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Prognostic value of T786C and G894T *eNOS* polymorphisms in sickle cell disease

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ABSTRACT

Endothelial Nitric Oxide Synthase (eNOS) is crucial for vascular homeostasis. Polymorphisms T786C and G894T affect eNOS regulation and have been related to various diseases. Sickle Cell Disease (SCD), a clinically diverse chronic hemolytic anemia, implies impaired nitric oxide bioavailability. Our aim was to determine *eNOS* genotype for T786C and G894T polymorphisms in Greek patients with SCD and to elucidate its consequences and effects if any on clinical phenotype. Seventy nine steady state cases, mostly compound heterozygous for Sickle Cell anemia/beta thalassemia and 48 controls were measured. Peripheral blood DNA was extracted and genotyped with PCR-RFLPs and Sanger sequencing. Total RNA was extracted from 18 patients and 9 controls and *eNOS* mRNA levels were determined by real-time PCR. Genotypes, allele distribution and *eNOS* mRNA levels did not differ between patients and controls, or among patients with different beta globin gene mutations. The 786CC genotype was more common in S/S and β^0/S patients with retinopathy. Moreover, 894TT S/S and β^0/S patients tended to have a higher hematocrit than 894GG and GT ones. However, the T786C *eNOS* genotype does not seem to affect peripheral blood cell-derived *eNOS* mRNA levels, at least in steady state conditions. This work is the first one describing the effects of *eNOS* polymorphisms on different forms of SCD, the first enrolling SCD patients of Caucasian origin and the first determining *eNOS* mRNA levels in peripheral blood from steady-state SCD patients.

1. Introduction

Structural and functional integrity of vasculature depends on a variety of signaling molecules; nitric oxide (NO) produced by NO synthase (NOS) plays a crucial role among them, regulating vascular tone control, vascular remodeling and proliferation. Three NOS isoforms are recognized, namely neuronal NOS (nNOS) expressed in neurons, inducible NOS (iNOS) expressed in innate immunity cells and endothelial NOS (eNOS) [1,2]. This latter isoform produces the majority of NO signals in the cardiovascular system; it is expressed mainly in endothelial cells and, to a lower extent, in granulocytes, monocytes, lymphocytes and erythrocytes [3–5]. The *eNOS* gene is located on chromosome 7q35–36 consisting of 26 exons. It has a total length of 21kb [1]. The cell-specific expression of *eNOS* is mediated by an epigenetic, DNA-based mechanism [6], while eNOS activity regulation takes place in the transcriptional, translational and post-translational level, the latter probably being the most important. Localization to the caveolae, dimerization, interaction with caveolin-1, calmodulin, hsp90,

Abbreviations: NO, Nitric Oxide; NOS, Nitric Oxide Synthase; eNOS, endothelial Nitric Oxide Synthase; kb, kilobases; HUVEC, Human Umbilical Vein Endothelial Cells; NO_x, nitrite and nitrate; SCD, Sickle Cell Disease; HbS, Sickle Hemoglobin; VNTR, Variable Number of Tandem Repeats; ACS, Acute Chest Syndrome; HACs, Hemolysis-Associated Complications; WBC, White Blood Cells; PLT, platelets; LDH, Lactate Dehydrogenase; CRP, C-Reactive Protein; VEGF, Vascular Endothelial Growth Factor.

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hemoglobin A, phosphorylation in various sites are, among others, the main post-translational mechanisms of eNOS activity regulation [7]. The eNOS enzyme catalyzes the production of NO from L-arginine, with NADPH as a co-factor and L-citrulline as a by-product. NO, a gas with a very short half-life, is rapidly diffused and interacts with soluble guanylate cyclase to activate it and enhance cGMP production. The latter activates Protein Kinase G and mediates smooth muscle relaxation and vasodilation [1]. Polymorphisms of *eNOS* gene have been detected and implicated in many diseases. The rs2070744 SNP occurs in the position −786 in the 5' flanking region of the gene, influencing promoter's activity when cytosine (C) substitutes the wild type thymine (T). Initially this polymorphism (T786C) was correlated with coronary spasm [8], coronary artery disease [9], arterial hypertension [10] and ischemic stroke [11]. The way this polymorphism affects mRNA and protein levels is controversial. Nakayama et al. [8] using a luciferase reporter gene assay in HUVEC cells found decreased promoter activity (52 ± 11%) corresponding to the 786C genotype [8]; Salvi et al. [10] using a luciferase reporter gene expression assay in HeLa and HEK293T cells, reported decreased activity of the wild-type 786T allele from 20% to 40% respectively in these cell lines [10]. Sim et al. [12] using a luciferase reporter gene assay in HepG2 cells found no difference [12]. Senthil et al. [13] found decreased mRNA levels in CC genotype cultured cells but no difference in protein levels or enzyme activity [13].

The other clinically relevant polymorphism of *eNOS* gene concerns the substitution of guanine, G at position +894 by thymine T (G894T) in exon 7. This SNP, rs1799983, leads to a change in the translated amino acid, missense variant. Glutamate is changed by aspartate at position 298 (Glu298Asp) [14]. Although originally thought to create a novel cleavage site [15] or increase plasma NO_x levels [16], this nucleotide substitution was associated with deficient caveolar localization and deficient shear stress response of eNOS [17] resulting in reduced enzymatic activity. In some populations, this polymorphism was found to be more prevalent in patients with coronary artery disease [9], ischemic stroke [18], and arterial hypertension [19].

SCD results from the change of glutamate by valine in the sixth amino acid position of the beta globin chain (Glu6val) due to a point mutation A > T in the beta globin gene. This substitution renders hemoglobin S (HbS) prone to polymerization when deoxygenated, leading to red blood cell structure deformation, early destruction and blood vessel occlusion. This occlusion involves interactions among red cells, white cells, platelets and endothelial cells. Following red cell destruction inside blood vessels, arginase and hemoglobin get released, thus reducing the available NO and promoting a prothrombotic status [20].

Although classically SCD is considered monogenic disorder, other modifying genes apart from globin genes, contribute to a varying clinical severity. One may suppose that polymorphisms affecting eNOS quantity/activity and NO levels could also affect clinical presentation and severity of SCD. Few studies assess *eNOS* polymorphisms T786C and G894T in sickle cell disease patients, and the results are varying or contradictory [21–27]. Here, we examined *eNOS* polymorphisms, T786C and G894T in adult SCD patients and we assessed their prevalence and potential effect on clinical course/prognosis.

2. Materials and methods

2.1. Study population

Seventy nine consecutive Greek SCD patients (mean age: 48.8 ± 11.5 years, 25–76 years, 25 male, 54 female) and forty eight Greek healthy controls were enrolled. The patients are regularly followed at the Hemoglobinopathies Unit of 1st Department of Internal Medicine, University of Athens Medical School at Laikon Hospital. The study was approved by the Hospital Ethics Committee in accordance to Helsinki Declaration. All patients were at steady state at the time of sample collection (no painful crises or other acute complications during the last three months) and they had not been transfused for the last three months. A detailed medical history and physical examination was recorded. Occurrence of severe crises requiring hospitalization during the last year (or the last year before starting hydroxyurea/blood apheresis, for patients receiving the above-mentioned treatment modalities) and history of specific clinical complications (i.e. acute chest syndrome, femoral head necrosis) were recorded. Patients were divided into two groups according to their beta globin gene mutation. The first group, group 1, included patients carrying beta globin gene mutations related to severe clinical course (S/S and β^0/S). The second group, group 2, included those carrying beta globin gene mutations predisposing to a milder clinical course (β^+/S δβ/S, Lepore/S).

All patients of our cohort were receiving acetylsalicylic acid (ASA, 100mg) and folate supplementation as standard therapy, 29 were under hydroxyurea treatment and 18 under regular blood apheresis. *Chronic pain* was defined as a pain that persists for at least three consecutive months and/or requires pain-relieving medications for at least three consecutive months, irrespective of the cause. *Femoral head necrosis* was diagnosed on the basis of clinical and MRI findings. *Acute Chest Syndrome* (ACS) was defined as the presence of new infiltrates in chest radiography with fever and/or respiratory tract symptoms (cough, sputum production, dyspnea). The term *SCD retinopathy* refers to the proliferative retinal involvement. SCD retinopathy was diagnosed on the basis of either neovascularization findings at retinal (fundus) examination or history of laser treatment. Other potential causes of retinal pathology were excluded. The term *Hemolysis-Associated Complications (HACs) is* used to encompass cutaneous leg ulcers, priapism and pulmonary hypertension. All the above-mentioned complications occur both in SCD and in other forms of hemolytic anemia and are a consequence of intravascular hemolysis [28]. Patients with a history of at least one of the above were considered as suffering from *HACs.*

2.2. DNA extraction and eNOS genotyping

Genomic DNA was isolated from peripheral blood using the PureLink Genomic DNA extraction kit (Invitrogen). Nucleotide substitutions T786C and G894T were detected using Sanger sequencing and PCR-RFLPs techniques respectively. The PCR primers used were as follows: for T786C: 5'*ATGCTCCCACCAGGGCATCA*3' (forward), 5'*GTCCTTGAGTCTGACATTAGGG*3' (reverse) covering a fragment of 237 bp and for G894T: 5'*CATGAGGCTCAGCCCCAGAAC*3' (forward), 5'*AGTCAATCCCTTTGGTGCTCAC*3' (reverse) covering a fragment of 206 bp.

PCR conditions for T786C were: 92 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s repeated for 34 cycles. For G894T, we applied the previous conditions decreasing the annealing temperature to 58 °C. Presence of 786C polymorphism was determined by Sanger sequencing of the PCR product using the above mentioned PCR primers. The 894T polymorphism was detected by PCR-RFLPs, digesting with the appropriate restriction enzyme, MboI, revealing two fragments of 119 base pairs and 87 base pairs.

2.3. RNA extraction and quantitative real-time PCR

Eighteen SCD patients (5 male, 13 female) from both groups of beta genotypes were selected to undergo eNOS expression study. Total RNA was isolated from peripheral blood of the above patients