図３　ヒト血清プロテオームマップと HSAC34 フラグメントペプチドピークの溶出位置

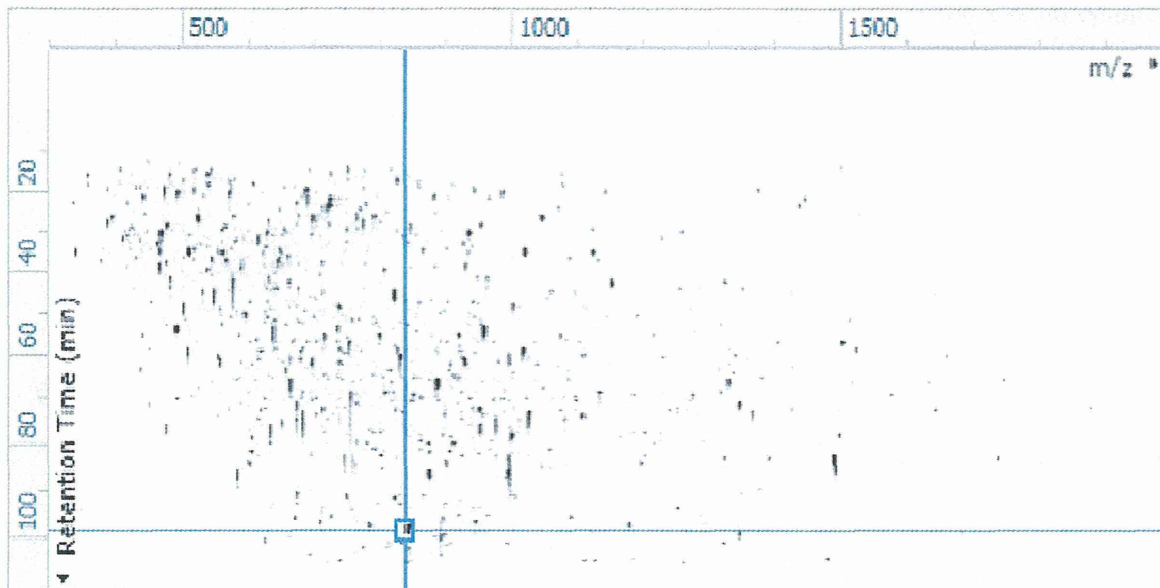


図４　HSA システイン 34 含有ペプチドの MS/MS スペクトルと付加体候補ペプチドの MS/MS スペクトルの比較

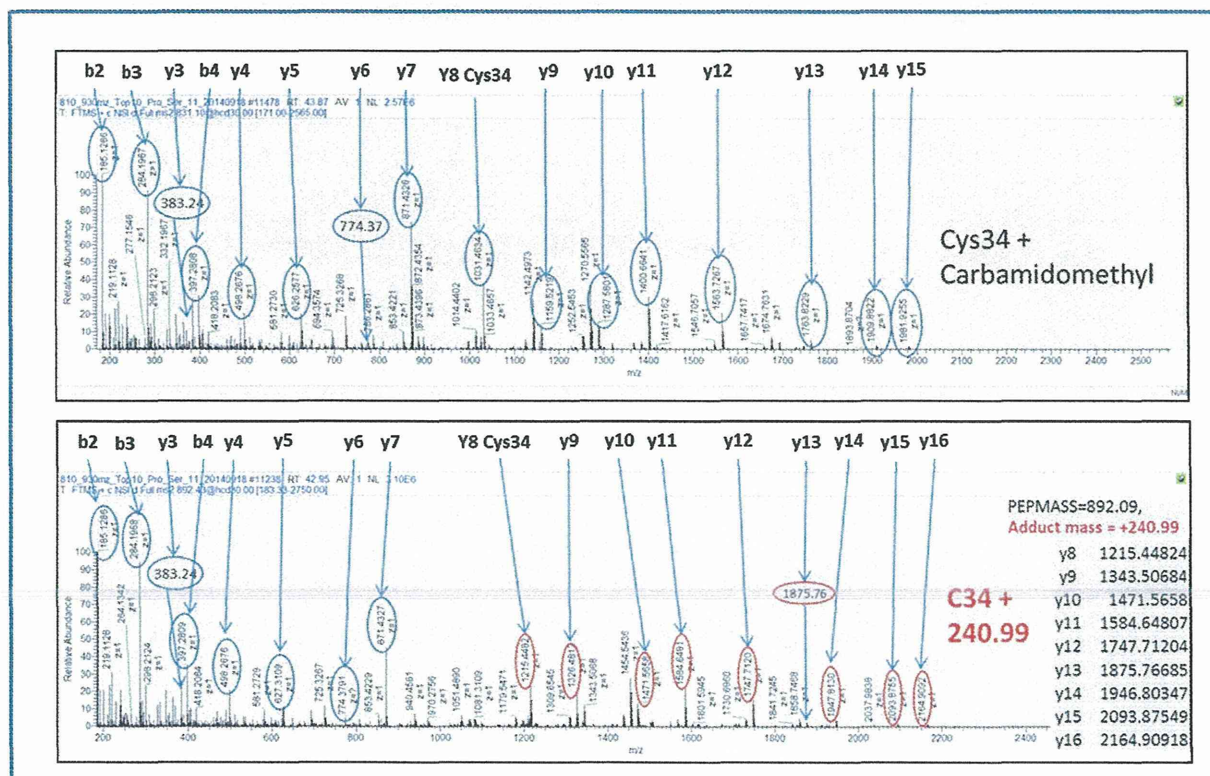
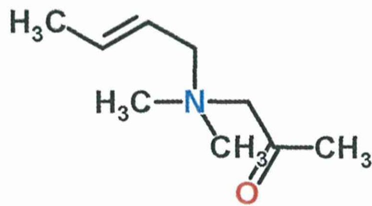


図５　元素組成より推定される化合物の構造

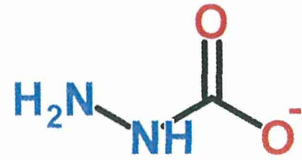
1-[[(E)-but-2-enyl]-dimethyl-λ5-azanyl]propan-2-one

Molecular Formula: C9H18NO
Average mass: Da
Monoisotopic mass: Da

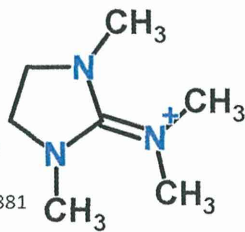


Carbazate

Molecular Formula: CH3N2O2
Average mass: 75.047 Da
Monoisotopic mass: 75.020004 Da

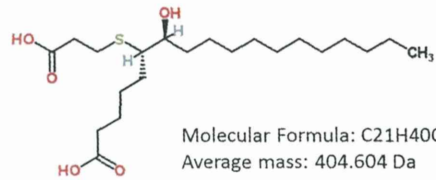


N,N,1,3-Tetramethyl-2-imidazolidiniminium



Molecular Formula: C7H16N3
Average mass: 142.221 Da
Monoisotopic mass: 142.133881 Da

(6S,7S)-6-[(2-Carboxyethyl)sulfanyl]-7-hydroxy-octadecanoic acid



Molecular Formula: C21H40O5S
Average mass: 404.604 Da
Monoisotopic mass: 404.259644 Da

図６　付加体候補ペプチドの発現量のサンプル間でのばらつき

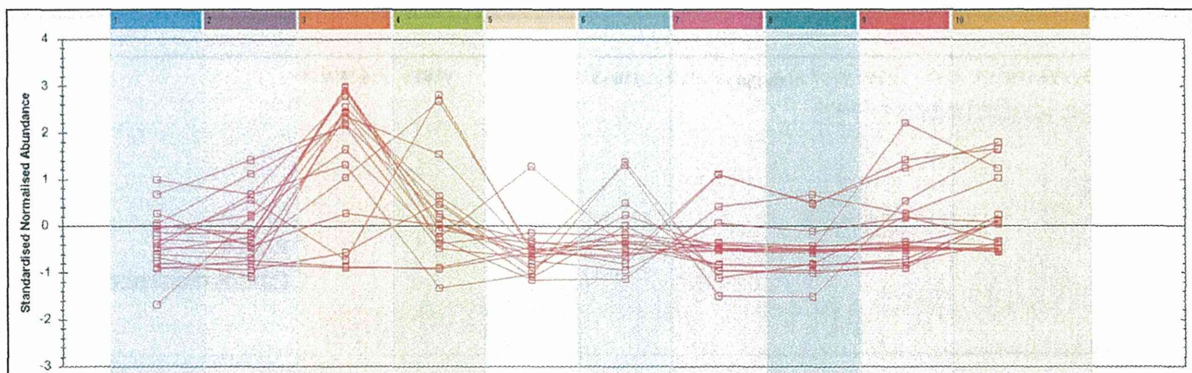


表 1　システイン 34 部位の既知の付加体候補

Modification details - UNIMOD						
Peptide mass	**2480.2449**	**Delta**	47.983	**PPM error**	0.4032	
Accession #	345		PSI-MS Name	Trioxidation	Interim Name	Cysteic_acid
Description	cysteine oxidation to cysteic acid					
Composition	O(3)		Monoisotopic	47.9847	Average	47.9982
Specificity						
Site	C		Position	Anywhere	Classification	**Chemical derivative**
Peptide mass	**2536.2519**	**Delta**	103.99	**PPM error**	11.8285	
Accession #	902		PSI-MS Name		Interim Name	DimethylArsino
Description	**dimethylarsinous (AsIII) acid**					
Composition	H(5) C(2) As		Monoisotopic	103.9607	Average	103.9827
Specificity						
Site	C		Position	Anywhere	Classification	**Post-translational**
Peptide mass	**2641.2869**	**Delta**	209.025	**PPM error**	2.6502	
Accession #	893		PSI-MS Name		Interim Name	CarbamidomethylDTT
Description	Carbamidomethylated DTT					
Composition	H(11) C(6) N O(3) S(2)		Monoisotopic	209.0180	Average	209.2864
Specificity						
Site	C		Position	Anywhere	Classification	**Artefact**

表 2　システイン 34 部位の未知の付加体候補の質量数と推定元素組成

Peptide Mass	Adduct mass	Elemental	PPM		Peptide Mass	Adduct mass	Elemental	PPM
2464.288	32.002	HO2	343.8		2521.169	88.884	CNS2	747.3
2485.236	53.983	C2HNO	287.5		2572.405	140.119	C7H15N3	4.2
2492.197	59.912	CHOS	915.3		2586.418	154.133	C9H17NO	12.8
2496.244	63.959	HS2	108.3		2588.375	156.090	C11H11N	5.41
2499.222	66.937	C3S	413.0		2629.212	196.927	C7H2OS3	1.12
2505.288	73.003	CH2N2O2	567.0		2675.700	240.990	C10H2N4O2S	0.63
2505.300	73.015	C6H2	8.8		2908.526	476.241	C23H35N5O4S	0.05
2511.260	78.974	O5	7.2		2835.537	403.252	C21H40O5S	18.9

別添５

III.　研究成果の刊行に関する一覧表

別紙4

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
鈴木孝昌	コンパニオン診断薬の現状と課題	技術情報協会	「最先端バイオマーカーを用いた診断薬/診断装置開発と薬事対応」	技術情報協会	東京	2015	271-275

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Horibata K, Ukai A, Honma M.,	Evaluation of rats' in vivo genotoxicity induced by N-ethyl-N-nitrosourea in the RBC Pig-a, PIGRET, and gpt assays.	Genes and Enviromment	36	199-202	2014
Matsumoto, M., Masumori, S., Hirata-K, M., Ono, A., Honma, M., Yokoyama, K. Hirose, A.	Evaluation of in vivo mutagenicity of hydroquinone in Muta™ mice.	Mutat Res	775-776	94-98	2014
Morita, T., Miyajima, A., Hatano, A., Honma, M.	Effects of the proposed top concentration limit on an in vitro chromosomal aberration test to assay sensitivity or to reduce the number of false positives	Mutat Res	769	34-49	2014
Onami, S., Cho, Y., Toyoda, T., Horibata, K., Ishii, Y., Umemura, T., Honma, M., Nohmi, T., Nishikawa, A. Ogawa, K.	Absence of in vivo genotoxicity of 3-monochloropropane-1,2-diol and associated fatty acid esters in a 4-week comprehensive toxicity study using F344 gpt delta rats.	Mutagenesis	29	295-302	2014

Takeiri A, Wada NA, Motoyama S, Matsuzaki K, Tateishi H, Matsumoto K, Niimi N, Sassa A, Grúz P, Masumura K, Yamada M, Mishima M, Jishage K, Nohmi T	*In vivo* evidence that DNA polymerase kappa is responsible for error-free bypass across DNA cross-links induced by mitomycin C	DNA Repair	24	113-21	2014
Takahashi-Y, F., Yoshihara, T., Jingushi, K., Igawa, K., Tomooka, K., Watanabe, Y., Morimoto, S., Nakatsu, Y., Tsuzuki, T., Nakabeppu, Y. Sasaguri, T.	DIF-1 inhibits tumor growth *in vivo* reducing phosphorylation of GSK-3b and expressions of cyclin D1 and TCF7L2 in cancer model mice	Biochem. Pharmacol.	89	340-348	2014
Ohno, M., Sakumi, K., Fukumura, R., Furuichi, M., Iwasaki, Y., Hokama, M., Ikemura, T., Tsuzuki, T., Gondo, Y. and Nakabeppu, Y.	8-Oxoguanine causes spontaneous *de novo* germline mutations in mice	Scientific Reports	4	4689 DOI: 10.1038/srep04689	2014
Isoda, T., Nakatsu, Y., Yamauchi, K., Piao, J., Yao, T., Honda, H., Nakabeppu, Y., Tsuzuki,T.	Abnormality in Wnt signaling is causatively associated with oxidative stress-induced intestinal tumorigenesis in MUTYH-null mice	Int. J. Biol. Sci.	10 (8)	940-947	2014
Kawamura Y, Hayashi H, Masumura K, Numazawa S, Nohmi T	Genotoxicity of phenacetin in the kidney and liver of Sprague-Dawley gpt delta transgenic rats in 26-week and 52-week repeated-dose studies	Toxicology	324	10–17	2014

IV.　研究成果の刊行物・別刷

Genes and Environment, Vol. 36, No. 4 pp. 199–202 (2014)

Short communication

Evaluation of Rats' *In Vivo* Genotoxicity Induced by *N*-ethyl-*N*-nitrosourea in the RBC *Pig-a*, PIGRET, and *gpt* Assays

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Received June 23, 2014; Revised August 14, 2014; Accepted August 25, 2014
J-STAGE Advance published date: August 30, 2014

The emerging *Pig-a* gene mutation assay, a powerful and promising tool for evaluating *in vivo* genotoxicity, is based on flow cytometric enumeration of red blood cells (RBCs), which are deficient in glycosylphosphatidylinositol anchored protein. Various approaches for measuring *Pig-a* mutant cells have been developed, particularly those focused on peripheral RBCs and reticulocytes (RETs). Previously, it had been reported that *Pig-a* and *gpt* mutant frequencies were relatively increased in *N*-ethyl-*N*-nitrosourea (ENU)- and benzo[*a*]pyrene (BP)-treated mice. The capacity and characteristics of the *Pig-a* assay relative to transgenic rodent (TGR) mutation assays, however, are unclear in rats. Here, using transgenic *gpt* delta rats, we compared the *in vivo* genotoxicity of single oral doses of ENU (40 mg/kg) in the *gpt* gene mutation assay in bone marrow and liver, and *Pig-a* gene mutation assays on RBCs and RETs in the same animals. The *Pig-a* gene mutation assays were conducted at 1, 2, and 4 weeks after treatment, whereas *gpt* assays were conducted on tissues collected at the 4-week terminal sacrifice. Consequently, we detected that *Pig-a* and *gpt* mutant frequencies were clearly increased in ENU-treated rats, indicating that both the *Pig-a* and TGR gene mutation assays can detect *in vivo* ENU genotoxicity equally.

Key words: transgenic rodent mutation assays; glycosylphosphatidylinositol anchor; red blood cells; reticulocyte

Introduction

Because gene mutations are implicated in the etiology of cancer and other human diseases, *in vivo* genotoxicity tests are important as public health management tools. One such tool is the transgenic rodent (TGR) mutation assay, which permits quantitative and accumulative evaluation of genotoxicity in all organs (1). The TGR mutation assay fulfills a need for a practical and widely available *in vivo* test for assessing gene mutation, and this assay has been recommended by regulatory authorities for safety evaluations (2,3) and international guidelines have been published describing the conduct of the assay (4).

The emerging *Pig-a* gene mutation assay, a powerful and promising tool for the evaluation of *in vivo* genotoxicity, was recently developed (5–7). Because the *Pig-a* gene is X-chromosome linked and involves the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, the forward mutation can result in the loss of GPI-anchored protein expression (7). Additionally, *Pig-a* mutation appears to function in a neutral manner whereby the accumulated effects of repeat exposures can be evaluated.

Various approaches for measuring *Pig-a* mutant cells have specifically focused on peripheral red blood cells (RBCs) and reticulocytes (RETs) (8–18). In these reports, although SYTO 13 dye or an antibody against the rat erythroid marker, HIS49, was used to label RETs in whole bloods, there was a limited capacity for counting RETs by flow cytometer. Conversely, a recently developed assay for measuring *Pig-a* mutant RETs, that is the PIGRET assay, is capable of flow cytometric cell counting $>1\times10^6$ RETs for the *Pig-a* mutant by concentrating RETs in whole bloods (19) and the approaches can be technically transferred between laboratories (20).

In this study, we performed the *gpt* and *Pig-a* gene mutation assays on RBCs (RBC *Pig-a* assay) and RETs (PIGRET assay) in the same animals, and we compared their performance in detecting ENU genotoxicity. This report describes the performance, effectiveness, and advantages of the RBC *Pig-a* and PIGRET assays in comparison with the *gpt* assay.

Materials and Methods

Preparation of chemicals: We dissolved ENU (Sigma-Aldrich Japan, Tokyo) in phosphate-buffered saline (PBS; pH 6.0) at 10 mg/mL.

Antibodies: We obtained anti-rat CD59 (clone

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TH9, FITC-conjugated), anti-rat CD71 (clone OX-26, PE-conjugated), and anti-rat erythroid marker (clone HIS49, APC-conjugated) antibodies from BD Biosciences (Tokyo, Japan).

Treatment of rats: Animal experiments were conducted humanely according to the regulations of the Animal Care and Use Committee of the National Institute of Health Sciences, Tokyo, and with their permission. *gpt* delta Wistar Hannover transgenic male rats were obtained from Japan SLC (Shizuoka, Japan). They were housed individually under specific pathogen-free conditions with a 12-h light-dark cycle and given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., Ltd., Tokyo) *ad libitum*. At 8 weeks of age, 5 rats per group were given a single oral administration of ENU (40 mg/kg) or PBS (negative control). Peripheral blood (120 μL) was withdrawn from a tail vein 1, 2, and 4 weeks after the treatments and used for the RBC *Pig-a* and PIGRET assays. At 4 weeks, all rats were sacrificed and the bone marrow and liver samples were collected for the *gpt* assay.

***Pig-a* mutation assays:** The RBC *Pig-a* and PIGRET assays were performed as previously described (5,19,20). Peripheral blood was withdrawn and immediately transferred into EDTA (dipotassium salt)-coated Microtainer® Tubes (BD Biosciences). For the RBC *Pig-a* assay, 3 μL of blood was suspended in 0.2 mL PBS and labeled with anti-rat CD59 (1 μg) and anti-rat erythroid marker (0.133 μg) antibodies. The cells were incubated for 1 h in the dark at room temperature, centrifuged (1,680 × g, 5 min), resuspended in 1 mL PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences). After gating for single cells, approximately 1×10^6 erythroid marker-positive cells were analyzed for the presence of surface CD59 and the *Pig-a* mutant frequency (MF) was calculated as previously described (19,20). For the PIGRET assay, 80 μL of blood was suspended in 0.2 mL PBS and labeled with 1 μg of PE-conjugated anti-rat CD71 antibody. The cells were incubated for 15 min in the dark on ice. After washing with 2 mL of 1 × IMag Buffer (BD Biosciences) and centrifuged (1680 × g, 5 min), the cells were mixed with 50 μL of BD IMag PE Particles Plus-DM (BD Biosciences) and incubated for 15 min in a refrigerator (4°C). The samples were enriched for CD71-positive cells by processing with a BD IMagnet magnetic stand (BD Biosciences) according to the manufacturer's instructions. The enriched samples were labeled with HIS49 and anti-CD59 antibodies as indicated for total RBC labeling, with the exception that the incubation time for labeling enriched RETs was half that for the total RBCs. The final cell suspension volume was 500 μL. *Pig-a* MF of CD71-positive RETs was examined using a FACS Canto II flow cytometer (BD Biosciences) as previously described (19,20).

***gpt* mutation assay:** We extracted high molecular weight genomic DNA from the liver and bone marrow samples using a Recover Ease DNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA), rescued lambda EG10 phages using Transpack Packaging Extract (Agilent Technologies), and conducted the *gpt* mutation assay as previously described (1). *gpt* Mutant frequencies (MFs) were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of colonies with rescued plasmids (1).

Statistical Analyses: The Mann-Whitney U-test was used for comparisons between PBS- and ENU-treated groups at each time point. Statistical analyses were performed using Prism 6 for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA). For these analyses, a p-value of <0.05 was considered significant and one-tailed tests were performed.

Results

The *Pig-a* assay: *Pig-a* MFs of whole RBCs (RBC *Pig-a* MF) in the ENU-treated rats were significantly increased, and the increase was modestly time-dependent (mean ± SD for rats treated with PBS: pre-treatment, $3.20 \pm 1.79 \times 10^{-6}$; 1 week after treatment, $1.80 \pm 1.30 \times 10^{-6}$; 2 weeks after treatment, $0.80 \pm 0.45 \times 10^{-6}$; and 4 weeks after treatment, $2.20 \pm 2.28 \times 10^{-6}$; mean ± SD for rats treated with 40 mg/kg ENU: pre-treatment, $2.80 \pm 2.28 \times 10^{-6}$; 1 week after treatment, $4.60 \pm 1.67 \times 10^{-6}$; 2 weeks after treatment, $31.4 \pm 5.86 \times 10^{-6}$; and 4 weeks after treatment, $52.80 \pm 8.84 \times 10^{-6}$) (Fig. 1A).

In the case of *Pig-a* MFs of RETs (RET *Pig-a* MF), significant increases were detected 1 week after treatment (mean ± SD for rats treated with PBS: pre-treatment, $3.00 \pm 4.81 \times 10^{-6}$; 1 week after treatment, $1.80 \pm 1.92 \times 10^{-6}$; 2 weeks after treatment, $2.80 \pm 1.64 \times 10^{-6}$; and 4 weeks after treatment, $0.80 \pm 0.84 \times 10^{-6}$; mean ± SD for rats treated with 40 mg/kg ENU: pre-treatment, $3.60 \pm 3.21 \times 10^{-6}$; 1 week after treatment, $107.6 \pm 10.5 \times 10^{-6}$; 2 weeks after treatment, $126.6 \pm 15.6 \times 10^{-6}$; and 4 weeks after treatment, $162.6 \pm 54.89 \times 10^{-6}$) (Fig. 1B).

The *gpt* assay on the bone marrow and liver samples

Compared with the solvent control animals (MF for PBS control group, $17.05 \pm 12.10 \times 10^{-6}$), significant increases in bone marrow *gpt* MFs were observed in ENU-treated rats [$87.39 \pm 60.55 \times 10^{-6}$ (Fig. 2)]. *gpt* MFs were also increased in the liver samples (MF for PBS control group, $7.56 \pm 7.97 \times 10^{-6}$; ENU, $79.04 \pm 31.62 \times 10^{-6}$) (Fig. 2).

Discussion

Here, we showed ENU genotoxicity using three different methods: the RBC *Pig-a*, PIGRET, and TGR

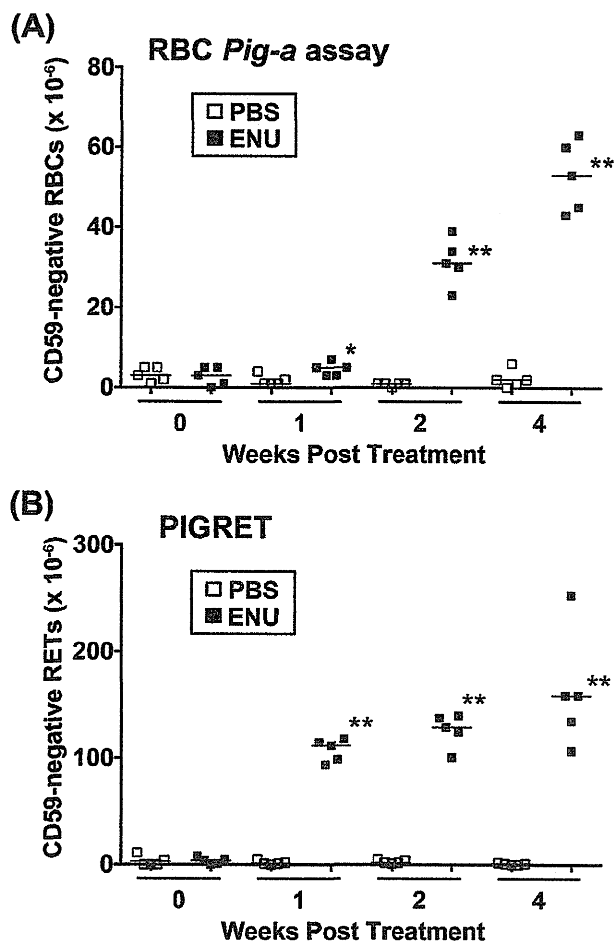

Fig. 1. Comparative analyses of the RBC *Pig-a* (A) and PIGRET (B) assays. At 1, 2, and 4 weeks after treatment with 40 mg/kg ENU or PBS solvent, peripheral blood was withdrawn from the tail vein and analyzed by flow cytometry for the presence of surface CD59 on RBCs or RETs. $^{*}p<0.05$, $^{**}p<0.01$.

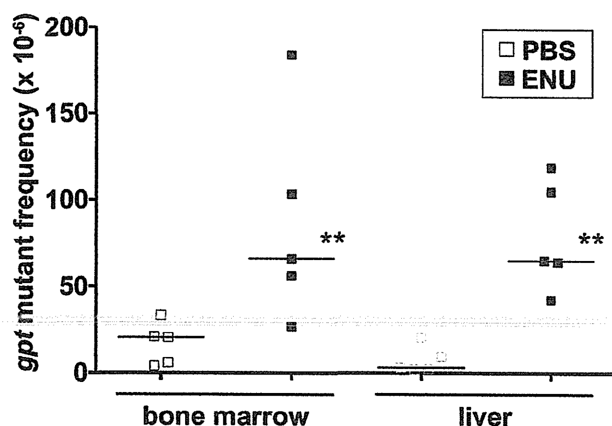

Fig. 2. *gpt* Mutation frequencies in the bone marrow and liver samples. Four weeks after treatment, all rats were sacrificed and their bone marrow and liver samples were collected and analyzed by the *gpt* assay. $^{**}p<0.01$.

mutation assays in rats. Although TGR mutation assays, such as the *gpt* gene mutation assay performed here, are well established methods and permit the *in vivo* evaluation of genotoxicity in more than one organ concurrently (1,4,21), they are costly and need TGR animals. While the *Pig-a* gene mutation assays, including the PIGRET assay, analyze only one type of cells (i.e., blood cells), these assays have the advantage of not using transgenic animals (5,6) and strong potential to be integrated into repeat-dose toxicology studies because accumulated effects can be evaluated (8–10). Additionally, compared with the RBC *Pig-a* assay, the PIGRET assay can detect increases in *Pig-a* MF sooner after exposure (20).

The results of our RBC *Pig-a* and PIGRET assays indicated that the latter more consistently detected ENU-induced increases in *Pig-a* MF at early sampling times than the former (Fig. 1). These results obtained using a single oral administration of ENU were consistent with those previously reported (15,19,20). The ENU-induced *gpt* MFs on the bone marrow and liver samples were well detected as MFs of the RBC *Pig-a* and PIGRET assays (Fig. 2), suggesting that both assays were equally able to detect ENU genotoxicity.

The OECD guideline for TGR assays recommends a tissue sampling time of 3 days after 28 consecutive daily treatments (4), making it difficult to integrate TGR assays into standard repeat-dose toxicology studies. Because the *Pig-a* gene is an endogenous gene, the *Pig-a* assay can be combined with a TGR assay as was done in this present and a previous study (14), and it also can potentially be integrated into repeat-dose toxicology studies that do not use TGRs (8,9,11,12,16,17,22–24). Additionally, the PIGRET assay has strong potential to detect genotoxicity in an early stage of the study, e.g., at 1 week after exposure. Currently, however, we need additional studies that compare mutational responses in the *Pig-a* gene and TGR transgenes to help validate the *Pig-a* assays.

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Disclosure statement: The authors declare no conflict of interest.

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