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Spectrum of benzo[*a*]pyrene-induced mutations in the *Pig-a* gene of L5178Y*Tk*$^{+/-}$ cells identified with next generation sequencing☆

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ABSTRACT

We used Sanger sequencing and next generation sequencing (NGS) for analysis of mutations in the endogenous X-linked *Pig-a* gene of clonally expanded L5178Y*Tk*$^{+/-}$ cells. The clones developed from single cells that were sorted on a flow cytometer based upon the expression pattern of the GPI-anchored marker, CD90, on their surface. CD90-deficient and CD90-proficient cells were sorted from untreated cultures and CD90-deficient cells were sorted from cultures treated with benzo[*a*]pyrene (B[*a*]P). *Pig-a* mutations were identified in all clones developed from CD90-deficient cells; no *Pig-a* mutations were found in clones of CD90-proficient cells. The spectrum of B[*a*]P-induced *Pig-a* mutations was dominated by basepair substitutions, small insertions and deletions at G:C, or at sequences rich in G:C content. We observed high concordance between *Pig-a* mutations determined by Sanger sequencing and by NGS, but NGS was able to identify mutations in samples that were difficult to analyze by Sanger sequencing (e.g., mixtures of two mutant clones). Overall, the NGS method is a cost and labor efficient high throughput approach for analysis of a large number of mutant clones.

1. Introduction

The endogenous, X-linked phosphatidyl inositolglycan class A (*Pig-a*) gene can serve as a reporter of mutation in mammalian cells [1]. The enzyme encoded by the *Pig-a* gene is involved in the synthesis of glycosyl phosphatidylinositol (GPI), which anchors a number of protein markers to the exterior surface of the cytoplasmic membrane [2]. In mammalian species, the *Pig-a* gene is present as a single functional copy (in males there is only one physical copy of the gene; in females there are two gene copies, but one copy resides on the transcriptionally inactivated X-chromosome). Mutation of the functional *Pig-a* gene results in the disruption of GPI synthesis, rendering the cell deficient in surface markers that are anchored by GPI (i.e., the cell acquires a mutant phenotype). The fraction of mutant-phenotype cells (potential *Pig-a* mutants) can be quantitated by flow cytometry in samples labeled with fluorescent antibodies against GPI-anchored markers: wild-type cells in such samples are labeled with antibodies and fluoresce, but GPI-deficient mutant cells do not.

Over the last decade, the *Pig-a* gene has been successfully employed as a reporter of in vivo mutation in human and rodent hematopoietic cells [3]. Recently, efforts have begun to design complementary in vitro models using common cell lines. Litron Laboratories has described a flow cytometry-based model for detecting *Pig-a* mutation in the mouse lymphoma L5178Y*Tk*$^{+/-}$ cell line [4]. This cell line is used for the mouse lymphoma assay, a standard test for regulatory safety evaluations of industrial and pharmaceutical compounds [5]. In Litron's model, an antibody against the GPI-anchored CD90 cell surface marker is used to identify potential *Pig-a* mutant cells. We followed the Litron approach to identify and enumerate mutant-phenotype cells in cultures of the L5178Y*Tk*$^{+/-}$ mouse lymphoma cells used in our laboratory. In addition, we sorted individual L5178Y*Tk*$^{+/-}$ CD90-deficient cells, expanded them into clones and performed Sanger sequencing to demonstrate that flow cytometry-identified mutant-phenotype cells possess bona fide mutations in the *Pig-a* gene [6].

Although mutations are an important metric for regulatory safety evaluations (e.g., of novel drugs intended for use by humans), mutational spectra are rarely, if ever, used in safety assessments, though such data might be informative in certain situations. It is rather difficult to generate mutational spectra even using multi-channel fluorescence capillary analyzers, the standard Sanger sequencing platform for processing a large number of samples. Amplifying fragments of the gene, setting up multiple individual sequencing reactions for each fragment, various purification steps, data processing, etc., may become an overwhelming task.

☆ The views and opinions presented in this article do not necessarily reflect the official position of the US Food and Drug Administration.

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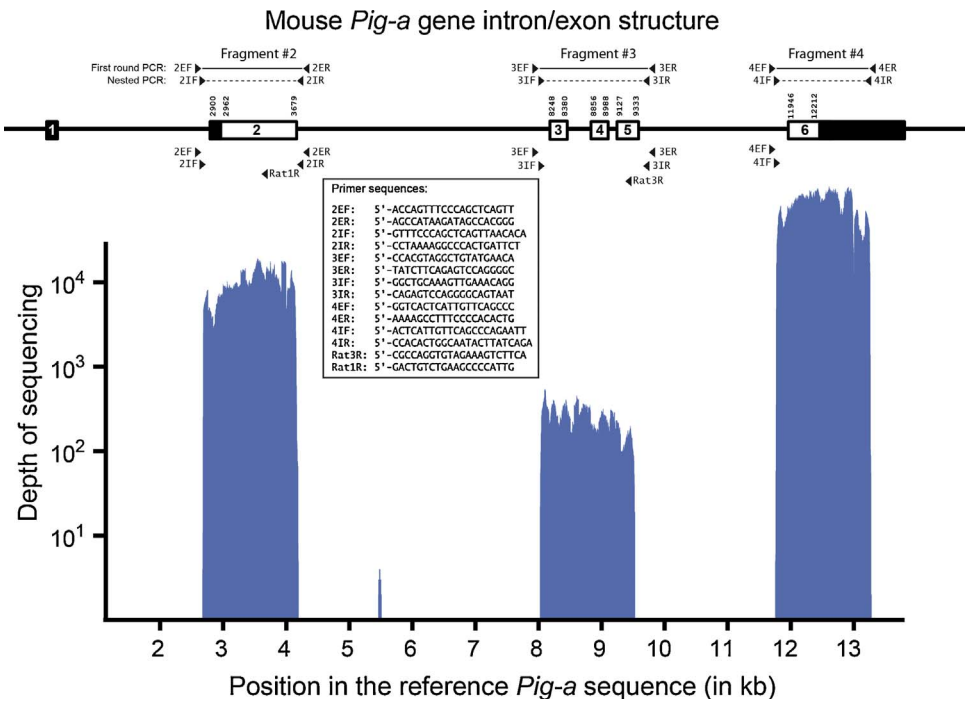

Fig. 1. Amplification and sequencing of the mouse *Pig-a* gene. At the top, exons of the *Pig-a* gene are represented by numbered boxes. White background within the exons indicates protein coding sequence. Small triangles above and below the exons represent appropriately oriented primers that were used for amplification and sequencing of the fragments of the *Pig-a* gene. The numbers above the exons designate the first and the last bases of the relevant exons relative to the reference sequence. Also shown are the positions of A of the ATG initiation codon (position 2962) and of G of the TAG stop codon (position 12212). The first *Pig-a* exon is non-coding; it was not sequenced in our analyses. The end position of the last *Pig-a* exon is not precisely known. The histogram at the bottom shows sequencing depth for each base within the covered *Pig-a* fragments as determined by NGS for clone 143. For this clone, all bases within fragments #2, #3 and #4 were present in multiple reads aligned to the reference mouse genomic *Pig-a* sequence. A G > A variant at 8381 was present in 100% of reads covering this particular position in clone 143. Primer sequences are listed in the insert.

In the present study we optimized the use of Next Generation Sequencing (NGS) for efficient analysis of *Pig-a* mutations in a large number of clones developed from sorted CD90-deficient L5178Y*Tk*$^{+/-}$ cells. Importantly, in our approach, each clone was analyzed individually so that the relationship between the sorted cell and its *Pig-a* mutation was preserved, enabling accurate estimation of the frequencies of independently induced mutations.

2. Materials and methods

2.1. Cell culture, mutagenesis, flow cytometry analyses and sorting

All manipulations with L5178Y*Tk*$^{+/-}$ 3.7.2C mouse lymphoma cells were described previously [6]. Benzo[*a*]pyrene (B[*a*]P; Sigma-Aldrich, St. Louis MO) was dissolved in DMSO at 2 mM and added to cells in S9-supplemented medium to achieve a final concentration of 20 μM as described in [6]. After 4 h of incubation at 37 °C, the treated cells were washed, re-suspended in 20 ml of the standard growth medium and maintained in culture for 8 days, resupplied daily with fresh medium in order to keep the cells in log phase growth. Control cell cultures (not treated with B[*a*]P and not exposed to S9) were processed in the same manner.

2.2. Antibody labeling and magnetic processing of cells

After an 8-day mutant phenotype manifestation period, cell aliquots were sampled from the cultures for analytical labeling and for sorting. For analytical labeling, 2 × 10^6 cells from control and B[*a*]P-treated cultures were labeled with 10 μg/ml of phycoerythrin (PE)-conjugated rat anti-mouse CD90 antibody and 10 μg/ml of allophycocyanin (APC)-conjugated rat anti-mouse CD45 antibody (both antibodies were purchased from BD Biosciences, Milpitas CA). Mutant-mimic control cells were labeled with APC anti CD45 only. The remaining cells were used for 25 × scaled-up labeling and enrichment for mutants on LS magnetic columns (Miltenyi Biotec, Auburn CA) with anti PE magnetic beads (Miltenyi) as described previously [6].

2.3. Flow cytometry, sorting and clonal expansion

The labeled analytical and enriched cell samples were processed on a FACSAria III cell sorter (BD Biosciences). Using the sorter's single-cell precision mode, cells were sorted according to the manufacturer's instructions at one cell per well into flat-bottom 96-well tissue culture plates (96WPs) pre-filled with 200 μl of growth medium. The following types of cells were sorted: CD45-positive (CD45$^+$) CD90-negative (CD90$^-$) mutant-phenotype cells from magnetically enriched control and B[*a*]P-treated cultures, and CD45$^+$ CD90$^+$ wild-type (WT) phenotype cells from an unenriched sample of the control cell culture. The gating strategy for sorting was the same as previously described [6]. Plates with sorted cells were incubated at 37 °C in a humidified incubator in the presence of 5% CO_2. Clones developed from sorted cells were scored on an inverted tissue culture microscope following 7–9 days of culture. After initial expansion in 96WPs some clones were further expanded in larger volumes of growth medium, while others were collected directly from 96WPs for genomic DNA extraction.

2.4. Molecular analysis of mutant clones

For clones that were expanded beyond 96WPs, DNA was extracted from 1 × 10^6 cells using an AllPrep DNA/RNA mini kit (Qiagen, Valencia CA) following the manufacturer's instructions. For the clones collected from 96WPs, the contents of the wells with a developing clone (estimated to consist of > 1 × 10^5 cells) were processed for DNA extraction using a QIAamp DNA Micro kit (Qiagen). Purified genomic DNA was eluted in a final volume of 40 μl in both cases. Three fragments (#2, #3 and #4) of the *Pig-a* gene containing coding exons and adjacent intron sequences were amplified using the primers shown in Fig. 1. Each fragment was amplified individually using pairs of external primers in a 20-μl reaction containing 2 μl of extracted genomic DNA, 1 μM of each primer, 0.2 mM of each dNTP, 1 unit of HotStarTaq polymerase and 1 × buffer from Qiagen's HotStarTaqPlus kit, and using a temperature profile of 95 °C × 5 min + (94 °C × 30 s + 58 °C × 30 s + 72 °C × 2min30 s) × 35 + 4 °C soak. Three microliters of the reaction were analyzed on a 1% agarose gel and the remainder was purified for subsequent sequencing [6]. If the amount of an amplified fragment was deemed insufficient for sequencing, the first-