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Analysis of Erythrocyte and Platelet Membrane Proteins in Various Forms of β-Thalassemia

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Abstract—Major membrane proteins have been quantitatively analyzed in erythrocytes and platelets from patients with homozygous (splenectomized and non-splenectomized) and heterozygous forms of β-thalassemia depending on severity of clinical manifestation of this disease. Quantitative analysis of erythrocyte membrane proteins revealed increase in α- and β-spectrin. (In non-splenectomized patients with homozygous β-thalassemia the amount of this protein was lower than in corresponding controls.) Besides spectrin, the increase of 2.1-2.3 fractions of ankyrin and the decrease of band 3 protein (anion-transport protein), 4.1, palladin, and glyceraldehyde-3-phosphate dehydrogenase were also found. Analysis of major platelet membrane proteins revealed significant increase in gelsolin. This increase was found in all forms of β-thalassemia irrespective of gender. Significant changes in platelet membrane protein fractions were found in patients (especially non-splenectomized) with homozygous β-thalassemia. These included significant decrease in myosin, profilin, and γ-actin and increase in actin-binding protein in both male and female patients. The content of other protein fractions (α-actinin, tubulin, tropomyosin) remained unchanged. Changes in protein fractions of erythrocytes and platelets correlated with severity of clinical manifestation of the disease.

Key words: erythrocytes, platelets, β-thalassemia, membrane proteins

Thalassemia is a heterogeneous group of inherited diseases that stem from impairments of synthesis of hemoglobin chains. Defects in the erythrocyte membrane—increased asymmetry of its phospholipid bilayer found in patients with β-thalassemia—cause increased hemolysis [1]. Good experimental evidence exists that under natural conditions of blood flow erythrocytes interact with platelets. Consequently, erythrocyte hemolysis may result in activation of platelet surface structures accompanied by increased reactivity of these cells [2]. Erythrocyte and platelet membranes exhibit both metabolic and structural functions; the latter is required for maintenance of unique shape, integrity, deformability, and rigidity of these cells [3]. A genetic defect in the β-globin chains seen in β-thalassemia influences other components of the hemopoietic system and causes anemia; the development of this diseases is often accompanied by thromboses, causing thromboembolism syndrome and hypercoagulation [2]. Changes in structure–function properties of platelets depend on membrane proteins of these cells. So the composition and content of structure–contractile platelet proteins under normal and pathological conditions require detailed investigation.

Studies by several laboratories have provided convincing evidence that manifestations of clinical and metabolic changes in patients with β-thalassemia depend on genetic variations of this disease and also on the "presence of spleen" (i.e., whether patients were subjected to splenectomy or not) [4, 5]. From this viewpoint, the study of the relationship between structure–function impairments of erythrocyte and platelet membranes in various forms of β-thalassemia is especially interesting.

MATERIALS AND METHODS

We investigated venous blood from 71 patients with β-thalassemia whose age ranged from 8 to 18 years. Forty-six patients were homozygous in β-thalassemia (20 patients had been splenectomized and 26 patients were non-splenectomized) and 25 patients were carriers of heterozygous β-thalassemia. The patients were from 8- to 18-year-old. The control group included healthy people ($n = 21$) of corresponding age.

Blood was taken from ulnar vein. Erythrocyte "ghosts" were obtained by the method of Dodge et al. [6]. Platelet membrane proteins were obtained after homogenization in a Teflon–glass homogenizer. For separation of

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membrane proteins, erythrocytes and platelets were treated with a lysing buffer containing 2% Triton X-100, 100 mM Tris-HCl, 10 mM EGTA, pH 7.4, and centrifuged in the cold (4°C) at 12,000 rpm for 5 min. The resulting particulate fraction and supernatant were then treated with denaturing solution containing 2% SDS, 2% mercaptoethanol, 10% glycerol, 1 mM EDTA, 4 mM EGTA, 150 mM Tris-HCl, pH 6.8. Protein components of erythrocyte and platelet cytoskeleton were separated by SDS-electrophoresis in Laemmli system [7] using 7.5% polyacrylamide gel. Gels were stained with Coomassie R-250. After electrophoresis gels were subjected to densitometry and protein content in bands was expressed as percent of total protein. Protein concentration was determined by the method of Lowry using bovine serum albumin as standard.

Results were statistically treated using the unpaired Wilcoxon–Mann–Whitney test.

RESULTS AND DISCUSSION

Erythrocyte membrane fractions. Electrophoresis of erythrocyte membrane proteins yielded 16 major protein fractions. According to literature data, spectrin, ankyrin, band 3 protein, actin, and band 4.1 protein are the most studied and functionally important erythrocyte membrane proteins. Spectrin tetramers bound to band 4.1 protein (and, possibly, band 4.9 protein) interact with actin core and form a continuous flexible cytoskeletal network [3].

Analysis of protein composition of erythrocyte membrane of patients with β-thalassemia revealed significant changes in relative (percent) content of major membrane proteins (Table 1). In all clinical forms of this disease, a significant decrease in band 3 protein and glyceraldehydes-3-phosphate dehydrogenase (G-3-PD), tendency to a decrease of tropomyosin and glutathione-S-transferase (G-S-T), and an increase in ankyrin 2.1-2.3 fractions were found.

The study of erythrocyte membranes in patients with β-thalassemia also revealed significant gender differences in protein composition of red cells. More pronounced disintegration of membrane protein content was found in boys. This included significant increase in α- and β-spectrin (especially in heterozygous β-thalassemia) and also 2.1, 2.2, and 2.3 fractions of ankyrin. Study of gender differences of protein composition of erythrocyte membrane content in various forms of β-thalassemia also revealed significant decrease in G-3-PD in all boys, whereas in girls the content of this protein insignificantly changed compared with the corresponding control.

Table 1. Quantitative changes in content of erythrocyte membrane proteins in β-thalassemia

Erythrocyte membrane proteins	Groups of patients with β-thalassemia			
	control	homozygous		heterozygous
		non-splenectomized	splenectomized	
α-Spectrin	11.6 ± 0.6	7.6 ± 1.0**	12.7 ± 1.3	17 ± 1.6**
β-Spectrin	11.0 ± 0.5	9.1 ± 0.5*	10.7 ± 0.8	14.8 ± 1.2*
2.1	3.0 ± 0.1	4.4 ± 0.5***	4.3 ± 0.5*	6.2 ± 0.6***
2.2	4.0 ± 0.4	6.0 ± 0.5**	6.0 ± 0.5**	6.8 ± 0.5***
2.3	3.6 ± 0.2	6.3 ± 0.8*	5.8 ± 0.4***	5.6 ± 0.4***
2.4	4.1 ± 0.3	5.1 ± 0.6	5.4 ± 0.6	4.3 ± 0.4
2.5	3.7 ± 0.3	4.8 ± 0.5	4.5 ± 0.4	3.6 ± 0.7
3	20.6 ± 0.7	15.6 ± 0.9*	14.0 ± 0.8***	14.3 ± 0.9***
4.1	4.8 ± 0.4	4.2 ± 0.5	3.1 ± 0.4***	3.4 ± 0.5*
4.2 (palladin)	6.5 ± 0.9	6.1 ± 0.5	5.0 ± 0.3*	3.3 ± 0.3***
4.5	7.8 ± 0.5	9.1 ± 1.1	8.4 ± 0.6	6.2 ± 0.8
4.9	3.1 ± 0.5	3.0 ± 0.5	3.0 ± 0.4	1.7 ± 0.2**
5 (actin)	7.1 ± 0.7	7.9 ± 0.8	6.4 ± 0.7	6.7 ± 0.8
6 (G-3-PD)	5.7 ± 0.4	4.2 ± 0.4*	4.8 ± 0.5*	3.9 ± 0.3**
7 (tropomyosin)	4.8 ± 0.3	4.2 ± 0.5	3.4 ± 0.2***	4.4 ± 0.5
8 (G-S-T)	3.7 ± 0.6	2.5 ± 0.2	2.5 ± 0.3	3.1 ± 0.8

* $p < 0.05$ (compared with control); ** $p < 0.01$; *** $p < 0.001$.

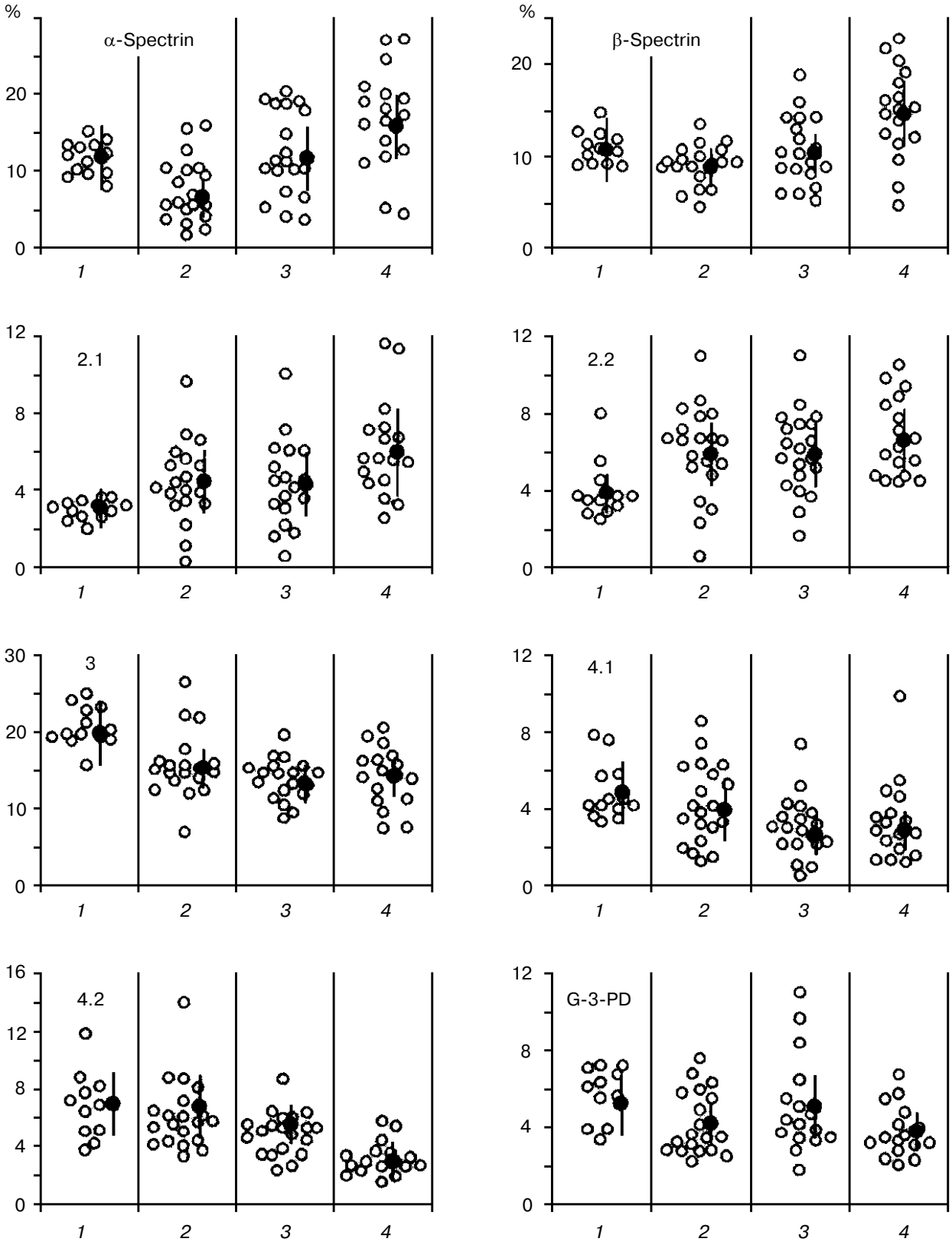

Fig. 1. Content of some erythrocyte membrane proteins in various forms of β-thalassemia (open circles show individual values and closed circles show mean for the whole group of patients): *1*) control, *2*, *3*) homozygous (non- (*2*) and splenectomized (*3*) patients); *4*) heterozygous.

In contrast to other forms of β-thalassemia, non-splenectomized patients with homozygous β-thalassemia are characterized by significantly reduced amount of α- and β-spectrin (Fig. 1). Decrease in spectrin content may significantly influence the mechanical properties of the erythrocyte membrane of non-splenectomized patients and reduce erythrocyte elasticity and deformability [3, 8]. The passage of such erythrocytes through the spleen microcapillary system causes partial loss of the erythrocyte membrane. In contrast to non-splenectomized patients, spectrin level varies within control values in splenectomized patients. Nevertheless, these patients have a higher level of ankyrin fractions. In splenectomized patients imbalance between spectrin and ankyrin results in impaired interactions between these proteins. Heterozygous β-thalassemia was characterized by significant increase in spectrin fractions. Higher content of α- and β-spectrin and 2.1, 2.2, and 2.3 ankyrin fractions in the group of patients with heterozygous β-thalassemia suggests tighter packaging of spectrin molecules in the structure of the membrane protein. Spectrin is bound to the membrane via band 2.1 protein. Ankyrin is a peripheral protein of the erythrocyte cytoskeleton; it binds to spectrin at stoichiometric ratio 1 : 1 [3, 8]. It is possible that increase in spectrin amount is accompanied by compensatory increase in various ankyrin fractions (2.1, 2.2, 2.3) in patients with heterozygous β-thalassemia as evidenced by moderate hemolysis in these patients. It should be noted that some proportion of ankyrin (10-15%) is bound to band 3 protein. Changes in content spectrin and ankyrin also cause conformational changes in the band 3 anion-transport protein. Patients with homozygous β-thalassemia are also characterized by reduced level of band 3 protein and G-3-PD compared with control (Fig. 1). In splenectomized patients with homozygous β-thalassemia and heterozygous β-thalassemia, significant decrease in band 4.1 protein, palladin, and tropomyosin was observed (Fig. 1). Proteins G-3-PD, palladin, and, possibly, band 4.1 protein are also bound to band 3 polypeptides. In the erythrocyte membrane, palladin modulates the activity of the band 3 anion-transport protein. On shortage of band 4.1 protein and palladin, the interactions spectrin–actin–band 3 protein are disturbed. Band 4.1 protein and band 4.9 protein play the key role in spectrin attachment to actin and band 3 anion-transport protein [2, 9, 10]. Reduction in band 4.9 protein seen in patients with heterozygous β-thalassemia may possibly be related to significant changes in content of spectrin fractions and 2.1 and 2.2 ankyrin fractions. Decrease in band 3 protein, G-3-PD, and palladin may strongly influence mechanisms of transmembrane cation transport. This results in accumulation of calcium ions in erythrocytes, which promotes increased degradation of erythrocyte membrane proteins by Ca^{2+}-dependent proteases.

Deficit of band 3 protein seen in various forms of β-thalassemia leads to reduction in the aminophospholipid phosphatidylserine, and this correlates with thrombotic risk [11, 12]. Erythrocyte hemolysis results in release of large amounts of biologically active compounds (e.g., ADP, free heme, etc.), which may influence the functional state of platelets. Free heme tightly binds to the lipid bilayer of platelet membrane or cytoskeleton and this results in significant imbalance in platelet protein spectrum.

Platelet membrane proteins. Electrophoresis of platelet membrane proteins from patients with β-thalassemia revealed 20 fractions. We have identified eight main protein fractions that play structure–contractile function (Table 2). Using 2D-electrophoresis, some

Table 2. Structure–contractile proteins in platelets of patients with β-thalassemia

Protein	Groups of patients with β-thalassemia			
	control	homozygous		heterozygous
		splenectomized	non-splenectomized	
Actin-binding protein	8.4 ± 0.7	9.8 ± 0.6	10.5 ± 0.7*	9.0 ± 0.7
Myosin	12.0 ± 0.7	10.0 ± 0.5*	9.1 ± 0.6**	11.7 ± 0.5
α-Actinin	13.5 ± 1.2	13.4 ± 0.8	13.3 ± 1.2	13.2 ± 0.6
Gelsolin	2.6 ± 0.3	7.4 ± 0.6***	12.4 ± 0.7***	4.9 ± 0.8**
Tubulin	12.1 ± 1.1	12.6 ± 1.0	13.1 ± 1.1	12.0 ± 0.7
γ-Actin	18.1 ± 0.7	15.6 ± 1.4	12.8 ± 0.8***	16.9 ± 1.1
Tropomyosin	14.3 ± 0.8	15.1 ± 0.9	13.0 ± 0.9	13.6 ± 0.9
Profilin	19.5 ± 1.4	19.7 ± 1.8	16.2 ± 0.9*	18.5 ± 0.8

* $p < 0.05$ (compared with control); ** $p < 0.01$; *** $p < 0.001$.

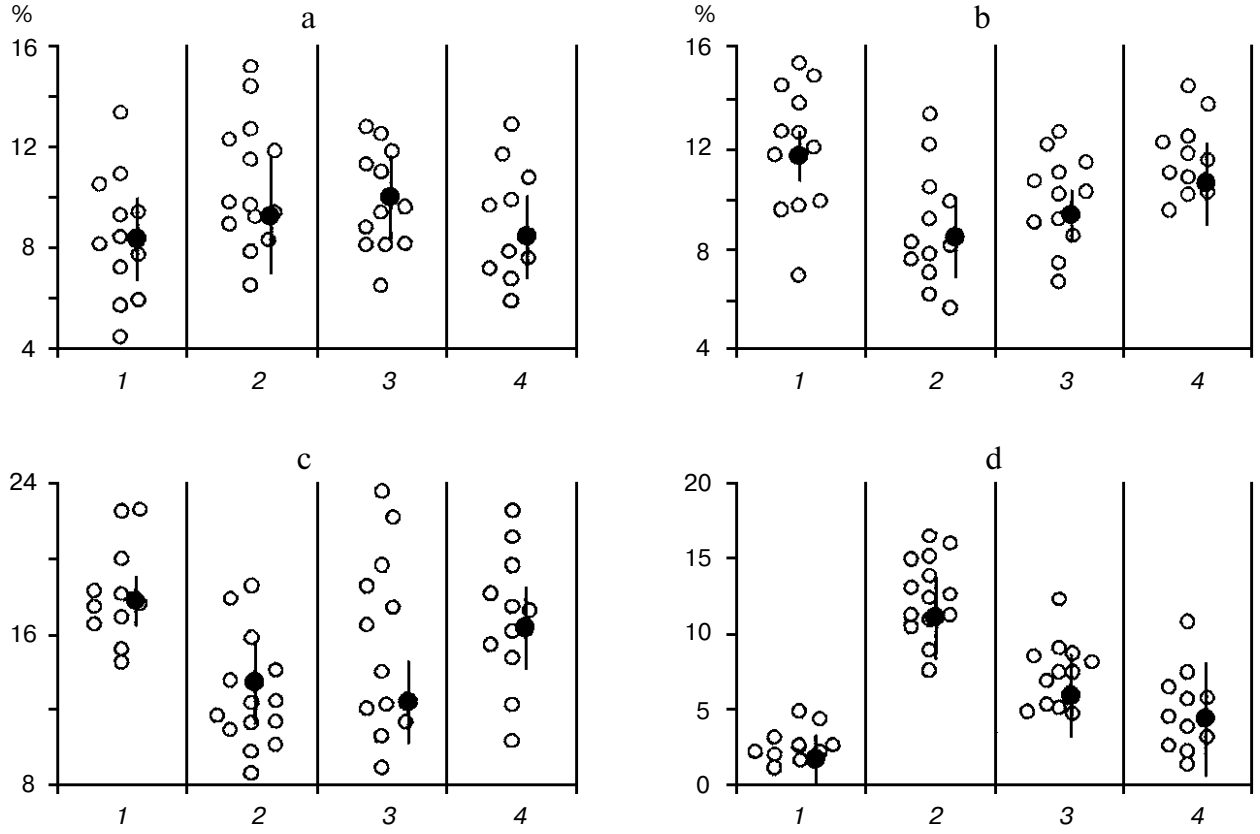

Fig. 2. Content of some structure–contractile platelet proteins (actin-binding protein (a), myosin (b), γ-actin (c), gelsolin (d)) in various forms of β-thalassemia (open circles show individual values and closed circles show mean for the whole group of patients): *1*) control; *2*, *3*) homozygous (non- (*2*) and splenectomized (*3*) patients); *4*) heterozygous.

authors were able to detect 105 constantly present fractions [13].

Besides the main contractile proteins (actin and myosin) that represent 30-50% of the total amount of protein, some other (additional) proteins have been recognized; these include actin-binding protein, α-actinin, profilin, gelsolin [14, 15], etc. Platelets (like muscles) possess their own contractile system and proteins involved in the contraction process.

Table 2 shows that platelets of patients with homozygous β-thalassemia are characterized by significant reduction in myosin fractions, whereas levels of γ-actin and profilin are mainly reduced in platelets of patients with homozygous β-thalassemia.

Separate analysis of gender differences revealed a tendency to myosin reduction and significant reduction in γ-actin in non-splenectomized boys with homozygous β-thalassemia. In girls, there was significant reduction in myosin level in patients with homozygous β-thalassemia; reduction in γ-actin and profilin was mainly found in non-splenectomized patients.

Myosin is the main factor of contractile capacity of platelets. Reduction of myosin level in patients with homozygous β-thalassemia suggests reduction in the contractile potential of platelets.

Contraction requires transition of monomeric actin (γ-actin) into polymeric actin (φ-actin), which is potentiated by Ca^{2+} ions [16, 17]. The polymeric φ-actin bound to myosin is involved in contraction.

Results of our studies suggest that cytoskeleton of intact platelets contains only 32.4% of total platelet actin and even less of myosin (15.3%). Activation of intact platelet cytoskeleton with ADP results in significant increase in the amount of actin and myosin in the cell [17]. Reduction in actin and myosin in platelets of patients with homozygous β-thalassemia (Fig. 2) apparently represents one of the main reasons for functional impairments of these cells. Reduced amount of profilin (which forms a complex with monomeric actin, known as profilactin) in platelets of patients with β-thalassemia possibly reflects reduced level of γ-actin.

Gelsolin forms centers of actin polymerization during platelet activation [14]; increased content of this protein in patients with β-thalassemia (for both genders) probably reflects a compensatory response of the cell to total transition of γ-actin into φ-actin,

which participates in contraction in the complex with myosin.

Increased level of actin-binding protein found in platelets of patients with β-thalassemia (Fig. 2) suggests tighter interaction of this protein with the membrane surface (actin-binding protein may link ends of actin filaments with platelet membrane surface). Phosphorylated actin binding protein may link the end of actin filaments into parallel bundles (stress-fibers). Pseudopodia formation is accompanied by increased length of microfilaments. They form stress-fibers, which "stick out" of plasma membrane sites and constitute a backbone for the forming pseudopodia [18]. This may explain the predominance of spherical shape of platelets with formed pseudopodia over normal discoid form of platelets in patients with β-thalassemia [19].

Results of the present study suggest the existence of insignificant tendency to increase in tubulin level in patients with homozygous β-thalassemia. Microtubules formed by polymers of this protein play a major structural role in platelets. In patients with β-thalassemia (especially in non-splenectomized patients), increased platelet volume was observed [20]. This possibly reflects increased content of this protein.

Thus, results of the study of structure–contractile platelet proteins suggest impairment of the relationship between fractions of membrane proteins in patients of both genders with homozygous β-thalassemia. In patients with heterozygous β-thalassemia, insignificant changes in the level of structure–contractile protein fractions were observed.

Thus, results of studies of erythrocyte and platelet membrane proteins suggest impairments of both absolute and relative content of the protein fractions in patients with β-thalassemia. These changes were especially notable (for both genders) in patients with homozygous β-thalassemia. This may be one of the main reasons for structure–function impairments found in blood cells.

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