

Endothelial Nitric Oxide Synthase Gene Polymorphisms in a Tunisian Deep Vein Thrombosis Group

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Abstract

Deep vein thrombosis (DVT) is a multi-factorial disease involving both genetic and acquired risk factors. The objective of this study was to determine the frequencies of endothelial nitric oxide synthase (eNOS) gene polymorphisms *G894T* (rs1799983) and *T-786C* (rs2070744) to assess the role of these polymorphisms as a potential risk factor in the development of DVT. Methods: In this case-control study, we included 32 patients with deep vein thrombosis (DVT) and 31 healthy control subjects. Clinical characteristics were collected. Lipids plasma concentrations were determined by the colorimetric method. Genotyping for the polymorphisms was performed by restriction fragment length polymorphism (PCR-RFLP) method. Results: We had found that the eNOS *G894T* genotype G/T was significantly increasing the risk of DVT ($P = 0.042$, OR = 3.9; 95% CI = 1.09 to 13.92). But no association of the eNOS *T-786C* variant and DVT was found. For the eNOS *T-786C* polymorphism, the frequency of the T/T genotype was 87.5% in patients (with an allelic frequency of T Allele equal to 91%). No significant difference was noted between the two groups ($P > 0.05$). Conclusion: The eNOS *G894T* polymorphism seems to be in association with DVT and may be considered as a risk factor, but this is not the case for the *T-786C* polymorphism.

Keywords

Deep Vein Thrombosis, eNOS, NO, *G894T* Polymorphism, *T-786C* Polymorphism

1. Introduction

Deep vein thrombosis, commonly referred to as “DVT,” occurs when a blood clot or

thrombus develops in the large veins of the legs or pelvic area. Some DVTs may cause no pain, whereas others can be quite painful. With prompt diagnosis and treatment, the majority of DVTs are not life threatening. However, a blood clot that forms in the invisible “deep veins” can be life threatening. A clot that forms in the large, deep veins is more likely to break free and travel through the vein. It is then called an embolus. When an embolus travels from the legs or pelvic areas and lodges in a lung artery, the condition is known as a “pulmonary embolism” or PE, a potentially fatal condition if not immediately diagnosed and treated [1].

The PE justifies the concept of venous thromboembolism (VTE). The latter remains a cause of morbidity and mortality whose incidence is on average around 1 to 2/1000 per year. Many factors favour the occurrence of VTE and can be genetic (deficits in antithrombin, protein C deficiency, protein S deficiency, resistance to activated protein C etc...) or acquired [2].

For many years, the basis for the understanding of venous thrombosis pathophysiology was Virchow's triad, which postulates that venous thrombosis may be generated by changes in blood composition, blood flow, or alterations in the blood vessel wall [3].

Clinical signs and symptoms that can be present with DVT are calf tenderness or tenderness along the course of the veins involved, pain on dorsiflexion of the foot, unilateral leg swelling, warmth and erythema. Other signs include distension of superficial veins and appearance of prominent venous collaterals [4].

During the past decade, considerable progress has been made in the study of risk factors for VTE [5] in particular genetic abnormalities.

It has been described that a deficit of nitric oxide (NO) can be considered as one of VTE risk factors. Indeed, NO is a key determinant of vascular homeostasis. It can regulate many physiological processes. The malfunction of one of these processes may result in thrombotic diseases. The endothelial NO is released due to the action of endothelial nitric oxide (eNOS).

Several polymorphisms in the eNOS gene have been described. This *G894T* and the *T-786C* variants have been reported to be associated with an increased risk of coronary artery disease and increased risk of death after coronary stenting

The eNOS is encoded by nitric oxide synthase 3 (NOS₃) located at the 7q36 chromosome and whose NOS3 mutations may affect the production of NO in endothelial cells and vascular homeostasis.

In this context, we have proposed in this study to assess the frequency of two eNOS gene polymorphisms (*T-786C* and *G894T*) in a group of patients with DVT and a group of witnesses in order to assess the role of these polymorphisms as a potential DVT risk factor.

2. Material and Methods

This study was conducted at the Research Unit of Biochemistry service in collaboration with the internal medicine department of the Main Military Hospital of Instruction of Tunis (HMPIT) during a period from March until the month of December 2015.

2.1. The Study Population

In this case-control study, we recruited 63 subjects divided into two groups:

- Group of patients: We collected 32 subjects with DVT for the first time or recurrent, admitted in internal medicine department of HMPIT.
- Control population: We collected 31 control subjects apparently healthy age and sex-matched with the patient population. The subjects in this group were selected from voluntary donors of blood at the military blood bank and among medical and paramedical personnel.

The ethics Committee of the Internal Medicine department of the Main Military Hospital of Instruction of Tunis approved the study protocol. All of the patients gave oral informed consent.

The study objectives and procedure were explained to all participants, before obtaining their consent to enrol into the study.

2.2. Biochemical Analysis

Blood samples for high density lipoprotein cholesterol (HDL-C), triglycerides, and total cholesterol were obtained after 12 hours of fasting.

Serum triglycerides, serum cholesterol and high-density lipoprotein (HDL)-cholesterol were assayed by an enzymatic-colorimetric technique using Unicell DXC 800 analyzer (Beckmann Coulter).

The low density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula for values of TG \leq 4.5 mmol/l. $LDLc = CT - (HDL + TG/2.18)$.

Serum concentration of hs-CRP, ApoA1, ApoB and Lp(a) were assayed by an immunonephelometric method using the BN II nephelometer Analyzer. Analysis was done according to manufacturing instructions.

2.3. Molecular Analysis

DNA was isolated from venous blood samples. eNOs 897G/C and-786T/C, polymorphisms were analyzed by the polymerase chain reaction restriction fragment length polymorphism technique (PCR-RFLP) with the following primers and enzymes (**Table 1**).

A final reaction volume of 50 μ L for the Polymerase Chain Reaction (PCR) was constituted, which contained 100 ng of genomic DNA, 10 μ M of each primer, 25 mM of

Table 1. Primers and enzymes used for polymerase chain reaction.

Polymorphism		Primers	Enzyme
G894T(rs1799983)	F	5'-AAGGCAGGAGACAGTGGATGGA-3'	Ban II
	R	5'-CCCAGTCAATCCCTTTGGTGCTCA-3'	
T-786C(rs2070744)	F	5'-GCAGGTCAGCAGAGAGACTA 3'	Msp I
	R	5'-GACACAGAACTACAAACCCC-3'	

each deoxynucleotide triphosphate (dNTP), 5 U/ μ L of Paq 5000 DNA polymerase (Agilent, USA), 10X Paq5000 Reaction Buffer (Agilent, USA) and 40 μ L of distilled water.

The PCR was realized under the following conditions (**Table 2**).

In order to genotype the two polymorphisms, 10 μ L of the resulting PCR products were digested at 37°C for 5 minutes (*T786C* polymorphism) and for 4 - 16 h (*G894T* polymorphism) with 10 U/ μ L of MspI and Ban II respectively (Thermo scientific). The enzymes are inactivated by incubation at 65°C for 30 min.

The reaction volume was set to 30 μ L, containing 10 μ L of PCR reaction mixture, 2 μ L of 10X Buffer, 1 μ L of the enzyme, and 18 μ L of nuclease free water.

2.4. Statistical Analysis

Statistical analysis of different data was performed by computer using SPSS Version 19 statistical software. For continuous variables, we expressed the results as the arithmetic mean and standard deviation. Statistical comparison was performed by the Student t test.

Comparison between non-continuous variables, genotype distribution, and Hardy-Weinberg Equilibrium were tested by the chi 2 test (χ^2).

$P < 0.05$ was considered statistically significant.

3. Results

Out of the 32 patients 19 were men, and mean age was 47.9 years. The control group (n = 31) consisted of 18 men, and mean age was 46.8 years. The two groups are age and sex matched. The epidemiological and biological characteristics of the study population are shown in **Table 3**. In our study, no statistically significant difference was shown for cholesterol, TG, HDLc and LDLc between the two groups.

Table 2. Reaction mixture of eNOS gene polymorphisms.

Polymorphism	Cycles	Temperature	Duration
<i>G894T</i>	1	95°C	2 minutes
		95°C	20 seconds
	35	55°C	20 seconds
		72°C	20 seconds
	1	72°C	5 minutes
	<i>T-786C</i>	1	95°C
95°C			30 seconds
35		56°C	30 seconds
		72°C	20 seconds
1		72°C	10 minutes

Table 3. Baseline characteristics and laboratory findings of the study groups.

Parameters	Patients	Controls	P
Age (years)	47.9 ± 16.8	46.8 ± 14.9	0.785
Sex Ration (M/W)	1.46	1.38	0.917
BMI (Kg/m ²)	27.10 ± 8.42	28.00 ± 4.70	0.614
Smoking (%)	50	25.8	0.05
Alcoholism (%)	15.6	3.2	0.96
Physical activity	34.4	51.6	0.170
Chol (mmol/l)	4.39 ± 0.95	4.54 ± 0.93	0.536
TG (mmol/l)	1.35 ± 0.74	1.72 ± 1.05	0.131
HDLc (mmol/l)	1.13 ± 0.51	1.08 ± 0.31	0.661
LDLc (mmol/l)	2.89 ± 0.86	2.70 ± 0.79	0.401

Chol: Cholesterol, TG: Triglyceride, HDLc: High Density Lipoprotein Cholesterol, LDLc: Low Density Lipoprotein Cholesterol.

Genotype Distribution

The *G894T* and *T-786C* eNOS gene polymorphisms are in agreement with Hardy-Weinberg equilibrium (HWE Eq = 0.711 < 3.841 and HWE Eq = 4.932 < 5.991 respectively).

The distribution of genotypes and allelic frequency are represented in **Table 4**.

For the *G894T* single nucleotide polymorphism (SNP), the T allele frequency is significantly higher in patients compared to controls. The odds ratio = 3.9 (P = 0.035; IC 95% = 1.09 - 13.92)

We compared two populations according to genotype for different models (dominant, recessive and additive) Results are shown in **Table 5**.

We have no significant difference between patients and controls for the three models.

4. Discussion

Deep vein thrombosis (DVT) is a multi factorial disease involving both genetic and acquired risk factors

We determined for each patient and control genotypes *G894T* (rs1799983) and *T-786C* (rs2070744) of the eNOS gene polymorphisms. We calculated the allele frequencies for each polymorphism.

4.1. The 894G/T Polymorphism of the eNOS Gene

The analysis of our results shows an allelic frequency of 93.35% and 6.56% for the G and T allele respectively in control group. According to the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs1799983), our results are similar to that of the African population (T allele = 7% and G allele = 93%).

Table 4. Distribution of genotypes in both groups.

SNP	Genotype frequency (%)			χ^2	Allele frequency (%)		χ^2
	GG	GT	TT	P value	G Allele	T Allele	P value
<i>G894T</i>							
Patient	62.5	37.5	0	0.042	85	15	0.035
Control	86.7	13.3	0		93.35	6.65	
<i>T-786C</i>							
Patient	87.5	6.3	6.3	0.85	91	9	0.76
Control	90.3	6.5	3.2		94	6	

Table 5. Statistical comparison by genotypes model of *T-786C* polymorphism in both groups.

Genotypes	Controls	Patients	P
Dominant Model			
CC + TC	9 (29%)	4 (12.5%)	0.129
TT	22 (71%)	28 (87.5%)	
Recessive Model			
TT + TC	30(96.8%)	30 (93.8%)	0.95
CC	1 (3.2%)	2 (6.2%)	
Additive Model			
Allele T	29 (93.5%)	29 (90.6%)	0.98
Allele C	2 (6.5%)	3 (9.4%)	

According to Akhter *et al.* [6], the frequency of G allele is 83% in Chinese population. Similarly, Ben Ali *et al.* [7] report a G allele frequency of 81.8% in a Tunisian healthy group.

In our patients group, the allelic frequency was 85% and 15% for G and T alleles respectively. The statistical comparison between patients and controls showed a significant difference ($P = 0.025$) with an OR = 3.9 (95% CI = 1.09 to 13.92). Our results were in agreement with those of Li *et al.* [8] who studied the relationship between the polymorphism (*G894T*) and DVT in a Chinese population.

Similarly, Akhter *et al.* [6] reports a statistically significant difference in allelic frequencies of *G894T* polymorphism between controls and DTV patients with an OR of 3.93.

Several other prospective studies have shown that the polymorphism *G894T* increase the risk of DVT [9] [10].

However, Ying *et al.* [11] demonstrated that this polymorphism has no connection to the DTV.

This polymorphism leads to an amino acid substitution, glutamate in position 298 by an amino acid Aspartate, which attributes to the enzyme cleavage by an endogenous

protease. Therefore, this polymorphism could decrease the levels of NO released by eNOS [9]. This loss of NO synthesis was described at the origin of cardiovascular diseases including DVT [12].

Vascular nitric oxide (NO) is produced continuously by endothelial cells. The reaction is catalysed by eNOS. NO is a molecule described as critical in the vascular system regulation including the inhibition of aggregation and platelet activation, also in leukocyte adhesion to the endothelium, in reducing the proliferation of vascular smooth muscle cells and therefore, NO plays a very important anti thrombotic role [13].

A malfunction in the release of NO can therefore promote DVT by inhibiting the protective effects. Several polymorphisms of eNOS gene can participate in this dysfunction including *G894T* polymorphism.

Indeed, the mutated eNOS protein becomes unstable. It has been demonstrated by an in vitro study that eNOS protein with aspartate at position 298 is cleaved, suggesting that variant *G894T* eNOS gene has a functional effect on eNOS protein [14]. Therefore, the polymorphism can reduce the eNOS protein function leading to a reduction of the NO synthesis in endothelial cells. This increases the risk of thrombotic and atherosclerotic disease, including deep vein thrombosis by reducing the protective effect of NO [8].

The relationship between the eNOS activity, hyper homocysteinemia and reactive oxygen species (oxidative stress main generator) has been demonstrated [9] [12].

4.2. The *T-786C* Polymorphism of the eNOS Gene

We found that the frequency of T/T genotype was 87.5% and 90.3% in patients and controls respectively. The allelic frequency of the T allele was 91% in patients and 94% in controls.

Our results show no significant difference in genotype distribution and allelic frequencies between the two groups.

Our results are consistent with the study of Akhter *et al.* [6]. The authors have shown that there is not a significant difference between controls and patients in an Asian population.

However, the *T-786C* polymorphism has been shown to repress transcription of the eNOS gene [15].

5. Conclusions

Deep Vein Thrombosis is a common disease which the vital and functional prognosis can be serious.

According to our preliminary results, the *G894T* eNOS gene polymorphism appears to be a potential risk factor for DVT. Subjects with the T allele seem to have a risk 3.9 times higher than patients in the GG genotype wild.

The *T-786C* eNOS gene polymorphism seems not to be involved in the occurrence of thromboembolic events.

These results seem to be interesting, and encourage us to expand the cohort of the

study to better draw conclusions.

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