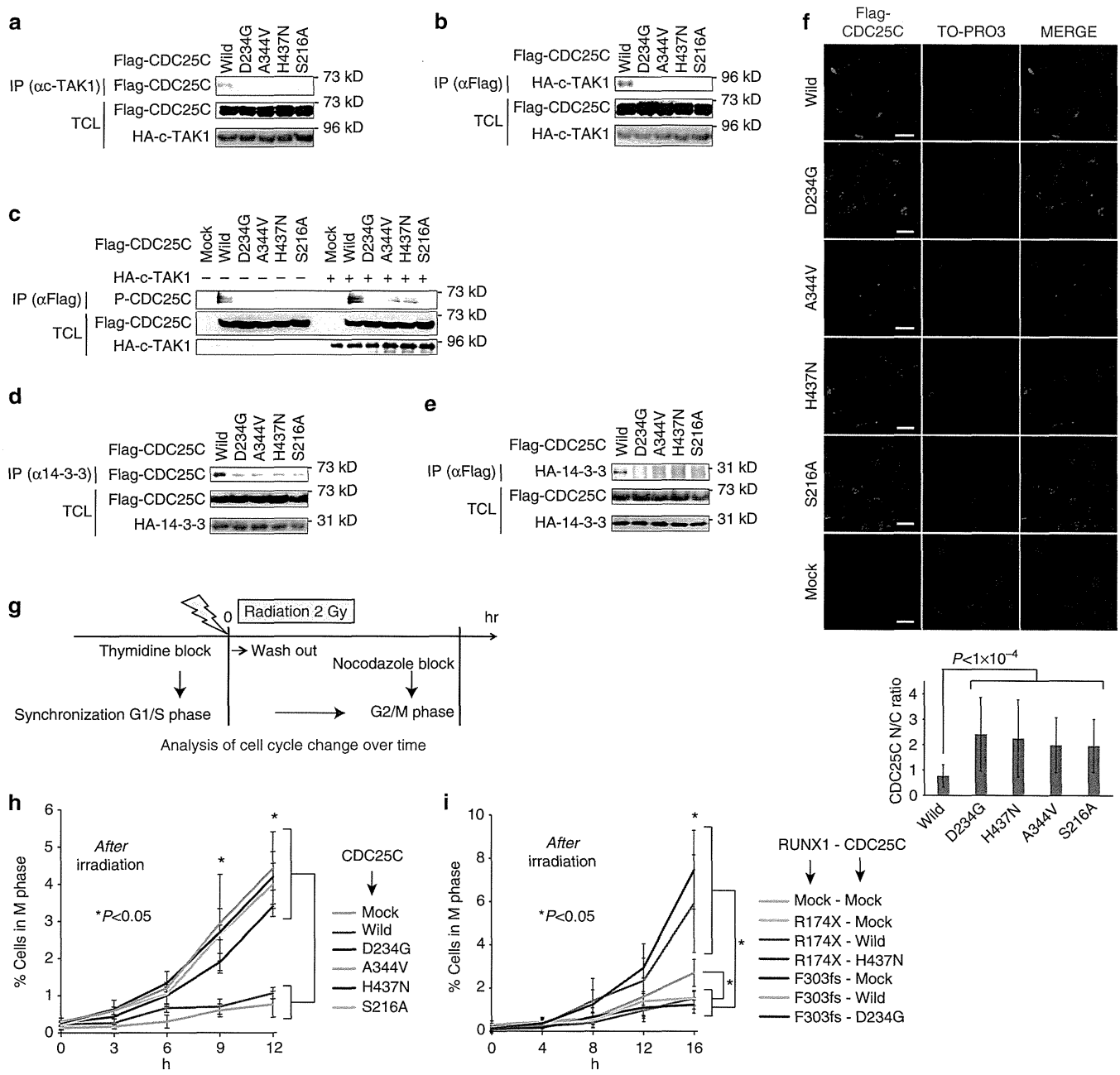

Figure 4 | Mutated CDC25C enhances mitotic entry. (**a**) HEK293T cells were transiently transfected with constructs encoding Flag-tagged CDC25C wild type or mutants, as indicated, and cell lysates were immunoprecipitated with anti-c-TAK1 antibody. Binding capacity of CDC25C was evaluated by western blotting. IP, immunoprecipitation; TCL, total cell lysate. (**b**) Reciprocal immunoprecipitation of **a** using anti-Flag (CDC25C) antibody for immunoprecipitation. (**c**) Left half; cell lysates were immunoprecipitated with anti-Flag antibody. Phosphorylation levels of CDC25C were assessed by phosphorylated-Ser216-specific anti-CDC25C antibody. Right half; the same experiment was performed with cell lysates from HEK293T cells transfected with constructs encoding Flag-tagged CDC25C wild type or mutants and HA-tagged c-TAK1. (**d**) Mutated CDC25C showed reduced capacity for binding to 14-3-3. Cell lysates were immunoprecipitated with anti-14-3-3 antibody and binding capacity of CDC25C was evaluated. (**e**) Reciprocal immunoprecipitation of **d** using anti-Flag (CDC25C) antibody for immunoprecipitation. (**f**) Localization of CDC25C or its mutants was visualized by immunofluorescence. Anti-Flag antibody and Alexa Fluor 555 antibody was used for visualization of CDC25C. N/C ratio of each cell was calculated as detailed in Supplementary Methods and Supplementary Fig. 10. The mean and s.d. of the N/C ratio is presented. Statistical significance of difference was determined by unpaired Student's *t*-test ($n>30$ for each). Scale bar, 10 μm. (**g**) Schematic description of the method used for evaluation of mitotic entry. (**h**) Mitotic entry of CDC25C-mutated cells. Percentage of mutated CDC25C-transduced cells in the M phase was compared with that of wild-type CDC25C-transduced cells. *P* values were calculated using Student's *t*-test and the differences between groups, as indicated, were all statistically significant ($^*P<0.05$) at 10 and 12 h after irradiation ($n=3$). The average and s.d. is presented. (**i**) Mutated RUNX1 and CDC25C were co-expressed in Ba/F3 cells, as indicated, and mitosis entry of these cells was evaluated. The differences between groups, as indicated, were all statistically significant ($^*P<0.05$ at 16 h after washout of thymidine ($n=3$). *P* values were determined using the Student's *t*-test. The average and s.d. is presented.

a germline *RUNX1* mutation. In addition, as Turowski and colleagues reported that CDC25C was involved in S phase entry in addition to mitotic entry[23], release from thymidine-induced G1/S block may be affected by some unknown machinery mediated by mutated CDC25Cs, which might affect the results when we observed G2/M phase fraction of these cells. It is not clear why *CDC25C* mutations are repetitively documented in FPD/AML, but not in sporadic MDS or AML cases. One possibility is that in the presence of a *RUNX1* mutation, as an initial event, an extended period is required before an additional *CDC25C* mutation is acquired. This proposal is supported by the clinical observation that ~40% of patients with FPD/AML develop leukaemia in their 30s[5]; however, the mutational status in *CDC25C* in the reported cohort was unknown.

One of the important problems in the research of FPD/AML is that definitive diagnostic criteria have not been established yet. For this purpose, more extensive studies are required for accumulating clinical characterization, genetic information and functional examination as to whether a *RUNX1* variant in families with thrombocytopenia and/or haematological malignancy is causal[24]. We clarified tentative diagnostic criteria for FPD/AML, which was used in this study (in Methods). Regarding the three missense variants in our study (p.Ser140Asn in pedigree 54, p.Gly172Glu in pedigree 57 and p.Leu445Pro in pedigree 32), Ser140 and Gly172 have been reported to be mutated in sporadic AML and/or MDS cases[25,26]. In addition, induced pluripotent stem cells from a FPD/AML pedigree with p.Gly172Glu recapitulate the phenotype of FPD/AML after hematopoietic differentiation[27]. Ser140 has been also shown to be important for RUNX1 conformation, and a mutation of this site affects hydrogen bonds and results in functional loss[28,29]. Furthermore, all the three missense variants have not been reported in the following SNP database: SNP database (dbSNP) (http://www.ncbi.nlm.nih.gov/projects/SNP), the 1000 Genomes Project (http://www.1000genomes.org), HGVB (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html). They were also predicted as 'damaging' by Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and PROVEAN (http://provean.jcvi.org/index.php). Therefore, we regarded the pedigrees with these *RUNX1* variants as having FPD/AML in this study. However, regarding pedigree 32 with p.Leu445Pro, we could not completely exclude the possibility of incidental co-occurrence of a possible non-causal *RUNX1* germline variant and hairy cell leukaemia, although co-occurrence of them is supposed to be rare. In addition, we should bear in mind the somatic as well as germline LOH of *RUNX1*, which contributes to thrombocytopenia and/or leukemogenesis in FPD/AML.

In conclusion, our results indicate that FPD/AML-associated leukaemic transformation is due to stepwise acquisition of mutations and clonal selection, which is initiated by a *CDC25C* mutation in the pre-leukaemic phase, and is further driven by mutations in other genes including *GATA2* (Supplementary Fig. 14). The identification of *CDC25C* as the target gene responsible for the leukaemic transformation will facilitate diagnosis and monitoring of individuals with FPD/AML, who are at an increased risk of developing life-threatening haematological malignancy.

Methods

Subjects. Studies involving human subjects were done in accordance with the ethical guidelines for biomedical research involving human subjects, which was developed by the Ministry of Health, Labour and Welfare, Japan; the Ministry of Education, Culture, Sports, Science, and Technology, Japan; and the Ministry of Economy, Trade, and Industry, Japan, and enforced on 29 March 2001. This study was approved by ethical committee of the University of Tokyo and each participating institution. Written informed consent was obtained from all patients whose samples were collected after the guideline was enforced. All animal experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments. The clinical data, peripheral blood sample and buccal mucosa of the patients whose pedigree contained two or more individuals with thrombocytopenia and/or any haematological malignancies were collected from participating institutions. Platelet threshold depended on each institution's judge and any haematological malignancies were allowed. The diagnoses were self-reported. When all the following four criteria were fulfilled, the patient was considered as having FPD/AML in this study: (1) the pedigree has two or more individuals with thrombocytopenia and/or any haematological malignancies; (2) a germline *RUNX1* variant, including missense, nonsense, frameshift, insertion and deletion, is confirmed by Sanger sequencing and a synchronized quantitative-PCR method in at least one family member; (3) the *RUNX1* variant has not been reported in public dbSNP; (4) no germline mutations were detected in the following 16 genes: *GATA2, GATA1, CEBPA, MPL, MYH9, MYL9, GP1BA, GP9, MASTL, HOXA11, CBL, DIDO1, TERT, ANKRD26, GFI1B* and *SRP72*. Regarding the last criterion, 16 genes were selected because they have been reported to be responsible for familial thrombocytopenia and/or haematological malignancies.

Whole-exome sequencing. Genomic DNA was extracted from samples using the QIAamp DNA Mini kit (Qiagen). Exome capture was performed. Enriched exome fragments were subjected to sequencing using HiSeq2000 (Illumina). We removed any potential somatic mutations that were observed in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) or in the 1000 Genomes Project (http://www.1000genomes.org) data. All candidate single-nucleotide variations and indels, which were predicted to be deleterious by the Polyphen-2 algorithm, were validated by deep sequencing and Sanger sequencing. Genomic DNA samples from the buccal mucosa of the two patients (subject 20 and subject 21) were used as references. All candidate somatic mutations were validated by Sanger sequence and deep sequencing using primers listed in Supplementary Tables 3 and 4.

Deep sequencing. Using genomic DNA of the patients as template, each targeted region was PCR amplified with specific primers (Supplementary Table 4). The amplification products from an individual sample were combined and purified with the AMpure XP Kit (Beckman Coulter) and library preparation was carried out using the Ion Xpress Fragment Library Kit (Life Technologies) according to the manufacturer's instructions. The Agilent 2100 Bioanalyzer (Agilent Technologies) and the associated High Sensitivity DNA kit (Agilent Technologies) were used to determine quality and concentration of the libraries. The amount of the library required for template preparation was calculated using the template dilution factor calculation described in the protocol. Emulsion PCR and enrichment steps were carried out using the Ion OneTouch 200 Template Kit v2 DL (Life Technologies). Sequencing was undertaken using Ion Torrent PGM and Ion 318 chips Kit v2 (Life Technologies). The Ion PGM 200 Sequencing Kit (Life Technologies) was used for sequencing reactions, following the recommended protocol. The presence of *CDC25C* and *GATA2* mutations was also validated by a subclone strategy for DNA sequence analysis.

Single-cell sequencing and genome amplification. Single cells were separated from the bone marrow of subject 20 at AML phase using FACSAria II (BD biosciences) (Supplementary Fig. 15a). Each cell was deposited into individual wells of a 96-well plate. Single cells were lysed and whole genome from single cell was amplified using GenomePlex Single Cell Whole-Genome Amplification Kit (Sigma-Aldrich). Mutation status of each gene was analysed by direct sequencing with specific primers (Supplementary Table 5). To improve the sensitivity of this procedure, we used multiple primer sets for detecting a single-nucleotide variation. We estimated the false-negative rate of this procedure based on the ratio of RUNX1 mutation, which is supposed to be observed in all of the cells. The false-negative rate was estimated to be 35% (22 cells out of 63 cells, Supplementary Table 2), which is consistent with the manufacturer's bulletin reporting the allelic dropout of 30%. In light of these results, we regard those cells with at least one gene mutation in a mutational group (coloured in red, orange, green, blue or purple) as being positive for gene mutations of the corresponding group. To assess whether mutations in *LPP, FAM22G, COL9A1* and *GATA2* and mutations in *AGAP4, RP1L1, DTX2* and *CHEK2* were mutually exclusive, we performed a statistical analysis as follows. First of all, we determine a matrix **A** that virtually represents the mutational status of eight genes (1: *LPP*, 2: *FAM22G*, 3: *COL9A1*, 4: *GATA2*, 5: *AGAP4*, 6: *RP1L1*, 7: *DTX2* and 8: *CHEK2*) of 57 cells. Concretely, **A** is defined as follows:

$$\mathbf{A}=\begin{pmatrix} a_{1,1} & \cdots & a_{8,1} \\ \vdots & \ddots & \vdots \\ a_{1,57} & \cdots & a_{8,57} \end{pmatrix} \quad a_{i,j}=\begin{cases} 0 : \text{if gene i of cell j is wildtype} \\ 1 : \textit{if} \text{ gene i of cell j is mutated} \end{cases} \qquad (1)$$

On the other hand, a matrix **R** indicates data from the actual experimental results of mutational analysis as shown in Fig. 2c. Elements of **R** is provided in

Supplementary Table 2.

$$\mathbf{R}=\begin{pmatrix} r_{1,1} & \cdots & r_{8,1} \\ \vdots & \ddots & \vdots \\ r_{1,57} & \cdots & r_{8,57} \end{pmatrix} \quad r_{i,j}=\begin{cases} 0: \text{if gene i of cell j is wild type} \\ 1: \text{if gene i of cell j is mutated} \\ 2: \text{if mutational status of gene i of cell j is undetermined} \end{cases} \tag{2}$$

Then we assumed two hypotheses: H_0 and H_1.

H_0: the mutational status of genes $1\sim4$ and genes $5\sim8$ is independent. Each matrix elements of $\mathbf{A}$ are randomly assigned 0 or 1 (at ratio of 1:1) independently of each other.

H_1: mutations in genes $1\sim4$ and genes $5\sim8$ are mutually exclusive, and cells $1\sim40$ harbour mutations of genes $1\sim4$, while cells $41\sim57$ harbour mutations of genes $5\sim8$. In mathematical representation,

$$a_{i,j}=\begin{cases} 0: (5\le i\le 8 \text{ and } 1\le j\le 40) \text{ and } (1\le i\le 4 \text{ and } 41\le j\le 57) \\ 0 \text{ or } 1 \text{ randomely}: (1\le i\le 4 \text{ and } 1\le j\le 40) \text{ and } (5\le i\le 8 \text{ and } 41\le j\le 57) \end{cases} \tag{3}$$

We assumed matrices $\mathbf{A}_0$ and $\mathbf{A}_1$ that represent virtually generated mutational status under the hypotheses H_0 and H_1, and calculate the probability of substantializing $\mathbf{R}$ for given $\mathbf{A}_0$ and $\mathbf{A}_1$.

$P_0(\mathbf{R}/\mathbf{A}_0)$ and $P_1(\mathbf{R}/\mathbf{A}_1)$ can be calculated for given matrices $\mathbf{A}_0$ and $\mathbf{A}_1$ under the condition as follows:

Probability that we cannot determine whether a cell has mutation in gene X when the cell does not actually have a mutation; 28% (based on our data shown in Supplementary Table 2).

Probability that we judge that a cell has a mutation in gene X when the cell does not actually have a mutation; 5% (because it is very unlikely to happen).

Probability that we can judge correctly that a cell does not have a mutation in gene X when the cell does not actually have a mutation; 67% ($100-28-5=67\%$).

Probability that we cannot determine whether a cell has mutation in gene X when the cell actually has a mutation; 28% (based on our data shown in Supplementary Table 2).

Probability that we judge that a cell has a mutation in gene X when the cell actually has a mutation; 35% (the estimated false-negative rate based on the ratio of *RUNX1* mutation).

Probability that we can judge correctly that a cell has a mutation in gene X when the cell actually has a mutation; 37% ($100-28-35=37\%$).

Put it simply, $\mathbf{P}_0$ represents the probability that one can get the mutational profile R when a cell harbours mutations independently of each other, while $\mathbf{P}_1$ indicates the probability that R is realized under the condition where mutations in gene groups $1\sim4$ and $5\sim8$ are exclusive. Because $\mathbf{A}_0$ and $\mathbf{A}_1$ that meet the hypotheses H_0 and H_1 can be generated innumerably, we conducted a computational simulation to acquire the distribution of P_0 and P_1 by generating $\mathbf{A}_0$ and $\mathbf{A}_1$ 100,000 times. For visibility, horizontal axis is converted to $-\ln(P)$.

Synchronized quantitative-PCR. These experiments were performed mostly as described previously[6]. Briefly, genomic DNA was denatured 95 °C for 5 min and iced immediately. Using the LightCycler 480 Instrument II (Roche), thermal cycling was performed with denatured genomic DNA, forward and reverse primers (Supplementary Table 6), THUNDERBIRD SYBR qPCR mix (TOYOBO). Threshold cycle scores were determined as the average of triplicate samples. We designed 27 primers for *RUNX1* and 3 reproducible primers (that is, primer RUNX-9, RUNX-19 and RUNX-20) were chosen by preparatory experiments. RPL5-2 and PRS7-1 primers, which were authorized previously[6], were also utilized as controls. In addition, genomic DNA extracted from the bone marrow sample of a MDS patient with a chromosome 21 deletion was also examined with the same primers as a control of *RUNX1* locus copy-number loss. Crossing points (Cps) of designed primers were examined by quantitative PCR. *RUNX1* locus copy-number relative to RPL5-2 was calculated using Cps of RUNX-9 and RPL5-2, with RPL5-2 values set at 2. Similar results were obtained when Cps of RUNX-19, RUNX-20 or RPS7-1 values were used.

LOH detection with SNP sequencing. To examine the existence of uniparental disomy, we designed four specific primers to detect nine SNPs in *RUNX1*, which are frequently seen (>40%) (Supplementary Table 7). Direct sequencing was performed with the primers, and heterogeneity of SNPs was examined.

Chemicals and immunological reagents. Thymidine and nocodazole were purchased from Sigma-Aldrich. Anti-CDC25C, anti-phospho-CDC25C (Ser216) and anti-beta-actin antibodies were purchased from Cell Signaling Technology. Anti-HA monoclonal antibody was purchased from MBL. Rabbit anti-Flag monoclonal antibody was purchased from Sigma-Aldrich. Anti-HA was purchased from Roche. Mouse anti-phospho-histone H2AX (Ser139) antibody and Alexa Fluor 488 mouse anti-phospho-H3 (Ser10) antibody were purchased from Merck Millipore. Alexa Fluor 488 rabbit anti-mouse immunoglobulin (Ig)G, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-rabbit IgG were purchased from Invitrogen. TO-PRO3 was purchased from Molecular Probes. Rabbit anti-14-3-3 Sigma antibody was purchased from Bethyl laboratories. Sheep anti-c-TAK1 antibody was purchased from Exalpha Biologicals. Anti-sheep IgG-HRP was purchased from RSD. Nonviable cell exclusion was performed by 7-AAD Viability Staining Solution (BioLegend).

Subclone strategy and direct sequencing. Using genomic DNA of the patients as template, each targeted region was amplified by PCR with specific primers (Supplementary Table 4). PCR products were purified with illustra ExoStar (GE Healthcare) and subcloned into *Eco*RV site of pBluescript II KS(−) (Stratagene). Ligated plasmids were transformed into *E. coli* strain XL1-Blue by 45 s heat shock at 42 °C. Positive transformants were incubated on LB plates containing 100 μg ml^{-1} ampicillin supplemented with X-gal (Sigma-Aldrich) and isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich). For colony PCR, a portion of a white colony was directly added to a PCR mixture as the DNA template. Insert region was amplified by PCR procedure with T3 and T7 universal primers, purified with illustra ExoStar (GE Healthcare Life Sciences), and sequenced by the Sanger method with T3 and T7 primers using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and ABI Prism 310 Genetic Analyzer (Life Technologies).

Immunoprecipitation and western blotting. These experiments were performed as described previously[30]. Briefly, HEK293T cells were transiently transfected with mammalian expression plasmids encoding Flag-tagged CDC25C and its mutants, HA-tagged 14-3-3 or c-TAK1. All plasmids were sequence verified. After 48 h, cell lysates were collected and incubated with an antibody (anti-HA antibody (1:200, 3 h), anti-Flag antibody (1:200, 3 h), anti-c-TAK1 antibody (1:150, 3 h) and anti 14-3-3 antibody (1:150, 3 h)). After incubation, the cell lysates were incubated with protein G-Sepharose (GE Healthcare) for 1 h. The precipitates were stringently washed with high salt-containing wash buffer and analysed by western blotting. Anti-Flag (HRP-conjugated, Sigma-Aldrich), anti-HA (MBL), anti-HA (HRP-conjugated, Roche), anti-CDC25C (Cell Signaling Technology), anti-phospho-CDC25C (Ser216) (Cell Signaling Technology), anti-c-TAK1 antibody (Exalpha Biologicals) or anti-14-3-3 antibody (Bethyl laboratories) antibodies and Immunostar LD (Wako) was used for detection. Original gel images of western blot analysis are shown in Supplementary Fig. 16.

Cell cycle synchronization and analysis for mitosis entry. After transduction of wild-type CDC25C or its mutated forms to murine lymphoid cell line Ba/F3 cells (RIKEN BioResource Center), double-thymidine block was performed to obtain cell cycle synchronization at G1/S phase. In brief, 2 mM of thymidine was added to the medium. After 16 h, cells were washed and released from the first thymidine for 8 h. A second block was initiated by adding 2 mM of thymidine, and cells were maintained for 16 h. Then thymidine was washed out and the cells were incubated with 1 mM nocodazole with or without 2 Gy of irradiation (Supplementary Fig. 10a). Ba/F3 cells were fixed over time with 75% ethanol in phosphate-buffered saline (PBS) at 4 °C overnight and permeabilized with 2% Triton-X at 4 °C for 15 min. The cells were stained with anti-phospho-H3 (Ser10) Alexa Fluor 488 conjugated antibody (dilution, 1:200) in PBS with 2% fetal calf serum at 4 °C for 30 min and then treated with 5% propidium iodide and 1% RNase in PBS at room temperature (RT) for 30 min. Cell cycle was analysed using a BD LSR II Flow cytometer (BD biosciences) (Supplementary Fig. 15b). To assess the cooperation of *CDC25C* and *RUNX1* mutation, wild-type or mutant (D234G, H437N) pMXs-neo-Flag-CDC25C and mutant (F303fsX566, R174X) pGCDNsam-IRES-KusabiraOrange-Flag-RUNX1 were retrovirally transduced into Ba/F3 cells.

Immunofluorescent microscopic analysis. These experiments were performed as described previously[30]. Briefly, Ba/F3 cells were fixed, permeabilized and blocked. Staining for phosphorylated histone H2AX was performed with anti-phospho-histone H2AX (Ser139) antibody (dilution, 1:500; Merck Millipore) at RT for 3 h. After washing with PBS three times and with 1% bovine serum albumin in PBS, the cells were treated with Alexa Fluor 488 rabbit anti-mouse IgG (dilution, 1:500; Invitrogen) and TO-PRO3 (dilution, 1:1,000; Molecular Probes) for 1 h. The proteins were visualized using FV10i (Olympus) or BZ-9000 (Keyence). The percentage of γH2AX foci-positive cells was determined by examining 100 cells per sample. Three independent experiments were performed. To evaluate the localization of CDC25C, Ba/F3 cells were treated with 2 mM thymidine for 12 h and stained. Staining was underwent with anti-Flag antibody or anti-CDC25C antibody at RT for 3 h. After washing, the cells were treated with Alexa Fluor 488 or 555 antibody and TO-PRO3 for 1 h. The mean intensity of CDC25C in the nucleus and cytoplasm of each cell was measured within a region of interest placed within the nucleus and cytoplasm (Supplementary Fig. 10). Similarly, the background intensity was quantified within the region of interest placed outside the cells. All the measurements were performed using the Fluoview FV10i software or ImageJ. The background-subtracted intensity ratio of the nucleus to cytoplasm was calculated in >30 cells in each specimen.

Retrovirus production. The procedures were performed as described previously[30]. Briefly, Plat-E packaging cells were transiently transfected with each retroviral construct using the calcium phosphate precipitation method, and supernatant

containing retrovirus was collected 48 h after transfection and used for infection after it was centrifuged overnight at 10,000 r.p.m.

Statistical analysis. To compare data between groups, unpaired Student's *t*-test was used when equal variance were met by the *F*-test. When unequal variances were detected, the Welch *t*-test was used. Differences were considered statistically significant at a P value of <0.05.

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Author contributions

A.Y., T.T., M.I. and M. Kurokawa analysed genetic materials and performed functional studies. A.T., H.I., M.N., Y.N. and S.A. were involved in sequencing and/or functional studies. M. Kawazu, T.U. and H.M. took part in whole-exome sequencing, deep sequencing and bioinformatics analyses of the data. A.Y., T.T., M.I., H.H., K.U., Y.H., E.I., K.K. and H.N. collected specimens. A.Y. and T.T. generated figures and tables. M. Kurokawa designed and led the entire project. A.Y., T.T. and M. Kurokawa wrote the manuscript. All authors participated in the discussion and interpretation of the data.

Additional information

Accession codes: Sequence data for FPD/AML patients has been deposited in GenBank/EMBL/DDBJ sequence read archive (SRA) under the accession code SRP043031

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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Baseline characteristics and disease burden in patients in the International Paroxysmal Nocturnal Hemoglobinuria Registry

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria is a rare, acquired disease associated with hemolytic anemia, bone marrow failure, thrombosis, and, frequently, poor quality of life. The International PNH Registry is a worldwide, observational, non-interventional study collecting safety, effectiveness, and quality-of-life data from patients with a confirmed paroxysmal nocturnal hemoglobinuria diagnosis or detectable paroxysmal nocturnal hemoglobinuria clone, irrespective of treatment. In addition to evaluating the long-term safety and effectiveness of eculizumab in a global population, the registry aims to improve diagnosis, optimize patient management and outcomes, and enhance the understanding of the natural history of paroxysmal nocturnal hemoglobinuria. Here we report the characteristics of the first 1610 patients enrolled. Median disease duration was 4.6 years. Median granulocyte paroxysmal nocturnal hemoglobinuria clone size was 68.1% (range 0.01-100%). Overall, 16% of patients had a history of thrombotic events and 14% a history of impaired renal function. Therapies included anticoagulation (31%), immunosuppression (19%), and eculizumab (25%). Frequently reported symptoms included fatigue (80%), dyspnea (64%), hemoglobinuria (62%), abdominal pain (44%), and chest pain (33%). Patients suffered from poor quality of life; 23% of patients had been hospitalized due to paroxysmal nocturnal hemoglobinuria-related complications and 17% stated that paroxysmal nocturnal hemoglobinuria was the reason they were not working or were working less. This international registry will provide an ongoing, valuable resource to further the clinical understanding of paroxysmal nocturnal hemoglobinuria. (*Clinicaltrials.gov identifier:01374360*)

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired disease characterized by chronic intravascular hemolysis caused by uncontrolled complement activation.[1] The cellular abnormality in this life-threatening disease originates from a mutation in the phosphatidylinositol glycan class A (*PIGA*) gene, resulting in a deficiency of glycosylphosphatidyl-inositol (GPI)-anchored complement regulatory proteins, including CD55 and CD59, on the surface of blood cells.[1] Patients with chronic hemolysis experience a marked increased risk of thromboembolism (TE), which may ultimately lead to target organ damage and death.[2,3] Retrospective analyses have reported that, despite best supportive care, the 10-year survival rate in patients with PNH ranged from 50% for patients diagnosed between 1940 and 1970[3-5] to 75% in a more recent series.[6] TE is the leading cause of mortality in patients with PNH, accounting for between 40% and 67% of deaths with known causes.[7] Patients with PNH also experience symptoms including fatigue, abdominal pain, headache, shortness of breath, dysphagia, and erectile dysfunction.[1] These symptoms can be debilitating and significantly reduce the quality of life (QoL) of patients with PNH.[8] PNH may develop in the absence of another bone marrow disorder (BMD), as a condition secondary to BMDs such as aplastic anemia (AA) or myelodysplastic syndrome, or as subclinical PNH.[1]

The only potentially curative therapy for PNH is allogeneic bone marrow transplantation; however, this procedure is associated with substantial morbidity and mortality[9-11] and, consequently, is not an appropriate therapeutic option for most patients. Historically, management of PNH was limited to the use of supportive measures such as blood transfusions and anticoagulation therapy. However, it has been reported that the risk of TE in patients with PNH remains high even in patients who have no clinical evidence of TE or are receiving prophylactic anticoagulation,[7,12] which is itself associated with an increased risk of bleeding complications.[13] In one

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study, spontaneous, long-term remission was observed in approximately 15% of patients.[4]

In 2007, eculizumab (Soliris®, Alexion Pharmaceuticals, Inc., Cheshire, CT, USA), a humanized monoclonal antibody that inhibits terminal complement activation, was approved for the treatment of patients with PNH. A phase II study (PILOT)[14] and two phase III studies (TRIUMPH and SHEPHERD)[15,16] demonstrated that eculizumab was well tolerated and provided a rapid, sustained, and clinically meaningful reduction in hemolysis, fatigue, and transfusion requirements, along with improved QoL. Other studies have shown that eculizumab therapy in patients with PNH is also associated with improvements in pulmonary hypertension and renal function.[2,17] Subsequent studies have demonstrated that eculizumab is associated with a 92% reduction in the risk of TE ($P<0.001$)[7] and with a highly significant improvement in patient survival to a level comparable to that of age-matched healthy controls.[3]

The natural history of PNH is highly variable and has previously been investigated by retrospective analyses involving relatively small patient populations.[4,6,18,19] The burden of disease from the patient's perspective has not been previously documented. The International PNH Registry was implemented to evaluate the safety and effectiveness of eculizumab, to gather comprehensive data on the natural history of PNH and the management of patients with PNH, and to evaluate the clinical symptoms and outcomes of the disease in order to better understand its progression and variability on a global scale. The long-term aim of the registry is to improve diagnosis and therapeutic strategies, optimize patient management and outcomes, enhance knowledge of pregnancy and related issues, and better understand the natural history of the disease.

In this first publication of data from the International PNH Registry, we report on the cross-sectional analysis of demographic and clinical characteristics of patients enrolled through June 30, 2012, and describe disease-associated morbidities commonly experienced in patients with PNH. In addition, we also report initial findings from a subset of patients who had completed base-line study questionnaires relating to impacts of the disease on various measures, including QoL, symptomatology, and employment status.

Methods

Patient population

The International PNH Registry is a prospective, non-interventional, observational study. It collects information from patients monitored in current medical practice irrespective of past, present, or future treatment. The registry was approved by the institutional review boards (or equivalent) of participating centers and all patients provided written informed consent prior to inclusion. The registry is sponsored by Alexion Pharmaceuticals, Inc., and is overseen by an independent executive committee of international PNH experts.

Patients of any age with a clinical diagnosis of PNH (by any applicable diagnostic method) and/or detectable PNH clone of 0.01% or over were eligible for inclusion in the registry. A PNH clone was defined as a population of granulocytes and/or erythrocytes deficient in GPI.

Data collection

Data captured in the registry include patients' demographics, medical and treatment history, comorbid conditions, PNH clone size, disease characteristics and outcomes, symptoms, PNH-specific treatments, PNH-related events, morbidity (including myeloproliferative disease, other malignancies, and infections), mortality, pregnancy (maternal and fetal outcomes), patient QoL, and health resource utilization. Clinical data captured include lactate dehydrogenase (LDH) levels, hemoglobin levels, transfusion requirements, thrombotic events (identified using major adverse vascular event categories), physician-reported renal dysfunction, and other laboratory data. Specific information collected for eculizumab-treated patients includes dosage and dose adjustments, meningococcal vaccination status, infusion reactions, reasons for treatment discontinuation, and outcomes associated with discontinuation.

Patient medical information and study questionnaire data are collected at study enrollment and every six months thereafter. The patients' questionnaires are collected at study enrollment and during routine office visits close to the 6-month follow-up time window and include QoL data based on the validated Functional Assessment of Chronic Illness Therapy (FACIT)-Fatigue[20] and European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire version 3.0 (EORTC QLQ-C30)[21] instruments, common PNH symptoms, use of health care resources including hospitalizations, work status and time lost from work due to PNH, and treatment satisfaction.

Statistical analysis

Continuous variables were described using standard summary statistics; categorical variables were described using frequencies and percentages. Group differences were determined using analysis of variance or the Student's t-test for continuous variables and χ^2 tests for categorical variables. Non-parametric Wilcoxon and Kruskal-Wallis tests were used for laboratory results (e.g. granulocyte clone size and LDH) with non-normal distributions. $P<0.05$ was considered statistically significant..

Patient-reported symptoms, history of TE, and transfusion requirements were stratified by PNH clone size at enrollment (<10%, 10-49%, and ≥50%), diagnosis (current or prior) of AA or other BMD, and LDH level (<1.5 or ≥1.5 x upper limit of normal (ULN), the higher levels having been shown to be associated with significantly increased risks of TE and mortality[22,23]). Clone size categories were used for analyses, as their bimodal distribution limits their suitability for consideration as a continuous variable.

For the EORTC QoL and FACIT-Fatigue assessments, lower scores indicate a poorer QoL and increased levels of fatigue. Differences in average scores between groups of 5 points or over[24] or 3 points or over,[25] respectively, are considered clinically meaningful.

Results

Patients' demographics and clinical characteristics

As of June 30, 2012, 1610 patients from 273 centers in 25 countries were enrolled in the International PNH Registry (*Online Supplementary Table S1*). Overall, 92.5% of patients were from Europe and North America and 87.5% of patients were Caucasian. The remaining patients were Asian/Pacific Islanders (5.0%), of African descent (3.5%), Native/Aboriginal (0.2%), or of other/unknown ethnicity/race (3.9%).

Table 1. Patients' demographics and clinical characteristics at enrollment into the International PNH Registry.

Parameter	Patients (n=1610)
Age, years, median (range)	42 (3-99)
Females, n (%)	857 (53.2)
Age at disease start, years, median (range)	32 (3-87)
Disease duration, years, median (range)	4.6 (<1-47)
Lactate dehydrogenase, ×ULN, median (5th, 95th percentile)[a]	1.96 (0.65, 10.32)
Hematologic parameters, median (5th, 95th percentile)	
Hemoglobin, g/L (n=1425)	106 (70, 145)
Platelets, ×10⁹/L (n=1430)	131 (19, 271)
Absolute neutrophils, ×10⁹/L (n=1275)	1.7 (0.00, 5.10)
Absolute reticulocytes, ×10⁹/L (n=971)	113 (27, 400)
Reticulocytes, % (n=1024)	3.6 (0.82, 13.06)
Granulocyte clone size, %, median (5th, 95th percentile)	68.1 (0.36, 99.30)
<10%, n (%)	280 (17.4)
10-49%, n (%)	247 (15.3)
≥50%, n (%)	832 (51.7)
Unknown, n (%)	251 (15.6)
History of thrombotic events, n (%)[b]	250 (15.5)
1	169 (10.5)
2	45 (2.8)
3	19 (1.2)
4+	10 (0.6)
Unknown	7 (0.4)
Time (years) since most recent thrombotic event, median (range)	3.8 (<1-45)
History of impaired renal function, n (%)[b]	223 (13.9)
Anticoagulation therapy, n (%)[b,c]	501 (31.1)
Immunosuppressive therapy, n (%)[b,c]	301 (18.7)
Pain medication, n (%)[b,c]	133 (8.3)
Eculizumab, n (%)[c,d]	411 (25.5)

[a]Includes only patients who had not received eculizumab in the year prior to enrollment and who had LDH value available (n=900). [b]Data not available for 60 patients (3.7%). [c]Any use in the 12 months prior to enrollment. [d]An additional 80 patients (5.0%) were reported as having received treatment with eculizumab, though the dates of administration were unknown. PNH: paroxysmal nocturnal hemoglobinuria; ULN: upper limit of normal; BMD: bone marrow disorder; AA: aplastic anemia.

Table 2. Treatment received[a] prior to enrollment into the International PNH Registry by history of AA.

Treatment	Number (%) of patients Ever diagnosed with AA (n=701)	With no history of BMD (n=776)
Anticoagulation therapy[b]	**147 (21.0)**	**326 (42.0)**
Immunosuppressive therapy[b,c]	**270 (38.5)**	**22 (2.8)**
Eculizumab therapy[b]	**131 (18.7)**	**262 (33.8)**
Red blood cell transfusion[d]	262 (37.4)	266 (34.3)
Immunosuppressive therapy[c] plus:		
Anticoagulation	**35 (5.0)**	**6 (0.8)**
Eculizumab	**37 (5.3)**	**8 (1.0)**
Red blood cells	**123 (17.6)**	**13 (1.7)**
Anticoagulation plus:		
Eculizumab	**53 (7.6)**	**108 (13.9)**
Red blood cells	**63 (9.0)**	**124 (16.0)**
Eculizumab plus red blood cells	60 (8.6)	84 (10.8)

Bold values indicate statistically significant difference between groups (P<0.001) calculated from χ^2 test for categorical variables and Student's t-test for continuous variables. [a]*Patients may have received more than 1 type of treatment.* [b]*In prior 12 months.* [c]*Immunosuppressive therapy includes cyclosporine and/or anti-thymocyte globulin.* [d]*In prior 6 months. PNH: paroxysmal nocturnal hemoglobinuria; AA: aplastic anemia; BMD: bone marrow disorder.*

Patients' demographics and clinical characteristics of the 1610 enrolled patients are provided in Table 1. Median patient age at enrollment was 42 years (range 3-99 years), and 53.2% of patients were female. Median PNH duration from disease start to enrollment was 4.6 years, with a minimum of less than 1 year and a maximum of 47 years. Overall, 774 (48.1%) of the patients had been diagnosed with one or more types of BMD, including AA or hypoplastic anemia (n=701; 43.5%), myelodysplastic syndromes (n=93; 5.8%), myelofibrosis (n=7; 0.4%), and/or acute myeloid leukemia (n=6; 0.4%). In the 900 of 1610 patients who had not received eculizumab in the 12 months prior to enrollment and for whom LDH values were available, the median LDH level at the time of enrollment was approximately twice the ULN. A history of any prior thrombotic event was reported in 250 patients (15.5%), with the majority of these patients (169 of 250, 67.6%) having experienced just one event. A similar number of patients (n=223,13.9%) had a history of impaired renal function. At enrollment, 31.1% of patients were receiving anticoagulation therapy, 18.7% immunosuppressive therapy, and 8.3% pain medication; approximately 25% of the patients were being treated with eculizumab (Table 1). At the time of analysis, completed base-line patients' questionnaires relating to symptoms of PNH, QoL, and work were available for 856 of the 1610 (53%) enrolled patients.

Due to the observational nature of this study, the number of patients contributing data to each assessment was not consistent. In addition, as eculizumab reduces hemolysis and prevents release of LDH, patients who had received eculizumab at any time in the 12 months prior to enrollment were excluded from all data summaries assessed by LDH levels. A graphical summary of the number of patients included in each assessment, along with the criteria defining each subpopulation, is provided in *Online Supplementary Figure S1*.

PNH clone size

The median granulocyte clone size at enrollment was 68.1% (Table 1), and the entire range of clone sizes was represented, from 0.01% (the minimum inclusion criterion for enrollment) through 100%. The median clone size was significantly larger in patients without a history of BMD than in patients who had at any point been diagnosed with AA (83% *vs.* 35%; $P<0.001$ by Wilcoxon test). The distribution of clone sizes varied with history of BMD: clone sizes of 50% or over and less than 10% were reported in, respectively, 34% and 40% of patients with a history of AA compared with 76% and 8% of patients without a diagnosis of BMD other than PNH.

In patients who had not received eculizumab in the 12 months prior to study enrollment, median LDH levels generally increased with PNH clone size category ($P<0.001$ by Kruskal-Wallis test) and in patients with no history of BMD other than PNH compared with those patients ever diagnosed with AA (Figure 1A). There was also a signifi-

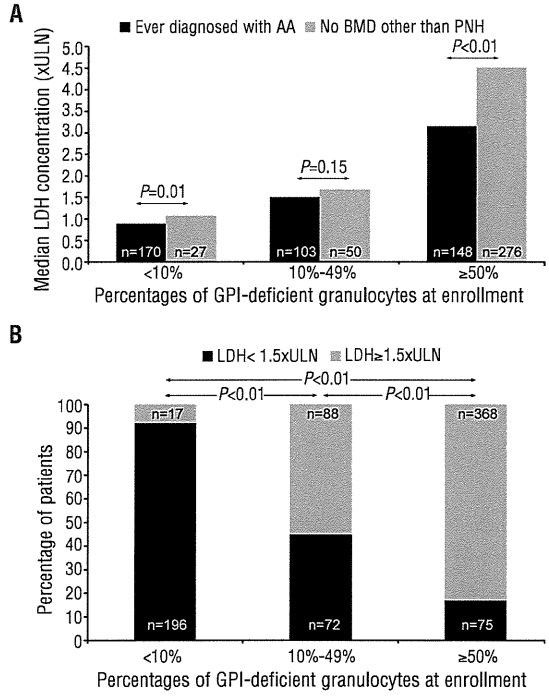

Figure 1. LDH concentration. (A) Median LDH concentration at enrollment by PNH clone size and diagnosis. (B) Percentage of patients with LDH <1.5×ULN or ≥1.5×ULN by PNH clone size. Only includes patients who had not received eculizumab in the 12 months prior to study enrollment.

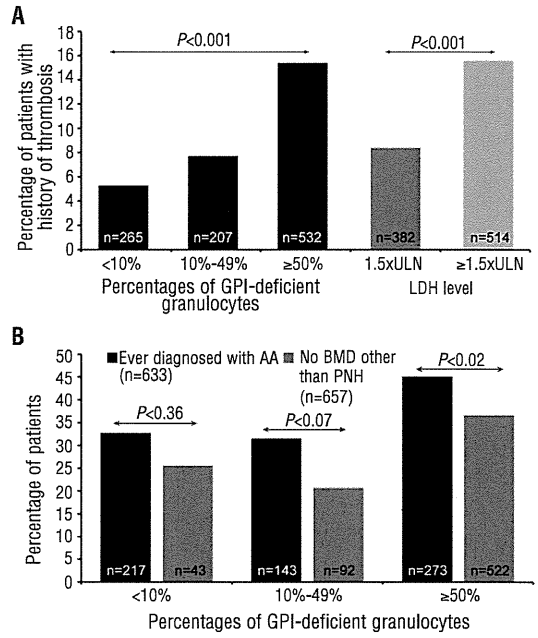

Figure 2. Thrombosis and red blood cell transfusion history. (A) Percentage of patients with a history of thrombosis by PNH clone size and LDH level at enrollment. (B) Percentage of patients receiving red blood cell transfusion in the year prior to enrollment by PNH clone size and diagnosis. Only includes patients who had not received eculizumab in the 12 months prior to study enrollment.

cant increase in the percentage of patients with LDH levels ≥1.5 x ULN as PNH clone size increased, going from 8% of patients with a clone size less than 10% to 55% of patients with a clone size of 10-49% and 83% of patients with a clone size 50% or over (Figure 1B).

In patients who had not received eculizumab in the 12 months prior to enrollment, there was a positive correlation between history of thrombosis and clone size at enrollment: 5.3% of patients with clone size less than 10% had a history of thrombosis and 7.7% of patients with clone size 10-49% had such a history, whereas 15.4% of patients with clone size 50% or over had such a history (P<0.001). In addition, a larger percentage of patients with LDH ≥1.5 x ULN at enrollment, compared with LDH <1.5 x ULN at enrollment reported a history of thrombosis (15.6% *vs.* 8.4%; P<0.001) (Figure 2A).

Concomitant therapies

The number of patients receiving anticoagulation therapy or immunosuppressive therapy in the 12 months prior to enrollment and the number of patients receiving red blood cell transfusions in the six months prior to enrollment are shown in Table 2. Compared with patients with no history of BMD, patients who had at some point been diagnosed with AA were less likely to have received treatment with anticoagulants (21.0% *vs.* 42.0%; P<0.001) or with eculizumab (18.7% *vs.* 33.8%; P<0.001), but they were more likely to have received immunosuppressive therapies such as cyclosporine and/or anti-thymocyte globulin (38.5% *vs.* 2.8%; P<0.001). There was no significant difference between these patient groups in the percentage who had received red blood cell transfusions in the six months before study enrollment (P=0.21), though patients who had at some point been diagnosed with AA received a significantly greater mean number of units of packed red blood cells during this time frame compared with patients with no history of BMD (9.0 *vs.* 6.8; P=0.001).

Table 3. Anticoagulant use (within 12 months prior to enrollment) by selected characteristics.

	N. (%) of patients	P
History of thrombotic events		
No (n=1300)	318 (24.5)	<0.01
Yes (n=250)	176 (70.4)	
Granulocyte clone size[a]		
<10% (n=280)	26 (9.3)	<0.01
10%-49% (n=247)	42 (17.0)	
≥50% (n=832)	359 (43.1)	
Lactate dehydrogenase[a,b]		
<1.5×ULN (n=384)	70 (18.2)	<0.01
≥1.5×ULN (n=516)	201 (39.0)	

[a]Recorded at time of enrollment. [b]Excludes patients who received eculizumab in the year prior to enrollment.

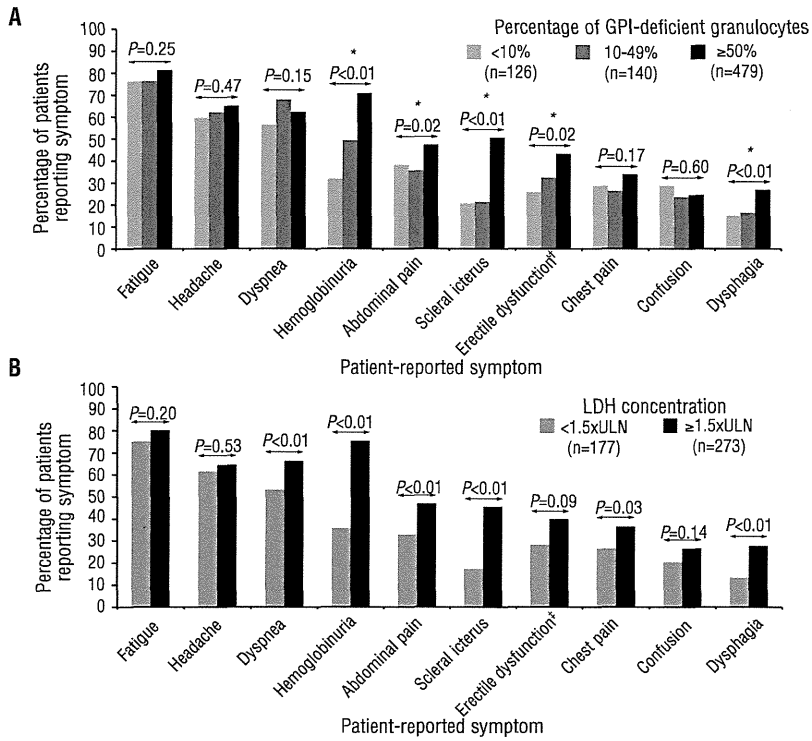

Figure 3. Patient-reported PNH symptoms. (A) Patient-reported symptoms by PNH clone size. (B) Patient-reported symptoms by LDH level. Only includes patients who had not received eculizumab in the 12 months prior to study enrollment. *Statistically significant. †Male patients only; n=62, 68, and 222. ‡Male patients only; n=82 and 134.

Compared with patients with no history of BMD, a significantly greater percentage of patients who had at some point been diagnosed with AA had received treatment with immunosuppressive therapies plus anticoagulants, eculizumab, or red blood cell transfusions (all $P<0.001$) (Table 2). In contrast, significantly more patients with no history of BMD had received treatment with anticoagulation therapy plus eculizumab or red blood cell transfusions (both $P<0.001$) (Table 2).

Patients were significantly more likely to have received anticoagulation therapy in the 12 months prior to enrollment if they had a history of thrombosis, a granulocyte PNH clone size 50% or over, or an LDH level ≥1.5 x ULN (Table 3). Figure 2B shows the percentage of patients who received red blood cell transfusions by diagnosis and PNH clone size. Patients who had at some point been diagnosed with AA were more likely to have received a blood transfusion in the six months prior to enrollment. This difference reached statistical significance in patients with clone sizes over 50% ($P=0.02$).

PNH symptoms

Of the 856 patients for whom self-reported symptom data were available (i.e. patients with a base-line questionnaire), a total of 799 patients (93.3%) reported at least one symptom associated with PNH. The percentage of patients reporting common symptoms associated with PNH in the past six months is shown in *Online Supplementary Figure S2*. Commonly reported symptoms included fatigue (80%), dyspnea (64%), headache (63%), and hemoglobinuria (62%), and 38% of male patients had experienced erectile dysfunction. Although fatigue was the most frequently reported symptom, 91.4% of patients (782 of 856) reported at least one symptom other than fatigue, whereas only 2.0% of patients (17 of 856) reported fatigue and no other symptoms.

Although each of the common PNH-related symptoms were reported in all categories of clone size, patients with clone sizes 50% or over reported significantly more hemoglobinuria, dyspnea, abdominal pain, scleral icterus, erectile dysfunction, and dysphagia (Figure 3A). There were no significant differences in the prevalence of fatigue, headache, dyspnea, or confusion between clone size categories. The most frequently reported symptom was fatigue, reported in over 75% of patients in each clone size category. However, 83.3% of patients with clone size less than 10%, 89.3% with clone size 10-49%, and 93.3% with clone size 50% or over reported at least one symptom other than fatigue, whereas only 4.0%, 3.6%, and 0.8% of patients, respectively, reported fatigue and no other symptoms. In patients who had not been treated with eculizumab, the prevalence of dyspnea, hemoglobinuria, abdominal pain, scleral icterus, chest pain, and dysphagia was significantly greater in patients with LDH ≥1.5 x ULN at enrollment than in patients with lower LDH levels (Figure 3B). There were no significant differences in the prevalence of confusion, headache, fatigue, or erectile dysfunction between patients with LDH levels ≥1.5 x ULN and those with LDH levels <1.5 x ULN. Overall, 95.9% of patients with LDH ≥1.5 x ULN reported at least one symptom other than fatigue, whereas only 0.6% of patients with elevated LDH levels reported fatigue and no other symptoms. Similarly, 88.7% of patients with LDH <1.5 x ULN had symptoms other than fatigue, while only 2.0% reported fatigue alone.

For the majority of the patient-reported PNH-related

symptoms, history of BMD was not associated with prevalence of the symptom. However, hemoglobinuria, scleral icterus, and dysphagia were significantly more common in patients never diagnosed with BMD (all $P<0.001$).

QoL, hospitalization, and employment

Patient-reported EORTC QLQ-C30 QoL and FACIT-Fatigue assessments indicated that, compared with patients without a history of thrombosis, patients with a prior TE had significantly lower global health status/QoL ($P=0.01$), physical functioning ($P<0.01$), and social functioning (P=0.02) and significantly greater fatigue ($P=0.04$) (*Online Supplementary Figure S3*).

Statistically significantly lower QoL scores for all EORTC domains were reported by patients who had reported a clinical symptom of abdominal pain, chest pain, confusion, dysphagia, dyspnea, erectile dysfunction, fatigue, headache, hemoglobinuria, or scleral icterus in the six months prior to completing the baseline questionnaire compared with patients who had not experienced the symptom. All results were highly significant ($P<0.0001$) with the exception of emotional functioning for patients with erectile dysfunction ($P=0.039$), physical functioning for patients with hemoglobinuria ($P=0.003$), and emotional and cognitive functioning in patients with scleral icterus ($P=0.001$ and $P=0.007$, respectively).

In the six months prior to completion of the base-line questionnaire, 194 of 856 patients (22.7%) reported being hospitalized due to their PNH. Patients were significantly more likely to be hospitalized if they had a history of thrombosis or had experienced self-reported PNH-related symptoms of scleral icterus, chest pain, dysphagia, abdominal pain, hemoglobinuria, dyspnea, or fatigue in the past six months (all $P<0.01$) (*Online Supplementary Figure S4*).

The impact of PNH on a patient's employment was assessed in patients aged 18-59 years. Eighty-eight of 506 patients (17.4%) reported PNH as the reason they were either not working or working less (e.g. part time rather than full time). Of the 312 full-time or part-time workers, 82 (26.3%) had missed work in the past six months for reasons related to PNH.

Discussion

As of June 30, 2012, the International PNH Registry had enrolled 1610 patients from 5 continents, with completed patient questionnaires at enrollment available for 856 patients. Collection of outstanding data, as well as new recruitment into the registry, is ongoing. The current analysis evaluated the base-line characteristics of the enrolled population and investigated the burden of disease in terms of symptomatology and clinical outcomes. As the questionnaires completed at enrollment are largely concerned with the patients' medical history, much of the data are retrospective. However, these base-line data are an important foundation for future analyses on prospectively collected data.

Assessing data from an extensive population is important, particularly for rare conditions, as the disease course can vary significantly among patients; at enrollment our population has an age range spanning almost 100 years, a disease duration of less than one to 47 years, and granulocyte PNH clone sizes ranging from less than 1% to 100%. Given these variations, associations between patients' characteristics and outcomes and determination of robust therapeutic strategies can be achieved only via an international registry.

The level of morbidity was high in our patient population: at enrollment, 43.5% had a history of AA or hypoplastic anemia, 15.5% had experienced at least one TE, and 13.9% had a history of impaired renal function. The majority of patients not treated with eculizumab had LDH concentrations ≥1.5 x ULN, a level associated with significantly increased risks of TE and mortality.[22,23] Our analyses suggested an association between PNH granulocyte clone size and LDH levels; in patients with a clone size less than 10%, median LDH levels were towards the ULN, with only 8% of patients having LDH ≥1.5 x ULN, whereas in patients with a clone size 50% or over, median levels were to >4.0 x ULN and 62% of patients had LDH ≥1.5 x ULN. Elevated LDH levels were also associated with higher prevalence of TE and symptoms such as abdominal pain, chest pain, and hemoglobinuria, all significant risk factors for TE.[23]

It is perhaps not surprising that a significantly larger percentage of patients who at some point have been diagnosed with AA had received immunosuppressive therapy but less often received anticoagulation compared with patients with no history of BMD. This latter group of patients was significantly more likely to have been treated with eculizumab; however, the percentage of patients requiring red blood cell transfusions appeared to be independent of underlying BMD.

Not only is thrombosis, the leading cause of death in PNH,[7] associated with a significant risk for mortality, but patients experiencing a TE are 5-fold more likely to experience subsequent thrombotic events.[5,6,26] Our findings demonstrate that PNH patients at all clone sizes may have a history of thrombosis, and patients with larger clones were more likely to have a history of thrombosis. These analyses suggest that all patients with PNH are at risk for thrombosis. This finding indicates that all patients should be routinely monitored for signs and symptoms of TE, including clinically evident hemolysis and abdominal pain.

Overall, 31.5% of patients with a PNH clone size less than 10% had required at least one red blood cell transfusion in the year prior to enrollment. As the majority of patients with this PNH clone size for whom LDH assessments at enrollment were available did not show signs of elevated hemolysis, the reason for transfusions is most likely related to an underlying BMD. These and other concomitant conditions may also be the cause of some of the symptoms classified as "PNH related". It should be noted that not all of the transfusion, symptom and other data collected at enrollment may be directly related to hemolysis due to PNH, though collection and analysis of all such data are important in order to provide greater insight into the course of the disease and help to identify patients at risk of TE.

Our results showed that patients were significantly more likely to have been prescribed anticoagulant therapy if they had a history of TE, a larger granulocyte clone size, or elevated LDH concentrations. It must be remembered that these factors are not mutually exclusive but interrelated.

Common PNH-related symptoms were experienced by over 93% of patients for whom data was available. Although significantly more patients with a clone size

50% or over experienced hemoglobinuria, abdominal pain, scleral icterus, dysphagia, and erectile dysfunction, a substantial number of patients with a clone size less than 10% also experienced these symptoms, indicating that clone size alone is not a good indicator of burden of disease. The percentage of patients reporting PNH-related symptoms was greater in those with LDH levels ≥1.5 x ULN, with, for example, abdominal pain, a symptom associated with a 3.6-fold greater risk of thrombosis,[27] being significantly more common in patients with elevated LDH levels. Overall, our results confirm that patients with PNH and elevated LDH levels are at increased risk of experiencing complications of PNH associated with increased risk of TE, mortality, and poor QoL.

Reference values for FACIT-Fatigue[28] give a mean fatigue score of 43.6 in the general population and 40.0 in non-anemic cancer patients. The lower scores of 35.9 and 33.4 in patients without and with a history of thrombosis, respectively, indicate that PNH patients have a clinically meaningful greater level of fatigue[29] than either of these other 2 populations. Similarly, the EORTC global health QoL assessments indicated that patients with PNH had a clinically meaningful worse QoL[22] (with mean scores of 63.7 and 57.5 in patients without and with a history of thrombosis, respectively) than the general population (reference score 71.230) and similar QoL scores to those seen in cancer patients.[28] These findings show that PNH has a clinically meaningful impact on QoL in all patients, with the occurrence of a thrombotic event having a significantly greater impact on patient levels of fatigue and overall QoL.

The EORTC scores also showed that global health status in patients with PNH is most affected by fatigue, confusion, and chest pain; these symptoms also significantly impact physical, role, and emotional functioning. Abdominal pain significantly affects global health status, particularly in terms of role, emotional, and cognitive functioning. This is not surprising given that abdominal pain and chest pain have been associated with a greater risk of thrombosis and mortality.[27]

PNH has wide-ranging effects on patients' lives. The symptoms associated with the disease have serious consequences and are frequently devastating; one-quarter of PNH patients in the registry had been hospitalized and one-third had missed work due to PNH. Approximately one in 7 patients were not working or worked less due to PNH.

This large, global PNH registry of patients observed in clinical practice will continue to prospectively evaluate disease burden and the long-term natural history of PNH and treatment outcomes. The breadth and diversity of the registry provides an excellent basis for investigating research questions that may not be answerable from a single institution or country, and the registry has the additional advantages of collecting both clinical and patient-reported data and collecting these data both at enrollment and prospectively. The International PNH Registry will provide a valuable ongoing resource to further the clinical understanding of this rare, life-threatening disease.

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ORIGINAL ARTICLE

Increased glycosylphosphatidylinositol-anchored protein-deficient granulocytes define a benign subset of bone marrow failures in patients with trisomy 8

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Abstract

Trisomy 8 (+8), one of the most common chromosomal abnormalities found in patients with myelodysplastic syndromes (MDS), is occasionally seen in patients with otherwise typical aplastic anemia (AA). Although some studies have indicated that the presence of +8 is associated with the immune pathophysiology of bone marrow (BM) failure, its pathophysiology may be heterogeneous. We studied 53 patients (22 with AA and 31 with low-risk MDS) with +8 for the presence of increased glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) cells, their response to immunosuppressive therapy (IST), and their prognosis. A significant increase in the percentage of GPI-AP$^-$ cells was found in 14 (26%) of the 53 patients. Of the 26 patients who received IST, including nine with increased GPI-AP$^-$ cells and 17 without increased GPI-AP$^-$ cells, 14 (88% with increased GPI-AP$^-$ cells and 41% without increased GPI-AP$^-$ cells) improved. The overall and event-free survival rates of the +8 patients with and without increased GPI-AP$^-$ cells at 5 yr were 100% and 100% and 59% and 57%, respectively. Examining the peripheral blood for the presence of increased GPI-AP$^-$ cells may thus be helpful for choosing the optimal treatment for +8 patients with AA or low-risk MDS.

Key words trisomy 8; bone marrow failure; GPI-AP$^-$ cells; immunosuppressive therapy

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Karyotypic abnormalities in patients with bone marrow failure are generally regarded as a hallmark of clonal hematopoietic disorders with the propensity toward transformation into acute myeloid leukemia (AML). The incidence of cytogenetic abnormalities in aplastic anemia (AA) and myelodysplastic syndromes (MDS) is approximately 4% and 50%, respectively (1, 2). Trisomy 8 (+8), one of the most frequent chromosomal abnormalities found in patients with MDS, is occasionally seen in patients with otherwise typical AA (3–8). A recent study based on 2072 MDS patients showed that 8% of these patients had +8 in isolation (9). For both AML and MDS, +8 is listed in the 'intermediate-risk cytogenetic group' (6, 9, 10). Several studies have shown that MDS patients with +8 are highly responsive to immunosuppressive therapy (IST) (3, 7, 11). However, +8 in AA patients is associated with an increased risk of evolving into MDS/AML (5, 8). Thus, the prognostic significance of +8 in patients with AA or low-risk MDS remains unclear.

Small populations of glycosylphosphatidylinositol-anchored protein-deficient (GPI-AP$^-$) blood cells are often detected in the peripheral blood (PB) of patients with AA or low-risk MDS, such as refractory anemia (RA) and refractory cytopenia with multilineage dysplasia (RCMD) in the FAB classification (12–14). The GPI-AP$^-$ blood cells are detectable even in patients with BM failure who have chromosomal abnormalities, including +8 (15–17). Parlier and Longo

reported the first patient with GPI-AP$^-$ blood cells and + 8 who had ringed sideroblasts (18, 19). Our recent study showed a close association of del(13q) with the presence of increased GPI-AP$^-$ cells as well as a favorable response to immunosuppressive therapy (IST) (16). In patients with AA or RA possessing +8, the presence of GPI-AP$^-$ cells may affect response to IST as well as prognosis. To test this hypothesis, we analyzed clinical data of 53 BM failure patients with +8 whose blood cells were examined for the presence of GPI-AP$^-$ cells.

Patients and methods

Patients

This study included retrospective analysis of clinical records for 1228 BM failure patients: 733 with AA and 495 with low-risk MDS, including 286 with refractory cytopenia with unilineage dysplasia (RCUD), 149 with RCMD, and 60 with unclassified MDS (MDS-U). In all patients, blood samples were examined for the presence of GPI-AP$^-$ granulocytes and erythrocytes at our laboratory between May 1999 and July 2010. BM smear slides and trephine biopsy specimens were reviewed by two independent hematologists. BM cellularity was expressed as the percentage of BM volume occupied by hematopoietic cells in the trephine biopsy specimens. Hypocellular marrow was defined as <30% cellularity in patients <70 yr, or <20% cellularity in patients ≥70 yr (20). Chromosomal analysis was conducted using the G-banding method, and the presence of +8 clones was confirmed by fluorescent *in situ* hybridization (FISH) when the number of +8 revealed by G-banding was less than or equal to two. The results of G-banding were described according to the International System for Human Cytogenetic Nomenclature (ISCN) (21). The Ethics Committee of Kanazawa University Graduate School of Medical Science approved the study protocol, and all patients provided informed consent prior to sampling.

Therapy and response criteria

Horse anti-thymocyte globulin (ATG, Lymphoglobulin, Genzyme, Cambridge, MA, USA) in combination with cyclosporine (CsA) was given to patients with severe aplastic anemia (SAA). Four to 6 mg/kg of CsA was administered to patients with moderate AA (MAA) or MDS. Trough levels of CsA were maintained between 150 and 250 ng/mL. Six patients (four with AA and two with MDS) received 10–20 mg/d of methenolone acetate in addition to CsA. Responses to IST were defined according to the established criteria (22, 23).

Monoclonal antibodies

Monoclonal antibodies (mAbs) used for flow cytometry were FITC-conjugated anti-CD59 (P282E, IgG2a; Beckman Coulter, Brea, CA, USA), FITC-conjugated anti-CD55 (IA10, IgG2a; BD PharMingen, San Diego, CA, USA), PE-conjugated anti-CD11b/Mac-1 (ICRF44, IgG1; BD PharMingen), and PE-conjugated anti-glycophorin A (JC159, IgG1; Dako, Glostrup, Denmark).

Detection of GPI-AP$^-$ cells by flow cytometry

All blood samples were analyzed within 24 h of collection to avoid false-positive results due to cell damage. Staining with each mAb was performed according to the lyse-stain protocol as previously described (24, 25). The presence of CD55$^-$CD59$^-$glycophorin A$^+$ erythrocytes at the level of ≥0.005% and/or CD55$^-$CD59$^-$CD11b$^+$ granulocytes at the level of ≥0.003% was defined as an abnormal increase ('positive') based on results obtained from 183 healthy individuals (26). With careful handling of samples and elaborate gating strategies, cutoff values can be lowered to these levels without producing false-positive results (24, 27, 28).

Statistical analysis

Prevalence of increased GPI-AP$^-$ cells among different patient populations was compared using the chi-squared test. The Kaplan–Meier method and the Cox proportional hazards model were used to estimate time-to-event analysis. Overall survival (OS) was calculated in months from date of diagnosis until date of death or last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to AML evolution or death. Two-sided *P*-values were calculated, and $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using the EZR software package (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) (29).

Results

Incidence of BM failure patients with +8

Of 754 patients with AA, 22 (2.9%) possessed +8; instead, of 483 patients with low-risk MDS, 31 (6.4%) possessed +8. Their clinical features are summarized in Table 1. The median age of patients with +8 was 61, and BM was hypocellular in 32 patients, normocellular in 15, and hypercellular in six. Thirty-five patients had trisomy 8 alone (+8 alone), while 18 patients had additional chromosomal abnormalities (+8 others). The median percentage of +8 cells in karyotyped cells was 15%. Diagnoses of 31 MDS patients according to the 2008 WHO classification included nine patients with RCUD, 16 with RCMD, and six with MDS-U. None of the patients with +8 had ringed sideroblasts. All MDS patients were classified as Int-1 according to the International Prognostic Scoring System (IPSS).

Table 1 Clinical features of bone marrow failure patients with trisomy 8

UPN	Age	Sex	Dx	Cellularity	IPSS	% of +8 cells	Other abnormalities	% GPI-AP$^-$ Granulocytes	% GPI-AP$^-$ Erythrocytes	GPI-AP$^-$ cells	Treatment	Response	Outcome	Cause of death	AML transformation
1	69	M	SAA	Hypo	NE	5	–	0.034	0.038	Positive	ATG+CsA	PR	Death	Infection	No
2	21	F	SAA	Hypo	NE	25	+	39.124	2.106	Positive	ATG+CsA	PR	Alive		No
3	50	F	SAA	Hypo	NE	15	–	0.002	0.026	Positive	ATG+CsA →BMT	NR	Alive		No
4	26	F	SAA	Hypo	NE	10	–	0	0	Negative	ATG+CsA	PR	Death	Infection	No
5	16	M	SAA	Hypo	NE	20	–	0	0	Negative	ATG+CsA →BMT	NR	Death	Infection	No
6	26	M	SAA	Hypo	NE	15	–	0	0	Negative	Allo-BMT	NA	Death	Infection	Yes
7	68	M	SAA	Hypo	NE	5	–	0.002	0.002	Negative	Allo-BMT	NA	Alive		Yes
8	46	F	SAA	Hypo	NE	55	+	0	0.004	Negative	CsA→ Allo-BMT	NR	Death	GVHD	Yes
9	66	F	MAA	Hypo	NE	10	–	0.007	0	Positive	CsA	PR	Death	Infection	No
10	61	M	MAA	Hypo	NE	10	–	6.201	8.657	Positive	CsA	PR	Alive		No
11	50	M	MAA	Hypo	NE	10	–	0.033	0.039	Positive	CsA+AS	PR	Alive		No
12	71	M	MAA	Hypo	NE	10	–	0.049	0.092	Positive	CsA+AS	PR	Alive		No
13	68	M	MAA	Hypo	NE	5	–	0.363	0.045	Positive	No treatment	NA	Alive		No
14	65	F	MAA	Hypo	NE	80	–	0.64	0.327	Positive	No treatment	NA	Alive		No
15	69	M	MAA	Hypo	NE	15	–	0	0.001	Negative	CsA	NR	Death	Lung cancer	No
16	35	M	MAA	Hypo	NE	5	–	0	0	Negative	CsA	NR	Alive		No
17	79	M	MAA	Hypo	NE	45	+	0	0	Negative	CsA	NR	Alive		No
18	71	F	MAA	Hypo	NE	30	–	0	0.002	Negative	CsA	PR	Alive		No
19	10	M	MAA	Hypo	NE	80	–	0	0.004	Negative	CsA+AS	NR	Death	Pneumonia	No
20	33	M	MAA	Hypo	NE	35	–	0	0	Negative	CsA+AS	PR	Alive		No
21	60	F	MAA	Hypo	NE	25	–	0	0.001	Negative	No treatment	NA	Alive		No
22	31	F	MAA	Hypo	NE	NE	–	0	0	Negative	No treatment	NA	Alive		No
23	42	F	RCUD(RA)	Normo	Int-1	50	+	0	0	Negative	CsA	NR	Death	Heart failure	No
24	87	F	RCUD(RA)	Hypo	Int-1	35	+	0	0.003	Negative	CsA+AS	NR	Death	Infection	No
25	70	M	RCUD(RA)	Hyper	Int-1	5	–	0	0	Negative	CsA+AS	NR	Death	Heart failure	No
26	81	M	RCUD(RA)	Normo	Int-1	5	+	0	0	Negative	AS,VitK	Progression	Death	Progression	No
27	51	M	RCUD(RA)	Hypo	Int-1	70	+	0	0	Negative	Allo-BMT	NA	Alive		No
28	56	F	RCUD(RA)	Normo	Int-1	85	–	0	0.001	Negative	PSL	SD	Alive		No

(continued)

Table 1 (continued)

UPN	Age	Sex	Dx	Cellularity	IPSS	% of +8 cells	Other abnormalities	% GPI-AP$^-$ Granulocytes	% GPI-AP$^-$ Erythrocytes	GPI-AP$^-$ cells	Treatment	Response	Outcome	Cause of death	AML transformation
29	75	M	RCUD(RA)	Normo	Int-1	15	–	0	0.001	Negative	AS	Progression	Death	Progression	No
30	72	F	RCUD(RA)	Hypo	Int-1	75	+	0	0	Negative	No treatment	NA	Alive		No
31	66	F	RCUD(RA)	Normo	Int-1	10	+	0	0.01	Negative	No treatment	NA	Alive		No
32	81	M	RCMD	Hyper	Int-1	5	–	0.034	0	Positive	NA	NA	Alive		No
33	88	F	RCMD	Hyper	Int-1	50	–	0.142	0.23	Positive	AS	PR	Alive		No
34	18	F	RCMD	Hypo	Int-1	55	+	0.003	0.008	Positive	Allo-PBSCT	NA	Alive		No
35	59	F	RCMD	Hyper	Int-1	5	–	0.001	0.001	Negative	CsA	CR	Alive		No
36	65	M	RCMD	Hypo	Int-1	5	–	0	0.001	Negative	CsA	HI-1	Alive		No
37	28	F	RCMD	Normo	Int-1	20	–	0	0	Negative	CsA	HI-1	Alive		No
38	61	F	RCMD	Hyper	Int-1	100	–	0	0	Negative	CsA	HI-2	Death	Bleeding	No
39	59	M	RCMD	Hypo	Int-1	35	+	0.001	0.003	Negative	CsA	Progression	Death	Progression	Yes
40	51	F	RCMD	Hypo	Int-1	13	+	0	0.002	Negative	PSL	SD	Death	Infection	No
41	89	M	RCMD	Normo	Int-1	50	–	0	0	Negative	AraC	Progression	Death	Progression	No
42	72	M	RCMD	Normo	Int-1	10	+	0	0	Negative	VitK	NA	Death	Pneumonia	No
43	50	F	RCMD	Normo	Int-1	NE	–	0	0.001	Negative	AS,VitK	SD	Alive		No
44	77	F	RCMD	Normo	Int-1	10	+	0	0.003	Negative	AS	Progression	Death	Progression	Yes
45	7	M	RCMD	Normo	Int-1	10	–	0	0.002	Negative	Allo-BMT	NA	Death	TMA	No
46	63	F	RCMD	Normo	Int-1	NE	+	0	0	Negative	No treatment	NA	Death	Progression	No
47	56	F	RCMD	Normo	Int-1	40	–	0	0.002	Negative	No treatment	NA	Alive		No
48	70	F	MDS-U	Hypo	Int-1	5	+	0.005	0.023	Positive	CsA	CR	Alive		No
49	81	M	MDS-U	Hypo	Int-1	95	+	6.851	0.272	Positive	CsA	NA	Alive		No
50	75	M	MDS-U	Hyper	Int-1	5	–	0	0	Negative	AS	HI-3	Death	Infection	No
51	77	M	MDS-U	Normo	Int-1	35	+	0	0	Negative	AS,VitK	Progression	Death	Progression	No
52	34	F	MDS-U	Hypo	Int-1	NE	–	0	0	Negative	Allo-PBSCT	NA	Alive		No
53	55	F	MDS-U	Normo	Int-1	45	–	0	0	Negative	No treatment	NA	Alive		No
Median	61					15									

AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; RCMD, refractory cytopenia with multilineage dysplasia; RCUD, refractory cytopenia with unilineage dysplasia; SAA, severe aplastic anemia.

Prevalence of patients possessing increased GPI-AP$^-$ cells

As shown in Table 1, 14 (26.4%) of patients with +8 had GPI-AP$^-$ cells that accounted for 0.003% to 39.124% (median, 0.049%) of granulocytes. One patient who possessed 0.002% GPI-AP$^-$ granulocytes was judged positive because 0.026% of the patient's erythrocytes were GPI-AP$^-$ cells (Figure S1). None of the patients evolved into clinical PNH during the observation period of 2–10 yr. The prevalence of increased GPI-AP$^-$ cells was lower than that (43%) in 937 BM failure patients (637 with AA and 300 with MDS) with normal karyotype (16). Of 22 AA patients with +8, nine (41%) had increased GPI-AP$^-$ cells; instead, of 31 low-risk MDS patients with +8, five (16%) had increased GPI-AP$^-$ cells ($P = 0.04$).

Response to IST in BM failure patients with +8

Twenty-six patients (49%) were treated with IST, and 25 of these had evaluable responses. IST included CsA alone in 15 patients, CsA and ATG in five patients, and CsA and methenolone acetate in six patients. The overall response rate to IST in the +8 patients was 56% (14/25 patients). Of 16 AA patients with +8 treated with CsA and ATG (5) or CsA ± methenolone acetate (11), nine (56%) responded. Nine MDS patients with +8 were treated with CsA ± methenolone acetate, and five (56%) improved ($P = 0.97$). Of eight patients positive for GPI-AP$^-$ cells treated with IST, 7 (88%) responded; instead, of 17 patients negative for GPI-AP$^-$ cells, 7 (41%) responded ($P = 0.03$). Comparison of patients with +8 with 141 BM failure patients (120 with AA and 21 with MDS) with normal karyotypes that were included in our previous study (16) showed that +8 patients had lower response rates to IST than patients with normal karyotypes, 56% in +8 AA patients vs. 81% in normal karyotype AA patients ($P = 0.03$) and 56% in +8 MDS patients vs. 62% in normal karyotype MDS patients ($P = 0.75$), although the differences were not statistically significant in MDS patients (16).

Prognosis in BM failure patients with +8

None of the 14 + 8 patients with increased GPI-AP$^-$ cells progressed to advanced MDS or AML during the follow-up period of 2–239 months (median, 67 months). On the other hand, five of the 39 + 8 patients without GPI-AP$^-$ cells developed AML. The 5-yr OS and EFS rates of the 53 patients with +8 patients were 69.4% and 68.1%, respectively (Fig. 1A). The 5-yr OS/EFS rates of +8 patients with increased GPI-AP$^-$ cells were 100%/100%; instead, the 5-yr OS/EFS rates of +8 patients without increased GPI-AP$^-$ cells were 58.6%/56.9% ($P = 0.0347$, $P = 0.0269$, respectively; Fig. 1B). The 5-yr OS rates of +8 patients with +8 alone were 81.7%; instead, the 5-yr OS rates of +8 patients with +8 with other abnormalities were 45.5% ($P = 0.0196$; Fig. 1C). When age, gender, diagnosis, cellularity, clone size, karyotype complexity, and GPI-AP$^-$ cells were included in the multivariate analysis, higher age (60 yr or older) and the absence of GPI-AP$^-$ cells represented independent negative predictors for OS (Table 2).

To further evaluate the significance of GPI-AP$^-$ cells in +8 patients, the 5-yr OS rates of BM failure patients with +8 were compared with those of 246 BM failure patients (179 with AA and 67 with MDS) with normal karyotype who were included in our previous study (16). There was no significant difference in the survival rates between the two groups with increased GPI-AP$^-$ cells (100% vs. 92.7% $P = 0.914$; Fig. 2A), while the survival rate of +8 patients without increased GPI-AP$^-$ cells (58.6%) was lower than that of patients with normal karyotype not possessing increased GPI-AP$^-$ cells (79.5%, $P = 0.0007$; Fig. 2B).

Discussion

The current retrospective study of a large number of BM failure patients revealed distinctive clinical features of BM failure patients with +8 abnormalities. Of the 483 patients with low-risk MDS, 31 (6.6%) possessed +8, which was comparable to the 8% reported in a recent study of 2072 MDS patients (2). That study did not provide any detailed diagnoses of the patients with +8. The present study detected GPI-AP$^-$ cells in 26.4% of patients with +8, and the prevalence of increased GPI-AP$^-$ cell percentages was higher in AA patients (41%) than in those with low-risk MDS (16%). This study is the first to reveal the prevalence of increased GPI-AP$^-$ cell percentages based on a large number of AA and low-risk MDS patients with +8.

Approximately half of the patients with +8 were treated with IST, with an overall response rate of 56%. The relatively high response rate was probably achieved because IST was only administered to patients who had clinical features associated with a good response to IST, such as a short disease duration and the presence of thrombocytopenia with decreased megakaryocytes (30). The response rates were similar between AA (56%) and low-risk MDS patients (56%). However, there was a significant difference in the response rate between the patients with and those without increased GPI-AP$^-$ cells (88% vs. 41%).

Consistent with our current data, several studies demonstrated that AA and MDS patients with +8 are likely to respond to IST (3, 7, 11). There may thus be a common mechanism underlying the preferential commitment of hematopoietic progenitor clones with +8 in immune-mediated BM failures. One study revealed an increased expression of the WT1 gene by BM mononuclear cells from MDS patients with +8, which may elicit specific T-cell responses to WT1 peptides and lead to the suppression of non-+8 hematopoietic

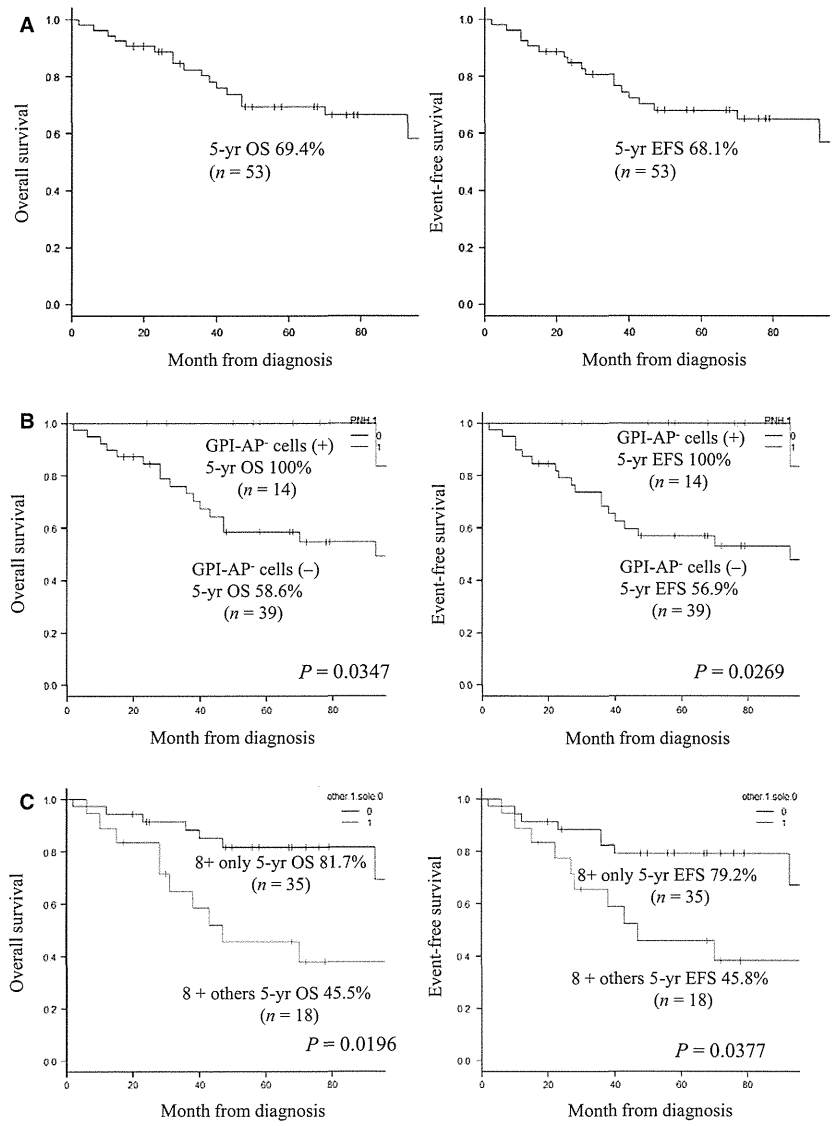

Figure 1 Overall and event-free survival rates of BM failure patients with trisomy 8. (A) Five-year overall survival (OS) and event-free survival (EFS) rates of +8 patients. (B) Five-year OS and EFS rates of +8 patients with and without increased GPI-AP$^-$ cells. (C) Five-year OS and EFS rates of +8 patients with +8 alone and +8 with other abnormalities (8+ others). The EFS was defined as the time from diagnosis to acute myeloid leukemia (AML) evolution or death.

Table 2 Results of multivariate analysis of prognostic factors for overall survival of patients with BM failure with trisomy 8

		BMF with trisomy 8	
Variable	Categories	Hazard ratio (95% CI)	*P*-value
Age	≥60 yr vs. <60 yr	3.9 (1.1–13.6)	<0.05
Sex	Male vs. female	1.5 (0.6–4.2)	0.42
Diagnosis	AA vs. MDS	1.3 (0.3–5.8)	0.74
Cellularity	Hypocellular vs. others	0.5 (0.1–1.9)	0.31
Karyotype complexity	8+ alone vs. 8+ others	0.4 (0.1–1.3)	0.12
Clone size (% of +8 cells)	≥15% vs. <15%	0.5 (0.2–1.3)	0.16
GPI-AP$^-$ cells	Positive vs. negative	0.1 (0.02–0.7)	<0.05

GPI-AP$^-$ cells, glycosylphosphatidylinositol-anchored protein-deficient blood cells; AA, aplastic anemia; MDS, myelodysplastic syndrome; CI, confidence interval; BMF, bone marrow failure.

progenitor cells by bystander effects of activated T cells (11). The same group proposed that BM CD34$^+$ cells of +8 patients exhibit resistance to apoptosis and increased myc expression as the mechanisms underlying the proliferative advantage of +8 clones (31). We were unable to examine WT1 gene expression and the number of WT1-specific T cells in our +8 patients

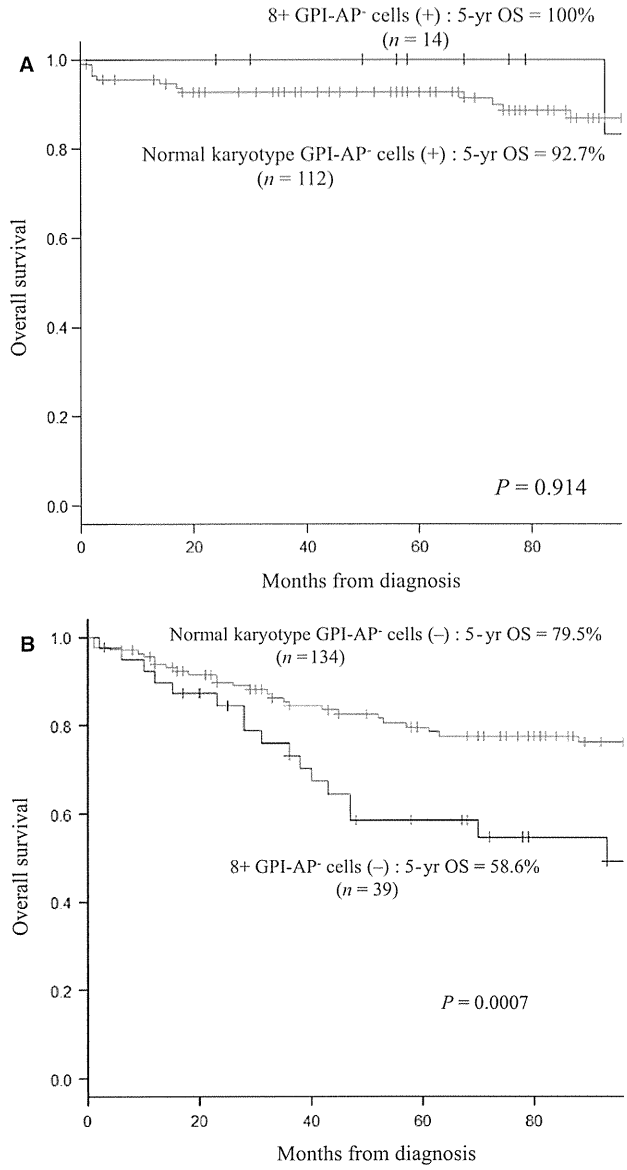

Figure 2 Overall survival rates of BM failure patients with trisomy 8 and normal karyotype. (A) Five-year overall survival (OS) rates of +8 patients and normal karyotype patients with increased GPI-AP$^-$ cells. (B) Five-year OS rates of +8 patients and normal karyotype patients without increased GPI-AP$^-$ cells.

who were responsive to IST. However, we believe that the specific immune responses to +8 clones may not be the main mechanism underlying the immune-mediated BM failure, for the following reasons: First, if the immune response is directed against +8 clones, successful T-cell suppression by IST should lead to the expansion of the abnormal clone. In reality, the changes in the percentage of +8 clones in patients responding to IST were highly variable and did not show a steady increase (Figure S2). Second, the likelihood of responding to IST was determined by the presence of GPI-AP$^-$ cells, not by the +8 clones; the +8 patients did not respond better to IST than patients with a normal karyotype (56% of AA patients with +8 vs. 81% of AA patients with a normal karyotype and 56% of MDS patients with +8 vs. 62% of MDS patients with a normal karyotype). Third, leukocytes with copy number-neutral loss of heterozygosity in the short arm of chromosome 6 (6pLOH) should be detected in patients with +8 if they are targets of cytotoxic T-cell attacks, based on our previous study showing that leukocytes with 6pLOH are detectable in 13% of AA patients (32). However, none of the six patients with +8 studied in the present population had leukocytes with 6pLOH (data not shown).

The IPSS classifies +8 as an intermediate risk factor for the progression of MDS (10, 33). The prognostic significance of +8 was confirmed by recent studies that involved MDS with at least 5% blasts (34). However, its significance in patients with AA and low-risk MDS with less than 5% blasts has not been extensively studied. In contrast to previous reports (3, 11), this study revealed that AA and MDS with less than 5% blasts comprise a subset of patients with a propensity to evolve into AML. Recently, Schanz *et al.* studied 2902 MDS patients including 133 patients with +8 who had a median blast percentage of 4% in their BM and revealed that the median overall survival of the 133 patients was 23 months (6). However, this study included 1190 (42.7%) patients with blast percentages >5% in the BM. The median overall survivals in our 53 patients with +8 were 78 months in AA and 43 months in MDS patients. This study is the first to estimate the overall survival in AA and low-risk MDS patients with +8 whose blast percentage in the BM is less than 5% based on a large number of patients.

On the other hand, the finding that the 5-yr EFS of +8 patients with an increased GPI-AP$^-$ cell percentage was 100% suggests that this subset of +8 BM failures is a benign type of BM failure similar to that of AA patients with normal karyotypes possessing increased GPI-AP$^-$ cells rather than a clonal disorder associated with a high risk of developing AML. The median age (66 yr vs. 59 yr) and prevalence of hypercellular marrow (14% vs. 10%) in patients with and without GPI-AP$^-$ cells were similar.

By comparing clinical courses between +8 patients and normal karyotype patients, both patient groups with increased GPI-AP$^-$ cells proved to have good prognosis regardless of the presence of +8, while in patients without increased GPI-AP$^-$ cells, the survival rate of +8 patients was significantly lower than that of patients with normal karyotype, strongly suggesting the importance of detecting GPI-AP$^-$ cells in predicting the prognosis of +8 patients. The WHO 2008 classification defined +8 as an intermediate-risk abnormality of MDS. The BM failure patients with +8 possessing an increased number of GPI-AP$^-$ cells may therefore be treated in an inappropriate way such as with hypomethylating agents and allogeneic stem cell transplantation from unrelated donors. Therefore, our present

findings suggest that it is important to determine whether increased GPI-AP$^-$ cells are detectable when BM failure patients are found to have +8. The significance of detecting GPI-AP$^-$ cells in +8 patients needs to be confirmed by prospective studies involving a large number of BM failure patients.

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Authorship contributions

K.H. and N.S. contributed equally to this work and participated in designing and performing the research. K.H. conducted statistical analysis; N.S., T.K., Y.S., C.S, K.M., H.Y., and A.T. contributed patient samples and data; S.N. initiated and designed the study; K.H. wrote the manuscript with contributions from N.S. All authors critically reviewed the final manuscript.

Disclosure of conflict of interest

The authors report no potential conflict of interests.

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