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Advances in the Diagnosis and Therapy of Paroxysmal Nocturnal Hemoglobinuria

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Summary

PNH is an uncommon acquired hemolytic anemia that often manifests with hemoglobinuria, abdominal pain, smooth muscle dystonias, fatigue, and thrombosis. The disease results from the expansion of hematopoietic stem cells harboring a mutation in a gene, *PIG-A*, that is required for the biosynthesis of a lipid moiety, glycosylphosphatidylinositol (GPI), that attaches dozens of different proteins to the cell surface. Thus, PNH cells are deficient in cell surface GPI anchored proteins; this deficiency on erythrocytes leads to intravascular hemolysis since certain GPI anchored proteins normally function as complement regulators. Free hemoglobin released from intravascular hemolysis leads to circulating nitric oxide depletion and is responsible for many of the clinical manifestations of PNH, including fatigue, erectile dysfunction, esophageal spasm, and thrombosis. Interestingly, rare *PIG-A* mutations can be found in virtually all healthy control subjects leading to speculation that *PIG-A* mutations in hematopoietic stem cells are common benign events. However, recent data reveals that most of these mutations in healthy controls are not derived from stem cells. The recently FDA approved complement inhibitor eculizumab has been shown to decrease hemolysis, decrease erythrocyte transfusion requirements, decrease the risk for thrombosis and improve quality of life for PNH patients.

Keywords

paroxysmal nocturnal hemoglobinuria; *PIG-A*; nitric oxide; eculizumab; FLAER; proaerolysin; complement; glycosylphosphatidylinositol anchored protein

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic stem cell disorder that has fascinated hematologists for more than a century due to its protean clinical manifestations and fascinating pathophysiology.¹ One of the earliest descriptions of PNH was by Dr. Paul Strübing, who in 1882 described a 29-year-old cartwright who presented with fatigue, abdominal pain, and severe nocturnal paroxysms of hemoglobinuria that were exacerbated by excess alcohol, physical exertion, and iron salts.² Later reports by Marchiafava and Micheli led to the eponym, Marchiafava-Micheli syndrome, but it was Enneking, in 1925, who introduced the term “paroxysmal nocturnal hemoglobinuria.”³

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In 1937, Thomas Ham found that PNH erythrocytes were hemolyzed when incubated with normal, acidified serum.⁴ This seminal discovery resulted in the first diagnostic test for PNH, the acidified serum or Ham test. Red cell lysis following acidified serum appeared to be complement dependent since heat inactivation abrogated the reaction; however, it wasn't until 1954, with the discovery of the alternative pathway of complement activation, that increased complement sensitivity was formally proven to cause the hemolysis of PNH red cells.⁵ Following the emergence of specific diagnostic tests, additional disease manifestations such as venous thrombosis, smooth muscle dystonias and other clonal stem cell disorders were found to be associated with PNH. These non-erythroid manifestations of the disease foreshadowed the discovery that PNH results from the clonal expansion of a mutated hematopoietic stem cell.

In the 1980s, it was discovered that PNH cells display a global deficiency in a group of proteins attached to the cell surface by a GPI moiety. Interestingly, two of these missing proteins (e.g., CD55 and CD59) are important complement regulatory proteins. A few years later, the genetic mutation (*PIG-A*) responsible for the GPI-anchor protein deficiency was discovered⁶ and most recently, a humanized monoclonal antibody that inhibits terminal complement activation has been shown to ameliorate hemolysis and disease symptoms in PNH patients.^{7;8} Although the pathophysiology of many manifestations of PNH are now understood, the mechanism of clonal dominance, and the close association of PNH and acquired aplastic anemia continue to be areas of intense investigation.

GPI-anchor biosynthesis

Covalent linkage to GPI is an important means of anchoring dozens of cell surface glycoproteins to the cell membrane. GPI is synthesized in the endoplasmic reticulum and transferred *en bloc* to the carboxyl terminus of a protein that has a GPI-attachment signal peptide. Biosynthesis of GPI anchors involves at least 10 reactions and more than 20 different genes (Figure 1 and Table 1).⁹ The common core structure of GPI consists of a molecule of phosphatidylinositol and a glycan core that contains glucosamine, three mannoses and an ethanolamine phosphate. The first step in GPI anchor biosynthesis is the transfer of N-acetylglucosamine (GlcNAc) from uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc) to phosphatidylinositol (PI) to yield GlcNAc-PI. This step is catalyzed by GlcNAc:PI α 1-6 GlcNAc transferase, an enzyme whose subunits are encoded by 7 different genes: *PIG-A*,⁶ *PIG-C*,¹⁰ *PIG-H*,¹¹ *GPI1*,¹² *PIG-Y*,¹³ *PIG-P* and *DPM2*.¹⁴ The second step is the de-N-acetylation of GlcNAc-PI to GlcN-PI.¹⁵ Later, three mannoses from dolichol-phosphate-mannose and an ethanolamine phosphate from phosphatidylethanolamine are added sequentially. The core is modified with side groups during or after synthesis. The last step in GPI anchor processing is the attachment to the newly synthesized proprotein via a transamidase-like reaction (involves at least 5 gene products, see Table 1) during which a C-terminal GPI attachment signal is released.⁹ The GPI anchored proteins then transit the secretory pathway to reach their final destination at the plasma membrane where they reside in 50 to 350-nm microdomains known as lipid rafts (Figure 1).

The *PIG-A* Gene Mutation

To date, all PNH patients have been shown to have genetic mutations in an X-linked gene known as *PIG-A* (phosphatidylinositol glycan class A).^{6;16–18} The *PIG-A* gene product is required for the first step in the synthesis of GPI-anchored proteins;¹⁹ thus, *PIG-A* mutations block the biosynthesis of the GPI anchor. Failure to synthesize a mature GPI anchor causes the cognate protein to be degraded intracellularly and results in an absence of all GPI anchored proteins from the cell surface. The fact that PNH is an acquired disorder and that *PIG-A* is on the X chromosome explains well why PNH patients all have *PIGA* mutations. A single mutation can generate a PNH phenotype since males and females have only 1 active X chromosome per

cell; in females, one X chromosome is inactivated through lyonization. All other genes involved in GPI anchor biosynthesis are found on autosomes; thus, inactivating mutations in these genes would have to occur on both alleles in the same cell, a statistically unlikely occurrence.

The role of complement in intravascular hemolysis

Hemolysis in PNH results from the increased susceptibility of PNH red cells to complement.^{20–23} The complement system consists of more than 20 serum proteins that interact in a precise series of enzymatic cleavages and membrane binding events leading to the generation of products with immunoprotective, immunoregulatory, proinflammatory, and cytolytic properties. There are 3 pathways by which complement is activated: the classical pathway, the lectin pathway, and the alternative pathway which all result in the generation of C3 convertase complexes that mediate the cleavage of C3 to C3a and C3b.²⁴ PNH red cells are vulnerable to complement activation through any of these pathways; however, the alternative pathway is in a state of continuous, low-level activation which explains why most PNH patients have chronic hemolysis.

PNH red cells are more vulnerable to complement-mediated lysis due to a reduction, or complete absence, of two important GPI-anchored, complement regulatory proteins (CD55 and CD59). CD59 is a 19,000 molecular weight glycoprotein that directly interacts with the membrane attack complex (MAC) to prevent lytic pore formation by blocking the aggregation of C9.^{23;25} Absence of this protein results in chronic complement-mediated intravascular hemolysis, a process that is integral to the morbidity and mortality of PNH. CD55, a 68,000 molecular weight glycoprotein, functions to accelerate the rate of destruction of membrane bound C3 convertase.²² Hence, CD55 reduces the amount of C3 that is cleaved and CD59 reduces the number of MAC that are formed. Of the two, CD59 is more important in protecting cells from complement.

Nitric oxide scavenging in PNH

Nitric oxide is a major regulator of vascular physiology and many clinical manifestations of PNH are readily explained by depletion of nitric oxide at the tissue level (Figure 2).^{26;27} Normally, oxygen and nitric oxide react with nitric oxide synthase in the endothelium to produce nitric oxide and citrulline. Nitric oxide then acts on the vascular wall to maintain normal tone and limit platelet activation. Free hemoglobin in the plasma has enormous affinity for nitric oxide and serves as a potent nitric oxide scavenger. Under normal conditions hemoglobin is sequestered by the red cell membrane, minimizing the scavenging of nitric oxide. In PNH, failure to regulate complement on the red cell membrane can lead to massive intravascular hemolysis and release of large amounts of free hemoglobin and erythrocyte arginase into the plasma. This leads to scavenging of nitric oxide and also decreases the substrate (arginine) for nitric oxide synthesis.^{28;29} Haptoglobin is one compensatory mechanism for free hemoglobin removal, but the concentration of plasma hemoglobin in PNH exceeds the capacity of haptoglobin to remove the hemoglobin from the plasma.²⁶ Depletion of nitric oxide at the tissue level manifests clinically as fatigue, abdominal pain, esophageal spasm, male erectile dysfunction, and possibly thrombosis.³⁰ Indeed, hemoglobinuria, thrombosis, male erectile dysfunction, and esophageal spasm are much more common in patients with large PNH populations.³⁰

Thrombophilia in PNH

Thrombosis is an ominous complication of PNH and the leading cause of death from the disease. It occurs in about 40% of PNH patients and predominantly involves the venous system. Patients with a large percentage of PNH cells and classical symptoms (hemolytic anemia and hemoglobinuria) have a greater propensity for thrombosis than patients with a small percentage

of PNH cells.^{30–33} Patients with PNH granulocyte clones of greater than 60% appear to be at greatest risk for thrombosis.³⁰ While the mechanism of thrombosis in PNH is not entirely understood, it is almost certainly related to intravascular hemolysis and other consequences of GPI anchor protein deficiency. Indeed, nitric oxide depletion has been associated with increased platelet aggregation, increased platelet adhesion, and accelerated clot formation.²⁷ In addition, in an attempt to repair complement-mediated damage, PNH platelets undergo exocytosis of the complement attack complex.³⁴ This results in the formation of microvesicles with phosphatidylserine externalization, a potent *in vitro* procoagulant. These prothrombotic microvesicles have been detected in the blood of PNH patients.³⁵ Fibrinolysis may also be perturbed in PNH given that PNH blood cells lack the GPI anchored urokinase receptor.³⁶ Lastly, tissue factor pathway inhibitor (TFPI), a major inhibitor of tissue factor, has been shown to require a GPI anchored chaperone protein for trafficking to the endothelial cell surface.³⁷ Due to the increased risk for venous thrombosis, especially in patients with a large percentage of PNH cells, prophylactic anticoagulation has been recommended by several experts. However, the high risk of serious bleeding associated with anticoagulation in PNH patients and the recent data suggesting that anticoagulation may not adequately prevent thrombosis in PNH has tempered enthusiasm for prophylactic anticoagulation in PNH patients.³⁸

The PNH Stem Cell and Clonal Expansion

In PNH patients, GPI anchor protein deficient erythroid, myeloid, and lymphoid cells are clonal and harbor matching *PIG-A* mutations; thus, the PNH stem cell (i.e., the cell where the *PIG-A* mutation originates) is a multipotent hematopoietic stem cell (HSC). Interestingly, granulocytes from the blood of healthy controls also harbor *PIG-A* mutations at a frequency of approximately 1 in 50,000.^{39;40} In contrast to the *PIG-A* mutations associated with PNH, most *PIG-A* mutations in healthy controls arise from a more differentiated colony forming cell rather than a multipotent HSC.⁴⁰ Unlike HSC, colony forming cells have no self-renewal capacity; hence, mutations arising at this level of hematopoietic differentiation will not be propagated beyond their terminally differentiated progeny and do not result in human disease. However, it remains unclear whether *PIG-A* mutations that do occur in a multipotent HSC are sufficient to cause the disease; immune selection or secondary mutations may be necessary to give the PNH stem cell a survival advantage. Thus, *PIG-A* mutations are necessary, but possibly not sufficient to cause the disease - another step may be required to promote expansion of the *PIG-A* mutant HSC.

Several hypotheses have been proposed to explain clonal expansion in PNH. A two step model for developing PNH proposes that *PIG-A* mutations are benign events in HSCs and that these cells only undergo clonal expansion in the setting of immune selection that targets normal stem cells but spares PNH stem cells. This would help explain the close association between acquired aplastic anemia (an autoimmune disease) and PNH. A simplistic explanation for the immune escape proposes that normal HSC have GPI anchored antigen(s) that are the target of immune attack in acquired aplastic anemia; however, there is no direct evidence for a GPI anchored protein serving as the target of an autoimmune attack in aplastic anemia or PNH. Others have suggested that the *PIG-A* mutation *per se* confers resistance to apoptosis, and that under immune selection or bone marrow stress, PNH cells may have a survival advantage in this hostile environment.⁴¹ Yet another hypothesis proposes that additional mutations occurring in *PIG-A* mutant stem cells are required for clonal expansion PNH. Recently, two patients were described with a gain of function t(12;12) that leads to increased *HMGA2* expression.⁴² *HMGA2* is a member of the high mobility group of regulatory transcription factors. While overexpression of *HMGA2* likely contributed to the clonal expansion in these two patients, the mechanism of clonal expansion in the majority of PNH patients without *HMGA2* overexpression remains unclear.

Diagnosis

Modern laboratories use monoclonal antibodies against specific GPI anchored proteins in conjunction with flow cytometry to diagnose PNH.⁴³ Anti-CD59 is most commonly used because it is widely expressed and is displayed on all hematopoietic lineages. Flow cytometry measures the size of the PNH clone in the various cell lineages and is very sensitive and specific if performed on granulocytes. Ideally, at least two different monoclonal antibodies, directed against two different GPI anchored proteins, on at least 2 different cell lineages, should be used to diagnose a patient with PNH. Solely screening red cells for PNH can lead to falsely negative tests, especially in the setting of a recent hemolytic episode or a recent blood transfusion. Since granulocytes and monocytes have a short half-life and are not affected by blood transfusions, the percentage of PNH cells in these lineages best reflects the size of the PNH clone.

A fluorescein-labeled proaerolysin variant, FLAER, is increasingly being used as a flow cytometric assay to diagnose PNH.^{44;45} Aerolysin is the principal virulence factor of the bacterium *Aeromonas hydrophila*. It is secreted as an inert protoxin, proaerolysin, that binds selectively and with high affinity to the GPI anchor. After binding to the glycan portion of the GPI anchor, the C-terminal peptide of proaerolysin is cleaved by cell proteases. This activates the toxin and leads to the formation of heptameric channels that insert into the membrane and kill the cell. PNH cells are resistant to aerolysin and proaerolysin because PNH cells lack cell surface GPI anchors.⁴⁶ FLAER is a proaerolysin variant that binds to the GPI anchor without forming channels and gives a highly accurate assessment of the GPI anchor deficit on PNH cells (Figure 3). Since the GPI anchor is the major determinant for binding FLAER, it allows for the direct assessment of GPI anchor expression on most cell lineages. Red cells are a notable exception; this may be because both normal and PNH red cells express large amounts of glycophorin, a protein shown to bind aerolysin weakly.

Classical PNH versus Aplastic Anemia/PNH Overlap

Many patients with acquired aplastic anemia have small to moderate populations of cells in the peripheral blood that are missing GPI-anchored proteins.^{45;47;48} This often leads to confusion concerning the diagnosis – PNH versus aplastic anemia. The fact that there is no threshold PNH population that predicts for hemolysis or thrombosis contributes to the confusion. The classical definition of PNH requires evidence of intravascular hemolysis; thus, patients with a hypocellular bone marrow and a small to moderate PNH population without intravascular hemolysis are often referred to as having aplastic anemia/PNH overlap (AA/PNH). Some, but not all, of these AA/PNH patients exhibit continued expansion of the *PIG-A* mutant clone and evolve into classical PNH.

Classical PNH manifests with overt hemolysis, an elevated reticulocyte count, an elevated LDH, and a normocellular to hypercellular bone marrow. Patients with AA/PNH have a hypocellular bone marrow and seldom demonstrate signs and symptoms of hemolysis because the population of PNH cells is small; symptoms from anemia, thrombocytopenia and neutropenia are usually a consequence of bone marrow failure.⁴⁵ Patients with AA/PNH often manifest with pancytopenia, a low reticulocyte count, and a normal to mildly elevated LDH. Distinguishing between classical PNH and AA/PNH is particularly important since eculizumab is more likely to be beneficial in patients with classical PNH. Immunosuppressive therapy or bone marrow transplantation is often employed to treat AA/PNH in cases with severe pancytopenia.⁴⁹

PNH treatment

Allogeneic hematopoietic stem cell transplantation is the only curative therapy for PNH. Younger patients with severe pancytopenia or life-threatening thrombosis who have an HLA-

identical sibling are the best candidates for this approach.^{50;51} Eradication of the PNH clone has been achieved with both myeloablative and non-myeloablative conditioning regimens.^{52;53} Unfortunately, most PNH patients are not suitable transplant candidates and most of those who lack a suitable donor. The use of bone marrow transplantation to treat PNH has become less common now that there is an effective drug therapy.

Eculizumab is a humanized monoclonal antibody against C5 that inhibits terminal complement activation (Figure 4) and is the first effective drug therapy for PNH. The drug was approved by the Food and Drug Administration in 2007. Eculizumab has been evaluated in three separate parent studies – a Phase 2 pilot study and two Phase 3 studies. All patients participating in these trials were allowed to continue eculizumab in an extension trial. The phase II pilot study in 11 PNH patients demonstrated that the drug reduced intravascular hemolysis and transfusion requirements over a 12 week time period.⁷ Shortly after, an international, double blind, randomized, placebo-controlled trial known as TRIUMPH demonstrated that eculizumab was effective in stabilizing hemoglobin levels and reduced transfusion requirements in patients with classical PNH.⁸ Eligible patients were required to have received at least 4 red cell transfusions in the year prior to enrollment, an LDH that was at least 1.5 times the upper limit of normal and a platelet count of at least 100,000/ μ l. This study randomized 87 PNH patients to receive either placebo (N=44) or eculizumab (N=43) administered intravenously at 600 mg weekly for four weeks, 900 mg the following week, and then 900 mg every two weeks for a total of six months. The primary endpoints were hemoglobin stabilization and reduction in units of transfused blood. Hemoglobin stabilization was maintained by a median of 48.8% patients in the eculizumab-treated group and 0% in the placebo-treated group ($p<0.001$). A median of 0 units of packed red cells were transfused in the eculizumab-treated group compared with a median of 10 units in the placebo-treated group ($p=0.001$). The eculizumab-treated group also showed significant improvements in quality of life and a significant decrease in LDH levels. The most common adverse events reported for eculizumab treated patients were headache, nasopharyngitis, back pain, and upper respiratory tract infections. Blockade of the complement cascade at C5 preserves the early activity of the complement cascade that is necessary for the opsonization of microorganisms and clearance of immune complexes; however, since terminal complement blockade may be associated with an increased risk for *Neisseria* infections, all patients treated with eculizumab must be vaccinated against *Neisseria meningitidis* before receiving the drug.

Following initiation of the TRIUMPH study, eculizumab was evaluated in SHEPHERD, an open-label, non-placebo controlled, phase 3 clinical safety and efficacy study in a more diverse PNH patient population. PNH patients with minimal transfusion requirements and a platelet count as low as 30,000/ μ l were eligible for SHEPHERD.⁵⁴ Eculizumab was administered for a total of 52 weeks using a dosing schedule identical to that of TRIUMPH. Ninety-seven patients at more than 30 international sites were enrolled. Adverse events were similar to those observed in the TRIUMPH study. Hemolysis was significantly reduced leading to improved anemia with reduced transfusion and increased transfusion independence, lessened fatigue, and improved overall health-related quality of life. Thus, the results of the SHEPHERD trial confirmed the safety and efficacy of eculizumab in a broader and more typical population of PNH patients.

Of the 195 patients entering the pilot, TRIUMPH or SHEPHERD studies, 187 patients completed these studies and elected to receive eculizumab in a 2-year Phase 3b open-label extension study. Overall, there was no evidence of an increased incidence of infection across PNH studies with eculizumab as compared to placebo, including serious infections, severe infections or multiple infections. Two patients experienced meningococcal sepsis after vaccination; they recovered without clinical sequelae. Importantly, the design of the Phase 3b extension study specified that the thrombosis rate for each patient would be compared before

and during eculizumab treatment. The thromboembolism rate with eculizumab treatment was 1.07 events/100 patient-years compared to 7.37 events/100 patient-years ($P < 0.001$) before eculizumab treatment.³⁸ This translates into an 85% absolute reduction in the risk for thrombosis while on eculizumab treatment. Ideally, one would like to have results from a prospective randomized control trial to determine whether eculizumab treatment prevents thrombosis, but given the rarity of PNH, this trial will probably never be performed. Nevertheless, these studies strongly suggest that eculizumab greatly reduces the risk for thrombosis in PNH patients.

Thus, in patients with classical PNH, eculizumab is highly effective in decreasing intravascular hemolysis; the drug greatly improves quality of life, reduces or eliminates the need for blood transfusions, and reduces the risk for thrombosis. Importantly, eculizumab also mitigates the smooth muscle dystonias that are often associated with PNH by reducing plasma free hemoglobin levels. Patients with AA/PNH are less likely to respond to eculizumab, since bone marrow suppression, rather than complement-mediated hemolysis is the major mechanism of anemia.

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Figure 1. Biosynthesis of GPI-anchored proteins

GPI-anchored biosynthesis takes place in the endoplasmic reticulum. *PIG-A* is one of 7 genes required for the first step (see Table 1), the transfer of N-acetylglucosamine (GlcNAc) from uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc:PI) to phosphatidylinositol (PI) to yield GlcNAc-PI. After synthesis of the mature GPI precursor, the cognate protein is attached and then transported to the plasma membrane where the GPI-anchored protein resides in membrane rafts. *PIG-A* mutations lead to a defect in the first step in GPI anchor biosynthesis resulting in intracellular degradation of the cognate protein and a lack of cell surface GPI anchored proteins.

Figure 2. Nitric oxide scavenging in PNH

Under normal conditions (A) Nitric oxide synthase (NOS) combines with oxygen (O_2) and arginine to form nitric oxide (NO) and citrulline. (B) Intravascular hemolysis releases free hemoglobin into the plasma. Oxygen bound Fe^{2+} from free hemoglobin enters the plasma and converts NO to inert nitrite and oxidizes hemoglobin to methemoglobin. In addition, intravascular hemolysis releases erythrocyte arginase, which depletes arginine, the substrate for NOS. Depletion of NO at the tissue level leads to many of the symptoms of PNH including smooth muscle dystonias.

Figure 3. Flow cytometric diagnosis of PNH. (A)

Cell surface GPI anchored proteins can be detected using individual monoclonal antibodies (e.g., anti-CD55, anti-CD59, anti-CD14 etc.) or FLAER. Monoclonal antibodies bind to the protein portion of the cell which may vary based on the cell type and level of differentiation. FLAER binds to the glycan portion of the GPI anchor and binds to virtually all GPI anchored proteins. **(B)** Flow cytometric analysis of granulocytes from a PNH patient showing GPI anchor deficient cells (left peak) and small population of normal GPI anchor replete cells (right peak).

Figure 4. Blockade of terminal complement activation

Classical, alternative and lectin pathways converge at the point of C3 activation. The lytic pathway is initiated with the formation of C5 convertase and leads to the assembly of the C5b, C6, C7, C8, (n) C9 membrane attack complex. Eculizumab binds to C5 and prevents the formation of the membrane attack complex by reducing cleavage of C5 into C5a and C5b.

Table 1

Genes involved in GPI anchor biosynthesis.

1ST STEP	Name of gene	Location
UDP-GlcNAc → GlcNAc-PI	PIG-A	Xp22.1
	PIG-C	1q23.3
	PIG-H	14q11-q24
	PIG-P	21q22.2
	GPI1 (PIG-Q)	16p13.3
	DPM2	9q33
	PIG-Y	4q21
Intermediate steps	PIG-L	17p12
	PIG-M	1q22
	PIG-N	18q21
	PIG-B	15q21-q22
	PIG-F	2q16-p21
	PIG-O	9
	DPM1	20q13.1
	DPM3	1q21.2
	SL15(MPDU1)	17p13.1
	PIG-V	1p36.11
	PIG-W	17p12
Transamidase reaction	PIG-X	3q29
	GPI8 (PIG-K)	1p22.2-p22.3
	GAA1 (GPAA1)	8q24.3
	PIG-S	17
	PIG-T	20q12-q13
	PIG-U	20q11