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ORIGINAL PAPER

Red Cells and Iron

Biallelic hexokinase 1 (*HK1*) variants causative of non-spherocytic haemolytic anaemia: A case series with emphasis on the *HK1* promoter variant and literature review

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Summary

The hexokinase (HK) enzyme plays a key role in red blood cell energy production. Hereditary non-spherocytic haemolytic anaemia (HNSHA) caused by HK deficiency is a rare disorder with only 12 different disease-associated variants identified. Here, we describe the clinical features and genotypes of four previously unreported patients with hexokinase 1 (HK1)-related HNSHA, yielding two novel truncating *HK1* variants. The patients' phenotypes varied from mild chronic haemolytic anaemia to severe infantile-onset transfusion-dependent anaemia. Three of the patients had mild haemolytic disease caused by the common *HK1* promoter c.-193A>G variant combined with an intragenic *HK1* variant, emphasizing the importance of including this promoter variant in the haemolytic disease gene panels. HK activity was normal in a severely affected patient with a homozygous *HK1* c.2599C>T, p.(His867Tyr) variant, but the affinity for ATP was reduced, hampering the HK function. In cases of HNSHA, kinetic studies should be considered in the functional studies of HK. We reviewed the literature of previously published patients to provide better insight into this rare disease and add to the understanding of genotype–phenotype correlation.

KEYWORDS
anaemia, haemolysis, hexokinase deficiency, *HK1*, promoter variant

INTRODUCTION

Here, we present four patients with hereditary non-spherocytic haemolytic anaemia (HNSHA) caused by a defect in the hexokinase (HK) enzyme. This is the first enzyme in the glycolytic pathway and plays a critical role in the regulation of glucose consumption, as 90% of glucose is metabolized through glycolysis.

There are four HK enzymes encoded by four different genes (*HK1, HK2, HK3* and *HK4*) with different tissue-specific

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expression patterns. Red blood cells (RBCs) contain two HK isoenzymes, the ubiquitously expressed hexokinase 1 (HK-1) and the RBC hexokinase called HK-R.[1,2] Both isoforms are encoded by *HK1*, but transcription of HK-R is initiated from an erythroid-specific promoter and the transcript includes an erythroid-specific exon (exon R).[3,4] HK-R has an important role in immature RBCs (i.e. erythroblasts, reticulocytes and young erythrocytes), but the activity declines in aging RBCs.[2]

Since 1967, approximately 35 patients with recessively inherited HK deficiency have been reported.[1,3,5–13] Less than half have been characterized at the molecular level, with a total of 12 different disease-associated variants identified.[1,5–11,13]

HK deficiency leads to chronic non-spherocytic haemolytic anaemia, ranging from mild to severe.[1] The mechanism of haemolysis is incompletely understood. It is thought that reduced ATP levels hamper RBC properties and function, eventually causing premature RBC destruction in the spleen (extra-vascular haemolysis).[14]

In this paper, we describe four previously unreported patients and review earlier published patient data with *HK1*-related HNSHA.

MATERIALS AND METHODS

Patient recruitment and clinical evaluation

The patients were recruited from the departments of clinical genetics at Oulu University Hospital (Patients 1–3) and Helsinki University Hospital (Patient 4). Clinical information was collected by a retrospective review of medical records, family history, and genetic and other laboratory results.

Genetic testing and data analysis

Peripheral blood samples were collected, and genomic DNA was extracted using routine methodologies in accredited laboratories. Exome-based next-generation sequencing (NGS) gene panels (Patients 1–2) or whole exome sequencing (Patient 3) were performed by Blueprint Genetics Laboratory, Espoo, Finland (Comprehensive Haematology Panel; version 5, updated on Oct 30, 2021). Patient 4's sample was analysed by Fulgent Genetics (Temple City, California, USA) using a customized NGS gene panel. The genes included in the two NGS gene panels are listed in Table S1. As the customized gene panel did not include the erythroid-specific promoter region of *HK1*, further Sanger sequencing was requested to check for the presence of a known promoter region variant in *HK1* (i.e. NM_033496.2:c.-193A>G, also known as rs187500777).

Hexokinase enzyme kinetics

Hexokinase enzyme activities (Vmax) and kinetic measurements (i.e. affinity for ATP and glucose) were performed in University Medical Centre Utrecht in the Netherlands as described in an article by Koralkova et al.[1] HK activity assays comprises the measurement of HK activity under optimal (Vmax) conditions of substrate, whereas affinity for ATP and glucose comprises a series of measurements at varying concentrations of ATP and glucose. The assay is performed on purified RBCs, depleted from WBC and PLT, which express their own HK. Kinetic measurements were performed on the patient-specific young red blood cell/reticulocyte fraction obtained from whole blood by density separation.[15]

RESULTS

Clinical description of patients

The clinical details of the patients are shown in Table 1 and more comprehensive clinical description in Supplementary Data.

Patient 1, the daughter of Patient 2, has severe infantile-onset transfusion-dependent haemolytic anaemia (Figure 1). She was referred to Oulu University Hospital at 2.5 months of age. After birth, she needed nasal oxygen therapy and was considered having had a fetomaternal haemorrhage because of low haemoglobin and reticulocytosis. However, fetal haemoglobin (HbF) measured from the mother was low. During follow-up, her Hb levels were between 6.7 and 8.7 g/dL (ref 10.0–13.6 g/dL), reticulocytes 22–68 × 10E9/L (ref 30–108 × 10E9/L) and she needed regular red blood cell (RBC) transfusions. In-depth diagnostic studies yielded negative results. During close follow-up, hyperbilirubinaemia and reticulocytosis slowly increased. Now, at the age of 2 years, she is on iron chelation because of iron overload in the liver and heart. She needs RBC transfusions every 2–3 weeks and she is planned to proceed to allogeneic haematopoietic stem cell transplantation.

Patient 2, who is the father of Patient 1, is a 31-year-old male, who was diagnosed with haemolytic anaemia causing icterus at the age of 3. His laboratory investigations showed mild haemolytic anaemia and splenomegaly, which slowly progressed. Splenectomy was performed at the age of 23 years. The operated spleen was 30 × 18 cm in diameter. Histopathology showed a normal white pulp of the operated spleen. There was a diffuse marked hyperplasia of the red pulp and the blood vessels in the red pulp were engorged with RBCs. After splenectomy, his laboratory investigations showed repeatedly normal haemoglobin.

Patient 3 is a 37-year-old male, with no family history of haemolytic anaemia. At the age of 11, he presented with abdominal pain and icterus; laboratory tests showed underlying haemolytic anaemia. Abdominal ultrasound revealed gallstones, and the spleen was relatively large at 11.2 cm (normal range for this age is 8.6–10.9 cm). Cholecystectomy was performed at 19 years of age. At the age of 37 years, an enlarged spleen at 20.9 cm × 13.8 cm × 9.2 cm and chronic haemolytic anaemia with thrombocytopenia were observed. A splenectomy has been recommended, but the patient has refused the operation.

Patient 4, a 26-year-old male, was diagnosed with an uncomplicated gallstone disease, with well-compensated

TABLE 1 Clinical and laboratory results of the patients presented in this study.

	Patient 1 at diagnosis	Patient 2 before splenectomy	Patient 2 after splenectomy	Patient 3	Patient 4[a]
Age and gender	0.2 y, female	23 y, male	31 y, male	37 y, male	26 y, male
Haemoglobin (g/dL) (children 2 m–1 y 10.0–13.6; adult males 12.4–16.7)	6.7	12.1	15.9	11.9	12.6 (ref 13.4–16.7)
RBC ($\times 10^{12}$/L) (3.8–5.5)	2.2	3.6	4.9	3.5	3.6 (ref 4.25–5.7E12/l)
Reticulocytes % (0.6–2)	7.3	4.6	0.7	3.9	1.6 (ref 0.7%–2.3%)
Bilirubin (μmol/L) (newborn 0–250; >30d < 25)	33	103	14	175	59 (ref <20)
Ferritin (μg/L) (22–322)	1055	77	N/A	131	379 (ref 20–195)
Haptoglobin (g/L) (0.29–2)		<0.10	0.70	<0.01	<0.01
Lactate dehydrogenase (U/L) (105–205)	238	471	184	1151	228 (ref 115–235)
Hexokinase (0.8–1.5 U/gHb)	1.1	N/A	0.8	0.52	N/A
Pyruvate kinase (6.1–12.3 U/g Hb)	9.3	N/A	10	7.17	18.9 μmol/(min.gram Hb) (ref 21.6–40.4)
HK1 kinetic studies; K_MATP [0.846 ± 0.074mMATP (mean ± SD)]	>2.5	N/A	1.333	0.85	N/A
HK1 kinetic studies; K_MGluc [0.074 ± 0.012mMGluc (mean ± SD)]	0.091	N/A	0.075	0.057	N/A
First symptoms (age)	Haemolytic anaemia at birth	Haemolytic anaemia, icterus (3 y)		Haemolytic anaemia, hyperbilirubinaemia, cholelithiasis (11 y)	Cholelithiasis, hyperbilirubinaemia
Transferrin saturation (0.15–0.45)		0.39		0.18	60% (ref 17%–52%)
Splenomegaly	No	Yes		Yes	No
Splenectomy	No	Yes (23 y)		No	No
Iron accumulation (MRI)	Yes (Liver/Heart)	N/A		Yes (Kidneys)	Yes (Liver)
Transfusions	Yes	No		No	No
HK1 variant 1 (NM_033496.2)	c.2599C>T, p.(H867Y)	c.2599C>T, p.(H867Y)		c.2361_2362del, p.(Q788Dfs*4)	c.372+1G>A
HK1 variant 2 (NM_033496.2)	c.2599C>T, p.(H867Y)	c.-193A>G		c.-193A>G	c.-193A>G

Abbreviations: MRI, magnetic resonance imaging; N/A, not available; RBC, red blood cell count; y, year.

[a]The reference values of Patient 4 differ from those of Patients 1–3 because Patient 4 was treated in a different hospital.

non-immunological haemolytic disease at the age of 19. He had suffered from uncomplicated neonatal icterus and a transient icterus following viral gastroenteritis, as a toddler. Hereditary cause of haemolysis was suspected. Pyruvate kinase (PK) activity was slightly lowered, but no pathogenic variants in *PKLR* were found. At the age of 25 years, his ferritin was 379 μg/L (ref 20–195 μg/L) and he had a mild iron overload in his liver. *HFE* gene test was negative. His blood picture has remained stable, and chelation therapy has been started.

Genetic results

Genetic testing results are shown in Table 1 and Figure 1 with nomenclature based on the erythroid-specific HK1 transcript (NM_033496.2). Patient 1, daughter of Patient 2, is homozygous for the *HK1* variant c.2599C>T, p.(His867Tyr). Her healthy mother is a heterozygous carrier of this variant. Her mildly affected father, Patient 2, is compound heterozygous for the *HK1* variant c.2599C>T, p.(His867Tyr) and c.-193A>G. Patient 3 is heterozygous for the *HK1* variants c.2361_2362del, p.(Gln788Aspfs*4) and c.-193A>G. His father carries neither of the variants and his mother is a healthy carrier of the *HK1* c.-193A>G heterozygous variant. Patient 4 is compound heterozygous for the *HK1* variants c.372+1G>A and c.-193A>G. His mother and healthy brother are heterozygous carriers of the *HK1* variant c.-193A>G. His father is a carrier of the *HK1* variant c.372+1G>A. Table 2 shows the details of the identified HK1 variants, including their nomenclature based on the MANE Select transcript (NM_000188.3) and variant classification.

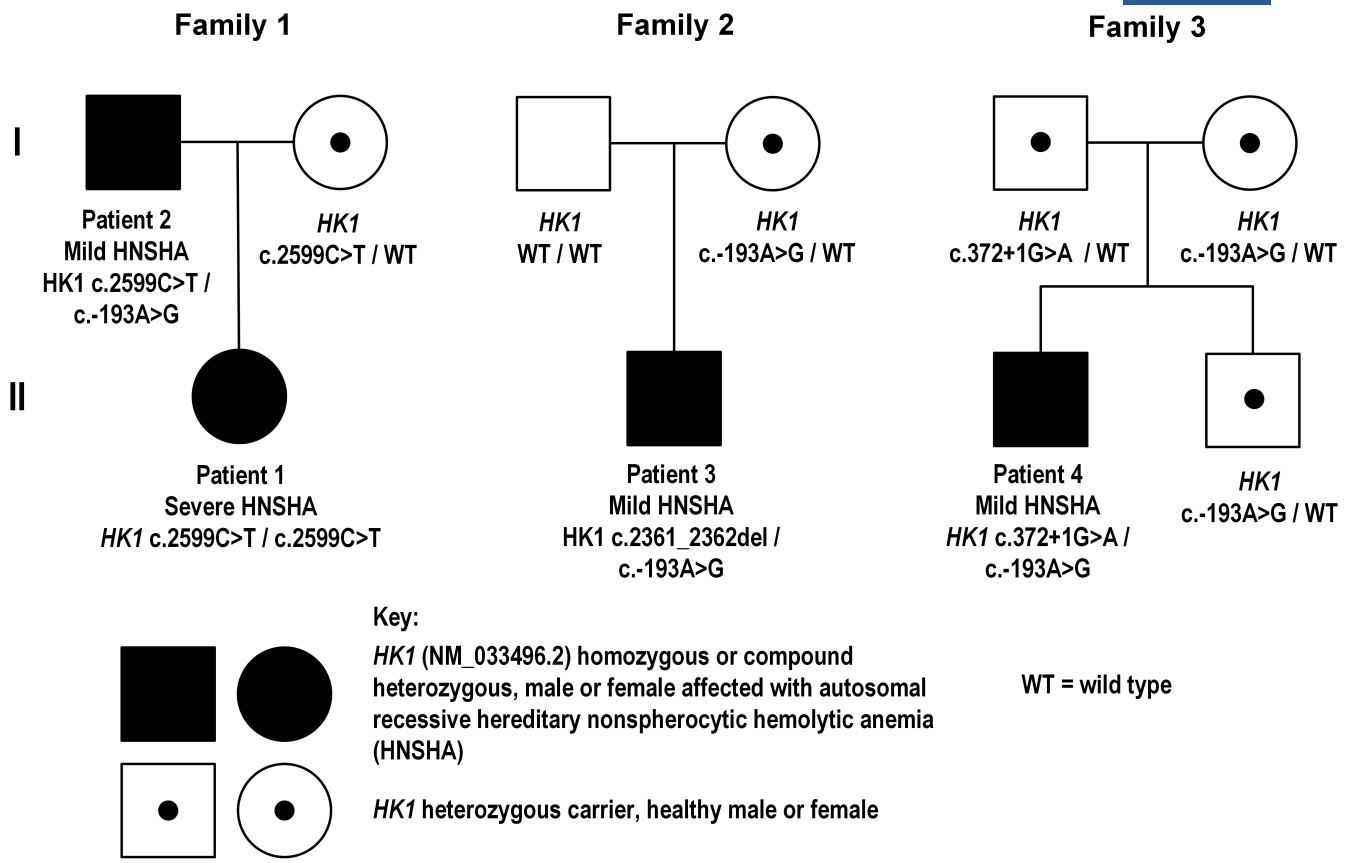

FIGURE 1 Pedigrees of the Families 1–3. Patient 1, daughter of Patient 2, has severe transfusion-dependent HNSHA and genetic testing showed that she has inherited the same *HK1* missense variant c.2599C>T, p.(His867Tyr) from both parents. Patients 2–4 all have mild HNSHA, and genetic testing identified two heterozygous *HK1* variants: the *HK1* promoter variant (c.-193A>G) in combination with an intragenic *HK1* variant. Patient 3 (Family 2) has inherited the *HK1* c.-193A>G promoter variant from his mother, *HK1* c.2361_2362del variant is likely a result of a de novo event, although germline mosaicism or non-paternity cannot be ruled out. *HK1* reference sequence NM_033496.2.

HK activity and kinetic testing results

The results of HK activity and kinetic studies are shown in Table 1. The HK activity was normal in the three patients investigated. Patients 2 and 3 had low HK activities, but no significant differences between patient/parents and healthy shipment and local controls were identified in the HK activity measurements. The HK activity was compared to PK activity to correct for reticulocytosis, but the PK:HK ratio was also normal.

HK kinetic studies of Patient 1 showed a strongly reduced affinity for ATP and a normal affinity for glucose in the patient-specific reticulocyte fraction (Supplementary Figure in Data S1). The reduced affinity for ATP severely hampers HK function and further confirms the pathogenic nature of the *HK1* c.2599C>T, p.(His867Tyr) variant. HK kinetic testing of Patient 2 showed that K_M value for ATP (K_MATP) was slightly increased, suggesting mildly reduced affinity for ATP, but there were no marked differences between the patient and control samples. HK kinetic testing of Patient 3 showed no differences between the patient and control samples.

Literature review

Published cases of hereditary HNSHA, where biallelic disease-associated *HK1* variants have been identified, are summarized in Table 3.

DISCUSSION

In this study, we describe four previously unreported patients with *HK1*-related non-spherocytic anaemia. The phenotypes of the patients varied from severe infantile-onset transfusion-dependent anaemia to mild chronic haemolytic anaemia. We report two novel truncating *HK1* variants and emphasize the importance of analysing a common promoter region variant to increase the diagnostic yield in patients with milder forms of haemolysis.

Interestingly, Patients 2–4, with a mild phenotype, were heterozygous for the *HK1* c.-193A>G promoter variant in *trans* with a truncating variant or a known pathogenic missense, suggesting that *HK1* c.-193A>G is a hypomorphic variant associated with a mild phenotype. The *HK1* c.-193A>G

TABLE 2 Disease-associated *HK1* variants identified in this study according to the erythroid-specific *HK1* transcript (NM_033496.2) and the canonical transcript (NM_000188.3)/MANE Select transcript (NM_000188.3). In addition, variants' location in the gene, variant identification (ID) number and genomic co-ordinates are shown. Variant classification based on the ACMG criteria and GnomAD frequencies is also shown.

***HK1* variants identified in this study**	**Variant 1**	**Variant 2**	**Variant 3**	**Variant 4**
NM_033496.2	c.2599C>T, p.(His867Tyr)	c.-193A>G	c.2361_2362del, p.(Gln788Aspfs*4)	c.372+1G>A
NM_000188.3	c.2602C>T, p.(His868Tyr)	c.-3186A>G	c.2364_2365del, p.(Gln789Aspfs*4)	c.375+1G>A
Location in the gene	exon 17 of 18	promoter	exon 16 of 18	intron 3 of 17
ID number	rs1461475795	rs187500777	NA	NA
Genomic co-ordinates (GRCh38)	g.10:69398821C>T	g.10:69315762A>G	g.10:69395088delTC	g.10:69360046G>A
Variant classification (ACMG)	Likely pathogenic	N/A (hypomorphic variant)	Likely pathogenic	Likely pathogenic
GnomAD (v.4.0.0) allele frequencies	1.373e-6	0.002034	6.841e-7	1.591e-6

Abbreviations: ACMG, American College of Medical Genetics and Genomics; GnomAD v.4.0.0, Genome Aggregation Database, version 4.0.0, https://gnomad.broadinstitute.org/; N/A, not available.

promoter variant is enriched in the Finnish population; its allele frequency is ~4 times higher in Finns (0.011) than in non-Finnish Europeans (0.002985) in the gnomAD v.2.1.1.[16]

The *HK1* variant c.2361_2362del, p.(Gln788Aspfs*4) has not been reported in the medical literature before, but the deletion generates a frameshift leading to a premature stop codon. As the stop codon appears at the very beginning of exon 17 (out of 18 exons), nonsense-mediated decay most likely takes place, but production of a truncated protein remains an alternative option. The novel splice site variant c.372+1G>A disrupts the canonical splice donor site of exon 3 and presumably results in exon skipping and disruption of the reading frame. However, the diagnostic laboratory initially reported the splice site variant as a variant of uncertain significance because of the inadequate number of reported patients with *HK1* loss-of-function variants in the literature. Our report now reinforces the association of loss-of-function *HK1* variants with recessively inherited haemolytic anaemia/haemolysis and should facilitate gene-disease validity curations and proper classification of *HK1* variants in the future.

Patient 1, with a severe haemolytic anaemia, was homozygous for the *HK1* c.2599C>T, p.(His867Tyr) variant. Her HK activity was normal, but the HK kinetic studies showed strongly reduced affinity for ATP. This finding confirms the pathogenic nature of p.(His867Tyr) and the genetic diagnosis. Koralkova et al. described a patient with the same homozygous *HK1* c.2599C>T, p.(His867Tyr) variant and a normal hexokinase activity.[1] Our findings are in line with the previous report on a patient with the same genetic diagnosis, transfusion-dependent haemolytic anaemia and decreased hexokinase ATP affinity.[1] The HK activity is not always decreased under the Vmax conditions (i.e. high substrate levels) as commonly used in the diagnostic enzymatic assay, even if the enzymatic function on the protein level is impaired.

The *HK1* promoter variant (c.-193A>G) has previously been described by de Vooght et al. and Koralkova et al.[1,6] Except for one child with severe transfusion-dependent haemolytic anaemia, these patients had mild (compensated) haemolysis/haemolytic anaemia (Table 3). Based on previous functional studies with promoter reporter constructs, the c.-193A>G *HK1* variant leads to strongly reduced promoter activity, resulting in a reduced expression of HK-R (8% compared to the wild type), but normal expression of HK-1.[6] In our study, Patients 2–4 have the common c.-193A>G *HK1* promoter variant and another intragenic variant. Patients 2 and 3 had mild but not significant changes in hexokinase activities/kinetics, suggesting that the *HK1* c.-193A>G low expression allele is likely able to produce enough HK to maintain sufficient HK activity levels in the RBC populations analysed. This result is significant in the clinical practice, as the diagnosis of *HK1*-related HNSHA should not be excluded based on normal hexokinase activity measurements without proper genetic testing, including the erythroid specific promotor region of *HK1*.

The American College of Medical Genetics and Genomics (ACMG) criteria are not well suited for the classification of hypomorphic variants.[17] However, the pathogenicity of the promoter region variant is supported by functional data (promoter activity decreased to 8%)[6] and publication of the variant in combination with altogether five different *HK1* variants in six patients with haemolysis including three previously published patients[1,6] and three patients presented in this publication (Table 3). ClinVar also lists the variant as likely pathogenic in one affected patient (analysed by GeneDx, Gaithersburg, Maryland, USA).

Gene panels and WES platforms do not routinely analyse promoter regions. However, some laboratories have included clinically relevant promoter region variants in their regions of interest. This explains why the promoter region variant was easily detected in the genetic analysis of Patients 2 and 3. In contrast, the promoter region variant would have been missed in the analysis of Patient 4 unless specifically requested and analysed by Sanger sequencing. Our findings demonstrate how crucial it is to analyse all clinically

TABLE 3 Known variants of the *HK1* gene described in association with HNSHA and clinical findings of cases reported to date.

PMID and first author	Patient/family origin	Variant nomenclature (according to the erythroid-specific HK1 isoform NM_033496.2); exonic/intronic/upstream location; mode of inheritance		Age at diagnosis; family relation	Clinical phenotype and treatment
		Variant 1	Variant 2		
This study	Finnish	c.2599C>T p(His867Tyr) Exon 17 AR (homozygous)	c.2599C>T p(His867Tyr) Exon 17	3-month-old female, proband	Severe infantile-onset transfusion-dependent haemolytic anaemia. Haematopoietic stem cell transplantation planned.
		c.2599C>T p(His867Tyr) Exon 17 AR (compound heterozygous)	c.-193A>G Promoter	30-year-old, father	Mild chronic haemolytic anaemia first diagnosed at age 3; splenomegaly; normalization of Hb level after splenectomy performed at age 23.
	Finnish	c.2361_2362del p(Gln788Aspfs*4) Exon 16 AR (compound heterozygous)	c.-193A>G Promoter	37-year-old male	Mild chronic haemolytic anaemia first diagnosed at age 11; splenomegaly; cholecystectomy at age 19.
	Finnish	*HK1* c.372+1G>A Intron 3 (splice site mutation) AR (compound heterozygous)	c.-193A>G Promoter	26-year-old male	Cholelithiasis/gallstone disease with compensated haemolytic disease first diagnosed at age 19.
34532855 Sasaki	Romanian Romani	c.278G>A p(Arg93Gln) Exon 3 AR (homozygous)	c.278G>A p(Arg93Gln) Exon 3	Three children: two siblings and one cousin (dizygotic twin I)	One of the siblings: death on Day 2 of life. Additional malformations. Coexistent 16p13.11 microdeletion syndrome. Other sibling and cousin: non-spherocytic haemolytic anaemia associated with developmental delay, intellectual disability, epilepsy, microcephaly, white matter atrophy, recurrent apnoeic episodes. Another child from this family, showing non-spherocytic haemolytic anaemia, developmental delay, intellectual disability and white matter atrophy, died without genetic testing.
33361148 Dongerdiye	Indian	c.2711C>A p(Thr904Lys) Exon 18 AR (homozygous)	c.2711C>A p(Thr904Lys) Exon 18	4.5-year-old female	Severe congenital haemolytic anaemia with mild hepatosplenomegaly, requiring repeated transfusions, in association with developmental delay, microcephaly, hypotonia and seizures. 4th-degree consanguineous parents.
31119733 Jamwal	Indian	c.34C>T p(Arg12Ter) Exon 1 AR (homozygous)	c.34C>T p(Arg12Ter) Exon 1	9-month-old male	Severe congenital haemolytic anaemia, delayed milestones, hepatosplenomegaly, death at age 1. Consanguineous parents and grandparents. 14 blood transfusions, starting with exchange transfusion on postnatal Day 4.
27297791 Khazal	Hispanic	c.1837G>A p(Gly613Arg) Exon 13 (first nucleotide substitution, leading to missense mutation with splice site disruption) AR (homozygous)	c.1837G>A p(Gly613Arg) Exon 13	4-year-old male	Hydrops fetalis, preterm birth at 32 weeks' gestation, severe congenital haemolytic anaemia. Exchange transfusion in the first hours of life; repeated PRBC transfusions; splenectomy in the second year of life; chelation therapy; allogenic bone marrow transplantation. 2 siblings had died of severe anaemia and hydrops after a few hours of life.

TABLE 3 (Continued)

PMID and first author	Patient/family origin	Variant nomenclature (according to the erythroid-specific HK1 isoform NM_033496.2); exonic/intronic/upstream location; mode of inheritance		Age at diagnosis; family relation	Clinical phenotype and treatment
		Variant 1	Variant 2		
27282571 Koralkova	Irish	c.873-2A>G Intron 7 (splice site mutation). AR (compound heterozygous)	c.-193A>G Promoter	2-year-old male, proband	Severe haemolytic anaemia. Transfusions and chelation therapy until age 3.
		c.873-2A>G Intron 7 AR (compound heterozygous)	c.-193A>G Promoter	4-year-old brother	Asymptomatic (compensated haemolytic anaemia).
	Czech Romani	c.278G>A p(Arg93Gln) Exon 3 AR (homozygous)	c.278G>A p(Arg93Gln) Exon 3	8-month-old male	Severe haemolytic anaemia, CNS bleeding (hypoxia during delivery) with psychomotor retardation and secondary epilepsy. Transfusions and chelation therapy. Consanguineous parents.
	Danish	c.2599C>T p(His867Tyr) Exon 17 AR (homozygous)	c.2599C>T p(His867Tyr) Exon 17	2-year-old male	Severe haemolytic anaemia. Transfusions and chelation therapy.
19608687 de Vooght	Dutch	c.278G>A p(Arg93Gln) Exon 3 (altered splicing) AR (compound heterozygous)	c.-193A>G Promoter	33-year-old male	Mild chronic haemolytic anaemia.
12393545 van Wijk	Dutch	c.2036C>G p(Thr679Ser) Exon 15. Described as 'HK Utrecht'. AR (homozygous)	c.2036C>G p(Thr679Ser) Exon 15	19-year-old female (at the time of case description in 1983)	Congenital haemolytic anaemia. Several transfusions in early infancy; splenectomy at age 2; cholecystectomy at age 15. Case originally reported in PMID 6848140. Consanguineous parents.
12211198 Kanno	Japanese	c.493-481_1028+454del Deletion of introns 4 through 8/exons 5 through 8, causing frameshift mutation and premature termination of translation. AR (homozygous)	c.493-481_1028+454del	29 weeks gestational age female fetus	IUGR fetus with severe haemolytic anaemia, stillbirth, periventricular leucomalacia. Parents apparently not consanguineous, though both carrying the same deletion.
7655856 Bianchi	Italian	c.1583T>C p(Leu528Ser) Exon 11 Described as 'HK Melzo'. AR (compound heterozygous)	c.493_588+1del Exon 5	27-year-old female (at the time of case description in 1985)	Severe congenital chronic haemolytic anaemia. Hepatosplenomegaly. Splenectomy performed at 11 months of age; repeated blood transfusions from early infancy to age 10. Clinical information reported in PMID 4027385.

Abbreviations: AR, autosomal recessive; CNS, central nervous system; IUGR, intrauterine growth restriction; PMID, PubMed Identifier; PRBC, packed red blood cell.

relevant areas of the *HK1* gene, including the promoter region. Another relevant point that should be considered in the genetic analysis of *HK1*-related HNSHA is the presence of multiple transcripts in *HK1*. As exemplified in a report of a severely affected HNSHA patient caused by a homozygous pathogenic variant *HK1* c.34C>T, p.(Arg12*) in exon 1 by Jamwal et al., variants can be missed during automated data analysis if the different isoforms are not properly considered.[8]

Splenectomy is considered the first line of treatment, but in the most severe cases with autohaemolysis, splenectomy only decreases the frequency of transfusions.[7] Khazal et al. described a four-year-old boy who was successfully treated with allogeneic haematopoietic stem cell transplantation.[10] More than 1 year after the transplantation, the boy was transfusion independent.[10] Because of possible iron accumulation, adequate follow-up is needed, even for mild cases of HNSHA, to allow the early initiation of iron chelation therapy, as demonstrated in Patient 4.

At first, the decreased PK activity in Patient 4 led to an erroneous clinical diagnosis of PK deficiency, as in one other patient with hexokinase deficiency and heterozygosity for a *PKLR* variant.[18] To our knowledge, patients with hexokinase deficiency typically show normal PK activity, with the exception of the above-mentioned patients and one patient reported by Koralkova et al.[1] The PK activity of our patient

was measured in two independent laboratories with similar results, which argues against technical artefacts in the enzymatic assay. Both hexokinase and PK function as rate-limiting enzymes in the glycolytic pathway (catalysing the first and last steps of glycolysis). In order to adjust the rate of glycolysis, PK is allosterically regulated by intermediate products of glycolysis, namely fructose 1,6-biphosphate (FBP). However, the underlying cause of the low PK activity in Patient 4 remains unknown.

In contrast to the mild presentation of Patients 2–4, we report an infant with severe early-onset transfusion-dependent anaemia caused by the homozygous *HK1* p.(His867Tyr) missense variant. Koralkova et al. described a similarly affected patient with the same homozygous *HK1* p.(His867Tyr) variant as our patient.[1] Both patients presented with early-onset transfusion-dependent haemolytic anaemia, normal HK activity and changes in the kinetic properties of HK, which caused the disease, thus demonstrating the importance of performing kinetic HK studies.

Here, we describe four previously unreported patients with *HK1* variants and HNSHA. Our findings highlight the importance of analysing the hypomorphic *HK1* variant in the promoter region and reinforce the association of loss-of-function *HK1* variants with hexokinase deficiency. Variant pathogenicity evaluations by functional studies should include the measurements of HK activity, as well as kinetic studies.

AUTHOR CONTRIBUTIONS

(CRediT definitions: https://casrai.org/credit/): Conceptualization: E-M.U., S.D.A. and E.R. Funding acquisition and investigation: S.D.A. Methodology and resources: E-M.U., A.H., U-W.K., S.K., M.K-T. and E.R. Software and supervision: E.R. Visualization and writing—original draft: E-M.U., S.D.A., A.H., M.K-T. and E.R. Writing—review and editing: E-M.U., S.D.A., A.H., U.W-K., S.K., M.R., B.O., M.K-T., R.W. and E.R.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Anonymized data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of the Northern Ostrobothnia Hospital District (EETTMK: 45/2015, amendment 2020) and Helsinki University Hospital.

PATIENT CONSENT STATEMENT

Written informed consent was obtained from all patients and all the participating healthy relatives and healthy control individuals.

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REFERENCES

1. Koralkova P, Mojzikova R, van Oirschot B, Macartney C, Timr P, Vives Corrons JL, et al. Molecular characterization of six new cases of red blood cell hexokinase deficiency yields four novel mutations in HK1. Blood Cells Mol Dis. 2016;59:71–6.
2. Murakami K, Blei F, Tilton W, Seaman C, Piomelli S. An isozyme of hexokinase specific for the human red blood cell (HKR). Blood. 1990;75(3):770–5.
3. Kanno H. Hexokinase: gene structure and mutations. Best Pract Res Clin Haematol. 2000;13(1):83–8.
4. Murakami K, Kanno H, Miwa S, Piomelli S. Human HKR isozyme: Organization of the Hexokinase I gene, the erythroid-specific promoter, and transcription initiation site. Mol Genet Metab. 1999;67(2):118–30.
5. Bianchi M, Magnani M. Hexokinase mutations that produce non-spherocytic hemolytic anemia. Blood Cells Mol Dis. 1995;21(1):2–8.
6. de Vooght KMK, van Solinge WW, van Wesel AC, Kersting S, van Wijk R. First mutation in the red blood cell-specific promoter of hexokinase combined with a novel missense mutation causes hexokinase deficiency and mild chronic hemolysis. Haematologica. 2009;94(9):1203–10.
7. Dongerdiye R, Jagadeesh S, Suresh B, Rajendran A, Devendra R, Warang P, et al. Novel pathogenic variant c.2714C>A (p. Thr905Lys) in the HK1 gene causing severe haemolytic anaemia with developmental delay in an Indian family. J Clin Pathol. 2021;74(10):620–4.
8. Jamwal M, Aggarwal A, Palodi A, Sharma P, Bansal D, Maitra A, et al. A nonsense variant in the Hexokinase 1 gene (HK1) causing severe non-spherocytic haemolytic anaemia: genetic analysis exemplifies ambiguity due to multiple isoforms. Br J Haematol. 2019;186(5):e142–e145.
9. Kanno H, Murakami K, Hariyama Y, Ishikawa K, Miwa S, Fujii H. Homozygous intragenic deletion of type I hexokinase gene causes lethal hemolytic anemia of the affected fetus. Blood. 2002;100(5):1930.
10. Khazal S, Polishchuk V, Manwani D, Gallagher PG, Prinzing S, Mahadeo KM. Allogeneic bone marrow transplantation for treatment of severe hemolytic anemia attributable to hexokinase deficiency. Blood. 2016;128(5):735–7.
11. Sasaki E, Phelan E, O'Regan M, Kassim AH, Miletin J, McMahon C, et al. HK1 haemolytic anaemia in association with a neurological phenotype and co-existing CEP290 Meckel–Gruber in a Romani family. Clin Genet. 2022;101(1):142–3.
12. Valentine WN, Oski FA, Paglia DE, Baughan MA, Schneider AS, Naiman JL. Hereditary hemolytic anemia with hexokinase deficiency. Role of hexokinase in erythrocyte aging. N Engl J Med. 1967;276(1):1–11.
13. van Wijk R, Rijksen G, Huizinga EG, Nieuwenhuis HK, van Solinge WW. HK Utrecht: missense mutation in the active site of human

hexokinase associated with hexokinase deficiency and severe non-spherocytic hemolytic anemia. Blood. 2003;101(1):345–7.
14. Koralkova P, van Solinge WW, van Wijk R. Rare hereditary red blood cell enzymopathies associated with hemolytic anemia - pathophysiology, clinical aspects, and laboratory diagnosis. Int J Lab Hematol. 2014;36(3):388–97.
15. Rijksen G, Schipper-Kester GPM, Staal GEJ, Veerman AJP. Diagnosis of pyruvate kinase deficiency in a transfusion-dependent patient with severe hemolytic anemia. Am J Hematol. 1990;35(3):187–93.
16. Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature. 2020;581(7809):434–43.
17. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405–24.
18. Bianchi P, Fermo E. Molecular heterogeneity of pyruvate kinase deficiency. Haematologica. 2020;105(9):2218–28.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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