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ORIGINAL ARTICLE

Erectile Dysfunction

Association of endothelial nitric oxide synthase polymorphisms with an increased risk of erectile dysfunction

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The purpose of our meta-analysis is to examine the associations between three single nucleotide polymorphisms of endothelial nitric oxide synthase (*eNOS*) gene, G894T, intron 4 and T-786C, and the risk of erectile dysfunction. An electronic database search was performed to identify case-control studies reporting the association between single nucleotide polymorphisms of *eNOS* gene and erectile dysfunction. Stringent inclusion and exclusion criteria were employed to select high-quality studies for this meta-analysis. Comprehensive Meta-analysis 2.0 software (Biostat Inc., Englewood, New Jersey, USA) was used for statistical analysis of the data extracted from the selected studies. From the initial 203 articles retrieved from database search, this meta-analysis finally selected 12 high-quality case-control studies that conformed to our inclusion criteria. The 12 studies contained a total of 1962 patients with erectile dysfunction and 1752 healthy controls. The results of our meta-analysis showed that G894T correlated with an increased risk erectile dysfunction under both the allele and dominant models (allele: OR = 1.556, 95% CI = 1.064–2.275, *P* = 0.023; dominant: OR = 1.613, 95% CI = 1.050–2.476, *P* = 0.029). A similar association was found between T-786C and erectile dysfunction under the allele model (OR = 1.679, 95% CI = 1.341–2.102, *P* < 0.001), but not under the dominant model (all *P* > 0.05). Our meta-analysis showed that the two single nucleotide polymorphisms in *eNOS* gene, G894T and T-786C, are strongly associated with the risk of erectile dysfunction.

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Keywords: endothelial nitric oxide synthase; erectile dysfunction; G894T; intron 4; meta-analysis; single nucleotide polymorphism

INTRODUCTION

Erectile dysfunction (ED) is defined as the inability to achieve or maintain penile erection sufficient for sexual performance.[1] It is most common suffering among men over 55 years old and its occurrence increases significantly with age, which seriously impacts the quality of life in patients and contributes to decreased self-confidence, panic and depression.[2,3] There is a significant number of men under 40 who experience ED and various diseases or medications contribute to ED in this age group. ED includes organic ED, characterized by a gradual onset, and psychogenic ED, which is considered as situational ED and varies in severity from mild to severe, according to the International Index of Erectile Function.[4,5] ED is a multifactorial disease involving organic, endocrine changes, smoking, and psychogenic factors, and in addition, an increased risk of ED is also associated with diseases such as diabetes mellitus, cardiovascular disease, genitourinary diseases, mental or psychological disorders, as well as other chronic diseases.[6–8] With respect to ED treatment, both pharmacological and psychological therapies are prevalent, and current evidence suggests that the majority of patients respond well to selective phoshodiesterase type-5 inhibitors, but at least 30%–35% of men with ED fail to respond to this therapy.[9–11] Considering the enormity of the negative influence of ED on the relationship between man and woman, it is important to investigate additional factors that may contribute significantly to ED risk.[12]

There are three major isoforms of nitric oxide synthase (NOS): inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS), and each of them is encoded by separate gene.[13] eNOS is located in the cytoplasm of cavernous endothelium and is activated by shear stress or mechanical stress via increased arterial blood flow.[14,15] eNOS is also a major regulator of angiogenesis, and low levels of the enzyme are constitutively expressed in endothelial cells, as well as other cell types.[16,17] eNOS gene is mapped to chromosome 7q35-36 and contains 26 exons that span 21 kb.[18,19] Three important single nucleotide polymorphisms (SNPs) in the eNOS gene (G894T, intron 4, and T-786C) have received substantial attention in the last few years, especially in relation to ED.[20–23] A few studies reported positive associations of G894T, T-786C, and intron 4 with the risk of ED.[24–26] However, other studies contend that this association of SNPs of *eNOS* gene appear to be a "bystander" effect for ED risk.[2,23,27] In this context, we used a meta-analysis framework to investigate the associations between G894T, intron 4, T-786C SNPs of *eNOS* gene, and the risk of ED.

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MATERIALS AND METHODS

Data sources and keywords

To identify potential relevant published studies that investigated the association between the SNPs of *eNOS* and ED, we systematically searched PubMed, SpringerLink, Web of Science, Wiley, Cochrane Library, CINAHL, and China National Knowledge Infrastructure (CNKI) using keywords related to ED and gene polymorphism. We applied the following keywords and MeSH terms in our literature search: ("polymorphism, genetic" or "polymorphisms" or "polymorphism" or "SNP" or "mutation" or "variants") and ("nitric oxide synthase, Type III" or "endothelial nitric oxide synthase" or "eNOS Enzyme" or "ECNOS Enzyme") and ("erectile dysfunction" or "impotence" or "male impotence" or "male sexual impotence"). Additionally, we also manually scanned the bibliographies of related articles to identify other potential relevant articles.

Study selection criteria and data extraction

Human case-control studies providing genotype data of the SNPs in *eNOS* gene, in both ED patients and healthy controls, were identified.

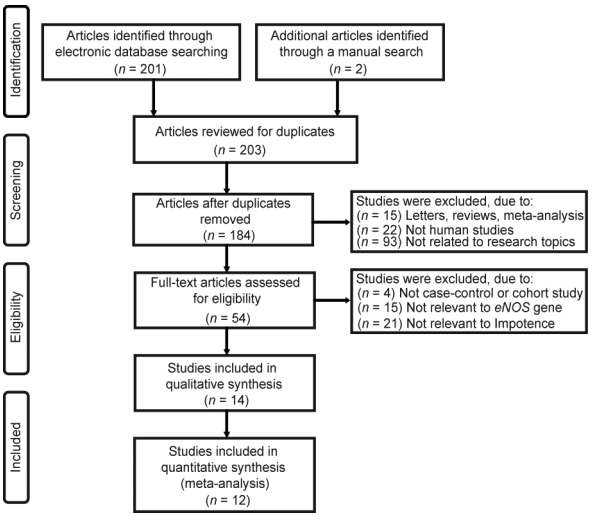

Figure 1: Flowchart showing the detailed steps of study screening procedure and reasons for exclusion. Twelve studies were included in this meta-analysis.

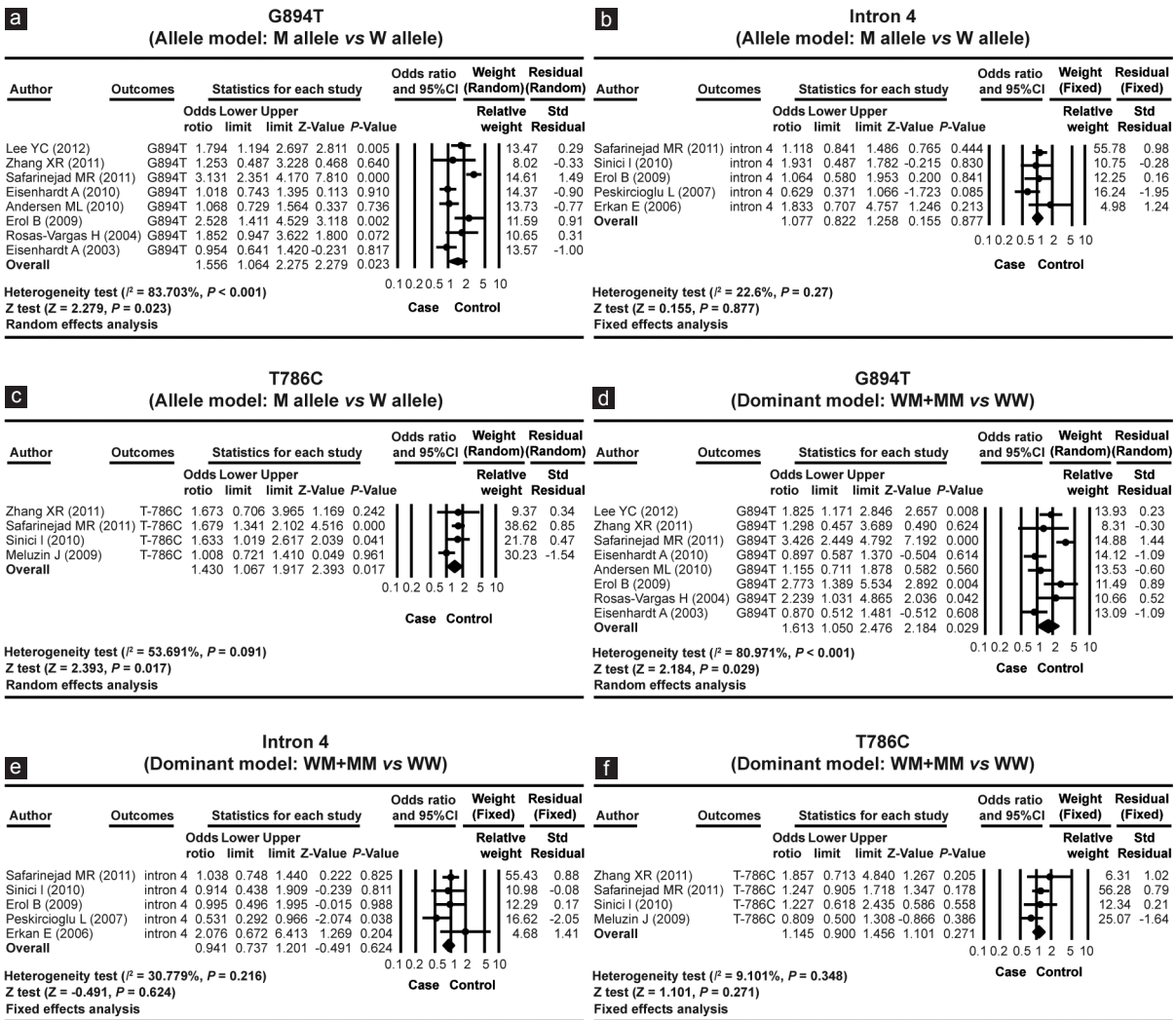

Figure 2: (**a–f**) Forest plot showing the differences of genotype frequencies among the SNPs of *eNOS* gene between case group and control group (G894T, intron 4, and T-786C).

We selected only those studies that contained larger sample size and extracted the required information on *eNOS* gene polymorphisms and other relevant data. A stringent study selection process was followed to exclude summaries, letters, meta-analyses, nonhuman studies, studies with incomplete, unavailable or irrelevant clinicopathologic data, and studies not relevant to the topic or those studies published in non-English or non-Chinese languages. In order to reduce bias and increase confidence in the data, two investigators separately extracted information from the selected articles and reach a consensus on all the items through discussion and reexamination. In case of disagreements between the two investigators, a third investigator was consulted to find a resolution. The following relevant data were extracted from each eligible study: first author, year of publication, country, ethnicity, language, number of samples, source of sample, age and SNPs.

Statistical analysis

We calculated the odds ratio (OR) with 95% confidence intervals (95% CIs), displayed by forest plots, to evaluate the differences in genotype frequencies of the SNPs of *eNOS* gene (G894T, intron 4 and T-786C) between cases and controls and employed *Z*-test to examine the significance of the overall effect size.[28] Heterogeneity among the enrolled studies was confirmed by Galbraith radial plot and Cochran's Q-statistic, and $P < 0.05$ indicated the existence of heterogeneity.[29,30] Subsequently, I^2 statistics was utilized to quantify the size of heterogeneity.[31] The value of I^2 ranged from 0% to 100%, where I^2 <50% indicated no significant heterogeneity and I^2 >50% suggested increasing heterogeneity. Random-effects model was applied if significant heterogeneity was detected ($P < 0.05$ or I^2 test exhibited >50%), otherwise fixed-effects model was utilized.[32] Sensitivity analyses were performed to evaluate whether removal of any one single study influenced the overall outcomes. Publication bias was evaluated by visual inspection of the funnel plots, further analyzed by both classic fail-safe N and Egger's linear regression test to demonstrate the reliability of the overall results.[33–35] We also estimated the expected power of individual study as determined by the probability of evaluating a definitive association between *eNOS* SNPs and the risk of ED.[36] All tests were two-sided ($P < 0.05$ was considered significant). All statistical data were analyzed with Comprehensive

Table 1: The baseline characteristics of each included literature in the present meta-analysis

First author	*Year*	*Country*	*Ethnicity*	*Disease*	*SNP*	*Genotyping method*	*n*		*Power*	*Age (years)*	
Lee *et al*.[26]	2012	China-Taiwan	Asians	ED	G894T	PCR-RFLP	297	293	0.762	54.5±3.3	56.1±4.6
Safarinejad *et al*.[25]	2011	Iran	Asians	ED	G894T, intron 4, T-786C	PCR-RFLP	322	318	1	55.7±12.2	53.2±13.6
Zhang *et al*.[45]	2011	UK	Caucasians	ED	G894T, T-786C	PCR-RFLP	47	53	0.058	40.6±5.4	41.0±4.6
Sinici *et al*.[44]	2010	Turkish	Asians	ED	Intron 4, T-786C	PCR-RFLP	72	71	0.793	54.3±9.2	55.4±8.2
Andersen *et al*.[2]	2010	Brazil	Caucasians	ED	G894T	AS-PCR	79	370	0.055	52.7±16.0	38.1±12.3
Eisenhardt *et al*.[43]	2010	Germany	Caucasians	ED	G894T	PCR-RFLP	455	108	-	56.9±11.7	57.1±2.2
Meluzin *et al*.[42]	2009	Czechoslovakia	Caucasians	ED	T-786C	PCR-RFLP	324	95	0.27	62±10	56±9
Erol *et al*.[41]	2009	Turkish	Asians	ED	G894T, intron 4	PCR-RFLP	64	82	0.864	51.3±7.2	50.2±7.6
Peskircioglu *et al*.[40]	2007	USA	Caucasians	ED	Intron 4	PCR-RFLP	96	167	0.634	52±15.2	-
Erkan *et al*.[39]	2006	Japan	Asians	ED	Intron 4	TaqMan	30	25	0.167	58.7±9.97	56.44±7.58
Rosas-Vargas *et al*.[38]	2004	Mexico	Caucasians	ED	G894T	PCR-RFLP	53	62	0.591	46.94±8.82	44.27±7.54
Eisenhardt *et al*.[37]	2003	Germany	Caucasians	ED	G894T	PCR-RFLP	113	108	0.08	53.1±11.4	57.1±2.2

PCR-RFLP: polymerase chain reaction with the restriction fragment length polymorphism; AS-PCR: allele-specific polymerase chain reaction; TaqMan: TaqMan probes are hydrolysis probes that are designed to increase the specificity of quantitative polymerase chain reaction; ED: erectile dysfunction; SNP: single nucleotide polymorphism

Table 2: Comparisons of genotype and allele frequencies between the case and the control groups

SNP	*eNOS G894T*			*eNOS intron 4*			*eNOS T-786C*		
	OR	*95% CI*	*P*	*OR*	*95% CI*	*P*	*OR*	*95% CI*	*P*
Gene model									
Allele model									
Overall	1.556	1.064-2.275	0.023	1.017	0.822-1.258	0.877	1.43	1.067-1.917	0.017
Ethnicity									
Asians	2.417	1.402-4.167	0.001	1.164	0.886-1.529	0.276	1.679	1.341-2.102	<0.001
Caucasians	1.26	0.945-1.681	0.116	0.825	0.588-1.159	0.267	1.289	0.894-1.858	0.173
WM + MM versus WW (dominant model)									
Overall	1.613	1.050-2.476	0.029	0.941	0.737-1.201	0.624	1.145	0.900-1.456	0.271
Ethnicity									
Asians	2.545	1.374-4.712	0.003	1.095	0.800-1.500	0.571	1.247	0.905-1.718	0.178
Caucasians	1.304	0.896-1.899	0.166	0.748	0.508-1.101	0.141	1.026	0.713-1.476	0.891
MM versus WW (homozygous model)									
Overall	1.358	0.847-2.176	0.204	3.836	2.566-5.735	<0.001	1.657	0.956-2.873	0.072
MM versus WM (heterozygous model)									
Overall	0.676	0.422-1.082	0.103	0.256	0.171-0.381	<0.001	0.505	0.293-0.872	0.014
MM versus WW + WM (recessive model)									
Overall	1.422	0.903-2.240	0.129	3.986	2.727-5.827	<0.001	1.862	1.111-3.122	0.018

OR: odds ratio; 95% CI: 95% confidential interval; SNP: single nucleotide polymorphism; *eNOS*: endothelial nitric oxide synthase

Meta-analysis 2.0 (CMA 2.0) software (Biostat Inc., Englewood, New Jersey, USA).

RESULTS

Included studies

A total of 203 studies were identified from electronic database search and manual searches, and after the study selection process was completed, only 12 studies published between 2003 and 2012 qualified for our meta-analysis. The study selection process excluded 19 articles for being duplicates, 130 for being irrelevant article types (letters, reviews, or meta-analyses), nonhuman studies or unrelated to research topics. After evaluating full texts, 36 studies were excluded for containing incomplete data or data of low relevance, and 4 were eliminated for the absence of required data. Besides, 2 low-quality studies were also excluded (**Figure 1**). Finally, 12 studies were enrolled into our meta-analysis.[2,25,26,37–45] The subjects in these 12 studies were Asians (5 studies) and Caucasians (7 studies), and the studies contained a combined total of 1962 ED patients and 1752 healthy controls. The available SNPs of *eNOS* gene in this meta-analysis were intron 4, T-786C, and G894T. The SNP detection methods used were polymerase chain reaction with the restriction fragment length polymorphism (PCR-RELP), allele-specific polymerase chain reaction (AS-PCR), and TaqMan assay. The baseline characteristics and power value of the studies are presented in **Table 1**.

The association between G894T and the risk of ED

Considering the significant heterogeneity detected among the eight studies reporting the association between G894T of *eNOS* gene and the risk of ED, random-effects model was applied (allele: $I^2 = 83.703\%$, $P < 0.01$; dominant: $I^2 = 80.971\%$, $P < 0.01$). The result of the meta-analysis suggested that G894T contribute significantly to the risk of ED (allele: OR = 1.556, 95% CI = 1.064–2.275, $P = 0.023$; dominant: OR = 1.613, 95% CI = 1.050–2.476, $P = 0.029$) (**Figure 2a**, **2d** and **Table 2**). Further subgroup analysis based on ethnicity suggested that G894T was significantly associated with risk of ED in Asians (allele: OR = 2.417, 95% CI = 1.402–4.167, $P = 0.001$; dominant: OR = 2.545, 95% CI = 1.374–4.712, $P = 0.003$), but not in Caucasians (allele: OR = 1.260, 95% CI = 0.945–1.681, $P = 0.116$; dominant: OR = 1.304, 95% CI = 0.896–1.899, $P = 0.166$) (**Figure 3a** and **3d**).

The association between intron 4 and the risk of ED

There was no significant heterogeneity among the 5 studies reporting

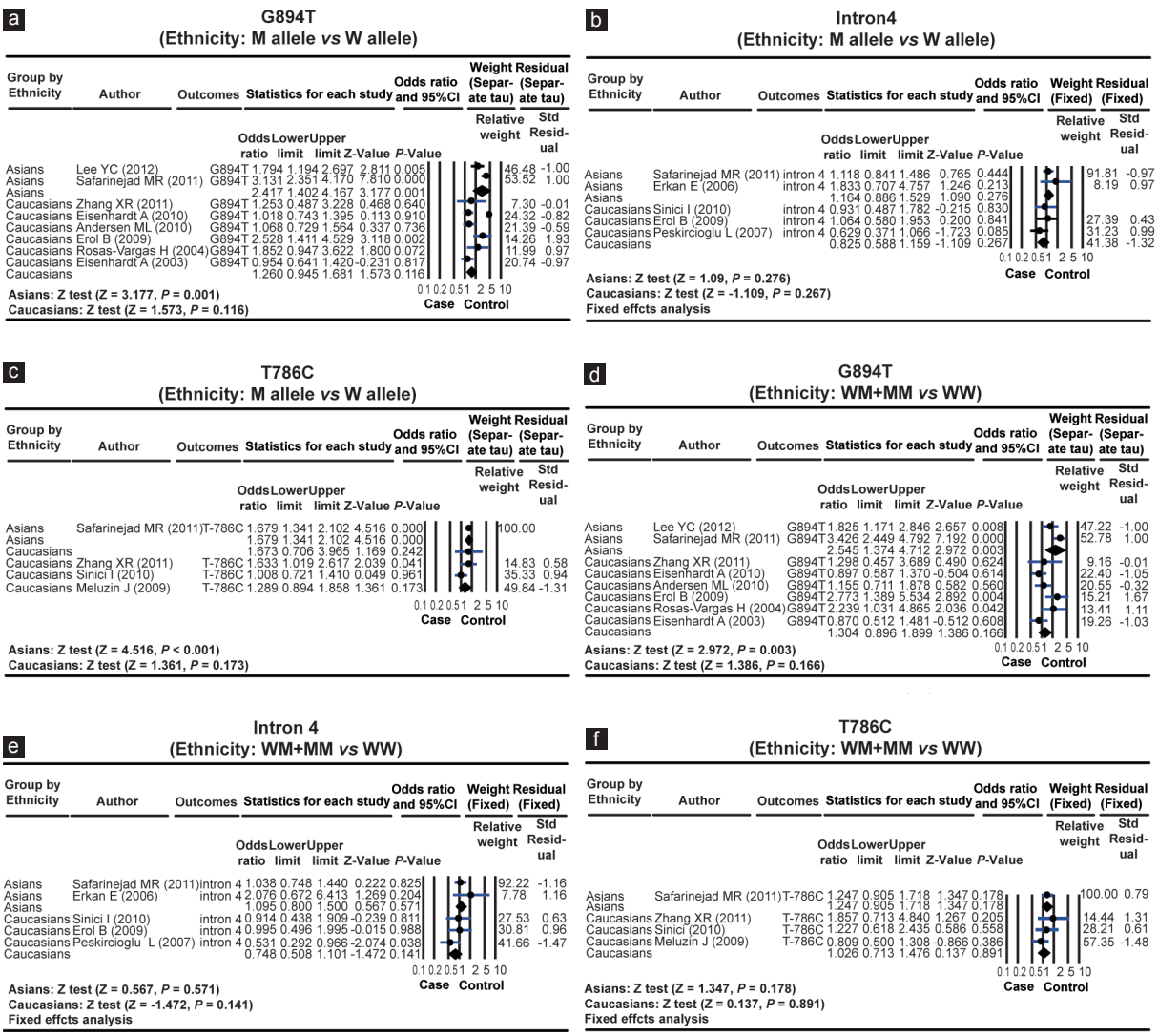

Figure 3: (**a–f**) Forest plot showing the differences of genotype frequencies among the SNPs of *eNOS* gene between case group and control group based on ethnicity (G894T, intron 4, and T-786C).

the association between intron 4 of *eNOS* gene and the risk of ED, thus fixed-effects model was used (allele: $I^2 = 22.600\%$, $P = 0.270$; dominant: $I^2 = 30.779\%$, $P = 0.216$). The result of the meta-analysis revealed that intron 4 was not relevant to the risk of ED (allele: OR = 1.017, 95% CI = 0.822–1.258, $P = 0.877$; dominant: OR = 0.941, 95% CI = 0.737–1.201, $P = 0.624$) (**Figure 2b**, **2e** and **Table 2**). Further subgroup analysis based on ethnicity suggested that intron 4 was not associated with the risk of ED either in the Asians (allele: OR = 1.164, 95% CI = 0.886–1.529, $P = 0.276$; dominant: OR = 1.095, 95% CI = 0.800–1.500, $P = 0.571$) or in Caucasians (allele: OR = 0.825, 95% CI = 0.588–1.159, $P = 0.267$; dominant: OR = 0.748, 95% CI = 0.508–1.101, $P = 0.141$) (**Figure 3b** and **3e**).

The association between T-786C and the risk of ED

Four studies reported the association between T-786C of *eNOS* gene and the risk of ED. The heterogeneity test indicated that heterogeneity existed among these studies under the allele model ($I^2 = 53.691\%$, $P = 0.091$), and random-effects model was used for this analysis while heterogeneity was absent under the dominant model ($I^2 = 9.101\%$, $P = 0.348$), and fixed-effects model was used for analysis. The result of meta-analysis revealed that T-786C significantly contributed to the risk of ED under the allele model (OR = 1.430, 95% CI = 1.067–1.917, $P = 0.017$), but not under the dominant model (OR = 1.145, 95% CI = 0.900–1.456, $P = 0.271$) (**Figure 2c**, **2f** and **Table 2**). Further, subgroup analysis based on ethnicity suggested that T-786C was associated with a significant risk of ED in the Asians (allele: OR = 1.679, 95% CI = 1.341–2.102, $P < 0.001$; dominant: OR = 1.247, 95% CI = 0.905–1.718, $P = 0.178$), but not in Caucasians (allele: OR = 1.289, 95% CI = 0.894–1.858, $P = 0.173$; dominant: OR = 1.026, 95% CI = 0.713–1.476, $P = 0.891$) (**Figure 3c** and **3f**).

Sensitivity analysis and publication bias

Under the allele model, removal of any single study, except Lee *et al*. and Erol *et al*. (removal led to adverse results), had minimal impact on the pooled OR values of G894T and the risk of ED (**Figure 4a**). Under dominant model, removal of any single study, except Lee *et al*. and Safarinejad *et al*. (removal led to adverse results), had minimal impact on the pooled OR values of G894T and the risk of ED (**Figure 4d**). For pooled OR values of intron 4 and the risk of ED, removal of any single study exerted no influence (**Figure 4b** and **4e**). However, except Safarinejad *et al*. (removal led to adverse results) under allele model, removal of any single study had minimal impact on the pooled OR values of T-786C and the risk of ED (**Figure 4c** and **4f**). Symmetrical funnel plots indicated the absence of publication bias, which was further confirmed by both classic fail-safe N and Egger's linear regression test (all $P > 0.05$) (**Figure 5**).

DISCUSSION

Using data from previous studies, we applied a meta-analysis approach to determine the associations between three SNPs of *eNOS* gene,

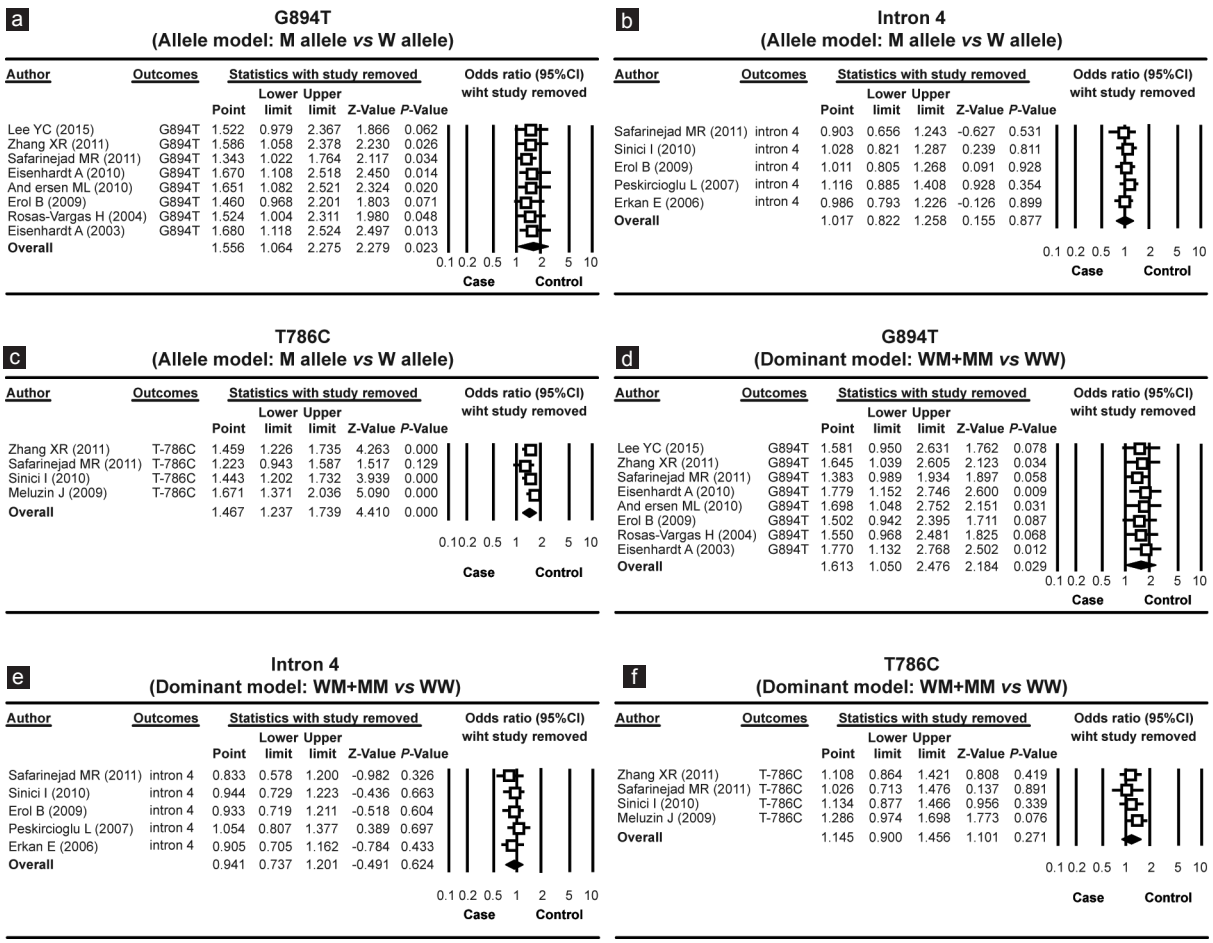

Figure 4: (**a–f**) Sensitivity analyses about the differences of genotype frequencies among the SNPs of *eNOS* gene between case group and control group (G894T, intron 4, and T-786C).

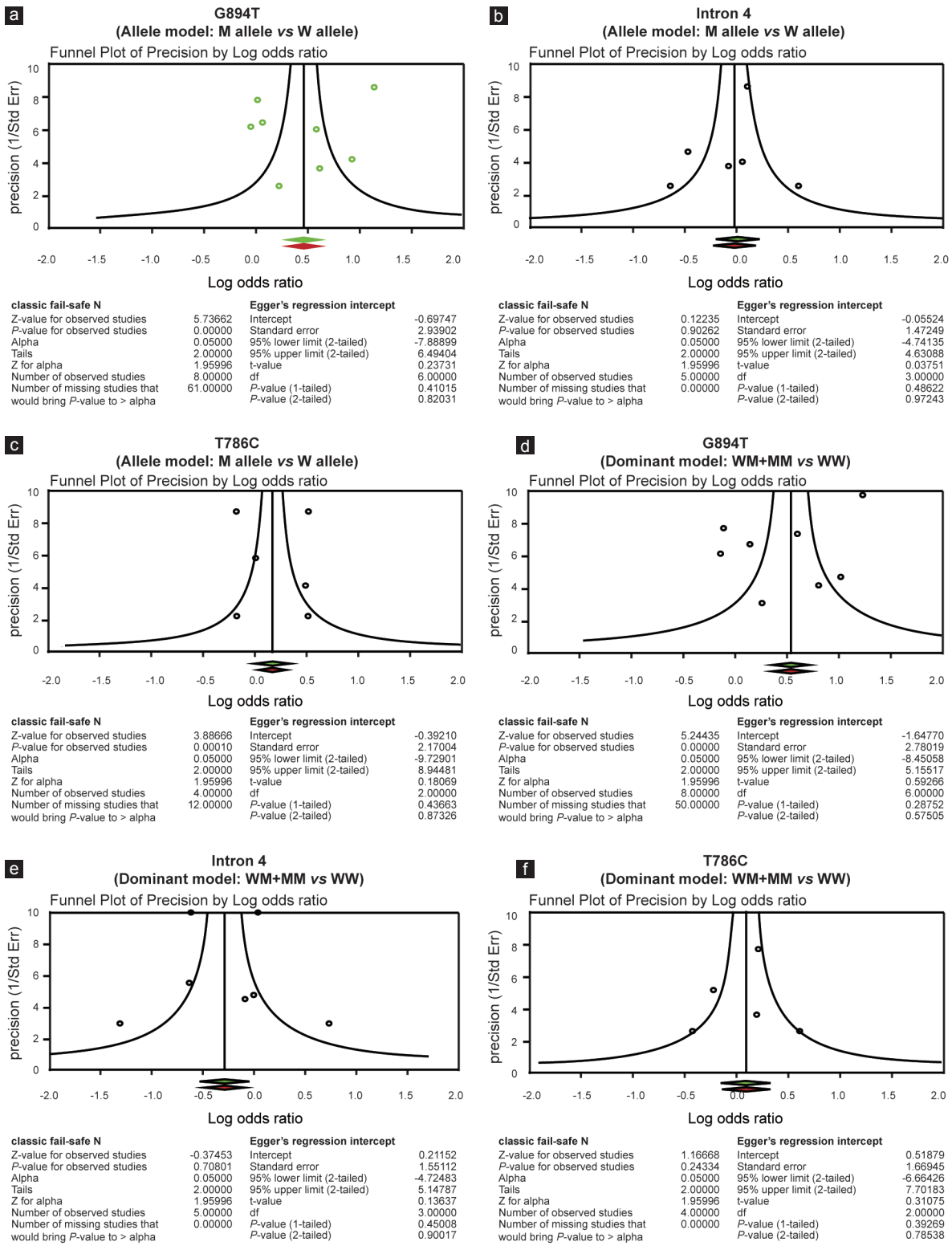

Figure 5: (**a–f**) Funnel plot demonstrating publication biases about the differences of genotype frequencies among the SNPs of *eNOS* gene between case group and control group (G894T, intron 4, and T-786C).

G894T, T-786C, and intron 4 and ED risk. Our findings showed that G894T and T-786C are tightly associated with an increased risk of ED. Penile tumescence begins with the release of NO from either nonadrenergic noncholinergic nerve terminals or endothelial cells, and inadequate NO production during sexual stimulation can result from down-regulation of NOS expression.[25] Our findings demonstrated the association between T-786C and the risk of ED. Low eNOS mRNA and serum nitrite levels were observed in individuals carrying the CC allele

of T-786C polymorphism and homozygosity in this polymorphism correlated with reduced eNOS expression in human endothelial cells and decreased NO-induced vasomotor function.[44] The G894T polymorphism results in an amino acid substitution in eNOS, altering its activity. Measurement of eNOS enzyme activity in T allele carriers revealed 20% lower activity compared to individuals homozygous for the G allele.[26] Erectile function depends on the relaxation and contraction of penis which is regulated by smooth muscle, and G894T polymorphism was found to interfere with this process, increasing ED predisposition.[41] The two SNPs (T-786C and G894T) variously affect either eNOS transcription rate or its enzyme activity to dramatically reduce NO production, resulting in decreased functioning of the eNOS/NO system to buffer blood pressure fluctuations.[21] Evidence shows that functional SNPs within *eNOS* gene interfering with normal eNOS functions down-regulate nitric oxide (NO) production and lower cyclic guanosine monophosphate (cGMP) concentrations, leading to alteration in vasoactive substances, endothelial function and erectile function. Thus, ED is closely associated with *eNOS* gene polymorphisms.[26,39] Based on the above analyses, we postulate that G894T and T-786C are the major genetic risk factors for ED. In support of this, Sinici *et al*. demonstrated that the frequency of CC genotype of T-786C SNPs was higher in ED patients, indicating that the T-786C *eNOS* polymorphism may be an independent risk factor in the pathogenesis of ED.[44]

To examine other factors that may influence the association between the of *eNOS* gene SNPs and the risk of ED, we conducted subgroup analysis based on ethnicity. In this analysis, G894T and T-786C were strongly associated with an increased risk of ED in Asians, but not in Caucasians. The intron 4 was not associated with the risk of ED either in Asians or in Caucasians. The possible reason could be that gene polymorphisms are not the only factors influencing ED and other environmental factors also play a very important role in the development and progression of ED. Further, genetic polymorphisms at other loci, such as in protein disulfide isomerase (PDI) or in the other members of the multi-subunit eNOS complex, geographical location, lifestyle, dietary habits, or limitations in the detection methods, could influence these findings. We intend to pursue further studies to address ethnic differences related to eNOS SNPs and the risk of ED. Therefore, our results should be interpreted taking into consideration the genetic backgrounds and the fact that the results need to be replicated in other ethnic populations.

There were limitations to the current meta-analysis: (1) although there were a total of twelve studies involved, three different SNPs were incorporated for analyses. Therefore, the sample size may not be adequate. (2) The studies employed multiple detection methods, which may have influenced our overall results. (3) Although there was no detectable publication bias in our study, the sample size was comparatively small and the outcomes were varied. (4) Our study may not have the sufficient statistical power to detect small difference between cases and controls Power values of some included literature were relatively low largely due to their small sample size or incompleteness of relevant data, which has a potential impact on the reliability of our study. These limitations may lower the validity and reliability of our overall results.

CONCLUSION

Our results strongly support that two SNPs of *eNOS* gene, T-786C and G894T, are associated with an increased risk of developing ED. Due to the multifactorial nature of ED, future studies in a larger population with diverse ethnic backgrounds are needed to confirm our results.

AUTHOR CONTRIBUTIONS

LG carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. ZZ carried out the immunoassays. FG participated in the sequence alignment. YL and JG participated in the design of the study and performed the statistical analysis. YZ and ZW conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

We declared that we have no competing interests.

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