Article

Association of VNTR 27-bp polymorphism in intron 4 of the eNOS3 gene and predisposition to Ischemic Heart Disease among Taif population in Saudi Arabia

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Abstract

Genetic variation and polymorphism became a hot spot for researches to study the link between societies and certain endemic diseases. This study is an attempt to examine the possible association of the incidence of ischemic heart disease with the genetic variations of 27-bp variable number of tandem repeats located in intron 4 of the eNOS3 gene among Taif population. A case-control study included 81 Ischemic Heart Disease (IHD) patients and 225 unrelated healthy participants from the population living in Taif City. Genotyping of the candidate sequence 27-bp repeat located in intron 4 VNTR of the eNOS3 gene in was conducted using the polymerase chain reaction technology. The minor allele (4a) was slightly less frequent among IHD patients and insignificantly linked to reduced relative risk for IHD. In addition, a significant difference in the distribution of both heterozygous genotype 4a4b between IHD patients and normal groups (p value = 0.012). Presented data suggest that the heterozygous genotype of eNOS3 gene intron 4a4b VNTR variation is might be associated with lowering the risk of IHD in the Taif population in the west of Saudi Arabia. While the minor allele (a) of the eNOS3 gene is insignificantly related to the predisposition of IHD.

Keywords Ischemic Heart Disease; VNTR 27-bp; eNOS; polymorphism; genetic variation.

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1 Introduction

Ischemic heart disease (IHD) is a medical term that describes impairment of oxygen supply to a part of the heart that causes about 30% of mortality worldwide which accounts for more than 17 million deaths and represents an 80% occurrence, especially in low-income countries (Wong, 2014). In the Kingdom of Saudi Arabia, the prevalence of ischemic heart disease IHD is about 5.5% (Aljefree and Ahmed, 2015). The term IHD describes different cardiac diseases such as myocardial infarction, stable or unstable angina and sudden cardiac death (Wong, 2014; Mocevic et al., 2015). Atherosclerosis is the major cause that leads to IHD which in turn caused by several factors such as hypertension, diabetes, tobacco smoking and hypercholesterolemia (Mocevic et al., 2015). Nowadays, several studies have shifted to understand the genetic principles that might underly the IHD. In 2007, J. Samani and his colleagues reported clear evidence for the association of genetic variation with the incidence of coronary heart disease and myocardial infarction (Samani et al., 2007). In parallel, it has clarified that there are 33 genetic variants were associated with IHD, 10 out of them were linked with lipids and hypertension (Samani et al., 2009; Dichgans et al., 2014; Martinez and Okoshi, 2018).

In another context, the physiological role of Nitric Oxide (NO) in cardiac functioning is being quite clear. NO protects the vascular system via inhibiting platelet aggregation and disrupts the adhesion between leukocyte and endothelium which in turn decreases susceptibility to atherosclerosis (Bredt and Snyder 1994; Davignon and Ganz 2004). In mammals, Nitric Oxide Synthase is the enzyme responsible for the endogenous NO production. Nowadays, three isoforms of Nitric Oxide Synthase have been identified; endothelial form (eNOS), inducible form (iNOS) and neural form (nNOS) (Davignon and Ganz, 2004). In humans, this enzyme is encoded by a gene located in chromosome 7 in the 7q35-7q36 region is called eNOS3 (Marsden et al., 1992). Several studies have been conducted on eNOS3 gene polymorphism and the association of that variation with different chronic disorders. The first study was published by Hingorani et al who reported that there is a substitution of guanine (G) with thymine (T) in the eNOS3 gene and glutamic acid was replaced by aspartic acid according to that mutation (Hingorani, 2003). G894-T mutation was reported to be associated with different chronic or acute diseases such as coronary spasm, myocardial infarction (MI) and hypertension (Colombo et al., 2002; Shimasaki et al., 1998). Nadaud and his colleagues have reported that there are two alleles in the fourth intron of the eNOS3 gene that have been designated as 4a and 4b (Nadaud et al., 1994). In these two alleles, there are four tandem repeats (27-bp) at 4a allele and five repeats at 4b allele (Nadaud et al., 1994). Other consequent studies investigated the possible link between the 4b/a polymorphism and some chronic diseases such as renal failure, diabetes, hypertension and coronary artery disease (Asakimori et al., 2001; Patkar et al., 2009; Al Fadhli, 2013; Ma et al., 2014). In Saudi Arabia particularly in the Taif region, the distribution of these two alleles is not in Hardy-Weinberg equilibrium (Alkhedaid et al., 2016). Therefore, the current study aims to examine the association between the genetic variation of the variable number of tandem repeats (VNTR) (27-bp) polymorphism the eNOS3 gene and the occurrence of ischemic heart disease among Taif population.

2 Materials and Methods

2.1 Sample collection

Whole blood specimens were extracted from 225 unrelated healthy individuals and 81 Ischemic Heart Disease (IHD) patients from people live in Taif. All participants were Saudi citizens, nonsmokers and their ages ranged between 40-95 years and the mean age was 76.87 ± 14.46 years. The procedures were approved by the Ethical and Scientific Research Committee (ESRC) at Turabah University College. All blood chemistry was assayed using Cobas600© system form Roche, Basel, Switzerland.

2.2 Genetic screening of the 27-bp repeats of the eNOS3 gene

The whole DNA was isolated from the blood specimens following the standard protocol of Sambrook et al, (Russell and David, 2001). Genotyping determination was carried out using traditional PCR amplification along the target region in the fourth intron of the eNOS3 gene and using a forward primer (5'- GCC CTATGG TAG TGC CTT -3') and reverse primer (5'-CTC TTAGTG CTG TGG TCA C -3'). Each PCR reaction sample contained 15 µl (4 µl of (60 ng/µl) genomic DNA + 7.5 µl 2X Taq complete master mix purchased from Promega, Madison, Wisconsin, United States + 0.75 µl (5 ρ moles) of each primers + 2 µl PCR grade H2O). Samples were heated for 5 minutes at 94°C and followed by 40 cycles of (94°C for 30 sec, 68°C for 30 sec and 72°C for 30 sec) with the final extension at 72°C for 3 minutes. The PCR products were investigated by electrophoresis on a 2% agarose gel stained with ethidium bromide. Both alleles 4b and 4a were identified at 420 bp and 393 bp respectively, as shown in Fig. 1.

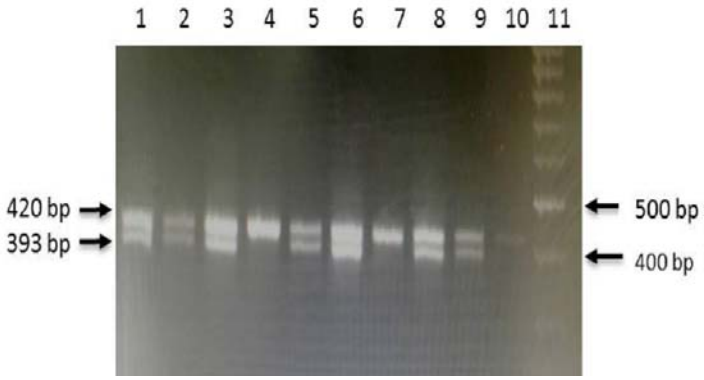

Fig. 1 The 420-bp band indicates five repeats and the 393-bp band indicates four repeats of the 27-bp consensus sequence. Lanes 1, 2, 3, 5, 6, 8 and 9 are a/b heterozygous; lanes 4, 7 and 10 are b/b homozygous. Lane 11: A 100-bp DNA marker.

2.3 Statistical analysis

Data obtained were analyzed using the Statistical Package for the Social Science software (SPSS) compatible with Windows 14. T-test was used to compare the distribution of genotypes between IHD cases and healthy individuals. Different statistical functions were applied to study different statistical concepts. The Chi-square test was applied to compare categorical data. Hardy-Weinberg equilibrium was used to study both genotypes and alleles frequencies of the 27-bp repeat polymorphism. The magnitude of association the polymorphism different genotypes (4a4a, 4a4b and 4b4b) and alleles (4a and 4b) and genetic susceptibility to IHD among participants were tested via Cochran - Armitage (CA) trend test and Odd ratios (ORs) and 95% confidence intervals (CIs) in different genetic models of inheritance were calculated. All P values of less than 0.05 were considered significant. Data were verified via repeating the PCR for at least three times**.**

3 Results

3.1 Characteristics findings of study subjects

The IHD patients ages were ranges between (40-95) years and the mean was (76.87 ± 14.46) years old. 49 (60%) are men and 32 (40%) are women, there is a significant difference between them (p-value = 0.044). The highest incidence of the onset of the disease was found among the age group (50 – 60 years). Table I shows a significant difference in serum glucose between the two examined categories in which the p-value was 0.006. Similar findings between IHD cases and controls in serum triglycerides l (p-value = 0.001) and serum

low-density lipoprotein (p-value = 0.001) while, there were no significant differences observed between IHD and normal participants in total cholesterol levels (p-value = 0.899).

Table 1 Comparison of characteristics findings in IHD patients and control groups.

Characteristics	Patients (Mean ± SD)	Controls (Mean ± SD)	p - value
AGE (Yrs)	66.87 ±13.70	62.00 ±20.20	0.095
Body weight (kg)	81.00 ±15.65	80.32 ±16.013	0.469
Body Mass Index (kg/m2)	28.90 ± 2.39	27.90 ± 5.39	0.222
Systolic BP	138.67 ± 25.43	137 ± 25.43	0.886
Diastolic BP	78.50 ± 12.39	77 ±8.2	0.431
TWBCs (cell/ dl)	8.20 ±3.00x10^3	7.29 ±2.49 x10^3	0.421
Hb (gm / dl)	13.46 ± 2.1	13.98 ±2.59	0.697
RBS (mmol/l)	11.71 ±5.44	9.82 ±3.60	0.006
Total Cholesterol Level (mmol/l)	4.21 ±1.29	4.08 ±0.88	0.899
Serum Triglycerides (mmol/l)	2.315 ±0.692	1.468 ±0.645	0.001
HDL (mmol/l)	1.170±0.380	1.265±0.380	0.599
LDL (mmol/l)	3.662 ±1.3	2.432 ±1.3	0.001

3.2 Evaluation of the link of 27-bp repeat variation in the fourth intron of the eNOS3 gene and ischemic heart disease incidence

Fig. 2 demonstrated the distribution of 27-bp repeat variation in intron 4 of the eNOS3 gene genotypes and allele frequencies of both IHD patients and healthy individuals. There was a significant difference in the distribution of both heterozygous genotype 4a4b between IHD patients and normal groups (p value = 0.012) as clearly shown in Fig. 2. The extent of the variation associated with susceptibility to IHD in the study subject was determined via relative risk and odds ratio for its penetrance in different genetic models of inheritance. Results of association and analysis show there are no association was found between eNOS3 alleles and IHD as shown in Table 2 as the 95% confidence interval for both relative risk and Odd ratio include 1. These data indicate that having a 4a allele is might be but insignificantly associated with reduced risk for IHD among the population of Taif.

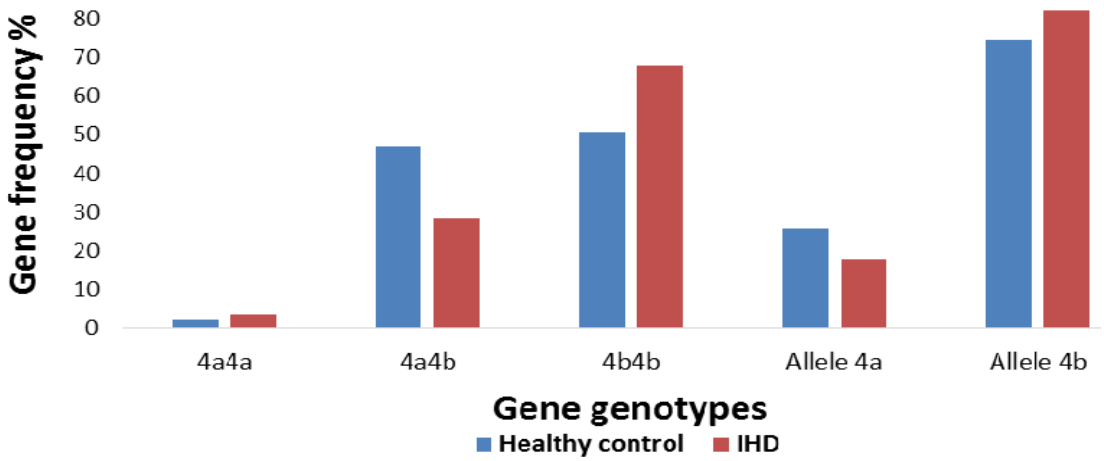

Fig. 2 The distribution of 27-bp repeat polymorphism in intron 4 of the e NOS gene genotypes (4a4a. 4a4b and 4b4b) and allele frequencies (4a and 4b) among IHD patients and healthy controls. Genotypes and alleles frequencies were expressed as percentage.

Table 2 Relative risk and odds ratio associated with eNOS3 alleles along with 95% confidence interval.

Allele	Relative Risk	Odds Ratio
a	0.95 (0.80, 1.14)	0.90 (0.60, 1.34)
b	1.05 (0.88, 1.25)	1.11 (0.75, 1.66)

4 Discussion

Ischemic heart disease is one of life threading chronic diseases over the world. The ischemic heart disease incidence differs between different races or ethnic groups and the frequencies of eNOS3 gene variations have been reported to vary in different populations (Matyar et al., 2005; Jemaa et al., 2009; Ichihara et al., 1998; Angeline et al., 2010). In Saudi Arabia is one of the major killer diseases which are mainly caused by diet and lifestyle.

Studying eNOS3 gene polymorphism is one of the genetic investigations that conducted to understand the association of that variation with IHD. The relationship between 4a allele and susceptibility to CAD was first reported by Wang and his colleagues (Wang et al., 1999). In Australia, they confirmed that its rule consistent with the recessive model of inheritance (Wang et al., 1999). It had been reported that the distribution of alleles and genotypes of VNTR 27-bp variation in intron 4 of the eNOS3 gene was not in Hardy-Weinberg equilibrium in the Saudi population from Taif region which means that the genetic variation and allele frequencies are not inheritance constantly as reported in a study published in 2016 (Alkhedaid et al., 2016). Herein, we investigated the rule of eNOS3 gene intron 4a4b VNTR polymorphism in predisposition to IHD among the Taif population. Data showed that the heterozygous genotypes and the minor allele (4a) might be associated with a reduced relative risk for IHD under both the dominant model and basic allelic as shown in Table 2 and Fig. 2. Several previous studies demonstrated the association between 4a/4b genotype and CAD (Angeline et al., 2010, Samani et al., 2007; Colombo et al., 2002; Ichihara et al., 1998; Ma et al., 2014). A significant association was reported between 4a allele of the NOS3 4a/4b gene polymorphism and

susceptibility to IHD in the Tunisian, Japanese and European populations (Jemaa et al., 2009; Ichihara et al. 1998; Gardemann et al., 2002; Casas et al., 2006). Other studies did not detect any link of the NOS3 4a/4b gene polymorphism and susceptibility to IHD in Taiwanese and German populations (Hwang et al., 2002; Gardemann et al., 2002).

The discrepancy reports in the rule of eNOS gene intron 4a4b VNTR polymorphism and predisposition to IHD from different populations may be attributed to differences in genetic background and gene-environment interactions among various populations (Gardemann et al., 2002; Yang et al., 2014; Abolhalaj et al., 2013). Therefore, our results cannot be considered contradictory to some of the previous studies from other populations. From all of these observations, it seems the link between eNOS3 intron 4b/a VNTR variation with CAD still not fully explained. However, the insignificant protective effect of the minor allele (a) of this polymorphism in this study might be due to a direct effect of another genetic variation in the vicinity of the gene that is linked to the minor allele as well as might be attributed to the small size of the investigated sample.

5 Conclusion

Our data concluded that the heterozygous genotype of eNOS3 gene intron 4a4b VNTR variation is might be associated with lowering the risk of IHD in the Taif population in the west of Saudi Arabia. While the minor allele (a) of the eNOS3 gene is insignificantly related to the predisposition of IHD. These data require further investigations with larger sample size.

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References

Abolhalaj M, Amoli MM, Amiri P. 2013. eNOS gene variant in patients with coronary artery disease. Journal of Biomarkers, 403783-403783

AlFadhli S. 2013. Influence of endothelial nitric oxide synthase gene intron-4 27bp repeat polymorphism on its expression in autoimmune diseases. Disease Markers, 34(5): 349-356

Aljefree N, Ahmed F. 2015. Prevalence of cardiovascular disease and associated risk factors among adult population in the Gulf region: a systematic review. Advances in Public Health, 2015: 105

Alkhedaid AQH, Merghani A, Ismael T. 2016. Distribution of endothelial nitric oxide synthase gene 27 bp-VNTR polymorphism in Turabah Region, Saudi Arabia. Imperial Journal of Interdisciplinary Research, 2: 595-599

Angeline T, Isabel W, Tsongalis GJ. 2010. Endothelial nitric oxide gene polymorphisms, nitric oxide production and coronary artery disease risk in a South Indian population. Experimental and Molecular Pathology, 89(3): 205-208

Asakimori Y, Yorioka N, Yamamoto I, Okumoto S, Doi S, Hirai T, Taniguchi Y. 2001. Endothelial nitric oxide synthase intron 4 polymorphism influences the progression of renal disease. Nephron, 89(2): 219-223

Bredt DS, Snyder SH. 1994. NITRIC OXIDE: A physiologic messenger molecule. Annual Review of Biochemistry, 63(1): 175-195

Casas JP, Cavalleri GL, Bautista LE, Smeeth L, Humphries SE, Hingorani AD. 2006. Endothelial nitric oxide synthase gene polymorphisms and cardiovascular disease: A HuGE Review. American Journal of Epidemiology, 164(10): 921-935

Colombo MG, Andreassi MG, Paradossi U, Botto N, Manfredi S, Masetti S, Rossi G, Clerico A, Biagini A. 2002. Evidence for association of a common variant of the endothelial nitric oxide synthase gene (Glu298-->Asp polymorphism) to the presence, extent, and severity of coronary artery disease. Heart, 87(6): 525-528

Davignon J, Ganz P. 2004. Role of Endothelial Dysfunction in Atherosclerosis. Circulation, 109(23_suppl_1): III-27-III-32

Dichgans M, Malik R, Konig IR, Rosand J, Clarke R, Gretarsdottir S, et al. 2014. Shared genetic susceptibility to ischemic stroke and coronary artery disease: a genome-wide analysis of common variants. Stroke, 45(1): 24-36

Gardemann A, Lohre J, Cayci S, Katz N, Tillmanns H, Haberbosch W. 2002. The T allele of the missense Glu298Asp endothelial nitric oxide synthase gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerotic risk profile. Atherosclerosis, 160(1): 167-175

Hingorani AD. 2003. Endothelial nitric oxide synthase polymorphisms and hypertension. Current Hypertension Reports, 5(1): 19-25

Hwang JJ, Tsai CT, Yeh HM, Chiang FT, Hsu KL, Tseng CD, Liau CS, Tseng YZ, Lai LP. 2002. The 27-bp tandem repeat polymorphism in intron 4 of the endothelial nitric oxide synthase gene is not associated with coronary artery disease in a hospital-based Taiwanese population. Cardiology, 97(2): 67-72

Ichihara S,Yamada Y, Fujimura T, Nakashima N, Yokota M. 1998. Association of a polymorphism of the endothelial constitutive nitric oxide synthase gene with myocardial infarction in the Japanese population. American Journal of Cardiology, 81(1): 83-86

Jemaa R, Ali SB, Kallel A, Feki M, Elasmi M, Taieb SH, Sanhaji H, Omar S, Kaabachi N. 2009. Association of a 27-bp repeat polymorphism in intron 4 of endothelial constitutive nitric oxide synthase gene with hypertension in a Tunisian population. Clinical Biochemistry, 42(9): 852-856

Ma ZJ, Chen R, Ren HZ, Guo X, Chen JG, Chen LM. 2014. Endothelial nitric oxide synthase (eNOS) 4b/a polymorphism and the risk of diabetic nephropathy in type 2 diabetes mellitus: A meta-analysis. Meta Gene, 2: 50-62

Ma ZJ, Chen R, Ren HZ, Guo X, Guo J, Chen LM. 2014. Association between eNOS 4b/a polymorphism and the risk of diabetic retinopathy in type 2 diabetes mellitus: a meta-analysis. Journal of Diabetes Research, 2014(3): 549747

Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, Michel T. 1992. Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Letters, 307(3): 287-293

Martinez PF, Okoshi MP. 2018. Genetic risk in coronary artery disease. Arquivos brasileiros de cardiologia, 111(1): 62-63

Matyar S, Attila G, Acarturk E, Akpinar O, Inal T. 2005. eNOS gene intron 4 a/b VNTR polymorphism is a risk factor for coronary artery disease in Southern Turkey. Clinica Chimica Acta, 354(1-2): 153-158

Mocevic E, Kristiansen P, Bonde JP. 2015. Risk of ischemic heart disease following occupational exposure to welding fumes: a systematic review with meta-analysis. International Archives of Occupational and Environmental Health, 88(3): 259-272

Nadaud S, Bonnardeaux A, Lathrop M, Soubrier F. 1994. Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. Biochemical and Biophysical Research Communications, 198(3): 1027-1033

Patkar S, Charita B, Ramesh C, Padma T. 2009. High risk of essential hypertension in males with intron 4 VNTR polymorphism of eNOS gene. Indian Journal of Human Genetics, 15(2): 49

Russell J, David W. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, USA

Samani NJ, Deloukas P, Erdmann J, Hengstenberg C, Kuulasmaa K, McGinnis R, et al. 2009. Large scale association analysis of novel genetic loci for coronary artery disease. Arteriosclerosis, Thrombosis, and Vascular Biology, 29(5): 774-780

Samani NJ, Erdmann J, Hall AS, Hengstenberg C, Mangino M, Mayer B, Dixon RJ, et al. 2007. Genomewide association analysis of coronary artery disease. New England Journal of Medicine, 357(5): 443-453

Shimasaki Y, Yasue H, Yoshimura M, Nakayama M, Kugiyama K, Ogawa H, Harada E, et al. 1998. Association of the missense Glu298Asp variant of the endothelial nitric oxide synthase gene with myocardial infarction. Journal of American College of Cardiology, 31(7): 1506-1510

Wang Y, Kikuchi S, Suzuki H, Nagase S, Koyama A. 1999. Endothelial nitric oxide synthase gene polymorphism in intron 4 affects the progression of renal failure in non-diabetic renal diseases. Nephrology Dialysis Transplantation, 14(12): 2898-2902

Wong ND. 2014. Epidemiological studies of CHD and the evolution of preventive cardiology. Nature Reviews Cardiology, 11(5): 276-289

Yang Y, Du K, Liu Z, Lu X. 2014. Endothelial nitric oxide synthase (eNOS) 4b/a gene polymorphisms and coronary artery disease: evidence from a meta-analysis. International Journal of Molecular Sciences, 15(5): 7987-8003