Genotype-Phenotype Correlation and Molecular Heterogeneity in Pyruvate Kinase Deficiency: Data from the PKD Natural History Study

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Background

- ✓ Pyruvate kinase (PK) deficiency is the most common enzyme defect of the glycolytic pathway causing hereditary non-spherocytic hemolytic anemia.
- ✓ PK deficiency is autosomal recessive, caused by both homozygous and compound heterozygous mutations in the *PKLR* gene with >300 mutations reported.

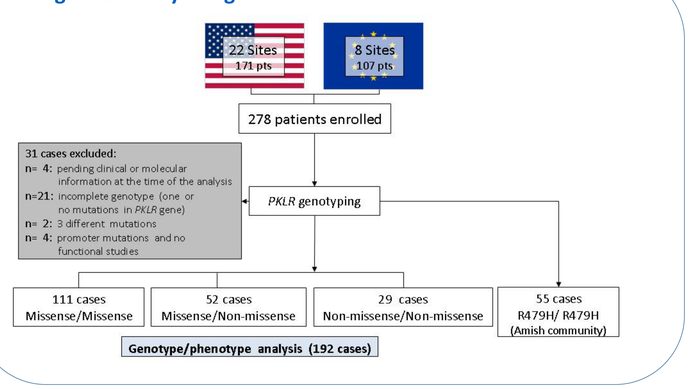
Aim

- ✓ To identify *PKLR* genotypes and determine the genotype-phenotype correlation in patients (pts) with PK deficiency enrolled in the PKD Natural History Study (NHS).

Patients and Methods

- ✓ 278 pts were enrolled on the PKD NHS, a retrospective and prospective international study, at 30 sites in North America and Europe. Molecular testing confirmed two *PKLR* mutations in 255 pts (Figure 1).
- ✓ In patients without prior *PKLR* gene sequencing results, the *PKLR* gene was analyzed by Sanger sequencing.
- ✓ To evaluate genotype-phenotype associations (Fisher's exact or Kruskal-Wallis test), pts were grouped according to genotype:
  - **M/M** : two missense mutations; 111 pts (58%)
  - **M/NM** : one missense/one non-missense; 52 pts (27%)
  - **NM/NM :** two non-missense mutations ; 29 pts (15%)
- ✓ Patients with three pathogenic variants or promoter mutations (n=6) were excluded from the genotype-phenotype analysis, and 2 pts were excluded for insufficient phenotype data.
- ✓ Patients from the Amish community (n=55, homozygous for the splicing mutation R479H) were analyzed separately.
- ✓ The Holm-Bonferroni method was used to adjust for multiple testing (significance level p<0.0055).

Figure 1. Study design



Results (I)- Molecular characterization

- ✓ 123 different mutations were identified: 79 missense, 36 non-missense mutations (12 splicing, 13 frameshift, 7 stop codons, and 4 large deletions), 5 inframe indel, and 3 promoter variants (Figure 2). Most pts had at least one missense mutation (85%).
- ✓ Fifty mutations, affecting PK structural domains, have not been reported previously (31 missense, 3 stop codon, 7 frameshift, 3 inframe indel, 5 splicing mutation, 1 promoter mutation); 22% of the patients (56/255) carried at least one new molecular variant (Table 1).
- ✓ The new missense mutations affected conserved residues in multiple domains of the *PKLR* gene, were not detected in 1000 Genomes and HGMD databases, and were considered pathogenic by different mutation algorithms.

Figure 2. Types of mutations

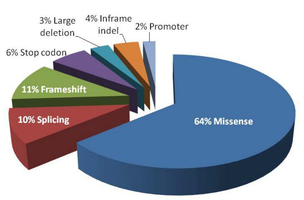


Table 1. Novel mutations

Missense	Effect	*Pred. SNP	Missense	Effect	*Pred SNP	Nonsense	Effect
c.92C>T	p. Ala31Val	51%	c.977 T>A	p. Ile326Asn	87%	c.1299C>A	p.Tyr433X
c.143C>A	p.Thr48Asn	74%	c.1015G>T	p. Asp339Tyr	87%	c.1357G>T	p.Glu453X
c.187G>A	p.Ala63Thr	76%	c.1076 G>C	p.Arg359Pro	87%	c.1574G>A	p. Trp525X
c.194T>C	p.Met65Thr	75%	c. 1097C>T	p.Pro366Leu	76%	**Frameshift**	**Effect**
c.353A>G	p.Asn118Ser	87%	c.1156 G>A	p.Ala386Thr	87%	c.119 del G	p.Arg41GlyfsX7
c.397A>G	p.Asn133Asp	63%	c.1241C>G	p.Pro414Arg	87%	c.439 delA	p.Ser147Ala fsX32
c.410 C>T	p. Ala137Val	68%	c.1388C>T	p. Ala463Val	63%	c.639-644insG	p.Gly215GlyfsX5
c.457 A>T	p Ile153Phe	68%	c.1487 T>G	p.Val496Gly	72%	c.1008 ins A	p.Arg337Thr fsX64
c.467 A>G	p.Asp156Gly	61%	c.1495T>C	p.Ser499Pro	65%	c.1542-1543insC	p.Leu516Ala fs4
c.664G>A	p.Gly222Arg	87%	c.1504G>C	p.Ala502Pro	61%	c.1573delT	p.Trp525GlyfsX5
c.665 G>A	p.Gly222Glu	87%	c.1510 C>T	p.Arg504Cys	87%	c.909gelGGAAGGAC	p.Glu304ArgfsX9
c.762 G>T	p. Leu254Phe	87%	c.1513C>G	p.Gln505Glu	65%	**Splicing**	**Effect**
c.764C>T	p.Pro255Leu	76%	c.1531 G>A	p.Gly511Arg	87%	c. 375(+1) g>a	Splicing
c.787G>C	p.Gly263Arg	76%	c.1600C>A	p. Gln534Lys	83%	c.965(+2)t>c	Splicing
c.804C>A	p. Asp268Glu	72%	c.1654G>T	p.Val552Leu	75%	c.965(+1)g>a	Splicing
c.878A>T	p.Ap293Val	87%	c.-70A>C	promoter	n.a.	c.966-9A>G	Splicing
Inframe Indel			**Effect**			c.1618+2t>c	Splicing
c1351_1356dupCCCACT			pThr452_glu453ProThr				
c.1553_1558delGTGAAC			p. Arg518-Glu519 del				
c.1137-1139delCAA			p.Lys380del				

*Prediction of the effects of mutations on protein function by PredictSNP1 analysis (Bendl et. al, 2014); % of expected accuracy. red: likely deleterious; green: likely benign.

Results (II)- Genotype-phenotype correlation

- **Age at Diagnosis.** There is a trend for pts with more severe mutations to be diagnosed at a younger age (p=0.049).
- **Splenectomy.** The NM/NM group had the highest rate of splenectomy (72%) vs. 50% in the M/NM and 44% in the M/M group (p=0.024). In pts splenectomized and not on regular transfusions, the Hb levels were significantly different between the groups (p=0.003).
- **Transfusion status.** There were significant differences in the transfusion status; 86% of the NM/NM group were previously or currently regularly transfused, versus 50% of the M/NM and 42% of the M/M group (p<0.0001). There were also significant differences in the total number of transfusions (p=0.0013); 96% in the NM/NM group received at least one lifetime transfusion compared to 81% and 75% of the M/NM and M/M groups, respectively.
- **Iron status.** The NM/NM group had the highest maximum ferritin (p<0.0001) and was more likely to have iron overload, defined as ferritin >1000 ng/ml or having received chelation in the year prior to enrollment (p=0.0013). However, iron overload was not uncommon in the M/M group (43%).
- **Enzyme activity.** There was no association of PK enzyme activity with the genotype.

Table 2: Genotype-Phenotype association in 192 patients with PK deficiency

	NM/NM, N=29 Median (Range)	M/NM, N=52 Median (Range)	M/M, N=111 Median (Range)	p-value[+]
Age at diagnosis (years)	0.4 (0-10.9) n=29	0.7 (0-42.3) n=50	1.3 (0-60.3) n=105	0.049
Hemoglobin (g/dl)**	7.9 (6.5-8.9) n=14	8.4 (6.4-12.8) n=21	9.2 (4.3-12.3) n=40	0.003*
Total number of lifetime transfusions	65 (3-991) n=27	25 (1-721) n=38	16 (1-1915) n=81	0.0013*
Maximum ferritin (ng/ml)	1787 (423-13,409) n=22	604 (22-8,220) n=37	573 (31-9,679) n=75	<0.0001*
PK enzyme activity normalized to patient-specific normal range (%)	-41.6 (-152.4-15.2) n=18	-51.9 (-211.1-64.4) n=24	-69.6 (-485.7-117.6) n=60	0.16
	n (%)	**n (%)**	**n (%)**	**p-value[++]**
Prenatal complications	11/27 (41)	15/48 (31)	28/98 (29)	0.47
Exchange transfusion	9/20 (45)	17/41 (41)	34/78 (44)	0.97
Rate of splenectomy	21/29 (72)	26/52 (50)	49/111 (44)	0.024
Rate of cholecystectomy	13/29 (45)	25/52 (48)	45/111 (41)	0.63
Extramedullary hematopoiesis	3/25 (12)	0/43 (0)	5/92 (5)	0.067
Iron overload***	21/25 (84)	20/38 (53)	33/77 (43)	0.0013*
Transfusions during pregnancy	3/3 (100)	3/4 (75)	8/12 (67)	0.77
Transfusion and splenectomy status******				0.002*[+++]
Currently on regular transfusions w/ SPL	7/29 (24)	4/52 (8)	8/111 (7)	
Currently on regular transfusion w/o SPL	6/29 (21)	7/52 (13)	13/111 (12)	
Historically on regular transfusion w/ SPL	12/29 (41)	11/52 (21)	23/111 (21)	
Historically on regular transfusion w/o SPL	0/29 (0)	1/52 (8)	2/111 (2)	
Occasionally transfused	3/29 (10)	16/52 (31)	37/111 (33)	
Never transfused	1/29 (4)	10/52 (19)	28/111 (25)	

SPL= splenectomy * statistically significant at p< 0.0055 [+]Kruskall –Wallis test; [++]Fisher's exact test; [+++] Monte Carlo estimation of Fisher's exact test. ** Hemoglobin in the splenectomized, not regularly transfused cohort. *** iron overload defined by maximum ferritin >1000ng/ml OR received chelation in prior 12 months **** Regular transfusions were defined as ≥6 transfusions within 12 months. Currently is defined as the 12 months prior the enrollment. Historically is defined as >1 year prior the enrollment

Results (III)- Analysis of the Amish cohort

- ✓ The Amish group has the homozygous missense mutation, R479H/R479H, causing abnormal splicing.
- ✓ Compared to the other genotypes, the rate of splenectomy was highest in this group, 93%, whereas the rate of cholecystectomy was the lowest, 27%.
- ✓ In this cohort, 67% had previously been on regular transfusions, but 18% had never been transfused.
- ✓ The maximum ferritin level was 615 ng/ml (range 126-3258) with 33% having iron overload.
- ✓ The median Hb of splenectomized and not regularly transfused pts was 9.4 g/dl (range: 8-11.2), similar to the M/M group.

Conclusions

- ✓ Genotype-phenotype associations were observed in a large international cohort of pts with PK deficiency.
- ✓ Pursuing molecular testing may be useful to discuss prognosis and to establish a monitoring plan in pts based on genotyping results.
- ✓ Fifty new mutations were identified, thus confirming the wide heterogeneity of the molecular genotype and diagnostic complexities in PK deficiency.

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