Indian J Med Res 146, September 2017, pp 362-368
DOI: 10.4103/ijmr.IJMR_195_14

Quick Response Code:

Value of CD16/CD66b/CD45 in comparison to CD55/CD59/CD45 in diagnosis of paroxysmal nocturnal haemoglobinuria: An Indian experience

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Received February 10, 2014

***Background & objectives*: Diagnosis of paroxysmal nocturnal haemoglobinuria (PNH), a rare haematopoietic stem cell disorder, is challenging in patients with bone marrow failure (BMF) syndrome like aplastic anaemia (AA). This study was conducted with the aim to test the efficacy of the newly recommended markers *viz.* anti-CD16 and CD66b antibody over the existing anti-CD55 and CD59 antibody for PNH diagnosis in India.**

***Methods*: This study was conducted on 193 suspected cases of PNH by flow cytometry using lyse wash technique to stain the granulocytes with CD16/CD66b and CD55/CD59.**

***Results*: Of the 193 suspected cases, 62 patients showed the presence of PNH clone. Forty six patients were detected by CD55/CD59/CD45, whereas 61 were detected by CD16/CD66b/CD45. CD16/CD66b detected 16 (25.8%) additional patients over CD55/CD59 ($P<0.05$) and was more sensitive in detecting the PNH clone with higher negative predictive value. Most of the patients (11/16) who were picked up by CD16/CD66b were of AA who had small clone sizes. Further, the PNH clones were more discreetly identified in CD16/CD66b plots than by CD55/CD59. Clone size assessed by CD16/CD66b which reflects the clinical severity of classical PNH (thrombosis/haemolysis), was more representative of the underlying clinical condition than CD55/59.**

***Interpretation & conclusions*: In our experience of 62 patients of PNH, CD16/CD66b proved to be more efficacious in detecting PNH. The new panel was especially useful in monitoring PNH associated with BMF which had small clone sizes.**

Key words Aplastic anaemia - CD16 - CD55 - CD59 - CD66b - flow cytometry - paroxysmal nocturnal haemoglobinuria - paroxysmal nocturnal haemoglobinuria associated with bone marrow failure

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare haematopoietic stem cell disorder characterized by a somatic mutation in the phosphatidylinositol glycan complementation class-A (*PIG-A*) gene, leading to a deficiency of proteins linked to the cell membrane via glycophosphatidylinositol

(GPI) anchors[1,2]. This deficiency of GPI-linked proteins is exploited for identifying PNH clones by immunophenotyping. Currently, flow cytometry is the method of choice for identifying cells deficient in GPI-linked proteins and is, therefore, necessary for the diagnosis of PNH. It is primarily based on the detection of PNH clone on granulocytes. Detection of at least two absent GPI-linked proteins in >1 per cent of cells confirms the diagnosis of PNH[3-6]. A number of antibodies against various GPI-linked antigens (CD14, CD16, CD24, CD48, CD52 and CD66b) have been developed[3-7]. Different studies have used different combinations of antibodies to detect PNH clones with different efficacy[3-7]. PNH diagnosis using CD55 and CD59 by flow cytometry as well as by gel card has been well established in Indian population[4]. It has fallen into disrepute due to lower sensitivity and weak antibody expression. The newer markers hence recommended include CD16 and CD66b[7]. However, there is little experience of these newly available antibodies on the Indian patients. This study was done to test the efficacy of new markers - CD16 and CD66b, for PNH diagnosis over the existing anti-CD55 and CD59 antibodies.

Material & Methods

This study was done on 193 cases of suspected PNH taken consecutively from February 2012 to December 2012. These patients were referred to the department of Hematology, All India Institute of Medical Sciences (AIIMS), New Delhi, India, for the diagnosis of PNH by flow cytometry. The cases were referred if they had thrombosis at unusual sites, non-spherocytic haemolytic anaemia, all cases of aplastic anaemia (AA) proven by bone marrow biopsy and some cases of unknown cytopaenias, in whom bone marrow showed hypocellularity with erythroid hyperplasia. Brief clinical details were collected and complete blood counts were done in addition to flow cytometric analysis for identifying PNH clone. Presence of PNH clone was defined by >1 per cent negative expression of anti-CD55 and anti-CD59 expression and/or anti-CD16 and anti-CD66b expression on granulocytes. In addition, the presence of PNH clone was substantiated by observing CD55/CD59 negative population on monocytes. The blood samples (7 ml) were freshly collected in EDTA-anticoagulated tubes (3 ml for haemogram and 4 ml for flow cytometry).

The study was approved by the ethics committee of AIIMS and written informed consent was obtained from all patients.

Processing for flow cytometric analysis: Total leucocyte count (TLC) of all samples was measured. If the TLC was in the normal range (TLC - 4000-11,000/µl), then the sample was processed directly. Otherwise, TLC was readjusted after centrifugation at 150 *g* for five minutes and excess plasma removed. Three tubes were processed for each sample: two 'stained' and one 'unstained'. The antibodies used were from BD Biosciences, USA. The following antibody cocktail was added in the stained tubes: (*i*) Tube A: FITC-anti-CD55 (BD Biosciences IA10); PE-anti-CD59 [p282 (BD Biosciences H19)]; PerCP-Cy 5.5-anti-CD45 (BD Biosciences 2D1); and (*ii*) Tube B: FITC-anti-CD66b (BD Biosciences G10F5); PE-anti-CD16 (BD Biosciences B73.1); PerCP-Cy5.5-anti-CD45 (BD Biosciences 2D1).

Titration of the antibodies was done at varying concentrations of 3, 5, 7, 10, 15 and 20 µl on the 20 PNH-negative controls and five PNH-positive controls (positive on gel card for CD55 and CD59). Concentration of 5 µl was found to be optimum. Thus, 5 µl each of anti-CD55, anti-CD59 and anti-CD45 was put in Tube A and 5 µl each of anti-CD16, anti-CD66b and anti-CD45 was put in Tube B along with 15 µl of whole blood and processed by lyse wash technique[6]. The samples were acquired on BD FACS CANTO II (BD Biosciences). The voltage threshold for acquiring was set. Moreover, 20,000 events were acquired in each sample. Dot blots were studied and gates for double negative were set after viewing the CD45/SSC in the unstained tube. Detailed dot blot analysis was done on both the stained tubes[7]. PNH clone was defined when >1 per cent double negative population was seen on both granulocytes and monocytes[7]. Granulocyte clone was defined as double negative population on CD55/CD59 or/and CD16/CD66b staining. Monocyte clone was defined as >1 per cent double negative population on CD55/CD59 staining. An expert opinion was taken for assigning each patient as negative or positive for PNH, after assessing the dot blots of both the sets of antibodies. Rest all were defined as PNH negative. A normal control sample (from a patient with non-haematological condition) was run in each batch and only when percentage cells in the double negative quadrant was 0-0.1 per cent, the test was considered acceptable. The patient was resampled on a later date and test repeated when the PNH clone was seen only in one tube, either CD16/CD66b or CD55/CD59, to rule out any technical or sampling error.

Statistical analysis: Data analyzed using Chi-square test (for categorical data), rank sum test (for continuous

data) and Pearson's correlation for finding linear correlation between two continuous variables. Sensitivity, specificity and related metrics were calculated with standard formulae.

Results

One hundred and ninety three patients of suspected PNH were evaluated by flow cytometry-based immunophenotyping. Sixty two (32.1%) patients showed the presence of PNH clone (defined by more than one double negative population by CD 55/59 and/or CD16/66b). The remaining 131 patients were negative for PNH clone. AA (38.3%) was the most common clinical condition for referral. Other conditions were myelodysplastic syndrome (MDS), haemolytic anaemia, thrombosis, unexplained transfusion-dependent anaemia and those cases where PNH study was advised on bone marrow examination. Table I gives the baseline characteristics in the two groups. Of all the clinical parameters, only presence of haemoglobinuria was significant difference between PNH-positive and PNH-negative patients (P<0.001).

The PNH-positive patients were classified into two groups: (*i*) Classic PNH: 16 patients (25.8%); and (*ii*) PNH associated with bone marrow failure (BMF) (PNH/BMF): 46 patients (74.2%).

The classic PNH *i.e.* thrombotic/haemolytic was present only in one-fourth of the patients. The maximum number of PNH patients was associated with BMF *i.e*. AA/PNH or MDS/PNH. Haemoglobinuria (P<0.001), thrombosis (P<0.01) and organomegaly (P<0.01) were significantly present in patients of classic PNH, whereas significant bleeding (P<0.001) and fever (P<0.01) characterized PNH/BMF. Further analysis was done on the PNH/BMF group, to evaluate the diagnostic efficacy of new panel comprising CD16/CD66b over existing panel of CD55/CD59. This was studied in terms of sensitivity of the new panel, assessment of the clone size, and better discretion of clone.

Sensitivity of CD16/CD66b versus CD55/CD59: Among 62 PNH-positive patients, CD55/CD59 detected PNH positivity in 46 (74.2%) patients and CD16/CD66b detected PNH positivity in 61 (98.3%) patients. Hence, 16 (25.8%) additional cases were picked up by CD16/CD66b. The difference was significant with 95 per cent confidence interval of 11.2-37.1 per cent (P=0.0003) (Table II). Comparison of two antibody panels is shown in Table III. Fig. 1 shows the flow cytometric images of patients who were detected as PNH positive by CD16/CD66b analysis only.

Clone size in CD16/CD66b versus CD55/CD59: Overall, the PNH clone size measured by CD16/CD66b

Table I. Clinical presentation of suspected positive and negative cases of paroxysmal nocturnal haemoglobinuria (PHN)

Clinical presentation	PNH negative (n=131), n (%)	PNH positive (n=62), n (%)
Age (median) (yr)	29	30
Sex (males)	79	44
Pallor	107 (81.7)	55 (90.2)
Fever	60 (45.8)	26 (42.6)
Bleeding	59 (45)	32 (52.5)
Jaundice	18 (14.2)	13 (22)
Only ↑ unconjugated serum bilirubin	8 (6.3)	6 (10.2)
Haemoglobinuria	1 (0.8)	10 (16.4)***
Thrombosis	20 (15.3)	7 (11.7)
Hepatomegaly	7 (5.4)	5 (8.3)
Splenomegaly	6 (4.6)	2 (8.3)
Hepatosplenomegaly	5 (3.8)	1 (1.7)

***P<0.001

Table II. Efficacy of CD55/CD59 & CD16/CD66b in detection of PNH in 62 PNH positive patients

Expression of antigen	PNH positive clone detected	PNH positive clones not detected
CD55/59	46	16
CD16/66b	61	1

P=0.0003 two-sample test for equality of proportions with continuity correction

Table III. Comparison in the efficacy of CD55/CD59 & CD16/CD66b in detection of 62 PNH positive patients

Parameters	CD55/ CD59 (%)	CD16/ CD66b (%)
Apparent prevalence	24	32
True prevalence	32	32
Sensitivity	74	98
Specificity	100	100
Positive predictive value	100	100
Negative predictive value	89	99

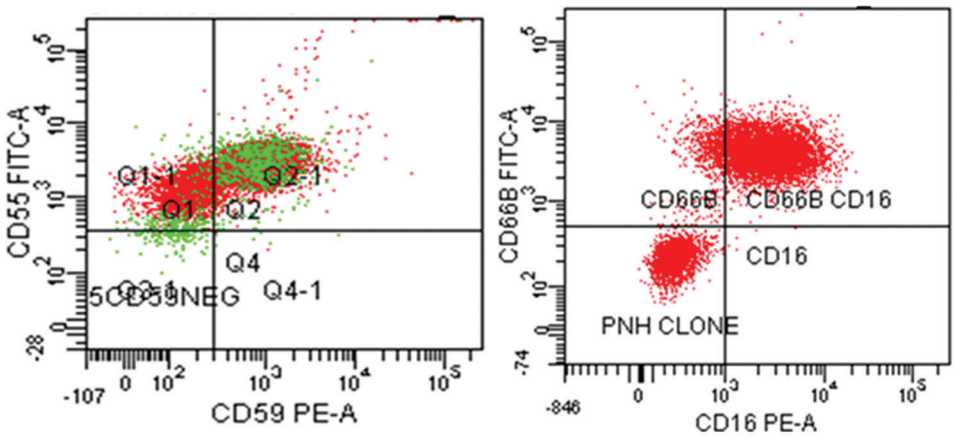

Fig. 1. Flow cytometric dot-blots of paroxysmal nocturnal haemoglobinuria (PNH) clone of patients with myelodysplastic syndrome -refractory cytopenia with multilineage dysplasia (MSD-RCMD) positive on CD16/CD66b; negative on CD55/CD59. Clone size detected by CD55/CD59 was 0.1 per cent and by CD16/CD66b was 26.1 per cent. Red population represents granulocytes, while green population represents monocytes that are gated on CD45/SSC.

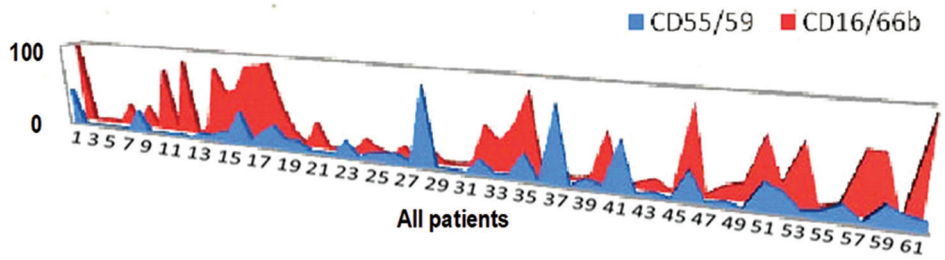

Fig. 2. Clone size difference in individual PNH-positive patients detected by CD55/CD59 and CD16/66b.

was significantly more than that measured by CD55/CD59 (P<0.0001). Although it was observed that mean clone size was more with CD16/CD66b (28.8±32.6) compared to CD55/59 (11.4±17.5) in PNH-positive patients (Fig. 2), the difference was not significant. This was probably because of the wide range in the clone sizes in different patients. However, individually, the clone size detected by CD16/CD66b group was more.

Classic PNH (PNH+with thrombosis): Detailed analysis of difference in clone size measured by the two tests in classic PNH who presented with thrombosis (n=7) showed that the mean and the median clone sizes detected by CD55/CD59 (17.5±21.3; 8.7) and CD16/CD66b (47.8±40.9; 45.5) were very different; however, due to high standard deviation and small numbers, the difference was not significant. Of the seven patients only one assessed by CD55/CD59 had clone size more than 50 per cent, but five patients detected by CD16/CD66b had clone size more than 50 per cent.

Classic PNH (PNH with haemolysis): Nine patients with PNH clone had features of haemolysis. Of these, one patient also had thrombosis and hepatosplenomegaly. There was no difference in the clinical presentation in the PNH-positive from PNH-negative patients. The presence of haemoglobinuria was the only significant clinical presentation seen in PNH-positive patients (P=0.003). Of the nine PNH patients with haemolysis, six had clone size more than 20 per cent by both the panels; however, the mean clone size was larger with CD16/CD66b (P<0.05).

PNH associated with bone marrow failure (BMF): Thirty patients were diagnosed as BMF syndromes (26 of AA and four of MDS). Sixteen patients in whom the exact diagnosis was not ascertained by bone marrow examination, were excluded from this analysis. Though there was a trend towards higher clone sizes detected on CD16/CD66b, but due to a significant overlap, the difference in the clone size measured by the two panels was not significant between PNH-positive and PNH-negative patients.

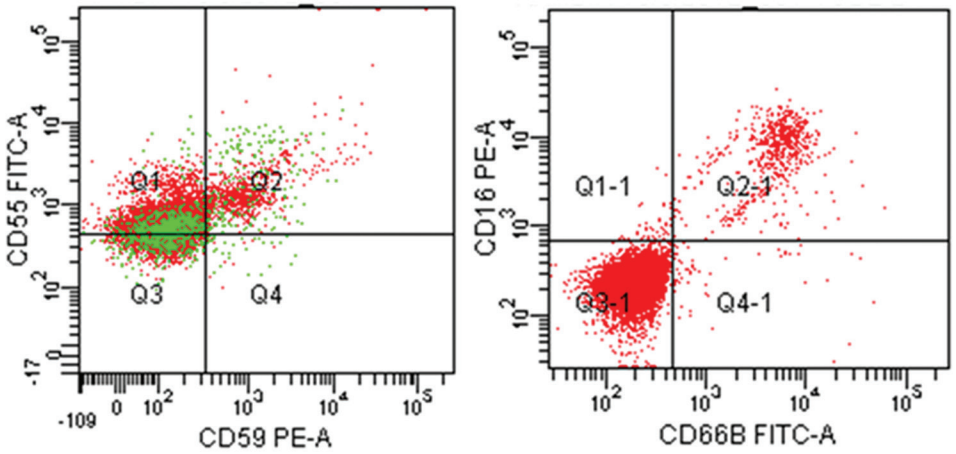

Fig. 3. Flow cytometric dot blots showing distinct separation of PNH-positive clone from PNH-negative clone when stained by CD45/CD16/CD66b. Red population represents granulocytes while green population represents monocytes that are gated on CD45/SSC.

Discretion of PNH clone in CD16/CD66b versus CD55/CD59: It was observed that the PNH clone was better separated from rest of the cells on analysis by CD16/CD66b, as illustrated in the flow cytometric graphs of two such patients (Figs. 1 & 3). Of the 61 PNH patients, 44 had two distinct populations by CD16/CD66b analysis as compared to CD55/CD59, in which only 14 cases had a distinct separation in PNH clone from normal population. This difference was significant (P=0.001).

Assessment of patients detected by CD16/CD66b only: Sixteen patients were missed by analysis with CD55/CD59 panel. Most of these cases were of PNH/BMF (11/16; 69%). In most of these patients, the clone size detected by CD16/CD66b was also low (<5%). However, there was one patient who was missed by CD16 and CD66b. He was a 19 yr old male who presented with pallor and bleeding for 14 months and was diagnosed as AA on bone marrow biopsy. On immunophenotyping, granulocytes showed 11.3 per cent of CD55/CD59 double negative population and 0.6 per cent of CD16/66b double negative population. CD55/CD59 double negative population on monocytes was 37.3 per cent.

Discussion

PNH is a rare haematopoietic stem cell disorder characterized by two major syndromes: classic PNH and PNH/BMF[8]. Classic PNH presents as a non-spherocytic haemolytic anaemia and/or thrombosis, especially at unusual sites. PNH/BMF, on the other hand, is characterized by the presence of AA or MDS concomitant or may develop subsequent or antecedent to the development of PNH clones. In the present study, the classic PNH was present in 25.8 per cent patients only. The majority of PNH patients were associated with BMF *i.e.* AA/PNH or MDS/PNH (51%). The remaining one-fourth were characterized by some form of BMF and hence reclassified into PNH/BMF category. In our study, 35.2 per cent AA patients revealed the presence of PNH clone. It has been shown that approximately 5 per cent of patients with AA develop PNH while about 20 per cent of those with PNH have antecedent AA[9,10]. Between 15 and 33 per cent of patients receiving anti-thymocyte serum for the treatment of AA have been shown to recover with evidence of PNH[9-15]. A multi-centre prevalence study on PNH detected granulocyte PNH clones ≥1 per cent in 199 of 5398 patients (3.7%), 93 of 503 AA patients (18.5%), 50 of 4401 of MDS patients (1.1%) and 3 of 130 of other BMF patients (2.3%)[16]. In our study, the incidence of PNH in AA was relatively higher. One of the reasons was strong selection bias being a referral institute and monitoring for PNH was a part of the workup for AA. Varma *et al*[4] also showed an incidence of 30.7 per cent PNH in patients with AA.

In our study 16 additional cases were picked up by CD16/CD66b. Most of these patients had AA (PNH/BMF). This group of patients is known to have small clone sizes and CD16/CD66b testing was helpful because CD55/59 was unable to pick up these small clones of PNH coexisting in AA. However, in RBC analysis CD59 is currently recommended and has shown superiority over CD55[17]. Dunn *et al*[18] measured

PNH clone by CD16/CD66b and effectively identified patients with subclinical PNH (AA and refractory anaemia). Wang *et al*[3] detected a larger and more distinctive PNH^+ granulocytic population, with the added advantage of clearly separating neutrophils from eosinophils using CD16/CD66b. However, they used CD15/SSC for gating neutrophils instead of CD45/SSC used in our study.

There were 16 patients of classic PNH, of whom seven presented primarily with thrombosis and nine with haemolysis. There was no difference in the clinical presentation of patients with thrombotic PNH from those who presented with thrombosis at non-deep venous thrombosis sites. The clone size detected by CD55/CD59 and CD16/CD66b was different but insignificant because the numbers were less and also because the standard deviation was large. Five out of seven detected by C16/CD66b had clone size >50 per cent unlike that detected by CD55/CD59, where only one case had clone size >50 per cent. It has been shown that clone size >50 per cent confers higher risk of thrombosis[9]. Hence, clone size assessed by CD16/CD66b was more representative of the underlying clinical condition. However, FLAER (Fluorescent- labelled proaerolysin variant) is considered better as it tends to measure much larger clone sizes. In a study by Agarwal *et al*[19], the average clone size measured by FLAER was >70 per cent.

In the classic PNH patients who presented with primarily haemolysis, the only feature which was significantly different between the two groups was the presence of haemoglobinuria in PNH category. In addition, clone size was higher when measured by CD16/CD66b than CD55/CD59 and this difference was significant. It was also observed that there were three patients who had haemolysis but had small clone sizes. PNH clones are known to exist in normal population[20], and it is possible that these cases might represent other causes of haemolysis and a small PNH clone as a coincidental finding. The prevalence of PNH clone in normal population is considered to be 1.7/million[20].

There were 16 patients which were missed by CD55/CD59. Eleven of these 16 were of AA having very small clone sizes which were only picked up by CD16/CD66b. This suggests that PNH/BMF is associated with low clone sizes which are better appreciated by CD16/CD66b. One of the limitations of this panel is CD16 polymorphisms. These polymorphic variants are, however, not identified by certain antibodies used for immunophenotyping. However, this problem is circumvented by combining it with CD66b while analysis.

In conclusion, CD16/CD66b was more efficacious in detecting PNH clone on granulocytes as compared to CD55/CD59 as it had greater sensitivity. It also characterized the PNH clone better as it measured greater clone size as well as separated the PNH clone better. This was especially useful in picking up cases with low clone sizes and hence can be used in following up the development of PNH clones in cases of BMF.

***Conflicts of Interest*:** None.

References

1. Moyo VM, Mukhina GL, Garrett ES, Brodsky RA. Natural history of paroxysmal nocturnal haemoglobinuria using modern diagnostic assays. *Br J Haematol* 2004; *126* : 133-8.
2. Rosse WF, Dacie JV. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. I. The sensitivity of PNH red cells to lysis by complement and specific antibody. *J Clin Invest* 1966; *45* : 736-48.
3. Wang SA, Pozdnyakova O, Jorgensen JL, Medeiros LJ, Stachurski D, Anderson M, *et al.* Detection of paroxysmal nocturnal hemoglobinuria clones in patients with myelodysplastic syndromes and related bone marrow diseases, with emphasis on diagnostic pitfalls and caveats. *Haematologica* 2009; *94* : 29-37.
4. Varma N, Garewal G, Varma S, Vohra H. Flowcytometric detection of PNH defect in Indian patients with aplastic anemia and myelodysplastic syndromes. *Am J Hematol* 2000; *65* : 264-5.
5. Brodsky RA, Mukhina GL, Li S, Nelson KL, Chiurazzi PL, Buckley JT, *et al.* Improved detection and characterization of paroxysmal nocturnal hemoglobinuria using fluorescent aerolysin. *Am J Clin Pathol* 2000; *114* : 459-66.
6. Piedras J, López-Karpovitch X. Flow cytometric analysis of glycosylphosphatidyl-inositol-anchored proteins to assess paroxysmal nocturnal hemoglobinuria clone size. *Cytometry* 2000; *42* : 234-8.
7. Borowitz MJ, Craig FE, Digiuseppe JA, Illingworth AJ, Rosse W, Sutherland DR, *et al.* Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. *Cytometry B Clin Cytom* 2010; *78* : 211-30.
8. Parker CJ. Management of paroxysmal nocturnal hemoglobinuria in the era of complement inhibitory therapy. *Hematology Am Soc Hematol Educ Program* 2011; *2011* : 21-9.
9. de Planque MM, Bacigalupo A, Würsch A, Hows JM, Devergie A, Frickhofen N, *et al.* Long-term follow-up of severe aplastic anaemia patients treated with antithymocyte globulin. Severe Aplastic Anaemia Working Party of the European Cooperative Group for Bone Marrow Transplantation (EBMT). *Br J Haematol* 1989; *73* : 121-6.

10. Griscelli-Bennaceur A, Gluckman E, Scrobohaci ML, Jonveaux P, Vu T, Bazarbachi A, *et al.* Aplastic anemia and paroxysmal nocturnal hemoglobinuria: Search for a pathogenetic link. *Blood* 1995; *85* : 1354-63.

11. Dacie JV, Lewis SM. Paroxysmal nocturnal haemoglobinuria: Clinical manifestations, haematology, and nature of the disease. *Ser Haematol* 1972; *5* : 3-23.

12. Najean Y, Haguenauer O. Long-term (5 to 20 years) evolution of nongrafted aplastic anemias. The Cooperative Group for the Study of Aplastic and Refractory Anemias. *Blood* 1990; *76* : 2222-8.

13. Nakakuma H, Nagakura S, Iwamoto N, Kawaguchi T, Hidaka M, Horikawa K, *et al.* Paroxysmal nocturnal hemoglobinuria clone in bone marrow of patients with pancytopenia. *Blood* 1995; *85* : 1371-6.

14. Schrezenmeier H, Hertenstein B, Wagner B, Raghavachar A, Heimpel H. A pathogenetic link between aplastic anemia and paroxysmal nocturnal hemoglobinuria is suggested by a high frequency of aplastic anemia patients with a deficiency of phosphatidylinositol glycan anchored proteins. *Exp Hematol* 1995; *23* : 81-7.

15. Richards SJ, Whitby L, Cullen MJ, Dickinson AJ, Granger V, Reilly JT, *et al.* Development and evaluation of a stabilized whole-blood preparation as a process control material for screening of paroxysmal nocturnal hemoglobinuria by flow cytometry. *Cytometry B Clin Cytom* 2009; *76* : 47-55.

16. Raza A, Ravandi F, Rastogi A, Bubis J, Lim SH, Weitz I, *et al.* A prospective multicenter study of paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure. *Cytometry B Clin Cytom* 2014; *86* : 175-82.

17. Tembhare P, Ramani M, Syed K, Gupta AD. Flow cytometric analysis of erythrocytes in paroxysmal nocturnal hemoglobinuria reveals superiority of CD59 as a diagnostic marker compared to CD55. *Indian J Pathol Microbiol* 2010; *53* : 699-703.

18. Dunn DE, Tanawattanacharoen P, Boccuni P, Nagakura S, Green SW, Kirby MR, *et al.* Paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure syndromes. *Ann Intern Med* 1999; *131* : 401-8.

19. Agarwal R, Chapple P, Brown M, Szer J, Juneja S. Analysis of abnormal clones by the fluorescent aerolysin method in paroxysmal nocturnal haemoglobinuria and other marrow disorders. *Int J Lab Hematol* 2015; *37* : 14-21.

20. Araten DJ, Nafa K, Pakdeesuwan K, Luzzatto L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. *Proc Natl Acad Sci U S A* 1999; *96* : 5209-14.

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