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Original scientific paper

Molecular Variation in Endothelial Nitric Oxide Synthase Gene (eNOS) in Western Mediterranean Populations

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A B S T R A C T

Endothelial nitric oxide synthase (eNOS or NOS3) is the main responsible for nitric oxide (NO) production in vascular system and different polymorphisms have been identified in epidemiological studies. Trying to test the eNOS genetic variation in general populations we studied the 27-bp VNTR in intron 4 and G894T substitution in exon 7 markers in 6 Western Mediterranean populations (3 from Iberian Peninsula, 1 from North Africa, and 2 from Sardinia) and a sample from Ivory Coast. The VNTR frequencies in Western Mediterranean and Ivory Coast fit well into the ranges previously described for Europeans and Sub-Saharans respectively, and a typical African allele has been detected in polymorphic frequencies in the Berber sample. The G894T substitution presents the highest frequencies described for the T allele in the North Mediterranean populations. Linkage disequilibrium is present between both markers in all populations except in the Ivory Coast sample. The variation found for these polymorphisms indicates that they may be a useful tool for population studies even at microgeographical level.

Key words: *eNOS, NOS3, nitric oxide, Mediterranean populations, haplotypes*

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Introduction

Nitric oxide (NO) is a gaseous molecule with important roles as a second messenger. All these actions are played by a single electron in its 2p-π antibonding orbital[1]. In cardiac muscle and vessel endothelium, NO inhibits platelet aggregation, relaxes smooth vascular musculature, reduces monocyte adhesion to endothelium[2,3], and attenuates atherogenic processes[4].

NO is endogenously produced by a family of three nitric oxide synthases (NOS). These enzymes are encoded by three different genes originated by duplications from a common ancestral gene[5] and received their names in the order they were discovered: NOS1 (or nNOS), NOS2 (or iNOS), and NOS3 (or eNOS). Although they share the same functional domains, their cellular expression and regulation is different for each NOS isoform. The NOS isoform mostly involved in cardiovascular system regulation is NOS3, and is the one with a well-known endothelium vessel expression (that is why is so called eNOS or endothelial NOS).

eNOS gene maps in chromosome 7 (7q35–7q36) and expands 22 Kb containing 26 exons. Different polymorphisms have been described all over the sequence, but no populational study has been made on them. The two most well-known markers on eNOS gene are ecNOS4a/b and G894T. ecNOS4a/b is a 27-bp VNTR located in intron 4 with two main alleles called ecNOS4*a and ecNOS4*b (with 4 and 5 repeats of the consensus 27-bp sequence, respectively) and two rare alleles found only in African-origin samples called ecNOS*c and ecNOS4*y (with 6 and 3 repeats, respectively). The other polymorphism is the G894→T substitution in exon 7, leading to an aminoacidic substitution from glutamate to aspartate.

Previous studies performed on eNOS polymorphisms focused on possible associations between them and many different diseases, most of them in the cardiovascular system (for a review, see Wang and Wang, 2000[6]). Moreover, the majority of these studies did not take into account the ethnical origin of their samples and only some of them considered the major origin of the individuals[7] (Caucasians, African-Americans, or Asians).

In a previous study of our group[8], frequencies for the G894T polymorphism in a Spanish population were significantly different from that described in other studies, except that published recently for an Italian population in an association study[9]. The samples used in these two studies belong to the same geographical area: the Mediterranean Basin. According to World Health Organization data, this area has one of the lowest cardiovascular disease incidences only comparable to far East populations (e. g. China and Japan) and the eNOS genetic variation in Mediterranean Basin has been scarcely investigated.

In this context, the aim of the present work is to explore variability in eNOS gene in a set of Western Mediterranean populations in order to analyze their genetic background and establish possible similarities among these populations.

Materials and Methods

Samples

A total of six Western Mediterranean populations were included in the present work. Three samples came from the Iberian Peninsula: one from Asturies (in the north, n=114), one from Center (n=120), and one from Andalusia (in the South, n=121). Two were from Sardinia: one from Nuoro (n=94) and one from Cabras (n=95). The last one was a Berber sample (n=120) from Khénifra in Morocco. As an outgroup, we analyzed a Sub-Saharan sample (n=112) from Ahïzi, in Ivory Coast. All individuals were healthy blood donors

with the four grandparents coming from the same geographical area. They were also informed on the objectives of the study and written consent was obtained for all individuals.

Genetic and statistical analyses

Determination of both polymorphisms included amplification by a PCR procedure and run in a 2% agarose gel electrophoresis as described previously[8]. For the G894T marker, prior to electrophoresis a digestion with 4U of BanII enzyme was performed.

Genotypic frequencies for both polymorphisms were computed by SPSS version 10.0 for Windows[10]. Allelic frequencies, Hardy-Weinberg adjustment, linkage disequilibrium, and haplotypic frequencies were calculated by Arlequin version 2.000[11]. Intrapopulational genetic variation, total gene diversities, and coefficients of gene differentiation were computed using the Genetix 4.02 software package.

Reynolds genetic distances between pairs of populations and neighbor joining trees were constructed using the PHYLIP 3.6 package[12]. The topology was assessed through 1000 bootstrap iterations. A principal components analysis (PCA) was performed using SPSS 10.0[10] to assess genetic relationships between populations.

For the comparative purposes other data on control samples as those from Tanus-Santos et al.[7], Salvarani et al.[9], Miyamoto et al.[13], Hibi et al.[14], Yoon et al.[15], Benjafield et al.[16], and Pulkkinen et al.[17] were included in some analyses.

Results

Allelic frequencies are shown in Table 1. In all populations, the most common allele for the 27-bp VNTR is ecNOS4*b, but its frequency ranges from 0.9121 (in the Cabras population) to 0.6116 (in Ivory Coast), showing lower values in the African samples (Ivory Coast and Berbers). The ecNOS4*c allele is present in polymorphic frequencies only in both African samples, although one individual from the Asturies sample was heterozygous for this allele. The ecNOS4*y allele was not present.

For the G894T substitution, frequencies for the T allele range from 0.5270 (in Nuoro) to 0.0759 (in Ivory Coast), showing the highest values in the North-Mediterranean populations.

TABLE 1
ALLELIC FREQUENCIES AND HARDY-WEINBERG PROBABILITIES FOR BOTH POLYMORPHISMS

	AST	CENT	AND	CABR	NUOR	BERB	IVOR
ecNOS4ab							
a	0.1409	0.1695	0.1154	0.0879	0.1351	0.1822	0.3571
b	0.8545	0.8305	0.8846	0.9121	0.8649	0.7944	0.6116
c	0.0045					0.0234	0.0313
HW p	0.1705	0.7424	0.6488	1.0000	0.1088	0.7193	0.7869
G894T							
G	0.5773	0.6441	0.5128	0.5055	0.4730	0.6355	0.9241
T	0.4227	0.3559	0.4872	0.4945	0.5270	0.3645	0.0759
HW p	0.0453	0.2313	0.2601	0.1456	0.0419	1.0000	0.4788

AST = Asturies; CENT = Center Spain; AND = Andalusia; CABR = Cabras; NUOR = Nuoro; BERB = Berbers from Khénifra; IVOR = Ivory Coast

TABLE 2
HAPLOTYPIC FREQUENCIES, LINKAGE DISEQUILIBRIUM PROBABILITY (P LD), LINKAGE DISEQUILIBRIUM COEFFICIENT (D'), AND TOTAL GENE DIVERSITY (GD)

	AST	CENT	AND	CABR	NUOR	BERB	IVOR
a-G	0.1152	0.1518	0.1027	0.0879	0.1013	0.1518	0.3571
a-T	0.0257	0.0177	0.0127		0.0338	0.0305	
b-G	0.4576	0.4923	0.4101	0.4176	0.3717	0.4604	0.5402
b-T	0.3970	0.3382	0.4745	0.4945	0.4932	0.3340	0.0714
c-G	0.0045					0.0234	0.0268
c-T							0.0045
D'	0.5814	0.7066	0.7741	1.0000	0.5253	0.5932	–
p LD	0.035	0.0072	0.0005	0.0005	0.0200	0.0218	0.1870
GD	0.6206	0.6226	0.5972	0.5765	0.6086	0.6516	0.5774

AST = Asturies; CENT = Center Spain; AND = Andalusia; CABR = Cabras; NUOR = Nuoro; BERB = Berbers from Khénifra; IVOR = Ivory Coast

For both markers, observed genotype values fitted well the expected frequencies under Hardy-Weinberg equilibrium assumptions with two minor exceptions for the G894T polymorphism, for which Asturies and Nuoro show probability values only slightly lower than 0.05 (0.0453 and 0.0419, respectively).

The most common haplotypes in all populations were b-G and b-T, except in the Ivory Coast sample, where b-G and a-G haplotypes presented the highest frequencies (see Table 2) as expected from both the low T and the high ecNOS4*a allele frequencies in this population.

Both polymorphisms showed linkage disequilibrium in all populations, except in the Ivory Coast sample (see Table 2). D' values range from 0.5253 (in Nuoro sample) to 1 (in Cabras sample).

Gene diversity was very similar in all populations, ranging from 0.6516 to 0.5765.

Intrapopulational genetic variation (Hs), total gene diversity (Ht), and coefficients of gene differentiation (Gst) are shown in Table 3. Ht for both markers is 0.397, and Gst is 0.0689. Ivory Coast population increase the total value of Gst because of its differentiation from all other populations here analyzed. Gst calculated without the Ivory Coast sample decreases to 0.0111.

From genetic distances analysis, a neighbor-joining tree with the seven analyzed populations was constructed (Figure 1). All the Mediterranean populations were joined at one side of the tree with the two Sardinian populations in the same sub-branch, while Berbers were closer to the Sub-Saharans than any other Mediterranean population.

In order to get a more general genetic picture, distance analyses were repeated adding seven populations from which data on the two polymorphisms were avail-

TABLE 3
INTRAPOPULATIONAL GENETIC VARIATION (HS), TOTAL GENE DIVERSITY (HT), AND COEFFICIENT OF GENE DIFFERENTIATION (GST) FOR EACH POLYMORPHISM AND FOR BOTH TOGETHER

	Hs	Ht	Gst
ecNOS4a/b	0.3015	0.3181	0.0522
G894T	0.4379	0.4760	0.0800
Total	0.3697	0.3970	0.0689

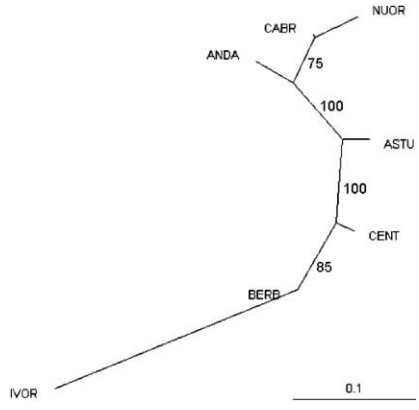

Fig. 1. Neighbor-joining tree of the seven analyzed populations.
AST = Asturies; CENT = Center Spain; AND = Andalusia; CABR = Cabras; NUOR = Nuoro; BERB = Berbers from Khénifra; IVOR = Ivory Coast

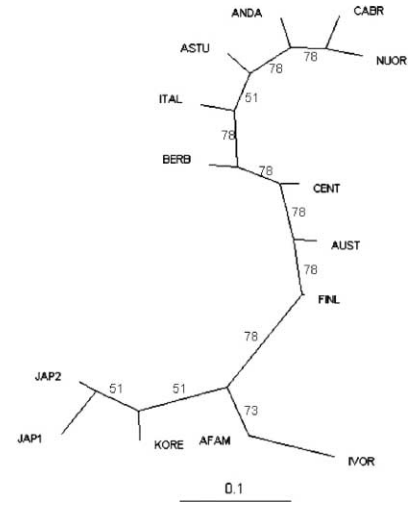

Fig. 2. Neighbor-joining tree of the seven analyzed populations together with seven populations taken from references.
AST = Asturies; CENT = Center Spain; AND = Andalusia; CABR = Cabras; NUOR = Nuoro; BERB = Berbers from Khénifra; IVOR= Ivory Coast; ITAL = Italy; AUST = Australia; FINL = Finland; AFAM = African-Americans; KORE = Korea; JAP1 and JAP2 = Japan

able. These populations were: Italians[9], African-Americans[7], two Japanese populations from Kyoto[13] and Kanagawa[14], Koreans[15], Australian Caucasians[16], and Finnish[17].

Population relationships among these populations are depicted in a neighbor-joining tree (Figure 2) and a PCA (Figure 3). Asian and Sub-Saharan populations appeared grouped in clearly particular clusters. European and North African samples were grouped together. PCA explained 85.803% of the total variation in allele frequencies for both markers and slight differences could be observed in the Europe/North-Africa cluster. North-Center European-origin populations (Australians and Finnish) are slightly separated from other samples and Berbers are slightly closer to African samples.

Discussion

The present study has examined the genetic distribution of the ecNOS4a/b VNTR and the G894T substitution in the eNOS gene in a set of six Mediterranean populations and in a Sub-Saharan population. These are the first genetic data on the populational variation of NO production genes. Our results are slightly different from that published for an epidemiological study in an Italian population[9], where ecNOS4*a allele was present in a frequency of 0.219, higher than any European Mediterranean population here analyzed. Furthermore, the present data confirm that ecNOS4*c allele is only present in polymorphic frequencies in African-origin populations, as previously suggested in African-American populations in association studies[7,18].

By the other hand, G894T frequencies in all European populations here analyzed (with the exception of Center

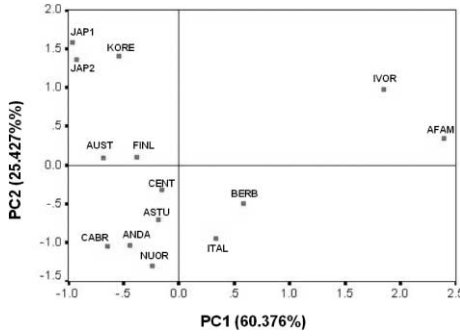

Fig. 3. Principal components analysis on fourteen populations.
AST = Asturies; CENT = Center Spain; AND = Andalusia; CABR = Cabras; NUOR = Nuoro; BERB = Berbers from Khénifra; IVOR = Ivory Coast; ITAL = Italy; AUST = Australia; FINL = Finland; AFAM = African-Americans; KORE = Korea; JAP1 and JAP2 = Japan

Spain) together with that previously described[8,9] confirm that European Mediterranean populations show the highest T allele frequencies ever described.

Cabras (with the lowest frequency for ecNOS4*a allele) and Nuoro (with the lowest frequency for G allele) presented the extreme values in genetic variation. That might be related to the effect of geographical isolation.

Linkage disequilibrium between both markers was expectable as the two markers are separated only 1.8 Kb. Ivory Coast sample shows no linkage disequilibrium and this fact is in accordance with previous reports on the lack of linkage disequilibrium between some polymorphisms in African populations[19]. That is, it might be attributed to the constrictions in the recent settlement of Europe and the Mediterranean Basin[20].

As expected, each principal component (PC) correlate with the information of each polymorphism, but PCA was performed in order to group the populations in a two-dimensional representation. Thus, PC1 resemble ecNOS4a/b variation and PC2 G894T variation with a correlation coefficient higher than 90%. PCA and neighbor-joining trees clearly differentiate Asian, Sub-Saharan, and European--Mediterranean populations in different clusters. In the Europe-Mediterra- nean cluster, two sub-groups can be observed: Northern-Central Europe origin, and Mediterranean populations.

PCA and neighbor-joining trees put Berber population together with the other Mediterranean populations with some trends towards Sub-Saharan origin populations. This might reflect a slight degree of genetic influence of Sub-Saharan populations in Berber genetic background.

As summary, the genetic variation showed in eNOS gene makes it a useful tool for populational studies, even at a microgeographical level. This variation is still poorly-known at population level, and further approaches should be done. Population studies on functional genes are showing an increasing importance in epidemiology and new disciplines, such as pharmacogenetics.

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MOLEKULARNE VARIJACIJE U GENU ZA ENDOTELIJALNU SINTAZU DUŠIKOVOG OKSIDA U POPULACIJAMA ZAPADNOG MEDITERANA

SAŽETAK

Endotelijalna sintaza dušikovog oksida (eNOS ili NOS3) u najvećoj je mjeri odgovorna za proizvodnju dušikovog oksida (NO) u vaskularnom sustavu i različiti polimorfizmi identificirani su u epidemiološkim studijama. Cilj ove studije je testirati varijacije eNOS gena u općoj populaciji ispitivanjem VNTR-a veličine 27-pb u intronu 4 i G894T supstitucije u exonu 7, u 6 populacija zapadnog Mediterana (3 iz Iberijskog poluotoka, 1 iz sjeverne Afrike, 2 iz Sardinije) te u uzorku iz Obale slonovače. Učestalosti VNTR-a u zapadnom Mediteranu i Obali slonovače odgovaraju rasponima koji su već opisani za europske, odnosno, sub-saharske populacije, a tipičan afrički alel je pronađen s polimorfnom učestalošću u uzorku populacije Berbera. Supstitucija G894T

pokazala je najveće učestalosti opisane za T alel u populacijama sjevernog Mediterana. Disekvilibrij vezanosti gena prisutan je između oba biljega u svim populacijama osim u uzorku populacije Obale slonovače. Varijacija koja je nađena za te polimorfizme ukazuje kako bi oni mogli biti korisno oruđe u populacijskim studijama čak i na mikrozemljopisnoj razini.