Hereditary xerocytosis - spectrum and clinical manifestations of variants in the PIEZO1 gene, including co-occurrence with a novel β-globin mutation

Karolina Maciak\textsuperscript{a}, Anna Adamowicz-Salach\textsuperscript{b}, Alicja Siwicka\textsuperscript{a}, Jarosław Poznanski\textsuperscript{a}, Tomasz Urasinski\textsuperscript{c}, Danuta Plochocka\textsuperscript{a}, Monika Gora\textsuperscript{a}, Beata Burzynska\textsuperscript{a,*}

\textsuperscript{a} Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland
\textsuperscript{b} Department of Pediatrics, Hematology and Oncology, Medical University of Warsaw, Poland
\textsuperscript{c} Department of Pediatrics, Hemato-Oncology and Gastroenterology, Pomeranian Medical University in Szczecin, Poland

\textbf{ARTICLE INFO}

Editor: Mohandas Narla

Keywords:
Hereditary xerocytosis
PIEZO1 gene
Hemolytic anemia
Molecular modeling

\textbf{ABSTRACT}

Hereditary xerocytosis (HX) is a rare, autosomal dominant congenital hemolytic anemia (CHA) characterized by erythrocyte dehydration with presentation of various degrees of hemolytic anemia. HX is often misdiagnosed as hereditary spherocytosis or other CHA. Here we report three cases of suspected HX and one case of HX associated with β-thalassemia.

Sanger method was used for sequencing cDNA of the PIEZO1 gene. Variants were evaluated for potential pathogenicity by MutationTaster, PROVEAN, PolyPhen-2 and M-CAP software, and by molecular modeling.

Four different variants in the PIEZO1 gene were found, including three substitutions (p.D669H, p.D1566G, p.T1732 M) and one deletion (p.745delQ). In addition, in the patient with the p.T1732 M variant we detected a 12-nucleotide deletion in the β-globin gene leading to a deletion of amino acids 62AHGK65. The joint presence of mutations in two different genes connected with erythrocytes markedly aggravated the presentation of the disease. Bioinformatic analysis and molecular modeling strongly indicated likely deleterious effects of all four PIEZO1 variants, but co-segregation analysis showed that the p.D1566G substitution is in fact non-pathogenic.

Identification of causative mutations should improve the diagnosis and management of HX and provide a new insight into the molecular basis of this complex red blood cell abnormality.

1. Introduction

Hereditary xerocytosis (HX), also known as dehydrated hereditary stomatocytosis (DHS), is a rare red blood cell membrane disorder resulting in hemolytic anemia with diverse presentation and iron overload. Dominantly inherited missense mutations in the PIEZO1 gene encoding a large mechanosensitive ion channel, affecting mainly its highly conserved COOH-terminus, have been identified in HX patients [1]. They increase the cation permeability of the membrane, which leads to erythrocyte dehydration. Red blood cells (RBCs) from HX patients are characterized by a higher Na\(^+\) content and a decreased K\(^+\) content relative to healthy RBCs [2]. Besides the PIEZO1 mutations, also mutations in \textit{KCNN4} encoding the Gardos channel have been detected in some HX cases [3,4]. The clinical presentation of hereditary xerocytosis is markedly heterogeneous, ranging from normal hemoglobin values to severe anemia, with borderline macrocytosis, increased cell hemoglobin content (MCH) and increased mean corpuscular hemoglobin concentration (MCHC) [5]. Another type of RBC disorder with well-understood genetic and molecular etiology is β-thalassemia. It is a heterogeneous group of hemoglobin disorders due to a decreased or absent production of normal β-globin chains. A special form of β-thalassemia defects, called dominant β-thalassemia, is caused by point mutations or small insertions or deletions leading to the production of highly unstable β-globin, which results in the formation of an unstable tetrameric α\(_2\)β\(_2\) protein. When such mutations generate a premature stop codon, the mutated mRNA may undergo degradation through a surveillance mechanism called nonsense-mediated mRNA decay (NMD). The NMD is not absolutely efficient and the mRNAs escaping degradation give rise to truncated β-globin chains, resulting in highly variable symptoms among carriers of the same variant. Heterozygotes for a dominant β-thalassemia mutation show typical anemia symptoms [6], whereas activation of NMD only leads to a mild anemia or is symptomless. Combined defects of the red cell membrane with other lesions affecting erythrocytes are very rare and difficult to diagnose properly. Only few HX cases associated with a thalassemia or RBC enzymopathy have been reported [7]. The aim of our study was to...
elucidate the molecular basis of hereditary xerocytosis in four Polish patients with suspected HX or an undiagnosed congenital hemolytic anemia. Four previously uncharacterized variants in the PIEZO1 gene were identified and, in one patient, a co-existing deletion in the β-globin gene. Homology modeling was performed in order to predict the effects of the identified variants on the structure of PIEZO1 and β-globin proteins and thereby their likely mechanism of pathogenicity. Family co-segregation studies were used to verify the predicted pathogenicity of two of the novel variants.

2. Materials and methods

2.1. Case presentation

2.1.1. Patient 1

A 16-year-old girl has been under the care of the outpatient department of a hematology division since birth due to hemolytic anemia of unknown etiology. She was hospitalized many times as she required packed red blood cells transfusions which occurred mainly during infections. Additionally, growth hormone deficiency, familial adenomatous polyposis (FAP) and Gilbert Syndrome were diagnosed. Mother and sister of the proband were asymptomatic. The father of the patient was unavailable for genetic studies. It is only known from an interview that he exhibited no symptoms of hemolytic anemia.

2.1.2. Patient 2

An 18-year-old girl with hemolytic anemia of unknown etiology has been observed since birth. She was hospitalized occasionally for packed red blood cells transfusions. Initially she was diagnosed with congenital dyserythropoietic anemia type II. A flow cytometry test with eosin-5'-maleimide (EMA) staining for red cell membrane disorders showed lowered fluorescence intensity compared with normal samples. At the age of 3, splenectomy was performed with no beneficial effect; no thrombotic event has been reported. Finally, other possible causes of anemia were excluded and diagnosis of HX was established. Mother, father and brother of the proband were asymptomatic.

2.1.3. Patient 3

A 59-year-old man complaining of weakness and fatigue since 2012. Lab studies revealed occasional mild anemia accompanied by elevated reticulocyte count and mild hyperbilirubinemia. In December 2017 he had an episode of severe abdominal pain located in the left upper quadrant of the abdomen. Gallstones were diagnosed and managed conservatively. Physical examination revealed no abnormalities. No family members were available for analysis. It is only known from an interview that the both parents exhibited no symptoms of hemolytic anemia.

2.1.4. Patient 4

A 3-year-old boy has been under the care of the outpatient department of a hematology clinic since birth due to hemolytic anemia of unknown etiology. Hereditary spherocytosis, enzymopathy of red cells and autoimmune hemolytic anemia were excluded. Since hemoglobin analysis showed elevated levels of hemoglobins F and A2, β-thalassemia was diagnosed. Red cell morphology demonstrated target cells in association with stomatocytes and microspherocytes (Fig. 1), and red cells displayed profoundly decreased osmotic fragility. The hematological data of the probands are shown in Table 1.

Informed consent has been obtained from the patients or their parents, as appropriate, and the study protocol approved by the Ethics Committee of the Medical University of Warsaw.

2.2. DNA isolation

The QIAquick* DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from peripheral blood samples.

2.3. RNA isolation and reverse transcription

Total RNA was isolated from peripheral blood samples using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany). Reverse transcription (RT) was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations.

2.4. DNA and cDNA sequencing

Genomic DNA was used to amplify the promoter region, entire coding sequence and flanking sequences of the β-globin gene by PCR. cDNA template was used for amplification of the coding sequence of the PIEZO1 gene. DNA fragments generated by PCR amplification were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced directly with BigDye Terminators and appropriate primers using an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA, USA). The mutations detected in cDNA were verified by sequencing of genomic DNA. Detected variants were evaluated for potential pathogenicity by MutationTaster [8], PROVEAN [9], PolyPhen-2 [10] and M-CAP software [11]. The sequences of all primers and the annealing temperatures used for PCR are available upon request.

2.5. Molecular modeling

The structure of the human PIEZO1 ion channel has not yet been determined experimentally, so it was modeled by homology using the recently determined cryo-electron microscopy structure of mouse Piezo1 (pdb6b3r). An initial structure of the protein was modeled using the SWISS-MODEL server [12], and more precise modeling was then performed for fragments T561-D710, E811-P960 and A1672-P1813 with the use of Yasara Structure [13] and further tuned with FoldX [14]. The thermodynamic effects of the D669H and T1732M substitutions were assessed with the use of FoldX.

The low-complexity region comprising the sequence 745QQQQQE-EEEEEE756 was modeled in the helical form, in agreement with the secondary structure predictions performed using the APSSP2 [15] and CFSSP [16] web servers.
A homology model of β-globin lacking the 62AHKG65 fragment was obtained using the SWISS-MODEL server [12]. As a template the 3D structure of human hemoglobin was used (PDB code: 1DXT0) [17].

**3. Results**

All the probands exhibited variable degrees of anemia with the presence of stomatocytes in the blood smear, and reticulocytosis. Sequencing of the PIEZO1 cDNA showed four previously uncharacterized variants, one in each patient (Table 2, Supplemental Figure 1S).

They were confirmed by sequencing corresponding regions of their genomic DNA. In patient 1 the c.2005 G > C variant causes a substitution of aspartic acid with histidine at position 669 (p.D669H). In this patient symptoms of anemia/jaundice have been noted since birth and she was occasionally transfused. The most significant laboratory findings were: hemoglobin (Hb) concentration of 8.1 g/dL and a significantly decreased number of red blood cells with slightly elevated red blood cell distribution width (RDW). Her mother and sister have never been anemic, and DNA sequencing did not show the presence of the c.2005 G > C substitution. p.D669H was categorized as a disease-causing mutation by all the bioinformatics software used. Molecular modeling showed that the D669H replacement destabilizes the protein structure only slightly (~0.4 kcal/mol, see Fig. 2). It should be mentioned, however, that the loop separating TM14 and TM15 (residues 650–690) carries a stretch of negatively charged residues D651, D664, E665, D669, E673 and E679, all of which are generally conserved in the homologous sequences and therefore are expected to be functionally important. Thus, the D669H substitution, which markedly changes the charge distribution along this loop could affect intermolecular interactions of PIEZO1 with an unidentified partner.

Patient 2 carries the c.4697 A > G substitution changing aspartic acid to glycine at position 1566 (p.D1566G). This variant was also bioinformatically classified as disease-causing, but less harmful than the variant in patient 1. Indeed, patient 2 showed a milder form of hemolytic anemia than patient 1. Since no reasonable template...
structures were available for the Q1298-E1677 region of PIEZO1, predicted as an extramembrane sub-domain, the effect of the D1566G replacement could not be studied by homology modeling. It is expected that the introduction of a glycine residue should destabilize the structure. Notably, sequencing of family members has revealed that the variant occurs also in the asymptomatic brother and father of the patient, indicating that it should be regarded as benign, in contrast to the bioinformatic predictions.

In patient 3 the c.2233_2235 CAG deletion removes one glutamine residue from the stretch of glutamines at positions p.745–749. This deletion was categorized as non-disease-causing, in accordance with the mild symptoms of compensated anemia presented by this patient. In spite of these results, molecular modeling showed that the Q deletion would change the size and consequently the charge distribution on a putative helical region, which could affect intermolecular interactions (Supplemental Figure 2S). No family members of the patients were available for analysis.

Patient 4 carries the c.5195C > T variant which results in the substitution of threonine with methionine at position 1732 (p.T1732 M). Patient 4 presents with macrocytosis, massively decreased MCH, and increased RDW. This variant was classified as disease-causing. Residue 1732 is located at the interface of transmembrane helices TM27 and TM28. The T1732 M substitution was found to stabilize the protein structure markedly, by -2 kcal/mol. Such excessive stability could compromise the functioning of PIEZO1. On the other hand, its paralog Piezo2 in the mouse, pongo and human does contain methionine in the corresponding position, which argues against a detrimental effect of the T1732 M variant (Supplemental Figure 3S). We were unable to perform co-segregation analysis of the patient's family.

Since patient 4 showed elevated levels of HbF and HbA2 and the presence of target cells in the blood smear, we sequenced his β-globin gene. A 12-nucleotide in-frame deletion (GCTCATGCAAG) involving codons 62–65 in the second exon, leading to a deletion of amino acids 62AHGK65, was found. Our modeling indicates that this deletion causes a deformation of the heme pocket, particularly on the distal side, likely affecting oxygen binding (Fig. 3).

Moreover, histidine 63 is engaged in an interaction with the heme moiety at its distal side, and its absence by itself should compromise the binding of oxygen.

4. Discussion

Hereditary xerocytosis syndromes are the most common disorder of erythrocyte volume homeostasis and are the most clinically heterogeneous [18]. The degree of anemia varies in severity, with an onset in the fetal period or late in life. Beside chronic anemia and its typical complications, such as iron overload, no other signs are observed with the exception of transient perinatal edema and hydrops fetalis [18]. Peripheral blood smear demonstrates stomatocytes - mouth-shaped erythrocytes. The reticulocyte count is elevated, and red cell mean corpuscular volume (MCHC) and mean corpuscular hemoglobin concentration (MCHC) are slightly increased. Spleenectomy should not be performed in the cases of hereditary xerocytosis due to an increased risk of venous thrombosis [19]. The majority (83%) of HX cases are caused by missense mutations in the PIEZO1 gene, and rare cases related to the KCNN4 gene, which encodes the Gardos channel, have also been reported [20].

In the present analysis of four unrelated putative or diagnosed HX cases with highly different presentations we found four different variants in the PIEZO1 gene, including three substitutions and one deletion. The p.D669H variant of patient 1 affects the same position as the causative D669Y mutation described recently [20,21] in three unrelated families. The p.D1566G (patient 2) and p.T1732 M (patient 4) variants, and the p.745delQ deletion (patient 3) are noted in dbSNP and scored of “unknown significance”.

The factors responsible for the strikingly different clinical manifestations of HX and its severity are still poorly understood. Some studies suggest that the clinical features of HX are, at least to some extent, related to the location of the causative mutation [20]. When non-critical parts of the PIEZO1 channel are affected, the resulting defect can vary greatly in strength. Such was the case for our patients 1 and 3 presenting with, respectively, typical strong and mild HX features. In both of them the mutations located to regions encoding peripheral helices of PIEZO1, exons 16 and 17, respectively [21].

Patient 4 was unusual as he carried two apparently unrelated mutations affecting the RBC: a missense mutation in the PIEZO1 gene and a 12-nucleotide in-frame deletion in the β-globin gene. Both these mutations were confirmed as pathogenic by different algorithms, and molecular modeling of the T1732 M mutated PIEZO1 protein showed its markedly higher stability compared to the normal variant. At face value this is not a typical signature of pathogenic mutations. However, one should bear in mind that protein functioning requires a considerable degree of conformational flexibility, therefore an excessive structural stability can actually be detrimental. Notably, de novo-designed proteins often show substantially higher stability than their natural counterparts, which suggests that their conformations (and the underlying amino acid sequences) shaped by evolution are not the most stable structures possible but rather represent a trade-off optimum between high stability and the flexibility required for functioning. On the other hand, one should note that a paralog of Piezo1, Piezo2, does contain a methionine at position corresponding to residue 1732 of Piezo1 in the mouse, pongo and human, which does not compromise its activity. Taken together, these informations suggest that the T1732 M substitution in PIEZO1 could affect the erythrocyte stability, albeit only functional studies of this PIEZO1 variant will prove that this is indeed so.

Dominantly inherited β-thalassemias are due to mutations that lead to production of a truncated or elongated and highly unstable β-globin chain. Mutations of this type have been found in many different ethnic groups, with very low frequency. Affected individuals have moderate to severe anemia, splenomegaly and the hematological features of β-thalassemia – elevated HbA2 and imbalanced globin chain synthesis [22]. Combined defects of red cell membrane and metabolism are very rare and difficult to diagnose properly, and carriership for a metabolic defect can modify the clinical picture of the patient. In fact, reports on the
impact of co-occurring mutations responsible for various defects of the red blood cell are inconclusive. It has been noted that the concomitance of β-thalassemia and hereditary spherocytosis can in fact reduce the degree of hemolysis [23]. In contrast, Fermo et al. [7] state that the hemolytic effect of hereditary spherocytosis associated with pyruvate kinase deficiency and the β-thalassemia trait does not differ from the typical HX cases. Furthermore, the coexistence of mutations in the erythrocyte membrane protein 4.2 gene and α-thalassemia-causing deletions results in a decreased hemoglobin concentration, microcytosis, and an increased red cell distribution width value [24]. Similarly, hereditary xeroctytosis due to a PIEZO1 mutation combined with the hemoglobin C (HbC) trait was associated with increased clinical severity [25]. Glogowska et al. [26] presented a patient with a PIEZO1 mutation co-inherited with heterozygous β-globin Cincinnati. A patient with an almost identical β-globin mutation (β-globin Geneva) exhibited only mild microcytic anemia [27], while in the former case the clinical severity was more profound and the patient had to be transfused regularly. In contrast, a deletion affecting codons 63–65 (c.189_195del TCATGGC) in a family from China leads to the β(0)-thal phenotype, but one should note that it causes a shift in the reading frame and a premature stop codon that causes nonsense-mediated decay of the mutant messenger RNA [28].

The severe clinical symptoms of patient 4 are unlikely to have been caused by the (previously not reported, therefore uncharacterized) β-globin defect alone. The deletion of the four amino acid residues 62–65 of β-chain results in a deformation of the heme pocket, particularly its distal side, indicating a consequent disorder in the oxygen-ion complex formation. The absence of the distal histidine important for the Fe-O-O coordination must itself have a similar effect. Whether the clinical picture of the patient is caused by a strong modifying effect of the otherwise non-detrimental mutation in PIEZO1, or is combined effect of two strongly deleterious mutations, cannot be decided at present.

To summarize, four previously clinically uncharacterized mutations in the PIEZO1 gene have been identified in as many patients with diverse forms of hemolytic anemia. For two of them the predicted clinical significance and molecular modeling consistently pointed to their pathogenicity, while in the other two cases the results were less clear-cut. Notably, family studies excluded a pathogenic effect of the p.D1566G variant predicted to be deleterious, therefore for this patient the genetic basis of the disorder remains to be identified.

Author contribution

KM, MG carried out experiments and wrote the manuscript, AAS, TU recruited the patients and were responsible for clinical care of the patients, JP, DP performed proteins modeling, AS performed erythrocytes staining and analysis, BB was responsible for the supervision of the study and manuscript revision.

Declaration of competing interest

None of the authors has any potential conflict of interest.

Acknowledgements

We are very grateful to Dr. J. Frank for a critical review of the manuscript. We would like to thank the patients and their families for their participation in this study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bclll.2019.102378.

References


