Title: A quantitative analysis of genomic instability in lymphoid neoplasms

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Abstract

It has been proposed that hypermutability is necessary to account for the high frequency of mutations in cancer. However, the mutation rate (μ) is difficult to measure directly, and increased cell turnover or selection could provide an alternative explanation. We recently developed an assay for μ using *PIG-A* as a sentinel gene and estimated that its average value is 10.6 x 10^{-7} mutations per cell division in normal B-lymphoblastoid cell lines (BLCLs). Here we determine that μ is elevated 10 fold, 50 fold, and >150 fold in cell lines derived from T cell acute lymphoblastic leukemia (ALL), mantle cell lymphoma, and follicular lymphoma in transformed phase, respectively, whereas μ was in the normal range in a marginal zone lymphoma-derived cell line. These data support the model of hypermutability in malignancy and provide a robust way to quantitate μ in individual malignancies.

Introduction

A model sentinel gene to measure spontaneous somatic mutations must be non-essential for growth or viability, and the mutant phenotype must be detectable among a vastly larger population of normal cells. Since a single loss of function mutation would be complemented by the wild type allele on the homologous chromosome, most autosomal genes are not suitable for this purpose. However, due to hemizygosity in males and X-inactivation in females, a single mutation is sufficient to inactivate X-linked genes. For example, among lymphocytes in normal adults, resistance to 6-thioguanine occurs as a consequence of a spontaneous inactivating mutation in *HPRT* (Xq26-q27.2), at a frequency (*f*) of ~ 2 - 10 per million. [1]

While *f* represents the proportion of mutants within a population, the mutation rate (μ) represents the probability of a *new* mutation per cell division. Indeed, if there is no selection, *f* will increase over time, such that $\Delta f = \mu \times \Delta d$, where *d* represents number of cell divisions. Using *HPRT,* by analysis of *f* values for populations of different ages and estimates for Δd, it has been proposed that μ is ~8.4×10^{-7} per cell division[2]. Similarly, based on population variance in *f* values for the *GPA* locus[3], μ has been estimated to be $2 - 4 \times 10^{-7}$. Given such data, Loeb[4] proposed that hypermutability would be essential for carcinogenesis, if the number of mutations required for malignancy (*n*) is >2 . It was subsequently shown that *n* is likely > 5, and it may be much higher[5, 6].

In support of hypermutability, a great increase in the frequency of an intronic p53 mutation has been demonstrated in diverse tumor types[7]. While an increase in *f* suggests

an elevation in μ, selection or an elevation in *d* could have the same effect. Measurements of μ in tumors by fluctuation analysis[8] have suggested hypermutability, but this technique has not been universally accepted for human tissue culture[9]. Others have calculated μ by the formula $\Delta f = \mu \times \Delta d$, after depletion of pre-existing *HPRT* mutants using hypoxanthine-aminopterin-thymidine (HAT)[10]. Whereas colon cancers have exhibited an elevated μ[11], results have been variable for hematologic malignancies[12], which some have attributed to metabolic cooperation[13,14].

We recently developed a robust measurement of μ in B-lymphoblastoid cell lines (BLCLs) using *PIG-A* (Xp22.1) as a sentinel[15]. *PIG-A* encodes an enzyme subunit involved in an early step in the synthesis of Glycosylphosphatidylinositol (GPI), such that mutants are unable to express any GPI –linked proteins (e.g. CD48, CD52, CD55, CD59) on their surface[16]. Acquired somatic *PIG-A* mutations are the hallmark of Paroxysmal Nocturnal Hemoglobinuria (PNH), and a broad spectrum of mutations can produce the GPI (-) phenotype in this condition. There is evidence that *PIG-A* mutations are growth neutral in humans, mice, and *in vitro*[17,15,18]. As for *HPRT*, normal individuals harbor rare cells with *PIG-A* mutations[19], which can also arise *in vitro* in cell lines[15,20]. By cytometry, pre-existing mutants can be depleted, and a large number of cells can be analyzed to determine both *f* and μ[15]. Here we have performed direct analysis of μ in lymphoid malignancies.

Methods

HBL2 (Mantle Cell lymphoma), PR1 (transformed follicular lymphoma), SSK41 (marginal zone lymphoma)[21], and Jurkat (T-cell ALL) were grown in RPMI, 15% FCS, non-essential amino acids, L-glutamine, and Penicillin-Streptomycin. A GPI (-) BLCL from a patient with PNH served as a control. μ in the *PIG-A* gene was determined as described[15,22]. Cells were first stained with an anti-CD59 antibody, and the upper 50th percentile of the distribution curve was collected by sorting, to deplete pre-existing mutants. The collected GPI (+) cells were counted by trypan blue exclusion and then expanded in culture. To determine *f,* cells were first stained on ice with a mixture of antibodies specific for multiple GPI-linked proteins (e.g. CD48, CD52, CD55, CD59, Serotec), followed by Rabbit anti-mouse-PE (DAKO-Cytomation), then a FITC-conjugated antibody specific for a transmembrane protein, either HLA-DR (BD Pharmingen) or CD45 (Serotec). Antibodies were added to pelleted cells, which were resuspended, repelleted, and again resuspended, to ensure that all cells came in contact with the antibody. Cells were washed twice between incubations. Flow cytometric analysis was performed on a FACScan using Cellquest and FlowJo software. Live cells were identified by FSC/SSC, exclusion of propidium iodide, and expression of transmembrane proteins. *f* was calculated by # events in the lower right quadrant divided by total # events analyzed. *d*, representing population doublings, was calculated by live cell counts before and after expansion [$d = LOG_2$ (# cells post-expansion / # cells pre-expansion)]. The mutation rate was calculated by the formula $\mu = f / d$. FLAER-Alexa 488 was obtained from Protox Biotech (Victoria, BC, Canada).

Results and Discussion

Preliminary studies demonstrated that HBL2 and PR1 had substantial GPI (-) populations; they were therefore cloned by limiting dilution and GPI (+) clones selected for further analysis. In all four malignant cell lines, analysis after expansion *in vitro* after sorting revealed that the vast majority expressed GPI-linked proteins as well as transmembrane proteins, registering in the upper right quadrant (figure 1B-E). A much smaller but distinct population of cells that expressed transmembrane proteins but not the GPI-linked proteins registered in the lower right quadrant (figure 1B-E) and appeared similar to a control *PIG-A (-)* BLCL from a patient with PNH (figure 1A). Because pre-existing mutants had been eliminated prior to expansion, these cells must have arisen due to new mutations occurring *in vitro*. We also stained cells as above except that we pre-incubated with the FLAER-Alexa 488 reagent— which binds to the GPI structure directly—and eliminated the last staining step. As expected, the spontaneously arising GPI (-) populations did not bind to FLAER, while the GPI (+) cells were FLAER (+) (figure 1F).

We divided the number of GPI (-) cells by the total number of cells analyzed to calculate *f*, which was greatly increased for PR1, HBL2, and Jurkat, in comparison with our previous data on normal BLCLs[15,22] and *ex vivo* cell populations[19](Table 1). We estimated μ to be 1726 x 10^{-7} per cell division for PR1, 562 x 10^{-7} for HBL2, 120 x 10^{-7} for Jurkat, and 11 x 10^{-7} for SSK41. In comparison, we previously calculated that μ in BLCLs from normal individuals ranges from 2.4 to 29.6 x 10^{-7} mutations per cell division[15,22]. We conclude that μ is in the normal range for SSK41, which, interestingly,

is derived from an indolent neoplasm and demonstrates low tumorigenicity in a nude mouse xenotransplantation model (unpublished observations). A normal μ value could be explained by a small *n* value or *in vivo* mutagen exposure.

In granulocytes,[19] lymphocytes,[23, 24] marrow precursors,[25] and BLCLs[15] from normal individuals, spontaneously arising populations of GPI (-) cells have been shown to harbor a broad spectrum of *PIG-A* mutations. This assay might also detect epigenetic silencing or large deletions. Hypermutability could well explain the frequent emergence of chemotherapy drug resistance, and indeed, *PIG-A* mutations could confer resistance to Campath—a therapeutic anti-CD52 antibody[24]. We believe that our measurement of μ using *PIG-A* reflects hypermutability in general and could contribute to resistance to other chemotherapy drugs. Ultimately we believe that it may be possible to quantitate genomic instability in individual malignancies with this approach.

Legend

FIGURE 1. GPI (-) populations arise spontaneously *in vitro* in malignant cell lines. Flow cytometry density dot plots. (**A**) Mixture of *PIG-A* (+) cells and *PIG-A* (-) BLCLs from a patient with PNH. The *PIG-A* (-) population does not express GPI-linked proteins but does express the transmembrane protein HLA-DR. (**B-E**) Cells were initially sorted to eliminate pre-existing mutants, and analyzed after expansion *in vitro*. The normal population expresses GPI-linked proteins and transmembrane proteins, registering in the upper right quadrant. Spontaneously arising mutants register in the lower right quadrant and appear similar to the *PIG-A* (-) cells from the patient with PNH. f is calculated as the number of events in the lower right quadrant divided by the total number events analyzed (see table). (**B**) Cell line PR1, follicular lymphoma in transformed phase. (**C**) HBL-2, mantle cell lymphoma. (**D**) SSK41, marginal zone lymphoma. (**E**) Jurkat, T-cell ALL. (**F**) Jurkat, as in (**E**), but with FLAER-Alexa-488 substituted for anti-CD45-FITC. Here the GPI (-) population registers in the lower left quadrant because it lacks surface expression of the GPI-linked proteins and does not take up the FLAER reagent, which binds to GPI directly. A similar pattern was seen for HBL2 and PR1 (data not shown).

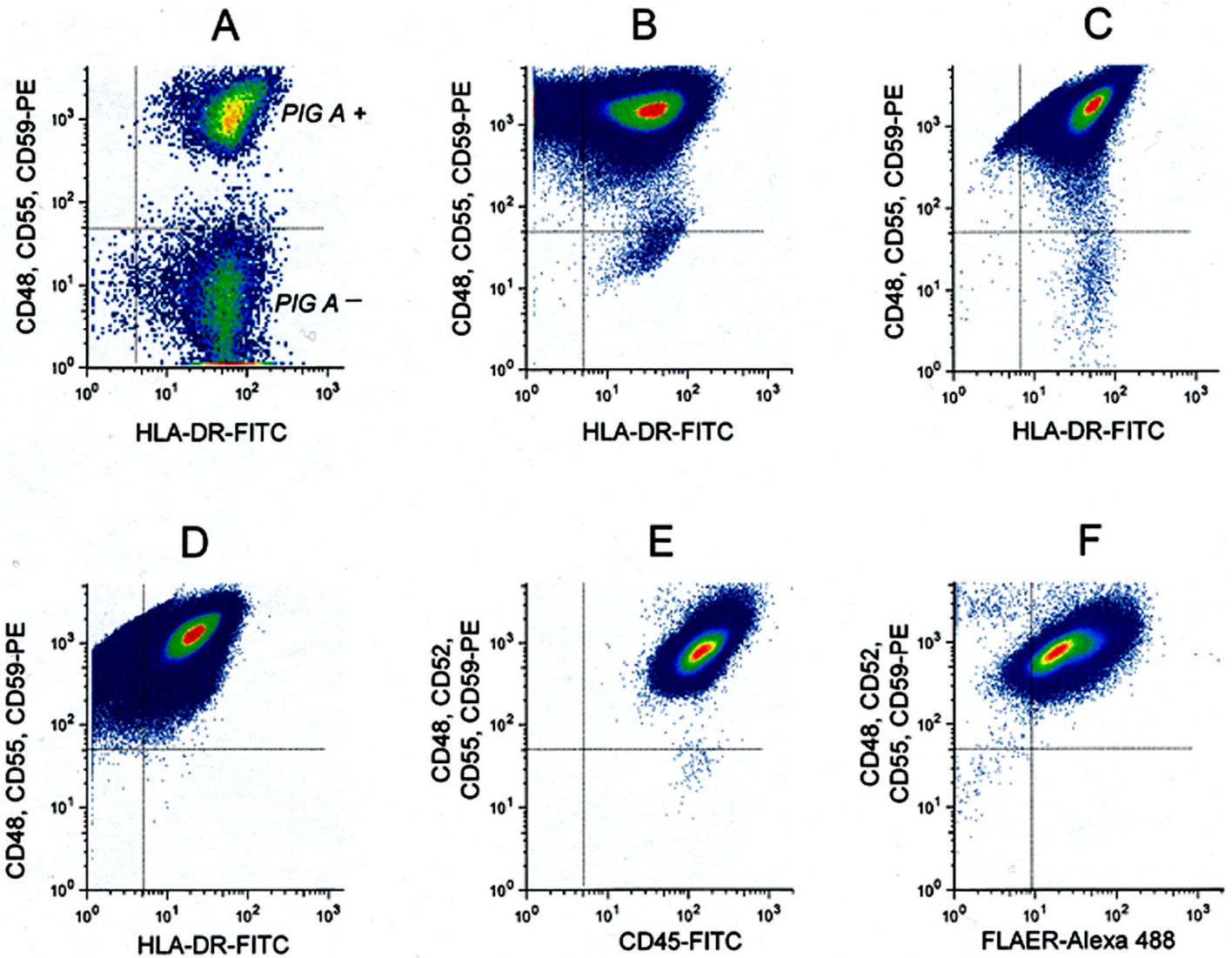
A
B
C
D
E
F
CD48, CD55, CD59-PE
CD48, CD52, CD55, CD59-PE
HLA-DR-FITC
CD45-FITC
FLAER-Alexa 488
PIG A +
PIG A −

Figure 1

Table 1. Determination of *f* and μ

Cell line	Starting # cells (x 10^{-6})*	# days in culture¶	# cell divisions, *d*¶¶	# GPI(–) cells**	Total # cells analyzed**	Mutant frequency *f* (x 10^6)‡	Mutation rate μ (x 10^7)‡‡
PR1	.73	21	6.45	1171	1,052,108	1113	1726
HBL2	3.3	21	5.41	635	2,086,003	304	562
JURKAT	1.14	41	10.15	76	624,373	121	120
SSK41	1.6	25	8.27	21	2,346,400	9	11

* Number of GPI (+) cells collected by sorting, in millions
¶ Number of days in culture between sorting and analysis
¶¶ Number of cell divisions occurring in culture between sorting and analysis
** Data shown graphically in figure 1
‡ *f* = # GPI (-) cells / # total cells analyzed. Results expressed as mutants per 10^6 cells.
‡‡ Mutation rate calculated by the formula $\mu = f / d$. Results expressed as mutations per 10^7 cell divisions

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