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In Vitro PIG-A Gene Mutation Assay in Human B-Lymphoblastoid TK6 Cells

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Abstract

The X-linked *PIG-A* gene is involved in the biosynthesis of glycosylphosphatidylinositol (GPI) anchors. *PIG-A* mutant cells fail to synthesize GPI and to express GPI-anchored protein markers (e.g., CD59 and CD55). In recent years, *in vitro PIG-A* assay has been established based on the high conservation of *PIG-A*/*Pig-a* loci among different species and the large data from the *in vivo* system. The purpose of this study was to extend the approach for *PIG-A* mutation assessment to *in vitro* human B-lymphoblastoid TK6 cells by detecting the loss of GPI-linked CD55 and CD59 proteins. TK6 cells were treated with three mutagens 7,12-dimethylbenz[a]anthracene (DMBA), *N*-ethyl-*N*-nitrosourea (ENU), etoposide (ETO), and two nonmutagens: cadmium chloride ($CdCl_2$) and sodium chloride (NaCl). The mutation rate of *PIG-A* gene within TK6 cells was determined on the 11th day with flow cytometry analysis for the negative frequencies of CD55 and CD59. The antibodies used in this production were APC mouse-anti-human CD19 antibody, PE mouse anti-human CD55 antibody, PE mouse anti-human CD59 antibody, and nucleic acid dye 7-AAD. An immunolabeling method was used to reduce the high spontaneous level of preexisting *PIG-A* mutant cells. Our data suggested that DMBA-, ENU-, and ETO-induced mutation frequency of *PIG-A* gene was increased by twofold compared with the negative control, and the effects were dose-dependent. However, $CdCl_2$ and NaCl did not significantly increase the mutation frequency of *PIG-A* gene, with a high cytotoxicity at a dose of 10 mmol/L. Our study suggested that the novel *in vitro PIG-A* gene mutation assay within TK6 cells may represent a complement of the present *in vivo Pig-a* assay, and may provide guidance for their potential use in genotoxicity even in cells with a significant deficiency of GPI anchor.

Keywords

- *PIG-A* gene mutation assay
- flow cytometry
- TK6 cells
- genotoxicity

Introduction

Genotoxicity tests are utilized to identify compounds with a potential risk for carcinogenicity and heritable mutations, which require a battery of tests to cover different genetic endpoints, such as DNA damage, gene mutation, as well as structural or numerical chromosomal abbreviation.[1] Currently, a series of standard test battery has been adopted for the detection of mutagens, including bacterial reverse mutation assay (Ames test), *in vivo* transgenic gene mutation

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assay (such as Muta Mouse, BigBlue mice, etc.), and *in vitro* gene mutation assay of mammalian cell lines (ICH 2012). The overall aim of these test systems is to generate a solid database for hazard identification with respect to mutagenicity and to assess the mechanisms of action of chemical carcinogens.

Currently, mutation assay for a rodent-based endogenous phosphatidylinositol class A gene (*PIG-A* in humans and *Pig-a* in rodents) has become a recognized method for detecting potential mutagenicity of exogenous compounds.[2] The *PIG-A/Pig-a* gene is involved in early synthesis of cell-surface glycosylphosphatidylinositol (GPI) anchors, which was usually employed by GPI anchor proteins (e.g., CD59, CD55, or CD90) to bind to the cell membrane. Of all the genes involved in the GPI anchor synthesis, only the *PIG-A/Pig-a* gene is located on the X chromosome. Therefore, in each cell, there is only one copy of the functional *PIG-A/Pig-a* gene, and a single inactivating mutation in this gene will result in the inactivation of the enzymatic function of the *PIG-A* protein and the subsequent deficiency of all GPI-linked surface proteins.[3,4]

Flow cytometry is the most common method for diagnosis of GPI(−) frequency (*PIG-A* gene mutation rate) in TK6 human lymphoblastoid cells.[5–9] From a throughput standpoint perspective, flow cytometry analysis of *Pig-a* mutations may be useful for *in vitro* analysis of gene mutation and development of high-density dose–response data.[10] Evidence suggests that an *in vitro* trial may also be conceived of as the value of testing hypotheses about the results of an *in vivo* assay (e.g., investigating negative *in vivo* responses) and of prescreening compounds for *in vivo* testing.[11] Compared with the existing *in vitro* gene mutation assays (such as mouse lymphoma assay [MLA] test and hypoxanthine-guanine phosphoribosyl transferase [HPRT] gene mutation test), the *in vitro PIG-A* gene mutation test has the following three advantages: (1) being able to select human cells with complete function of *P53* gene; (2) reducing the false-positive rate of *in vitro* genotoxicity test; and (3) shorter detection period (only 11 days) in comparison to MLA or HPRT gene mutation tests. Therefore, *in vitro PIG-A* gene mutation test using flow cytometry may be more objective, convenient, and high-throughput.[9,12]

TK6 cells, known as GPI-anchor-negative cells, grow in suspension, and have good practical usage in genotoxicity testing, and this may be attributed to its well-known characteristics for flow cytometric analysis, as well as the available access of the standardized cultures for genetic toxicological assessment from cell repositories.[13–15] In this study, a simple and efficient immunomagnetic separation was explored in TK6 cells to obtain a relative low and stable GPI(−) background frequency. A *PIG-A* gene mutation assay was then conducted through flow cytometry assay for detecting the loss of GPI-linked CD55 and CD59 using mutagen and nonmutagenic compounds with different mechanisms of action. Our data suggested that the development and testing of methods for *in vitro PIG-A* gene mutation assay may represent a promising strategy to investigate the potential mutagenicity of chemical or physical mutagens.

Materials and Methods

Reagents and Antibodies

Human TK6 cells were purchased from American Type Culture Collection (ATCC; http://www.atcc.com); benzopyrene (B[*a*]P; Cat. No. B1760), ethyl methanesulfonate (EMS; Cat. No. M0880), *N*-ethyl-*N*-nitrosourea (ENU; Cat. No. N3385), etoposide (ETO; Cat. No. E1383), paraformaldehyde (Cat. No. 158127), 7,12-dimethylbenz[a]anthracene (DMBA; Cat. No. D3254), and sodium chloride (NaCl; Cat. No. S9888) were purchased from Sigma-Aldrich (United States); cadmium chloride ($CdCl_2$; Cat. No. C11634) was obtained from Aladdin (China); RPMI-1640 medium, heat-inactivated horse serum, phosphate buffer solution (PBS), antibiotics, and *L*-glutamine were purchased from Gibco (United States); bovine serum albumin (BSA; Cat. No. 69003433) was purchased from Sinopharm Chemical Reagent Co., Ltd. (China); APC mouse anti-human CD19 antibody (Cat. No. 561742), PE mouse anti-human CD55 antibody (Cat. No. 341030), PE mouse anti-human CD59 antibody (Cat. No. 560953), and nucleic acid dye 7-amino-actinomycin D (7-AAD; Cat. No. 561080) were obtained from BD Bioscience (United States); anti-PE microbeads (Cat. No. 130–048–801) and LS Separation columns (Cat. No. 130–042–401) were purchased from Miltenyi Biotec (Germany); actinomycin-D (Cat. No. SLD-1366) was obtained from Nanjing Sunlida Bio-tech Co., Ltd. (China); rat liver S9 was purchased from Moltox (United States); liver S9 cofactor was a stock solution prepared in the laboratory. The cytometer used was BD Accuri C6 PLUS (Becton-Dickinson, United States). The cell counter was Chemometec (Nucleo-Counter NC-100, Denmark).

Cell Culture

TK6 cells (0.2×10^6 or 10^6 cells/mL) were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 2 mmol/L *L*-glutamine, and antibiotics (penicillin at 20 units/mL and streptomycin at 20 µg/mL). The cells were cultured at 37°C with 5% CO_2 in an incubator.

S9 Metabolic Activation System and Test Article Dose Formulation Preparation

The mammalian liver post-mitochondrial fraction (S9) was purchased from Molecular Toxicology, Inc. (Moltox Inc., Boone, North Carolina, United States), which was prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 at 500 mg/kg. The liver microsome enzyme system (S9 mixture) was the mixture of S9 and S9 cofactors containing NADP (4 mmol/L), glucose-6-phosphate (5 mmol/L), KCl (30 mmol/L), $MgCl_2$ (10 mmol/L), PBS at pH 7.4, and deionized water. The final concentration of S9 in the culture medium was 1.0% (v/v).

Antibody Labeling

TK6 cells (2×10^6) were washed with PBS (10 mL), antibody-labeled in a total volume of 175 µL staining solution (PBS with 2% BSA, mouse anti-human CD19-APC [40 µL/sample], mouse anti-human CD55-PE [24 µL/sample], and mouse anti-human CD59-PE [40 µL/sample]), and then incubated at room

temperature for 30 minutes in the dark. After washing with staining buffer (PBS with 2% BSA [w/v], precooled) (1 mL × 2), cell pellets were resuspended in 500 µL DNA staining solution (PBS with 2% BSA; 7-AAD for 2 µL/sample) for 10 minutes on ice to exclude dead cells, and then centrifuged. The supernatant was discarded and cells were fixed in 600 µL fixation buffer (PBS with 1% formaldehyde [w/v] and 2.5 µg/mL AD). Cells were placed on ice before flow cytometry analysis. All solutions or buffer was kept on ice after preparation.

To ensure the accuracy, four samples of 2×10^6 TK6 cells were separately prepared and labeled with individual antibody staining solution for further 30 minutes of incubation in the dark. The staining solutions included staining buffer with mouse anti-human CD19-APC (20 µL/sample), or staining buffer with mouse anti-human CD55-PE (12 µL/sample) and mouse anti-human CD59-PE (20 µL/sample), or DNA staining solution. After completion, all samples were rinsed with staining buffer (1 mL × 2), and cells were fixed in 600 µL fixation buffer and placed on ice before analysis.

Flow Cytometry Assay for GPI(−) Frequency Analysis

All flow cytometric analyses were conducted with an Accuri C6 PLUS from Becton-Dickinson. Excitation and emission detection measurements of the applied fluorescence dyes were as follows: APC (640–670/14 nm) and PE (488–575/26 nm). Samples were analyzed with a middle rate or 65 µL/min. For each determination of the GPI(−) frequency (*PIG-A* gene mutation rate), at least 10^6 cells were collected.

Cleansing of Preexisting GPI(−) TK6 Cells by Immunomagnetic Separation

For cleansing of preexisting GPI(−) TK6 cells, $\sim 4 \times 10^6$ TK6 cells were pelleted at 200 g for 5 minutes and resuspended in 2 mL staining solution. The cells were labeled at room temperature for 30 minutes in the dark. After labeling, the cells were washed with staining buffer twice, and then pelleted at 200 g for 5 minutes. The cell pellets were resuspended in 500 µL PBS (with 2% BSA and precooled) containing 40 µL anti-PE microbeads for 30 minutes on ice or at 2 to 8°C protected from light. After incubation, the cells were washed by 10 mL staining buffer (precooled), and resuspended in 2 mL staining buffer (precooled). LS columns were placed in the MACS Separator and prewetted with a 5 mL staining buffer (precooled) in a biosafety cabinet. Then 2 mL cell suspension was added into the LS columns and the cells were allowed to freely flow through the column by gravity. After all of the suspension was run out, 5 mL staining buffer (precooled) was loaded onto the LS column for three times. Then the LS column was taken out from the separator and 5 mL staining buffer (precooled) was added. The cells adsorbed in the LS column were extruded with a piston and collected. The cell suspension was centrifuged at 200 g for 5 minutes. Pellet was resuspended in 10 mL culture medium for expansion culture. After expansion, GPI(−) frequency was analyzed by flow cytometry. If GPI(−) frequency of TK6 cells was too high, the above steps were repeated and performed the cleaning steps again.

Determination of Doubling Times and Spontaneous Mutation Rate of TK6 Cells

Preexisting GPI(−)-cleansed TK6 cells were named $TK6^{GPI+}$ cells. For doubling time determination, $TK6^{GPI+}$ cells were seeded in triplicate at a density of 10^5 and 2×10^5 cells/mL in a total volume of 25 mL medium. Cell growth was followed for 3 days by determining cell numbers every day. The doubling time was calculated according to **Eq. (1)**:

$$D.T. = \frac{(t_2 - t_1) \times \log_{10} 2}{\log_{10} Ch - \log_{10} Ci} \quad (1)$$

where *D.T.* is the doubling time, t_2 the cell harvest time, t_1 the cell inoculation time, *Ch* the cell harvest density, and *Ci* the cell inoculation density.

$TK6^{GPI+}$ cells were cultured and passaged for 40 days and 10^6 cells were subcultured in 25 mL RPMI-1640 every 2 days. GPI(−) frequency was tested every 4 days, and every 8 days for the last two times. Based on the TK6 doubling time, the GPI(−) frequencies were plotted over the corresponding population doublings. After linear regression was applied (fit curve by GraphPad Prism 7), the resulting slope value was equivalent to the spontaneous mutation rate (μ) that was calculated according to **Eq. (2)**:

$$\mu = \frac{\alpha \times 10^{-6} \times D.T.}{24} \quad (2)$$

where α is the slope of the fit curve, *D.T.* the doubling time of $TK6^{GPI+}$ cell, and 24 the 24 hours.

Optimized Cytotoxicity Determination

The cytotoxicity of test chemicals was measured to design appropriate dose ranges for mutagenicity testing by relative increase in cell counts (RICC). Cell density was determined by cell counter methods and RICC was calculated at 24 and 48 hours in the group without S9. In the group with S9 (24 hour − S9 group), $TK6^{GPI+}$ cells were treated with articles for 3 hours (3 hour + S9 group) and rinsed with 10 mL PBS twice followed by cell density determination at 24 and 48 hours. Treatments which showed RICC between 10 and 20% of control and lower at 24 and 48 hours were selected as the top concentrations. If not limited by cytotoxicity, 10 mmol/L or precipitate concentration was chosen as the top concentration. RICC was calculated according to **Eq. (3)**:

$$\text{RICC (control \%)} = \frac{\text{Cell Density}_{\text{treatment}} - 3 \times 10^5}{\text{Cell Density}_{\text{control}} - 3 \times 10^5} \times 100 \quad (3)$$

Treatments

All experiments were performed with GPI(−)-cleansed $TK6^{GPI+}$ cells. In case of incubations, $TK6^{GPI+}$ cells were seeded at 3×10^5 cells/mL in 9.9 mL culture media in T25 flasks on day 1. A 100 µL formulation of test articles, positive control (200 µmol/L EMS or 8 µmol/L B[*a*]P), or appropriate solvent control was added into the flasks drop by drop. The details of every test article are shown in ►**Table 1**. Cell

Table 1 Test article list

Test chemical	Abb.	Cas No.	Molecular weight	Modes of action (MOA)
Ethyl methanesulfonate	EMS	62–50–0	124.16	Alkylating agent
Benzo[*a*]pyrene	B[*a*]P	50–32–8	252.31	Forms bulky adducts
7,12-Dimethyl-benz[*a*]anthracene	DMBA	57–97–6	256.34	Forms bulky adducts
Etoposide	ETO	33419–42–0	588.56	DNA topoisomerase II inhibitors
N-Ethyl-*N*-nitrosourea	ENU	759–73–9	117.11	Alkylating agent
Cadmium chloride	$CdCl_2$	10108–64–2	183.32	Nongenotoxic carcinogen
Sodium chloride	NaCl	7647–14–5	58.44	Salt

suspension was mixed by gentle vortex. Cells were directly cultured for 24 hours at 37°C, 5% CO_2 in a humidified environment for the 24 hour − S9 group. Then, the cells were rinsed with PBS and resuspended with 10 mL medium after 3 hours of treatment and with a continuous culture for 24 hours for the 4 hour + S9 group. After 24 hours, cells were washed with PBS. A total of 2×10^6 cells were subcultured every 2 days in 25 mL culture medium to allow phenotype expression. On day 11, flow cytometry was performed to detect GPI(−) frequencies.

Test Result Evaluation Criteria

The biological relevance of the results was carefully examined. The response was considered positive when the following conditions were met: the test agents induced a twofold increase over negative control in the frequency of GPI(−) cells in a dose-dependent manner with Microsoft excel 2007.

Results and Discussion

Template of GPI(−) Frequency Analysis by Flow Cytometry

The occurrence of GPI mutations is extremely rare, which requires very sensitive methods to detect. To achieve this, two independent GPI-anchored proteins, CD55 and CD59, were labeled. The GPI(+) cells were separated from GPI(−) cells by staining CD55 and CD59 with PE-conjugated antibodies, and an APC-conjugated CD19 antibody was used to identify most of the TK6 cells as B-lymphocytes.

In this experiment, the gating procedure for flow cytometric analyses was set up using parallel samples and the results are presented in ►**Fig. 1**. Cells were collected by FSC/SSC scatter plot (►**Fig. 1A**, gate *cells*) and agglomeration cells were excluded by gating in FSC-A versus FSC-H scatter plots (►**Fig. 1B**, gate *single cells*). Dead cells were excluded via the uptake of 7-AAD, and healthy cells were collected (►**Fig. 1C**). Finally, GPI(+) and GPI(−) cells were separated. The GPI(+) cells showed positive APC and PE-fluorescent signals and were presented in the upper right quadrant (►**Fig. 1D**). Cells with nonspecific events lacking the APC signal were presented in the upper left quadrant and lower left quadrant, and were excluded from analysis (►**Fig. 1D**). In this study, a total of 10^6 APC-positive cells were collected to analyze the GPI(−) frequency.

Cleansing of Preexisting GPI(−) TK6 Cells

Flow cytometric analysis showed that the spontaneous mutation rate of the GPI(−) frequency in TK6 cells was ~23.4% (►**Fig. 2A**), which is a very high rate, and the basic mutation frequency of GPI(−) cells was reduced to 0.18% (►**Fig. 2B**) after being cleared by the first magnetic bead adsorption, and 13.5×10^{-6} (►**Fig. 2C**) after the second clearance, suggesting that immunomagnetic separation is a very useful method to separate and cleanse the GPI(−) TK6 cells. The cells after cleansing of mutant GPI(−) cells are called $TK6^{GPI+}$ cells.

Doubling Time and Spontaneous Mutation Rate of $TK6^{GPI+}$ Cells

Cell doubling time has a decisive influence on the efficiency of genotoxicity testing. A stable cell doubling time is also a sign of good cell status. Our result showed that $TK6^{GPI+}$ cells had a doubling time of 15.5 ± 1.2 hours.

The spontaneous GPI(−) rate was determined by cell division in the culture. $TK6^{GPI+}$ cells were tested for spontaneous GPI(−) rate up to 40 days, and the mean spontaneous GPI(−)

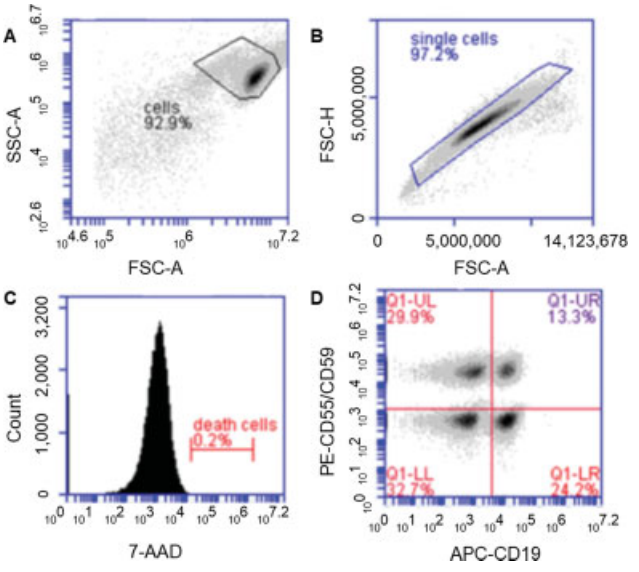

Fig. 1 TK6 cell detection template (parallel samples). (**A**) FSC-A versus SSC-A scatter plots for determining cell locations; (**B**) FSC-A versus FSC-H scatter plots for exclusion adhesive cells; (**C**) histogram of FL-3 fluorescence channel, used to exclude necrotic and late apoptotic cells; (**D**) APC-CD19 versus PE-CD55 CD59 scatter plot, using cross-gate to distinguish GPI(−) and CD19(+) cells.

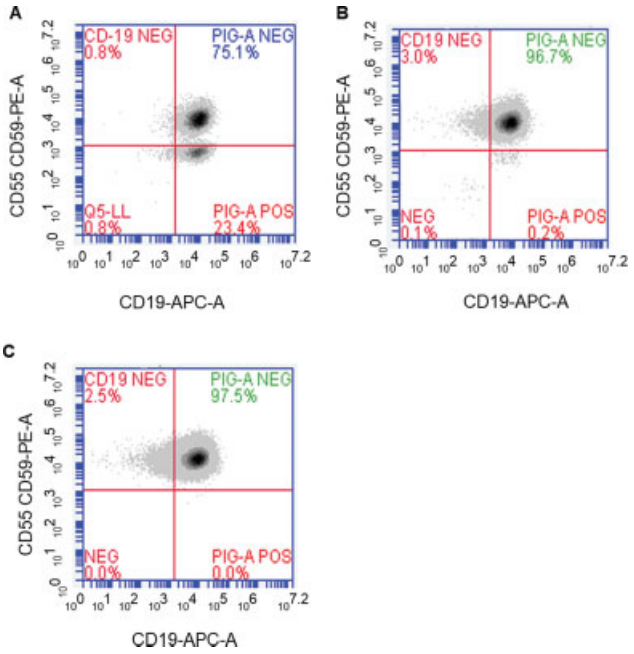

Fig. 2 Flow cytometric analysis of the background level of preexisting mutant cells. (**A**) Before the first cleansing; (**B**) after the first cleansing; (**C**) after the second cleansing. *PIG-A* NEG defines wild cells of *PIG-A* gene; *PIG-A* POS means the mutant cells of the *PIG-A* gene.

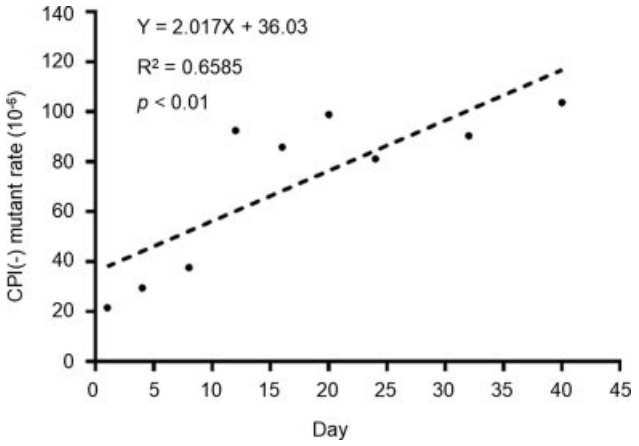

Fig. 3 Fitted curve of spontaneous *GPI(−)* gene mutation rates, where $\alpha = 2.017$.

rate was 1.37×10^{-6}/cell/generation for the $TK6^{GPI+}$ cell line (fitted curve in ►**Fig. 3**). This means that the mutation rate of TK6 cells increases by $\sim 22.2 \times 10^{-6}$ after 11 days of culture (doubling time of 15.5 hours). Therefore, we calibrated the tested mutation rate by subtracting the value of the spontaneous mutation in 11 days' culture (22.2×10^{-6}).

Optimized Cytotoxicity Determination

To detect the mutagenic effect, the dose of the treatment agents should be sufficiently high. However, the cytotoxicity should not be less than 10% for the highest concentration, otherwise the cells could not be recovered in 11 days' culture. In our study, the doses were selected according to the higher RICC in 24 or 48 hours after cell exposure to the test agents (►**Fig. 4**). If a reduction of RICC was not limited up to 10 mmol/L or precipitation concentration, as in the case of NaCl, 10 mmol/L was selected as the highest concentration. The cytotoxicity of ETO, DMBA, and $CdCl_2$ reached the maximum at 24 hours. Therefore, the concentrations of ETO and $CdCl_2$ were selected based on the RICC of 24 hours. However, the concentrations of ENU were selected based on the RICC of 48 hours (►**Table 2**). Furthermore, the kinetics of RICC tested in 11 days' culture of all test articles (including EMS and B[*a*]P as positive) were determined. Our data suggested that the trend of RICC initially rises, then falls, and finally returns gradually to normal level in 11 days (►**Fig. 5**).

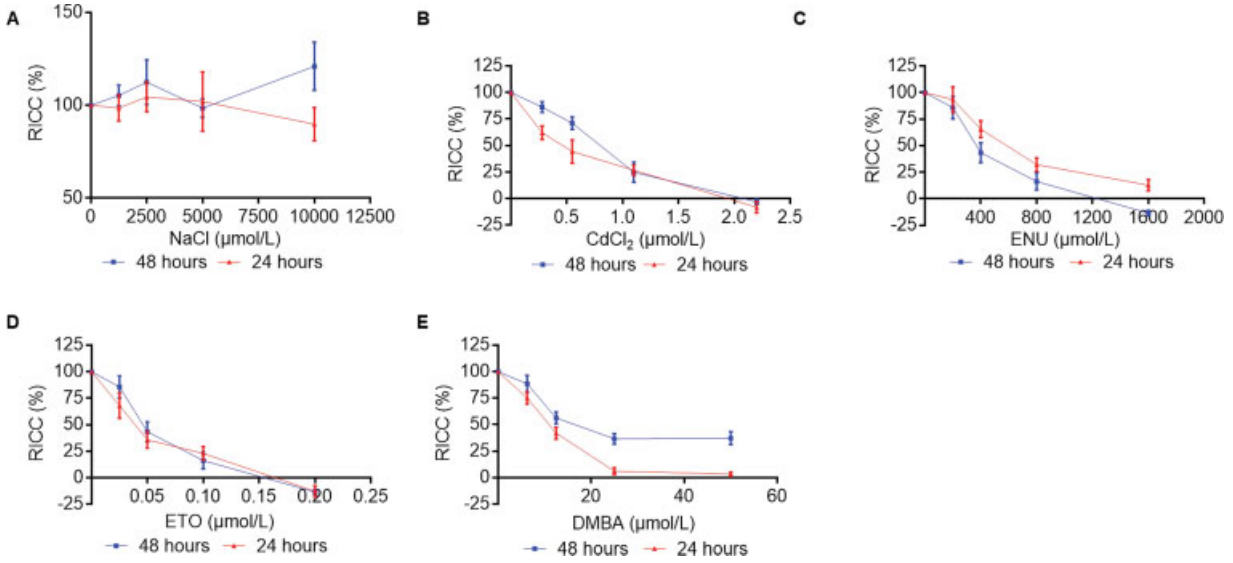

Fig. 4 The preliminary results of the dose range-finding study of the subjects with or without metabolic activation system. Pretest results of cytotoxicity of (**A**) NaCl; (**B**) $CdCl_2$; (**C**) ENU; (**D**) ETO, and (**E**) DMBA.

Table 2 Concentrations of test articles

Test chemical	Abb.	Metabolic activation	Solvent	Test Con. (μmol/L)
Ethyl methanesulfonate	EMS	No	DMSO	200[a]
Benzo[*a*]pyrene	B[*a*]P	Yes	DMSO	16[a]
7,12-Dimethyl-benz[*a*]anthracene	DMBA	Yes	DMSO	7.5, 15, 30
Etoposide	ETO	No	dH_2O	0.0275, 0.055, 0.11
N-Ethyl-*N*-nitrosourea	ENU	No	dH_2O	200, 400, 800
Cadmium chloride	$CdCl_2$	No	dH_2O	0.55, 1.1, 2.2
Sodium chloride	NaCl	No	dH_2O	1000, 3160, 10000

[a]Positive control for ± S9 treatment.

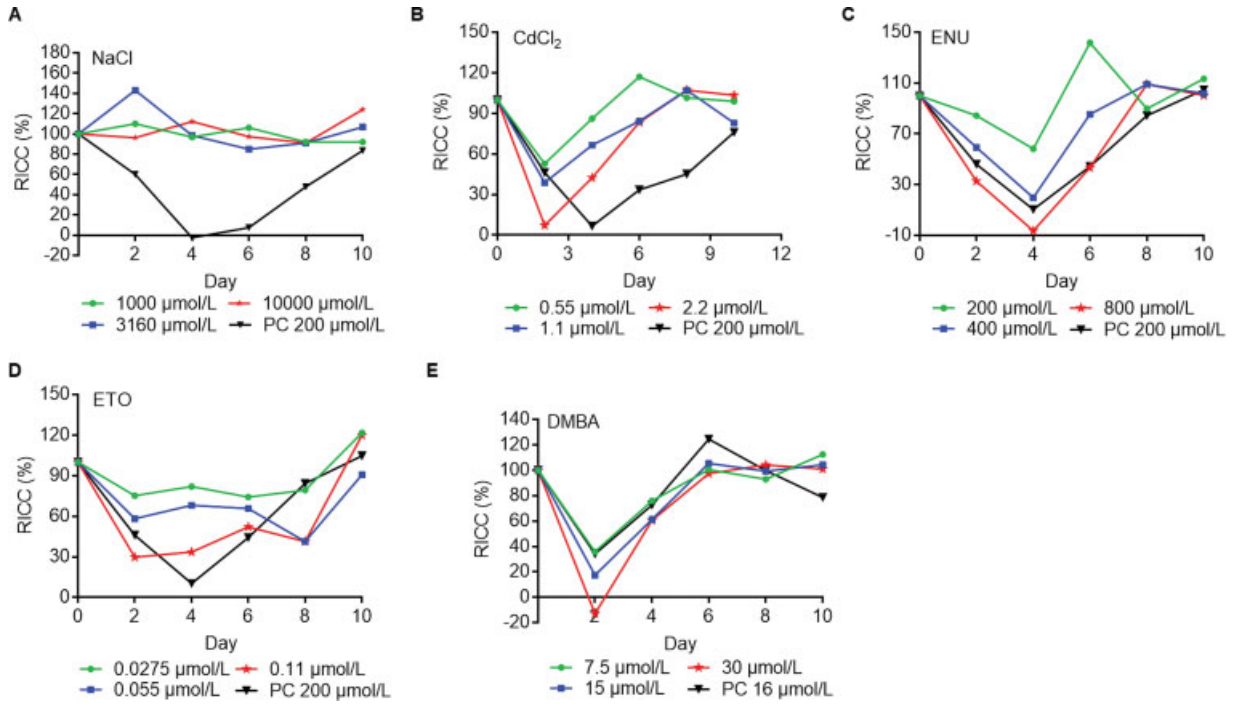

Fig. 5 The RICC of day 2–day 10 for test articles with or without a metabolic activation system. (**A**) NaCl; (**B**) $CdCl_2$; (**C**) ENU; (**D**) ETO; (**E**) DMBA. PC (positive control) of 200 μmol/L EMS (24 hour − S9) and 16 μmol/L B[*a*]P (4 hour + S9). RICC, relative increase in cell counts.

Effect of Chemicals on *PIG-A* Mutation

Negative and Positive Effects

Four experiments were performed in this study with 1% (v/v) dimethylsulfoxide (DMSO) or deionized water as negative control and 200 μmol/L EMS (24 hour − S9) or 16 μmol/L B[*a*]P (4 hour + S9) as positive control. The *PIG-A* mutation rate of the negative control groups ranged from 67.6 to 75×10^{-6} with an average of 71.4×10^{-6}, while the *PIG-A* mutation rate of the positive control groups ranged from 142.3 to 282.6×10^{-6} with an average of 228.2×10^{-6}. The high variability of the positive control groups may be attributed to the test article or manipulation (►**Fig. 6**).

Sodium chloride (NaCl): NaCl is a commonly used inorganic salt and a known nongenotoxic compound selected as a negative test article. NaCl was not cytotoxic to $TK6^{GPI+}$ cells

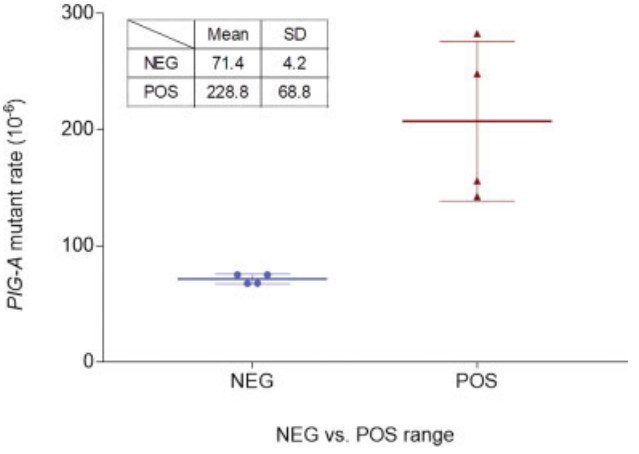

Fig. 6 *PIG-A* mutation ranger of negative and positive in this study.

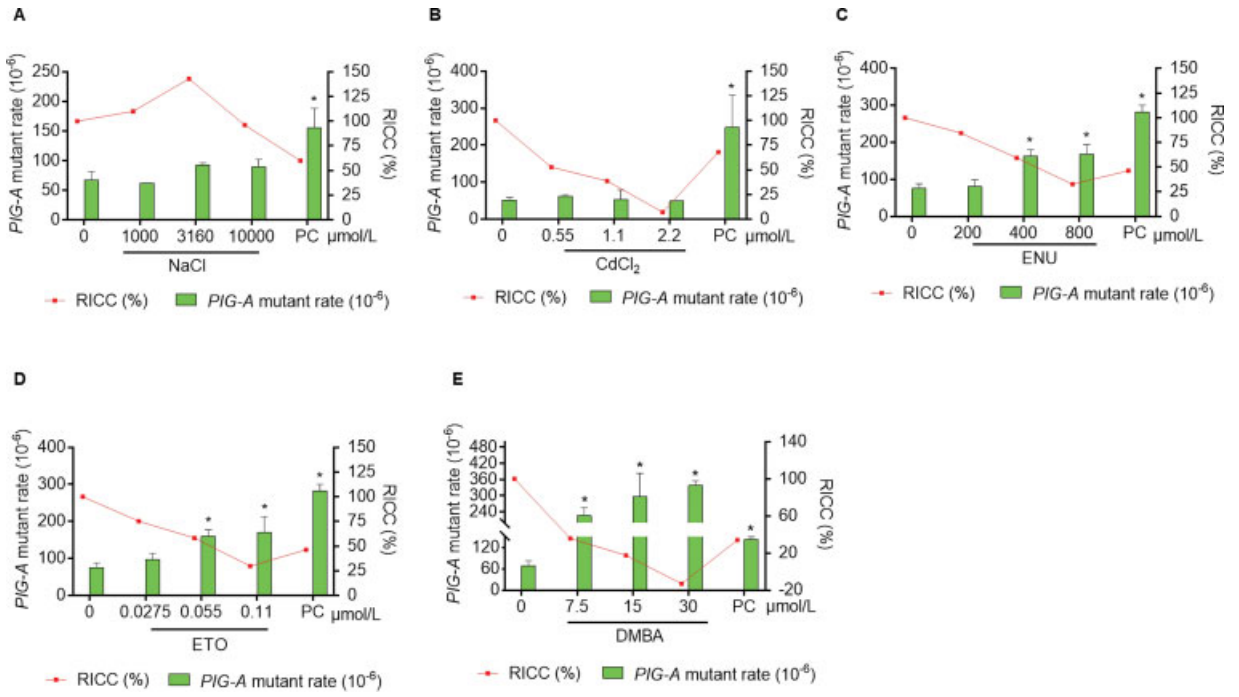

Fig. 7 Chemicals with or without metabolic activation system. *In vitro PIG-A* gene mutation detection results of (**A**) NaCl; (**B**) $CdCl_2$; (**C**) ENU; (**D**) ETO; and (**E**) DMBA. PC: positive control 200 μmol/L EMS (24 hour − S9) and 16 μmol/L B[*a*]P (4 hour + S9). **PIG-A* gene mutation rate represents at least a twofold increase compared with the negative control.

and the solubility of NaCl in the medium was higher than 10 mmol/L, so the highest concentration of NaCl was selected as 10 mmol/L. In this experiment with the treatment without *in vitro* −S9 for 24 hours, NaCl did not cause a concentration correlation of the *PIG-A* gene mutation rate (►**Fig. 7**). The results of this NaCl were classified as negative in this experiment, consistent with the results of Ames and MLA tests for NaCl.[16]

N-nitroso-N-ethylurea (ENU): ENU acts as a potent alkylating agent of the nitrosourea class and it is a direct mutagenic agent that does not require metabolic activation.[17] ENU is known to induce transitions and reversals after O^{2-} and O^{4-} alkylation of thymine residues and O^{6-} alkylation of guanine residues.[18] ENU was positive under mouse lymphoma TK gene mutation assay (MLA) test and HPRT gene mutation test in the absence of S9 conditions. In this experiment, 400 and 800 μmol/L of ENU induced a concentration-dependent increase of *PIG-A* gene mutation rate in $TK6^{GPI+}$ cells and more than twofold of the negative control (►**Fig. 7**), which was a positive result and was consistent with the published results of *in vivo*/*in vitro* *PIG-A* mutation assays.[7]

Etoposide (ETO): ETO is a topoisomerase II inhibitor that, upon entry into the nucleus, forms a drug–enzyme–DNA complex with DNA that interferes with DNA topoisomerase II, resulting in irreparable DNA damage.[19,20] ETO was classified as a carcinogen (group 2A) that may be carcinogenic to humans by International Agency for Research on Cancer (IARC). In *in vitro* genotoxicity studies, ETO was weakly positive for strain TA98 in the Ames test, induced high frequency of small and large colony mutants in the MLA test, and induced chromosomal structural aberrations in the chromosomal aberration test.[21,22] In the present experiments, the *PIG-A* gene mutations in TK6 cells induced by 0.055 and 0.11 μmol/L of ETO were more than twofold higher than those in negative controls under conditions without metabolic activation system and with a dose-related manner in the absence of the S9-mix (►**Fig. 7**), confirming the roles of ETO as a mutagen.

Cadmium chloride ($CdCl_2$): $CdCl_2$ is a cadmium heavy metal salt, and IARC classifies cadmium and cadmium-like compounds as Class I carcinogens (possibly carcinogenic to humans).[22] However, studies on $CdCl_2$ have found that it is not a direct DNA-acting agent, and the possible carcinogenic mechanism of $CdCl_2$ is to stimulate and promote cell division by inhibiting DNA repair mechanisms and upstream signals of apoptosis through epigenetic pathways.[23] The Ames test of $CdCl_2$ had both negative and positive results (without S9 metabolism), while the results of the Bhas42 cell transformation assay of $CdCl_2$ suggested that $CdCl_2$ is a cancer promoter and noninitiator.[24] Our data showed that a high cytotoxicity with RICC of 7.2% was induced by 2.2 μmol/L of $CdCl_2$ (►**Fig. 7**). However, no significant and concentration-dependent increase was noted at all tested concentrations of $CdCl_2$ in the *PIG-A* gene mutation rate. Thus, $CdCl_2$ was tested as a nonmutagen in this experiment.

***7,12-dimethyl-benz[a] anthracene (DMBA)*:** DMBA is a polycyclic aromatic hydrocarbon that is oxidized by P450 enzymes (CYP1A1 and 1B1) to form DNA adducts covalently with DNA, which can lead to DNA damage and gene mutations. DMBA is commonly used in animal tests to induce skin and mammary tumors, as well as leukemia and other

tumors.[25] A significant and concentration-dependent increase was noted in the *PIG-A* gene mutation rate in TK6 cells at concentrations of 7.5, 15, and 30 μmol/L DMBA in the presence of S9-mix (►**Fig. 7**), suggesting that DMBA is a mutagen, which is consistent with the published findings.

Conclusions

The aim of this study was to develop an *in vitro PIG-A* gene mutation assay in TK6 cells via detection of the loss of GPI-linked CD55 and CD59 proteins by flow cytometry. Since mutations occur at a low frequency, the sensitivity of mutation detection methods is particularly important. To establish a highly sensitive *PIG-A* gene mutation assay, cleansing of preexisting GPI(−) TK6 cells was performed, and $TK6^{GPI+}$ cells were obtained by immunomagnetic sorting. Subsequently, the doubling time and spontaneous mutation rate of $TK6^{GPI+}$ cells were characterized. We reduced the effect of the spontaneous mutation rate on the sensitivity of the assay by controlling the subculture time for $TK6^{GPI+}$ cells that had a higher spontaneous mutations rate. We also found that RICC was important in the assay, with some compounds showing greater differences in RICC at 24 and 48 hours. If we selected the RICC at 24 hours as the concentration selection criterion, some chemicals may induce all cells to die on day 4 or 5 of the formal assay. ENU, ETO, DMBA, $CdCl_2$, and NaCl were selected as subjects to validate the *in vitro PIG-A* assay, and they showed a positive result that each result of test articles was as expected.

TK6 cells have a normal P53 status which contributes to the maintenance of genomic integrity through recombinational repair.[26] The TK6 cell line is mainly used in *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene. The mutagens (X-rays, EMS, methyl methanesulfonate, and mitomycin C) have been proved to dose-dependently induce TK mutations in TK6 cells.[27] In contrast to the low spontaneous mutation frequency of TK mutation, the spontaneous mutation frequency of PIG-A mutation was high, and it still elevated during culture after cleansing of preexisting GPI(−) cells. Therefore, the passage time of TK6 cells should be controlled within 4 weeks. Besides the *PIG-A* mutations, *PIG-L* mutations also cause GPI-deficient isolates in the TK6 cell line, as *PIG-L* is heterozygous in these cells.[28] Thus, although the TK6 cell assay may not be totally analogous to the *in vivo Pig-a* assay, it has been shown that the sensitivity of the TK6 cell assay may benefit from having both an X-linked and an autosomal reporter of mutation in terms of increasing the types of mutations that the assay can detect.

In summary, an *in vitro PIG-A* gene mutation detection method based on flow cytometry and a $TK6^{GPI+}$ cell line was successfully established and preliminarily validated in this study. The method uses automated flow cytometry analysis to achieve high-throughput detection. The *in vitro PIG-A* gene mutation assay has a shorter experimental period and is relatively simple to perform in comparison to MLA assay and HPRT gene mutation assay. Therefore, the *in vitro PIG-A* mutation assay is expected to complement the *in vivo Pig-a* assay with some distinct advantages compared with other *in vitro* mammalian mutagenicity tests.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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