Mutation Research 808 (2016) 20–26

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Monitoring genotoxicity in patients receiving chemotherapy for cancer: application of the *PIG-A* assay

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ARTICLE INFO

Article history:
Received 25 May 2016
Received in revised form 5 August 2016
Accepted 8 August 2016
Available online 10 August 2016

Keywords:
Glycosylphosphatidylinositol anchor
Red blood cell
Chemotherapy
Genotoxicity

ABSTRACT

The recently introduced *Pig-a* in vivo gene mutation assay measures endogeneous mutations of *Pig-a* (human, *PIG-A*), an X-linked gene that is conserved across species from rodents to humans. Flow cytometric analysis enables the enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient erythrocytes, resulting from a mutation in *Pig-a*/*PIG-A*, in only a few microliters of peripheral blood. *Pig-a*/*PIG-A* mutations appear to function in a neutral manner, allowing evaluation of the accumulated genotoxic effects of repeated exposures. To date, most *Pig-a* studies have been conducted in rodents; only a few reports regarding human applications of the *PIG-A* assay have been published. We have conducted a *PIG-A* assay in the context of human genotoxicity monitoring. Peripheral blood was collected from healthy human donors and chemotherapy-treated cancer patients at Yamagata University Hospital. To investigate the *PIG-A* mutant frequency (MF) induced by chemotherapy, red blood cells were analyzed via flow cytometry following staining with allophycocyanin-conjugated anti-CD235ab (erythrocyte specific) and fluorescein isothiocyanate-conjugated anti-CD59 antibodies (GPI-anchored protein specific). Reticulocyte frequencies (%RET) were also analyzed using a phycoerythrin-conjugated anti-CD71 antibody to monitor bone marrow suppression and reticulocytosis. Two of 27 patients exhibited a significantly elevated frequency of *PIG-A* mutants. Although we observed either a reduced or an increased %RET in all patients, no association was observed between this factor and the *PIG-A* MF. Unfortunately, we could not analyze blood samples collected before treatment during therapeutic processes. Additionally, the sampling time point for some patients was too short to express the *PIG-A* mutant phenotypes. Therefore, the possibility of natively high *PIG-A* MFs prior to treatment must be considered. The human *PIG-A* assay shows promise as a human genotoxicity monitoring method.

1. Introduction

Gene mutations are implicated in the etiology of cancer and other diseases. Therefore, in vivo genotoxicity tests are important tools for management of public health. Several types of in vivo genotoxicity tests are available for rodents, including transgenic rodent mutation assays and micronucleus testing of appropriate organs. In contrast, few tests are available for human monitoring, given the requirement of minimal invasiveness. Furthermore, the exposures/doses encountered by human subjects in daily life are very low compared with those used in rodent in vivo genotoxicity tests; therefore, human tests should be able to measure the long-term accumulation of low-dose genotoxicities. These factors present challenges to the establishment of any human genotoxicity test.

The *Pig-a* gene mutation assay, a powerful tool for evaluating in vivo genotoxicity, was recently established in rodent models [1,2]. The availability and validity of this rodent assay have been thoroughly discussed [3]. This assay uses the endogenous *Pig-a* (human, *PIG-A*) gene. Because *Pig-a*/*PIG-A* is located on the X-chromosome and affects the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, mutation(s) of this gene can result in loss of expression of GPI-anchored proteins on the cell membrane, a phenotype that can be detected by flow cytometry

Abbreviations: CDHP, 5 chloro-2,4-dihydroxypyridine; 5-FU, 5-fluorouracil; %RET, percentage of reticulocytes; FITC, fluorescein isothiocyanate; GEM, gemcitabine; GPI, glycosylphosphatidylinositol; MF, mutant frequency; Oxo, monopotassium 1,2,3,4-tetrahydro-2,4-dioxo-1,3,5-triazine-6-carboxylate; PE, phycoerythrin; PNH, paroxysmal nocturnal hemoglobinuria; RBC, red blood cell; RET, reticulocyte.

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http://dx.doi.org/10.1016/j.mrgentox.2016.08.002
1383-5718/

[1,2]. Consequently, in vivo genotoxicity induced in hematopoietic organs can be conveniently detected. In humans, somatic mutation of *PIG-A* has been reported to result in paroxysmal nocturnal hemoglobinuria (PNH) [4]; however, clones of hematopoietic cells and granulocytes that harbor the *PIG-A* mutation (and corresponding phenotype) are present in normal individuals [5,6]. In addition, two key points regarding this assay are relevant to the human genotoxicity test. First, the assay is minimally invasive, because a small amount of peripheral blood is sufficient for analysis [1,2]. Second, the *Pig-a* assay could detect accumulated genotoxicity [7]. Recent studies have demonstrated that a *PIG-A* assay could be applied to in vivo human genotoxicity testing [8–10]. These findings have prompted us to establish and evaluate the performance of a human *PIG-A* assay.

Because anticancer drug therapies often induce genotoxicity and can be used as a p̈roving groundf̈or human genotoxicity testing, we have established a human *PIG-A* assay and applied it to monitor genotoxicity in surplus blood samples collected from chemotherapy-treated cancer patients during therapeutic procedures. To investigate chemotherapy-induced *PIG-A* mutant frequencies (MFs), red blood cells (RBCs) were analyzed using flow cytometry. Frequencies of reticulocytes (%RET) were also analyzed to monitor bone marrow suppression.

2. Materials and methods

2.1. Blood collection

Ethics approval for all studies was obtained from the ethics committees of the National Institute of Health Sciences and Yamagata University. Research was conducted according to the Principles of the Declaration of Helsinki. Written informed consent was obtained from each participant.

Blood samples were collected from volunteers and cancer patients at Yamagata University Hospital. Blood was collected into standard heparin-coated tubes. Preserved blood was chilled with cold packs and shipped to the NIHS facility on the same day, via an overnight carrier. All personal donor information associated with the blood samples was removed by the suppliers, with the exception of basic identifiers such as age, sex, and health status. Additionally, the suppliers provided information about the diagnosed cancer types and chemotherapy regimens of patients with cancer.

2.2. Antibodies

We obtained anti-human CD59 [clone p282 (H19), fluorescein isothiocyanate (FITC) conjugated] and anti-human CD71 [clone M-A712, phycoerythrin (PE) conjugated] antibodies from BD Biosciences (Tokyo, Japan). An anti-human CD235ab [clone HIR2, allophycocyanin conjugated] antibody was purchased from BioLegend Japan (Tokyo, Japan).

2.3. Human *PIG-A* mutation assay

Blood samples (3 μl) were labeled with anti-human CD59 (20 μl) and anti-human CD235ab (0.05 μg) antibodies. Approximately 1×10^6 CD235ab-positive cells were subjected to surface CD59 analysis using a FACSCanto II flow cytometer (BD Biosciences); *PIG-A* MFs were calculated as previously described [9,10]. Regarding the flow cytometer gating strategies, we refined the gate for *PIG-A* mutant RBCs as the area encompassing a maximum of $99.0 \pm 0.1\%$ of the lower RBC FITC staining intensities only, as previously described, to avoid inflating the *PIG-A* MFs with artifacts (Fig. S1) [11,12].

2.4. Analysis of percent of reticulocytes

Aliquots (3 μl) of blood were suspended in phosphate-buffered saline (PBS), 200 μl, and labeled with anti-human CD71 (20 μl) and anti-human CD235ab (0.05 μg) antibodies. The cells were incubated in the dark for 30 min at room temperature, centrifuged ($1{,}680 \times g$, 5 min), resuspended in 1 ml of PBS, and examined on a FACSCanto II flow cytometer (BD Biosciences).

2.5. Statistical analyses

Statistical analyses of *PIG-A* MF and %RET data were conducted using Prism 6 software for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA) as follows. A one-way analysis of variance was applied, followed by Dunnett's test (pair-wise comparisons of the mean frequencies of healthy volunteers and patients with cancer were one sided for *PIG-A* MF and two sided for %RET). The two-sided Student's *t*-test was used to compare time-dependent differences in both the *PIG-A* MF and %RET obtained from blood samples collected at multiple time points from patient 10.

3. Results

3.1. Cancer chemotherapy

We analyzed blood samples obtained from ten healthy volunteers [six men, four women; ages: mean (±standard deviation), 36.0 ± 9.7 y; range, 26–53 y] and 27 cancer patients (14 men, 13 women; ages: mean, 61.9 ± 13.7 y; range, 26–81 y). All samples were collected from a Japanese population. The diagnostic results and types of chemotherapies are summarized in Table 1 and Fig. 1.

3.2. Human *PIG-A* assay

In healthy volunteers, *PIG-A* MFs ranged from $0.00–5.00 \times 10^{-6}$ (mean, $2.60 \pm 1.51 \times 10^{-6}$). In contrast, the *PIG-A* MFs of cancer patients ranged from $0.00–49.67 \times 10^{-6}$. We compared the mean *PIG-A* MF of each cancer patient with that of all healthy volunteers and detected significant differences in patients 07 and 26 (Figs. 2 and S1 and Table 2).

3.3. Frequency of RETs

Among healthy volunteers, %RET ranged from 0.11–0.52% (mean, $0.31 \pm 0.12\%$). In contrast, these values ranged from 0.01–2.87% among cancer patients. We compared the values from each cancer patient with the mean value for all healthy volunteers and detected significant differences in some patients (Fig. 3). Variations in %RET are known to reflect toxicity, and recovery occurs in the bone marrow following chemotherapy. We were able to obtain blood samples over multiple time points from only one patient, patient 10. We compared the two %RET values for this patient and detected significant differences (Fig. 3).

4. Discussion

We conducted a *PIG-A* assay for human genotoxicity monitoring and analyzed ten healthy volunteers and 27 cancer patients. Although sex differences and a wide age range were observed among the healthy volunteers, we detected no significant difference between the *PIG-A* MFs (Fig. 2). Additionally, the *PIG-A* MFs of the healthy volunteers were consistent with previous reports, including the results of a rodent *Pig-a* assay; the background level of *Pig-a*/*PIG-A* MFs was expected to be approximately 0–10 per mil-