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RESEARCH ARTICLE

Endothelial Nitric Oxide Synthase Gene Polymorphism (T-786C) and Cerebrovascular Storke with or without Smoking

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Abstract

Background: Cerebrovascular Stroke (CS) is the second common cause of death and major cause of disability throughout the world. CS mainly originates mainly from ischemic cause secondary to multifactorial disorder. **Aim of the work**: to determine the association of serum nitric oxide and T-786C polymorphism of eNOS in cerebrovascular stroke patients with or without smoking. **Patients and Methods:** A total forty two patients with cerebrovascular stroke (25 with and 17 without cigarette smoking). Patients with diabetes mellitus, renal disease, chronic obstructive pulmonary disease and hepatitis were excluded from the study group. Their age ranged between 45 to 60 years. Genotyping analysis was done by combination of techniques PCR and sequencing. **Results and conclusions** the presence of the eNOS mutant allele reduces endothelial production of NO and may predispose the patients carrying the mutant allele to cerebrovascular stroke.

Introduction

Cerebrovascular Stroke (CS) is the second common cause of death and major cause of disability throughout the world. CS mainly originates mainly from ischemic cause secondary to multifactorial disorder. In addition to atherosclerosis, heart disease, vasculitis, hypertension, smoking, genetic susceptibility factors may be also involved in cerebral ischemia due to endothelial dysfunction. The endothelial nitric oxide synthase (*eNOS*) enzyme can synthesize nitric oxide (NO) from L-arginine. NO has several cerebral physiological activities including regulation of cerebral blood flow and modulation of neuronal activity (1). Most NO actions are mediated by cyclic guanosine monophosphate (cGMP) pathway. Any defect in endothelial NO synthesis leads to vascular endothelial dysfunction and impaired cerebral activities. Cerebrovascular disorders secondary to *eNOS* enzyme dysfunction is related to atherosclerosis, thromboembolic activity and various vascular wall defects (2).

The *eNOS* gene (21 kb) is located on chromosome 7q35–36 and consists of 26 exons (3). T-786C is one of *eNOS* gene polymorphism and is located in the promoter region. It was reported that *eNOS* gene polymorphism (T-786C) could reduce the *eNOS* gene promoter activity and decrease *NOS* gene expression (4). T-786C polymorphism has been additionally demonstrated to be correlated with CS. However, various studies designed to investigate the association between both this polymorphism and CS and showed contradictory results (5).

The aim of the present study was to determine the association of serum nitric oxide and T-786C polymorphism of eNOS in cerebrovascular stroke patients with or without smoking.

Patients AND METHODS

Patients: A total forty two patients with cerebrovascular stroke (25 with and 17 without cigarette smoking). Patients with diabetes mellitus, renal disease, chronic obstructive pulmonary disease and hepatitis were excluded from the study group. Their age ranged between 45 to 60 years were collected in Sohag University Hospital between December 2013 and October 2014, Faculty of Medicine, Egypt. Diagnosis of Cerbrovascular stroke was done on the basis of the current WHO criteria (6). All subjects gave their informed consent and the protocol was approved by the Ethical Committee of Scientific Research, Sohag Unit and the consent was taken from all patients.

Venous blood samples were collected in the morning after an overnight fast and were allowed to clot at room temperature for about 1 hour. Serum samples were separated by centrifugation at 1500 rpm for 10 min kept at -80˚C and stored at -20˚C until analysis.

Lipid measurements

Blood samples were obtained after an overnight fast. Serum levels of total cholesterol, triglycerides and HDL were measured by standardized enzymatic procedures using Olympus AU 400 using Olympus kit. LDL was calculated by Freidewald equation (*7*).

Nitric oxide (NO)

The levels of NO in serum were determined calorimetrically by Griess method (8). In this method, NO undergoes a series of reactions with several molecules present in biological fluids including O_2^-, O_2 and NO_2. The invivo final products of NO are nitrite (NO_2^-) and nitrate (NO_3^-). The relative proportion of NO_2^- and NO_3^- is variable and cannot be determined with certainty. Thus, the best index of total NO production is the sum of both NO_2^- and NO_3^-. The method used in this study provides an accurate and convenient measurement of nitrate/nitrite concentration in a simple two-steps process. The first step is conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of Griess reagent which converts nitrite into deep purple azo compound. Colorimetric measurement of the absorbance is due to this azo chromophore accurately determines nitrite concentration at 548 nm.

DNA analysis and genotyping

Genomic DNA was prepared from white blood cells by phenol/chloroform extraction technique as described by ***Sambrook et al., 1989*** (9). Using the polymerase chain reaction (PCR) and subsequent analysis of restriction fragment lengths, the T–786→C polymorphism was determined according to the method by ***Ghilardi et al*** (2003; 10) with modifications. For this procedure, a certain site on the promoter of the eNOS gene was amplified using a pair of specific primers: upstream (sense) – 5'-CAC CTG CAT TCT GGG AAC TGTA-3' and downstream (antisense) – 5'-GCC GCA GTA GCA GAG AGAC-3'. PCR was performed for 35 cycles in a 25 μL volume containing 40 ng of DNA, 5 μL 5× PCR buffer, 1.5 mM MgSO4, 200 μM of each dNTP, 20 pM of each primer and 0.5 U of Taq DNA polymerase (AmpliSense, Russia). PCR was performed in a thermocycler (Applied Biosystems 2700; Perkin Elmer, USA). Denaturation was performed at 94°C (1 min), annealing at 63°C (50 s) and extension at 74°C (1 min). Six microliters of the PCR products (125 bp) were digested with 5 U *PdiI* (*NaeI*; Fermentas, Lithuania) and incubated at 37°C for 18 h. The presence of thymine at position –786 of the promoter prevented restriction; in the case of thymine substitution by cytosine, *PdiI* cleaved the amplified fragment of the promoter into two fragments (95 bp and 30 bp in length).

DNA Sequencing:

Samples were run on 1.5% agarose gels and the bands corresponding to the predicted size were cut and purification was carried out using the gel extraction kit following the manufacturer protocol (QIA quick columns, Qiagen). Purified samples were subjected to cycle sequencing using Big Dye Terminator v3.1 Kit and injected to ABI 3100 Genetic Analyzer (Applied Biosystems, Germany).

Statistical analysis

Data was presented by means ± SD and percentages .The compiled data were computerized and analyzed by SPSS PC+, version 12. The following tests of significance were used: Analysis of variance (ANOVA) test between more than two means, t-test between means we used analyze mean difference, t-test between percentage to analyze percent difference and chi –square. A level of $p < 0.05$ was considered significant.

Results

This study population consisted of 42 smokers with CS, their mean age was 52.5±3.5 years and mean smoking index was 35±5. Baseline characteristics; Clinical and biochemical characteristics; were shown in table 1.

Table (1): Baseline Characteristics of all cases.

Parameters	Smoker n=42 mean ± SD
Age (years)	**52.5±3.5**
Total cholesterol (mg/dl)	**190±32**
LDL-c (mg/dl)	**128±25**
HDL-c (mg/dl)	**40±5**
Triglycerides (mg/dl)	**120±25**
NO (Nitric Oxide)	**11.7 ± 5.0**
Smoking index	**35± 5**

LDL-c: Low density lipoprotein cholesterol, **HDL-c**: High density lipoprotein cholesterol

Genotyping results:

Analysis the association between genotypes of T-786C polymorphism (CT, TT and CC) with lipid profile , Smoking index , NO (Nitric Oxide) and HbA1c in smoking subjects; it was found that, none of the variables (T-C, LDL-C and HDL-C) and HbA1c were associated with T-786C polymorphism. While significant difference were detected concerning Smoking index (Si) and NO (Nitric Oxide) and Si with these genotypes of T-786C polymorphism ($P<0.05$ and $P<0.05$. respectively) (Table 2).

Table (2): Relation between biochemical parameters and genotypes.

Variable	CT	TT	CC	*P* value (ANOVA)
Triglycerides	118± 40	114± 38	110±29	**0.642**
Cholesterol	207± 36	217± 37	207± 38	**0.625**
HDL	47± 9	51± 9	47±8	**0.310**
LDL	130± 39	132± 37	140± 40	**0.752**
NO	17± 3	10± 4	11± 5	**0.005***
Si	45± 4	35± 5	36± 3	**0.005***

LDL-c: Low density lipoprotein cholesterol, **HDL-c**: High density lipoprotein cholesterol, **Si**: Smoking index, **NO:** Nitric Oxide)

***P* <0.05 significant

Smoking index and NO showed significant difference just in case of smoker with stroke with genotypes (P = 0.005 and 0.002) . While there was no any statistically significant variation was recorded between Smoking index and NO and genotypes I in smoker without stroke (table 3).

Table (3) Relation between biochemical parameters and genotypes in smoker with stroke .

Variable	CT	TT	CC	P value (ANOVA)
NO	18±4.8	14±6	16±4.9	**0.002***
Si	**55±6**	**40±5.5**	**46±8**	**0.005***

NO: Nitric Oxide, **Si**: Smoking index

- ***P* <0.05 significant.

Discussion

The risk of thrombosis is also raised due to tobacco's effect on fibrinogen levels and its effects on increased platelet aggregation which makes the blood more stick (11; 12). It has been shown that smoking causes the body's blood vessels to constrict (vasoconstriction) by decreasing nitric oxide which dilates blood vessels and increasing endothelin-1 which causes constriction of blood vessels (13). The net result is raised blood pressure and a transient reduction in blood supply may be through inhibiting the release of endothelin-1 (vasoconstrictor) to regulate blood pressure. The positive correlations between smoking, hypertension and CS agree with that in previous publications (13).

It has been reported that endothelium-dependent vasorelaxation is diminished in cigarette smokers and in the brain of individuals with cerebral diseases such as dementia, optic neuropathy and cerebrovascular stroke. Cerebrovascular stroke primarily affects people who are elderly, smoking, ischemic heart disease or have a history of diabetes (1; 2). Nitric oxide (NO) has a role in regulating vascular tone and hemodynamic. It also, has anti-inflammatory ,anti-thrombotic and anti-proliferative effects (14). It can be synthesized in most tissue and cells and stimulates endothelial proliferation and angiogenesis, thereby playing an important role in microcirculation (2). Nitric oxide, released from the endothelium stimulates soluble guanylyl cyclase, producing increased concentrations of cGMP which activates cGMP-dependent kinases that decrease intracellular calcium, producing relaxation of vascular smooth muscle cells (1). NO is a major endogenous vasodilator that contributes to the low vascular resistance in the cerebral circulation. Smoking can also modulate the level NO by decreasing the activity of eNOS and promoting that of its inducible form (iNOS).In the current study, there is a significant negative correlation between nitric oxide and smoking index. These results are correlated with Zhang et al 2006, Ignarro, et al 1999, Napoli and Ignarro 2001 and Ignarro and Napoli 2004 (15; 16; 17; 18).

An impairment of the NOS isoform is one of the earliest events in atherogenesis (19; 20). Decreased NO bioavailability increases non-thrombogenic intimal factor such as circulating von-Willebrand factor, decreases in heparan sulfated glycosaminoglycans (19) and promotes platelet adhesion and aggregation, as well as deposition of platelets on the abnormal endothelial surface (20). The decreases in production or bioavailability of NO are associated with events that accelerate the development of atherosclerosis such as vasoconstriction, thrombocyte aggregation, migration of monocytes to the vascular wall, oxidized LDL and foam cell production. There is a positive significant correlation between cholesterol, triglyceride and LDL-cholesterol concentrations and smoking status but, no longer an association between HDL- cholesterol concentrations and smoking. These results agree with (21- 23)

The eNOS gene is a possible candidate gene for susceptibility to CS in that dysregulation of eNOS. Large epidemiological studies have estimated the relationship of T-786C polymorphism in smoking and susceptibility to CS, but the results are inconsistent. Therefore, we carried out the current study to discover that relation in a sector of Egyptian population. Several studies revealed that there are various polymorphisms on eNOS gene and these mutations might be a risk factor for CS. The polymorphisms differ largely among races. In this study, we investigated the relationship between T-786C mutation of eNOS gene and CS in the Egyptian population. To our knowledge, this polymorphism has never been investigated in this population. The results demonstrated an

association between C allele and CS in the Egyptian population. The polymorphism is a result of a thymidine being replaced by a cytosine at nucleotide -786 (T-786C).

Smoking is known to induce oxidative stress, which is a potent suppressor of eNOS activity. Also, others found an association between genetic variation in the eNOS gene and diabetes in CAD, therefore in this study all subjects selected were non-diabetic.

Rossi et al. (2003) and Rios et al. (2007) reported that they found a significant association between the T-786C mutation and smoking in CAD (24, 25). In the current study, high frequency of CC genotype of T-786C was noticed in smoking group (24, 25). The frequency of C allele was significantly higher in that group. The results are in agreement with those reported by others (Rossi et al, 2003 and Rios et al. 2007). The T-786C mutation in the promoter region of eNOS resulted in the reduction of eNOS promoter transcription rate, leading to the reduced NO production in blood vessels and endothelial dysfunction (1).

It was concluded that the presence of the eNOS mutant allele reduces endothelial production of NO and may predispose the patients carrying the mutant allele to cerebrovascular stroke.

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References

1- Liu R, Geng P, Ma M, Yu S, Wang X, Zhang W, Di H.(2014): Association between endothelial nitric oxide synthase gene polymorphism (T-786C) and ischemic stroke susceptibility: a meta-analysis. Liu R, Geng P, Ma M, Yu S, Wang X, Zhang W, Di H. Int J Neurosci. Sep;124(9):642-51.

2- **Wang M, Jiang X, Wu W, Zhang D. (2013):** Endothelial NO synthase gene polymorphisms and risk of ischemic stroke in Asian population: a meta-analysis. PLoSOne. 2013;8(3):e60472.

3- Yemişçi M, Sinici I, Ozkara HA, Hayran M, Ay H, Celtikçi B, Onder E, Büyükşerbetci G, Kaya EB, Tokgözoglu L, Dalkara T. (2009): Protective role of 27bp repeat polymorphism in intron 4 of eNOS gene in lacunar infarction. Free Radic Res. Mar; 43(3):272-9.

4- Moe KT, Woon FP, De Silva DA, Wong P, Koh TH, Kingwell B, Chin-Dusting J, Wong MC. (2008): Association of acute ischemic stroke with the MTHFR C677T polymorphism but not with NOS3 gene polymorphisms in a Singapore population. Eur J Neurol. Dec; 15(12):1309-14.

5- Lee CR, North KE, Bray MS, Avery CL, Mosher MJ, Couper DJ, Coresh J, Folsom AR, Boerwinkle E, Heiss G, Zeldin DC (2006): NOS3 polymorphisms, cigarette smoking, and cardiovascular disease risk: the Atherosclerosis Risk in Communities study. Pharmacogenet Genomics. Dec;16(12):891-9.

6- WHO (World Health Organization; 2005): STEP wise approach to stroke surveillance.

7- Friedewald WT, Levy RI, and Fredrickson DS. (1972): Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of preparative ultracentrifuge . Clin Chem., 18:499 – 502

8- Robak J, Marcinkiewicz E, Michalska Z, Gryglewski RJ. (1997): Nitric oxide donation and nitrite assays in the presence of thiols and albumin as determined byGriess' and Werringloer's methods. Pol J Pharmacol. 1997 Aug;49(4):255-62.

9- Sambrook J, Fritsch E F, and Maniantis T. (1989): Molecular cloning**.** A laboratory manual 2th edition, cold spring Harbor, Laboratory press, New York;1989.

10- Ghilardi G, Biondi M, Cecchini F, DeMonti M, Guagnellini E, Scorza R. (2003): Vascular invasion in human breast cancer is correlated to T-->786C polymorphism of NOS3 gene. Nitric Oxide. 2003 Sep;9(2):118-22.

11- Kannel WB, Wolf PA, Castelli WP, D'Agostino RB. (1987): Fibrinogen and risk of cardiovascular disease. The Framingham Study. JAMA 1987; 258:1183–6

12- Hunter KA, Garlick PJ, Broom I, Anderson SE, McNurlan MA. (2001): Effects of smoking and abstention from smoking in fibrinogen synthesis in humans. Clinical Science. 100(4): 459-65.

13- Kioski W, Linder L, Stoschitzky K, Pfisterer M, Burckhardt D, Burkart F, Bühler FR. (1994): Diminished vascular response to inhibition of endothelium-derived nitric oxide and enhanced vasoconstriction to exogenously administered endothelin-1 in clinically healthy smokers. Circulation. 90: 27–34

14- Brischetto CS, Connor WE, Connor SL, Matarazzo JD. (1984): Plasma lipid and lipoprotein profiles of cigarette smokers from randomly selected families: enhancement of hyperlipidemia and depression of high-density lipoprotein. Am J Cardiol. 983;52:675-80.

15- Zhang, W.Z.; Venardos, K.; Chin-Dusting, J. (2006): Kaye, D.M. Adverse effects of cigarette smoke on NO bioavailability: Role of arginine metabolism and oxidative stress. Hypertension. 48, 278-285.

16- Ignarro L.J., Cirino G., Casini A., Napoli C. (1999): Nitric oxide as a signaling molecule in the vascular system: an overview, J. Cardiovasc. Pharmacol. 34,876–884.

17- Napoli C. and Ignarro L.J. (2001): Nitric oxide and atherosclerosis, NitricOxide 5,88–97.

18- Ignarro L.J. and Napoli C. (2004): Novel features on nitric oxide, endothelial nitric oxide synthase and atherosclerosis, Curr. Atheroscler. Rep. 6,278–287.

19- Furchgott R.F. (1999): Endothelium-derived relaxing factor: discovery ,early studies, and identification as nitric oxide, Biosci. Rep. 19,235–251.

20- Napoli C., Lerman L.O. (2001): Involvement of oxidation-sensitive mechanisms in the cardiovascular effects of hypercholesterolemia, Mayo Clin. Proc. 76,619–631.

21- De Nigris F., Lerman A., Ignarro L.J., Ignarro-Williams S., Sica V., Baker A.H., Lerman L.O., Geng Y.J., Napoli C. (2003): Oxidation-sensitive mechanisms, vascular apoptosis and atherosclerosis, Trends Mol.Med. 9,351–359.

22- Freedman DS, Cresanta JL, Srinivasan SR, Webber LS, Berenson GS. (1985): Longitudinal serum lipoprotein change in white males during adolescence :the Bogalusa heart study. Metabolism. 354:396-403.

23- **Craig WY, Palomaki GE, Haddow JR. (1989):** Cigarette smoking and serum lipid and lipoprotein concentrations: an analysis of published data. Br Med J. 298:784-9.

24- Rossi GP, Cesari M, Zanchetta M, Colonna S, Maiolino G, Pedon L, Cavallin M, Maiolino P, Pessina AC. (2003): The T-786C endothelial nitric oxide synthase genotype is a novel risk factor for coronary artery disease in Caucasian patients of the GENICA study. J Am Coll Cardiol. Mar 19;41(6):930-7.

25- **Rios DL1, D'Onofrio LO, Souza JK, Queiroz AM, Raduy-Maron L, Silva-Neto N, Carvalho HG, Santos-Filho A, Galvão-Castro B. (2007):** Smoking-dependent and haplotype -specific effects of endothelial nitric oxide synthase gene polymorphisms on angiographically assessed coronary artery disease in Caucasian- and African-Brazilians. Atherosclerosis. Jul;193(1):135-41.