Table 1. Representative Test Classified by Endpoints

	DNA damage	Gene mutation	Chromosomal aberration
In vitro	Rec assay Umu assay ^{32}P-post labeling assay Alkaline elution assay Comet assay	Microbial reverse mutation assay (OECD TG 471)* HPRT gene mutation assay using Chinese hamster cells (OECD TG476) Mouse lymphoma TK assay (MLA, OECD TG is preparing)	Metaphase analysis using cell Line or primary culture (OECD TG473) *In vitro* micronucleus assay (OECD TG478) Mouse lymphoma TK assay (MLA, OECD TG is preparing)
In vivo	Unscheduled DNA sysnthesis Assay (OECD TG486) Alkaline elution assay comet assay (OECD TG is preparing)	Transgenic animal mutation model (OECD TG488)	Bone marrow metaphase analysis (OECD TG475) Rodent micronucleus assay (OECD TG474)

* Assay with underline is a component of standard test battery in fields of pesticides, agricultural chemicals, and pharmaceuticals

There are four independent Expert Committees for evaluation of agricultural chemicals under umbrella of the Executive Committee, one of which purpose is to make balance among four Expert Committees. The authors hope this paper will be a guidance to help makings evaluation fair and transparent across the working groups.

Basically, the FSC evaluates the safety of material as a single chemical and not as a complex mixture, to which people usually are exposed. There remains a great need to assess complex mixtures or combinations of chemicals to which humans are exposed. To consider such multiple chemicals exposure, the concept of threshold is important to evaluate overall risk if each chemical is evaluated separately, because, if each chemical has no threshold genotoxic effects the effects should be accumulating,and may reach the virtual human risk. Another important and difficult issue is the reliability of extrapolation from unrealistically high concentrations/dose experiments to the actual human exposure level. Generally, laboratory assays for genotoxicity are conducted at quite high concentrations/dose levels because of technical considerations.

Evaluation of the safety of agricultural chemicals is not simple. People usually consume vegetables, for example, after careful washing and cooking (heating). Therefore, the agricultural chemical and probably metabolite residues are diminished during preparation. Thus, actual exposures are often overestimated. This provides an additional margin of safety between our actual consumption and the estimated exposure, in relation to the ADI.

Genotoxicity Test Systems

To detect genotoxicity of chemicals many test methods have been developed and used in both research and regulatory science fields. There are two principal endpoints thought to be related directly to health risk—gene mutation and chromosomal aberration—among other supportive endpoints. The most important secondary endpoint is DNA damage that can lead to mutations or chromosomal aberrations. There is no assay system that detects all endpoints in one experiment. Therefore we use several assay systems to cover at least two major endpoints. This evaluation strategy is called a "battery" approach and has been accepted in the regulatory science field to avoid making false negative conclusions for the chemicals. **Table 1** shows representative assay systems classified by endpoint and test materials.

An important consideration is the choice of test systems. One major classification is *in vitro* vs. *in vivo* assays. Gene mutation was originally recognized and evaluated using plants by Mendel, and much basic mutation research has been conducted using microbial assays and plant cells. In the field of regulatory science, Bruce N. Ames developed the assay system to detect mutagenic chemicals using *Salmonella typhimurium* TA strain series in combination with an exogenous metabolic activation system to mimic mammalian metabolism (OECD TG471)[3]. These tester strains were modified to increase their sensitivity to detect either base-change gene mutation or frame-shift mutation. The so-called Ames assay is performed in Petri plates, and so we call this an *in vitro* assay although it uses a whole organism (bacterium). To detect another endpoint, chromosomal aberrations, cultured Chinese hamster cells are frequently used because of the small number of large chromosomes, which can be easily scored for chromosomal aberrations. Recently, the selection of target cells has become an important issue for regulatory science because it has been recognized that the species or origin and

also the status of the p53 gene can affect sensitivity/specificity[4]. The currently employed *in vitro* assay systems have high performance and relatively low cost, and have proven valuable as reliable screening methods to detect mutation.

There is a current trend toward more extensive use of exposure-related risk assessment of genotoxic agents, and therefore more emphasis is being placed on results from the *in vivo* assay systems. The rodent micronucleus assay to detect chromosomal aberrations is well-established in the field of regulatory science[5,6] and has been incorporated into the standard battery for assessing chemical mutagenicity. Although the micronucleus assay has been widely used, the target organ has been limited exclusively to hematopoietic cells; *e.g.*, bone marrow polychromatic erythrocytes and peripheral reticulocytes. The most important role of genotoxicity evaluation is to identify the mechanism of carcinogenicity and to determine whether it is dependent on genotoxicity or not. Accordingly, the use of only hematopoietic cells is insufficient to answer this question. The transgenic animal models (OECD TG488)[7] and the single cell gel electrophoresis assay (comet assay)(OECD TG in preparation) have become well recognized and are widely used because these assays can evaluate essentially any tissue of interest. Recently, the micronucleus assay has been extended to many organs (*e.g.*, testes, skin, liver, digestive tract)[8]. Thus three important endpoints of genotoxicity can be covered by *in vivo* assay systems: the comet assay detects DNA damage, the transgenic animal model detects gene mutation, and the micronucleus assay detects chromosomal aberrations. Because these assays can be applied to the organ that is the target for carcinogenicity, the genotoxicity assay can play an important role for chemical safety assessment for humans.

Although the importance of the *in vivo* assay systems is recognized, another important issue is animal welfare. Therefore, it is important to seek a balance between these two important issues, and attention has been given to minimizing animal use while at the same time obtaining the data necessary for risk assessment[6]. Examples include: 1) The micronucleus assay using a tiny amount of peripheral blood without killing experimental animals can provide time-course information after treatment[9]. This method can reduce animal usage. 2) Omission of the concurrently treated positive control animals in the assay is appropriate when a laboratory has demonstrated experience and pre-prepared scoring controls are included. 3) Several endpoints may often be combined into one assay; for example, the micronucleus assay and comet assay sharing animals for both endpoints[10]. This also reduces animal use. 4) The possible ultimate method is the integration of genotoxic endpoints into the general toxicological study[11]. This not only reduces the number of animals to be used but improves the overall assessment and interpretation of chemical safety to humans by providing toxicity, exposure, and metabolic information in the context of the genotoxicity assay.

Assay systems used for regulatory safety assessments should be well validated and widely used for a long period. On the one hand, standardization of methods for research oriented studies is not critical. On the other hand, for regulatory science, the assay methods to be used must be well-characterized and standardized, including characterization of limitations and the possibility of false-positive/false-negative results. Validation studies should be designed based on the established strategy and should evaluate the technical aspects of the method, intra- and inter-laboratory reproducibility, and performance to detect genotoxic chemicals and also not to detect non-genotoxic chemicals[12,13].

Strategy of Assessment and Interpretation

1) Data Quality

At the FSC, risk assessment of food and food related chemicals, including agricultural chemicals, is routinely undertaken. One of the most important criteria is that the assessment should be done using high quality test data, especially when the data are obtained from the public literature. Adherence to test guideline (TG) and good laboratory practice (GLP) cannot fully guarantee the quality of data, but these are minimum requirements of qualified data. In the case of assessment of new agricultural chemicals, the majority of data are obtained using TG and done under conditions of GLP compliance, while in the case of food additives, it is often necessary to use published data obtained without consideration of TG nor GLP. Moreover, some chemicals may need to be assessed without any final reports of tests on the chemicals, using only the evaluation reports conducted by overseas authorities.

2) Expert Judgment and Low-dose Assessment

Some low level food additive chemicals, especially flavoring chemicals that have been evaluated and used in overseas countries, require the use of a strategy that uses only information about structural alerts, mode of action (MOA), and overall weight of evidence (WOE)[14] together with an established threshold of toxicological concern (TTC)[15–17]. It is best to conduct risk assessments that consider mechanisms of all toxicological endpoints, referred to as mechanistic

risk characterization. It is, however, usually difficult to explain all toxicological effects by credible mechanisms. It is also difficult to assess weak/marginal effects biologically, although statistical evaluation can be done especially at very high dose levels. For risk assessment, exposure analysis is important especially at low dose levels, to establish an acceptable Margin of Exposure (MOE)[18].

3) Threshold

One important consideration in genotoxicity risk assessment is the theoretical lack of threshold for DNA-reactive chemicals. Theoretically, reactivity is based on the probability of collision between DNA and the chemical substance. This cannot result in a "0" probability of introducing a lesion that could theoretically cause, for example, a cancer or birth defect. Organisms, however, have many defense mechanisms against genotoxic events. For example, DNA repair mechanisms rapidly remove lesions on the DNA to restore normal DNA. Such protective mechanisms suggest that even agents that directly introduce DNA lesions may have a threshold of exposure below which the probability of producing a health effect is insignificantly small.

Kirsch-Volders, *et al.* (2000)[19] proposed simple definitions for "real" threshold and "alleged" threshold as follows:

"Real" threshold: a concentration/dose below which the measured effect does not occur.

"Alleged" threshold: a concentration/dose below which the measured effect does occur, but cannot be detected, because the system is not sufficiently sensitive to discriminate it from spontaneous events.

Now it is widely accepted that the genotoxic chemicals that do not target DNA directly have thresholds, at least a practical threshold[20]. For example, spindle poisons, which induce numerical chromosomal aberration, target mitotic apparatus[21], topoisomerase family, which disturbs the fidelity of DNA unwinding and resulting in infidelity of DNA duplication[22,23], the imbalance of nucleotides in cells which also disturbs the fidelity of DNA duplication[24,25], *etc.*, are thought to have such thresholds. Whenever the mechanism of mutagenesis is based on lesions in biomolecules other than DNA, it is generally accepted that a threshold can be identified.

For risk characterization, it is important how the threshold of genotoxicity is considered, especially in the case of direct-acting mutagens that interact with DNA. As mentioned above, mutagens that target macromolecules other than DNA can be assessed by considering exposure level relative to a defined threshold. Although international worldwide agreement has not yet been obtained to accept a threshold concept for direct DNA-reactive mutagens, appropriate approaches for estimating acceptable exposure levels to such mutagens are being discussed by international expert committees. As mentioned in the former section, there is already substantial evidence to demonstrate practical thresholds for at least some directly DNA-reactive mutagens[26,27]. We think it is possible to assess such mutagens in the same way as other toxicological effects that exhibit thresholds[28,29], by determining a virtually safe dose (VSD), ensuring levels As Low As Reasonably Achievable (ALARA) in most cases, and/or establishing appropriate TTCs.

There are several cases in which evidence has shown a threshold concentration/dose, at least a practical threshold, below which the measured effect does not occur although the exposure is enough for some other effects occur[26,27]. These cases include observations of DNA damage, bacterial gene mutation, and chromosomal aberrations. The *rec* assay provides a good example (**Fig. 1**). The test chemical is spotted on the filter paper (black circle on the right) and diffuses into the plate making a concentration gradient. Repair deficient *Bacillus subtilis* strain (M45) cannot survive in an area close to the test chemical spotted site where the concentration is high. In contrast, the repair proficient strain (H17) survives at the area even close to the spotted site where the chemical exists at higher concentration. This is evidence to show that there are protective mechanisms that would be expected to result in a threshold at low exposure levels.

Kirsch-Volders, *et al.*[19] discussed the alleged threshold because any assay system may not be sufficiently sensitive to detect the endpoint at very low concentration/dose levels. However, Asano, *et al.* showed that increasing the number of cells analyzed in the peripheral blood micronucleus assay up to 1 million cells per animal by flow cytometer on three model chemicals still did not demonstrate an effect at low concentration (**Fig. 2**)[30]. Sensitivity of course depends on the unit evaluated, numbers of cells or animals. Although it may be possible to increase sensitivity using more animals or cells, the important issue is the biological relevance of minimal effects. Animal welfare considerations also place a limit on the extent to which numbers of animals are increased to obtain small, and possibly biologically insignificant, increases in sensitivity.

These apparent threshold phenomena are also observed in the bacterial reverse mutation (**Fig. 3**). **Fig. 3** shows the outcomes of the Ames assay using TA1535 and YG7108, which is an O^6-methylguanine DNA methltransferase-deficient strain. The YG7108 strain induced reverse mutation by *N*-methyl-*N*-nitro-nitrosoguanidine at concentration levels where the TA1535 strain did not respond[26]. Finally, the authors would like to propose the following definitions of threshold, including the case of DNA direct-acting mutagens:

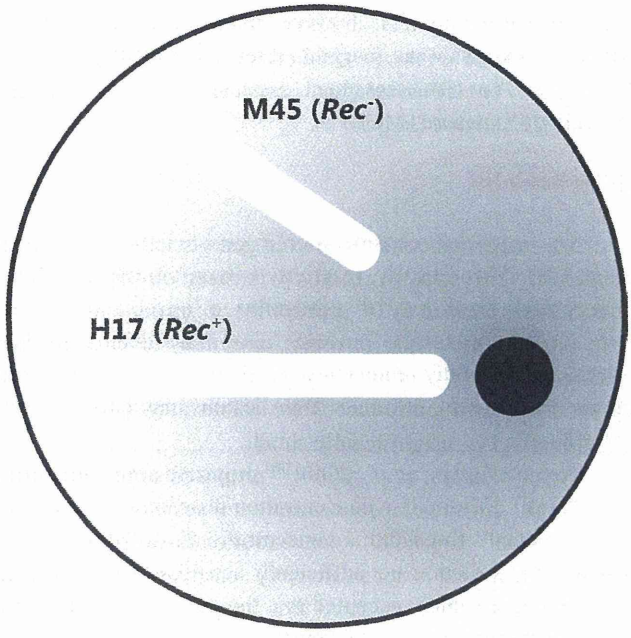

Fig. 1. *rec*-assay has been used widely in Japan to assess DNA damage using *Bacillus subtilis* wild strain H17 with repair capacity and strain M45 deficient DNA repair.

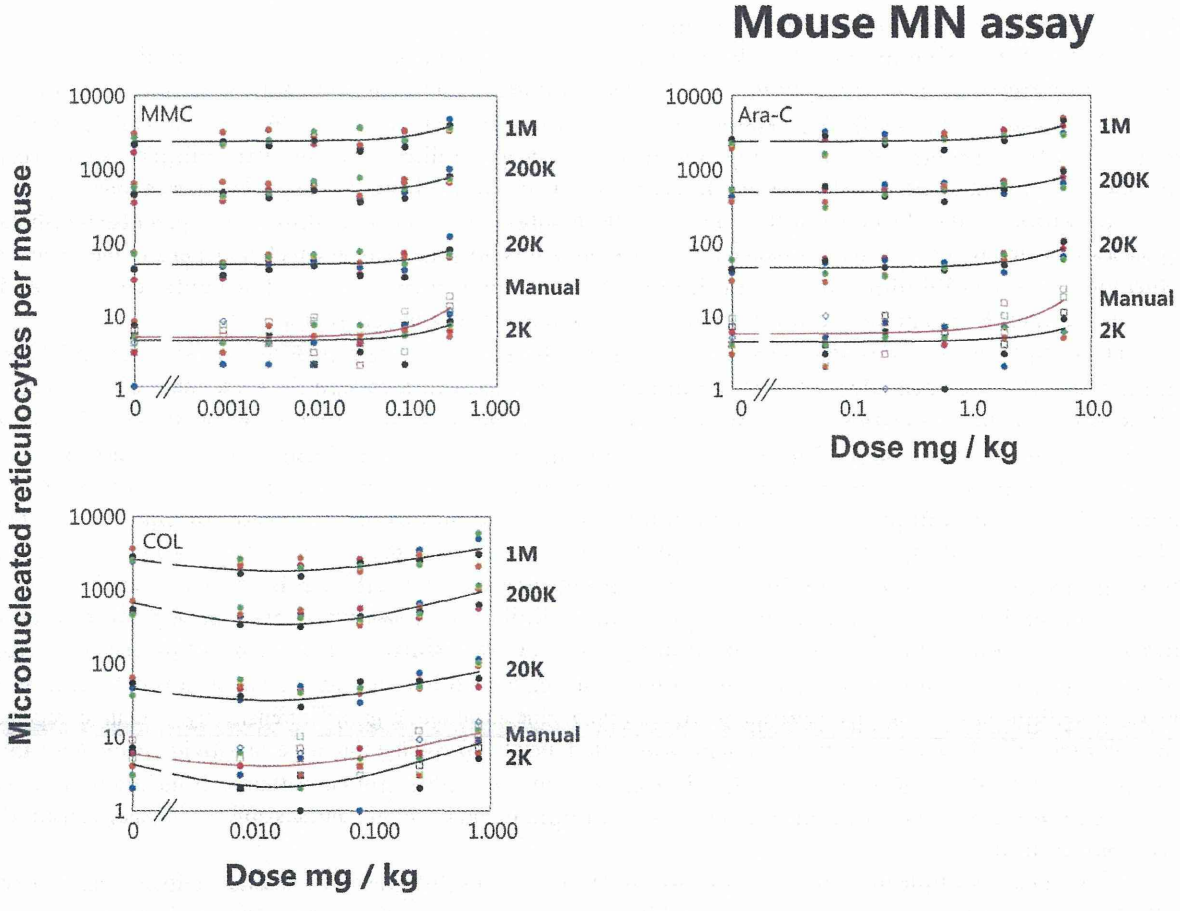

Fig. 2. Mouse micronucleus assay using 3 model chemicals with different mode of action analyzed from 2000 cells per animal manually (red) and flow-cytometry up 1 million cells per animal with flow cytometer (black).

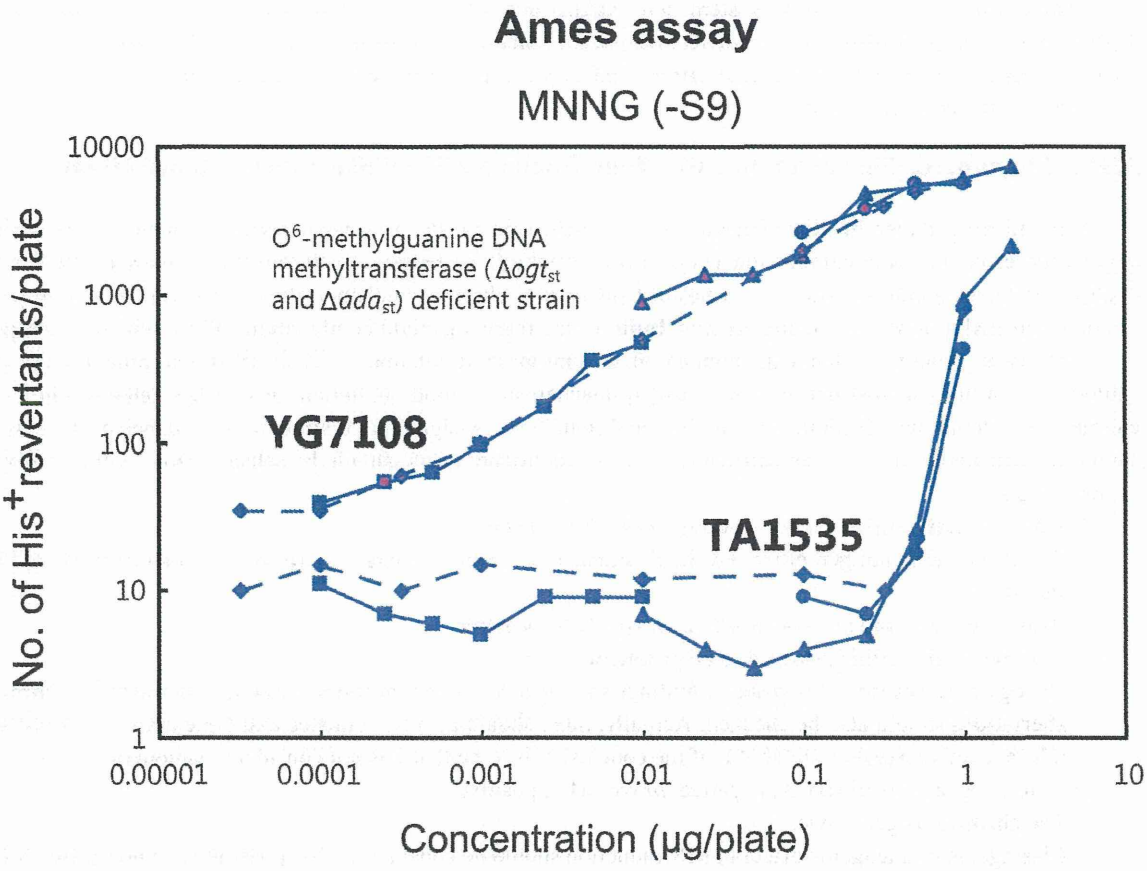

Fig. 3. Dose response curves of reverse gene mutation using TA1535 and $O^{6)}$-methylguanine DNA methltransferase deficient strain YG7108 on *N*-methyl-*N*-nitro-nitrosoguanidine

"Real" threshold: a concentration/dose below which the measured effect does not occur.

"Practical" threshold: a concentration/dose below which the measured effect is not observed (does not occur or cannot be detected by ordinary assay systems) and below which any effect is not considered biologically relevant.

According to the definition, for the purpose of risk characterization, we do not have to consider "no threshold" or "no risk" even for DNA direct acting mutagens. It may be necessary, however, to add larger safety-factors in the case of clearly mutagenic chemicals.

4) Standard Battery and Additional Studies

It is the basic strategy to apply a battery system for identification of hazard of agricultural chemicals. This battery includes a "microbial (*S. typhimurium* and *E. coli*) gene mutation assay (commonly called the Ames test) with and without an exogenous metabolic activation system (OECD TG471)", an "*in vitro* chromosomal aberration assay (OECD TG473)", and an "*in vivo* rodent micronucleus assay (OECD TG474)". The mouse lymphoma TK assay (MLA) (OECD TG476/under preparation) and *in vitro* micronucleus assay (OECD TG478) are alternatives to the *in vitro* chromosomal aberration assay and evaluate equally the induction of structural and numerical chromosomal aberrations by test chemicals. Recently, other *in vivo* assay systems have been, or are being, developed and validated. The transgenic animal model to detect gene mutation (OECD TG488) and the comet assay to detect DNA damages (OECD TG under preparation) have been introduced. Both assays can be applied to any organs of animals, and thus it is possible to assess these endpoints in the organ that is the target of carcinogenicity. Moreover, the development of the micronucleus assay in tissues other than bone marrow has been achieved; moreover it can target tissues other than hematopoietic tissue, *e.g.*, testes, skin, liver, and digestive tracts[8)].

There are many other assay systems to detect genotoxic events. For example, gene mutation assays using insects, the sperm abnormality assay, cytogenetic assays using plants, the gene-conversion assay using yeast, the sister chroma-

tid exchange assay, *etc.* These assay systems have neither been well validated nor used widely. Accordingly, the results of these assays are considered as supportive evidence of chemical genotoxicity and should be used on a case-by-case basis. The basic components of standard battery and assay systems with validated test guideline should be considered first to make risk characterization.

5) Identification of Genotoxic Hazards from Chemicals That May Impact Human Health

Standard hazard identification of chemicals for genotoxicity as the first step of risk assessment is shown in **Table 2**, which is based on the combination of the results from a standard test battery. As the current standard test battery consists of a bacterial gene mutation assay (*i.e.*, Ames test), an *in vitro* chromosomal aberration (CA) assay, and an *in vivo* rodent micronucleus (MN) assay (see section 4) and **Table 1**), the following eight combinations of test outcomes are possible:

In all cases, quality of data (*e.g.*, application of appropriate guidelines or GLP), chemical properties (*e.g.*, purity, solubility, volatility)/class/structure, study design, mechanism or mode of action, dose-effect relationship, or biological relevance should be taken into account. For additional tests, weight and/or strength of evidence of the existing data should be considered on a case by case basis, and the additional test(s) should be selected from within well-validated standard tests.

1. Ames, negative; *in vitro* CA, negative; *in vivo* MN, negative
   The chemical is not genotoxic. For final conclusion, quality of data (*i.e.*, reliability, relevance) should be taken into account.
2. Ames, negative; *in vitro* CA, positive; *in vivo* MN, negative
   The chemical is not considered to be genotoxic *in vivo*.
   Biological relevance of the positive findings should be taken into account. Structural or numerical chromosomal aberrations should also be checked. Actually, there should also be evidence that bone marrow is exposed to the relevant active agent or metabolite if the conclusion is to be that it is not considered genotoxic *in vivo*.
3. Ames, negative; *in vitro* CA, negative; *in vivo* MN, positive
   The chemical is genotoxic.
   Clastogenic or aneugenic effect in MN induction should be considered. The possibility of non-genotoxic mechanism in MN induction is also taken into account on a case by case basis. Expert judgment is needed.
4. Ames, negative; *in vitro* CA, positive; *in vivo* MN, positive
   The chemical is genotoxic.
   Germ cell mutagenicity test might be considered on a case by case basis. Or, evaluation of effect to the germ cells in data from reproductive toxicity studies might be considered. Expert judgment is needed.
5. Ames, positive; *in vitro* CA, negative; *in vivo* MN, negative
   The chemical is concluded to be neither genotoxic nor genotoxic *in vivo*.
   Consider specific bacterial metabolism of the chemical for positive results in the Ames test. The case needs further investigation *in vitro* and/or second *in vivo* test. If a negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic. If positive, the chemical is genotoxic and a germ cell mutagenicity test should be considered. Expert judgment is needed.
6. Ames, positive; *in vitro* CA, positive; *in vivo* MN, negative
   The chemical is concluded to be neither genotoxic nor genotoxic *in vivo*.
   Structural or numerical chromosomal aberrations should be checked. Need second *in vivo* test. If a negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic *in vivo*. If positive, the chemical is genotoxic and a germ cell mutagenicity test should be considered. Expert judgment is needed.
7. Ames, positive; *in vitro* CA, negative; *in vivo* MN, positive
   The chemical is genotoxic.
   A second *in vivo* test is required. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered to cause gene mutation *in vivo*. If positive, a germ cell mutagenicity test should be considered. Expert judgment is needed.
8. Ames, positive; *in vitro* CA, positive; *in vivo* MN, positive
   The chemical is genotoxic, and is a possible germ cell mutagen.
   A germ cell mutagenicity test should be considered. If a positive result is obtained, the chemical is a germ cell mutagen and possible heritable mutagen. Expert judgment is needed.

Table 2. Genotoxic Hazard Categorization of Chemical Based on the Results of the Test Battery

No.	Ames	*In vitro* CA	*In vivo* MN	Judgement	Need for additional test(s)	Explanation
1	−	−	−	Not genotoxic	No	The chemical is not genotoxic. Quality of data (*i.e.*, reliability, relevance) should be taken into account.
2	−	+	−	Not genotoxic	No	The chemical is not considered to be genotoxic *in vivo* . Structural or numerical chromosomal aberrations should be checked. Bone marrow exposure to the chemical or metabolite should also be checked.
3	−	−	+	Genotoxic	No	The chemical is genotoxic. Consider clastogenic or aneugenic effect in MN induction. The possibility of non-genotoxic mechanism in MN induction is taken into account on a case by case basis. Expert judgement is needed
4	−	+	+	Genotoxic	No	The chemical is genotoxic. Germ cell mutagenicity test might be considered on a case by case basis. Or, evaluation of effect to the germ cells in data from reproductive toxicity studies might be considered. Expert judgement is needed.
5	+	−	−	Not concluded yet	Yes	The chemical is not concluded to be either genotoxic or not genotoxic *in vivo* . Consider specific bacterial metabolism of the chemical for positive result in the Ames test. Need further investigation *in vitro* and/or second *in vivo* test. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic. If positive, the chemical is genotoxic and germ cell mutagenicity test should be considered. Expert judgement is needed.
6	+	+	−	Not concluded yet	Yes	The chemical is not concluded to be genotoxic *in vivo* or not. Structural or numerical chromosomal aberrations should bechekced. Need second *in vivo* test. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic *in vivo* . If positive, the chemical is genotoxic and germ cell mutagenicity test should be considered. Expert judgement is needed.
7	+	−	+	Genotoxic	Yes	The chemical is genotoxic. Need for second *in vivo* test. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered to cause gene mutation *in vivo* . If positive, germ cell mutagenicity test should be considered. Expert judgement is needed.
8	+	+	+	Genotoxic	Yes	The chemical is genotoxic, and is a possible germ cell mutagen. Germ cell mutagenicity test should be considered. If positive result is obtained, the chemical is a germ cell mutagen and possible heritable mutagen. Expert judgement is needed.

In all cases, quality of data (*e.g.*, application of guideline or GLP), chemical properties (*e.g.*, purity, solubility, volatility)/class/structure, study design, mechanism or mode of action, dose-effect relationship, or biological relevance should be taken into account. The weight and/or strength of evidence of existing data should be considered for futher testing on a case by case basis. Additional test(s) should be selected from among well-validated standard tests.

If the chemical with possible genotoxic hazard (*e.g.*, No. 3 to 8) is carcinogenic, it might be a genotoxic carcinogen. However, evaluation such as carcinogenic mode of action in the target organ or toxicokinetics data will be needed to determine that it is a "genotoxic" carcinogen. If the chemical is not carcinogenic, genotoxic hazrd of the chemical to human health will be low concern. Even if so, effects to germ cells should be considered on a case by case basis.

If a chemical that is a possible genotoxic hazard (*e.g.*, No. 3 to 8) is carcinogenic, it might be a genotoxic carcinogen. However, evaluation such as carcinogenic mode of action in the target organ or toxicokinetics data will be needed to determine that it is a "genotoxic" carcinogen. If the chemical is not carcinogenic, the potential genotoxic hazard of the chemical to human health will be of low concern. Even if so, its effect to germ cells should be considered on a case by case basis.

For risk assessment of genotoxic hazard with respect to human health, exposure assessment and mode of action should be taken into account. Determination of a VSD, the TTC, and MOA or MOE will be considered as appropriate.

Discussion

The risk characterization should include proper hazard identification, dose-response relationship analysis, and consideration of exposure levels. Genotoxicity has been evaluated mainly by hazard identification in the course of chemical risk assessment. However, qualitative assessments should be considered in combination with evaluation of actual exposure levels in the context of the exposure-response relationship and with consideration of the practical threshold concept and agreed-upon exposure thresholds of concern. Generally, there is tendency to detect genotoxicity of chemicals at high concentrations/dose levels. The biological relevance of observed effects and the possibility to extrapolate to the actual exposure levels to humans should also be considered. To detect genotoxicity, there are many *in vitro* assay systems but we do not have good tools to convert from the results in *in vitro* assays to the *in vivo* situation—especially the extrapolations required to estimate the human ADI. *In vitro* assays may play an important role in characterizing hazard and defining mechanisms, thereby supporting the principals of animal welfare, but for accurate risk characterization *in vivo* assay systems play a toxicologically more important role. New *in vivo* assay systems and integration of multiple genetic endpoints into individual assays, including standard toxicological assays, can minimize animal use while providing the information necessary for accurate risk characterization[7,31]. It is not necessary to discuss the importance of the concept of threshold. It is well accepted that there are thresholds for genotoxicants that target macromolecules other than DNA. While the existence of thresholds for genotoxicants that react directly with DNA is still under discussion, there is evidence showing the existence of thresholds, at least practical thresholds, for some direct acting genotoxic chemicals. In many cases in the field of toxicology, the TTC concept is accepted as a part of chemical safety assessment[20,32,33]. The TTC concept might also be applicable to the assessment of genotoxicity in the same manner as with other toxicological endpoints. The concept of threshold is also relevant to the strategy of assessing the safety of complex mixtures. People are exposed to many kinds of chemicals concomitantly, but it is general practice to assess each single chemical independently. There is no clear evidence to show that complex mixtures give more potent genotoxicity than additive effects. If we accept the existence of threshold, even practical one, it makes easy to expand the idea to the complex mixture simply based on the exposure levels of the component.

For risk characterization, sound scientific data and reasoning are critical, but fairness and transparency are also important. These factors are especially important for risk communication, which is just as important as risk assessment and risk management. Guidance for risk characterization must include fairness and transparency among the teams that perform risk-based assessments of chemical safety.

References

1. European Chemicals Agency (ECHA), CMR substances from Annex VI of the CLP Regulation registered under REACH and/or notified under CLP – a first screening, ECHA-12-R-01-EN, May 2012 Available at: http://echa.europa.eu/documents/10162/13562/cmr_report_en.pdf.
2. European Food Safety Authority (EFSA), Opinion of the Scientific Committee on a request from EFSA related to A Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic, The EFSA Journal (2005) 282, 1–31. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/282.htm.
3. OECD 1997. OECD Guidelines for the Testing of Chemicals. Bacterial reverse mutation test (TG 471). Available at: http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en.
4. Kirkland D, Pfuhler S, Tweats D, et al. How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM workshop. Mutat Res. 2007; **628**(1): 31–55.
5. OECD 1997. OECD Guidelines for the Testing of Chemicals. Mammalian Erythrocyte Micronucleus Test (TG 474). Available at: http://www.oecd-ilibrary.org/environment/test-no-474-mammalian-erythrocyte-micronucleus-test_9789264071285-en.

6. International Conference on Harmonization, S2(R1) Guideline, Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, step 4 version, 9 November, 2011. Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step4/S2R1_Step4.pdf.
7. OECD 2011. OECD Guidelines for the Testing of Chemicals: Transgenic rodent somatic and germ cell gene mutation assays (TG 488). Available at: http://www.oecd-ilibrary.org/environment/test-no-488-transgenic-rodent-somatic-and-germ-cell-gene-mutation-assays_9789264122819-en.
8. Morita T, MacGregor JT, Hayashi M. Micronucleus assays in rodent tissues other than bone marrow. Mutagenesis. 2011; **26**(1): 223–230.
9. The Collaborative Study Group for the Micronucleus Test Micronucleus test with mouse peripheral blood erythrocytes by acridine orange supravital staining: The summary report of the 5th collaborative study by CSGMT/JEMS·MMS. Mutat Res. 1992; **278**(2–3): 83–98.
10. Pfuhler S, Kirkland D, Kasper P, et al. Reduction of use of animals in regulatory genotoxicity testing: Identification and implementation opportunities—Report from an ECVAM workshop. Mutat Res. 2009; **680**(1–2): 31–42.
11. Rothfuss A, O'Donovan M, De Boeck M, et al. Collaborative study on 15 compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies. Mutat Res. 2010; **702**(1): 40–69.
12. Organisation for Economic Co-operation and Development (OECD) Series on testing and assessment Number 34, guidance document on the validation and international acceptance of new or updated test methods for hazard assessment, ENV/JM/MONO, 14 2005. Available at: http://search.oecd.org/officialdocuments/displaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2005)14.
13. Kirkland D, Kasper P, Müller L, Corvi R, Speit G. Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests: A follow-up to an ECVAM workshop. Mutat Res. 2008; **653**(1–2): 99–108.
14. Kirkland DJ, Aardema M, Banduhn N, et al. *In vitro* approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive *in vitro* genotoxicity results. Mutagenesis. 2007; **22**(3): 161–175.
15. Munro IC, Renwick AG, Danielewska-Nikiel B. The Threshold of Toxicological Concern (TTC) in risk assessment. Toxicol Lett. 2008; **180**(2): 151–156.
16. Munro IC, Ford RA, Kennepohl E, Sprenger JG. Correlation of structural class with no-observed-effect levels: a proposal for establishing a threshold of concern. Food Chem Toxicol. 1996; **34**(9): 829–867.
17. Munro IC, Kennepohl E, Kroes R. A procedure for the safety evaluation of flavouring substances. Joint FAO/WHO Expert Committee on Food Additives. Food Chem Toxicol. 1999; **37**(2–3): 207–232.
18. EFSA Scientific Committee Statement on the applicability of the Margin of Exposure approach for the safety assessment of impurities1 which are both genotoxic and carcinogenic in substances added to food/feed, EFSA Journal 2012;10(3):2578. Available at: http://www.efsa.europa.eu/en/efsajournal/doc/2578.pdf.
19. Kirsch-Volders M, Aardema M, Elhajouji A. Concepts of thresholds in mutagenesis and carcinogenesis. Mutat Res. 2000; **464**(1): 3–11.
20. EFSA Scientific Committee Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment, EFSA Journal 2011;9(9):2379. Available at: http://www.efsa.europa.eu/en/efsajournal/doc/2379.pdf.
21. Parry JM, Fielder RJ, McDonald A. Thresholds for aneuploidy-inducing chemicals. Advisory Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment of the UK Department of Health. Mutagenesis. 1994; **9**(6): 503–504.
22. ECETOC 1997. Aneuploidy. ECETOC Monograph No.27, August 1997. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels.
23. Aardema MJ, Albertini S, Arni P, et al. Aneuploidy: a report of an ECETOC task force. Mutat Res. 1998; **410**(1): 3–79.
24. Yajima N, Kurata Y, Sawai T, Takeshita Y. Comparative studies in induction of micronuclei by three genetically recombinant and urinary human erythropoietins. Mutagenesis. 1993; **8**(3): 237–241.
25. Yajima N, Kurata Y, Imai E, Sawai T, Takeshita Y. Genotoxicity of genetic recombinant human erythropoietin in a novel test system. Mutagenesis. 1993; **8**(3): 231–236.
26. Sofuni T, Nohmi T, Ohta T, Hayashi M. [Genotoxicity: Is a threshold concept applicable to evaluate the mutagenic activity of DNA-targeting substances!?] Environ Mutagen Res. 2005; **27**: 61–73. Japanese.
27. Gocke E, Müller L. *In vivo* studies in the mouse to define a threshold for the genotoxicity of EMS and ENU. Mutat Res. 2009; **678**(2): 101–107.
28. Gillespie Z, Pulido O, Vavasour E. Risk Assessment Approaches for Carcinogenic Food Contaminants. International Food Risk Analysis Journal. 2011; **1**: 1–18.
29. O'Brien J, Renwick AG, Constable A, et al. Approaches to the risk assessment of genotoxic carcinogens in food: a critical appraisal. Food Chem Toxicol. 2006; **44**(10): 1613–1635.
30. Asano N, Torous DK, Tometsko CR, Dertinger SD, Morita T, Hayashi M. Practical threshold for micronucleated reticulocyte induction observed for low doses of mitomycin C, Ara-C, and colchicine. Mutagenesis. 2006; **21**(1): 15–20.
31. OECD Test Guideline, *In vivo* Mammalian Alkaline Comet Assay (in preparation).
32. EFSA Scientific Committee Scientific Opinion on Exploring options for providing advice about possible human health risks based on the concept of Threshold of Toxicological Concern (TTC). EFSA Journal. 2012; 10(7): 2750. Available at: http://www.efsa.europa.eu/de/efsajournal/doc/2750.pdf.
33. Dewhurst I, Renwick AG. Evaluation of the Threshold of Toxicological Concern (TTC) – Challenges and approaches. Regul Toxicol Pharmacol. 2013; **65**(1): 168–177.

Environmental and Molecular Mutagenesis 54:747–754 (2013)

Research Article

Evaluation of *In Vivo* Genotoxicity Induced by *N*-Ethyl-*N*-nitrosourea, Benzo[*a*]pyrene, and 4-Nitroquinoline-1-oxide in the *Pig-a* and *gpt* Assays

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The recently developed *Pig-a* mutation assay is based on flow cytometric enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient red blood cells caused by a forward mutation in the *Pig-a* gene. Because the assay can be conducted in nontransgenic animals and the mutations accumulate with repeat dosing, we believe that the *Pig-a* assay could be integrated into repeat-dose toxicology studies and provides an alternative to transgenic rodent (TGR) mutation assays. The capacity and characteristics of the *Pig-a* assay relative to TGR mutation assays, however, are unclear. Here, using transgenic *gpt* delta mice, we compared the *in vivo* genotoxicity of single oral doses of *N*-ethyl-*N*-nitrosourea (ENU, 40 mg/kg), benzo[*a*]pyrene (BP, 100 and 200 mg/kg), and 4-nitroquinoline-1-oxide (4NQO, 50 mg/kg) in the *Pig-a* (peripheral blood) and *gpt* (bone marrow and liver) gene mutation assays. *Pig-a* assays were conducted at 2, 4, and 7 weeks after the treatment, while *gpt* assays were conducted on tissues collected at the 7-week terminal sacrifice. ENU increased both *Pig-a* and *gpt* mutant frequencies (MFs) at all sampling times, and BP increased MFs in both assays but the *Pig-a* MFs peaked at 2 weeks and then decreased. Although 4NQO increased *gpt* MFs in the liver, only weak, non-significant increases (two- or threefold above control) were detected in the bone marrow in both the *Pig-a* and the *gpt* assay. These findings suggest that further studies are needed to elucidate the kinetics of the *Pig-a* mutation assay in order to use it as an alternative to the TGR mutation assay. Environ. Mol. Mutagen. 54:747–754, 2013.

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

INTRODUCTION

Since 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 quantitatively measures the accumulation of mutations in all organs, including germ cells [Nohmi et al., 2000]. The TGR mutation assay fulfills a need for a practical and widely available *in vivo* test for the assessment of gene mutation; the assay has been recommended by regulatory authorities for safety evaluations [COM, 2011; ICH, 2011] and international guidelines have been published describing the conduct of the assay [OECD488, 2011].

The recently developed *Pig-a* gene mutation assay is a powerful and potentially useful tool for evaluating *in vivo* genotoxicity that may complement the TGR assay [Miura et al., 2008a,b,2009]. Because the *Pig-a* gene is on the X-chromosome and involves the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, single mutations in the *Pig-a* gene can result in the loss of

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expression of GPI-anchored proteins, a phenotype that can be detected by flow cytometric evaluation with only a few μL of peripheral blood cells [Miura et al., 2009]. Additionally, *Pig-a* mutation appears to function in an apparently neutral manner, and the accumulated effects of repeat exposures can be evaluated. A standardized protocol for conducting and interpreting the assay, as well as the accuracy of the assay, however, have not been established, and the target organ for the assay currently is limited only to blood cells.

In this study, we performed the *Pig-a* assay and the *gpt* assay in the same animals and compared the performance of the two assays in detecting three known mutagens. We also developed a flow cytometric strategy for defining *Pig-a* mutant cells. This report describes the performance, effectiveness and advantages of the *Pig-a* assay in comparison with the *gpt* assay.

MATERIALS AND METHODS

Preparation of Chemicals

We dissolved *N*-ethyl-*N*-nitrosourea (ENU, Sigma-Aldrich Japan, Tokyo) in phosphate-buffered saline (PBS) (pH 6.0) at 10 mg/mL. Benzo[*a*]pyrene (BP, Wako Pure Chemical, Osaka) was suspended in olive oil at 10 mg/mL (for 100 mg/kg treatment) or 20 mg/mL (for 200 mg/kg treatment). 4-Nitroquinoline-1-oxide (4NQO, Sigma-Aldrich Japan, Tokyo) was suspended at 5 mg/mL in olive oil.

Antibodies

We obtained anti-mouse TER119 antibody for erythroid cell staining (clone TER-119, PE-Cy7-conjugated) and anti-mouse CD24 antibody (clone M1/69, FITC-conjugated) from BioLegend Japan (Tokyo).

Treatment of Mice

Animal experiments were conducted humanely according to the regulations of the Animal Care and Use Committee of the National Institute of Health Sciences (NIHS), Tokyo, and with their permission. *gpt* Delta C57BL/6J transgenic male mice were bred and maintained at the NIHS animal facility. They were housed individually under specific pathogen-free conditions with a 12-hr light–dark cycle and given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., Tokyo) *ad libitum*. At 8 weeks of age, five mice per group were given a single oral administration of ENU (40 mg/kg), BP (100 mg/kg or 200 mg/kg), 4NQO (50 mg/kg), or PBS (the negative control). Peripheral blood (18 μL) was withdrawn from a tail vein 2, 4, and 7 weeks after the treatments and used for the *Pig-a* assay. At 7 weeks, all mice were killed and bone marrow and liver samples were collected for the *gpt* assay.

Pig-a Mutation Assay

The *Pig-a* assay was performed with some modification of previously described methods [Miura et al., 2008a; Phonethepswath et al., 2008; Horibata et al., 2011; Kimoto et al., 2011]. Briefly, EDTA (dipotassium salt) was dissolved in distilled water to make a 12% solution and used as an anticoagulant. Peripheral blood (18 μL) was mixed with 2 μL EDTA solution. Two microliters of the blood/EDTA mixture was suspended in 0.2 mL PBS, and labeled with 1 μg each of anti-mouse TER119 and anti-mouse CD24 antibodies. The cells were incubated for 1 hr in the dark at room temperature, centrifuged (1000*g*, 5 min), resuspended in 2 mL PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences Japan, Tokyo). After gating for single cells, about 1×10^6 TER119-positive cells were analyzed for the presence of surface CD24, and the *Pig-a* mutant frequency (MF) was calculated as previously described [Horibata et al., 2011].

Gating Strategy for *Pig-a* Assay

Red blood cells (RBCs) were stained with anti-TER119, an antibody that specifically recognizes RBCs, and anti-CD24, an antibody used to detect GPI-anchored protein, as previously reported [Keller et al., 1999; Phonethepswath et al., 2008; Horibata et al., 2011; Kimoto et al., 2011]. Single cells, including RBCs and white blood cells (WBCs), were gated by light scatter (Fig. 1A). TER119-positive cells from this population (Fig. 1B) were analyzed further for the presence of the GPI-anchored CD24 antigen on the cell surface. The FITC-fluorescence intensities of RBCs without FITC-conjugated anti-CD24 were distributed as shown in Figure 1C, defining a gate that included 100% of lower FITC intensities of RBCs as "*Pig-a* mutant RBCs." This gating most likely included a number of events that were not true RBC *Pig-a* mutants, and therefore, to avoid artifactually inflating *Pig-a* MFs, we refined the gate for "*Pig-a* mutant RBCs" as the area encompassing a maximum of 99.0% of the lower RBC FITC staining intensities only (Figs. 1D and 1E).

gpt Mutation Assay

We extracted high molecular weight genomic DNA from liver and bone marrow cells using a Recover Ease DNA Isolation Kit (Agilent Technologies, Santa Clara, CA), rescued lambda EG10 phages using Transpack Packaging Extract (Agilent Technologies) and conducted the *gpt* mutation assay as previously described [Nohmi et al., 2000]. *gpt* MFs were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of colonies with rescued plasmids [Nohmi et al., 2000].

Statistical Analyses

The Kruskal–Wallis nonparametric test was used for comparisons among multiple groups at each time point. When significant differences were observed in the Kruskal–Wallis test, the Steel test was performed as a *post hoc* analysis for comparisons between the responses in the negative control and each treated group. Statistical analyses were performed using GraphPad Prism6 (GraphPad Software, La Jolla, CA) and Excel Statistics 2012 (Social Survey Research Information, Tokyo, Japan). For these analyses, a P-value of <0.05 was considered significant and two-tailed tests were performed.

Power analyses were performed using GraphPad StatMate2 software. GraphPad StatMate2 estimates the statistical power of detecting a difference between two sets of observations using an unpaired t-test (two-tailed). For these estimates, observations from our historical vehicle control data ($n = 95$, male C57BL/6 mice, 3–12 weeks of age, mean ± SD: 0.41 ± 0.91) were used to generate the power estimates (at the 80 and 95% level) of detecting true increases of 2-, 3-, etc., fold over the historical vehicle control MF at an $\alpha = 0.05$. SigmaPlot 12.5 also was used to estimate the effect of increasing the number of animals assayed on the power.

We also hypothesized that the power of the assay would be influenced by the number of erythrocytes interrogated for each sample, with the variability in the measurements, reflected in the SDs of mean *Pig-a* MFs for groups. In order to evaluate this effect, we determined assay power when the number of animals per group was 5 and the SDs of the mean control MF ranged from 0.1 to 15, comparing to the historical vehicle control ($n = 95$, SD = 0.91). Again, the historical vehicle control

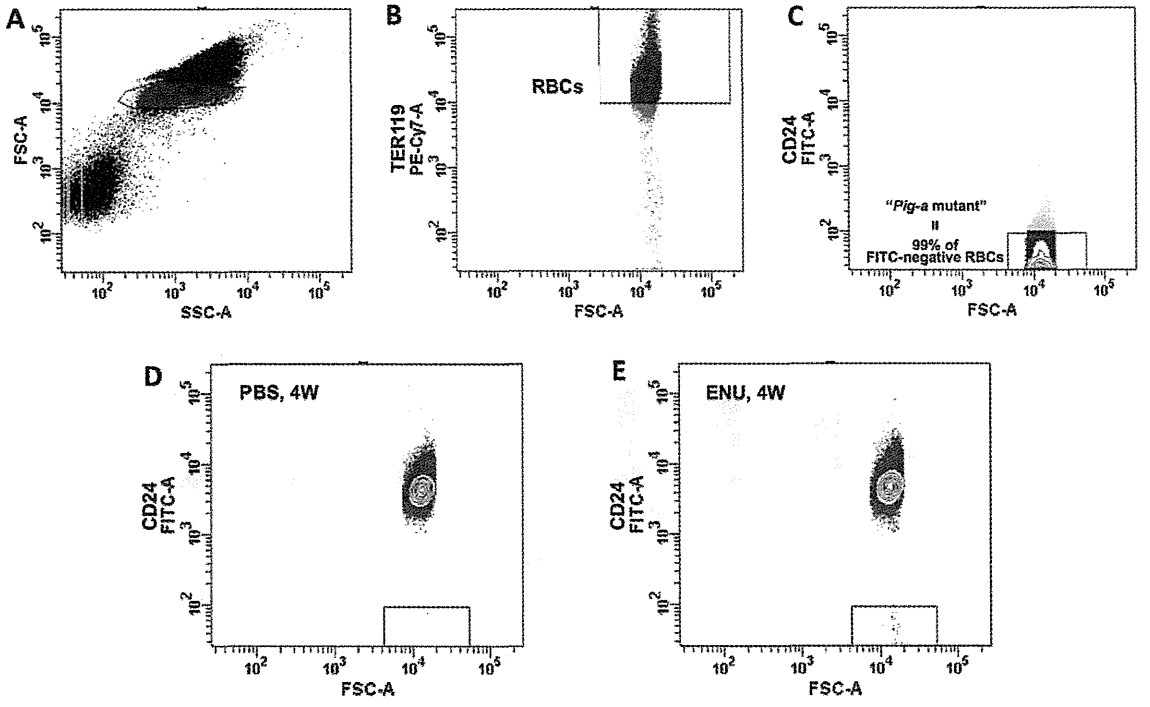

Fig. 1. Flow cytometeric analysis of mouse peripheral blood. A: Single cell populations were gated and further analyzed with anti-TER119 antibody. B: TER119-negative WBCs were excluded from the cell population gated in (A). TER119-positive RBCs were further analyzed with and without anti-CD24 antibody. C: TER119 positive cells were analyzed without anti-CD24 antibody staining so as to mimic *Pig-a* mutant RBCs. *Pig-a* mutant RBCs were defined by a gate encompassing at least 99% of lower intensities of FITC fluorescence associated with RBCs without anti-CD24 staining. D,E: Typical cytograms detecting *Pig-a* mutants. Approximately 1×10^6 TER119-positive cells derived from PBS- (D) or ENU- (E) treated mice were analyzed for CD24 expression.

MF (0.41×10^{-6}) was used as the control MF: minimal detectable fold-increases over vehicle control were calculated by dividing each estimated minimal detectable increase by the mean value of our historical vehicle control MF.

RESULTS

Pig-a Assay

The *Pig-a* mutant (CD24-negative) RBC population identified by flow cytometry after PBS or ENU treatment is shown in Figure 1D and 1E. MFs in the ENU-treated mice were significantly increased and the increase was modestly dependent upon time (mean ± SD for the PBS: 2 weeks after treatment, $0.80 \pm 0.45 \times 10^{-6}$; 4 weeks after treatment, $0.40 \pm 0.55 \times 10^{-6}$; and 7 weeks after treatment, $0.80 \pm 1.30 \times 10^{-6}$; mean ± SD for mice treated with 40 mg/kg ENU: 2 weeks after treatment, $23.00 \pm 6.96 \times 10^{-6}$; 4 weeks after treatment, $29.40 \pm 6.11 \times 10^{-6}$; and 7 weeks after treatment, $29.60 \pm 13.58 \times 10^{-6}$) (Fig. 2A).

In the case of 4NQO-treated mice, the average frequencies were more than twofold greater than controls at each of the time points studied (mean ± SD for mice treated with 50 mg/kg 4NQO: 2 weeks after treatment, $2.60 \pm 4.72 \times 10^{-6}$; 4 weeks after treatment, $1.00 \pm 1.00 \times 10^{-6}$; and 7 weeks after treatment, $2.20 \pm 4.38 \times 10^{-6}$) (Fig. 2B). Although there were no significant differences between the control and treated mice, there was relatively large range for the *Pig-a* MFs in individual 4NQO-treated mice (Fig. 2B, e.g. minimum MF was 0×10^{-6} and maximum MF was 11×10^{-6}). These results prompted us to conduct power analyses. At the 2-, 4-, and 7-week sampling times, the power of the assay to detect a significant increase in *Pig-a* MF after 4NQO-treatment was only 12%, 18%, and 10%, respectively, whereas the statistical power of the assay to reject the null hypothesis (i.e. no difference between control and treatment groups) and detect a significant increase in MF in the ENU-treated group was >95–99% at each of the sampling points.

Significant increases in *Pig-a* MF were observed for both BP doses at 2 weeks after treatment (Figs. 2C and 2D). For the mice treated with 100 mg/kg BP, the frequencies diminished with time, and no increase was evident 7 weeks after treatment (mean ± SD for mice treated with 100 mg/kg BP: 2 weeks after treatment, $9.25 \pm 2.63 \times 10^{-6}$; 4 weeks after treatment, $3.00 \pm 2.16 \times 10^{-6}$; and 7 weeks after treatment, $0.50 \pm 1.00 \times 10^{-6}$; with

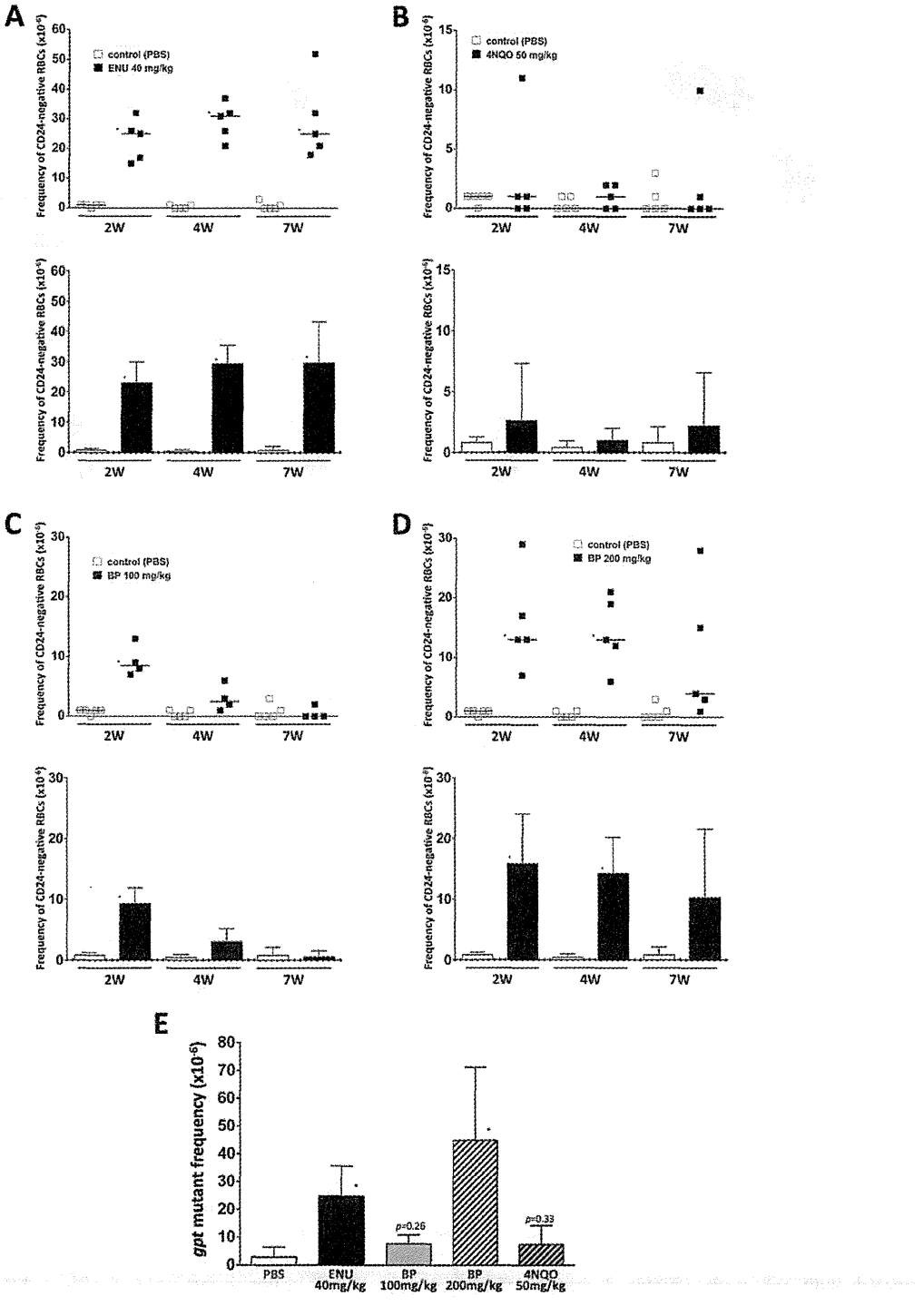

Fig. 2. Comparative analyses of *Pig-a* mutation in peripheral blood RBCs and *gpt* mutation in bone marrow cells. At 2, 4, and 7 weeks after treatment with (**A**) 40 mg/kg ENU, (**B**) 50 mg/kg 4NQO, (**C**) 100 mg/kg BP, or (**D**) 200 mg/kg BP, or PBS solvent, peripheral blood was withdrawn from the tail vein and analyzed by flow cytometry for the presence of CD24 on the surface of RBCs. Upper panels of (A)–(D): scatter plots with median bars. Lower panels of (A)–(D): the mean ± SD. (**E**) Seven weeks after treatment, all mice were killed and their bone marrow cells were isolated for the *gpt* assay. Frequencies are the mean ± SD of five animals per treatment group. $^{*}P < 0.05$.

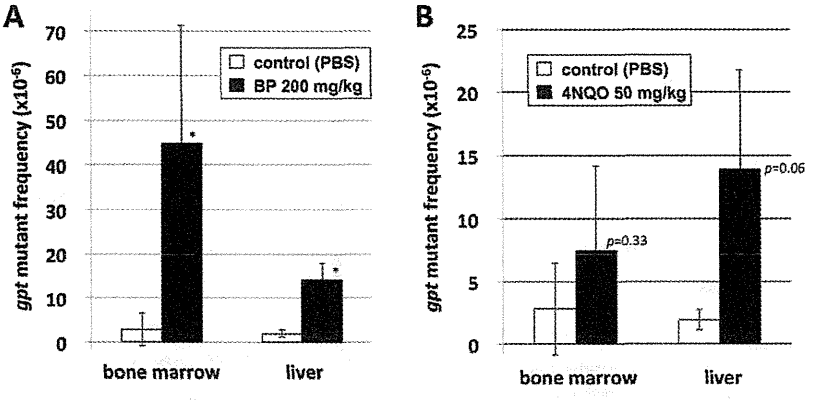

Fig. 3. Comparative analyses of *gpt* mutation in bone marrow and liver. Seven weeks after treatment, all mice were killed and liver samples were collected and analyzed by the *gpt* assay (bone marrow data are the same as in Fig. 2). The frequencies are the mean ± SD of data from five animals. $^*P < 0.05$.

power analyses indicating that the assay had probabilities of >99%, >60%, and <10% of rejecting the null hypothesis, respectively, i.e. to distinguish between the negative control and BP-treated group). Although significant increases were measured at all sampling times, a similar reduction with time was detected in the mice treated with 200 mg/kg BP (mean ± SD for mice treated with BP: 2 weeks after treatment, $15.80 \pm 8.20 \times 10^{-6}$; 4 weeks after treatment, $14.20 \pm 5.97 \times 10^{-6}$; and 7 weeks after treatment, $10.20 \pm 11.34 \times 10^{-6}$; with power analysis indicating that the assay had probabilities of >95%, >99%, and >30%, respectively, to distinguish between the treated and control groups) (Figs. 2C and 2D).

gpt Assay on Bone Marrow and Liver

Compared with the solvent control animals (MF for PBS control group, $2.83 \pm 3.68 \times 10^{-6}$), significant increases in bone marrow *gpt* MFs were observed in ENU- and high-dose BP-treated mice (ENU, $24.70 \pm 10.49 \times 10^{-6}$; BP 200 mg/kg, $44.86 \pm 26.37 \times 10^{-6}$) (Fig. 2E), but not in low-dose BP- or in 4NQO-treated mice (100 mg/kg BP, $7.64 \pm 3.12 \times 10^{-6}$; 4NQO, $7.45 \pm 6.75 \times 10^{-6}$) (Figs. 2B and 2C). *gpt* MFs also were increased in the liver of high-dose BP- and 4NQO-treated mice (MF for PBS control group, $1.97 \pm 0.83 \times 10^{-6}$; 200 mg/kg BP, $14.04 \pm 3.76 \times 10^{-6}$; 4NQO, $13.92 \pm 7.83 \times 10^{-6}$) (Fig. 3).

DISCUSSION

TGR mutation assays, such as the *gpt* gene mutation assay, are an established method for monitoring *in vivo* genotoxicity in multiple tissues concurrently [for review, see Nohmi et al., 2000; Lambert et al., 2005; OECD488, 2011]. While the *Pig-a* gene mutation assay analyzes only one type of cells, i.e., blood cells, it has the advantage of not requiring the use of transgenic animals [Miura et al., 2008a,b]. The *Pig-a* assay has been undergoing extensive development and validation studies are presently being conducted [see the special issue of *Environmental and Molecular Mutagenesis* 52, 2011]. Based on the recent studies, it has been suggested that the *Pig-a* assay could be integrated into repeat-dose toxicology studies. The majority of *Pig-a* work to date has been performed with rats, with very little data being generated in mice, and this data gap needs to be addressed. Therefore, in this study, we analyzed both transgene and *Pig-a* mutation using *gpt*-delta transgenic mice.

Our results using a single oral administration of ENU were consistent with those of previously reported mouse studies [Bhalli et al., 2011a; Horibata et al., 2011; Kimoto et al., 2011]. The ENU-induced MFs detected by the *Pig-a* and *gpt* assays were similar, suggesting that both assays were able to detect ENU genotoxicity equally well.

We also detected dose-dependent increases of *Pig-a* MF in BP-treated mice (Figs. 2C and 2D). The MFs in the mice treated with both low- and high- doses of BP peaked at 2 weeks after treatment and declined thereafter. These time-dependent reductions in *Pig-a* MF differed from the kinetics of ENU–induced *Pig-a* mutation. Phonethepswath et al. reported on the kinetics of *Pig-a* mutation in RBCs from Wister rats treated with ENU, 7,12-dimethyl-1,2-benz[*a*]anthracene, N-methyl-N-nitrosourea, 4NQO and BP [Phonethepswath et al., 2010]. While erythroid progenitors normally mature over a period of 7–14 days *in vitro* [Iscove and Sieber, 1975], it takes 4–6 months to re-establish stable hematopoietic stem cell numbers following bone marrow transplantation [Jordan and Lemischka, 1990]. These observations imply that ENU may effectively mutate hematopoietic stem cells, and that a large proportion of *Pig-a* mutants in BP-treated mice may be due to mutations induced in erythroid progenitors.

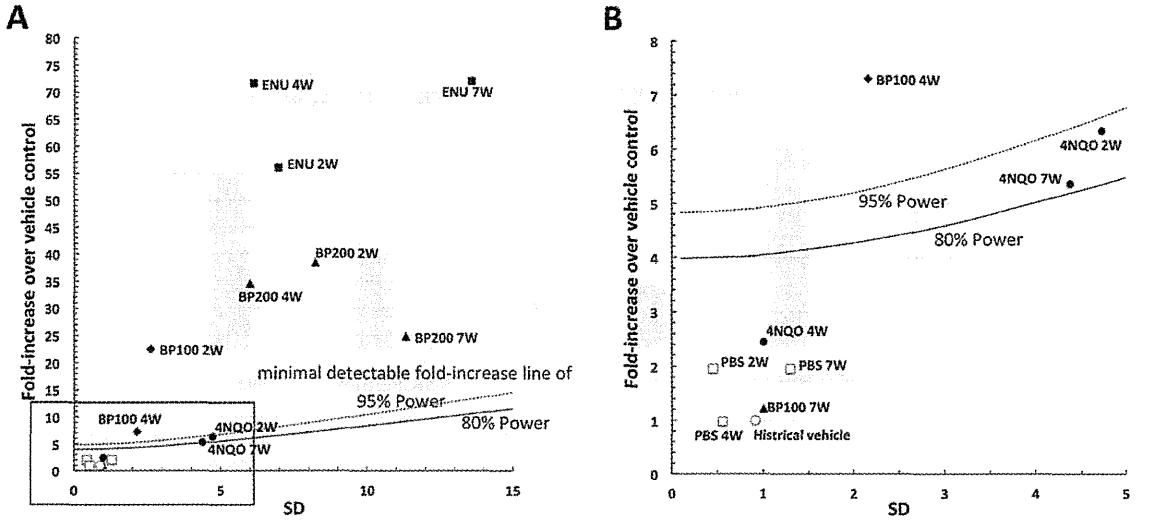

Fig. 4. Relationships between the power to detect fold-increases in *Pig-a* mutant frequency (MF) over the historical vehicle control MF and the effect of the SD of the mean measurements on the power of detection. The solid and dotted lines indicate the fold-increase over the historical vehicle control data detected with 80% and 95% power, respectively. All power estimates were based on an $n = 5$ comparing to the historical vehicle control ($n = 95$, SD = 0.91), $\alpha 5\%$, and use an unpaired two-tailed t-test. Closed square, closed triangle, closed diamond, closed circle, open square, and open circle indicate the fold-increase produced by treatment with ENU, high-dose BP, low-dose BP, 4NQO, and PBS and the historical vehicle control, respectively. The symbols located above each boundary line indicate that the fold-increases for these groups are detected with 95 and or 80% power by an unpaired t-test (two-tailed). Open-boxed area indicated in (A) is expanded in (B).

TABLE I. Power Analysis Using the Historical Control Data[a]

The number of animals of		Power to detect increase of				
experimental group	control group	twofold change over control	threefold change over control	fourfold change over control	fivefold change over control	sixfold change over control
5	5	10%	24%	47%	71%	88%
10	10	16%	48%	82%	97%	>99%
15	15	22%	66%	95%	>99%	>99%
20	20	28%	79%	>99%	>99%	>99%
25	25	35%	88%	>99%	>99%	>99%
80	80	81%	>99%	>99%	>99%	>99%

[a]The historical control data of *Pig-a* MF were $n = 95$, male mice of C57BL/6, 3–12 weeks of age and mean ± SD: 0.41 ± 0.91.

In this study, we found no significant increases in *Pig-a* MF in 4NQO-treated mice using the Steel multiple comparison test, although at each sampling time, the mean MF for the treated mice was greater than the negative control (Fig. 2B). Power analysis employing our historical background *Pig-a* MF ($n = 95$, male C57BL/6 mice, 3–12 weeks of age, mean ± SD: 0.41 ± 0.91) indicated that the assays conducted in this present study (sample size of 5, interrogating 1×10^6 cells/sample) lacked the ability to distinguish these two- to threefold differences between the treated and control groups. The plots shown in Figure 4 indicate that the assays had, at best, an 80–95% power to detect a true four- to fivefold increase of the control and that power depended on the variability of the measurements. When the SD for the MF measurement is small, that is, when the range in MFs for the individuals in the treatment group is small, the assay is capable of detecting about a fourfold increase in *Pig-a* MF with 80% of power (Fig. 4). But when the variability in within-group measurements increased, as it did for many of the treated groups and even some of the controls, then the power of the assay to detect true differences decreased. For instance, the range of MFs for the individual 4NQO-treated mice was relatively large, which contributed to the uncertainty in this measurement. It should be noted that the 2- and 7-week 4NQO responses fell into the 'gray area' for detection based on our power analysis. Performing unpaired t-tests comparing the historical vehicle control data and the responses produced by the 4NQO-treated groups indicated that the assays on 4NQO-

treated mice at 2 and 7 weeks were able to detect significant increases in *Pig-a* MF with over 80% of power (Fig. 4).

The statistical power of the assays can be increased by increasing either the number of mice per group, the number of RBCs interrogated per sample, or both. For instance, increasing the number of mice per group to 20 by itself will enable the assay to detect a threefold increase with nearly 80% power (Table I). Interrogating additional erythrocytes will reduce the number mice with "0" MFs (as can be seen in Fig. 2) and, potentially, the mouse to mouse variability in MF [Nowosiad et al., 2011]. With sufficient sample sizes, it is possible that 4NQO will test positive in the mouse *Pig-a* assay, even if the statistical analyses are conducted using multiple comparison tests. Similar to the *Pig-a* assay results, the mean bone marrow *gpt* MF in 4NQO-treated mice was only about two times higher than the frequency in the negative control, and these frequencies were not significantly different.

In contrast to the bone marrow *gpt* findings, we did detect a marginally significant increase in 4NQO-induced *gpt* MF in liver (Fig. 3B). Significant increases in liver mutagenicity also have been detected in MutaMouse following a single oral administration of 4NQO [Nakajima et al., 1999; Suzuki et al., 1999]. In these studies, the *lacZ* MF in the liver increased with time, but the MF in bone marrow peaked at 1 week and then decreased with time. These findings suggest that the peak bone marrow *gpt* MF in our present study could have been missed because the tissues were analyzed only 7 weeks after treatment. Additionally, in the previous MutaMouse studies, the *lacZ* MFs induced by BP and 4NQO were much higher in the bone marrow than in the liver [Hakura et al., 1998; Nakajima et al., 1999; Suzuki et al., 1999; Lemieux et al., 2011]. We also observed a greater response for BP in the bone marrow than liver. For 4NQO, however, we observed a higher *gpt* MF in the liver than in the bone marrow. The reason for these disparate results is unknown, but they may be related to the use of different TGRs.

Both the *Pig-a* and TGR assays have unique strengths and weaknesses. TGR assays are costly but they can be used for surveying mutation in various tissues. In this study, in fact, the *gpt* assay detected 4NQO genotoxicity in liver whereas the *Pig-a* assay did not detect the mutagenicity of 4NQO in peripheral blood. This differential response may, at least in part, be due to the target tissues for 4NQO mutagenesis because 4NQO also did not significantly increase *gpt*-mutation in bone marrow (although it is recognized that sampling times in the current study were not optimal for bone marrow mutation assessment).

The OECD guideline for TGR assays recommends a tissue sampling time of 3 days after 28 consecutive daily treatments [OECD488, 2011], making it difficult to integrate TGR assays it into standard repeat-dose toxicology studies. Since the *Pig-a* gene is an endogenous gene, the *Pig-a* assay does not require TGR animals. Thus, while the *Pig-a* assay can be combined with a TGR assay, as was done in this present study, it also potentially can be integrated into repeat-dose toxicology studies that do not use TGRs [Dertinger et al., 2010; Dobrovolsky et al., 2010; Bhalli et al., 2011b; Cammerer et al., 2011; Dertinger et al., 2011; Lemieux et al., 2011; Lynch et al., 2011; Schuler et al., 2011; Shi et al., 2011]. Currently, however, we need additional studies that compare mutational responses in the *Pig-a* gene and TGR transgenes in order to help validate the *Pig-a* assay.

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AUTHOR CONTRIBUTIONS

Drs. Horibata and Honma designed the study. Drs. Horibata, Kimoto, Masumura, Nohmi, and Honma critically discussed the study. Dr. Horibata, Ms. Ukai, Dr. Suzuki, and Ms. Kamoshita collected the data. Dr. Horibata and Ms. Ukai analyzed the data and prepared draft figures. Dr. Horibata prepared the manuscript draft with important intellectual input from Dr. Honma. All authors approved the final manuscript. Drs. Horibata and Honma had completed access to the study data.

REFERENCES

Bhalli JA, Pearce MG, Dobrovolsky VN, Heflich RH. 2011a. Manifestation and persistence of Pig-a mutant red blood cells in C57BL/6 mice following single and split doses of *N*-ethyl-*N*-nitrosourea. Environ Mol Mutagen 52:766–773.

Bhalli JA, Shaddock JG, Pearce MG, Dobrovolsky VN, Cao X, Heflich RH, Vohr H-W. 2011b. Report on stage III Pig-a mutation assays using benzo[a]pyrene. Environ Mol Mutagen 52:731–737.

Cammerer Z, Bhalli JA, Cao X, Coffing SL, Dickinson D, Dobo KL, Dobrovolsky VN, Engel M, Fiedler RD, Gunther WC, Heflich RH, Pearce MG, Shaddock JG, Shutsky T, Thiffeault CJ, Schuler M. 2011. Report on stage III Pig-a mutation assays using *N*-ethyl-*N*-nitrosourea—Comparison with other in vivo genotoxicity endpoints. Environ Mol Mutagen 52:721–730.

COM. 2011. Guidance on a strategy for genotoxicity testing of chemical substances.: Committee on mutagenicity of chemicals in food, consumers products, and the environment (COM).

Dertinger SD, Phonethepswath S, Franklin D, Weller P, Torous DK, Bryce SM, Avlasevich S, Bemis JC, Hyrien O, Palis J, MacGregor JT. 2010. Integration of mutation and chromosomal damage endpoints into 28-day repeat dose toxicology studies. Toxicol Sci 115:401–411.

Dertinger SD, Phonethepswath S, Weller P, Avlasevich S, Torous DK, Mereness JA, Bryce SM, Bemis JC, Bell S, Portugal S, Aylott M, MacGregor JT. 2011. Interlaboratory Pig-a gene mutation assay trial: Studies of 1,3-propane sultone with immunomagnetic

enrichment of mutant erythrocytes. Environ Mol Mutagen 52: 748–755.

Dobrovolsky VN, Miura D, Heflich RH, Dertinger SD. 2010. The in vivo Pig-a gene mutation assay, a potential tool for regulatory safety assessment. Environ Mol Mutagen 51:825–835.

Hakura A, Tsutsui Y, Sonoda J, Kai J, Imade T, Shimada M, Sugihara Y, Mikami T. 1998. Comparison between in vivo mutagenicity and carcinogenicity in multiple organs by benzo[a]pyrene in the lacZ transgenic mouse (Muta Mouse). Mutat Res 398:123–130.

Horibata K, Ukai A, Koyama N, Takagi A, Kanno J, Kimoto T, Miura D, Hirose A, Honma M. 2011. Fullerene (C60) is negative in the in vivo Pig-A gene mutation assay. Genes Environ 33:27–31.

ICH. 2011. The ICH S2(R1) Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use. International Conference on Harmonisation. Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step4/S2R1_Step4.pdf.

Iscove NN, Sieber F. 1975. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. Exp Hematol 3:32–43.

Jordan CT, Lemischka IR. 1990. Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev 4:220–232.

Keller P, Tremml G, Rosti V, Bessler M. 1999. X inactivation and somatic cell selection rescue female mice carrying a Piga-null mutation. Proc Natl Acad Sci USA 96:7479–7483.

Kimoto T, Suzuki K, Kobayashi XM, Dobrovolsky VN, Heflich RH, Miura D, Kasahara Y. 2011. Manifestation of Pig-a mutant bone marrow erythroids and peripheral blood erythrocytes in mice treated with *N*-ethyl-*N*-nitrosourea: Direct sequencing of Pig-a cDNA from bone marrow cells negative for GPI-anchored protein expression. Mutat Res 723:36–42.

Lambert IB, Singer TM, Boucher SE, Douglas GR. 2005. Detailed review of transgenic rodent mutation assays. Mutat Res 590:1–280.

Lemieux CL, Douglas GR, Gingerich J, Phonethepswath S, Torous DK, Dertinger SD, Phillips DH, Arlt VM, White PA. 2011. Simultaneous measurement of benzo[a]pyrene-induced Pig-a and lacZ mutations, micronuclei and DNA adducts in Muta™ mouse. Environ Mol Mutagen 52:756–765.

Lynch AM, Giddings A, Custer L, Gleason C, Henwood A, Aylott M, Kenny J. 2011. International Pig-a gene mutation assay trial (Stage III): Results with *N*-methyl-*N*-nitrosourea. Environ Mol Mutagen 52:699–710.

Miura D, Dobrovolsky VN, Kasahara Y, Katsuura Y, Heflich RH. 2008a. Development of an in vivo gene mutation assay using the endogenous Pig-A gene. I. Flow cytometric detection of CD59-negative peripheral red blood cells and CD48-negative spleen T-cells from the rat. Environ Mol Mutagen 49:614–621.

Miura D, Dobrovolsky VN, Mittelstaedt RA, Kasahara Y, Katsuura Y, Heflich RH. 2008b. Development of an in vivo gene mutation assay using the endogenous Pig-A gene. II. Selection of Pig-A mutant rat spleen T-cells with proaerolysin and sequencing Pig-A cDNA from the mutants. Environ Mol Mutagen 49:622–630.

Miura D, Dobrovolsky VN, Kimoto T, Kasahara Y, Heflich RH. 2009. Accumulation and persistence of Pig-A mutant peripheral red blood cells following treatment of rats with single and split doses of *N*-ethyl-*N*-nitrosourea. Mutat Res 677:86–92.

Nakajima M, Kikuchi M, Saeki K, Miyata Y, Terada M, Kishida F, Yamamoto R, Furihata C, Dean SW. 1999. Mutagenicity of 4-nitroquinoline 1-oxide in the MutaMouse. Mutat Res 444:321–336.

Nohmi T, Suzuki T, Masumura K. 2000. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res 455:191–215.

Nowosaid P, Collins JE, Giddings A, Aylott MC, Sumption ND, Lad A, Rees BJ, Dertinger SD, Kenny JD. 2011. *Pig-a* mutation assay: Enhancing flow cytometric throughput and statistical power through immunomagnetic wild type depletion. Mutagenesis 27: 132.

OECD488. 2011. Test guideline 488: OECD guideline for the testing of chemicals. Transgenic rodent somatic and germ cell gene mutation assays. Paris: Organisation for Economic Cooperation and Development.

Phonethepswath S, Bryce SM, Bemis JC, Dertinger SD. 2008. Erythrocyte-based Pig-a gene mutation assay: Demonstration of cross-species potential. Mutat Res 657:122–126.

Phonethepswath S, Franklin D, Torous DK, Bryce SM, Bemis JC, Raja S, Avlasevich S, Weller P, Hyrien O, Palis J, Macgregor JT, Dertinger SD. 2010. Pig-a mutation: Kinetics in rat erythrocytes following exposure to five prototypical mutagens. Toxicol Sci 114:59–70.

Schuler M, Gollapudi BB, Thybaud V, Kim JH. 2011. Need and potential value of the Pig-ain vivo mutation assay-A hesi perspective. Environ Mol Mutagen 52:685–689.

Shi J, Krsmanovic L, Bruce S, Kelly T, Paranjpe M, Szabo K, Arevalo M, Atta-Safoh S, Debelie F, LaForce MK, Sly J, Springer S. 2011. Assessment of genotoxicity induced by 7,12-dimethylbenz(a)anthracene or diethylnitrosamine in the Pig-a, micronucleus and Comet assays integrated into 28-day repeat dose studies. Environ Mol Mutagen 52:711–720.

Suzuki T, Itoh S, Nakajima M, Hachiya N, Hara T. 1999. Target organ and time-course in the mutagenicity of five carcinogens in Muta-Mouse: A summary report of the second collaborative study of the transgenic mouse mutation assay by JEMS/MMS. Mutat Res 444:259–268.

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Tracing the fates of site-specifically introduced DNA adducts in the human genome☆

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ABSTRACT

We developed a system for tracing DNA adducts in targeted mutagenesis (TATAM) and investigated the prevalence and types of consequent mutations. Targeted mutagenesis methods site-specifically replace endogenous DNA bases with bases carrying synthetic adducts using targeting vectors. The TATAM system was enabled by introduction of site-specific DNA double strand breaks (DSB), which strongly enhanced targeting efficiency through homologous recombination (HR), and a new polymerase chain reaction-based technique, which gives high yields of the target vectors carrying DNA adducts. Human lymphoblastoid TSCER122 cells are compound heterozygous for the thymidine kinase gene (*TK*−/−), and have a homing endonuclease I-SceI site in intron 4 of the *TK* gene. The TATAM system enabled targeting of the *TK*− allele with the I-*Sce*I site using a synthetic *TK*+ allele containing an 8-oxo-7,8-dihydroguanine (8-oxoG) adduct, a typical product of oxidative DNA damage. The targeted clones (*TK*+/−) were then isolated by drug selection. Site-specific HR for DSB induced by I-SceI improved targeted integration of the synthetic allele by five orders of magnitude (from 10^{-7} to 10^{-2}). Subsequent analyses of approximately 800 target clones revealed that 8-oxoG was restored to G in 86% clones, probably reflecting base excision repair or translesion synthesis without mutation. Lesions of the remaining clones (14%) were associated with mutations. The mutation spectrum corresponded closely with that of oxidative DNA damage inducers reported, in which G:C to T:A transversions (5.9%) were predominant. Over-expression of MutY homologs in cells, which prevents G:C to T:A transversions by removing 8-oxoG:A mispairing, significantly decreased the frequency of mutations to 2.6%, indicating that the 8-oxoG adducts introduced by the TATAM system are processed in the same manner as those generated by oxidative DNA damage.

1. Introduction

Humans are frequently exposed to thousands of potentially harmful dietary and environmental chemicals, many of which bind DNA and produce DNA adducts [1,2]. Furthermore, numerous known and unknown DNA adducts are constitutively present in human genomes, indicating exposure to DNA-reactive agents [3,4]. Owing to apoptotic and DNA repair mechanisms that eliminate premutagenic cells, not all DNA adducts lead to mutations [5–8]. However, numerous known mutagenic DNA-reactive agents produce specific gene mutations that are potentially carcinogenic.

Although relationships between specific DNA adducts and their characteristic mutagenic properties have been established, that between specific DNA adducts and mutations remain elusive. Indeed, multiple DNA adducts are often produced by single mutagens such as aflatoxin B1 and it remains unknown which of these are associated with major mutational events [9–11]. Studies of site-specific mutagenesis, DNA repair, and bypass using site-specifically modified oligonucleotides to monitor the fate of a DNA adduct induced by chemicals and radiation have been reviewed [9]. Numerous studies have employed extra-chromosomal approaches with episomal vectors, in which adduct-modified oligodeoxynucleotides are ligated into single- or double-stranded vectors

Abbreviations: TATAM, tracing DNA adducts in targeted mutagenesis; 8-oxoG, 8-oxo-7,8-dihydroguanine; *TK*, thymidine kinase gene; HR, homologous recombination; DSB, double-strand break; MYH, human MutY homolog.

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containing replication origins and are transfected into cells [9,12–17]. These episomal vectors independently replicate from the host genome. However, DNA adducts may be processed *via* different mechanisms to those occurring in the genome. To demonstrate direct association between DNA adducts and genomic mutations, DNA adducts must be directly introduced into host genomes for subsequent investigations of their effects.

Site-specific intra-chromosomal mutagenesis was first reported by Essigmann and colleagues [18,19]. In these studies, SV40 vectors containing single adducts were randomly integrated into the genomes of Chinese hamster ovary cells. Subsequently, clones were isolated by drug selection, and the DNA at the adduct sites was sequenced. In a more recent study, Izhar et al. developed a unique system using phage integrase-mediated integration of plasmid-borne synthetic DNA adducts with defined site-specific DNA lesions into mammalian genomes [20]. This unique system demonstrated the genetic consequences of the 6-4 photoproduct and benzo[a]pyrene-guanine adduct after processing of translesion DNA synthesis (TLS) and homology-dependent repair in the human genome.

We previously developed a system for tracing the genetic consequences of DNA double strand breaks (DSBs) by introducing the homing endonuclease I-*Sce*I site into intron 4 of the thymidine kinase gene (*TK*) in human lymphoblastoid TK6 cells [21–23]. These experiments showed that the I-SceI-inducing DSB significantly enhanced homologous recombination (HR). In the present study, we developed a unique system for tracing DNA adducts in targeted mutagenesis (TATAM) that can site-specifically replace endogenous DNA bases in intron 4 of *TK* gene with bases containing a synthetic DNA adducts (Fig. 1). Subsequently, we traced the genetic consequences of the integrated DNA adduct. DSB enabled high gene targeting efficiency for the TATAM system following enhanced site-specific HR for DSB repair [24,25]. Using the TATAM system, we also stably introduced an 8-oxo-7,8-dihydroguanine (8-oxoG) adduct, which is a typical product of oxygen radical-forming agents, into non-transcribed (NTS) and transcribed strands (TS) of *TK* gene at high frequency. This is the first report to trace the fate of a DNA adduct occurring in an endogenous single-copy gene in the human genome.

2. Materials and methods

2.1. Cell culture

Cells were cultured in RPMI 1640 medium (Nacalai Tesque Corp., Kyoto, Japan) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS, USA), 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin, and 10^5–10^6 cells/ml were maintained at 37 °C in 5% CO_2 and 100% humidity.

2.2. Construction of TSCER122 cells

TSCER122 human lymphoblastoid cells were isolated from TSCE105 cells [22], which were derived from TK6 cells [26]. TSCE105 (*TK*+/−) cells are heterozygous for a point mutation in exon 4 of the *TK* gene and have two I-*Sce*I recognition sites surrounding exon 5 of the *TK* allele. The I-SceI expression vector pCBASce (50 μg) was transfected into TSCE105 (5×10^6) cells that were suspended in 0.1 ml of Nucleofector solution V (Amaxa Biosystem, Koeln, Germany) using Nucleofector I according to the manufacturer's recommendations [27]. Subsequently, cells were seeded into 96-microwell plates at 1 cell/well. Two weeks later, *TK*-deficient mutants (*TK*−/−) were isolated in the presence of 2.0 μg/ml trifluorothymidine (TFT). Mutants were independently expended and DNAs were analyzed. DSBs occurring at the two I-*Sce*I sites in TSCE105 cells were correctly fused by error-free end-joining, and produced a new I-*Sce*I site with a 356-bp deletion containing the entire exon 5. The resulting *TK*-deficient mutant TSCER122 (Fig. 3A) was confirmed by drug resistance, I-SceI digestion, and DNA sequencing experiments.

2.3. Construction of MYH-overexpressing TSCER122 cells

The coding region of the human *MutY* homolog gene (MYH) was amplified from cDNA of TK6 human lymphoblastoid cells using polymerase chain reaction (PCR) with the primers 5′-TGG GAA TTC GCC ACC ATG AGG AAG CCA CG-3′ and 5′-TTT CAG TCG ACT CAC TGG GCT GCA CTG TT-3′. PCR products were digested using *EcoR*I/*Sal*I restriction enzymes and were cloned into the *EcoR*I/*Sal*I site of the pCI-neo Mammalian Expression Vector (Promega Corp., Madison, WI). The resulting pCI-MYHβ3 contained variant beta 3, which is known to localize in the nucleus [28,29]. TSCER122 cells (5×10^6) were transfected with 10 μg *Xmn*I-linearized pCI-MYHβ3 using Nucleofector I, were cultured for 48 h, and were then seeded into 96-microwell plates in the presence of 0.6 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA). G418-resistant clones were then isolated and MYH protein expression was quantified using western blotting analyses and ImageGauge software (Fujifilm, Tokyo, Japan). MYH protein expression was normalized to β-actin, and was compared to that in wild type cells.

2.4. PCR-based preparation of a site-specifically modified targeting vector containing a single 8-oxoG adduct

The targeting vector pvINT^{8OxG}, which contained a single 8-oxoG adduct, and the control vector pvINT^{G} were prepared as described previously [30]. In brief, the method involved the following: (i) primer design, (ii) PCR amplification, (iii) isolation and self-annealing of four single-stranded (ss) DNA fragments originating from four individual PCR amplicons, and (iv) T4 DNA ligation (Fig. 2A). The plasmid pTK15 (9-kb) was derived from pTK10 [21,22] and was used as a PCR template. This plasmid vector comprises 6.1-kb of the original *TK* gene, encompassing exons 5–7, and the part of I-*Sce*I sequence in intron 4, which carries a loss of function TTAT deletion. We inserted 8-oxoG into the pvINT^{8OxG} vector in place of the central guanine at the *Bss*SI site in the NTS (5′-CTCGTG in Primer 3F) of *TK* gene (Fig. 2B). Moreover, we labeled a 5′-TTCA-sequence (*Mse*I^{R}) near the 8-oxoG-modified *Bss*SI site that was resistant to *Mse*I digestion, and thereby distinguished between targeted and non-targeted revertants according inter-allelic recombination (Fig. 3B). Twenty primers were designed to amplify sequential 300-bp fragments from the end of the 6.1-kbp targeting vector, and the whole sequence was analyzed using an ABI 3730xl 96-capillary DNA analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. Transfection and cloning of TK revertant cells using the TATAM system

TSCER122 (5×10^6) cells were suspended in 0.1 ml Nucleofector Solution V and were co-transfected with 50 μg of pCBASce vector and 2 μg of targeting vector (pvINT^{8OxG} or pvINT^{G}) using Nucleofector I according to the manufacturer's recommendations [27]. Subsequently, cells were cultured for 72 h and were then seeded into 96-microwell plates in the presence of HAT (200 μM hypoxanthine, 0.1 μM aminopterin, and 17.5 μM thymidine) to isolate 8-oxoG-integrated revertant clones. Drug-resistant colonies were counted 2 weeks later, frequencies of *TK*

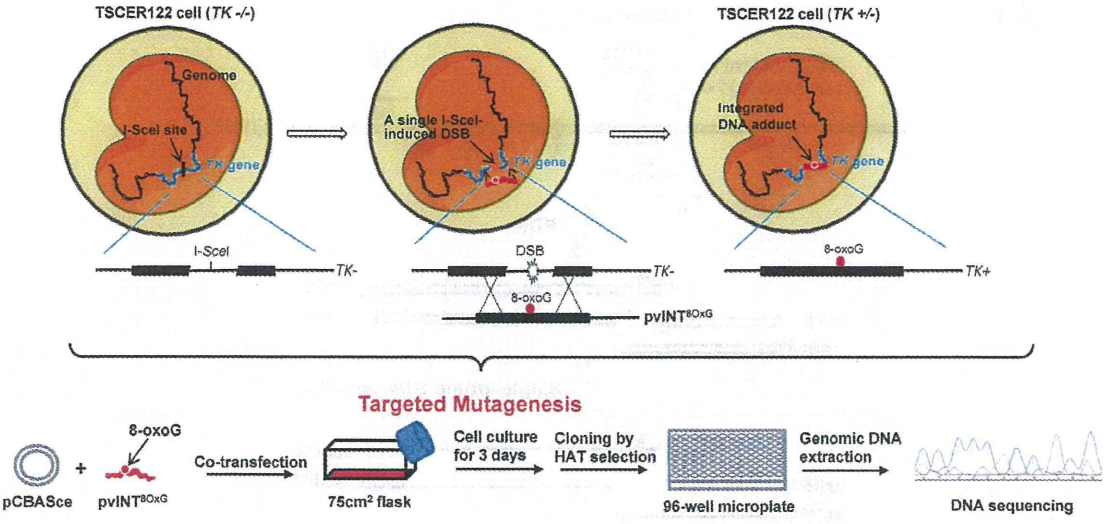

Fig. 1. Outline of the tracing DNA adducts in targeted mutagenesis (TATAM) system. The human lymphoblastoid cell line TSCER122 is compound heterozygous for thymidine kinase gene (*TK*−/−) and has a single I-*Sce*I endonuclease site. TSECR122 cells were co-transfected with the 8-oxoG-modified targeting vector pvINT^{8OxG} and the I-SceI expression plasmid pCBASce. Three days after transfection, cells were seeded into 96-microwell plates in the presence of HAT to isolate 8-oxoG-integrated *TK*-revertant clones. Subsequently, the *TK* gene containing 8-oxoG was sequenced. I-SceI-induced double strand breaks (DSB) enhanced the gene targeting efficiency by inducing homologous recombination (HR) and resulted in insertion of 8-oxoG into the genome.

revertants were calculated according to the Poisson distribution [31], and *TK* revertants were independently cultured for DNA analysis.

2.6. Mutation analysis at 8-oxoG adduct lesions

High-purity TSCER122 genomic DNAs were isolated from the *TK* revertant clones on 96-microwell plates using DNeasy 96 Blood & Tissue Kits (QIAGEN), and the mutation spectrum of 8-oxoG introduced by pvINT^{8OxG} integration was analyzed (Fig. 3). Subsequently, the *TK* gene fragments containing the 8-oxoG-integration site were amplified by PCR using KOD FX polymerase with the forward and reverse primers: (Pri#291, intron 4), 5′-GCT CTT ACG GAA AAG GAA ACA GG-3′; (Pri#292, intron 5), 5′-CTG ATT CAC AAG CAC TGA AG-3′, respectively. PCR amplification was performed with denaturation at 96 °C for 5 min followed by 25 cycles of 96 °C for 30s, 57 °C for 30s, and 68 °C for 1 min. Regions around the *Bss*SI and *MseI*^{R} sites were sequenced using an ABI 3730xl DNA analyzer and clones harboring the *MseI*^{R} sequence were counted to determine the frequency of 8-oxoG integration and numbers of mutations at the *Bss*SI site. Subsequently, the integration frequency of 8-oxoG adducts in the pvINT^{8OxG} targeting vector was calculated by dividing the number of *MseI*^{R} clones by the total number of revertant clones analyzed. Point mutation frequencies opposite the 8-oxoG site were also calculated by dividing the number of single-base substitutions, deletions, and insertions by the number of *MseI*^{R}-bearing clones (Table 1).

2.7. Non-targeting vectors for competitive assays of 8-oxoG repair

Double-stranded (ds) 100-bp DNAs [5′-GGT ACC GGG CCC CCC CTC GAG GTC GAC GGT ATC GAT AAG CTT AGC CTC XTGGGA CTG CAG CCC GGG GGA TCC ACT AGT TCT AGA GCG GCC GCC ACC GCG G (X = G or 8-oxoG)] with nonspecific sequences containing unmodified G or 8-oxoG in *Bss*SI recognition sequences, from Japan Bioservice Corp. (Saitama, Japan). Subsequently, noncompetitive (pvINT^{8OxG} vector and the unmodified 100-bps^{G} dsDNA) and competitive (pvINT^{8OxG} vector and the 8-oxoG-modified 100-bps^{8OxG} dsDNA) combinations of vectors and dsDNAs were co-transfected in the presence of the I-SceI expression vector (50 μg) into 5 × 10^{6} TSCER122 cells using Nucleofector I according to the manufacturer's instructions[27]. The molar ratio between targeting vector and non-targeting vector in both noncompetitive (pvINT^{8OxG}:dsDNA 100-bps^{G}) and competitive experiments (pvINT^{8OxG}:dsDNA 100-bps^{8OxG}) was approximately 1:60. After transfection, cells were cultured for 3 days and were then seeded into 96-microwell plates in the presence of HAT to isolate 8-oxoG-integrated revertant clones. Drug-resistant colonies were then counted 2 weeks later and were independently cultured for sequencing analysis.

2.8. The TATAM system for 8-oxoG adducts in TS of the TK gene

Initially, 8-oxoG was introduced into NTS of the *TK* gene according to the TATAM system. To compare the mutation properties of 8-oxoG between NTS and TS of the *TK* gene, we also prepared a pvIT^{8OxG} targeting vector for the TS of the *TK* gene. An oligonucleotide primer (R2) containing 8-oxoG was then synthesized (Supplementary Fig. S1) and the pvIT^{8OxG}-targeting vector for TS was prepared as described above.

3. Results

3.1. Preparation of the 8-oxoG-modified targeting vector

We prepared pvINT^{8OxG} and pvINT^{G} targeting vectors using synthetic 8-oxoG-modified and unmodified primers, respectively, as starting materials (Fig. 2). DNA sequencing confirmed the absence of errors in the entire 6.1-kbp targeting vectors sequence. A stalled peak was also detected at the 8-oxoG-neighboring base site of the pvINT^{8OxG} vector, but not at the same site in the pvINT^{G} vector (Supplementary Fig. S2), indicating that 8-oxoG adduct was present at the expected site in pvINT^{8OxG} vector, and pvINT^{G} vector can be used as control.

3.2. Frequency of TK revertant TSCER122 cells in the TATAM system

TSCER122 cells (*TK*−/−) are compound heterozygous for *TK* because of a 356-bp deletion of exon 5 on one *TK* allele, and a point