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A novel missense polymorphism of the *EPB41* gene

Nowy polimorfizm skutkujący zmianą sensu w genie *EPB41*

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SUMMARY

Defects in ankyrin-1 are implicated in approximately half of all cases with hereditary spherocytosis. Our studies aim to provide description of the molecular basis of this disease in some selected families in Poland. We report a case of a family with diagnosis of hereditary spherocytosis. The analysis of the erythrocyte membrane protein profile revealed a reduction of overall and 2.1 ankyrin in two HS patients father and one son. In the reported case, disease was manifested by symptoms typical of HS. The analysis of genes: *ANK1*, *SLC4A1*, *SPTB* and *EPB41* coding regions of the genomic and mRNA sequences revealed presence of a new polymorphism in the exon 13 of the *EPB41* gene (G358D). This heterozygous substitution was present in the father and both sons (HS and healthy), it was not detected in the mother and healthy control. We report here a novel polymorphism which occurs in HS patients *EPB41* gene coding for protein 4.1. The pattern of inheritance however, suggests that this polymorphism is either not associated or only partly associated with HS phenotype. In any case this seems interesting as most mutations found in this gene have been so for assigned to hereditary elliptocytosis and is not typical of North European population.

KEY WORDS: Protein 4.1 – Ankyrin – Erythrocyte membrane – Spectrin/actin interaction – Hereditary spherocytosis

STRESZCZENIE

Defekty ankiryny-1 są przyczyną prawie połowy wszystkich przypadków dziedzicznej sferocytozy. Celem prowadzonych przez nasz zespół badań jest określenie podłoża molekularnego tego schorzenia w wybranych polskich rodzinach. W niniejszej pracy opisujemy przypadek rodziny z diagnozą dziedzicznej sferocytozy. Analiza profilu białkowego błony erytrocytów wykazała ubytek zarówno ankiryny 2.1, jak i ankiryny całkowitej. W opisywanym przypadku, obraz kliniczny jest typowy dla dziedzicznej sferocytozy. Analiza sekwencji kodującej genomowego DNA oraz mRNA genów *ANK1*, *SLC4A1*, *SPTB* and *EPB41* wykazała obecność nowego polimorfizmu zlokalizowanego w eksonie 13 genu *EPB41* (G358D). Ta heterozygotyczna substytucja była obecna u ojca i syna (obaj z diagnozą HS) oraz u drugiego bezobjawowego syna, nie stwierdzono jej natomiast u matki i osób zdrowych (kontroli). W niniejszej pracy przedstawiamy jak dotąd nie opisywany przypadek dziedzicznej sferocytozy z towarzyszącą mu zmianą w genie kodującym białko 4.1 (*EPB41*). Wprawdzie wzór dziedziczenia sugeruje, iż zmiana sekwencji nukleotydowej nie jest lub jest tylko częściowo związana z fenotypem HS, interesujący jest jednak fakt, iż większość mutacji w tym genie, jak do tej pory, związana była jedynie z dziedziczną eliptocytozą, która nie jest typowa dla populacji północnoeuropejskiej.

SŁOWA KLUCZOWE: Białko 4.1 – Ankiryna – Błona erytrocytu – Interakcje spektryna/aktyna – Dziedziczna sferocytoza.

INTRODUCTION

Hereditary spherocytosis (HS) is a common inherited anaemia in northern Europe characterised by the presence of spherocytic red cells and by heterogeneous clinical presentation, and heterogenous molecular basis and inheritance. The primary molecular defects reside in the red blood cell membrane, particularly in proteins involved in the vertical interactions between the membrane skeleton and the lipid bilayer. Defects in these interactions lead to the loss of red cell surface area and to the spheroidal shape of the erythrocyte, in particular to the loss of the membrane elasticity and mechanical stability. Severe HS is often associated with a substantial reduction of, and/or dysfunction of, the affected membrane protein(s). Molecular diagnosis of HS cases focuses on the molecular characterization of the genomic defects and the resulting protein functional alterations. Mutations responsible for hereditary spherocytosis (HS) lie in a variety of genes encoding erythrocyte membrane proteins (ankyrin, band 3, α- and β-spectrin, protein 4.2) [1, 2]. Inheritance of HS is usually (75%) autosomal, dominant. Similar to HS, the hereditary elliptocytosis (HE) syndromes have been associated with α- and β-spectrin, protein 4.1 and glycophorin C [3]. The 4.1R protein of the erythrocyte membrane is critical for the membrane-associated cytoskeleton structure [4, 5, 6]. It binds tightly to β-spectrin and strengthens the otherwise weak spectrin/actin interaction. Therefore mutations in the *EPB41* gene might affect vertical interactions of skeletal attachment to membrane and have the potential to cause erythroid and non-erythroid defects [7, 8]. For example, in central neurons 4.1R regulates the stabilization of AMPA receptors on the neuronal surface at the postsynaptic density, and a defect of the 4.1R membrane protein is associated with neuroacanthocytosis. We report here a novel polymorphism which occurs in HS patients *EPB41* gene coding for protein 4.1. Previously we performed an analysis of the red cell membrane proteins in the patients from the family reported in this paper. Both patients – father and one son (G30 and G31), exhibited a reduction reaching more than 50% of overall and 2.1 ankyrin in the erythrocyte membrane [9]. Genomic DNA from both patients was found to be heterozygous for the AC repeat length (G30 – 11/14 AC repeats, G31 – 13/14 AC repeats) in the 3' untranslated region of ankyrin-1gene. Simultaneously we identified 14-nucleotide deletion, which was found to be heterozygotic (G31) and homozygotic (G30). This deletion is shifted by 5 nucleotides in relation to another 14-nucleotide deletion located in the 3' untranslated region of ankyrin-1 gene listed in SNP NCBI data base (rs 6150565). Both polymorphisms were found also in normal individuals; therefore, most probably, the polymorphisms are not the cause of spherocytic phenotype [10].

MATERIALS AND METHODS

Patients

A case of hereditary spherocytosis diagnosed in a Polish family (father G30 and son G31) was the subject of this report. Haematological examinations confirmed the presence of moderate haemolytic anaemia, an increased red cell osmotic fragility and the presence of spherocytes. The propositus (G31) underwent splenectomy. Mother and the other son were free of any haematological manifestations. Both patients showed a depressive status (G30 – heavy, G31 – moderate).

ANK1, *SPTB* and *EPB41* genes - Genomic DNA studies

For the purpose of the study the lymphocyte DNA was isolated by using Perfect gDNA for Blood Mini kit (Eppendorf AG, Germany) and PCR primers were designed[1]. The 42 exons and the 5-untrans-

[1] All primer sequences used in this study are available from corresponding Author on request.

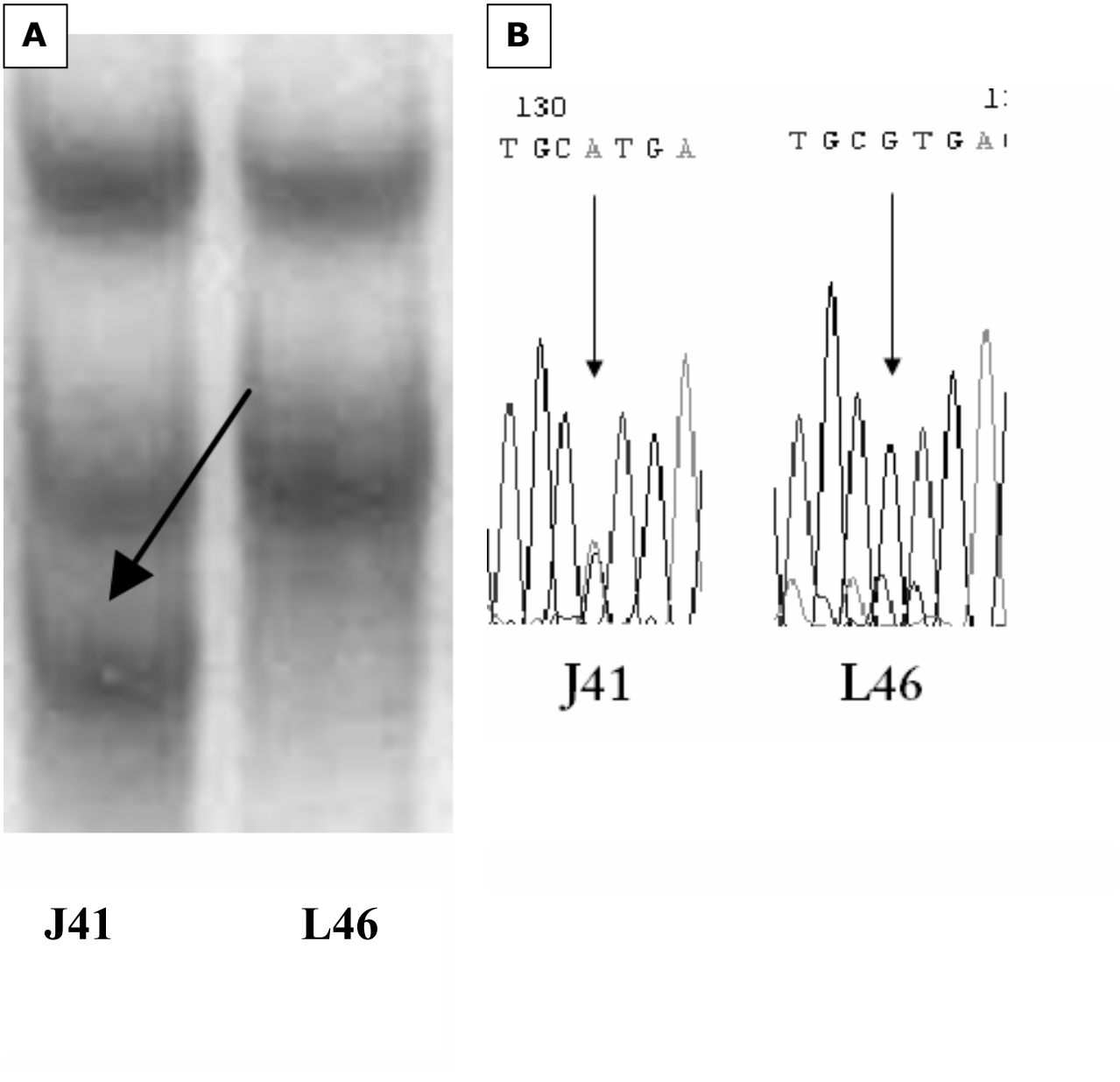

Fig. 1. Jaźwiński P. et al.

A. Example of MSSCP analysis of the PCR products covering the exon 41 of *ANK1* gene. Normal type (*L46)* and heterozygote (J41) are visible.

B. Example of the fragments of DNA sequence chromatograms showing the identified polymorphism (SNP NCBI: rs 516946) located in the sequence encoding regulatory domain of erythrocyte ankyrin (J41 – sense strand, heterozygotic case, L46 – sense strand, homozygotic case – normal type).

Ryc. 1. Jaźwiński P. i wsp.

A. Przykładowy elektroforetogram wykonany metodą MSSCP przedstawiający rozkład frakcji dla eksonu 41 genu *ANK1*. Widoczny jest fenotyp normalny (*L46*) i fenotyp ze zmianą w układzie heterozygotycznym *(J41)*.

B. Przykładowe fragmenty chromatogramów sekwencji DNA przedstawiające zidentyfikowany polimorfizm (SNP NCBI: rs 516946) zlokalizowany w sekwencji odpowiedzialnej za kodowanie domeny regulatorowej ankiryny erytrocytarnej (J41 – nić sensowna, fenotyp ze zmianą w układzie heterozygotycznym, L46 – nić sensowna, fenotyp bez zmiany – homozygotyczny).

lated region and also promoter of the *ANK1* gene was examined by Multitemperature Single Strand Conformation Polymorphism (MSSCP) technique using DNA Pointer System (Kucharczyk T.E., Poland) and, when appropriate, nucleotide sequencing. PCR products of the promoter and 42 exons of the *ANK1* gene, the 11, 12, 13 exons of the *SPTB* gene and the 13 exon of the *EPB41* gene were directly sequenced by using dideoxy chain termination method. Sequencing was carried out at least twice.

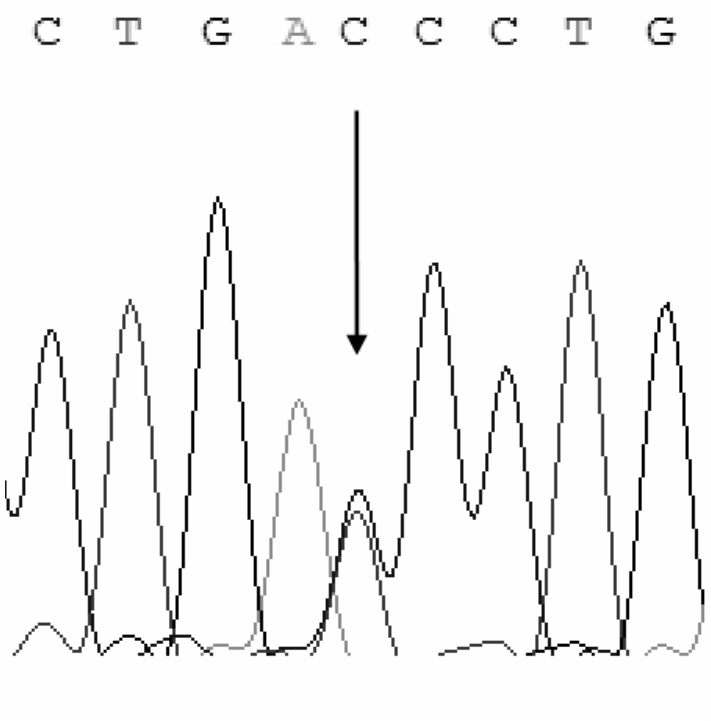

Fig. 2. Jaźwiński P. et al.
Chromatogram from an automated DNA sequencer with location for found missense polymorphism G358D (GGT→GAT) in exon 13 of *EPB41* gene in heterozygotic case.
Ryc. 2. Jażwiński P. i wsp.
Chromatogram z automatycznego sekwencera DNA. Zaznaczono zlokalizowany w eksonie 13 genu *EPB41* missensowny polimorfizm G358D (GGT→GAT) w układzie heterozygotycznym.

RNA studies

Total RNA was obtained from peripheral blood by using RNeasy Mini Kit (Qiagen GmbH, Germany). Reverse transcription (RT) was performed by using AMV Reverse Transcriptase (Finnzymes Oy, Finland). PCR reactions were performed using sequence specific primers and the products were analyzed by nucleotide sequencing. We amplified cDNA's from proband G31, and using the primers enabled the screening of the whole mRNA of the protein 4.1 and the mRNA of the cytoplasmic domain of band 3 (cdb3).

RESULTS AND DISCUSSION

Our studies aim to provide a description of the molecular basis of hereditary spherocytosis in several families from South-Western Poland. Here we report a case of dominant hereditary spherocytosis in a Polish family. Although both patients father and one son exhibited a reduction in total and 2.1 ankyrin in the erythrocyte membrane [9], the mutation was not present in any of the 42 exons and the 5-untranslated region and promoter of the *ANK1* gene, in the region encoding the ankyrin-binding site of the erythroid β-spectrin or in the region encoding the cytoplasmic domain of band 3 (cdb3). Figure 1 presents an example of the results showing the known polymorphism (SNP NCBI: rs 516946) located in the exon 41 of *ANK1* gene, identified with MSSCP analysis. Genomic DNA from both patients was found to be heterozygous for the AC repeat length as well as heterozygous and homozygous for 14-nucleotide deletion in the 3' untranslated region of ankyrin-1gene. Both polymorphisms were found also in normal individuals; therefore, most probably, the polymorphisms are not the cause of spherocytic phenotype [10]. Therefore, one should assume that in the examined family, the molecular basis of HS phenotypes is related to the mutation(s) in genes other than *ANK1*, *SPTB* or *SLC4A1*, coding for erythrocyte membrane proteins.

We started the analysis of the *EPB41* gene from the cDNA obtained from the patients or normal persons' reticulocytes. We found that codon 358 located in the exon 13 of genomic sequences could have been mutated. The analysis of the *EPB41* gene nucleotide sequence confirmed the presence of novel missense polymorphism (we propose to call it "protein 4.1 Legnica" as the patients are residents of this town) in codon 358: GGT→GAT (exon 13 of the *EPB41* gene, chromosome 1, contig position 12204022) (Fig. 2). This single-nucleotide substitution resulting in the replacement of G358 for D was present in the father and both sons (HS and healthy), and it was not detected in the mother and healthy subjects serving as controls. The identified missense polymorphism is inherited as an autosomal dominant trait. There is a possibility that in the case of this family we are probably dealing with a combined heterozygote and the identified polymorphism could be only one of them. Presence of this change in healthy patient's brother additionally excludes this change as a cause of the disease. Other causes of the disease are also possible in the examined cases.

New, missense polymorphism in exon 13 of *EPB41* gene was found in HS patients and asymptomatic patient's brother. The pattern of inheritance, however, suggests that this substitution is either not associated or only partly associated with HS phenotype. In any case this seems interesting as most mutations found in this gene have been so far assigned to hereditary elliptocytosis and is not typical for North European population.

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3. Permissions and consent: This study was carried out under the permission from Medical University of Wrocław Ethical Committee (project nr 96/2005) and patients' consent.

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