Next-Generation Sequencing Testing in Identification and Differential Diagnosis of Hereditary Anemia due to Erythrocyte Membrane Disorders, Enzymopathies and Related Disorders

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BACKGROUND

Our laboratory offers a hereditary anemia panel designed to use next generation sequencing genetic testing to help with the diagnosis of a series of anemia disorders. This panel focuses on red blood cell (RBC) membrane and enzyme disorders (PMID: 23664421, 29402830, 29803284; Table 1). Intrinsic RBC membrane disorders include membrane structural defects and membrane transport function defects. Glucose-6-phosphate dehydrogenase deficiency is the most common RBC enzymopathy. Glycolytic enzymopathies are relatively rare. This panel also comprises other RBC disorders for clinical differential diagnosis, such as hyporegenerative anemias and genetic conditions related to hyperbilirubinemia.

Table 1: Molecular characteristics, phenotype, and inheritance of fifty-one genes tested in this hereditary anemia NGS panel.

RBC defects	Genes	Disorders	Inheritance
RBC membrane structural defects	ANK1, SPTB, SPTA1, SLC4A1, EPB42	hereditary spherocytosis (HS)	AD, AR
	EPB41, SPTA1, SPTB, SLC4A1, GYPC	hereditary elliptocytosis (HE), hereditary pyropoikilocytosis (HPP)	AD, AR
RBC membrane transport function defects	PIEZO1, KCNN4	Dehydrated hereditary stomatocytosis (DHS) / Hereditary xerocytosis	AD
	RHAG	Overhydrated hereditary stomatocytosis (OHS)	AD
	ABCG8, ABCG5	Sitosterolemia	AR
	SLC2A1	Stomatin-Deficient Cryohydrocytosis with Neurologic Defects	AD
	ATP11C	Congenital hemolytic anemia	XL
Hyporegenerative anemias	CDAN1, C15orf41/CDIN1, SEC23B, KIF23, LPIN2	Congenital dyserythropoietic anemia (CDA)	AR
	RPS19, RPS24, RPL35A, RPL5, RPL11, RPS7, RPS10, RPS26	Diamond-Blackfan anemia (DBA)	AD
	ALAS2	Sideroblastic anemia	XL
	GATA1	Anemia with or without neutropenia and/or platelet abnormalities	XL
RBC enzymopathies	G6PD	Nonspherocytic hemolytic anemia due to G6PD deficiency (favism)	XLD
	PKLR	Pyruvate kinase deficiency	AR
	AK1, ALDOA, GCLC, GPI, GPX1, GSR, GSS, HK1, NT5C3A, PFKM, PGK1, TPI1	Hemolytic anemia due to enzyme deficiency	AR, XL (PGK1)
Hyperbilirubinemia	UGT1A1, UGT1A6, UGT1A7	Gilbert syndrome, Crigler-Najjar syndrome, familial transient neonatal hyperbilirubinemia	AR
	SLCO1B1, SLCO1B3	Hyperbilirubinemia, Rotor type	DR
Other	COL4A1	hereditary angiopathy with nephropathy, aneurysms, and muscle cramps	AD
	CYB5R3	Methemoglobinemia	AR
	XK	McLeod syndrome with or without chronic granulomatous disease	XL

METHODS

Fifty-one genes are included in this panel (Table 1). The exome of genomic DNA is enriched by an Agilent targeted sequence capture method. Direct sequencing of the amplified captured regions is performed on Illumina next-generation sequencing systems. Data analysis is performed using Illumina platform, internal ODIN software and NxClinical software. Genetic variants were classified according to the American College of Medical Genetics (ACMG) guidelines.

RESULTS AND DISCUSSION

A cohort of 340 reported cases of the panel are included in this study. Pathogenic and likely pathogenic (P/LP) variants were identified in approximately 35% (119/340) cases. The P/LP variants were detected in individuals as diagnostic findings, carrier status findings, and in some instances, in genes inherited as autosomal dominant or autosomal recessive, additional familial testing and clinical correlation may be needed. The most commonly identified genes with P/LP variants (more than 5 cases) are ANK1, G6PD, PIEZO1, PKLR, SEC23B, SPTA1 and SPTB. 104 cases were confirmed with molecular diagnostic findings, which indicated that disease-causing compound heterozygous, homozygous, hemizygous and/or autosomal dominant P/LP variants were identified in these cases. The molecular diagnostic rate is 31% (104/340 cases).

Cases identified with pathogenic/likely pathogenic variants (Table 2)

Hereditary spherocytosis (HS) is the most common RBC membrane disorder diagnosed by this panel testing. The majority cases are caused by P/LP variants in the ANK1, SPTB and SPTA1 genes. All P/LP ANK1 variants identified in 13 diagnosed cases are loss-of-function (LoF) variants. LoF ANK1 variants are definitively in association with autosomal dominant HS, while very limited evidence supports a recessive form of this gene-disease relationship (ClinGen Gene-Disease Validity 2021). All P/LP SPTB variants identified in 18 diagnosed cases are also LoF variants. LoF SPTB variants typically cause mild to moderate form of autosomal dominant HS; while autosomal recessive hereditary elliptocytosis and hereditary pyropoikilocytosis (HE/HPP) caused by SPTB are less commonly observed (PMID: 23664421, 29402830). Interestingly, SPTA1 variants were also detected in the three cases identified with missense P/LP SPTB variants, two of them with clinical suspicion of HS, no clinical information was provided in the third case. Thus, it is quite difficult to make a conclusion that which gene(s) contributed to the clinical manifestations in these individuals. Alpha-LELY and alpha-LEPRA polymorphic alleles in the SPTA1 gene are well known to be associated with HE/HPP or HS as in compound heterozygous with another P/LP SPTA1 variant (PMID: 8844207, 14692233). In 19 diagnosed cases of SPTA1, alpha-LELY allele was detected in 14 cases and alpha-LEPRA allele was detected in one case.

The PIEZO1 gene is in association with both autosomal dominant and autosomal recessive disorders but with different disease mechanisms. Typically, the gain-of-function PIEZO1 variants cause dominant dehydrated hereditary stomatocytosis (DHS; PMID: 28728825). Four such diagnosed cases were reported with a common pathogenic PIEZO1 in-frame duplication variant (p.Leu2495_Glu2496dup) and other two missense variants. The loss-of-function PIEZO1 variants has been suggested in association with recessive lymphatic malformation which may lead to nonimmune hydrops fetalis; however, unaffected carriers were reported to have a mild form of DHS (PMID: 26333996). There are two such cases with heterozygous LoF PIEZO1 variants identified, but no clear clinical evidence of DHS was indicated.

Table 2: Summary of the pathogenic/likely pathogenic variants identified by this panel testing.

Gene	OMIM Phenotype	Inheritance	Cases with diagnostic findings	Cases with carrier status
ALAS2	Anemia, sideroblastic, 1	XL	1	
ANK1	Spherocytosis, type 1	AD	13	
EPB41	Elliptocytosis-1	AD/AR	2	
G6PD	Hemolytic anemia, G6PD deficient (favism)	XLD	26	
KCNN4	Dehydrated hereditary stomatocytosis 2	AD	4	
PIEZO1	Dehydrated hereditary stomatocytosis 1 (DHS1); Lymphatic malformation 6 (LMPHM6)	AD (DHS1); AR (LMPHM6)	4	2
PKLR	Pyruvate kinase deficiency	AR	8	8
RHAG	Overhydrated hereditary stomatocytosis	AD	2	
RPS26	Diamond-Blackfan anemia 10	AD	1	
SEC23B	Congenital dyserythropoietic anemia	AR	2	3
SLC4A1	Spherocytosis, type 4; Ovalocytosis, Southeast Asian type; Cryohydrocytosis	AD	4	
SPTA1	Spherocytosis, type 3; Elliptocytosis-2; Pyropoikilocytosis;	AR/AD	19	
SPTB	Spherocytosis, type 2; Elliptocytosis-3	AD	18	
UGT1A1	Gilbert syndrome; Crigler-Najjar syndrome	AR	1	
ABCG8	Sitosterolemia 1	AR		1
HK1	Hemolytic anemia due to hexokinase deficiency	AR		1

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common genetic cause of chronic and acute drug-, food-, or infection-induced hemolytic anemia. The low-activity alleles of G6PD are thought to provide reduced risk for malaria. Only four P/LP G6PD variants were identified in 26 diagnosed cases. They are alleles of G6PD A- (p.[Val68Met; Asn126Asp] and p.[Leu323Pro; Asn126Asp]), G6PD Mediterranean (p.Ser188Phe), and G6PD KAIPING (p.Arg463His). The most common G6PD variant observed is G6PD A- p.[Val68Met; Asn126Asp], detected in 17 of 26 cases, and this variant is classified as pathogenic with reduced penetrance.

The most common glycolytic disorder is pyruvate kinase (PK) deficiency. The PKLR gene encodes PK isozymes in red blood cells and liver. RBC PK deficiency is the most common cause of hereditary nonspherocytic hemolytic anemia. PK deficiency is an autosomal recessive disorder, the same as most RBC enzymopathies. Amongst eight diagnosed cases, there are five cases with compound heterozygous and three cases with homozygous P/LP PKLR variants identified. While only one P/LP PKLR variant was detected in other eight cases, a second PKLR variant of uncertain significance was detected in three of these cases indicating the candidates for novel P/LP PKLR variants. The most common pathogenic PKLR variants observed are c.1529G>A(p.Arg510Gln) and c.721G>T(p.Glu241Ter).

The hereditary hyperbilirubinemias include unconjugated hyperbilirubinemia such as Gilbert syndrome and Crigler-Najjar syndrome type I and II, and conjugated hyperbilirubinemia such as Dubin-Johnson syndrome and Rotor syndrome (Digenic recessive, SLCO1B1 and SLCO1B3). The UGT1A1 c.-55_-54insAT variant, also known as A(TA)7TAA or UGT1A1*28 allele, is a polymorphic variant in the TATAA element of 5-prime promoter region of the UGT1A1 gene. This variant causes reduced enzymatic activity and is in association with Gilbert syndrome or Crigler-Najjar syndrome type II, which are mild unconjugated hyperbilirubinemia (PMID: 9621515, 15378351). Homozygous UGT1A1 c.-55_-54insAT variant was identified in one case with clinical diagnosis of Gilbert syndrome.

Cases identified with variants of uncertain significance (Table 3)

Variants of uncertain significance (VUS) were identified in approximately 61% (208) cases, including cases also reported with P/LP variants. There are 23 out of 51 panel genes with VUS reported in more than five cases. Some of these genes are in association with rare genetic conditions or with few reported variants, such as ALDOA, EPB41, GPX1, GSR, KCNN4, KIF23 and LPIN2. These identified VUS may be targets for further investigation in identifying the causative variant and/or mechanism responsible for the individual's clinical presentation.

Table 3: Summary of the variants of uncertain significance identified by this panel testing.

	Identified VUS number	Genes
Recessive gene with compound heterozygous or homozygous variants	23	ABCG5, ABCG8, CDAN1, PFKM, PKLR, SEC23B, SPTA1
Recessive gene with one heterozygous variant	94	ABCG5, ABCG8, AK1, ALDOA, CDAN1, CDIN1, CYB5R3, EPB42, GCLC, GPI, GPX1, GSR, GSS, HK1, KIF23, LPIN2, NT5C3A, PFKM, PKLR, SEC23B, TPI1, UGT1A1
Dominant gene	107	ANK1, COL4A1, KCNN4, PIEZO1, RHAG, RPL35A, RPL5, RPS26, SLC2A1, SLC4A1, SPTB
Gene with both dominant and recessive inheritance	19	EPB41, SPTA1
X-linked gene	13	ALAS2, ATP11C, G6PD, GATA1, XK

CONCLUSION

The differential diagnosis of anemia, which is commonly presented in clinic, can be challenging based on clinical features and pathological testing findings, while genetic testing can be the ultimate methodology for diagnosis.

To date, this hereditary anemia panel effectively facilitated clinicians to recognize and diagnose the genetic component of RBC disorders for proper treatment, monitoring, and supportive care.