



Endothelial nitric oxide synthase (eNOS) 4b/a polymorphism and the risk of diabetic nephropathy in type 2 diabetes mellitus: A meta-analysis[☆]



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ABSTRACT

Many studies have accessed the association between eNOS-4b/a polymorphism and the risk of diabetic nephropathy (DN) among type 2 diabetic subjects. However, the results are conflicting and inconclusive. The aim of current meta-analysis was to more precisely estimate the relationship. Pubmed, Embase, the China National Knowledge Infrastructure and the Wanfang Database were searched for articles published up to May 26th, 2013 that addressed eNOS-4b/a polymorphism and the risk of DN among type 2 diabetic subjects. 18 studies were included in this meta-analysis. eNOS-4b/a polymorphisms were associated with an overall significantly increased risk of DN (allele model: OR = 1.44, 95% CI = 1.14–1.82; additive model: OR = 2.03, 95% CI = 1.14–3.62; dominant model: OR = 1.34, 95% CI = 1.07–1.68; recessive model: OR = 2.01, 95% CI = 1.12–3.61). Subgroup analysis revealed a significant association between the eNOS-4b/a polymorphism and DN in Asian population, especially in Chinese population, but not in non Asian populations. Our meta-analysis supported an association between the 4b/a polymorphism of eNOS gene and increased risk of DN in type 2 diabetes among Asians, especially in Chinese population.

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Abbreviations: eNOS, endothelial nitric oxide synthase; DN, diabetic nephropathy; ESRD, end-stage renal disease; ACE, angiotensin-converting enzyme; MTHFR, methylenetetrahydrofolate reductase; CNKI, China National Knowledge Infrastructure; OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium; FEM, fixed-effects model; REM, random-effects model.

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Introduction

Diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes and is the leading cause of end-stage renal disease (ESRD) globally (Collins et al., 2012; Liao, 2012). It has become the primary diagnosis in almost 50% of patients starting renal replacement therapy in some Asian countries (Tang, 2010), in Europe and in the US. Asians, including Chinese type 2 diabetic subjects, have a higher prevalence of nephropathy, with 20% having clinical proteinuria and 40% microalbuminuria (Wu et al., 2005).

Why some type 2 diabetics develop nephropathy, whereas others do not, despite having a long-term hyperglycemia remains unknown. Accumulated evidences suggest that both environmental and genetic factors are related with the etiology of DN (Adler et al., 2003; McCarthy, 2010; Stanton and King, 2011). A number of genes have been suggested as diabetic nephropathy candidate genes, for example, angiotensin-converting enzyme (ACE) (Wang et al., 2012), eNOS, methylenetetrahydrofolate reductase (MTHFR) (Yang et al., 2013), among which eNOS may play a critical role because endothelial dysfunction is considered as an important pathophysiologic factor for DN (Futrakul et al., 2006).

Nitric oxide (NO), a vasodilator molecule, is produced through the oxidation of L-arginine by eNOS (Moncada and Higgs, 1993). NO can regulate endothelial function and is an important factor in the maintenance of homeostasis. The presence of eNOS polymorphisms might contribute to a decreased eNOS activity and a reduced NO level, and has been reported to be a potential factor in the pathogenesis and development of DN (Ahluwalia et al., 2008; Palm et al., 2009). This polymorphism consists of the two alleles of eNOS 4a with 4 tandem 27-repeats and eNOS 4b with 5 repeats (Bellini et al., 2007).

To date, many studies have been carried out to investigate the relationship between eNOS-4b/a polymorphism and the risk of DN among type 2 diabetic subjects, but results of these studies were conflicting and inconclusive. Some studies observed that there was an association between eNOS-4b/a polymorphism and the risk of DN in patients with type 2 diabetes (Ahluwalia et al., 2008); while others suggested that there was no significant association (Fujita et al., 2000; Shoukry et al., 2012). To draw a more reliable conclusion, we performed a meta-analysis of all available studies dealing with the relationship between the eNOS-4b/a polymorphism and DN among type 2 diabetic subjects. Subgroup analyses were performed according to different geographic location to investigate ethnicity-specific effects.

Materials and methods

Literature search strategy

Two investigators (ZJM and HZR) independently searched Pubmed, Embase, the China National Knowledge Infrastructure (CNKI) database and the Wanfang Database. The last updated search was performed on May 26th, 2013. We used any possible combinations of relevant keywords of “Endothelial nitric oxide synthase”, “eNOS”, “4b/a”, “polymorphism” and each term designating “T2DM-DN” (e.g., “type 2 diabetes”, “T2D”, “nephropathy”, “albuminuria”, “proteinuria”, and “ESRD”). We also reviewed all the references cited in these articles to identify additional relevant publications. There was no language limitation. For overlapping publications, only the most recent or complete study was included in this meta-analysis.

Inclusion and exclusion criteria

All studies included in the meta-analysis were required to meet the following criteria: (1) case-control, (2) studies investigating the association of eNOS-4b/a polymorphism with T2DM-DN as the outcome, (3) the control group with subjects who had T2DM but were free of diabetic kidney disease, (4) available data to estimate an odds ratio (OR) with 95% confidence interval (CI), and (5) genotype distribution among the control group must be in Hardy-Weinberg equilibrium (HWE).

Studies were excluded if one of the following existed: (1) review articles and editorials; (2) case reports; (3) repeat or overlapping publications; (4) no report about the genotype frequency, or insufficient information for data extraction.

Data extraction

Two investigators (ZJM and HZR) collected the data independently from all eligible publications. Disagreements about eligibility were resolved during a consensus with a third reviewer (RC) until consensus was achieved on every item. The following data were extracted from each study: name of the first author, year of publication, country of the study, ethnicity, number of cases and controls, genotype and allele frequencies, and genotyping method. Populations were categorized into Asian and non-Asian.

Statistical analysis

The strength of relationship between eNOS-4b/a polymorphism and DN risk was expressed with OR and corresponding 95% CIs. The association was evaluated with the use of the allelic model (a vs b), as well as the additive model (aa vs. bb), the dominant model (aa + ab vs bb) and the recessive model (aa vs ab + bb). Heterogeneity was assessed using the Cochran Q chi-square test and the I^2 test (DerSimonian and Laird, 1986). A P value less than 0.10 indicated significant heterogeneity. We considered I^2 values less than 25% to represent low heterogeneity, values between 25% and 50% to represent moderate heterogeneity, and values of 50% or greater to represent high heterogeneity. The fixed-effects model (FEM) was used when there was no heterogeneity among the included studies; otherwise, the random-effects model (REM) was used. In the subgroup analysis, statistical analysis was conducted according to different geographic location. Sensitivity analysis was performed to estimate the stability by omitting one study at a time to examine influence of one study on the overall summary estimate. Funnel plot and Egger's test were performed to assess publication bias (Begg and Mazumdar, 1994; Egger et al., 1997). An asymmetric plot or the P-value of Egger's test less than 0.1 suggested possible publication bias. Analyses were performed using the software Review Manager 5.2, publication bias was conducted using STATA software (version 12.0; Stata, College Station, TX). A two-sided $P < 0.05$ was considered statistically significant.

Results

Characteristics of the studies

A total of 59 articles were identified. The flow chart of study selection is shown in Fig. 1. Of these, 41 were excluded because they did not meet the criteria or were over-lapping publications. Finally, a total of 18 studies published between 2000 and 2013 were identified according to our inclusion criteria, involving 2972 cases and 2700 controls. The sample size in these case-control studies varied considerably (range from 84 to 903 individuals). All of the included studies used polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism (PCR-RFLP) to check genotypes. The main study characteristics were listed in Table 1. The genotype and allele distributions for each study and HWE in controls were summarized in Table 2. Of the 18 studies, three were non-Asian population (Degen et al., 2001; Ezzidi et al., 2008; Shoukry et al., 2012), 15 studies were Asian population, including eight studies conducted in the Chinese population (Dong et al., 2005; Guo, 2011; Huang et al., 2003; Lin et al., 2002; Luo, 2003; Sun et al., 2004; Xing et al., 2004; Zhang et al., 2005) and seven studies conducted in the non-Chinese population (Ahluwalia et al., 2008; Cheema et al., 2013; Fujita et al., 2000; Neugebauer et al., 2000; Rahimi et al., 2013; Shimizu et al., 2002; Taniwaki et al., 2001).

Overall meta-analysis and subgroup analyses

The summary of the meta-analysis for eNOS-4b/a gene polymorphisms and DN was shown in Table 3. Significant association was found between the 4a allele and increased risk of DN when compared with the 4b allele in overall populations (REM OR = 1.44, 95% CI = 1.14–1.82) (Fig. 2). Significant associations were also found under additive model (REM OR = 2.03, 95% CI = 1.14–3.62) (Fig. 3), dominant model (REM OR = 1.34, 95% CI = 1.07–1.68) (Fig. 4) and recessive model (REM OR = 2.01, 95% CI = 1.12–3.61) (Fig. 5).

Sub-group analysis was performed according to different geographic location (Asian and non-Asian) in this meta-analysis. For Asian population, the 4a allele was found contributing significantly to increased DN risk in

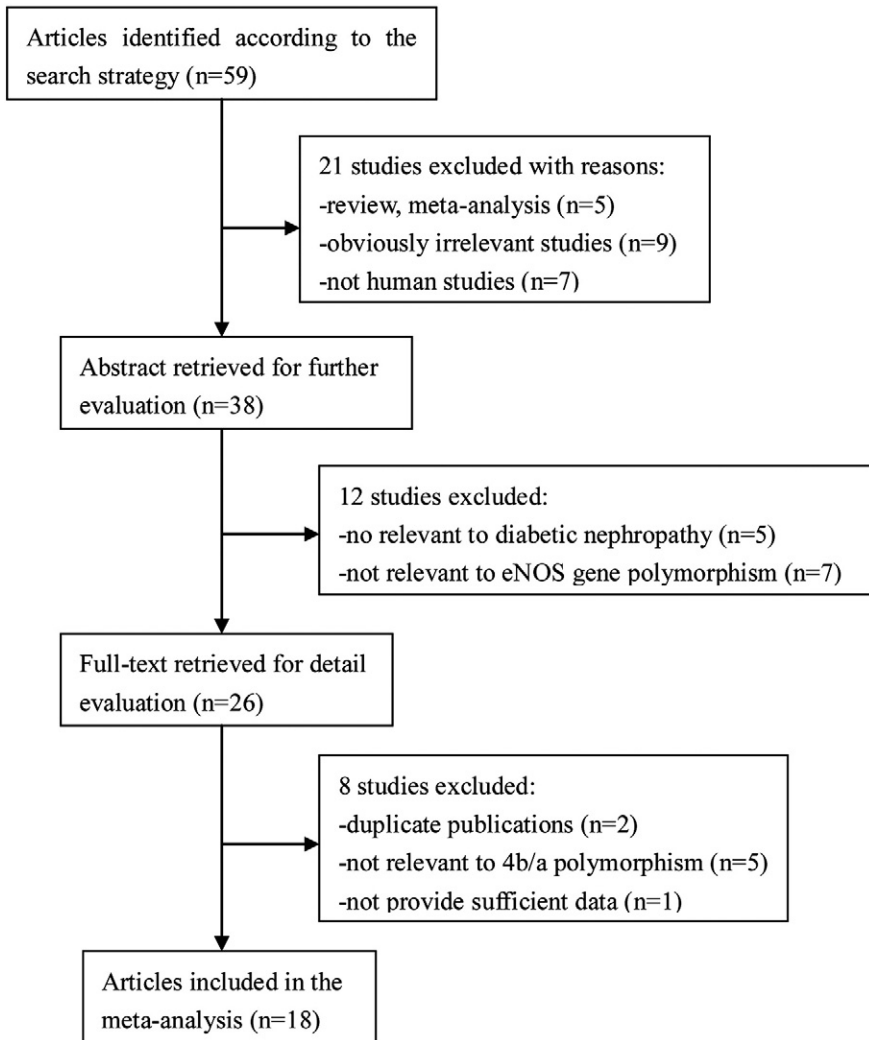


Fig. 1. Flow chart of included studies.

allelic model (REM: OR = 1.59, 95% CI = 1.22–2.09), additive model (FEM: OR = 3.94, 95% CI = 2.72–5.71), dominant model (REM: OR = 1.48, 95% CI = 1.12–1.95), and recessive model (FEM: OR = 4.01, 95% CI = 2.78–5.80), respectively. No significant association was found in any of the above mentioned models for non-Asian population (data were shown in Table 3).

Sub-group analysis according to different country (Chinese population and non Chinese population) in Asian populations was also performed in this meta-analysis. Of all, eight case–control studies with 906 cases and 685 controls were conducted in the Chinese population, seven case–control studies with 1164 cases and 1200 controls were examined in the non-Chinese population. Significant associations were found under allelic model (REM: OR = 2.02, 95% CI = 1.31–3.11), recessive (FEM: OR = 2.25, 95% CI = 1.22–4.13), dominant (REM: OR = 2.05, 95% CI = 1.25–3.36) and additive (FEM: OR = 2.25, 95% CI = 1.22–4.13) models among the Chinese population. For non-Chinese population, significant associations were found under recessive (FEM: OR = 5.52, 95% CI = 3.43–8.89) and additive (FEM: OR = 5.31, 95% CI = 3.29–8.56) models, but the

Table 1

Main characteristics of included studies in the meta-analysis.

Author	Year	Country	Ethnicity	Case/control	Genotyping methods
Ahluwalia et al.	2008	India	South-Asian	195/255	PCR-RFLP
Cheema et al.	2013	India	South-Asian	320/490	PCR-RFLP
Degen et al.	2001	Germany	Caucasian	197/217	PCR
Dong et al.	2005	China	East-Asian	70/64	PCR
Ezzidi et al.	2008	Tunisia	Arabs	505/398	PCR-RFLP
Fujita et al.	2000	Japan	East-Asian	86/69	PCR
Guo et al.	2011	China	East-Asian	69/73	PCR-RFLP
Huang et al.	2003	China	East-Asian	141/61	PCR
Lin et al.	2002	China	East-Asian	79/48	PCR
Luo et al.	2003	China	East-Asian	49/35	PCR
Neugebauer et al.	2000	Japan	East-Asian	133/82	PCR
Rahimi et al.	2013	Iran	West-Asian	121/52	PCR-RFLP
Shimizu et al.	2002	Japan	East-Asian	230/203	PCR
Shoukry et al.	2012	Egypt	Arabs	200/200	PCR-RFLP
Sun et al.	2004	China	East-Asian	188/114	PCR
Taniwaki et al.	2001	Japan	East-Asian	79/48	PCR
Xing et al.	2004	China	East-Asian	130/136	PCR
Zhang et al.	2005	China	East-Asian	180/154	PCR

association was not found in allelic model (REM: OR = 1.34, 95% CI = 0.98–1.83) and dominant model (FEM: OR = 1.20, 95% CI = 0.99–1.45).

Publication bias diagnostics

Begg's funnel plot and Egger's test were performed to evaluate the publication bias in this meta-analysis. The shapes of the funnel plots in all genetic models did not reveal any sign of obvious asymmetry (Figure not shown). The Egger's test showed that there was no significant publication bias for any of the four genetic models (for a versus b: $P = 0.837$; for aa versus bb: $P = 0.387$; for aa + ab versus bb: $P = 0.902$; for aa versus ab + bb: $P = 0.434$).

Table 2

The distribution of the 4b/a genotype and allele frequency for cases and controls.

Study	Distribution of 4b/a eNOS genotype						Allele frequency				HWE
	Cases			Controls			Cases		Controls		
	aa	ab	bb	aa	ab	bb	a	b	a	b	
Ahluwalia et al.	21	28	146	5	61	189	70	320	71	439	Yes
Cheema et al.	51	61	208	10	118	363	163	477	138	844	Yes
Degen et al.	2	53	142	6	50	161	57	337	62	372	Yes
Dong et al.	0	27	43	0	11	53	27	113	11	117	Yes
Ezzidi et al.	29	162	314	21	143	234	220	790	185	611	Yes
Fujita et al.	2	21	63	0	19	50	25	147	19	119	Yes
Guo et al.	6	7	56	3	0	70	19	119	6	140	Yes
Huang et al.	2	31	108	0	8	53	35	247	8	114	Yes
Lin et al.	0	8	71	1	6	41	8	150	8	88	Yes
Luo et al.	27	2	20	8	4	23	56	42	20	50	Yes
Neugebauer et al.	6	26	101	1	10	71	38	228	12	152	Yes
Rahimi et al.	3	32	86	0	14	38	38	204	14	90	Yes
Shimizu et al.	6	44	180	3	44	156	56	404	50	356	Yes
Shoukry et al.	12	64	124	9	60	131	88	312	78	322	Yes
Sun et al.	4	48	136	2	11	101	56	320	15	213	Yes
Taniwaki et al.	0	8	71	1	6	41	8	150	8	88	Yes
Xing et al.	4	16	110	0	8	128	24	236	8	264	Yes
Zhang et al.	0	21	159	2	21	131	21	339	25	283	Yes

HWE: Hardy–Weinberg equilibrium.

Table 3

Meta analysis of the association of eNOS-4b/a gene polymorphism with DN in type 2 diabetes.

Genetic model	Populations	Studies	Number of	Heterogeneity	I ² (%)	OR (95% CI)	P value
		(n)	cases/controls	Q test P-value			
a versus b	All	18	2972/2700	<0.00001	74	1.44 (1.14–1.82) (REM)	0.0002
	Non-Asian	3	902/815	0.52	0	0.99 (0.84–1.17) (FEM)	0.92
	Asian	15	2070/1885	0 < 0.0001	67	1.59 (1.22–2.09) (REM)	0.0007
	China	8	906/685	0.001	70	2.02 (1.31–3.11) (REM)	0.006
	Non-China	7	1164/1200	0.01	64	1.34 (0.98–1.83) (REM)	0.07
aa versus bb	All	18	2972/2700	0.0006	61	2.03 (1.14–3.62) (REM)	0.02
	Non-Asian	3	902/815	0.38	0	1.02 (0.64–1.61) (FEM)	0.95
	Asian	15	2070/1885	0.10	35	3.94 (2.72–5.71) (FEM)	<0.00001
	China	8	906/685	0.28	20	2.25 (1.22–4.13) (FEM)	0.009
	Non-China	7	1164/1200	0.18	32	5.31 (3.29–8.56) (FEM)	<0.00001
aa + ab versus bb	All	18	2972/2700	0.0004	61	1.34 (1.07–1.68) (REM)	0.010
	Non-Asian	3	902/815	0.41	0	0.98 (0.80–1.20) (FEM)	0.85
	Asian	15	2070/1885	0.002	60	1.48 (1.12–1.95) (REM)	0.006
	China	8	906/685	0.008	63	2.05 (1.25–3.36) (REM)	0.005
	Non-China	7	1164/1200	0.22	27	1.20 (0.99–1.45) (FEM)	0.06
aa versus bb + ab	All	18	2972/2700	0.0004	62	2.01 (1.12–3.61) (REM)	0.02
	Non-Asian	3	902/815	0.36	1	1.04 (0.66–1.64) (FEM)	0.87
	Asian	15	2070/1885	0.07	39	4.01 (2.78–5.80) (FEM)	<0.00001
	China	8	906/685	0.24	24	2.21 (1.21–4.05) (FEM)	0.010
	Non-China	7	1164/1200	0.18	33	5.52 (3.43–8.89) (FEM)	<0.00001

OR, odds ratio; CI, confidence interval.

Sensitivity analysis

Sensitivity analysis was performed by sequentially excluding one study at a time. The results indicated that fixed-effects estimates and/or random-effects estimates before and after sequential omission of each study were similar at large (data not shown), which suggested that the results of this meta-analysis were reliable and robust.

Discussion

DN is the major cause of end-stage renal disease globally and is the second cause of blood dialysis in China (13.5%) (Lu et al., 2010). The etiology of DN is multi-factorial and involves both environmental and genetic factors. It is still a major challenge for clinicians and researchers to elucidate the pathogenesis of DN and identify patients at DN risk. Genetic studies may provide valuable information with regard to the pathobiology of DN, and in the recent years, interest in the genetic susceptibility to DN has evoked a growing attention to the investigation of susceptibility polymorphisms of DN (Hellemons et al., 2012; Thomas et al., 2012).

Vascular endothelial dysfunction is prevalent in diabetes and has been shown to be an important pathophysiologic factor for DN (Cheng et al., 2012). eNOS plays a critical role in the regulation of endothelial function through production of NO (Moncada and Higgs, 2006). The mechanism responsible for the potential association between eNOS polymorphisms and risk of DN is still unclear. However, eNOS activity and NO levels have been suggested as one of the critical factor for whether patients with diabetes are at risk for developing DN. Increased availability of eNOS may be an effective strategy in restoring endothelial function in patients with diabetic nephropathy (Badal and Danesh, 2012). Polymorphisms in the eNOS gene may lead to defective NO synthesis and decreased NO levels, contribute to the development and progression of DN (Ahluwalia et al., 2008; Palm et al., 2009). Therefore, this metabolic pathway of diabetes may be associated with renal complications of diabetes.

In the past two decades, a number of case-control studies were performed to evaluate the association between eNOS-4b/a polymorphism and risk of DN in type 2 diabetes patients, but because of relatively small sample size and the low statistical power of individual studies, results remained inconclusive. A meta-analysis

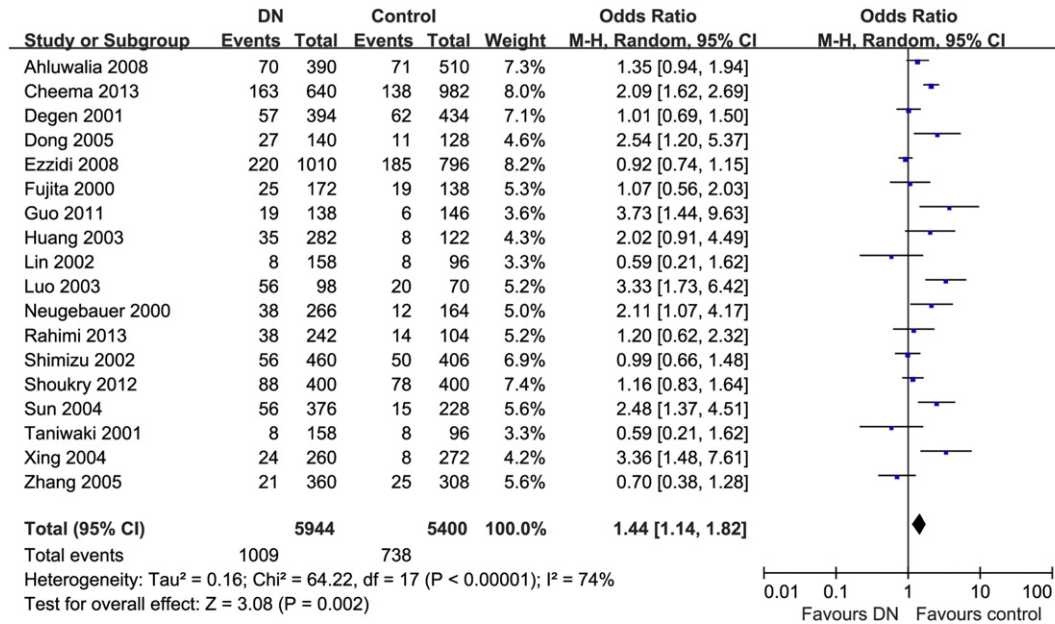


Fig. 2. The forest plot of a vs. b of eNOS polymorphism and overall DN risk.

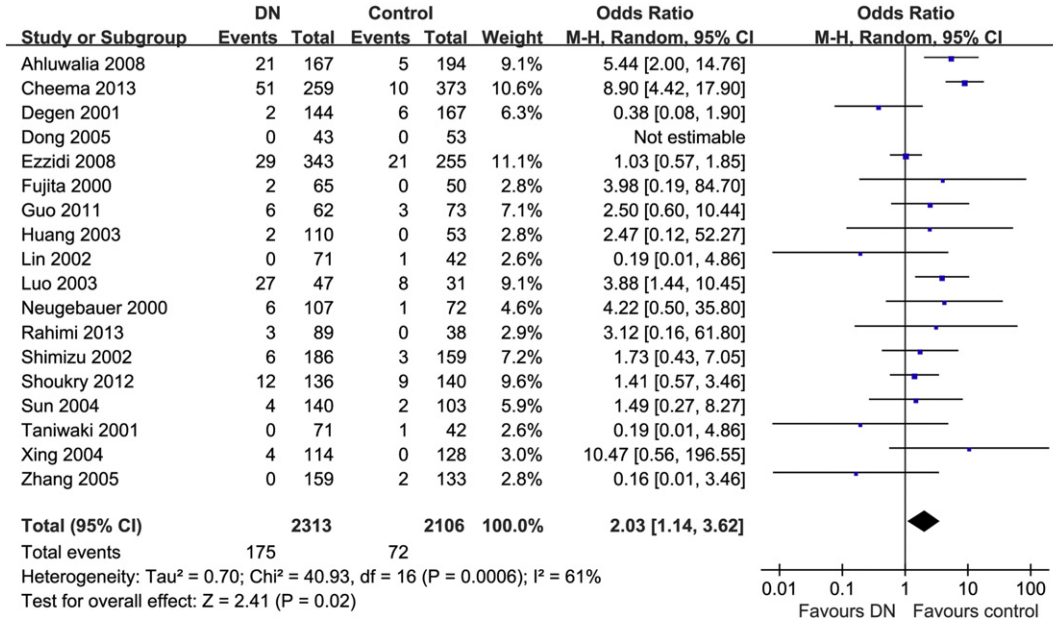


Fig. 3. The forest plot of aa vs. bb of eNOS polymorphism and overall DN risk.

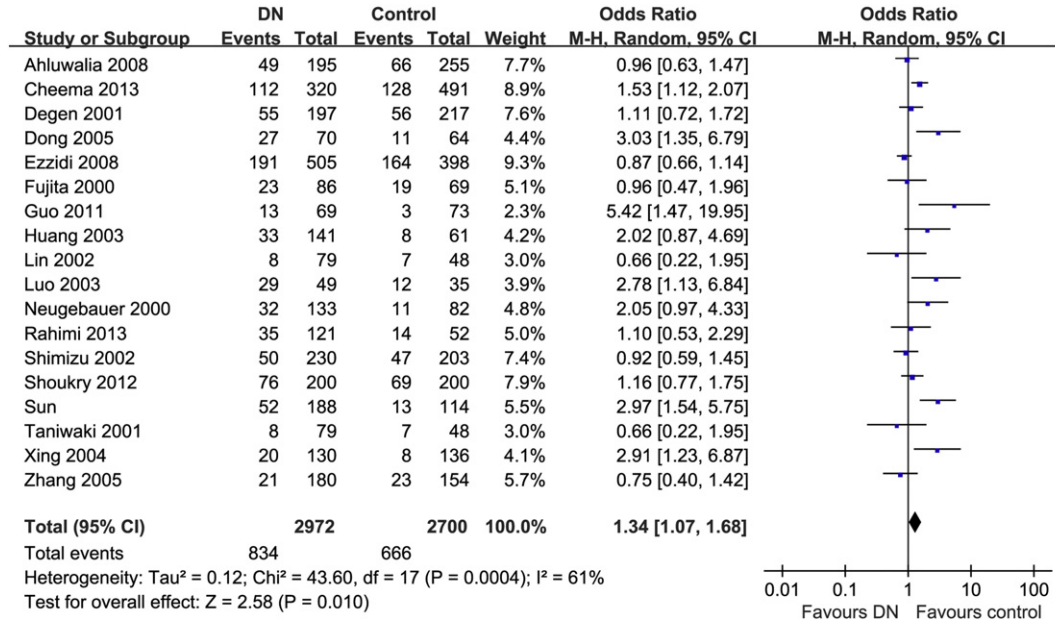


Fig. 4. The forest plot of aa + ab vs. bb of eNOS polymorphism and overall DN risk.

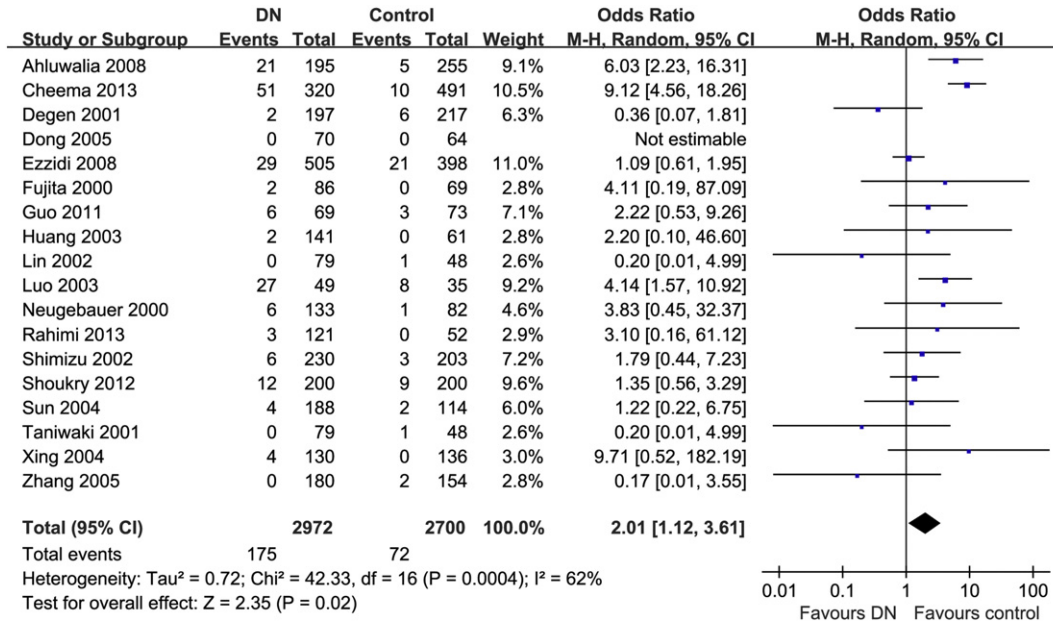


Fig. 5. The forest plot of aa vs bb + ab of eNOS polymorphism and overall DN risk.

was published in 2009 to assess the association between eNOS-4b/a polymorphisms and risk of DN in type 2 diabetes patients (Zintzaras et al., 2009). However, the meta-analysis didn't report an obvious association between eNOS-4b/a polymorphisms and risk of DN in type 2 diabetes patients. From 2009, several new studies have been performed to further investigate this association, but the association between the eNOS-4b/a polymorphisms and risk of DN is still unclear. To obtain a more reliable conclusion, we conducted the present meta-analysis of all available studies based on 18 studies published from 2000 to 2013, involving 2972 cases and 2700 controls. Significant association was found between the 4a allele and increased risk of DN when compared with the 4b allele in all populations from the present meta-analysis. According to those results, we conclude that eNOS-4b/a would be considered as an effective genetic factor, which contributed to the pathology of DN in type 2 diabetes patients.

The geographic and ethnic differences might be an important factor to effect the association of gene polymorphism with the susceptibility of DN in type 2 diabetes patients. The results of subgroup analyses indicated that significant association of eNOS-4b/a polymorphism with DN only existed in Asian population, but not in non-Asian population. Furthermore, such association appeared to be enhanced when subgroup studies were performed among Chinese population. The differences in ethnic backgrounds, lifestyle or public sanitation may partly explain this discrepancy (Hossain et al., 2007; Lee, 2003). Further ethnic-specific association studies should be performed to investigate the population discrepancy of DN susceptibility.

Heterogeneity is one of the important issues when interpreting the results of meta-analysis (Nakaoka and Inoue, 2009). Our results indicated that heterogeneity existed in the overall analysis. After subgroup analysis by ethnicity and country, the heterogeneity was decreased or removed, suggesting that certain effects of genetic variants are ethnic specific. Publication bias is an important factor to affect us to get a reliable conclusion for meta-analysis. In this meta-analysis, no significant publication bias for 4b/a polymorphism in any of the above-mentioned inherited models was found, suggesting the associations observed should be stable. Sensitivity analyses did not significantly alter the results, also suggesting that the association was reliable and stable.

Despite our efforts in performing a comprehensive analysis, several limitations exist in this meta-analysis. First, only published studies in English or Chinese were included for data analysis, some potential studies with other languages or unpublished could be missed. Second, the majority of studies involved Asian populations, whereas non-Asian populations were only seen in three studies, more studies needed to be performed to investigate the association in non-Asian populations. Third, the ORs obtained by all comparison models were unadjusted, and a more precise analysis should be conducted if all individual raw data were available. This would allow for adjustment by other covariates such as age, gender, glycemic control, hypertension, smoking status, family history, lifestyle, environmental factors, and so on. Fourth, genotyping methods were different among the selected studies, which might affect the results. Finally, DN is a complex disease, and many genes are related to DN, however, we could not address gene–gene interactions in our study due to the lack of the related information. Despite these limitations, our study provided a better understanding of the association between eNOS-4b/a gene polymorphisms and risk of DN in type 2 diabetes.

In conclusion, our meta-analysis supported an association between the 4b/a polymorphism of eNOS gene and increased risk of DN in type 2 diabetes. However, significant association only existed in Asian populations, especially in Chinese population. As for non-Asian populations, no significant association was found under all the genetic models. Taking into account the limitations of this meta-analysis, further larger well-designed studies involving different ethnic populations, particularly referring to gene–gene and gene–environment interactions are required to warrant and validate our findings.

Author contributions

ZJM and LMC conceived and designed the experiments. ZJM, XG and RC carried out the literature searching and data extraction. ZJM, HZR, JG and LMC analyzed the data. ZJM, XG, RC and JG contributed reagents/materials/analysis tools. ZJM, XG and LMC wrote the paper.

Conflict of interests

All authors declare that there are no conflicts of interest.

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