Expansion of donor-derived hematopoietic stem cells with PIGA mutation associated with late graft failure after allogeneic stem cell transplantation.

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Expansion of a donor-derived hematopoietic stem cell with *PIG-A* mutation associated with late graft failure after allogeneic stem cell transplantation.

Short title: *PIG-A* mutant stem cell expansion with LGF

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Abstract

A small population of $CD55^{-}CD59^{-}$ blood cells was detected in a patient who developed donor-type late graft failure after allogeneic stem cell transplantation (SCT) for treatment of aplastic anemia (AA). Chimerism and *PIG-A* gene analyses showed the paroxysmal nocturnal hemoglobinuria (PNH)-type granulocytes to be of a donor-derived stem cell with a thymine insertion in *PIG-A* exon 2. A sensitive mutation-specific PCR-based analysis detected the mutation exclusively in DNA derived from the donor bone marrow (BM) cells. The patient responded to immunosuppressive therapy and achieved transfusion independence. The small population of PNH-type cells was undetectable in any of the 50 SCT recipients showing stable engraftment. The *de novo* development of donor-cell derived AA with a small population of PNH-type cells in this patient supports the concept that glycosyl phosphatidylinositol anchored protein deficient stem cells have a survival advantage in the setting of immune mediated BM injury.

Introduction

Although small populations of $CD55^{-}CD59^{-}$ blood cells are often detectable in patients with aplastic anemia (AA), it remains unclear how such paroxysmal nocturnal hemoglobinuria (PNH)-type cells arise. [1] We recently encountered a patient with immune-mediated late graft failure (LGF) following allogeneic stem cell transplantation (SCT) for treatment of AA. Analyses of the the patient's peripheral blood (PB) and bone marrow (BM) showed an HSC of donor origin with mutant *PIG-A*, supporting the concept that glycosyl phosphatidylinositol anchored protein (GPI-AP) deficient stem cells have a survival advantage in the setting of immune mediated BM injury.

Materials and methods

Patients

A 59-year-old male underwent allogeneic PBSCT from an HLA-matched sibling donor after conditioning with fludarabin 120 mg/m^2, cyclophosphamide 1200 mg/m^2, and antithymocyte globulin 60 mg/kg for treatment of very severe AA in April 2002 (Table 1) and achieved complete donor chimerism with normal blood cell counts. In January 2006, he developed pancytopenia and was diagnosed as having LGF without residual recipient cells. The patient underwent a second PBSCT from the original donor without preconditioning on February 8, 2006. Pancytopenia

resolved completely by day 16 post PBSCT. However, at approximately day 60, the blood counts decreased gradually, and the patient became transfusion dependent. On day 196 after the second PBSCT, the WBC was $5.3x10^9$ /L with 17% neutrophils, the Hb concentration was 75 g/L, and the platelet count was $22x10^9$ /L. Treatment with horse antithymocyte globulin (ATG) and cyclosporine was started on day 205 after the second PBSCT. Transfusions were terminated after 88 days of the immunosuppressive therapy. Although he presently receives low-dose tacrolimus for treatment of chronic graft-versus host disease which developed 1 year after the second PBSCT, his pancytopenia has markedly improved as shown in Table 1. PB and BM of the patient were subjected to analyses of chimerism, flow cytometry to detect $CD55^-CD59^-$ cells, and *PIG-A* gene analysis.

As controls, the PB from 51 SCT recipients (48 with hematologic malignancies and 3 with AA) who achieved a complete recovery of donor-derived hematopoiesis were subjected to flow cytometric analysis for the screening of $CD55^-CD59^-$ cells. Of the 51 patients, 4 and 23, respectively had acute graft-versus-host disease (GVHD) ≥grade II and chronic GVGD at sampling.

BM aspirates were obtained from the patient's donor and 10 healthy individuals for *PIG-A* gene analysis. All patients and healthy individuals gave their informed consent according to the Declaration of Helsinki for blood examination, and the experimental protocol for *PIG-A* gene analysis

was approved by our participating institutional ethics committee (No.157).

Detection of PNH-type cells

To detect GPI-AP deficient (GPI-AP$^-$), PNH-type cells, we performed high-sensitivity 2-color flow cytometry of granulocytes and red blood cells (RBCs), as described previously. [1] The presence of ≥0.003% CD55$^-$CD59$^-$CD11b$^+$ granulocytes and ≥0.005% CD55$^-$CD59$^-$glycophorin-A$^+$ RBCs was defined as an abnormal increase based on the results in 183 healthy individuals. [2]

Cell sorting and chimerism analysis

CD3$^+$cells were isolated from the PB mononuclear cells of the patient using MACS CD3 Microbeads (Miltenyi Biotec, Auburn, CA). The CD55$^-$CD59$^-$CD11b$^+$ granulocytes were separated from the CD55$^+$CD59$^+$CD11b$^+$ granulocytes with a cell sorter (JSAN; Bay Bioscience Co., Ltd., Yokohama, Japan). More than 95% of the sorted cells were CD55$^-$CD59$^-$CD11b$^+$. The *D1S80* locus was amplified from DNA of different cell populations with an AmpliFLP D1S80 PCR Amplification Kit (Perkin-Elmer Cetus, Norwalk, CT).

***PIG-A* gene analysis**

The coding regions of *PIG-A* were amplified by semi-nested PCR or nested PCR from DNA extracted from the sorted PNH-type cells using 12 primer sets [3,4] (Supplemental table online), and 6 ligation reactions were used to transform competent *Escherichia coli* JM109 cells (Nippon Gene, Tokyo,

Japan). Five clones were selected randomly from each group of transfectants and subjected to sequencing with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Amplification refractory mutation system (ARMS)-PCR

On the basis of a mutant sequence detected in *PIG-A* of the patient, a nested amplification refractory mutation system (ARMS) forward primer with a 3'-terminal nucleotide sequence complementary to the mutant sequence was prepared [5] (Supplemental table online). To enhance the specificity, a mismatch at the penultimate nucleotide position of the mutation site was incorporated in the ARMS forward primer (P1).[6,7] P1 and a reverse primer (P3) were used to amplify a 127 bp fragment containing the mutant sequence from the exon 2 amplified product. PCR was conducted under the following conditions; denaturation for 30 seconds at 94 °C, annealing for 60 seconds at 64 °C and extension for 90 seconds at 72 °C for 20 cycles. Another forward primer (P2), complementary to the wild-type *PIG-A* sequence upstream of the mutation site, was used in combination with P3 to amplify an internal control according to the same condition of ARMS-PCR.

Results and discussion

PNH-type cells were not detected in the donor or the patient at the time

of development of the first LGF, whereas 0.147% PNH-type granulocytes and 0.019% PNH-type RBCs were detected in the PB obtained at the time of development of the second LGF (Figure 1A). Similar percentages of PNH-type blood cells were detectable in the PB of the patient 6 and 14 months later. When PB from 51 SCT recipients was examined, none of the patients were found to have detectable PNH-type cells (data not shown). PNH-type blood cells were also undetectable in a donor PB sample obtained 21 months later.

The *D1S80* locus allelic pattern of the PNH-type granulocytes in the patient was compatible to that of the donor (Figure 1B). The emergence of donor-derived PNH-type cells and hematologic improvement after immunosuppressive therapy suggest that LGF arises as a result of de novo development of AA which affects the donor-derived hematopoietic stem cells (HSCs).

PIG-A gene analysis of the DNA prepared from the sorted PNH-type cells of the patient obtained at the development of LGF and 6 months later showed an insertion of thymine at position 593 (codon 198) in 3 of 5 clones and 5 of 5 clones examined, respectively (Figure 1C). Mutations in other exons were not identified. The presence of a single *PIG-A* mutation in PNH-type granulocytes and its persistence over 6 months suggests that these PNH-type cells are derived from a mutant HSC rather than from a committed granulocyte progenitor cell. Moreover, an ARMS-PCR with a 5'

primer specific to the mutated sequence amplified a 127 bp fragment from DNA of the donor BM as well as of the recipient BM and PB while it failed to amplify the same fragment in donor PB and in BM of all 10 healthy individuals (Figure 1D).

These experiments demonstrate that the *PIG-A* mutant HSC was present in the BM of the donor in a dormant state and was transplanted into the recipient and provide, for the first time, *in vivo* evidence that *PIG-A* mutant, GPI-AP deficient HSCs have a survival advantage in the setting of immune mediated BM injury. Similarly, relative resistance to immune injury likely accounts for the high incidence of PNH observed in association with acquired AA.

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Authorship

Author contribution: K.M. and C.S. contributed equally to this work and participated in designing and performing the research. Z.Q. and X.L. performed experiments. K.M., C.S., and S.N. wrote the paper. C.S., A.T., K.I., Y.K., H.Y., and H.O. provided patient care. All authors have

approved the final version of the manuscript.

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Figure legends

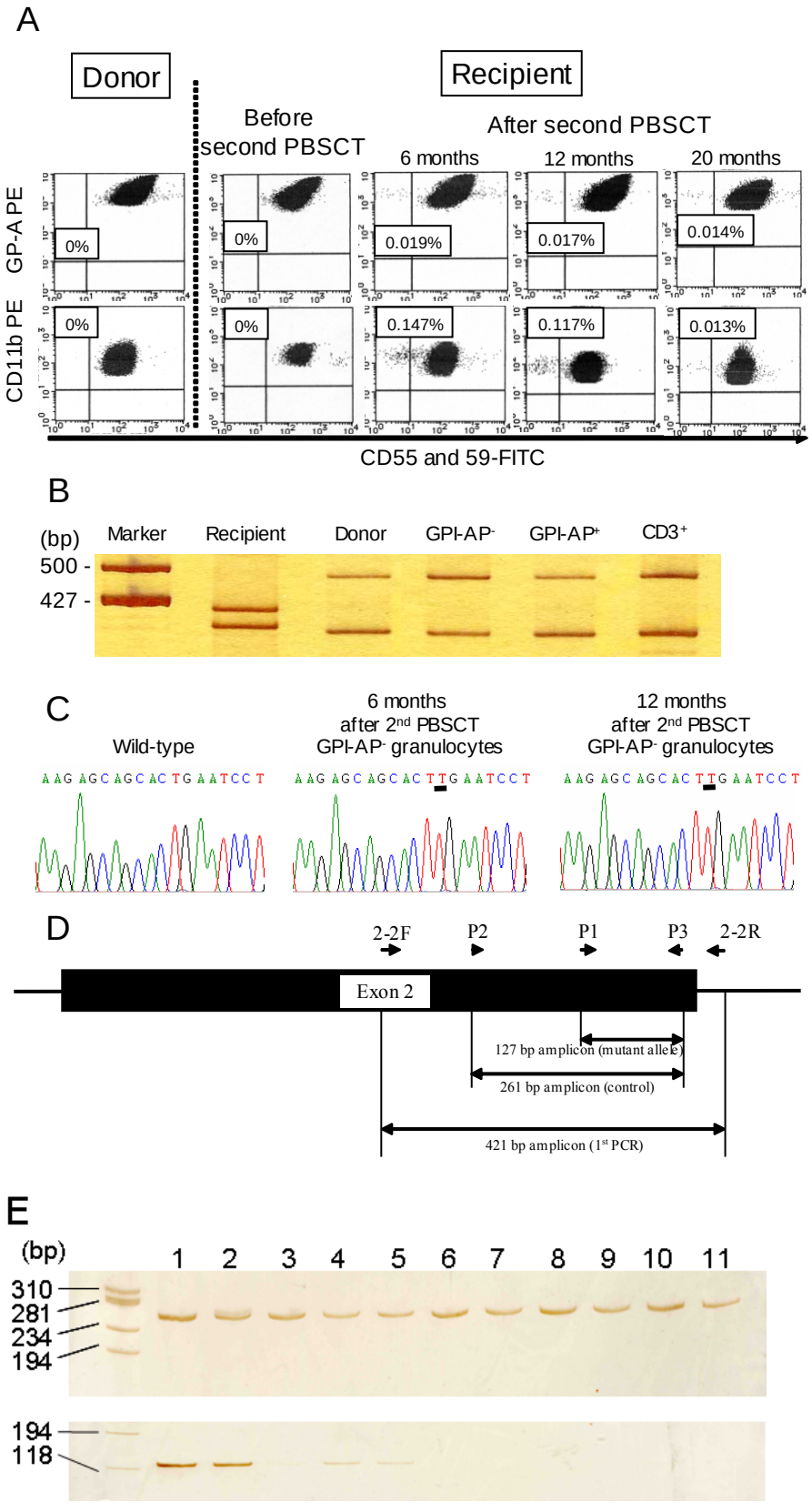
Figure1. Analysis of PNH-type cells after the second PBSCT. (A) High-sensitivity flow cytometry detected small populations of $CD55^{-}CD59^{-}$cells in both granulocytes and red blood cells at the development of the second LGF as well as in those obtained 6 and 12 months later, but did not detect PNH-type cells in the donor or in the recipient before the second PBSCT. The numbers denote the proportion of PNH-type cells in $CD11b^{+}$ granulocytes or glycophorin A^{+} RBCs. (B) *D1S80* allelic patterns of sorted GPI-AP^{-} granulocytes, GPI-AP^{+} granulocytes, and $CD3^{+}$ lymphocytes. The polymerase chain reaction (PCR) products were subjected to 8% polyacrylamide gel electrophoresis and visualized by silver staining. (C) Nucleotide sequences of *PIG-A* exon 2 in DNA from PNH-type granulocytes obtained 6 and 12 months after the second PBSCT. (D) A schematic illustration for ARMS-PCR is shown. Primer positions for the first, second are shown by short arrows. A black box and adjacent lines represent exon 2 and introns, respectively. (E) Amplified products of control PCR (the upper gel) and ARMS-PCR (the lower gel) were electrophoresed in 12.5% polyacrylamide gel and visualized by the silver staining. A pMD20-T vector containing the mutated exon 2 fragment was used as a positive control for ARMS-PCR. The template DNA derives from a plasmid containing the mutated exon 2 in lane 1, donor BM in lane 2, donor PB in lane 3, recipient BM in lane 4,

recipient PB in lane 5, and BM from healthy individuals in lanes 6-11. PCR with a 5'primer specific to the nucleotide sequence upstream of the mutated sequence amplified a 261 bp fragment from DNA of the donor and all healthy individuals.

Table1 Hematologic parameters of donor and recipient.

	Donor		Recipient			
			Before 1st SCT	Before 2nd SCT	at ATG therapy	20 months of ATG therapy
	Apr 2002	May 2007	Apr 2002	Jan 2006	Aug 2006	Apr 2008
White Blood Cell Count, $x10^9$/L	7.0	5.1	1.2	1.7	5.3	4.0
Neutrophil Proportions (%)	77	65	0	0	17	62
Red Blood Cell Count, $x10^{12}$/L	4.21	4.43	2.20	2.75	2.07	3.04
Reticulocytes, $x10^9$/L	not tested	35	2	3	26	61
Hemoglobin, g/L	146	150	72	89	75	120
Platelet Count, $x10^9$/L	261	230	19	52	22	54

Figure 1


A
Donor
Recipient
Before second PBSCT
After second PBSCT
6 months
12 months
20 months
GP-A PE
CD11b PE
0%
0%
0%
0%
0.019%
0.147%
0.017%
0.117%
0.014%
0.013%
CD55 and 59-FITC
B
(bp)
500 -
427 -
Marker
Recipient
Donor
GPI-AP-
GPI-AP+
CD3+
C
Wild-type
6 months after 2nd PBSCT GPI-AP- granulocytes
12 months after 2nd PBSCT GPI-AP- granulocytes
AAG AGC AGC ACT TG AATCCT
AAG AGC AGC ACT TTG AATCCT
AAG AGC AGC ACT TTG AATCCT
D
2-2F
P2
P1
P3
2-2R
Exon 2
127 bp amplicon (mutant allele)
261 bp amplicon (control)
421 bp amplicon (1st PCR)
E
(bp)
1 2 3 4 5 6 7 8 9 10 11
310
281
234
194
194
118

Supplemental table PCR primers

Primer	Sequence 5' → 3'	Location	Annealing Temperature, °C	Product size, bp
1st PCR primers				
2-1F*	GAGCTGAGATCCTGTTTTAC	intron1	58.0	625
2-1R*	GTATCACAAAGAGACACGG	exon2	58.0	-
2-2F*	CATTGCTCAGGTACATATTTGTT	exon2	58.0	421
2-2R*	AAAGTCTACAATGCAATTATAGC	intron2	58.0	-
3F*	AAGTGGATTCTCAGTCGTTCTGGT	intron2	58.0	247
3R*	CTTCTCCCTCAAGACAACATGAA	intron3	58.0	-
4F	ACTCCTTTCTTCCCCTCTCATT	intron3	58.0	206
4R	CACAGAAATCCCAACCATGAAT	intron4	58.0	-
5F†	TCTTCCTGAGGTATGATTATGGTG	intron4	58.0	306
5R	CATCAAGTAAGAGTTCAGACACAAT	intron5	58.0	-
6F†	GTCATTGTTTATCATGGGACAG	intron5	58.0	360
6R†	TCTTACAATCTAGGCTTCCTTC	exon6	58.0	-
2nd (nested or semi-nested) PCR primers				
2-1F*	GAGCTGAGATCCTGTTTTAC	intron1	60.0	598
2-1R2	TTGTTTGTAAGCACCGAG	exon2	60.0	-
2-2F2	GAGAGAGTCACGATAATCCA	exon2	60.0	395
2-2R*	AAAGTCTACAATGCAATTATAGC	intron2	60.0	-
3F2	ATTAATATCTCCAACATTCAACTTT	intron2	60.0	203
3R*	CTTCTCCCTCAAGACAACATGAA	intron3	60.0	-
4F	ACTCCTTTCTTCCCCTCTCATT	intron3	60.0	193
4R2	ACCATGAATGCCCTCAAAGC	intron4	60.0	-
5F2	GATTATGGTGGAGTATGTGTTC	intron4	60.0	269
5R2	ATCTTTTCTCAGCATGTGAC	intron5	60.0	-
6F2	TGATGGGTGAAAGTGCTC	intron5	60.0	329
6R†	TCTTACAATCTAGGCTTCCTTC	exon6	60.0	-
ARMS-PCR Primers				
P1	ACTGTACTAAGAGCAGCAGTT	exon2	64.0	127
P2	TTCAGACAGTCTTCACGG	exon2	64.0	261
P3	GTCTGCTGACAACAACAATAG	exon2	64.0	-

* primers derived from a reference by Kai et al.[3]

† primers derived from a reference by Mortazavi et al.[4]