1. Introduction

Nitric oxide (NO) is a soluble gas produced in the vascular endothelium from L-arginine by the constitutive calcium-calmodulin-dependent enzyme endothelial nitric oxide synthase (eNOS). NO plays a crucial role in retaining the normal endothelium function and maintaining vascular homeostasis. NO maintains the vasodilatation tone of the vascular endothelium, regulates its cell growth, and protects it from injuries caused by platelet aggregation (1). Previous investigations on the production of endogenous NO suggested that vascular endothelium diseases probably result from reduced production of endogenous NO. Several factors are known to affect the release of endothelium NO including: down-regulation of eNOS, increased bioavailability of Asymmetric dimethylarginine (ADMA) that down-regulates the activity of eNOS, decreased bioavailability of Tetrahydrobiopterin (BH4), which is an essential co-factor for NOS activity, and the availability of reactive oxygen species (ROS), e.g. superoxide (O$_2^-$) (2,3).

The eNOS gene (located on chromosome 7 at the 7q36.1 region) contains 26 exons and 25 introns spanning ~23.5 kb of genomic DNA. Although the gene is known to encode an mRNA of 4,052 nucleotides, some 10 splice variants of the gene have been reported (The Gene Cards human gene database index, ensemble). The gene has been the focus of intensive research to identify potentially functional polymorphisms or mutations that might affect mRNA
transcription and gene expression. To date more than 1,730 polymorphic sites have been identified in, or in the vicinity of the gene (http://www.ncbi.nlm.nih.gov/snp). However, no clear correlation could be detected between the presence of SNPs in the coding and/or non-coding (e.g. promoter) sequences of the eNOS gene and NO production, despite the fact that several SNPs have been associated with cardiovascular disease (CVD) (4).

One of the most studied clinically relevant genetic variants is a SNP in the promoter region (T-786C; rs2070744), frequently associated with the development of coronary artery disease (CAD) (5). A statistically significant decrease in average eNOS expression has been reported for the T-786C polymorphism and was suggested to contribute to the vascular, contractile, and autonomic responses in failing human myocardium (6). Another study provided evidence that the same SNP is associated with the presence and severity of angiographically defined CAD in the Italian population (7).

We have previously investigated the distribution of several reported SNPs in a number of genes involved in the NO signaling pathway, including DDAH2 SNP1 (−1151 C/A, rs805304) and SNP2 (−449 C/G, rs805305) (8), eNOS (Glu298Asp polymorphism) (9), NADPH oxidase (C242T polymorphic site of p22 phox gene) (10) and paraoxonase (PON1 Q192R) (11), in a large number of DNA samples extracted from Egyptian healthy subjects and myocardial infarction (MI) patients. In these studies, however, no clear correlation could be detected between the reported SNPs and other relevant biochemical parameters.

Thus, the main goal of the present study is to investigate the effect of the T-786C polymorphism in the 5'-flanking promoter region of the eNOS gene on the gene expression level in the vascular endothelium, using a sample of Egyptian cardiovascular patients. To accomplish this goal, promoter sequences of eNOS carrying three reported SNPs were extracted from Egyptian cardiovascular patients before being cloned into a promoter-less vector (pGL3-basic) carrying the luciferase gene as a reporter. Luminescence levels were then recorded after transfecting human endothelial cell line (EA.hy926).

### 2. Materials and Methods

#### 2.1. Samples

The T-786C SNP was examined in blood samples collected from acute myocardial infarction patients recruited for the study from the intensive care unit of the National Heart Institute, Imbaba, Giza and El Demerdash Hospital, Cairo, Egypt. Written informed consent were obtained from all participants in the study. The study protocol was approved by the German University in Cairo ethics committee and complied with Declaration of Helsinki. The main target is to classify the samples into TT, TC, or CC according to the genotype distribution of the eNOS T-786C SNP.

Blood samples were collected into EDTA coated vacuum tubes and stored at 4°C until DNA extractions were performed. Extracted DNA was used as a template for PCR amplification of the eNOS gene.

#### 2.2. Purification and amplification of DNA by polymerase chain reaction (PCR)

DNA purification was done using Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit (Rockford, USA). The purified DNA was used directly in the PCR. For PCR amplification of DNA, oligonucleotide primers were designed to flank either short or extended fragments upstream of the translation start site. An extended fragment represents the complete eNOS promoter (1,594 bp), while the short fragment (345 bp) is a basal central part of the promoter. Recognition sequences for restriction endonucleases were inserted at both ends of the corresponding oligonucleotide (Table 1). All PCR amplifications mixtures were carried out in a total volume of 100 µL containing 50 µL EmeraldAmp GT PCR Master Mix (TAKARA, Japan), 2 µg genomic DNA as a template, 40 pmol final concentrations of both forward and reverse primers, and the volume was completed to 100 µL using nuclease-free sterile water. The PCR amplification programs for the long and short fragments are listed in Tables 2 and 3, respectively. The amplified PCR products were purified using PureLink PCR Purification Kit (Thermo Fisher Scientiﬁc, Rockford, USA) and sequenced.

#### Table 1. Primer sequences

| Primer name                        | Sequence (Underlined Restriction Sites)       | Amplicon size |
|-----------------------------------|-----------------------------------------------|---------------|
| Forward eNOS primer – extended insert | GAAGATCTATCTGATGCTGCTGTCACCTTGACCCCTGAG (Bgl II) | 1,612bp       |
| Reverse eNOS primer – extended insert | ATTAAGGCTTCCGCTGTTGCCACGAGGCCCCTGCCCCTTTC (Hind III) |               |
| Forward eNOS primer – extended insert | GAAGATCTCTGATGCTGCTGTCACCCCTGAG (Bgl II) | 1,594bp       |
| Reverse eNOS primer – extended insert | ATTAAGGCTTCCGCTGAGGCCCCTGCCCCTTTC (Hind III) |               |
| Forward eNOS primer – short insert (primers use for sequencing) | GAAGATCTAGATGGC ACGAGACTCACAAACC (Bgl II) | 345bp         |
| Reverse eNOS primer – short (primers use for sequencing) | ATTAAGGCTTCCCTT GAGCTGACATTAGG (Hind III) |               |
Statistical analysis

Average luminescence levels were determined for three independent measurements and differences were analyzed using analysis of variance (ANOVA) using GraphPad Prism v5.

3. Results

3.1. Effect of T-786C SNP on luciferase expression using pGL3 eNOS promoters

EA.hy926 cells were transfected with plasmid constructs carrying the different promoter fragments. pSV-β-galactosidase was co-transfected into the cells as a control for transfection efficiency. Both luminescence intensity and β-galactosidase activities were measured in cell lysates 48 hours post-transfection. Luminescence levels were normalized to β-galactosidase activities. Luminescence levels for EA.hy926 cells harboring the CC_short-786 eNOS promoter constructs were not significantly different (*p = 0.5903) from those obtained for cells transfected with empty plasmid vectors. On the other hand, the CT_short and TT_short SNP genotypes resulted in luminescence levels that were 1.6 and 1.8-fold higher than their respective controls (*p < 0.0001). Luminescence results obtained for constructs carrying CC_short-786, CT_short, TT_short were significantly different from each other's as shown in Figure 1.

3.2. Effect of eNOS promoter sequence length on luciferase expression

Luminescence results obtained for EA.hy926 cells transfected with constructs carrying the extended version of eNOS promoter (eNOS_long) with the CC-786 genotype

Scientific, Rockford, USA) and their concentrations estimated using Qubit dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific). The amplified fragments were then digested using FastDigest Bgl II and Hind III (Thermo Fisher Scientific, Rockford, USA) before being ligated into a pGL3-basic vector (Promega, Madison, USA); Luciferase reporter vector (E1741, Promega, USA) previously restricted using the same endonucleases. After an overnight incubation at 23°C, the ligation mixtures were transformed into competent E. coli XL1 cells (Stratagen, USA). All recombinant plasmid sequences were confirmed by Sanger chain termination sequencing.

2.3. Transfection of EA.hy926 cells using the polyethylenimine (PEI) reagent

Procedures were done following the protocols of a previous study (12). Basically 600 ng of plasmid DNA (e-NOS TT SNP, e-NOS TC SNP, e-NOS CC SNP long, or e-NOS CC SNP short, or the control plasmids pGL3-basic or pSV-β-galactosidase) were added to 1.8 µg of PEI reagent and completed to 40 µL using Dulbecco’s Modified Eagle’s Medium (DMEM) without serum. Transfection mixtures were incubated at room temperature for 15 minutes. Each transfection mixture (40 µL) was transferred to 24-well plate previously seeded with 10^5 cells/well; where each well contained 400 µL of total liquid volume (360 µL of complete medium and 40 µL of transfection mixture). The plate was incubated at 37°C for 48 hours.

Cell lysis and luminescence assays were performed using 1× Glo Lysis Buffer, Steady-Glo, and Beta-Glo Luciferase Assay Systems (Promega, Madison, USA). Luminescence was measured using GloMax® Multi Detection System (Promega, Madison, USA).

| Protocol step | Temperature | Time | Number of cycles |
|---------------|-------------|------|------------------|
| Denaturation   | 95°C        | 10 min | 1                |
| Annealing     | 60°C        | 30 sec | 35               |
| Extension     | 72°C        | 2 min  |                  |
| Final elongation | 72°C     | 10 min | 1                |
| Final hold    | 4°C         | Hold  |                  |

| Protocol step         | Temperature | Time  | Number of cycles |
|-----------------------|-------------|-------|------------------|
| Initial denaturation  | 95°C        | 5 min | 1                |
| Denaturation          | 95°C        | 30 sec| 35               |
| Annealing             | 57°C        | 30 sec|                  |
| Extension             | 72°C        | 1 min |                  |
| Final elongation      | 72°C        | 10 min| 1                |
| Pause                 | 4°C         | Hold  |                  |

Table 2. PCR thermo-cycling conditions for the long insert (1,612 bp)

Table 3. PCR thermo-cycling conditions for the short insert (345 bp)

Figure 1. Comparison between luminescence levels obtained for EA.hy926 cells transfected with plasmid constructs of short eNOS promoter sequences carrying the three different SNPs. Control luminescence is the Luminescence produced by EA.hy926 cells transfected with the empty plasmid vector pGL3-Basic. Differences in luminescence emission were analyzed using independent t test, where different letters (a, b, or c on bars) indicate significant differences at ***p < 0.0001. Error bars represent standard error of the mean (SEM).
mRNA and protein expression were present in TT and CT genotype cells but absent in cells with CC genotype. They concluded that the T-786C SNP of eNOS gene constitutes a genetic risk factor for CAD, presumably due to binding of an inhibitory transcription factor to the C-type promoter blocking shear stress-dependent maintenance of eNOS expression (13).

In their trials to elucidate the molecular mechanism behind the reduced eNOS gene expression associated with the T-786C mutation, Miyamoto et al. were able to identify and purify a protein called replication protein A1 (RPA1) that contributes to the diminished eNOS expression in T-786C placenta cells. Diminished eNOS expression was revealed by the finding that serum nitrite/nitrate levels among individuals carrying the T-786C SNP were significantly lower than among the T variant subjects. They concluded that RPA1 apparently functions as a repressor protein in the T-786C polymorphism-related reduction of eNOS gene transcription associated with the development of CAD (14).

AliReza et al. investigated the frequency of T-786C polymorphism of the eNOS gene in non-smoker and non-diabetic CAD patients in North West Iran. Compared to the healthy control group, the eNOS T-786C genotype was associated with reduced serum levels of NO (16). In a sample of Japanese population, Nakayama et al. assessed the influence of the same polymorphism on coronary spasm development. They found that the T-786C mutation resulted in a significant reduction in eNOS gene promoter activity that reduces the endothelial NO synthesis and predisposes the patients to coronary spasm (13). Moreover, Popov et al. demonstrated that T-786C polymorphism contributes to a higher prevalence of postoperative mortality after emergency cardiac surgery. Thus, the eNOS T-786C polymorphism could serve to differentiate high risk subgroups in individuals with cardiac disease who need cardiac surgery with cardiopulmonary bypass (17).

In contrast, however, Kincl et al. found no impact of T-786C polymorphism on CAD prognosis (death or AMI, unstable angina, necessity of percutaneous or coronary artery bypass graft, heart failure, or cardioverter/defibrillator implantation) (18). Similarly, Jaramillo et al. and Alp et al. observed that T-786C polymorphism of the eNOS gene was not associated with CAD in a studied sample of Chilean and Turkish individuals, respectively (19, 20). To our knowledge, no parallel investigations were performed on Egyptian population regarding the association of T-786C polymorphism of eNOS with the incidence of CAD.

Two stretches of eNOS promoter were tested in the study; a short sequence (345 bp) carrying the CC genotype and a longer promoter sequence (1,594 bp) carrying the same genotype. Luminescence levels obtained for EA.hy926 cells transfected with constructs carrying the longer promoter version were significantly higher than those obtained from cells bearing the shorter (CC <sub>long</sub> = 1,594 bp) were significantly higher than those obtained for cells transfected with constructs of the shorter promoter version (CC <sub>short</sub>) that carries the same SNP site (Figure 2).

4. Discussion

To date more than 1,730 polymorphic sites have been identified in, or in the vicinity of the eNOS gene (http://www.ncbi.nlm.nih.gov/snp). However, no clear correlation could be detected between the presence of SNPs in the coding or non-coding sequences of the gene and the amount of NO produced to reflect the activity of the encoded enzyme.

Here, we extended our studies on eNOS by performing in vitro gene expression analyses of the T-786C polymorphism in the promoter region of the eNOS gene, with the ultimate goal of shedding more light on the mechanisms by which genetic variations of eNOS might affect the expression of eNOS and therefore the vascular level of NO.

DNA sequencing of amplified regions of the eNOS gene revealed the presence of three different genotypes; T/T, T/C, and C/C. Our results show significantly higher expression levels of eNOS for the T/T genotype compared to the other two genotypes; a T to C mutation resulted in 1.7–1.9-fold lower expression levels of the gene. In agreement with our findings, earlier in vitro observations suggested that the replacement of a T at position – 786 by a C resulted in ~50% lower transcriptional activity (13,14).

In harmony, Cattaruzza et al. (2004) investigated the effect of T-786C SNP on the shear stress-induced NO release from endothelial cells isolated from umbilical cords. They found that shear stress-induced eNOS gene transcription associated with CAD in a studied sample of Chilean and Turkish individuals, respectively (19, 20). To our knowledge, no parallel investigations were performed on Egyptian population regarding the association of T-786C polymorphism of eNOS with the incidence of CAD.

Two stretches of eNOS promoter were tested in the study; a short sequence (345 bp) carrying the CC genotype and a longer promoter sequence (1,594 bp) carrying the same genotype. Luminescence levels obtained for EA.hy926 cells transfected with constructs carrying the longer promoter version were significantly higher than those obtained from cells bearing the shorter
inserts ($p < 0.0001$).

A study made by Xing et al. using DNA sequence deletion, concluded that 68% of the basal activity of the eNOS promoter was controlled by the region from $–1$ to $–166$ bp and the rest was dependent on the region from $–1,033$ to $–1,600$ bp (27). The study strongly suggested that the main functional region of eNOS promoter is from $–1$ to $–166$ bp that binds to the upstream activator transcription factor 1 (AP1). This explains the increased gene expression using the extended sequence for eNOS promoter (long version = 1,594 bp) observed in our study as compared to the gene expression using the short version (345 bp) that lacks the binding site to transcription factor AP1.

In conclusion, our study suggests that a $T$ allele at the $–786$ site of eNOS gene increases genetic expression of the enzyme, while the $C$ genotype at the same site could be a predisposing factor to CVD through marked down expression of eNOS.

**Acknowledgements**

We would like to thank Dr. Frank Rosenau, Head of Ulm Center for Pharmaceutical Peptides, for hosting Aliaa El-Akkad in his lab and Suzanne Nour El-Din for helping with the luminescence determination experiments.

**References**

1. Tousoulis D, Kampoli AM, Tentolouris C, Papageorgiou N, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol. 2012; 10:4-18.
2. Jones L, C’Hingorani AD. Genetic regulation of endothelial function. Heart. 2005; 91:1275-1277.
3. Gad MZ, Hassanein SI, Abdel-Maksoud SM, Shaban GM, Abou-Aisha K. Assessment of serum levels of asymmetric dimethylarginine, symmetric dimethylarginine and L-arginine in coronary artery disease. Biomarkers. 2010; 15:746-752.
4. Oliveira-Paula GH, Lacchini R, Tanus-Santos JE. Endothelial nitric oxide synthase: From biochemistry and gene structure to clinical implications of NOS3 polymorphisms. Gene. 2016; 575:584-599.
5. Zhao GL, Li QJ, Lu HY. Association between NOS3 promoter was controlled by the region from $–1$ to $–166$ bp and the rest was dependent on the region from $–1,033$ to $–1,600$ bp (27). The study strongly suggested that the main functional region of eNOS promoter is from $–1$ to $–166$ bp that binds to the upstream activator transcription factor 1 (AP1). This explains the increased gene expression using the extended sequence for eNOS promoter (long version = 1,594 bp) observed in our study as compared to the gene expression using the short version (345 bp) that lacks the binding site to transcription factor AP1.

In conclusion, our study suggests that a $T$ allele at the $–786$ site of eNOS gene increases genetic expression of the enzyme, while the $C$ genotype at the same site could be a predisposing factor to CVD through marked down expression of eNOS.

**Acknowledgements**

We would like to thank Dr. Frank Rosenau, Head of Ulm Center for Pharmaceutical Peptides, for hosting Aliaa El-Akkad in his lab and Suzanne Nour El-Din for helping with the luminescence determination experiments.

**References**

1. Tousoulis D, Kampoli AM, Tentolouris C, Papageorgiou N, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol. 2012; 10:4-18.
2. Jones L, C’Hingorani AD. Genetic regulation of endothelial function. Heart. 2005; 91:1275-1277.
3. Gad MZ, Hassanein SI, Abdel-Maksoud SM, Shaban GM, Abou-Aisha K. Assessment of serum levels of asymmetric dimethylarginine, symmetric dimethylarginine and L-arginine in coronary artery disease. Biomarkers. 2010; 15:746-752.
4. Oliveira-Paula GH, Lacchini R, Tanus-Santos JE. Endothelial nitric oxide synthase: From biochemistry and gene structure to clinical implications of NOS3 polymorphisms. Gene. 2016; 575:584-599.
5. Zhao GL, Li QJ, Lu HY. Association between NOS3 variants and coronary artery disease in the Han population. Genet Mol Res. 2016; 15:gmn3044.
6. Doshi AA, Ziolo MT, Wang H, Burke E, Lesinski A, Binkley P. A promoter polymorphism of the endothelial nitric oxide synthase gene is associated with reduced mRNA and protein expression in failing human myocardium. J Card Fail. 2010; 16:314-319.
7. Colombo MG, Paradossi U, Andreassi MG, Botto N, Manfredi S, Masetti S, Biagini A, Clerico A. Endothelial nitric oxide synthase gene polymorphisms and risk of coronary artery disease. Clin Chem. 2003; 49:389-395.
8. Gad MZ, Hassanein SI, Abdel-Maksoud SM, Shaban GM, Abou-Aisha K. Association of DDHA2 gene polymorphism with cardiovascular disease in Egyptian patients. J Genet. 2011; 90:161-163.
9. Gad MZ, Abdel Rahman MF, Hashad IM, Abdel-Maksoud SM, Farag NM, Abou-Aisha K. Endothelial nitric oxide synthase (G894T) gene polymorphism in a random sample of the Egyptian population: Comparison with myocardial infarction patients. Genet Test Mol Biomarkers. 2012; 16:695-700.
10. Hashad IM, Abdel Rahman MF, Abdel-Maksoud SM, Amr KS, Effat LK, Shaban GM, Gad MZ. C242T polymorphism of NADPH oxidase p22phox gene reduces the risk of coronary artery disease in a random sample of Egyptian population. Mol Biol Rep. 2014; 41:2281-2286.
11. Rahman MF, Hashad IM, Abou-Aisha K, Abdel-Maksoud SM, Gad MZ. Addressing the link between paraoxonase-1 gene variants and the incidence of early onset myocardial infarction. Arch Med Sci. 2015; 11:513-520.
12. Longo PA, Kavran JM, Kim MS, Leahy DJ. Transient mammalian cell transfection with polyethyleneimine (PEI). Methods Enzymol. 2013; 529:227-240.
13. Nakayama M, Yasse H, Yoshimura M, Shimasaki Y, Ogawa H, Kugiyama K, Mizuno Y, Harada E, Nakamura S, Ito T, Saito Y, Miyamoto Y, Ogawa Y, Nakao K. T(-786)->C mutation in the 5`-flanking region of the endothelial nitric oxide synthase gene is associated with myocardial infarction, especially without coronary organic stenosis. Am J Cardiol. 2000; 86:628-634.
14. Miyamoto Y, Saito Y, Nakayama M, et al. Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T-->C mutation associated with coronary spastic angina. Hum Mol Genet. 2000; 9:2629-2637.
15. Cattanuza M, Guzik TJ, Sladowski W, Pelvan A, Becker J, Halle M, Buchwald AB, Shannon KM, Hecker M. Shear stress insensitivity of endothelial nitric oxide synthase expression as a genetic risk factor for coronary heart disease. Circ Res. 2004; 95:841-847.
16. Ali-Reza Y, Fatemeh KK, Nosratallah Z, Mohammad RN, Mohammad P. T-786C Polymorphism of Endothelial Nitric Oxide Synthase Gene and Serum Level of Nitric Oxide in Nonsmoker and Nondiabetic Patients Suffering from Coronary Artery Disease. J Biotechnol Biomaterial. 2012; 2:125. doi:10.4172/2155-952X.1000125
17. Popov AF, Henker C, Schmitto JD, Wiese CH, Coskun KO, Moerer O, Danner BC, Schoendube FA, Quintel M, Hinz J. Clinical relevance of eNOS T-786C polymorphism for hospital mortality and morbidity in cardiac surgical patients. J Cardiovasc Surg (Torino). 2010; 51:265-272.
18. Kincel V, Machal J, Droz dov a A, Panovsky R, Vasku A. The relation between eNOS -786 C/T, 4 a/b, MMP-13 rs640198 G/T, eotaxin 426 C/T, -384 A/G, and 67 G/A polymorphisms and long-term outcome in patients with coronary artery disease. Dis Markers. 2015; 2015:230248.
19. Jaramillo PC, Lanas C, Lanas F, Salazar LA, -786T>C polymorphism of the endothelial nitric oxide synthase gene in Chilean subjects with coronary artery disease and controls. Clin Chim Acta. 2008; 387:105-108.
20. Alp E, Mencese S, Tulmac M, Kan D, Yalcin R, Erkan AF, Cengel A. Lack of association between matrix metalloproteinase-9 and endothelial nitric oxide synthase gene polymorphisms and coronary artery disease in Turkish population. DNA Cell Biol. 2009; 28:343-350.
21. Xing F, Jiang Y, Liu J, Zhao K, Mo Y, Qin Q, Wang J, Ouyang J, Zeng Y. Role of AP1 element in the activation of human eNOS promoter by lysophosphatidylcholine. J Cell Biochem. 2006; 98:872-884.

(Received December 30, 2016; Revised February 11, 2017; Accepted July 17, 2017)