Enhancing the sensitivity of the thymidine kinase assay by using DNA repair-deficient human TK6 cells

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Abstract
The OECD guidelines define the bioassays of identifying mutagenic chemicals, including the thymidine kinase (TK) assay, which specifically detects the mutations that inactivate the TK gene in the human TK6 lymphoid line. However, the sensitivity of this assay is limited because it detects mutations occurring only in the TK gene but not any other genes. Moreover, the limited sensitivity of the conventional TK assay is caused by the usage of DNA repair-proficient wild-type cells, which are capable of accurately repairing DNA damage induced by chemicals. Mutagenic chemicals produce a variety of DNA lesions, including base lesions, sugar damage, crosslinks, and strand breaks. Base damage causes point mutations and is repaired by the base excision repair (BER) and nucleotide excision repair (NER) pathways. To increase the sensitivity of TK assay, we simultaneously disrupted two genes encoding XRCC1, an important BER factor, and XPA, which is essential for NER, generating XRCC1−/−/XPA−/− cells from TK6 cells. We measured the mutation frequency induced by four typical mutagenic agents, methyl methane sulfonate (MMS), cis-diaminedichloro-platinum(II) (cisplatin, CDDP), mitomycin-C (MMC), and cyclophosphamide (CP) by the conventional TK assay using wild-type TK6 cells and also by the TK assay using XRCC1−/−/XPA−/− cells. The usage of XRCC1−/−/XPA−/− cells increased the sensitivity of detecting the mutagenicity by 8.6 times for MMC, 8.5 times for CDDP, and 2.6 times for MMS in comparison with the conventional TK assay. In conclusion, the usage of XRCC1−/−/XPA−/− cells will significantly improve TK assay.

KEYWORDS
DNA-damaging agent, OECD guideline, thymidine kinase assay, TK assay, TK6 cells

1 INTRODUCTION
Genotoxicity assessment is essential for developing medicines and ensuring the safety of industrial chemicals. In vitro assessment of genotoxicity precedes its in vivo evaluation in the development of drugs (Corvi and Madia, 2017). The Organization for Economic Co-operation and Development (OECD) has provided guidelines for the testing of chemicals using various in vitro genotoxicity tests including Ames test, the micronucleus test, the mouse lymphoma assay (MLA) and the thymidine kinase (TK) assay (TG-471; TG-487; TG-490). The TK assay uses human lymphoblastoid TK6 cells harboring heterozygous for mutation at the TK gene (TK+/−) and detects various mutations that...
inactivate the intact TK allelic gene including point mutations, long deletion, DNA recombination, and chromosome loss (Liber and Thilly, 1982; Koyama et al., 2006). The specificity of the TK assay is likely to be very high for the following reasons. The TFT selection works with extremely high specificity to kill the cells that lost the TK activity (Moore-Brown et al., 1981). The TK gene is a stably expressed house-keeping gene, and mutations of the TK gene, but not other mechanisms, cause the complete irreversible inactivation of the TK activity by the four-hour exposure to chemicals in the TK assay (Clements, 1995). However, the sensitivity of this assay is very low especially in the detection of the chemicals that generate point mutations due to the following reasons. The size of the exons in the TK gene is 0.7 kb, which accounts for only $1.1 \times 10^{-7}$ in the whole genome ($6 \times 10^{6}$). BER normally removes over 50% of the chemical-induced base damage within 0.5 hr (Hoch et al., 2017), replication forks in an asynchronous population of cells encounter only 4% (0.5/13) of the chemical-induced base damage considering 13 hr cell cycle time of TK6, and less than 0.04% ($4 \times 10^{-5}$) of the chemical-induced base damage causes mutations considering that the error-rate of translesion DNA synthesis (TLS) polymerases is approximately $10^{-2}$ per base (McCulloch and Kunkel, 2008). Thus, chemical-induced base damage causes mutations in the coding sequences of the TK gene with less than $4.4 \times 10^{-11}$ probability ($1.1 \times 10^{-7} \times 4 \times 10^{-5}$). Approximately 20% of the missense and nonsense mutations in cancer-related genes affect oncogenesis (Scott and Meldrum, 2005). If these mutations inactivate the TK gene also at ~20% frequency, chemical-induced base damage inactivates the TK gene with less than $10^{-11}$ probability. We therefore need to damage more than $10^5$ nucleotides per cell to inactivate the TK gene at least in a single cell of $~10^6$ cells used for a TK assay. Collectively, the sensitivity of the TK assay is very low as we need to damage such a large number of nucleotides to detect the mutagenic potential of chemicals. We therefore hypothesized that the usage of DNA repair-deficient cells might increase the sensitivity of the currently used genotoxic assays, including TK assay.

Chemicals induce point mutations by damaging nucleotides, inaccurate replication of such damaged nucleotides often happened during error-prone TLS (Sale et al., 2012). The vast majority of damaged nucleotides are repaired by the two major pathways, base excision repair (BER) and nucleotide excision repair (NER). BER removes lesions caused by the alkylation, hydrolysis, and oxidation of nucleotides. Typical BER is initiated by the incision of the DNA strand 5’ to the damaged bases, generating single-strand breaks (SSBs) (Krokan and Bjoras, 2013). Their repair is facilitated by the x-ray repair cross-complementing group 1 (XRC1) protein, which provides docking sites for various BER effecter enzymes (Thompson et al., 1990; Caldecott, 2003). Hypersensitivity of XRC1 deficient cells to alkylating agents and H2O2, indicates the vital role of XRC1 in BER (El-Khamisy et al., 2003). A typical alkylating agent is MMS, which modifies both guanine (to 7-methylguanine) and adenine (to 3-methyladenine) generating base mispairing and replication blocks, respectively (Beranek, 1990). NER removes helix-destabilizing bulky adducts generated by crosslinking agents and UV (Aboussekha et al., 1995). XPA recognizes such bulky adducts and is essential for initiating NER (De Vries et al., 1995). The capability of NER is evaluated by measuring cellular sensitivity to UV and crosslinking agents such as MMC and CDDP, which induce protein-DNA crosslinks, intra-strand, and interstrand crosslinks (Weng et al., 2010; Dasari and Bernard, 2014). Both XRC1 and XPA prevent the induction of point mutations through absolutely accurate and error-free repair mechanisms (Lindahl, 1999). We therefore hypothesized that XRC1/XPA double knockout cells may accumulate a higher number of mutations following a wide variety of base-damaging substances in comparison with wild-type cells.

In this study, we disrupted both XRC1 and XPA genes in the TK6 cell line and used the resulting XRC1$^{-/-}$/XPA$^{-/-}$ cells for the TK assay. We measured the mutagenicity of four mutagenic alkylating agents, MMS, CDDP, MMC, and CP following the OECD guideline for TK assay (TG-490). This TK assay using XRC1$^{-/-}$/XPA$^{-/-}$ cells detected 2–8 times higher numbers of mutations when compared with the conventional TK assay using wild-type TK6 cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and culture conditions

The cell lines used in this study were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 $\mu$g/ml sodium pyruvate, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin, and maintained at $10^5$ to $10^6$ cells/ml at 37°C in a 5% CO$_2$ atmosphere with 100% humidity (Koyama et al., 2006). To accurately measure spontaneously arising mutations in TK assay, we incubated a population of cells with CHAT (20 $\mu$M 2'-deoxyctydine, 200 $\mu$M hypoxanthine, 0.1 $\mu$M aminopterin, T: 17.5 $\mu$M thymidine)-containing medium for 3 days to kill TK$^{-/-}$ cells before starting TK assay (Lorge et al., 2016).

### 2.2 | Test chemicals

We purchased MMS and CDDP from Nacalai Tesque Inc (Kyoto, Japan), MMC from Sigma-Aldrich Inc. (CA), and CP from FUJIFILM Wako Pure Chemical Co. (Tokyo, Japan). We purchased liver S9 prepared from SD rats treated with phenobarbital and 5,6-benzoflavone from BoZo Research Center Inc (Tokyo, Japan). All test chemicals were dissolved in phosphate-buffered saline PBS purchased from Takara Bio Inc. (Shiga, Japan). All test chemicals were prepared immediately before the TK test.

### 2.3 | TK gene mutation assay

We examined mutation frequencies as described (Koyama et al., 2011) and following the OECD guidelines (TG-490). In brief, we
incubated cells for 4 hr either with CDDP, MMC, or MMS in the absence of S9 mix or with CP together with S9 mix, washed with PBS, resuspended in a fresh medium and cultured for 3 days to allow for the expression of the TK deficient phenotype. To determine the plating efficiency of cells treated with mutagens, we seeded cells at 1.6 cells/well in 96-microwell plates in the absence of TFT at day 0 (PE0) and day 3 (PE3). To count the number of cells carrying TK−/− allelic genes, we seeded the cells at day three into 96-microwell plates at 40,000 cells/well in a medium containing 3.0 μg/ml trifluorothymidine (TFT), which kills only TK+ cells carrying an intact TK gene. All plates were incubated at 37°C in 5% CO2 in a humidified incubator. To determine the relative survival (RS), we scored the number of colonies in the PE3 plates at days 14 and 17 for wild-type and XRCC1−/−/XPA−/− TK6 cells, respectively. As the doubling time of XRCC1−/−/XPA−/− was longer (16–18 hr) than that of wild-type TK6 cells (11–12 hours), we found that day 17 was the suitable timing for counting surviving clones. For the scoring of mutation frequency (MF), we determined the date to count TFT-resistant clones based on the doubling-time of cells. We counted the number of colonies in TFT plates on day 14, then re-supplied with TFT media, and counted again on day 28 for wild-type cells. We counted the number of XRCC1−/−/XPA−/− colonies on day 17, resupplied with TFT media, and counted on day 31 after plating. Mutation frequencies were calculated based on the assumption that the occurrence of mutations follows the Poisson distribution. Statistical analysis was calculated using two-way ANOVA to analyze the statistical significance between wild-type and XRCC1−/−/XPA−/− TK6 cells after comparing the slopes of minimum dose responses. The statistically significant difference between spontaneously arising MFs and induced ones was calculated by the Student’s t test. Data were generated from at least three independent experiments.

2.4 Disruption of XRCC1 gene in XPA−/− TK6 cells

To generate XRCC1−/−/XPA−/− TK6 cells, we transfected XRCC1 targeting vector (Saha et al., 2018) into previously generated XPA−/− TK6 cells (Mohiuddin et al., 2018) after marker excision by a transiently expressed CRE-recombinase. As we described in (Saha et al., 2018), we generate a pair of TALEN expression plasmids against the XRCC1 gene, using a Golden Gate TALEN kit and a TAL effector kit (Addgene) (Cermak et al., 2011; Sakuma et al., 2013). A pair of the TALENs target sites recognizes the sequences shown in Figure 1a, which localize at the first exon of the XRCC1 gene. We generated the gene-targeting constructs using DT-A-pA/loxP/PGK-hisDR-pA/loxP and DT-A-pA/loxP/PGK-BsrR-pA/loxP vectors. Note that these vectors were generated from DT-A-pA/loxP/PGK-NeoR-pA/loxP (Riken Center for Life Science Technology, Japan). The genomic DNA was amplified with primers: F2 5′-GGCTATCCGGCGAGAGAAGAAGGATGAGGT-3 and R2 5′-CTGGGGCTCGAGGGGGGGGGCCTGGCCA GAAGGATGAGGT-3′ of which have 15-nt sequences identical to the upstream and downstream of the Apol site in both the DT-A-pA/loxP/PGK-hisDR-pA/loxP and DT-A-pA/loxP/PGK-BsrR-pA/loxP vectors. The 3′-arm was amplified using the primers: F3 5′-TGGGAAG TCTGTCGACCTAAAGACGTTAGGGAATTATGAG-3′ and R3 5′- CACTAGTGGGGCCGGCTTTACACCCACATCCCCTGCTATT-3′, of which have 15-nt sequences identical to the upstream and downstream of the AflII site in these vectors.

The DT-A-pA/loxP/PGK-hisDR-pA/loxP or DT-A-pA/loxP/PGK- BsrR-pA/loxP vector was digested with both Apol and AflII, which cut at the 5′ and 3′ of the selection marker genes, respectively. We combined the digested DNAs with the 5′- and 3′-arms using the Seamless Cloning and Assembly Kit (Thermo Fisher Scientific). We transfected 6 μg each of the TALEN-expression plasmids and 2 μg each of the two gene-targeting vectors carrying hisD and BsrR into 4 × 106 TK6 cells using the Neon Transfection System (Life Technologies) with three times 1,350 V pulse with a 10 ms pulse width.

![FIGURE 1](image-url) Generation of XRCC1−/− in XPA−/− TK6 cells. (a) Schematic representation of the human XRCC1 locus, base sequences of TALEN-recognition sites, and structure of the targeting constructs containing genomic fragments and a selection-marker gene (hisDR or bsrR) with loxP sites on both sides. The x-mark indicates the TALEN-induced DSB site. A pair of TALEN was designed to target exon 1. Schematic representation of the XRCC1 locus (upper) and configuration of the targeting construct carrying a marker gene (lower) flanked by ~1 kb of genomic sequence on either side. The closed boxes represent exon sequences. Note that the size of the schematic representation does not reflect the actual size of the DNA. (b) Western blot analysis of XRCC1 disrupted TK6 clones using the antibody against XRCC1 in XPA−/− human TK6 cell line.
After electroporation, we released cells into a 20 ml drug-free medium containing 10% horse serum and incubated them for 48 hr. We seeded cells into 96-well plates with both histidinol and blasticidin S antibiotics and incubated for 2 weeks. We confirmed the loss of XRCC1 protein expression in individual clones by western blot analysis (Figure 1b).

2.5 | Generation of XRCC1−/−/XPA−/− heterozygous for the TK gene for TK assay

We disrupted the XRCC1 gene in XPA−/− TK6 cells derived from a TSCER2 subline carrying compound heterozygous mutations of the TK allelic gene (TK−−) (Honma et al., 2007). Cells (5 × 10^5) of XRCC1−/−/ XPA−/− TSCER2 (TK−−) were suspended in 0.1 ml Nucleofector Solution V and were cotransfected with 50 μg of pCBASce vector and 2 μg of targeting vector pTK15 plasmid (Honma et al., 2003) using Nucleofector I according to the manufacturer’s recommendations (Maasho et al., 2004). Subsequently, the cells were cultured for 72 hr 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 TK+/− revertant TSCER2 clones. The drug-resistant colonies were isolated 2 weeks later and were independently cultured for DNA analysis.

2.6 | Western blot

Cells (1 × 10^6) were lysed in 100 μl sodium dodecyl sulfate (SDS) buffer, containing Tris–HCl (25 mM, pH 6.5), SDS (1%), β-mercaptoethanol (0.24 mM), bromophenol blue (0.1%), and glycerol (5%). Whole-cell extracts were separated by electrophoresis, transferred onto polyvinylidene difluoride membranes, and blocked in 5% skimmed milk dissolved in Tween-20 (0.1%) in TBS. Membranes were incubated with primary antibodies (Rabbit polyclonal α-XPA, Santa Cruz) overnight at 4°C, followed by washing in Tween-20 (0.1%) in TBS. Membranes were incubated with appropriate HRP-linked secondary antibodies (Goat polyclonal α-rabbit HRP, Santa Cruz) at room temperature for 1 hr and washed thrice before signal detection. Membranes were developed by chemiluminescence using ECL reagent.

FIGURE 2 The mutation frequency (MF) of wild-type and XRCC1−/−/XPA−/− TK6 cells induced by CP. The percentage survival of cells following exposure of cells to the indicated concentrations of CP (a). Hundred percentage is the survival of untreated cells in (a). The average MF of cells to the indicated concentrations of CP (b). Error bars represent SD from at least three independent experiments. The statistically significant difference between spontaneously arising MFs and induced ones was calculated by the Student’s t test. We defined p-value < .05 as statistically significant and mark such difference with *. (ns) p-value was not significant.

3 | RESULTS

3.1 | The TK assay using XRCC1−/−/XPA−/− TK6 cells detected a higher CP-MF response without a significant increase in the TK assay sensitivity

To determine if the usage of XRCC1−/−/XPA−/− cells enhances the sensitivity of the TK assay, we firstly examined the mutagenicity of CP in the presence of 59 mix. The mutation frequencies of wild-type and XRCC1−/−/XPA−/− TK6 cells linearly increased with the dose of CP (Figure 2b). Since the highest dose (5 μg/ml) killed over 95% of the XRCC1−/−/XPA−/− mutant (Figure 2a), we chose 3 μg/ml CP as of the highest dose for XRCC1−/−/XPA−/−. The average frequency of spontaneously arising mutation (hereafter called spontaneous MF) of wild-type TK6 cells was 4.1 ± 1.2 × 10−6 and their MF was 5.7 ± 0.7 × 10−6 at 1 μg/ml CP and 6.6 ± 0.8 × 10−6 at 3 μg/ml CP (Figure 2b). Spontaneous MF of XRCC1−/−/XPA−/− cells was 9.5 ± 1.3 × 10−6, and their MF was 12.7 ± 1.3 × 10−6 at 1 μg/ml CP and 20.9 ± 6.1 × 10−6 at 3 μg/ml CP (Figure 2b). Collectively, after comparing the slopes of the minimum dose–response, we found that the usage of XRCC1−/−/ XPA−/− cells for the TK assay increases the detection of the mutagenicity associated with 1 and 3 μg/ml CP by 4.5 folds in comparison with the conventional TK assay, p-value = .02 (Table S1). However, the difference between spontaneously arising MFs and induced ones at the minimum concentration of CP was not statistically significant in wild-type or XRCC1−/−/XPA−/− cells (Figure 2b).
3.2 The usage of XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 cells allows for detecting a ~three times higher number of MMS-induced mutations than the conventional TK assay

We tested the new TK assay using XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 cells for detecting mutagenicity of MMC, CDDP, and MMS without adding S9 mix (Honma et al., 1997) as they did not require exogenous metabolic activation to induce their mutagenicity in our experiments. This protocol was in agreement with the latest recommendations proposed by the international workshop for genotoxicity testing (IWGT) (Gollapudi et al., 2019). The spontaneous MF was 7.0 ± 1.4 × 10<sup>−6</sup> for wild-type and 13.3 ± 1.1 × 10<sup>−6</sup> for XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 cells without S9 mix, in which MFs were ~2 times higher than the spontaneous MF with S9 mix. The higher spontaneous MFs might be due to the usage of different batch of horse serum since spontaneous MF is not supposed to be affected by the absence or presence of S9 mix as previously described (Koyama et al., 2006).

We measured MMS-induced mutagenesis comparing the new TK assay using XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 cells with the conventional TK assay. The relative survival (RS) was comparable between wild-type and XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 cells; 70% for wild-type and 50% for XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> at 0.25 μg/ml MMS (Figure 3a). The MF for wild-type TK6 cells was 8.9 ± 2.8 × 10<sup>−6</sup> at 0.25 μg/ml MMS and 12.6 ± 2.4 × 10<sup>−6</sup> at 0.5 μg/ml MMS (Figure 3b). The MF of XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> was 15.8 ± 2.3 × 10<sup>−6</sup> at 0.25 μg/ml MMS and 27.9 ± 3.7 × 10<sup>−6</sup> at 0.5 μg/ml MMS (Figure 3b). Comparing the slopes of the dose–response showed that the usage of XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> cells for the TK assay increases the sensitivity of detecting the mutagenicity associated with 0.25 and 0.5 μg/ml MMS by 2.6 folds in comparison with the conventional TK assay, p-value < .0001 (Table S2). To determine the minimum concentrations of MMS that induced mutations in a statistically significant manner, we compared the difference between spontaneously arising MFs and induced ones. XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 shows a significant detection of mutations at 0.25 μg/ml MMS (p-value = .036), while wild-type TK6 shows a significant induction of mutations at 0.5 μg/ml MMS (p-value =.02) but not at 0.25 μg/ml MMS (p-value = .23) (Figure 3b).

3.3 TK assay using XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 cells detected several times higher number of mutations induced by crosslinking agents, MMC and CDDP, than the conventional TK assay

The RS was comparable between wild-type and XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 cells; 80% at 0.025 μg/ml MMC for wild-type and 70% at 0.025 μg/ml MMC for XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> (Figure 4a). The MF of wild-type was 11.4 ± 1.1 × 10<sup>−6</sup> at 0.025 μg/ml MMC and 13 ± 1.1 × 10<sup>−6</sup> at 0.05 μg/ml MMC (Figure 4b). The MF of XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> was 48 ± 6.1 × 10<sup>−6</sup> at 0.025 μg/ml MMC and 65 ± 4.5 × 10<sup>−6</sup> at 0.05 μg/ml MMC. The usage of XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> cells increases MF response by 8.6 folds after comparing the slopes of the dose–response at 0.025, 0.05 μg/ml MMC with wild-type TK6 cell line, p-value < .0001 (Table S3). After comparing the difference between spontaneously arising MFs and induced ones, XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> TK6 shows a significant detection of mutations at 0.025 μg/ml MMC (p-value = .001), while wild-type TK6 shows a significant induction of mutations at 0.05 μg/ml MMC (p-value = .030) but not at 0.025 μg/ml MMC (p-value = .063) (Figure 4b).

We then examined CDDP-induced mutagenicity at 0.25 μM and 0.5 μM CDDP, which decreased RS by less than 50% in both genotypes (Figure 5a). The MF for wild-type was 7.7 ± 0.4 × 10<sup>−6</sup> at 0.25 μM CDDP and 9.3 ± 1.7 × 10<sup>−6</sup> at 0.5 μM CDDP (Figure 5b). The MF of XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> was 14.3 ± 1.1 × 10<sup>−6</sup> at 0.25 μM CDDP and 33 ± 6.7 × 10<sup>−6</sup> at 0.5 μM CDDP (Figure 5b). RS was more than 50% (Figure 5a). The dose–response slope for XRCC1<sup>−/−</sup>/XPA<sup>−/−</sup> showed 8.5 fold increase than wild-type TK6 at
0.25, 0.5 μM CDDP, p-value <.0001 (Table S4). After comparing the difference between spontaneously arising MFs and induced ones, XRCC1−/−/XPA−/− TK6 detected a significant detection of mutations at 0.5 μM CDDP (p-value = .039), while the TK assay using wild-type TK6 detected a significant induction of mutations at 1.0 μM CDDP (p-value = .002) but not at 0.5 μM CDDP (p-value = .106) (Figure 5b).

4 | DISCUSSION

In the present study, we showed that the usage of XRCC1−/−/XPA−/− cells for the TK assay increased its sensitivity in detecting the mutagenicity of various DNA cross-linking and alkylating agents. Using this new TK assay, we studied four typical mutagens, CP, MMS, MMC, and CDDP. The usage of XRCC1−/−/XPA−/− cells gives a higher number of MF response by 4.5 times for CP, 2.6 times for MMS, 8.6 times for MMC, and 8.5 times for CDDP in comparison with the conventional TK assay.

To check the ability of XRCC1−/−/XPA−/− cells to detect weak mutagens with more sensitivity, we determine the minimum doses of the genotoxic agents whose doses increased the MF in a statistically significant manner. We found that the TK assay using XRCC1−/−/XPA−/− TK6 enhanced a significant detection of mutations at the minimum concentrations of DNA cross-linking agents MMC (0.025 μg/ml) and CDDP (0.5 μM) while the wild-type TK6 could not detect similar significant increase (Figures 4b and 5b). Similarly, the TK assay using XRCC1−/−/XPA−/− TK6 detected a significant number of mutations at the minimum concentration of DNA alkylating agent MMS (0.5 μg/ml) while the wild-type TK6 could not detect similar significant increase (Figure 3b). We could not detect a similar enhanced sensitivity for detecting CP because the slope of dose-MF was similar between wild-
type and XRCC1−/−/XPA−/− cells (Figure 2b). CP generates DNA cleavage, crosslinks, and adducts (reviewed in Ozolinsć, 2010), and MMS is a mono-alkylating agent (Sobol et al., 2002). MMC and CDDP, on the other hand, are DNA cross-linking agents generating a variety of lesions, including protein-DNA crosslinks, intrastrand crosslinks, and interstrand crosslinks (Tomasz, 1995; Jordan and Carmon-Fonseca, 2000; Lorenti García et al., 2009). XPA and XRCC1 participate in the repair of crosslinks, and these pathways have an overlapping role in removing a fraction of the crosslink DNA lesions (Zheng et al., 2003; Mustra et al., 2007; Zhang and Walter, 2014; Semailow et al., 2016). This overlapping role may explain why the usage of XRCC1−/−/XPA−/− cells increased the sensitivity of the TK assay for detecting the mutagenicity of MMC and CDDP to greater extents when compared with MMS, which induces the DNA lesions that are repaired exclusively by XRCC1-dependent BER (Op Het Veld et al., 1998). In summary, the usage of XRCC1−/−/XPA−/− cells is advantageous, particularly when the TK assay examines the mutagenicity of crosslinking agents.

Enhancing the performance of the in vitro genotoxicity testing would lead to more reliance on the in vitro tests and less of a need to use in vivo tests. The EURL ECVAM has requested the improvement of the individual in vitro genotoxicity detection assays to increase their overall performance and as a consequence, minimize or even prevent the use of animals for detecting mutagenic chemicals (Corvi and Madia, 2017; EURL ECVAM, 2013a). A significant concern of the mammalian cell-based gene mutation tests is their limited sensitivity, and these tests need very high concentrations of chemicals, whose genotoxicity might not be extrapolated to the genotoxicity of environmentally appropriate lower levels of the substances (Elespuru et al., 2009). The usage of DNA repair-deficient cells may solve this problem by increasing the sensitivity of the metazoan cell-based gene mutation tests (Ji et al., 2009; Yamamoto et al., 2011; Nishihara et al., 2016). Moreover, this usage gives an insight into molecular mechanisms underlying the mutagenicity of chemicals. For example, higher induced MF in XRCC1−/−/XPA−/− cells than wild-type cells indicate that relevant compounds cause the DNA lesions that are repaired by either XRCC1-dependent BER or XPA-dependent NER. We propose the following two-step examination of genotoxic chemicals; first, the identification of a wide variety of genotoxic chemicals using a few mutants such as XRCC1−/−/XPA−/− and double-strand-break repair mutant cells (Hsieh et al., 2019), and subsequently, characterization of molecular mechanisms underlying the identified genotoxicity using cells deficient in individual repair pathways that repair specific lesions. Our proposal for the multistep process is not for routinely evaluating genotoxicity but for investigating mutagenic mechanisms for special purposes, for example, the elucidation of molecular mechanisms underlying the mutagenesis of different chemicals in academic research. In conclusion, we propose that the usage of XRCC1−/−/XPA−/− cells will improve the sensitivity of the TK assay; however, further validation is still needed before requesting it for regulatory use.

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AUTHOR CONTRIBUTIONS

Shunichi Takeda and Mahmoud Abdelghany Ibrahim designed the basic framework of this study. Mahmoud Abdelghany Ibrahim performed all experiments. Mahmoud Abdelghany Ibrahim prepared the manuscript draft with intellectual input from Shunichi Takeda, Masamitsu Honma, Manabu Yasui, and Shunichi Takeda finalized the manuscript. Manabu Yasui, Liton Kumar Saha provided information for designing some of the experiments. Masamitsu Honma provided isogenic TK6 wild-type cells. Hiroyuki Sasanuma and Manabu Yasui advised Mahmoud Abdelghany Ibrahim about technical issues during the experimentation. All authors have approved the final manuscript.

REFERENCES

Aboussekhra, A., Biggerstaff, M., Shivji, M.K.K., Vlite, J.A., Moncollin, V., Podust, V.N., Protić, M., Hübischer, U., Egly, J.-M. and Wood, R.D. (1995) Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell, 80, 859–868.

Berenek, D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutation Research, 231, 11–30.

Caldecott, K.W. (2003) DNA single-strand break repair and spincerebellar ataxia. Cell, 112, 7–10.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somalia, N.V., Bogdanove, A.J. and Voytas, D.F. (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Research, 39, e82–e82.

Clements, J. (1995) Gene mutation assays in mammalian cells. Methods in Molecular Biology, 43, 277–286.

Corvi, R. and Madia, F. (2017) In vitro genotoxicity testing—can the performance be enhanced? Food and Chemical Toxicology, 106, 600–608.

Dasari, S. and Bernard, T.P. (2014) Cisplatin in cancer therapy: molecular mechanisms of action. European Journal of Pharmacology, 740, 364–378.

El-Khamisy, S.F., Masutani, M., Suzuki, H. and Caldecott, K. (2003) A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage. Nucleic Acids Research, 31, 5526–5533.

Elespuru, R.K., Agarwal, R., Atrakchi, A.H., Bigger, C.A.H., Hefflich, R.H., Jagannath, D.R., Levy, D.D., Moore, M.M., Ouyang, Y., Robison, T.W., Sotomayor, R.E., Cimino, M.C. and Dearfield, K.L. (2009) Current and future application of genetic toxicity assays: the role and value of in vitro mammalian assays. Toxicological Sciences, 109, 172–179.

EURL ECVAM (2013a) EURL ECVAM strategy to avoid and reduce animal experimentation. All authors have approved the final manuscript.

Gollapudi, B.B., White, P.A. and Honma, M. (2019) The iWG in vitro mammalian cell gene mutation (MCCM) assays working group—Introductory remarks & consensus statements. Mutation Research, 848, 403061.

Hoch, N.C., Hanzlikova, H., Ruiten, S.L., Têtreault, M., Komulainen, E., Ju, L., Hornyak, P., Zeng, Z., Gitters, W., Rey, S.A., Staras, K., Mancini, G.M.S., McKinnon, P.J., Wang, Z.Q., Wagner, J., Care4Rare
Canada Consortium, Yoon, G. and Caldecott, K.W. (2017) XRCC1 mutation is associated with PARP1 hyperactivation and cerebellar ataxia. Nature, 541, 87–91.

Honma, M., Hayashi, M. and Sofuni, T. (1997) Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis, 374, 89–98.

Honma, M., Izumi, M., Sakuraba, M., Tadokoro, S., Sakamoto, H., Wang, W., Yataagi, F. and Hayashi, M. (2003) Deletion, rearrangement, and gene conversion; genetic consequences of chromosomal double-strand breaks in human cells. Environmental and Molecular Mutagenesis, 42, 288–298.

Honma, M., Sakuraba, M., Koizumi, T., Takashima, Y., Sakamoto, H. and Hayashi, M. (2007) Non-homologous end-joining for repairing I-SceI-induced DNA double strand breaks in human cells. DNA Repair (Amst), 6, 781–788.

Hsieh, J.-H., Smith-Roe, S.L., Huang, R., Sedykh, A., Shockley, K.R., Auerbach, S.S., Merrick, B.A., Xia, M., Tice, R.R. and Witt, K.L. (2019) Identifying compounds with Genotoxicity potential using Tox21 high-throughput screening assays. Chemical Research in Toxicology, 32, 1384–1401.

Ji, K., Kogame, T., Choi, K., Wang, X., Lee, J., Taniguchi, Y. and Takeda, S. (2009) A novel approach using DNA-repair-deficient chicken DT40 cell lines for screening and characterizing the genotoxicity of environmental contaminants. Environmental Health Perspectives, 117, 1737–1744.

Jordan, P. and Carmo-Fonseca, M. (2000) Molecular mechanisms involved in cisplatin cytotoxicity. Cellular and Molecular Life Sciences, 57, 1229–1235.

Koyama, N., Sakamoto, H., Sakuraba, M., Koizumi, T., Takashima, Y., Hayashi, M., Matsufuji, H., Yamagata, K., Masuda, S., Kinae, N. and Honma, M. (2006) Genotoxicity of acrylamide and glycylamide in human lymphoblastoid TK6 cells. Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 603, 151–158.

Koyama, N., Yasui, M., Oda, Y., Suzuki, S., Sato, T., Suzuki, T., Matsuda, S., Masuda, S., Kinae, N. and Honma, M. (2011) Genotoxicity of acrylamide in vitro: Acrylamide is not metabolically activated in standard in vitro systems. Environmental and Molecular Mutagenesis, 52, 11–19.

Krokan, H.E. and Bjoras, M. (2013) Base excision repair. Cold Spring Harbor Perspectives in Biology, 5, a012583.

Liber, H.L. and Thilly, W.G. (1982) Mutation assay at the thymidine kinase locus in diploid human lymphoblasts. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 94, 467–485.

Lindahl, T. (1999) Quality control by DNA repair. Science (80-), 286, 1897–1905.

Lorenti García, C., Mechilli, M., Proietti De Santis, L., Schnipper, A., Katarzyna, K. and Palitti, F. (2009) Relationship between DNA lesions, DNA repair and chromosomal damage induced by acetaldehyde. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis, 662, 3–9.

Lorge, E., Moore, M.M., Clements, J., O’Donovan, M., Fellows, M.D., Honna, M., Kohara, A., Galloway, S., Armstrong, M.J., Thybuad, V., Gollapudi, B., Aardema, M.J. and Tanir, J.Y. (2014) Standardized cell sources and recommendations for good cell culture practices in genotoxicity testing. Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 809, 1–15.

Maasho, K., Marusina, A., Reynolds, N.M., Coligan, J.E. and Borrego, F. (2004) Efficient gene transfer into the human natural killer cell line, NKL, using the Amaxa nucleofection system™. Journal of Immunological Methods, 284, 133–140.

McCulloch, S.D. and Kunkel, T.A. (2008) The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. Cell Research, 18, 148–161.

Mohiuddin, M., Evans, T.J., Rahman, M.M., Keka, I.S., Tsuda, M., Sasanuma, H. and Takeda, S. (2018) SUMOylation of PCNA by PIA51 and PIA54 promotes template switch in the chicken and human B cell lines. Proceedings of the National Academy of Sciences, 115, 12793–12798.

Moore-Brown, M.M., Clive, D., Howard, B.E., Batson, A.G. and Johnson, K. O. (1981) The utilization of trifluothymidine (TFT) to select for thymidine kinase-deficient (TK−/−) mutants from LS178Y/TK+/− mouse lymphoma cells. Mutation Research/Environmental Mutagenesis and Related Subjects, 85, 363–378.

Mustra, D.J., Warren, A.J., Wilcox, D.E. and Hamilton, J.W. (2007) Preferential binding of human XPA to the mitomycin C-DNA interstrand crosslink and modulation by arsenic and cadmium. Chemico-Biological Interactions, 168, 159–168.

Nishihara, K., Huang, R., Zhao, J., Shahane, S.A., Witt, K.L., Smith-Roe, S.L., Tice, R.R., Takeda, S. and Xia, M. (2016) Identification of genotoxic compounds using isogenic DNA repair deficient DT40 cell lines on a quantitative high throughput screening platform. Mutagenesis, 31, 69–81.

Op Het Veld, C.W., Jansen, J., Zdzieckia, M.Z., Vrieling, H. and Zeeland, A.A.v. (1998) Methyl methanesulfonate-induced hprt mutation spectra in the Chinese hamster cell line CH09 and its xrc1-deficient derivative EM-C11. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 398, 83–92.

Ozolińc, T.R.S. (2010) Cyclophosphamide and the teratology society: an awkward marriage. Birth Defects Research Part B: Developmental and Reproductive Toxicology, 89, 289–299.

Saha, L.K., Kim, S., Kang, H., Akter, S., Choi, K., Sakura, T., Sasunuma, H., Hirota, K., Nakamura, J., Honma, M., Takeda, S. and Dertinger, S. (2018) Differential micronucleus frequency in isogenic human cells deficient in DNA repair pathways is a valuable indicator for evaluating genotoxic agents and their genotoxic mechanisms. Environmental and Molecular Mutagenesis, 59, 529–538.

Sakuma, T., Ochiai, H., Kaneko, T., Mashino, T., Tokumasu, D., Sakane, Y., Suzuki, K., Miyamoto, T., Sakamoto, N., Matsura, S. and Yamamota, T. (2013) Repeating pattern of non-RVD variations in DNA-binding modules enhances TALEN activity. Scientific Reports, 3, 3379.

Sale, J.E., Lehmann, A.R. and Woodgate, R. (2012) Y-family DNA polymerases and their role in tolerance of cellular DNA damage. Nature Reviews. Molecular Cell Biology, 13, 141–152.

Scott, R.J. and Meldrum, C.J. (2005) Missense mutations in cancer predisposing genes: can we make sense of them? Hereditary Cancer in Clinical Practice, 3, 123–127.

Semlow, D.R., Zhang, J., Budzowska, M., Drohat, A.C. and Walter, J.C. (2016) Replication-dependent unhooking of DNA Intersand cross-links by the NEIL3 Glycosylase. Cell, 167, 498–511.e14.

Sobol, R.W., Watson, D.E., Nakamura, J., Yakes, F.M., Hou, E., Horton, J.K., Ladapo, J., Van Houten, B., Swenberg, J.A., Tindall, K.R., Samson, L.D. and Wilson, S.H. (2002) Mutations associated with base excision repair deficiency and methylation-induced genotoxic stress. 99, 6860–6865.

TG-471. Test No. 471: Bacterial Reverse Mutation Test. OECD.

TG-487. Test No. 487: in vitro Mammalian Cell Micronucleus Test - see OECD.

TG-490. Test No. 490: in vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene. OECD.

Thompson, L.H., Brookman, K.W., Jones, N.J., Allen, S.A. and Carrano, A.V. (1990) Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. Molecular and Cellular Biology, 10, 6160–6171.

Tomasz, M. (1995) Mitomycin C: small, fast and deadly (but very selective). Nature, 377, 169–173.

Weng, M.W., Zheng, Y., Jasti, V.P., Chapelle, E., Tomasz, M., Wang, Y., Basu, A.K. and Tang, M.S. (2010) Repair of mitomycin C mono- and
interstrand cross-linked DNA adducts by UvrABC: A new model. *Nucleic Acids Research*, 38, 6976–6984.

Yamamoto, K.N., Hirota, K., Kono, K., Takeda, S., Sakamuru, S., Xia, M., Huang, R., Austin, C.P., Witt, K.L. and Tice, R.R. (2011) Characterization of environmental chemicals with potential for DNA damage using isogenic DNA repair-deficient chicken DT40 cell lines. *Environmental and Molecular Mutagenesis*, 52, 547–561.

Zhang, J. and Walter, J.C. (2014) Mechanism and regulation of incisions during DNA interstrand cross-link repair. *DNA Repair (Amst)*, 19, 135–142.

Zheng, H., Wang, X., Warren, A.J., Legerski, R.J., Naim, R.S., Hamilton, J.W. and Li, L. (2003) Nucleotide excision repair- and polymerase- mediated error-prone removal of mitomycin C interstrand cross-links. *Molecular and Cellular Biology*, 23, 754–761.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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