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Endothelial Nitric Oxide Synthase intron 4 VNTR Gene polymorphisms in European and African populations

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Abstract

Nitric oxide is an endogenous molecule that play an important role in the regulation of the systemic, cardiac and pulmonary circulations. We investigated the frequency of intron 4 VNTR *ecNOS* gene polymorphism in Ivorian and Italian populations. The frequencies of the bb, ab and aa genotypes were 0.422, 0.476, and 0.102, respectively, for the Ivorian sample, and 0.683, 0.300, and 0.017, respectively, for the Italian sample. The frequencies of 4b and 4a alleles were 0.660 and 0.340, respectively, for the Ivorian group, and 0.833 and 0.167, respectively, for the studied Italian population. The genotype frequencies were in Hardy-Weinberg equilibrium in both populations. No differences were found in *ecNOS* genotype and allele frequencies between sexes. The Ivorian population showed a significantly higher frequency of the 4a allele with respect to North African populations (Tunisia, Algeria and Morocco) and to the African-Brazilian sample. *Viceversa*, the Italian population did not show any significant differentiation with respect to other European

populations, with exception of the Holland and Turkey samples, confirming the high genetic homogeneity of Italy and Europe in the distribution of this polymorphism. In the Ivorian sample the 4a allele was prevalently found in a heterozygous status. A possible explanation could be that the heterozygote individuals produce a sufficient level of NO metabolites, although in lower amount with respect to the bb genotypes. As a consequence, the 4a allele is maintained prevalently in a heterozygous status and does not provoke an effective selective disadvantage for the carriers.

Key words: EcNOS; Nitric Oxide; genetic polymorphism; Italy; Ivory Coast

Introduction

Nitric oxide (NO) is an endogenous highly reactive molecule that play an important role in the regulation of vascular smooth muscle cell proliferation and migration, in the endothelial permeability, and in the endothelial-leucocyte interaction. Because of these properties, NO has been established as being a regulator of the systemic, cardiac and pulmonary circulations [1], with an important antithrombotic and antimicrobial role [2, 3]. An impairment of NO production has been showed to be related to many diseases, including coronary artery disease [4], ischemic stroke [5], hypertension [6], renal disease [7], and Alzheimer's disease [8]. At cellular level, an overproduction of NO has been described to be associated to DNA damage, cell cycle arrest, and cellular apoptosis [9], whereas reduction in basal NO release may predispose to hypertension, thrombosis, vasospasm and atherosclerosis [10].

NO is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS) [11]. Three distinct isoforms of NOS, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial constitutive NOS (ecNOS) have been identified in humans. ecNOS produces the majority of circulating NO [12] and, with nNOS, it is constitutively expressed in both airway and vascular endothelia. The continuously generated NO serve to maintain basal vascular NO production necessary for a good endothelial function and blood flow regulation, particularly coronary flow.

The ecNOS is encoded by a gene located on chromosome 7q35-q36 (13-15) and composed of 26 exons that span more than 21 kb of the genome [13]. Several polymorphisms were reported in the promotor region, exons, and introns of this gene, and an important one is represented by a 27-base pair core consensus VNTR (variable number of tandem repeats) polymorphism in the intron 4 [14]. The common wild-type 'b-allele' (4b allele) has five tandem 27-bp repeats and has been designated as *ecNOS4b*, while a rare 'a-allele' (4a allele) has been detected with only four repeats. Tsukada et al. (1998) [15] reported that *ecNOS* gene polymorphisms correlate with the circulating NO concentrations and individuals with bb and aa genotypes exhibit the highest and the lowest activity, respectively. The four repeats of VNTR, influencing the plasma NO levels and ecNOS protein

expression [12, 15], were reported to be involved in the pathogenesis of several diseases including coronary heart disease [4, 16,17], lung disease [18], hypertension [6, 19], atopic asthma [20], and early-onset colorectal cancer [21].

Published papers about this gene polymorphism refer mostly to case-control studies, and only few population genetic studies were published in order to assess the frequency of this polymorphism in populations worldwide. The aim of this study was to investigate the genetic variability at *ecNOS* locus in an African population from Ivory Coast and in an European population from Italy, for which no data has been published about *ecNOS* intron 4 VNTR polymorphism. We also compared our results with those reported from other control populations sampled worldwide, in particular from African and European groups.

Materials and Methods

Population studied

The African sampling was performed in Ouangolodougou, a rural village of 20.000 inhabitant located on the Northern Ivory Coast. One hundred twenty-eight unrelated individuals (59 males and 69 females, mean age 31.9±14,5) were analyzed. The Italian sampling was performed in Piedmont, a region of Northern Italy. Two hundred and forty individuals (112 males and 128 females, mean age 58.36±6.7) were analyzed. All the subjects were healthy volunteers and had received information about the study. The study has been approved by the local ethics committee and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

PCR Analysis

Peripheral blood samples (5-10 ml venipuncture) were collected in heparinized vacutainers and stored at –20°C. To extract DNA we used Chelex® solution protocol [22]. To determine the ecNOS genotype, a genomic DNA fragment of intron 4 of the ecNOS gene was amplified by PCR, using

the following primers: forward primer 5′-AGGCCCTATGGTAGTGCCTTT-3′ and the reverse primer 5′-TCTCTTAGTGCTGTGGTCAC-3′. PCR reactions were carried out in a total volume of 25 μl containing 10 ng of DNA (template), with a final concentration of 1X Reaction Buffer, 1.5 mM of MgCl2, 5% of DMSO, 250 μM of dNTPs, 0.5 μM of each primer, and 1U/sample of Taq DNA polymerase (Fischer, U.S.). The reaction profile was: 30 cycles of 94°C for 1 min, 56°C for 1 min., 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on a 4% Nusive high resolution agarose gel and visualized by ethidium bromide staining. Amplification yielded either a 420 bp (five repeats of the 27-bp sequence) fragment or a 393 bp (four repeats of the 27-bp sequence) fragment or both (heterozygous genotype). A 10% of the samples were amplified twice for checking the accuracy of the results. Moreover, in order to confirm that the amplified products represent genuine *ecNOS* intron 4 regions, selected PCR amplified segments, corresponding to a/a and b/b genotypes, were completely sequenced at both strands.

Statistical analysis

The allele and genotype frequencies were determined using GENEPOP program. Hardy-Weinberg equilibrium was evaluated by the Chi-square (χ^2) test using a 95% confidence interval. Allele frequencies obtained in the studied samples were compared with those reported for other European and African populations, through contingency tables analyzed with a χ^2-test.

Results and Discussion

The frequency distribution of *ecNOS* 27-bp repeat polymorphism of intron 4 within the study groups was presented in Table 1. The frequencies of the bb, ab and aa genotypes were 0.422, 0.476, and 0.102, respectively, for the Ivorian sample, and 0.683, 0.300, and 0.017, respectively, for the Italian sample. The frequencies of 4b and 4a alleles were 0.660 and 0.340, respectively, for the Ivorian group, and 0.833 and 0.167, respectively, for the studied Italian population.

The genotype frequencies distribution of this polymorphism was in Hardy-Weinberg equilibrium in both populations. Because of existing gender differences in NO production [23, 24], allele and genotype frequencies of the *ecNOS* polymorphisms were analyzed separately in men and women. No differences were found in *ecNOS* genotype and allele frequencies distribution between sexes (Table 1).

The *ecNOS* intron 4 VNTR polymorphism has been analyzed in several human populations (Table 2), mostly in order to ascertain its association with several type of diseases. Results showed a significant ($P<0.001$) decreasing gradient of the 4a allele frequency from Africa to Europe, America and Asia, probably ascribed to a founder effect due to ancient migrations of geographically distinct and isolated groups from Africa to other continents. In addition, being this polymorphism involved in the pathogenesis of several diseases, this different distribution pattern of the allele frequencies could also be the result of a selective pressure exerted on different populations.

Our Ivorian sample showed similar allele frequency values with respect to the other Ivorian sample genotyped for the same polymorphism, but a significantly ($p<0.001$) higher frequency of the 4a allele with respect to North African populations (Tunisia, Algeria and Morocco) and to the African-Brazilian sample (Table 2).

This different distribution pattern among Africans could probably be attributed to the different evolutionary histories of the different ethnic groups, in particular of the Northern Africa populations with respect to West and South Africa groups. In Africa, the genetic structure of many otherwise genetically "neutral" systems, such as mitochondrial DNA variation, has already been demonstrated [25]. Therefore, it is not surprising to discover differences between west and north African populations.

Viceversa, the Italian population, did not show any significant level of differentiation with respect to other European populations previously analysed for the same genetic marker, with exception of the Holland and Turkey samples ($p<0.001$), confirming the high genetic homogeneity of Italy and Europe in the distribution of this polymorphism.

According to many global surveys of genetic markers demonstrating that the African populations have consistently greater diversity than other populations [26, 27], our African sample showed an high frequency of heterozygous genotypes, indicating an high level of heterozygosity to the studied locus. It was found that the four repeats of VNTR influence the plasma NO levels. Indeed, the 4a allele of the *ecNOS* gene was related to low NO metabolite levels, and subjects homozygous for the 4a allele exhibit about 20% lower levels of NO metabolites than subjects with the b/b genotype [15]. Among Ivorians, the 4a allele was found prevalently in a heterozygous status. A possible explanation could be that the heterozygote individuals produce a sufficient level of NO metabolites, although in minor amount with respect to the bb genotypes. As a consequence, the 4a allele is maintained prevalently in a heterozygous status and does not provoke an effective selective disadvantage for the carriers.

In conclusion, we reported on novel frequency data as regards *ecNOS* intron 4 VNTR polymorphism in Ivorian and European populations, thereby extending previous observations obtained from other worldwide populations. Considering the implication of this polymorphism in many diseases and the scarcity of data about the prevalence of these diseases principally among African populations, further studies are needed to address the impact of selective constraints on the variability associated to this polymorphism in different populations and ethnic groups.

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Table 1 – Allele and Genotype Frequencies of intron 4 VNTR *ecNOS* gene polymorphism in the studied populations

Population	N	Genotype (frequency)			Allele (frequency)	
		bb	ba	aa	b	a
Ivory Coast	128	54 (0.422)	61 (0.476)	13 (0.102)	169 (0.660)	87 (0.340)
Male	58	28 (0.483)	22 (0.379)	8 (0.138)	78 (0.672)	38 (0.328)
Female	70	26 (0.372)	39 (0.557)	5 (0.071)	91 (0.650)	49 (0.350)
Italy	240	164 (0.683)	72 (0.300)	4 (0.017)	400 (0.833)	80 (0.167)
Male	112	78 (0.696)	34 (0.304)	0 (0.000)	190 (0.848)	34 (0.152)
Female	128	86 (0.672)	38 (0.297)	4 (0.031)	210 (0.820)	46 (0.180)

Table 2 – Allele and Genotype Frequencies of intron 4 VNTR *ecNOS* gene polymorphism in the studied and other populations worldwide distributed

Population	N	Allele (frequency) b	a	References
Africa				
Ivory Coast	128	169 (0.660)	87 (0.340)*	Present Study
Tunisia	250	431 (0.862)	69 (0.138)*	[28]
Algeria	122	202 (0.828)	42 (0.172)*	[29]
Morocco (Berber)	117	190 (0.812)	44 (0.188)*	[30]
Ivory Coast	109	138 (0.633)	80 (0.367)	[30]
African-Brazilians	121	194 (0.802)	48 (0.198)*	[31]
African-Americans	58	81 (0.698)	35 (0.302)	[32]
Pooled Africa	*905*	*1405 (0.776)*	*405 (0.224)*^{a,b,c}	
Europe				
Italy	240	400 (0.833)	80 (0.167)$^{\&}$	Present Study
Italy (Sardinia)	189	336 (0.889)	42 (0.111)	[30]
Spain	499	859 (0.861)	139 (0.139)	[30, 33]
Holland	466	839 (0.900)	93 (0.100)$^{\&}$	[34]
Turkey	488	873 (0.894)	103 (0.106)$^{\&}$	[35, 36, 47]
Polish	321	553 (0.861)	89 (0.139)	[38]
Germany	941	1572 (0.835)	310 (0.165)	[39, 40]
Austria	133	228 (0.857)	38 (0.143)	[41]
Czech Republic	316	517 (0.818)	115 (0.182)	[42]
Finland	110	184 (0.836)	36 (0.164)	[43]
Scotland	300	526 (0.877)	74 (0.123)	[44]
Greece	295	492 (0.834)	98 (0.166)	[45]
Greece, Crete	160	279 (0.872)	41 (0.128)	[46]
Pooled Europe	*4458*	*7658 (0.859)*	*1258 (0.141)*^{a,d}	
America				
Amerindians	170	326 (0.959)	14 (0.041)	[47]
Colombia	859	1474 (0.858)	244 (0.142)	[48]
Pooled America	*1029*	*1800 (0.875)*	*258 (0.125)*^{b}	
Asia				
South Korea	224	412 (0.920)	36 (0.080)	[49, 50]
China	216	392 (0.907)	40 (0.093)	[51, 52]
India	769	1274 (0.828)	264 (0.172)	[53, 54, 55]
Japan	1329	2409 (0.906)	249 (0.094)	[17, 56, 57, 58, 59]
Pooled Asia	*2538*	*4487 (0.884)*	*589 (0.116)*^{c,d}	

* = significantly different (P<0.001) with respect to our studied Ivorian sample

$^{\&}$ = significantly different (P<0.001) with respect to our studied Italian sample

^{a,b,c,d} = P<0.001