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Association of endothelial nitric oxide synthase (eNOS) gene G894T polymorphism with hypertension risk and complications

Abeer A. ALrefai[1] · Mona Salah El-din Habib[1] · Rehab I. Yaseen[2] · Mahmoud K. Gabr[2] · Rabab M. Habeeb[3]

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Abstract This study evaluated the association of NOS3 polymorphisms with hypertension risk and complications. eNOS (G894T) SNP was performed by RT-PCR on 70 hypertensive patients (25 were hypertensive, 25 were hypertensive with CAD, and 20 were diabetic with hypertension) and 30 age- and gender-matched individuals. Lipid and glucose profile were assessed by standard colorimetric assay. Our results revealed that combination of (GT + TT) genotype and T allele significantly increases the risk of hypertension (OR = 3.86 and 4.33), respectively. Subgroup analysis showed significant association between CAD with eNOS (G894T) mutant genotype ($P = 0.002$) and allele frequency ($P < 0.001$). Moreover, the mutant homozygous and heterozygous eNOS genotype together were significantly associated with higher TC, LDLc, ($P < 0.001$), and TG ($P = 0.001$). Thus, hypercholesterolemia ($P < 0.001$ and OR = 12.48) increases the risk of hypertension among T carrier. These results indicated that the T carriers significantly increase hypertension risk and complication (CAD), mainly with hypercholesterolemia and in elderly.

Keywords *eNOS* (G894T) · Hypertension · CVD

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✉ Abeer A. ALrefai
drabeer1975@hotmail.com

[1] Department of Medical Biochemistry, Faculty of Medicine, Menoufia University, Shebeen El-Kom, Egypt

[2] Departments of Cardiology, Faculty of Medicine, Menoufia University, Shebeen El-Kom, Egypt

[3] Departments of Anesthesia, Faculty of Medicine, Menoufia University, Shebeen El-Kom, Egypt

Introduction

Hypertension is a worldwide health challenge as a result of its high prevalence and concomitant risks of cerebrovascular accident and cardiovascular disease [1]. The pathogenesis of hypertension is multifactorial, influenced by numerous genetic, environmental factors, their interaction, and biological systems [2]. The vascular endothelium regulates the vascular tone, structure, and function through release of active or signaling agents [3]. In particular, the vasodilator effect of endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) is the most imperative activities for maintenance of vascular function [4]. Accumulating evidences showed that NO assumed an important role in the control of BP as hypertension is the most obvious phenotype in eNOS-deficient mice (eNOS-KO), proposing that other physiologic systems regulating BP cannot compensate for eNOS deficiency [5]. NO is quite related with lipid metabolism, and it may take part in the regulatory effects of blood lipid on blood pressure [6]. In addition, variable studies have reported that NO plays an important protective role against the onset and progression of cardiovascular disease through inhibition of smooth muscle proliferation, migration, platelet adhesion and aggregation, lastly LDL oxidation [7]. The association between eNOS polymorphism with reduced eNOS expression, activity and subsequently reduced NO production has been reported [8]. Among these polymorphisms, the G894T polymorphism (Glu298Asp or rs1799983) at exon 7 of the eNOS gene, influencing NOS3 expression or activity, could be a potential candidate marker for hypertension development [9]. But, the relationship between eNOS (Glu298Asp) variant and hypertension risk among Asp 298 carriers remains unclear in various populations [10, 11]. This controversy opens the field for conducting further studies. Thus, we investigated the interaction

of NOS3 polymorphisms with hypertension conventional risk factors and complications.

Subjects and methods

Subjects: A total of 70 hypertensive patients, selected among individuals referred to Internal Medicine department and Cardiac Catheterization Unit of Cardiology Department in Menoufia University Hospital, were enrolled in this case–control study. These patients were known to have long-standing hypertension [systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg in at least two separate measurements or regular use of antihypertensive medication] [12]. Hypertensive patients were sub-gathered by associated comorbidity; 25 (35.7 %) of them had angiographically proven CAD (>50 % stenosis affecting at least one coronary vessel) [11], 20 (28.6 %) were diabetics, and 25 (35.7 %) of them were hypertensive without complications (essential hypertension EH). Type 2 diabetes mellitus is defined as a fasting plasma glucose (FBG) ≥126 mg/dL, a 2-h postprandial glucose (2HPPG) ≥200, HbA1C ≥6.5 %, or random plasma glucose ≥200 mg/dL in a patient with classic symptoms of hyperglycemia [13]. Age- and gender-matched apparently healthy individuals [n = 30] who remained free of hypertension were also enrolled in the study as a control group. Hypertensive patients with systemic disease, cancer, bleeding disorders, and acute disease (including infections) were excluded from this study. Ethical approval for this research was obtained from the Research Ethics Committee, Faculty of Medicine, Menoufia University and informed consent was obtained from all participants. A thorough medical and demographic history (Hypertension, diabetes mellitus, cardiovascular diseases and smoking status) was obtained from all participants. Blood pressure was measured while the subjects were at rest. Venous blood samples were collected after an overnight fasting for determination of lipid panel [serum total cholesterol (TC), triglycerides (TG), HDL-C levels] and blood glucose profile [Fasting blood glucose and 2-h postprandial glucose], by the standard enzymatic colorimetric kits (SPINREACT, Spain) [14, 15]. Glycemic control was assessed by glycosylated hemoglobin (HbA1c) utilizing quantitative colorimetric measurement kits as a percentage of total hemoglobin supplied by Teco Diagnostics, Lakeview Ave, Anaheim, CA, USA [16]. LDLc was calculated by Friedewal formula [17].

DNA extraction and genotyping

Genomic DNA was extracted and purified from whole blood (collected in EDTA tube) utilizing QIAamp DNA Mini Kit (QIAGEN, USA, 2012) [18] according to the manufacturer's protocol, and quantified by a spectrophotometer. Extracted DNA samples were stored at—20 till analysis. Genotyping of eNOS3 Glu298Asp (rs1799983) single nucleotide polymorphism (SNP) was carried out by real-time PCR (RT-PCR) with allele discrimination using TaqMan®SNP Genotyping assay kit on applied biosystem 7500 real-time PCR with software version: 2.0.1, supplied by (Applied Biosystem, USA, 2012). During TaqMan SNP Genotyping Assay experiment, DNA polymerase from the TaqMan Universal PCR Master Mixture amplifies target DNA using sequence-specific primers supplied with the kit. The two TaqMan fluorogenic minor groove binder probes provide a fluorescence signal for allelic discrimination [19] utilizing the dyes 6-carboxyfluorescein (FAM; excitation, 494 nm) and (VIC; excitation, 538 nm) which are easily differentiated in RT-PCR system. The reaction mixture (25 µl total volume per single well reaction) containing 12.5 µl of TaqMan 2× universal master mix (Applied Biosystems, USA, 2012), 1.25 µl of 20× SNP genotyping assay [which contains forward and reverse sequences of primers and TaqMan Probe (VIC/FAM) dye mix], [CCCTGCTGCTGCAGGCCCCAGATGA[G/T]CCCCCAGAACTCTTCCTTCTGCCCC], 10.25 µl of RNase- and DNase-free water, and 1 µl of DNA template. DNase-free water (negative control) was included in each assay run. Briefly, the cycling conditions for eNOS G894T polymorphism detection include 10 min of pre-denaturation (AmpliTaq Gold® DNA polymerase activation), followed by 50 cycles with a fast denaturation at 95 °C for 15 s, annealing of the TaqMan MGB probes to its complementary sequence, and extension of the primers by AmpliTaq Gold® DNA polymerase for 1 min at 60 °C. After assay completion, we read the 96-well PCR plate in Applied Biosystems 7500 Real-Time PCR System with endpoint analysis mode of the SDS v1.3.1, which uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample and then convert allele calls to genotypes [VIC® dye detects the Allele 1 sequence and FAM™ dye detects the Allele 2 sequence].

Statistical analysis

Results were statistically analyzed by SPSS version 16. Student's *t* test and F (ANOVA) were used for parametric data. Mann–Whitney was used for nonparametric data. Chi-Squared (χ^2) and fisher tests were used for qualitative variables. *P* value <0.05 is considered significant.

Results

Demographic, clinical, and biochemical data of the study population are summarized in Table 1. A total of 70 hypertensive patients [36 (51.4 %) males; 34(48.6 %) females]

and 30 healthy control individuals [16 (63.3 %) males and 14 (46.7 %) females] were involved in this study. Their mean ages were 54.07 ± 7.64 and 52.46 ± 5.17 years for hypertensive and control groups, respectively, with no significant difference regarding age ($P = 0.296$) and gender (0.861) among studied groups (Table 1). Of hypertensive patients, 35.7 % were proven to have CAD [mean age was (53.68 ± 8.13)] and 28.6 % were diabetics [mean age (53.65 ± 8.86)]. Demographic and clinical data regarding smoking status [30 % vs. 3.3 % ($P = 0.01$)], systolic (146.79 ± 15.22 vs. 114.67 ± 7.76), and diastolic blood pressure (90.86 ± 10.49 vs. 73.66 ± 6.68) ($P < 0.001$) were significantly higher in hypertensive patients compared to control group (Table 1). Similarly, subgroup analysis of hypertensive patients revealed a significant difference regarding smoking status [24, 52, and 15 % ($P = 0.007$)] among them (not shown). Moreover, TC (191.03 ± 27.22 vs. 159.47 ± 6.11), TG (149.92 ± 59.04 vs. 93.30 ± 10.06), HDLc (35.19 ± 4.56 vs. 41.08 ± 2.74), and LDLc (124.35 ± 21.9 vs. 104.44 ± 9.83) were significantly higher in hypertensive group (Table 1) and subgroups (not shown) compared to control group ($P < 0.001$). Additionally, fasting blood glucose (105.99 ± 33.57 vs. 84.26 ± 7.35) 2-PPBG (139.89 ± 76.16 vs. 89.90 ± 6.96) were significantly higher in hypertensive group compared to control group ($P < 0.001$), while both hypertensive and control groups exhibited nonsignificant difference regarding HbA1C ($P = 0.38$) (Table 1). In this study, the association of eNOS (G894T) polymorphism with hypertension was analyzed by comparing hypertensive patients with normotensive volunteers, Table 2. Genotypic distribution of G894T was 70.0 % GG, 22.9.0 % GT, and 7.1 % TT for hypertensive cases and 90.0 % GG, 10.0 % GT, and 0.0 % TT for control, revealing nonsignificant association with hypertension [GG ($P = 0.058$), (GT) ($P = 0.219$), and (TT) ($P = 0.319$)] or in hypertension without diabetes ($P = 0.167$), while the combination of both heterozygous and mutant homozygous genotype (GT + TT) increases the risk of hypertension [OR = 3.86 (95 % CI = 1.05 ~ 14 & $P = 0.032$). The G allele of the rs1799983 (G > T) (G894T) SNP was significantly less common in hypertensive patients than in controls (5 vs.95 %), while the T allele of this SNP was significantly more common in hypertensive patients than in controls (81.4 vs. 18.6 %) ($P = 0.012$). This finding suggested that G allele was protective, while individuals with T allele were at high risk of hypertension (OR = 4.33; 95 % CI = 1.26–14.29) (Table 2). Our results also showed a significant association of [GT (40 %) + TT (12 %) genotype] ($P = 0.002$) and *T* (32 %) allele ($P < 0.001$) frequencies with CAD accompanying hypertensive group but not with diabetes subgroup ($P = 0.167$) (Table 3). Also, DM accompanying hypertension had no significant association with mutant genotype or allele frequency when compared to control group ($P = 0.167$) (Table 3). Thus GT + TT

Table 1 Demographic, Clinical, and Biochemical data of the studied groups

	Groups				Test	*P* value
	Patients ($N = 70$)		Controls ($N = 30$)			
Age (Y) X ± SD	54.07 ± 7.64		52.46 ± 5.17		$t = 1.05$	0.296
Sex: no %						
Male	36	51.4	16	53.3	$\chi^2 = 0.03$	0.861
Female	34	48.6	14	46.7		
Smoking: no %						
Smoker	21	30.0	1	3.3	*$\chi^2 = 9.30$*	*0.010(S)*
Ex-smoker	8	11.4	3	10.0		
Nonsmoker	41	58.6	26	86.7		
Comorbidity						
CAD	25	35.7	0	0.0	–	–
Diabetes mellitus	20	28.6	0	0.0		
SBP (mmHg) X ± SD	146.79 ± 15.22		114.67 ± 7.76		$t = 13.92$	$<0.001^{(HS)}$
DBP (mmHg) X ± SD	90.86 ± 10.49		73.66 ± 6.68		$t = 9.82$	$<0.001^{(HS)}$
FBG (mg/dl) X ± SD	105.99 ± 33.57		84.26 ± 7.35		$t = 5.13$	$<0.001^{(HS)}$
PPBG(mg/dl) X ± SD	139.89 ± 76.16		89.90 ± 6.96		3.59°	$<0.001^{(HS)}$
HbA1c % X ± SD	5.91 ± 2.62		4.70 ± 0.15		0.87°	0.381
TC(mg/dl) X ± SD	191.03 ± 27.22		159.47 ± 6.11		$t = 9.17$	$<0.001^{(HS)}$
TG(mg/dl) X ± SD	149.92 ± 59.04		93.30 ± 10.06		5.16°	$<0.001^{(HS)}$
LDL(mg/dl) X ± SD	124.35 ± 21.9		104.44 ± 9.83		$t = 6.27$	$<0.001^{(HS)}$
HDL(mg/dl) X ± SD	35.19 ± 4.56		41.08 ± 2.74		$t = 6.56$	$<0.001^{(HS)}$

° Mann whitney test

Table 2 eNOS gene polymorphism data of the studied patients and controls

	Groups				Test	*P* value	Odds ratio
	Patients ($N = 70$)		Controls ($N = 30$)				
	No	%	No	%			
Genotype					*Z*		
GG	49	70.0	27	90.0	1.89	0.058	–
GT	16	22.9	3	10.0	1.23	0.219	TT + GT
TT	5	7.1	0	0.0	1.0	0.319	3.86 (1.05–14.12)
G	114	81.4	57	95.0		χ^2	
T	26	18.6	3	5.0	6.24	0.012 (*S*)	4.33(1.26–14.92)

Table 3 eNOS gene polymorphism data of the studied hypertensive subgroups and controls

	Patients						Controls (II) ($N = 30$)		χ^2 test	*P* value	OR (CI 95 %)
	Htn (Ia) ($N = 25$)		Htn + CAD(Ib) ($N = 25$)		Htn + DM(Ic) ($N = 20$)						
	No	%	No	%	No	%	No	%			
Genotype											9.75
GG	22	88.0	12	48.0	15	75.0	27	90.0	Ia vs. II = 0.06•	1.0	(2.33−40.65)
GT	3	12.0	10	40.0	3	15.0	3	10.0	Ib vs. II = 12.18	0.002 (S)	
TT	0	0.0	3	12.0	2	10.0	0	0.0	Ic vs. II = 3.57	0.167	
Alleles									Ia vs. II = 0.05•	1.0	8.94
G	47	94.0	34	68.0	33	82.5	57	95.0	Ib vs. II = 13.91	<0.001 (HS)	(2.42−32.94)
T	3	6.0	16	32.0	7	17.5	3	5.0	Ic vs. II = 3.17•	0.084	

• Fisher exact test

genotype was a potential risk factor for CAD [OR = 5.01 (95 % CI 1.68–14.98)] among hypertensive patients (Table 4). The effects of mutant genotypes (GT + TT vs. GG) on some clinical and biochemical parameters were evaluated in the hypertensive patients (Table 5) and hypertensive subgroups (not shown). The mutant G894T genotypes were found to have an increasing effect on TC, LDLc, and TG ($P = 0.001$) with nonsignificant effect on HDLc ($P = 0.068$) among hypertensive patients (Table 5). The mutant G894T genotypes significantly associated with low HbA_{1C} level as compared to wild genotype ($P = 0.025$) (Table 5). Thus, the risk ratio of GT + TT against GG for the increased incidence of hypertension was 3.86 (95 % CI 1.05–14.12) in all hypertensive patients and was 12.48 (95 % CI 3.68–42.33) in hypercholestromic patients (Table 6). Also, GT + TT genotype was a potential risk factor for hypertension among elderly (Table 6).

Discussion

Hypertension, a major independent risk factor for CAD, is influenced by numerous genetic and environmental factors, lifestyle, and their interactions [2]. In addition, hypertension is commonly associated with other cardiovascular risk factors "obesity, diabetes, and dyslipidemia" [20]. The presence of these cardiovascular risk factors and the resulting endothelial dysfunction may play a role in the pathophysiology of hypertension [21]. Accumulating evidence suggested an association between blood pressure (BP) levels and gene regulatory mechanisms at the NOS3 loci, providing new insights on the prediction, progression, and severity of the disease [8]. Several polymorphisms in the eNOS gene have been identified, and much attention has been focused on G894T with a controversial variant on its association with blood pressure, lipid profile, and blood glucose in different ethnic groups [10, 11]. Therefore, in this study, we analyzed the association of *NOS3* (Glu298Asp) polymorphisms with hypertension risk, its conventional risk factors, and complications. In our study, the genotypic distribution of G894T of GG, GT, and TT was 70.0 %, 22.9.0 %, and 7.1 %, respectively, for hypertensive cases and 90.0, 10.0, and 0.0 % TT for control, revealing a nonsignificant association with hypertension. However, the combination of both heterozygous and mutant homozygous genotype (GT + TT) and T allele frequency was significantly prevalent in hypertensive compared to control group ($P = 0.032$), and the risk ratio of GT + TT for increased incidence of hypertension was 3.86 (1.05–14.12). Srivastava et al. found higher prevalence of GT + TT genotypes

Table 4 eNOS gene polymorphism data of the studied hypertensive patients with and without complications

	All patients				Test	*P* value	OR (CI 95 %)
	GG ($N = 49$)		GT + TT ($N = 21$)				
	No	%	No	%			
CAD							5.01
Yes	12	24.5	13	61.9	χ^2	0.002	(1.68–14.98)
No	37	75.5	8	38.1	8.96	(S)	–
Diabetes mellitus							
Yes	15	30.6	5	23.8	χ^2	0.563	
No	34	69.4	16	76.2	0.33		

Table 5 The effect of human eNOS E298D genotypes on clinical and biochemical parameters among all patients

Variables	eNOS gene among all patients		*t* test	*P* value
	GG ($N = 49$) Mean ± SD	GT + TT ($N = 21$) Mean ± SD		
SBP	145.41 ± 14.64	150.0 ± 16.43	1.15	0.251
DBP	90.10 ± 10.33	92.61 ± 10.91	0.91	0.362
FBG	105.24 ± 33.69	107.71 ± 34.05	0.28	0.780
PPBG	141.37 ± 79.53	136.43 ± 69.36	1.71°	0.086
HbA1c	6.15 ± 2.68	5.34 ± 2.46	2.23°	0.025
TC	180.47 ± 20.84	215.67 ± 24.55	5.74	<0.001(HS)
TG	132.29 ± 44.48	191.04 ± 68.86	3.36°	0.001(S)
LDL	116.65 ± 19.14	142.3 ± 17.08	5.29	<0.001(HS)
HDL	35.66 ± 5.29	34.09 ± 1.70	1.85	0.068

° Mann Whitney test

Table 6 Potential risk factors related to genetic polymorphism among hypertensive patients

Variables	All patients				Test	*P* value	OR (CI 95 %)
	GG (N = 49)		GT + TT ($N = 21$)				
	No	%	No	%			
Age					Fisher's exact = 5.0	0.027(S)	–
>45	39	79.6	21	53.3			
≤45	10	20.4	0	46.7			
Sex:					$\chi^2 = 0.03$	0.875	0.51 (0.19–0.39)
Male	27	55.1	12	57.1			
Female	22	44.9	9	42.9			
Smoking: no, %					χ^2	0.403	1.74 (0.62–4.87)
Smoker	13	26.5	9	42.9	1.81		
Ex-smoker	6	12.2	2	9.5			
Nonsmoker	30	61.2	10	47.6			
Hypercholesterolemia					χ^2	<0.001(HS)	12.48 (3.679–42.33)
Yes	10	20.4	16	76.2	19.59		
No	39	79.6	5	23.8			

in eHT patients as compared to the controls [22], agreeing with our results. Additionally, Alkharfy et al. revealed that the GT + TT genotype of 894G > T polymorphism was significantly associated with pre-hypertension status in Saudi population [23]. The correlation between the T allele of 894G > T SNP and hypertension risk confirms previous

findings from analyses performed in Saudi and Japanese subjects [23, 24]. A comprehensive meta-analysis by Niu and Qi ascertained that the comparison of allele "894T" to "894G" yielded "16 % increased risk for hypertension (odds ratio [OR] = 1.16) and particularly a 32 % increased risk for hypertension in Asians and a 40 % increased risk in Chinese," suggesting a heterogeneous association of G894T polymorphism in ethnicity-specific populations [25]. Also, Men et al. demonstrated a significant and independent association between a eNOS-G894T polymorphism and EH in the Chinese patients [26]. Periaswamy et al. reported that eNOS Glu298Asp polymorphism is associated with hypertension, particularly in hypertensive female when compared to their hypertensive male cases (Asp allele 22 vs. 16 %) [27]. However, a broad age range of Caucasian-based cohort study revealed that eNOS G894T polymorphism is neither associated with prevalent hypertension nor with systolic or diastolic BP [28]. Also, lack of association of the G894T polymorphism with hypertension status or blood pressure levels was reported in Malaysian and Tunisian population [29, 30]. Moreover, Sandrim et al. reported a nonsignificant difference in eNOS genotype [(T786C) (Glu298Asp)] and allele distribution among HT patients with or without T2DM [31]. The effects of eNOS with hypertension are studied in Turkish patients by Kayhan et al. who reported that the eNOS G894T polymorphism may increase the risk of hypertension when associated with high serum total-cholesterol levels [11]. In patients with hypercholesterolemia, long-term existence of LDL in the circulatory system leads to its increase in oxidation, resulting in blood pressure elevation and simultaneously suppressing the expression of eNOS. Hypercholesterolemia also causes the reduction of NO by producing oxygen free radicals [32]. The current study demonstrated that the GT + TT genotypes of 894G > T SNP increased the hypertension risk [OR = 12.48 (3.679–42.33)] with hypercholesterolemia. These data coincided with other studies which demonstrated that the GA/AA genotypes of the Glu298Asp polymorphism may increase the risk of hypertension when associated with hypercholesterolemia [32]. Mc-Enery has reported that age could modulate genetic effects in blood pressure regulation and susceptibility to HT [33]. Similarly, our results revealed a significant association of GT + TT with increased hypertension risk among elderly (>45Y). Additionally, long-term administration of atorvastatin improves age-related endothelial dysfunction in aged rats via normalization of eNOS/iNOS imbalance [34]. Besides, established risk factors, genetic factors may have an important role in the pathogenesis of coronary atherosclerosis. Given the major role played by NO in the cardiovascular system and the effects of NOS3 polymorphisms on NOS3 expression, activity, and circulating levels of markers of endogenous NO formation, numerous studies evaluated the effect of these polymorphisms in cardiovascular diseases. Patients with the rs1799983 variant were more likely to have coronary artery disease or stroke which is consistent with our result [35]. Numerous association studies have investigated the relation between the eNOS G894T polymorphism and the risk of coronary artery disease (CAD), MI, coronary spasms, and hypertension in different populations with controversial results. Meta-analysis study by Zhang et al. [36] and Luo et al. [37] reported that eNOS 894 G/T polymorphism may play an important role in CHD development and is associated with increased MI risks among Asia population. Abdel-Aziz et al. [38] concluded that the TT genotype of Glu298Asp polymorphism of eNOS gene is an independent risk factor of premature coronary artery diseases (PCAD) and its association with established risk factors such as smoking, obesity, dyslipidemia, and/or metabolic syndrome increased the risk of the development of PCAD in Egyptians population. However, Abdelhedi et al. [39] found no association of ACE4 and ACE-I/D polymorphism in the ACE gene, T786C, and Glu298Asp in the NOS3 gene with CAD among the Tunisian population, persisted after adjusting for several potential confounding factors. Similarly, Taqddus A et al. [40] Jaramillo et al. [41] and Ragia et al. [42] reported no association of the eNOS gene polymorphisms with coronary artery disease in subjects from Multan-Pakistan, South Chilean, and Greece.

Conclusions

These results indicated that the T carriers [(GT + TT combination) and T allele] of Glu298Asp (rs1799983) polymorphism of eNOS3 are significantly associated with hypertension a major risk factor of atherosclerotic diseases (cardiovascular diseases), mainly with hypercholesterolemia and advanced age. However, further large sample studies with a broad range of age are necessary for confirming the association of the Enos Glu298Asp (rs1799983) with increased hypertension risk and complications differentiating pulmonary hypertension from other hypertensives which are not associated with CAD or diabetes. In addition, skewed smoking samples selection need to be avoided by extensive studies.

Authors' contribution Alrefai A and Habib M researched literature and conceived the study. Yaseen R, Habib M and Habeeb R gained ethical approval, and contributed to data collection and analysis. Alrefai A wrote the first draft of the manuscript. ALrefai A and Habib M reviewed and edited the manuscript and all authors approved the final version of this manuscript.

Compliance with ethical standards

Conflict of interests No conflicts of interests.

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