a crucial role for the increased MFs found in the neonatal mice. We sequenced approximately 8 mutants from each animal. Eighty-nine of 90

mutations from ENU-treated neonatal mice and 41 of 47 mutations from ENU-treated adults were clearly of independent origin.

ENU reacts with oxygen in DNA, and results in modification of the O^6 position of guanine and the O^2 and O^4 positions of thymine. O^6-ethylguanine, which induces G:C → A:T transition, can be efficiently repaired in normal mammalian systems. A:T → T:A transversion induced by O^2-ethylthymine andA:T→ G:Ctransitioninducedby O^4-ethylthymine become the major types of mutation because O^2- and O^4-ethylthymine adducts are relatively persistent in normal mammalian cells [27–32]. In this study, the overall pattern of mutations induced by ENU in the neonate and adult mice was very similar (p =0.17). A:T → T:A transversion and A:T → G:C transition were the predominant mutations in both the spectra (33 and 18% in neonate, and 29 and 17% in adult). The percentages of ENU-induced mutation at A:T base pairs in this study (65% in neonates and 51% in adults) are consistent with the ENU-induced mutation spectrum for the liver *lacZ* gene of adult Muta mouse (56.7% at A:T [19]). Hence, our spectra results suggest that the DNA repair system is similar in neonate and adult mice and may not contribute to the higher MFs induced by ENU in neonates.

The mechanisms for the age-related mutagenesis and carcinogenesis are still unknown although a number of factors have been suggested as determining susceptibility at different stages [33]. Cell proliferation has been regarded as a major factor since mutations are fixed by DNA replication [34,35]. It has been reported that each liver cell in the neonatal mouse divides an average of 3 times, resulting in an 8- to 10-fold increase in liver mass and about a 3-fold increase in the number of cells. Hepatocytes rarely divide in the adult mouse, less than one division every 3 months [36–38]. Genotoxic carcinogens are generally more effective in rapidly dividing tissues because the higher rate of cell division increases the number of cells in S phase. This rapid division makes the cell population more vulnerable to the genotoxic effects of chemicals and leaves less time for DNA repair prior to fixation of the damage as a mutation [39,40]. This reasoning is substantiated by studies using partial hepatectomy, which increases liver cell turnover rate in adult animals. Hepatoecotmyzed mice treated with ENU have enhanced hepatocarcino- genesis [41] and liver MFs [42]. The same principle may also apply to neonatal mice, considering the much faster rate of cell division in neonatal animals. In apparent contradiction with this theory, mice treated with ENU perinatally had lower liver MFs than neonates although fetal and newborn mice have a faster rate of cell proliferation. One explanation for this may be that most of the liver cells are hematopoietic rather than hepatic at these developmental stages and these cells will largely be absent from the liver when MF assays are conducted and/or these hematopoietic cells may respond differently to the ENU insult from hepatocytes [25]. Another possibility is that the level of liver damage in fetal or PND 1 mice is less than the level of damage in neonates. It is also conceivable that DNA repair activity is greater in embryonic cells compared to neonatal cells.

It also seems a paradox that ENU induced significantly higher tumor incidences and lower MFs in male mice than in females at nearly every age (Table 5). For example, the *cII* liver MFs for mice treated with ENU at PND 15 were 416×10^{-6} in males and 531×10^{-6} in females, whereas the tumor incidences were 94% in males and 67% in females. This pattern

of mutagenicity and carcinogenicity has also been found in other chemical-treated male and female mice [43–45]. The functions of sex hormones may be a reason for this inconsistency. The hormonal environment plays an important role in the development of liver tumors. Experimental and clinical data have shown that both estrogens and androgens have important effects in controlling liver cell proliferation and promoting liver tumors [46]. Androgens have been implicated in hepatic carcinogenesis because men develop liver tumors with a much greater frequency and worse prognosis than women. Males tend to have a lower survival rate and an increased recurrence of the disease after treatment. In rodents, carcinogen-induced and spontaneous liver tumors also occur at a higher incidence in males than in females [47,48]. The role of estrogens in tumor incidence, however, is unclear. Although estrogens also have been suggested as cancer promoters in rat liver [49–51], a number of subsequent studies using animal models observed opposite findings. In some studies, estrogens have shown unexpected suppressive effects on spontaneous and chemical-induced liver tumors [52–54].

In summary, the mutagenicity of ENU in the liver *cII* gene of Big Blue mice was significantly modified by the age at which the animals were exposed. Neonatal Big Blue mice were the most vulnerable to the mutagenicity of ENU while adults displayed much less sensitivity. Although there was a significant difference between the MFs of the neonatal and adult mice, the overall patterns of mutations inducedby ENU in the neonates and adults were very similar. The age-dependent MF responses are comparable to the tumor incidence observed in carcinogenesis studies, suggesting that mutation induction is a major factor for the high susceptibility of neonates to tumor formation by genotoxic carcinogens.

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Abbreviations:

ENU	*N*-ethyl-*N*-nitrosourea
PNDs	postnatal days
MF	mutant frequency
DMSO	dimethylsulfoxide

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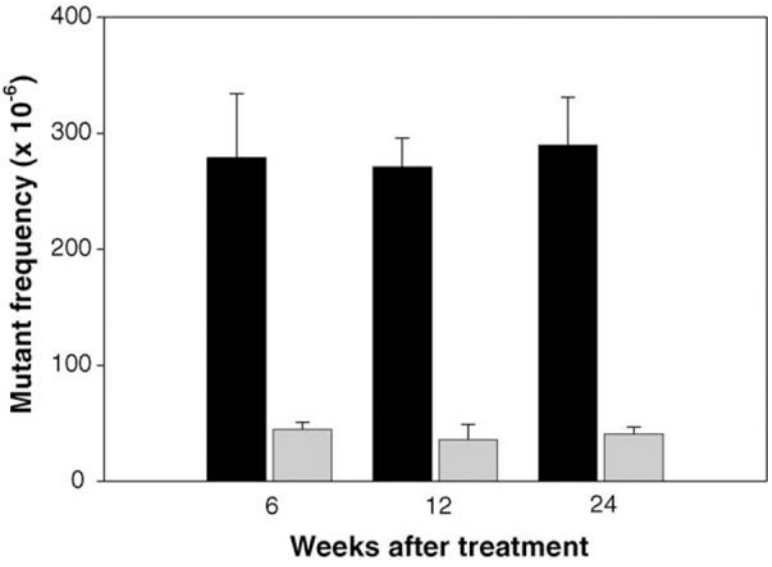

Fig. 1.

Mutant frequencies in the liver *cII* gene at different times after dosing male Big Blue mice with ENU transplacentally. The dark bars represent the MFs for the mice treated with ENU and the light bars are the data from the concurrent controls. ENU increased MFs at all of the time points over the controls ($p < 0.001$), whereas no significant difference was found among the treatment groups ($p \geq 0.79$).

Table 1

Liver *cII* mutant frequencies in ENU-treated and control Big Blue male mice

Group name	Age at treatment (postnatal day)	Treatment[a]	Mouse ID	Total plaques screened (× 10^3)	Mutant plaques	Mutant frequency (×10^{-6})	Average MF ± S.E. (×10^{-6})
Control 1[b]	8	DMSO	C6W1M	330	12	36	45 ± 6
			C6W2M	284	10	35	
			C6W3M	373	24	64	
			C6W4M	301	10	33	
			C6W5M	317	18	57	
Control 2	42	DMSO	C12W1M	323	9	28	36 ± 13
			C12W2M	428	32	75	
			C12W3M	853	16	19	
			C12W4M	330	8	24	
Control 3	126	DMSO	C24W1M	287	12	42	41 ± 6
			C24W2M	274	17	62	
			C24W3M	326	13	40	
			C24W4M	309	7	23	
			C24W5M	201	8	39	
PND –3	–3[c]	ENU	EP1M	252	112	444	279 ± 55[*]
			EP2M	274	61	223	
			EP3M	636	149	234	
			EP4M	313	67	214	
PND 1	1	ENU	E1D1M	223	68	305	206 ± 50[*]
			E1D2M	202	32	159	
			E1D3M	313	32	102	
			E1D4M	357	42	118	
			E1D5M	244	84	344	
PND 8	8	ENU	E8D1M	561	78	139	442 ± 87[*,#]
			E8D2M	232	112	483	
			E8D3M	308	204	662	

Group name	Age at treatment (postnatal day)	Treatment[a]	Mouse ID	Total plaques screened (× 10^3)	Mutant plaques	Mutant frequency (×10^{-6})	Average MF ± S.E. (×10^{-6})
			E8D4M	190	76	400	
			E8D5M	253	133	526	
PND 15	15	ENU	E15D1M	466	149	320	416 ± 65 *,#
			E15D2M	353	127	360	
			E15D3M	382	99	259	
			E15D4M	689	160	232	
			E15D5M	265	178	672	
			E15D6M	250	154	616	
			E15D7M	201	91	452	
PND 42	42	ENU	E6W1M	245	27	110	158 ± 24 *
			E6W2M	211	45	213	
			E6W3M	292	44	151	
			E6W4M	445	46	103	
			E6W5M	289	61	211	
PND 126	126	ENU	E18W1M	193	19	99	136 ± 30 *
			E18W2M	226	26	115	
			E18W3M	213	48	225	
			E18W4M	519	55	106	

[a] All mice were sacrificed 6 weeks after the treatment.

[b] Control 1 serves as the concurrent control for PNDs –3, 1, 8 and 15 treatment groups. Controls 2 and 3 serves as the concurrent controls for PNDs 42 and 126, respectively.

[c] The mice were treated transplacentally at 3 days prior to birth (gestational day 18). Five pregnant female mice were used for the treatment.

* There is a significant difference between the treatment group and its concurrent control ($p < 0.01$).

There is a significant difference between the treatment group and mice treated with ENU at PNDs 42 or 126 ($p < 0.05$).

Table 2

Liver *cII* mutant frequencies in ENU-treated and control Big Blue female mice

Group name	Age at treatment (postnatal day)	Treatment[a]	Mouse ID	Total plaques screened (× 10^3)	Mutant plaques	Mutant frequency (×10^{-6})	Average MF ± S.E. (×10^{-6})
Control	8	DMSO	C6W1F	396	15	38	39 ± 6
			C6W2F	412	13	32	
			C6W3F	269	12	45	
			C6W4F	263	6	23	
			C6W5F	193	11	57	
PND –3	–3[b]	ENU	EP1F	188	60	319	382 ± 59 *,#
			EP2F	423	110	260	
			EP3F	328	174	530	
			EP4F	245	103	420	
PND 1	1	ENU	E1D1F	348	50	143	201 ± 32 *
			E1D2F	387	112	289	
			E1D3F	202	33	163	
			E1D4F	347	72	207	
PND 8	8	ENU	E8D1F	386	193	500	646 ± 107 *,&
			E8D2F	315	117	371	
			E8D3F	308	233	756	
			E8D4F	244	241	988	
			E8D5F	248	152	616	
PND 15	15	ENU	E15D1F	224	139	620	531 ± 44 *,&
			E15D2F	257	127	494	
			E15D3F	235	108	460	
			E15D4F	274	178	650	
			E15D5F	291	126	432	
PND 42	42	ENU	E6W1F	301	92	306	388 ± 76 *
			E6W2F	326	75	230	
			E6W3F	272	157	577	

Group name	Age at treatment (postnatal day)	Treatment[a]	Mouse ID	Total plaques screened (× 10^3)	Mutant plaques	Mutant frequency ($\times 10^{-6}$)	Average MF ± S.E. ($\times 10^{-6}$)
PND 126	126	ENU	E6W4F	280	123	439	145 ± 7 *
			E18W1F	365	62	170	
			E18W2F	339	45	133	
			E18W3F	275	38	138	
			E18W4F	271	39	144	
			E18W5F	297	41	138	

[a] All mice were sacrificed 6 weeks after the treatment.

[b] The mice were treated transplacentally at 3 days prior to birth. Five pregnant female mice were used for the treatment.

[*] There is a significant difference between the treatment group and its concurrent control ($p < 0.001$).

[#] There is a significant difference between the treatment group and mice treated with ENU at PND 126 ($p < 0.01$).

[&] There is a significant difference between the treatment group and mice treated with ENU at PNDs 1 or 126 ($p < 0.001$).

Table 3

Mutations in the liver *cII* gene from ENU-treated neonate and adult Big Blue male mice

Position[a]	Mutation[b]	Amino acid change	Sequence context 5′→ 3′[c]	Number of mutations (independent)	
				Neonate	Adult
–14	G → T	N/A	ctaaggaaa	1	
1	A → T	Met → Leu	catATGgtt	1	
2	T → A	Met → Lys	catATGgtt	2(1)	
2	T → C	Met → Thr	catATGgtt	1	
5	T → A	Val → Asp	atgGTTcgt		1
25	G → A	Glu → Lys	aacGAGgct		1
25	G → T	Glu → Stop	aacGAGgct	1	
29	C → A	Ala → Asp	gagGCTcta	1	
32	T → C	Leu → Pro	gctCTAcga	1	
34	C → T	Arg → Stop	ctaCGAatc	2 (2)	
35	G → A	Arg → Gln	ctaCGAatc	1	
35	G → T	Arg → Leu	ctaCGAatc	1	
38	T → A	Ile → Asn	cgaATCgag		1
40	G → A	Glu → Lys	atcGAGagt	1	
40	G → T	Glu → Stop	atcGAGagt		3(1)
41	A → C	Glu → Ala	atcGAGagt	1	
41	A → T	Glu → Val	atcGAGagt		1
42	G → T	Glu → Asp	atcGAGagt	1	
53	T → C	Leu → Pro	ttgCTTaac	2 (2)	
55	A → T	Asn → Tyr	cttAACaaa	1	
58	A → T	Lys → Stop	aacAAAatc	2 (2)	
58	A → C	Lys → Gln	aacAAAatc	1	
61	A → T	Ile → Phe	aaaATCgca	1	
62	T → C	Ile → Thr	gcaATCgca		1
64	G → A	Ala → Thr	atcGCAatg		3 (2)
68	T → A	Met → Lys	gcaATGctt		1
74	G → A	Gly → Glu	cttGGAact	1	

Position[a]	Mutation[b]	Amino acid change	Sequence context 5′→ 3′[c]	Number of mutations (independent)	
				Neonate	Adult
84	G → C	Lys → Asn	gagAA<u>G</u>aca	1	
85	A → C	Thr → Pro	aag<u>A</u>CAgcg	1	
85	A → G	Thr → Ala	aag<u>A</u>CAgcg	1	
86	C → A	Thr → Lys	aag<u>A</u>C<u>A</u>gcg		1
89	C → T	Ala → Val	acaG<u>C</u>Ggaa		1
92	A → G	Glu → Gly	gcgG<u>A</u>Agct		1
94	G → A	Ala → Thr	gaa<u>G</u>CTgtg		1
98	T → A	Val → Glu	gctG<u>T</u>Gggc	2 (2)	
99–100	GG → AT	ValGly → ValCys	gctG<u>T</u>G<u>G</u>GCgtt		1
103	G → A	Val → Ile	ggc<u>G</u>TTgat	1	
103	G → T	Val → Phe	ggc<u>G</u>TTgat	1	
104	T → C	Val → Ala	ggcG<u>T</u>Tgat	1	
108	T → A	Asp → Glu	gttGA<u>T</u>aag	1	1
109	A → G	Lys → Glu	gat<u>A</u>AGtcg	1	
110	A → C	Lys → Thr	gatA<u>A</u>Gtcg	1	
112	T → C	Ser → Pro	aag<u>T</u>CGcag		2 (2)
113	C → A	Ser → Stop	aag<u>T</u>C<u>G</u>cag	2 (2)	
118	A → C	Ile → Leu	cag<u>A</u>TCagc	1	
118	A → T	Ile → Phe	cag<u>A</u>TCagc	1	
119	T → G	Ile → Ser	cagA<u>T</u>Cagc	1	
127	T → A	Trp → Arg	agg<u>T</u>GGaag	1	
129	G → A	Trp → Stop	aggTG<u>G</u>aag	1	
130	A → G	Lys → Glu	tgg<u>A</u>AGagg	1	
132	G → T	Lys → Asn	tggAA<u>G</u>agg		1
133	A→G	Arg → Gly	aag<u>A</u>GGgac	1	
137	A→G	Asp → Gly	agg<u>G</u>A<u>C</u>tgg	1	
139	T→A	Trp → Arg	gac<u>T</u>GGatt	1	
141	G→A	Trp → Stop	gacTG<u>G</u>att		3 (3)
142	A→T	Ile → Phe	tgg<u>A</u>TTcca	2(2)	
143	T→C	Ile → Thr	tgg<u>A</u>TTcca	1	

Position[a]	Mutation[b]	Amino acid change	Sequence context 5′→ 3′[c]	Number of mutations (independent)	
				Neonate	Adult
145	C→T	Pro → Ser	attCCAaag	2 (2)	
	C→T	Pro → Leu	attCCAaag	1	
152	T→C	Phe → Ser	aagTTCtca	1	
155	C→A	Ser → Stop	ttcTCAatg	1	
161	T→A	Leu → Gln	atgCTGctt	1	
164	T→C	Leu → Pro	ctgCTTgct	1	
169	G→C	Val → Leu	gctGTTctt	1	
170	T→A	Val → Asp	gctGTTctt		1
170	T→C	Val → Ala	gctGTTctt	1	
176–177	–A	Frameshift	cttGAAtgg		1
178/185	+G	Frameshift	gaaTGGGGGGTCgtt	3 (3)	7 (4)
178	T→A	Trp → Arg	gaaTGGggg	2 (2)	
180	G→A	Trp → Stop	gaaTGGggg	1	
185	T→A	Val → Asp	gggGTCgtt	1	1
185	T→C	Val → Ala	gggGTCgtt	1	
191	A→C	Asp → Als	gttGACgac	1	1
193	G→A	Asp → Asn	gacGACgac	2 (2)	
194	A→G	Asp → Gly	gacGACgac		1
197	A→C	Asp → Ala	gacGACatg	1	
197	A→T	Asp → Val	gacGACatg	1	
200	T→A	Met → Lys	gacATGgct	1	
203	C→T	Ala → Val	atgGCTcga		1
209	T→A	Leu → Stop	cgaTTGgcg	1	
212	C→A	Ala → Glu	ttgGCGcga	2 (2)	
212	C→T	Ala → Val	ttgGCGcga	1	1
214	C→T	Arg → STOP	gcgCGAcaa	3 (3)	1
221	T→C	Val → Ala	caaGTTgct	1	1
221	T→G	Val → Gly	caaGTTgct	1	
230	T→A	Ile → Asn	gcgATTctc	2 (2)	
235	A→C	Thr → Pro	ctcACCaat	2 (2)	1

Position[a]	Mutation[b]	Amino acid change	Sequence context 5′→ 3′[c]	Number of mutations (independent)	
				Neonate	Adult
241	A→T	Lys → Stop	aat<u>A</u>AAaaa	1	1
245	A→C	Lys → Thr	aaaA<u>A</u>Acgc	1	
289	T→A	Phe → Ile	gag<u>T</u>TCtga		1
290	T→A	Phe → Tyr	gagT<u>T</u>CtgaTCtga	1	2 (2)
292	T→A	Stop → Arg	ttc<u>T</u>GAggt	1	1
294	A→G	Stop → Trp	ttcTG<u>A</u>ggt		1
294	A→T	Stop → Cys	ttcTG<u>A</u>ggt	2 (2)	
Total				90 (89)	47 (41)

Abbreviations: –, deletion; +, insertion.

[a] Position 1 is the first base of the start codon in the *cII* coding sequence.

[b] Presented in term of sequence change on nontranscribed DNA strand.

[c] Uppercase indicates target codon and target bases are underlined.

Table 4

Comparison of spontaneous and ENU-induced mutations in the liver *cII* gene of neonate and adult Big Blue mice

Type of mutation	Neonate		Adult	
	Control (%)[a]	ENU (%)	Control (%)[a]	ENU (%)
G:C → C:G	5	2	9	0
G:C→A:T	50	18	41	27
G:C → T:A	21	12	6	7
At CpG site	43	21	41	17
A:T → T:A	0	33	0	29
A:T → C:G	9	14	9	5
A:T → G:C	2	18	9	17
Frameshift	13	3	22	12
Others	0	0	3	2
Total mutants screened	56	89	32	41

[a]Control mutations from literature [18]. Control animals were treated with DMSO at approximately 2 μl/g body weight.

Table 5

Comparison of mutant frequencies in the liver *cII* gene and incidence of liver tumors from the Big Blue or generic B6C3F1 mice treated with 120 mg ENU/kg body weight[a]

Sex	Age[b] (PND)	Mutant frequency ($\times 10^{-6}$)	Tumor incidence[c] (%)
	Control	41	11
	–3	279	67
	1	206	85
Male	8	442	–
	15	416	94
	42	158	35
	126	136	–
	Control	39	0
	–3	382	33
	1	201	60
Female	8	646	–
	15	531	67
	42	388	8
	126	145	–

[a] ENU was administrated to pregnant mother on day 18 of gestation (PND –3) or to offspring (postnatal days, PNDs).

[b] Age at which animals were given ENU.

[c] Tumor incidence data were from [25,26].