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RESEARCH ARTICLE

Evaluation of protein Z plasma level in beta-thalassemia major patients in Ahvaz city in Iran

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OBJECTIVES: Thrombotic episodes occurred frequently in beta-thalassemia major (BTM) patients, leading to hypercoagulability of plasma. Protein Z (PZ) is a vitamin-K-dependent anti-coagulation factor that plays a role in the human homeostatic process. The objective of the current study is to investigate the distribution pattern of PZ plasma concentrations between BTM patients and the normal population in Ahvaz city, the center of Khuzestan province, southwest of Iran.

MATERIAL and METHODS: Forty confirmed BTM patients and 40 healthy volunteers were evaluated for complete blood count (CBC) indices and PZ plasma levels. CBC samples were measured using an automated cell counter, and PZ was assayed with an immunoassay method. Statistical analysis was conducted using SPSS software. The ROC curve and binary logistic regression estimated the sensitivity, specificity, and Odd's ratio for PZ measurement.

RESULTS: The mean±SD of the PZ plasma level in normal individuals was 1.68±0.63 μg/mL, and in BTM patients, it was 1.10±0.52 μg/mL. This shows a significant reduction of PZ in BTM patients statistically (CI = 0.99; $p < 0.001$). Further, the mean±SD of the PZ plasma levels in BTM patients who received washed red blood cells was not significantly different from that of patients undergoing packed red blood cell therapy (CI = 0.95; $p = 0.320$). The area under the curve (AUC) for PZ was 0.759 ($p = 0.00$). The cut-off value = 1.4 μg/mL of the PZ plasma level had at least 70% sensitivity and specificity in BTM patients.

DISCUSSION: Several epidemiologic studies have shown thromboembolism episodes in BTM patients. In the current study, PZ was reduced significantly in BTMs.

CONCLUSION: We noticed that BTMs have lower plasma PZ concentration might be predisposed to BTM.

Keywords major beta-thalassemia, protein Z, thrombosis, immunoassay, anti-coagulation factor

Introduction

The deficiency or absence of the beta chain of hemoglobin leads to major beta-thalassemia, which is a kind of severe and chronic anemia (Weatherall, 1976). Thalassemia affects various organs and is associated with a considerable increase in morbidity and the mortality rate (Cunningham et al., 2004).

Beta-thalassemia major is a severe form of thalassemia, and patients are dependent on blood transfusion throughout their life spans (Steinberg, 2001). Thalassemia is a vastly heterogeneous disease because of various mutations of the beta globin gene and other disease-associated factors. It is well known that BTM patients undergo an increase in the rate of thrombotic episodes. These episodes include transient ischemia, stroke, and arterial and vascular thrombosis, which in turn result in an increased rate of hypercoagulability (Michaeli et al., 1992). The relatively high prevalence of co-inherited α-thalassemia and hemoglobinopathies among β-thalassemia carriers reveals the importance of molecular

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analysis to diagnose double heterozygous for prenatal diagnostic purposes (Alizadeh et al., 2014). A lack of information about prenatal testing can cause thalassemia major births, which can be prevented if we increase awareness of screening and prenatal genetic diagnosis services (Mendiratta et al., 2017). Various hemostatic disturbances mediate these episodes, including abnormalities of the red blood cell (RBC) membrane and charge (Kuypers and de Jong, 2004), the presence of activated platelets (Winichagoon et al., 1981), a low or high plasma level of coagulation, and anti-coagulation factors (Eldor and Rachmilewitz, 2002). It is shown that a decreased level of anti-coagulation factors such as anti-thrombin III (AT-III), protein C (PC), and protein S (PS) induces the coagulation process (Schettini et al., 1987). Protein Z (PZ) is a vitamin-K-dependent anti-coagulation factor (Broze and Miletich, 1984) that is necessary for the function of the Z-dependent protease inhibitor (ZPI) (Yin et al., 2000). In the presence of protein Z, ZPI inactivates the coagulation factor Xa (Han et al., 2000). In the presence of Z protein, the inhibitory function of ZPI on the Xa factor increases by about 1000 times (Han et al., 1999). Published studies proposed that a decrease in the plasma level of PZ may have an important role in the occurrence of bleeding episodes (Kemkes-Matthes and Matthes, 1995), deep vein thrombosis (DVT) (Kemkes-Matthes et al., 2002), early fetal loss (Gris et al., 2002), and stroke (Vasse et al., 2001).

The current study showed that PZ plasma levels are significantly decreased in BTM patients compared to those of local normal cases. There are some questions about PZ:

1. Does a decrease in the level of PZ play a role in coagulation induction and the occurrence of thrombosis episodes?
2. How much of a decrease in the PZ plasma level causes clinical complications?
3. Is PZ necessary as an anti-thrombosis prophylactic agent for thromboembolism prevention in BTM patients? If yes, then at which cut-off point should PZ therapy be started as treatment?

To address these questions, normal PZ ranges should be determined for each local area. In addition, more studies are necessary among various subpopulations of beta thalassemia according to the presence or absence of thrombosis or splenectomy history.

According to what we mentioned Protein Z existence can decrease bleeding, and decrease in protein Z can be one reason of bleeding in thalassemia patients. Our goal is to run a study to evaluate the protein Z level in thalassemia patients. Also there are limited studies on PZ plasma levels in beta thalassemia patients in Iran. Therefore, we studied and compared the PZ plasma levels and complete blood count (CBC) indices between cases and normal control individuals to estimate Odd's ratio and to propose PZ as a probable hypercoagulability index in BTM patients.

Material and methods

Study groups

In the current study, 40 beta-thalassemia major (BTM) patients (18 males and 22 females) and 40 control individuals were evaluated for CBC indices and plasma levels of PZ. The patient's age was 17.78 ± 7.76 years, and the control's age was 18.38 ± 8.75 years. All patients and controls who participated in this work completed consent forms and permitted us to use their samples for analyzing and publishing data.

Patients and samples

Forty BTM cases were selected from the research center of thalassemia and hemoglobinopathy hospital of Ahvaz Jundishapur Medical University. Inclusion criteria were chosen according to the hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), Hb-electrophoresis in alkaline pH, family history, and beta-globin gene analysis by molecular technique. All of these data were present in the patients' history documents. Exclusion criteria were current fever, infection, or increased serum activities of alanine amino transferase (ALT) and aspartate aminotransferase (AST).

Forty normal controls were selected from patients referred to the hospital laboratory according to the inclusion criteria Hb $>$ 12 g/dL, MCV $>$ 80 fl, MCH $>$ 30 pg, and a negative history of bleeding, thrombosis, cardiovascular, or hepatic problems. Control individuals were matched with BTM patients for age and sex. All BTM patients were transfusion dependent. Some of them received washed red cells and the ot $56+$ hers packed cell on a monthly basis. Plasma samples were collected from patients at least 30 days after the last transfusion to minimize the transfused blood effect on the PZ and CBC indices. Whole blood samples were collected in EDTA-K2 containing tubes. Complete blood counts (CBC) were done using an automated procedure (Mindray apparatus). Plasma samples were separated by centrifugation and kept at −70 ºC until the day of the test.

ELISA method and statistical analysis

Plasma PZ concentrations were determined by using an enzyme-linked immunosorbent assay (ELISA) kit (ZYMUTEST Protein Z, HYPHEN Biomed) and according to the operating manual of the kit insert.

First of all, there are numbers of principles as mentioned in the kite the immunoconjugate, which is a polyclonal antibody specific for PZ coupled to horse radish peroxidase (HRP), is introduced into the microwells coated with a polyclonal antibody specific to PZ. Then, the diluted test sample is immediately introduced, and the immunological reaction

starts. When present, PZ binds onto the polyclonal-antibody-coated solid phase through one epitope, and fixes the polyclonal antibody coupled to HRP through free epitopes. Following a washing step, the peroxidase substrate, 3,3′,5,5′–Tetramethylbenzidine (TMB), in the presence of hydrogen peroxide (H_2O_2), is introduced, and a blue color develops. When the reaction is stopped with sulfuric acid, a yellow color is obtained. The amount of color developed is directly proportional to the concentration of human PZ in the tested sample.

According to the kite, we used 50 microliters (μL) of conjugate anti (h)-PZ-HRP to introduce the anti-(h)-PZ- HRP immunoconjugate in the micro ELISA plate wells. Then we added 200 μL of the tested sample introduced immediately, or the tested samples in the corresponding micro ELISA plate well. Next, we mixed them gently on a plate shaker or manually, and incubated them for 1 h at room temperature (18–25 ºC). Then, 300 μL of wash solution was used five times in a wash procedure using a washing instrument. Immediately after the washing, we introduced 200 μL of TMB/H_2O_2 substrate. We incubated for exactly 5 min at room temperature (18–25 ºC). We stopped the color development by introducing 50 μL of 0.45 M sulfuric acid. We waited for 10 min to allow the color to stabilize, and measured the absorbance at 450 nm (A450). We subtracted the blank value first.

Data analysis was conducted using SPSS Ver. 20 software for descriptive statistics, a comparison of mean±SD between groups, correlations, and Odd's ratio estimations. Furthermore, a receiver operating characteristic (ROC) curve was plotted to estimate a cut-off value of the PZ protein for the hypercoagulability state in BTM patients.

Results

Odd's ratio (OR) value for PZ (OR = 5.40; 2.20 to 13.22) suggests that a PZ assessment is valuable for BTM patients. PZ could be about 2.2- to 13.22-fold lower in BTM patients than in normal people. Furthermore, a cut-off value of 1.4 μg/mL PZ could be considerable in clinical evaluations. A lower value indicates a predisposition to hypercoagulability, and higher values may indicate a normal state of coagulation cascade.

The mean±SD for plasma levels of PZ were 1.10±0.524 and 1.68±0.63 for BTM and control individuals, respectively. There was a significant difference between the two groups statistically for PZ plasma levels (CI = 0.95, p = 0.000) (Fig. 1).

The mean of the PZ plasma levels was lower in BTM patients who received washed red cell therapy than patients who received packed cell therapy. However, the difference was not statistically significant (CI = 0.95, p = 0.32). Table 1 lists CBC data and PZ plasma levels in BTM and normal individuals. Furthermore, the table shows comparative P-vales and Odd's ratio from binary logistic regression. A high value of Odd's ratio for PZ was obtained, in addition to some CBC indices.

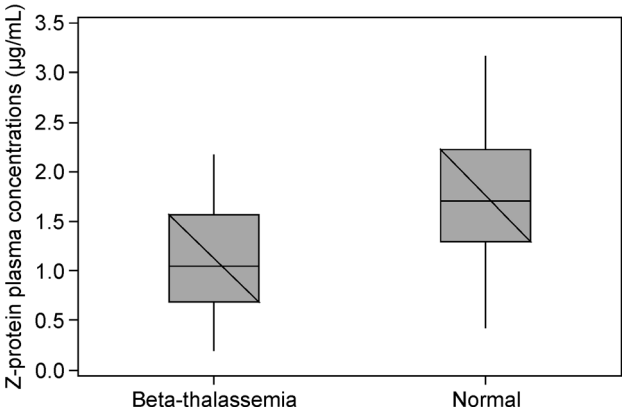

Figure 1 Comparative chart of protein Z plasma levels in beta-thalassemia major (BTM) patients and normal controls.

The average platelet count was not significantly different in the two studied groups (CI = 0.95, p = 0.365), but the difference was significant for Hb, hematocrit, and MCV, which were lower in BTM patients (Table 1). There was no meaningful correlation between PZ and other hematologic indices for BTM or normal individuals. In addition, the duration of transfusion therapy did not show a correlation with PZ protein (Table 2).

Figure 2 shows the ROC curve of PZ. The area under the curve (AUC) for PZ was 0.759 (p = 0.00). ROC analysis showed that when considering a PZ plasma level of 1.4 μg/mL, the sensitivity and specificity of the test is about 70%. In fact, 28 cases (70% of BTM patients) and 10 control individuals (28% of controls) had PZ plasma levels that were less than 1.4 μg/mL. Therefore, this value is proposed as a predictor for the hypercoagulability state in BTM patients.

Discussion

Current therapeutic approaches have the potentiated life expectancy and survival of BTM patients (Olivieri and Brittenham, 1997). However, these approaches are accompanied with new complications such as thromboembolism, pulmonary embolism, deep vein thrombosis, and portal vein thrombosis (Michaeli et al., 1992; Gillis et al., 1999; Kemkes-Matthes et al., 2002). Several epidemiologic studies have shown thromboembolism episodes in BTM patients (Borgna-Pignatti et al., 1998; Moratelli et al., 1998). Current studies showed that PZ was reduced significantly in BTM patients over normal individuals (Schettini et al., 1987; Cappellini et al., 2000; Singer et al., 2006; Hassan et al., 2010). A decrease in PZ may predispose these BTM patients to thromboembolism episodes.

Thus, PZ can be a prophylactic index for hypercoagul-

Table 1 Hematological parameters of thalassemia patients and control individuals

Hematological index	Category ($N = 40$)	Mean±SD (μg/mL)	*p*-value	Odds ratio
WBC	Case	8.19±3.10	0.075	0.86 (0.73, 1.02)
	Control	7.06±2.44		
Hb	Case	8.22±1.22	0.000	130.86 (0.64, 26849.24)
	Control	13.13±1.40		
RBC	Case	3.05±0.44	0.000	606.19 (19.79, 18571.33)
	Control	4.71±0.45		
HCT	Case	23.96±3.53	0.000	3.53 (1.10, 11.34)
	Control	38.78±3.72		
MCV	Case	78.68±4.54	0.000	1.23 (1.08, 1.40)
	Control	82.44±4.66		
MCH	Case	26.93±2.17	0.036	1.29 (1.01, 1.64)
	Control	27.91±1.92		
MCHC	Case	34.30±1.63	0.132	0.76 (0.52, 1.10)
	Control	33.82±1.10		
RDW-SD	Case	47.53±5.99	0.000	0.51 (0.37, 0.68)
	Control	40.66±2.34		
Platelet	Case	292.48±142.51	0.365	1.00 (0.99, 1.00)
	Control	268.5±85.38		
Protein Z	Case	1.10±0.52	0.000	5.40 (2.20, 13.22)
	Control	1.68±0.63		

ability states in BTM patients. Hepatic sidrosis could be the reason for a reduction in PZ production in BTM patients. It was investigated that erythrocytes of splenectomized BTM patients have more phosphatidyl serine in their outer membranes (Kuypers and de Jong, 2004). This could result in the sequestration of anti-coagulation proteins such as protein Z by the phosphatidyl serine, and in turn increase the PZ turnover, as reported for proteins C and S (Cappellini et al., 2000; Eldor and Rachmilewitz, 2002).

There are conflicting reports on the plasma level of PZ and its role in coagulation induction, especially in BTM patients. Studies have indicated a significant association between decreased levels of PZ and thromboembolism episodes such as vascular-arterial thrombosis and DVT (Santacroce et al.,

Table 2 Correlation values between hematological indices and protein Z plasma levels

Hematological index	Category ($N = 40$)	Correlation values with protein Z plasma levels	*p*-value
WBC	Case	0.155	0.339
	Control	0.318	0.046
Hb	Case	–0.008	0.962
	Control	–0.064	0.693
RBC	Case	0.039	0.809
	Control	–0.102	0.530
HCT	Case	–0.087	0.595
	Control	–0.133	0.414
MCV	Case	–0.311	0.051
	Control	–0.049	0.762
MCH	Case	–0.067	0.682
	Control	0.032	0.845
MCHC	Case	0.251	0.117
	Control	0.164	0.312
RDW-SD	Case	0.332	0.036
	Control	–0.091	0.577
Platelet	Case	–0.037	0.820
	Control	0.104	0.522
Duration of transfusion therapy	Case	0.217	0.178

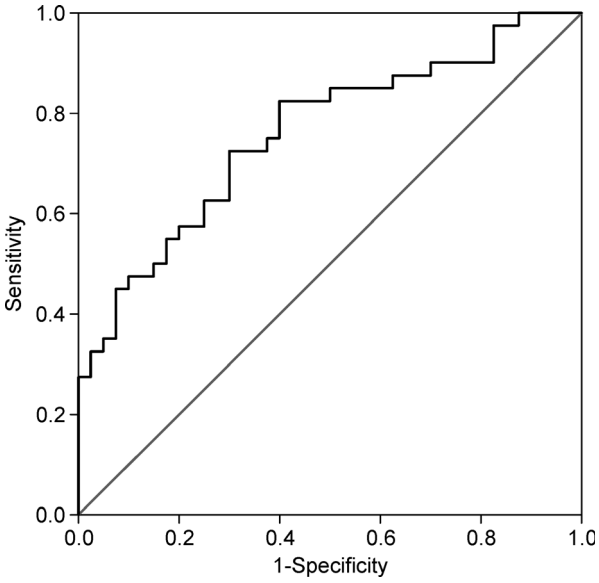

Figure 2 ROC curve of PZ plasma levels plotted according to BTM patients and normal individuals. Area under curve (AUC) for PZ was 0.759 (p = 0.00). Then, assessment of PZ could be valuable for BTM patients.

2006; Pardos-Gea et al., 2008). However, such findings do not agree with other investigations (Al-Shanqeeti et al., 2005; Elkhateeb et al., 2009). One of the most important differences between these studies is the employment of different cut-off points for reporting decreased levels of PZ. An obvious difference in cut-off points could be seen in Elkhateeb et al. (2009) (1.13 μg/mL).

Such differences in the cut-off value could cause a bias in reporting Odd's ratio (OR) and falsely overestimate reports of the thrombosis rate. These problems may stem from the wide reference range intervals that have been reported for PZ plasma concentrations. This phenomenon could be a result of vast intraindividual variability, which in turn arose from gene polymorphism in different investigated populations. As a result, it would be rational to determine reference ranges of PZ for each population according to their ethnicity and polymorphism. Such determinations help to find proper cut-off values to evaluate PZ plasma levels. This result was confirmed by Miletich and Broze (1987) but is not in accordance with Heeb et al. (2002).

Conclusion

In our study, age and PZ plasma levels did not have a significant association. However, we suggested 1.4 μg/mL cut-off values in the current study. More investigation should be performed to find a more precise cut-off value and the benefits of PZ determination. Then, comprehensive studies with larger sample sizes, in addition to polymorphism determination, are necessary to clarify such discrepancies. We reported that there was no association between most CBC indices, the duration of transfusion therapy, the type of blood product for transfusion therapy, and decreased levels of PZ in BTM patients. Significant differences in CBC indices between the case and control categories were not novel findings and were expected.

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Compliance with ethics guidelines

The authors declare no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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