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ORIGINAL ARTICLE

Markers of Thrombin Generation and Inflammation in Patients with Paroxysmal Nocturnal Hemoglobinuria

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Abstract Paroxysmal nocturnal hemoglobinuria (PNH) presents with intravascular hemolysis, bone marrow failure and thrombosis. Various studies have reported geographic and ethnic variation in prevalence of thrombosis in PNH. There is limited data on thrombosis in PNH from the Indian subcontinent. In this study we describe disease burden and risk factors for thrombosis in 18 Indian PNH patients. We studied markers of thrombin generation (Thrombin-antithrombin complexes; TAT and D-Dimer), endothelium and platelet activation (soluble P-selectin) and inflammation (interleukin-6; IL-6) in PNH patients and compared their levels with healthy controls. Thrombosis was identified in 17% of PNH patients. TAT, sP-selectin and D-Dimer levels were significantly elevated in PNH patients (TAT: 5.06 ± 1.08 ng/ml; sP-selectin: 80.57 ± 19.5 ng/ml; D-Dimer mean: 936 ng/ml 95% CI 559, 1310) compared to control population (TAT: 3.39 ± 0.769 ng/ml $P = 0.016$; sP-selectin: 44.67 ± 5.17 ng/ml $P = 0.002$). Using Youden's J statistic, the cut-off values for TAT and sP-selectin in our cohort of PNH patients were 2.90 ng/ml and 58.41 ng/ml respectively. TAT, sP-selectin and D-Dimer levels were elevated beyond the cut-off values in PNH patients with thrombosis compared to those without thrombosis. A positive correlation was noted between TAT, sP-selectin and D-Dimer levels. Increased TAT, sP-selectin, and D-Dimer levels may indicate impending thrombosis in PNH.

Keywords Paroxysmal nocturnal hemoglobinuria · Thrombosis · Inflammation · Thrombin–antithrombin complexes · P-selectin · D-Dimer

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare haematological illness acquired at the multipotent stem cell level [1]. Its clinical manifestations are consequences of intravascular hemolysis, bone marrow failure, and thromboembolism [2, 3]. Thromboembolism rates in PNH range from 16 to 44% [4–10]. Visceral thrombosis precedes the diagnosis of PNH in 19% of patients [11]. While venous thrombosis predominates, arterial thrombosis has also been reported [6, 12, 13]. Thrombosis is the leading cause of mortality in PNH [5–9]. Various studies have reported geographical and ethnic variation in prevalence of thrombosis in PNH [4, 8, 14, 15].

Patients with PNH have elevated markers of hemostatic activation e.g. D-Dimers and Thrombin-antithrombin complexes (TAT), inflammation e.g. interleukin-6 (IL-6) and endothelium and platelet activation e.g. soluble P-selectin (sP-selectin) which decrease upon treatment with complement inhibitor eculizumab [16, 17].

We undertook this study in Indian patients with PNH to chronicle the clinical presentation and study the spectrum of complications of PNH with special emphasis on thromboembolic complications. We studied markers of thrombin generation (TAT, D-Dimer), endothelium and platelet activation (sP-selectin) and inflammation (IL-6) in PNH patients and compared their levels with healthy

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controls in order to better understand the development of thromboembolic complications in PNH.

Patients and Methods

We studied disease characteristics, with emphasis on thromboembolic complications in 18 patients with PNH who were registered in our clinic between January 2004 and January 2014. Patients with more than 5% Glycosyl phosphatidylinositol-anchored protein (GPI-AP) deficient leukocytes (monocytes and granulocytes) on flow cytometry were diagnosed to have PNH. As per International PNH Interest Group recommendations (IPIG), patients were divided into classic PNH and PNH in the setting of another specified bone marrow (BM) disorder e.g. PNH-Aplastic Anemia (PNH-AA) [18]. PNH-AA patients had BM cellularity $< 30\%$ in conjunction with clinical and lab evidence of intravascular hemolysis. Classical PNH patients had clinical and lab evidence of intravascular hemolysis in absence of any BM abnormality. Anemia, thrombocytopenia and neutropenia were defined as per International Agranulocytosis and Aplastic Anemia Study Group (IAAAG) criteria; haemoglobin < 100 g/l platelet count $< 50 \times 10^9$/l, neutrophil count $< 1.5 \times 10^9$/l [19]. BM aspiration and biopsy was performed for all patients with PNH presenting with cytopenias. In other patients, BM examination was done at discretion of the treating physician.

Patients were sampled for markers of hemostatic activation; thrombin-antithrombin complex (TAT), D-Dimers, soluble P-Selectin (sP-selectin) and inflammatory cytokine Interleukin-6 (IL-6). Samples for TAT, sP-selectin and IL-6 were also collected from 35 age and sex matched healthy controls in order to determine the cut-off values for these assays in an Indian population. A standardized quantitative immunoturbidimetric D-Dimer assay is available at our institution (cut off value in healthy individuals < 500 ng/ml). Hence D-Dimer levels were not quantified in healthy control population. Blood samples were collected by non-traumatic peripheral vein sampling into tubes containing buffered 3.2% sodium citrate. Patients who had suffered an acute thrombotic episode had their blood samples drawn when at least four weeks had elapsed after the latest thrombotic episode. Platelet poor plasma was prepared by centrifugation at 2500 *g* for 15 min, aliquoted and frozen at minus 80 °C until testing. Plasma TAT, sP-selectin and IL-6 levels were measured using commercial enzyme-linked immunoassays (Abcam TAT ELISA, Abcam Inc, MA, USA; Human sP-selectin/CD62P ELISA & Human IL-6 Quantikine HS ELISA, R & D Systems Inc, Minneapolis, MN, USA respectively). D Dimer was measured with automated STA LIATEST immunoturbidimetric D-dimer assay performed on the STA-Compact analyzer (Diagnostica Stago, France).

Statistical Analysis

Description of patients at presentation is presented as N(%) for qualitative variables and as either mean (standard deviation, S.D) or median (range) for continuous variables. The distribution of presentation characteristics between various subcategories were compared by chi-squared test or Fisher exact test and by Kruskal–Wallis or Mann Whitney test for continuous variables. Pearson correlation was used to calculate correlation coefficient (r) and P values between different clinical characteristics, LDH levels and different markers of thrombin generation and inflammation. Accuracy of TAT, sP-selectin and IL-6 assays in discriminating PNH patients from age and sex matched healthy controls was evaluated by receiver operating characteristic (ROC) analysis. Sensitivities, specificities and Youdens J statistic were calculated for identifying cut-off values of respective assays. Statistical analysis was performed with SPSS version 22.0 software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). A P value less than 0.05 was taken as statistically significant.

Results

A total of 18 patients (13 males and 5 females) with PNH were included in this study. Seven (four males and three females) of these patients, were enrolled and followed up prospectively. The clinical and follow up data of remaining 11 patients (nine males and two females) was retrieved and they were followed up prospectively after inclusion in the study. The median duration of follow up of 18 patients in PNH cohort was 54 months (range 6–247). Overall 17 patients had classic PNH and one patients had PNH-AA. Disease characteristics of 18 patients enrolled in our study are summarized in Table 1.

A total of 3 (17%) patients with PNH had thrombotic events in our study. All three patients had classic PNH. Two of these patients had cerebral venous thrombosis and chronic portal vein thrombosis respectively at the time of presentation. Another patient developed cerebral venous thrombosis six months after the diagnosis. One patient (Patient-14) who developed cerebral venous thrombosis later developed light chain amyloidosis involving the heart at presentation. The three patients with thrombosis had granulocyte PNH clone size of 85%, 95% and 98% respectively.

Bone marrow findings were available for 13 patients. Erythroid hyperplasia, defined as a predominance of erythroid (E) precursors over myeloid (M) cells (M:E ratio:

Table 1 Clinical and lab parameters of 18 patients with paroxysmal nocturnal hemoglobinuria (PNH)

Characteristic	N (%) or median (range)
Sex	
Male	13 (72%)
Female	5 (28%)
Age at diagnosis (years)	30.5 (18–52)
Disease duration (years) prior to diagnosis	4 (0–15)
Presentation	
Classical PNH	17 (95%)
PNH-AA	1 (5%)
Signs and symptoms	
Fatigue	18 (100%)
Hemoglobinuria	13 (72%)
Abdominal pain	6 (33%)
Splenomegaly	4 (22%)
Erectile dysfunction, N (%)males	8 (62%)
Infections	1 (5%)
Renal failure	4 (22%)
Thrombosis	3 (17%)
Portal vein thrombosis	1 (5%)
Cerebral venous thrombosis	2 (11%)
Diagnostic test	
Size of PNH clone % (FLAER)	85% (52–99.5)
LDH, fold above ULN	
Median (range)	11 (2.18–32.66)
Peripheral blood abnormalities	
Anemia alone	11 (61%)
Anemia and thrombocytopenia	2 (11%)
Pancytopenia at diagnosis	3 (17%)
Bone marrow cellularity (13 patients)	
Hypercellular	7 (54%)
Normocellular	5 (38%)
Hypocellular	1 (8%)

PNH paroxysmal nocturnal haemoglobinuria, *PNH-AA* PNH-aplastic aknemia, *LDH* lactate dehydrogenase, *FLAER* fluorescently labeled aerolysin, *ULN* upper limit of normal

$\leq$ 1), was seen in 12 patients. The cellularity of marrow was increased in seven (54%) patients (hypercellular marrow), normal in five (38%) patients (normocellular marrow) and decreased in one (8%) patient (hypocellular marrow). Megaloblastoid erythropoiesis was seen in 10 (77%) patients. Five patients had peripheral blood (PB) cytopenias in two or more lineages as per IAAAG criteria. Three of these five patients had pancytopenia. Of these three, one patient having pancytopenia with decreased BM cellularity and normal ME ratio was diagnosed with PNH-AA. The remaining two patients with pancytopenia had myeloid hypoplasia in addition to erythroid hyperplasia and increased BM cellularity.

Anemia and thrombocytopenia as per IAAAG criteria were present in the remaining two of the five patients with PB cytopenias. Both had increased BM cellularity with hyperplastic erythropoiesis. The combination of myeloid hypoplasia and erythroid hyperplasia was also seen in two patients having normal PB neutrophil and platelet counts and normal BM cellularity. As per IPIG criteria, 12 patients had classical PNH and one patient had PNH-AA. BM was not performed for five patients. None of these patients had PB cytopenias fulfilling IAAAG criteria.

Markers of hemostatic activation; TAT, D-Dimer, sP-selectin and inflammatory cytokine, IL-6, were measured in all the 18 patients including three patients with thrombosis. None of these patients were receiving anticoagulation at the time of the sampling. All patients were started on prednisolone therapy for florid hemolysis at presentation.

TAT and sP-selectin levels were significantly elevated in PNH patients (TAT: 5.06 ± 1.08 ng/ml; sP-selectin: 80.57 ± 19.5 ng/ml) compared to control population (TAT: 3.39 ± 0.769 ng/ml $P = 0.016$; sP-selectin: 44.67 ± 5.17 ng/ml $P = 0.002$) (Table 2. There was no difference in IL-6 levels between PNH patients (2.75 ± 1.34 pg/ml) and healthy control population (2 ± 0.596 pg/ml $P = 0.327$). Mean D-Dimer level in PNH cohort was almost twice the upper limit of normal; 936 ng/ml (95% CI 559–1310).

Cut-off values to distinguish PNH patients from normal population were determined for TAT and sP-Selectin using Youden's J statistic. The cut-off value obtained for TAT was 2.90 ng/ml (Youden index: 0.432, 89% sensitive and 54% specific) and for sP-selectin was 58.41 ng/ml (Youden index: 0.552, 67% sensitive and 89% specific). Using Pearson correlation coefficient (r) a significant correlation of TAT levels with sP-Selectin levels (r: 0.37, $P = 0.006$) and D-Dimer levels (r: 0.5, $P = 0.033$) was noted in PNH patients.

We compared levels of markers of thrombin generation (TAT, sP-selectin, D-Dimer) and inflammation (IL-6) in the three PNH patients with thrombosis to 15 PNH patients without thrombosis. Although TAT, sP-selectin and D-Dimer levels were increased above cut-off values in all the three PNH patients with thrombosis, this difference was not significant. In one patient (Patient-13) who developed cerebral venous thrombosis 4 weeks after the sampling, TAT, sP-selectin and D-Dimer levels were highly elevated (> 2–3 times the cut-off; TAT: 7.83 ng/ml, sP-selectin: 138.95 ng/ml, D-Dimer: 3320 ng/ml).

Discussion

The median age at diagnosis of PNH in our study was 30 years (range 18–52) and a male preponderance was noted. These findings are similar to that reported in other series on PNH from Asia [8, 14, 20–22]. Studies on Caucasian PNH patients show that disease usually gets diagnosed in the fourth decade of life and females are predominantly afflicted [5, 6, 8, 10]. This suggests a geographical difference in presentation of PNH between Caucasians and Asians. The reasons for these differences are not known. Median disease duration prior to diagnosis of PNH was 4 years, similar to that reported in the International PNH registry (4.6 years) [10]. Fatigue, intermittent hemoglobinuria, erectile dysfunction and abdominal pain were present in 100%, 72%, 62% and 33% of the patients respectively. All patients had granulocyte PNH clone sizes more than 50% and lactate dehydrogenase (LDH) levels were elevated in all the patients at the time of diagnosis, reflecting active ongoing hemolysis. In the international PNH registry study involving 1610 patients with PNH, a significantly more number of patients with clone sizes > 50% reported symptoms of hemoglobinuria, erectile dysfunction and abdominal pain. PNH-related symptoms were common in patients with elevated LDH [10]. A retrospective analysis on 301 PNH patients from South Korean National Registry identified significantly increased risk of thrombosis in patients with abdominal pain and elevated LDH [12].

Whereas cytopenias fulfilling the IAAAG diagnostic criteria for AA were seen in five (28%) patients, only one patient had bone marrow findings suggestive of aplastic anemia. The latter patient was classified as PNH-AA. Rest of the patients in our study were classic PNH (95%). Two other studies on PNH from India report a higher prevalence of classic PNH compared to PNH-AA [21, 22]. Other

Table 2 Comparison of markers of thrombin generation and inflammation in PNH patients and controls

	PNH (N = 18) Mean (95% CI)	Controls (N = 35) Mean (95% CI)	PNH versus controls *P* value
TAT (ng/ml)	5.06 (3.98, 6.14)	3.39 (2.62, 4.16)	0.016
sP-selectin (ng/ml)	80.57 (61.5, 99.7)	44.67 (39.5, 49.8)	0.002
IL-6 (pg/ml)	2.75 (1.41, 4.09)	2.0 (1.4, 2.6)	0.327
D-Dimer (ng/ml)	936 (559, 1310)		

Normal control values: TAT: < 2.90 ng/ml; sP-selectin: < 58.41 ng/ml; D-Dimer: < 500 ng/ml

PNH paroxysmal nocturnal hemoglobinuria, *TAT* thrombin–antithrombin complex, *sP-selectin* soluble P-selectin, *IL-6* interleukin-6

Asian countries report a higher prevalence of PNH-AA and subclinical PNH with AA compared to classic PNH [8, 14, 20].

In our study 17% of patients had thrombosis. This is similar to prevalence of thrombosis reported in other Indian studies and is likely related to higher prevalence of classic PNH reported in these studies [21, 22]. Studies on PNH from Asia report greater prevalence of bone marrow failure and lower risk of thrombosis compared to Western patients with PNH [8, 14, 15, 20]. South Korean National Registry data on PNH challenges this perception. Majority of patients in this study had haemolytic PNH and prevalence of thrombosis was 17.9% [12]. Most of the studies on PNH from Asia are single centre retrospective analysis involving relatively small number of patients. Comprehensive multicentre registry-based data on PNH from Asia are needed for better understanding of clinical presentation and natural course of this disease in Asians vis-à-vis Caucasians.

In order to better understand thromboembolic phenomena in PNH we compared markers of thrombin generation (TAT, D-Dimer), platelet activation (sP-selectin) and inflammation (IL-6) in PNH patients and healthy control population. TAT, sP-selectin and D-Dimer levels were significantly elevated in PNH patients. We did not find significant elevation in IL-6 levels in PNH patients in our study. All our 18 patients were receiving prednisolone at the time of study. Prednisolone is known to inhibit the release of IL-6 [23]. Positive correlation of TAT with D-Dimer levels is understandable as both are markers of hemostatic activation. Significant correlation between TAT and sP-selectin levels noted in our study suggests contribution by platelet and endothelial activation towards thrombosis [17]. The high thrombosis rates and elevated markers of thrombin generation and endothelial and platelet activation observed in PNH patients in our study strengthens the recommendation for prophylactic anticoagulation in Indian patients with classic PNH.

The cut-off values for TAT (2.9 ng/ml) & sP-selectin (58.41 ng/ml) in our cohort of PNH patients were similar to that reported by Weitz et al. [17] in their cohort of Caucasian PNH patients (TAT: 2.9 ng/ml Vs. 2.8 ng/ml and sP-selectin: 58.41 ng/ml vs. 40 ng/ml). We did not find significant difference in levels of markers of thrombin generation and inflammation in PNH patients with and without thrombosis. This is likely because our study was underpowered to detect this difference. One of the patients in our study had very high TAT, sP-selectin and D-Dimer levels in the weeks preceding thrombosis. This suggests a role of these markers in predicting thrombosis in PNH patients. However we need to confirm this finding in a larger cohort of PNH patients with longer follow up before a firm recommendation can be made in this regard. Eculizumab therapy decreases the levels of these markers and also leads to decline in thrombosis risk in hemolytic PNH [16, 17, 24]. PNH patients with minimal hemolysis are also at risk for thrombosis. A recent study reported thrombosis risk to be as high as 50% in patients with PNH granulocyte clone more than 30% and no hemolysis [13] Such patients are not candidates for eculizumab therapy and there are no firm recommendations for prophylactic anticoagulation in this group of patients. Studying markers of thrombin generation and inflammation in this group of PNH patients may identify the subgroup of patients at risk for developing thrombosis and who may benefit from prophylactic anticoagulation.

The major limitation of our study is the relatively small PNH patient sample size. This is not surprising as PNH is a rare illness. However the clinical presentation of PNH in our Indian cohort of patients is comparable to that reported in large registry based studies [10, 12]. None of the patients in our study received eculizumab. In this regard our study represents real world data as majority of PNH patients do not have access to eculizumab. In a recently published study by International PNH Registry, only 779 (16%) of the 4948 patients with hemolytic PNH received Eculizumab and had non-missing data for analysis [25]. To the best of our knowledge this is the first comprehensive analysis of thrombosis in PNH patients from India. An adequately powered study is needed to study discriminant values of TAT, sP-selectin and D-Dimer levels in predicting thromboembolic phenomena in PNH. Nevertheless our study provides important suggestions for further research in this area.

In conclusion thrombosis rates in Indian PNH patients are comparable to that reported in Caucasians. There is significant elevation of markers of thrombin generation and endothelial and platelet activation in PNH patients in our study. These role of these markers in predicting thrombosis in PNH needs further study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in this study.

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