

Prothrombin Gene G20210A Mutation in Acute Deep Venous Thrombosis Patients with Poor Response to Warfarin Therapy

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Abstract: *Aim:* The pathogenesis of deep venous thrombosis (DVT) involves an interaction between hereditary and acquired factors. Prothrombin gene mutation is one of the hereditary risk factors. We evaluated the frequency of the prothrombin gene mutation in patients with DVT and its relation to oral warfarin anticoagulant therapy response.

Methods: Prothrombin gene mutation was looked for in 40 DVT patients with poor response to warfarin. The results were compared with 40 DVT patients with a normal response to warfarin and 30 healthy blood donors. Blood samples were also assessed for protein C, protein S, anti-thrombin III and anticardiolipin antibodies (ACA) levels.

Results: Prothrombin gene mutation was found in normal and poor DVT responders (6/40 and 13/40, respectively; $p = \text{NS}$) as well as in healthy controls (1/30). Patients with recurrent DVT or a family history of DVT were significantly ($p < 0.0001$) more likely to have the prothrombin mutation than other DVT patients. Non prothrombin abnormalities (protein C, anti-thrombin III and ACA) were more common in poor responders than controls ($p < 0.0037$) as were ACA ($p < 0.034$).

Conclusions: Prothrombin gene mutation is present in several DVT patients, especially those with recurrent DVT or a family history of DVT. This mutation may contribute to a poor response to warfarin.

Keywords: Deep venous thrombosis, gene mutation, prothrombin, warfarin.

INTRODUCTION

Deep venous thrombosis (DVT) and its complications [pulmonary embolism and post-thrombotic syndrome] are not only common preventable causes of hospital death but also a source of substantial long-term morbidity [1].

The pathogenesis of DVT involves an interaction between hereditary and acquired conditions [2-4]. Generally, DVT occurring in the setting of a recognized risk factor is defined as secondary, whereas that occurring in the absence of risk factors is termed primary or idiopathic [5].

In primary DVT, tendency toward venous thrombosis could arise from hyperactive coagulation pathway, hypoactive anticoagulant mechanisms, or hypoactive fibrinolysis [2, 6]. With the identification of the well-characterized risk factors associated with secondary DVT, there is increasing interest in the laboratory identification of primary thrombotic risk factors. Identification of these risk factors may affect treatment and identify other affected family members before the onset of symptoms. This could justify the use of prophylactic anticoagulant therapy during high-risk periods [7].

Over the past few years, studies have focused on the role of mutations in genes that encode proteins involving thrombosis pathways. Prothrombin (factor II) is one of these proteins. It is the precursor of the serine protease thrombin, a key enzyme acting as a procoagulant, through platelet activation and the generation of fibrin and factors Va, VIIIa, and XIIIa, and subsequently as an anticoagulant, by activating circulating protein C [2, 6]. Therefore, regulation of thrombin activity is crucial for maintaining hemostatic balance [7]. The gene encoding prothrombin is 21-kb-long located on chromosome 11, position 11p11-q12 [8, 9]. The prothrombin gene is organized in 14 exons, separated by 13 introns with the 5' upstream untranslated (UT) region and the 3'-UT region which may play regulatory roles in gene expression [6, 10]. One genetic variation in the 3'-UT region of the prothrombin gene is the G to A transition at nucleotide position 20210, at or near the cleavage site of the mRNA precursor to which poly A is added [8]. This is termed as the factor II G 20210 A mutation.

In 1996, Poort *et al.* reported that the factor II G20210A mutation is associated with an increased risk of venous thrombosis [6]. Several studies confirmed this initial observation [10, 11].

The prevalence of carriers of factor II G20210A mutation in healthy Northern Europeans is 1.7% whereas in Southern

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Europeans the prevalence is nearly double (3%) [12]. In contrast, factor II G20210A mutation was found in only 1 of 441 African Americans [13] and was completely absent among 231 Amerindians from Brazil and 210 Japanese subjects [11, 14]. Patients with this heterozygous gene mutation have increased prothrombin levels and therefore, a thrombotic tendency [2].

In this study we assessed the presence of the factor II G20210A mutation in DVT patients especially in those who showed poor response to warfarin therapy.

PATIENTS AND METHODS

This is a prospective comparative study. The studied population included 80 patients with acute DVT and 30 healthy blood donor controls of both sexes, residing in the Suez Canal area in Egypt.

Clinical diagnosis of acute DVT (Department of Surgery – Suez Canal University Hospital) was confirmed by Duplex scan. The Department of Surgery was also responsible for the initial and maintenance anticoagulation therapy.

Patients with previous history of DVT (in the same or other site) were defined as recurrent DVT, and those with DVT history in first degree relatives were defined as having a positive family history.

Data collection included other DVT risk factors such as: complete bed rest for more than 3 days, recent surgery (thoracic, abdominal, pelvic or major lower extremity orthopedic procedure within the previous week), major trauma (fracture [e.g. pelvic, femoral or tibial], spinal cord injuries or associated with major venous injury), use of contraceptive pills or hormone replacement therapy and the coexistence of varicose veins or neoplasia [5]. Drug history (specially interacting with anticoagulants) was checked.

Exclusion criteria: Patients receiving drugs that interact with warfarin were excluded, as well as pregnant females as oral anti-coagulants are contraindicated during pregnancy [5].

All patients received low molecular weight heparin enoxaparin (Clexane) 1 mg/kg/12h subcutaneously for 5 days, with a dose of 5 mg for 2 days. Warfarin dose was adjusted to reach International Normalized Ratio (INR) of 2.0-3.0 [5].

The study population was divided into 2 groups: the first group was the Poor Responders, defined as the DVT patients who fail to reach the intended level of INR with the usual dose of warfarin (usually 1 - <9 mg/day), those patients usually require more than 9 mg/day to reach the therapeutic INR level [15-19]; we recruited 40 patients during the study period. This first group was compared with a second group of 40 randomly selected DVT Normal Responders, defined as those who reached an INR between 2 and 3. In addition, a control group of 30 healthy blood donors living in the same area were randomly selected; none of them had a previous history of DVT.

Factor II gene mutation, protein S, protein C, anti-thrombin III and anti-cardiolipin antibodies were assessed in all DVT patients and controls. Prothrombin time and INR were assessed in DVT patients before induction of therapy.

Detection of Factor II G20210A Mutation by Real-time PCR

Detection was done by real-time PCR assay [20] using the LightCycler prothrombin G20210A mutation detection kit (Roche Molecular Biochemicals, Catalog No. 2 236 842) [21]. The 165 bp fragment of prothrombin gene is monitored by adjacent hybridization probes that are designed to bind on 1 amplicon strand. The 3' end of 1 probe is labeled with fluorescein (FLU), whereas the 5' end of an adjacent probe is labeled with LightCycler-Red640 (Roche Molecular Biochemicals) as the anchor probe. When both probes hybridize in close proximity, only after hybridization to the template DNA, fluorescence resonance energy transfer (FRET) occurs, producing a specific fluorescence emission of LC-Red as a result of FLU excitation. Increasing the temperature during fluorescence reading yields a temperature/fluorescence curve from which the melting point of the probe can be derived. When the appropriate conditions are chosen, the mismatch under the detection probe caused by a single point mutation leads to a substantial decrease in the melting point of the probe [16]. DNA was isolated from 200 µL of EDTA-treated blood with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted in 200 µL of elution buffer and stored at -20 °C. PCR reactions were performed in a final volume of 20 µL in the LightCycler glass capillaries, which contained 2 µL of 1x Light Cyler prothrombin G20210A mutation detection mix, 2µL of 1x Light Cyler prothrombin G20210A mutation reaction mix, and 5 µL of DNA solution. PCR-grade water was added to a final volume of 20 µL. Each run included a positive control, which is heterozygous DNA control, and negative control, which is PCR-grade water.

Statistical Analysis

Results are expressed as mean and proportion values for baseline characteristics for patients and control subjects. Differences were tested with the Student's t and Fisher exact tests using SPSS version15.

RESULTS

Sex and age distribution in the studied groups are shown in Table 1. Median age in the 3 groups was similar.

Regarding the site of DVT, the superficial femoral vein was the affected in 35% and 30 % of the poor responders and normal responders, respectively, followed by the popliteal vein in 25% and 27.5 % and only 3% and 5 % of the patients had iliac DVT. In the remaining 37% of the poor responders and 37.5% of the normal responders the affected vein was either the anterior or the posterior tibial vein.

Forty five percent of the poor responders and 40% of the normal responders had no risk factors. In the remaining patients of both groups, these risk factors were comparable and not significant. Except for recurrent DVT which was higher in the poor responders group (25%) than the normal responders (10%); (Table 2).

The laboratory results of the inherited and acquired thrombophilic risk factors in the studied groups are shown in Table 3. Protein C, S and antithrombin III deficiencies

Table 1. Sex and Age Distribution in the Deep Venous Thrombosis (DVT) Patients and Control Subjects

	Poor DVT Responders (n = 40)	Normal DVT Responders (n = 40)	Control Subjects (n = 30)
Male/Female	16/24	18/22	18/12
Median (range) age in years	41.5 (22-61)	42.5 (20-65)	43.1 (20-48)

No differences were significant.

Table 2. Risk Factors in the Deep Venous Thrombosis (DVT) Patients

Risk Factor	Poor DVT Responders (n)	Normal DVT Responders (n)	P
None	18	16	NS
Prolonged bed rest	0	0	
Recent surgery	8	6	NS
Major trauma	6	4	NS
Contraceptive pill /hormone therapy	2	4	NS
Varicose veins	0	0	
Neoplasia	2	2	NS
Recurrent DVT	10	4	NS
Family history	6	4	NS

NS = not significant.

were observed only in the poor responders group. The non Prothrombin mutation abnormalities (Protein C, anti-thrombin III and ACA) were present in 10 poor responders vs 0 controls ($p = 0.0037$). ACA was significantly higher in poor responder than in the control subjects ($p < 0.034$). Regarding the prothrombin gene mutation, homozygous mutation was observed in only 1 patient in the poor responders group. Heterozygous mutation of the PT gene was observed in the 3 studied groups, but it was higher in the poor responders than in the normal responder and control groups (12, 6 and 1 subject, respectively). The difference between the poor responders and the normal controls was highly significant $p = 0.002$ (Table 3). This table also shows the distribution of other thrombophilic risk factors.

Table 4 shows that the positive factor II G20210A mutation was significantly ($p < 0.0001$) more frequent among patients with a history of recurrent DVT or a positive family history of DVT compared with the other DVT patients.

DISCUSSION

On treating patients with acute DVT, we faced patients who did not show the usual elevation of PT/INR in response to usual doses of warfarin. This may reflect an elevated prothrombin level due to a genetic mutation [19]. This mutation may, not only, cause the low response to oral anticoagulant, but may also contribute to the recent and future episodes of DVT [2]. Patients with this gene mutation may need higher doses of warfarin (to reach INR target levels) for longer periods [15, 22].

In the present study, analysis of risk factors showed that recurrent DVT was numerically higher but not significant in the poor responders than for the normal responders. Family history was also higher in the poor responders, but not significantly so. This may reflect the presence of a specific risk factor for DVT in the poor responders group. The rest of the risk factors showed similarity with no significant differences between the poor and normal responders. This agrees with the fact that these are recognised risk factors that may cause DVT in all patients. It also suggests that multiple risk factors might be necessary before clinically evident DVT develops even in patients with thrombotic gene mutation [23].

We detected the factor II G20210A mutation in 20 individuals in our study population ($n = 110$). Homozygous mutation was found in only 1 patient in the poor responders group, while heterozygous mutation was found in the 3 groups. The gene mutation was recognized in 1 (3.4%) of the normal population, which agrees with others who found this mutation in 1%, 1.7% and 3% of the normal Pakistan, Northern and Southern European population, respectively [11, 12, 24]. However, our finding only represents the 30 studied healthy blood donors. Larger studies are needed to define the exact prevalence in our community.

Heterozygous gene mutation was found in 30% of the poor responders and 15% of the normal responders. This supports the concept that factor II G20210A mutation may cause high prothrombin levels [6] which may, in turn, be responsible for resisting oral anticoagulants. The presence of the same mutation in some normal responders may be

Table 3. Distribution of the Inherited and Acquired Thrombophilic Risk Factors Alone or in Combination with Prothrombin Gene Mutation

Risk Factors	Poor DVT Responders (n = 40)	Normal DVT Responders (n = 40)	P	Control Subjects (n = 30)
Protein C deficiency	2	0	NS	0
Protein S deficiency	0	0	NS	0
Anti - thrombin III	2	0	NS	0
Homo FII A mutation	1	0	NS	0
Hetero FII A mutation	12	6	0.002**	1
ACA	6	4	0.034*	0
F II A + Anti-thrombin III	0	0	NS	-
F II A + Protein C	1	0	NS	-
F II A + Protein S	1	0	NS	-
F II A + ACA	2	2	NS	-

* Poor DVT responders vs control subjects

** FII A mutation in poor DVT responders vs control subjects; includes the homozygous mutation in the poor responders

NS = not significant

ACA = anticardiolipin antibodies

F II A = factor II G20210A mutation.

Table 4. Relation Between Factor II G20210A Mutation and Recurrent or Family History of Deep Venous Thrombosis (DVT)

	Recurrent DVT	Non – Recurrent DVT	P
Mutation +ve	12	7	<0.0001
Mutation -ve	2	59	
	+ve Family History	-ve Family History	
Mutation +ve	8	11	<0.0001
Mutation -ve	2	59	

explained by the presence of different degrees of mutation that did not clinically affect the prothrombin levels. This gene mutation with less clinical significance was previously suggested [25].

Our findings agree with previous reports that suggested 3 to 6 fold higher risk of developing DVT in prothrombin gene mutation patients than the normal population [2, 9]. This also points to the importance of screening the patient's family as they may be at increased risk of developing DVT. However, the interpretation of our results is limited by the small sample size and selection bias.

Protein C, anti-thrombin III and ACA abnormalities were found in more poor responders DVT patients than controls. Bernd *et al.* [26] suggested that special caution is needed when giving anticoagulants to patients with protein C deficiency. Because protein C is a vitamin K dependent factor, the administration of warfarin could lead to sudden decrease in protein C before any noticeable decrease in coagulation factors. This could cause enhanced thrombosis and diffuse skin necrosis. Also, ACA was significantly more common

in poor responders than control subjects. It has also been suggested that patients with ACA and venous thromboembolism are resistant to usual intensities of warfarin therapy, which may prompt more intense anticoagulation of these patients. However, no substantial difference was seen between the ACA-positive and ACA-negative patients, for several thromboplastins [27].

To add to the dilemma of DVT and genetic disorders, pharmacogenetic studies should be ideally be carried out in DVT patients before warfarin anticoagulant therapy as Leung *et al.* 2007 [28] reported that patients with Cytochrome P-450 CYP2C9*2, CYP2C9*3, or VKORC1*2 genotype (c.-1639G>A) in presence of Factor V Leiden and/or factor II G20210A mutation require significantly reduced doses, and are at a higher risk of serious bleeding.

In conclusion, our findings suggest that factor II G20210A mutation may be more common in DVT patients who are poor responders to warfarin. Factor II G20210A mutation was more common in patients with recurrent DVT or positive family history of DVT.

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