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Short Communication

Mean fluorescence intensity rate is a useful marker in the detection of paroxysmal nocturnal hemoglobinuria clones

Zsuzsa Hevessy[1], Béla Nagy Jr[1], Flóra Kiss[1], Attila Kiss[2] and János Kappelmayer[1,*]

[1] Department of Clinical Biochemistry and Molecular Pathology,
[2] 2nd Department of Medicine,
Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disorder of the pluripotent stem cell resulting from the somatic mutation of the X-linked *PIG-A* gene, involved in the synthesis of the glycosylphosphatidyl-inositol (GPI) anchor of membrane proteins such as CD55, CD59 and CD14. In the past decade, flow cytometry has become a valuable diagnostic tool in the detection of deficient expression of the GPI-anchored proteins. We report the diagnosis of PNH in four patients confirmed by flow cytometry. Red blood cells, granulocytes and monocytes were classified as PNH types I, II and III according to the mean fluorescence intensities (MFI) of membrane proteins. The MFI rate is a numerical data reflecting the severity of decreased antigen expression, and it is obtained by dividing the MFI of the type II or type III cells by the MFI of the respective cells obtained for a normal sample. We found that the investigation of granulocytes and monocytes was more informative than red blood cells when percent negativity was evaluated. In addition, the lowest MFI rate (mean 0.011) was obtained for CD14 on monocytes while CD59 and CD55 gave higher values on all three investigated cell types (0.021–0.34). Thus, CD14 on monocytes seems to be the most reliable marker for establishing the PNH clone size and the severity of antigen deficiency.

Keywords: CD14; CD55; CD59; mean fluorescence intensity; paroxysmal nocturnal hemoglobinuria.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disorder of the pluripotent stem cell resulting from the somatic mutation of the X-linked *PIG-A* gene (1–3). This gene is involved in the synthesis of the glycosylphosphatidyl-inositol (GPI) anchor of several membrane proteins, such as CD55 (decay accelerating factor), CD59 (membrane inhibitor of reactive lysis) and CD14 (lipopolysaccharide receptor), also known as a monocyte marker [reviewed in Ref. (4)]. Mutation of the *PIG-A* gene aborts synthesis of the GPI-anchored proteins on the surface of erythrocytes, leukocytes and platelets (5). Recently it was verified that clonal populations of hemopoietic cells with PNH genotype and phenotype are present in normal bone marrow samples (6, 7). The development of PNH requires the simultaneous presence of two factors: the mutated stem cells and an abnormal, hypoplastic bone marrow environment (8, 9). The relative growth advantage of the PNH clone in patients is partly explained by the fact that non-mutant stem cells from PNH bone marrow have a higher apoptotic rate than *PIG-A* mutant stem cells, resulting in the clonal expansion of the mutant clone in the hostile marrow environment (10, 11). Alternatively, the *PIG-A* mutation might confer a relative advantage to PNH clones over normal cells. This theory is also supported by current experimental data indicating that *PIG-A* mutant cells are relatively resistant to cytotoxic attack by natural killer cells and cytotoxic T-lymphocytes (12, 13). Due to the increased susceptibility of red blood cells (RBC) to complement-mediated lysis, the leading clinical symptoms are nocturnal hemolysis and morning hemoglobinuria (14). The pathophysiology of other clinical signs, like immune-mediated bone marrow failure and thrombotic tendency are not fully elucidated (15). Despite the lack of membrane-bound CD14, monocytes from PNH patients can respond to bacterial lipopolysaccharides through an activation pathway dependent on soluble CD14, present in normal amounts in their sera (16–18).

Before the widespread use of flow cytometric assays, PNH patients with hemolysis were diagnosed with the Ham test (19) and the sucrose test (20). These tests rely on the differential sensitivity of PNH red blood cells to hemolysis after the activation of the complement system. In the past decade, flow cytometry has emerged as a valuable diagnostic tool in the detection of deficient expression of the GPI-anchored proteins in PNH patients (21–27). The advantage of flow cytometry is, that even in non-hemolysing patients, small PNH clones can be detected and the proportion of type III (complete deficiency), type II (partial deficiency) and type I (normal) cells in each investigated population can be determined (23–26).

We tried to find reliable numerical data characterizing the severity of GPI-anchored protein deficiency

*Corresponding author: János Kappelmayer, MD, PhD, Department of Clinical Biochemistry and Molecular Pathology, Medical and Health Science Center, University of Debrecen, Nagyerdei krt 98, Debrecen, Hungary
Phone: +36-52-340-006, Fax: +36-52-417-631,
E-mail: kappelmayer@dote.hu

Table 1 Laboratory parameters of the four patients with PNH.

	Patient 1	Patient 2	Patient 3	Patient 4	Ref. range
Sex	Male	Male	Female	Female	
Hemoglobin, g/L	86	109	113	97	120–175
Total bilirubin, μmol/L	63	145	25	50	<17
Conjugated bilirubin, μmol/L	20	10.6	5.6	7	<4
LDH, U/L	3682	5711	2187	1498	280–460
Haptoglobin, g/L	<0.26	<0.26	<0.26	<0.26	0.5–2.3
Reticulocyte, %	7.2	6.8	3.9	7.9	0.5–1.7

on blood cells that can be calculated separately for PNH type I, II and III cells in each population.

In the period of January 2001 to December 2004, 80 samples were analyzed in our laboratory from patients with clinical evidence of hemolysis, and the diagnosis of PNH could be confirmed by flow cytometry in only four cases. In the medical history of these patients, several episodes of abdominal pain with vomiting, dark urine, fever and back pain could be explored. Detailed examinations could not detect malignancy or ulceration in the gastrointestinal tract and bone marrow examination showed minimal dysplastic alteration without signs of neoplasia. Laboratory tests displayed anemia, elevated lactate dehydrogenase (LDH) activity, unconjugated hyperbilirubinemia, low haptoglobin and high reticulocyte percentage measured after attacks (Table 1). Hemolysis was evident and a positive sucrose test suggested the possibility of PNH.

Venous blood samples from patients and healthy controls were collected into K_3EDTA-containing tubes for whole blood count (Technicon H3 hematology analyzer, Bayer Diagnostics, Tarrytown, NY, USA) and for flow cytometric analysis (FACScan flow cytometer, Becton Dickinson, San Jose, CA, USA). For RBC analysis, whole blood was diluted with phosphate-buffered saline (PBS) and the RBC count was set to 1×10^7/mL. One hundred μL diluted blood was mixed with 5 μL phycoerythrin-conjugated anti-CD55 or anti-CD59 (both from Pharmingen, San Diego, CA, USA) monoclonal antibody. After a 30-min incubation at room temperature in the dark, cells were centrifuged at $300\times g$ for 5 min and the pellet was resuspended in 1 mL PBS. After the second wash, the pellet was resuspended in 0.5 mL 1% paraformaldehyde (PFA).

For granulocyte and monocyte analysis 50 μL blood was mixed with a saturating concentration of phycoerythrin-conjugated anti-CD55 or anti-CD59 or anti-CD14 (Becton Dickinson) monoclonal antibody. After a 15-min incubation period at room temperature, 1 mL BD lysing solution at working dilution was added to each tube. Samples were incubated for 10 min and then centrifuged for 5 min at $300\times g$. The pellet was resuspended in 1 mL PBS and after the second washing step the cells were resuspended in 0.5 mL 1% PFA. PE-conjugated mouse IgG_1 was used as negative control. Granulocytes and monocytes were analyzed from their respective gates created in the forward and side scatter dot plots. Cells were classified as PNH I, PNH II and PNH III according to their mean fluorescence intensities. In the case of CD55 and CD59 on RBCs, granulocytes and monocytes were classified as PNH III when MFI was below 10, PNH II when MFI was 34 ± 11 and PNH I when MFI was 280 ± 150. In the case of CD14 on monocytes, type III cells had MFI 39 ± 36, no type II cells were found and type I cells had MFI 2060 ± 1000, in accordance with the higher CD14 antigen number on monocytes. Quantitative measurements were performed using Quantibrite beads (Becton Dickinson) according to the instructions of the manufacturer. The number of PE molecules bound per cell was calculated from the MFI of the unknown cell population using a calibration curve created from given values for PE molecules per bead plotted against FL2 MFI. Monoclonal antibodies in this study were monovalent and the PE:mAb ratio was 1:1; this was the basis of the antibody binding capacity (ABC) calculation.

The characteristic histograms of a normal and a PNH granulocyte population are shown in Figure 1. The result report contains the MFI of the CD55- or CD59-deficient – type II or type III cells separately – and the normal cell population together with the percent of the negative cells (Figure 1B and Table 2). The average of the percent negative cells in the case of RBCs was one third of that obtained in the case of granulocytes and monocytes. This phenomenon is well known in PNH patients and can be explained by the premature destruction of these PNH erythrocytes. The size of the PNH clone can be better assessed by immunophenotypic analysis of monocytes and granulocytes rather than of erythrocytes (26, 28).

However, these report forms do not express the heterogenous expression of the GPI-anchored proteins when compared to a normal cell population. We recommend a new parameter, the MFI rate, which can be obtained by dividing the MFI of the PNH type II or PNH type III cells by the MFI of the respective cell population obtained for the normal sample (Figure 1C). In all four cases, the expression of CD55 and CD59 on the remnant normal cell population (PNH type I), represented by the MFI value, was similar to that of the normal sample (Figure 1A). The smaller the MFI rate, the better the discrimination between the GPI-negative and the normal cells can be achieved. Our results are in accordance with previous reports (21) describing that anti-CD55 does not delineate the erythrocyte population as well as anti-CD59 does. This finding is evidenced numerically by the calculation of MFI rates, being much lower for CD59 (0.011–0.031 in four different patients) than for CD55 (0.049–0.096 respectively) in erythrocytes.

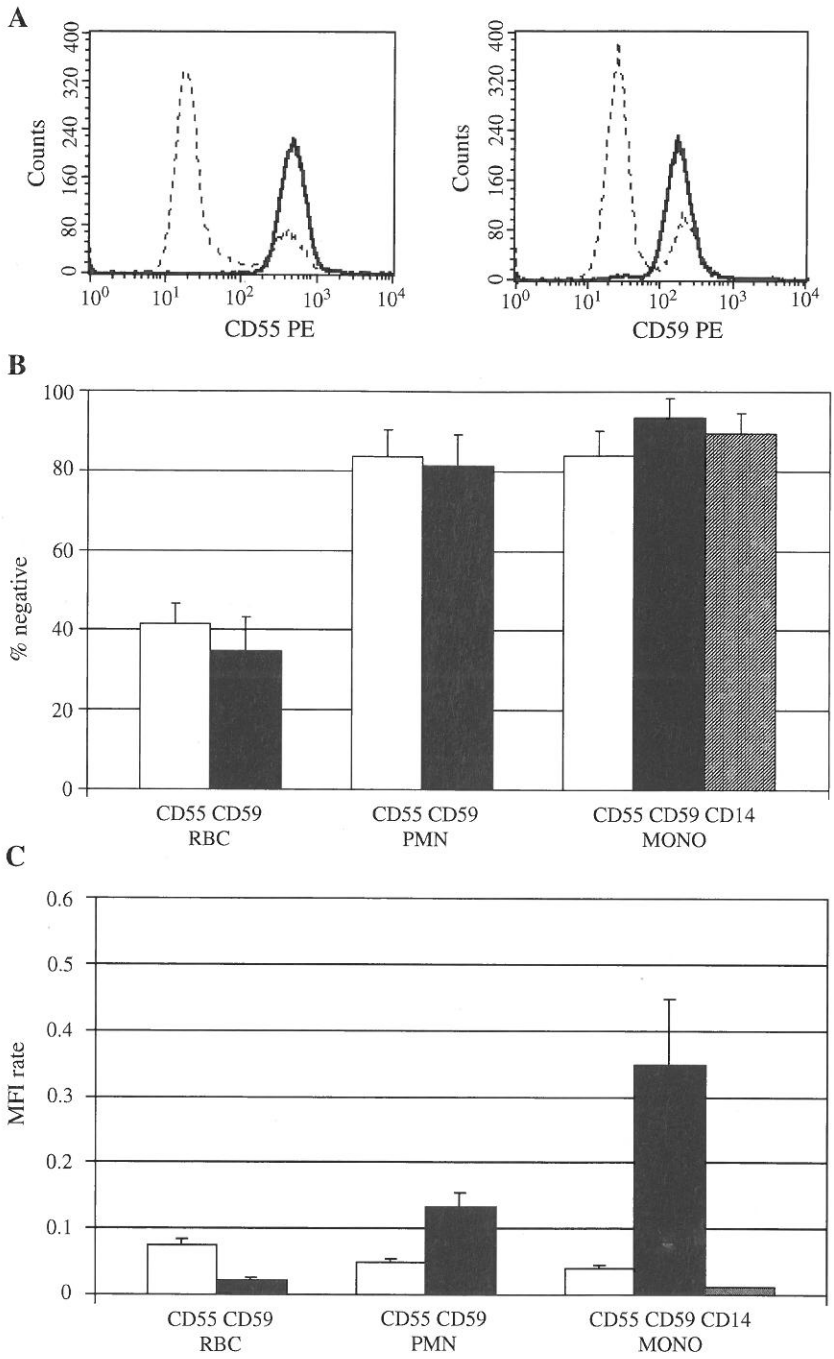

Figure 1 Expression of CD55 (left panel) and CD59 (right panel) on granulocytes (A). Fluorescence intensity is represented on the x-axis and count of the positive events on the y-axis. The solid line indicates RBCs from a normal sample while the dotted line represents the RBCs of a PNH patient. Percentage (B) and mean fluorescence intensity rate (C) of type II or type III cells were detected with PE-labeled monoclonal antibodies against CD55 (white bars), CD59 (black bars) or CD14 (dashed bars) on RBCs, granulocytes (PMN) and monocytes (MONO). MFI rate: MFI of the type II or type III population divided by the MFI of the relevant population in normal peripheral blood. Data represent the average calculated from four different patients, error bars indicate standard error of mean (SEM).

Although molecular biological techniques are extremely sensitive in the detection of *PIG-A* mutations, the transiently detected mutant clones in healthy individuals (5, 6) indicate that these clones have no selective advantage in a normal marrow environment and PNH is not manifested. Flow cytometry is now the laboratory investigation of choice.

Besides the percent negative cell ratio in each population, we report the MFI rates that provide more information about the decreased antigen expression of PNH cells compared to normals. Furthermore, in the case where the patient has PNH type II and type III cells at the same time (Table 2, patient 4), the MFI rate can be calculated separately for the appropriate populations. We found this approach reliable and cost-effective, while the use of REDQUANT and CELLQUANT assays only provide another degree of confidence for the differentiation of normal and PNH cells (29, 30). According to the literature, quantitative analysis of CD55 and CD59 on normal blood cells gave

Table 2 Rate of type I, type II and type III PNH cells in patients upon admission.

% Negative	Patient 1	Patient 2	Patient 3	Patient 4
RBC CD55 type III	34	39	19	74
RBC CD55 type II	0	0	0	0
RBC CD55 type I	64	60	80	26
RBC CD59 type III	25	45	16	54
RBC CD59 type II	0	0	0	17
RBC CD59 type I	75	55	81	27
GRAN CD55 type III	0	0	0	92
GRAN CD55 type II	82	98	65	0
GRAN CD55 type I	18	0	35	7
GRAN CD59 type III	0	0	60	93
GRAN CD59 type II	78	94	0	0
GRAN CD59 type I	22	6	39	7
MONO CD55 type III	0	0	0	90
MONO CD55 type II	84	98	67	0
MONO CD55 type I	16	0	31	10
MONO CD59 type III	0	0	0	100
MONO CD59 type II	80	98	100	0
MONO CD59 type I	19	0	0	0
MONO CD14 type III	93	98	75	92
MONO CD14 type II	0	0	0	0
MONO CD14 type I	7	0	25	8

extremely heterogenous results. While Kinoshita et al. reported 3.3×10^3 molecules CD55 per red blood cell (31), Plesner et al. found 30×10^3 CD55/red blood cell (32) and Stewart reported 4.7×10^3 CD55/red blood cell (33). We found that normal RBC display 5×10^3 CD55 and 20×10^3 CD59 molecules per cell. Quantitative analysis data also diverge greatly in the polymorphonuclear population, ranging from 16 to 80×10^3 CD55/granulocyte, respectively (31, 33). Our results were 7×10^3 CD55/granulocyte and 11×10^3 CD55/monocyte in healthy individuals. The explanation of the heterogenous results concerning CD55 and CD59 receptor numbers on different cell populations might be the lack of standardization in monoclonal antibody clones and optimal assay conditions. The majority of results providing ABC values in PNH patients utilize indirect immunofluorescence techniques. This does not comply with the usually applied routine in flow cytometry laboratories where directly conjugated antibodies are used.

Besides red blood cells, granulocytes and monocytes, we detected lower expression of CD55 and CD59 on lymphocytes and platelets as well, but that was not informative concerning clone size and severity of antigen deficiency. It has been previously shown in PNH patients that monocytes have the lowest expression of GPI-anchored molecules (34). We also found that the most informative cell type for the detection of GPI-negative cells is the monocyte population, because the CD14 expressing and non-expressing cells can be easily separated, as indicated by the lowest MFI rates (0.007–0.015 determined in four different patients). Thus, we recommend reporting the CD14-negative monocyte percent along with the MFI rate in order to determine the PNH clone size and the severity of antigen deficiency on the cell surface, respectively.

Another application of flow cytometry is in the detection of small PNH clones, like in aplastic anemia (35). Flow cytometry can also be used after CAMPATH-1H therapy in the detection of PNH-like lymphocytes (36). In these cases flow cytometry is the only method for the follow-up of the small clone size and the calculation of the MFI rate would be an extra data in the diagnosis and monitoring of patients.

In conclusion, a novel, calculated parameter, the MFI rate, is a numerical data reflecting the severity of decreased antigen expression. Based on MFI rate results, monocyte CD14 seems to be the most valuable marker in the determination of PNH clone size.

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References

1. Bessler M, Hillmen P, Longo L, Luzzatto L, Mason PJ. Genomic organization of the X-linked gene (PIG-A) that is mutated in PNH and of a related pseudogene mapped to 12q21. Hum Mol Genet 1994;3:751–62.
2. Miyata T, Takeda J, Iida Y, Yamada N, Inoue N, Takahashi M, et al. The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. Science 1993;259: 1318–20.
3. Bessler M, Mason PJ, Hillmen P, Miyata T, Yamada N, Takeda J, et al. Paroxysmal nocturnal hemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. EMBO J 1994;13:110–7.
4. Antal-Szalmas P. Evaluation of CD14 in host defence. Eur J Clin Invest 2000;30:167–79.
5. Rosse WF, Ware RE. The molecular basis of paroxysmal nocturnal hemoglobinuria. Blood 1995;86:3277–86.
6. Araten DJ, Nafa K, Pakdeesuwan K, Luzzato L. Clonal populations of hemopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. Proc Natl Acad Sci 1999; 96:5209–14.

7. Hu R, Mukhina GL, Piantadosi S, Barber JP, Jones RJ, Brodsky RA. PIG-A mutations in normal hematopoiesis. Blood 2005;105:3848–54.
8. Johnson RJ, Hillmen P. Paroxysmal nocturnal hemoglobinuria: nature's gene therapy? Mol Pathol 2002;55: 145–52.
9. Rosse WF. New insights into paroxysmal nocturnal hemoglobinuria. Curr Opin Hematol 2001;8:61–7.
10. Chen G, Kirby M, Zeng W, Young NS, Maciejewski JP. Superior growth of glycophosphatidylinositol-anchored protein-deficient progenitor cells in vitro is due to the higher apoptotic rate of progenitors with normal phenotype in vivo. Exp Hematol 2002;30:774–82.
11. Heeney MM, Ormsbee SM, Moody MA, Howard TA, DeCastro CM, Ware RE. Increased expression of anti-apoptosis genes in peripheral blood cells from patients with paroxysmal nocturnal hemoglobinuria. Mol Genet Metab 2003;78:291–4.
12. Nakakuma H, Kawaguchi T. Pathogenesis of selective expansion of PNH clones. Int J Hematol 2003;77:121–4.
13. Marsh JC, Elebute MO. Stem cells in paroxysmal nocturnal hemoglobinuria and aplastic anaemia: increasing evidence for overlap of haemopoietic defect. Transfus Med 2003;13:377–86.
14. Nakakuma H. Mechanism of intravascular hemolysis in paroxysmal nocturnal hemoglobinuria (PNH). Am J Hematol 1996;53:22–9.
15. Young NS. Paroxysmal nocturnal hemoglobinuria: current issues in pathophysiology and treatment. Curr Hematol Rep 2005;4:103–9.
16. Sundan A, Ryan L, Brinch L, Espevik T, Waage A. The involvement of CD14 in stimulation of TNF production from peripheral mononuclear cells isolated from PNH patients. Scand J Immunol 1995;41:603–8.
17. Golenbock DT, Bach RR, Lichenstein H, Juan TS, Tadavarthy A, Moldow CF. Soluble CD14 promotes LPS activation of CD14-deficient PNH monocytes and endothelial cells. J Lab Clin Med 1995;125:662–71.
18. Schutt C, Schilling T, Grunwald U, Stelter F, Witt S, Kruger C, et al. Human monocytes lacking the membrane-bound form of the bacterial lipopolysaccharide (LPS) receptor CD14 can mount an LPS-induced oxidative burst response mediated by a soluble form of CD14. Res Immunol 1995;146:339–50.
19. Ham T. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: study of the mechanism of hemolysis in relation to the acid–base equilibrium. N Engl J Med 1937;915:915–7.
20. Hartmann RC, Jenkins DE. The "sugar-water" test for paroxysmal nocturnal hemoglobinuria. N Engl J Med 1966;275:155–7.
21. Schubert J, Alvarado M, Uciechowski P, Zielinska-Skowronek M, Freund M, Vogt H, et al. Diagnosis of paroxysmal nocturnal hemoglobinuria using immunophenotyping of peripheral blood cells. Br J Haematol 1991;79:487–92.
22. Kwong YL, Lee CP, Chan TK, Chan LC. Flow cytometric measurement of glycosylphosphatidyl-inositol-linked surface proteins on blood cells of patients with paroxysmal nocturnal hemoglobinuria. Am J Clin Pathol 1994; 102:30–5.
23. Hall SE, Rosse WF. The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. Blood 1996;87:5332–40.
24. Richards SJ, Rawstron AC, Hillmen P. Application of flow cytometry to the diagnosis of paroxysmal nocturnal hemoglobinuria. Cytometry (Comm Clin Cytometry) 2000;42:223–33.
25. Piedras J, López-Karpovitch X. Flow cytometric analysis of glycosylphosphatidylinositol-anchored proteins to assess paroxysmal nocturnal hemoglobinuria clone size. Cytometry (Comm Clin Cytometry) 2000;42:234–8.
26. Borowitz MJ. Flow cytometry in PNH. How much is enough? Cytometry (Comm Clin Cytometry) 2000;42: 221–2.
27. Hernandez-Campo PM, Martin-Ayuso M, Almeida J, Lopez A, Orfao A. Comparative analysis of different flow cytometry-based immunophenotypic methods for the analysis of CD59 and CD55 expression on major peripheral blood cell subsets. Cytometry (Comm Clin Cytometry) 2002;50:191–201.
28. Meletis J, Terpos E. Recent insights into the pathophysiology of paroxysmal nocturnal hemoglobinuria. Med Sci Monit 2003;9:161–72.
29. Oelschlaegel U, Besson I, Arnoulet C, Sainty D, Nowak R, Naumann R, et al. A standardized flow cytometric method for screening paroxysmal nocturnal hemoglobinuria (PNH) measuring CD55 and CD59 expression on erythrocytes and granulocytes. Clin Lab Haem 2001;23: 81–90.
30. Hsi ED. Paroxysmal nocturnal hemoglobinuria testing by flow cytometry. Evaluation of the REDQUANT and CELLQUANT kits. Am J Clin Pathol 2000;114:798–806.
31. Kinoshita T, Medof ME, Silber R, Nussenzweig V. Distribution of decay-accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. J Exp Med 1985;162: 75–92.
32. Plesner T, Hansen NE, Carlsen K. Estimation of PI-bound proteins on blood cells from PNH patients by quantitative flow cytometry. Br J Haematol 1990;75:585–90.
33. Poncelet P, Besson-Faure I, Lavabre-Bertrand T. Clinical application of quantitative immunophenotyping. In: Stewart CC, Nicholson JK, editors. Immunophenotyping. New York: Wiley-Liss, 2000:117–20.
34. Maciejewski JP, Young NS, Yu M, Anderson SM, Sloand EM. Analysis of the expression of GPI-anchored proteins on platelets from patients with PNH. Thromb Res 1996;83:433–47.
35. Azenishi Y, Ueda E, Machii T, Nishimura J, Hirota T, Shibano M, et al. CD59-deficient blood cells and PIG-A gene abnormalities in Japanese patients with aplastic anemia. Br J Haematol 1999;104:523–9.
36. Rawstron AC, Rollinson SJ, Richards S, Short MA, English A, Morgan GJ, et al. The PNH phenotype cells that emerge in most patients after CAMPATH-1H therapy are present prior to treatment. Br J Haematol 1999;107: 148–53.

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