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Study of Relationship between G894T Variant of Endothelial Nitric Oxide Synthase Gene and Acute Ischemic Stroke

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ABSTRACT

Background: Cerebral stroke (CS) is considered as one of the major causes of death and disability worldwide**.** Ischemic stroke is the most common type of stroke in Egypt, as in other countries, accounting for 43% to 79% of all stroke types .There are some traditional factors that increase the risk of ischemic stroke such as hypertension and smoking, but genetic risk factors, suggested by evidence from inheritance-based studies, might contribute to predisposition to ischemic stroke.Among investigated genetic variations of eNOS is G894T polymorphism that leads to a change of glutamate to aspartate at site 298 and is said to have increased susceptibility to cleavage of eNOS enzyme which contribute to the development of stroke.

Patients and methods: The study was conducted on a group of thirty **(30)** Egyptian patients admitted to the Neurology Department and Stroke Units of Ain Shams University hospitals (Al-Demerdash hospital).They were presented with acute ischemic stroke in the first 48 hours and diagnosed according to clinical neurological and radiological examinations. Oral informed consents were obtained either from the patients or their relatives before enrollment in the study according to the Ethical Committee of Faculty of Medicine, Ain Shams University. In addition, ten (10) Egyptian apparently healthy, age and sex- matched subjects were investigated as control group. All individuals included in this study were subjected to full history taking, thorough general, neurological and radiological assessment. The laboratory investigations included complete blood count, coagulation profile, and blood glucose level assay. Furthermore, eNOS G894T gene polymorphism was assessed by PCR- RFLP. Results: In this study, we couldn't find a significant association between eNOS G894Tgene polymorphism and acute stroke Egyptian patient.

Keywords: acute ischemic stroke , endothelial nitric oxide.

INTRODUCTION

Cerebral stroke **(CS)** is considered as one of the major causes of death and disability worldwide **[(1)].**Ischemic stroke is the most common type of stroke in Egypt, as in other countries, accounting for 43% to 79% of all stroke types **[(2)]**.There are some traditional factors that increase the risk of ischemic stroke such as hypertension and smoking, but genetic risk factors, suggested by evidence from inheritance-based studies, might contribute to predisposition to ischemic stroke**[(3)]**.Endothelium derived nitric oxide **(NO)** is critical to vascular homeostasis. **NO** has potent vasodilator and anti-proliferative effects, as well as antithrombotic properties. It stimulates smooth muscle cell relaxation and inhibits platelet aggregation and leukocyte adhesion. Impaired endothelium-dependent vasodilation is a general attribute of atherosclerotic vessels, which to some extent could be due to the reduction in the activity of vascular endothelial nitric oxide synthase (eNOS). This impaired NO-dependent vasomotor reactivity has been implicated in the patho-physiology of stroke**[(4)]**.Previously published studies have indicated that the **(eNOS**) gene plays a crucial role in thepathogenesis of many diseases including stroke, essential hypertension and it has an important role in development of coronary artery disease **[(5,6)]**. The eNOS gene is situated on chromosome 7 (7q35–q36), it consists of 26 exons and codes for an enzyme that produces Nitric Oxide **(NO)** in the vascular endothelium**[(7)]**.Different genetic variations of eNOS were identified to be associated with development of stroke as (4b⁄ a, 786T>C, A-922G and Glu298Asp) gene polymorphisms **[(8)]**.Among investigated genetic variations in eNOS is G894T polymorphism that leads to a change of glutamate to aspartate at site 298 and is said to have increased susceptibility to cleavage of eNOS enzyme which contribute to the development of stroke **[(9)]**.

AIM OF THIS WORK

Explore the relationship between G894T polymorphism of endothelial nitric oxide synthase gene and acute ischemic stroke.

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SUBJECT AND METHODS

This study was conducted at Clinical Pathology and Stroke Units of Ain Shams University hospitals from period between November **2016** and July **2017**.A total number of **40** subjects were included (30 patients and 10 controls).Patient group included 30 patients presented with acute ischemic stroke (13 females and 17 males), diagnosed according to clinical, neurological and radiological examinations.

A control group of ten (10) apparently healthy, age and sex matched subjects were included in the study (2 females and 8 males). Under complete aseptic conditions, 5mL of venous blood were obtained by a clean vein-puncture into vacutainer tube with EDTA .

The Samples were stored at -20°C for DNA extraction and detection of eNOS point mutation by PCR-RFLP. It depends on amplification of a target region of the gene of interest by PCR then the amplified DNA product was digested by restriction endonuclease enzyme (**BSP143I)** Restriction endonucleases can digest DNA with a high degree of nucleotide sequence specificity. If the point mutation is present within the recognition sequence, the DNA of interest acts as the substrate for the specific restriction enzyme.

The size of the digestion products was detected by agarose gel electrophoresis with ethidium bromide staining and ultraviolet trans-illumination. In this case the position of the bands varies between subjects according to absence or presence of the recognition site for the restriction enzyme used detected by the by ladder.

eNOS gene polymorphism	Primer	Restriction enzyme
G894T	5AAGGCAGGAGACAGTGG ATGGA-**(forward)** 5'-CCAGTCAATCCCTTTGGTG CTCA-3' **(reverse)**	**(BSP143I)**

RESULTS

Table (1): shows comparative statistics of different studied parameters between acute ischemic stroke patients and healthy control which include age, serum levels of total cholesterol (TC), Triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), and glycated hemoglobin (HbA1C).A significant increase in levels of TC, TG and glycated hemoglobin (HbA1C) was observed in acute ischemic stroke patients compared to control group (P value = 0.009, 0. 004 and 0.0 respectively). However, no statistical significant difference was observed between the two groups regarding age, serum level of HDL, LDL (P value = 0.310, 0.06, and 0.221 respectively).

Among controls when the different genotypes of eNOS G894T gene polymorphism (mutant genotypes & wild type) compared with the studied parameters no statistical significant difference was found as shown in **Table (2).**

However, among acute ischemic stroke patients when the different genotypes of eNOS.G894T gene polymorphism(mutant genotypes & wild type) compared to the studied parameters, there were statistical significant difference between different genotypes and length of the hospital stay as well as stroke score (NIH stroke scale score) (P value = 0.001 and 0.0 respectively) **Table (3) &Table (4).**

Table (1): Comparison of studied parameters between the acute ischemic stroke patients and healthy controls

Parameters	Control Group (n=10) Median (IQR)	Patients Group (n=30) Median (IQR)	z	P	Sig
Age (years)	67(38-80)	60.5(49.5-70)	-1.016	0.310	NS
HBA1C (%)	4.4(4-5.1)	6.2(5.4-6.9)	-4.396	0	HS
TC(mg/dL)	136(120-145.5)	177(134.75-207)	-2.609	0.009	HS
HDL(mg/dL)	43.5(40-48.25)	51(41.5-67)	1.862	0.063	NS
LDL(mg/dL)	75.5(55.25-84)	89(57-141.5)	-1.223	0.221	NS
TG(mg/dL)	75.5(53.5-92)	126.5(96.5-146.5)	-2.858	0.004	HS

Table (2): comparison of laboratory parameters between different genotypes of eNOS G894T gene polymorphism among healthy controls

Parameters	Control (N=10) Median(IQR)		Z	P	Sig
	Mutant genotypes (n=5)	Wild type (n=5)			
Age(years)	28(23-38.5)	28(24.5-34.5)	0	1	NS
HBA1C (%)	4.7(3.9-5.2)	4.2(3.9-4.8)	-0.731	0.465	NS
TC(mg/dL)	138(117-147)	133(120-153.5)	-0.314	0.753	NS
HDL(mg/dL)	45(41-48)	42(39-48.5)	-0.525	0.599	NS
LDL(mg/dL)	79(61-84)	70(54.5-96.5)	-0.314	0.753	NS
TG(mg/dL)	80(51-94)	74(58-133.5)	-0.104	0.917	NS

Table (3): comparison of laboratory parameters between different genotypes of eNOS.G894T gene polymorphism among acute ischemic stroke patients

Parameters	Patients (N=30) Median (IQR)		Z	P	Sig
	Mutant genotypes (n=12)	Wild type (n=18)			
Age(years)	59.5(47.75-67)	61.5(52.25-71.5)	-0.53	0.596	NS
HBA1C (%)	5.9(5.3-6.7)	6.3(5.95-6.975)	-1.125	0.26	NS
TC(mg/dL)	177(129.7-193)	175.5(137.7-238.5)	-0.593	0.553	NS
HDL(mg/dL)	49(36-61)	54.5(49-76.5)	-1.081	**0.28**	NS
LDL(mg/dL)	87 (61-113.5)	112(54.5-156.5)	-0.177	0.859	NS
TG(mg/dL)	127.5(81.5-151.5)	122(99.25-144)	-0.148	0.882	NS
Hospital stay(days)	2(2-3)	4(3-4.75)	-3.329	0.001	HS
NIH Stroke Scale score	4(3-6)	10.5(8.25-17.5)	-3.744	0.001	HS

Table (4) comparison of laboratory parameters between different genotypes of eNOS.G894T gene polymorphism (Hetero &Wild) among acute ischemic stroke patients

Parameters	Patients (N=30) Median(IQR)		Z	P	Sig
	Hetero (N=9)	Wild (N=18)			
Age(years)	**60(78.5-70)**	**59.5(47.75-67)**	**-0.103**	**0.918**	NS
HBA1C (%)	**6.6(5.8-7.2)**	**5.9(5.3-6.7)**	**-0.1.083**	**0.279**	NS
TC(mg/dL)	**173(140.5-256.5)**	**177(129.75-193)**	**-0.772**	**0.44**	NS
HDL(mg/dL)	**55(49-81)**	**49(36-61)**	**-1.16**	**0.246**	NS
LDL(mg/dL)	**120(55-167)**	**87(61-11.3.5)**	**-0.162**	**0.872**	NS
TG(mg/dL)	**130(116.5-161)**	**127.5(81.5-151.5)**	**-0.849**	**0.396**	NS
BUN(mg /dl)	**19(10.5-23.5)**	**13(8-16.25)**	**-1.677**	**0.094**	NS
CREAT (mg /dl)	**1(0.8-1.3)**	**0.8(0.7-1)**	**-1.907**	**0.057**	NS
Sodium (mEq/L)	**138(135-138)**	**136.5(134.75-138)**	**-0.132**	**0.895**	NS
Potassium (mEq/L)	**4.1(3.9-4.35)**	**4.2(3.85-4.425)**	**-0.363**	**0.713**	NS
Hospital stay(days)	**3(3-4)**	**2(2-3)**	**-2.775**	**0.006**	HS
NIH Stroke Scale score	**10(7.5-14.5)**	**4(3-6)**	**-3.157**	**0.002**	HS

Table (6) and **figure (1)** summarize the allelic distribution of T and G alleles of **eNOS.G894T** gene polymorphism in acute ischemic stroke patients and healthy controls groups. No statistical significant difference was detected between the two groups as regard to allelic distribution (P> 0.05).

Table (7) and **figure (2)** show descriptive Statistics of the frequency of different genotypes of **eNOS.G894T** gene in acute ischemic stroke patients and controls. We found that the mutant genotypes was higher in healthy control group, also the wild genotype was higher in acute ischemic stroke patients , however no statistical significant association was detected between the studied groups (p>0.05).

Table (6): Descriptive statistics of allele frequencies of eNOS.G894T Gene Polymorphism in acute ischemic stroke patients and healthy controls

Allele	Cases (n = 30)			Control (n = 10)			Z	P	Sig.
	N	(%)	Total	N	(%)	Total			
G allele	15	25%	60	8	40%	20	1.28	>0.05	NS
T allele	45	75%		12	60%				

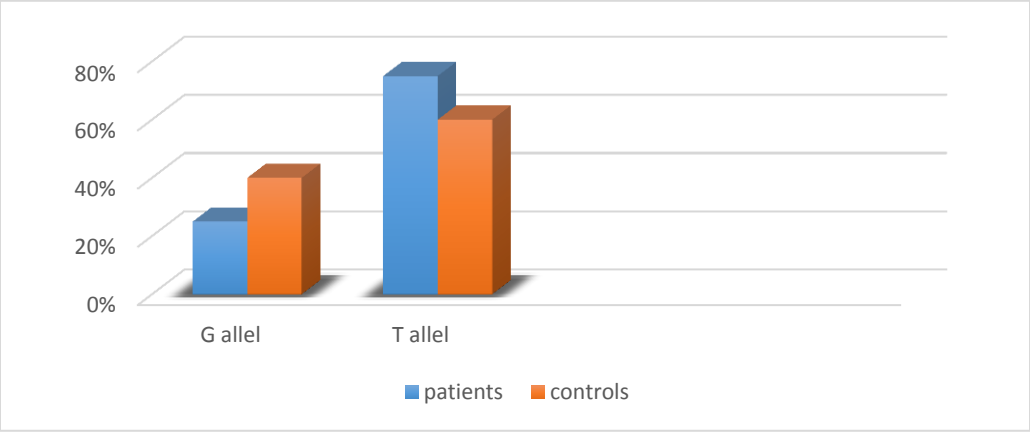

Figure (1): Allele Frequencies of **eNOS.G894T Gene Polymorphism** in acute ischemic stroke patients and controls

Table (7): Descriptive statistics of different genotypes of eNOS.G894Tgene polymorphism in acute ischemic stroke patients and healthy controls

eNOS.G894T	control (n=10)	Patients (n=30)	Total Count (%)	Pearson Chi-Square X^2	P	Sig
Mutant genotypes (GT +TT)	5 (50%)	12 (40%)	17(42.5%)	0.307	0.580	NS
Wild type (GG)	5 (50%)	18(60%)	23(57.5%)			
Total Count (%)	10(100%)	30(100%)	40(100%)			

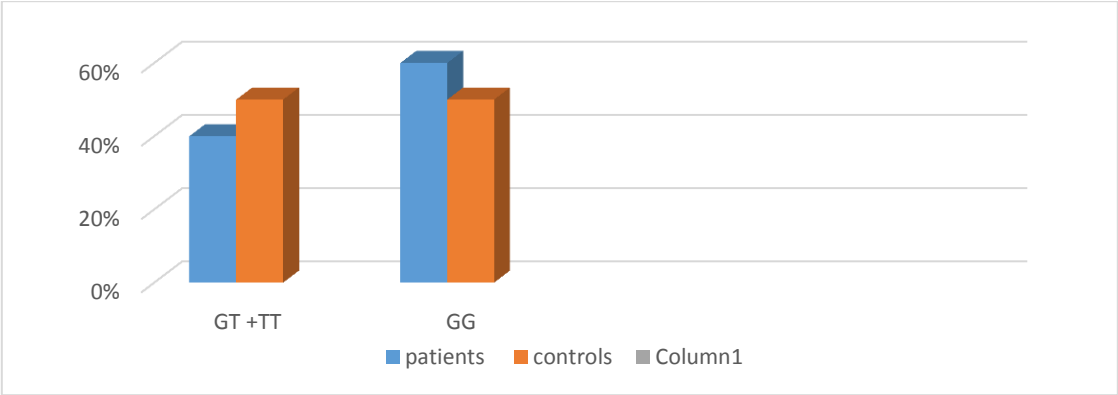

Figure (2): Frequency of eNOS.G894Tgene polymorphism in acute ischemic stroke patients and healthy controls
GG: Wild type , GT: Heterozygous mutant type ,TT: Homozygous mutant type

Table (8): Describes the effect of different genotypes of **eNOS.G894T** gene polymorphism on the severity of the stroke (stroke score). There were a significant difference between different genotypes and severity of the stroke (P=0.001).

Table (8): Descriptive statistics of different genotypes of eNOS.G894T gene polymorphism among acute ischemic stroke patients regarding severity of the stroke

eNOS.G894T	Minor (1-4)	Moderate (5-15)	Sever (16-20)	Total Coun (%)	Pearson Chi-Squar X^2	P	Sig
Mutant genotypes (GT +TT)	**13 (72.2%)**	**5(27.8%)**	**3 (25%)**	**21 (100%)**	**13.3**	**0.001**	**HS**
Wild type (GG)	**1(8.3%)**	**8 (66.7%)**	**0 (0%)**	**9 (100%)**			
Total Count (%)	**14(46.7%)**	**13 (43.3%)**	**3 (10%)**	**30 (100%)**			

DISCUSSION

Cerebro-vascular stroke is the second most common cause of death (after ischemic heart diseases) and the leading cause of disability worldwide [(2)]**.** According to the WHO media center, ischemic stroke is one of the leading causes of death in Egypt*;* accounting for 43% to 79% of all stroke types [(10)]**.**

Acute Ischemic stroke is diagnosed by history, general and neurological examination, emergent brain imaging for confirming the diagnosis including CT &MRI, and the conventional baseline laboratory testing that is often limited to blood glucose, coagulation studies, complete blood count (CBC) and lipid profile. Additional laboratory tests may include cardiac biomarkers, homocysteine level and high- sensitivity C-reactive protein (hsCRP) [(11)]**.**

Several factors have been implicated in the risk of ischemic stroke such as hypertension, diabetes mellitus and hyperlipidemia [(3)]**.** The contribution of genetic factors have been suggested by various investigators as major risk in the development of acute ischemic stroke [(8)]**.**Among these genes the endothelial nitric oxide synthase NOS gene which is responsible for endothelial nitric oxide (NO) production [(4)]**.**NO is known as a potent endogenous vasodilator which inhibits the platelet aggregation and reduces the adherence of leukocyte to vascular endothelium. It also suppresses the proliferation of vascular smooth muscle cells [(7)]**.**Impaired endothelium-dependent vasodilation has been suggested to be an attribute of atherosclerotic vessels, which to some extent could be due to the reduction in the activity of vascular endothelial nitric oxide synthase (eNOS) [(7)]**.**For this purpose, our study tried to find a possible association between eNOS G894T gene polymorphism and acute ischemic stroke.

The study was conducted on a group of thirty **(30)** Egyptian patients admitted to the Neurology Department and Stroke Units of Ain Shams University hospitals (Al-Demerdash hospital).They were presented with acute ischemic stroke in the first 48 hours and diagnosed according to clinical neurological and radiological examinations. Oral informed consents were obtained either from the patients or their relatives before enrollment in the study according to the Ethical Committee of Faculty of Medicine, Ain Shams University. In addition, ten **(10)** Egyptian apparently healthy, age and sex- matched subjects were investigated as control group.

All individuals included in this study were subjected to full history taking, thorough general, neurological and radiological assessment. The laboratory investigations included complete blood count, coagulation profile, and blood glucose level assay. Furthermore eNOS **G894T** gene polymorphism was assessed by PCR- RFLP.

Comparative analysis between acute ischemic stroke patients and healthy controls showed that there was a statistically significant increase in levels of total cholesterol (TC), triglyceride (TG) and HbA1c in patients compared to healthy control group. Although, no statistical significant difference was found in serum levels of HDL and LDL. Such findings were very close to the data of **Ozcelik [(13)],** who found higher levels of total cholesterol, triglyceride, and LDL-cholesterol, among group of Turkish ischemic patients and

lower HDL-cholesterol levels. It was previously justified an association between the strong contribution of diabetes mellitus and hyperlipidemia as risk factors of acute ischemic stroke.

Our genetic analysis revealed that acute ischemic stroke patients, the most frequent genotype of the eNOS G894T gene polymorphism was the wild GG genotype (60%) followed by mutant genotypes GT +TT (40%). Our results are in agreement with those obtained by **Kaur** [9] who performed their study on North Indian population They observed that most of stroke patients have the wild type genotype GG, followed by mutant genotypes GT+TT respectively.

On the contrary, a similar retrospective case–control study on Tunisian population by **Saidi**[8] **,**reported increased frequency of the mutant genotypes GG+GT of eNOS G894Tgene among patients followed by wild GG genotype.

Among controls, the percent of wild GG genotype was equal to that of mutant genotypes GT+TT; 50% each. Our Findings agree with those carried out on Moroccan population [14] ,who noticed a slight difference between the mutant genotypes G T + TT and the wild genotypes GG of eNOS G894T .However **Ozcelik** [13]**,**demonstrate more frequent mutant genotypes GT+TT among their control.

When we compared the frequency of the eNOS **G894T**polymorphism genotypes between patients and controls, our results showed that the wild GG genotype frequencies were higher in patients group (60%) than control group (50 %), yet, the difference between the studied groups was insignificant. Moreover, the mutant genotypes GT+TT were higher in control group (50%). Regarding alleles frequency, the results showed that T allele was more frequent than G one in both studied groups and that the frequency of T allele hadn't differed significantly between cases and controls (P value >0.05).

Previously ***In 2010*** **Majumdar**[15] and his co-workers, found more frequent GG genotype of the eNOS **G894T** gene among control than patients group and that the mutant GT+TT was higher among their studied Indian patients compared to control group. However the difference was insignificant.

In this study, we couldn't find a significant association between eNOS **G894T**gene polymorphism and acute stroke among our studied Egyptian patients. However, but We found that greater number of patients with ICA and MCA occlusion were found carrying the *T* allele (the *TT/GT* genotype).Also we found the acute ischemic stroke patient with high **NIH Stroke Scale** Score carrying the T allele.In addition, significant results were obtained when the risky genotypes G894T studied with other polymorphism such as intron 4 VNTR or *eNOS* -786T polymorphism.

However, the results of association of G894T polymorphism with ischemic stroke are quite contradictory; a positive association has been reported between eNOS3 G894T polymorphism and ischemic stroke risk [16]**,** On the other hand, some studies showed no association between eNOS3 G894T polymorphism and ischemic stroke [15]**.**

Due to functional relevance ofG894T variant and published reports on its association with ischemic stroke, several studies positively correlated G894T polymorphism with various cardiovascular diseases including Hypertension [17]**,** ischemic heart disease [18]**,** and coronary artery dysfunction [19]**.**

The explanation for these apparently contradicting association may reside in the ethnic origin of the studied population, selection of study subjects and sample size.

CONCLUSION

In conclusion we couldn't find a significant association between eNOS **G894T**gene polymorphism and acute stroke in the studied population.

RECOMMENDATION

1. Repetition of the study with inclusion of larger sample size to To justify the present results.
2. The ethnic variation between different populations should be taken in consideration.
3. It was worthy considering the presence the presence of other functional variants of eNOS gene.

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