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ORIGINAL ARTICLE

PROTHROMBIN GENE G20210A MUTATION IN PATIENTS WITH ACUTE DEEP VENOUS THROMBOSIS

By
Fadia Attia,[1] Sherif Reffat[2]
[1]Department of clinical Pathology, [2]Department of Surgery, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Correspondence to: Sherif Reffat, Email: shrefat@hotmail.com

Aim: *Deep venous thrombosis (DVT) is an interaction between hereditary and acquired factors. Prothrombin gene mutation is one of these hereditary risk factors that may cause DVT through elevation of the Prothrombin level and therefore, requires special attention. In this study we tried to have an idea about frequency of this gene mutation in patients with DVT.*
Methods: *Prothrombin gene mutation was looked for in forty Warfarin-Resistances DVT patients. The results were compared to another forty Warfarin-Sensitive DVT patients and thirty healthy blood donors.*
In addition blood samples were assessed for the levels of protein C, protein S, antithrombin III and anticardiolipin antibodies.
Results: *Recurrent DVT and positive family history were more frequent in the Warfarin-Resistance group. Prothrombin gene mutation was found in DVT patients as well as healthy controls, but with different percentages. The higher frequency of this gene mutation in Warfarin-Resistance individuals may confirm its mechanism in causing DVT.*
Conclusion: *This study supports that Prothrombin gene mutation is present in our population, especially DVT patients. The study also suggests that patients with Warfarin-Resistance should be tested for the presence of this gene mutation.*

Keywords: *Venous thrombosis, Gene mutation, Prothrombin.*

INTRODUCTION

Deep venous thrombosis is an important disease. Its complications (pulmonary embolism and post-thrombotic syndrome) are not only the most common preventable cause of hospital death but also a source of substantial long-term morbidity.(1)

The origin of deep vein thrombosis is 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 an increasing interest in the laboratory identification of the primary thrombotic risk factors. Identification of these risk factors has significant importance. First it may affect therapy of the patient. Second it may identify other affected family members before the onset of symptoms. This could justify the use of prophylactic anticoagulant therapy during high-risk periods, potentially avoiding the first episode of venous thrombo-embolism.(7)

Over the past few years, studies have focused on the role of

mutations in genes that encode proteins in thrombosis pathways and its role in the predisposition to venous thrombosis. 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 localized 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.[8] This is termed as the Prothrombin G 20210 A.

The prevalence of carriers of factor II G20210A in healthy Northern Europeans was 1.7% whereas in Southern Europeans the prevalence was nearly twice (3%).[11] In contrast, factor II G20210A was found in only 1 of 441 African Americans[12] and was completely absent among 231 Amerindians from Brazil and 210 Japanese subjects.[13,14]

In 1996, Poort et al., described Prothrombin G20210A mutation to be associated with an increased risk of venous thrombosis.[6] Several other studies later confirmed this initial observation.[10,13] This thrombotic tendency was explained by increased Prothrombin levels[2] and therefore, these patients need special attention to guard against DVT during surgery. During an attack of DVT, Patient with this gene mutation and Warfarin resistance will require longer period of anticoagulant therapy (up to 2 years), with higher doses of Warfarin (up to 30 mg/day). If still unsuccessful to reach INR between 2.0 and 3.0, Low Molecular Weight Heparin alone should be used for the whole therapeutic period.[15]

In this study we assessed the existence of the G20210A mutation in DVT patients with some attention to those patients who showed resistance to Warfarin therapy, which may refer to an increased Prothrombin level.[16]

PATIENTS AND METHODS

This study is a descriptive prospective comparative study.

Inclusion criteria: The studied population included 80 patients with acute DVT in addition to 30 healthy blood donor controls of both sexes, residing in Suez Canal area.

Patients were diagnosed to have acute DVT by the Department of Surgery – Suez Canal University Hospital, diagnosis was confirmed by Duplex scan. The surgical department 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 the first degree relatives of the family were defined as 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 pelvis / femur / tibia, 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 from this study, as well as pregnant females as oral anti-coagulant is contraindicated during their pregnancy.[5]

All patients received Low Molecular Weight Heparin, Enoxaparin (Clexan) 1mg/kg/12 hours subcutaneously for 5 days. Warfarin was started from the first day, with a dose of 5 mg for 2 days. Warfarin dose was adjusted to reach INR of 2.0-3.0.[5]

Therefore, the study population included tow groups: the first group was the Warfarin-Resistanance, defined as those 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 were able to recruit 40 patients with this criterion over 36 months, (between June 2004 and June 2007). This first group was compared to a second group of 40 Warfarin-sensitive DVT patients, defined as: those who reached INR between 2.0 and 3.0 with the usual doses (1-<9 mg of Warfarin).[17-19]

Both groups were compared to a third control group of 30 healthy blood donors, living in the same residing area, None of them had previous history of DVT.

Prothrombin 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.

The rationale for blood tests was explained for the studied individuals, blood samples were taken after being sure that they understand and agree to participate.

Detection of G20210A mutation by Real-time PCR: Detection was done by real-time PCR assay[20] in the Hematology Department, using the LightCycler prothrombin G20210A mutation detection kit (Roche Molecular Biochemicals, Cat. No. 2 236 842.[21] The 165 bp fragment of prothrombin gene is monitored by adjacent hybridization probes that are designed to bind on one amplicon strand. The 3′ end of one 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.[21] DNA was isolated from 200 µL of EDTA-treated blood with the QIAamp DNA Mini Kit (Qiagen) according to the instructions of the manufacturer. 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 Cycler prothrombin G20210A mutation detection mix, 2µL of 1x Light Cycler 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: Mean and proportion values for baseline characteristics were calculated for patients and control subjects, and differences were tested for significance using the student's t test or qui square analysis. Fisher exact test was used for sample size less than 5.

RESULTS

Sex and age distribution in the studied groups are shown in Table 1. Mean ages in the three groups were nearly similar.

The First group of DVT patients (Warfarin Resistance) includes 16 males and 24 females. Their age ranged from 22-61 years with a mean age of 41.5 years. The second group of DVT patients (Warfarin-sensitive) includes 18 males and 22 females. Their age ranged from 20-65 years with mean of 42.5 years. Finally the third group (Healthy Blood donors) includes 18 males and 12 females. Their age ranged from 20- 48 years with mean of 43.1 years.

Regarding the site of DVT, the superficial femoral vein was the affected vein in 35% and 30 % of the Warfarin-resistance and the Warfarin-sensitive groups 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 Warfarin-resistance and 37.5% of the Warfarin-sensitive the affected vein was the either the anterior or the posterior tibial vein.

Forty five percent of the Warfarin resistance and 40% of the Warfarin sensitive had no circumstantial risk factors. In the remaining patients of both groups, these risk factors were comparable and statistically insignificant. Except for the recurrent DVT which was significantly higher in the Warfarin-resistance (25%) than the Warfarin-sensitive group (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 anti thrombin III deficiencies were observed only in the Warfarin-resistance group. Regarding the PT gene, homozygous mutation was observed in only one patient of the Warfarin-resistance group. In contrast, heterozygous mutation of the PT gene was observed in the three studied groups, but it was higher in the Warfarin-resistance patients than the Warfarin-sensitive and the normal control group (12, 6 and one patient respectively). The difference between the Warfarin-resistance and the Warfarin-sensitive groups was statistically significant as shown in Table 3. This table also shows the coexistence between the heterozygous state and other thrombophilic risk factors. This mutation was found with Protein C deficiency in one patient and found in another patient with protein S deficiency in the Warfarin-resistance group only. This was statistically significant.

Out of the 20 individuals found to have Prothrombin Gene mutation 13 of them were females (65%) and 7 of them were males (35), shown in table 4. This difference was statistically significant ($P < 0.05$).

Examples of the heterozygous and homozygous mutation of the F II gene 20210 are shown in (Figs. 1-3).

The correlation between F II A gene mutation state and recurrent or family history of DVT is shown in Table 4. It is observed that patients with recurrent or family history of DVT were more frequent in F II A mutation patients than patients without this gene mutation. This observation is statistically significant.

Table 1. Sex and age distribution in the studied groups of patients.

	Warfarin-Resistance N= 40	Warfarin-Sensitive N= 40	Normal control N= 30
Male / Female	16/24	18/22	18/12
Mean age / range	41.5 (22-61)	42.5 (20-65)	43.1 (20-48)

Table 2. DVT risk factors in the DVT patients.

Risk factor	Warfarin-resistance % (no)	Warfarin-sensitive % (no)	P value
None	45 (18)	40 (16)	P > 0.05
Prolonged bed rest	0	0	
Recent surgery	20 (8)	15 (6)	P > 0.05
Major trauma	15 (6)	10 (4)	P > 0.05
Contraceptive pills/ hormone therapy	5 (2)	10 (4)	P > 0.05
Varicose veins	0	0	
Neoplasia	5 (2)	5 (2)	P > 0.05
Recurrent DVT	25 (10)	10 (4)	P < 0.05*
Family history	15 (6)	10 (4)	P > 0.05

P* = 0.035 (Significant).

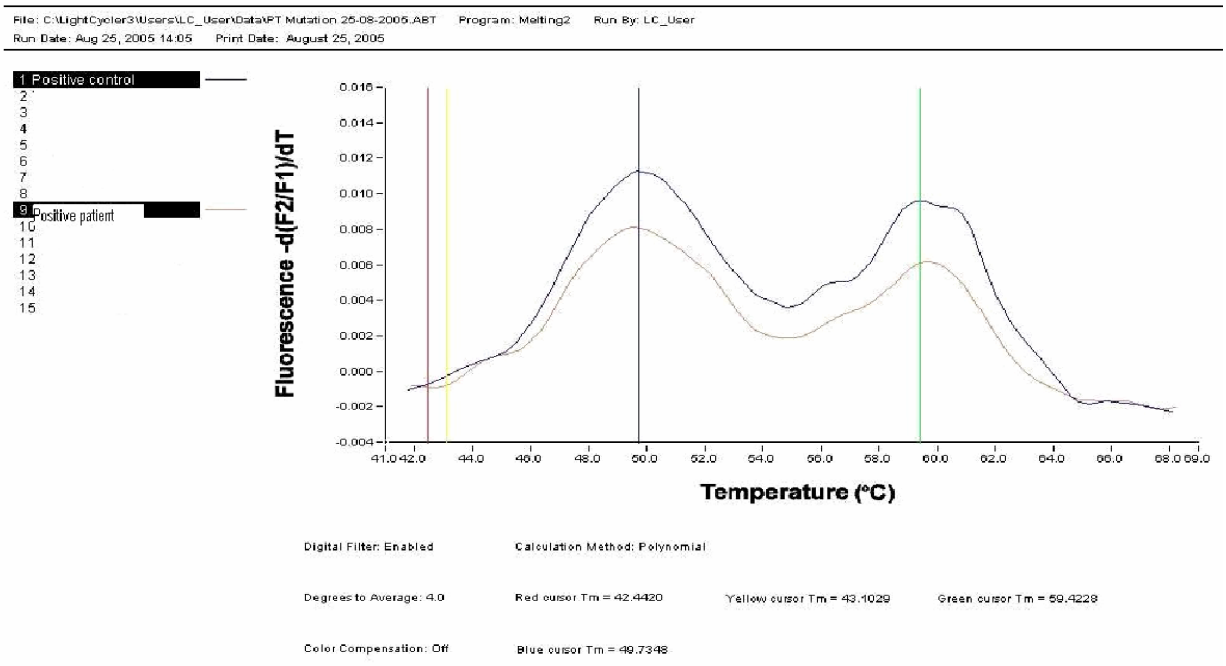
Table 3. Distribution of the inherited and acquired thrombophilic risk factors alone or in combination with Prothrombin gene mutation.

Risk factors	Warfarin-resistance % (no) N= 40	Warfarin-sensitive % (no) N= 40	P value	Normal control N= 30
Protein C deficiency	5 (2)	0	P<0.05	0
Protein S deficiency	0	0	p>0.05	
Anti thrombin III deficiency	5 (2)	0	P<o.05	0
Homozygous FII A 20210 mutation	2.5 (1)	0	-	0
Heterozygous FII A 20210 mutation	30 (12)	15 (6)	P <0.05	3.4 (1)
Anticardiolipin antibodies	15 (6)	10 (4)	P0.055	0
F II A + Antithrombin III	0	0	P >0.05	-
F II A + Protein C	2.5 (1)	0	P <0.05	-
F II A + Protein S	2.5 (1)	0	P <0.05	-
F II A + ACA	5 (2)	5 (2)	P >0.05	-

Table 4. Correlation between F II A mutation state and recurrent, Family history of DVT and the sex.

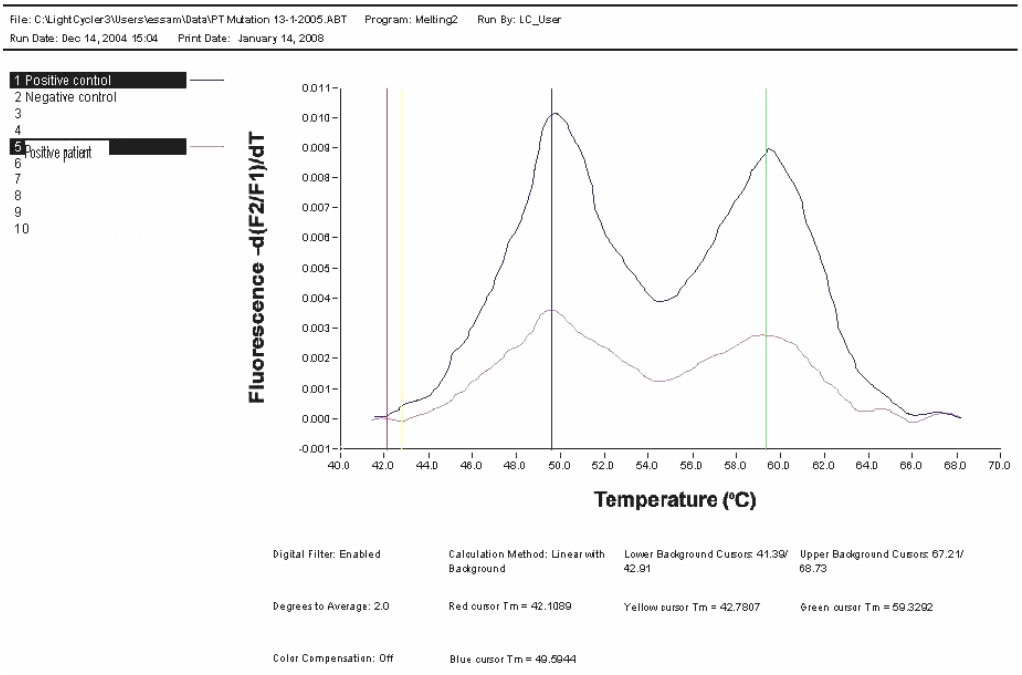
F II A mutation	Recurrent DVT % (No.)	Family History of DVT % (No.)	Sex in numbers		
			Male	Female	P value
+ ve	86 (12)	80 (8)	7	13	0.041
- ve	14 (2)	20 (2)	45	45	-
P value	0.001	0.002	52	58	Total

+ ve: patients with either Homogenous or heterogeneous Prothrombin gene mutation.
- ve: patients with neither Homogenous nor heterogeneous Prothrombin gene mutation.



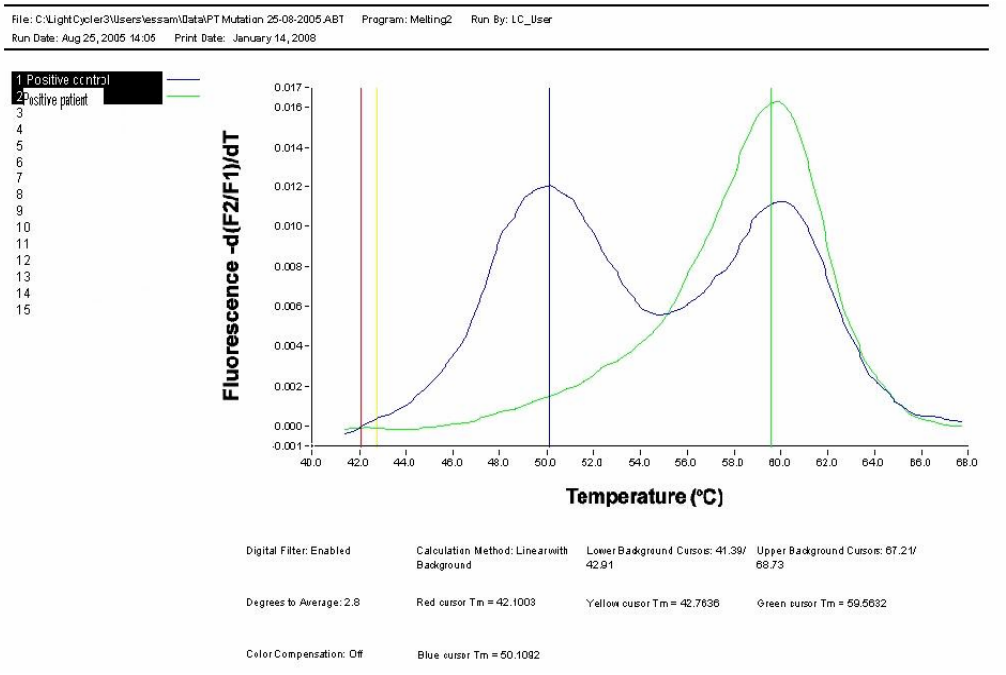
* Patients names were removed for ethical considerations.

Fig 1. The heterozygous gene mutation in a male patient with DVT.



* Patients names were removed for ethical considerations.

Fig 2. The heterozygous gene mutation in a female patient with DVT.



* Patients names were removed for ethical considerations.

Fig 3. The homozygous gene mutation in one patient with DVT.

DISCUSSION

On treating patients with acute DVT, we were faced with certain patients who do not show the normal elevation of PT/INR in response to the usual therapeutic doses of Warfarin. This may reflect an elevated prothrombin level due to a Genetic mutation.[19] This may, not only, cause the resistance to Warfarin but may also contribute to the recent attack and may predispose to further episodes of DVT.[2] Patients affected with this gene mutation should receive higher doses of Warfarin (to reach the intended levels of INR) for a longer periods.[15,22]

In the present study, analysis of the circumstantial risk factors showed that recurrent DVT was higher in the Warfarin-resistance than the Warfarin-sensitive groups (25% versus 10%), which was statistically significant. Family history was also higher in the Warfarin-resistance group, but statistically non-significant. This may reflect the presence of a specific risk factor for DVT in the Warfarin-resistance group that has a genetic background.

The rest of the circumstantial risk factors showed similarity with no statistical differences between the Warfarin-resistance and Warfarin-sensitive groups. 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 thrombosis is likely to develop even in patients with thrombotic gene mutation. This has been early suggested by Jackson C M.[23]

We were able to demonstrate FIIA 20210 gene mutation in 20 individuals of the studied population (18%). Homozygous mutation was found in only one patient, who was in the Warfarin-resistance group, while heterozygous mutation was found in the three subgroups. The gene mutation was recognized in one out of the 30 healthy blood donors (3.4%), which agrees with Muhammad Abdul Naeem et al who found this mutation in 2 individuals out of 200 normal population from Pakistan (1%).[25] It also agrees with Miletich JP et al., Arruda VDet al. and Rosendaal FR, who found this mutation in 1.7% and 3% of the normal Northern European and Southern European population respectively.[2,11,12] However, our finding represents only the 30 studied healthy blood donors, larger random sample size is needed to define the exact prevalence in the community.

Heterozygous gene mutation was found in 30% of the Warfarin-resistance and only 15% of the Warfarin-sensitive group. This difference was statistically significant and may support the theory that F II A gene mutation may cause high prothrombin level as early suggested by Poort S R et al.[2] This high prothrombin level may be responsible for resisting the normal response to Warfarin with the usual doses.

However, the presence of the same mutation in certain patients of the Warfarin-sensitive group may be explained by the presence of different degrees of mutation that did not clinically affect the Prothrombin levels and therefore the response to Warfarin in these patients. This gene mutation with less extent of clinical significance was previously suggested by Souto J C et al.[24]

Male to female ratio for patients with positive gene mutation was 7:13. This was statistically significant ($P < 0.05$), which may suggest a sex relation with this type of mutation.

The relation between this gene mutation and recurrent DVT or family history was interesting. Most of the recurrent DVT patients (86%) and those with positive family history (80%) were found to have prothrombin gene mutation. Both findings are statistically significant.

This confirms that patients with Prothrombin gene mutation are at a higher risk of developing recurrent DVT, which was estimated by Miletich et al. and Royle et al. to be 3-6 folds higher than the normal population.[2,9]

It may also point to the importance of screening other members of the family as they may be at increased risk of developing DVT.

However, the significance of the present study results are limited by the small sample size and to some extent the selection bias.

In conclusion: This study suggests that F II A gene mutation may be present in certain DVT patients, especially in Warfarin-Resistance patients. The study suggests that Prothrombin gene mutation should be tested in a DVT patient who is recurrent or has a family history or showing Warfarin-resistance.

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