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ORIGINAL ARTICLE

Association Of Prothrombin G20210 Gene Polymorphism And Risk Of Thrombosis In B.Thalassemic Children In Egypt

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ABSTRACT

Background: Hypercoagulable condition is linked to an increased risk of thromboembolic events in thalassemic children. There are multiple factors that contribute to hypercoagulable state in thalassemia patients.

Methods: This is a case control study. A total of 40 thalassemic patients and 40 healthy controls were included in the study. All subjects were tested for prothrombin gene polymorphism by by restriction fragment length polymerase chain reaction (RFLP PCR) and evaluated for other clinical and laboratory risk factors for TE including; age, sex, consanguinity, family history of TE, clinical presentation, level of serum protein C(PC), serum protein S(PS) and D-Dimer.

Results: The thalassemic group show significant decrease in PS, PC and D-dimer, compared to control group. The frequency distribution of GA genotype of prothrombin gene was 25% in thalassemic group while 15% in control. There was non-significant difference between studied groups regarding prothrombin gene polymorphism but there was significant difference regarding A allele. There were non-significant relation between prothrombin gene polymorphism and either gender, age , family history of TE

Conclusions: The natural coagulation inhibitors PC and PS were significantly reduced in patients with β-thalassemia major and were thus important risk factors for the hypercoagulable state, but prothrombin polymorphism do not seem to be significant risk factors for thromboembolic events.There was no significant difference in prothrombin genotype distrubuation between thalassemic children and control .Presence of A allele may consider additional risk of thrombosis in children with thalathemia .

Keywords:thalassemia**;** prothrombin; thromboembolism, polymorphism.

INTRODUCTION

Beta-thalassemia is a monogenic condition in which the beta-globin portion of adult haemoglobin is synthesised insufficiently or not at all **[1,2].** It is characterized by Ineffective erythropoiesis, chronic haemolytic anaemia, and clinical consequences define it **[1].** The most frequent chronic hemolytic anaemia in Egypt is beta thalassemia (85.1 percent). In 1000 normal random subjects from various geographical areas of Egypt, a carrier rate of 9-10.2 percent has been estimated **[3].**

Although the magnitude of thrombosis risk is difficult to assess, thalassemia major and intermedia are considered hypercoagulable conditions. In thalassemia intermedia, the risk of thromboembolic events is higher than in thalassemia major **[4].** The incidence of thromboembolism in patients with thalassemia diseases ranges from 1.7 to 9.2 percent **[5].** As a result, the incidence of thromboembolism in patients with thalassemia diseases is roughly 10 times higher than the general population **[6].**

Multiple factors are contributing to hypercoagulable state in thalassemia patient such as, increased endothelial activation produced by the activation of monocytes and granulocytes leads to endothelial damage and increased levels of endothelial adhesion proteins and tissue factor. Furthermore, greater activation of the hemostatic system is associated with a rise in endothelial cell, platelet, and white blood cell (WBC) and red blood cell (RBC) micro particles, which are shedded fragments having high PS with a size of 0.1–2 m from activated and dying cells **[7].**

Prothrombin, also known as factor II, is a vitamin K-dependent blood coagulation factor that serves as a precursor to thrombin, the major coagulation enzyme **[8].** The F2 gene produces thrombin, which has both procoagulant and anticoagulant

properties in its active form **[9].** G20210A is a missense mutation of a nucleotide in the 3rd untranslated region of the prothrombin gene **[10].** The polymorphism in the 3'UTR of PT G20210A (PT G20210A) was found to be strongly linked to a high risk of thrombosis **[11].** In 1–6% of the population, this mutation was discovered. 2016 **[12].** The prothrombin G20210A mutation has also been found to be a factor in 6–20 percent of confirmed thrombotic events **[13].**

The Prothrombin G20210A mutation was discovered to induce high blood prothrombin levels (by one-third above normal; 133%), which is more than the additional 15% required to develop VTE (venous thromboembolism). This mutation has also been shown to result in enhanced prothrombin mRNA and protein expression **[14],** resulting in an elevated thrombin level and hypercoagulable condition **[15].** Furthermore, elevated prothrombin levels may cause a rise in a protein known as thrombin-activatable fibrinolysis inhibitor (TAFI), which is a fibrinolysis inhibitor. Therefore, an increase in TAFI may disrupt the fibrinolysis process, allowing for the formation of clots that contribute to VTE **[16,17].** Meltzer Methylenetetrahydrofolate reductase (MTHFR), prothrombin (PT), and Factor V Leiden (FVL) gene polymorphism were found to be predisposing factors for thromboembolic symptoms in -thalassemia in previous investigations. As a result, our study attempted to assess the frequency of prothrombin gene polymorphisms in thalassemic patients as an additional risk factor for thromboembolism in thalassemic children **[19].** The existence of thrombophilic mutations in instances, together with additional risk factors such as splenectomy and anaemia (Hb 9 g/dl), may indicate that antithrombotic medication is required to prevent thrombotic events in these patients **[18].**

METHODS

Subjects

Written informed consent was obtained from all participants. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. This case –control study included forty β-thalassemia major children were evaluated in the pediatric and medical biochemistry departments of Zagazig university Hospitals for this case-control research. As controls, forty healthy children of similar age and gender were recruited from the same demographic region. This study comprised children who had been diagnosed as thalassemic patients by hemoglobin electrophoresis, had received at least ten blood transfusions, and had been on chelation therapy for at least six months.

Informed written consent was given to all participants. They were all given a thorough medical history, which included their age, thalassemia family history, positive consanguinity, blood transfusion type and amount, iron chelation therapy, prior thromboembolism history, and physical examination. BMI (body mass index) (weight in kilos, height in meters) (BMI). BMI is calculated as follows: weight (kg) / height (m) (m2). B-thalassemia has skeletal and abdominal characteristics. All of the patients were thoroughly examined for signs and symptoms of thromboembolism, such as thigh or calf pain or soreness, leg swelling (edema), warm skin on contact, and sudden unexplained shortness of breath. In all cases, liver function tests such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were performed, as well as urea, creatinine, complete blood count, PT, PTT, International normalized ratio (INR), Protein C, Protein S and d-dimer.

Blood Sampling

Five microliter venous blood was taken and divided as follows: 1.5 mL with ethylenediaminetetraacetic acid K2 for DNA extraction). and about 3.5ml collected in sodium citrate tube for estimation of protein c PC and protein S PS.

Estimation of serum protein C and S

Blood collected into one 3.2% Sodium citrate tube was centrifuged to separate platelet poor plasma which was aliquoted and frozen at –70 °C until the time of assay. Citrated sample was used for measurement of PC, PS using The AssayMax Human Enzyme-Linked Immunosorbent Assay (ELISA) kit according to manfucture instruction.

Genotyping of prothrombin G20210A gene polymorphism

Genomic DNA was extracted from whole blood using the commercially available Gspin TM Total DNA Extraction Kit (iNtron bio-tehnology, Seongnam-Si, Korea). The primers used for prothrombin G20210A amplification were 5’TCAGGCAGGAACAACACCAT3 (forward) and 5’GGTTACTTCAAGGACAAAATACCTGTAA AGCT3′(reverse).

The samples were initially denatured at 94°C for 5 min, followed by 35 cycles including 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. Samples were maintained for final extension at 72°C for further

10 min. PCR product was visualized by gel electrophoresis on 2% agarose gel (iNtRON Biotechnology, Korea) stained with Ethidium Bromide. Then 10 µl of each PCR product was digested with 10 units of Hind III in NEB Buffer for 18 hours at 37°C. Results of enzymatic digestion was separated by electrophoresis on 2% agarose gel (iNtRON Biotechnology, Korea) stained with Ethidium Bromide and the products were visualized in UV Transiluminator.

Statistical analysis

Data collected throughout history, basic clinical examination, laboratory investigations and outcome measures coded, entered and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) (Statistical Package for the Social Sciences) software for analysis. According to the type of data qualitative represent as number and percentage, quantitative continues group represent by mean ± SD, the following tests were used to test differences for significance; difference and association of qualitative variable by Chi square test (X2). Differences between quantitative independent groups by t test. P value was set at <0.05 for significant results &<0.001 for high significant result.

RESULTS

This study comprised 40 children patients with thalassemia and thromboembolism with a median age of 11±3 yearsThere are 18 males and 22 females in the group. There was no discernible difference in age or sex distribution between patients and controls (P = 0.09 and 0.9, respectively) (Table 1). Clinical presentation and complications of studied groups shown in Table 2. There was significant difference in Hb,iron,ALT,AST. PT and PTT levels between thalssemic group and control.There was significant decrease in PC and Ps level in thalssemic group compared to conrol (Table 3).

In control group, the frequencies of GG, GA, AA genotypes were 85, 15 and 0%, respectively, and in Patients group, the frequencies were 62.5,25, and 12.5 %, respectively. The frequencies of G and A allele in control were 87.5 and 12.5%; while in patients group were 75 and 25%. Chi-square revealed no significant difference regarding the distribution of Prothrombin genotypes GA and AA between patients and control (X2 = 3.015, P=0.082) (Table 4). There was significant increase in A allele carriage in patient group than control group (P = 0.0043) There was non-significant correlation between the prothrombin genotyping and the clinical characteristics of the cases group including: jaundice, splenomegaly, splenectomyand hepatomegaly (Table 5).

There was non-significant correlation between the prothrombin genotyping and the laboratory data of the cases groupincluding**:** hemoglobin, TLC, Platelet count, reticulocyte, ferritin, PT, PTT, Bilirubin, AST, AT, Urea, Creatinine except with platelets where there was decrease associated with GG polymorphism (174.96 ± 22.36$^{\infty}$ (×10³/mm³) (Table 6).

Table 1: Comparison between the studied groups regarding the demographic data

Parameter	Groups		Test	
	Case group N=40 (%)	**Control group N=40 (%)**	**Z/χ^2**	**P**
Age: Median (range)	11 (3 – 18)	5 (3 – 18)	-1.695	0.097
Gender Female Male	 18 (45) 22 (55)	 18 (45) 22 (55)	0	>0.999

Z Mann Whitney test *p<0.05 is statistically significant χ^2 Chi squaretest

Table 2: Clinical presentation and complications of studied groups.

			Group		**P**
			Case	**Control**	
Pallor	**No**	**N**	**0**	**40**	
		%	**0.0%**	**100.0%**	
	Yes	**N**	**40**	**0**	**0.00****
		%	**100.0%**	**0.0%**	
Jaundice	**No**	**N**	**0**	**40**	
		%	**0.0%**	**100.0%**	
	Tinge	**N**	**14**	**0**	**0.00****
		%	**35.0%**	**0.0%**	

			Group		P
			Case	Control	
	Yes	N	26	0	
		%	65.0%	0.0%	
Splenomegaly	No	N	2	40	
		%	5.0%	100.0%	
	Splenectomized	N	11	0	0.00**
		%	27.5%	0.0%	
	Splenomegaly	N	27	0	
		%	67.5%	0.0%	
Hepatitis	No	N	35	40	
		%	87.5%	100.0%	
	Yes	N	5	0	0.021*

Table 3: Laboratory data of the studied groups.

	Case	Control	P
HB (g/dl)	7.16±0.71	12.20±0.66	0.00**
WBCS (/mm³)	10018.6±3034.0	5885.45±1167.5	0.00**
Platelets (×10³/mm³)	182.20±17.51	384.42±16.83	0.00**
Reticulocyts (%)	2.82±0.46	0.58±0.07	0.00**
S ferritin (ng/ml)	1599.83±554.6	37.75±8.85	0.00**
PT (seconds)	11.77±0.95	10.05±0.71	0.00**
PTT (seconds)	29.65±3.34	28.07±1.71	0.010*
AST (u/l)	31.57±11.07	22.52±1.46	0.00**
ALT (u/l)	32.52±10.99	23.52±1.61	0.00**
S Bilirubin (mg/dl)	2.04±0.33	0.53±0.09	0.00**
Urea (mg/dl)	24.80±5.35	13.57±1.98	0.00**
Creatinine (mg/dl)	0.83±0.11	0.63±0.10	0.00**
Protein C activity %	76.54±16.32	82.3±13.55	0.032*
Protein S activity %	80.42±10.76	97.32±8.19	0.002*
D -dimer	3.01 ± 5.04	0.57 ± 0.36	<0.001*

Table 4: Comparison between the studied groups regarding prothrombin genotypes and alleles.

Prothrombin	Groups		Test		COR (95% CI)
	Case group N=40 (%)	Control group N=40 (%)	χ^2	p	
Genotypes:					
AA	5 (12.5)	0 (0)			3.2(0.57– 7.89)
GA	10 (25)	6 (15)	3.015	0.082	2.13(0.68– 6.67)
GG	25 (62.5)	34 (85)			1 (reference)
Alleles					
A	20 (25)	10 (12.5)	4.103	0.043*	2.3(1.01 – 5.37)
G	60 (75)	70 (87.5)			

COR crude odds ratio CI confidence interval *p<0.0 is statistically significant χ^2 chi square test

Table 5: Relation between prothrombin gene polymorphisms and clinical characters of the case group.

Parameter	Prothrombin genotype			P
	GG N=25(%)	GA N=10(%)	AA N=4(%)	
Age Mean ± SD	11 (3 – 18)	11 (4 – 18)	10 (4 – 16)	0.932
Gender:				
Female	11 (44)	5 (50)	2 (50)	>0.999
Male	14 (56)	5 (50)	2 (50)	
Family history:				
Negative	17 (68)	9 (90)	4 (80)	0.079
Positive	8 (32)	1 (1)	1 (20)	

Parameter	Prothrombin genotype			P
	GG	GA	AA	
	N=25(%)	N=10(%)	N=4(%)	
Consanguinity:				
Negative	11 (44)	6 (60)	3 (75)	0.475
Positive	14 (56)	4 (40)	1 (25)	
Spleen:				
Splenomegaly	19 (76)	6 (60)	4 (80)	0.85
Splenectomy	6 (24)	4 (40)	1 (20)	
Hepatomegaly:				
No	8 (32)	5 (50)	3 (75)	0.325
Yes	17 (68)	5 (50)	1 (25)	
Disease duration:				
<10 years	12 (48)	5 (50)	3 (60)	>0.999
≥10 years	13 (52)	5 (50)	2 (40)	
Transfusion frequency				
More than once/month	12 (48)	5 (50)	4 (80)	0.225
Once/month	13 (52)	5 (50)	1 (20)	

Table 6: Relation between prothrombin genotypes and laboratory data of the case group.

Parameter	Prothrombin genotype			p
	GG	GA	AA	
	Mean ± SD	Mean ± SD	Mean ± SD	
Hemoglobin:	7.1 ± 0.79	7.2 ± 0.79	8.0 ± 0.82	0.122
TLC	10220.04±3129.17	10031.2±3330.86	8351.5±3068.7	0.541
Platelet	174.96 ± 22.36$^{\infty}$	182.2 ± 12.97	206.25 ± 29.64$^{\infty}$	0.031*
Reticulocyte$^{¥}$	3 (2 – 4)	2 (2 – 4)	2 (2 – 3)	0.092
Ferritin$^{¥}$	1500 (545 – 5000)	1155 (200 - 3500)	982.5 (514-3428)	0.255
PT	11.68 ± 0.95	11.7 ± 0.95	11.75 ± 0.96	0.99
PTT	29.88 ± 3.22	28.9 ± 3.73	29.5 ± 4.04	0.747
S. bilirubin	2.28 ± 0.32	2.04 ± 0.44	2.38 ± 0.14	0.124
AST$^{¥}$	26 (21 – 55)	25.5 (21 – 53)	43.5 (21 – 56)	0.671
ALT$^{¥}$	24.5 (20 – 61)	26 (21 – 56)	47.5 (24 – 62)	0.395
Urea	26.0 ± 4.92	23.0 ± 5.54	23.75 ± 6.55	0.284
Creatinine	0.84 ± 0.13	0.83 ± 0.09	0.83 ± 0.05	0.978

DISCUSSION

Hypercoagulable condition is a well-known consequence of -thalassemia that is linked to an elevated risk of thromboembolic problems in these patients, although the underlying processes are yet unknown. (21). In the etiology of thrombosis in thalassemia, several etiologic variables may play a role. Changes of the red cell membrane may explain their enhanced aggregation and capacity to enhance thrombin generation. Platelets and endothelial cells are eventually activated and tissue factor released. All these factors enhance the thrombotic process. The generation of free oxygen radicals is induced by the disintegration of unstable alpha globin chains in RBC, leading in the accumulation of intracellular labile iron and oxidation of membrane lipids and proteins, culminating in more stiff and misshapen RBC and premature death **[20].**

In our study we investigate the frequency or prothrombin gene polymorphism, one of thrombophilic mutations, in thalssemic patients. In the current study, all of the participants in this study had regular blood transfusions. The observation that thromboembolic manifestations are more common in less developed countries with limited transfusion resources, as well as ex vivo and in vitro experiments showing normal RBC eliminating the abnormal aggregation seen with thalassemic RBC, point to a possible beneficial role of regular blood transfusions in lowering the incidence of thromboembolic complications **[21].**

In the present study, the serum ferritin level was significantly greater in thalassemic patients in the current investigation, with a range of (200 ng/ml to 5000 ng/ml) and a median value of (1250 ng/ml), A study found that a serum ferritin level of greater than 1000 ng/ml is possibly

contributing to hypercoagulability and thrombosis in thalassemic patients **[22].** Furthermore, is associated with the prognosis of poor long-term survival. This is caused by the buildup of iron in erythrocytes and other tissues such as the endothelium, liver, and heart into a complex condition excess iron is exceedingly hazardous to all human tissues, causing severe morbidity and mortality in -thalassemic individuals as well as other iron-overload disorders such cirrhosis, liver fibrosis, heart disease, and endocrine problems **[23].**

As platelets play an important role in the pathogenesis of thrombosis, In the current study, the platelet count was significantly lower in cases than controls, which mean value was (179.6± 22.57(×10³/mm³) and (384.43 ± 16.84 (×10³/mm³) respectively but was within normal range. There was a significant decrease in platelet count when compared with controls. This could be due to the occurrence of recurrent and chronic platelet consumption in patients with major β-thalassemia **[24].**

This not coincide with **[25],** who reported that thrombocytosis, chronic platelet activation, and enhanced aggregation were implied in the development of hypercoagulable state in thalassemic patients. This can be explained by presence of more cases with splenomegally with hypersplenism, another study by **[26]** had estimated the prevalence of thrombotic events in β-thalassemia in 9% of the patients. The enhanced platelet function and the higher platelet number comprise a double risk for hypercoagulability in splenectomized patients. In agreement with our findings, other studies also reported thromboembolic complications in splenectomized patients **[27, 28].**

Protein C and protein S work together to prevent the clotting cascade. The levels of protein C and S in the case in this study were lower than those in the control, low levels of protein C, protein S were detected, which is in agreement with **[29]** who reported that protein C was low in 26.2% of patients, protein S was low in 28.6%, Furthermore**, [30]** reported that protein C and protein S levels were below normal in most their patients with thalassemia. In general, causes of decreased protein C and protein S in major patients with beta thalassemia include vitamin K deficiency, iron overload leading to liver impairment, because protein C and S are vitamin K-dependent factors, and other reasons protein C and protein S consumption may be increased .In addition to decreased levels of protein C and protein S in severe thalassemia patients, this study also showed that compared with the control group, the level of D-dimer was significantly increased. These results are consistent with researches conducted in Egypt **[31]** and Iraq **[32]**.

After chronic activation of the coagulation system, the lower content of protein C and protein S in these species may be due to increased consumption **[33]** believes that due to abnormal liver function, low-level natural anticoagulant proteins (such as protein C) present in the study are possible because protein C, protein S and antithrombin are synthesized in the liver, the defect is highly hepatotoxic and even mild. The liver impairment was not the only reason for decreased natural anticoagulant proteins in beta thalassemia patients. Another explanation for the significant decrease in the proteins may be and perhaps this type of protein is related to phosphatidylserine, or other negatively charged phospholipids, abnormally located in the outer membrane of thalassemia RBCs **[34]. Penday et al., [35]** shows that the occurrence of heterozygous PT G20210A polymorphism was not associated in patients in the β-thalassemia major and controls groups. Limited studies demonstrated that the increased frequency of thrombophilic mutation has not been associated with thalassemia patients **[36].**

In the current study, there was statistically non-significant difference between the studied groups regarding prothrombin G20210A gene polymorphisms but there was significant difference between them regarding prothrombin alleles. This agrees with, study by **[37],** who found no association between MTHFR C677T, PT G20210A, and FVL G1691A gene polymorphisms with β-Thalassemia major patients and controls. Also, they concluded that the MTHFR C677T, PT G20210A, and FVL G1691A gene polymorphisms may not play an important role in the pathogenesis of a thromboembolic events in thalassemia. On contrast, **[38]** founded that 1.7% (1/60) of adult thalassemia patients were heterozygous for the PT 20210A mutation, and none were homozygous.

In our study we reported higher prevalence AA genotype in patients compared to healthy individuals although there was no significant difference. Further, there was statistically significant difference of A allele distribution between cases and control groups. This disagree with, **[39]**, found that the A allele frequencies of the PT G20210A mutation was 3% and 2.5% in β-thalassemia major and healthy controls,

respectively and the differences in between were not statistically significant**.**For numerous reasons, the findings of this study cannot be applied to other situations. To begin with, ethnic differences may be a significant factor influencing genetic studies of this sort. Second, bigger sample sizes may make it easier to understand the prevalence of thrombophilic mutations and their connection with thromboembolic events.

In conclusion, with the prolonged life expectancy of patients with β-thalassemia, more caution of thromboembolic complications should be considered. The presence of mutant A allele of the PT G20210A in cases along with the presence of additional risk as low protein C and protein S levelsshould be investigated for congenital thrombophilia, and prophylactic antithrombotic agents may be recommended.we did not find an association between PT G20210A gene polymorphism with β-Thalassemia major patients as compared to controls. Overall, it can be concluded from general and subgroup analyses that the PT G20210A gene polymorphism may not play an important role in the pathogenesis of a thromboembolic event. Still, there is not a sufficient amount of relevant studies to give a safe and good assumption. In the future, however, more well-designed studies with a bigger sample size and metacentric studies will be necessary to validate the current outcomes.

CONFLICT OF INTEREST: None

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