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The rate of spontaneous mutations in human myeloid cells

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Abstract

The mutation rate (μ) is likely to be a key parameter in leukemogenesis, but historically, it has been difficult to measure in humans. The *PIG-A* gene has some advantages for the detection of spontaneous mutations because it is X-linked, and therefore only one mutation is required to disrupt its function. Furthermore, the *PIG-A*-null phenotype is readily detected by flow cytometry. Using *PIG-A*, we have now provided the first *in vitro* measurement of μ in myeloid cells, using cultures of CD34+ cells that are transduced with either the AML-ETO or the MLL-AF9 fusion genes and expanded with cytokines. For the AML-ETO cultures, the median μ value was $\sim 9.4 \times 10^{-7}$ (range ~ 3.6 to 23×10^{-7}) per cell division. In contrast, few spontaneous mutations were observed in the MLL-AF9 cultures. Knockdown of p53 or introduction of mutant *NRAS* or *FLT3* alleles did not have much of an effect on μ . Based on these data, we provide a model to predict whether hypermutability must occur in the process of leukemogenesis.

Keywords

Mutation Rate; Spontaneous Somatic Mutations; Human Myeloid Cultures; *PIG-A* gene; GPI-linked proteins; Myeloid Leukemia

1. Introduction

In a large population, the mean frequency (f_x) of cells harboring a mutation in any gene “x” will be the product of the mutation rate per cell division for that gene (μ_x) and the number of cell divisions (d) that have occurred to produce that population. If n different mutations are required to produce a malignancy, the probability (P) of these all occurring in the same cell

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by chance will be $f_1 \times f_2 \times f_3 \dots f_n$. By substitution, this probability can be expressed as $P = (\mu_1 d)(\mu_2 d)(\mu_3 d) \dots (\mu_n d)$, and using $\bar{\mu}$ as the geometric mean of $\mu_1, \mu_2, \mu_3, \dots, \mu_n$, this can be reduced to $P = (\bar{\mu} d)^n$. This model rests on the assumption that the mutations are, individually, growth neutral, that they are independent of each other, and that the rates of mutation are constant. As long as n is >1 , this predicts a greater than linear relationship between cancer and d -and presumably age, because d must increase over time. In fact, the incidence of most types of cancers does increase much more than linearly with age [1-3].

While μ is predicted to have an equally strong effect as d , this parameter has been very difficult to measure historically, due to the stringent requirements for a mutation reporter (sentinel) gene. Indeed, the vast majority of genes are not candidates at all, due to the diploid genome, which allows the effect of a single mutation to be complemented by the unmutated allele on the homologous chromosome. Even for the X-chromosome, where this would not be an issue, most mutations would either produce no detectable phenotype, or conversely, could be lethal. *HPRT* (Xq26-q27.2) represents a notable exception, since mutations in this gene disrupt a non-essential function—the purine salvage pathway, conferring resistance to 6-thioguanine [4]. Using *HPRT*, it is possible to quantitate f in circulating lymphocytes [4-6], and Seshadri *et al* [7] have used fluctuation analysis to arrive at estimates of the human mutation rate using *HPRT*. However, translation of this technique from microorganisms to human tissue cell culture has been questioned [8] because of the slower rate and lower density at which mammalian cells grow. It may be possible to deplete pre-existing *HPRT* mutants using HAT media, permitting an estimate of μ from the slope of f as a function of cell divisions in mammalian cells [9]. However, it requires 2 weeks for this pre-incubation step, which may be less effective if metabolic cooperation occurs between cells [10]. By taking the slope of median f values in large population studies as a function of age, Green *et al* have estimated that μ in lymphocytes in the human population is approximately 8×10^{-7} per cell division [11]. This approach, however, does not allow an estimate of μ in individual samples.

We have previously shown that *PIG-A* [12] is also suitable as a mutation reporter gene [13], [14,15]. Expanded populations of hematopoietic stem cells with acquired somatic mutations in *PIG-A* are the hallmark of paroxysmal nocturnal hemoglobinuria (PNH), and from this condition, it is known that a very broad spectrum of mutations can inactivate this gene. It has also been shown that apart from PNH, mutations in *PIG-A* are growth neutral in almost all circumstances-- *in vitro* and *in vivo*, in humans and animals [14,16-18]. *PIG-A* encodes an enzyme subunit necessary for the production of the glycosylphosphatidylinositol (GPI) anchor, which is required for linking a subset of proteins to the cell membrane. Like *HPRT*, *PIG-A* is on the X-chromosome, and it has the further advantage that its associated mutant phenotype entails loss of all GPI- anchored membrane proteins and is thus detected by flow cytometry. We have reported that in granulocytes from normal donors [13], the mean frequency of spontaneously arising GPI (-) cells is about 22×10^{-6} . We have detected *PIG-A* mutations in these cells, and others have confirmed these finding in bone marrow cultures from normal individuals [19].

A further advantage of using *PIG-A* as a mutation reporter gene is that pre-existing mutants can be eliminated by flow cytometry, which allows the measurement of μ as well as f . In B-

lymphoblastoid cell lines (BLCLs) from normal individuals, we have reported that μ ranges from about 2.5 to 30×10^{-7} mutations per cell division [14,15], which is similar to the results provided by Green et al [11], and it is elevated in a significant proportion of malignant cell lines [20]. Recently, we have shown an elevation in f in approximately 50% of *ex vivo* blast samples derived from children with ALL [21].

While these data pertain to lymphoid cells, the establishment of a technique for expanding populations of human CD34+ cells using fusion oncoproteins associated with leukemia represented the first opportunity to measure μ in myeloid cells [22-26]. The AML-ETO fusion gene transforms murine hematopoietic bone marrow cells in cooperation with secondary mutagenic events [27,28]; it induces an increase in AP-1 dependent transcription [29], and it acts in a dominant-negative manner by interfering with AML1 dependent transcriptional activation [30]. AML-ETO also reduces the transcription of C/EBP α [31] and directly antagonizes the PU.1 [32] and the MEF transcription factors [33]. The net effect is to increase proliferation and survival and to block terminal differentiation [25]. AML-ETO transduced human cord blood cells maintain CD34 expression and multilineage potential, and will proliferate for about 8 months *in vitro* [23,34], though they can not form a leukemia when transferred to an immunodeficient mouse.

The MLL-AF9 fusion gene can also transform murine hematopoietic stem cells as well as committed progenitors using a bone marrow retroviral transduction approach [35], and transgenic mice expressing MLL-AF9 will develop myeloid leukemia with a minimal latency of 4 months [36]. Human CD34+ cord blood cells transduced with MLL-AF9 express telomerase, expand indefinitely *in vitro* in the presence of FLT3 ligand, and can form a myeloid leukemia with a latency of 5-10 weeks in immunocompromised mice [26]. MLL-AF9 may transform cells by upregulating HOXA genes, which may be mediated by H3K4 and H3K79 methylation [37].

Here we have measured μ in myeloid cultures of human CD34+ cord blood cells transduced with these fusion oncogenes, allowing us to reconcile μ with the estimated number of hematopoietic stem cells, their estimated replication rate, estimates of n , and the incidence of AML.

2. Materials and Methods

2.1 Cell cultures and media for myeloid cells

Long term cultures of human myeloid cells derived from umbilical cord CD34+ cells transduced with the AML-ETO fusion gene ("AE" cell lines) were established as described [23,34]. Cells were grown in IMDM with HEPES, BIT9500 (Stem Cell Technologies), 0.1 mMolar beta mercaptoethanol, l-glutamine, and penicillin-streptomycin. IL-6, FLT3 ligand, SCF, and TPO were added at a final concentration of 20 ng/ml and IL-3 at 10 ng/ml. Cells were grown in a humidified incubator at 37°C with 5% CO₂. The original AE cell lines, derived from 4 different cord blood donors, are designated AE30, AE31, AE33, and AE42. The cell lines derived from AE30 and AE31 cultures that are described here were transduced with a retrovirus containing the HPV E6/E7 viral oncogenes (a gift from Dr. Susanne Wells and Dr. Denise Galloway [38]), the pBABEneo-p53^{DD} plasmid (which has a deletion

mutation of mouse p53 that acts in a dominant fashion and transforms human cells [39,40]), a p53 specific shRNA, or if not otherwise specified, either a scrambled shRNA or the empty LXS vector [22,38]. Knockdown of p53 levels was confirmed by Western Blot. Cell lines MA9.3 and MA9.6 were derived from CD34+ cord blood cells and transduced with the MLL-AF9 gene as described [24,26]. We also analyzed μ in the parental MA9.3 and MA9.6 cultures compared with sub-cultures that had been transduced with a construct containing a mutant NRAS^{G12D} allele or a FLT3 internal tandem duplication; in these experiments the transduced and parental cultures were grown with cytokines in IMDM media, 10% fetal bovine serum, l-glutamine, and penicillin-streptomycin.

2.2 Control cell lines

BLCLs and the AML cell line KG-1 were grown in RPMI with 15% fetal calf serum, L-glutamine, penicillin streptomycin, and nonessential amino acids. A BLCL from a normal donor served as a positive control for the expression of GPI-anchored proteins. A BLCL from a patient with PNH which initially had GPI (+) and GPI (-) populations was treated with proaerolysin (Protox Biotech, Victoria, BC), a recombinant bacterial pore-forming toxin that kills cells in a GPI-dependent manner. The surviving proaerolysin-resistant cells served as a negative control.

2.3 Analysis of f in normal granulocytes and determination of μ in BLCL's

Healthy volunteers donated samples of whole blood collected by venipuncture into heparinized tubes after signing informed consent in accordance with institutional protocols. Granulocytes were isolated and stained for flow cytometry as previously described [13] using antibodies specific for CD55 and CD59, Rabbit anti-mouse-PE, and anti-CD11b. Dead cells were excluded using propidium iodide. As a comparison for the measurement of μ in myeloid cells, BLCL's were analyzed as previously described [14,15].

2.4 Sorting to eliminate pre-existing GPI (-) cells and in vitro expansion of myeloid cultures

Determination of μ was performed as described for BLCL's [14,15], with some modifications for cells of the myeloid lineage. Cells first were stained with anti-CD59-FITC antibody (Serotec) and sorted on a Cytomation Moflow instrument. Events within the upper 50th percentile of the distribution were collected, a procedure that we have shown consistently eliminates GPI (-) cells with a purity of >98%. The collected GPI (+) cells were counted by trypan blue and returned to culture under logarithmic growth conditions. After expansion, the cells were recounted and the number of cell divisions estimated by the formula $d = \text{LOG}_2 [\text{cell number post expansion} / \text{cell number prior to expansion}]$.

2.5 Preparation of cells and analysis by flow cytometry

To prepare cells for flow cytometry, cells were first incubated at 37°C for 30 minutes with biotinylated FLAER reagent (Protox biotech, Victoria BC) at a concentration of approximately 10^{-7} Molar. FLAER is a derivative of proaerolysin that binds to cells in a GPI-dependent manner but which does not form a pore and is therefore non-toxic. The cells were then placed on ice for the remainder of the procedure and were incubated with a mixture of unconjugated murine anti-CD59 and anti-CD55 antibodies (Serotec) at a dilution

of 1:40 and 1:10 respectively. Cells were then washed twice with Hanks Buffered Saline Solution with 0.1% BSA (HBSS-BSA) to remove any biotin in the media, followed by incubation with a mixture of Streptavidin-PE (Becton Dickinson) at a dilution of 1:2.5 and R-phycoerythrin-conjugated F(ab')₂ fragment rabbit anti-mouse Ig (RAM-PE, Dako-Cytomation) at a 1:5 dilution. Cells were washed twice again in HBSS-BSA and then incubated with FITC-conjugated anti-CD45 antibody (Serotec) at a 1:10 dilution. In initial experiments, instead of anti-CD45, we used FITC conjugated-w6/32 antibody, which recognizes HLA-class I molecules. Cells were washed once again and resuspended in HBSS-BSA. Cells were passed through a 40µM filter (Falcon) prior to flow cytometry analysis on a Becton Dickson Facscan instrument. To ensure that the entire cell population came in contact with the antibodies, we added antibodies prior to resuspension of the cells, which were then briefly recentrifuged and resuspended before incubation on ice.

Live cells were identified based on forward and side scatter characteristics, expression of CD45, and by exclusion of propidium iodide, which was added at a final concentration of 0.1 to 0.15 µg/ml immediately prior to analysis. By this approach, GPI (+) cells appear in the upper right quadrant and GPI (-) cells appear in the lower right quadrant.

To standardize the instrument settings, QuantiBrite PE beads and CaliBrite beads (Becton Dickinson) were analyzed with each experiment. Voltage on the FL2 photomultiplier tube was set so that the brightest of the four QuantiBrite PE populations expressed a mean fluorescence of approximately 300. Because the number of PE molecules on each of the bead populations is provided by the manufacturer, it is possible to develop a regression curve by which the number of PE molecules bound to each cell is determined for each experiment. In order to confirm that this calibration curve would be valid for cells that also fluoresced in the FL1 channel, we mixed QuantiBrite PE beads with CaliBrite FITC beads and gated on bead doublets; likewise, we also stained GPI (+) and GPI (-) BLCLs with either anti-CD59-PE alone or anti-CD59-PE and anti-HLA-DR-FITC. With the assumption that after compensation there would be no effect of the presence of the FITC-conjugated antibody on the fluorescence or binding of the PE-conjugated antibody, we developed a modified regression curve specifically for cells that had been gated for FITC-positive events. The spontaneously arising GPI (-) population for each AML-ETO-transduced culture was generally defined as having <4% of the number of PE molecules on the surface of the cell as the parental GPI (+) population from which it arose, a cutoff that we have found useful for other types of cells as well.

We calculated f as the number of GPI (-) cells that register in the lower right quadrant divided by the total number of cells analyzed. A large number of cells were stained with the aim of collecting at least 10^6 total events, which we achieved in most cases. The mutation rate was then calculated by the formula $\mu = f / d$.

2.6 Modeling the acquisition of mutations that cause AML

Since the formula $P = (\mu d)^n$ represents the probability for an individual cell to undergo transformation, we considered that the cumulative probability of leukemia, L_p , would equal P times the number of total “at risk” cells, C , that are capable of giving rise to the malignancy. We considered that $d = AR$ where R equals rate of cell division per year, and A

equals chronological age. We therefore derived the formula $L_P = C(\mu AR)^n$. Based on epidemiological data for L_P , using our experimental values for μ as an estimate for $\underline{\mu}$, and the estimates for n described below, we solved this equation for C using different possible values for R .

We estimated n as the minimum number of mutations required to produce a cytogenetically normal AML, by derivation from L_P , the cumulative incidence of this type of leukemia as a function of age, based on the epidemiologic data of Moorman *et al* [3]. L_P was calculated by multiplying the annual incidence rate in each binned age group by the number of years in the bin. We then plotted the cumulative incidence of this subtype of AML as a function of age on a log-log scale. This is based on the approach of Armitage and Doll, which makes the assumption that R , C , and μ in the “at risk” pool of cells are constant over time. By taking the logarithm of both sides of the equation above one can derive that $\text{LOG}[L_P] = \text{constant} + n\text{LOG}[A]$. The slope of the plot of the $\text{LOG}[L_P]$ vs $\text{LOG}[A]$ can then be taken as an estimate for n .

3. Results

3.1 Demonstration of GPI (+) and GPI (-) control cells by flow cytometry

BLCL cells from a normal donor express transmembrane proteins, GPI-anchored proteins (such as CD55 and CD59), and take up the FLAER reagent (by virtue of having GPI on their surface). As expected, in an FL2 versus FL1 bi-variate dot plot, normal control BLCL cells registered in the upper right quadrant (figure 1A). Likewise, the control GPI (-) BLCL cells derived from a patient with PNH express transmembrane proteins but not CD55 or CD59, and they do not take up the FLAER reagent, and they therefore register in the lower right quadrant (figure 1B). For the AML-derived cell line KG-1, most cells expressed CD45 brightly (with a sub-population of CD45-dim cells). The vast majority of these were GPI (+) and appeared in the upper right quadrant (figure 1C). Within this cell line there is also a sub-population of spontaneously arising CD45 (+) cells with the GPI (-) phenotype, registering in the lower right quadrant, at a frequency of $\sim .0023$ (figure 1C). This value is about 2 orders of magnitude above the mutant frequency in granulocytes from normal donors, and we believe that this represents a manifestation of widespread hypermutability across the genome, including the *PIG-A* gene. This is in keeping with our previous report that a large proportion of cell lines derived from hematologic malignancies demonstrate an elevated μ value [20]; in the KG-1 cell line, this may be due to a mutation in *hOGG1* and a reduction in base excision repair [41].

3.2 Mutant frequency in normal granulocytes and AML-ETO transduced cell lines

To serve as a comparison, we demonstrated that normal adults harbor populations of spontaneously arising GPI (-) granulocytes (figure 1E,F) that appear similar to GPI (-) granulocytes from a patient with PNH (figure 1D) as we previously described [13]. We then demonstrated a similar pattern in granulocytes from an umbilical cord blood sample (figure 1G); the corresponding frequencies of GPI (-) granulocytes in these samples were 54×10^{-6} , 4.5×10^{-6} , and 4.9×10^{-6} respectively, which is consistent with our previously reported range. In an analysis of the AE30 and AE31-derived cell lines, we next demonstrated a

distinct population of spontaneously arising GPI (-) cells at a frequency of 17.8×10^{-6} and 6.7×10^{-6} respectively (figure 1H and 1I). These values are consistent with the f values corresponding to circulating granulocytes from normal donors and also to marrow progenitor cultures from normal donors reported by others [19]. This suggested that μ would not likely be affected much by the AML-ETO fusion gene, the cytokines used to grow the cells, or their undifferentiated state.

3.3 Direct Measurement of the Mutation Rate in AML-ETO and MLL-AF9 transduced cell lines

To measure μ , we next established purified GPI (+) cultures of the AE cell lines by sorting. A total of 10 cultures were established using AE cell lines derived from 4 different cord blood donors. The cells were analyzed after a median of 20 days post sorting, during which time they underwent a median of 6.1 cell divisions (Table 1). Populations of spontaneously arising GPI (-) cells were then observed in the lower right quadrant after this expansion period (figure 1J).

Using the formula $\mu = f / d$, we calculated μ values that ranged from $\sim 3.6 \times 10^{-7}$ to 23×10^{-7} mutations per cell division (table 1) with a median value of $\sim 9.4 \times 10^{-7}$. We also measured μ in two representative BLCLs from normal donors, and the values were 24.6×10^{-7} and 9.1×10^{-7} mutations per cell division. These values for μ are very close to our previous determinations [14,15] and are also very close to mathematical models of the *in vivo* mutation rate based on the *HPRT* gene [11]. CD34+ cell lines transduced with the MLL-AF9 fusion oncogene were also analyzed, and here μ was lower—below the limit of detection in one cell line and $\sim 2 \times 10^{-7}$ mutations per cell division in another cell line (figure 1L, Table 1).

3.4 Effect of p53 knockdown on μ

To predict whether decreasing p53 levels would affect spontaneous mutagenesis, the mutant frequency in subcultures of AE30 and AE31 expressing a p53 specific shRNA was estimated to be 7.5×10^{-6} and 10×10^{-6} respectively (data not shown). These values were not different from the mutant frequency of the p53-wild type cell lines from the same donors. We then analyzed μ directly in 5 subcultures of AE30, 3 of them transduced with a p53 specific shRNA construct, one with a dominant-negative p53 mutation, and one expressing the HPV E6/E7 viral oncoproteins. For these cell lines we confirmed by Western blot at least a 70% knockdown of p53. For AE30-derived cell lines, there was only a modest increase in the number of spontaneously appearing GPI (-) cells (example shown in Figure 1K, table 1) in the cells with disrupted p53. In none of these cultures was the mutation rate more than twice the value obtained from the parental AE30 cell lines. The median μ value for these 5 cultures was 10.8×10^{-7} , representing a 30% increase ($p < 0.02$, two sided Mann Whitney test), though this value is still well within the range for the AE cell lines overall. For subcultures of AE31 expressing a p53-specific shRNA, there was no appreciable difference in μ compared with AE31 expressing a scrambled construct (Table 1), despite $\sim 50\%$ knockdown of p53 expression. We also analyzed μ in Kasumi-1, an AML cell line harboring an 8;21 translocation and a known mutation in p53 [42]. Kasumi-1 therefore shares with these cell lines both the AML-ETO fusion protein and the loss of p53.

Interestingly, for Kasumi-1, μ was very similar to the data we obtained for the AE cell lines and the BLCLs from normal donors (table 1).

3.5 Effect of $NRAS^{G12D}$ and $FLT3^{ITD}$ on μ

The $FLT3^{ITD}$ is known to cooperate with MLL-AF9 in the induction of leukemia [43], and both the $FLT3^{ITD}$ mutation and $NRAS$ mutations are late cooperating mutations in the transition from myelodysplasia to AML [44]. Likewise, both $NRAS^{G12D}$ [45] and the $FLT3^{ITD}$ will confer cytokine independence upon MA9 cell lines (unpublished observations). We therefore investigated whether either of these oncogenes would increase μ in the MA9 cell lines. For the MA9.3 culture, there was no measurable effect of either oncogene on the acquisition of spontaneously arising GPI (-) cells. For the MA9.6 culture, the subculture expressing $NRAS^{G12D}$ demonstrated a μ value of 12.9×10^{-7} per cell divisions, which was higher than control MA9.6 cells but still well within our estimates of the normal mutation rate.

3.6 Modeling the acquisition of mutations that cause AML

The data points representing the log of the cumulative incidence of cytogenetically normal AML [3] as a function of the log of chronological age almost completely fell on a straight line, with a Pearson r value of 0.998. The regression line had a slope of 2.73 and an intercept of -8.548. This implies that on average, in the general population, a minimum of 2.73 independent events must occur to generate a cytogenetically normal AML. This number may be similar to estimates of the number of required driver mutations in AML based on large scale sequencing projects [46], although a high rate of cell turnover, selection, and drift may result in more mutations in advanced cases [47]. We then considered that from the data of Moorman *et al* [3], the cumulative incidence of cytogenetically normal AML at age 70 is approximately 0.00033. Given that $L_p = 0.00033$ when $A = 70$, and using $n = 2.73$, and $\mu = 12 \times 10^{-7}$ per cell division (as per our data here), the formula $L_p = C(\mu AR)^n$ was solved for C (the number of “at risk” cells) for different estimates of R (the number of annual cell divisions) in this population (figure 2, red square data points). When $n = 2.73$, it is evident that the values for C must be higher than the estimated number of human hematopoietic stem cells (14,000 to 80,000 per individual, highlighted in yellow [48,49]) unless R is at least 10-- a rate of proliferation that is considerably higher than estimates for hematopoietic stem cells [50].

We next generated additional curves, hypothetically setting $n = 2, 5$, or 8. If $n = 8$, C would have to be higher than the total number of cells in the body, commonly estimated to be $\sim 10^{13}$. This suggests that a significant elevation of the μ values above our estimate of 12×10^{-7} must occur to produce any leukemic cell population that requires 8 or more mutations. If $n = 5$ it would be possible for AML to arise from within a population close to the total number of marrow cells, about 1.5×10^{12} cells [48], provided that their annual rate of cell division is sufficiently high. Only if $n = 2$ would the model be readily consistent with pathogenic mutations occurring at a low mutation rate within an “at risk” number of cells [48] and an annual rate of cell division that is expected of hematopoietic stem cells [50]. This can be seen in that only the curve corresponding to $n = 2$ traverses through the highlighted region at low values for R .

4. Discussion

4.1 Considerations related to the use of *PIG-A* as a reporter gene

The advantages of *PIG-A* as reporter gene include the fact that it is X-linked, that the associated mutant phenotype is detectable by flow cytometry, that multiple reagents can be used simultaneously to confirm the phenotype, and that sorting will allow an estimate of μ as well as f without the need for fluctuation analysis or limiting dilution cloning [14,15]. A further advantage is that there is evidence *in vitro* and *in vivo*, in humans [16] and animals [17], that *PIG-A* mutations are growth-neutral in almost all circumstances, and that a very broad spectrum of mutations across the coding region [51], including very large deletions [52], can inactivate the gene. In theory, transcriptional silencing of *PIG-A* [or any of the other genes involved in GPI-anchor biosynthesis or trafficking] could produce the *PIG-A* null phenotype, and therefore, this assay could detect epigenetic instability as well. Of note, the coding sequence of *PIG-A* has 59 sites where a T→G transversion (a consequence of oxidative damage) could produce a stop codon, and there are two CpGA codons where a spontaneous deamination of methylcytosine could produce a stop codon. However, we can predict that gene duplications, translocations of an intact gene, or loss of heterozygosity events resulting from gene conversion or mitotic recombination will not be detected. It is possible that inherited polymorphisms, such as reported for *APC* [53] and *JAK2* [54] will predispose to somatic mutations at those loci in cis but not in reporter genes. Mutational showers, (kataegis), perhaps accounting for 1% of spontaneous mutations in reporter genes in transgenic mice, have been observed in human malignancy [55-60]: it is not known whether *PIG-A* is susceptible to kataegis. In spite of these caveats, we believe that *PIG-A* represents quite an appropriate system for the mutations that inactivate tumor suppressor genes, and many of the mutations that activate oncogenes as well.

4.2 Comparison with previous data on f in myeloid cells

There have been only a few previous studies of spontaneous mutagenesis in cells of the myeloid lineage. Using 6-thioguanine resistance in colonies growing in methylcellulose as a marker for spontaneous *HPRT* mutations, Grant *et al.* have determined that the mutant frequency for CD34+ cells is of the order of 1.8 to 9.2×10^{-6} for neonatal cord blood samples, and 1.4 to 8.6×10^{-6} for adult bone marrow samples [61]. We previously identified *PIG-A* mutations in circulating granulocytes from normal donors [13] and showed by flow cytometry that the mean f value is $\sim 22 \times 10^{-6}$. Hu *et al.* performed similar studies of *in vitro* colonies from marrow from normal donors and showed that f was $\sim 14 \times 10^{-6}$ and somewhat higher for mobilized circulating progenitors [19]. Among red cells from adults, the frequency of spontaneous glycophorin A allele loss is ~ 7 to 10×10^{-6} [4,62], and we have determined that the frequency of red cells with spontaneous acquisition of the *XK*-mutant (McLeod) phenotype is $\sim 39 \times 10^{-6}$ in adults and $\sim 9 \times 10^{-6}$ in neonates [63]. Our initial estimate of f in CD34+ AE cells was ~ 6.7 to 17.8×10^{-6} , which was in the range of these prior estimates.

4.3 Comparison with previous data on μ in myeloid cells

There is even less comparative data for μ in myeloid cells, though our estimates matched our previous data in B-lymphoblastoid cell lines [14,15] and previous mathematical models

pertaining to lymphocytes [11]. Welch *et al* [46] have recently estimated the rate of mutation in exons in stem cells based on large scale sequencing of hematopoietic colonies from normal donors of different ages. They estimated that there would 0.13 new somatic mutations per year of life and quoted estimates of one division per stem cell every 25 to 50 weeks. Assuming that there are 2×10^4 genes and that *PIG-A* is of average length, and correcting for the fact that sequencing can detect mutations on either of 2 alleles, the corresponding mutation rate based on their data would be between 16×10^{-7} and 31×10^{-7} in our assay. Of note, this estimate is very close to our experimental *in vitro* data.

4.4 Reasons why *in vitro* estimates could differ from *in vivo* mutation rates

It should be said that our estimates could differ from the *in vivo* mutation rate in myeloid progenitor cells for several reasons: (i) our culture media uses beta-mercaptoethanol rather than physiologic anti-oxidants; (ii) *in vivo*, mature myeloid cells are capable of an oxidative burst, a potential source of free radicals; (iii) conversely, the normal marrow environment is relatively hypoxic and resides in a radio-shielded niche; (iv) as mentioned above, gene duplications will not be detected; (v) in some malignancies, multiple oncogenic mutations may occur in a short time due to transient hypermutability, whereas our assay is designed to measure a basal mutation rate. In spite of these caveats, we believe that our estimates for μ are a good approximation of the probability of many of the important mutations in leukemogenesis.

4.5 Effect of oncogenes on the mutation rate

We cannot completely rule out the possibility that in this study, the introduction of a fusion oncoprotein (or growth under cytokine stimulation) affects μ . There is a precedent for this, in that the BCR-ABL fusion protein induces genomic instability through an effect on oxidative free radicals [64]. However, transgenic AML-ETO mice do not develop leukemia without mutagen exposure [28], suggesting that any effect of the AML-ETO fusion on spontaneous mutagenesis might be small. From one of our laboratories it has been reported that AML-ETO reduces the levels of certain repair enzymes--including FANCA, BRCA2, ATM, and hOGG1-- and increases the level of phosphorylated histone γ H2A.x and p53 [22,65]. Furthermore, the frequency of reporter lacZ mutations in bone marrow cells expressing AML-ETO was increased two-fold relative to control cells, though some of this effect might be due to an increased number of cell divisions rather than an increase in μ [22]. Based on this and our current data, we believe that any effect of AML-ETO itself on μ is likely to be subtle. Interestingly, we found an even lower μ in CD34+ cells transduced with the MLL-AF9 fusion gene, suggesting that this fusion oncoprotein also does not induce hypermutability. A low μ in these cells is consistent with recent data showing that lymphoid leukemias harboring an MLL fusion gene often carry only one additional mutation [66]. The expression of activated *NRAS* and *FLT3* oncogenes did not serve to elevate μ in cells with an MLL-AF9 fusion; this suggested that leukemogenesis would be possible without genomic instability. This conclusion is also supported by our mathematical model for the case where $n = 2$, and is supported by our data showing a low μ for the Kasumi-1 cell line. It should be noted, however, that even if a specific oncogene does not increase the mutation rate, it could serve to increase f simply by increasing d .

DNA damage, acting through ATM and ATR, results in phosphorylation of p53, which in turn plays a role in the regulation of nucleotide excision repair [67] and base excision repair [68] in addition to cell cycle arrest and apoptosis. However, it has been difficult to demonstrate an effect of p53 deficiency on spontaneous mutations in mice using a lacI reporter transgene [69,70] or the endogenous *Hprt* gene [71], or in human cells using *HPRT* [72]. Increases in the frequency of DNA damage-induced mutations have been seen in p53 null cells compared with p53 wild-type cells, but the magnitude of the effects have been small [69-71]. We found that in absolute terms, the effect of p53 knockdown on spontaneous mutations in the AE cells was small. Likewise, for the Kasumi-1 cell line, which harbors a p53 mutation [42], we did not demonstrate an increase in μ compared with normal B-lymphoblastoid cell lines. Our findings, then, would be consistent with work based on mice with inducible p53, suggesting that p53 is more important in eliminating cells that have sustained an activating mutation in an oncogene than in preventing mutations [73]. Since the mutations in *PIG-A* that we are observing are presumably not oncogenic, it may not be surprising that p53 plays little or no role in eliminating or preventing them.

4.6 Hypermutability and the number of mutations required for leukemogenesis

Lawrence Loeb has proposed that hypermutability may be required for the development of cancer in a multi-hit model [74]. In this model the number of required mutations is a critical question, and there are two ways to estimate this number: the epidemiologic approach of Armitage and Doll [75], and large scale sequencing projects. Our analysis based on the former approach suggests that the number of required mutations for a cytogenetically normal AML would be, on average, 2.73, whereas the data provided by Welch *et al* [46] suggest that almost all AML samples have at least 2 driver mutations, and that 63% of samples have at least 4 “likely” driver mutations. Based on these numbers and the model that we have provided (figure 2) it is possible that hypermutability would be required in some cases of AML, but not in others, as our recent data in ALL has suggested [21].

5. Conclusion

Here we have provided the first *in vitro* data on the rate of spontaneous somatic inactivating mutations in human myeloid cells, and this parameter seems to be of the order of 3.6 to 23×10^{-7} . The influence of stimulatory cytokines, the introduction of 2 specific fusion oncoproteins, and the introduction of cooperating mutations in p53, *NRAS*, and *FLT3* did not serve to increase the rate of mutation above that which we previously measured in non malignant human lymphoid cells, or which has been derived from mathematical models. Based on the experimental data here, hypermutability would probably have to occur as an early event to produce a leukemia that required 5 or more mutations, but not if only 2 mutations were required.

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Abbreviations

PNH	Paroxysmal nocturnal hemoglobinuria
GPI	Glycosylphosphatidylinositol
BLCLs	B-lymphoblastoid cell lines
AML	Acute myelogenous leukemia

Highlights

- We provide the first measurement of the mutation rate (μ) in human myeloid cells
- μ is measured to be 3.6 to 23×10^{-7} per cell division
- The AML-ETO and MLL-AF9 fusions do not seem to increase μ
- Cooperating mutations in *NRAS*, *FLT3* and *p53* not seem to increase μ
- Hypermutability may be required to explain leukemogenesis

Figure 1.

Bivariate pseudocolor dot plot analysis demonstrating GPI (+) and GPI (-) cells. **(A)** BLCL from a normal donor. These cells express GPI-anchored surface proteins such as CD55 and CD59 and bind to the FLAER reagent and also express transmembrane proteins such as CD45; **(B)** Control GPI (-) BLCL cells from a patient with PNH do not express CD55 or CD59 and do not take up the FLAER reagent but do express transmembrane proteins; **(C)** KG-1, an AML-derived cell line. There is a large subpopulation of spontaneously arising cells with the GPI (-) phenotype (lower right quadrant); **(D)** Granulocytes from a patient with PNH, which do not express CD55 or CD59 but do express transmembrane proteins such as CD11b; **(E and F)** Granulocytes from two normal donors. The vast majority of the cells are GPI (+) and appear in the upper right quadrant, and a small population of spontaneously arising GPI (-) cells are seen in the lower right quadrant. The mutant frequency is 54×10^{-6} and 4.5×10^{-6} respectively, representing the high and low end of our previously reported range in normal donors. **(G)** Granulocytes from an umbilical cord blood sample. As in the adult samples, the vast majority of the cells are GPI (+) and appear in the upper right quadrant, and a small population of spontaneously arising GPI (-) cells are seen in the lower right quadrant at a frequency of 4.9×10^{-6} . **(H and I)** AE30 and AE31-derived

myeloid cell cultures. As for mature granulocytes, the vast majority are GPI (+) and there is a small population of spontaneously arising GPI (-) cells at a frequency of 17.8×10^{-6} and 6.7×10^{-6} respectively (**J**) AE30-derived myeloid cultures, shown after expansion after sorting to eliminate pre-existing GPI (-) cells. New GPI (-) cells have spontaneously arisen in culture and appear in the lower right quadrant (see Table 1 for calculations of μ); (**K**) AE30-derived myeloid cells with an shRNA targeting p53, analyzed after expansion after sorting. f and μ are only modestly higher in this example than for the AE30-derived cell line with normal p53, shown in panel J. (**L**) CD34+ cell line transduced with the MLL-AF9 fusion gene, analyzed after expansion after sorting. This cell line has very low f and μ values.

Figure 2.

The number of stem or progenitor cells (C) that must be capable of giving rise to AML (shown on a log scale) is calculated for different estimates of their annual rate of cell replication (R) based on different estimates of n . The solid lines represent the calculations for C and R based on the mean μ value for the AE cell lines ($\sim 12 \times 10^{-7}$ per cell division), and the dotted lines represent the C and R values based on the lower and upper range of the calculated μ values for the AE cell lines (3.6×10^{-7} and 23×10^{-7}). The yellow highlighted region indicates the estimated number of hematopoietic stem cells (14,000 to 80,000 [1,2]). If $n = 8$, C is greater than the number of cells in the body, indicating that hypermutability must occur at some point in the process of malignant transformation. Only in the case where

$n = 2$ is it possible that AML could arise from within a true hematopoietic stem cell without any elevation in μ .

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Table 1

Determination of μ in myeloid cell cultures

Cell line	Starting # cells (in millions)*	# days in culture [†]	# cell divisions, d^{eff}	# GPI(-) cells	Total # cells analyzed	Mutant frequency (μ) per 106cells [‡]	Mutation rate (μ) per 107 cell divisions ^{§§}
<i>AE lines p53 -wt</i>							
AE30	0.58	20	7.08	6	1,654,792	3.63	5.12
AE30.1-culture 1	2.25	51	8.24	8	1,135,922	7.04	8.55
AE30.1-culture 2	2.25	22	5.59	4	833,079	4.80	8.59
AE30.1-culture 3	1.43	20	6.02	7	1,436,441	4.88	8.09
AE31- culture 1	1.13	20	6.86	16	1,230,982	13.00	18.95
AE31- culture 2	3	22	5.13	9	1,098,688	8.19	15.97
AE 33	2.02	22	5.06	14	1,204,729	11.62	22.97
AE42 -replicate 1	1.56	19	6.17	6	960,811	6.24	10.12
AE42 -replicate 2	1.56	19	6.23	2	905,293	2.21	3.55
AE42 -replicate 3	1.56	19	6.00	8	935,713	8.55	14.25
<i>AE lines-- p53 inactivation</i>							
AE30.1 p53 shRNA-1**	2.9	51	8.67	9	1,108,130	8.12	9.38
AE30.1 p53 shRNA-2**	2.9	22	5.26	7	1,175,898	5.95	11.32
AE30.1 p53 shRNA-3**	0.61	20	7.40	11	1,589,571	6.92	9.35
AE30 E6/E7**	0.74	20	7.02	11	1,452,625	7.57	10.79
AE30.1 p53 ^{DD} **	1.93	20	5.87	12	1,282,579	9.36	15.94
AE31 p53 shRNA-1**	1.66	20	5.87	23	1,452,135	15.83	26.98
AE31 p53 shRNA-2**	3.0	22	5.18	5	1,006,182	4.97	9.59
<i>MLL-AF9 lines</i>							
MA 9.3- culture 1	2.44	22	5.04	1	968,822	1.03	2.05
MA 9.3- culture 2	1.9	22	3.89	0	1,439,777	0	0
MA 9.3 NRAS G12D ^{§§}	1.51	22	4.93	0	617,200	0	0
MA 9.3 FLT3 ITD ^{§§}	2.33	22	4.76	1	1,145,961	0.87	1.83

Cell line	Starting # cells (in millions)*	# days in culture [†]	# cell divisions, d ^{††}	# GPI(-) cells	Total # cells analyzed	Mutant frequency (f) per 10 ⁶ cells [‡]	Mutation rate (μ) per 10 ⁷ cell divisions ^{‡‡}
MA 9.6-culture 1	2.06	22	3.38	0	489,749	0	0
MA 9.6-culture 2	2.44	51	7.79	0	1,554,049	0	0
MA 9.6-culture 3	2.14	22	4.72	0	1,621,185	0	0
MA 9.6-culture 4	2.91	22	5.51	2	3,090,407	0.65	1.17
MA 9.6 NRAS G12D ^{§§}	1.96	22	4.32	17	3,050,299	5.57	12.90
<i>Kasumi-1</i>							
Kasumi-1-culture 1	4.5	26	2.96	2	699,150	2.86	9.66
Kasumi-1-culture 2	3.0	20	3.86	8	782,354	10.2	26.49
<i>Normal B-lymphoblastoid cell lines</i>							
Normal Donor 1	2.07	21	4.6	21	1,852,194	11.3	24.65
Normal Donor 2	1.09	20	6.56	7	1,170,152	5.98	9.12

* 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

[‡] Number of cells in the upper right quadrant plus number of cells in the lower right quadrant

^{‡‡} f = number of GPI (-) cells divided by total number of cells analyzed. Results expressed as mutants per 10⁶ cells.

[§] Mutation rate calculated by the formula $\mu = f$ divided by d . Results expressed as mutations per 10⁷ cell divisions Each line in the table represents the results obtained from a single culture.

^{§§} indicates cell lines with introduction of constructs to interfere with p53.

^{§§§} indicates cell lines with constructs introducing an activated form of the oncogene listed.