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Defects of erythrocyte membrane proteins in Polish patients with hereditary spherocytosis

Defekty erytrocytarnych białek błonowych u polskich pacjentów ze sferocytozą wrodzoną

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S u m m a r y

Introduction. Hereditary spherocytosis (HS) is the most common red blood cell (RBC) membrane disorder. This disorder is highly heterogeneous in clinical presentation, inheritance, molecular basis and biochemical phenotype. The molecular defect involves inherited deficiency of one or several of membrane/cytoskeleton proteins: α-spectrin, β-spectrin, ankyrin, anion exchanger 1 (AE1, protein band 3) or/and protein 4.2.

Aim. The aim of this study was to investigate alterations of red cell membrane proteins in previously HS diagnosed Polish patients.

Material and methods. To identify the RBC membrane defects, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by densitometric analysis was used. Blood samples were from 41 HS patients.

Results. Protein deficiency related to HS were showed in 40 from 41 samples.

Conclusions. In analyzed population ankyrin deficiency was of the highest percentage of occurrence (73%). The next frequent deficiency was spectrin α (35%) followed by protein 4.2 (27%), AE1 (27%) and spectrin β (24%). Most (85%) of the analyzed HS samples revealed combined deficiency. Only 15% (6/40) of HS patients showed isolated protein deficiency: ankyrin (4/41) and spectrin α (2/41). We also analyzed distribution of the protein deficiencies among members of 6 families. Only in one family the pattern of deficiencies was the same in both family members.

S t r e s z c z e n i e

Wstęp. Sferocytoza wrodzona (HS) jest najczęstszą membranopatią krwinek czerwonych. Jest to zaburzenie bardzo niejednorodne zarówno na poziomie objawów klinicznych, sposobu dziedziczenia, jak i podstaw molekularnych. Defekt molekularny związany jest z wrodzonym niedoborem jednego lub kilku białek błonowych/cytoszkieletu: α-spektryny, β-spektryny, ankiryny, białka pasma 3 lub/i białka 4.2.

Cel pracy. Celem niniejszej pracy było zbadanie zmian białek błonowych/cytoszkieletu krwinek czerwonych leżących u podłoża HS u polskich pacjentów.

Materiał i metody. W celu jakościowego i ilościowego określenia defektów białek błonowych erytrocytów wykorzystano metodę elektroforezy w żelu poliakrylamidowym w obecności siarczanu dodecylu sodu (SDS-PAGE), a następnie analizę densytometryczną. Próbki krwi pochodziły od 41 pacjentów z HS.

Wyniki. Defekty białkowe związane z HS zaobserwowano w 40 z 41 próbek.

Wnioski. W analizowanej populacji zdecydowanie najczęściej występującym deficytem białkowym był niedobór ankiryny (73%). Kolejnym często identyfikowanym deficytem białkowym był niedobór α-spektryny (35%), a następnie białka 4.2 (27%), AE1 (27%) i β-spektryny (24%). Większość (85%) badanych próbek HS wykazała tzw. mieszany defekt białkowy. Natomiast tylko u 14,6% (6/41) badanej populacji zidentyfikowano tzw. izo-

lowany deficyt białkowy, tj. niedobór ankiryny (4/40) i α-spektryny (2/40). Analizowano także rozkład niedoborów białkowych wśród członków 7 rodzin. Tylko w jednym przypadku wzór deficytów białkowych był taki sam u obu członków rodziny. Porównanie dostępnych wartości testu EMA ze zidentyfikowanymi deficytami białkowymi nie wykazało korelacji.

INTRODUCTION

Hereditary spherocytosis is the most common red blood cell (RBC) membrane disorder, that occurs in all ethnic groups, in Caucasians with the prevalence ranging from 1:2000 to 1:5000 (1). This disorder is highly heterogeneous in clinical presentation, inheritance, molecular basis and biochemical phenotype. HS is clinically characterized by anemia, jaundice, splenomegaly and gallstones. However the clinical severity of HS varies from symptom-free carrier to severe anemia. During microscopic examination of peripheral blood smear, RBC reveal spheroidal shape and the number of reticulocytes is significantly increased. The abnormal red cell morphology results in shortened cell survival due to RBC protein deficiency. Subjects with HS are characterized by inherited deficiency of one or several of membrane/cytoskeleton proteins: α-spectrin, β-spectrin, ankyrin, anion exchanger 1 (AE1, protein band 3) or/and protein 4.2. The molecular defect in one of the protein underlying HS is a cause of so-called primary deficiency. In some cases of HS more than one RBC membrane protein is deficient. This is because the primary protein defect "triggers" secondary protein deficiencies (2). Either a deficiency or dysfunction of one or more of RBC membrane proteins leads consequently to the detachment of the lipid bilayer from the spectrin-based cytoskeleton resulting in weakening of the vertical protein interaction (3, 4).

Single or combined protein deficiency in RBCs can be determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (5).

In this study we report red cell membrane proteins alterations in previously HS diagnosed patients of Polish population by densitometric analysis of SDS-PAGE separated proteins.

AIM

In our study we aimed: 1) to identify RBC membrane protein defects in Polish HS patients, 2) to analyze the distribution of membrane protein defects among members of analyzed HS families, and 3) to correlate the defined by SDS-PAGE protein deficiencies with the values of EMA test.

MATERIAL AND METHODS

In this study 41 consecutive HS patients and 60 healthy blood donors as controls were investigated. Peripheral blood was collected from patients and controls during diagnostic procedures after obtaining informed consent and approval from the Bioethics Committee of the Centre of Postgraduate Medical Education. The procedures were followed in accordance with the Helsinki international ethical standards on human experimentation.

Patients were from 31 unrelated families: 19 males and 22 females, range of age 1.5-74 years; 31 patients were aged < 18 and 10 were adults. Only four (three adults and one < 18 y.a) patients were splenectomized before the time of the study. In all but two cases, HS was diagnosed on the basis of the clinical history, physical examination ant the results of the laboratory tests: positive EMA (eosin-5-maleimide) binding assay, complete blood count, blood smear examination, reticulocyte count. None of the patients had had transfusions within the 3 months preceding the study.

Blood samples of healthy blood donors were used as a controls. For each RBC ghost preparation blood of three blood donors was taken and after separation from plasma and WBC, RBC were pooled and treated as a control sample.

Peripheral venous blood samples (4-10 ml) drawn from the patients and controls were collected on EDTA as anticoagulant and if needed stored in 4°C until processed, but no longer than 24 h. Erythrocyte ghost were prepared according to the Dodge et al. (6) with minor modifications. Briefly, erythrocyte suspension was washed with phosphate buffered saline (pH 7.2), and then cells were lysed with 5 mM phosphate buffer (pH 8.0) supplemented with 0.2M PMSF and spun at 15 000 x g for 15 min, 4°C. The supernatant was removed and cells were washed by using lysis buffer until haemoglobin-free ghosts were obtained. Purified RBC ghost were frozen in small aliquots in -70°C. Sample protein concentration was determined using Roti-Quant (Roth). Isolated membranes were subjected to SDS-PAGE in the Laemli buffer system (7). Gels were loaded with 20 μg of total membrane proteins. Before loading samples were denatured for 45 min in 37°C and reduced with sample buffer containing 5% β-mercaptoethanol. Polyacrylamide gels (8%, Rothiphorese, Roth), were stained with 0.012% Coomassie blue (R-250, Roth) in 10% ethanol, 5% acetic acid and destained in 10% ethanol and 5% acetic acid. The electrophoretic analysis for each RBC membrane protein sample from HS patient was performed in duplicate gels. Each gel was loaded as follows: controls, n = 3 and HS n = 6. Quantitative analyses were performed using ImageJ 1.48v Windows Application (8). Each band was quantified as an area under densitometry curve and its quantity was expressed as percent of total, when for total the sum of bands: α-spectrin, β-spectrin, ankyrin, anion exchanger1 (AE1), protein 4.1, protein 4.2 and actin was accounted. The level of each analyzed HS protein was compared to controls and presented as percent of control, assuming the level of control as 1.

RESULTS

In this study 41 membrane protein samples of HS diagnosed patients have been analyzed. Quantitative analyses were performed using ImageJ 1.48v Windows Application (8). Each band was quantified as an area under densitometry curve and its quantity was expressed as percent of total, when for total the sum of bands: α-spectrin, β-spectrin, ankyrin, anion exchanger 1 (AE1), protein 4.1, protein 4.2 and actin was accounted. The level of analyzed RBC membrane protein was compared to controls and presented as proportional value, assuming the level of control as 1. As a relevant value of protein deficiency the deficit ≥ 5% was adopted.

Almost 100% (40/41) of HS patients clinically qualified to this analysis revealed abnormal level of RBC membrane proteins. In analyzed population, ankyrin deficiency was of the highest percentage of occurrence (73%). The next frequent deficiency defined was spectrin α (35%) followed by protein 4.2 (27%), AE1 (27%) and spectrin β (24%). Only one of the samples revealed no protein defects (tab. 1). Mean protein deficiency was the highest for ankyrin (22%; range 5-47%) followed by AE1 (16%; range 6-18%) and spectrin α (14%; range 4-14%). The deficit was the slightest in case of spectrin β, not exceeding 10% wit the average loss of protein amount at 5%. Most (85%) of the analyzed HS samples revealed combined deficiency. The distribution of types of protein defects combination is presented in table 2. The most common combined deficiencies were ankyrin + spectrin α (16% of analyzed population) and AE1 + ankyrin (13.5%) which is correlated with the highest percentage of occurrence of this three protein deficiencies. Only 15% (6/41) HS patients showed isolated protein deficiency: ankyrin (4/41) and (2/41).

We also analyzed distribution of the protein deficiencies among members of 7 families (tab. 3). Only in one case (family 5) the pattern of deficiencies was the same in both family members.

Correlations of available EMA binding assay results (available from coauthor, performed as in (9)) with protein deficiencies defined by SDS-PAGE are presented in table 4.

DISCUSSION

In our study we aimed to identify the distribution of RBC membrane defects in Polish HS patients. Until the 1980s spectrin deficiency-the largest and most abundant component of the erythrocyte membrane skeleton was the best known protein abnormality reported in HS (10, 11). Further researches revealed many other protein deficiencies, such as ankyrin, band 3, and protein 4.2 defects (alone or combined) being responsible for HS (12, 13). In analysis of Polish population we defined spectrin deficiency in 59% of cases (α-spectrin in 35% and β-spectrin in 24%). According to recent data, ankyrin-1 mutation is the most common cause of hereditary spherocytosis (HS), accounting for approximately 35-65% cases in Northern European populations (14-17). Our analysis demonstrate even higher than European average prevalence of ankyrin deficiency in Polish HS patients (73%).

Recessive HS caused by protein 4.2 deficiency accounts for less than 5% of all HS cases and is common in Japan but rare in other populations (18). There is a wide difference between the occurrence of protein 4.2 deficit reported from different populations: ranging from 45% in Japan, 17% in Italy, 8% in Turkey and 1% in Greece (19-22). It is interesting that representation of protein 4.2 deficiency – 27% – in analyzed Polish population is much higher than reported till now for any oth-

Table 1. Appearance of red cell membrane protein deficiencies in Polish population

Defective proteins	% of analyzed population with deficient protein	Number of patients	Mean protein deficiency	Range of protein deficiency
α-spectrin	35	13	14%	4-14%
β-spectrin	24	9	5%	5-10%
ankyrin	73	27	22%	5-47%
AE1	27	10	16%	6-18%
protein 4.2	27	10	10%	8-23%
no protein defect	3	1	–	–

Table 2. Distribution of combined protein deficiencies in Polish population

	Combined deficiency									
	ankyrin + α-spectrin	ankyrin + β-spectrin	ankyrin + protein 4.2	β-spectrin + protein 4.2	α + β-spectrin	AE1 + α-spectrin	AE1 + ankyrin	α-spectrin + protein 4.2	α + β-spectrin + protein 4.2	AE1 + ankyrin + protein 4.2
no of patients	6/37	4/37	4/37	1/37	1/37	2/37	5/37	1/37	1/37	2/37
% of analyzed population	16	10	10	2.7	2.7	5.4	13.5	2.7	2.7	5.4

Table 3. Distribution of the protein deficiencies determined by SDS-PAGE and the quantitative analysis among members of the same family

Protein level in comparison to control (= 1)		α-spectrin	β-spectrin	Ankyrin	AE1	Protein 4.2
family 1	child 1	1.04	0.97	0.86	1.00	1.03
	child 2	0.87	1.11	0.73	1.03	0.92
	mother	0.86	0.98	1.01	1.06	1.02
family 2	child 1	0.99	1.01	0.95	1.02	0.77
	child 2	0.88	0.94	NA	1.03	0.85
family 3	child 1	0.97	0.94	0.89	1.05	0.96
	father	0.90	0.97	0.90	1.04	1.00
family 4	child 1	0.98	0.96	0.69	1.05	0.96
	child 2	0.91	1.04	0.72	1.05	1.03
family 5	child1	1.16	1.08	0.78	0.88	0.95
	mother	1.09	1.20	0.82	0.82	0.90
family 6	child 1	1.01	0.99	0.77	1.01	0.90
	child 2	0.99	1.02	0.85	0.99	1.02
family 7	child 1	0.95	1.08	0.68	1.06	0.91
	child 2	0.93	1.01	0.82	1.07	1.00

*NA – not analyzed

Table 4. Comparison of the protein deficiencies with the value of EMA binding assay

	Protein deficiency determined by SDS-PAGE					EMA [%]
	α-spectrin	β-spectrin	ankyrin	AE1	protein 4.2	
P18	0.99	1.01	0.95	1.02	0.77	78.1
P19	0.88	0.94	NA	1.03	0.85	76.9
P20	0.95	1.08	0.68	1.06	0.91	74.8
P21	0.93	1.01	0.82	1.07	1.00	72.39
P23	0.94	1.02	0.92	1.02	1.05	75.3
P24	0.96	1.04	0.88	1.00	1.00	64.09
P25	1.00	0.95	1.04	1.03	0.81	61.2
P26	0.99	0.97	1.32	1.00	1.03	98.0
P35	0.98	0.96	0.69	1.05	0.96	77.08
P36	0.91	1.04	0.72	1.05	1.03	68.6
P37	0.88	1.04	0.71	1.07	0.97	70.7
P49	1.01	0.99	0.77	1.01	0.90	73.2
P50	0.99	1.02	0.85	0.99	1.02	72.0
P51	0.96	0.95	1.00	1.04	1.00	96.2

*NA – not analyzed

er western populations. The observed range of protein 4.2 deficiency in Polish population, 8-23% suggests that this are heterozygous traits or rather secondary than primary protein defect (18).

Analyses of family distribution revealed, except one case (family 5) dispersion of protein deficiencies among family members. Such different pattern familial protein deficiencies was already reported in literature (14, 23). The observed difference can be explained by distinctive influence of levels of expression of other membrane proteins on mutual interaction of all membrane and cytoskeletal proteins. Only in one case, members of family 5 (mother and a child) represent identical pattern of protein deficiencies, having decreased amount of ankyrin, AE1 and protein 4.2. Previously described phenomenon of impaired interaction of protein 4.2 with cytoplasmic HS mutants of AE1 (24) may indicate that in this family we observe effects of mutation in SLC4 gene (coding for AE1) having influence on binding of protein 4.2 and also 4.2 with ankyrin. In case of family 1, both children besides HS are also heterozygous for $-\alpha^{3.7}$ α-thalassemia mutation (results presented in this issue, K. Koza et al.). Still, neither homologous protein deficiencies profile among the members of the family was found, nor was the protein deficits characterization of this family clearly different from six other families tested.

In further step we aimed to correlate the defined by SDS-PAGE protein deficiencies with the values of EMA test. A flow cytometric-based analysis measures fluorescence intensity of RBC labeled with EMA dye, which reacts covalently with lysine-430 on the first extracellular loop of band 3 protein (13). In EMA test the level of protein abnormalities is expressed by diminished binding of EMA dye to band 3 protein and its fluorescence emission (13, 26). Since the cytoplasmic domain of band 3 interacts with ankyrin and protein 4.2, which interact with the spectrin-based cytoskeleton, and stabilizes the membrane lipid bilayer (27), absent or decreased expression of red blood cell membrane proteins observed in HS causes a disruption of the cytoskeleton network and reduces normal presentation of AE1 protein at the erythrocyte membrane (28). This results in a reduced binding of EMA dye to band 3 protein and depletion of its fluorescence emission (13, 26).

In analyzed Polish population no visible correlation of EMA binding efficiency with the pattern of protein deficiencies was found. Although the highest value of EMA binding assay (98%) was in accordance with our results, detecting no protein deficiency in SDS-PAGE (tab. 4, P26).

CONCLUSIONS

This study showed that ankyrin deficiency is the most common biochemical finding in Polish HS patients, and protein 4.2 deficiency represented more frequent cause of HS than in any other Caucasian populations reported previously.

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