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

Chang-Hui Zhou¹# Chun-Rong Yu¹# Peng-Cheng Huang¹ Ruo-Wan Li¹ Jing-Ting Wang¹ Tian-Tian Zhao¹ Ze-Hao Zhao¹ Jing Ma¹* Yan Chang¹*

¹Shanghai Innostar Bio-tech Co., Ltd., China State Institute of Pharmaceutical Industry, Shanghai, People’s Republic of China

Address for correspondence Jing Ma, PhD, Shanghai Innostar Bio-tech Co., Ltd., 199 Guoshoujing Road, Pudong New Area, Shanghai 201203, People’s Republic of China (e-mail: jingma2019@163.com).

Yan Chang, PhD, Shanghai Innostar Bio-tech Co., Ltd., 199 Guoshoujing Road, Pudong New Area, Shanghai 201203, People’s Republic of China (e-mail: ychang@innostar.cn).

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 (CdCl2) 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 CD59 and CD55. 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, CdCl2 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

# These authors contributed equally to this work.

DOI https://doi.org/10.1055/s-0041-1735146.
ISSN 2628-5088.

© 2021. The Author(s).
This is an open access article published by Thieme under the terms of the Creative Commons Attribution License, permitting unrestricted use, distribution, and reproduction so long as the original work is properly cited. (https://creativecommons.org/licenses/by/4.0/)
Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany
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. 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.

Flow cytometry is the most common method for diagnosis of GPI(−) frequency (PIG-A gene mutation rate) in TK6 human lymphoblastoid cells. From a throughput 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. 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. Compared with the existing in vitro gene mutation assays (such as mouse lymphoma assay [MLA] test and hypoxanthine-guanine phosphoribosyltransferase [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 testing using flow cytometry may be more objective, convenient, and high-throughput.

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. 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 (DBMA; Cat. No. D3254), and sodium chloride (NaCl; Cat. No. S9888) were purchased from Sigma-Aldrich (United States); cadmium chloride (CdCl2; 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. S561080) 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 cytomter used was BD Accuri C6 PLUS (Becton-Dickinson, United States). The cell counter was Chemometec (NucleoCounter NC-100, Denmark).

#### Cell Culture

TK6 cells (0.2 × 10⁶ or 10⁶ 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% CO₂ 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), MgCl₂ (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 × 10⁶) 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 × 10⁶ 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 10⁶ cells were collected.

Cleansing of Preexisting GPI(−) TK6 Cells by Immunomagnetic Separation
For cleansing of preexisting GPI(−) TK6 cells, ~4 × 10⁶ 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 TK6GPI+. For doubling time determination, TK6GPI+ cells were seeded in triplicate at a density of 10⁵ and 2 × 10⁶ 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):

\[
D.T. = \frac{(t_2-t_1) \times \log_2 \frac{C_2}{C_1}}{\log_2 \frac{C_i}{C_0}}
\]

where D.T. is the doubling time, t₂ the cell harvest time, t₁ the cell inoculation time, Ch the cell harvest density, and Ci the cell inoculation density.

TK6GPI+ cells were cultured and passaged for 40 days and 10⁵ 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):

\[
\mu = \frac{a \times 10^{10} \times D.T.}{24}
\]

where a is the slope of the fit curve, D.T. the doubling time of TK6GPI+ 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), TK6GPI+ 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):

\[
RICC (\text{control %}) = \frac{\text{Cell Density}_{\text{treatment}}}{\text{Cell Density}_{\text{control}}} \times 10^5 \times 100
\]

Treatments
All experiments were performed with GPI(−)-cleansed TK6GPI+ cells. In case of incubations, TK6GPI+ cells were seeded at 3 × 10⁵ 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
suspension was mixed by gentle vortex. Cells were directly cultured for 24 hours at 37°C, 5% CO₂ 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 x 10⁶ 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 10⁶ 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 x 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 TK6GPI⁻ cells.

Doubling Time and Spontaneous Mutation Rate of TK6GPI⁻ 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 TK6GPI⁻ cells had a doubling time of 15.5 ± 1.2 hours.

The spontaneous GPI(−) rate was determined by cell division in the culture. TK6GPI⁻ cells were tested for spontaneous GPI(−) rate up to 40 days, and the mean spontaneous GPI(−) rate up to 40 days, and the mean spontaneous GPI(−)
rate was \(1.37 \times 10^{-6}/\text{cell/generation}\) for the TK6GPI\(^+\) cell line (fitted curve in Fig. 3). This means that the mutation rate of TK6 cells increases by \(~22.2 \times 10^{-6}\) 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 \times 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).
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% with an average of 71.4%.

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

Table 2 Concentrations of test articles

| Test chemical                      | Abb. | Metabolic activation | Solvent | Test Con. (μmol/L) |
|-----------------------------------|------|----------------------|---------|-------------------|
| Ethyl methanesulfonate            | EMS  | No                   | DMSO    | 200^               |
| Benzo[a]pyrene                    | B[a]P| Yes                  | DMSO    | 16^               |
| 7,12-Dimethyl-benz[a]anthracene   | DMBA | Yes                  | DMSO    | 7.5, 15, 30       |
| Etoposide                         | ETO  | No                   | dH2O    | 0.0275, 0.055, 0.11|
| N-Ethyl-N-nitrosourea             | ENU  | No                   | dH2O    | 200, 400, 800      |
| Cadmium chloride                  | CdCl2| No                   | dH2O    | 0.55, 1.1, 2.2    |
| Sodium chloride                   | NaCl | No                   | dH2O    | 1000, 3160, 10000 |

^Positive control for S9 treatment.
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.

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. ENU is known to induce transitions and reversals after O2 and O4 and O6 alkylation of thymine residues and O6 alkylation of guanine residues. 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 TK6GPI+ 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.

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. 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. 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 (CdCl2): CdCl2 is a cadmium heavy metal salt, and IARC classifies cadmium and cadmium-like compounds as Class I carcinogens (possibly carcinogenic to humans). However, studies on CdCl2 have found that it is not a direct DNA-acting agent, and the possible carcinogenic mechanism of CdCl2 is to stimulate and promote cell division by inhibiting DNA repair mechanisms and upstream signals of apoptosis through epigenetic pathways. The Ames test of CdCl2 had both negative and positive results (without S9 metabolism), while the results of the Bhas42 cell transformation assay of CdCl2 suggested that CdCl2 is a cancer promoter and noninitiator. Our data showed that a high cytotoxicity with RICC of 7.2% was induced by 2.2 μmol/L of CdCl2 (Fig. 7). However, no significant and concentration-dependent increase was noted at all tested concentrations of CdCl2 in the PIG-A gene mutation rate. Thus, CdCl2 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. 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 pre-existing GPI(−) TK6 cells was performed, and TK6GPI− cells were obtained by immunomagnetic sorting. Subsequently, the doubling time and spontaneous mutation rate of TK6GPI− cells were characterized. We reduced the effect of the spontaneous mutation rate on the sensitivity of the assay by controlling the subculture time for TK6GPI− cells that had a higher spontaneous mutation 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, CdCl₂, 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. The TK6 cell line is mainly used 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. 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.

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 TK6GPI− 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.

Funding

This study was financially supported by the Major Projects Foundation of the National Health Commission of the People’s Republic of China (Grant No. 2018ZX09201017-008).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Cimino MC. Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. Environ Mol Mutagen 2006;47(05):362–390
2. Miyata T, Takeda J, Iida Y, et al. The cloning of Pig-A, a component in the early step of GPI-anchor biosynthesis. Science 1993;259(5099):1318–1320
3. Zurzolo C, Simons K. Glycosylphosphatidylinositol-anchored proteins: membrane organization and transport. Biochim Biophys Acta 2016;1858(04):632–639
4. Kinoshita T. Biosynthesis and deficiencies of glycosylphosphatidylinositol, Proc Jpn Acad, Ser B, Phys Biol Sci 2014;90(04):130–143
5. Krüger CT, Hofmann M, Hartzwig A. The in vitro PIG-A gene mutation assay: mutagenicity testing via flow cytometry based on the glycosylphosphatidylinositol (GPI) status of TK6 cells. Arch Toxicol 2015;89(12):2429–2443
6. Rees BJ, Tate M, Lynch AM, et al. Development of an in vitro Pig-a gene mutation assay in human cells. Mutagenesis 2017;32(02):283–297
7. Bemis JC, Avlasevich SL, Labash C, et al. Glycosylphosphatidylinositol (GPI) anchored protein deficiency serves as a reliable reporter of Pig-a gene mutation: Support from an in vitro assay based on L5178Y/Tk− cells and the CD90.2 antigen. Environ Mol Mutagen 2018;59(01):18–29
8. Wang Y, Revollo J, McKinzie P, et al. Establishing a novel Pig-a gene mutation assay in L5178Y/Tk− mouse lymphoma cells. Environ Mol Mutagen 2018;59(01):4–17
9. David R, Talbot E, Allen B, Wilson A, Arshad U, Doherty A. The development of an in vitro Pig-a assay in L5178Y cells. Arch Toxicol 2018;92(04):1609–1623
10. Johnson GE, Slob W, Doak SH, et al. New approaches to advance the use of genetic toxicology analyses for human health risk assessment. Toxcol Res 2015;4:667–676
11. Bemis JC, Heßlich RH. In vitro mammalian cell mutation assays based on the Pig-a gene: a report of the International Workshops on Genetic Toxicology (IWGT) workshop. Mutat Res Genet Toxcol Environ Mutagen 2019;847:403039
12. Ellis P, Fowler P, Booth E, et al. Where will genetic toxicology testing be in 30 years’ time? Summary report of the 25th Industrial Genotoxicity Group Meeting, Royal Society of Medicine, London, November 9, 2011. Mutagenesis 2014;29(01):73–77
13. OECD Library. OECD Guideline for the testing of chemicals, Section 4. In vitro mammalian cell gene mutation tests using the Hprt and xprt genes. Accessed July 2, 2021 at: https://www.oecd-ilibrary.org/docserver/9789264264809-en.pdf?family=1624388874&amp;id=2acacname=guest&amp;checksum=48A3A1AD7D05C668BB8E902EFC0F0D3.
14. OECD Library. OECD Guideline for the testing of chemicals, section 4. In vitro mammalian cell gene mutation tests using the Thymidine Kinase gene. Accessed July 2, 2021 at: https://www.oecd-ilibrary.org/docserver/9789264264908-en.pdf?family=1624388821&amp;id=2acacname=guest&amp;checksum=655A70F3F7AB4295BBEE05274C46532
15. Lorge E, Moore MM, Clements J, et al. Standardized cell sources and recommendations for good cell culture practices in genotoxicity testing. Mutat Res 2016;808:1–15
16. 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
17 Kimoto T, Horibata K, Miura D, et al. The PIGRET assay, a method for measuring Pig-a gene mutation in reticulocytes, is reliable as a short-term in vivo genotoxicity test: summary of the MMS/JEMS-collaborative study across 16 laboratories using 24 chemicals. Mutat Res 2016;811:3–15
18 Zielenska M, Beranek D, Guttenplan JB. Different mutational profiles induced by N-nitroso-N-ethylurea: effects of dose and error-prone DNA repair and correlations with DNA adducts. Environ Mol Mutagen 1988;11(04):473–485
19 Ezoe S. Secondary leukemia associated with the anti-cancer agent, etoposide, a topoisomerase II inhibitor. Int J Environ Res Public Health 2012;9(07):2444–2453
20 van Maanen JM, Lafleur MV, Mans DR, et al. Effects of the ortho-quinone and catechol of the antitumor drug VP-16-213 on the biological activity of single-stranded and double-stranded phi X174 DNA. Biochem Pharmacol 1988;37(19):3579–3589
21 Ashby J, Tinwell H, Glover P, et al. Potent clastogenicity of the human carcinogen etoposide to the mouse bone marrow and mouse lymphoma L5178Y cells: comparison to Salmonella responses. Environ Mol Mutagen 1994;24(01):51–60
22 Suzuki H, Nakane S. Differential induction of chromosomal aberrations by topoisomerase inhibitors in cultured Chinese hamster cells. Biol Pharm Bull 1994;17(02):222–226
23 Kirkland D, Kasper P, Martus HJ, et al. Updated recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests. Mutat Res Genet Toxicol Environ Mutagen 2016;795:7–30
24 Sakai A, Sasaki K, Muramatsu D, et al. A Bhas 42 cell transformation assay on 98 chemicals: the characteristics and performance for the prediction of chemical carcinogenicity. Mutat Res 2010;702(01):100–122
25 Han EH, Hwang YP, Jeong TC, Lee SS, Shin JG, Jeong HG. Eugenol inhibit 7,12-dimethylbenz[a]anthracene-induced genotoxicity in MCF-7 cells: Bifunctional effects on CYP1 and NAD(P)H:quinone oxidoreductase. FEBS Lett 2007;581(04):749–756
26 Honma M, Hayashi M, Sofuni T. Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells. Mutat Res 1997;374(01):89–98
27 Honma M, Momose M, Tanabe H, et al. Requirement of wild-type p53 protein for maintenance of chromosomal integrity. Mol Carcinog 2000;28(04):203–214
28 Nicklas JA, Carter EW, Albertini RJ. Both PIGA and PIGL mutations cause GPI-a deficient isolates in the Tk6 cell line. Environ Mol Mutagen 2015;56(08):663–673