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Natural history of paroxysmal nocturnal hemoglobinuria clones in patients presenting as aplastic anemia

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Abstract

Objective—Investigate the natural history of PNH clones in patients with acquired aplastic anemia (AA).

Patients and Methods—Twenty-seven patients with AA and a detectable PNH clone were monitored for a median of 5.7 years (range 1.5 to 11.5 years). Twenty-two patients received high dose cyclophosphamide (HiCy) therapy. The erythrocyte and granulocyte PNH clone sizes were measured using flow cytometry and analyzed via CellQuest software. PE-conjugated anti-glycophorin A, anti-CD15, FITC-conjugated anti-CD59, and FLAER staining were used to define GPI-AP deficient cells.

Results—We found a linear relationship between PNH clone size and the development of intravascular hemolysis, assessed by LDH values (Pearson Correlation Coefficient=0.80, $P<0.001$ for erythrocyte PNH clones; and Pearson Correlation Coefficient=0.73, $P<0.0001$ for granulocyte PNH clones). An erythrocyte PNH size of 3~5% and granulocyte PNH size of 23% were the thresholds to predict hemolysis as measured by an elevated LDH (ROC analyses with AUC=0.96 for erythrocyte PNH clone sizes and AUC=0.88 for granulocyte PNH clone sizes). Patients with small ($\leq 15\%$) initial PNH clone sizes were less likely to develop an elevated LDH (mean \pm SD: 236.9 \pm 109.9 vs 423.1 \pm 248.8; $P=0.02$). Over time, the PNH clone sizes remained stable in 25.9% of patients; 48.1% experienced a rise in the PNH clone size and 25.9% experienced a decrease.

Conclusion—The risk of developing clinically significant PNH after HiCy therapy appears to be low in AA patients with PNH clones, especially for those with small initial PNH clones and for those who respond to HiCy therapy.

Keywords

Aplastic Anemia (AA); Paroxysmal Nocturnal Hemoglobinuria (PNH); High dose cyclophosphamide (HiCy); Fluoresceinated Aerolysin Variant (FLAER); Lactate Dehydrogenase (LDH).

Introduction

Acquired aplastic anemia (AA) and paroxysmal nocturnal hemoglobinuria (PNH) are closely related bone marrow failure disorders^{1,2}. Most cases of AA result from an

autoimmune attack directed against hematopoietic stem/progenitor cells^{3;4}. PNH is an acquired clonal hematopoietic stem cell disorder that manifests following the expansion and differentiation of a hematopoietic stem cell harboring a *PIG-A* mutation. The *PIG-A* gene product is necessary for the synthesis of glycosylphosphatidylinositol (GPI), a glycolipid moiety that anchors dozens of proteins to the cell surface. Consequently, PNH cells are missing a variety of GPI-anchored proteins (GPI-APs), two of which (CD55 and CD59) are important complement regulatory proteins. The absence of these proteins on PNH cells contributes to many manifestations of the disease such as hemolytic anemia, thrombosis and smooth muscle dystonias. Both PNH and AA can be cured by allogeneic bone marrow transplantation (BMT), but only a minority of patients is offered this approach due to the potential morbidity and mortality of BMT and the availability of other less toxic approaches⁵. AA can be treated effectively with immunosuppressive therapy^{6,7} and high dose cyclophosphamide (HiCy).⁸ PNH can be controlled by a monoclonal antibody that interferes with terminal complement activation⁹⁻¹¹.

PNH often arises from AA. It has been estimated that more than 50% of AA patients harbor small, but expandable PNH populations at diagnosis^{12;13}. The natural history of patients with classical PNH has been well documented¹⁴⁻¹⁷; recently, a retrospective study investigated 83 severe aplastic anemia (sAA) patients with PNH clones following standard immunosuppressive therapy such as antithymocyte globulin and cyclosporine (ATG/CsA).¹⁸ However, the natural history of PNH clones in AA patients following HiCy therapy has not been documented. We developed a highly sensitive and specific assay to detect PNH blood cells based on the properties of the bacterial toxin proaerolysin^{19;20}. Since 1998, we have used this assay to detect small PNH clones in patients with AA¹³. The purpose of this study is to determine the fate and clinical relevance of these PNH clones in patients with AA, especially those patients treated with HiCy.

Patients and Methods

Patients and patient selection

The patient selection criteria for this study were as follows: 1) having an initial diagnosis of acquired AA; 2) having at least three positive values ($\geq 0.02\%$) on PNH FLAER assay in the course of follow-up; 3) having been monitored for at least 18 months after detecting a PNH clone. These patients were treated and monitored at every 3 to 6 months between November, 1998 and March, 2010.

Venous peripheral blood was drawn into heparin-containing tubes after informed consent as approved by the Joint Committee on Clinical Investigation of The Johns Hopkins Hospital. Acquired aplastic anemia was diagnosed as per clinical criteria^{21;22}. Severe aplastic anemia (SAA) was defined as 1) $<25\%$ bone marrow cellularity and 2) at least two of the following three peripheral blood counts: neutrophils $<0.5 \times 10^9/L$; platelets $<20 \times 10^9/L$; and anemia with corrected reticulocytes $<1\%$; patients with very severe aplastic anemia (vSAA) met the same criteria except that the neutrophil count was below $0.2 \times 10^9/L$. Non-severe aplastic anemia (nSAA) was defined as marrow hypoplasia with pancytopenia not severe enough to meet the above criteria²³.

Methods

Detection of GPI anchor expression deficiency was assessed via flow cytometry as previously described.^{16, 19} Briefly, erythrocytes and granulocytes were identified on the basis of forward and side scatter and by staining with PE-conjugated anti-phosphorin A and anti-CD15 (Immunotech). The gates used to define CD59⁻ erythrocytes and FLAER-

negative granulocytes were set up by using normal erythrocytes and granulocytes as control. 17, 19

CellQuest software was used to collect and analyze the flow cytometry study data. PNH clone size was defined as percentage of GPI-AP deficient erythrocytes or granulocytes in whole peripheral blood. Serum lactate dehydrogenase (LDH) values were measured by Johns Hopkins Hospital core laboratory.

Statistical analysis

Association between PNH and LDH was characterized by Pearson correlation and the statistical significance was determined using a mixed effect model with longitudinal PNH size and LDH value data. To evaluate the accuracy of PNH clone size to discriminate normal and abnormal LDH, receiver operating characteristic (ROC) analysis based on logistic regression was used, and sensitivities and specificities were computed for identifying PNH clone size thresholds. Random coefficient models were applied to examine the association of baseline PNH clone size and LDH over time. Two sample t-test for the numeric LDH and Fisher's exact test for binary LDH status were used to compare LDH values at five years after diagnosis between patients with low and higher initial PNH clone sizes. Separate linear regression of PNH size on elapsed time since the initiation of treatment was constructed for each subject, and the slope parameter of the regression line was used to estimate the change rate of PNH size over the course of follow-up. All analyses were carried out using statistical package SAS®, Version 9.1.

Results

Patient characteristics

Twenty-two patients who received HiCy therapy and met selection criteria were screened from 67 AA patients treated on our HiCy protocol. 8 Five AA patients who did not receive HiCy therapy were recruited from our AA clinic. Patient characteristics are outlined in Table 1. All patients were initially diagnosed with acquired AA with a median granulocyte count of $0.15 \times 10^9/L$ (range: $0.03-2.30 \times 10^9/L$), a median platelet count of $9 \times 10^9/L$ (range: $4-21 \times 10^9/L$), a median corrected reticulocyte count of 0.3 % (range: 0.1-2.6%), and a median bone marrow CD34 count of 0.1% (range: 0.02%-0.24%). All patients had a bone marrow cellularity <20%. Twenty-one patients met criteria for SAA, two patients met criteria for vSAA, and four patients met criteria for nSAA. Based on initial granulocyte PNH clone sizes, patients were arbitrarily divided into three groups: small ($\leq 15\%$, n=16), medium (15~40%, n=7), and large ($\geq 40\%$, n=4). There were fifteen patients initially treated with HiCy therapy; seven patients initially treated with common immunosuppressive regimens, then treated with HiCy due to either refractory or relapsed SAA; two patients only received immunosuppressive therapy (one received CsA and another received ATG/CsA), and three patients (all nSAA) did not require treatment during the period of observation. Patient demographics and response to therapy are shown in Table 2.

Relationship between PNH size and serum LDH value

In order to determine the minimum PNH clone size that is associated with the onset of intravascular hemolysis, we plotted all available serum LDH values with paired PNH clone sizes, which were measured at the same time point for individual patients (Fig. 1A). We found a significant linear relationship between PNH clone sizes and LDH values in both erythrocytes and granulocytes (Pearson Correlation Coefficient=0.804, $P < 0.001$ for erythrocytes; and Pearson Correlation Coefficient=0.72788, $P < 0.0001$ for granulocytes). Using a receiver operating characteristic curve (ROC) analysis, we demonstrated that the area under the ROC curve (AUC) for erythrocytes was 0.96 and for granulocytes was 0.88,

which indicated that the size of the PNH clone in both erythrocytes and granulocytes had the predictive value to discriminate patients with normal and abnormal LDH values (Fig.1B). The subsequent sensitivity and specificity analysis demonstrated that a PNH clone size of 3~5% for erythrocytes and of 23% for granulocytes had threshold effects to distinguish normal and abnormal LDH, which in turn would classify a patient as with or without hemolysis. Next, we investigated the probability of developing intravascular hemolysis, using LDH as a surrogate, based on initial PNH size at diagnosis (Figure 2). We compared serum LDH values five years after diagnosis of AA among the patients who initially presented with a small percentage of PNH granulocyte (n=15), to patients who presented with medium and large percentages of PNH granulocytes (n=9). We found that the AA patients in the small size group had significantly lower LDH levels than those in other two groups (mean±SD=236.9±109.9 vs 423±248.8; P=0.02). Furthermore, using random coefficient models, we found that LDH values over time have a significant relationship with the baseline PNH sizes. Higher LDH values over the period of follow-up are significantly associated with larger granulocyte PNH clone size at diagnosis (P=0.03). Finally, we arbitrarily chose a LDH value of 1.5x the upper limit of normal LDH value (330mg/dL) as a surrogate for clinically relevant intravascular hemolysis. This is the LDH value that has been used in prior PNH studies for eligibility to receive eculizumab^{9;10}. Fisher's exact test showed that patients with high initial PNH clone size had a greater chance to develop hemolysis (P=0.06). Among the AA patients with granulocyte PNH size ≤15%, only two of fifteen patients (13.3%) acquired LDH values greater than 330mg/dL five years after diagnosis. In contrast, among those AA patients with granulocyte PNH size >15% at diagnosis, five of nine patients (55.6%) had LDH values > 330mg/dL at five years.

Trends of PNH clone size

We classified the PNH clone size as stable if the percentage change of PNH granulocytes was < 2.5% of the original value. In this study, 25.9% (7/27) patients remained stable, 48.1% (13/27) patients increased their clone size, and 25.9% (7/27) patients decreased their clone size (Table 1). Each patient had his/her unique PNH clone size expansion pattern, even within his/her own group (Fig.3A to C). Among patients in these three groups, those with initial PNH clones size of ≤15% were the most stable over time (Table 1 and Fig.3D).

Using separate linear regression analyses, we determined that the average monthly PNH clone size change rates following HiCy therapy for each patient group were 7.47±27.32%, 8.75±9.8%, and 33.07±33.74% respectively (Fig. 3D). These data suggests that patients with large initial PNH clones (size ≥ 40%) are more likely to experience expansion of their PNH clones (p=0.08). In addition, we found that PNH clone size expanded more rapidly following relapse of AA (P=0.029) (Table 3). Interestingly, no patient in this study had clinical signs or symptoms of thrombosis during the period of observation.

Influences of therapy on PNH granulocyte clone size

There were fifteen patients who received HiCy as initial therapy. Fourteen of them (93.3%) achieved remissions. One patient, a non-responder, was observed to have continuous PNH size expansion following HiCy therapy (23% to 52%). She eventually received an Allogeneic BMT for refractory SAA 30 months after HiCy and remains in remission without detectable PNH granulocytes. Late expansion of the PNH clone was observed in seven of these fifteen patients (46.6%), who were initially managed with HiCy. Five of these patients eventually required intermittent supportive care such as blood transfusion, but only one patient developed symptomatic hemolysis necessitating eculizumab therapy (Table 3). The median time to granulocyte PNH clone expansion after HiCy was 38 (range: 18 to 86) months.

There were nine patients who initially were treated with immunosuppressive therapies other than HiCy: four patients received ATG/CsA, four patients received oral CsA alone, and one patient received oral prednisone. Two patients (one had nSAA and was managed with CsA alone, and another one had SAA and was treated with ATG/CsA) achieved a remission following immunosuppressive therapy and did not require further medical intervention. Interestingly, that latter patient kept a normal CBC profile and was asymptomatic while her PNH clone size expanded to 50% after 37 months observation. Two nSAA patients in the small PNH group spontaneously recovered their own hematopoiesis during the observation periods of 84 months and 56 months respectively, neither had expansion of their PNH clones. Expansion of the PNH clone was observed in seven of nine (77.8%) patients who were initially managed with immunosuppressive therapy at a median of 3 (range: 2 to 87) months after immunosuppressive therapy. Seven of these patients eventually received HiCy salvage therapy because of refractory pancytopenia. After HiCy salvage, all of them became transfusion independent and four had no further expansion of their PNH clones.

A nSAA patient who remained transfusion independent with a hematocrit >35% never required any medical intervention; her granulocyte PNH size increased from 25% to 51% and her LDH level increased to over 500 U/L after 36 months of observation.

Discussion

Detecting PNH cells at diagnosis in AA is increasingly common with the advent of newer and more sensitive flow cytometric assays to detect PNH cells;^{13,20,25} however, the clinical relevance of these often diminutive populations is not well studied. We and others have previously shown that more than 50% of patients with acquired aplastic anemia harbor PNH cells at diagnosis.^{19,20,25} The purpose of this study is to uncover the natural history of these PNH cells over time, especially for those patients who received HiCy therapy.

In this study, laboratory evidence of intravascular hemolysis, demonstrated by a rise in LDH greater than 1.5 times the upper limit of normal value, correlated closely with the granulocyte and red cell PNH clone size. Most patients with greater than 23% PNH granulocytes (or greater than 3~5% PNH erythrocytes) had laboratory evidence of hemolysis. The PNH clone size in granulocyte population is usually larger than in erythrocyte population for following reasons: 1) many of these patients were receiving erythrocyte transfusions, 2) erythrocytes have a longer half-life than granulocytes, and 3) PNH erythrocytes are more vulnerable to complement mediated destruction. AA patients with small initial PNH clones were unlikely to develop laboratory evidence of hemolysis over time. Only 15% of patients who initially had fewer than 15% PNH granulocytes developed an abnormal LDH (LDH > 1.5x control) within 5 years of diagnosis. In contrast, most patients with larger PNH clones at diagnosis experienced a rise in LDH within 5 years. Furthermore, following HiCy therapy the patients with initial PNH clone size larger than 40% were prone to expansion of their PNH clones (Fig.3D). Only one patient developed clinically significant intravascular hemolysis that required treatment with eculizumab and none of the patients developed thrombosis over the period of observation.

There are now a few studies that have investigated the natural history of PNH clones in AA patients.^{17, 18, 24,26} The major difference between our study and previous reports is that the majority patients in this study received HiCy therapy.⁸ In this study, we found that expansion or contraction of the PNH clone size appeared to be stochastic. Roughly 50% of AA patients demonstrated an increase in their PNH clone with time; whereas, 25% experienced a decrease in clone size and another 25% remained stable. These findings are in agreement with Sugimori et al., who used flow cytometry to follow the fate of small PNH clones in 75 Japanese patients with AA over a 5 year period²⁴. They found that the PNH

clone increased in 17%, persisted in 59%, and disappeared in 24% of patients; however, the median starting size of the PNH clone in Sugimori's study was 0.178 (range, 0.003 to 94.2%). We chose to exclude patients with less than 0.02% of granulocyte PNH size since we and others have shown that healthy control subjects can have 0.002 to 0.003% PNH granulocytes that are the consequence of non-clonal *PIG-A* mutations that arise in colony forming cells.^{25,27} Nevertheless, both our study and that of Sugimori et al demonstrated that AA patients with very small PNH clones at diagnosis are less likely to experience expansion of the PNH clone than those with larger PNH clones. Our study is also in agreement with a more recent report from Sheinberg *et. al*¹⁸, demonstrating a correlation between PNH size and LDH value. Similar to the Sugimori study, the patients reported by Sheinberg and his colleagues were also predominantly treated with antithymocyte globulin and cyclosporine.

It is impossible to know what, if any, influence HiCy had on the expansion or regression of the PNH clone; however, while expansion of the PNH clone may occur after HiCy and other immunosuppressive therapies²⁸, progression to clinically relevant PNH is not common within 10 years in patients who respond to treatment.^{8,18,29} Interestingly, expansion of the PNH clone appeared to accelerate at the time of AA relapse (Table 3), which suggests that the PNH clone preferentially survives the immune mediated killing.^{30,31}

In conclusion, the risk of developing clinically significant PNH after HiCy therapy appears to be low in AA patients with detectable PNH clones. Patients with very small PNH clones at diagnosis (granulocyte PNH size \leq 15%) are especially unlikely to experience expansion of their PNH clones or a rise in their LDH values.

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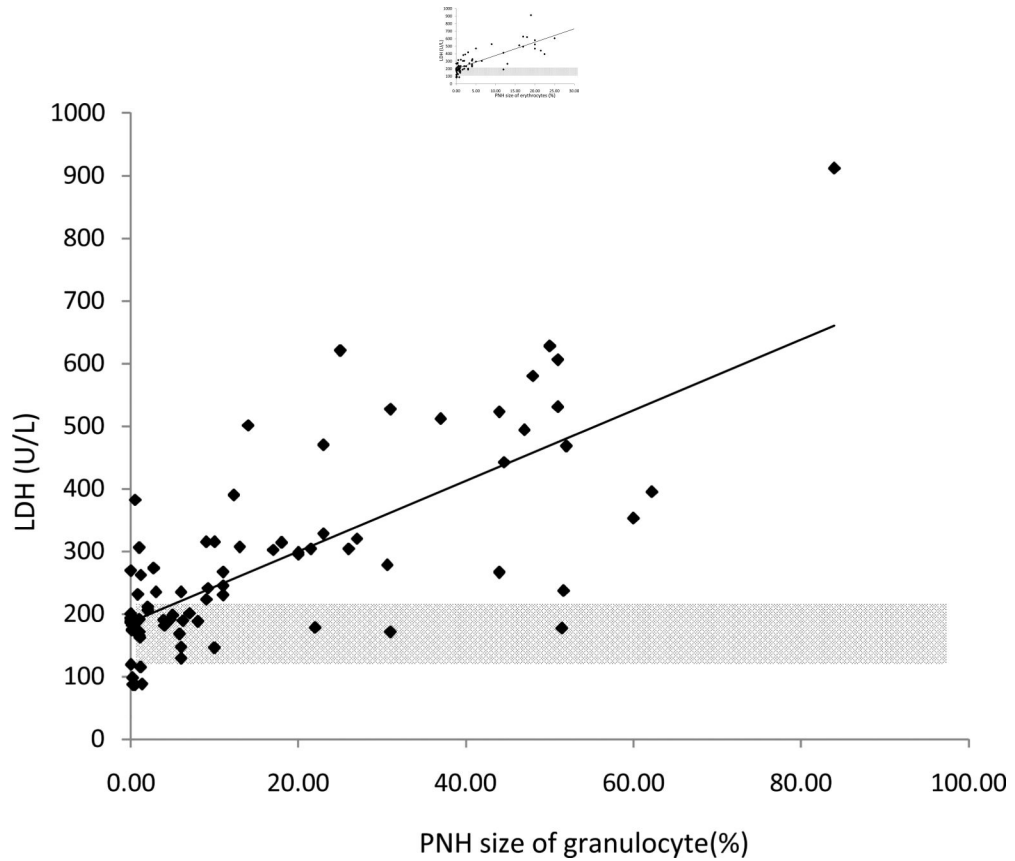
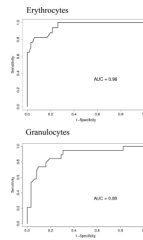


Fig. 1A. The correlation between PNH-like clone size and LDH value. (Pearson Correlation Coefficient=0.80; $P < 0.001$ for erythrocytes; and Pearson Correlation Coefficient=0.73; $P < 0.0001$ for granulocytes. The partial transparent gray color areas represent normal LDH ranges.)



Erythrocytes

Red threshold	Sensitivity	Specificity
0	1.000	0.000
0.01	1.000	0.077
0.03	1.000	0.092
0.04	1.000	0.169
0.05	1.000	0.185
0.06	1.000	0.200
0.08	1.000	0.215
0.1	1.000	0.231
0.12	1.000	0.262
0.18	1.000	0.277
0.3	1.000	0.292
0.35	1.000	0.323
0.36	1.000	0.338
0.4	1.000	0.354
0.49	1.000	0.369
0.5	1.000	0.385
0.6	1.000	0.477
0.74	1.000	0.523
0.79	1.000	0.538
0.8	1.000	0.554
0.9	1.000	0.569
1	1.000	0.631
1.11	1.000	0.677
1.15	1.000	0.692
1.6	1.000	0.708
1.69	1.000	0.723
1.78	1.000	0.738
2	0.941	0.738
2.26	0.941	0.785
2.5	0.882	0.785
3	0.882	0.800
3.3	0.824	0.831
4	0.824	0.846
4.1	0.824	0.923
5	0.824	0.938
6.5	0.765	0.954
9	0.765	0.969
12	0.706	0.969
13	0.647	0.985
16	0.647	1.000
17	0.588	1.000
18	0.471	1.000
19	0.412	1.000
20	0.353	1.000
21.5	0.176	1.000
22.44	0.118	1.000
25	0.059	1.000

Granulocytes		
PNH threshold	Sensitivity	Specificity
1	0.947	0.754
2	0.947	0.723
3	0.947	0.693
4	0.947	0.663
5	0.947	0.633
6	0.947	0.603
7	0.947	0.573
8	0.947	0.543
9	0.947	0.513
10	0.947	0.483
11	0.947	0.453
12	0.947	0.423
13	0.947	0.393
14	0.947	0.363
15	0.947	0.333
16	0.947	0.303
17	0.947	0.273
18	0.947	0.243
19	0.947	0.213
20	0.947	0.183
21	0.947	0.153
22	0.947	0.123
23	0.947	0.093
24	0.947	0.063
25	0.947	0.033
26	0.947	0.003
27	0.947	0.000
28	0.947	0.000
29	0.947	0.000
30	0.947	0.000

Fig. 1B. Receiver operating characteristic curve (ROC) analysis. We define LDH ≥ 330 mg/dl (1.5x of normal upper limit) as abnormal. The aim is to evaluate the predictive value of PNH to discriminate normal and abnormal LDH. The area under the ROC curve (AUC) is 0.96 for erythrocyte PNH and is 0.88 for granulocyte PNH. The erythrocyte PNH size of 3~5% and granulocyte PNH size of 23% are the optimal threshold at which the PNH clone sizes can best classify patients as with and without hemolysis.

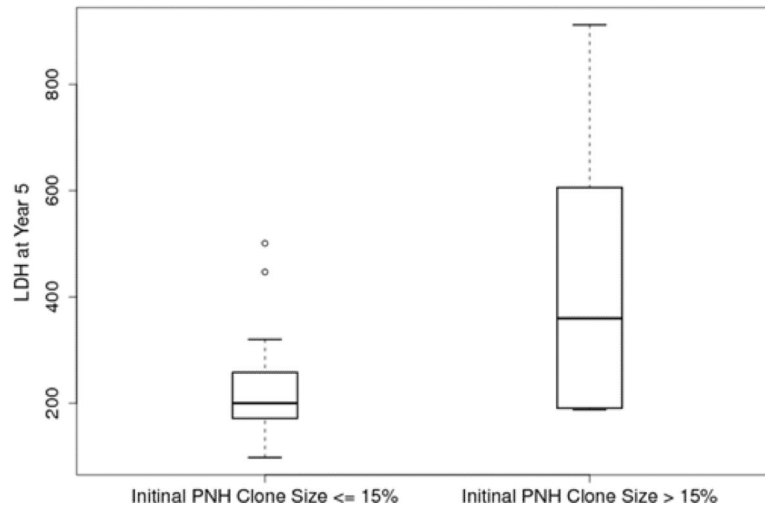


Fig. 2. Box plot comparison of LDH values at year 5 after diagnosis by initial PNH size. The LDH at 5 years after diagnosis is significantly higher for the patients whose initial granulocyte PNH size was larger than 15% (mean±SD=423.1±248.8, n=9) compared to those with granulocyte size PNH smaller than 15 (mean=236.9±109.9, n=15) (P= 0.02).

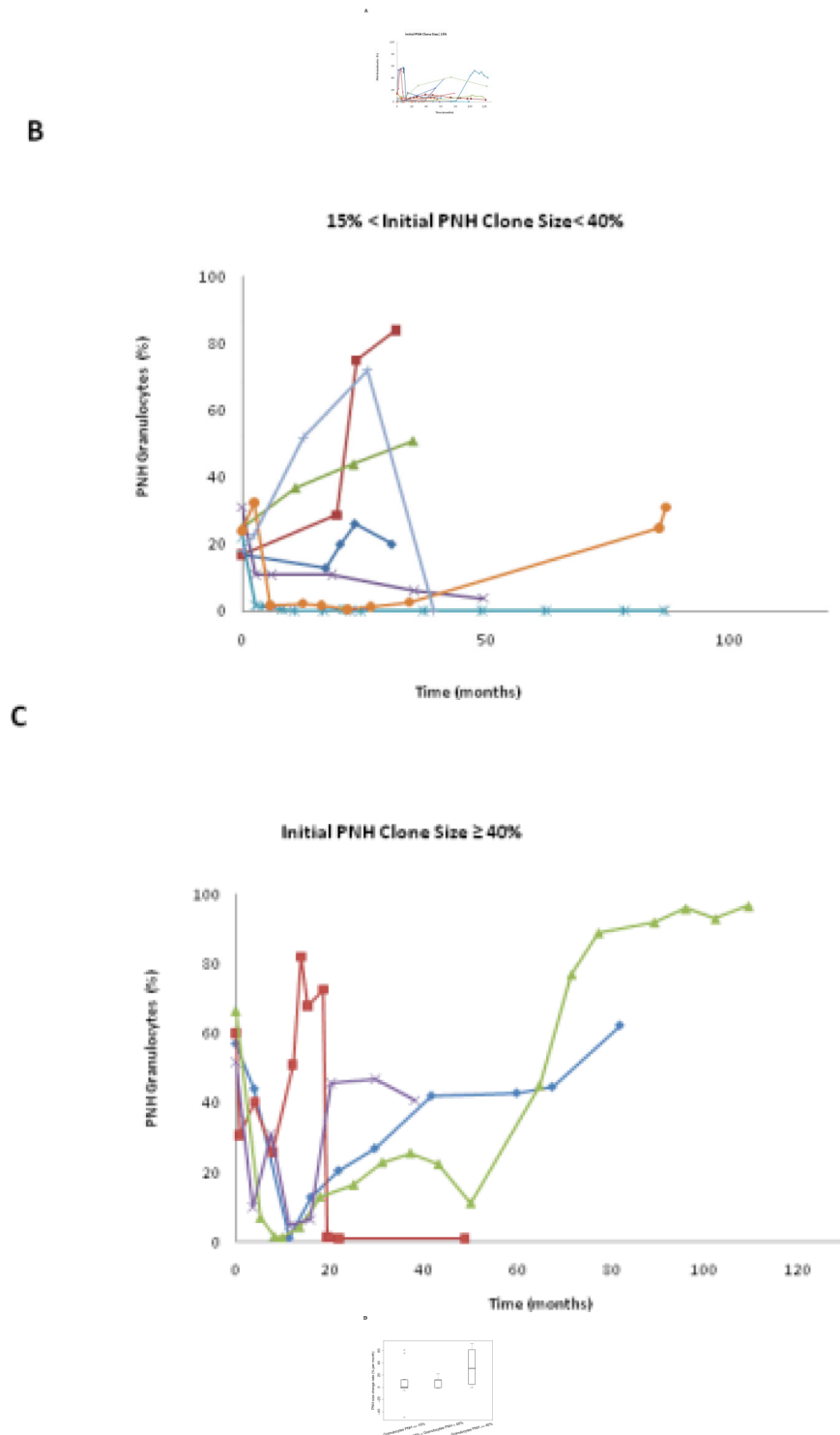


Fig. 3. The PNH size change trends in each patient group. A,B,C show the granulocyte PNH size change of individual patient in the course of this study. D shows the PNH size expansion rates of each patient group after receiving HiCy treatment.

Table 1

Patient characteristics (n=27)

Characteristics	Initial PNH clone size (granulocytes)			Total
	≤15%	>15% and <40%	≥40%	
Sex				
Male, n (%)	6 (22.2)	2 (7.4)	1 (3.7)	9 (33.3)
Female, n (%)	10 (37)	5 (18.5)	3 (11.1)	18(66.7)
Age at diagnosis, Median (range)				
Male	29 (20-41)	30 (24-36)	41 (41)	24(20-41)
Female	21 (10-62)	48 (15-68)	31 (19-32)	29(10-68)
Therapy				
IST only	1	1	0	2
IST→HiCy	5	0	2	7
HiCy only	7	4	2	13
HiCy → BMT	0	1	0	1
HiCy → Eculizumab	1	0	0	1
No therapy	2	1	0	3
Follow up years, Median (range)	6.3 (1.5-11.5)	3.3 (2.5-7.3)	5.5 (3.2-9)	5.1(1.5-11.5)
PNH clone size				
Increase, n (%)	6 (22.2)	5(18.5)	2 (7.4)	13 (48.1)
Decrease, n (%)	3 (11.1)	2 (7.4)	2 (7.4)	7 (26)
Stable, n (%)	7 (25.9)	0 (0)	0 (0)	7 (26)
Eventually developed PNH symptoms (n)	3	3	1	7
Transfusion dependent	3	3	1	
Worsening Fatigue	1	3	0	
Abdominal pain	1	0	0	
Hemoglobinuria	1	0	0	
Thrombosis	0	0	0	

Table 2

Clinical characteristics of the cohort.

Patient	Sex/Age (yrs)	Initial Diagnosis	Follow-up (mos)	PNH size (%) (initial/ current)*	LDH (mg/dl) (initial/current)	Therapy	Post-treatment AA status
1	F/41	SAA	114	0.5/0.2	87/98	HiCy	Remission
2	F/22	SAA	129	0.16/50	186/628	HiCy	Remission for 108 mos, then transformed to PNH
3	F/68	vSAA	86	22/0.18	178/172	HiCy	Remission
4	F/15	SAA	87	24/31	382/527	HiCy	Remission for 84 mos, then transformed to PNH
5	M/37	SAA	78	12.3/14	390/501	HiCy	Remission
6	M/9	SAA	109	66.5/96.7	536/507	HiCy	Remission for 60 mos, then asymptomatic PNH expansion
7	F/19	SAA	63	0.1/38	181/447	HiCy	Remission
8	F/19	SAA	52	0.12/23	193/285	HiCy	Remission for 52 mos, then AA relapse
9	M/22	SAA	50	5/0.8	198/162	HiCy	Remission
10	M/36	SAA	49	31/3.9	171/190	HiCy	Remission
11	M/20	SAA	37	0.06/1	200/269	HiCy	Remission
12	F/32	SAA	38	51.67/40.7	237/191	HiCy	Remission with asymptomatic PNH expansion
13	M/20	SAA	18	0.34/0.46	180/172	HiCy	Remission
14	M/24	SAA	30	17/84	302/912	HiCy	AA relapse after 3 mos remission, then transformed to PNH after 18 mos
15	F/52	SAA	39	23/72	314/229/188	HiCy, then BMT	Refractory, then remission post BMT
16	F/34	SAA	138	0.14/3	235/182	CSA, then HiCy	AA relapse after 6 mos remission, then remission again post HiCy
17	F/18	vSAA	123	6/26	147/266	Prednisone, then HiCy	Refractory, then remission post HiCy
18	F/10	SAA	134	0.02/0	178/208	ATG/CSA, then HiCy	AA relapse after 30 mos remission, then remission again post HiCy
19	F/31	SAA	82	57/62.2	267/395	ATG/CSA, then HiCy	Refractory, then remission post HiCy
20	M/41	SAA	81	0.12/1	174/171	ATG/CSA, then HiCy twice	AA relapse after 7 mos remission, then relapse again 72 mos after HiCy, then remission again post second HiCy
21	F/62	SAA	60	15/6.2	189/189	CSA, then HiCy	Refractory, then remission post HiCy
22	F/19	SAA	49	60/1	353/306	CSA, then HiCy	Refractory, then remission post HiCy
23	F/46	NSAA	131	0.5/6	223/235	CSA	Remission

Patient	Sex/Age (yrs)	Initial Diagnosis	Follow-up (mos)	PNH size (%) (initial/ current)*	LDH (mg/dl) (initial/current)	Therapy	Post-treatment AA status
24	F/27	SAA	38	17/50	307/ 600	ATG/CSA	Remission for 30 mos, then asymptomatic PNH expansion
25	M/39	NSAA	84	0.3/1.5	206/231	No treatment	Self recovery
26	F/17	NSAA	56	15/4.2	419/ 189	No treatment	Self recovery
27	F/48	NSAA	36	25/51	512/ 606	No treatment	Asymptomatic PNH expansion

Remission was classified as HCT $\geq 30\%$ /independence of transfusion/ANC ≥ 500 /cumm/Platelet count $\geq 20K$ /cumm. PNH transformation was classified as presenting classical PNH symptoms and developing apparent hemolysis with LDH value $> 1.5\times$ of normal upper limit.

* initial PNH size means the PNH size before treatment, and current PNH size means the PNH size in the most recent visit.

Table 3

PNH clone size change in five patients received HiCy only treatment, who first went in remission and then relapsed

Patient ID	PNH clone size (%)change with time	
	during remission	during relapse
2	0.84 (0.16 to 1) in 86 mos	49 (1 to 50) in 35 mos
4	1.02 (1.68 ⁺ to 2.7) in 30 mos	28.3 (2.7 to 31) in 53 mos
7	0.3 (0.1 to 0.4) in 53 mos	37.6 (0.4 to 38) in 56 mos
8	1.88 (0.12 to 2) in 38 mos	21 (2 to 23) in 14 mos
14	3 mos*	55.3 (28.7 to 84) in 12 mos
Mean±SD	1.01±0.7 in 51.8±24.7 mos	38.2±14.2 in 34±20.8 mos

P=0.029

* no PNH size data available during those 3 months of remission period.

⁺ this patient's initial (treatment naïve) PNH granulocyte clone size was 24% (see table 2) and her initial post HiCy treatment PNH granulocyte clone size was 1.68%. Other 4 patients didn't have obvious PNH size change right after HiCy treatments, which might be due to their original PNH clone sizes were very small already.