

First familial cases of type 2 congenital erythrocytosis (ECYT2) with a Chuvash pathogenic variant in *VHL* gene in Poland: example of the clinical utility of next-generation sequencing in diagnostics of orphan diseases

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Abstract

In this article, we report familial cases of type 2 congenital erythrocytosis (ECYT2) in two siblings, a 2-year-old boy and his younger sister. Both patients were diagnosed based on laboratory findings including erythrocytosis, elevated hemoglobin levels, and hematocrit. Acquired erythrocytosis was excluded based on the clinical features and genetic analysis of *JAK2/CALR/MPL* genes. Next-generation sequencing was employed for older brother revealing NM_000551.4: c.598C>T, p.Arg200Trp homozygous variant in the *VHL* gene, the similar variant was detected in the younger sibling. Sequencing analysis confirmed the *VHL* c.598C>T heterozygous variant in both parents. To the best of our knowledge, these are the first confirmed cases of familial erythrocytosis type 2, also known as Chuvash type, in Poland.

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Keywords:

erythrocytosis, ECYT2, *VHL* variant, next-generation sequencing

Introduction

Erythrocytosis may be caused by several factors, including congenital or acquired genetic defects and conditions resulting in abnormal erythropoietin (EPO) production, such as chronic obstructive pulmonary disease, high altitude exposure, and EPO producing tumors. Erythrocytosis can be divided into two major groups: primary erythrocytosis, with low levels of EPO in the serum, with an intrinsic defect in the erythroid lineage and secondary erythrocytosis, when EPO production is increased due to acquired condition or genetic defect. Both primary and secondary erythrocytoses are mostly acquired. Polycythemia vera is the most common type of acquired primary erythrocytosis and associated with a canonical pathogenic variant in the Janus Kinase 2 (*JAK2*) gene, namely c.1849G>T, p.Val617Phe (p.V617F) [1, 2].

Familial (congenital) erythrocytosis is a rare disease and may belong to a primary or secondary type. The underlying genetic defects may affect different genes but ultimately lead to the elevated levels of EPO or hypersensitivity of erythroid progenitors to EPO. In contrast to the primary erythrocytosis, secondary erythrocytosis can be caused

by additional abnormalities related to cardiac, pulmonary, or renal dysfunction. In addition, changes in the anatomical structure of the liver and spleen may be observed [1, 3].

Familial erythrocytosis may result from pathogenic variants in the *EPOR*, *VHL*, *EGLN1*, *EPAS1*, or *EPO* genes. Five types of familial erythrocytosis (ECYT) have been defined, from ECYT 1 to ECYT 5 depending on which one of these genes is defective (Tab. 1) [4]. In addition, variants in hemoglobin (*HBB*) and bisphosphoglycerate mutase (*BPGM*) genes which increase oxygen affinity may cause erythrocytosis. ECYT1 is the only primary congenital erythrocytosis and is caused by gain-of-function in the *EPOR* gene encoding erythropoietin receptor. ECYT1 is characterized by low serum EPO levels in contrast to other familial erythrocytoses. On the other hand, ECYT 2-5 belongs to secondary erythrocytosis and is caused by defects in genes involved in oxygen sensing pathways or due to aberrant Hb variants with high oxygen affinity. This results in higher EPO production and serum levels [1, 3, 4].

Familial type 2 erythrocytosis (ECYT2), also known as Chuvash type polycythemia, has been described for the first time almost 20 years ago in Chuvashia, one of the republics in Russia, where it is endemic [5].

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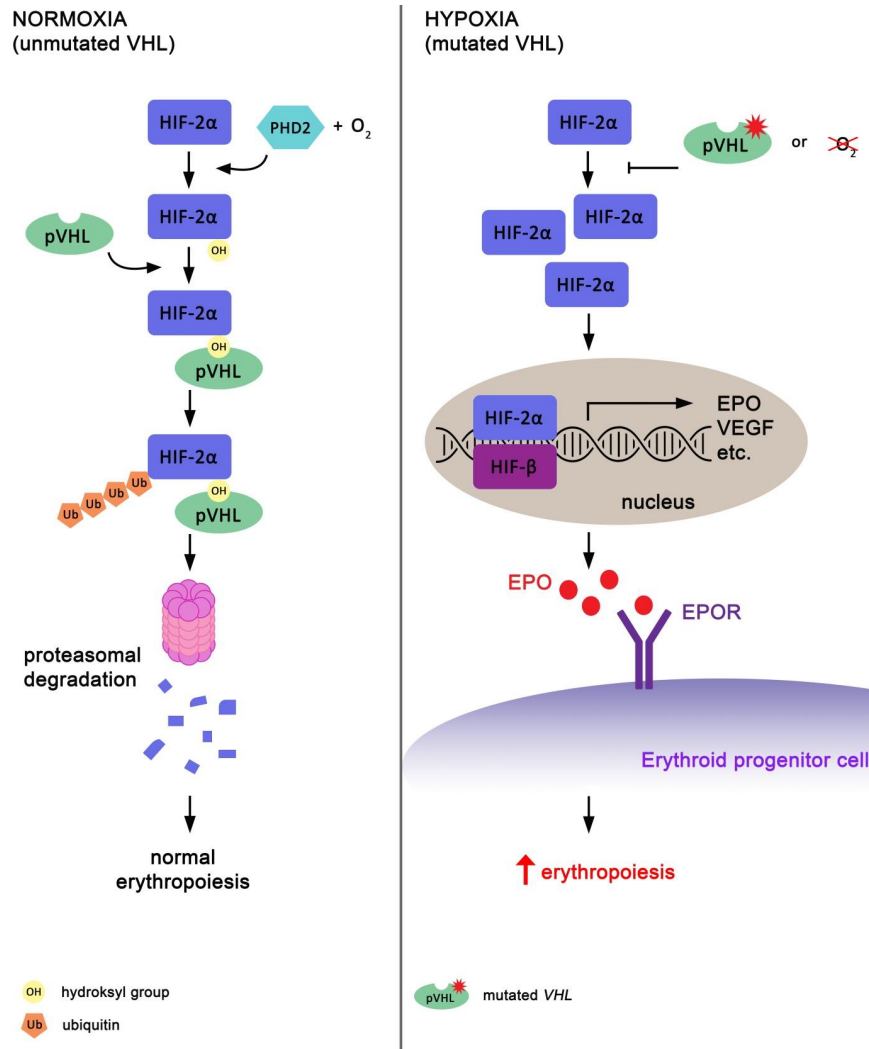


Fig. 1. The role of the VHL in the pathogenesis of ECYT2

Table I. Five major types of congenital erythrocytoses

Disease name	Gene	Protein	Inheritance
ECYT1	EPOR	EPOR (erythropoietin receptor)	Dominant
ECYT2	VHL	VHL (von Hippel-Lindau)	Recessive
ECYT3	EGLN1	PHD2 (prolyl hydroxylase domain-containing protein 2)	Dominant
ECYT4	EPAS1	HIF2α (hypoxia Inducible factor 2α)	Dominant
ECYT5	EPO	EPO (erythropoietin)	Dominant

Further studies revealed this type of erythrocytosis in the population of Ischia, Italian island, and also in other parts of the world [6, 7]. ECYT2 is an autosomal recessive form of erythrocytosis associated with the c.598C>T (p.Arg200Trp) homozygous variant in the von Hippel-Lindau gene (*VHL*) [5, 8, 9]. *VHL* gene is located on chromosome 3 and encodes VHL protein, which functions as an E3 ubiquitin ligase for hypoxia inducible factor (HIF). In normoxia, HIF-2α

subunit is hydroxylated by prolyl hydroxylase domain-containing protein 2 (PHD2) and becomes a target for ubiquitination and proteasomal degradation mediated by VHL. Mutated *VHL* mimics hypoxia condition when HIF-1α is stabilized, translocates to nucleus and forms with HIF-1β active transcription factor. This leads to upregulation of downstream target genes such as *EPO*, transferrin (*TF*), transferrin receptor (*TFRC*), vascular endothelial growth receptor A (*VEGF*), solute carrier family 2 member 1 (*SLC2A1*) and consequently to dysregulation of blood homeostasis (Fig. 1) [10–13]. Although homozygous mutation causing Chuvash erythrocytosis which has been associated with a higher risk of thrombosis, heterozygosity has been linked with some degree of protection against anemia [14]. This phenomenon may explain the prevalence of heterozygous alleles despite the higher mortality of homozygotes. Here, we describe the clinical and genetic characteristics of two siblings who were diagnosed with ECYT2 after the exclusion of the most common acquired types of erythrocytosis and employing next-generation sequencing (NGS).

Table II. Laboratory results of blood morphology of patients #1 and #2 at the time of diagnosis, after 12 (or 4 for #2) months of observation and maximum values

	Patient #1			Patient #2		
	Diagnosis	After 12 months of observation	Maximum values	Diagnosis	After 4 months of observation	Maximum values
WBC/ μ l	7,540	7,740	9,700	14,570	9,850	17,630
Neutrophils/ μ l	3,580	3,180	4,080	6,860	3,290	6,860
Lymphocytes/ μ l	3,140	3,900	4,500	6,420	5,600	6,420
RBC/ μ l	6,810,000	6,320,000	6,870,000	7,330,000	6,560,000	7,330,000
Hb [g/dL]	18.7	12.7	19.1	20.3	18.1	20.3
Ht [%]	55.4	41.7	55.5	58.4	52.6	58.4
MCV [fL]	81.4	66.0	81.4	79.7	80.2	83.8
MCH [pg]	27.4	20.1	27.9	27.7	34.4	35.1
PLT/ μ l	227,000	519,000	519,000	382,000	305,000	415,000

Material and methods

Blood was drawn after receiving written consent from patients' parents according to institutional guidelines and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki), and patients' material was kept anonymous. Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Targeted sequencing of patient #1 was performed using the SeqCap EZ Choice Library (Roche, Basel, Switzerland) covering coding sequences of 193 genes implicated in hematological diseases (see Suppl. Data 1). DNA library was prepared from 550 ng DNA using Kapa Library Preparation Kit for Illumina (Roche), multiplexed with others before solution-based capture (Roche NimbleGen), and sequenced on Illumina MiSeqFGx. The mean depth of coverage was 85.61x. The variant discovery was performed according to GATK best practices. Variants more frequent than 1% in public databases (1,000 genomes, NHLBI ESP, and gnomAD) and 5% in our internal database were discarded from further analysis. Five bioinformatics predictors were used to identify possible protein-damaging variants: CADD, FATHMM, SIFT, PolyPhen2, and Mutation Taster.

NM_000551.4:c.598C>T variant in patient #2 was revealed by direct Sanger sequencing. In the polymerase chain reaction (PCR), the 206 bp fragment of the *VHL* exon 3 was amplified using HotStarTaq Plus DNA Polymerase (Qiagen) and specific primers flanking codon 200: F-CAGGAGACTGGACATCGTCA and R-AAGGAAGGAACCAGTCCTGT. The PCR product was purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) and sequenced on Genetic Analyzer 3500 using BigDye chemistry (Applied Biosystems, Foster City, CA, USA). Sanger sequencing was also used to confirm the *VHL* c.598C>T heterozygous variant in parents.

Results and discussion

Patient #1. A 2-year-old boy was referred to the Department of Paediatric Oncology and Haematology, State Hospital No 2 in Rzeszow (south-eastern region of Poland, historically known as

Galicja) due to elevated red cell values from 24 months of age (Tab. II). The boy was born G1P1 at 35 weeks of gestation. The perinatal period was uncomplicated apart from severe jaundice. His family history is unremarkable—neither thromboembolic events nor spine hematomas and arterial hypotonia have occurred among close relatives. But the parents have reported distant kinship in the fifth generation back. Until his admission, the boy had been developing properly and had not any significant illness. Physical examination was unremarkable apart from erythema on the cheeks. Among others, high values of red cell parameters in peripheral blood morphology (RBC = 6.87 million/ μ l; Hb = 19.1 g/dL; Ht = 55.6%) were found. A bone marrow biopsy with trepanobiopsy was performed. The biopsy and myelogram obtained did not show features of a proliferative process or any other structural abnormalities within the red blood cell system. None of the canonical pathogenic variants in *JAK2*, *CALR*, and *MPL* genes was found. The EPO level was within the reference range. Furthermore, any significant deviations were found in biochemical tests. A chest X-ray and abdominal ultrasound did not show any pathological lesions.

Due to clinical uncertainty, diagnostics was expanded and NGS was performed. A homozygous variant in the *VHL* gene (NM_000551.4:c.598C>T, p.Arg200Trp) was detected which is typical for type 2 erythrocytosis also known as Chuvash polycythemia (Fig. 2A). NGS results were additionally confirmed with Sanger sequencing (Fig. 2B). Then, both parents were examined for the genetic status of the *VHL* variant and Sanger sequencing confirmed the *VHL* c.598C>T heterozygosity in both parents (Fig. 2B).

ECYT2 is characterized by elevated hemoglobin level and hematocrit together with normal or elevated levels of EPO, as well as normal oxygen affinity. Clinical manifestations of the disease are associated with elevated levels of red blood cells as well as increased circulating blood volume. In children, they may include hyperviscosity syndrome characterized by headaches and dizziness, fatigue, drowsiness, blurred vision, hearing impairment, paresthesia, and myalgia. Hypoxia and peripheral hypoperfusion are associated with erythrocytosis, which also increases thromboembolic risk [15]. At a later age, an increase in the incidence of strokes, heart attacks, and a paradoxically increased frequency of bleeding are often

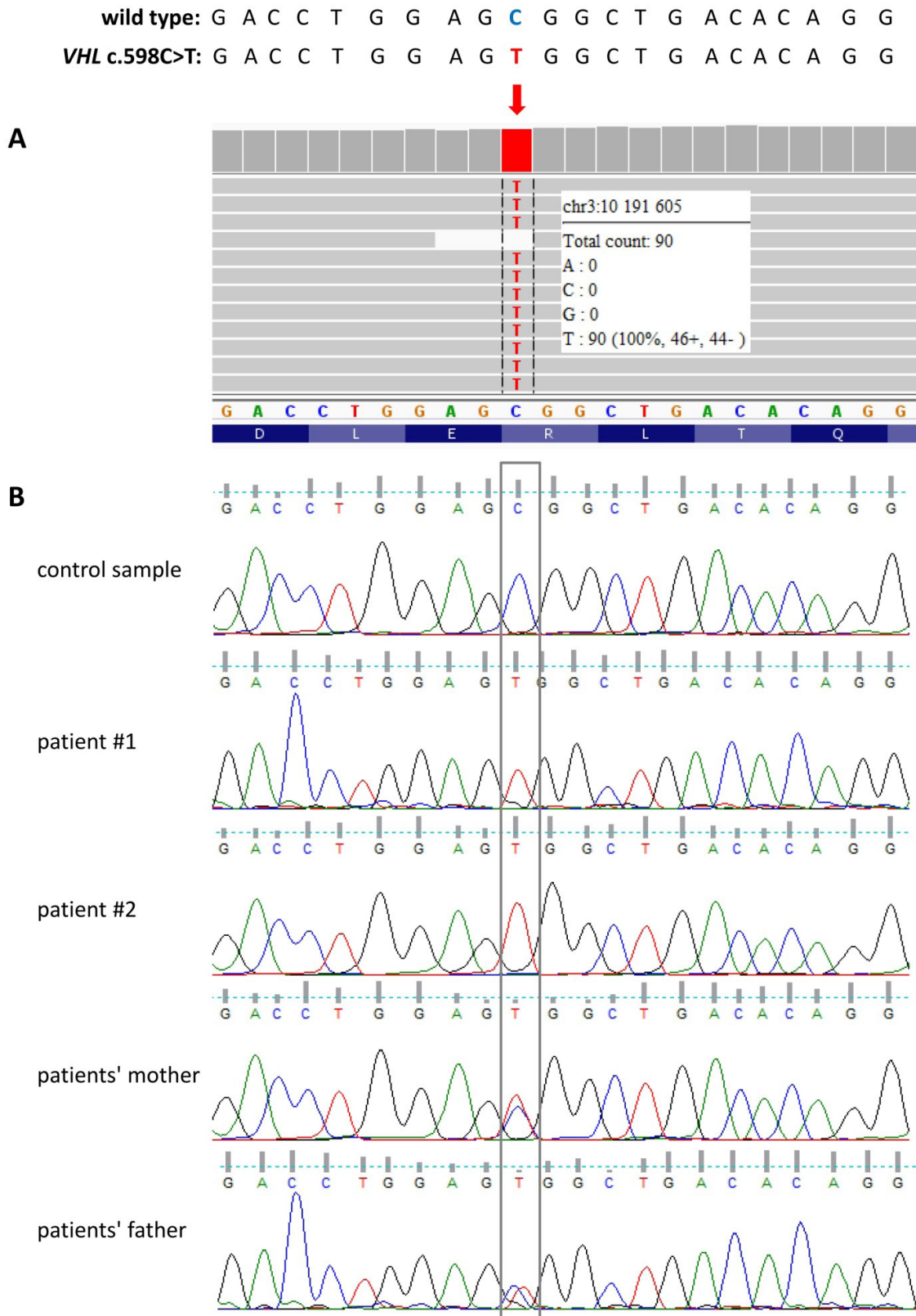


Fig. 2. VHL c.598C>T pathogenic variant detected by NGS and Sanger sequencing. (A) NGS results of patient #1 presented as a screenshot from the IGV software. Chromosomal coordinate, the total number of reads, and variant allele frequency (VAF) are provided in the box. (B) Screenshot from the FinchTV software showing the VHL c.598C>T variant detected by Sanger sequencing in patient #1 (confirmation of NGS results), in patient #2 and patients' parents. The control sample represents the reference sequence without the variant c.598C>T

observed. Other complications include hemangiomas and pulmonary hypertension also occur [16]. Compared with other germline mutations that predispose to hemangioblastomas in the retina and central nervous system, renal tumors, pheochromocytoma, pancreatic tumors, and so on [17], *VHL* c.598C>T variant is not associated with tumor development [18].

In the described patient, phlebotomy was used as a treatment, which resulted in a temporary decrease in Hb and Ht. In total, eight treatments were performed over for 6 months. The boy remained in good general condition, no significant symptoms associated with erythrocytosis were observed, and his development was normal. After 9 months of observation, peripheral blood red cell values gradually decreased (RBC = 6.32 million/ μ l; Hb = 12.7 g/dL; Ht = 41.7%). Currently, the boy is 4 years old and does not present any clinical symptoms of polycythemia.

Patient #2. A 9-month-old girl (G2P2 at 39 weeks of pregnancy, complicated by gestational diabetes), with a remarkable family history (her brother was diagnosed with a family type 2 erythrocytosis confirmed by a genetic test), was admitted to the clinic due to persistently elevated red blood cell values (RBC = 5.9 million/ μ l; Hb = 16.8 g/dL; Ht = 47.2%), which had been observed from 5 months of age. In the child's previous medical history, recurrent mild upper respiratory tract infections and cow's milk intolerance had also been found. At the time of admission, no abnormalities were found on physical examination. In peripheral blood morphology, similarly to her brother, increased red blood cell values were found (RBC = 7.33 million/ μ l; Hb = 20.3 g/dL; Ht = 58.4%). Further laboratory tests showed no other relevant abnormalities. Since NGS analysis in older brother revealed a pathogenic variant in *VHL* gene typical for familial erythrocytosis type 2, direct Sanger sequencing of the target amplicon was performed and revealed the presence of the same homozygous variant in the *VHL* gene (c.598C>T) as detected in her brother (Fig. 2B).

Treatment in the form of repeated courses of phlebotomy was scheduled. In total, 14 treatments have been performed for over 4 months. The girl remains in good general condition, no significant symptoms associated with erythrocytosis were observed, and development was normal. Currently, the girl is 13 months old and does not present any pathological symptoms. Recently, peripheral blood red blood cell values remain elevated (RBC = 5.56 million/ μ l, Hb = 18.1 g/dL, Ht = 52.6%), so further phlebotomy treatments are necessary.

Conclusions

1. The course of familial type 2 erythrocytosis in infants and young children seems to be mild. The abnormal values of the red blood cell system in peripheral blood counts are the basis for diagnosis.
2. In the boy (patient #1) a spontaneous normalization of hemoglobin and hematocrit values was observed and currently, he does not require any treatment. Due to the small number of cases described in the literature, the duration of remission and the course of the disease are difficult to predict.
3. In such rare cases with an unclear cause of erythrocytosis, advanced genetic diagnostics, such as NGS, allows to reveal the underlying genetic defect and help to establish a proper diagnosis, choose therapeutic options, and assess the prognosis.
4. Phlebotomy in infants and young children appears to be a safe and effective procedure, without serious complications.

Authors' contributions

MP – prepared NGS libraries, carried out Sanger sequencing, analyzed data, and wrote the manuscript. WB – provided and analyzed clinical data, wrote the manuscript. MRa – monitored and treated both patients. MMM – prepared NGS libraries, carried out Sanger sequencing, and analyzed NGS data. MK – analyzed NGS data. MRy, AP – sequenced NGS libraries. PS – performed bioinformatic analysis of NGS data. RP – analyzed NGS data and supervised MRy, AP, PS. RC – conceived the idea of NGS testing, coordinated clinical part, and supervised WB, MRa. TS – conceived the idea of NGS testing, analyzed data, supervised MP, MK and MMM and wrote the manuscript. All authors provided critical feedback and contributed to the final manuscript.

Conflicts of interest

None.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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