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Endothelial nitric oxide synthase VNTR (intron 4 a/b) polymorphism association with nonsyndromic oral clefts

[Endotelyal nitric oksit sentaz VNTR (intron 4 a/b) polimorfism ve semptomatik olmayan dudak yariklari ile iliskisi]*

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ABSTRACT

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ABSTRACT

Objective: Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a common birth defect with substantial clinical and social impact, and whose causes include both genetic and environmental factors. Folate and homocysteine metabolism have been indicated to play a role in the etiology of NSCLP. The aim of this study was to determine the prevalence of *NOS3* 27-bp VNTR and to evaluate whether this polymorphism contributed to the risk of NSCLP.

Material and methods: We studied the 27 base pair tandem repeat polymorphism in intron 4 of the endothelial nitric oxide synthase (*NOS3*) gene in 230 unrelated individuals belong to 7 Indian populations along with 141 NSCLP cases and 142 unrelated controls. The genotyping was performed by polymerase chain reaction and electrophoresis. The data were statistically analysed using the χ^2-test.

Results: The *NOS3* 27-bp VNTR 4a allele is present in six of the seven populations analysed and allele frequencies range from 6.8% in Sugali to 23.5% in Madiga populations. *NOS3* showed protective association with predisposition towards NSCLP for the heterozygous (4b/a) genotypes (4b/b vs. 4b/a: OR =0.58, 95% CI =0.34 to 0.99, p=0.044).

Conclusions: The current study suggests significant differences in the frequency of the *NOS3* VNTR allele across the populations. There is protective association between *NOS3* 27-bp VNTR polymorphism and NSCLP in the Indian population.

Key Words: nitric oxide, *NOS3*, VNTR polymorphism, orofacial clefts, Indians

Conflict of Interest: The authors declare that they have no competing interests..

ÖZET

Amaç: Semptomatik olmayan damak yarıklı veya yarıksız yarık dudak (NSCLP) olguları sosyal ve klinik etkisi ağır olan ve çok sık rastlanan bir doğum bozukluğu olup, genetik ve çevresel etkenlere bağlıdır. NSCLP etiyolojisinde folat ve homosistin metabolizmalarının etkin olduğu endike edilmiştir. Bu çalışmanın amacı *NOS3* 27 bp VNTR sıklığının belirlenmesi ve bu polimorfizmin NSCLP için bir risk faktörü olma olasılığının araştırılmasıdır.

Materyal ve metod: Hindistanda bulunan 7 toplumdan 141 NSCLP ve 142 ilişkisiz kontrol olmak üzere 230 ilişkilendirlmemiş bireyde nitrik oksit sentaz geninin intron 4 bölgesinde bulunan 27 bazlık ardışık tekrar polimorfizmi incelenmiştir. Genotiplendirme polimeraz zincir reaksiyonu ve elektroforez ile gerçekleştirilmiştir. Istatistiksel veri analizi χ^2-testi ile yapılmıştır.

Bulgular: *NOS3* 27-bp VNTR 4a alleli incelenen 7 toplumdan altısında bulunmakta ve allel sıklıkları Sugali toplumunda %6.8'den Madiga toplumunda %23.5'e kadar değişmektedir. *NOS3* koruyucu assosiyasyon, heterezigot 4b/a genotipleri (4b/b vs. 4b/a: OR = 0.58, 95% CI = 0.34 to 0.99, p=0.044) için NSCLP'e yatkınlık gözlemlenmiştir.

Sonuç: Bu çalışmada toplumlar arasında NOS3 VNTR allel sklığında belirgin farklılıklar göstermektedir. Hint toplumu için *NOS3* 27-bp VNTR polimorfismi ve NSCLP arasında koruyucu asosiyasyon bulunmaktadır.

Anahtar Kelimeler: nitrik oksit, NOS3, VNTR polimorfizmi, orofasiyal cepler, Hintliler

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Our perception on the etiology of nonsyndromic cleft lip with or without cleft palate is relatively poor because it is a complex multi-factorial trait, exhibiting varying levels of penetrance, gender disparities and environmental overlays involved in its pathogenesis [1]. Nonsyndromic cleft lip and palate (NSCLP) is multifactorial trait and may be influenced by the additive effects of several **gene**s corresponding to the pathways that regulate transcription factors, growth factors, cell signaling, folate and detoxification metabolisms [2]. Several studies have shown a strong association between homocysteine and nitric oxide (NO) synthesis [3, 4]. Methionine synthase converts cellular homocysteine to methionine and is a major enzyme in the biosynthetic pathways for folates, S-adenosylmethionine and biological methylations. Nitric oxide-induced inactivation of methionine synthase alters the levels of homocysteine [5] and could therefore provide a connection between the plasma homocysteine and orofacial clefts. The gene coding for the nitric oxide synthase (*NOS3*) is located on chromosome 7q36, spans 21 kb, and contains 26 exons that encode 1203 amino acid, 133-kDa protein. The exon 7 Glu298Asp missense variant and the intron-4 27-base pair (bp) variable number of tandem repeat (VNTR) polymorphism of *NOS3* are postulated to be associated with altered eNOS function, leading to impair NO synthesis [6].

Since its identification several studies have been designed to evaluate associations between NOS3 intron4b/a genotypes and essential hypertension, coronary risk, osteomyelitis, sepsis, diabetic retinopathy and diabetic nephropathy but the results were inconsistent because of phenotypic heterogeneity, population stratification and the interaction other risk factors [7-10]. As the elevated homocysteine levels are involved in the impaired palatogenesis, the present study was aimed to investigate the association between cleft lip and palate and *NOS3* intron4a/b genotypes. The ethnic variations in the *NOS3* 27-bp VNTR in South Indian populations were also examined.

Materials and Methods

A total of 230 unrelated individuals belong to 7 Indian populations and additional 141 cases with NSCLP and 142 unrelated control individuals without clefts were included in this study. The populations samples included from seven ethnic groups of south India were apparently normal healthy volunteers. The subjects with nonsyndromic clefts and unrelated control individuals without clefts or family history of clefting, were ascertained at Sri Ramachandra cleft and craniofacial centre, Sri Ramachandra University, Chennai, India. To determine their individual cleft status all the subjects with clefts were examined clinically twice by two surgeons independently and also through their medical records. All the subjects in the case group are isolated nonsyndromic clefts, and none of them is presented with other congenital malformations or major developmental disorders. The study was approved by the Institutional Ethics Committee of Sri Ramachandra University, Chennai, India. As many of the children are under 15 years of age, consent was requested from their parents. Three ml of blood sample was collected from all the participants after obtaining the informed consent. Genomic DNA of the above samples was isolated by standard protocols with phenol-chloroform extraction and ethanol precipitation [11]. Genotyping for NOS3 27-bp VNTR polymorphism was performed following published PCR-based methods [12]. Amplifications were performed in a 10-µl volume containing 2x Ampliqon master mix, 1 pmol of forward primer, one pmol of reverse primer, and 40 ng of genomic DNA. All PCR products were examined by agarose gel electrophoresis, and the genotypes were scored by two researchers independently to minimize error. Allele frequencies were determined by direct counting of alleles at each locus. The genotype distribution for each site in each group was evaluated for Hardy–Weinberg's equilibrium (HWE). The observed and expected genotype distributions and allele frequencies were computed using the HWSIM program [13]. The association between *NOS3* 27-bp VNTR polymorphism and NSCLP was analysed using χ^2-test. Odds ratios and 95% CI were calculated. All statistical analyses were performed with SPSS statistical software version 17.0 (SPSS Inc, Chicago, Illinois) for Windows.

Results

Population specific frequencies and counts of *NOS3* 27-bp VNTR genotypes among different populations were shown in Table 1. The *NOS3* 4a allele is present in six of the seven populations analysed and allele frequencies range from 6.8% in Sugali to 23.5% in Madiga populations. Genotype distributions are following Hardy Weinberg equation in all populations. The *NOS3* 4a allele frequencies that collected from various studies conducted on several human populations were presented in table 1. Data showed significant differences in the frequency of the *NOS3* 4a allele across the populations. The *NOS3* 27-bp VNTR genotypes are following the Hardy-Weinberg equilibrium in control group as well as in NSCLP group (Table 2). The *NOS3* VNTR mutation was found in 49 of 141 control subjects and in the NSCLP group 35 of 142 subjects. The *NOS3* 4a allele frequencies are 18.4% and 13.7% respectively for control and NSCLP group. Analysis pertaining to the assessment of risks associated with individual mutant genotypes of the *NOS3* VNTR with regards to risk of NSCLP depicted protective association with predisposition towards NSCLP for the heterozygous genotypes (4b/a) (4b/b vs. 4b/a: OR =0.58, 95% CI =0.34 to 0.99, p=0.044). The *NOS3* VNTR did not show significant association with NSCLP at allele level (4b vs. 4a: OR = 0.70, 95% CI =0.45–1.11, p = 0.127) (Table 2).

Table 1. The frequency of *NOS3* 27-bp VNTR polymorphism in the current study compared to different ethnic groups in different studies.

Population	4 b/b	4 b/a	4 a/a	MAF %	HWp
Balija (Present study)	23(74.2)	6(19.4)	2(6.5)	16.1	0.11
Irula (Present study)	26(76.5)	7(20.6)	1(2.9)	13.2	0.54
Reddy (Present study)	28(71.8)	10(25.6)	1(2.6)	15.4	0.92
Muslim (Present study)	32(76.2)	8(19.0)	2(4.8)	14.3	0.15
Madiga (Present study)	11(64.7)	4(23.5)	2(11.8)	23.5	0.154
Mala (Present study)	45(100.0)	0(0.0)	0(0.0)	0.0	NC
Sugali (Present study)	19(86.4)	3(13.6)	0(0.0)	6.8	0.73
Tunisian [9]	274(69.4)	112(28.4)	9(2.3)	16.5	0.534
Tunisian [8]	511(68.3)	217(29.0)	20(2.7)	17.2	0.593
African Americans [14]	64(47.8)	53(39.6)	17(12.7)	32.5	0.256
African Americans [23]	78(51.0)	58(37.9)	17(11.1)	30.1	0.22
Italian [10]	392(72.1)	138(25.4)	14(2.6)	15.3	0.657
Italian [7]	388(69.0)	152(27.0)	22(3.9)	17.4	0.15
Italian [24]	345(69.3)	135(27.1)	18(3.6)	17.2	0.295
Italian [25]	314(71.2)	114(25.9)	13(2.9)	15.9	0.5
Brazilian [26]	100(70.4)	39(27.5)	3(2.1)	15.8	0.722
Spanish [27]	70(64.8)	34(31.5)	4(3.7)	19.4	0.959
Turkish [28]	97(72.9)	35(26.3)	1(0.8)	13.9	0.254
Iranian [29]	128(81.0)	29(18.4)	1(0.6)	9.8	0.639
Saudi Arabian [30]	281(59.0)	169(35.5)	26(5.5)	23.2	0.928
Indian [31]	105(78.9)	23(17.3)	5(3.8)	12.4	0.018
Indian [32]	181(64.0)	89(31.4)	13(4.6)	20.3	0.628
Indian [33]	583(71.8)	203(25.0)	26(3.2)	15.7	0.112
Chinese [34]	81(81.8)	18(18.2)	0(0.0)	9.1	0.319
Chinese [35]	402(82.9)	80(16.5)	3(0.6)	8.9	0.648
Korean [36]	217(78.6)	54(19.6)	5(1.8)	11.6	0.448
Korean [37]	171(81.0)	40(19.0)	0(0.0)	9.5	0.128
Korean [38]	177(80.5)	41(18.6)	2(0.9)	10.2	0.824
Japanese [39]	284(79.6)	68(19.0)	5(1.4)	10.9	0.687

Data from previous studies is denoted by the citations. 4b: *NOS3* VNTR wild type allele; 4a: *NOS3* VNTR mutant allele; MAF: Minor allele frequency; HWp: Hardy-Weinberg equilibrium p value.

Table 2. Results of association tests with *NOS3* 27-bp VNTR polymorphism in cleft lip and palate.

Genotype	Control	NSCLP	OR (95%CI)	p value
4bb	92 (65.2)	107 (75.4)	Reference	
4ab	46 (32.6)	31 (21.8)	0.58 (0.34-0.99)	0.044
4aa	3 (2.1)	4 (2.8)	1.14 (0.25-5.25)	0.860
4b	230 (81.6)	245 (86.3)	Reference	
4a	52 (18.4)	39 (13.7)	0.70 (0.45-1.11)	0.127
HWp	0.315	0.349		

4b: *NOS3* VNTR wild type allele; 4a: *NOS3* VNTR mutant allele; HWp: Hardy-Weinberg equilibrium p value.

Discussion

An analysis of 7 populations has shown significant difference in allele frequencies among populations. The *NOS3* 27-bp VNTR 4 repeat allele occurs more frequently among Africans than the Europeans and other populations [14]. The current study suggests that *NOS3* 27-bp VNTR is associated with nonsyndromic cleft lip with or without cleft palate in the Indian population.

Several polymorphisms in the *NOS3* gene may be associated with reduced eNOS activity and basal NO production [15]. The 27-bp VNTR has been associated with variations in plasma levels of nitric oxide and its metabolites [16, 17]. The association between eNOS mRNA levels and the 4-repaet allele appeared to be dose-dependent [18]. The 27-bp VNTR plays a cis-acting role in *NOS3* promoter activity and inhibits eNOS expression [19]. Endothelial cells containing the 4-repeat (4a) allele produce a higher level of eNOS mRNA compared with cells containing more common 5-repeat (4b) allele [19]. Till date *NOS3* 27-bp VNTR was not studied for cleft lip and palate, but several other polymorphisms were analysed to assess the association between NSCLP and *NOS3* gene. Analysis of *NOS3* -922A>G homozygotes showed a 60 percent increased risk of cleft lip and palate but not the cleft palate only [20]. In non-Hispanic white group the interaction between *CRISPLD2 and NOS3* polymorphisms altered the transmission of *NOS3* alleles and exhibited association with NSCLP [21]. The interaction between *NOS3* and other folate gene polymorphisms in risk of NSCLP varies in Hispanic and non-Hispanic populations [22]. Unfortunately our study has not included the other polymorphism of *NOS3* genes to validate the above findings.

In conclusion, our results support the involvement of *NOS3* 27-bp VNTR polymorphisms in NSCLP. Although, limited data that available on folate and homocysteine metabolism would not support their direct role in NSCLP pathogenesis. As maternal hyperhomocysteinemia is a key risk factor in the aetiology of oral clefts and folate supplementation is the most important matter in prevention of oral clefts, it is very important to study interaction of folic acid or 5-MTHF in production of nitric oxide from endothelial nitric oxide synthase.

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Conflict of Interest: The authors declare that they have no competing interests.

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