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Dr. Nehal Salah Hasan
Department of Clinical and Chemical Pathology,
National Research Center, Cairo, Egypt

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Genetic Mutation in Thrombophilic Egyptian Patients

[1]Nehal Salah Hasan, [1]Hesham Gamal El Din,
[1]Mona Abd El Kader Awad and [2]Hesham Abou Aisha

Factor V Leiden and the prevalence of PAI-1 promoter 4G/5G polymorphism were examined in 48 Egyptian patients who presented with deep venous thrombosis. They were investigated for the presence of APCR, where positive cases were further subjected to DNA analysis by PCR for detection of factor V Leiden mutation. PAI-1 polymorphism was also studied in patients and 40 healthy individuals matching in age and sex as controls by PCR-RFLP to demonstrate the influence of 4G/5G dimorphism of the PAI-1 promoter gene on thrombotic risk. Present results revealed the presence of APCR in 14/48 (29.2%) cases out of the whole thrombotic cases by DNA analysis, only 10/14 (71.4%) were found carrying the mutant factor V gene ($p<0.05$). No synergistic effect was observed between the 2 polymorphisms when analyzed together. There was an association between the 4G allele of the PAI-1 gene and the risk of thromboembolism in patients suffering from DVT compared with healthy control (41.7% vs. 20%; $p = 0.04$, OR 2.85; 95% CI 1.089-7.493). The prevalence of 5G homozygous carriers was significantly lower in patients than in controls (12.5% vs. 40%; $p = 0.006$, OR 0.214, 95% CI 0.074-0.621). In conclusion, APCR as a result of factor V Leiden is the most commonly recognized inherited risk factor for thromboembolism. In patients with deep vein thrombosis the 4G polymorphism of PAI-1 gene promoter should be taken into consideration as a facilitating condition for pathological fibrinolysis together with other environmental and genetic factors.

Key words: Thromboembolism, DVT, APCR, factor V Leiden, PAI-1

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[1]Department of Clinical and Chemical Pathology, National Research Center, Cairo, Egypt
[2]Department of General Surgery, Faculty of Medicine, Cairo University, Cairo, Egypt

INTRODUCTION

Vascular haemostasis results from the regulated interaction of coagulation and fibrinolytic systems and that these two systems are in dynamic equilibrium. The net fibrinolytic activity of cells and blood appears to reflect the balance between the plasminogen activators on one hand and PAI-1 on the other. Thus changes both in the PAs or PAI-1 may alter this balance and lead to thrombotic problems or a tendency to develop a bleeding diathesis (Stiko *et al*., 2001).

A few years ago up to 70% of thrombotic patients with no identifiable risk factors, were termed idiopathic. Now, molecular diagnostics combined with existing laboratory techniques allow accurate classification of at least half of patients with inherited thrombotic disorders.

Inherited thrombophilia is of a multifactor nature. A point mutation at nucleotide position 1691 of the factor V molecule (factor V Leiden) accounts for the most common cause of inherited thrombophilia (Svensson and Dahlback, 1994), this disorder was initially described by Dahlback *et al*. (1993).This mutation alters factor V molecule such that it is no longer degraded by activated protein C (APC), yet it still retains its procoagulant activity favoring thrombosis (Bertina *et al*., 1994).

Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (serpine) super family, is the principle inhibitor of t-PA and u-PA in the fibrinolytic system (Wu and Zhao, 2002). Reduction of active PAI-1 may lead to a decreased tendency of thrombosis (Gils *et al*., 2002).

This research aims to study the molecular diagnosis for the possible most common inherited causes of thrombosis (G1691A in the factor V gene and the 4G/5G dimorphism of PAI-1 gene promoter) to determine their role in hereditary thrombophilia. Correlation between the hereditary causes of thrombosis, clinical presentation and laboratory findings of the patients was also studied.

MATERIALS AND METHODS

The current study included 48 cases with deep venous thrombosis. Their ages ranged between 20-53 years. These patients were selected from the emergency unit of Kasr El Aini Hospital, Cairo University (January 2004 - July 2004) before receiving the anticoagulant medication.

All patients were submitted to full history including personal and family history of thrombosis, complete general and local examination. Routine laboratory investigations were done including CBC, platelet count, fasting blood sugar and lipid profile. Also coagulation profile was done including PT, PTT (Diamed), protein C and S (ELISA from Corgenix), ATIII (Stago) and lupus anticoagulant (Stago). Patients were tested for APCR phenotype by modified activated partial thromboplastin time.

Positive cases for APCR were further subjected to DNA analysis by PCR followed by reversed hybridization for detection of factor V Leiden. PAI-1 polymorphism was studied in patients and controls by restriction fragment length polymorphism (PCR-RFLP) to demonstrate the influence of 4G/5G dimorphism of the PAI-1 promoter on thrombotic risk. Genomic DNA was prepared by standard salting-out technique on the method of Miller *et al*. (1988). The above DNA analysis was conducted at Clinical and Chemical Department in National Research Center.

Factor V Leiden amplification and detection: Patient sample PCR reactions were prepared by mixing 5 μL of patient DNA, 35 μL primer/nucleotide mix (containing biotinylated forward primer and biotinylated reverse primer), 0.5 μL Taq polymerase and 5 μL $MgCl_2$. Amplification was carried out in 50 μL in a Perkin Elmer thermocycler 9600 (Applied Biosystems) using the following profile: 95°C for 5 min; followed by 30 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C; followed by 5 min at 72°C. Then, the amplified DNA fragments are subsequently used for the reverse hybridization assay. The reverse hybridization assay starts with the denaturation of amplicon DNA. Subsequently, hybridization buffer is added and a strip coated with probes for the wild type and the mutated sequences of the gene loci analyzed as well as a number of control zones is added to each sample. During incubation in a shaking water bath the various single-stranded amplicons are hybridizing with respective complementary gene probes. A highly specific washing step removes any unspecifically bound DNA. After addition of streptavidin conjugated alkaline phosphatase to the hybrid consisting of probe and biotin-labeled amplicon, the complex is visualized by the addition of NBT/BCIP (a substrate for alkaline phosphatase). The provided commercial reading strips (Thrombo Type) ensure the fast and easy interpretation of the banding pattern obtained.

PAI-1 amplification and detection: Amplification was carried out in Pekrin Elmer Thermocycler 9600 with a final volume of 50 μL (Applied Biosystems). Each PCR sample contained 5 μL of genomic DNA in: 5 μL of 10× Taq polymerase buffer minus Mg, 8 μL dNTPs (2.5 Mm L^{-1}; Q Biogene USA), 1 μL of each primer (30 pmol μL^{-1}; Pharmacia Biotech. USA) and 0.5 μL Taq polymerase

(5 μL μL^{-1}; Promega comp. USA), 4 μL of $MgCl_2$ (50 Mm L^{-1}) and 25.5 μL distilled water.

Forward sequence: 5'-CACAGAGAGAGTCTGGCCACGT-3'
Reverse sequence: 5'-CAGCCACGTGATTGTCTAGGT-3'

A first denaturation at 94°C for 5 min was followed by 35 cycles of the following conditions: 57°C for 30 sec (annealing), 72°C for 30 sec (extension); 94°C for 30 sec (denaturation) then 72°C for 5 min and a 4°C hold temperature.

Electrophoresis was applied for detection of the 4G allele in amplified DNA band at 142 base pair. While as the 5G allele was detected by the restriction fragment length polymorphism (PCR-RFLP), in which the amplified DNA fragment is further subjected to a digesting restriction endonuclease enzyme (BslI enzyme from *Bacillus* species with a recognition sequence

5'...CCNNNN/NNGG...3' and a concentration of 10 U μL^{-1}). Since the polymorphism changes the recognition sequence for the restriction enzyme, the number of DNA fragments after digestion will change depending on the presence or absence of this polymorphism. The amount and sizes of the resulting fragments are visualized by gel electrophoresis.

Statistical analysis: The data were coded and entered on the computer and statistical analysis was performed by using computer software package SPSS 11.0. Data were summarized using the mean; as a measure of central tendency and standard deviation; as a measure of dispersion was calculated for continuous variables. While frequency and percent distribution were calculated for categorical ones. Statistical differences for categorical variables were assessed using the Chi-Square test.

RESULTS

Results of Table 1 showed that out of 48 DVT patients, 62.5% had their thrombotic event for the first time, while the thrombotic event recurred two times in 25% and recurred three times in 12.5% of DVT cases, 37.5% had positive family history of thrombosis, 25% were smokers, 33.3% were diabetics and 45.8% were hypertensive. Out of the 48 patients, only 16.7% had past history of pulmonary embolism.

Table 2 represents the coagulation profile done to our 48 DVT patients with their mean, standard deviation, minimum and maximum ranges and APC ratio.

APCR was indicated when the ratio was below or equal the cut-off value. The cut-off value in this study was considered to be 2.

Table 1: Frequencies of clinical parameters of DVT patients (No. 48)

	Item	No.	Percentage
Sex	Female	32	66.7
	Male	16	33.3
Recurrence of	1	30	62.5
attacks	2	12	25.0
	3	6	12.5
Family history	Positive	18	37.5
Of thrombosis	Negative	30	62.5
Smoking	Positive	12	25.0
	Negative	36	75.0
Diabetes	Positive	16	33.3
	Negative	32	66.7
Hypertension	Positive	22	45.8
	Negative	26	54.2
Pulmonary	Positive	8	16.7
embolism	Negative	40	83.3

Table 2: Coagulation profile of DVT patients and their APCR coagulation test (no.: 48)

Item	Mean	±SD	Minimum	Maximum
PT(sec)	12.80	0.80	11.7	14.10
PC (%)	87.30	11.20	70.0	100.00
PTT (sec)	38.40	7.90	24.0	53.00
Ptn S (%)	95.30	20.70	60.0	120.00
Ptn C (%)	102.80	17.90	60.0	120.00
ATIII (%)	111.80	10.80	84.0	120.00
LA (sec)	50.60	17.60	33.0	106.00
APTT with APC/$CaCI_2$ (sec)	96.90	31.30	37.3	150.40
APTT with $CaCl_2$ (sec)	42.30	6.30	29.6	53.50
Ratio	2.28	0.65	1.1	3.28

Table 3: Frequencies of APCR and DNA analysis of factor V in DVT patients (No. 48)

	Item	Number	Percentage
APCR	Positive	14	29.2
	Negative	34	70.8
Factor V	Normal	38	79.2
	Heterozygous mutant	9	18.8
	Homozygous mutant	1	2.0

Table 4: Genotype frequencies of PAI-1 4G/5G polymorphism in DVT patients and controls

Genotype	Cases (%) No. 48)	Controls (%) (No. 40)	Odds ratio	95% CI	p-value
4G4G	20(41.7)	8(20)	2.875	1.089-7.493	0.04
4G5G	22(45.8)	16(40)	1.269	0.542-2.970	0.70
5G5G	6(12.5)	16(40)	0.214	0.074-0.621	0.006

Table 3 shows that 29.2% of our DVT patients were resistant to APC and by DNA analysis, 20.8% were found carrying the mutant factor V gene, the mutation was heterozygous in 18.8% of patients, only 2% of patients showed homozygous mutation. Out of our 14 APCR patients, 10 were carrying mutant factor V gene (71.4%, $p<0.05$).

Table 4 shows that out of the 48 DVT, 20 patients (41.7%) were of the 4G/4G genotype vs. 8 healthy controls (20%) ($p = 0.04$). While 22 patients (45.8%) were having 4G/5G compared with 16 healthy controls (40%) ($p = 0.7$). The 5G/5G allele frequency was significantly higher in control group (40%) than in the DVT patients (12.5%) ($p = 0.006$).

DISCUSSION

Venous thrombosis is a multi-factorial disorder that arises from the combined effects of acquired and inherited risk factors (Lane and Grant, 2000) of which, genetic risk factors became a frequent predisposing cause of venous thromboembolism (Emmerich and Aiach, 2002).

The factor V Leiden mutation, an Arg to Gln change at amino acid 506, leads to resistance to cleavage by activated protein C (APCR). It is the most common inherited risk factor for venous thrombosis and is found in 20 to 40% of cases (Bertina, 1997; Sheppard, 2000; Elmer *et al*., 2005).

Among candidate genes that contribute to thrombosis, plasminogen activator inhibitor-1 (PAI-1) is of interest. Elevated levels of PAI-1 are associated with venous thrombosis (Green and Humphries, 1994). A single-base insertion/deletion at nucleotide-675 (4G/5G) in the upstream regulatory region of PAI-1has associated with increased levels of PAI-1 protein (Dawson *et al*., 1993). The-675 4G/5G polymorphism affects the binding of nuclear proteins involved in the regulation of PAI-1 gene transcription (Erikson *et al*., 1995) and is being associated with increased transcription of the PAI-1 gene (Henry *et al*., 1998; Sartori *et al*.,1998).

The aim of this research is to study the possible most common inherited causes of thrombosis; G1691A in the factor V gene and PAI-1gene at molecular basis, to determine their role and frequency in hereditary thrombophilia and to demonstrate the correlation between causes of thrombosis, clinical presentation and laboratory findings of these patients.

In this study, 14/48 (29.2%) cases were found to be resistant to activated protein C and by DNA analysis, 10/14 (71.4%) were found carrying the mutant factor V gene. We found that the prevalence of factor V Leiden in our DVT patients was 10/48 (20.8%). Of the cases found to have mutation 90% were heterozygous and 10% were homozygous.

In study done by Eid (2002) on 602 patients with suspected thrombosis, protein C deficiency was found in 3.8%, protein S deficiency in 2.3% and antithrombin deficiency in 1.4%. An APC-R problem was seen in 23%. Out of the APC-R patients, 75% had the factor V Leiden mutation by DNA analysis. These results confirm that APCR, as a result of factor V Leiden mutation, is the most prevalent cause of thrombosis.

Fisher *et al*. (1996) and van der Bom *et al*. (1996) documented that activated protein C resistance can occur due to an acquired state. Acquired APC resistance is common in the antiphospholipid syndrome and has been suggested to play a major role in the pathogenesis of thrombosis in patients with this syndrome (Hanly, 2003).

Recent evidence suggests that the antiphospholipid antibodies disrupt phospholipids-dependent anticoagulant mechanisms or that these antibodies induce the expression of procoagulant and proadhesive molecules on endothelial cells (Rand, 2003). The detection of lupus anti coagulants and, possibly, of immunoglobulin G (IgG) anticardiolipin antibodies at medium or high titers helps to identify patients at risk for thrombosis (Galli *et al*., 2003; Meroni *et al*., 2003; Lawrence and Leung, 2005). This explanation and other causes for acquired APCR can be applied to the 4 patients with positive APCR phenotype and normal DNA analysis of factor V in this study.

In this study, no statistically significant difference was found between mutant and normal factor V patients as regards the recurrence of thrombotic attacks. This agrees with Eichinger *et al*. (2002) and Hoppensteadt *et al*. (2005) moreover, Buyru *et al*. (2005) found no evidence of association between factor V Leiden mutation and ischemic stroke.

Both factor V Leiden mutation and homozygous mutation G20210A in prothrombin gene may constitute concomitant risk factors for deep venous thrombosis but, alone, are not high risk factors for venous thrombosis and together may increase 5-10 fold risk of venous thrombosis (Kurkowska-Jastrzebska *et al*., 2003; Doggen *et al*., 2004; Brown and Yuen, 2005).

Plasminogen activator inhibitor-1 (PAI-1) is the main regulator for the endogenous fibrinolytic system and modulates the thrombosis progression (Lopes *et al*., 2003).

In this study, there was an association between the 4G allele of the PAI-1 gene and the risk of thromboembolism in patients suffering from DVT compared with healthy controls (41.7% vs. 20%; $p = 0.04$, OR 2.85; 95% CI 1.089-7.493). The 5G allele frequency was significantly reduced in patients than in controls (12.5% vs. 40%; $p = 0.006$, OR 0.214, 95% CI 0.074-0.621).

Several studies reported associations between PAI-1 levels and venous thromboembolism (Francis, 2002), coronary disease and ischemic stroke (Smith *et al*., 2005). Moreover, studies have focused on genetic polymorphisms, particularly the 4G/5G insertion/deletion in the promoter region, affecting transcription rates. Most of them, have reported higher PAI-1 levels in individuals with homozygous PAI-1 gene mutation 4G/4G (Sartori *et al*., 2003; Glueck *et al*., 2004).

A similar result was reported in a group of young Asian Indians who smoke, where the PAI-1 4G allele was found to be a mild risk factor for the development of myocardial infarction (Pegoraro and Ranjith, 2005).

On the other hand, Segui *et al*. (2000) found no differences between the 4G/5G allele distribution in the

DVT group and in the control group. However, the presence of the 4G allele significantly increased the risk of thrombosis in patients with other thrombophilic defects.

The prevalence of PAI-1 5G allele in healthy subjects found by Sartori *et al.* (1998) goes with our results suggesting that 5G could be considered as a protective allele against thromboembolism. However, Gerhardt *et al.* (2005) found that women carrying the PAI-1 5G/5G genotype are at risk for early onset of severe pre-eclampsia.

From the present study, we can conclude that, the prevalence of the 4G allele in DVT patients was higher than that in controls with a statistically significant difference and carrying the homozygous 4G allele increases the risk of thrombosis in the presence of other genetic or acquired risk factors. Moreover, APCR can occur due to causes other than factor V Leiden. Also, family history of thrombosis, pulmonary embolism, recurrence rates and lupus anticoagulant were not related to the presence or absence of laboratory evidence of heritable thrombophilia (PAI-1dimorphism or FVL) ($p>0.05$). However, present results support the hypothesis that factor V Leiden and PAI-1 probably interact with other genetic or environmental risk factors to induce a thromboembolic event.

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