Next-generation sequencing for the diagnosis of hereditary hemolytic anemias including pyruvate kinase deficiency: Report from a no-cost diagnostic program

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BACKGROUND

- Hereditary hemolytic anemias (HHAs) are a heterogeneous group of rare blood disorders, with clinical presentation varying from asymptomatic to severe anemia[1]
- HHAs are characterized by red blood cell destruction, some of which can be caused by enzyme deficiencies such as pyruvate kinase (PK) deficiency (as a result of mutations in the *PKLR* gene)[1]
- The wide range of natural histories and management approaches for different HHAs means there is a significant need to accurately diagnose them
- Given the rarity of PK deficiency and the overlap of clinical presentation with other HHAs, routine diagnostic techniques can be inconclusive in many patients[2]
- Next-generation sequencing (NGS) may allow for more sensitive and specific diagnosis of HHAs, including PK deficiency[3]
- A no-cost diagnostic program for patients with suspected HHA has been available since 2018 (ARUP® 2018–2020, AnemiaID®/PerkinElmer® 2020–present) to provide diagnosis using an NGS panel of anemia-associated genes

OBJECTIVE

- **To describe the results from patients tested using a no-cost diagnostic program and who were identified as carrying ≥1 reportable variant(s) in the *PKLR* gene**

METHODS

- The NGS panel used in this analysis includes ~50 genes (**Table 1**)
- Genes included in the panel encode cytoskeletal proteins and enzymes relating to HHA, including enzymopathies such as PK deficiency and similar disorders with overlapping clinical features
  - The panel covers the complete coding region, splice site junctions, and, where appropriate, deep intronic or regulatory regions
- Targeted gene capture and library construction for NGS were performed using a Whole Blood and Saliva kit (PerkinElmer®) and sequenced on Illumina® NGS systems
- Samples were sequenced using 150 base pair paired-end sequencing at target average coverage of 80×
- NGS output data were summarized descriptively for all patients

RESULTS

- Samples from 1007 patients were run by the program from July 2018 to May 2022
- 74 (7%) probands patients were identified with ≥1 reportable *PKLR* variant and included in this analysis
  - 18/74 (24%) were homozygous for *PKLR* variants (including 3 patients homozygous for large deletions), 33/74 (45%) were heterozygous for 2 *PKLR* variants, and 23/74 (31%) samples had just 1 *PKLR* variant
- From the 74 patients, 127 variants were identified
  - 91/127 (72%) *PKLR* variants were classified as likely pathogenic (LP)/pathogenic (P)
  - 36/127 (28%) *PKLR* variants were classified as variants of uncertain significance (VUSs)
- 37/74 (50%) patients had available PK enzyme levels
  - PK enzyme levels ranged from <1.1 to 8.5 U/g hemoglobin (Hb)
  - Most patients (21/37; 57%) with available PK enzyme levels had <2.0 U/g Hb
  - 3 patients had a molecular diagnosis in the PKLR gene and normal PK enzyme levels (>5.5 U/g Hb), 2 of whom had received a transfusion in the prior 2 weeks (the transfusion status of the other patient was unknown)
- Adult (≥18 years) and pediatric patients (<18 years) were distributed approximately evenly in the cohort
  - 29/74 (39%) were ≥30 years at testing, of whom 18/29 (62%) were ≥50 years

Table 1. Genes included in the NGS panel for a no-cost diagnostic program, and their associated disorders

Genes included in NGS panel	Associated disorder
ABCG5	Sitosterolemia
ABCG8	Sitosterolemia
ADA	ADA deficiency
AK1	AK1 deficiency
ALAS2	Sideroblastic anemia 1; erythropoietic protoporphyria
ALDOA	ALDOA deficiency
ANK1	Spherocytosis
ATP11C	Congenital hemolytic anemia
CDAN1	CDA type Ia
CDIN1	CDA type Ib
COL4A1	COL4A1-related disorders
CYB5R3	Methemoglobinemia type 1; methemoglobinemia type 2
EPB41	Elliptocytosis-1
EPB42	Spherocytosis type 5
G6PD	G6PD deficiency
GATA1	GATA1-related anemia and/or thrombocytopenia
GCLC	Hemolytic anemia due to GCL deficiency
GPI	Acute/chronic hemolytic anemia due to GPI deficiency
GPX1	Hemolytic anemia due to GPX deficiency
GSR	Hemolytic anemia due to GSR deficiency
GSS	Hemolytic anemia due to GSS deficiency
GYPC	Elliptocytosis; glycophorin C deficiency
HK1	Hemolytic anemia due to HK deficiency; neurodevelopmental disorder with visual defects and brain anomalies; hereditary motor and sensory neuropathy, Russe type; retinitis pigmentosa 79
KCNN4	Dehydrated hereditary stomatocytosis 2
KIF23	CDA type III
LPIN2	Majeed syndrome
NT5C3A	Hemolytic anemia due to UMPH1 deficiency
PFKM	Glycogen storage disease 7
PGK1	PGK1 deficiency
PIEZO1	Dehydrated hereditary stomatocytosis with or without pseudohyperkalemia and/or perinatal edema; lymphatic malformation 6
PKLR	Elevated ATP of erythrocytes; PK deficiency
RHAG	Overhydrated hereditary stomatocytosis; Rh-null, regulator type hemolytic anemia
RPL11	Diamond–Blackfan anemia 7
RPL35A	Diamond–Blackfan anemia 5
RPL5	Diamond–Blackfan anemia 6
RPS10	Diamond–Blackfan anemia 9
RPS19	Diamond–Blackfan anemia 1
RPS24	Diamond–Blackfan anemia 3
RPS26	Diamond–Blackfan anemia 10
RPS7	Diamond–Blackfan anemia 8
SEC23B	CDA type II
SLC2A1	SLC2A1-related disorders
SLC4A1	SLC4A1-related disorders
SLCO1B1	Hyperbilirubinemia (rotor type); Rotor syndrome
SLCO1B3	Hyperbilirubinemia (rotor type); Rotor syndrome
SPTA1	Elliptocytosis-2; spherocytosis type 2; pyropoikilocytosis
SPTB	Elliptocytosis-3; spherocytosis type 2
TPI1	Hemolytic anemia due to TPI1 deficiency
UGT1A1	Crigler–Najjar syndrome 1 & 2; hyperbilirubinemia (unconjugated); Gilbert syndrome
XK	McLeod syndrome with or without chronic granulomatous disease

Genes included in the ARUP® NGS panel only
Genes included in the PerkinElmer® NGS panel only
Genes included in both the ARUP® and PerkinElmer® NGS panels

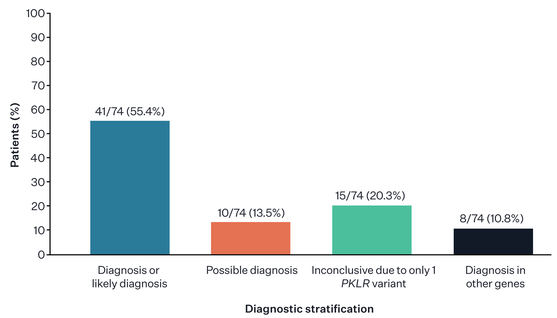
ADA, adenosine deaminase; AK1, adenylate kinase 1; ALDOA, aldolase A; CDA, congenital dyserythropoietic anemia; COL4A1, collagen, type IV, alpha 1; G6PD, glucose-6-phosphate dehydrogenase; GCL, gamma-glutamylcysteine synthetase; GPI, glucose phosphate isomerase; GPX, glutathione peroxidase; GSR, glutathione reductase; GSS, glutathione synthetase; HHA, hereditary hemolytic anemia; HK, hexokinase; NGS, next-generation sequencing; PGK1, phosphoglycerate kinase 1; PK, pyruvate kinase; SLC2A1, solute carrier family 2 member 1; SLC4A1, solute carrier family 4 member 1; TPI1, triosephosphate isomerase; UMPH1, uridine 5-prime monophosphate 1

Diagnostic stratification based on genetic testing results

- Of the 74 probands patients, 41 patients had a diagnostic or likely diagnostic result in the *PKLR* gene (**Figure 1**)
  - 25 patients were homozygous or compound heterozygous for LP/P variants, including 3 patients homozygous for a large deletion
  - 13 patients had 2 LP/P *PKLR* variants of unknown phase
  - 3 patients were compound heterozygous for an LP/P variant and a VUS
- Possible diagnostic *PKLR* findings were identified in 10 patients
  - 4 patients were homozygous or compound heterozygous for *PKLR* VUS variants
  - 6 patients had LP/P sequence variants and a VUS with phase unknown
- There were 15 patients whose diagnosis was inconclusive because only 1 *PKLR* variant was identified
- 8 patients' diagnosis was identified in other genes; these genes were *SPTA1* (n=2), *UGT1A1* (n=2), *G6PD*, *ANK1*, *EPB41*, *PIEZO1*,[a] and *SLC4A1*[a] (all n=1)

[a]Dual diagnosis in *PIEZO1* and *SLC4A1* was found in the same patient

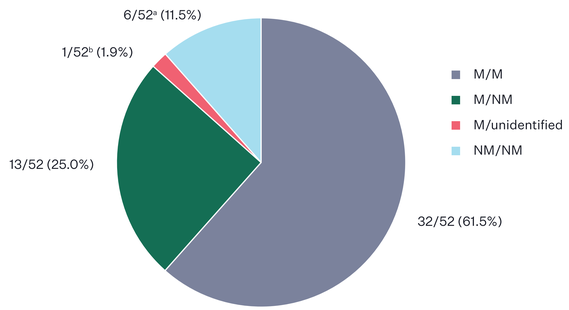
Figure 1. Diagnostic stratification based on genetic testing results of patients with ≥1 reportable *PKLR* mutation



Diagnostic outcome from combined genetic and clinical testing results

- Of the 25 patients with a possible or inconclusive diagnosis in *PKLR* based on genetic testing results, 11 patients had clinical or biochemical data supporting a diagnosis of PK deficiency (ie, were likely to have PK deficiency)
- These 11 patients, in addition to the 41 patients with a diagnosis in *PKLR* based on genetic testing, resulted in a total of 52 patients who were likely to have PK deficiency
  - The 52 patients were classified as missense/missense (M/M), missense/non-missense (M/NM), non-missense/non-missense (NM/NM), or missense/variant unknown (M/unidentified, for which a second *PKLR* variant was not found)
  - All 22 other patients with just 1 *PKLR* variant had either another molecular diagnosis (n=8) or there was insufficient clinical evidence to consider them as patients with PK deficiency (n=14)
- Of the 52 patients likely to have PK deficiency (**Figure 2**):
  - 32/52 (62%) patients had an M/M genotype
  - 13/52 (25%) patients had an M/NM genotype
  - 1/52 (2%) patient had only 1 missense variant identified and 1 unknown variant, but based on *PKLR* enzyme data (the patient had a PK enzyme level of 2.6 U/g Hb, below the normal range) they were considered by clinicians as likely to have PK deficiency (M/unidentified genotype)
  - 6/52 (12%) patients had an NM/NM genotype, including 3 homozygous for multi-exon deletions, 2 with other loss-of-function variants (truncating or splice), and 1 with 2 amino acid deletions as well as the insertion of 3 amino acids

Figure 2. Classification of *PKLR* variants in patients likely to have PK deficiency



[a]Includes 3 patients with homozygous multi-exon deletions, 2 patients with loss-of function truncating or splice variants, and 1 patient with 2 amino acid deletions as well as the insertion of 3 amino acids; [b]M/unidentified represents patients with 1 *PKLR* variant reported, but with PK enzyme data supporting a clinical diagnosis of PK deficiency
M/M, missense/missense; M/NM, missense/non-missense; M/unidentified, missense/variant unknown; NM/NM, non-missense/non-missense; PK, pyruvate kinase

SUMMARY

- **NGS is an effective platform for the diagnosis of PK deficiency**
- **Most pathogenic variants identified were small sequence variants, but large deletions in *PKLR* are not uncommon and should be part of the NGS panel**
- **Approximately 39% of the patients tested were ≥30 years of age, some of whom were without a previous diagnosis, emphasizing the complexity of a PK deficiency diagnosis in patients with lifelong anemia**
- **NGS, through this no-cost diagnostic testing program, may assist in making a genetic diagnosis, particularly in situations when PK enzyme levels are inconclusive or potentially confounded (eg, patients receiving frequent transfusions)**

With the recent advances in drug development and the approval of a new therapy for patients with PK deficiency, improved diagnosis may increase awareness of the disease and allow for appropriate treatment and counseling for patients with this condition

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