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Endothelial Nitric Oxide Synthase Gene Polymorphism and Coronary Artery Disease in Asian Populations

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Background and Objectives: Glu298Asp endothelial Nitric Oxide Synthase (eNOS) gene polymorphism results in a reduced bioavailability of Nitric Oxide (NO) and prone to atherosclerosis. Even though there were more than 15 studies conducted on Asian populations investigating the role of this polymorphism in Coronary Artery Disease (CAD), however; the representative data from Malaysia still not clear. The aim of this study was to assess the association between Glu298Asp polymorphism and CAD by conducting a cross-sectional comparative study; in addition to a meta-analytic approach of review to overcome the contradicting evidence arising from conflicting studies in East Asia and South East Asia. **Methodology:** The study included 185 CAD patients and 188 control participants. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was used to identify the genotype of the study participants. The results were confirmed by nucleotide sequencing of selected genotypes and the association with CAD was tested using SPSS version 19 with Chi-square test. Execution of meta-analysis involved 23 Asian case-control studies using Revman meta-analysis software version 5.3. **Results:** The study has demonstrated a significant association between Glu298Asp genotype variants and CAD patients ($p<0.001$). Asp allele was a significant predictor for CAD ([$p = 0.001$, OR = 2.186 (1.53-3.125; CI = 95%)]. Through meta-analysis the combined cohort data showed significant Glu298Asp risk to CAD [$p<0.0001$, OR = 2.41 (1.61-3.59); Heterogeneity test ($I^2 = 10\%$, $p = 0.33$)]. Conclusion: It was concluded that homozygous Asp genotype of Glu298Asp polymorphism potentiates a risk for CAD.

Key words: Endothelial nitric oxide synthase, coronary artery disease, single nucleotide polymorphism, Glu298Asp eNOS, meta-analysis

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INTRODUCTION

Coronary Artery Disease (CAD) is the most common type of cardiovascular diseases[1]. It is the leading cause of death worldwide with an anticipated growing figure to reach 11.1 million deaths in 2020[2]. This disease has a multifactorial etiology including environment, physiological and genetic factors[3]. Among the genetic factors, there are spectrums of susceptible genes that exist in different forms potentially participating as risk factors for CAD by encoding for incompetent forms of proteins which are originally vital in the regulation of haemodynamic functions.

The endothelial Nitric Oxide Synthase (eNOS) is one of these potential risk proteins that synthesizes a free radical molecule known as endothelial Nitric Oxide (NO)[4]. This free radical molecule is a potent anti-oxidant that contributes to the regulation of the vascular tone[4]. In addition, it protects against atherosclerosis by inducing vascular smooth muscles relaxation, inhibiting the sympathetic vasoconstriction and inhibiting platelet aggregation and leukocyte adhesion within the atherosclerotic lesion[5-7]. The NO also processes an essential role in neurons and blood vessels development[8,9].

The eNOS gene located in chromosome 7q36 contains many vulnerable sites of polymorphisms recognized during the human eNOS gene sequencing in the mid-1990s[10]. These polymorphisms include three general classes: those in intron regions, those in 5-flanking DNA (promoter) and those within the open reading frame[11]. One of the most important and well-studied polymorphism that code for a variant form of the enzyme is the Single Nucleotide Polymorphism (SNP) in exon 7 at position 894, resulting in an exchange of glutamate (Glu) with aspartate (Asp) amino acid at position 298[11]. This alteration affects the enzymes activity and its bioavailibility due to the selective proteolytic cleavage in endothelial cells and vascular tissues[12,13]. Although some evidence might indicated the increased expression of eNOS in CAD in response to the accumulated Reactive Oxygen Species (ROS); however, this is also accompanied by an accelerated degradation of NO by the ROS[14]. Therefore, the presence of Asp298 form of eNOS would have a stronger impact on the reduction of NO levels in CAD patients.

Multiple molecular and epidemiological studies had examined the association between Glu298Asp polymorphism and cardiovascular diseases in various population ethnicities[15,16]. However, the inferential findings of these studies are inconsistent and occasionally contradictory. Therefore, the aim of this study was to assess this association in Malaysian sample of population and to merge these findings with studies of compatible ethnic groups in the region through meta-analytic approach to get a better understanding about the possible relation between this polymorphism and CAD.

MATERIALS AND METHODS

Participants: The study included unrelated 185 CAD patients (152 males and 33 females) and 188 healthy controls (122 males and 66 females). The recruitment of the CAD patients took place at the Tengku Ampuan Afzan Hospital in Kuantan, Pahang. The diagnosis was confirmed by the physician for at least three months before the selection. The healthy controls were volunteers with no history of CAD or chest pain recruited from different locations. The study received its ethical approval from the Ministry of Health, Malaysia (NMMR 10-495-5071) and the IIUM Research Ethical Committee (IREC). The sample size was calculated using the OpenEpi software adapting the results from Chu *et al*.[17] for unmatched case-control study. The study laboratory experiments were conducted in the Molecular Laboratory, Faculty of Medicine, International Islamic University Malaysia between June 2014 and December 2015.

Genotyping: The collected peripheral blood samples from participants were subjected to DNA extraction using QIAmp Blood purification kit (Qiagen, Germany) following the manufacturer's protocol. Quantity and quality of DNA were measured using bio-photometer plus (Eppendorf, USA). Glu298Asp NOS gene polymorphism was genotyped following the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay method. The amplification took place in 15 µL reaction volume that included; 0.25 µM of each primer, 200 µM of dNTPs, I U of One *Taq*™ Polymerase (New England Biolabs), 1X reaction buffer, 3 mM of $MgCl_2$ and 30 ng of DNA. The primers utilized in this study were adopted from Zheng *et al*.[18]. The thermal setting of the PCR reaction included initial denaturation 94°C for 30 sec followed by 35 cycles of denaturation, annealing and extension steps each lasted for 30 sec at 94, 60 and 68°C, respectively followed by final extension at 68°C for 5 min. Upon successful amplification, the products were further digested with Ban II restriction enzyme.

At the end of endonuclease digestion, the products were stained with ethidium bromide followed by electrophoretic separation on 3.2% agarose gel and visualized using the gel documentation system (Bio-Rad, USA). The genotypes of wild Glu/Glu, heterozygous Glu/Asp and recessive Asp/Asp were represented as 163 and 85 bp, 248, 163 and 85 bp and 248 bp fragments respectively.

Validation of results by sequencing analysis: Validation of the genotyping results took place by direct sequencing of the PCR products as shown in Fig. 1. The PCR products from four randomly chosen samples with different genotypes were

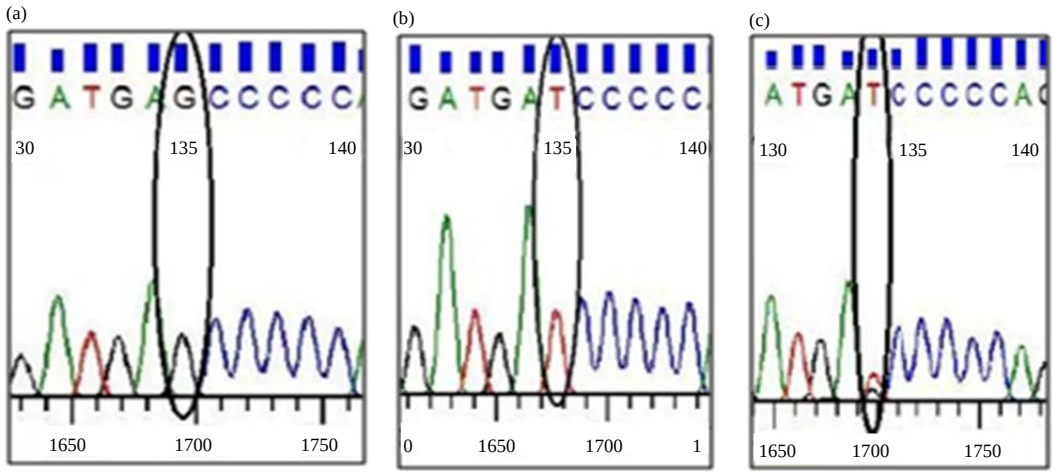


Fig. 1(a-c): Interpretation of Glu298Asp polymorphisms nucleotide sequences, (a) Homozygous Glu/Glu, (b) Homozygous Asp/Asp and (c) Heterozygous Glu/Asp)

Table 1: Demographic data

[#]Variables	CAD (n = 185) n (%)	Controls (n = 188) n (%)	p-value
Race			
Malay	139 (74.3)	166 (88.3)	
Chinese	31 (16.6)	14 (4.3)	0.02*
Indian	17 (9.1)	8 (7.4)	
Sex			
Male	154 (82.4)	66 (35.4)	<0.001*
Female	33 (17.6)	122 (64.9)	
[+]Variables	CAD (n = 185) Mean (SD)	Controls (n = 188) Mean (SD)	p-value
Age (years)	60 (10.5)	47 (5.7)	<0.001

[#]Chi-square test, p<0.05 is taken as statistically significant at 95% confidence interval. [+]Independent t-test. *Significant difference

subjected to automated direct DNA sequencing using ABI PRISM 3130 genetic analyzer (Applied Biosystems, USA). The PCR products were first purified using QIAquick Gel Extraction Kit Protocol (Qiagen, Germany) followed by cycle sequencing of the product using the forward primer and Big Dye Terminator v3.1 cycle sequencing kit. The cycle sequenced products were purified using ethanol precipitation method. The nucleotide sequence interpretation was completed using the reference sequences from NCBI (rs1799983).

Statistical analysis: The statistical analysis of primary data was completed using SPSS software version 19.0 for Windows; SPSS (Inc., Chicago, IL) and online SHEsis program[19]. The Hardy-Weinberg test of equilibrium for genotypes or alleles distribution in the cases and controls were done by the chi-square (X^2) method to evaluate the potential bias of genotype aberration from a normal distribution. A p-value of less than 0.05 was considered statistically significant[20].

Meta-analysis: The meta-analysis approach involved scrutinizing Web of Science, Scopus, PubMed and Google databases for studies with similar case-control design in East-Asian or Southeast Asian countries.

While screening the studies, any report with incomplete genotypes or allele data was excluded from the analysis. After exclusion of the non relevant data, a total of 23 studies[17,21-43] were integrated with the current study for meta-analysis. The collected sample conveniently yielded a data of 3583 CAD cases and 8710 controls with more than 95% sampling power to detect a genetic risk factor of OR = 1.2 ($\alpha = 0.05$) based on the minor allele frequency in the study of Chu *et al.*[17]. In the meta-analysis, the odds ratios were calculated based on the presence of homozygous Asp genotype (recessive allele model) and the minor allele model. The further sub-group analysis was conducted only when the pooled heterogeneity test exceeded the substantial heterogeneity (1^2>50%). The sub-grouping was based on the geographical location of the populations. In addition, a sensitivity analysis was conducted in both types of the meta-analysis by omitting each of the individual studies each time to make sure none of the studies have imposed any undue influences on the pooled results. The meta-analysis was performed using the Revman meta-analysis software version 5.3.

RESULTS

Socio-demograpgic data: The demographic data of the subjects are as shown in Table 1. There was a significant difference in race, gender and age between CAD and control groups (p<0.05).

Table 2: Distribution of eNOS Glu298Asp genotypes and allele frequencies in CAD patients and controls

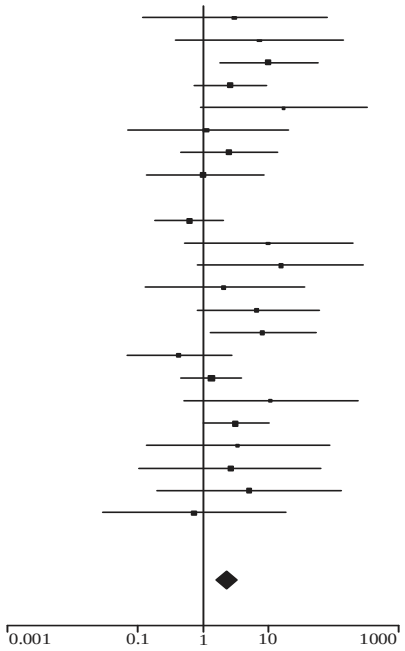
	eNOS Glu298Asp genotype n (Frequency (Hz))				Minor allele (Asp)	OR	
Groups	Glu/Glu	Glu/Asp	Asp/Asp	p-value	n (Frequency)	(95% CI)	p-value
CAD (n = 185)	90 (0.486)	83 (0.449)	12 (0.065)		107 (0.289)		
Control (n = 188)	133 (0.707)	51 (0.271)	4 (0.021)	<0.001	59 (0.157)	3.19 (1.01-10.08)	<0.001

Chi-square test, OR: Odds ratio, CI: Confidence interval, p<0.05 is taken as statistically significant at 95% confidence interval

Table 3: Forest plot of the effect estimate for Asp/Asp genotype of eNOS3 gene. Meta-analysis of the combined sub-groups data showed OR = 2.41 (1.61-3.59; 95% CI), p<0.0001, I2 = 10%, (Meta-analysis by Review Manager Software; version 5.3)

	CAD patients		Control participants				
Study or subgroups	Events	Total	Events	Total	Weight (%)	Odds ratio IV, fixed, 95% Cl	Odds ratio IV, fixed 95% Cl
Bae *et al.*[21]	1	192	0	196	1.6	3.08 [0.12, 76.03]	
Chang *et al.*[22]	3	108	0	112	1.8	7.46 [0.38, 146.24]	
Chao *et al.*[23]	5	41	2	150	5.7	10.28 [1.92, 55.13]	
Chu *et al.*[17]	17	220	3	98	10.3	2.65 [0.76, 9.27]	
Hibi *et al.*[24]	5	226	0	357	1.9	17.75 [0.98, 322.63]	
Ji *et al.*[25]	1	165	1	190	2.1	1.15 [0.07, 18.57]	
Jo *et al.*[26]	2	129	5	803	5.9	2.51 [0.48, 13.09]	
Katakami *et al.*[27]	1	226	15	3593	3.9	1.06 [0.14, 8.06]	
Kim *et al.*[28]	0	147	0	222		Not estimable	
Lin *et al.*[29]	6	120	6	78	11.8	0.63 [0.20, 2.03]	
Min *et al.*[30]	4	242	0	270	1.9	10.21 [0.55, 190.58]	
Qi *et al.*[31]	9	107	0	81	2.0	15.72 [0.90, 274.21]	
Shimasald *et al.*[32]	1	285	1	607	2.1	2.13 [0.13, 34.24]	
Song *et al.*[33]	7	114	1	104	3.6	6.74 [0.81, 55.73]	
Tamemoto *et al.*[34]	3	54	2	283	4.9	8.26 [1.35, 50.70]	
Wang *et al.*[35]	2	332	3	218	5.0	0.43 [0.07, 2.62]	
Wang *et al.*[36]	12	58	7	43	15.2	1.34 [0.48, 3.75]	
Wei *et al.*[37]	2	51	0	108	1.7	10.96 [0.52, 232.56]	
Wisam *et al.*[38]	12	185	4	188	12.2	3.19 [1.01, 10.08]	
Yoon *et al.*[39]	1	110	0	128	1.6	3.52 [0.14, 87.30]	
Yoshimura *et al.*[40]	1	113	0	100	1.6	2.68 [0.11, 66.53]	
Yoshimura *et al.*[41]	1	201	0	345	1.6	5.17 [0.21, 127.50]	
Yu *et al.*[42]	0	120	1	264	1.6	0.73 [0.03, 18.02]	
Zhan *et al.*[43]	0	37	0	172		Not estimable	
Total (95% Cl)		3583		8710	100.0	2.41 [1.61, 3.59]	
Total events	96		51				

Heterogeneity, chi square = 23.22, df = 21 (p = 0.33), I^2 = 10%
Test for overall effect: Z = 4.28 (p<0.0001)



CAD: Coronary artery disease

Genotype analysis: The genotype distributions for Glu298Asp of eNOS gene are shown in Table 2. There was no deviation of genotype frequencies from the Hardy-Weinberg equilibrium for both cases and controls group. The genotypes and alleles frequencies of eNOS Glu298Asp were significantly (p<0.05) associated with CAD a shown in Table 2.

Meta-analysis: The assimilated sample has shown a significant association between homozygous Asp genotype and Asp allele with CAD.

The recessive model of analysis elucidated that the frequency of homozygous Asp genotype was significantly higher in CAD patients than in the control group [p<0.0001; OR = 2.41 (1.61-3.59) CI = 95%; I^2 = 10%] as shown in Table 3. The pooled data revealed a non-significant small degree of heterogeneity among the studies included in the meta-analysis[17,21-43] [Cochran's Q test (p = 0.33), I^2(10%)]. By this low level of heterogeneity fixed model meta-analysis was used.

The Asp minor allele model of meta-analysis has also demonstrated a combined significant association with CAD [p<0.0001, OR = 1.77 (1.50-2.08) CI = 95%. I^2 = 62%)]. There was a marked intergroup heterogeneity (62%) and therefore, the random effect model was used in the analysis followed by subgroup meta-analysis as presented in Table 4.

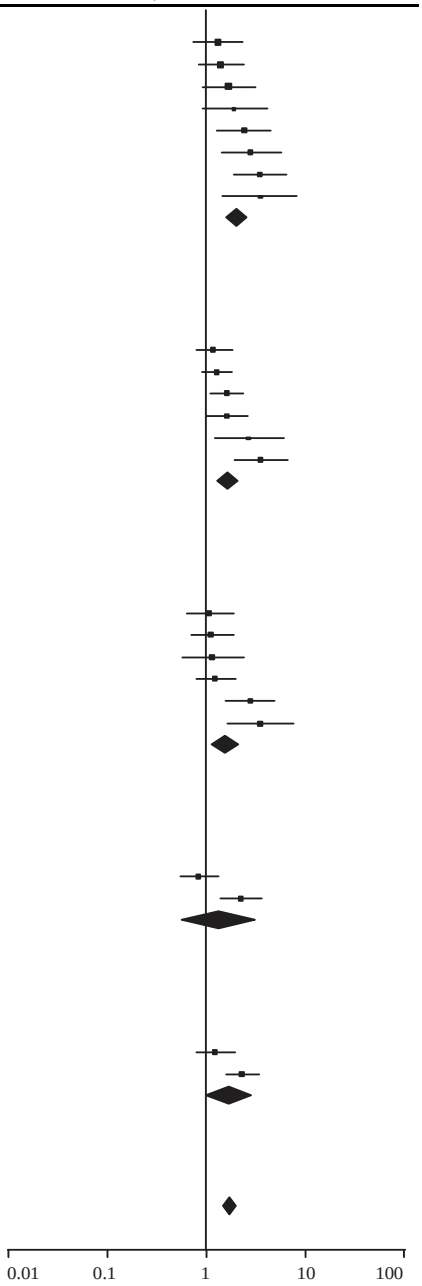
DISCUSSION

In the current study, the socio-demographic characteristics including gender, age and ethnicity were significantly associated with CAD (p<0.05) as shown in Table 1. The secondary analysis indicated a non-significant association between these socio-demographic factors and the genotypes distribution (p>0.05).

As demonstrated in Table 2, Glu allele was more frequent than Asp allele in patients and controls groups. However, Asp allele was found significantly more frequent in the patients group than in the controls group [OR = 3.19 (1.01-10.08; 95% CI); p<0.05]. Similarly, Glu/Asp and Asp/Asp genotypes were significantly more frequent in the patient's group than in the controls group (p<0.05).

Table 4: Forest plot of the effect estimate for Asp Allele of eNOS gene. Meta-analysis of the combined sub-groups data showed OR = 1.77 (1.50-2.08; 95% CI); p<0.00001; I^2 = 62%. (Meta-analysis by Review Manager Software; version 5.3)

	CAD		Control				
Study or subgroups	Events	Total	Events	Total	Weight (%)	Odds ratio IV, random, 95% Cl	Odds ratio IV, random 95% Cl
3.2.1 China							
Wang *et al.*[36]	57	116	36	86	3.9	1.34 [0.76, 2.35]	
Ji *et al.*[25]	42	330	35	380	4.5	1.44 [0.89, 2.31]	
Yu *et al.*[42]	22	240	29	528	3.8	1.74 [0.98, 3.09]	
Than *et al.*[43]	12	74	31	344	3.0	1.95 [0.95, 4.01]	
Song *et al.*[33]	39	228	16	208	3.6	2.48 [1.34, 4.58]	
Qi *et al.*[31]	43	214	13	162	3.4	2.88 [1.49, 5.57]	
Chao *et al.*[23]	26	82	35	300	3.8	3.52 [1.96, 6.30]	
Wei *et al.*[37]	15	102	10	216	2.5	3.55 [1.54, 8.21]	
Subtotal (95% Cl)		1386		2224	28.6	2.12 [1.61, 2.78]	
Total events	256		205				
Heterogeneity, Tau^2 = 0.05; Chi square = 10.98, df = 7 (p = 0.14); p = 36%							
Test for overall effect: Z = 5.41 (p<0.00001)							
3.2.2 Japan							
Hibi *et al.*[24]	47	452	62	714	5.1	1.22 [0.82, 1.82]	
Katakami *et al.*[27]	46	452	578	7186	5.7	1.30 [0.94, 1.78]	
Shimasaki *et al.*[32]	62	570	83	1214	5.5	1.66 [1.18, 2.35]	
Yoshimura *et al.*[40]	41	402	44	690	4.7	1.67 [1.07, 2.60]	
Yoshimura *et al.*[41]	26	226	9	200	2.7	2.76 [1.26, 6.04]	
Tamemoto *et al.*[34]	22	108	38	566	3.9	3.55 [2.01, 6.30]	
Subtotal (95% Cl)		2210		10570	27.5	1.73 [1.30, 2.31]	
Total events	244		814				
Heterogeneity, Tau^2 = 0.08; Chi^2 = 12.89, df = 5 (p = 0.02); p = 61%							
Test for overall effect: Z = 3.72 (p<0.0002)							
3.2.3 Korea							
Kim *et al.*[28]	28	294	38	438	4.3	1.11 [0.66, 1.85]	
Bae *et al.*[21]	37	384	33	392	4.4	1.16 [0.71, 1.90]	
Yoon *et al.*[38]	18	220	18	256	3.2	1.18 [0.60, 2.32]	
Jo *et al.*[26]	29	258	146	1606	4.9	1.27 [0.83, 1.93]	
Min *et al.*[30]	48	484	20	540	4.1	2.86 [1.67, 4.90]	
Chang *et al.*[22]	28	204	10	236	2.9	3.60 [1.70, 7.60]	
Subtotal (95% Cl)		1844		3468	23.8	1.59 [1.09, 2.32]	
Total events	188		265				
Heterogeneity, Tau^2 = 0.14; Chi^2 = 14.30, df= 5 (p = 0.01); p = 65%							
Test for overall effect: Z = 2.39 (p<0.02)							
3.2.4 Taiwan							
Wang *et al.*[35]	59	664	44	436	5.0	0.87 [0.58, 1.31]	
Lin *et al.*[29]	107	240	41	156	4.8	2.26 [1.46, 3.50]	
Subtotal (95% Cl)		904		592	9.8	1.40 [0.55, 3.56]	
Total events	166		85				
Heterogeneity, Tau^2 = 0.41; Chi^2 = 9.71, df = 1 (p = 0.002); p = 90%							
Test for overall effect: Z = 0.70 (p<0.48)							
3.2.5 Malaysia							
Chu *et al.*[17]	101	454	37	202	4.9	1.28 [0.84, 1.94]	
Wisam *et al.*[38]	119	370	63	376	5.4	2.36 [1.66, 3.33]	
Subtotal (95% Cl)		824		578	10.4	1.75 [0.96, 3.20]	
Total events	220						
Heterogeneity, Tau^2 = 0.15; Chi^2 = 4.86, df = 1 (p = 0.03); p = 79%							
Test for overall effect: Z = 1.83 (p<0.07)							
		7168		17432	100.0	1.77 [1.50, 2.08]	
Total events	1074		1469				
Heterogeneity, Tau^2 = 0.10; Chi^2 = 59.89, df = 23 (p = 0.0001); p = 62%							
Test for overall effect: Z = 6.75 (p<0.00001)							
Test for subgroup differences: Chi^2 = 2.15, df = 4 (p = 0.71); I^2 = 0%							



The current meta-analytic approach involved studies from Southeast Asian and East-Asian regions. This is attributed to the assumption of similarity among different Asian ethnicities that was suggested by the Singapore Genome Variation Project groups[44] and the other report about people similarity of origin in SEA and EA[45]. This meta-analysis elucidated a significant association between homozygous Asp genotype and CAD in Asian populations [OR = 2.41 (1.61-3.59; 95% CI); p<0.0001], with low level of heterogeneity [Cochran's Q test (p-value = 0.3), I^2 = 10%] which is consistent with previous meta-analytic evidence[15]. As demonstrated in Table 3, two studies were in line with the current original research findings including Chau[23] and Tamemoto *et al.*[34] from China and Japan respectively that had demonstrated a significant increase in the susceptibility to CAD among homozygous Asp carriers (p<0.05). However, most of the remaining individual studies

from the East Asian and South East Asian regions demonstrated a non-significant association between the homozygous Asp genotype and CAD as shown in Table 3. In the studies conducted by Wang *et al*.[36] and Lin *et al*.[29], homozygous Asp genotype was found to be more frequent in the control group as shown in Table 3 [OR = 0.63(0.2, 2.03; 95% CI), 0.43(0.07, 2.62; 95% CI) respectively]. These studies carry some controversial issues including small sample size and the inclusion criteria that involved CAD patients and patients with hypertension or stroke. In addition, the participants were confounded by other risk factors such as diabetes, smoking and hypertension. At the same time, the control participants included patients admitted to the same hospital due to other complaints. This approach carries a bias in the selection and a probability to affect the distribution of Asp allele and renders doubts on the validity of findings. Therefore, these two studies carry less inferential significance in the meta-analysis.

In the minor allele model of meta-analysis, the results had shown a significant overall association between Asp allele and CAD as shown in Table 4 ($p<0.05$). However, there was a marked heterogeneity among the studies included in the meta-analysis [Cochran's Q test ($p<0.001$), $I^2 = 62\%$). The variability was unlikely due to any publication bias as was indicated by the symmetrical funnels plot. Further subgroup meta-analysis had shown a significantly high degree of heterogeneity in China, Japan, Korea, Taiwan and Malaysia subgroups with an I^2 value of 36, 61, 65, 90 and 79%, respectively. The considerably less heterogeneity in the meta-analytic findings among homozygous Asp genotype model than what was found in the Asp minor allele model might indicate the recessive genetic behavior of Asp allele.

To understand the role of eNOS in CAD, it is essential to know that this disorder is primarily caused by atherosclerosis in the coronary arteries accompanied by endothelial dysfunction, vascular smooth muscle proliferation and spasm and oxidation reaction involving deposited LDL molecules associated with inflammatory cells infiltration[46]. NO protects against these pathological events by inhibiting the changes within the vascular smooth muscles, limiting the oxidative changes and reducing the inflammatory responses[4]. Therefore, a reduction in the bioavailability of vascular NO will precipitate atherosclerosis process. Functional studies were used to validate these protective effects by using gene disrupted mice that confirmed the role of NO in limiting the vascular atherosclerotic damage[14,47,48]. However, the causal relation between eNOS gene polymorphisms and CAD can't be simply confirmed in human because the aetiology for CAD is stratified by other risk factors such as obesity, smoking, hypertension, diabetes and lifestyle[49,50]. Therefore, the presence of eNOS gene polymorphisms with other risk factors may cause an earlier onset of CAD or even more severe disease consequences[50,51]. For this reason it might be necessary to identify the status of eNOS genotype in susceptible individuals to provide them with more preventive measures against CAD and its complications.

CONCLUSION AND FUTURE RECOMMENDATION

Meta-analysis and the comparative study elucidated a significant risk for CAD among Asian populations carrying the Asp homozygous genotype or Asp allele. The presence of some controversial issues, related to the selection criteria among the individual studies might explain the variation in their findings. The effect of environmental factors might be the source of discrepancy as well. Therefore, more efforts are needed to examine the interactions of eNOS gene polymorphism with other genetic factors and or the environmental factors precipitating CAD to clearly explain the role of genetics in this complex disease.

SIGNIFICANCE STATEMENT

This study highlights the relation between Glu298Asp eNOS gene polymorphism and coronary artery disease among Asian populations. The findings of this study would help to identify people with high-risk for CAD to provide them with more intensive screening and to assess for other cardiovascular complications. These preliminary findings determine the need to uncover the multiple consequences causing the hemodynamic deterioration due to the reduced bioavailability of nitric oxide among CAD patients. Thus, more studies are needed to complete the understanding about the link between nitric oxide level and the pathophysiological changes in coronary artery disease.

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