

# Current Insights into Paroxysmal Nocturnal Hemoglobinuria

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**P**aroxysmal nocturnal hemoglobinuria (PNH) has long been recognized as a hemolytic disorder by its predominant clinical features of chronic intravascular hemolysis i.e. chronic anemia with exacerbations and intermittent dark urine from hemoglobinuria. With recent studies for the better understanding of the disease, PNH is recognized as an acquired hemolytic anemia, which has originated through the clonal expansion of a hematopoietic progenitor cell that has a somatic mutation of the X linked, phosphatidylinositolglycan class A (PIG-A) gene. PNH can also be classified as an acquired non-malignant, clonal hematopoietic stem cell disorder. It is characterized by chronic intravascular hemolysis with paroxysms, a tendency to form venous thrombosis, and features of bone marrow failure as well as a close association with aplastic anemia.<sup>1,2</sup>

Although PNH occurs in all populations around the world, it is rare in the western countries. PNH is not so uncommon in East Asia, including Thailand, probably because of a considerable high incidence of aplastic anemia in the region.<sup>3</sup> New insights have recently been developed into better understanding PNH in various aspects including its pathophysiology, particularly biochemical and molecular abnormalities and underlying genetic pathology, leading to the development of diagnosis and novel treatment.

## The GPI anchor and genetics of PNH

Intravascular hemolysis, the major cause of anemia in PNH, occurs as a result of the increased sensitivity of PNH red cells to the lytic action of complement. It is now clear that the abnormal complement-sensitivity of the red cells occurs through the missing of two complement-regulatory membrane proteins, CD55 (decay accelerating factor; DAF) and CD59 (membrane inhibitor of reactive lysis; MIRL). It turns out that these two membrane proteins as well as the others missing from PNH cells, such as erythrocyte acetylcholine esterase and leukocyte alkaline phosphatase are expressed on the cell membrane by their carboxy-terminal linkage to a glycolipid, glycosylphosphatidylinositol (GPI) anchor.<sup>4,5</sup> Up to now, at least 27 different GPI-anchored proteins (GPI-APs) have been found to be

expressed on blood cells, with a multitude of functions such as complement inhibitors, ecto-enzymes, accessory molecules for growth receptors, and adhesion molecules.

GPI is synthesized through step-wise reactions in endoplasmic reticulum, starting from phosphatidylinositol. In patients with PNH, GPI biosynthesis in the affected blood cells is defective as a result of a somatic mutation of the X linked, phosphatidylinositolglycan class A (PIG-A) gene,<sup>6,7</sup> encoding the catalytic subunit of N-acetylglucosamine transferase which mediates the first step of GPI biosynthesis. As PIG-A is an X-chromosome gene and only one X chromosome is active in a hematopoietic stem cell (HSC) either in a male or female (through lyonization), a single "hit" of the PIG-A in a HSC can generate a PNH phenotype in all types of blood cells.<sup>8</sup> At present, more than 180 somatic mutations of the PIG-A gene have been identified in blood cells from patients with PNH. These mutations are mostly small ones such as single base deletions/insertions, single base substitutions, few bases insertions/deletions.<sup>9,10</sup> The majority of these mutations are frame shift mutations leading to an inactive PIG-A product and a complete loss of glycosyltransferase activity with predictable absence of GPI-APs from PNH blood cells (type III PNH cells). Some point mutations of single base substitutions confer to a PIG-A protein with some residual function which explains a partial expression of GPI-APs on blood cells (type II PNH cells.)<sup>9-12</sup>

## Cellular phenotypes and clinical implications

PNH is characterized by the presence of blood cells that are deficient in GPI-APs (PNH cells). All cell lineages including their progenitors are involved. As the GPI-APs carry out various functions, deficiencies of these surface proteins potentially confer particular clinical consequences and manifestations in patients with PNH. The most prominent clinical consequences are the complement-mediated destruction (hemolysis) of the PNH erythrocytes from their increased sensitivity to complement due to the absence of the two surface proteins, CD55 and CD59, which are responsible for regulating early and terminal complement activities, respectively. In addition to anemia, free hemoglobin

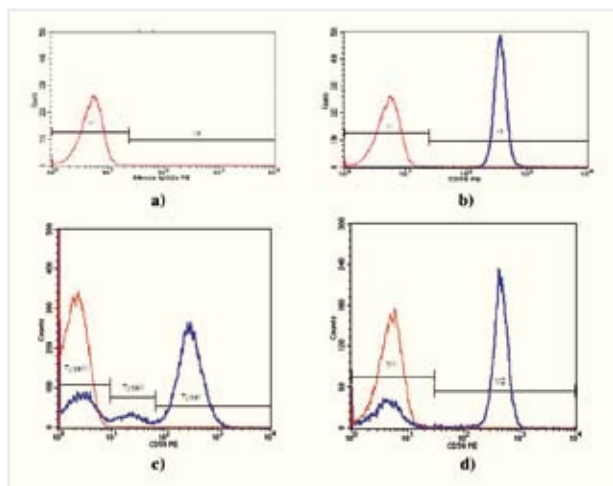
from complement-mediated intravascular hemolysis (IVH) can lead to other manifestations. Hemoglobin readily binds stoichiometrically to nitric oxide (NO) and removes it from tissues.<sup>13</sup> The depletion of tissue NO is suggested to be responsible for a group of symptoms related to smooth muscle contraction, including erectile dysfunction, abdominal pain, esophageal spasm and dysphagia.<sup>14</sup> These symptoms are intensified during paroxysms of IVH. Moreover, NO has been shown to inhibit platelet aggregation and platelet adhesion through its activity of increasing cGMP.<sup>15</sup> Therefore, the hemoglobin-induced NO depletion, through its increased platelet aggregation and adhesion on prothrombotic surfaces (such as injured vessel walls), may be one of the contributing factors to the thrombotic tendency observed in patients with PNH. Decreased expression of the GPI-linked urokinase plasminogen activator (CD87) on PNH granulocytes may also increase the thrombotic risk in the patients with PNH due to the failure of the fibrinolytic process.<sup>16</sup>

Detection of circulating blood cells with deficiency of the GPI-linked proteins is the hallmark and is essential for the diagnosis of PNH. Previously, diagnosis of PNH has traditionally been based on tests which demonstrate the complement sensitivity of red cells, such as acidified serum (Ham) test and sucrose lysis test. Recently, these tests have been preferably replaced by flow cytometric analysis of GPI-APs deficient blood cells, as it directly determines the percentage of PNH cells. It is recommended that at least two monoclonal antibodies to GPI-APs are used in order to exclude the possibility of inherited deficiency of a single GPI-AP. Flow cytometric analysis, especially of erythrocytes, also determines the degree of GPI-anchor deficiency into PNH III, PNH II and PNH I, representing blood cells with completely lack, partial expression, and normal expression of the GPI-APs respectively (Fig 1). Typically, normal (PNH I) and PNH (PNH III or PNH II) blood cells co-exist in patients with PNH. Apart

from detection of PNH erythrocytes, the analysis of GPI-APs on granulocytes is also suggested, as the GPI-AP deficient granulocytes are not complement-sensitive and have a normal half-life in circulation. Therefore, the proportion of GPI-AP deficient granulocytes, is not affected by both hemolysis and transfusion, which reflects the actual size of the PNH clone or the status of PNH hematopoiesis.<sup>17</sup> In addition to diagnostic application, flow cytometry analysis, especially on granulocytes, plays an important role in disease categorization and decision making for proper treatment, predicting the severity of anemia<sup>12</sup> and also the risk of thrombosis.<sup>18</sup>

### Bone marrow failure and selection of PNH clone

Association of PNH with aplastic anemia is well recognized.<sup>1</sup> Approximately, one-third of PNH patients have had a precedent history of aplastic anemia, months or years before developing clinical IVH of PNH.<sup>19</sup> Up to 70% of patients with aplastic anemia, even without clinical PNH, exhibited PNH clones.<sup>20,21</sup> On the other hand, a proportion of patients with PNH have varying degrees of leucopenia and/or thrombocytopenia, suggesting an underlying condition of bone marrow failure. Moreover, even patients with hemolytic PNH and without clinical features of leucopenia/thrombocytopenia or marrow hypoplasia may have evidence of deficient hematopoiesis.<sup>22</sup> Taken together, it is suggested that aplastic anemia or bone marrow failure is closely associated with or underlies the occurrence of PNH. The mutation of PIG-A with the resultant deficiency of GPI-anchored molecules does not confer any absolute growth advantage to the mutant hematopoietic stem cells and, therefore, does not explain the expansion of the PNH clone.<sup>23</sup> It is likely that it is autoimmunity to the hematopoietic stem cells that causes aplastic anemia which mediates clonal selection of the PIG-A mutant cells. These mutant stem cells may be more resistant than the non-mutant ones to the cytotoxic lymphocytes that are responsible for aplastic anemia. The immune-mediated clonal selection is proposed to be the second step following the first step of PIG-A mutation in the pathogenesis of PNH. Whether there are additional mechanisms operating clonal expansion following the clonal selection is one of the current research issues in PNH.



**Fig 1.** Flow cytometric analysis of red blood cells using fluorescently labeled CD59 to determine PNH cells and also degree of GPI-anchor deficiency, a: negative control, b: a normal person with normal expression of CD59 on red cells (PNH I), c: a patient with three populations of red cells with normal (PNH I), partially (PNH II), and completely deficient (PNH III) in surface expression of CD59, d: a patient with type I and type III cells.

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