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Evolution patterns of paroxysmal nocturnal hemoglobinuria clone and clinical implications in acquired bone marrow failure

Yu Lian, Jun Shi, Neng Nie, Zhendong Huang, Yingqi Shao, Jing Zhang, Jinbo Huang, Xingxin Li, Meili Ge, Peng Jin, Min Wang, and Yizhou Zheng

State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China

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The paroxysmal nocturnal hemoglobinuria (PNH) clone often presents in acquired bone marrow failure (aBMF), which is involved in more than half of aplastic anemia (AA) cases and about 10%–20% of myelodysplastic syndrome (MDS) cases. PNH clone expansion patterns and clinical implications, however, remain obscure. We conducted a large retrospective study of 457 aBMF patients with positive PNH clones to explore the wide spectrum of clone architecture, evolution patterns, and clinical implications. PNH clone size at diagnosis in AA or MDS was significantly smaller than that in clinical PNH ($p < 0.001$); the main clone patterns in AA and MDS were granulocyte dominant, with the remaining cases having a granulocyte–erythrocyte balance pattern in clinical PNH. In 131 AA patients at follow-up, there was no obvious difference in response rates between those with the aggressive pattern of clone evolution (73.7%) and those with the stable pattern (81.1%). A quarter of AA patients evolved into clinical hemolysis within a median interval of 11 months. AA cases progressing into clinical hemolysis after immunosuppressive therapy had significantly larger clones (granulocytes: 12.3% vs. 2.6%; erythrocytes: 5.7% vs. 1.3%) at diagnosis and presented mainly an aggressive pattern, especially the granulocyte–erythrocyte aggressive model. Clone sizes reaching 37% for erythrocytes and 28% for granulocytes were indicators of the onset of hemolysis in AA. In conclusion, aBMF patients presented significantly various PNH clone patterns at diagnosis. AA patients with either an aggressive or stable evolution pattern can achieve a response, but patients with an aggressive evolution pattern, especially the granulocyte–erythrocyte aggressive model, tend to evolve into clinical hemolysis.

The riddle raised by Dr. Dameshek in 1967 focused on a provocative question: What do aplastic anemia (AA), paroxysmal nocturnal hemoglobinuria (PNH), and "hypoplastic" leukemia have in common [1]? The riddle was based on the following clinical observation: frequent development of PNH in AA patients; the overlap between the symptomatic PNH and AA; and the similar high prevalence of both PNH and AA in the Orient. In 1992, Young addressed the issue and restated the riddle relevant to the hematopoietic cell clonality, a single "insult" of PNH clone occurring in AA, PNH, and a preleukemic condition of myelodysplastic syndrome (MDS) [2]. In 2018, Luzzatto also reinforced that PNH was so different from AA in clinical nosography, but from the point of view of pathogenesis, the majority of PNH patients were a subset of AA patients [3]. All the questions pointed to the core issue of PNH clone evolution in the context of acquired bone marrow failure (aBMF), especially in AA and MDS.

YL completed the collection of the clinical data and analyzed and interpreted the data. NN, ZH, YS, JZ, JH, XL, MG, PJ, MW, and YZ contributed to the clinical data collection. JS designed the research, wrote the paper, and approved the final article.

Offprint requests to: Jun Shi, State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, China; E-mail: shijun@ihcams.ac.cn

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PNH is a rare clonal hematopoietic stem cell (HSC) disorder with three clinical features: hemolytic anemia, bone marrow failure, and risk of thrombosis. PNH originates from clonal expansion of HSCs as a consequence of an inactivating mutation of the X-linked gene *PIG-A*, leading to deficiency in glycosylphosphatidylinositol-anchored proteins (GPI-APs) of mature differentiated cells [4–6]. The red blood cells (RBCs) arising from PNH clones, which are deficient in surface membrane complement-regulatory proteins CD55 and CD59, are exquisitely sensitive to lysis by activated complement, and complement blockade with the anti-C5 monoclonal antibody eculizumab is highly effective in reducing hemolysis, improving quality of life, and lowering the risk for thrombosis. The neutrophils or monocytes with CD24, CD16, and CD14 deficiency, which contribute to the damage of endothelium cells and increase the risk of thrombosis, are highly activated [7,8]. We usually screen PNH clones by flow cytometry using the antibodies CD55 and CD59 for RBCs and the fluorescein-labeled proaerolysin variant (FLAER) for granulocytes with standard protocols [9–11]. Because of high-sensitivity resolution, it is known that PNH clones can be present in the setting of aBMF, in about 40%–70% of AA patients [8,12,13] and 12.2%–17.6% of MDS cases, especially in refractory anemia [14,15]. However, the clone expansion patterns and clinical implications of clone evolution in AA or MDS remain obscure, and more detailed evidence is required [16,17].

We were interested in the issue of PNH clone evolution in the context of AA, MDS, and classic PNH. We focused on clone architecture on diagnosis of AA, MDS, and classic PNH; clone evolution patterns and changes in clone size; and aggressive models from the presence of clones to the onset of clinical hemolysis. We then analyzed clone evolution related to hematological response in AA patients with sequential clone screening during follow-up. Collectively, we sought to partly clarify Dameshek's riddle.

Methods

Patients and design

Four hundred fifty-seven aBMF patients with positive PNH clones were enrolled in this study at the Blood Diseases Hospital, Chinese Academy of Medical Sciences, from 2012 to 2017. These patients were diagnosed into three main categories on the basis of AA, MDS, and classification of PNH [18]: (1) patients with classic PNH, in whom there was clinical evidence of intravascular hemolysis but no evidence of another defined bone marrow abnormality; (2) patients with subclinical PNH, in whom small PNH clones were present, but there was no evidence of hemolysis, and there was concomitant BMF, such as AA (termed as AA with PNH clones) and MDS (termed as MDS with PNH clones); (3) patients with PNH in the context of other primary bone marrow disorders, for whom there was clinical and laboratory evidence of hemolysis, but who also had concomitantly or a history of a defined underlying BMF, called AA-PNH syndromes or MDS-PNH syndromes.

Bone marrow aspiration and biopsy for morphology and cytogenetics, the Ham test, levels of lactate dehydrogenase (LDH) and indirect bilirubin for identifying intravascular hemolysis, and other routine examinations were required for diagnosis and differentiation. Children and young adults underwent chromosome assays and even more specific tests to exclude inherited BMF. Of note, AA patients received cyclosporine A alone or combined with an antithymocyte globulin regimen. All MDS and PNH patients were provided supportive care and erythropoiesis-stimulating agents. For AA patients, the response to cyclosporine A or antithymocyte globulin was evaluated according to the published criteria [19]. Written informed consent was obtained from all patients.

PNH clone screening and definition

Peripheral blood samples were collected into heparin tubes to detect PNH clone cells by flow cytometric assay with a cut-off value of 0.01%. Erythrocytes were analyzed for PNH clones with CD55 and CD59 antibodies in a CD235-positive population, while granulocytes were determined by FLAER staining positivity or negativity. For each patient enrolled in our study, PNH clones in both erythrocytes and granulocytes were measured. PNH clone screening was sequentially followed in 131 AA patients every 6–12 months with a median follow-up of 18 months (range: 6–56 months), including 34 events less than 6 months, 115 events from 6 to 12 months, 189 events from 1 to 3 years, and 43 events longer than 3 years. As outlined in Table 1, we defined the three PNH clone sizes, PNH clone patterns, and different aggressive models.

Statistical analysis

Statistical analyses were carried out in Microsoft Office Access 2016, SPSS version 23.0, and GraphPad PRISM version 7.0. The normality of the data was evaluated with the Kolmogorov–Smirnoff test. Continuous variables without a normal distribution were expressed as medians (min, max). The differences between the quantities of PNH cells were compared with a t test or the Mann–Whitney U test, and differences in hematological response rates among different subgroups were compared with the χ^2 test. P values < 0.05 were considered to indicate statistical significance.

Results

PNH clone architecture in different settings of acquired bone marrow failure

As outlined in Table 2, 457 patients who had screened positive for the PNH clone at least once were enrolled; these included patients with AA ($n = 252$, 55.1%), AA-PNH syndromes ($n = 30$, 6.7%), MDS ($n = 48$, 10.5%), MDS-PNH syndromes ($n = 8$, 1.8%), and classic PNH ($n = 119$, 26.0%). All patients with classic PNH, AA-PNH syndromes, and MDS-PNH syndromes had positive PNH clones with different size distributions. We compared the initial PNH clone sizes at the time of diagnosis in the three settings of patients (Figure 1A, B). As compared with classic PNH patients, AA with PNH clones and MDS with PNH clones had significantly lower burdens of PNH clones, all in granulocytes (AA: 3.7% vs. 92.1%, $p < 0.001$; MDS:

Table 1. Classifications and definitions of PNH clone patterns and aggressive models

Terminology	Definition
PNH clone size	
Small	PNH clone size <10%
Medium	PNH clone size range 10%–50%
Large	PNH clone size >50%
PNH clone patterns at diagnosis	
Granu-dominant	(1) Clone size of one lineage involved at a higher hierarchy of clone size or (2) one lineage positive but the other lineage negative.
Ery-dominant	
Granu-Ery balance	Two lineages at the same hierarchy of clone size
PNH clone aggressive model	
Granu-aggressive	In a small group, clonal expansion reaches a higher hierarchy and clone size doubles; in a medium group, clone expansion reaches a higher hierarchy, or clone size doubles. Otherwise, the status is stable.
Ery-aggressive	
Granu-Ery aggressive	
Stable	

Granu=granulocyte; *Ery*=erythrocyte.

Table 2. Characteristics of 457 patients with positive PNH clones

Clinical data	AA with PNH clones	AA-PNH syndromes	MDS with PNH clones	MDS-PNH syndromes	Classic PNH
N	252	30	48	8	119
Age, y (range)	32 (7–74)	37 (9–70)	36 (7–90)	42 (18–63)	36 (12–70)
Sex, male/female	138/114	15/15	25/23	4/4	75/44
Granulocytes-PNH clone size, % (min, max)	3.69 (0.00, 94.50)	81.05 (5.53, 99.76)	2.1 (0.00, 59.89)	67.48 (1.62, 98.80)	92.10 (0.00, 100.00)
Erythrocyte-PNH clone size, % (min, max)	1.20 (0.00, 37.00)	28.20 (9.23, 86.93)	0.00 (0.00, 23.90)	25.99 (7.38, 52.66)	54.30 (0.00, 98.20)
Median LDH, U/L (range)	195.5 (21, 1,176)	507 (32, 3,120)	202.5 (127, 451)	960.5 (369, 1,355)	1,443 (125, 5,144)

2.1% vs. 92.1%, $p < 0.001$) and erythrocytes (AA: 1.2% vs. 54.3%, $p < 0.001$; MDS: 1.0% vs. 54.3%, $p < 0.001$). Compared with MDS, AA with PNH clones had a higher clone burden in granulocytes ($p < 0.01$), but similar burden in erythrocytes.

The two subclassifications of PNH in the setting of another specified bone marrow disorder, AA-PNH syndromes and MDS-PNH syndromes, exhibited expansion of PNH clone size in granulocytes and erythrocytes as compared with the subclinical PNH forms, AA or MDS with PNH clones. Interestingly, the clone size in granulocytes increased to the same levels of classic PNH, but clone burdens in erythrocytes were still significantly lower than those in classic PNH ($p < 0.01$).

With respect to clone architecture (Figure 1C), AA patients had clone-involved patterns distinct from those of MDS patients ($p = 0.022$). Briefly, 113 of the 252 AA patients (44.8%) had PNH clones involved in both granulocytes and erythrocytes, 99 (39.3%) in granulocytes alone and 3 (1.2%) in erythrocytes alone; the other 37 cases were negative at the time of diagnosis. In contrast, the proportion of granulocyte–erythrocyte double clones was only 22.9% (11/48) in the MDS group, but there was a high prevalence (52.1%) of granulocyte-alone clones and a similar proportion of erythrocyte-alone clones. Thirty-seven of 252 AA cases (14.7%) and 11 of 48 MDS cases (22.9%) did not manifest PNH clones at the time of diagnosis but presented with PNH clones during follow-up. The median interval from diagnosis to the occurrence of PNH clones was 6 months (range: 3–36 months) for AA and 6 months (range: 4–36 months) for MDS, respectively.

PNH clone patterns in different settings of acquired bone marrow failure

We defined PNH clone size as small (<10%), medium (10%–50%), and large (>50%) according to potential risk of hemolysis and thrombosis [4,12,20]. We further identified granulocyte-dominant, erythrocyte-dominant, and granulocyte–erythrocyte balance patterns based on the hierarchy of PNH clone size. As illustrated in Figure 2A, the granulocyte-dominant or erythrocyte-dominant pattern fulfilled the criteria for clone size of one lineage involved at a higher hierarchy; otherwise one was lineage positive but the other was lineage negative. The granulocyte-dominant pattern fulfilled the criteria for PNH clone size of granulocyte at a higher hierarchy than erythrocyte PNH clone size (i.e., medium vs. small, large vs. medium or large vs. small), or granulocyte clone was positive while erythrocyte clone negative. The criteria for erythrocyte-dominant pattern was just opposite. In contrast, two-thirds of classic PNH cases manifested the granulocyte–erythrocyte balance pattern, and one-third had the granulocyte-dominant pattern (Figure 2B). Although patients with AA-PNH or MDS-PNH syndromes

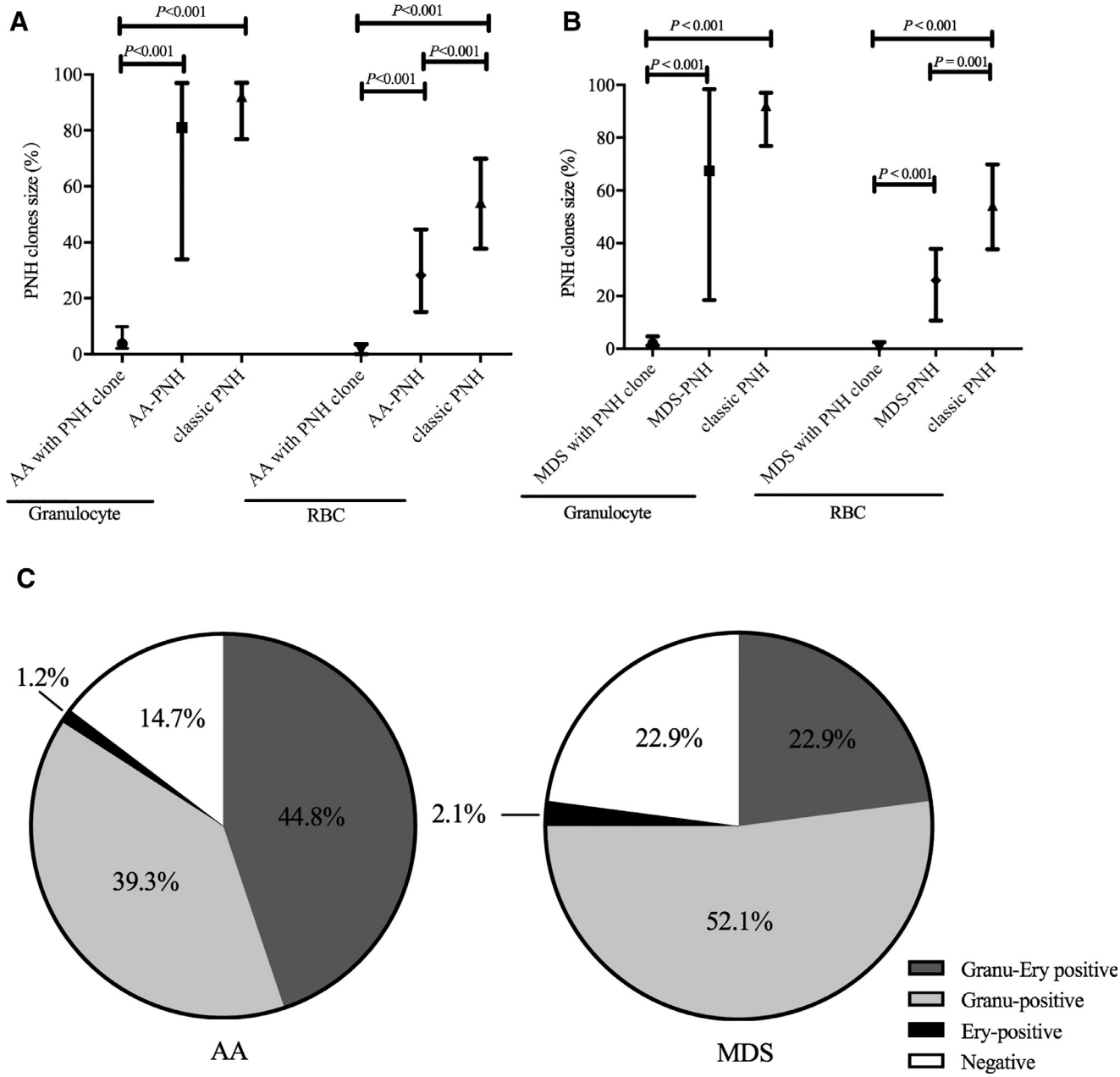

Figure 1. PNH clone architecture at diagnosis in different settings of acquired bone marrow failure. Comparison of the initial PNH clone size at diagnosis in AA with PNH clones, AA-PNH, and classic PNH **(A)**, and in MDS with PNH clones, MDS-PNH, and classic PNH **(B)**. Pie graphs illustrate the proportions of PNH clone architecture at diagnosis in AA and MDS, respectively **(C)**. *Granu*=granulocyte; *Ery*=erythrocyte.

experienced hemolysis, the erythrocyte-dominant pattern was seldom observed (3.3% in AA-PNH; 12.5% in MDS-PNH), and half of these patients had the granulocyte-dominant pattern.

PNH clone evolution from the occurrence of clones to the onset of clinical hemolysis

Of 131 AA patients with sequential PNH clone screening and clinical data, 34 developed clinical hemolytic symptoms within a median interval of 11 months (range: 3–50 months). First, we observed the distribution of PNH clone patterns at the time of diagnosis among the 34 patients and the other 97 cases. As illustrated in Figure 3A, there were similar proportions of granulocyte-dominant, erythrocyte-dominant, and granulocyte–erythrocyte balance patterns. Second, we analyzed the baseline clone size at the time of occurrence of PNH clones and found that the 34 cases that ultimately presented with clinical hemolysis had higher burdens of clones as compared with the 97 patients without symptomatic hemolysis (granulocytes: 12.3% vs. 2.6%, $p < 0.001$; erythrocytes: 5.7% vs. 1.3%, $p \leq 0.001$) (Figure 3B). Third, we observed changes of clone size

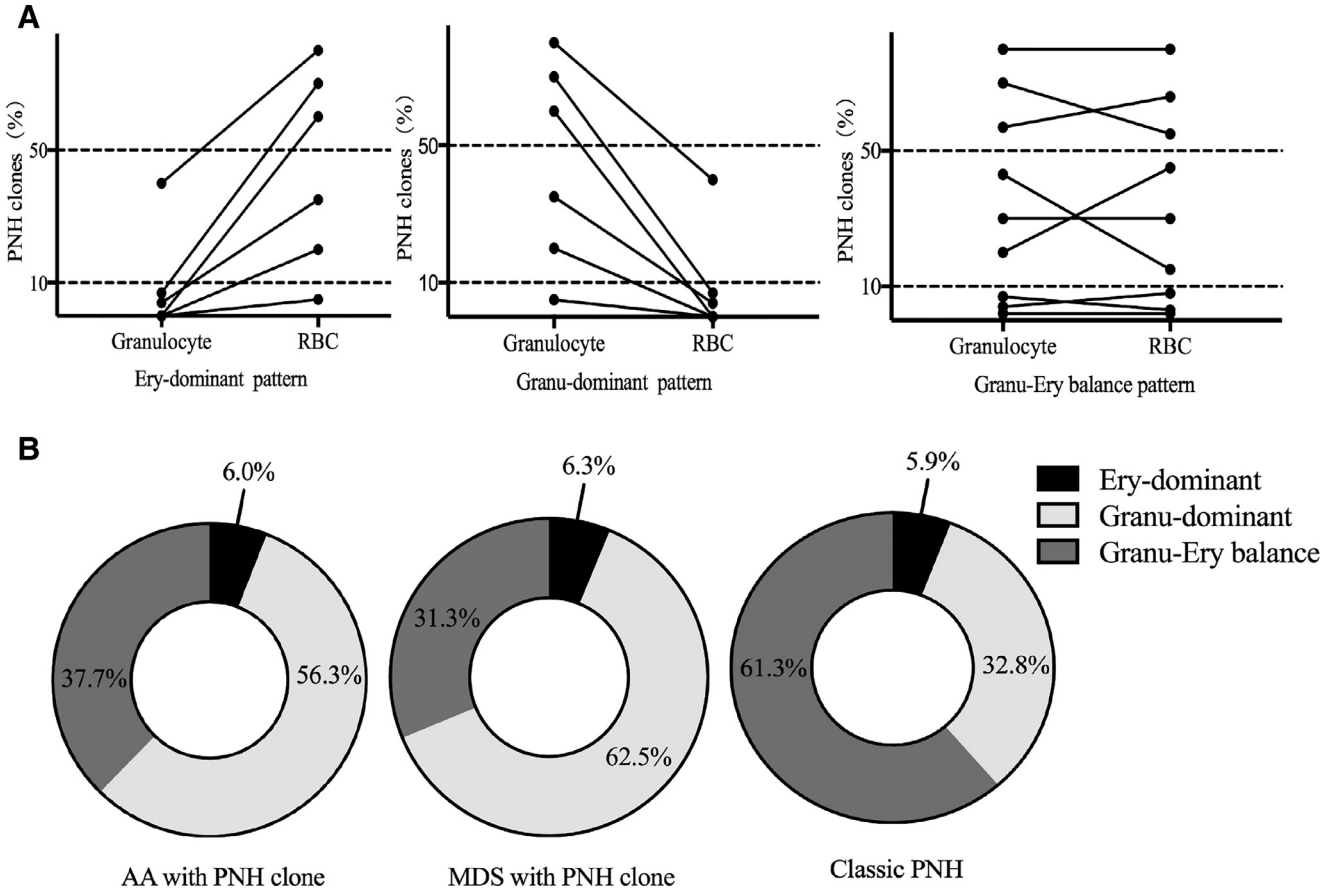

Figure 2. PNH clone patterns at diagnosis in different settings of acquired bone marrow failure. **(A)** We defined PNH clone size as small (<10%), medium (10%–50%), and large (>50%). The Granulocyte-dominant or erythrocyte-dominant pattern fulfilled the criteria for clone size of one lineage involved at a higher hierarchy; otherwise one was lineage positive but the other lineage negative. The Granulocyte-Erythrocyte balance pattern was defined as two lineages at the same hierarchy. **(B)** Pie graphs illustrate the proportions of PNH clone patterns at diagnosis in AA with PNH clones, MDS with PNH clones, and classic PNH. *Granu*=granulocyte; *Ery*=erythrocyte.

during the progression from nonhemolysis to symptomatic hemolysis, and indeed noted an obvious increase in the granulocytes and erythrocytes of PNH clones (Figure 3C). Twenty-one of 34 (61.8%) patients experienced hemolysis in less than 1 year, 29.4% cases in from 1 to 3 years, and 8.8% cases in more than 3 years (Figure 3D). Lastly, we focused on the patterns in the progress of clone evolution. We defined the aggressive model as follows: In a small group, clone expansion reached a higher hierarchy and clone size doubled; in a medium group, clone expansion reached a higher hierarchy or clone size doubled. Otherwise, we named it stable status. Among the 34 patients, 16 cases were classified with the granulocyte-dominant form, 8 (50.0%) with the granulocyte–erythrocyte aggressive pattern, 5 (31.2%) with the erythrocyte-aggressive pattern, and 2 (12.5%) with the granulocyte-aggressive pattern. Of 14 patients with the granulocyte–erythrocyte balance form, 10 (71.4%) also exhibited the granulocyte–erythrocyte aggressive pattern, 1 case (10.0%) had the erythrocyte-aggressive pattern, and the other 3 cases had stable status. Two of 4 patients with the erythrocyte-dominant form were erythrocyte-aggressive and two had stable status (Figure 3E). Furthermore, we found the median clone size of erythrocytes (37.3%, interquartile range: 18.9%–48.9%) and granulocytes (27.8%, interquartile range: 13.7%–62.5%) when these patients progressed to symptomatic hemolysis (Figure 3F).

PNH clone evolution related to hematological response in AA patients

A total of 131 AA patients were followed up after immunosuppression therapy with sequential PNH clone screening. One hundred two of 131 cases (77.9%) exhibited a response, including 35 complete responses (CRs) and 67 partial responses (PRs). For 60 nonsevere AA (NSAA) patients, the response rates were 90.0% and 67.6% in 71 SAA cases. We were interested in clone evolution after immunosuppression therapy. Sixty-six of 131 (50.4%) patients had granulocyte-dominant clones, and 57 (43.5%) had granulocyte–erythrocyte balance clones before treatment. Interestingly, in 66 granulocyte-dominant clone cases, more than one half exhibited the stable model; the

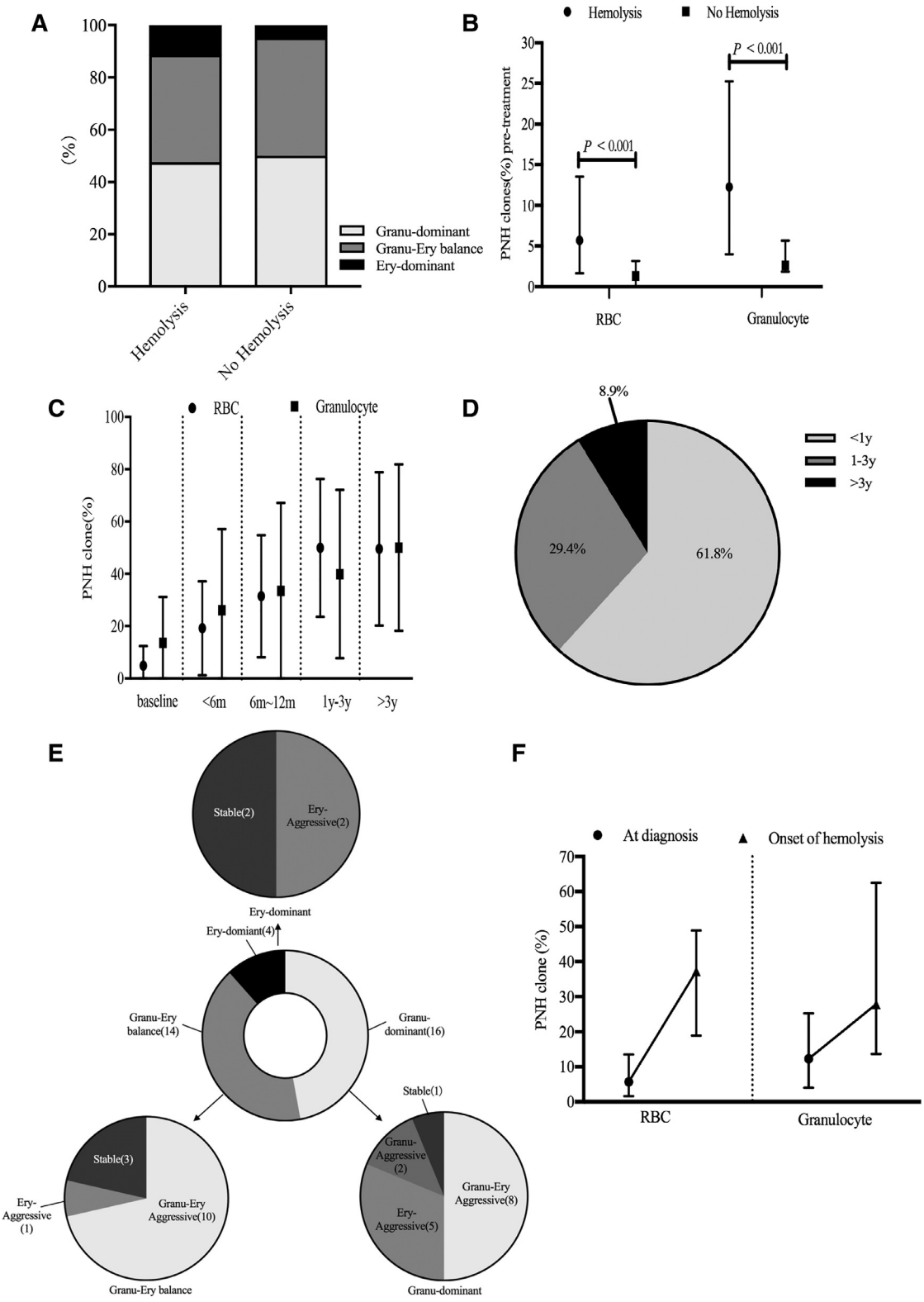

Figure 3. PNH clone evolution from the occurrence of clones to the onset of clinical hemolysis. **(A)** The proportion of PNH clone patterns at diagnosis in 34 follow-up AA patients who developed clinical hemolysis after IST. **(B)** Comparison of PNH clone size at diagnosis between 34 patients with AA with clinical hemolysis and 97 patients without hemolysis. **(C)** PNH clone size increased over time in the 34 AA patients with hemolysis. **(D)** Proportions of the interval between occurrence of PNH clones and onset of hemolysis. **(E)** The clone evolution patterns of 34 AA patients from diagnosis to onset of symptomatic hemolysis. **(F)** PNH clone size at the time of occurrence of PNH clones and the onset of hemolysis.

erythrocyte-aggressive, granulocyte–erythrocyte aggressive, and granulocyte-aggressive models each accounted for about one-sixth of cases. For 57 granulocyte–erythrocyte balance AA patients, also more than one half exhibited the stable model, and 11 (19.3%) exhibited the granulocyte–erythrocyte aggressive model. The proportions for the erythrocyte-aggressive and granulocyte-aggressive models were 15.8% and 3.5% (Figure 4A). We further explored the clone evolution models with respect to hematological response. There were no obvious differences in response rates between aggressive and stable models ($p = 0.312$). Similar response rates were seen in patients with the aggressive model (73.7%) and those with the stable model (81.1%). Therefore, patients with either aggressive or stable clone evolution can all achieve a response (Figure 4B).

Discussion

An understanding of the association between PNH clone evolution and aBMF seems to be an important part of solving Dameshek's riddle. Our large retrospective study of 457 aBMF patients with positive PNH clones over a 5-year period allowed us to explore the wide spectrum of clone architectures, expansion patterns, and the relationships between evolution patterns of PNH clone and hematological response in aBMF.

Because of flow cytometry, our detection of PNH clones was highly sensitive, at the level of 0.01% for both erythrocytes and granulocytes [9]. Several previous studies focused on PNH granulocytes only, because of the possibly reduced detection of PNH erythrocytes affected by hemolytic waves or transfusion [6,11,21]. In practice, although total clone size on erythrocytes was usually less than that seen on granulocytes, some cases were the exact opposite. In a large Italian series of 414 newly discovered PNH clones, tens of cases had greater PNH clone size in erythrocytes than in granulocytes [22]. It was also seen in our study that around 6% of cases exhibited the erythrocyte-dominant pattern at diagnosis, and one-third of AA or MDS cases with PNH clones and two-thirds of classic PNH cases exhibited the granulocyte–erythrocyte balance pattern. What is more, the clone size of erythrocytes contributes greatly to clonal evolution, especially in those aBMF patients with clinical hemolysis.

As for clone size at diagnosis, our study indicated that AA or MDS with PNH clones had significantly lower burdens than classic PNH, which was consistent with a few previous studies. A retrospective single-center study at Johns Hopkins reported that 61 AA patients had a PNH clone at diagnosis; the median (with ranges) erythrocyte clone size was 0.06% (0.02%–37%) and the granulocyte clone size 0.8% (2.01%–94%) [13]. In a prospective multicenter study in the United States, granulocyte PNH clones were detected in 93 cases of AA with a median clone size of 5.1% and 50 MDS patients with a median clone size of 17.6% [11]. Data from the International PNH Registry revealed that the median granulocyte clone size was significantly larger in classic PNH than in AA (83% vs. 35%, $p < 0.001$) [12]. Interestingly, there were significantly fewer erythrocyte clones in AA-PNH or MDS-PNH than in classic PNH, while they had almost the same proportions of granulocyte clones in our study.

Further exploration of the architecture of baseline PNH clones revealed differences in patterns between AA and MDS cases. AA cases had a similar proportion of granulocyte–erythrocyte positive and granulocyte-positive, while the proportion of granulocyte-positive was more than twice that of the granulocyte–erythrocyte positive pattern in MDS cases. And erythrocyte clones occurred more frequently in AA than MDS, which may partially explain the phenomenon that some AA patients with PNH treated with immunosuppressive therapy could develop hemolysis, but MDS patients seldom did [9,23]. It is worth mentioning that 14.7% of AA cases and 22.9% of MDS cases did not manifest PNH clones at diagnosis, but presented with PNH clones during follow-up with median intervals of 6 months (3–36 months) for AA and 6 months (range: 4–36 months) for MDS. The results remind us that serial PNH clone monitoring annually [9] or every 6 months is warranted, even in aBMF patients without PNH clones for a long time.

For better research and accurate characterization of the PNH clone pattern and evolution model, we first defined PNH clone size as small (<10%), medium (10–50%), and large (>50%) according to potential risk of hemolysis and thrombosis. Hall et al. reported a 44% 10-year risk of venous thrombosis in PNH patients with a granulocyte clone size of >50%, while the risk in those with smaller clone sizes was only 5.8% [24]. Schrezenmeier et al. stated that patients with 50% or even larger clone sizes experienced more hemoglobinuria, dyspnea, abdominal pain, scleral icterus, erectile dysfunction, and dysphagia. More specifically, 5.3% of patients with a clone size <10% had a history of thrombosis, 7.7% of patients with a clone size of 10%–49% had thrombosis, and 15.4% with a clone size ≥50% had such a history ($p < 0.001$) [12]. Another study recommended a PNH granulocyte clone size >50% as an indicator for consideration of vitamin K antagonist prophylaxis and possibly Eculizumab [4,24]. Additionally, detection of <10% of PNH granulocytes was typical in AA and did not contribute to hemolysis [25,26]. Three-quarters of both AA and MDS patients in our study with <10% granulocyte PNH clones at diagnosis further confirmed the method of classification.

Several previous studies had found that the presence of PNH clones in AA suggested a superior response rate to IST. In a Russian prospective study of 125 patients with AA with IST, the response of patients with PNH clones was higher than that of patients without PNH clones (after first- and second-line IST: 68% vs. 45% and 53%

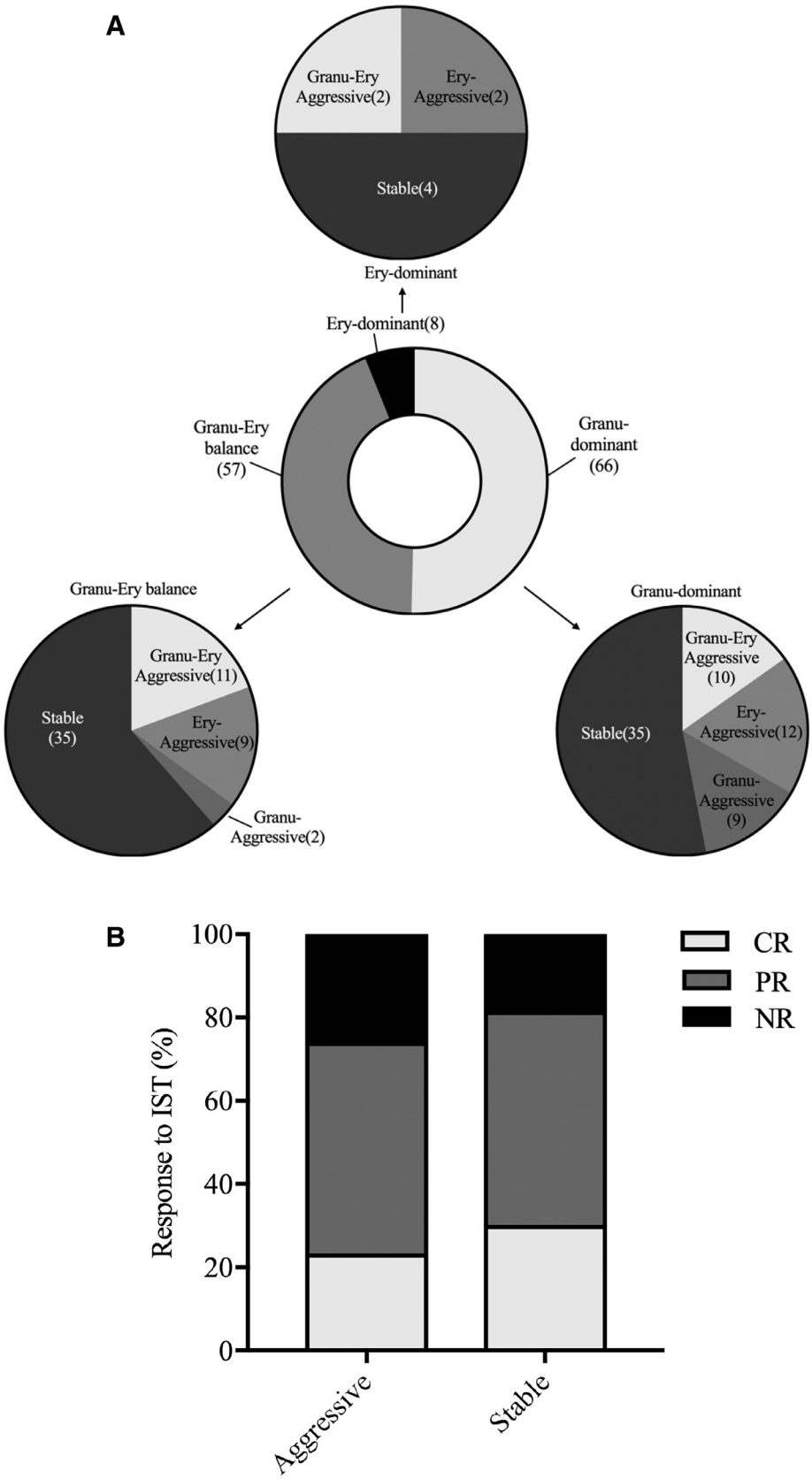

Figure 4. Relationship of PNH clone evolution patterns to hematological response in AA patients. **(A)** Clone evolution patterns of 131 follow-up AA patients. **(B)** Comparison of hematological response in patients with aggressive or stable evolution patterns.

vs.13%, respectively) [27]. A good predictor of response was also confirmed in both adults and children [28,29]. A similar phenomenon was also observed in MDS patients [15,30]. It could be interpreted that the clone of PNH is a benign clonal expansion and the deficiency of GPI-AP, which is the target of the immune attack, leads to a survival advantage and allows their clonal outgrowth over the normal hematopoiesis in aBMF [7]. However, PNH clonal expansion varied considerably among those patients with PNH clones. A series of research studies suggested that 15%–50% of cases had clonal expansion, 10%–25% clone disappearance, and 25%–60% unchanged clone size [26,31,32]. In a recent large-scale multicenter Italian study, among 151 cases with more than 1-year of follow-up, clones in 88.7% of patients remained stable, clones in 2.7% increased in size, and clones in 8.6% decreased [23]. Therefore, what confused us is whether the different evolutional patterns influence the hematological response of AA, because of the various and nonuniform assessment criteria for clonal expansion and the lack of studies on its association with therapeutic response. Based on the architecture of baseline PNH clones mentioned above and to gain a keen awareness of clonal expansion, we defined clone evolution patterns as clone expansion reaching a higher hierarchy and doubling of clone size in small groups, and reaching a higher hierarchy or doubling of clone size in medium groups. We found that more than one half exhibited the stable model, and that the majority of patients in the aggressive model were both granulocyte–erythrocyte and erythrocyte-aggressive. Furthermore, there were no obvious differences in response rates between aggressive and stable models.

However, the evolution of PNH clones is sometimes a nasty thing. Clinical PNH is a late complication occurring in 10%–15% of AA cases with IST therapy, which results in clonal expansion [23]. In a German multicenter study of 84 AA patients with IST over a median of 11.3 years, 32% developed clonal diseases, including all hematological and nonhematological clonal processes. Of note, clinical PNH was a major clonal disease, accounting for more than one-third of clonal events and was diagnosed 4.3 to 9.4 years after the diagnosis of aplastic anemia (actuarial probability: 10% at 11 years) [33]. A single-center Spanish study with a 40-year follow-up reported that the median interval from AA to the advent of clinical PNH was 8.5 years (range: 4 months to 30 years) [34]. Our study found that about a quarter of AA patients with PNH developed clinical hemolysis in a median interval of 11 months (range: 3–50 months), and taking previous studies mentioned above into consideration, we speculated that we would have found more cases of AA with IST developing into clinical hemolysis with a longer follow-up. A comparison of 34 AA patients with hemolysis after IST with 97 AA patients without hemolysis suggested that patients with AA with hemolysis have significantly larger clones in both granulocyte and erythrocytes at diagnosis, and the aggressive clone model is mainly the granulocyte–erythrocyte aggressive model; the stable model occurs seldomly, which is completely different from the whole follow-up of AA patients.

Conclusions

Patients with aBMF had significantly varied PNH clone architecture at diagnosis. AA patients with either aggressive or stable clone evolution can achieve a response, but an aggressive pattern, especially the granulocyte–erythrocyte aggressive pattern, tends to be associated with clinical hemolysis, and granulocyte clone size >28% or erythrocyte clone size >37% is an indicator of the onset of symptomatic PNH.

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Authorship contributions

YL completed the collection of the clinical data and analyzed and interpreted the data. NN, Z-DH, Y-QS, JZ, J-BH, X-XL, M-LG, PJ, MW, and Y-ZZ contributed to the clinical data collection. JS designed the research, wrote the paper, and approved the final article.

References

1. Dameshek W. Riddle: what do aplastic anemia, paroxysmal nocturnal hemoglobinuria (PNH) and "hypoplastic" leukemia have in common? Blood. 1967;30:251–254.
2. Young NS. The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. Blood. 1992;79:1385–1392.
3. Luzzatto L, Risitano AM. Advances in understanding the pathogenesis of acquired aplastic anaemia. Br J Haematol. 2018;182:758–776.
4. Brodsky RA. Paroxysmal nocturnal hemoglobinuria. Blood. 2014;124:2804–2811.
5. Luzzatto L. Recent advances in the pathogenesis and treatment of paroxysmal nocturnal hemoglobinuria. F1000Res. 2016;5.
6. Parker CJ. Paroxysmal nocturnal hemoglobinuria. Curr Opin Hematol. 2012;19:141–148.
7. DeZern AE, Brodsky RA. Paroxysmal nocturnal hemoglobinuria: a complement-mediated hemolytic anemia. Hematol Oncol Clin North Am. 2015;29:479–494.
8. Brodsky RA. How I treat paroxysmal nocturnal hemoglobinuria. Blood. 2009;113:6522–6527.
9. Borowitz MJ, Craig FE, Digiuseppe JA, et al. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. Cytometry B Clin Cytom. 2010;78:211–230.
10. Fletcher M, Sutherland DR, Whitby L, et al. Standardizing leucocyte PNH clone detection: an international study. Cytometry B Clin Cytom. 2014;86:311–318.

11. Raza A, Ravandi F, Rastogi A, et al. A prospective multicenter study of paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure. Cytometry B Clin Cytom. 2014;86:175–182.
12. Schrezenmeier H, Muus P, Socié G, et al. Baseline characteristics and disease burden in patients in the International Paroxysmal Nocturnal Hemoglobinuria Registry. Haematologica. 2014;99:922–929.
13. DeZern AE, Symons HJ, Resar LS, Borowitz MJ, Armanios MY, Brodsky RA. Detection of paroxysmal nocturnal hemoglobinuria clones to exclude inherited bone marrow failure syndromes. Eur J Haematol. 2014;92:467–470.
14. Wang SA, Pozdnyakova O, Jorgensen JL, 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.
15. Wang H, Chuhjo T, Yasue S, Omine M, Nakao S. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. Blood. 2002;100:3897–3902.
16. Brodsky RA. How do PIG-A mutant paroxysmal nocturnal hemoglobinuria stem cells achieve clonal dominance? Expert Rev Hematol. 2009;2:353–356.
17. Young NS. Paroxysmal nocturnal hemoglobinuria and myelodysplastic syndromes: clonal expansion of PIG-A-mutant hematopoietic cells in bone marrow failure. Haematologica. 2009;94:3–7.
18. Parker C, Omine M, Richards S, et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood. 2005;106:3699–3709.
19. Killick SB, Bown N, Cavenagh J, et al. Guidelines for the diagnosis and management of adult aplastic anaemia. Br J Haematol. 2016;172:187–207.
20. Parker CJ. Management of paroxysmal nocturnal hemoglobinuria in the era of complement inhibitory therapy. Hematology Am Soc Hematol Educ Program. 2011;2011:21–29.
21. Illingworth A, Marinov I, Sutherland DR, Wagner-Ballon O, DelVecchio L. ICCS/ESCCA consensus guidelines to detect GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and related disorders: part 3. Data analysis, reporting and case studies. Cytometry B Clin Cytom. 2018;94:49–66.
22. Cannizzo E, Raia M, De Propris MS, et al. Features, reason for testing, and changes with time of 583 paroxysmal nocturnal hemoglobinuria clones from 529 patients: a multicenter Italian study. Ann Hematol. 2019;98:1083–1093.
23. Parker CJ. The pathophysiology of paroxysmal nocturnal hemoglobinuria. Exp Hematol. 2007;35:523–533.
24. Hall C, Richards S, Hillmen P. Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH). Blood. 2003;102:3587–3591.
25. Mukhina GL, Buckley JT, Barber JP, Jones RJ, Brodsky RA. Multilineage glycosylphosphatidylinositol anchor-deficient haematopoiesis in untreated aplastic anaemia. Br J Haematol. 2001;115:476–482.
26. Scheinberg P, Marte M, Nunez O, Young NS. Paroxysmal nocturnal hemoglobinuria clones in severe aplastic anemia patients treated with horse anti-thymocyte globulin plus cyclosporine. Haematologica. 2010;95:1075–1080.
27. Kulagin A, Lisukov I, Ivanova M, et al. Prognostic value of paroxysmal nocturnal haemoglobinuria clone presence in aplastic anaemia patients treated with combined immunosuppression: results of two-centre prospective study. Br J Haematol. 2014;164:546–554.
28. Sugimori C, Chuhjo T, Feng X, et al. Minor population of CD55−CD59− blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. Blood. 2006;107:1308–1314.
29. Sakaguchi H, Nishio N, Hama A, et al. Peripheral blood lymphocyte telomere length as a predictor of response to immunosuppressive therapy in childhood aplastic anemia. Haematologica. 2014;99:1312–1316.
30. Ishiyama K 1, Chuhjo T, Wang H, Yachie A, Omine M, Nakao S. Polyclonal hematopoiesis maintained in patients with bone marrow failure harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. Blood. 2003;102:1211–1216.
31. Sugimori C, Mochizuki K, Qi Z, et al. Origin and fate of blood cells deficient in glycosylphosphatidylinositol-anchored protein among patients with bone marrow failure. Br J Haematol. 2009;147:102–112.
32. Pu JJ, Mukhina G, Wang H, Savage WJ, Brodsky RA. Natural history of paroxysmal nocturnal hemoglobinuria clones in patients presenting as aplastic anemia. Eur J Haematol.201;87:37−45.
33. Frickhofen N, Heimpel H, Kaltwasser JP, Schrezenmeier H. German Aplastic Anemia Study Group. Antithymocyte globulin with or without cyclosporin A: 11-year follow-up of a randomized trial comparing treatments of aplastic anemia. Blood. 2003;101:1236–1242.
34. Muñoz-Linares C, Ojeda E, Forés R, et al. Paroxysmal nocturnal hemoglobinuria: a single Spanish center's experience over the last 40 yr. Eur J Haematol. 2014;93:309–319.