Original Article

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Methylenetetrahydrofolate Reductase Polymorphisms in Iranian Patients with Glanzmann's Thrombasthenia

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Abstract

Background: The most common polymorphisms identified in the Methylenetetrahydrofolate reductase (MTHFR) gene, C677T and A1298C lead to defective activity of this enzyme and increase the risk of venous and arterial thrombosis. There are limited investigations regarding the effects of thrombogenic polymorphisms on the clinical phenotypes of rare hereditary hemorrhagic disorders like Glanzmann's thrombasthenia (GT) and the exact correlation between MTHFR polymorphisms and GT is not well established. This calls for further studies in populations with a large number of such patients. So, this study was performed to question whether coinheritance of MTHFR polymorphisms and GT can modulate the clinical phenotype of GT.

Material and Methods: In the present case-control study which performed at Pathology and Stem Cell Research Center at Kerman University of Medical Sciences, 65 patients with GT and 100 normal voluntary blood donors as the control group were evaluated. The mean (SD) age of patients and the control group were 2.33±1.54 years (range 0-5 years) and 2.6±1.72, respectively. The detection of MTHFR C677T and A1298C polymorphisms was carried out using a Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) method. In accord with the Glanzmann's Thrombasthenia Italian Team (GLATIT) Protocol, the clinical severity of bleeding in patients with GT was determined. Two tests of descriptive statistics (i.e. frequencies) and Chi-square, using the SPSS version 19, were employed to analyze the data.

Results: Based on results, there were no significant statistical differences in the prevalence of the MTHFR C677T polymorphism (P=0.703) or the MTHFR A1298C polymorphism (P=0.187) between patients and the control group. In addition, no association between the severity of bleeding and these polymorphisms was found (P=0.385).

Conclusions: It was concluded that the thrombogenic mutations of MTHFR do not solely modulate the severity of clinical symptoms in patients with GT.

Keywords: Glanzmann's thrombasthenia, Polymorphism, Methylenetetetrahydrofolate reductase, Rare hereditary bleeding disorders.

Introduction

Glanzmann's thrombasthenia (GT) is a rare autosomal recessive disorder that results in a lifelong bleeding tendency due to defective synthesis and association of fibrinogen binding glycoprotein IIb/IIIa (GpIIb/IIIa) receptors on the platelet membrane (1). GT has been sub-classified into types I, II, and III. Patients with less than 5% of normal GPIIb/IIIa are classified as type I. Type II is less severe with 5% to 20% of normal GPIIb/IIIa. Type III variants usually have dysfunctional receptors with near-normal GPIIb/IIIa levels (2). The bleeding tendency in GT is variable, some

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individuals have minimal bruising, while others have frequent, severe, potentially fatal hemorrhages. Moreover, platelet αIIbβ3 levels correlate poorly with hemorrhagic severity, as virtually undetectable αIIbβ3 levels may be associated with negligible bleeding symptoms, while 10-15% of normal levels may correlate with severe hemorrhage (3). Unidentified factors other than the platelet defect itself may also play important roles in the severity of hemorrhage. As many genes have been confirmed to be highly associated with hypercoagulability (thrombophilia), it seems that genetic differences between individuals are likely to be the most important factors. Thrombophilia refers to a series of acquired and inherited conditions that confer a tendency to thrombus formation (4). Inherited thrombophilia may be caused by mutations and polymorphisms in a variety of genes mainly involved in haemostatic pathways such as coagulation, fibrinolysis, and platelet glycoprotein receptor function and homocysteine metabolism. Thrombophilic genes are well recognized as common risk factors for venous thromboembolic disorders (5-8). However, the relationship between inherited thrombophilias and the bleeding diathesis of hereditary hemorrhagic disorders is still unclear. One of the most common thrombophilic factors, methylenetetrahydrofolate reductase (MTHFR) plays a critical role in the B12-dependent methylation of homocysteine to methionine (9). The most common MTHFR polymorphism, C677T, substitutes alanine for valine, and is associated with enzyme thrombolability and reduced activity (10). Another prevalent mutation is MTHFR A1298C (11). As the exact correlation between MTHFR polymorphisms and GT is not well established and the number of studies on patients with hereditary defects of platelet function addressing this issue is limited, this study was performed to question whether coinheritance of MTHFR polymorphisms and GT can modulate the clinical phenotype of GT.

Materials and Methods

In the present case-control study, 63 Iranian patients with GT were included. A total of 100 volunteer blood donors, consisting of 50 females and 50 males, were randomly allocated to the control group. The patients were selected among those visiting the Iranian Blood Transfusion Organization (IBTO) and the Iranian Comprehensive Hemophilia Care Centre (ICHCC) from early 2000 to September 2010. None of the members of the control group had any history of spontaneous bleeding, or showed any abnormality in their blood analyses. The inclusion criteria for selection of patients were based upon aggregometry and flow cytometry findings as noted in the patients' records. Patients with recurrent bleeding episodes whom their disease had not been confirmed by aggregometry and flow cytometry analysis were excluded from this study. Written informed consent was obtained from all patients before they were entered into the study. This study was performed at Pathology and Stem Cell Research Center at Kerman University of Medical Sciences from June 2014 to March 2015.

Analysis of Bleeding Severity

To determine the bleeding severity in patients with GT, the Glanzmann's Thrombasthenia Italian Team (GLATIT) Protocol was applied. Based on this protocol, the patients were divided into three groups with mild, moderate and severe clinical symptoms. Based on these classification criteria, mild bleeders were patients with mild clinical symptoms like petechiae or bleeding after surgery. Patients with spontaneous bleeding and rare life-threatening haemorrhage such as gastrointestinal bleeding were classed in the moderate group, and patients with severe cases of bleeding who urgently needed to receive platelet concentrates and

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hospital care, were included in the severe group (12).

Quantitative Flow Cytometry

The EDTA blood samples of both patient and control group were quantitatively and qualitatively analyzed through mono- and dichromatic analyses by a Partec Flow Cytometry System (Cyflow Space Model). For this purpose, conjugated CD41, CD61 and CD42b antibodies, as well as the control isotopes were used for qualitative analysis. The non-conjugated form of these antibodies, the Qifikit Dako kit and indirect staining were used for quantitative measurement of glycoproteins on the platelet membranes. Finally, based on the GpIIb/IIIa levels, patients were divided into three types. Type I and II GT patients had GpIIb/IIIa surface expression levels of 0-5% and 6-20% of normal levels, respectively, whereas type III (variant type) patients showed normal or a little less than normal levels of glycoprotein (13, 14).

Molecular Analysis

Five ml samples of venous blood were taken from subjects into EDTA, and genomic DNA was extracted from blood leukocytes after centrifugation. Using the primers and restriction enzymes listed in Tables I and II, the polymorphisms of the MTHFR enzyme (A1298C and C677T) were determined by PCR-RFLP (15). To confirm the existence and accuracy of amplified sequences, the PCR products were electrophoresed on a 2% agarose gel stained by Sybr® safe (Invitrogen TM, USA), and detected on a UV transilluminator (GelDocTM XR). To digest PCR products by restriction enzymes, 10µl of PCR products was mixed with the enzymes and incubated at 37°C overnight. Electrophoresis was then performed on 8% polyacrylamide gel (PAGE) with the relevant DNA marker.

Results

Clinical and Demographic Characteristics of Patients

Sixty-three patients with GT, including 31(49.2%) males and 32 (50.8%) females, were investigated. The mean (SD) age of these patients was 2.33±1.54 years (range 0-5 years) at the time of sampling. First-degree consanguinity was present in all patients except four. The mean (SD) age at diagnosis was 0-3 years. A history of petechiae, epistaxes, and prolonged bleeding after trauma were the most common symptoms obtained. However, petechiae in 90% of cases, epistaxes in 49%, and gum bleeding in 30% of both genders, were the major bleeding manifestations. Based on the severity of bleeding and the score assigned according to the Italian protocol, 10 (15.9%) patients had mild, 22 (34.9%) had moderate, and 31 (49.2%) had severe bleeding. No significant correlation was found between bleeding severity and different types of GT.

Quantitative Measurement of Platelet Integrin IIb3

The results of this study revealed different patterns of GPIIb/IIIa expression among the patients compared with normal controls. Varying results were also obtained for GT types. Forty (65.1%) patients presented the classical type of GT (type I), characterized by absence or extreme reduction (<5%) of both GPIIb (CD41) and GPIIIa (CD61) levels, as compared with normal platelets (Figure 1a). Fifteen (22.2%) patients whose GPIIb/IIIa levels ranged between 6-20% were classified as type II (Figure 1b), and eight (12.7%) patients were diagnosed with type III or variant type, where they exhibited a normal or near-normal level of GPIIb/IIIa complexes, corresponding to approximately 80-100% of control levels (Figure 1c).

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Molecular Findings

The molecular analysis carried out in order to determine MTHFR genotypes (C677T and A1298C) in patients with GT showed that 33 subjects (52.4%) had the normal form (CC), 26 subjects (41.3%) had the heterozygous form (CT), and only 4 subjects (6.3%) had a homozygous form (TT) of the C677T polymorphism. However, regarding the A1298C polymorphism, the prevalence of the normal form (AA), heterozygous form (AC), and homozygous form (CC) were 31.7%, 44.4% and 23.8%, respectively (i.e. among 20, 28, and 15 subjects). On the other hand, among the control group, 52 subjects (52%) showed the normal form, 38 had the heterozygous form (38%) and 10 (10%) had a homozygous form of the C677T polymorphism. However, 33 subjects (33%) showed the normal form, 54 had the heterozygous form (54%) and 13 (13%) had a homozygous form of the A1298C polymorphism (Table III). Moreover, the molecular data showed that both of MTHFR genotypes are equally distributed among patients and control groups (figure 2).

Statistical Findings

Two tests of descriptive statistics (i.e. frequencies) and Chi-square, using the SPSS (version 19.0, SPSS, Inc., Chicago, IL, USA) software package, were employed to analyze the data. A P value of less than 0.05 was considered statistically significant. Based on the results of the chi-square test, no significant association was found between genotype and severity of bleeding.

Table I. Primers Used for Replication of Parts of MTHFR Gene

Polymorphism	Primer Sequence	Amplified Product (bp)	Ref
MTHFR C677T	5'-TGAAGGAGAAGGTGTCTGCGGGA-3' 5'-AGGACGGTGCGGTGAGAGTG-3'	198 bp	(16)
MTHFR A1298C	5'-CAAGGAGGAGCTGCTGAAGA-3' 5'-CCACTCCAGCATCACTCACT-3'	128 bp	(16)

Table II. Restriction Enzymes Used in RCP-RFLP Test

Polymorphism	Restriction Enzyme	Wild Type	Heterozygote	Homozygote	Ref
MTHFR C677T	Hinf I	198 bp	23, 175, 198 bp	23, 175 bp	(16)
MTHFR A1298C	Mbo II	28, 28, 72 bp	28, 72, 100 bp	28, 100 bp	(16)

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Table III. Frequency of MTHFR1298A>C and MTHFR677C>T genotypes in the patients group according to severity of bleeding

Genotype							Genotype				
	Ge A1298C						C677T				
AA n(%)	AC n(%)	CC n(%)	P-value	χ^2	Bleeding score	n	CC n(%)	CT n(%)	TT n(%)	P-value	χ^2
1 (10)	7 (70)	2 (20)			Mild	10	5 (50)	5 (50)	0		
9 (40.9)	5 (22.7)	8 (36.4)			Moderate	22	13 (59.1)	9 (40.9)	0		
10 (32.3)	16 (51.6)	5 (16.1)			Severe	31	15 (48.4)	12 (38.7)	4 (12.9)		
20 (31.7)	28 (44.4)	15 (23.8)	0.074	**8**	Total	63	33 (52.4)	26 (41.3)	4 (6.3)	0.324	8

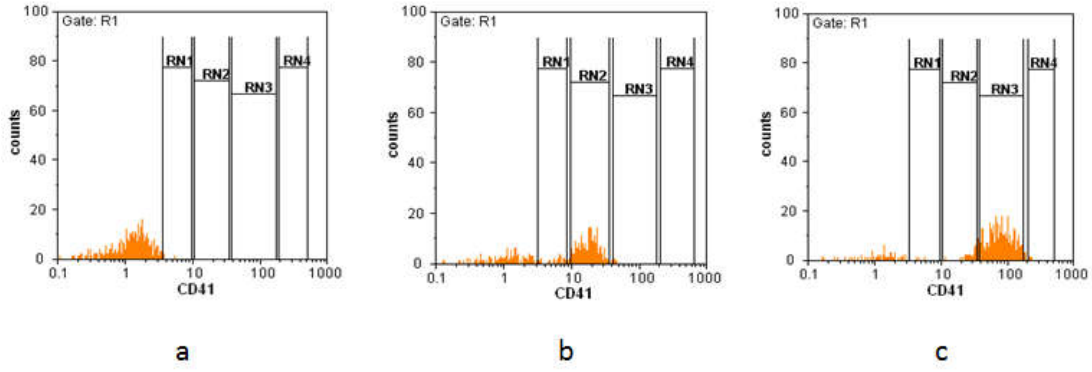


Figure 1. Expression of CD41 (GPIIb) in groups of GT (a: lack of CD41 expression on the surface of platelets in patients with type I GT; b: reduced CD41 expression on the surface of platelets in patients with type II GT; c: relatively normal CD41 expression on the surface of platelets in patients with variant type of GT).

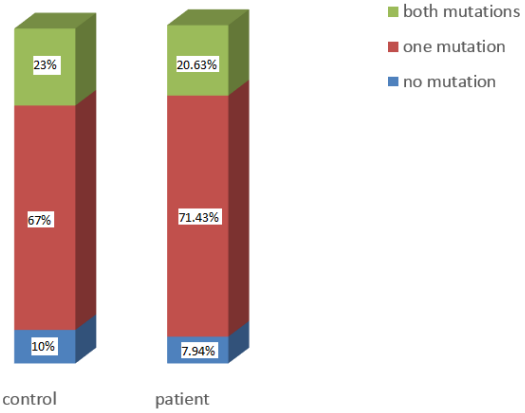


Figure 2. Histogram of MTHFR677 and MTHFR1298 genotypes in the patient and control group

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Discussion

According to the results of the present study, the greatest prevalence of the MTHFR677 polymorphism in the patient group was associated with a normal genotype (52.4%) and the lowest abundance was associated with a heterozygous mutant genotype (6.3%), while the highest prevalence of the MTHFR1298 polymorphism, was associated with a heterozygous mutant genotype (44.4%) and the lowest prevalence was related to a homozygous mutant genotype (23.8%). Statistical analysis indicated that there was no significant difference in the prevalence of genotypes of the MTHFR C677T polymorphism between the patient and control group (P=0.703). In addition, no statistically significant difference was observed in the prevalence of the MTHFR A1298C polymorphism, between the two groups (P=0.187). As a result, it was suggested that in GT the two common mutations of MTHFR are inherited separately, and since GT is a dominant autosomal disorder, one would expect to find a common expression pattern of the mutations in question among these individuals. Comparing the results obtained, and the epidemiologic findings, it can easily be understood that the allele frequency of these two polymorphisms in Iranian patients with GT is higher than those reported in most other studies (17, 18). Considering the high prevalence of consanguineous and inter-ethnic marriages in most parts of Iran, this difference is understandable. On the other hand, there was no association between the severity of bleeding in GT and these two polymorphisms (P=0.324 and P=0.074). In other words, these potentially thrombogenic polymorphisms cannot protect GT patients from the development of bleeding, but it may well be that the synergistic effect of other polymorphisms and mutations may result in improvement of the clinical signs of GT. A review of the literature shows that there are varying and sometimes paradoxical findings in the field of prothrombotic mutations and clinical symptoms of hemorrhagic disorders. This can make it difficult for investigators to establish a definite association between various polymorphisms and clinical signs (19). The first attempts to investigate the genetic factors involved in moderating the bleeding phenotype of patients with severe hereditary hemorrhagic disorders began when cases were found in whom bleeding was clinically less marked than expected. A number of authors consequently began evaluating the role of mutations and polymorphisms of platelet glycoproteins in this regard. According to these studies, none of the polymorphisms of glycoprotein Ib (GPIb) and glycoprotein VI (GPVI) result in increased survival of GT patients (14). In contrast, Ghosh et al. (2002) suggested that GT patients homozygous for Human Platelet Antigen 1b/1b (HPA-1b/1b) had higher levels of platelet aggregation and fibrinogen binding, as well as a milder bleeding tendency, as evidenced by infrequent epistaxis and no clinical requirement for transfusion (20). In addition, the concentration of α2β1 receptors on platelets was suggested to be a determining factor affecting the severity of clinical symptoms in GT. D'Andrea et al studied 25 patients with GT and proposed that the level of α2β1 receptors on platelets might be an additional factor influencing GT clinical expression, because the platelet α2C807T gene polymorphism is associated with α2β1receptor density on the platelet surface (21).

Several studies were conducted to investigate the modulation of clinical phenotypes of rare hereditary hemorrhagic disorders by thrombogenic mutations such as Factor V Leiden (FV-Leiden), Factor V HR2 (FV-HR2), prothrombine 20210, MTHFR C677T and A1298C polymorphisms (22). It is worth mentioning that the presence of the factor

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V Leiden mutation appears to decrease the severity of severe haemophilia quite consistently. However, findings in relation to other prothrombotic mutations were inconclusive and paradoxical (23). For instance, Tüten et al (2012) hypothesized that prothrombotic mutations are effective in decreasing annual factor consumption in children with hemophilia. They evaluated the effect of Factor V Leiden (G1691A), prothrombin (PT) G20210A, MTHFR C677T and A1298C mutations in 51 children with moderate to severe hemophilia A. Paradoxically, only patients who were homozygous for MTHFR C677T were found to have increased factor consumption, whereas no decrease in factor consumption was observed in hemophilic patients found to have the other prothrombotic mutations. As a result, they could not confirm a significant decrease in consumption of factor concentrates in children with both hemophilia and prothrombotic mutations (24). Ahmad et al (2012) studied 114 patients with Von Willebrand's disease (VWD) and suggested that patients carrying the defective alleles of different thrombogenic markers show milder phenotypes than expected (25). Moreover, Kannan et al (2009) screened 45 patients with GT for the thrombogenic polymorphisms and suggested that the coinheritance of heterozygous FV Leiden alone or homozygous HPA 1b alone, or the combined heterozygosity of MTHFR and HPA-1 were predicted to alter the clinical phenotype. However, the inheritance of heterozygous MTHFR alone or heterozygous HPA-1 alone did not significantly alter the clinical phenotype. Hence FV Leiden and MTHFR C677T polymorphisms along with PLA-1 and HPA-1 phenotypes may have been the ameliorating factors in mild GT phenotypes (22).

Due to different genetic predispositions in hereditary bleeding disorders and the large number of patients in this study, it seems that this study confirmed the results which had suggested the lack of correlation between MTHFR polymorphisms and GT. As GT is a rare bleeding disorder and the distribution of patients with GT is very high in Iran, the process of sampling was so difficult and time-consuming. This prolonged the study and was the main limitation of this investigation.

Conclusion

Iranian patients with GT and healthy individuals have a higher prevalence of common polymorphisms in the MTHFR gene compared with most parts of the world. These polymorphisms do not independently influence the clinical phenotype of GT patients. However, one cannot simply neglect their role in the reduced severity of bleeding in these patients. It is possible to suggest that these polymorphisms, together with other genetic alterations, may play a role in modulation of clinical symptoms of hemorrhagic diseases. Therefore, further investigations are recommended to identify thrombogenic polymorphisms either in coagulation factors or platelet glycoproteins in regions where hemorrhagic disorders are prevalent, so as to precisely analyze the interactions of these factors.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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