Original Article

Haematol J Bangladesh 2023;7(1):18-25
https://journal.hematologybd.org/index.php/haematoljbd
ISSN: 2523-1219 (print)
ISSN: 2707-1405 (online)

Prevalence of Paroxysmal Nocturnal Haemoglobinuria Clone in Aplastic Anaemia: A Single Centre Study

A. Q. M. Ashraful Haque[1], Salmun Nahar[2], A. H. M. Aslam-Ul- Haque[3], Md. Arif-Ur- Rahman[1], A B M Yunus[1], Tanjina Afrin[1], Md. Abdul Aziz[1], Md. Rafiquzzaman Khan[1], Md. Salahuddin Shah[1], Nusrat Jahan[1]

[1]*Department of Haematology, Bangabandhu Sheikh Mujib Medical University, Shahbagh, Dhaka.*
[2]*Directorate General of Health Services (DGHS), Mohakhali, Dhaka.*
[3]*Department of Medicine, Dhaka Medical College and Hospital, Dhaka.*

Citation: Haque AQMA, Nahar S, Haque AHMA, Rahman MAU, Yunus ABM, Afrin T, Aziz MA, Khan MR, Shah MS, Jahan N. Prevalence of Paroxysmal Nocturnal Haemoglobinuria Clone in Aplastic Anaemia: A Single Centre Study. Haematol J Bangladesh. 2023;7(1):18-25.

DOI: https://doi.org/10.37545/haematoljbd2023100

Received: 10 July 2022

Accepted: 07 December 2022

Published: 20 March 2023

*Correspondence: Dr. A. Q. M. Ashraful Haque, Resident Physician (Medicine), Colonel Maleque Medical College Hospital, Manikgonj, Bangladesh. Email: draqmhq@gmail.com

ABSTRACT

Background: *Flow cytometry assay for PNH clone is a compulsory routine test for all aplastic anaemia patients.* ***Objective:*** *To estimate the frequency of PNH clone in aplastic anaemia.* ***Method:*** *Twenty-two known cases of aplastic anaemia patients were enrolled for the study. Flow cytometric quantitation of glycosyl phosphatidyl-inositol (GPI)-anchored proteins deficiency using markers CD14, CD24, CD45, CD59, Fluorescent Aerolysin (FLAER), CD235a (6 markers) were performed.* ***Result:*** *PNH clone was identified in 8(36.4%) of the study population. Among PNH clone positive patients 7(87.5%) were suffering from non-severe aplastic anaemia.* ***Conclusion:*** *PNH clone is significantly associated with aplastic anaemia and PNH clone assay should be regularly assayed in aplastic anaemia patients for specific management.*

Key words: *PNH, aplastic anaemia, flowcytometry, FLAER, glycosyl phosphatidyl-inositol (GPI).*

Introduction

Paroxysmal nocturnal haemoglobinuria is an acquired haematopoietic disorder characterized by the clonal expansion of PIG-A mutated stem cell and consequent defective synthesis of glycosyl phosphatidyl-inositol-anchored proteins, complement-mediated haemolysis, increased incidence of thrombosis, bone marrow failure. Paroxysmal nocturnal haemoglobinuria and acquired aplastic anaemia are closely related and a reciprocal progression is possible.[1] Aplastic anaemia is a gross reduction or absence of haematopoietic precursors in all 3 cell lineages in bone marrow resulting in pancytopenia in peripheral blood. Although this encompasses all situations in which there is myelosuppression, the term is generally used to describe those in which spontaneous marrow recovery is unusual.

High resolution flow cytometry analysis (FCA) has revealed a high incidence of minor paroxysmal nocturnal haemoglobinuria (PNH) clones in adult aplastic anaemia (AA) patients at diagnosis.[2] The minimum prevalence of PNH is estimated to 1-1.5 cases per million.[3] PNH has the same general epidemiology as aplastic anaemia (e.g. in Thailand the coincidence has been clearly shown).[4] Both are most common in young adults with a later secondary increase in the 7th decade.[5,6] The diagnosis is made often in East Asian countries but it is not certain whether the incidence is greater in that region.[7] During long-term observation, the transformation of AA into classic PNH is likely.[8] The proportion of patients with aplastic anaemia who subsequently develop PNH varies widely among studies, in part because patients with clinical PNH can be divided into the following two groups: those without a preceding history of aplastic anaemia (classic PNH); and those with an antecedent history of aplastic anaemia who subsequently develop PNH (PNH/aplastic anaemia).[9] An association between aplastic anaemia and PNH has been recognized at least since 1961, and numerous subsequent studies have confirmed the association. The probability is negligible that these two diseases would occur together so frequently by chance. Therefore, a pathophysiologic link between PNH and aplastic anaemia must exist.

In one study, PNH granulocytes and PNH RBCs were 42.96% (10.04%-59.50%) and 48.87% (15.02%-90.80%), respectively.[10] Pathogenetic relationship of AA and PNH was registered a long time ago. Approximately in 50-60% of patients with the criteria for diagnosis of PNH are not uniform. When there is no sign of haemolysis in aplastic anaemia, flow cytometry assay for PNH clone is recommended yearly.[11]

Bone marrow injury may play a central role in the development of PNH by providing the conditions that favour the growth/survival of PIGA-mutant, GPI-AP–deficient stem cells. Currently, there is no evidence that the types of PIGA mutations that occur in PNH/aplastic anaemia are different from those observed in classic PNH.[12]

Methods

This observational study was conducted in the Department of Haematology, Bangabandhu Sheikh Mujib Medical University (BSMMU, Dhaka, Bangladesh. From February 2018 to January 2019, total twenty-two confirmed aplastic anaemia patients had been selected by purposive sampling technique. Inherited aplastic anaemia, myelodysplastic syndrome (MDS) and transient bone marrow aplasia were excluded from the study. Informed consent was taken from the participants. Before this study protocol was submitted to and approved by the Institutional Review Board (IRB) of Bangabandhu Sheikh Mujib Medical University (BSMMU). All participants were informed about the objectives and purpose of the study in an easy understandable way. Data obtained from the participants were used only for the research purpose, confidentiality of all study information was maintained strictly. Aplastic anaemia was diagnosed by history, physical examination, relevant tests, and then, finally confirmed by bone marrow study. Flow cytometry is the gold standard diagnostic test for PNH, performed on peripheral blood, demonstrating deficiency of GPI-linked proteins from granulocytes/monocytes/red blood cells according to the revised guidelines for diagnosis issued by the International Clinical Cytometry Society (ICCS) in 2010 (defined as identifying an abnormal population of 1% or more) and high-sensitivity analysis (in which as few as 0.01% PNH cells are detected).[13] Granulocyte analysis provides better estimate of size of PNH clone than RBC analysis. Routine red cell analysis is not recommended without white cell analysis. CD59 is the best single RBC reagent; CD55 is not acceptable by itself. FLAER and CD24/CD16/CD66b are recommended as preferred

granulocyte reagents, CD14 for monocytes. Lymphocyte analysis is not recommended because of lifespan of lymphocytes.

FLAER (FLuorescent AERolysin) binds to the GPI-anchor itself, rather than to a single protein such as CD55 or CD59. FLAER provides much greater signal and better accuracy than an antibody against a single target.

Antibody Panel for RBC: CD235a, CD59; WBC: CD45, CD24, CD14 and FLAER.

Flow Cytometer: FACSCalibur/Canto II

A pre-designed semi-structured data collection sheet was used for data collection. The statistical analysis was carried out using the Statistical Package for Social Sciences version 24.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Qualitative variables of this study were expressed as percentage. Quantitative variables were expressed as mean ± standard deviation. Chi-Square test was used for categorical variables. To compare means of two groups' unpaired t-test was used in case of normally distributed data. For all statistical tests, p-value less than 0.05 was considered as statistically significant.

Results

In this study, PNH clone was detected in 8 (36.4%) out of 22 patients of acquired aplastic anaemia (Figure I). The overall mean (mean ± SD) age of the patient was 50.2±18.1 and range of age distribution was 16-90 years. The majority (50%) patients belonged to 51-70 years age group. Male patients were predominant 13 (59.1%) whereas females were 9 (40.9%). Among the respondents, 16 (72.7%) had non severe aplastic anaemia, 3 (13.6%) severe aplastic anaemia and 3 (13.6%) very severe aplastic anaemia.

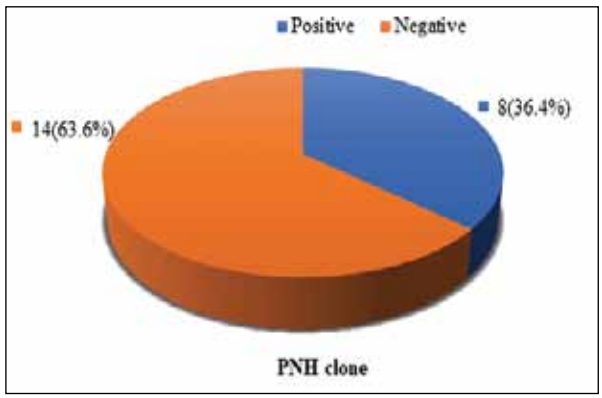

Figure 1: Pie chart showing PNH clone status of the study population (n=22)

All patients had fatigue irrespective of PNH cone status. Abdominal pain, haemoglobinuria and dysphagia were present in 5 (62%), 4 (50%) and 3 (37.5%) of PNH clone identified group respectively, these clinical features were found statistically significant. However, fever, infection, erectile dysfunction, jaundice, haemorrhage, and thrombosis were not found to be statistically significant (p>0.05) between two groups (Table I).

Table I: Association between clinical manifestations with PNH clone (n=22)

Clinical features	PNH clone				
	Positive (n=8)		Negative (n=14)		
	n	%	n	%	P value
Fatigue	8	100.0	14	100.0	-
Infections	4	50.0	6	42.9	0.546 ns
Abdominal pain	5	62.5	2	14.3	0.032 s
Haemoglobinuria	4	50.0	0	0.0	0.010 s
Dysphagia	3	37.5	0	0.0	0.036 s
Erectile dysfunction	2	25.0	0	0.0	0.121 ns
Jaundice	2	25.0	0	0.0	0.121 ns
Haemorrhage	1	12.5	0	0.0	0.364 ns
Thrombosis	1	12.5	0	0.0	0.364 ns

s = significant, ns = not significant; P value reached from chi square test

Mean clone was highest in granulocytes and lowest in Monocytes. Measurement of clone size in granulocytes has most vital role in PNH diagnosis, management and prognosis (Table II).

Table II: PNH clone size according to ICCS recommended flow cytometry protocol.

PNH clone series	Mean±SD
RBC type II	9.13±23.45
RBC type III	0.63±1.77
Granulocytes	70.02±43.29
Monocytes	63.45±42.74

Out of 8 patients with PNH clone positive, non-severe aplastic anaemia was found in 7 (87.5%) with a mean clone size in granulocyte 67.4±45.4% and very severe aplastic anaemia was in 1 (12.5%) with a mean clone size in granulocyte 96.4±0.0% (Table III).

Table III: PNH clone in aplastic anaemia (n=8) with their clone size in granulocytes

	Aplastic anaemia Frequency (%)	PNH clone Mean±SD
Non severe	7 (87.5%)	67.4±45.4
Very severe	1 (12.5%)	96.4±0.0

Complete blood count had no statistically significant role between PNH clone positive & negative group. Reticulocyte count and S. LDH level were higher in PNH clone positive group, the difference was statistically significant (P value <0.05). In PNH, severity of haemolysis can be assessed from Reticulocyte count & S. LDH level assessment (Table IV).

Table IV: Association between investigation profile with PNH clone (n=22)

	PNH clone				P value
	Positive (n=8)		Negative (n=14)		
	Mean	±SD	Mean	±SD	
Hb (gm/dl)	7.87	±1.32	8.73	±1.48	0.193 ns
WBC (x10^9/L)	2.34	±0.66	2.37	±0.80	0.935 ns
Absolute neutrophil count (x10^9/L)	1.09	±0.79	0.90	±0.65	0.550 ns
Platelet count (x10^9/L)	48.63	±43.69	40.14	±19.62	0.535 ns
Reticulocyte count (%)	2.78	±3.71	0.54	±0.55	0.036 s
S. LDH (U/L)	521.63	±181.40	268.23	±170.0	0.004 s

s = significant, ns = not significant; P value reached from unpaired t-test.

Non severe aplastic anaemia was found in 16 cases of total participants. Among them 7 (43.75%) were in PNH positive group and 9 (56.25%) were in PNH negative group. Severe aplastic anaemia (SAA) was found in 3 cases, all of them (100.0%) belonged to PNH negative group. Very severe aplastic anaemia (VSAA) was found in 3 cases. Among them 1 (33.3%) in PNH positive group and 2 (66.7%) in PNH negative group. The difference was not statistically significant (p>0.05) between two groups (Table V).

Table V: Association between type of aplastic anaemia with PNH clone (n=22)

Type of aplastic anaemia	Total	PNH clone				P value
		Positive (n=8)		Negative (n=14)		
		n	%	n	%	
Non severe	16	7	43.75	9	56.25	
Severe	3	0	0.0	3	100.0	0.349ns
Very severe	3	1	33.3	2	66.7	

ns = not significant; P value reached from chi square test

Discussion

PNH clone in aplastic anaemia has versatile features from silent to typically symptomatic. In this study it was observed that majority 11 (50.0%) patients belonged to age group 51-70 years with mean 50.2±18.1 ranging from 16 to 90 years. Almost similar study conducted by Urbano-Ispizua et al. median age was found to be 45.0 years with age range from 18 to 99 years.[14] Scott reported that the disease presents most commonly in people between the ages of 15 and 25 years.[15] Another study conducted by Shilova et al. where they documented that median age was 46 years with range from 23 to 54 years.[16]

In our study PNH clone was detected in 8 (36.4%) participants and not found in the rest 14 (63.6%). Shilova et al. (2016) reported that out of 81 patients, 47 patients (58%) were found AA/PNH-positive.[16]

Kulagin et al. (2014) consisted that PNH clones were present in 74 patients (59.2%).[17] Another study conducted by Gupta et al. (2010) found that out of 46 aplastic anaemia patients, PNH clone was detected in 23 (50%) patients.[18]

In this study, PNH clone was identified in 8 patients, non-severe aplastic anaemia was found in 7 (87.5%) with mean clone size was 67.4±45.4% and very severe aplastic anaemia in 1 (12.5%) with mean clone size was 96.4±0.0%. Scheinberg et al. (2010) documented that clone was present in about 40-50% of patients with severe aplastic anemia.[19]

In this current study it was observed that abdominal pain was found in 5 (62.5%) patients in PNH clone positive group and 2 (14.3%) in PNH negative group. Haemoglobinuria was found in 4 (50.0%) in PNH clone positive group but not found in PNH negative group. Dysphagia was found 3 (37.5%) in PNH clone positive group. Which were statistically significant. However, fever, infection, abdominal pain, dysphagia, erectile dysfunction, jaundice, haemorrhage, and thrombosis were not found to be statistically significant ($p > 0.05$) between two groups. Gupta et al. (2010) reported that the common signs and symptoms noted in PNH patients were pallor (91.3%), bleeding (43.5%), cola-colour urine (30.4%), icterus (26.1%) and fever (26.1%).[18]

Among 8 PNH positive patients, non-severe aplastic anaemia was found in 7 (87.5%) and very severe aplastic anaemia was found in 1 (12.5%). PNH clone was not detected in 14 patients, 9 (64.3%) of them had non severe aplastic anaemia, 3 (21.4%) and 2 (14.3%) belonged to severe aplastic anaemia and very severe aplastic anaemia group respectively. The difference was not statistically significant ($p>0.05$) between two groups. In a study conducted by Kulagin et al. (2014) where they showed that moderate aplastic anaemia (MAA) found 29.7% in PNH positive group and 29.4% in PNH negative group. Severe aplastic anaemia (SAA) was found in 41.9% and 41.2% in PNH positive and PNH negative group respectively.17 Very severe aplastic anaemia (VSAA) was found in 28.4% in PNH positive group and 29.4% in PNH negative group. The difference was not statistically significant ($p>0.05$).

In our study RBC II mean clone size was 9.13±23.24%. Mean RBC III clone size was 0.63±1.77%. In a study of Scott where they found in the 83 patients (68%) with PNH cells, defined as CD55-negative/CD59-negative, the proportion of PNH cells ranged from 0.005% to 23.1%.[15] Fidarova et al. consisted that median clone size on the Red Blood Cells (RBC: type II + type III) was 0.25% (0.03-25.3%).[20] Kulagin et al. documented that the median baseline clone size was 0.15% (IQR, 0.033-0.51%, range 0.01-9.15%) PNH-type RBCs.[17]

Regarding monocyte, majority 6 (75%) had >8% PNH clone on monocyte and their mean clone size was 63.45±42.74%. Regarding granulocyte, majority 6 (75%) had >10% PNH clone on granulocyte and their mean was 70.02±43.29%. Study conducted by Scott (2018) where they found that for aplastic anaemia patients with PNH clones at subclinical levels (≤5% of granulocytes), it is important to monitor levels of PNH cells every 6 months to provide early evidence of clonal expansion.15 Fidarova et al. consisted that median (Me) clone size on the Granulocytes (GR) -1.7% (0.02- 93.92%), Monocytes (Mon) -23.2% (0.05-95.66%).[20] Kulagin et al. reported that the median baseline clone size was 0.6% (IQR, 0.1-3.99%, range 0.01-51.48%) PNH-type granulocytes.[17] Eleven patients (15%) were classified as having AA/PNH, defined by PNH clone size >1% in granulocytes (median of 10%, IQR 5.2-46, range 1.8-51.48). There is another

study documented by Gupta et al. (2010) where they observed on flow cytometry, the PNH clone size (on granulocytes) in PNH patients varied from 7% to 97%. Sixteen (69.6%) patients had >50% clone size, six (26.1%) had 10-50%, and one patient had >50% clone size, six (26.1%) had 10-50%, and one patient had <10% clone size.[18] The PNH clone size on monocytes varied from 2.8% to 92.2% in these patients between two groups. These finding were consistent with our study.

In our study it was observed that mean reticulocyte count was found 2.78±3.71% in PNH clone positive group and 0.54±0.55% in PNH clone negative group. Mean serum LDH was 521.63±181.40 U/L and 268.23±170.0 in PNH clone positive and negative group respectively. Reticulocyte count and serum LDH were significantly higher in PNH clone positive group than negative group. However, differences in Hb, WBC, absolute neutrophil count and platelet count were statistically significant (p>0.05) between two groups. Kulagin et al. also found in their study no statistically significant differences in baseline white blood cell count (WBC), absolute neutrophil count (ANC), absolute lymphocyte counts (ALC), lactate dehydrogenase (LDH) level between the PNH-positive and PNH-negative groups.[17] However, in contrast to previous data we did not find statistically significant correlations between response rate and WBC, ALC, ANC, gender or interval between diagnosis and treatment.[19,21,22] There is another study documented by Gupta et al. (2010) where they observed on comparing patients of AA with (n=23) and without (n=23) PNH clone, no significant difference in Hb, ANC and platelet count in two groups.[18] These finding were consistent with the previous study.

Conclusion:

This study showed that PNH clone was significantly associated with aplastic anaemia. Our data confirm the need for regular PNH clone assay in aplastic anaemia patients for early specific management with Eculizumab and Haematopoietic Stem Cell Transplantation (HSCT) to save life. Flow cytometry for PNH clone must be done in all patients of aplastic anaemia routinely. More modern laboratories for flow cytometry assays should be established for this purpose.

Limitation

The study population was selected from one hospital – BSMMU, Dhaka. So, the results of the study may not reflect the exact picture of the country. The present study was conducted in a very short period. Patients were not sorted as children and adults, which may exert certain influences on the conclusions.

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